

**THE PROTEOLYTIC ACTIVITY IN RAW MILK AND THE EFFECT OF  
SUCH ACTIVITY ON THE STABILITY OF MILK PROTEINS**

**BY**

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# CHAPTER 1

## 1.1 Introduction

Milk is regarded as a complex food and is described as a colloidal suspension since it consists of three phases namely an emulsion of fat globules, a dispersion of casein micelles and an aqueous/serum phase that consists of dissolved and suspended components such as lactose, whey proteins, vitamins and minerals. It is a sensitive product since the properties can be altered by various factors (Gaucher *et al.*, 2008; Hallén, 2008; Raikos, 2010). Milk contains numerous nutrients, hormones, immunoglobulins, protective agents and growth factors (Tamime, 2008). Milk is regarded as an important part in the human diet due to its high nutritional value, therefore it can be defined as a nutrient dense product (Cilliers, 2007; Raikos, 2010).

The composition of milk may vary according to several factors such as breed of the cow, genetic factors, stage of lactation, diet, environmental factors and health status of the cow (Le Roux *et al.*, 2003). The principal constituents of milk are 87.3% water, 3.7% fat, 8.9% solids-non-fat, 4.6% lactose and 3.25% proteins (Thompson *et al.*, 2008). The milk fat contains 70% saturated fatty acids, 2% polyunsaturated fatty acids and 12.5% glycerol (Oupadissakoon, 2007). Phospholipids, cholesterol, free fatty acids and diglycerides are also present in the lipid fraction of milk (Pulkkinen, 2014). Lactose is regarded as the main carbohydrate in milk and is a disaccharide that contains glucose and galactose molecules (Oupadissakoon, 2007).

There are two main types of proteins present in milk which are the caseins and the whey proteins. The caseins represent 80% of the total proteins within milk. There are four types of casein proteins present namely; alpha-s<sub>1</sub> ( $\alpha_{s1}$ ), alpha-s<sub>2</sub> ( $\alpha_{s2}$ ), beta-casein ( $\beta$ -casein) and kappa-casein (K-casein). The whey proteins represent the remaining 20% of the proteins in milk and the main whey proteins are beta-lactoglobulin ( $\beta$ -LG), alpha-lactalbumin ( $\alpha$ -lactalbumin) and serum albumin (Oupadissakoon, 2007). The quantity and quality of proteins within milk has a substantial influence on the quality of milk (Forsbäck, 2010).

Other minor components within milk are enzymes, non-protein nitrogenous substances, fat- and water-soluble vitamins, peptides, inorganic elements and gases (Oupadissakoon, 2007). Milk is rich in micronutrients such as calcium, vitamin B<sub>5</sub>, vitamin A, iodine, magnesium, phosphorus, potassium and zinc (Pulkkinen, 2014). The nine essential amino acids are also present in milk which is tryptophan, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine (Oupadissakoon, 2007).

Fresh (raw) milk refers to milk that is in its natural, unpasteurized state (Hassan *et al.*, 2009). Good quality raw milk is essential for the production of milk products with a good quality (Cilliers, 2007). This type of milk can be contaminated with harmful bacteria since no heat treatments are applied to raw milk. There are various heat treatments that can be applied to milk in order to inactivate bacteria and enhance the shelf-life of milk such as pasteurization, sterilization and ultra-high temperature (UHT) treatment. Heat treatments are beneficial for milk, however it can result in problems such as the development of off-flavours and destabilisation processes such as coagulation, flocculation and age gelation. These problems limit the shelf-life of milk (Hassan *et al.*, 2009). Milk is therefore regarded as being an unstable medium (Reiffers-Magnani *et al.*, 2000).

## **1.2 Background to the study**

Milk is regarded as an unstable medium since it is very fragile, especially the proteins within milk. The proteins in milk undergo major changes during prolonged heating which can lead to the destabilization of milk. The proteins susceptible to changes are mainly the caseins and whey proteins such as  $\beta$ -LG. The casein proteins can be destabilized through heat, enzymes and acid. During the heat treatment of milk complexes are formed between K-casein and  $\beta$ -LG which result in modifications in the properties of the casein micelle and affects the way in which K-casein provides stabilization. This causes the casein micelle to lose its stability and thus destabilization of the casein proteins occur. Prolonged heating of milk result in  $\beta$ -LG being denatured since this protein unfolds and forms aggregates.

The flocculation of milk can occur due to heat treatments applied to milk or due to enzymes present within milk such as plasmin and microbial protease. Heat damage can cause problems in milk such as flocculation which in turn lead to gelation of milk. This ultimately results in milk that is not suited for consumption. The flocculation of milk occurs in fresh (raw), pasteurized and UHT milk.

Milk flocculation is presently a major concern for the dairy industry since it affects the quality of milk in a negative manner. There are various factors that influence the prevalence of milk flocculation hence certain methods for controlling this phenomenon is needed that can be applied during milk processing. Methods for controlling flocculation is beneficial to the dairy industry since it can result in prevention thus producing milk of substantial quality hence not rejected by consumers and that is suitable for consumption. Detection methods for milk flocculation are needed in order to establish the cause and possibly combat this problem.

## CHAPTER 2

### **Literature review**

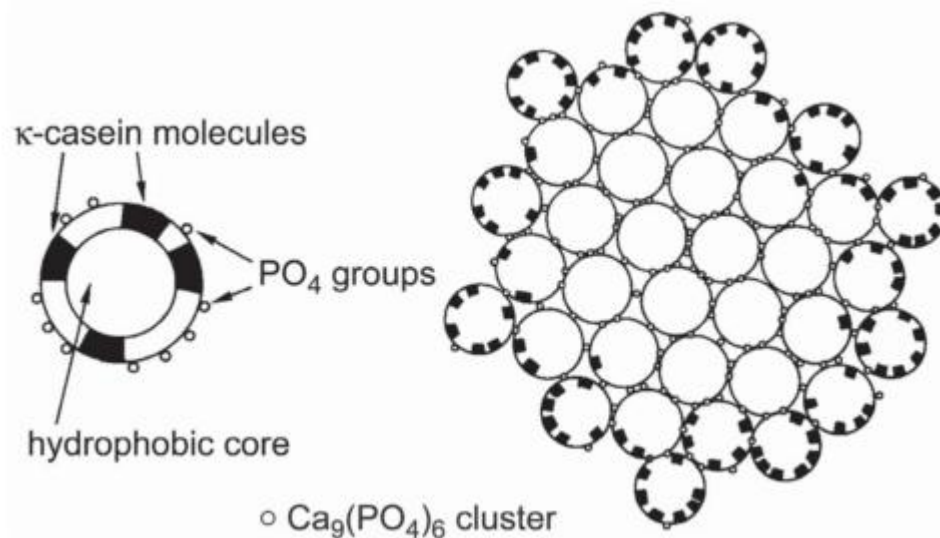
#### ***Objectives***

This in depth literature review demonstrates the definition and background information of milk flocculation. It was also important to differentiate between the two mechanisms of milk flocculation which are the enzymatic and non-enzymatic/chemical mechanisms, factors that influence milk flocculation, susceptibility of milk towards flocculation, methods of control as well as detection techniques for milk flocculation. Other points given attention to in the literature review that have an impact on milk flocculation were milk production factors, role of  $\beta$ -LG and most important the different enzymes present within milk as well as their roles in milk flocculation. The susceptibility of various types of milk to flocculation was also stated in the literature review. Attention was also given to methods for controlling milk flocculation as well as detection methods such as the Alizarol test, Reverse-phase high performance liquid chromatography (RP-HPLC) and assays for proteases, plasmin and plasminogen.

### **2. Stability of milk**

Milk is regarded as complex in nature due to its various components. The protein content, especially the casein micelle, is of great significance for the stability of milk. The structure of casein micelles is governed by self-associating forces between caseins, a balance of attractive hydrophobic and repulsive electrostatic forces and calcium-mediated interactions (Federation, 2007).

A casein micelle is regarded as a colloidal particle which is comprised of thousands of small nano-clusters. These nano-clusters are the building blocks of the casein micelle which is self-assembling of nature. Figure 2.1 below is a model of the casein micelle and presents the components which are present within the micelle. The casein micelle is characterized by the presence of K-caseins at the surface which extend beyond the solvent. There are 169 amino acids present within the casein micelle of which 63 are present on the external part of the micelle whereas 106 are present in the internal part and are regarded as being hydrophobic (Tuinier & De Kruif, 2002).



**Figure 2.1. The submicellar model for casein micelles, also proteinaceous structures and surface arrangement of K-casein (Qi, 2007).**

The stability of the casein micelles in milk is maintained by four parameters namely surface K-casein, colloidal calcium phosphate, surface potential (also zeta-potential) and steric stabilization (Datta & Deeth, 2001). Steric stabilization of the casein micelles is provided by the external part of K-casein (Tuinier & De Kruif, 2002). The calcium phosphate creates a partition against aggregation. The interaction between casein micelles are promoted and aggregated by any change at the surface of the casein micelle during UHT processing as well as storage and can be observed through an increase in viscosity of the UHT milk. In general, these changes occur slowly during the preliminary phase of storage, however after certain degrees of surface changes, the rapid occurrence of aggregation is common which result in the formation of a gel network (Datta & Deeth, 2001).

The K-casein network in the casein micelle is transformed by the formation of  $\beta$ -LG-K-casein complexes ( $\beta$ K-complex) which lead to weak associations between this  $\beta$ K-complex complex and other caseins such as  $\alpha$ s<sub>1</sub>-casein. The  $\beta$ K-complex is released due to disturbances of these weak associations. These complexes can be observed as protuberances and tendrils on the surface of the casein micelle. This disruption of K-casein can be enhanced through enzymatic or non-enzymatic actions which are commonly known as the two mechanisms for age gelation (Datta & Deeth, 2001).

There are several manufacturing processes applied to milk which lead to destabilisation processes and are described below (Federation, 2007).

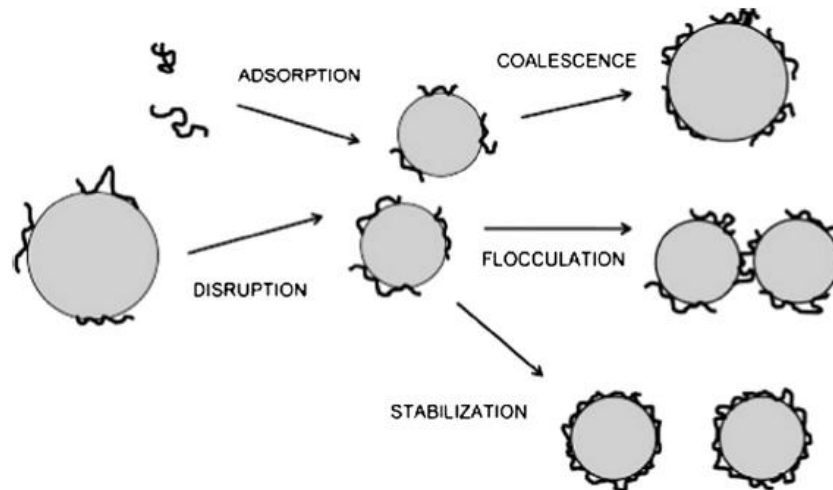
## **2.1 Destabilisation of milk**

### **2.1.1 Definitions of coagulation**

It is important to consider the context in which the term coagulation is used in order to understand the specific meaning. According to the Concise Oxford Dictionary, coagulation is described as the process where liquids change into semi-solid masses (Allen, 1991). In Science, the term coagulation is used when denaturation of proteins occurs due to protein which become insoluble and either remain in suspension or precipitates into a curd (Isaacs & Uvarov, 1979). In Food Science coagulation is random aggregation reactions with denaturation where protein-protein interactions normally dominate over protein-solvent interactions which ultimately lead to the formation of a coarse coagulum (Cheftel *et al.*, 1985).

With regard to dairy products, the term coagulation does not necessarily mean protein denaturation. Other terms used in the dairy industry to describe similar events are precipitation, flocculation and gelation. According to Cheftel *et al.*, 1985, precipitation can be used to cover all aggregation reactions which cause total or partial losses in solubility. This source also describes flocculation as a process which involves random interactions and aggregation reactions, but excludes any denaturation. The process where denatured molecules aggregate in order to form a network is referred to as gelation according to Cheftel *et al.*, 1985. A clear differentiation between aggregation, flocculation and coagulation is provided by Walstra, 2003. According to this source, aggregation is when two particles tend to stay together for longer than they normally would without colloidal interaction forces whereas the term flocculation is used to describe reversible aggregation and coagulation is used in situations where aggregation is irreversible. Separating any of these terms is only a matter of opinion.

Milk is regarded as a fragile product and being unstable hence it can be destabilized. The destabilisation of milk can occur in various ways. The main destabilisation process of milk is flocculation. Figure 2.2 below illustrates the various destabilisation processes that occur in milk. The most common destabilisation mechanisms are discussed below.



**Figure 2.2. Illustration of milk destabilisation processes (Raikos, 2010).**

### **2.1.1.1 Destabilisation by heat**

Milk proteins are precipitated during prolonged heating hence milk is regarded as unstable upon heating. The formation of complexes between  $\beta$ -LG and K-casein is common during the heat treatment of milk. It occurs through the formation of hydrophobic interactions as well as formation of intermolecular covalent, disulphide bonds. This process results in the alteration of properties of the micelle surface and it affects steric stabilization (Federation, 2007).

Whey proteins also undergo major changes during heat treatment such as self-aggregation. The whey protein,  $\beta$ -LG, dissociate into monomers, unfolds and forms aggregates by disulphide cross-linking and non-covalent interactions. This protein can also form aggregates with  $\alpha$ -lactalbumin during heat treatments (Federation, 2007).

### **2.1.1.2 Destabilisation by enzymes**

Casein can be precipitated by enzymes such as rennet. Destabilisation by rennet occurs in two phases. The first stage involves losses in K-casein that result in a reduction in the zeta potential of the micelles hence causes a loss in the steric stabilization. The second phase starts when 60-85% of the total K-casein has been hydrolysed. A protein network is then formed which consists of entrapped fat and moisture. A third stage may also exist which includes further cross-linking that result in the formation of a gel. Major factors that affect enzymatic destabilisation are the protein and rennet concentration, addition of  $\text{CaCl}_2$  and destabilisation temperature (Federation, 2007).

### **2.1.1.3 Destabilisation by other proteolytic enzymes**

Milk destabilisation can be induced by the usage of other proteolytic enzymes. Chymosin that is produced by bacteria can serve as an alternative for rennet. Pepsin is an acid protease that can also be used for destabilisation. This enzyme originates from microbial sources such as psychrotrophic

bacteria. Proteolytic enzymes have the ability to split other bonds of K-casein, not just the Phe<sub>105</sub> - Met<sub>106</sub> bond. Enzymes extracted from fruits can also induce destabilisation (Federation, 2007).

#### **2.1.1.4 Destabilisation by acid**

Acid coagulation of milk is used in various cheese making processes and as the basis for the production of yoghurt. Starter cultures are responsible for the production of acid. Calcium phosphate within micelles is dissolved and released into the milk serum during acidification. Destabilisation of caseins occurs as soon as the isoelectric point has been reached which is at pH 4.6. The production of a structure of clusters and chains of casein particles is normal practice during acidification. A coagulum is produced due to a reduction in pH. The destabilisation of milk by acid is usually done in combination with heat or enzymes such as heat and acid or enzymes and acid (Federation, 2007).

#### **2.1.1.5 Destabilisation by ethanol**

Ethanol can also be used for the precipitation of milk proteins. The primary effect of ethanol is the removal or reduction of the steric stabilization provided by the calcium phosphate within the micelle. The net negative charge on the caseins is also reduced hence precipitation of calcium phosphate occurs. Destabilisation by ethanol is influenced by various factors such as pH, ionic strength and calcium concentration (Federation, 2007).

#### **2.1.1.6 Destabilisation by higher storage temperatures**

Milk stored at temperatures that range between 8-12°C may develop sediment of precipitated protein which is mainly casein. The time of precipitation varies and the stability of milk depends greatly on the storage temperature. The caseins are mainly destabilized by calcium phosphate cross-links. The precipitate, mainly casein, generally becomes harder as the storage time progresses (Federation, 2007).

#### **2.1.1.7 Proteolytic coagulation of whey proteins**

The usage of proteinases may induce gelation of whey proteins through partial proteolysis that creates a decrease in electrostatic repulsion and in turn allows the formation of a gel. The mechanism by which this process occurs is considered that partial proteolysis of specific sections of whey proteins play a role in order to create a reduction in electrostatic repulsion that in turn allows attractive forces to predominate and ultimately lead to the formation of a gel (Federation, 2007).

#### **2.1.1.8 Destabilisation by polysaccharides**

Milk precipitation can occur when milk is mixed with polysaccharide ingredients such as carrageenan and pectin. Systems can follow certain trends when mixed such as co-solubility where the solutions remain homogeneous, incompatibility where precipitation occurs and the solution separates into

protein-rich/polysaccharide-poor and protein-poor/polysaccharide-rich phases and complexation where the protein and polysaccharide precipitate together (Federation, 2007).

## **2.2 Milk flocculation**

### **2.2.1 Definition**

Milk flocculation is the process where the association between several droplets occur due to unbalanced repulsive and attractive forces. It can also be referred to as reversible aggregation that takes place when droplets associate as a result of unbalanced attractive and repulsive forces (Reiffers-Magnani *et al.*, 2000). Flocculation also describes the process of spontaneous aggregation by bacteria in order to form flakes with sediments in milk (Abdel-Aziz *et al.*, 2012). The word flocculation can be replaced with aggregation or destabilisation without the loss of any meaning. Milk is regarded as flocculated when droplets are not randomly dispersed in the solution (Dickinson, 2010).

### **2.2.2 Background information**

Flocculation cause protein stabilized emulsions to be unstable. It reduces product quality in a drastic manner since it results in accelerated creaming, an increase in viscosity and sedimentation. A weak flocculation is characterized by closely packed structures (Dickinson, 2010). The stabilization against flocculation of droplets in milk products is challenging especially for infant formula milk as well as UHT milk (Liang *et al.*, 2013).

Heat-induced flocculation results in rapid creaming due to the formation of protein-linked droplet clusters that increases the diameter of the droplets and then in turn influences the creaming behaviours of emulsions (Raikos, 2010; Liang *et al.*, 2013). Milk protein types play a role in the physicochemical properties of an oil-in-water emulsion. The emulsifying abilities of milk proteins are influenced by the casein aggregation state, the protein pre-heat treatment as well as the calcium content (Liang *et al.*, 2013).

Casein micelles are essential for the production of flocculated milk products such as yoghurt and cheese (Tuinier & De Kruif, 2002). These micelles occur as colloidal particles in milk and they are stabilized against flocculation by K-casein (Payens, 1982). Milk flocculation is temperature dependent and mostly reversible (Dickinson, 2010).

Milk flocculation occurs in three phases namely the enzymatic degradation of K-casein, flocculation of the casein micelle and lastly gel formation. Each phase is characterized by a different pattern. The pattern of the second phase (flocculation of the casein micelle) is influenced by the cooperative nature of micellar flocculation. The properties of the gel formed are influenced by the type of action of the proteases, the type of milk and the pattern of casein proteolysis. Different factors such as pH

and temperature influence the overall milk flocculation process (Ageitos *et al.*, 2006). Flocculation does not take place until 75% of the K-casein in milk is hydrolysed (Carlson *et al.*, 1987). However, it occurs rapidly after 85-90% of K-casein have been hydrolysed (Lagaude *et al.*, 2004).

Reduction in pH of cow's milk results in flocculation of the casein micelles. Flocculation occurs when the steric stabilization of the micelles is destroyed (De Kruif & Roefs, 1996). The onset of flocculation starts at a pH of 4.7-4.8 (Tuinier & De Kruif, 2002) whereas the process will occur at 8°C during the heating of milk. Therefore, flocculation is regarded to be temperature dependent and irreversible below a pH of 4.9. Milk acidification and increased temperatures results in irreversible flocculation (De Kruif & Roefs, 1996).

Flocculation occurs in most types of milk and normally at a pH below 5.5. Decreasing the pH of milk below the isoelectric point will reverse the flocculation process (Van Aken *et al.*, 2011). Heat treatment with increased pH from 6.5 to 7.0 results in decreased flocculation. The pH at which heat treatment is performed has an influence on the rate of flocculation (Vasbinder & De Kruif, 2003). The flocculation of casein micelles in heated milk are attributed to whey protein denaturation however, the denaturation of whey proteins does not induce flocculation but it does inhibit the process (Vasbinder *et al.*, 2003).

During cold storage of milk, flocculation occurs due to unstable casein proteins. However, lower storage temperature will ensure that milk can be stored for a longer duration of time. The lower the temperature, the longer the milk can be stored without the occurrence of flocculation. Flocculation will occur naturally after 4 months of storage even if the milk was stored below 20°C (Nakanishi & Itoh, 1970).

The state of flocculation is regarded as sensitive since it is affected by various factors (Dickinson, 2010). Factors that influence the flocculation process are pH, temperature, casein content, ionic strength, enzyme concentration, calcium content, the presence of homogenized fat globules, the concentration of denatured whey proteins and casein hydrolysis by proteinases such as plasmin (Thompson *et al.*, 2008). Therefore, the flocculation process is a complex mechanism since it is influenced by so many different factors. The flocculation state can be weak and reversible or strong and irreversible. It all depends on the intermolecular forces and the colloidal interactions that are involved (Dickinson, 2010).

## **2.3 Age gelation in Ultra-High Temperature treated milk**

### **2.3.1 Ultra-High Temperature treated milk**

#### **2.3.1.1 Definition and conditions of Ultra-High Temperature treated milk**

UHT treatment of milk is used in most countries and is a well-established technology (Chavan *et al.*, 2011). UHT treatment of milk refers to a heating process at very high temperatures for a short time. This treatment has the ability to prolong the shelf-life of products and makes milk commercially sterile. UHT milk can be stored at ambient temperatures without deteriorating effects (Chove *et al.*, 2013; Rauh *et al.*, 2014).

The UHT process consists of various stages which are pre-heating with heat regeneration, holding at pre-heat temperature, heating to sterilization temperature, holding at sterilization temperature, cooling and aseptic packaging (Tamime, 2008). Generally, UHT treatments are carried out at temperatures between 140-142 °C for 4 seconds or at 138°C for 2-5 seconds. There are two types of UHT treatments which refer to as direct heat treatment at 142°C for 5 seconds and indirect heat treatment at 145°C for 3 seconds (Chove *et al.*, 2013; Rauh *et al.*, 2014). Direct heat treatment consists of the usage of steam injection or infusion with vacuum-flash cooling which involves the mixture of superheated steam with milk whereas the indirect heat treatment system uses high-performance heat exchangers that transfers heat across a divider between the milk and the heating medium, normally steam or hot water (Tamime, 2008).

The direct UHT treatment process consists of two stages which is an indirect pre-heat treatment at 75°C for 1 second using heat exchangers before the steam injection high-temperature heater (Newstead *et al.*, 2006). Both direct and indirect heat treatment increases the shelf-life of milk. Heat treatment with temperatures above 150°C for 2 seconds results in reduction of bacteria but does not damage the milk components. However, UHT treatment does have an influence on the stability of caseins, interact with whey proteins and ultimately lead to milk proteolysis (Chove *et al.*, 2013; Rauh *et al.*, 2014). Heat treatment at 145°C normally produces low quality UHT milk which may result in increased proteolysis during storage (Topcu *et al.*, 2006), the occurrence of gelation as well as result in bitterness (Chove *et al.*, 2013).

#### **2.3.1.2 Background information on Ultra-High Temperature treated milk**

UHT milk is packed under sterile conditions and in sterile packaging thus resuming its freshness for several months (Oupadissakoon, 2007). The whole purpose of UHT treatment is to produce commercially sterile milk hence it should not contain bacteria that normally grow during storage (Tamime, 2008). This type of milk should be able to stay commercially sterile for several months when stored at ambient temperatures (Gaucher *et al.*, 2008). UHT treatment results in a larger

production of small sized casein micelles when compared to raw and pasteurized milk. UHT milk also appears whiter when compared to other types of milk due to the changes in casein micelle size and the denaturation of whey proteins. Biochemical processes involved during UHT treatment are heat resistance and reactivation of native and microbial proteases and the survival of microbial spores (Hassan *et al.*, 2009).

UHT processing is a severe heat treatment that may cause some changes to milk (Gaucher *et al.*, 2008). Changes include whey protein denaturation, protein-protein interaction, lactose-protein interaction, isomerization of lactose, Maillard browning, compound formation, formation of flavoursome compounds and the formation of insoluble substances (Datta *et al.*, 2002). Chemical changes to UHT milk have a negative impact on the flavour, nutrients and physical stability (Tran *et al.*, 2008). UHT treatment also results in decreased pH (Hassan *et al.*, 2009).

The flavour of UHT milk is affected by the severity of heat treatment, storage time and storage temperature (Oupadissakoon, 2007). Oxygen and storage temperature may result in the evolution of components in UHT milk during storage (Valero *et al.*, 2001). Minor changes in macro and micro nutrients are common during UHT processing (Rehman & Salariya, 2005). The extent of these changes depends on various factors such as the type of UHT processing and temperature used (Datta *et al.*, 2002). The severity of changes progresses when heat treatments become more severe (Hassan *et al.*, 2009). Changes that occur in UHT milk during storage are due to proteolytic, lipolytic and oxidative reactions (Richards *et al.*, 2014). Chemical and biochemical reactions may take place during the storage of UHT milk thus modifying and influencing the components within the milk (Dupont *et al.*, 2007).

Changes to milk are desirable or undesirable. Destruction of bacteria, reduced plasmin activity and inactivation of enzymes are desirable changes whereas decreased sensory acceptability, browning, nutrient losses, whey protein denaturation, sedimentation, fat separation and off-flavours are undesirable changes that take place during UHT processing (Enright *et al.*, 1999; Chavan *et al.*, 2011).

Undesirable changes in the sensory characteristics of UHT milk are due to the decomposition of milk fat and protein that occur as a result of lipolysis and proteolysis during storage (Rehman & Salariya, 2005). UHT milk undergoes higher lipolysis during storage due to activity of heat-resistant lipases thus causing an increase in free fatty acids (Fernandez *et al.*, 2008). Residual proteolytic systems which survive heat treatment hydrolyse casein and ultimately result in age gelation during storage (Dupont *et al.*, 2007). Other changes to UHT milk during storage include changes in colour, odour, texture, flavour, pH and sediment. Prolonged storage may result in off-flavours (Celestino *et al.*, 1997) such as bitter tastes, stale and lipolyzed flavours due to active heat-stable enzymes such as lipases. Lipolyzed flavours are characterized as rancid, butyric and bitter tastes (Oupadissakoon, 2007).

Off-flavours are associated with the release of tyrosine in UHT milk whereas the hydrolysis of caseins is responsible for changes in the viscosity. The release of  $\beta$ K-complexes, which are formed during heat treatment, from the casein micelle is responsible for increase in viscosity (Richards *et al.*, 2014). A reduction in the sweet taste of UHT milk is also common during storage (Hassan *et al.*, 2009). Increased storage time result in increased free fatty acid concentration, viscosity as well as proteolysis however it also result in decreased protein content (Fernandez *et al.*, 2008). Changes that occur during storage depend on temperature of storage, extent of exposure of milk to light and oxygen (Hassan *et al.*, 2009).

These undesirable changes may lead to the destabilisation of UHT milk in the form of a gel or sediment during storage (Gaucher *et al.*, 2008) thus reducing the quality and affecting the shelf-life of UHT milk in a negative manner (Datta *et al.*, 2002). The usefulness of UHT milk can be enhanced by addressing the above mentioned limitations which cause defects during storage and finding solutions for it (Tran *et al.*, 2008).

Shelf-life of UHT milk refers to the storage time before the quality starts to deteriorate and eventually leads to an unacceptable level (Chavan *et al.*, 2011). The shelf-life of UHT milk can vary between 3-9 months when stored at temperatures ranging between 20-30°C (Celestino *et al.*, 1997; Richards *et al.*, 2014). The shelf-life of UHT milk depends on the development of various physicochemical and biochemical changes after processing. The sensory properties of UHT milk determine the shelf-life as well as the acceptability (Richards *et al.*, 2014).

UHT treatment consists of many advantages however it can also be disadvantageous since proteolysis can occur (Newstead *et al.*, 2006). The advantages of UHT milk is reduced energy consumption, extended shelf-life, ambient storage and distribution conditions (Chavan *et al.*, 2011). Another advantage of UHT milk is convenience and it can be stored for several months without refrigeration (Oupadissakoon, 2007).

UHT milk is successful, however commercial acceptance depends on post-process contamination, consumer acceptance, chemical and physical changes due to heat treatment as well as extended storage (Chavan *et al.*, 2011). The cooked flavour produced during UHT processing is one of the main reasons for consumers to reject this type of milk (Oupadissakoon, 2007). A major disadvantage is age gelation which limits the shelf-life of UHT milk (Chavan *et al.*, 2011).

Therefore, the selection of good quality raw milk for UHT manufacture is essential in order to produce UHT milk with an enhanced shelf-life which is the whole purpose of UHT milk (Tamime, 2008). The severe heat treatment and aseptic packaging makes this possible for UHT milk (Oupadissakoon, 2007).

### **2.3.2 Definition of age gelation**

Age gelation is a common defect that occurs specifically in UHT milk after a long storage time. This phenomenon is referred to as the physical change in UHT milk which is characterized through a decrease in fluidity and an increase in viscosity due to the formation of a three-dimensional protein network (Datta & Deeth, 2001). Gelation can be visually observed as a thick white gel at the bottom of UHT milk within the container (Kelly & Foley, 1997).

### **2.3.3 Background information on age gelation in Ultra-High Temperature treated milk**

Age gelation is a big problem for the dairy industry since it results in a shorter shelf-life of UHT milk and influences the market potential of this type of milk in a negative manner (Datta & Deeth, 2001). UHT milk is regarded as being microbiologically stable, however it is not considered as shelf stable for a long duration of time due the formation of a gel (Kohlmann & Nielsen, 1988).

There are various factors that influence the commencement of age gelation such as heat treatment, homogenization, sequence of processing steps, content of milk solids, milk composition, quality of milk and storage temperature (Cilliers, 2007). Changes in the pH of milk also have an influence on gelation properties (Vasbinder & De Kruif, 2003).

Age gelation occurs at temperatures that range between 25-30°C, however it can be delayed by higher or lower temperatures (Holland *et al.*, 2011). The intense heat treatment used for UHT processing is responsible for the occurrence of age gelation (Celestino *et al.*, 1997). Therefore, gelation is temperature dependant (Djabourov *et al.*, 1988) since gelation occurs rapidly at higher temperatures (Esteves *et al.*, 2003).

The starting point of gelation is enhanced by changes at the surface of casein micelles when the micelles lose its colloidal stability and then in turn form a three-dimensional gel network. Interaction between the micelles is enhanced by these changes. Protease activity is responsible for these changes (Cilliers, 2007). One of the causes of age gelation is proteolysis of caseins (Datta & Deeth, 2001).

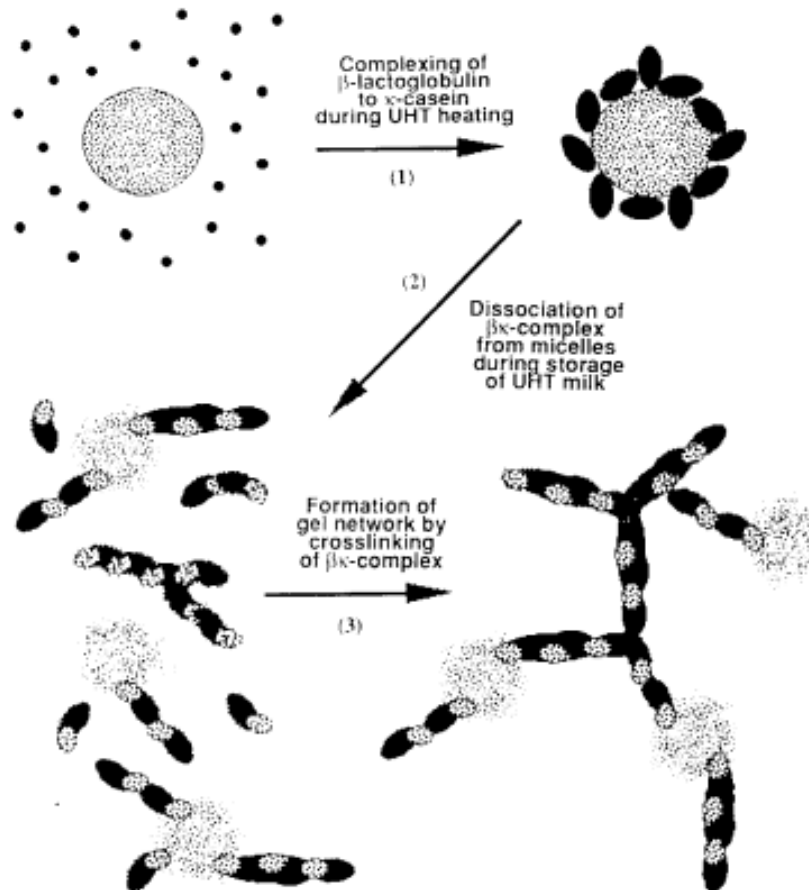
Increases in protease activity cause the breakdown of casein as well as protein denaturation in UHT milk. Gelation pattern is associated with the formation of  $\gamma$ -casein and is therefore related to plasmin activity within the milk. Age gelation results in various changes in UHT milk during storage (Cilliers, 2007). These changes in UHT milk is caused by casein hydrolysis which releases the  $\beta$ K-complex, formed during heat treatment, from the micelle. This released complex aggregates in order to form a three-dimensional network which consists of cross-linked proteins. This ultimately results in the formation a gel (McMahon, 1996).

A change in the physical state of UHT milk is increased viscosity which is the result of progressive denaturation and unfolding of proteins within milk and is also associated with casein micelle aggregation which results in the formation of a coagulum (Celestino *et al.*, 1997). Viscosity changes are divided into four stages. The first stage consists of a short period where thinning of the product is taking place whereas the second stage takes a longer period and is characterized through small changes in viscosity. A sudden viscosity change occurs during the third stage as well as eventual gel formation. During the final stage there is a decrease in viscosity due to a broken gel matrix which ultimately leaves a serum layer as well as protein curds (Datta & Deeth, 2001). The viscosity of UHT milk is stable for up to 30 days of storage and then increases after 60 days. Gelation may occur after 90 days of storage (Fernandez *et al.*, 2008).

Structural changes of the casein micelles play a more substantial role in age gelation than proteolysis (Celestino *et al.*, 1997). Age gelation occurs through the association of micelle surface proteins (Cilliers, 2007). Proteins that are extensively degraded are not able to form a gel matrix (Chavan *et al.*, 2011). Therefore, any acceleration, caused by processing or storage conditions, which result in the release of the  $\beta$ K-complex from the micelle will cause the acceleration of age gelation or delay it (Datta & Deeth, 2003). Another cause of age gelation is polymerization that is initiated by Maillard-type reactions (Celestino *et al.*, 1997).

The ease at which age gelation occurs depend on three processes that lead to gelation namely, the interaction between  $\beta$ -LG and K-casein, the release of the  $\beta$ K-complex from the casein particle and the cross-linking of the  $\beta$ K-complex and associated proteins (Datta & Deeth, 2001; Chavan *et al.*, 2011). Refer to Figure 2.3 for the illustration. The separation of milk into curds and whey and decreases in viscosity is normal practice during the gelation process (Datta & Deeth, 2003).

Consumers sometimes reject UHT milk due to the changes that occur during storage such as development of bitter tastes, increased viscosity and acidity, decreased pH, becoming translucent and the formation of a sediment. This affects the shelf-life, quality and consumer acceptance of UHT milk in a negative manner (Newstead *et al.*, 2006; Hassan *et al.*, 2009).



**Figure 2.3. Model of age gelation in UHT milk where 1 represents the formation of the  $\beta$ K-complex, 2 shows its dissociation from micelles during storage and 3 shows the subsequent gelation of the milk through cross-linking of the  $\beta$ K-complex (Datta & Deeth, 2001).**

### **2.3.4 Factors that affect age gelation in Ultra-High Temperature treated milk**

#### **2.3.4.1 Mode and severity of heat treatment**

Prior to UHT processing, raw milk is normally pre-heated to 80-95°C for 30-60 seconds in order to stabilize the  $\beta$ -LG before heating the milk to 135-150°C for a few seconds during UHT treatment. This step is regarded as important since  $\beta$ -LG is denatured during the pre-heat treatment and hence will enable this protein to remain stable in order to protect the high temperature heating section from deposits of this protein. This pre-heat step also ensures the precipitation of whey proteins thus decreasing the number of sites that are available for clotting. An increase in a heat treatment of 72°C for 30 seconds to a heat treatment of 80°C for 30 minutes has the ability to delay age gelation during storage (Datta & Deeth, 2001).

An increase in temperature from 142°C to 152°C may allow UHT milk to be stored longer without the occurrence of age gelation since heating temperatures between 135-140°C results in increased gelation times. The increased level of whey protein denaturation serves as the explanation behind the resistance to age gelation of severe heat-treated milks. UHT milk with a 28% whey protein denaturation tend to gel after 115 days whereas the more severe heat-treated UHT milk with a 66% of denatured whey proteins tend to gel after 150 days. Therefore, the formation of complexes between whey proteins and caseins plays an important role in the onset of age gelation. More severe heat treatments tend to delay age gelation due to the enhancement of the extent of chemical cross-linking within casein micelles (Datta & Deeth, 2001).

#### **2.3.4.2 Proteolysis**

Proteolysis can affect the quality of dairy products in a positive or negative manner depending on the processing purposes and conditions (Prado *et al.*, 2006). Proteolysis may be positive for some dairy products such as cheese, however it has a negative impact on UHT milk since it results in age gelation and bitterness which is undesirable. Proteolysis of caseins and age gelation are in correlation since proteolysis is the cause behind age gelation during the storage of UHT milk (Datta & Deeth, 2001). The extend of proteolysis is not necessarily directly related to the onset of age gelation (Enright *et al.*, 1999) however the rate of proteolysis has a direct effect on the rate of age gelation (Esteves *et al.*, 2003). Proteolysis is a major factor that limits the shelf-life of UHT milk since it results in changes in flavour and texture. These changes affect the quality of UHT milk in a negative manner (Datta & Deeth, 2003; Hassan *et al.*, 2009).

Both indigenous milk proteases as well as heat-stable proteases produced by psychrotrophic bacteria are responsible for the occurrence of proteolysis (Celestino *et al.*, 1997; Datta & Deeth, 2001). The psychrotrophs responsible for proteolysis are mainly *Pseudomonas fluorescens* (Gaucher *et al.*, 2011; Richards *et al.*, 2014). There are other enzymes that also contribute to proteolysis such as cathepsins and elastase. However, the enzymes from psychotropic origin contribute the most to proteolysis (Forsbäck, 2010).

Proteolysis promotes the disaggregation of casein micelles (Fernandez *et al.*, 2008). The breakdown of  $\beta$ -casein is more rapid than  $\alpha$ <sub>1</sub>-casein during storage due to the presence of plasmin activity (Datta & Deeth, 2001). Proteolysis therefore causes a decrease in the percentage of caseins such as the  $\alpha$ <sub>1</sub>-casein as well as  $\beta$ -casein (Bavarian *et al.*, 2010).

Proteolysis causes a reduction in milk secretion. Two mechanisms are involved. The first mechanism is referred to as "contact proteolysis" which includes the activity of acidic proteases that destroy caseins at the neutral pH of milk. The second mechanism is referred to as "proteolysis by internalization" which is the ingestion of granules into the vesicles of casein micelles or fat globules (Le Roux *et al.*, 2003).

Indirect heat-treated UHT milk is less at risk of proteolysis due to higher heat treatment and slower cooling procedures (Newstead *et al.*, 2006). Direct heat-treated UHT milk normally shows high levels of proteolysis thus a shortened shelf-life is expected for this type of UHT milk (Topçu *et al.*, 2006; Newstead *et al.*, 2006).

Proteolysis has a negative impact on some of the properties in milk such as sensory properties, texture, consistency and flavour (Cilliers, 2007). Proteolysis results in the formation of bitter off-flavours, fat separation, sedimentation and age gelation hence limiting the shelf-life of UHT milk (Datta & Deeth, 2003; Hassan *et al.*, 2009). Proteolysis of UHT milk and the release of tyrosine in milk are correlated with each other and may contribute to the formation of off-flavours (Richards *et al.*, 2014). It also causes an increase in the viscosity of UHT milk thus resulting in the formation of a gel (Topçu *et al.*, 2006).

Proteolysis occurs at storage temperatures between 20-30°C, however higher levels of proteolysis occurs in milk at 40°C than milk that is stored at 20°C (Gaucher *et al.*, 2008). The storage of UHT milk at 40°C does delay the onset of age gelation. The storage of temperatures at 20°C will result in decreased proteolysis, bitterness and age gelation (Datta & Deeth, 2001). High temperature heat treatments have a positive effect on proteolysis since it can reduce the susceptibility of UHT milk to proteolysis during storage (Chove *et al.*, 2013).

The occurrence of proteolysis in UHT milk can be reduced by the processing of milk at higher temperatures such as 150°C as well as extended holding times. However, this may result in milk having a more intense cooked flavour thus affecting the acceptability of UHT milk by consumers (Topçu *et al.*, 2006). The homogenization of milk prior to UHT treatment may result in a reduction of enzyme activity which is responsible for proteolysis during storage. The presence of the  $\beta$ K-complex at the surface of casein micelles has the ability to reduce the degree of proteolysis and age gelation since it can inhibit the access of proteases to caseins (Chove *et al.*, 2013).

A method that can be used to monitor proteolysis by microbial proteases is the determination of free sialic acid. This method can also serve as a warning for the onset of age gelation (Datta & Deeth, 2001). Proteolysis can also be measured by monitoring changes in nitrogen levels such as decreases in casein nitrogen or increases in non-protein nitrogen. These changes are well correlated with functionality changes such as microstructural changes as well as increases in viscosity of UHT milk (Bavarian *et al.*, 2010).

The addition of certain inhibitors which inhibit plasmin may protect UHT milk from the occurrence of proteolysis therefore the addition of plasminogen activator inhibitor can enhance the shelf-life of UHT milk since it reduces the activity of plasmin (Datta & Deeth, 2001).

### **2.3.5 Susceptibility of various types of milk to flocculation or age gelation**

#### **2.3.5.1 Raw milk**

Raw milk is regarded as a perishable product since it only has a shelf-life of approximately three to five days. This type of milk is unpasteurized and thus storage under refrigerated conditions is essential in order to prevent spoilage. There are several factors that influence the shelf-life of raw milk such as milk collection, handling techniques, the hygiene of the milking environment, storage temperature, somatic cell count and amount of bacteria present (Vijayakumar, 2012).

Raw milk consists of a very diverse system of bacteria due to many possibilities for recontamination which includes milking equipment, air, water, feed, grass, soil and teats of cow (Baur *et al.*, 2015). The dominant microbial population within raw milk is both Gram-positive (*Bacillus*, *Clostridium*, *Corynebacterium*, *Micrococcus*, *Microbacterium*, *Staphylococcus*, *Streptococcus* and *Lactobacillus*) as well as Gram-negative (*Pseudomonas*, *Aeromonas*, *Acinetobacter*, *Alcaligenes*, *Enterobacter* and *Flavobacterium*) (Baur *et al.*, 2015). Extended storage of raw milk at temperatures between 2-6°C has an enormous influence on the composition of the natural microbial population (Samaržija *et al.*, 2012) as well as enables growth of psychrotrophic bacteria (Baur *et al.*, 2015). It is normal practice that the initially dominant Gram-positive organisms are replaced by Gram-negative and Gram-positive psychrotrophic organisms within cooled raw milk. It is therefore clear that psychrotrophs are the dominant organisms within raw milk since they comprise more than 90% of the total microbial population in cooled raw milk (Samaržija *et al.*, 2012).

Gram-positive spore-forming bacteria are also present within raw milk, however the presence is less than Gram-negative psychrotrophs due to these organisms consisting of a longer generation time (8.5 hours) and longer lag phase at temperatures between 2-7°C. The presence of bacteria within raw milk results in certain degrees of spoilage, therefore it is essential that the total count of mesophiles within raw milk is lower than 30 000 CFU/mL<sup>-1</sup> and the total count for psychrotrophs must be lower than 5000 CFU/mL<sup>-1</sup>. The most common spoilage bacteria within raw milk are species from *Pseudomonas* and *Bacillus* (Samaržija *et al.*, 2012).

Flocculation does occur in raw milk that is incubated with proteolytic enzymes. Flocculation in raw milk results in changes in particle size of the fat globules (Ye *et al.*, 2011). Raw milk has a higher rate of proteolysis than other types of milk due to high activity of native enzymes hence this type of milk being susceptible to flocculation (Chove *et al.*, 2013).

#### **2.3.5.2 Direct and indirect Ultra-High Temperature treated milk**

Age gelation normally does not occur in UHT milk that is processed by the indirect heat treatment method (Celestino *et al.*, 1997) since this type of UHT milk is more stable than UHT milk produced by the direct heat treatment method (McMahon, 1996). Indirect heat-treated UHT milk involves a

greater heat intensity thus inactivating the proteases to a greater extent (Datta & Deeth, 2001). However, plasmin activity is normally higher in this type of UHT milk (Cilliers, 2007).

UHT milk processed by the direct heat treatment method are less stable hence more susceptible to age gelation due to reduced heat load, rapid heating and cooling when compared to UHT milk processed by indirect methods (Rauh *et al.*, 2014). Since direct processed UHT milk is exposed to more severe heat treatments, it is considered to be more susceptible to age gelation than indirect processed UHT milk (Kohlmann & Nielsen, 1988). However, more severe heat treatment can also delay gelation (Topçu *et al.*, 2006). This type of UHT milk also contains higher activity of plasmin and plasminogen than indirect heat-treated UHT milk (Datta & Deeth, 2001). Including a pre-heat step or increasing the temperature of UHT treatment may result in this type of UHT treated milk to be more stable (Rauh *et al.*, 2014).

The difference in gelation time for both direct- and indirect processed UHT milk is that during indirect processing, a larger portion of enzyme and more  $\beta$ -LG is denatured which inhibits the proteolytic activity of native proteases (Kohlmann & Nielsen, 1988). It is therefore clear that direct processed UHT milk is more susceptible to age gelation than indirect processed UHT milk.

#### **2.3.5.3 Skimmed and full cream milk**

The susceptibility of milk towards age gelation varies according to fat content of milk such as skimmed- and full cream milk. Research shows that skimmed milk has a shorter shelf-life than full cream milk. The difference in spoilage rates for these two types of milk may be ascribed to different rates of growth of psychrotrophic bacteria and different handling and storage conditions. The effects and spoilage patterns of psychrotrophic bacteria on skimmed and full cream milk are also different. The bacteria responsible for the spoilage of skimmed and full cream milk are also different since the different compositions of these milks result in the selection of different bacteria. The spoilage organisms for both milks include *Pseudomonas* spp. which is responsible for the production of proteases (Deeth *et al.*, 2002).

The homogenization of milk prior to UHT treatment may result in lower levels of enzymes that contribute to proteolysis during storage. However, homogenization may also cause proteins to be more susceptible to proteolytic attack since it causes attachment of caseins and whey proteins to the fat globule membrane hence giving rise to smaller micellar particles (Chove *et al.*, 2013). Skimmed milk reflects higher levels of proteolysis thus causing this milk to have a shorter shelf-life than full cream milk. The higher levels of proteolysis in skimmed milk can be attributed to the production of higher protease activity in this type of milk (Deeth *et al.*, 2002). The fat in full cream milk has a protective effect against proteolytic attack by proteases (Valero *et al.*, 2001). Lipase production occurs in both types of milk however the activity of lipases tends to be lower in skimmed milk than in full cream milk. The higher levels of lipase in full cream milk are due to the presence of their

substrate that is triglycerides which are responsible for lipolysis. Lower levels of lipase in skimmed milk are attributable to most lipases being hydrolysed by proteases thus inactivating these enzymes (Deeth *et al.*, 2002).

The production of bitter and sour tastes is more common in skimmed milk than in full cream milk. The prevalence of bitter flavours in skimmed milk is attributed to the occurrence of higher levels of proteolysis. The degradation of protein and destabilisation of casein micelles is more prevalent in skimmed milk due to higher protease activities (Deeth *et al.*, 2002). Skimmed UHT milk is considered to deteriorate more than UHT full cream milk due to proteolysis and the formation of bitter flavours (Valero *et al.*, 2001). The major difference between skimmed and full cream milk is that skimmed milk consists of higher protease activity hence result in higher levels of proteolysis thus causing this type of milk to have a shorter shelf-life than full cream milk. It is therefore clear that skimmed milk is more susceptible to age gelation than full cream milk (Deeth *et al.*, 2002).

### **2.3.6 Methods of controlling age gelation in Ultra-High Temperature treated milk**

Age gelation in UHT milk can be prevented or delayed by the application of certain methods which is based on minimizing proteolytic activity by proteases and plasmin, delaying the dissociation of the  $\beta$ K-complex from the casein micelle and inhibiting the cross-linking and protein network formation. These ways of controlling age gelation also involves the usage of high quality fresh milk, manipulation of processing or storage conditions and the usage of additives (Datta & Deeth, 2001). Controlling age gelation of UHT milk is advantageous since it results in the stability of UHT milk hence extended shelf-life for this type of milk (Chavan *et al.*, 2011). Methods of controlling age gelation in UHT milk are discussed below.

#### **2.3.6.1 Quality of fresh milk**

In order to achieve UHT milk with an improved shelf-life, it is very important to use raw milk of a high quality. Storage temperatures of 4°C for no more than 48 hours results in minimum growth of psychrotrophic bacteria as well as decreased amounts of extracellular microbial proteases. The usage of milk with low somatic cell counts is also of utmost importance in order to ensure low levels of plasmin and plasminogen activators in milk (Datta & Deeth, 2001; Chavan *et al.*, 2011).

#### **2.3.6.2 Inactivation of proteases by low temperatures**

Heat-resistant enzymes can be inactivated at heat treatments of 55°C for prolonged holding times such as 30-60 minutes. Low temperature inactivation of proteases is regarded as effective and effectiveness does not depend on the concentration of proteases. This treatment also does not have any negative effect on the flavour and protein content of milk and can be applied prior to or after sterilization and is most effective when applied to UHT milk after one day of UHT treatment. This method of inactivation of proteases is most effective for up to 60 minutes of heating at 55°C since proteases undergo a unique conformational change at this temperature which is followed by

aggregation of proteases with casein to form an enzyme-casein complex which in turn inactivate enzymes. The reactivation of proteases after this treatment is not possible. Combining this treatment with UHT treatment will enhance shelf-life of UHT milk. This treatment has the ability to inhibit proteolysis and delay age gelation of UHT milk for up to 146 days. This treatment is regarded as the best solution for the problem of heat-stable proteases. Low temperature inactivation of proteases may be limited due to the resistance of proteases to heat treatments above 55°C (Datta & Deeth, 2001; Chavan *et al.*, 2011).

#### **2.3.6.3 Heat treatment during pre-heating and sterilization**

An important consideration in delaying age gelation of UHT milk is the severity of heat treatment during pre-heating and sterilization since gelation is induced by proteolysis. Adequate heat treatments result in  $\beta$ -LG denaturation as well as the complexation of denatured whey proteins with caseins. Plasmin is also inactivated by very high heat treatments. Another practical method for delaying age gelation is the indirect heat treatment of UHT milk. This approach is considered as a very practical method of delaying age gelation and does not result in cooked flavours within UHT milk (Datta & Deeth, 2001; Chavan *et al.*, 2011).

Increase in sterilization temperature from 142 to 152°C may delay the onset of gelation since milk can be then stored for a longer period without the occurrence of age gelation. However, an increase in sterilization temperature from 135 to 140°C may result in an increase in gelation time. A sterilization temperature of 150°C extends the shelf-life of UHT milk since it reduces the occurrence of proteolysis, gelation and bitterness (Chavan *et al.*, 2011). A heat treatment of 75°C for 15 seconds together with a storage temperature of 30°C will result in the onset of age gelation at 7 months. Heat treatments above 90°C for 30-60 seconds have the ability to inhibit age gelation however, pre-heat treatments at 80°C for 30 seconds enhances age gelation (Newstead *et al.*, 2006).

#### **2.3.6.4 Manipulating the storage temperature of UHT milk**

The storage temperature of UHT milk has a significant influence on the time of age gelation. Age gelation normally occurs at temperatures between 20-25°C rather than temperatures between 35-40°C or temperatures below 4°C. Reduced gelation at higher temperatures is still not fully understood, however it may be due to an increase in proteolytic activity at higher storage temperatures. The high degree of protein denaturation, which results in the proteins being unable to form a stable gel matrix, at higher heat treatments may be the reason behind the lack of age gelation at higher temperatures. Age gelation are inhibited at a temperature of 37°C since regions of proteins that have a part in protein-protein interactions are blocked by casein-lactose interactions that involve lysine residues. These interactions result in browning in UHT milk that is stored at temperatures above 30°C (Datta & Deeth, 2001; Chavan *et al.*, 2011). Therefore, the Maillard browning reaction

may inhibit the hydrolysis of K-casein since the blockage of lysine and arginine residues of K-casein molecules result in the loss of sensitivity to rennet coagulation (Chavan *et al.*, 2011).

#### **2.3.6.5 Addition of sodium hexametaphosphate and other additives**

Another effective approach to delay gelation is the addition of sodium hexametaphosphate to milk prior to UHT treatment, however the addition of additives may result in negative consumer reactions. Therefore, it is essential to consider the legal aspects of this approach. Sodium hexametaphosphate has the ability to delay the release of the  $\beta$ K-complex by holding the K-casein more tightly, thus delaying age gelation. The addition of this additive is effective since it inhibits the second stage of age gelation and results in the coagulation of proteins (Datta & Deeth, 2001; Chavan *et al.*, 2011). The addition of hexametaphosphate prior to UHT treatment is preferable since this additive is eliminated during UHT processing (Kocak & Zadow, 1980).

The addition of sodium citrate or disodium ethylenediaminetetraacetic acid (EDTA) to cold-stored raw milk prior to UHT treatment induces age gelation whereas calcium chloride has the ability to delay age gelation. The addition of sodium polyphosphate also increases the stability of UHT milk thus protecting it against age gelation (Kocak & Zadow, 1980).

The stability of milk can be increased by the addition of sodium chloride prior to UHT processing which in turn delays gelation for up to 500 days when the milk is stored at 25°C, however the addition of too much calcium may enhance gelation. The addition of disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) after UHT processing does not affect proteolysis to a great extent. These additives stabilize the proteins within the milk since they associate with the caseins and in turn alter the net charge on the surface of the casein micelles thus protecting the milk against age gelation (Datta & Deeth, 2001).

#### **2.3.6.6 Addition of the sulfhydryl (SH) group-blocking agent**

The denaturation of whey proteins and the interaction of them with caseins can be inhibited by the addition of a SH blocking agent such as N-ethylmaleimide to milk prior to heat treatments. Direct heat-treated UHT milk that contains this additive normally shows age gelation later than indirect heat-treated UHT milk with the same additive (Datta & Deeth, 2001; Chavan *et al.*, 2011).

#### **2.3.6.7 Lysine**

Lysine has the ability to inhibit plasminogen activation since it competes for lysine-binding sites that are present in plasmin and plasminogen. Plasmin and plasminogen can also be dissociated from casein micelles by lysine. The usage of lysine has some limitations such as not being able to completely inactivate plasminogen (Chavan *et al.*, 2011).

#### **2.3.6.8 Treatment of milk with carbon dioxide or nitrogen**

The addition of carbon dioxide to milk serves as a preservative. Carbon dioxide consists of antimicrobial properties against psychrotrophic bacteria. Carbon dioxide has the ability to reduce protease secretion at low temperatures. Therefore, milk can have a longer shelf-life when the air in the sealed container is replaced with carbon dioxide (Tamime, 2008). The growth of various psychrotrophs can also be inhibited by the treatment of milk with nitrogen (Chavan *et al.*, 2011).

#### **2.3.6.9 ISI heating**

A new type of steam injection that involves high temperatures with shorter holding times is referred to as ISI heating. The temperatures involved range between 150-180°C with a holding time of less than 0.2 seconds thus enables milk to be cooled rapidly. During the use of this equipment, the product is pumped through a pipe with a narrow end as well as a wall which contains several small openings where the high-pressure steam is injected hence allowing fast heating of a product. The pre-heat treatment of milk can occur prior to or after heat treatment with the ISI heater. Treatment through the usage of this equipment has the ability to inactivate plasmin and denature  $\beta$ -LG. The post-heat treatment is most successful in order to reduce plasmin sufficiently. The usage of this new approach can result in UHT milk which consist of a longer shelf-life, will lead to less product degradation such as <50% denaturation of  $\beta$ -LG and also result in improved taste characteristics (Chavan *et al.*, 2011).

#### **2.3.6.10 Membrane processing of Ultra-High Temperature treated milk**

Modern membrane technology can be applied to UHT milk thus enhancing its shelf-life such as ultrafiltration, nano-filtration and reverse osmosis. Milk with a higher fat content results in decreased heat stability as well as the formation of flakes. Storage stability of milk can be enhanced by the usage of electrodialysis, ultrafiltration and nano-filtration (Chavan *et al.*, 2011).

#### **2.3.6.11 Cooling the milk during storage**

Rapid cooling of milk is necessary since contamination by psychrotrophic bacteria is unavoidable. The storage of milk at a temperature of 2°C is more effective than storage at temperatures between 4-7°C. The storage of milk at 2°C also has a positive effect on the quality of UHT milk since milk stored at this temperature ensures that UHT milk have a longer shelf-life (Tamime, 2008).

#### **2.3.6.12 Lactic acid bacteria**

The addition of lactic acid to raw milk can inhibit the growth of psychrotrophic bacteria. The amount of lactic acid bacteria needed in order to obtain complete inhibition of the growth of gram negative rods is 108 cfu/mL<sup>-1</sup>. Lactic acid bacteria act against psychrotrophic bacteria in two ways. Firstly, lactic acid production occurs at refrigerated temperatures and therefore effectively acts against

proteases that normally grow under these conditions. Secondly, lactic acid bacteria also adjust nutrients within milk thus acting as competitors to the proteases. This method of protection against protease attack can be effective for UHT milk as well (Tamime, 2008).

#### **2.3.6.13 Multi-target attack/integrative approaches**

The usage of two or more bio-preservatives in order to achieve a synergistic effect on psychrotrophic bacteria can be an effective method for extending the shelf-life of milk. This method has the ability to expand the range of bacteria that may be inhibited. An example of this approach is the usage of natural antimicrobials with other non-thermal preservation methods such as high pressure processing, pulsed electric fields, ultra-sonication and irradiation. The combination between pulsed electric field technology and pasteurization can extend the shelf-life of milk to up to 78 days (Tamime, 2008; Vijayakumar, 2012).

#### **2.3.6.14 Other**

The complex formation between  $\beta$ -LG and K-casein consists of the ability to protect casein proteins against protease attack. The fat in UHT milk may also play a role in the protection of proteins within milk against protease attack (Celestino *et al.*, 1997).

Legume protease inhibitors have the potential to prevent or reduce proteolytic activity of *Bacillus* proteases. These inhibitors are not able to reduce defects caused by proteases produced by *Pseudomonas* spp. however they do consist of inhibitory activities against *Pseudomonas* proteases. Legume protease inhibitors have the ability to reduce the activity of *Bacillus* proteases to a great extend in UHT treated milk (Richards *et al.*, 2014).

### **2.4 The two mechanisms of age gelation**

The mechanism of age gelation is complex hence it is not fully understood (Pulkkinen, 2014). The process of age gelation consists of two stages. The first stage involves structural changes whereas the second stage involves physicochemical reactions that cause decreased stability and result in gel formation (Datta & Deeth, 2001). The first stage is characterized through the release of the micelle from a complex formed between denatured  $\beta$ -LG and K-casein during the UHT-sterilization of milk. Proteolytic activity of heat-resistant enzymes, which are active during storage, is responsible for the first stage hence age gelation is the result of an enzymatic process (Gaucher *et al.*, 2008).

The second stage is characterized by non-enzymatic physicochemical changes which are the separation of proteins from casein micelles and the association of proteins to the surface of the micelle, which result in aggregation and the formation of a three-dimensional protein network (Gaucher *et al.*, 2008). Age gelation is related to aggregation of the casein micelle however it is not always caused by this action since the casein particles are connected by the  $\beta$ K-complex. It is known

that proteolytic activity causes the release of the  $\beta$ K-complex. This disruption of K-casein can be induced by enzymatic action or by non-enzymatic action (Datta & Deeth, 2001). Therefore, both enzymatic as well as non-enzymatic/chemical reactions are regarded as the two mechanisms for milk flocculation/age gelation (Pulkkinen, 2014). Both mechanisms of age gelation will be discussed in detail in the sections that follow.

#### **2.4.1 Enzymatic mechanism of age gelation**

This mechanism suggests that proteases are responsible for the release of the  $\beta$ K-complex which forms a protein network and eventually result in the formation of a gel. The proteases cleave the peptide bonds which in turn anchor the K-casein to the casein micelle and thus facilitate release of the  $\beta$ K-complex, however it does not have a direct reaction on this complex. This mechanism of age gelation consists of two stages and this separation of  $\beta$ K-complexes by proteases is considered to be the first stage. Aggregation of  $\beta$ K-complexes and the formation of a three-dimensional cross-linked protein network are considered to be the second stage. A gel is produced as soon as a subsequent concentration of cross-linked  $\beta$ K-complexes as well as entrained proteins is reached (Datta & Deeth, 2001; Chavan *et al.*, 2011).

##### **2.4.1.1 Enzymes in milk**

###### **2.4.1.1.1 Definition of enzymes**

Enzymes can be defined as proteins that have certain biological functions. They also originate from various sources such as milk itself, microbial contamination and from the somatic cells within milk (Guinee & O'Brien, 2010).

###### **2.4.1.1.2 Background information**

Milk is regarded as a very biologically active product since it consists of many different native enzymes (Chen *et al.*, 2003). There have been more than 70 indigenous enzymes characterized in milk (Gazi *et al.*, 2014). Enzymes within milk can be indigenous or produced by some bacteria (Vijayakumar, 2012). This determines their characteristics and also their mechanism of inactivation (Chavan *et al.*, 2011; Chove *et al.*, 2013). The indigenous enzymes present in milk are associated with casein micelles, the fat globule membrane, milk serum, somatic cells, blood and the cell cytoplasm (Cilliers, 2007). The native enzymes present in milk originate from four main sources such as the blood plasma, secretory cell cytoplasm, milk fat globule membrane and somatic cells from the blood, however most enzymes are normally associated with the fat globule membrane within the milk (Vijayakumar, 2012).

The enzymes present within milk have different functions such as stability to processing, impact on dairy products as well as significance for consumer safety. Some enzymes such as lactoperoxidase

are especially of interest for their beneficial activity and preservation of milk quality. Some enzymes are used as indices of processing as well as the thermal history of milk such as alkaline phosphatase. Some serve as indices of mastitic infection such as catalase, while certain enzymes can serve as indices of antimicrobial activity such as lysozymes, and others for the effects on quality of dairy products which may either be positive or negative depending on the product such as plasmin and lipase (Kelly & Fox, 2006; Fox & Kelly, 2006).

The growth of psychrotrophic bacteria is the cause behind the occurrence of proteolytic enzymes within milk such as proteases and lipases (Topçu *et al.*, 2006). Native milk enzymes are regarded as inactive due the absence of substrates and the presence of inhibitors or unsuitable environmental conditions. Few of the indigenous enzymes serve a biologically important role after the secretion of milk, however they do not play a role in the synthesis and secretion of milk (Vijayakumar, 2012).

The levels of enzymes in milk are not constant and the factors that influence the variability of enzyme levels in milk are the breed of cow, age of the cow, lactation stage, season, somatic cells, mastitis, diet and health status of the cow. The presence of enzymes in milk has an impact on the quality and shelf-life of milk (Chen *et al.*, 2003; Larsen *et al.*, 2006). The level of activity for all enzymes also tends to differ due to different origins of the enzymes (Kelly & Fox, 2006). Milk enzymes can serve as an indicator of cow's health or heat treatment history of the milk since they can cause deterioration of quality or induce changes in milk. Some enzymes also offer protective effects towards milk (Tamime, 2008). Enzymes that cause deterioration in milk quality are lipases, proteases, acid phosphatase and xanthine oxidase (Vijayakumar, 2012).

The indigenous enzymes that are characteristic to milk are plasmin, plasminogen, plasminogen activators, plasmin inhibitors, plasminogen activator inhibitors, thrombin, cathepsin D, acid milk proteases and amino-peptidases (Aslam & Hurley, 1997; Newstead *et al.*, 2006; Cilliers, 2007). Enzymes are characterized into these above mentioned groups through to their mechanism of action (Chen *et al.*, 2003). There can be distinguished between indigenous enzymes from that of microbial origin since plasmin produce larger and more hydrophobic peptides than enzymes produced by psychrotrophs (Le *et al.*, 2006). Plasmin and microbial proteases consists of different affinities for the different caseins (Datta & Deeth, 2001). Proteases can also be derived from somatic cells, the bacteria present in milk and in the serum phase within milk (Cilliers, 2007).

#### **2.4.1.1.3 Psychrotrophic bacteria**

Psychrotrophic bacteria present in milk can be categorized into two groups namely pathogenic and spoilage organisms (Tamime, 2008). Microbial contamination of milk is mainly due to psychrotrophs such as the *Pseudomonas* species (spp.). Three main sources of this contamination have been attributed to the interior of the udder, teats and milking and storage equipment (Chen *et al.*, 2003).

Bacteria can also enter milk from the udder of the cow, during milking, milk handling practices and the environment (Vijayakumar, 2012). The most common cause of spoilage to heat-treated milk is through psychrotrophic organisms. Species from psychrotrophic origin are capable to stick to solid surfaces and form biofilms on the inner surfaces of dairy equipment which are difficult to remove. Therefore, biofilms are regarded as stubborn sources of permanent contamination caused by psychrotrophs (Samaržija *et al.*, 2012).

Psychrotrophic bacteria are regarded as the most important group of bacteria in dairy products due to the gigantic effect they have on the quality of milk (Pulkkinen, 2014). Characteristics of psychrotrophic bacteria are that they are motile and Gram-negative (Chen *et al.*, 2003). The activity of psychrotrophic organisms depend on storage-length and temperature conditions (Celestino *et al.*, 1997). The optimal metabolic activity of psychrotrophs is at temperatures that range between 20-30°C (Samaržija *et al.*, 2012). These bacteria have the ability to grow at a temperature of 7°C and lower hence they tend to grow during the cold storage of milk (Pulkkinen, 2014). The presence of psychrotrophic bacteria are ascribed to poor cleaning practices, poor hygiene on farms and in factories, extended cold storage of milk at temperatures below 4°C and post-pasteurization contamination (Cilliers, 2007).

The psychrotrophic bacteria isolated from cooled milk belong to the Gram-negative and Gram-positive genera and can be classified into seven classes. Pathogenic organisms isolated from cooled milk belong to different genera such as *Bacillus*, *Stenotrophomonas*, *Acinetobacter* and *Pseudomonas* (Samaržija *et al.*, 2012). The main psychrotrophic bacteria in milk are the gram negative rods such as *Pseudomonas* spp. since they consist of 50% of the total bacteria in milk hence they are considered to be the major spoilage agents in milk (Cilliers, 2007). The *Pseudomonas* genus is characterized by being motile, Gram-negative, catalase positive and mostly strict aerobes. The *Pseudomonas* spp. produces extracellular, alkaline metalloproteinases that need divalent cations like Ca and Zn for stability and activity (Fajardo-Lira, 1999). The optimum pH of proteases produced by *Pseudomonas* spp. is neutral (below pH 7) or alkaline (pH 7-9) and the majority of these proteases are heat-resistant (Samaržija *et al.*, 2012). Examples of *Pseudomonas* spp. are *Pseudomonas putida*, *Pseudomonas fragi* and *Pseudomonas fluorescens* (Cilliers, 2007).

There are other bacteria present in milk such as *Achromobacter*, *Aeromonas*, *Alcaligenes*, *Chromobacterium* and *Flavobacterium*. The *Pseudomonas* spp. have the ability to produce heat-resistant proteases and lipases which result in quality deterioration since they are the main cause behind flavour and quality defects in milk (Champagne *et al.*, 1994).

Gram-positive organisms are also present in milk, however they consist of smaller amounts than the Gram-negative organisms (Champagne *et al.*, 1994). The Gram-positive psychrotrophs within milk include the following genera such as *Bacillus*, *Clostridium*, *Corynebacterium*, *Microbacterium*, *Micrococcus*, *Arthobacter*, *Streptococcus*, *Staphylococcus* and *Lactobacillus*. These genera are

considered to be heat-resistant with the exception of *Arthobacter* and *Lactobacillus* (Samaržija *et al.*, 2012). The *Bacillus* spp. is widely distributed in the environment and is introduced into milk during production, handling and processing (Chen *et al.*, 2003). This specie is classified as a spore former which is able to survive heat treatments applied to milk such as pasteurization since they are heat-resistant (Champagne *et al.*, 1994).

The *Bacillus* spp. is a heterogeneous group of organisms and is characterized by different nutritional requirements as well as consists of the ability to grow at a wide range of temperatures and pH values. Due to these different properties, it is more difficult to isolate and inactivate these bacteria from milk. The most commonly isolated species from raw and heat-treated milk are *B. stearothermophilus*, *B. licheniformis*, *B. coagulans*, *B. cereus*, *B. subtilis* and *B. circulans*. *Bacillus cereus* expresses both proteolytic and lipolytic activities and is the most common contaminant of milk. Certain species of the genus *Bacillus* has the ability to produce more than one type of proteases simultaneously as well as forming more than one type of extracellular and intracellular proteases (Samaržija *et al.*, 2012).

There are four main sources that cause defects in milk namely the growth of psychrotrophic bacteria prior to pasteurization, the activity of heat-resistant enzymes, the growth of heat-resistant psychrotrophs and post pasteurization contamination (Champagne *et al.*, 1994).

#### **2.4.1.1.4 Effect of heat treatment on psychrotrophic bacteria**

The Gram-positive psychrotrophic bacteria are regarded as being heat-stable since they can survive heat treatment. The shelf-life of heat processed milk is reduced and processes such as gelation can be observed due to the ability of Gram-positive psychrotrophs to continue their growth in cooled heat-treated milk. It is therefore clear that the presence of psychrotrophs has a negative impact on the quality of milk due to their ability to grow and multiply rapidly (Samaržija *et al.*, 2012).

Psychrotrophic bacteria can be eliminated by heat treatments (Pulkkinen, 2014). Proteases and lipases produced by these organisms are resistant against heat therefore are not inactivated during heat treatment (Champagne *et al.*, 1994). Therefore, heat treatment has an influence on protease activity. It can result in decreased activity by the unfolding of the proteases such as denaturation, heat enhances self-digestion of proteases such as auto-proteolysis and it may also result in the irreversible non-enzymatic covalent modification such as deamination. Losses in activity of proteases normally occurs at heating temperatures that range between 55-60°C rather than temperatures above 60°C. Protease activity in milk cannot be entirely inactivated by severe heat treatments or by low temperatures thus proteases remain active in milk. The presence of proteases and lipases in pasteurized milk is due to their ability to survive heat treatments since they are heat-resistant hence they remain active during storage of milk and thus affecting the quality of milk since it results in functional and flavour defects (Chen *et al.*, 2003).

The pasteurization of milk destroys all the heat-labile psychrotrophs at temperatures between 72-75°C, however some bacteria may survive this process (Tamime, 2008). Heat-resistant enzymes also have the ability to survive UHT treatments (Richards *et al.*, 2014) and ultimately cause age gelation (Celestino *et al.*, 1997; Newstead *et al.*, 2006). Bacteria that survive the pasteurization process are referred to as being thermotolerant such as the *Bacillus* species. These species are also present in UHT milk hence the level of enzymes is used as an indicator of the quality for this type of milk (Chen *et al.*, 2003).

Most bacteria are inactivated during the UHT process, however heat-stable enzymes of microbial origin can survive (Valero *et al.*, 2001; Topçu *et al.*, 2006). Small amounts of bacteria survive the UHT treatment process thus causing spoilage through the action of their proteolytic and lipolytic enzymes. The spores that can survive the UHT treatment process as well as the most common isolated bacteria in UHT milk are *Geobacillus stearothermophilus*, *B. subtilis*, *B. cereus*, *B. licheniformis*, *B. megaterium*, *B. sporothermodurans* and *Paenibacillus lactis* (Tamime, 2008). These heat-stable enzymes cause serious defects during storage and are also the cause of gelation and off-flavours such as bitter, stale and oxidized flavours (Valero *et al.*, 2001; Topçu *et al.*, 2006).

#### **2.4.1.1.5 Contamination of milk by psychrotrophs after heat treatments**

Stored milk contains heat-resistant enzymes (Chen *et al.*, 2003) hence there are several factors that influence the shelf-life of pasteurized milk such as the contamination by the psychrotrophic organisms and the storage temperature as well as storage time of the milk. Contamination is a major factor that must be considered during the handling of milk after the UHT treatment process has been applied and it results from the seals of the homogenizer as well as the air supply to the aseptic packaging unit. The main species responsible for UHT contamination is *Bacillus* spp. such as *B. stearothermophilus*, *B. subtilis*, *B. cereus* and *B. licheniformis*. These *Bacillus* species can result in flocculation of milk as well as off-flavours and aromas. Further spoilage of UHT milk results from the on-going activities of heat-stable proteases and lipases that are produced by psychrotrophs such as *Pseudomonas* spp. and *Alcaligenes* spp. Spoilage bacteria may also result from the processing equipment and during the packaging process (Tamime, 2008). These bacteria also result in decreased shelf-life of milk and cause technological problems (Champagne *et al.*, 1994).

Pasteurized milk also contains spoilage bacteria such as post-process contaminants which enter the milk after the heating process and heat-resistant organisms which survive the heating process. Psychrotrophic bacteria are the biggest contributor to the spoilage of pasteurized milk due to their ability to re-contaminate the milk after heat treatments (Tamime, 2008).

#### **2.4.1.1.6 Effect of refrigerated storage on the growth of psychrotrophic bacteria**

The cold storage of milk accelerates the growth of psychrotrophs which is regarded as a problem for the dairy industry since storage at cold temperatures is common and regarded as normal practice for milk (Tamime, 2008; O'Brien & Guinee, 2011). Refrigerated storage of milk is necessary during holding in the bulk tanks however it can be disadvantageous to milk since it supports the growth of psychrotrophic bacteria (Guinee & O'Brien, 2010; Samet-Bali *et al.*, 2013). It is clear that refrigerated storage does not prevent microbial growth in milk since it favours and promotes the growth of psychrotrophic bacteria (Tamime, 2008), however storage at temperatures between 4-6°C can slow their growth as well as their proteolytic activity (Samaržija *et al.*, 2012).

The temperature and duration of refrigerated storage influences the extent to which these bacteria increase since large amounts of organisms are the result of prolonged refrigerated storage (Guinee & O'Brien, 2010). Psychrotrophic bacteria have the ability to dominate the microflora in milk within two to three days under refrigeration conditions (Fajardo-Lira, 1999). Poor hygiene with regard to the refrigerated farm bulk tank, also induce the growth of psychrotrophic bacteria (Guinee & O'Brien, 2010). Psychrotrophs induce the production of heat-resistant proteases and lipases thus refrigerated storage has a negative, deteriorative effect on milk (Montanhini *et al.*, 2013).

Another specie that normally grows under refrigerated conditions is *Bacillus* spp. which also has the ability to produce heat-resistant proteases and lipases. The *Bacillus* species consists of higher extracellular and intracellular proteolytic activity than any other bacteria and are introduced into milk during production, handling and processing. Other species that are prevalent to the cold storage of milk are *Clostridium*, *Arthrobacter*, *Microbacterium*, *Streptococcus* and *Corynebacterium* (Chen *et al.*, 2003).

#### **2.4.1.1.7 Characteristics of enzymes produced by psychrotrophic bacteria (Proteases)**

Proteases have an optimum pH range of pH 7-8, however maximum activity can be observed at pH 6.5. Proteases produced by psychrotrophic bacteria have an optimum temperature of 45°C (Chavan *et al.*, 2011).

The most common Gram-negative isolated pathogenic bacteria in raw and pasteurized milk is *Pseudomonas fluorescens* which is heat-resistant and consists of distinct enzymatic extracellular proteolytic, lipolytic and phospholipolytic activity. This specie also has great multiplication ability (Keogh & Pettingill, 1982; Samaržija *et al.*, 2012) and is characterized by its production of a fluorescent pigment during the growth in an adequate media. The optimum growth temperature for *Pseudomonas* is 25-30°C however this specie is able to grow at temperatures just above freezing. The proteases produced by *Pseudomonas fluorescens* is characterized with optimum activity in the pH

range of 6-8, optimum temperatures at 37-45°C and is normally sensitive towards EDTA (Fajardo-Lira, 1999).

*Bacillus* spp. normally grows at a temperature of 2°C (Champagne *et al.*, 1994). Proteases produced by *Bacillus* spp. consists of an alkaline pH and a temperature optimum that range between 30-37°C. (Chen *et al.* 2003). These spore formers are activated at high temperatures hence pasteurization does not always lead to increased shelf-life of milk. It can therefore be said that Gram-positive organisms are the main cause of milk spoilage after heat treatments. There are other bacteria that can also grow at a temperature of 7°C such as *Enterococcus*. They have proteolytic activity and are present in pasteurized milk since they are considered to be resistant against pasteurization (Champagne *et al.*, 1994). The *Bacillus* spp. are considered to be the main microbial causes for milk spoilage as well as the main reason for economic losses in the dairy industry (Samaržija *et al.*, 2012).

#### **2.4.1.1.8 Effect of enzymes produced by psychrotrophs on milk**

The quality and stability of milk is affected by the presence of protease enzymes (Larsen *et al.*, 2006; O'Brien & Guinee, 2011). The presence of enzymes produced by psychrotrophs is associated with certain problems such as technological problems. It also causes certain defects in dairy products such as sensory, rheological and functional defects (Němečková *et al.*, 2009). Proteases produced by psychrotrophic bacteria result in the biochemical degradation of milk (Gaucher *et al.*, 2011). Heat-resistant protease enzymes cause the breakdown of major milk components (Samaržija *et al.*, 2012). These enzymes remain active after heat treatments since they withstand pasteurization, low temperature inactivation as well as UHT treatments (Fajardo-Lira, 1999) hence they cause serious defects in UHT milk (Chavan *et al.*, 2011) such as changes in viscosity within UHT milk (Richards *et al.*, 2014). Enzymes produced by psychrotrophs play an important role in the shelf-life and sensory quality of milk since they cause the formation of off-flavours, thickening and eventually age gelation (Němečková *et al.*, 2009). These enzymes result in protein and lipid breakdown, therefore leads to the spoilage of milk during storage (Chen *et al.*, 2003). Proteolytic enzymes contribute more to the degradation of milk during storage than the indigenous enzymes (Forsbäck, 2010).

The proteases of *Bacillus* spp. also affect the quality of milk in a negative manner. This psychrotrophic bacterium grows under very cold temperatures and it also produces proteases and lipases thus causing defects in milk when stored at cold temperatures. This specie is of great importance since it results in the formation of undesirable sensory qualities in UHT milk as well as decreased shelf-life of milk (Montanhini *et al.*, 2013).

The enzymes produced by *Pseudomonas* spp. and *Bacillus* spp. (*Bacillus cereus*) are most significant to the dairy industry since it result in proteolysis and age gelation (Samaržija *et al.*, 2012). *Pseudomonas* is the most common cause of post-pasteurization contamination whereas *Bacillus* spp. is regarded as the most important organism which limits the shelf-life of pasteurized milk (Němečková *et al.*, 2009).

#### **2.4.1.2 Enzymes responsible for age gelation**

The enzymes that play a role in age gelation are indigenous plasmin and proteases produced by psychrotrophs and will be discussed in detail in the sections that follow.

##### **2.4.1.2.1 Proteases**

Proteases are considered as the most important enzymes in the dairy industry. Most of the proteases within milk are produced from microbial sources (Radha *et al.*, 2011). This enzyme can be classified into four classes based on the mechanism of action namely as an acid, a cysteine, a neutral or an alkaline protease (Chen *et al.*, 2003). These enzymes are produced by a wide variety of microorganisms such as bacteria, fungi and yeast. Other strains for proteases include moulds of genera *Aspergillus*, *Penicillium* and *Rhizopus* (Radha *et al.*, 2011).

Proteases produced by psychrotrophs prefer to attack the caseins instead of the whey proteins. Some casein is more susceptible to protease attack such as the  $\beta$ -casein and K-casein (Chen *et al.*, 2003). Caseins are hydrolysed by proteases from native or microbial origin and this accelerates the physical-chemical process of age gelation (Kohlmann & Nielsen, 1988). The K-casein, casein micelles and colloid calcium phosphate is affected by *Pseudomonas* spp. (Němečková *et al.*, 2009). The proteases of *Pseudomonas* spp. can degrade K-casein, producing para-K-casein,  $\alpha_1$ -casein as well as  $\beta$ -casein which can result in the destabilisation of casein micelles and in turn cause milk flocculation (Samaržija *et al.*, 2012). The  $\beta$ -caseins and  $\alpha$ -caseins are also attacked however  $\beta$ -casein is hydrolysed more rapidly. Since K-casein stabilize casein micelles, their degradation normally results in aggregation and gelation of milk. The preferential attack on K-casein can be explained since this casein is located on the micelle surface, and is more available for initial degradation. Whey proteins are generally not sensitive towards psychrotrophic proteases due to the globular nature of these proteins when compared to the random non-helical structure of caseins. The caseins have more open structures than whey proteins which cause that their peptide bonds are more exposed more to enzymatic cleavage (Fajardo-Lira, 1999).

Casein hydrolysis by proteases causes changes in the functional properties of milk (Cilliers, 2007), such as a reduction in protein content (Datta & Deeth, 2001), which influence the quality of milk in a negative manner (Cilliers, 2007). The breakdown of proteins by proteases is the cause behind the formation of bitter tastes and other unpleasant flavours (Datta & Deeth, 2001; Vijayakumar, 2012).

Proteases play a major role in age gelation of UHT milk since they are capable to induce proteolysis (Champagne *et al.*, 1994). Age gelation can be caused by both indigenous milk protease and proteases produced by psychrotrophic bacteria which are heat-resistant (Datta & Deeth, 2001; Le *et al.*, 2006). Age gelation by proteases are accelerated when the proteases destabilizes the system by hydrolysing the K-casein (Pulkkinen, 2014). Microbial proteases normally result in gels with tighter

protein networks and thicker strands. These gels also contain more intact casein micelles and micelle aggregates. The time when age gelation occurs in milk is strongly dependant on the activity of the proteases present (Datta & Deeth, 2001). Proteases therefore play a role in age gelation during storage of various types of milk such as pasteurized milk and UHT milk (Cilliers, 2007).

#### **2.4.1.2.2 Lipases**

Lipolytic enzymes are referred to as carboxylesterases that hydrolyse acylglycerols (Chen *et al.*, 2003). Lipoprotein lipase is produced in the secretory cells of the mammary gland. It is a glycoprotein that has 450 amino acid residues and has a molecular weight that range between 30-50 kDa. Lipase is active at an optimum temperature of 37°C and has an optimum pH of 8-9.2 (Tamime, 2008). Lipases can be produced by *Pseudomonas* spp. as well as *Bacillus* spp. Lipases produced by *Bacillus* are considered to be more stable against heat (Samaržija *et al.*, 2012).

This enzyme is normally more active in the lipid-water interface than in the aqueous phase. Lipolytic enzymes are classified according to their specificity for certain substrate features such as fatty acid position and fatty acid chain length. They can be divided into three groups namely the non-specific group, the specific group and the enzymes in the last group has a preference for certain fatty acids. The enzymes in the first group release free fatty acids from all the three positions of the acylglycerol moiety whereas the second group of enzymes release free fatty acids from the outer first and third positions of the triacylglycerol moiety. In general, lipases do not belong to the last group (Chen *et al.*, 2003).

Lipases are associated with the casein micelles within milk, some are associated with the serum phase of milk whereas a small amount of this enzyme is associated with the milk fat globule membrane (Tamime, 2008). The level of lipases in milk depends greatly on the breed, lactation stage, diet and nutrition of the cow and season. These enzymes can be indigenous to milk or produced by psychrotrophic bacteria. Factors that influence the production of such lipases are temperature, pH, lipid sources, concentration of inorganic salts and availability of oxygen. Milk is regarded to be a good medium for the production of lipases (Chen *et al.*, 2003).

Lipases are responsible for the lipolysis of fatty acids in milk (Forsbäck, 2010). Lipolysis can be described as induced or spontaneous depending on the means of activation of lipases. Induced lipolysis is ascribed to both mechanical damage and temperature changes to milk whereas spontaneous lipolysis develops in milk during cold storage (O'Brien & Guinee, 2011). During lipolysis, this enzyme liberates fatty acids from the first and third positions in di and tri-monoglycerides in two stages. The first stage of lipolysis is when lipase is absorbed at the lipid-water interface. The second stage is when this enzyme aligns its active site at the substrate molecule and hydrolyses it. Free fatty acids are released during lipolysis which results in the development of hydrolytic rancidity in milk

(Tamime, 2008). Lipolytic activity within milk is shown by increases in free fatty acid concentration (Celestino *et al.*, 1997). Lipases from psychrotrophic origin have the ability to disrupt the native membrane structure of fat globules which in turn makes milk fat susceptible to degradation by native milk lipases. This ultimately results in the physical degradation of the emulsion within milk (Samaržija *et al.*, 2012).

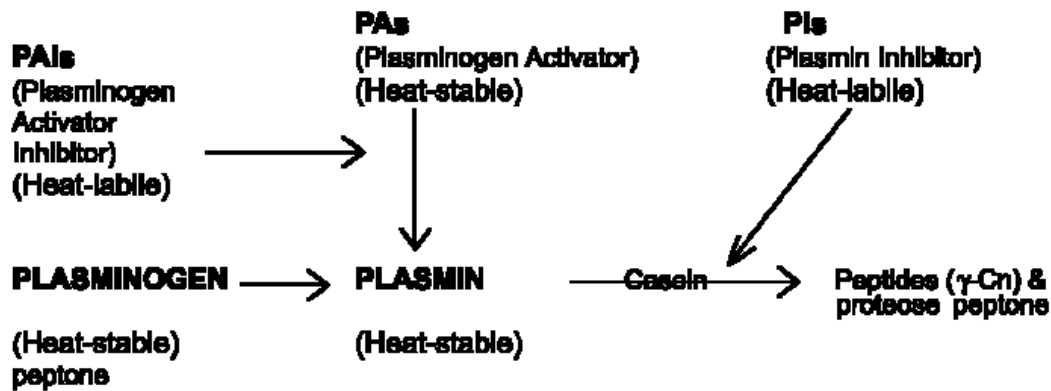
Lipases are normally inactivated during pasteurization since they are regarded as being heat labile and unstable enzymes (Chen *et al.*, 2003). They are not fully inactivated by heat treatments since very small amounts of this enzyme can survive pasteurization. They can be fully inactivated by severe heat treatments such as UHT processing (Champagne *et al.*, 1994). However, lipases produced by psychrotrophic bacteria such as *Pseudomonas* spp. are considered to be heat-resistant which normally survive UHT treatments (O'Brien & Guinee, 2011). These enzymes remain active during storage (Champagne *et al.*, 1994). They are also active during the cold storage of milk (Forsbäck, 2010).

Lipases produce off-flavours in milk (Champagne *et al.*, 1994). The hydrolysis of fatty acids during lipolysis are responsible for the formation of off-flavours such as rancid, oxidized, bitter, unclean and metallic (Richards *et al.*, 2014). Lipolysis also gives rise to the formation of soapy and tangy flavours. Therefore, lipases are capable of causing the spoilage of milk (Corrêa *et al.*, 2011), but does not play a role in age gelation.

#### **2.4.1.2.3 Plasmin**

##### **2.4.1.2.3.1 The plasmin system**

This system is regarded as a complex system since it consists of plasmin, plasminogen (which is the plasmin precursor and also the inactive zymogen of plasmin), plasminogen activators (which converts plasminogen to plasmin), plasmin inhibitors (which inhibit plasmin activity) and inhibitors of plasminogen activators (which inhibit plasminogen activity), Refer to Figure 2.4 for an illustration of the plasmin system (Hamed *et al.*, 2012; Richards *et al.*, 2014; Gazi *et al.*, 2014). Plasmin, plasminogen and plasminogen activators are associated with the casein micelles whereas plasmin inhibitors and inhibitors of plasminogen activators are soluble in the milk serum (Němečková *et al.*, 2009; Vijayakumar, 2012). The association of plasmin and plasminogen with casein micelles depends on the pH since a reduction in the pH leads to the release of plasmin and plasminogen from the micelles (Gazi *et al.*, 2014). The inhibitors might appear in various forms within milk due to formation of complexes with milk proteins (Chavan *et al.*, 2011).



**Figure 2.4. The plasmin-plasminogen system (Datta & Deeth, 2001).**

There are many factors that affect this system thus making this a complex system (Prado *et al.*, 2007; Schroeder *et al.*, 2008). The factors that affect this system, in various ways, are mainly processing conditions such as heating, pH manipulation and storage. The activity of the plasmin system is also influenced by proteases produced by psychrotrophs during storage of milk (Schroeder *et al.*, 2008).

Researchers therefore claim that a relationship exists between proteases from psychrotrophic bacteria and the plasmin system since the growth of psychrotrophs could affect this system. The growth of *Pseudomonas* spp. has the ability to damage the integrity of the casein micelles hence cause the release of plasmin and plasminogen from the casein micelle into the whey fraction of milk. The growth of psychrotrophic bacteria in milk could affect the pH and cause changes in the pH levels which in turn affect the stability of casein micelles that can result in the release of plasmin into the whey fraction. It can therefore be said that proteases produced by psychrotrophic organisms may act as plasminogen activators. It is therefore clear that the interaction between psychrotrophic bacteria and the plasmin system has a negative impact on the quality of milk since proteases from psychrotrophic origin causes a loss of plasmin in the casein fraction thus increasing the plasmin activity within the whey fraction of milk (Fajardo-Lira, 1999).

Refrigerated storage of milk may also have an influence on this system since plasmin undergoes more autolysis during storage. Increased plasminogen activation is also common during the cold storage of milk (Schroeder *et al.*, 2008). The final plasmin activity in milk depend on the activity of plasminogen, plasminogen activators and plasminogen activators inhibitors (Chavan *et al.*, 2011).

The components consist of variations within their stability towards heat. Plasmin, plasminogen and plasminogen activators are stable against heat whereas the plasmin inhibitor is heat labile, therefore plasmin and plasminogen have the ability to survive pasteurization (Guinee & O'Brien, 2010). The plasmin system undergoes some changes during heat treatment such as the heat-induced inactivation of plasmin inhibitor and plasminogen activator inhibitor (Gazi *et al.*, 2014). Enhanced activation of plasminogen due to the inactivation of plasminogen activator inhibitor leads to increased plasmin

levels after the pasteurization of milk (Prado *et al.*, 2007). The natural balance between the activators and inhibitors are altered through the heat treatment of milk which may result in enhanced proteolysis in heated milk (Chavan *et al.*, 2011).

The components of the plasmin system interact with each other as well as with the components within milk (Pulkkinen, 2014). Interactions between the components of the plasmin system can have positive or negative effects on the quality of milk (Prado *et al.*, 2007). These interactions can stimulate or inhibit the occurrence of proteolysis (Pulkkinen, 2014).

#### **2.4.1.2.3.2 Plasminogen activation**

Plasminogen is the inactive form of plasmin and is considered to be more resistant to heat than its active form. It can be activated by the cleavage of a single peptide bond by various activators which are also resistant against heat (Datta & Deeth, 2001; Richards *et al.*, 2014). Bovine plasminogen consists of 786 amino acid residues and has a molecular weight of 88 kDa (Cilliers, 2007). The optimum temperature for plasminogen activation is 37°C (Pulkkinen, 2014). Little plasminogen activation occurs at low temperatures such as temperatures below 4°C (Crudden *et al.*, 2005; Cilliers, 2007). Plasminogen may also be inactivated during the heat treatment of milk (Cilliers, 2007). Heat treatment at 72°C for 15 seconds can decrease plasminogen activity by 10% (Fajardo-Lira, 1999).

Plasminogen consists of cysteine residues and triple-loop structures, which are referred to as kringles and are stabilized by three disulphide bonds. These kringles contains a lysine binding site whereas one site has a high affinity for lysine, however the other four sites have a lower affinity. These binding sites are essential for the regulation of plasminogen activation (Bastian & Brown, 1996). Lysine has the ability to inhibit plasminogen activation since it competes for the lysine binding sites that are present in the kringles. It can also cause the dissociation of plasmin and plasminogen from the casein micelles (Datta & Deeth, 2001). Plasmin is secreted in milk as plasminogen and is activated during storage (Bastian & Brown, 1996). Plasminogen concentration varies according to different types of milk since it is normally higher in colostrum than in late lactation milk (Cilliers, 2007).

Plasminogen does not originate from within the mammary gland, since it is activated by the hydrolysis of Arg<sup>557</sup>-Ile<sup>558</sup> in bovine plasminogen (Bastian & Brown, 1996). The Ile<sup>558</sup> amino group can be replaced with Val<sup>562</sup> in order to activate plasminogen (Cilliers, 2007). It is also associated with the casein micelles within milk. Plasminogen consists of an N-terminal aspartic acid which is converted to an N-terminal arginine by plasmin through the removal of the first 77 residues. This N-terminal 77 residue fragment of plasminogen is also referred to as the pre-activation peptide. The release of this terminal does not cause the activation of plasminogen however it does cause a conformational change in plasminogen (Bastian & Brown, 1996). The formation of a salt bridge

between Val<sup>562</sup> and Asp<sup>740</sup> plays a role in these conformational changes in plasminogen in order to form plasmin (Cilliers, 2007).

The activation of plasminogen is the result of a cascade of reactions (Cilliers, 2007). Plasminogen activation occurs when plasmin releases the pre-activation peptide and leaving a structurally modified, inactive protein. The Arg-Ile is cleaved by certain activators in order to give rise to an active two-chain molecule that consists of a heavy chain that is joined to a light chain by a single disulphide bond. The light chain is the active site for plasmin and consists of His<sup>598</sup>, Asp<sup>641</sup> and Ser<sup>736</sup>. Plasminogen activation normally results in casein degradation (Bastian & Brown, 1996). The Arg<sup>557</sup>-Ile<sup>558</sup> cleavage results in the conversion of plasminogen to plasmin (Vijayakumar, 2012).

Casein may serve as a matrix for plasminogen activation since multimers of K-casein and dimers of  $\alpha_2$ -casein have been reported as binding components for certain activators. Casein may cause enhanced plasminogen activation since it plays a role in the activation process (Bastian & Brown, 1996). The caseins that cause enhanced plasminogen activation are  $\alpha_1$ -casein,  $\alpha_2$ -casein,  $\beta$ -casein and K-casein (Cilliers, 2007). Plasminogen activity is affected by plasmin, the inhibitors, caseins,  $\beta$ -LG and  $\alpha$ -lactalbumin (Heegaard *et al.*, 1994).

Plasminogen within milk can also be activated by a protein from *Streptococcus uberis*. Other bacteria that consist of a receptor plasminogen are *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium*. Plasminogen receptors have the ability to enhance plasminogen activation (Le Roux *et al.*, 2003). Constituents of somatic cells can also convert plasminogen to plasmin, however plasminogen activation cannot be accelerated by leukocytes, milk coagulants and extracellular microbial enzymes. Plasminogen activation can occur in the udder prior to milking or during storage of milk (Verdi & Barbano, 1991). The plasminogen activation system has important functions in the mammary gland (Heegaard *et al.*, 1994).

#### **2.4.1.2.3.3 Activators and inhibitors of plasminogen**

Plasminogen is activated to plasmin by plasminogen activators (Gazi *et al.*, 2014). Plasminogen activators are enzymes that are responsible for the conversion of plasminogen to plasmin (Crudden *et al.*, 2005). These activators act on the Arg<sup>557</sup>-Ile<sup>558</sup> bond in plasminogen and are associated with the casein micelles (Gazi *et al.*, 2014). Plasminogen activators are regarded to be stable against heat since they are not affected by pasteurization (Datta & Deeth, 2001). They are slightly inactivated during UHT processing due to very high temperatures (Datta & Deeth, 2003).

There are two main types of plasminogen activators which are referred to as tissue type (t-PA) and urokinase type (u-PA) (Aslam & Hurley, 1997; Enright *et al.*, 1999). These activators have the ability to convert inactive plasminogen to active plasmin during storage of milk (Datta & Deeth, 2001). The

t-PA is associated with the casein micelles whereas u-PA is mainly associated with the somatic cells within milk (Bastian & Brown, 1996; Crudden *et al.*, 2005; Hamed *et al.*, 2012). These two activators consist of different amounts of amino acids since u-PA contains 413 amino acids and t-PA has 566 amino acids (Cilliers, 2007). Both activators are regarded as heat-resistant since they normally survive heat treatments that are applied to milk such as pasteurization. However, heating milk at temperatures above 75°C can result in decreased t-PA activity (Prado *et al.*, 2007).

These two activators have different effectiveness for plasminogen activation (Wang *et al.*, 2007). The u-PA is produced by epithelial cells and this production is under hormonal control. Certain factors have an influence on u-PA production such as increased production caused by insulin whereas inhibition of production is caused by glucocorticoids (Baldi *et al.*, 1997). The u-PA is secreted in a single-chain zymogen form and is processed to its active two-chain form by plasmin. The u-PA also has the ability to bind to casein (Wang *et al.*, 2007), however t-PA can also bind to casein micelles due to electrostatic forces. Plasminogen activation by t-PA is stimulated by the binding of t-PA to fibrin and fibrinogen fragments. The t-PA also has non-fibrinolytic functions (Heegaard *et al.*, 1994). The t-PA is considered to be the major enzyme which contributes to plasminogen activation (Cilliers, 2007), however according to Prado *et al.*, 2007, u-PA plays the more significant role in the activation of plasminogen.

Inhibitors are also present in milk such as inhibitors of plasminogen activation and plasmin inhibitors, also referred to as  $\alpha_2$ -antiplasmin (Datta & Deeth, 2001). These two inhibitors consist of different molecular masses since plasminogen activator inhibitor has a molecular mass of 55 kDa whereas the molecular mass of plasmin inhibitor is 60 kDa (Cilliers, 2007). They inhibit the activity of plasmin and plasminogen activators (Prado *et al.*, 2006; Wang *et al.*, 2007). The final plasmin activity in milk greatly depends on the action of these inhibitors (Fajardo-Lira, 1999). These inhibitors are present in the serum phase of the milk (Chen *et al.*, 2003). The natural balance between the activators and inhibitors are altered by the heat treatment of milk (Datta & Deeth, 2001).

The inhibitors are not regarded as heat-resistant since they are unstable and also inactivated during heat treatments (Newstead *et al.*, 2006; Prado *et al.*, 2006; Vijayakumar, 2012; Chove *et al.*, 2013). These inhibitors consist of different heat stabilities since the plasminogen activator inhibitor is less resistant to heat than plasmin inhibitor (Prado *et al.*, 2006). Heat treatment results in increases in plasminogen activators due to the inactivation of the inhibitors that occur during heat treatment (Datta & Deeth, 2003). Therefore, plasmin activity is higher in stored pasteurized milk than in fresh milk due to the heat inactivation of inhibitors (Fajardo-Lira, 1999).

#### **2.4.1.2.3.4 Factors that influence plasminogen activation**

There are many factors that have an influence on plasminogen activation since these factors either inhibit or enhance the activation of plasminogen. The inactivation of the plasmin inhibitor and plasminogen activator inhibitor during heat treatment result in increased plasminogen activation (Gazi *et al.*, 2014). Heat treatment of milk normally enhances plasminogen activation (Newstead *et al.*, 2006; Prado *et al.*, 2007). Intense heat treatments such as UHT processing result in the irreversible inactivation of inhibitors thus cause decreased plasminogen activation (Enright *et al.*, 1999). The covalent bonding between plasminogen and  $\beta$ -LG may also cause decreased plasminogen activation (Prado *et al.*, 2006).

Milk production factors also have an influence on plasminogen activation since increased activation is observed in mastitis milk and late lactation milk (Bastian & Brown, 1996; Larsen *et al.*, 2006; Cilliers, 2007). Less frequent milking may result in increased plasminogen activation (Stelwagen *et al.*, 1994). Increased somatic cell counts also result in increased plasminogen activation (Kelly & Foley, 1997). Plasminogen activation is also influenced by the breed of cow since higher activity is more common in Friesian milk than in Jersey and Ayrshire milk (Cilliers, 2007).

Other factors that increase plasminogen activation are the epidermal growth factor as well as the insulin-like growth factor which increases u-PA activity. Prostaglandin has a stimulating effect on u-PA and t-PA. The action of plasminogen activators can also be stimulated by serine proteases. The action of u-PA and t-PA can be inhibited by glucocorticoids (Le Roux *et al.*, 2003).

#### **2.4.1.2.3.5 Effects of plasminogen activation**

Plasminogen activation has certain effects on milk and its components since it can lead to the breakdown of milk proteins, especially the caseins such as  $\beta$ -casein and  $\alpha$ -casein. Peptides are also formed during activation such as  $\gamma$ -casein and proteose-peptones. Some proteins are resistant to plasminogen activation such as K-casein. The breakdown of proteins due to activation is regarded as a negative aspect since it has a negative influence on the texture, physical- and chemical properties of milk. Plasminogen activation result in increased plasmin within milk that ultimately leads to proteolysis of casein (Cilliers, 2007).

Plasmin in UHT milk increases during storage due to plasminogen activation. The alteration of the natural balance between activators and inhibitors during heat treatment can result in enhanced proteolysis (Datta & Deeth, 2001). Plasminogen activators also play a role in age gelation. It is therefore clear that the activation of plasminogen has a negative impact on milk since it promote age gelation due to increased plasmin during storage and the occurrence of proteolysis (Kohlmann & Nielsen, 1988; Cilliers, 2007).

#### **2.4.1.2.3.6 Activated form of plasminogen (Plasmin)**

Plasmin is defined as the major native protease in milk (Gaucher *et al.*, 2008). This enzyme belongs to the peptidase/trypsin family (Fajardo-Lira, 1999). It is present in bovine blood and milk (Prado *et al.*, 2006). This enzyme is indigenous to milk however it is also active in UHT milk (Gaucher *et al.*, 2008). It is secreted into milk from the blood plasma through the mammary cells (Vijayakumar, 2012).

The origin of this enzyme is the blood serum however plasmin is also associated with the casein micelles within milk rather than the whey fraction (Chen *et al.*, 2003; Tamime, 2008). This enzyme is also associated with the fat globule membrane in milk (Datta & Deeth, 2003). It has been hypothesized that plasmin is associated with casein via lysine binding sites on the enzyme molecule, comparable to the way that plasmin binds to fibrin in blood. However, plasmin's association with casein is pH dependent, with little loss of plasmin activity from micelles occurring in the pH range 4.8-6.6, but very little or no plasmin activity is found in the casein fraction at pH values below 4.6 (Fajardo-Lira, 1999).

Plasmin is often referred to as a serine protease with trypsin-like activity hence it can be inhibited by certain trypsin inhibitors (Cilliers, 2007). Plasmin can be inhibited by disopropylfluorophosphate, trypsin inhibitors, Cu, Zn, Hg, bovine colostrum, tosyl-lysine, chloromethylketone and  $\beta$ -LG. This enzyme cannot be inhibited by EDTA or iodoacetic acid. Bovine plasmin has a high specificity for peptide bonds involving lysyl residues, and to a lesser degree, arginine (Fajardo-Lira, 1999).

This enzyme consists of certain characteristics such as being active at an optimum temperature of 37°C and optimum pH 7.5 (Bastian & Brown, 1996; Chen *et al.*, 2003). Autolysis of plasmin occurs at 37°C thus increased plasmin activities can be observed at this temperature (Crudden *et al.*, 2005). The pH of bovine milk ranges between 6.5-6.8 which serves as the ideal environment for plasmin activity. Plasmin activity can be delayed by the storage of milk under refrigerated conditions (Vijayakumar, 2012) however it has the ability to remain active at temperatures as low as 4°C (Crudden *et al.*, 2005). Plasmin has a molecular mass of 58 kDa which suggests that it might exist as a dimer under certain conditions (Cilliers, 2007).

Various factors have an influence on the levels of plasmin within milk such as seasonal variations, animal health, breed and age of cow, milk yield of cows, nutritional status of cows, lactation stage, presence of mastitis, inflammation and storage of milk (Heegaard *et al.*, 1994; Cilliers, 2007). Plasmin tend to increase during the first 6 months of lactation, however levels remain constant towards the end of the lactation period. Higher plasmin activity is observed in milk from older cows as well as with the presence of mastitis. Plasmin activity also varies between different breeds of cows since higher activity is observed in milk from Holstein-Friesian cows when compared to Jersey cows (Bastian & Brown, 1996).

This enzyme can be active on all the types of casein however it has a preference for certain caseins. Plasmin cleaves  $\beta$ -casein as well as  $\alpha_{s2}$ -casein and has the ability to hydrolyse  $\alpha_{s1}$ -casein (Chen *et al.*, 2003; Gaucher *et al.*, 2008). The  $\alpha_{s1}$ -casein as well as  $\alpha_{s2}$ -casein is susceptible to proteolysis by plasmin (Silanikove *et al.*, 2006). Plasmin cleaves  $\beta$ -casein particularly at positions Lys28-Lys29, Lys105-His106 and Lys107-Glu108 in order to give rise to three  $\beta$ -casein fragments ( $\gamma_1$ -casein,  $\gamma_2$ -casein and  $\gamma_3$ -casein) and proteose-peptones (Chen *et al.*, 2003). It hydrolyses the other caseins, such as  $\alpha_{s1}$ -casein at a slower rate, however K-casein is resistant to hydrolysis by plasmin (Bastian & Brown, 1996).

Plasmin-induced hydrolysis of the other caseins may result in K-casein being disrupted and also result in the formation of bitter peptides in milk (Gazi *et al.*, 2014). The dissociation of plasmin from casein micelles are affected by pH, casein hydrolysis, ionic strength and storage temperature. This dissociation is enhanced by a lower pH (Pulkkinen, 2014). This enzyme does not hydrolyse whey proteins such as  $\alpha$ -lactalbumin and  $\beta$ -LG. These two whey proteins along with bovine serum albumin have inhibitory effects on plasmin activity (Datta & Deeth, 2001; Chen *et al.*, 2003; Tamime, 2008).

Plasmin is regarded as a heat-resistant enzyme (Chove *et al.*, 2013). This enzyme has the ability to survive heat treatments such as pasteurization but it can be inactivated during treatments at higher temperatures (Tamime, 2008). Pasteurization of milk at 72°C for 15 seconds can increase plasmin activity by 30-40% (Fajardo-Lira, 1999). Plasmin activity can only be reduced by UHT treatment and not fully inactivated since up to 30% of plasmin activity is present after UHT processing (Newstead *et al.*, 2006).

However, heat treatments at a temperature of 120°C have the ability to inactivate plasmin completely such as the sterilization of milk (Gazi *et al.*, 2014). The conditions needed in order to inactivate plasmin activity are 120°C for 15 min for bottled milk, and 142°C for 18 sec for UHT milk (Fajardo-Lira, 1999). Heat treatments at high temperatures result in the reversible denaturation of plasmin. This denaturation occurs in two phases, namely by unfolding at high temperatures and then by refolding to an active form after cooling, hence referred to as reversible denaturation since this enzyme is resistant to degradative reactions. Plasmin activity can also increase after heat treatment due to the destruction of inhibitors and activators during heat treatment (Chen *et al.*, 2003; Vijayakumar, 2012).

The presence of  $\beta$ -LG has an influence on the heat inactivation of plasmin since this whey protein promotes the inactivation of this enzyme through sulphhydryl-disulphide interchange reactions (Tamime, 2008). Denaturation through heat of  $\beta$ -LG cause low plasmin activity in UHT milk since this whey protein is exposed to the reactive sulphhydryl group, which undergoes disulphide-sulphydryl interchange reactions with the disulphide bonds of plasmin, during denaturation of  $\beta$ -LG. This process has a weakening effect on plasmin since it inhibits the refolding of this enzyme thus inhibiting

its activity (Vijayakumar, 2012). Plasmin is more effectively inhibited by denatured  $\beta$ -LG rather than native  $\beta$ -LG (Bastian & Brown, 1996). The addition of  $\text{KIO}_3$  has the ability to protect plasmin from inactivation against  $\beta$ -LG (Enright *et al.*, 1999). This native protease is considered to play a role in flavour defects, bitterness as well as age gelation of UHT milk (Tamime, 2008).

#### **2.4.1.2.3.7 Effect of plasmin on age gelation**

Plasmin is regarded as an important enzyme since its presence may affect the quality of milk in a negative manner. This enzyme is also present in UHT treated milk and has a negative impact on this type of milk since it may accelerate age gelation during storage due to this enzyme being able to cause decreases in the viscosity of milk (Le Roux *et al.*, 2003). Plasmin activity in UHT milk also results in the formation of bitter and off-flavours (Kelly & Foley, 1997). Increased plasmin and plasminogen activity is the result of increased somatic cell count due to the presence of mastitis in milk. Therefore, age gelation occurs in mastitis milk due to high activity of this enzyme (Topçu *et al.*, 2006).

The explanation for the occurrence of age gelation caused by plasmin follows: Plasmin is heat-resistant thus survives the UHT process and remains active during storage. The concentration of plasmin increases during storage thus affecting the quality of milk and resulting in negative occurrences such as proteolysis (Cilliers, 2007). Proteolysis leads to age gelation and plasmin has the ability to induce proteolysis (Chen *et al.*, 2003) since this enzyme stimulates most of the proteolytic activities in milk (Aslam & Hurley, 1997). Plasmin is also responsible for releasing the  $\beta$ K-complex which is considered to be a preliminary step in the process of age gelation (Crudden *et al.*, 2005). It is therefore clear that plasmin activity plays a role in the sedimentation and age gelation of UHT milk (Newstead *et al.*, 2006).

Plasmin induced proteolysis can be prevented by severe heat treatments of milk such as heating milk at temperatures that range between 115-120°C (Chen *et al.*, 2003) since a temperature range of 80-85°C result in increased plasmin activity thus leading to age gelation (Newstead *et al.*, 2006). Decreased plasmin activity is desirable since increased plasmin activity is related to increased viscosity and reduced stability of milk (Bavarian *et al.*, 2010).

It is therefore clear that plasmin activity is strongly affected by temperature (Crudden *et al.*, 2005). Age gelation caused by plasmin greatly depends on the rate of casein hydrolysis (Rauh *et al.*, 2014). Casein hydrolysis by plasmin can be prevented by keeping milk cold throughout the whole handling and processing procedure, therefore enhancing the shelf-life of milk thus delaying the process of age gelation (Crudden *et al.*, 2005).

### **2.4.2 Non-enzymatic/Chemical mechanism of age gelation**

Age gelation is sometimes referred to as a non-enzymatic mechanism that involves physicochemical processes due to the lack of a relationship between gelation time and proteolytic activity. The three-dimensional protein network is formed during storage by the interaction between  $\beta$ -LG and K-casein within the casein micelle due to heat treatment which eventually results in the formation of a gel. Age gelation tends to cause changes at the surface of the casein micelle. The polymerization of casein and whey proteins, due to high storage temperatures, by Maillard reactions also causes non-enzymatic age gelation, however this suggestion is not accepted due to the lack of gel formation at storage temperatures above 35°C (Datta & Deeth, 2001; Chavan *et al.*, 2011).

Non-enzymatic age gelation may also be the result of changes in the free energy of casein micelles due to the lowered surface potential of the casein micelles (Pulkkinen, 2014). Aggregation of casein micelles is endorsed by differences in potential energy and the extent of this depends on the probability of contact and the number of low-potential micelles. Increased viscosity of UHT milk is the result of micelle aggregation (Datta & Deeth, 2001; Chavan *et al.*, 2011).

#### **2.4.2.1 Two distinct types of chemically induced flocculation**

There are two main types of chemically induced milk flocculation. High-molecular-weight biopolymers can adsorb to two or more droplets at a very low concentration and then in turn form bridges. This phenomenon is referred to as bridging flocculation. Depletion flocculation occurs due to the presence of an unabsorbing biopolymer in the continuous phase which promotes aggregation of droplets through inducing an osmotic pressure gradient in the continuous phase which surrounds the droplets. When the biopolymer is either poorly or unabsorbed, it is squeezed out of the area between two droplets. This may result in osmotic imbalance when the concentration of the biopolymer between the emulsion droplets decreases below its overall solution concentration. The end result is flocculation since the particles are attracted towards each other (Thompson *et al.*, 2008).

Depletion flocculation involves spherical particles in the presence of macromolecules and this type of flocculation is characterized by weak, flexible and reversible bonds. Low protein-to-oil ratios may result in bridging flocculation since there is not enough protein to fully cover the oil-water interface during homogenization. Very high protein-to-oil ratios may result in depletion flocculation due to the presence of excess and unabsorbed protein. Sufficient protein content protects droplets from flocculation hence emulsions are normally stable for weeks however excess unabsorbed protein results in depletion flocculation (Thompson *et al.*, 2008).

Normally, bridging flocculation is observed in emulsions that are formed with aggregated milk protein products such as calcium-caseinate and milk protein concentrates (MPC) where the droplets are bridged by casein aggregates or micelles, whereas depletion flocculation is observed in sodium-

caseinate based emulsions and not emulsions formed with calcium-caseinate, MPC or whey proteins due to the absence of suitably sized protein particles at the required concentrations in the aqueous phase. The casein micelles in MPC are too large to induce depletion flocculation and the molecular size of the whey proteins are less than the optimum. Calcium-caseinate does contain casein aggregate mixtures however the concentration of aggregates are too low in order to induce depletion flocculation (Thompson *et al.*, 2008).

The difference between bridging and depletion flocculation is clear since bridging flocculation is associated with a low droplet packing density due to strong attractive interactions whereas depletion flocculation is associated with high packing density due to weak attractive interactions (Dickinson, 2010).

## **2.4.2.2 The contribution of milk production factors to age gelation**

### **2.4.2.2.1 Mastitis**

Mastitis is regarded as an important disease of dairy cows since it has a great influence on the quality and quantity of milk. It can be described as the inflammation of the udder (Fernandez *et al.*, 2008; Forsbäck, 2010). Mastitis is the result of a microbial infection or poor milking practices (Cilliers, 2007). Changes can be observed in the udder due to mastitis such as leakage of ions, proteins and enzymes from the blood into the milk. These changes take place due to increased porousness, invasion of phagocytising cells into the milk compartment and a decrease in synthetic capacity of the mammary gland. Substances such as acute phase proteins may also be produced by the affected quarter (Sharif & Muhammad, 2008).

The somatic cell counts increase during mastitis hence the usage of these cells for monitoring the udder health of the cow as well as the quality of milk (Bavarian *et al.*, 2010). High somatic cell counts expose milk to greater risk of contamination with pathogenic bacteria and antibiotic residues (Sharif & Muhammad, 2008). High somatic cell counts reduce the acceptance of dairy products due to the changes it causes within milk (Fernandez *et al.*, 2008). The factors that have an effect on the amount of somatic cells in milk are mammary gland infection, age of cow, season and stage of lactation. Mastitis can therefore be characterized by increased somatic cells (Sharif & Muhammad, 2008; Forsbäck, 2010).

Mastitis causes a decline in milk quality, lower animal wellbeing, increased antibiotic treatments and increased labour costs (Forsbäck, 2010). This disease has several negative effects on milk such as reduced shelf-life and decreased viscosity in UHT milk (Fernandez *et al.*, 2008). The health status of the udder has an influence on the composition of milk. Mastitis leads to changes in the components and properties of milk due to increased somatic cells. This disease has an effect on the production of lactose, fat and protein and also milk yield. Low somatic cell counts result in milk that have better quality and yield (Sharif & Muhammad, 2008). The processing properties of milk are also influenced by mastitis (Forsbäck, 2010).

High somatic cell counts result in increased concentrations of serum albumin, whey proteins and immunoglobulin. A decrease in caseins is a normal occurrence during mastitis (Sharif & Muhammad, 2008) due to increased somatic cells (Fernandez *et al.*, 2008). These changes lead to a reduction in the heat stability of milk and ultimately cause milk flocculation during pasteurization and UHT treatment thus result in milk having a shorter shelf-life (Sharif & Muhammad, 2008).

Plasmin increases during mastitis thus causing increased proteolysis (Chen *et al.*, 2003; Forsbäck, 2010). The activity of plasmin is higher in mastitis milk than in normal milk due to increased activity of plasminogen activators and other proteolytic enzymes present in the somatic cells (Bastian & Brown, 1996). Increased somatic cell counts are directly associated with increased levels of proteolysis. It is therefore clear that mastitis milk is more susceptible to age gelation due to a greater potential for the occurrence of proteolysis (Fernandez *et al.*, 2008).

UHT mastitis milk is more likely to undergo age gelation than normal UHT milk. The reason for this is increased proteolytic activity due to high levels of plasmin. UHT mastitis milk tends to show higher proteolysis when stored at 20°C. Milk that contains high somatic cell counts normally gel the fastest. Indirect heat-treated UHT milk with high or low somatic cell counts reveal little plasmin activity hence it shows a low tendency to gel. Increased plasminogen levels result in a higher tendency of age gelation for milk with both high and low somatic cell counts. Therefore, the presence of plasmin is responsible for enhanced age gelation in milk with high somatic cell counts (Datta & Deeth, 2001; Chavan *et al.*, 2011).

#### **2.4.2.2.2 Milk composition variations**

##### ***Factors that influence milk composition variations***

The composition of milk varies from time to time for certain reasons. There are several factors that influence milk composition variations which can be divided into genetic, physiological, environmental and pathological factors (Hallén, 2008; Forsbäck, 2010). The factors that influence composition changes of milk are the breed of cow, health status and age of cow, lactation stage, season, diet, genetic factors, milking conditions and frequency and the amount of somatic cells and bacteria present within milk. Some of these factors are correlated with other parameters such as season, geographic region of production and social practice in herd management (Gaucher *et al.*, 2008; Chen *et al.*, 2014). Milk composition variations have a direct effect on the processing of milk and manufacturing properties hence affecting the quality of the end product (O'Brien & Guinee, 2011).

- **Age of the cow**

The activity of plasmin tends to increase as the age of the cow increases. The activity of plasmin remains constant throughout lactation of first lactation cows but increases during the lactation in the milk that is collected from older cows. The milk from older cows tends to exhibit age gelation faster than that of younger cows (Datta & Deeth, 2001).

- **Diet of the cow**

The diet of the cow has a direct influence on the fatty acid composition of milk. Fresh pasture, conserved forage and concentrates influence the fatty acid composition since milk of cows fed with

fresh pasture tends to be rich in unsaturated fatty acids such as linoleic acid and trans-fatty acids. The milk produced from cows fed with conserved forage normally contains lower levels of linoleic acid than that of cows fed with fresh pasture. Other fatty acids such as stearic and oleic acid tend to be lower in milk from cows fed with fresh pasture than that of cows fed with conserved forage. A low fibre diet is associated with decreased milk yield as well as a decline in milk fat and protein (Mel'uchová *et al.*, 2008; Ostrovsky *et al.*, 2009). The fat content of milk tends to decrease when cows are fed with pelleted food with high concentrates. The underfeeding of cows reduces milk yield, total solids and protein content. Underfeeding with protein result in decreased milk yield whereas overfeeding with protein will not necessarily lead to increased protein content of milk (Laben, 1963). However, the final protein composition is changed through the amount and source of protein and fat in the diet of the cow (Schönfeldt *et al.*, 2012).

- **Stage of lactation**

The stage of lactation can also cause changes in the composition of milk. Lactation changes in milk composition take place during the period of milk production between parturition and drying-off. Early lactation is correlated with decreased levels of total solids, protein, caseins and fat whereas an increase in lactose concentration is common. Late lactation milk tends to show a decrease in milk yield and increased levels of total solids, fat, protein, casein. Decreased lactose concentration is normal for late lactation milk (O'Brien & Guinee, 2011). Late lactation milk consists of lower levels of casein and higher levels of free fatty acids (Guinee & O'Brien, 2010).

An association between the plasminogen activators (PA)–plasminogen–plasmin system and gradual involution exists which is the decline phase of lactation. Increased plasmin and PA activity in milk are correlated with gradual involution. Treatment with bovine somatotropin prevents the increase in plasmin during gradual involution, indicating that bovine somatotropin interferes with the conversion of plasminogen to plasmin. The  $\beta$ -casein is highly negatively correlated with milk yield in the declining phase of lactation. The production of  $\beta$ -casein by plasmin during the storage of milk in the udder represents between 8% and 12% of the total proteose–peptone fraction in whey.  $\beta$ -casein is resistant to further degradation by plasmin. All these characteristics make  $\beta$ -casein an ideal candidate for negative feedback control of milk secretion (Silanikove *et al.*, 2006).

Reinitiating milk removal can reverse the first stage of involution, but the second stage of involution is irreversible and is characterized by activation of proteases that destroy the globular–alveolar structure of the gland by degrading the extracellular matrix and basement membrane, and cause massive loss of alveolar cells (Silanikove *et al.*, 2006).

Involution in cows is normally completed by 21–30 days after drying-off. The compositional changes include a dramatic decrease in the concentrations of lactose and fat, and parallel increases in the

concentrations of lactoferrin and immunoglobulins. Plasmin activity in mammary secretion tends to increase gradually and become substantially higher within 13 days in cows reaching involution while still producing a lot of milk, which is then followed by cessation of milking (Silanikove *et al.*, 2006).

The infusions of casein hydrolysates (CNH's) which contain products of plasmin activity, dramatically increase the rate of involution, to the extent that it is completed within 3 days. These responses are accentuated in late lactation when there is natural increase in the activity of the PA–plasminogen–plasmin system. All glands treated with CNH tend to show normal lactation (Silanikove *et al.*, 2006).

The different caseins in milk therefore vary according to stage of lactation since  $\beta$ -casein levels are low at the initial stage of lactation, but increases thereafter. The levels of  $\beta$ -casein tend to decrease as  $\gamma$ -casein levels increases (O'Brien & Guinee, 2011). The degradation of caseins is common in late lactation milk (Hallén, 2008). The total proteose-peptone fraction generally increases during lactation (O'Brien & Guinee, 2011).

The susceptibility to age gelation is higher for early lactation milk than that of late lactation milk. However, plasmin-induced proteolysis is normally higher in late lactation milk than in early lactation milk due to increased levels of  $\beta$ -LG in late lactation milk (Datta & Deeth, 2001; Chavan *et al.*, 2011).

- **Stress**

A mechanism that connects stress with the PA–plasminogen–plasmin system exists. Stress is considered to activate this system and thus causing an increased plasmin activity as well as the formation of  $\beta$ -casein. A reduction in milk production is also common during stress and is also correlated with plasmin activity within milk (Silanikove *et al.*, 2006).

- **Season**

Seasonal variations have an influence on the composition of milk. The components within milk as well as the physicochemical properties of milk vary with season (Chen *et al.*, 2014). Seasonal variations refer to as the changes in the composition, quality and suitability of milk throughout the year (O'Brien & Guinee, 2011). Seasonal changes cause problems in milk but can also create opportunities for dairy producers (Chen *et al.*, 2014). Seasonal variations also have a direct influence on the gelation of UHT milk during storage. Milk from summer is regarded as being more stable than winter milk, especially in the case of UHT milk. In general, spring and autumn milk has more gelation problems than milk from summer and winter since they are more prone to gel during storage (Datta & Deeth, 2001; Chavan *et al.*, 2011).

The composition of milk also varies according to season since spring milk consists of higher values of pH and lactose than autumn milk (Gaucher *et al.*, 2008). Summer milk is considered to have the

highest heat stability whereas spring, autumn and winter milk has low heat stability (O'Brien & Guinee, 2011). The pH of milk also varies according to season since summer milk has the highest pH when compared to spring and autumn milk (Celestino *et al.*, 1997).

There are also changes in the concentrations of fat, protein, casein and lactose within milk during different seasons (O'Brien & Guinee, 2011). Environmental factors normally affect the protein content of milk (Guinee & O'Brien, 2010). Summer milk contain less protein and fat than winter milk due to different temperatures and feed composition since cows consume more dry food during winter and more fresh pasture during summer. Higher protein content is more common for spring milk than autumn milk (Chen *et al.*, 2014). Spring milk has lower values of fat (Chavan *et al.*, 2011) whereas autumn milk tends to have a higher fat content (Chen *et al.*, 2014). Changes in casein micelle size during seasons are also common since spring milk contain larger casein micelles than autumn milk (Gaucher *et al.*, 2008).

- **Milking frequency**

The frequency of milking also has an influence on the composition of milk as well as milk yield. Once a day milking produce milk with a decrease in levels of lactose, plasmin concentrations and increased somatic cell counts. Once a day milking also produce milk with higher protein and fat contents than milk from twice a day milking. Milk from once a day milking has higher casein contents as well as higher gel strength (Guinee & O'Brien, 2010). Once-daily milking, in comparison with twice-daily milking reduce milk yield by 30% (Silanikove *et al.*, 2006).

A decreased milking frequency is directly associated with an increased activity of the PA-plasminogen-plasmin system. Thrice-daily milking, in comparison to twice-daily milking, is considered to prevent increased casein degradation that is normally associated with late lactation. Part of this effect is due simply to reduced exposure to plasmin as a result of the reduced storage time in the udder, but it is partly due to a better maintenance of epithelial tight junction integrity as lactation advances. Increased milking frequency also dilutes the content of  $\beta$ -casein f (1-28) (Silanikove *et al.*, 2006).

- **Geographic region**

The composition of milk is influenced by geographic locations since it varies amongst countries. The components of milk that varies according to country are the fatty acid composition, amino acid content and mineral- and vitamin content. Research shows that the retinol content of milk is higher in South Africa than in the United States. The amino acid content and profile of milk varies among countries but also within the same country due to seasons and changes in feeding regimes of cows. The differences in milk composition among countries can be attributed to different breed and farming systems of dairy cows between countries (Schönfeldt *et al.*, 2012).

- **Calving**

The production of milk may be divided in two systems where the first system involves an even milk production pattern throughout the year. The second system involves the production of milk that is at peak during a specific time of the year and decreases towards the end of lactation. The second system is known as the seasonal milk production system and is characterized by compact calving patterns where cows calve at a specific time of the year. Dairy farmers who use fresh pasture as feeding tend to use this seasonal milk production system in order to maximize pasture utilization where the calving date starts during the grass herbage growing season. The calving date is adjusted in order for cows to calve during spring and be dried off during winter (O'Brien & Guinee, 2011).

The seasonal milk production system maximizes cost efficiency however it may lead to the irregular supply of milk during winter as well as an irregular milk quality during autumn and winter. This system also leads to seasonal variation in the composition of milk however seasonal variation is also prevalent for the consistent milk production systems. This system has an influence on the gelation properties of milk since feeding spring-calved cows with grass silage improves the gelation properties of milk (O'Brien & Guinee, 2011).

#### **2.4.2.3 The role of $\beta$ -LG on age gelation**

##### **2.4.2.3.1 Background information of $\beta$ -LG**

The whey protein,  $\beta$ -LG is a globular protein studied in detail due to its numerous influences on the quality of milk. This protein is adsorbed at a neutral pH to oil-water interface and has a denser and thinner monolayer than the caseins. This protein is unfolded, denatured and aggregated during heat treatments of milk. During the protein unfolding of  $\beta$ -LG, a two-dimensional gel-like layer is formed which is followed by the strengthening of a non-bonded physical intermolecular interaction and slow covalent cross-linking. It is therefore clear that the properties of  $\beta$ -LG are sensitive to the conditions of thermal processing (Dickinson, 2010).

High-pressure treatments of  $\beta$ -LG have an influence on protein aggregation and the stability of milk. This protein becomes reactive during unfolding hence they are more prone to be flocculated due to the influence of hydrophobic association and disulphide bond formation between the  $\beta$ -LG molecules. The concentration of free protein also has an influence on the flocculation behaviour of milk that contains high amounts of  $\beta$ -LG since unabsorbed proteins within milk leads to extensive flocculation. However, excess protein in the aqueous phase may lead to milk being more stable against droplet aggregation. The occurrence of depletion flocculation is not common for  $\beta$ -LG emulsions. These types of emulsions are flocculated in the presence of calcium ions. The flocculation behaviour of  $\beta$ -LG emulsions are also affected by interactions with an ionic surfactant and the addition of non-ionic solutes such as ethanol and sucrose (Dickinson, 2010).

The heat treatment of milk also results in  $\beta$ -LG being more susceptible to enzymatic cleavage due to heat induced conformational change. This protein is less susceptible to conformational changes at an acidic pH rather than a neutral pH, therefore the pH of milk is important since it also influences the level of interaction between denatured  $\beta$ -LG and casein micelles. Denatured  $\beta$ -LG along with K-casein is normally released from the casein micelles at a pH above 6.8. The interaction between  $\beta$ -LG and casein micelles occurs faster at a lower pH and higher temperature. This interaction normally occurs in the serum phase or on the casein micelles (Thompson *et al.*, 2008).

The enzymes, plasmin and plasminogen, is regarded as being heat labile in the presence of  $\beta$ -LG. The denaturation of  $\beta$ -LG is correlated with the inactivation of plasmin and plasminogen (Newstead *et al.*, 2006) since higher degrees of  $\beta$ -LG denaturation results in lower plasmin and plasminogen activity (Rauh *et al.*, 2014). Highly reactive free SH-groups become available during  $\beta$ -LG denaturation due to the unfolding of  $\beta$ -LG, this SH-groups cause the irreversible denaturation of plasmin. It is therefore clear that a certain amount of  $\beta$ -LG denaturation is necessary for the increased inactivation of plasmin and plasminogen (Chavan *et al.*, 2011).

#### **2.4.2.3.2 Mechanism by which $\beta$ -LG induces age gelation**

The interaction between  $\beta$ -LG and K-casein is known to have serious effects on the physical properties of milk and play a role in the stability of milk (Crudden *et al.*, 2005). During the heat treatment of milk, a complex is formed due to the denaturation of  $\beta$ -LG and aggregation of K-casein which is known as the  $\beta$ K-complex (McMahon, 1996). This association between  $\beta$ -LG and K-casein occurs at pH 6.5 and a temperature of 70°C (Pulkkinen, 2014).

The cause for age gelation is still unknown, however the release of the  $\beta$ K-complex from the casein micelle is known to play a role since this released complex forms a three-dimensional protein network and a gel when aggregated (McMahon, 1996) thus causing gelation of milk (Richards *et al.*, 2014). The other caseins such as  $\alpha$ <sub>1</sub> and  $\alpha$ <sub>2</sub>-casein do not interact with  $\beta$ -LG during the heat treatment of milk. The interaction of  $\beta$ -LG with K-casein depends on time, temperature and rate of heating, milk pH and concentration of milk salts (Thompson *et al.*, 2008). Proteolysis induces the release of the  $\beta$ K-complex by casein hydrolysis (Datta & Deeth, 2001). High proportions of the  $\beta$ K-complex on the surface of the casein micelles can reduce the degree of proteolysis and age gelation (Chove *et al.*, 2013).

#### **2.4.2.3.3 Protection of milk against $\beta$ -LG induced flocculation/age gelation**

There are ways to protect milk against flocculation such as adding less salt to the  $\beta$ -LG emulsion since the addition of salt induces droplet flocculation. The heating of milk at temperatures above 65°C leads to increased  $\beta$ -LG denaturation which results in more reactive  $\beta$ -LG molecules thus making milk

more susceptible to flocculation. Therefore, the heating of milk at temperatures below 65°C result in milk that is less prone to be flocculated (Dickinson, 2010). However, according to Kelly & Foley (1997), increased  $\beta$ -LG denaturation reduces the occurrence of age gelation in UHT milk.

The formation of intermolecular disulphide bonds between  $\beta$ -LG molecules leads to the flocculation of milk. The blocking of thiol groups with N-ethyl maleimide prevents the formation of these bonds, thus inhibits milk flocculation (Dickinson, 2010). An increase in the  $\beta$ -LG concentration of milk prior to homogenization also retards flocculation (Blijdenstein *et al.*, 2003).

## **2.5 Detection methods for flocculation in milk**

The various milk flocculation detection methods are discussed in detail which is the Alizarol test, RP-HPLC, azo-casein assay for protease activity, plasmin and plasminogen assays.

### **2.5.1 The Alizarol test**

The Alizarol test is popular due to the ease at which it can be performed as well as the immediate availability of useful results (Robertson, 2010). The Alizarol test is a parameter to determine the quality of milk. This test serves as the first screening test for raw milk (Aquino, 2013) since it gives valuable information regarding the quality of raw milk (Kurwijila, 2006). This test is used to establish the acceptability of raw milk for further processing (Robertson, 2010). The Alizarol test is regarded as practical and well-suited for the determination of raw milk quality due to its simplicity and ability to point out more than one defect of milk (Milkman, 2010), however this can complicate the interpretation of the result and can thus serve as a weakness of this test. This test is 75 years old and was developed in 1940 by Schwarz and Hagemann. The outcome of the test is controlled by two factors namely the development of acidity and the chemical instability of the milk protein. The Alizarol test is normally performed at the point of raw milk collection by the tanker drivers (Robertson, 2010).

The main objective of this test is the indication of the development of an unaccepted level of acidity or alkalinity in milk or pH that falls outside the acceptable norms. It can therefore be said that the purpose of the Alizarol test is to determine the acceptability of milk based on its acidity or pH (Robertson, 2010). Alizarin is a pH-indicator since it changes colour according to the level of acidity and will thus indicate whether milk is abnormally sour or alkaline (Milkman, 2010). This solution is commercially available or it can be prepared by the addition of 0.4g alizarin to one litre of 68% alcohol solution. An advantage of this test is that it serves as an indication of milk flocculation since the colour of the milk turns violet. The Alizarol test detects milk with a pH of 6.4 and lower. Colostrum and mastitis milk results in a positive Alizarol test (Kurwijila, 2006).

Casein stability in milk depends on the casein particles' degree of hydration. Partial dehydration of the casein micelles are caused by development of acidity in milk. Addition of alcohol results in further

dehydration and destabilisation of casein (Kurwijila, 2006) since alcohol serves as the dehydrator. The dehydration of casein micelles will result in protein flocculation and eventually lead to the coagulation of milk. Slightly unstable proteins in sour milk will lead to the precipitation of proteins in the form of flakes. Acid destabilizes the protein complex in milk. A positive Alizarol test will also serve as an indication of the heat stability of milk hence the popularity of this test during processing of UHT milk or milk powder where the heat stability of milk proteins is of great importance (Milkman, 2010). Thus this test can also be used to indicate the heat stability of the proteins within milk (Robertson, 2010).

No flocculation, coagulation or precipitation will occur if the milk is of good quality. At a pH of 6.4, milk normally flocculates. Milk fails the Alizarol test when it flocculates or precipitates since this test is very sensitive (Kurwijila, 2006).

### **2.5.1.1 Factors that affect the Alizarol test**

- **Acidity**

The most important cause for a positive Alizarol test is the presence of lactic acid which results from microbial action and lowers the pH of milk (Robertson, 2010). The proteins in milk are less stable at a higher acid level hence the occurrence of protein flocculation when acid milk is mixed with alcohol. Therefore, acidity influences the test (Milkman, 2010).

- **Mastitis**

The proteins and minerals within mastitis milk differs from that of normal milk therefore the proteins in mastitis milk will flocculate more rapidly than in normal milk. The pH of mastitis milk is also higher than normal milk thus the alizarine colour will turn violet when tested (Milkman, 2010).

- **Sweet curdling**

Sweet curdling refers to viscosity increases of milk and result from certain spoilage bacteria which are capable of producing rennin-like enzymes and even precipitate casein in the absence of acid at a normal pH (Milkman, 2010). These organisms enter milk through dust, hay and soil. Examples are *Streptococcus liquefaciens*, *Bacillus subtilis* and *Bacillus cereus* (Robertson, 2010).

- **Colostrum**

Milk with high colostrum content is unstable against alcohol due to high contents of albumin and globulin. Colostrum is present in milk which is secreted within the first 3 to 6 days after calving (Robertson, 2010).

- **Mineral imbalance**

Mineral imbalances such as too high or too low levels of calcium, phosphate and citrate increase the ease of which casein will flocculate in the presence of alcohol. Supplementing cows with mono-calcium phosphate may be a solution to this phenomenon (Milkman, 2010).

- **Stage of lactation**

Early and late lactation milk differs from normal milk in terms of chemical composition since it may contain higher levels of albumin, globulin and chloride, therefore the protein matrix in milk is unstable against alcohol. During early lactation, milk is relative unstable against alcohol but gradually becomes more stable. A decrease in stability is common towards the end of lactation since late lactation milk has a higher content of albumin, globulin and chloride (Milkman, 2010; Robertson, 2010).

- **Churned milk fat**

Churned milk fat granules clinging to the inside of the test container may be mistaken as protein flocculation (Robertson, 2010).

- **Low milk urea nitrogen (MUN) levels**

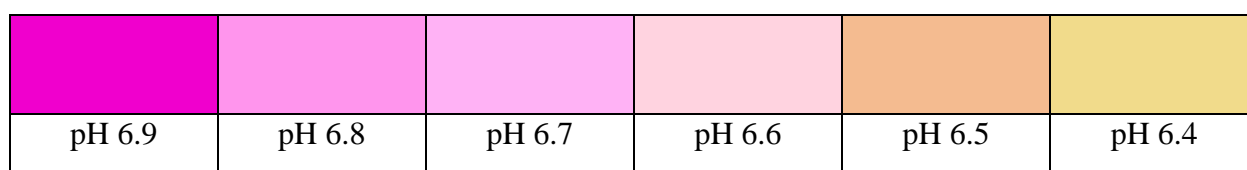
Diets which contain as much as 99% of the variation in the non-protein-nitrogen (NPN) content of milk may result in low MUN levels in the blood. This phenomenon may result in a positive Alizarol test (Robertson, 2010).

### 2.5.1.2 Interpretation of the Alizarol test

Table 2.1 and Figure 2.5 are parameters used for the interpretation of the Alizarol test. These parameters makes interpretation easier since it clearly states the pH levels as well as clear descriptions of colour guidelines to be followed during the evaluation of samples for milk flocculation.

**Table 2.1. Interpretation of the Alizarol test (Kurwijila, 2006).**

Parameter	Normal milk	Slightly acidic milk	Strongly acidic milk	Alkaline milk
<b>pH</b>	6.6-6.7	6.4-6.5	6.3 or lower	6.8 or higher
<b>Colour</b>	Red brown	Yellowish-brown	Yellowish	Violet
<b>Appearance</b>	No flocculation and flakes	No flocculation	Flocculation: yellowish in colour with small flakes or completely flocculated	Flocculation: violet in colour, clots and flakes indicate mastitis milk



**Figure 2.5. Colour chart range for the Alizarol test (Robertson, 2010).**

### **2.5.2 Reverse-phase High Performance Liquid Chromatography**

Chromatography is the movement of a sample through a system. Molecules within a sample have different affinities and interactions with the system that will result in separation of the molecules. HPLC refers to High Performance Liquid Chromatography which is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in a solution. It is used to separate molecules based on their differences in structure or composition. RP-HPLC is used to determine the specific compound in a solution. Essential equipment for HPLC includes a pump, injector, column, detector and integrator. Separation of molecules occurs in the column. Columns differ in length, size and packing materials. Integrators and data-processing equipment are used to collect, store and analyse the chromatographic data. The pumps used in HPLC have a pressure of 1000-2000 psi. The injectors can be a single injection or an automated injection system and the injector for HPLC should provide injection of the sample within the range of 0.1-100 $\mu$ L. There are different types of detectors for HPLC which each has their own advantages and limitations. Computers serve as the data acquisition system for HPLC. Different types of HPLC require different types of solvents. The solvent is normally a mixture of water and polar organic solvent for RP-HPLC (Kupiec, 2004).

The purpose of using RP-HPLC in this study is to establish enzymatic peptide profiles in order to differentiate between microbial proteases and plasmin within milk. RP-HPLC will also be compared with other tests in order to establish the most effective test for detection of milk flocculation.

### **2.5.3 Azo-casein assay for protease activity**

Kohlmann *et al.*, 1991, performed a study regarding the purification and characterization of an extracellular protease produced by *Pseudomonas fluorescens* M3/6. They inoculated this strain into reconstituted non-fat dry milk and incubated it at 7°C for 46 days. It was established that a significant amount of extracellular protease was produced during the latter part of the culture's life cycle. They purified the protease by using ammonium sulphate fractionation, ion-exchange chromatography and gel filtration. It was confirmed that the isolate protease had activity on azo-casein,  $\alpha$ ,  $\beta$  and K-caseins as well as a plasmin substrate, however did not have plasminogen activator activity. This protease had a molecular weight of 45 kDa, and isoelectric point of pH 8.25, a broad temperature and pH range for activity and was less heat-stable in the isolated form than in the cell-free extract (Kohlmann *et al.*, 1991).

### **2.5.4 Plasminogen and plasmin assay**

A characteristic of the psychrotrophic bacteria within milk is that they produce heat-stable proteases which in turn have a negative impact on the plasmin system as well as the quality of processed milk. According to a study performed by Fajardo-Lira & Nielsen (1998), microbial strains such as

*Pseudomonas fluorescens* M3/6, *Pseudomonas* spp. SRM21A as well as SRM28A can be inoculated into milk and incubated at 4°C for approximately 9 days. They obtained casein and whey fractions from each of the samples and electrophoresed it in order to visualize protein hydrolysis as well as plasmin activity. Colorimetric assays were also used to quantify plasmin-related activities. They established the presence of caseinolytic activity, at the same molecular mass range as commercial bovine plasmin which they used as a control, within the whey fractions of all three *Pseudomonas* strains through casein SDS-PAGE gels. Through this study they confirmed that plasminogen as well as plasmin activity in the casein samples decreased as incubation time increased, however activity for both plasminogen and plasmin increased in the whey samples. They also established that cross-activity exists between anti-bovine plasminogen and plasmin and plasminogen from the whey samples through protein immune-detection. The growth of the *Pseudomonas* strains tested and their concomitant production of proteases caused the release of plasmin and plasminogen from the casein micelle into the whey fraction (Fajardo-Lira & Nielsen, 1998).

#### **2.5.4.1 Plasmin assay**

The measurement of plasmin activities in the casein fractions was done by the colorimetric method described above however with some modifications. Activity of plasmin in the whey fractions was measured in a similar way. Whey samples were mixed with Spectrozyme plasmin as well as casein samples however some casein samples were also mixed with Tris buffer. Both the whey and casein samples were placed in microcentrifuge tubes and then incubated in a water bath at 37°C for 1 hour. Incubation was followed up with centrifugation at 15600 RPM for 10 minutes. The supernatant were transferred to a microtiter plate and all the samples were measured for absorbance at 405 nm and 490 nm with an ELISA plate reader. Absorbance at 490 nm was subtracted from absorbance at 405 nm to account for the turbidity in the samples (Fajardo-Lira *et al.*, 2000).

#### **2.5.4.2 Plasminogen assay**

This assay was similar to the assay described above but included the addition of a urokinase-type plasminogen activator prior to incubation in order to activate plasminogen to plasmin. Standard curves with bovine plasminogen consisted of a Spectrozyme plasmin solution, a urokinase-type plasminogen activator solution, a plasminogen solution as well as modified Tris buffer. Samples were also incubated in microcentrifuge tubes at 37°C for 1 hour. Assays were also carried out in microtiter plates. Samples were also centrifuged for 10 minutes and absorbance was read using an ELISA plate reader. Values from the plasminogen assay account for total plasminogen and plasmin activity (Fajardo-Lira *et al.*, 2000).

## **2.6 Conclusions**

Milk flocculation/age gelation is regarded as a major problem in the dairy industry since it reduces the shelf-life of milk. This phenomenon can occur either through proteolytic enzymatic action or chemical action. There are several factors that have a substantial influence on age gelation however methods for controlling it can be used in order to delay the occurrence of flocculation. Presently, the detection methods for milk flocculation are considered to be out-dated since flocculation is still a common problem. Therefore, the need arose to develop new and improved detection methods in order to minimize the risk of flocculation and age gelation in the dairy industry.

The stability of milk casein micelles is very complex and the destabilisation thereof occurs through various mechanisms. The focus in this study was on flocculation/gelation through proteolytic action.

Chapter 3 that follows is mainly focused on the milk flocculation detection techniques and the effectiveness of the various techniques regarding detection of flocculation/proteolytic activity and differentiation of proteolytic enzymes.

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## CHAPTER 3

### **Techniques: Validation of methods for the determination of proteolytic activity in raw milk**

#### ***Objectives***

Raw milk was incubated with Plasmin, *Bacillus* and *Pseudomonas* proteases as well as a combination of all three proteolytic enzymes (cocktail sample). The treated milk samples were subjected to a protease assay in order to establish activity values. The Alizarol test was done on these samples in order to establish the effectiveness of this test regarding the detection of milk flocculation. A modified milk agar plate technique was tested in order to investigate whether this technique was capable of detecting proteolytic activity. The milk samples were analysed using RP-HPLC in order to establish enzymatic protein peptide profiles for each proteolytic enzyme and to distinguish between bacterial protease and indigenous plasmin. A computer assisted programme was also incorporated in order to assist with RP-HPLC interpretation (MILQC software).

#### **3.1 Introduction**

Milk flocculation is currently a major problem in the dairy industry since it causes milk to have a shorter shelf-life and also results in a negative consumer acceptance towards milk (Datta & Deeth, 2001). The starting point of gelation is enhanced by changes at the surface of casein micelles when the micelles lose their colloidal stability and then in turn form a three-dimensional gel network. Protease activity is responsible for these changes which include casein breakdown as well as protein denaturation in milk (Cilliers, 2007). Proteolysis of caseins is known to be one of the causes of milk flocculation while plasmin as well as proteases produced by psychrotrophic bacteria is known to play a role (Datta & Deeth, 2001).

Both plasmin and plasminogen are heat-resistant thus are unscathed by the UHT process and remain active during storage (Cilliers, 2007). The concentration of plasmin increases during storage due to autolysis of plasmin (Schroeder *et al.*, 2008) and the activation of plasminogen by psychrotrophs (Fajardo-Lira, 1999). Plasmin has the ability to induce proteolysis which leads to age gelation (Chen *et al.*, 2003). The main psychrotrophic bacteria present in milk are the Gram-negative rods such as *Pseudomonas* spp. They make up 50% of the total bacteria in milk and are considered to be the major spoilage agents thereof (Cilliers, 2007). Heat-resistant proteolytic enzymes are produced by *Pseudomonas fluorescens* (Chen *et al.*, 2003). Another common isolated bacterial species that normally grows under refrigerated conditions in milk is *Bacillus* spp. (such as *Bacillus licheniformis*) which also has the ability to produce heat-resistant proteases (Chen *et al.*, 2003). Heat-resistant proteases produced by *Pseudomonas fluorescens* and *Bacillus licheniformis* are known to play a

substantial role in the destabilisation of UHT milk during storage (Gaucher *et al.*, 2011) which results in proteolysis and age gelation and therefore causing enormous economic losses in the dairy industry (Samaržija *et al.*, 2012).

The optimum growth temperature for *Bacillus* is 20-40°C (Tamime, 2008) while 17.5-30°C is the optimum growth temperature for *Pseudomonas* (Chen *et al.* 2003). The proteases produced by *Pseudomonas fluorescens* is characterized with optimum temperatures at 37-45°C (Fajardo-Lira, 1999). Plasmin has an optimum activity temperature of 37°C (Chen *et al.* 2003).

The Alizarol test is used to determine the quality of milk with regard to milk flocculation. The Alizarol test is popular due to the ease at which it can be performed as well as immediate availability of results needed in the dairy industry. The main objective of this test is the indication of the development of an unacceptable level of acidity or alkalinity in milk that falls outside the acceptable norms (detects milk with a pH of 6.4 and lower). It can therefore be said that the purpose of the Alizarol test is to determine the acceptability of milk based on its acidity or pH (Robertson, 2010). Alizarin is a pH-indicator since it changes colour according to the level of acidity and will thus indicate whether milk is abnormally sour or alkaline (Milkman, 2010).

The milk agar plate technique for protease detection is a modification of the milk agar plate technique used during the classification of microbes (Himedia Laboratories, 2015). The clear halos that appear on the milk agar are indicative of proteolytic enzymes that hydrolyse the milk casein (Himedia Laboratories, 2015).

The impact of proteases is therefore regarded as important for the dairy industry due to their enormous impact on the quality of milk (off-flavours and gelation). It is therefore important to establish effective detection methods. Rapid and sensitive techniques are of utmost importance and are highly desirable (Vijayaraghavan & Vincent, 2013). The already established techniques for milk flocculation such as the Alizarol test are not suited to differentiate between proteolysis since this test only has the ability to detect milk flocculation thus not the presence of proteolytic enzymes. New techniques have to be developed. In the current study the Alizarol test will be weighed up against other methods that may detect milk flocculation, specifically by free proteases. These methods involve a milk agar plate technique, protease assays and analysis of protease produced peptides. Chromatography (RP-HPLC) and a computer assisted interpretation (MILQC software) will be applied.

**Keywords:** Flocculation, Plasmin, *Bacillus*, *Pseudomonas*, Detection, Techniques

## **3.2 Materials and reagents**

### **3.2.1 Milk**

The milk used for the self-production of enzymes was Pick and Pay Choice Low Fat Long Life UHT milk and was obtained from Pick and Pay in Langenhovenpark, Bloemfontein, Free State, South Africa. UHT milk was used instead of raw milk since all the indigenous enzymes are destroyed during the UHT process, except plasminogen.

The milk used for the Alizarol test was Full Cream Ultra-Pasteurised milk from Clover SA and was obtained from Checkers Langenhovenpark in Bloemfontein, Free State, South Africa. Ultra-pasteurised milk was used in order to have milk without the presence of active microorganisms.

The milk used for the evaluation of the protease assay was Pick and Pay Choice Full Cream Long Life UHT milk and was obtained from Pick and Pay in Langenhovenpark, Bloemfontein, Free State, South Africa. UHT milk was used in order to have milk with a long shelf-life. A large number of containers of the same batch of milk were purchased in order to have the same batch available for various tests and experiments.

The milk used for the preparation of the milk agar plates was Pick and Pay Choice Low Fat Long Life UHT milk and was obtained from Pick and Pay in Langenhovenpark, Bloemfontein, Free State, South Africa. Fat serves as a steric hindrance for proteases hence low fat milk were chosen (Datta & Deeth 2001). The reason for using UHT milk was to have milk with a long shelf-life and a large number was purchased in order to have the same batch of milk available for various tests and experiments.

The milk used for the RP-HPLC technique (proteolytic peptide profiles) was raw milk and was supplied by Dairy Corporation in Bloemfontein, Free State, South Africa. Raw milk was used since heat treatments applied to milk limit the capability of proteases to hydrolyse casein within milk and therefore the usage of untreated milk was essential.

### **3.2.2 Reagents**

Glycerol, obtained from Sigma Aldrich, was used for the storage of self-produced enzymes to conserve activity during freezing. Bacteriological agar powder was supplied by Quantum Biotechnologies, Randburg, South Africa. The nutrient broth was supplied by Merck, South Africa.

The 68% Alizarol solution used for the Alizarol test was obtained from Selectech, South Africa.

The protease assay kit (Calbiochem no. 539125) was obtained from Merck Millipore. Reagents within this kit consisted of Fluorescein thiocarbamoyl (FTC) casein, the incubation buffer, the assay buffer and the protease positive control. Trichloroacetic acid (TCA) was obtained from Merck, South Africa.

All the following reagents used for the proteolytic peptide profiles (RP-HPLC) were supplied by Merck, South Africa. Acid solutions were used to precipitate samples prior to centrifugation. TCA as well as Hydrochloric acid (HCl) was used. According to literature TCA precipitates almost or high pH proteins in milk (large molecules) whereas HCl precipitate only proteins soluble at pH 4.6 (isoelectric point) (Datta & Deeth, 2001). The 12% TCA was used since the peptides produced by bacterial protease are less hydrophobic and elute early in the RP-HPLC, while the peptides produced by native plasmin are more hydrophobic and elute later. Acid precipitation with 12% TCA allows the peptides to be fractionated in a similar manner (Datta & Deeth, 2003). A reference for retention time, vanillyl-alcohol, was used for all the samples in order to achieve more reliable results since this reference will show on the opposite side of the peaks in the RP-HPLC chromatogram.

### **3.2.3 Proteolytic enzymes used in all experiments**

#### **3.2.3.1 Commercial enzymes**

The commercial enzymes used were obtained from Sigma Aldrich. The two proteolytic enzymes had different activities. The plasmin used was 5 U/mL and originate from bovine plasma. The Microbial protease Type VIII (*Bacillus licheniformis*) was in freeze dried powder form. The activity of *Bacillus* protease was 11 U/mg. Commercial *Pseudomonas fluorescens* protease was also evaluated, however it was inactive within two batches of enzymes received from Sigma Aldrich. Therefore, the sole source of *Pseudomonas fluorescens* protease had to be self-produced.

The enzyme used in the Alizarol section was Neutrased protease (1.5 U/mg) and was supplied by Novozymes, Denmark.

#### **3.2.3.2 Self-produced enzymes**

Two protease extracts were produced from *Bacillus licheniformis* as well as *Pseudomonas fluorescens*.

##### ***Bacteria***

Two bacterial species were used in order to self-produce proteolytic enzymes. *Bacillus licheniformis* and *Pseudomonas fluorescens* were selected since these organisms are the culprits in the milk flocculation problem (Gaucher *et al.*, 2011; Samaržija *et al.*, 2012). These bacteria were supplied by the UFS bacterial collection maintained in the Department of Food Science, Faculty of Natural and Agricultural Sciences, University of the Free State, South Africa.

### **3.3 Methods**

#### **3.3.1 Commercial and self-produced proteolytic enzymes**

##### **3.3.1.1 Preparation of stock solutions for commercial proteolytic enzymes**

Stock solutions of commercial enzymes were prepared in DH<sub>2</sub>O to ensure ease of handling and ensure a uniform level of activity over the time of research and were stored at -20°C. *Bacillus* protease was prepared as two stock solutions and sub divided in 500µL aliquots; stock solution 1 at 11 U/mL and stock solution 2 at 110 U/mL and stored at -20°C. For experimental use, the *Bacillus* protease stock solutions were diluted 10x so that 5µL could be added to reaction vessels at final respective activities of 0.0055 U and 0.055 U. A volume of 10µL of commercial plasmin, which contained 0.05 U, was used for experimental purposes.

A stock solution was also prepared for the Neutrase protease by dissolving 0.5g enzyme in 10mL DH<sub>2</sub>O which was 75 U/mL.

##### **3.3.1.2 Self-produced proteolytic enzymes**

###### ***Maintenance of bacterial cultures and preparation of pre-inoculum and nutrient agar slants***

The *Bacillus* and *Pseudomonas* were maintained and pre-inoculations prepared in nutrient broth (1.6%) and agar (1%) slants. The nutrient broth was prepared by dissolving 3.2g in 200mL of DH<sub>2</sub>O of which 10mL was used in McCartney bottles as pre-inoculum. For the nutrient agar slants, 5g agar along with 8g nutrient broth was dissolved in 500mL of DH<sub>2</sub>O and thoroughly mixed and 20mL used for each slant in McCartney bottles. All the McCartney bottles were sterilized by autoclaving for 20 minutes at 121°C. The pre-inoculum bottles, along with the nutrient broth agar slants, were stored in a refrigerator at 4°C after sterilization until usage.

###### ***Procedure for the self-production of proteolytic enzymes***

Two separate Erlenmeyer flasks were filled with 250mL of low fat UHT milk, the beak of the flasks were plugged with cotton wool wrapped in cheese cloth, followed by autoclave at 121°C for 20 minutes.

An eye needle was aseptically used to inoculate *Bacillus* and *Pseudomonas* from the respective nutrient agar slants into the pre-inoculums which were subsequently incubated at 32°C for 24 hours. 1mL of each pre-inoculum was inoculated into separate Erlenmeyer flasks which contained sterile UHT milk. The flasks were slowly agitated (30 rpm) and incubated at 25°C for three days until bacteria growth reached the stationary phase. Finally, the flasks containing the bacteria in their

stationary phase were incubated at 4°C, without agitation, for three days in order to cause milk flocculation (Gaucher *et al.*, 2011).

In order to obtain a clear supernatant for both *Bacillus* and *Pseudomonas*, the UHT milk with the cultures were precipitated. Half of the preparation was precipitated with 12% TCA as well as 0.1 N HCl (250µL to 1mL of the milk and bacteria mixture) for peptide profile determination. The other half (250mL) was precipitated with 0.1 N HCl (1mL) for enzyme production. The samples were subsequently centrifuged (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan) at 10 000 rpm for 20 minutes to obtain a clear supernatant. These supernatants containing proteolytic enzymes were used in all the self-produced protease experiments to follow.

It is known that activity of the *Pseudomonas* enzyme is lost during freezing and thawing, therefore the enzyme preparations were frozen in 25% glycerol in order to preserve activity. Both the self-produced proteases from *Pseudomonas* supernatant (30mL) and *Bacillus* supernatant (30mL) were stored in glycerol as 10mL aliquots at -20°C.

Initially 100µL of the self-produced enzyme preparation (*Pseudomonas* and *Bacillus*) was used since activity of these two enzymes was unknown at this stage (Refer to Table 3.5 in Section 3.4.2). When the activity proved sufficient or close to similar than the commercial enzymes, the amount of 100µL was used throughout all experiments.

### **3.3.2 Alizarol test**

Milk was artificially flocculated using Neutrase protease in order to induce flocculation and in turn evaluate the effectiveness of the Alizarol test.

The milk samples were incubated in a water bath at 37°C. Two samples (in duplicate) were prepared of which the first control contained 19mL of the full cream ultra-pasteurised milk and 1mL DH<sub>2</sub>O, whereas the second sample contained 19mL of ultra-pasteurised milk and 1mL of the Neutrase enzyme stock solution (75 U/mL) (Section 3.3.1.1). Samples from both reaction volumes were retrieved prior to incubation in the water bath to act as control (prior to heat treatment). The Alizarol test was performed directly after samples were retrieved. Incubation lasted 1 hour. Samples (400µL) from both the incubated and non-incubated reaction volumes were retrieved at time intervals of 15 minutes for up to 1 hour. The remaining samples were further incubated for 1 hour. The purpose of this study was to detect whether protease treated milk samples will test positive with the Alizarol test and also to visualise the degradation process (protease) of the milk with the Alizarol test.

Different alcohol levels may be used for the Alizarol test; a 68% is used for an ordinary Alizarol test whereas a 70-72% is used for a more sensitive Alizarol test (Robertson, 2010). In the current study 68% alcohol was used. Equal volumes (400µL) of Alizarol solution were transferred to the various milk samples. The milk and Alizarol solution was thoroughly mixed by inversion (important not to shake). Evaluation of samples followed, specifically for the presence of flakes (degree of flocculation) and colour changes, Refer to Figure 2.5 (Robertson, 2010).

### **3.3.3 Protease assay**

The protease assay was performed on various levels of activity for commercial *Bacillus* protease as well as on the self-produced enzymes in order to evaluate the effectiveness of this assay in comparison to the Alizarol test and peptide analysis by RP-HPLC.

The protease assay kit contained the following; FTC-casein that consisted of 10mL of 0.6% FTC-casein in 50 mM Tris-HCL (pH 7.3), the incubation buffer that was 10mL of 200 mM Tris-HCL (pH 7.8), 20 mM CaCl<sub>2</sub>, 0.1% NaN<sub>3</sub>, the assay buffer which was 120mL of 500 mM Tris-HCL (pH 8.8), 0.1% NaN<sub>3</sub> and the protease positive control that consisted of 1mL of 1 mg/mL trypsin in phosphate-buffered saline (PBS) with 10 mg/mL bovine serum albumin (BSA).

The principle for this protease assay is based on the hydrolysis of the FTC-casein in the milk sample when incubated at 37°C for 24 hours. The protease activity in the sample cleaves FTC-casein into smaller, TCA soluble, FTC-peptides to release the yellow chromophore. TCA (5%) was added to the reaction mixture in order to precipitate any remaining FTC-casein and to stop enzymatic reactions. The chromophore was measured spectrophotometrically at 492nm. The intensity of colour produced is directly proportional to the total protease activity in the sample (Twining, 1984; Wiesner & Troll, 1982).

Samples were prepared according to the assay protocol (Calbiochem user protocol, 2007) and incubated in a water bath at 37°C for 24 hours. The purpose of this assay was to determine the activity of the various enzymes mentioned above and samples such as the reagent blank and protease-positive control was prepared. Finally, there were 16 samples in total (Refer to Table 3.2 on page 78), of which the first sample was the reagent blank, the second sample was the protease-positive control, four samples for commercial *Bacillus* protease (2 samples for 0.0055 U and 2 samples for 0.055 U), three samples for self-produced *Bacillus* protease, the three samples for self-produced *Pseudomonas* protease and lastly two samples for the Alizarol test and two samples for RP-HPLC analysis.

**Table 3.2. Description of samples used for the protease assay.**

<b>Sample</b>	<b>Full Cream UHT Milk</b>	<b>FTC-casein*</b>	<b>Incubation buffer*</b>	<b>DH<sub>2</sub>O</b>	<b>Enzyme sample</b>	<b>Protease positive control mixture</b>	<b><i>Bacillus</i> protease 0.0055 U</b>	<b><i>Bacillus</i> protease 0.055 U</b>
<b>Reagent blank (x2)</b>	0	25µL	25µL	50µL	0	0	0	0
<b>Protease positive control (x2)</b>	0	0	0	90µL	0	10µL	0	0
<b>Commercial <i>Bacillus</i> protease samples (x2)</b>	0	25µL	25µL	0	0	0	50µL	0
<b>Commercial <i>Bacillus</i> protease samples (x2)</b>	0	25µL	25µL	0	0	0	0	50µL
<b>Self-produced <i>Bacillus</i> protease samples (x3)</b>	0	25µL	25µL	0	50µL	0	0	0
<b>Self-produced <i>Pseudomonas</i> protease samples (x3)</b>	0	25µL	25µL	0	50µL	0	0	0
<b>Alizarol test sample for lower activity <i>Bacillus</i> (x1)</b>	900µL	0	0	95µL	0	0	5µL	0
<b>Alizarol test sample for higher activity <i>Bacillus</i> (x1)</b>	900µL	0	0	95µL	0	0	0	5µL
<b>RP-HPLC sample for lower activity <i>Bacillus</i> (x1)</b>	900µL	0	0	95µL	0	0	5µL	0
<b>RP-HPLC sample for higher activity <i>Bacillus</i> (x1)</b>	900µL	0	0	95µL	0	0	0	5µL

\*Part of the protease assay kit

The 16 samples were then incubated in a water bath at 37°C for 24 hours, after which the reaction was stopped by addition of 250µL 5% TCA and mixed with a vortex mixer for 10 seconds. This was followed by incubation at 37°C in a water bath for another 10 minutes. Samples were then centrifuged (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan) at 10 000 rpm (standard rotor head) for 20 minutes in order to obtain a clear supernatant. Two hundred µL of each was transferred into separate reaction tubes. The assay buffer (300µL) was added and carefully mixed. The absorbance was read at 492 nm in a Cecil (CE 2021, 2000 series) spectrophotometer (supplied by Lasec SA) against the reagent blank sample in order to determine the total protease activity. No calculations were needed since the protocol states that the value obtained with the spectrophotometer is the activity value for the sample (activity in units/mL).

The four samples prepared for the RP-HPLC analysis and the Alizarol test were handled separately after incubation for 24 hours. The same experimental procedures were followed as described in Sections 3.3.2 and 3.3.5. The results of these three chemical tests were compared with one another.

### **3.3.4 Milk agar plate technique for protease detection**

In this study, an attempt was made to use milk agar plates for the rapid detection of protease activity.

A total volume of 500mL of milk agar (bacteriological) solution (2x 250mL Erlenmeyer flasks) was prepared containing 250mL low fat UHT milk and 250mL contained 1% agar (5g agar dissolved in 250mL DH<sub>2</sub>O). The first flask contained the UHT milk and was heated to 55°C in a water bath. The second flask, containing the agar, was heated in a boiling water bath for 15 minutes in order to dissolve the agar, after which it was placed in a water bath at 55°C for 30 minutes to cool. Thereafter the first flask containing the UHT milk was mixed with the agar solution in a 1L Schott bottle and subsequently sterilized by autoclaving at 121°C for 20 minutes. After sterilization the milk-agar solution was placed in a water bath at 55°C for approximately 20 minutes in order to cool down. The sterile milk-agar solution ( $\pm 10$ mL) was then aseptically poured into petri dishes. After cooling and setting for  $\pm 15$  minutes, the petri dishes were stored upside down on the bench top at room temperature for 24 hours to dry and point out any contamination. The petri dishes were then wrapped in plastic and stored in a refrigerator at 4°C until further usage.

The total volume of each enzyme added on top of milk agar plates was 10 $\mu$ L containing plasmin (0.05 U), commercial *Bacillus* protease (0.0055 U), self-produced *Bacillus* protease (0.00085 U) and self-produced *Pseudomonas* protease (0.00097 U) activity respectively.

For the commercial proteolytic enzymes (Plasmin and *Bacillus* protease) as well as self-produced enzymes (*Pseudomonas* and *Bacillus* protease), 10 $\mu$ L of each enzyme were pipetted on top of milk agar in the petri dishes. The plates were then incubated upside down at 32°C for 24 hours and observed for possible halo formation.

### **3.3.5 Proteolytic peptide analysis by Reverse-phase High Performance Liquid Chromatography**

#### **3.3.5.1 Preparation of milk peptide hydrolysates**

Raw milk was treated with proteolytic enzymes, plasmin, commercially obtained and self-produced proteases from *Bacillus licheniformis* and self-produced proteases from *Pseudomonas fluorescens* and the resulting peptides analysed with RP-HPLC in order to establish peptide profiles for each enzyme.

All incubations were performed at 37°C in a water bath. The various samples were prepared by the addition of 900µL raw milk in each Eppendorf tube and subsequently placed into the water bath until 37°C was reached (approximately 10 minutes). The different enzyme preparations were added to the raw milk in the Eppendorf tubes. The reaction volume of all the samples consisted of 900µL of raw milk, different volumes of enzyme and the final reaction volume made to 1000µL with DH<sub>2</sub>O except for the two cocktail samples which was slightly more than 1000µL as indicated in Table 3.3. The first cocktail sample had plasmin, self-produced *Bacillus* protease and self-produced *Pseudomonas* protease whereas the second cocktail sample was the same with the exception of plasmin. There were 23 samples in total (Refer to Table 3.3 below). All the samples were incubated for 6 hours (Crudden *et al.*, 2005), after which they were transferred to a boiling water bath (90°C) for 10 minutes as described by Denis *et al.* (2001) in order to stop all enzymatic actions.

**Table 3.3. Description of samples analysed by RP-HPLC.**

<b>Sample</b>	<b>Raw milk</b>	<b>Plasmin</b>	<b>Commercial <i>Bacillus</i> protease</b>	<b>Self-produced <i>Bacillus</i> protease</b>	<b>Self-produced <i>Pseudomonas</i> protease</b>	<b>DH<sub>2</sub>O</b>
<b>Control (x1)</b>	900µL	0	0	0	0	100µL
<b>Plasmin samples (x5)</b>	900µL	10µL	0	0	0	90µL
<b>Commercial <i>Bacillus</i> protease samples (x5)</b>	900µL	0	5µL of 10x dilution	0	0	95µL
<b>Self-produced <i>Bacillus</i> protease samples (x5)</b>	900µL	0	0	100µL	0	0
<b>Self-produced <i>Pseudomonas</i> protease samples (x5)</b>	900µL	0	0	0	100µL	0
<b>Cocktail sample 1 (x1)</b>	900µL	10µL	0	100µL	100µL	0
<b>Cocktail sample 2 (x1)</b>	900µL	0	0	100µL	100µL	0

### **3.3.5.2 Reverse-phase High Performance Liquid Chromatography analysis of peptide profiles**

TCA (12%) or HCL (0.1 N) (250 $\mu$ L) was added to the samples followed by vortex mixing in order to precipitate all the proteins, or the caseins, respectively. The samples were then centrifuged (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan) in an Eppendorf centrifuge tube for 15 minutes at 10 000 rpm (standard rotor head). A clear supernatant was obtained (no filtration was performed) and 150 $\mu$ L of the clear supernatant was collected and placed into vials for RP-HPLC analysis. A stock solution of 0.25g/100mL vanillyl-alcohol in DH<sub>2</sub>O was prepared and a 100x dilution was used. Vanillyl-alcohol (50 $\mu$ L of the 100x dilution) was added as reference of retention time to the supernatant of all of the samples.

The elution solvent used was acetonitrile (CH<sub>3</sub>CN) and the acetonitrile concentration gradient ranged between 0 and 65% over a time interval of 90 minutes. The elution speed was set at 1 mL/min at 200 bar. Samples were injected by an automatic injector (capable of injecting 20 $\mu$ L). A 150mm C 18 column with internal diameter of 4.6mm was used. The temperature of the column was maintained at 40°C. UV detection was at a wavelength of 214nm and the data was integrated using data-reprocessing software capable of measuring peak areas (International Standard, 2005).

### **3.3.6 Computer assisted identification of the proteolytic peptide profiles using MILQC software**

The neural network software is the best option to process the data imported from RP-HPLC proteolytic peptide profiles. The neural network software forms the backbone in the programming of the MILQC software. Neural network is a form of a multiprocessor computer system that works on the same basis as human information processing regarding memory/processing. This type of network consists of simple processing elements as well as an adaptive interaction between the elements. Neural network is also helpful when identification of conserved data and behaviour patterns are required. This network also has the ability to form a model from the training data which is useful when working with data from a complex process where there may be an algorithm which is not known nor has too many variables. In this case, it is easier to let the network learn from certain examples (Smith, 1996).

The purpose of this study was to distinguish between the microbial proteases and plasmin by using the peptide profile fingerprints after they were processed by the MILQC software.

The RP-HPLC peptide profiles, obtained in Section 3.4.4, were integrated into the MILQC software using the Wolfram Mathematica 10 programme in order to obtain representative profiles for all the proteolytic enzymes evaluated. All the data from the graphs obtained were based on the data of 20 repetitions per peptide profile.

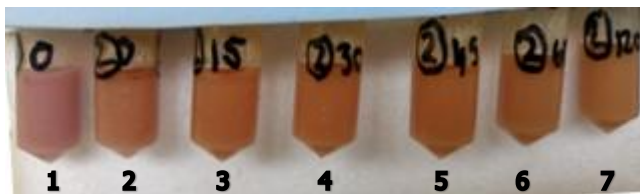
### 3.4 Results and discussions

The results obtained from the various techniques described in the methods section follow.

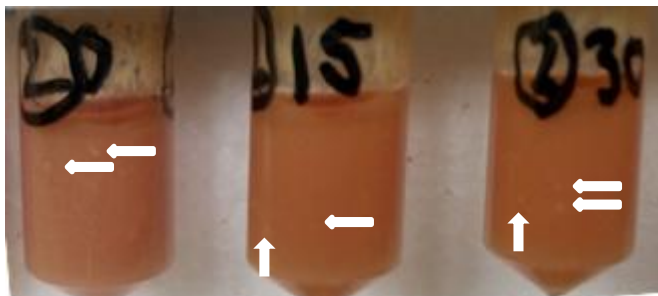
#### 3.4.1 Alizarol test

Milk was artificially flocculated using Neutrased protease in order to induce flocculation and in turn evaluate the effectiveness of the Alizarol test.

The results of the artificially flocculated milk with a proteolytic Neutrased enzyme, which was subsequently evaluated with the Alizarol test, are depicted in Figure 3.6. The numbers on all the samples refers to the amount of time that the milk samples were exposed in the water bath [from 0 minutes up until 120 minutes (indicated by tube numbers 1-7 on the bottom of the figure)]. The first sample on the left (Tube 1) served as the control (without Neutrased, thus no flocculation) whereas the rest of the samples (Tube 2-7) were treated with Neutrased enzyme. Tube 2 also served as the 0 time incubation for the Neutrased enzyme. The variation in colour from that of the sample without Neutrased enzyme (Tube 1 with violet colour) to that of the samples treated with Neutrased enzyme (Tube 2-7 with brown-yellow colour) is a further indication that the Alizarol test effectively detected milk flocculation. The visible flakes within the Alizarol positive samples are indicated with white arrows in Figure 3.7. Flocculation occurred instantly within the time 0 sample treated with Neutrased enzyme (Tube 2 in Figure 3.7).



**Figure 3.6. Alizarol test samples treated with Neutrased enzyme. The numbers on all the samples refers to the amount of time that the milk samples were exposed in the water bath [from 0 minutes up until 120 minutes (indicated by tube numbers 1-7 on the bottom of the figure)].**



**Figure 3.7. Alizarol positive samples with visible flakes as indicated by the white arrows.**

In this study the focus was only on the formation of flakes during enzymatic flocculation/destabilisation of milk and not on the change in pH, since the pH will not be affected by the enzyme alone. In the case of fresh milk, the growth of microbial organisms is linked to protease production, which in turn causes the flocculation, while simultaneously lead to a decrease in pH which is due to H<sup>+</sup> production by lactic acid.

### 3.4.2 Protease assay

The protease assay was performed on various levels of activity for commercial *Bacillus* protease as well as on the self-produced enzymes in order to evaluate the effectiveness of this assay in comparison to the Alizarol test and peptide analysis by RP-HPLC. No calculations were needed since the protocol states that the value obtained with the spectrophotometer is the activity value for the sample (activity in units/mL).

Table 3.4 shows the activity values for the samples (duplicate) relevant to the protease assay. The reason for using two activities of commercial *Bacillus* protease was that the sensitivity level was unknown for this enzyme. The results for the protease-positive control are at an acceptable level, being lower than 0.5 U/mL. The level of activity for the 0.0055 U *Bacillus* protease sample was 0.041 U/mL however the activity was 10x higher than expected for the 0.055 U *Bacillus* protease sample which was 0.461 U/mL. The delta absorption difference between the reagent blank sample and the 0.0055 U *Bacillus* protease sample was 0.009 U/mL whereas the delta absorption difference was much higher (0.429 U/mL) between the reagent blank sample and the 0.055 U *Bacillus* protease sample which is an indication of a higher level of activity for the 0.055 U *Bacillus* protease sample.

**Table 3.4. Absorption/activity levels for protease assay samples.**

Sample	Activity (U/mL)		
	1	2	Average activity (U/mL)
Reagent Blank	0.032	0.032	0.032
Protease positive control	0	0	0
Commercial <i>Bacillus</i> protease (0.0055 U)	0.041	0.042	0.041
Commercial <i>Bacillus</i> protease (0.055 U)	0.460	0.462	0.461

Table 3.5 below represents the activity values for both the self-produced enzymes that were obtained through the protease assay. The assay was done in triplicate. Numbers 1 to 3 indicates the value for each sample, whereas the column on the right represents the average value for all three samples. The activity for *Pseudomonas* protease was  $0.097 \pm 0.001$  U/mL whereas activity for *Bacillus* protease was  $0.085 \pm 0.001$  U/mL. The delta absorption difference between the reagent blank sample (Refer to Table 3.4) and the self-produced *Pseudomonas* protease was 0.065 U/mL whereas the delta absorption difference between the reagent blank sample and the self-produced *Bacillus* protease was 0.053 U/mL which is an indication of a higher level of activity for the self-produced *Pseudomonas* protease.

**Table 3.5. Activity of self-produced enzymes as determined by the protease assay.**

Self-produced enzyme	Activity (U/mL)			
	1	2	3	Average activity $\pm$ Standard deviation (U/mL)
<i>Pseudomonas fluorescens</i>	0.097	0.098	0.097	$0.097 \pm 0.001$
<i>Bacillus licheniformis</i>	0.084	0.085	0.085	$0.085 \pm 0.001$

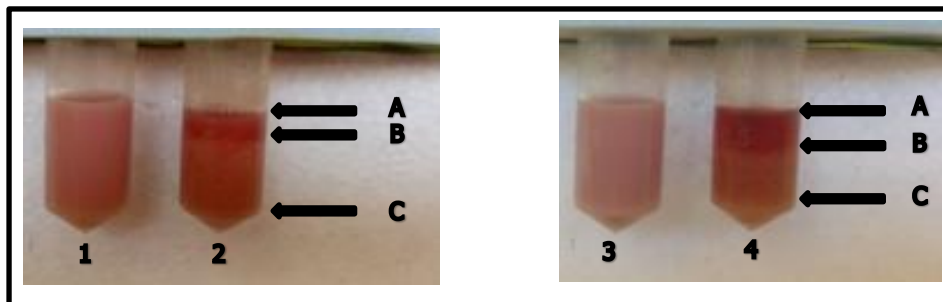
### 3.4.2.1 Alizarol test in comparison with the protease assay

The samples discussed in this section were incubated simultaneously with the samples of the protease assay.

Figure 3.8 show the results from the Alizarol test that was done in combination with the protease assay (Refer to Table 3.4).

In Figure 3.8, the tube on the left (Tube 1) is the control which was UHT milk without any addition of *Bacillus* protease, the tube on the right (Tube 2) was incubated with the protease assay samples and contained *Bacillus* protease (0.0055 U). In tube 2, the milk separated into a supernatant (fraction between arrows A and B) as well as a flaky precipitate between arrows B and C. Tube 3 is the control (UHT milk) and did not contain any *Bacillus* protease, whereas Tube 4 was incubated with the protease assay samples and contained *Bacillus* protease (0.055 U). In tube 4 the milk separated into a supernatant (fraction between arrows A and B) as well as a flaky precipitate between arrows B and C.

The results for the Alizarol samples were similar to that of the protease assay data in Table 3.4, which tested Alizarol positive. The only visible difference between the two enzyme concentrations was the higher level of syneresis that was observed after proteolysis visible with the enzyme with higher activity (Tube 4 which contained 0.055 U *Bacillus* protease).

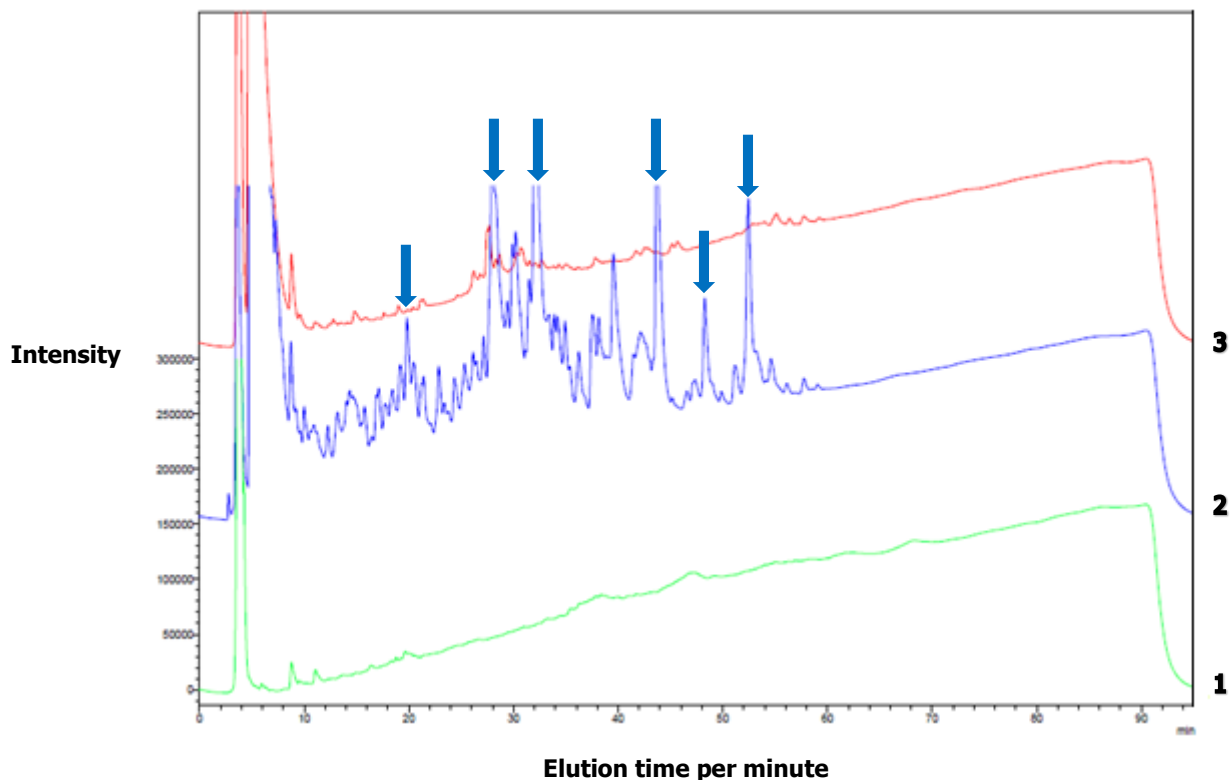


**Figure 3.8. Results from the Alizarol test in comparison with the protease assay for sample with 0.0055 U of *Bacillus* protease (Tube 2) and 0.055 U *Bacillus* protease (Tube 4). Tubes 1 and 3 are the control without enzyme. The level of syneresis (between A and B) and precipitation (between B and C) is indicated by the black arrows in Tubes 2 and 4.**

### 3.4.2.2 Reverse-phase High Performance Liquid Chromatography in comparison with the protease assay and the Alizarol test

The samples discussed in this section were incubated simultaneously with the samples of the protease assay.

In Figure 3.9 the peptide profiles for the low activity of *Bacillus* protease (0.0055 U), are indicated by profile 3, and the high activity of *Bacillus* protease (0.055 U), and are indicated by profile 2. The control is indicated by profile 1. These results are in correlation with both the protease assay as well as the Alizarol test, since it shows distinguishable differences between the various peptide profiles obtained from the two enzyme levels. Figure 3.9 shows that there were differences between the peptide peaks liberated by samples 2 and 3. It was evident that the higher the enzyme activity, the higher the peaks as indicated by the blue arrows (peptide profile 2).



**Figure 3.9. Peptide profiles for the low activity *Bacillus* protease (0.0055 U), as indicated by profile 3, and the high activity *Bacillus* protease (0.055 U), indicated by profile 2. The control is indicated by profile 1. The blue vertical arrows are indicative of prominent peaks for the higher enzyme load peptide profile.**

### 3.4.3 Milk agar plate technique for protease detection

In this study, an attempt was made to use milk agar plates for the rapid detection of protease activity.

After the incubation of the plates for 24 hours at 32°C, the following results were obtained. The commercial and self-produced milk agar plates are combined in Figure 3.10 and all four plates exhibited clear zones of hydrolysis of the milk casein. In the case of the plasmin, the halos had a sharp edge (indicated by PL), while the commercial *Bacillus* protease had a cloudy edge (indicated by B). This is important since the milk agar plate technique may be used to distinguish between native milk plasmin and microbial proteases. From literature, there is a difference between the affinities towards casein degradation between plasmin and microbial proteases (Datta & Deeth, 2001). The plasmin hydrolyses  $\beta$ -casein,  $\alpha$ <sub>1</sub>-casein and  $\alpha$ <sub>2</sub>-casein (Chen *et al.*, 2003; Gaucher *et al.*, 2008) whereas proteases from microbial origin generally attack  $\beta$ -casein and K-casein (Chen *et al.*, 2003). It is still unknown whether this is the reason for the difference in halo edges. This warrants further investigation.

The milk agar plates for both the self-produced *Pseudomonas* protease (indicated by SCP) and self-produced *Bacillus* protease (indicated by SCB) also indicate that halos had been formed. This indicated that the self-produced enzymes were active and gave the same hydrolysis results as the commercial enzymes. Since this is a cheaper source of specific bacterial protease, the self-produced enzymes were used for all the further work and also because no commercial enzyme from *Pseudomonas* with acceptable activity could be obtained.



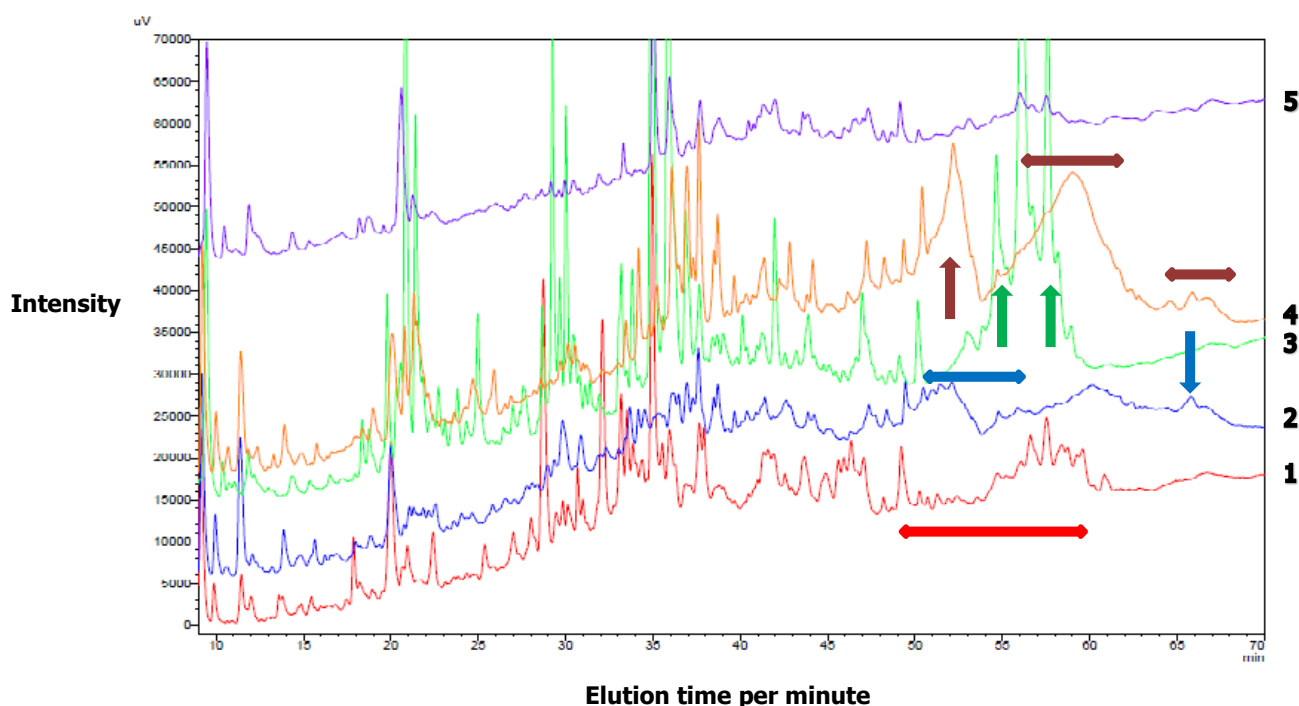
**Figure 3.10. Milk agar plates containing commercial Plasmin (indicated by PL), commercial *Bacillus* protease (indicated by B), the self-produced *Pseudomonas* protease is indicated by SCP and the self-produced *Bacillus* protease is indicated by SCB.**

To conclude, this plate technique is very simple, sensitive, rapid (results observed within 1 hour), cost effective and it can be performed in a very basic dairy laboratory or on the farm. Its sensitivity, however, needs to be optimized in terms of agar layer thickness, % agar and whether milk should be used directly or be diluted.

### 3.4.4 Proteolytic peptide analysis by Reverse-phase High Performance Liquid Chromatography

Various proteolytic enzymes were used in this section. Commercial proteolytic enzymes are easy accessible hence the usage of these enzymes. Cocktail samples (plasmin, *Bacillus* protease and *Pseudomonas* protease) were prepared in order to see if the characteristic peaks are evident for all the enzymes. The self-produced proteolytic enzymes were used since this was the sole source for *Pseudomonas* protease and also to depict comparisons between commercial *Bacillus* protease and self-produced *Bacillus* protease. Raw milk was treated with the various proteolytic enzymes and the resulting peptides analysed with RP-HPLC in order to establish peptide profiles for each enzyme.

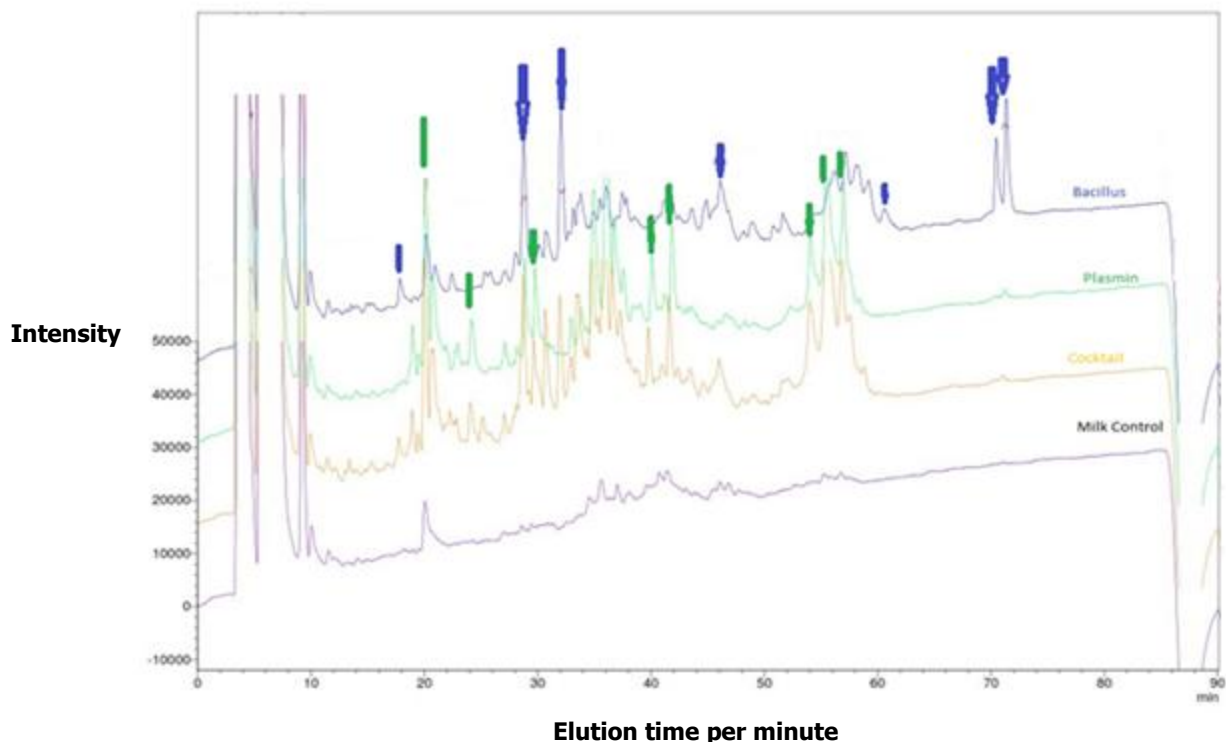
The data in Figure 3.11 shows the peptide profiles liberated by commercial plasmin and commercial *Bacillus* protease precipitated with TCA or HCl. The numbers **1** to **5** on the graph represent the various peptide profiles where **1** is *Bacillus* protease (0.0055 U) precipitated with 12% TCA, **2** is *Bacillus* protease (0.0055 U) precipitated with 0.1 N HCl, **3** is plasmin (0.05 U) precipitated with 12 % TCA, **4** is plasmin (0.05 U) precipitated with 0.1 N HCl. Number **5** is raw milk, which served as the control sample precipitated with 12% TCA.



**Figure 3.11. Chromatograms of milk hydrolysed by commercial plasmin and commercial *Bacillus* protease and precipitated by TCA or HCl. Number 1: *Bacillus* protease (0.0055 U) precipitated with 12% TCA, 2: *Bacillus* protease (0.0055 U) precipitated with 0.1 N HCl, 3: Plasmin (0.05 U) precipitated with 12% TCA, 4: Plasmin (0.05 U) precipitated with 0.1 N HCl. Number 5 is raw milk control precipitated with 12% TCA. The arrows indicate the various prominent peaks.**

These results confirm that there are clear differences between the peptides precipitated by TCA and HCl. For *Bacillus* protease, the distinct peaks for TCA precipitation (number 1) was at an elution time between 55 and 60 minutes (indicated by the red arrow) whereas HCl precipitation (number 2) resulted in distinct peaks at an elution time of 50-55 and at 65 minutes (indicated by the blue arrows). There was also a difference between TCA and HCl precipitation for plasmin since TCA (number 3) resulted in distinct peaks at an elution time between 55 and 60 minutes (indicated by the two green arrows), whereas the distinct peaks for HCl precipitation (number 4) was between an elution time of 50 and 55 minutes (indicated by the upwards maroon arrow), again between 55 and 60 minutes (indicated by a horizontal maroon arrow) and the last prominent peak between 65 and 70 minutes (indicated by a horizontal maroon arrow). Secondly, this data also depicted distinguishable peptide chromatograms (HCl precipitation) between plasmin (peptide profile number 4) and protease from *Bacillus* (peptide profile number 2) which was evident at an elution time between 50 and 55 minutes and again between 55 and 60 minutes.

The RP-HPLC chromatogram depicted in Figure 3.12 shows the enzymatic peptide profiles liberated by commercial plasmin, commercial *Bacillus* protease as well as the cocktail sample (self-produced enzymes) precipitated with 12% TCA. The activity volumes used for each enzyme were as follows; 0.0055 U of *Bacillus* protease, 0.05 U of plasmin and the cocktail sample (the first one which contained 0.0085 U of self-produced *Bacillus* protease, 0.0097 U of self-produced *Pseudomonas* protease and 0.05 U of plasmin). The highlighted markers on the various profiles show the characteristic peaks which differ among the various proteolytic enzymes. It is visibly clear that there are distinguishable differences between the different enzymatic peptide profiles for each enzyme.

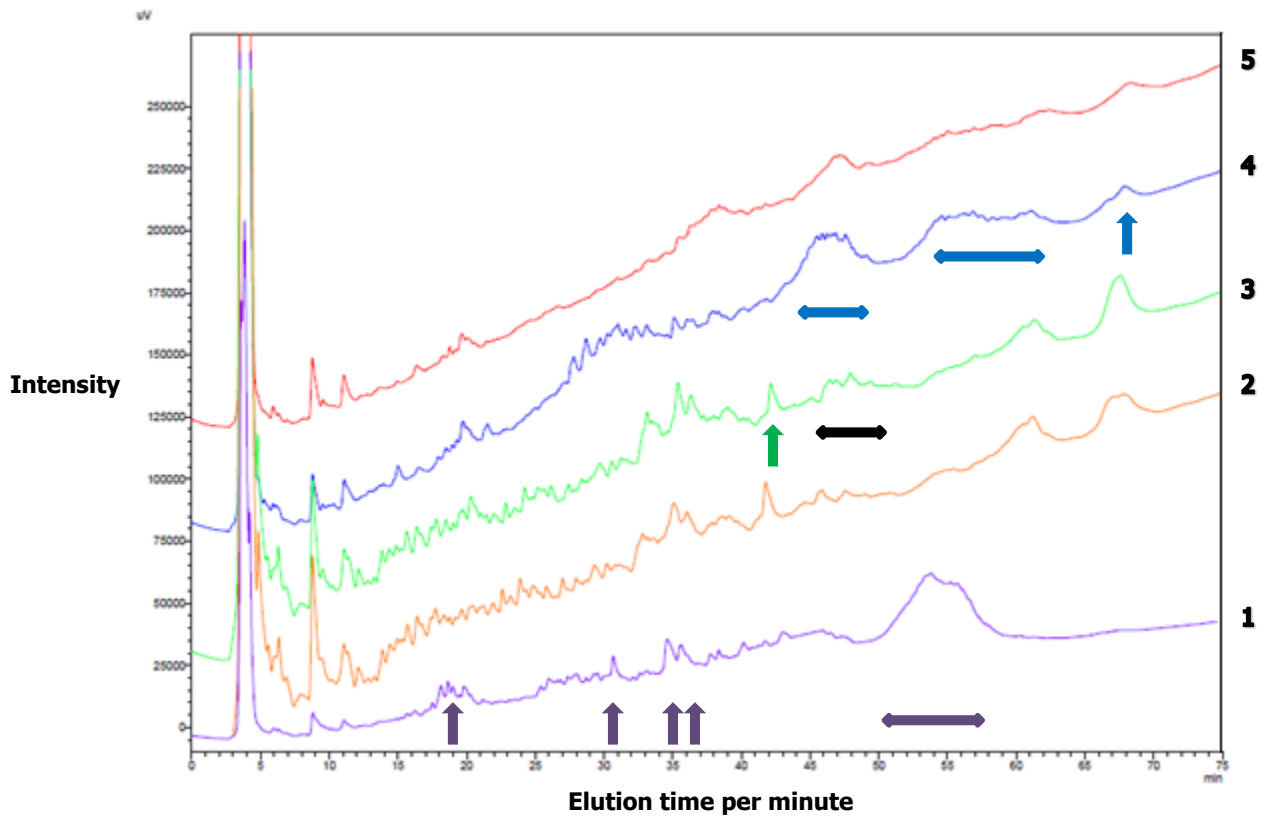


**Figure 3.12. Chromatograms of TCA precipitated peptides. The milk control is indicated by the purple line, orange indicates the cocktail sample, plasmin is indicated by green and commercial *Bacillus* protease is indicated by blue.**

From the peptide profile of milk treated by *Bacillus* protease (blue) the unique characteristic peaks were visible around the elution times of 20, 30, 32, 45, 60 and 70 minutes. In the case of the plasmin derived peptide profile (green) the characteristic peaks were at the elution times of 20, 25, 30, 40, 42 and between 50 and 58 minutes. The plasmin and *Bacillus* protease peptide profile overlapped to a great extent in the cocktail sample (orange peptide profile) where both these enzymes were used. For reasons unknown, the last peaks at 70 minutes for *Bacillus* protease derived peptides disappeared within this cocktail sample.

According to Datta & Deeth, 2001, it was recommended to use both TCA and HCl for peptide precipitation. The peptide profiles of both TCA and HCl precipitation gave distinguishable results, however, the latter gave more distinguishable chromatograms.

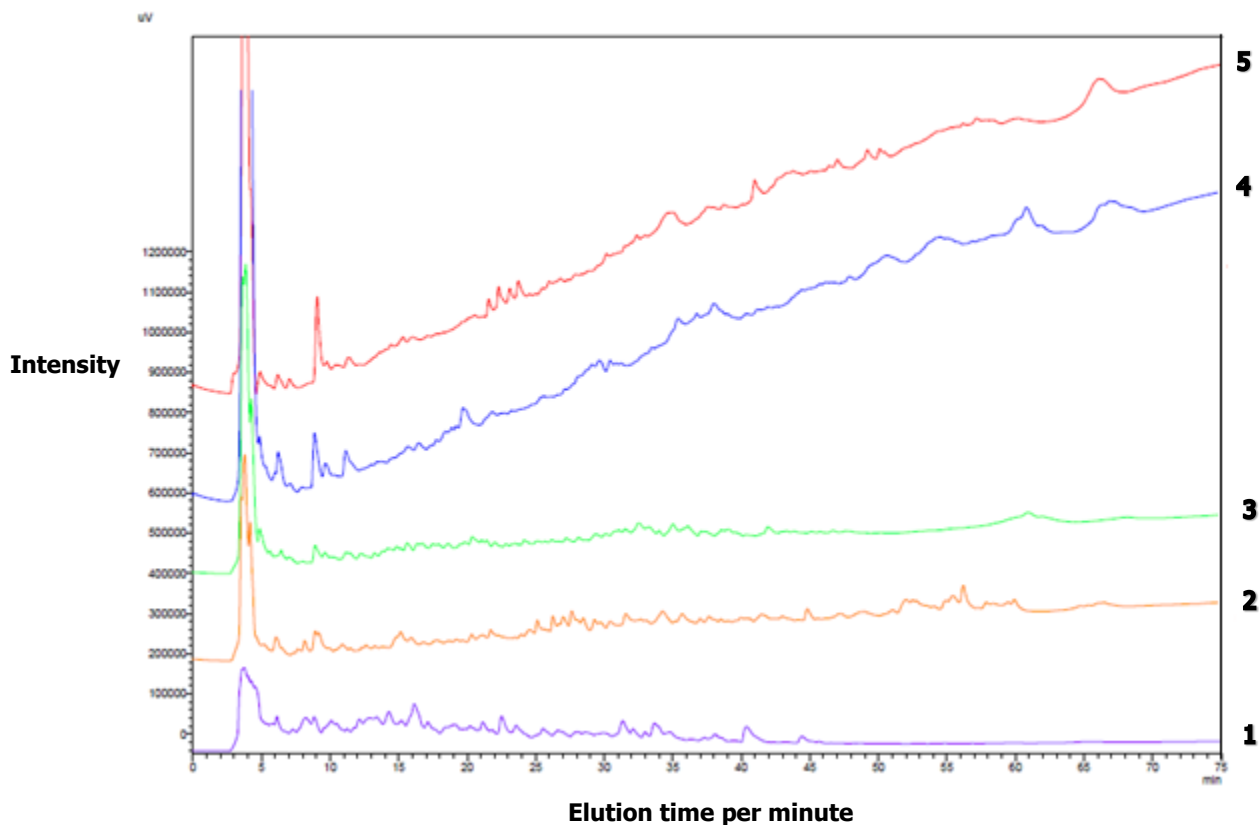
The enzymatic peptide profiles in Figure 3.13 are liberated by commercial plasmin, commercial *Bacillus* protease and the two cocktail samples precipitated with 0.1 N HCl. Numbers **1** to **5** on the graph represent the various peptide profiles where **1** is commercial plasmin (0.05 U), **2** is a cocktail sample that consisted of self-produced *Bacillus* protease and self-produced *Pseudomonas* protease, **3** is also a cocktail sample that consisted of 0.05 U commercial plasmin, 0.0085 U self-produced *Bacillus* protease and 0.0097 U self-produced *Pseudomonas* protease, **4** is commercial *Bacillus* protease (0.0055 U) and **5** is raw milk, which served as the control.



**Figure 3.13. Chromatograms of HCl precipitated peptides. Number 1: Commercial plasmin (0.05 U), 2: Cocktail sample that contained 0.0085 U of self-produced *Bacillus* protease and 0.0097 U of self-produced *Pseudomonas* protease, 3: Cocktail sample that contained 0.05 U of commercial plasmin, 0.0085 U of self-produced *Bacillus* protease and 0.0097 U of self-produced *Pseudomonas* protease, 4: Commercial *Bacillus* protease (0.0055 U) and 5: Raw milk control. The arrows are indicative of the various prominent peaks.**

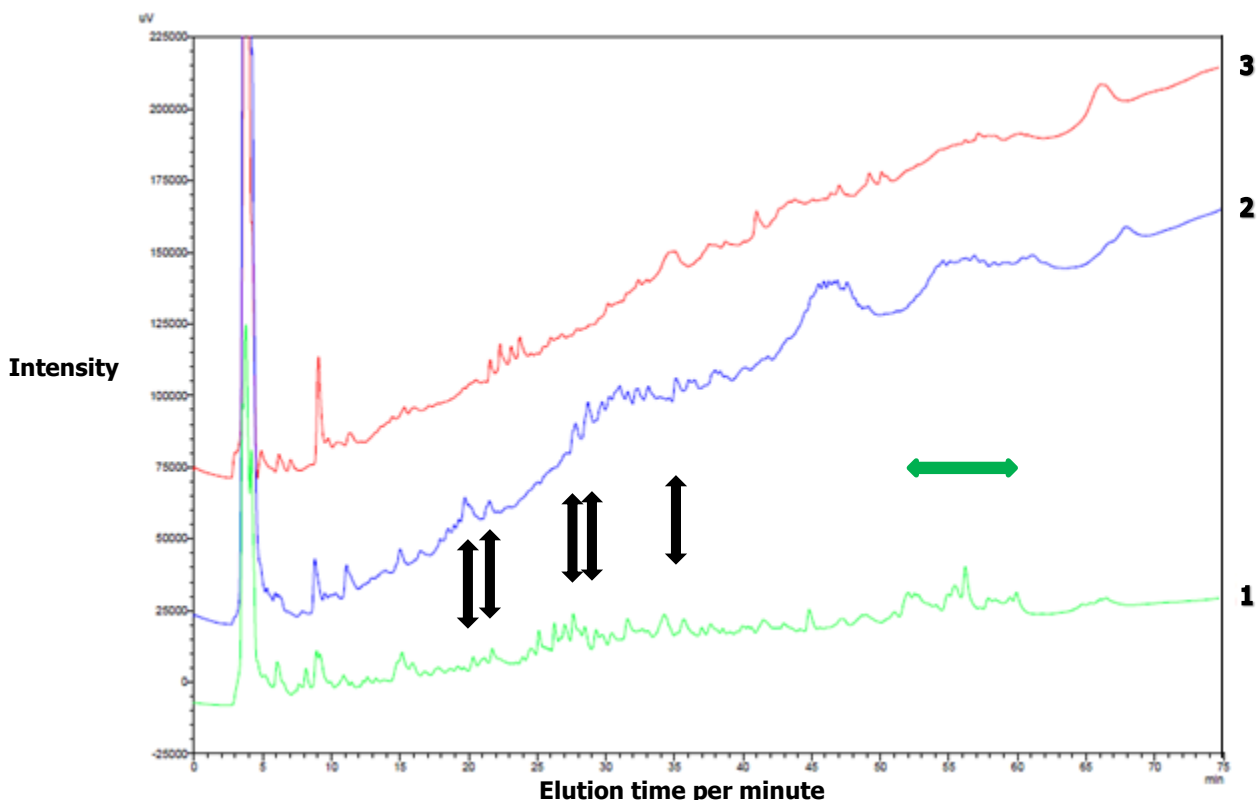
These results also depict distinguishable variations between plasmin and *Bacillus* protease. In the case for plasmin (number 1) the characteristic peaks are between the elution time of 20 and 50 minutes (indicated by purple arrows) whereas a prominent continuous peak was visible between 50 and 60 minutes (indicated by the horizontal purple arrow). For commercial *Bacillus* protease (number 4) the prominent peaks are between an elution time of 45 and 50 minutes (indicated by a horizontal blue arrow), again between 55 and 60 minutes (indicated by a horizontal blue arrow) and lastly at 70 minutes (indicated by an upwards blue arrow). The two peptide profiles for the cocktail samples (numbers 2 and 3) are more or less the same except for the characteristic peak for plasmin which is evident at an elution time of 40 minutes for the cocktail sample that contained plasmin (number 3) as indicated by the green arrow and the characteristic peak for *Bacillus* protease which is visible between an elution time of 45 and 50 minutes within both cocktail samples as indicated by the black arrow.

The data in Figure 3.14 shows the RP-HPLC chromatogram for enzymatic peptide profiles liberated by self-produced *Bacillus* protease and self-produced *Pseudomonas* protease precipitated with 0.1 N HCl. Due to the absence of peptide profiles for *Pseudomonas* protease it was essential to obtain peptide profiles for the self-produced enzymes. Numbers **1** to **5** on the graph represent the various peptide profiles where **1** is the self-produced protease of *Pseudomonas* after milk hydrolysis during incubation (Direct digestion), **2** is the self-produced protease of *Bacillus* after milk hydrolysis during incubation (Direct digestion), **3** is the self-produced protease of *Pseudomonas* harvested after incubation (Refer to Section 3.3.5.1 for details), **4** is the self-produced protease of *Bacillus* harvested after incubation (Refer to Section 3.3.5.1 for details) **5** is UHT milk, which served as the control. These results indicate clear differences between the two self-produced proteolytic enzymes (*Bacillus* and *Pseudomonas*). The peptide profiles for the self-produced enzymes (after milk hydrolysis during incubation (numbers 4 and 5) also differ significantly from that of the peptide profiles from the self-produced enzymes harvested after incubation (numbers 2 and 3) since the profiles of the latter shows far less peaks.



**Figure 3.14. Chromatograms of peptides liberated by the self-produced proteolytic enzymes. Number 1 is self-produced protease of *Pseudomonas* after milk hydrolysis during incubation (Direct digestion), 2: Self-produced protease of *Bacillus* after milk hydrolysis during incubation (Direct digestion), 3: Self-produced protease of *Pseudomonas* harvested after incubation, 4: Self-produced protease of *Bacillus* harvested after incubation and 5 is UHT milk, which served as the control.**

The peptide profiles in Figure 3.15 depicts the comparison between the profiles liberated by commercial *Bacillus* protease and self-produced *Bacillus* protease. Numbers **1** to **3** on the graph represent the various peptide profiles where **1** is the peptide profile liberated by the self-produced protease of *Bacillus*, **2** is the peptide profile liberated by the commercial *Bacillus* protease, **3** is the peptide profile liberated by UHT milk, which served as the control. The black vertical arrows (peaks) (between an elution time of 20 and 25 minutes, 25 and 30 minutes and at 35 minutes) and the green horizontal arrow (area), between an elution time of 50 and 60 minutes, are indicative of similar characteristic marker peaks for both commercial and self-produced *Bacillus* protease (numbers 1 and 2). Unfortunately in order for comparison purposes, the peptide profile for self-produced *Bacillus* protease was flattened down, therefore the peaks are not that pronounced as in the original peptide profile in Figure 3.14. Thus in conclusion, it is evident that the peptide profiles of both the commercial and self-produced *Bacillus* protease are similar, therefore we can safely continue using the commercial or self-produced *Bacillus* protease. Unfortunately, due to the unavailability of a commercial *Pseudomonas* protease, the comparison between commercial and self-produced *Pseudomonas* protease peptide profiles is not possible.



**Figure 3.15. Chromatograms of peptides liberated by commercial *Bacillus* protease and self-produced *Bacillus* protease. Peaks of differences are indicated by the black and green arrows. Number 1 is the peptide profile liberated by self-produced protease of *Bacillus*, 2: Peptide profile liberated by the commercial *Bacillus* protease, 3: Peptide profile liberated by UHT milk as the control.**

### **3.4.5 Computer assisted identification of the proteolytic peptide profiles using MILQC software**

The computer assisted MILQC software was used to identify the differences in peaks within the chromatograms from RP-HPLC as they are not always visible with the naked eye and small differences may occur between different preparations. Chromatograms of 20 repeats of a specific experiment were drawn into the programme. From these, which may contain a difference in some peak sizes, or presence/absence of some peaks, an average chromatogram was constructed for the specific experiment. This was repeated for all the different protease assays described above, such as proteolysis by plasmin, commercial *Bacillus* protease or both the self-produced enzymes. The MILQC-created chromatograms are therefore representative of conserved peptide peaks liberated by each enzyme, which makes it possible to effectively distinguish between the various peptide profiles without the non-sense peaks.

The data in Figure 3.16 shows the peptide profiles for the self-produced proteolytic enzymes that were derived from the RP-HPLC chromatograms and integrated into the MILQC software. The green circle on the graph indicates the area where *Pseudomonas* protease-produced peptides showed distinguishable differences from that of *Bacillus* protease-produced peptides which are indicated by the red circle. Figure 3.16 shows that the peptides originating from self-produced *Pseudomonas* protease differ significantly from those of the self-produced *Bacillus* protease. This MILQC software thus makes it possible to distinguish between the peptides generated during milk hydrolysis by the two different microbial proteases.

Intensity

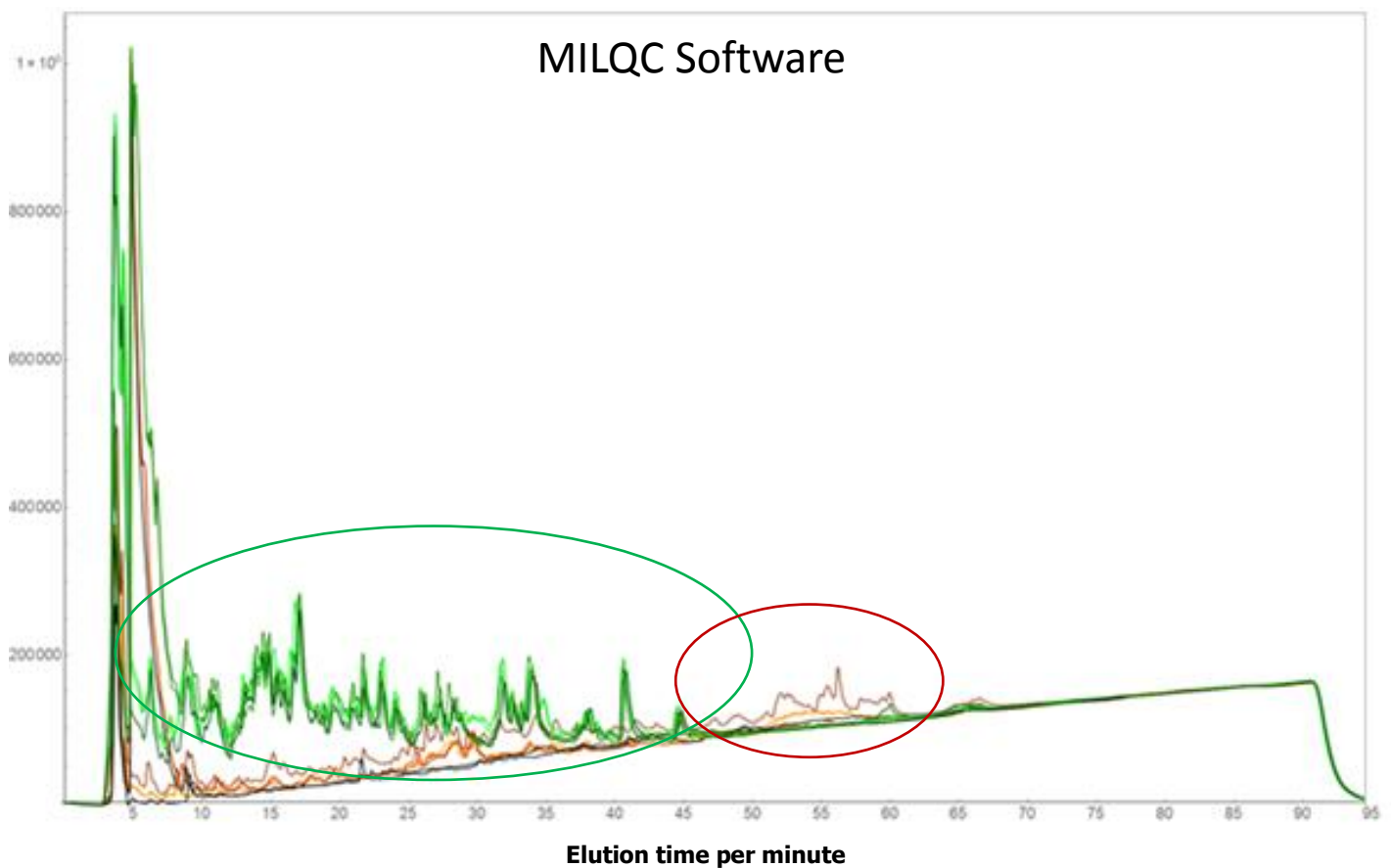
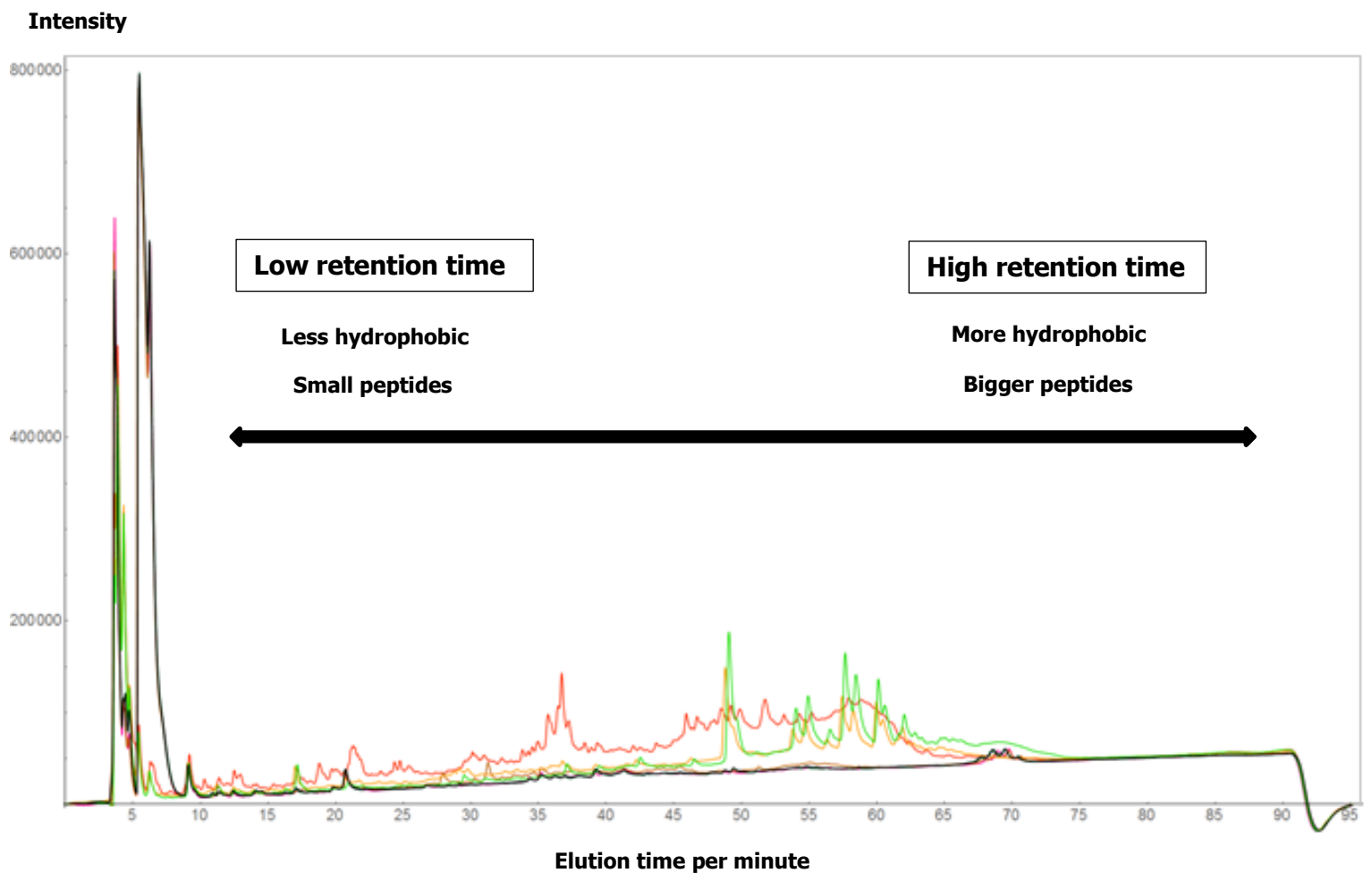


Figure 3.16. MILQC software created chromatograms of peptides liberated by *Pseudomonas* protease versus *Bacillus* protease. The green circle is representative of a conserved area for *Pseudomonas* protease produced peptides whereas the distinct area for *Bacillus* protease is indicated by the red circle.

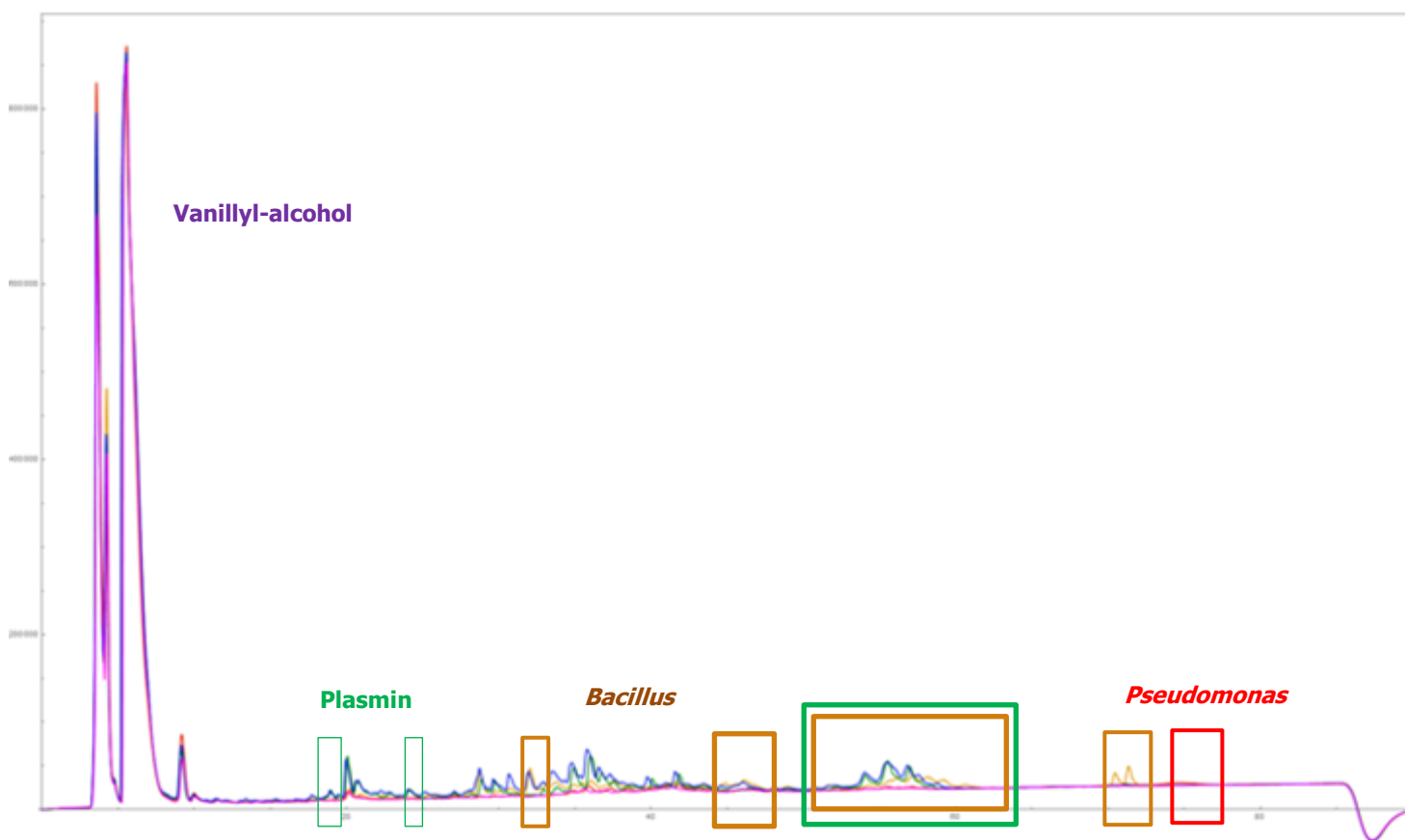
Figure 3.17 shows that the peptide profile liberated by plasmin (red) has a larger and continuous peptide between at an elution time of 45 and 60 minutes, whereas the peptide profiles from microbial proteases (green and orange) shows slimmer and sharper peptides between an elution time of 50 and 60 minutes. As indicated on the diagram, the high retention time results in larger peptides which are more hydrophobic. These results are in correlation with literature which states that plasmin produces larger and more hydrophobic peptides in comparison to enzymes produced by psychrotrophic bacteria (Le *et al.*, 2006). It can therefore be concluded that this MILQC software can accurately distinguish between milk being hydrolysed by plasmin and microbial protease.



**Figure 3.17. MILQC software created chromatograms of peptides liberated by plasmin (red) in comparison with microbial proteases (green and orange peptide profiles).**

The coloured blocks on the diagram (Figure 3.18) highlights the distinct areas of peptides liberated by the three different enzymes. Green represents plasmin-derived peptide profiles, orange by *Bacillus* protease whereas red by *Pseudomonas* protease. From this graph it is evident that, after the MILQC processing, there are distinct conserved areas which display unique peptide profiles for each proteolytic enzyme.

The previous graph (Figure 3.17) reflects results in accordance with literature (Le *et al.*, 2006), however in Figure 3.18 it is evident that peaks are formed which are more hydrophobic and larger in size than plasmin and this can be attributed to the fact that in literature the researchers used TCA during the precipitation step whereas in this study HCL was used. HCL was selected because the resulting peptides led to a better distinction between the different enzymes, and also to simplify the procedure.



**Figure 3.18.** MILQC software created chromatograms of peptides liberated by Plasmin, *Bacillus* protease and *Pseudomonas* protease. The green blocks represent distinct conserved areas for plasmin, orange for *Bacillus* protease and red for *Pseudomonas* protease.

### **3.5 Concluding discussion**

All the milk flocculation detection techniques in Chapter 3 have the ability to effectively detect flocculation within milk which was the Alizarol test, the protease assay, milk agar plate technique, RP-HPLC and the MILQC software.

The Alizarol test detects milk flocculation effectively, however results are based on interpretation by the human eye. The mere fact that the time 0 which was treated with Neutrase enzyme flocculated with the addition of Alizarol solution raised some concern over the credibility of the Alizarol test.

The protease assay kit successfully determines the level of protease activity in U/mL, however this test is very expensive (R7000 for 100 samples) and takes a long time, 24 hours, to complete.

The milk agar plate technique is very cost effective, simple and clear halos may be observed within 1 hour. This technique is effective to prove the presence of proteolytic enzyme activity and can distinguish between plasmin (sharp edge of halo) and microbial protease (cloudy edge of halo).

RP-HPLC is useful to detect differences in peptides liberated by plasmin and microbial proteases such as from *Bacillus* or *Pseudomonas*. Computer assistance with MILQC software simplifies the interpretation of the chromatograms. This technique can therefore not only explain the cause of milk flocculation and ascribe it to proteolytic activity, but also detect the culprit source of enzyme.

Chapter 4 that follows focuses on the application of the various milk flocculation detection techniques in Chapter 3 in the form of a real life scenario in order to investigate whether the various techniques can detect high risk milk with high proteolytic activity that is prone to flocculation.

### **3.6 References**

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## CHAPTER 4

### **Evaluation of proteolysis tests on milk**

#### ***Objectives***

Raw milk samples were collected from 10 commercial milk producers. The milk samples were incubated at 7°C until flocculation occurred. The milk samples were then evaluated with the protease assay, the Alizarol test and with RP-HPLC (MILQC software) in order to compare the results of the different techniques and to establish the most effective technique that can possibly differentiate between various proteolytic enzymes.

#### **4.1 Introduction**

In Chapter 3, the various analytic detection techniques for milk flocculation were described. In this Chapter, the purpose is to validate and to test the practicality of the application of proteolysis tests. An ideal situation would be to apply these tests by screening a large number of milk samples from commercial milk producers. The hygiene of milk producers is normally of high standard, and milk samples of partial decay due to the actions of microorganisms or enzymes are hard to come by. It was therefore decided to simulate a partial decay process in milk that may (or may not) have been contaminated by psychrotrophic organisms as obtained from commercial milk producers.

**Keywords:** Application, Simulation, Protease assay, Plates, RP-HPLC, MILQC

#### **4.2 Materials and methods**

##### **4.2.1 Materials**

###### **4.2.1.1 Milk**

The milk used was raw milk and was collected from 6 different farms of commercial milk producers for 6 successive weeks (36 samples in total) in the Bloemfontein area, Free State, South Africa.

The milk used for the preparation of the milk agar plates was Pick and Pay Choice Low Fat Long Life UHT milk and was obtained from Pick and Pay in Langenhovenpark, Bloemfontein, Free State, South Africa. Fat serves as a steric hindrance for proteases, hence low fat milk was chosen (Datta & Deeth 2001). The reason for using UHT milk was to consist of milk with a long shelf-life in order to have the same batch of milk available for various tests.

#### **4.2.1.2 Reagents**

The reagents used for the milk agar plates were the same as in Section 3.2.2 however with the addition of sodium azide (supplied by Merck, South Africa), to prevent microbial growth, to the milk agar solution (Refer to Section 3.3.4).

The same protease assay kit was used as in Section 3.2.2 for the performance of the protease assay.

The same reagents were used for RP-HPLC as described in Section 3.2.2, however with the addition of sodium azide to the HCl solution to inhibit microbial growth.

#### **4.2.2 Methods**

In order to simulate contamination with psychrotrophic organisms, the milk samples collected from the 6 different commercial milk producers were incubated at 7°C for 7 days, the optimum growth conditions of psychrotrophs. This was continued until flocculation occurred and the milk tested positive with the Alizarol test (72% Alizarol test). The milk samples therefore contained high psychrotrophic bacterial levels. Microbiological analysis confirmed that all the samples under study contained psychrotrophic organisms, of which at least *Bacillus* and *Pseudomonas* were present. This was part of a study which fell outside the borders of the current study and was performed by someone else, hence these data will not be shown here (Prof. Celia Hugo and co-workers, University of the Free State, 2016). The only observation to be made here was whether proteases were present and whether the source may be identified. The Alizarol positive samples were immediately frozen in order to prevent further microbial and enzymatic contamination. The milk samples were thawed as needed and evaluated for proteolytic activity using the milk agar plate technique, the protease assay, RP-HPLC and the MILQC software.

##### **4.2.2.1 Milk agar plate technique for protease detection**

The method for the milk agar plate technique is the same as described in Section 3.3.4, however 0.5g of sodium azide was added to the 500mL milk agar solution after sterilization in order to prevent microbial growth on the plates, either by the organisms that were cultivated in the milk or secondary contaminants.

All 36 milk samples which were incubated until Alizarol-positive were tested with the milk agar plate technique. The samples were precipitated using 0.1 N HCl containing the 0.05g sodium azide (to inhibit microbial growth) followed by gentle mixing with a vortex. Thereafter it was centrifuged (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan) at 10 000 rpm (standard rotor head) for 20 minutes in order to obtain a clear supernatant. The supernatant of each sample (10 $\mu$ L) was placed on top of the milk agar plates. The plates were then incubated upside down at 37°C for 24 hours and inspected for possible halo formation.

#### **4.2.2.2 Protease assay**

The protease assay was performed in duplicate on all 36 milk samples which tested Alizarol positive. Samples were prepared and incubated according to the assay protocol (Calbiochem user protocol, 2007) (Refer to Section 3.3.3 for the detailed description).

#### **4.2.2.3 Reverse-phase High Performance Liquid Chromatography analysis**

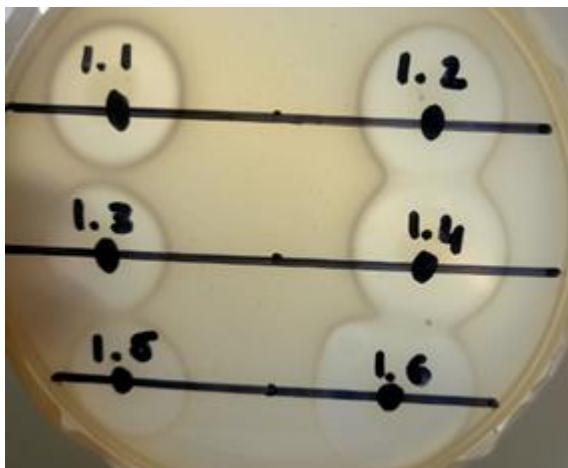
Of the 36 milk samples, two from each commercial milk producer, specifically those taken during the first and last week were subjected to chromatography. The samples were precipitated with 250 $\mu$ L of the 0.1 N HCl and sodium azide (0.05g) solution (to inhibit microbial growth) and gently mixed by vortex. It was subsequently centrifuged (Table Top Eppendorf Centrifuge, Harmonic series, model PLC-012, supplied by Gemmy Industrial Corporation, Taiwan) for 20 minutes at 10 000 rpm (standard rotor head) up until a clear supernatant was obtained. These supernatants were further analysed by RP-HPLC in order to establish proteolytic peptide profiles.

The MILQC software was used in order to possibly detect the culprit bacteria which is responsible for the production of proteases, within the various milk samples collected from the commercial milk producers, by importing the RP-HPLC chromatograms. Chromatograms from Chapter 3 (Section 3.4.4) for plasmin, *Bacillus* protease and *Pseudomonas* protease were used for comparison purposes.

## **4.3 Results and discussions**

### **4.3.1 Milk agar plates**

The data in Figure 4.19 shows the results (halos) obtained for the various milk samples that were incubated until Alizarol positive. The results and observations for all the 36 samples tested with the milk agar plate technique were similar for all the consecutive weeks and all the 6 different commercial milk producers, therefore only one plate is shown. Since the milk had already flocculated, the presence of proteolytic enzymes could be expected, but this was confirmed by the formation of clear halos. Clear halos were visible in less than 1 hour of incubation and no microbial growth was visible due to the presence of sodium azide. The method is therefore quick, which makes it beneficial to the dairy industry because it is possible to detect protease activity (plasmin as well as microbial protease) during milk delivery whilst the tanker is waiting at the milk depot.



**Figure 4.19. Milk agar plate that indicates clear halos for all the milk samples collected from all six commercial milk producers after one week and incubated until Alizarol positive. The numbers represent the following: 1.1 where 1 refer to week 1 and the second refer to producer 1. The numbering is the same for all the producers to follow.**

### 4.3.2 Protease assay

The results from the protease assay that was performed on all the milk samples collected from the 6 different commercial milk producers over a period of 6 weeks, are documented in Table 4.6. The results obtained from this protease assay prove that proteases were present in all the samples which were confirmed by the milk agar plates and also by RP-HPLC (Refer to Section 4.3.3 below).

The protease activity values for the milk samples collected from all the 6 different commercial milk producers over the 6 week period are also depicted in Figure 4.20. The purpose of this study was to simulate a decay process within the various milk samples collected from the commercial milk producers, therefore the conditions were extraordinary since these values were obtained after the various milk samples tested positive with the Alizarol test. Therefore, the protease activity values will be much higher than under normal circumstances and the activity values cannot act as a good indicator for the hygiene practices on the various farms of the commercial milk producers, however the protease assay successfully detected proteolytic activity values for all the 36 milk samples.

**Table 4.6. Results for the protease activities for the milk samples collected from all six commercial milk producers evaluated by the protease assay.**

Week	Protease assay activity values (U/mL)*					
	Producer 1	Producer 2	Producer 3	Producer 4	Producer 5	Producer 6
1	0.131	0.083	0.069	0.150	0.085	0.068
2	0.093	0.174	0.070	0.171	0.166	0.066
3	0.147	0.083	0.086	0.072	0.075	0.089
4	0.141	0.081	0.078	0.167	0.063	0.067
5	0.125	0.065	0.113	0.069	0.072	0.110
6	0.128	0.070	0.151	0.087	0.084	0.083

\*Average of duplicate determinations

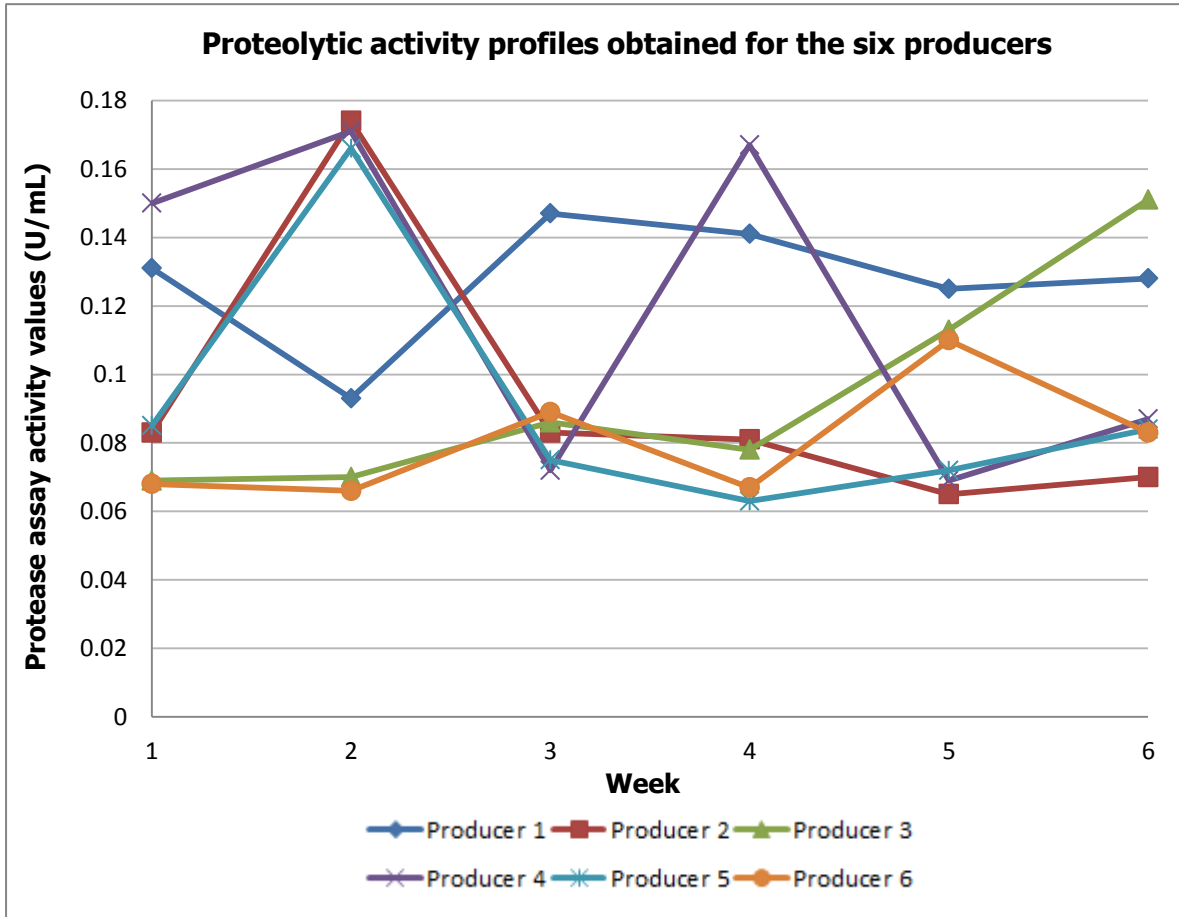
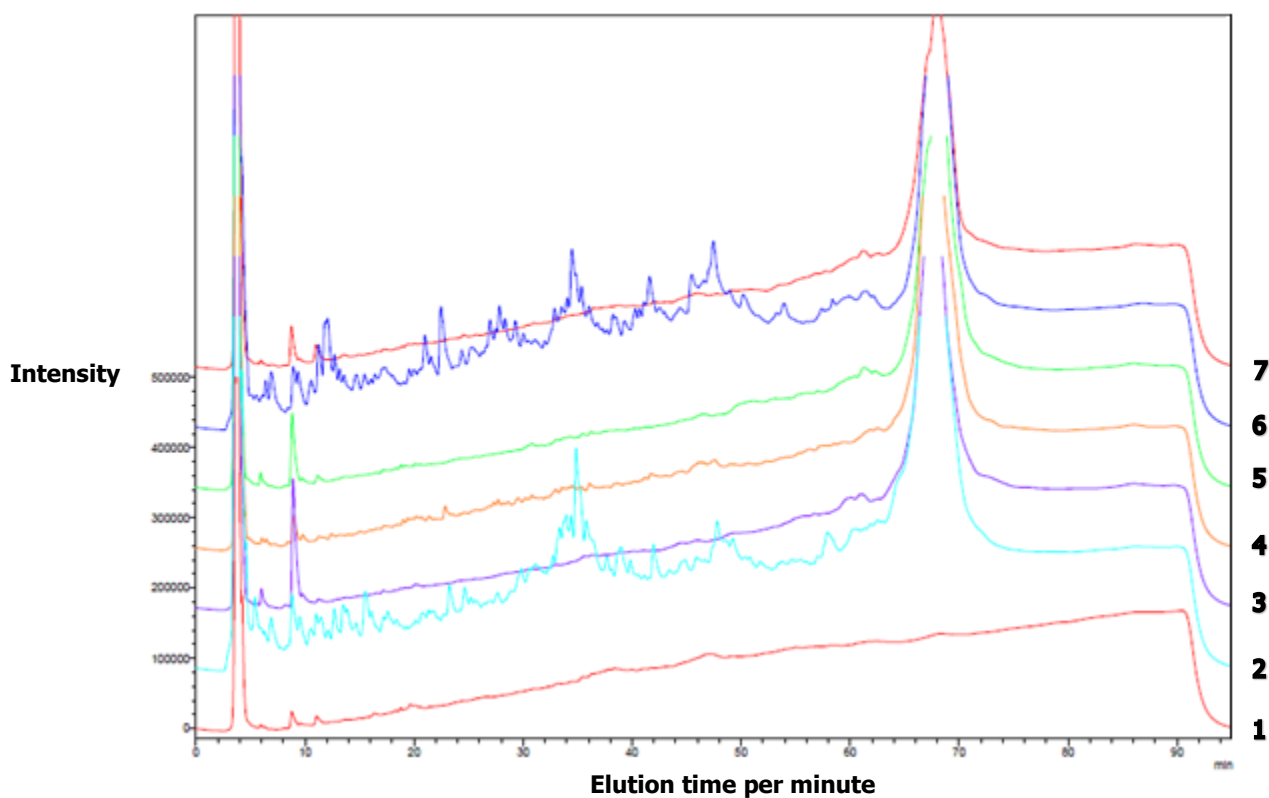


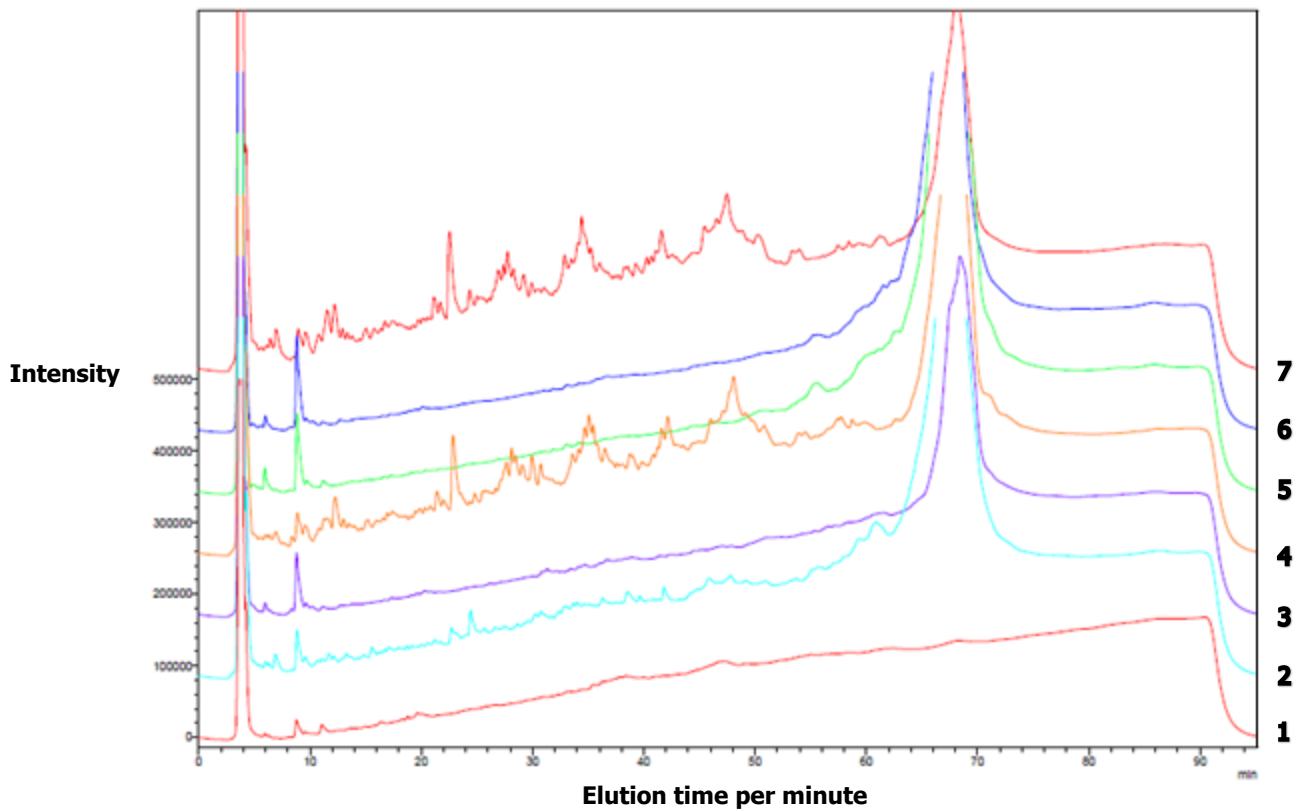
Figure 4.20. Proteolytic activity profiles obtained for the milk samples collected from all six commercial milk producers over a period of six weeks followed by an incubation period at 7°C until Alizarol positive.

### 4.3.3 Reverse-phase High Performance Liquid Chromatography

The RP-HPLC chromatograms for all the milk samples collected from the 6 commercial milk producers for the first and last week are depicted in Figures 4.21 and 4.22. The peptide profiles were further analysed using the MILQC software (Refer to Figure 4.23).



**Figure 4.21. Chromatograms of peptides liberated by the milk samples collected from commercial milk producers 1, 2 and 3. Number 1: Raw milk which served as the control, 2: Sample from producer 3 (week 6), 3: Sample from producer 3 (week 1), 4: Sample from producer 2 (week 6), 5: Sample from producer 2 (week 1), 6: Sample from producer 1 (week 6) and 7 is sample from producer 1 (week 1).**

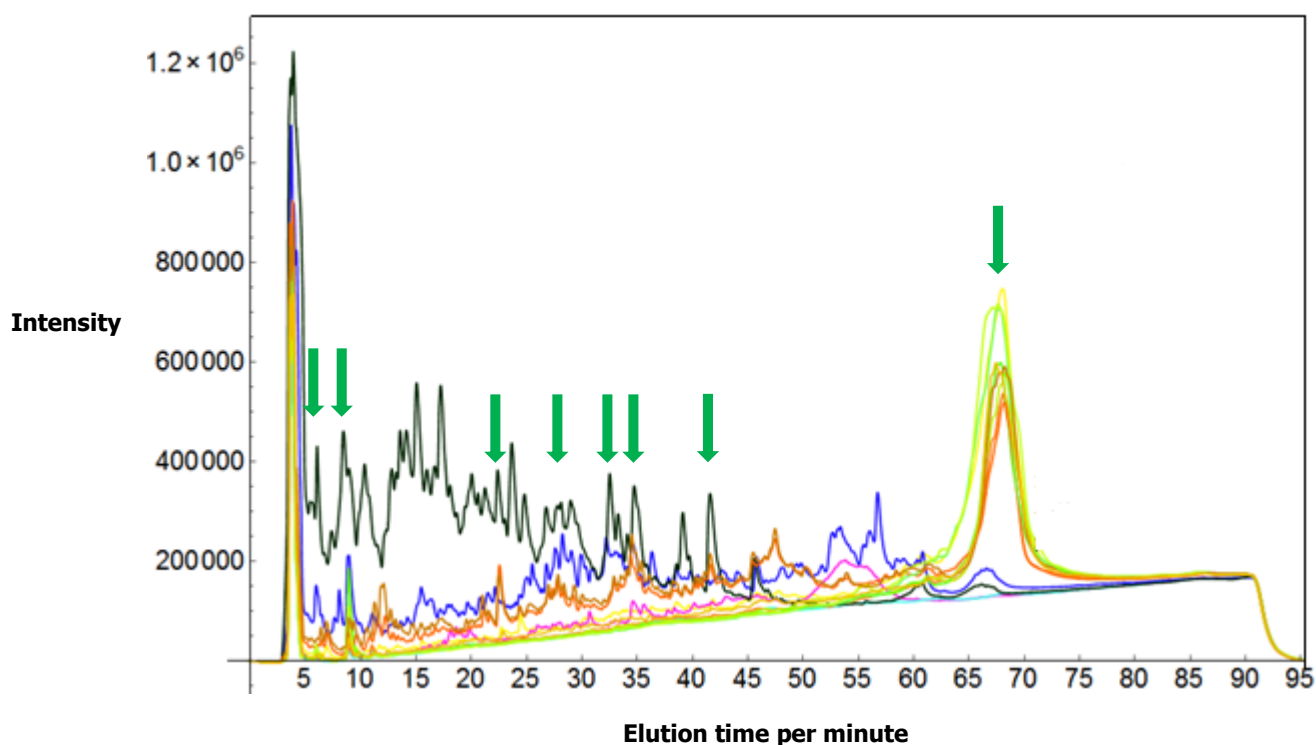


**Figure 4.22. Chromatograms of peptides liberated by the milk samples collected from commercial milk producers 4, 5 and 6. Number 1: Raw milk which served as the control, 2: Sample from producer 6 (week 6), 3: Sample from producer 6 (week 1), 4: Sample from producer 5 (week 6), 5: Sample from producer 5 (week 1), 6: Sample from producer 4 (week 6) and 7 is sample from producer 4 (week 1).**

#### 4.3.4 MILQC software

The MILQC software was used in order to detect the culprit bacteria responsible for production of proteases within the milk samples collected, from the various commercial milk producers, by importing the RP-HPLC chromatograms (Figures 4.21 and 4.22). Chromatograms from Chapter 3 (Section 3.4.4) for plasmin, *Bacillus* protease and *Pseudomonas* protease were used for comparison purposes.

The MILQC peptide profiles for the milk samples collected from all 6 commercial milk producers are depicted in Figure 4.23. The purpose of this was to clearly distinguish between the peptide profiles of the milk samples collected from the various commercial milk producers and the peptide profiles liberated by the proteolytic enzymes of *Pseudomonas*, *Bacillus* and plasmin. The colour scheme for the milk samples collected from the commercial milk producers is yellow, green and orange (due to overlapping the specific peptide profiles are not visible). Black represents the peptide profile liberated by *Pseudomonas* protease, blue is the peptide profile liberated by *Bacillus* protease whereas pink indicates the conserved peptide profile for plasmin. The green arrows in Figure 4.23 indicate distinct peaks for the peptide profile of *Pseudomonas* protease that are in correlation with the peaks of the peptide profiles of the milk samples collected from the various commercial milk producers.



**Figure 4.23. MILQC software created chromatograms of peptides liberated by the milk samples collected from all six commercial milk producers. The green arrows are indicative of distinct peaks that are in correlation with the peptide profiles for the milk samples of the various commercial milk producers and the peptide profile liberated by *Pseudomonas* protease.**

#### **4.4 Concluding discussion**

The milk in this study was deliberately flocculated until tested positive with the Alizarol test, the milk agar plates showed halos for all the samples and the protease assay detected activity. The RP-HPLC peptide profiles for the various milk samples collected from all the commercial milk producers are in correlation with the peptide profile liberated by the proteolytic action of *Pseudomonas* protease. The MILQC software was used to establish the *Pseudomonas* protease as the main culprit of flocculation between all the milk samples. From this study it was clear that all the established techniques developed and optimized for the detection of milk flocculation in Chapter 3 work effectively and each test by itself could also point out the risk of possible flocculation within the milk samples.

#### **4.5 References**

Calbiochem user protocol. (2007). Protease assay kit no. 539125. Retrieved from:  
[http://search.cosmobio.co.jp/cosmo\\_search\\_p/search\\_gate2/docs/CBC\\_/539125.20080523.pdf](http://search.cosmobio.co.jp/cosmo_search_p/search_gate2/docs/CBC_/539125.20080523.pdf).

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## CHAPTER 5

### **General conclusions**

Due to the fact that milk flocculation is such a major issue in the dairy industry, it is beneficial to have rapid, reliable and sensitive detection methods. All the chemical detection techniques described in Chapter 3 can effectively detect the presence of milk flocculation and some techniques can distinguish between plasmin and microbial proteases.

The Alizarol test is commonly used in dairy laboratories even though it is 75 years old. This test can be used to assess the risk of flocculation in milk. The conclusions of the test are questionable since interpretation relies on the visual interpretation through the human eye. The researchers were also surprised that milk flocculation (colour also changed from violet to yellow-brown) occurred instantly with addition of enzyme which is not ideal. The Alizarol test is currently used for the screening of milk which raises doubt regarding its effectiveness since milk flocculation is still a major problem worldwide.

The protease assay kit was very accurate and the results were highly repeatable. This assay can detect the enzyme activity values for a wide spectrum of proteolytic enzymes (enzymes such as plasmin, the protease produced by *Bacillus* and *Pseudomonas*). The operating skills needed to perform this assay are low, and the operator must only have basic skills on the reading of absorption values using a spectrophotometer. A major disadvantage is that this assay kit is very expensive (R70/sample) and the test is time consuming, since results take 24 hours to obtain.

The milk agar plate technique can be used to successfully detect proteolytic activity through halo formation. Reliable results were obtained even though conditions for this technique are not yet optimized. This technique can distinguish between the enzymatic action of plasmin and microbial proteases based on the difference in halo edges. Clear halos were visible in less than 1 hour. A drawback however was that the addition of sodium azide was essential to inhibit microbial growth when working with bacterial cultures cultivated in milk. The search for a safer preservative will form part of future research. This technique is simple, accurate and very cost effective (less than R20/petri dish). A milk producer on a farm will be able to perform this technique easily after proper training.

The RP-HPLC technique is highly sensitive and presented reliable results. This technique can identify milk with a high potential to flocculate and it has the capability to distinguish between the actions of the proteolytic enzyme peptide profiles for plasmin, *Bacillus* protease and *Pseudomonas* protease. Currently it is possible to detect the major culprits responsible for milk flocculation (plasmin, *Bacillus* protease and *Pseudomonas* protease) however in future, more microbial proteases need to be evaluated in order to obtain additional peptide profiles. Drawbacks of this technique are that the

HPLC operator requires high operating skills to perform a HPLC analysis and the running costs are extremely high (R200/sample), however it is acceptable when taken in consideration that more than one parameter is established within the peptide profiles. It was possible to obtain peptide profiles for the various proteolytic enzymes, however the interpretation of the data was confusing and therefore the need arose to process the data to a more understandable format which simplify the interpretation. For this purpose, the MILQC software was developed.

The MILQC software directly imports and process the data generated by the RP-HPLC. This MILQC software combines the data of many samples into an average peptide profile with conserved peaks which makes it possible to distinguish between plasmin and microbial proteases.

Finally, the focus of this research study was to evaluate the effectiveness of the various analytical detection techniques that was performed and documented in Chapter 4. Raw milk was collected from various commercial milk producers and incubated until Alizarol (72%) positive which indicates proteolytic activity (flake formation). All the analytical detection techniques evaluated were capable to detect proteolytic activity with ease which serves as an indication of milk flocculation. In future, all these techniques will be performed prior to UHT processing in order to flag high risk milk which result in age gelation.

It is important to note that the onset of flocculation occurs in the early stage of milk storage (fresh milk) whereas gelation (mainly in UHT milk) occurs during the final stage.

This research will contribute tremendously to the dairy industry since milk flocculation is such a major problem and it result in product losses, economic losses and consumers reject this type of milk. It will also contribute significantly to the overall quality of milk since milk that is not prone to flocculation has a better quality and consumers will have access to milk with a better quality (lower levels of proteolytic enzymes that cause age gelation).

## CHAPTER 6

### Summary

Milk flocculation/age gelation is regarded as a major problem for the dairy industry since it has a negative impact on milk quality. Flocculation can be observed as a physical change in fresh milk when milk is exposed to extreme destabilisation conditions such as low storage temperatures and heat exposure (milk added to boiling water during coffee preparation). The end result is decreased fluidity and increased viscosity due to the formation of a three-dimensional protein network and the formation of visible flakes. This type of milk is totally rejected by the consumers.

Flocculation can occur through chemical or enzymatic action. The chemical mechanism is when the three-dimensional protein network is formed during the storage of milk by the interaction between  $\beta$ -LG and K-casein within the casein micelle due to heat treatment which eventually results in the formation of a gel. During this interaction, a complex is formed between  $\beta$ -LG and K-casein. The enzymatic mechanism involves proteases which are responsible for the release of this  $\beta$ K-complex which forms a protein network and eventually results in the formation of a gel. The two main enzymes that play a role in milk flocculation are native plasmin and proteases from psychrotrophic bacteria.

Detection methods for milk flocculation are needed in order to establish the cause and possibly combat this problem. The already established milk flocculation detection techniques are the Alizarol test and the protease assay. The techniques developed in this study to detect milk flocculation/age gelation included RP-HPLC (MILQC software) and the milk agar plate technique for protease detection. All the techniques can effectively detect high risk milk prone to flocculation/gelation and some of the techniques can even distinguish between the proteolytic action of indigenous plasmin and microbial proteases.

## HOOFSTUK 6

### Opsomming

Melk flokkulasie/ouderdomsgelering is huidiglik 'n groot probleem vir die suiwel industrie aangesien dit 'n negatiewe impak het op die kwaliteit van melk. Flokkulasie is die fisiese verandering wat plaasvind in melk as gevolg van blootstelling aan ekstreme destabilisasie toestande soos lae opbergings temperature en blootstelling aan hitte (melk wat by kookwater gevoeg word tydens bereiding van koffie). Die eind resultaat is die afname in vloeistof en terselfde tyd 'n toename in viskositeit wat gevolglik lei tot die vorming van 'n drie-dimensionele proteïen netwerk. Dit lei daartoe dat melk flokkies bevat wat verbruikers onaanvaarbaar vind en verwerp.

Flokkulasie kan deur middel van chemiese- of ensiematiese aksie plaasvind. Die chemiese aksie word toegeskryf daartoe dat die drie-dimensionele proteïen netwerk gevorm word as gevolg van interaksies wat plaasvind (tussen  $\beta$ -LG en K-kaseïen) tydens die opberging van melk. Die interaksies vind binne die kaseïen missel plaas as gevolg van hitte behandelings wat op melk uitgevoer word. Tydens hierdie interaksies word 'n kompleks gevorm tussen  $\beta$ -LG en K-kaseïen. Die ensiematiese aksie behels die vrystelling van hierdie kompleks wat gevolglik lei tot die vorming van 'n gel. Proteases van mikrobiese oorsprong is hiervoor verantwoordelik. Daar is hoofsaaklik twee ensieme wat 'n rol speel by melk flokkulasie, naamlik plasmien en bakterieë van 'n psigotrofiese oorsprong.

Opsporings metodes vir melk flokkulasie is noodsaaklik om ten einde die oorsaak te kan bepaal en moontlik 'n oplossing te vind vir die probleem. Tegnieke wat reeds ontwikkel is vir die opsporing van melk flokkulasie is die Alizarol toets en die protease toets. Die tegnieke wat ontwikkel is met die doel om flokkulasie/gelering te bepaal sluit die RP-HPLC (MILQC sagteware) en die melk agar plaat tegniek vir protease bepaling in. Al die bogenoemde tegnieke kan hoë risiko melk wat geneig is tot flokkulasie/gelering effektief aandui en sommige van die tegnieke kan selfs 'n onderskeid tref tussen plasmien en mikrobiese protease.