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Cold microfiltration as an enabler of innovative dairy protein ingredient composition and techno-functionality

Thesis presented by

Thomas C. France

B.Sc. Food Science, University College Cork

for the degree of

Doctor of Philosophy

in

Food Science and Technology

August 2022

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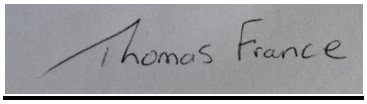
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Declaration

I hereby declare that the work submitted is entirely my own and has not been submitted to any other university or higher education institute, or for any other academic award in this university.

Signature:



Thomas France Date:19th August 2022

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This thesis is dedicated to my dad,

Mike, and my grandmother,

Kathleen.

Abstract

Microfiltration (MF) of skim milk at warm temperatures (i.e., 45-55 °C) is common practise in the dairy industry; however, high rates of fouling and short run times due to fouling and microbial quality can occur when operating at these temperatures. The adoption of cold temperatures (i.e., ≤20 °C) provides opportunities to improve efficiency and overall sustainability of the process. However, it can affect the protein, mineral and enzyme partitioning during MF which can ultimately impact the functional properties of the final ingredients, although this has not been sufficiently studied within the cold MF range. This thesis systematically studies the effects of different temperatures within the cold MF range on processing performance and fouling, protein, mineral and enzyme partitioning and some of the consequences on the physicochemical properties of the resulting process streams. Lower processing temperatures, in the range from 20 to 4 °C, resulted in significantly higher concentrations of β-casein in the permeate, with κ-casein, α-lactalbumin and β-lactoglobulin not significantly impacted by alterations in processing temperature. Lower processing temperature also resulted in lower rate of permeate flux decline during MF although higher mechanical (pump) and thermal energy consumption were measured for the lower processing temperature (i.e., 4 °C). Altering processing temperature had minor effects on plasmin activity in permeate streams although extensive plasmin-mediated hydrolysis was observed on storage; such plasmin-mediated hydrolysis had significant impacts on the temperature-dependent self-association of β-casein. Extensive diafiltration (DF) with water was performed at 4 °C, resulting in a β-casein-depleted micellar casein retentate which displayed superior heat stability when compared to a micellar casein retentate produced at 50 °C. The learnings obtained were applied to the final study in which different DF media were used, i.e., water and two simulated milk ultrafiltrates (SMUF) designed to resemble the mineral profile of ultrafiltration permeate produced at 10 and 50 °C. The use of both SMUFs resulted in similar protein partitioning and significantly improved the heat stability of the micellar casein retentates generated, compared to when water was used as the DF medium. The findings presented in this thesis constitute a significant advance in the understanding of the effects of processing temperature within the cold MF range on parameters of relevance in the optimisation of next generation protein ingredient development in the dairy industry.

Publications

France, T. C., Barone, G., O'Regan, J., Kelly, A. L., & O'Mahony, J. A. (2020). Physical and colloidal stability of conventional and micronised calcium citrate ingredient powders in the formulation of infant nutritional products. *Colloids and Surfaces B: Biointerfaces*, *194*, 111125.

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France, T. C., Kelly, A. L., Crowley, S. V., & O'Mahony, J. A (2022). Influence of processing temperature on plasmin activity and proteolysis in process streams from cold microfiltration of skim milk. *International Dairy Journal (submitted).*

Introduction and research objectives

Microfiltration (MF) within the dairy industry is a key unit operation which has traditionally been performed at warm temperatures (i.e., 45-55 °C). Performing MF at 45-55°C results in high initial permeate flux due to reduced viscosity of the process streams (i.e., permeate). In addition to this, warm temperatures allow for the retention of the casein fraction resulting in a permeate stream relatively free from caseins, as these high temperatures ensure that β-casein does not dissociate from the micelle, as hydrophobic interactions remain strong; thus, a whey protein permeate stream of high purity can be obtained. However, there has been increased interest in performing MF at cold temperatures (i.e., ≤ 20 °C), with associated benefits of reduced fouling, improved microbiological quality and opportunities to produce ingredients with tailored protein profiles (O'Mahony *et al*., 2014; Steinhauer *et al*., 2015; Schiffer & Kulozik, 2020).

The cold MF range encompasses a broad temperature range from 4-20 °C. As temperature directly impacts membrane performance, mineral, protein and potentially plasmin partitioning, altering temperature within this range could have large effects of the above parameters. The extent to which the different temperatures within the cold MF range impacts the extent of protein and mineral partitioning has not been studied in-depth in the scientific literature. Many of the studies published in the scientific literature have examined the difference between single temperatures within the cold (i.e., 8.9 °C) and warm (i.e., 50 °C) temperature ranges (Steinhauer *et al*., 2015; McCarthy *et al*., 2017; Hartinger *et al*., 2019). Therefore, the need to conduct an in-depth study on how small changes in processing temperature, within the cold MF range, ultimately effects protein partitioning (mainly β-casein) and mineral partitioning (mainly calcium) is required.

The abundance of plasmin and plasminogen in membrane filtration-derived milk protein ingredients can also be of significance, affecting both the functionality of the ingredient and final product quality. There have been previous studies which report the presence of plasmin in sweet whey and ultracentrifugal supernatants of skim milk, indicating that plasmin may also be present in whey generated from MF (Crudden *et al*., 2005; Crudden & Kelly, 2003; Hayes & Nielsen, 2000). Performing MF of skim milk at cold temperatures (i.e., 4 °C) results in a decrease in the strength of hydrophobic interactions, which in turn results in the dissociation of β-casein from the casein micelle and into the serum phase. This phenomenon during cold MF may also result in the release of small quantities of plasmin, as β-casein is the principal substrate for plasmin (Andrews, 1983; Andrews & Alichanidis, 1983). As plasmin can result in the hydrolysis of β-casein, reducing the levels of intact protein which is required for its functional properties (e.g., emulsification and foaming properties), the presence of plasmin in permeates becomes of even greater significance during the cold MF of skim milk.

Research objectives

The overall objective of this thesis was to perform novel research to determine the effects of temperature, within the cold MF range, on membrane performance and partitioning of milk constituents during the MF of skim milk. The new knowledge obtained on the effects of temperature within this range will provide important information on the key considerations (e.g., permeate flux, rate of fouling, protein partitioning, plasmin system partitioning, energy consumption) of importance when selecting the most suitable temperature to tailor ingredient composition and techno functionality for intended applications.

The initial two objectives of this thesis were as follows:

1 To review the state of the art with reference to cold microfiltration within the dairy industry, reviewing the effects of processing temperature on milk constituent partitioning, membrane performance (i.e., fouling and permeate flux) and dairy ingredient innovation.

2 To review the state of the art with regards to the plasmin system in milk.

The specific aims of the research conducted throughout this thesis are:

- To examine membrane performance (i.e., selectivity, permeate flux, fouling) at selected temperatures within the cold MF range (4, 8, 12, 16 and 20 °C).
- To study the partitioning of proteins, minerals and plasmin during MF of skim milk and determine how altering temperature (and pressure) can influence these.

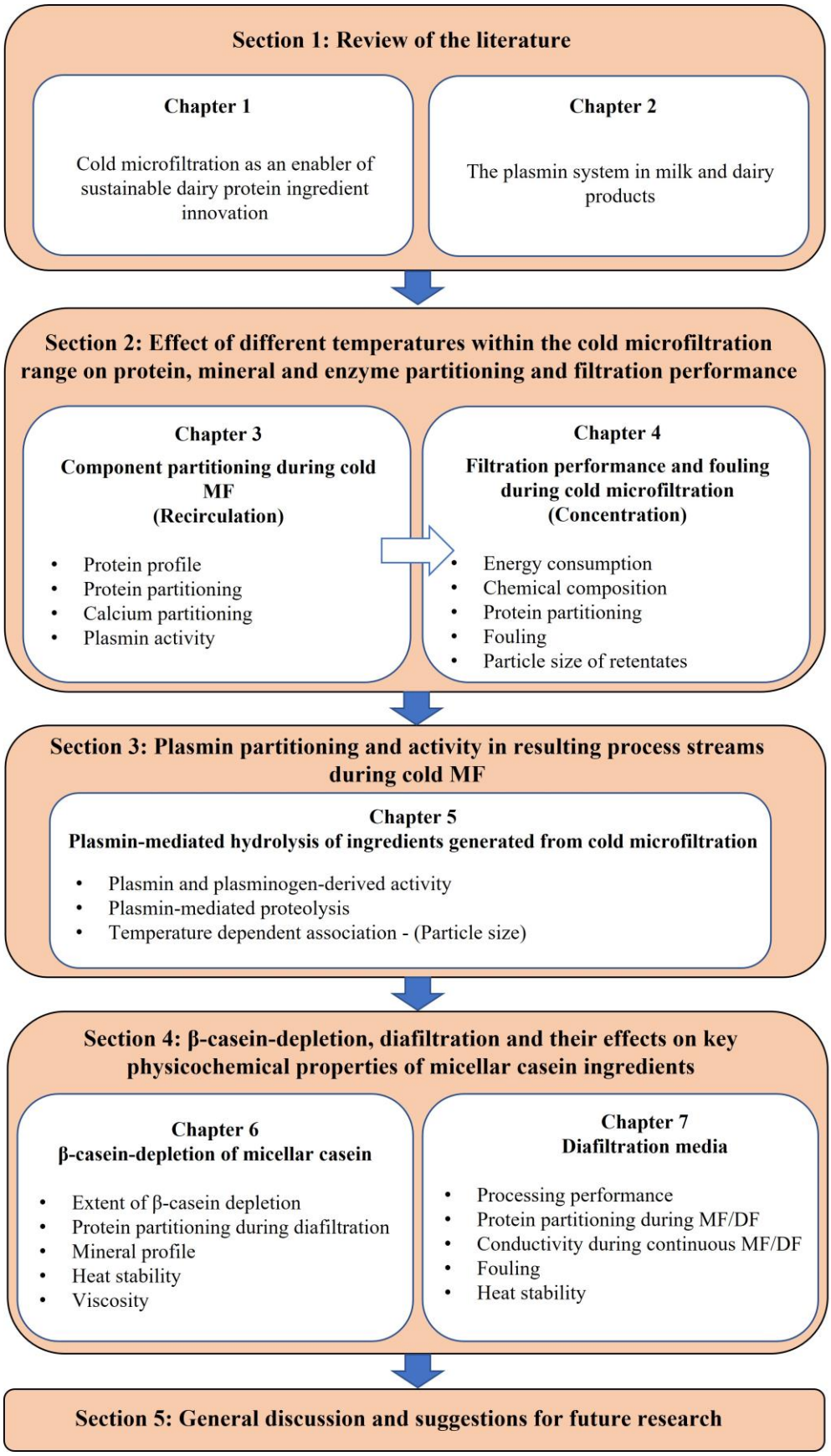
- To determine the enzymatic stability of micellar casein concentrate and β-casein-enriched whey ingredients produced following MF at different temperatures within the cold microfiltration range.

- To determine how plasmin-mediated hydrolysis of β-casein alters its temperature dependent self-association.

- To investigate the extent of β-casein depletion from skim milk following extensive diafiltration and to determine key physicochemical properties of the resultant β-casein-depleted micellar casein retentate.

- To determine how altering the composition of the diafiltration media effects protein partitioning during cold MF and whether changes in mineral profile of the diafiltration media impact heat stability of micellar casein retentate.

A schematic representation of the work conducted in this thesis is presented below.


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The plasmin system in milk and dairy products
Section 2: Effect of different temperatures within the cold microfiltration range on protein, mineral and enzyme partitioning and filtration performance
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Component partitioning during cold MF
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Protein partitioning
Calcium partitioning
Plasmin activity
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Section 5: General discussion and suggestions for future research

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Chapter 1

Cold microfiltration as an enabler of sustainable dairy protein ingredient innovation

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Declaration

This chapter was written by author Thomas C. France and reviewed by all co-authors.

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1.1 Introduction to microfiltration

Membranes contain small pores of a defined average size, which permit molecules smaller than the pore size to pass through into the permeate stream, while larger molecules that cannot pass through the membrane are concentrated in the retentate. Membrane separation processes have numerous industrial applications, with the pressure-driven processes of microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) being the most well established in the dairy industry with pore sizes of 10–0.1 µm, 0.10–0.01 µm, 10–0.1 nm and <0.1 nm, respectively (Pouliot, 2008). Membrane filtration technology is widely used in dairy processing to concentrate, fractionate, isolate, demineralise, defat, and purify different target macro-molecules, such as proteins, phospholipids and lactose.

MF is a key unit operation in dairy processing, in which micrometre-scale particles (e.g., fat globules and bacterial spores) are retained while smaller molecules such as water, minerals, lactose and whey proteins permeate through the membrane. MF membranes cover a wide range of pore sizes, ranging from 0.1–10 µm, and are also associated with the lowest processing pressures (0.01–0.20 MPa) (Pouliot, 2008; Saboya & Maubois, 2000). In comparison, higher operating pressures are required for UF, NF and RO, with pressures ranging from 0.1–1.0, 1.5–3.0 and 3.0–5.0 MPa, respectively (Pouliot, 2008). MF is increasingly being used in the dairy industry to reduce the bacterial load by removing bacteria cells (0.4–2.0 µm) and spores from skim milk prior to subsequent processing (Gésan-Guiziou, 2010; Wang *et al*., 2019). Based on the size difference between casein micelles (50–500 nm) and whey proteins (4–8 nm), MF technology is capable of fractionating the major proteins in milk in a chemical-free (e.g., no acids or bases required for pH adjustment) manner which is perceived as a “clean” approach when compared to alternative methods for protein

fractionation (e.g., precipitation techniques). Thus, tight MF membranes (e.g., 0.1 µm) allow for the retention of casein micelles while serum proteins are removed, producing a casein-rich concentrate containing micelles in their native form, known as micellar casein concentrate (MCC), and a permeate enriched in whey proteins in their native form, known as 'ideal' or 'native' whey. This review will examine the effects of performing MF at low temperatures and how altering processing temperature ultimately affects membrane performance, fouling, protein and mineral partitioning, as well as microbial biodiversity and growth. The potential for ingredient innovation and the overall sustainability of the process are also considered. The studies cited throughout the review report the pasteurisation of feed material prior to filtration, with filtration operated under concentration mode, unless otherwise stated

1.2 Choice of membrane

There are a wide variety of MF membranes available to the dairy industry, differing both in the configuration and membrane construction material. Both the choice of membrane material and configuration depend on the intended application.

1.2.1 Membrane structures and configurations

The internal structure of MF membranes can be symmetric (isotropic) or asymmetric (anisotropic); isotropic membranes have pores of uniform size over the entire cross-section of the membrane, while anisotropic membranes have multiple layers, each with different structures and pore size (Fig. 1.1) (Cheryan, 1998; Strathmann *et al*., 2011). Due to the uniform structure of isotropic membranes, particles are often retained within the internal structure, resulting in reduced flux due to plugging of the pores (Smith, 2013a). Asymmetric membranes consist of a 'skin'

layer on the surface of the membrane which acts as the actual selective barrier; below this thin 'skin' layer is a thicker porous sub-layer which has little effect on separation but provides mechanical support as well as allowing improved flux (Smith, 2013a). Asymmetric membranes are the most common commercially available membranes (Wang & Zhou, 2013).

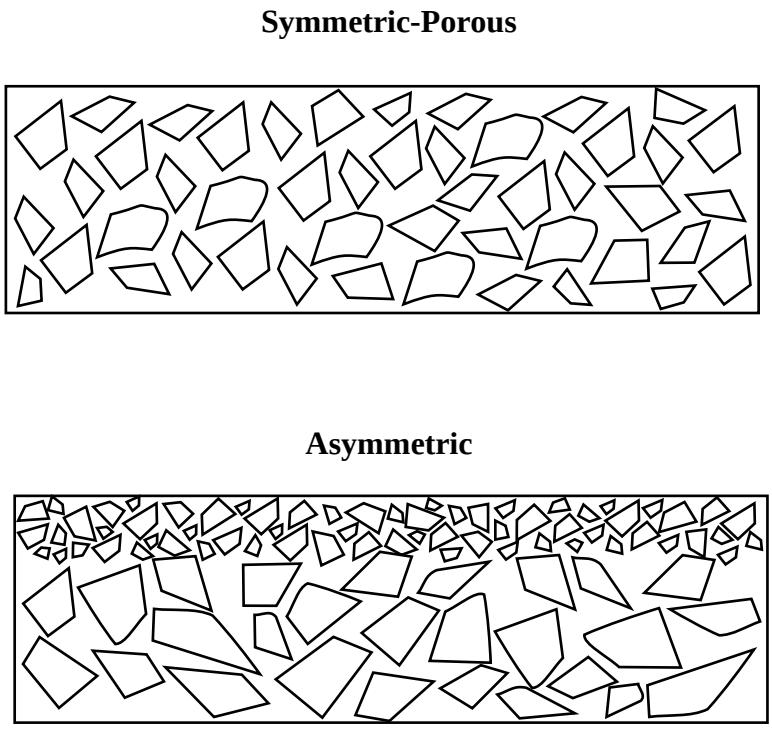


Figure 1.1: *Schematic representation of the difference in structure of a symmetric (porous) and asymmetric membrane (modified from Smith (2013a)).*

Membranes are available in hollow fibre, tubular, capillary and flat sheet geometries. Hollow fibre and tubular membranes have similar geometries, differing in terms of the tube diameters, with the former having outer diameters ranging from 0.05–0.5 mm while the latter have outer diameters of 5–25 mm (Smith, 2013b). For continuous membrane operations, these membranes are arranged into membrane elements/modules, with the type of module design depending on the membrane type

(e.g., flat sheet membranes can be arranged into plate and frame configuration or spiral wound modules). There are four general categories of membrane configuration/design: spiral-wound, tubular, plate and frame and hollow fibre with both ceramic and polymeric materials available for use (Wang & Zhou, 2013). The choice of membrane module used depends on multiple factors, such as the capital costs associated with the module, resistance to fouling, ease of cleaning and the properties of the feed material (e.g., viscosity).

1.2.2 Choice of membrane material

The membrane material used in the MF of dairy streams is primarily polymeric, although ceramic materials are also used. There are a wide variety of organic (polymeric) and inorganic (ceramic) materials that can be used for the manufacture of membranes, although the choice of material used depends on the intended application, as each has specific advantages and disadvantages. Besides the choice of membrane material, the cost associated with purchasing the membrane, cost of installation and operation, and the expected membrane lifetime are often the main factors determining membrane choice. The differences between polymeric and ceramic membranes in terms of milk protein fractionation has received some attention in recent years (Espina *et al*., 2009; Karasu *et al*., 2010), with the efficiency of removal of serum proteins often being higher for ceramic membranes (Zulewska *et al*., 2009; Carter *et al*., 2021a).

1.2.2.1 Polymeric

The main polymers used to create MF membranes include polysulphone/polyethersulphone (PS/PES) and polyvinylidene fluoride (PVDF). PS

and PES membranes have wide pH, temperature and chemical tolerances, as well as having the advantage of being able to be manufactured into a wide variety of configurations with a wide range of pore sizes. However, they are often limited to low operating pressures due to low mechanical strength. PVDF membranes possess many of the advantages of PES membranes, while they have narrower temperature and pH tolerances. Polymeric membranes have a broader pore size distribution compared to ceramic membranes and are relatively hydrophobic, which can facilitate protein adsorption (Baruah *et al*., 2006). The surface of these membranes can also be modified to alter the surface chemistry, modifying the way in which the membrane interacts with components of the feed system. Alternatively, hydrophobic polymers such as PVDF can be directly modified during the membrane production process to reduce hydrophobicity through the addition of hydrophilic polymers (Liu *et al*., 2011). A hydrophilic membrane is most desirable in the processing of dairy streams to minimise binding of hydrophobic proteins to the membrane surface.

1.2.2.2 Ceramic

Ceramic, also known as "inorganic", membranes are formed by combining metals such as titanium or aluminium in different forms (e.g., oxides) with support materials (e.g., aluminium, stainless steel, silicon carbide). Generally, ceramic membranes are available in tubular form as either a single-channel tube or, more commonly, a multichannel element, with the latter design increasing packing density and mechanical strength. However, ceramic membranes have fewer disadvantages when compared to polymeric membranes. Due to the inorganic nature of the materials used to manufacture these membranes, they are inert to many chemicals and have wide temperature (>100 °C) and pH (0.5–13.5) tolerances (Baruah *et al*., 2006). As ceramic

membranes can tolerate high temperatures, they can be sterilised, unlike polymeric membranes. However, ceramic membranes are both more brittle and heavier than their polymeric counterparts. In addition, the higher capital and running costs for ceramic membranes have made polymeric membranes an attractive alternative for milk processers. Inorganic membranes have longer life spans than polymeric membranes, with the life span of polymeric membranes depending on the properties of the feed stream (e.g., viscosity), frequency of use and the nature of cleaning (e.g., temperature at which cleaning is performed, type and strength of chemicals). Ceramic membranes also have the capability of allowing backflushing in which backpressure is applied to remove foulants from the surface of the membrane, helping to restore flux. In addition to this, to reduce fouling on ceramic MF membranes during filtration, a high cross-flow velocity can be used (e.g., 5–7 m/s), which increases wall shear stress and therefore improves the efficiency of removal of loose foulant material (Carter *et al*., 2021b).

1.3 Cold microfiltration in dairy processing

Both MF and UF at low temperatures (i.e., <20 °C) have become increasingly common in the dairy industry due to evidence of reduced fouling, which may allow for longer run times (Luo *et al*., 2015; Steinhauer *et al*., 2015; McCarthy *et al*., 2017), the opportunity to produce new ingredients with targeted protein profiles (i.e., β-casein-enriched ingredients), and reduced levels of denatured whey protein (i.e., higher levels of native whey protein) (O'Mahony *et al*., 2014). The differences between warm and cold MF in terms of operating conditions, membrane performance and partitioning are discussed below.

1.3.1 Temperature range

Traditionally, both MF and UF of milk are performed at temperatures of 45–55 °C (O'Mahony & Tuohy, 2013), with associated advantages of higher initial fluxes, resulting from lower viscosity and efficient removal of whey proteins (McCarthy *et al*., 2017; Hurt *et al*., 2015). However, the filtration of milk within this temperature range can cause issues with fouling and the growth of thermophilic bacteria. As a result, some dairy processors are transitioning from traditional warm MF (45–55 °C) towards cold MF. The term "cold", in the dairy industry, when applied to MF (and also UF), covers a broad temperature range, with temperatures <20 °C generally being considered as low temperature filtration (Crowley *et al*., 2015). Selecting the temperature at which MF is performed is an important consideration, as it can have significant effects on membrane performance, the degree and type of fouling, partitioning of milk constituents and energy requirements of the plant, as well as the microbial quality of the ingredients produced.

1.3.2 Membrane performance and fouling

Operating at low temperatures is associated with lower permeate flux due to higher viscosity of the permeate stream, in agreement with Darcy's law:

$$J = \frac{TMP}{\eta \times R} \qquad [1.1]$$

where J is the permeate flux, TMP is the transmembrane pressure, η is the viscosity of the fluid permeating the membrane at a specific processing temperature and R is the overall hydraulic resistance, consisting of the sum of resistances contributed by the clean membrane, reversible and irreversible fouling. Thus, when performing filtration at cold temperatures, longer run times may be required to achieve the same volume

concentration factor compared to operating at higher temperatures. One of the biggest challenges of membrane filtration is fouling, in which solutes accumulate at the membrane surface or within the membrane itself (Tan *et al*., 2014). Although, during warm MF, initial flux levels are higher than for cold MF, fouling is more extensive with the former process (McCarthy *et al*., 2017). McCarthy *et al*. (2017) filtered unpasteurised skim milk using a 0.14 µm pore size ceramic membrane at 8.9 (i.e., cold) and 50 °C (i.e., warm) and reported that permeate flux was lower for cold MF compared to warm MF, although flux decreased rapidly during the warm MF process due to higher levels of fouling. Hartinger *et al*. (2019) reported that MF of skim milk at 50 °C, under total recirculation mode, using a 0.1 µm spiral-wound PVDF membrane, resulted in a more rapid decline in flux and a more significant reduction in protein permeation throughout filtration, compared to MF at 10 °C. Similar findings have been reported for UF membranes, with Luo *et al*. (2015) and Ng *et al*. (2018) observing lower fouling at low processing temperatures (i.e., 10–15 °C) compared to higher temperatures (i.e., 50 °C). Barukčić *et al*. (2014) measured water flux before and after MF of whey under total recirculation mode, using ceramic membranes with different pore sizes (0.1, 0.5 and 0.8 µm), at 20 and 50 °C, and reported that fouling intensity was lower at the lower processing temperature for each pore size. Therefore, even though initial flux levels are lower for cold MF (Fig. 1.2), longer processing times with a more stable flux over time, as a result of lower fouling, could result in a reduced number of cleaning cycles and therefore reduced downtime during cleaning in place (CIP) and associated consumption of water and cleaning chemicals (Schiffer & Kulozik, 2020).

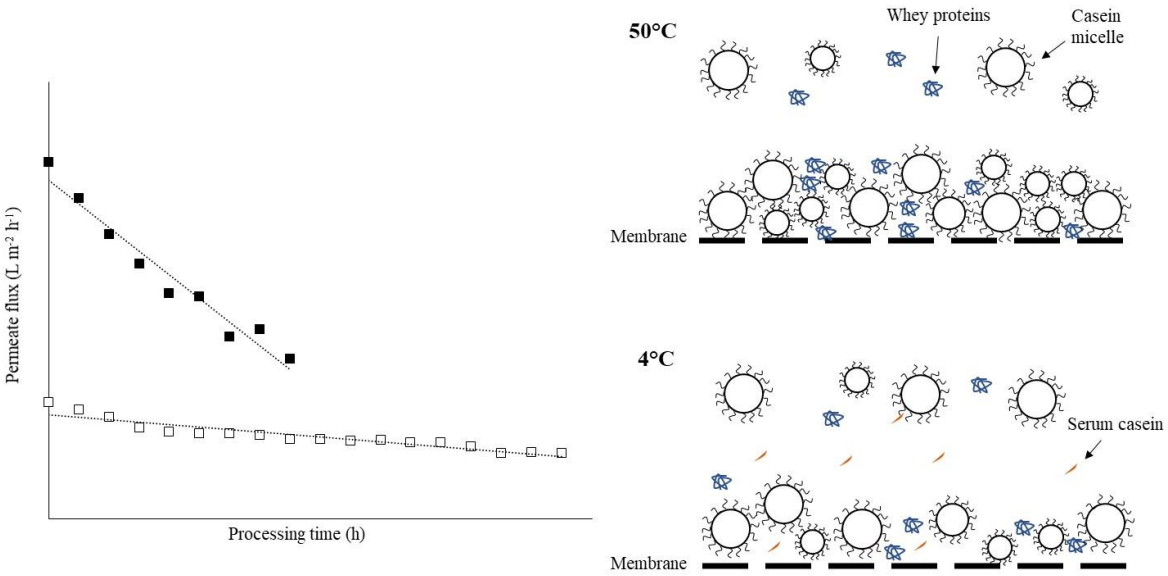


Figure 1.2: *Schematic representation of permeate flux decline during warm (50°C; ■) and cold (4°C: □) microfiltration of skim milk (left; modified from McCarthy et al. (2017)) and differences in structural arrangement and compactness of the deposit layer on the membrane surface after MF at the respective temperature (right).*

These studies all indicate that more extensive fouling occurs when filtration is performed at higher processing temperatures (i.e., 50 °C) compared to lower processing temperatures (i.e., 10 °C). MF of dairy streams at these high processing temperatures (45–55 °C) can also result in partial unfolding of whey proteins, particularly β-lactoglobulin, which can lead to more extensive fouling. β-lactoglobulin has a free cysteine group which becomes more accessible as the protein unfolds. At temperatures up to 60 °C, reversible dissociation of β-lactoglobulin dimers to monomers and reversible unfolding occurs, resulting in increased sulfhydryl group reactivity (de Wit, 2009), which can increase protein–protein interactions. Studies have shown that performing filtration at higher temperatures (i.e., 50 °C) results in a more compact fouling layer compared to low temperatures (i.e., 10 °C; Fig. 1.2) (Steinhauer *et al*., 2015; Hartinger *et al*., 2019; Ng *et al*., 2018). Steinhauer *et al*.

(2015) reported that MF of β-lactoglobulin suspensions at 10 °C, with a 0.1 µm pore size, resulted in the formation of a loosely packed and highly permeable fouling layer; on the other hand, MF at temperatures ≥40 °C resulted in the formation of a thicker and more compact fouling layer with higher fouling resistance, which was attributed to increased protein hydrophobicity and sulfhydryl group reactivity.

The differences in membrane performance and the extent of fouling at high and low temperatures is also evident when comparing permeate flux before and after increasing TMP. Hartinger *et al*. (2019) examined the effect of a stepwise increase in TMP and the subsequent stepwise reduction in TMP on filtration resistance during the MF of skim milk at 10 and 50 °C. At 50 °C, a distinct hysteresis was observed in the flux data, indicating extensive accumulation of deposits on the membrane, whereas at 10 °C there was no distinct hysteresis in flux after the pressure cycle. The results suggest that the compression of the deposit layer at 10 °C is largely reversible, in contrast to the deposit layer formed at 50 °C. The high pressure, coupled with the high processing temperature, may allow for increased protein–protein interactions and crosslinking of foulant components, resulting in a more compressed fouling layer than that generated at lower processing temperatures. Thus, although warm MF is associated with higher initial fluxes, extensive fouling can reduce membrane life and alter filtration performance, while cold MF can provide stable flux with less extensive fouling, albeit with lower flux.

1.3.3 Composition of the fouling layer

During the MF of skim milk and other dairy streams (e.g., whey), fouling occurs, resulting in the formation of a cake layer varying in composition, with proteins and minerals (e.g., calcium phosphate) being the principal fouling materials (Cheryan,

1998; Tan *et al*., 2014; Beckman & Barbano, 2013; Koh *et al*., 2013). Protein fouling is caused by the deposition and adsorption of proteins (whey proteins and casein) onto the surface of the membrane or within the pores of the membrane (Koh *et al*., 2013; James *et al*., 2003), while mineral fouling is largely due to calcium, facilitating protein–protein and protein-membrane interactions. Tan *et al*. (2014) found both whey protein and casein to be the primary foulants during cold MF of raw skim milk using a 1.4 µm ceramic membrane, with only a minor contribution of minerals to membrane fouling. The solubility of calcium phosphate is inversely proportional to temperature; thus, calcium phosphate deposition/scaling on the membrane surface or within pores during cold MF is likely to be less significant than during warm MF. Extensive membrane fouling by proteins during MF not only reduces the permeation of proteins through the membrane but is also associated with reduced membrane selectivity, reduced yield, and increased membrane cleaning times and associated costs (Hartinger *et al*., 2019; France *et al*., 2021a).

1.3.4 Microbial growth

MF with nominal pore sizes of 0.8–1.4 µm can be used to reduce the microbial load of milk, such as in the production of extended shelf-life milk (Schmidt *et al*., 2012) and in the production of infant formula-grade milk powders (Li *et al*., 2019a). It also provides the dairy industry with MF permeate with improved microbial quality prior to the generation of milk-derived protein ingredients. However, if this is not performed, and membranes with pore sizes of 0.1 µm are employed in the fractionation of milk proteins, bacteria present are retained and concentrated in the retentate. In the dairy industry, MF systems are operated in continuous mode for long periods of time, which can result in the accumulation of microorganisms and support the formation of

biofouling on the MF membrane. Filtration in the dairy industry is often performed at temperatures suitable for the growth of microorganisms; in addition to this, high TMP, lack of mechanical cleaning and constant flow of feed through the membrane make membranes susceptible to biofilm formation. Therefore, the temperature at which filtration is performed is an important consideration for processors, as it influences microbial growth and the bacterial community that can grow (Fig. 1.3).

In order to delay and/or limit biofilm formation and microbial growth, the temperature at which MF is performed can be reduced. Filtration at high temperatures (i.e., 45–55 °C) can provide optimum conditions for the growth of thermophilic bacteria, which can proliferate at temperatures between 45–70 °C (Burgess *et al*., 2010; Seale *et al*., 2015a). Common thermophilic bacteria such as *Anoxybacillus flavithermus*, Bacillus spp. and Geobacillus spp. can produce heat-stable proteases and lipases, with important implications for the quality of dairy products; their spore-forming and biofilm-forming abilities also present challenges, as they are difficult to remove and/or inactivate and are also capable of producing acid on growth, reducing pH (Schiffer & Kulozik, 2020; Burgess *et al*., 2010; Seale *et al*., 2015b). Studies have indicated that operating at high temperatures can result in a faster rate of microbial growth and biofilm formation than at low temperatures. Chamberland *et al*. (2019) reported that the rate of biofilm formation on UF membranes during skim milk processing at 15 °C was slower than at 50 °C. These authors reported that bacterial growth was significantly higher at 50 °C, with the number of 16S rRNA gene copies on the membrane increasing from 3.21 ± 0.12 to 8.83 ± 1.58 $\log_{10}$ gene copies per cm^2 after 15 h of processing, while at 15 °C, the number of gene copies on the membrane only increased from 3.31 ± 0.24 to 3.86 ± 0.58 $\log_{10}$ gene copies per cm^2 after 48 h of filtration.

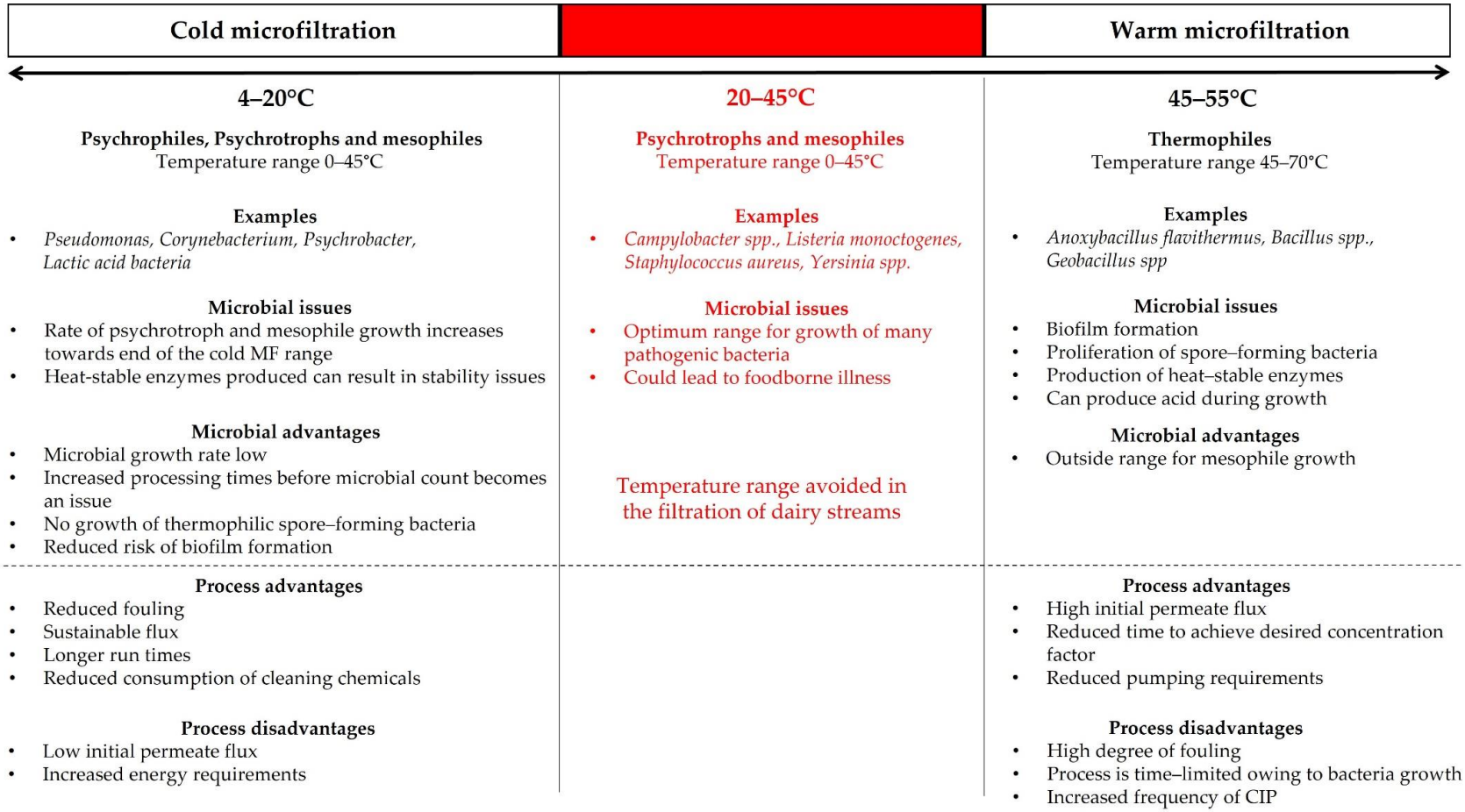


Figure 1.3: *Schematic representation of some of the microbial considerations when selecting microfiltration processing temperature and the implications of such processing temperatures for processing efficiency and finished product quality.*

During filtration of milk at low temperatures (<15 °C), psychrophilic and psychrotrophic bacterial genera, such as Pseudomonas, Psychrobacter and Corynebacterium are capable of growing (Chamberland *et al*., 2019). Facultative psychrotrophs can grow within a broad temperature range (0–40 °C), occurring at higher numbers in milk compared to obligate psychrophiles (0–15 °C) (Seale *et al*., 2015a). The optimal growth temperature for most psychrotrophic bacteria is between 20–30 °C; therefore, the extent of proliferation during cold MF is low (Samaržija *et al*., 2012). Operating at the upper end of the cold MF range can result in the growth of some mesophilic organisms (Seale *et al*., 2015a). However, the growth rate of such mesophilic bacteria (e.g., lactic acid bacteria) is low at these temperatures (i.e., 15 °C). As these higher temperatures (15–20 °C) facilitate the growth of mesophilic bacteria and are close to the optimal metabolic activity of psychrotrophic bacteria, such temperatures are generally avoided for MF in dairy processing. Schiffer and Kulozik (2020) examined the effect of temperature (10, 14, 16, 20 °C and 55 °C) on microbial growth during MF of skim milk under feed-and-bleed mode (permeate and 10 L/h of retentate was continuously removed during MF and replaced with skim milk), using a 0.1 µm PES membrane; the authors reported that filtration at 55 °C was deliberately stopped after 10 h due to a sudden drop in pH, attributing this to microbial activity. Within the cold MF range, microbial growth was highest at 20 °C, followed by 16 °C and 14 °C taking 8, 10 and 17 h, respectively, to reach the critical colony forming unit level of 105 cfu/mL; at 10 °C, microbial counts remained almost constant over 24 h of MF. The bacterial communities responsible for the increasing microbial counts at 16 and 20 °C in the aforementioned study were not determined, and so it was not clear whether mesophilic bacteria made a considerable contribution towards microbial counts at the upper end of the cold MF range.

In order to improve the microbial quality of ingredients produced using filtration, there has been a trend towards cold MF, as the rate of microbial growth and biofilm formation is lower than that during warm MF. Therefore, performing MF at low temperatures (i.e., ≤10 °C) may allow for longer run times before microbial growth becomes an issue for hygiene standards. However, reducing the temperature at which filtration is performed results in reduced flux and can also increase energy demands. Therefore, research on finding the optimum temperature for maintaining microbial growth and biofilm formation low while also optimising filtration performance (e.g., permeate flux) is still required.

1.3.5 Protein partitioning

During the MF of dairy streams, a cake layer is formed, which results in the membrane becoming less permeable; this fouling layer is known to act as a secondary membrane, altering the permeate flux and the selectivity of the membrane. There have been numerous studies comparing the effect of temperature, as well as differences between ceramic and polymeric membranes, on the efficiency of protein fractionation (Karasu *et al*., 2010; Zulewska *et al*., 2009; Steinhauer *et al*., 2015; Hartinger *et al*., 2019; Jarto *et al*., 2018). Hartinger *et al*. (2019) studied the influence of temperature and TMP on protein fractionation during MF of skim milk using a 0.1 µm membrane. The authors reported that, at 50 °C, the initial rate of reduction in protein permeation was higher than that at 10 °C, with MF at 50 °C also being associated with a higher mean reduction in the permeation of protein (2.8% h^{-1} vs. 1.2% h^{-1} at 50 and 10 °C, respectively) between 40 and 180 min of MF; this was attributed to a more extensively fouled membrane and temperature-induced aging of the deposit layer at 50 °C.

Although many of these studies have examined the effects of temperature on protein partitioning at either one temperature or at two different temperatures (often involving a comparison between low and high temperatures), there is limited information available on the effect of different temperatures within the cold MF range on protein partitioning and fouling. Jarto *et al*. (2018) reported that MF of skim milk at 13 °C resulted in higher whey protein permeation compared to MF at 5 °C. Similar results were reported by France *et al*. (2021a), who observed that MF of skim milk at 12 °C resulted in higher concentrations of β-lactoglobulin and α-lactalbumin in permeate, compared to at 4 and 8 °C, although differences between temperatures were not significant. Thus, temperature affects protein partitioning during filtration primarily through differences in the form and extent of fouling. These studies indicate that processing temperature influences whey protein permeation. MF of milk at low temperature (i.e., 4 °C) also results in the permeation of casein, in particular β-casein, through the membrane and into the permeate, resulting in the generation of a β-casein-enriched whey, a phenomenon that is not associated with MF performed warm. Therefore, not only does the temperature at which MF is performed influence whey protein permeation, but also the protein profile of the resulting permeate and retentate.

The choice of membrane material can also impact the efficiency of whey protein permeation and the composition of the final permeate and retentate streams. Zulewska *et al*. (2009) examined the efficiency of serum protein removal from the MF of skim milk at 50 °C, using both spiral-wound polymeric (0.3 µm) and two ceramic membranes (uniform transmembrane pressure and graded permeability MF systems) of equivalent pore size (0.1 µm). The authors reported that permeate generated using ceramic membranes had a higher concentration of protein than that generated using the polymeric membrane. The use of ceramic MF membranes resulted in higher levels

of β-lactoglobulin in the permeates, with an overall higher efficiency of whey protein removal than the spiral-wound polymeric membrane (64.4–61.0% vs. 38.6%). The authors suggested that the differences observed were, in part, due to the hydrophilic nature of ceramic membranes resulting in lower protein adsorption to the membrane when compared to the hydrophobic polymeric membrane. Similar results have since been reported by Carter *et al*. (2021a) and Beckman *et al*. (2010).

1.3.6 Mineral partitioning

Performing MF at low temperatures changes the composition of final permeate, not just in terms of the protein profile, but also of the mineral profile. As temperature decreases, colloidal calcium phosphate (CCP) within casein micelles solubilises, increasing the concentration of calcium and other minerals in the serum phase (Fox *et al*., 2015). Therefore, when MF is performed at low temperatures (i.e., 4 °C), there is a higher concentration of soluble minerals (e.g., calcium) that can permeate the membrane, resulting in a higher concentration of ash in the permeate (France *et al*., 2021b). There have been numerous studies detailing the effect of processing temperature on the partitioning of calcium during both UF and MF of skim milk; the authors of these studies report that reducing the temperature at which filtration is performed increases the concentration of total and/or ionic calcium in the permeate (Luo *et al*., 2015; France *et al*., 2021b; Lin *et al*., 2015; Zulewska *et al*., 2018). Crowley *et al*. (2018) analysed MCC powder produced by MF of skim milk at low (<10 °C) or high temperature (50 °C) and reported that the MCC powder generated at cold temperatures had lower levels of calcium and phosphorus compared to that made from warm MF, while levels of monovalent ions were relatively unchanged. The authors also studied the effect of MF on the rehydration characteristics of MCC

powders and reported that MCC generated from cold MF had superior dispersion characteristics, with 50–60% less sedimentation following centrifugation; this was partially attributed to the lower Ca content of the cold MCC.

The presence of high concentrations of calcium in β-casein-enriched whey following cold MF (0.1–0.5 µm) of skim milk can lead to challenges with subsequent downstream processing of such novel whey streams. On warming the β-casein-enriched whey, the strength of hydrophobic interactions increases, causing the self-association of molecular β-casein (7–8 nm) and an increase in particle size, ultimately resulting in the formation of β-casein micelles (20–30 nm in diameter); this phenomenon is normally reversible on cooling, with β-casein returning to a monomeric state (Dauphas *et al*., 2005; Crowley *et al*., 2019; Li *et al*., 2019b). However, in the presence of calcium (e.g., 8–15 mM at 37 °C), these β-casein micelles can aggregate via calcium-mediated crosslinking, resulting in a particle size of >1 µm, and leading to precipitation of the aggregated β-casein (Dauphas *et al*., 2005; Huppertz, 2013). Therefore, following cold MF of skim milk, a demineralisation step is often applied to the permeate stream to reduce the concentration of soluble minerals, allowing for stable downstream processing or indeed allowing for the controlled, reversible, thermal aggregation of β-casein without incurring precipitation (O'Mahony *et al*., 2014). Thus, MF of skim milk at low temperatures results in increased concentrations of total divalent cations (e.g., calcium) in the permeate, altering the mineral composition of both the permeate and retentate compared to that produced from warm MF, which can have important implications for ingredient functionality.

1.3.7 Enzyme partitioning

The principal indigenous milk proteinase, plasmin, can result in challenges for the proteolytic stability of filtration-derived milk protein ingredients. During warm MF of milk using a 0.1-µm membrane, casein micelles are concentrated in the retentate, while whey proteins are removed in the permeate, with such permeate containing many inhibitors of the plasmin system, including β-lactoglobulin. As plasmin is primarily associated with casein micelles through lysine binding and to a lesser extent through electrostatic interactions (Korycha-Dahl *et al*., 1983; Richardson, 1983), it becomes concentrated in the retentate. Consequently, plasmin activity in the retentate increases, with subsequent diafiltration resulting in further increases in plasmin activity as inhibitors are further removed (Aaltonen & Ollikainen, 2011).

During cold MF, β-casein dissociates from the casein micelles into the serum phase, where it passes through the membrane into the permeate stream. Therefore, there remains the question whether temperature-induced changes in casein micelles during cold MF also affect the association of plasmin with the casein micelles. As the principal substrate for plasmin is β-casein, plasmin activity in such permeates may lead to issues with β-casein hydrolysis on further downstream processing and in products incorporating the ingredient. France *et al*. (2021b) studied the effect of temperature (4, 8, 12, 16 or 20 °C) on enzyme partitioning during MF of skim milk under total recirculation mode and reported that temperature had no significant effect on plasmin activity in β-casein-enriched permeate; therefore, the effect of temperature on plasmin activity in permeates is not as pronounced as that seen for β-casein. Furthermore, in the purification of β-casein, the serum phase containing many of the inhibitors are removed; thus, in the absence of such inhibitors, plasmin activity may be even greater. The presence of plasmin in permeate streams containing β-casein

could result in negative consequences, with plasmin-induced hydrolysis of β-casein potentially reducing levels of intact β-casein, thus reducing its functional properties of value in food formulations.

1.4 Microfiltration as an enabler of dairy ingredient innovation

MF of skim milk allows for the generation of specific ingredients, with the temperature at which MF is performed strongly influencing the composition and, therefore, the functionality and applications of the ingredients generated, as discussed below.

1.4.1 Warm microfiltration applications in the dairy industry

MF of dairy streams has many different applications depending on the feed material, processing temperature, and membrane pore size. One of the first applications of warm MF in the dairy industry was the removal of residual fat and denatured whey proteins from whey in the production of whey protein isolate (WPI). MF membranes with a pore size of less than 1.0 µm retain any residual fat (i.e., that from the cheesemaking process in sweet whey), phospholipids, and denatured protein, thereby allowing for a higher protein content to be achieved in the final WPI product. This MF process creates a co-product with a high content of protein, fat and phospholipid which can be further processed to produce a whey protein phospholipid concentrate (WPPC). A standard for the composition of WPPC was established in 2015 by the American Dairy Products Institute which states that such products must have a minimum of 50% protein on a dry basis, minimum 12% fat, maximum of 8% ash and maximum of 6% moisture. WPPC is a functional dairy ingredient that can add functionality to ingredients at a low cost (e.g., in cakes as an egg replacement) (Levin

et al., 2016), with its biggest potential use being in nutritional applications due to its rich phospholipid profile. Although MF for WPI production has been traditionally performed at 45–55 °C, transitioning to cold processes could allow for reduced fouling and higher native whey protein levels.

Warm MF in the dairy industry has also been used for the removal of bacteria from milk using a typical membrane pore size of ~1.4 µm (Saboya & Maubois, 2000; Maubois, 2002). Here, the objective is to remove bacterial cells, somatic cells and endospores, while minimising the retention of proteins to maintain the composition of the MF permeate as close to that of the milk feed. Therefore, 1.4 µm membranes are most widely used, allowing for the right balance between high flux, bacteria removal and the permeation of other milk components with little or no rejection. A reduction in the pore size (e.g., 0.5–0.8 µm) of MF membranes results in improved reduction in bacteria but can lead to a more rapid decline in flux and increased retention of casein micelles (Lindquist, 1998). In the dairy industry, the removal of bacteria is primarily performed at high temperatures (i.e., 50 °C). Such temperatures allow for the growth of thermophilic spore-formers and can lead to the formation of resistant biofilm formation, while using low-temperature MF can reduce the risk of bacterial growth and proliferation of thermophilic spore-formers. Studies have shown that cold MF of skim milk using ceramic membranes effectively reduces bacterial cells and somatic cells (Wang *et al*., 2019; Fritsch & Moraru, 2008; Griep *et al*., 2018); therefore, cold MF can be used to reduce bacterial load and somatic cell count while reducing the risk of thermophilic bacterial growth and biofilm formation, which could otherwise negatively impact on final product quality. Within the dairy industry, cold MF is now also used to improve the microbial quality of the feed material prior to the production of ingredients, e.g., Micelate Prestige™, a product currently produced by

FrieslandCampina, is a cold-processed, native micellar casein isolate (MCI) which has undergone cold MF to improve the microbial quality of the ingredient.

Following MF of whole milk using a 1.4 µm membrane, as described above, fat globules (0.1–15 µm) are also retained in the retentate. The milk fat globule membrane (MFGM) retained in this MF retentate can be isolated to produce MFGM-enriched material. A recent study reported that MF of raw milk resulted in the production of high purity MFGM material (Hansen *et al*., 2020). As MFGM material is often isolated from heavily processed dairy streams (e.g., buttermilk and high fat retentate generated following MF of WPC), the process mentioned offers the dairy industry a practical method to produce MFGM material from a less heavily processed stream.

Warm MF of skim milk, when performed with a 0.1 µm pore size membrane, results in two streams, a casein micelle-enriched retentate and a whey protein-enriched permeate (Fig. 1.4). Performing MF at these high temperatures ensures that β-casein does not dissociate from the micelle and into the serum phase as hydrophobic interactions remain strong. The permeate produced from warm MF of milk is often referred to as 'ideal' or 'native' whey, with a composition similar to that of sweet whey but is free of many of the contaminants present in sweet whey, such as glycomacropeptide, starter culture, colour residues, lipid material, lactic acid and coagulant. In addition, ideal whey has lower fat content, improved protein quality and higher levels of native whey proteins when compared to sweet whey, as sweet whey is typically subjected to more intense thermal treatment (Heino *et al*., 2007; Evans *et al*., 2009). The retentate stream or co-product following diafiltration (UF permeate or RO water) is known as MCC and is enriched in native micellar casein. The production of MCC has multiple applications in nutritional and clinical products and in sports

nutrition products, providing a slow release of amino acids (Jäger *et al*., 2017). MCC can also be used as a starting feed material in the production of β-casein. Warm MF of cheese milk is also used in the manufacture of cheese, enhancing cheese yield and output. MF instead of UF also provides the added benefit of depleting most of the serum proteins prior to reaching the cheese vat and therefore the whey is not contaminated with colour residues (e.g., annatto) and other residues from the cheese making process (Soodam & Guinee, 2018)

1.4.2 Cold microfiltration

There has been an increasing trend in the use of cold MF in the dairy industry. One of the opportunities cold MF offers is the production of new ingredients with selected protein profiles (e.g., β-casein ingredients), with an overview of some in the following sub-section.

1.4.2.1 Influence of temperature on β-casein dissociation

The adoption of cold MF offers dairy processors the opportunity to produce ingredients from skim milk with differentiated protein profiles. Of the four caseins (α_{s1}-, α_{s2}-, β- and κ-), β-casein enrichment/isolation is the most viable and widely practised. Bovine β-casein is held within the casein micelle primarily via hydrophobic interactions (De Kruif & Holt, 2003). Reducing temperature results in a decrease in the strength of hydrophobic interactions, resulting in the dissociation of β-casein from the interior to the surface of the micelle, from where it dissociates into the serum phase of milk (Creamer *et al*., 1977).

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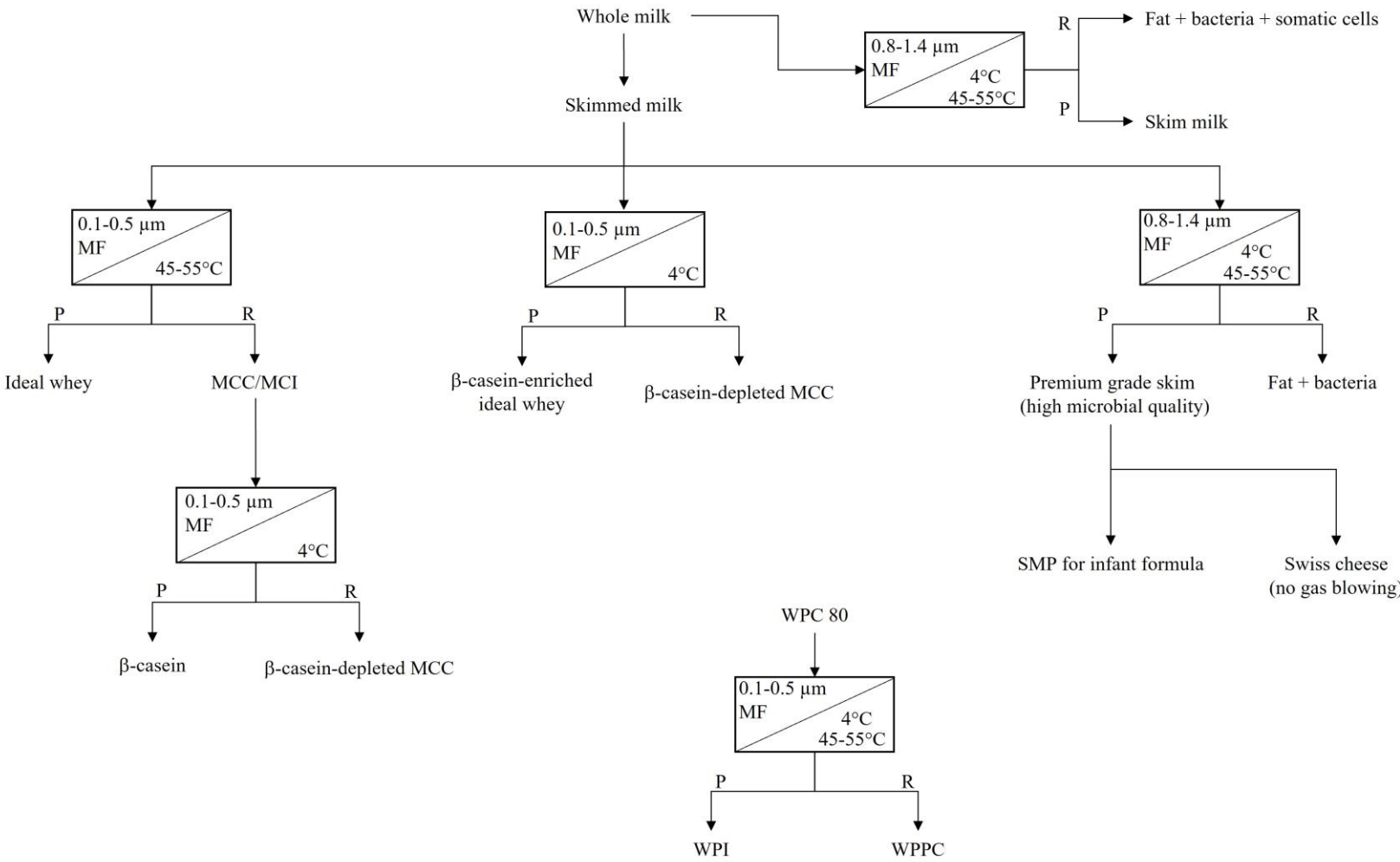


Figure 1.4: *Schematic representation of the use of microfiltration (MF) to improve microbiological quality and generation of different milk protein-derived ingredients as influenced by processing temperature. MCC, micellar casein concentrate; MCI, micellar casein isolate; SMP, skim milk powder; WPC, whey protein concentrate; WPI, whey protein isolate; WPPC, whey protein phospholipid concentrate; P, permeate; R, retentate.*

At low temperature (i.e., ≤4 °C), β-casein primarily exists in a monomeric state (Payens & Van Markwijk, 1963; Andrews *et al*., 1979), although even at these temperatures polymeric structures are not entirely absent (Farrell Jr *et al*., 2001). This phenomenon can be exploited in the production of β-casein-enriched whey during cold MF of milk, because at these low temperatures (i.e., ≤4 °C), monomeric β-casein can permeate through the MF membrane, producing a β-casein-enriched permeate and a β-casein-depleted retentate (Fig. 1.4).

The temperature at which filtration is performed at within the cold MF range has a significant impact on serum β-casein concentration, with increasing processing temperature significantly reducing β-casein concentration in the permeate stream. A study by France *et al*. (2021b) reported that increasing processing temperature in the range 4–20 °C during the cold MF of skim milk, under total recirculation mode, reduced the concentration of β-casein in the permeate stream, from 2.02 to 0.53 mg/mL at 4 and 16 °C, respectively, under sub-critical flux conditions. By controlling temperature, the strength of hydrophobic interactions can be manipulated, ultimately influencing the concentration of β-casein in the serum phase that can permeate across the membrane.

The complete retention of caseins is often desired in milk protein fractionation to obtain a whey protein stream free from serum casein. Therefore, the increasing trend towards cold MF presents challenges to dairy processors in the production of native whey, as the purity of the whey protein fraction is affected. A recent study by Schiffer *et al*. (2021) examined the effect of added calcium and pH on milk protein fractionation during the MF of skim milk, under total recirculation mode, using a 0.1 µm ceramic membrane at different temperatures within the cold MF range (10–20 °C).

The authors hypothesised, and the results confirmed, that the addition of calcium to skim milk prior to cold MF would reduce casein concentration in the permeate as it has been shown that calcium concentration influences the ratio of soluble and micellar casein, partly by increasing milk protein hydrophobicity (Rose, 1968; Philippe *et al*., 2003), and thereby reducing the amount of casein in the permeate. At 10 and 14 °C, the strongest effect for β-casein was observed at a level of 5 mM calcium and above; however, addition of >5 mM calcium negatively affected membrane performance and resulted in more extensive deposit formation on the membrane, as well as reducing whey protein permeation.

1.4.2.2 Production of β-casein-enriched ingredients via cold microfiltration

There are different approaches that can be used to generate β-casein and β-casein-enriched whey. There have been numerous studies investigating the permeation of β-casein during cold MF of different feed materials (O'Mahony *et al*., 2014; Le Berre & Daufin, 1994; Schäfer *et al*., 2019). The most common starting material for the generation of β-casein-enriched whey is skim milk; however, other materials/ingredients can also be used, such as whole milk, MCC and sodium caseinate, although the latter is rarely used.

There are two common MF approaches that can be utilized in the manufacture of β-casein (Fig. 1.4). One approach is referred to as 'cold-then-warm' MF, in which skim milk is first subjected to cold MF, often holding at ≤4 °C to facilitate, and maximise, the release of β-casein into the serum phase. Most commonly, a 0.1 µm membrane is used, allowing the serum proteins and serum β-casein to permeate the membrane while the casein micelles are retained, generating a β-casein-depleted

retentate (O'Mahony *et al*., 2014). The β-casein-enriched whey can be used as an ingredient without further fractionation, or it can undergo further processing to produce β-casein concentrate or isolate (van der Schaaf *et al*., 2021). The β-casein-enriched whey can be ultrafiltered and diafiltered to further enrich and demineralise the permeate; for example, O'Mahony *et al*. (2014) achieved this at low temperature (i.e., 1–6 °C) using a 10-kDa molecular weight cut-off membrane and chilled water as the diafiltration media. As previously mentioned, the removal of calcium and other soluble minerals prevent extensive calcium-mediated crosslinking of β-casein and subsequent precipitation. Following this, the concentrated and demineralised permeate is heated (25–50 °C) to increase hydrophobic interactions, resulting in the controlled self-association of β-casein and an increase in particle size (Li *et al*., 2019b). O'Mahony *et al*. (2014) warmed the permeate to 25 °C and passed it through a 0.5 µm polymeric membrane, resulting in the removal of serum proteins while the micellised β-casein was retained, yielding two streams, a β-casein concentrate retentate and an 'ideal' whey permeate.

The second approach can be referred to as 'warm-then-cold' MF, wherein warm MF (~45–55 °C; 0.1–0.5 µm) of the skim milk is performed, removing the serum proteins (permeate) while retaining the casein micelles (retentate) and the β-casein due to hydrophobic interactions within the micelles. Following this, cold MF (i.e., 4 °C) of the micellar casein retentate is performed, yielding a β-casein-depleted MCC retentate and a β-casein permeate. The application of membrane filtration allows for the production of high purity β-casein (up to 96% of total casein); however, yields are typically low (≤20%), although yields can be increased through additional β-casein dissociation and recovery of the β-casein depleted retentate (Atamer *et al*., 2017).

If the feed material for cold MF is MCC rather than skim milk, a β-casein permeate relatively free of serum proteins can be obtained. Schäfer *et al*. (2019) microfiltered MCC at ≤5 °C using a 0.3 µm pore size spiral-wound membrane, heated the β-casein permeate to 50 °C to achieve self-association of the β-casein, and then subjected the heated permeate to UF (10 kDa) to achieve further enrichment of β-casein. As the original permeate contained little or no whey proteins, an additional MF step to remove whey proteins was not required. Thus, cold MF can be exploited by dairy processors to generate innovative ingredients with tailored protein profiles when feed material containing casein is used.

Polymeric membranes such as PVDF, polyacrylonitrile (PAN) or PES are commonly used for the fractionation of caseins. Crowley *et al*. (2015) studied the processing and protein fractionation characteristics of different polymeric membranes during cold MF of skim milk. The authors reported that PES and PVDF membranes, with equivalent pore size, had essentially identical selectivity; however, the PES membrane resulted in higher flux and lower fouling.

1.5 Sustainability

The sustainability of a process must consider its effects on environmental, social, economic, and technical aspects, with capital and operational costs, consumption of energy, footprints, consumption of chemicals, product loss, valorisation of co-products and membrane fouling, some examples of the various criteria that must be considered when assessing the sustainability of membrane processing (Chen, 2018; Kamali *et al*., 2019). In terms of protein fractionation, MF yields less chemical waste, as alternative techniques involve the addition of chemicals (e.g., pH adjustment) or

the generation of large quantities of waste buffer (e.g., chromatography). Considering the factors mentioned and the increasing demand for more sustainable dairy processing, cold MF offers both opportunities and challenges in meeting such requirements. Maximising the active production time of a membrane filtration plant reduces the frequency of cleaning and reduces the consumption of cleaning chemicals and the associated environmental impact. As mentioned throughout this review, cold MF of skim milk offers increased processing times due to reduced fouling and a slower rate of decline in permeate flux. Schiffer and Kulozik (2020) used the data obtained from their study regarding flux stability, pH, and bacterial counts at different temperatures (10, 14, 16, 20 and 55 °C) during the MF of skim milk to approximate the number of production cycles and overall annual process times. The authors reported that possible production times were highest for MF at 10 °C (>24 h), while operation for only 7 h was possible for MF at 55 °C; thus, the number of annual production cycles, and therefore the number of cleaning cycles, was calculated to decrease with decreasing processing temperature. The study highlights that, even though permeate flux at low temperatures is not comparable to that at higher temperatures, the improved microbial quality and sustainable flux enables longer run times and reduced cleaning cycles. Reducing the number of CIP cycles reduces the quantity of water and cleaning agents consumed, reducing the impact of the process on the environment, resulting in a more sustainable process. In addition to this, other factors such as product loss resulting from mixing of product and cleaning solutions and energy requirements need to be considered.

The extent and type of fouling also affects the severity of cleaning required to restore membrane performance to acceptable limits. As discussed, performing MF at high temperatures can result in extensive fouling, as well as the formation of biofilms

produced by thermophilic bacteria such as Bacillus spp., which have been shown to be one of the species most resistant to CIP solutions (Anand & Singh, 2013). Therefore, cold MF of dairy streams may result in shorter cleaning times and use of reduced amounts or strengths of cleaning agents, ultimately reducing the environmental impact of the process, as a more loosely packed fouling layer is produced.

Another important consideration in the sustainability of a process is the energy requirement. One of the factors influencing the mechanical and thermal energy requirements during membrane filtration is the temperature at which filtration is performed. As the temperature of skim milk is decreased, the apparent viscosity increases, which increases the pumping energy requirements for feed and retentate recirculation. Méthot-Hains *et al*. (2016) reported that UF of skim milk required to reach a volume concentration factor of 3.6 at 10 °C resulted in a higher total energy consumption (0.32 kWh/kg) compared to at 50 °C (0.28 kWh/kg); the authors also reported that, at 10 °C, pumping energy was 2.3 times higher than at 50 °C, which can be attributed to the higher viscosity of the feed and retentate during filtration at low temperatures. France *et al*. (2021a) studied the effect of temperature on the mechanical and thermal energy consumption during cold MF of skim milk, and reported that operating at 4 °C resulted in considerably higher (~10.6%) mechanical and thermal energy requirements compared to 12 °C. Therefore, although cold MF offers opportunities to improve the sustainability of the process through reduced CIP cycles and associated lower consumption of chemicals and water as a result of reduced protein and bio-fouling, increased electrical input will contribute to increased operational costs.

1.6 Conclusions

MF has become an integral unit operation within the dairy industry, with the emergence of cold MF driven by increased demand for novel ingredients with enhanced functional properties, improved microbial quality, and improved membrane performance. However, a better understanding of the effect of temperature on deposit formation on membranes, in particular different temperatures within the cold MF range, which can ultimately lead to improved protein permeation and flux, is required. Cold MF can result in improved microbiological quality, longer, more sustainable, processing times and reduced fouling, extending the life span of membranes as well as providing dairy processors opportunities to produce new functional milk-derived protein ingredients, all acting as an incentive to shift from warm to cold MF. However, although increased processing times and reduced frequency of CIP can be achieved through cold MF, high operational costs associated with higher energy consumption, means research is required to reduce the energy consumption of such processes. Recent literature reports promising concepts for improving the MF of dairy streams, such as developments in surface modification (e.g., charged membranes), offering the potential to resolve challenges relating to fouling. For the MF of dairy streams, these and other approaches will form a future role in improving efficiency and therefore the sustainability of cold MF in dairy processing.

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Chapter 2

The plasmin system in milk and dairy products

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2.1 Introduction

The presence of a proteolytic enzyme in fresh, raw milk was first reported by Babcock and Russell (1897) as they studied the decomposition of skim milk and proteolysis of cheese during ripening. The observed proteolysis was attributed to an indigenous enzyme, galactase, with additional researchers reaching a similar conclusion in time (Thatcher and Dahlberg, 1917; Somer, 1938). Other researchers, however, concluded that the observed proteolysis was most likely from bacterial enzymes present in milk (see Humbert and Alais, 1979). Later work verified that the proteolytic activity in milk was not a result of bacterial proteases, but due to a naturally present, indigenous milk proteinase, subsequently named plasmin (Harper *et al*., 1960; Fox, 1982). Bovine plasmin is an alkaline serine proteinase identical to plasmin isolated from blood when compared kinetically (Bastian and Brown 1996), and is analogous to human plasmin in terms of pH stability, inhibition patterns and amino acid sequence (Kaminogawa *et al*., 1972; Reimerdes *et al*., 1981). In blood, the physiological role of plasmin is to break down blood clots; in milk, however, its role is not as clear.

2.2 Plasmin and plasminogen

Bovine plasmin (EC 3.4.21.7), the principal indigenous milk proteinase, displays trypsin-like activity (Harper *et al*., 1960; Kaminogawa *et al*., 1969; Reimerdes *et al*., 1975) with similarities in its activity characteristics (e.g., sensitivity to inhibitors). Plasmin (48,000 Da) exhibits optimum activity at pH 7.4–7.5 and a temperature of 37 °C (Warner and Polis, 1945; Kaminogawa *et al*., 1969, 1972; Richter *et al*., 1979). Hence, the physiological conditions in the mammary glands of bovines are almost ideal for plasmin activity. Bovine plasminogen, the inactive

zymogen of plasmin, is a glycosylated protein containing 786 amino acid residues with a molecular weight of 88,092 Da, based on its amino acid sequence and carbohydrate content (Schaller *et al*., 1985).

Bovine plasminogen consists of two carbohydrate moieties and therefore contains two specific glycosylation sites, like human plasminogen (Schaller *et al*., 1985). Plasminogen contains five characteristic, triple-loop structures, known as kringles, each stabilised by three disulphide bonds, as shown in Fig. 2.1. These kringles contain lysine-binding sites which possess different affinities for lysine but are all critical in the regulation of activation of plasminogen to plasmin, as shown in human plasminogen (Markus *et al*., 1978; Wiman and Collen, 1978; Collen, 1980). The affinity for lysine residues and ω-aminocarboxylic acids such as ε-amino-n-caproic acid (EACA) has been attributed to the structure of these kringles (Wiman and Wallén, 1977; Sottrup-Jensen *et al*., 1978).

The concentration of plasmin in bovine milk is influenced by a number of environmental factors, in addition to the various processing conditions to which milk is subjected (discussed in Section 2.5). Richardson and Pearce (1981) found pasteurised, bovine milk to contain 0.55–2.75 μg plasminogen/ml and 0.14–0.73 μg plasmin/ml, determined based on an enzymatic activity assay. These results were found to be in agreement with those of previous studies (Dulley, 1972; Halpaap *et al*., 1977), with the concentration of plasminogen decreasing and plasmin increasing during storage as plasminogen is converted to plasmin by plasminogen activators. A later study by Benfeldt *et al*. (1995) quantified the amount of plasminogen present in skimmed milk using an enzyme-linked immunosorbent assay (ELISA), and reported values of 1.5 μg plasminogen/ml, comparable to those previously reported.

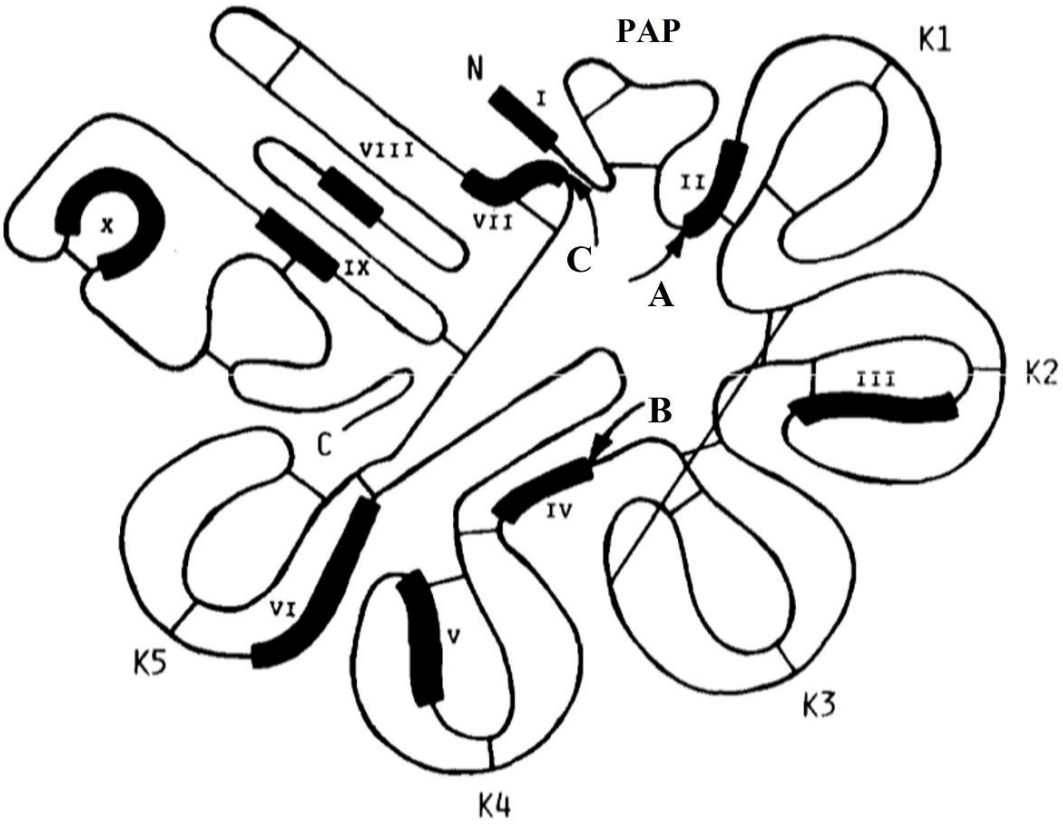


Figure 2.1: *Domain structure of plasminogen isolated from bovine milk. The sequences identified by Benfeldt et al. (1995), approximately 15% of the sequence, are represented by bold lines and Roman numerals. Disulphide bridges are predicted according to homology with human plasminogen. The three arrows, A, B and C, correspond to the sites cleaved by plasmin or by plasminogen activators. Arrow C: The activation cleavage site between kringle 5 and the catalytic domain, cleaved by plasminogen activators (t-PA and u-PA). Arrow A: The autocatalytic cleavage site by plasmin (intra or intermolecular) between* Lys_{77}-Arg_{78}*, releasing the preactivation peptide (PAP). Arrow B: Plasmin cleavage site (intra or intermolecular) in bovine system, creating midi-plasmin (kringle 4 + kringle 5 + catalytic domain) which is probably the most abundant form of active, bovine plasmin (modified from Benfeldt et al. 1995)*

2.2.1 Proteolytic specificity of plasmin on milk proteins

Plasmin has an affinity for lysine and arginine residues, hydrolysing Lys-X and Arg-X bonds, with a preference for peptide bonds containing lysine. Plasmin acts on

all caseins; however, the susceptibility of the caseins to plasmin action varies, with β-casein being the principal substrate for plasmin, followed by α_{s2}-casein.

β-casein

β-Casein is a single polypeptide chain consisting of 209 amino acid residues (Huppertz, 2013) which is hydrolysed by plasmin, producing γ-caseins and proteose peptones (Snoeren and van Riel, 1979; Andrews and Alichanidis, 1983). Plasmin preferentially cleaves β-casein at three specific sites: Lys_{28}-Lys_{-29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}, with Lys_{29}-Ile_{30}, Lys_{113}-Tyr_{114} and Arg_{183}-Asp_{184} also cleaved by plasmin, but to a lesser extent (Fox *et al*., 1994). The cleavage of the three primary sites produces three γ-caseins; γ_1 (β-CN f29-209), γ_2 (β-CN f106-209) and γ_3 (β-CN f108-209), in addition to the corresponding proteose peptones; proteose peptone (PP) 8_{fast} (β-CN f1-28), PP 5 (β-CN f1-105/107) and PP 8_{slow} (β-CN f29-105/107) (Ribadeau-Dumas *et al*., 1972; O'Mahony *et al*., 2013).

α_{s2}-casein

α_{s2}-Casein contains a greater number of plasmin-sensitive peptide bonds compared to β-casein. Plasmin cleaves α_{s2}-casein at eight specific sites, seven of type Lys-X and one Arg-X: Lys_{21}-Gln_{22}, Lys_{24}-Asn_{25}, Arg_{114}-Asn_{115}, Lys_{149}-Lys_{150}, Lys_{l50}-Thr_{15l}, Lys_{181}-Thr_{182}, Lys_{188}-Ala_{189} and Lys_{197}-Thr_{198} (Le Bars and Gripon, 1989; Visser *et al*., 1989). The hydrolysis of α_{s2}-casein by plasmin results in the formation of approximately 14 peptides, with three of the peptides arising from the C-terminus region displaying high hydrophobicity, and therefore potentially being bitter peptides (α_{s2}-CN f198-207, f182-207 and f189-207) (Le Bars and Gripon, 1989).

α_{s1}-casein

Plasmin has also been shown to act on α_{s1}-casein, albeit to a lesser extent than β- and α_{s2}-casein. Aimutis and Eigel (1982) found the action of plasmin on α_{s1}-casein resulted in the formation of λ-casein, a minor casein component. McSweeney *et al*. (1993) found the principal cleavage sites in α_{s1}-casein to be Arg_{22}-Phe_{23}, Arg_{90}-Tyr_{91}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Lys_{105}-Val_{106}, Lys_{124}-Glu_{125} and Arg_{151}-Gln_{152}, with up to 11 additional cleavage sites.

κ-casein

κ-casein is very resistant to plasmin, despite κ-casein containing several lysyl residues; Chen and Ledford (1971) reported that κ-casein was unaffected by plasmin. Eigel (1977) reported no hydrolysis of κ-casein when incubated in the presence of bovine plasmin at 37ºC, conditions which resulted in the complete hydrolysis of α_{s1}-casein. However, Andrews and Alichanidis (1983) reported little hydrolysis of κ-casein by indigenous plasmin in pasteurised milk incubated at 37ºC for 7 d, with 4% of the peptides produced originating from κ-casein. Pihlanto-Leppälä *et al*. (1993) reported the release of some peptides from the N-terminus of κ-casein by plasmin; however, the specificity of plasmin on κ-casein is yet to be determined. Differences in results are most likely due to differences in experimental conditions of the studies, but indicate that limited hydrolysis of κ-casein can occur when exposed to adequate amounts of plasmin for sufficient periods of time.

Whey proteins

Due to the tight, globular structure of whey proteins, they are less susceptible to plasmin action than caseins (Kelly and McSweeney, 2003). A number of studies have reported that β-lactoglobulin and α-lactalbumin are not hydrolysed by plasmin (Chen and Ledford, 1971; Yamauchi and Kaminogawa, 1972; Aslam and Hurley, 1997). However, considerable hydrolysis of β-lactoglobulin by plasmin was reported by Caessens *et al*. (1999a, b), with significant disulphide-sulphydryl exchange occurring during hydrolysis. Aslam and Hurley (1997) identified peptides produced by plasmin-mediated hydrolysis of lactoferrin during involution of the mammary gland. The susceptibility of β-lactoglobulin and α-lactalbumin to plasmin-mediated hydrolysis can be affected by photooxidation and lactosylation (Dalsgaard *et al*., 2007; Dalsgaard *et al*., 2008), causing changes in the structure of the protein and the modification of lysine residues.

2.3 The plasmin system in bovine milk

The plasmin system in milk is a complex protease-protease inhibitor system consisting of the active enzyme (plasmin), the inactive zymogen (plasminogen), plasminogen activators (which convert plasminogen to plasmin), plasminogen activator inhibitors and plasmin inhibitors (with the latter both able to inhibit the activity of plasmin) (Fig. 2.2).

Plasminogen is not expressed in the bovine mammary gland or mammary cells (Berglund *et al*., 1995); therefore, the origin of plasmin and plasminogen in milk is the cow's blood. Plasmin is transferred to bovine milk from blood as the inactive zymogen, plasminogen. As the mammary glands have the ideal physiological

conditions (i.e., correct temperature and pH), there is often sufficient time for plasminogen to be activated and for plasmin to act on its substrate within the mammary gland (Schaar, 1985; Kelly and Fox, 2006) or during storage (Driessen and van der Waals, 1978). The occurrence of γ-caseins, the products of casein hydrolysis as mentioned above, directly after milking is an indication of plasminogen activation and plasmin activity within the udder. The conversion of plasminogen to plasmin is controlled by two main types of plasminogen activators present in milk (Heegaard *et al*., 1993), urokinase-type plasminogen activators (u-PA) and tissue-type plasminogen activators (t-PA). Plasminogen to plasmin ratios can be used to express the extent of activation of plasminogen to plasmin at different times (e.g., different stages of processing or lactation). Plasminogen activator inhibitors and plasmin inhibitors both act to reduce plasmin activity; however, their effect on the plasmin system is often dependent on processing conditions such as thermal treatments (see Section 2.4).

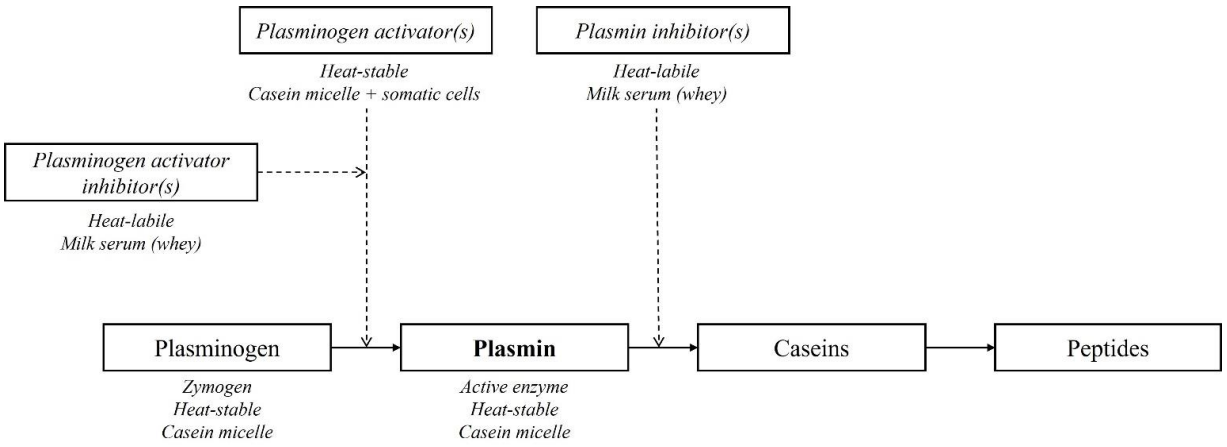


Figure 2.2: *Schematic representation of the plasmin system in bovine milk*

2.3.1 Activators of the plasmin system

Richardson (1983a) found the concentration of plasminogen in milk to decrease during storage at 37 °C for 3 d, with a subsequent increase in plasmin concentration. This phenomenon is caused by the presence of plasminogen activators, which hydrolyse the inactive zymogen (plasminogen) to plasmin. Since plasmin and plasminogen pass from the blood into bovine milk, plasminogen activators and inhibitors present in blood also leak into milk. As plasminogen activators convert plasminogen to plasmin and therefore control the plasmin activity in milk, there has been considerable interest in developing a better understanding of them; particularly t-PA and u-PA, the main plasminogen activator types present in both blood and bovine milk (Lu and Nielsen, 1993a; Bastian and Brown, 1996; Nielsen, 2002).

u-PA is the best characterised plasminogen activator, consisting of two subunits linked by a disulphide bond. It has been shown by researchers, including Ismail *et al*. (2006), that u-PA causes greater plasminogen activation than t-PA. Ismail *et al*. (2006) observed that a small amount of u-PA can cause more plasminogen activation than that caused by a larger amount of t-PA. Thus, urokinase is often the preferred plasminogen activator for the determination of plasminogen in milk. The activities of u-PA and t-PA in milk have been shown to increase in the presence of casein (Markus *et al*., 1993; Politis *et al*., 1995), while the presence of fibrin increases the activity of t-PA (Karlan *et al*., 1987). Markus *et al*. (1993) and Politis *et al*. (1995) reported that all the casein fractions (α-, β- and κ-casein) enhanced plasminogen activation, depending on their relative concentration, with the α-casein fraction inducing the greatest rate of plasminogen activation by both u-PA and t-PA. A later study conducted by Heegaard *et al*. (1997) reported that α_{s2}-casein, specifically dimeric α_{s2}-casein, resulted in a concentration-dependent enhancement of plasminogen activation

by t-PA. From ligand-blotting experiments, the authors showed that both plasminogen and t-PA bound to dimeric α_{s2}-casein, suggesting that the formation of a ternary complex between dimeric α_{s2}-casein, plasminogen and t-PA enhances plasminogen activation. Plasminogen activators are heat-stable molecules, with u-PA being more heat-stable than t-PA (Prado *et al*., 2007), and often survive heat treatments used in dairy processing such as pasteurisation and ultra-high temperature (UHT) treatments. Lu and Nielsen (1993a) showed that plasminogen activators may be even more heat-stable than plasmin and plasminogen. Therefore, plasmin activity can increase on heat treatment, as inhibitors of the plasmin system are often heat-labile.

t-PA and u-PA are serine proteinases (Grufferty and Fox, 1988a), which activate bovine plasminogen by hydrolysing the Arg_{557}-Ile_{558} bond between kringle 5 and the catalytic domain (arrow c in Fig. 2.1) (Schaller *et al*., 1985; Benfeldt *et al*., 1995). Bovine plasminogen contains an N-terminal aspartic acid and therefore native bovine plasminogen is commonly referred to as Asp-plasminogen (Fig. 2.3). Plasmin autocatalytically cleaves off the first 77 residues of bovine plasminogen at the Lys_{77}-Arg_{78} bond, leaving an N-terminus arginine (Schaller *et al*., 1985), producing the proteolytically-modified Arg-plasminogen. However, plasmin cannot cleave the Arg_{557}-Ile_{558} bond to activate plasminogen. Instead, the release of the 77-residue fragment of plasminogen, known as the preactivation peptide, causes a conformational change in Arg-plasminogen (Grufferty and Fox, 1988a). This change in conformation of plasminogen exposes the Arg_{557}-Ile_{558} bond, making it more accessible to plasminogen activators, subsequently allowing activation of Arg-plasminogen into active plasmin (Wallén and Wiman, 1975; Collen *et al*., 1985; Schaller *et al*., 1985). The cleavage of Arg_{557}-Ile_{558} bond by u-PA or t-PA produces an active, disulphide-linked, two-chain molecule (plasmin), consisting of a heavy and light chain (Fig. 2.1).

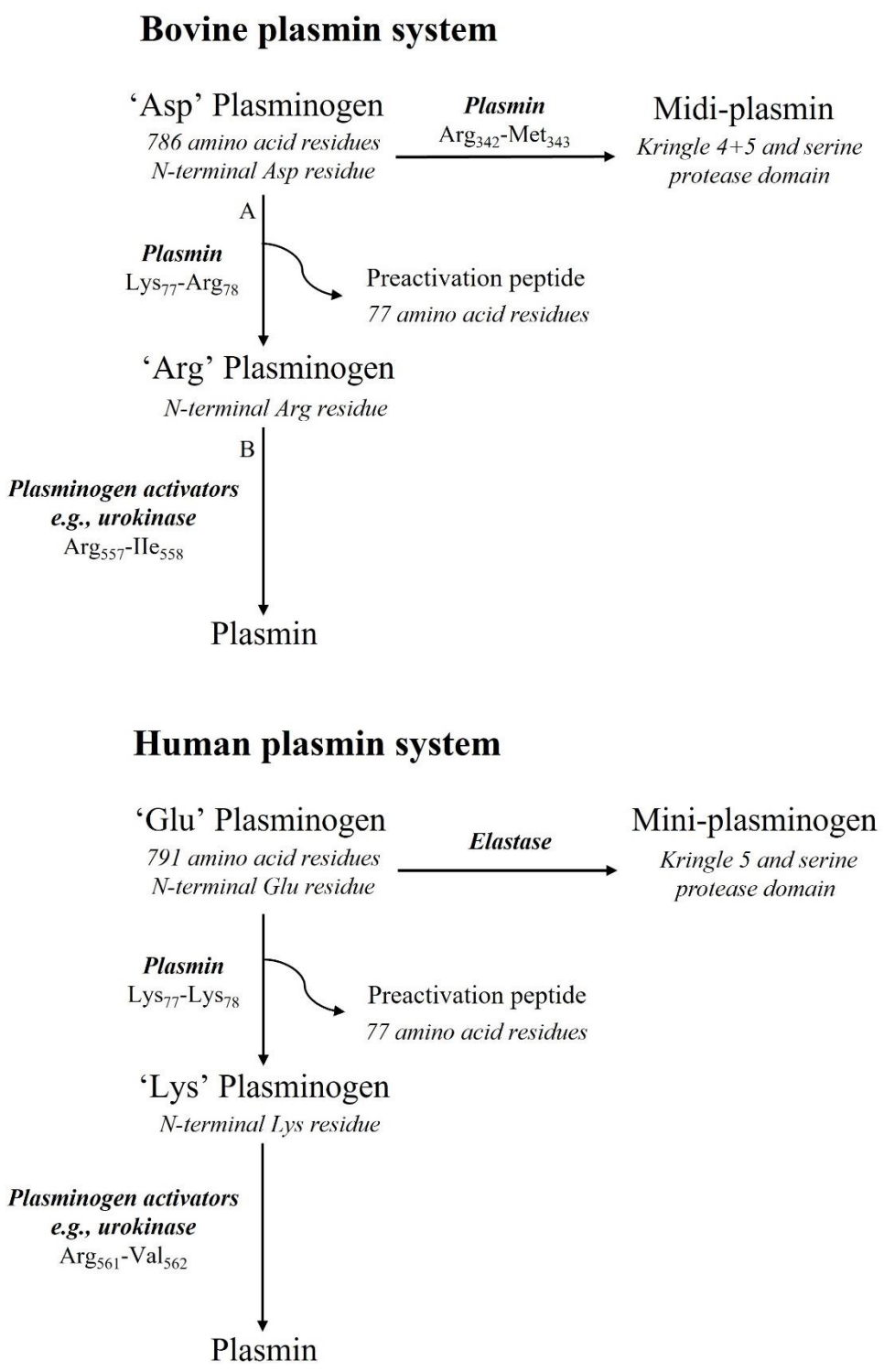


Figure 2.3: *Schematic representation of the activation of bovine and human plasminogen as a comparison. Cleavage of A can occur before (inter-molecular) or after (intra-molecular) cleavage at B. Details of cleavage sites in human plasminogen obtained from Castellino and Ploplis (2005) and Booth and Backmann (2006).*

The active site of plasmin is located on the light chain (Wallén, 1978), consisting of His_{598}, Asp_{641}, and Ser_{736}, (Schaller *et al*., 1985), like that of trypsin; the heavy chain consists of the kringles. Benfeldt *et al*. (1995) reported that bovine plasmin (midi-plasmin) kringles 4 and 5 and the light chain had relative molecular weights of 50,000, 30,000 and 26,000 Da, respectively, determined from sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); bovine plasmin appeared to be smaller than human plasmin.

In addition to the activation of plasmin, limited hydrolysis and autolysis of plasminogen/plasmin can result in the formation of micro-, mini- or midi-plasmin/plasminogen. Cleavage of human Lys-plasminogen by plasmin results in the formation of micro-plasminogen, which consists of only the serine proteinase domain (light chain) (Shi and Wu, 1988). In addition, autolytic cleavage of human plasmin results in the formation of micro-plasmin, which consists of the serine proteinase domain and a 31-residue peptide from the heavy chain connected by disulphide bonds (Wu *et al*., 1987). Mini-plasminogen consists of kringle 5 and the serine proteinase domain (Sottrup-Jensen *et al*., 1978), produced by limited hydrolysis of plasminogen by elastase. Bovine midi-plasmin contains kringle 4, kringle 5 and the serine proteinase domain (Benfeldt, 1992; Christensen *et al*., 1995b), produced from the cleavage of the Arg_{342}-Met_{343} bond between kringle 3 and 4 by plasmin (arrow b in Fig. 2.1); Benfeldt *et al*. (1995) suggested that midi-plasmin is likely to be the naturally occurring form of plasmin in bovine milk.

2.3.2 Inhibitors of the plasmin system

The plasmin system in milk is regulated by both plasminogen activator inhibitors and plasmin inhibitors. Plasminogen activator inhibitors slow down the conversion of plasminogen to plasmin, while plasmin inhibitors directly inhibit plasmin activity. Plasmin system inhibitors are present in the serum phase of milk (whey) (Reimerdes *et al*., 1976; Korycha-Dahl *et al*., 1983), and their activity is affected by pH and heat treatments to a greater extent than the activators of the plasmin system. The mechanism of action of proteinase inhibitors, many of which belong to the serpin family, is not yet fully understood. Despite this, it is known that exposed, reactive-site loops in the C-terminus of the serpin interact with the active site of plasmin, potentially blocking or altering the shape of the active site (Christensen and Sottrup-Jensen, 1994).

Unlike plasminogen activators, less information is available on the inhibitors of the plasmin system in milk. However, it can be assumed that the plasmin inhibitors controlling the plasmin system in milk are similar to those in the blood stream, as the source of the plasmin system is the blood stream. Many of the serine proteinase inhibitors that have been studied in detail are trypsin inhibitors (Reimerdes *et al*., 1976). Precetti *et al*. (1997) partially purified two serine proteinase inhibitors from bovine milk, plasminogen activator inhibitor (plasminogen activator inhibitor 1) and a plasmin inhibitor (α_2-antiplasmin), finding both inhibitors to be present in several forms, which they attributed to the possible formation of complexes with other milk proteins.

Several blood-derived proteinase inhibitors have been detected in bovine milk, many of which are serpins, including C1-inhibitor (C1 Inh), antithrombin-III, inter-α-

trypsin inhibitor, α_2-macroglobulin (α_2-M), α_1-proteinase inhibitor and α_2-antiplasmin (α_2-AP) (Lindberg, 1979; Weber and Nielsen, 1991; Christensen and Sottrup-Jensen, 1992; Christensen *et al*., 1995a). The principal inhibitor of plasmin is considered to be α_2-AP (Wiman and Collen, 1978; Levi *et al*., 1993). α_2-AP is a single-chain glycoprotein consisting of two disulphide bridges with a molecular weight of 70,000 Da. Bovine α_2-AP shares close homology with human α_2-AP, especially at the reactive site and N-terminus (Christensen and Sottrup-Jensen, 1992). The effective plasmin-inhibiting ability may be due to the strong complex formed between plasmin and α2-AP through the lysine-binding sites of plasmin (Wiman and Collen, 1978). Christensen and Sottrup-Jensen (1994) isolated a proteinase inhibitor of trypsin (TI) from bovine plasma and milk, which had the ability to inhibit plasmin in addition to trypsin, even though it showed little similarity to α_2-AP. Interactions between plasminogen activators and plasminogen activator inhibitors, as well as between plasmin inhibitors and plasmin, provide a complex mechanism for the control of plasmin activity in bovine milk.

2.4 Distribution of plasmin system components in milk

The components of the plasmin system are distributed between the serum phase (whey) and the micellar phase (casein) of milk (Bastian and Brown, 1996; Politis *et al*., 1992). The distribution of plasminogen activators and plasminogen activator inhibitors in bovine milk is known; however, there have been many conflicting results over the years as to which plasminogen activator is primarily associated with casein micelles.

Lu and Nielsen (1993a) indicated that plasminogen activators u-PA and t-PA are associated with the casein micelle, while other studies have suggested that both u-PA

and t-PA are associated with somatic cells (Politis *et al*., 1991; Zachos *et al*., 1992). Many researchers have agreed that the plasminogen activators, u-PA and t-PA, are predominantly associated with somatic cells (*via* a urokinase receptor) and casein micelles, respectively (Heegaard *et al*., 1993, 1994a, b; White *et al*., 1995); Heegaard *et al*. (1994b) assessed the binding of t-PA to native casein micelles and purified preparations of the individual caseins; they concluded that t-PA bound to dimeric α_{s2}-casein and multimeric κ-casein, suggesting that t-PA binds to casein micelles *via* electrostatic interactions. White *et al*. (1995) isolated the plasminogen activators from both the casein micelles and somatic cells, confirming that t-PA is the main plasminogen activator associated with micelles and that u-PA is associated with the somatic cells. Ismail *et al*. (2006) showed u-PA activity to be highest in milk with high somatic cell count (SCC), confirming results from previous researchers that u-PA is associated with somatic cells. Results also confirmed that both u-PA and t-PA associate with caseins, and that u-PA has the ability to dissociate from somatic cells and re-associate with micelles.

Unlike plasminogen activators, plasmin inhibitors are found only within the serum phase (whey) (Reimerdes *et al*., 1976; Korycha-Dahl *et al*., 1983; Christensen and Sottrup-Jensen, 1994; Christensen *et al*., 1995a; Precetti *et al*., 1997). Korycha-Dahl *et al*. (1983) found that plasminogen activators were only associated with casein micelles while inhibitors of both plasmin and plasminogen activators were found solely in the serum phase. Both plasmin and plasminogen are primarily associated with the casein micelles (Kaminogawa *et al*., 1972; Korycha-Dahl *et al*., 1983; Richardson, 1983a). Politis *et al*. (1992) found that 82% of total plasminogen was associated with casein micelles, while the remainder was present in the serum phase. The conversion of plasminogen to plasmin does not cause dissociation of plasmin from the casein

matrix (Baer *et al*., 1994); therefore, the presence of plasmin in whey is due to certain factors causing its dissociation from the micelle (see Section 2.4.1). Numerous researchers have conducted plasmin activity assays on different milk fractions, all finding plasmin-casein interactions (Reimerdes *et al*., 1975; Snoeren and van Riel, 1979; Fox, 1981; Politis *et al*., 1992). Hofmann *et al*. (1979) found plasmin associated with the milk fat globule membrane (MFGM): components of the MFGM displayed protease activity, with plasmin being the dominant protease present. Politis *et al*. (1992) also found plasminogen to be present within the MFGM; however, the membrane-associated plasminogen is believed to be a result of small amounts of casein present within the MFGM, as plasminogen is strongly associated with casein micelles; this was later confirmed by Benfeldt *et al*. (1995).

Early studies showed that plasmin remains associated with casein micelles after centrifugation (Reimerdes and Klostermeyer, 1974), isoelectric precipitation (acid casein) (Warner and Polis, 1945; Kiermeier and Semper, 1960) and rennet coagulation (i.e., rennet casein) (Richardson and Elston, 1984), indicating strong plasmin-casein interactions. Plasminogen, plasmin and plasminogen activators bind to casein micelles through lysine-binding sites and, to a lesser extent, through electrostatic interactions (Grufferty and Fox, 1988a; Baer *et al*., 1994). In blood, lysine-binding is also important for the plasminogen-fibrin complex (Wiman and Collen, 1978). The amount of plasminogen bound to the casein micelles depends on the type of casein, with Baer *et al*. (1994) observing that immobilised α-casein bound twice as much plasminogen as immobilised β- and κ-casein.

2.4.1 Factors affecting the distribution of plasmin system components within milk

The distribution of plasmin system components within the different milk fractions is affected by several environmental and non-environmental factors, some of which cause complete dissociation from the micelle. The addition of lysine or 6-aminohexanoic acid/ε-amino-n-caproic acid (EACA) (a lysine derivative) at concentrations up to 50 mM dissociates plasmin from casein (Korycha-Dahl *et al*., 1983; Richardson, 1983a); however, at this concentration, it can increase the rate of plasminogen activation, as both ligands bind to plasminogen, altering its conformation, thereby enhancing cleavage of the preactivation peptide (Walther *et al*., 1975; Searls, 1980).

Plasmin-casein association is strongly influenced by pH. The acidification of fresh bovine milk to pH 4.6–4.7 caused almost complete dissociation of plasmin from the casein micelles, with plasmin beginning to dissociate from the micelle at pH 5.7 (Richardson and Elston, 1984). Grufferty and Fox (1988b) also found that, at pH 4.6, most of the plasmin was dissociated; however, they observed no reduction in plasmin-micelle activity between pH 6.6 and pH 4.8. The discrepancies observed lie in the preparation of the casein micelles, as Richardson and Elston (1984) isolated casein by isoelectric precipitation before assaying the casein for plasmin activity, while Grufferty and Fox (1988b) utilised ultracentrifugation to isolate the casein micelles. The dissociation of plasmin from the casein micelle to the serum phase can potentially cause negative effects on the quality of food products containing whey protein ingredients due to residual plasmin activity. Hayes and Nielsen (2000) and Crudden and Kelly (2003) studied plasmin activity in acid and sweet whey, finding significantly higher plasmin activity in acid whey compared to sweet whey. Hayes and Nielsen (2000) suggested the higher plasmin activity in acid whey may be due to the disruption

of the casein micelles at the low pH. Crudden and Kelly (2003) analysed plasminogen-derived activity in whey, finding low levels of plasminogen in acid whey, suggesting that activation of plasminogen may have partly contributed to the high plasmin activities. Crudden and Kelly (2003) also reported an inverse relationship between plasmin activity in whey and the rate of acidification. The effect of pH on the dissociation of plasmin can help explain the higher levels of plasmin observed in acid whey (44 μg/g protein), compared to sweet whey (4 μg/g protein) (Ismail and Nielsen 2010).

The addition of NaCl to milk has also been reported to cause the dissociation of plasmin from casein micelles (Johnson, 1974; Grufferty, 1986). The effect of temperature on plasmin dissociation is unclear, with conflicting evidence regarding its effect. A report by Reimerdes and Herlitz (1979) found an increase in plasmin activity in milk stored at 4 °C. However, Donnelly and Barry (1983) concluded that temperature had no significant influence on plasmin-casein interactions, with the work of Grufferty and Fox (1988b) supporting such findings.

2.5 Factors affecting plasmin activity and consequent proteolysis

The concentration of plasmin and plasminogen in bovine milk is affected by numerous factors including environmental factors (Politis *et al*., 1989a), processing conditions (Korycha-Dahl *et al*., 1983; Kelly and Foley, 1997; Aaltonen and Ollikainen, 2011; García-Risco *et al*., 2003), storage conditions (Crudden *et al*., 2005a) and the presence of microbial proteases (Nielsen, 2002). The plasmin system is complex, involving a multitude of interactions that ultimately result in the activation of plasminogen to plasmin. The system is made more complex by the interference of

milk components with plasmin and plasminogen under different conditions (pH, storage temperature, heating). The conditions mentioned ultimately affect the kinetics of the plasmin system, altering the rate/extent of plasminogen activation, or hydrolysis of caseins by plasmin. The effect of these conditions and the subsequent interactions with the different plasmin system components are discussed below.

2.5.1 Mastitis

Mastitis is an inflammation of the mammary gland caused by injury to the udder, trauma or bacterial infection (Oliver *et al*., 2011). Plasmin activity is higher in milk during mastitis (Barry and Donnelly, 1981; de Rham and Andrews, 1982; Andrews, 1983a; Schaar and Funke, 1986; Politis *et al*., 1989a). Barry and Donnelly (1981) observed that the increased proteolytic activity observed in mastitic milk was primarily due to plasmin, with proteinases from other sources (such as leukocytes) representing only a small fraction of total proteolytic activity. Studies conducted by de Rham and Andrews (1982) and Andrews (1983a) also reported that plasmin activity increased in mastitic milk, but did not have as great a contribution to total proteolysis as previously reported by Barry and Donnelly (1981). Politis *et al*. (1989b) reported an increase in the concentration of plasmin and plasminogen, by 105 and 74%, respectively, as the SCC increased from less than 250,000/ml to over 1,000,000/ml. Previous work also showed an increase in plasmin activity as SCC increased from 100,000 to 1,300,000/ml (Politis *et al*. 1989a).

The increased plasmin activity in mastitic milk can be attributed to a number of factors, often due to the leakage of blood components into milk as a result of a compromised blood-milk barrier. During mastitis, there is an increase in the SCC (i.e., white blood cells) of milk to help combat the infection/inflammation. Leucocytes

contain plasminogen activators (Christman *et al*., 1977), which they can secrete; therefore, the secretion of these plasminogen activators may contribute to some of the increased plasmin activity observed in mastitic milk. Plasminogen activators are also produced by activated neutrophils and macrophages (Mullins and Rohlich, 1983), which can increase the rate at which plasminogen is converted to plasmin in mastitic milk, thereby increasing plasmin activity. Heegaard *et al*. (1994a) reported a 10- to 20-fold increase in the content of plasminogen activators in mastitic milk. The authors also observed an increase in both u-PA and t-PA in mastitic milk; somatic cells appeared to have a larger number of u-PA receptors compared to that in uninfected milk. The large increase in SCC during mastitis, coinciding with an increase in plasminogen activators, helps explain increased plasmin activity in mastitic milk. Bacterial mastitis can also contribute to increased plasmin activity, as specific bacteria such as *Streptococcus uberis* can contain/secrete plasminogen activators (discussed in Section 2.5.9).

2.5.2 Stage of lactation

Throughout lactation, the plasmin activity fluctuates. Late lactation is associated with a higher level of plasmin activity (Barry and Donnelly, 1980; Andrews, 1983a; Donnelly and Barry, 1983; Richardson, 1983b; Schaar, 1985; Politis *et al*., 1989a, b), resulting in increased proteolysis of caseins. A study by Barry and Donnelly *et al*. (1980) showed that there is less β- and α_{s1}-casein, and more γ-casein, in late-lactation milk than in mid-lactation milk, indicative of higher plasmin activities. The reason for this increased plasmin activity in late-lactation milk has been disputed between researchers. Richardson (1983b) reported that plasminogen and plasmin concentration in late-lactation milk increased and suggested that the increased plasmin activity

observed was due to more plasmin entering the mammary gland, rather than increased activation of plasminogen. On the other hand, Korycha-Dahl *et al*. (1983) reported that the increased plasmin activity is likely due to increased plasminogen levels, observing a twofold increase in plasminogen concentrations in late-lactation milk compared to mid-lactation. Schaar (1985) reported similar findings to Richardson (1983b), observing a gradual increase in plasmin activity with advancing lactation. Politis *et al*. (1989b) reported an increase in plasmin activity in late-lactation milk; however, they concluded that this increase was predominately due to an increase in plasminogen activators. They observed a decrease in the ratio of plasminogen to plasmin from early lactation (6.55) to late lactation (3.29). Bastian *et al*. (1991a) found plasmin activity to increase steadily at the beginning of lactation, before increasing rapidly towards the end of lactation, with a corresponding increase in the percentage of plasminogen activated.

These studies indicate that there is an increase in the rate of plasminogen activation to plasmin in late-lactation milk, potentially due to increased plasminogen activators, and suggested that the leakage of plasmin from the blood into milk increases in early lactation but remains constant throughout lactation. It is known that there is an increase in the permeability of epithelial cells in mammary glands during late lactation, resulting in increased permeability of somatic cells (known to contain plasminogen activators) and other constituents originating from blood. Therefore, it is likely that increased plasminogen activation, caused by increased permeability of mammary gland tissue due to a compromised blood-milk barrier, have a significant impact on the higher plasmin activity seen in late-lactation milk.

2.5.3 Age of cow

Plasmin activity is higher in milk from older cows (Schaar, 1985; Politis *et al*., 1989a; Bastian *et al*., 1991a) but, although plasmin activity is higher in milk from older cows, plasminogen levels remain relatively constant. Therefore, similar to late-lactation milk, the increase in plasmin activity is likely due to increased permeability of the secretory tissue, increasing the presence of plasminogen activators. In addition to this, older cows are likely to have experienced more mastitis in their lifetime; it has been reported that plasmin activity in milk does not return to levels prior to the onset of mastitis (Saeman *et al*., 1988), and therefore this may be responsible for the higher plasmin activity in milk from older cows.

2.5.4 Breed of cow

The concentration of plasmin in milk can vary depending on the breed of the cow. Richardson (1983b) found milk of Holstein-Friesian cows (0.27–0.53 mg/L) to have a higher plasmin activity than milk of Jersey cows (0.15–0.37 mg/L). Schaar (1985) reported similar findings, with Jersey cows producing milk with the lowest plasmin activity, while cross-bred Swedish Friesian-Swedish Red and White cows possessed the highest activities. However, when the effect of casein content was considered, it was found that the differences in plasmin between breeds was mainly due to differences in their relative amount of casein. Schaar (1985) reported a negative correlation between casein content and plasmin activity, previously observed by Richardson and Pearce (1981), and suggested that this finding was due to the competition between casein and the synthetic substrate used for the analysis of the enzyme. A study by Bastian *et al*. (1991b) reported that casein interferes with plasmin assays when synthetic substrates are used, acting as a competitive inhibitor. However,

they found that, by reducing the casein to substrate ratio, such interference could be avoided. Bastian *et al*. (1991a) found no significant differences in the plasmin activity of milk collected from Jersey and Holstein-Friesian cows once the casein to substrate ratio was adjusted to prevent any interference by casein.

2.5.5 Milk pH

The activity of plasmin depends strongly on the pH of its environment (i.e., milk, cheese). There have been several studies conducted to determine the optimum pH of plasmin over the years (Kiermeier and Semper, 1960; Carini and Bozzolati, 1970; Chen and Ledford, 1971; Kaminogawa *et al*., 1972), with many reporting different optimum pH ranges (see Humbert and Alais, 1979). It is now generally accepted that the optimum pH for plasmin is between 7.5–8 (Fox, 1981), but it is recognised that plasmin remains active and stable over a broad pH range. Decreasing the pH causes a decrease in the activity of plasmin as it moves away from its optimum, as well as causing the dissociation of plasmin from the micelle (as discussed in Section 2.4.1).

2.5.6 Heat treatment

Plasmin is a thermostable enzyme capable of surviving many of the heat treatments applied to dairy products (Kiermeier and Semper, 1960; Dulley, 1972; Prado *et al*., 2006). The severity of the heat treatment of milk depends on various factors including the desired shelf life of the product and the end use of the milk. The wide range of heat treatments that can be applied to milk, in addition to plasmin system components varying in their heat stabilities, further highlights the complexity of this system. Pasteurisation is a mild heat treatment (72 °C for 15–30 s) employed in the dairy industry to kill vegetative pathogenic microorganisms, thereby extending the

shelf-life of the milk. Noomen (1975) reported an increase in the rate of proteolysis of 30–40% and 8–24% after milk had been heated to 75 °C for 15 s and 63 °C for 30 min, respectively. Richardson (1983a) and Korycha-Dahl *et al*. (1983) reported that pasteurisation of fresh milk at 72 °C for 15 s decreased plasmin content by 17 and 10%, respectively. Although there may be a reduction in the plasmin content on heating, plasmin activity after such a heat treatment increases (de Rham and Andrews, 1982; Korycha-Dahl *et al*., 1983; Richardson, 1983a). Richardson (1983a) suggested that the increase in plasmin activity was due to the inactivation of plasminogen activator inhibitors (Prado *et al*., 2006), resulting in increased plasminogen activation, as the decrease in the concentration of plasminogen was greater than the increase in plasmin activity.

Although the increase in plasmin activity on mild heat treatments has been attributed to the inactivation of plasminogen activator inhibitors and plasmin inhibitors, other factors such as improved efficiency of activation of denatured plasminogen or the thermal stabilities of the plasminogen activators (u-PA and t-PA), could contribute to the increased plasmin activity (Ismail and Nielsen, 2010). The denaturation temperature of plasminogen has been reported to range from 50.1–61.6 °C (Castellino *et al*., 1981; Burbrink and Hayes, 2006). In this temperature range, plasminogen becomes more accessible to the action of plasmin, due to conformational changes and the partial unfolding of the kringles, exposing the Lys_{77}-Arg_{78} bond (Burbrink and Hayes, 2006). Prado *et al*. (2006) found almost complete inactivation of plasminogen activator inhibitors on heating milk to 75 °C for 15 s, with plasmin inhibitors being less affected by the heat treatment. A later study by Prado *et al*. (2007) explored the difference in the thermal stabilities of the plasminogen activators u-PA and t-PA. u-PA displayed greater heat stability than t-PA when milk was heated to

85 °C for 30 s, with t-PA losing half of its activity, while u-PA lost only 30%. The sensitivity of plasmin inhibitors to mild heat treatments, in addition to the higher heat stability of plasminogen activators, may explain the increased plasmin activities in milk subjected to pasteurisation-like conditions.

As discussed earlier (Section 2.4.1), plasmin activity in acid whey is higher than in sweet whey, with Crudden *et al*. (2005b) having assessed the thermal behaviour of plasmin in acid and sweet whey. Plasmin inactivation, in both acid and sweet whey, followed first-order inactivation kinetics, which was consistent with the findings of plasmin inactivation in buffers, model milk systems and milk (Alichanidis *et al*., 1986; Rollema and Poll, 1986; Kennedy and Kelly, 1997; Saint Denis *et al*., 2001a). Crudden *et al*. (2005b) reported D-values (the time required for a 90% reduction in activity) of 108 ± 29 min and 0.021 ± 0.006 min for the inactivation of plasmin in acid and sweet whey at 90 °C, respectively. In comparison, D-values for plasmin inactivation in milk of 59 s at 90 °C (Kennedy and Kelly, 1997) or 71 s at 90 °C (Saint Denis *et al*., 2001a) had previously been reported. Crudden *et al*. (2005b) attributed the higher heat stability of plasmin in acid whey to reduced sulphydryl/ disulphide interchange between plasmin and β-lactoglobulin, due to higher heat stability of β-lactoglobulin in acid whey, and to structural changes in plasmin.

More severe heat treatments applied to milk include ultra-high-temperature (UHT) processing and in-container sterilisation. In these processes, the heat treatment applied, involves time-temperature combinations of 135–150 °C for 1–5 s and 115–120 °C for 12–20 min for UHT and in-container sterilisation, respectively (Spreer, 1998). Driessen and van der Waals (1978) reported D-values of 7 s for plasmin at 142.5 °C, indicating that plasmin is capable of surviving most UHT treatments. They

found that, in order to fully inactivate plasmin, temperatures as high as 142 °C for 18 s were needed. de Koning *et al*. (1985) found no proteolysis in UHT-sterilised, evaporated milk produced from milk that was pre-heated for 3 min at 120 °C. A later study by Saint Denis *et al*. (2001a) reported D-values of 13 and 11 s for indigenous plasmin and plasminogen in bovine milk, respectively, at 140 °C. Studies have also shown that plasminogen activators not only survive mild heat treatments but also survive UHT treatments (Kelly and Foley, 1997); D-values of 16 s at 140 °C have been reported for plasminogen activators in bovine milk. Kelly and Foley (1997) concluded that the increase in plasminogen activation in UHT milk with a high SCC was most likely due to u-PA. Later work by Prado *et al*. (2007) supported these conclusions, reporting that u-PA is more heat stable than t-PA.

It can be concluded that the plasmin system is not completely inhibited by high temperature treatments; the system in which plasmin occurs (i.e., milk or acid whey) also has an influence on its thermal stability. The presence of remaining plasminogen and plasminogen activators, in addition to the destruction of plasmin inhibitors, indicates that some residual plasmin activity remains after heat treatment. The studies mentioned above give an indication of the complexity with which plasmin activity is influenced by heat treatments, and how the shelf-life of milk-based products, from an enzymatic perspective, can ultimately be influenced by the specific heat treatment applied.

2.5.7 Presence of β-lactoglobulin

As discussed previously (Section 2.5.6), heat treatment has a significant impact on the plasmin system, with the components of the system varying in their thermal

stabilities. However, heating also results in the interaction of plasmin system components with milk constituents. Evidence in the literature states that whey proteins have the ability to reduce plasmin activity (Rollema and Poll, 1986; Bastian *et al*., 1993; Grufferty and Fox, 1986; Kelly and Foley, 1997; Enright and Kelly, 1999; van Asselt *et al*., 2008). A study by Alichanidis *et al*. (1986) showed that, in the absence of β-lactoglobulin, both plasmin and plasminogen are heat-stable, indicating that β-lactoglobulin interacts with plasmin. β-Lactoglobulin, the predominant whey protein in bovine milk, contains a free sulphydryl (-SH) group buried within its globular structure. On heating to >55 °C, the 3-dimensional structure begins to unfold, exposing the sulphydryl group on Cys121 (Sawyer 2003). The subsequent decrease in plasmin activity is believed to be due to the free sulphydryl group of β-lactoglobulin interacting with the disulphide bonds which link subunits within the plasmin molecule *via* thiol disulphide interchange (Grufferty and Fox, 1988a; Kelly and Foley, 1997). Chemical modification of β-lactoglobulin, such that there are no free -SH groups, results in no inhibition of plasmin, verifying the role of -SH groups in plasmin inhibition. It has been shown that, at temperatures lower than 65 °C, plasmin is reversibly inactivated, only becoming irreversibly inactivated once temperatures surpass 65 °C (Metwalli *et al*., 1998).

Both native β-lactoglobulin (Kaminogawa *et al*. 1972; Rollema and Poll, 1986; Bastian *et al*., 1993) and denatured β-lactoglobulin (Snoeren *et al*., 1980; Bastian *et al*., 1993) inhibit the action of plasmin, with some studies reporting native β-lactoglobulin to have a greater inhibitory effect (Bastian *et al*., 1993). Rollema and Poll (1986), on the other hand, showed that heat denatured β-lactoglobulin inhibited plasmin activity to a greater extent than native β-lactoglobulin, in a solution consisting of casein micelles suspended in simulated skim milk ultrafiltrate (SMUF). β-

Lactoglobulin has also been shown to affect plasminogen. The conformational change that occurs in plasminogen on heating may promote interactions with denatured β-lactoglobulin (Burbrink and Hayes, 2006). Burbrink and Hayes (2006) observed a decrease in plasminogen-derived plasmin at high temperatures (>62 °C) and concluded that the formation of plasminogen-β-lactoglobulin complexes was responsible; β-lactoglobulin, when bound to plasminogen, can interfere with the activation of plasminogen to plasmin, in addition to affecting the accessibility of plasminogen activators to plasminogen.

In a milk system comprising of proteins with free -SH groups (i.e., β-lactoglobulin), plasminogen activators have been found to be as thermally sensitive as plasmin and plasminogen on heating (Saint Denis *et al*., 2001a). Rippel *et al*. (2004) studied the effect of native and denatured proteins on plasminogen activators, concluding that both α-lactalbumin and β-lactoglobulin increased plasminogen activator activity. Kinetics experiments indicated that both native and denatured α-lactalbumin, and denatured β-lactoglobulin, increased the catalytic efficiency of u-PA, suggesting that the whey proteins have the ability to facilitate the release of u-PA from active plasmin - a phenomenon which occurs much more slowly in their absence.

The effect of β-lactoglobulin on the plasmin system depends on the concentration of native or denatured β-lactoglobulin present, and its effect on plasmin inactivation is reduced during severe heat treatments, as the presence of -SH groups available for disulphide bonding decreases.

2.5.8 Storage temperature of milk

Many dairy-based products are refrigerated (≤5 °C) to reduce microbial growth and extend shelf life, while some products in which microbial growth is not of concern are stored at room temperature (20–25 °C). The temperature at which these dairy based products are stored can have a significant impact on plasmin activity and can influence the extent of proteolysis.

2.5.8.1 Cold storage

The temperature at which milk is stored prior to processing can greatly affect plasmin activity and the quality of products made from the resulting milk. In industry, milk is primarily stored at low temperatures (2–5 °C) prior to processing. Milk is also rapidly cooled from 37 to ≤5 °C on the farm immediately after milking, and stored at this temperature until collection. Proteolysis of caseins by plasmin action before processing can impact products made from that milk (i.e., proteolysis prior to cheesemaking can reduce cheese yield). After pasteurisation, milk is also cooled and stored at refrigeration temperatures for extended periods of time. The extended storage of milk at low temperatures can therefore affect milk quality by affecting the plasmin system, and also due to endogenous proteinases produced as a result of the growth of psychrotrophic microorganisms.

The optimum temperature for plasmin activity is 37 °C; however, plasmin can remain active at cold temperatures. A study by Donnelly and Barry (1983) characterised the rate of conversion of β-casein to γ-casein at 4 and 37 °C, finding the activity in milk stored at 37 °C to be approximately 20 times higher than that stored at 4 °C. However, a previous study indicated that plasmin activity in milk stored at 4 °C is higher than that stored at 26 °C (Reimerdes and Herlitz, 1979). They came to the

conclusion that the higher activity at 4 °C was due to the dissociation of β-casein and plasmin from the micelle into the serum phase, thus allowing increased proteolysis due to closer proximity of plasmin and its substrate. Subsequent studies have confirmed that, during cold storage, dissociation of β-casein from the micelle does occur (Davies and Law, 1983; Dalgleish and Law, 1989). Crudden *et al*. (2005a) studied plasmin activity in raw milk at 37, 20 and 5 °C, reporting plasmin activity to be highest at 37 °C initially, but decreasing more rapidly during storage than at the other temperatures. This was in agreement with the findings of Huppertz *et al*. (2004a), who studied plasmin activity of high pressure-treated bovine milk stored at 37 °C, attributing the decrease to the autolysis or self-hydrolysis of plasmin. The greatest degree of dissociation of β-casein was found at 5 °C; however, hydrolysis of β-casein was highest at 37 °C, with higher amounts of intact β-casein at the lower temperatures. Like plasmin, plasminogen activators can be active at refrigeration temperatures. The initial high plasmin activity at 37 °C, corresponding to high initial proteolysis, was attributed to conversion of plasminogen to plasmin by plasminogen activators.

Schroeder *et al*. (2008) evaluated the effects of both cold storage temperature and calcium on the plasmin system. Milk was stored at 2.2, 3.3 and 4.4 °C, with the latter resulting in higher plasmin activity and plasminogen activation. Under cold storage conditions, the solubilisation of colloidal calcium phosphate within casein micelles occurs, increasing the concentration of calcium (Ca) in the serum phase (Fox *et al*., 2015). The higher serum Ca level found at the coldest temperature (2.2 °C) corresponded to decreased plasminogen activation (Schroeder *et al*., 2008). Subsequent reaction kinetics experiments showed that calcium had no impact on plasmin activity kinetics; however, Ca was found to lower the affinity of plasminogen

activators for plasminogen, potentially through a competitive inhibition mechanism, ultimately reducing plasminogen activation and plasmin activity at the lowest temperature (2.2 °C).

2.5.8.2 Warm (room temperature) storage

The storage of milk and milk-based products at room temperature is not commonly practised due to both microbiological concerns and plasmin activity. UHT milk and sterilised milk-based products are normally stored at room temperature. Plasmin activity in these products can be high, as plasminogen activator inhibitors are already inactivated due to the high heat treatment applied to such products; in addition, room temperature (20–25 °C) conditions are close to the optimum temperature (37 °C) for plasminogen activation and plasmin activity. This can ultimately lead to more extensive casein hydrolysis, resulting in impaired functional properties due to reduced intact protein and quality issues, e.g., bitterness and gelation of UHT milk (see Section 2.7.2).

2.5.9 Microbial proteases

Microbial proteases (often metalloproteases) produced by psychrotrophic microorganisms can cause proteolysis during refrigerated storage (Nielsen, 2002). Over the years there have been several studies indicating that the presence of microbial proteases can affect the enzyme system in milk. Some bacteria have the ability to enhance plasminogen activation or, in some instances, actually produce plasminogen activators during growth. *Streptococcus uberis* has been shown to secrete proteinases capable of activating bovine plasminogen; Leigh (1994) purified a 57,000 Da plasminogen activator from culture filtrates of *S. uberis* strain 0140J. Johnsen *et al*.

(1999) identified a 45,000 Da bovine plasminogen activator secreted from *Streptococcus uberis* strain SK880, which was isolated from a case of mastitis. Plasminogen activators produced by *Streptococcus uberis* such as PauA and PauB are thought to enhance proteolysis in the mammary gland during mastitis, thereby providing the bacterium with essential nutrients through the hydrolysis of caseins (Rosey *et al*., 1999; Ward and Leigh, 2002). Larson *et al*., (2006) showed that a protease produced by *Bacillus polymyxa* has the ability to act as a plasminogen activator by cleaving bovine plasminogen to produce a product with plasmin-like activity.

Pseudomonas strains produce heat-stable proteases of concern to the food industry, as they are capable of surviving pasteurisation. Pseudomonas strains can grow at refrigeration temperatures and have been shown to disrupt the plasmin system (Fajardo-Lira *et al*., 2000). The growth of pseudomonas resulted in an initial increase in plasmin in the whey fraction and a subsequent decrease in the casein fraction. The authors concluded that the extracellular proteases secreted from *Pseudomonas fluorescens* caused the release of plasmin from the casein micelles. Further studies into proteases produced by *Pseudomonas fluorescens* M3/6 have shown that it can enhance plasminogen activator activity, therefore increasing plasmin activity (Frohbieter *et al*., 2005). The presence of M3/6 proteases increased bovine plasminogen activation 2.5-fold and increased the catalytic efficiency of bovine u-PA four-fold. Some microbial proteases can have the opposite effect, acting as inhibitors of the plasmin system. *Escherichia coli* produces ecotin, a periplasmic protein, which has the ability to inhibit u-PA (Eggers *et al*., 2004).

These studies suggest that extracellular bacterial proteases have the ability to interact and interfere with the plasmin system, altering the rate of proteolysis and the location of plasmin, potentially affecting the quality of milk, cheese and other foods that utilize these ingredients in functional applications.

2.5.10 Membrane filtration

The filtration of milk can greatly influence plasmin activity (Benfeldt, 2006). Ultrafiltration (UF) of milk prior to cheese making is commonly practised in the dairy industry (Soodam and Guinee, 2018). Studies have shown that cheese produced from UF milk exhibits slower proteolytic action during storage, with reduced hydrolysis of β- and α_s-caseins (Lelievre and Lawrence, 1988; Benfeldt, 2006). This has been attributed to increased levels of native whey protein in UF-cheeses compared to traditional cheese, which inhibits plasmin, thereby affecting cheese maturation. Benfeldt (2006) reported lower plasmin activity in cheese produced from UF milk, and several reasons were given as to the lower plasmin activity, including the increased incorporation of plasmin inhibitors into the cheese matrix, and the inactivation of plasminogen activators during UF concentration.

Microfltration (MF) can also affect the plasmin system due to the separation of certain components within the plasmin system. The MF of milk often involves the separation of casein from whey proteins. Whey contains many inhibitors of the plasmin system and therefore alters the equilibrium of the plasmin system (see Sections 2.3.2 and 2.4). Aaltonen and Ollikainen (2011) studied the effect of MF on the activities of plasmin and plasminogen in the retentate of MF skim milk. As expected, plasmin and plasminogen were retained in the retentate, as they are both

associated with casein micelles (see Section 2.4). Subsequent diafiltration resulted in additional removal of serum proteins from the retentate, resulting in increased activation of plasmin. Plasminogen levels decreased due to increased conversion of plasminogen to plasmin, as inhibitors of plasmin and plasminogen activators were removed. Consequently, proteolysis of casein was enhanced, with an accumulation of proteolysis products in the retentate. During diafiltration, the concentration of β-lactoglobulin in retentate decreased, resulting in increased plasmin activity. Aaltonen and Ollikainen (2011) found that plasmin activity in MF retentate was strongly correlated with the concentration of β-lactoglobulin, with plasmin activity increasing with decreasing levels of β-lactoglobulin.

2.5.11 High pressure

The treatment of milk with new and emerging technologies is becoming more popular, with high pressure treatment gaining increased consumer acceptance as a food processing technique. High pressure treatment of milk is a non-thermal treatment which can extend shelf-life by inactivating microorganisms and denaturing enzymes (Patterson *et al.*, 1995; García-Risco *et al*., 1998) without negatively affecting the sensory and nutritional qualities (Cheftel, 1995). García-Risco *et al*. (1998) subjected milk to high pressure (400 MPa at 25 °C for 30 min) and monitored microbiological and biochemical changes during subsequent cold storage. Plasmin was not fully inactivated by the pressure treatment, as indicated by the formation of γ-caseins on storage; however, they estimated that the treatment caused a 25% reduction in total plasmin activity. Rademacher *et al*. (1998) found pressures as high as 800 MPa for 8 min were required to completely inactivate plasmin in milk, with a treatment at 500 MPa for 90 min and 600 MPa for 10 min only causing 50% inactivation of

plasmin. A later study by García-Risco *et al*. (2003) gave similar findings, with the pressure treatments not causing the inactivation of plasmin and only decreasing total plasmin activity by 20–30% after plasminogen activation. García-Risco *et al*. (2000) showed that pressurisation at higher temperatures (400 MPa at 60 °C for 15 min) caused greater inactivation of plasmin (86.5% reduction), thereby reducing proteolysis and improving the overall organoleptic properties of the milk. The decreased plasmin activity cannot be attributed to one single phenomenon, but rather to a series of complex changes upon pressurisation, besides the effects on plasmin and plasminogen directly. Casein micelles, when subjected to very high pressure, can dissociate (López-Fandiño *et al*., 1998; Needs *et al*., 2000a, b), due to the disruption of hydrophobic and electrostatic interactions (Mozhaev *et al*., 1996), which may result in the dissociation of plasmin and plasminogen from the micelle into the serum phase. This would result in increased contact with inhibitors of the plasmin system present in the serum, resulting in enhanced inactivation and lower activity (Korycha-Dahl *et al*., 1983; Politis *et al*., 1992).

In addition to the aforementioned effects, high pressure treatment causes the denaturation of whey proteins (Garía-Risco *et al*., 2000; Scollard *et al*., 2000a, b; García-Risco *et al*., 2003; Huppertz *et al*., 2004a, b), which have been shown to act as proteinase inhibitors. The denaturation of β-lactoglobulin during high pressure treatment exposes a free -SH group that can interact with plasmin, causing irreversible inactivation of plasmin. Scollard *et al*., (2000a) showed that, in a buffer system, plasmin is capable of resisting pressures of 600 MPa at 20 °C for 20 min in the absence of β-lactoglobulin, but plasmin activity is reduced at 400 MPa in its presence. Scollard *et al*. (2000b) found that applied pressures of ≥400 MPa reduced plasmin activity in milk. However, they observed increased proteolysis in milk subjected to pressures of

300–500 MPa, possibly due to the changes in the structure of casein micelles and dissociation of some caseins from the micelle, increasing the accessibility of protein for proteolysis; Huppertz *et al*. (2004a) reported similar findings, with increased proteolysis in milk treated at 300–400 MPa also suggesting the more extensive proteolysis was due to high pressure-induced disruption of casein micelles.

Although high pressure treatment can inactivate microorganisms while maintaining the nutritive and sensory profiles of milk, the effect on plasmin activity, micelle integrity and subsequent proteolysis in milk could be significant, from a quality and sensory perspective, for the manufacture of products from high-pressure-treated milk

2.6 Characterisation of plasmin and plasminogen

Plasminogen-derived activity is defined as the plasmin activity resulting from the addition of a defined amount of urokinase. There are various methods used to determine both plasminogen-derived and plasmin activity, using both indirect and direct methods. These methods vary in their sensitivity, sample preparation and accuracy. Multiple analytical techniques are also used to observe plasmin-mediated proteolysis, as well as identifying and quantifying proteolysis products.

2.6.1 Assay methods for plasmin and plasminogen

There are several methods used for determining plasmin and plasminogen-derived activity in milk, varying from monitoring degradation of milk proteins, to fluorometric and chromogenic assays. Methods originally employed for determining plasmin activity involved monitoring degradation of milk proteins, such as quantifying

the rate of formation of γ-caseins. Driessen and van der Waals (1978) determined plasmin activity in sterilised milk by monitoring γ-casein formation using polyacrylamide gel electrophoresis (PAGE) and densitometry. Andrews (1983a) employed similar techniques, using quantitative PAGE on gel rods and qualitative PAGE on gel slabs to monitor plasmin activity in mastitic milk. The increase in the number of free amino acids has also been used to determine plasmin activity (Chism *et al*., 1979; McKellar, 1981); milk was treated with trichloroacetic acid (TCA) and filtered, and the filtrate was analysed for free amino acids by monitoring absorbance at 420 nm, with an increase in free amino acids (μmoles/ml milk) indicating increased proteolysis and therefore higher plasmin activity (McKellar, 1981).

Dulley (1972) determined plasmin activity by incubating plasmin and casein (substrate) at 37 °C for 16 h before precipitating out the protein with 10% TCA. The supernatant obtained after centrifugation was assayed for the amino acid tyrosine and tyrosine-containing peptides by monitoring absorbance at 750 nm using Folin-Ciocalteu phenol reagent and alkaline copper, as described by Lowry *et al*. (1951). The increase in absorbance at 750 nm was used as a measure of proteolysis and therefore plasmin activity.

Other researchers have used labelled proteins such as ^{14}C-methylated β-casein to determine plasmin activity (Donnelly *et al*., 1980; Donnelly and Barry, 1983). Reductive methylation of β-casein provides an ideal substrate for monitoring plasmin-like activities with the proteolysis product, ^{14}C γ-casein, easily detected by diethylaminoethyl (DEAE)-cellulose chromatography. Igarashi (1989) used DEAE-cellulose chromatography to separate γ-caseins from milk before measuring absorbance at 280 nm to determine the rate of formation of γ-caseins.

Andrews (1982) developed a novel method for detecting and measuring both proteinase and proteinase inhibitor activities. Substrate (casein) was immobilised to Sepharose 4B which was linked *via* glutaraldehyde or *m*-maleimidobenzoyl N-hydroxysuccinimide ester to β-glucosidase, a reporter enzyme resistant to the action of plasmin. The cleavage of the substrate by plasmin releases the reporter enzyme into the supernatant, where it acts on its substrate molecules. The action of the reporter enzyme results in a significant amplification of the initial hydrolysis caused by plasmin, ultimately increasing the sensitivity of the analysis. Many of these assays described require extensive sample preparation and are not specific for plasmin (e.g., many other proteinases can cause the formation of peptides and free amino acids).

Pierzchala *et al*. (1979) developed and described a new fluorogenic peptide, 7-(N-succinoylalanylphenylalanyllysylamido)-4-methylcoumarin trifluoroacetate salt, for the detection of plasmin in cells. Richardson and Pearce (1981) modified the method described by Pierzchala *et al*. (1979) to develop a rapid, fluorometric, amidolytic assay for the detection of plasmin in dairy products using N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin, a non-fluorescent substrate. Plasmin hydrolyses the lysine-coumarin bond releasing 7-amido-4-methyl coumarin (AMC), a fluorescent product. Fluorescence intensity, using excitation and emission wavelengths of 380 and 460 nm, respectively, is proportional to plasmin activity. Recordings of intensity over time are taken at regular intervals with one AMC unit of plasmin activity releasing one nanomole AMC per min. Using this method, they reported plasmin activities of 0.037–0.195 AMC units/ml, and plasmin and plasminogen concentrations of 0.14–0.73 μg/ml and 0.55–2.75 μg/ml, respectively, in pasteurised bovine milk.

Chromogenic assays are also used to characterise proteolytic activity in milk. Reimerdes *et al*. (1979) experienced low sensitivity when using the chromogenic substrate, amino acid-4-nitroanilides. The chromogenic substrate H-D-Valyl-L-Leucyl-L-Lysine-*p*-nitroanilide dihydrochloride (S-2251) has been used for the detection of plasmin associated with casein fractions (Snoeren and van Riel, 1979; Rollema and Poll, 1986). Plasmin hydrolyses the lysine-nitroanilide bond, releasing *p*-nitroanilide, a chromogenic product; absorbance at 405 nm is used to detect *p*-nitroanilide, with a change in absorbance per unit of time taken as a measure of plasmin activity (Politis *et al*. 1993). Plasminogen concentrations of between 0.7–2.4 mg/L were reported for skim milk, which agrees with those reported by Richardson and Pearce (1981), who used a fluorogenic substrate.

For accurate measurement of plasmin, the use of synthetic substrates is often advised due to their purity, defined structures and their ability to bind to fluorescent and chromogenic groups. In addition, the proteolytic products of these substrates are easy to detect and the specificity of enzymes for the substrate is often well-defined. The advantages of these fluorometric and chromogenic assays over the previously presented methods include reduced sample preparation and increased sensitivity and limits of detection. However, it has been reported that whey proteins and caseins present in milk can interfere with these methods (Schaar, 1985; Bastian *et al*., 1991b), leading to the underestimation of true plasmin levels.

Plasminogen can also be detected by fluorometric and chromogenic assays. Plasminogen is activated by the addition of urokinase, a plasminogen activator, which converts all plasminogen present to plasmin (Politis *et al*., 1993). Sufficient time, along with the correct temperature, allows plasminogen present to be converted to

plasmin, which can then be measured with the two methods described previously. The amount of plasminogen present can be calculated by subtracting plasmin activity with no added urokinase (i.e., only plasmin) from plasmin activity with added urokinase (i.e., plasmin + plasminogen).

[Plasmin + plasminogen (urokinase added)] - [plasmin (no urokinase added)] = plasminogen

Plasmin and plasminogen can also be determined in skim milk or for different fractions of milk (i.e., whey (serum) and casein micelles). Determination of plasmin and plasminogen in milk serum and casein fractions involves defatting milk, ultra-centrifuging (100,000 × g for 1 h at 4 °C) and obtaining the supernatant (milk serum) and pellet (casein micelles) (Korycha-Dahl *et al*., 1983). As previously discussed, casein micelles can interfere with some assays that use synthetic peptides. Therefore, plasmin and plasminogen can be dissociated from the casein micelles into the milk serum using sodium citrate (Politis *et al*., 1989a) or ε-aminocaproic acid (EACA) (Korycha-Dahl *et al*., 1983; Politis *et al*., 1989b). However, as many of the inhibitors of the plasmin system reside in the serum phase, measurement of plasmin activity in the milk serum could be questionable. To avoid this issue, Politis *et al*. (1993) ultra-centrifuged skim milk prior to EACA addition to obtain a casein pellet with plasmin and plasminogen still associated; the pellet was then reconstituted and EACA was added, resulting in the dissociation of plasmin and plasminogen into a whey protein-free serum.

Collin *et al*. (1988) determined plasmin using ELISA. They reported higher plasmin activities than those determined by Richardson and Pearce (1981), attributing

these differences to the role of caseins and other inhibitors in fluorometric and chromogenic assays, which utilize synthetic substrates. Later studies by Politis *et al*. (1992) and Benfeldt *et al*. (1995) determined the concentration of plasminogen in bovine milk using ELISA methodology, reporting results comparable to those of Richardson and Pearce (1981) and one another.

Many of these assays require the clarification of samples, due to interference of some milk components (i.e., casein and fat globules) with plasmin and plasminogen-derived activities. However, these clarification/purification steps can alter the true plasmin activity in the sample, leading to over or under-estimation of plasmin or plasminogen-derived activities. Therefore, assays such as the ELISA described above have been developed to try overcome the interference of some milk components. Ozen *et al*. (2003) successfully utilised Fourier-transform infrared spectroscopy (FTIR), combined with multivariate statistical analysis, to measure and differentiate between plasminogen and plasmin concentrations in the presence/ absence of whey proteins and casein. These studies indicate that the plasmin enzyme system can be studied in the presence of casein and whey proteins.

In addition to the more "traditional" fluorometric and chromogenic assays, researchers have optimised methods for specific products. Rauh *et al*. (2014a) optimised a spectrophotometric method for the measurement of both plasmin and plasminogen-derived activity in dairy products, specifically in turbid, fat-containing products, combining spectrophotometric and sample preparation methods from Rollema and Poll (1986) and Saint Denis *et al*. (2001b), respectively. A reproducible assay was optimised, allowing for the measurement of cheese extracts, whole milk and

micellar casein solutions, with improved repeatability and levels of detection, compared to previous spectrophotometric assays.

As the turbidity of dairy products can affect spectrophotometric measurements, and clarification steps (i.e., filtration and centrifugation) are time-consuming and may impact the results, the use of a 'clarifying reagent' may provide a solution. The clarifying reagent can be used at the end of enzymatic colorimetric assays, retarding the reaction and producing a transparent solution due to the disruption of fat globules and solubilisation of casein micelles (Saint Denis *et al*., 2001a, b). This allows for direct spectrophotometric measurements without the need for extensive sample preparation (Saint Denis *et al*., 2001a; Humbert *et al*., 2006).

2.6.2 Assay methods for activators and inhibitors of the plasmin system

Several different plasminogen activator measurements in blood and cells have been developed (Rånby *et al*., 1982; Verheijen *et al*., 1982). However, as described by Lu and Nielsen (1993b), these assays often need to be adapted to measure plasminogen activators in bovine milk, for various reasons. Plasmin, plasminogen and plasminogen activators are all associated with casein micelles, and therefore plasmin and plasminogen are difficult to remove in the purification of plasminogen activators. The concentration of plasminogen activators in bovine milk is also very low, and therefore highly sensitive assays are required for their detection.

Lu and Nielsen (1993b) developed a colorimetric assay for the measurement of plasminogen activators using the synthetic substrate Spectrozyme™, which formed a yellow chromophore *p*-nitroanilide on cleavage by plasmin, which could be quantified by measuring absorbance at 405 nm by ELISA (Baldi *et al*., 1993; Fajardo-Lira *et al*.,

2000). The measurement of plasminogen activators is thus based around plasmin activity resulting from the activation of plasminogen by plasminogen activators. In order to eliminate the effect of turbidity from casein micelles, plasminogen activator activity was calculated from the difference in absorbance measured at 405 nm (from *p*-nitroanilide and turbidity) and 490 nm (turbidity only). They also reported the use of casein-plasminogen SDS-PAGE assay to visualise and determine the molecular weight of plasminogen activators. These assays were developed by modifying techniques used to quantify plasminogen activators in blood and other sources.

Saint Denis *et al*. (2001a, b) assessed plasminogen activator activity by measuring the conversion of exogenous inactive plasminogen into active plasmin, adapting methods previously described by Lu and Nielsen (1993a, b) and Baldi *et al*. (1993). Untreated milk sample containing plasminogen activators was added to a solution consisting of human plasminogen, starting the reaction. The increase in plasmin activity due to plasminogen activators was quantified using the synthetic substrate N-succinyl-L-alanyl-L-phenylalanyl-L-lysyl-7-amido-4-methyl coumarin; the increase in the AMC fluorescent product was measured using a spectrofluorometer with excitation and emission wavelengths set to 370 and 440 nm, respectively.

The measurement of plasmin and plasminogen activator inhibitors has involved adaptations from plasmin activator activity assays (Lu and Nielsen, 1993b; Precetti *et al*., 1997). The first step in determining plasmin and plasminogen inhibitors involves their partial isolation from bovine milk (Precetti *et al*., 1997). Determining their activities involves indirect assays which assess the differences in activity in their absence and presence, therefore calculating activities by difference. Prado *et al*. (2006) measured plasminogen activator inhibitor activity using a direct assay for u-PA, with

the substrate Spectrozyme™ UK. Plasmin inhibitor activity was measured using a similar method, with plasmin assays being carried out according to those described by Fajardo-Lira *et al*. (2000) using Spectrozyme™ PL as a substrate (as outlined above). The plasminogen activator inhibitory activity and plasmin inhibitor activity was then calculated as follows:

$$\text{Inhibitory Activity}_{\mu PA/PL} = \text{Activity}_{\mu PA/PL} - \text{Activity}_{(\mu PA+PAI) \text{ or } (PL+PI)}$$

2.6.3 Monitoring of plasmin-mediated proteolysis

There are several ways in which plasmin-mediated proteolysis can be monitored using numerous analytical techniques.

2.6.3.1 Soluble protein in supernatant

An easy, but indirect, method to determine the action of plasmin is to measure the protein content of the supernatant following the addition of 12% TCA to the milk sample (Kelly and Foley, 1997). An increase in the level of TCA-soluble nitrogen over storage gives an indication of the extent of plasmin action, due to an increase in proteolysis products (i.e., free amino acids, small peptides, PPs). The protein content of the supernatant after centrifugation can be measured using techniques such as the Kjeldahl or Lowry method (Lowry *et al*., 1951). However, this technique is relatively crude and does not take into consideration the action of other indigenous proteinases present which produce such products (i.e., PP).

2.6.3.2 Analysis of free amino groups

Free amino groups can be determined by mixing TCA-soluble extracts (Section 2.6.3.1) with 1 M potassium borate buffer (pH 9.2) and 5 mM trinitrobenzenesulfonic acid (TNBS). After the mixture is incubated at 25°C for 30 min, 2 M monobasic sodium phosphate (containing 18 mM sodium sulphite) is added (McKellar, 1981; Chove *et al*., 2011). Absorbance is then read at 420 nm and converted to micromoles of free amino groups/ml milk using a standard curve. The increase in the concentration of TCA-soluble free amino groups per millilitre of milk can then be used as an indication of proteolysis.

2.6.3.3 Urea-polyacrylamide gel electrophoresis

As previously mentioned, the principal substrate for plasmin in dairy products is β-casein. The hydrolysis of β-casein yields γ-caseins and proteose peptones. The formation of γ-caseins, in addition to the degradation of α_s-caseins, can be monitored using urea-polyacrylamide gel electrophoresis (urea-PAGE) (Andrews, 1983b; Kelly and Foley, 1997; Enright *et al*., 1999). Urea-PAGE allows qualitative analysis of plasmin-mediated proteolysis over time, giving an indication of the plasmin activity in that sample. It therefore provides additional information regarding plasmin activity measurements, providing information on the rate of proteolysis on each specific substrate, allowing comparisons between the proteolytic activity and specificity of different samples.

2.6.3.4 Reversed-phase high performance liquid chromatography

Reversed-phase high performance liquid chromatography (RP-HPLC) is a highly sensitive technique that provides both qualitative and quantitative analysis of plasmin mediated-proteolysis. pH 4.6-soluble extracts of the samples are generally run on HPLC (Kelly and Foley, 1997; Enright *et al*., 1999), with samples acidified to pH 4.6, generally with acetic acid, and centrifuged to obtain a supernatant containing proteolytic products such as PPs, peptides (i.e., α_s-casein peptide fragments) and γ-caseins. Plasmin action can also be monitored by measuring the levels of intact casein in milk (Visser *et al*., 1991; Enright *et al*., 1999), with lower levels of intact casein (particularly β-casein) indicating higher proteolytic activities. The proportion of intact casein and proteolysis products can be quantified by determining peak area, allowing for improved comparisons between the proteolytic profile of samples/products.

2.6.3.5 Mass spectrometry

The specific peptide fragments produced by plasmin-mediated hydrolysis can be identified and quantified using mass spectrometry (Rauh *et al*., 2014a). Although generally an expensive technique compared to other methods used to monitor proteolysis, mass spectrometry can provide very specific information, such as the rate of formation of specific peptides at different storage times; therefore, information on the bonds most susceptible to cleavage within the protein can be obtained.

2.7 Applications of plasmin and its significance in various dairy products

Plasmin can have both positive and negative impacts on a wide range of dairy products. Some of these effects of plasmin on the processability and quality of dairy products are outlined here.

2.7.1 Cheese

Plasmin can have numerous effects on the cheesemaking process and on the sensory properties of the resulting cheese. There have been several studies on the effects of plasmin-mediated proteolysis on curd formation and ripening of cheese during maturation (Fox, 1989; Farkye and Fox, 1991, 1992; Bastian and Brown, 1996).

2.7.1.1 Cheese-making properties

It is well known that late-lactation milk results in poor clotting properties, with weaker gel formation, resulting in longer clotting times and reduced curd firmness (Grufferty and Fox, 1988c; Mara *et al*., 1998). It has been reported that plasmin can be attributed with the poor clotting properties of cheese milk due to increased proteolysis of caseins (Okigbo *et al*., 1985). Farkye (1986) found that the addition of plasmin to milk did not alter rennet coagulation times, but resulted in poor curd firming rates and reduced curd firmness (Grufferty and Fox, 1988c). Bastian *et al*. (1991a), however, found no relationship between milk clotting properties and plasmin in milk, although 90% of milk samples contained less than 300,000 somatic cells/ml. As high SCCs are often associated with increased plasmin levels (see Section 2.5.1), the effect of high SCCs and plasmin activity on milk coagulation parameters was not explored.

Mara *et al*. (1998) reported similar findings to Farkye (1986) and Grufferty and Fox (1988c), observing that the addition of plasmin to milk prior to rennet coagulation increased curd firming time and reduced the final curd firmness. In general, these results suggest that, although plasmin may not affect rennet coagulation times, it has a significant impact on the gel strength and resulting curd firmness.

The moisture content and yield of cheese have also been reported to be affected by plasmin-mediated hydrolysis of caseins, but to a lesser extent than the properties discussed above, despite their great importance from both an economic and quality perspective. A number of studies have found that cheese made from late-lactation milk and milk with high SCCs have higher moisture contents; many have partially attributed this to plasmin-mediated hydrolysis of caseins, resulting in higher levels of serum casein, reduced syneresis and therefore higher moisture levels (O'Keeffe, 1984; Barbano *et al*., 1991). These studies involved the analysis of late-lactation milk or milk with high SCCs with no plasmin addition; therefore, the role of plasmin in contributing to the high moisture observed in these studies was only hypothesised. A study conducted by Mara *et al*. (1998), in which different concentrations of plasmin were added to milk prior to rennet coagulation, found curd moisture to be unaffected by the addition of plasmin to milk; the addition of plasmin also increased protein losses, with increased protein levels in whey, thus reducing cheese yield, which was attributed to increased hydrolysis of caseins.

These studies indicate that plasmin can have a significant effect on milk coagulation and cheese yield; however, the need to further investigate the influence of high plasmin levels on such parameters remains.

2.7.1.2 Cheese ripening

Unlike the role of plasmin in milk coagulation and cheese yield, there have been numerous studies which examine the importance of plasmin during cheese ripening. Plasmin is of great importance to the organoleptic properties of several cheeses (Ollikainen and Kivelä, 1989; Farkye and Fox, 1991, 1992; Farkye and Landkrammer, 1992), producing peptides which are precursors for the production of free amino acids by starter bacteria and enzymes (i.e., peptidases) (Fox, 1989). Farkye and Fox (1991) demonstrated the importance of plasmin in cheese ripening, as the addition of a plasmin inhibitor to curds at salting resulted in significantly lower proteolysis of β-casein compared to cheese with no added plasmin inhibitor.

The contribution of plasmin to cheese ripening varies between different cheese varieties and depends on factors such as the pH of the cheese during maturation, cooking temperature of the coagulum and ripening temperature (Richardson and Pearce, 1981; Lawrence *et al*., 1987; Farkye and Fox, 1990; Bastian and Brown, 1996). Richardson and Pearce (1981) found plasmin activity to be significantly lower in Cheddar cheese compared to Swiss cheese, a finding confirmed by Farkye and Fox (1990), who found Cheddar cheese to have much lower plasmin activity compared to other cheese varieties. Lawrence *et al*. (1987) attributed differences in plasmin activities in different cheese varieties to the different pH at drainage of the cheeses. The later study by Farkye and Fox (1990) showed that plasmin activity in rennet curds drained at different pH values were identical, suggesting that pH at drainage does not affect plasmin activity. These findings were in agreement with the results of Grufferty and Fox (1988b), who found the association of plasmin with casein micelles to be independent of pH between pH 4.8 and 6.6. If the results of Grufferty and Fox (1988b) are correct, the pH range relevant to rennet coagulated cheeses (i.e., >5.0) at drainage

should not affect plasmin retention within the curd. The final pH of many cheese varieties also does not favour plasmin activity, with pH values much lower than the pH optimum of plasmin (pH 7.5); however, plasmin action can be more significant in cheese varieties in which pH increases during ripening (e.g., blue cheeses) (Fox, 1989).

The pH at drainage during cheese manufacture may not account for the different plasmin activities observed in different varieties of cheese; Fox (1989) suggested that some differences between cheese varieties may be due to different salting procedures, as salt causes the dissociation of plasmin from the micelle, resulting in the loss of some plasmin into the whey. However, Farkye and Fox (1990) disproved this theory, finding that plasmin activity in Cheddar, Emmental and Cheshire cheese was not influenced by the method of salting, instead reporting that increasing cooking temperatures during the manufacture of the cheeses caused higher plasmin activities.

As higher cooking temperatures are used for the manufacture of Swiss cheese (~52 °C) compared to other cheeses such as Cheddar (~38 °C), cooking temperature is likely to contribute to the different plasmin activities observed between cheese varieties. The contribution of plasmin to primary proteolysis is most significant in high-cooked cheese varieties, as the coagulant is extensively denatured (Sheehan *et al*., 2007). Increasing cooking temperature increases plasmin activity in the resulting cheese; Choi *et al*. (2006) observed increased plasmin, plasminogen and urokinase-type PA activities extracted from curds cooked at higher temperatures, with Somers and Kelly (2002) concluding that the higher plasmin activity is due to increased activation of plasminogen. Heat treatment of milk prior to cheese manufacture can also impact proteolysis during cheese ripening. Benfeldt *et al*. (1997)

subjected milk to different heat treatments prior to cheesemaking, and reported that plasmin activity in the resulting cheese decreased as the temperature and holding time increased. Reduced casein hydrolysis during ripening of cheese made from heated milks was attributed to reduced plasmin activity, due to thermal inactivation of plasminogen activators, and interactions between β-lactoglobulin and components of the plasmin system on heating (see Section 2.5.7).

Plasmin activity in cheese is also strongly influenced by ripening temperature. As mentioned in earlier sections, the optimum temperature for plasmin activity is 37 °C. As some cheese varieties are ripened at low temperatures, the rate of plasmin activity, and therefore proteolysis, is reduced. Ripening temperatures for Cheddar (6–8 °C) and Dutch varieties (12–14 °C) are much lower than the optimum temperature for the action of plasmin, with ripening temperatures reaching as low as 4 °C (Fox *et al*., 1996). Increasing ripening temperatures increases the rate of proteolysis (Fox *et al*., 1996; Feeney *et al*., 2001). This has gained a lot of interest, especially in Cheddar cheese, as ripening is an expensive and time-consuming process. However, as not all biochemical reactions increase at the same rate with increasing temperature, high temperatures can cause limitations such as unbalanced flavour profiles (Fox *et al*., 1996).

Plasmin-mediated hydrolysis decreases during cheese ripening, often due to variations in plasmin activity (Bastian and Brown, 1996; Benfeldt *et al*., 1997). Therefore, some researchers have added plasmin (Farkye and Fox, 1992) and plasminogen activators (i.e., urokinase) (Bastian *et al*., 1991a, 1997) to cheese milk to maintain high plasmin activity throughout cheese ripening; these studies resulted in increased proteolysis of the cheese with improved flavour and quality of some cheese.

Studies have also shown that the addition of exogenous plasmin to accelerate cheese ripening is possible while avoiding defects such as bitterness and off-flavours (Farkye and Fox, 1992; Scherze *et al*., 1994; Kelly, 1995). Upadhyay *et al*. (2004) added a preparation of streptokinase (a plasminogen activator produced by *Streptococcus uberis*) to cheese milk, reporting increased plasmin activity and proteolysis in the resulting cheese compared to cheese with no added streptokinase. A later study by Upadhyay *et al*. (2006) accelerated the ripening of Cheddar-type cheese using a streptokinase-producing strain of *Lactococcus lactis*. Using this strain, they found an approximately three-fold increase in plasmin activity compared to cheese manufactured with a control strain; this resulted in increased hydrolysis of β-casein, with higher levels of γ_1-, γ_2- and γ_3-caseins. The addition of exogenous plasminogen activators to cheese milk results in higher plasmin activity in the resulting cheese due to an increase in the rate of conversion of plasminogen to plasmin. Many of these results suggest that the addition of exogenous plasmin to cheese milk could be used to accelerate cheese ripening. Although the addition of exogenous plasmin is effective, from an economic perspective it is not practical, due to its high cost.

2.7.2 Milk-based UHT products

UHT treatment of milk involves heating milk at very high temperatures (~140 °C) for short periods of time (4–6 s) (Rauh *et al*., 2014b). Plasmin is a heat-stable enzyme capable of surviving many UHT treatments and therefore plasmin-mediated proteolysis can occur in many UHT-treated dairy beverages. UHT-treated milk provides an ideal environment for the action of remaining plasmin, as they are often stored at room temperature (close to the optimum temperature for plasmin activity). Storage temperature, processing conditions (i.e., time-temperature

combinations), other ingredients used in the product and plasmin levels in the milk prior to processing, have all been shown to be major factors influencing age gelation (Manji *et al*., 1986; Ismail and Nielsen, 2010; Rauh *et al*., 2014b, c; Anema, 2017). Kohlmann *et al*. (1988) found that the addition of low levels of plasmin and plasminogen to UHT-treated milk resulted in greater proteolytic activity and faster milk gelation, respectively, compared to milk with no added enzyme or zymogen. Kelly and Foley (1997) found the addition of potassium iodate to milk prior to UHT treatment resulted in considerably higher plasmin activity during storage, compared to UHT milk with no added potassium iodate. As discussed in earlier sections, denatured β-lactoglobulin can form complexes with plasmin *via* disulphide bonds, inactivating plasmin. Therefore, these results suggest that denaturation of β-lactoglobulin has a significant role in reducing plasmin activity in UHT-treated milk. Studies by Rauh *et al*. (2014b, c) confirmed that plasmin action contributes to the age gelation of UHT milk. An increase in plasmin activity, with a simultaneous decrease in plasminogen-derived activity was observed during storage, with a corresponding increase in casein hydrolysis. Rauh *et al*. (2014c) suggested that the hydrolysis of casein-calcium phosphate and casein-casein interaction sites destabilises the casein micelles, causing age gelation in UHT milk. In both studies, age gelation occurred with extensive hydrolysis of β- and α_s-caseins (plasmin's principal substrates), indicating that plasmin contributes to the age gelation of UHT milk.

Plasmin-mediated proteolysis of caseins has been shown to produce a soft and fragile gel in UHT-treated milk on storage (Kelly and Foley, 1997; Zhang *et al*., 2018). Zhang *et al*. (2018) added plasmin to skim UHT milk-based model systems, finding that plasmin induced the formation of soft gels when approximately 60% of both β- and α_{s1}-casein were hydrolysed and the degree of hydrolysis reached 2.1%.

Milk processed by direct and indirect UHT heating systems differs in terms of their susceptibility to plasmin activity and age gelation. Studies have shown that plasmin activity is lower in indirect-treated UHT milk, compared to direct-treated milk (Corradini and Pecchini, 1981; Manji *et al*., 1986). Kohlmann *et al*. (1988) observed UHT milk processed by direct heating, with added plasminogen (0.3 mg protein/L), to gel after 155 d, while milk processed by indirect heating took longer to gel (195 d). Manji *et al*. (1986) reported directly processed milk to contain 37 and 19% of the original plasminogen and plasmin activities, respectively, while indirect heat-treated milk contained 19% of the original plasminogen activity and no plasmin activity. The longer heating-up and cooling-down times in indirectly heat-treated milk is believed to contribute to the higher degree of plasmin loss (Stoeckel *et al*., 2016). Therefore, in many direct-heating treatments, a preheating step is implemented to adequately reduce plasmin activity in the final UHT-treated product and ensure a suitable shelf life is achieved (Newstead *et al*., 2006; van Asselt *et al*., 2008), in addition to stabilising β-lactoglobulin before high temperature heating to 135–150 °C (Datta and Deeth, 2001). Anema (2017) found that proteolysis could be prevented, and gelation slowed, by increasing pre-heating severity (90 °C for 60 or 120 s), increasing UHT holding times (143 °C for 15 or 30 s), and combinations of the two. However, these conditions do not reflect those currently used, as such extensive heat treatment would cause finished product quality issues such as ‘cooked’ flavours. These results suggest that mild heat treatments before UHT treatments may induce plasmin-β-lactoglobulin interactions, reducing plasmin’s ability to hydrolyse caseins, and therefore reducing plasmin-mediated gelation.

The high heat treatment applied to UHT milk and dairy-based beverages can result in changes in protein conformation, protein aggregation and Maillard reactions

(van Boekel 1998). These physicochemical changes can ultimately affect the action of plasmin and alter the susceptibility of caseins to plasmin-mediated hydrolysis. The susceptibility of caseins to plasmin action can be affected by micellar structure. Enright and Kelly (1999) found that increasing the severity of heat treatments applied to milk resulted in lower levels of plasmin-mediated proteolysis, and suggested that the binding of denatured proteins to the surface of casein micelles results in steric blockage of plasmin to susceptible peptide bonds (i.e., Lys-X bonds), thus restricting hydrolysis. In addition to the modification of micellar structure through the adsorption of whey proteins, the susceptibility of caseins to plasmin can also be affected by the modification of lysine residues on caseins (i.e., lactosylation) (Bhatt *et al*., 2014, 2017). Lactosylation is the first step in the Maillard reaction in which lactose and the ε-amino groups of lysine residues react to form lactulosyllysine (the Amadori rearrangement product) (van Boekel, 1998). Dalsgaard *et al*. (2007) examined the effect of lactosylation on five different milk proteins (α-casein, κ-casein, β-casein, β-lactoglobulin and lactoferrin), finding that lactosylation reduced hydrolysis of these proteins by plasmin. A later study by Bhatt *et al*. (2014) studied the effect of lactosylation on plasmin-induced hydrolysis of β-casein, demonstrating that lactosylation resulted in modification of the ε-amino groups of lysine residues, making casein unrecognisable to the substrate-binding pocket of plasmin, thereby reducing hydrolysis of β-casein by plasmin.

The role of plasmin in the destabilization of UHT milk-based products is likely due to the loss of colloidal stability of the casein micelles due to extensive plasmin-mediated hydrolysis of the caseins. However, the effect of plasmin on UHT products is complicated and depends on various factors, with processing conditions, storage

temperature and initial plasmin levels (depending on stage of lactation, mastitis, age of the cow, etc.) being some of the most important.

2.7.3 High protein milk products

The use of milk protein ingredients in the formulation of products and beverages is widely practised. However, depending on the composition and production method of the protein ingredient, the presence of plasmin in these ingredients must also be considered, as it can potentially affect the functional and stability properties of such final products. These ingredients are often added to products to provide functionality such as foaming, gelation and emulsification and for nutritional purposes (e.g., to support high protein label claims). However, these properties are largely influenced by the structure and physicochemical properties of the proteins. Plasmin-mediated hydrolysis can subsequently affect the functional properties of these protein ingredients, in addition to potentially hydrolysing other proteins present in the formulated product.

2.7.3.1 Whey protein-containing ingredients

The abundance of plasmin in whey is generally lower than that of milk, as plasmin and plasminogen are associated with the casein micelles. However, the dissociation of plasmin into the serum phase (whey) could be of significance, as it could affect the quality of food products such as high protein beverages, which incorporate whey products such as whey protein concentrate and isolate. As previously discussed in Section 2.4.1, plasmin activity in acid whey is higher than that of sweet whey; therefore, food products that incorporate whey protein manufactured from acid whey may experience more issues than if whey proteins manufactured from sweet

whey were used. Hayes and Nielsen (2000) reported lower plasmin activities in whey protein isolates (WPIs) than in sweet- and acid whey-derived protein concentrates, attributing the lower levels observed to the ion-exchange process used in the manufacture of WPIs. They also observed variability in plasmin activity associated with the commercial whey protein products, with the highest plasmin activity being from an acid whey derived protein concentrate ingredient (~80% protein). Therefore, the source of whey used to manufacture protein ingredients, in addition to processing conditions, are likely to cause variations in plasmin concentration and/or activity in these ingredients.

The presence of plasmin in whey protein ingredients and whey-derived products may have negative effects on products that utilise these ingredients for nutritional or functional purposes.

2.7.3.2 Casein-containing ingredients

The use of casein-containing milk protein ingredients (e.g., sodium caseinate, milk protein concentrate/isolate and micellar casein concentrate/isolate), like whey protein ingredients, are widely applied in formulation of food systems (e.g., beverages, coffee creamers, yoghurt). The presence of residual plasmin activity in these ingredients can lead to flavour and stability issues. However, there is limited knowledge on plasmin activity in the different milk protein ingredients mentioned above and how their manufacturing processes affect plasmin activity. Gazi *et al*. (2014) measured plasmin and plasminogen-derived activity in a range of milk protein ingredients following reconstitution; the study included 14 milk protein concentrate, 2 micellar casein isolate, 19 sodium caseinate and 13 calcium caseinate samples. They found higher levels of plasmin and a higher occurrence of plasmin in micellar casein

isolate and milk protein concentrate, compared to sodium and calcium caseinates. They observed the activation of plasminogen to plasmin during storage, which resulted in extensive plasmin-mediated casein hydrolysis. Micellar casein ingredients are often produced by the microfiltration of skim milk using a 0.1 μm pore size membrane, resulting in the permeation of whey proteins, soluble constituents and low molecular weight nitrogenous compounds (i.e., the serum phase) through the membrane (into the permeate). Intact casein micelles are retained by the membrane (retentate) where they are further concentrated by diafiltration, removing additional serum phase components. Therefore, the process of manufacturing micellar casein ingredients essentially removes the inhibitors of the plasmin system, present in the serum phase, while plasmin, plasminogen and plasminogen activators are concentrated with the casein micelles. Such ingredients can therefore possess high plasmin activities which can impact their functionality when added as an ingredient to other products.

The results from this study suggest that residual plasmin activity should be an important factor when selecting milk protein ingredients for food formulation.

2.8 Conclusions and areas for additional research

Knowledge of the plasmin system and factors affecting the distribution and activity of different plasmin system components continues to grow. However, there remain gaps in our understanding of the plasmin system in various dairy products. In addition to this, little research has been conducted on residual plasmin activity in membrane filtration-derived milk protein ingredients, although these ingredients are widely used in dairy and non-dairy products for their functional properties, where

residual plasmin activity could have negative consequences for the resulting quality and functional properties of that product.

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Chapter 3

The influence of temperature and transmembrane pressure on protein, calcium and plasmin partitioning during microfiltration of skim milk

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Declaration

This chapter was written by author Thomas C. France (TCF) and reviewed by all co-authors. TCF co-designed the study with co-authors and performed all of the experimental work.

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3.1 Abstract

Dissociation of β-casein from casein micelles at low temperature is exploited in the manufacture of β-casein-enriched ingredients. In this study, the effects of selected temperatures within the cold processing range (4, 8, 12, 16 and 20°C), and different transmembrane pressures (0.05 and 0.30 bar), on protein, mineral and plasmin partitioning during microfiltration were determined. Significantly higher ($p < 0.05$) total and ionic calcium concentrations were measured in permeate generated at 4°C under sub-critical flux conditions (0.05 bar) compared to limiting flux conditions (0.30 bar). Under the former, the highest concentration of β-casein in permeate (2.02 mg mL^{-1}) was achieved at 4°C, under sub-critical flux conditions; however, these conditions also led to the highest plasmin activity (0.023 AMC units mL^{-1}) in permeate. Lower processing temperature and reduced fouling contribute to higher yields of β-casein, although the concomitantly higher plasmin activity may contribute to protein stability challenges during down-stream processing of such permeates.

3.2 Introduction

Microfiltration (MF) of milk is commonly used for the removal of micro-organisms (wide-pore; 1.4 µm) and the fractionation of whey proteins from caseins (tight-pore; 0.1-0.5 µm) (Crowley *et al*., 2015b; Rosenberg, 1995). Traditionally, filtration of milk, using both MF and ultrafiltration (UF), was, and to a limited extent still is, performed at temperatures of 45-55°C (Hurt *et al*., 2010; O'Mahony & Tuohy, 2013; Zulewska *et al*., 2009), mainly due to the advantage of higher initial permeate flux, resulting from lower permeate viscosity. However, there is increased interest in the use of cold MF at 4-15°C (Coppola *et al*., 2014; Lawrence *et al*., 2008; Seibel *et al*., 2015). Cold MF offers the potential for longer processing times due to reduced fouling (Steinhauer *et al*., 2015) and the opportunity to produce ingredients with novel targeted protein profiles (e.g., β-casein-enriched whey protein streams) (Crowley *et al*., 2015b; O'Mahony *et al*., 2014).

Bovine β-casein, representing approximately 35% of total casein in milk (Huppertz, 2013), exists primarily within the casein micelle where it is associated *via* hydrophobic interactions (De Kruif & Holt, 2003). By reducing temperature, hydrophobic interaction strength decreases, resulting in the partial dissociation of β-casein, in addition to smaller quantities of κ-casein, from the micelle into the serum phase of milk (Creamer *et al*., 1977; McMahon & Oommen, 2013). This phenomenon has been exploited to fractionate β-casein from the micelle to produce β-casein-enriched whey protein ingredients, β-casein concentrates or isolates (O'Mahony *et al*., 2014; Woychik, 1992). The increasing interest in the fractionation, enrichment and isolation of β-casein is attributed to a number of factors, such as the increasing need by manufactures of infant milk formula to best reproduce the protein profile of human

milk, as β-casein comprises ~80-85% of total casein in human milk, compared to 35% in bovine milk (Hambraeus & Lönnerdal, 2003). In addition, there is increased interest in the unique technological and functional properties of β-casein (e.g., emulsification, foaming and mineral-binding properties) (Crowley *et al*., 2019; Li *et al*., 2016) and use of bioactive peptides derived from β-casein hydrolysates (Phelan & Kerins, 2011; Turpeinen *et al*., 2009).

However, milk is a complex heterogenous system consisting of macromolecules, micronutrients and enzymes, such as plasmin. There have been few studies conducted on how filtration affects the partitioning of plasmin in milk systems and how this may ultimately influence plasmin activity in the resultant protein ingredients (Aaltonen & Ollikainen, 2011; Gazi *et al*., 2014). Gazi *et al*. (2014) studied a range of milk protein ingredients and reported the levels of plasmin and plasminogen-derived activity to be highest in milk protein concentrates (MPCs) and micellar casein concentrates (MCCs), compared with sodium and calcium caseinate ingredients. It is well established that plasmin associates primarily with the casein micelles (Politis *et al*., 1992). However, the generation of fractionated and/or enriched protein streams during filtration can be associated with higher levels of proteolysis, due to either the concentration of caseins (e.g., preparation of MPC or MCC) or the removal of plasmin and plasminogen activator inhibitors (e.g., β-lactoglobulin) in such permeates (Aaltonen & Ollikainen, 2011). Plasmin primarily acts on β-casein (Bastian & Brown, 1996), yet little is known about how temperature-induced changes in casein micelles during the production of β-casein affect the partitioning of plasmin.

Another key processing parameter during MF that may influence separation performance is transmembrane pressure (TMP) (Hartinger *et al*., 2019). Critical flux

can be defined as the point at which the flux-TMP relationship starts to deviate from linearity (Bacchin *et al*., 2006; Youravong *et al*., 2003). Therefore, sub-critical flux is the flux/TMP below the critical flux, at which the flux-TMP relationship remains linear (pressure-dependent region). Conversely, limiting flux represents the point at which increases in TMP yield no additional increase in flux (pressure-independent region) (Wu *et al*., 1999). It is preferable to maximise flux by increasing TMP, but excessive increases are associated with significant fouling. Operating at critical or limiting flux is associated with extensive fouling, including irreversible fouling, and the formation of a more compact, less permeable cake layer. In contrast, operating at sub-critical flux can confer lower rates and extents of fouling (Howell, 1995; Steinhauer *et al*., 2011).

In the dairy industry, cold MF refers to a broad temperature range, and can include any MF processes performed at temperatures <20°C (Crowley *et al*., 2015b). The objective of this study was to understand and predict how the partitioning of β-casein and plasmin are influenced by MF temperature in the range 4-20°C and to determine whether there is a threshold temperature above which the permeation of β-casein decreases considerably. In addition, to obtain further insight into the influence of membrane fouling on filtration performance, MF was performed under two different operating conditions, low TMP (sub-critical flux; 0.05 bar) and high TMP (limiting flux; 0.30 bar), to determine the influence of TMP on protein, mineral and plasmin partitioning.

3.3 Materials and methods

3.3.1 Materials

Low-heat skim milk powder was kindly provided by Arrabawn Co-op (Nenagh, Co. Tipperary, Ireland). Reconstituted skim milk was prepared by adding low-heat skim milk powder to ultrapure water over 2 h at 22°C to attain a 3.20% (w/v) protein solution at a native pH of 6.70. The skim milk was then stored at 4°C for 48 h under gentle constant magnetic stirring to ensure complete rehydration. All chemicals, reagents and high-performance liquid chromatography (HPLC) standards, unless otherwise stated, were sourced from Sigma-Aldrich (Wicklow, Ireland) and were of analytical grade.

3.3.2 Microfiltration setup, operating parameters and cleaning

Microfiltration (MF) experiments were carried out on a lab-scale, pressure-driven, tangential-flow filtration device (Pellicon 2 mini-holder; Merck Millipore, Tullagreen, Carrigtwohill, Ireland) as described by Crowley *et al*. (2015a), with minor modifications. A 1000 kDa molecular weight cut-off (equivalent to a 0.1 µm pore size MF membrane), V-screen, polyethersulfone (PES) membrane cartridge (Biomax, Merck Millipore) with a total membrane area of 0.1 m^2 was used for all filtration experiments.

Critical and limiting flux were determined in triplicate, at each of the five selected temperatures (4, 8, 12, 16 and 20°C), with a freshly prepared sample used for each temperature (Wu *et al*., 1999). Transmembrane pressure (TMP) excursions at each temperature were performed as outlined by Crowley *et al*. (2015a) with values of critical flux determined as described by Youravong *et al*. (2003); the maximum permeate flux reached on increasing TMP was selected as the limiting flux (Bacchin

et al., 2006). The TMP value at which permeate first started to be produced was selected as the value for operation in the sub-critical region.

The membrane filtration unit was set up such that both permeate and retentate lines were returned to the feed (2 L) (i.e., total recirculation mode), resulting in no concentration. A plate heat exchanger was used to control the temperature of recirculating retentate at each of the five target temperatures (4, 8, 12, 16 and 20°C) with 40 min equilibration time allowed at each temperature before sampling to ensure stable conditions. After equilibration, equal volumes of both permeate and retentate were collected at the same time intervals while still in recirculation mode, to eliminate concentration effects. Not all five temperatures were run on the same day; instead, three temperatures were run on one day and two on the next. After each temperature, TMP with no backpressure applied was compared with original TMP; deviations from this resulted in cleaning of the membrane as described below and measurement of normalised water permeability (NWP; Section 3.3.3). The order of each temperature during filtration were randomised to avoid bias. Two independent trials were conducted at sub-critical and limiting flux at each temperature.

The filtration system was fully cleaned after each trial according to the method described by Crowley *et al*. (2015a), with minor modifications. After conducting trials at limiting flux, the strength of NaOH had to be increased from 0.10 to 0.50 N, along with the addition of 1 L of 2.5% ethylenediamine tetra-acetic acid (EDTA; recirculated for 30 min at 25°C), to retore membrane performance, due to the membrane being more extensively fouled.

3.3.3 Membrane fouling

NWP measurements were performed before filtration, immediately after processing and after cleaning in place, to determine the overall extent of fouling when operating at sub-critical or limiting flux and the efficiency of cleaning. The NWP (L m^{-2} h^{-1} bar^{-1}) was calculated as follows:

$$NWP = \frac{R \times F}{A \times TMP} \quad [3.1]$$

where R is permeate flow rate (L h^{-1}), F is the temperature correction factor based on water fluidity relative to 25°C (-), A is the membrane area (m^2), and TMP is the transmembrane pressure (bar). The extent of membrane fouling when processing was performed under sub-critical or limiting flux conditions was reported as the reduction in NWP following MF (i.e., difference between NWP measured before and after MF of skim milk).

3.3.4 Composition and physicochemical analyses of process streams

Protein content (% w/w) of the starting skim milk feed solution, retentates and permeates were determined using the Kjeldahl method with a nitrogen-to-protein conversion factor of 6.38 (IDF, 2001). The permeation of protein through the membrane was expressed as sieving coefficient (S_o) (-), which was calculated as follows:

$$S_o = \frac{C_p}{C_r} \quad [3.2]$$

C_p and C_r are the concentrations of protein (% w/w) in the permeate and retentate streams, respectively. Ash content was determined after heating samples to 650°C until a white ash was obtained (IDF, 2008). Apparent viscosity of the feed at each

temperature was measured at a shear rate of 1000 s^{-1} using a rotational viscometer (HAAKE RotoVisco 1, Thermo Fisher Scientific, MA, USA) with a cylindrical double-gap cup and rotor (DG43, Thermo Fisher Scientific, MA, USA). A standardised volume (13 mL) of sample was added to the cup for each run. A standard pH meter with a PHM210 electrode (MeterLab, Radiometer Analytical, Lyon, France) was used to measure pH of the samples at 20°C.

3.3.5 Total and ionic calcium

The total calcium content of the feed and permeate solutions was determined using flame atomic absorption spectroscopy (AAS) (SpectrAA, 55B, Atomic Absorption Spectrophotometer, Varian, CA, USA) fitted with a calcium hollow cathode lamp (Activion, Halstead, Essex, England) according to the International Dairy Federation Standard 119:2007 (IDF, 2007). The instrument was calibrated using standard solutions of known calcium concentration (0.00, 2.00, 4.00, 6.00, 8.00 and 10.0 mg calcium L^{-1}) prepared from a reference solution of known calcium concentration (1000 mg L^{-1}) with 2% addition level of a 10% lanthanum chloride solution.

A Titrando 907 autotitrator with Tiamo v2.2 software, equipped with a calcium (Ca)-ion-selective electrode (Metrohm Ireland Ltd, Co. Carlow, Ireland), was used to measure the ionic calcium concentration of feed, permeate and retentate samples according to the method of Crowley *et al*. (2014), with minor modifications. The probe was calibrated at 25°C using buffer solutions of known calcium-ion concentration (0.50, 1.00, 2.50, 5.00 and 10.0 mM). Samples were equilibrated with the probe for 1 min prior to taking measurements.

3.3.6 Reversed-phase high performance liquid chromatography

The protein profile of the feed, retentate and permeate samples was measured quantitatively using reversed-phase high performance liquid chromatography (RP-HPLC) as detailed by Crowley *et al*. (2015b), with minor modifications. A Varian Prostar system (Varian Associates Inc., Walnut Creek, CA, USA) was used to carry out the separation of proteins. The elution gradient was as described by Bonfatti *et al*. (2008) and Bot *et al*. (2020) using solvents A (10.0% HPLC-grade acetonitrile, 89.9% ultrapure water and 0.10% TFA) and B (89.9% HPLC-grade acetonitrile, 10.0% ultrapure water and 0.10% TFA); the sample injection volume was 40 µL, and detection was carried out at 214 nm. Prior to analysis, samples were filtered through 0.45 µm filters (Minisart® RC25, Göttingen, Sartorius AG, Germany). Standard curves for β-lactoglobulin, α-lactalbumin, β-casein and κ-casein were prepared by dispersing a known concentration of the desired protein in ultra-pure water and diluting to a range of concentrations with buffer A.

3.3.7 Plasmin activity

The plasmin activity of feed, permeate and retentate samples was measured according to the method described by Richardson and Pearce (1981), with minor modifications, using a Varioskan flash multiwell plate reader (Thermo Fisher Scientific Oy, Finland). Samples were added to a black, flat-bottomed 96 well plate with tris-HCl buffer and coumarin peptide substrate (N-Succinyl-Ala-Phe-Lys-7-amido-4-methylcoumarin acetate salt) (Sigma-Aldrich, Wicklow, Ireland). Standard curves were prepared by dissolving 7-amino-4-methyl coumarin (AMC) in dimethyl sulphoxide followed by diluting to desired AMC concentration with tris-HCl buffer (0.05 M; pH 7.5). Excitation and emission wavelengths were set to 380 and 460 nm,

respectively, with the fluorescence intensity measured at regular intervals over a 60 min period. Plasmin activity was expressed in AMC units (nanomoles of AMC released per minute) mL^{-1} of sample.

3.3.8 Statistical data analysis

Two independent trials were conducted under sub-critical and limiting flux conditions at each temperature on separate days, with three independent replicates performed for all experimental analyses. Results are expressed as mean ± standard deviation, unless otherwise stated. Analysis of variance (one-way ANOVA; Tukey's HSD test) was performed using R i386 version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) to compare a response (e.g., protein content) at the five different temperatures performed for a specific pressure (i.e., either at sub-critical or limiting flux). Independent t-test was used to identify statistically significant differences between samples generated under sub-critical and limiting flux conditions at the same processing temperature. The level of significance was determined at $p < 0.05$ to determine whether statistical differences existed between the mean values.

3.4 Results and discussion

3.4.1 Process design and operational parameters

The pressures at which membrane filtration is carried out determine whether processing is performed under sub-critical (J_{sub}), critical (J_{crit}) or limiting (J_{lim}) flux (volume of permeate per unit membrane surface area per unit time) conditions (Bacchin *et al*., 2006), as evident in Fig. 3.1. In this study, J_{crit} and J_{lim} values decreased significantly ($p < 0.05$) as processing temperature decreased. J_{crit} values of 13.5 ± 0.19 and 9.30 ± 0.35 L m^{-2} h^{-1} were determined at 20 and 4°C, respectively. J_{lim} also

decreased with decreasing processing temperature, with values of 14.9 ± 0.12 and 10.6 ± 0.19 L m^{-2} h^{-1} at 20 and 4°C, respectively.

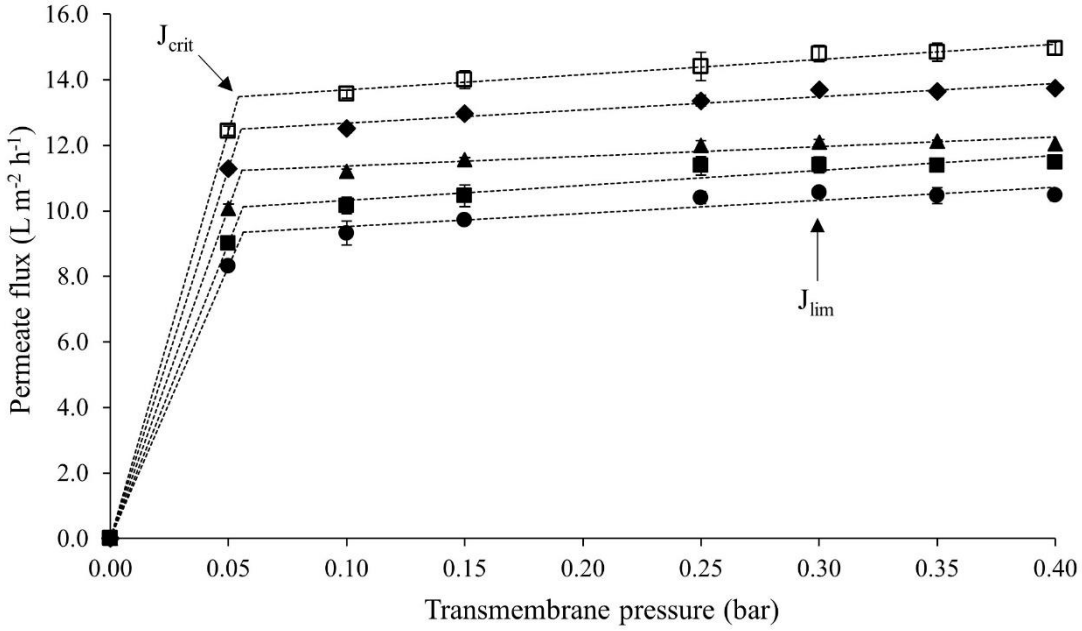


***Figure 3.1**: Permeate flux as a function of transmembrane pressure during microfiltration of skim milk (3.20%, w/v protein) in total recirculation mode at 4 (●), 8 (■), 12 (▲), 16 (◆) and 20°C (□). Critical flux (J_{crit}) values were determined as the point of intersection between linear fits of the pressure-dependent and pressure-independent regions. Limiting flux (J_{lim}) values were determined as the maximum permeate flux achieved on increasing transmembrane pressure.*

The viscosity of the feed was measured at each of the processing temperatures, with values of 1.95, 2.18, 2.68, 3.03 and 3.85 mPa.s at 20, 16, 12, 8 and 4°C, respectively. The higher viscosity associated with performing membrane filtration of skim milk at low temperatures has been shown to result in lower initial permeate flux (Jarto *et al*., 2018; McCarthy *et al*., 2017). Therefore, filtration at low temperatures is costly, not only due to the refrigeration and associated electrical energy costs required to maintain the plant at the low temperature, but also due to the increased energy required to pump higher viscosity feed, permeate and retentate streams. In addition, if

performing membrane filtration at low temperatures under J_{sub} conditions, the TMP that can be applied during the process is reduced, ultimately reducing flux and thereby increasing processing times.

For the subsequent trials aimed at determining the effect of temperature and TMP on protein and enzyme partitioning, TMP values of 0.05 and 0.30 bar were selected for operating at J_{sub} and J_{lim}, respectively. J_{sub} was selected as it is known to limit the effects of fouling (Bacchin *et al*., 2006), while operating above J_{sub} (i.e., J_{crit} or J_{lim}) is known to cause extensive irreversible fouling (Diagne *et al*., 2013).

3.4.2 Fouling analysis

Fouling during the filtration of dairy streams can have a significant impact on membrane performance (e.g., flux, processing times) and on the selectivity of separation (Beckman & Barbano, 2013; Vyas *et al*., 2000). Fouling of the membrane was significantly higher ($p < 0.05$) when filtration was performed at J_{lim} compared to J_{sub}. The reduction in normalised water permeability (NWP; Eq. (3.1)), from original NWP before MF to that after the MF of skim milk was 146 ± 32.8 and 270 ± 15.4 L m^{-2} h^{-1} bar^{-1} for J_{sub} and J_{lim}, respectively. The higher TMP associated with operating at J_{lim} resulted in more extensive fouling of the membrane. These findings are similar to those of Diagne *et al*. (2013), who studied the effect of operating at J_{crit} or J_{lim} on the cleanability of a polyethersulfone (PES) membrane. The authors reported that after filtration of skim milk at J_{lim} and J_{crit}, lower water flux values were measured at J_{lim}, indicating a more extensively fouled membrane when filtration was performed under J_{lim} conditions. Operating at high TMP (i.e., J_{lim}) during the MF of skim milk causes the structure of the membrane deposit layer to become more compact and less permeable (Heidebrecht *et al*., 2018; Steinhauer *et al*., 2011), thereby reducing protein

transmission, due to a reduction in the porosity of the fouling layer and the membrane itself (Hartinger *et al*., 2019).

3.4.3 Composition of the process streams

The composition of permeate and retentate streams (Table 3.1) on MF of skim milk was dependent on processing temperature and TMP. A reduction in processing temperature resulted in an increase in ash content in the permeate streams, under J_{sub} and J_{lim} conditions. The ash content of permeate streams at 20 and 4°C were 0.46 and 0.50%, respectively, under J_{sub} conditions, and 0.46 and 0.51%, respectively, under J_{lim} conditions. As temperature decreases, solubilisation of colloidal calcium phosphate (CCP) within casein micelles occurs, increasing the concentration of calcium and other minerals in the serum phase of milk (Fox *et al*., 2015). Therefore, at 4°C, the concentration of soluble minerals that can permeate the membrane is higher (Méthot-Hains *et al*., 2016), contributing to the higher ash levels measured in the permeate streams. In agreement with this, Crowley *et al*. (2018) reported that micellar casein concentrate (MCC) powder produced using MF of skim milk at cold temperatures (<10°C) had lower levels of calcium compared to MCC powder generated at warm temperatures (50°C).

Significantly higher ($p < 0.05$) protein content in the retentate streams was observed when filtration was performed at higher TMP. Filtration under J_{lim} conditions resulted in a significantly higher ($p < 0.05$) protein content in the retentates compared to retentate generated at the same temperature under J_{sub} conditions (i.e., 3.78 and 4.02% protein at 20°C under J_{sub} and J_{lim}, respectively). As expected, a decrease in processing temperature resulted in a significant decrease ($p < 0.05$) in the protein content of retentates. McCarthy *et al*. (2017) reported higher protein contents in both

MF and ultrafiltration (UF) retentates generated from skim milk at 50°C compared to 10°C. A similar effect was observed by Luo *et al*. (2015), when studying the effect of temperature on the protein content of permeate streams generated from UF of skim milk at 15, 30 and 50°C, using a membrane with a molecular weight cut-off (MWCO) of 20 kDa; the authors also reported that retentate generated at the highest temperature had the highest protein content. In the present study, under J_{sub} conditions, the protein content in permeate increased significantly ($p < 0.05$) from 0.485 to 0.563% (w/w) as processing temperature decreased from 20 to 4°C, respectively. The higher protein content observed in permeates generated at the lower temperatures may be attributed, at least in part, to the higher concentrations of serum β-casein at low temperature (McMahon & Oommen, 2013).

It is evident that protein sieving coefficient, calculated using Eq. (3.2), increased as processing temperature decreased (Fig. 3.2). A reduction in processing temperature resulted in an increase in protein sieving coefficients from 0.13 at 20°C to 0.17 at 4°C, under J_{sub} conditions. As previously mentioned, the higher protein content in retentates generated at higher processing temperatures, coupled with the lower protein content in the permeate, resulted in lower sieving coefficients measured at the higher processing temperature (i.e., 20°C).

Protein sieving coefficients were also higher following MF under J_{sub} conditions, e.g., sieving coefficients of 0.18 and 0.16 were measured under J_{sub} and J_{lim} conditions, respectively, at 8°C. This was expected following analysis of the fouling data (Section 3.4.2), as MF under J_{lim} conditions resulted in the greatest reduction in NWP due to a more extensively fouled membrane. In addition, filtration under J_{lim} conditions has been shown to increase the thickness and compaction of the fouling layer, reducing protein transmission (Heidebrecht *et al*., 2018).

Table 3.1: *Protein, ash and pH values for feed, permeate and retentate streams during microfiltration of skim milk at 4, 8, 12, 16 and 20ºC under both sub-critical and limiting flux conditions.*

Sample	Processing temperature (°C)	Protein (% w/w)			Ash (%)			pH		
		Sub-critical flux	Limiting flux	*p*-value	Sub-critical flux	Limiting flux	*p*-value	Sub-critical flux	Limiting flux	*p*-value
Feed	-	3.16 ± 0.02	3.19 ± 0.03	n.s.	0.71 ± 0.01	0.71 ± 0.01	n.s.	6.70 ± 0.02	6.70 ± 0.01	n.s.
Retentate	4	3.27 ± 0.02^{a}	3.26 ± 0.01^{a}	n.s.	0.73 ± 0.01^{ab}	0.75 ± 0.02^{a}	n.s.	6.79 ± 0.03^{a}	6.80 ± 0.02^{a}	n.s.
	8	3.23 ± 0.03^{a}	3.42 ± 0.01^{b}	*	0.70 ± 0.01^{a}	0.76 ± 0.01^{a}	*	6.81 ± 0.02^{a}	6.79 ± 0.01^{a}	n.s.
	12	3.57 ± 0.03^{b}	3.54 ± 0.02^{c}	n.s.	0.73 ± 0.01^{ab}	0.75 ± 0.01^{a}	n.s.	6.81 ± 0.03^{a}	6.79 ± 0.02^{a}	n.s.
	16	3.69 ± 0.01^{c}	3.79 ± 0.04^{d}	*	0.76 ± 0.01^{b}	0.75 ± 0.03^{a}	n.s.	6.78 ± 0.03^{a}	6.81 ± 0.02^{a}	n.s.
	20	3.78 ± 0.02^{d}	4.02 ± 0.07^{e}	*	0.73 ± 0.02^{ab}	0.76 ± 0.01^{a}	n.s.	6.80 ± 0.03^{a}	6.80 ± 0.02^{a}	n.s.
Permeate	4	0.563 ± 0.005^{d}	0.571 ± 0.004^{d}	n.s.	0.50 ± 0.01^{b}	0.51 ± 0.01^{c}	n.s.	6.78 ± 0.03^{a}	6.76 ± 0.02^{a}	n.s.
	8	0.572 ± 0.005^{d}	0.556 ± 0.003^{c}	*	0.50 ± 0.02^{b}	0.49 ± 0.01^{bc}	n.s.	6.74 ± 0.01^{a}	6.74 ± 0.02^{a}	n.s.
	12	0.549 ± 0.003^{c}	0.517 ± 0.007^{b}	*	0.47 ± 0.01^{ab}	0.48 ± 0.01^{ab}	n.s.	6.74 ± 0.03^{a}	6.74 ± 0.01^{a}	n.s.
	16	0.529 ± 0.006^{b}	0.476 ± 0.006^{a}	*	0.47 ± 0.01^{ab}	0.47 ± 0.01^{ab}	n.s.	6.76 ± 0.03^{a}	6.73 ± 0.02^{a}	n.s.
	20	0.485 ± 0.003^{a}	0.465 ± 0.006^{a}	*	0.46 ± 0.01^{a}	0.46 ± 0.01^{a}	n.s.	6.73 ± 0.03^{a}	6.72 ± 0.02^{a}	n.s.

Values followed by different superscript letters in a column for each analysis within a specific sample set (e.g., protein content for retentate under sub-critical flux conditions) are significantly different ($p < 0.05$).

Significance of difference between sub-critical and limiting flux conditions at individual temperatures for a specific analysis was identified with independent t-test. * indicates significant difference ($p < 0.05$); n.s., no significant difference

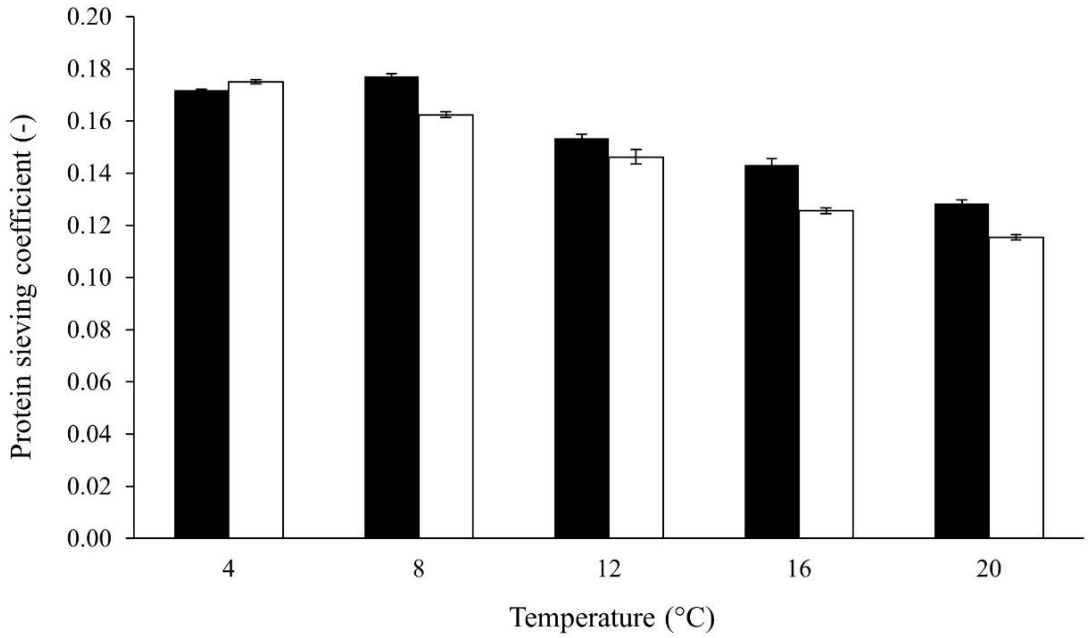


Figure 3.2: *Protein sieving coefficient (S_o) during microfiltration of skim milk at 4, 8, 12, 16 and 20°C under both sub-critical (■) and limiting flux conditions (□). Mean values and standard deviations are presented.*

3.4.4 Influence of temperature and transmembrane pressure on calcium distribution

In order to further investigate the effect of temperature and TMP on the partitioning of minerals between retentate and permeate during MF of skim milk, total calcium and ionic calcium of all filtration streams (Fig. 3.3) were measured. Total calcium content of the skim milk feed (1245 ± 7.64 mg L^{-1}) was found to be similar to that of fresh skim milk (Gaucheron, 2005). Reducing processing temperature resulted in a significant increase ($p < 0.05$) in the total calcium content of permeates generated under both J_{sub} and J_{lim} conditions. Total calcium contents of 414 and 397 mg L^{-1} were measured for permeates produced under J_{sub} and J_{lim} conditions, respectively, at 20°C. When filtration was performed at 4°C, total calcium content of the MF permeate increased significantly ($p < 0.05$) to 451 and 438 mg L^{-1} for J_{sub} and J_{lim}, respectively. Similarly, Davies and White (1960) reported that total calcium concentration

increased in skim milk ultrafiltrates from 350 to 374 mg L^{-1} as processing temperature was decreased from 20 to 3°C, respectively. Méthot-Hains *et al*. (2016) also reported increased permeation of calcium during UF of skim milk at 10°C, compared to 50°C. Crowley *et al*. (2018) produced MCC powders from the retentate stream arising from MF of skim milk at low (<10°C) and high temperature (50°C); the authors reported that the MCC powder generated at cold temperatures had lower levels of calcium compared to that made from warm MF, indicating increased permeation of calcium into the permeate at the low temperature.

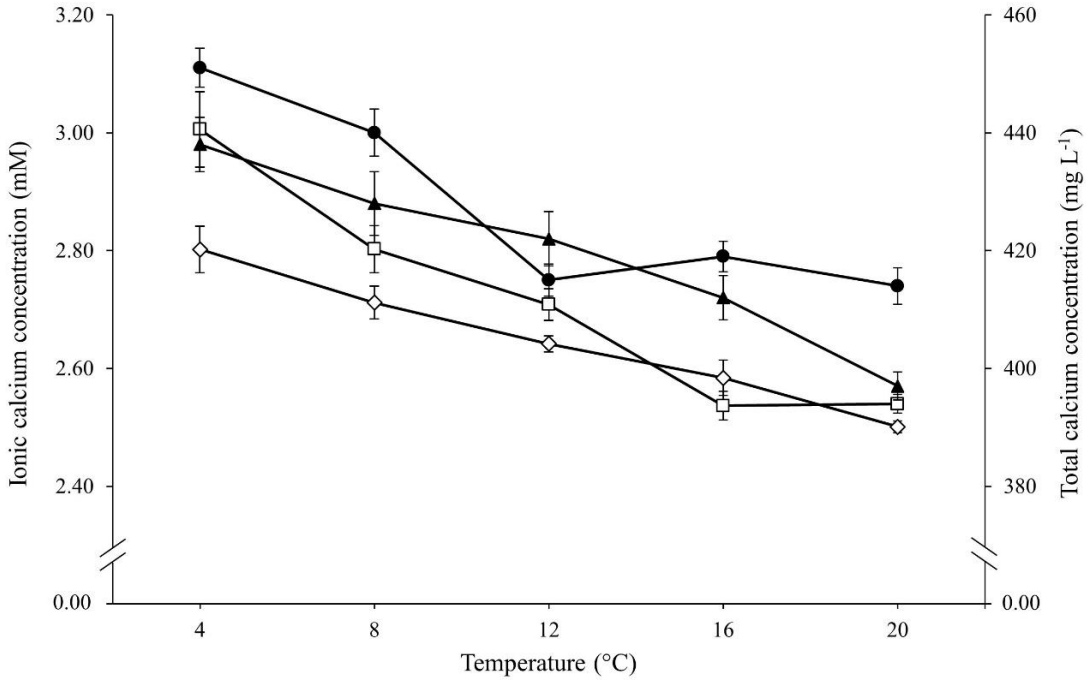


Figure 3.3: *Total calcium and ionic calcium concentrations of permeate streams generated by microfiltration of skim milk at 4, 8, 12, 16 and 20ºC under both sub-critical and limiting flux operating conditions; total calcium content values produced under sub-critical (●) and limiting flux (▲) conditions; ionic calcium values produced under sub-critical (□) and limiting flux (◊) conditions. Mean values and standard deviations are presented.*

The concentration of ionic calcium in feed and retentate streams ranged from 2.27-2.35 mM under J_{sub} and J_{lim} conditions, resembling those of milk (Lewis, 2011), with the concentration of ionic calcium being unaffected by temperature. This was to be expected, as the off-line analysis of ionic calcium was performed at 25°C; thus, the solubility of calcium phosphate in retentates decreases as temperature increases, causing a shift in the mineral equilibrium towards the micellar phase (Fox *et al*., 2015; On-Nom *et al*., 2010). However, although CCP is present in the skim milk feed and retentates, no CCP is present in the permeates; therefore, this phenomenon is relevant only to the feed and retentate, resulting in significant differences in concentrations of ionic calcium between permeate streams generated at different temperatures.

Reducing processing temperature resulted in a significant increase ($p < 0.05$) in ionic calcium concentration in the permeate streams generated under J_{sub} conditions; the concentration of ionic calcium increased from 2.54 to 3.01 mM as processing temperature decreased from 20 to 4°C, respectively. For milk, it is known that ionic calcium concentration increases as temperature decreases (Lewis, 2011; On-Nom *et al*., 2010) due to solubilisation of CCP at lower temperatures (Fox *et al*., 2015).

Under J_{lim} conditions, the same magnitude of increase in ionic calcium was not observed as processing temperature was decreased. In order to operate at J_{lim}, filtration is performed at a high TMP and operating at high TMP results in more extensive membrane fouling, which ultimately affects efficiency of separation (Heidebrecht *et al*., 2018). Therefore, the more extensive fouling when operating under J_{lim} conditions may have resulted in lower ionic calcium content in permeates compared to those permeate samples generated under J_{sub}, where a lower degree of fouling was recorded (Section 3.4.2). As there was no statistically significant ($p > 0.05$) difference in pH values for permeate streams generated at different temperatures (Table 3.1), the

measured differences in ionic calcium concentration were attributed to differences in processing temperature and membrane performance. Such an increase in total and ionic calcium concentration of permeate streams generated at low temperatures could potentially lead to subsequent processing issues, such as more extensive fouling on heating (Visser & Jeurnink, 1997) or greater demineralisation requirements.

3.4.5 Effects of temperature and transmembrane pressure on protein profile of permeates

The concentrations of individual caseins and whey proteins in permeates generated under J_{sub} and J_{lim} conditions at 4, 8, 12, 16 and 20°C are presented in Table 3.2. The highest concentration of β-casein was measured in permeate generated at 4°C under J_{sub} conditions. No β-casein was detectable following MF at 20°C under both J_{sub} and J_{lim} conditions. Decreasing processing temperature resulted in significant increases ($p < 0.05$) in the concentration of β-casein in permeate streams, generated under J_{sub} conditions, with values of 0.53 and 2.02 mg mL^{-1} for 16 and 4°C, respectively. The concentration of β-casein in permeate generated under J_{lim} conditions increased significantly ($p < 0.05$) from 0.45 to 1.68 mg mL^{-1} as temperature decreased from 16 to 4°C, respectively. The lower concentration of β-casein in the permeates generated under J_{lim} conditions is most likely due to the increased fouling observed at the higher TMP, impairing permeation of β-casein through the membrane. Therefore, operating at high pressure, although more attractive in terms of initial flux, can have negative consequences for the selectivity and efficiency of separation during membrane filtration (Steinhauer *et al*., 2011).

Permeate generated at 4°C under J_{sub} conditions contained 19.3% of the original concentration of β-casein in the skim milk feed; O'Mahony *et al*. (2014) achieved 10-20% depletion of the original β-casein following MF of skim milk at 2-5°C. The increase in concentration of β-casein in permeate as processing temperature decreased is evident from the reversed-phase high performance liquid chromatography (RP-HPLC) chromatograms in Fig. 3.4. Crowley *et al*. (2015b) assessed the processing and protein fractionation characteristics of three different polymeric UF and MF membranes during the filtration of skim milk at 3-4°C and reported a similar protein profile for permeate using the same 1000 kDa PES membrane used in this experiment. In the present study, a small quantity of κ-casein was identified in the permeate generated at each temperature, with Crowley *et al*. (2015b) also observing κ-casein in the permeate from the 1000 kDa membrane. However, Crowley *et al*. (2015b) detected small amounts of α_{s1}-casein, which were not observed in the chromatograms from the present study, potentially due to differences in processing conditions, as they concentrated skim milk to a volume concentration factor of 3. Although the predominant casein in human milk is β-casein, κ-casein is also present, acting as a colloidal stabiliser (Hambraeus & Lönnerdal, 2003). The κ- and β-casein-enriched whey stream is a potentially useful ingredient for application in infant milk formula, especially in whey protein-dominant first-age infant formula (O'Callaghan *et al*., 2011).

Table 3.2: *Concentration of β-casein, β-lactoglobulin, α-lactalbumin and κ-casein in permeate samples, determined using reversed-phase high performance liquid chromatography, produced during microfiltration of skim milk at 4, 8, 12, 16 and 20°C under both sub-critical and limiting flux conditions.*

Protein	Processing conditions					
	Temperature	Sub-critical flux	Limiting flux	*p*-value	Sub-critical flux	Limiting flux
	(°C)	Concentration of protein (mg mL^{-1})		-	Proportion of respective protein in feed (%)	
β-Casein	4	2.02 ± 0.20^{d}	1.68 ± 0.02^{c}	*	19.3	16.1
	8	1.66 ± 0.08^{c}	1.59 ± 0.12^{c}	n.s.	15.9	15.3
	12	1.19 ± 0.04^{b}	1.02 ± 0.05^{b}	*	11.4	9.80
	16	0.53 ± 0.08^{a}	0.45 ± 0.07^{a}	n.s.	5.12	4.31
	20	n.d.	n.d.	n.a.	n.a.	n.a.
β-Lactoglobulin	4	2.29 ± 0.05^{a}	2.35 ± 0.05^{a}	n.s.	95.0	97.4
	8	2.30 ± 0.06^{a}	2.28 ± 0.02^{a}	n.s.	95.4	94.8
	12	2.26 ± 0.04^{a}	2.25 ± 0.14^{a}	n.s.	93.7	93.2
	16	2.08 ± 0.14^{a}	2.15 ± 0.09^{a}	n.s.	86.4	89.0
	20	2.16 ± 0.08^{a}	1.97 ± 0.24^{a}	n.s.	89.4	81.6
α-Lactalbumin	4	1.27 ± 0.03^{a}	1.35 ± 0.09^{a}	n.s.	86.1	91.2
	8	1.27 ± 0.06^{a}	1.29 ± 0.04^{a}	n.s.	86.3	87.6
	12	1.23 ± 0.04^{a}	1.17 ± 0.27^{a}	n.s.	83.2	79.5
	16	1.22 ± 0.04^{a}	1.12 ± 0.14^{a}	n.s.	82.6	75.7
	20	1.15 ± 0.14^{a}	1.14 ± 0.13^{a}	n.s.	78.2	77.0
κ-Casein	4	0.36 ± 0.01^{a}	0.38 ± 0.01^{a}	n.s.	7.96	8.35
	8	0.35 ± 0.01^{a}	0.35 ± 0.01^{a}	n.s.	7.64	7.67
	12	0.34 ± 0.04^{a}	0.35 ± 0.01^{a}	n.s.	7.56	7.68
	16	0.35 ± 0.10^{a}	0.34 ± 0.01^{a}	n.s.	7.80	7.54
	20	0.35 ± 0.08^{a}	0.34 ± 0.02^{a}	n.s.	7.76	7.42

Values followed by different superscript letters for the concentration of a specific protein in a column are significantly different ($p < 0.05$). n.d., not detectable; n.a., not applicable. Significance of difference between sub-critical and limiting flux conditions at individual temperatures, for a specific protein, was identified with independent t-test. * indicates significant difference ($p < 0.05$); n.s., no significant difference.

β-casein, β-lactoglobulin, α-lactalbumin and κ-casein concentration as a function of the original respective protein in the skim milk feed solution are also presented.

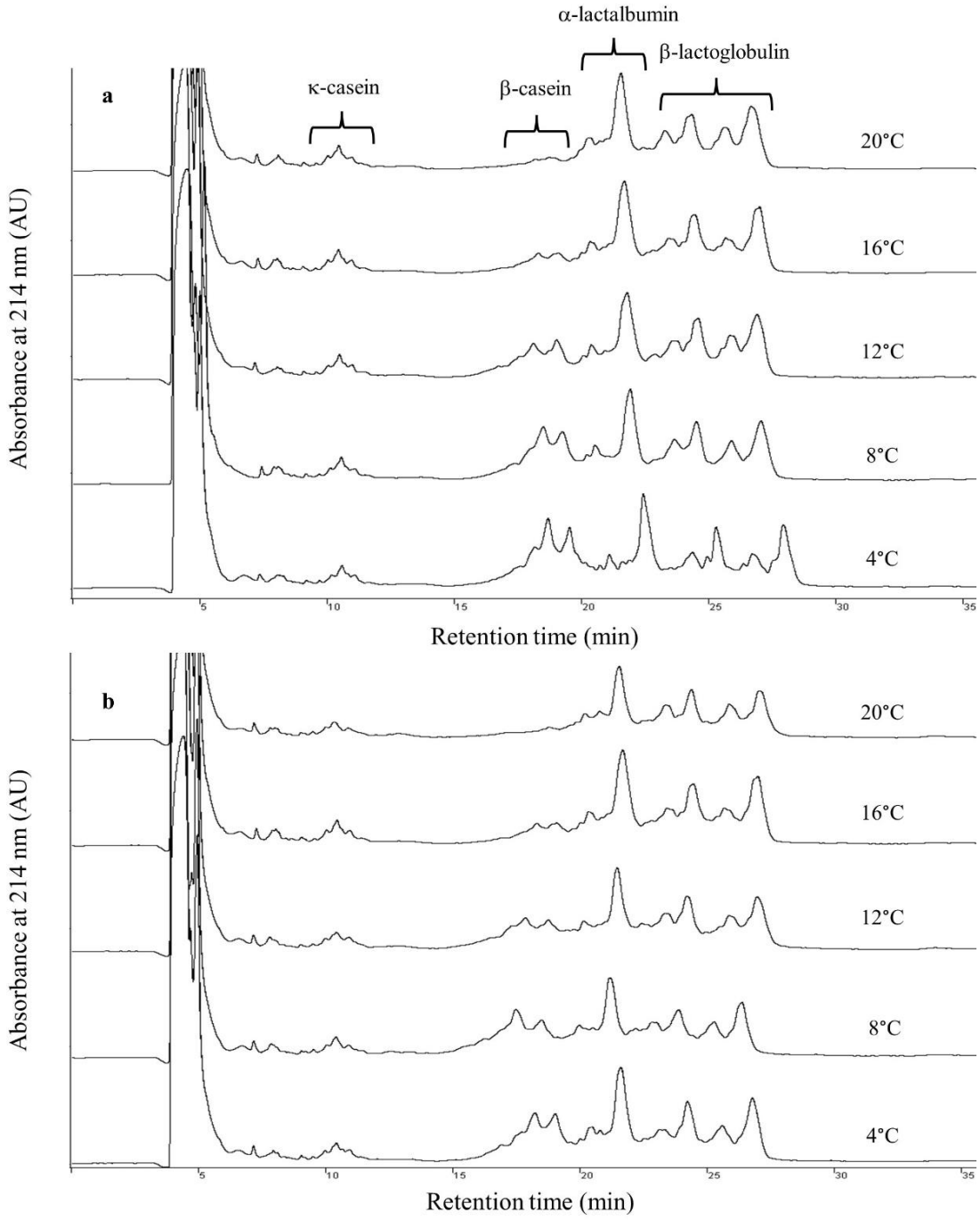


Figure 3.4: *Chromatograms from reversed-phase high performance liquid chromatography analysis of permeate samples produced during microfiltration of skim milk at 4, 8, 12, 16 and 20°C under (a) sub-critical flux and (b) limiting flux conditions.*

The concentrations of β-lactoglobulin (β-lg), α-lactalbumin (α-lac) and κ-casein showed similar trends as for the permeation of β-casein, increasing in the permeate streams as processing temperature decreased from 20 to 4°C, although the observed differences were not statistically significant ($p > 0.05$). Interestingly, and in contrast to the results for β-casein, the concentrations of all three proteins were higher in the permeate generated at 4°C under J_{lim} than under J_{sub} conditions, although not statistically different ($p > 0.05$). However, the concentrations of β-lg and α-lac decreased more extensively in permeates generated under J_{lim} conditions as temperature increased, compared to permeates generated under J_{sub} conditions. The greater fouling that occurs at higher processing temperatures, in addition to the higher fouling associated with operating at J_{lim}, is likely to have contributed to these decreases (Hartinger *et al*., 2019). Hartinger *et al*. (2019) reported the permeation of β-lg and α-lac to be higher at 10°C compared to 50°C, due to the formation of a more compact and crosslinked deposit layer on the membrane at 50°C. In the present study, operating under J_{lim} conditions resulted in NWP decreasing by 270 ± 15.4 L m^{-2} h^{-1} compared to just 146 ± 32.8 L m^{-2} h^{-1} under J_{sub} conditions, indicating more extensive fouling of the membrane under the former conditions (Section 3.4.2). Therefore, when combining the effect of higher TMP and higher processing temperatures (i.e., 20 compared with 4°C), protein permeation is reduced due to the formation of a thicker, more compact, fouling layer, altering the selectivity of the membrane as it effectively acts as a secondary filtration layer (Hartinger *et al*., 2019; Steinhauer *et al*., 2011).

3.4.6 Plasmin activity

Plasmin activity was analysed in the feed, retentate and permeate streams (Fig. 3.5), to assess how plasmin partitioned between feed and permeate as a function of processing temperature. The plasmin activity in the feed and retentate streams were significantly higher ($p < 0.05$) than in permeate, as plasmin is primarily associated with casein micelles (Bastian & Brown, 1996; Politis *et al*., 1992). There were no statistically significant ($p > 0.05$) differences in plasmin activity between retentates generated under J_{sub} and J_{lim} conditions.

Plasmin activity in permeate streams was not statistically different ($p < 0.05$), although plasmin activity was slightly higher at 4°C, under both J_{sub} and J_{lim} conditions (Fig. 3.5a). Plasmin activity values of 0.023 and 0.021 AMC units mL^{-1} were measured in permeate generated at 4°C under J_{sub} and J_{lim} conditions, respectively. There is conflicting evidence regarding the effect of temperature on the dissociation of plasmin from casein micelles; Reimerdes and Herlitz (1979) reported an increase in plasmin activity in milk stored at 4°C, suggesting that higher enzyme activity was due to the dissociation of plasmin and β-casein from casein micelles into the serum phase. However, later studies concluded that temperature had no significant influence on interactions between plasmin and casein in bovine milk (Donnelly & Barry, 1983; Grufferty & Fox, 1988). In this study, changes in processing temperature and TMP had no significant effect on plasmin activity measured in the different permeate streams.

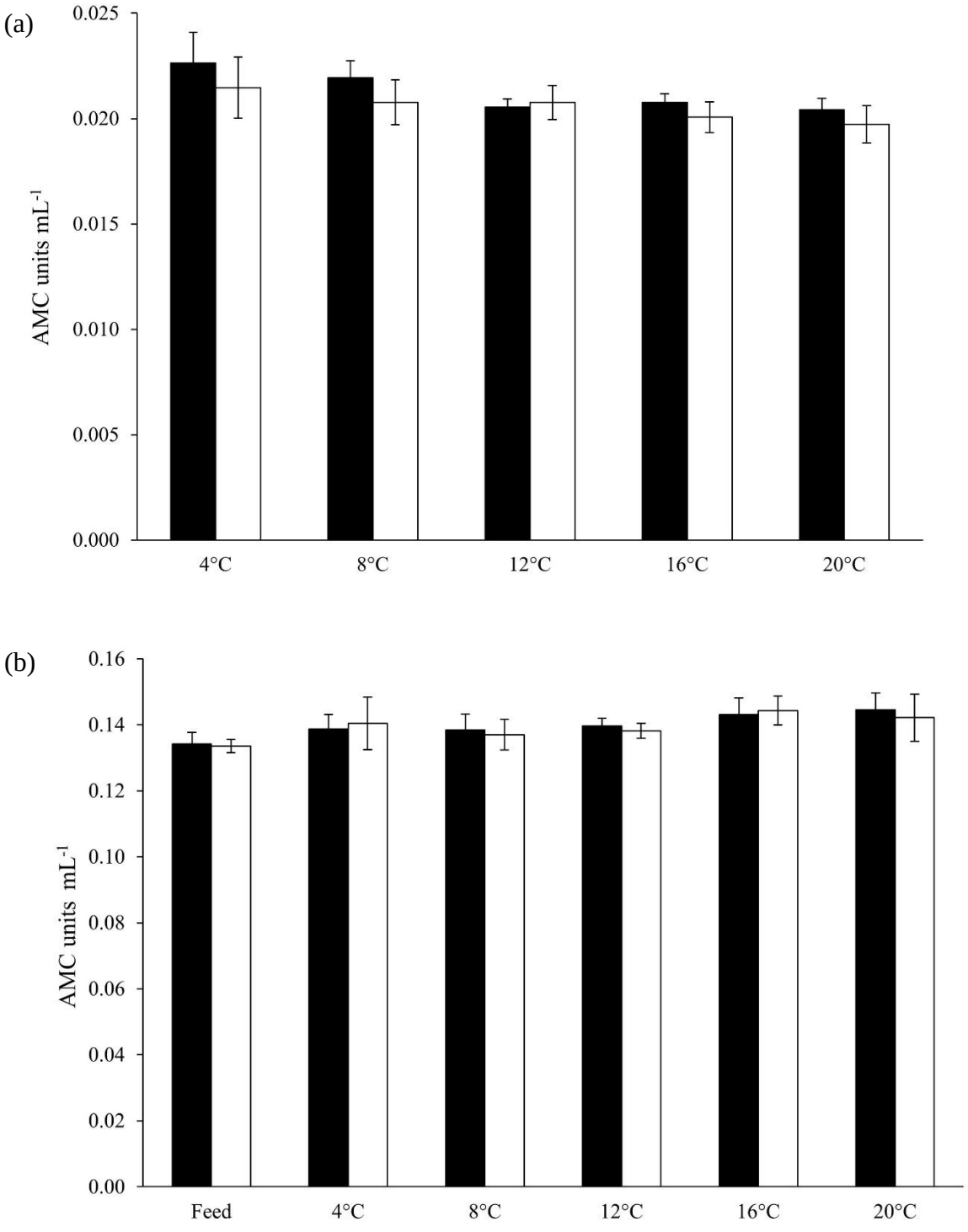


Figure 3.5: *Plasmin activity (expressed as AMC units mL^{-1}) of (a) permeates and (b) feed and retentate samples during microfiltration of skim milk at 4, 8, 12, 16 and 20°C under both sub-critical (■) and limiting flux (□) conditions. Mean values and standard deviations are presented.*

Concentration, instead of total recirculation, may result in greater plasmin activity of permeate and retentate samples, as differences in fouling become more pronounced, ultimately affecting protein partitioning (e.g., β-lg removal). Aaltonen and Ollikainen (2011) studied the effect of MF on plasmin activity and reported that plasmin activity in retentate was proportional to the concentration of β-lg; plasmin activity increased during diafiltration as β-lg was progressively removed in the permeate stream. The principal substrate for plasmin is β-casein (Bastian & Brown, 1996); therefore, plasmin activity in β-casein-enriched permeates could have negative consequences for the stability of the subsequent ingredient, particularly if specific functional properties of β-casein (i.e., emulsification, foaming or mineral binding properties) are desired in that ingredient.

3.5 Conclusion

The temperatures and pressures that microfiltration of skim milk is performed under significantly influence the partitioning of proteins and minerals. Operating at 4°C resulted in the highest yield of β-casein in the permeate; however, it was also associated with the highest plasmin activity, which could have negative implications for downstream processing and the colloidal stability and technological functionality of ingredients derived therefrom. Operating at high transmembrane pressure resulted in extensive membrane fouling, which consequently altered partitioning, reducing transmission of protein and plasmin to the permeate. These results provide new information on how adjusting membrane processing conditions can alter protein, mineral and enzyme partitioning during cold microfiltration of skim milk.

3.6 Acknowledgements

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Chapter 4

The influence of temperature on filtration performance and fouling during cold microfiltration of skim milk

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Declaration

This chapter was written by author Thomas C. France (TCF) and reviewed by all co-authors. TCF co-designed the study and performed all of the experimental work.

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4.1 Abstract

Changes in the physicochemical properties and distribution of constituents in skim milk during microfiltration (MF) at low temperature influence filtration performance and product composition. In this study, the influence of processing temperature within the cold MF range (4, 8 and 12°C) on filtration performance, fouling and partitioning of proteins was investigated. MF at 4°C required the greatest energy input due to the significantly higher ($p < 0.05$) viscosity of feed and retentate streams, compared to processing at 8 and 12°C. The greatest and lowest extents of reversible and irreversible fouling during MF were observed on filtration at 12 and 4°C, respectively. Chemical analysis of the cleaning solutions post-processing demonstrated that protein was the major foulant; the lowest protein content in the recovered cleaning solutions (50°C water and 55°C alkali) was measured after MF at 4°C. The concentration of β-casein, β-lactoglobulin and α-lactalbumin in the permeate all decreased throughout MF, due to fouling of the membrane. The greatest decrease in concentration of β-casein in the permeate during MF was observed at 12°C (18.1%) followed by 8°C (17.1%) and 4°C (13.6%). The results of this study provide new and valuable information on processing efficiency (i.e., energy consumption and protein yield) and membrane fouling during the processing of skim milk in the cold MF temperature range.

4.2 Introduction

Microfiltration (MF) is a technology used for the separation of whey proteins from casein micelles in skim milk, allowing for the generation of functional ingredients such as ideal whey (i.e., that produced using filtration) and micellar casein concentrate (Maubois, 2002). MF of skim milk can be performed using polymeric or ceramic membranes (Beckman *et al*., 2010; Coppola *et al*., 2014; Jørgensen *et al*., 2016), with the choice of membrane material influencing the efficiency of separation (Zulewska *et al*., 2009). The temperature at which MF is performed is an important consideration, having significant implications on membrane performance, extent of membrane fouling and composition of the process streams and final ingredients generated. For example, MF of skim milk at high processing temperatures (i.e., 50°C) results in the generation of ideal whey, containing essentially no β-casein; conversely, MF of skim milk at low temperatures (i.e., 4°C) results in the generation of ideal whey enriched in caseins, specifically β-casein (i.e., β-casein enriched whey) (Coppola *et al*., 2014). This ability to selectively modify protein profile, coupled with lower rates of fouling, has prompted increased research and commercial attention in low-temperature MF approaches for processing of milk (Luo *et al*., 2015; Ng *et al*., 2018; Schäfer *et al*., 2019).

Fouling remains one of the main challenges during membrane filtration of milk and, in addition to process performance-related challenges, membrane selectivity is also often adversely affected by fouling (Beckman and Barbano, 2013). The extent of fouling is influenced by a variety of factors including the composition of the feed, pre-treatment of the product, membrane pore size, transmembrane pressure (TMP) and the

temperature of filtration (Barukčić *et al*., 2014; Tan *et al*., 2014). During filtration of dairy streams such as milk and whey, it is generally accepted that proteins are the major foulants, due to their interactions with each other and the membrane, with the former often exacerbated by calcium-mediated cross-linking (James *et al*., 2003; Tan *et al*., 2014). Tan *et al*. (2014) investigated the composition of internal and external foulants after the cold MF (i.e., 6°C) of skim milk and reported elevated levels of whey proteins in the foulant material, suggesting a higher affinity of whey proteins, compared to caseins, for the membrane material.

It has been shown that the temperature at which filtration is performed can significantly influence the extent of fouling (Luo *et al*., 2015; Steinhauer *et al*., 2015; Hartinger *et al*., 2019). Steinhauer *et al*. (2015) studied the effect of temperature on membrane fouling during the filtration of whey and whey proteins (i.e., β-lactoglobulin; β-lg), reporting that filtration at 50°C resulted in greater membrane fouling compared to at 10°C. Méthot-Hains *et al*. (2016) reported that, during the UF of skim milk, operating at 50°C resulted in a more rapid rate of flux decline compared to at 10°C; the authors also reported that higher membrane resistance and greater reversible fouling were measured at 50°C, with similar results reported by Luo *et al*. (2015). However, although the higher degree of fouling experienced during filtration at high temperatures (i.e., 50°C) can largely be attributed to proteins, the precipitation of colloidal calcium phosphate (CCP) on the surface, or within the pores, of the membrane during the filtration of dairy streams, could also contribute to fouling at the higher processing temperatures (Hanemaaijer *et al*., 1989; Rice *et al*., 2009).

Membrane fouling during MF of dairy streams can impede the permeation of proteins through the membrane, thereby altering the partitioning dynamics and yield of individual proteins in the permeate (Beckman and Barbano 2013; Hartinger *et al*., 2019). Le Berre and Daufin (1994) reported that, during cold filtration (i.e., 4°C) of sodium caseinate suspensions, the permeability of β-casein was limited by the formation of a reversible fouling layer. Beckman and Barbano (2013) studied the effect of concentration factor (CF) on serum protein removal during MF of skim milk at 50°C, and reported that fouling of the membrane hindered the permeation of whey proteins into the permeate at the different CFs (i.e., 1.50, 2.25 and 3.00x). The authors reported that the protein concentration in the permeate decreased by 0.022, 0.034 and 0.035% per hour at 1.50, 2.25 and 3.00× CF, respectively.

Although operating at low temperature (i.e., 4°C) allows for the generation of novel ingredients (i.e., enriching permeate in a particular casein such as β-casein), changes in the physicochemical properties (i.e., increased viscosity) and distribution of milk constituents can alter membrane performance and composition of process streams. Therefore, the objective of this study was to investigate the impact of low temperatures at 4, 8 and 12°C on the processing performance, protein partitioning, fouling and energy consumption during the MF of skim milk. The results of this study provide valuable information on processing efficiency (i.e., energy consumption and protein yield) and membrane fouling in the cold MF range, which can be applied in developing and optimising filtration conditions for the generation of innovative milk protein ingredients.

4.3 Materials and methods

4.3.1 Materials

Low-heat skim milk powder was provided by a local Irish dairy company (Arrabawn Co-op, Nenagh, Co. Tipperary, Ireland). Reconstituted skim milk was prepared by adding low-heat skim milk powder to ultrapure water over 2 h at 22°C to attain a 3.20% (w/v) protein solution, at a native pH of 6.70. The skim milk was then stored at 4°C for 48 h under gentle constant magnetic stirring to facilitate complete rehydration. Prior to microfiltration (MF), the reconstituted skim milk was filtered using filter paper (Whatman No. 113; pore size 30 µm). All chemicals, reagents and high-performance liquid chromatography (HPLC) standards, unless otherwise stated, were sourced from Sigma-Aldrich (Wicklow, Ireland) and were of analytical grade.

4.3.2 Microfiltration set-up

MF experiments were performed on a lab-scale, pressure-driven, tangential-flow filtration device (Pellicon 2 mini-holder; Merck Millipore, Tullagreen, Carrigtwohill, Ireland) as described by Crowley *et al*. (2015a), using a 1000 kDa molecular weight cut-off (equivalent to a 0.1 µm pore size MF membrane), V-screen, polyethersulfone membrane (Biomax, Merck Millipore) with 0.1 m^2 total membrane area. MF was performed at 4, 8 and 12°C, at a cross flow velocity of 0.24 m/s, with a plate heat exchanger (PHE) recirculating the feed/retentate used to control the temperature throughout filtration. An equilibration time of 40 min was allowed at each of the specific temperatures prior to concentration to ensure stable conditions by returning both permeate and retentate lines to the feed vessel (2.5 L; i.e., full recirculation mode). During concentration, the retentate stream was recycled back to the feed vessel,

while the permeate was continuously removed until a volume concentration factor (VCF) of 3 was achieved.

In order to assess changes in the partitioning of proteins during MF, permeate samples were taken at different stages throughout filtration, aliquoted in 2 mL Eppendorf tubes and frozen at -80°C. Samples were collected as soon as permeate started to be produced (taken as VCF 1) and at a VCF of 2 and 3. The protein profile of these permeate samples were then analysed using reversed-phase high performance liquid chromatography (RP-HPLC) to determine the effect of fouling on the permeability of the membrane (detailed in Section 4.3.6).

4.3.3 Microfiltration processing performance

4.3.3.1 Transmembrane pressure and permeate flux

TMP (bar) and permeate flux were measured at 5 min intervals during MF. TMP was calculated according to the following equation:

$$TMP = \frac{P_i + P_o}{2} \quad [4.1]$$

where P_i and P_o are inlet and outlet pressure, respectively. Permeate flux ($L/m^2/h$) was recorded during processing by measuring the volume of permeate generated in a graduated cylinder over 30 s.

4.3.3.2 Energy consumption

Power meters (Energenie, Essex, UK) were used to measure the energy consumption (kWh) of both the feed/recirculation pump (i.e., mechanical energy) and the water bath (i.e., thermal energy) controlling the temperature of the PHE at each of

the processing temperatures. For thermal energy calculations, it was assumed that the system had ideal thermal insulation and the dissipation of thermal energy was considered negligible. The total energy at each of the processing temperatures was taken as the sum of the thermal and mechanical energy consumption required to reach a VCF of 3.

4.3.4 Determination of fouling and membrane cleaning

The extent of fouling, and the efficiency of cleaning, of the membrane was assessed by measuring normalised water permeability (NWP) after filtration, and after each of the cleaning solution cycles for comparison to reference values. The NWP (L m^{-2} h^{-1} bar^{-1}) was calculated as follows:

$$NWP = \frac{R \times F}{A \times TMP} \quad [4.2]$$

where R is permeate flow rate (L/h), F is the temperature correction factor based on water fluidity relative to 25°C (-), A is the membrane area (m^2), and TMP is the transmembrane pressure (bar). Immediately after completion of filtration, 1 L of ultrapure water was flushed through the membrane to remove any remaining skim milk within the membrane and tubing. Following this, NWP was measured using 1.4 L of ultrapure water. This value was taken as the NWP of the fouled membrane after filtration and the water used to perform the NWP was collected and taken as the initial water clean. After this, 1 L of ultrapure water was recirculated through the membrane at 50°C for 50 min (i.e., 50°C water) with an applied TMP of 0.2 bar; this cleaning solution was collected and taken as the 50°C water clean and a second NWP measurement was performed using 0.5 L of ultrapure water. Finally, 1 L of 0.4 N

NaOH was recirculated through the membrane at 55°C for 50 min (i.e., 55°C alkali) with an applied TMP of 0.2 bar; this cleaning solution was collected and taken as the 55°C alkali clean, and a final NWP measurement was performed on the cleaned membrane using 0.5 L of ultrapure water. Prior to each new trial, an NWP test was also performed on the clean membrane, to allow both the extent of fouling, and the efficiency with which each of the cleaning solutions restored NWP after filtration, to be calculated.

In addition to the cleaning procedures detailed above, the membrane was further cleaned on a separate day to ensure that the membrane was in the best condition for each subsequent trial. The cleaning steps applied were as follows; 2 L of 50°C ultrapure water recirculated for 30 min, followed by 1 L of 55°C 0.4 N NaOH recirculated for 50 min, 2 L of ultrapure water flushed to waste and 1 L of 3% (w/v) ethylenediamine tetra-acetic acid recirculated for 30 min at 25°C. A TMP of 0.2 bar was applied for all cleaning steps. This additional cleaning procedure ensured that the NWP for all processing trials was >84% of that of the new membrane. For cleaning of the PHE, the membrane element of the unit was bypassed and a step-wise cleaning cycle was performed, consisting of ultrapure water (2 L), 1.0 N NaOH (1 L), ultrapure water (2 L), 1.0 N HNO_3 (1 L), followed by a final flush with ultrapure water (2 L).

4.3.5 Composition and rheological properties of process streams

Retentate samples were collected at 12 min intervals during MF and the total solids were determined using standard International Dairy Federation methodology (IDF, 2010). Total nitrogen content of each of the cleaning solutions and process streams (i.e., feed, retentate and permeate) was determined using the Kjeldahl method

using a nitrogen-to-protein conversion factor of 6.38 for the calculation of crude protein (IDF, 2001); ash content was determined after heating samples to 650°C until a white ash was obtained (IDF, 2008).

The ionic calcium concentration of feed, permeate and retentate samples was measured using a Titrando 907 autotitrator with Tiamo v2.2 software equipped with a calcium (Ca)-ion-selective electrode (Metrohm Ireland Ltd, Co. Carlow, Ireland) according to the method reported by Crowley *et al*. (2014), with minor modifications. The probe was calibrated at 25°C using buffer solutions of known calcium-ion concentration (0.50, 1.00, 2.50, 5.00, 10.0 mM). Samples were equilibrated with the probe for 1 min prior to taking measurements.

Apparent viscosity of feed, permeate and retentate streams was measured at a shear rate of 1000 s^{-1} using a rotational viscometer (HAAKE RotoVisco 1, Thermo Fisher Scientific, MA, USA) with a cylindrical double gap cup and rotor geometry (DG43, Thermo Fisher Scientific, MA, USA). The temperature at which the viscosity of the samples was determined was the same as the processing temperature used to generate the samples (i.e., 4, 8 and 12°C).

4.3.6 Protein profile of process streams

The protein profile of the feed, retentate and permeate (final bulk permeate and individual in-process permeate at VCF 1, 2 and 3) samples were measured using RP-HPLC (Agilent 1220 Infinity II LC, Santa Clara, CA 95051 United States) with a C18 column (3.6 µm x 250 mm x 4.6 mm, Aeris Widepore, Phenomenex, UK) as described by Bonfatti *et al*. (2008), Crowley *et al*. (2015b) and Bot *et al*. (2020), using solvents

A (10.0% HPLC-grade acetonitrile, 89.9% ultrapure water and 0.10% TFA) and B (89.9% HPLC-grade acetonitrile, 10.0% ultrapure water and 0.10% TFA); the injected volume was 40 µL, and detection was carried out at 214 nm. Prior to analysis, samples were filtered through 0.45 µm filters (Minisart® RC25, Göttingen, Sartorius AG, Germany). The concentrations of individual proteins were determined by preparing standard curves of the respective proteins ($R^2 > 0.99$) and results are expressed as mg protein per mL of sample.

4.3.7 Statistical data analysis

All experimental analyses were conducted in triplicate, with samples being produced from three independent trials for each processing temperature, unless otherwise stated. Results are expressed as mean ± standard deviation. Analysis of variance (one-way ANOVA; Tukey's HSD test) was performed using R i386 version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) on mean values. The level of significance was determined at $p < 0.05$ to determine whether statistical differences were present between the mean values. Linear regression analysis by least squares minimisation was performed using Microsoft Excel 2007. The goodness-of-fit was evaluated based on the correlation coefficient (R^2), with an $R^2 > 0.99$ indicating a strong correlation.

4.4 Results and discussion

4.4.1 Processing performance

The temperature at which filtration is performed influenced both the permeate flux and the rate of permeate flux decline (Fig. 4.1). Initial permeate flux values of 13.2, 12.0 and 11.0 L/m^2/h were measured at 12, 8 and 4°C, respectively. Permeate flux decreased in a linear manner throughout processing, resulting in final flux values of 7.4, 7.0 and 6.2 L/m^2/h at 12, 8 and 4°C, respectively. The rate of decline in flux, determined by calculating the slope of the flux-time profiles, was highest at 12°C (-0.059), followed by 8°C (-0.049) and 4°C (-0.042).

From Fig. 4.1, it is evident that, at the beginning of MF, there are large differences in permeate flux at the different temperatures; however, after 45 min, differences in permeate flux are considerably less, with permeate flux reaching similar values towards the end of filtration. The higher flux recorded at 12°C was expected, due to the significantly lower ($p < 0.05$) apparent viscosity (2.06 mPa.s) of the permeate at 12°C than at 8 and 4°C, with values of 2.21 and 2.48 mPa.s, respectively (Table 1). McCarthy *et al*. (2017) reported higher initial flux values when MF of skim milk was carried out at 50°C, compared to 8.9°C; in agreement with the results from the present study, those authors also observed that flux decline was more rapid at the higher processing temperature, and attributed this to greater fouling. Similar results have been previously reported by Luo *et al*. (2015) and Méthot-Hains *et al*. (2016), who reported that ultrafiltration (UF) of skim milk at low processing temperatures (i.e., 10-15°C) results in a lower extent of flux decline than at high processing temperatures (i.e., 50°C).

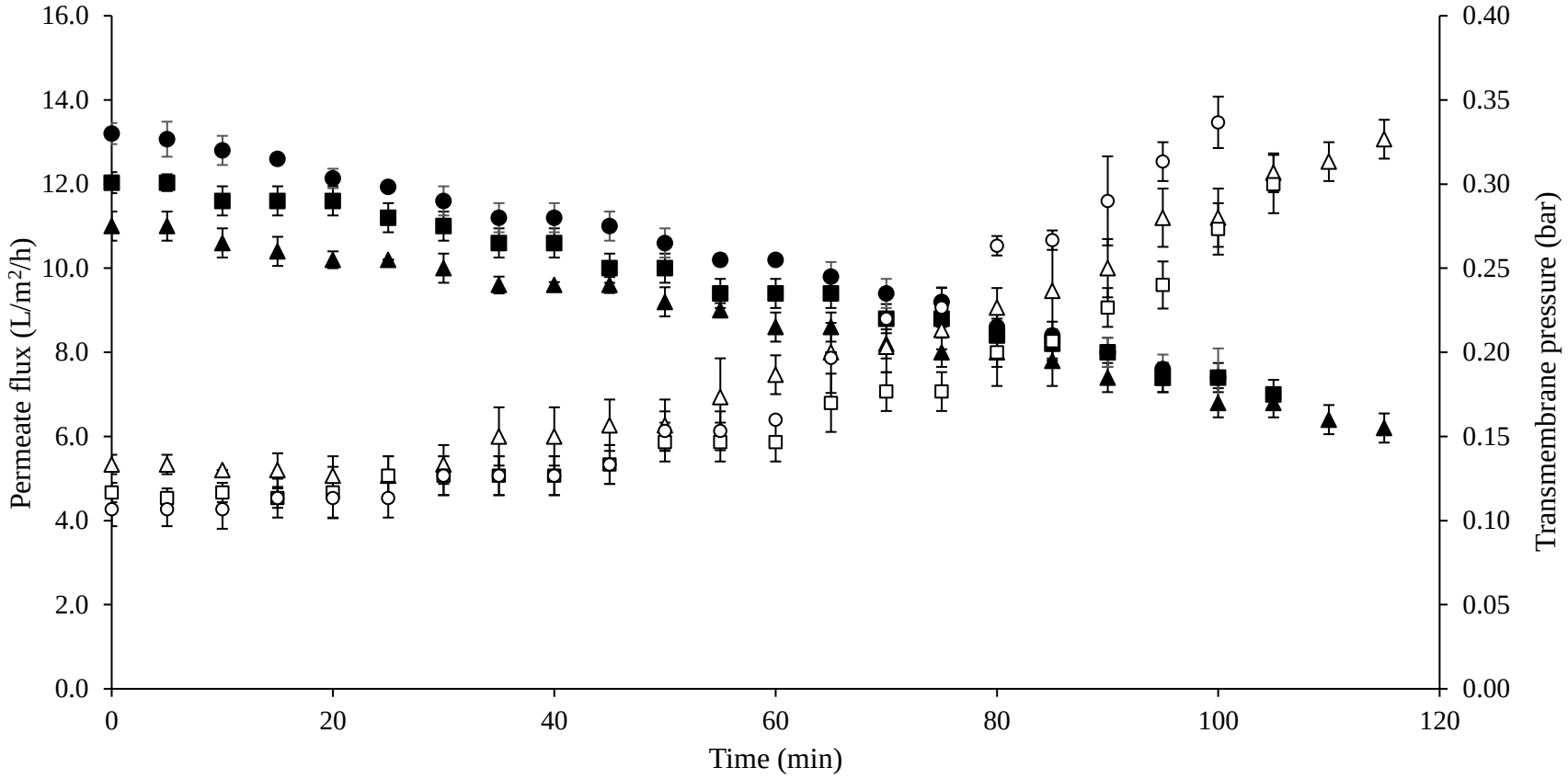


Figure 4.1: *Permeate flux (closed symbols) and transmembrane pressure (open symbols) as a function of time during the microfiltration of skim milk at 4, 8 and 12°C: permeate flux at 4°C (▲), 8°C (■) and 12°C (●); transmembrane pressure at 4°C (△), 8°C (□) and 12°C (○). Filtration was continued until a volume concentration factor of 3 was achieved.*

TMP is a key processing parameter determining filtration process performance. Initial TMP values of 0.11, 0.12 and 0.14 bar were measured at 12, 8 and 4°C, respectively. During MF at each of the processing temperatures, TMP increased gradually for the first 70 min of filtration and after this, the greatest increase in TMP was observed at 12°C.

4.4.2 Effect of processing temperature on energy consumption during filtration

The mechanical and thermal energy requirements during the MF of skim milk are strongly dependent on processing temperature (Table 4.1). At 4°C, significantly higher ($p < 0.05$) mechanical energy requirements were measured compared to at 8 and 12°C, with values of 27.6, 24.6 and 22.9 $\times 10^{-3}$ kWh, respectively. This can be attributed to the significantly higher ($p < 0.05$) viscosity of the skim milk feed and retentate at 4°C; the viscosity of the final retentate generated at 4, 8 and 12°C was 9.82, 8.58 and 7.11 mPa.s, respectively. The energy required to maintain the MF plant at 4 and 8°C was also significantly higher ($p < 0.05$) than at 12°C (Table 4.1). These results are in accordance with those of Méthot-Hains *et al*. (2016), who reported that optimal conditions, in terms of total energy consumption, were achieved when UF of skim milk was performed at a higher temperature (i.e., 50°C), compared to a lower temperature (i.e., 10°C). In addition to higher process stream viscosity, the higher energy requirements at 4°C may be partly attributed to the generation of heat through frictional forces (i.e., pumping), which would increase the energy required to maintain the lower temperature; longer processing times required to reach a VCF of 3 at 4°C also contributed to higher energy requirements.

Table 4.1: *Mechanical and thermal energy requirements to reach a volume concentration factor of 3 during the microfiltration of skim milk and the apparent viscosities of skim milk feed, permeate and retentate at 4, 8 and 12°C.*

Processing temperature (°C)	Energy consumption (10^{-3} kWh)			Viscosity (mPa.s)		
	Mechanical	Thermal	Total	Feed	Retentate	Permeate
4	27.6 ± 1.10^{c}	872 ± 21.0^{b}	900	3.69 ± 0.10^{c}	9.82 ± 0.32^{c}	2.48 ± 0.16^{b}
8	24.6 ± 0.60^{b}	846 ± 17.0^{b}	871	3.13 ± 0.09^{b}	8.58 ± 0.46^{b}	2.21 ± 0.21^{a}
12	22.9 ± 0.50^{a}	791 ± 30.0^{a}	814	2.70 ± 0.08^{a}	7.11 ± 0.48^{a}	2.06 ± 0.05^{a}

[a-c]Values followed by different superscript letters within a column are significantly different ($p < 0.05$).

4.4.3 Influence of processing temperature on retentate and permeate composition

MF processing at 12°C resulted in the greatest increase in total solids in retentate throughout filtration and the highest total solids in the retentate at the end of MF (14.1% w/w), compared to at 8 and 4°C, with values of 13.9 and 13.8% (w/w), respectively. However, higher total solids were measured in permeate generated at 4°C, with values of 6.56, 6.51 and 6.49% (w/w) for permeates generated at 4, 8 and 12°C, respectively. MF of skim milk to a VCF of 3 resulted in significant ($p < 0.05$) increases in levels of protein in retentates, with the highest protein content in the retentate generated at 12°C (7.80%) (Table 4.2). For permeates, a significantly higher ($p < 0.05$) protein content was measured at 4°C, followed progressively by permeates generated at 8 and 12°C. The higher degree of dissociation of β-casein into the serum phase of milk at lower temperatures (McMahon and Oommen, 2013), allowing for increased partitioning thereof into the permeate, is likely to have resulted in the higher protein content, in addition to the higher total solids measured in the permeate generated at 4°C.

Ash content of retentates after MF was also significantly ($p < 0.05$) higher than that of the initial skim milk, due to the concentration of casein micelles and associated CCP (Table 4.2). As expected, a lower ash content was measured in permeates than retentates, while the ash content was significantly ($p < 0.05$) higher in the permeate generated at 4°C, with values of 0.51, 0.47 and 0.46% for permeates generated at 4, 8 and 12°C, respectively. A similar trend was observed for ionic calcium, with the highest concentration of ionic calcium measured in the permeate generated at 4°C (3.07 mM). The solubilisation of CCP at low temperatures (Fox *et al*., 2015) has been shown to result in increased calcium concentration in permeate following the MF of skim milk (McCarthy *et al*., 2017).

Table 4.2: *Ash, protein and ionic calcium concentrations in skim milk feed, retentate and permeate samples generated from the microfiltration of skim milk at 4, 8 and 12°C.*

Sample	Processing temperature (°C)	Ash (%)	Protein (% w/v)	Ionic calcium (mM)
Feed	n.a.	0.74 ± 0.02	3.18 ± 0.04	2.45 ± 0.09
Retentate	4	1.16 ± 0.01^{ab}	7.59 ± 0.08^{a}	2.52 ± 0.07^{a}
	8	1.18 ± 0.03^{b}	7.77 ± 0.13^{b}	2.48 ± 0.08^{a}
	12	1.14 ± 0.02^{a}	7.80 ± 0.17^{b}	2.52 ± 0.12^{a}
Permeate	4	0.51 ± 0.01^{b}	0.634 ± 0.006^{c}	3.07 ± 0.16^{b}
	8	0.47 ± 0.01^{a}	0.617 ± 0.007^{b}	2.93 ± 0.07^{b}
	12	0.46 ± 0.02^{a}	0.585 ± 0.007^{a}	2.77 ± 0.04^{a}

$^{a\text{-}c}$Values followed by different superscript letters within a column for each analysis within a specific sample set (e.g., ash content for retentate) are significantly different ($p < 0.05$).

n.a. = not applicable

4.4.4 Effect of processing temperature on membrane fouling and composition of cleaning solutions

The extent of fouling after MF of skim milk at 4, 8 and 12°C was assessed by measuring the NWP after filtration and after each individual cleaning step, in order to obtain information on the extent of fouling and the nature of the foulant (Fig. 4.2). The greatest decrease in NWP after MF was at 12°C, with NWP decreasing to 68.6% of the original NWP measured prior to filtration. Lower decreases in NWP were measured after MF at 8 and 4°C, decreasing to 75.0 and 73.1% of the original NWP, respectively, indicating less fouling compared to that on MF at 12°C. Barukčić *et al*. (2014) measured water flux before and after MF of whey with different mean pore sizes (0.1, 0.5 and 0.8 μm), at 20 and 50°C, and reported that fouling intensity was reduced at the lower processing temperature.

After determining the NWP of the fouled membrane, the membrane was cleaned using ultrapure water at 50°C and the NWP was measured, yielding information on the loosely bound fouling material. Following cleaning with water at 50°C, the NWP for the membrane increased at all selected processing temperatures, approaching that of the original NWP. The NWP of the membrane after MF at 4°C (93.7% of the original NWP) was significantly higher ($p < 0.05$) than that of the membrane following MF at 12°C (85.2% of the original NWP). The largest increase in NWP after the water clean at 50°C was for the membrane following MF at 4°C, with a 20.6% increase in NWP, while the smallest increase in NWP after the 50°C clean was at 8°C, followed by 12°C, with NWP increasing by 15.1 and 16.6% at those temperatures, respectively. Therefore, these results indicated that MF at 4°C resulted in the greatest amount of weakly attached foulant.

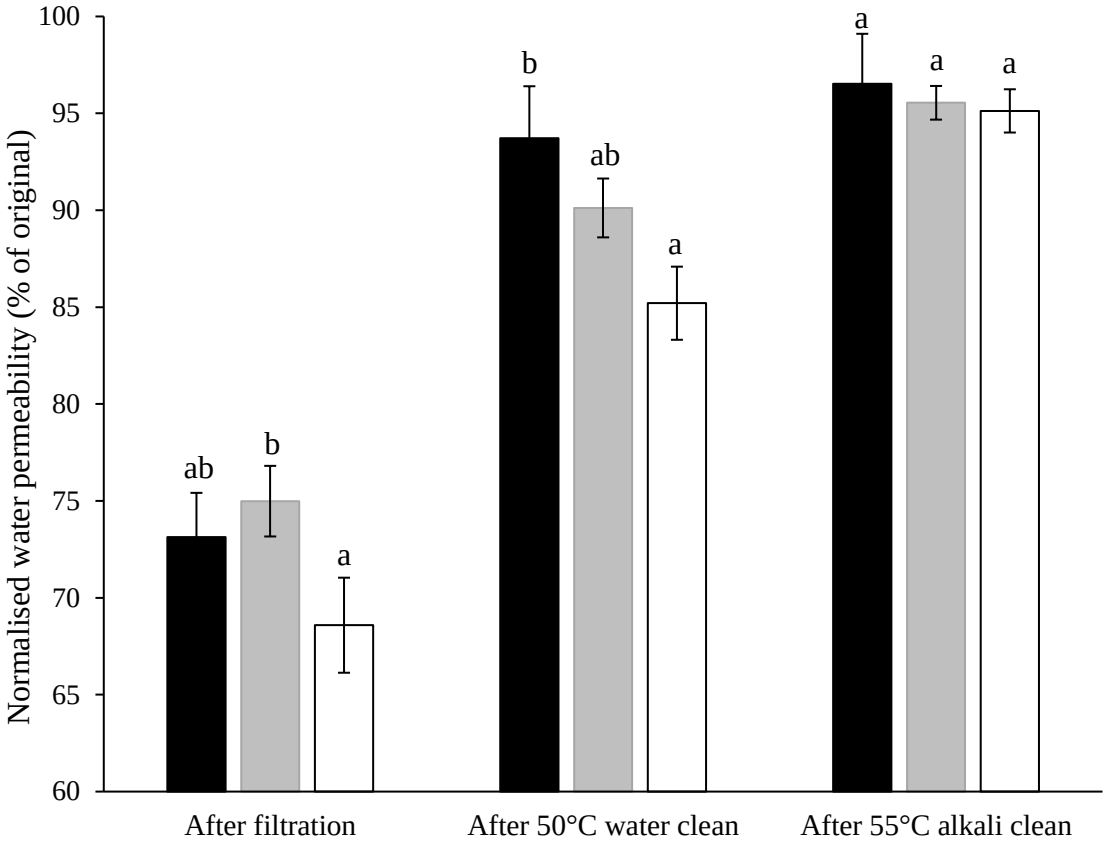


Figure 4.2: *Normalised water permeability, measured after filtration, after water clean at 50°C and after alkali clean at 55°C, as a percentage of the original normalised water permeability measured prior to filtration at 4 (■), 8 (■) and 12°C (□).* [a,b] *Mean values with different superscript letters within each individual cleaning step (e.g., after filtration) are significantly different ($p < 0.05$).*

The NWP after the 55°C alkali cleaning step was measured to provide information on the amount of strongly attached fouling material. Only a small increase in NWP was observed for the membrane following MF at 4°C, compared to 8 and 12°C with a 2.81, 5.42 and 9.92% increase in NWP, respectively; although more loosely bound fouling material was observed at 4°C, lower amounts of strongly bound material were present. Cleaning of the membrane with water at 50°C following MF at 4°C restored NWP to values close to those prior to MF, suggesting that mild cleaning regimes may be adequate at 4°C. The results indicate that more strongly bound fouling

material was present following MF at 8 and 12°C. Steinhauer *et al*. (2015) studied the effect of temperature on membrane fouling during MF and UF of whey and individual whey proteins and the authors reported that the flux decrease during MF at temperatures ≤20°C was due to the formation of a loosely packed fouling layer, whereas at higher temperatures (i.e., 40°C) a more densely packed fouling layer was formed. The formation of a loosely packed fouling layer at 4°C in the present study is supported by the effectiveness of water in the removal of foulant material.

The difference between the original and the final NWP after cleaning (data not shown) provided an index of the extent of irreversible fouling. Although the differences were not significant ($p > 0.05$), the lowest extent of irreversible fouling was measured at 4°C, followed by 8 and 12°C, with values of 3.47, 4.46 and 4.84%, respectively. As expected, irreversible fouling was considerably less prominent than reversible fouling, as the former is often associated with operating at limiting flux (high TMP) conditions (Steinhauer *et al*., 2011; Hartinger *et al*., 2019). During MF, TMP remained low until the last 30-40 min of filtration and is therefore unlikely to have allowed sufficient time for extensive irreversible deposition (Fig. 4.1).

Fouling of the membrane after filtration has been shown to be predominately caused by interactions between proteins and the polymeric membrane material and the formation of protein-protein aggregates that can result in pore blocking (James *et al*., 2003). In terms of fouling mechanisms of different milk proteins, casein micelles/aggregates have been shown to cause pore blockage, while whey proteins cause internal fouling and adsorption to the surface of the membrane during the MF of skim milk (Brans *et al*., 2004). Therefore, the composition (ash and protein levels)

of the cleaning solutions were analysed to determine the influence of processing temperature on the composition of the fouling layer (Fig. 4.3a, b). Higher levels of protein and ash were measured in the initial clean (ultrapure water) following MF at 4°C, although no significant ($p > 0.05$) differences between the selected processing temperatures were evident. The significantly higher ($p < 0.05$) viscosity of the feed and retentate at 4°C is likely to have reduced turbulence at the membrane surface, thereby increasing concentration polarisation and reducing the efficiency of foulant removal from the surface of the membrane (Gill *et al*., 1988; Aimar and Field, 1992; Jarto *et al*., 2018).

Protein and ash levels following the initial clean were significantly lower ($p < 0.05$) in the 50°C water solution, with the lowest level of protein and ash following MF at 4°C, with values of 0.15 and 0.03%, respectively. Similar results were observed in the 55°C alkali solution, with significantly less ($p < 0.05$) protein removed from the membrane following MF at 4°C, compared to that at 8 or 12°C. Although proteins are the major foulants during filtration of dairy streams, minerals such as calcium phosphate can also contribute to fouling, affecting membrane performance (Gésan-Guiziou *et al*., 1993). The low levels of ash measured in the cleaning solutions in this study suggest that the contribution of minerals to membrane fouling were not as significant as that of proteins. However, the solubilisation of CCP at the low temperatures may have indirectly contributed to membrane fouling, as the solubilisation of CCP at low temperature results in an increase in the concentration of ionic calcium, which can facilitate the formation of protein aggregates and cause blocking of membrane pores. In addition, cleaning of membranes at high temperatures

(i.e., 50-55°C) may have resulted in some precipitation of CCP onto the membrane, preventing its removal (Rice *et al*., 2009).

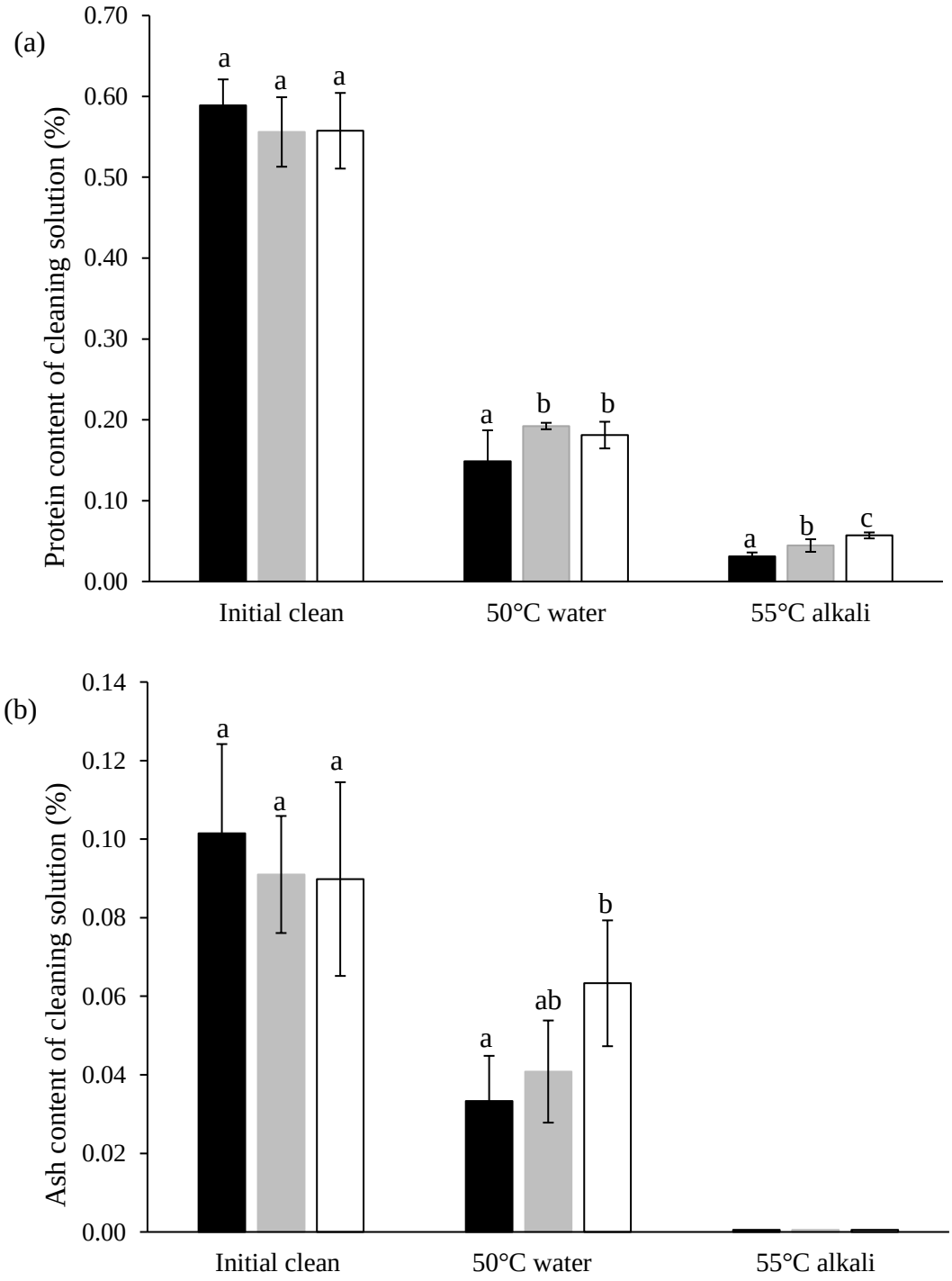


Figure 4.3: *Concentration of (a) protein and (b) ash in the initial water clean, water clean at 50°C and alkali clean at 55°C collected after the microfiltration of skim milk at 4 (■), 8 (■) and 12°C (□). Protein and ash values are related back to the total volume of cleaning solution used.* ^{a,b,c} *Mean values with different superscript letters within each individual cleaning step (e.g., after filtration) are significantly different ($p < 0.05$).*

4.4.5 Influence of temperature and volume concentration factor on protein partitioning

To understand how the partitioning of proteins changes throughout an MF run, as a function of temperature, permeate samples were collected at different VCFs (Table 4.3). α_s-Caseins were not detected in permeates, while the concentration of κ-casein did not change throughout MF (data not shown). Concentrations of β-casein, β-lg and α-lactalbumin (α-lac) in permeates all decreased throughout MF, with the greatest decrease measured for β-casein. The extent of decrease in concentration of β-casein in permeate from the beginning of MF (VCF 1) to the end (VCF 3) was in the order 12°C (18.1%), followed by 8°C (17.1%) and 4°C (13.6%). At low temperatures (i.e., 4°C), β-casein partially dissociates from the micelle into the serum phase, with β-casein in the serum primarily existing in monomeric form (Payens and Van Markwijk, 1963; Andrews *et al*., 1979). As temperature increases, β-casein monomers begin to self-associate, yielding micelles of β-casein, with a larger diameter than that of the monomeric form (Andrews *et al*., 1979; Li *et al*., 2019). Paynes and Van Markwijk (1963) reported that β-casein exists as a monomer at 4°C but, as temperature increased to 8.5°C, the degree of association increased to approximately 22. Similarly, subsequent research has shown that reducing temperature from 20 to 0°C decreases the tendency to associate and hydrodynamic diameter of β-casein micelles, with β-casein existing primarily in a monomeric state at 4°C (Leclerc and Calmettes, 1997; Farrell *et al*., 2001; O'Connell *et al*., 2003).

Table 4.3: *Concentration of β-casein, α-lactalbumin and β-lactoglobulin in permeates collected at different stages throughout the microfiltration of skim milk at 4, 8 and 12°C. Permeate was collected at a volume concentration factor (VCF) of 1, 2 and 3 and the percentage decrease in concentration of each protein, from the beginning to the end of microfiltration, was calculated.*

		Protein content (mg/mL)			
Protein	Processing temperature (°C)	VCF 1	VCF 2	VCF 3	% decrease
β-casein	4	2.24 ± 0.06^{b}	2.09 ± 0.09^{ab}	1.93 ± 0.06^{a}	13.6 ± 0.52^{A}
	8	1.67 ± 0.02^{b}	1.50 ± 0.05^{a}	1.38 ± 0.06^{a}	17.1 ± 1.88^{B}
	12	1.05 ± 0.03^{c}	0.94 ± 0.01^{b}	0.87 ± 0.03^{a}	18.1 ± 2.73^{B}
α-lactalbumin	4	0.80 ± 0.005^{b}	0.79 ± 0.009^{b}	0.72 ± 0.003^{a}	9.96 ± 1.03^{B}
	8	0.82 ± 0.013^{c}	0.79 ± 0.007^{b}	0.77 ± 0.007^{a}	6.22 ± 2.24^{AB}
	12	0.84 ± 0.010^{b}	0.81 ± 0.008^{a}	0.81 ± 0.006^{a}	3.19 ± 1.39^{A}
β-lactoglobulin	4	2.16 ± 0.03^{a}	2.15 ± 0.06^{a}	2.11 ± 0.03^{a}	2.30 ± 0.68^{A}
	8	2.25 ± 0.04^{b}	2.17 ± 0.04^{a}	2.17 ± 0.04^{a}	3.39 ± 1.11^{A}
	12	2.35 ± 0.03^{b}	2.33 ± 0.03^{ab}	2.28 ± 0.02^{a}	2.61 ± 1.13^{A}

[a-c]Values followed by different superscript letters for the concentration of protein within a row are significantly different ($p < 0.05$).

[A-B]Values followed by different superscript letters for the percentage decrease in protein content during filtration within a column for individual proteins (i.e., β-casein) are significantly different ($p < 0.05$). Values are means ± standard deviations from two independent trials at each processing temperature.

Therefore, it can be hypothesized that fouling during MF is likely to have affected the permeation of β-casein in polymeric state (i.e., at 8 and 12°C) to a greater extent than that in the monomeric state (i.e., at 4°C). In addition, the higher rate of decrease in β-casein concentration at 12°C can be attributed to the greater extent of fouling (Fig. 4.2) at that temperature, reducing the permeability of the membrane.

The extent of decrease in the respective concentrations of β-lg and α-lac in the permeates during MF was lower than that observed for β-casein (Table 4.3). Unlike the trend observed for β-casein, the greatest percentage decrease in the concentration of α-lac was observed during MF at 4°C, while MF at 12°C resulted in a significantly ($p < 0.05$) lower decrease in the concentration of α-lac during MF. The largest decrease in β-lg during MF was observed at 8°C (3.39%), while the lowest decrease was observed at 4°C (2.30%). The reduced transmission of proteins as a result of altered membrane permeability due to fouling has been reported by numerous researchers (Gésan-Guiziou *et al*., 1999; Lawrence *et al*., 2008). It is well known that the whey proteins, in particular β-lg, are strongly responsible for membrane fouling during the filtration of skim milk and whey streams at low (i.e., <10°C) (Tan *et al*., 2014) and high temperatures (45-55°C) (Ng *et al*., 2018; Hartinger *et al*., 2019).

The differences in the transmission of β-casein and the whey proteins during MF at the different processing temperatures, may be attributed to the mechanism responsible for dissociation of β-casein. β-Casein is located primarily in the interior of the casein micelle and, at low temperature, β-casein first migrates to the surface of the casein micelle, from where it dissociates into the serum phase. At 4°C, the rate of dissociation of β-casein into the serum phase is greater than at 8 and 12°C, due to

lower hydrophobic interactions (Creamer *et al*., 1977; McMahon and Oommen, 2013); therefore, β-casein physically removed in the permeate stream during MF is, at least partially, replenished by further dissociation from the micelle. As there is an equilibrium between micellar and serum β-casein (Downey and Murphy, 1970), the greater rate of dissociation of β-casein from the micelle at 4°C, as free β-casein permeates the membrane, may have resulted in the lower rate of decrease in β-casein during MF at 4°C. On the other hand, for α-lac and β-lg, their serum phase concentrations are not in an equilibrium or dependent on the processing temperature; therefore, the differences observed for α-lac during MF are likely to be attributed to interactions with the membrane and fouling. α-Lac has the ability to bind Ca^{2+} (Anderson *et al*., 1997), which could explain the influence of temperature on transmission of α-lac, as there was more calcium ions present at 4°C, which may result in greater potential for calcium-mediated interactions between α-lac molecules and salt bridging between α-lac and the membrane.

4.4.6 Influence of temperature on the protein profile of process streams

The profile and concentrations of individual proteins in the permeate and retentate streams were analysed to provide further insight into the influence of processing temperature on partitioning of individual proteins (Fig. 4.4). As expected, the concentrations of all individual caseins were higher in the retentates than in the feed. No significant ($p > 0.05$) differences were observed in the concentrations of κ- and α_s-casein, in addition to α-lac and β-lg, between retentates produced at the different processing temperatures; however, significant differences were observed for β-casein. The concentration of β-casein was significantly lower ($p < 0.05$) in retentate

produced at 4°C compared to retentates produced at 8 and 12°C, with values of 24.4, 28.3 and 29.7 mg/mL, respectively. This was expected, as at low temperatures, hydrophobic interaction strength decreases, facilitating the dissociation of β-casein from the micelle into the serum phase of milk (McMahon and Oommen, 2013), where it can subsequently be transmitted through the membrane, into the permeate. As expected from the concentration of β-casein in the retentate, a significantly ($p < 0.05$) higher concentration of β-casein was measured in permeate following MF at 4°C (2.09 mg/mL), followed by 8°C (1.46 mg/mL) and 12°C (0.93 mg/mL) (Fig. 4b). The β-casein content of the permeate generated at 4°C represented 17.7% of the β-casein concentration of the original skim milk feed. No significant ($p > 0.05$) differences were observed between the concentrations of α-lac and β-lg in permeates generated at different temperatures, although the highest concentration of both was in permeate generated at 12°C.

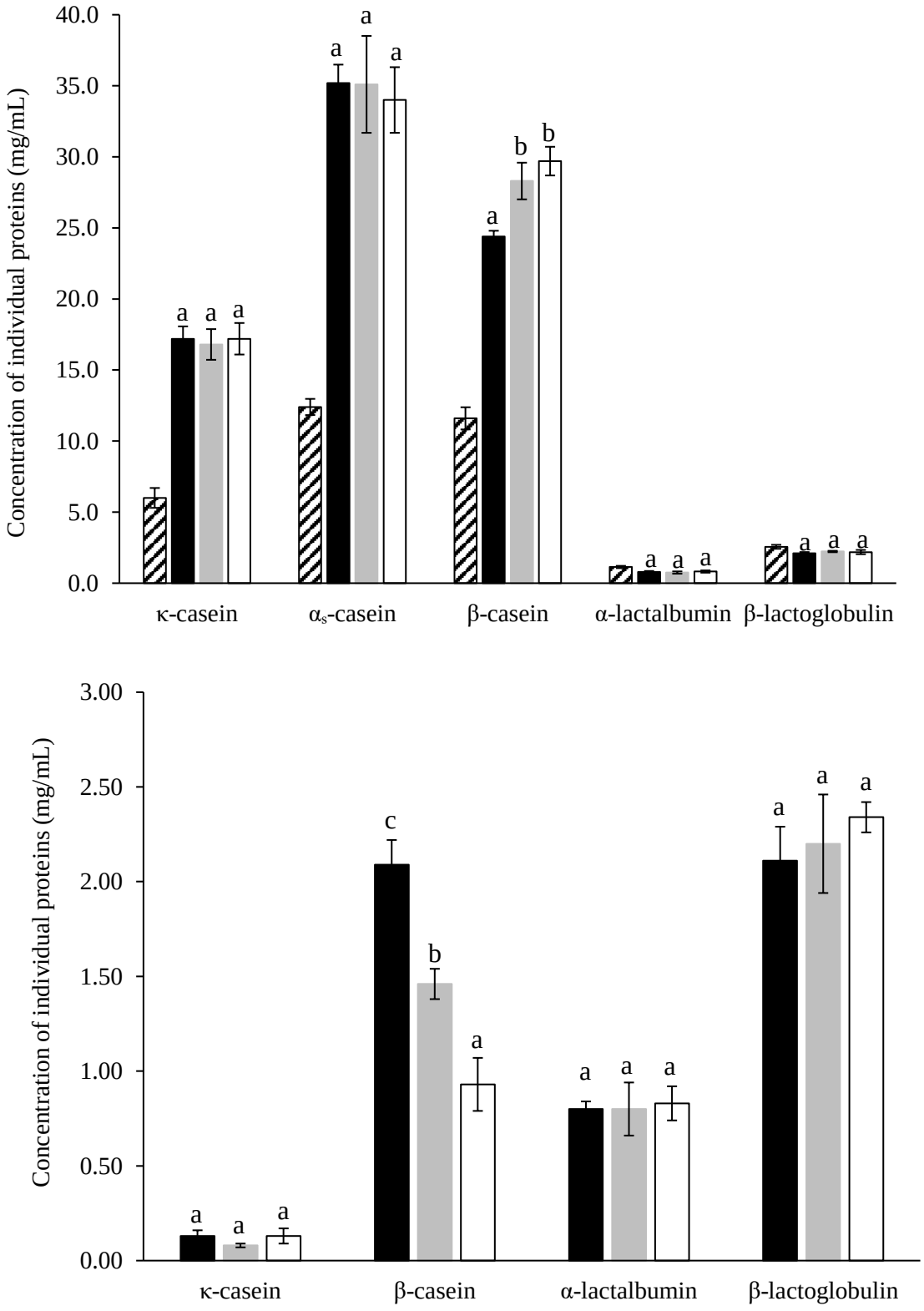


Figure 4.4: *Concentrations of individual proteins in (a) feed (skim milk: ▨) and retentate, and (b) permeate after microfiltration of skim milk at 4 (■), 8 (■) and 12°C (□) as measured using reversed phase-high performance liquid chromatography.* ^{a,b,c} *Mean values with different superscript letters within each individual protein are significantly different ($p < 0.05$). Different scale values were used between figure (a) and (b).*

4.5 Conclusions

The specific temperature, in the range 4-12°C, at which cold microfiltration of skim milk is performed significantly impacts membrane performance, fouling and protein partitioning. Performing microfiltration at 4°C resulted in lower initial fluxes, compared to 8 or 12°C, but ultimately a slower decline in permeate flux during processing was achieved. Filtration at 4 and 8°C resulted in the lowest extent of reversible and irreversible fouling, although these processing temperatures were associated with greatest mechanical and thermal energy requirements. Microfiltration at 4°C resulted in permeate with the highest concentration of β-casein and the lowest decrease in the concentration of β-casein in permeate throughout microfiltration. The results of this study provide new information on the influence of temperature on membrane performance, fouling and protein partitioning during the cold MF of skim milk, and demonstrate that performing microfiltration at lower temperatures may enable production of next-generation dairy streams with novel protein fractions, due to enhanced membrane performance and improved yields of β-casein.

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Chapter 5

Influence of processing temperature on plasmin activity and proteolysis in process streams from cold microfiltration of skim milk

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Declaration

This chapter was written by author Thomas C. France (TCF) and reviewed by all co-authors. TCF co-designed the study and performed all of the experimental work.

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5.1 Abstract

The presence of plasmin in process streams derived from microfiltration of skim milk can result in hydrolysis of caseins, and potentially affect the quality and functionality of ingredients produced therefrom. In this study, the influence of different temperatures (4, 8 and 12 °C) during cold microfiltration of skim milk were investigated to determine whether the partitioning of plasmin into permeates was influenced by processing temperature. Permeate generated at 4 °C had the highest plasmin activity (0.0185 AMC units/mL), followed by permeates generated at 8 and 12 °C. When plasmin activity was expressed relative to β-casein content, differences in the extent of dissociation of β-casein, at the different processing temperatures, did not influence plasmin activity in the resulting permeate streams. Particle size measurements before and after plasmin-mediated hydrolysis of permeates indicated that the proteolysis products of β-casein, γ-caseins, retained the ability to self-associate on heating, with particle size increasing with increasing temperature.

5.2 Introduction

During microfiltration (MF) of bovine milk at warm temperatures (i.e., 45-55 °C), using membranes with pore sizes of ~0.1 µm, casein micelles are retained (retentate), while the serum phase passes through the membrane (permeate). This results in casein-enriched retentate and whey protein-enriched permeate streams. However, performing MF of skim milk at low temperatures (i.e., ≤20 °C), alters the composition of such permeate and retentate streams; at low temperatures, β-casein dissociates from the casein micelle into the serum phase, from where it passes through the membrane into the permeate stream (France, Kelly, *et al*., 2021; O'Mahony *et al*., 2014). The lower the processing temperature used during MF of skim milk, the greater the extent of β-casein dissociation and the higher the concentration of β-casein in the permeate stream (France, Bot, *et al*., 2021). As a result, a β-casein-enriched permeate stream is generated, with a corresponding retentate depleted in β-casein.

MF of skim milk also alters the proteolytic activity, particularly that of plasmin, the principal indigenous proteinase in milk. In bovine milk, plasmin and its zymogen, plasminogen, are primarily associated with the casein micelles, while the inhibitors of the plasmin system are present in the serum phase (Korycha-Dahl *et al*., 1983; Politis *et al*., 1992). As the serum phase contains the inhibitors of the plasmin system (e.g., β-lactoglobulin and α_2-antiplasmin), its removal alters the equilibrium of the plasmin system. Aaltonen & Ollikainen (2011) studied the influence of MF and diafiltration of milk at 50 °C on plasmin activity; they observed that plasmin activity in retentate increased during diafiltration, suggesting that this was primarily due to the progressive removal of β-lactoglobulin, as a strong correlation was observed between plasmin activity and β-lactoglobulin concentration. In addition to the removal of inhibitors of the plasmin system during MF, plasminogen activators in bovine milk are

concentrated in the retentate, as the two main plasminogen activators in bovine milk, urokinase- and tissue-type plasminogen activators, are predominately associated with somatic cells and casein micelles, respectively (Heegaard *et al*., 1994b; Heegaard *et al*., 1994a; White *et al*., 1995). Increased plasmin activity can lead to undesirable plasmin-mediated hydrolysis of casein, resulting in defects such as age-gelation and bitterness, and also reduce yield due to loss of intact casein as peptides (Kelly & Foley, 1997; Rauh *et al*., 2014). In addition, the incorporation of casein-derived ingredients generated from such retentates in formulated food systems (e.g., clinical nutritional beverages) may also result in undesired plasmin-induced proteolysis, affecting the quality of products that include such ingredients.

Plasmin and plasminogen-derived activity in sweet whey and ultracentrifugal supernatants from skim milk have also been reported (Crudden *et al*., 2005; Crudden & Kelly, 2003; Hayes & Nielsen, 2000), indicating that plasmin is present in whey and permeate, respectively, albeit at low concentrations and activities (due to high levels of inhibitors of the plasmin system). Therefore, the presence of plasmin in whey-derived ingredients is of significance as it may affect the quality of products incorporating such ingredients. However, the presence of plasmin in permeate becomes even more significant during the cold MF of skim milk, as β-casein is also present in such permeate streams. Of the four major caseins (α_{s1}-, α_{s2}-, β- and κ-), β-casein is preferentially cleaved by plasmin, resulting in the loss of intact β-casein and the production of γ-caseins and proteose peptones (Andrews, 1983; Andrews & Alichanidis, 1983). With an ever-increasing drive towards cold MF (France, Kelly, *et al*., 2021), plasmin activity in β-casein-enriched permeates could have negative implications for the quality of such streams where intact β-casein is desired for its technological, functional and nutritional properties (e.g., emulsification, foaming and

encapsulation properties) (Li *et al*., 2016; Murphy & Fox, 1991; Shapira *et al*., 2010). The plasmin-mediated hydrolysis of β-casein in β-casein concentrate (BCC) manufactured using cold MF indicates that plasmin can affect the quality of the final ingredient (Kelly *et al*., 2021).

Therefore, the aim of the present study was to assess plasmin and plasminogen-derived activity in permeate and retentate streams generated by MF of skim milk, at different temperatures (4, 8 and 12 °C) within the cold MF range, to determine whether temperature, and consequential differences in the extent of β-casein dissociation during MF influenced plasmin partitioning. A related aim was to assess the influence of plasmin activity on quality and storage stability of the process streams generated. The results of this study provide information into the partitioning of plasmin during the cold MF of skim milk and the consequential plasmin-mediated proteolysis of β-casein in the process streams generated, during storage.

5.3 Materials and methods

5.3.1 Materials

Samples of skim milk, permeates and retentates were produced as described by France, Bot, *et al*. (2021; Chapter 4). In short, microfiltration (MF) of skim milk, using a 1000 kDa polyethersulfone membrane, was performed at 4, 8 or 12 °C until a volume concentration factor of 3 was achieved. The permeates generated consisted of whey proteins, in addition to various levels of β-casein, as influenced by processing temperature. The β-casein content of permeates generated at 4, 8 and 12°C was 2.09, 1.46 and 0.93 mg/mL, respectively, while the β-casein content of the corresponding retentates was 24.4, 28.3 and 29.7 mg/mL, respectively. More detailed composition of the permeates and retentates can be found in France, Bot, *et al*. (2021; Chapter 4). All chemicals, reagents and standards, unless otherwise stated, were sourced from Sigma-Aldrich (Wicklow, Ireland) and were of analytical grade.

5.3.2 Plasmin and plasminogen-derived activity

Plasmin and plasminogen-derived activity of skim milk, retentate and permeate samples were measured according to the method described by Richardson & Pearce (1981), with minor modifications, using a Varioskan flash multiwell plate reader (Thermo Fisher Scientific Oy, Finland). Skim milk feed and retentate samples were diluted with water (1:5 dilution) to reduce interference in the assay by casein. The diluted feed, retentate and undiluted permeate samples were mixed in a 3:1 ratio (milk:citrate) with 0.4 M trisodium citrate (TSC).

For plasminogen-derived activity, urokinase solution (200 Ploug U mL^{-1}) was added to the TSC-treated samples in a 1:1 ratio, resulting in a final concentration of 100 Ploug U/mL. The samples were then incubated at 37 °C for 60 min prior to

assaying for total plasmin activity. Following all sample preparation, samples were added to a black, flat-bottomed 96 well plate with tris-HCl buffer (0.05 M, pH 7.5) and coumarin peptide substrate (N-Succinyl-Ala-Phe-Lys-7-amido-4-methylcoumarin acetate salt) (Sigma-Aldrich, Wicklow, Ireland), a non-fluorescent substrate which is cleaved by plasmin to yield a fluorescent product, 7-amino-4-methylcoumarin (AMC). Standard curves were prepared by dissolving AMC in dimethyl sulphoxide, followed by diluting to desired AMC concentration with tris-HCl buffer (0.05 M; pH 7.5). Excitation and emission wavelengths were set to 380 and 460 nm, respectively, with the fluorescence intensity being measured at 1 min intervals over a 30 min period. Plasmin activity was expressed in AMC units (nanomoles of AMC released per min)/mL of sample. Plasminogen-derived activity was calculated according to the following equation:

$$\text{Plasminogen-derived activity} = [\text{plasmin activity (urokinase added)}] - [\text{plasmin activity (no urokinase added)}] \quad [5.1]$$

5.3.3 Incubation of samples to determine extent of plasmin-mediated proteolysis

After MF, sodium azide was added to all samples. Skim milk feed, permeate and retentates were added to a shaking incubator (Stuart shaking incubator, SI600, Bibby Scientific Ltd, Staffordshire, UK) at 37 °C for 7 d. The samples were gently shaken continuously to ensure the protein always remained in suspension. Due to the aggregation and partial precipitation of β-casein from β-casein-enriched permeates, TSC-treated permeates (~9 mM) were also incubated, with the TSC effectively disrupting the supra-micellar β-casein aggregates, thus maintaining the β-casein micelles in suspension. Samples were taken on d 0, 1, 2, 3, 5 and 7 and immediately frozen at -80 °C. Samples were taken in a laminar flow hood (Holten HH 48) which

was cleaned with 70% ethanol prior to sampling. Sterile plastic syringes were used to take samples to avoid contamination.

5.3.4 Electrophoretic and densitometric analysis of plasmin-mediated proteolysis

Samples were diluted with double strength buffer before analysis by urea-polyacrylamide gel electrophoresis (urea-PAGE; Andrews, 1983; O'Mahony *et al*., 2005). Skim milk feed, retentate and permeate samples were diluted in a 3:5, 1:5 and 1:1 ratio (sample:buffer), respectively. Gels were stained with Coomassie Brilliant Blue G250 (Blakesley & Boezi, 1977) and destained in distilled water. Densitometric analysis of the bands was carried out using Total-Lab Quant 13.1v software (Totallab, Newcastle, UK).

5.3.5 Measurement of particle size distribution

The particle size distribution of the β-casein-enriched permeates, before and after storage at 37 °C for 7 d, was measured by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Samples were syringe-filtered (0.45 µm) prior to measurements. A temperature ramp from 10 to 55 °C with 5 °C increments was performed, with an equilibration time of 9 min at each temperature; dry air was flushed through the instrument to avoid condensation at low temperature. A refractive index value of 1.45 was used for protein and a total of 5 measurements were taken at each individual temperature using a back-scattering angle of 173°. The particle size was expressed as the intensity-weighted mean hydrodynamic diameter (Z-average).

5.3.6 Turbidity

The turbidity of permeates was measured at 600 nm using a Cary 300 bio UV-Visible spectrophotometer (Varian Inc., CA, USA) which was equipped with a temperature control system. Permeate samples were measured for turbidity immediately after MF and at the end of incubation (at 20 °C). Permeate samples were also heated prior to analysis to facilitate micellisation of β-casein according to the methods of Crowley *et al*. (2019) and Li *et al*. (2019), with minor modifications; samples were heated to 50 °C for 30 min to allow for the self-association of β-casein while avoiding denaturation of whey proteins.

5.3.7 Statistical analysis

Samples were generated from three independent trials at each processing temperature. All experimental analyses were conducted in triplicate, unless otherwise stated. Results are expressed as mean ± standard deviation. The data generated was subjected to one-way analysis of variance (ANOVA) using R i386 version 4.0.2 (R foundation for statistical computing, Vienna, Austria). A Tukey's paired-comparison *post-hoc* test was used to determine statistically significant differences ($p < 0.05$) between mean values for different samples, with mean values deemed to be significantly different from each other at a 95% confidence level.

5.4 Results and discussion

5.4.1 Effect of processing temperature on plasmin and plasminogen-derived activity

Table 5.1: *Plasmin (PL) and plasminogen (PLG)-derived activities in skim milk feed, retentate and permeate samples after microfiltration of skim milk at 4, 8 and 12 °C*

Sample	Processing temperature	Plasmin activity	Plasminogen-derived activity	PLG:PL ratio
	°C	AMC units/mL	AMC units/mL	
Feed		0.147 ± 0.011	1.623 ± 0.092	11.0 ± 0.63
Retentate	4	0.294 ± 0.018^{a}	2.81 ± 0.089^{a}	9.56 ± 0.30^{a}
	8	0.324 ± 0.016^{b}	3.06 ± 0.089^{b}	9.44 ± 0.28^{a}
	12	0.328 ± 0.019^{b}	3.12 ± 0.126^{b}	9.51 ± 0.39^{a}
Permeate	4	0.0185 ± 0.0018^{b}	0.159 ± 0.006^{b}	8.59 ± 0.33^{a}
	8	0.0179 ± 0.0026^{ab}	0.154 ± 0.008^{ab}	8.60 ± 0.47^{a}
	12	0.0150 ± 0.0018^{a}	0.145 ± 0.005^{a}	9.65 ± 0.34^{b}

$^{a-b}$Values followed by different superscript letters within a column for a specific process stream are significantly different ($p < 0.05$)

The temperature at which MF was performed influenced the plasmin and plasminogen-derived activities in the β-casein-enriched permeate and casein-enriched retentate streams (Table 5.1). The plasmin and plasminogen-derived activities measured in retentates were higher than in the feed; as plasmin and plasminogen are primarily associated with the casein micelles, they are concentrated in the retentate during MF (Aaltonen & Ollikainen, 2011). This, combined with the removal of inhibitors in the permeate stream, results in higher activities measured in all retentates relative to the feed. The protein contents of retentates generated following the MF of skim milk at 4, 8 and 12 °C (R4, R8 and R12) were 7.59, 7.77 and 7.80%, respectively (France, Bot, *et al*., 2021). Plasmin and plasminogen-derived activities in retentates increased with increasing processing temperature. The significantly higher ($p < 0.05$)

plasmin and plasminogen-derived activities measured in R8 and R12 may partly be attributed to the significantly higher ($p < 0.05$) protein content, compared to R4. An alternative hypothesis for the lower plasmin and plasminogen-derived activity in R4 is the co-migration of plasmin with β-casein into the permeate stream; therefore, the plasmin and plasminogen-derived activities were also measured in the β-casein-enriched permeate streams.

The plasmin activity of permeates generated at 4, 8 and 12 °C (P4, P8 and P12) was lower than both the skim milk feed and corresponding retentates. The plasmin activity in P4 (0.0185 AMC units/mL) was significantly higher ($p < 0.05$) than for P12 (0.0150 AMC units/mL), with similar trends observed for plasminogen-derived activities (Table 5.1). Similar values have been reported for sweet whey (0.011 AMC units/mL; Crudden *et al*., 2005) and ultracentrifugal supernatants from skim milk (0.038 AMC units/mL; Crudden & Kelly, 2003). The lower plasmin activity measured at 12 °C could be attributed to the higher β-lactoglobulin concentration in these permeates (2.34 mg/mL) compared to P8 (2.20 mg/mL) and P4 (2.11 mg/mL), as the concentrations of both native and denatured β-lactoglobulin have been shown to be negatively correlated with plasmin activity in casein micelle suspensions in simulated milk ultrafiltrate (Bastian *et al*., 1993; Rollema & Poll, 1986). Bastian *et al*. (1993) suggested that in unheated mixtures (no heat treatment applied to plasmin/β-lactoglobulin mixtures), the inhibition of plasmin by unheated native and heat denatured β-lactoglobulin occurred through competitive inhibition. Aaltonen & Ollikainen (2011) observed a strong correlation between plasmin activity and the concentration of β-lactoglobulin in retentates during warm MF (50 °C) of milk, with plasmin activity increasing as β-lactoglobulin concentration decreased. A number of authors have attributed the reduced plasmin activity in the presence of β-lactoglobulin,

on heating, to the interaction of sulfhydryl groups with disulphide groups, which link the subunits in the plasmin molecule, *via* thiol-disulphide interchange, with acceleration of inactivation of plasmin in the presence of cysteine confirming this (Grufferty & Fox, 1986; Metwalli *et al*., 1998; Rollema & Poll, 1986).

However, as mentioned previously, the higher plasmin activity measured in P4 may also be due to the greater dissociation of β-casein during MF at this temperature, affecting plasmin-casein interactions to a greater extent. Therefore, the plasmin activity was also expressed as a function of β-casein content, to establish if there was a relationship between plasmin activity and extent of β-casein dissociation. The plasmin activity of P4, P8 and P12 was 8.83, 12.2 and 16.2 AMC units/g β-casein, respectively. When the β-casein concentration of the permeates is considered, the opposite trend is observed compared to when plasmin activity is expressed as AMC units mL^{-1}. This indicates that, at 4 °C, the greater extent of β-casein dissociation, and therefore the higher β-casein concentration, in permeate does not contribute directly to the higher plasmin activity. Therefore, it can be concluded that, although the temperature at which MF is performed within the cold MF range (≤20 °C) greatly impacts the β-casein content in the resulting permeate, the same effect is not observed for plasmin activity and is therefore most likely due to other factors, such as β-lactoglobulin concentration. However, the presence of plasmin and plasminogen-derived activity in such permeates is of significance as it may result in undesirable plasmin-mediated proteolysis of β-casein.

5.4.2 Proteolysis in feed and retentate samples following microfiltration at different temperatures

Urea-PAGE electrophoretograms of incubated skim milk feed and retentate samples (Fig. 5.1a; Fig. 5.2a) showed extensive plasmin-mediated hydrolysis of both β- and α_{s2}-casein during storage at 37 °C. Storage of skim milk at 37 °C resulted in only 30.6% of initial β-casein content remaining after 7 d of incubation, with the rate of hydrolysis decreasing towards the latter stages of storage (Fig. 5.1b). The decrease in the extent of hydrolysis in the later stages of storage could be attributed to the autolysis of plasmin (Gazi *et al*., 2014; Huppertz *et al*., 2004; Ueshima *et al*., 1996). Crudden *et al*. (2005) reported similar findings to those in the present study, observing that the greatest extent of β-casein hydrolysis occurred within the first 4 d of storage at 37 °C, before reducing significantly in the later days of storage; the authors reported that the decreased β-casein hydrolysis after 4 d of storage corresponded to a rapid decrease in plasmin activity. Although the decrease in hydrolysis is often attributed to autolysis of plasmin, recent findings highlight the complexity of the plasminogen/plasmin system. Nurup *et al*. (2020) examined the plasminogen/plasmin system in acid whey, reporting that the autolysis products of plasminogen/plasmin (e.g., midi-, mini-, and micro-plasmin) contributed to the total plasminogen/plasmin activity. Therefore, autolysis of plasminogen/plasmin, at least initially, is not likely to be the sole contributor to the decline in hydrolysis during storage, as the autolytic products of plasminogen/plasmin remain proteolytically active.

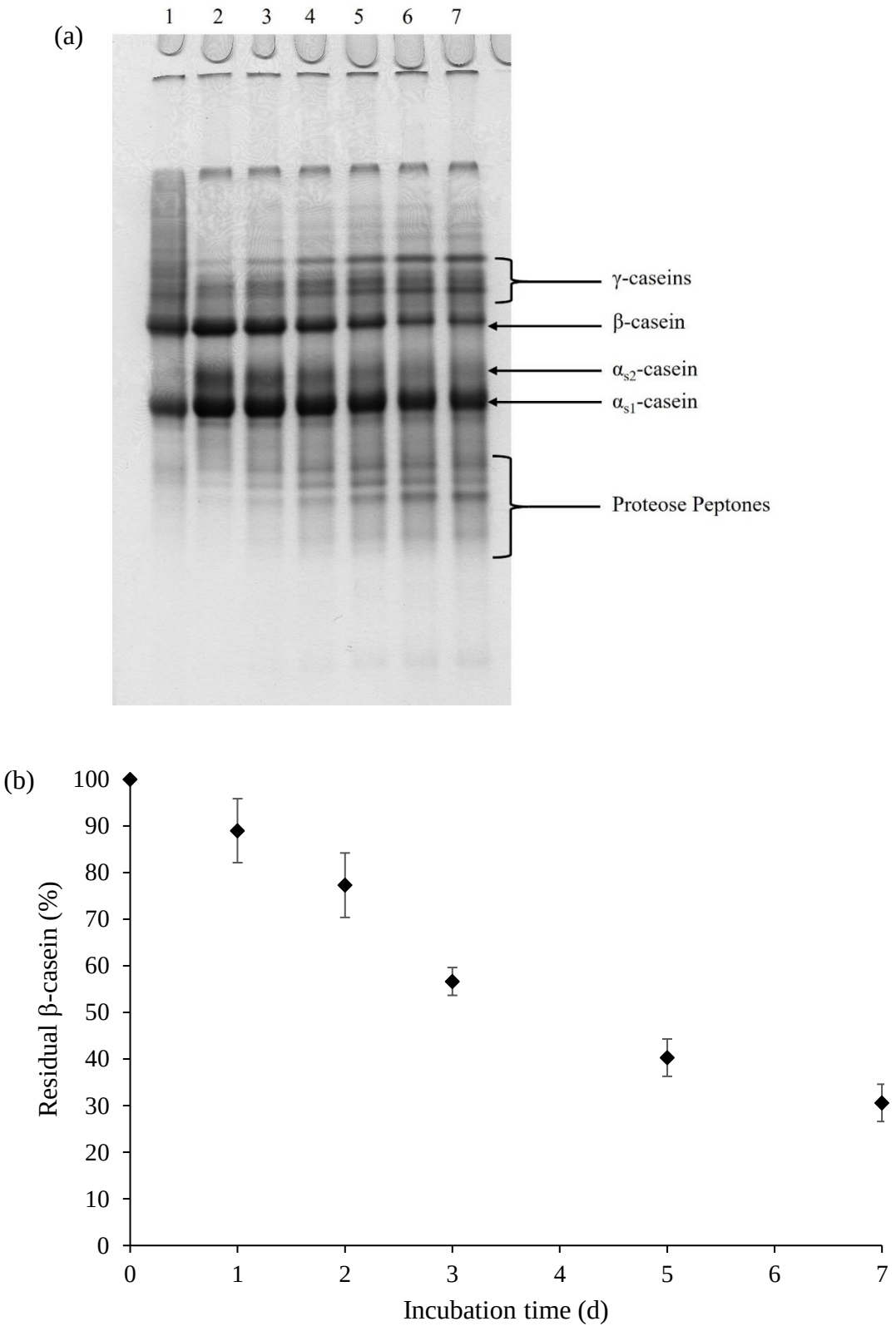


Figure 5.1: *(a) Urea-PAGE electrophoretograms of skim milk stored at 37 °C for 7 d; lane 1, sodium caseinate standard; lane 2, d 0; lane 3, d 1; lane 4, d 2; lane 5, d 3; lane 6, d 5; lane 7, d 7 and (b) hydrolysis of β-casein in skim milk (expressed as % of the level of β-casein in skim milk at day 0) during storage at 37 °C.*

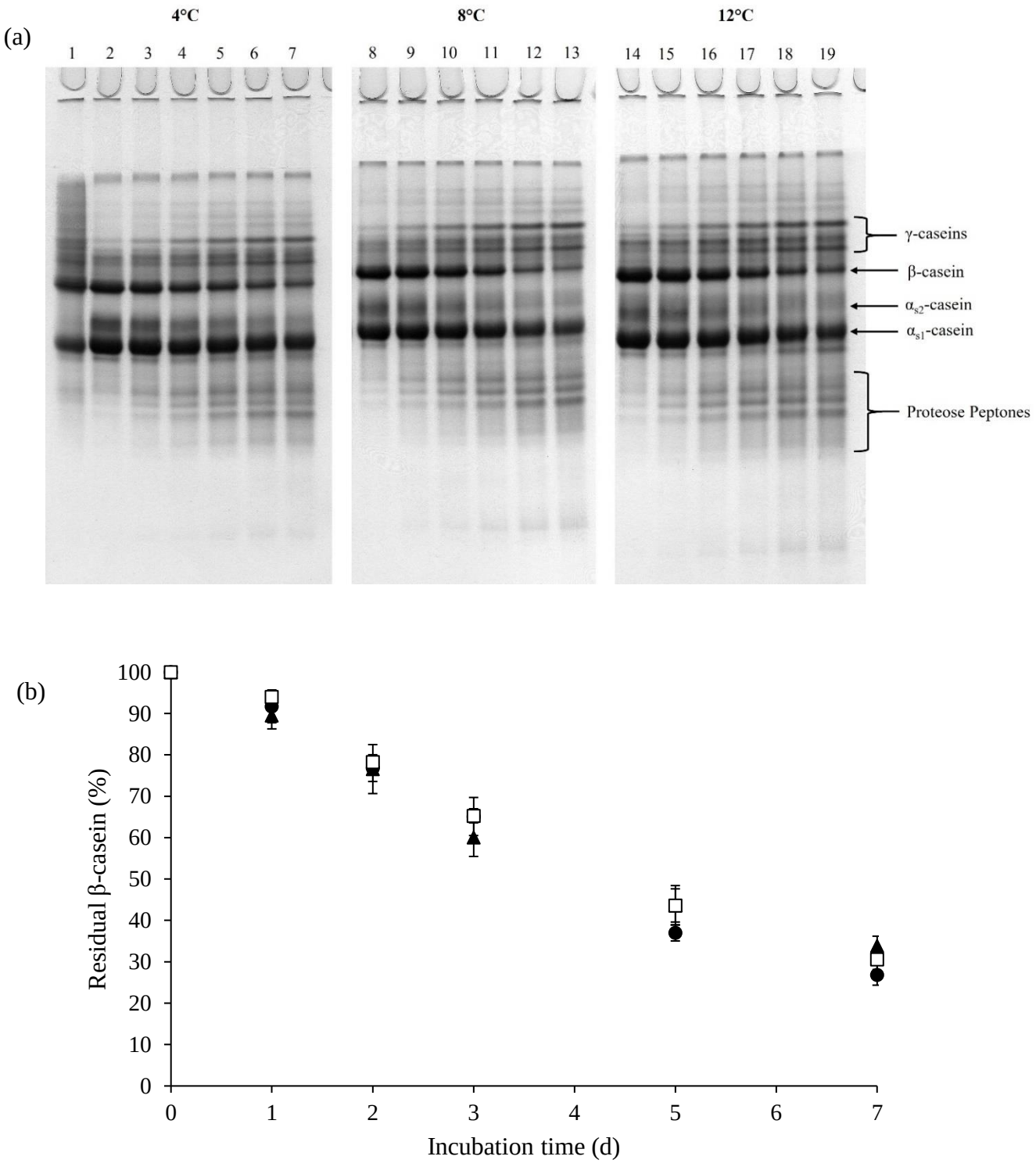


Figure 5.2: *(a) Urea-PAGE electrophoretograms of retentates stored at 37 °C for 7 d. Left gel (4 °C retentate); lane 1, sodium caseinate standard; lane 2, d 0; lane 3, d 1; lane 4, d 2; lane 5, d 3; lane 6, d 5; lane 7, d 7. Middle gel (8 °C retentate); lane 8, d 0; lane 9, d 1; lane 10, d 2; lane 11, d 3; lane 12, d 5; lane 13, d 7. Right gel (12 °C retentate); lane 14, d 0; lane 15, d 1; lane 16, d 2; lane 17, d 3; lane 18, d 5; lane 19, d 7 and (b) hydrolysis of β-casein in retentates (expressed as % of the level of β-casein in retentate at day 0), generated on microfiltration of skim milk at 4 (▲), 8 (●) and 12 °C (□), during storage at 37 °C.*

Similar results were observed for retentates, with the lowest amount of residual β-casein after 7 d of storage measured in R8 (26.8%), followed by R12 (30.6%) and R4 (33.8%), respectively (Fig. 5.2b). R4 had significantly higher ($p < 0.05$) residual β-casein levels compared to that produced at 8 °C. The higher amount of residual β-casein in R4 may be due to the significantly lower ($p < 0.05$) plasmin and plasminogen-derived activities measured. Nonetheless, extensive hydrolysis was observed in all retentates (Fig. 5.2a), indicating that during MF of skim milk the concentration of plasmin, plasminogen and plasminogen activators with the casein micelles, together with the removal of inhibitors, may affect the quality (i.e., proteolytic quality) of the milk protein ingredients generated from these streams. As the ingredients manufactured from such retentates are depleted of whey proteins, plasmin will remain stable even after heat treatment and therefore plasmin-induced hydrolysis of casein may be of significance for products incorporating such ingredients (e.g., clinical nutrition products). Therefore, from an industrial perspective, extensive plasmin-mediated hydrolysis is observed in retentates regardless of whether the temperature of MF is performed at 4, 8 or 12°C.

5.4.3 Proteolysis in β-casein-enriched permeates generated following microfiltration at different temperatures

The presence of plasmin and plasminogen-derived activity in permeates resulted in the hydrolysis of β-casein during storage at 37 °C (Fig. 5.3). From Fig. 5.3, it is evident that, during storage, there was an increase in the concentration of γ-caseins, the proteolysis products of β-casein. However, accurate determination of the extent of β-casein hydrolysis was not possible as incubation of the β-casein-enriched permeates resulted in the self-association of β-casein into micelles and further association of

these micelles into supra-micellar aggregates *via* calcium; as a result, partial precipitation of β-casein occurred during incubation, in addition to some of these β-casein aggregates adhering to the container walls, most notably at 4 °C. However, continuous rocking of samples ensured the protein remained in suspension during incubation. Studies have shown that heating of β-casein results in increased hydrophobic interactions and the formation of β-casein micelles which in the presence of calcium, specifically ionic calcium, can result in the association of these β-casein micelles to form supra-micellar β-casein aggregates (Li *et al*., 2019).

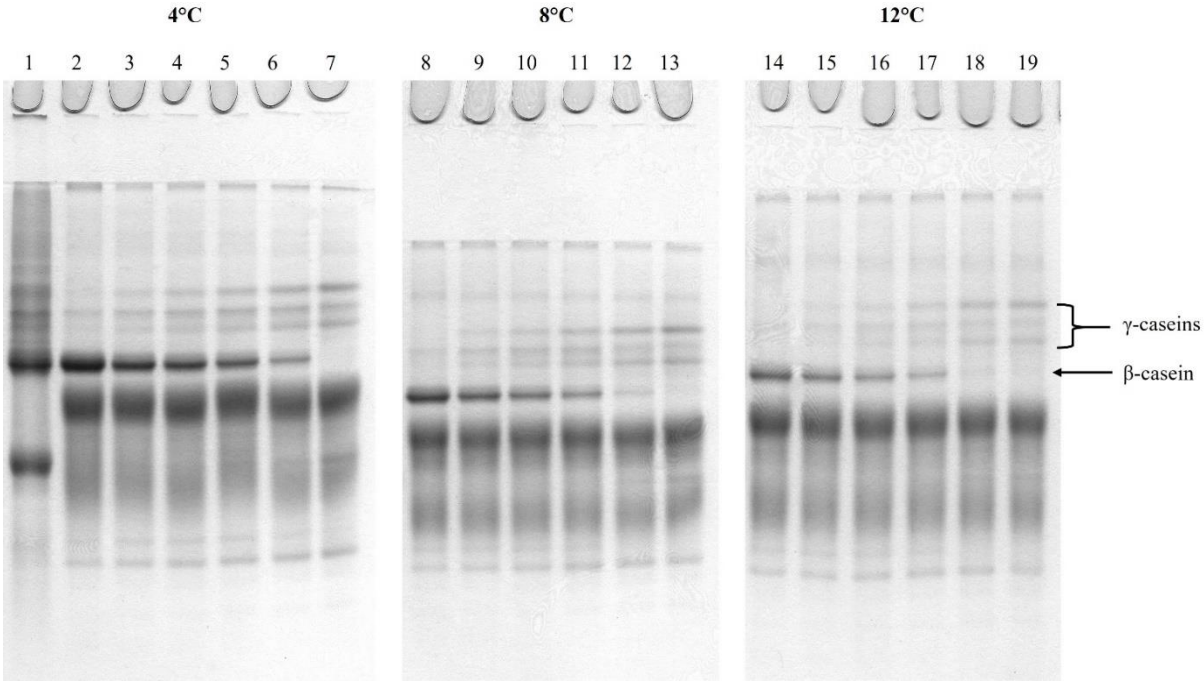


Figure 5.3: *Urea-PAGE electrophoretograms of permeates generated on microfiltration of skim milk at 4, 8 and 12 °C, stored at 37 °C for 7 d. Left gel (4 °C permeate); lane 1, sodium caseinate standard; lane 2, d 0; lane 3, d 1; lane 4, d 2; lane 5, d 3; lane 6, d 5; lane 7, d 7. Middle gel (8 °C permeate); lane 8, d 0; lane 9, d 1; lane 10, d 2; lane 11, d 3; lane 12, d 5; lane 13, d 7. Right gel (12 °C permeate); lane 14, d 0; lane 15, d 1; lane 16, d 2; lane 17, d 3; lane 18, d 5; lane 19, d 7. Note: the apparent band under β-casein is not* α_s*-casein but was found to be poor separation of the whey proteins as a result of freezing the sample with the buffer, prior to loading the gel.*

As a result, permeate samples were also incubated in the presence of TSC (Fig. 5.4a), which maintained β-casein in suspension through the binding of ionic calcium, allowing accurate determination of the rate of β-casein hydrolysis. P4 and P8 had significantly higher rates of β-casein hydrolysis compared to P12, with residual β-casein contents of 42.7, 44.0 and 53.9%, respectively (Fig. 5.4b). As there were no significant differences ($p > 0.05$) in the pH of TSC-treated permeates (data not shown), the lower extent of β-casein hydrolysis in P12 can be attributed to significantly lower ($p < 0.05$) plasmin and plasminogen-derived activity (Table 5.1). From Fig. 5.3, it is clear that extensive proteolysis occurred even in cases where there was the formation of supra-micellar β-casein aggregates in suspension, with no visible intact β-casein after 7 d of incubation at 37 °C. This suggests that the formation of these complexes does not inhibit the action of plasmin. The addition of TSC prevented the aggregation of β-casein; this was also confirmed as addition of TSC to permeates resulted in no turbidity development when heated to 50 °C and no significant increase in particle size (data not shown). As bovine plasminogen is capable of binding to β-casein (Baer *et al*., 1994; Haïssat *et al*., 1994), it is possible that, in permeates with no added TSC, the formation of β-casein micelles and aggregates may have reduced contact between both plasminogen and plasmin and the inhibitors present in the serum phase. As such phenomena do not occur in TSC-treated permeates, plasmin would be expected to be in greater contact with the inhibitors present within the serum phase, possibly resulting in reduced plasmin-mediated hydrolysis during storage of TSC-treated permeates.

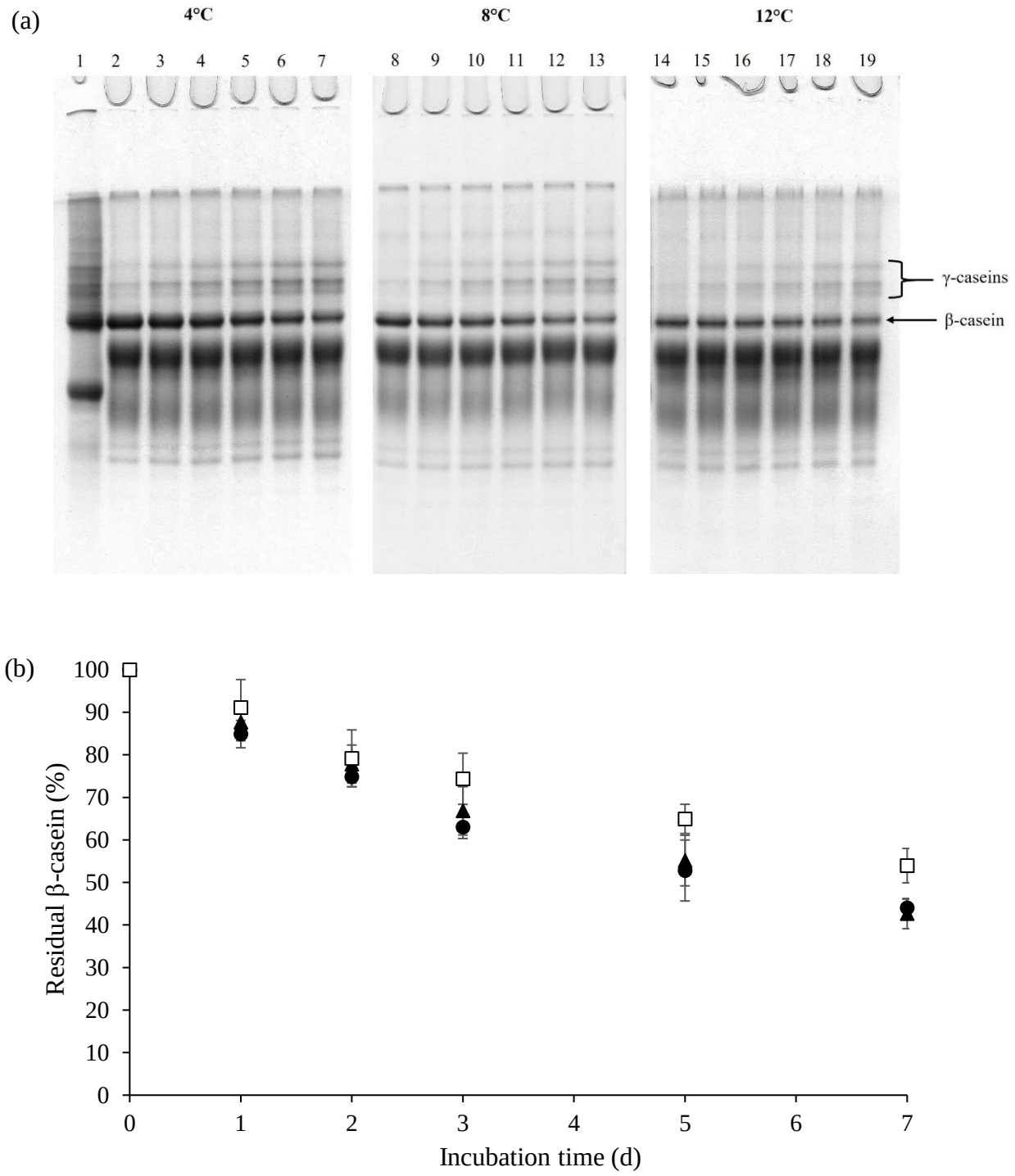


Figure 5.4: *(a) Urea-PAGE electrophoretogram of permeates with added trisodium citrate stored at 37 °C for 7 d. Left gel (4 °C permeate); lane 1, sodium caseinate standard; lane 2, d 0; lane 3, d 1; lane 4, d 2; lane 5, d 3; lane 6, d 5; lane 7, d 7. Middle gel (8 °C permeate); lane 8, d 0; lane 9, d 1; lane 10, d 2; lane 11, d 3; lane 12, d 5; lane 13, d 7. Right gel (12 °C permeate); lane 14, d 0; lane 15, d 1; lane 16, d 2; lane 17, d 3; lane 18, d 5; lane 19, d 7 and (b) hydrolysis of β-casein in permeates with added trisodium citrate (expressed as % of the level of β-casein in permeate at day 0), generated on microfiltration of skim milk at 4 (▲), 8 (●) and 12 °C (□), during storage at 37 °C. Note: the apparent band under β-casein is not α_s-casein but was found to be poor separation of the whey proteins as a result of freezing the sample with the buffer, prior to loading the gel.*

β-Casein-enriched whey is generated following the MF (0.1-0.5 µm) of skim milk at low processing temperatures (i.e., 4 °C) and this can be used as an ingredient itself or it can undergo further processing to produce β-casein concentrate/isolate (France, Kelly, *et al*., 2021). In the production of β-casein, the β-casein-enriched permeate is often heated (i.e., 25-50 °C), facilitating the self-association of β-casein, which can then be isolated from the whey proteins using tight pore MF (e.g., 0.1 µm; O'Mahony *et al*., 2014). The combined effects of β-casein self-association and the removal of plasmin inhibitors may have the negative effect of increasing relative plasmin activity in the final β-casein ingredient. Extensive plasmin-mediated hydrolysis has previously been reported in BCC produced using cold MF, indicating the process used to isolate and concentrate β-casein can result in the co-enrichment of plasmin (Kelly *et al*., 2021). As microfiltration (0.1 µm) of this heated β-casein-enriched permeate should remove plasmin (~48 kDa) and plasminogen (~88 kDa) from the resulting β-casein retentate, this suggests that plasmin and plasminogen bind to the β-casein micelles or are already bound to the β-casein prior to self-association. Therefore, the presence of plasmin and plasminogen-derived activity in permeates generated following the MF of skim milk at low temperatures could be of significance where intact β-casein is required, as our data (Fig. 5.3) indicates that extensive plasmin-mediated hydrolysis occurs in the permeates produced at each filtration temperature.

5.4.4 Effect of temperature and plasmin-mediated hydrolysis on the particle size of β-casein-enriched permeates

Particle size measurements were performed to provide information on the differences in association of β-casein before and after incubation at 37 °C (Fig. 5.5). There were no significant differences ($p > 0.05$) in particle size between permeates when measured at 10 °C, prior to storage at 37 °C (Fig. 5.5a), with particle sizes of 71.7, 68.4 and 74.8 nm for P4, P8 and P12, respectively. Volume mean particle sizes of 9.3, 8.6 and 8.4 nm were measured for P4, P8 and P12 at 10°C, respectively, indicative of monomeric β-casein. Heating of these permeates from 10 to 30 °C resulted in little change in particle size; however, at temperatures >30 °C, particle size increased rapidly, reaching the largest particle size at 55 °C. At 55 °C, significantly higher ($p < 0.05$) particle sizes were measured for P4 and P8, compared to P12, with values of 281, 279 and 242 nm, respectively.

Studies have shown that heating solutions of β-casein results in an increase in particle size (Crowley *et al*., 2019; Dauphas *et al*., 2005; Li *et al*., 2019). At low temperatures, β-casein exists largely in the form of monomers, although some polymeric structures still exist (O'Connell *et al*., 2003). On increasing temperature, hydrophobic interactions strengthen and β-casein self-associates, resulting in what are commonly referred to as β-casein micelles (Aschi *et al*., 2009; O'Connell *et al*., 2003). The lower particle size measured at 55 °C for P12 could be attributed to the significantly lower ($p < 0.05$) concentration of β-casein compared to P8 and P4. In addition, P12 had the lowest concentration of ionic calcium (2.77 mM), compared to P8 (2.93 mM) and P4 (3.07 mM) (France, Bot, *et al*., 2021). As ionic calcium can further promote self-association of β-casein micelles and facilitate the formation of supra-micellar β-casein aggregates, resulting in larger particle size, the lower ionic

calcium levels in P12 may also have contributed to the smaller particle size (Li *et al*., 2019). The increase in particle size on heating was also associated with a decrease in the polydispersity index (PDI). The high PDI at low temperatures (i.e., 10 °C) indicates a multi-modal distribution of particle sizes which is likely due to the presence of β-casein particles in both monomeric and polymeric forms, in addition to the different whey proteins. At the higher measurement temperatures (i.e., 45-55 °C), the reduction in PDI indicates that there was a shift towards a system with a more mono-modal distribution of particle size.

The particle size of these permeates were also analysed after storage at 37 °C for 7 d to provide information on the effect of plasmin-mediated hydrolysis on self-association of β-casein (Fig. 5.5b). The particle size of P4 (171 nm) and P8 (173 nm) were significantly higher ($p < 0.05$) than that of P12 (129 nm), when measured at 10 °C. After heating to 55 °C, P4 and P8 still had significantly higher particle sizes ($p < 0.05$) compared to P12, with values of 365, 341 and 274 nm, respectively. Interestingly, these permeates, following storage at 37 °C for 7 d, had higher particle sizes than those prior to storage at 37 °C. As can be seen from Fig. 5.3, the samples of permeate collected after 7 d of storage at 37 °C contained mainly γ-caseins, with no visibly intact β-casein. This result, combined with the fact that particle size still increased in all permeates after storage at 37 °C on heating suggests that γ-caseins may display similar properties to β-casein, in terms of their ability to self-associate. β-Casein contains an N-terminal part which is highly hydrophilic, while the C-terminal part is mainly comprised of neutral and hydrophobic amino acids (Atamer *et al*., 2017). The three γ-caseins, γ1 (β-CN f29-209), γ2 (β-CN f106-209) and γ3 (β-CN f108-209), retain the C-terminal part, while the N-terminal part is cleaved by plasmin, resulting in the formation of the γ-caseins and corresponding proteose peptones (Eigel

et al., 1984). The γ-caseins contain a high percentage of hydrophobic amino acids and are more hydrophobic than β-casein (Swaisgood, 1982). In addition to the γ-caseins, proteose peptones may also display the ability to associate. Proteose peptone 5 (β-CN f1-105-107) contains the N-terminal phosphorylation clusters and may show sensitivity to calcium which could promote further interactions with γ-caseins or other proteose peptones. Thus, it is likely that large peptides derived from plasmin hydrolysis of β-casein can interact with one another, although additional work is required to confirm this.

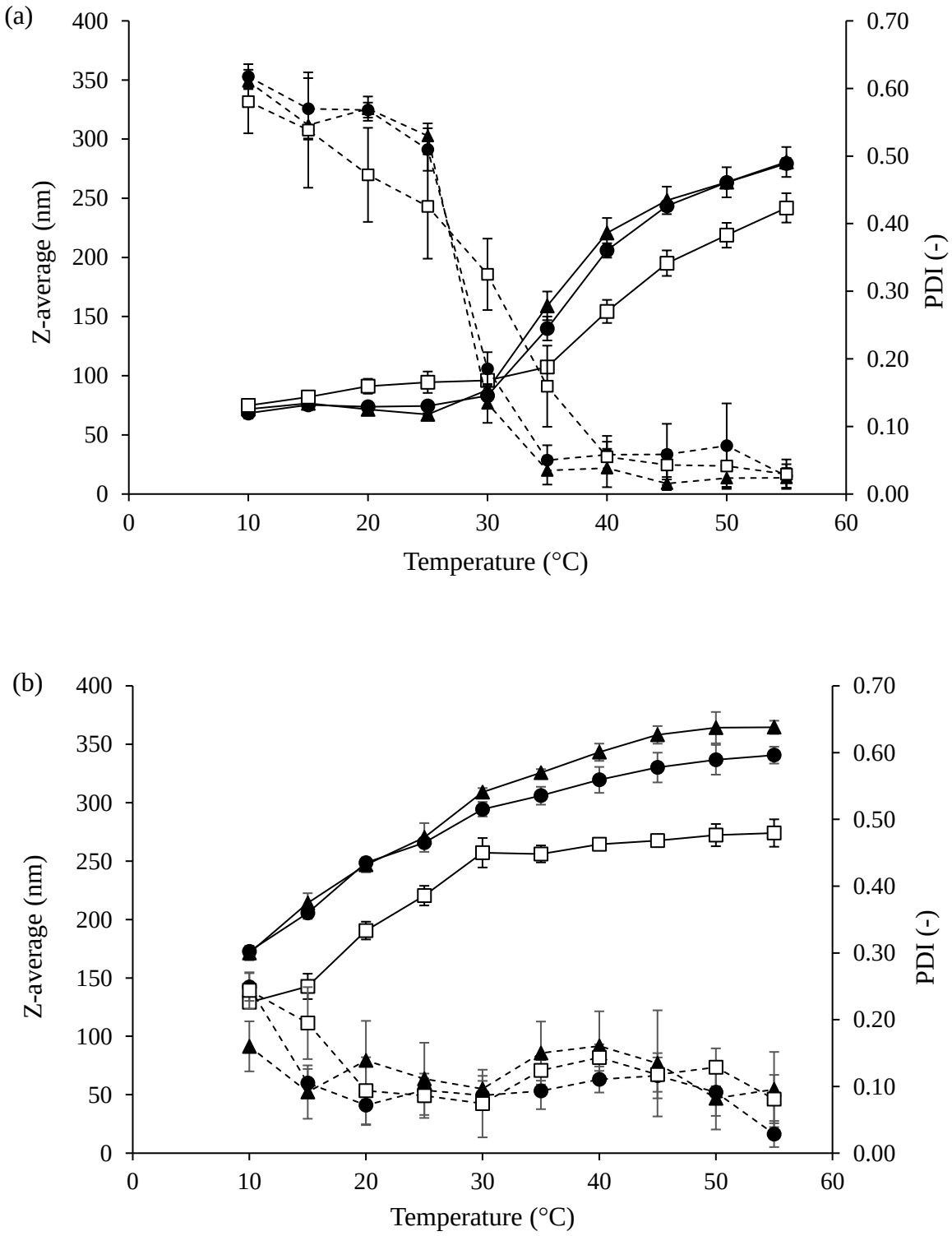


Figure 5.5: *Z-average particle diameter (solid line) and polydispersity index (PDI; broken line) as a function of temperature of β-casein-enriched permeates, generated on microfiltration of skim milk at 4 (▲), 8 (●) and 12 °C (□), before (a) and after storage at 37 °C for 7 days (b).*

5.4.5 Influence of plasmin-mediated hydrolysis of β-casein on turbidity development

The results from turbidity analysis of β-casein-enriched permeates before and after plasmin-mediated hydrolysis were in good agreement with the particle size measurements (Fig. 5.6). In the unheated permeates (Fig. 5.6a), the turbidity of permeates after storage at 37 °C for 7 d was higher than for permeates prior to storage at 37 °C. This can be attributed to the higher particle size of all permeates after storage at 37 °C compared to those prior to storage at 37 °C, when measured at 20 °C.

The turbidity of heated (50 °C) β-casein-enriched permeates before incubation resulted in turbidity values of 1.09, 0.77 and 0.40, for P4, P8 and P12, respectively (Fig. 5.6b); these results reflect the β-casein and ionic calcium concentration of the permeates, with the turbidity decreasing as β-casein and ionic calcium concentration decreases, as was also observed with particle size data (Fig. 5.5a). After storage of these permeates at 37 °C for 7 d, these permeates were also heated. The turbidity values indicated that, although extensive plasmin-induced hydrolysis of β-casein occurred (Fig. 5.3), the turbidity remained essentially unchanged, with the turbidity of P8 and P12 exceeding that of the corresponding permeates before incubation. As mentioned earlier the samples of permeate collected after 7 d incubation at 37 °C contained no visual intact β-casein. Considered together with the particle size data, this suggests that γ-caseins and/or proteose peptones display the ability to self-associate and potentially form ‘micelles’ in a similar way to β-casein, with increased hydrophobic interactions at high temperatures promoting interactions between γ-caseins. Thus, the temperature at which filtration was performed only gave marginal differences in particle size and turbidity, with the temperature-dependent association changing as β-casein is hydrolysed into γ-caseins and proteose peptones.

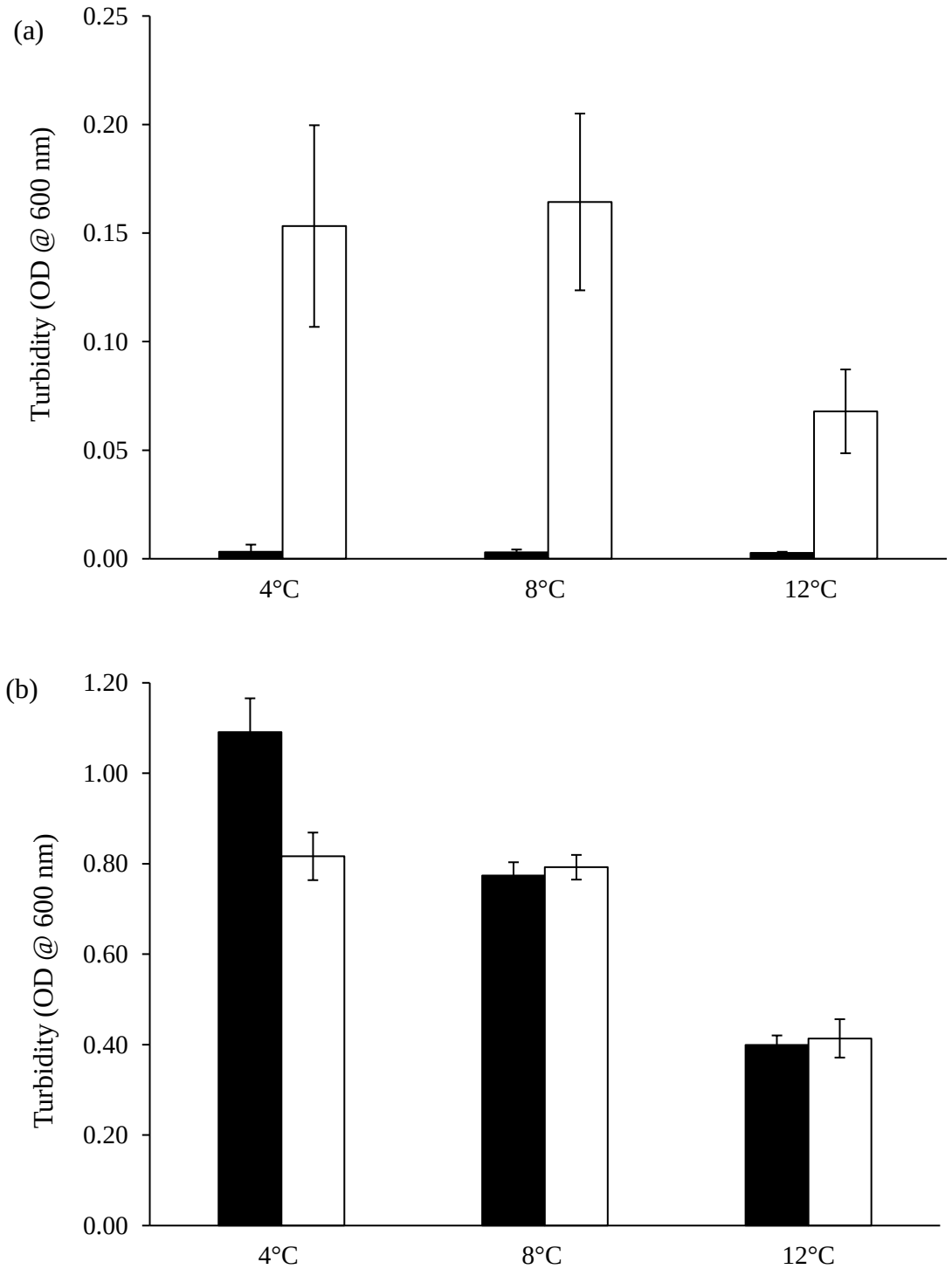


Figure 5.6: *Turbidity of β-casein-enriched permeates generated on microfiltration of skim milk at 4, 8 and 12 °C, before (■) and after storage at 37 °C for 7 d (□); measurement of permeates at 20 °C (unheated; a) or heated to 50 °C for 30 min (b).*

5.5 Conclusion

During microfiltration of skim milk, both plasmin and plasminogen are mostly concentrated in the retentate together with the casein micelles, resulting in extensive plasmin-mediated hydrolysis of such process streams on storage at 37 °C. However, microfiltration at low temperatures also results in the permeation of β-casein and plasmin into the permeate stream. Although the effect of temperature on plasmin partitioning is not as pronounced as that of β-casein during cold microfiltration, plasmin activity in β-casein-enriched permeates results in hydrolysis of β-casein, with the highest rates of hydrolysis observed for permeates generated at 4 and 8 °C. Heating of permeates prior to plasmin-induced hydrolysis resulted in an increase in particle size, with permeate generated at 4 °C displaying the highest particle size at 55 °C. Similar results were observed for permeates following storage at 37 °C, indicating that the hydrolysis products of β-casein, γ-caseins, may also be able to self-associate on heating. Therefore, during the cold microfiltration of skim milk, at different temperatures, plasmin-meditated hydrolysis can occur, resulting in undesirable extensive hydrolysis of caseins in both retentate and permeate streams, as well as altering the self-association properties of caseins in β-casein-enriched permeates.

5.6 Acknowledgements

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Chapter 6

Influence of microfiltration temperature on protein profile, mineral profile, thermal stability and rheological properties of micellar casein retentates

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Declaration

This chapter was written by author Thomas C. France (TCF) and reviewed by all co-authors. TCF co-designed the study and performed all of the experimental work with the exception of mineral analysis of process streams, which was performed by an external laboratory (FBA Laboratories, Waterford, Ireland).

6.1 Abstract

Temperature has a significant influence on the composition and functionality of the resulting process streams following microfiltration (MF) of skim milk. In this study, MF and diafiltration (DF) were performed at 4 or 50 °C to produce β-casein-depleted and control (i.e., native casein profile) micellar casein isolate retentates, respectively. Extensive DF resulted in a 40.3% depletion of β-casein when performed at 4 °C, while no β-casein depletion occurred at 50 °C. MF at 4 °C resulted in greater transmission of calcium into permeates, with retentate generated at 4 °C containing a lower amount of total calcium (1863 mg/780 mL) compared to retentate generated at 50 °C (2351 mg/980 mL), based on the volume of retentate remaining. The highest heat stability was measured for retentates generated at 4 °C compared to those at 50 °C, across all pH values measured. Retentates generated at 4°C also had significantly lower ionic calcium values at each pH compared to retentates generated at 50 °C. Higher viscosities, at 300 s^{-1}, were measured for retentates generated at 4 °C compared to retentate generated at 50 °C, suggesting increased voluminosity of β-casein-depleted casein micelles. The results of this study provide information into how changing the composition of MF retentates, by selection of processing temperature and extensive DF, can alter physicochemical properties of casein micelles, with potential implications for ingredient functionality.

6.2 Introduction

Cross-flow microfiltration (MF) has become a key unit operation in the processing of bovine milk, particularly in the production of milk-protein derived ingredients, where MF of skim milk using tight pore sizes (0.1-0.5 µm) allows for the separation of serum proteins from casein micelles (Carter *et al*., 2021). In the separation of casein micelles and serum proteins, MF is commonly performed at warm temperatures (45-55°C). This has associated advantages of high flux levels, lower process stream viscosities, greater retention of caseins, and a whey protein fraction of high purity (Zulewska *et al*., 2014; McCarthy *et al*., 2017). The two streams generated following warm MF of skim milk are referred to as micellar casein concentrate (MCC; retentate) and 'ideal' whey (permeate).

Increasingly, MF of dairy streams is being performed at cold temperatures (i.e., ≤20°C), primarily due to associated benefits of improved microbial quality and reduced fouling (Schiffer and Kulozik, 2020; France, *et al*., 2021a). However, operating at lower temperatures results in changes to the composition of retentate and permeate streams. At low temperatures (i.e., 4°C), β-casein dissociates from the interior of the casein micelle to the surface, from where it dissociates into the serum phase of milk (Downey & Murphy, 1970; Creamer *et al*., 1977). During cold MF of dairy streams containing casein micelles, this serum β-casein permeates the membrane into the permeate stream, a phenomenon that is exploited in the production of β-casein enriched whey protein or high purity β-casein (O'Mahony *et al*., 2014; Seibel *et al*., 2015; McCarthy *et al*., 2017; France, Bot, *et al*., 2021). In the generation of β-casein-enriched whey protein or β-casein concentrate, through the cold MF of skim milk or other feed materials, a β-casein-depleted MCC co-product is produced. A similar

phenomenon is observed for colloidal calcium phosphate, with the concentration of both total and ionic calcium being higher in permeates generated from filtration of skim milk at low temperatures (Davies and White, 1960; Luo *et al*., 2015; Méthot-Hains *et al*., 2016; France, *et al*., 2021b).

These differences in mineral and protein profile between retentates generated following warm and cold MF are likely to result in differences in functional properties such as thermal stability and rheological properties. There have been numerous studies detailing the different potential uses of MCC, such as in cheese-making, high-protein beverages, protein bars and Greek-style yoghurt (Hogan *et al*., 2012; Bong and Moraru, 2014; Lu *et al*., 2017); however, few studies examined the potential uses of β-casein-depleted MCC generated from cold MF. Studies have reported that β-casein-depletion during cold MF results in modifications in the functional properties of MCCs, such as rennet and acid-induced gelation (O'Mahony *et al*., 2008; 2009; Seibel *et al*., 2015). Seibel *et al*. (2015) reported that cheese making from MCC with reduced β-casein:α_s-casein ratio (0.66) resulted in weaker rennet-induced gels compared with cheese made from warm processed MCC (no β-casein depletion; β-casein:α_s-casein ratio of 0.74). Moynihan *et al*. (2015) reported that altering the β-casein:α_s-casein ratio in milk protein concentrate solutions impacted on rennet coagulation properties; reduced β-casein:α_s-casein ratio (0.64) resulted in stiffer, more elastic gels with lower loss tangent and higher yield stress compared to gels made with higher β-casein:α_s-casein ratio (0.82), in agreement with results previously reported by O'Mahony *et al*. (2009). However, Xia *et al*. (2021) reported that rennet coagulation properties (coagulation time and curd firming rate) were not influenced by β-casein concentration, although there was only a small difference in depletion between cold (4.25% depletion) and warm (1.83% depletion) generated MCC.

Although studies have evaluated MCC generated *via* warm or cold MF in terms of their functionality, e.g., cheesemaking properties and rehydration characteristics, there is limited knowledge on how viscosity and thermal stability of these MF process streams are affected and how differences in composition affect such properties. In the present study, the focus was on micellar casein isolate (MCI) retentates as opposed to MCC. Therefore, the aim of this study was to assess the differences between cold and warm generated MCI retentate streams in terms of composition and physicochemical properties such as heat stability and rheology, to understand how changes in protein and mineral profile impact these properties.

6.3 Materials and methods

6.3.1 Materials

Low-heat skim milk powder was provided by a local Irish dairy company (Arrabawn Co-op, Nenagh, Co. Tipperary, Ireland). Reconstituted skim milk was prepared by adding low-heat skim milk powder to ultrapure water over 2 h at 22 °C to attain a 3.20% (w/v) protein solution, at a native pH of 6.70. The skim milk was then stored at 4 °C for 48 h under gentle constant magnetic stirring, to facilitate complete rehydration. All chemicals, reagents and high-performance liquid chromatography (HPLC) standards, unless otherwise stated, were sourced from Sigma-Aldrich (Wicklow, Ireland) and were of analytical grade.

6.3.2 Membrane filtration

MF experiments were performed on a lab-scale, pressure-driven, tangential-flow filtration device (Pellicon 2 mini-holder; Merck Millipore, Tullagreen, Carrigtwohill, Ireland) as described by Crowley *et al*. (2015). A 1000 kDa molecular weight cut-off (equivalent to a 0.1 µm pore size MF membrane) polyethersulfone membrane (Biomax, Merck Millipore) was used. A detailed illustration of the MF procedure at 4 and 50 °C is presented in Fig. 6.1. Filtration was performed over two days, with each MF/ diafiltration (DF) step performed with the same 1000 kDa PES membrane at a cross flow velocity of 0.22 m/s. Deionised water was used as the DF media for all DF steps.

Prior to MF at 4 °C, skim milk was stored at 4 °C for 48 h prior to filtration, to facilitate rehydration and to maximise the release of β-casein from the micelle and into the serum phase. After 48 h of storage at 4 °C, an equilibration time of 40 min at 4 °C

was applied prior to concentration, to ensure stable conditions (i.e., flux and transmembrane pressure; TMP) by returning both permeate and retentate lines to the feed vessel (i.e., total recirculation mode). After this, skim milk was microfiltered to a volume concentration factor (VCF) of 3.3, and the retentate (retentate 1) was diluted with water (dilution ratio 1:4) and recirculated through the membrane for a further 40 min. Extensive DF was performed to remove enough lactose and whey protein, as micellar casein isolate (MCI) was the target. The diluted retentate was subjected to cold MF again until a VCF of 5.83 was achieved. This concentrated retentate (retentate 2) was again diluted with water (dilution ratio 1:2) and stored for ~18 h at 4 °C under constant magnetic stirring, to facilitate further dissociation of β-casein. The following day, a 40 min equilibration step at 4 °C was performed to condition the membrane (i.e., total recirculation mode). After completion of same, cold MF was performed on the diluted retentate (retentate 2) until a VCF of 3 was achieved. Following this, the final DF/MF step was performed, in which the retentate (retentate 3) was diluted (dilution ratio 1:2), equilibrated for 40 min, and concentrated to a final VCF of 3 (retentate 4).

MF of skim milk at 50 °C followed the same procedure, with one exception. The final MF/DF step was stopped when a VCF of 2.25 was achieved, to achieve final retentates with similar protein contents. Cleaning of the membrane was performed at the end of each day of filtration, with the extent of membrane fouling assessed according to France, Bot, *et al*. (2021).

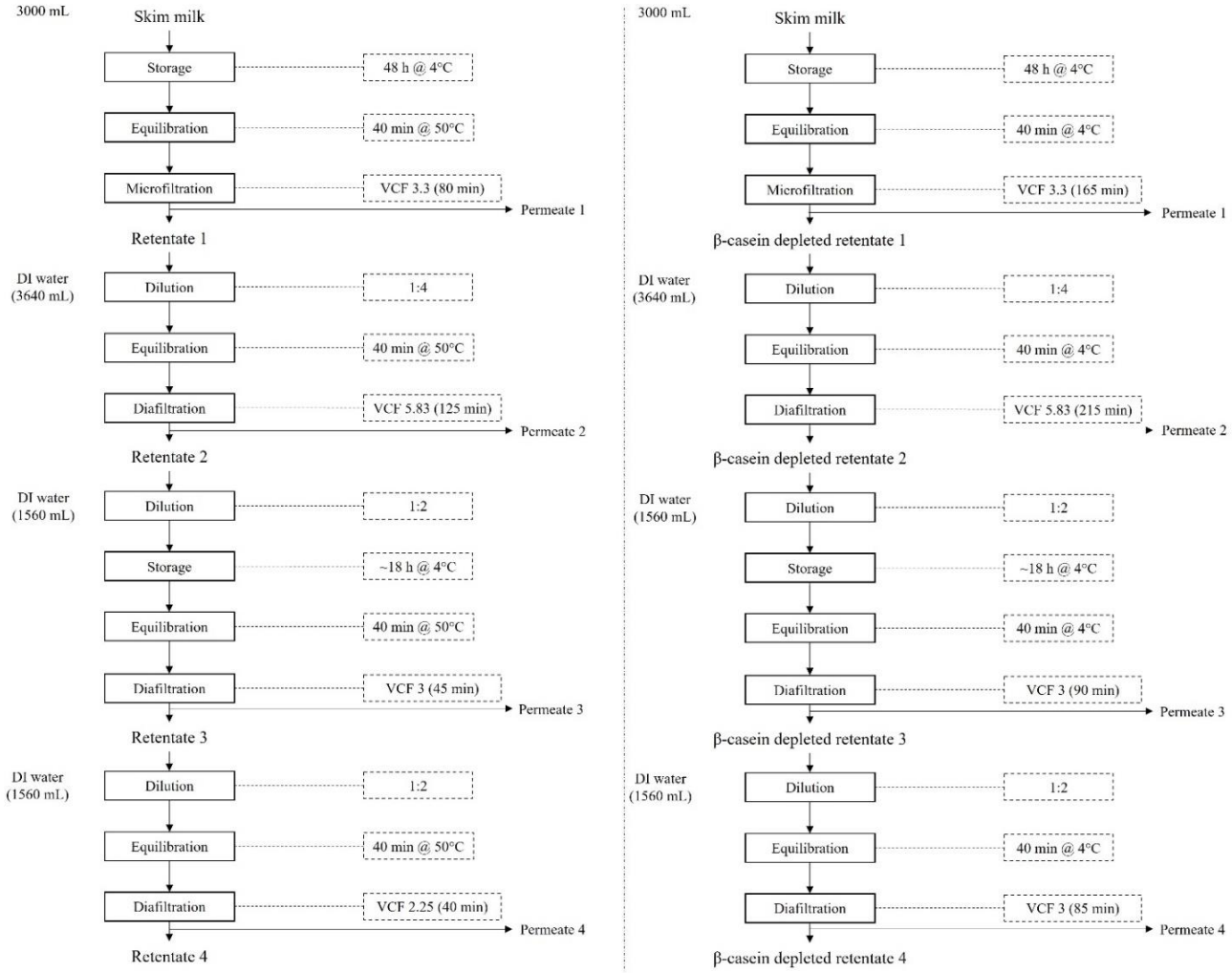


Figure 6.1: *Schematic representation of the procedure developed and applied for the microfiltration and diafiltration of skim milk at 4 and 50 °C; VCF, volume concentration factor.*

6.3.3 Composition of the process streams

Total nitrogen content of process streams (i.e., retentate and permeates) was determined using the Kjeldahl method using a nitrogen-to-protein conversion factor of 6.38 for the calculation of crude protein (IDF, 2001). Non-casein nitrogen was determined according to the method of Wojciechowski and Barbano, (2015), based on the AOAC International method (AOAC International, 2010 method number 998.05) as described by Lynch *et al*. (1998). The concentration of buffers was unchanged; however, the volume of buffers was increased from 1 mL, as described by Lynch *et al*. (1998), to 5.5 mL of both acetic acid (10% v/v) and sodium acetate (1.0 mol/L). The increase in buffer volumes was required to provide sufficient buffering capacity to compensate for the higher protein content of the retentates.

6.3.4 Protein profile analysis of process streams

The protein profile of the feed, retentate and permeates (P1, P2, P3 and P4) were measured using RP-HPLC (Agilent 1220 Infinity II LC, Santa Clara, CA 95051 United States) with a C18 column (3.6 µm x 250 mm x 4.6 mm, Aeris Widepore, Phenomenex, UK) as described by Bonfatti *et al*. (2008) and Bot *et al*. (2020), using solvents A (10.0% HPLC-grade acetonitrile, 89.9% ultrapure water and 0.10% TFA) and B (89.9% HPLC-grade acetonitrile, 10.0% ultrapure water and 0.10% TFA); the injected volume was 40 µL, and detection was performed at 214 nm. Prior to analysis, samples were filtered through 0.45 µm filters (Minisart® RC25, Göttingen, Sartorius AG, Germany). The concentrations of individual proteins were determined by preparing standard curves of the respective proteins ($R^2 > 0.99$) and results are expressed as mg protein per mL of sample.

The extent of β-casein depletion was determined by calculating the relative amount of β-casein in each of the four permeates relative to the total amount of β-casein in the feed material according to the following equations:

$$\beta\text{-CN}_F = \beta\text{-CN concentration (mg/mL)} \times \text{volume feed (mL)} \quad [6.1]$$

$$\beta\text{-CN}_{Pn} = \beta\text{-CN concentration (mg/mL)} \times \text{volume permeate (mL)} \quad [6.2]$$

$$\beta\text{-CN}_{depletion} = \frac{\sum_1^4 \beta\text{-CN}_{Pn}}{\beta\text{-CN}_F} \times 100 \quad [6.3]$$

6.3.5 Mineral profile analysis of process streams

Mineral profile of feed, retentate and permeate samples (P1, P2, P3 and P4) were measured for macro (Ca, P, K, Na, and Mg) and trace (S, Zn, Co, Mn, Cu, Mo, and Se) elements using inductively coupled plasma mass spectrometry (ICP-MS). Mineral analysis was performed by FBA Laboratories Ltd. (Cappoquin, Waterford, Ireland).

6.3.6 Particle size measurements

The particle size distribution of the retentates after each MF/DF step was measured by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Retentate samples were diluted with calcium-imidazole buffer (5 mM $CaCl_2$, 20 mM imidazole and 30 mM NaCl, pH 7.0) as described by Anema and Li (2003), with a refractive index of 1.45 for protein. Measurements were carried out at 25 °C after temperature equilibration for 120 s, using a backscattering configuration of 173°. The particle size was expressed as the intensity-weighted mean hydrodynamic diameter (Z-average).

6.3.7 Measurement of pH and ionic calcium

The pH of permeate and retentate streams were measured using a standard pH meter at 20 °C (Metler Toledo, SevenGo Duo, SG23, Switzerland). The pH of retentate streams, at 8% protein, were adjusted to pH 6.8, 6.9, 7.0, 7.1 and 7.2 using 0.5 N NaOH and HCl as appropriate. Retentates were adjusted to the desired pH prior to overnight storage at 4 °C. The following day samples were equilibrated to 20 °C and re-adjusted to the desired pH, if needed, prior to analysis of ionic calcium and heat coagulation time (HCT) measurements.

The ionic calcium concentration of permeate and retentate samples were measured using a calcium (Ca)-ion-selective polymer membrane electrode (Metrohm Ireland Ltd, Co. Carlow, Ireland). The probe was calibrated at 25 °C using standard calcium solutions (0.50, 1.00, 2.50, 5.00, 10.0 mM), prepared with $CaCl_2$ in a KCl-imidazole buffer; correlation of log ionic calcium [mM/L] against the electrical output (mV) yields a linear relationship, according to the Nernst equation. Samples were equilibrated with the probe for 1 min prior to taking measurements.

6.3.8 Heat coagulation time as a function of pH

HCT of retentates (8% protein), generated after MF was determined at 120 °C as a function of pH (pH 6.8-7.2) using the method of Davies and White (1966). Samples (2 mL) were added to heat-resistant glass tubes and stoppered using a rubber bung, placed in a steel rack, and immersed in a silicone oil bath (Hettich ESP oilbaths; Hettich Benelux BV). The HCT was recorded as the time elapsed between immersing the sample in the oil bath and the visible detection of aggregates within the sample.

6.3.9 Rheological properties of retentate stream

The rheological properties of retentates generated at 4 and 50 °C, at 8% protein and pH 6.9, were measured using a rotational viscometer (HAAKE RotoVisco 1, Thermo Fisher Scientific, MA, USA) with a cylindrical double gap cup and rotor geometry (DG43, Thermo Fisher Scientific, MA, USA). Measurements were performed at 4 and 25 °C with a standardised volume (13 mL) added to the cup; to determine the Power Law parameters, shear rate sweeps (up and down ramps) were performed between 1 and 1500 s^{-1} as described by Kieferle *et al*. (2019). Various models have been used to describe the non-linear flow behaviour of concentrated milk and high protein retentates following filtration of bovine milk, including the Power Law and Herschel-Bulkley model (Sauer *et al*., 2012; Anema *et al*., 2014; Kieferle *et al*., 2019).

Shear stress-shear rate data was modelled according to the Power Law equation (Anema *et al*., 2014):

$$\tau = K\dot{\gamma}^n \quad [6.4]$$

where τ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the consistency coefficient (Pa s^n) and n is the flow behaviour index. The Power Law model was chosen due to its good applicability to the data.

6.3.10 Statistical data analysis

All experimental analyses were conducted in triplicate, with samples produced from three independent trials for each processing temperature, unless otherwise stated. Results are expressed as mean ± standard deviation. All data were normally distributed, except for ionic calcium measurements, with the Mann-whitney Wilcoxon

test being used for analysis of these data. Independent t-tests were performed for all other data using R i386 version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) to identify statistically significant differences between samples generated at 4 and 50°C. The level of significance was determined at $p < 0.05$ to determine whether statistical differences existed between the mean values.

6.4 Results and discussion

6.4.1 Composition of liquid micellar casein isolate retentates and permeates

MF of skim milk was performed at 4 and 50 °C to produce a β-casein-depleted retentate and a retentate with no β-casein depletion (i.e., native casein profile), respectively. Significantly higher ($p < 0.05$) protein content was measured in all permeates (P1, P2, P3 and P4) produced at 4 °C, compared to the corresponding permeates produced at 50 °C (Table 6.1). The higher protein content in each of the permeates produced at 4 °C can be attributed to these permeates containing β-casein (France *et al*., 2021b). As a result of this, different VCFs were used for the MF process step at 50 °C such that the target protein content of the final retentates was the same (Table 6.1). On processing at 50 °C, filtration was stopped once a VCF of 2.25 was achieved, compared to a VCF of 3 at 4 °C (Fig. 6.1), resulting in protein contents of 8.29 and 8.40% for retentates produced at 4 (R4) and 50 °C (R50), respectively. Extensive DF resulted in R4 and R50 containing 97.6 and 97.1% casein and a whey protein:casein ratio of 0.025:1 and 0.029:1, respectively. Thus, retentate with a casein content representative of micellar casein isolate (MCI) was produced.

The mineral profile of permeates and retentate was influenced by processing temperature and the extent of DF (Table 6.2). R4 contained slightly lower calcium concentration compared to R50. However, a lower volume of retentate was produced at 4 °C, as the final filtration step was performed to a VCF of 3, compared to 2.25 at 50°C. Therefore, when a mass balance is performed and the volume is taken into account, a significantly lower ($p < 0.05$) amount of calcium is present in R4 (1863 mg/780 mL), compared to R50 (2351 mg/980 mL). Therefore, changing the temperature at which MF is performed can alter the mineral profile, in particular calcium content, of both the permeate and retentate streams. Calcium is of particular significance in the processing of dairy streams, as it can result in difficulties in subsequent downstream processing (e.g., promoting fouling though calcium-mediated protein aggregation).

Higher calcium contents were measured in each of the permeates generated at 4 °C compared to the corresponding permeates produced at 50 °C (e.g., 288 and 239 mg/kg calcium for P1 produced at 4 and 50 °C, respectively). At low temperatures (i.e., 4 °C), there is an increase in the solubility of colloidal calcium phosphate (CCP), increasing the concentration of both calcium and phosphorous in the serum phase (Gaucheron, 2005; Lewis, 2011; Fox *et al*., 2015). As a result, this calcium can permeate the membrane, resulting in higher calcium in permeates produced at low processing temperatures (Davies and White, 1960; Méthot-Hains *et al*., 2016; France *et al*., 2021b). These results are also reflected in the ionic calcium concentration of the samples, with significantly higher ($p < 0.05$) ionic calcium measured in permeates produced at 4 °C, compared to the corresponding permeate produced at 50 °C (Table 6.1).

Table 6.1: *Protein content, pH and ionic calcium of permeates and retentate generated following microfiltration and diafiltration of skim milk at 4 and 50 °C. Permeates 1-4 represent the permeate collected following each microfiltration/diafiltration step.*

Sample	Protein (%)			Native pH			Ionic calcium (mM)		
	4°C	50°C	*p*-value	4°C	50°C	*p*-value	4°C	50°C	*p*-value
Permeate 1	0.640 ± 0.010	0.543 ± 0.006	*	6.77 ± 0.02	6.53 ± 0.02	*	3.12 ± 0.14	1.60 ± 0.08	*
Permeate 2	0.249 ± 0.006	0.149 ± 0.009	*	7.07 ± 0.02	6.85 ± 0.02	*	2.04 ± 0.13	1.20 ± 0.03	*
Permeate 3	0.240 ± 0.004	0.084 ± 0.005	*	7.24 ± 0.02	7.05 ± 0.03	*	2.24 ± 0.08	1.13 ± 0.03	*
Permeate 4	0.135 ± 0.006	0.051 ± 0.002	*	7.38 ± 0.03	7.18 ± 0.02	*	1.34 ± 0.10	1.00 ± 0.05	*
Retentate	8.29 ± 0.45	8.40 ± 0.30	n.s.	7.11 ± 0.02	7.23 ± 0.01	*	n.a.	n.a.	n.a.
NCN	0.20 ± 0.02	0.24 ± 0.01	*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
% Casein	97.6 ± 0.08	97.1 ± 0.12	*	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Significance of difference between samples generated at 4 and 50 °C was identified with independent t-test or Mann Whitney-Wilcoxon test in the case of ionic calcium measurements. *indicates significant difference ($p < 0.05$); n.s., no significant difference. Means are from three independent trials

n.a., not applicable.

The mineral profile of permeates and retentate was influenced by processing temperature and the extent of DF (Table 6.2). R4 contained slightly lower calcium concentration compared to R50. However, a lower volume of retentate was produced at 4 °C, as the final filtration step was performed to a VCF of 3, compared to 2.25 at 50°C. Therefore, when a mass balance is performed and the volume is taken into account, a significantly lower ($p < 0.05$) amount of calcium is present in R4 (1863 mg/780 mL), compared to R50 (2351 mg/980 mL). Therefore, changing the temperature at which MF is performed can alter the mineral profile, in particular calcium content, of both the permeate and retentate streams. Calcium is of particular significance in the processing of dairy streams, as it can result in difficulties in subsequent downstream processing (e.g., promoting fouling though calcium-mediated protein aggregation).

Higher calcium contents were measured in each of the permeates generated at 4 °C compared to the corresponding permeates produced at 50 °C (e.g., 288 and 239 mg/kg calcium for P1 produced at 4 and 50 °C, respectively). At low temperatures (i.e., 4 °C), there is an increase in the solubility of colloidal calcium phosphate (CCP), increasing the concentration of both calcium and phosphorous in the serum phase (Gaucheron, 2005; Lewis, 2011; Fox *et al*., 2015). As a result, this calcium can permeate the membrane, resulting in higher calcium in permeates produced at low processing temperatures (Davies and White, 1960; Méthot-Hains *et al*., 2016; France *et al*., 2021b). These results are also reflected in the ionic calcium concentration of the samples, with significantly higher ($p < 0.05$) ionic calcium measured in permeates produced at 4 °C, compared to the corresponding permeate produced at 50 °C (Table 6.1).

Table 6.2: *Mineral profiles of skim milk feed, retentates at 8% protein, and permeates obtained following microfiltration (Permeate 1) and diafiltration (Permeates 2, 3 and 4) of skim milk at 4 and 50 °C.*

Mineral		Feed	Retentate			Permeate 1			Permeate 2			Permeate 3			Permeate 4		
			4°C	50°C	*p*-value	4°C	50°C	*p*-value	4°C	50°C	*p*-value	4°C	50°C	*p*-value	4°C	50°C	*p*-value
Calcium		972 ± 18.1	2271 ± 56.5	2293 ± 85.9	n.s.	288 ± 7.02	239 ± 4.51	*	97.7 ± 12.7	70.7 ± 11.9	n.s.	61.0 ± 3.00	40.7 ± 1.15	*	33.7 ± 0.58	22.7 ± 6.11	*
Phosphorus		915 ± 42.0	1584 ± 65.5	1454 ± 67.6	n.s.	414 ± 25.3	453 ± 46.9	n.s.	130 ± 18.5	110 ± 21.0	n.s.	78.3 ± 2.08	61.7 ± 1.15	*	40.0 ± 2.00	30.0 ± 9.54	n.s.
Potassium	mg/kg	1470 ± 51.5	120 ± 4.85	81.0 ± 2.49	*	1392 ± 41.7	1504 ± 30.2	*	354 ± 44.2	320 ± 50.3	n.s.	145 ± 10.2	122 ± 2.00	*	56.7 ± 1.53	45.3 ± 14.8	n.s.
Magnesium		89.3 ± 2.89	101 ± 1.71	89.2 ± 2.98	*	58.7 ± 1.53	63.0 ± 1.00	*	16.3 ± 2.31	14.3 ± 2.89	n.s.	9.23 ± 0.55	6.70 ± 0.25	*	4.40 ± 0.08	2.98 ± 1.34	n.s.
Sodium		312 ± 9.24	47.3 ± 26.6	67.9 ± 2.61	n.s.	294 ± 10.0	315 ± 7.57	*	76.0 ± 9.64	67.7 ± 11.0	n.s.	29.3 ± 1.53	25.3 ± 0.58	*	12.3 ± 0.58	11.3 ± 1.53	n.s.
Sulphur		232 ± 8.14	486 ± 21.1	475 ± 21.0	n.s.	103 ± 2.65	102 ± 2.08	n.s.	49.0 ± 5.29	40.7 ± 5.77	n.s.	40.7 ± 8.14	28.3 ± 3.21	n.s.	33.3 ± 3.06	35.3 ± 11.5	n.s.
Cobalt		0.37 ± 0.04	0.90 ± 0.34	0.79 ± 0.140	n.s.	0.23 ± 0.07	0.29 ± 0.06	n.s.	0.13 ± 0.04	0.15 ± 0.11	n.s.	0.25 ± 0.08	0.13 ± 0.07	n.s.	0.20 ± 0.08	0.10 ± 0.02	n.s.
Copper		71.7 ± 4.04	249 ± 5.69	248 ± 13.3	n.s.	21.0 ± 5.20	21.3 ± 4.93	n.s.	12.1 ± 5.14	13.9 ± 5.10	n.s.	10.4 ± 3.98	22.0 ± 8.72	n.s.	19.7 ± 5.86	7.83 ± 2.41	*
Manganese	ug/kg	27.7 ± 2.31	71.6 ± 6.26	65.0 ± 2.67	n.s.	11.9 ± 6.17	6.91 ± 1.86	n.s.	6.97 ± 2.36	5.45 ± 1.14	n.s.	7.73 ± 1.51	4.01 ± 0.47	*	8.53 ± 2.71	3.86 ± 1.66	n.s.
Molybdenum		31.7 ± 1.53	12.4 ± 0.79	5.24 ± 0.15	*	25.3 ± 0.58	30.7 ± 0.58	*	7.79 ± 0.88	6.75 ± 0.99	n.s.	3.80 ± 0.24	3.13 ± 0.69	n.s.	1.73 ± 0.27	3.63 ± 2.20	n.s.
Selenium		16.0 ± 0.00	46.0 ± 3.37	41.7 ± 3.08	*	2.60 ± 0.13	2.60 ± 0.23	n.s.	1.27 ± 0.24	0.92 ± 0.06	*	1.21 ± 0.20	0.53 ± 0.08	*	0.68 ± 0.13	0.40 ± 0.17	n.s.
Zinc		3175 ± 112	12237 ± 230	10479 ± 551	*	119 ± 33.7	116 ± 18.7	n.s.	96.3 ± 8.39	125 ± 27.2	n.s.	101 ± 36.5	71.0 ± 29.4	n.s.	91.7 ± 10.3	70.0 ± 27.1	n.s.

Significance of difference between the samples generated at 4 and 50 °C for a specific mineral was identified with independent t-test. *indicates significant difference ($p < 0.05$); n.s., no significant difference.

6.4.2 Protein profile and extent of β-casein depletion

All permeates produced at 4 °C contained β-casein (Fig. 6.2), with no detectable β-casein in permeates produced at 50 °C (data not shown). The highest concentration of β-casein was measured in P1, after skim milk had been stored at 4 °C for 48 h; after this, P3 contained the highest concentration of β-casein, which was expected, as overnight storage and dilution promote dissociation of β-casein. At low processing temperatures (i.e., 4 °C), hydrophobic interactions are weakened, resulting in the dissociation of β-casein from the interior of the micelle to the surface, from where it releases into the serum phase; this serum β-casein can then permeate the membrane into the permeate stream (Creamer *et al*., 1977; France, Bot *et al*., 2021). However, at warm temperatures (i.e., 50°C), hydrophobic interactions are strong, maintaining β-casein within the casein micelles, and therefore a permeate free from β-casein can be obtained.

Filtration at 4 °C resulted in 40.3% depletion of β-casein (Fig. 6.2b). O'Mahony *et al*. (2014) achieved between 10 and 20% depletion of β-casein from skim milk, subjected to cold MF (2-5 °C). Schäfer *et al*. (2019) reported 19.89% depletion of β-casein in retentate following cold MF and DF (≤ 5°C) of MCC. In the present study, extensive DF at 4 °C was performed with 48 h storage at 4 °C prior to filtration and overnight storage at 4 °C between the two days of filtration. Similar extents of β-casein depletion have been achieved following centrifugation of casein micelles at low temperatures; Duerasch *et al*. (2020) achieved a 43.2% depletion of β-casein following stepwise depletion (3 steps) of a micellar casein suspension *via* ultracentrifugation and cooling at 6 °C. As expected, retentate produced at 4 °C contained significantly lower ($p < 0.05$) β-casein compared to retentate produced at 50 °C, with values of 24.2 and 33.6 mg/mL, respectively (Fig. 6.3b). Retentates produced at 4 °C also contained

significantly higher ($p < 0.05$) levels of α_s-casein, while no significant differences ($p > 0.05$) in κ-casein were measured between retentates produced at 4 and 50 °C.

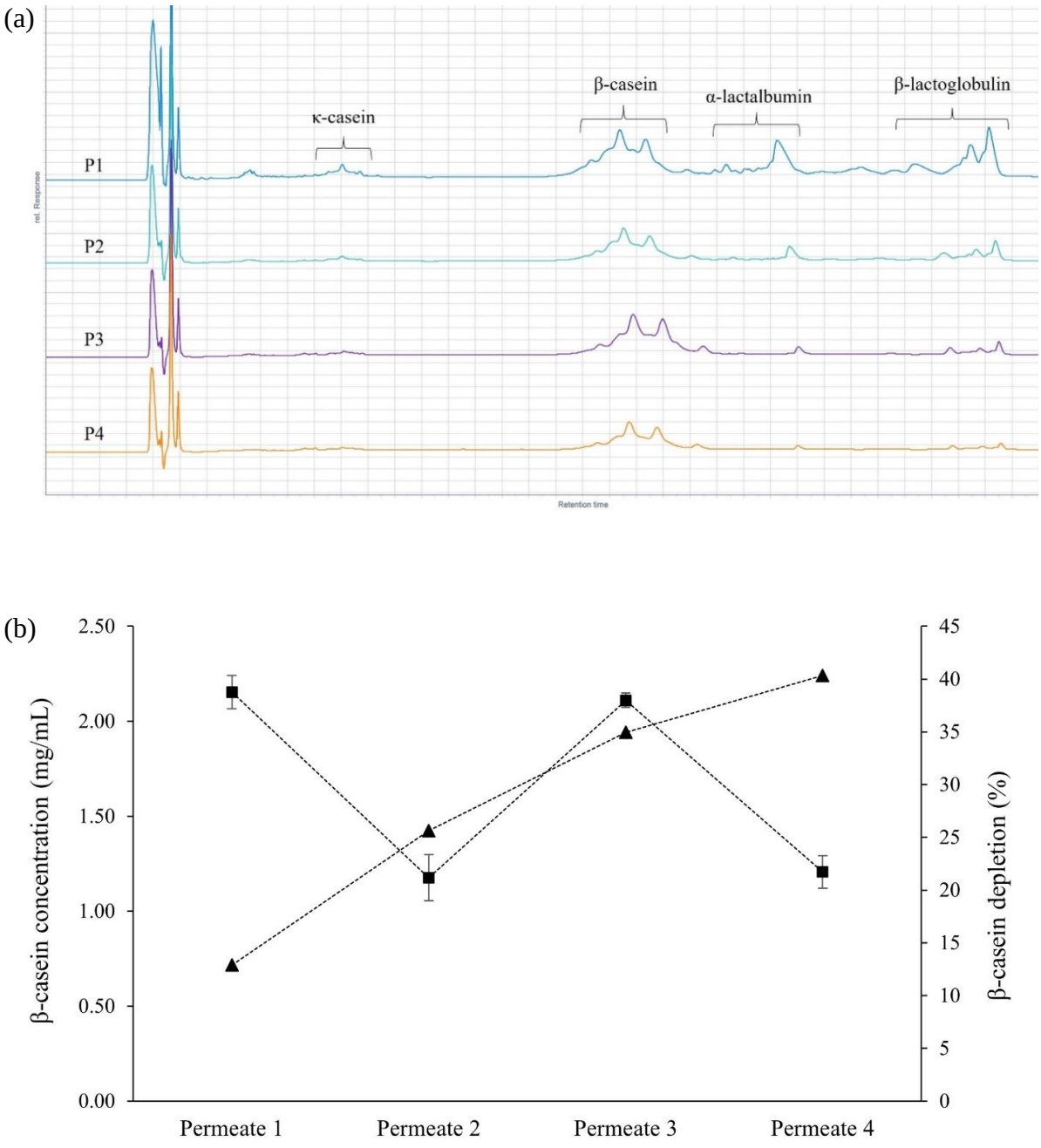
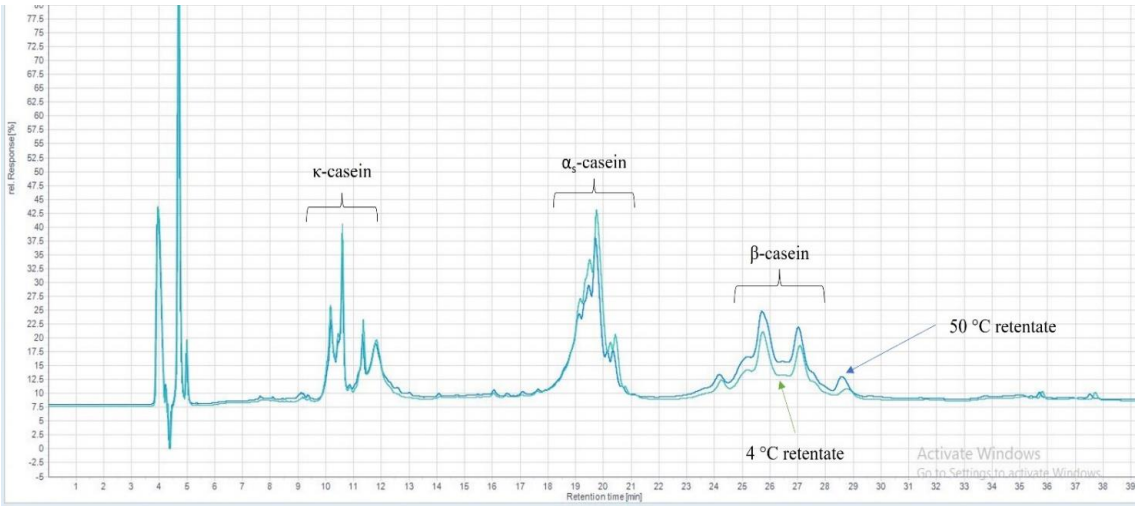


Figure 6.2: *(a) Chromatograms from reversed-phase high performance liquid chromatography analysis of permeate samples (P1, P2, P3 and P4) produced during MF of skim milk at 4°C and (b) β-casein content of the permeates (■) and corresponding cumulative percentage of β-casein depletion (▲) following each filtration step (lines are only to guide the eye).*

(a)



(b)

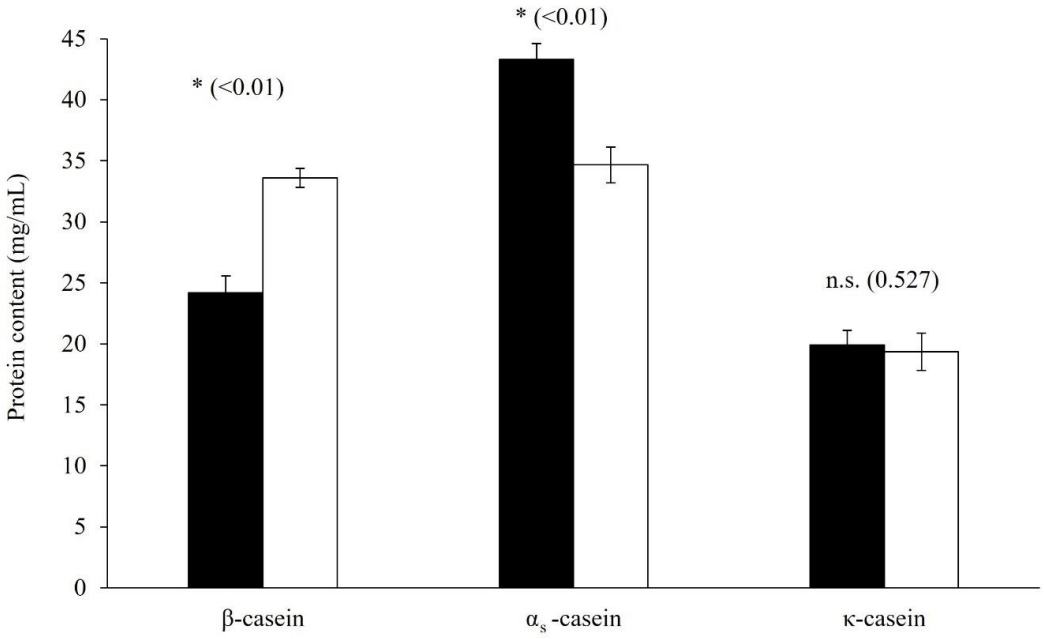


Figure 6.3: *(a) Chromatograms of retentate produced at 4 and 50 °C and (b) concentration of β-casein, α_s-casein and κ-casein in retentates after microfiltration and diafiltration of skim milk at 4 (■) and 50 °C (□), at 8% protein. Significant differences between retentates for a specific protein were identified with independent t-test: * indicates significantly different ($p < 0.05$); p-values in parentheses; n.s., not significantly different.*

After each filtration step, particle size measurements were performed to evaluate the effect of different extents of diafiltration and β-casein depletion on casein micelle size (Table 6.3). MF/DF at 4 °C resulted in no change in particle size, with particle sizes of 164 and 165 for skim milk and the final retentate (retentate 4), respectively. At 50°C, particle sizes decreased with subsequent diafiltration steps, resulting in a particle size of 158 nm in the final retentate (retentate 4). Thus, MF/DF at 4°C resulted in a significantly larger ($p < 0.05$) particle size in the final retentate, when compared to 50 °C, albeit differences were very small. Crowley *et al*. (2018) reported similar findings, with MCC generated following cold MF (<10 °C) having larger particle sizes when compared to MCC generated following warm MF (50° C). At 4 °C hydrophobic interactions are weaker/less numerous resulting in more swelling of casein micelles and increased voluminosity (Nöbel *et al*., 2012).

Table 6.3*: Particle size (z-average) of skim milk and retentates after microfiltration/diafiltration at 4 and 50 °C*

	Particle size (nm)		
	4 °C	50 °C	*p*-value
Skim milk	164 ± 1.18	162 ± 0.87	n.s.
Retentate 1	164 ± 3.75	161 ± 0.90	n.s.
Retentate 2	166 ± 1.58	160 ± 1.26	*
Retentate 3	164 ± 3.01	159 ± 1.31	*
Retentate 4	165 ± 3.23	158 ± 0.61	*

Significance of difference between samples generated at 4 and 50 °C was identified with independent t-test. *indicates significant difference ($p < 0.05$); n.s., no significant difference.

6.4.3 Thermal stability of liquid micellar casein isolates

The heat coagulation time (HCT) of the retentates were measured to assess whether the differences in protein and mineral profile altered heat stability (Fig. 6.4). The HCT increased with increasing pH for both retentates produced at 4 and 50 °C. At pH 7.2, the highest HCT was measured, with values of 56.4 and 46.6 min for retentate produced at 4 and 50 °C, respectively. At each pH, significantly higher ($p < 0.05$) HCTs were observed for retentates produced at 4 °C compared to retentates produced at 50 °C. As whey protein:casein ratios of 0.025:1 and 0.029:1 were achieved for R4 and R50, respectively, differences in heat stability due to whey proteins was avoided. Sauer and Moraru (2012) evaluated the heat stability of MCC by heating reconstituted MCC (8%, w/v, casein) to different temperatures (110 to 150 °C), at different pH values (pH 6.5 to 7.3); the authors reported that samples with pH lower than 6.7 displayed poor heat stability, with visible aggregation or coagulation for all heat-treated samples, whereas at pH > 6.9 samples did not coagulate, with no or minor changes observed. In the present study it was also found that increasing the pH resulted in improved heat stability, with the HCT increasing significantly ($p < 0.05$) with each pH increase. De Kort *et al.* (2012) investigated the effect of calcium chelators on the heat-induced changes of concentrated MCI (9% w/v protein) at 126°C; the authors reported similar findings to those in the present study with no chelator addition; they reported that the HCT increased with increasing pH with values of 2, 40 and 55 min at pH 6.7, 7.0 and 7.3, respectively.

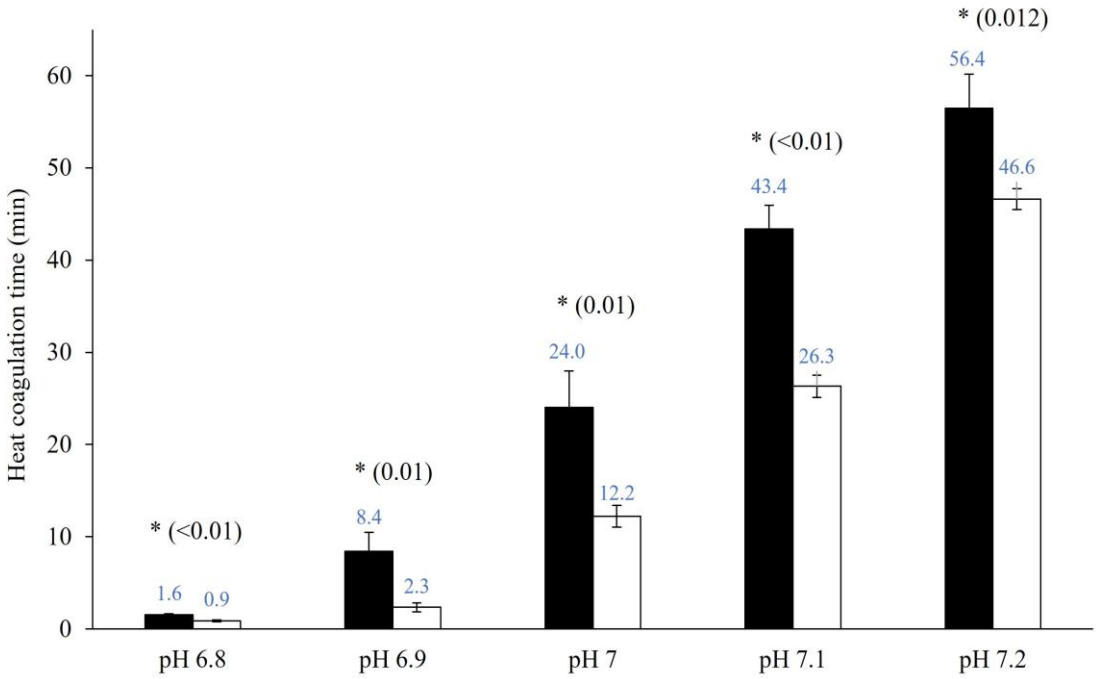


***Figure 6.4**: Heat coagulation time of retentates at different pH, produced after microfiltration and diafiltration of skim milk at 4 (■) and 50 °C (□), standardised to 8% protein. Values for the heat coagulation times are presented in blue. Significant differences between retentates for a specific pH were identified with independent t-tests: * indicates a significant difference ($p < 0.05$); p-values in parentheses; n.s., not significantly different.*

To assess whether differences in heat stability observed between retentates were influenced by differences in ionic calcium, the ionic calcium content of retentates at different pH values was measured (Fig. 6.5). Ionic calcium decreased with increasing pH in both retentates with values of 6.28 and 7.67 mM at pH 6.8 and 3.2 and 3.8 mM at pH 7.2 for retentates produced from MF at 4 and 50 °C, respectively. The equilibrium between colloidal and soluble calcium is strongly dependent on both pH and temperature, with low temperature and low pH both shifting the equilibrium towards the soluble phase (Le Graët and Gaucheron, 1999; Lewis, 2011). In the current filtration procedure, extensive DF was performed, altering the equilibrium of minerals in the resulting retentates. When MF and DF are performed at low temperatures (i.e., 4 °C) additional calcium is removed through partial solubilisation of CCP and removal

into the permeate stream (Table 6.2). This is likely to have contributed to the differences in ionic calcium at each pH, as the micelles in retentate generated at 4 °C are likely to have the ability to bind higher amounts of soluble calcium, as partial solubilisation (and removal) of CCP occurred during cold MF/DF. De Kort *et al.* (2012) reported higher calcium-ion activity in MCI solutions at lower pH, with no chelator addition. Similar findings were reported by Sauer and Moraru (2012), with the concentration of soluble calcium increasing in MCC solutions as pH decreased, following heating to the desired temperature (i.e., 110-150 °C). Therefore, the solubilisation of CCP in both retentates at low pH is likely to have contributed to the destabilisation of the casein micelles, resulting in the low HCT measured at these lower pH values.

However, as retentate generated by cold MF was considerably depleted in β-casein (40.3%), it is likely that other confounding factors contributed to the differences observed. The zeta-potential of retentates, measured at their native pH, were similar, with values of -22.1 and -23.8 mV, for retentates produced at 4 and 50 °C, respectively. As pH decreases (i.e., pH 7.2-pH 4.7) the net negative charge on casein micelles decreases, reducing electrostatic repulsion between micelles, and so it is possible that differences in zeta potential between the retentates at each pH contributed to the differences observed. Further studies could explore how the heat stability is affected if calcium content is standardised after filtration, which could clarify whether β-casein depletion alone affects heat stability, as it is worth noting that the differences in heat stability were much greater than the difference in ionic calcium. In addition to this, the calcium-binding potential of β-casein-depleted MCI warrants investigation.

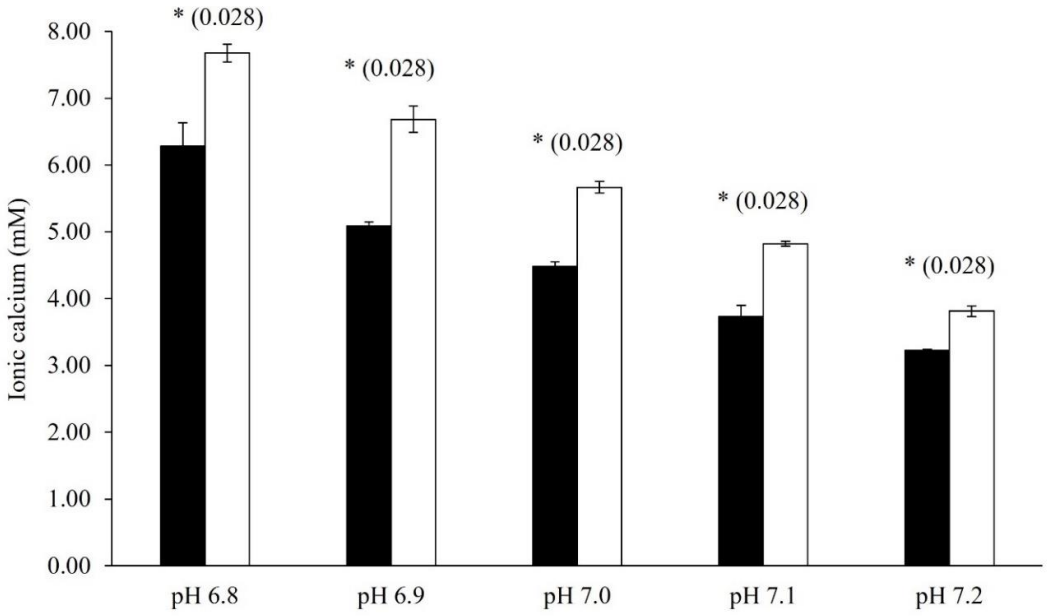


Figure 6.5: *Ionic calcium content of retentates produced from microfiltration and diafiltration of skim milk at 4 (■) and 50 °C (□), at 8% protein, at different pH values. Significant differences between retentates at a specific pH were identified with Mann Whitney-wilcoxon test: * indicates significant differences ($p < 0.05$); p-values in parentheses*

6.4.4 Flow behaviour of micellar casein isolates

To determine whether the differences observed in protein and mineral profile affected flow properties of retentates generated at 4 and 50 °C, the consistency coefficient and flow behaviour index were measured (Table 6.4a). Significantly higher ($p < 0.05$) consistency coefficient was measured for R4 compared to R50, when measured at 4 °C. At 4 °C, a lower flow behaviour index value was also determined for R4 (0.914), compared to R50 (0.923), indicating that R4 displayed greater shear-thinning behaviour; a similar trend was observed when measured at 25 °C. It has been shown that milk protein-derived concentrates are non-Newtonian and generally display shear-thinning properties (Sauer *et al*., 2012; Kieferle *et al*., 2019). As both samples were at the same protein content (8% protein) and pH, the differences in

protein profile and mineral profile are likely to have contributed to the differences observed between R4 and R50 at the specific temperatures tested.

To evaluate the effect of composition and temperature on viscosity of the retentates, comparisons were made using apparent viscosity (η_{app}) at a shear rate of 300 s^{-1} (Table 6.4b). R4 had significantly higher ($p < 0.05$) η_{app} compared to R50, with values of 9.73 and 8.42 mPa.s, respectively, when measured at 4 °C; measurements at 25 °C showed similar differences in η_{app}. At 4 °C, the η_{app} was higher on the shear down ramp compared to the shear up ramp, for both R4 and R50, indicating structure formation in the samples; similar results were reported by Kieferle *et al*. (2019) for fresh milk protein concentrates. In the present study, no significant differences were observed for the increase in η_{app} between shear up and shear down ramps, when measured at 4 °C, for R4 and R50.

Casein is the main contributor to the viscosity of high purity MCC (Sauer *et al*., 2012); as both retentates only differed by approximately 0.04% casein, when at 8% protein, the differences observed can be attributed to the differences in casein micelle composition and/or structure. Snoeren *et al*. (1982) reported that, at high concentrations of casein, the rheological properties of concentrated skim milk are affected by changes in the voluminosity of casein micelles. As temperature decreases, the voluminosity of casein micelles increases (Whitnah & Rutz, 1959; Nöbel *et al*., 2012). As R4 was depleted in β-casein, it is likely that this resulted in differences in voluminosity compared to R50, at 4°C. In addition to this, R50 had significantly lower particle size, compared to R4, at 25 °C (Table 6.3). Therefore, higher viscosity was measured for retentate produced at 4 °C compared to retentate produced at 50 °C.

***Table 6.4**: (a) Consistency coefficient (K) and flow behaviour index (n) and (b) apparent viscosity at 300 s^{-1} on the shear up and shear down ramp of retentates generated from microfiltration and diafiltration of skim milk at 4 and 50 °C, measured at 4 and 25 °C.*

(a)

Assay temperature (°C)		Sample		*p*-value
		4 °C Retentate	50 °C Retentate	(-)
4	K ($Pa.s^n$)	0.017 ± 0.003	0.012 ± 0.001	*
	n	0.914 ± 0.023	0.923 ± 0.008	n.s.
25	K ($Pa.s^n$)	0.005 ± 0.001	0.003 ± 0.001	n.s.
	n	0.981 ± 0.010	0.980 ± 0.010	n.s.

(b)

Assay temperature (°C)		η at 300 s^{-1}		*p*-value
		4 °C Retentate	50 °C Retentate	(-)
4	shear up	9.73 ± 0.57	8.42 ± 0.57	*
	shear down	10.4 ± 0.59	9.12 ± 0.19	*
25	shear up	4.33 ± 0.08	3.53 ± 0.20	*
	shear down	4.43 ± 0.49	3.49 ± 0.35	n.s.

Significance of difference between samples generated at 4 and 50 °C was identified with independent t-test. *indicates significant difference ($p < 0.05$); n.s., no significant difference.

6.5 Conclusion

Microfiltration of skim milk at 4 and 50 °C resulted in different protein and mineral profiles in the resulting retentates. A 40.3% depletion in β-casein was achieved using microfiltration and extensive diafiltration at 4 °C, with prolonged storage at 4 °C in between filtration steps. Retentates generated from cold microfiltration displayed higher heat stability across the pH range studied compared to retentates produced from warm microfiltration. Higher ionic calcium content was also measured in the retentates generated from cold microfiltration at each pH, with ionic calcium increasing with decreasing pH, corresponding to decreased heat

stability. Similar consistency coefficient and flow behaviour index were measured for retentates; however, apparent viscosity at 300 s^{-1} was higher for retentates at 4 °C compared to retentates generated at 50°C. These findings suggest that β-casein-depleted micellar casein concentrate or isolate could be used in similar applications as micellar casein concentrate produced following warm microfiltration, where heat stability and rheological properties are important. Further work is suggested to more deeply understand the factors contributing to the differences in heat stability and whether restoring calcium to β-casein-depleted retentates will provide information into whether protein profile alone provides similar results. In addition, there is further scope to explore/exploit the calcium-binding capacity of β-casein-depleted micellar casein isolate systems.

6.6 Acknowledgements

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Chapter 7

The influence of diafiltration medium on protein partitioning and thermal stability of micellar casein retentates obtained by microfiltration of skim milk

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Declaration

This chapter was written by author Thomas C. France (TCF) and reviewed by all co-authors. TCF co-designed the study and performed all of the experimental work.

7.1 Abstract

During microfiltration (MF) of skim milk, diafiltration (DF) is often performed to facilitate the additional removal of target constituents. Water is widely used as a DF media in industry; however, ultrafiltration (UF) permeate also provides an ideal medium as it can minimise changes in mineral equilibria. Therefore, in this study, the effect of different DF media, namely water and simulated milk ultrafiltrate resembling the mineral profile of UF permeates obtained at 10 and 50 °C (i.e., SMUF 10 and 50, respectively), on processing performance, protein partitioning, and heat stability of the resulting retentates was investigated following the MF and DF of skim milk at 8 °C. The use of water resulted in significantly lower ($p < 0.05$) conductivity and higher pH of retentates compared to use of SMUF 10 or 50. The different DF media had no significant impact on the transmission of β-lactoglobulin, α-lactalbumin or β-casein throughout filtration. However, significantly longer ($p < 0.05$) heat coagulation times (HCT) were measured for retentates generated using SMUF 10 and 50 as the DF medium which corresponded to significantly lower ($p < 0.05$) concentrations of ionic calcium, compared to when water was used. The results of this study indicate that the use of model lactose-free UF permeate (i.e., SMUF) provides similar protein partitioning characteristics to when water is used; however, a final micellar casein retentate with superior thermal stability can be produced.

7.2 Introduction

When tight-pore microfiltration (MF; 0.1-0.5 µm) of skim is performed, the separation of the two major milk protein fractions, casein micelles and serum proteins, can be achieved (Carter *et al*., 2021; France, Kelly, *et al*., 2021). During the MF of skim milk, diafiltration (DF) can be used to further remove serum proteins, minerals and lactose while the casein micelles are retained, resulting in micellar casein concentrate (MCC) or isolate (MCI), depending on the extent of DF applied.

DF involves washing the retentate with a DF media, either stepwise (i.e., batch) or gradually throughout MF (i.e., continuously). However, the type of DF media that can be used varies from deionised water to water with varying ionic composition to ultrafiltration (UF) permeate (Renhe *et al*., 2019; Reitmaier *et al*., 2021). When deionised water is used as a DF medium, changes in micelle integrity, pH and mineral composition must be considered, as the continuous addition of water causes a shift in the mineral equilibrium towards the serum phase, resulting in the solubilisation of colloidal calcium phosphate (CCP; Kulozik, 1998; France *et al*., 2022; Chapter 4). On the other hand, UF permeate contains lactose and a mineral profile comparable to that of the serum phase of skim milk (Dumpler *et al*., 2017). Therefore, when UF permeate is used as a DF medium, there are minimal changes in the mineral equilibrium of the casein micelles, maintaining the structural integrity of the micelles (Beliciu and Moraru, 2009).

Alterations in micelle structure and composition, as affected by different DF media, can result in changes in the voluminosity and size of casein micelles, which in turn can alter the functional properties of the final ingredient generated (e.g., viscosity and heat stability; Le Berre & Daufin, 1998; Renhe *et al*., 2019; Reitmaier *et al*., 2020). In addition to changes in the structure and composition of casein micelles, it has been

reported that differences in pH, calcium content and ionic strength of water can alter flux and deposit resistance during MF, with the calcium level being the main contributing factor (Reitmaier *et al*., 2021).

In the dairy industry, MF is performed at either warm (i.e., 45-55 °C) or cold (i.e., ≤20 °C) temperatures, which can further alter the natural mineral equilibrium (France, Kelly, *et al*., 2021). At low temperatures (e.g., 4 °C), the solubility of CCP increases, resulting in an increase in the concentration of calcium and phosphate in the serum phase (Fox *et al*., 2015). In addition, at low temperatures (i.e., ≤20 °C), β-casein dissociates from the micelle, into the serum phase, from which it passes into the permeate stream (Creamer *et al*., 1977; O'Mahony *et al*., 2014). Therefore, DF at low temperatures could have even greater consequences for the integrity and mineral content/profile of the micellar phase, as well as potentially impacting the rate and/or extent of β-casein dissociation. In addition, UF of skim milk can be performed at either warm or cold temperatures, altering the mineral profile of the UF permeate generated; thus, differences in the mineral profile of UF permeate, as a result of different processing temperatures, could have implications on retentate properties and processing performance if used as DF media during MF of skim milk.

Many researchers use water, usually deionised water, as the DF medium in order to achieve ingredients of a higher purity, as selective components can continually be removed without the addition of other solids (e.g., lactose in UF permeate; Evans *et al*., 2010; Coşkun *et al*., 2022; Schäfer *et al*., 2021). This is also likely the case in industry as high-quality recovered water is now readily accessible (e.g., reverse osmosis (RO)-treated, water). Therefore, there remains the question as to whether model salt solutions (i.e., SMUF) can be used in place of water, allowing for purified protein ingredients to be produced while minimising changes in the natural mineral

equilibrium of skim milk and MF retentates. In previous work (Chapter 6) DF with water resulted in extensive β-casein dissociation and partitioning into the permeate stream during the MF of skim milk, as well as causing the depletion of minerals in the serum phase. Therefore, the influence of different DF media on β-casein partitioning at low temperature and compositional consequences for ingredients remains to be investigated. The objective of this study was to perform MF of skim milk at low temperature (i.e., 8 °C) with continuous DF using three DF media (water and two different SMUFs designed to resemble the mineral profile of UF permeate produced from skim milk at 10 or 50 °C) and characterise the effects of the different DF media on composition, protein partitioning and heat stability of the resultant retentates.

7.3 Materials and methods

7.3.1 Materials

Low-heat skim milk powder was provided by a local Irish dairy company (Arrabawn Co-op, Nenagh, Co. Tipperary, Ireland). Reconstituted skim milk was prepared by adding low-heat skim milk powder to ultrapure water over 2 h at 22 °C to attain a 3.20% (w/v) protein solution. The skim milk was then stored at 4 °C for 48 h under gentle constant magnetic stirring to facilitate complete rehydration. All chemicals, reagents and high-performance liquid chromatography (HPLC) standards, unless otherwise stated, were sourced from Sigma-Aldrich (Wicklow, Ireland) and were of analytical grade.

7.3.2 Diafiltration media

Three different diafiltration (DF) media were used during the microfiltration (MF) of skim milk, ultrapure water and simulated milk ultrafiltrate (SMUF) designed

to resemble the mineral profile of ultrafiltration (UF) permeate produced at 10 (SMUF 10) and 50 °C (SMUF 50). SMUF was prepared according to the method of Dumpler *et al*. (2017) with the pH of SMUF simulating UF permeate at 10 °C (pH 6.79) and 50 °C (pH 6.64) also being typical of the pH of UF permeate produced at these temperatures (Dumpler *et al*., 2017). A detailed breakdown of the composition of SMUF 10 and SMUF 50 is provided in Appendix 3.

7.3.3 Microfiltration of skim milk

MF experiments were performed on a lab-scale, pressure-driven, tangential-flow filtration device (Pellicon 2 mini-holder; Merck Millipore, Tullagreen, Carrigtwohill, Ireland) as described by Crowley *et al*. (2015). A 1000-kDa molecular weight cut-off (equivalent to a 0.1 µm pore size MF membrane) polyethersulfone membrane (Biomax, Merck Millipore) was used.

Prior to MF, a 40 min equilibration time was used to ensure stable conditions (e.g., temperature, transmembrane pressure (TMP) and permeate flux) in which retentate and permeate lines were returned to the feed vessel (3 L; i.e., total recirculation mode). The first stage of MF was carried out with continuous DF (Fig. 7.1). For every 100 mL of permeate removed, 100 mL of DF media was added to the feed vessel, until 4 L of DF media had been added, resulting in the generation of permeate 1 (P1). After this, a concentration step was carried out until a volume concentration factor (VCF) of 3.5 was achieved, resulting in a concentrated micellar casein retentate and permeate 2 (P2).

Cleaning of the membrane and plate heat exchanger was performed at the end of each day of filtration, with the extent of membrane fouling assessed as described by France, Bot *et al*. (2021).

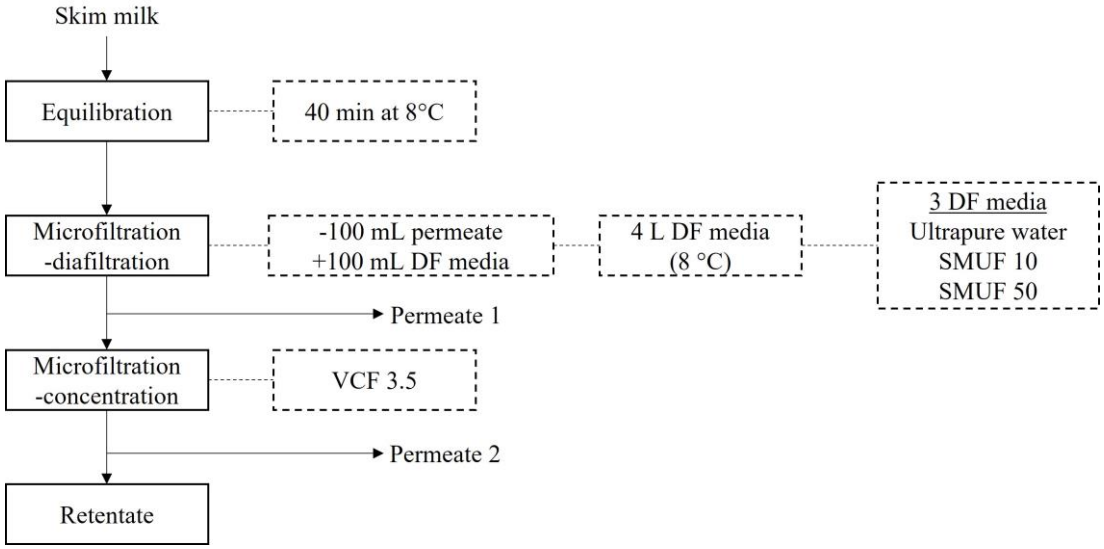


Figure 7.1: *Schematic representation of the microfiltration and diafiltration of skim milk at 8 °C; DF, diafiltration; VCF, volume concentration factor; SMUF, simulated milk ultrafiltrate.*

7.3.4 In-process measurements

7.3.4.1 Transmembrane pressure and permeate flux

TMP and permeate flux were measured at 15 min intervals during MF. TMP was calculated according to the following equation:

$$TMP = \frac{P_i + P_o}{2} \quad [7.1]$$

where P_i and P_o are inlet and outlet pressure, respectively. Permeate flux ($L/m^2/h$) was recorded by measuring the volume of permeate generated in a graduated cylinder over 1 min.

7.3.4.2 Conductivity and pH

Conductivity and pH of the retentate was measured at 15 min intervals during filtration using a portable pH/conductivity meter (Metler Toledo, SevenGo Duo, SG23, Switzerland). The conductivity probe (InLab® 738-ISM, Mettler Toledo,

Schwerzenbach, Schweiz) was calibrated with a standard solution of known conductivity of 12.88 mS/cm.

7.3.5 Sieving coefficient

Samples of permeate and retentate were taken every 45 min during filtration and the protein profile analysed. Permeation behaviour of selected proteins during MF/DF were characterised using the sieving coefficient (S_o):

$$S_o = \frac{C_P}{C_R} \quad [7.2]$$

where C_P and C_R are the concentrations of the protein of interest in the permeate and retentate streams, respectively.

7.3.6 Composition of process streams

Total nitrogen content of process streams (i.e., retentate and permeates) was determined using the Kjeldahl method using a nitrogen-to-protein conversion factor of 6.38 for the calculation of crude protein (IDF, 2001). Non-casein nitrogen was determined as described by Wojciechowski and Barbano, (2015), using a method based on the AOAC International method (AOAC International, 2010 method number 998.05). The concentration of buffers was unchanged; however, the volume of buffers was increased from 1 mL, as described by Lynch *et al*. (1998), to 5.5 mL of both acetic acid (10% v/v) and sodium acetate (1.0 mol/L). The increase in buffer volumes was required to provide sufficient buffering capacity to compensate for the higher protein content of the retentates. Ash content was determined by heating samples to 700 °C until a white ash was obtained (IDF, 2008).

7.3.7 Protein profile of process streams

The protein profile of the retentates and permeates (P1 and P2) were measured using RP-HPLC (Agilent 1220 Infinity II LC, Santa Clara, CA 95051 United States) with a C18 column (3.6 µm x 250 mm x 4.6 mm, Aeris Widepore, Phenomenex, UK) as described by Bonfatti *et al*. (2008) and Bot *et al*. (2020), using solvents A (10.0% HPLC-grade acetonitrile, 89.9% ultrapure water and 0.10% TFA) and B (89.9% HPLC-grade acetonitrile, 10.0% ultrapure water and 0.10% TFA); the injected volume was 40 µL, and detection was performed at 214 nm. Prior to analysis, samples were filtered through 0.45-µm filters (Minisart® RC25, Göttingen, Sartorius AG, Germany). The concentrations of individual proteins were determined by preparing standard curves of the respective proteins ($R^2 > 0.99$) and results are expressed as mg protein per mL of sample.

7.3.8 pH adjustment

The pH of permeate and retentate streams were measured using a standard pH meter at 20 °C (Metler Toledo, SevenGo Duo, SG23, Switzerland). The pH of retentate streams, at 8% protein, were adjusted to pH 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1 and 7.2 using both 0.5 N NaOH and HCl. Retentates were adjusted to the desired pH prior to overnight storage at 4 °C. The following day samples were equilibrated to 20 °C and re-adjusted to the desired pH, if needed, prior to analysis of ionic calcium and heat coagulation time (HCT) measurements.

7.3.9 Ionic calcium

A Titrando 907 autotitrator with Tiamo v2.2 software, equipped with a calcium (Ca)-ion-selective electrode (Metrohm Ireland Ltd, Co. Carlow, Ireland), was used to

measure the ionic calcium concentration of feed, permeate and retentate samples according to the method of France *et al*. (2022) (Chapter 6).

7.3.10 Heat coagulation time as a function of pH

HCT of retentates (8% protein), generated after MF, as a function of pH (6.5-7.2), was measured at 120 °C according to Davies and White (1966), with modifications. Sample (2 mL) was added to heat-resistant glass tubes, stoppered using a rubber bung, placed in a steel rack, and immersed in a silicone oil bath (Hettich ESP oilbaths; Hettich Benelux BV), with continuous rocking at motor speed setting of 3. The HCT was recorded as the time elapsed between immersing the sample in the oil bath and the visible detection of aggregates within the sample.

7.3.11 Particle size and zeta potential

The particle size distribution (PSD) of the different samples was measured after heating at 120 °C for 10 min. PSD was measured by dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Retentate samples were diluted with water and measured immediately using a refractive index of 1.45 for protein. Measurements were carried out at 25 °C after temperature equilibration for 80 s, using a backscattering configuration of 173°.

The zeta-potential (ζ-potential) of the retentates at each pH, prior to heating, was also measured using a Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Each retentate was diluted 1 in 100 with ultrapure water and allowed to equilibrate at 25 °C for 80 s in the cuvette prior to analysis. The measurement was performed using an automatic voltage selection and calculated using the Smoluchowski model (Kirby & Hasselbrink, 2004).

7.3.12 Statistical analysis

All experimental analyses were conducted in triplicate, with samples produced from three independent trials for each processing temperature, unless otherwise stated. Results are expressed as mean ± standard deviation. Analysis of variance (ANOVA; Tukey's HSD test) was performed using R i386 version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) to identify statistically significant differences between mean values for different samples. The level of significance was determined at $p < 0.05$ to determine whether statistically significant differences existed between the mean values.

7.4 Results and discussion

7.4.1 Filtration performance

The effect of different diafiltration (DF) media on the resulting permeate flux and transmembrane pressure (TMP) was monitored throughout microfiltration (MF). Continuous DF was performed until 4 L of DF medium had been added, after which MF was continued until a volume concentration factor (VCF) of 3.5 was achieved. DF using water resulted in the shortest processing time (420 min) compared to when SMUF 50 and SMUF 10 were used, both taking approximately 450 min (Fig. 7.2). Throughout DF, TMP remained stable before rapidly increasing during concentration, with final TMP values of 0.325, 0.325 and 0.338 bar for water, SMUF 50 and SMUF 10, respectively. Following MF/DF, the extent of fouling was assessed by determining the water flux through the membrane; the results indicated that using water as a DF medium resulted in a significantly ($p < 0.05$) lower reduction in water flux (18.3%) compared to when SMUF 50 (23.7%) and SMUF 10 (25.7%) were used.

It has previously been reported that changes in the ionic composition of the DF media used can alter permeate flux (Reitmaier *et al*., 2021). Reitmaier *et al*. (2021) investigated the effect of several different diafiltration media (e.g., tap water with different degrees of hardness, softened water and deionised water) on the structure of a milk-derived deposit layer and filtration performance; they reported that water containing lower concentrations of divalent cations (e.g., deionised water) displayed higher flux over time compared to water containing higher levels (e.g., tap water of 18 ° dH). It has also previously been described that the reduction of serum ions by DF, using deionised water, improves filtration performance, as the removal of soluble calcium available for casein-casein interactions is reduced, resulting in a more loosely packed fouling layer and thus lower flow resistance (Kulozik, 1998). Therefore, in the current study, the significantly ($p < 0.05$) shorter processing time observed when water was used as DF medium could be partly attributed to the lower extent of fouling due to the reduction in soluble ions, mainly calcium, available for casein crosslinking and for possible interaction with the membrane.

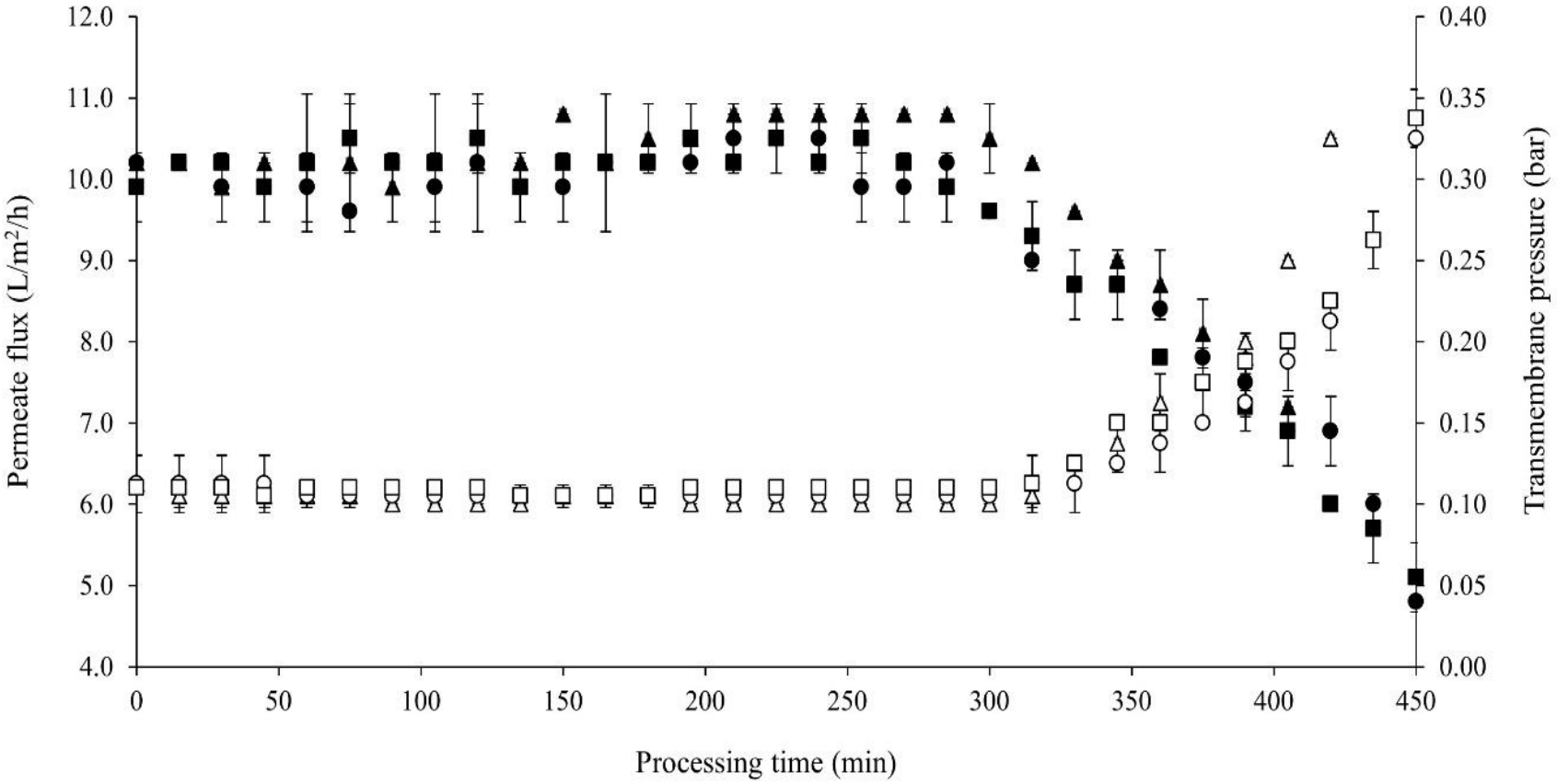


Figure 7.2: *Permeate flux (closed symbols) and transmembrane pressure (open symbols) as a function of processing time during the microfiltration and diafiltration of skim milk at 8 °C using different diafiltration media: permeate flux using water (▲), SMUF 50 (●) and SMUF 10 (■); transmembrane pressure using water (Δ), SMUF 50 (○) and SMUF 10 (□).*

7.4.2 In-line measurements

During MF and DF, pH and conductivity were measured to provide information on the effects of each DF medium on the ionic content of the retentate during filtration (Fig. 7.3a, b). The use of water as the DF medium resulted in an increase in the pH of retentate during DF, before plateauing during the concentration step (i.e., after 270 min). However, when SMUF 50 (native pH = 6.64) and SMUF 10 (native pH = 6.79) were used, the pH remained essentially constant throughout DF and concentration. At the end of the concentration step, significantly ($p < 0.05$) higher pH was measured for retentate diafiltered with water (6.97), followed by SMUF 10 (6.74) and SMUF 50 (6.70). A similar trend was also observed for conductivity, with only a small change in conductivity throughout filtration when SMUF 50 and SMUF 10 were used. Conductivity decreased from 5.27 mS/cm at the beginning of filtration to 4.60 or 4.58 mS/cm, at the end of filtration, for SMUF 50 or SMUF 10, respectively. However, the conductivity of the retentate during diafiltration with water decreased rapidly, reaching a final value of 1.64 mS/cm.

The addition of water during DF results in the continuous removal of minerals present in the serum phase and subsequently alters the natural mineral equilibrium present in milk (Ferrer *et al*., 2014; Reitmaier *et al*., 2021). As a result, DF using water can result in changes in the level of colloidal minerals (i.e., micellar bound calcium), with colloidal calcium and phosphate levels reducing with progressive DF (Alexander *et al*., 2011; Ferrer *et al*., 2014; Reitmaier *et al*., 2021). Performing MF at low temperatures (i.e., 10 °C) could result in more pronounced changes in colloidal calcium during DF, as the solubility of CCP increases with decreasing temperature (Liu *et al*., 2014). When SMUF is used, the minerals removed in the permeate stream are restored and therefore the mineral equilibrium will remain relatively unchanged,

as is evident in Fig. 7.3b. As it has been shown that such alterations can result in changes in the structural integrity of the casein micelle, affecting the structure of deposit formation, micelle size and voluminosity, it is likely that the different DF media could also impact protein transmission during MF/DF.

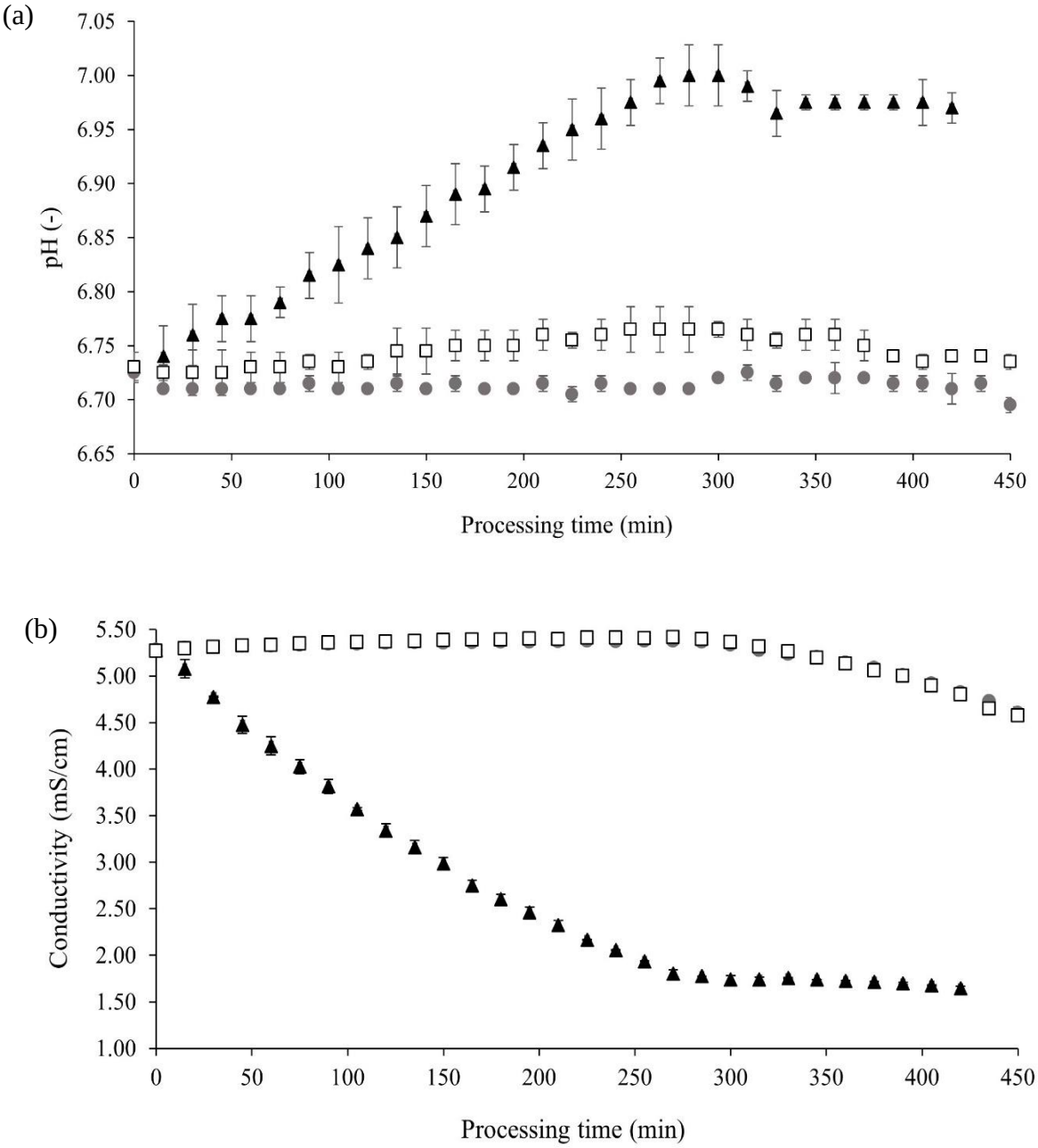


Figure 7.3: *pH (a) and conductivity (b) of retentates as a function of processing time during the microfiltration and diafiltration of skim milk at 8 °C using different diafiltration media; water (▲), SMUF 50 (●) and SMUF 10 (□).*

7.4.3 Protein transmission during filtration

During warm MF of skim milk using membranes of tight pore size (i.e., 0.1 µm), the casein micelles are retained while the serum proteins permeate the membrane into the permeate stream. Fig. 7.4 illustrates the concentration of individual proteins in the permeate stream during filtration of skim milk using different DF media and the corresponding sieving coefficient (S_o).

The concentration of β-lactoglobulin in permeates decreased during DF from 2.27 to 0.668 mg/mL for water, 2.29 to 0.83 mg/mL for SMUF 50 and 2.45 to 0.756 mg/mL for SMUF 10 (Fig. 7.4a). During the concentration step, the concentration of β-lactoglobulin in permeates remained constant with no significant differences ($p > 0.05$) in the concentrations present at the final sampling point, between the different DF media. In addition, only minor differences were observed for the S_o of the different DF media, although higher S_o were observed throughout MF/DF when using SMUF 10.

A similar trend was observed for α-lactalbumin concentration in permeates, decreasing during DF before plateauing during the concentration step (Fig. 7.4c). As expected, the S_o for α-lactalbumin decreased during filtration, with only minor differences between the different DF media. These results indicate that the different DF media studied had no significant effects on the transmission of either β-lactoglobulin or α-lactalbumin.

When MF of skim milk is performed at low temperature (i.e., 8 °C), the strength of hydrophobic interactions decreases, resulting in the release of β-casein from the micelle and into the serum phase, from where it can pass into the permeate stream (Creamer *et al*., 1977; France, Bot *et al*., 2021). Fig. 7.4e illustrates the effects of different DF media on the rate of β-casein dissociation and removal. Unlike the whey

proteins, β-lactoglobulin and α-lactalbumin, the concentration of β-casein showed only a small decrease during DF, with the concentration actually increasing during the concentration step. Again, only small differences were evident between the different DF media, although a significantly higher ($p < 0.05$) concentration of β-casein was measured in the final permeate collected when water was used as the DF media (1.63 mg/mL), compared to SMUF 10 (1.40 mg/mL) and SMUF 50 (1.38 mg/mL).

The addition of calcium to skim milk has been shown to reduce the extent of β-casein dissociation and concentration of β-casein in the resulting permeate stream (Schiffer, Scheidler, *et al*., 2021; Schiffer, Vannieuwenhuyse, *et al*., 2021). Therefore, it was our hypothesis that the addition of SMUF 10 would result in lower concentrations of β-casein throughout DF, as it had higher concentrations of calcium and ionic calcium (1.82 mM), when compared to SMUF 50 (1.18 mM) and water (0 mM). However, from the results, such reductions in β-casein concentration were not observed during the DF and concentration of skim milk using the different DF media.

Schiffer, Vannieuwenhuyse, *et al*. (2021) studied the effect of added calcium on milk protein fractionation during the MF (0.1 µm) of skim milk at low temperatures (i.e., 10, 14 and 20 °C); the authors reported that 1 mM of added calcium resulted in a small decrease in β-casein concentration in the permeate at 10 °C, while the addition of 5, 10 and 20 mM caused further large decreases in permeate β-casein concentration. It has also been reported that dilution of skim milk can facilitate further dissociation and solubilisation of β-casein (McCarthy *et al*., 2017). Thus, in the present study, the continuous removal of β-casein followed by the addition of DF media during DF is likely to have resulted in the rate of β-casein dissociation, due to dilution, outweighing the effect of added calcium when SMUF was used as the diafiltration media.

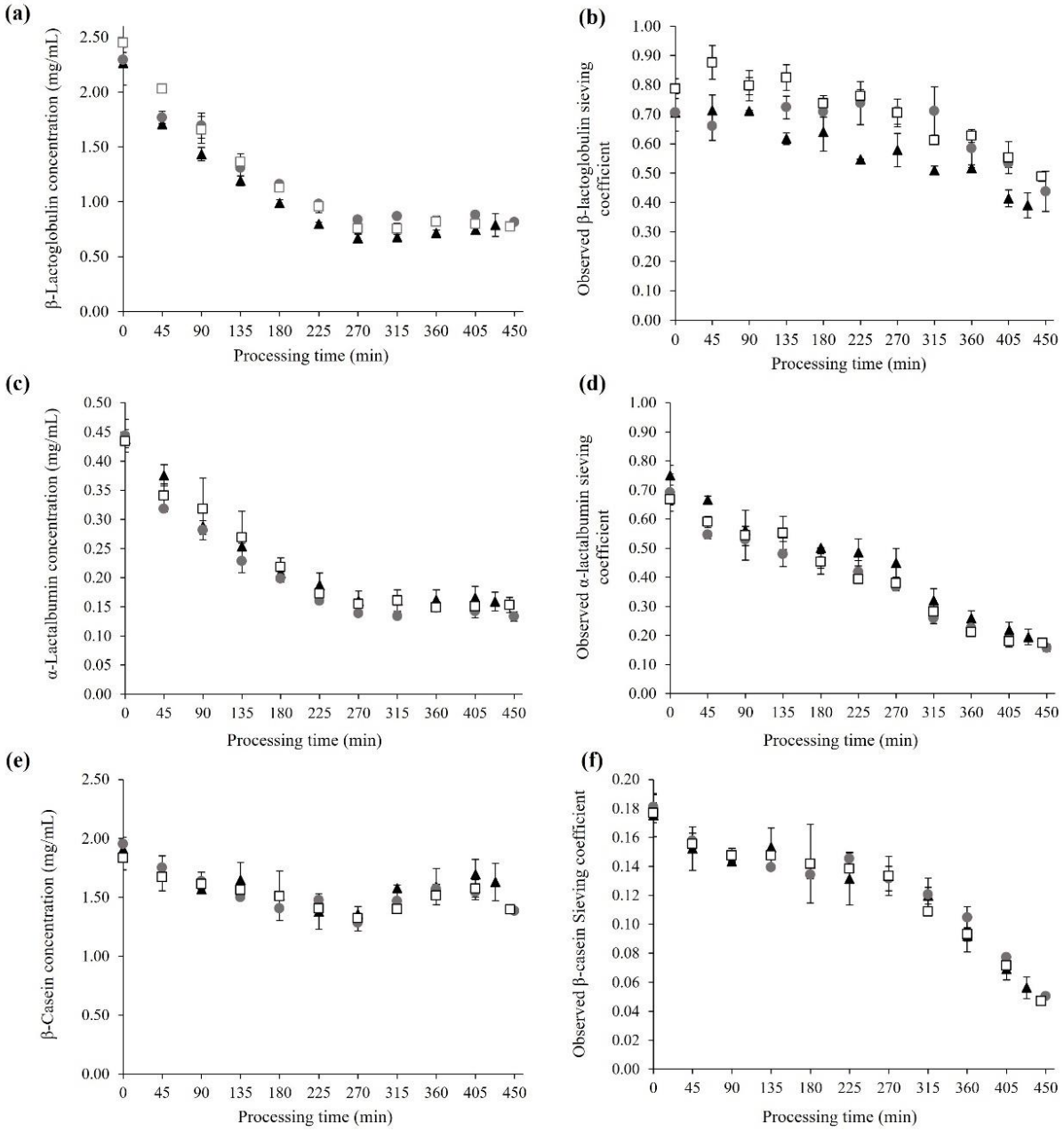


Figure 7.4: *Concentration of β-lactoglobulin (a), α-lactalbumin (c) and β-casein (e) in permeates and sieving coefficients of β-lactoglobulin (b), α-lactalbumin (d) and β-casein (f) as a function of processing time during the microfiltration and diafiltration of skim milk at 8 °C using different diafiltration media; water (▲), SMUF 50 (●) and SMUF 10 (□).*

7.4.4 Effect of diafiltration media on the composition of process streams

Changing the DF media used during the DF of skim milk can result in changes to the final composition of the resulting process streams (Table 7.1). Similar protein contents were obtained for retentates following the DF and concentration step. As expected from protein transmission results, the total protein content of permeates were similar. In both permeates, the highest protein content was measured when SMUF 50 was used as the DF media, followed by water and SMUF 10.

Although no large differences were observed for protein content, the different DF media had significant impacts on the ash content of the resulting streams (Table 7.1). DF with water resulted in a significantly lower ($p < 0.05$) ash content of the retentate (0.840%) compared to retentates generated following DF with SMUF 50 (1.059%) and SMUF 10 (1.074%). The same trend was observed for both permeates (permeate 1 and 2), with the highest concentration of ash in permeates generated when SMUF 50 was used as the DF medium, followed by SMUF 10 and water. As SMUF 50 and SMUF 10 resemble the mineral profile of milk permeate generated following UF of skim milk at 50 and 10 °C, respectively, their use as DF media maintains the mineral profile during DF of skim milk. On the other hand, when water is used as a DF medium, the minerals present in the serum phase are continuously removed and the mineral profile of the serum phase is not restored, consequently resulting in lower ash content of the final retentate.

As discussed in Section 7.4.2, the different DF media used resulted in large differences in pH throughout filtration. Significantly higher ($p > 0.05$) pH was measured for retentate generated using water as the DF media (6.91), followed by SMUF 10 (6.73) and SMUF 50 (6.70). The same trend was also observed in the two permeates generated (permeate 1 and 2). Therefore, altering the DF media has

consequences for the ash content of retentates which is likely to alter its functional properties.

Table 7.1: *Protein content, ash content and pH of process streams generated during the microfiltration and diafiltration of skim milk at 8 °C using different diafiltration media.*

Analysis	Sample	Diafiltration media		
		Water	50 SMUF	10 SMUF
Protein content (%w/w)	Retentate	8.490 ± 0.202^{a}	8.287 ± 0.196^{a}	8.707 ± 0.071^{b}
	Non-casein nitrogen	0.313 ± 0.014^{ab}	0.304 ± 0.013^{a}	0.330 ± 0.011^{b}
	% Casein	96.32 ± 0.09	96.33 ± 0.08	96.21 ± 0.04
	Permeate 1	0.435 ± 0.009^{a}	0.436 ± 0.009^{a}	0.431 ± 0.007^{a}
	Permeate 2	0.275 ± 0.007^{ab}	0.280 ± 0.007^{b}	0.268 ± 0.001^{a}
Ash (%w/w)	Retentate	0.840 ± 0.024^{a}	1.059 ± 0.060^{b}	1.074 ± 0.044^{b}
	Permeate 1	0.218 ± 0.005^{a}	0.432 ± 0.057^{b}	0.400 ± 0.006^{b}
	Permeate 2	0.094 ± 0.012^{a}	0.405 ± 0.052^{b}	0.373 ± 0.011^{b}
pH (-)	Retentate	6.91 ± 0.01^{c}	6.70 ± 0.01^{a}	6.73 ± 0.00^{b}
	Permeate 1	6.85 ± 0.01^{c}	6.74 ± 0.01^{a}	6.77 ± 0.01^{b}
	Permeate 2	7.01 ± 0.01^{c}	6.74 ± 0.01^{a}	6.79 ± 0.02^{b}

Values followed by different superscript letters within a row are significantly different ($p < 0.05$).

7.4.5 Changes in heat stability as a function of pH

In order to assess the effects different DF media have on the heat stability of the final retentate generated, the heat coagulation time (HCT) of the retentates was determined at different pH values (Fig. 7.5). Using water as a DF medium resulted in retentates with significantly lower ($p < 0.05$) HCTs, compared to when SMUF 50 and SMUF 10 were used, across each pH studied. Retentates generated using SMUF 50 and SMUF 10 displayed very high heat stability, with retentates generated using SMUF 50 displaying the highest stability at each pH studied. The HCT of retentates generated using water were strongly dependent on pH, varying from 1.50 to 59.4 min over the pH range (pH 6.5-7.2). A study by Renhe *et al*. (2019) compared the heat stability of protein concentrates generated from the ultrafiltration (UF; 10 kDa), MF (800 kDa) and DF of skim milk, using both water and UF permeate as DF media. The

authors reported that retentate generated from the MF and DF of skim milk, using water, had significantly longer HCT compared to retentate generated using UF permeate as the DF media. Their results are in contrast to the results found in the present study. The retentates obtained by Renhe *et al*. (2019) following MF and DF were not standardised for pH prior to determining the HCT, with retentates generated using water as the DF media having a pH of 6.9, while retentates generated using UF permeate had a pH of 6.7. In addition to this, their MF/DF retentates contained very high levels of whey proteins (e.g., 86.6% whey protein retained in retentates after MF and subsequent DF with water). It has been shown that increasing pH of casein concentrates, depleted of whey proteins, results in an increase in heat stability (De Kort *et al*., 2012; France *et al*., 2022; Chapter 6). In the present study, the retentates were extensively depleted of whey proteins with all retentates containing 96% casein (Table 7.1).

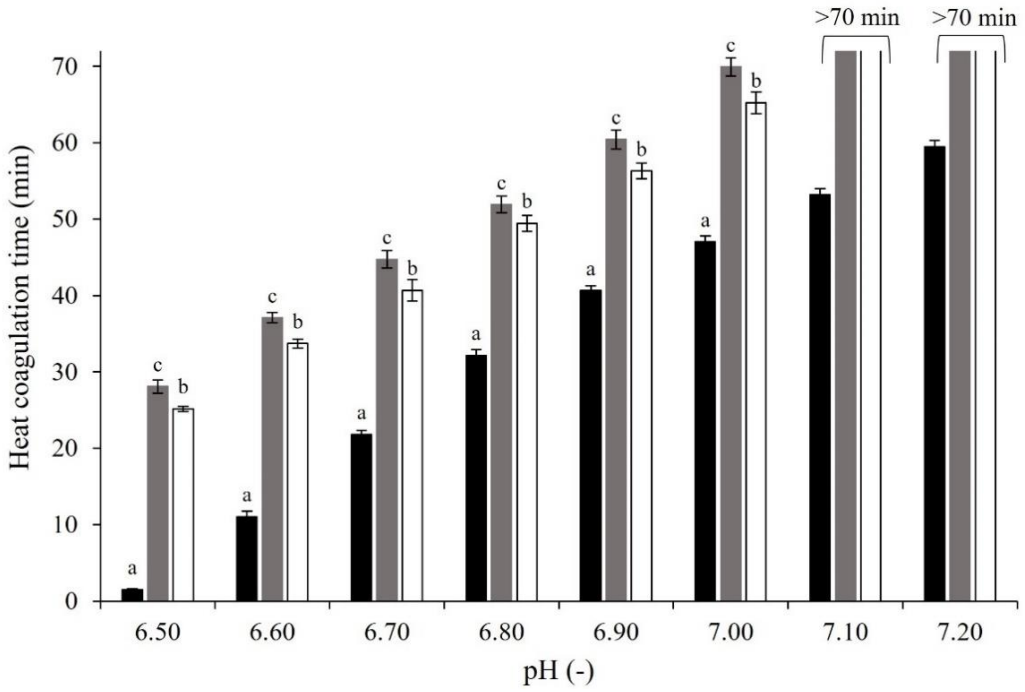


Figure 7.5: *Heat coagulation time of retentates generated following the microfiltration and diafiltration of skim milk at 8 °C using different diafiltration media; water (■), SMUF 50 (■) and SMUF 10 (□).* ^{a,b,c} *Mean values with different superscript letters within each pH are significantly different ($p < 0.05$).*

Zeta (ζ)-potential was measured to provide an indication on the effect of both pH and DF media on the colloidal stability of retentates (Fig. 7.6). ζ-potential ranged from -22 to -29 mV for all samples across all pH values measured. Although no statistical significance ($p > 0.05$) was detected between the retentates at each pH, the results show the increase in ζ-potential with increasing pH, contributing to the improved heat stability observed for all retentates at higher pH.

The particle size of retentates following heating at 120 °C for 10 min, at different pH values, provided an insight into the stability of the casein micelles when subjected to sterilization-type heat treatment (Fig. 7.7). All the retentates showed similar particle size from pH 6.8 to 7.2, with only minor differences. However, the particle size of retentates generated using water as the DF media showed large increases in size, as the pH was reduced below pH 6.8. The particle size of retentates generated using water as the DF media at pH 6.7 and 6.6 was 210 and 380 nm, respectively, with no particle size determined for pH 6.5 as it coagulated within 2 min. Retentates generated using SMUF 50 and SMUF 10 as the DF media showed significantly smaller ($p < 0.05$) particle sizes across this pH range compared to when water was used as the DF media.

These results, together with the HCTs, indicate that using a model UF permeate without lactose (i.e., SMUF) as the DF media, during the MF of skim milk, improves the thermal stability of retentates over a wide pH range, comparted to when water is used. Furthermore, as there were no significant differences in ζ-potential between the retentates, it is likely that the differences in mineral equilibrium and profile present in the retentates, as a result of the different DF media, may have contributed to the observed differences in HCTs.

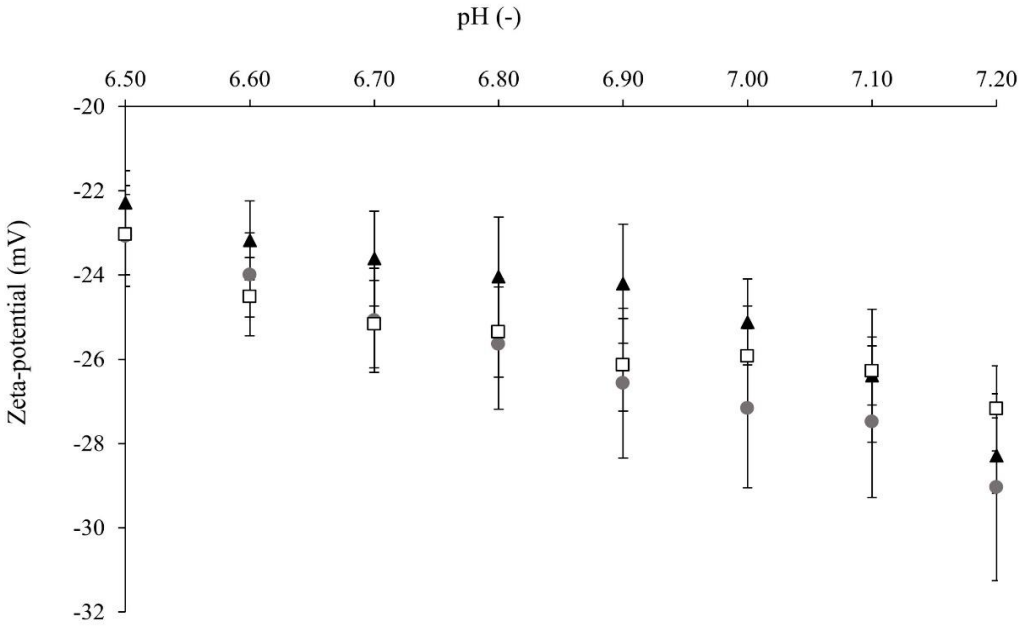


Figure 7.6: *Zeta-potential of retentates as a function of pH generated following the microfiltration and diafiltration of skim milk at 8 °C using different diafiltration media; water (▲), SMUF 50 (●) and SMUF 10 (□).*

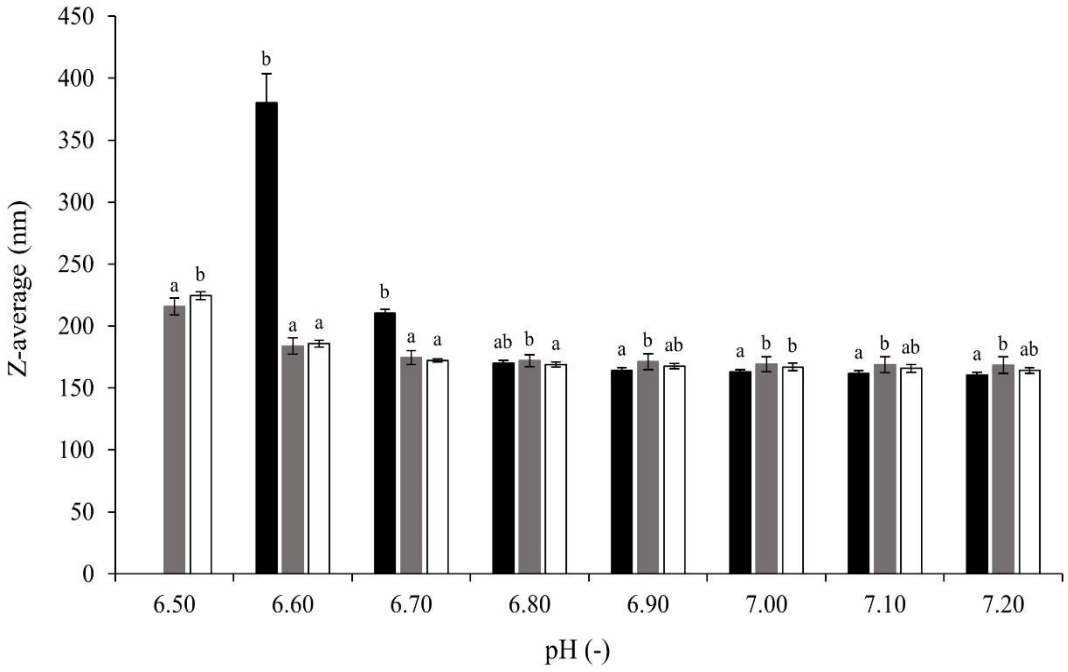


Figure 7.7: *Z-average of particles in retentates, heated to 120 °C for 10 min at different pH values, generated following the microfiltration and diafiltration of skim milk at 8 °C using different diafiltration media; water (■), SMUF 50 (■) and SMUF 10 (□).* ^{a,b} *Mean values with different superscript letters within each pH are significantly different ($p < 0.05$). No size was determined for retentate generated using water as the DF media at pH 6.5 due to coagulation within the 10 min.*

7.4.6 Effect of diafiltration media on ionic calcium content of retentates

To provide further understanding into the differences observed in heat stability, the ionic calcium content of the retentates was measured across the same pH range (Fig 7.8). Significantly higher ($p < 0.05$) ionic calcium was measured for retentates generated using water as the DF media, compared to those in which SMUF 50 and SMUF 10 were used, at each pH. The ionic calcium concentrations at pH 6.5 was 4.03, 2.45 and 2.54 mM for retentates generated using water, SMUF 50 and SMUF 10, respectively, as the DF medium. Increasing the pH to 7.2 resulted in a decrease in the concentration of ionic calcium, with values of 1.27, 0.82 and 0.82 mM, for retentates generated using water, SMUF 50 and SMUF 10 as the DF medium, respectively. Ionic calcium is strongly dependent on pH, with the solubility of CCP increasing as pH decreases, resulting in an increase in ionic calcium (Lewis, 2011).

In milk, calcium phosphate exists in an equilibrium between the colloidal (micellar) and serum phases, with the serum phase containing a large proportion of the minerals (e.g., one-third of calcium and half the inorganic phosphate are present in the aqueous phase of milk; Gaucheron, 2005; Holt, 1985). Calcium in the aqueous phase is present as ionic calcium and as stable complexes with citrate, inorganic phosphate or chloride (Gaucheron, 2005). The use of water as a DF medium during the MF of skim milk results in the continuous removal of minerals. On the other hand, when SMUF 50 and SMUF 10 are used, the minerals lost in the permeate stream are largely restored. As the minerals (e.g., calcium, phosphate, citrate) present in the aqueous phase of retentates generated using water have been removed, the equilibrium is altered, resulting in a shift from the colloidal phase to the aqueous phase. As a result, it is likely that some CCP is solubilised to compensate for the reduction in serum calcium and phosphate. However, now there are no anions (i.e., citrate) present to bind

the calcium and therefore it can be assumed most of the calcium in the serum phase is present as free ions (i.e., ionic calcium). Thus, this is likely the reason for the significantly higher ionic calcium measured in retentate samples generated using water as the DF medium.

The results of ionic calcium concentration reflect the HCTs reported in Section 7.4.5. The higher the ionic calcium concentration in retentates, the shorter the HCT. As the protein profile of retentates was comparable, and no significant differences ($p > 0.05$) in ζ-potential were detected, it can be suggested that the observed differences in heat stability are mainly as a result of differences in mineral profile. France *et al*. (2022; Chapter 6) examined the protein profile and physico-chemical properties of micellar casein retentates produced from the MF and DF of skim milk at 4 and 50 °C, using water as the DF medium; similar findings were found to those reported in the present study, with the HCT of retentates increasing rapidly with increasing pH, which coincided with a decrease in ionic calcium.

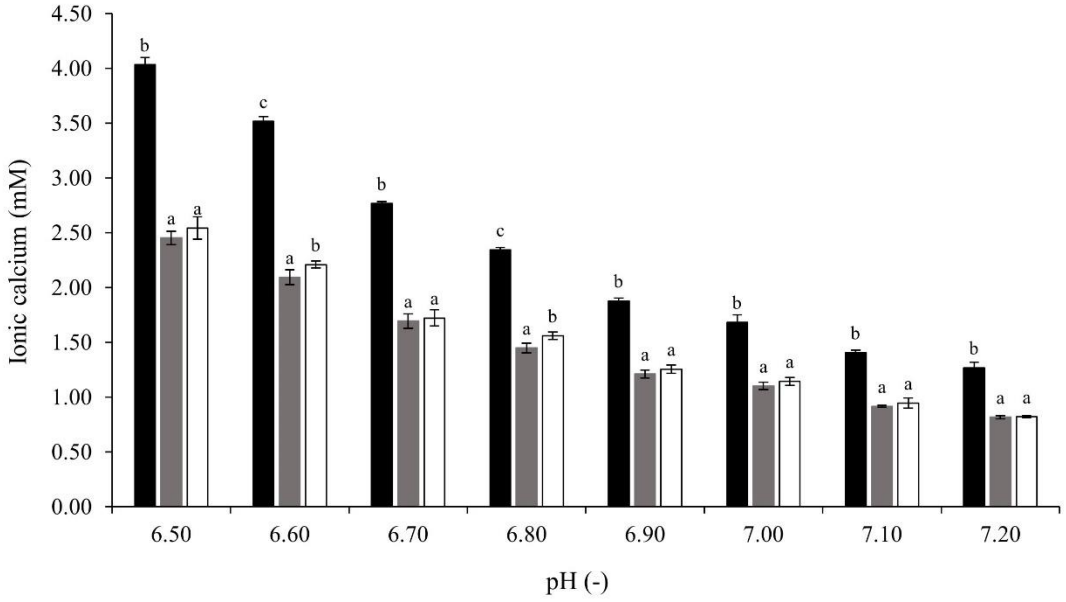


Figure 7.8: *Ionic calcium content of retentates generated following the microfiltration and diafiltration of skim milk at 8 °C using different diafiltration media; water (■), SMUF 50 (■) and SMUF 10 (□).* ^{a,b,c} *Mean values with different superscript letters within each pH are significantly different ($p < 0.05$).*

7.5 Conclusion

The findings presented in this study indicate that the use of simulated milk ultrafiltrates, designed to resemble the mineral profile of UF permeate generated at 10 and 50 °C, result in similar processing performance, protein transmission and retentate properties. Longer heat coagulation times were measured for retentates generated using simulated milk ultrafiltrate 10 and 50 as the DF medium which corresponded to significantly lower concentrations of ionic calcium, compared to when water was used. During cold microfiltration of skim milk, extensive β-casein depletion can still be obtained when simulated milk ultrafiltrate is used with the extent of removal of β-casein unchanged. Importantly though, a β-casein-depleted micellar casein co-product with high thermal stability across a broad pH range is also produced when simulated milk ultrafiltrate is used as the diafiltration medium. Furthermore, if lactose-free UF permeate is used instead of water, as a diafiltration medium, a retentate with improved heat stability with a similar protein profile can be achieved.

7.6 Acknowledgements

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Chapter 8

General discussion and suggestions for future research

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Declaration

This chapter was written by author Thomas C. France.

General discussion

Microfiltration (MF) in the dairy industry is traditionally performed at warm temperatures (i.e., 45-55 °C), with associated advantages of high initial permeate flux and retention of the entire casein fraction, resulting in a whey protein stream of high purity (France *et al*., 2021; **Chapter 1**). However, with increasing focus on sustainable processing and new ingredient innovation, as discussed in **Chapter 1** (cold MF review), the use of lower temperatures (i.e., ≤20 °C) has gained interest among dairy processors and researchers. Reducing the temperature at which MF is performed offers the opportunity to improve processing efficiency through more sustainable permeate flux, consequently resulting in increased processing times (McCarthy *et al*., 2017; Schiffer & Kulozik, 2020). This was observed in **Chapter 4** and **Chapter 6,** with lower processing temperatures resulting in a lower rate of flux decline during MF of skim milk. The ability to extend processing times provides the opportunity to reduce the number of cleaning cycles, reducing the consumption of cleaning chemicals and water, as well as reducing the loss of product remaining in the filtration system prior to cleaning.

Cold MF also offers the ability to generate β-casein-enriched whey and isolate β-casein of high purity at industrial scale (O'Mahony *et al*., 2014; Schäfer *et al*., 2019). However, bovine milk is a complex system with many of the components in an equilibrium between the colloidal (micellar) and serum phases; changing processing temperature during MF can alter this equilibrium, ultimately influencing protein (i.e., β-casein), mineral (i.e., calcium) and enzyme (i.e., plasmin) partitioning. The extent to which different temperatures within the cold MF range affect the partitioning of these milk constituents has not been studied in detail in the published scientific

literature; many of the published studies have compared the differences between warm (i.e., 50°C) and a single temperature within the cold MF range (e.g., 10 °C) (Steinhauer *et al*., 2015; Hartinger *et al*., 2019). It was therefore important to investigate the changes in the partitioning of milk constituents and membrane performance (i.e., permeate flux and fouling) as influenced by processing temperature. In addition, the influence of changing composition (i.e., mineral and protein profile) on the techno-functionality of the final ingredients was important to determine. The objectives of the research in this thesis were thus to provide new information on the effects of different processing temperatures, within the cold MF range, on partitioning of milk constituents and membrane performance during the MF of skim milk and to determine the effects of compositional changes on the techno-functionality of micellar casein ingredients. In particular, the focus was on high purity micellar casein concentrate/isolate (MCC/MCI) as these have received little attention to date. Some of the key findings from the work completed as part of this thesis may be integrated as follows.

In the first part of this thesis (**Chapters 3, 4 and 5**), the effects of selected temperatures, within the cold MF range, on membrane fouling and protein, calcium, and plasmin partitioning were investigated. Performing MF in total recirculation mode allowed for a range of different temperatures (4, 8, 12, 16 and 20 °C) and transmembrane pressures (sub-critical and limiting flux) to be examined. Lowering processing temperature, in the range from 20 to 4 °C, resulted in increased concentrations of β-casein and both total and ionic calcium in the permeate streams. Narrowing the selected temperatures (4, 8 and 12 °C) and operating MF under concentration, instead of total recirculation, provided additional new information on the effect of temperature on flux, membrane fouling and the rate of protein partitioning

during MF. Performing MF at 4 °C resulted in the lowest initial permeate flux, due to the associated high viscosity, but a slower rate of flux decline during MF. The rate of decrease in β-casein concentration from the start of MF, to the end, was lowest when MF was performed at 4°C, which can be attributed to a low extent of fouling. Additionally, the mechanism responsible for β-casein dissociation could contribute to the lower rate of decrease, as the rate of dissociation of β-casein into the serum phase at 4 °C is higher than at 8 and 12 °C, due to weaker hydrophobic interactions.

The casein-dominant retentate and β-casein-enriched whey streams obtained by cold MF at different temperatures showed extensive plasmin-mediated hydrolysis on storage, with the hydrolysis of β-casein in the permeates resulting in the formation of γ-caseins and proteose peptones. Interestingly, these plasmin-mediated hydrolysis products also displayed the ability to self-associate on heating. The learnings generated in **Chapters 3, 4 and 5** provided information on how temperature impacted the partitioning and the composition of the resulting permeate streams and highlighted the need to further investigate the thermal stability and rheological properties of the resulting β-casein-depleted co-product, as described in **Chapters 6 and 7**.

In **Chapter 6**, a β-casein-depleted micellar casein isolate ingredient and a micellar casein ingredient containing the innate protein profile (i.e., casein profile unchanged from that of skim milk) was successfully made using cold (i.e., 4 °C) and warm (i.e., 50°C) MF, respectively. Extensive diafiltration (DF) was performed to facilitate and maximise the dissociation and removal of β-casein during cold MF/DF. Both micellar casein ingredients displayed similar rheological properties; however, the β-casein-depleted micellar casein ingredient displayed superior heat stability at 120 °C across a broad pH range compared to that generated at 50 °C. The learnings

obtained in **Chapters 3 and 4** indicated that performing MF at low processing temperatures increases the solubility of colloidal calcium phosphate, with the solubilised minerals ultimately passing through the membrane, resulting in increased total and ionic calcium concentrations in the permeate stream. This was observed in **Chapter 6**, with each DF step removing more calcium when MF was performed at 4 °C, compared to 50°C. The higher heat stability of the β-casein-depleted micellar casein ingredient was attributed to the removal of more calcium (serum and colloidal) when DF was performed at low temperature.

In **Chapter 7**, the learnings from **Chapter 6** were used to design an experiment to understand how altering the DF media used can impact protein partitioning and how the heat stability of the ingredient can be influenced. Three different DF media were used: ultrapure water and simulated milk ultrafiltrate (SMUF) resembling the mineral profile of ultrafiltration (UF) permeate produced from skim milk at 10 or 50 °C. No differences were observed between the different DF media studied on protein partitioning during DF and concentration. However, when both SMUFs were used, a micellar casein ingredient with a higher heat stability was obtained compared to when water was used as the DF medium. The data demonstrated that the use of SMUF resulted in similar protein partitioning and permeate flux to when water was used as the DF medium, while a final micellar casein ingredient with high thermal stability across a wide pH range can be obtained.

In summary, the studies reported in this thesis have generated new insights into how processing temperature can influence the partitioning of different milk constituents, specifically β-casein, calcium and plasmin. Understanding the effects of processing temperature on membrane performance and the extent and type of

membrane fouling is important in order to predict the efficiency of such processes. These findings provide greater understanding of the key considerations that must be made by dairy scientists and engineers when selecting the most suitable processing temperatures to tailor ingredient composition and techno-functionality, while also enabling sustainable dairy ingredient innovation.

Suggestions for future research

Follow-up studies that would be complementary to the work presented in this thesis include:

Mineral loading/ hydrophobic binding of β-casein-depleted micellar casein retentate

During the generation of β-casein-enriched whey, a β-casein-depleted co-product is produced; however, the mineral profile, especially in terms of calcium, of the β-casein-depleted retentate is altered. In **Chapter 6** it was found that the reduction of calcium improved heat stability of β-casein-depleted retentate compared to control retentate (no β-casein depletion). A gap remains in understanding how the heat stability of the β-casein-depleted MCI is affected if the calcium content is standardised after filtration, which would clarify whether β-casein-depletion alone affects heat stability. In addition, the calcium-binding potential of the β-casein-depleted casein ingredient is an attribute that requires investigating as it could be exploited by dairy scientists.

In addition to calcium loading, the hydrophobic binding capacity of this β-casein-depleted co-product warrants exploration. As up to 40.3% of the hydrophobic β-casein is removed from the interior of the casein micelle, there is the potential for

the binding of alternative hydrophobic compounds, such as polyphenols. Recent research has shown that polyphenols can potentially improve the structural stabilisation of casein micelles, possibly by binding to the hydrophobic patches and making them less prone to disintegration (van de Langerijt *et al*., 2022). Therefore, β-casein-depleted MCC/MCI could provide an ideal ingredient for polyphenol enrichment of milk-protein beverages, although research is required to fully understand the implications of adding polyphenols on the stability of the β-casein-depleted micelles.

Influence of processing parameters on the composition of milk permeate used for diafiltration

Given that milk permeate generated from the ultrafiltration (UF) of skim milk is sometimes used as a DF media during the production of micellar casein concentrate, changes in its composition can alter properties of the final ingredient (**Chapter 7**). Studies have shown that milk permeate generated at cold (i.e., ≤20 °C) and warm (i.e., 45-55 °C) temperatures contain different mineral profiles and lactose concentrations compared to those generated at warm temperatures, with the pH of skim milk also contributing to such differences (Dumpler *et al*., 2017; St-Gelais *et al*., 1992). However, there is limited knowledge on how differences in transmembrane pressure alter the composition of the UF milk permeate. Therefore, it is worth investigating how altering the transmembrane pressure, such that UF is performed at sub-critical, critical or limiting flux conditions, alters the mineral and lactose concentrations of the milk permeate produced.

Use of alternative DF media

In **Chapter 7**, DF of skim milk was performed with deionised water and two different simulated milk ultrafiltrates (SMUF) resembling the mineral profile of UF permeate produced at 10 or 50 °C. The use of both SMUFs resulted in micellar casein retentates with improved heat stability compared to when water was used as the DF medium. Thus, there remains the scope to further explore the potential use of simple salt solutions that could be used easily, to minimise the effects of using water on the mineral composition, and therefore improve the functional properties of the final ingredient. In addition, there is the opportunity to further utilise side streams currently available in dairy processing plants (e.g., delactosed permeate; DLP, lactose derived MF permeate of UF whey permeate, reverse osmosis (RO) permeate). These streams provide an opportunity to improve the circular economy and sustainability considerations of the process. These streams could be used successfully as alternative DF media; however, research into their effects on the properties of the resultant retentates is required. A recent study by Reitmaier and Kulozik (2022) examined the composition of dairy side streams, assessing their applicability as diafiltration media; 89 industrial samples from 38 different processes were analysed, including UF, NF and RO permeate from different sources, vapour condensate and thermo-curd whey. Their results indicated that there was greater than expected compositional variability for dairy side streams, although all were generally acceptable for use as DF media. However, there requires the need to further explore how these different dairy side streams impact the functionality of the final dairy ingredient and the potential microbiological risks of using such streams.

The effects of different DF media on the downstream processability of permeate

In **Chapter 7**, the focus was on the effect of different DF media on membrane performance, protein partitioning and the thermal stability of the resultant retentates. However, it must also be noted that, by changing the DF media, the processability of the permeate is also likely to be affected. It has been shown that high levels of calcium, specifically ionic calcium, can facilitate protein-protein interactions, resulting in fouling, as well as directly contributing to fouling in the form of calcium phosphate particles (Visser and Jeurnink, 1997). Therefore, it is likely that the use of DF media containing high concentrations of minerals (e.g., SMUF) could result in permeates requiring an additional demineralisation step in order to improve its processability (i.e., improve thermal stability).

Rehydration characteristics of micellar casein concentrate/isolate powders generated using different diafiltration media.

In **Chapter 7** it was demonstrated that changing DF media impacted the heat stability of high protein casein-dominant retentates. There remains scope to examine the physicochemical properties of these ingredients in powder physical form as well to understand how the different retentates behave on drying. Crowley *et al*. (2018) examined the rehydration behaviour of micellar casein concentrates produced following MF at warm (50 °C) and cold (< 10 °C) temperatures; they reported pronounced differences in dispersion characteristics, resulting in cold produced micellar casein concentrate displaying reduced sedimentation during accelerated physical stability testing. Therefore, it is likely the effects of different DF media on the mineral profile of the resulting retentates will cause differences in rehydration characteristics of the micellar casein ingredient.

Sensory analysis of MCI powders generated from warm and cold microfiltration

Studies have shown that depletion of β-casein from casein micelles *via* cold MF affects rennet coagulation properties (e.g., curd forming rate and curd strength) and certain physicochemical properties such as heat stability and rheology (**Chapter 6**) (O'Mahony *et al*., 2008, 2009; Seibel *et al*., 2015). However, there remains the question as to whether the powders generated from cold (β-casein-depleted MCC/MCI) or warm microfiltration (MCC/MCI; native casein profile) differ in terms of their sensory properties. One potential application of these ingredients could be in the formulation of high-protein clinical beverages. Therefore, the depletion of β-casein may result in reduced bitterness, especially if hydrolysed, or flavour profiles different to micellar casein ingredients containing the innate protein profile. The combination of sensory analysis and mass spectrometry to identify peptides present would provide an in-depth understanding of how cold and warm generated MCC/MCI differ from a sensory perspective or formulations made using such ingredients.

Digestion properties

The simulated gastrointestinal digestion properties of β-casein-depleted retentate and the control micellar casein retentate (native casein profile) generated at 4 and 50 °C, respectively, in **Chapter 6** remains an area for further investigation. Recent work from colleagues evaluated the rennet and acid-induced coagulation properties of the ingredients generated in **Chapter 6** (Li *et al*., 2022). Longer rennet coagulation times were found for the β-casein-depleted retentate compared to the control retentate. For acid coagulation, both retentates showed gelation pH values of >5. Therefore, investigating the curd-forming properties of these ingredients in the gastric phase would provide valuable information into the properties of the curd

formed and the extent to which the curd breaks down. In addition to this, differences in curd strength and structure could result in differences in satiety.

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Appendix 1

Physical and colloidal stability of regular and micronised calcium citrate ingredients powders in the formulation of infant nutritional products

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Declaration

This chapter was written by author Thomas C. France (TCF) and reviewed by all co-authors. TCF and Giovanni Barone co-designed the study and performed all of the experimental work.

1.1 Abstract

The fortification of food systems with calcium remains difficult; one challenge is to maintain the colloidal stability of insoluble calcium salts during processing and shelf life. Particle size reduction of insoluble salts may result in improved product stability. In this study, insoluble calcium citrate in two different particle sizes, conventional calcium citrate and micronised calcium citrate, were first evaluated in terms of physical and bulk handling properties, followed by protein adsorption and colloidal stability when dispersed in two dairy-based nutritional beverages differing in composition, infant milk formula stage 1 and 3. Particle size distribution analysis showed micronised calcium citrate (volume-weighted diameter = 5.10 μm) to have significantly smaller ($p < 0.05$) particle size than conventional calcium citrate (volume-weighted diameter = 88.2 μm). The adsorption of dairy proteins onto particles of calcium citrate resulted in caseins having greater affinity for both salts, followed by β-lactoglobulin. The smaller particle size of the micronised citrate resulted in higher affinities for casein and greater colloidal stability when dispersed in both infant milk formula solutions compared to conventional. The results of this study provide knowledge on the application of micronised insoluble calcium salts in the fortification of nutritional dairy-based products.

1.2 Introduction

The fortification of food products with micronutrients, including calcium, is widely practised within the food industry (WHO, 2004). The main dietary sources of calcium vary depending on the individual and between countries, although dairy products are generally the most important sources of dietary calcium (Fleming and Heimback, 1994; Alexy and Kersting, 2003; EFSA, 2015; Kumssa *et al*., 2015). Despite this, a large proportion of the global population does not reach the recommended daily intake (RDI) of calcium (Balk *et al*., 2017), with potential consequences for human health such as osteoporosis (Cashman, 2006a, 2006b; Heaney, 2013). Therefore, there is increasing research in how to increase levels of calcium fortification in formulated dairy-based beverages.

The fortification of infant formula with calcium is vital, as calcium is required for normal growth and development of infants (O'Callaghan *et al*., 2011). However, necessary technological and formulation considerations must be made when fortifying with insoluble or soluble salts, or a combination of both. Soluble calcium salts used in the fortification of infant formula include calcium lactate, chloride and gluconate, while calcium carbonate, phosphate and citrate are the most widely used insoluble calcium salts (Gerstner, 2003; Chmura *et al*., 2008; Guo and Ahmad, 2014). Technological and nutritional considerations when fortifying protein-based beverages with soluble calcium salts include the adsorption and bioavailability of calcium, effects on the heat stability and pH of the system during processing as well as the potency of the calcium salt (Vyas and Tong, 2004; Crowley *et al*., 2014a); for insoluble salts, organoleptic defects such as chalkiness and sedimentation are often the

limiting factors when selecting the appropriate salt type and addition level for fortification (Gerstner, 2005; Singh *et al*., 2007).

Increasing the level of fortification of calcium using insoluble calcium salts without incurring sedimentation issues is challenging in fortified milks and infant formula (Vyas and Tong, 2004; Crowley *et al*., 2014a; Tercinier *et al*., 2017). Insoluble, or sparingly soluble, salts sediment out of solution over time, leading to phase separation which results in losses in both nutritional and organoleptic quality/acceptance (perceptions of grittiness) (Singh *et al*., 2007). Applying the principles of Stoke's law, there are three main approaches to reduce the rate of separation of two or more immiscible phases, two of which apply to reducing the sedimentation rate of insoluble calcium salts (McClements, 1998). Firstly, the viscosity of the continuous phase (i.e., the phase in which the insoluble salts are dispersed) can be increased to reduce the rate of sedimentation, maintaining a stable suspension. Therefore, hydrocolloids are often added to improve the suspension stability of insoluble salts in the dispersant phase due to high intrinsic viscosity and their relatively low cost (Nelson *et al*., 1987; Gerstner, 2005). In some types of formulated nutritional products (e.g., infant formula or growing up milks), however, this is not possible due to regulatory controls, the desire for clean label requirements (EU, 2016) and the negative perceptions of consumers (Hollowood *et al*., 2002; Varela and Fiszman, 2013). The second approach involves reducing the particle size of the dispersed phase (i.e., micronisation) (Baláž and Dutková, 2009) to reduce the sedimentation rate, therefore maintaining the calcium salt in suspension for extended periods of time without sedimentation. Micronisation of insoluble calcium salts can overcome these challenges experienced with colloidal stability during fortification by reducing the particle size of the salts; this can be achieved by applying techniques such

as dry milling followed by selective sieving, or by the rapid expansion of supercritical fluids (Knights and Kjelsberg, 1998; Gerstner, 2003; Rasenack and Müller, 2004; Vemula *et al*., 2010; Esfandiari and Ghoreishi, 2015).

The different particle size distribution (PSD) of insoluble calcium salts may also influence the adsorption of proteins onto the surface of the suspended calcium salt particles, potentially improving colloidal stability of such calcium-protein systems. A number of studies have been conducted on the ability of milk proteins to adsorb onto the surfaces of insoluble calcium salt particles (mainly hydroxyapatite) when in simple protein systems (Knights and Kjelsberg, 1998; Iafisco *et al*., 2011; Wen *et al*., 2016; Tercinier *et al*., 2017), thereby enhancing the colloidal stability of calcium particles.

The impact of micronisation on the bulk handling properties or stabilisation and suspension stability of added calcium citrate in complex heterogenous systems, such as milk-based infant formula, remains an area that requires additional research. Therefore, these properties of calcium citrate powder ingredients with two different PSDs were examined, followed by the suspension of the calcium salts in two infant formulas: a whey-dominant stage 1 (IMF-S1), and a casein-dominant stage 3 (IMF-S3), for protein adsorption and colloidal stability analysis. The aim of this study was to provide knowledge on the physical and morphological characteristics of calcium citrate in two forms, conventional and micronised, and determine the potential benefits of micronisation for the colloidal stability of calcium citrate in the calcium fortification of infant nutritional products.

1.3 Materials and methods

1.3.1 Materials, composition and reconstitution conditions

Tri-calcium citrate 4-hydrate conventional (CCit-C) and tri-calcium citrate 4-hydrate micronised (CCit-M), produced by milling, followed by sieve classification, were sourced from Kirsch Pharma GmbH (Salzgitter, Germany). Two infant milk-based formulas (IMF), stage 1 (IMF-S1) and stage 3 (IMF-S3), were donated by Nestlé Ireland (Askeaton, Co. Limerick, Ireland). The protein content of IMF-S1 and IMF-S3, determined by the Kjeldahl method using a nitrogen to protein conversion factor of 6.38 (IDF, 2001), was 10.0 ± 0.11% and 13.3 ± 0.28%, respectively. As stated by the manufacturer, whey and casein contents were 6.55 and 3.52 g/100 g for IMF-S1, respectively, and 5.43 and 8.15 g/100 g for IMF-S3, respectively. The fat, total carbohydrate and ash contents for IMF-S1 were 27.1, 56.4 and 3.96 g/100 g and 14.5, 64.5 and 4.50 g/100 g for IMF-S3, respectively. The manufactures recommended reconstitution rate for IMF-S1 and IMF-S3 was 133 g/L and 173 g/L, respectively. IMF was reconstituted at 70°C using ultra-pure water according to guidelines set out by the WHO (2007) for infant formula rehydration.

1.3.2 Physical properties of calcium citrate powder ingredients

Moisture content was determined by oven drying using a Memmert universal oven UN160 (Memmert GmbH and Co.KG, Schwabach, Germany) in accordance with ISO (2004). Water activity (a_w) was determined at 20°C using a HygroLab C1 bench-top indicator (Rotronic AG, Bassersdorf, Switzerland), equilibrating samples with the probe for 20 min prior to measurements. Specific surface area (SSA) was determined using a Micromeritics Gemini 2375 (Micromeritics, Norcross, GA) volumetric analyser as described by Raynaud *et al*. (2002) and Crowley *et al*. (2014b),

with minor modifications. Degassing was performed at 60°C overnight with high purity nitrogen before each analysis. Values of SSA were calculated from sorption isotherms using the Brunauer-Emmett-Teller (BET) equation, as described by Brunauer *et al*. (1938). The particle size distribution of CCit-C and CCit-M was analysed by laser diffraction using a Malvern Mastersizer 3000 with Aero S dry dispersion unit (Malvern Instruments Ltd, Worcestershire, UK) as described by Crowley *et al*. (2014b) and Amagliani *et al*. (2016) using particle refractive and absorption indices of 1.627 and 0.1, respectively. An AccuPyc II 1340 gas pycnometer (Micromeritics, Norcross, Georgia, USA) was used to determine the absolute density of the powders using helium with 99.9% purity supplied at a pressure of 21-25 psig.

1.3.3 Bulk handling properties

Flowability, compressibility index (CI), bulk density (ρ_b) and wall friction angle (φ_w) of the powders were analysed using a Brookfield Powder Flow Tester (PFT; Brookfield Engineering Laboratories, Inc., Middleboro, Massachusetts, USA) as reported by Crowley *et al*. (2014b). Flowability was assessed based on Jenike's classification (Jenike, 1964). Prior to analysis, both CCit-C and CCit-M were sieved using a 710 μm sieve to remove any over-sized material arising from spontaneous clumping.

1.3.4 Scanning electron microscopy

Powder microstructure and particle morphology were analysed using a JSM-5510 scanning electron microscope (Jeol Ltd., Tokyo, Japan), operated at a voltage of 5 kV. The powders were mounted on aluminium stubs using double-sided adhesive carbon tape. A Quorum Q150R ES sputter coating unit (Quorum Technologies Ltd.,

Sussex, UK) was used to sputter-coat the powders with a 5 nm layer of gold/palladium (Au:Pd = 80:20). Images were captured at a magnification of 1000x.

1.3.5 Protein adsorption onto calcium citrate powder particles

Stock solutions of IMF-S1 and IMF-S3 were rehydrated in ultra-pure water at 3.10% (w/w) protein on a dry powder basis following the manufacturer's guidelines and adjusted to pH 6.90 with sodium hydroxide and hydrochloric acid prior to analysis. Suspensions of CCit in the two IMF solutions prepared at a range of different protein contents were prepared according to the method detailed by Tercinier *et al*. (2017), with some minor modifications. Solutions of IMF with different protein concentrations (i.e., 0.00, 0.10, 0.20, 0.40, 0.60, 0.75, 1.50 and 3.00% w/w) were prepared by diluting the stock solution (i.e., 3.10%, w/w protein) with the required proportions of ultra-pure water. Dispersions of a fixed quantity of CCit-C and CCit-M (3.00%, w/w) were added to the IMF solutions at the different protein concentrations outlined above. The suspensions were stirred using low-speed magnetic agitation for 2.5 h at 20°C and re-adjusted to pH 6.90 prior to centrifugation at 4,500 g for 20 min. The Langmuir model was fitted to the adsorption data, as described by Iafisco *et al.* (2011), using Origin data analysis software (OriginLab Corporation, Northampton, MA, US), thereby providing data for the calculations of maximum surface coverage (q_m) and the affinity constant (K) for the interactions between milk proteins and CCit in the different samples.

1.3.6 Electrophoretic and densitometric analysis of protein profile

The protein profile of the supernatant (obtained from Section 1.3.5) of the IMF formulas (i.e., IMF-S1 and IMF-S3) at 0.10, 0.40, 0.75 and 1.50% (w/w) protein with added calcium citrate (3.00%, w/w) was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970), with minor modifications. Prepared samples were analysed using pre-cast mini-protean Tetra Cell TGX 4-20% acrylamide (Bio-Rad Laboratories, Inc., Hercules, CA, US) at a constant voltage of 180 V, under reducing conditions. The loading volume injected in the pre-cast SDS-PAGE gel varied in accordance with the protein content of the supernatant, in order to have a constant protein load of 5 µg in the gel wells. All gels were Coomassie-stained, before being de-stained with a solution of water, methanol and acetic acid, 50:40:10, respectively, until a bright background was reached. Stained SDS-PAGE gels were scanned using a desktop flatbed scanner (HP Scanjet G4010, HP, Leixlip, Ireland), and densitometric analysis of the bands was carried out using Total-Lab Quant 13.1v software (Totallab, Newcastle, UK).

1.3.7 Zeta potential

The CCit pellets, obtained after centrifugation (outlined in Section 1.3.5), were resuspended at 0.05% (w/w) in ultra-pure water according to the method of Tercinier *et al*. (2013). A Malvern Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) with DTS 1070 folded capillary cells (Malvern Instruments Ltd, Malvern, UK) was used to determine the zeta potential (ζ) of the CCit in suspension using automatic voltage selection, and the Smoluchowski model (Kirby and Hasselbrink, 2004).

1.3.8 Colloidal stability and rheological properties of calcium-fortified infant formula

Colloidal stability of CCit-C and CCit-M in the two different IMFs was assessed using a light scattering vertical scan analyser (Turbiscan^{LAB}, Formulaction, Toulouse, France) as described by Yang *et al*. (2017). The IMFs were rehydrated according to the manufacturer's recommended reconstitution rate (i.e., not standardised to protein content as detailed in Section 1.3.5) using low-speed magnetic agitation to prevent disruption of fat globules. CCit-C and CCit-M (3% w/w) was added to the IMF's and stirred as per Section 1.3.5. Samples (20 ml) were placed into glass holding cells and the analysis was performed at 25°C for 3 h. The rate of sedimentation was determined using the TurbiSoft software and reported as turbiscan stability index (TSI) values. Sediment thickness at the base of the vial over time was also determined from backscattering profiles. Apparent viscosity of the IMF solutions was measured at a shear rate of 1000 s^{-1} at 25°C using a rotational viscometer (HAAKE RotoVisco 1, Thermo Fisher Scientific, MA, USA) with a cylindrical double gap cup and rotor geometry (DG43, Thermo Fisher Scientific, MA, USA).

1.3.9 Statistical data analysis

All experimental analyses were conducted in triplicate, unless otherwise stated. Results are expressed as mean ± standard deviation. T-test and analysis of variance (ANOVA; Tukey's HSD test) were performed using R i386 version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) on mean values for different samples. The level of significance was determined at $p < 0.05$.

1.4 Results and discussion

1.4.1 Powder physical properties

The physical properties of a powder can be important attributes influencing properties such as bulk handling, storage stability and processability (Teunou *et al*., 1999). Data for the composition and physical properties of conventional calcium citrate (CCit-C) and micronised calcium citrate (CCit-M) are provided in Table 1.1. The density of CCit-M (1.98 g/cm^3) was slightly, but significantly higher ($p < 0.05$) than that of the CCit-C (1.95 g/cm^3). The size of powder particles in CCit-M was significantly smaller ($p < 0.05$) compared to CCit-C with Dv(50) values of 3.05 and 85.0 µm, respectively. The surface-weighted mean particle diameter D[3,2] was also significantly different ($p < 0.05$), with values of 2.16 and 10.4 µm for CCit-M and CCit-C, respectively. These results were similar to those reported by Mejía and Bolaños (2013), who reported a reduction in the average particle diameter of calcium citrate from 80 to 30 µm, albeit using supercritical carbon dioxide for micronisation as opposed to milling. Following sieving of the CCit-M, any slight compression of the powder caused the formation of small aggregates and clumping; this aggregation may explain the second peak of the particle size distribution (PSD) as the collision of particles occurs during the sieving analysis. This type of interaction between powder particles has been shown to lead to issues such as agglomeration due to the generation of triboelectric charge (Zhang *et al*., 2015).

Table 1.1: *Moisture content, water activity (a_w), density, specific surface area (SSA), particle size distribution parameters, compressibility index (CI), flow index (i) and Jenike classification of conventional calcium citrate (CCit-C) and micronised calcium citrate (CCit-M).*

		Unit	CCit-C	CCit-M
Composition and physical analysis	Moisture	% w/w	7.15 ± 0.01[a]	7.21 ± 0.18[a]
	a_w	-	0.556 ± 0.001[a]	0.561 ± 0.001[b]
	Density	g/cm^3	1.971 ± 0.001[a]	1.981 ± 0.001[b]
	SSA	m^2/g	7.34 ± 0.13[a]	12.6 ± 2.04[b]
Particle size distribution parameters	D[4,3]	µm	88.2 ± 0.78[a]	5.10 ± 0.10[b]
	D[3,2]	µm	10.4 ± 0.06[a]	2.16 ± 0.02[b]
	Dv(10)	µm	3.58 ± 0.01[a]	0.97 ± 0.01[b]
	Dv(50)	µm	85.0 ± 0.64[a]	3.05 ± 0.03[b]
	Dv(90)	µm	183 ± 1.53[a]	10.3 ± 0.38[b]
Flow Properties	CI	%	53.8 ± 0.68[a]	74.1 ± 1.02[b]
	i	-	7.16 ± 1.12[a]	1.77 ± 0.35[b]
	Jenike classification	-	Easy Flowing	Very Cohesive

[a-b] Mean values followed by different superscript letters in the same row are significantly different ($p < 0.05$).

D[4,3] = volume-weighted mean particle diameter.

D[3,2] = surface-weighted mean particle diameter.

Dv(10) = particle size below which 10% of sample volume is found.

Dv(50) = particle size below which 50% of sample volume is found.

Dv(90) = particle size below which 90% of sample volume is found

From the micrographs (Fig. 1.1), it is evident that CCit-M contained significantly smaller particles than CCit-C, with the particles in the former having a more jagged, non-uniform morphology. At 1000x magnification, one large CCit-C particle is visible, while a mass of small particles is seen for CCit-M. It appears that the micronised particles in the sample have an ability to become interlocked with one another due to the jagged nature of the powder particle surfaces. As expected from PSD analysis, the smaller mean particle size resulted in significantly higher ($p < 0.05$) specific surface area (SSA) values for CCit-M (12.6 m^2/g) compared to CCit-C (7.34 m^2/g). The tendency of CCit-M to re-compact and cause clumping may contribute to the large standard deviation observed in the measurement of SSA for the CCit-M sample.

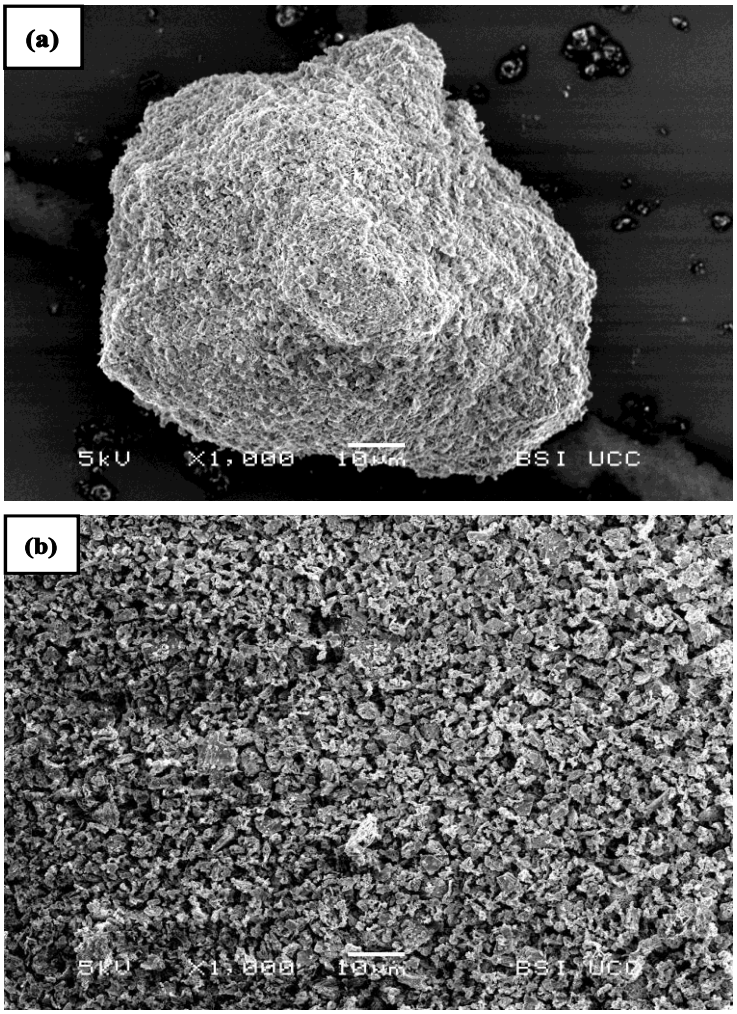


Figure 1.1: *Scanning electron micrographs of conventional calcium citrate (CCit-C; (a)) and micronised calcium citrate (CCit-M; (b)) at 1000x magnification.*

1.4.2 Bulk handling properties

The flowability of powders is influenced by a large number of variables such as particle size, composition and microstructure (Fitzpatrick *et al*., 2007; Schulze *et al*., 2008). The effect of particle size on the flowability of CCit is illustrated in Fig. 1.2, with CCit-C exhibiting good flowability. At low consolidating stress, CCit-C was cohesive but became easy-flowing (*i* of 7.16) as the stress applied increased. Conversely, CCit-M displayed impaired flowability (*i* of 1.77) and at very high shear there was a significant decrease ($p < 0.05$) in the unconfined failure strength, most

likely due to the extensive compaction of the micronised powder. This may lead to difficulties in handling of the micronised powder or in storage of the powder in silos, where high pressures experienced within the silo could lead to powder arching (Fitzpatrick *et al*., 2007). The poor flowability observed for CCit-M was most likely due to the smaller particle size, higher surface area and the distinct morphological properties of the powder itself compared to CCit-C; all of these factors facilitate greater particle-particle interactions, resulting in a very cohesive powder (Teunou *et al*., 1999; Fitzpatrick *et al*., 2007; Crowley *et al*., 2014b).

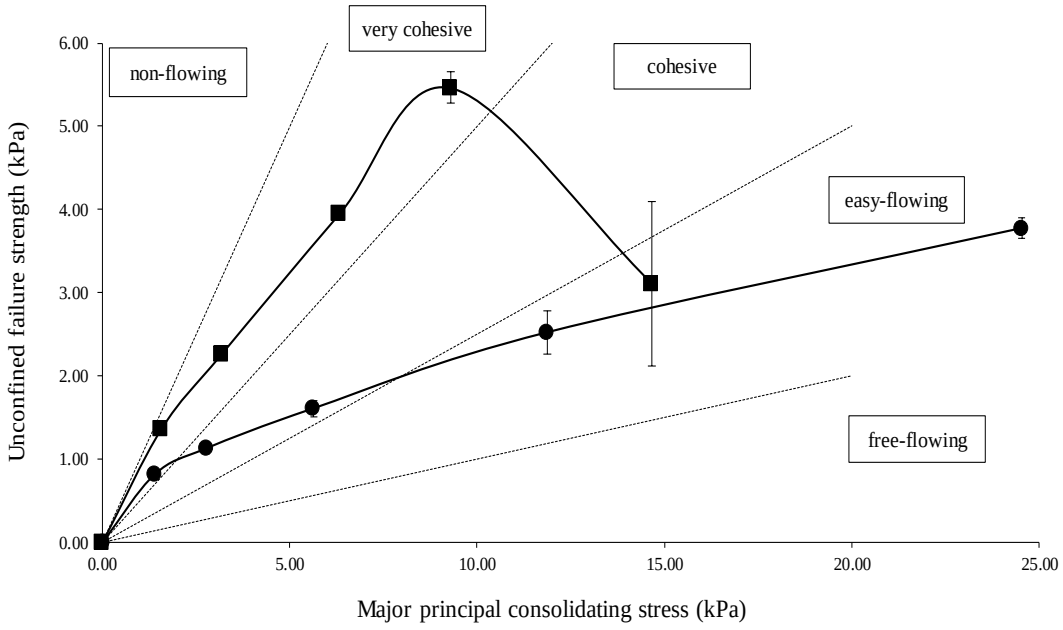


Figure 1.2: *Flow function curves showing unconfined failure strength as a function of major principal consolidating stress for conventional calcium citrate (CCit-C; ●) and micronised calcium citrate (CCit-M; ■).*

The angle of wall friction is an important parameter to consider in the storage of powders (Fitzpatrick *et al.*, 2004; Iqbal and Fitzpatrick, 2006; Fitzpatrick *et al.*, 2007; Schulze *et al*., 2008). As expected from flowability analysis, a higher angle of wall friction was measured for CCit-M (Fig. 1.3a). The wall friction angle (φ_w) represents the minimum hopper angle required to ensure mass flow. Values of φ_w for CCit-M were significantly higher ($p < 0.05$) than those for CCit-C with initial φ_w values of 36.0º and 30.5º, respectively. The higher φ_w measured for CCit-M is most likely due to greater particle-wall interactions as a result of the higher surface area, as has been previously reported by Garg *et al*. (2018) for calcium sulphate and dicalcium phosphate. Both CCit powders compressed upon the application of increasing stress, with a corresponding increase in bulk density (Fig. 1.3b). No change in density was observed for CCit-M after 9.30 kPa due to the complete compaction of the powder, a result also reflected in the flow function curve (Fig. 1.2). The bulk density of a powder is influenced significantly by the way in which the particles pack on the application of stress. Generally, cohesiveness is expected to decrease as particle size increases due to decreased SSA and associated reduction in interparticle interactions (Fitzpatrick *et al*., 2004). Significantly higher ($p < 0.05$) compressibility index (CI) values for CCit-M (74.1%) were observed compared to CCit-C (53.8%; Table 1.1). Compressibility is related to the change in bulk density with higher CI values indicating a more cohesive powder (as evident in Fig. 1.2). The smaller particle diameter, lower bulk density and higher SSA of CCit-M, compared to CCit-C suggests greater interstitial air, allowing for more voids to be filled by the particles on compression, resulting in a greater change in bulk density (Garg *et al*., 2018). From the SEM micrographs (Fig. 1.1), the ability for CCit-M particles to interlock and compact is clear and is likely to be the reason for the higher CI.

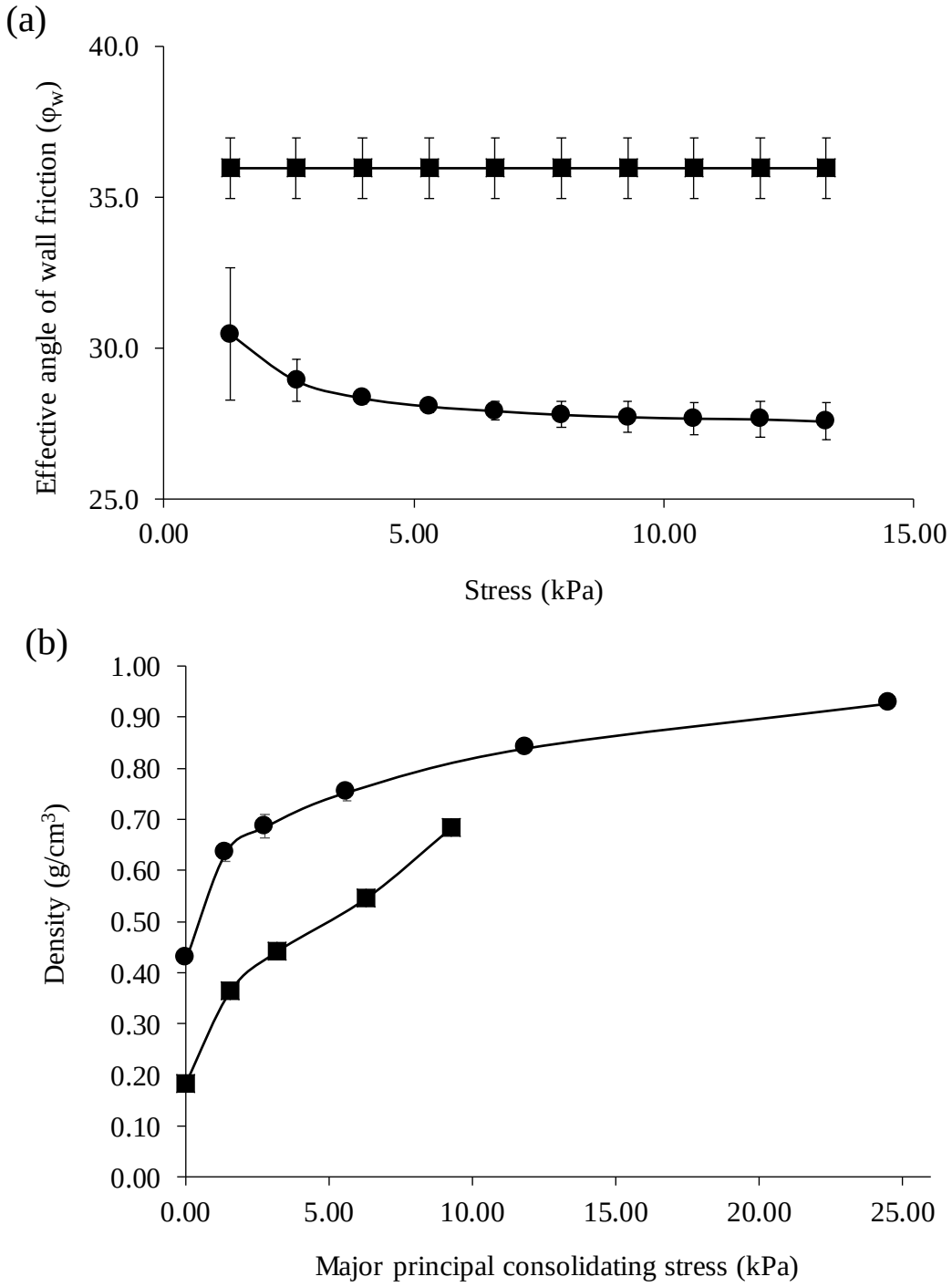


Figure 1.3: *Effective angle of wall friction for conventional calcium citrate (CCit-C; ●) and micronised calcium citrate (CCit-M; ■) (a) and bulk density as a function of major principal consolidating stress for conventional calcium citrate (CCit-C; ●) and micronised calcium citrate (CCit-M; ■) (b)*

1.4.3 Protein adsorption and affinity properties

To determine whether the significantly smaller particle size in the micronised powder results in increased protein-calcium citrate interactions, protein adsorption analysis was carried out on CCit-C and CCit-M suspended in both infant formula stage 1 (IMF-S1) and stage 3 (IMF-S3) (Fig. 1.4). The surface protein concentration on the CCit particles increased gradually with increasing protein content up to 0.50% (w/w). The Langmuir-Freundlich model was used to describe the adsorption process (Iafisco *et al.*, 2011; Tercinier *et al.*, 2017) as it considers some heterogeneity of the adsorbent (i.e., rough surface and inconsistency of the particle) in addition to potential interactions between the proteins present once they are adsorbed to the surface of the CCit particles.

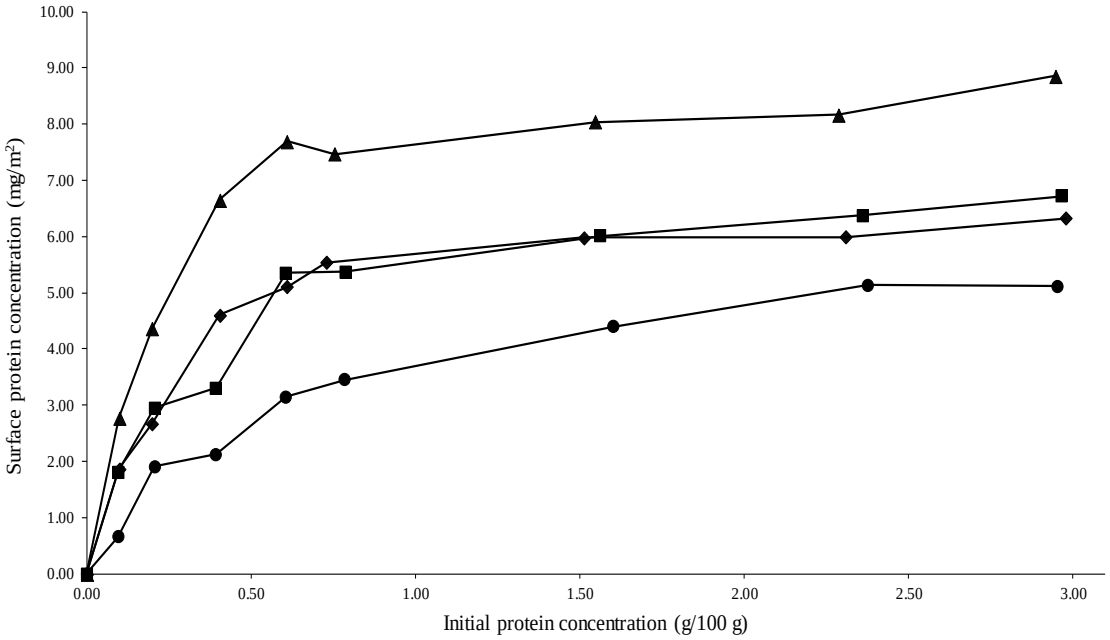


Figure 1.4: *Surface protein concentration (mg/m^2) of conventional calcium citrate (CCit-C) and micronised calcium citrate (CCit-M) as a function of the initial protein concentration of infant formula stage 1 (IMF-S1) and infant formula stage 3 (IMF-S3): IMF-S1 CCit-C (■), IMF-S1 CCit-M (●), IMF-S3 CCit-C (▲), IMF-S3 CCit-M (♦)*

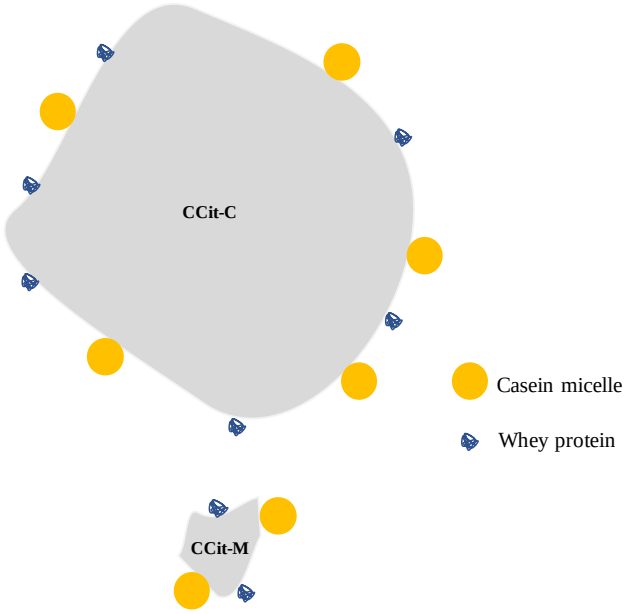


Figure 1.5 *Schematic representation of the effect particle size of calcium salt on the adsorption of proteins onto its surface. Conventional calcium citrate (CCit-C), micronised calcium citrate (CCit-M).*

CCit-C possessed higher maximum surface coverage (q_m) values compared to CCit-M for IMF-S1, with values of 7.39 and 6.36 mg/m^2, respectively. Similar results were also observed for the IMF-S3 samples with CCit-C and CCit-M having q_m values of 8.67 and 6.42 mg/m^2, respectively. In addition, the affinity constant (K) for CCit-C was higher than CCit-M for both IMF-S1 (3.13 and 1.56, respectively) and IMF-S3 (11.2 and 7.91). The lower q_m and K measured for CCit-M is due to the smaller particle size compared to CCit-C, reducing the total amount of protein that can adsorb onto the surface of an individual calcium salt particle (Fig. 1.5).

However, for both IMF-S1 and IMF-S3, a greater quantity of protein was present in the pellet of CCit-M compared to CCit-C after centrifugation (Table 1.2). In the complete fortified system, a greater proportion percentage of total protein is bound to CCit-M, as there is a greater number of micronised particles per unit mass and this results in increased surface area for protein-CCit particle interactions, compared to

CCit-C. This result is supported by the SDS-PAGE analysis (Fig. 1.6), whereby the CCit-M sample had a lower quantity of casein in the supernatant compared to CCit-C at the same protein concentration. The SDS-PAGE gels also showed that the casein proteins preferentially adsorb to the CCit particles to a greater extent than whey proteins in both IMF-S1 and IMF-S3, especially at the lower protein contents studied. Similar results were reported by Tercinier *et al*. (2017), showing that caseins have a greater preference for binding to the surface of hydroxyapatite (HA) particles, compared with α-lactalbumin (α-lac) and β-lactoglobulin (β-lg). Additionally, more casein adsorbed to the surface of CCit-M particles compared to CCit-C particles when dispersed in both IMFs, as indicated by a higher whey protein to casein ratio in the supernatant following centrifugation (i.e., 4.65 and 1.58 for CCit-M and CCit-C in IMF-S3 at 0.1% protein, respectively; Table 1.3). For both IMF-S1 and IMF-S3, caseins had a greater affinity for the surface of CCit particles compared to whey proteins, with almost all caseins adsorbed to the surface of CCit-M at 0.1% (w/w) protein (Fig. 1.6). As the protein content of the original solutions was increased to >0.4% prior to centrifugation, the whey protein to casein ratio in the supernatant decreased, returning to values close to the original IMF solution with no added salt, indicating that the CCit particles were saturated with protein and that the adsorption process plateaued, in agreement with protein adsorption analysis (Fig. 1.4). A similar trend was observed for the ratio of α-lac to β-lg, with a higher proportion of β-lg being adsorbed to CCit particles in both IMF-S1 and IMF-S3 compared to α-lac; this trend was even more evident in the whey-dominant formula (IMF-S1) (Table 1.3). These findings were expected and reflect those observed by Tercinier *et al*. (2017), who found that β-lg adsorbed to the surface of HA particles to a greater extent than α-lac.

Table 1.2: *Protein content (% w/w) of micronised calcium citrate (CCit-M) and conventional calcium citrate (CCit-C) pellets after centrifugation of the calcium salts suspended in infant formula stage 1 (IMF-S1) or stage 3 (IMF-S3). Results are presented as mean ± standard deviation of data from three independent trials.*

	0.00	**0.10**	**0.20**	**0.40**	**0.60**	**0.75**	**1.50**	**2.25**	**3.00**
					%				
IMF-S1 CCit-C	0	0.039 ± 0.001^{b}	0.066 ± 0.005^{a}	0.073 ± 0.001^{a}	0.117 ± 0.010^{a}	0.118 ± 0.016^{a}	0.132 ± 0.009^{a}	0.140 ± 0.015^{a}	0.148 ± 0.019^{a}
IMF-S1 CCit-M	0	0.023 ± 0.001^{a}	0.069 ± 0.005^{a}	0.077 ± 0.006^{a}	0.115 ± 0.008^{a}	0.126 ± 0.016^{ab}	0.161 ± 0.012^{b}	0.188 ± 0.020^{ab}	0.187 ± 0.030^{ab}
IMF-S3 CCit-C	0	0.061 ± 0.001^{c}	0.096 ± 0.001^{b}	0.146 ± 0.004^{b}	0.169 ± 0.004^{b}	0.164 ± 0.018^{bc}	0.177 ± 0.006^{b}	0.180 ± 0.037^{ab}	0.195 ± 0.026^{ab}
IMF-S3 CCit-M	0	0.068 ± 0.003^{d}	0.098 ± 0.004^{b}	0.168 ± 0.002^{c}	0.187 ± 0.007^{b}	0.203 ± 0.017^{c}	0.219 ± 0.008^{c}	0.219 ± 0.011^{b}	0.232 ± 0.010^{b}

Values followed by different superscript letters in the same column are significantly different ($p < 0.05$).

Table 1.3: *Densitometry analysis of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of stage 1 (IMF-S1) and stage 3 (IMF-S3) supernatants at different protein contents, expressed as whey protein:casein and α-lactalbumin (α-lac):β-lactoglobulin (β-lg) ratios of the supernatants of samples containing micronised calcium citrate (CCit-M) and conventional calcium citrate (CCit-C). Results are presented as mean ± standard deviation of data from three independent trials.*

Sample	Ratio	IMF	0.1% protein		0.4% protein		0.75% protein		1.5% protein	
			CCit-C	CCit-M	CCit-C	CCit-M	CCit-C	CCit-M	CCit-C	CCit-M
IMF-S1	Whey:casein	1.36 ± 0.05^{a}	2.89 ± 0.17^{bc}	13.0 ± 0.51^{d}	2.12 ± 0.11^{ac}	3.03 ± 0.21^{c}	1.92 ± 0.17^{ac}	2.03 ± 0.17^{ac}	1.60 ± 0.10^{ab}	1.58 ± 0.01^{a}
IMF-S3	Whey:casein	0.79 ± 0.04^{a}	1.58 ± 0.14^{bc}	4.65 ± 0.15^{d}	1.46 ± 0.19^{bc}	1.89 ± 0.19^{c}	1.31 ± 0.14^{b}	1.51 ± 0.15^{bc}	1.18 ± 0.12^{ab}	1.18 ± 0.14^{ab}
IMF-S1	α-lac:β-lg	1.44 ± 0.17^{a}	2.35 ± 0.35^{cd}	4.63 ± 0.31^{e}	2.05 ± 0.22^{bcd}	2.49 ± 0.06^{d}	2.12 ± 0.08^{cd}	1.86 ± 0.13^{ac}	1.55 ± 0.13^{ab}	1.48 ± 0.19^{a}
IMF-S3	α-lac:β-lg	0.39 ± 0.02^{bc}	0.31 ± 0.03^{ab}	0.26 ± 0.06^{a}	0.37 ± 0.01^{bc}	0.35 ± 0.02^{bc}	0.38 ± 0.01^{bc}	0.37 ± 0.03^{bc}	0.40 ± 0.01^{c}	0.40 ± 0.01^{c}

Values followed by different superscript letters in the same row are significantly different ($p < 0.05$).

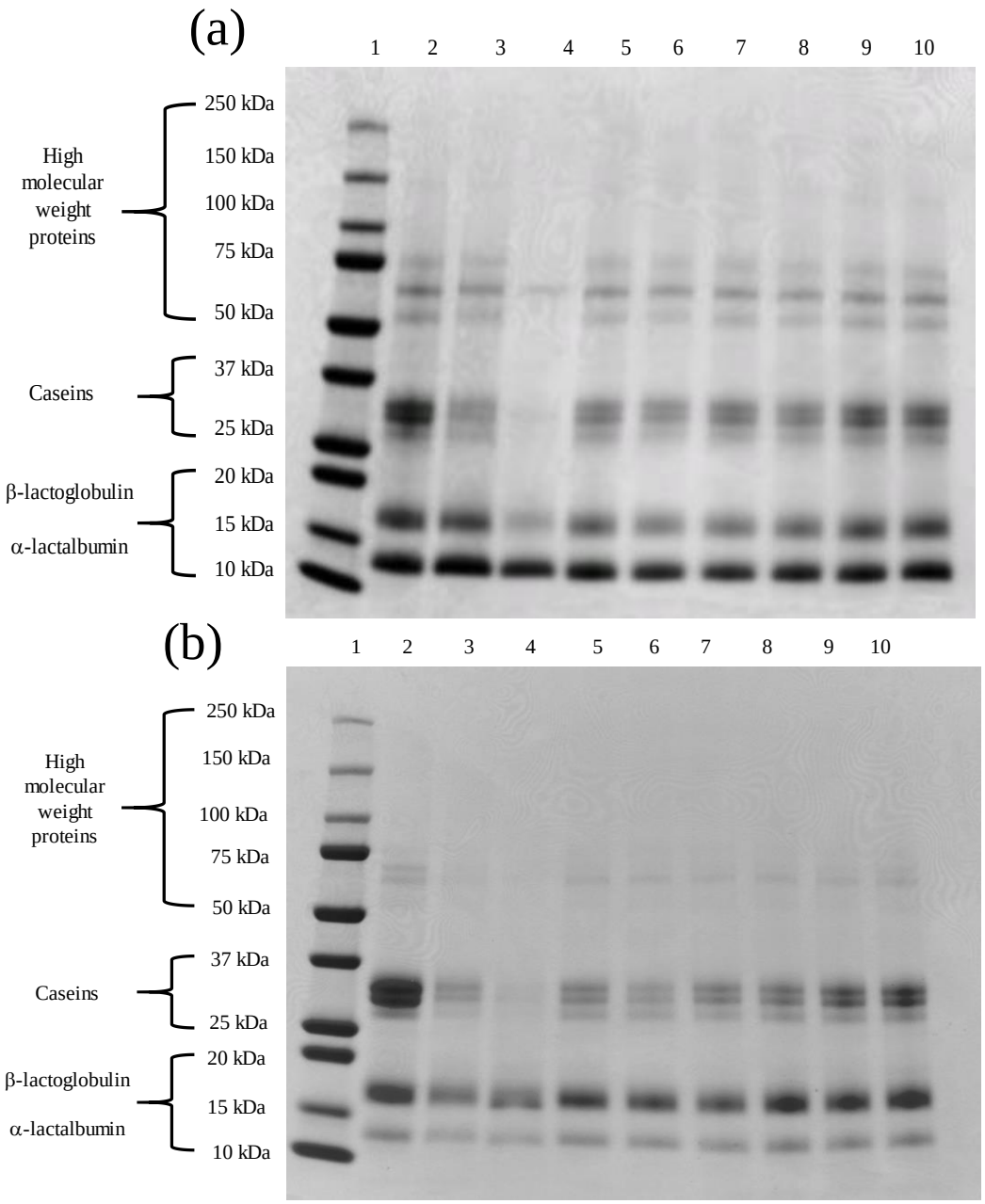


Figure 1.6: *Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoretograms of supernatants of micronised calcium citrate (CCit-M) and conventional calcium citrate (CCit-C) samples following centrifugation of (a) infant milk formula stage 1 (IMF-S1) and (b) infant milk formula stage 3 (IMF-S3), with added calcium: lane 1, molecular weight marker; lane 2, IMF with no added CCit; lanes 3-10, 0.10% protein CCit-C, 0.10% protein CCit-M, 0.40% protein CCit-C, 0.40% protein CCit-M, 0.75% protein CCit-C, 0.75% protein CCit-M, 1.50% protein CCit-C and 1.50% protein CCit-M.*

The zeta potential (ζ) of CCit-C and CCit-M suspended in water was -11.9 and -9.56 mV, respectively, while the ζ for IMF-S1 and IMF-S3 with no added salt was -30.9 and -27.6 mV, respectively. In general, an increase in the net negative charge of insoluble calcium particles improves suspension stability due to greater repulsive electrostatic forces reducing phenomena such as aggregation and sedimentation (Anema, 2009; Tercinier *et al*., 2013). The adsorption of protein onto the surface of CCit particles when suspended in IMF-S1 and IMF-S3 significantly increased ($p < 0.05$) the net negative charge of the particles (Fig. 1.7). At 0.4% (w/w protein) in IMF-S3, there was a significantly higher ($p < 0.05$) zeta potential (more negative value) for CCit-M particles (-21.5 mV) compared to CCit-C particles (-18.7 mV), which is likely due to greater amounts of protein adsorbed to the surface of CCit-M particles when suspended in IMF-S3. In contrast, in IMF-S1 there was no significant difference ($p > 0.05$) in zeta potential for CCit-M (-21.4 mV) and CCit-C particles (-21.9 mV) at 0.4% (w/w protein). At a protein concentration of 0.75% (w/w) a plateau was observed for both salts (i.e., CCit-C and CCit-M) with CCit-M particles displaying slightly higher zeta potential than CCit-C particles when dispersed in both IMFs; values of -23.0 and -19.9 mV were measured for CCit-M particles when dispersed in IMF-S1 and IMF-S3, respectively, while values of -22.3 and -18.5 mV were measured for CCit-C particles when dispersed in IMF-S1 and IMF-S3, respectively

The zeta potential of the resuspended CCit particles in IMF-S1 was higher (more negative) than that of IMF-S3 overall. The results observed are in accordance with those of Reynolds and Wong (1983), who reported that the adsorption of milk proteins onto calcium particles caused them to acquire a net negative charge, with Tercinier *et al*. (2013) reporting similar results. Differences in calcium salt binding properties can be observed between the two different formulas in which the calcium citrate powders

were suspended. Numerous studies have detailed the different binding affinities of milk proteins for calcium salts (Tercinier *et al*., 2014; Tercinier *et al*., 2017) due to their different structural or physicochemical properties (Bernardi and Kawasaki, 1968; Nakanishi *et al*., 2001), with globular proteins binding to solid surfaces to a lesser extent than flexible, linear proteins (Norde, 1986; Wahlgren and Arnebrant, 1991). This explains why β-lg and α-lac adsorbed to CCit particles to a lesser extent than caseins, and why β-lg adsorbed to a greater extent than α-lac, as α-lac has a more compact bilobal structure (Brew, 2003). The protein profile of IMF changes to meet the changing nutritional demands of the neonate, as IMF-S1 is a whey-dominant system, while follow-on formulae (i.e., IMF-S3) have a casein-dominant protein profile (O'Callaghan *et al.*, 2011). This difference in protein profile can help explain the greater adsorption measured for proteins in IMF-S3 for both CCit-C and CCit-M, as this formula is a casein-dominant system; as previously discussed, caseins have been shown to have a greater preference for adsorbing to the surface of calcium salts compared to whey proteins, which are dominant in the IMF-S1. The greater adsorption of caseins to CCit-M could lead to improved colloidal stability, as caseins possess a high net negative charge at pH 6.8 (Huppertz, 2013).

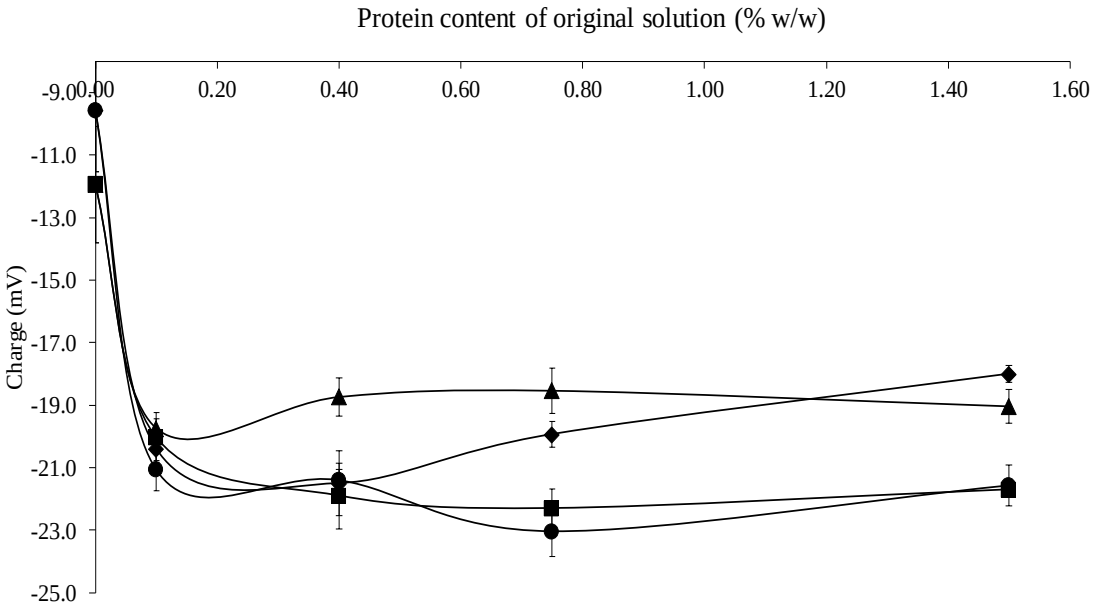


Figure 1.7: *Effect of adsorbed protein on the zeta potential of conventional calcium citrate (CCit-C) and micronised calcium citrate (CCit-M) particles resuspended in water after centrifugation: Infant milk formula stage 1 (IMF-S1) coated CCit-C particles (■), Infant milk formula stage 3 (IMF-S3) CCit-C particles (▲), IMF-S1 CCit-M particles (●), IMF-S3 CCit-M particles (♦)*

1.4.4 Colloidal stability of the suspended calcium salt

Maintaining insoluble calcium salts in suspension over the shelf life of fortified liquid nutritional products is a challenge. Therefore, the colloidal stability of CCit-M and CCit-C dispersed in both IMF's was assessed to determine whether the smaller particle size of CCit-M would improve suspension stability. The rate of destabilisation (i.e., sedimentation) for CCit-C and CCit-M suspended in IMF is represented in Table 1.4, while the increase in the height of the sediment layer over time is shown in Fig. 1.8. The viscosity of IMF-S1 and IMF-S3 was 2.44 and 3.05 mPa.s, respectively. An increase in the instability index values (i.e., TSI) over time indicates an increase in the destabilisation of the sample. CCit-M displayed a statistically significant ($p < 0.05$) lower rate of sedimentation compared to CCit-C after 15 min in both IMF-S1 and

IMF-S3 (Table 1.4). At 45 min, CCit-M remained more colloidally stable than CCit-C with TSI values of 1.00 and 1.50 in IMF-S1, respectively, and 0.55 and 1.30 in IMF-S3, respectively. Significantly higher ($p < 0.05$) colloidal stability was measured for IMF-S3 compared to IMF-S1, potentially due to the higher apparent viscosity of IMF-S3 caused by a higher protein content and a higher reconstitution rate. This results in the CCit particles remaining in suspension longer, in accordance with Stoke's law, especially as the ζ of IMF-S3 (-27.6 mV) was lower than that of IMF-S1 (-30.9 mV), indicating that ζ does not significantly contribute to the higher stability seen for CCit particles when suspended in IMF-S3 compared to IMF-S1.

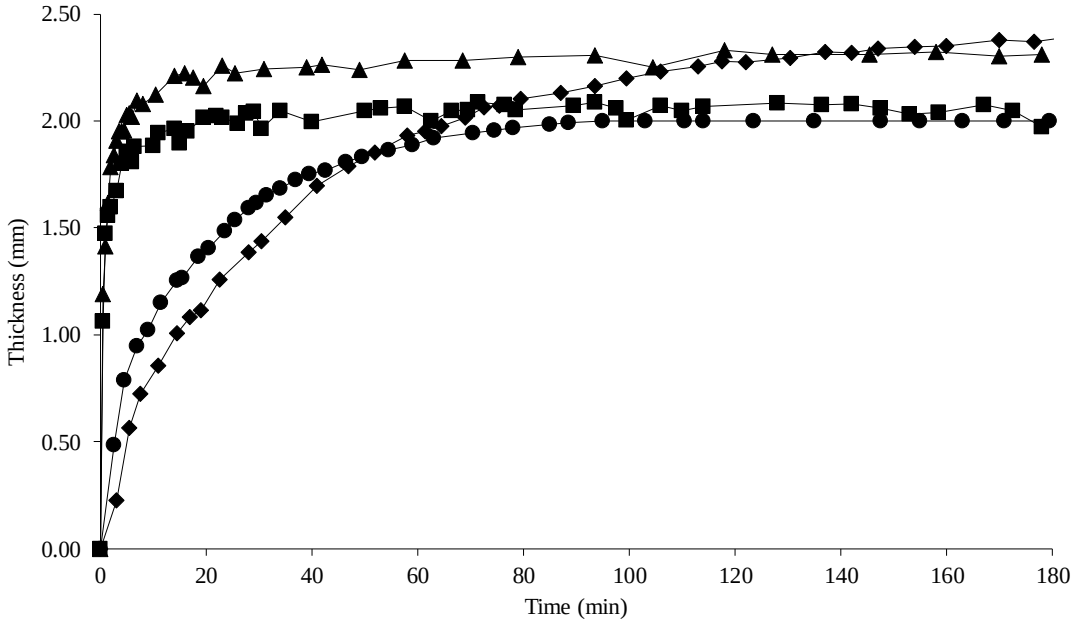


Figure 1.8 *Thickness (mm) of sediment layer at the base of the test vial as a function of time for conventional calcium citrate (CCit-C) and micronised calcium citrate (CCit-M) dispersed in infant formula stage 1 (■ and ●, respectively) and infant formula stage 3 (▲ and ♦, respectively).*

The height of the sediment layer (Fig. 1.8) increased rapidly for CCit-C suspended in both IMF-S1 and IMF-S3, with sediment heights of 1.89 and 2.21 mm after 15 min, respectively. In comparison, after 15 min the height of the sediment layer for CCit-M in IMF-S1 (1.27 mm) and IMF-S3 (1.03 mm) was significantly lower ($p < 0.05$), with a greater amount of time required to fully sediment out of solution (indicated by the plateau in sediment layer thickness). For the same quantity of CCit added (3% w/w) to either IMF, a greater amount of protein is adsorbed to the surface of CCit-M particles compared to CCit-C (Table 1.2), resulting in a more negative ζ and therefore greater repulsion between the protein-coated particles. This may also explain why the height of the sediment layer (Fig. 1.8), after complete sedimentation (i.e., 180 min), was higher for both CCit-C and CCit-M when suspended in IMF-S3, as there is more protein adsorbed when suspended in IMF-S3 compared to IMF-S1 (Table 1.2). Therefore, the improved stability of CCit-M cannot solely be attributed to reduced particle size but also to the impact of adsorbed proteins (Wen *et al*., 2016).

Table 1.4: *Turbiscan stability index (TSI) values for conventional calcium citrate (CCit-C) and micronised calcium citrate (CCit-M) dispersed in infant formula stage 1 (IMF-S1) and stage 3 (IMF-S3). Higher TSI values indicate increased destabilisation within the sample. Results are presented as mean ± standard deviation of data from three independent trials*

Time (min)	IMF-S1 CCit-C	IMF-S1 CCit-M	IMF-S3 CCit-C	IMF-S3 CCit-M
15	1.05 ± 0.07^{b}	0.45 ± 0.07^{a}	1.00 ± 0.01^{b}	0.25 ± 0.07^{a}
45	1.50 ± 0.01^{d}	1.00 ± 0.01^{b}	1.30 ± 0.01^{c}	0.55 ± 0.07^{a}
180	2.30 ± 0.14^{b}	2.10 ± 0.14^{b}	1.80 ± 0.14^{ab}	1.35 ± 0.07^{a}

Values followed by different superscript letters in the same row are significantly different ($p < 0.05$)

1.5 Conclusion

Decreasing the particle size of calcium citrate salts has the potential to improve the colloidal stability of calcium-fortified nutritional products. Micronised calcium citrate had greater particle-particle interactions due to smaller particle size, in conjunction with a more irregular particle morphology, resulting in a cohesive powder with lower bulk density and a higher angle of wall friction, compared to conventional calcium citrate. The results obtained demonstrate that micronised, insoluble calcium salts improve suspension stability compared to conventional calcium citrate due to higher protein affinity, increased protein adsorption and lower net charge of the calcium citrate particles when added to a complex system such as infant formula. Findings from this study represent valuable information regarding the effects of micronisation on interactions between insoluble calcium salts and milk proteins, as well as the benefits of micronised salts on colloidal stability in fortified nutritional products.

1.6 Acknowledgements

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Appendix 2

Enzymes indigenous to milk: Plasmin system in milk

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Declaration

This chapter was written by author Thomas C. France and reviewed by all co-authors.

Abstract

Plasmin, the principal indigenous proteinase present in milk, is part of a complex protease-protease inhibitor system, including its inactive zymogen plasminogen, plasminogen activators, plasminogen activator inhibitors and plasmin inhibitors. The components of the plasmin system are affected by cow-related factors (e.g., stage of lactation), processing conditions (e.g., heat treatment) and storage conditions (e.g., temperature of storage). The rate of plasmin-induced hydrolysis is affected by numerous factors, such as heat treatment, pH, presence of whey proteins and storage conditions. Plasmin-induced proteolysis of milk proteins can have either desirable (e.g., cheese ripening) or detrimental (age gelation of UHT milk) effects, depending on the type of dairy product.

Introduction

Proteolysis in milk-based systems can have desirable or detrimental effects depending on the dairy product. Such proteolysis can be attributed to both indigenous enzymes (e.g., plasmin; PL) and exogenous proteases produced by bacteria, mainly psychrotrophic bacteria, during the storage of milk. PL-mediated proteolysis is desirable in some products such as cheese, where hydrolysis results in textural changes and flavour development, ultimately enhancing the quality of the product. However, proteolysis in other products, such as ultra-high temperature (UHT) treated milk and milk protein ingredients, can result in undesirable changes such as hydrolysis of intact proteins, alteration of functional properties and gelation. This article focuses on the molecular properties of the components of the PL system, factors affecting PL activity, regulation of the PL system, and the consequences of PL-mediated proteolysis in dairy products.

Plasmin and plasminogen

Plasminogen (PG) and PL found in bovine milk are identical to those found in bovine blood in terms of their amino acid sequences, pH optimum, pH stability and inhibition patterns. In blood, PL is part of the clotting mechanism where it breaks down blood clots; however, in bovine milk its role is unclear. PG is not expressed in the mammary gland and therefore PL is transferred to bovine milk from blood as the inactive zymogen (PG), *via* the mammary cell wall lining. The concentration of PL in bovine milk is influenced by a number of environmental factors, in addition to the various processing conditions to which milk is subjected. Bovine milk contains between 0.55-2.75 μg PG mL^{-1} and 0.14-0.73 μg PL mL^{-1} (Richardson and Pearce,

1981). Bovine PG is a glycosylated protein containing 786 amino acid residues with a molecular weight of ~88 kDa (Schaller *et al*., 1985). PG contains five triple-loop structures, known as kringles, each stabilised by three disulphide bonds.

Bovine PL (EC 3.4.21.7), the principal indigenous milk proteinase, displays trypsin-like activity with similarities in its activity characteristics (e.g., sensitivity to inhibitors). PL exhibits optimum activity at 37°C and pH ~7.5. PL has an affinity for lysine and arginine residues, hydrolysing Lys-X and Arg-X bonds, with a preference for peptide bonds containing lysine. PL acts on all caseins; however, the susceptibility of the caseins to PL action varies, with β-casein being the principal substrate, followed by α_{s2}- and α_{s1}-casein. Much of the work on PL action on casein has focused on β-casein, which is preferentially cleaved at three primary sites producing three γ-caseins (γ1 (β-CN f29-209), γ2 (β-CN f106-209) and γ3 (β-CN f108-209)), in addition to the corresponding proteose peptones. Whey proteins, such as β-lactoglobulin (β-lg) and α-lactalbumin, are less susceptible to PL action due to their tight, globular structure.

The plasmin system

The PL system in milk is a complex protease-protease inhibitor system consisting of the inactive zymogen (PG), the active enzyme (PL), plasminogen activators (PAs), plasminogen activator inhibitors (PAIs) and plasmin inhibitors (PIs) (Fig. 2.1). The conversion of PG to PL is controlled by two main types of PAs present in milk, urokinase-type plasminogen activators (u-PA) and tissue type plasminogen activators (t-PA).

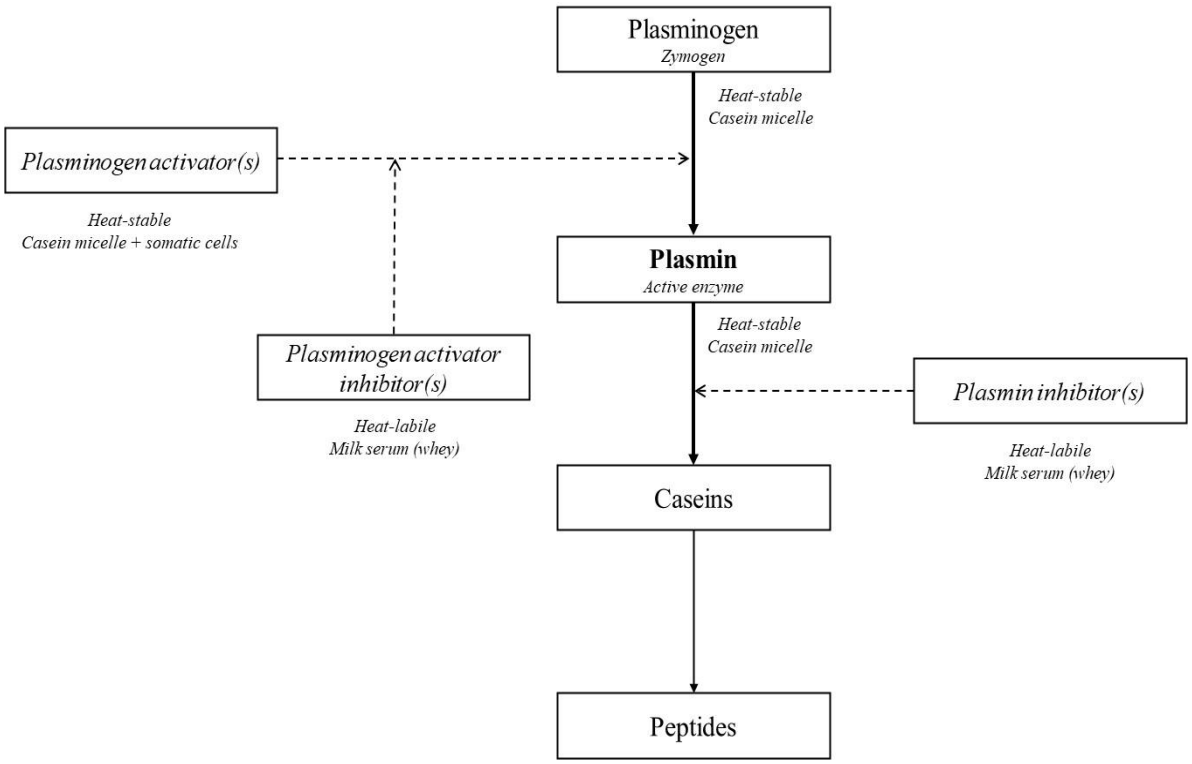


Figure 2.1: *Schematic representation of the plasmin system in bovine milk (adapted from France et al., 2021).*

Plasminogen activators

The concentration of PG in milk decreases during storage, with a consequent increase in PL concentration, caused by the activity of PAs which hydrolyse PG to PL. Since PG and PL pass from the blood into bovine milk, PAs and inhibitors present in blood also leak into milk; t-PA and u-PA are the main PAs present in bovine milk. It has been shown that the activities of μ-PA and t-PA increase in the presence of casein, in particular α-casein, while t-PA activity is also significantly enhanced by fibrin (Politis *et al*., 1995).

t-PA and u-PA are serine proteinases, which activate bovine PG by hydrolysing the Arg_{557}-Ile_{558} peptide bond. Bovine PG contains an N-terminal aspartic acid and

therefore native bovine PG is commonly referred to as Asp-PG. PL autocatalytically cleaves off the first 77 residues of bovine PG at the Lys_{77}-Arg_{78} bond, producing a 77-residue fragment known as the preactivation peptide (Fig. 2.2). The cleavage of the preactivation peptide results in the exposure of an arginine residue producing Arg-PG. However, PL cannot cleave the Arg_{557}-Ile_{558} bond to activate PG. Instead, the release of the preactivation peptide causes a conformational change in Arg-PG which exposes the Arg_{557}-Ile_{558} bond, making it more accessible to PAs, subsequently allowing activation of Arg-PG into active PL.

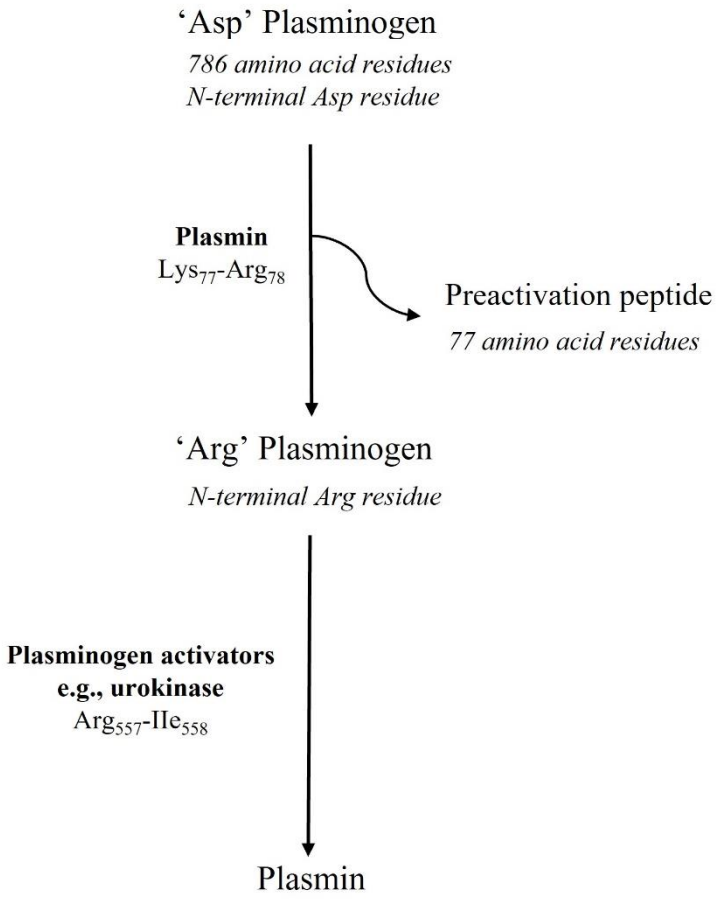


Figure 2.2: *Activation of bovine plasminogen (adapted from France et al., 2021).*

Inhibitors of the plasmin system

The PL system in milk is regulated by both PAIs and PIs. PAIs slow down the conversion of PG to PL, while PIs directly inhibit PL activity, reducing PL-mediated hydrolysis of caseins. The activity of PAIs and PIs are affected by pH and heat treatment, with inhibitors of the PL system being more heat-labile than PAs. The mechanism of action of proteinase inhibitors is not fully understood. Despite this, it is known that exposed reactive-site loops in the C-terminus interact with the active site of PL, potentially blocking or altering the conformation of the active site (Christensen and Sottrup-Jensen, 1994).

Many of the serine proteinase inhibitors that have been studied in detail are trypsin inhibitors. Several blood-derived proteinase inhibitors have been detected in bovine milk, many of which are serpins, including C1-inhibitor, α_2-macroglobulin (α_2-M), α_1-proteinase inhibitor and α_2-antiplasmin (α_2-AP). Interactions between PAs and PAIs, as well as between PL and PIs, provide a complex mechanism for the control of PL activity in bovine milk.

Distribution of plasmin system components in milk

The components of the PL system are distributed between the serum phase (whey) and the micellar phase (casein) of milk (Table 2.1). PG and PL are primarily associated with casein micelles, although dissociation from the micelle into the serum phase of milk can occur. There is conflicting evidence with regards to which PA is predominately associated with the casein micelle; however, many researchers have concluded that u-PA and t-PA are predominantly associated with somatic cells (*via* a urokinase receptor) and casein micelles, respectively (Heegaard *et al*., 1994a, b; Politis

et al., 1995; White *et al*., 1995). u-PA also has the ability to dissociate from somatic cells and re-associate with micelles. The association of t-PA with somatic cells has been attributed to the presence of casein entrapped within the somatic cell extract; removal of such casein material from extracts indicated that only u-PA was present (White *et al*., 1995). Electrostatic forces have been shown to play a significant role in t-PA-casein interactions with lysyl-binding sites also involved in such interactions (Heegaard *et al*., 1994b; Heegaard *et al*., 1997). Unlike PAs, PAIs and PIs are found only within the serum phase (whey). PG and PL bind to casein micelles through lysine-binding sites and, to a lesser extent, through electrostatic interactions.

Table 2.1: *Primary location of the components of the plasmin system within milk and the interactions involved in their association with milk constituents.*

Components of the plasmin system	Abbreviations	Primary location in milk	Interactions involved
Plasmin	PL	Casein micelles	Lysine-binding sites + electrostatic interactions
Plasminogen	PG	Casein micelles	Lysine-binding sites + electrostatic interactions
Urokinase-type plasminogen activators	u-PAs	Somatic cells	*via* a urokinase receptor (u-PAR)
Tissue-type plasminogen activators	t-PAs	Casein micelles	Electrostatic interactions + lysine-binding sites
Plasmin inhibitors	PIs	Serum phase (whey)	n.a.
Plasminogen activator inhibitors	PAIs	Serum phase (whey)	n.a.

n.a. = not applicable

The distribution of PL system components within the different milk fractions is affected by several environmental and non-environmental factors, such as NaCl concentration, pH and hydrolysis of casein by PL. Although the effect of temperature on PL-casein association is unclear, PL-casein association is strongly influenced by pH. The acidification of fresh bovine milk to pH 4.6-4.7 causes almost complete dissociation of PL from the casein micelles. The effect of pH on PL dissociation explains, at least in part, the higher levels of PL observed in acid whey (44 μg g^{-1} protein), compared to sweet whey (4 μg g^{-1} protein) (Ismail and Nielsen 2010). The addition of NaCl (1 mol L^{-1}) to milk, or long exposure to ethanol, also causes the dissociation of PL from casein micelles. The dissociation of PL from the casein micelle to the whey fraction may potentially cause negative effects on the quality of food products that incorporate whey protein ingredients, as PL may hydrolyse other proteins present in the system.

Factors affecting plasmin activity

The concentration of PG and PL in bovine milk is affected by numerous factors, including cow-related factors, processing conditions, storage conditions and the presence of microbial proteases (Fig. 2.3). The PL system is complex, involving a multitude of interactions that ultimately result in the activation of PG to PL. The system is made more complex by the interference of milk components with PL and PG under different conditions (pH, storage temperature, heating), collectively affecting the kinetics of PG activation and PL-induced hydrolysis.

Cow-related factors

Cow-related factors such as mastitis, age, stage of lactation and breed impact PL activity and the rate of PL-induced proteolysis. PL activity is higher in milk during mastitic infection and can be attributed to a number of factors, often due to the leakage of blood components into milk as a result of a compromised blood-milk barrier. The large increase in somatic cell counts during mastitis, coinciding with an increase in PAs, helps explain increased PL activity in mastitic milk.

Throughout lactation, the level of PL activity fluctuates, with a higher level of PL activity associated with late lactation, resulting in increased proteolysis of caseins. PL activity is higher in milk from older cows but, although PL activity increases, PG levels remain relatively constant. Therefore, similar to late-lactation milk, the increase in PL activity is likely due to increased permeability of the secretory tissue, increasing the presence of PAs. In addition to this, it has been reported that PL activity does not return to levels prior to the onset of mastitis (Saeman *et al*., 1988). This may explain the higher PL activity in milk from older cows, as older cows are likely to have experienced more mastitis in their lifetime.

Milk pH

The activity of PL strongly depends on the pH of its environment (i.e., milk, cheese). PL has an optimum pH of ~7.5. The average pH of bovine milk is ~6.8, thus, the pH of milk is close to the optimum for PL activity. Decreasing the pH causes a decrease in the activity of PL, due in part to the dissociation of PL from the micelle.

Heat treatment

The severity of the heat treatment of milk depends on various factors, including the desired shelf life of the product and the end use of the milk. Pasteurisation is a mild heat treatment (72ºC for 15-30 s) employed in the dairy industry to kill vegetative pathogenic microorganisms, thereby extending the refrigerated shelf-life of milk. Both PL and PG survive pasteurisation conditions. D-values (the time required for a 90% reduction in activity) for inactivation of PL in milk of 59-71 s at 90°C have been reported. A consequential increase in the rate of proteolysis can be associated following pasteurisation. Although the increase in PL activity on mild heat treatments has been attributed to the inactivation of PAIs and PIs, other factors such as higher thermal stabilities of the PAs (u-PA and t-PA), compared to inhibitors, and improved efficiency of activation of denatured PG, could contribute to the increased PL activity. The denaturation temperature of bovine PG has been reported to range from 50.1 to 61.6ºC (Burbrink and Hayes, 2006). In this temperature range, PG becomes more accessible to the action of PL, due to conformational changes and the partial unfolding of the kringles, exposing the Lys_{77}-Arg_{78} bond. Inhibitors of the PL system are heat-labile, with researchers reporting almost complete inactivation of PAIs on heating milk to 75ºC for 15 s, with PIs being less affected by the heat treatment (retains two-thirds of its activity) (Prado *et al*., 2006). Unlike the heat-labile inhibitors, PAs are more heat-stable and are capable of surviving pasteurisation conditions. Researchers have reported that u-PA displays greater heat stability than t-PA when milk is heated to 85ºC for 30 s, with t-PA losing half of its activity, while only 30% of u-PA activity is lost (Prado *et al*., 2007). The sensitivity of PAIs and PIs to mild heat treatments, in addition to the higher heat stability of PAs, may explain the increased PL activities in milk subjected to pasteurisation-like conditions.

More severe heat treatments applied to milk include UHT processing and retort sterilisation. In such processes, more severe heat treatments are applied, with time-temperature combinations of 135-150ºC for 1-5 s and 115-120ºC for 12-20 min for UHT and sterilisation, respectively. D-values of 13 and 11 s have been reported for PL and PLG in bovine milk, respectively, at 140°C, indicating that PL can survive most UHT treatments (Saint Denis *et al*., 2001). Studies have also shown that PAs not only survive mild heat treatments but are also capable of surviving UHT treatments; D-values of 16 s at 140°C have been reported for PAs in bovine milk. The presence of remaining PG and PAs, in addition to the destruction of inhibitors of the PL system, indicates that some residual PL activity remains after heat treatment.

Heating also results in the interaction of PL system components with milk constituents, namely β-lg, the predominant whey protein in bovine milk. β-Lg contains a free sulphydryl (-SH) group, buried within its globular structure and, on heating at >55ºC, the 3-dimensional structure begins to unfold, exposing the sulphydryl group on Cys_{121}. The subsequent decrease in PL activity is believed to be due to the free sulphydryl group of β-lg interacting with the disulphide bonds which link subunits within the PL molecule, *via* thiol disulphide interchange (Kelly and Foley, 1997). β-Lg has also been shown to affect PG; the conformation change that occurs in PG on heating may promote interactions with denatured β-lg. Burbrink and Hayes (2006) observed a decrease in PG-derived PL activity on heating milk to high temperatures (>62°C), with no added u-PA; the authors concluded that the formation of PG-β-lg complexes on heating may interfere with the activation of PG to PL, in addition to affecting the accessibility of PAs to PG. Thus, PL activity can ultimately be influenced

by the specific heat treatment directly or indirectly by the interactions of milk constituents with the components of the PL system on heating.

Cold storage

Many dairy-based products are refrigerated (≤5°C) to reduce microbial growth and extend shelf-life. The temperature at which milk is stored prior to processing can greatly affect PL activity and the quality of products made from the resulting milk. In industry, milk is primarily stored at low temperatures (2-5ºC) prior to processing. Milk is also rapidly cooled from 37 to <5ºC on the farm immediately after milking, and stored at this temperature until collection. Although the optimum temperature for PL activity is 37ºC, PL can remain active at cold temperatures; indeed, PL-induced proteolysis occurs during the storage of milk at low temperatures albeit to a lower extent at higher temperatures (i.e., 37°C). Although PL activity is higher at 37 ºC than at lower temperatures (i.e., 5°C), the decrease in PL activity is more rapid during storage at this temperature compared to at the lower temperatures; this can be attributed to the autolysis of PL (Crudden *et al*., 2005). Storage of milk at refrigeration temperatures causes the dissociation of β-casein from the casein micelle into the serum phase of milk. This phenomenon results in closer proximity of PL and its principal substrate, β-casein; however, studies have shown that higher levels of intact β-casein are present at low temperature compared to at 37°C, due to lower plasmin activity and therefore lower rates of casein hydrolysis.

Ambient storage

UHT milk and sterilised milk-based products are normally stored at room temperature. In these products, the inhibitors of the PL system are already inactivated

due to the high heat treatment applied to such products; in addition to this, they are stored at room temperature (20-25ºC) conditions, which are close to the optimum temperature (37ºC) for PG activation and PL activity. Residual PL activity in these products can lead to casein hydrolysis, ultimately resulting in age gelation (formation of a soft, fragile gel) of UHT milk.

Microbial proteases

Microbial proteases (often metalloproteases) produced by psychrotrophic microorganisms can cause proteolysis during refrigerated storage. Some bacteria have the capacity to enhance PG activation or, in some instances, actually produce PAs on growth (e.g., *Streptococcus uberis*; Johnsen *et al*., 1999). Additionally, some extracellular bacterial proteases have the ability to interact with and disrupt the distribution of PL system components, altering the rate of proteolysis and the location of PL, potentially affecting the quality of milk-based systems (Fajardo-Lira *et al*., 2000).

Membrane filtration

The filtration of milk can greatly influence PL activity. Cheese produced from UF milk exhibits slower proteolytic action during storage, with reduced hydrolysis of β- and α_s-caseins, due to increased levels of native whey protein in UF-cheeses compared to traditional cheese, which inhibits PL.

Microfiltration (MF) can also affect the PL system due to the separation of certain components within the PL system. The MF of milk often involves the separation of casein from whey proteins. As many of the inhibitors of the PL system

are present in the serum phase of milk (whey), removal of whey can alter the PL system. During the MF of skim milk with a 0.1 µm pore size membrane, PL and PG are retained in the retentate, as they are both associated with casein micelles and the removal of whey proteins in the permeate results in increased PG activation, resulting in increased PL activity in the retentate (Aaltonen and Ollikainen, 2011).

High pressure

High pressure treatment is a non-thermal treatment which can extend shelf-life by denaturing enzymes and inactivating microorganisms without negatively affecting the sensory and nutritional qualities of food products. High pressure has been shown to reduce PL activity in milk, with pressures of 600 MPa for 10 min causing 50% inactivation of PL. Pressurisation at higher temperatures (400 MPa at 60°C for 15 min) has been shown to result in greater inactivation of PL (86.5% reduction in PL activity), subsequently reducing PL-mediated proteolysis (García-Risco *et al*., 2000). Casein micelles, when subjected to high pressure, can dissociate, due to the disruption of hydrophobic and electrostatic interactions, which may result in the dissociation of PL and PG from the micelle into the serum phase. This would result in increased contact with inhibitors of the PL system present in the serum, resulting in enhanced inactivation and lower activity. In addition, high pressure treatment causes the denaturation of whey proteins, in particular β-lg, which have been shown to act as proteinase inhibitors. However, increased proteolysis in milk can result when subjected to pressures of 300-500 MPa, possibly due to the changes in the structure of casein micelles and dissociation of some caseins from the micelle, increasing the accessibility of protein for proteolysis (Scollard *et al*., 2000).

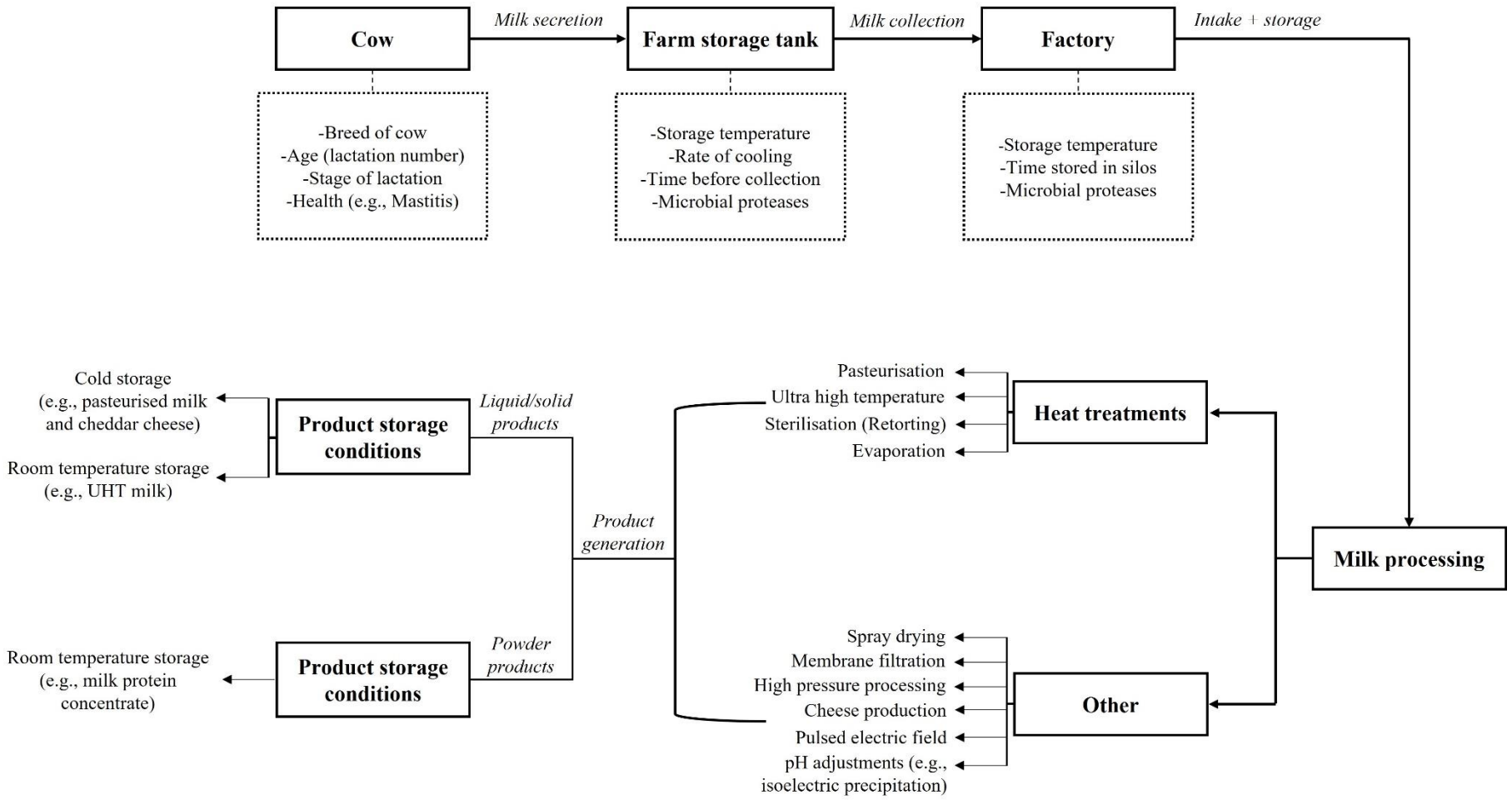


Figure 2.3: *Schematic representation of factors affecting plasmin activity from milk secretion to the final*

Measurement of plasmin and plasminogen

There are several methods used for determining PL and PG-derived activity in milk, varying from indirect monitoring of degradation products of milk proteins, to fluorometric and chromogenic assays for direct measurement of plasmin activity. Methods originally employed for determining PL activity involved monitoring degradation of milk proteins, such as quantifying the rate of formation of γ-caseins. Chromogenic and fluorometric assays using synthetic substrates are routinely used for measuring PL and PG-derived activity. PG-derived activity is defined as the PL activity resulting from the addition of a defined amount of urokinase. The amount of PG present can be calculated by subtracting PL activity with no added urokinase (i.e., only PL) from PL activity with added urokinase (i.e., PL + PG). Other methods used in the measurement of PL activity include enzyme-linked immunosorbent assay **(**ELISA) methodology and spectrophotometric assays. Indirect assays for determining PL-mediated proteolysis involve measuring the proteolysis products by reversed-phase high performance liquid chromatography (RP-HPLC) or mass spectrometry

Significance of plasmin and its applications

PL can have both positive and negative impacts on a wide range of dairy products, with UHT milk and cheeses the principal dairy products that have been studied. Understanding the components of the PL system and their interaction with milk constituents is important to optimise the quality of dairy products.

UHT products

PL is a heat-stable enzyme capable of surviving UHT treatment. There have been many studies into the role of PL in the destabilisation of UHT milk-based products (e.g., age gelation) with processing conditions, storage conditions, other ingredients used in the products and initial PL level all being shown to be major factors influencing age gelation. PL-mediated proteolysis of caseins (β- and α_s-caseins) has been shown to result in age gelation of UHT milk. Hydrolysis of caseins by PL during the storage of UHT milk results in the production of a soft, fragile gel. In order to reduce PL-mediated gelation, as well as stabilise β-lg, an integrated preheating step (80-95°C for 30-60 s) is often implemented prior to final UHT treatment (Datta and Deeth, 2001; Newstead *et al*., 2006). Researchers have reported that implementing mild heat treatments reduces PL activity in the final UHT-treated product through the formation of PL-β-lg interactions, reducing PL-mediated hydrolysis of casein and age gelation.

Cheese

Research has shown that PL can influence cheesemaking properties. Although studies have shown that PL may not affect rennet coagulation times, high PL activity has been shown to result in poor curd forming rates and reduced curd firmness. Unlike the role of PL in UHT products, PL is of considerable beneficial significance in cheese ripening. PL-mediated hydrolysis during cheese ripening leads to the production of peptides which contribute to the organoleptic profile of the cheese as well as texture development. The contribution of PL to cheese ripening depends on various factors, such as the variety of cheese, cooking temperature of the coagulum, pH of the cheese during maturation and the ripening temperature. PL activity is high in Gouda,

Emmental and Romano-type cheeses, while Cheddar and Cheshire cheeses generally exhibit lower PL activity. The higher PL activity in Swiss compared to in Cheddar cheese was initially thought to be due to differences in pH at drainage or due to different salting procedures, as salt has been shown to cause the dissociation of PL from the micelle, and thus some PL is lost in the whey. However, later research found that the higher PL activity in Swiss-type cheese was due to higher cooking temperatures. Researchers have found that increasing curd cooking temperature results in increased plasmin activity in the resulting cheese, due to increased activation of PG (Somers and Kelly, 2002). Therefore, in high-cooked cheese varieties, the contribution of PL to primary proteolysis is most significant as the coagulant (i.e., chymosin) is extensively inactivated (Sheehan *et al*., 2007). During cheese ripening, β-casein is hydrolysed by PL, resulting in the formation of γ-caseins and proteose peptones.

Cheese ripening is an expensive and time-consuming process, and therefore acceleration of ripening is desirable. Increasing ripening temperature increases the rate of PL-mediated hydrolysis; however, unbalanced flavour profiles have resulted in limitations. On the other hand, the addition of exogenous PL has been shown to increase PL-mediated hydrolysis in cheese while avoiding defects such as off-flavour (Farkye and Fox, 1992; Kelly, 1995); however, from an economic perspective it is not feasible, due to its high cost. The use of streptokinase-producing *Lactococcus lactis* (a PA producing strain) in the production of Cheddar-type cheese, has been shown to result in increased plasmin activity (~three-fold increase compared to cheese manufactured with control strain) and higher levels of plasmin-mediated hydrolysis of β-casein (Upadhyay *et al*., 2006).

Milk protein ingredients

Whey protein and casein-containing milk protein ingredients are widely used in the formulation of food systems (e.g., beverages and coffee creamers). As PL and PG are primarily associated with casein micelles, the presence of PL in whey is generally lower than that of milk. PL activity is higher in acid whey compared to sweet whey, due to the dissociation of PL from the casein micelle and into the serum (whey). Therefore, studies have shown that higher PL activity is associated with acid whey-derived protein ingredients compared to sweet whey-derived protein ingredients (Gazi *et al*., 2014).

As with whey protein ingredients, residual PL activity in casein-containing ingredients can lead to flavour and stability issues. Studies have shown extensive PL-mediated casein hydrolysis following storage of reconstituted casein-containing milk protein ingredients (e.g., micellar casein isolate and milk protein concentrate). The presence of PL in whey protein ingredients and casein-containing ingredients may have consequences for the quality of food products that incorporate such ingredients and, therefore, should be a factor when selecting milk protein ingredients for food formulation.

Future trends

Extensive research has been conducted on identifying and characterising the components of the PL system and understanding the factors that affect the different components of the PL system. However, to facilitate greater control of PL activity, our understanding of the PL system and the interactions between PL and other constituents in dairy streams/products needs to improve. The mechanisms behind PL inhibition, factors that influence PA kinetics, and the mechanisms responsible for interactions

between β-lg and PL/PG need to be further studied. Additional research is also required into PL activity in milk protein ingredients, as residual activity in these ingredients can have negative consequences when applied in other products. Manipulation of heat treatments and storage temperature, along with additional research into PL activity in dairy products, might allow for improved control of the PL system, allowing for improved quality and tailored functionality of these dairy products.

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Appendix 3

Composition of simulated milk ultrafiltrate resembling the mineral profile of ultrafiltration permeate generated from skim milk at 10 and 50 °C

Table 3.1: *Quantities of salts required for the preparation of simulated milk ultrafiltrate (SMUF) resembling the mineral profile of ultrafiltration permeate produced from skim milk at 10 and 50 °C as described in Dumpler et al. (2017).*

Component	Weight (g L^{-1})		Amount (mM; SMUF 10 °C/SMUF 50 °C)							
	SMUF 10 °C	SMUF 50 °C	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	PO_4^{3-}	Citrate	SO_4^{2-}
KH_2PO_4	0.933	0.833		6.9/6.1				6.9/6.1		
K_2HPO_4	0.867	0.753		10.0/8.65				5.0/4.3		
$K_3C_6H_5O_7 \cdot H_2O$	0.7	0.533		4.9/3.7					2.1/1.6	
$Na_3C_6H_5O_7 \cdot 2H_2O$	1.2		12.2						4.0	
$Mg_3(C_6H_5O_7)_2 \cdot 9H_2O$	0.6					2.9			1.9	
$C_6H_8O_7 \cdot H_2O$	0.057								0.3	
KCl	0.633	0.933		8.5/12.5			8.5/12.5			
NaCl	0.167		2.9				2.9			
K_2SO_4	0.2			2.3						1.15
$CaCl_2 \cdot 2H_2O$	1.233	0.933			8.4/6.35		17.7/12.7			
KOH	0.112	0.056		2.0/0.7						
Sum			15.1	34.5/35.2	8.4/6.34	2.9	29.0/28.1	11.9/10.5	8.4/7.9	1.15

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