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Process and ingredient interactions in the processing of advanced dairy based nutritional formulae

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I dedicate this work to my ever-patient husband Matthew.

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List of Abbreviations

Abbreviation	Description
%	Percent
°C	Degrees Celsius
α	Alpha
α - lac	Alpha lactalbumin
aWPC	Acid whey protein concentrate
β	Beta
β - lg	Beta lactoglobulin
Ca^{2+}	Ionic calcium
$CaCl_2$	Calcium chloride
$CaHPO_4$	Calcium hydrogen phosphate
CCP	Colloidal calcium phosphate
cP	Centipoise
cWP	Cheese whey permeate
cWPC	Cheese whey protein concentrate
cWPI	Cheese whey protein isolate
DLS	Dynamic light scattering
DM	Dry matter
et al.	Et alia
Eq.	Equation
FDA	Food and Drugs Authority
FFMP	Fat filled milk powders
Fig.	Figure
g	grams

g	g force
G′	Elastic / storage modulus
G″	Viscous / loss modulus
GMP	Glyco-macro-peptide
H^+	Hydrogen ion
HC aWPC	High calcium acid whey protein concentrate
HCl	Hydrochloric acid
HCT	Heat coagulation time
HTST	High temperature short time
Hz	Hertz
ICP-OES	Inductively coupled plasma - optical emission spectrometry
IDF	International Dairy Federation
IF	Infant formula
ISE	Ion-selective electrode
ISO	International standards office
κ	Kappa
kcal	Kilo calorie
KCL	Potassium chloride
kDa	Kilo daltons
kg	Kilo grams
KOH	Potassium hydroxide
kPa	Kilo pascals
L	Litre
L*	Whiteness index (colour L*a*B*) scale
LC-FLD	Liquid chromatograph fluorescence detection

LVE	Linear viscoelastic region
M	Molar
m^2	Metres squared
mg	Milligrams
min	Minute
mL	Milli litres
mm	Milli metre
mM	Milli molar
MP	Milk Permeate
mPa.S	Milli Pascal seconds
MPC	Milk protein concentrate
mV	Milli Volts
NaOH	Sodium hydroxide
NPN	Non protein Nitrogen
nsCWP	Non salty cheese whey permeate
NSI	Nitrogen solubility index
P	Phosphorous
Pa	Pascals
PDI	Poly dispersity index
R^2	The correlation coefficient
RPM	Revolutions per minute
RTD	Ready to drink
S	Supplementary materials
s	Seconds
s^{-1}	Shear rate

sCWP	Salty cheese whey permeate
SMP	Skim milk powder
SMUF	Simulated milk ultrafiltrate
TSC	Tri sodium citrate
UF	Ultrafiltration
UHT	Ultra-high temperature
v/v	Volume per volume
VCF	Volume concentration factor
w/w	Weight per weight
WPC	Whey protein concentrate
$WPC_{UF4.6}$	Acid WPC which was ultrafiltered at pH 4.6
$WPC_{UF6.5}$	Acid WPC which was ultrafiltered at pH 6.5
WPNI	Whey protein nitrogen index

Declaration

Process and ingredient interactions in the processing of advanced dairy based nutritional formulae.

This is to certify that the work I am submitting is my own and has not been submitted for another degree, at either University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property. All the work described herein is the original work of the author.

Sinead Mc Entee Date:

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Publications and Awards

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Mc Entee, S. A., Kelly, A. L., Lawless, F. N., McCarthy, N. A., & Murphy, E. G., (2024). Effect of ultrafiltration of acid whey at pH 4.6 or 6.5 on acid whey concentrate composition and resultant powder properties. *International Journal of Dairy Technology*, 0.

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Abstract

Advances in dairy protein fractionation and purification have resulted in a wide range of protein ingredients available for use in nutritional products such as infant formula with very different functionalities. In addition, the increased demineralisation of protein ingredients has concomitantly resulted in the production of large quantities of high-mineral containing permeates, which can be challenging to valorise into food applications. However, one major application for these permeates is in protein standardisation of fat-filled milk powders. A key requirement for many nutritional formulations, especially those targeted to infant nutrition or long shelf-life powders, is that they must be able to withstand high temperature processing. Typically, this is related to protein aggregation during heating; however, the content and ionic state of minerals present has a key influence on this aggregation. In this thesis, the thermal stability of protein ingredients with varying mineral contents were examined in model infant formula systems. Meanwhile, to understand the impact of permeate mineral content on the ionic environment and thermal stability of low protein skim concentrates, concentrates standardised with five different standardisation media, each with differing mineral contents and profiles depending on their source and processing history, were produced and subjected to simulated HTST treatment. Through this, a deeper understanding was developed of the interactions of proteins with other ingredients and mitigation strategies to control aggregation during heating.

The amino acid profile of acid whey makes it highly suitable for infant formula (IF); however, due to a higher calcium content than cheese whey, acid whey protein concentrate (WPC) is considered to have poor thermal stability, which has limited its incorporation into IF. During this study, it was found that the pH and temperature of ultrafiltration are key to determining the final calcium and phosphorous content of acid WPC, with increases in these parameters resulting in higher calcium phosphate levels in the final product. Subsequent

manufacture of acid WPC 35, with elevated calcium and phosphorus levels, highlighted the challenges faced during downstream processing, such as high viscosity following evaporation. In addition, when added to a model IF system, this high level of innate calcium caused significant viscosity increases during heating. However, it was also shown that further demineralisation of acid WPC 35 to an acid WPC 60 significantly improved its thermal stability and so could be an option for increased usage of acid whey proteins in IF applications.

While much of this thesis focused on protein-mineral interactions in model IF systems, these interactions affect all nutritional dairy processes. The impact of minerals in permeate on the thermal stability of low protein skim concentrates could clearly be seen, with higher-mineral concentrates undergoing significant gelation during HTST treatment. However, by increasing pH, the viscosity of all permeate-standardised concentrates were significantly reduced following heat treatment, proving that, by controlling the ionic environment, heat-stable low-protein concentrates with a high mineral content are feasible.

In conclusion, throughout these studies, the role of minerals and their ionic state has been shown to play a key part not only in formulation thermal stability, but also in the composition and functionality of individual protein ingredients. The results of the work have shown that there can be significant benefits to the IF industry by using existing acid whey protein streams, in terms of amino acid and mineral profile. Tailoring the mineral content of acid whey during membrane processing could also further enhance the usage of this ingredient by improving high heat stability in IF applications. Finally, permeates, derived from MPC and WPC manufacture, have major applications in fat filled milk powder production, but vary widely in their impact on heat stability and viscosity, due to the significant variation in their mineral profile. Therefore, an in-depth understanding of the ionic state of minerals present, their potential interactions with proteins during processing, and possible interventions that

could minimise protein aggregation is imperative to ensure high quality nutritional formulae can be produced efficiently.

Introduction and Research Objectives

The pursuit of the humanisation of IF has driven significant advances in dairy protein fractionation and purification, with ingredients now available which are widely different in terms of composition and functionality. However, despite these advances, IF is fundamentally a low protein, typically whey-dominant product with an appreciable high proportion of minerals and so understanding the interactions of ingredients in this environment is key. It is known that minerals, in particular ionic calcium, can significantly promote whey protein aggregation; however, the ionic state of these minerals at the point of thermal processing is instrumental in determining stability. A range of membrane-concentrated casein and whey protein ingredients are available for IF with both high and low levels of minerals. However, typically highly purified protein ingredients such as milk protein concentrate or whey protein isolate are more expensive per gram of protein than lower protein, higher mineral skim, or WPC 35; thus, given that these ingredients will be combined with significant amounts of minerals to produce an IF, the benefit of taking on the additional cost of using purified, low mineral protein sources may be questioned. A key objective of the studies reported in this thesis was to understand the effect of the innate mineral profile and content of casein and whey protein ingredients on thermal stability of a model IF system.

In addition, the amino acid profile of acid whey, due to a lack of glyco-macro-peptide is known to be better suited to IF applications than cheese whey sources; however, due to a higher calcium content, it is considered more reactive in IF processing and prone to instability. Part of this research aimed to gain a greater understanding of the impact of the mineral profile of acid WPC 35 on IF stability and also to investigate the potential for optimisation of the ultrafiltration of acid whey which could support the demineralisation and improved thermal stability of this protein ingredient.

Finally, while highly purified protein ingredients may be better suited to certain applications, their purification results in the production of large quantities of permeate rich in minerals which in many cases are low value and often considered a waste stream. With the dairy industry coming under increased pressure to become more sustainable, there is increased focus on finding uses for these side streams, one of which is in the protein standardisation of fat-filled milk powders. Fat filled milk powder (FFMP) is generally manufactured by combining protein-standardised skim milk with vegetable fat prior to spray drying to produce a more affordable dairy powder suitable for use in a range of food applications as an alternative to milk. While permeate is considered a low cost option to standardise the protein level in FFMP and affordable low protein FFMP powders, differences in the mineral profile of the permeate due to its source and processing history can significantly influence the thermal stability of low protein concentrates prior to spray drying. Therefore, as part of this thesis, another objective was to assess the suitability of permeates from different sources and with different processing histories on the thermal stability of low protein skim concentrates which could be used in FFMP manufacture.

Thus, the overall aim of this research focused on understanding interactions of ingredients during processing of advanced dairy-based nutritional products and the impact of minerals and their ionic state on their thermal stability. In order to achieve this, the research objectives of this work were as follows:

- To review the published literature on the ingredients used in the manufacture of IF and their impact on heat stability (Chapter 1);
- To investigate the effect of innate mineral composition of protein ingredients on their thermal stability when blended to ratios typical for infant formula, alone or with the addition of lactose (Chapter 2);

- To understand the effect of processing conditions during ultrafiltration on the effectiveness of the removal of calcium and phosphorus from acid WPC (Chapter 3);
- To investigate the effect of a high calcium content following ultrafiltration on heat treatment, evaporation and spray-drying of acid WPC 35 and the properties of the subsequent powder (Chapter 4);
- To examine the impact of whey protein ingredient mineral content and composition on the thermal stability of concentrated model IF systems with major minerals standardised through addition of suitable ingredients prior to heat treatment (Chapter 5);
- To examine the effect of the mineral content of standardisation media on the thermal stability of low protein skim concentrates suitable for FFMP manufacture (Chapter 6).

A schematic of the research conducted is shown below.

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Chapter 1

Infant formula – Effect of protein and mineral composition and interactions on heat stability during manufacture.

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Declaration

This chapter was written by Sinead Mc Entee (SME) and reviewed by all co-authors.

1.1. Abstract

Infant formula is an acceptable alternative source of nutrition for infants when human milk is not available. As a result, research into infant formula (IF) is continually evolving to develop bovine milk-derived ingredients and technologies which will better emulate the nutritional and functional quality of human milk. As IF formulations are whey dominant (whey: casein, 60: 40), the whey fraction has been of particular interest. For whey in general, some research has focused on the effect of the original casein removal step e.g., cheese-making vs. acid precipitation on amino acid profile and mineral profile of resulting whey. In recent years, significant advances have been made in the commercialisation of whey protein fractions, such as lactoferrin and α-lactalbumin, which have provided a significant step towards matching the protein profile of human milk. Likewise, formulations can now be enriched in the most prominent human-milk casein (β-casein) via means of enriched bovine derived ingredients. However, the thermal stability and interactions of these new ingredients with other proteins, minerals, and lactose in an IF process must be considered in order to produce a shelf-stable product. This review discusses a number of modifications which can be made to the protein content of infant formulations to more closely imitate the functional/ nutritional content of human milk and how these may affect the mineral balance and subsequent thermal stability of formulations.

1.2. Introduction

Infant formula (IF) is a breast milk substitute which is suitable as the sole source of nutrition for an infant until appropriate complimentary food can be introduced (Codex Alimentarius, 2007; European Commission, 2006). It is available in a liquid ready-to-drink format or as a powder which is reconstituted using sterile water prior to feeding. IF is typically manufactured through blending protein streams with lactose, fat, minerals, and vitamins at the required nutritional levels for each stage of infant development. The protein source for IF is most commonly bovine-based; however, plant-based offerings using non-dairy proteins such as soya are available. The ingredients used in IF manufacture can vary depending on the specific dietary needs of the infant; Table 1.1 outlines the typical ingredients that can be used in IF manufacture.

Table 1.1. Ingredients used in IF manufacture.

Nutritional component	Source ingredient
Protein – Casein	Skim milk powder, skim milk concentrate, acid casein, sodium /potassium/calcium caseinate, milk protein concentrate (MPC), milk protein isolate (MPI)
Protein – Whey Protein	Demineralised whey powder (DWP), whey protein concentrate (WPC) from cheese or acid casein production, whey protein isolate (WPI), hydrolysed whey ingredients, lactoferrin, α- lactalbumin, β-casein
Protein – Alternative sources	Soy milk, soy protein isolate, locust bean protein, amino acids (L form)
Fat / Oils	Soy, corn, safflower, sunflower, colza, palm, copra, structured lipids, milk fat
Carbohydrates	Lactose, starch, sucrose, corn syrup, corn syrup solids, maltodextrin
Major minerals (non-exhaustive)	Calcium carbonate, calcium phosphates, dibasic magnesium phosphate, potassium citrate, magnesium chloride
Minor minerals (non-exhaustive)	Potassium iodide, ferrous sulphate, manganese sulphate, copper sulphate, zinc sulphate
Vitamins	A, D, E, K, B1, B2, B6, B12, niacin, folic acid, pantothenic acid, biotin, choline, inositol
Functional Ingredients	Soy lecithin, mono- and di-glycerides

(Murphy, 2014)

While each IF manufacturer may modify their process to optimise the nutritional quality and processing efficiency, most powder manufacturing processes can be broadly classified into one of 3 groups: wet mixing, dry blending or a combination of wet and dry blending (Kent et al., 2015). In a wet-mix process, ingredients in a liquid or powder format are rehydrated and

blended to create a wet-mix formula. The wet-mix will then undergo a series of heat treatment steps, homogenisation, and, in the case of an IF powder process, concentration and spray-drying before packaging. By combining all the ingredients and subjecting the formula to heat treatment at pasteurisation or UHT temperatures, the IF manufacturer can ensure the microbial safety of the product before packaging (Murphy, 2014).

A dry blend process uses only powdered ingredients, which are blended in powder form to the ratios required before packaging. This process requires significantly less capital investment than a wet mix process, as the final blend does not undergo heat treatment or drying prior to packaging. However, as there is no microbial reduction step, all potential contaminants must be strictly controlled to ensure microbial safety of the final product (Kent et al., 2015). A combination process uses a mix of wet mix processing and dry blending of ingredients following spray-drying.

A critical factor in any IF manufacturing process is ensuring the microbial safety of the product. For this reason, processes for manufacturing IF-grade ingredients or final IF products often have multiple high heat treatment steps. As a result, ingredients and formulations must be able to maintain functionality after high temperature processing. Therefore, an understanding of the effect of heat treatment on ingredients and the reactions that take place between ingredients once blended, concentrated, and heat-treated is key to developing a stable final product.

The aim of this review is to discuss recent advances in the humanisation of IF protein content and potential challenges faced in incorporating these ingredients into products where interactions with other components (e.g., minerals, carbohydrates) could negatively affect thermal stability.

1.1.1 IF Nutrition and regulations

As IF is the sole source of nutrition for an infant, its composition is tightly regulated to ensure it meets the nutritional needs for healthy growth and development, with detailed compositional standards published by the FDA (2017), European Commission (2006) and Codex Alimentarius (2007). A key area of scrutiny for these regulations is the product's protein content and amino acid profile, which play a vital role in growth and development. As human milk is the benchmark, all regulatory authorities stipulate that IF must at least match the essential and semi-essential amino acid content of human milk (Codex Alimentarius, 2007). However, as the protein profile and bioavailability of amino acids in bovine or non-dairy protein differs from that of human milk protein, the regulated minimum protein content for IF is currently higher than that of human breast milk, in order to ensure that these essential requirements for growth and development are met.

Mature human milk typically has an average protein content of ~0.9 g 100 $mL^{-1}$with a casein: whey protein ratio of 40:60, which delivers the optimum amino acid quantity for infant growth and development (Feng et al., 2016; Gurr, 1981). However, depending on a number of factors, this can range from between 0.6 to 1.3 protein g/100 mL with the whey protein: casein ratio varying from approximately 90:10 in early lactation, 60:40 in mature breast milk to 50:50 in late lactation milk, with a high degree of variation between mothers (Gurr, 1981; Kunz & Lönnerdal, 1992; Liao et al., 2017; Thompkinson & Kharb, 2007). This is significantly different to bovine milk, which typically has a protein content of 3.3 – 3.5% and a casein: whey protein ratio of 80:20 with some variation across lactation and season particularly in pasture-based feeding systems where weather can affect milk composition (Gurr, 1981). Table 1.2 shows a comparison of the amino acid profile of human milk as outlined by Codex and the European commission compared to bovine milk, casein, and whey protein. It can clearly be seen that, if bovine milk with its native 80:20 casein: whey protein ratio was used in IF, the

minimum requirements for some essential amino acids such as cysteine and tryptophan would not be achieved. However, as bovine whey protein contains higher levels of these essential amino acids, by increasing the proportion of whey protein in the formula, the minimum essential amino acid requirements can be achieved. It is on this basis that IF manufacturers blend bovine casein and whey protein ingredients to develop a formulation with a casein: whey protein ratio which enables a reduction in total protein content while still ensuring amino acid requirements are met. While many commercial IF products are whey-dominant, with a casein: whey protein ratio of 40:60 commonly targeted, a wide range of IF and follow-on products are available on the market with broad array of casein: whey protein ratios depending on the targeted nutritional stage of the infant (Thompkinson & Kharb, 2007).

Table 1.2. Comparison of amino acid profiles of human and bovine milk

	Human milk	Bovine milk	Bovine Casein	Bovine Whey protein
		mg g protein^{-1}		
Cysteine	19	10	12	17
Histidine	23	24	25	16
Isoleucine	54	57	60	48
Leucine	97	100	108	91
Lysine	63	70	63	78
Methionine	16	23	21	22
Phenylalanine	45	46	46	40
Threonine	44	43	40	57
Tryptophan	21	13	8	19
Tyrosine	42	54	56	43
Valine	55	58	54	56

(Codex Alimentarius, 2007; European Commission, 2006; Heine, 1994; Rafiq et. al., 2016; Swaisgood, 1982)

Often when discussing protein in IF, the casein: whey ratio and protein type is referenced. However, due to differences in the casein and whey protein composition of bovine milk, many formulae on the market can have significantly higher total protein contents than human milk, with the maximum allowable protein content as high as 2 g 100 mL^{-1} (based on a 67 kcal formula) (Codex Alimentarius, 2007; European Commission, 2006; Food and Drug Administration, 2017). Regulatory authorities stipulate that bovine IF must contain a minimum of 1.2 g 100 mL^{-1} protein (based on a 67 kcal formula) in order to ensure sufficient quantities of available amino acids, which is still 33% higher than the typical protein content of mature human breast milk (Codex Alimentarius, 2007; European Commission, 2006; Food and Drug Administration, 2017). Recent studies have shown that the higher protein content in IF is linked to increased risk of obesity in later life and so significant research is ongoing to humanise IF and bring it more closely in line with human milk (Layman et al., 2018; Luque et al., 2015).

1.3. Protein

1.3.1. IF protein composition and the importance of protein type from a nutritional and physicochemical viewpoint

As previously mentioned, whey-dominant formulations are most common in IF as this allows for a product with a lower protein content while still achieving the minimum levels of essential amino acids required. A high proportion of whey protein can bring issues with high heat processing during IF manufacture, due to its sensitivity to temperatures > 70 °C (Joyce et al., 2018; Simmons et al., 2007). Bovine whey proteins are compact globular structures which unfold during denaturation, exposing thiol groups of β-lactoglobulin (β-lg) which can form disulphide bonds with other proteins, leading to aggregation, increased viscosity in the formula, and in extreme cases sedimentation of protein (Crowley et al., 2016; Singh, 2009). This denaturation can be controlled by adapting the formula to contain increased levels of casein,

which has been found to have a protective effect against whey protein aggregation whilst still achieving the minimum essential amino acid requirements. An alternative approach involves incorporating pre heat treatment steps to the whey stream prior to mineral addition so as to pre-denature the whey proteins, thereby reducing the risk of aggregation during UHT heat treatment (Beaulieu et al., 1999; Kelleher et al., 2020).

However, while IF manufacturers have the ability to achieve a degree of similarity between bovine IF and human milk on a casein: whey protein ratio basis, differences in the amino acid profile remain. These differences are due to differences in the sub-class of casein and whey protein found in human and bovine milk in particular β-casein, α-lactalbumin and lactoferrin (Table 1.3). In addition, many IF manufacturers use whey sourced from cheese manufacture. Cheese whey contains glyco-macro peptide (GMP) which has been cleaved from the casein micelle by chymosin during cheese manufacture, and therefore transfers to the whey stream, and can make up approximately 12 % of the protein in whey, resulting in a significant alteration in the amino acid profile (Dutta et al., 2017). GMP is rich in branch-chained amino acids; however, it is deficient in the essential amino acid tryptophan (Dutta et al., 2017). To compensate for this, manufacturers using cheese-sourced whey have to use higher proportions of whey in the formulation in order to meet the minimal regulatory requirements for tryptophan, which results in an increase in the total protein content. This overdosing of protein in IF has been suggested to cause increased metabolic stress on the immature kidneys and liver of the infant and has been linked with increased secretion of insulin and insulin-like growth factor, resulting in increased weight gain and risk of obesity in later life (Layman et al., 2018; Luque et al., 2015). While human milk has an average protein content of 0.6-1.3 g 100 mL^{-1}, many IF products have a protein content of 1.3-1.5 g 100 mL^{-1}, which is up to 40 % higher than that of human milk (Layman et al., 2018; Trabulsi et al., 2011). However, there is scope to further humanise the protein profile of IF through the avoidance of GMP through the use of whey from

non-cheese sources such as mineral acid whey and addition of α-lactalbumin, lactoferrin and β-casein. If this could be achieved, there is potential to develop an IF with a lower overall protein content which still delivers all the essential amino acids to the infant needed for safe growth and development.

Table 1.3. Protein compositions of human and bovine milk

Constituent	Human milk	Bovine milk
Total Protein %	1.00	3.4
Casein % (% of total protein)	0.3	2.6
Whey %	0.7	0.8
Casein: Whey ratio	30:70	80:20
Caseins (% of total)		
α_{s2} - Casein	-	40
α_{s1} - Casein	-	8
β – Casein	85	38
κ – Casein	15	12
Whey Proteins (% of total)		
α– lactalbumin	26	17
β-lactoglobulin	-	43
Lactoferrin	26	Trace
Serum Albumin	10	5
Lysozyme	10	Trace
Immunoglobulins	16 (IgA)	10 (IgG)
Other minor proteins	12	24

(Guo, 2009)

In recent years, significant advances in bovine protein fractionation in particular the isolation of whey proteins such as α-lactalbumin (α-lac) and lactoferrin have allowed for the advancement of the protein profile of IF to more closely align with human milk. While the objective if the industry is to humanise IF, these changes in protein profile significantly alter the processing characteristics of the formulations with each protein type having unique physiochemical characteristics.

1.3.2. Acid whey – benefits to IF and processing challenges

Whey produced as a by-product of the acidification of milk either to produce acid casein, Greek style yoghurt or cottage cheese is known as acid whey. Unlike cheese whey where GMP is released from casein through the cleavage of κ-casein by chymosin, acid whey does not contain GMP as the acidification process does not affect κ-casein (Menchik et al., 2019; Singh, 2009). Therefore, in an IF formulation, acid whey contains higher levels of tryptophan and cysteine per gram of protein than cheese whey and so could enable the formulation to achieve the essential amino acid targets at a lower overall protein content (Tinghäll Nilsson et al., 2023). Research published in Mc Entee, et al., (2023b) indicates that IF produced with acid whey could achieve the minimum tryptophan levels at 1 g protein per 100 mL formula while IF produced from cheese whey sources requires 1.2 g protein per 100mL to meet the required tryptophan levels. Despite this, cheese whey remains the most prevalent choice for whey protein in IF. This is because formulations produced with acid whey are considered to be more heat sensitive and can have issues with high viscosity during processing when compared against formulations with cheese whey (Mc Entee, et al., 2023b).

When milk is acidified, colloidal calcium phosphate is released from casein into the liquid phase as ionic calcium and phosphate. Therefore, acid whey contains significantly higher levels of calcium than cheese whey with unprocessed mineral acid whey containing up to 3

times more calcium than cheese whey (Bylund, 1995; Singh, 2009). However, the mineral composition of acid whey can vary depending on the method of manufacture. Acid whey from acid casein manufacture is produced by the rapid acidification of skim milk to close to or below the isoelectric point of casein (pH 4.6) using either an organic acid such as lactic, citric, or acetic acid, or a mineral acid such as hydrochloric or sulphuric acid. In this scenario, calcium phosphate is released rapidly from casein into the whey phase, resulting in the majority of the calcium and phosphate present in casein being released into the serum whey phase (Bylund, 1995). This high calcium content negatively affects the thermal stability of acid whey as high levels of ionic calcium promote calcium-mediated aggregation of whey proteins at temperatures above 70 °C which may be the reason for the lower thermal stability observed in acid-whey containing IF formulations compared to cheese whey containing IF (Joyce, Kelly, & O'Mahony, 2018). In addition, depending on the type of acidulant used, mineral acid whey can also contain elevated levels of other minerals such as chloride which are undesirable in protein ingredients targeted to IF applications (Rocha-Mendoza et al., 2021). In this thesis, mineral acid whey produced by acidification with hydrochloric acid was examined and modified for incorporation into IF applications.

Other sources of acid whey such as the manufacture of Greek style yoghurt, soft cheese, etc typially have much slower, fermentation-based, acidification processes which results in the formation of a casein gel structure rather than a precipitated curd. In this scenario, calcium can form part of the casein gel structure therefore remaining with the casein stream and resulting in a whey with a lower calcium and phosphorous content than an acid casein produced whey (70 mg calcium 100 g^{-1} in cottage cheese whey compared to 120 mg calcium 100 g^{-1} in HCL precipitated acid casein whey (Bylund, 1995; Mencik et al., 2019; Rocha-Mendoza et al., 2021).

Prior to inclusion in IF whey, including acid whey, may be concentrated by membrane ultra filtration to reduce mineral content so that subsequent IF formulations can meet regulatory specifications. This process will also improve the thermal stabiltiy of the acid whey by removing some of the excess calcium (Caussin, et al., 2003; Hendrix, Griko, & Privalov, 2000; Wijayanti, Bansal, & Deeth, 2014). However, the high calcium content of acid whey can pose challenges due to calcium phosphate fouling during membrane filtration resulting in lower membrane flux rates when compared to cheese whey (Mc Entee, et al., 2023a; Steinhauer et al., 2015). This in turn can affect the degree of calcium removal during ultrafiltration, with acid WPC products typically having a higher calcium content than cheese WPC's of the same protein concentration due to a combination of higher starting calcium content and poorer transmission of calcium.

In an IF formulation the minerals are balanced and so in theory it might be expected that once all the minerals have been added, formulations produced with cheese whey would have a similar thermal stability. However, as IF produced using cheese whey has a lower proportion of calcium coming from the protein streams, a manufacturer has the option to add insoluble forms of calcium such as tri calcium phosphate which will not react with protein during thermal processing. In contrast, IF produced from acid whey has a higher proportion of calcium coming from the protein stream which may be predominantly ionic calcium and therefore highly likely to associate with whey proteins during heat treatment (Barone et al., 2022). Chelating agents such as citrates and phosphate can be added to the formulation to minimise calcium mediated aggregation however as these can also alter pH it can result in a more challenging and complicated process flow (Hebishy et al., 2019). Therefore, while there may be a nutritional advantage for using acid WPC as the whey protein source in IF, the processing challenges which must be overcome to avoid viscosity issues during heat treatment can limit its application.

1.3.3. α-lactalbumin – methods of manufacture and its impact on IF

A number of studies have shown that, by enriching α-lac in IF to human milk levels, the protein content of IF can be reduced while ensuring sufficient delivery of essential amino acids to the infant (Davis et al., 2008; Lien et al., 2004; Trabulsi et al., 2011). Trabulsi et al. (2011) performed a randomised control clinical study comparing the growth of healthy term formula fed infants fed a standard formula with a protein content of 14.1 g L^{-1} against an experimental formula with a protein content of 12.8 g L^{-1} which had been enriched with α-lac to human milk levels. A reference control group of breast-fed infants were also examined. In this study, it was observed that infants fed the lower protein α lac-enriched formula had age-appropriate growth and weight gain and, perhaps more interestingly, their growth pattern was similar to that of the breast-fed infants. This suggests that, not only is it safe to reduce the protein content of IF provided the essential amino acid requirements are met but, by doing so, the risk of excessive weight gain and metabolic stress in infants can be reduced. Additional benefits in terms of digestibility have also been observed when formulae have been enriched in α- lac, with reduced incidences of gastrointestinal intolerance reported (Davis et al., 2008; Lien et al., 2004).

Also, a study by Nielsen et al. (2020) examined differences in the gut microbiota of preterm pigs fed a standard formula and a formula enriched in α– lac. In this study, it was found that enriching in α-lac results in a prebiotic effect on the gut microbiota by promoting the growth of certain bacterial groups in a manner similar to those in breast milk, such as *Bifidobacterium infantis* and *Bifidobacterium breve*. However, interestingly, it was also found that feeding α-lac at a dosage beyond human milk levels does not offer significant advantages to overall gut health or immunity. Therefore, while it has been shown that enriching IF in α-lac can have a positive effect on the gut, enrichment beyond human milk levels would not offer an advantage.

In recent years, commercial methods of enriching α-lac have been developed using membrane filtration, selective precipitation of α-lac followed by centrifugation or ion exchange technologies (Barone, O'Regan & O'Mahony, 2019). Membrane filtration or selective precipitation results in a concentrate where between 30 and 45% of total protein is α-lac (Barone et al., 2019; Layman et al., 2018; Marella et al., 2011). In contrast, the ion-exchange method, which applies ion-exchange chromatography to separate α-lac and β-lg in liquid whey, results in a highly purified α-lac ingredient in which 93 % of total protein is α-lac (Barone et al., 2019; Layman et al., 2018). The method used to isolate α-lac results in significant differences in protein composition and physiological properties (Barone et al., 2020). If cheese whey is used as the starting material, membrane concentration of α-lac will also co-enrich glyco-macro-peptide (GMP) and so reduce the beneficial impact of enriching α-lac on the amino acid profile. Selective precipitation, on the other hand, will not co-enrich GMP and so this product will contain more tryptophan per gram of protein and will more closely align with the amino acid profile of human whey protein. However, as the protein has been aggregated, surface hydrophobicity is increased, which can affect its solubility in water. Ion exchange yields the highest purity of α-lac per gram of protein without aggregation; therefore, in theory this method of α-lac enrichment could be most suitable for IF applications, but the high cost of manufacture can be prohibitive (Barone et al., 2020).

From a processing perspective, bovine whey proteins are dominant in β-lactoglobulin (β-lg) which has very poor heat stability and will denature at temperatures in excess of 65°C, exposing reactive sulphydryl groups. These exposed groups can form disulphide bonds with other proteins and calcium-binding sites leading to aggregation and destabilisation of the formula, especially in high ionic calcium environments typical of IF (Donovan & Mulvihill, 1987; Roefs & De Kruif, 1994, Simmons et al., 2007). In contrast, α-lac will also begin to denature at temperatures in excess of 65 °C (Nielsen et al., 2018); however, unlike β-lg, α-lac

does not contain a free sulphydryl group and so protein-protein interactions are less pronounced during heat treatment (Barone et al., 2020; Donovan & Mulvihill, 1987). α-Lac also contains Ca^{+}-ion-binding sites which can alter the structure of protein resulting in increased protein stability to heat treatment (Permyakov & Berliner, 2000). Buggy et al. (2017) found that model IF with increased proportions of α- lac appeared to be less susceptible to heat-induced aggregation when compared against control formulae prepared using WPI and skim milk. Therefore, increasing α-lac content in IF appears to have a stabilising effect during thermal processing, as formulas with higher levels of α-lac showed reduced viscosity after heat treatment. This is also in line with findings by Crowley et al. (2016) that protein-protein interactions were reduced in whey-dominant IFs with higher levels of α-lac, resulting in lower viscosity after heat treatment. Therefore, whey streams and IF blends with increased proportions of α-lac typically show improved protein stability to high heat treatment processing.

1.3.4. Lactoferrin– methods of manufacture and its impact on IF

Lactoferrin is the second most prevalent whey protein in human milk, making up approximately 26 % of total whey protein, but it is present in only trace amounts in bovine milk (Guo, 2014). As such, its absence in IF will affect the amino acid profile, however; more significantly, lactoferrin is believed to play a key role in the development of the gut microflora of the infant. Lactoferrin is an iron-binding glyco-protein and is considered an important host defence molecule during infant development, as it has been shown to have strong antimicrobial and antiviral properties (Chierici & Vigi, 1994; Jenssen & Hancock, 2008). This is achieved through a number of mechanisms:

- the ability to sequester iron from bacterial pathogens to inhibit bacterial growth;
- interaction with gram-negative bacteria and damage the bacterial membrane;

- inhibition of biofilm formation, preventing bacteria from becoming resistant to host cell mechanisms and antibiotics;
- blocking virus interactions with host cells and so inhibiting viral infection (Jenssen & Hancock, 2008).

Lactoferrin has also been shown to have a prebiotic effect as it promotes the growth of bifidobacteria in the gut. Studies have shown that the faeces of breast-fed infants have significantly higher levels of bifidobacteria than infants fed formula without added lactoferrin (Balmer et al., 1989; Chatterton et al., 2013; Chierici et al., 1992). This difference in gut microflora has been attributed to the high levels of lactoferrin in human milk, along with other components such as oligosaccharides, and it has been shown that IF enriched with lactoferrin increases bifidobacteria levels in the gut, thereby offering a protective effect against bacterial infection (Oda et al., 2014).

While lactoferrin is present in only trace amounts in bovine milk, it can be isolated and purified from cheese whey on an industrial scale, with 95 % pure lactoferrin being available on the market (Tomita et al., 2002). This is purified by using cation-exchange membranes that adsorb lactoferrin to the membrane, followed by a series of elution steps with salt solutions. The eluted lactoferrin is then desalted, concentrated, pasteurised or micro-filtrated before freeze-drying or spray-drying to produce the end powder (Chierici et al., 1992).

Unlike many proteins lactoferrin has a high isoelectric point of pH 8.7 and at neutral pH (pH 7), it has a net positive charge therefore making it immune to calcium-induced aggregation (Bengoechea et al., 2011; Lönnerdal, 2003). During thermal denaturation, carboxylic groups of sialic acid in lactoferrin become exposed and bind calcium ions in solution, which reduces hydrophobic reactions between lactoferrin molecules. Therefore, not only is lactoferrin immune to calcium-mediated thermal aggregation, but it is more heat-stable in high calcium environments (Rossi et al., 2002). This positive charge also results in

electrostatic attraction to other negatively charged caseins including β-casein and whey proteins. Lactoferrin binds to the negatively charged phosphate sites on casein and whey proteins to form a soluble complex with positively charged lactoferrin on the surface. This reduces the number of available locations for calcium binding, creating increased steric repulsion between these newly formed lactoferrin: casein/ whey complexes and positively charged calcium ions resulting in improved thermal stability of the formulation (Bengoechea et al., 2011; McCarthy et al., 2014). Evidence of this was observed by McCarthy et al. (2014), who examined the effect of thermal heat treatment and $CaCl_2$ in emulsions stabilised with β-casein, lactoferrin or a β-casein: lactoferrin mix. In this study, it was observed that emulsions stabilised with β-casein were significantly more sensitive to thermal aggregation in the presence of 30 mM $CaCl_2$ than emulsions stabilised with lactoferrin; however, the most heat-stable emulsions were those stabilised with a 1:1 blend of lactoferrin and β-casein. These emulsions gelled at a higher temperature and a slower rate than lactoferrin-only emulsions, suggesting delayed protein aggregation of lactoferrin in the presence of β-casein. Also, the gel strength of β-casein-stabilised emulsions containing $CaCl_2$ was significantly higher than that of β-casein/ lactoferrin emulsions containing $CaCl_2$ indicating that lactoferrin played a role in hindering Ca^{2+}- induced aggregation of β-casein. Therefore, in an IF type application, which is rich in calcium, increasing lactoferrin content could have a stabilising effect during processing.

The advancements in the isolation of α-lac and lactoferrin have allowed for significant inroads to be made in terms of humanising IF and there are now formulas available on the market which have protein contents close to 1.2 g mL^{-1}; however, this is still 33 % higher than that of human milk. This difference in protein content is due to the continued differences in the casein protein profile of human milk and IF derived from bovine milk, most notably the lack of β-casein enrichment.

1.3.5. β-casein– methods of manufacture and its impact on IF

Human milk casein contains 85% β-casein, whereas bovine casein comprises only 38% β-casein (Guo, 2014). Aside from the obvious nutritional benefits of enriching β-casein in IF, it has also been shown to have a protective chaperone effect on whey proteins including β-lg, α-lac and bovine serum albumin (BSA), resulting in reduced turbidity and gelation of whey protein solutions when heat-treated at UHT temperatures (Kehoe & Foegeding, 2011). Therefore, in a whey-protein-dominant formula such as is common in IF, enrichment with β-casein could have a positive effect on whey protein stability.

A number of techniques have been developed at laboratory or pilot scale for isolation of β-casein. These methods typically apply acid or calcium to trigger a precipitation reaction, followed by centrifugation, cold microfiltration or a combination of these, resulting in a β-casein fraction with up to 95% purity (Atamer et al., 2017; Ram et al., 1993; Thekkilaveedu et al., 2020). However, the high cost of manufacture and the large volume of waste, coupled with poor β-casein recovery rates, has restricted the uptake of these processes at industrial scale.

In recent years, a method of cold microfiltration of skimmed milk at <10 °C has been developed which results in a β-casein and whey-protein-enriched permeate stream with α_{s1}- and α_{s2}- caseins and a large proportion of κ-casein being retained (Crowley, 2016; McCarthy et al., 2017; O'Mahony et al., 2008). At low temperatures, β-casein will solubilise and will pass through a microfiltration membrane along with native whey protein, lactose and minerals. However, at ambient temperature, β-casein will form micelles, thereby allowing for separation from the whey components present in the above mentioned permeate. Crowley (2016) subjected the β-casein and whey enriched permeate to microfiltration at 26 °C, resulting in the retention of β-casein in the microfiltration retentate stream while the whey protein, lactose and minerals were removed in the permeate. This isolated β-casein from the whey stream and resulted in a retentate with a casein: whey protein ratio of 77:23 %, with 95 % of the casein

fraction consisting of β-casein. While, as a β-casein ingredient, the purity using this method is not as high as other methods, this process could prove cost-effective for the enrichment of β-casein in a whey protein dominant IF, as impurities in the casein such as whey protein; lactose and milk minerals are common-place in IF. Also, the β-casein-depleted casein fraction retained during cold microfiltration remains a functional micellar casein, which has use in other applications such as cheese-making, therefore avoiding the loss of a significant percentage of protein to waste (O'Mahony et al., 2008).

There is an argument that, for IF applications, it may not be necessary to isolate β-casein from whey protein and so to reduce costs the warm microfiltration step could be avoided. A process of cold microfiltration of skim milk using ceramic membranes with single-stage diafiltration using RO water at pilot scale was developed by McCarthy et al. (2017). The resulting permeate then under-went cold UF concentration to produce a concentrate with a casein: whey protein ratio of 35.5: 65.5 %, of which the casein fraction was 100 % β-casein. This ingredient aligns with the casein: whey protein ratio of human milk; however, the whey protein fraction is β-lg-rich native whey. If this ingredient was applied in IF as the sole source of casein, there is little scope for addition of other whey ingredients such as α-lac or lactoferrin and so, while the casein fraction would be closer to that of human milk, the whey fraction would not be aligned. Liu et al. (2019) applied cold microfiltration with 5 stages of diafiltration with water to skimmed milk, which yielded a β-casein enriched whey protein with a ratio of 49% β-casein: 45% whey protein: 6% other caseins. Additional diafiltration stages add processing costs through additional water usage and also subsequent concentration steps; however, this casein: whey protein ratio would allow for the addition of α-lac and lactoferrin. As human milk contains almost no β-lg, use of this ingredient will still restrict the humanisation of whey proteins. Therefore, while warm microfiltration may result in additional capital and

operational costs, from a nutritional perspective it would allow for a more complete humanisation of the protein profile of bovine-based IF.

β-casein is a hydrophobic protein and therefore will form micelles or aggregates when isolated from the casein micelle (Crowley, 2016; O'Connell et al., 2003). This characteristic presents a number of issues from an ingredient processing perspective. It has been shown that, at low temperatures, β-casein exists as a monomer and is soluble; however, the hydrophobicity of β-casein increases with increasing temperature, and it will begin to form micelles at ambient temperatures (O'Connell et al., 2003). The hydrophobicity of β-casein increases with increasing temperature, decreasing pH, and increasing ionic strength, and eventually aggregation will occur in these environments (Kehoe & Foegeding, 2011; Post et al., 2012). This poses an issue for β-casein ingredient processing, as heat treatment through traditional pasteurisation or evaporation, or changes to pH as a result of concentration, could cause aggregation. These issues are not, however, insurmountable; it has been shown that temperature-induced aggregation of β-casein is thermo-reversible, and so cooling the product after heat treatment could aid further processing (Crowley et al., 2019). Alternatively, concentration and sterilisation methods which do not require heating, such as the use of membrane filtration / concentration, could be applied, and a cold processing chain could be maintained until drying; however, these processes add significant manufacturing costs when compared to SMP production. Therefore, while replacement of SMP with a β-casein ingredient as the casein source in IF may result in a product with a casein profile more closely aligned to human milk, the increased costs of manufacture may be prohibitive.

These issues for ingredient processing will also follow through to the end applications where β-casein is added at high levels. Additionally, as IF is a complex product in terms of formulation and processing, changes in the ionic environment or pH through mineral addition

and concentration or UHT treatment will all affect the hydrophobicity of β-casein, potentially adding an extra layer of complexity to processing of β-casein enriched IF.

It has been observed that, in solutions super-saturated with calcium phosphate (9 mM Ca and 12 mM P), such as could be expected in an IF formulation, calcium phosphate exacerbates non-reversible self-association of β-casein at elevated temperatures (Crowley, 2016). A study by Crowley et al. *(*2016) suggested that calcium phosphate forms complexes with exposed hydrophobic groups of β-casein that remain in place after cooling. Importantly, during this work, it was noted that the increased opacity of the β-casein solutions in a high-calcium environment was reduced in the absence of phosphate or the presence of calcium-sequestering agents such as tri-sodium citrate. Therefore, utilisation of a phosphate-free calcium-sequestering agent may be beneficial in β-casein enriched IF.

It is these thermal stability issues that have limited the development of commercial scale β-casein ingredients, as thermal processing is currently typically a critical control point during manufacturing to ensure microbial safety of the ingredient. However, a recent study by Hailu et al. (2022) found that by reintroducing some micellar casein through the addition of skim milk to a liquid β-casein-enriched whey protein concentrate significantly reduced protein aggregation and precipitation during evaporation and drying. In this study, a β-casein-enriched whey protein concentrate was produced by performing cold microfiltration of dilute skim milk. The permeate from this process subsequently underwent UF processing to produce a concentrate with a casein: whey protein ratio of 25:75 where all of the casein present was β-casein. Skim milk was then added to this concentrate at a dosage rate of 0, 0.1, 0.3 or 0.5 % of total protein before evaporation and spray-drying. It was observed that, after evaporation, as expected, concentrates which did not have skim milk added had significant protein aggregation however, as increasing levels of skim milk were added prior to evaporation, protein aggregation reduced with significant reductions observed with 0.3 or 0.5 % added skim milk. This

improvement in thermal stability was attributed to the ability of the micellar casein present in skim milk to incorporate the isolated β-casein and so provide colloidal stability to protect against thermal precipitation. While this technique reduces the purity of β-casein present in the casein fraction of the concentrate, at 0.5 % skim milk addition 81 % of the casein present was β-casein. In comparison to skim milk, this is a significant improvement in aligning the casein profile to human milk and may be an acceptable compromise if it enables the production of a commercially viable source of β-casein enrichment for commercial scale IF manufacturing.

In conclusion, significant advances have been made in aligning the protein profile of bovine IF with that of human milk. Commercially viable methods for the isolation of α-lac and lactoferrin have been developed which have allowed significant inroads to be made into matching the whey protein profile of IF to human milk. However, significant differences remain in the casein profile, most notably the lack of β-casein in bovine IF, which prevents reduction in the protein content to human milk levels. Much research has been conducted into isolating β-casein, with a number of lab- and pilot-scale methods developed. However, the high cost of manufacturing a highly pure β-casein ingredient has prohibited its application in IF. While further research is required to allow for a cost-effective means of isolating β-casein at industrial scale for use in IF, recently developed methods using cold microfiltration to develop a β-casein-enriched whey protein show the most promise for industrial acceptance, with the potential for improved thermal stability through colloidal stabilisation with micellar casein before heat treatment (Hailu et al., 2022).

1.4. Minerals

The mineral profile of a protein source is an important consideration when selecting ingredients for IF. Minerals play an essential role in the growth and development of the infant, including skeletal development and regulating certain body functions; however, bovine milk

contains significantly higher quantities of minerals than human milk and so care must be taken to ensure that the mineral profile of bovine based IF does not exceed safe levels (Mc Dowell, 2003; Mc Sweeney, O'Regan, & O'Callaghan, 2013). Technologies such as membrane filtration which are primarily applied to increase the protein content of casein and whey protein ingredients can also help to reduce the innate mineral content. Typically, mineral contents are reduced to well below the levels stipulated by legislation, allowing IF manufacturers to fortify the product with controlled amounts of mineral salts, which ensures a consistent mineral profile is achieved in the final IF (Bylund, 1995). This also allows manufacturers the opportunity to add inert forms of minerals such as insoluble calcium phosphate or sequestering agents such as tri-sodium citrate to improve the stability of the formulation during thermal processing while still meeting regulatory mineral requirements.

It is well known that the salt concentration and equilibria between the colloidal and serum phase in a bovine-milk-based formulation have an important role in maintaining the integrity of casein micelles (Aoki et al., 1990). As such, minerals can have a significant impact on the processability and final functionality of dairy proteins, with multivalent ions such as calcium, magnesium, phosphate, and citrate having the most significant impact.

During manufacture of IF and other nutritional products, the protein streams will undergo various heat treatments, pH adjustment and, in some cases, membrane processing steps to achieve the desired protein composition, functionality and processing efficiency. However, with each of these processing steps, the balance of minerals in the colloidal and serum phase can become altered, which can have a significant effect on the process and final product functionality.

1.4.1. Calcium and Magnesium

In bovine milk, there is ~120 mg calcium and ~ 11 mg magnesium per 100 mL milk (based on a specific gravity of milk of 1.035 kg L^{-1}) which exists in the colloidal or serum phase depending on the environmental conditions. However, in its natural state, approximately two thirds of the magnesium and calcium present in bovine milk are associated within the colloidal casein environment (Holt, 2004). In comparison, IF contains a minimum of 30 mg calcium 100 mL^{-1} sourced from the protein streams or added to the formulation to meet regulatory requirements (Codex Alimentarius, 2007).

Calcium is essential to the structure and stability of the casein micelle, as it not only forms colloidal calcium phosphate (CCP) which is integral to maintaining the internal conformation of the casein micelle; but also positively charged calcium ions can form calcium bridges between negatively charged residues of caseins, which helps the protein to remain suspended in solution. However, when in excess, calcium ions can have a detrimental effect on the stability of milk proteins as they can bind to negatively charged whey proteins such, as β-lg, during heating, promoting the formation of covalent bridges between proteins. These covalent bonds can result in increased aggregation of the proteins which then precipitate out of solution (Joyce et al., 2018; Nicolai et al., 2011). In the case of IF, where whey protein can make up 60 % of the total protein, free ionic calcium can cause significant issues with aggregation and fouling during processing at the high temperatures required for IF manufacture. Therefore, it is essential that levels of free ionic calcium be minimised prior to heat treatment.

In addition, a high ionic calcium environment can result in increased levels of casein aggregation and sedimentation after high temperature treatment especially UHT treatment. UHT processing causes dissociation of hydrophilic κ-casein from the casein micelle, exposing hydrophobic β- and α-casein. If this occurs in a high ionic calcium environment, calcium

bridges can form between the exposed hydrophobic micelles in an attempt to stabilise them. However, if high levels of ionic calcium remain in the serum phase or the electrostatic repulsion between caseins remains low due to low pH, for example, this can lead to significant aggregation and eventual sedimentation of the κ-casein -depleted casein micelles (Gaur et al., 2018).

There is significantly less research on the impact of magnesium on protein solutions compared to calcium, however limited studies suggest that magnesium behaves and is released from the casein micelle in a similar way to ionic calcium (Dalgleish & Law, 1989). While magnesium is naturally present in much smaller quantities than calcium (Holt, 2004), in order to achieve the required levels in IF, it may have to be added to the formulation. As such, it is important to be aware of the effect it can have on the system in its ionic form.

1.4.2. Phosphate and Citrate

Phosphate and citrate are both ion-sequestering agents. The key function of sequestering agents is to shift the mineral equilibria to favour the reduction of free ions (De Kort et al., 2011). Therefore, in an IF formulation, they can bind to free ions such as calcium and magnesium and, in doing so, offer increased protection against calcium-mediated protein aggregation.

Over half of the phosphate present in bovine milk exists in the serum phase, while in the case of citrate about 90 % of it is non- colloidal (Holt, 2004). This is an important consideration if a dairy protein being used in IF manufacture has previously undergone a separation process such as UF, as much of the native phosphate and citrate will be lost to the permeate stream. This in turn can impact the stability of the formulation if it undergoes a process triggering the release of calcium and magnesium ions from casein, such as pH adjustment, heat treatment or increased total solids concentration; or if additional ions are

added to the formulation. In this scenario, additional phosphate or citrate may need to be added to the formulation to avoid calcium-mediated protein aggregation.

Phosphate exists in the colloidal phase mainly in the form of colloidal calcium phosphate. In the serum phase, it can exist bound to calcium as $CaHPO_4$ or as free inorganic phosphate in the form of HPO_4^{2-} and $H_2PO_4^-$ (Holt, 2004). In the serum phase, calcium and phosphate exist in equilibria as per the below equation (Holt, 2004):

$$Ca^{2+}{}_{(aq)} + 0.7HPO_4^{2-}{}_{(aq)} + 0.2PO_4^{3-}{}_{(aq)} \leftrightarrow Ca(HPO_4^{2-})_{0.7}(PO_4^{3-})_{0.2(s)} \quad \text{(Eq. 1)}$$

During heat treatment, calcium phosphate $Ca(HPO_4^{2-})$ becomes increasingly insoluble and will precipitate out of the serum phase (O'Connell & Fox, 2001; Singh, 2004). When this occurs, in order to balance the equilibrium in the serum phase, phosphate ions will bind to free calcium ions to form calcium phosphate, resulting in a release of H^+ ions and a decrease in the pH of the serum phase. By binding to free calcium, phosphate can reduce the risk of increased denaturation and aggregation of whey proteins, thereby improving the heat stability of a formulation. However, this reduction in free calcium ions in the serum phase could result in increased electrostatic repulsion between casein micelles and an overall increased negative charge within the micelle, resulting in swelling of the casein micelles and increased viscosity of the formulation (De Kort et al., 2011).

For IF formulations where high levels of ionic calcium may be present, a phosphate-based chelator may be added to prevent calcium-induced aggregation. However, each calcium phosphate binding reaction releases a hydrogen ion, resulting in a decrease in pH. This is an important consideration as colloidal calcium phosphate dissociates at lower pH values (Jeswan Singh et al., 2015). Therefore, as more insoluble calcium phosphate is formed and the pH declines, CCP will dissociate from the casein micelle, resulting in destabilisation of the casein, dissociation of κ-casein from the micelle, potential sedimentation of κ-casein-depleted casein micelles, and an increase in free ionic calcium levels. This then begins the cycle again, as

phosphate ions will attempt to bind to calcium ions in order to return the serum phase to a state of equilibrium, resulting in a further decline in pH (De Kort et al., 2011; Karlsson et al., 2019).

Evidence of this phenomena was observed by Karlsson et al. (2019), who investigated the impact of adding calcium on the stability of UHT milks. They found that milks with higher levels of added calcium which were stored at 37 °C showed a decline in pH over storage and increased levels of sediment, which suggests that there was formation of calcium phosphate, resulting in a decrease in pH, which triggered the destabilisation of casein and release of κ-casein into the serum phase. Interestingly, they also found that, when the samples were stored at 4°C, a decline in pH was not observed. This is in line with studies carried out by O' Connell & Fox (2001) and Singh (2004), who found that the solubility of calcium phosphate is lower at elevated temperatures. This reduction in solubility would trigger the binding of any remaining phosphate ions to calcium so as to maintain a stable equilibrium, resulting in a decrease in pH at elevated temperatures. While the study by Karlsson et al. (2019) did find high levels of sediment even at 4 °C, this was thought to be as a result of β-casein destabilisation and not calcium phosphate formation.

Citrate is also a sequestering agent and will bind to calcium as per the below equation (Holt, 2004):

$$Ca^{2+}{}_{(aq)} + Cit^{3-}{}_{(aq)} \leftrightarrow Cacit^{-}{}_{(aq)} \qquad \text{(Eq. 2)}$$

Unlike phosphate, the binding of calcium and citrate does not result in the release of an H^+ ion, and so pH will not decrease. In fact, binding of citrate to calcium results in an increase in pH (Gaur et al., 2018). This can be beneficial as, if the pH is not declining, then CCP will not dissociate from the casein micelle; however, if the pH increases too much, other reactions such as casein micelle swelling and Maillard browning may occur.

Another benefit of using citrate as a sequestering agent is that, unlike calcium phosphate, calcium citrate complexes remain soluble in the serum phase and so will not

sediment if present in excess amounts (Holt, 2004). However, as with all calcium-binding salts, this will increase electrostatic repulsion between caseins potentially leading to casein swelling and gelation of the formulation (De Kort et al., 2011; Gaur et al., 2018; Singh, 2004). Therefore, in many cases, a mix of citrate and phosphate-based calcium- chelating agents such as disodium hydrogen phosphate and trisodium citrate are used to prevent calcium-mediated aggregation (De Kort et al., 2011).

1.4.3. Effect of processing on the mineral balance and formulation stability

Processing conditions applied during heat treatment and concentration can significantly affect the salt equilibria within a formulation. All nutritional products must undergo heat treatment to a minimum of 72 °C for 15 seconds to kill microorganisms but in many cases this temperature may be increased to ensure the safety of the product particularly in the case of IF where the consumer (infant) may not have a fully developed immune system. It has been previously shown that, as temperature increases, the solubility of calcium phosphate declines significantly (O' Connell & Fox, 2001; Singh, 2004), resulting in increased formation of insoluble calcium phosphate and a decrease in pH. However, it has been found that, at temperatures below 100 ºC, the calcium phosphate formed has a similar structure to CCP and so, upon cooling, the salt equilibrium could revert to its pre-heated state (Wang & Ma, 2020). However, if exposed to severe heat treatment (over 120 ºC), the structure of the casein micelle is irreversibly altered, resulting in the formation of insoluble calcium phosphate which cannot be returned to its native state (Fox, 1981; Wang & Ma, 2020).

It is well established that, when dairy systems are heated, a pH decrease will be observed (Fox, 1981; Jeswan Singh et al., 2015). Fox (1981) estimated that a milk solution at pH 6.5 and 20 °C could have a pH of 5.8 at 140 °C. This pH decline on heating can have a significant impact on the salt equilibria and changes can occur at these high temperatures that

could have a knock-on effect during further processing. Fox (1981) proposed three principal reactions that may account for the decline in pH at high temperatures:

a) production of organic acids from lactose;

b) precipitation of primary and secondary calcium phosphate as tertiary phosphate, with the concomitant release of H^+;

c) hydrolysis of CCP and its subsequent precipitation as $Ca_3(P0_4)^2$, with release of H^+.

Fox (1981) found that, if the pH of a milk solution is maintained close to its native pH (~6.7) during heating, it could be heated at temperatures of 140 °C indefinitely without coagulation. This suggests that, while high temperature heat treatment results in significant changes to the protein conformation and salt equilibrium, it is the pH decrease as a result of the heat treatment that triggers coagulation and not the temperature applied. Therefore, if a formulation maintains a stable pH throughout processing, it can perhaps survive multiple high heat treatment steps without coagulation.

Depending on the process, IF may be exposed to multiple lower temperature heating steps prior to the final high temperature pasteurisation / sterilisation, for example, in order to aid rehydration of added powder ingredients. Murphy (2014) examined the relationship between temperature and pH in model IF formulations. As part of this work, a linear decrease in pH was observed in all formulations as the temperature increased from just 30 °C to 60 °C. Therefore, without in-line pH monitoring after each processing step and controlled pH adjustment through the addition of acidic or alkaline ingredients, the pH of a formulation could be decreasing throughout the process and not just during the high heat treatment step which could affect the stability of the casein micelle particularity if the pH falls below pH 6.2 resulting in the release of CCP. Similarly, a more recent study by Aydogdu *et al*., (2022) examined the change in pH of a range of casein and whey protein ingredients rehydrated in either distilled water or simulated milk ultrafiltrate (SMUF) at temperatures up to 120 °C. In this study, a

linear decline in pH with increasing temperature was observed across all samples regardless of protein type and serum phase mineral profile (R^2 ranged from 0.94 to 0.995 across all samples) with this pH decline tending to be more pronounced for solutions prepared in simulated milk ultrafiltrate (SMUF) due to the higher level of soluble minerals and to be non-reversible upon cooling in whey dominant solutions.

Water removal, as occurs during evaporation, was also found to have important effects on pH by Murphy (2014) who found that as dry matter increased in a model IF, pH decreased concomitantly. Model IF samples at 20 % solids had a pH of ~6.75; 50 % solids had a ~pH 6.37. pH was also found to decrease linearly when heat was applied (R^2= 0.93). This decrease in pH with increased concentration is most likely due to a higher concentration of minerals within a confined volume, forcing a higher level of reactivity and ionic strength. As pH is a measure of the hydrogen ion activity of a solution, which is defined by both the concentration of hydrogen ions and the activity coefficient of a solution, when the aqueous phase in a solution decreases, the ionic strength increases, which increases the activity coefficient resulting in an increased hydrogen ion activity and so a lower pH value (Aydogdu et al., 2023). In addition, as mentioned previously, the solubility of calcium phosphate is affected by concentration and so as the total solids of a formulation increase, the solubility of calcium phosphate will diminish resulting in precipitation with the release of H^+ ions and a decline in pH as a result.

1.4.4. Effect of pH on the mineral balance and formulation stability

The pH of a formulation is a critical factor in the release of free calcium ions, as it affects electrostatic repulsion between the caseins (De Kort et al., 2012; Fuquay et al., 2011; Singh, 2004; Zadow, 1971). As pH declines, electrostatic repulsion within the casein micelle reduces and CCP begins to dissociate (Fox, 1981; Fuquay et al., 2011; Singh, 2004; Zadow, 1971), resulting in the release of calcium and phosphate ions into the serum phase, formation

of insoluble calcium phosphate, and a further decline in pH. As the pH continues to decline and calcium ion activity continues to increase, further hydrolysis of CCP occurs, eventually leading to the dissociation of κ-casein, destabilisation of the micelles and coagulation of the formulation. However, conversely, if pH is increased, electrostatic repulsion between caseins increases because of the increased net negative charge, causing swelling of the micelle. Calcium ion activity is reduced as a result of calcium phosphate formation and increased calcium bridging between caseins, which results in an increase in viscosity of the formulation but also increased heat stability (De Kort et al., 2012).

However, it is important to bear in mind that, while high pH may aid mineral stabilisation, other components such as proteins and lactose may be affected. For example, at pH values above 7, κ-casein is almost fully dissociated and, while calcium bridging could prevent coagulation of the formulation, the viscosity may have increased to such a level that it has formed a gel (Singh, 2004). Also, Maillard browning reactions are accelerated at pH values above neutral, leading to discolouration and destabilisation of the formula (Ajandouz & Puigserver, 1999). Therefore, it is essential that pH is monitored and where possible controlled throughout processing, to stay within a range where calcium-induced aggregation is minimised, but also other components of the formula are not negatively affected.

In summary, minerals particularly multivalent minerals such as calcium and magnesium, and more importantly their ionic state play an important role in determining thermal stability of formulations. In the colloidal phase, calcium is essential to maintaining the integrity of casein through colloidal calcium phosphate bridging while, in the serum phase, ionic calcium can have a detrimental effect on whey proteins during heat treatment as it promotes whey protein aggregation. Calcium-sequestering agents such as citrates and phosphates can be used to reduce the concentration of ionic calcium in the serum phase;

however, these agents have a knock-on effect on the formulation, with phosphate tending to reduce the pH of a formula, while excessive use of citrates can lead to high pH.

Importantly, processing conditions such as temperature and concentration also affect the mineral balance and subsequent protein stability of formulations. This is typically observed as a reduction in pH with increasing temperature and concentration which, if uncontrolled, can result in casein destabilisation and increased calcium ion activity in the serum phase, leading to accelerated calcium-mediated whey protein aggregation during heat treatment. While pH control can minimise these effects, a high pH can also lead to undesirable outcomes such as high viscosity, through increased negative charge repulsion of caseins and accelerated Maillard browning of the formula.

1.5. Lactose

Lactose is the main source of carbohydrate in mammalian milk, with IF regulatory authorities stipulating that lactose or glucose polymers should be the preferred carbohydrates in bovine based IF (Codex Alimentarius, 2007; Gurr, 1981). During the early stages of life, an infant undergoes rapid growth and development, which are energy-intensive processes; therefore, IF must contain sufficiently high levels of carbohydrate to meet this demand. Regulations currently state that IF must contain between 9 and 14 g carbohydrate / 100 kcal, (Codex Alimentarius, 2007). This high lactose content can pose a number of challenges to the IF manufacturing process including Maillard browning, powder stickiness and destabilisation of the formula as a result of crystallisation during storage due to the low glass transition temperature (T_g). These effects have been studied in detail by a number of experts in the area and further information on the mechanisms and effects of lactose on powder stickiness and stability during storage can be found in the following publications, Hogan et al., (2010); Roos, (2007) Chuy & Labuza, (1994), Gianfrancesco, et al., (2009) and Lin et al., (2018).

1.5.1. Lactose during heat treatment

The effect of lactose on IF processing and formula stability is very much dependant on the heat treatment temperature. In some studies, lactose has been suggested to have a protective effect against protein denaturation when heated under HTST conditions (<100 °C) (Mc Entee, Murphy, et al., 2023; Murphy et al., 2014). This phenomenon is thought to be due to an increase in free energy of the system as a result of the high lactose content. In this environment, whey protein is more thermodynamically stable in its native globular form and so heat induced denaturation is inhibited (Anema et al., 2006). However, a major concern for IF manufacturers when adding lactose to the wet mix is the Maillard reaction, which is accelerated at elevated temperatures (>100 °C) or high pH (> pH 7) (Ajandouz & Puigserver, 1999; Xing et al., 2020). In this reaction, reducing sugars such as lactose interact with lysine residues in protein resulting in reduced bio-availability of lysine in the formula and reduced protein quality of the product (Friedman, 1999; Yilmaz & Toledo, 2005, Erbersdobler & Hupe, 1991). However, as the reaction advances, non-enzymatic browning and acid formation occurs resulting in an unsatisfactory discolouration of the formula and a reduction in pH which can affect protein stability and mineral reactivity during further processing (Davidek et al., 2006). Importantly, once initiated, the cascading reactions will continue, albeit at a slower rate, during storage, resulting the destabilisation of the formula over time (Nasirpour et al., 2006). The Maillard reaction is well known and further details on the mechanisms of the reactions can be found in a number of publications including; Al-Saadi et al.,(2013); Nursten, (2005); and Xiang et al., (2021).While heat treatment during manufacture is essential to ensure the microbiological safety of the final product; manufacturers must minimise product exposure to elevated temperatures for prolonged times in order to minimise Maillard reactions. One of the early products of the Maillard reaction is furosine and this can be used by IF manufactures as an

indication of the extent of heat treatment to which the formulation has been exposed (Rufián-Henares et al., 2004).

In summary, the high lactose content in IF required to meet the energy demands of the infant pose a number of challenges to the manufacturing process, including Maillard browning, powder stickiness and crystallisation during storage. Therefore, manufacturers must carefully monitor the entire IF process in order to maintain product quality and safety.

1.6. Dairy ingredient seasonality

As outlined previously, IF is typically manufactured from a blend of bovine based ingredients such as skim milk and whey protein. As these are produced from bovine milk, which is a dynamic biological raw material, the composition can vary depending on a number of factors, including animal health, genetics, stage of lactation and feeding practices (Magan et.al., 2021; Timlin et al., 2021). In particular, in countries where a predominantly pasture-based feeding system is applied, such as Ireland and New Zealand, significant variation in raw milk and, therefore, subsequent dairy ingredient composition can be experienced throughout the year. In these systems, the majority of the herd is typically calved in the springtime to ensure they are grazing on pasture for the larger part of the milk production window (8-10 months of the year). As a result, the majority of the milk pool will be early lactation in springtime, mid lactation during the summer months and then enter late lactation in the autumn / winter period (O'Brien, Moran and Shalloo, 2018; Magan et. al., 2021).

The composition of bovine milk, similar to that of human milk, adapts to meet the nutritional requirements of the calf throughout each stage of lactation with protein, fat, lactose minerals and other micronutrients all changing across each stage of lactation. Early lactation milk typically contains lower levels of protein, which increases as lactation progresses to mid and late lactation, with the protein level ranging from as low as 2.8% w/w in early lactation

and reaching up to 4.6% w/w in late lactation (Timlin et al., 2021). In addition, the protein profile and casein: whey protein ratio of the milk can vary depending on stage of lactation, with studies showing increased proportions of whey protein in late lactation milk. Meanwhile, early lactation milk contains elevated levels of α-casein and lower amounts of β- casein with the ratio of α- casein to β- casein continuing to fall as lactation progresses (Kroeker et al., 1985; Mackle et al., 1999; Bernabucci et al., 2015; O'Callaghan et al., 2016).

With regard to fluctuations in mineral profile, variation in calcium, phosphorous and citrate are of particular concern for protein functionality and heat stability in IF applications with these variations influenced by lactation stage and also diet of the animal. Typically, calcium, magnesium, sodium and phosphorous content have been shown to be positively correlated with stage of lactation, with levels increasing towards late lactation. Meanwhile, citrate level decreases from early to late lactation, therefore resulting in a loss of calcium-chelating species which could influence heat stability (Timlin et al., 2021).

The variation in raw milk composition in turn alters the composition of protein ingredients produced across the year especially for ingredients where the protein is not highly purified or fractionated e.g. skim milk. This can result in variation in the protein content, mineral content and functionality of protein ingredients, which in turn can impact their acceptability for IF applications. A number of strategies can be employed to counteract this, however. In the case of skim milk, when the protein levels are high, the protein content can be standardised through addition of lactose or de-proteinated milk permeate to reduce the total protein content (Murphy et al., 2018). In addition, where IF manufacturers are using powdered protein ingredients, these can be produced at specific points of the year where it will meet the required specification and stored until needed. However, this can also affect supply as there is a limited window where product can be made which meets the required specification and, in some cases, due to a number of seasonal factors, a shortage of supply may occur. It is for this

reason that, in some cases, more highly purified protein ingredients may be more desired for IF applications as, by increasing the protein purity, particularly through membrane filtration, total minerals, and therefore mineral variation is reduced (Bylund, 1995). This then allows IF manufacturers to add the required minerals in a controlled fashion ensuring that the final IF product meets the required mineral targets.

1.7. Fat filled milk powders

The increased purification of dairy protein streams for high value applications such as IF has also resulted in the production of large volumes of low value, protein depleted side streams, such as milk and whey permeate which are by products of MPC and whey protein concentration respectively. One application for these streams which are rich in lactose and minerals, is in the protein standardisation of fat filled milk powders (Rattray & Jelen, 1996). Fat-filled milk powders (FFMPs) are dairy powders where the milk fat has been replaced with a vegetable fat source. In this process, skimmed milk is typically concentrated by evaporation to high solids, with heat treatment taking place either before or after evaporation, followed by the addition of vegetable oil to the concentrate and homogenisation to form an emulsion prior to drying (Finnegan, Mahomud, & Murphy, 2012). The protein content of these products is standardised by increasing the lactose content, typically by adding milk permeate or lactose to the skim milk prior to evaporation to offset the natural variation of protein in cow's milk across the lactation cycle or to produce a low protein vegetable-fat-filled powder as an inexpensive alternative to whole milk powder. As permeate side streams are relatively low cost in comparison to purified lactose, they are an economically advantageous option for incorporation into FFMPs.

Depending on the source and the prior history of the permeate, such as the operating conditions during protein concentration (pH, temperature, membrane pore size, target protein

concentration), and mechanism of permeate concentration (membrane concentration, or evaporation and spray drying), these various types of standardisation media can have very different mineral profiles, which can also vary across the production season due to seasonality changes in the initial milk. As a result, when combined with skim milk for FFMP manufacture, the ionic environment of the product can vary significantly, resulting in changes in thermal stability and physico-chemical properties during processing (Murphy et al., 2018; Sikand, Tong, & Walker, 2010). A particular issue is fouling and high viscosity after evaporation, which can potentially affect the physical properties of the final powder such as bulk density, powder moisture and solubility, as a result of high viscosity prior to drying (McCarthy et al., 2021).

However, while there may be processing challenges associated with using permeates in FFMP manufacture, it is becoming increasingly important that these challenges are overcome to avoid the environmental and economic cost of sending nutritionally valuable permeate to waste and to enable the sustainable growth of purified protein ingredients for IF applications. As part of this thesis, the impact of permeate composition and source on the ionic environment and thermal stability of low protein skim concentrates suitable for FFMP manufacture is examined, using permeates with varying mineral compositions depending on source and prior processing history. In addition, mechanisms to improve thermal stability through controlling pH are explored with a view to understand the impact of ionic environment and encourage greater use of permeate from various protein concentration sources in FFMP manufacture.

1.8. Conclusion

In conclusion, significant advancements have been made in altering the protein profile of bovine based IF through whey protein fractionation which may also facilitate a reduction in overall protein content. However, IF protein levels are still above typical human milk

concentrations due to differences in casein profile, with research continuing to develop commercially viable technologies for the enrichment of β-casein in IF applications.

Aside from protein differences, controlling the pH and ionic calcium balance can be challenging for manufacturers with careful monitoring and controlled chelator addition required throughout processing, to minimise the risk of protein destabilisation and sedimentation. In addition, IF typically contains five times more lactose than protein, which, if not correctly managed, can pose a number of challenges, related to Maillard browning, powder stickiness during drying and lactose crystallisation during storage.

This review shows that significant improvements in the functional and nutritional properties of IF are possible through modification of the protein fraction. However, great care must be exercised when changing the protein profile as the inherent properties of formulations will also change. In particular, it is important to determine the effect of such changes on mineral balance and protein: mineral interactions as these will determine the heat stability of formulations.

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Chapter 2

Effect of casein-whey ingredient blends on the protein stability of model infant formulas

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Declaration

This chapter was written by Sinead Mc Entee (SME) and reviewed by all co-authors.

2.1. Abstract

In this study, milk protein concentrate (MPC 80), skim milk powder (SMP), acid whey protein concentrate (aWPC 35), cheese whey protein concentrate and isolate (cWPC 35 and cWPI) were assessed for suitability in IF applications at a total solids similar to commercial ready-to-drink applications. Serum-phase compounds such as phosphate, citrate and chloride in casein-rich streams were shown to play an important role in controlling whey protein thermal aggregation, especially in calcium-rich aWPC 35. Meanwhile, replacing cWPC 35 with cWPI improved protein stability at UHT temperatures, indicating that removal of minerals from whey protein streams could increase thermal stability of formulations. At HTST temperatures (95 °C), lactose appeared to have a protective effect against protein denaturation; however, at 140 °C, lactose contributed to cascading Maillard reactions, which triggered complete protein destabilisation. Such reactions occurred most rapidly in MPC 80 formulations which were comparatively low in serum-phase calcium-binding species such as phosphate and citrate. These findings highlight the impact of innate minerals and lactose on protein stability during IF manufacture.

2.2. Introduction

Codex Standard 72-1981 (2007 revision) defines infant formula (IF) as "a breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding" (Codex Alimentarius, 2007). Human breast milk is considered the best source of nutrition for infants, although IF provides a suitable alternative in cases where breast milk is unavailable. As formula is the sole source of nutrition for some infants, the industry is tightly regulated, with a number of standards in place defining the compositional requirements (Codex Alimentarius, 2007; European Commission, 2006; Food and Drug Administration, 2017).

Human breast milk protein typically consists of approximately 40 % casein to 60 % whey protein (Gurr, 1981; Thompkinson & Kharb, 2007). Many IFs use bovine milk as a base; however, as bovine milk has a casein to whey protein ratio of 80:20, the formulation must be modified in order to more closely mimic human milk (Thompkinson & Kharb, 2007). This is achieved by blending bovine-based casein and whey protein sources to the desired protein composition. The most common casein source used in IF is skim milk (Nasirpour, Scher, & Desobry, 2006) while whey protein can be obtained from, sweet whey (by-product of cheese/rennet casein manufacture), acid whey (by-product of acid casein/ Greek-style yoghurt manufacture) or in more recent year's ideal whey which is extracted via membrane fractionation. Without further processing, these whey streams have a prohibitively high ash: protein ratio and so are often subjected to further filtration / separation processes to increase the protein: total solids ratio, producing a whey protein concentrate (WPC) with 35 to 85 % protein or whey protein isolate (WPI) with up to 95 % protein (Singh, 2009). In some cases, whey can undergo a process of demineralisation *via* diafiltration, nanofiltration, ion exchange, or electro dialysis making it more suitable for use in IF (Gernigon, et al., 2011). In order to complete the formulation, fat, lactose, vitamins and minerals are added during the wet

processing stage or, in some cases for IF powder manufacture, additional carbohydrate, vitamins, minerals or probiotics can be dry-blended with the finished powder (Masum et al., 2021).

During the manufacture of both liquid and powder IF, a series of heat treatments are applied, which may include ultra-high temperature (UHT) treatment (Murphy, 2014). In both cases, the heat treatment applied is a key step in the process, primarily as a means of ensuring microbial safety, but also due to its effect on physical properties of the formula such as viscosity and protein stability (Masum et al., 2020). There are a number of challenges faced by IF manufacturers with regard to protein stability during thermal processing. Bovine whey proteins tend to have poor heat stability with irreversible whey protein aggregation occurring at temperatures >70 °C (Joyce et al., 2017). While casein has been shown to reduce whey protein aggregation during thermal processing, in formulations where whey protein is dominant, this effect is lower than in casein-dominant formulae (Masum et al., 2020). In addition, as IF is the sole source of nutrition, a significant amount of minerals such as calcium are required to meet dietary needs. These minerals can be innate to the protein streams or added during processing (Masum et al., 2020). If present innately, these minerals have a significant impact on the heat stability of the protein stream. For example, WPC 35 sourced from acid whey contains high levels of ionic calcium; it is known that ionic calcium has a significant destabilisation effect on both casein and whey proteins during heat treatment (De Kort et al., 2011; Joyce, Kelly, & O'Mahony, 2018). Therefore, to avoid issues during thermal processing, counter ions such as phosphate and citrate are added to bind calcium ions (Hebishy et al., 2019; Holt, 2004).

In this study, a selection of casein and whey-protein rich streams available in the dairy industry have been combined to form model IF at a concentration similar to ready-to-drink (RTD) IF products. The samples chosen contain varying protein and mineral contents and, while they are all potentially suitable for use in IF, it was hypothesised that they would impart

different functionalities in particular heat stability in a model IF system due to differences in mineral composition. Some key research questions for this study were, what impact, if any will the mineral profile of protein ingredients have on the thermal stability of model IF systems and, given that there is significant differences in cost of these ingredients, why an IF manufacturer might choose to use a more purified source of casein or whey protein over a lower cost, less purified protein ingredient. Heat stability, viscosity, particle size and ionic environment were examined to determine the potential impact of protein source and innate mineral profile on processability during high temperature processing. The overall aim of this study was to determine the impact of protein source and innate mineral levels on the stability of model IF during high heat processing and explore the potential suitability of less commonly used ingredients such as aWPC 35 and MPC 80 for IF.

2.3. Materials and methods

2.3.1. Materials

Unstandardised skim milk powder (SMP), milk protein concentrate (MPC 80), whey protein isolate (cWPI), acid whey protein concentrate produced from hydrochloric acid (HCL) precipitation of acid casein (aWPC 35), cheese whey protein concentrate from Cheddar cheese processing (cWPC 35) and lactose (L) were obtained from Tirlán, Kilkenny, Ireland.

2.3.2. Compositional analysis

Compositional analysis on all protein ingredients is shown in Table S2.1 and was performed by Teagasc technical services (Moorepark, Fermoy, Co. Cork). Protein and non-protein nitrogen (NPN) were analysed by Kjeldahl (ISO/IDF, 2014, 2016), fat by Rose Gottlieb (ISO/ IDF, 2010), ash by furnace (GEA Niro A 25a) and moisture using an oven at 102 ºC (ISO/ IDF, 2004). Mineral analysis (Fig. 2.2) was completed by Eurofins Food Testing Ireland Ltd.

using an accredited ICP-OES method. Samples were acid digested using nitric acid or a nitric acid/HCL mix prior to analysis by ICP-OES.

2.3.3. Amino acid analysis

Eurofins Steins Laboratorium (Vejen, Denmark) performed amino acid profiling on all protein ingredients, as per the method outlined in ISO 13903:2005 (ISO, 2005) (Fig. S2.2). Samples underwent acid hydrolysis in aqueous HCl in order to break peptide bonds. Subsequently the amino acids were separated in an amino acid analyser and the detection carried out using post column derivatisation with ninhydrin reagent and 440 and 570 nm UV detection. For cysteine and methionine analysis, samples were oxidised with hydrogen peroxide and formic acid prior to acid hydrolysis with HCL. Tryptophan was analysed by alkaline hydrolysis followed by quantification using liquid chromatograph fluorescence detection (LC-FLD). For quantification purposes, a one-point calibration was used, with a control internal standard included in every run.

2.3.4. Preparation of non-fat model infant formulae

Based on the composition of the protein ingredients, non-fat model IF were prepared by adding MPC 80 or SMP to either cWPI, cWPC 35, or aWPC 35 to give a whey protein to casein ratio of 60:40. When compared against Codex regulations, Codex values were converted from g 100 mL $^{-1}$ to g 100 g $^{-1}$ based on a calculated density value of 1.028 g mL $^{-1}$. This was calculated based on a formulation composition containing; 1.5 % w/w protein (density 1.35 g mL $^{-1}$), 7.7 % w/w lactose (density 1.52 g mL $^{-1}$), 3.9 % w/w fat (density 0.93 g mL $^{-1}$) and 86.93 % w/w water (density 1 g mL $^{-1}$) (Belitz, Grosch, & Schieberle, 2009; Elversson & Millqvist-Fureby, 2005; Watson & Tittsler, 1960). This translates to a dry matter formulation of 12 % w/w protein, 60 % w/w lactose and 30 % w/w fat. All protein and model IF solutions

contained 1.5 % protein (w/w) and were produced both without or with added lactose (7.7 %, w/w).

For formulae with added lactose, the carbohydrate was added to ultra-pure water (Elga purelab flex 2, High Wycombe, UK.) at 50 ºC and stirred until fully dissolved, after which the casein and whey protein sources were added under gentle agitation and left to stir for 2 hours. Samples were then pH-adjusted to pH 7 using 0.1 M HCl / NaOH at 25 °C before being left to equilibrate for at least 30 min and used for experiments within 1 hour. For formulae without added lactose, protein powders were added directly to ultra-pure water (at 50 ºC) and prepared as described above.

2.3.5. Whey Protein Nitrogen Index (WPNI)

The WPNI of MPC 80 and SMP was assessed using the GEA Niro A 21a method (GEA, 2009). Samples were re-constituted to 3.5 % protein with distilled water to ensure accurate comparison between MPC and SMP. Casein and heat-denatured whey proteins were removed by precipitation with NaCl followed by filtration. Un-denatured proteins present in the filtrate were then precipitated by adding 2 drops of 10 % HCl. Turbidity of the filtrate was measured as % transmittance using a UV-spectrophotometer (20 Genesys, Thermo Fischer Scientific, Massachusetts, USA.) at a wavelength of 420 nm. Using a standard curve, this reading was converted to mg un-denatured whey protein nitrogen g $^{-1}$ of powder (WPNI). Based on the WPNI value, the powders were classified as having undergone high (WPNI < 1.5), medium (WPNI > 1.5- < 6.0) or low (WPNI > 6.0) levels of heat treatment.

2.3.6. Nitrogen Solubility Index (NSI)

Nitrogen solubility index at pH 4.6 was assessed on cWPI, aWPC 35 and cWPC 35 35 to determine the whey protein denaturation level as per the method outlined in ISO 15323:2002

(ISO/IDF, 2002). Powder (5 g) was added to 70 mL of sodium chloride solution (0.1 M) and left to hydrate for 30 min. The pH was adjusted to 4.6 with hydrochloric acid, samples were stirred for a further 20 min, and the pH re-checked, before transferring to a 100 mL volumetric flask, filling to the mark with 0.1 M NaCl and mixing thoroughly. A 30 mL aliquot of sample was then centrifuged at 12,000 *g* for 30 min and filtered through Whatman No. 1 filter paper. Samples of the initial dispersion and the supernatant were then analysed for nitrogen content by the Kjeldahl method.

Nitrogen solubility index was calculated as follows:

$$\text{Nitrogen Solubility Index (NSI) \%} = N_{supernatant} / N_{dispersion} \times 100 \quad (1)$$

$$\text{\% Denaturation} = 100\text{-NSI \%} \quad (2)$$

2.3.7. Simulated high temperature short time (HTST) heat treatment.

Protein solutions and model IF solutions underwent simulated HTST heat treatment using a HR-1 Discovery Hybrid Rheometer (TA Instruments, New Castle, DE, USA) equipped with a starch pasting cell geometry, similar to the studies of Hebishy et al. (2019) and Murphy et al. (2014). Samples were prepared as outlined in section 2.3.4. above (1.5 %, w/w, protein) Viscosity measurements of protein solutions (28 g) were carried out at a constant shear rate of 16.8 s^{-1}. A temperature sweep was performed over a heating step from 25 to 40 ºC, held at 40 °C for 2 min before heating to 95 °C at a rate of 19.5 °C min^{-1}, and held at 95 °C for 5 min before cooling back to 40 °C at a rate of 22 °C min^{-1}. Samples were held at 40 °C for 2 min to obtain viscosity data following heat treatment prior to further cooling to 25 °C.

2.3.8. Measurement of pH and apparent ionic calcium concentration

Samples were prepared and pH-adjusted to pH 7 as per the method outlined in section 2.3.4. The pH was measured using a standard pH Meter (WTW pH 3310 with a Sentix 41

probe, VWR International, Blanchardstown, Dublin, Ireland). Apparent ionic calcium (Ca^{2+}) concentration was estimated using a Ca^{2+}-ion selective electrode (Sension^{+} 9660C combination calcium ion-selective electrode (ISE), Hach Ireland, Cork, Ireland) using the method outlined by Crowley et al. (2014). Prior to analysis, the electrode was activated by immersion in a calcium chloride ($CaCl_2$; 10 mg L^{-1}) solution for at least 30 min. An ISE calibration curve was then generated using a series of 10 mL $CaCl_2$ solutions prepared to calcium concentrations of 0.5, 1.0, 2.5 and 5 mM, which enabled the estimation of apparent calcium ion concentration based on electrical potential (Bier, 2009).

The electrode measures the activity of calcium ions in solution, which is correlated with calcium ion concentration (Cf) *via* the activity coefficient (Rundle, 2016), where;

$$Cf = \frac{\text{activity}}{\text{activity coefficient}} \quad (3)$$

In order to mitigate against variation of the activity coefficient as a result of differences in ionic strength between samples, the ionic strength of each standard and sample was standardised by adding 1 % (v/v) of 3 M KCl prior to analysis. In doing so, the total ionic strength was increased to a level where variance in the activity coefficient due to differences in ionic strength was negligible, enabling an estimation of apparent ionic calcium concentration based on the calibration curve (Rundle, 2016).

2.3.9. Particle size analysis

Particle size distributions of the model IF before and after HTST treatment were analysed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, England). Samples were analysed at 25 °C, using a refractive index of 1.45 for milk protein. Samples were diluted in ultra-pure water (1 in 50) and added to disposable polystyrene cuvettes for analysis.

Dynamic particle size of each suspension was assessed by monitoring zeta average (weighted mean particle size), and poly-dispersity index (indication of the breadth of particle size) of the solution (Malvern Panalytical, 2021).

2.3.10. Heat coagulation time (HCT)

Samples were analysed using the method outlined by Davies and White (1966) with some modifications. Protein ingredient and model IF solutions (1.5 %, w/w, protein) were prepared as described in section 2.3.4. prior to pH adjustment. Samples were divided into seven 30 mL aliquots and then pH adjusted to pH 6.2, 6.4, 6.6, 6.8, 7.0, 7.2 or 7.4 using 0.1 M HCl or NaOH. All samples were kept at 4 °C overnight under constant agitation. Samples were then equilibrated at 20 °C and pH adjustments made if necessary. Prior to analysis, samples (3 g) were transferred into individual glass tubes with rubber stoppers before securely placing in the oil bath rack. The rack was then placed into an oil bath at 140 °C and oscillated at a speed of 8 rpm with a swing angle of 35 °. The time taken for large coagulates to form was recorded. This test was conducted in triplicate for each sample by the same individual.

2.2.11. Statistical analysis

Samples and formulations were prepared and analysed in triplicate unless stated otherwise. SPSS statistical package was used to perform one way ANOVA with Bonferonni *post hoc* analysis (SPSS Inc., Chicago, IL, USA).

2.4. Results

2.4.1. Powder ingredient composition

The composition of casein and whey protein rich ingredients are shown in Table 2.1 and Fig. 2.1 with results obtained in line with typical compositions for these ingredients

(Affertsholt & Watson, 2020, 2021). Protein is calculated as Nitrogen X 6.25 so as to align with Codex Alimentarius recommendations (Codex Alimentarius, 2007). Comparing the casein-rich ingredients, SMP and MPC 80, the latter had higher protein and moisture content and lower level of NPN. Fig. 2.1 shows that, compared to SMP, the majority of minerals removed during MPC 80 manufacture are monovalent, particularly potassium, sodium, and chloride.

Table 2.1. Composition of milk protein concentrate (MPC 80), skim milk powder (SMP), cheese whey protein isolate (cWPI), cheese whey protein concentrate (cWPC 35) and acid whey protein concentrate (aWPC 35).

		MPC 80	SMP	cWPI	cWPC 35	aWPC 35
Fat	%	1.29	0.72	0.81	12.9	2.01
Protein (N×6.25)	%	78.8	37.3	86.2	35.3	33.3
Moisture	%	4.91	2.79	5.92	3.10	4.49
Lactose (estimated)	%	5.64	50.4	2.46	43.0	51.1
Ash	%	7.70	7.99	2.78	6.01	8.46
Ca: P ratio		1.77	1.28	1.88	0.90	1.72
NPN	%	0.04	0.25	0.81	0.41	0.26
NPN as % of total N	%	0.34	4.16	5.88	7.44	4.82
WPNI	mg / g powder	3.5	4.6			
Denaturation at pH 4.6	% of total N			1	31	29

Ca: P – Calcium: Phosphorus; NPN – non-protein nitrogen; WPNI – whey protein nitrogen index; N – Nitrogen

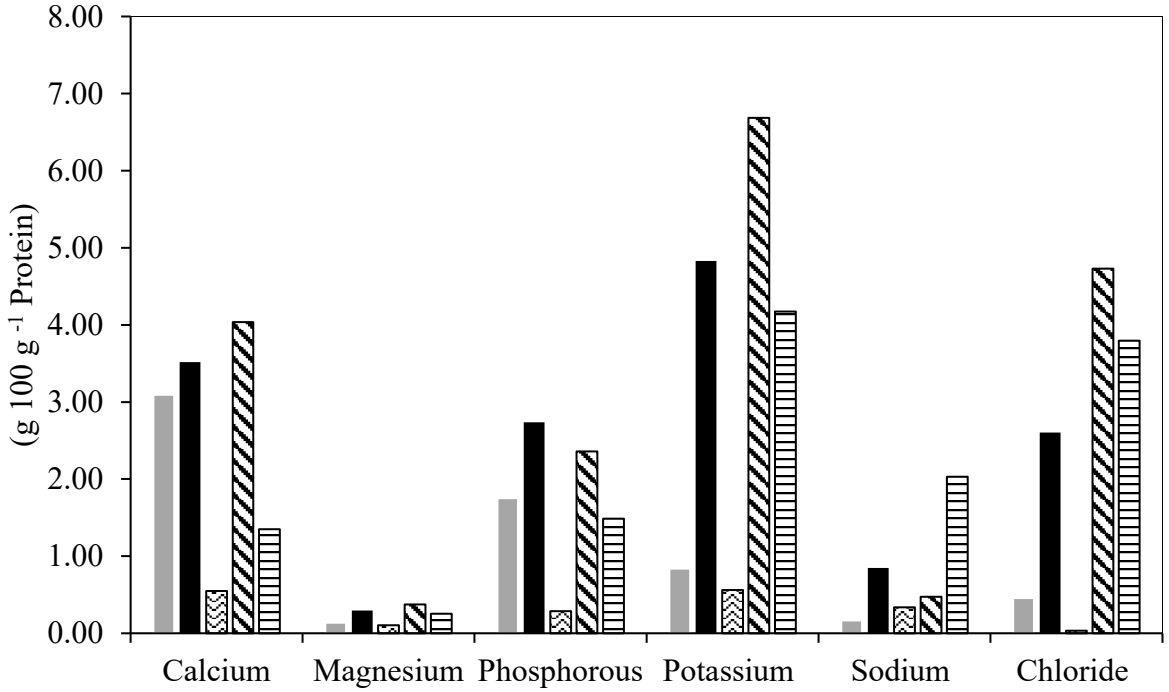

Fig. 2.1. Mineral profile (g 100 g^{-1} protein) of milk protein concentrate (▒), skim milk powder (■), cheese whey protein isolate (▒), acid whey protein concentrate (▒), cheese whey protein concentrate (▒)

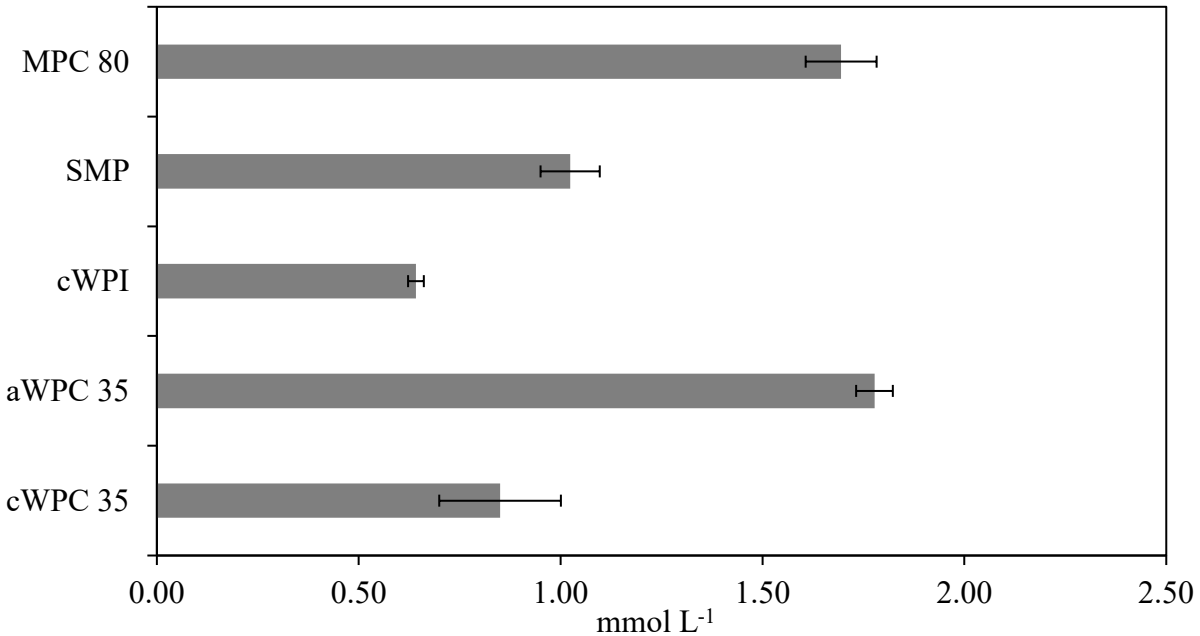

Fig. 2.2. Apparent ionic calcium concentration of suspensions (1.5%, w/w, protein) of skim milk powder (SMP), milk protein concentrate (MPC 80), cheese whey protein isolate (cWPI), acid whey protein concentrate (aWPC 35), cheese whey protein concentrate (cWPC 35) reconstituted in MQ water;

Interestingly, the calcium content was similar between SMP and MPC 80, but the phosphorus level was significantly lower in MPC 80, suggesting that, prior to ultrafiltration, a considerable amount of the phosphorus content in skim milk exists in the serum phase and susceptible to removal during MPC manufacture (Singh, 2009). The calcium: phosphorus ratio of SMP and MPC 80 was 1.28 and 1.77, respectively, with the latter having a significantly higher apparent ionic calcium concentration, at 1.70 mM, compared to SMP at 1.02 mM (Fig. 2.2).

Table 2.1 also shows the composition of all three whey protein ingredients, with both aWPC 35 and cWPC 35 containing ~35% protein. However, the ash and calcium: phosphorus ratio was significantly higher in aWPC 35 than cWPC 35 (Table 2.1). aWPC 35 also had a significantly higher apparent ionic calcium concentration (1.78 mM) compared to cWPC 35 (0.85 mM) (Fig. 2.2), suggesting there was insufficient phosphorus available in aWPC 35 to bind calcium ions. Further differences in the mineral profile are shown in Fig. 2.1, with aWPC 35 containing higher levels of calcium, phosphorus, potassium and chloride, and lower levels of sodium, compared to cWPC 35. cWPI contained 86.2 % (w/w) protein and was significantly depleted in minerals compared to the WPC samples. It was also observed that aWPC 35 had a lower NPN content than either cWPC 35 or cWPI, while cWPI contained almost no denatured whey proteins. Also of note is the difference in amino acid profile of aWPC 35 and cWPC 35 or cWPI with aWPC 35 containing higher levels of the essential amino acids tryptophan and cysteine per gram of protein than its cheese whey counterparts. (Fig 2.3)

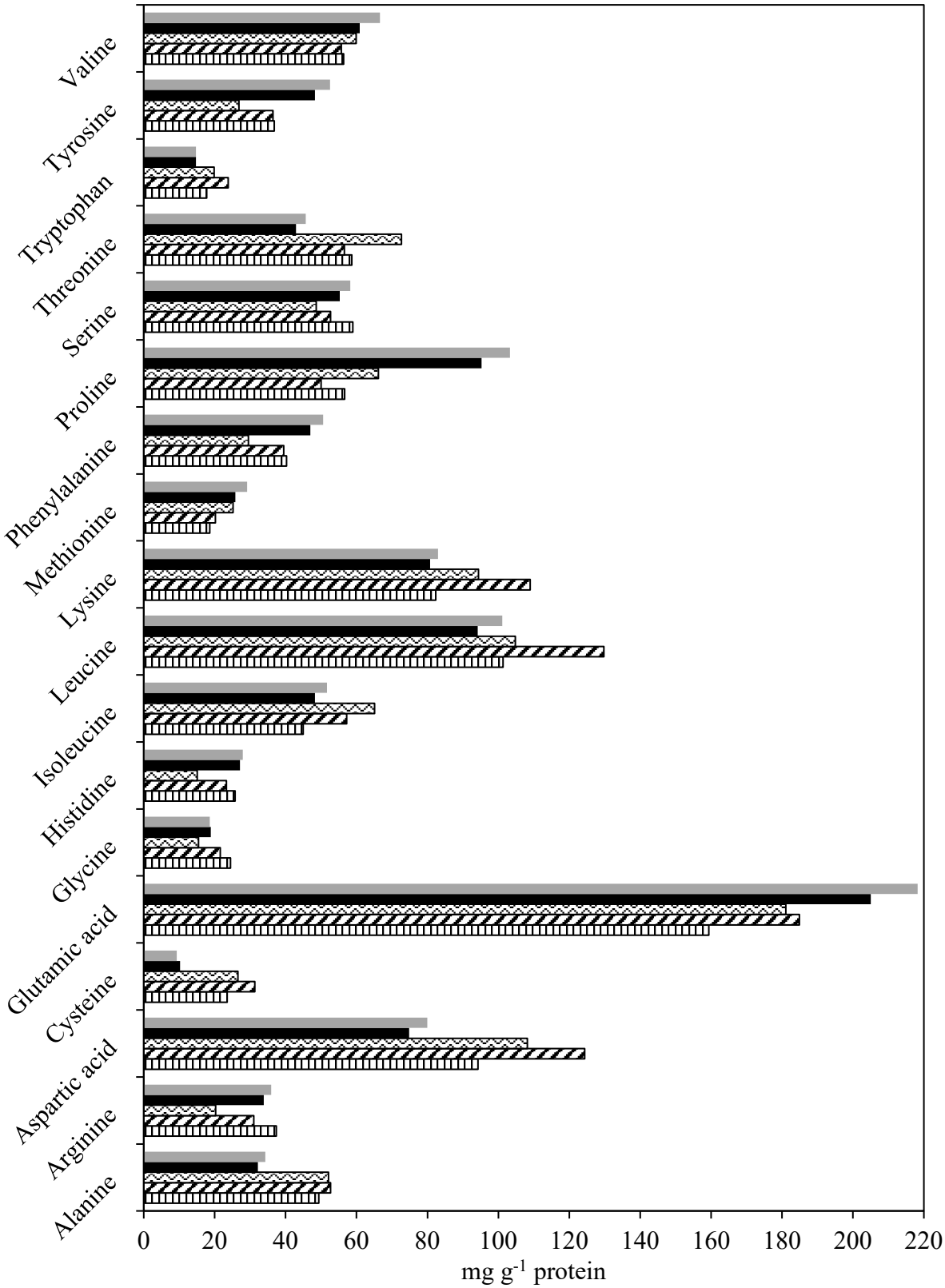

Fig. 2.3. Amino acid profiles of protein ingredients from (A) casein sources and (B) whey protein sources on a g 100 g $^{-1}$ protein basis (protein calculated as N*6.25) MPC 80 (▒), SMP ■, cWPI ▨, aWPC 35 ▨, cWPC 35 ▤

2.4.2. Model infant formulae composition

The composition of model IF was calculated based on measured ingredient compositions when combined to a 40:60 casein: whey protein ratio and a total protein content of 1.5 % w/w (Table 2.2). The calculated amino acid profile of the model IFs is shown in Fig. 2.4 and compared against codex requirements (Codex Alimentarius, 2007). Each blend meets the minimum requirements according to Codex; however, formulations using cWPC 35 and cWPI barely exceed the minimum requirements for tryptophan and cysteine, with aWPC 35 formulations offering a slightly larger margin. However, there were a number of amino acids that exceeded the minimum levels required by a significant amount, such as isoleucine, leucine, lysine, threonine, valine and methionine.

Table 2.2. Composition of model infant formulae containing blends of milk protein concentrate (MPC 80), skim milk powder (SMP), cheese whey protein isolate (cWPI), cheese whey protein concentrate (cWPC 35) and acid whey protein concentrate (aWPC 35),

	MPC 80 & cWPI	MPC 80 & WPC35	MPC 80 & aWPC35	SMP & cWPI	SMP & cWPC35	SMP & aWPC35
	g 100 g^{-1} formula					
Protein (N×6.25)	1.5	1.5	1.5	1.5	1.5	1.5
Casein	0.6	0.6	0.6	0.6	0.6	0.6
Whey protein	0.9	0.9	0.9	0.9	0.9	0.9
NPN (% of total N)	3.0	3.8	2.5	4.9	5.7	4.4
Total solids	9.3	9.6	9.5	9.4	9.8	9.6
Lactose	7.7	7.7	7.7	7.7	7.7	7.7
Lactose from protein ingredients	0.1	1.0	1.2	1.0	2.0	2.0
Fat	0.02	0.26	0.06	0.02	0.26	0.06
Ash	0.09	0.18	0.25	0.18	0.27	0.33
Ca: P ratio	1.79	1.37	1.73	1.34	1.15	1.48

NPN – non-protein nitrogen; N- Nitrogen, Ca: P- Calcium: phosphorus

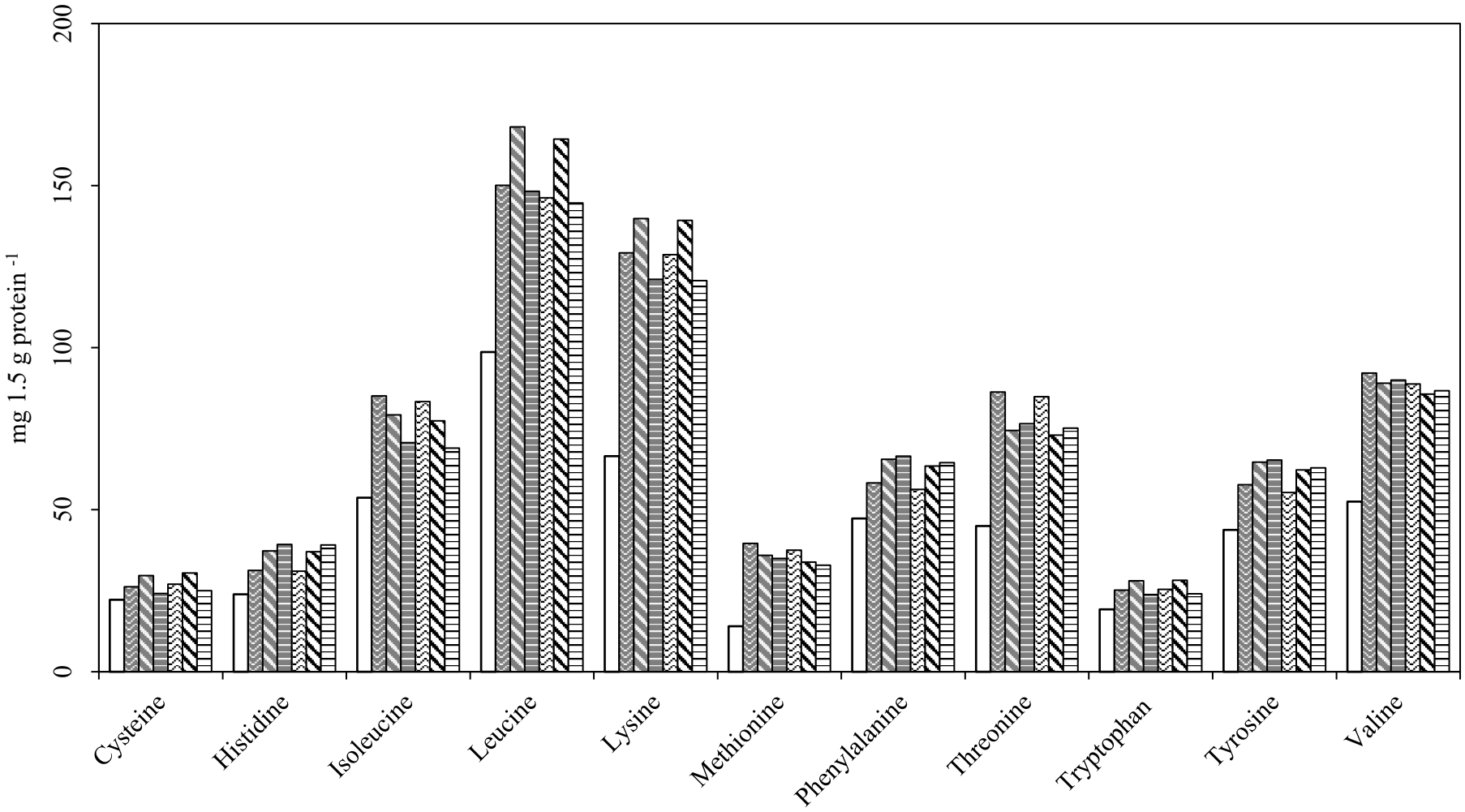

Fig. 2.4. The minimum level of each amino acid (□) as outlined by Codex Alimentarius (2007) comapted to the calculated amino acid profile of protein blends prepared to a concentration of 1.5% (w/w) protein with a casein: whey ratio of 40:60 from MPC 80 and cWPI (▨), MPC 80 and aWPC 35 (▨), MPC 80 and cWPC 35 (▨), SMP and cWPI (▨), SMP and aWPC 35 (▨), SMP and cWPC 35 (▨) .

Compositional differences in NPN of ingredients result in significantly different calculated NPN values between blends, with model formulae of MPC 80 / aWPC 35 (2.5 % of total nitrogen) having the lowest NPN content while the SMP / cWPC 35 blend had the highest NPN content (5.7 % of total nitrogen).

The calculated ash content also varied significantly between blends with MPC 80-cWPI having the lowest ash content at 0.09 g 100 g^{-1} formula while the blend of SMP-aWPC 35 had the highest ash content of 0.33 g 100 g^{-1}. The estimated mineral profile of all model IFs is shown in Fig. 2.5 relative to current Codex regulations, with all IF blends being below the maximum levels allowed for each of the major elements. However, it can be seen that the IF blend with SMP-aWPC 35 had the highest innate mineral content; notably, it was high in potassium, chloride and phosphorus. The SMP-aWPC 35 and MPC 80-aWPC 35 blends contained the highest level of total calcium and the highest apparent ionic calcium concentration (Fig. 2.5 and 2.6), but the MPC 80-cWPI blend was below the minimum levels for all the major elements with the exception of phosphorus. To be confident of not exceeding regulatory limits, it is often desirable to have the innate mineral profile significantly lower than the targeted levels, which in turn are often significantly lower than the upper regulatory limits. This provides IF manufacturers with the flexibility to supplement minerals in a form, or at a processing point, where any negative impact on processing can be minimised without exceeding mineral targets.

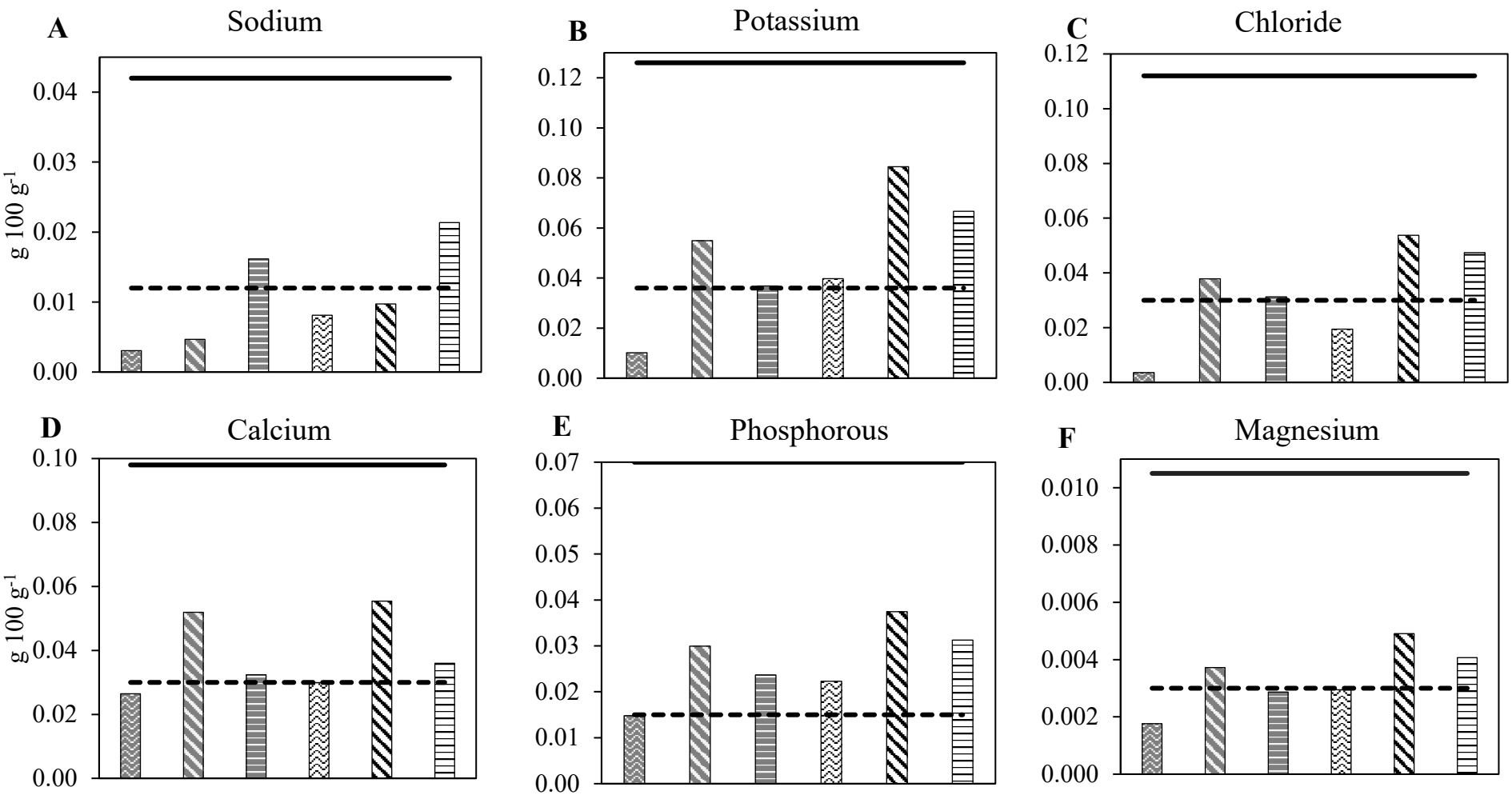

Fig. 2.5. Calculated mineral profiles of protein blends (1.5%, w/w, protein) with a casein: whey ratio of 40:60; MPC 80 and cWPI, MPC 80 and aWPC 35, MPC 80 and cWPC 35, SMP and cWPI, SMP and aWPC 35, SMP and cWPC 35 on a g $100g^{-1}$ basis. Blends were compared to Codex standards (Codex minimum - - -, Codex maximum, ——) (Codex Alimentarius, 2007)

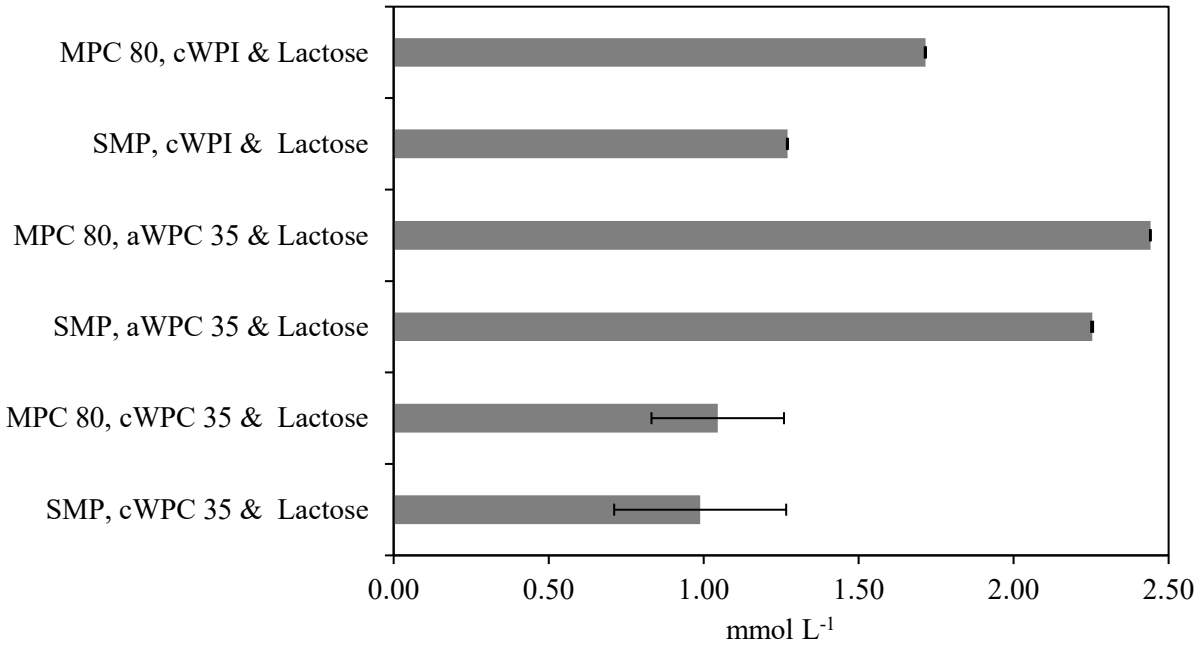

Fig. 2.6. Apparent ionic calcium concentration of 1.5% (w/w) protein and 7.7% (w/w) lactose model infant formulae prepared to a casein: whey protein ratio of 40:60.

2.4.3. Heat stability

The heat coagulation profiles at 140 °C for protein ingredients are shown in Fig. 2.6A. When assessed individually, SMP followed a typical type A HCT curve across the pH range examined, with a HCT max observed at pH 7.0 followed by a decrease in HCT at pH 7.2 and 7.4. MPC 80, which is low in lactose (Table 2.1) and serum phase calcium-binding compounds (Fig. 2.1), followed a type B heat coagulation profile, with HCT increasing with increasing pH. As expected, due to the high apparent ionic calcium concentration (Fig. 2.2), heat-induced aggregation of aWPC 35 occurred rapidly at pH values below pH 7.0; however, interestingly, aggregation appears to be delayed above pH 7.0. In comparison, cWPC 35 showed improved heat stability at pH values in excess of pH 6.8, with heat stability increasing steadily with increasing pH. Interestingly, cWPI, which is depleted in minerals and lactose, showed a higher heat stability up to pH 7.0 than the other whey protein concentrates (Fig. 2.7A).

When the casein and whey protein streams were combined to IF ratios (Fig. 2.7B), the mineral-depleted blend of MPC 80 and cWPI had the longest heat coagulation time in the range

pH 6.8-7.4, with a type B curve of increasing heat stability with increased temperature, similar to that of MPC 80 alone. The SMP- cWPI blend also followed a type B trend at pH values above 6.8; however, coagulation occurred more rapidly than the MPC 80-cWPI blend. Fig. 2.7C shows all blends with lactose standardised to IF levels, and it can be clearly seen that lactose significantly reduced the heat stability of all formulations across all pH ranges, with the worst affected being MPC 80-cWPI and MPC 80-aWPC 35 blends.

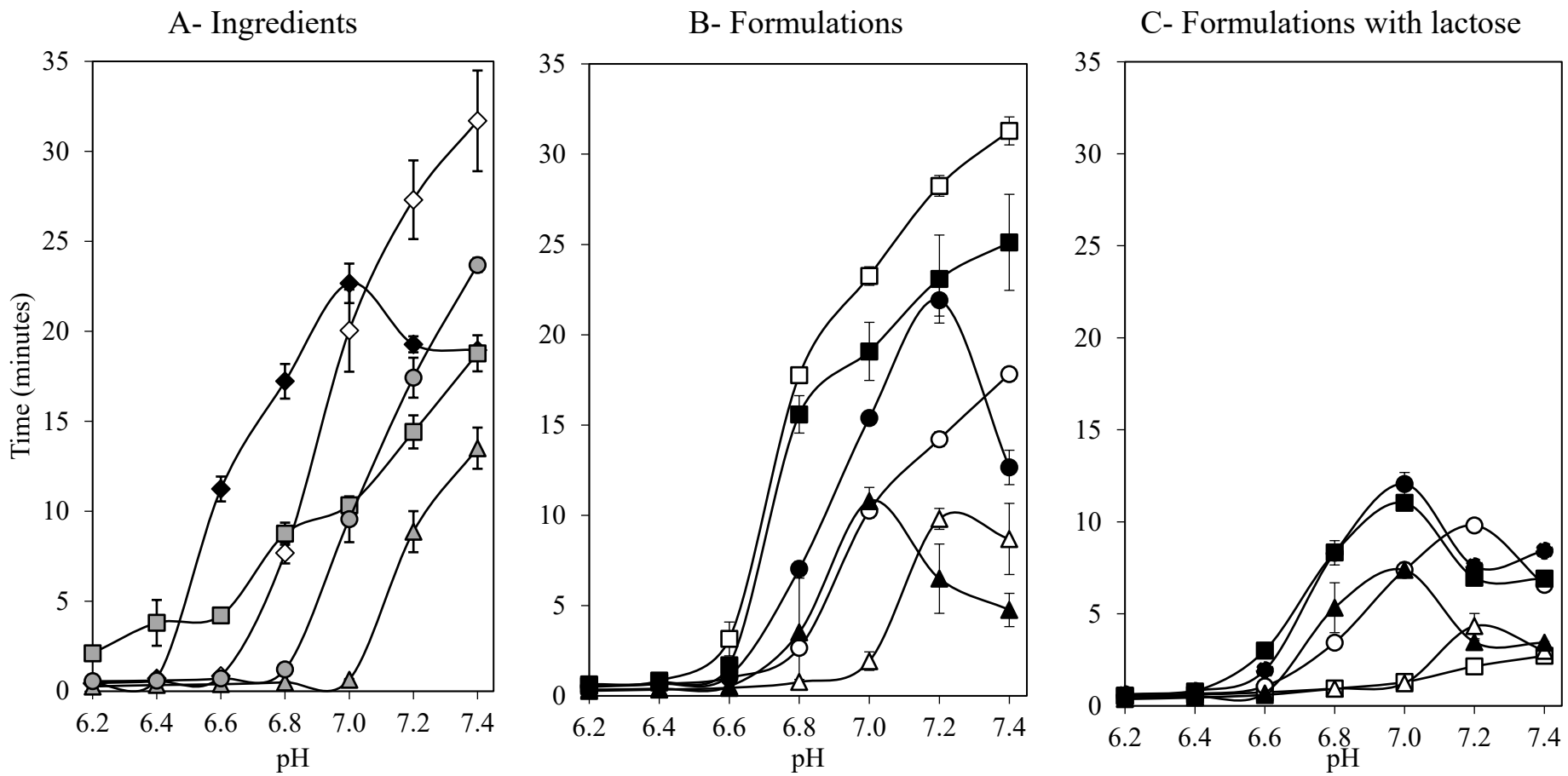

Fig. 2.7. Heat coagulation time of (A) protein ingredients: milk protein concentrate (MPC 80) ◇, skim milk powder (SMP) ◆, cheese whey protein isolate (cWPI) □ , acid whey protein concentrate (aWPC 35) △ and cheese whey protein concentrate (cWPC 35) ○, (B) formulations and (C) formulations with added lactose measured as a function of pH at 140 °C. Formulations containing MPC 80 are depicted with a white coloured symbol while formulations with SMP are shown in black. Formulation whey protein type is differentiated by symbol shape; cWPI □, aWPC 35 △ and cWPC 35 ○.

2.3.3 Changes in viscosity and particle size during simulated HTST

The viscosity of each protein ingredient and model IF before, during and after HTST treatment (95 °C for 5 min) are shown in Table 2.3. The formulations were subjected to this heating profile (95°C for 5 min) so as to stress the system by subjecting it to high temperature for a longer hold time than would typically be applied at industrial scale and therefore emphasise any gelation reactions that could occur during HTST treatment. All protein ingredients were relatively stable after HTST treatment, with the exception of aWPC 35, which showed an increase in viscosity at 74 °C and a peak viscosity of 145.7 mPa.s at 95 °C. Interestingly, all IF blends and blends standardised for lactose were stable during HTST treatment with only a slight increase in peak viscosity at 95 °C observed for blends of MPC 80-aWPC 35 and SMP-aWPC 35 in the presence of lactose; however these blends returned to pre-heat-treatment levels of viscosity during cooling (Table 2.3).

Table 2.3. Viscosity changes during simulated HTST treatment of protein suspensions (1.5%, w/w, protein) containing milk protein concentrate (MPC 80), skim milk powder (SMP), cheese whey protein isolate (cWPI), cheese whey protein concentrate (CWPC 35), acid whey protein concentrate (AWPC 35), and formulations at 40:60 casein: whey ratios without and with added lactose. Values presented are the means and standard deviations of data from triplicate trials; values within each sample category (i.e., ingredients, formulations without added lactose and formulations with added lactose) not sharing a common superscript differ significantly ($P < 0.05$).

	Initial Viscosity at 40°C	Temperature of inflection	Peak Viscosity at 95°C	Viscosity post cooling at 40°C
	mPa.s	°C	mPa.s	mPa.s
Ingredients				
MPC	12.0 ± 0.1 ^{ab}	87.3 ± 2.3 ^{ac}	13.3 ± 2.5 ^{ab}	11.4 ± 0.1 ^{a}
SMP	12.2 ± 0.3 ^{ab}	85.1 ± 1.5 ^{ac}	11.9 ± 2.1 ^{ab}	11.6 ± 0.1 ^{a}
cWPI	11.2 ± 0.04 ^{a}	87.8 ± 2.5 ^{a}	12.6 ± 1.2 ^{b}	11.5 ± 0.1 ^{a}
aWPC	12.9 ± 0.9 ^{b}	74.0 ± 1.4 ^{b}	145.7 ± 26.8 ^{c}	22.8 ± 2.8 ^{b}
cWPC	12.0 ± 0.1 ^{ab}	81.6 ± 1.2 ^{c}	11.5 ± 0.7 ^{a}	12.0 ± 0.7 ^{a}
Formulations (no added lactose)				
MPC/cWPI	11.9 ± 0.3 ^{a}	81.8 ± 2.7 ^{a}	18.4 ± 3.6 ^{a}	11.6 ± 0.1 ^{a}
SMP/cWPI	11.5 ± 0.05 ^{a}	88.1 ± 0.4 ^{b}	14.0 ± 2.7 ^{a}	11.6 ± 0.1 ^{a}
MPC/aWPC	11.9 ± 0.04 ^{a}	83.9 ± 2.6 ^{ab}	14.4 ± 2.0 ^{a}	12.1 ± 0.2 ^{bc}
SMP/aWPC	12.7 ± 0.6 ^{b}	84.0 ± 5.3 ^{ab}	18.3 ± 5.8 ^{a}	12.2 ± 0.1 ^{c}
MPC/cWPC	11.9 ± 0.1 ^{a}	86.5 ± 1.6 ^{ab}	11.6 ± 0.5 ^{a}	11.6 ± 0.03 ^{a}
SMP/cWPC	11.8 ± 0.2 ^{a}	87.9 ± 1.0 ^{b}	10.7 ± 0.2 ^{a}	11.9 ± 0.2 ^{b}
Formulations with added lactose				
MPC/cWPI	12.6 ± 0.1 ^{a}	90.6 ± 1.8 ^{a}	10.6 ± 0.5 ^{a}	12.5 ± 0.1 ^{ab}
SMP/cWPI	12.3 ± 0.2 ^{a}	89.7 ± 0.9 ^{a}	10.3 ± 0.6 ^{a}	12.4 ± 0.1 ^{a}
MPC/aWPC	14.7 ± 0.9 ^{b}	89.8 ± 1.0 ^{a}	36.6 ± 17.9 ^{b}	13.5 ± 0.7 ^{c}
SMP/aWPC	12.6 ± 0.4 ^{a}	84.6 ± 3.9 ^{a}	15.2 ± 3.7 ^{ab}	12.9 ± 0.01 ^{abc}
MPC/cWPC	13.8 ± 0.01 ^{b}	89.3 ± 4.7 ^{a}	11.5 ± 0.5 ^{a}	12.7 ± 0.1 ^{ab}
SMP/cWPC	12.6 ± 0.04 ^{a}	87.8 ± 0.5 ^{a}	11.4 ± 0.4 ^{a}	13.2 ± 0.05 ^{bc}

Changes in particle size and poly-dispersity index (PDI) as a result of HTST treatment are shown in Table 2.4 Particle size before and after HTST treatment for MPC 80, SMP and cWPC 35 were stable with little change occurring; however, WPI was found to be too polydisperse prior to heat treatment and was seen to contain visible flecks after HTST treatment, while aWPC 35 was not measurable due to significant gelation occurring during HTST treatment. Similar to the viscosity data in Table 2.3, the particle size of IF blends and blends with added lactose were all similar after HTST treatment, with no major changes in size.

Table 2.4. Zeta average particle size before and after HTST treatment of protein suspensions (1.5%, w/w, protein) containing milk protein concentrate (MPC 80), skim milk powder (SMP), cheese whey protein isolate (cWPI), cheese whey protein concentrate (cWPC 35), acid whey protein concentrate (aWPC 35) and formulations at 40:60 casein: whey ratios without and with added lactose.

	Before HTST		After HTST		Particle size Difference
	d.nm	(PDI)	d.nm	(PDI)	%
Ingredients					
MPC	179 ± 13.3	(0.16)	187 ± 9.5	(0.15)	5% [a]*
SMP	221 ± 13.1	(0.12)	193 ± 9.0	(0.12)	-15% [a]
cWPI	**		Flecked		N/A
cWPC	286 ± 18.6	(0.40)	293 ± 11.4	(0.41)	2.3% [a]
aWPC	194 ± 12.5	(0.15)	Gelled		N/A
Formulations					
MPC/ cWPI	186 ± 16.4	(0.20)	205 ± 12.1	(0.22)	9% [a]
SMP/ cWPI	216 ± 16.2	(0.12)	219 ± 11.0	(0.13)	1% [a] *
MPC/ cWPC	228 ± 48.9	(0.31)	231 ± 8.6	(0.29)	1.3% [a]
SMP/ cWPC	250 ± 9.8	(0.26)	232 ± 8.2	(0.26)	-7.1% [b]
MPC/aWPC	197 ± 8.0	(0.22)	213 ± 15.6	(0.29)	7% [a]
SMP/aWPC	218 ± 13.6	(0.12)	220 ± 10.9	(0.10)	1% [a]
Formulations with added lactose					
MPC/ cWPI	218 ± 24.5	(0.31)	231 ± 19.2	(0.26)	6% [a]
SMP/ cWPI	235 ± 15.5	(0.14)	232 ± 18.0	(0.16)	-1% [ab]
MPC/ cWPC	241 ± 15.0	(0.32)	245 ± 12.0	(0.32)	1.6% [ab]
SMP/ cWPC	253 ± 8.6	(0.25)	234 ± 9.9	(0.26)	-7.5% [b]
MPC/aWPC	***		***		N/A
SMP/aWPC	229 ± 15.7	(0.12)	242 ± 18.8	(0.13)	6% [a]

Values presented are the means and standard deviation of data from triplicate trials; values within each subsection not sharing a common superscript differ significantly ($P < 0.05$).
N/A – not applicable
**sample too polydisperse for accurate measurement.
*** sample did not meet quality criteria for accurate measurement

2.5. Discussion

It is essential that any IF meets the correct amino acid requirements for the healthy development of infants. As the only source of amino acids, it is critical that the protein content and profile of the base formulation meets the minimum essential amino acid levels as outlined by regulatory authorities (Codex Alimentarius, 2007). Typically, tryptophan and cysteine levels are the most difficult to meet, and so IF manufacturers have to increase overall protein content in order to achieve these minimum requirements (Dutta, et al., 2017). Cheese whey protein tends to have a lower proportion of these amino acids due to the presence of tryptophan-depleted glyco-macro peptide (GMP), which results in a need to increase the protein content of formulas using cheese whey-derived protein in order to meet amino acid requirements (Dutta et al., 2017). Some indications of this can be seen in Fig. 2.3, with aWPC 35 having a 20 % and 34 % higher tryptophan content than cWPI and cWPC 35 on a mg g^{-1} protein basis. The model IF blends in this study were prepared at 1.5 % w/w (1.54 g per 100 mL) protein. However, in recent years, there has been much discussion around reported links between high protein content in IF and increased risk of obesity, which has driven a focus towards reducing the protein content of IF to closer to human milk levels without addition of free amino acids (0.9-1.3 g per 100 mL) (Kunz & Lönnerdal, 1992; Layman, Lönnerdal, & Fernstrom, 2018; Luque, et al., 2015). It is clear that, using cheese-whey-derived ingredients, it would be difficult to reduce the overall protein content and still consistently achieve the minimum requirements for tryptophan and cysteine.

According to Codex Alimentarius (2007), IF must contain a minimum of 33 mg tryptophan per 100 kcal (19.8 mg per 100 mL). Based on the results shown in Figure S1, both casein-rich ingredients (MPC 80 and SMP), contain 14.7 mg tryptophan g^{-1} protein, acid-whey protein 23.8 mg tryptophan g^{-1} protein and cheese-whey protein 17.8 mg tryptophan g^{-1} protein. With these figures, the minimum acceptable protein content of IF prepared using cWPC 35 is 1.2 g per 100 mL (19.8 mg tryptophan), while formulating using aWPC 35 could meet tryptophan

requirements at a protein content of 1 g per 100 mL (20 mg tryptophan). Therefore, increasing the use of aWPC 35 in IF manufacture could allow for a reduction in protein content while maintaining sufficient delivery of all essential amino acids required for growth and development.

Differences in NPN content of ingredients, notably the observed lower NPN content of aWPC 35 and MPC 80 compared to other whey protein and casein rich ingredients analysed (Table 2.2), is also relevant as a high NPN content, if not accounted for, could lead to variation in measured protein and nutritional composition of the formula (Depeters & Ferguson, 1992; Dupont, Grappin, Pochet, & Lefier, 2011). Also, the difference in composition of NPN between ingredients needs to be considered, In SMP, NPN is predominantly low molecular weight compounds such as urea, which is non-nutritive, however, in cheese whey it can be due to a large amount of GMP that is still soluble at 12% trichloroacetic acid and so is composed of nutritive peptides (Kalan & Woychik, 1965).

However, the processability of these ingredients during IF manufacture often defines their suitability. A good indicator of ingredient behaviour in complex systems is their inherent heat stability measured over a range of pH values. SMP displayed greater heat stability compared to MPC 80 at pH values below 7.0 (Fig. 2.7A). The lower heat stability of MPC 80 in this pH range may be due to increased concentration of ionic calcium caused by the loss of serum phase calcium-binding ions such as phosphate, citrate and chloride during the ultrafiltration process. This theory aligns with research published by Ho et al. (2018) who found a correlation between apparent ionic calcium levels and HCT for MPC samples, the pH of which was adjusted with HCL or citric acid. An estimation of the apparent calcium ion concentration is shown in Fig. 2.2 which shows MPC to be significantly higher than SMP at the same protein concentration (1.5 g 100 g^{-1}). However, it's important to note that standardisation of pH to pH 7 (section 2.3.4.) and base ionic activity through the addition of KCl (section 2.3.8.) in order to improve the repeatability and robustness of the measurement will alter the natural calcium ion activity of the

solution. Therefore, this should be treated as an indication of differences in apparent calcium ion concentration between samples rather than a strictly quantitative measurement.

Temperature-viscosity profiles showed that, when assessed as individual ingredients, casein-dominant MPC 80 and SMP had a high temperature of inflection and show very little change in viscosity following HTST treatment (Table 2.3). This suggests that they are highly heat-stable, with little to no aggregation taking place during HTST treatment. There was also no statistical difference between MPC 80 and SMP for any of the viscosity or particle size characteristics analysed, which suggests that, in a casein-dominant environment, the loss of soluble compounds and negatively charged counter ions has little effect on protein stability, in this temperature range. As expected, calcium-rich aWPC 35 had a lower temperature of inflection for viscosity (i.e., 74 °C; Table 2.3) than all other protein ingredients, indicating that the onset of denaturation/aggregation occurred earlier, with complete gelation occurring during holding at 95 °C and cooling back to 40 °C. The high calcium and ionic calcium content of aWPC 35 (Fig. 2.1 and 2.2) is caused by the release of calcium ions from casein micelles during acidification which then remain with the acid whey protein stream during further processing (Jeswan Singh, et al., 2015).

In comparison, cWPC 35, which had a similar level of protein denaturation and ash content to aWPC 35, displayed good protein stability, with a relatively high temperature of inflection and no significant change in viscosity or particle size post HTST treatment, due to its comparatively lower level of total and apparent ionic calcium concentration (Fig. 2.1 and Fig. 2.2, respectively). Interestingly, a high PDI value was observed in cWPC 35 (0.40, Table 2.4), which may be due to the higher fat content of this ingredient (12.9%, Table 2.1) than other samples analysed, suggesting the presence of fat globules in the higher particle size range. It is important to note that, during HCT testing, the coagulation of cWPI differed from other samples in that, instead of forming large aggregated coagulates, the coagulates formed were much smaller

flecks. This is most likely due to the low level of minerals (Table 2.1; Fig. 2.1) present in cWPI, preventing extensive protein-protein aggregation from occurring (Petit et al., 2016). Similarly, this was also seen in the particle size analyses, where cWPI flecked under HTST conditions. This is in contrast to cWPC 35 which remained relatively stable throughout HTST treatment. The high degree of flecking (formation of very small, fine particles which adhere to the glass tube, a typical precursor to coagulation) observed in cWPI may be a result of the high level of native whey protein in cWPI as opposed to cWPC 35, which contains pre denatured whey protein (Table 2.1) (Ryan & Foegeding, 2014).

In general, all protein blends analysed had good thermal stability under heat treatment at temperatures representative of HTST pasteurisation or UHT treatment (Table 2.3 and Fig. 2.7B). Interestingly, despite the poor thermal stability of aWPC 35 when analysed alone, blends containing aWPC 35 remained stable throughout HTST processing with only a slight increase in viscosity at 95ºC observed in blends containing aWPC 35 and lactose which returned to pre heat treatment viscosity during cooling. This indicates that while there may be slight differences in the thermal stability of these blends at 95 °C, the reactions were insufficient to trigger significant protein aggregation. It was also observed that blends of SMP and aWPC 35 have significantly longer HCT profiles than MPC 80-aWPC 35 at pH values below pH 7.0 (Fig. 2.7B). This indicates that serum phase minerals present in SMP play a role in maintaining protein thermal stability in a calcium-rich environment, likely *via* calcium binding, resulting in reduced calcium-mediated protein aggregation at high temperatures (Aydogdu, et al., 2021). Overall, it was observed that blends containing SMP tended to have better thermal stability at UHT temperatures, and a reduced particle size increase post HTST treatment than those containing MPC 80, with the exception of the MPC 80-cWPI blend, which was the most stable blend. This suggests that serum phase compounds present in SMP, such as phosphorus, citrate and chloride, may play an important role in maintaining thermal stability when combined with mineral-rich

whey ingredients during IF manufacture. The exceptional stability in the case of MPC 80-cWPI blends is likely due to its low mineral content, i.e., 50-73% lower than all other blends (Table 2.2). This high stability of MPC 80-cWPI also indicates that, in a lactose and mineral-depleted environment, where protein- protein interactions dominate, casein may have a protective effect against whey protein aggregation (Kelleher et al., 2020). Numerous studies have observed this protective or chaperone effect of casein proteins against whey protein aggregation, with theories such as stearic hindrance where casein simply inhibits access to hydrophobic whey protein aggregation sites exposed during denaturation being suggested as the mechanism for limiting aggregation (Kehoe and Foegeding, 2011; Kelleher et al., 2020).

The addition of lactose to IF blends resulted in very different outcomes for HCT and HTST treatment. HCT data clearly showed that lactose addition decreased heat stability in all formulations at 140 °C; whereas at HTST temperatures, lactose addition seemed to delay the onset of viscosity increase during the temperature ramp (Table 2.3). At elevated temperatures (>100 °C), the interaction of lactose with amino acid lysine-residues in protein *via* the Maillard reaction triggers protein cross-linking and, in advanced stages, formation of acetic and formic acid (Al-Saadi, Easa, & Deeth, 2013; Davidek, Devaud, Fabien, & Imre, 2006, Yilmaz & Toledo, 2005). This reaction is accelerated with increasing temperature and so, at 140 °C, it is likely that the Maillard reaction cascaded quickly from protein cross-linking to acid formation and protein destabilisation (Angel Rufián-Henares, Guerra-Hernández, & García-Villanova, 2006; Xing et al., 2020). During HTST treatment (<100 °C), lactose can retard whey protein aggregation due to preferential hydration of the sugar and the exclusion of lactose from the protein, leading to an unfavourable increase in the free energy of the system. As contact between carbohydrate and protein is thermodynamically unfavourable, whey proteins have a greater tendency to remain in native, compact form when heated in the presence of lactose which may explain the observed

delay in viscosity increase during heating to HTST temperatures of formulations with added lactose (Anema, Siew, & Klostermeyer, 2006; Murphy et al., 2014).

When it comes to ingredient selection for IF manufacture, there are obviously a number of characteristics, such as amino acid profile, mineral profile/content, and physico-chemical properties to be considered; all of which are balanced against cost of ingredients. For instance, the additional processing of MPC 80 results in a product which is more expensive per kg of protein than SMP, with MPC 80 market sale prices from 2015 to 2020 on average 15% higher than SMP on a per kg protein basis and also requires additional lactose and minerals, which further increases manufacturing costs (Affertsholt & Watson, 2021). Similarly, the price of WPI from 2015-2020 was approximately 45% higher per kg protein than that of WPC 35 (Affertsholt & Watson, 2020). Based on the figures provided by Affertsholt and Watson (2020 & 2021), the base formulation of MPC, cWPC 35 and lactose used in this study without minerals balanced would cost approximately 14% more per litre than the same formulation using SMP instead of MPC (\$0.16 L^{-1} using MPC compared to \$0.14 L^{-1} using SMP). Meanwhile, a base formulation consisting of SMP, WPI and lactose (\$0.19 L^{-1}) would cost an additional 34% compared to the same formulation manufactured using cWPC 35 (\$0.14 L^{-1}). However, from an IF perspective, the reduced levels of innate minerals in MPC 80 and cWPI (Fig. 2.1) could allow for better control of the mineral content in the final product and so, while it may be more expensive to formulate, it could enable a reduction in manufacturing out of specification material. In addition, the low lactose content of these ingredients could in theory facilitate the production of a low lactose IF product where alternative carbohydrate sources could be added, which may have benefits for infants who have sensitivity to high lactose IF products. Variation in innate mineral profile is another important factor when selecting a protein source. The seasonal nature of the mineral profile of bovine milk can pose challenges, as an ingredient with a high level of innate minerals may have a high degree of variability across the year. IF products must be within an

acceptable range of the stated mineral content on the product label. Therefore, a high degree of innate mineral variation requires IF manufacturers to adapt their formulation on a batch-by-batch basis, which adds significant complications for processing. An alternative option is to put in place a detailed product specification such that ingredient suppliers only supply product that grades into the acceptable mineral profile range; however, this may affect product supply if seasonal changes result in suppliers being unable to meet these tight specifications and also restricts manufacturers to using powdered ingredient sources. Therefore, while protein ingredients with a lower ash content such as MPC 80, cWPI or a higher protein WPC such as WPC 80 would be more expensive compared to SMP or WPC 35 (15 %, 45 % and 31 % premium per kg protein respectively), they could facilitate a more consistent mineral profile, with savings achieved through the avoidance of ongoing formulation changes and out of specification product (Affertsholt & Watson, 2020, 2021).

In the use of ingredients with a high mineral content and relatively low heat stability, such as SMP and aWPC 35 blends, negatively charged counter ions in the form of tri-sodium citrate, tri-potassium citrate or disodium hydrogen phosphate may be added to reduce the level of free positively-charged divalent ions (Hebishy et al., 2019), although their addition rates may be limited if sodium and potassium levels are inherently high in the protein ingredients. Therefore, the use of aWPC 35 in IF production can make it difficult to control calcium-mediated aggregation during processing while remaining within the mineral regulatory limits. In addition, these ingredients, as they will remain in the product and are not removed during processing, are considered additives and must be included on the ingredient listing of the final product. On this basis, it could be preferable to use protein sources with a lower calcium content and add an insoluble calcium source such as calcium phosphate or calcium carbonate, which may have less of an impact on processing; however, these will also be considered food additives and will have to be include in the ingredients list (Joyce et al., 2018; Karlsson et al., 2019).

While these model formulations were not formulated to meet regulatory mineral requirements and, in a commercial setting, additional minerals would be added to meet Codex requirements, which would further alter the functionality and thermal stability of the formulation, these results offer an insight into the baseline stability of key IF ingredients. These findings, in particular with regard to the roles of ionic calcium, serum phase compounds and lactose during thermal processing, highlight the importance of understanding not only the composition of an ingredient but also its physico-chemical properties in various pH, temperature and ionic environments when selecting protein ingredients for use in IF applications. Additionally, these formulations were modelled on a RTD formulation (12-14 % total solids) and so have been heat treated at a lower concentration than typical powder manufacturing processes (>20 % total solids). Therefore, while the differences in heat stability and protein aggregation observed in these formulations will provide indicative guidance on how these ingredients will perform at a higher concentration, increased total solids level will impact the rate and magnitude to which thermal aggregation occurs.

2.6. Conclusions

This study examined the impact of ingredient composition on thermal stability of base IF formulations at RTD concentrations. A number of factors need to be considered when choosing a protein ingredient for IF applications; however, some key considerations include the protein profile, total mineral content, ionic mineral balance and lactose concentration with a high lactose content potentially having a positive or negative effect, depending on temperature. Interestingly, despite the high levels of minerals present, notably calcium, these results suggest that aWPC35 could replace cheese whey-based ingredients in IF provided the correct mineral balance is achieved, either through use of a casein source such as skim milk or through the addition of calcium-binding agents during wet processing. Further work would be required to build the

formulations to meet mineral requirements through addition of minerals and assess subsequent functionality. However, the work presented here could be used to guide this research with a view to enabling the increased use of aWPC35 in IF applications and subsequent reduction of overall protein level as a result of the more suitable amino acid profile when compared against cheese whey sources.

2.7. Acknowledgment

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Chapter 3

Effect of calcium phosphate precipitation on ultrafiltration of acid whey

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Declaration

This chapter was written by Sinead Mc Entee (SME) and reviewed by all co-authors.

3.1. Abstract

The high calcium content of acid whey can pose challenges to protein concentration using UF membranes due to calcium-induced fouling. This study examined the effect of pH and temperature on calcium precipitation, UF permeation efficiency and the removal of calcium through UF processing of acid whey across a pH range from 4.6 to 7.0 (15 °C) and a temperature range from 10 to 35 °C (pH 6.0). An apparent increase in calcium-phosphate precipitation was identified with increasing temperature and pH (from pH 5.6 onwards) which was correlated with an increased retention of calcium in the UF retentate indicating these parameters play a key role in determining the calcium content of the final product. Finally, examining the reversibility of calcium-phosphate solubility showed that, while reducing pH (from 7.0 to 4.6) can fully resolubilise calcium-phosphate, reducing temperature (50 to 10 °C) will only partially reverse calcium-phosphate precipitation. Therefore, from a manufacturing perspective, pH adjustment immediately prior to UF seems to be an effective tool for controlling calcium precipitation, which can be used to control process efficiency as well as product composition.

3.2. Introduction

Mineral acid whey is produced as a by-product of acid casein. In this process, the pH of milk is reduced through addition of mineral acid such as hydrochloric or sulphuric acid to the isoelectric point of casein (pH 4.6) causing it to separate from the serum phase in the form of casein aggregates. Once separated, the remaining liquid phase is known as acid whey, consisting of whey protein, lactose, and a high level of ash, notably calcium (Bylund, 1995; Singh, 2009). During the acidification process, colloidal calcium phosphate within casein micelles becomes solubilised and enters the serum phase, which results in a higher proportion of calcium in acid whey compared to whey produced by cheese manufacture or so-called ideal whey produced by microfiltration. This in turn has a negative effect on the thermal stability of acid whey during further processing, as high levels of ionic calcium promote calcium-mediated aggregation of whey proteins at temperatures above 70 °C (Joyce, Kelly, & O'Mahony, 2018). Crude acid whey typically has quite a low protein content and so undergoes further processing such as membrane filtration to increase protein concentration and remove excess lactose and minerals, including calcium. Alongside increasing protein concentration, ultrafiltration also removes excess minerals, including calcium, which can improve the thermal stability of the product (Caussin, Famelart, Maubois, & Bouhallab, 2003; Hendrix, Griko, & Privalov, 2000; Wijayanti, Bansal, & Deeth, 2014).

In the aqueous phase of a dairy stream, calcium can exist in ionic form (Ca^{2+}) or bound to counter ions such as phosphate, citrate or chloride. An equilibrium exists between ionic calcium and phosphate and bound calcium phosphate (Holt, 2004). Changes to the aqueous environment, such as pH, temperature, and calcium concentration, can cause shifts in this equilibrium. While calcium phosphate can be found in a soluble form in acid whey, its solubility is limited and so it will precipitate out of solution with increased concentration, in the form of a white crystalline material (Chandrapala et al., 2015). When this occurs, it creates a shift in the

serum phase salt equilibria and, in order to maintain a balance, ionic calcium and phosphate will bind to form additional calcium phosphate, which will continue to precipitate out of solution until a balance has been achieved between soluble calcium phosphate and ionic calcium and phosphate (Holt, 2004).

The solubility of calcium phosphate and the form in which calcium is present at each step of acid whey processing is key to maintaining processing efficiency and minimising fouling. During the concentration of whey protein by ultrafiltration (UF), ionic calcium permeates the UF membrane and so the calcium: whey protein ratio is reduced (Purkait & Singh, 2018). Conversely, insoluble calcium phosphate may not permeate through UF membranes and in fact can contribute to membrane fouling, which reduces permeation flux and membrane efficiency (Fei et al., 2021; Hausmann et al., 2013). In this scenario, it would be preferable to maintain the calcium in acid whey in the ionic form so as to minimise membrane fouling and maximise calcium removal and protein concentration. However, as mentioned previously, temperature is also a key factor in determining salts equilibria in dairy systems, as well as playing a determinant role in UF membrane performance and flux (Wagner, 2001). Therefore, optimum conditions of temperature and pH are required to reduce the negative effects of calcium phosphate precipitation and ensure efficient protein concentration and calcium removal.

Some studies have been published aimed at understanding the chemical and physical properties of acid whey, how it differs from other whey sources, and its impact on membrane performance during protein concentration, with calcium phosphate formation in particular highlighted as being a significant factor in determining the efficiency of protein concentration during UF (Konrad, Kleinschmidt and Faber, 2012; Chandrapala et al., 2015). In addition, alternative processes aimed at reducing the calcium loading of acid whey through forced precipitation of calcium phosphate and subsequent clarification using hydrocylcone technology have also been proposed which could improve the processing efficiency and thermal stability of

acid whey concentrates; however, this also results in the production of an additional by product rich in calcium phosphate, which will require further processing in order to minimise waste (Crowley et al., 2018).

In this study, the effect of pH and temperature on calcium phosphate precipitation in acid whey and the subsequent impact on UF membrane performance and calcium removal were assessed. The overall aim was to determine if processing efficiency of acid whey and the calcium content of the final product can be altered by controlling these conditions. This is significant for industrial acid whey processing as through optimising pH and temperature during UF processing, there is an opportunity to not only improve the processing efficiency of acid whey protein concentration, but also develop acid WPC's with a tailored calcium content which could be suitable to a wide range of applications.

3.3. Materials and methods

3.3.1. Acid whey preparation

Acid whey was manufactured from skim milk using the method outlined by Magan et al. (2019); fresh pasteurised skim milk obtained from a local supermarket (~5 L) was acidified to pH 4.6 using 1M HCl. The precipitated curd was then separated from the whey using cheese cloth and the whey was then further clarified using a 0.1 μm Sartocon Slice polyethersulfone cassette membrane (Sartorius AG, Göttigen, Germany) as per the method outlined by (Magan et al., (2019). The resultant acid whey consisted of approximately 6.13 % w/w^{-1} total solids, 0.67% w/w^{-1} protein, 0.8 % w/w^{-1} ash, 0.12 % w/w^{-1} calcium and 0.06 % w/w^{-1} phosphorous.

3.3.2. Alkalisation buffering index

The buffering index of acid whey was measured using a titration method. The titration was conducted using an auto titrator (Titrando 842 Autotitrator with a TIAMO v.2.2 software

package, Meterohm, Ireland Ltd., Carlow, Ireland) with a pH electrode which was calibrated using standards at pH 4, 7 and 10 prior to use. Acid whey (50 g) at 25 °C was initially acidified from native pH to pH 2 using 0.5 M HCL, and then alkalised using 0.5 M NaOH from pH 2 to pH 8. The buffering index was calculated based on the amount of titrant required to increase or decrease the pH of the sample using following equation (Van Slyke, 1922):

$$\frac{dB}{dpH} = \frac{\text{(volume of acid or base added) x (normality of acid or base)}}{\text{(volume of sample)x (pH change produced)}} \quad \text{(Eq. 1)}$$

3.3.3. Sample preparation

The clarified acid whey was divided into 1 L batches and pH-adjusted using 35 % KOH to give pH values (at constant temperature; 15 °C) of 4.6 (unchanged), 5.6, 6.0, 6.2, 6.5 and 7.0. KOH was added drop wise while the sample was stirred on a stirring plate. Samples were then left to equilibrate at 15 °C for at least 30 minutes and, if necessary, readjusted to the target pH before subsequent analysis. The pH was measured using a standard pH Meter (WTW pH 3310 with a Sentix 41 probe, VWR International, Blanchardstown, Dublin, Ireland).

The effect of temperature was assessed at pH 6. Samples were equilibrated at the following temperatures: 10 °C, 15 °C, 25 °C and 35 °C. Once samples had achieved the target temperature, they were adjusted to pH 6 (as outlined above) and allowed to equilibrate for a further 30 minutes before analysis.

3.3.4. Compositional analysis

Compositional analysis was completed by Eurofins Food Testing Ireland Ltd using accredited methods. Protein was analysed by the Kjeldahl method (ISO/IDF, 2014, 2016), and

ash via the furnace method (GEA Niro A 25a). Total solids was measured using a CEM smart 5 total solids analyser (CEM Corporation, Matthews, NC, USA) (ISO/ IDF, 2004).

Calcium and phosphorus analysis was performed as described in Chapter 2, Section 3.2.

3.3.5. Sedimentation

Samples were filled into 50 mL centrifuge tubes and centrifuged at 12,000 × g for 30 min using a centrifuge with a fixed angle rotor (Thermoscientific Multifuge X1R, Fiberlite™ F15-8 x 50cy Fixed Angle Rotor, Massachusetts, USA). Once centrifuged, the supernatant was gently poured into a clean beaker; the weights of the sediment and supernatant were measured and compared against the weight of the initial dispersion.

$$\% \text{ insoluble material} = \text{wt. sediment/ wt. dispersion} \times 100 \quad \text{(Eq. 2)}$$

$$\% \text{ soluble material} = \text{wt. supernatant/ wt. dispersion} \times 100 \quad \text{(Eq. 3)}$$

The supernatant was then filtered through Whatman filter paper No 1 before further analysis.

3.3.6. Colour

The CIELAB colour space of each sample and centrifuged supernatant was analysed using a CM-600d spectrophotometer (Konica Minolta, Shanghai, China). This instrument plots colour on a three-dimensional space where L* measures white (0) to black (100), a* measures from green (-60) to red (+60) and B* indicates blue (-60) to yellow (+60).

3.3.7. UF membrane filtration

Samples underwent UF using a lab scale, pressure-driven, crossflow polyethersulfone membrane filtration device (Vivaflow 200, Sartorius AG, Göttigen, Germany) with a 10 kDa molecular weight cut-off and 0.2 m^2 total membrane area at approximately 2.5 bar pressure

(Sartorius Stedim Lab Ltd, 2016). UF concentration was performed on 500 mL of sample under recirculation for 1 hour with composite retentate and permeate samples retained.

3.3.8. Hysteresis- pH

Acid whey (1 L) at 15 ºC was adjusted to pH 7 using 35 % KOH as per the method outlined in section 3.3.3. Once equilibrated, sedimentation and colour tests were performed as per Sections 3.3.5 and 3.3.6 respectively. To test the reversibility of pH-induced changes, the material was re-acidified stepwise back to the original pH, i.e., the acid whey after colour and sediment analysis was acidified from pH 7 to pH 6.2 using 2 M HCL and allowed to equilibrate at the target pH for 30 minutes before analysing. Subsequently, the acid whey was acidified to pH 6.0, 5.6 or 4.6 with 30 minutes equilibration at each target pH prior to colour and sedimentation testing.

Calcium and phosphorus contents of the starting acid whey and supernatant from each sedimentation test were analysed as described in Chapter 2, Section 3.2.

3.3.9. Hysteresis- temperature

Acid whey (1 L) was pH adjusted to pH 6.0 at 10 ºC as per section 3.3.3. To assess the reversibility of temperature-induced changes, the sample was then either gradually heated to 50 ºC with pH, colour and sedimentation testing performed after 30 minutes equilibration at 10 ºC, 15 ºC, 25 ºC, 35 ºC or 50 ºC or rapidly heated to 50 ºC and gradually cooled back to 10 ºC with measurements taken at each of the target temperatures during cooling.

Calcium and phosphorus contents of the starting acid whey and supernatant from each sedimentation test were analysed as per section 3.3.2.

3.3.10. Statistical analysis

Each experiment was completed in triplicate unless stated otherwise with One-way Anova with Tukey's *post hoc* statistical analysis and regression analysis performed using Minitab® 20 statistical analysis package (Minitab Ltd., Coventry, UK).

3.4. Results & Discussion

3.4.1. Effect of pH on acid whey colour, buffering index, sedimentation, and ultrafiltration performance

The composition of the acid whey produced as described in Section 3.3.1 was 11 %, w/w dry matter, protein, 13%, w/w dry matter, ash, 1.9%, w/w dry matter, calcium, 1%, w/w dry matter, phosphorous and had a total solids of 6.13%, w/w. The effect of adjusting pH through the addition of KOH on the colour of the acid whey is shown in Fig. 3.1. Fig. 3.1A shows that there was no significant change in L*-value (whiteness) from pH 4.6 to 5.6; however, there was a significant linear increase in L*-value of acid whey with increasing pH, from 5.6 – 7.0 (R^2 value = 0.89). The increased level of whiteness observed in acid whey during alkalization is indicative of calcium phosphate formation. During the acidification of skim milk, colloidal calcium phosphate dissociates from the casein micelle into the liquid whey in the form of ionic calcium (Ca^{2+}) and phosphate (HPO_4^{2-}, $H_2PO_4^-$ or PO_4^{3-}) (Aydogdu, Mahony, & McCarthy, 2022; Fox, 1981). Ionic calcium, and phosphate exist in equilibrium with soluble calcium phosphate ($CaPO_4^-$ or $CaHPO_4$) within the aqueous phase; this equilibrium is strongly affected by pH and temperature among other factors (Chandrapala, et al., 2010; Fox, 1981; Holt, 2004). Above its solubility limit, calcium phosphate will precipitate out of solution in the form of a white crystalline substance (Lei, et al., 2018; Van Kemenade & de Bruyn, 1987). As the acid whey produced in this study displayed a strong yellow, translucent, colour at pH 4.6, the calcium

phosphate precipitation with increasing pH can be clearly observed through an increase in whiteness of the material.

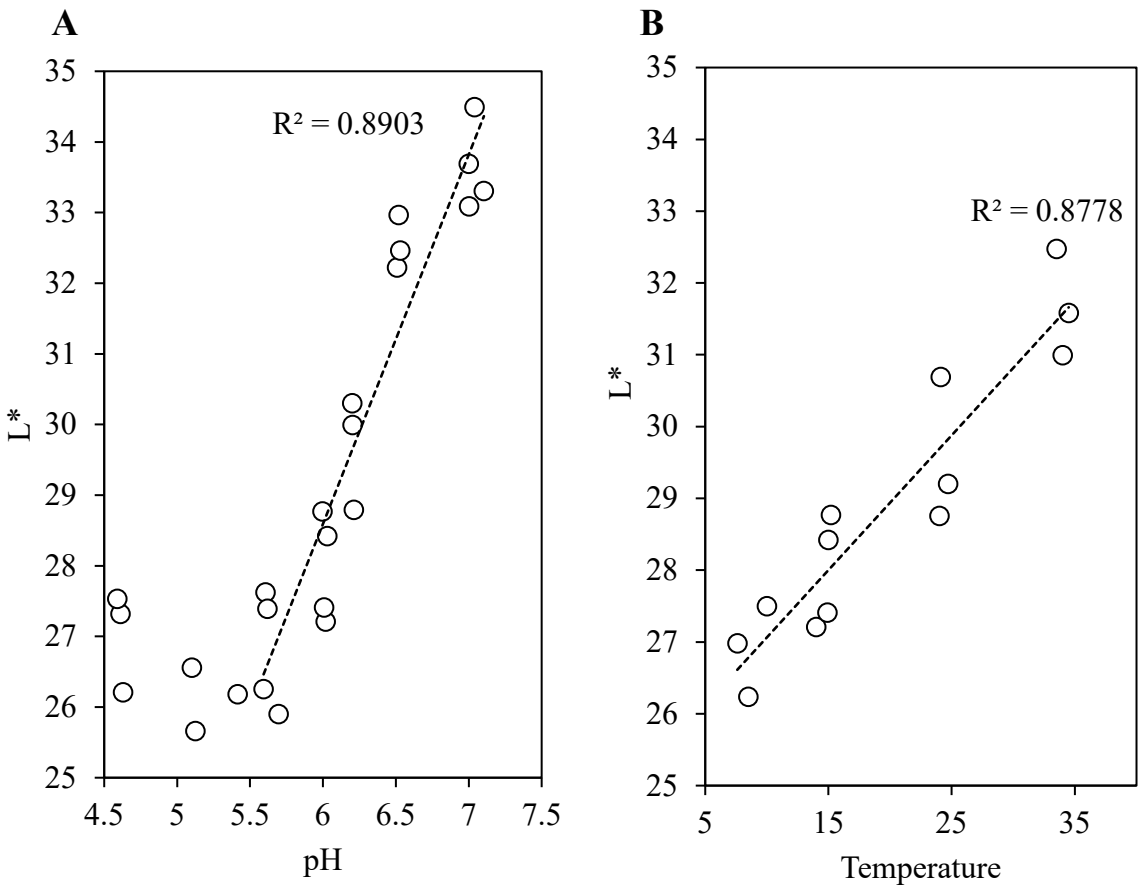

Fig. 3.1. CIE Colour *L** (whiteness index) values of acid whey as a function of (A) pH when measured at a constant temperature of 15 °C (R^2 value taken from data points from pH 5.6-7.0) and (B) temperature when measured at a constant pH of 6.0.

In line with the colour changes observed in acid whey during pH adjustment, Table 3.1 shows the level of sediment also increased although only from pH 6 and above. This aligns with a decrease in soluble calcium and phosphorus (Table 3.1) and suggests that the white sediment isolated during centrifugation is predominantly insoluble calcium phosphate. The observed increase in calcium phosphate precipitation from pH 6 onwards is in line with findings by Mekmene et al., (2009) who found calcium phosphate precipitation efficiency increased with increased pH.

Table 3.1. Effect of pH and temperature on sedimentation of acid whey (calculated as a % of starting whey on a w w^{-1} basis) and concentration of calcium and phosphorus in the supernatant (calculated as % of the original concentration in unfiltered whey at pH 4.6 * on a g g protein $^{-1}$ basis) after centrifugation.

	Sediment	Supernatant Calcium	Supernatant Phosphorous
	% w w^{-1}	% of original concentration	
pH @ 15°C			
4.6	1.4 ± 0.61^{ab}	98.04 ± 6.6^{ab}	93.97 ± 0.6^{a}
5.6	0.8 ± 0.62^{b}	100.76 ± 6.9^{a}	95.09 ± 0.7^{a}
6	1.2 ± 0.26^{ab}	100.81 ± 4.16^{a}	95.11 ± 2.3^{a}
6.2	2.0 ± 0.12^{ac}	83.78 ± 6.0^{b}	80.86 ± 5.1^{a}
6.5	2.6 ± 0.54^{cd}	54.79 ± 6.2^{c}	56.37 ± 9.1^{b}
7	3.4 ± 0.19^{d}	37.13 ± 2.8^{d}	40.54 ± 5.71^{c}
Temperature (°C) @ pH 6.0			
10	1.2 ± 0.1^{a}	101.08 ± 4.6^{a}	98.79 ± 7.3^{a}
15	1.2 ± 0.3^{a}	101.87 ± 5.1^{a}	100.34 ± 3.4^{a}
25	1.6 ± 0.3^{a}	91.2 ± 6.5^{ab}	89.29 ± 5.3^{a}
35	2.4 ± 0.4^{b}	78.88 ± 6.4^{b}	73.05 ± 0.5^{b}

* Original unfiltered whey at pH 4.6 average calcium concentration 0.17 g g protein^{-1}, average phosphorous concentration 0.09 g g protein^{-1}

The precipitation of insoluble calcium phosphate creates an imbalance of the calcium equilibrium mentioned above, triggering calcium phosphate binding reactions aimed at maintaining a stable salt balance in the liquid phase. With each binding of ionic calcium (Ca^{2+}) and phosphate (HPO_4^{2-} and $H_2PO_4^-$) to form calcium phosphate, a H^+ ion is released as a by-product, leading to a drop in pH of the solution (Fuquay, Fox, & McSweeney, 2011; Holt, 2004). This phenomenon can be used to pinpoint the pH where the calcium equilibrium shifts in favour of calcium phosphate formation by assessing the buffering index of the acid whey during

alkalisation from pH 2- 8 as described in section 3.3.2. A high buffering index suggests that constituents in the product counteract the effect of alkali on pH and so a larger volume of alkali is required to produce an increase in pH (Salaün, Mietton, & Gaucheron, 2005). From Fig. 3.2, it can be seen that, during alkalisation, a large buffering index peak was observed starting at pH 5.6 with the maximal point occurring at pH 6.18. This aligns with observed increases in whiteness starting from pH 5.6 and sedimentation from pH 6.0 and so further indicates that pH 5.6 is the point where the acid whey began to form calcium phosphate. From Fig. 3.2, it appears that significant buffering continues from pH 5.6 to pH 7, suggesting that a significant number of calcium phosphate formation reactions are taking place within this pH range. The low level of buffering observed after that point is likely an indication that the majority of phosphate present has already bound to calcium and precipitated out of solution.

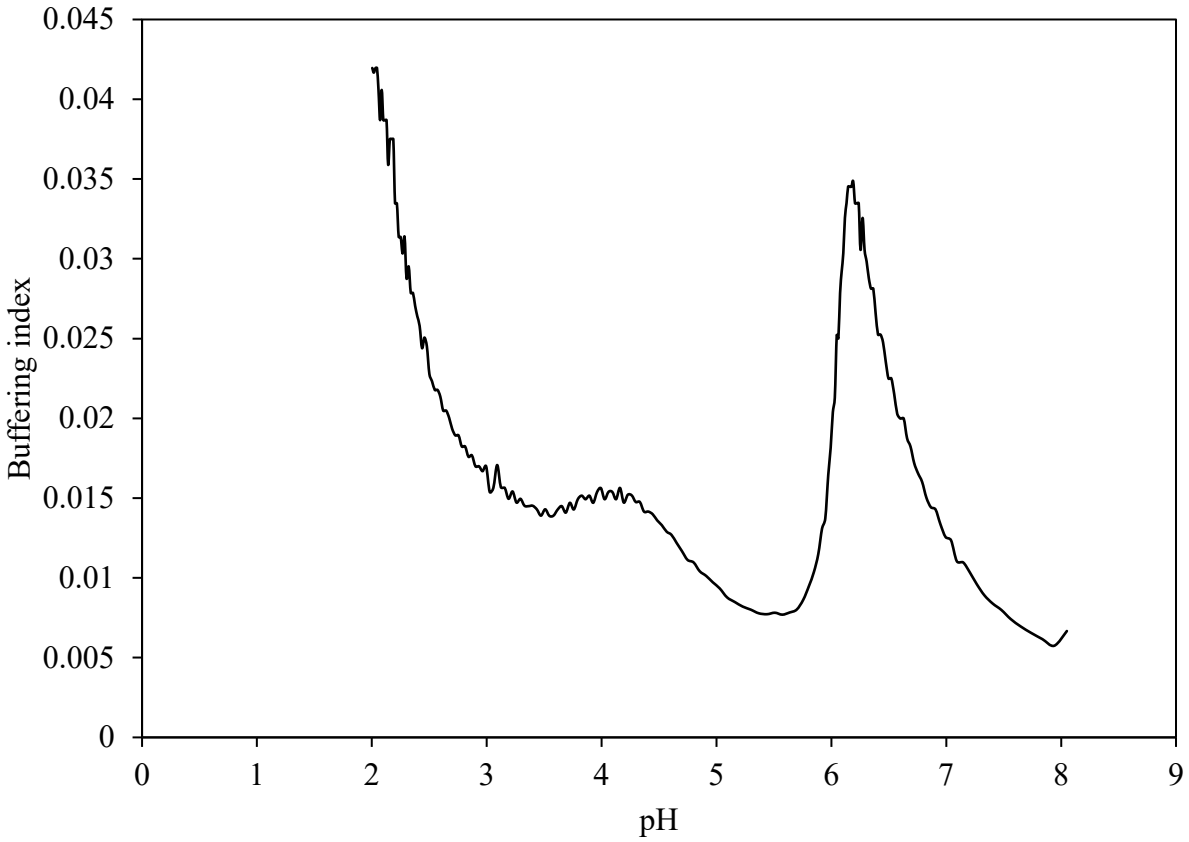

Fig. 3.2. Buffering index of acid whey during alkalisation from pH 2 to pH 8 using 0.5M NaOH (Average of duplicate testing, with no statistically significant difference between replicates, P > 0.05)

The impact of acid whey pH on UF membrane performance is shown in Fig. 3.3A. Regarding pH, a linear decrease in membrane permeation was observed with increasing pH. Analysis of the final UF retentate after 60 minutes of UF also found that samples produced from acid whey with a starting pH above pH 6 had a significantly higher calcium and phosphorus content (Table 3.2) per gram of protein than samples produced with a lower pH. In its soluble ionic form, calcium will permeate through UF membranes; however, crystalline calcium phosphate has a significantly larger particle size (Crowley et al., 2018), which is less likely to permeate during UF and can pose a number of issues with membrane fouling, as calcium phosphate crystals become trapped within the membrane pores (Hausmann et al., 2013). The observed reduction in UF permeation and subsequent increase in calcium and phosphorous in the retentate stream correlates with the observed increase in acid whey sedimentation and L* value, indicating the precipitation of insoluble calcium phosphate, which affected membrane performance (Fig. 3.1A & Table 3.1).

Table 3.2. Effect of pH at constant temperature (15 °C) and temperature at constant pH (pH 6.0) on the concentration of calcium and phosphorus in acid whey UF retentate, expressed as a % of the original concentration in unfiltered whey at pH 4.6 (on a g g protein^{-1} basis)

	Retentate Calcium	Retentate Phosphorous
	% of original concentration	
pH @ 15°C		
4.6	56.49 ± 6.9^{a}	55.01 ± 5.9^{a}
5.6	58.16 ± 4.1^{a}	53.24 ± 2.4^{a}
6	64.60 ± 7.4^{a}	59.65 ± 4.3^{a}
6.2	83.34 ± 7.9^{b}	78.22 ± 2.9^{b}
6.5	93.58 ± 9.1^{b}	87.39 ± 11.7^{b}
7	95.51 ± 3.6^{b}	92.87 ± 3.6^{b}
Temperature (°C) @ pH 6		
10	70.81 ± 6.3^{a}	68.47 ± 5.7^{a}
15	67.11 ± 7.7^{ab}	64.44 ± 4.6^{ab}
25	80.42 ± 4.5^{ab}	72.89 ± 6.5^{ab}
35	91.95 ± 0.7^{b}	85.25 ± 5.1^{b}

*Starting whey at pH 4.6 average calcium concentration 0.17 g g protein^{-1}, average phosphorous concentration 0.09 g g protein^{-1}

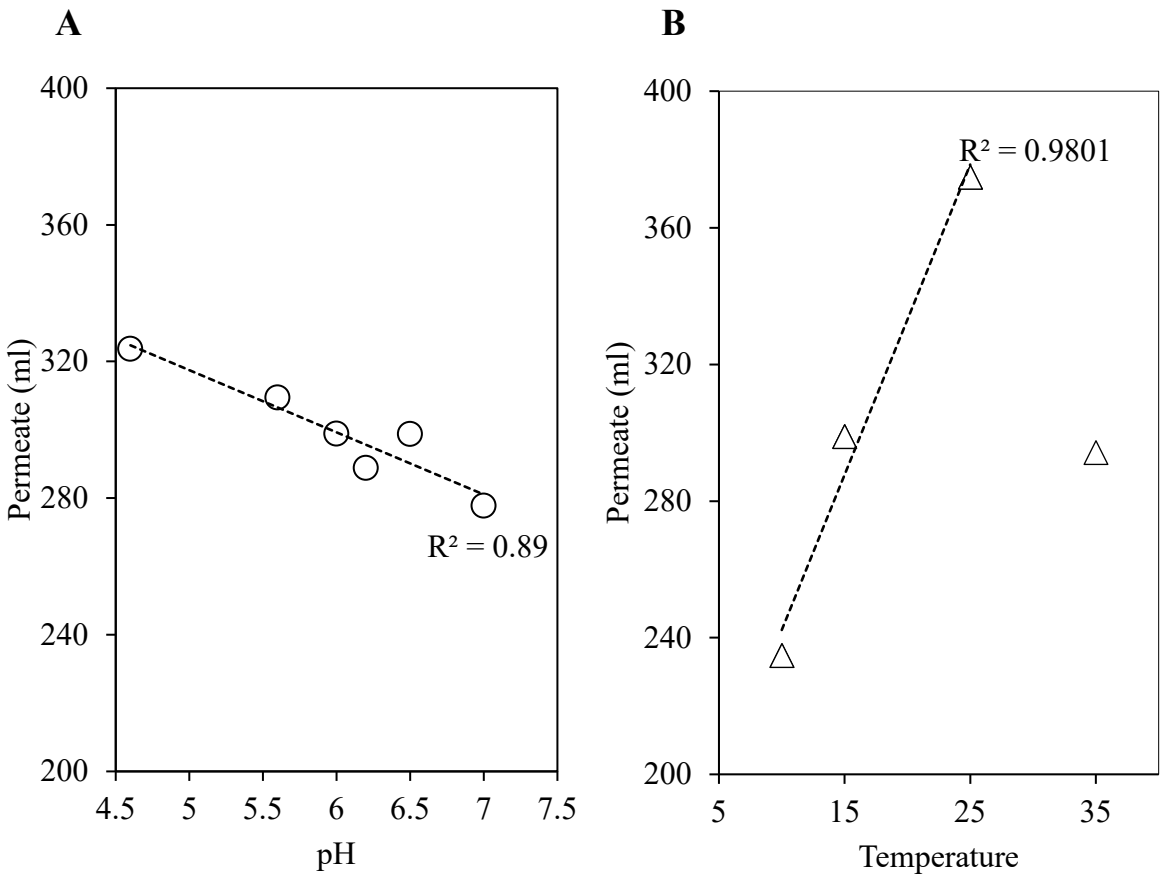

Fig. 3.3. Average total UF permeate volume following 60 minutes UF membrane protein concentration of 500 mL of acid whey, calculated based on the average area under the curve of UF flux over time of 3 replicates, as a function of (A) starting acid whey pH at constant temperature (15 °C, ○) and (B) starting acid whey temperature at constant pH (pH 6.0, △ , R^2 calculated based on data from 10 °C – 25 °C).

Crude acid whey contains a high protein: ash ratio, with samples produced during this study containing a protein: ash ratio of 1:1.2. Therefore, additional processing such as membrane filtration is required to increase the protein content and remove excess minerals and lactose. This process will not only increase the whey protein content but reduce the calcium content, reducing the risk of calcium-mediated aggregation during further thermal processing or in final product applications (Barone, O'Regan, & O'Mahony, 2019; Liu et al., 2022; Simons, et al., 2002). However, depending on the downstream processing of acid whey or final product mineral specifications of the product, it may be necessary to increase the pH prior to UF so as to retain calcium in the retentate stream at the expense of UF permeation efficiency. The findings from this study highlight the importance of consistent acid whey pH prior to UF processing not only

on UF membrane performance but also on the composition of the final product, as fluctuations in pH can alter the calcium equilibrium, leading to variation in calcium content and consequently functionality of the final product.

In addition, the pH of acid whey prior to ultrafiltration will not only impact the composition of the UF retentate (acid whey concentrate) but also the permeate produced from this process, as performing UF at lower pH values results in greater partitioning of ionic calcium and phosphorus into the permeate stream. Typically, permeate will undergo further processing such as membrane or evaporation concentration and a high calcium content in the permeate will result in increased fouling in these processes, due to the formation and precipitation of calcium phosphate.

3.4.2. Effect of temperature on acid whey colour, sedimentation, and ultrafiltration performance.

Linear correlations were observed between temperature and whiteness (Fig. 3.1B) as well as temperature and sedimentation (Table 3.1), indicating a reduction in calcium phosphate solubility with increasing temperature. The effect of temperature was examined at a constant pH of 6.0 as, based on the buffering index analysis, this pH was found to be at a point where significant calcium phosphate formation occurs at 25 °C (Fig. 3.2). Notably, samples at 10 °C retained a yellow colour and had minimal sedimentation despite being at pH 6.0. This suggests that, at lower temperatures, the solubility of calcium phosphate is increased and so for an industrial processor it is key not only to be aware of the pH of the product but also the temperature of pH adjustment as this can also affect calcium phosphate formation.

While reducing the temperature of pH adjustment (and subsequent processing) may appear to be a viable option to minimise calcium phosphate formation and maximise calcium removal from acid whey during UF (Table 3.2), it was observed that, UF permeation rates with

increasing temperature were not primarily affected as a function of calcium phosphate sedimentation. In the region 10 to 25 °C, permeation increased with increasing temperature despite increased sedimentation and whiteness in the acid whey; however, permeation rate was found to be reduced when the temperature was further increased to 35 °C (Fig. 3B). This behaviour may be a result of the viscosity-dependence of membrane filtration, i.e., the initial increase in temperature from 10 to 25 °C decreased viscosity despite the increased sedimentation; as a result, permeation increased due to a reduction in the thickness of the boundary layer in contact with the membrane (Kapsimalis & Zall, 1981). Therefore, at low temperatures, while there will be effective removal of soluble calcium to the permeate stream (Table 3.2), the efficiency of protein concentration is reduced. However, as temperature was increased to above 25 °C, the increased level of sedimentation seems to have resulted in greater fouling, which was enough to cancel and supersede any potential positive effects of temperature induced viscosity reduction with poor permeation and poor calcium removal observed (Fig. 3B, Table 3.2). Closer investigation of the flux over time during UF concentration (Fig. 3.4) shows that, initially, UF at 35 °C gave a higher flux rate than other temperatures analysed, but this declined rapidly after 10 minutes of concentration. As mentioned above, this is likely due to the excessive precipitation of calcium phosphate at 35 °C, causing increased membrane fouling with similar observations made by Barukcic, Bozanic, & Kulozik (2015) when examining ultrafiltration of sweet whey at 50 °C. Therefore, an optimum temperature must be found which maximises membrane porosity and flux but remains below the temperature threshold for excessive calcium phosphate formation. It is also important to consider the impact of temperature on microbial growth on the membranes. Typically, mesophilic bacterial growth increases in the temperature range between 20 and 40°C and while UF permeation performance can be improved by increasing temperature, industrial scale processes avoid operating in this temperature range as it negatively affects the microbial quality of the final product (Suresh et al., 2021).

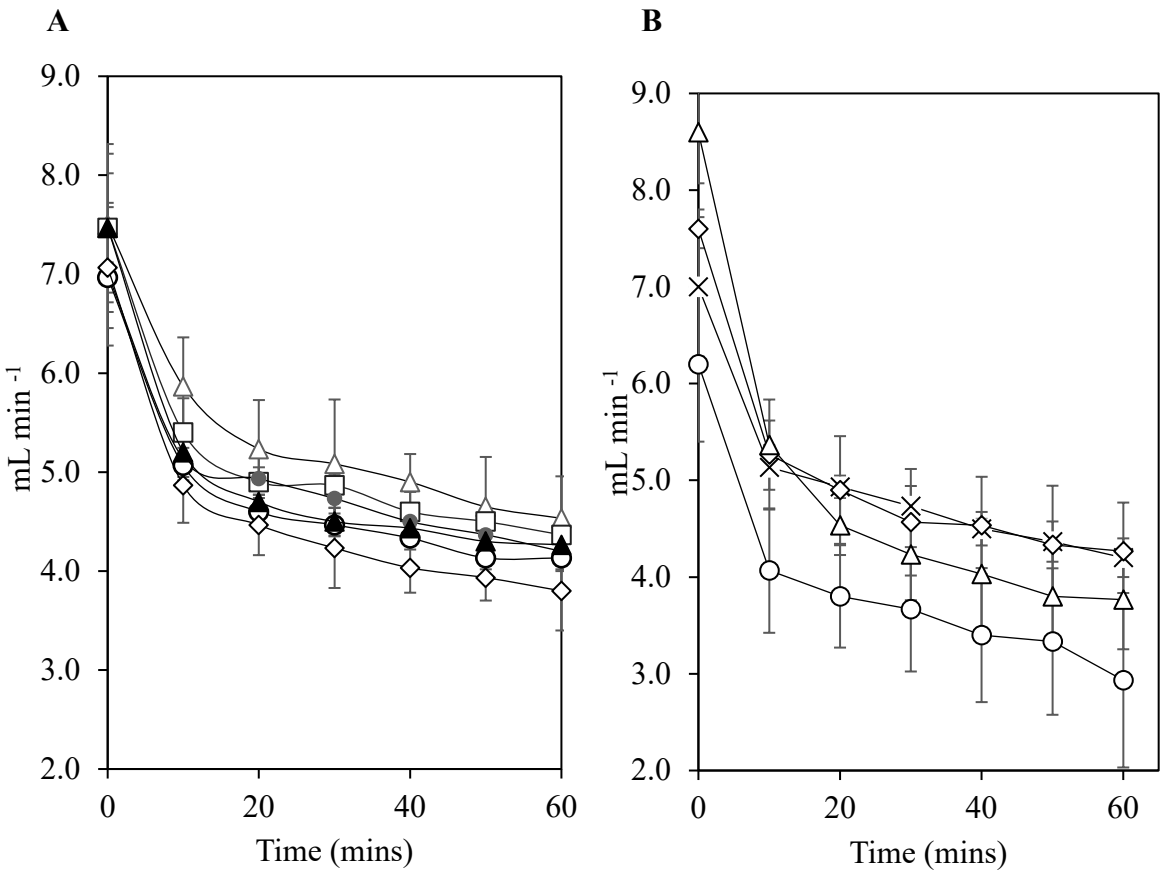

Fig. 3.4. Average UF flow rate (mL permeation min^{-1}) over time during UF membrane protein concentration of (A) acid whey at pH 4.6 -△-, 5.6 -□-, 6.0 -●-, 6.2-○-, 6.5 –▲- and 7.0 -◇- at constant temperature (15 °C) and (B) acid whey at 10 °C -○-, 15 °C -X-, 25 °C -◇-, and 35 °C -△- at constant pH (pH 6) (Average of 3 replicates, error bars depict standard deviation between replicates).

3.4.3. Hysteresis effect of pH and temperature on calcium phosphate formation in acid whey

The reversibility of the effects shown above are also of interest in industrial applications. Therefore, it was examined whether re-acidifying or re-cooling of the acid whey could reverse the effects i.e., could calcium phosphate be resolubilised. Fig. 3.5 shows the change in L* (whiteness) as a function of heating, cooling, or reducing pH. A strong linear correlation was observed between declining pH and reducing L* value between pH 7 and pH 5.6, with an R^2 value of 0.98 (Fig. 3.5A). This trend compares with the increase in L* with increasing pH shown in Fig. 3.1A, where an R^2 value of 0.89 was observed between increasing pH from pH 5.6- 7.0. These findings also correlate with sedimentation test data (Fig. 3.6A), which show a rapid decline

in sedimentation as the pH is reduced from pH 7 to 5.6, after which minimal sedimentation is observed. This suggests that the precipitation of insoluble calcium phosphate as a result of increasing pH can be reversed simply by reducing the pH back to a level where the aqueous salt balance favours ionic calcium over insoluble calcium phosphate. Based on the L* values for these samples, maintaining the pH at pH 5.6 or lower appears to minimise the presence of insoluble calcium phosphate at the total solids concentration used in this study.

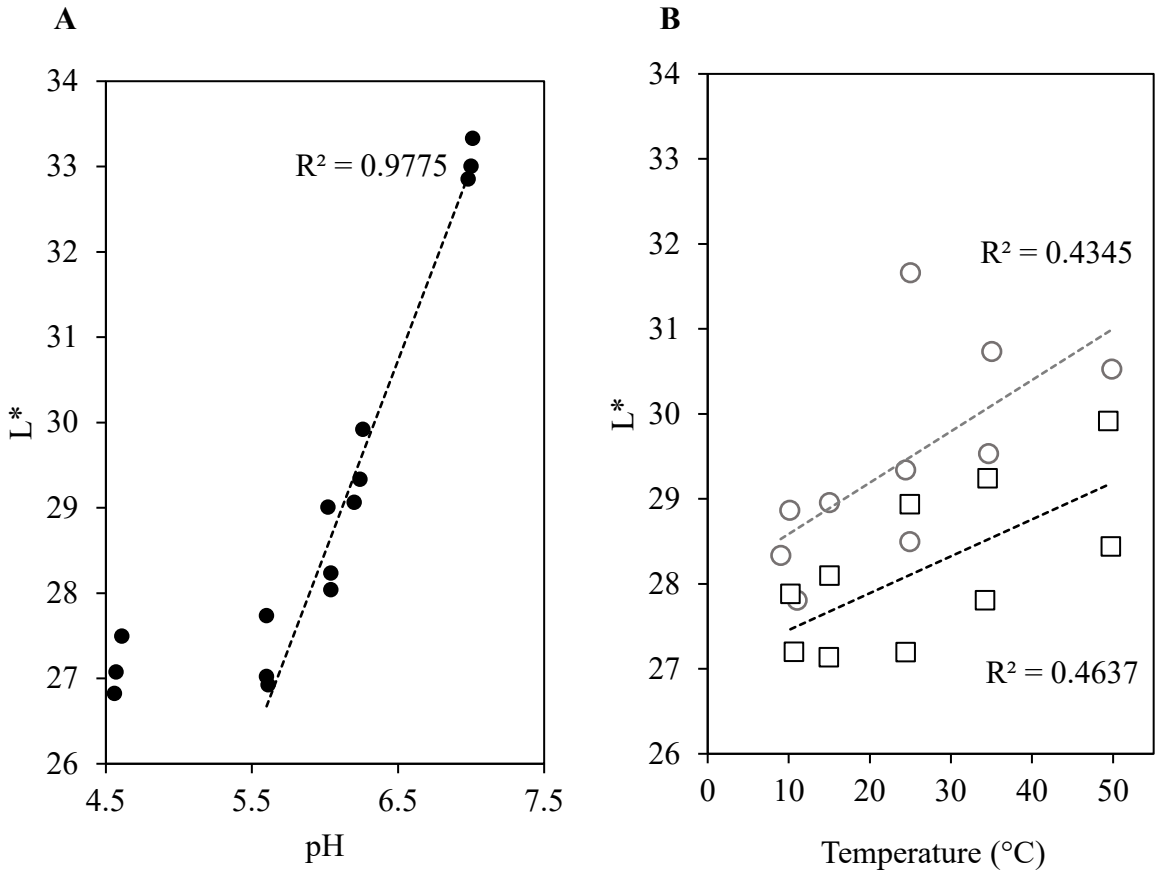

Fig. 3.5. Hysteresis effect of L* (whiteness index) of acid whey during (A) acidification from pH 7 to pH 4.6 (●, R2 calculated from pH 5.6-7.0) and (B) heating (□) and cooling (○) with a starting pH of 6.0 at 10 °C, which was allowed to vary as a function of temperature.

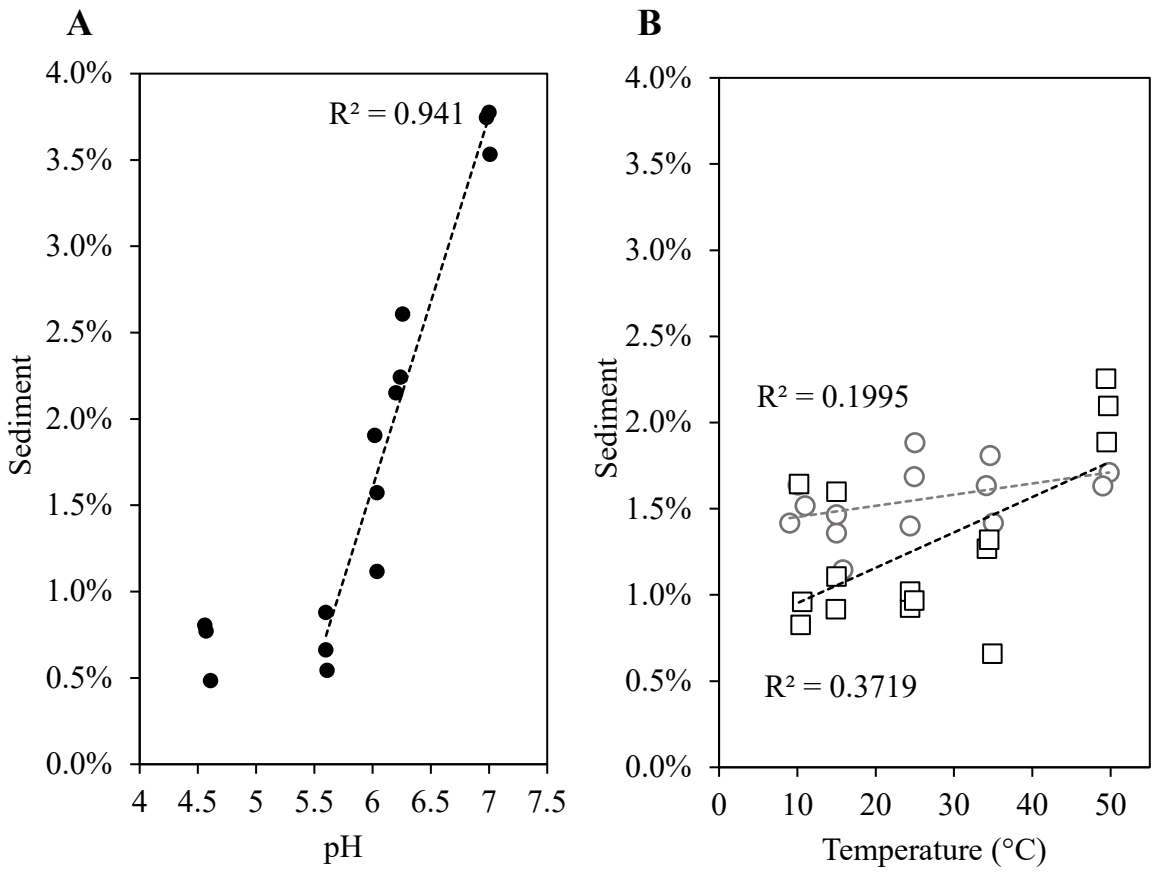

Fig. 3.6. Hysteresis effect of sedimentation as a percent of total centrifuged volume of acid whey during (A) acidification from pH 7 to pH 4.6 at a constant temperature (15 °C, ●, R^2 calculated from 5.6-7.0) and (B) heating (□) and cooling (○) with a starting pH of 6.0 at 10 °C which was allowed to vary as a function of temperature.

In order to determine if temperature adjustment alone could alter the balance of insoluble calcium in acid whey, samples pH adjusted to pH 6.0 at 10 °C were heated and subsequently cooled with no pH control. While a positive correlation was found between the temperature of acid whey and whiteness development during heating and cooling (Fig. 3.5B), the correlation during heating at un-controlled pH was quite weak (L* R^2 = 0.43). This is much weaker than the observed correlation between temperature and whiteness development when the pH was controlled to pH 6 during heating (L* R^2 =0.95, Fig. 3.1B). Similarly, a weak correlation was also observed between acid whey sedimentation and increasing temperature (Fig. 3.6 B). This may be due to the uncontrolled pH changes observed with increasing and decreasing temperature

during the hysteresis study (Fig. 3.7). Fig. 3.7 clearly shows a linear decline in pH with increasing temperature, with acid whey at 50 °C displaying a pH of 5.5. As shown in Fig. 3.6A, the solubility of calcium phosphate increases with decreasing pH and so this corresponding decline in pH with increasing temperature may have counteracted the effect of temperature on insoluble calcium phosphate formation.

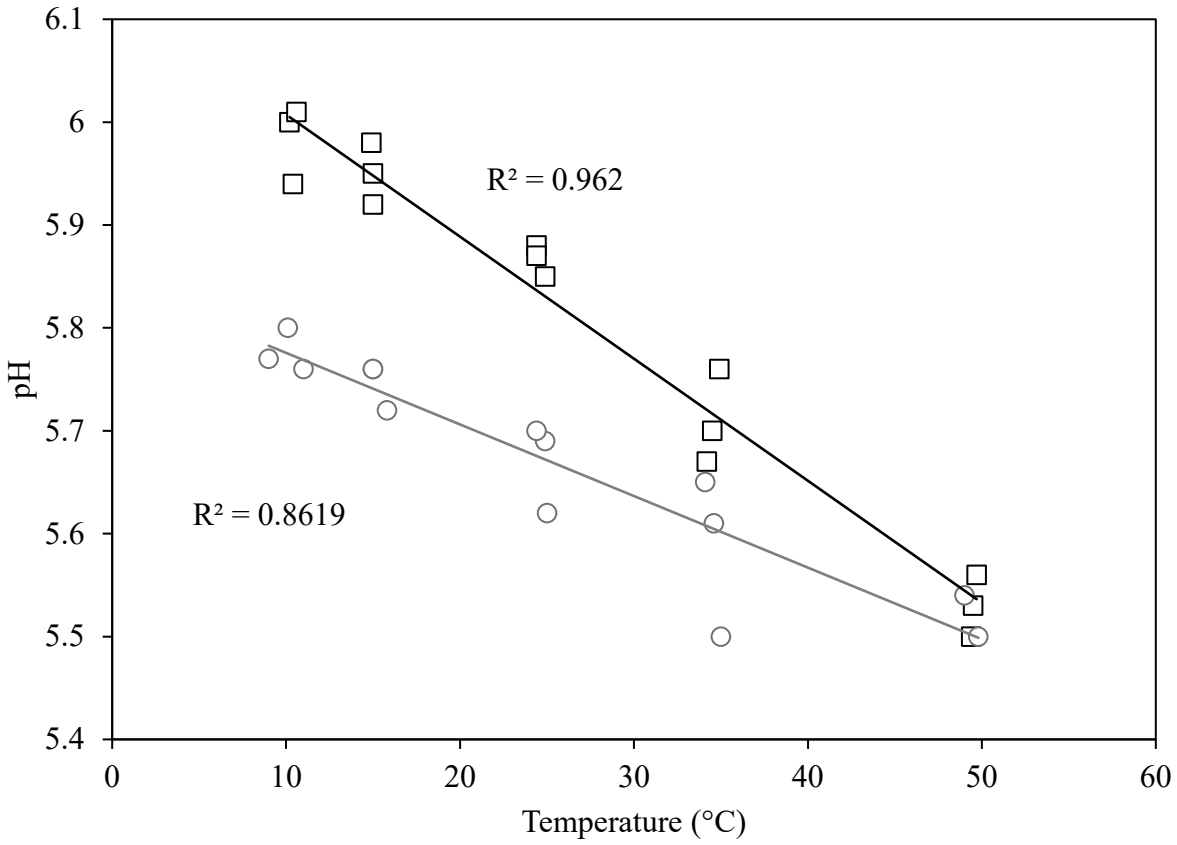

Fig. 3.7. Hysteresis effect of acid whey pH during heating (□) and cooling (○) based on a starting pH of 6.0 at 10 °C.

This decline in pH can mainly be attributed to the association reaction of ionic calcium and phosphate to form calcium phosphate in liquid dairy streams which results in the release of H+ ions and a decrease in pH (Fuquay et al., 2011; Holt, 2004). As the temperature of acid whey increased, the solubility of calcium phosphate decreased (Chaplin & Lyster, 1988), triggering the formation of insoluble calcium phosphate and a pH reduction, which consequently reduced

the effect of further temperature increases on insoluble calcium phosphate formation. Interestingly, when the sample was cooled, a partial reversal in pH decline was observed (Fig. 3.7), with pH increasing from pH 5.5 at 50 °C back to a final pH of 5.8 at 10 °C. This is in line with an observed reduction in whiteness, indicating the re-solubilisation of calcium phosphate which would consequently trigger an increase in pH. Therefore, the formation of insoluble calcium phosphate as a result of increasing temperature appears to be partially reversible with reducing temperature; however, pH appears to be the dominant characteristic which could be adjusted to control calcium phosphate precipitation.

3.5. Conclusion

From this study, it was evident that increasing temperature and pH had significant effects on membrane filtration behaviour, mostly as a function of the extent of calcium phosphate precipitation of acid whey. Despite increased calcium phosphate precipitation, it was noted that UF permeation flux increased with increasing temperature up to 25 °C possibly due to the positive effect of temperature induced viscosity reduction on membrane filtration.

When the reversibility of calcium phosphate precipitation was examined, a stronger correlation was found as a function of pH compared to as a function of temperature. Therefore, it is suggested that, while both temperature and pH can be used to modulate UF of acid whey, through manipulation of the salt equilibria, pH adjustment provides for more predictable control and should be used in preference over temperature where possible. This finding may be more in keeping with industrial practice, i.e., it is easier for processors to maintain material at a constant temperature and adjust pH than *vice versa*. More specifically, the results show that for optimum flux and calcium removal UF should be carried out at lower pH values.

3.6. Acknowledgment

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Chapter 4

Effect of ultrafiltration of acid whey at pH 4.6 or 6.5 on acid whey concentrate composition and resultant powder properties.

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Declaration

This chapter was written by Sinead Mc Entee (SME) and reviewed by all co-authors.

4.1. Abstract

This study investigated the effect of modifying the mineral composition through adjustment of pH prior to ultrafiltration on the functionality of acid whey protein concentrate (WPC; pH 4.6). Increasing pH to 6.5 prior to ultrafiltration produced a concentrate with a significantly higher calcium and phosphorous content, compared to ultrafiltration at pH 4.6. WPC produced at pH 6.5 also had significantly more calcium-phosphate precipitation following pasteurisation and evaporation and had a higher viscosity at 30% solids (300 s^{-1}, 50 °C). In addition, both reconstituted powders (5 %, w/w, protein) had strong gelation properties following heat treatment at 90 °C; however, the WPC produced at pH 6.5 had a significantly firmer gel strength, but such gels appeared to be more sensitive to breakage at increased frequency and oscillation strain. Overall, the pH prior to UF had a significant impact on the processing of acid whey and final product functionality.

4.2. Introduction

Acid whey is the byproduct of the acidification of milk, either through addition of organic or mineral acids until the isoelectric point of casein is achieved (pH 4.6, mineral acid whey) or the acid fermentation of skim milk during the manufacture of Greek style yoghurt or acid cheeses such as cottage cheese. Acid whey typically contains whey protein, lactose, minerals and vitamins; however, depending on the method of manufacture, the mineral composition can vary, with acid whey produced by mineral acidification tending to have higher levels of calcium and phosphorous compared to acid whey produced during Greek yoghurt manufacture (Bylund 1995; Singh 2009; Menchik *et al*. 2019)

Acid whey has numerous applications, such as, fermented milk beverages, biofuel production, and valorisation in the form of membrane filtration to produce high protein ingredients and lactose (Rocha-Mendoza *et al.* 2021). Acid whey is of particular interest to the infant formula (IF) industry as, unlike whey from cheese production, acid whey does not contain glyco-macro-peptide (GMP), a peptide derived from κ-casein which is depleted in tryptophan. GMP is also depleted in phenylalanine, which makes this peptide one of the few suitable protein sources for those suffering from phenylketonuria; however, the lack of tryptophan has a negative effect for other applications, in particular infant nutrition, as sufficient levels of tryptophan are essential for the growth and development of the infant (Dutta *et al*. 2017; Pena *et al*. 2018). The absence of GMP in acid whey, translates to a nutritional benefit in its use in infant formula, due to an increase in the levels of essential amino acids, tryptophan and cysteine, per gram of protein, therefore allowing a formulation to achieve the required amino acid levels with a lower overall protein content (Dutta *et al*. 2017; Mc Entee *et al.* 2023b, Nilsson *et al*. 2023).

However, the functionality of acid whey is different to cheese whey, with acid whey tending to be more prone to gelation during thermal processing, which can limit its use in certain applications (Mc Entee *et al.* 2023a). This difference in functionality can be attributed to the

differences in the mineral profile, in particular the calcium and phosphorous content, with acid whey containing three times more calcium per gram of protein than whey from cheese production (Bylund 1995). Depending on pH, calcium concentration and pre pasteurisation temperature, much of this calcium can exist in its ionic form, which promotes whey protein aggregation during thermal processing as it can create calcium bridges between whey proteins in particular β-lactoglobulin by binding to negatively charged sites exposed during the denaturation of whey proteins at temperatures > 70 °C (Joyce *et al.* 2018). Therefore, it is the proportion of ionic calcium to whey protein during thermal processing that determines the degree of aggregation.

Calcium exists in a balance between dissociated ionic calcium and bound to calcium phosphate which is affected by pH, temperature, and concentration (Holt 2004; Mekmene *et al*. 2009; Wang and Ma 2020). At low pH, low temperature and low calcium concentration, the majority of calcium in the liquid phase tends to exist as ionic calcium. However, any increase in any of these parameters can tip the balance to promote the formation of calcium hydrogen phosphate (Holt 2004; Mekmene *et al.* 2009). As acid whey is rich in ionic calcium and phosphorous, this balance between dissociated and undissociated calcium phosphate plays an important role in determining the processing efficiency as precipitated calcium phosphate can result in significant fouling of membranes and other equipment within the process, such as heat exchangers and evaporators (Tanguy *et al.*, 2016; Fei *et al.*, 2021). In addition, this balance also impacts the functionality of acid whey products, with the ionic environment often being manipulated through adjustments in pH, temperature and concentration to promote the formation of ionic calcium or calcium hydrogen phosphate depending on the processing step (Bylund 1995).

Increasing the whey protein content of acid whey is usually performed using ultrafiltration to reduce the lactose and mineral content and produce whey protein concentrates ranging from 35 to 80 % protein, w/w on a dry matter basis (Bylund, 1995; Menchik *et al.* 2019).

However, a number of considerations must be accounted for prior to the filtration of acid whey, including the pH of the material and temperature of processing as this will affect the balance of dissociated and undissociated calcium phosphate during filtration and can not only affect membrane performance due to calcium phosphate fouling, but will also determine the calcium content in the final product (Mc Entee *et al.* 2023a). Typically, following ultrafiltration, the acid whey concentrate will undergo heat treatment and concentration by evaporation followed by spray drying, with other processes such as pH adjustment also being potentially applied (de la Fuente et al., 2002). As the ultrafiltration step is the only point where calcium can be removed from acid whey, the calcium content after this stage has a significant impact on the downstream processing of the concentrate, with a risk of increased fouling and protein gelation during heat treatment and evaporation due to calcium-mediated protein aggregation (Simons *et al.* 2002; Joyce *et al.* 2018).

While acid whey is recognised as having nutritional benefits over cheese whey, in particular for infant formula applications, the challenges during processing and differences in heat-induced functional properties in end applications have in some cases limited the valorisation of this ingredient. While studies have been published examining the compositional, functional and nutritional properties of acid whey and mechanisms to remove calcium, as well as studies into effective ultrafiltration of acid whey, few have directly examined the impact of calcium content following ultrafiltration on downstream heat treatment and drying processes and subsequent ingredient functionality (Konrad, Kleinschmidt and Faber, 2012; Chandrapala et al., 2015; Crowley et al., 2018; Rocha-Mendoza et al., 2021; Corrigan, O'Mahony and Fenelon, 2023) . The objectives of this study were:

A) to investigate the effect of modifying the mineral composition, predominantly calcium and phosphorous content, through manipulation of pH prior to ultrafiltration, on the functional

properties of the resultant concentrate following subsequent heat treatment, evaporation and spray drying processes and

B) To assess the impact of such steps on the final acid whey protein concentrate functionality during heat treatment.

As ionic calcium-mediated protein aggregation during heat treatment is generally associated with an observed increase in gelation in the finished product, gel formation, and gel strength during and after heat treatment of reconstituted acid WPC solutions were chosen as key indicators of the impact of innate calcium concentration on the thermal stability of acid whey solutions (Kharlamova, Nicolai and Chassenieux, 2018).

4.3. Materials and methods

4.3.1. Materials

Liquid mineral acid whey (1800 L; pH 4.6; 5.8%, w/w, total solids) was obtained from Tirlán (Ballyragget, Kilkenny, Ireland) with a composition of 12.1% protein, 11.5% ash, 1.98% calcium and 1.02% phosphorous on a dry matter basis (w/w).

4.3.2. Manufacture of acid WPC powders

Liquid acid whey (1800 L) was divided in to two separate 900 L batches. The first aliquot remained at pH 4.6 ($WPC_{UF4.6}$) and the second aliquot was pH adjusted to pH 6.5 ($WPC_{UF6.5}$) using a 35% KOH solution (pH meter TW pH 3310 equipped with a Sentix 41 probe, VWR International, Blanchardstown, Dublin, Ireland). Ultrafiltration (at 15°C) of acid whey (900 L) was carried using a pilot-plant ultrafiltration unit (Complete Filtration Resources, Wisconsin, USA) equipped with a single spiral wound 10 kDa UF membrane with a surface area of 30 m^2 (Alfa Laval GR70PE 8038). Ultrafiltration was performed in batch-mode with the retentate returning back to the feed tank and providing agitation to the feed. The membrane inlet pressure

was maintained at approximately 137 kPa during processing, with an outlet pressure of approximately 20 kPa. The permeate flux was monitored volumetrically during filtration until a volume concentration factor (VCF) of 9 was achieved. After filtration, protein and total solids analysis was conducted on the final retentate using a LECO protein analyser (LECO corporation, Michigan, USA) and CEM Smart 5 total solids analyser (CEM Corporation, Matthews, NC, USA), respectively. The protein content was standardised to 35% (w/w, dry matter basis), by adding permeate back to the retentate.

Prior to pasteurisation, the whey concentrates were adjusted to pH 6.7 (10-15°C) using 35% KOH to manipulate the calcium balance in favour of calcium phosphate formation and reduce the risk of ionic calcium mediated protein aggregation during pasteurisation. Heat treatment was carried out using a tubular heat exchanger (MicroThermics, Raleigh, NC, USA) at 73 °C × 15 s, followed by evaporation to 30% w/w solids using a Tetra Scheffers® single-stage falling film evaporator operating in recirculation mode at 60 °C (Tetra pak, Pully, Switzerland). The whey protein concentrates were subsequently spray dried at an approximate feed temperature of 50 °C using an anhydro Lab 3 dryer (SPX Flow Technology A/S, Soeborg, Denmark), equipped with a nozzle atomiser at an inlet and outlet temperature of 185 and 85°C, respectively. The pH of the final powder on rehydration to 10% solids using deionised water was measured at 25°C.

4.3.3. Compositional analysis

Compositional analysis was completed by Eurofins Food Testing Ireland Ltd using accredited methods. Protein and non-protein nitrogen was analysed by the Kjeldahl method (ISO/IDF, 2014, 2016), ash using the furnace method (GEA Niro A 25a), fat by Rose Gottlieb (ISO/ IDF, 2010), and moisture using an oven at 102 °C (ISO/ IDF, 2004). Calcium and phosphorus analysis were performed as described in chapter 2, section 3.2.

4.3.4. Colour measurements of liquid acid whey and whey protein concentrates

The CIE Lab colour space measurement of each liquid sample was analysed using a CM-600d spectrophotometer (Konica Minolta, Shanghai, China), where L* measures white (0) to black (100), a* measures from green (-60) to red (+60) and b* indicates blue (-60) to yellow (+60).

4.3.5. Viscosity of reconstituted whey protein concentrates across a shear rate sweep of 0.1 – 300s^{-1}

The final powder was reconstituted to 30% w/w solids using Milli -Q water at 50 °C for 2 hours before analysing to produce a concentrate of similar solids to the evaporated concentrate prior to spray drying. Total solids level was confirmed by measurement using a CEM Smart 5 total solids analyser. Viscosity at 50 °C was measured using a HR-1 Discovery Hybrid Rheometer (TA Instruments, New Castle, DE, USA) equipped with a 40 mm stainless steel parallel plate geometry. Each sample was subjected to a logarithmic shear sweep from 0.1- 300 s^{-1}, with 5 measurements per decade, followed by a peak flow hold at 300 s^{-1} for 30 s.

4.3.6. Native whey protein level

The total whey protein and native whey protein concentration was measured using reversed phase high performance liquid chromatography (RP-HPLC) by the method outlined by Mounsey and O' Kennedy (2009). The percentage native whey protein as a function of total protein was calculated as:

$$\frac{\%\ \text{whey native protein}}{\%\ \text{total whey protein}} \qquad \text{Eq. 1}$$

4.3.7. Level of calcium precipitation

Calcium precipitation was measured in liquid $WPC_{UF4.6}$ and $WPC_{UF6.5}$ after pH standardisation to pH 6.7, after pasteurisation and after evaporation. Samples (50 g) underwent centrifugation at 10,000×g for 30 min, and the calcium content of the supernatant was measured by method outlined in the compositional analysis section.

Precipitated calcium was calculated as follows:

$$\frac{\text{supernatant calcium } (\text{g } 100\ \text{g}^{-1})}{\text{dispersion calcium } (\text{g } 100\ \text{g}^{-1})} \text{ X } 100 \qquad \text{Eq. 2}$$

4.3.8. Powder particle size distribution and bulk density

The particle size distribution of the final powder was measured using a Malvern 3000 equipped with an automated Aero S dry powder dispersion unit (Malvern Instruments Ltd., Worcestershire, England). Samples were measured at an air pressure of 0.7 bar with a refractive index of 1.45 and absorption index of 0.0001.

The tapped bulk density (100 taps) of the powder was analysed using a Stampf-volumeter (Engelsmann, Germany) using the method outlined by Gea Niro (2006) and calculated as g ml^{-1}.

4.3.9. Gel strength

The final powder was reconstituted to 5% protein using Milli -Q water at 50 °C. Samples were hydrated for 2 hours before pH adjustment to 6.8 using 1M and 0.1M NaOH. Samples were then allowed to equilibrate at 25°C for a minimum of 30 minutes; if required pH was adjusted again to reach the target of pH 6.8 before analysis.

Dynamic oscillatory measurements, at a frequency of 1 Hz, and amplitude strain of 0.25% (which was within the LVE of both samples, Fig. 4.8A), were conducted using a HR-1 Discovery

Hybrid Rheometer (TA Instruments, New Castle, DE, USA) equipped with a concentric cylinder geometry similar to that described by Peng *et al.* (2019). Samples (19 g) were heated within the rheometer from 25 to 90 °C at a rate of 3 °C per minute, held at 90 °C for 30 min before cooling back to 25 °C (3 °C per min) and holding at 25 °C for 10 min. To prevent evaporation during heating, each sample was covered in a thin layer of tetradecane (Sigma Aldrich, Missouri, US) and a solvent trap was also used. Changes in the elastic modulus (G') and viscous modulus (G'') were monitored, with the gelation point being determined as the point where $G' = 1$. Following gelation, a logarithmic frequency sweep was conducted between 0.01 and 100 Hz at 25 °C and 0.25 % strain. Additionally, an amplitude strain sweep was performed at a frequency of 1 Hz with a logarithmic strain increase from 0.01 to 100 %.

4.3.10. Measurement of Ionic strength and apparent calcium ion concentration at 25°C followed by pH monitoring with increasing temperature.

WPC samples were reconstituted to 5 % (w/w) protein and adjusted to pH 6.8 as using the same methodology as applied for gel strength testing. Apparent ionic calcium (Ca^{2+}) concentration at 25 °C was determined as described in chapter 2, section 3.8. Conductivity was measured using a VWR® CO 310/CO 310 M conductivity meter (VWR International, Blanchardstown, Dublin, Ireland). The samples (30 g, w/w) were then placed in a water bath and heated to from 25 – 90°C with pH measured at 25°C, 40, 50, 60, 70, 80 and 90 °C. Samples were allowed to stabilise at the target temperature for 5 minutes before recording pH.

4.3.11. Statistical analysis

Each trial was completed in triplicate unless stated otherwise, with one-way ANOVA and Tukey's *post-hoc* analysis or paired T-test being performed using a Mini-tab® 20 statistical analysis package (Minitab Ltd., Coventry, UK).

4.4. Results and Discussion

4.4.1. Effect of pH adjustment on calcium partitioning during ultrafiltration and final composition

Adjusting pH prior to ultrafiltration significantly affected the mineral composition of acid whey concentrates (Table 4.1). The ash content decreased from 11.5 % to 7.48 % (w/w dry matter) in the $WPC_{UF4.6}$ but increased in the $WPC_{UF6.5}$ system to 15.4 % when using a VCF of 9 during ultrafiltration. This in turn slightly affected protein concentration efficiency, which increased from 12.1 % in the original acid whey to 45.1 % (w/w dry matter) in $WPC_{UF4.6;}$ however, it was only possible to achieve 42.3 % in the $WPC_{UF6.5}$, despite it undergoing the same UF processing conditions. However, the ash: protein ratio in the original acid whey was 0.95: 1.0, compared to 0.17: 1.0 in the $WPC_{UF4.6}$ and 0.36:1.0 in the $WPC_{UF6.5}$ product. In the $WPC_{UF4.6}$ retentate, calcium and phosphorus made up 16 % and 9 % of the total ash, respectively, while, in the $WPC_{UF6.5}$ retentate, 25 % of the total ash was calcium and 12 % was phosphorous. These differences in composition following UF indicate that increasing pH prior to processing significantly reduced the removal rate of ash from the retentate of $WPC_{UF6.5}$, in particular calcium and phosphorus. This in turn negatively impacted protein concentration, with $WPC_{UF6.5}$ having a 3 % lower protein content despite undergoing the same VCF concentration. This is attributed to the precipitation of calcium phosphate at pH 6.5, which is not only unable to pass through a 10-kDa membrane due to its larger particle size but will also contribute to the formation of a fouling layer which will further negatively impact protein concentration (Barukcic, Bozanic and Kulozik, 2015; Mc Entee *et al.*, 2023). During this study, it was observed that the average permeate flux was lower for $WPC_{UF6.5}$ than $WPC_{UF4.6}$ with average flux values of 4.6 L min^{-1} and 5.0 L min^{-1} (paired T-test, $P< 0.05$), respectively, despite their being processed under the same conditions, resulting in a longer time taken to achieve the target VCF 9 and indicating increased fouling when processing $WPC_{UF6.5}$ compared to $WPC_{UF4.6.}$

Table 4.1. Composition and pH of the initial acid whey, $WPC_{UF4.6}$ and $WPC_{UF6.5}$ streams and subsequent finished powder. Results shown are the average of three samples with a statistically significant difference between $WPC_{UF4.6}$ and $WPC_{UF6.5}$ indicated by different letters.

	Total solids	Moisture	Protein	Ash	Ca	P	Na	K	Mg	Cl	Native Whey protein	pH at 25°C
	g 100 g^{-1}		% w/w dry matter								% w/w protein	
Acid whey	5.81 ±0.6	-	12.11 ±1.4	11.53 ±1.0	1.98 ±0.1	1.02 ±0.1	0.44 ±0.1	2.44 ±0.2	0.19 ±0.0	4.67 ±0.3	n/a	4.59 ±0.05
UF retentate												
$WPC_{UF6.5}$	12.14 ±2.2[a]	-	42.33 ± 0.8[a]	15.41 ± 2.4[a]	3.89 ± 0.2[a]	1.92 ±0.1[a]	0.21 ±0.0[a]	2.08 ±0.1[a]	0.17 ±0.0[a]	2.05 ±0.1[a]	n/a	6.19 ±0.13[a]
$WPC_{UF4.6}$	10.32 ±1.2[a]	-	45.05 ±0.5[b]	7.48 ±1.5[b]	1.19 ±0.1[b]	0.7 ±0.0[b]	0.28 ±0.0[a]	1.36 ±0.1[b]	0.11 ±0.0[b]	2.66 ±0.0[b]	n/a	4.58 ±0.05[b]
Final powder												
$WPC_{UF6.5}$	-	3.61 ±0.4[a]	38.97 ± 0.8[a]	16.19 ± 1.3[a]	3.82 ± 0.5[a]	1.98 ±0.2[a]	0.29 ±0.0[a]	2.56 ±0.3[a]	0.18 ±0.0[a]	2.29 ±0.1[a]	49 ± 4[a]	6.35 ±0.07[a]*
$WPC_{UF4.6}$	-	3.11 ±0.7[a]	38.42 ± 2.0[a]	11.01 ± 0.5[b]	1.29 ±0.1[b]	0.73 ±0.0[b]	0.30 ±0.0[a]	3.25 ±0.2[b]	0.12 ±0.0[b]	2.84 ±0.1[b]	48 ± 2[a]	6.50 ±0.06[b]*

*Final powder pH was measured following reconstitution to 10% solids, n/a: not analysed

Despite adjusting the pH of the starting whey to pH 6.5 before ultrafiltration, it was observed that the pH of the final retentate had dropped to pH 6.19 while the pH of $WPC_{UF4.6}$ retentate remained similar to that of the starting acid whey at pH 4.58 (Table 4.1).The drop in pH observed in $WPC_{UF6.5}$ may be due to the re-equilibration of the ionic environment during partitioning of the retentate and permeate. Ionic calcium and phosphorous exists in an equilibrium with calcium phosphate which adjusts in favour of calcium phosphate formation with increasing pH, temperature and or concentration leading to the precipitation of calcium phosphate out of solution in the form of a white crystalline material once the solubility limit has been exceeded (Mekmene *et al.* 2012; PubChem, 2024). This conversion of ionic calcium and phosphorus to calcium phosphate also results in the release of a H^+ ion and an observed drop in pH of the solution (Holt 2004). The observed drop in pH of $WPC_{UF6.5}$ indicates continued calcium phosphate formation during filtration. This is in comparison to whey at pH 4.6, where minimal change in pH was observed in the final retentate, indicating that, in this low pH environment, despite increased concentration during ultrafiltration, calcium remained in an ionic form with minimal calcium phosphate formation. A previous study by Mc Entee, *et al*. (2023a) showed that the buffering capacity of acid whey increases at pH values above pH 6.2 and has been attributed to increased calcium phosphate formation in this pH range, resulting in the release of H^+ ions counteracting the pH impact of added NaOH.

After ultrafiltration, both retentates were protein-standardised to 38% protein w/w dry matter and pH adjusted to pH 6.7 prior to pasteurisation. The material was pH adjusted to pH 6.7 prior to pasteurisation so as to favour the formation of calcium hydrogen phosphate, and therefore minimised aggregation reactions caused by ionic calcium during heat treatment. However, despite this, the pH of the final $WPC_{UF6.5}$ powder on rehydration to 10 % solids at 25 °C was lower than that of the $WPC_{UF4.6}$ sample, with a pH of 6.35 and 6.50, respectively,

perhaps indicative of further calcium phosphate formation during pasteurisation, evaporation and drying (Table 4.1).

The percentage of calcium partitioning between retentate and permeate streams following UF, was calculated based on the total calcium of the starting acid whey. For acid whey, where the pH was maintained at pH 4.6 during UF, approximately 94 % of the calcium present in the starting whey passed through the membrane to the permeate side. Meanwhile, where the pH of the starting acid whey was increased to pH 6.5, only ~ 57 % of the calcium present in the starting whey passed through to the permeate stream. Therefore, increasing the pH prior to UF processing resulted in a high calcium product with three times more calcium than $WPC_{UF4.6}$ (Table 4.1). Also, it is important to note that approximately 24 % of the calcium in the $WPC_{UF6.5}$ sample was unaccounted for in either the retentate or permeate stream and so was most likely retained on the membranes as calcium fouling.

The effect of calcium phosphate fouling can clearly be seen in the composition of the final UF retentate in Table 4.1, with $WPC_{UF6.5}$ containing approximately 3 % less protein per 100 g of dry matter. This suggests that increasing pH to alter the ionic environment reduces the efficiency of protein concentration and impacts the final product composition. Previously Hausmann *et al.* (2013) showed that milk salts, and in particular calcium phosphate, play a key role in membrane fouling with the rate of decline in membrane flux more pronounced in whey solutions with added salts resulting in a fouling layer comprised of a combination of whey protein and salt deposits (predominantly calcium).

Further confirmation of the conversion of ionic calcium to calcium phosphate is the observed increase in L* whiteness index of the acid whey during processing (Fig. 4.1). As mentioned previously, calcium phosphate precipitates in the form of a white crystalline material, which results in increased opacity and whiteness in liquid whey (Holt 2004; Mc Entee et al. 2023a). pH adjustment of the starting acid whey to pH 6.5 resulted in a slight increase in

L* value, which was further exacerbated after ultrafiltration with $WPC_{UF6.5}$ having an L* that was 75% higher than $WPC_{UF4.6.}$ Interestingly, standardising protein back to 35% (w/w, dry matter) and pH adjusting to pH 6.7 did not appear to significantly alter the L* value of either $WPC_{UF4.6}$ or $WPC_{UF6.5.}$ However, a significant increase in L* values was observed after pasteurisation, with both $WPC_{UF4.6}$ and $WPC_{UF6.5}$ having a similar degree of whiteness. This is likely due to a combination of calcium phosphate formation at elevated temperatures and whey protein denaturation.

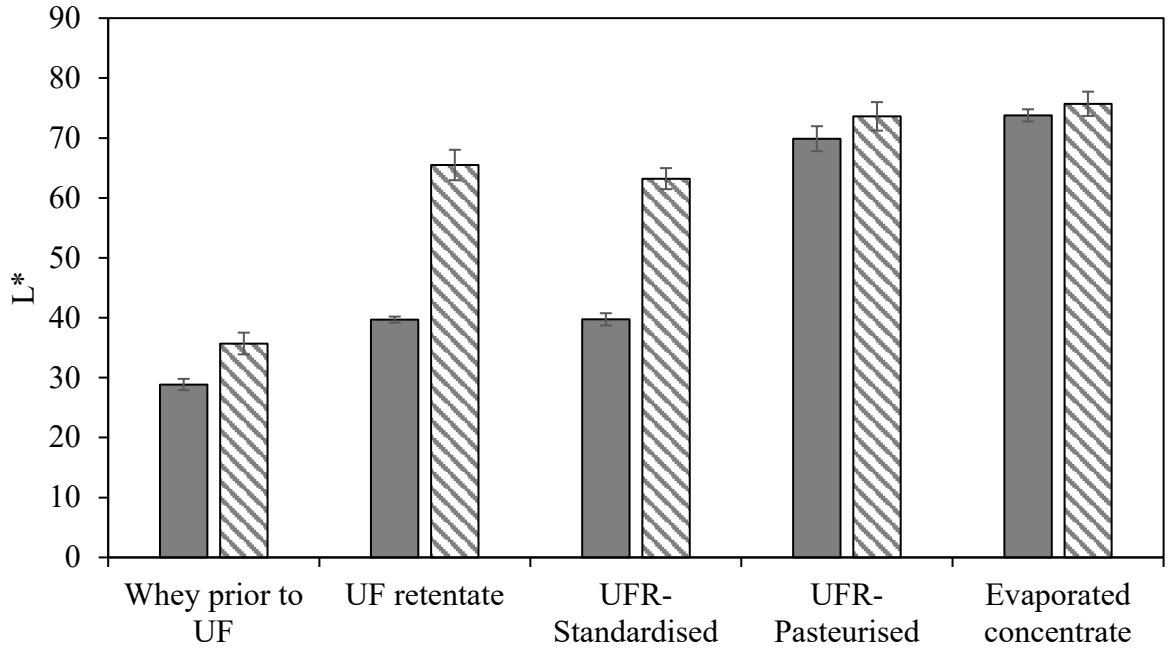


Fig. 4.1. Average L* colour whiteness index of liquid samples throughout processing of $WPC_{UF4.6}$ (■)and $WPC_{UF6.5}$ (▨). Error bars indicate the standard deviation across 3 replicates.

4.4.2. Calcium sedimentation following pH standardisation, pasteurisation, and evaporation.

Changes in the calcium equilibria during processing can pose a number of challenges not only through altering the ionic environment but also because precipitated calcium phosphate can build up on pipes and pumps, affecting heat transfer capacity and resulting in increased wear and tear on equipment due to the abrasive nature of calcium phosphate (Tanguy *et al.* 2016). This in turn leads to increased costs due to a requirement for more frequent and

intensive cleaning of equipment and more onerous preventative maintenance requirements. This issue is most prevalent during and after heat treatment and concentration as these processes in themselves promote the formation of calcium phosphate.

Fig. 4.2 shows the proportion of sedimentable calcium after standardisation to 38% protein (w/w dry matter) and adjustment to pH 6.7, and after subsequent pasteurisation and evaporation. Significant differences were observed between $WPC_{UF4.6}$ and $WPC_{UF6.5}$ solutions when the pH was adjusted to 6.7, with ~90 % of the total calcium in $WPC_{UF6.5}$ precipitating, compared to only ~27 % of the total calcium in the $WPC_{UF4.6}$ solution. Pasteurization and evaporation had no effect on the level of sedimentable calcium for $WPC_{UF6.5}$, mainly because approximately 90% of the total calcium appeared to be in an insoluble form once adjusted to pH 6.7 and so was not further altered by heat treatment and concentration. However, in contrast the level of sedimentable calcium continued to increase for $WPC_{UF4.6}$ after each processing step. This suggests that, in the case of $WPC_{UF4.6}$, which had a higher percentage of soluble calcium following standardisation, heat treatment and concentration altered the calcium balance through either calcium phosphate precipitation or calcium binding to protein, thereby reducing the proportion of soluble calcium.

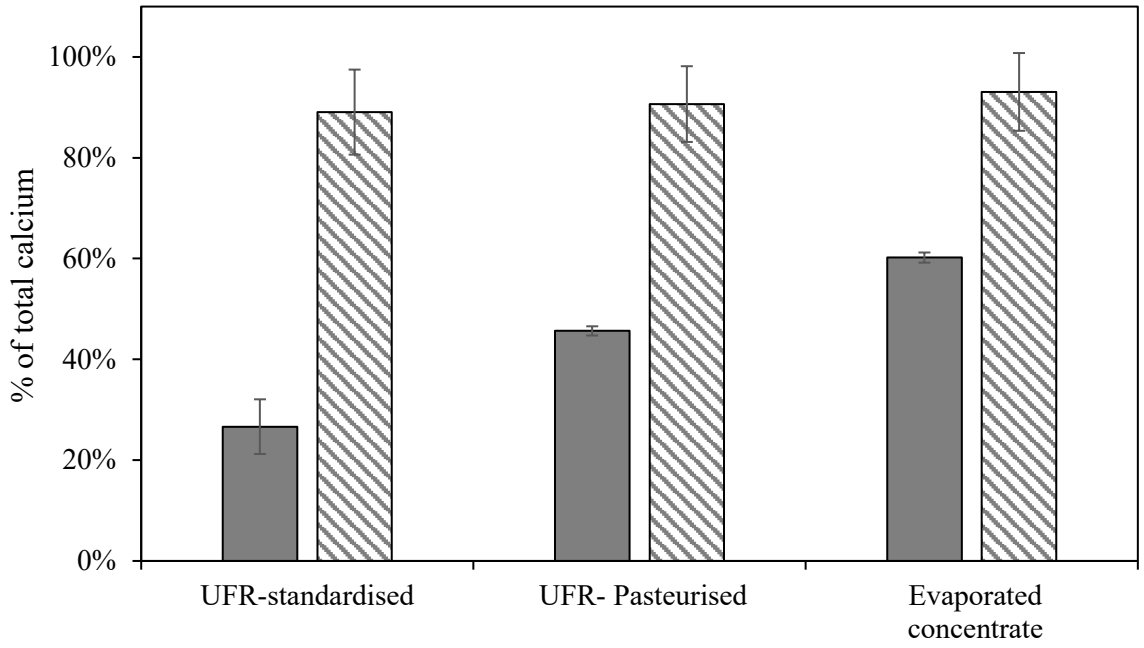
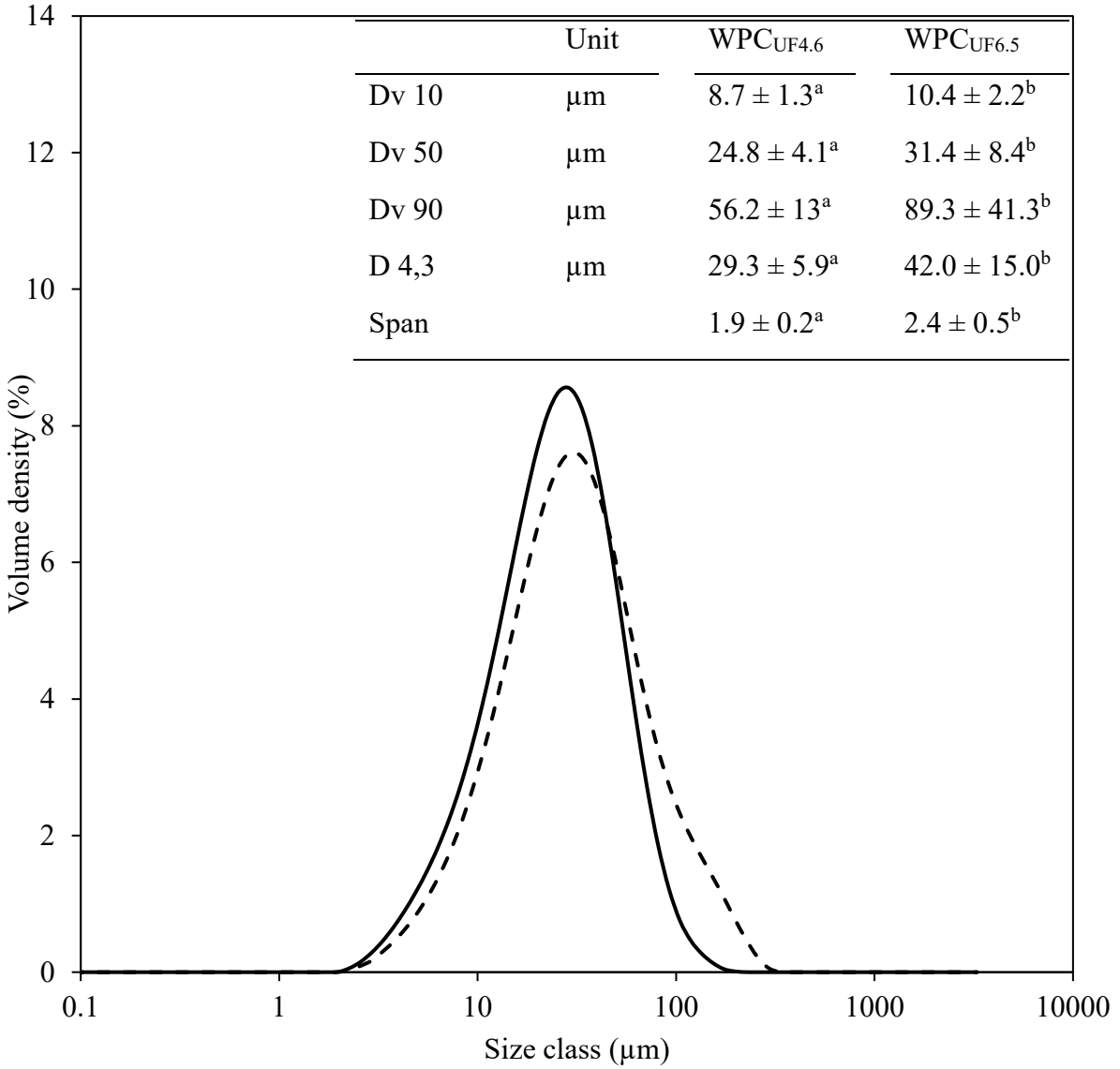


Fig. 4.2. Average percentage sedimentable calcium of $WPC_{UF4.6}$ ( )and $WPC_{UF6.5}$ ( ) following protein and pH adjustment (38 % protein w/w DM, pH 6.7), pasteurisation and evaporation based on the calcium content of the supernatant following centrifugation at 10,000 g × 30 min as a percentage of the material before centrifugation. Error bars indicate the standard deviation across triplicate measurements.

After pasteurisation and evaporation, the level of sedimentable calcium in $WPC_{UF4.6}$ increased from 27 % (w/w) to 60 % (w/w), with ~ 0.52 g (40 % w/w) of the total calcium remaining in a soluble form after evaporation to 30 % (w/w) solids (Fig. 4.2). This difference in behaviour between $WPC_{UF4.6}$ and $WPC_{UF6.5}$ can in part be attributed to the large differences in total calcium between these concentrates, with $WPC_{UF6.5}$ containing 3.82 g of calcium per 100 g^{-1} dry matter (3.44 g sedimentable after pH adjustment to pH 6.7) while $WPC_{UF4.6}$ contained only 1.29 g calcium per 100g^{-1} dry matter (0.35 g sedimentable after pH adjustment to 6.7) (Table 4.1 and Fig. 4.2). Given that the total calcium content and proportion of insoluble calcium was so high prior to heat treatment and concentration of $WPC_{UF6.5,}$ the calcium balance of this material had already progressed to calcium phosphate formation at pH 6.7. Meanwhile for $WPC_{UF4.6}$, for which the total calcium content was significantly lower, a significant proportion of the calcium remained in a soluble ionic form at pH 6.7 and therefore the effects of heat treatment and concentration had a greater impact on the calcium equilibria.

4.4.3. Properties of acid whey protein concentrate powders

The powder particle size distribution of both $WPC_{UF4.6}$ and $WPC_{UF6.5}$ powders are shown in Fig. 4.3. $WPC_{UF6.5}$ had a larger powder particle size than $WPC_{UF4.6}$, with D_{90} values on average 37 % larger which correlated with an observed slightly higher tapped bulk density (0.8 g ml^{-1} compared to 0.7 g ml^{-1} for $WPC_{UF4.6}$) and. The $WPC_{UF6.5}$ powder also had a greater span of particles compared to $WPC_{UF4.6}$, indicating a more heterogeneous particle size distribution. Fig. 4.3 shows the volume distribution of the powder particle size, and it can be clearly seen that, while both powders have a monomodal peak which broadly overlap each other, the high calcium powder contains some larger particles between 100 and 1000 µm. The larger particle size observed in $WPC_{UF6.5}$ can be attributed a higher viscosity prior to drying. It was observed that following evaporation to 30 % solids, $WPC_{UF6.5}$ appeared to be significantly more viscous than $WPC_{UF4.6}$ and gelled quickly if held for a period of time. However, due to the sensitivity of this product to gelation it was not possible to accurately measure viscosity mid process. Therefore, the final powder was reconstituted to 30 % w/w solids and viscosity was measured at 50 °C, 300 s^{-1} using a rheometer to compare the differences between samples (Fig. 4.4). From this, it is clear that $WPC_{UF6.5}$ had much higher viscosity than the $WPC_{UF4.6}$ and validates the observed differences in the concentrates prior to spray drying, Viscosity prior to drying has been shown to play a vital role in determining the final powder properties and morphology (Bista *et al.* 2022). High viscosity concentrate (>100 cP prior to atomisation) tends to result in bigger droplets forming during atomisation, which are not only more difficult to dry, resulting in reduced efficiency and increased risk of high moisture in the finished powder but can also affect the powder physical properties (Keogh *et al.* 2003; Pisecký 2012).



	Unit	$WPC_{UF4.6}$	$WPC_{UF6.5}$
Dv 10	µm	8.7 ± 1.3^a	10.4 ± 2.2^b
Dv 50	µm	24.8 ± 4.1^a	31.4 ± 8.4^b
Dv 90	µm	56.2 ± 13^a	89.3 ± 41.3^b
D 4,3	µm	29.3 ± 5.9^a	42.0 ± 15.0^b
Span		1.9 ± 0.2^a	2.4 ± 0.5^b

Fig. 4.3. Average powder particle size distribution (Volume Density %) of acid whey protein concentrate (WPC) 35 powders which underwent ultrafiltration at either pH 4.6 ($WPC_{UF4.6}$, ——) or pH 6.5 ($WPC_{UF6.5}$, – –) (average of 3 replicates). Insert table shows the average particle size distribution and standard deviation across 3 replicates. Dv10, 50 and 90 give the diameters (µm) below which 10, 50 and 90 % of the distribution (by volume) lie. D 43 is Sauter Mean Diameter. Span = (Dv90-Dv10)/Dv50. Samples with a statistically significant difference between $WPC_{UF4.6}$ and $WPC_{UF6.5}$ are indicated by different letters.

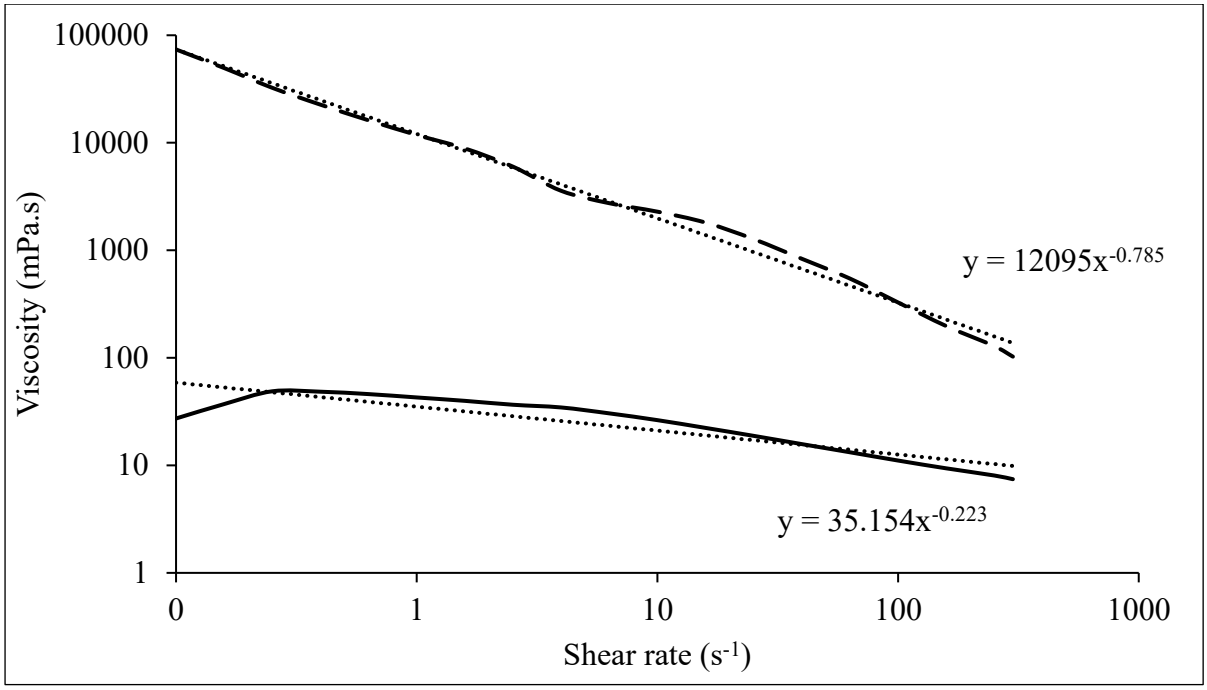


Fig. 4.4. Average viscosity (mPa.S) of $WPC_{UF4.6}$ (——) and $WPC_{UF6.5}$ (— —) aWPC powders reconstituted to 30 % total solids and measured at 50 °C as a function of shear rate (s^{-1}). Power law modelling trend curve and equation for each sample is also shown (.....). Profiles shown are the average of triplicate measurements.

4.4.4. Heat-induced gelation of reconstituted whey protein powders

Fig. 4.5 shows the storage modulus of whey protein solutions (5 %, w/w, protein; pH 6.8) during heating to 90 °C, holding for 30 min and subsequent cooling to 25 °C. It can clearly be seen that the storage modulus of the $WPC_{UF6.5}$ solution increased rapidly on heating, with gelation commencing at 68 °C (i.e., $G' > 1$ Pa), while the G' of $WPC_{UF4.6}$ did not increase during heating and only began to rise after 5 min of holding at 90 °C. Also, it was of note that the G' value for $WPC_{UF6.5}$ reached 230 Pa after 30 min at 90 °C, compared to 66 Pa for $WPC_{UF4.6}$ indicating that a firmer gel was formed in $WPC_{UF6.5}$. Gel strength increased further during cooling, with a G' of 192 Pa at 25°C for $WPC_{UF4.6}$ and a G' of 1080 Pa for $WPC_{UF6.5}$ after 30 min holding at 25 °C. Given that the pH of both $WPC_{UF4.6}$ and $WPC_{UF6.5}$ was adjusted to 6.8 prior to rheological analysis, the greater gel strength associated with $WPC_{UF6.5}$ may be due to the higher total ash, calcium and phosphorous contents, compared to $WPC_{UF4.6}$ (Table 4.1). This may have promoted protein-mineral interactions during heat treatment and enhanced gel

strength. However, conductivity and apparent ionic calcium concentration was higher in $WPC_{UF4.6}$ than in $WPC_{UF6.5}$ solutions (Fig. 4.6). This appears to be counterintuitive, given the higher gel strength that occurred in $WPC_{UF6.5}$ during heating.

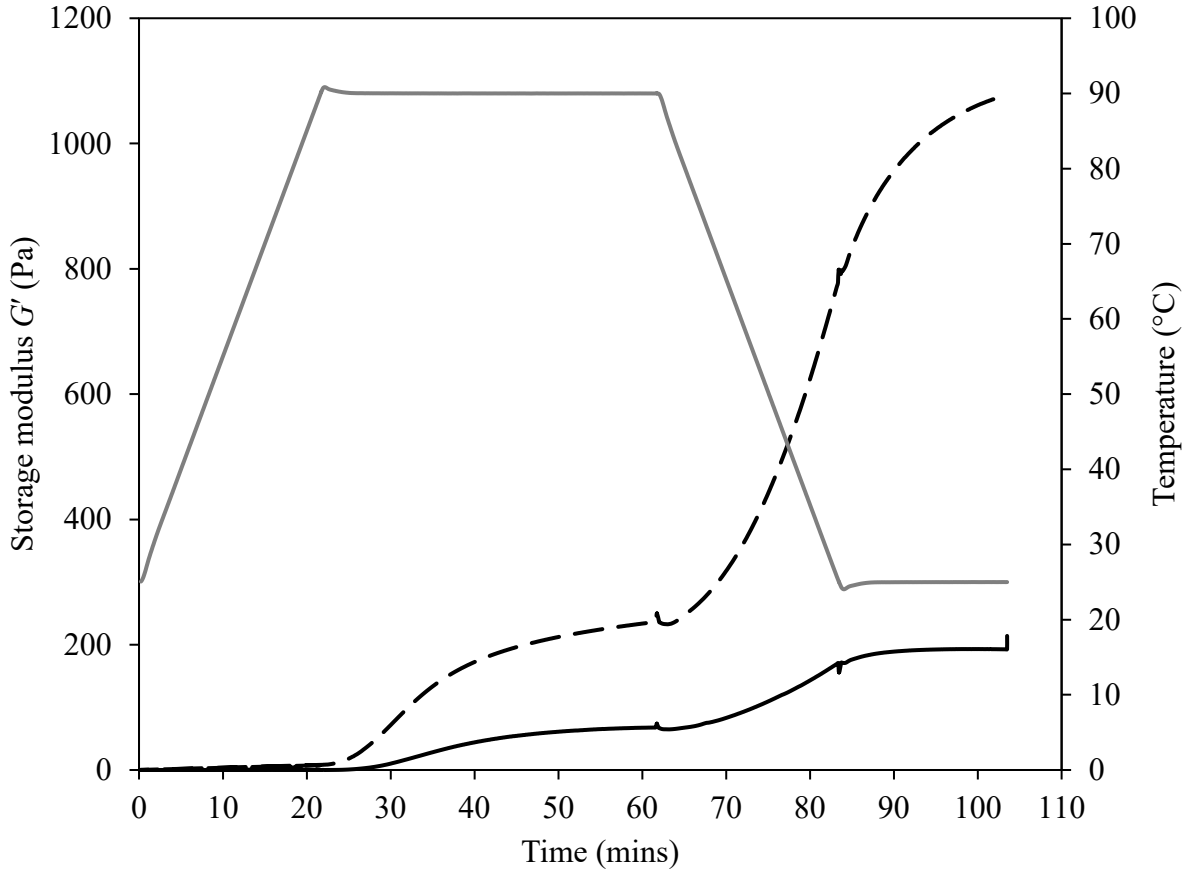


Fig. 4.5. Storage modulus (G') of reconstituted $WPC_{UF4.6}$ (——) and $WPC_{UF6.5}$ (– –) protein solutions (5 %, w/w, protein) measured during heating and cooling (Temperature profile shown as ——) at 5 % protein. Profiles shown are the average of triplicate measurements.

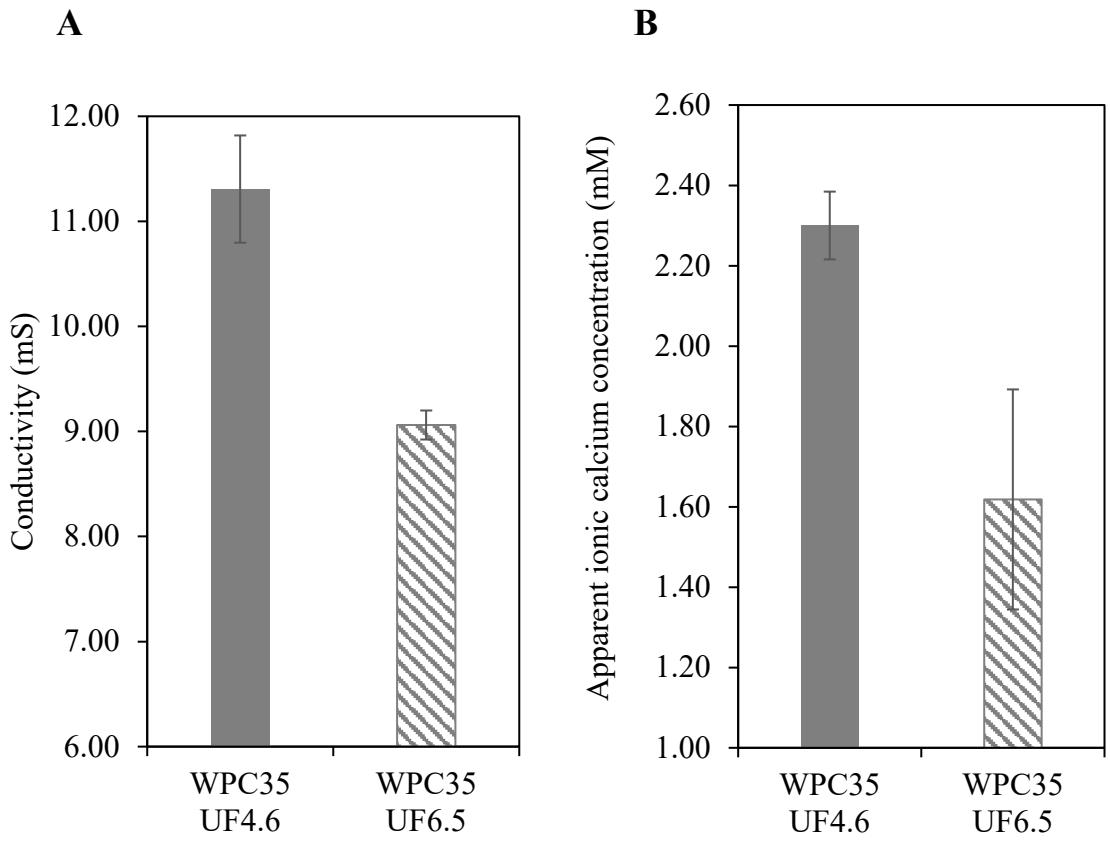


Fig. 4.6. Conductivity (A) and apparent ionic calcium concentration (B) of 5 % protein solutions of $WPC_{UF4.6}$ (■)and $WPC_{UF6.5}$ (▨) at 25 °C following pH adjustment to pH 6.8 (average of triplicate measurement)

To further assess the differences in gelation properties, the change in pH of WPC solutions during heat treatment was examined (Fig. 4.7). A clear decrease in pH was observed in both $WPC_{UF6.5}$ and $WPC_{UF4.6}$ with increasing temperature, resulting in a pH of 5.8 and 6.0 at 90 °C, respectively. The decrease in pH of solutions during heat treatment is related to increased hydrogen ion activity, described by the Van Hoof equation (Aydogdu *et al.* 2022). It is worth noting that $WPC_{UF6.5}$ decreased below pH 6.0 at 70 °C, which also aligns with its onset of gelation (Fig. 4.5), while the pH of $WPC_{UF4.6}$ only reached 6.0 at 90 °C also coinciding with its temperature of gelation. While a number of factors are probably involved in gelation kinetics, the release of hydrogen ions from mineral equilibration during heat treatment and subsequent impact on pH is pivotal.

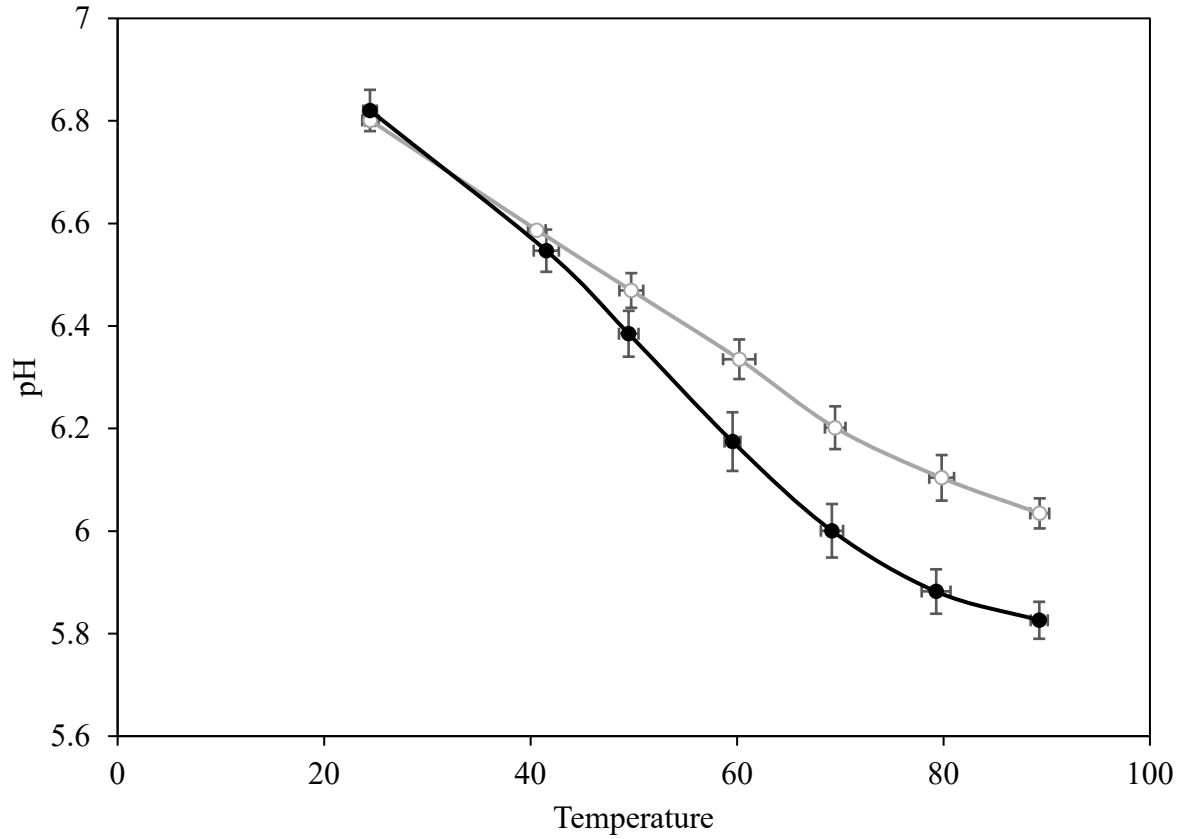


Fig. 4.7. pH profiles of $WPC_{UF4.6}$ (–○–) and $WPC_{UF6.5}$ (–●–) protein solutions (5 %, w/w) as a function of temperature. Profiles shown are the average of triplicate measurements.

The strength of the final gel networks was assessed by performing a frequency sweep from 0.1 to 100 Hz and an oscillation strain sweep from 0.01 to 100 % strain (Fig. 4.8A and B). Fig. 4.8A indicates that $WPCUF_{6.5}$ appeared to be more sensitive to increases in oscillation strain (linear viscoelastic region between 0.1 and 1.0 % strain; Fig. 4.8B), with gel breakage occurring above 1% strain, while the gel structure of $WPC_{UF4.6}$ did not break until close to 10 % strain (Anton Paar 2024). Fig. 4.8B shows a linear increase in *G′* with increasing frequency for both $WPC_{UF4.6}$ and $WPC_{UF6.5.}$ Increases in G' with increased frequency and a gel breakage at a low oscillation stain can indicate a brittle / non elastic gel (Chen 2023). Therefore, while the gel formed from $WPC_{UF6.5}$ may have had a firmer structure, its sensitivity to increases in oscillation indicate it was also more brittle than the weaker gel formed by $WPC_{UF4.6}$.

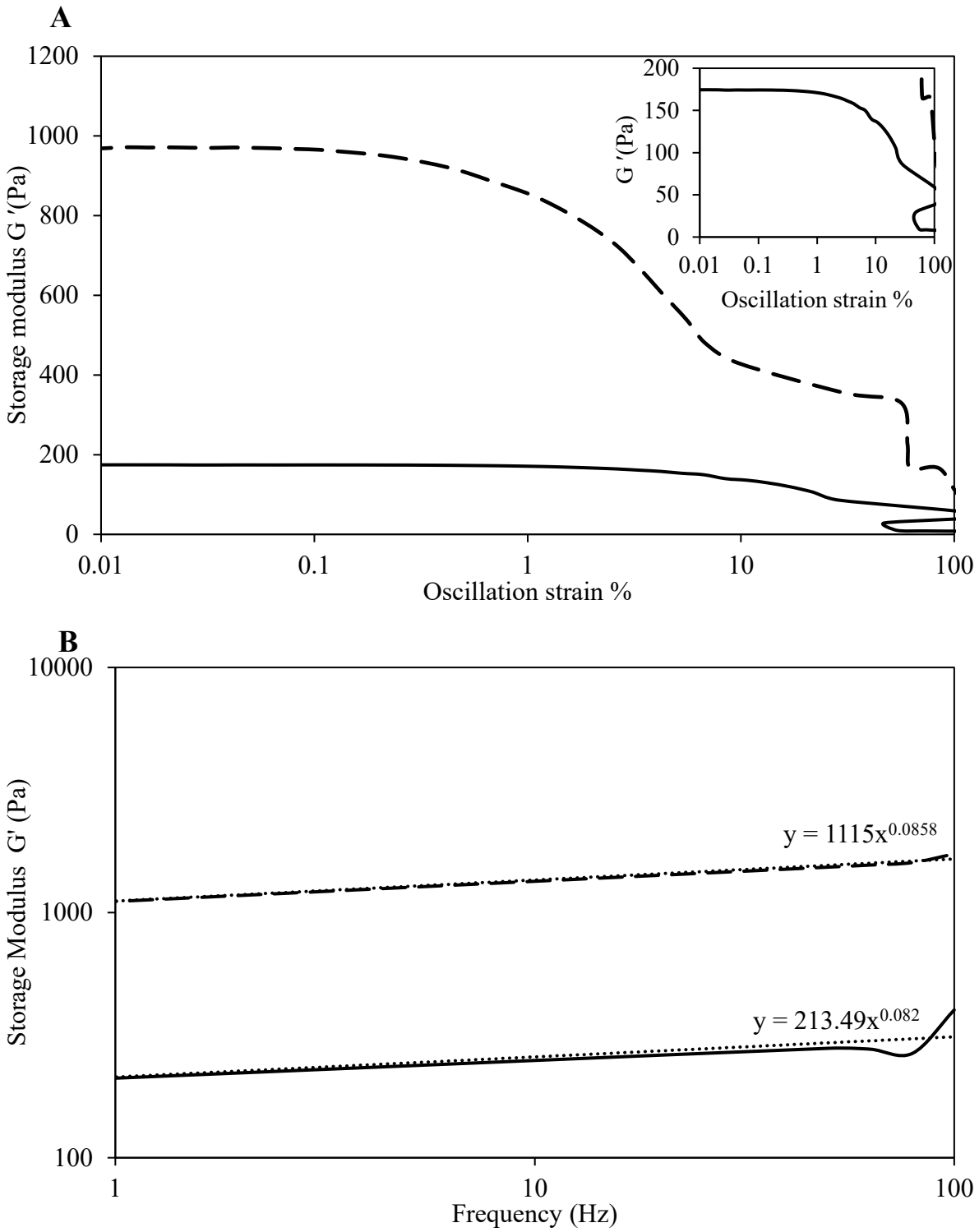


Fig. 4.8. Amplitude sweep ranging from 0.01% - 100 % oscillation strain (A) and frequency sweep ranging from 0.1 to 100 Hz (A) and an of $WPC_{UF4.6}$ (——) and $WPC_{UF6.5}$ (– –) protein solutions (5%, w/w) measured after heat treatment at 90 °C × 30 min. Fig 8A Inset shows $WPC_{UF4.6}$ (——) on a y axis scale of 0- 200 Pa. Power law modelling trend curve and equation for each sample across the frequency sweep is also shown (.....**,** B**)** Profiles shown are the average of triplicate measurements.

4.5. Conclusion

The mineral composition and functionality of acid whey concentrate is significantly affected by the pH at which filtration is performed. Filtration at acidic or neutral pH conditions have both advantages and disadvantages when it comes to processing efficiency and end-product functionality. pH neutralization prior to filtration results in greater calcium phosphate retention but increased membrane fouling, compared to filtration at acidic conditions where more calcium phosphate is removed. Retaining more calcium phosphate in the WPC also adds functionality in the case of greater heat-induced gel strength, ideal for certain applications. Therefore, modifying the pH of acid whey prior to filtration allows for the production of WPC ingredients with tailored mineral composition and functionality.

4.6. Acknowledgment

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Chapter 5

Effect of whey protein source and innate mineral profile on thermal stability of model infant formulae

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Declaration

This chapter was written by Sinead Mc Entee (SME) and reviewed by all co-authors.

5.1. Abstract

Advances in dairy technology have produced a variety of ingredients with varying mineral profiles suitable for infant formula (IF). High protein/ low mineral options allow greater control of the final mineral content, with more scope to add techno-functional minerals, e.g., chelating agents. However, these highly purified proteins tend to be more expensive and likely will have different thermal stability during IF processing. In this study, the impact of acid- and cheese- whey- derived protein ingredients with varying mineral contents on the ionic environment and thermal stability of high-solids model IF with key minerals formulated to target levels was examined. Formulations where the majority of minerals were innately present alongside the protein had higher apparent ionic calcium concentration, less negative zeta potential and significantly poorer thermal stability than those where minerals were added separately. In particular, formulations where all the calcium and phosphorus were derived from the protein ingredients showed significant increases in viscosity when heated to high temperatures (90°C). This indicates that despite added costs when using purified protein sources, improved stability during high heat processing is an advantage.

5.2. Introduction

Dairy-based infant formula (IF) is a breast milk substitute made by combining casein and whey protein ingredients to meet a target amino acid profile, with, carbohydrate, fat and minerals being added to produce a formulation which meets the nutritional requirements for healthy growth and development of an infant (Thompkinson & Kharb, 2007). Typically, the casein source is skim milk or skim milk powder, while the whey protein can come from the by-products of cheese manufacture (cheese whey) or acidification of milk to pH 4.6 to produce acid casein, cottage cheese or Greek style yoghurt (acid whey) (Singh, 2009).

Whey ingredients used in IF manufacture can have very different compositions depending on the source. Notably, the process of cheese manufacture results in the cleavage of glyco-macro-peptide (GMP) from κ-casein, which transfers to the whey (Oliveira, Fox, & O'Mahony, 2019). GMP lacks tryptophan and cysteine and can contribute up to 12 % of the total protein in the whey (Dutta, et al., 2017). As IF is manufactured based on an amino acid profile, the presence of GMP in cheese whey means that a higher proportion of whey protein is required to meet the tryptophan and cysteine requirements.

On that basis, acid whey may be more desirable as it will allow a manufacturer to achieve the required amino acid content with a lower protein content. However, the acidification of skim milk results in the release of colloidal calcium phosphate into the acid whey. As a result, acid whey has up to 3 times more calcium per gram of protein than cheese whey, which can significantly affect its thermal stability, as acid whey is more sensitive to calcium-mediated aggregation at elevated temperatures (Barone, et al., 2022; Bylund, 1995).

Ultrafiltration, along with diafiltration, is commonly employed to increase protein content of whey streams, and the resulting products are called whey protein concentrates (WPC) with protein content typically ranging from 35 to 90% w/w, on a dry basis (Gernigon, et al., 2011). This process also has the added benefit of reducing ash content and so results in

a product that is less sensitive to calcium-mediated aggregation during thermal processing. However, as infant formula is the sole source of nutrition for an infant, there is a requirement for a significant amount of minerals to support growth and development (Codex Alimentarius, 2007; Masum, et al., 2020). Typically, formulators design a recipe so that the innate minerals present in the protein ingredients are significantly below targeted levels, which allows for subsequent addition of defined quantities of mineral salts to meet the requirements. However, as established above, the innate mineral content of whey sources can vary substantially. Therefore, an IF manufacturer must decide where these minerals should come from, i.e., whether they are sourced from cost-effective low protein WPC 35 or if a high protein whey source such as WPC 80 or WPI be used with minerals added in a format and at a stage of processing, where they are less likely to affect thermal stability, should be used (Barone et al., 2022).

In this study, the impact of whey protein ingredient mineral composition on IF formulation functionality when all minerals are formulated to target levels was examined. Model IF concentrates at high solids (35.5 % w/w) were produced using different whey protein ingredients. The whey protein ingredients examined had varying mineral contents and compositions, with the major minerals being formulated to target levels in the final IF formulation prior to analysis.

5.3. Materials and methods

5.3.1. Materials

Skim milk powder (SMP), lactose (L), WPC 35 made from cheese whey (cWPC 35) and WPI from cheese whey (cWPI) were obtained from Tirlán, Kilkenny, Ireland. Two other types of WPC 35 (aWPC 35 and HC aWPC 35) were produced at pilot scale by performing

ultrafiltration at either pH 4.6 (aWPC 35) or pH 6.5 (HC aWPC 35), as described in Chapter 4, Section 4.3.2 using acid whey sourced from Tirlán, Kilkenny, Ireland.

5.3.2. Manufacture of acid WPC 60

Acid WPC 35 as described in Section 5.3.1. was reconstituted to 10% solids using ultra-pure water (Elga purelab flex 2, High Wycombe, UK) and pH-adjusted to pH 5.5 with 1 M HCl (VWR International, Blanchardstown, Dublin, Ireland) to ensure that any calcium phosphate present was in ionic form (Mc Entee, Kelly, Lawless, Murphy, & McCarthy, 2023). The material subsequently underwent ultrafiltration (1 L batches) to a target protein in dry matter of 60% w/w using a lab scale, pressure-driven, crossflow polyethersulfone membrane filtration device (Vivaflow 200, Sartorius AG, Göttigen, Germany) with a 10 kDa molecular weight cut-off and 0.2 m^2 total membrane area. Milli-Q water (500 ml) was added in 5 x 100 mL aliquots throughout processing to maintain a total solids of 8-11% (diafiltration volume was 50 % of the starting whey). UF continued until an approximate volume concentration factor (VCF) of 3 had been achieved. Total solids levels were monitored using a CEM smart 5 total solids analyser (CEM Corporation, Matthews, NC, USA) and final protein content was measured by the Dumas method using a LECO protein analyser (LECO corporation, Michigan, USA). The final protein level was standardised to 60% w/w DM by adding permeate obtained from the early stages of UF processing, i.e., before diafiltration water was added. pH of the retentate was then readjusted to pH 6.7 using 35% KOH before freeze drying.

5.3.3. Compositional analysis

Compositional analysis was completed by Eurofins Food Testing Ireland Ltd using accredited methods as described in chapter 4, section 3.3.

5.3.4. Model IF Preparation

High solids formulations were prepared to a target of approximately 35.5 % total solids, 5 % w/w protein with a 40: 60 casein: whey protein ratio and lactose content of 28.46 % w/w. Target pH was 6.9 at 25 °C, when diluted back to 10% solids. Initially, lactose was reconstituted in ultra-pure water at 70°C and stirred until fully dissolved; to minimise evaporation, the sample was covered whilst agitating. The solution was then allowed to cool to 55 °C before addition of SMP and either cWPC 35, cWPI, aWPC 35, HC aWPC 35 or aWPC 60, with the formulations labelled according to the whey protein ingredient added. Minerals were formulated to target levels using EFSA and Codex approved mineral additives- tri-calcium phosphate, calcium hydroxide, tri-potassium citrate, potassium chloride, sodium chloride, tri-sodium citrate and magnesium hexachloride (European Commission, 2006; Codex Alimentarius, 2007). Citric acid was added to reduce the pH to the target of pH 6.9 (25 °C) at 10 % solids where required. Minerals were sourced from VWR International, Blanchardstown, Dublin, Ireland. After mineral addition, formulas were agitated for 2 hours and allowed to cool to 25°C, with further minor pH adjustment using citric acid, if required, 30 minutes before analysis.

5.3.5. Measurement of pH, apparent ionic calcium concentration and conductivity

The pH was measured using a standard pH Meter (WTW pH 3310 with Sentix 41 probe, VWR International, Blanchardstown, Dublin, Ireland). Apparent ionic calcium (Ca^{2+}) level was determined as described in chapter 2, section 3.8. Conductivity was measured using a VWR® CO 310/CO 310 M conductivity meter (VWR International, Blanchardstown, Dublin, Ireland). The pH, apparent ionic calcium ion concentration and conductivity of each sample at 35.5% solids were analysed at 25 °C.

5.3.6. Zeta potential

Zeta potential (mV) analysis was conducted on the model formulations using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, England). Zeta potential was measured on samples at 25 °C after dilution in ultra-pure Milli Q water at a ratio of 1: 100 before loading into folded capillary cells (DTS1070, Malvern Instruments Ltd., Worcestershire, England) for measurement.

5.3.7. Simulated high temperature short time (HTST) heat treatment.

The stability of samples prepared at 5 % w/w protein as outlined in Section 5.3.4 to simulated high temperature short time (HTST) treatment was assessed using a HR-1 Discovery Hybrid Rheometer (TA Instruments, New Castle, DE, USA) equipped with a starch pasting cell geometry, using a similar method to that in Chapter 2, Section 3.7, with some modifications to the temperature sweep. Viscosity measurements of model IF concentrates (28 g) were carried out at a constant shear rate of 16.8 s^{-1}. A temperature sweep was performed consisting of the following steps: (i) heating from 25 to 40ºC, (ii) holding at 40 °C for 2 min, (iii) heating to 90 °C at a rate of 19.5 °C min^{-1}, (iv) holding at 90 °C for 5 min and (v) cooling back to 40°C at a rate of 22 °C min^{-1}. Samples were held at 40 °C for 2 min to obtain viscosity data following heat treatment prior to further cooling to 25 °C.

5.3.8. Soluble composition

Samples (50 g) of each formulation before and after heat treatment were centrifuged using a fixed angle rotor at 15,000 *g* for 30 min (Thermoscientific Multifuge X1R, Fiberlite™ F15-8 x 50cy Fixed Angle Rotor, Massachusetts, USA). Once centrifuged, the supernatant was gently poured off and filtered through Whatman filter paper No. 1 before conducting compositional analysis as described in section 4.3.3.

5.3.9. Statistical analysis

Each trial was completed in triplicate unless stated otherwise, with One-way Anova statistical analysis with Tukey's *post hoc* analysis being performed using Mini-tab® 20 statistical analysis package (Minitab Ltd., Coventry, UK).

5.4. Results

5.4.1. Formulation composition

Model formulations were prepared by adding SMP and either cWPC 35, cWPI, aWPC 35, HC aWPC 35 or aWPC 60 to a reconstituted lactose solution followed by mineral addition to the target composition. All formulations were based on a model, fat-free infant formulation containing 1.3 % w/w protein (casein to whey ratio 40: 60) and 7.4 % w/w lactose at ready to drink (RTD) concentrations. The target calcium, phosphorus, sodium, potassium, chloride, and magnesium contents of the formulations were set based on the maximum value obtained for each mineral across all combinations of ingredients. For example, the blending of SMP and cWPC 35 to the required protein content and ratios resulted in an innate sodium content of 30 mg 100 ml^{-1} formula at RTD concentrations, and so all other model IF were formulated to this sodium content through the addition of tri sodium citrate (TSC) or sodium chloride (Fig. 5.1) where appropriate. However, when innate mineral levels were already at the target value, as for sodium in cWPC35, this limited the addition of certain minerals e.g., tri-sodium citrate. As certain mineral salts can also have technological effects, such limitations may also affect behaviour during heating and further processing etc., (see Discussion section). Table 5.1 shows the quantities of mineral salts added to the formulations. In general, the formulations produced using higher protein, lower ash whey proteins sources (aWPC 60 and cWPI) had significantly higher addition rates of all minerals to the formulation when compared to their WPC 35

counterparts, which contained higher levels of minerals provided by the protein source (aWPC 35 and cWPC 35).

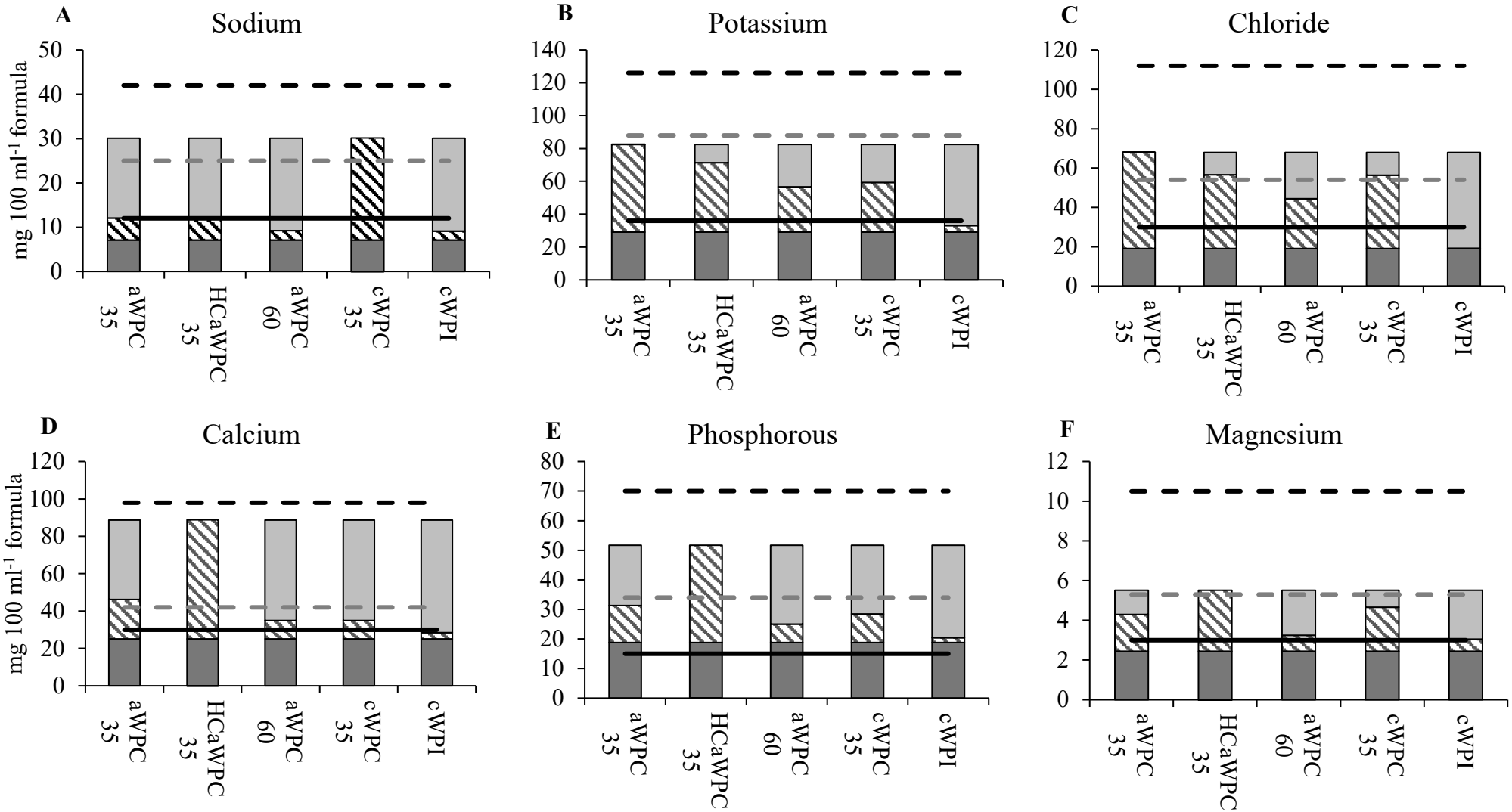

Fig. 5.1. Contribution of SMP (■), whey protein ingredient (▨), and added minerals (□) to the model formulation target mineral composition with comparison to codex regulatory maximum (– –) and minimum (—) limit and commercial IF (- -), Aptamil first milk) on a mg 100 ml^{-1} ready to drink formula basis (model recipe based on a 1.3 % w/w protein, 7.4%w/w lactose and 3.4% w/w fat formulation). (Aptamil, 2024; Codex Alimentarius, 2007)

Table 5.1. Quantities of mineral salts and citric acid added to model formulation on a dry basis (values adjusted to minerals at 100 % purity)

Ingredient	aWPC 35	HCaWPC 35	aWPC 60	cWPC 35	cWPI
	mg 100 g^{-1} solids				
Tri-calcium phosphate	1065	-	1407	1217	1620
Calcium hydroxide	67	-	41	176	22
Potassium chloride	16	226	334	252	645
Tri-potassium citrate	-	-	261	282	534
Tri-sodium citrate	660	697	614	-	368
Sodium chloride	28	20	141	-	337
Magnesium chloride hexahydrate	37	-	68	25	75
Total added minerals	1873	943	2866	1951	3601
Citric acid	42	22	-	113	-

Fig. 5.1 shows the contributions of SMP, the relevant whey protein ingredient and added mineral salts within the various formulations to the mineral content and level of added minerals required to achieve the target mineral content of the formulations at RTD concentrations compared to Codex maximum limits and a commercial IF product; (Aptamil first milk, Aptamil, 2024). It can clearly be seen that while the mineral profiles of the model formulations were within Codex limits, sodium, chloride, phosphorus and calcium were all higher than the commercial Aptamil product. Further scrutiny of Fig. 5.1 shows that the innate levels of calcium and phosphorous in HC aWPC 35 resulted in the higher target levels for these minerals across all formulations. Similarly, the high targets for sodium and chloride were set

by the innate levels in cWPC35 and aWPC35, respectively. Most notably, the calcium content of the model IF recipe was 89 mg 100 ml^{-1} which was below the codex maximum of 98 mg 100 ml^{-1} but was over 2 times higher than that of the commercial Aptamil product, which had a calcium content of 42 mg 100 ml^{-1}. Sodium, chloride and phosphorous levels are all within codex limits but are 20 % (w/v), 26 % (w/v) and 52 % (w/v) higher than the Aptamil product respectively. In addition, the calcium to phosphorous ratio was also quite different, with the model formula having a calcium: phosphorus ratio of 1.72: 1 while the Aptamil product had a much lower ratio (1.24), due to the significantly lower calcium content of that product.

5.4.2. Ionic activity of model formulae following hydration.

Table 5.2 shows the conductivity, ionic calcium activity and zeta potential of formulations following hydration at 35.5% solids (samples diluted 1:100 with Milli Q water for zeta potential analysis). The conductivity of all samples was broadly similar (5.27 to 5.55 mS per cm). Significant variation was observed in ionic calcium activity, with aWPC 60 and cWPI formulations having the lowest ionic calcium activity at 1.30 mM and 1.09 mM, respectively. Interestingly, cWPC 35 appeared to have the highest ionic calcium activity, at 1.88 mM. This may be due to the necessity to add increased amounts of calcium hydroxide to fully balance the calcium levels (Table 5.1) which is more soluble in water than tri calcium phosphate (National Research Council (US) Committee on Water Treatment Chemicals., 1982). Another point to note is that the ionic calcium activity of aWPC 35 and HCaWPC 35 appear to be similar, despite 44% of the calcium present in aWPC 35 being added in the form of insoluble tri calcium phosphate (Fig. 5.1); HCaWPC35, in contrast, contained no added calcium salts (Table 5.1).

Table 5.2. Ionic properties of formulations before heat treatment. Different letters beside values indicate they are statistically significantly different (P<0.05)

Formula	Conductivity	Ionic calcium	Zeta potential
	mS	mM	mV
aWPC 35	5.50± 0.01 ^{ab}	1.64± 0.12 ^{b}	-26.58± 2.12 ^{b}
HC aWPC35	5.40± 0.16 ^{ab}	1.62± 0.15 ^{b}	-24.71± 1.20 ^{a}
aWPC 60	5.27± 0.07 ^{b}	1.30± 0.06 ^{c}	-29.83± 1.45 ^{c}
cWPC 35	5.46± 0.11 ^{ab}	1.88± 0.11 ^{a}	-26.18± 1.50 ^{ab}
cWPI	5.55± 0.10 ^{a}	1.09± 0.17 ^{c}	-34.28± 1.64 ^{d}

Formulations produced using low mineral whey protein ingredients (aWPC 60 and cWPI) also had the strongest negative charge, with aWPC 60 and cWPI having a zeta potential of -29.83 and -34.28 mV respectively. In contrast, formulations where the whey protein ingredients had a high innate mineral content, had weaker negative charge, with the HCaWPC 35 formulation having the least negative zeta potential at -24.71.

5.4.3. Reactions during heat treatment

The model formulations were subjected to a HTST heat treatment of 90 °C for 5 min with continuous viscosity monitoring as described in Section 5.3.7. Fig 5.2 shows the change in viscosity during heating and cooling of the model formulations. The formulation produced with HC aWPC 35 displayed a sharp increase in viscosity during heating upon reaching 90 °C and increased steadily during the 90 °C hold. aWPC 35 and cWPC 35 were somewhat more

heat-stable; however, the viscosity of the aWPC 35 formulation began to increase after 1 minute at 90 °C, with cWPC 35 formulations increasing after 2 minutes at 90 °C. In contrast, the aWPC 60 and cWPI formulations displayed minimal viscosity increase across the 5 min hold at 90 °C. Upon cooling to 40 °C after heat treatment, all formulations showed increased viscosity; however, the increase was most extreme in the HCaWPC 35 formulation which was 4.7 times more viscous on cooling to 40 °C after heat treatment when compared to the viscosity at 40 °C prior to heat treatment. The lowest viscosity increases were observed for aWPC60 and cWPI, which were 58 % and 62 % higher than before HTST, respectively. The final viscosities at 40 °C after heat treatment were significantly different between the formulations and were in the following order:

HCaWPC35 >> aWPC 35 > cWPC 35 >> aWPC 60 = cWPI.

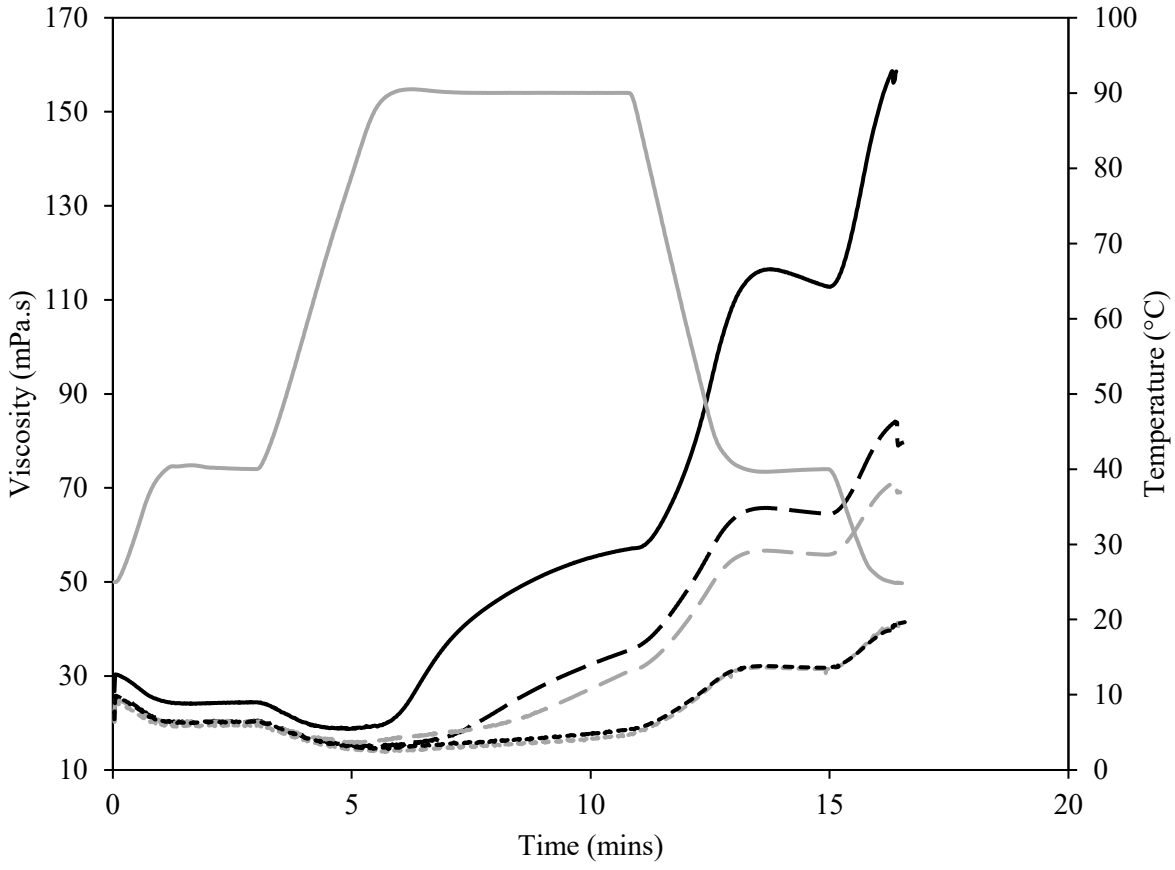

Fig. 5.2. Change in viscosity (mPa.s) under HTST conditions of model IF concentrates containing aWPC 35 (— –), HC aWPC 35 (——), cWPC35 (— –), cWPI (- - -)or aWPC 60 (- - -). Temperature during testing is shown as (——). Data are averages of triplicate analysis.

5.4.4. Changes in ionic environment as a result of heat treatment

All formulations were prepared to a target pH of 6.9 ± 0.03 at 25 °C when diluted back to 10% solids, as is typical in IF products. Citric acid was used, where required, to control pH, with the addition rates as shown in Table 5.1. Fig. 5.3 shows the pH of all formulations before and after HTST at 35.5% solids (Fig. 5.3A) and following dilution back to 10 % solids with Milli Q water (Fig. 5.3B). As expected, the pH at 35.5 % solids was significantly lower than at 10% solids, due to increased hydrogen ion activity and calcium phosphate formation at higher solids levels (Anema, 2009; Aydogdu, O' Mahony, & McCarthy, 2023). Interestingly, while the pH was similar at 10% solids across all formulations, there was variation in the formulations at 35.5% solids with HCaWPC35 having a statistically significantly lower pH and cWPC 35 having a slightly higher pH than the other formulations. Heat treatment reduced the pH of all formulations at 35.5% solids; however, the effect was most pronounced in the HCaWPC 35 formulation, which dropped from an average pH of 6.51 before heat treatment to pH 6.40 after heat treatment. Interestingly, for aWPC 60 and cWPI, a slight but statistically significant decline in pH was observed when measured at 35.5% solids, however, this difference was not significant when diluted back to 10% solids.

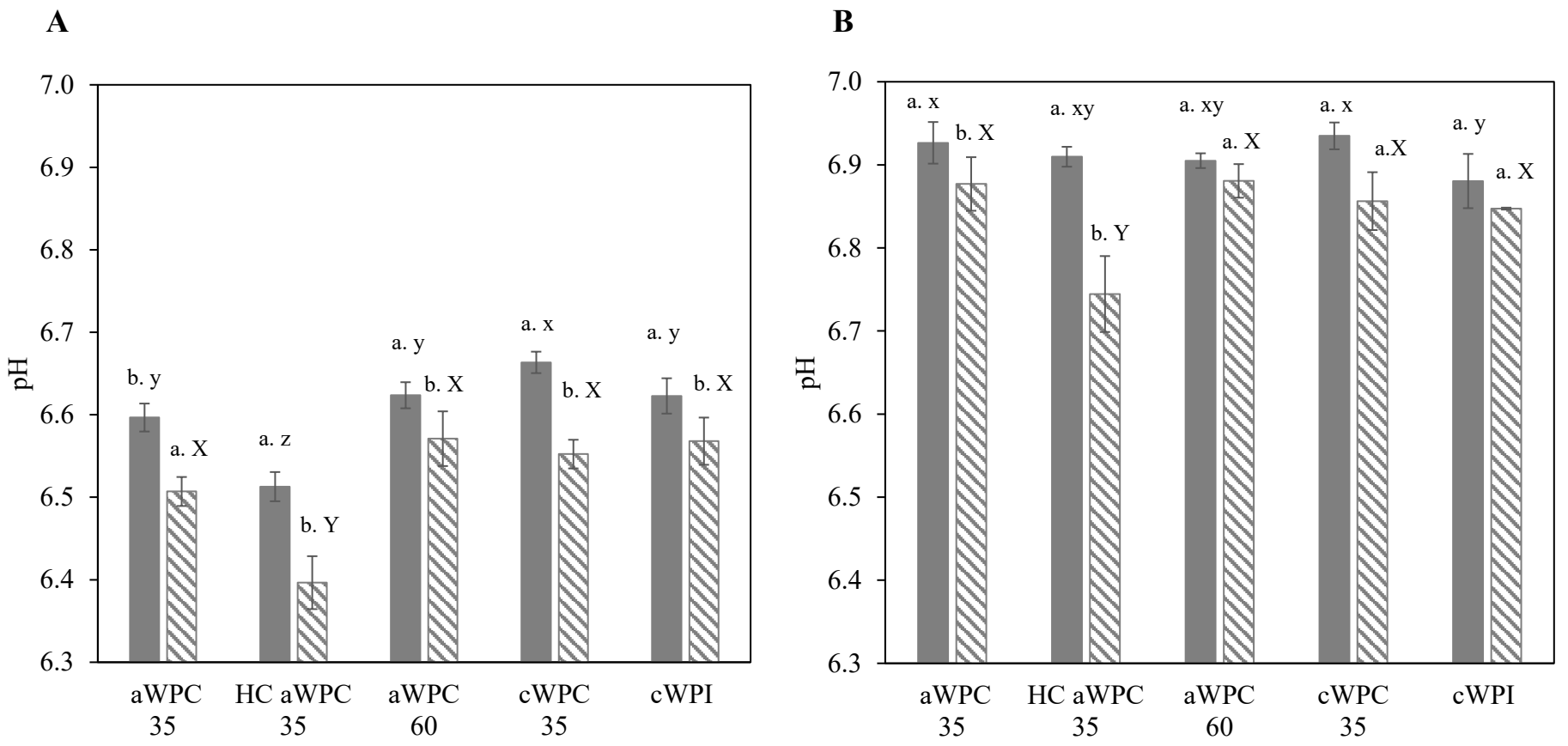

Fig. 5.3. pH of model infant formulae before ( )and after ( )heat treatment at 90 °C × 5 min at 35.5% solids. (A) and dilution to 10% solids with Milli- Q water (B) (Average of triplicate analysis). Statistical difference ($P < 0.05$) of the same formulation before and after heat treatment is shown by different lower-case letters (a, b). Statistical difference between different formulations before heat treatment are shown by lower case x. y, z letters, while statistical difference between different formulations after heat treatment is shown by capital X, Y, Z letters.

Table 5.3 shows the soluble protein, ash, calcium, and phosphorus content of each model formulation on a % (w/w) dry matter basis before and after heat treatment. Prior to heat treatment, aWPC 60 and cWPI had both the highest level of soluble protein and lowest soluble calcium levels of all the formulations tested, resulting in soluble protein: soluble calcium ratios of 34.3: 1 and 38.5: 1, respectively. In contrast, cWPC 35 had a soluble protein: soluble calcium ratio of 28.3 :1, while aWPC 35 and HC aWPC 35 had ratios of 23.9: 1 and 26.8: 1, respectively. Following heat treatment, all formulations experienced a reduction in soluble protein level; however, aWPC 60 and cWPI retained the highest levels of soluble protein, with 88.5% of the total protein remaining in solution following centrifugation. Similarly, a drop in soluble calcium and phosphorous was also observed for all formulations after heat treatment; however, this change was least severe in aWPC 60 and cWPI formulations, which had a lower proportion of these minerals in a soluble form before heat treatment (Table 5.3).

Table 5.3. Composition of soluble components of model formulations before and after heat treatment. Soluble components were extracted by centrifugation at 15,000 g for 30 minutes. Values are expressed as a percentage of the initial recipe on a dry matter basis. Lower case letters indicate statistical difference ($P<0.05$) between samples at the same stage of processing, e.g., before heat treatment or after heat treatment.

	aWPC 35	HCaWPC 35	aWPC 60	cWPC 35	cWPI
			%		
Before heat treatment					
Protein	84.3 ± 2.1^{b}	82.3 ± 6.7 ^{b}	103.4 ± 8.2 ^{a}	89.9 ± 0.8^{ab}	101.5 ± 7.1^{a}
Ash	78.0 ± 5.8^{ab}	83.3 ± 8.4 ^{a}	68.42 ± 2.4 ^{b}	72.2 ± 2.8^{ab}	68.7 ± 5.0^{b}
Calcium	53.1 ± 0.5^{a}	53.7 ± 7.8 ^{a}	45.5 ± 3.0 ^{ab}	47.9 ± 1.2^{ab}	39.5 ± 2.0^{b}
Phosphorous	58.4 ± 1.0^{a}	59.2 ± 4.9 ^{a}	49.1 ± 4.1 ^{b}	52.9 ± 1.4^{ab}	46.4 ± 2.4^{b}
After heat treatment					
Protein	66.1 ± 0.1^{y}	49.4 ± 1.2 ^{z}	88.4 ± 8.0 ^{x}	67.9 ± 3.7^{y}	88.5 ± 2.4^{x}
Ash	71.8 ± 8.1^{x}	75.1 ± 3.4 ^{x}	63.1 ± 5.9 ^{x}	71.5 ± 3.1^{x}	70.0 ± 4.6^{x}
Calcium	42.6 ± 2.3^{x}	33.7 ± 1.0 ^{y}	40.0 ± 4.2 ^{x}	33.5 ± 2.1^{y}	32.6 ± 1.3^{y}
Phosphorous	48.9 ± 2.1^{x}	42.5 ± 1.0 ^{y}	43.0 ± 3.9 ^{xy}	38.9 ± 3.3^{y}	37.7 ± 2.0^{y}

Interestingly, while a decline in soluble calcium and phosphorus was observed in all formulations after heat treatment, it appears that calcium and phosphorus precipitated in different amounts depending on the formulation, as shown by the soluble calcium to

phosphorus ratios (Fig. 5.4). The soluble calcium: phosphorus ratios of aWPC 60, and cWPI were not significantly different after heat treatment when compared to the values before heat treatment, indicating that calcium and phosphorus precipitated in equal amounts, perhaps indicative of calcium phosphate precipitation. However, the soluble calcium: phosphorus ratios of aWPC 35, cWPC 35 and HC aWPC 35 showed a statistically significant decline after heat treatment, with HCaWPC 35 showing the most significant change; an initial ratio of 1.5:1 which fell to 1.35:1 after heat treatment. This suggests that calcium also precipitated in some form other than bound, to phosphorus, possibly by binding to protein *via* calcium bridging.

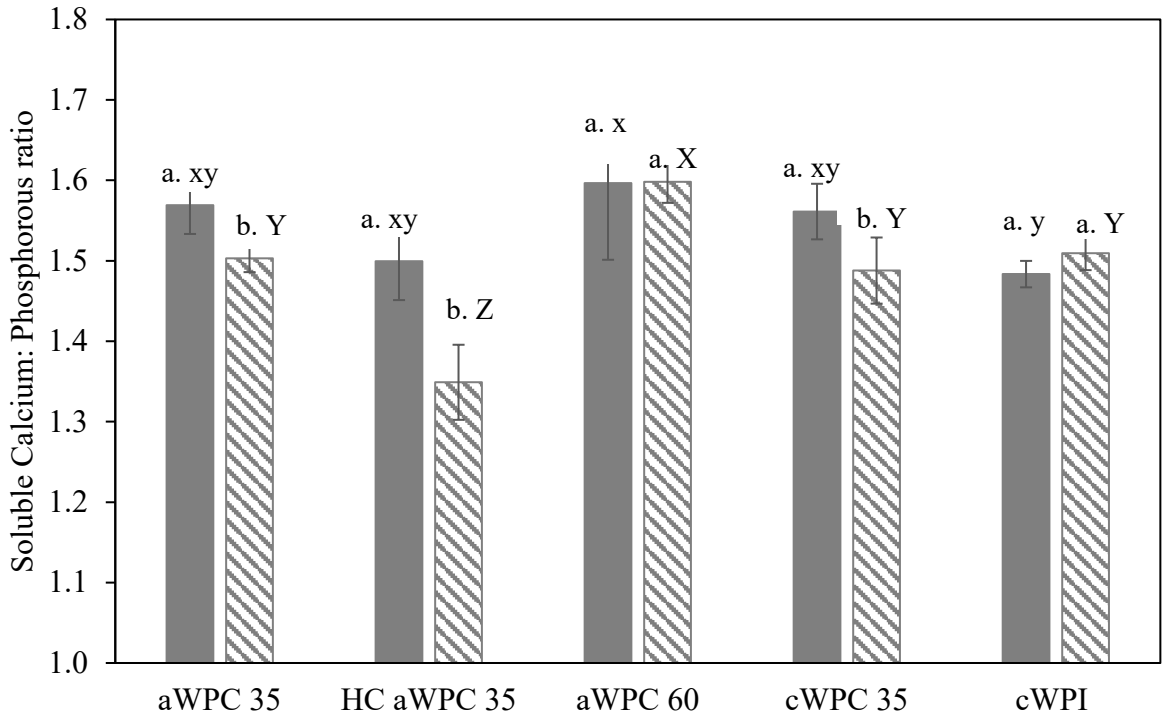

Fig. 5.4. Ratio of soluble calcium to soluble phosphorous based on the supernatant compositions following centrifugation of model formulations before (▓) and after (▨) heat treatment (Average of triplicate analysis). Statistical difference ($P < 0.05$) of the same formulation before and after heat treatment is shown by different lower-case letters (a, b). Statistical difference between different formulations before heat treatment are shown by lower case x. y, z letters, while statistical difference between different formulations after heat treatment is shown by capital X, Y, Z letters.

5.5. Discussion

In this study, five high solids model IF formulations were prepared to the same protein, lactose, and major mineral composition prior to simulated HTST treatment. While all formulations were standardised to the same composition, their thermal stability varied significantly. Formulations where the majority of the minerals were obtained from the whey protein source (aWPC35, HCaWPC35, cWPC 35) appeared to be more sensitive to thermal aggregation than those where minerals were predominantly added separately to protein (aWPC 60 and cWPI formulations). By using low mineral protein sources, increased levels of both mineral chelators such as tri-potassium citrate and tri-sodium citrate, and inert minerals such as tri-calcium phosphate could be added (Table 5.1) (Chow, 2001; El Moussaoui, et al., 2023; Hebishy et al., 2019). The resultant aWPC60 and cWPI formulations had significantly lower ionic activity (Table 5.2), which may have contributed to higher thermal stability. Similarly, these formulae possessed a stronger negative charge, which is indicative of greater dispersion stability (Clogston & Patri, 2010), i.e., greater repulsion between proteins in these solutions, which may have resulted in lower levels of protein – protein aggregation during heating. These results highlight the real-life impact of whey protein source and innate mineral content on IF processing.

Low mineral protein sources are generally used in IF because they remove the possibility of exceeding their target mineral levels and allow for great flexibility in terms of the mineral salts which can be added. Furthermore, the present study illustrates an added benefit of increased thermal stability which can be attributed to such whey protein sources. It is also noteworthy that these findings were recorded at high solids content and in a relatively concentrated ionic environment, where sodium, chloride, phosphorous and, in particular, calcium levels were higher than found in a typical IF (Fig. 5.1). Therefore, similar observations could be expected in IFs with typical mineral profiles. However, such whey sources typically

require more processing and are more expensive; for example, WPI commands approximately a 45% premium over WPC 35 per kg of protein (Affertsholt & Watson, 2020). In addition, higher mineral salt requirements may also marginally increase the cost of an IF recipe. It is also important to consider the bioavailability of these inert or insoluble minerals when consumed by the infant. Therefore, a balance between protein/ mineral content, cost, in-process behaviour and nutritional quality must be found.

The source of calcium and phosphorus in formulations appeared to be key in determining thermal stability, as can be illustrated by examining the behaviour of the high mineral content formulations (HCaWPC35, aWPC35 and cWPC 35). In the case of the HCaWPC35 formulation, all of this calcium and phosphorus came from the protein ingredients and so was in an uncontrolled state. This formulation was highly heat sensitive and suffered extreme gelation during HTST treatment (Fig. 5.1). In contrast, the aWPC 35 formulation which appeared to have a similar apparent ionic calcium activity and level of soluble calcium prior to heat treatment, was 50 % less viscous than the HCaWPC 35 formulation when cooled to 40°C after heat treatment (Fig. 5.2, Table 5.2 & 5.3). The key difference in these formulations is the form of calcium which was present; for the aWPC 35 formula, 44% of the calcium present was in the form of added tri calcium phosphate, which is highly insoluble and relatively inert during thermal processing (Chow, 2001). Meanwhile, as alluded to above, all of the calcium present in HCaWPC 35 came from the protein ingredients (Fig. 5.1). Given that the apparent ionic calcium activity (Table 5.2) and level of soluble calcium (Table 5.3) of HC aWPC 35 was not significantly higher than other samples prior to heat treatment, this would suggest that some of the calcium in this formulation may have been in a bound form such as in the form of calcium hydrogen phosphate. In this state, at 25 °C the calcium would be non-ionic; however, with increasing temperature the solubility of calcium hydrogen phosphate decreases, and it will precipitate out of solution (Mc Entee et al., 2023; Wang & Ma, 2020). This reaction

also results in the release of a hydrogen ion and a subsequent drop in pH similar to what was observed in the HCaWPC 35 formulation after heat treatment (Fig 5.3). This decline in pH has a knock-on effect on the ionic environment and can result in the release of colloidal calcium phosphate from casein, thus increasing the ionic calcium content at elevated temperatures (Holt, 2004; Singh, 2004). This, in turn, may lead to increased protein aggregation *via* calcium bridging and an increase in viscosity of the material. Evidence of this pH decline can be seen in the lower pH values observed in the HCaWPC 35 formulation after heat treatment at both 35.5 % solids and 10 % solids (Fig. 5.2). In addition, the decline in soluble protein and calcium levels, which also altered the soluble calcium: phosphorus ratio after heat treatment, also corroborates the theory that ionic calcium played a role in protein aggregation and sedimentation.

Interestingly, the formulation with the highest apparent ionic calcium activity prior to heat treatment, cWPC 35, appears to have been slightly more heat-stable than aWPC 35. This could be due to a number of reasons. The high apparent calcium ion activity of this formulation is believed to be due to the addition of calcium hydroxide to the formulation, which was added to balance the calcium requirements without exceeding phosphorous limits. However, this solution also had higher levels of citric acid added to control pH which may also have aided the chelation of calcium in the form of calcium citrate (Table 5.1). Also, it was observed that cWPC 35 contributed not only a lower level of calcium but also phosphorus to the model formula when compared with aWPC 35. This facilitated the increased addition of tri-calcium phosphate to this formula, which could have reduced the impact of reactions related to balancing the calcium phosphorous equilibria as the sample was heated. In addition, the level of soluble protein prior to heat treatment was slightly higher in cWPC 35 than aWPC 35 which resulted in a higher ratio of soluble protein: soluble calcium (28.3: 1 compared to 23.9: 1 for

aWPC 35). This may have limited pH decline, as calcium became bound to protein rather than being involved in calcium phosphate reactions.

Another factor to consider is the presence of GMP in cWPC 35, which can make up anywhere from 12-25% of total protein in cheese whey and may have contributed to the reduced viscosity following heat treatment when compared to formulations manufactured with aWPC 35 (Thomä-Worringer, Sørensen and López-Fandiño, 2006; Dutta et al., 2017). It has been reported in a number of studies that the presence of GMP alters the kinetics of denaturation and aggregation of β-lactoglobulin during heat treatment, with many studies indicating that, at pH values where both β-lactoglobulin and GMP are negatively charged (neutral pH), GMP hinders aggregate formation by β-lactoglobulin (Martinez, Farías and Pilosof, 2010; Gaspard et al., 2021).

While the exact mechanism is yet to be determined, it is clear that cWPC 35 is more stable than aWPC 35, despite having increased calcium ionic activity and similar levels of soluble minerals before heat treatment. In addition, Fig. 5.2 clearly shows the limitations of using all WPC35 and, in particular acid WPC35 ingredients; poor associated thermal stability, which when combined with a high mineral content, limits their application from both a processing and a formulation perspective. However, when comparing the WPC35 results obtained, it is clear that controlling the form of calcium phosphate is key. Furthermore, it can be seen from Fig. 5.1 that the innate levels of minerals in aWPC35 are only marginally in excess of typical IF calcium and chloride targets. Therefore, further concentration of aWPC35 through membrane filtration to aWPC60 was investigated as a means of reducing mineral content and achieving greater control over the form of calcium phosphate. To allow for comparison with the other formulations, the levels of minerals in aWPC60 formulations were maintained at the same high level for sodium, chloride, calcium and potassium, Fig. 5.2 shows that the innate levels in this formulation were below typical values, thereby increasing usability

in IF. Finally, it should also be noted that acid whey has a more favourable amino acid profile, which can allow for reduced protein content in IF and associated nutritional and cost benefits (Kunz & Lönnerdal, 1992; Mc Entee, et al., 2023b).

Concentration of acid whey to a WPC 60 significantly improved the thermal stability of the model IF formulations and resulted in a product that retained a low viscosity at 35.5 % solids after being heated to 90 °C for 5 min (Fig. 5.2). While protein concentration may result in increased premiums for acid WPC 60 when compared against acid WPC 35 which, along with costs associated with adding minerals to the formula, will increase the overall IF manufacturing cost, this option results in a product that could have a lower overall protein content which is more in line with human milk and supports the aim of producing IF products which mimic the composition of human milk protein.

5.6. Conclusion

This study highlights the importance of understanding the ionic state and mineral composition of an IF and its impact on thermal processing. Despite standardising protein, lactose and mineral composition, significant differences in viscosity following HTST treatment were observed depending on the proportion of minerals sourced from the whey protein ingredients or added separately. Formulations where the majority of minerals in particular calcium, were sourced from the protein ingredients, suffered a loss of soluble protein and increased viscosity following heat treatment, likely as a result of increased ionic calcium, and less negative zeta potential prior to heat treatment.

In contrast, formulations which used acid and cheese whey ingredients with a low ash content (aWPC 60 and cWPI), where inert minerals or calcium chelators could be added, were shown to result in a less acute rise in viscosity following heat treatment.

This study has shown that while demineralised whey ingredients may be more costly to use in IF, these ingredients produce a formulation that is more stable to high temperature processing and could facilitate increased incorporation of acid whey in low mineral forms which could support an overall reduction in IF total protein through the avoidance of GMP.

5.7. Acknowledgment

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5.8. References

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Chapter 6

Effect of protein standardising media on heat stability of skim milk concentrates prior to oil addition during the manufacture of low protein fat-filled milk powders

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Declaration

This chapter was written by Sinead Mc Entee (SME) and reviewed by all co-authors.

6.1. Abstract

Increased production of milk and whey protein concentrates means that finding applications for permeate side-streams is more critical than ever. One application is the protein standardisation of fat-filled milk products. In this study, standardisation media from five different sources (i.e., lactose, two different milk permeates, cheese whey permeate, and salty cheese whey permeate), were combined with skim milk to produce concentrates (35% dry matter DM; 24% protein on DM basis). Concentrates standardised with permeates displayed significant increases in viscosity after heating, with gelation occurring during cooling, meanwhile, in contrast, concentrates standardised with lactose displayed only a slight increase in viscosity following heating. However, increasing the pH of all concentrates to 6.5 prior to heating prevented gelation. Overall, the functionality of skim concentrates standardised with either milk or whey permeate was dependent on mineral profile, but the negative effect of high viscosity and gelation can be off-set by pH adjustment.

6.2. Introduction

Finding applications for low value, protein depleted dairy streams, such as milk permeate (by -product of MPC protein concentration) and whey permeate (by-product of whey protein concentration), are critical for the environmental sustainability of the dairy industry. Valorisation of de-proteinated permeate streams is becoming increasingly important, with the ever-expanding growth of both cheese, which is forecast to have a volume growth of 2.6% per year over the next 4 years, and MPC which has experienced a compound annual growth rate (CAGR) of 13% since 2018 (3A Business consulting, 2022; Statistica, 2023). One common use for milk permeate is standardisation of protein content in certain products such as fat-filled milks and coffee creamers (Rattray & Jelen, 1996). Fat-filled milk powders (FFMPs) are dairy powders where the milk fat has been replaced with a vegetable fat source. In this process, skimmed milk is often concentrated by evaporation to high solids, with heat treatment taking place either before or after evaporation, followed by the addition of vegetable oil to the concentrate and homogenisation to form an emulsion prior to drying (Finnegan, Mahomud, & Murphy, 2012). The protein content of these products is standardised by increasing the lactose content, typically by adding milk permeate or lactose to the skim milk prior to evaporation to offset the natural variation of protein in cow's milk across the lactation cycle or to produce a low protein vegetable-fat-filled powder as an inexpensive alternative to whole milk powder. As permeate side streams are relatively low cost in comparison to purified lactose, they are an economically advantageous option for incorporation into FFMPs. In skim and whole milk powder production, only milk permeates or lactose may be used for protein standardisation; however, in FFMP production, whey permeates can also be used.

Typically, permeate will undergo some form of concentration to reduce transport costs and the energy requirements for water removal during FFMP production (Metzger & Marella, 2016). This can be achieved using membrane filtration techniques such as nanofiltration or

reverse osmosis to concentrate permeates from ~6.0% dry matter (DM) up to 15-25% DM; further water removal may take place through evaporation and spray drying. Alternatively, isolation of lactose from permeates is another common valorisation path within the dairy industry. This is instigated by the evaporation of permeate to the point where lactose becomes super saturated, followed by controlled cooling to allow lactose crystallization and sedimentation, resulting in a product with a very low mineral content and high level of sugar purity (Wong & Hartel, 2014). The end applications of lactose vary largely, from a source of carbohydrate in infant formula, to use as an excipient in pharmaceutical applications or, as mentioned above, as protein standardization media in FFMPs.

Composition of permeates is quite variable, depending mainly on the process from which they originate. However, permeates typically contain significant levels of minerals, which can react with the protein in skim milk during heat treatment (Tsermoula et al., 2021). Milk permeate is a by-product from MPC production, where the protein in skim milk is concentrated using ultrafiltration membranes with lactose and ash removed in milk permeate (Oliveira, Fox, & O'Mahony, 2019). Another major source of protein-depleted permeates is from protein concentration of cheese whey. In this case, the composition of the cheese whey permeate can vary significantly depending on the variety of cheese originally manufactured, whether it is from a single source of whey or from a mixture of whey from multiple cheese processes. In addition, the composition of cheese whey permeate can vary depending on where in the cheese production cycle the whey was taken from. This is particularly the case during Cheddar cheese manufacture, e.g., if collected after the salting step where salt is added to the cheese curd prior to pressing and moulding into a cheese block; this permeate will be classified as salty cheese whey permeate, as some of the salt added to the curd will be lost to the removed permeate during the pressing stage (McSweeney, 2007).

Depending on the source and the prior history of the permeate, such as the operating conditions during protein concentration (pH, temperature, membrane pore size, target protein concentration), and mechanism of permeate concentration (membrane concentration, or evaporation and spray drying), these various types of standardisation media can have very different mineral profiles, which can also vary across the production season due to seasonality changes in the initial milk. As a result, when combined with skim milk for FFMP manufacture, the ionic environment of the product can vary significantly, resulting in changes in thermal stability and physico-chemical properties during processing (Murphy et al., 2018; Sikand, Tong, & Walker, 2010). A particular issue is fouling and high viscosity after evaporation, which can potentially affect the physical properties of the final powder such as bulk density, powder moisture and solubility, as a result of high viscosity prior to drying (McCarthy et al., 2021).

The objective of this study was to assess the impact of the ionic environment, as determined by standardisation media and pH on the stability of low protein skim milk concentrates under high temperature short time (HTST) heat treatment conditions.

6.3. Materials and methods

6.3.1. Materials

Low heat skim milk powder (SMP) was manufactured at pilot scale in Teagasc Moorepark as described by Magan et al. (2019). The composition of the SMP was 38.6% w/w protein dry matter, 49% lactose w/w dry matter and 8.2 % w/w ash dry matter. Lactose powder and the following types of liquid permeate were obtained from Tirlán (Kilkenny, Ireland); milk permeate concentrated to 20% solids via nanofiltration membrane concentration (MP 1), milk permeate concentrated to 25% solids by reverse osmosis (MP 2), and salty cheese whey permeate (sCWP) at 15% solids taken from a Cheddar cheese process. Non-salty cheese whey

permeate (nsCWP) taken from a continental cheese process and concentrated to 25% solids using reverse osmosis was obtained from Royal – Aware (Lopik, Netherlands). In the case of the conentrated permeates (MP1, MP2 and nsCWP), citric acid was added prior to membrane concentration to minimise mineral fouling. The composition of the lactose and whey permeate ingredients are shown in Table 6.1.

6.3.2. Compositional analysis

Compositional analysis on all permeates is shown in Table 6.1 and was completed by Eurofins Food Testing Ireland Ltd using accredited methods as described in Chapter 4, Section 3.3. Lactose analysis was performed by Eurofins Food Testing Ireland Ltd using HPAE-PAD analysis (ISO 22184 / IDF 244: 2021). Total solids levels were measured using a CEM smart 6 rapid total solids analyser (CEM Corporation, Matthews, NC, USA) (ISO/ IDF, 2004). Citric acid analysis was performed by an r-biofarm enzyme assay and UV detection (ISO 17025:2018).

6.3.3. Sample preparation

All permeates were adjusted to 15% total solids using deionised (DI) water prior to further analysis. Similarly, lactose powder was rehydrated and dissolved in DI water at a dry matter content of 15% (w/w) at 70 °C, to ensure complete solubilisation. All five standardisation media were tempered at 50 °C prior to the direct addition of skim milk powder (SMP) to the permeate or rehydrated lactose solution under agitation using a stirring plate, producing a concentrate at approximately 35.5 % solids content (i.e., protein content standardised from 38.6 %, w/w, to 24%, w/w, dry matter basis). The samples were then left to mix on a stirring plate for 1 hr before storing overnight at 4 °C to ensure complete hydration.

Following overnight storage, the samples were heated to 25 °C and sub-divided into two aliquots, with the first aliquot maintained at its native pH, and the second pH-adjusted to pH 6.5 using 1 M NaOH. The pH-adjusted samples were left to equilibrate at pH 6.5 for at least 30 min before analysis.

6.3.4. Ionic environment

Readings of pH, conductivity and ionic calcium were taken for the standardisation media at 15% solids and the skim concentrates before heat treatment. pH was measured using a standard pH meter (Metrohm 691 pH meter, Metrohm Ireland Ltd., Carlow, Ireland). Conductivity was measured using a VWR CO310M conductivity probe (VWR international, Pennsylvania, USA). Apparent ionic calcium (Ca^{2+}) concentration at 25 °C was measured as described in Chapter 2, Section 3.8.

6.3.5. Simulated high temperature short time (HTST) heat treatment

The low protein skim concentrates underwent simulated HTST heat treatment using a Kinexus Pro+ rheometer (Malvern Instruments Ltd., Worcestershire, England) equipped with a starch pasting cell geometry, similar to the method used by Hebishy et al. (2019) and Murphy et al. (2014). Viscosity measurements of protein solutions (70 g) were carried out at a constant shear rate of 16.78 radians sec^{-1}. Viscosity was monitored at 25 °C initially for 1 minute before a temperature sweep was performed over a heating step from 25 to 40 ºC at a rate of 15 °C min^{-1}. Samples were then held at 40 °C for 1 min before heating to 95 °C at a rate of 15 °C min^{-1} and holding at 95 °C for 5 min, followed by cooling back to 40 °C at a rate of 15 °C min^{-1}. Samples were held at 40 °C for 1 min to obtain viscosity data after heat treatment prior to further cooling to 25 °C.

6.3.6. Particle size

Particle sizes of the concentrates before and after HTST treatment (Section 6.3.5) were measured using a Malvern Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, England) at a refractive index of 1.38 with water used as the dispersant.

6.3.7. Statistical analysis

Each trial was completed in at least triplicate unless stated otherwise. One-way ANOVA statistical analysis with Tukey's *post hoc* analysis was performed using the Mini-tab® 20 statistical analysis package (Minitab Ltd., Coventry, UK).

6.4. Results & Discussion

6.4.1. Compositional properties of standardization media

The compositional profile of lactose and permeates are shown in Table 6.1, with the lactose used containing only trace amounts of major minerals and consisting of > 99% lactose on a dry matter basis (including chemically bound moisture). The composition of the two milk permeates was significantly influenced by the method of concentration used, i.e., using either nanofiltration (MP 1) or reverse osmosis (MP 2) to concentrate to 20 and 25% (w/w) solids, respectively. The differences between these two permeates is reflected in the lower level of chloride, potassium, sodium and NPN in the NF-concentrated permeate, compared to RO-concentrated permeate (MP2), as the former filtration process facilitated increased permeation of these components. However, calcium, phosphorous (indicating phosphate) and magnesium levels were similar between MP1 and MP2, indicating that the permeation of multivalent ions during NF was negligible, and comparable to RO filtration. This is in-line with a study conducted by Suárez et.al., (2009) who found minimal partitioning of divalent ions during nanofiltration of milk permeates.

Comparing the composition of the cheese whey permeates, there were some obvious, and expected differences, with higher levels of sodium and chloride in sCWP compared to the other permeates analysed, but interestingly there was a lower content of potassium in sCWP, compared to nsCWP, which was comparable to MP 2 (Table 6.1). NPN levels were similar across MP2, nsCWP, sCWP at ~0.37%, (w/w), and were higher than in MP1 at 0.25% (w/w). Citric acid was added to the concentrated permeates (MP 1, MP2 and nsCWP) as a processing aid during reverse osmosis and nanofiltration concentration to minimise calcium fouling (Metzger & Marella, 2016). Citric acid levels were highest in MP1 (3.76 g 100 g^{-1}), while the lowest levels were in sCWP (2.67 g 100 g^{-1}), which was not subjected to membrane concentration following whey UF separation.

Overall, of the permeates analysed, MP 1 had the lowest total ash content with 6.96 g 100 g^{-1} (dry matter basis) while sCWP had the highest overall ash content at 14.02 g 100 g^{-1}, as it had significantly higher levels of sodium and chloride (3.06 and 4.66 g 100 g^{-1}, respectively), due to the addition of salt in the final stages of Cheddar cheese manufacture, which carried through to the whey and the subsequent whey permeate stream. MP2 and nsCWP, which were both concentrated using reverse osmosis, had a similar mineral content and generally similar protein levels. These differences in mineral profile carried through to significant differences in the mineral profile of the skim concentrates, with concentrates standardised with sCWP containing over 2 fold more sodium than other concentrates (1.22 g 100 g^{-1} dry matter compared to 0.50 g 100g^{-1} dry matter or less for other concentrates). Meanwhile, concentrates standardised with MP1 had lower levels of monovalent minerals than concentrates standardised with MP 2, sCWP or nsCWP, and concentrates standardised with lactose had the lowest levels of all minerals analysed.

Table 6.1. Composition of permeate standardisation media on a dry matter basis. Different lower-case letters adjacent to the value indicate statistical difference (P<0.05) between standardisation media type. Results are the average of at least triplicate analysis.

	Lactose	MP 1	MP2	Salty CWP	Non- Salty CWP
			g 100 g^{-1} dry matter		
Ash	0.35 ± 0.0 ^{d}	6.96 ± 0.64 ^{c}	9.57 ± 0.9 ^{b}	14.02 ± 1.9 ^{a}	10.03 ± 0.6 ^{b}
Lactose	99.38 ± 01^{a}	89.59 ± 1.9 ^{b}	87.56 ± 1.0 ^{b}	83.31 ± 2.0 ^{c}	87.15 ± 0.7 ^{b}
Non-Protein Nitrogen	< 0.04 ± 0.0 ^{c}	0.25 ± 0.0 ^{b}	0.37 ± 0.0 ^{a}	0.37 ± 0.0 ^{a}	0.38 ± 0.0 ^{a}
Protein (Nx6.38)	0.28 ± 0.1^{b}	3.16 ± 0.9 ^{a}	2.5 ± 0.1 ^{a}	2.30 ± 0.1 ^{a}	2.57 ± 0.0 ^{a}
Chloride	0.04 ± 0.0 ^{c}	0.37 ± 0.2 ^{c}	1.83 ± 0.0 ^{b}	4.66 ± 1.5 ^{a}	2.7 ± 0.2 ^{b}
Calcium	0.05 ± 0.0 ^{b}	0.64 ± 0.21 ^{a}	0.61 ± 0.1 ^{a}	0.68 ± 0.1 ^{a}	0.6 ± 0.2 ^{a}
Magnesium	0.01 ± 0.0 ^{c}	0.14 ± 0.0 ^{ab}	0.13 ± 0.0 ^{b}	0.14 ± 0.0 ^{ab}	0.16 ± 0.0 ^{a}
Phosphorus	0.03 ± 0.0 ^{b}	0.74 ± 0.0 ^{a}	0.74 ± 0.1 ^{a}	0.75 ± 0.2 ^{a}	0.66 ± 0.1 ^{a}
Potassium	0.04 ± 0.0 ^{d}	1.58 ± 0.1 ^{c}	2.84 ± 0.1 ^{ab}	2.38 ± 0.5 ^{b}	3.28 ± 0.1 ^{a}
Sodium	0.02 ± 0.0 ^{d}	0.33 ± 0.0 ^{cd}	0.62 ± 0.0 ^{bc}	3.06 ± 0.4 ^{a}	0.76 ± 0.1 ^{b}
Citric acid	0.08 ± 0.0 ^{d}	3.76 ± 0.1 ^{a}	3.31 ± 0.1 ^{b}	2.67 ± 0.1 ^{c}	3.52 ± 0.1 ^{ab}

6.4.2. Ionic environment

The pH of all standardising media was below pH 6.2, with nsCWP having the lowest pH, of 5.7. It is expected the sCWP and nsCWP samples would have a lower pH due to the fermentation process during cheese manufacture, resulting in a decline in pH (Mc Sweeney, 2007). In comparison, permeate produced from MPC manufacture would be expected to have a pH similar to that of original skim milk (pH 6.6 - 6.7 at 25 °C, Chaplin & Lyster, 1988). However, as shown in Table 6.2, the pH of the MP samples was between pH 6.0 and 6.2, significantly lower than that of skim milk. As mentioned previously, the permeates from MPC manufacture had previously been concentrated by nanofiltration or reverse osmosis to increase the total solids level. To prevent calcium phosphate precipitation during permeate concentration, citric acid was added to reduce the pH and prevent mineral fouling. This approach has been previously shown to be effective by Kravtsov, et al. (2021). Similarly, sCWP and nsCWP permeates had a low pH of 6.0 and 5.7, respectively. However, this in part can be attributed to fermentation during cheese manufacture, which reduces the pH (Bylund, 1995). Given that sCWP did not undergo membrane concentration following UF separation and had a lower citric acid content than the other permeates (Table 6.1), it is likely that pH 6.0 was achieved purely from the cheese- making process. In contrast nsCWP had an even lower pH of 5.7 and also had elevated levels of citric acid, indicating, that the low pH may not only be as a result of the cheese-making process but also the addition of citric acid during membrane concentration to control calcium fouling (Table 6.1)

Conductivity was found to vary greatly between the standardisation media, with lactose having extremely low conductivity due to the negligible mineral content, and sCWP having the highest values likely due to the high sodium and chloride content (Table 6.1). Despite total calcium levels being similar across all permeates, apparent ionic calcium concentration varied; however, this may be influenced by conductivity, as sCWP had both the highest conductivity

and apparent ionic calcium concentration (4.5 mmol), both when analysed as permeate and when combined with SMP (Table 6.1).

It was observed that the native pH of each of the skim concentrates produced with permeate (MP 1, MP2, sCWP and nsCWP) was between pH 6.0 and 6.1, while the concentrate produced with lactose had a pH of 6.3 (Table 6.2). These concentrates with a pH of 6.1 or less, also showed higher apparent ionic calcium concentration when compared against concentrates prepared with lactose. As pH decreases, increasing amounts of CCP are released from casein micelles, which increases ionic calcium levels (Gonzalez-Jordan, Thomar, Nicolai, & Dittmer, 2015; Holt, 2004). This may have contributed to the higher observed apparent ionic calcium concentration of the concentrates prepared with permeate when compared with the concentrate standardised with lactose. Interestingly, pH adjustment to 6.5 with NaOH did not significantly alter the conductivity of the concentrates; however, the apparent ionic calcium concentration was significantly reduced for all concentrates indicating that, by controlling pH, ionic calcium levels can be reduced. Given that nsCWP permeate had a significantly lower pH than the other permeate samples (pH 5.7, compared to a pH between 6.0-6.2 for MP1, MP2 and sCWP), this sample was also assessed after its pH had been increased to pH 6.1, using NaOH, prior to its addition to the skim milk concentrate. This change in pH did not appear to significantly alter the conductivity of the permeate; however, it did result in a reduction in apparent ionic calcium concentration from 3.1 mmol at pH 5.7 to 2.6 mmol at pH 6.1, which also followed through to a slight but not statistically significant reduction in apparent ionic calcium concentration when combined with skim milk concentrate (Table 6.2).

Table 6.2. Measurements of the ionic environment of the standardisation media (15 % w/w solids) and low protein skim concentrates (35%, w/w solids) at natural pH and pH adjusted to pH 6.5. Lower case letters indicate statistical difference between sample type e.g. Lactose, MP 1, MP 2, sCWP, and nsCWP) while upper case letters indicate a statistical difference between the same sample type at either natural pH or pH 6.5. Results shown are the average of triplicate analysis.

	Standardisation media			Skim concentrate (natural pH)			Skim concentrate (pH 6.5)		
Sample type	pH	Conductivity (mS)	Apparent Ca Ion concentration (mmol)	pH	Conductivity (mS)	Apparent Ca Ion concentration (mmol)	pH	Conductivity (mS)	Apparent Ca Ion concentration (mmol)
Lactose	6.0 ±0.1^{bc}	0.4 ±0.0^{d}	0.6 ±0.2^{b}	6.3 ±0.0^{a}	5.8 ±0.2$^{d\ A}$	1.7 ±0.1$^{b\ A}$	6.5 ±0.0^{a}	5.8 ±0.1$^{d\ A}$	1.4 ±0.2$^{ab\ B}$
MP 1	6.0 ±0.1^{c}	5.5 ±0.1^{c}	3.3 ±1.5^{a}	6.1 ±0.1^{b}	7.5 ±0.3$^{c\ A}$	1.6 ±0.3$^{b\ A}$	6.5 ±0.0^{a}	7.9 ±1.4$^{cd\ A}$	1.0 ±0.3$^{b\ B}$
MP 2	6.2 ±0.0^{a}	11.3 ±0.3^{b}	2.8 ±0.6^{ab}	6.1 ±0.0^{b}	9.8 ±0.3$^{b\ A}$	1.7 ±0.3$^{b\ A}$	6.5 ±0.0^{a}	10.1 ±0.3$^{bc\ A}$	1.1 ±0.2$^{b\ B}$
sCWP	6.0 ±0.1^{ab}	19.4 ±3.0^{a}	4.5 ±1.5^{a}	6.1 ±0.2^{b}	13.9 ±2.0$^{a\ A}$	2.5 ±0.6$^{a\ A}$	6.5 ±0.0^{a}	14 ±2.1$^{a\ A}$	1.6 ±0.3$^{a\ B}$
nsCWP	5.7 ±0.0^{d}	14.6 ±0.0^{b}	3.1 ±0.0^{ab}	6.0 ±0.0^{b}	11.7 ±0.3$^{ab\ A}$	2.0 ±0.1$^{ab\ A}$	6.5 ±0.0^{a}	11.6 ±0.6$^{ab\ A}$	1.1 ±0.1$^{ab\ B}$
nsCWP (pH 6.1)*	6.1 ±0.0^{abc}	14.7 ±0.1^{b}	2.6 ±0.2^{ab}	6.1 ±0.0^{ab}	11.7 ±0.3$^{ab\ A}$	1.83 ±0.1$^{ab\ A}$	6.5 ±0.0^{a}	11.5 ±0.1$^{ab\ A}$	1.1 ±0.0$^{ab\ B}$

*nsCWP was also examined following pH adjustment of the permeate to pH 6.1 with 1M NaOH to align with the pH of the other samples.

6.4.3. *Heat treatment - Effect of standardisation media and pH on viscosity and particle size*

Viscosity profiles of protein standardised skim milk concentrates measured at both native pH and after pH was adjusted to 6.5 are shown in Fig. 6.1A and B. At native pH, all viscosity profiles displayed a decrease in viscosity with increasing temperature from 25 up to 95°C. Skim milk concentrates with added lactose had significantly lower viscosity compared to all other skim milk concentrates. Skim standardised with permeate began to show a sharp increase in viscosity during holding at 95 °C, indicating the onset of significant protein aggregation. MP2 had the earliest onset of viscosity increase, with a sudden increase observed after 90 sec at 95 °C (Fig. 6.1A). Once viscosity started to increase, skim standardised with MP2 underwent extensive gelation, probably due to the higher levels of monovalent ions present compared to MP1 (Table 6.1), as explained earlier in Section 6.4.1, due to differences in the filtration processes. The addition of nsCWP (pH 5.7, Table 6.1) to skim milk concentrate resulted in a pH of 6.0 and resulted in a sharp increase in viscosity during HTST treatment, with extensive gelation occurring during cooling (Fig. 6.1A). Interestingly, the same sample with the permeate pH adjusted to 6.1 prior to addition to skim milk was significantly more heat-stable and had a lower viscosity than the concentrates prepared with MP 1 or MP 2, despite having a similar concentrate pH before heat treatment. In addition, while significant differences were observed in the pH and apparent ionic calcium concentration between each of the standardisation media, these differences were less obvious when the permeates were combined with skim milk, indicating some re-equilibration of the ionic environment upon reformulation, which impacted the subsequent thermal stability.

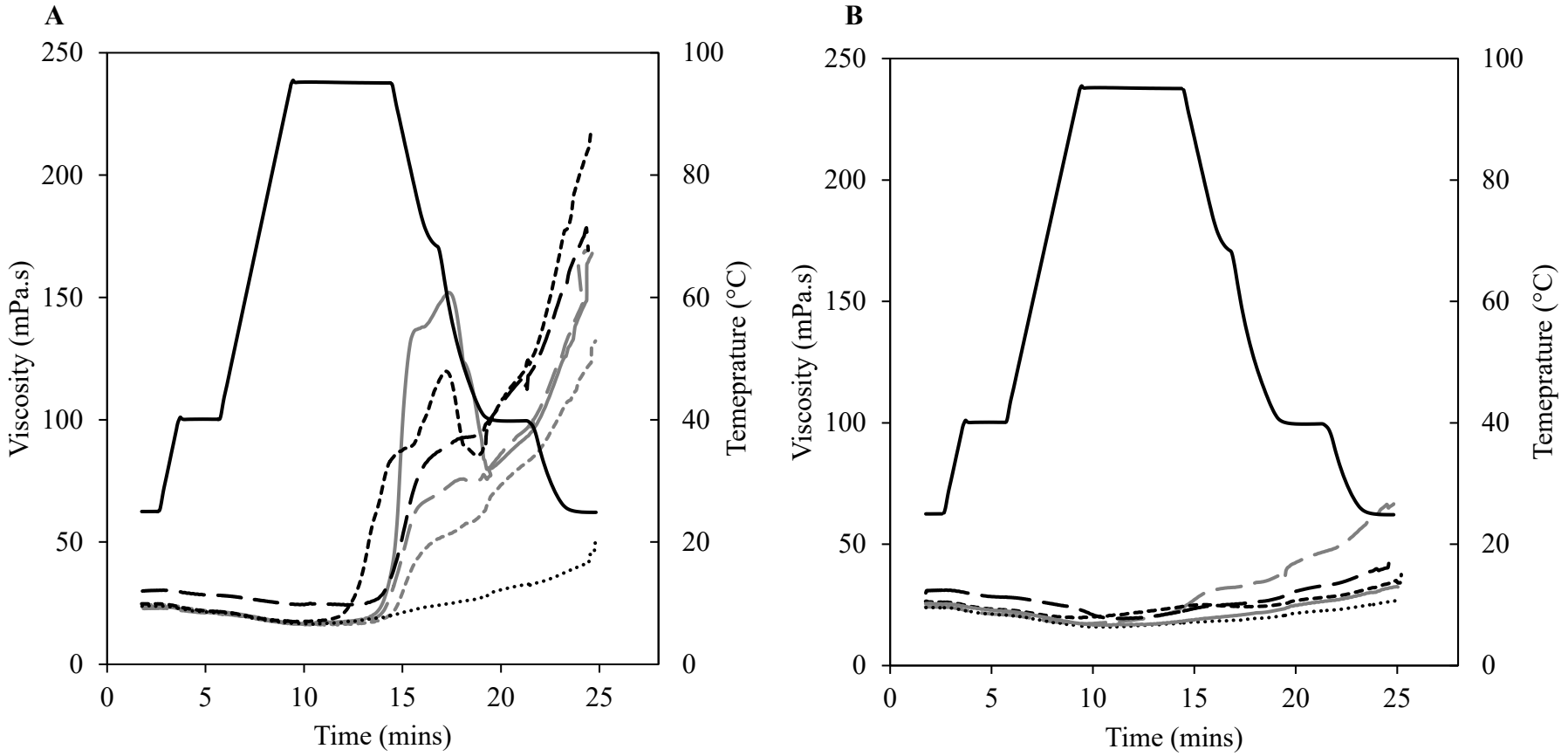

Fig. 6.1. Viscosity during HTST treatment (temperature (——)) of low protein skim concentrates standardised with lactose (.....), MP 1 (— —), MP2 (- - - -), sCWP (— — —), nsCWP (——) or nsCWP pH adjusted to 6.1 (- - - -) at either (A) native Ph, or (B) pH adjusted to pH 6.5 with NaOH. Results shown are the average of triplicate analysis.

Adjusting the pH of all samples to pH 6.5, prior to HTST treatment, resulted in significantly lower viscosity profiles for all samples (Fig. 6.1B), compared to their corresponding low pH samples shown in Fig. 6.1A. The viscosity of skim milk standardised with lactose was almost the same before and after heat treatment (~24 - 27 mPa.s; Fig. 6.1B), indicating no major thermal instability, even after heat treatment at 90 °C. Skim milk with added sCWP was the only sample that showed a significant increase in viscosity after HTST treatment (31 - 66 mPa.s; Fig. 6.1B).

The influence of pH on skim milk concentrate stability is highlighted in the particle size data shown in Fig. 6.2, with concentrates heat treated at native pH having large volumes of particles >1.0 μm , with the exception of skim milk with added lactose, which had a monomodal size distribution after heat treatment at both native (Fig. 6.2A) and adjusted pH values (Fig. 6.2B). As displayed in the viscosity profiles, adjusting the pH of concentrates to 6.5 prior to heat treatment resulted in less aggregation, as shown by the lower volume of particles in the 1.0 - 1000 μm range; although some aggregation was still visible (Fig. 6.2B). Casein micelles become increasingly unstable as pH declines, due to the increased dissociation of colloidal calcium phosphate (CCP) with decreasing pH, which not only weakens the structural integrity of casein, but also results in increased levels of ionic calcium in solution which will promote increased calcium-mediated whey protein aggregation during heat treatment (Holt, 2004; Sinaga, Bansal, & Bhandari, 2017). By pH-adjusting the concentrates to pH 6.5, the negative charge in the solution was increased due to increased OH- ions, which increased the charge repulsion between casein micelles, prevented the dissociation of CCP and therefore likely aided stability during thermal processing (Sinaga et al., 2017). In comparison to native pH, a significant drop in apparent ionic calcium concentration was observed across all concentrates after adjusting to pH 6.5, this is likely due to the inhibition of CCP dissociation from casein and also the increased conversion of serum phase ionic calcium to calcium phosphate at

elevated pH values, which would have further reduced the frequency of calcium mediated whey protein aggregation during subsequent HTST treatment and so increased the thermal stability of the concentrates (Holt, 2004).

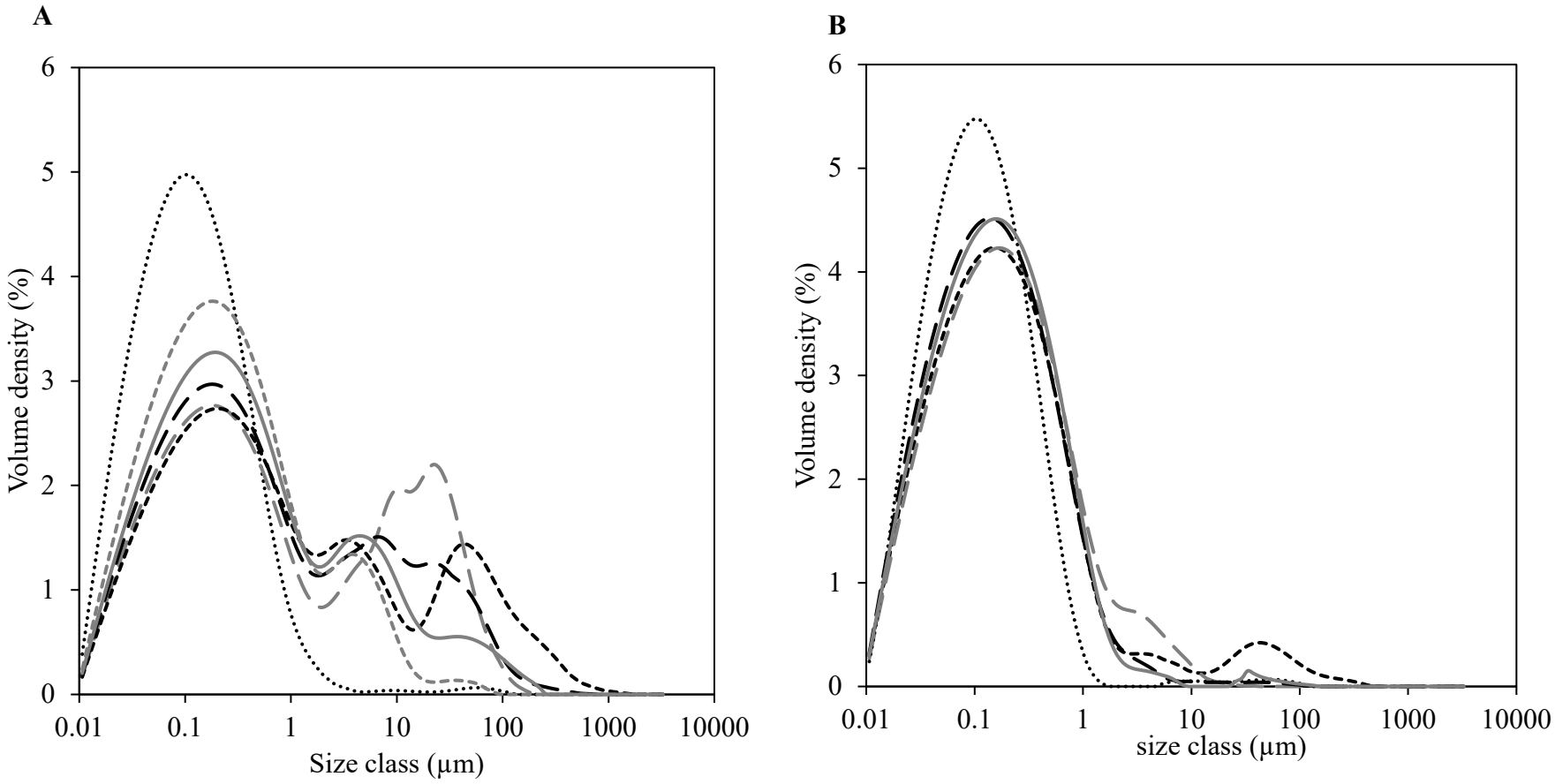

Fig 6.2. Particle size distribution of low protein skim concentrates of low protein skim concentrates standardised with lactose (.....), MP 1 (— —), MP2 (- - - -), sCWP (— — —), nsCWP (——) or nsCWP pH adjusted to 6.1 (- - - -) after HTST treatment at either (A) native pH (B) or skim concentrate pH adjusted to pH 6.5.

6.4.4. Change in ionic environment following HTST treatment

The changes in pH, conductivity, and apparent ionic calcium concentration across all concentrates both at native pH and pH adjusted to pH 6.5 as a result of HTST treatment was examined to determine if changes in the ionic environment could explain observed differences in viscosity during HTST treatment. However, no significant difference was found in conductivity and, due to the high viscosity of the skim concentrates, especially at native pH following heat treatment, it was not possible to accurately measure apparent ionic calcium concentration after heat treatment. Therefore, only changes in pH as a result of HTST treatment are shown (Fig. 6.3).

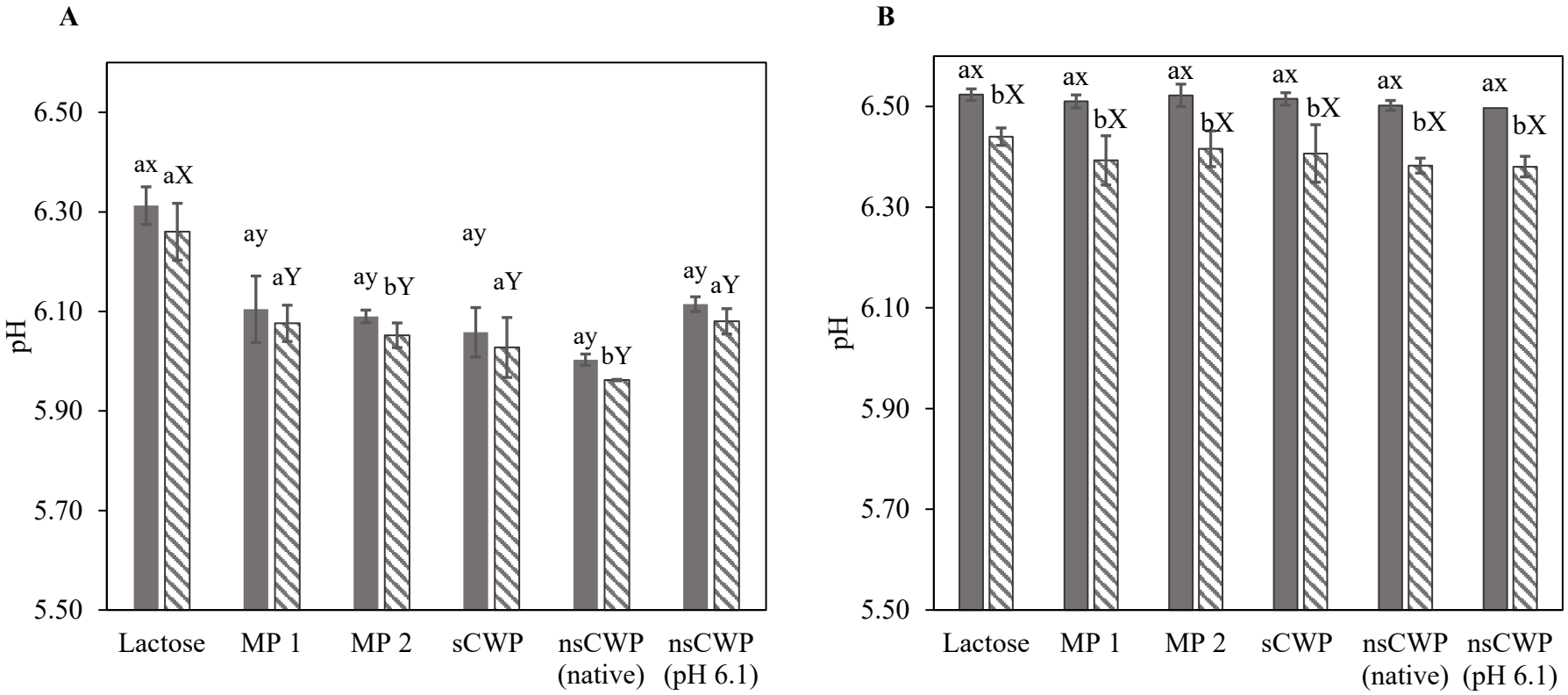

Fig. 6.3. pH of low protein skim concentrates at (A) natural pH and (B) pH adjusted to pH 6.5, before (■) and after heat treatment (▨) to 95 °C for 5 min. Statistical difference ($P<0.05$) is shows using different letters. Statistical difference between the same sample before and after heat treatment is shown by different a, b letters. Statistical difference between samples is shown by lower case x, y letters while statistical difference between samples after heat treatment is shown by uppercase X, Y, letters.

At natural pH, there was no significant decline in pH after heat treatment, indicating that heat treatment did not cause any major increase in free H+ ion activity, such as that associated with calcium phosphate precipitation. However, as mentioned previously, the pH in the non-adjusted pH concentrates was likely low enough to counteract extensive calcium phosphate formation. Concomitantly, the high ionic calcium level may have played a key role in the high viscosity and eventual gelation during HTST treatment.

Concentrates which were pH-adjusted to pH 6.5 prior to heat treatment, displayed a slight decrease in pH after HTST; however, the pH remained above 6.3 for all concentrates. This decline in pH does, however, indicate that reactions took place during heat treatment which caused some release of H^+ ions. This may have been due to a number of factors, one of which may have been the precipitation of calcium phosphate, which is known to precipitate out of solution at elevated pH values with increasing temperature, resulting in the release of H^+ ions (Holt, 2004; Mc Entee, et al., 2023). While this reaction results in a decline in pH, it also lowers the amount of ionic calcium available to react with whey protein during heat treatment. Therefore, provided the pH remains above the point where significant CCP is released from casein, the precipitation of calcium phosphate at elevated pH and temperature could be expected to have a stabilising effect on the concentrates, and this correlates with the observed lower viscosity following HTST of concentrates, which had been pH-adjusted to 6.5 prior to heat treatment.

6.5. Conclusions

Numerous low-value side streams are produced from cheese manufacture or milk protein production, with most finding value as protein standardisation media in skim milk and fat-filled milk powders. There were some obvious differences between the use of milk and cheese whey permeates or lactose, but essentially standardisation media with low pH (i.e., <

6.2) and high in minerals, caused significant viscosity and gelation issues when combined with skim milk concentrate during heating. Interestingly, the method of permeate concentration also had an effect on standardised skim milk concentrate viscosity, with concentration by reverse osmosis retaining more monovalent ions, compared to nanofiltration, and therefore causing lower heat stability in skim milk concentrates. However, by adjusting the pH of the standardisation media or adjusting after addition to skim milk heat-induced gelation at 90°C could be prevented.

While this study shows that, with correct pH control, salty whey permeate is feasible for use as a protein standardisation media, the high sodium content may be an issue from a nutritional point of view with many consumer products targeting a reduction in sodium due to health concerns. Also, the high salt content may result in an undesired salty flavour of the final product and so, while from a functional perspective, it is a viable option, in reality demineralisation may need to be considered before using this permeate stream for protein standardisation.

6.6. Acknowledgment

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Chapter 7

General discussion and suggestions for future research

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Declaration

This chapter was written by Sinead Mc Entee (SME) and reviewed by all co-authors.

7.1. General Discussion

The dairy ingredients industry is large and varied, providing an array of products intended for further use in a wide range of applications. In particular, the composition of protein ingredients can be highly variable depending on their origin e.g., whey protein from cheese production compared to whey protein from acid casein manufacture. Mineral content is one of the ways in which composition varies and can lead to particular challenges in terms of formulation and processing. Advances in dairy protein purification such as membrane filtration have led to the development of a variety of lower mineral, purified protein ingredients, which in theory could be used as a protein source for nutritional formulae. However, significant variations in mineral content still exist in these streams, which may result in very different processability and functionality in finished products. In addition, the increased demand for highly purified protein ingredients has led to significant volumes of de-proteinated permeates which, while they contain nutritionally valuable minerals and carbohydrates, are often considered a waste stream, and pose a challenge to ingredient manufacturers to extract value from. This PhD which was completed as part of a dairy-ingredient industry-based fellowship project, aimed to investigate the impact of dairy protein ingredient composition on functionality in nutritional formulae and also to examine the impact of incorporation of de-proteinated permeates from different processes on the functionality of standardised low protein skim milk concentrates.

Infant formula was chosen as the model system for assessing protein ingredient functionality as due to the complex nature of the formulation, a predominantly whey protein profile and an appreciably high mineral to protein ratio, it can be considered quite challenging to process into a shelf-stable powder or liquid formulation (Thompkinson and Kharb, 2007). Significant research has been conducted into not only isolating dairy protein fractions to yield an IF with a protein profile which more closely aligns with that of human milk, but also, into

understanding the reactions that take place between protein, lactose, and minerals during processing (Tomita et al., 2002; Crowley, 2016; Barone et al., 2020, **Chapter 1**). However, despite these advances, controlling the pH and ionic environment during IF processing remains a significant challenge and often plays a key part in determining which protein source is used (Zadow, 1971; Fuquay, Fox and McSweeney, 2011, **Chapter 1**).

Chapter 2 showcases the impact of protein ingredient mineral composition on heat stability in IF systems. In this study, a selection of available casein (MPC and SMP) and whey protein ingredients (cheese WPC 35, cheese WPI and acid WPC 35) with both high and low mineral contents were combined to IF protein ratios (1.5% w/w protein, 40:60 casein: whey ratio) and subjected to HTST and UHT treatment as protein blends and also with lactose added to IF ratios (**Chapter 2**). The careful balancing of pH and mineral addition through the use of protein ingredients with a narrow variance in mineral content, addition of chelators and inclusion of reactive minerals such as calcium in relatively inert formats, i.e., tri-calcium phosphate, has allowed for the successful manufacture of shelf-stable IF products (**Chapter 1**). However, as it is a fine balance, manufacturers can be hesitant to change protein sources and minerals due to the risk of a negative impact on thermal stability. In addition, infant formula typically contains five times more lactose than protein which, under high temperature processing, may cause Maillard reactions that can result in a reduction in pH, further exacerbating negative interactions between protein and other formulation components (**Chapter 1,** Ajandouz and Puigserver, 1999; Codex Alimentarius, 2007). **Chapter 2** clearly shows some of the challenges associated with modifying the protein source in infant formulations and highlights why manufacturers may be hesitant to change protein source despite the potential nutritional and or economic benefits.

While it is generally considered that a high mineral content negatively affects protein stability during thermal processing, **Chapter 2** showed that the interrelationship of protein and

mineral content in determining thermal stability of formulations is more complex. With regard to whey proteins, the use of low mineral WPI did result in a more heat stable formulation particularly when combined with low mineral MPC. However, combining MPC with cheese or acid WPC 35, resulted in faster coagulation than when these whey proteins were combined with higher mineral SMP. This is likely due to differences in the mineral profile of these blends with SMP containing serum phase calcium chelators, such as phosphate and citrate, which could help to delay calcium mediated whey protein aggregation at elevated temperatures. This suggests that while MPC has a lower mineral content, the loss of these serum phase minerals negatively impacts its suitability for whey dominant low protein formulations such as IF and therefore is a much less feasible casein source than SMP for IF. This highlights the benefits of screening protein ingredients prior to incorporation into IF products and also shows why manufacturers can be hesitant to modify formulations through the inclusion of certain protein ingredients, despite their potential nutritional benefits.

These challenges with ionic environment have limited the use of acid whey in infant formula, despite it containing up to 30 % more of the essential amino acid tryptophan per gram of protein than cheese whey due to a lack of glyco-macro-peptide (GMP), therefore, potentially allowing for the reduction in total protein of an IF to more closely align with human milk (**Chapter 2,** Chandrapala et al., 2015; Dutta et al., 2017). However, acid whey protein ingredients tend to have a higher calcium content when compared to cheese whey protein ingredients, which if not correctly controlled via chelation or pH adjustment can result in poorer thermal stability and increased whey protein aggregation during IF processing (Holt, 2004; De Kort *et al.*, 2012). However, in **Chapter 2**, it was noted that, while blends of aWPC 35 and SMP were less heat stable than those containing cheese WPC 35, these blends were relatively heat-stable at both UHT and HTST temperatures at pH 7 indicating that, despite the high calcium content of aWPC 35, there is potential for increased usage of acid whey in IF

applications provided the ionic environment is controlled, which could support a reduction in total protein of the formulation through replacement of cheese whey protein, as discussed in **Chapter 1** and **Chapter 2**.

While the nutritional advantages of acid whey protein over cheese whey protein for IF applications are clear (i.e., >30% tryptophan per gram of total protein), the poorer thermal stability due to a higher innate calcium content remains a roadblock to its incorporation into IF applications. However, as acid whey protein is typically concentrated by ultrafiltration, the calcium and phosphorous content could be controlled during the protein concentration step by manipulating the ionic calcium balance through adjusting pH and temperature at this point in the process (**Chapters 3 and 4)**. By adjusting pH and temperature a greater understanding of the impact of the ionic state of acid whey on membrane performance and calcium removal during ultrafiltration was obtained with a rapid decline in permeation efficiency and calcium removal observed with increasing pH. While this study intentionally altered the calcium balance to promote the formation of calcium phosphate by adjusting temperature and pH, these observations are of particular relevance to ensuring consistency of the mineral content and profile of acid WPC 35 ingredients. Given acid WPC 35 is a by-product of other processes (acid casein manufacture) the calcium concentration and pH could fluctuate due to processing changes upstream which if not identified prior to ultrafiltration could negatively impact membrane performance and also affect the functionality of the final acid WPC 35 ingredient in application.

The impact of calcium content on evaporation, drying and the subsequent powder properties of acid WPC 35 was further investigated in **Chapter 4** by producing standard calcium and high calcium acid WPC 35 through pH adjustment prior to ultrafiltration. In this study it could clearly be seen that a high calcium content can have a negative impact on downstream processing and in high temperature applications such as IF, with a higher viscosity

observed following evaporation and significant gelling occurring when the final powder was subjected to HTST treatment.

To really understand the impact of calcium content on IF processing when using an acid WPC 35 and to advance the learnings from **Chapter 2**, these aWPC 35 powders with different calcium contents were then examined in concentrated model infant formulations without any added fat (35.5%, w/w, solids) with all major minerals formulated to target and compared against formulations prepared with cheese WPC 35 and WPI (**Chapter 5**). In addition, acid WPC 60 was manufactured by applying ultrafiltration and diafiltration to rehydrated aWPC 35 to produce a low mineral acid whey protein ingredient for comparison in the model formulation. As with the findings from **Chapter 1 and 2**, this study also highlights the benefits of using low mineral protein sources to facilitate the addition of minerals *via* inert or chelating forms to enhance formulation thermal stability. Formulations prepared with high protein whey ingredients (cheese WPI and acid WPC 60) were more heat stable and less viscous following HTST treatment than low protein ingredients when at a standardised protein and mineral content. The increase in viscosity following heat treatment was most severe in formulations containing the high-calcium acid WPC 35 produced in **Chapter 4,** where all the calcium and phosphorus present originated from the protein sources. This emphasises the importance of understanding the ionic state of minerals present in a formulation and their impact on protein stability during high temperature processing of concentrated solutions. In addition, it suggests that by further demineralising acid whey protein to a WPC 60, much of the challenges regarding heat induced protein aggregation can be eliminated therefore potentially enabling greater incorporation of acid whey into IF applications and perhaps a reduction in the overall protein content of IF.

While in **Chapters 2 and 5**, it was shown that highly purified protein ingredients are more stable in IF applications, as mentioned previously, this purification produces significant

volumes of high-mineral permeate which, due to its low value, can be considered a waste stream (Risner et al., 2020). One application for permeates is in the protein standardisation of skim milk for fat filled milk powder; however, as with IF applications, variations in the mineral profile of the protein standardisation media can impact thermal stability of standardised skim milk. In **Chapter 6,** the ionic environment and thermal stability of low protein skim concentrates standardised with either low mineral lactose or permeates with different mineral profiles depending on the source (MPC, cheese whey, salty cheese whey) and mechanism of permeate concentration (reverse osmosis or nanofiltration) was examined. As pH has been shown to play a significant role in thermal stability (**Chapter 1 and Chapter 2**), the concentrates were assessed at their native pH (pH 6.0 – 6.3) and after adjustment to pH 6.5.

Similar to the observations in **Chapter 2** and **5**, skim concentrates standardised with lactose which had a lower innate mineral content tended to have a lower viscosity following simulated HTST treatment compared to concentrates standardised with permeate which suffered significant gelling following heat treatment at native pH regardless of permeate type. Interestingly, when the pH of these concentrates was increased to pH 6.5, all concentrates prepared with permeate displayed a significant reduction in concentrate viscosity following HTST treatment, which was more in line with concentrates standardised with lactose. This is attributed to a reduction in apparent ionic calcium concentration following pH adjustment, which likely reduced calcium mediated protein aggregation reactions during heat treatment. In addition, increasing the pH would increase the net negative charge of the concentrate resulting in increased charge repulsion between caseins aiding protein stability during thermal processing (Sinaga, Bansal and Bhandari, 2017). Therefore, this study shows that, by controlling pH, high mineral permeate from a range of sources can produce skim concentrates which are stable to HTST treatment.

Overall, this thesis provides important insights into the role of minerals and their ionic state on the processing of dairy based nutritional formulae such as infant formula and low-protein skim concentrates. In addition, it has been shown that demineralisation of proteins, in particular acid whey protein, can result in significant improvements in thermal stability, allowing for greater application of these proteins in high heat nutritional formulae, such as infant formula. Ideally, the next steps for this research would be to promote and communicate these findings to within the dairy ingredient processing industry and also to the wider scientific community to support further advancements in process optimisation of advanced dairy based nutritional formula and the ingredients used therein.

7.2. Suggestions for future research

Some suggestions for additional research that would advance the work presented in this thesis are as follows:

- Examine the impact of protein source on formulation stability following fat addition to understand the effect on emulsion stability in nutritionally complete IF systems (Chapter 2, Chapter 5).
- Examine formulations produced with MPC where all minerals are balanced to determine whether the addition of chelators to balance the final mineral content results in a formulation with improved thermal stability compared to formulations with SMP. While it was shown that SMP is more suitable for IF, due to the demineralisation of MPC, it is less prone to seasonal variation in mineral content and therefore, if correctly stabilised, it could be added at times in the season where the composition of skim milk is fluctuating (Chapter 2, Chapter 5).
- Further investigate opportunities to increase the incorporation of acid whey protein ingredients in IF applications through further demineralisation and also examine its

functionality and stability when combined into a nutritionally complete IF system where the required essential amino acid content could be achieved at a lower total protein content than formulations made using cheese whey protein (Chapter 3 and 4)

- Examine opportunities to valorise / increase the use of dairy permeates which are a natural source of nutritionally valuable minerals and lactose in food applications (Chapter 6).

7.3. References

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Appendix

Published Literature