Goat and cow casein derived ingredients and their interactions with iron

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Food Technology

Massey University, Palmerston North, New Zealand

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2017

Abstract

The objective of this study was to gain a fundamental understanding of how goat casein micelles and the products of casein proteins behave when fortified with iron.

Iron fortified skim milk was characterised by analysing the mass balance of micellar/non micellar fractions, chemical changes, micellar size changes and internal structure. Two treatments were examined to determine where in the processing line the addition of iron might best be added to a milk system. On average, at least 72% of the iron is bound to the micellar phase across the treatments and iron concentrations. Small angle X-ray scattering (SAXS) indicated that internal changes, mainly at the location of the colloidal calcium phosphate, occurred with iron addition.

Casein was extracted from goat milk using isoelectric precipitation however the extraction was more difficult than using cow milk. Iron fortification of the caseinates resulted in a tendency for oxidation and precipitation of the proteins to occur causing the formation of large aggregates. The caseinates could not stabilise the same amounts of iron to that of an intact casein micelle.

Phosphopeptides were isolated by adding calcium and ethanol to caseinate digests. There was an increase in serine, glutamic acid and isoleucine residues compared to caseinate. There was an increase in phosphorus from 7.8 ± 0.3 mg P/ g solids to 45.4 ± 2.4 mg P/ g solids in the isolate. The phosphopeptides were composed of smaller, more hydrophilic peptides compared to the full digest prior to precipitation. Ferrous sulfate was then investigated for use as the precipitant, instead of calcium. The peptides produced similar trends in terms of amino acid profile changes, phosphorus concentration increase and yield. Immobilised metal affinity chromatography was also investigated however this had a low throughput that may not be effective at process scale.

The effect of heating, cooling, ionic strength of the solution, holding time, iron loading, processing order and use in a model milk system were investigated to simulate potential industrial processing conditions using the calcium - extracted phosphopeptides. It was found that goat peptide isolates were able to bind 54.4 ± 0.5 mg Fe/ g protein compared to goat milk of 4.3 ± 0.1 mg Fe/ g protein. The optimal conditions for binding were found to be at pH 6.7 in a low ionic strength solution,

around 37 ºC. There was a potential synergistic effect of adding the peptides to milk in terms of iron binding capacity. There were few differences in the amount of iron that could be bound comparing cow and goat derived phosphopeptides under the tested conditions.

The oxidation potential of ingredients was determined using malondialdehyde (MDA) as an oxidation product marker. There was a reduction in oxidation when iron was bound to milk or peptides compared to free ferrous sulfate in solution with intact goat milk performing the best producing 0.46 ± 0.04 μg MDA/mL after 3 days at 30 ºC compared to the blank of 1.25 ± 0.16 μg MDA/mL. The goat peptides produced non-significantly different levels of MDA compared to the blank containing no ferrous sulfate.

Caco-2 cell lines are a way of approximating how systems may function in an intestine in terms of nutrient absorption. Iron absorption was improved in the order of casein hydrolysates > caseinate > skim milk for goat milk. In contrast, cow milk appeared to perform better without any modifications to the proteins. On an equal iron filtrate basis after the digestion and intestinal phase, calcium- precipitated goat phosphopeptides produced a response of 9.64 ± 0.94 ng ferritin/ nM iron. This response was greater than all other treatments with the exception of goat milk fortified with 5 mM iron and ascorbic acid with 12.30 ± 1.23 ng ferritin/ nM iron.

This work covers a wide range of milk products and iron interactions and has helped to build a fundamental understanding of goat milk protein functionality. The underpinning considerations to a manufacturing setting may allow further development of large scale ingredient production for the improved stability of iron fortified systems.

Acknowledgments

I would firstly like to thank my supervisor Dr. Alistair Carr for allowing me to pursue a PhD. His discussions and ideas have made for a very interesting and exciting project. This sense of humour made the long stints in the Synchrotron labs and during the whole project a lot more colourful. Thank you for carefully reading my thesis and asking valid questions which have enriched the discussion.

To my co-supervisor Dr. Lara Matia-Merino, thank you for your hard work and support during my studies. She has provided focus to the project and kept me motivated when I was struggling. Her directness helped move work along which I am grateful for while her advice and personal support showed her true caring nature.

Dr. Bridget Ingham: my co-supervisor who had her work cut out for her. I am thankful that I was able to incorporate techniques like SAXS in my work as it allowed me to experience a whole different world in science, not to mention travelling to the Synchrotrons. It has to be mentioned that she has great patience in explaining the theory, equations and models on numerous occasions and assessing my fitted models many times over.

I would like to acknowledge Dairy Goat Co-operative, specifically Dr. Colin Prosser for giving me the opportunity to undertake a PhD by providing the funding for the whole project.

Thanks to Bob (Dr. Robin Stewart) for firstly performing the Caco-2 cell assays for the part of the project and also showing me how the method works (and making me thankful that none of my work was as temperamental and demanding). Also, for being a great office buddy and sharing a few good yarns.

Thank you to the Laboratory Technicians in the School: Steve Glasgow, Micelle Tamehana, Janiene Gilliland, Warwick Johnson, Garry Radford and John Edwards (SEAT) for help using equipment, making orders and running methods (as well as some amusing chats). Trevor Loo from the Fundamental Sciences department taught me how to carry out digestions and gave some good pointers in this area so I must acknowledge how much this helped me. Along the same track, thanks to Don Otter for helping me with mass spectrometry, if anything, I have learnt how to read the

output even if results didn't eventuate. Finally, thank you to Maggie Zou who helped me run the HPLC and showing me the ins and outs of this technique.

Thank you to Prof. Dick FitzGerald for the useful discussions regarding IMAC; this helped me immensely to achieve results for this section of work.

Thank you to the Australian Synchrotron and the Lawrence Berkeley Advanced Light Source, specifically Nigel Kirby and Cheng Wang for helping our team in running the equipment and input in analysis.

Thank you to Circo Acrofit Studios and the people there that make it so much fun. My time in Palmerston North has been made more fulfilling when I knew class was coming up. This place was great for exercise and humour and I have made some awesome friends here.

Thank you to Kate Donohue who initially convinced me to carry out a postgraduate degree and being an excellent support through the shared experience of doing a PhD. Also, for turning me to the 'R side' of statistics, making me pretty confident in plot coding but also giving insight into statistical analysis. Our time together as flatmates was super fun as evidenced by us being inseparable during weekend shenanigans.

I need to also thank my parents for motivating and encouraging me (along with financial support) during this time. Thanks for being proud of me and being okay with me delaying becoming a 'real adult' and stretching out student life for as long as possible.

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1 Introduction

Iron deficiency is a worldwide issue with an estimated 2 billion people suffering from an insufficient iron status; in New Zealand over a third of teenage girls and 80 % of toddlers do not meet intake requirements. Iron deficiency has increased from the years 1997 to 2008/2009 (MacPhail, 2007; Ministry of Health, 2007; University of Otago & Ministry of Health, 2011). This has many repercussions from decreased energy levels and anaemia to premature death. In children low iron can affect neurological development, immunity and growth. Due to the importance of iron in general wellbeing, further investigations into the improvement of iron delivery systems is required for those at risk of iron deficiency (MacPhail, 2007; Ministry of Health, 2007). Improvements in the methods of iron fortification into milk based products is important. Currently iron fortification levels in food are high as non-heme iron bioavailability is low, for example infant formula can contain 6-12 mg Fe/L (Moy, 2000) while iron in human milk contains 0.2 to 0.8 mg/L due to the much higher bioavailability (Yeung et al., 2002). Iron fortification is difficult; it can lead to problems in organoleptic quality, increased rancidity of fats, solubility problems and degradation of vitamins (Mehansho, 2006).

Goat milk is becoming an increasingly popular source of dairy (Carlson et al., 2011). Goat milk has different levels of whey proteins, composition of fat containing medium chain fatty acids, ascorbic acid and vitamin A concentration which may facilitate the absorption of iron more effectively than cow milk (López-Aliaga et al., 2009). Many rat model studies have shown that iron fortified goat's milk allows a better delivery system than cow's milk due to these various differences in composition (López-Aliaga et al., 2000; Barrionuevo et al., 2002; Alférez et al., 2006; Nestares et al., 2008).

Casein phosphopeptides are peptides produced by proteolytic breakdown of casein; these contain electronegative polar acidic sequences of residues- phosphoseryl or phosphothreonine that have a high affinity to cations (Emery, 1992). In caseins the phosphoserine clusters are made up of three phosphoserine residues followed by two glutamic acid residues (Reynolds et al., 1994; Bouhallab & Bougle, 2004; Miquel et al., 2006b). Studies have shown that these regions have the ability to bind calcium via ionic bonding and iron via co-ordination bonds (Crichton, 2012) allowing transport through the digestive tract (Yeung et al., 2002). The addition of iron to phosphopeptides has been studied in their ability to bind iron and allow the iron to remain soluble during digestion;

rat models and Caco-2 cells have often been employed and have shown an improvement in absorption (Pérès et al., 1999; Ait-Oukhatar et al., 2002; Bouhallab et al., 2002; Yeung, Glahn & Miller, 2002; Bouhallab & Bougle, 2004; Ani-Kibangou et al., 2005; Kibangou et al., 2005; Miquel et al., 2006a; García-Nebot, Barberá & Alegeía, 2013). The short peptides allow a different pathway of iron delivery than an iron salt fortified sample (Bouhallab & Bougle, 2004) as it has been thought that the peptides can deliver the iron to the membrane more efficiently by keeping the iron soluble through digestion (Pérès et al., 1999; Bouhallab et al., 2002; Yeung, Glahn & Miller, 2002; Ani-Kibangou et al., 2005; Kibangou et al., 2005) or causing an endocytosis mechanism to occur at the gut wall (Bouhallab & Bougle, 2004).

While there has been extensive research on cow casein phosphopeptides; limited work on goat derived peptides has been found. Therefore work carried out herein aims to provide a direct comparison of goat peptides to cow, employing methodology from cow milk based research and creating modifications where necessary based on the known chemistry of goat milk. Due to the limitations of information for goat milk and casein products fundamental analysis was also performed on skim milk and caseinates to assess the differences in characteristics which will help underpin the analysis of the phosphopeptide isolates.

The aims of the thesis, along with the fundamental characterisation of New Zealand produced goat milk casein (produced by Dairy Goat Co-operative, Hamilton), is to characterise goat peptide isolates and compare to cow isolates produced under the same conditions using two techniques commonly used for extraction. The focus was placed on methodologies that could be feasible for use under industrial scale food manufacturing conditions however due to the limitations of literature the thesis focused on the fundamental understanding of the casein phosphopeptides. Understanding the iron fortification of the goat caseinate derived peptides was the core aim of the project and this was assessed by looking at the mechanism of binding as well as binding studies under predicted simulated manufacturing conditions.

Several techniques were used to probe a variety of aspects required for the potential development of an end product. The project is outlined in a way that follows the isolation of the peptides from the starting cow or goat skim milk. Initially, cow and goat skim milk with addition of 0, 5, 10, 15 and 20 mM ferrous sulfate as well as the effect of spray

drying iron fortified milk was examined. While iron fortified cow's milk has been studied in various ways, goat milk has been less frequently therefore the effect of iron fortification on the physicochemical properties was assessed by mass balance and small angle X-ray scattering (SAXS). This technique allowed the internal structure of the casein micelle to be thoroughly investigated by looking at the changes to the structure with the addition of iron.

The different composition of goat caseinate affects isolation of the caseinate; the effect of calcium and iron fortification of the caseinates was then investigated. The stability and changes in particle size of the aggregates was investigated in chapter five to determine how the removal of soluble milk salts, whey proteins and colloidal calcium phosphate affects caseinate functionality.

Chapter six looks at the effects of caseinate digestion with optimisation of the use of trypsin and analytical comparisons of the resultant digests by using techniques such as reversed phase-high performance liquid chromatography (RP-HPLC), degree of hydrolysis and iron binding. Isolates from the digests were prepared with calcium or iron precipitation, as well as immobilised metal affinity chromatography (IMAC) separation. Calcium and ethanol precipitation methodologies were adapted from Reynolds et al., (1994); Adamson & Reynolds, (1995); Adamson & Reynolds, (1996); Cross et al., (2005); Strader et al., (2006) and the resultant peptides were compared using phosphorus assay, amino acid profiling, RP-HPLC and mass balance. An adaptation to the method was carried out using iron for the precipitation and the products were assessed similarly.

Various clean up techniques of the peptides and analysis of the binding ability of the peptides under simulated manufacturing conditions were then performed in chapter seven. The peptide isolate was then tested in stability trials by fortification of iron under manufacturing conditions to measure iron binding. The isolates were also tested for oxidative stability using the TBA reagent in chapter eight. Assessment of the potential bioavailability using Caco-2 cells to elucidate the usefulness as an ingredient was also investigated and compared to and compared to milk and caseinate samples fortified with 5 mM iron.

There is still a need to scale up the methodology and test the ingredient in a food model due to the current work only being undertaken in lab-scale and highly idealised

conditions. This would help build a more realistic understanding of the phosphopeptide iron complex functionality in an end product.

2 Literature review

The purpose of this literature review is to provide a framework for the development of an iron fortified milk product and a plan for identifying and assessing the relevant physicochemical characteristics of milk proteins. Milk is a complex mixture of components containing a specific balance of protein and minerals that can be unstable when the environment changes therefore an understanding of these changes is important. Key to this project is the development of casein phosphopeptides; the covered literature includes the isolation of the peptides as well as how these fortified systems affect iron absorption in cell or animal models. Finally, several techniques used in the project have been outlined in their principles of use.

2.1 Casein micelle composition and structure

2.1.1 Casein

The casein micelle serves three functions in milk; a) it prevents calcium phosphate precipitation by sequestering the calcium phosphate via the phosphate centres on the protein which prevents calcification in the mammary gland; b) the micelle suppresses amyloid fibril formation in the mammary gland by interaction of the different caseins (Thorn et al., 2005; Thorn et al., 2008; Holt et al., 2013; Holt et al., 2016) and c) milk delivers nutrition to the neonate by providing a mixture of lipids, proteins, carbohydrates, vitamins and minerals (Swaisgood, 2008).

The casein micelle is a complex association of proteins and minerals of which the structure is still disputed (Horne, 2006; Dalgleish, 2011). An average bovine micelle contains 10^4 individual casein molecules of α_{s1}-, α_{s2}-, β- and κ- in a 4:1:3.5:1.5 ratio that make up approximately 93 % of the micelle by dry weight. These form a colloidal suspension with 6-8 % milk salts of mainly: calcium, phosphate and small amounts of magnesium, sodium, potassium and citrate (Holt et al., 1986; Fox, 2003; de Kruif et al., 2012).

Micelles are held together by hydrophobic and electrostatic interactions between the caseins as well as interactions with the colloidal calcium phosphate (CCP) via phosphoserine groups (Horne, 2002; Swaisgood, 2008). Micelles remain stable in milk by κ- casein protruding out of the micelle. The κ- casein layer is considered to be a salted polyelectrolyte brush that causes steric and electrostatic stabilization preventing the micelles from flocculating (Walstra, 1979; de Kruif & Holt, 2003). In milk, there is a

distribution of casein micelle sizes. As the micelle size decreases the κ- casein concentrations increase while the β- and α_{s2}- casein decrease indicating that κ- casein is involved in terminating the growth of the casein micelle (Davis and Law, 1983).

The assembly structure of the CCP and protein fractions is still not understood (Walstra & Jenness, 1984; Dalgleish, 2011). Several models have been proposed over the history of casein micelle research. Notably, Schmidt & Payens (1976) and Walstra (1999) suggested the subunit model in which the overall micelle was made up of discrete sub-micelles. Horne (2003, 2006) offered the dual binding model where caseins cross-link via hydrophobic regions of the protein and also bridge across the calcium phosphate nano-clusters, while Holt et al., (2003) proposed the nano-cluster model in which the micelle was more uniform in structure. The nano-clusters of CCP are dispersed through the micelle with a homogeneous protein matrix and the phosphorylated proteins bind to the nano-clusters and the proteins are held together by weak interactions. Holt et al., (2003) calculated that there are 830 calcium phosphate clusters in a 108 nm radius casein micelle.

Caseins undergo post-translational phosphorylation which creates anionic clusters in the calcium sensitive caseins which are α- and β- casein while κ casein also undergoes modification but only one group is formed (Swaisgood, 2008). These phosphorylated residues cause the caseins to become insoluble in the presence of calcium ions. For a phosphate centre to exist there needs to be at least three phosphorylated regions and at least two other acidic residues occurring within a short sequence in order to bind calcium phosphate (de Kruif & Holt, 2003; Swaisgood, 2008). The caseins have an amphipathic structure as the anionic clusters producing a polar charge are clustered in separate domains to the hydrophobic regions. The polar domains are dominated by the phosphoseryl residues and therefore carry a large net negative charge at the native pH of milk (Swaisgood, 2008). At these sites the CCP is able to form (de Kruif & Holt, 2003).

Table 2-1 shows the amino acid sequence of some anionic regions on the calcium sensitive caseins along with the charge for the section. The sequences share a common motif of three phosphorylated serine groups followed by glutamic acid. Phosphorylation occurs at serine or threonine residues which have the sequence of Ser/Thr-X-Y (where X is any amino acid and Y is an acidic residue). When glutamate (or aspartate) occupies the third position of this sequence, the site is termed a primary phosphorylation site (Ginger & Grigor, 1999).

Table 2-1: Anionic clusters of calcium sensitive caseins with charge by Swaisgood et al., (2008)

Peptide sequence	Charge
α_{s1} –casein (f 61-70)	
Glu•Ala•Glu•SerP•Ile•SerP•SerP•SerP•Glu•Glu	-12
α_{s2}-casein (f 5-12); α_{s2}-casein(f 56-63)	
Glu•His•Val•SerP•SerP•SerP•Glu•Glu	-9
SerP•SerP•SerP•Glu•Glu•SerP•Ala•Glu	-11
β-casein (f 14-21)	
Glu•SerP•Leu•SerP•SerP•SerP•Glu•Glu	-11

The casein micelles are dynamic structures and are sensitive to changes in temperature and pH due to the individual casein characteristics. The temperature has an effect on the hydrophobic bonds of the caseins in particular with respect to β-casein (Davis & Law, 1983; Law and Leaver, 1998). β- casein is amphiphilic and contains a large region of hydrophobic residues which causes dissociation at low temperatures as the forces decrease. Low temperatures have a large effect on the overall milk protein partition. At native pH at 4 ^{o}C the β- casein dissociates while at 30 ^{o}C no casein dissociates from the micelle even when the calcium phosphate has been removed from the micelle (Davis & Law, 1983; Law and Leaver, 1998; Dalgleish & Law, 1988).

2.1.2 Milk Salts

The mineral content of bovine milk is 8-9 g/L and milk salts consist primarily of chlorides, phosphates, citrates, sodium, potassium, calcium and magnesium (Holt et al., 1986; Fox, 2003; de Kruif et al., 2012). Both inorganic and organic salts are present in milk and can be in the form of free ions and ion complexes (Vegarud et al., 2000). Multivalent ions such as Ca^{2+} and Mg^{2+} generally exist as complexes such as Ca-citrate and Mg-citrate. Only 20-30 % of the ultra-filtratable calcium and magnesium ions exist as free divalent ions, whereas the univalent ions exist almost entirely as free ions. Approximately one third of calcium, half the inorganic phosphate, two thirds of magnesium and over 90 % of citrate are in the aqueous phase of milk (Gaucheron, 2005).

Milk salts in the colloidal phase can bind to the proteins as individual ions or as part of a complex structure, such as CCP (Swaisgood, 2008).

2.1.2.1 Colloidal calcium phosphate

About two thirds of the total calcium in skim milk binds within the micelle, specifically in the form of colloidal calcium phosphate (Jenness & Patton, 1959; Dalgleish, 2011). It has been well established that the colloidal calcium phosphate is involved in stabilising the casein micelle however it is only partially responsible for maintaining the structure (Dalgleish and Law, 1988). CCP is essentially amorphous and exists as 2.0:1 Ca to P_i having these clusters a diameter of 4.6 nm (de Kruif & Holt, 2003). It has been proposed that the CCP resembles the structure of brushite with the clusters homogeneously scattered in the matrix which are linked to the Ser (P) groups (Chaplin, 1984; Holt, 1992; Le Graët & Gaucheron, 1999).

Changing the environment of milk solutions such as during industrial processing can have a large impact on the migration of minerals. When milk is acidified the casein molecule charges are neutralised and the solubilisation of calcium phosphate, magnesium and citrate ions occurs (Dalgleish et al., 1989; Gaucheron et al., 1996; Law & Leaver, 1998; Le Graët & Gaucheron, 1999; Holt, 2003). The Ca^{2+} and P_i dissociate from the casein micelle at pH 4.6 and 5.2, respectively, and the amount of Ca and P_i that dissociated at 20 ºC across all pH levels tested (from pH 4.9 to 6.7) resulted in the same dissociation constant of 1.75/1.85 Ca/P_i (Dalgleish & Law, 1988). The forces that maintain the colloidal state of the micelle are not fully dependent on colloidal calcium phosphate as at 30 ºC most of the phosphate can be removed without affecting the micelle structure. This means that other types of bonds hold the micelle together such as hydrophobic and electrostatic (Dalgleish & Law, 1988). The CCP is sensitive to changes in the environment and respond rapidly to manipulation. Law and Leaver (1998) showed that few changes occurred after 30 minutes when the pH or temperature was altered. The mineral solubilisation was different depending on the casein concentration of the system but was not affected by the ionic strength of the system (Le Graët & Gaucheron, 1999).

2.1.3 Milk processing

2.1.3.1 Spray drying

Spray drying is a method to rapidly dry liquid solutions with minimal heat damage. Preheating is carried out on milk before evaporation to reduce the microbiological load

and also to control the final functional properties of the powder (Kelly, O'Connell & Fox, 2003). It is usually performed using direct or indirect heat exchangers and is classified as being low, medium or high heat: 75 ^{o}C, 15 sec; 75 ^{o}C, 1-2 min; 80 ^{o}C, 30 min/ 120 ^{o}C, 1min, respectively. The milk is then evaporated to 42-48 % total solids usually using a multiple effect falling-film evaporator. Other techniques can also be used such as ultra-filtration, reverse osmosis or nano-filtration. Spray drying is then carried out which causes the rapid drying of a liquid by atomising the liquid in a hot convective medium. The liquid feed enters the spray drier by an atomizer nozzle that allows the droplets to become very small (Blanchard et al., 2013). The rate of drying is very important as it can influence the final moisture of the particle, the particle size and the particle wettability (Kelly, O'Connell & Fox, 2003). The spray drying process can cause some irreversible changes such as whey denaturation (Martin et al., 2007). During the evaporation step prior to spray drying more soluble calcium, casein and whey proteins are forced into the micellar phase due to the change in the aqueous phase. In the spray drying step more whey proteins attach to the surface of the micelle proportionally to the severity of heating, causing the resultant micelle size to increase. Irreversible denaturation occurs for major and minor whey proteins mainly in the pre-heating step (Oldfield, Taylor & Singh, 2005) whereas the effect of evaporation and spray drying is minor. Preheating at 100-120 ^{o}C for 52 s is known to cause extensive denaturation (70-90 %) of β-lg and BSA.

2.1.3.2 Reconstitution of skim milk

The reconstitution of skim milk powder or whole milk powder depends on its degree of agglomeration, water temperature, heat treatment of powder, wettability, shrinkability and solubility (Kelly, O'Connell & Fox, 2003). Anema & Li (2003) examined changes that occurred when skim milk powder was rehydrated. The turbidity of the reconstituted skim milk was always greater than fresh milk and the micelle size was greater as the evaporation and spray drying causes the turbidity to increase as the micelles become more optically dense however this is slowly reversed upon reconstitution. Soluble calcium and phosphate reached the final concentration level within a few minutes of reconstitution and there was an approximate 1 mmol/kg and 2 mmol/kg change for calcium and phosphate after 32 hours. Low heat skim milk powders took slightly less time to re-equilibrate than medium and high heat skim milk (Anema & Li, 2003).

2.2 Comparison of cow and goat milk composition

The most common type of milk consumed in New Zealand is cow milk however there is an increasing trend of farming goats for milk. Production increased 15 % on average each year from 2006 to 2010 (Carlson et al., 2011). The protein fraction within goat milk contains 80 % casein by weight and makes up approximately 2.40-2.50 % of goat milk (Jandal, 1996; Park et al., 2007). There are four main protein fractions within casein: α_{s1}-, α_{s2}-, β-, and κ- casein that vary in amounts depending on the species. There have been several studies examining the difference in composition of goat milk compared to cow milk and it has also been proven that there are large variations between the different breeds of goat (Jenness, 1980).

2.2.1 Protein

Table 2-2 shows the composition of goat and cow milk adapted from Jandal (1996) and Park et al., (2007). There are differences in the composition which may be due to variations in the breed used or other environmental factors. Notably, there is a large difference between the values of goat protein which are 2.9 % and 3.4 % reported by the two authors. In general, milk composition also changes over time; at the beginning of lactation the total solids, fat and protein are high but then fall quickly and reach a minimum after two to three months of lactation (Park, 2006).

A study by Sanz Ceballos et al., (2009) compared Holstein Fresian cows and Granadina goats stabled in the same area looking at differences in milk composition. Feeding was carried out to the animals' dietary requirements. The goat milk had higher levels of protein, total solids, fats and minerals, and this remained when expressed as dry matter compared to the cow milk. There was no significant difference in the casein content in milk, α_{s2}- and whey proteins. The goat milk had 62.8% less α_{s1} casein and 19.7 % more β- and κ casein than cow milk. In general, goat milk contains less α_s casein than cow milk and usually has more α_{s2}- than α_{s1}- (Jandal, 1996; Park et al., 2007).

Table 2-2: Composition of goat, cow and human milk by Jandal, (1996) and Park et al., (2007)

Component (%) unless stated otherwise	Goat	Goat	Cow	Cow	Human
Fat	3.80	3.8	3.67	3.6	4.0
Solid-non-fat	8.68	8.9	9.02	9.0	8.9
Lactose	4.08	4.1	4.78	4.7	6.9
Protein	2.90	3.4	3.23	3.2	1.2
Casein	2.47	2.4	2.63	2.6	0.4
Whey proteins	0.43	0.6	0.60	0.6	0.7
Non-protein nitrogen		0.4		0.2	0.5
Total ash	0.79	0.8	0.73	0.7	0.3
Ca	0.194		0.184		
P	0.270		0.235		
Cl	0.154		0.105		
Vitamin A (IU g^{-1} fat)	39.0		21.0		
Vitamin B_1 (mg per 100mL)	68.0		45.0		
Vitamin B_{12} (mg per 100mL)	210.0		159.0		
Vitamin C (mg per 100mL)	20.0		2.0		
Vitamin D (IU g^{-1} fat)	0.70		0.70		
Energy (Cal. Per 100mL)	70.0	70	69.0	69	68
	Jandal, 1996	Park, et al., 2007	Jandal, 1996	Park, et al., 2007	Park, et al., 2007

Goat milk proteins are similar to cow milk with the major types of caseins and whey proteins present however they have differing genetic polymorphisms due to different amino acid substitutions on the protein chain (Haenlein, 2004). The amino acid compositions of the caseins are therefore slightly different from cow milk (Jenness, 1980). There are eight different types or alleles of α_{s1} casein in goat- A, B, C, E, F, G, H and null (the absence of the α_{s1} casein) that result in high levels of α_{s1} (3.5 g/L milk), medium (1.1-1.7 g/L) and low levels (0.45 g/L) (Park et al., 2007). Seven alleles have also been identified for α_{s2} which produce normal, reduced and null amounts of α_{s2} casein (Park et al., 2007). α_{s1} has the same amino acid length as cow while β- and α_{s2}-casein are one longer and two shorter in amino acid residues, respectively. κ- Casein is 171 residues long with some substitutions of residues in the chain, while cow κ-casein contains 169

amino acids (Park et al., 2007). Table 2-3 shows the variation in the amount of caseins present in cow and goat milk. The composition varies greatly between authors and this can be attributed to the breed of goat that the milk came from. The number of phosphorylation sites for cow milk in α_{s1}, β and κ- is 9, 5 and 3, while in goat milk it is 11, 6 and 3, respectively (Martin et al., 2003). The α_{s2} was not measured, but was predicted to be 17 and 16, for cow and goat, respectively.

Table 2-3: Comparison of casein compositions between cow and goat milk expressed as % of total casein

Casein type	Cow %	Goat %			
cited	Clark & Sherbon, (2000)	Clark & Sherbon, (2000)	Sanz Ceballos et al., (2009)	Tziboula (1997)	Selvaggi et al., (2014)
α_{s1}-	36-40	4-26	22.8	1.9-19.1	5.6
α_{s2}-	5-19	5-19	10.3	15.1-19.3	19.2
β-	34-41	42-64	66.8(incl. κ-)	51.3-58.5	54.8
κ-	10-24	10-24		12.1-14.9	20.4

2.2.2 Physicochemical properties of goat casein micelles

There are physicochemical characteristics of goat milk casein micelles that vary from cow casein. The casein micelles are less hydrated, more mineralised, less heat stable and lose β-casein more readily than bovine micelles (Haenlein, 2004; Park et al., 2007; Raynal-Ljutovac et al., 2007; Raynal-Ljutovac et al., 2008).

The casein micelle from goat milk is smaller than cow milk micelles, which is due to the differences in casein composition (Haenlein, 2004). Remeuf (1993) looked at the characteristics of goat milk with different α_{s1}- casein compositions. The three goat milk samples had a homozygous genotype at the α_{s1}- casein locus of AA, EE or FF. These genotypes had a significant effect on the fat, total nitrogen, protein, casein and casein number (casein N/ total N) in the order of AA> EE> FF. There was a large influence of the casein micelle size in the opposite order (AA< EE< FF) while the genotype had little influence on the mineral and salt equilibrium. There was no significant difference on mineral content of A and null variants (Pierre at al., 1995). Pierre et al., (1995) used photon correlation spectroscopy and TEM to look at goat milk from the genotype with a high level of α_{s1} (4.7 g/kg) and the null genotype, producing no α_{s1}. The null genotype had an average micelle diameter of 280 nm and the α_{s1} rich milk had an average size of

199 nm and gave a narrow distribution of sizes unlike the null allele, while Jenness (1980) reported a size distribution mostly below 80 nm, with only some micelles reaching 200 nm in diameter. They concluded that from compositional analysis the main difference was only the α_{s1} content and this therefore governed the size of the micelles.

Goat milk is slightly alkaline compared to cow milk caused by the higher content of protein and a different arrangement of phosphates (Jandal, 1996). The isoelectric point of goat milk is pH 4.1 rather than pH 4.6 for bovine milk (Sanz Ceballos et al., 2009). At 20 ^{o}C, 10 % of the goat β- casein is soluble while under the same conditions the solubility is 1 % for cow milk (Jenness, 1980). The hydration is reported to be between 1.60 g H_2O/g pellet on average and ranges from 1.28 to 1.97 g H_2O/g pellet, compared to 1.90 g H_2O/g pellet for cow. This may also be due to the low level or absence of α_{s1} along with the difference in mineral distribution (Jenness, 1980).

2.2.3 Minerals

The mineral content of bovine milk of 563 mg/100 g milk is lower compared to caprine with 646 mg/100 g (Park, 2006). The amount of calcium and phosphorus in goat milk varies between authors which again, must be due to the different breeds (Haenlein, 2004). Table 2-4 shows the reported compositions of cow and goat milk. Goat casein micelles contain more calcium, inorganic phosphorus and non-centrifugal casein than cow milk (Jenness, 1980; Haenlein, 2004; Park et al., 2007; Raynal-Ljutovac et al., 2007; Raynal-Ljutovac et al., 2008) which contains approximately 30 mM calcium and 20 mM phosphorus, respectively (Law, 1996; Udabage et al., 2000).

Jenness (1980) concluded that there is an inverse relationship between the mineralization of the micelle and the micelle hydration. Park et al., (2007) reported that cooling led to a partial solubilisation of colloidal calcium phosphate and β casein. The high ionic calcium content and low micellar solvation in goat milk may contribute to heat instability (Haenlein, 2004).

Table 2-4: Calcium and phosphorus content of cow and goat milk in mg/100g or mg/100mL

Species	Calcium	Phosphorus	Total mineral*	Author
Goat	134 mg/ 100 g	121 mg/100 g	671.1 mg/100 g	Park et al., 2007
Goat	194 mg/100 g	270 mg/ 100 g	790 mg/100 g	Jandal, 1996
Goat	126 mg/100 mL	97 mg/100 mL	624.4 mg/100 mL	Raynal-Ljutovac et al., 2008
Goat (range of breeds)	118-198 mg/100 mL	95-153 mg/100 mL	710-880 mg/100 g	Jenness, 1980
Goat	120 mg/100 g	90 mg/100 g		Raynal-Ljutovac et al., 2007
Goat- A variant (Colloidal phase only)	78.6 mg/ 100 g	50.1 mg/ 100 g		Pierre et al., 1995
Goat- O variant (Colloidal phase only)	64.1 mg/ 100 g	47.7 mg/ 100 g		Pierre et al., 1995
Goat (Colloidal phase only)	94.2 mg/ 100 g	57.6 mg/ 100 g		Pierre et al., 1995
Cow	122 mg/ 100 g	119 mg/100 g	595.71 mg/100 g	Park et al., 2007
Cow	120 mg/ 100 g	97 mg/ 100 g	528.5 mg/100 mL	Raynal-Ljutovac et al., 2008
Cow	125.5 mg/100 g	68.7 mg/100 g		Udabage et al., 2000
Cow	143.2 mg/100 mL	66.0 mg/100 mL		Law, 1996

* Summation of minerals measured

2.2.4 Nutritional value differences of cow and goat milk

Goat milk has been substituted for cow milk in a study by Haenlein (2004) of children over 5 months old. They gained more weight, height, and skeletal mineralisation and higher blood serum content of vitamin A, calcium, thiamine, riboflavin, niacin and haemoglobin were also detected when taking the goat milk compared to cow milk. It has also been shown that there are fewer problems with allergic reactions of goat milk compared to cow milk. The composition of goat milk fat is quite different from cow as it

contains a higher level of medium-chain triglycerides, 36 % compared to 21 % in cow milk and contains less soluble and more insoluble volatile fatty acids than cow milk fat (Jandal, 1996). The medium chain triglycerides are absorbed and metabolised faster (Haenlein, 2004). García Unciti (1996) has proposed that this could increase the synthesis of carrier proteins and therefore the absorption of iron.

Goat milk contains a higher concentration of vitamin C which is able to chelate iron allowing it to remain soluble in the higher pH levels within the small intestine aiding the absorption of iron by forming a chelate of iron and ascorbic acid (Jandal, 1996; Lopez-Aliaga et al., 2000; Barrionuevo et al., 2002). In addition, goat milk contains more vitamin D, 250 ng/ 100 mL versus 63 ng/ 100 mL for cow milk and vitamin A (Lopez-Aliaga et al., 2000; Nestares et al., 2008). However, goat milk is deficient in vitamin B_{12} and folic acid, and pure diets of goat milk will lead to megaloblastic anaemia as these are necessary for the synthesis of haemoglobin; cow milk contains fivefold more of these nutrients (Park et al., 2007). While goat milk may contain higher levels of vitamin C and D than cow milk, these are still deficient for the human diet and these, in addition to vitamin B_6 must be supplemented in infant formula, for example (Park et al., 2007). Lactose is an important nutrient as it favours intestinal absorption of calcium, magnesium and phosphorus as well as the utilization of vitamin D. The lactose content in goat milk is 0.2-0.5 % less than in cow milk. Apart from lactose, the other carbohydrates in goat milk are oligosaccharides, glycopeptides, glycoproteins and nucleotide sugars in small amounts.

Goat milk contains higher levels of cysteine, reduced cysteinyl peptides (Glahn & Van Campen, 1997) and lysine and is capable of solubilising Fe^{3+} or Fe^{2+} which may aid absorption of iron by forming tridentate chelates (Barrionuevo et al., 2002). Goat milk also contains more nucleotides/ nucleosides than cow milk which may allow the iron to be more bioavailable in goat milk as they increase iron absorption in the intestine (Schlimme et al., 2000). Calcium from cow milk interferes with the absorption of iron in the diet. Goat milk may decrease this calcium-iron interference and may increase the bioavailability of iron (Barrionuevo et al., 2002). The goat milk casein is more soluble and also contains a higher level of soluble whey proteins which could support iron absorption. Goat milk is able to reduce calcium- iron interactions due to the different levels of components compared to cow milk that aid iron absorption despite the presence of calcium (López- Aliaga et al., 2009).

2.3 Natively Occurring Iron in milk

2.3.1 Iron Concentration in milk

The concentration of native iron in cow milk is on average 0.2 mg/kg but varies between breeds and season according to Gaucheron (2000). However Park (2006) stated that there is 0.8 mg/kg iron in cow milk and 0.7 mg/kg in goat milk. Table 2-5 by Gaucheron (2000) shows where the native iron is located in both whole and skim milk. The proportions of casein and whey bound iron change in the skimming process which could be due to whey denaturation. Estimations of the distribution of iron within the casein fraction are 72 % bound to α_s- casein and 21 % to β casein (Kitts, 2005).

Table 2-5: Proportions of where native iron exists in whole and skim milk, by Gaucheron (2000)

Location	Amount in whole milk	Amount in skim milk
Milk fat membrane	14 %	-
Casein	24 %	50-65 %
Whey proteins	29 %	18-33 %
Low molecular weight fraction	32 %	15-33 %

2.3.2 Native Iron chelates in milk- Lactoferrin and Transferrin

Transferrin and lactoferrin are two types of iron binding proteins in milk. Mammals are able to produce both of these but at different concentrations. Lactoferrin is a single chain protein with cow lactoferrin having slightly different glycosylation features. The tertiary structure forms two lobes, each having an iron binding site. When there is no iron bound the protein is in a more open conformation which changes when iron binds. The most common metal ion that it binds is iron in the ferric form (Lönnerdal, 2003). In human milk the concentration of lactoferrin is greater than 2000 µg/mL while cow and goat produce between 20-200 µg/mL. Transferrin has the same concentration in cow and goat while in human milk there is less than 50 µg/mL (Packard, 1982; Hambræus & Lönnerdal, 2003). Whey proteins also have an affinity to bind iron (Gaucheron et al., 1997) and Jackson, (1992) states that it is present at a concentration of 1-2 mg/mL in human milk and 0.01-0.1 mg/mL in cow while Park et al., (2007) claims that there is no more than 2 mg/mL lactoferrin in human milk while cow and goat milk have an equal amount of lactoferrin present at 0.02-0.2 mg/mL.

It has been suggested that the lactoferrin aids absorption of iron due to the high bioavailability of iron in human milk with a low concentration of iron present. There has also been evidence that there is a lactoferrin receptor on the brush-border membrane of the small intestine of some species (Hambræus & Lönnerdal, 2003). Lactoferrin may have a bacteriostatic effect in various ways, one being that iron is removed from the system so it cannot be utilised by bacteria. In human milk, a major proportion of iron is bound to lactoferrin however it has not been confirmed that lactoferrin causes a higher bioavailability of iron (Lönnerdal, 2003).

2.4 Iron Fortification of Milk

Scarce information could be found on the fortification of iron in goat milk therefore emphasis will be placed on bovine milk fortification.

2.4.1 Iron chemistry

Iron is defined by IUPAC as a transition metal where "*an element whose atom has an incomplete d sub-shell or which can give rise to cations with an incomplete sub-shell*". Iron binds strongly with anions, has low mobility in solution and is often involved in biochemical systems as redox catalysts and oxygen chemistry (Crichton, 2012). Conversely, Ca^{2+} is a moderate ligand/ anion binder, is semi-mobile in aqueous solutions and its function is a trigger, transfer or structural ion. Calcium therefore mostly interacts with ligands via ionic bonding (electrostatic interactions) which is caused by a difference in electronegativity of the cation and anion. The electrons are transferred between atoms and occur when the difference in electronegativity is greater than 1.7. This causes a transfer of electrons from an unfilled shell of one atom to an unfilled shell of another and these bonds are highly polarised. Iron interacts with ligands via covalent bonds, specifically co-ordination bonds. Covalent bonds are defined as an orbital overlap in which electrons are shared and atoms are in close proximity with significant overlap causing a stable bond. There is a balance between attractive and repulsive forces and the electrons are shared unequally between the atoms. These bonds occur when the difference in electronegativity between the atoms is less than 1.7. Co-ordination bonds arise when there is a central atom surrounded by electron rich ligands. These ligands are directly co-ordinated to the central atom and the number of ligands can range from 2 to 9 and can be a single atom, ion or molecule. The electrons for sharing are supplied by one atom and there is often a fractionated positive charge of the donor atom and fractional negative charge on the acceptor atom (Crichton, 2012).

Iron can be in various oxidation states from –II to +VI however the common ionisation states are II (d^6) and III (d^5). Fe^{3+} is insoluble in water with a solubility product of $K_{sp}= 10^{-39}$ M and at pH 7.0 the concentration of $[Fe^{3+}] =10^{-18}$ M, while Fe^{2+} is very soluble. Fe^{3+} is considered a hard metal acid while Fe^{2+} is considered an intermediate acid. A hard acid is defined as having a high charge density, small ionic radius and no easily excited outer shell electrons. Hard acids prefer to bind with hard ligands, such as PO_4^{3-} while a ligand such as imidazole is an intermediate ligand. Fe^{2+} can accommodate hard and soft ligands such as histidine.

Hard acids tend to form ionic interactions due to the large difference in electronegativity while intermediate acids tend to form orbital bonds. When metal ions are dissolved in water they complex to water ligands and these can be displaced with other ligands causing the formation of complexes. This chelate is due to a favourable entropic contribution regardless of the enthalpy of the reaction. The three dimensional structure of a protein is important with regard to metal ion binding capacity as it can improve the co-ordination geometry on the metal ion and determines the ligands available for co-ordination, the stereochemistry and the local environment (Crichton, 2012). Histidine, glutamic acid and aspartic acid are able to bind metal ions when in the deprotonated form (Crabb and Moore, 2010).

Ferrous iron is more bioavailable than the ferric form (Davidsson et al., 2000) and therefore work needs to be carried out on ensuring the iron fortified ingredient contains the ferrous form. However if free Fe^{2+} ions are exposed to oxygen which is likely to occur in the fortification step, there is a chance of oxidation occurring. Fe^{3+} is a strong Lewis acid and will therefore react in water and will produce highly insoluble $Fe(OH)_3$ amongst other complexes shown in Equation 2-1 (Crabb and Moore, 2010).

Equation 2-1

$$4Fe^{2+}_{(aq)} + O_{2(g)} + 4H^{+}_{(aq)} \rightarrow 4Fe^{3+}_{(aq)} + 2H_2O_{(l)}$$

$$Fe^{3+}_{(aq)} + 3OH^{-}_{(aq)} \rightarrow Fe(OH)_{3\,(s)}$$

The reactions are very dependent on pH; Fe^{3+} is very soluble at low pH but will form a precipitate at pH 7.0. Free Fe^{2+} ions are unlikely to be present in the solution as they are very reactive (forming a superoxide radical anion) so they will be in a compound or complex form such as bound to protein or citrate in milk.

2.4.2 Iron salts for fortification

When iron is added to milk it may not only bind to the casein proteins; associations could form with caseins, colloidal calcium phosphate or citrate molecules (Gaucheron, 2000). Fe^{2+} or Fe^{3+} ions have the ability to interact with a variety of components within milk. These interactions may not necessarily be beneficial. A metallic after taste can arise along with an unacceptable taste of rancid fat due to oxidation caused by the iron. Undesirable colour changes and the degradation of vitamins can also occur (Mehansho, 2006). Iron compounds can have a high or low bioavailability however those with high availability tend to have more detrimental effects while low available iron types are usually more compatible with food systems.

Table 2-6 shows the different compounds of iron that are permitted to be used in food fortification according to Australia New Zealand Food Standards Code (2013) with the bioavailability stated for each from Hurrell (1985). The bioavailability is compared with ferrous sulfate which is the most common form of iron salt for fortification. It is interesting to note that ferrous or ferric chloride do not appear in the list of permitted forms of iron, however extensive work has been carried out using these forms indicating there may be variation in permitted additives between countries (Gaucheron, Famelart & Le Graët, 1996; Gaucheron et al., 1997; Raouche et al., 2009a; Raouche et al., 2009b; Mittal et al., 2016).

Table 2-6: Iron forms permitted in the use of food according to Hurrell (1985), Allen et al., (2006) and Australia New Zealand Food Standards Code (2013).

Iron form	Approximate Fe Content (%)	Solubility	Average relative bioavailability (Humans)*
Ferric ammonium citrate	18	Freely in water	51
Ferric ammonium phosphate			
Ferric citrate	16.5-18.5	Slow in cold water, rapid in hot water	
Ferric hydroxide			
Ferric phosphate	29 (dihydrate form)	insoluble	25-32
Ferric pyrophosphate	25	Almost insoluble or insoluble	21-74
Ferric sodium EDTA (not breakfast cereals or formulated supplementary food for young children)	12- 14 (hydrate form)	Soluble	>100
Ferric sulfate	21-23 (hydrate form)	Freely in water	
Ferrous carbonate			
Ferrous citrate	24	Poor	74
Ferrous fumarate	33	Poor	101
Ferrous gluconate	12	Freely in water	89
Ferrous lactate	19	Freely in water	106
Ferrous succinate	35	Poor	123
Ferrous sulfate. $7H_2O$	20	Freely in water	100
Ferrous sulfate dried	33	Slow	100
Iron, reduced (ferrum reductum)	>99	Insoluble in water	13-148

*Compared to ferrous sulfate, where 100 is the equivalent to ferrous sulfate bioavailability.

Blanks indicate no information could be found.

2.4.3 Iron binding and effects on casein

Milk proteins are often used for the delivery of nutrients in complex nutritional systems. Caseins are a suitable vehicle for iron delivery and have been studied by various authors in terms of iron binding capacity and physicochemical effects on the milk with iron fortification.

Approximately 80-90 % of $FeCl_2$/$FeCl_3$ or ferripolyphosphate bind to the colloidal phase of skim milk (Gaucheron et al., 1997; Hekmat & McMahon, 1998). When iron binds, it neutralises the negative charges and therefore increases the hydrophobicity. The charge of the iron added is important (2^+ or 3^+) as the caseins will aggregate at concentrations of 2 or 4 mM when $FeCl_2$ or $FeCl_3$ are added (Gaucheron et al., 1997; Hekmat & McMahon, 1998).

Gaucheron et al., (1996) examined the iron-casein stability at different pH and heat treatments. The casein concentration of the supernatant up to the addition level of 4 mM iron remained constant at 25 g/L, indicating no precipitation of protein. At 7.5 mM addition, there was no casein or iron detected in the supernatant indicating poor solubility of the caseins. The pH dropped by 0.12 pH units with a 1.5 mM iron addition, likely due to the exchange of iron and micellar H^+ ions. When the pH was adjusted from 3.7 to 6.5 at 1.5 mM iron there was no change in the amount of bound iron after ultrafiltration. Heat treatments of 50, 70 and 90 ºC for 15 minutes did not alter the amount of bound iron indicating iron forms a stable casein- iron complex at 1.5 mM iron. Similarly, Baumy & Brule (1988) looked at the effect that pH and ionic strength have on the binding of divalent ions to bovine β- casein. The binding capacity of β- casein to Fe^{2+} was studied from pH 5.0-8.0 and at different ionic strengths (0, 0.05 and 0.10). The amount of Fe^{2+} bound was not dependent on the ionic strength or pH range tested. Between pH 5.4 and 8.0 the number of bound Fe^{2+} ions per mole of protein was 6.6. In general, the change in pH and ionic strength decreases the binding strength of the casein for the ions due to the weakening of the ionic links which are sensitive to pH, however at the pH and ionic strength range used in this study, the iron binding to β-casein did not change. When iron binds to the β-casein it is estimated that 7 iron ions bind however there are only 5 phosphoserine groups in this casein, therefore iron may also be able to bind to other amino groups. Baumy & Brule (1988) observed zinc binding to two carboxylic sites and this may explain the two extra binding irons.

When ferrous iron is added to milk the Fe^{2+} oxidises to Fe^{3+} upon binding with the casein proteins (Emery, 1992), as opposed to oxidising first in solution via oxygen and then binding to the protein. The author also determined that the iron must bind to the phosphoserine groups as dephosphorylation of the casein reduced the amount of iron bound. When the concentration of casein was increased the acceleration of iron oxidation changed indicating that the oxidation of iron acted as a 'thermodynamic trap' to which more ferrous iron became oxidised. The reaction was second order for the casein oxidising the ferrous iron (Emery, 1992).

Sugiarto et al., (2009) studied the binding of iron to sodium caseinate and determined which fractions the iron binds to and how the binding is affected by pH changes and different levels of ferrous sulfate (0-20 mM). The study focused on the solubility of iron and protein. Similarly, Gaucheron et al., (1997) added iron to skim milk to determine the iron binding. They used $FeCl_2$ or $FeCl_3$ and a lower fortification level of 0-1.5 mM which was added drop-wise with rapid mixing. In a 1 % sodium caseinate solution with an iron concentration of 0-4 mM over 90 % of the iron added was in the supernatant (along with soluble protein) and above this, the solubility of iron decreased which Sugiarto et al., (2009) hypothesised to be due to all the binding sites being full. Above 4 mM the sodium caseinate began to precipitate and when 20 mM of iron was added less than 10 % of the sodium caseinate was soluble. Under 4 mM there was nearly no free iron in the permeate indicating that all the iron was bound. Gaucheron et al., (1997) also supported these findings as the binding sites were not saturated within their study in which up to 1.5 mM of iron was added. The amount of iron in the supernatant from centrifugation decreased as the added iron increased from 11.0-9.3 % for $FeCl_2$ and 9.8-7.8 % in $FeCl_3$. Therefore as the concentration of iron added increased the amount bound to the colloidal phase increased.

The maximum amount of bound iron was 25.5 mg iron/g protein according to Surigato et al., (2009). There were two regions of binding; firstly, a high affinity for iron due to specific binding sites and secondly, non-specific binding sites when the molar ratio of iron to protein was high. Fourteen binding sites for iron on a sodium caseinate molecule were calculated. It is likely that iron is able to bind via other co-ordination bonds such as COOH, CONH and H_2O rather than just through phosphoserine. This was supported by the findings that pH did not affect the iron binding ability. Raouche et al., (2009b) found differing results; when the pH was changed between pH 3-7 the least amount of soluble

iron was between pH 4-5.5. Outside the range of pH 4-5.5 there was an increase of (soluble) iron in the serum phase (obtained from ultra-centrifugation). The authors determined that the mineral composition of the casein micelles was changing with the addition of iron with subsequent pH adjustment. A greater amount of iron was able to be incorporated into the micelle in some way, i.e. as colloidal iron phosphate, between pH 4-5.5 when the calcium was solubilised as calcium citrate etc.

Hekmat & McMahon (1998) adjusted the pH of skim milk to determine the binding distribution of iron. The 83 % of the iron bound to the casein fraction within the pH range of 5.3-6.7 with a binding ratio of 72: 21: 4 for α_{s1}-: β-: κ-casein which correlates with the amount of phosphoseryl residues present in the caseins. It was hypothesised by the author that iron may form complexes with the colloidal calcium phosphate as the addition of iron affected how the calcium phosphate dissociated from the micelles as the pH decreased. As the pH dropped less calcium phosphate dissociated compared to a non-fortified sample with the biggest difference being at pH 5.3 where 40 % more calcium and 19 % more phosphate were present in the fortified samples.

2.5 Casein Phosphopeptides

Phosphopeptides are the breakdown products from the fractionation of casein protein. Within these products there are a range of bioactive peptides including opioid antagonist peptides, inhibitory angiotensin-I-converting enzyme peptides, antibacterial, antithrombotic and mineral binding peptides (FitzGerald & Meisel, 2003). Peptides can be characterised by their mode of formation by digestion in vitro by proteinases/ exopeptidases in vivo using gastrointestinal proteinases and exopeptidases or by fermentation using bacterial proteinases/ exopeptidases. Casein phosphopeptides from digestion may function as carriers for minerals and play an important role in the bioavailability of the minerals (Bouhallab et al., 2002; Miquel et al., 2006b).

Mineral binding peptides are characterised by containing a phosphate group covalently bound to seryl residues via monoester linkages. Casein phosphopeptides from α_{s1}-, α_{s2}- and β caseins have a common motif of a sequence of three phosphorylated residues followed by two glutamic acid residues (Figure 2-1), (SpSpSpEE) (Reynolds et al., 1994; Bouhallab & Bougle, 2004; Miquel et al., 2006b). The high mineral binding affinity has been attributed to the highly polar acidic domain that is created by this cluster. For calcium, the apparent binding constant (K_{app}) for tryptic digested peptides were 629, 328,

841 L/mol for β-CN (f 1-25) 4P, α_{s1}-CN (f 43- 58) 2P and α_{s1}-CN (f 59-79) 5P, respectively (FitzGerald & Meisel, 2003). The differences in binding could be due to the different surrounding residues to the clusters.

Meisel (1997) postulated that the clusters were able to maintain calcium in a soluble state by first binding the mineral, namely calcium, and then shielding the calcium using the hydrophobic regions of the protein to prevent further interaction, mainly for formation of insoluble calcium phosphate complexes. This makes them stable and soluble in different physicochemical conditions, such as changes in pH (Bouhallab & Bougle, 2004; Miquel et al., 2006b). The cation binding capacity of the phosphoserine residue cluster decreases in the order α_{s2}> α_{s1}> β> κ casein which is related to their decreasing phosphoserine contents (Gaucheron, 2005).

The higher the number of phosphoseryl residues the stronger the interaction strength with colloidal calcium phosphate is (Gagnaire et al., 1996). Baumy, (1989) proposed that the calcium binding capacity may be enhanced when the SerP residues are present in clusters. There is a minimum number of phosphate groups in a cluster to keep the structure intact (West, 1986); Aoki, (1992) estimated this minimum number to be three while Heganauer, Saltman and Nace (1979) determined it was two.

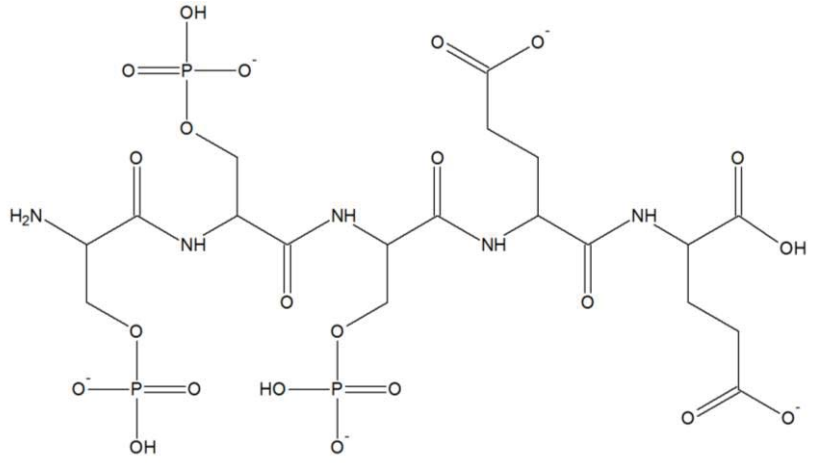


Figure 2-1: Phosphoserine cluster containing three phosphorylated serines followed by two glutamates inferred by Bouhallab, & Bougle (2004).

2.5.1 Digestion of casein and peptide analysis

Digestion or hydrolysis of protein occurs when an enzyme breaks the peptide bonds of the primary structure. When the enzyme breaks down the protein and the degree of hydrolysis increases the zeta potential becomes more negative (above the p*I*) which is due

to the exposure of more charged amino groups with more carboxylic and amino groups being liberated (Mahmoud et al., 1992).

Mahmoud et al., (1992) used porcine pancreatin to hydrolyse casein which hydrolysed the casein rapidly in the initial 10 minutes of digestion and then slowed down for the remaining time until the end point of 100 minutes. More than 95 % of the hydrolysates formed were smaller than 500 Da. When β casein was digested using typical digestive enzymes some of the peptides contained four or five phosphate groups with the unique cluster sequence SpSpSpEE (Miquel et al., 2006b).

Table 2-7 by Kibangou et al., (2005) shows the phosphopeptides formed with the sequence and molecular mass. β-CN (1-25) is the phosphorylated N-terminal fragment of β casein of molecular mass 3124 containing four phosphoseryl residues and is widely used in bioavailability and iron binding studies (Gaucheron et al., 1995; Pérès et al., 1999; Ait-Oukhatar et al., 2000; Bouhallab et al., 2002; Ani-Kibangou et al., 2005; Kibangou et al., 2005).

Table 2-7: Characteristics of purified casein phosphopeptides (CPP) adapted from Kibangou et al., (2005)

Casein	Sequence	Molecular mass (Da)	Number of phosphate groups
β CPP	f(1-25)	3124	4
α_{s1} CPP	f(59-79)	2721	5
α_{s1} CPP	f(2-21)	2747	4

Gagnaire et al., (1996) carried out a tryptic digestion on whole casein micelles to isolate phosphopeptides that interact with colloidal calcium phosphate. Large casein micelles were isolated from raw skimmed milk and digested using L-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin and maintained at 37 ^{o}C to maintain cross-linking of the CCP to caseins. Samples were ultra-centrifuged to obtain the pellet and serum. Analysis of the peptides was carried out using RP-HPLC-ESI-MS. The calcium and phosphate colloidal fraction, nitrogen and micelle size decreased as the hydrolysis progressed. There was a high retention of organic phosphorus remaining in the colloidal fraction while more of the inorganic phosphate moved to the supernatant fraction in direct proportion to the calcium removal. After 1 hour of hydrolysis the casein micelles had completely disappeared. The soluble fraction therefore had a large increase in peptides

after hydrolysis. There were 14 phosphopeptides identified that came mostly from β-CN A^1, β-CN A^2, α_{s1}- CN B and α_{s2}-CN A in the ultra-centrifugation pellet. The pellet peptides were further purified on a hydroxyapatite column and again purified on RP-HPLC and sequenced by Edman degradation. Six fractions were obtained where the first three were weakly bound and were non-phosphorylated. Most of the phosphopeptides present in the pellet contained four or seven to eight residues of SerP; this is in agreement with the condition that at least three SerP residues are needed to interact with CCP.

Reynolds et al., (1994) carried out a tryptic digest of casein to isolate the phosphoseryl containing peptides. Sodium caseinate was adjusted to pH 8.0 and digested with trypsin at 20 ^oC for 18 hours and then the pH was lowered to pH 4.6. Centrifugation was carried out to remove insoluble material and then the pH was either adjusted to 3.5, 8.0 or remained at 4.6. A calcium solution of 20 mol/mol protein was added and the protein was precipitated with 50 % final volume ethanol with slow addition. At pH 3.5 all the peptides precipitated contained the sequence: Glu-Ser(P)-Xaa- Ser(P)-Ser(P)-Ser(P)-Glu-Glu where Xaa is Ile in α_{s1}- peptides and Leu in β- peptides of α_{s1}(59-79) and β(1-25). The recovery of the peptides containing the Ser (P) clusters (multiple) was 73-85 %. At pH 4.6 all of the peptides in the pH 3.5 fraction were recovered also along with di-phosphorylated peptides α_{s1} (43-58) and α_{s2} (126-136). At pH 8.0 all of these peptides were recovered plus a monophosphorylated peptide. The mono or di- phosphorylated peptides were not precipitated at the lowest pH due to the protonation of the side chain carboxyl's of the glutamyl residues preventing cross linking by Ca^{2+}. At pH 8.0 all of the phosphorylated peptides were recovered except for two monophosphorylated peptides. The method achieved very high recoveries of the required peptides.

Alcalase was investigated for use in the production of caseinophosphopeptides by Adamson & Reynolds, (1996). Alcalase has a main enzyme component of subtilisin Carlsberg which is an endoproteinase and has broad specificity and cleaves at peptide bonds at Gln^4-His^5, Ser^9-His^{10}, Leu^{15}-Tyr^{16}, and Tyr^{26}-Thr^{27}. The yield of CPP was 12.27±0.24 % from the Alcalase digest which was similar to trypsin of 10.5±0.4 %. There were some peptides that were not detected from the Alcalase digest that are normally detected in a trypsin digest that contained diphosphorylated and monophosphorylated regions. Alcalase produced shorter peptides and bound less Ca^{2+} than trypsin and this is likely to be due to the broader specificity and affinity to hydrophobic residues which means it can reach further into the sites of the protein.

2.5.2 Phosphopeptides and their interaction with iron

The phosphoserine residues create a polar, acidic domain that favours the sequestering of divalent metal ions. Phosphoseryl residues promote iron oxidation from the ferrous to the ferric state. This makes the complex highly stable and CPPs can bind up to 4 mM of iron and can bind iron down to pH 2.5 (Yeung et al., 2002; Kitts, 2005). The binding of ferric iron by casein is decreased by dephosphorylation of the phosphoserine groups suggesting that the affinity of casein for ferric iron is related to the presence of phosphoserine residues (Yeung et al., 2002). Phosphopeptides are able to bind ions via both, ionic and coordination bonds (Peres et al., 1999). The coordination bonds with Fe formed by the phosphorylated serines on proteins do not get dissociated by changes in pH. Conformational changes occur to the peptide where the iron attaches to the oxygen on the phosphoserine residue in a tetrahedral (Kitts, 2005) or octahedral (Raouche et al., 2009a) co-ordination. β CN-(1-25)4P has been shown to bind four or five iron atoms and the complexes are resistant to further breakdown in the gut (Ani-Kibangou et al., 2005; Miquel et al., 2006b).

Gaucheron et al., (1995) used RP-HPLC on line with ESI-MS to look at the iron-phosphopeptide β (1-25) complex. β- casein was purified and digested by TPCK- treated trypsin which was analysed before and after iron addition which had an iron to β-CN (1-25) ratio of about 6. Prior to iron addition the β (1-25) peptide had a molecular mass of 3123.0 Da. After iron was added the peptide profile had changed with one peak disappearing and another appearing. Three different iron-β (1-25) complexes were obtained. It was predicted that the iron binds to the oxygen of the phosphate group via a co-ordination bond and causes a release of three protons for every iron atom bound. The three forms of iron bound phosphopeptide differed by the number of iron atoms bound from 4 to 6 with the majority of the species binding 5 iron atoms. The peak shift that occurred indicated that the peptide became more hydrophobic when the iron was bound which indicated a structural shift changed causing hydrophobic regions to be exposed. They suggested that not only is the oxygen of the phosphoseryl group important for bonding but also the steric conformation of the peptide to allow proper binding. Peptides with no phosphoseryl groups showed no change in the peptide profiles indicating they had no ability to bind iron.

2.5.3 Animal and human studies using casein phosphopeptide- iron complexes and their absorption

The hydrolysis of caseins has improved iron absorption however different caseins give different outcomes (Kibangou et al., 2005). Hydrolysed β caseins improved absorption in rat and human studies while α- caseins inhibit absorption (Kibangou et al., 2005). Casein phosphopeptides are able to bind iron preventing free iron to form ferric hydroxides which are not absorbed well. Absorption of β-(1-25) bound with iron is partially through endocytosis due to the smaller molecular weight as well as passive transport (Ait-oukhatar et al., 1999; Ait-oukhatar et al., 2002). The absorption mechanism is more efficient than gluconate iron (Peres et al., 1999). The peptides allow the iron to remain in a soluble form rather than salts which can form insoluble ferric hydroxide groups in the small intestine, preventing absorption (Martinez-Torres & Layrisse, 1970).

Studies have shown that iron bound to β-CN (1-25)4P displayed better iron absorption and uptake than inorganic salts (Miquel et al., 2006a). Ait-oukhatar et al., (2002) used rat as a model to look at the absorption of iron using iron-bound β-(1-25); this complex displayed better gut uptake and net absorption than ferric ascorbate; these findings were also supported by Peres et al., (1999). Pérès et al., (1999) fortified β-CN (1-25) with $FeCl_2$ and the peptides were compared to a control of iron-gluconate and the rats were either iron deficient or controls for both treatments. The net iron absorption was higher in Fe-β CPP animals than the iron gluconate fed animals while the absorption was higher in Fe-deficient rats than in control rats. This indicates that the β-CN (1-25)-bound iron is able to be absorbed more by the intestine due to the increased ability for the peptide to be absorbed. This peptide can bind four to five iron atoms with high affinity (Bouhallab & Brule, 2004). Alpha caseins may bind the iron too strongly to release the iron when necessary due to the different phosphorylation of the peptides. Bouhallab et al., (2002) looked at the effects of using different types of CPPs for the effectiveness of iron absorption: whole hydrolysed β casein, a mixture of CPPs, and β casein (1-25). Iron absorption was improved only by the iron-CPPs complex and not when free peptides were added to iron. Iron bound to the phosphopeptide produced by digestion of β-casein gave a better absorption than whole CPPs. The fractions derived from phosphopeptides rich in β-casein had better iron absorption than the α_s caseins. The best iron absorption was from β-CN (1-25). It has been shown that binding iron induced structural changes that differed between α- and β- casein and this may explain the differences in binding affinity between

different sequences of phosphopeptides (Bouhallab et al., 2002). Ani-Kibangou et al., (2005) postulated that the CPP could release iron close to the brush boarder membrane allowing absorption.

Ait-oukhatar et al., (1999) compared the ability of deficient rats to restore haemoglobin levels using a low molecular weight phosphopeptide compared to $FeSO_4$ and an intact β-casein. Two doses of iron were used: 40 mg/kg and 200 mg/kg and fed to iron deficient and normal rats. β-CN (1-25) bound iron improved the iron tissue storage and the haemoglobin levels during the repletion of the deficient rat compared to $FeSO_4$. The whole casein bound to iron also had an improvement but not to the same extent. This shows that the smaller peptide is able to be absorbed easier. Part of β-CN (1-25) bound iron is absorbed by endocytosis; enterocytes are able to uptake large peptides up to 30 kDa (Ait-oukhatar et al., 1999).

Hurrell et al., (1989) looked at the effect of hydrolysis of various proteins on the iron absorption. Native casein inhibited the most the iron dialysability of the iron out of the protein sources, however if the casein was hydrolysed the dialyzable iron increased from 0.19 % to 17.2 % for 90 % acid hydrolysed casein. When tested in human studies the iron absorption did not increase markedly, only a 2 fold increase compared to the intact casein, regardless of the large increase in dialysability.

2.5.4 Current commercial phosphopeptide ingredients

Table 2-8 shows some examples of casein phosphopeptide derived ingredients for various purposes. Recaldent has the single purpose of the re-mineralisation of teeth and can be found in toothpastes and chewing gum while Tekkotsu Inryo is in a Japanese drink. Lacprodan®, Capolac® and Hyvital are sold as ingredients which can be added into products.

Table 2-8: Commercial products derived from casein phosphopeptides for various purposes

Name	Company	Purpose
RECALDENT	Cadbury Enterprises Pte Ltd	Re-mineralisation of teeth
Lacprodan®, Capolac®	Arla Food Ingredients	Mineral delivery- calcium and zinc specified
Tekkotsu Inryo	Suntory Group (Japan)	CPP containing fruit drink containing 200 mg of calcium, 56 mg of CPP, 2.0 mg of iron
Hyvital	DOMO Friesland Campina	Mineral delivery- calcium, iron and zinc specified

2.6 Techniques

2.6.1 Small angle X-ray scattering

Recently in casein micelle analysis the use of high powered techniques: small-angle neutron scattering (SANS) and small angle X-ray scattering (SAXS) have been employed to probe its internal structure (de Kruif, 2014). SAXS is a non-destructive method where information can be collected on a length scale of 1-100 nm by measuring the samples at small angles of 0.1 to 10^{o} (Schnablegger & Singh, 2011). The principle of SAXS is that structural data can be obtained by measuring the X-rays scattering from areas with different electron density contrasts while SANS is based on neutrons scattering in a similar way but according to nuclear cross sections (Ingham et al., 2015).

The measurements are made by forming high energy X-ray radiation through a series of magnets to tune the energy and a monochromator to select the required wavelengths (collimator system), shown in Figure 2-2. The collimated X-ray beam passes through the sample, usually in a quartz capillary, in transmission mode (Kikhney & Svergun, 2015). When X-rays irradiate the sample all the atoms cause scattering, called background scattering but clusters of atoms making up the particles will produce excess scattering and the different electron density will give contrast. The atoms cause scattering that produce waves with interference patterns which are collected by the detector which produces an extremely high resolution image of patterns of light and dark at the detection plane which

can then be analysed (Schnablegger & Singh, 2011). A beam stop is used to prevent direct X-rays from reaching the detector as they are high energy and would damage it.

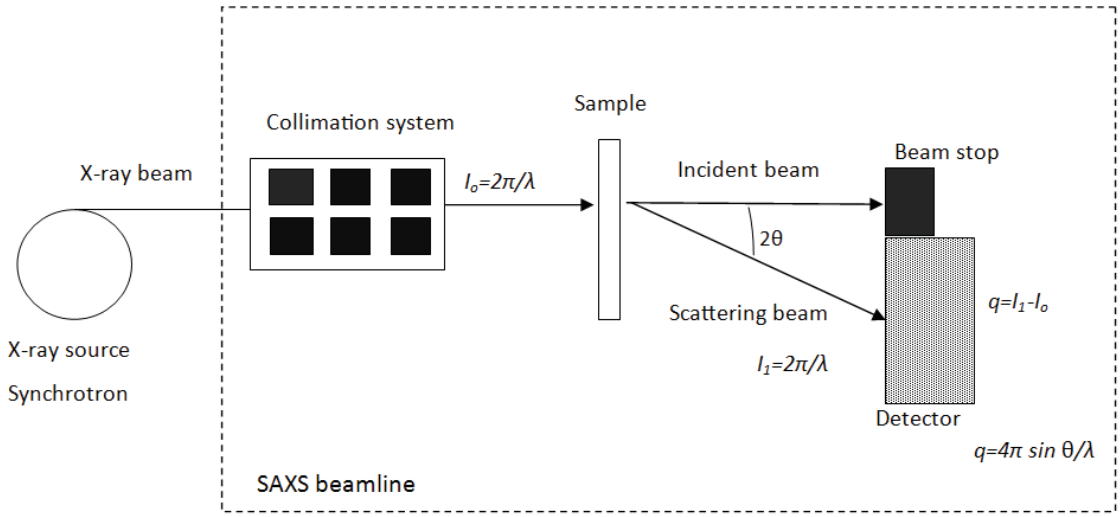


Figure 2-2: Schematic of SAXS adapted from Schnablegger & Singh, (2011) and Kikhney & Svergun, (2015).

Four features have been reported in native casein micelle analysis using SAXS and SANS over the scattering vector, q. The feature at q= 0.05 nm^{-1} that is seen in both SANS and SAXS is attributed to the overall micelle diameter and is approximately 100 nm in diameter (Bouchoux et al., 2010). The second feature (still disputed) is seen at q= 0.1 nm^{-1} and has been linked to mini-micelles (Horne, 2002; Müller-Buschbaum, 2007; Gebhardt, 2008), a second oscillation of a core-shell form factor, or other structure (Horne, 1998). The third feature at q=0.35 nm^{-1} is often seen in SANS data but not in SAXS data- unless using dried powders- and has been speculated to be the sub-micelle size or interaction distance (Stothart, 1989; Holt et al., 2003; Mata et al., 2011). Lastly, the feature at q=0.8-1 nm^{-1} is considered to be the CCP (Gebhardt, 2008; Bouchoux et al., 2010; Mata et al., 2011) however has been suggested to be the protein inhomogeneities with a length scale of 1-3 nm (de Kruif et al., 2012).

Holt et al., (2003) used SANS and SAXS analysis to measure diluted casein micelles in serum and found the q=0.35 nm^{-1} feature in SANS analysis, attributing it to the inter-particle interference of the CCP as the feature disappeared in water rich solvents but was apparent in heavy water. In support of this model de Kruif et al., (2012) concluded that the feature at q=0.35 nm^{-1} must be the CCP interaction distance as if the feature was protein-based the shoulder would disappear in D_2O rich solvents. When casein was cross-

linked and 6 M urea was added the CCP was more prominent in the SANS spectra at q= 0.35 nm^{-1}. The CCP clusters are 18.6 nm apart due to the hydrophilic parts of the casein binding to the CCP and the hydrophobic tails sticking out forming hydrophobic associations with other caseins about 2 nm in size. They predicted that there are 285 CCP clusters in a casein micelle of a median particle radius of 55 nm.

2.6.2 Particle characterisation

2.6.2.1 Dynamic Light Scattering (DLS)

The Zetasizer is an instrument developed by Malvern Instruments, UK that incorporates a variety of applications including particle sizing and zeta potential. Dynamic light scattering is the interaction of light with a particle causing radiated energy in the form of scattered light over a period of time. It is based on the diffusion of particles that dominates under 1 μm sizes, "Brownian motion"- the random movement of particles caused by collisions due to bombardment of solvent molecules. The diffusion is influenced by the size of the particle, viscosity of the solution and temperature; small particles move faster than large ones (Pecora, 2000; Kaszuba & Connah, 2006). The laser illuminates the particles and analyses the scattering to determine the size distribution. The intensity of the light produced is proportional to the particle diameter ($I \propto d^6$) therefore large particles scatter exponentially more light than small ones. Dynamic light scattering calculates particles size using the Stokes Einstein equation based on Brownian motion. The translational diffusion coefficient allows the calculation of the hydrodynamic diameter via deconvolution of the measured intensity correlation function. The distribution of the intensity is obtained by fitting algorithms such as CONTIN (Min et al., 2002):

Equation 2-2

$$d_H = \frac{kT}{3\pi\eta D}$$

Where k is the Boltzmann's constant, T is the absolute temperature, η is the viscosity and D is the diffusion coefficient. The hydrodynamic diameter is the diameter of an equivalent hard sphere that diffuses at the same speed as the particle being measured. The volume and number distributions of the particle size are calculated from the intensity data using the Mie theory however should only be used when the samples are spherical, homogenous, have known optical properties and no errors in the intensity data are detected (Malvern Instruments, 2011).

2.6.2.2 Zeta-potential

Particles such as casein micelles have a surface charge which can be manipulated by changing the environment (Dalgleish, 1984). In general, a charge further away from a neutral charge indicates that the colloids are more stable against aggregation so a good electrostatic stabilization is already achieved at ±30 mV. Charged particles can be described using the electrical double layer theory. The particles have a surface charge where counter ions are strongly attracted which is called the Stern layer and these have limited mobility and drive away co-ions. The co-ions make up the diffuse layer which is more loosely attached to the particle (Kirby & Hasselbrink Jr, 2004). The zeta-potential is measured via electrophoretic mobility, in the region of the diffuse layer and the medium in which the particles are dispersed in.

The zeta potential is measured using a technique called Laser Doppler microelectrophoresis. An electric field is applied across the sample cell which then causes the charged particles to move toward an electrode and the speed of this movement is measured using a laser and converted to the zeta potential. In other words, the velocity of the particle movement relates to the electrophoretic mobility which is converted to the zeta-potential (Dalgleish, 1984; Malvern Instruments, 2011).

2.6.3 Immobilised metal ion affinity chromatography

Immobilised metal affinity chromatography is a separation technique that allows proteins or peptides to be separated based on the affinity to a particular ion. Analysis of phosphopeptides is difficult because they are in low abundance compared to other peptides in the system and due to their high electronegativity caused by the phosphoseryl group are also poorly ionised in the positive ion mode so are difficult to detect using mass spectrometry. As in most mixtures of peptides the relative abundance of the phosphorylate peptides are low, and they may not be ionized due to the competition of the more ionisable peptides, which results in low ionisation efficiency. A pre-concentration step is therefore useful (Raska et al., 2002).

IDA is a common chelator as it is tri-dentate; the metal binds via a nitrogen atom and two oxygen atoms from the carboxylate group. When IDA binds to a metal ion it forms a double five membered ring with metals that are tetra- and hexa- coordinated, such as iron (Porath, 1992). Spacer arms such as epoxypolymers are often used to attach the matrix to the chelating ligand to reduce steric effects (Porath, 1992).

2.6.3.1 Iron as a chelating ion interacting with amino groups

Iron is classed as a hard Lewis acid which means it forms strong bonds, forming the strongest with hard bases. The affinity between the iron group and the peptide of interest depends on the groups that come into close proximity of the iron. There are three groups of hard bases that therefore form strong interactions with iron: oxygen, aliphatic nitrogen and phosphorus which in terms of amino residues relate to carboxylates, asparagine & glutamine and phosphorylated amino acids, respectively (Ueda et al., 2003). As a Lewis acid, the metal ion always acts as the electron acceptor. The water molecules are weakly associated with the iron when bound to the chelator and will be removed by competition when the peptide passes through the column. Histidine, tryptophan and cysteine residues have the best metal chelating abilities. Histidine and cysteine contain imidazole and thiol groups respectively while aromatic groups can also have affinity to metal. The primary and secondary structure can also influence the binding however for the purpose of this study the secondary structure will likely be destroyed. Iron has most affinity for carboxyl and phosphate groups.

2.6.4 Lipid oxidation

Oxidation of foods can have a negative effect on the quality as well as forming potential hydroxyl radicals which are harmful; lipid peroxidation is a major cause of food deterioration (Yen et al., 1999). Hydroperoxides are the first product of oxidation which are stable and when these breakdown they form the secondary products at the end of the oxidation process (Schaich, 2016). Divalent metal ions act as pro-oxidants and therefore it is beneficial to chelate these in a way that prevents interaction with fats. It has been shown that caseins are able to bind metals via the phosphoserine residues and therefore have an antioxidant effect in various systems (Diaz et al., 2003). The use of casein phosphopeptides as anti-oxidants would prevent the addition of additives in food products that have a negative consumer response.

2.6.4.1 Mechanism of oxidation

Oxidation of fats involves three steps: initiation, propagation and termination in unsaturated fatty acids. The first stage, initiation, is where hydroperoxide is formed which is odourless and tasteless (O'Connor & O'Brien, 1995). The initiation of the oxidation can occur in several ways: (i) auto-oxidation, a free radical mechanism that is non-enzymatic and is a radical chain reaction, (ii) photo-oxidation, a singlet oxygen

mechanism that requires light and a high energy oxygen molecule, and (iii) an enzymatic mechanism where lipoxygenase initiates the reaction.

Auto-oxidation occurs when a hydrogen atom is removed from a methylene group next to a double bond and requires either an external energy source or reaction with an existing free radical which is an unpaired electron highly reactive (O'Connor & O'Brien, 1995). When a free radical reacts with a ground state molecular oxygen a peroxy radical forms. Photo-oxidation involves metal ions as the catalysts or pro-oxidants; the metal allows the formation of singlet oxygen which then reacts with unsaturated fatty acids producing hydroperoxide.

Propagation is where these free radicals keep reacting with oxygen then form more hydroperoxides. Equation 2-3 shows the whole chain reaction with the different ways that the reaction can be terminated (O'Connor & O'Brien, 1995). Hydroperoxides breakdown to give oxidation products, they can split at either the oxygen- oxygen bond or on either side of the alkoxy group. Metals are involved by acting as pro-oxidants where they help to decompose the hydroperoxides to give new free radicals (Yen et al., 1999). A major pathway of lipid oxidation involves a self-catalytic free radical chain reaction and can be catalysed by several factors including divalent metals. Fe^{2+} has shown the strongest effects on pro-oxidation on linoleic acid compared to other forms of iron. Metals react with hydroperoxides causing the decomposition into peroxy radicals (Equation 2-4), the Fe^{2+} ions are oxidised to Fe^{3+} which in turn can react with the product and become reduced through the redox cycle of the reaction allowing a cycle of oxidation. The reaction involving Fe^{2+} is much faster (Yen et al., 1999).

Propagation occurs until it reacts with another radical and the reaction is terminated. The breakdown products are converted to carbonyls which include aldehydes, ketones etc. which are easy to detect organoleptically at very low concentrations and give a rancid taste. The reaction chain is not straightforward, there are many pathways in which products can form and these products can form in parallel with the hydroxyperoxides such as aldehydes. Lipid alkoxyl radicals can undergo alternative reactions (Schaich, 2016).

Equation 2-3

Initiation

$$RH + \text{Initiator} \rightarrow R\bullet + H\bullet$$

Propagation

$$R\bullet + O_2 \rightarrow ROO\bullet$$

$$ROO\bullet + RH \rightarrow ROOH + R\bullet$$

Termination

$$R\bullet + R\bullet \rightarrow R\text{-}R$$

$$ROO\bullet + R\bullet \rightarrow ROOR$$

$$ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$$

Where RH is any unsaturated fatty acid, R• is a free radical formed by removing a hydrogen atom from a carbon atom next to a double bond, ROOH is a hydroperoxide which is a major initial oxidation product.

Equation 2-4

$$ROOH + Fe^{2+} \rightarrow RO\bullet + OH^- + Fe^{3+}$$

$$ROOH + Fe^{3+} \rightarrow ROO\bullet + H^+ + Fe^{2+}$$

2.6.4.2 Methods of oxidation quantification

Initial products (conjugated dienes and hydroperoxides) can be measured directly. Conjugated dienes are formed by free radicals which cause a shift in the double bonds forming conjugated double bonds; these are stable products of oxidation that can be measured spectrophotometrically.

Hydroperoxides which are stable primary products can be measured in a variety of ways. Iodometric titration uses potassium iodide with starch and an indicator whereby the reduction of iodide to I_2 by hydroxyperoxides causes a change in colour when iodine molecules form. With this method it can be quite difficult to achieve good sensitivity and reproducible results and must be in an oxygen free environment with a dimly light room.

In addition, the endpoint can be quite difficult to detect (Schaich, 2016). Xylenol orange is another assay that is more sensitive than the iodometric titration. Ferrous ions are used to reduce the hydroperoxides to form ferric ions which are complexed by xylenol orange which forms a blue purple colour (Schaich, 2016).The ferric thiocyanate method involves adding ferrous iron which is oxidised to ferric ion by the hydroperoxides, setting off a chain of reactions with the products forming more ferric ions which in turn reacts with thiocyanate to form a red colour. This can be measured spectrophotometrically (Schaich, 2016). Other compounds that can be measured using a variety of techniques include the measurement of epoxides, carbonyls and TBA products.

2.6.4.2.1 TBA or Thiobarbituric acid reactive substances

The TBA reaction measures the secondary product, malondialdehyde. This product reacts with thiobarbituric acid when heated under acidic conditions. This forms a pink coloured Schiff base adduct however is not overly specific and can react with over alkanals, alkenals etc., but absorb at lower wavelengths than at 532 nm, the wavelength at which the colour is measured at. The benefit of this method is that the lipids do not have to be extracted and can be directly measured in the sample. There has been speculation that the long heating time and the presence of iron can artificially cause a higher value of decomposition products rather than measuring the true oxidation products (Schaich, 2016).

The TBARS test can give erroneous results as other substances can react with TBA, other than just MDA (St. Angelo, 1996). The TBARS method can accurately measure vegetable oils, unprocessed meat and fish but is not accurate with processed meat, pork, fish, dry nuts, cheese and potato chips (Papastergiadis et al., 2012). Results can be overestimated due to interference from other compounds and, while not tested in the study; dairy systems may also be affected.

2.6.4.3 Characteristics of cow and goat casein derived products on oxidation reactions

De Gobba et al., (2014) hydrolysed goat milk, permeate and retentate using Subtilisin, trypsin or a combination of both. The milk and retentate digest had a high 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity compared to permeate which was attributed to a high tyrosine content. Peptides rich in phenylalanine prevented the formation of secondary products in the retentate fraction and

milk however, the permeate was more effective overall. This was due to the permeate having a higher iron binding activity which was attributed to non-protein components binding iron in addition to protein. Partition of the iron away from the fat prevented the cleavage of hydroperoxides. There was a strong positive correlation between the degree of hydrolysis and the scavenging activity due to the increased number of peptides while having an enzyme with broad specificity.

Goat and cow milk and their digests via pepsin were assessed for their superoxide and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (Ahmed et al., 2015). When there was a high level of superoxide present of 5 mU XOD (xanthine oxidase) the peptides of goat milk showed very strong scavenging ability while all other treatments had no effect. When there was a lower level of 2.5 mU XOD, the goat milk and goat peptides showed good scavenging along with the cow peptides, however the cow milk was inactive. The whey peptides from goat had a strong $O_2^{\cdot -}$ scavenging activity compared to the casein peptides with a 80 % and 65 % reduction of $O_2^{\cdot -}$, respectively. The peptides were also able to scavenge DPPH radical; over time they could quench the free radicals and turning them into stable products. The hydrolysis allows an increase in accessibility of the amino acid residues which allows the donation of protons to the free radicals. The smaller peptides had better scavenging activity than larger or intact peptides/ proteins. There were particular fractions of peptides that had superior scavenging activity in both fractions. In casein, there were some fractions that had a masking effect and therefore when these were removed the activity greatly improved. Peptides that were amphiphilic appeared to be better due to an increased solubility allowing proton exchanges with free radicals.

Cesa, (2004) measured the malondialdehyde (MDA) content of various commercial infant formula and cow milk; there was a range of 200- 1200 ppb MDA for all samples. Samples enriched with long chain polyunsaturated fatty acids had the highest MDA levels while cow milk had the lowest. This showed that the amount of unsaturated fatty acids had a significant effect on the oxidation while the age, oil content and if the product was hydrolysed or not, were also factors that increased oxidation. There was no indication whether the iron content in the samples affected the rate of oxidation in this study however Burg et al., (2010) analysed two commercial cow and soy infant formulae for MDA content showing that the iron content did have an effect on the oxidation. The soy milks contained 7.7 mg Fe/ 100 g formula, while the cow based formula contained 5.25 and

3.66 mg Fe/ 100 g, with the soy based products also containing more polyunsaturated fatty acids and vitamin C that contributed to the oxidation. Samples with higher levels of vitamin E reduced oxidation due to its antioxidant behaviour.

Kim et al., (2007) measured the peroxyl and hydroxyl scavenging activity of CPPs precipitated at different pHs. The peroxyl scavenging activity increased with increasing CPP concentration from 10 to 50 μg/mL but the activity decreased with increasing pH; at pH 3.0 the scavenging activity was greater. The precipitation pH caused differences in the amino acid compositions of peptides and at pH 3.0 there was an increased concentration of cysteine, aspartic acid, glycine, histidine, proline, tyrosine and leucine which indicated κ-caseins were being precipitated. When the hydroxyl scavenging activity of the CPPs were tested those precipitated at pH 7.0 performed better. Hydroxyls are compounds that are most damaging in biological systems. The CPPs at this pH were likely to perform better as they were able to chelate metal ions as well as scavenge the hydroxyl compound itself therefore terminating the reaction. While there was more serine and glutamic acid in the precipitate there was no trend for the phosphorus content compared to the other CPPs to which the authors concluded that the glutamic acid residues played a significant role in scavenging activity. This indicates that CPPs can function in specific ways toward different oxidation products and this makes it difficult to determine the most effective method in the production of the peptides, as there are numerous pathways that oxidation can occur.

In other study using bovine casein derived products, Díaz & Decker (2004) looked at the effect of CPP concentration on ground beef oxidation. 100 μM of CPP caused a decrease of 82 % of TBARS production compared to the control however increasing the concentration to 300 μM caused no significant difference to the control and increasing the concentration to 500 μM caused a pro-oxidative effect on the oxidation. Therefore the ratio of casein phosphopeptides in a mixture may have to be optimised to ensure a pro-oxidant effect does not occur in formulations. Intact caseins were shown to prevent lipid oxidation when copper ions were added to a trilinolein mixture (Allen & Wrieden, 1982). After 30 hours of incubation at 25 ºC the absolute oxidation rate of the casein emulsion was negligible and when sunflower oil was used, in a ratio to casein that was double of that in milk, the casein was able to protect against oxidation with a 10 and 100 μM Cu^{2+} addition. This may indicate that copper does not have such a strong pro-oxidant effect as iron.

2.6.5 Caco-2 cells

Caco-2 cells are derived from cancer cells that mimic the cells of the intestine. They allow approximations for the absorption of iron by measuring the production of ferritin proteins which store iron. This method avoids the use of animal models. It is not intended to mimic a human model but rather to compare between the samples. Several steps are required to mimic the full digestion process including the stomach digestion and intestinal digestion then passing of the sample through the mucus layer of the intestine for cell absorption.

1.1.1.1 Iron storage proteins in cells

When iron is ingested any excess iron absorbed is stored in proteins called ferritin. This functions to store the iron because free iron is toxic to cells due to the production of radicals with oxygen. Apoferritin is 450 kDa and is made up of 24 sub-units of 170 amino acids (Rehder, 2014). It is a hollow protein made up of a wall of mostly α-helical peptide chains. The inner surface of the protein is lined with carboxylate groups of Glu and Asp where the Fe^{3+} can co-ordinate (Crabb & Moore, 2010; Rehder, 2014). The mechanism of iron being incorporated to the ferritin occurs when Fe^{2+} migrates to the centre through channels into the interior of the sphere which is rich in carboxylate residues. There residues coordinate the Fe^{2+} where it is oxidised to Fe^{3+} and the crystal structure grows. While the iron enters in the ferrous form it must be oxidised to the ferric form to become stable (Crabb & Moore, 2010). Each Fe atom is surrounded by and inner shell of 6 to 7 oxygen atoms and a second shell of 7 or 8 Fe atoms. One ferritin protein can store up to 4500 Fe^{3+} ions or up to 20 % total mass of the protein (Crabb & Moore, 2010). The ions are interconnected by bridging oxido and hydroxido groups and are stored as a crystal type structure of hydrated ferrihydrite.

1.1.1.2 Studies investigating iron-casein phosphopeptide bioavailability using Caco- 2 cells

Yeung, Glahn and Miller (2002) compared caseinate and phosphopeptides in the presence or absence of $FeSO_4$ using Caco-2 cells. The dialysability of the iron fortified peptides was the same as iron and ascorbic acid alone. The dialysability was significantly better than on the non-hydrolysed caseinate. It was speculated that the iron solubility was greater in the hydrolysed samples and could therefore pass through the membrane more efficiently. Whey proteins have a higher availability than caseinate, since the whey proteins do not hold iron as strongly as casein therefore more iron can be absorbed. The peptides enhanced absorption of iron in foods with low availability of iron however in the

presence of iron ligands such as citrate there was no enhancement. Several authors have shown that the phosphopeptide extract with no further purification had no significant enhancement of iron availability and only some peptides promote ferritin production. García-Nebot et al., (2013) compared pooled casein phosphopeptides and peptide isolates of α- and β- casein. The isolates improved production of ferritin while the pooled peptides were not significantly different to the controls. β-casein-(1-25) had the best response of ferritin production and it was speculated that it was probably due to the conformation of the α- and β- casein derived peptides being different when iron is bound, causing a difference in absorption and ferritin production.

The matrix that the peptides are in could also be important. García-Nebot et al., (2010) looked at the effects of adding milk or casein phosphopeptides with added iron, to fruit juice. When the fruit juice contained milk, the sample had the highest uptake and retention of iron compared to fruit juices that contained no milk or phosphopeptides. The authors believed that the milk may have been able to protect the iron from iron inhibitors found in juice such as polyphenols.

2.7 Conclusion

The literature review shows that iron and milk interactions have been extensively studied and are not limited to intact casein micelles. It is evident however that cow milk based systems have been extensively studied compared to goat milk. This presented the first interesting research question of whether the physicochemical differences of cow and goat milk affect how iron binds to the casein proteins of each species. Another question that rises from the literature is how iron fortified milk systems are affected by processing conditions, such as changes in temperature and the order of iron addition in the process. It would seem likely that heat treatments could affect the stability and binding of iron which may result in reduced functionality. An analysis of the changes in structure with the addition of iron and processing conditions can be carried out using small angle X-ray scattering to monitor changes on a structural level.

The literature has indicated that by reducing the protein size by isolating phosphopeptides the bioavailability of iron bound to these will be improved. The affinity for iron to these peptides is strong due to the concentration of phosphoserine clusters that can covalently bind iron. In addition to this, binding iron directly to the high affinity sites will reduce interactions with other sensitive components in milk. While studies have assessed the

bioavailability of goat casein derived peptides in animal models, technological considerations have been neglected. Looking at the isolation, fortification and stability in terms of processing feasibility are important factors with regard to producing an ingredient. The literature has revealed that there are several techniques to isolate these peptides from a digest mix such as the use of calcium and ethanol to precipitate the peptides or immobilised metal affinity chromatography.

The stability in formulation is important when fortifying with iron due to the strong pro-oxidant ability of the ferrous forms. The use of a linoleic acid emulsion to measure the TBARS formation has been used to measure the oxidative stability of iron- protein complexes. This has not been performed on iron bound phosphopeptides and is of interest to compare this to intact casein proteins. Similarly, Caco- 2 cells have been used as absorption models for iron uptake by milk proteins however the use of phosphopeptides and goat derived proteins in these models to determine iron bioavailability is scarce in literature. The opportunity for research has been shown to be on developing an ingredient from goat milk that can form a peptide- iron complex with a focus on considerations for industrial processing feasibility and looking at the potential bioavailability and stability of the ingredient.

3 Materials and methods

3.1 Composition of skim milk powders

Low heat skim milk powder was purchased from Fonterra Co-operative Ltd, New Zealand. Analysis was performed by Massey University Nutrition Laboratory.

Table 3-1: Composition of cow skim milk powder

Cow skim milk powder Manufactured by Fonterra Co-operative Manufactured: 9/11/2011 Batch number: B1654/DV09	
Nutrient	Quantity
Moisture %	4.4
Ash %	7.8
Protein %	33.5
Fat %	1.2
Calcium mg/kg	13000
Sodium g/100g	0.37
Phosphorus mg/kg	10700
Iron mg/kg	<5
Chloride g/100g	1.01
Analysis by the Massey Nutrition laboratory	

Goat skim milk powder was gifted by Dairy Goat Co-operative Ltd, New Zealand with the following specification.

Table 3-2: Composition of goat skim milk powder

Goat Skim milk powder Manufactured by Dairy Goat Co-operative (N.Z.) Ltd Batch number: 1700 JX17 Z8812/ TDO600M	
Nutrient	Quantity
Moisture %	3.3
Ash %	9.7
Protein %	36.3
Fat %	0.9
Analysis by Dairy Goat Co-operative (N.Z.) Ltd	

3.2 Studies on Iron fortified- skim milks: - sample preparation

3.2.1 Nomenclature

Dry blended milk refers to reconstituted skim milk powder with iron added which is tested without any further processing.

Wet blended milk refers to reconstituted skim milk powder with iron added which is then spray dried; the samples are reconstituted for analysis.

While the definition for dry blended milk does not exactly match the technical definition, it was used for simplicity to contrast the process of wet blending which is more accurate to what is used in industry.

3.2.2 Iron fortified dry blended milk

The method was adapted from previous studies that fortified milk with iron (Gaucheron et al., 1996; Raouche et al 2009a; Raouche et al 2009b). Cow and goat skim milk powders were rehydrated in Milli-Q water (5 % w/w protein content) for 2 hours at room temperature and chilled overnight at 4 ^{o}C to allow full powder hydration and mineral equilibration. This overnight hydration would allow an adequate time for full equilibration according to Anema & Li (2003). Chilled 0.5 M ferrous sulfate was added drop-wise to the milk solutions with rapid mixing to 5, 10, 15 or 20 mM final concentration. The pH was adjusted to 6.7±0.05 with 1 M NaOH and mixed for 2 hours at 4 ^{o}C; the pH was adjusted again if necessary. The solutions were made up to 3.5 % (w/w) protein concentration. True dry blending was not carried out due to the high levels of iron being added into solution. When reconstituted the pH would decrease which would affect the micelle stability. This method also allowed better comparison with the literature as many authors have studied iron binding in this way.

3.2.3 Iron fortified wet blended milk

Cow and goat skim milk powders were rehydrated in deionised water (30 % w/w solids content) for 2 hours at room temperature and chilled overnight at 4 ^{o}C. Chilled 1 M ferrous sulfate was added drop-wise to the rapidly mixed milk solutions to achieve 5, 10, 15, 20 mM final concentrations on a 3.5 % (w/w protein) basis. The pH was adjusted using 5 M NaOH drop-wise to reach a pH of 6.7±0.05 with rapid stirring in an ice bath and mixed for 1 hour.

3.2.3.1 Spray drying

A counter current spray drying system was used to dry the solutions (Mobile Minor™, GEA, Denmark). The solution was pre-heated to 55 ºC in a water bath via the feed line prior to spray drying. The inlet temperature of the spray dryer was 190 ºC and the outlet was maintained at 78-85 ºC by adjusting the flow rate of the feed. The upper and lower powder outlets were collected and kept separate.

3.2.4 Casein micelle partition

Casein partition of iron fortified skim milk powders was performed using an ultra-centrifuge (ThermoScientific, Sorvall WX+ Floor Ultra Centrifuge, rotor type: T-860, Germany). All samples were centrifuged at 90,000 xg at 20 ºC for 1 hour. Dry blended milks were centrifuged immediately after fortification and adjustment as stated in 3.22. Wet blended powders (3.2.3) were rehydrated to 3.5 % (w/w) protein content and mixed for 1 hour before ultra-centrifugation.

The centrifuged samples resulted in the supernatant containing the free iron and soluble proteins and the pellet containing the casein and bound iron. The supernatant was carefully decanted off for further analysis.

3.2.5 Moisture content of pellet

The pellets obtained from ultra-centrifugation were placed in accurately weighed moisture dishes and placed in a forced air oven set at 108 ºC for 3 hours. Sample weights were accurately measured and the difference in weights over the initial weight was the calculated moisture content.

Equation 3-1

$$Moisture\ content\ \% = \frac{Initial\ sample\ weight - Final\ sample\ weight}{Initial\ sample\ weight}\ x\ 100\%$$

3.2.6 Calcium removal from cow and goat skim milk

Amberlite IRC-50 (BDH Chemicals, England) is a weakly cationic resin that can be charged with sodium allowing ion exchange with calcium. The resin was prepared by hydrating the dry resin in 3 volumes of 1 M NaCl for 1 hour and then rinsing with Milli-Q water. The meq/L of the IRC-50 resin is 3.0 meq/ L therefore one litre of resin can bind 3.0 M of sodium ions or 69 g sodium. A 10 % (w/w) milk solution contains approximately

1.3 g of calcium per litre. The equivalent of calcium is 20.03 Eq; which means that the resin binds 60.09 g calcium per litre of resin. Cow and goat milk solutions (10 % w/w solids) were prepared and resin was added to achieve volume fraction of 0.025, 0.05, 0.075 and 0.25 mL in the milk. The milk with the resin was incubated at 4 ^{o}C for 5 hours and the milk was decanted off from the resin.

3.3 Studies on sodium caseinates- Isoelectric precipitation of sodium caseinate from cow and goat skim milk powders

3.3.1 Cow caseinate preparation

Isoelectric precipitation of caseins was carried out according to the information supplied by Munro & Carr (2011) at pilot plant scale. Cow skim milk powder was rehydrated in deionised water (10 % w/w solids) and chilled overnight at 4 ^{o}C. The casein was acid precipitated using 0.5 M H_2SO_4; the sulfuric acid was dumped into the solution with a predetermined volume found by titration. The pH was lowered to pH 4.6 at 15 ^{o}C, no stirring was carried out at this stage and the solution was held for 30 minutes at 15 ^{o}C. The solution was gently heated to 30 ^{o}C and held for 30 minutes then further heated to 40 ^{o}C. The precipitate was collected by filtration or manual collection of the large aggregates. The whey solution was heated to 40 ^{o}C to allow any lost solids to precipitate and be recovered again. The curd was washed twice in deionised water. The curd was filtered or manually collected and pressed to remove excess water. The precipitate was milled and solubilised with 1 M NaOH to pH 6.7 at 60 ^{o}C and then freeze dried.

3.3.2 Goat casein preparation

The method of precipitation was carried out similar to the cow milk. Skim milk was reconstituted by adding 4 kg of milk powder to 36 kg of deionised water at 20 ^{o}C with constant stirring. The solution was mixed for 2 hours and then chilled overnight at 4 ^{o}C. An aliquot of 0.5 M H_2SO_4 was then dumped in the milk. The pH was lowered to pH 4.2 at 15 ^{o}C and held for 1 hour with no mixing. The solution was heated to 30 ^{o}C. Further modifications to the methods will be discussed in section 5.2.1.2.

3.3.3 Iron fortification of caseinates

Caseinates were prepared by dissolving powders in Milli-Q water to (5 % w/w protein) by heating the solutions to 50 ^{o}C to aid solubilisation and the pH was adjusted to pH 6.7 when required. Samples were chilled overnight at 4 ^{o}C to allow hydration. Chilled ferrous

sulfate (100 mM) was added slowly to the required concentrations of 5, 11 or 22 mM with rapid mixing and the pH was adjusted with 1 M NaOH and made up to a final 3.5 % (w/w) protein concentration. Samples were kept chilled.

3.3.3.1 Solubility of caseinate protein with iron addition

The iron fortified caseinates were centrifuged at 10,800 xg for 20 min at room temperature and the supernatant was carefully removed for analysis.

3.4 Studies on cow and goat caseinate hydrolysates

3.4.1 Hydrolysis of cow and goat caseinate using trypsin

Hydrolysis of the goat and cow caseinate was performed with modifications from the methods of (Reynolds et al., 1994; Adamson & Reynolds, 1996; Strader et al., 2006). Sodium caseinates (35 mg/mL) were dissolved in 50 mM ammonium bicarbonate solution, pH 8.0. Commercial grade trypsin (6.0 PTN, Novozymes hydrated in 1 mM HCl at 4 mg/mL) was added to the solution at an enzyme to protein ratio of 1:50 or 1:200 with rapid stirring. Digestion was maintained at 37 ^{o}C overnight and samples were withdrawn at 0, 2, 4, 8 and 18 hours during hydrolysis. The reaction was stopped by heating the solutions to 75 ^{o}C for 10 minutes or by addition of formic acid at 1 % (v/v) final concentration.

3.4.2 Selective precipitation of phosphopeptides using calcium chloride

The hydrolysed caseinates after 18 hours of digestion were adjusted with 1 M NaOH to pH 4.6±0.1 and centrifuged at 12,000 xg for 10 minutes to removed undigested material. The serum was carefully separated and a 0.5 mL aliquot was taken for RP-HPLC profiling. The serum pH was increased to pH 6.74±0.03 and a 10 % $CaCl_2$ was added slowly at room temperature to a final 100 mM concentration and the solution was incubated at room temperature for 1 hour. Ethanol (95 % purity) was added to a final 50 % (v/v) concentration by slow addition. The solution was centrifuged at 12,000 xg for 15 minutes and the pellet and serum were collected and freeze dried.

The protocols described in the sections above are summarized in the following Figure 3-1.

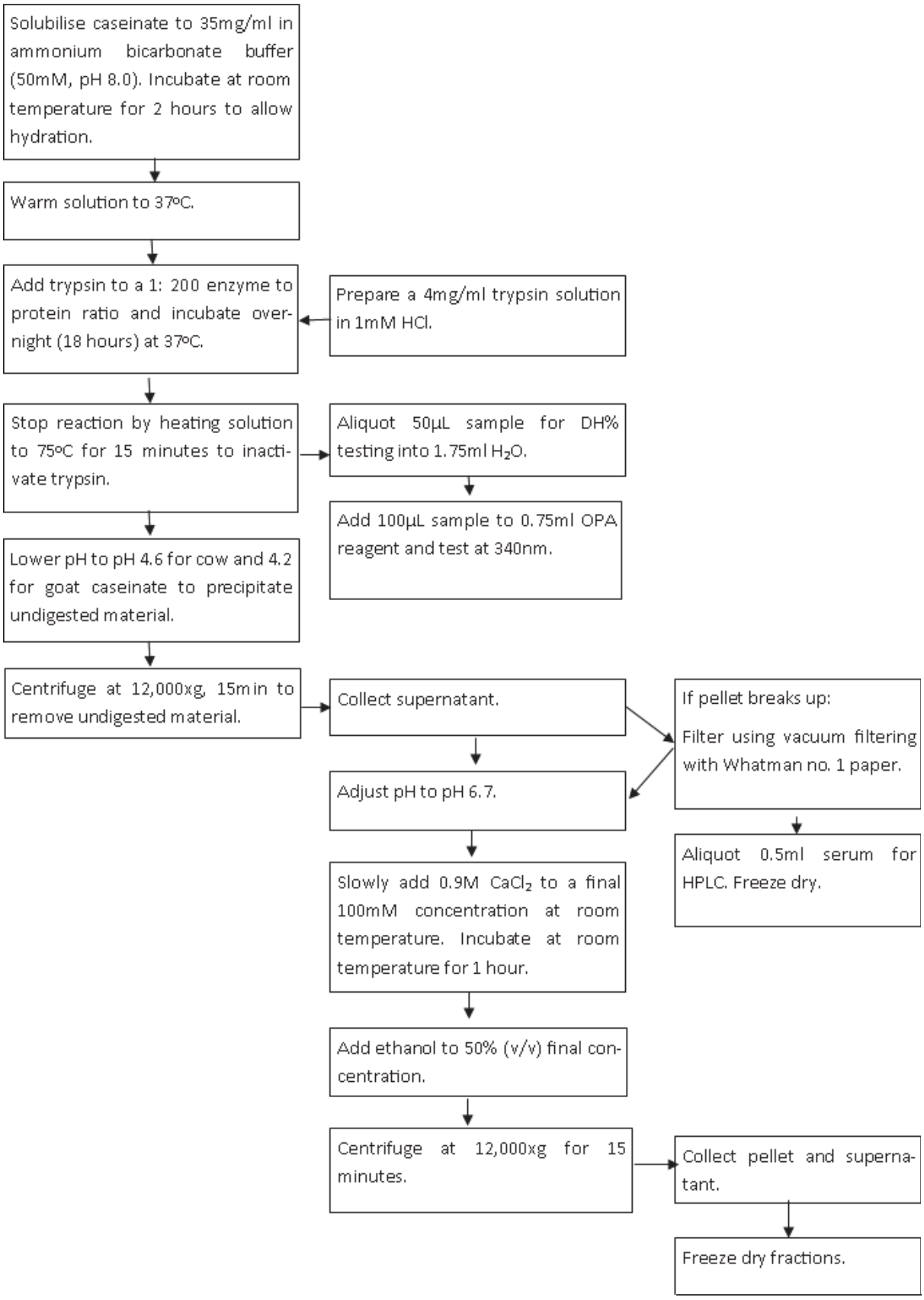


Figure 3-1: Protocol for hydrolysis and for the isolation of calcium precipitated phosphopeptides.

3.4.3 Selective precipitation of phosphopeptides using ferrous sulfate

The same method in section 3.4.1 was carried out for digestion, termination of the reaction and removal of insoluble material after digestion. The serum was adjusted to pH 6.67±0.02 and chilled to 4 ºC. A 10% (0.9 M in 1 mM HCl) $FeSO_4$ solution was prepared and chilled to 4 ºC. Under rapid mixing the ferrous sulfate solution was slowly added to a final 90 mM concentration and the solution was immediately chilled for 1 hour at 4 ºC; the pH was not adjusted. Ethanol (95 %) was added to a final 50 % (v/v) concentration. The solution was centrifuged at 12,000 xg for 15 minutes and the pellet and serum were collected and freeze dried.

3.4.4 Immobilised metal affinity chromatography for phosphopeptide isolation

Cow and goat caseinate hydrolysates (35 mg/mL) were dialysed against sodium formate (5 mM) at pH 4.0 overnight at 4 ºC to remove buffers and excess salt from hydrolysis. The protein content was measured using the Modified Lowry reagent and the required volume of protein solution was determined.

The resin (16 mL) was charged by adding 5 mL of 200 mM $FeCl_3$ (de la Hoz et al., 2014a; de la Hoz et al., 2014b) and washed with 5 bed volumes of MilliQ water followed by the elution buffer (water adjusted to pH 9.0 with ammonium solution) and washing buffer (water adjusted to pH 4.0 with formic acid). The resin was then divided into two equal volumes for the cow and goat caseinate samples.

The samples (containing 48 mg total protein) were incubated for 15 min with gentle swirling of the flask in the washing buffer and then the solution above the resin was carefully pipetted off. Approximately 5 bed volumes of washing buffer were applied to the resin and the first two washes of 5 mL and 10 mL were collected from this for analysis. The elution buffer was applied and the first three washes were collected for further analysis of 8 mL each. This was followed by a stronger elution buffer of 100 mM NaH_2PO_4 buffer, in which four fractions of 8 mL were collected. Sample fractions were frozen for amino acid and RP-HPLC analysis.

3.5 Studies on cow and goat phosphopeptides

3.5.1 Sample preparation

3.5.1.1 Dialysis to remove calcium from peptide precipitate

Sodium formate buffer was prepared by dissolving 1.5 mL of concentrated formic acid and 0.748 g sodium chloride in 900 mL MilliQ water and adjusting the pH to 3.0 with 1 M NaOH and made up to 1 L. Cow and goat calcium precipitated phosphopeptides were weighed out accurately to 10 mg and dissolved in 5 mL buffer. The solutions were pipetted into 0.1-0.5 kDa MWCO cellulose ester membrane (Spectrum Laboratories Inc., USA) and placed in the reservoir with 200 volumes of buffer. The buffer was chilled at 4 ^{o}C to reduce potential spoilage and to reduce the ionic interaction bonds between the calcium and protein. The buffer was changed 2 more times with buffer and then a final 2 hour dialysis with MilliQ. The total dialysis time was 40 hours.

3.5.1.2 Ion exchange of calcium using Amberlite IRC-50

The ion exchange capacity found in section 3.2.6 using milk was used to estimate the amount of resin required to remove the calcium from the phosphopeptides. Adding charged resin to 20% (v/v) in milk at 10 % solids removed approximately 80 % of the calcium. The phosphopeptides contained 6.5 % (w/w) calcium and therefore a 12.1 mg sample contained $7.9x10^{-4}$ g Ca. To fully remove the calcium, 0.11 mL resin was added to the solution. The solutions were chilled overnight although it was previously found that a 5 hour binding time was sufficient, concern was raised that the phosphopeptides may structure the calcium differently and may therefore have a stronger binding affinity. The samples were decanted from the resin.

3.5.2 Iron chelation measurement

Phosphopeptide retentates were diluted to 0.2 mg/mL with a total volume of 0.925 mL in a modified simulated milk ultra-filtrate buffer (20 mM imidazole, 30 mM NaCl, pH 6.7); the buffer was described by Anema & Li (2003) as a substitute for milk ultra-filtrate however the calcium was omitted. Ferrous sulfate (25 µL, 1 mM in 1 mM HCl) was added, vortexed and incubated at room temperature for 3 minutes. Ferrozine (0.04 mL of 6.09 mM) was added to stop the binding reaction and held for 10 minutes to allow colour development and then read at 562 nm. The spectrophotometer used throughout was Pharmica UV/visible Ultrospec II, (LKB Biochem, England).

3.5.2.1 Methodology variations to determine the iron binding properties of the phosphopeptides

3.5.2.1.1 Effect of ionic strength, pH and temperature on iron binding

Samples were heated at 4, 20, 37, 50, 60 and 72 ^{o}C in buffers at pH 2.75 (sodium formate), 4.6 (sodium acetate) or 6.7 (imidazole) with an ionic strength of either 50 mM or 400 mM; the ionic strength was adjusted using sodium chloride. Chilled ferrous sulfate was added to samples and allowed to bind for 3 min followed by ferrozine reagent.

3.5.2.1.2 Effect of different buffers at pH 6.7 on iron binding

Different buffers were tested at pH 6.7 to assess if the buffer type had an effect on iron binding. 50 mM HEPES and water adjusted to pH 6.7 were tested along with 50 mM imidazole at 4, 20, 37 and 72 ^{o}C. Chilled ferrous sulfate was added to samples and allowed to bind for 3 min followed by ferrozine reagent.

3.5.2.1.3 Effect of temperature ramping

Imidazole (pH 6.7, 50 mM) was used to look at the effect of increasing the temperature of the solution followed by cooling on iron binding. This was to determine if iron remained bound to the protein during cooling. Samples were heated to 72 ^{o}C from 4 ^{o}C and cooled to 4 ^{o}C in 100 min. Aliquots were taken during the heating and cooling process.

3.5.2.1.4 Effect of holding time

Samples at 20, 37, 50 and 72 ^{o}C were held for 3, 10, 15, 20 and 25 min in imidazole buffer (pH 6.7, 50 mM) to determine if the length of binding time affects the iron chelation and to also assess the stability of the peptide- iron mixture in holding phases in industrial processing.

3.5.2.1.5 Effect of iron loading

Different ratios of peptides and iron were tested in imidazole buffer (pH 6.7, 50 mM) at 20, 37 and 50 ^{o}C. The loading of iron to peptide was tested to determine the maximum binding capacity of the peptides.

3.5.2.1.6 Addition of peptides to a milk system

Different weight fractions of peptides were added to milk from 0.1 to 0.5 to determine what ratio could be used for ingredient use. Milk was hydrated in water (10 % w/w solids) for 2 hours and chilled overnight at 4 ^{o}C then diluted to 0.2 mg/mL in imidazole buffer

and the phosphopeptides were added to the required concentration. The order of addition of iron to the solution was also varied. The iron was either added to the peptide and held for 3 minutes and then added to the milk solution or the peptide and milk solution were mixed then the iron was added to this. This was done to determine if there is any difference in the binding capacity of the final solution depending on whether the iron is exposed only to the peptides first. Iron binding was carried out at 20 ^{o}C to limit the effects of temperature on oxidation.

3.5.2.2 Iron chelation equation

The iron chelation was calculated as follows:

Equation 3-2

$$Fe\ chelation = \frac{Abs\ blank - Abs\ sample}{Abs\ blank}$$

Equation 3-3

$$Fe\ chelation\ activity = \frac{Fe\ chelation\ x\ \mu M\ Fe\ added}{\frac{mg}{ml}\ protein}$$

The value was then converted from µM iron bound to mg Fe/ g protein via the required factors.

Equation 3-4

$$Fe\ bound = \frac{Fe\ chelation\ activity}{1000} * 55.8 g/mol$$

3.5.2.3 Method considerations

A blank was run containing only buffer under the same respective treatments. This was to show the stability of ferrous iron under the conditions set. As the ferrozine chelates and emits a purple colour only from free ferrous ions there is a false positive result in binding if iron in the solution oxidises to ferric iron. Using a blank therefore allows a better estimate of what proportion of iron oxidises due to the conditions. This does not allow for any potential interactions between the iron and protein or protein and buffer promoting

oxidation. The peptides tested were cow and goat phosphopeptides that contained calcium after dialysis and peptides that had calcium extensively removed by ion exchange.

Throughout the trials the ferrous iron concentration of the stock solutions was checked in buffer at 20 ^{o}C to ensure that the iron solution was not oxidising over the course of the experiment. Iron solutions were prepared fresh immediately before experimental runs had begun.

3.6 Mineral and protein estimation

3.6.1 Kjeldahl method

0.5 g of cow or goat skim milk powder was weighed into a digestion tube followed by two Kjeltabs and 15 mL concentrated H_2SO_4. The samples were heated to 420 ^{o}C for 45 minutes or until the solution was clear. 70 mL of hot distilled water was added with gentle shaking. 25 mL of 4% boric acid (400 g boric acid dissolved in 6 litres hot distilled water + 100 mL 0.1% bromocresol green made up in alcohol + 70 mL 0.1% methyl red solution made up in alcohol added, all diluted to 10 litres with distilled water) was added to a 250mL conical flask and placed in the distilling unit (Kjeltec 1030 System, Tecator Sweden) and the digestion tube connected. After running in automatic mode the sample was titrated with 0.1 M HCl until the end point (mauve-grey colour) was reached.

Equation 3-5

$$\%\ Nitrogen = \frac{(A\ x\ B)\ x\ 14\ x\ 100}{1000\ x\ C}$$

Where:

A = mLs HCl used

B = exact molarity (normality) of HCl

C = weight in g of original sample used

% of crude protein was calculated by a multiplying factor of 6.38.

The Kjeldahl nitrogen analysis with a multiplying factor of 6.38 gave the following results for the crude protein content:

Table 3-3: Cow and goat skim milk powder crude protein content

Milk powder sample	Protein content
Cow skim milk	31.3±0.01%
Goat skim milk	34.8±0.04%

3.6.2 Lowry method

The protein concentrations of dilute solutions were determined using the Lowry method with the Ohnishi and Barr modification (TP0200, Sigma, MO, USA) according to the manufacturer's directions. Samples were diluted in 0.85% sodium chloride solution in the range of 150-1000 µg/mL and 0.2 mL of sample was combined with 2.2 mL of Biuret reagent and incubated for 10 minutes followed by 0.1 mL of Folin Ciocalteau's reagent and incubated for 30 minutes. The samples were measured against a standard curve prepared from a stock protein standard provided. The absorbance was measured at 550 nm.

3.6.3 Iron binding analysis

All glassware was washed with mild detergent in hot water and rinsed thoroughly with RO water. Washed glassware was placed in a 10 % nitric acid bath for (18 hours) and then rinsed three times with RO water and then rinsed once with Milli-Q water (Millipore Milli Q system, Bedford, MA). Rinsed glassware was placed in a drying rack and covered until use.

The ferrozine method by Carter (1971) has a measurement range of $1x10^{-6}$ to $1x10^{-2}$ g/L iron. Samples were diluted to be within this range in water. 0.5 mL of sample was pipetted into an Eppendorf tube followed by 0.5 mL of ascorbic acid (20 mg in 100 mL + 1.67 mL conc. HCl) with rapid mixed followed by incubation at room temperature for 5 minutes. 0.5 mL of 11% tricholoracetic acid was added, mixed and centrifuged at 3,000 xg for 10 minutes to removed insoluble material. A 1 mL aliquot of the supernatant was taken and 0.4 mL of 10 % ammonium acetate was added followed by 100 µL of ferrozine colour reagent (25 mg ferrozine + 25 mg neocuporine + 0.025 mL conc. H_2SO_4, made up to 25 mL), mixed and incubated for 5 minutes at room temperature. The solutions were measured at 562 nm and the iron concentration determined against a standard curve.

3.6.3.1 Ferrozine method validation

The ferrozine iron assay was validated by measuring the detected iron content over three days to ensure that the response did not decrease over time from ageing of the reagents, namely the ascorbic acid solution. A linear response was formed that varied non-significantly confirming that the reagents are stable over at least three days, Figure 3-2.

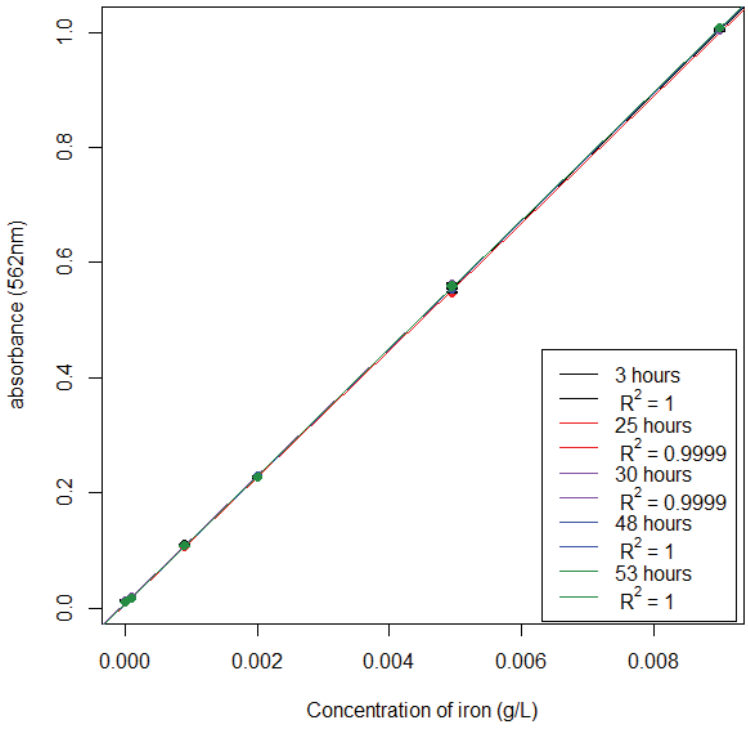


Figure 3-2: Response of iron detection over three days to assess stability of reagents.

3.6.4 Phosphorus assay

The phosphorus assay was performed according to Bartlett (1959). Samples were prepared by dissolving powders in water to a 0.7 mg/mL protein concentration and cation precipitated peptides were diluted to 0.07 mg/mL. 2 mL of the solution was pipetted into boiling tubes. Standards were prepared by diluting a 1.5 mM potassium phosphate solution in a concentration range from 0 to 0.4 mM, Figure 3-3. An aliquot of 0.5 mL of 5 M sulfuric acid was added to this, and heated at 155 ^{o}C for 3 hours. Hydrogen peroxide was added (0.1 mL, or until the solution was close to clear) and the solution was further heated for 90 minutes. Ammonium molybdate (0.22 %, 4.6 mL) was added followed by 0.2 mL of Fiske reducer (0.0625 g 1-amino-2-napthol-sulfonic acid and 0.125 g sodium sulphite in 25 mL 15 % sodium bisulfite (3.75 g), filtered). The solution was heated at 100 ^{o}C for 7 minutes and then read at 830 nm.

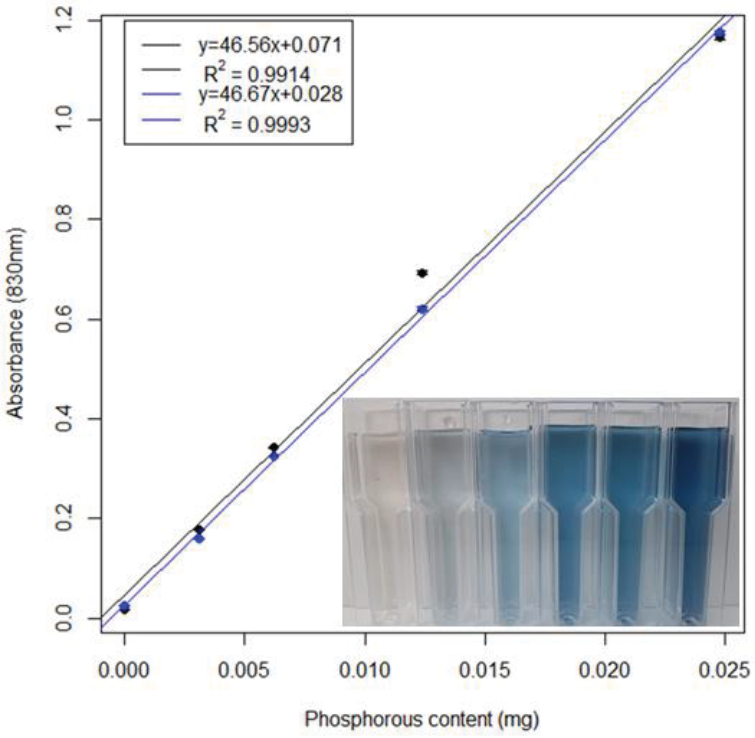


Figure 3-3: Phosphorus standard curve showing colour profile.

3.6.5 Calcium content

3.6.5.1 NM-BAPTA

The calcium content of the calcium precipitated samples was performed by the Massey University Nutrition Laboratory using the chromophore (5-nitro-5′-methyl-(1,2-bis(o-aminophenoxy)ethan-N,N,N′,N′-tetraacetic acid) NM-BAPTA.

3.6.5.2 Complexiметric Titration with EDTA

Total calcium was determined using EDTA titration with Eriochromeblack T indicator. Borate buffer was prepared by dissolving 3.332 g sodium tetraborate decahydrate in 80 mL water. Sodium hydroxide (0.54 g) and sodium sulphide (0.4165 g) were dissolved and added to the borate solution and made up to 100 mL. The indicator solution was made by dissolving 0.1 g of Eriochromeblack T powder in 3 mL water and 0.1 mL of 1 N sodium carbonate and made up to 10 mL with 2-propanol. A 1 mM or 5 mM EDTA titrant was prepared with disodium EDTA dehydrate. For a 25 mL sample, 0.5 mL of borate buffer was added followed by 0.025 mL indicator. The endpoint was achieved when a light blue colour developed.

3.7 Characterisation Techniques

3.7.1 Dynamic Light scattering

Particle size measurements were taken at 25 ^{o}C with 173 ^{o}C backscatter using the Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) based on intensity, volume and number . Three readings, each an average of 13 measurements, were carried out automatically by the instrument. Samples were not diluted as the instrument could read the solutions at the concentrations used in this study. The average diffusion coefficients of protein particles were calculated from changes in light intensity and were converted to average particle diameters following the Stokes-Einstein correlation for spheres (Anema & Li, 2003).

3.7.2 Zeta potential

The zeta potential (ζ) values of the protein particles were measured using a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK), through a combination of laser Doppler velocimetry (LDV) and phase angle analysis light scattering (M3-PALS) techniques. The zeta potential is related to particle electrophoresis. Samples were diluted in simulated milk ultra-filtrate (SMUF) (20 mM imidazole, 30 mM NaCl, 5 mM $CaCl_2$, pH 6.7) to the required dilution factor, usually around 100 fold. One millilitre of the sample was injected into one of the ports of a universal folded capillary cell (DTS 1060, Malvern Instruments Ltd., UK) equipped with platinum electrodes and any air bubbles were removed, before inserting the stopper. The measurements were carried out at 25 ^{o}C. Three readings were taken, each the result of up to 100 measurements, this was done automatically by the instrument in zeta potential mode.

3.7.3 Turbidity

Samples were measured at 515 nm according to Pearce & Kinsella (1978) using a 1cm light path.

Equation 3-6

$$\tau = \frac{2.303\ x\ absorbance}{path\ length\ (cm)} \quad cm^{-1}$$

3.7.4 Small angle X-ray Scattering (SAXS)

3.7.4.1 Theory

SAXS determines the structure of a particle based on averaged particle sizes or shapes (Schnablegger, & Singh, 2011). X-rays on the length scale of <0.3 nm are used which when passed through a sample scatter light from areas with different electron density contrasts, producing scattering patterns which can be transformed into SAXS profiles.

The magnitude of the scatting vector, Q, is defined as

Equation 3-7

$$Q = \frac{4\pi}{\lambda} sin\theta$$

Where λ is the wavelength and 2θ is the scattering angle.

The method used for fitting the data in the study was based on calculating the intensity based on the size distribution $n(r)$; the form factor $f(qr)$, describing the shape of the particles; and the structure factor $S(qr)$, describing the interactions between the particles. The intensity $I(q)$ is calculated for each population of scattering objects by fitting the four populations to the model using the following equation for scattering:

Equation 3-8

$$I(q) = \sum_{populations} (\Delta\rho)^2 a \int n(r).(f(qr))^2.V(r)^2.S(qr).dr$$

3.7.4.2 Iron fortified milk powders

Native cow and goat iron fortified skim milk solutions were prepared according to 1.2.2 and reconstituted milk was prepared from the spray dried milk in 1.2.3 (3.5 % w/w protein). Samples were held for 1 hour and the pH adjusted to pH 6.7. Samples were immediately tested after this holding time at 4, 25 or 37 ºC using a double walled beaker circulating thermostatically controlled heated or cooled water.

The background samples were made by filtering reconstituted milk samples through a 0.2 μm Whatman filter followed by a 20 nm Whatman filter to remove the casein micelles.

The background is important as the background need to be subtracted from the sample scattering and is necessary in the high q region.

3.7.4.3 Instrumentation

Data was collected using the SAXS/ WAXS beam-line with a detector distance of 7 m at the Australian Synchrotron (Clayton, Melbourne) and an X-ray wavelength of 1.7712 Å (7.000 keV) was used. One second exposures were collected in a continuous fashion while the solution was flowing through a quartz capillary placed in the X-ray beam path with a peristaltic pump. Twenty exposures were averaged together.

Data was transformed via *scatterBrain* (developed in-house at the Australian Synchrotron). The background (milk filtrate) was subtracted and data fitted using the Irena macros in Igor (Ilavsky & Jemian, 2009). Scattering curves were plotted as functions of intensity versus Q ($Å^{-1}$).

3.7.5 OPA for Degree of hydrolysis

The degree of hydrolysis was determined by the method of Nielsen et al., (2001). Samples were diluted to 0.8 mg/mL and a 100 µL aliquot was mixed with 0.75 mL OPA solution (1.905 g di-Na-tetraborate + 50 mg SDS dissolved in 37.5 mL H_2O + 40 mg o-phthaldialdehyde in 1mL ethanol added + 44 mg dithiothreitol, all made up to 50 mL). Samples were held for exactly 2 min before being measured at 340 nm using an Ultrospec Pharmacia UV/ visible spectrophotometer. Calculations were carried out using the casein value constants 1.039, 0.383 and 8.2 for α, β and h_{tot}, respectively. The standard used was 0.01 % (w/v) serine solution with a milli equivalence of 0.9516 meqv/L.

Equation 3-9

$$Serine - NH_2 = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} x \frac{0.9516 meqv}{L} x\ 0.1\ x \frac{100}{X}\ x\ P$$

Where X is the g of sample, P is the protein (%) in the sample and 0.1 is the sample volume in litres.

Equation 3-10

$$H = \frac{serine - NH_2 - \beta}{\frac{\alpha\ meqv}{gprotein}}$$

Equation 3-11

$$DH\ \% = \frac{H}{h_{tot}}\ x\ 100\%$$

3.7.6 RP-HPLC analysis

RP-HPLC was performed with a 1200 Series apparatus from Aligent Technologies (Santa Clara, CA, 95051 United States) equipped with an G1311A Quaternary pump and 1200 Series sample injector and UV detector. The chromatographic software used was EZChrom (Santa Clara, CA, 95051 United States). The apparatus was equipped with a RP column, Prevail C18 (150 x 4.6 mm i.d., 5 µm pore size; Chromservis, CZ-109 00 Prague, Czech Republic).

3.7.6.1 Caseins and caseinates

Caseins and caseinates were dissolved to 2 mg/mL in buffer A. The samples were centrifuged for 10 min at 13,000 xg and the supernatant was collected for analysis. A linear gradient was performed with buffer B; with 20 mL/L buffer in 5 min followed by 20 to 650 mL/L buffer within 25 min followed by a further 650 mL/L buffer for 5 min (refer to Figure 3-4) (Buffer A: 95% H_2O, 5% MeCN, 0.1% TFA; Buffer B: 95% MeCN, 5% H_2O, 0.08% TFA). At a flow rate of 1 mL min^{-1}, 30 µL of sample was injected onto the column, maintained at 25 ºC and the absorbance was measured at 214 nm and 280 nm.

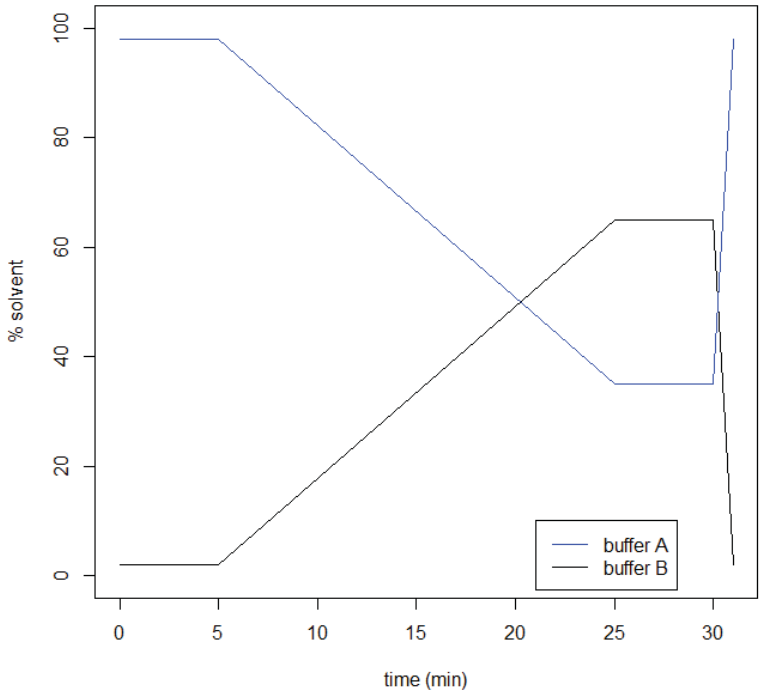


Figure 3-4: Buffer gradient for RP-HPLC elution of casein and caseinates, buffer A: 95% H2O, 5% acetonitrile, 0.01% trifluroacetic acid; Buffer B: 95% acetonitrile, 5% H2O, 0.08% trifluroacetic acid.

3.7.6.2 Caseinate digests

Samples were diluted by 1:10 with 95% MilliQ water and 5% acetonitrile. A linear gradient was performed with 50 mL/L buffer B in 1 min followed by 50 to 450 mL/L buffer B within 8 min followed by 450 to 900 mL/L within 14 min (Figure 3-5). At a flow rate of 1 mL min^{-1}, 5 µL of sample was injected onto the column, maintained at 25 ^{o}C and the absorbance was measured at 214 nm and 280 nm.

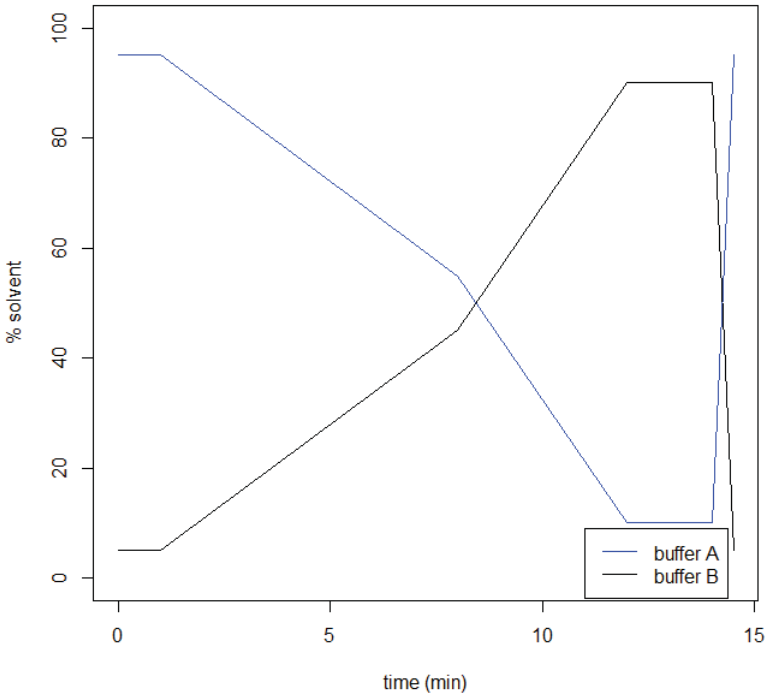


Figure 3-5: Buffer gradient for RP-HPLC elution of caseinate digests, buffer A: 95% H2O, 5% acetonitrile, 0.01% trifluroacetic acid; Buffer B: 95% acetonitrile, 5% H2O, 0.08% trifluroacetic acid.

3.7.6.3 Hydrolysates and phosphopeptides

Samples were dissolved to 2 mg/mL in buffer A. EDTA was used to an approximate 1:1 ratio to remove metal ions when present. Samples were centrifuged for 10 minutes at 13,000 xg and the supernatant was collected for analysis. A linear gradient was performed with 50 mL/L buffer B in 5 min followed by 50 to 600 mL/L buffer B within 120 min followed by 600 to 900 mL/L within 121 min (Figure 3-6). At a flow rate of 1 mL min^{-1}, 5 µL of sample was injected onto the column, maintained at 25 ºC and the absorbance was measured at 214 nm and 280 nm.

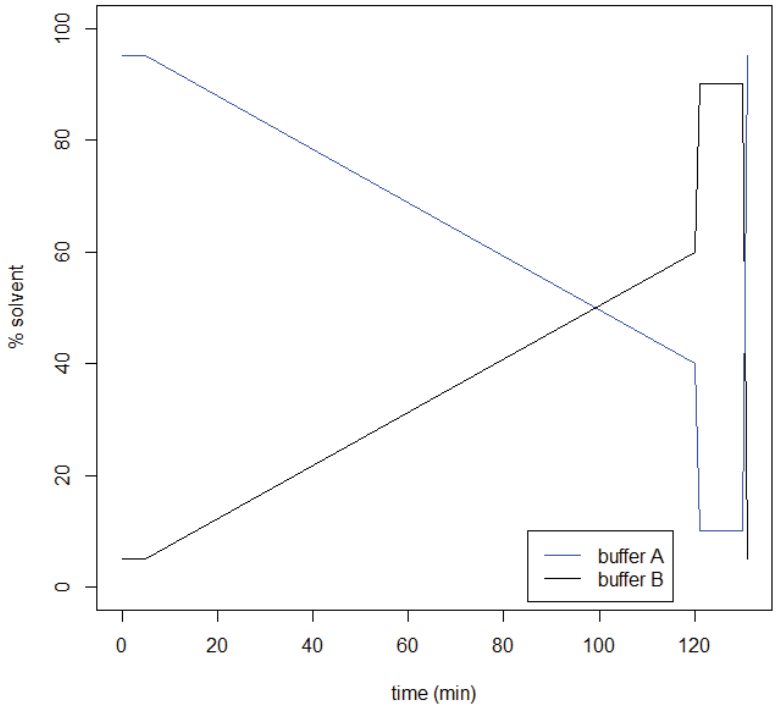


Figure 3-6: Buffer gradient for HPLC elution of hydrolysates, buffer A: 95% H2O, 5% acetonitrile, 0.01% trifluroacetic acid; Buffer B: 95% acetonitrile, 5% H2O, 0.08% trifluroacetic acid.

3.7.7 Amino acid composition

The amino acid analysis was performed by the Massey University Nutrition Laboratory for the cow and goat caseinate and phosphopeptides. The methodology used was Standard AAA Hydrochloric acid hydrolysis followed by RP-HPLC separation using AccQ Tag derivatization, AOAC 994.12.

3.7.8 Protein- mineral oxidation activity

Samples were analysed according to Sugiarto (2010) and Hegenauer (1979a) using linoleic acid as the lipid to be oxidised. The emulsion was prepared with linoleic acid (1.402 g) and Tween 20 (1.402 g) in 250 mL phosphate buffer (0.2 M, pH 7.4) using a homogeniser (11,000 xg, 2 min) (Ultra-Turrax, IKA-Werke GmbH & Co., KG). Protein samples were prepared by dissolving skim milk or peptides to 2 % protein content. The iron and protein solutions were combined at equal volume to produce a final 1 mM Fe and 1 % protein solution. A blank control and a positive control were included in the trial. The positive control contained 1 mM iron in 50 mM imidazole buffer while the blank contained buffer only. Solutions (8 mL) were added to 40 mL of the emulsion and

incubated at 30 ºC for up to 72 hours. 5 mL aliquots were taken at the required time for analysis.

Thiobarbituric acid reagent (TBA) was prepared by dissolving 0.036 g in 1 M NaOH and this was made up to 10 mL with water. 10 mL of phosphoric acid/ citric acid (2 M H_3PO_4/ 2 M) was added. An aliquot of 2.5 mL of TBA reagent was added to 5 mL sample and placed in a water bath at 90 ºC for 10 min. The solution was immediately cooled on ice. This produces a red colour from the malondialdehyde (MDA). A 1.9 mL aliquot of this was placed in a new centrifuge tube and 1.25 mL cyclohexanone and 0.25 mL ammonium sulfate (4 M) was added and then samples mixed for 2 min then centrifuged at 3000 xg for 10 min. The coloured complex was pipetted off and measured at 532 nm.

A standard curve was prepared by the acid hydrolysis of 1,1,3,3 tetraethoxypropane (TEP). TEP breaks down into malondialdehyde; 73.2 mg of TEP was weighed into a test tube and dissolved in 10 mL of 0.1 N HCl. The solution was heated in a water bath at 90ºC for 5 min and cooled by running under water. The solution was diluted to 239 µg/mL by diluting the solution in 100 mL water and this was then diluted to give concentrations from 0 to 1.9 µg/mL (Figure 3-7).

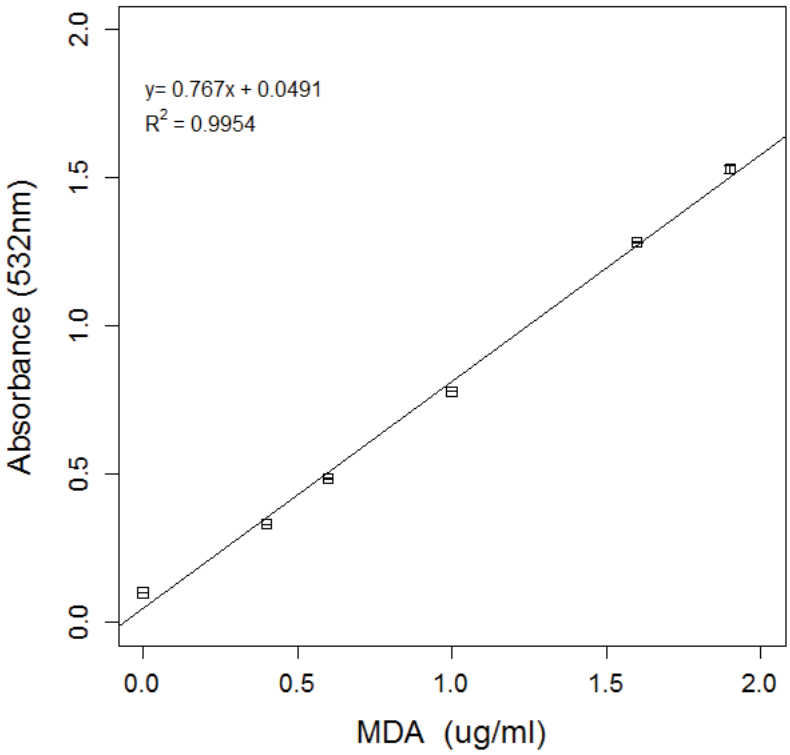


Figure 3-7: Standard curve of acid hydrolysed 1,1,3,3 tetraethoxypropane (TEP) for the determination of malondialdehyde content.

3.7.9 Caco-2 cell lines

The Caco-2 cell line work was performed by Dr. Robin Stewart; the method is described briefly here from unpublished work. The iron concentration of the samples were determined by the ferroin assay and standardised by diluting the sample to a 1:10 ratio and adjusting the weight of sample accordingly to 1mL. The diluted sample was then made up to 5 mL with saline at pH 2.0 which was then combined with activated pepsin and the pH was checked to pH 2.0. The solutions were digested at 37 ^{o}C for 2 hours to simulate the gastric phase followed by neutralisation with bicarbonate and activated pancreatin and bile salts added and titrated to pH 6.7. The volumes were made up to 15 mL with saline solution, maintaining the pH at 6.7. The samples were incubated at 37 ^{o}C for 120 min to simulate the intestinal phase then passed through a 10 kDa filter to mimic the mucosa wall and the iron content was measured. The uptake phase involved the use of the Caco-2 cells; these had been incubated with FBS-free DMEM for 24 hours and the samples were combined with the DMEM at a 1:1 ratio; 1 mL of sample was required. The cells with samples were incubated for 24 hours. After this incubation the cells were washed, lysed and ferritin and total protein concentration assayed.

3.8 Statistical analysis

Data was analysed using one-way, two-way ANOVA or a two sample t-test after checking for homogeneity of variances using F-test where appropriate (Harrell et al., 2014). Analysis was carried out using R version 3.1.1 for Windows, 32 bit.

4 Iron fortification of goat and cow skim milk

4.1 Introduction

Casein micelles are a complex association of four types of casein proteins and minerals of which the structure is still disputed (Walstra & Jenness, 1984; Horne, 2002; Horne, 2006; Dalgleish, 2011). These proteins: α_{s1}-, α_{s2}-, β- and κ- casein make up approximately 80 % of the protein content in bovine milk (Holt et al., 1986) and have a rheomorphic protein conformation, with some secondary structure. The average micelle hydrodynamic diameter is 200 nm and the proteins are held together by colloidal calcium phosphate and weak interactions. The micelles are stabilised mostly by steric forces from κ- casein protruding out of the micelle. On a dry weight basis 7 % of the micelle is inorganic material including calcium, phosphate, citrate and magnesium (de Kruif et al., 2012).

Many studies have investigated iron fortification of cow skim milk (Demott and Dincer, 1976; Hegenauer, Saltman & Nace, 1979; Gaucheron et al., 1997; Hekmat and McMahon, 1998; Raouche et al., 2009a; Raouche et al., 2009b), fractionated milk, (Demott & Park, 1974; Hekmat & McMahon, 1998); casein micelle suspensions, (Philippe et al., 2005), caseinates (Gaucheron et al., 1996; Sugiarto et al., 2009), and individual caseins (Baumy & Brule 1988; Emery, 1992) while only one paper, investigating processed caseins and whey, reports on both iron binding and digestion studies (Hurrell et al., 1989). Iron binds predominantly to the casein fraction, with some whey and low molecular weight compounds such as citric acid binding iron (Raouche et al., 2009a). Casein micelles have a high capacity to incorporate iron into the structure by binding via the phosphoserine groups as well as acidic residues of tyrosine, tryptophan, glutamic acid and aspartic acid (Gaucheron, 2000). About 85 % of added $FeCl_2$ iron is bound to casein with 72 % binding to the α_s- caseins. The preferential binding by α_s- caseins is thought to be due to the phosphoserine residues via co-ordination bonds (Demott & Dincer, 1976, Gaucheron et al., 1997; Hekmat & McMahon, 1998; Raouche et al., 2009a). Many studies have been carried out that measure the iron binding capacity of various milk solutions under different conditions (Demott & Park, 1974; Gaucheron et al., 1996; Gaucheron et al., 1997; Hekmat & McMahon, 1998; Raouche et al., 2009b). Additionally three studies focused on iron absorption using phosphopeptides (Pérès et al., 1999; Yeung et al., 2002; Kibangou et al., 2005). While these authors speculated where the iron is binding using partition studies (Hekmat & McMahon, 1998; Philippe et al., 2005), few studies have

experimentally identified the binding sites directly. Raouche et al., (2009a) used ^{57}Fe Mossbauer spectroscopy which provides information on structures based on electron density. There has been argument against the iron binding to the CCP with Gaucheron (2000) stating that iron does not dissociate at the same pH as calcium and phosphate. Silva et al., (2001) used enzymatic digestion to show that iron was bound to the peptide backbone as opposed to Ca, Mg and Zn that are associated with the CCP.

For the current work it was important to understand the characteristics (casein micelle size, charge and isoelectric point) of the intact milk proteins for both species under study. These characteristics were explored first in non-fortified cow and goat milk focusing on the casein properties. The effects of iron were then studied with the casein micelle characterised in terms of the micelle size, iron partition, structural and physicochemical changes. Small angle X-ray scattering was used to investigate the internal structure of the iron fortified milk systems in both dry blended iron fortified milks and wet blended milks that had been spray dried after fortification. This was to gain a fundamental understanding of the effects of the species on iron binding. These studies also give insight into the casein micelle structure. While iron fortification of milk has been analysed extensively in cow milk there has been less work reported using goat milk therefore this work is important for understanding if iron binding in milk is affected by the species origin of the milk.

4.2 Results and Discussion

4.2.1 Cow and goat skim milk characterisation

4.2.1.1 Protein content of skim milk powders

The Kjeldahl nitrogen analysis with a multiplying factor of 6.38 gave the results in Table 4-1 for the crude protein content.

Table 4-1: Cow and goat skim milk powder crude protein content, n=3

Milk powder sample	Protein content
Cow skim milk	31.3±0.01 %
Goat skim milk	34.8±0.04 %

Proximate values were also obtained from the Massey University Nutrition Laboratory for the cow skim milk powder and from the manufacturer for goat skim milk powder of 33.5 % and 36.3 %, respectively. The protein content of cow and goat milk differs in terms of quantity and composition (Jandal, 1996; Haenlein, 2004; Park et al., 2007). Jandal (1996) and Park et al., (2007) reported the total protein content of liquid cow milk to be 3.2-3.3 % while there is much more spread in the goat protein content values, ranging from 2.90 % to 4.6 % (Jandal, 1996; Park et al., 2007).

4.2.1.2 Casein micelle hydrodynamic diameter

Figure 4-1 shows the particle size distribution from an average of three readings for cow and goat milk. Samples were measured according to the method in section 3.7.1. There was a significant difference between the cow and goat skim milk Z- average micelle size ($p<0.001$) with the goat milk having a smaller casein micelle size of 133.5±1.2 nm compared to cow milk of 180.7±1.2 nm, Table 4-2. On average, cow milk has a mean diameter of 200 nm (Horne & Dalgleish, 1985) however there is a range of micelle sizes ranging from 53 to 149 nm in radius (Dalgleish, Horne & Law, 1989). There is high variability in the literature on the average micelle size of goat milk therefore an average size is difficult to compare (Jenness, 1980; Pierre et al., 1995; Pierre et al., 1998). Jenness (1980) determined the goat casein micelle was smaller than cow micelles; this was supported by Haenlein (2004). Jenness (1980) reported a size distribution of goat micelles with most being smaller than 80 nm in diameter with only a small fraction reaching 200 nm. In contrast, Remeuf and Lenoir (1985) found the average diameter to be 260 nm for

goat and 180 nm for cow casein micelles. Raynal-Ljutovac et al., (2008) also stated that goat casein micelles are larger than cow. Pierre et al., (1995) used photon correlation spectroscopy and TEM to look at goat milk casein micelle size. Samples containing a high content of α_{s1} had an average size of 199 nm while α_{s1} depleted milk had an average diameter of 280 nm. They concluded that from compositional analysis the main difference was the α_{s1}-casein content and this therefore governed the size of the micelles. The large distribution of sizes reported by different authors was due to the genetic polymorphisms of the goat caseins which would influence the size of the micelles, as well as the sample preparation and measurement techniques used. Overall it would seem that the goat milk used in this study has a composition that produces relatively small micelles compared to literature values.

Table 4-2: Z- average size of cow and goat skim milk after centrifugation 10,000 xg, 45 minutes and filtration at 0.22 µM, n=3

Sample	Z-average ± standard error (d. nm)
Cow skim milk	180.7±1.2
Goat skim milk	133.5±1.2

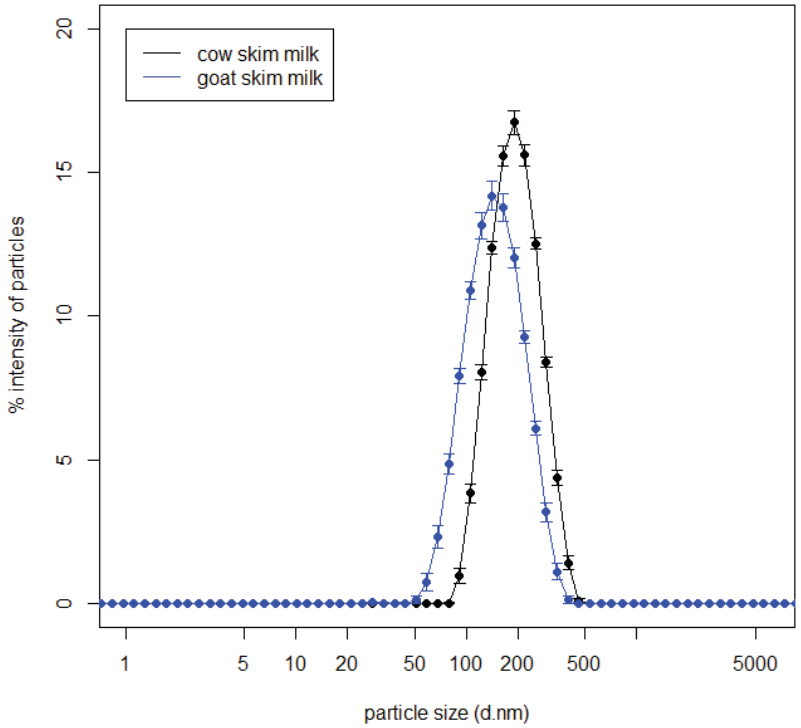


Figure 4-1: Cow and goat skim milk casein micelle size distribution by intensity; error bars indicate standard error, n=3.

4.2.1.3 Zeta potential

The zeta potential of the skim milks was -19.5 ± 7.7 for cow milk and -20.4 ± 8.6 for goat milk. There was no significant difference between the two species at 95 % confidence. The zeta potential values are similar to literature values: Anema & Klostermeyer (1996) reported a zeta potential of -19 mV at pH 6.7 for cow milk.

Table 4-3: Zeta potential of cow and goat skim milk at 1 mg/mL in SMUF, n=3

Sample	Zeta potential ± standard error
Cow milk 1mg/mL	-19.5±7.7
Goat milk 1mg/mL	-20.4±8.6

4.2.2 Iron fortification of goat and cow skim milk

Two methods of iron fortification using a ferrous sulfate solution were carried out to determine if different processing techniques affected the iron binding capacity of protein. The two methods, addition to the reconstituted skim milk or addition to reconstituted skim milk prior to spray drying and rehydration, were carried out to mimic the dry or wet

blending processes respectively that can be used in nutritional formulation manufacture. The methods are detailed in section 3.2.2 and 3.2.3.

4.2.2.1 Effect of iron addition to skim milk solutions

Iron is an acidic compound and therefore when ferrous sulfate is added the pH of the solution decreases. In addition to this when the iron binds to the caseins there is a release of H^+ ions (Gaucheron et al., 1997). As shown in Figure 4-2, the initial pH of the cow milk was pH 6.82 while the goat milk was pH 6.70. With 20 mM ferrous sulfate the pH dropped to pH 5.89 in cow milk while with goat milk the pH dropped to pH 6.05. There was no significant difference between the species in terms of the rate of pH drop ($p=0.346$). Gaucheron et al., (1997) measured the pH decrease with the addition of ferrous chloride to cow skim milk to the addition level of 1.5 mM giving a pH of 6.63. At the same iron addition level by fitting a linear regression our results gave a pH level of 6.65 for cow milk indicating that the rate of pH decrease was similar.

Park (1991) found that Nubian goat milk had a higher buffering capacity upon titration with acid compared to cow milk. Components that govern the buffering of milk are the protein, amino acid composition, primary caseins and phosphates. The milk samples prepared in this trial were made up to the same protein concentration (3.5 % w/w) and it is therefore likely that the different concentrations of P_2O_5 and amino acid composition caused the different buffering capacity with the addition of ferrous sulfate.

In the proceeding comparison of the milks upon the addition of iron the pH of the milks were adjusted to pH 6.7 to eliminate the factor of the different acidities with treatment. With increases in iron concentration there is a concomitant decrease in pH and thus the amount of NaOH required to adjust the pH of the milk to 6.7. This results in variation in the ionic strength of the pH adjusted iron fortified milks of about 20 mM. This ionic strength variation is assumed not to impact iron binding based on the study of Baumy & Brule (1988) who reported that iron binding is not affected by the ionic strength up to 100 mM for caseins.

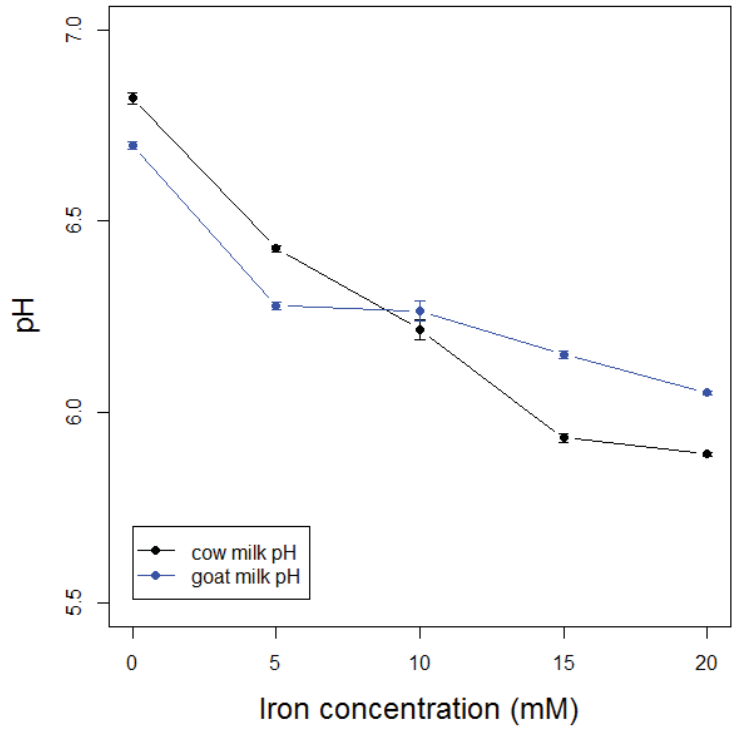


Figure 4-2: pH of cow and goat skim milk at 3.5 % (w/w) protein as 0, 5, 10, 15 and 20 mM of ferrous sulfate are added; error bars indicate standard error, n=3.

4.2.2.2 Particle sizing of iron fortified milks at pH 6.7

The particle sizes of the iron fortified samples were measured to determine if the addition of iron causes the micelle to change hydrodynamic diameter from the increase in ionic strength and/or iron binding to the casein micelle.

4.2.2.2.1 Particle sizing of dry blended cow and goat milk fortified with iron

Figures 4-3, 4-4, 4-5 and Table 4-4 present the micellar size distribution of cow and goat milk fortified with iron with dry blending. Although there was a significant difference ($p<0.001$) in the average micelle size between cow and goat milks, there was no evidence ($p=0.9257$) that iron fortification affects the micelle size of either species at pH 6.7. There was some evidence that the effect of the iron fortification level on the response of the Z-average size was influenced by the species type ($p=0.0597$).

The cow and goat milk controls had a Z-average diameter of 416.0 ± 3.1 nm and 321.5 ± 2.4 nm while with iron the average particle size increased to around 420 nm for cow and 340 nm for goat. This was not statistically significant for cow milk however the goat milk size did increase with iron, in terms of the average diameter, compared to the control

(based on Duncan's test at α= 0.05). Philippe et al., (2005) and Gaucheron et al., (1997) measured the micelle size of cow milk when ferric iron was added up to 8 mmol/kg and also found that there was no significant change in the size – micelles remained constant at 196 ± 17 nm in diameter. The results presented here are much larger than the typical sizes and those shown in Figure 4-1 as the milk solutions were not filtered to remove large particles which therefore caused an increase in size. Checking against the number distribution showed that there were no smaller populations of micelles present therefore the data is not measuring larger micelles or fat only. A search in the literature did not reveal any studies that measured goat casein micelles with iron fortification.

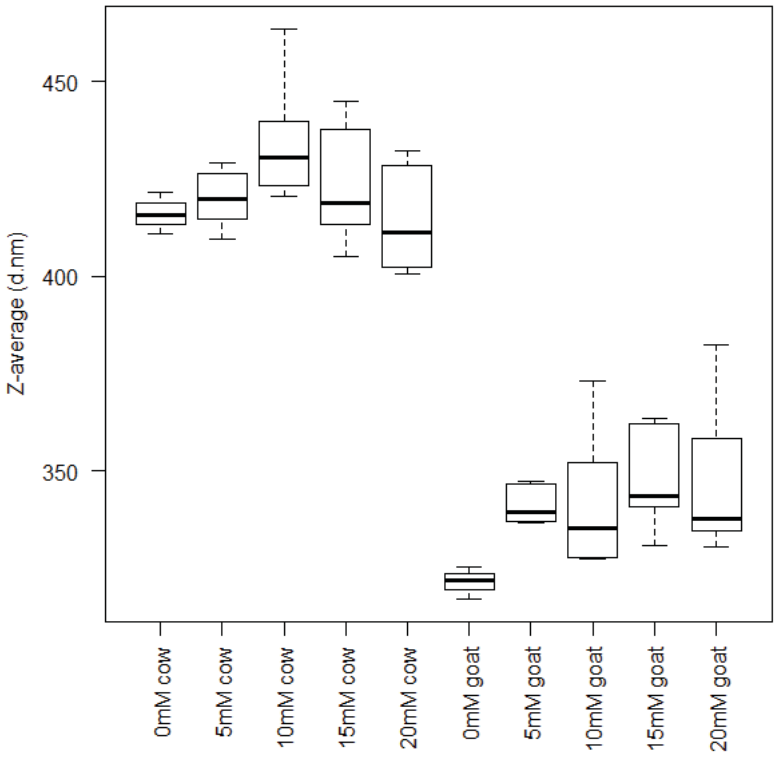


Figure 4-3: Box plot of dry blended cow and goat milk fortified with 0, 5, 10, 15 or 20 mM ferrous sulfate showing particle size distribution, significant differences are indicated in Table 4-4, n>3.

Table 4-4: Z-average particle diameter (nm) of cow and goat milk fortified with ferrous sulfate; superscripts with different letters indicate significantly different values at 95% confidence, n>3

Iron concentration	Cow milk nm ± standard error	Goat milk nm± standard error
0 mM	416.0 ± 3.1^{ab}	321.5 ± 2.4^{d}
5 mM	419.8 ± 3.6^{ab}	341.4 ± 2.3^{c}
10 mM	434.6 ± 6.5^{a}	341.8 ± 7.3^{c}
15 mM	423.1 ± 6.3^{ab}	347.3 ± 5.3^{c}
20 mM	414.3 ± 5.4^{b}	347.5 ± 5.8^{c}

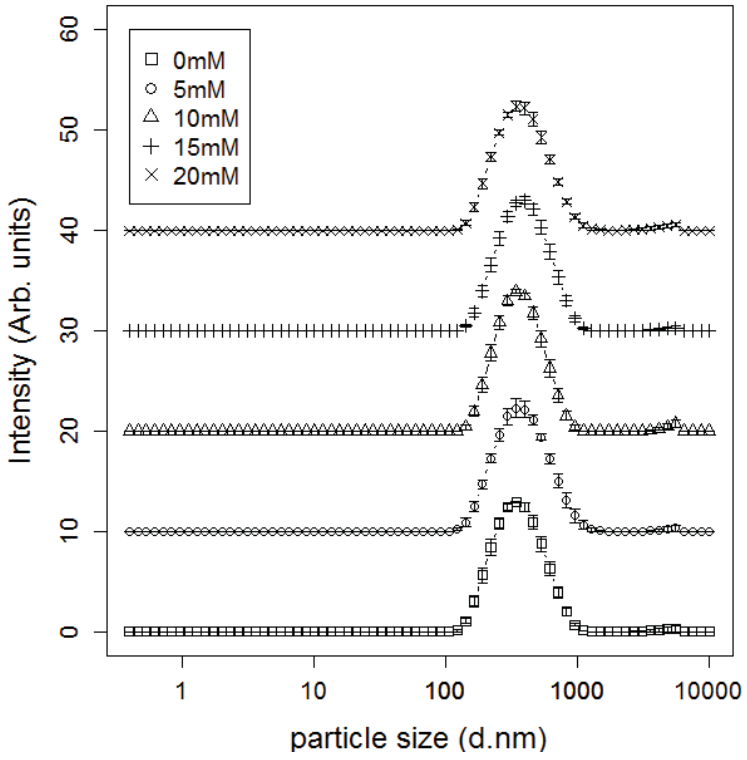


Figure 4-4: Size distribution of goat milk fortified with 0, 5, 10, 15 and 20 mM iron by Intensity in dry blended iron fortified milk; error bars indicate standard error, n>3.

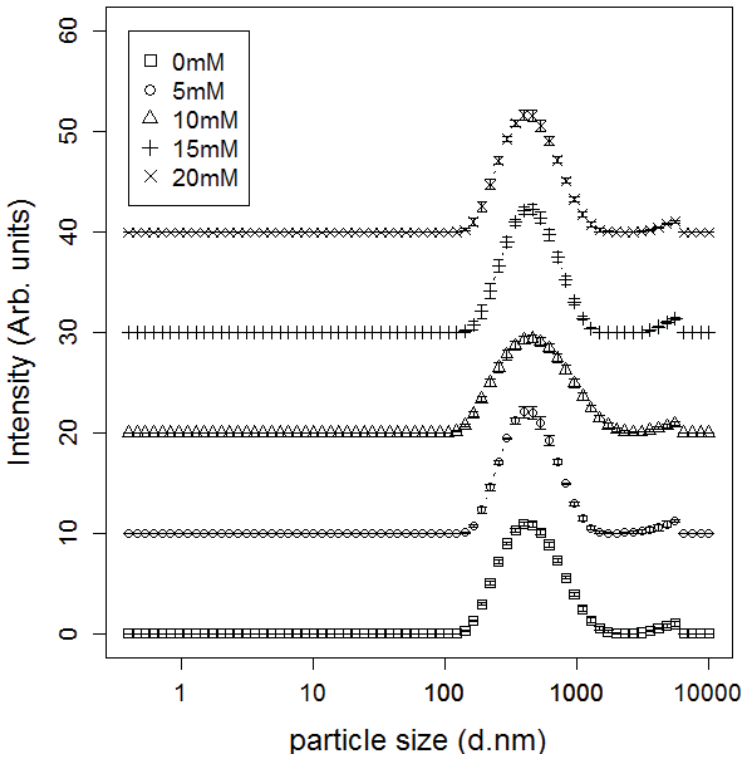


Figure 4-5: Size distribution of cow milk fortified with 0, 5, 10, 15 and 20 mM iron by Intensity in dry blended iron fortified milk; error bars indicate standard error, n>3.

4.2.2.2.2 Particle sizing of wet blended cow and goat milk fortified with iron

Figure 4-6, 4-7, 4-8 and Table 4-5 presents the particle size distribution of cow and goat milk wet blended with ferrous sulfate. When iron was added to milk, spray dried and then reconstituted the iron fortification level ($p=0.0136$) and the species type ($p<0.001$) were significant in determining the Z-average size of the casein micelles. The variation in the goat milk micelle size was considerably less varied in terms of micelle size. The only significantly different sample in terms of micelle size was at 5 mM iron of 368.7 ± 1.7 nm, compared to the control of 350.5 ± 2.8 nm ($p=0.005$). The cow milk samples were all significantly larger in diameter than the goat milk samples. There was a large variation of sizes with respect to the cow milk samples. The 5 mM and 10 mM iron fortified cow milks were significantly larger than the same treatment in dry blended milk at 502.8 ± 8.5 nm ($p<0.001$) and 463.3 ± 4.1 nm ($p=0.022$), respectively. At 15 mM and 20 mM iron addition there was a smaller average particle size than the control (423.9 ± 1.7 nm) at 394.0±2.8 nm ($p=0.001$) and 403.7 ± 5.7 nm ($p=0.054$), respectively. It is unlikely that the conditions used in pre-heating and spray drying of the milks was in the temperature range that would have caused whey protein denaturation leading to larger micelles

(Martin et al., 2007). It is unclear why there was considerably more variation in the micelle size of the cow milk samples in this treatment group and it is unknown if it was due to experimental variation or a real unidentified variable under these conditions.

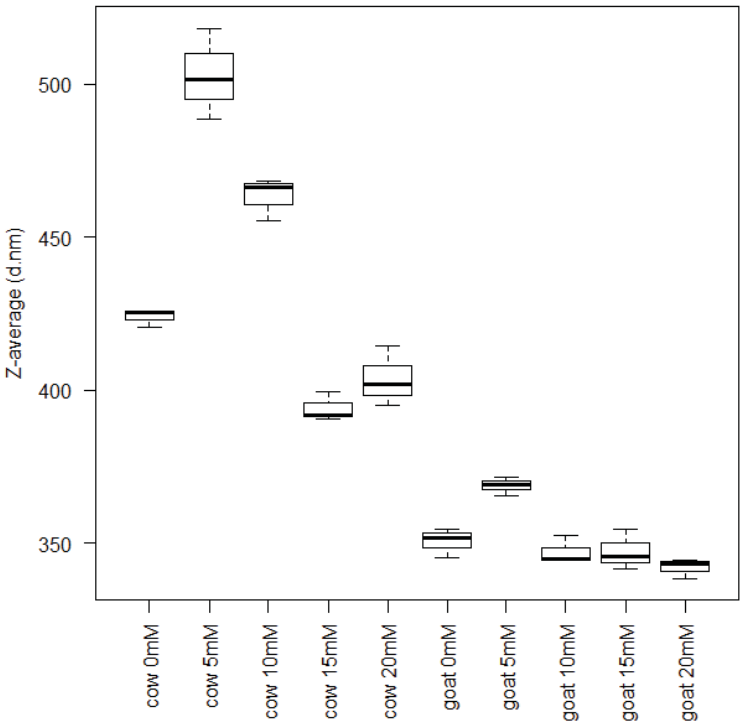


Figure 4-6: Boxplot of Z-average diameter of wet blended iron fortified cow and goat milk; significant differences are indicated in Table 4-5, n >3.

Table 4-5: Z-average particle diameter (nm) of wet blend cow and goat milk fortified with ferrous sulfate; superscripts with different letters indicate significantly different values at 95% confidence, n>3

Iron concentration	Cow milk nm± standard error	Goat milk nm± standard error
0 mM	423.9 ± 1.7^{c}	350.5 ± 2.8^{f}
5 mM	502.8 ± 8.5^{a}	368.7 ± 1.7^{e}
10 mM	463.3 ± 4.1^{b}	347.1 ± 2.6^{f}
15 mM	394.0 ± 2.8^{d}	347.2 ± 3.8^{f}
20 mM	403.7 ± 5.7^{d}	342.0 ± 1.8^{f}

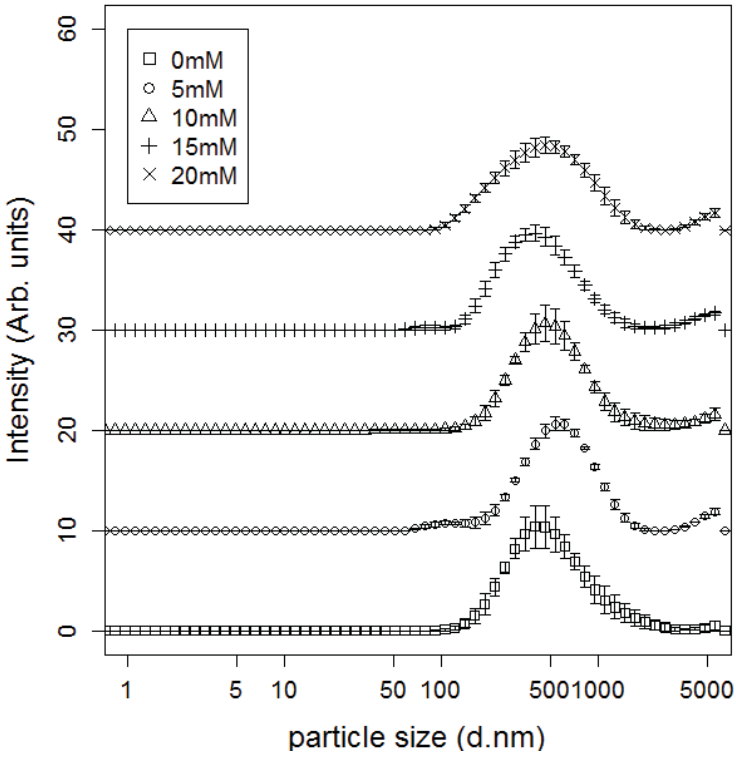


Figure 4-7: Size distribution of cow milk fortified with 0, 5, 10, 15 and 20 mM iron by Intensity in wet blended iron fortified milk; error bars indicate standard error, n>3.

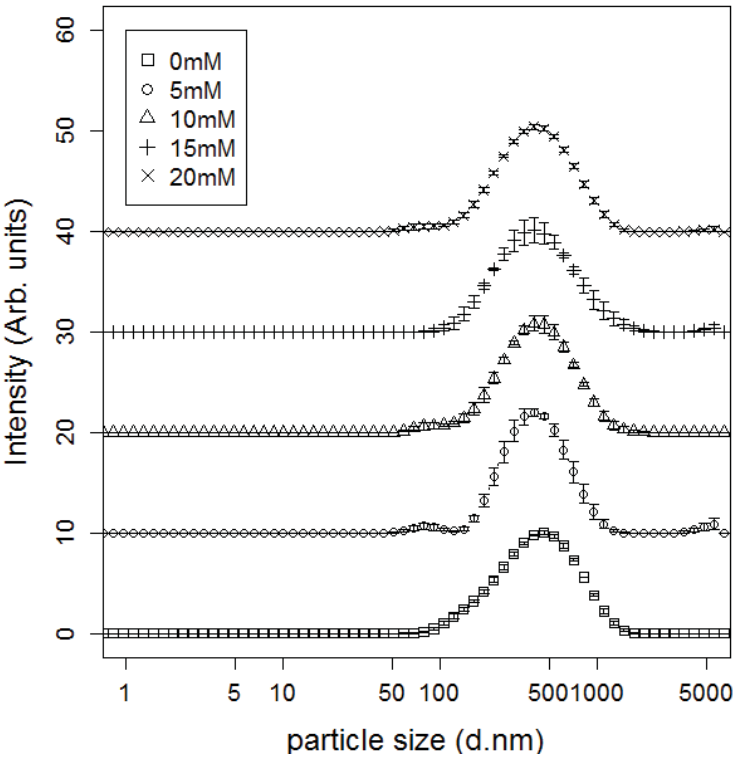


Figure 4-8: Size distribution of goat milk fortified with 0, 5, 10, 15 and 20 mM iron by Intensity in wet blended iron fortified milk; error bars indicate standard error, n >3.

4.2.2.3 Iron binding of goat and cow micellar phase by partition

The pellet and supernatant phase were partitioned according to the method in section 3.2.4. The amount of iron bound to the micellar fraction was determined by mass balance; the total and supernatant iron concentrations were determined and the difference was assumed to be the micellar content (Gaucheron et al., 1997; Philippe et al., 2005; Raouche et al., 2009a; Raouche et al., 2009b). The iron binding characteristics of cow and goat milk prepared under the different conditions are presented in terms of the fraction bound to the pelleted phase and on a basis of mg iron bound/ g of hydrated pellet.

Figures 4-9 and 4-10 show the amount of iron bound with increasing ferrous sulfate addition in dry and wet blend formulations. The ratio of iron that bound to the micellar phase was different with the different processing methods. The binding of dry blended cow milk was on average 0.73 while the wet blended milk was 0.80 ($p=0.003$). Similarly, the fraction of iron bound in dry blended goat milk was on average 0.72 while the wet blended milk gave a binding ratio of 0.86 ($p<0.0001$). The iron fortification level ($p<0.0001$) and processing method ($p=0.0491$) significantly affected the fraction of iron that bound to the micellar phase while the species type was not ($p=0.7127$). There is always a fraction of iron that does not bind to the micelles (Gaucheron et al., 1997; Hekmat & McMahon, 1998; Raouche et al., 2009a; Raouche et al., 2009b). This is evident when the milks for all treatments were analysed without the addition of any iron; the fraction of total native milk iron bound to the pellet phase was low at around 0.25 for dry blended cow and goat milk. For cow and goat wet blended milk this increased to 0.3 and 0.5, respectively. In contrast to the dry blended milk (where skim milk powder was rehydrated), de la Fuente et al., (1997) found that in raw goat milk 56% of the iron was in the colloidal phase of the milk indicated that the wet blended goat milk was closer to the literature value for the distribution of natively occurring iron. The low colloidal binding indicates that there is a higher affinity for the natively occurring iron to bind to low molecular weight compounds (such as free amino acids and citrates) or whey proteins, namely lactoferrin, that are not pelleted via ultra-centrifugation (Gaucheron et al., 1997). As these iron chelators are low in concentration the majority of the added iron binds to the casein micelles, however it appears there is an equilibrium between the micelle and serum phase as a similar proportion at all fortification levels occurs. In the wet blended cow and goat milk with no added iron there is a higher pelleted iron content than the dry blended milks. This may be due to the spray drying process forcing a

redistribution of the iron to the micellar phase – presumably through a combination or concentration (ex-evaporator) and/or heat treatment during the spray drying process.

The fraction of added iron bound to the micellar phase from the wet blended milks was similar to Raouche et al., (2009a) where 84-95 % of iron bound to the micellar phase and Raouche et al., (2009b) who found that micelles could bind 84-86 % of the ferrous chloride added. Both studies added up to 20 mmol/kg iron to milk. Philippe et al., (2005) only added up to 8 mmol/kg ferric chloride however found 99 % of the added iron bound to the colloidal phase. In contrast, Gaucheron et al., (1997) and Hekmat & McMahon (1998) added lower levels of iron to milk. Gaucheron et al., (1997) added up to 1.5 mM at which point 89 % of the iron bound to the micellar phase. Hekmat & McMahon (1998) added 1.8 mM iron which resulted in 83 % of the iron binding to the micellar phase. In the current study, the iron was added at 5 mM at the lowest concentration which makes comparisons with the authors' results difficult.

The weight of the pellets of the different treatments varied with iron fortification level and milk species and therefore the results were also presented in terms of mg iron/ g hydrated pellet. The iron bound to the micelles displayed a positive linear trend indicating that the casein micelle is able to bind increasing amounts of iron for both the dry and wet blended samples. The iron binding was greater with goat milk than cow milk in both processing conditions of wet blended milk ($p=0.0369$) and dry blended milk ($p=0.0003$). Mora-Gutierrez et al., (2010) also found that goat caseins were able to bind more iron than cow caseins. The authors found the result to be unexpected due to the higher β-casein content of goat milk and therefore fewer phosphoserine groups present. The comparison of the processing methods revealed that the interaction of the species type and iron fortification level effect is different for the different processing methods ($p=0.0007$). Therefore the processing method does have an effect on how much iron can be bound to the micellar phase of each species. The binding of the dry blended milk from cow milk was the poorest out of all the treatments with 6.92 ± 0.05 mg Fe/ g pellet when close to 20 mM iron was added, wet blended goat milk displayed an iron binding capacity of 10.64 ± 0.36 mg Fe/ g pellet with similar iron addition levels.

The wet blended samples were prepared by adding the iron when the milk solution was at high solids concentration (30 % w/w) which simulates the pre-evaporation step which can reach up to 50 % solids. Le Graët & Gaucheron, (1999) found that increasing the

casein concentration did not cause a change in the mineral content of the aqueous phase at pH 6.7 therefore the mineral content of the micellar phase increased as the mass of water decreased. Similarly, Liu et al., (2012) evaporated skim milk and determined that water was removed preferentially from the serum phase and soluble calcium and casein moved into the micellar phase. It is likely that when the iron was added into this environment there was a stronger driving force for the ferrous ions to either bind to the micelle or precipitate onto the casein micelles.

Hegenauer et al., (1979), Gaucheron et al., (1997) and Hekmat and McMahon (1998) carried out iron binding trials with fresh skim milk while Philippe et al., (2005) and Raouche et al., (2009a); Raouche et al., (2009b) used reconstituted skim milk powder. Due to the different iron loadings and methodology used in the literature it is hard to draw conclusions on whether the use of skim milk powder has an effect on the iron binding capacity as fresh skim milk was not used in the present study as comparison. Skim milk powder has the ability to mostly restore protein and mineral equilibrium after hydration (Anema & Li, 2003; Martin et al., 2007) and therefore the use of skim milk powder in the present study should not affect the iron binding capacity. No literature was found on the iron binding behaviour of milk spray dried after iron addition.

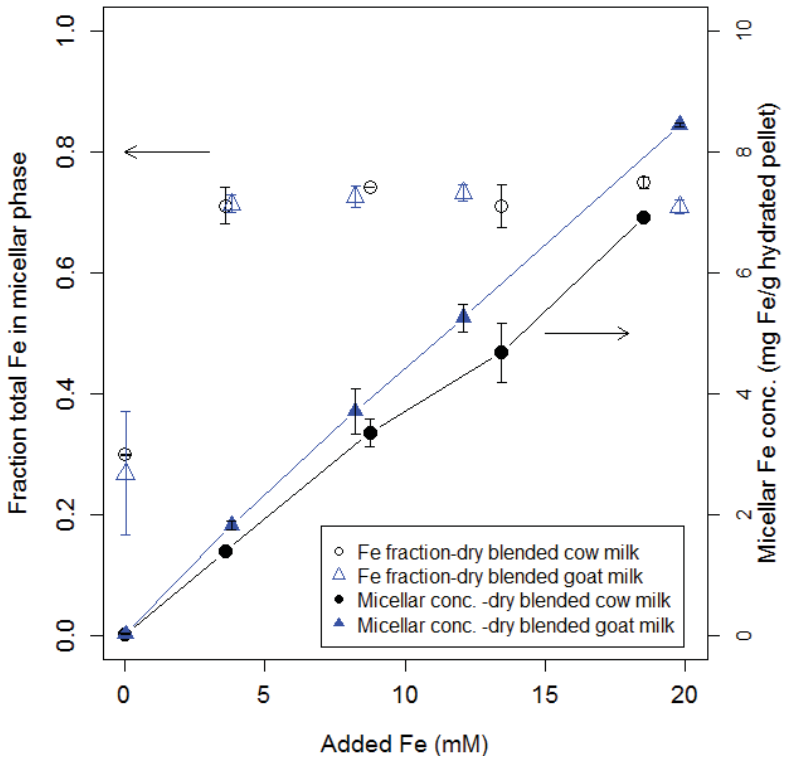


Figure 4-9: Partition of iron in micellar phase after ultra-centrifugation at 90,000 xg, 1 hour, 20 ºC shown as a fraction of the total iron detected in the micellar phase (open shapes) and the amount of iron bound to the micellar fraction (filled shapes) in dry blended iron fortified milk; error bars indicate standard error, n=3, arrows indicate related axis to points.

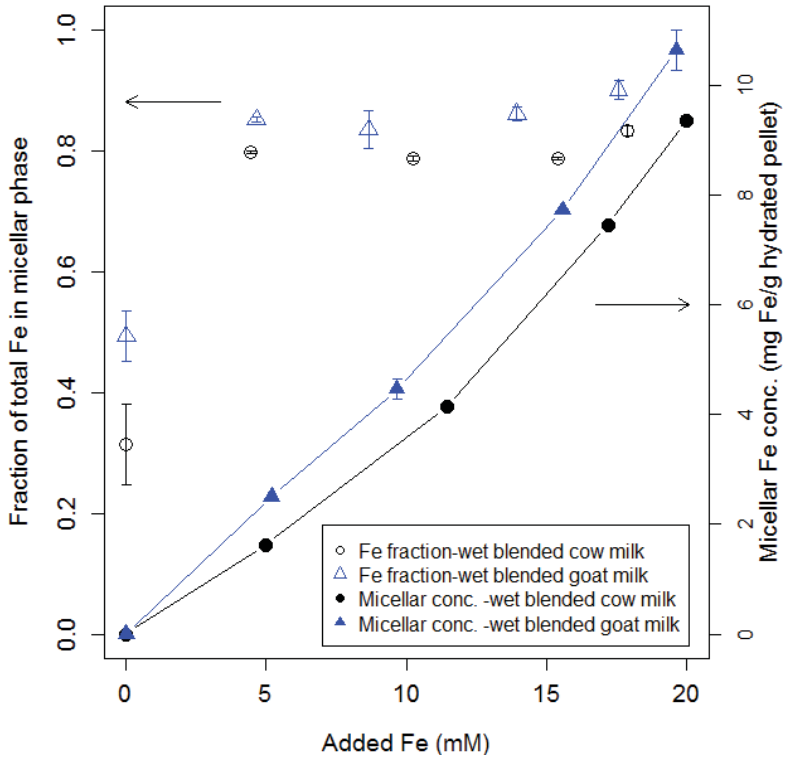


Figure 4-10: Partition of iron in micellar phase after ultra-centrifugation at 90,000 xg, 1 hour, 20 ºC shown as a fraction of the total iron detected in the micellar phase (open shapes) and the amount of iron bound to the micellar fraction in (filled shapes) wet blended iron fortified milk; error bars indicate standard error, n=3, arrows indicate related axis to points.

4.2.2.4 Moisture content of goat and cow micellar phase by partition

The moisture content of the micellar phase was determined according to section 3.2.5. The micellar phase became less hydrated with increasing iron from both cow and goat milk; the goat milk micelles had a lower water content overall than the cow milk micelles as shown in Figure 4-11. The species ($p<0.05$) and iron concentration ($p<0.001$) were significant factors in the moisture content of the micellar phase for each treatment. The moisture content was the same for both cow ($p=0.318$) and goat ($p=0.111$) within species when no iron was added with an average of 70.7 ± 0.2 % (2.4 g H_2O/ g dry pellet) for cow milk and 67.1 ± 0.3 % (2.1 g H_2O/ g dry pellet) for goat milk. The hydration of goat micelles ranged from 1.28 to 1.97g H_2O/g pellet as reported by Jenness (1980). The difference in hydration was attributed variations α_{s1} along with the mineral distribution.

Both the dry and wet blended goat milk micelle hydration decreased to a significantly lower moisture content than the control ($p<0.01$) to 64.4 ± 0.9 % and 60.4 ± 0.4 %, respectively. The addition of iron causes the solvent properties of the system to change causing water to be released from the amino acids resulting in a drier pellet (Gaucheron et al., 1997) but could also be due to a reduction in the electrostatic charge of the κ-casein allowing a denser pellet to form (Raouche et al., 2009a). Reddy & Mahoney (1991) and Gaucheron et al., (1996), using UV and fluorescence spectroscopy, have shown that there is a change in structure when iron is bound which is likely due to the neutralisation of charged groups which would allow increases in hydrophobic bonds. The moisture content of the dry blended cow milk decreased to 66.6 ± 0.3 % at 20 mM which was significantly drier than the control ($p<0.001$). An exception to the decreasing moisture content occurred at 10 mM where the moisture content increased to 71.1 ± 0.9 %; however this was not significantly greater than the unfortified milk ($p=0.695$). Similarly, this occurred at 5 mM for the wet blended cow milk sample where the moisture content increased to 74.0 ± 0.3 % with 5 mM iron addition ($p<0.001$) and then decreased to 62.0 ± 0.5 % at 20 mM ($p<0.001$). It is unclear why the moisture content would increase with 5 mM iron and 10 mM iron at each respective treatment however this correlates well with the micelle size of these samples which both display the largest Z-average out of the treatments. This indicates that moisture holding capacity could be governed by the micelle size with iron fortification in cow milk. Raouche et al., (2009a) reported cow milk had a hydration of 2.13 g water/ g dry pellet that decreased to 1.61 g water/ dry pellet with 20 mmol/kg iron ferrous chloride. In the current study, the micelles were more hydrated with the exception

of the 20 mM wet blended cow milk sample which had a moisture content of 1.63 g water/ g dry pellet. The authors used ultra-centrifugation conditions of 149,000 xg for 55 min which may have changed the pellet properties compared to our methodology.

A comparison between the two treatment types showed that the effect of iron content on the response of micellar phase moisture content depends on the dry or wet blended form ($p=0.0002$). At 5 mM iron addition the wet blended cow milk showed a significantly higher water holding capacity than the dry blended form at 5 mM ($p=0.0312$). At 10 mM the water holding capacity for cow milk changes with the dry blended milk having a higher water holding capacity ($p=0.0644$). In contrast, this cross over occurs at 20 mM iron addition ($p=0.0013$). This indicates that the treatment of the milk only has a significant effect on the moisture content at high iron addition levels. It appears that the dry blended milk has a better ability to minimise water loss at higher iron fortification levels as the rate of moisture loss is lower. Jenness, (1980) showed that there was an inverse relationship between the mineralisation of the micelle and hydration; this is evident when the iron concentration and binding of the milks increase the moisture content decreases. This correlates well with the iron binding characteristics of the treatment types shown in section 4.2.2.3 where wet blended milk could bind more iron. In terms of food formulation the levels of iron addition are significantly greater than what would be added to a food product. This indicates that iron can be added to milk either before or after spray drying without influence on the micelle hydration.

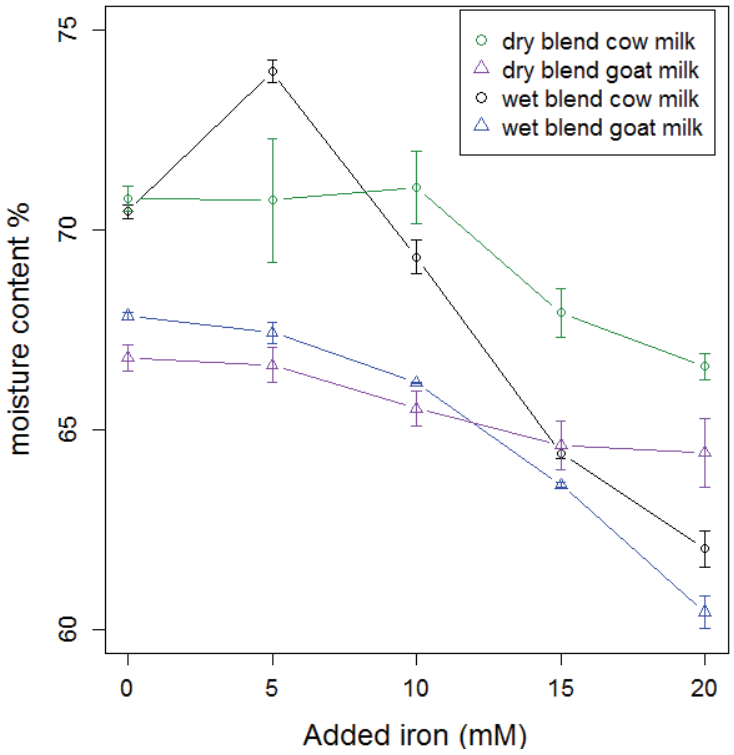


Figure 4-11: Moisture content (%) of pellet from dry blended and wet blended cow and goat milk after ultra-centrifugation at 90,000 xg for 1 hour at 20 °C; error bars indicate standard error, n=3.

4.2.3 Elemental analysis of wet blended goat and cow milk

The elemental partition of cow and goat wet blended iron fortified milk was analysed after ultra-centrifugation to determine if the addition of iron caused any effects on the movement of other minerals. The analysis was carried out by Hill's Laboratories (Hamilton, New Zealand) and due to the extensive analysis required only the wet blended milk samples were analysed. Errors although not shown on the plots are ±15 % at 95 % confidence, as reported by Hill's Laboratories.

From section 4.2.2.4 it was found that the moisture content of the pellet phase decreased with increasing iron concentration. Compared to the controls, the decrease in moisture for the wet blended samples with 20 mM iron addition was 32 % and 28 % for cow and goat, respectively. The results of the elemental analysis are reported on a weight basis and therefore do not account for changes in moisture content in the micellar phase. This needs to be taken into consideration when determining whether changes in the partition are a result of this.

In Figure 4-12, sodium increased in both the supernatant (350 mg/kg to 680 mg/kg for cow milk and 510 mg/kg to 810 mg/kg for goat milk) and pellet (500 mg/kg to 910 mg/kg for cow milk and 480 mg/kg to 690 mg/kg for goat milk, refer to Figure 4-13), this is due to the pH adjustment of the solutions using sodium hydroxide when iron was added. As sodium has no strong affinity to proteins it is likely that it was in equilibrium with the supernatant and micelle phase. As the pellet became drier the sodium content in the pellet levelled off as there was less serum in the micelles.

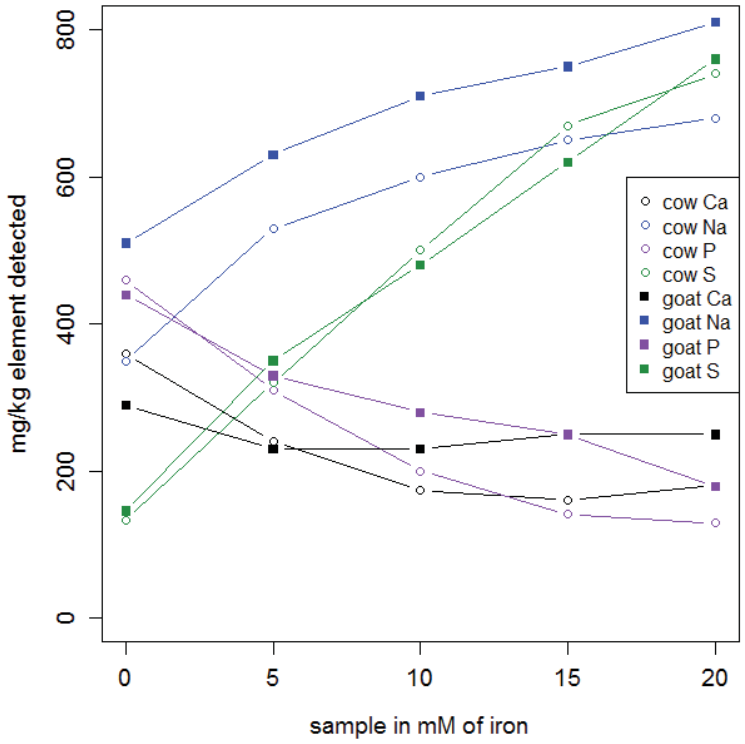


Figure 4-12: Elemental analysis of supernatant from wet blended cow (open circles) and goat milk (closed squares) after ultra-centrifugation at 90,000 xg for 1 hour showing calcium, sodium, phosphorus and sulfur.

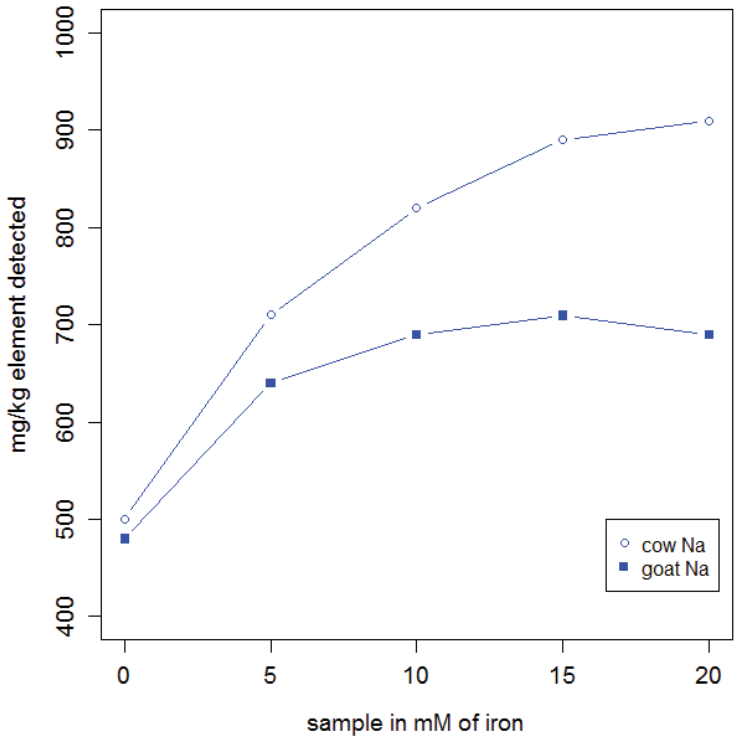


Figure 4-13: Elemental analysis of pellet from wet blended cow (open circles) and goat milk (closed squares) after ultra-centrifugation at 90,000 xg for 1 hour showing sodium.

The iron content increased linearly with increasing addition in both the supernatant and micelle phase (refer to Figure 4-14 and 4-15, respectively). Significantly more iron was separated into the micelle phase by weight (and on a protein basis). There is always a fraction of iron that does not bind to the micelles and is likely due to other components in milk having an affinity to iron; this was also found in section 4.2.2.3. At 20 mM there was a decrease in serum iron which may indicate that there was an excess of iron resulting in some precipitation of the iron which was not seen in the previous analysis (section 4.2.2.3). It was not visually obvious that the samples contained precipitated protein, however it is the best explanation for this decrease.

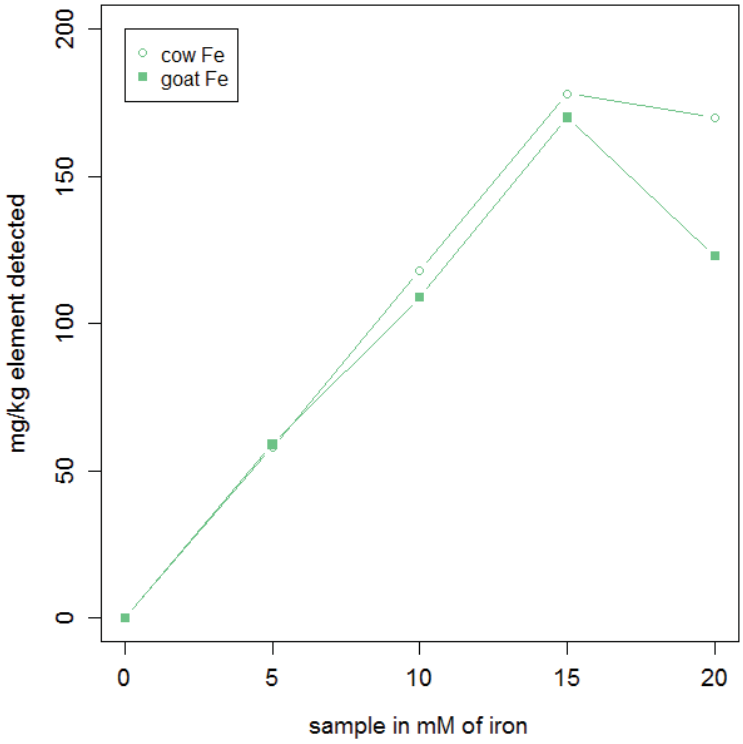


Figure 4-14: Elemental analysis of supernatant from wet blended cow (open circles) and goat milk (closed squares) after ultra-centrifugation at 90,000 xg for 1 hour showing magnesium and iron.

The sulfur increased in concentration with increasing iron as this was the anion of the iron salt added. It would be expected that the concentration of sulfur in the supernatant would increase as the salt dissociates in solution (refer to Figure 4-12). The supernatant content increased by 82 % from the control to the maximum concentration added and the pellet content increased by 25 % in both species from 0 to 20 mM added iron (refer to Figure 4-15). The increase in the micellar phase sulfur content can be explained by the decrease in pellet moisture content as the percentage change of sulfur is lower than the moisture content loss.

Figure 4-12 shows the calcium content of the cow supernatant decreased from 0 mM to 10 mM iron and then plateaus after this. The goat supernatant calcium content remained relatively stable over the iron addition range. The calcium content of the cow and goat micelles did appear to increase however this is likely to be due to the dehydration of the pellet (refer to Figure 4-15). The percentage increase of micellar goat calcium was 9.5 % while the micellar cow milk increased by 27 %. This is below the amount of moisture lost and therefore ionic calcium is not precipitating or moving into the micellar phase with the

addition of iron. Rather, the iron is either precipitating around the existing CCP or forming a new structure with the calcium and phosphorus.

Figure 4-15 shows the phosphorus content in the micellar phase increased linearly with increasing iron. The initial phosphorus content of the control cow milk was 5300 mg/kg and the goat milk micellar phase content was 6600 mg/kg. This increased to 9000 mg/kg and 9500 mg/kg for cow and goat milk, respectively. The supernatant content decreased in concentration from approximately 440 mg/kg to just under 200 mg/kg for both samples (refer to Figure 4-12). This appears to be quite significant as there is a two-fold reduction in sera concentration. Phosphorus makes up the CCP and therefore if the iron is adding to or precipitating around these clusters it may be that the phosphorus is migrating to balance the chemical equilibria (Mittal et al., 2016). Raouche et al., (2009a) showed via a ^{57}Fe Mossbauer study that inorganic phosphate migrated from the soluble phase to the micellar phase to interact with iron. This provides evidence that the iron is interacting with the CCP as this causes a significant shift in the phosphorus equilibria. Alternatively, non-protein bound iron could be binding to ionic phosphorus and precipitating as iron phosphate. This is quite likely at 20 mM iron addition as seen by the decrease in supernatant concentration.

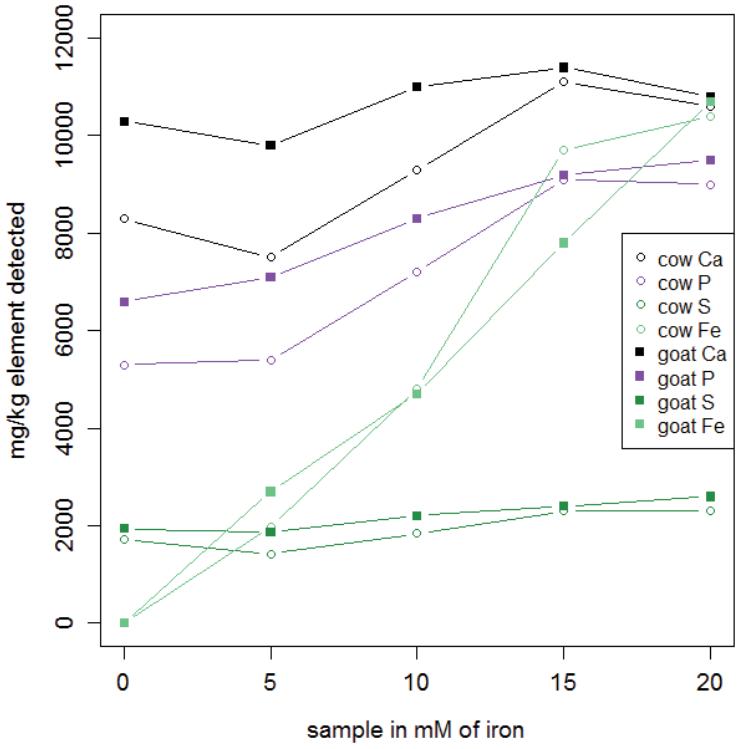


Figure 4-15: Elemental analysis of pellet from wet blended cow (open circles) and goat milk (closed squares) after ultra-centrifugation at 90,000 xg for 1 hour showing calcium, phosphorus, sulfur and iron.

The study therefore indicated that the most significant change in the equilibria of the measured minerals occurred with the phosphorus when iron is added to the milks while there may be some migration of calcium into the micelle when lower (5- 10 mM) amounts of iron are added. Phosphate ions are likely to be involved in the stabilisation of iron acting as a counter ion and allowing incorporation of the iron into the micelle.

4.2.4 SAXS analysis of dry blended and wet blended iron fortified goat and cow milk

4.2.4.1 Explanation of Small angle X-ray scattering and similar techniques

Small angle X-ray scattering (SAXS) is a technique used to look at milk systems (Holt et al., 2003; Pignon et al., 2004; Shukla et al., 2009; Mata et al., 2011; de Kruif et al., 2012; Sørensen et al., 2013). SAXS uses X-rays to determine the structure of a substance with differences in electron densities on the 1-1000 nm scale. The X-rays, when passed through a sample, produce a scattering pattern collected on a detector that can then be transformed into a scattering curve. Milk, namely the casein micelle, can be studied using this

technique as there are differences in electron density contrast between the protein of the micelle and the surrounding serum phase, and differences in electron density within the micelle.

Equation 4-1 describes the SAXS intensity (I) as a function of the magnitude of the scattering vector *q* as:

Equation 4-1

$$I(q) \sim V^2(\Delta\rho)^2 \int_0^\infty [f(qr)]^2 n(r) S(qr) dr$$

Where V is the total volume of scatterers, Δρ is the electron density contrast and the integral is performed over all radii. *f(qr)* is the form factor which is a function that describes the scattering from one particle of a shape. These shapes could be core-shell, ellipsoids, cubes, discs, aggregates, flexible polymers and so on; different equations are used to fit these. *n(r)* describes the size distribution of the scattering particles and there are different distribution functions used; the form factor and the size distribution cannot be obtained independently. *S(qr)* is a structure factor which describes inter-particle interactions (Ingham, 2015). In a scattering pattern containing a structure factor there will be peaks evident which measure the distance between the centres of electron density.

For hierarchical structures with dramatically different length scales, it may also be appropriate to use a model with separate populations to describe each hierarchical level (Ingham et al., 2016).

Resonant soft X-ray scattering (RSoXS) uses longer wavelengths that scan close to the L_2 and L_3 edges of both calcium and iron (hard X-ray scattering measures at the K edge). Peaks are observed at 708 eV and 710 eV for iron and 349.2 and 352.4 eV for calcium. Soft X-rays are 'soft' as the photon energy is much lower than the hard X-rays of SAXS and these match the energy of the core levels of the atoms of interest and specific spectral transmission. This allows the contrast and intensity to be enhanced, compared to hard X-rays (Swaraj et al., 2010). This technique complements the previous SAXS technique; SAXS determines the structure of the micelle in this case while RSoXS gives specific information on the calcium and iron location in this structure.

Based on our previously published findings (Ingham et al., 2015, 2016) it is believed that the features of these scattering profiles have been correctly assigned to structure in the micelle allowing the following iron fortified milk data to be interpreted.

The use of these techniques will help to gain a fundamental understanding of where the iron binds in the milk system through looking at whether there are any changes in the scattering profile of the data when more iron is added into the system as well as determining if spray drying affects this binding, distribution or location of the iron binding in the micelle. This method allows investigations into the internal structure of the micelle in a non-destructive way. Looking at two different species of cow and goat will also show if there is a difference in the binding characteristics of iron caused by differences in casein composition and milk constituents. Adding iron which has a higher electron density will help to prove that the feature at 0.035 $Å^{-1}$ is the CCP and high q is more likely to be a protein inhomogeneity. The results will explain each feature of the micelle in terms of the scattering profile and how the processing conditions of the iron fortification may have affected the structure.

4.2.4.2 Identification of features in SAXS scatting patterns

Samples were prepared and measured according to section 3.7.4. Four populations were identified from the background subtracted data of the scattering patterns from all the milk data sets. Figure 4-16 shows the full range data of cow skim milk which encompasses the whole casein micelle; in the low q range the turnover is reached such that the size of the casein micelles can be determined. There are three commonly identified features in SAXS data however these have often been assigned to different structures of the micelle, these are seen as bumps, shoulders or peaks in the scattering profile. The first feature occurs in the range of 0.001-0.004 $Å^{-1}$ which occurs in the low q range and this is assigned as the overall micelle and this feature is not disputed. The next feature occurs at 0.01-0.02 $Å^{-1}$, and is in the intermediate q range and this is sometimes discussed and has been assigned differently by different authors. At the highest q range of 0.08-0.1 $Å^{-1}$ there is more debate on what this represents. Finally there is also a feature at 0.035 $Å^{-1}$; this is not often seen or explained well in SAXS patterns. The micelle scattering profile has shown that the micelle is a hierarchical structure where each feature helps to form the next or is the building blocks for the next structure to form. An additive model by Ingham et al., (2016) was used where each feature was fitted with the respective model and then added together to produce the overall model of the micelle structure.

In the following data the wide range was not measured as it was not a priority to measure the overall micelle size. The Kratky plot exaggerates the turnover and the other features for better comparison between treatments (Bouchoux et al., 2010; Ingham et al., 2016).

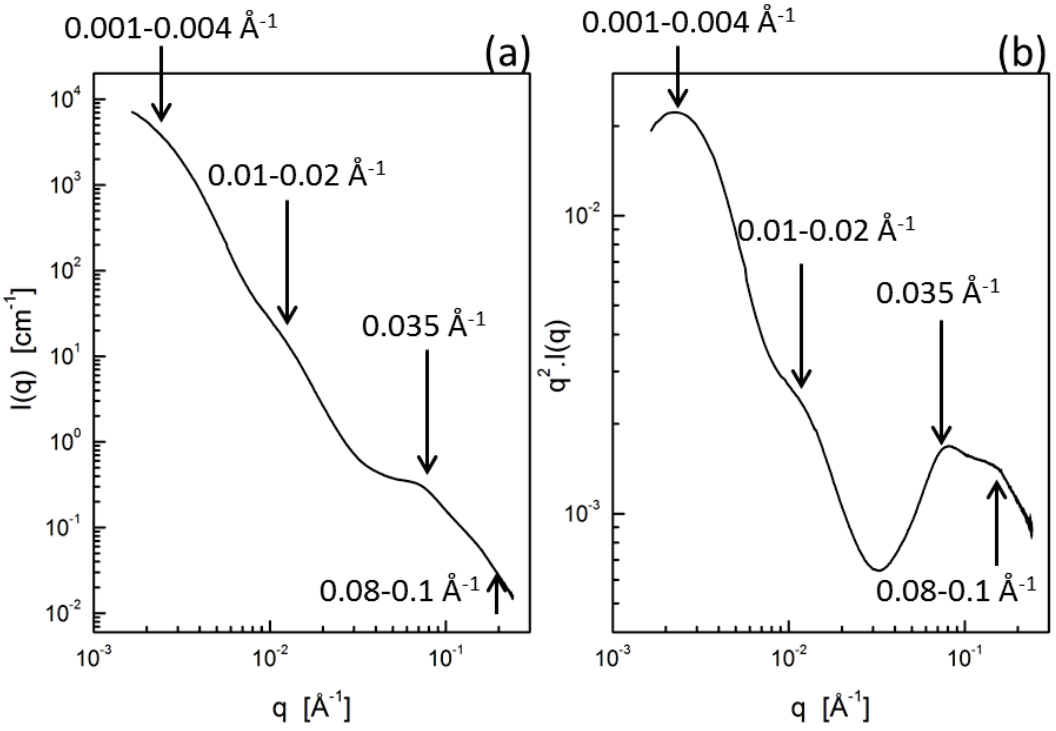


Figure 4-16: Background-subtracted scattering of wet blended skim cow milk collected over a wide q-range, in both log-log (a) and a Kratky plot (b).

4.2.4.3 Overall micelle

The feature that describes the overall micelle size and shape occurs in the range of 0.001-0.004 $Å^{-1}$ in the scattering pattern and was modelled as a non-interacting sphere. It is known that in milk the casein micelles are roughly spherical and they are dilute in terms of volume fraction and therefore are not in close proximity to each other; here the structure factor equals $S(qr) \equiv 1$. Casein micelles are sterically separated and cannot merge into each other and therefore it may be thought that a hard sphere structure factor would be required, however the definition of the structure factor is that the presence of one particle affects the location of another particle, which is not the case (Ingham et al., 2016). Figure 4-17 shows a diagrammatic representation of what the model would be. It would see an electron density difference between the protein matrix and the serum phase of the milk. Due to the camera length of 7 m used in these trials the micelle size cannot be accurately determined as the turnover of the curve does not occur; the beginning of the

feature however can be seen and this occurs at around 0.005 $Å^{-1}$. The focus of the experiment was not on this feature as there is no dispute on what this is as the size can be measured using a variety of other techniques and it is well known that the micelle size is around 100 nm in diameter. From previous data in section 4.2.2.2 dynamic light scattering showed that the size of the casein micelle did not change with increasing iron content for cow milk, however there may be a slight swelling with goat milk.

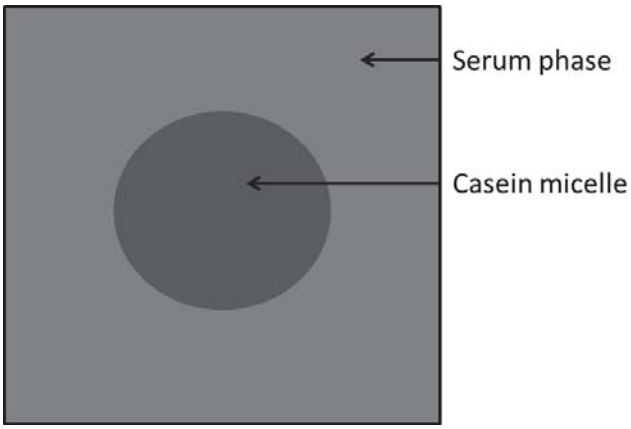


Figure 4-17: Representation of how a casein micelle looks in a SAXS model.

4.2.4.4 Incompressible region

The next feature can be seen around 0.01-0.02 $Å^{-1}$ as a slight bump or wide peak in some of the scattering profiles; refer to Figure 4-24 for all scattering plots. This has been termed the incompressible region based on Bouchoux's analysis. A casein micelle was described as a sponge by Bouchoux et al., (2010) where the sponge models consist of compressible (void/ serum) and incompressible (protein) regions. The protein cannot be easily compressed however the void spaces can be squeezed and the water can be displaced easily. In the micelle, the sponge is the protein matrix while the water is the serum. This is a robust model as it is known that the casein micelle is highly solvated at 3.9 g water/ g dry casein (Walstra, 1979) (with goat micelles being less hydrated (Park et al., 2007)). It is logical that there would be large compressible regions where serum is able to move in the micelle and the casein network would have areas of protein strands which would be denser in terms of electron density. This was modelled as a population of spheres with no structure factor and this is mainly because the location of a protein cluster does not affect the location of another. They are able to be in close proximity without repulsion.

The data did not fit perfectly as a sphere however to reduce over-parameterisation it was adequate as opposed to fitting an ellipsoid or other suitable shape (Ingham et al., 2016).

The intermediate feature is still a point of discussion as there are many points of view. Often this feature is ignored however work during this experimental project has indicated that this is quite relevant to the structure of the micelle. Shukla et al., (2009) attributed the intermediate feature to being a residual feature of the casein micelle based on the type of model they used which was a core-shell form factor which produces oscillations. Gebhardt et al., (2008) explained these as small micelles and therefore separate entities to the large micelles and Bouchoux et al., (2010) proposed that these were part of the micelle that were more resistant to compression than the overall micelle. From the previous work by Ingham et al., (2015) it was concluded that Bouchoux's model was most consistent with the findings in the data. Gebhardt's hypothesis was proved by Ingham et al., (2016) when milk was filtered to remove large micelles and the intensity of the feature was proportional to the low q feature and therefore the intermediate feature was related to the large micelles and not a separate entity, which would otherwise cause a significant increase in intensity of this feature in relation to the large micelles. Dynamic light scattering of filtered milk also showed that there was no secondary peak of small micelles and that there is only a single population of a distribution of micelle sizes which are above the approximate size of the intermediate feature (section 4.2.1.2). Figure 4-18 shows an approximation of what these incompressible and compressible regions would look like with a diagrammatic representation of the networks of protein strands that leave channels of serum throughout the micelle and how the SAXS model would describe these as chains or networks of connected spheres.

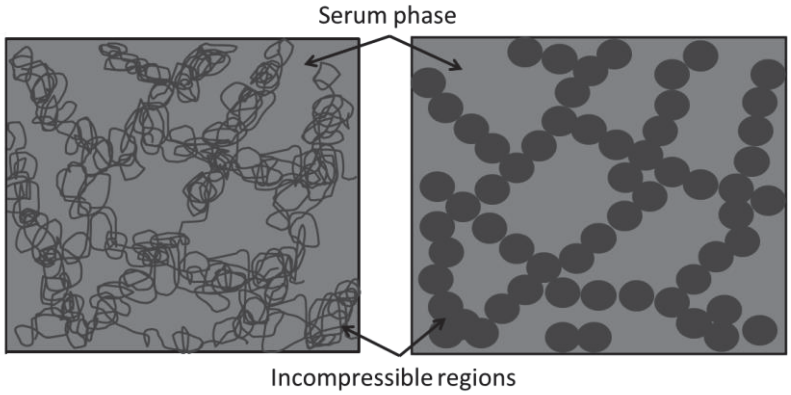


Figure 4-18: Left; a diagrammatic representation of the incompressible protein matrix and compressible serum phase, right; a representation of how the SAXS models the protein and serum.

4.2.4.4.1 Effect of iron fortification on the incompressible and compressible structure

The incompressible feature on the scattering profile is very subtle and is therefore best viewed using a Kratky plot. The Kratky plot transforms the data by multiplying the I(q) by q^2 (Figure 4-24), it allows the features to become easier to see than the usual log- log plot and was used by Bouchoux et al., (2010) for this purpose. The intermediate feature is only very strongly present in the dry blended cow milk at 4 ^{o}C. The scattering profile of the incompressible feature of dry blended cow milk at 4 ^{o}C shows that the peak is in a different location and has a different shape and intensity. This indicates that the addition of iron causes an effect on the incompressible feature. In all other sample types of dry blended and wet blended milks this intermediate feature is difficult to detect with the exception of wet blended cow milk at 37 ^{o}C where the sample containing 5 mM iron shows a very prominent feature and there is a concomitant decrease in the feature intensity as the iron concentration increases. The 5 mM iron sample had a larger particle size and moisture content (refer to section 4.2.2.2.2 and 4.2.2.4) and the SAXS data indicates that there is also a difference in the internal structure which would explain the larger size and increased moisture content. In the other samples tested it may be that the feature is being washed out by the tailing of the overall micelle size feature. This would be expected to occur if there was a large distribution of micelle sizes or broad distribution of incompressible region sizes. As this population is a measure of the electron density contrast between the protein and serum it may be that the serum has a higher electron density when iron is present. Goat milk has more mineralised casein micelles (Park, 2006)

and therefore the sera may have a higher electron density than cow which may reduce the contrast between the two phases. The elemental analysis of the supernatants of the iron fortified milks also show that there is generally a higher mineral content in the goat milk samples compared to cow milk, as evidenced in Figure 4-12.

When the cow and goat milk were prepared with spray drying the heat treatment may have changed the properties of the protein matrix and therefore caused a prolonged swelling time and thus the contrast may not have developed. Hydration typically takes about 2 hours for mineral equilibration in non- fortified spray dried milk (Anema & Li, 2003); however a higher ionic strength and iron loading may have affected this and may have caused protein to swell at a slower rate. The intensity of the feature in cow wet blended milk at 4 ^{o}C is low and is at a higher q range which may indicate that the size of the incompressible region is reduced (depending on actual position) compared to the dry blended milks. A higher temperature at 37 ^{o}C for reconstitution may have sped up the swelling causing the intermediate feature to become more obvious, the lower iron content may have allowed more contrast between the protein and serum as the overall concentration of iron in the serum was lower than the higher iron loadings. The wet blended milks with no iron added do not show an obvious feature here compared to the dry blended cow milk samples. The two-fold spray drying that occurred may have also inhibited the rate of hydration and the protein does not expand as much as the dry blended milks. This phenomena was observed in a separate experiment published as Ingham et al., (2016) wherein milk was rehydrated and the rate of hydration was measured over 10 hours during which time the feature became more intense and moved to a lower q indicating that the incompressible regions take time to swell.

4.2.4.5 Colloidal calcium phosphate spacing

The feature at 0.035 Å^{-1} is usually only seen in SANS experiments via contrast matching of the serum and is a structure factor peak related to the separation distance between the CCP particles (Holt et al., 2003; de Kruif et al., 2012). Mata et al., (2011) were able to observe this feature in dry powders and the feature was identified in our experiments in liquid systems published as Ingham et al., (2015) using RSoXS. A characteristic of a structure factor is a sharp peak, called the correlation peak, which is more evident in the iron fortified samples that have been spray dried and are rendered more prominent in the Kratky plots. The CCP was modelled as a core-shell form factor with a structure factor of a hard sphere. This means that the CCPs are interacting such that there is a distance at

which they can no longer come any closer to each other. The CCPs consist of the calcium phosphate and iron clusters which make up the core and are surrounded by a shell of protein, rich in glutamic acid, glutamine, serine and phosphoserine groups that hold the CCP cluster (Holt, Davis & Law, 1986). This shell prevents other CCPs from moving closer due to protein repulsion forces. The SAXS model diagram (refer Figure 4-19) shows approximately what the modelled feature is: the CCP core designated as a dark sphere and the shell shown as a homogeneous surrounding layer of dense protein. The scattering pattern shows the distances between two particles via the hard spheres structure factor. While it is also measuring the size of the particles with the form factor it is hidden under the strong structure factor scattering, which has been described in Figure 4-20, hence why the particle size can no longer be directly measured.

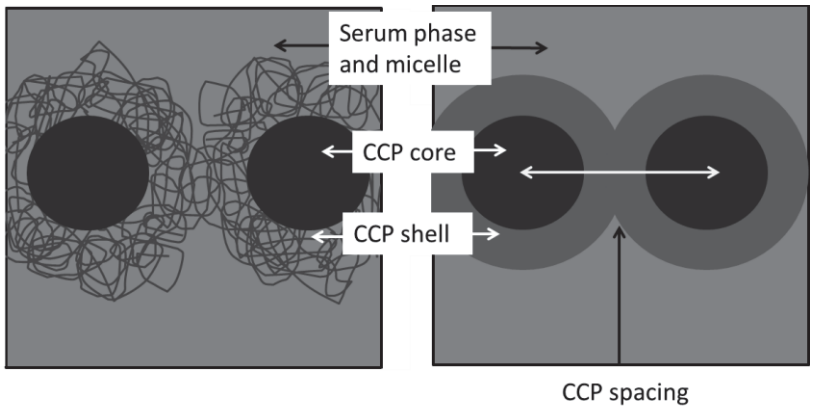


Figure 4-19: Left; diagrammatic representation of two CCP core particles with protein shells, right; SAXS mode of CCP core and shell.

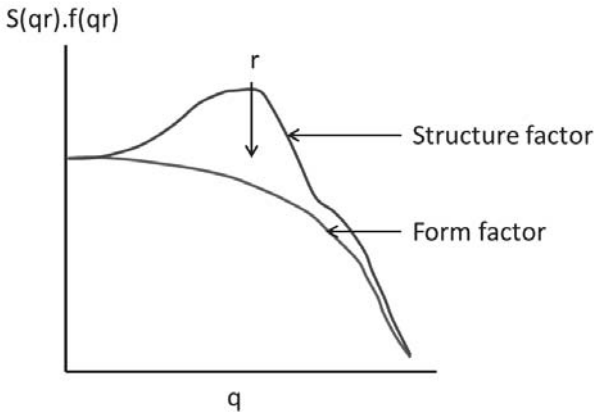


Figure 4-20: Representation of the form factor and structure factor of a population and how the structure factor hides the form factor.

4.2.4.5.1 Effect of iron fortification on the colloidal calcium phosphate structure

There is strong evidence from the iron addition data that the iron is binding to the CCP either by precipitation around the calcium phosphate or an association close to the CCP. Analysis of the phosphorus content of the wet blended milks showed that there was a decrease in the serum phosphorus indicating that there was a precipitation of more phosphorus in the pellet phase with the iron (section 4.2.3) indicating phosphorus is involved in the binding. Similar results were reported by Mittal et al., (2015). At 0.035 $Å^{-1}$ there is a small peak developing in the scattering profile with increasing iron fortification. A structure factor would be suitable for use here, as the iron concentration increases the intensity of the peak increases which supports the hypothesis that the iron is moving into the CCP. This is evident as the iron has a higher electron density than the other atoms involved in the feature. This explains why with no iron the feature is very difficult to observe using this technique.

Preliminary studies using RSoXS to identify the location of calcium and iron in the milk have support this argument. Milk samples were scanned at various energy levels over a range that covered the on and off resonance energy of calcium and iron. When wet blended cow milk fortified with 15 mM iron was tested, a strong peak was observed on the length scale expected, 0.035 $Å^{-1}$ for both calcium and iron. The plots show the resonance of the calcium and iron as the scan was run through different energies (refer to Figure 4-22) while the red curves show (Figure 4-21) the scattering curve with the peak at 0.035 $Å^{-1}$. As they occur at the same q range this strongly indicates that the iron is binding with the CCP, either incorporating into the structure or precipitation around the CCP. This also provides further evidence that the calcium is in this feature range.

In every sample set tested the CCP peak becomes more intense (refer to Figure 4-24) with increasing iron loading in the SAXS data and typically the peak cannot be seen in the 0 mM milks as there is not enough electron density contrast between the phases.

The goat milk appears to behave in the same way as cow milk in terms of incorporating iron into the structure and this was expected as similar fractions of iron were able to be incorporated in the micelle according to the partition studies (refer to section 4.2.2.3). At 20 mM in particular but also in the lower fortification levels it appears that the peak is more intense in the goat milk data than cow at the same level. This may mean that either there are a higher number of CCP clusters or there is a greater difference in the electron

density contrast. The precise reason for the increase in intensity cannot however be distinguished as the number of CCP and the electron density contrast are confounded in the intensity equation $I \propto V(r).\ \Delta\rho$; where the intensity is proportional to the product of the total volume and electron density contrast.

The wet blended milks also appear to produce a more intense peak than the dry blended milks, and data suggest that the spacing of the CCPs has become more regular. Therefore it seems likely that the heating and/or spray drying process have changed the structuring of the calcium phosphate and iron particles. The high concentration of the milk during the pre-heat step or spray drying operation may have put more stress on the CCP and iron complex to move into more energetically favourable positions. The wet blended milks were prepared by dissolving milk powder into a high solids solution; this would have caused a high ionic strength solution to develop thus a high osmotic stress on the micelles. This hypothesis of a higher osmotic stress was supported by Le Graët & Gaucheron, (1999) who dissolved casein at high concentrations in milk ultra-filtrate which caused a different mineral equilibrium than native milk. The wet blended iron solution at high solids content may have caused some rearrangement of the structure so that these interacting particles were in a more energetically favourable position therefore creating a less random spacing distance. The increase of iron would have increased the ionic strength of the solution, not only with the ferrous salt added but due to the addition of alkali required to ensure a consistent pH between samples. Additionally the higher iron loading would create a larger electron density contrast.

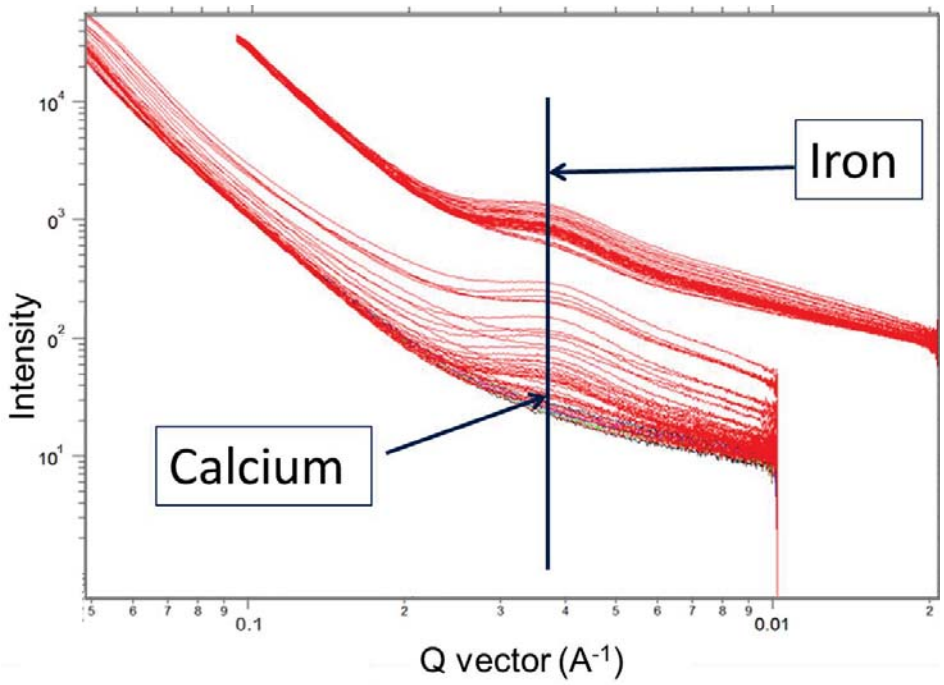


Figure 4-21: Scattering curves of calcium (bottom) and iron (top) of energy scans.

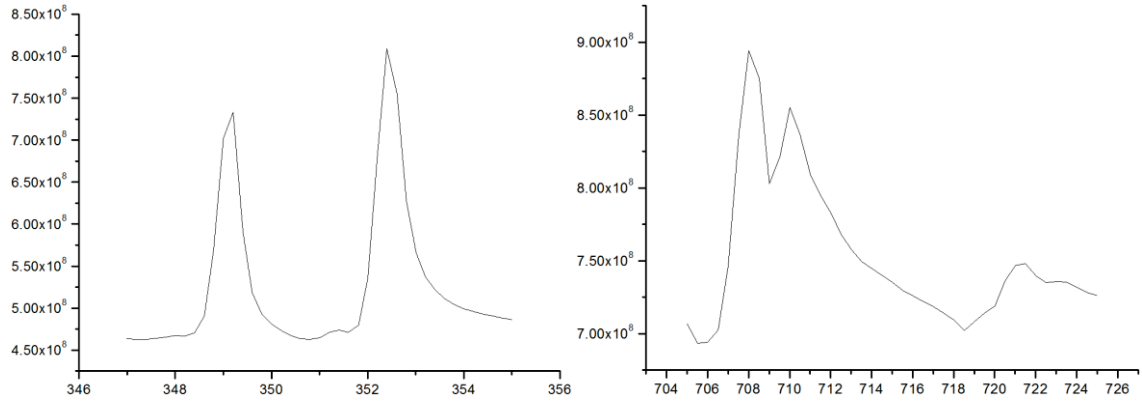


Figure 4-22: Resonance of calcium L-edge with 15 mM iron (left), Resonance of iron L-edge with 15 mM iron (right).

4.2.4.6 Protein inhomogeneities

The high q feature (0.08-0.1 $Å^{-1}$) is usually attributed to the form factor scattering from the CCP particles (Marchin et al., 2007; Gebhardt et al., 2008; Shukla et al., 2009; Bouchoux et al., 2010; Mata et al., 2011) however there is evidence that this is incorrect and the feature is in actually the result of small dense regions of overlapping protein termed protein inhomogeneities (De Kruif et al., 2012; Ingham et al., 2015). De Kriuf et al., (2012) calculated that the CCP would not be detected at the intensity that was seen in the scattering plots. These protein inhomogeneity strands are casein proteins that are

aligned via weak interactions such as van der Waals and hydrophobic interactions that form fibrils or worm shaped aggregates that make up the network of the casein micelle. De Kruif estimated these to be 1-3 nm in size.

Figure 4-23 attempts to show how protein strands may align for a short section, overlaying to form a worm like structure. α_{s1} casein has three sections of hydrophobic residues, 1-40, 90-110 and 130-199 while β- casein is hydrophobic with the exception of the sequence of 1-50 (Swaisgood, 1982). These interacting sections of hydrophobic protein strands would interact then move apart in the hydrophilic section to form further networks with other binding such as the CCP.

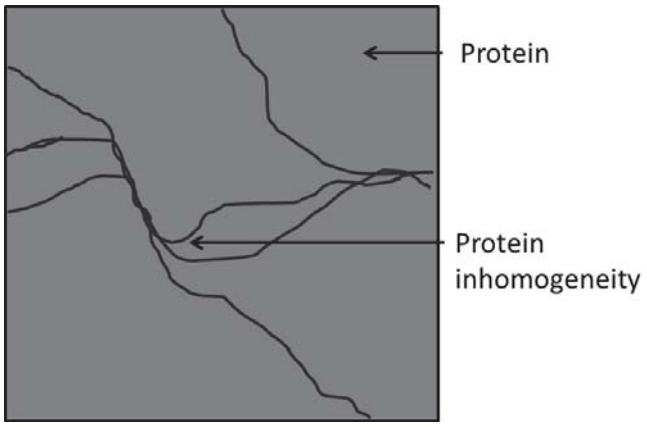


Figure 4-23: Diagrammatic representation of strands of protein associating by hydrophobic bonding.

Thus in this study we have fitted the high q feature (0.08-0.1 $Å^{-1}$) as a protein inhomogeneity. This feature was fitted using the form factor calculated by Sorensen with a hard sphere structure factor; this was shown to have the best fit by Ingham et al., (2016). A mild structure factor was required as there is some regularity to the spacing of the protein inhomogeneities. This is based off the Debye-Büche term which includes a term for the surface texture of the protein determined by the Porod scattering from the surface which fits into the form factor expression of equation 4-2, where ξ is the correlation length and σ is the surface roughness.

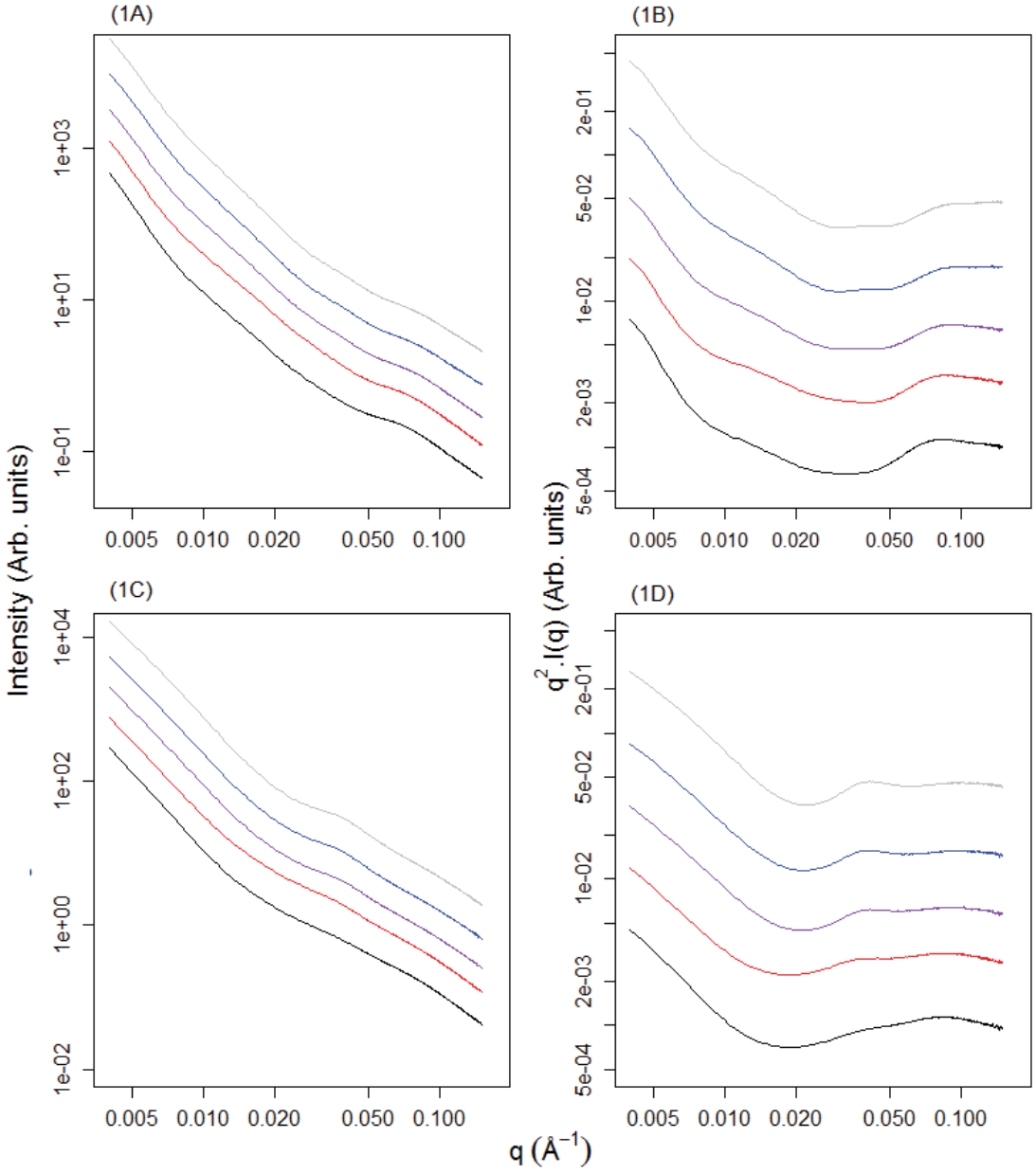
Equation 4-2

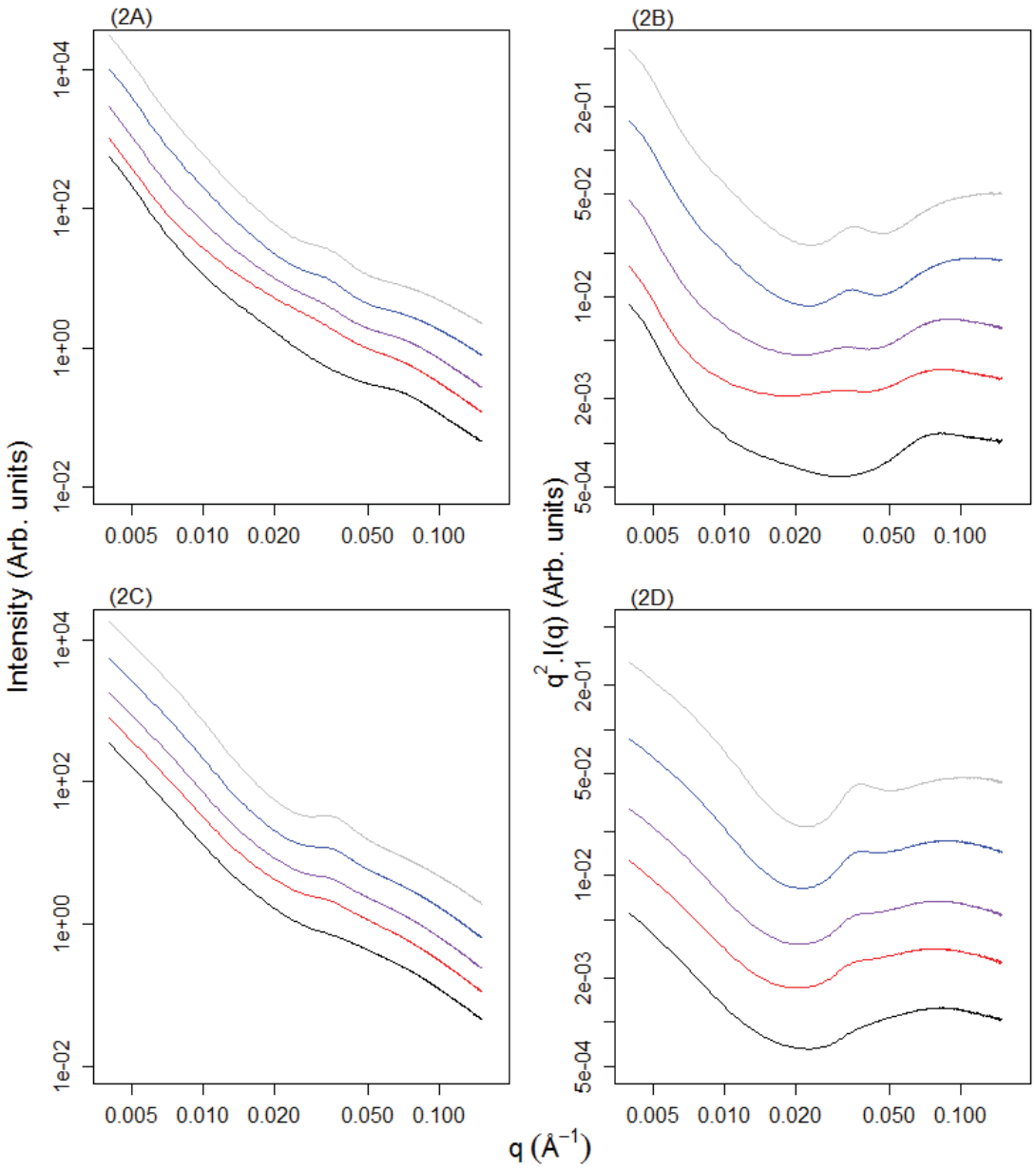
$$P(q,\xi,\sigma) = [f(q,\xi,\sigma)]^2 = \frac{1}{1+(q\xi)^2} exp(-\sigma^2 q^2)$$

4.2.4.6.1 Effect of iron fortification on the structure of the protein inhomogeneities

In most of the sample sets the slope of the protein inhomogeneity feature does not change over the iron addition range of 0-10 mM. The exception to this generalised observation is the 4 ^{o}C and 37 ^{o}C wet blended goat milks wherein the sample with 15 mM iron also has the same slope. In all other samples slope flattens with 15 mM iron addition and becomes flatter again with 20 mM addition. The slope of this feature is the p factor which indicates the shape of the object. Through rough fitting of the data the shape of the proteins seems to be a fuzzy worm-like structure and the shallower slopes indicate that the inhomogeneity regions become slightly rounder. This may have been caused by the protein structure changing with increasing iron content. This could be caused by the increase in ionic strength altering the weak interactions causing the shape of the protein strands to change. Another possibility is that the change in slope is influenced by the enhanced CCP scattering.

4.2.4.6.2 Scattering patterns of goat and cow milks at various temperatures and processing type




(2A)
(2B)
(2C)
(2D)
Intensity (Arb. units)
q².I(q) (Arb. units)
q (Å⁻¹)
1e-02
1e+00
1e+02
1e+04
5e-04
2e-03
1e-02
5e-02
2e-01
0.005
0.010
0.020
0.050
0.100

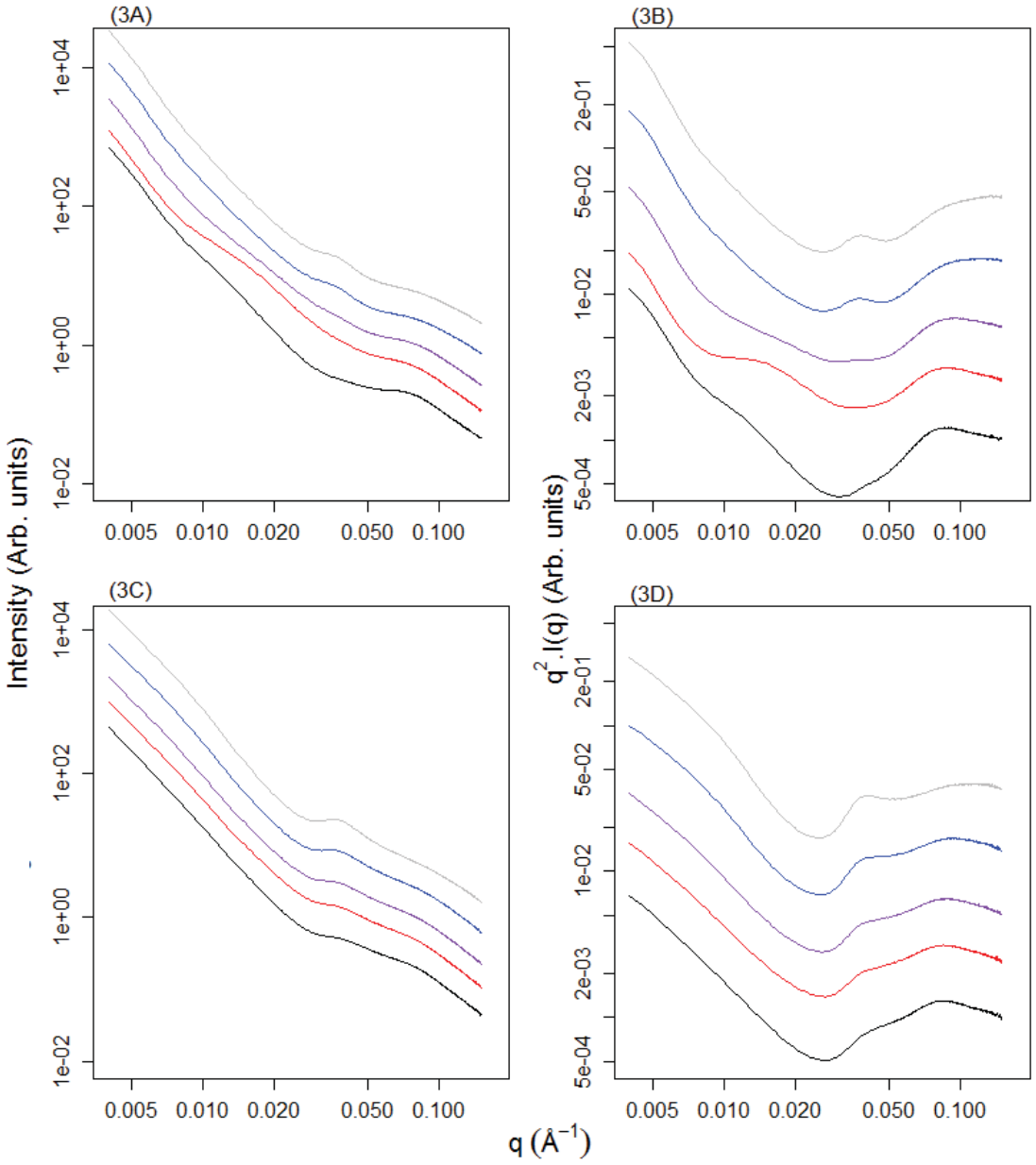


Figure 4-24: Intensity plots (A, C) and Kratky plots (B, D) of milk treatments from SAXS. Figure 1 plots show dry blended milks measured at 4°C; Figure 2 plots show wet blended milks at 4°C; Figure 3 plots show wet blended milks at 37°C. For all Figures, A and B are cow milk samples and C and D are goat milk samples. Colours; black= 0 mM, red= 5 mM, purple= 10 mM, blue= 15 mM and grey= 20 mM, indicate the level of iron addition.

4.2.4.7 Effect of processing and iron concentration on casein micelle structure

Typically nutritional formulations come in a dry form that has been spray dried after the required ingredients have been added, known as wet blending. The study aimed to replicate this process by adding iron to the liquid milk which was then spray dried and reconstituted at 4 ^{o}C prior to testing. Although cow milk is the most widely used dairy source, goat milk is becoming increasingly popular and therefore this was concurrently studied (Carlson et al., 2011). Iron fortified skim milk cow and goat wet blended powders were produced to test the effects of typical commercial processing on the binding of iron to the protein. Dry blended milks were defined as cow and goat skim milk powder that were hydrated in water and then had ferrous iron added at 0, 5, 10, 15 and 20 mM and then tested at 4 ^{o}C. This temperature (4 ^{o}C) was chosen as chilled temperatures have been shown to improve iron binding (Raouche et al., 2009b). This was attributed to the greater stability of the κ-casein at low temperature, preventing micelle aggregation. The models of the dry blended milks simulated adding ferrous iron salts dry to a dry blend of milk.

Wet blended milk SAXS runs were carried out at temperatures of 4 ^{o}C and 37 ^{o}C; the cold temperature was used to compare with the dry blended milks at an ideal temperature for iron binding and 37 ^{o}C was to mimic body temperature to understand how the solution may behave prior to infant feeding, for example. The most obvious difference in the dry blended and wet blended milks is the increased intensity of the CCP spacing feature. This may be due to differences in the processing conditions.

4.2.4.7.1 Iron fortification and location of binding in the micelle

In section 4.2.2.3 when iron was added to cow and goat milk from 5 to 20 mM, around 72-86 % of the iron bound to the micellar fraction which indicates that this iron is binding or associating to the casein micelle in some way. This supports the hypothesis that iron is moving into the casein micelle and the iron is binding to the phosphoseryl groups that are located on the α- and β- caseins or interacting with CCP. The κ- casein makes up the hairy layer producing the steric repulsion so it is unlikely that the iron is only associating with the surface of the micelle, rather it is moving into the micelle.

SAXS has shown that the main feature that is altered by an increase of iron is the CCP feature which increases in intensity and occurs with all sample types. It is very likely

that the iron is associating with the colloidal calcium phosphate by either precipitating around it or incorporating into the structure (refer to Figure 4-25). The addition of iron does cause a change in the structure as the CCP distances become more ordered. At present, no more information on this change can be stated as only qualitative analysis has been performed. The dry blended milks may not cause a large change in CCP structure in terms of ordering compared to the wet blended which may be of importance however it is not known if this structural change has a negative impact on the functionality of the micelle or overall nutrition value of the system.

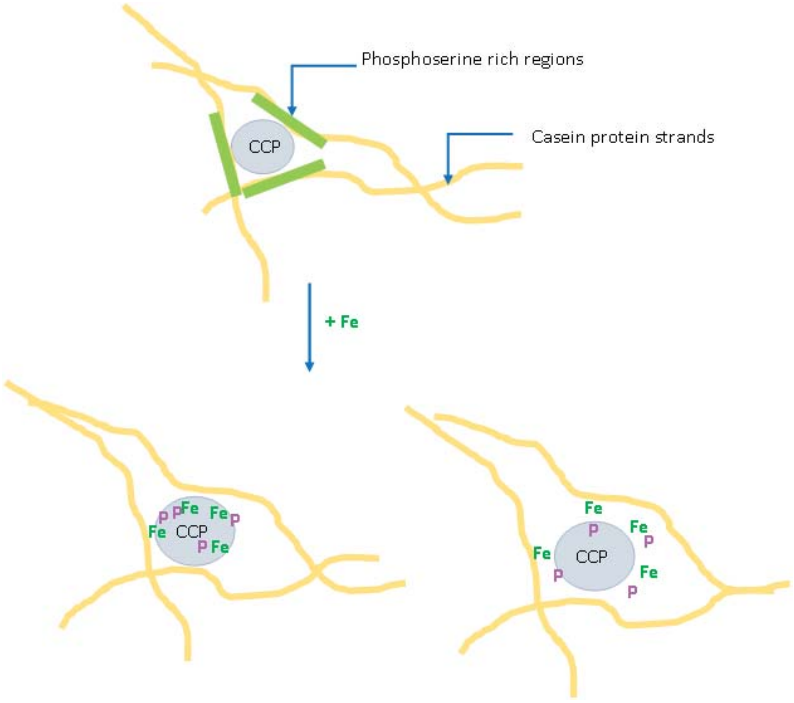


Figure 4-25: Schematic of how casein protein strands associate in native milk (top) and iron may interact with the colloidal calcium phosphate (bottom), either with the iron becoming part of the CCP amorphous structure (left) or precipitating onto the CCP (right).

The elemental analysis data showed that with the addition of iron the phosphorus increased in concentration within the micelle. If the mechanism of iron binding to the CCP regions was primarily competitive in nature then one would expect that the divalent cations (Ca, and Mg) would decrease in concentration while the P concentration, as part of the phosphate anion, would either remain constant if the iron remained in the ferrous form or increase if the iron oxidised to the ferric form. It is likely that along with the moisture loss in the micellar phase there was a concentration

of the elements which may have instead of moving out of the casein precipitated onto the micelle to different binding sites such as carboxyl residues.

4.2.5 Characterisation of iron fortified goat and cow milk with calcium depletion

4.2.5.1 Calcium removal of cow and goat milk

The SAXS studies confirmed that iron and calcium have the same binding location within the micelle. This led to the research question of determining the nature of the relationship between the impacts of calcium on iron binding. To this end trials were carried out investigating iron addition to micellar systems wherein the calcium level was depleted. The amount of calcium removed, via ion exchange (refer to section 3.2.6), was 69.65 ± 1.03 % for cow milk and 70.65 ± 0.94 % for goat milk ($p=0.3232$). The opacity of both the cow and goat milk was significantly reduced indicating that the micelle structure had largely though not completely broken down.

4.2.5.2 Particle sizing of iron fortified milks after calcium depletion

Figure 4-26 shows the particle size analysis of the cow milk indicated a bi-modal distribution for all iron treatments with a peak intensity ranging from 396 nm to 300 nm and a second minor peak at on average 40 nm. In terms of a number distribution it was found that the average peak particle size was 28 nm. This indicated that most of the micelle structure has been destroyed with the removal of calcium however there may be a few remaining intact micelles. The Z-average diameters of the milk decreased from 251.0 ± 3.1 nm at 0 mM to 175.6 ± 1.5 nm at 20 mM iron ($p<0.001$), Figure 4-27. Adding a high concentration of iron did not allow a reformation of the micelles. Rather, the decrease in size is likely due to the charge neutralisation of the iron binding to the phosphoserine groups therefore causing the aggregates to become more hydrophobic (Reddy & Mahoney, 1991; Gaucheron et al., 1996).

In contrast to the cow milk samples goat milk did not produce distributions with clear trends with respect to the amount of iron added (refer to Figure 4-26). At 5 mM and 20 mM iron addition there was a bi-modal distribution while the other treatments appeared to produce a mono-modal distribution by intensity. The peak intensities were on average, 420 nm. By number distribution the particle sizes compared to the cow milk samples are much larger with peaks of on average, 240 nm. The Z-average particle sizes of the goat milk treatments were significantly larger than the cow milk

samples ($p<0.001$). The diameters (Figure 4-27) decreased with increasing iron content from 368.9 ± 3.5 nm at 0 mM to 303.3 ± 2.7 nm at 20 mM iron ($p<0.001$). This indicates that the majority of the micelles or aggregates are either maintaining the micellar structure even after calcium removal with some breakdown or the caseins are reforming into larger aggregates that were not seen in the cow milk. The differences in cow and goat milk micelle breakdown resistance may be due to the higher β-casein content of goat micelles (Park, 2007). This may allow hydrophobic forces to dominate in stabilising the goat micelles as opposed to binding via the CCP. α_s-caseins contain more phosphorylated binding sites and therefore the cow micelles are likely to be more sensitive to calcium removal (refer to Table 2-4).

Mittal et al., (2015) removed calcium from milk using a similar resin achieving different levels of calcium depletion to which iron was added. At the highest iron addition level of 25 mM the protein solubility decreased to 85 % and 80 % of the iron remained in the supernatant phase. The Z-average diameter of the particles decreased with increasing iron fortification from 158 nm with no iron addition to 119 nm with 25 mM iron. The number diameter of the particles obtained by Mittal et al., (2015) at the high depletion level was 20.1 nm which is similar to what was found here for cow and 94 % of the protein was soluble. The protein and iron in Mittal and co-workers samples were more soluble with a similar level of calcium removal. The Z-average was larger in the current work which may explain why more protein, and therefore iron, sedimented.

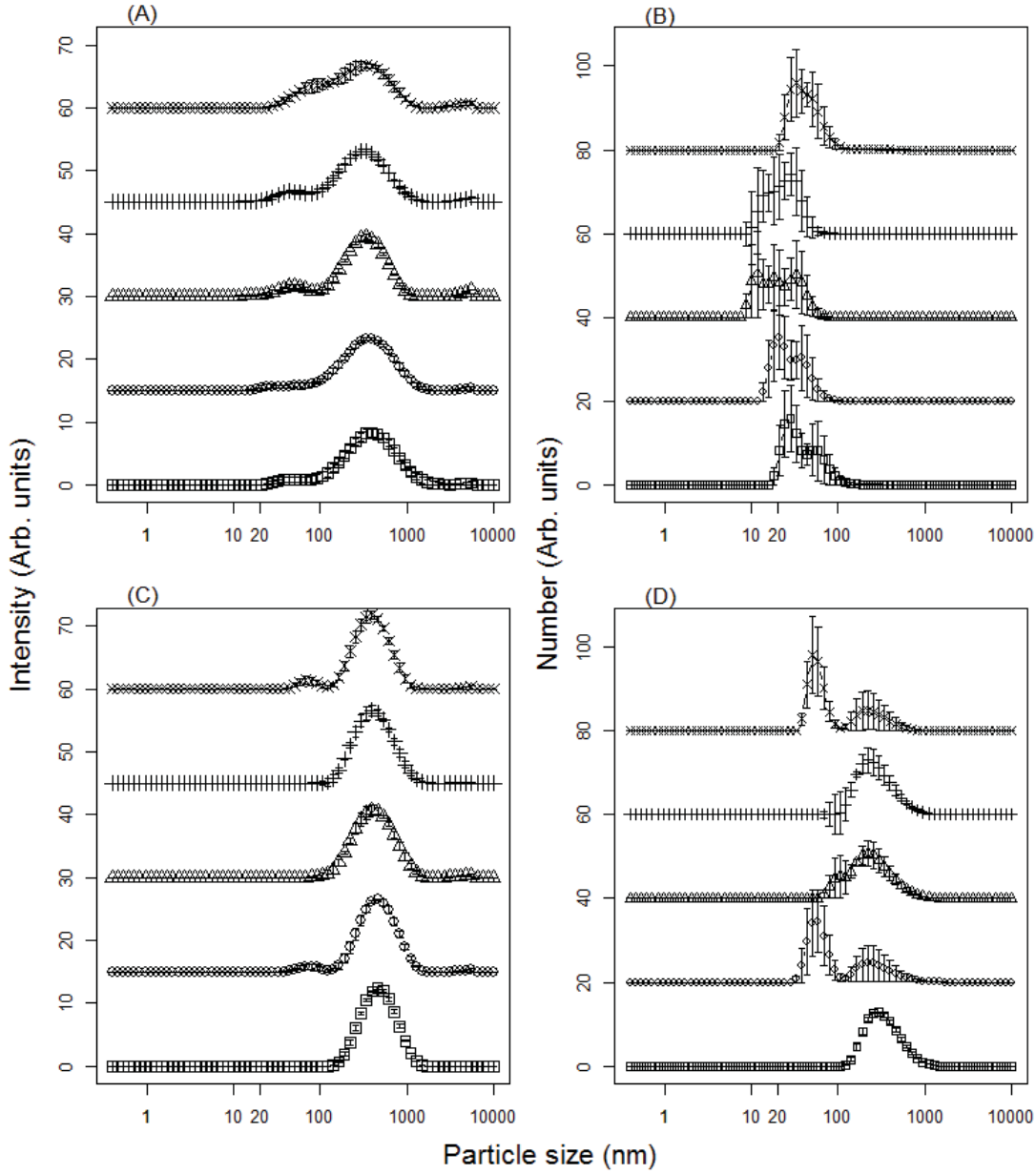


Figure 4-26: Particle size of cow milk particle size by intensity (Figure A) and number (Figure B) and goat milk by intensity (Figure C) and number (Figure D) with 0 (□), 5 (○), 10 (Δ), 15 (+) and 20 (x) mM iron; error bars indicate standard error, n=3.

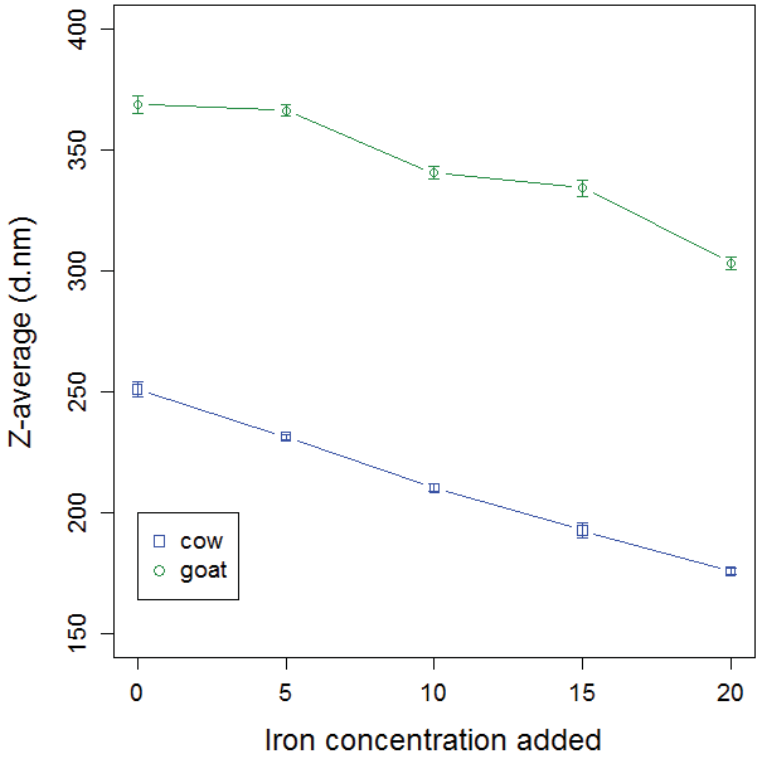


Figure 4-27: Z-average particle sizes of calcium depleted cow and goat milks fortified with iron; error bars indicate standard error, n=3.

4.2.5.3 Partition of iron and protein in calcium depleted goat and cow milk

The goat milk produced a greater fraction of sedimentable material compared to cow and the pellet mass increased with increasing iron content for both species after ultra-centrifugation at 90,000 xg for 1 hour at 20 ºC; this can be seen in Figure 4-28 where the amount pelleted goat milk is much larger than the cow milk. With no added iron the amount of sedimented material was 0.5 % and 2.7 % milk of total sample weight; this increased to 4.4 % and 6.6 % at 20 mM added iron for cow and goat, respectively. The difference in sedimentation is expected after the large differences in particle size where the goat milk samples were still in the micellar size range therefore, based on Stokes law, more likely to sediment. The fraction of pellet increased with increasing iron concentration however the moisture content of the pellet did not change. This indicates that the iron caused the formation of the protein aggregates of sufficient size to settle under the ultracentrifugation conditions used in the experiment and that the aggregates formed had the same density/hydration level as the initial aggregates/micelle remnants found in the control milks. At similar depletion levels

(68-72 %) as carried out here, Mittal et al., (2015) found there was complete dissociation of the micelle whereby no protein was sedimented.

Figure 4-29A shows the cow pellet contained more moisture than the goat milk pellet with 82.0 ± 0.3 % and 74.3 ± 0.6 % water content, respectively. Previously (in section 4.2.2.4) the samples became drier as the iron expelled the water when binding to casein. In the calcium depleted system, the particles became smaller with increasing iron however the moisture content did not change, but more protein sedimented. The addition of iron into the system may have caused the casein particles to become denser without significantly affecting the moisture content allowing more particles to sediment with ultra-centrifugation. There was less protein in the serum from goat milk than cow milk as shown in Figure 4-29B. This was due to the larger micelle and amount of protein sedimented. With no iron added the serum protein content was 19.4 mg/mL and 13.2 mg/mL which decreased to 11.9 mg/mL and 3.7 mg/mL with 20 mM iron addition, for cow and goat milk, respectively. Analysis of the iron shows that in cow milk nearly all the added iron is in the soluble fraction with the exception of 20 mM, at 15 mM 92.8 % of the iron was soluble while at 20 mM the soluble iron decreased to 77.4 % (refer to Figure 4-29C and 4-29D). There is considerably less iron in the goat milk serum: at 5 mM the highest amount of soluble iron occurred with 80.8 % which halved with 20 mM iron addition to 40.3 % soluble iron. The greater serum phase iron content in the cow milk was likely due to the higher serum phase protein content and therefore added iron is more likely to bind to the free casein proteins than to partially aggregated or micellar and sedimentable casein.

a b c d e f g h i j

Figure 4-28: Pellets of cow and goat, 0, 5, 10. 15 and 20 mM iron addition; from left to right: (a) 0 mM cow, (b) 0 mM goat, (c) 5 mM cow, (d) 5 mM goat, (e) 10 mM cow, (f) 10 mM goat, (g) 15 mM cow, (h) 15 mM goat, (i) 20 mM cow, (j) 20 mM goat.

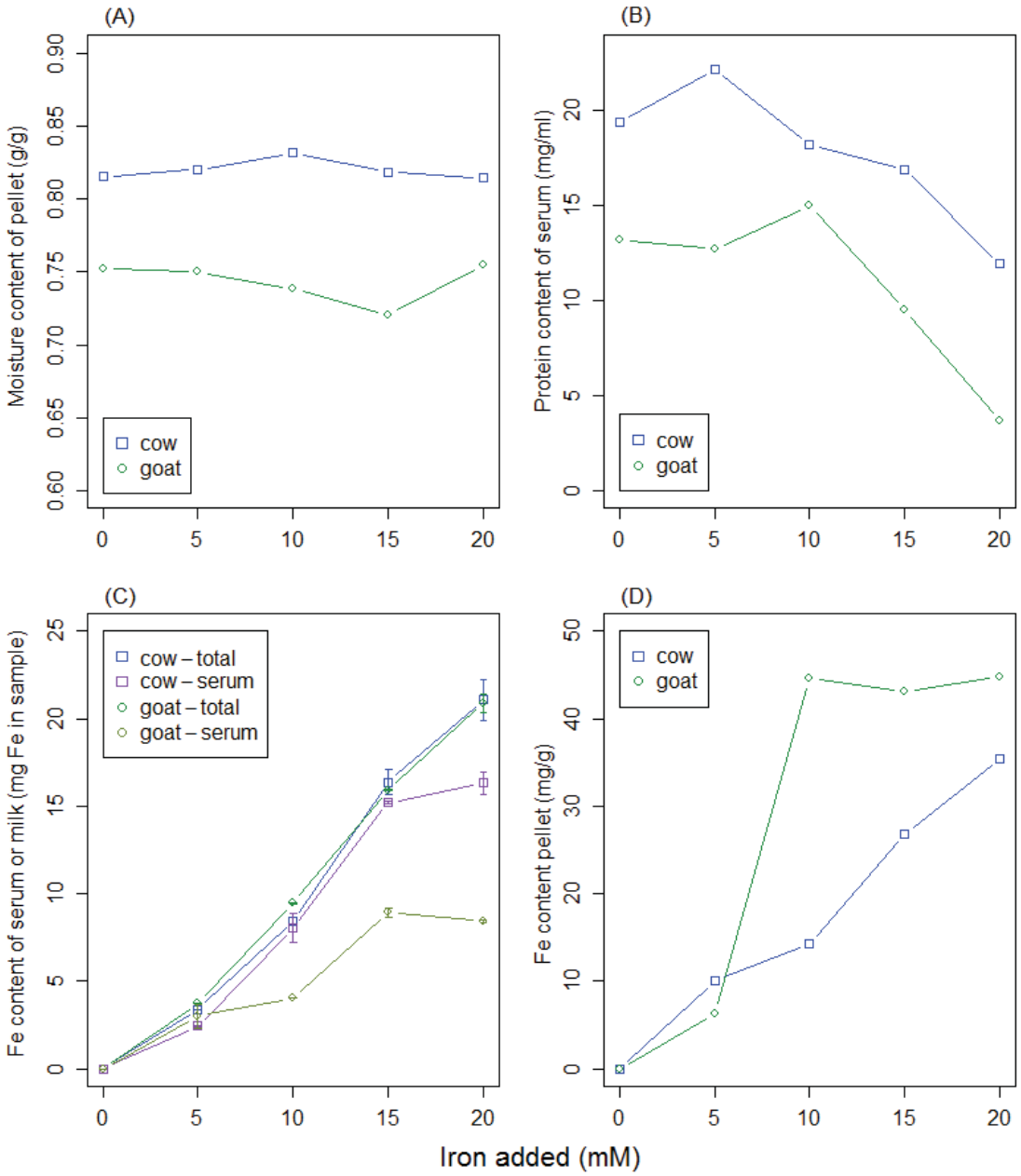


Figure 4-29: Moisture content (g water/ g pellet), A; protein content of serum (mg protein/ mL serum, B; iron content of either serum phase or starting milk solution, C; and iron content of pellet (mg Fe/ g pellet), D, of cow and goat calcium depleted milk after ultra-centrifugation at 90,000 xg for 1 hour.

4.3 Conclusion

This chapter looked at the characteristics of cow and goat milk in the presence and absence of iron as well as the effect of different processing techniques and chemical modifications. When the effect of wet blending and dry blending was considered, the wet blended milks were capable of binding more iron however it was not distinguishable if this was due to the processing method or due to the high solids

concentration. The moisture content of iron fortified goat casein micelles was found to be lower with the micelle size remaining smaller. Elemental analysis indicated that the phosphorus content of the pellet phase increased with increasing iron fortification which could be attributed to a co-precipitation or iron-phosphorus- CCP interaction to stabilise iron binding to the micelle. SAXS revealed that the processing method influenced the internal structure of the casein micelles. Not only was it confirmed that the iron binds to the same feature assigned as the CCP but the incompressible region was found to be affected in dry blended samples. In particular cow milk at 4 ^{o}C and wet blended cow milk at 37 ^{o}C with 5 mM iron addition showed a strong response at this feature. The impact is unclear at this stage. Calcium was removed from cow and goat milk to determine the effect on the external structure of the micelles and the effect of iron binding. The goat milk casein micelles were more resistant to micelle breakdown indicated by the large micelle size. The addition of iron had little effect on the moisture content of the micelles however caused further sedimentation of the serum proteins with increasing iron fortification. It appeared that more iron could bind in the pellet phase of goat milk; however this is attributed to the greater amount of sedimented protein due to the micelles being more intact.

5 Isolation and Addition of iron to caseinate solutions

5.1 Introduction

Sodium caseinate is widely used in food manufacturing and therefore has been extensively studied and reviewed for its properties and functionality (Modler, 1985; Carr & Golding, 2016). Caseins can be isolated from milk by several methods which can be categorised into insoluble (acid precipitation, renneting) and soluble (membrane technology) techniques (Carr & Golding, 2016). For example, acidification of milk produces a precipitate which can then be isolated from the serum phase. There are several parameters that must be controlled to produce a functional caseinate including temperature and pH during acidification, dewheying which aids in removing whey from the casein aggregates, washing, dewatering, mincing and finally, drying, tempering and drying (Carr & Golding, 2016).

When acid is added to cow milk the pH that needs to be obtained is between pH 4.6 and 4.7 to precipitate the casein and allows the curd to be easier to handle. This also dissolves the calcium phosphate and destroys the casein micelle structure (Dalgleish & Law, 1988; Law, 1996; Carr & Golding, 2016). The pH of goat milk needs to be lowered to pH 4.2 to allow casein precipitation (Sousa & Malcata, 1998; Park et al., 2007). Heating the acidified milk causes an aggregation of the protein allowing collection of the whey and mineral depleted caseinate. The solubility of the caseinates reduces as the temperature increases to 30 ^{o}C (Dalgleish & Law, 1988). Dewheying allows the interstitial serum containing whey and salts to be removed by pressing, centrifugation or a combination of techniques. Washing is an important step that further removes residual whey, calcium, lactose and salts from the casein precipitate (Carr & Golding, 2016). A series of washes using water can be performed in various ways at different temperatures to remove the required components but also maintain casein functionality. In the final processes of drying, tempering and grinding sodium hydroxide can be used to solubilise the casein forming sodium caseinate.

Adding calcium to caseinate solutions causes a reduction in the electrostatic repulsion allowing aggregation to occur, producing micellar type structures (Guo et al., 2003; Pitkowski, Nicolai & Durand, 2009). Due to the absence of citrate and phosphate ions in calcium caseinate the nature of calcium caseinate micellar structures is significantly

different to native milk micelles. Additionally a major use of calcium caseinates and sodium caseinates is in nutritional applications wherein iron often forms part of the formulation. Iron fortified sodium caseinate has been studied previously (Gaucheron 1996; Sugiarto, Ye & Singh 2009; Mittal et al., 2016). Sugiarto, Ye & Singh (2009) looked at the factors that influence iron binding to sodium caseinate and 90 % of the iron remained soluble from 0- 4 mM iron after which there was a precipitation of protein. They reported that adding iron up to 8 mM led to a reduction of binding to the proteins. Gaucheron (1996) found similar results where sodium caseinate precipitated at 4 mM iron. Similarly, a 2 % sodium caseinate solution could allow 90 % of up to 5 mM iron to remain soluble at pH 6.8 while above 10 mM iron there was significant protein and iron precipitation likely due to the shielding of the negative charges on the caseinate leading to aggregation and precipitation (Mittal et al., 2016). The iron binding is lower in sodium caseinate compared to milk due to an absence of phosphorus and minerals in the casein micelle. There is comparatively little data available on iron binding to goat caseinates and thus an investigation into how goat caseinates behave in the presence of iron is warranted.

This chapter compares cow and goat caseinate initially by how the composition of the caseins affect the characteristics of acid precipitated aggregates. The caseinates were fortified with ferrous sulfate at 5, 11 and 22 mM and the changes with increasing iron addition were monitored by particle sizing, protein solubility and iron partition.

5.2 Results and Discussion

5.2.1 Isoelectric precipitation of sodium caseinate from cow and goat milk

The production of cow sodium caseinate is produced on an industrial scale however goat caseinate production is restricted to a few published reports (Tavaria et al., 1997; Sousa & Malcata, 1998; Minervini et al., 2003). Casein micelles are sterically stabilised by κ-casein which coat the micelle extending out into the solvent however the stability is related to the environment. The stability can be affected by renneting, pH, salt ions and solvent quality (de Kruif, 1999). Reducing the charge density of the solution, where the pH is decreased, will cause the κ-casein to collapse on the surface of the micelle. The steric stabilisation is lost causing flocculation and van der Waals forces predominate. This allows the casein micelles to be obtained as they remain intact in an aggregated form.

5.2.1.1 Isoelectric precipitation of casein from cow milk

An investigation into the production of a precipitate of cow casein was therefore carried out at pilot scale (50 kg), with the method detailed in section 3.3.1. Precipitation of the casein began soon after sulfuric acid was added and the precipitate increased in mass as the solution was warmed. Figure 5-1 shows that the curd is very prominent with large aggregation from the serum while the separated curd in Figure 5-2 is shown to be very dry retaining a firm structure. The isoelectric precipitation allowed a strong curd to form that allowed a straight forward separation of the curd. The curd could be removed by hand or with a coarse sieve that did not result in much curd break up. A recovery of the whey with further heating allowed a clear separation of the whey and casein.

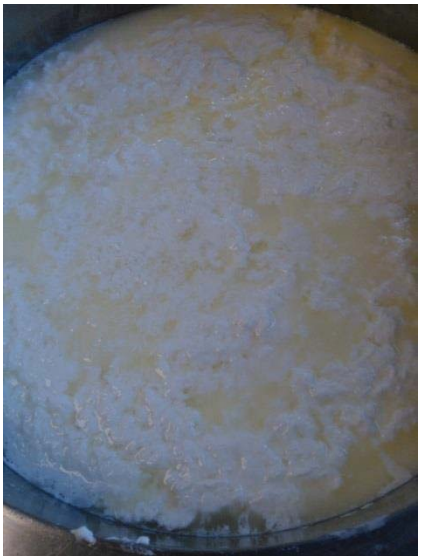


Figure 5-1: Cow skim milk precipitating at pH 4.6.

Figure 5-2: Cow casein curd after collection.

5.2.1.2 Isoelectric precipitation of casein from goat milk

The goat milk did not form a precipitate in the same manner as the cow milk. After acidification, according to section 3.3.2, the pH was checked and found to be at pH 5.0 which was slightly higher than the required pH of 4.2-4.3 (Sanz Ceballos et al., 2009; Sousa & Malcata, 1998), this indicates that the buffering capacity was higher than cow milk (Park et al., 2007). The pH was further lowered to the required pH of 4.2 with the addition of more acid. At 30 ºC a precipitate was not developing while in cow milk precipitation had occurred. The temperature was then increased to 40-45 ºC at which point the casein and whey were separating producing a strong green/ yellow clear liquid in the serum phase, Figure 5-3. However, as soon as the vat was disrupted to remove the casein precipitate it would break up turning the disrupted area back into a milk colour and the separated slurry would lose structure (Figure 5-4). The goat milk had very different properties than cow due to the casein composition.

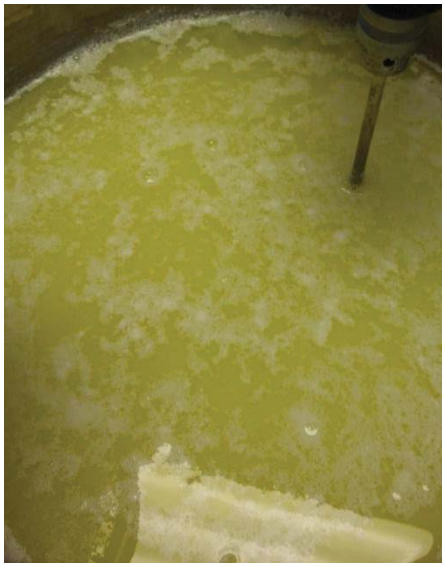


Figure 5-3: Goat skim milk precipitating at pH 4.2.

Figure 5-4: Goat casein slurry after collection.

A search of patents revealed that there were no available specific processes for industrial goat caseinate production and few about the curding characteristics of goat milk. Rheological studies have been conducted on renneting milk for cheese production and direct acidification for acid gel studies. Remeuf at al., (1989) and Park (2007) summarised that goat milk has a weaker curd structure due to its physicochemical properties of casein micelle composition (α_s to β casein ratio), size and hydration. The total casein content and casein/ total nitrogen for goat milk is lower at 75 % versus 78 % for cow which produces a more fragile rennet curd than cow. When the casein content of cow and goat milk are standardised the rennet curd is softer than from cow. Goat milk that contains overall higher casein content allows a stronger curd to form and the α_{s1} content also influences the curd structure (Clark & Sherbon, 2000). Milks with different α_{s1} contents produce very different rennet coagulates. The A variant contains the most α_{s1} casein and the null allele produces no α_{s1} casein (Pierre et al., 1995). When the total nitrogen was equal between the two variants the null allele performed worse in terms of firming time and firmness of the curd. It was believed that the smaller micelle size of the A variant and higher α_{s1} casein content favoured aggregation and enhanced performance. The gel strength and gel strengthening rate was largely influenced by the genotype in the order of AA> EE > FF, with the AA variant producing the stronger gels (Remeuf, 1993).

Storing goat milk at 4 ^{o}C for 0 to 48 hours had little effect on the renneting properties such as curd formation time and gel strength, whereas significant changes occurred to

the cow milk (Raynal & Remeuf, 2000). Cow milk increased the soluble casein fraction 4 fold after 48 hours compared to goat that increased by 10 %. The soluble calcium fraction increased by 10 % for cow whereas for goat this increased by 7 %. This indicates that the storage of goats' milk is far less affected than cow milk for the renneting of milk. The differences in the coagulating properties of goat milk can be explained by several physicochemical parameters: "casein content, total and colloidal calcium content, soluble calcium/total calcium ratio, mean diameter of casein micelles, α_s/β- casein ratio."

5.2.1.2.1 Lab scale precipitation of goat casein

A lab scale production was carried out whereby the casein curd was separated by centrifugation rather than sieving due to the weak curd. On a laboratory scale, Tavaria et al., (1997) and Sousa & Malcata (1998) isoelectrically precipitated goat casein by lowering the pH of milk to pH 4.2 – 4.3 with 6.0 M HCl followed by incubation at 37 ^{o}C for 30 minutes. The precipitate was extracted by centrifugation at 6,000 xg for 10 minutes followed by washing with water. Minervini et al., (2003) carried out an isoelectric precipitation of various species of milk with the curd being separated using centrifugation. Milks were acidified at pH 4.6 and then heated to 35 ^{o}C for 10 minutes and then centrifuged at 5000 xg for 10 minutes in their method. There is variation in the pH that was chosen for acidification between Sousa & Malcata (1998) and Minervini et al., (2003) indicating that the milk species or breed may affect the optimal pH for precipitation. A similar method of Tavaria et al., (1997) and Sousa & Malcata (1998) was employed in the present study which allowed the serum and pellet to be separated. The curd was found to separate very well using centrifugation with a clear serum containing the whey (Figure 5-5). After the second wash the serum layer was clear indicating that the majority of the whey had been removed. On a large scale, a separator would have to be used.

Figure 5-5: Goat casein curd after isoelectric precipitation and centrifugation.

5.2.1.3 RP-HPLC of cow caseinate

Cow caseinate was checked for purity using RP-HPLC by comparing the chromatograph to Alanate 180 (a commercial cow Na-Caseinate manufactured by Fonterra Co-operative Ltd) eluted under the same conditions and protein concentration. Section 3.7.6.1 details the RP-HPLC methodology used. Gagnaire et al., (1996) used a C_{18} column with similar buffers to monitor the elution profile of casein micelles. κ- casein eluted first followed by α_{s2}-casein, α_{s1}-casein and then β-casein. It can be assumed that the profiles produced will elute in the same order due to the same stationary phase used. A direct comparison cannot be done as the column length and buffer conditions were different therefore the peaks would not be expected to elute at the same time. The chromatograms of the pilot scale caseinate and Alanate 180 were very similar such that there are no significant peaks outside of the major casein protein peaks indicating that most of the whey proteins have been removed, Figure 5-6, 5-7. In the Alanate 180 elution profile there is a small peak at 18 minutes which is likely to be the κ- casein. This does not appear in the lab-scale cow caseinate indicating that the κ-casein may have been washed away or is masked by the large α_{s2}-casein peak. The concentration of β-casein may have also been reduced as Alanate 180 had a peak of about 650 mAu while the precipitated caseinate had a β-caseinate peak of 590 mAu and the α_{s1}-casein peak intensity remained similar between the samples. The separation was not very clear due to limitations of the column as the column length may not have been long enough to allow the casein proteins to separate fully.

The small peak eluted after β-casein may be α- lactalbumin which appears in both chromatograms indicating complete removal was not achieved (Moatsou et al., 2008). During preheating for spray drying denatured whey can attach to the casein micelles and are predominantly β- lactoglobulin and bovine serum albumin (Oldfield, Taylor & Singh, 2005). In comparison to the other peaks however, the area is very small and indicates that the majority of the whey proteins were removed.

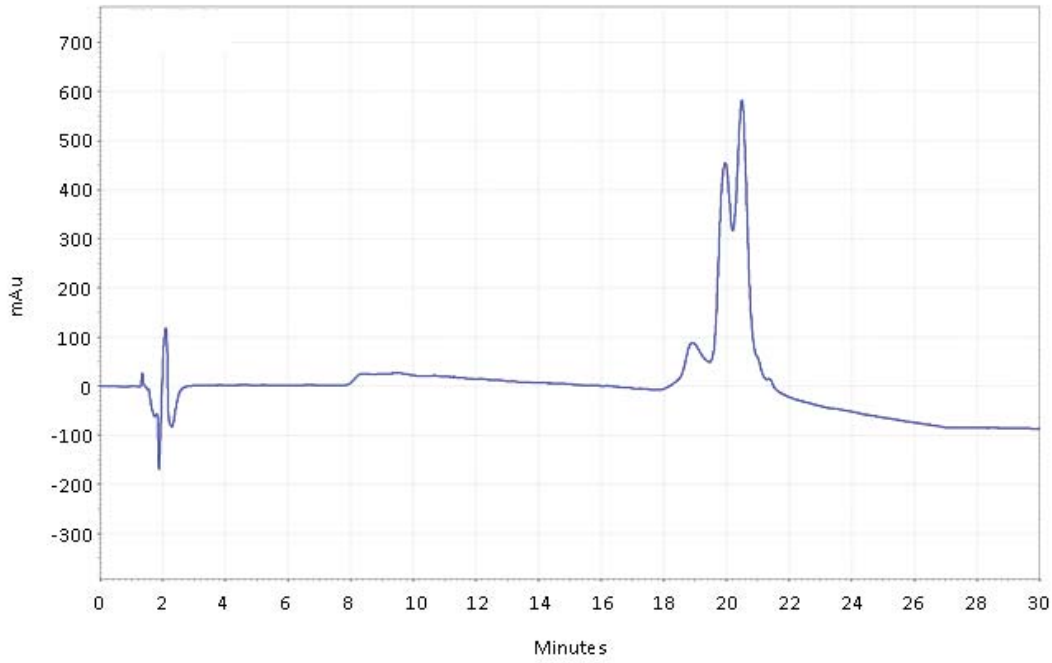


Figure 5-6: RP-HPLC chromatogram of isoelectric precipitated cow caseinate using a 5 % to 60 % buffer B gradient at 214 nm.

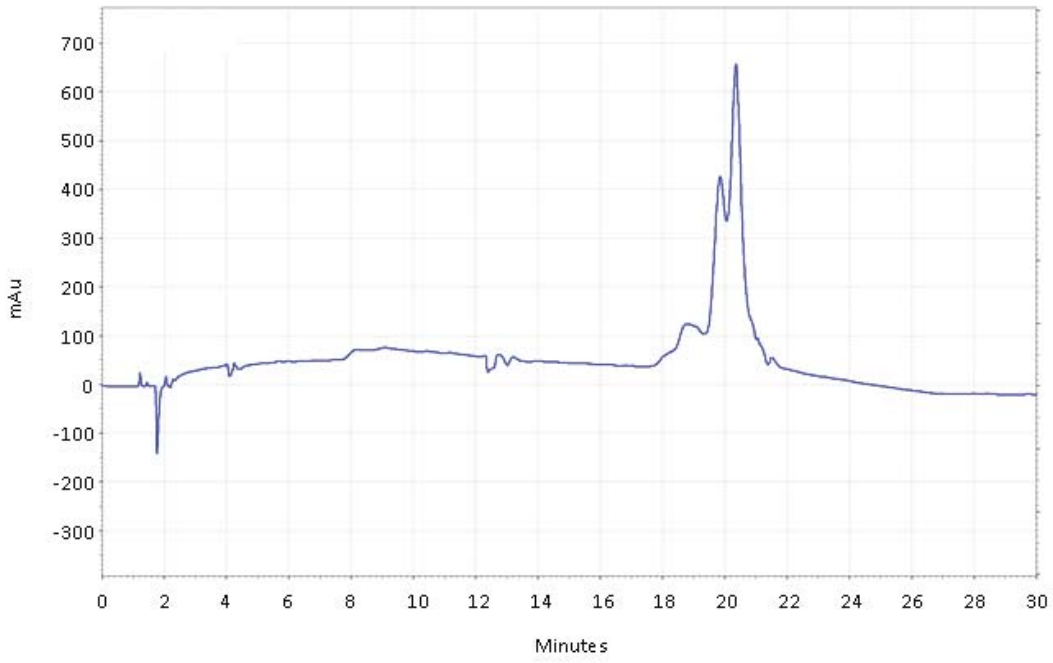


Figure 5-7: RP-HPLC chromatogram of Alanate 180 using a 5 % to 60 % buffer B gradient at 214 nm.

5.2.1.4 RP-HPLC of goat caseinate prepared at laboratory scale

Figure 5-8 shows the elution profile of the goat caseinate was very different to the cow caseinates when performed under the same elution conditions. There are four peaks which can be assigned as κ-, α_{s1}-, α_{s2}-, and β- caseins, respectively. The κ- casein has a much stronger response which may indicate that either this is in higher concentration than the cow caseinates or the precipitation process on a lab scale is gentler compared to large scale precipitations. Unlike the cow caseinates, the α_{s1}- casein response is a lot lower and is not overly well separated from the β-casein which has a stronger response than cow. This supports Tziboula (1997); Clark & Sherbon, (2000); Sanz Ceballos et al., (2009); Selvaggi et al., (2014) indicating that goat casein is much higher in β-casein concentration compared to cow casein. This confirms why goat casein is harder to precipitate than cow casein as a higher β casein concentration forms a weaker curd due to a lower phosphorylation than the α- caseins. Moatsou et al., (2004) ran different breeds of goat caseins on a C_4 column and the caseins did elute in the same order as cow caseins and that κ-, α_{s1}-, and α_{s2}- showed elution peaks at approximately equal heights with some variation between breeds, while the β- casein had a much greater peak height. There did not appear to be any whey protein peaks appearing after the β- caseinate peak. This is similar to what was found although the

separation of α_{s1}- casein was poor the shoulder is about equal to the κ- casein peak height. The α_{s2}- casein peak is significantly smaller and Moatsou et al., (2008) showed that some breeds do produce caseinate profiles with α_{s2}- casein that is lower in peak height than the κ- and α_{s1}- casein peaks.

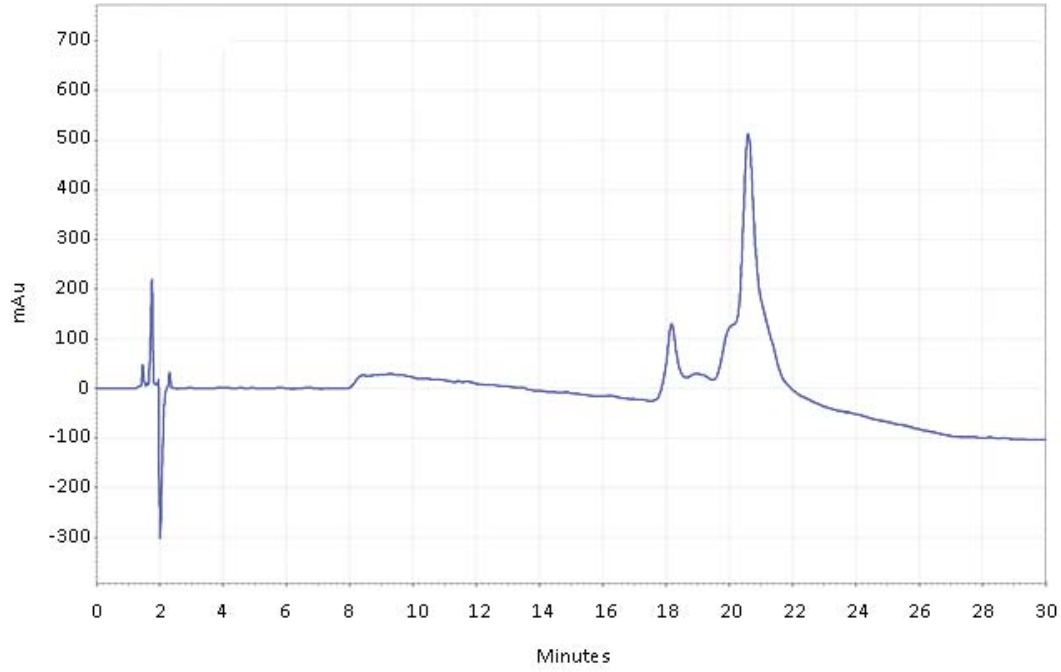


Figure 5-8: RP-HPLC chromatogram of isoelectric precipitated goat caseinate from a lab scale extraction at 214 nm.

5.2.2 Iron fortification of cow and goat caseinate

5.2.2.1 Addition of ferrous sulfate to cow and goat caseinate

Ferrous sulfate was added to caseinate mixtures to determine if caseinate behaves similarly to when calcium is added (Smialowska et al., 2017). The particle size and turbidity were measured for the caseinate mixtures to compare the characteristics of the solutions. Cow and goat caseinate were fortified with 5 mM, 11 mM and 22 mM ferrous sulfate (according to the method detailed in section 3.3.3) to match the work carried out using calcium on the cow caseinate samples. Samples were adjusted back to pH 6.7 with sodium hydroxide or left unadjusted.

5.2.2.1.1 Observations of cow and goat caseinate fortified with iron

The samples that were adjusted to pH 6.7 after ferrous addition (Figure 5-9c, d) became more orange than the unadjusted samples (Figure 5-9a, b). This is due to the

ferrous ions becoming oxidised with the increased alkalinity of the samples. The cow samples appear more oxidised than the goat samples while the 11 mM samples appear more orange than the 22 mM samples in both cow and goat samples. This can be explained by the differences in opacity between the 11 mM and 22 mM samples. At 22 mM the samples were more opaque than the 11 mM and therefore it appeared that there was a greater extent of oxidation in the clearer, 11 mM samples. There was more extensive aggregation in the 22 mM samples which follows the trends that occurred in the calcium where the opacity increased after 11 mM addition. The turbidity could not be measured accurately due to the colour change and formation of large gelatinous aggregates. The caseinates were not stable in the presence of iron, samples were chilled prior to measurement and once exposed to ambient air the oxidation increased quickly to form a strong orange colour with precipitation. Over time period of approximately three hours a gelatinous layer formed for all the samples which indicated that caseinate solutions are very unstable in the presence of high iron, unlike skim milk. This was expected as the iron fortification levels were above the iron fortification level used by Gaucheron (1996) and Sugiarto et al., (2009) who reported instability at around 4 mM iron.

Figure 5-9a: Cow caseinate (3.0 %) fortified with 5 mM, 11 mM and 22 mM ferrous sulfate with an unadjusted pH after fortification.

Figure 5-9b: Goat caseinate (3.0 %) fortified with 5 mM, 11 mM and 22 mM ferrous sulfate with an unadjusted pH after fortification.

Figure 5-9c: Cow caseinate (3.0 %) fortified with 5 mM, 11 mM and 22 mM ferrous sulfate with an adjusted pH to 6.7 after fortification.

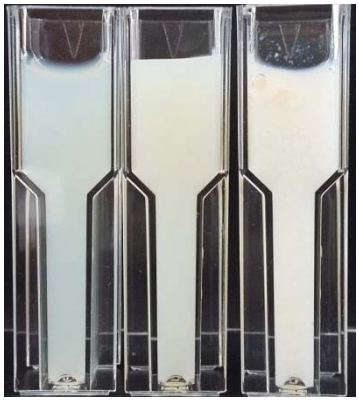


Figure 5-9d: Goat caseinate (3.0 %) fortified with 5 mM, 11 mM and 22 mM ferrous sulfate with an adjusted pH to 6.7 after fortification.

5.2.2.1.2 Dynamic light scattering of cow and goat caseinate fortified with iron at pH 6.7

Cow caseinate fortified with 5 mM, 11 mM and 22 mM iron produced particle size distributions by intensity that were bi-modal with the size increasing as the iron content increased as shown in Figure 5-10. At 5 mM the dominant peak was at a maximum at 530 nm with a minor peak at 21 nm. At 11 mM the major peak at 299 nm was smaller however the minor peak increased to 50 nm. At 22 mM the major peak was close to the 5 mM addition at 531 nm however the minor peak was much larger at 141 nm. The control (0 mM) containing no added iron displayed a tri-modal distribution with peaks at 6, 23 and 190 nm. This may indicate that the minor peak in the intensity distribution is dependent on the iron concentration while the larger peak is the aggregation of these smaller particles. In terms of the number distribution the means increased from 15 nm, 24 nm and 91 nm with increasing iron concentration

with the control having a peak at 4 nm. The major peaks were no longer present as they were in low concentration while these distributions represent the dominant particle size distribution.

The goat caseinate with increasing levels of iron did not produce consistent results as with cow caseinate. At 5 mM and 11 mM, by intensity distribution there was a uni-modal size distribution while at 22 mM there was a bi-model distribution. With no added iron the peak intensity was 190 nm. At 5 mM the peak intensity was at 220 nm which did not change at 11 mM however the distribution was much larger. Increasing to 22 mM caused a major peak at 396 nm. Due to the spread of 11 mM and the bi-modal distribution of 22 mM the size by number distribution trend was that the particle sizes were not as consistent. At 5 mM the particle size was 91 nm, at 11 mM the size decreased to 24 nm and then increased to 43 nm at 22 mM. The control had a peak particle size of 33 nm. The polydispersity was high, this indicates that the formation of aggregates may have been quite random rather than forming a structure, or a broad distribution of aggregates were formed. In terms of volume distribution it can be seen that the spread is large indicating various particles are formed. This may indicate that the goat caseinate is more unstable than cow in forming aggregates that may grow as iron is added.

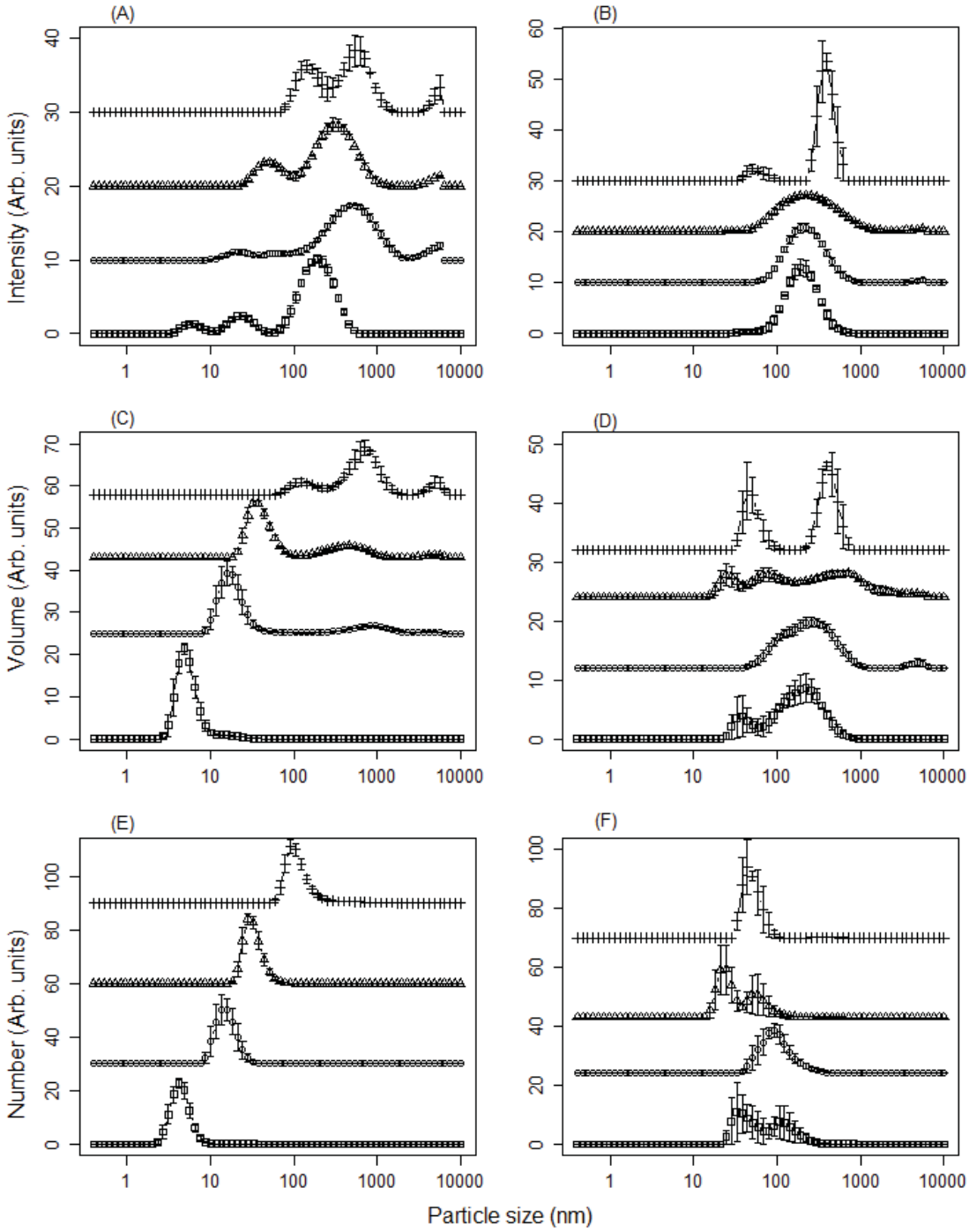


Figure 5-10: Particle size by intensity (A, B), volume (C, D) and number (E, F) of cow (A, C, E) and goat (B, D, F) caseinate fortified with 0 (□), 5 (○), 11 (Δ), and 22 (+) mM ferrous sulfate; error bars indicate standard error, n=3.

5.2.2.1.3 Protein solubility of cow and goat caseinate with addition of iron

The solubility of the iron fortified caseinate solutions was tested according to Sugarito et al., (2009). After fortification the samples were centrifuged at 10,800 xg for 20 minutes at room temperature and the soluble protein in the serum layer was measured.

The samples were analysed before the onset of precipitation. Figure 5-11 showed that with no iron added all the cow caseinate protein was soluble while 0.97 ± 0.02 of the goat caseinate protein was soluble (p=0.432). With the addition of 5 mM iron the protein solubility did not decrease significantly, 0.95 ± 0.01 of the cow caseinate remained in the supernatant and 0.99 ± 0.03 of the goat caseinate remained soluble (p=0.301). With 11 mM iron addition the cow caseinate was mostly soluble with 0.92 ± 0.01 of the protein in the supernatant while the goat caseinate solubility decreased significantly to 0.64 ± 0.02 soluble protein (p=0.008). With 22 mM iron the amount of soluble caseinate was the same for the species with 0.39 ± 0.01 of cow caseinate being soluble and 0.41 ± 0.01 of the goat caseinate being soluble (p=0.796).

Sugiarto et al., (2009) added ferrous sulfate to a 1 % sodium caseinate solution up to 20 mM. The soluble protein content remained stable to 4 mM iron and then decreased to less than 10 % soluble protein at the maximum iron addition therefore the protein remained soluble with up to 22 mg Fe/ g protein. Gaucheron et al., (1996) used a sodium caseinate concentration of 25 g/L with a maximum of 7.5 mM ferrous chloride added. The protein remained soluble to about 5 mM but became completely insoluble at 7.5 mM. The partition method was different with the centrifugation being 500 xg for 15 minutes and therefore is difficult to compare but the amount of iron added that allowed the protein to remain soluble was 9 mg Fe/ g protein. Purified β- casein was able to bind 16.62 mg Fe/ g protein (Baumy & Brule, 1988) while Nelson & Potter (1979) found the precipitation point of ferrous sulfate and sodium caseinate was 20 mg Fe/ g protein. In comparison, the cow sodium caseinate in this study with 11 mM iron, added the protein bound 16.15 mg Fe/ g protein before the solubility of the protein decreased. The iron binding capacity of the caseinates in the present study is lower than the value obtained by Nelson & Potter (1979) and Sugiarto et al., (2009) which may indicate that the solution is less stable under the conditions used. The goat caseinate bound 7.34 mg Fe/ g protein with 5 mM addition before the solubility decreased. An explanation for this decreased solubility is the particle sizes of the aggregates. At 11 mM it was found that the goat caseinates produced a broad distribution of particle sizes and therefore these large aggregates may have sedimented. A greater number of iron fortification levels would allow for a better prediction of the solubility as the protein may remain soluble with a greater level of fortification before 22 mM for cow and 11 mM for goat. The iron saturation point of

the goat caseinate is lower than cow caseinate which would mainly be governed by the higher amount of β-casein. As Baumy & Brule (1988) found that the solubility of β-casein is lower than the other reported values of sodium caseinate it may be that the goat caseinate has a lower stability due to the higher β-casein content. No literature was found where iron was added to goat derived caseinate.

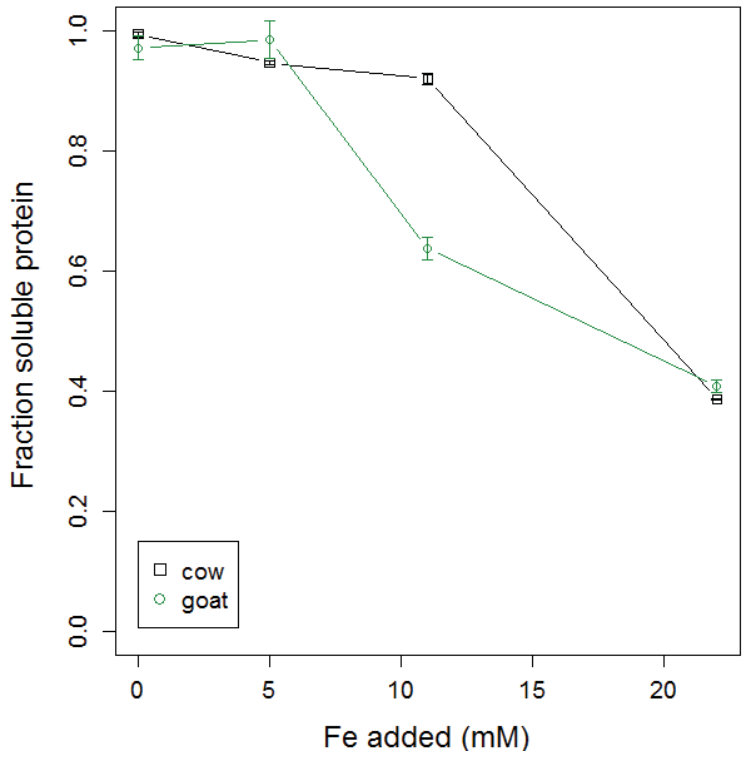


Figure 5-11: Protein solubility of cow and goat caseinate with iron addition of 0, 5, 11 and 22 mM ferrous sulfate after centrifugation at 10,800 xg for 20 minutes; error bars indicate standard error, n=3.

5.2.2.1.4 Iron partition of cow and goat caseinate

Very small quantities of iron were found in the unfortified caseinates and this was present in the full amount in the supernatant as essentially all the protein was soluble with no iron addition.

The solubility of iron in this section refers to iron that remains in the supernatant after centrifugation. At 5 mM iron addition the solubility of the iron in cow and goat caseinate is over 100 %; Figure 5-12 (due to variation in the assay). This indicates that the iron is soluble along with the caseinate proteins. The iron is presumably binding to the caseinates (or in ionic form in solution) as this is below the saturation point for iron binding (Nelson & Potter, 1979; Gaucheron et al., 1996; Sugiarto et al., 2009).

With 11 mM iron the iron solubility with cow caseinate was 0.40±0.11. A similar trend occurred by Sugiarto et al., (2009) with cow caseinate where the iron solubility decreased and the protein remained soluble, for example, at the lowest solubility of 20 % iron about 60 % of the protein was soluble. The saturation of iron and protein was reached and there was a precipitation of oxidised iron that was able to sediment along with protein with centrifugation. Interestingly, when 11 mM iron was added to goat caseinate the iron solubility was 0.83±0.07 which is significantly higher than the cow caseinate (0.40±0.11) but more so that the protein solubility was lower at 0.64±0.02 soluble protein compared to 0.92±0.01 for the equivalent cow caseinate system. The amount of soluble iron bound to the soluble phase of the protein is 20.95 mg Fe/ g protein, similar to Nelson & Potter (1979) and Sugiarto et al., (2009); the soluble iron and protein binding for cow caseinate was 6.87 mg Fe/ g protein at 11 mM addition.

With 22 mM iron addition 0.32±0.09 and 0.43±0.08 of the iron remains soluble for cow and goat caseinate, respectively. About 40 % protein remains in solution for both caseinates indicating that when there is an excess of iron the caseinates behave similarly. It is likely that there is a co-precipitation of the iron and protein when the charges are neutralised along with excess iron that has oxidised.

When comparing skim milk to a caseinate digest the iron binding of the caseinate can be explained by an absence of other non-casein constituents that have been removed from the system. Whey proteins, citrates and inorganic phosphate have an ability to bind iron and therefore molecules and compounds that are not taken into account on a protein basis could increase the iron binding (Gaucheron, 2000) and increase stability through a stronger buffering capacity.

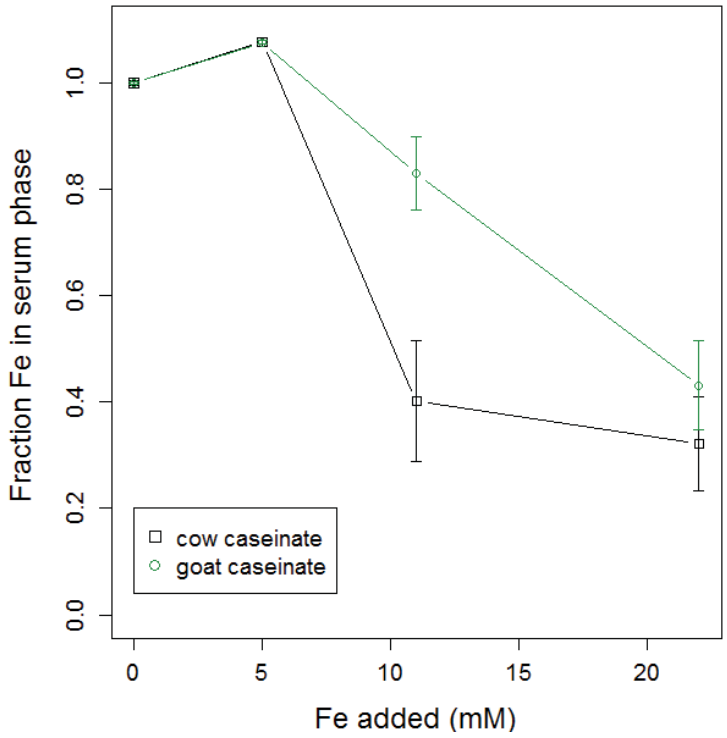


Figure 5-12: Iron partition of iron fortified cow and goat caseinate; error bars indicate standard error, n=3.

5.3 Conclusion

From the observational results the curd structures of the two milk species are very different. The cow curd is significantly firmer than the goat which was found to produce the characteristically soft curd. Further investigation into whether the conditions are required to be changed for the isoelectric precipitation of goat casein should be carried out, for example the optimal temperature of precipitation and the true acid consumption. It is also obvious that manual type separation is not suitable for goat casein collection and some form of separator would be required in an industrial setting if this is the strength of curd that can be produced. RP-HPLC showed that a lab scale isoelectric precipitation of goat casein was effective at removing whey proteins. The goat caseinate was found to contain significantly more β-casein than cow caseinate.

Particle size analysis, protein and mineral partition as well as observations on the iron fortified cow and goat caseinates have shown that the caseinate- iron complex is not stable at room temperature. The caseinates show obvious iron oxidation, precipitation and gelation over a short time period (approximately three hours); in 22 mM iron fortified samples this was within an hour. The particle size analysis showed that a

broad distribution of particles sizes. In terms of number distribution the cow caseinates increased in size from 15 nm, 24 nm and 91 nm with increasing iron however no trend was found for goat caseinates of 91 nm, 24 nm and 43 nm, with 5 mM, 11 mM and 22 mM iron addition. The partition studies indicate that 5 mM of iron allows the iron-caseinate complex to remain soluble as all of the iron remained in the supernatant phase and 0.95 ± 0.01 and 0.99 ± 0.03 of the cow and goat caseinate, respectively, remained soluble. With 11 mM variation in the iron and protein solubility occurred with the iron becoming progressively more insoluble while at 22 mM less than half of the iron and protein in cow and goat caseinate solutions remained soluble.

6 Isolation and characterisation of phosphopeptides by selective precipitation and IMAC

6.1 Introduction

Some digestion products of milk caseins facilitate the absorption of iron in the small intestine (Aít-Oukhatar et al., 1999; Pérès et al., 1999; Bouhallab et al., 2002; Kibangou et al., 2005). Iron bound to β-CN (1-25), a phosphoserine containing peptide, improved the iron tissue storage and the haemoglobin levels during the repletion of deficient rats compared to $FeSO_4$ (Aít-Oukhatar et al., 1999). Hurrell et al., (1989) showed that a casein digest could improve iron absorption compared to intact casein. The dialysability and absorption of the hydrolysed product was most likely improved by the difference in size allowing it to pass through a membrane, simulating the intestine, while whole caseins cannot. Phosphopeptides from α_{s1}-, α_{s2}- and β caseins contain highly polar acidic sequences of three phosphoseryl groups followed by two glutamic acid residues, Ser(P)Ser(P)Ser(P)EE (Reynolds et al., 1994; Bouhallab & Bougle, 2004; Miquel et al., 2006b). These mineral binding peptides contain a phosphate group covalently bound to seryl residues via monoester linkages. The cation binding capacity of the phosphoserine residue cluster decreases in the order α_{s2}> α_{s1}> β> κ casein which is related to their decreasing phosphoserine contents (Gaucheron, 2005).

Phosphoseryl residues promote iron oxidation from the ferrous to the ferric state in a complex which appears to be highly stable. Unlike the ferric form in a simple salt solution, ferric ions in the phosphoseryl-iron complex do not reduce to the ferrous form when the pH is lowered to pH 2.5 and remain bound (Emery, 1992; Yeung et al., 2002; Kitts, 2005). This indicates that the interaction is via covalent bonding; however Pérès et al., (1999) postulated that phosphopeptides can bind iron with ionic bonding in addition to coordination bonds. Dephosphorylation of phosphoserine results in a reduction of iron binding suggesting that the affinity of casein for ferric iron is caused by phosphoserine residues (Yeung et al., 2002). Conformational changes occur to the peptide where the iron attaches to the oxygen on the phosphoserine residue in a tetrahedral co-ordination (Kitts, 2005). β CN-(1-25)4P has been shown to bind four or five iron atoms and the complexes are resistant to further breakdown in the gut (Ani-Kibangou et al., 2005; Miquel et al., 2006b).

Phosphopeptides have been isolated using the method by the group Adamson and Reynolds (Reynolds et al., 1994; Adamson & Reynolds, 1995; Adamson & Reynolds, 1996; Cross et al., 2005) and also has been used several times by different authors (Aoki, et al., 1998; McDonagh & FitzGerald, 1998; Corsetti, et al., 2003; Kim et al., 2007; Zhao, Wang, Xu, 2007). Most of the work carried out using this method uses cow caseinate with trypsin as the enzyme however other enzymes including Alcalase have been used (Adamson & Reynolds, 1996; Zhao, Wang, Xu, 2007). Extensive investigations by McDonagh & FitzGerald, (1998) were carried out on the efficacy of 28 commercial enzymes who found that there were no relationships between degree of hydrolysis, CCP yield and calcium binding. Only two papers (Corsetti et al., 2003; Mora-Gutierrez et al., 2007) were found that investigated caseinates derived from non-cow milks, including goat caseinate using calcium and ethanol precipitation. Mora-Gutierrez, et al., (2007), like much of the work of Reynolds and co-workers, used trypsin to generate phosphopeptides from bovine and caprine caseinates. Following the trypsin digest, Mora-Gutierrez et al., (2007), investigated calcium intestinal absorption in rats. Corsetti et al., (2003), used a different enzyme system (a peptidase isolated from *Lactobacillus helveticus* PR4) and reported that goat caseinate produced the highest phosphopeptide yield and that these goat derived phosphopeptides had a lower calcium binding capacity compared to bovine caseinate derived CCP. Trypsin was chosen for further study in the current research to enable comparison with the major body of phosphopeptide work.

The objectives of this chapter are as follows:

1. To determine the effect of the trypsin to substrate ratio on the degree of hydrolysis from cow and goat caseinate at a 1:50 and 1:200 enzyme to protein ratio.
2. To determine how the degree of hydrolysis affects the iron chelation capacity of the resultant peptide solution at the two different enzyme ratios.
3. To characterise the isolated peptides that are isoelectrically precipitated by calcium according to the methods of Adamson & Reynolds (1996).
4. To investigate whether the methodology of Reynolds and co-workers can be adapted to utilise iron in place of calcium for the precipitation step. Other workers have manufactured iron containing phosphopeptides but these have started from isolated phosphopeptides followed by adding iron and then

filtering the solution (Ait-Oukhatar et al., 2002; Bouahallab et al., 2002; Chaud et al., 2002, Kibangou et al., 2005; Wang et al., 2011).

5. To compare calcium and iron precipitated peptides to determine if similar compositions of isolated peptides could be obtained from an iron derived peptide isolate.
6. Use IMAC to isolate iron binding peptides for caseinate digests (Nuwaysir & Stults, 1993; Cao & Stults, 1999; Zhang et al., 2007) to determine if a pure phosphoserine fraction of peptides can be due to the reported higher sensitivity of the resin to phosphoserine groups.

6.2 Results and Discussion

6.2.1 Digestion of cow and goat caseinate- Rate of hydrolysis

6.2.1.1 1:50 enzyme to protein ratio

The rate of change in the degree of hydrolysis profile with time is shown in Figure 6-1 according to the method detailed in section 3.4.1. The DH-time profiles for both cow and goat caseinates are typical of hydrolysate reactions (Wang et al., 2011) and there was no significant difference ($p>0.05$) between the profiles. The pH of the solutions after 18 hours of hydrolysis was pH 7.05 ± 0.04 for cow caseinate and pH 7.03 ± 0.10 for goat caseinate. The activity of trypsin begins to decrease below pH 7.5 and therefore the rate of digestion would be slower near the end of the digestion period which may explain why the DH levelled off by 8 hours (Sipos & Merkel, 1970). No previous studies have been reported comparing the rate of hydrolysis of goat caseinate with cow caseinate using trypsin as the proteolytic enzyme.

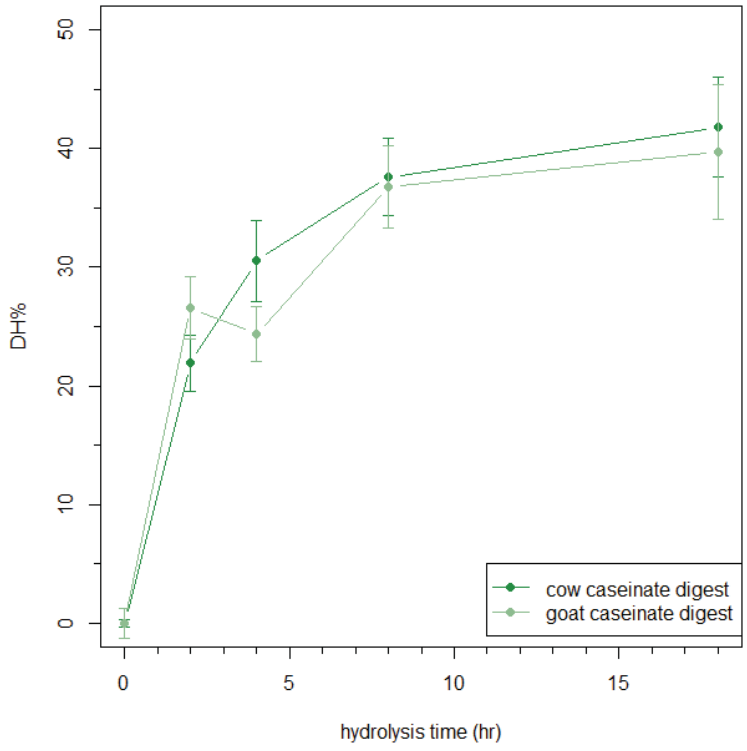


Figure 6-1: Degree of hydrolysis versus time of 1:50 enzyme to protein cow and goat caseinate samples; error bars indicate standard error, n=3.

6.2.1.2 1:200 enzyme to protein ratio

Decreasing the enzyme to substrate ratio resulted in a typical DH-time profile and due to the linear increase of DH with time from 2 hours to 18 hours it was evident that the reaction had not reached completion as shown in Figure 6-2. Through comparison with the 1:50 E: S data it would seem that only 55-60 % of hydrolysable bonds have been cleaved. The degree of hydrolysis was 25.0 ± 1.0 % for cow and 22.6 ± 0.8 % for goat ($p=0.448$) at the end of the reaction time. As the hydrolysis was carried out under pH non-stat conditions the final pH at the cessation of the experiment (pH 7.44 ± 0.01 for cow and pH 7.29 ± 0.02 for goat caseinate) was, as expected, higher than in the 1:50 E:S experiment.

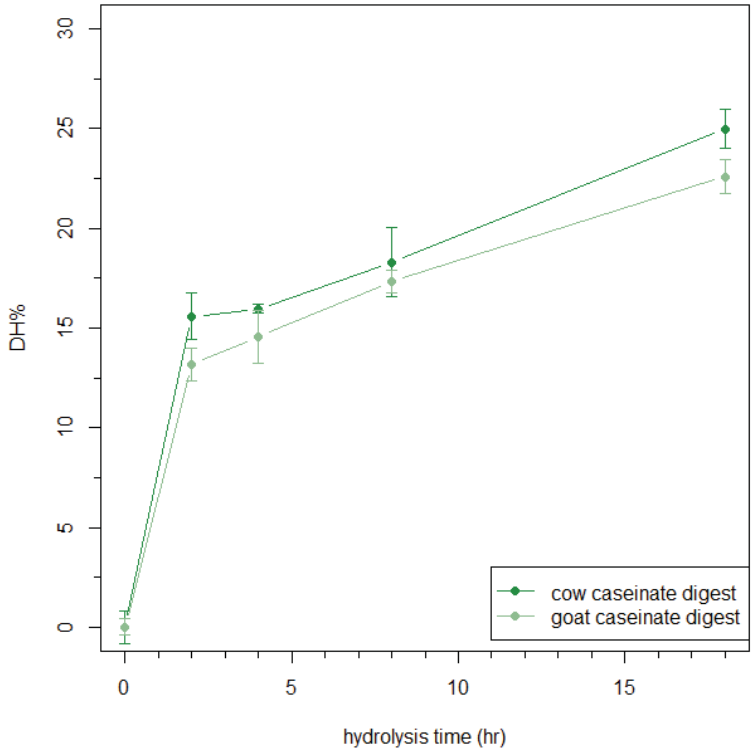


Figure 6-2: Degree of hydrolysis curve of cow and goat caseinate with a 1:200 enzyme to protein ratio; error bars indicate standard error, n=3.

Although trypsin was used by many researchers (Léonil et al., 1994; Ono, Ohotawa & Takagi, 1994; Reynolds et al., 1994; Gagnaire et al., 1996; Aít-Oukhatar et al., 1999; Bouhallab et al., 2002; Kibangou, 2005) to hydrolyse bovine casein there is little data published on the degree of hydrolysis of the digest. Ellegård et al., (1999) found the degree of hydrolysis was 18.8±0.6 % from caseinate using trypsin. This is lower than

the results obtained here however the solids content of the Ellegård et al., (1999) sample was 10 % (w/w) caseinate while this trial used 3.5 % (w/w) which may have caused a difference in cleavage. McDonagh and FitzGerald (1998) found a degree of hydrolysis of sodium caseinate using trypsin of 8 % when the reaction was maintained at 50 ^{o}C and allowed to proceed until no more base consumption occurred. Adamson and Reynolds (1997) compared various enzymes including TPCK-treat trypsin and Novo trypsin 3.0S at different protein to enzyme ratios from 1:50 to 1:1600 with a 2 hour hydrolysis time. It was found that the degree of hydrolysis of a 1:50 ratio was 20 % while a 1:200 was close to 15 %; the hydrolytic activity of pure trypsin was greater (where no more base was consumed) than the Novo trypsin indicating that the contaminating enzymes caused further non-specific cleavage. It is likely that the enzyme used in this work contained contaminant enzymes as it is a commercial grade enzyme rather than bio-reagent grade which would explain the higher degree of hydrolysis. The curvilinear trend displayed in Figure 6-1 indicates that the reaction rate decreased which is likely due to both binding sites for cleavage becoming less common in addition to the pH decreasing such that the enzyme is no longer in optimal conditions. In contrast, the pH at 1:200 did not drop to the same extent as 1:50 therefore enzyme activity was greater over the hydrolysis time.

Nielson et al., (2001) used sodium caseinate as a model for the hydrolysis process and tested the method using both endo-peptidases (Alcalase, and Neutrase) and exo-peptidases (Flavourzyme). The degree of hydrolysis found after 0, 2, 3 and 20.5 hours was 2.36, 37.69, 42.86 and 60.33 %, respectively. Similarly, Mahmoud et al., (1992) used porcine pancreatin on casein and found the degree of hydrolysis to be around 70 % at the end of the reaction. A broad range of enzymes have been used to digest casein to obtain the peptides of interest. The lower DH results reported here reflect the reduced specificity of the enzyme used. The differences in the degree of hydrolysis relate directly to the sizes of the final peptide mix. It would be expected that a high DH would result in a peptide profile containing predominantly small peptides. While small peptides may result in a final isolate of high phosphoserine content the overall functionality of the peptides may be reduced in terms of iron binding and protection of the iron from fats. These investigations into changes in functionality were beyond the scope of the project due to the vast number of enzymes that could be utilised and various hydrolysis conditions that could be applied. Trypsin was used due to the

extensive application by Reynolds and co-workers under various conditions using cow sodium caseinate.

The goat caseinates produced a lower DH (although not significantly lower) than the cow caseinates. In contrast, Almaas et al., (2006) found that goat milk degraded faster than cow milk using a two stage digestion of human digestive enzymes of the gastric and intestinal stages. The author attributed this mainly to the β-LG in bovine milk which was more resistant to digestion. However, variations in the protein content of casein, difference in minerals, carbohydrate content, buffering capacity and the enzyme activity on the proteins were stated to cause the faster pH drop in goat milk. As the current study was using caseinate only, many of these factors were eliminated and therefore it is most likely that the structure of the casein proteins was the significant factor that altered how the digestion occurred. The goat caseinate may be more resistant to digestion than cow caseinate, compared to a full milk hydrolysis such as Almaas et al., (2006) performed.

The literature indicates that the degree of hydrolysis is extremely variable depending on the enzyme used, the enzyme to protein ratio, the hydrolysis environment and reaction time. It is difficult to assess whether the hydrolysis performed as expected because of this, however it can be stated that a lower enzyme to protein ratio appears to produce a lower degree of hydrolysis which is closer in range to Adamson & Reynolds (1997); McDonagh & FitzGerald (1998); Ellegård et al., (1999) who reported their values of a tryptic digestion. This may indicate that the higher ratio of 1:50 over the hydrolysis time used may cause more non-specific cleavage from the trypsin (Lorenzen, Fisher & Schlimme, 1994; Adamson & Reynolds, 1997) which may compromise the phosphoserine clusters in the peptides that will be isolated.

6.2.2 RP-HPLC of hydrolysed cow and goat caseinates

6.2.2.1 1:50 time series of cow and goat caseiante hydrolysates

RP-HPLC was performed according to section 3.7.6.2. Figure 6-3 shows the RP-HPLC chromatograms at 0 hours of hydrolysis show that there are broad peaks for cow and goat caseinate confirming that the caseinate was intact. After 2 hours of hydrolysis the caseinates have already broken down significantly for both species; this is in good agreement with the degree of hydrolysis data which shows a sharp increase

within the first two hours of reaction. As the hydrolysis continues over the 18 hour reaction time the number of peaks increases and they elute earlier in the chromatogram. Appendix A shows the chromatograms of the full times series of 0, 2, 4, 8 and 18 hours of digestion. The RP-HPLC column causes hydrophilic peptides to elute early on and hydrophobic peptides or larger peptides and proteins to be retained longer indicating that smaller and more hydrophilic peptides are being produced with hydrolysis. The peak that appears in all the chromatograms at approximately 16.5 minutes is an acetonitrile peak caused by the elution profile.

The intact casein is largely digested by the end of the hydrolysis time however there may be some un-digestible protein present as peaks remain. The cow caseinate has two intense peaks after two hours of digestion which only disappear at 18 hours, and different peptide peaks become more intense. This indicates that large or hydrophobic protein is quite resistant (presumably due to potential cleavage sites being either sterically hidden from the enzyme or that the sites are energetically less favourable to the binding pockets of the enzymes) to breakdown and require a long hydrolysis time. The small peptides and hydrophilic peptides become more numerous at 18 hours.

In contrast, in Figure 6-4, goat caseinate produced smaller or more polar peptides relatively quickly. The degree of hydrolysis at 2 hours was found to be significantly higher in goat caseinate compared to cow which is in agreement with the pattern seen in the chromatogram. The degree of hydrolysis data indicated that digestion still occurred after 2 hours and it can be seen in the chromatogram that changes did occur at 18 hours of digestion, with more peaks appearing in the earlier and later stages of elution.

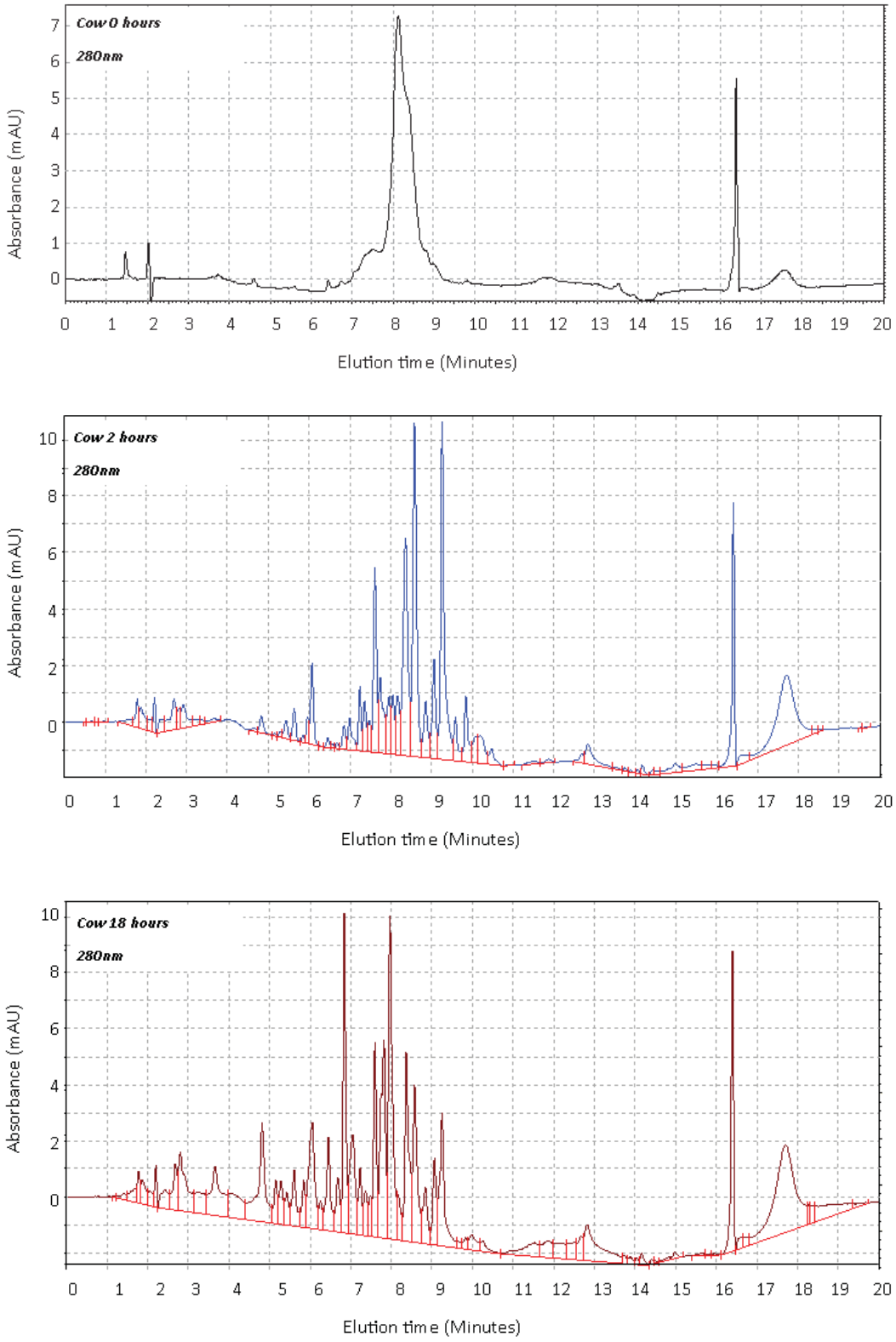


Figure 6-3: Chromatograms of cow caseinate digests at 0, 2, and 18 hours with 1:50 enzyme to protein ratio measured at 280 nm, bandwidth 8 nm.

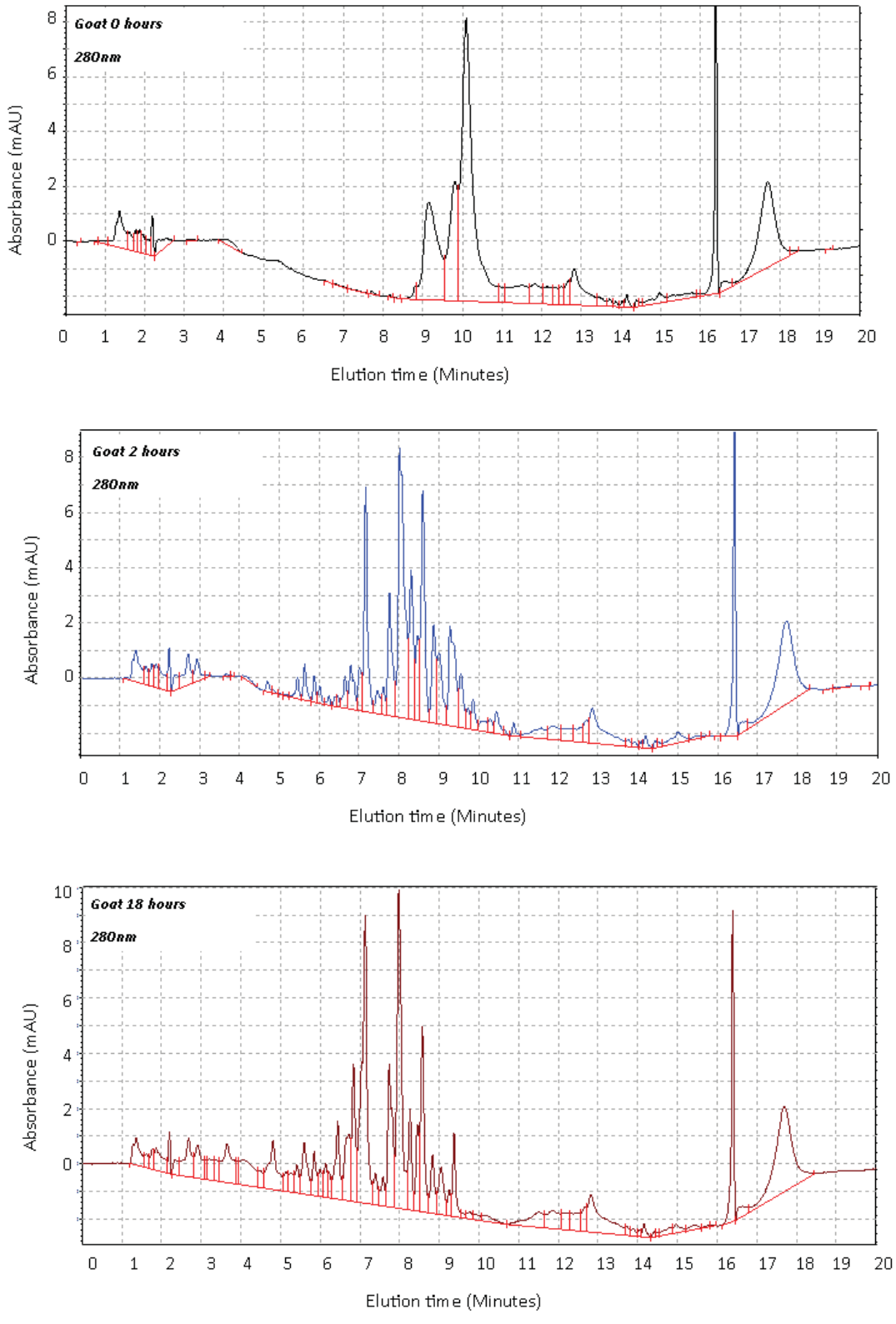


Figure 6-4: Chromatograms of goat caseinate digests at 0, 2 and 18 hours with 1:50 enzyme to protein ratio measured at 280 nm, bandwidth 8 nm.

6.2.2.2 1:200 time series of cow and goat caseinate hydrolysates

A similar trend occurred at 1:200 compared to 1:50 enzyme in terms of the pattern of protein breakdown. After 2 hours of hydrolysis there is a rapid digestion of the caseinate even at the lower trypsin concentration which correlates well to the degree of hydrolysis values. The peptide number increased over the hydrolysis time and shifted to a shorter elution time. The full time series of the digestion is in Appendix A. In Figure 6-5, there was a major peak at 8.5 and 9.5 minutes in the cow caseinate digests in all the hydrolysed samples indicating that there was also protein resistant to digestion. The goat caseinate produced more peaks, as shown in Figure 6-6, at the same time compared to cow caseinate at the same hydrolysis time indicating it is more liable to tryptic digestion; this does not correlate well to the degree of hydrolysis values which indicated the cow and goat caseinate were very similar.

As the run time of the elution was quite short the resolution of the peaks in the chromatogram is not optimal. Ideally the run time would have been longer, created by a slower gradient of buffer B. This makes it difficult to compare the chromatograms of the 1:50 and 1:200 however the number of peaks and therefore peptides produced is greater, over the same sampling time, when more enzyme is present.

In terms of protein breakdown, a similar trend occurred with Gagnaire et al., (1996) who followed the hydrolysis of bovine casein micelles up to 8 hours in both the supernatant and pellet phase after ultracentrifugation. Peptides began to appear in the sedimentable phase after 30 minutes along with the undigested caseinates however by 1 hour of digestion the intact caseinates had been broken down. In the supernatant phase the soluble peptides became abundant after 1 hour and increasing in frequency over the 8 hours. The peptides became smaller and more hydrophilic. Ono, Ohotawa & Takagi, (1994) monitored the digestion of casein micelles by trypsin at a 1:300 enzyme to trypsin ratio and reported that the digestion was completed after 30 minutes by monitoring urea-PAGE patterns and finding no change in the profile. Again, examining literature chromatograms of caseinate tryptic digests showed that the peptide profiles of the resultant hydrolysates were varied due to differences in the method of digestion. There is a lot of variation between the resultant peptide profiles in the literature and the rate of digestion is highly dependent on the enzyme to protein ratio and the digestion time as well as the specificity of the enzyme and steric hindrance of the protein for enzyme accessibility. There may be peptide bonds that

can only be reached once other parts of the protein have been hydrolysed. In addition to this, the use of commercial grade trypsin will likely cause unspecific cleavage due to contaminants such as chymotrypsin (Titani et al., 1982) which will allow the rate of digestion to increase as more sites can be digested. Lorenzen, Fisher and Schlimme (1994) confirmed this when they showed that different enzyme to protein ratios do cause a difference in the peptide profile when measured after a fixed hydrolysis time. When there is a lower concentration of enzyme the trypsin will preferentially cleave high affinity sites and therefore the expected cleavage sites of trypsin.

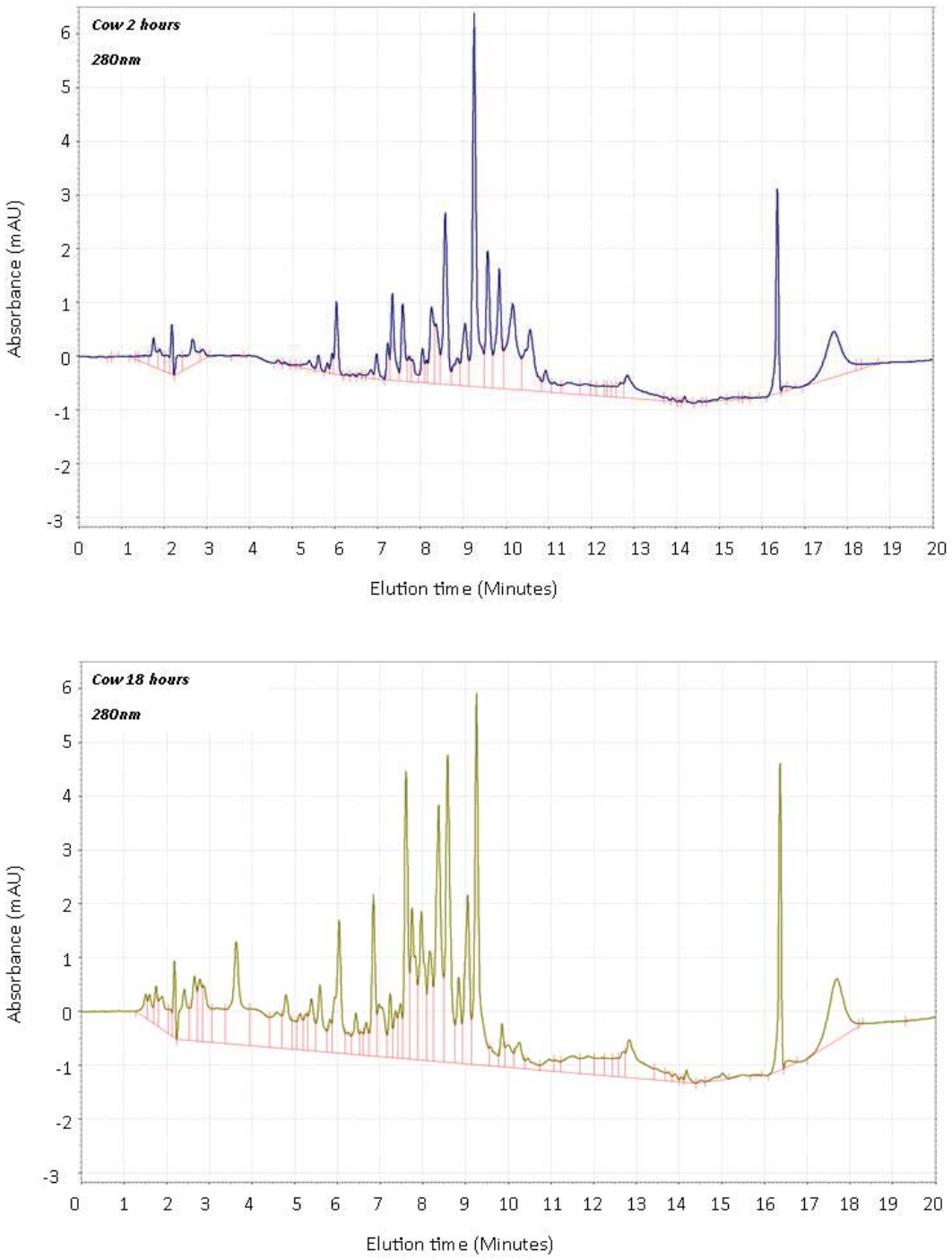


Figure 6-5: Chromatograms of cow caseinate digests at 2 and 18 hours with a 1:200 enzyme to protein ratio measured at 280 nm, bandwidth 8 nm.

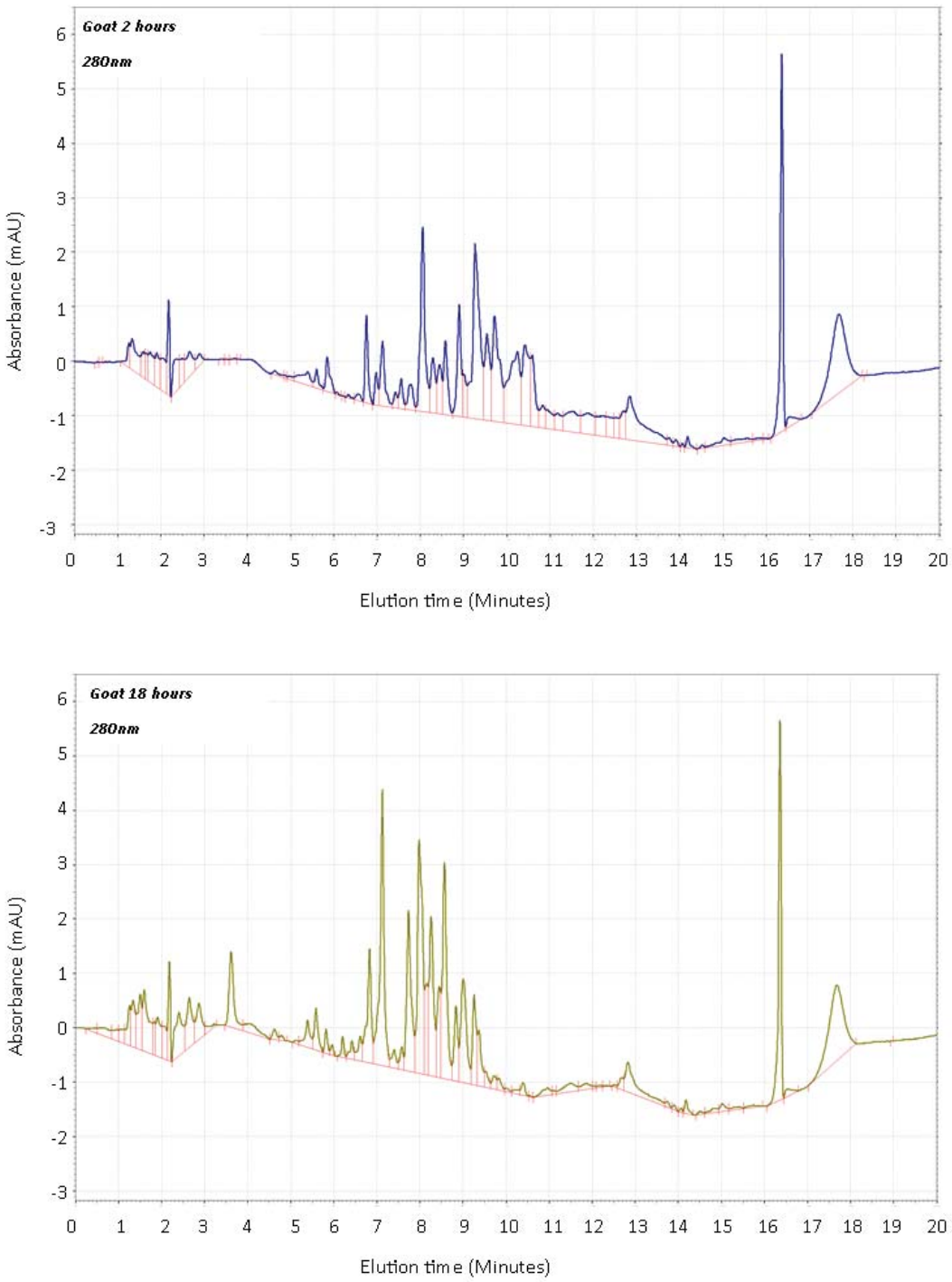


Figure 6-6: Chromatograms of cow caseinate digests at 2 and 18 hours with a 1:200 enzyme to protein ratio measured at 280 nm, bandwidth 8 nm.

6.2.3 Iron chelation of digests using Ferrozine

Ferrozine was used to measure the iron binding capacity of the hydrolysates at 0, 2, 4, 8 and 18 hours and performed according to section 3.6.3. The results of Figure 6-7 and 6-8 are presented in terms of the degree of hydrolysis that was found for each

extraction time. The ferrozine can only chelate free ferrous (Fe^{2+} form) iron and therefore the difference from the control is the amount of iron that has bound to the protein. The rate of oxidation at this iron concentration and environmental conditions was minimal and therefore it can be assumed that ferric ion formation should not be a significant confounding factor. This method was used as the sample volumes obtained were small and other partition methods would have been difficult to carry out on such a small scale.

6.2.3.1 Fe chelation at 1:50

For both species, the iron binding capacity is inversely related to the degree of hydrolysis. Goat caseinate hydrolysate had significantly lower iron binding capacity than cow caseinate ($p<0.001$). The results of this study may have underestimated the iron binding capacity of the peptides as it is possible that the addition of formic acid could neutralise the negative charges on the peptides. This could reduce the affinity of the iron to the peptides and would therefore remain in the serum phase where ferrozine would bind to the free ions. The trend of an iron binding decrease was similar for both species however it can only be partially explained by differences in the protein content. The protein content of the goat caseinate was 4.3 % less than cow however on average there was 27 % less iron bound to the goat proteins. It may be that the goat caseinates were more sensitive to the presence of formic acid.

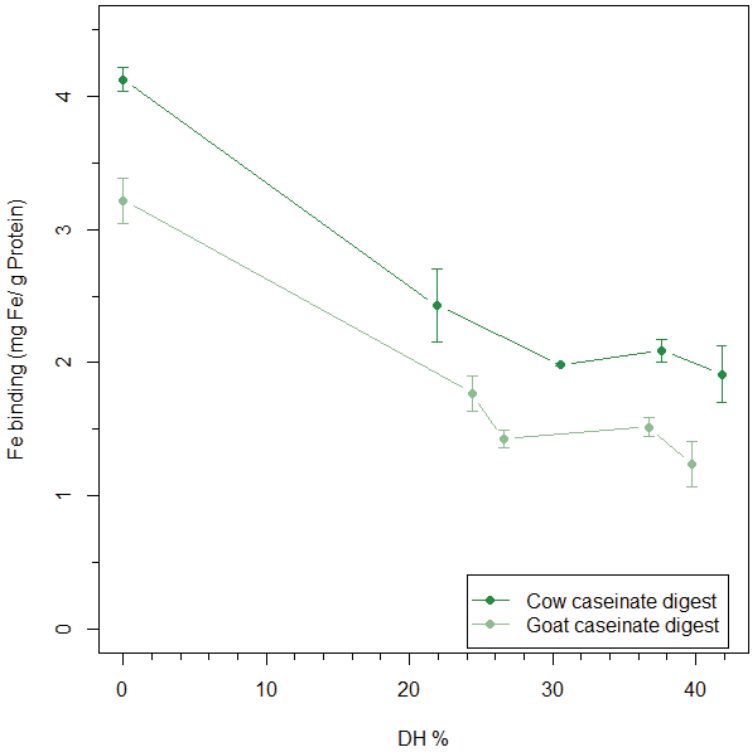


Figure 6-7: Fe binding capacity versus degree of hydrolysis of goat and cow caseinate hydrolysate at a 1:50 enzyme to protein ratio; error bars indicate standard error, n=3.

6.2.3.2 Fe chelation at 1:200

Figure 6-8 shows the amount of iron bound to cow or goat caseinate hydrolysates with up to 18 hours of digestion. The iron binding of the goat caseinate hydrolysate increased as the hydrolysis proceeded while the cow caseinate hydrolysate remained constant. These samples were heat inactivated to inactivate the trypsin as opposed to the 1:50 samples that were acidified (the method was altered due to problems associated with acidification). There was a significant difference between the species ($p=0.0264$) in terms of iron binding. Hydrolysis may have improved the iron binding capacity for the goat peptides by approximately 0.37 mg Fe/ g protein however the cow peptides after the full hydrolysis decreased by 0.11 mg Fe/ g protein.

The iron binding capacity of the 0 hour 1:200 caseinates was lower than the 1:50 caseinate treatments; it would be expected that these would be the same as no hydrolysis had occurred. The samples with 1:200 enzyme added at the 0 hour were heat treated to keep all sample treatments the same however it is likely that the heating

time affected the solubility via protein precipitation and therefore functionality of the caseins under the buffer conditions reduced.

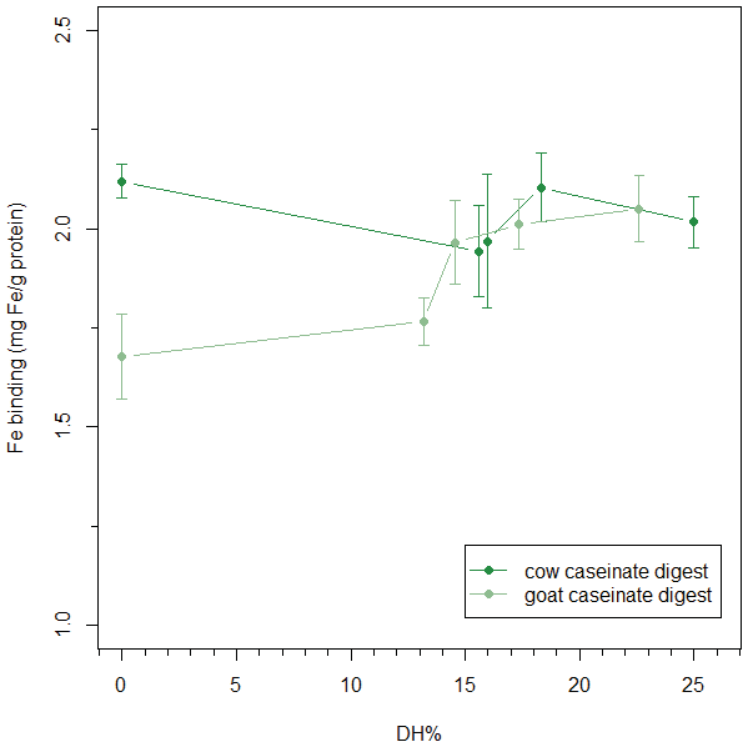


Figure 6-8: Fe binding capacity versus degree of hydrolysis of goat and cow caseinate hydrolysate at a 1:200 enzyme to protein ratio; error bars indicate standard error, n=3.

The initial studies of iron chelation to caseinate digests have indicated that the iron binding is poor, and increasing the degree of hydrolysis does not improve iron binding for most samples. This can be explained by the overall small change in amino acid composition. The digests have only been partially purified through removal of sedimentable material resulting in an average of 10 % solids loss. The amino acid profile would not have changed significantly from the casein proteins. It would be expected then that the iron binding would not be any greater than milk as there would not be a large increase in the proportion of mineral binding peptides. While the iron binding of the peptides was around 2 mg Fe/ g protein, Emery (1992) reported iron binding 5 times greater than the data presented here with values of 10 mg/ g casein for intact casein therefore the peptides from the current study have performed significantly worse. The salt concentration and buffers of the digest may have increased the ionic strength of the solution from pH adjustments carried out throughout the hydrolysis protocol. This may have caused some salt shielding of the protein which

would increase competition for the iron that the milk system did not have. With this in consideration it is clear that a purification of the digest is required to improve the iron binding capacity so that the phosphoserine and iron binding amino acids increase significantly in concentration compared to the overall digest.

6.2.4 Selective precipitation of phosphoserine containing peptides using calcium

In the previous section the hydrolysis was monitored over time and the iron binding capacity of these digests was assessed. The iron binding capacity of the peptides was found to be negatively affected by formic acid addition and an increased degree of hydrolysis at a 1:50 enzyme to protein concentration. The iron binding capacity of peptides hydrolysed with 1:200 enzyme to protein did not change for goat caseinate as the degree of hydrolysis increase while the cow caseinate peptides decreased slightly. This was due to the amino acid composition of the digest most likely being similar to the original caseinate material (some sedimentable material was removed) and the digests being under more favourable conditions in the absence of formic acid. To improve the iron binding of the peptides the phosphopeptides were isolated by the method of Reynolds et al., (1994); Adamson & Reynolds, (1995) and Adamson & Reynolds, (1996), with some modifications. The peptides are precipitated with calcium followed by ethanol and have been shown to produce an isolate that contains a high concentration of phosphoserine residues. The method is detailed in section 3.4.2. The peptides isolated using this method were characterised by measuring the calcium and phosphorus content, amino acid compositional change and HPLC profile change to confirm the isolation of a phosphoserine rich fraction.

6.2.4.1 Observations

The goat caseinate hydrolysate was cloudy while the cow hydrolysate was clear after acidification to pH 4.6 and centrifugation as shown in Figure 6-9. Both solutions were filtered through Whatman no. 1 paper to remove any insoluble material that may have re-suspended in the serum however the cloudiness of the goat caseinate digest persisted. When the calcium was added to begin the selective precipitation method there was no obvious visual change. This was expected as the peptides are low in molecular weight and even with chelation around the calcium the particles would not be large enough to reach the micron size range where turbidity is observed. When ethanol was added precipitation occurred quickly (refer Figure 6-10) and started to

settle after 30 minutes of standing. The final solids content of the cow and goat peptides was the same despite the difference in turbidity.

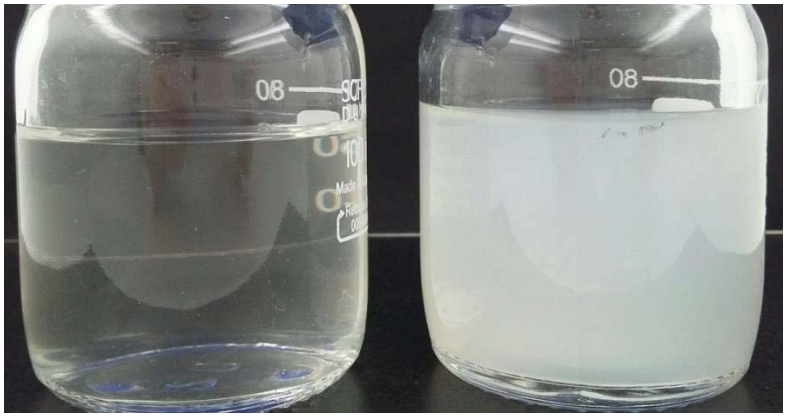


Figure 6-9: Isoelectric precipitated cow (left) and goat (right) caseinate hydrolysate after filtration.

Figure 6-10: Isoelectric precipitated cow (left) and goat (right) caseinate hydrolysate after filtration and calcium addition at 100 mM final concentration and ethanol addition to 50 % (v/v) final volume.

6.2.5 Yield of phosphopeptides from the caseinate digests

The yield of the phosphopeptide was 16.1 ± 0.3 % from the cow caseinate digest and 15.3 ± 0.2 % for the goat caseinate digest (p=0.1033). The yield obtained is greater than that of Reynolds et al., (1994) who obtained a 10.5 ± 0.4 % (w/w) yield at pH 3.5. Adamson & Reynolds (1996) obtained a yield of 12.27 ± 0.24 % (w/v) using Alcalase enzyme at pH 4.6 while Adamson & Reynolds (1995) obtained a yield of 11.04 ± 0.30 % using trypsin at pH 4.6 which increased to 13.85 ± 0.48 % when the pH was increased to pH 8.0. The composition of the precipitates differed in terms of the phosphopeptide clusters of the peptides with changes in pH. At pH 4.6 the peptides mainly contained the phosphopeptide cluster of Ser(P)- Ser(P)- Ser(P)-Glu- Glu with some di-phosphorylated peptides while at pH 8.0 more di-phosphorylated peptides and mono-phosphorylated peptides were obtained which is the reason for the higher yield

obtained in the current study. At higher pH the extraction loses specificity whereby di-phosphopeptides and mono-phosphopeptides can also be isolated (Reynolds, Riley & Adamson, 1994). While the pH for the isolation in this study was set at pH 6.7 the yield is higher than the literature which would indicate that there was some unspecific binding and more di-phosphorylated and mono-phosphorylated peptides were extracted also. In addition to this, Zhu & FitzGerald (2010) found that CPPs were more stable against heating when the peptides were isolated at pH 7.0; this may be beneficial in the present study as the peptides would be expected to be heat treated in an industrial process. Therefore there may be a trade-off for heat stability and purity of the peptides at the near neutral pH.

In addition to these studies using trypsin, McDonagh & FitzGerald (1998) used a wide range of enzymes along with calcium and ethanol precipitation and found a yield range from 3.4 % to 16.0 %. Consequently, McDonagh & FitzGerald (1998) found that there was no relationship between the degree of hydrolysis, molecular weight and calcium binding when different enzymes were tested, however Adamson & Reynolds (1996) reported a slight increase in yield for a digest held at pH 4.6 by substituting trypsin for Alcalase. The differences in calcium binding and solubility of the resultant phosphopeptides were attributed to the different cleavage sites of the enzymes causing a different formation of sequences of the peptides (McDonagh & FitzGerald, 1998). This indicates that some cleavage sites may not be beneficial in producing the optimal yield of peptides that can chelate calcium or ultimately iron. The amount of enzyme used also affects the resultant peptide yield; non-specific cleavage due to a high enzyme to substrate ratio may reduce the yield via cutting at important phosphoserine cluster areas which will also reduce mineral binding. Adamson & Reynolds (1997) found that when using Novo trypsin with a 2 hour digestion time, the phosphopeptide yield was dependent on the degree of hydrolysis of the casein obtained with different enzyme to substrate ratios. When the DH was low, at 9-15 %, the yield of α_s- casein derived peptides were negatively affected however β-casein derived peptides did not show a significant difference to higher DH yields. Increasing the DH to 17 % produced the greatest yield while the phosphopeptide yield decreased at 21 % DH.

Corsetti et al., (2003) was the only author found to report the yield of CPPs from goat caseinate, this being 1.86 % (w/w). These workers used Lactobacillus *helveticus PR4* on different species of sodium caseinate and found a yield of 0.6- 1.86 % (w/w); the

highest yield being from goat caseinate. The overall low yield compared to the other authors may have been due to the use of a different culture and digestion conditions.

6.2.6 Calcium content

The calcium content of the solids after calcium and ethanol precipitation was 7.05 ± 0.09 % for cow and 7.19 ± 0.19 % for goat peptides on a dry weight basis. Ellegård et al., (1999) reported a calcium content of calcium precipitated phosphopeptides of 6.97 ± 0.53 %. Based on a molar ratio, the amount of Ca to serine groups obtained by Ellegård et al., (1999) was 1.78 mol/mol. In the current study, this was calculated to be 1.02 mol/mol for the cow phosphopeptides and 0.84 mol/mol for goat phosphopeptides however only 16 amino acids were measured (refer to section 6.2.8) and it is therefore likely that the ratio of calcium to serine would increase as the relative concentration of serine would likely decrease.

6.2.7 Phosphorus content

The phosphorus content of cow and goat caseinate was 7.03 ± 0.8 mg/g solids and 7.84 ± 0.31 mg/g solids, respectively ($p=0.3967$). This is close to the reported value by McMeekin & Polis (1949) of 8.6 mg/g casein; Díaz et al., (2003) found bovine casein contained 7 mg/g and Dickson & Perkins (1971) found that the phosphorus made up 1 %, 0.5 % and 0.2 % of α_{s1}, β and κ, respectively. After digestion and calcium and ethanol precipitation the phosphorus content of the samples increased to 40.08 ± 1.57 mg P/ g solids for cow and 45.39 ± 2.37 mg P/ g solids for goat ($p=0.1096$); this was a 5.8 and 5.9 fold increase in phosphorus for cow and goat, respectively. The increase in the phosphorus content clearly indicates that the purification step to concentrate the phosphopeptides is effective. Kim et al., (2007) reported a phosphorus content of approximately 5 % for phosphopeptides obtained at pH 6.7; in this case the content is similar with contents for cow and goat of 4.01 % and 4.64 % respectively. This is also similar to Ellegård et al., (1999) of 3.29 ± 0.06 % and found that the ratio of Ca/P was 1.64 ± 0.10 mol/mol. In the present study the ratio for the cow peptides was 1.36 ± 0.03 mol/mol and the goat peptides bound 1.22 ± 0.03 mol/mol indicating that less calcium was able to bind to the peptides in terms of the phosphorus content. This is likely to correlate with the higher yield of peptides isolated with less specificity. Díaz et al., (2003) found bovine casein phosphopeptides contained 2.8 % phosphorus. The variation of phosphorus content is likely due to the average peptide length.

6.2.8 Amino acid analysis

The results presented in Table 6-1 and Figures 6-11 and 6-12 show the difference in composition of the amino acid residue content of cow and goat phosphopeptides compared to the starting material of the respective caseinates. The majority of the amino acids decreased in concentration which indicates that there was an exclusion of the non-binding peptides. Serine residues increased by 72.3 % for cow and 77.7 % for goat derived peptides. Aspartic acid, glutamic acid, glycine, threonine and isoleucine also increased in concentration for cow derived peptides while glutamic acid, glycine, histidine, threonine and isoleucine increased in concentration for goat derived peptides. According to the sequence of caseinates from UniProtKB (Apweiler, Bairoch & Wu, 2004), serine clusters that make up the phosphoseryl clusters are usually surrounded by acidic residues such as glutamic acid (Reynolds, Riley & Adamson, 1994). Comparing the theoretical amino acid profile and the expected cutting pattern of trypsin indicated that the same trends for the relative increases and decreases of the residues of the phosphoserine containing peptides occurred. This gives supporting evidence in the absence of mass spectrometry analysis that the isolated fraction contains the phosphoserine containing clusters and that there has been significant removal of the peptides that contain no phosphoseryl groups. Ellegård et al., (1999) found the serine content of the CPP was 10.3 % which was lower than what was found in this study however the authors obtained a phosphorus to serine ratio of 1.06 ± 0.03 mol/mol; in the present study the same ratio was 0.75 ± 0.03 for the cow peptides and 0.68 ± 0.04 for the goat peptides indicating that not all the serine groups are phosphorylated in to isolated fraction. This may have been due to some de-phosphorylation caused by some step in the reaction or natively de-phosphorylated serine was isolated, however these occur in relatively low concentration.

Table 6-1: Difference in amino acid composition of cow and goat caseinate versus cow and goat calcium precipitated peptides

Amino acids	Cow caseinate	Goat caseinate	Cow phospho-peptide	Goat phospho-peptide	% change cow	% change goat
Aspartic Acid	7.09	6.71	7.54	3.85	5.70	-41.52
Threonine	4.09	5.08	4.28	6.47	4.20	22.98
Serine	5.00	5.11	18.08	22.49	72.29	77.68
Glutamic Acid	19.00	19.53	31.12	27.43	38.81	30.10
Proline	10.77	12.24	4.05	3.53	-62.51	-70.62
Glycine	1.82	1.52	2.19	2.06	16.75	27.41
Alanine	2.957	2.72	2.74	2.74	-7.14	2.39
Valine	6.99	8.03	6.43	5.84	-8.24	-25.86
Methionine	2.80	2.83	0.46	0.90	-83.46	-67.52
Isoleucine	5.22	5.01	11.25	8.32	53.51	40.87
Leucine	9.22	9.65	3.44	4.56	-62.81	-51.88
Tyrosine	5.60	4.44	0.96	0.91	-82.92	-79.21
Phenylalanine	5.02	5.12	0.84	0.96	-83.29	-80.97
Histidine	2.81	2.84	0.67	5.82	-76.05	52.10
Lysine	7.78	7.93	2.42	2.05	-68.96	-73.63
Arginine	3.63	3.11	3.53	2.06	-2.94	-32.29
Units	mg/100 mg	mg/100 mg	mg/100 mg	mg/100 mg	%	%

On an equal weight basis (100 mg/100 mg)

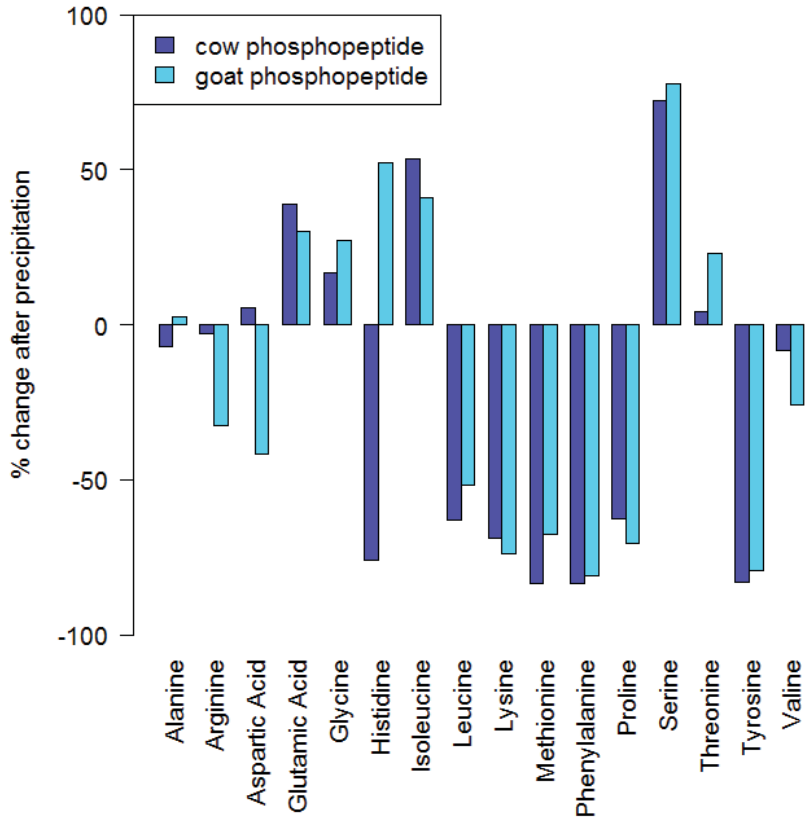


Figure 6-11: Percentage change of amino acids after calcium and ethanol precipitation.

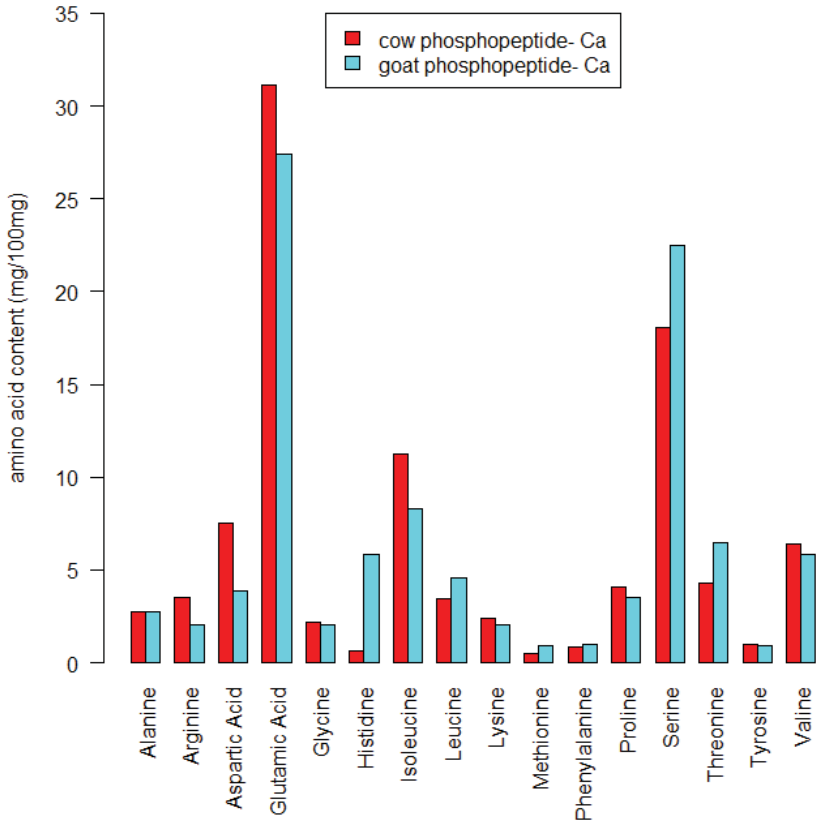


Figure 6-12: Amino acid composition of cow and goat calcium and ethanol precipitated peptides.

6.2.9 RP-HPLC of cow and goat calcium precipitated digests

RP-HPLC is a powerful tool to analyse peptide solutions. At 214 nm the peptide bonds are the absorbing species and are significantly more sensitive than at 280 nm, which detect aromatic residues. Few peaks appeared at 280 nm as aromatic amino acids are detected. Figure 6-13 and 6-14 shows the chromatograms comparing caseinate digests and selectively precipitated digests of cow and goat caseinate (the method for sample preparation is in section 3.7.6.3). At the 214 nm wavelength (Figure 6-13), the digested caseinate chromatograms show there are approximately 75 peaks for cow and 70 for goat; this is very similar to the number of theoretical peptides produced in a tryptic digest according to SwissProt^{TM}; Léonil et al., (1994) calculated that a cow caseinate digest would contain 72 peptides with complete digestion. After calcium and ethanol precipitation in this study the number of peaks reduced for both cow and goat caseinate isolate. The cow isolate produced one very intense peak, about 7 strongly signalling peaks and approximately 35 in total while goat produced one intense peak, about 6 major peaks and about 33 peaks in total. Interestingly, the intense peak for each species was in the same position indicating that this peptide occurs in high abundance in both samples of phosphopeptide. Ellegård et al., (1999) found 13 major peaks after precipitation of tryptic digested sodium caseinates.

When the detector was run at 280 nm very little was detected in the phosphopeptide solutions (Figure 6-14). Seven peaks for cow and 3 for goat were found. This indicates that the peptides in the phosphopeptide fraction contain very few aromatic residues (Matsudaira, 1990; Højrup, 2004; Mennella, D'Alessandro & Francese, 2010). The peaks that appear in the phosphopeptide chromatograms finish at approximately 45 min at 214 and 48 min at 280 nm; this is much shorter than the full digest which is up to 75 min at 214 nm and approximately 70 min at 280 nm. This indicates that the eluted phosphopeptides are small and more hydrophilic than the overall digest, as expected with the characteristics of phosphoserine. It can be concluded that there is a reduction of peptides from the calcium ethanol precipitation which appear to have the characteristics of being either smaller or more hydrophilic overall than the whole caseinate digest that they originated from. There are few aromatic residues present in these peptides, as shown by the 280 nm chromatograms and supported by the amino acid analysis: phenylalanine and tyrosine decreased by 83.3 % and 81.0 %, and 82.9

% and 79.2 % for cow and goat, respectively while for histidine cow decreased by 76.1 % while goat increased by 52.1 % (tryptophan was not measured).

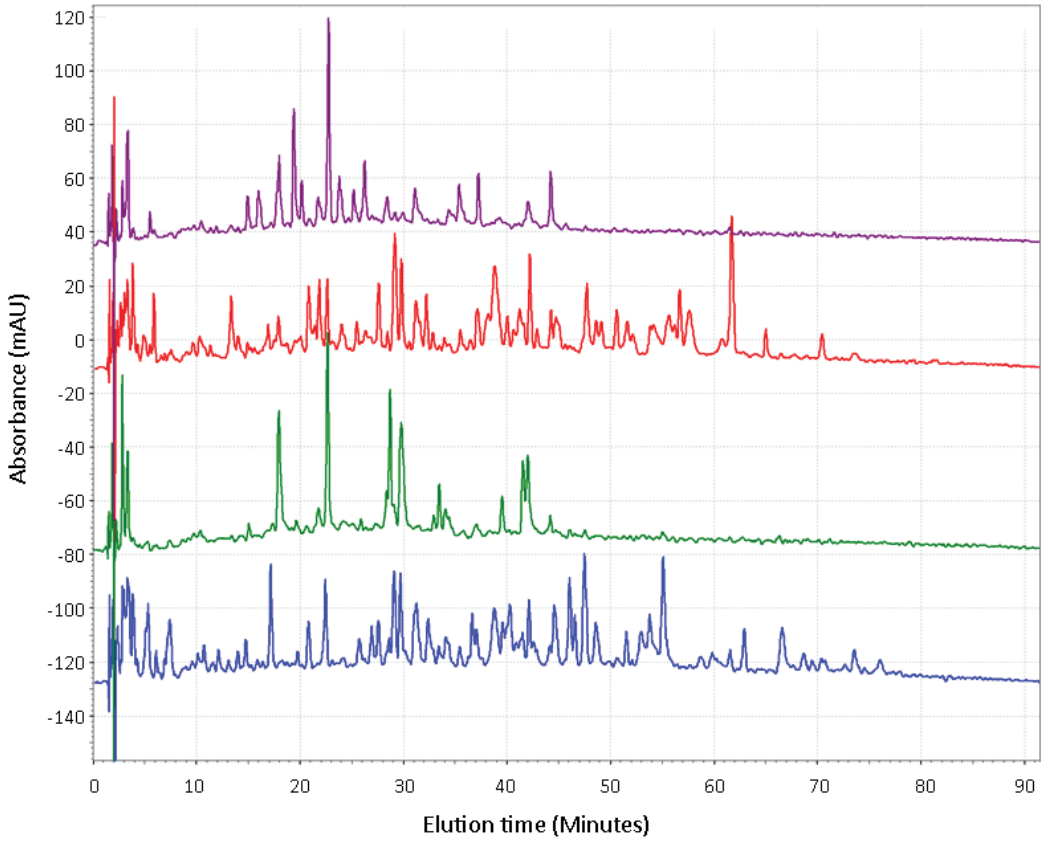


Figure 6-13: Chromatograms of cow caseinate (blue), cow phosphopeptide (green), goat caseinate (red) and goat phosphopeptide (purple) at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

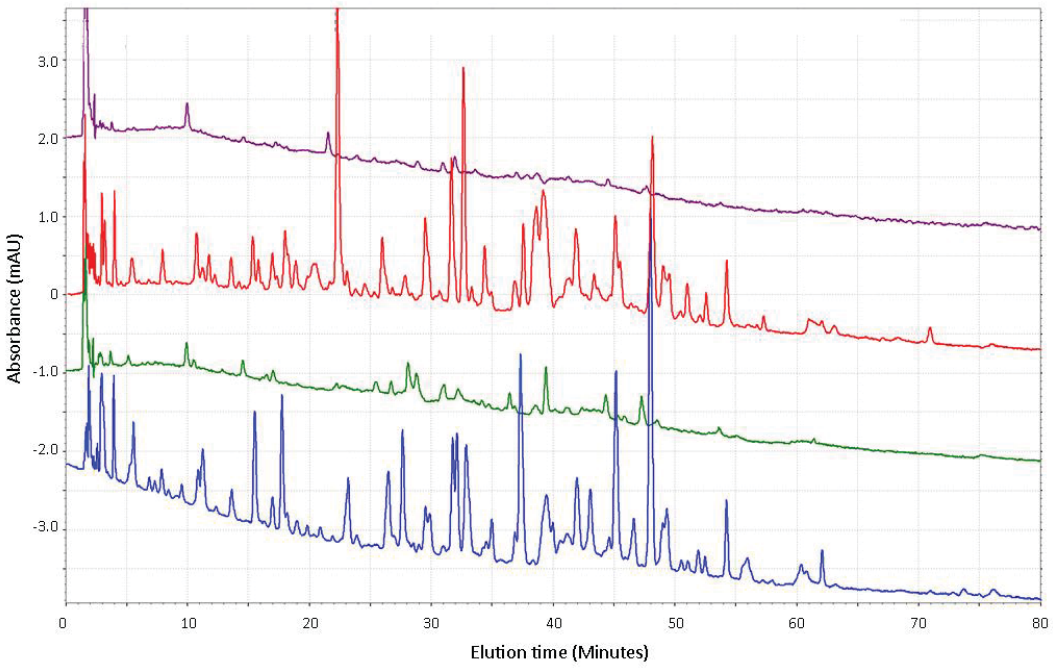


Figure 6-14: Chromatograms of cow caseinate (blue), cow phosphopeptide (green), goat caseinate (red) and goat phosphopeptide (purple) at 280 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

6.2.10 Selective precipitation of phosphoserine containing peptides using iron

6.2.10.1 Description of experiment

After demonstrating that the method of Reynolds is adequate for isolating phosphopeptides from goat caseinate a series of experiments were then conducted to extend the basis of the Reynolds method to enable iron binding potential to be determined in the absence of potentially competing divalent cations (i.e. calcium). In terms of an industrially relevant process this would avoid several steps where calcium and ethanol would have to be added for precipitation and then potentially removed again to add iron in place as the functional cation.

Ferrous sulfate was used in place of calcium chloride as the precipitating cation to isolate peptides, however no pH adjustment was used which would promote oxidation of the iron (refer to section 3.4.3 for methods). Figures 6-15 and 6-16 show the changes in the solution appearance with the iron addition followed by ethanol. After iron addition both cow and goat peptide solutions were clear, with a green colour (refer Figure 6-15). The goat peptide solution also has a slight cloudy appearance, a

phenomenon that was also observed with calcium (refer section 6.2.4.1). After ethanol addition; the iron immediately oxidised and large precipitates formed that were much coarser than the calcium precipitants (refer Figure 6-15).

The oxidation of ferrous sulfate on the addition of ethanol is expected as all forms of iron approved for fortification are insoluble in ethanol (Kim et al., 2016). This reaction is unavoidable. When ferrous sulfate was added to each of the peptide solutions the pH dropped to 5.44 ± 0.02 (a pH decrease of 1.23 ± 0.04 units). The pH was not adjusted due to the potential promotion of the ferrous iron oxidising prior to ethanol addition due to the high iron concentration. Even in the acidic environment the ethanol promoted oxidation. In the original method of Reynolds precipitation does not occur as $CaCl_2$ is readily soluble in ethanol at 25.8 g/100g. The implication for this work is that precipitated iron would likely settle during centrifugation resulting in an overestimate of the iron binding capacity of phosphopeptides.

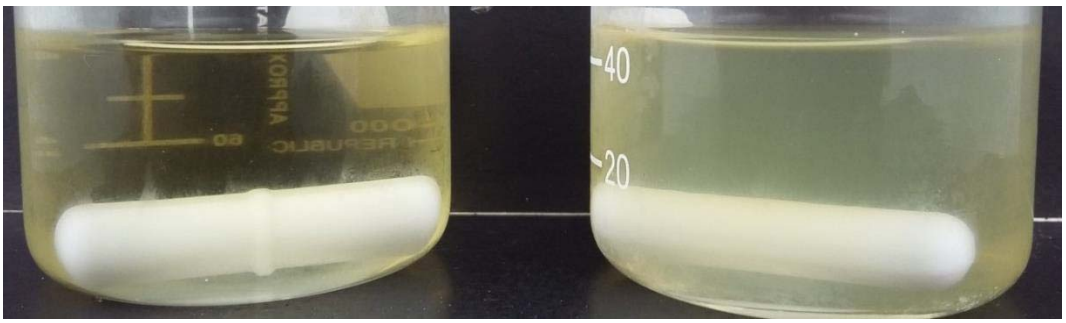


Figure 6-15: Acid clarified cow (left) and goat (right) caseinate digests after ferrous sulfate addition of 90 mM final concentration.

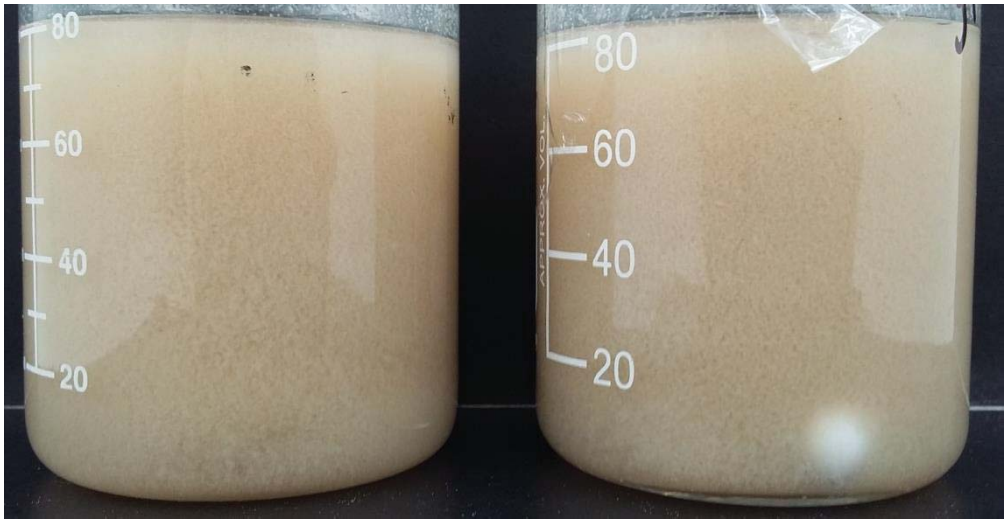


Figure 6-16: cow caseinate (left) and goat caseinate (right) digests after iron and ethanol addition.

6.2.11 Iron content

The digests contained 12.3 ± 1.0 % iron in the cow caseinate digest precipitate while the goat caseinate digest contained 10.2 ± 1.9 % iron in the solids (p=0.3883); the apparent difference between the species was not statistically significant. On a protein basis the iron binding or co-precipitation was 65.4 ± 5.1 mg of iron/ gram peptide for cow caseinate and 53.9 ± 9.2 mg iron/gram peptide for goat caseinate. Assuming that the amino acid residues that have iron binding capacity, serine, glutamic acid, aspartic acid and histidine, the amount of iron that binds to the isolated fraction would be 1.22 g Fe/ g peptide for the cow isolate and 0.98 g Fe/ g goat isolate (see section 6.2.13 for the amino acid composition). It therefore seems unlikely that all the iron is bound to the peptide. Hegenauer, Saltman & Nace (1979) estimated only two phosphoserine groups are required to stabilise the ferric iron as these groups are multiply charged. Based on the phosphorus content shown in section 6.2.12, the molar ratio of iron to phosphorus is 1.90 ± 0.78 mol/mol for cow peptides and 1.45 ± 0.78 mol/mol for goat peptides indicating there is significantly more iron present than the binding ratio described by Hegenauer, Saltman & Nace (1979). Therefore it is likely that some of the iron in the peptide complex is unbound and present as precipitated ferric oxides. Despite this, the overall peptide binding is close to the reported value by Bouahallab et al., (2002) of 70 mg/g phosphopeptide. Chaud et al., (2002) obtained a casein hydrolysate Fe^{3+} complex containing 51.5 mg/ g protein prepared by the digestion of casein with no further purification and mixing ferric chloride at 27 ^{o}C, pH 7.8.

6.2.12 Phosphorus content

The phosphorus content was more difficult to measure compared to the calcium precipitated samples as the data contained a relatively high standard error (SE= ±6.0 for cow and 6.1 for goat). This may be due to potential interference with the iron content. The cow precipitate contained 35.99±10.6 mg P/ g solids and goat precipitate contained 38.91 ± 13.5 mg P/ g solids (p=0.873). The phosphorus content was less than the calcium precipitated samples which were 40.08 ± 1.57 mg P/ g solids for cow and 45.39 ± 2.37 mg P/ g solids for goat; however with the high error of the iron precipitated samples there is no difference in the phosphorus content. Based on the molar ratio calculated by Ellegård, et al (1999) for P/Ser, the average molar ratio for the cow peptides would be 1.35 and the goat peptides, 1.54 (refer to section 6.2.13 for amino acid composition). It is unlikely that there are other phosphorus compounds present other than those on the casein as the starting material was caseinate. When the lower bounds of the phosphorus assay was used the ratio for cow and goat was 0.96 and 1.00, respectively therefore indicating that the phosphorus content would more likely be 25.39 mg P/ g solids and 25.41 mg P/ g solids for cow and goat peptides, respectively.

6.2.13 Amino acid composition

The cow and goat peptides had similar amino acid compositions as shown in Figure 6-17 to 6-19. Glutamic acid was the most abundant amino acid from both peptide species while serine and aspartic acid were the next most abundant residues present. There were some similarities of the amino acid composition of the iron precipitated cow and goat peptides compared to the calcium precipitated samples. Proline, alanine, valine, methionine, leucine, tyrosine, phenylalanine, lysine and arginine all decreased in concentration after cation precipitation; most with similar percentage decreases. In terms of concentration increases the increase in serine was not as large compared to the calcium precipitation with a concentration of 9.0 mg/100 mg and 8.6 mg/100 mg resulting in a percentage increase of 44.5 % and 41.4 % for cow and goat, respectively, compared to 72.3 % and 77.7 % for the calcium data. This smaller increase in serine content correlated with the lower phosphorus content compared to the calcium precipitated peptides (35.99 ± 10.6 mg P/ g solids and 38.91 ± 13.5 mg P/ g solids versus 40.08 ± 1.57 mg P/ g solids and 45.39 ± 2.37 mg P/ g solids for cow and goat peptides, respectively). There was a larger increase in glutamic acid with a final

concentration of 33.5 mg/100 mg and 34.2 mg/100 mg which was a 43.1 and 43.9 % increase for cow and goat, respectively. Other increases in amino acid content for the iron precipitated peptides were aspartic acid which gave a 23.5 % and 25.0 % increase; in the calcium precipitated samples, aspartic acid only increased slightly with cow and dropped by 41 % for goat while with iron there is a high affinity for peptides containing this amino acid. The opposite trend occurred for isoleucine, with calcium the increase in isoleucine was much greater at 50 % and 43 % for cow and goat, respectively while with iron there was a 19.2 % and 10.9 % increase, for cow and goat respectively. Similarly, with calcium there was an increase of 50 % of histidine for goat while with iron there was only a 14.2 % increase indicating the affinity was not as high. Glycine showed a significant difference in concentration; there was an increase in glycine content with calcium of 16.8 % and 27.4 % while with iron the content decreased by 12.0 % and 7.6 % for cow and goat, respectively.

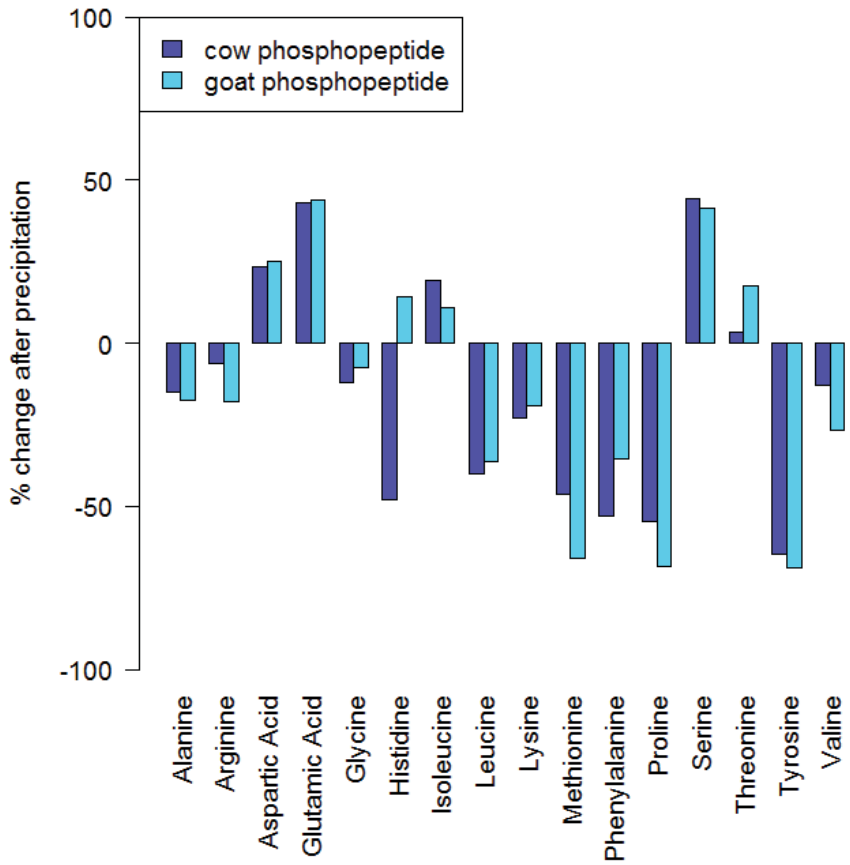


Figure 6-17: Percentage change of amino acid composition of cow and goat iron precipitated peptides with respect to their respective caseinate starting material.

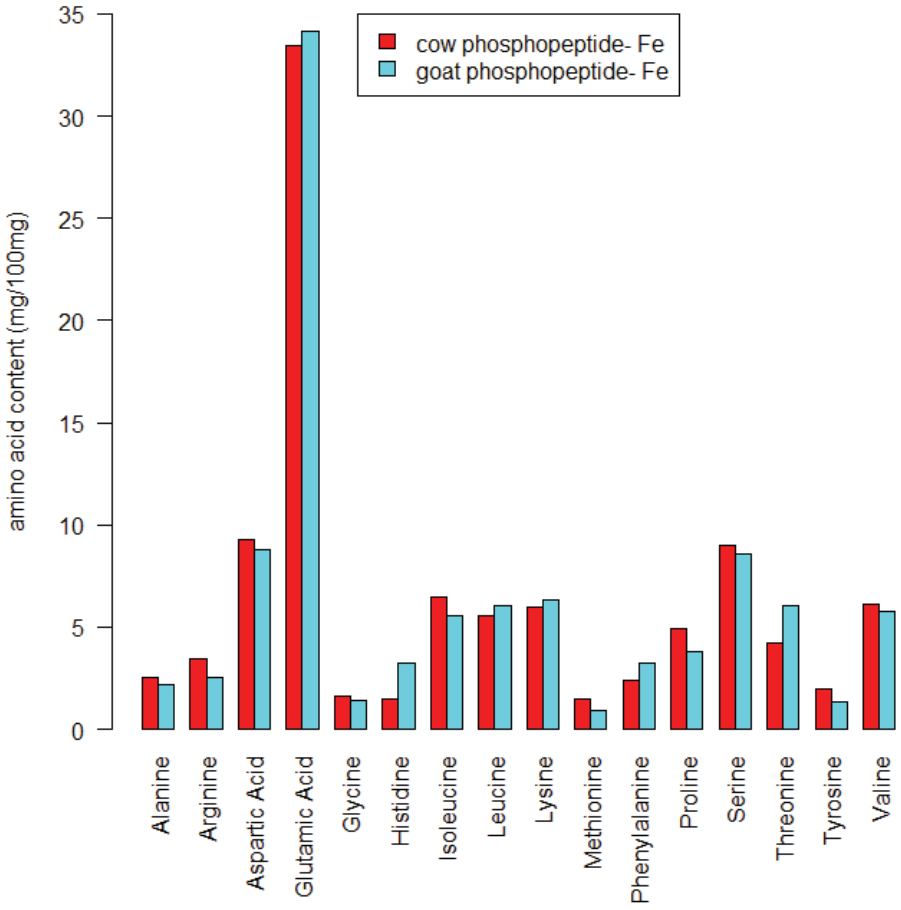


Figure 6-18: Comparison of amino acid composition of cow and goat iron precipitated peptides in mg/ 100 mg protein.

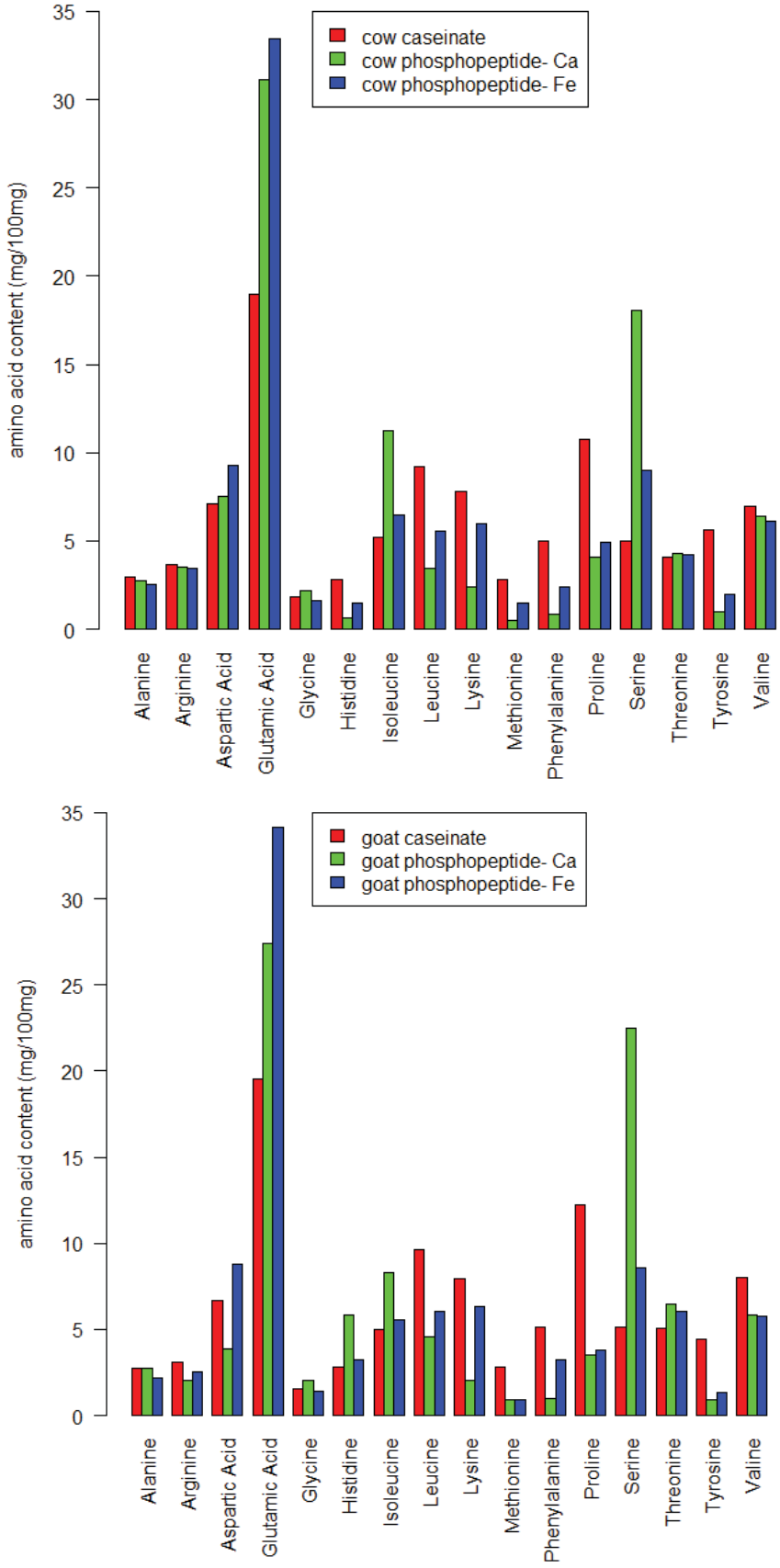


Figure 6-19: Compositions of cow caseinate, calcium precipitated and iron precipitated peptides (top) and composition of goat caseinate, calcium precipitated and iron precipitated peptides (bottom), in mg/ 100 mg protein.

The comparison of calcium and iron precipitated peptides shows that the type of cation does cause a difference in the profile of the precipitated peptides and the iron does not allow a high phosphoserine containing fraction to be extracted. This is somewhat unexpected due to numerous studies showing that the binding affinity of iron is much stronger than calcium (Hegenauer, Saltman & Nace, 1979). When iron binds to phosphoseryl groups the oxidation state changes to the trivalent form and binds via coordination bonds (refer to Figure 6-20). In contrast, calcium remains in the same ionic state and binds only via ionic bonding to the casein (refer to Figure 6-21). The change in oxidation state of iron may cause a shift in equilibrium and result in secondary binding with the glutamic acid residues in addition to the higher affinity to carboxylic acids compared to calcium. If the glutamic acid is omitted from the composition, due to its large increase, the serine composition increases by 63.1 % for cow and 61.0 % for goat. This becomes more similar to the calcium precipitation results as the large change in glutamic acid skews the results. Sugiarto et al., (2009) determined that there were two different types of binding for iron to casein proteins. One is the high affinity binding of the phosphoserine groups and the second is non-specific binding which could be the acidic residues, namely glutamic acid. Similarly, Baumy & Brule (1988) determined that on β-casein there are 5 binding sites of phosphoserine and 2 sites of carboxylic groups and with regard to Zn^{2+} ions the binding of phosphoserine is 4.5 times stronger than the carboxylic groups. There was also a difference in affinity between cations, and while not measured, iron may have a higher affinity for carboxylic groups compared to calcium. In the study, zinc had a higher affinity than calcium (Baumy & Brule, 1988).

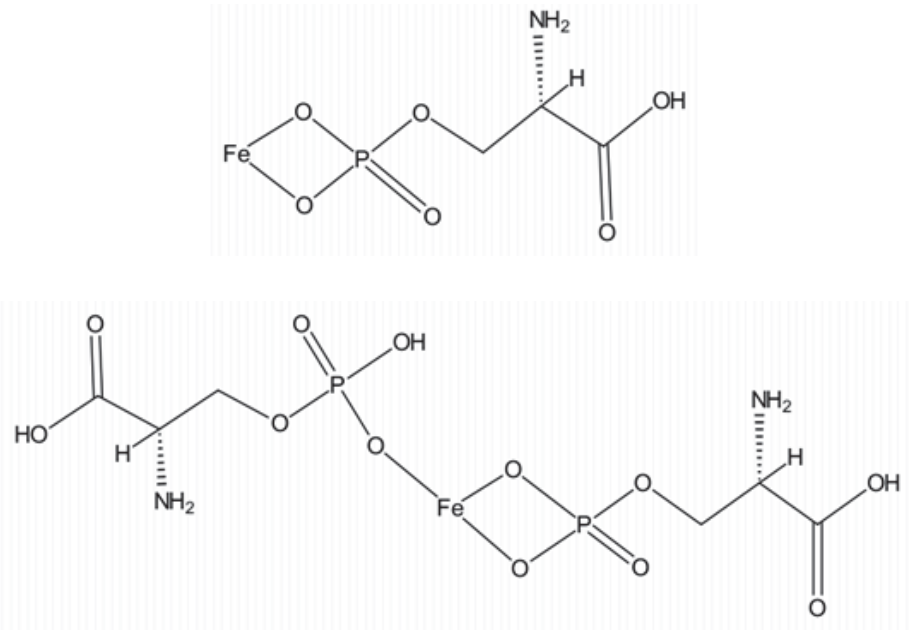


Figure 6-20: Possible covalent bonding arrangements of iron to phosphoserine. A minimum of two oxygen groups are required to stabilise the iron (top) however the iron could bind to three oxygen atoms and could join two residues (bottom).

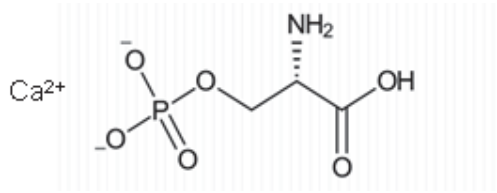


Figure 6-21: Ionic bonding of calcium and the phosphoserine group.

6.2.14 HPLC of cow and goat iron precipitated digests

Figure 6-22 shows the chromatograms of iron precipitated cow and goat peptides. The chromatograms produced a longer elution profile than the calcium precipitated samples under the same conditions; while the digest for the calcium samples finished by approximately 75 minutes, the digest for iron precipitated samples finished at 85 minutes. This may have been caused by slight differences in the buffer composition or peptides produced by iron precipitation were larger or more hydrophobic. The amino acid data showed relative increases in some residues such as glutamic acid that are so significant that it seems more plausible that new peptides are being precipitated and that these peptides may be the cause of the elongated elution time.

The phosphopeptide chromatogram elution profile is shorter than the overall digest, similar to what occurred with the calcium precipitated samples. The iron precipitation caused a large reduction in peaks with some similarities between the species. The

largest peaks in each sample eluted at 26 minutes for both samples. Comparing the cow phosphopeptide produced by the calcium precipitation (section 6.2.9) to the iron precipitated sample there is a similar pattern in eluted peaks however the iron appears more separated. There are three clusters of peaks in the calcium sample after the largest peak while in the iron sample there is one peak at 24 minutes followed by two clusters of peaks. There is not so much similarity in the goat samples other than that there are several smaller peaks; the last peak of each chromatogram are both large, however larger in iron but may have similar characteristics.

The phosphopeptides isolated from cow and goat caseinate digests using calcium and iron were compared under identical conditions within the same run such that the chromatograms can be compared without potential variations in the elution conditions (Figure 6-23). The chromatograms were scaled as the iron- precipitated samples had a much stronger injection peak due to the iron and therefore the height of the peaks cannot be accurately compared between samples (although best attempts were made to scale the injection peaks to be equal). The same large peak occurred in the chromatograms at approximately 24.5 minutes with the small minor peak occurring just before it. Another peak that occurred in all profile was at 54 minutes which is more prominent in goat; in particular the iron precipitated but still occurs in the other samples. At 33 minutes there are peaks that appear more prominent in cow and are minor peaks in goat. It still appears that there are more similarities within the species rather than the type of ion used.

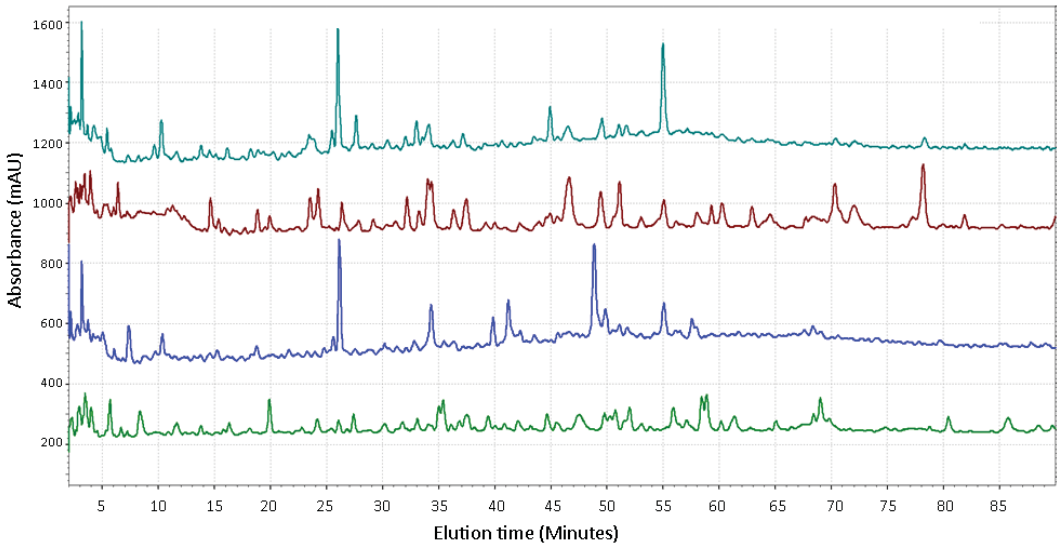


Figure 6-22: cow caseinate digest (green) and phosphopeptide (blue); goat caseinate digest (maroon) and phosphopeptide (teal) at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

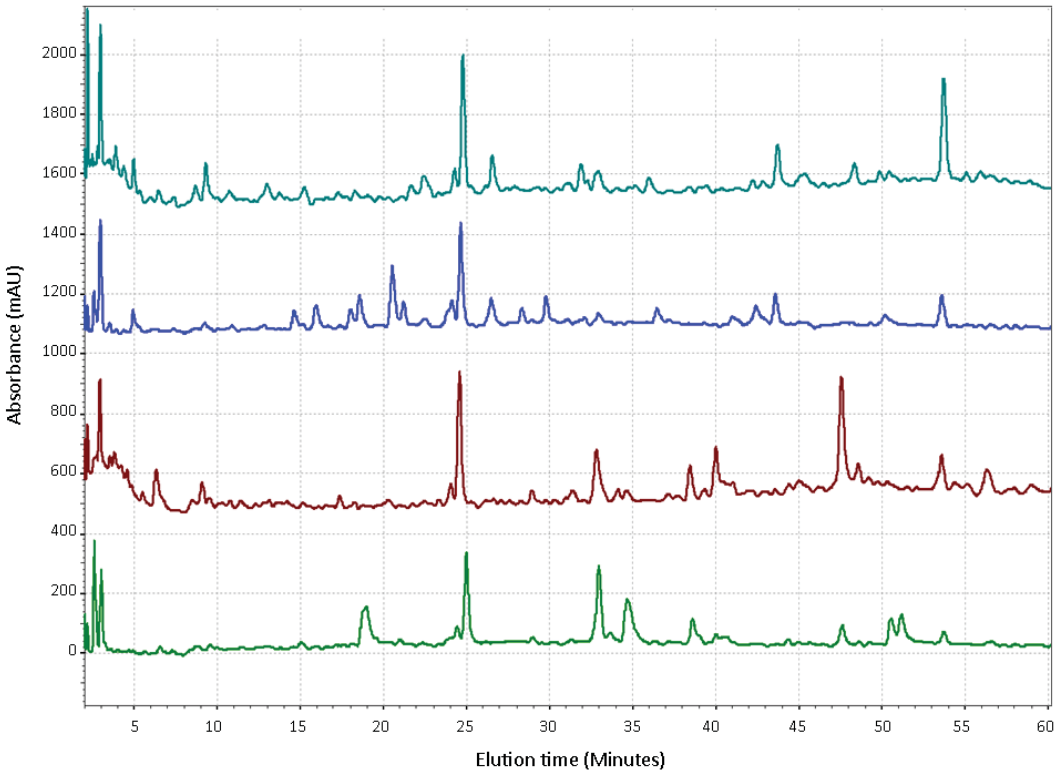


Figure 6-23: Combined chromatograms of calcium and iron precipitated phosphopeptides: calcium- cow caseinate (green), iron- cow caseinate (maroon), calcium- goat caseinate (blue), iron- goat caseinate (teal) at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

6.2.15 Isolation of phosphopeptides using IMAC

An alternative method of isolating phosphopeptides, immobilised metal affinity chromatography (IMAC), was explored due to the reported high selectivity of the

technique (Andersson & Porath, 1986; Nuwaysir & Stults, 1993; Holmes & Schiller, 1997; Cao & Stults, 1999). The technique employs resin with functional ligand groups attached that can interact with iron, for example, IDA binds iron via a nitrogen atom and two oxygen atoms from the carboxylate group (Porath, 1992). The iron can then interact with peptides containing hard bases (as iron is a hard acid) which are oxygen, aliphatic nitrogen and phosphor which in terms of amino residues relate to carboxylates, asparagine & glutamine and phosphorylated amino acids, respectively (Ueda et al., 2003). IMAC was performed on cow and goat caseinate digests as prepared in section 6.2.1.2 and separation according to section 3.4.4. Based on the literature it was hypothesised that the separation would produce a phosphopeptide fraction that would be significantly more pure than the cation selective precipitation technique of section 6.2.4 and 6.2.10.

6.2.15.1 Protein content of eluted fractions

Figure 6-24 shows the protein content of each fraction as determined by the Lowry method outlined in section 3.6.2. There was an initial large quantity of protein in the first fraction of the washing buffer of 1.95 ± 0.02 mg/mL and 2.44 ± 0.06 mg/mL of peptide from the cow and goat digest, respectively. Therefore significant amounts of non-binding peptides were removed. Analysis of the eluted fractions after the addition of ammonia buffer indicated that there was very little protein eluting off which shows that the buffer is poor at eluting bound peptides. The first elution fraction from the cow digest contained 0.112 mg/mL and decreased to 0.005 mg/mL protein, while the goat elution fraction contained no more than 0.028 mg/mL protein. A 100 mM NaH_2PO_4 buffer was applied to the resin according to De la Hoz et al., (2014a) and De la Hoz et al., (2014b); these authors used a 100 mmol/L ammonium phosphate buffer, pH 4.5 to elute the bound peptides after an acidic washing buffer. This has also been used by other authors with various concentrations (Andersson & Porath, 1986; Lund & Ardö, 2004; Tsai et al., 2008; Storcksdieck, Bonsmann & Hurrell, 2007). The phosphate buffer works by out-competing the phosphopeptides for the iron binding sites (Holmes & Schiller, 1997). The phosphate buffer showed that there was an increase in concentration for both cow and goat peptides which had a peak concentration of 0.152 ± 0.020 mg/mL and 0.27 ± 0.023 mg/mL, respectively.

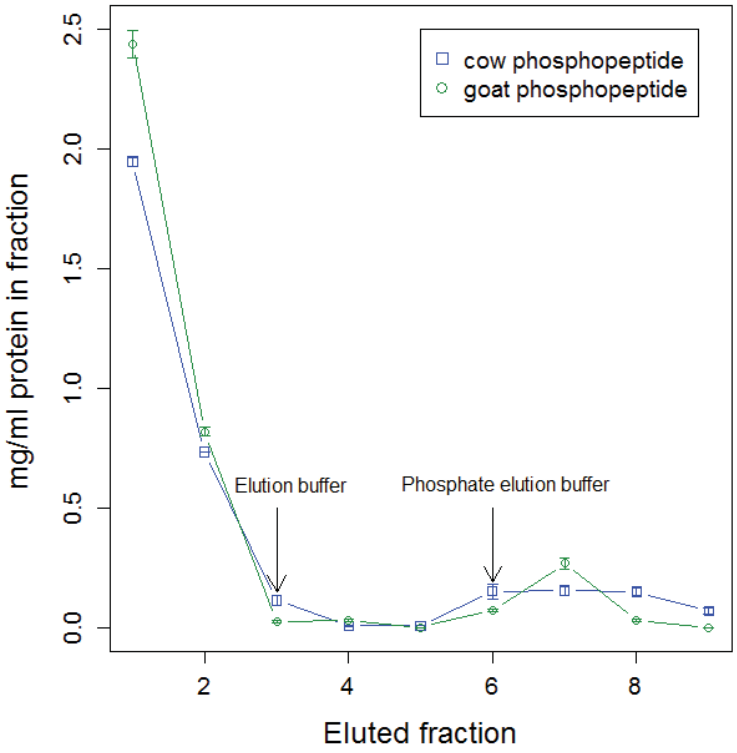


Figure 6-24: Protein content of cow and goat peptide eluted fractions in mg/mL; error bars indicate standard error.

6.2.15.2 RP-HPLC of cow and goat peptides eluted with IMAC

6.2.15.2.1 Caseinate digests and washing buffer fractions

The washing buffer fractions have very similar chromatographic profiles to the original digest when comparing Figure 6-25 and 6-27 (cow caseinate digests), and 6-26 and 6-28 (goat caseinate digests). Appendix B contains all chromatograms for the washing and elution fractions. This may indicate that the concentration of peptide added to the resin was in excess to the binding capacity of the resin. However, some of the relative heights of the peaks have decreased particularly in the earliest eluting peptides which may show a binding of these peptides. It was previously seen in section 6.2.9 and 6.2.14 that the phosphopeptides from calcium and iron precipitation eluted faster than a whole caseinate digest.

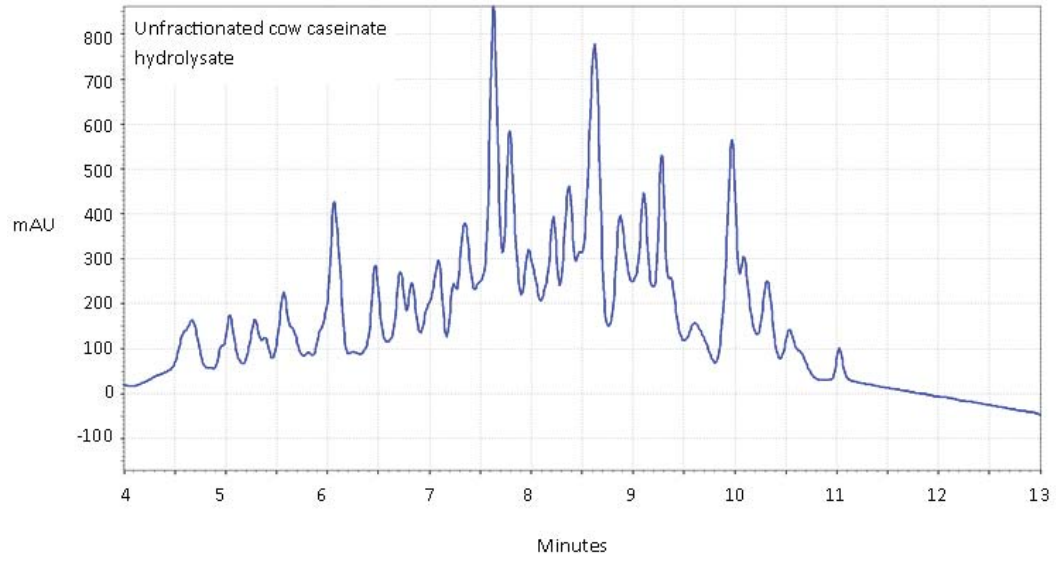


Figure 6-25: Chromatogram of cow caseinate digest at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

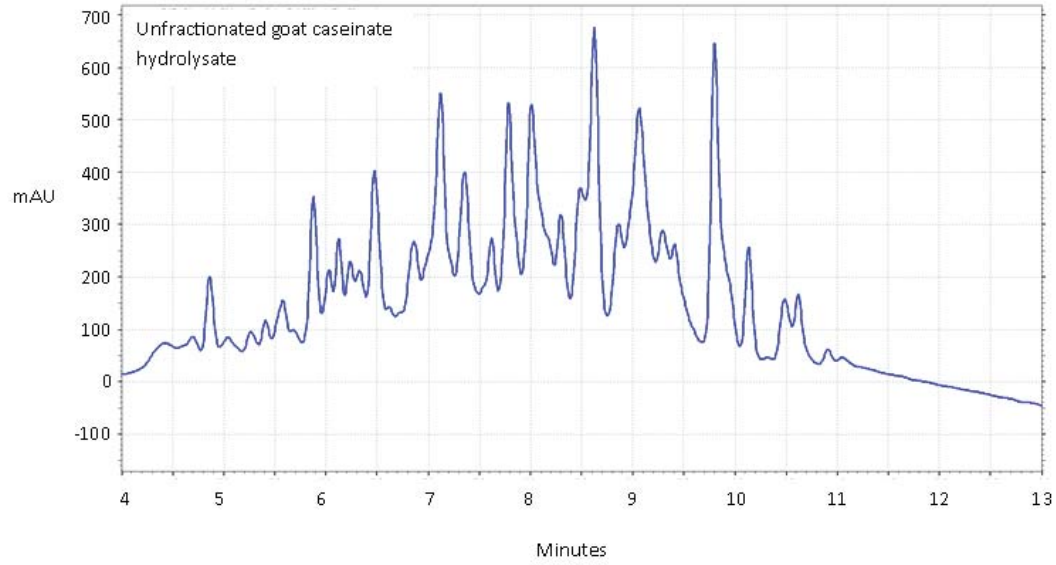


Figure 6-26: Chromatogram of goat caseinate digest at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

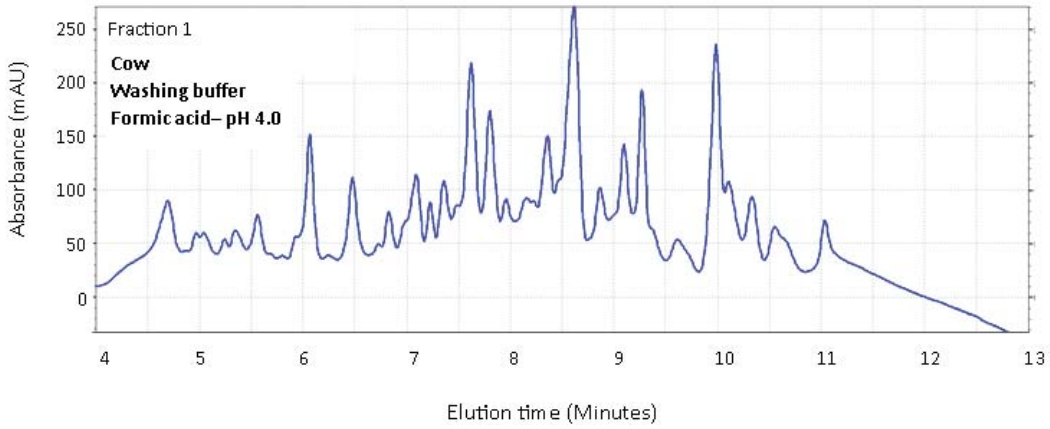


Figure 6-27: Chromatogram of cow washing buffer peptides at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

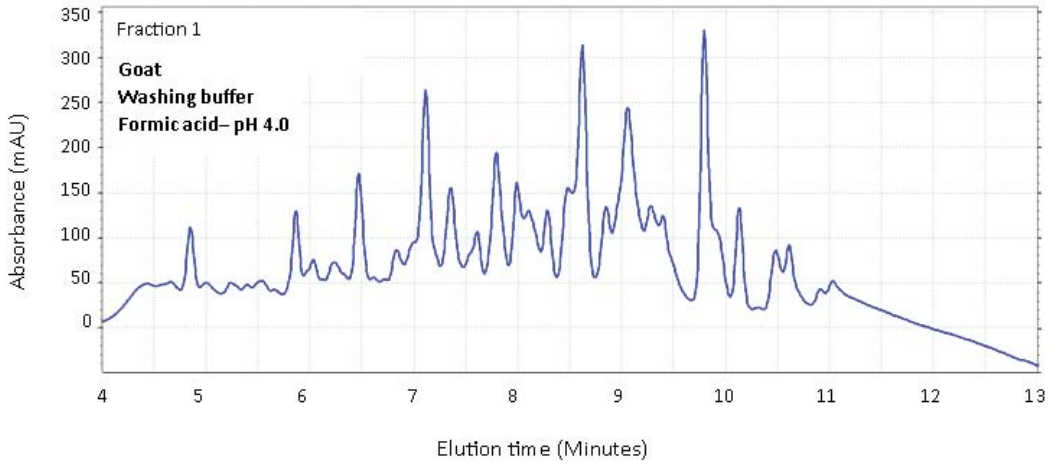


Figure 6-28: Chromatogram of goat washing buffer peptides at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

6.2.15.2.2 Elution buffer

The elution buffer of ammonia solution at pH 9.0 was found to elute very few peptides, as shown in Figures 6-29 and 6-30. The change in mAU scale from the washing buffer showed that the absorbance was significantly lower and the peaks are far over-shadowed by the changes in absorbance from the buffer gradient, left and right in the chromatogram.

The original ammonium buffer may not have worked due to the low ionic strength and low buffering capacity. As the elution buffer was water adjusted to pH 9.0 with no added salts for buffering, it is likely that when it interacted with the peptides and resin which contained the washing buffer the pH would have dropped significantly and

therefore the phosphopeptides would not be able to elute. Phosphopeptides are eluted in the pH range of 6.9-7.5 and therefore a significant drop of pH would have occurred to cause this elution step to be ineffective (Holmes & Schiler, 1997). This was not seen in the initial testing of the low ionic strength buffers as the pH was not measured as the focus was the leaching of iron (Appendix B). This was confirmed when the elution fractions were tested using RP-HPLC and there was very little signal caused by protein; thus this essentially turned into an extended washing phase.

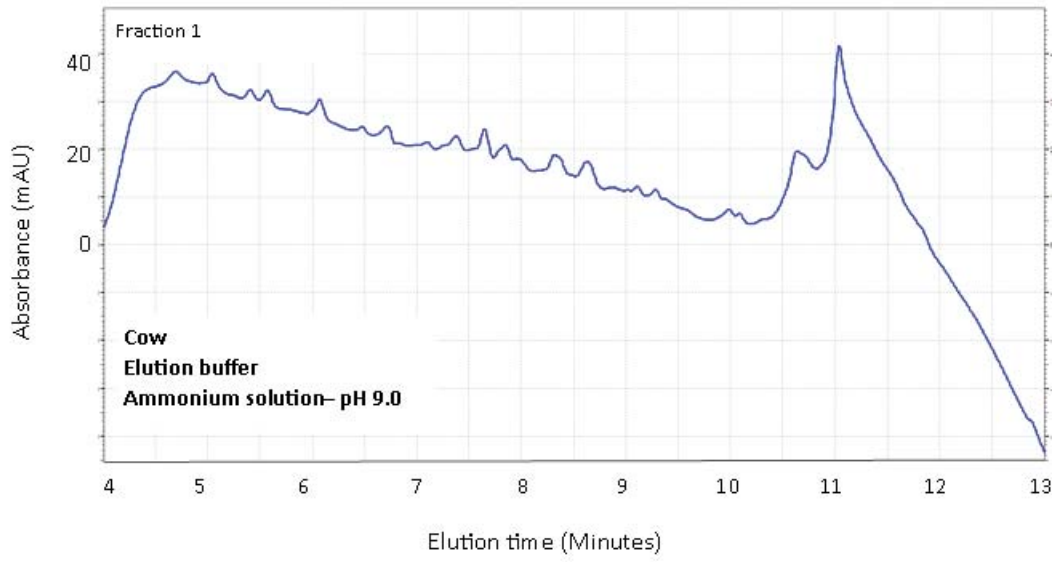


Figure 6-29: Chromatogram of cow elution buffer peptides at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

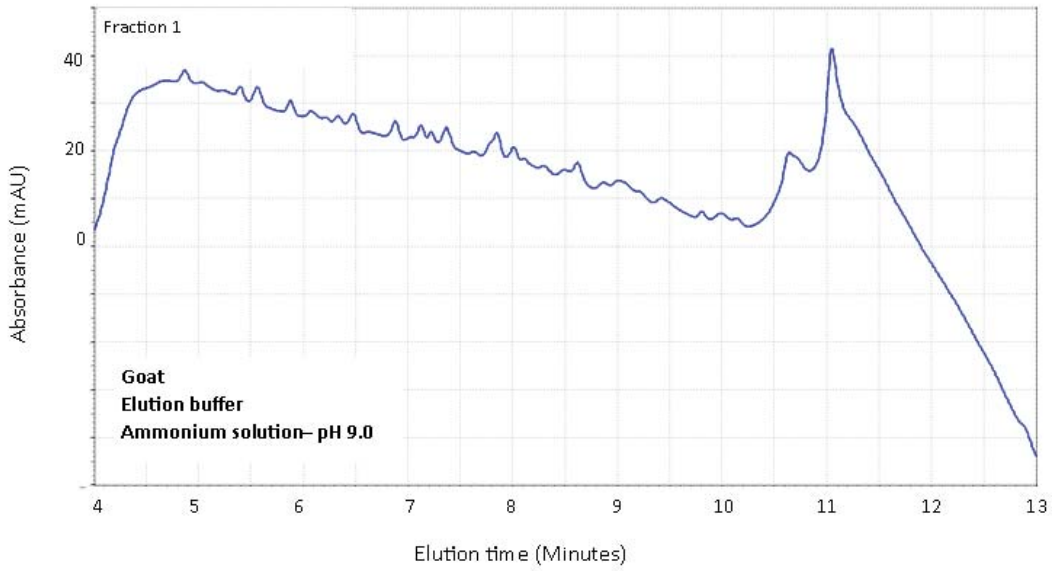


Figure 6-30: Chromatogram of goat elution buffer peptides at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

6.2.15.2.3 Phosphate elution buffer

The phosphate buffer was effective at eluting peptides after the initial elution buffer. There was a strong response in the four fractions with the peaks eluting at the same time, Figures 6-31 and 6-32. The first two fractions were diluted 2 fold prior to running on the RP-HPLC so therefore the response would be larger than what is shown compared to the third and fourth fraction. The distinct peaks for the cow samples elute between 5:45 and 8:15 minutes while for goat the peaks elute at 5:45 and 8 minutes. The elution profile shows that the peaks are generally eluting slightly faster than the overall mix possibly indicating a smaller and more hydrophilic fraction as seen in section 6.2.9 and 6.2.14. It can be seen that there is a significant reduction in peaks after elution compared to the original mix and due to the long washing stage, in part due to the elution buffer that showed little peptide content. Therefore it can be concluded that these peptides were bound to the IMAC resin via the chelated iron.

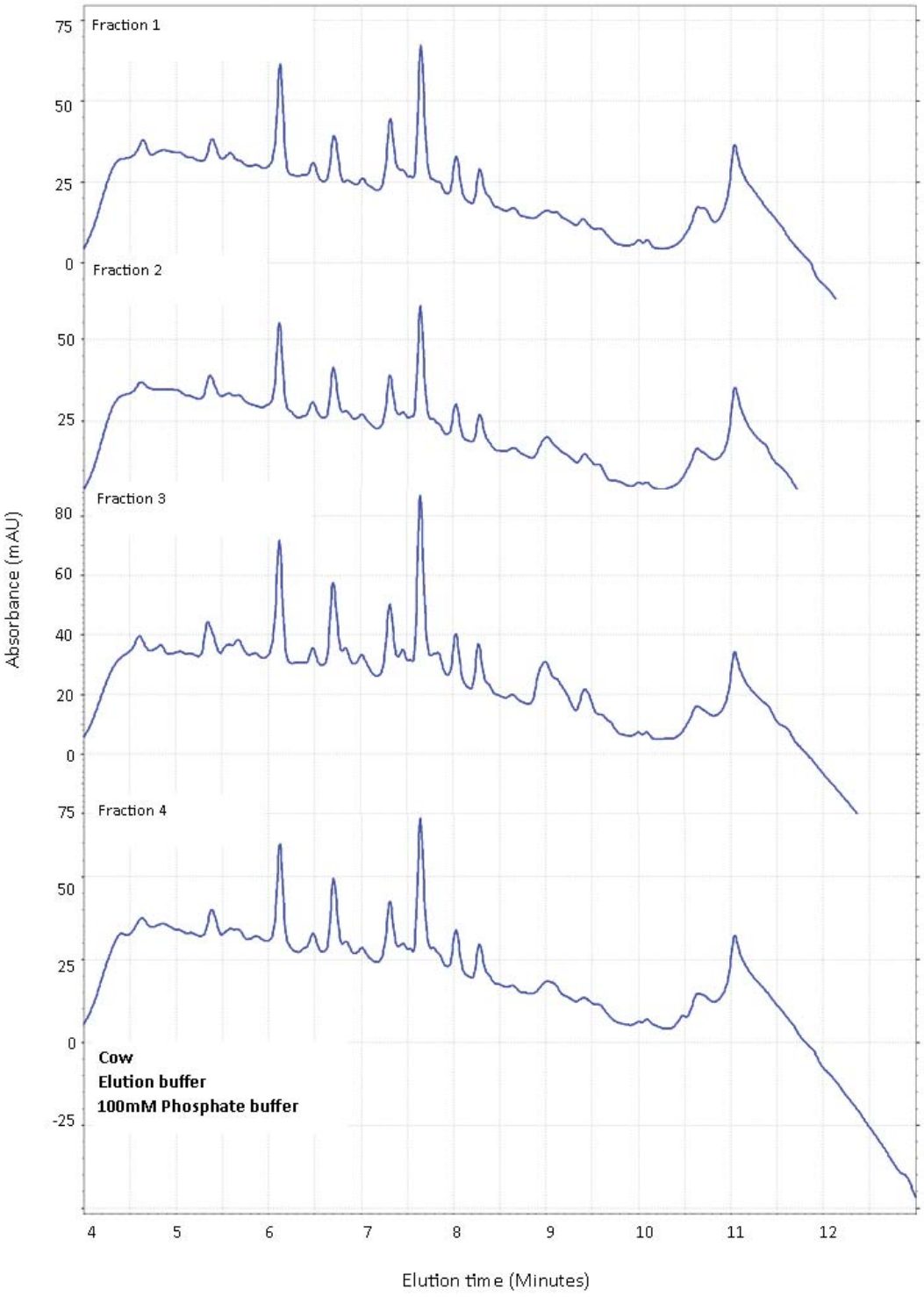


Figure 6-31: Chromatogram of cow phosphate buffer peptides at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

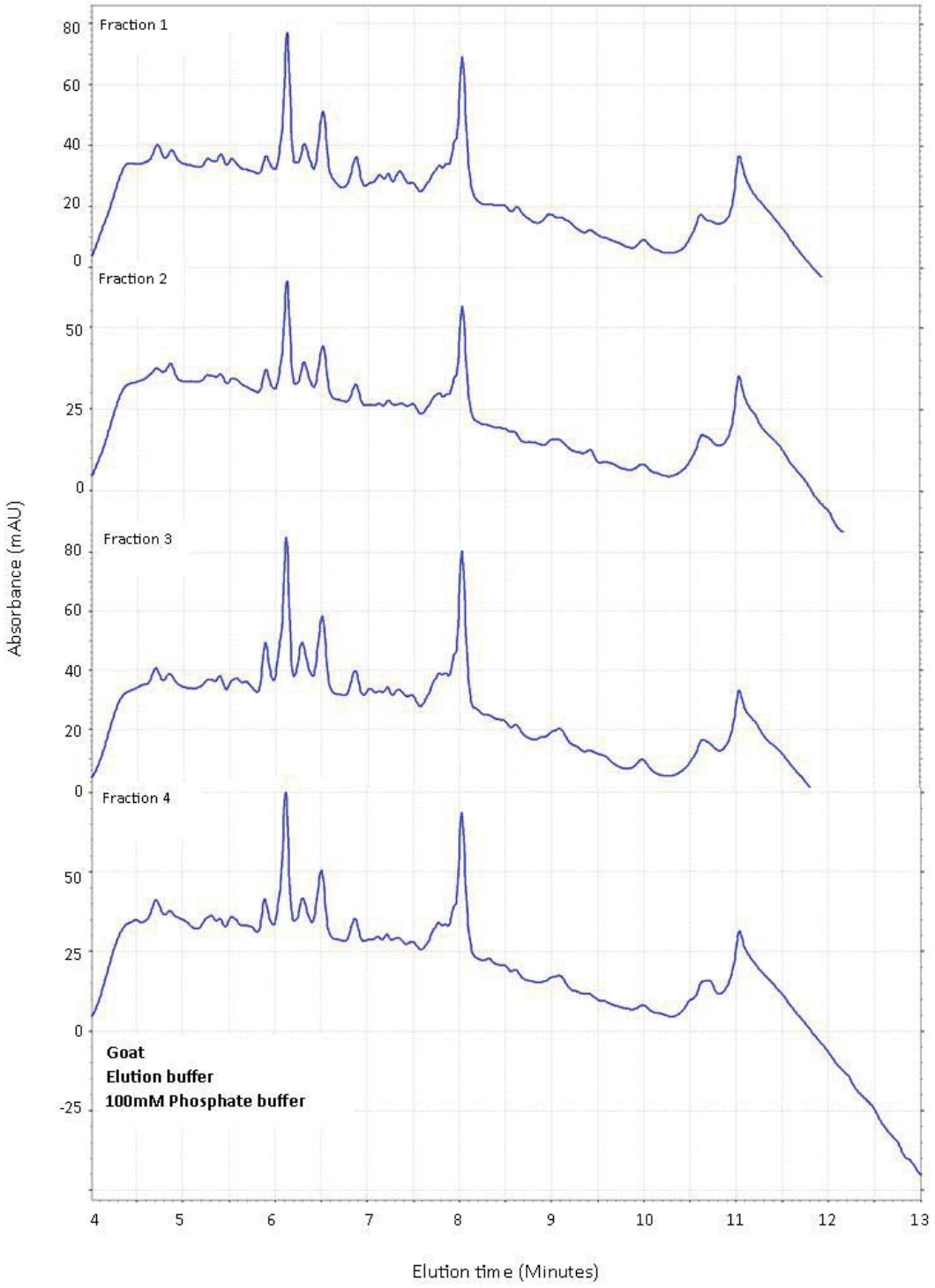


Figure 6-32: Chromatogram of goat phosphate buffer peptides at 214 nm, bandwidth 8 nm, reference 360 nm, bandwidth 50 nm.

6.2.15.3 Iron leaching analysis

The iron leaching followed a similar trend to the protein content as shown in Figure 6-33. There was an initial spike in the iron content which may suggest that the washing buffer was not flushed through long enough prior to peptide addition or the change in ionic strength may have caused some iron to unbind. This may have caused some

binding peptides to elute off in the washing fraction. The elution buffer of ammonia caused minimal leaching to occur and there was a slight increase in iron concentration in the phosphate buffer, however significantly less than in the washing buffer fractions. This shows that the large change in ionic strength of the phosphate buffer did not cause iron to be released. Rather, the phosphate buffer caused an exchange between the bound peptides and the phosphate group allow to peptide to be released, acting as a competing ligand, while the iron remained bound to the resin (Porath, 1992).

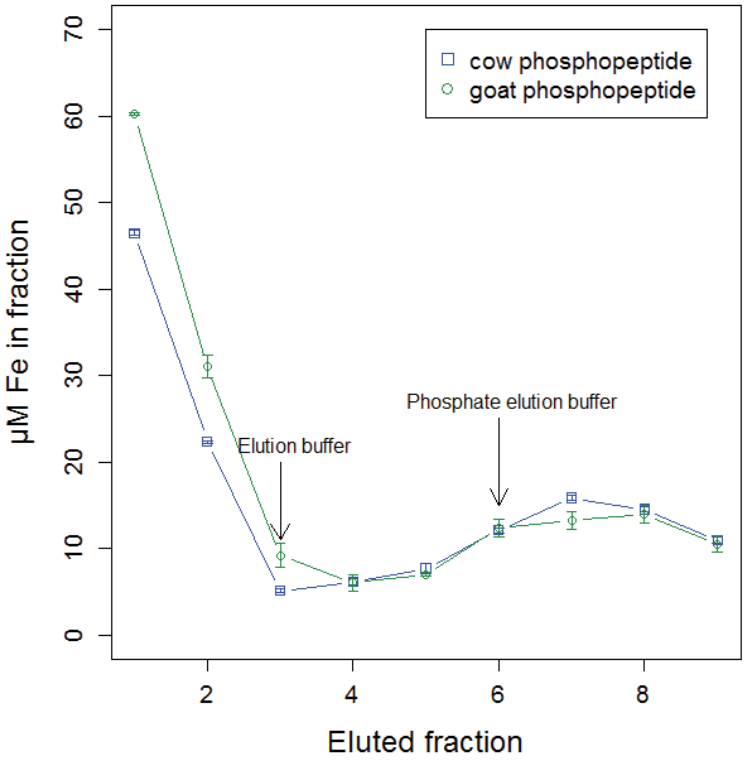


Figure 6-33: Iron content of cow and goat eluted fractions in µM; error bars indicate standard error.

6.2.15.4 Amino acid analysis

The amino acid analysis showed that there was smaller change between the original caseinate digests and the phosphate eluted fractions compared to the precipitation technique as shown in Figure 6-34. While the IMAC caused a decrease in alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tyrosine and valine for both cow and goat peptides as well as threonine for cow, the magnitude of the change was not as large as the selective precipitation technique for calcium. The largest increase in amino acid content for both species was glutamic acid of 37.7 % and 39.5 % for cow and goat, respectively which is known to have affinity to iron

while serine showed the next largest increase for only cow peptides of 24.2 % while threonine for goat peptides had the second largest change in content with an increase of 18.8 % with serine only having the fifth largest change in concentration of 13.7 %. This may relate to the β- casein which has an extra phosphorylated threonine whereas cow peptides do not. A similar trend was seen with calcium precipitated peptides, however it would be expected that the serine would increase along with it.

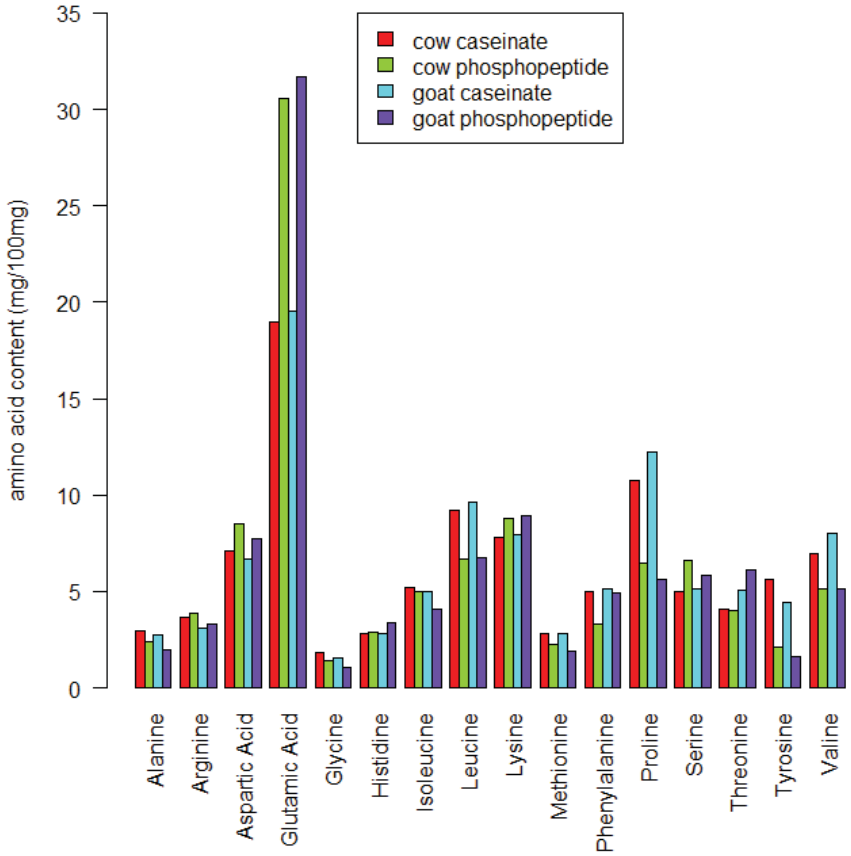


Figure 6-34: Amino acid content (mg/100mg protein) of caseinates and IMAC phosphate buffer eluted peptides.

6.3 Conclusion

The selective precipitation using both iron and calcium confirmed that there was a reduction in the number of peptides in the final isolate as shown by HPLC. Most of the aromatic containing peptides were eliminated indicating that these have low affinity to calcium or iron. Calcium precipitated peptides contained the greatest increase in serine groups indicating that the phosphoserine content did increase with isolation. The iron precipitated peptides had a significant increase in the amount of

glutamic acid compared to the calcium precipitated peptides however when the glutamic acid relative change was omitted the increase in serine was close to the calcium profile. When iron binds to the caseins they oxidise to the ferric form therefore increasing the oxidation state while when calcium is added there is no change. This change in state by iron therefore changes the equilibrium of the ions as there is now an unbalance of counter ions of sulfate. It is therefore likely that the excess ferrous ions are also binding to glutamic acid residues which can act as a counter ion due to their affinity with cations. This would cause the increase in glutamic acid that was not seen with calcium. Baumy & Brule (1988) showed that calcium and iron have different affinities to the binding sites of β-casein and therefore a different isolation of peptides would be expected.

While there has been extensive literature on the use of IMAC to isolate phosphopeptides to high purity this was not achieved in the current work. Significantly larger increases in serine were obtained using calcium or iron via precipitation. There were also significant differences in the amino acid profile between the calcium precipitated peptides and the IMAC peptides. The amino acid composition of the peptides obtained from selective iron precipitation and IMAC were more similar and therefore may bind to similar peptides. Calcium appears to be more selective for serine compared to iron as shown by the larger increase in content compared to the original starting material.

7 Characterisation of iron binding capacity of cow and goat phosphopeptides

7.1 Introduction

Phosphopeptides from α_{s1}-, α_{s2}- and β caseins contain highly polar acidic sequences of three phosphoseryl groups followed by two glutamic acid residues, SpSpSpEE (Bouhallab & Bougle, 2004; Miquel et al., 2006b). These are able to bind cationic minerals and are stable and soluble at different physicochemical conditions, such as changes in pH (Aít-Oukhatar et al., 2000). It has been shown by Pérès et al., (1999) that iron fortified phosphopeptides increase iron absorption in rat models compared to iron gluconate. Therefore, the hydrolysis products of casein proteins may provide a good, naturally (clean label) derived vehicle for iron fortification.

Typically, iron binding in milk systems is measured using partition of the iron bound and non-bound phases via ultra-centrifugation, dialysis or filtration (Demott & Park, 1974; Gaucheron, Famelart & Le Graët, 1996; Gaucheron et al., 1997; Hegenauer et al., 1979; Raouche et al., 2009b; Sugiarto et al., 2009). As the peptides are small, the separation process of the iron-bound peptides using these techniques presents a problem and a more direct method, where the components of the sample are not separated, was employed in the present study; a modified ferrozine method by Carter (1971). As ferrozine only binds and emits colour with free (unbound) ferrous iron, an estimation of the iron chelated (bound) to protein can be made. Many authors, such as Decker & Welch (1990); Emery, (1992); Kim et al., (2007); Farvin et al., (2010); De Gobba et al., (2014); Jaiswal et al., (2015) and O'Loughlin et al., (2015) have used methods based on iron chelation using ferrozine in dairy based systems to measure the iron binding capacity of the protein of interest.

This chapter will explore how the iron binding capacity of isolated goat phosphopeptides (and cow for comparison) is affected by various simulated industrial conditions of heating, cooling and holding times. A comparison of iron binding properties of the peptides in the presence and absence of calcium will also be made as it is likely that nutritional foods will contain this mineral. As calcium has been shown to bind in the same location of iron it is important to understand if calcium reduces

iron binding. It is hypothesised that calcium present in the solution will inhibit iron binding as there will be increased competition for binding sites.

7.2 Results and Discussion

7.2.1 Calcium content

The calcium content of the solids after calcium and ethanol precipitation of the peptide solution was 7.05 ± 0.09 % (w/w) for cow peptides and 7.19 ± 0.19 % (w/w) for goat peptides on a dry weight basis, as shown in section 6.2.6. In order to reduce the calcium present in the precipitated peptides, dialysis was carried out in acidic conditions as detailed in section 3.5.1.1. After dialysis at pH 3.0, the calcium content on dry weight basis was reduced to 5.14 ± 0.37 % for cow and 4.93 ± 0.29 % for goat (or 3.67 ± 0.09 mM and 3.53 ± 0.09 mM at 1 % w/w solids) resulting in a calcium reduction of 27.1 ± 4.3 % and 31.4 ± 2.2 %, respectively. Clearly, there was still a high calcium content detected in the sample even after extensive dialysis for 48 hours which proved to be ineffective at these particular conditions.

A lower pH of 1.7 was also tested for the dialysis buffer, as this pH is expected to cause greater protonation of the side chains and carboxylic acid groups (since it is below their p*Ka's*), resulting in the unbinding of ionic- bound calcium. The phosphoserine group is the most relevant residue as this is where the calcium binds and needs to be protonated to release the ionic calcium. This residue has several p*Ka* values due to the three oxygen groups. The p*Ka* value where the overall charge of the residue is neutral is 2.19 (Śmiechowski, 2010) however histidine, which has been shown to bind divalent ions, (Torres-Fuentes, Alaiz & Vioque, 2012) has a carboxylic p*Ka* of 1.77. In any case, even if the tested pH of 1.7 was below these values, a considerable amount of calcium was detected after dialysis in the cow and goat phosphopeptide retentates; 3.22 ± 0.24 mM and 3.13 ± 0.33 mM (1% w/w solids), respectively. Surprisingly, the calcium content did not change significantly by dialysing at this low pH compared to a higher dialysis pH of 3.0. Explanations for this include possible precipitation of the protein causing the migration of the calcium to become inhibited (Taborsky, 1980), or that the functional binding groups of calcium were protonated equally at pH 3.0 as pH 1.7 and therefore similar amounts of calcium movement were observed.

Ion exchange was performed on the peptides to remove the calcium which is outlined in section 3.5.1.2. The use of an ion exchange resin for the isolated phosphopeptides was found to be an efficient method to remove calcium as its final level was below the

detectable limits of the titration (0.007 mM Ca, method in section 3.6.5.2). The almost calcium-free phosphopeptides were therefore prepared in this manner for the study.

7.2.2 Iron binding of phosphopeptides at various calcium levels

If the calcium content in the peptides is high, it may cause binding of iron to be lower as there could be increased competition for binding sites (section 4.2.4.5.1). Existing literature has also described how the ferrous iron (Fe^{2+}) can displace Ca^{2+} from the phosphoseryl groups and then can oxidise to form the Fe^{3+}-protein complex (Emery, 1992; Hegenauer et al., 1979b). This would indicate that the iron should still be able to displace the calcium ions and occupy the binding site, so theoretically the presence of calcium should not make a great difference to the overall iron binding. This is relevant as it determines whether a calcium removal step is required in a scale up process and whether other divalent ions in a system would create competition for the iron. In order to investigate this, the iron binding of peptides at different levels of calcium was measured by comparing the original starting peptide material containing approximately 7 % (w/w) calcium, the dialysed peptides (calcium 5 % w/w) and the ion exchanged peptide samples with very low calcium content (below detectable levels).

Results confirm (Table 7-1), that there was some difference between removing calcium partially or almost completely from the peptides, with regards to their iron binding properties. The ferrozine test revealed that removing calcium partially (dialysis) or almost completely (ion exchange) from the peptides, increased the iron binding capacity significantly for both cow and goat peptides, respectively compared to the control, with the exception of the goat retentate. The controls, which did not have any treatment to remove calcium, produced the lowest iron binding. It is important to notice that the calcium content may not be the only factor that can affect the iron binding. The controls had the lowest binding (14.42 ± 0.26 for cow peptides and 14.62 ± 0.07 mg Fe/ g peptide for goat peptides) which could be caused by the impurities or salts remaining in the sample; proceeding work shows that ionic strength can greatly influence iron binding. The increase in sodium via the calcium/sodium exchange for the ion exchanged peptide samples may have led to an unfavourable ionic strength for iron binding. There was no significant difference in the amount of iron bound between cow (16.06 ± 0.43 mg Fe/ g peptide) and goat (15.94 ± 0.08 mg Fe/ g peptide) ion exchanged peptides. In contrast, the dialysed samples would present

a lower ionic strength (with the small free ions passing through the membrane) though still a considerable amount of bound calcium was present. When only part of the calcium was removed by dialysis, the cow peptides (17.79 ± 0.37 mg Fe/ g peptide) seemed to bind statistically more iron compared to goat peptides (15.57 ± 0.51 mg Fe/ g peptide). Based on the existing literature and the results obtained here, the subsequent iron binding studies were performed with the retentates after dialysis in pH 3.0 buffer and for some of the testing also the ion exchanged samples were used.

Table 7-1: Comparison of the iron binding capacity of peptides before and after dialysis in 40 mM sodium formate, pH 3.0 and ion exchanged samples using Amberlite IRC-50, letters that are the same indicate no significant difference at 95 % confidence, n=3

Sample	Mean ± Standard error (mg Fe/ g peptide bound)
Cow control	14.42 ± 0.26^{d}
Goat control	14.62 ± 0.07^{cd}
Cow retentate	17.79 ± 0.37^{a}
Goat retentate	15.57 ± 0.51^{bc}
Cow ion exchanged	16.06 ± 0.43^{b}
Goat ion exchanged	15.94 ± 0.08^{b}

7.2.3 Determination of isoelectric point of ion-exchanged peptides

As observed in Figure 7-1, the cow peptides had an isoelectric point of 3.67 which is slightly higher than the goat peptides with an isoelectric point of 3.55. Phosphate groups are highly charged and therefore a low p*I* is expected. Xu et al., (2007) compared two software programmes that predicted the p*I* for phosphorylated peptides from α_{s1}, α_{s2} and β casein. The range of p*I* values for one programme (*Scansite,* MIT, MA) was 1.93-4.26 while the other (*pIMethylation*, written by the authors) generated values between 1.02 and 4.25. The p*I* found here for the peptide mix falls within this range. This is the average isoelectric point of the sample; individual peptides may have very different charges. It is also expected that the pH drop (to pH 4.6) that occurred after digestion would remove any undigested material or peptides that have a p*I* close to pH 4.6, so that mostly negatively charged peptides remained.

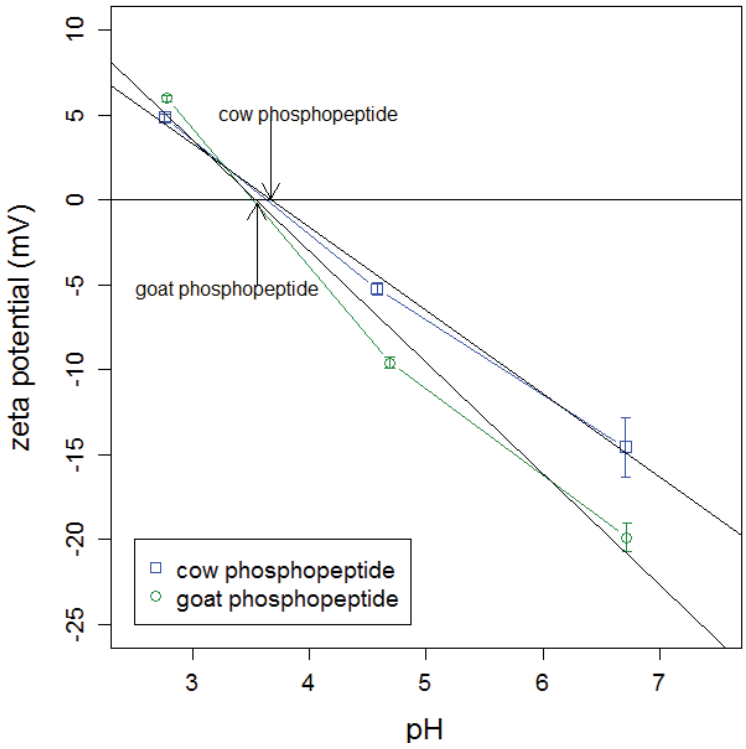


Figure 7-1: Zeta potential of cow and goat phosphopeptides (0.2 mg/mL) after calcium removal with ion exchange; error bars indicate standard error, n=3.

7.2.4 Iron binding at various pH, ionic strength and temperatures

7.2.4.1 Ferrous iron availability in the absence of peptides

Iron is liable to oxidation under various conditions; therefore for the correct calculation of the iron binding capacity, the iron that is oxidised due to the environment (and that is not picked up in this technique) must be differentiated from the iron bound by protein. The initial trial intended to cover a wide range of parameters to give a general understanding of the behaviour of iron in a range of acidities, ionic strength and temperatures; the temperatures tested were 4 ^{o}C, 20 ^{o}C, 37 ^{o}C, 50 ^{o}C, 60 ^{o}C and 72 ^{o}C; the pH levels tested were 2.75, 4.6 and 6.7; the two levels of salt providing low and high ionic strength were 50 mM and 400 mM NaCl. An acidic pH was used to understand the effect of protonation of proteins on the binding strength of iron. Basic pH promoted rapid precipitation of ferrous complexes where a dark green precipitate was formed (data not shown) therefore this pH range (> 6.7) was not tested. The ionic strength was tested at a low strength of 50 mM NaCl and high strength (400 mM NaCl) to determine the effect of salt shielding; the upper limit for allowable osmolality in infant formula (as an example of a nutritional food) is 400 mOsm/kg

(Packard, 1982; Steele et al., 2013). The ionic strength of 400 mM tested here was roughly double this osmolality so to investigate extreme effects of salt and to encompass real food conditions found in products such as "follow up" infant formula or other drinks which may have a higher osmolality. The temperatures are typical processing temperatures: 4 ºC (chiller), 50 ºC/ 60 ºC (evaporation) and 72 ºC (pasteurisation), while 20 ºC and 37 ºC are room and infant feeding/ body temperature.

As observed in Figure 7-2, an acidic environment of pH 2.75 was found to allow more free ferrous ions to remain in solution with approximately 93-97 % of the total iron being in the ferrous form at 50 mM across all temperatures and slightly less present at 400 mM with 93-96 %. Similarly, at pH 4.6 approximately 91-95 % of the iron remained ferrous for both ionic strengths across all temperatures with slightly lower values at 400 mM NaCl when compared to 50 mM NaCl. A significant effect occurred at pH 6.7 when the amount of ferrous iron rapidly decreased above 37 ºC for both ionic strengths, due to the increasing oxidation of iron to ferric iron above this temperature. The decrease of the total ferrous iron present was linear, dropping to 83 % and 76 % for 50 mM and 400 mM NaCl, respectively at 72 ºC. At 4 ºC there was little influence caused by ionic strength and pH for all the systems. The pH of the solutions did not change with the addition of the iron solution indicating that the buffering strength was adequate for the amount of iron added.

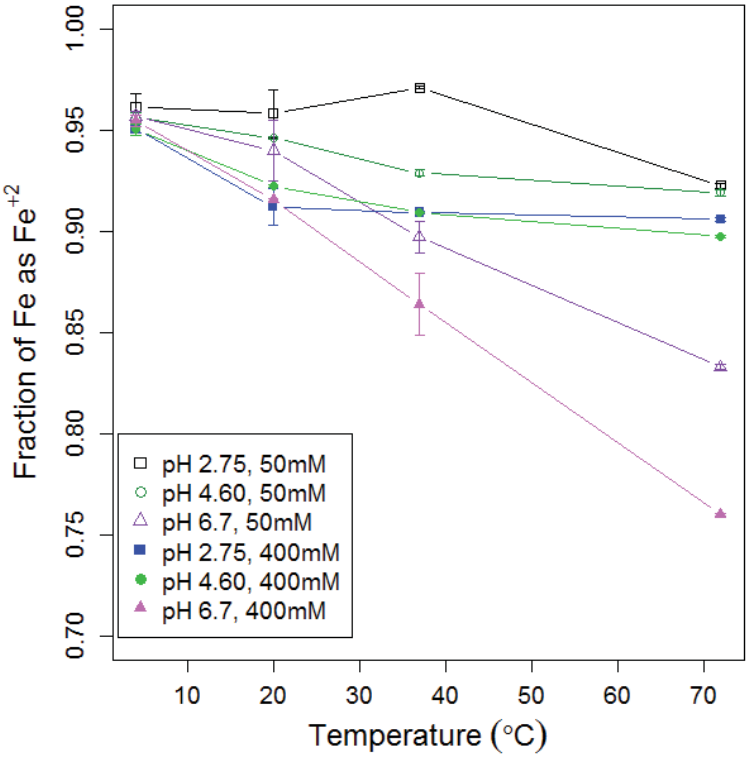


Figure 7-2: Fraction of available ferrous iron out of the total iron added as a function of temperature in buffers at pH 2.75, 4.60, 6.7 with 50 mM and 400 mM NaCl in the absence of protein; error bars indicate standard error, n=3.

7.2.4.2 Iron binding capacity of cow and goat phosphopeptides in the presence of calcium (dialyzed peptides)

The iron chelation activity of the protein solutions was calculated by the difference between the blank (section 7.2.4.1) and the absorbance of the protein + iron solution under similar conditions, generating mg of bound iron per gram of protein. This attempted to take into account the iron oxidation that occurred under the conditions which may be unfavourable for iron solubility. Due to the nature of the technique it is difficult to assess what iron is oxidised or bound other than doing this baseline calculation. Overall, the interactions of temperature and pH ($p=0.005$), temperature and ionic strength ($p=0.044$), and pH and ionic strength ($p=0.034$) were significant on the iron binding capacity. There were strong similarities between the cow and goat phosphopeptide samples however the goat derived peptides bound less iron, in some cases at 5 % significance level.

As observed in Figure 7-3 and 7-4, at ***pH 2.75*** nearly all the iron remained free and in the reduced form over the entire temperature range tested and at both low and high

ionic strength. Overall, iron binding was not affected by ionic strength or temperature at this pH ($p>0.05$). The values found for bound iron were below the level of resolution of the method (*i.e.* at 20 ºC and 50 mM, values such as 0.01 ± 0.08 mg Fe/g goat protein and 0.12 ± 0.05 mg Fe/g cow protein, $p=0.6984$, were obtained). This indicates that the iron did not bind to the protein under high acidic conditions. This may be due to either (i) protein precipitation (Taborsky, 1980) which could result in steric hindrance to the binding site or to (ii) protonation of the serine phosphates groups which in turn would also prevent binding (Castellani et al., 2004); (iii) iron is also more soluble at low pH. The isoelectric points of the isolates were found to be 3.67 and 3.55 for cow and goat peptides, respectively (section 7.2.3). This confirms that the peptides would be positively charged at pH 2.75 and therefore the protonation of the side chains is likely to inhibit iron binding. Interestingly, some studies have found that binding the iron to peptides such as β- casein-(1-25) prior to lowering the pH to 2.5, did not cause a release of iron (Aít-Oukhatar et al., 2000). This would suggest that the acidification of the peptides before or after addition of iron is critical for keeping the iron bound. This would have important implications from a processing and nutritional point of view.

At ***pH 4.6*** the iron binding by the peptides increased and was particularly sensitive to temperature, at low ionic strength; in a 50 mM ionic strength buffer the binding at 20 ºC was 0.64 ± 0.06 mg Fe/ g protein for goat and 0.77 ± 0.03 mg Fe/ g protein for cow ($p=0.6268$) which increased at 37 ºC to 2.92 ± 0.43 mg Fe/ g protein and 1.47 ± 0.09 mg Fe/ g protein ($p=0.3757$), and then to a further 6.17 ± 0.01 mg Fe/ g protein and 8.42 ± 0.01 mg Fe/ g protein at 72 ºC for goat and cow respectively. The effect of temperature on the iron binding capacity will be discussed below since a similar effect was found at neutral pH. At pH 4.6 the peptides are slightly negatively charged as this is above the isoelectric point therefore there would be a greater iron affinity for the phosphate and side chain groups. Increasing the ionic strength to 400 mM however, reduced iron binding (0.53 ± 0.06 mg Fe/ g protein for goat peptides and 0.80 ± 0.09 mg Fe/ g protein for cow at 37 ºC, $p=0.5283$) and made it temperature independent, possibly due to salting out of the protein thus preventing iron from accessing the binding sites.

At ***pH 6.7*** the iron binding capacity was greater compared to the more acidic buffers as the proteins have more available binding sites, being away from the isoelectric point

(Castellani, 2004). At low ionic strength the iron binding increased with temperature in a curvilinear fashion, with a sharp increase in iron binding from cold temperature, ~5-5.5 mg Fe/ g protein at 4 ºC, to 10.03 ± 0.03 mg Fe/ g protein at 20 ºC for the cow peptides. Above this temperature the values almost reached a plateau, only rising to 11.60 ± 0.11 mg Fe/ g protein at 72 ºC. Increasing the ionic strength to 400 mM resulted in an overall lower amount of bound iron as well as a temperature shift for the occurrence of the sharp increase in bound iron. In this case, the values almost doubled from 4.72 ± 0.04 at 20 ºC to 8.24 ± 0.008 mg Fe/ g protein at 37 ºC for cow peptides, above which only a very gentle increase was observed at 72 ºC. Similar trends were found for goat peptides. Clearly, the binding seemed to increase with temperature at this neutral pH, as it happened at pH 4.6 and low ionic strength. Nelson & Potter (1979) obtained similar results by looking at iron binding with casein at 25 ºC and 60 ºC where more iron could bind to casein at the warmer temperature (for example, 11.6 mg iron/g protein bound at 60 ºC versus 5.2 mg iron/ g protein bound at 25 ºC within 30 minutes). Having already discussed the increase in iron oxidation above 40 °C, which has already been taken into account, Nelson & Potter (1979) attributed the increase in binding due to differences in protein solubility of intact casein. The authors determined that this was due to an increase in insolubility of the caseins which decreased from 68 % at 25 ºC to less than 25 % at 60 ºC therefore a greater proportion of ferrous iron bound and moved into the pelleted phase. No changes in insolubility were observed in the present study, due to low protein concentrations, however Nelson & Potter's results suggest that the iron may have a higher affinity to casein at warmer temperatures, as casein is not heat sensitive at 60 ºC, causing large complexes to form allowing sedimentation. This may indicate that within short binding times the increased temperature may allow a greater interaction between the iron and peptides allowing more iron binding. These results indicate that under pasteurisation conditions the binding of iron will not be negatively affected since iron binding is not inhibited after heating up the peptides at 72 ºC for 3 minutes.

Over the range of parameters studied in this work (pH 2.75-6.7, high and low ionic strength of the buffer, and temperature 4-72 °C) the maximum iron binding was measured at neutral pH in a low ionic strength buffer (50 mM NaCl) and at warm temperatures (40 °C and above). This is likely to be due to the lower competition of salt for the binding sites and the high level of protein ionization in the environment.

In contrast, a high acidic solution inhibited the binding of iron probably due to the protonation of the phosphoserine and acidic residue binding sites causing repulsion of the iron. Castellani et al., (2004) found that the optimum conditions for iron binding using phosvitin from egg yolks were pH 6.5 and low ionic strength (0.15 M) also suggesting that at this pH the phosphates are completely ionised and there is little salt to compete with the iron for binding sites. Additionally, Wang et al., (2011) found optimal iron binding conditions for yak casein digests at pH 6.0 and at 40 ºC. It seems that all results agree with the fact that iron will bind to the peptides in solutions that are of neutral acidity, ideally of low ionic strength and at warm temperatures.

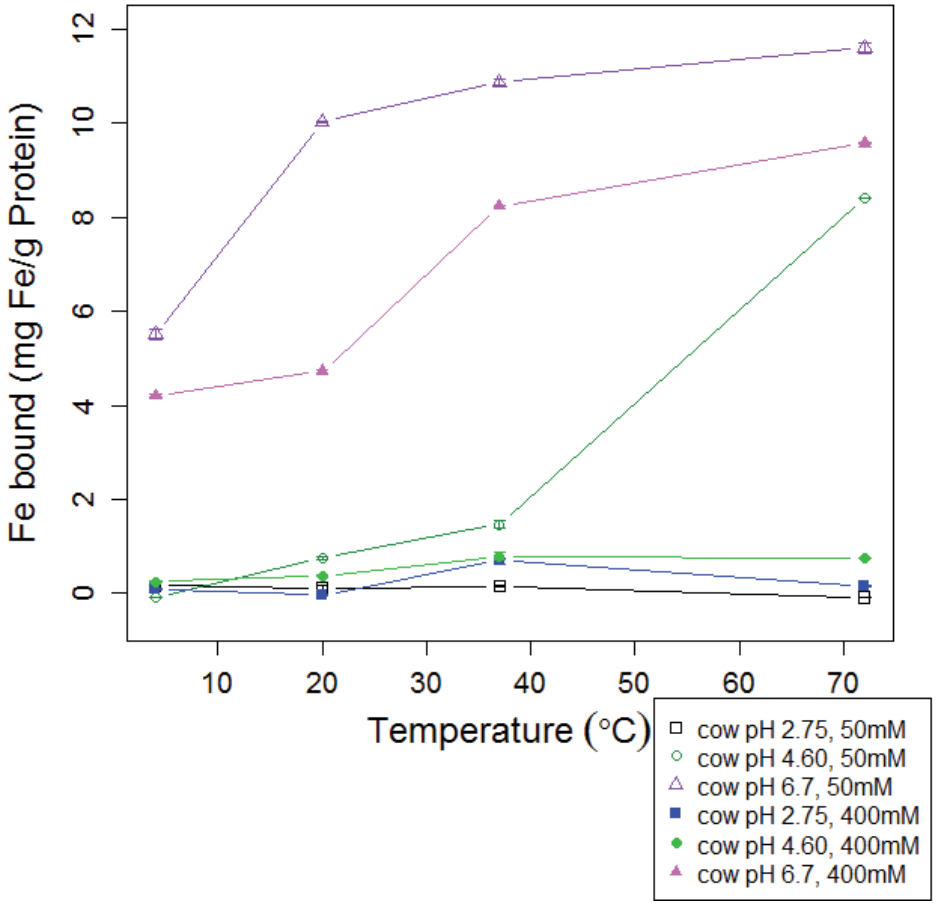


Figure 7-3: Iron bound by cow phosphopeptides as a function of temperature in buffers at pH 2.75, 4.60, 6.70 at 50 mM and 400 mM NaCl after blank subtraction of iron in respective buffer; error bars indicate standard error, n=3.

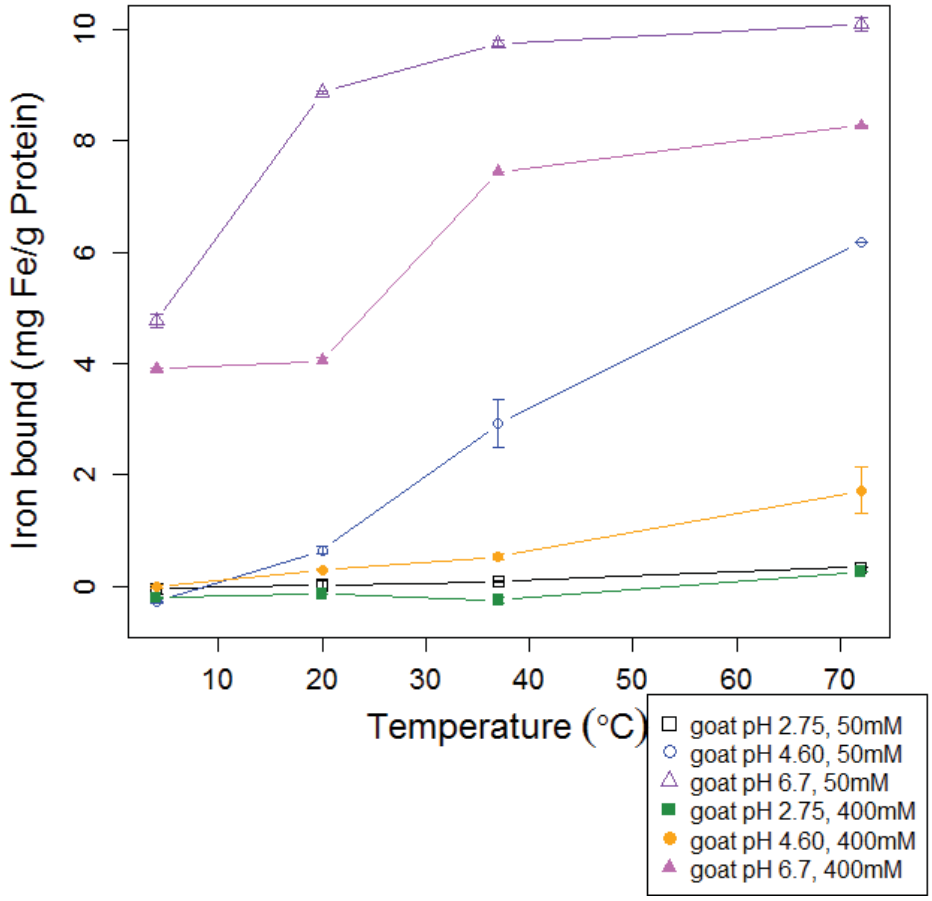


Figure 7-4: Iron bound by goat phosphopeptides as a function of temperature in buffers at pH 2.75, 4.60, 6.70 at 50 mM and 400 mM NaCl after blank subtraction of iron in respective buffer; error bars indicate standard error, n=3.

7.2.4.3 Iron binding capacity of cow and goat phosphopeptides in the absence of calcium (ion exchanged peptides)

As the samples at acidic pH's (2.75, 4.6) in the previous section 7.2.4.2 displayed a lower amount of iron binding, analysis at pH 6.7 was carried out with peptides in the absence of calcium. Similar trends found for the dialyzed peptides occurred here with the phosphopeptides in imidazole buffer at pH 6.7 and 50 mM NaCl (Figure 7-5); the binding of iron increased sharply from 4 ºC (8.8 ± 0.1 and 8.23 ± 0.36 mg Fe/ g protein (p=0.266) for cow and goat, respectively) to 20 ºC, levelling off as the samples were heated up to 72 ºC (12.85 ± 0.72 and 11.80 ± 0.02 mg Fe/ g protein (p=0.383), for cow and goat, respectively). However at high ionic strength (400 mM NaCl) the iron binding increased linearly with increasing temperature up to 50 ºC, reaching values not significantly different from iron binding values at 72 ºC and low ionic strength for both species (between ~11.5 and 12.5 mg Fe/ g protein). Overall, iron binding in the

absence of calcium, was greater than for dialyzed peptides (with a considerable amount of calcium present). The difference was especially significant at lower temperatures and at high ionic strength (*i.e.* iron binding capacity for goat peptides doubled at 20 ºC and high ionic strength from ~4 to ~8 mg Fe/g protein when comparing dialyzed versus ion exchanged peptide, respectively). There was a statistically significant interaction of temperature and ionic strength on the iron binding ($p=0.0028$). This trial therefore indicates that the removal of calcium can improve the iron binding ability of the peptides.

Finally, to confirm the trends observed at acidic pHs, iron binding was also tested at pH 2.75 and pH 4.6 and high and low ionic strength at 37 ºC (data not shown). There was no significant difference in the iron binding capacity between samples containing calcium and those which were ion exchanged (calcium free) at 95 % confidence. This reinforces that only at near neutral conditions does the calcium have an effect on iron binding capacity.

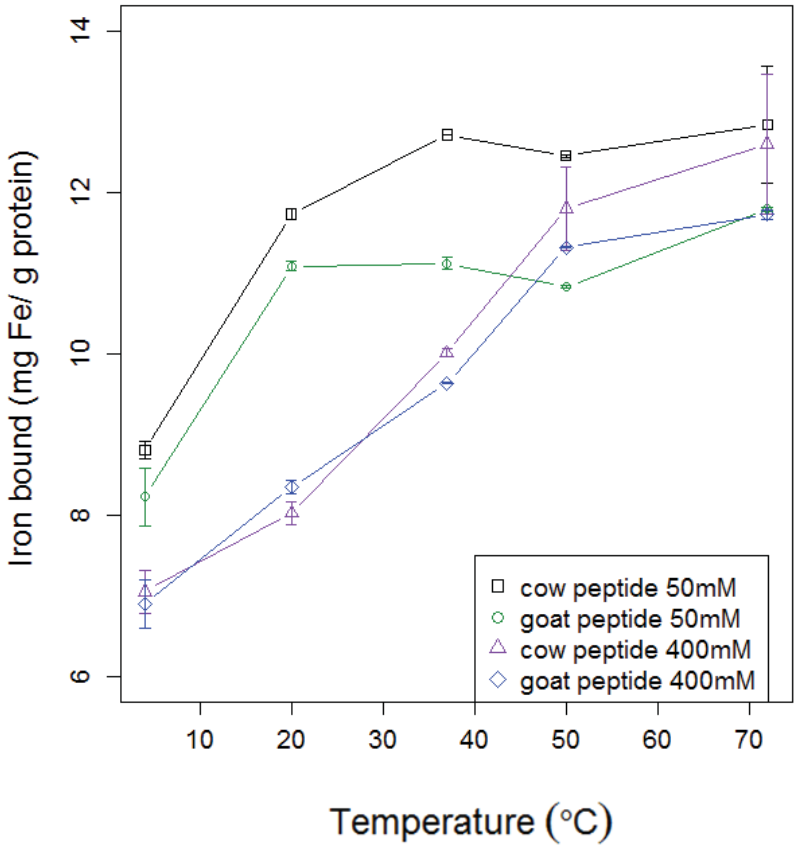


Figure 7-5: Iron binding of cow and goat phosphopeptides after ion exchange with non-detectable calcium content in imidazole buffer, pH 6.7, 50 mM or 400 mM NaCl; error bars indicate standard error, n=3.

7.2.5 Effect of different buffer solutions at pH 6.7

7.2.5.1 Ferrous iron availability in the absence of peptides

In the previous section (7.2.4) it was found that peptides at pH 6.7 show better iron binding capacity, therefore the influence of the type of buffer at this pH was investigated. Three solutions were used at pH 6.7, and at low ionic strength (50 mM NaCl): imidazole buffer, used in the previous section, HEPES buffer which has been used in previous binding studies and water adjusted to pH 6.7. All solutions were adjusted to 50 mM ionic strength, ruling out any test at high ionic strength given the binding inhibition shown at 400 mM in the previous section.

Figure 7-6 shows the fraction of ferrous iron present when using various buffers. The iron was stable between 4 and 37 ^{o}C in all buffers (and up to 72 ^{o}C when using imidazole) since the free ferrous iron fraction was above 85 % in all cases. However, at high temperatures (72 ^{o}C) there was a significant drop in Fe^{2+} for HEPES buffer ($p<0.001$) and a less substantial decrease with water. The interaction of temperature and buffer was significant ($p=0.0124$) on the amount of ferrous iron, that is, the effect of temperature on iron oxidation depends on the type of buffer. HEPES buffer has been found to have an effect on iron auto-oxidation. Tadolini (1987) found that the pH of the buffer, the iron concentration and to a small extent, the buffer concentration, can affect the rate of iron auto-oxidation. While temperature was not tested in Tadolini's study, it would be expected that with increasing temperature the rate of reaction would increase therefore explaining the disappearance of ferrous iron at 72 ^{o}C. Water is not a very good buffer, with the solution becoming more acidic when the iron was added. It is unclear why this would promote iron oxidation shown as a decrease in available Fe^{2+}. Several replications on different days were carried out achieving similar results.

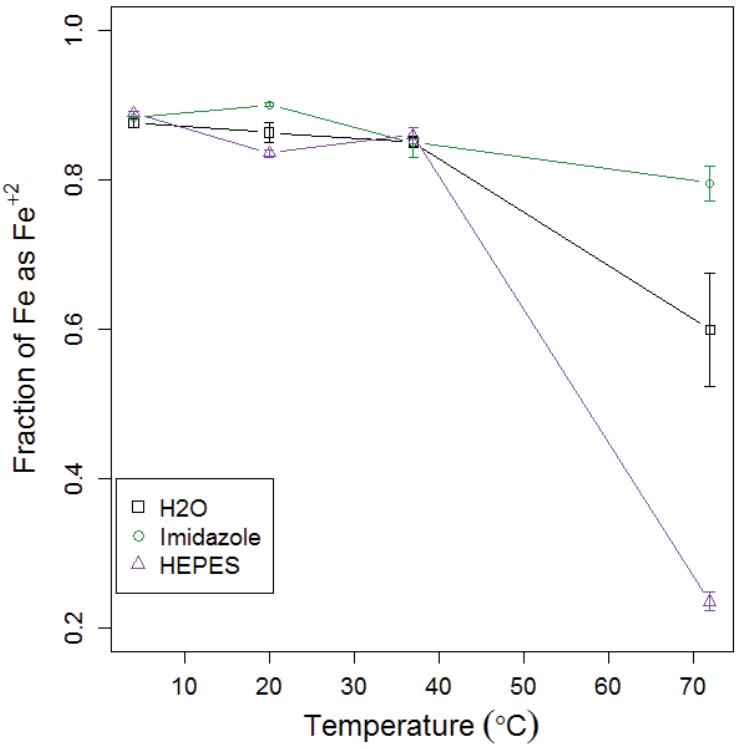


Figure 7-6: Fraction of available ferrous iron out of the total iron added as a function of temperature in various buffers adjusted at pH 6.70, 50 mM NaCl; error bars indicate standard error, n=3.

7.2.5.2 Iron binding capacity of cow and goat phosphopeptides (dialyzed peptides)

The iron binding capacity of cow and goat derived peptides using different buffers at pH 6.7 was tested. Similar results were found for both phosphopeptides species (Figure 7-7) in terms of iron binding, with goat phosphopeptides consistently exhibiting lower values than the cow phosphopeptides. The binding capacity was calculated after deducting the oxidation of iron obtained with the blank buffers. The imidazole buffer produced very similar trends as found in the previous section (7.2.4). The HEPES buffer however, followed similar increase in iron binding with temperature from 4 to 37 ºC as imidazole buffer, but then dropped significantly at higher temperatures, from 8-11 mg Fe/g protein (at 37 ºC) to 2-3 mg Fe/g protein (at 72 ºC). This is likely to be attributed to the lower levels of available Fe^{2+} for binding in the presence of this buffer at high temperatures. The iron binding in water was very low compared to the other buffers especially below 60 ºC. It is not understood why the goat peptides in water at 60ºC exhibited an increase in iron binding. The imidazole and HEPES buffers had little change in pH after the addition of iron while the water

dropped by approximately 0.4 pH units. The phosphopeptides in the more acidic water with no buffering salts were probably more protonated, decreasing their binding capacity, as shown earlier for peptides at low pH. Overall, analysis of the data showed that the there was a significant interaction of the effect of temperature on the response of iron binding which was dependent on the type of buffer ($p<0.001$).

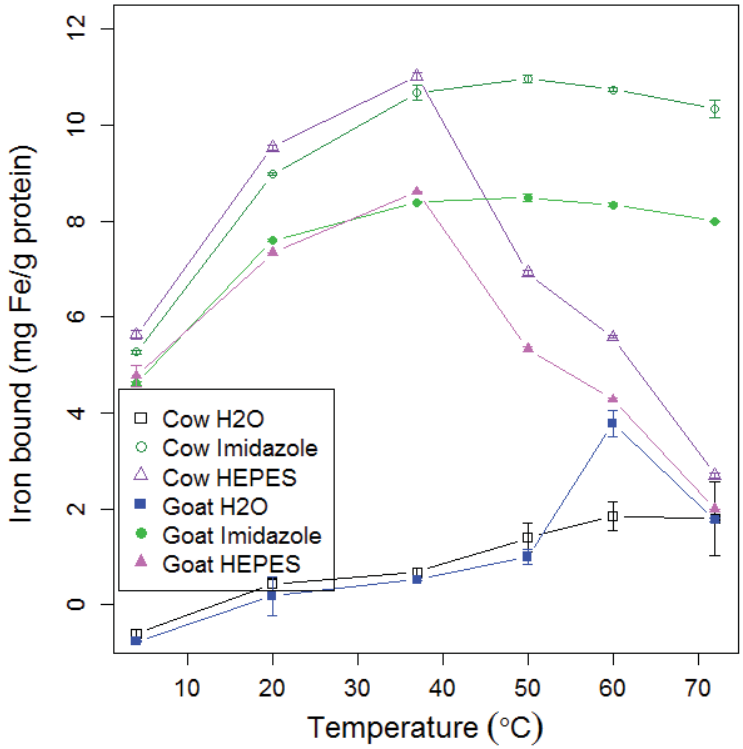


Figure 7-7: Iron binding of cow and goat phosphopeptides with various buffers at pH 6.70, 50 mM NaCl; error bars indicate standard error, n=3.

7.2.6 Effect of holding time on iron binding

Holding times could readily occur in an industrial setting where an iron solution could be in a holding stage prior to addition to the protein. Also the point at which iron is added will determine what temperature/time profiles the iron-peptide mixture will be subjected to. It is therefore critical to understand the effect that process delays (holding time) would have on iron peptide mixtures held at constant temperatures. The effects of holding times were measured up to 25 minutes at 20 ºC, 37 ºC, 50 ºC and 72 ºC. At 50 ºC the temperature would mimic a pre-evaporation holding step, 72 ºC is the temperature at which pasteurisation occurs while 20 ºC and 37 ºC mimic potential temperatures in an industrial process (or in food preparation steps by the consumer). Therefore the following section investigates the iron oxidation and binding at a given

temperature held for a period of time. In terms of the holding time parameters used, Wang et al., (2011) determined that the maximum amount of iron bound in their system using yak casein hydrolysates occurred after 20 minutes and therefore extending the time much beyond this time was not carried out.

7.2.6.1 Oxidation of iron in buffer held at constant temperature over a period of time

As shown in Figure 7-8, at 20 ºC there was very little oxidation over 25 minutes with the available ferrous iron decreasing only from 97 % to 93 %, the decrease becoming slightly more significant at 37 ºC, from 93 % to 80 % ferrous iron after 25 minutes. At 50 ºC there was a substantial oxidation with a near linear decrease in ferrous iron from 88 % to 60 % ($p=0.0003$), becoming a dramatic fall at 72 ºC where the oxidation was extremely rapid, decreasing linearly from 84 % to 13 % after 25 minutes ($p<0.0001$). This indicates that the iron at or above 50 ºC is unstable and can oxidise quickly lowering the potential availability of the added iron. Sung & Morgan (1980) looked at the effect of temperature on the rate of ferrous iron disappearance from 5 to 30 ºC. In buffered solutions at pH 6.76-6.88 the half life of ferrous iron in solution dropped from 315.6 minutes at 5 ºC to 4.1 min at 30 ºC therefore it is evident that at 50 ºC the rate of oxidation of ferrous iron will be high however the rate will likely be different due to different environmental conditions compared to the literature results.

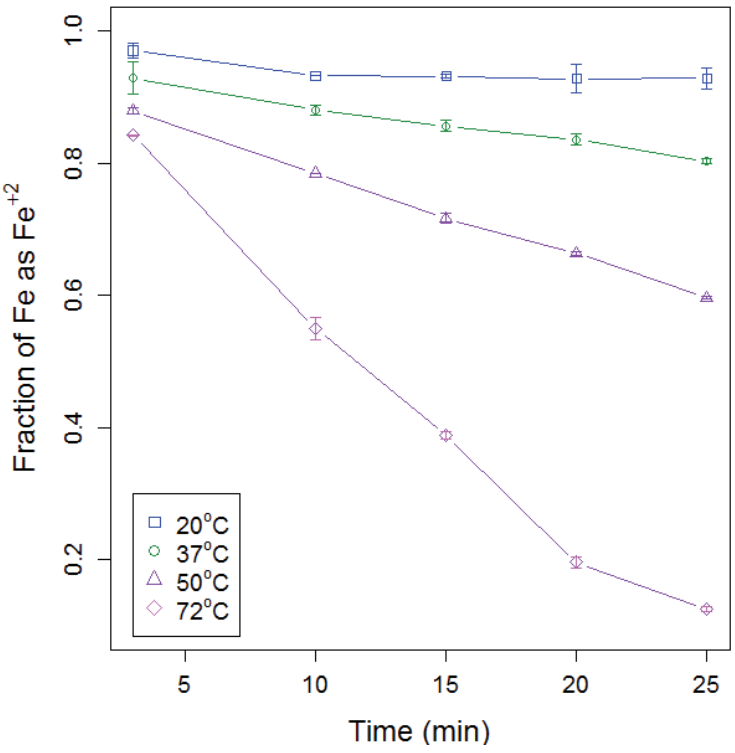


Figure 7-8: Effect of holding time at different temperatures on blank imidazole, pH 6.70, 50 mM NaCl on the availability of ferrous iron; error bars indicate standard error, n=3.

7.2.6.2 Effect of holding time on iron binding of cow and goat peptides

The peptides were analysed under the same conditions as the blanks in section 7.2.6.1. When there was a presence or absence of calcium the effect of temperature on the response of iron binding depends on the holding time ($p<0.001$). The removal of the calcium improved the binding capacity of the peptides and this indicates that the affinity to the iron increased when the binding sites are not occupied with calcium.

As shown in Figure 7-9 at 20 ºC the iron binding increased for both cow and goat peptides reaching 17.51 ± 0.44 mg Fe/ g protein and 15.80 ± 0.27 mg Fe/ g protein after 25 minutes, respectively. The same trend occurred in the ion exchanged samples (Figure 7-10) however more iron was able to bind after 25 minutes with the cow peptides binding 20.29 ± 0.10 mg Fe/ g protein and goat peptides binding 19.46 ± 0.01 mg Fe/ g protein. At 37 ºC, in the presence of calcium, the cow peptides were able to bind more iron than goat peptides however there was no significant change in the amount of iron bound over the holding time. In contrast, the binding over the 25 minutes increased signifcantly for both treatments without calcium. For example, cow

peptides with calcium bound 15.47 ± 0.05 mg Fe/ g protein while without calcium 16.29 ± 0.35 mg Fe/ g protein; moreover the goat peptides were able to bind more iron in the absence of calcium. At 50 ºC, across all treatments, the amount of iron bound decreased with time. For example, goat peptides in the presence of calcium decreased from 12.55 ± 0.12 at 3 minutes to 10.47 ± 0.13 mg Fe/ g protein (p=0.002). This trend was more prominent at 72 ºC across all treatments. In the absence of calcium goat peptides decreased from 17.31 ± 0.76 mg Fe/ g protein which decreased to 6.65 ± 0.26 mg Fe/ g protein (p=0.6852) after 25 minutes. Nelson & Potter (1979) monitored the ferrous iron binding of casein for up to 180 minutes and found that after this time period, at 25 ºC the iron binding had not plateaued while in the present study the slope began to decrease after only 25 minutes. At 60 ºC, Nelson & Potter (1979) found that maximum binding had been achieved after 30 minutes, as opposed to the decrease in binding as observed in the present study at 50 ºC and 72 ºC.

As previously discussed, the large decrease at the high temperatures over time is likely to be due to the influence of the blanks at the respective temperature. At 50 ºC and 72 ºC the samples have a confounding factor of the ferrous iron oxidising and therefore this has to be taken into consideration. Based on Figure 7-9 and 7-10, at 72 ºC the iron would appear to dissociate from the protein as time progresses. It is certain that any free iron in the solution would oxidise but it is unclear how much iron really remains bound to the protein at high temperatures.

Emery (1992) determined that the mechanism of iron binding to casein results in an equilibrium shift of the remaining iron in solution. This is because when ferrous (Fe^{2+}) iron is added to a casein solution the iron that binds to the casein is oxidised to the ferric (Fe^{3+}) form and increases the oxidation of the ferrous iron, therefore allows more iron to bind to the protein via the phosphate groups. Emery (1992) stated that this did not mean that the casein acted as a catalyst for more iron to bind to the casein as the oxidation state changed, rather it was a stoichiometric interaction. It can be stated with certainty that ferrous iron solutions should not be incubated at temperatures higher than 50 ºC due to oxidation, and/or dissociation of ferric iron could occur.

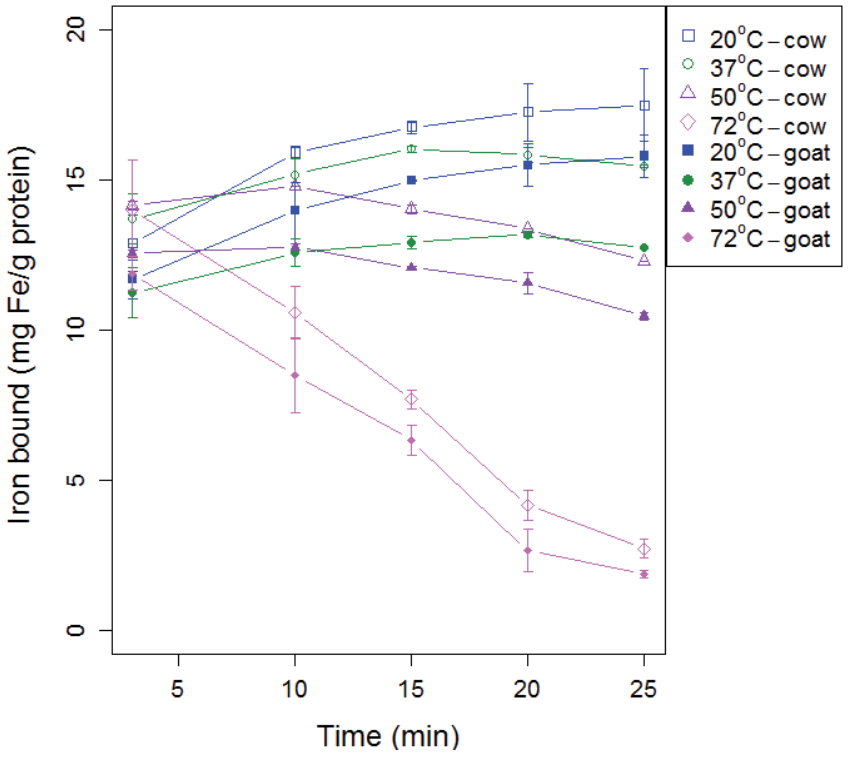


Figure 7-9: Effect of holding time on the iron binding capacity at various temperatures at pH 6.7, 50 mM NaCl, in the presence of calcium; error bars indicate standard error, n=3.

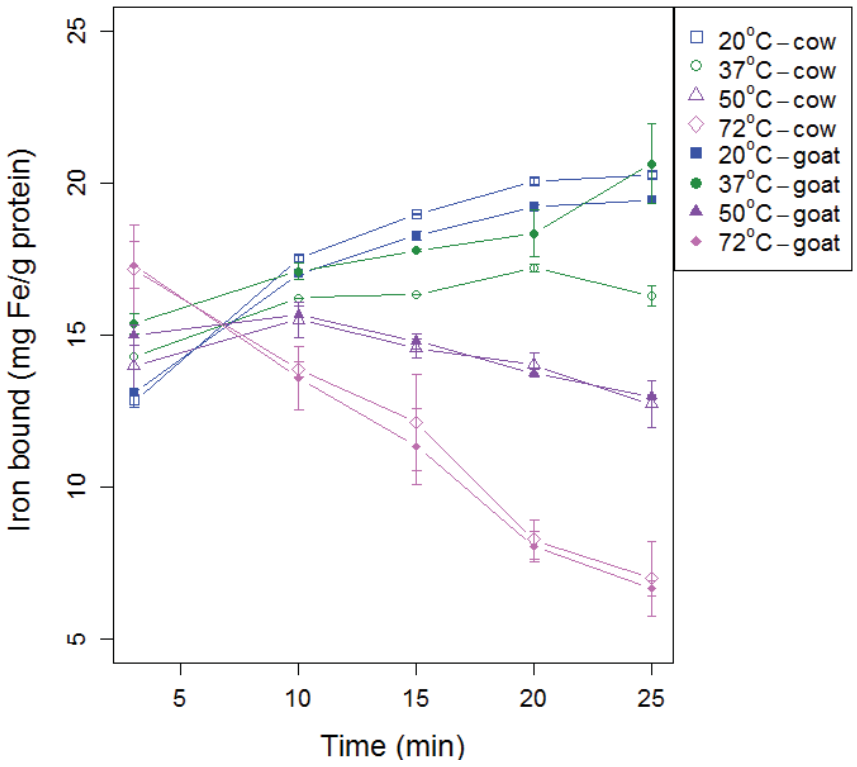


Figure 7-10: Effect of holding time on the iron binding capacity at various temperatures at pH 6.7, 50 mM, in the absence of calcium; error bars indicate standard error, n=3.

7.2.6.3 Effect of temperature ramping on iron binding at pH 6.7, 50mM NaCl

A temperature ramp was investigated to determine how iron would behave in an industrial setting; this replicated if iron were to be added to a cold solution and then heated during pasteurisation or evaporation prior to spray drying. Therefore the solutions were heated up first and then cooled back to 4 ºC and analysed for free iron during this cycle. The aim of the ramp was to determine if the iron was bound irreversibly by the peptides or if the bound iron can be unbound with a decrease of temperature.

7.2.6.3.1 Effect of temperature ramping in the absence of peptides

The buffer blank used was imidazole. The iron profile in the presence of the buffer showed that the iron oxidised with increasing temperature (a decrease in available Fe^{2+}) and occurred in a sigmoidal fashion with respect to time (Figure 7-11). At 4 ºC nearly all the iron was present in the ferrous form and there was a slight reduction as the temperature was increased to 50 ºC in about 30 minutes. The fraction of ferrous iron present at 72 ºC after 45 minutes had dropped to 50 %. When the temperature was then ramped down, the fraction of ferrous iron continued to drop until about 37 ºC when there was approximately only 4 % ferrous iron remaining in solution, staying like this until 4 ºC. Tadolini (1987) prepared 5 mM HEPES at pH 7.0 with 150 µM Fe^{2+} and found that by 30 minutes the autoxidation of the iron resulted in all the ferrous ions being oxidised, also following the lag phase that was observed in the present study. Cooling down the sample back to 4 ºC did not stop the oxidation process since there was still an overall decrease in ferrous iron that plateaued below 50 ºC.

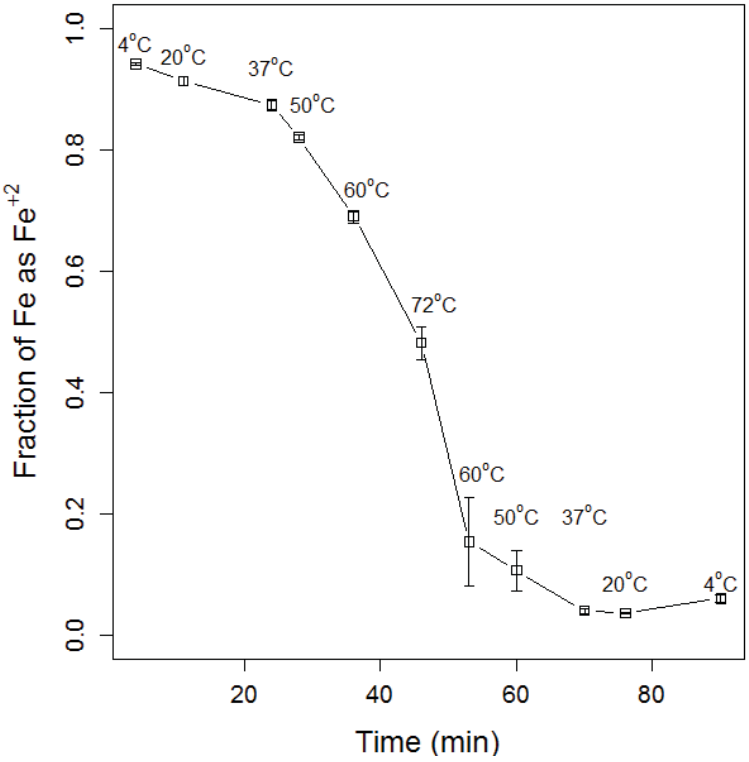


Figure 7-11: Effect of temperature ramping on blank buffers imidazole, pH 6.70, 50 mM with time with respect to iron oxidation; error bars indicate standard error, n=3.

7.2.6.3.2 Effect of temperature ramping on the iron binding capacity of cow and goat phosphopeptides

Figure 7-12 shows that the iron binding of the peptides reached a maximum at 37 ºC after a heating time of 20 minutes in imidazole buffer at pH 6.7 and low ionic strength. The binding of iron to protein then decreased as the temperature increased to 72 ºC and continued to do so upon cooling. This seems to indicate that as the temperature increased above 37 ºC the iron would appear to dissociate from the peptides. From the blank trial in Figure 7-11 the massive oxidation of iron is clear upon heating. Above 50 ºC, the oxidation of iron takes over. No more iron can probably be bound because it gets oxidised, appearing as a false decrease in bound iron which in fact is just an increase in oxidized iron. Alternatively, the iron binds to the protein and as the temperature increases there is an equilibrium shift where some iron unbinds from the protein and immediately oxidises, or dissociates from the protein in the ferric iron. Iron has been shown to dissociate from saturated lactoferrin upon fast-rate heating using calorimetry when using a monoferric species in simulated milk ultrafiltrate (Rüegg, Moor & Blanc, 1977). However this is a structured protein so there may be

differences in the mechanism of binding and dissociation between lactoferrin and the peptides.

Section 7.2.6 shows that it is difficult to differentiate between the existence of ferric iron (due to oxidation) and true iron binding to the peptides, even when subtracting the blank reading. Any holding times beyond the 3 minute time period found in the literature may have to be treated with caution, in particular at higher temperatures where the liability of ferrous iron is clearly increased. What can be concluded from this work is that ferrous iron in a buffer solution is not stable when heated at elevated temperatures for prolonged periods of time (though it is relatively stable up to 3 minutes as shown in the sections above). When ferrous iron is added to peptides there is binding and oxidation occurring and it seems likely that within the first 20 minutes the binding would be predominant as the iron is quite stable in the buffer at temperatures below 37 °C. Above 37 °C at extended times, the oxidation of iron takes over and the process seems irreversible as shown upon cooling, also by the blank.

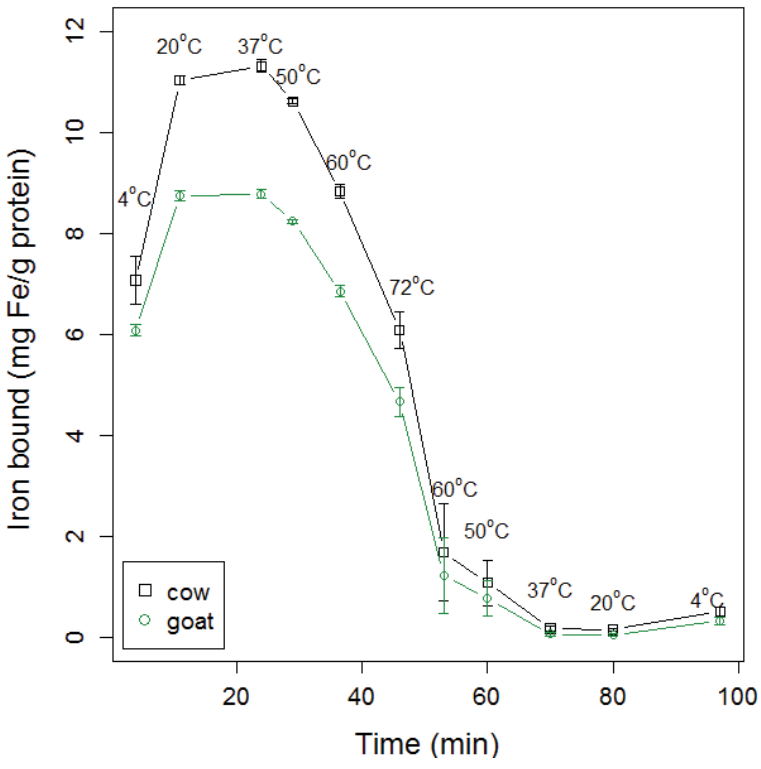


Figure 7-12: Effect of temperature ramping on cow and goat phosphopeptides over time, in imidazole buffer, at pH 6.7 and 50 mM NaCl; error bars indicate standard error, n=3.

7.2.7 Testing the maximum capacity of iron binding

7.2.7.1 Ferrous iron oxidation in imidazole buffer at pH 6.7, 50mM NaCl

A quantification of how much iron can be added to the peptide mixture before significant amounts of iron remain unbound and therefore susceptable to binding to other components is relevant to this study. Therefore various iron loadings were investigated to determine a possible iron saturation point for the peptide mixture. Increasing amounts of iron were added starting at 25 μM, at temperatures of 20, 37 and 50 ºC. From Figure 7-13, it can be observed that increasing the iron content in the buffer solution caused an increase in iron oxidation. As detected earlier, the oxidation also augmented with increasing temperature. Both the iron loading ($p=0.005$) and temperature of the buffer ($p<0.001$) had a significant effect on the iron oxidation. The ferrous iron remaining in solution decreased with a higher iron loading; Tadolini (1987) added ferrous iron at concentrations from 50 to 300 μM Fe^{2+} in buffer at pH 7.3 and found a sigmoidal curve developed with a decrease of ferrous iron present in solution. Within approximately 10 minutes, at 300 μM, most of the iron had oxidised while after 20 minutes at 50 μM, some ferrous iron remained at room temperature. Adding high levels of ferrous iron in aqueous solutions may therefore promote oxidation to the ferric form.

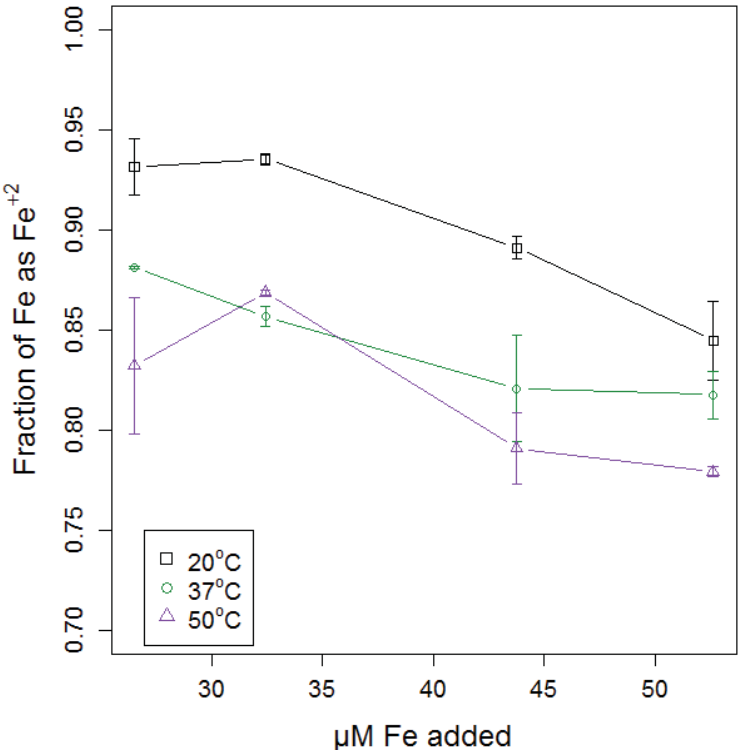


Figure 7-13: Effect of iron loading in blank imidazole buffer on fraction of ferrous iron availability with increasing temperature; error bars indicate standard error, n=3.

7.2.7.2 Maximum iron binding capacity of cow and goat phopshopeptides in the presence and absence of calcium

Increasing volumes of ferrous iron were added to the cow and goat phosphopeptide solutions in the presence (Figure 7-14) and in the absence of calcium (Figure 7-15). In the presence of calcium, the results show that as more iron was added, the binding increased linearly, with a pronounced slope up to 25 mg Fe^{2+}/ g protein added. Further additions up to 58 mg Fe^{2+}/ g protein decreased the slope of the linear increase with the binding almost plateauing for some temperatures, between 58 and 125 mg Fe^{2+}/g protein additions. The iron binding by the peptides with increasing iron loading followed a similar trend as with calcium present; however more iron was able to bind with the same iron concentration (Figure 7-15). The iron concentration ($p<0.001$) and temperature ($p<0.001$) were significant on the iron binding when calcium was present however the interaction of temperature and iron concentration was significant on the iron binding ($p<0.001$) in the absence of calcium. In contrast to treatments in the presence of calcium, the iron concentration was increased to 125 mg Fe^{2+}/g protein as

a plateau had not been reached with 100 mg Fe^{2+}/g protein addition when calcium was removed.

At 20 ºC the maximum iron bound at 100 mg Fe^{2+}/g protein for cow and goat was 37.4 ± 1.3 and 32.8 ± 0.04 mg Fe/ g protein, respectively in the presence of calcium. When calcium was removed the binding was greater at 40.67 ± 1.46 and 39.18 ± 1.02 mg Fe/ g protein at 125 mg Fe^{2+}/g protein addition, for goat and cow peptides respectively. In both cases, there was a decrease in binding above 100 mg Fe^{2+}/g protein indicating that a maximum binding had been achieved at this iron concentration. At 37 ºC, in the presence of calcium, the binding for cow and goat was 44.2 ± 0.7 and 41.3 ± 1.4 mg Fe/ g protein, respectively and there still appeared to be potential for further binding. A similar trend was found at 37 ºC, in the absence of calcium, with maximum binding for goat peptides at 50.16 ± 0.63 mg Fe/ g and for cow peptides at 51.10 ± 0.07 mg Fe/ g protein at 100 mg Fe^{2+}/g protein loading which may indicate that increasing the concentration in the previous treatment may not improve the iron binding capacity. At 50 ºC the binding for cow and goat had almost plateaued at 45.2 ± 0.2 and 43.4 ± 0.1 mg Fe/ g protein, respectively in the presence of calcium. The iron binding capacity was greater in the absence of calcium. At 125 mg Fe^{2+}/g protein the iron binding was 55.76 ± 2.48 mg Fe/ g protein for goat peptides and 57.93 ± 3.69 mg Fe/ g protein for cow peptides in the absence of calcium however this was not significantly different from the lower addition level of 100 mg Fe^{2+}/g protein.

The results show that adding 100 mg Fe^{2+}/g protein to peptides in the presence or absence of calcium appears to allow for the greatest amount of binding, in general, as few differences were seen at the higher addition level at 125 mg Fe^{2+}/g protein in the second treatment when calcium was removed. It appears that using a warmer temperature, such as 50 ºC, would allow more iron to bind to the peptides. However, from a manufacturing perspective and knowledge on the holding time phenomena the iron is not stable at 50 ºC for longer holding times and therefore may not be the optimal binding temperature in terms of oxidation. Certainly, what this work shows is that the removal of calcium does improve the iron binding capacity. A short reaction time (3 minutes) used here may allow iron to bind at a faster rate than when the calcium is present. Adding iron to caseins has been shown to cause precipitation at 4mM iron (Gaucheron, Famelart & Le Graët, 1996) however Raouche et al., (2009b) found that milk could bind 20 mmol/kg milk under chilled conditions. Hekmat & McMahon

(1998) added 100 mg/L $FeCl_3$ to milk and 83 % remained in the casein pellet within the pH range of 5.3 to 6.7. More than 90 % of added iron could be bound to a calcium depleted milk solution at 11 % (w/w) which roughly corresponds to about 26 mg Fe/g protein at 5 ºC (Mittal et al., 2015). These results indicate that there is significantly more binding of iron using peptides compared to intact casein proteins. On the assumption that in section 4.2.2.3 the casein was able to bind all the iron that remained in the pellet phase, it was calculated that the intact milk protein would bind 6, 12, 18 and 26 mg Fe/ g protein with 5, 10, 15 and 20 mM ferrous iron addition taking into account that ~75 % iron bound to the pellet phase (refer to Figure 4-10). The casein phosphopeptides are therefore able to bind more iron at higher addition rates as evidenced by Figure 7-15a and 7-15b. Also compared to caseinate, (25.5 mg/g protein at 20 ºC as shown by Surigato, Ye and Singh (2009) the quantities detected in this study, doubled that iron load.

While it appears that the peptides in this study have significantly improved iron binding capacity over milk and caseinate systems it has not matched Bouhallab et al., (2002) who reported binding of 70 mg iron per gram of phosphopeptide. These authors used ferrous chloride with a molar ratio of 4 which was bound to β-(1-25) at 37 ºC at pH 6.5. This indicates that using a single peptide produces complexes with higher binding capacities compared to an extracted peptide mix with no further purification. Chaud et al (2002) bound ferric iron with a digest and the resultant precipitate contained 5.61 % ferric chloride hexahydrate by weight or 51.6 mg Fe/ g protein.

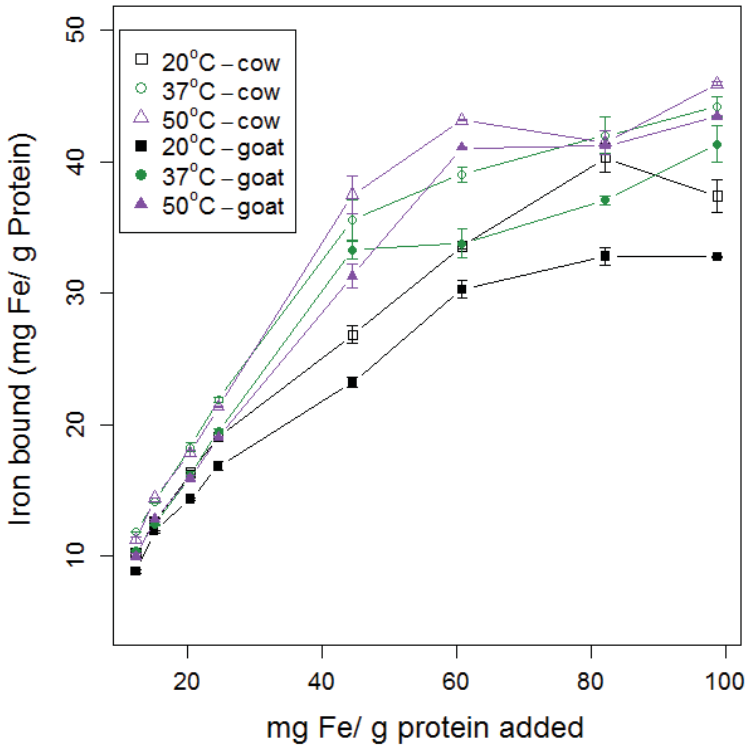


Figure 7-14: Effect of higher iron loading on cow and goat phosphopeptides, pH 6.70 50 mM NaCl with blank buffer subtracted with respect to iron concentration in the presence of calcium; error bars indicate standard error, n=3.

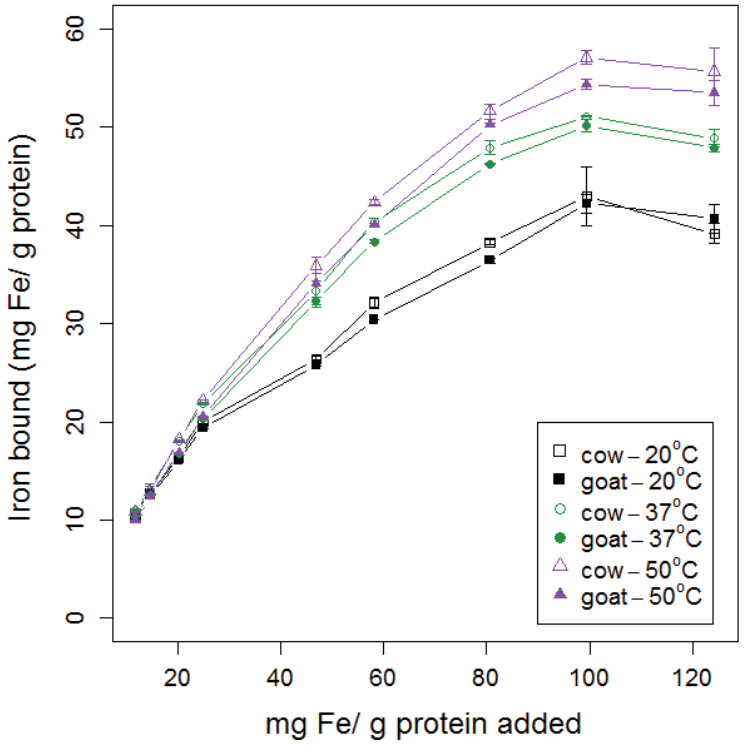


Figure 7-15: Effect of higher iron loading on cow and goat phosphopeptides, pH 6.70 50 mM with blank buffer subtracted with respect to iron concentration in the absence of calcium; error bars indicate standard error, n=3.

7.2.8 Iron binding in a milk system

7.2.8.1 Iron binding of cow and goat phosphopeptides containing calcium (dialyzed) in a milk system

The ability of the phosphopeptides to bind iron in a milk system was investigated to determine their effectiveness at delivering iron in a model commercial-style application. After overnight storage milk dissolved in imidazole buffer was mixed with several aliquots of peptides (also solubilised in imidazole buffer) to produce different ratios of milk protein to peptide to achieve a final 0.2 mg/mL protein concentration. Iron binding was carried out at 20 ^{o}C to limit the effects of temperature on oxidation and an equal iron fortification of 28 μM was used across all the samples. The order of addition of iron to the peptide solution was varied to reflect two possible process options and whether these have an impact on iron binding by the peptides. Ferrous sulfate was either added to the peptide solution and held for 3 minutes and then added to the milk solution or the peptide and milk solution were mixed first then the iron was added to this. It was hypothesised that adding the iron to a peptide solution first would, in the absence of competition from intact casein, increase the probability for iron peptide interactions and thus result in a more effective iron binding in the final milk system.

The iron binding increased with increasing peptide content (Figure 7-16); the order ($p<0.0001$) and peptide concentration ($p<0.0001$) were significant factors for the amount of iron bound. The iron binding of cow and goat milk was 4.13 ± 0.11 mg Fe/g protein and 4.24 ± 0.04 mg Fe/ g protein ($p=0.425$). The iron binding at 0.5 peptide/total protein (maximum concentration), where the milk and peptide were mixed first, increased to 11.28 ± 0.21 mg Fe/ g protein and 11.32 ± 0.02 mg Fe/ g peptide ($p=0.871$) for cow and goat, respectively. At the same peptide level, with the peptide and iron mixed first, the binding was 10.65 ± 0.13 mg Fe/ g protein and 10.93 ± 0.06 mg Fe/ g protein ($p=0.192$) for cow and goat, respectively.

As expected, as more peptide was added into the milk solution more iron was able to be bound. However, against the hypothesis, adding the iron to a pre-mixed peptide and milk solution allowed more binding than when the iron and peptide were pre-mixed and then added to the milk solution. When the fraction of peptide was 0.3 for cow, there was 11.9 % greater binding when the peptide and milk were pre-mixed (8.91 ±

0.02 mg Fe/ g protein compared to 7.97 ± 0.01 mg Fe/ g protein ($p=0.0005$)). The greatest difference for goat peptides was at 0.1 and 0.2 fraction addition where there was a 13.7 % and 13.0 % difference in the two treatments.

The unexpected nature of the results could be attributed to the presence of calcium. When the iron was added to the peptide containing calcium there would potentially be competition with the calcium, even more so than with normal milk as the calcium is in higher concentration than in milk (6 % calcium by weight versus 6-7 % total minerals in milk). Therefore the affinity may be different than the system used by Hegenauer et al., (1979b) who found is not inhibited by calcium in terms of binding in casein micelles. When the peptide was dispersed in the milk solution the calcium bound peptide may have dissociated some calcium into solution and also interacted with the milk binding sites therefore reducing the competition. As the milk was dissolved in simulated milk ultrafiltrate with no calcium present it is likely that the equilibrium calcium state would result in a depleted calcium micelle to which the peptide calcium would move into, reaching equilibrium across all proteins and peptides. This would then allow the added iron to move to the preferential binding sites on the peptide with the reduced competition within the 3 minute binding period causing a significant difference compared to the peptide and iron mixture reaction.

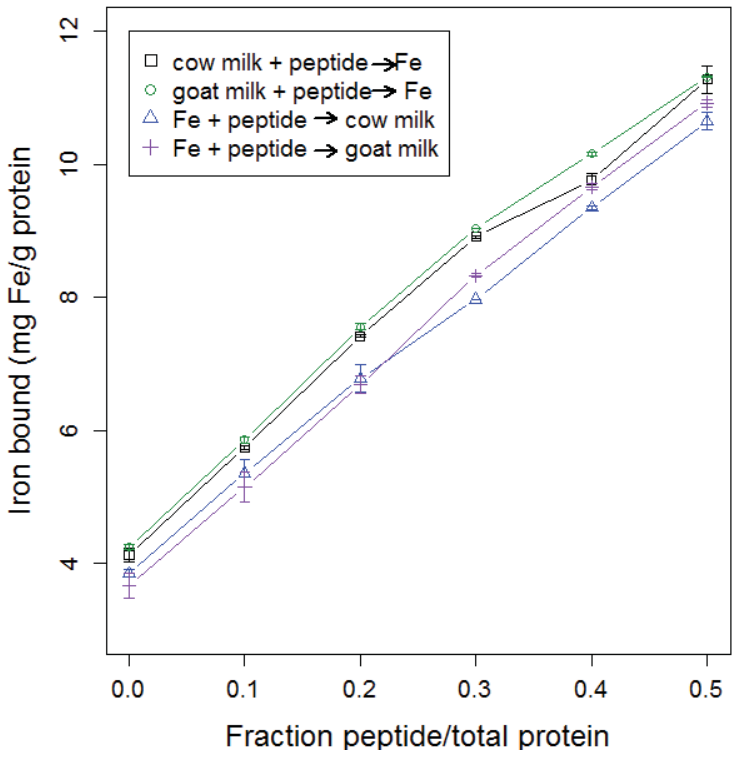


Figure 7-16: Binding capacity of cow and goat peptide and milk mixtures with different fractions of peptides and different ordering of addition at a fixed iron loading of 28 µM in the presence (dialyzed) of calcium; error bars indicate standard error, n=3.

7.2.8.2 Iron binding of cow and goat phosphopeptides in the absence of calcium (ion exchanged) in a milk system

Milk and peptide solutions were prepared in the same way as the previous trial. The species, fraction of peptide and the order in which the iron was added to the peptide and milk were statistically significant factors on the iron binding ($p<0.0001$) (Figure 7-17). The iron binding of milks containing no peptides was 3.73±0.01 mg Fe/ g protein (cow milk) and 3.83±0.01 mg Fe/ g protein ($p=0.06214$) (goat milk), slightly lower than in the previous section (7.2.8.1), indicating some variability, however non-significant statistically.

At all levels of peptide addition there was an improvement in the amount of iron bound when the iron was added to the peptide solution first compared to addition directly to a peptide + milk mixture. This supports the hypothesis that iron binding can be improved by iron addition to a peptide stream prior to addition to milk. The goat peptide and iron mix that were added to the milk produced the greatest binding out of the treatments while the cow peptide and milk mix where the iron was added produced

the lowest binding as the ratio was increased. The binding between these two samples was significantly different from 0.1 to 0.5 ($p<0.019$). Iron added to the pre-mixed milk and peptide solution at 0.5 peptide addition was 11.11 ± 0.04 mg Fe/ g protein which was slightly less than when the peptide and iron were mixed first (11.27 ± 0.02 mg Fe/g protein) for goat proteins ($p=0.076$). At 0.5 peptide addition for cow peptides, the pre-mixed milk and peptide solution produced iron binding of 10.75 ± 0.01 mg Fe/ g protein while mixing the peptide and iron first, gave binding of 11.23 ± 0.03 mg Fe/ g protein ($p=0.03$). The percentage improvement in iron binding in the final milk solution varied with peptide concentration. With both cow and goat, the greatest difference in binding occurred when the peptide addition was low and the difference decreased as the peptide concentration approached 0.5.

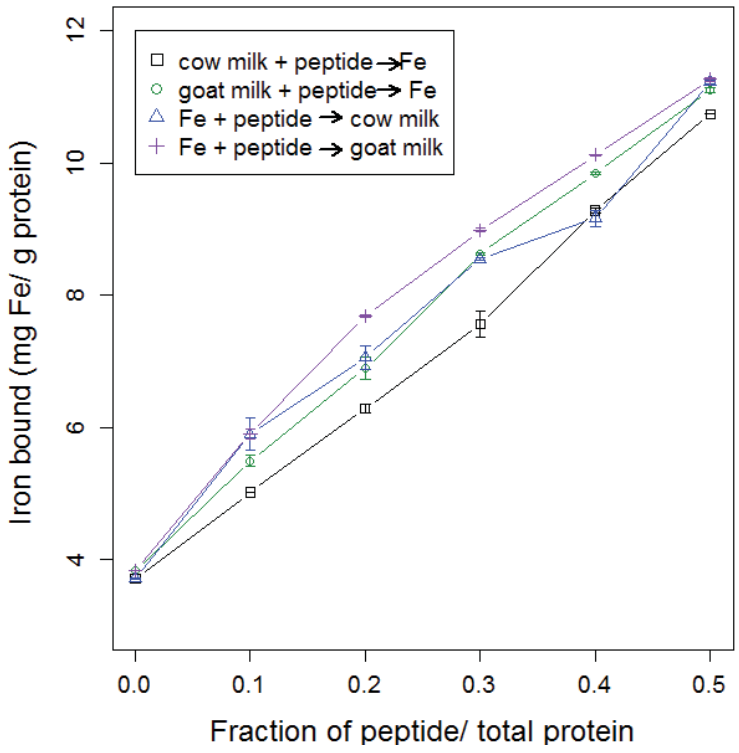


Figure 7-17: Binding capacity of cow and goat peptide and milk mixtures with different fractions of peptides and different ordering of addition at a fixed iron loading of 28 µM in the absence (ion exchanged) of calcium; error bars indicate standard error, n=3.

7.2.8.2.1 Comparison of iron binding in casein phosphopeptides in the presence (dialyzed) and absence (ion exchanged) of calcium in a milk system

When there was a smaller fraction of peptide present making up the total protein content in solution, for both calcium treatments, there was a greater difference in binding between the addition orders compared to high peptide levels. This can be explained by the change in the likelihood of peptide- iron interaction. At low peptide concentration when the iron is added first there is a higher change for interaction to occur compared to when the milk and peptide are mixed first, due to the dilution effect. However when the peptide concentration increases the probability for interaction increases and therefore the order of addition is not as important. This would be an important consideration when a known amount of peptide is required in a formulation as to which method of addition is best.

In the presence of calcium when the milk and peptides were mixed and the iron subsequently added, the binding tended to be greater than when calcium was removed. This was significant (at 95 % confidence) for cow at the peptide addition levels of 0.1, 0.2, 0.3 and 0.4 peptide/ total protein. In the goat samples there was a significant difference at 0.3 and 0.4 peptide/ total protein. As stated previously in section 7.2.8.1, this is likely due to the dilution effect of the milk solution allowing reduced competition of calcium.

Changing the order of the mixture additions did have an effect on the iron binding capacity. When the peptide and iron was mixed first, in the absence of calcium, the binding of iron was significantly greater for cow at 0.3 and 0.5 additions and at 0.2, 0.3 and 0.4 additions for goat compared to when calcium was present. This is likely due to the high concentration of calcium not being diluted, unlike in the previous treatment. This could be an important finding as it shows that there may be some influence of calcium on iron binding under these idealised conditions, and therefore may have an impact on food systems. In a system with no calcium the results indicate that it may be advantageous to mix the iron and peptide prior to addition to a bulk formulation to ensure there is binding between these two ingredients to limit interactions with other components. However it appears that when there is calcium already bound to the peptide, dilution into another system to re-distribute the calcium may allow better binding. Considering other factors found in the previous trials, it

would seem that removing the calcium first and allowing concentrated interaction and also limiting potential interactions with other components within the food system could be advantageous. When the peptide is introduced into a complex nutritional system there are more factors that can influence the binding of the iron. Milk contains free phosphates, citrates, casein and whey proteins that all have the potential to bind iron and therefore there are many other factors that have to be considered other than calcium which would therefore influence the ability of the peptides to bind iron. The addition order may still be important in terms of preferential binding so that the iron is not exposed to other compounds that can bind iron, such as fat and salts (oxidation test).

While increasing the peptide concentration improved iron binding a significant amount of peptide has to be added into the solution before the iron binding is significantly more than normal milk. The food would have to contain about 10 % of the protein to bind 5mg iron/g protein. Statistically there appears to be a difference in the order of addition however in a realistic situation there would be little difference under these conditions. If a 20 % mass fraction of phosphopeptide ingredient was added then a 10 % increase in binding may improve the stability of the product however it is likely that this level of addition is commercially uneconomical.

7.2.8.3 Synergistic effect of milk and peptide

An observation was made that if the iron binding of the milk and peptide mixture was extrapolated to containing 100 % peptide, the iron binding would be greater than in previous trials (at 20 ^{o}C, with the same concentration of iron added and three minutes of binding allowed). Therefore, a theoretical iron binding trend was constructed for both the milk and peptide alone. In the current trial, the iron binding of 100 % milk was 4.13 mg Fe/ g protein for cow and 4.24 mg Fe/ g protein for goat milk and the binding would reduce to no binding without any protein present. Similarly, for the peptides, the average iron binding at 100 % peptide based on the previous trials was 11.7 mg Fe/ g protein for cow peptide and 11.8 mg Fe/g protein for goat peptide, which would result in no iron binding when no peptide was present. These were added in Figure 7-18 and 7-19 which show the expected iron binding if the binding of the peptide and milk proteins was purely additive (plotted as blue dashed line). At different ratios of peptide and milk the theoretical iron binding is the expected amount of iron that could bind if no interactions occurred.

The milk model results (peptides in the absence of calcium) were added to this plot and it was found that the amount of iron bound was greater than the theoretical iron binding. This provides strong evidence that there is a synergistic interaction between the phosphopeptides and the milk. In section 4.2.3 when iron was added to milk micelles there was an increase in the pelleted phosphorus concentration. Raouche et al., (2009a) found similar trends when adding iron to milk. It was concluded that there was an interaction between the iron, phosphorus and CCP to stabilise the added iron. The peptides are rich in phosphorus and therefore may act as phosphate ions in stabilising the iron around the CCP in milk. This explains how the binding increases with increasing peptide concentration as there is an increasing 'phosphorus' content to interact with the milk CCP (milk at increasing concentrations in buffer was also tested and there was only a small increase in iron binding and therefore cannot be attributed to milk phosphate alone). It would be expected that when the percentage of peptide increase to 100 % the curves would return to the theoretical linear regression point, as this value was obtained from real data; however it is not known how the milk and peptides behave in this untested area. Due to technological considerations the analysis was not performed beyond 50 % as this is an expensive ingredient and would likely be added at far lower concentrations that the maximum ratio tested. The reduction of the slope in iron binding with more peptide may be due a lower concentration of CCP that could aid in stabilising the iron and peptides. Figure 7-20 is a diagrammatic representation of how the milk, peptides and iron could interact to allow more iron binding. As it has been shown that iron binds to the phosphoserine clusters where the CCP is present, it is likely that the iron will go to the binding sites, close to the CCP (for clarity, the milk peptides are drawn further away from the CCP) and to stabilise the extra positive charge the phosphopeptides will precipitate on the iron- protein interaction. This is favourable for the use of an iron-peptide ingredient in a milk model as the peptides become more functional in the presence of unaltered milk. It is unlikely that the peptide and iron mixture would be an isolated ingredient and interactions would occur with other components in food and therefore using it in a milk system would be beneficial.

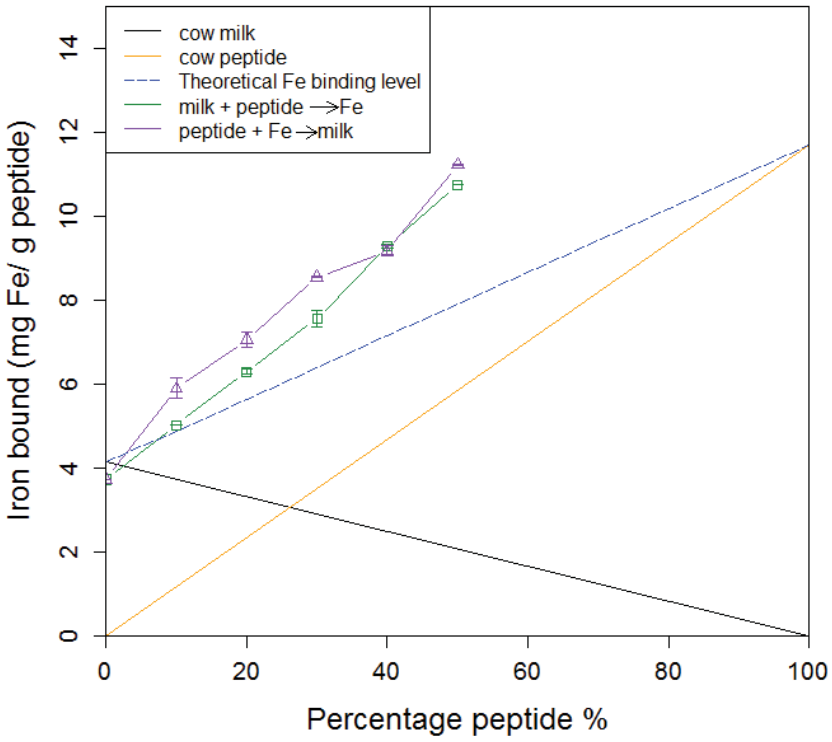


Figure 7-18: Comparison of milk model iron binding data and theoretical additive plot (blue dashed line) of milk (black line) and peptides (light purple line) of cow milk derived proteins.

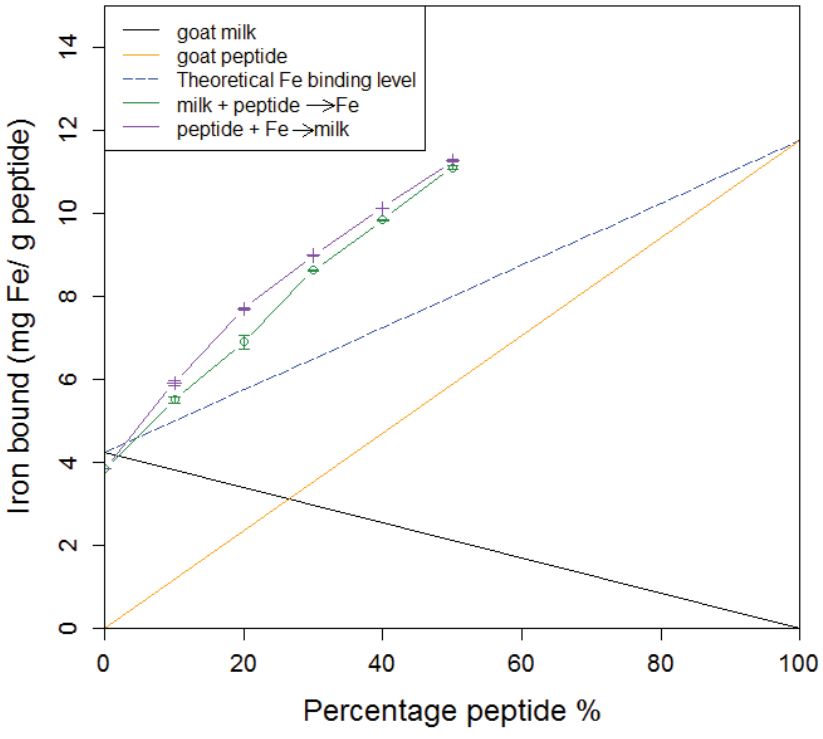


Figure 7-19: Comparison of milk model iron binding data and theoretical additive plot (blue dashed line) of milk (black line) and peptides (light purple line) of goat milk derived proteins.

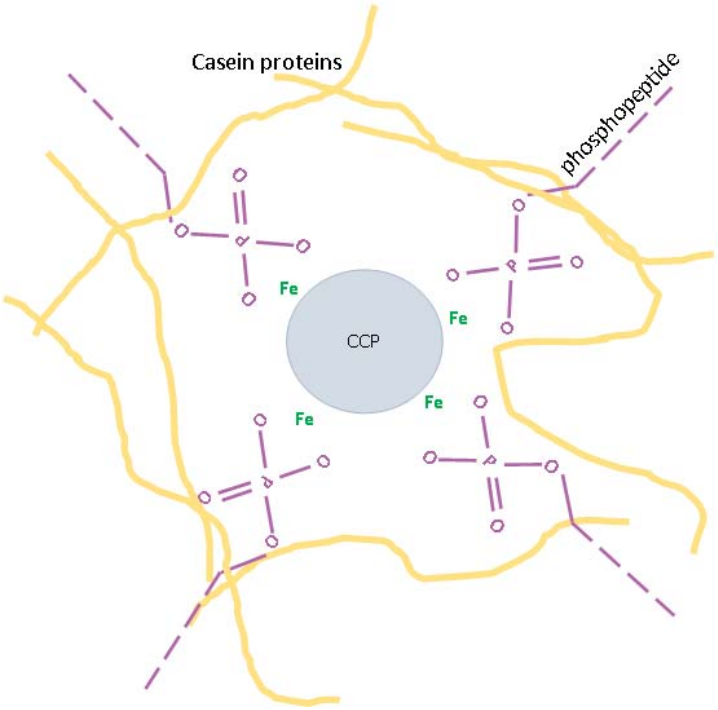


Figure 7-20: Schematic of how the milk containing CCP, iron and phosphoserine groups interact to increase the iron binding of the mixture.

7.3 Conclusion

A fundamental understanding of how an iron- phosphopeptide compound would behave in an industrial setting with various processing conditions placed on the ingredient is important in determining where in the line it could be added to the bulk mix. The study has shown that the iron- phosphopeptide complex has potential for binding considerable amounts of iron however can be sensitive to temperature, ionic strength and holding times. It was also found that removing calcium completely from the system has the ability to improve the amount of iron that can be bound to the peptides. The optimal maximum binding was around 37 ^{o}C, with the highest iron loading tested at 125 mg Fe/g protein where 56 mg Fe/ g protein was bound for goat and 58 mg Fe/ g protein for cow peptides. It has also been shown that at elevated temperatures there may be detrimental effects for the rate of iron oxidation therefore a lower temperature may reduce potential oxidation of unbound iron. At temperatures below 37 ^{o}C the iron and peptide compound can be held for up to 25 minutes without any significant potential detrimental effects. The order of iron and peptide addition to a milk system had no significance in terms of industrial relevance however even the

addition of 0.1 peptide in a milk system improved the iron binding by 27- 33 %. There is evidence that there is a synergistic effect between the peptides and the intact milk proteins where the phosphopeptides may act as a phosphorus source to aid in iron binding in the CCP region.

8 Stability and absorption of iron- phosphopeptide complexes

8.1 Introduction

For a phosphopeptide ingredient to be an effective vehicle for iron in food systems, in addition to functionality in terms of iron binding in different environments, must protect and deliver the iron to the intestinal wall for absorption. The iron must be prevented from oxidising in solution, as ferric ions can form insoluble ferric polymers preventing absorption (Jacobs & Miles, 1969). Additionally iron must be partitioned from fat components in a food system as iron can catalyse fat oxidation (Zhu et al., 2006; Schaich, 2016). This could be very detrimental in nutritional formulations such as infant formula which contain higher levels of oxidation prone polyunsaturated fats (Blanchard et al., 2013). Divalent metal ions act as pro-oxidants and therefore it is beneficial to chelate these in a way that prevents interaction with fats (Díaz et al., 2003). Caseins are able to bind metals strongly via the phosphoserine residues and have been shown to have an antioxidant effect both as intact proteins and peptides including phosphopeptides (Diaz et al., 2003; Mora-Gutierrez et al., 2010; Sugiarto et al., 2010; De Gobba et al., 2014). Ahmed et al., (2015) showed that smaller sized peptides from cow and goat milk allowed higher scavenging activity than large peptides or intact caseins and Kim et al., (2007) showed phosphopeptides can scavenge free peroxyl radicals. Díaz & Decker (2004) looked at the effect of phosphopeptides on ground beef oxidation and low levels of these peptides reduced thiobarbituric acid reactive substances (TBARS) production by 82 %. The methodology used in this study followed Hegenauer et al., (1979a) whereby thiobarbituric acid was used to measure the malondialdehyde (MDA) content, a secondary oxidation product. Oxidation of the emulsion was measured over a period of 72 hours at 30 ^{o}C.

Caco-2 cells are derived from cancer cells that mimic the cells of the intestine (Glahn et al., 1998). They allow approximations for the absorption of iron by measuring the production of ferritin proteins which store the iron. It is not intended to fully mimic a human model but rather to compare between the samples and give some indication to bioavailability. Yeung, Glahn & Miller (2001); García-Nebot et al., (2010) and García-Nebot et al., (2013) investigated casein phosphopeptide-iron absorption in

Caco- 2 cells and found mixed results with the isolates. The iron solubility in the peptides was greater than intact casein indicating that the smaller size allowed the complex to pass through the membrane (simulating the intestinal mucous membrane) however whey proteins produced a higher ferritin response. Only isolates from α_s-casein and β-casein digests improved iron absorption, rather than a pooled peptide mix (García-Nebot et al., 2013). However in another study by Kibangou, (2005) only β-derived caseins were reported to improve absorption in Caco- 2 cells and α_s- casein had an inhibitory influence, which was thought to be due to the strong binding capacity of the peptides to iron which prevented release at the absorption site. Compared to egg phosvitin, which is highly phosphorylated and inhibits iron absorption, the binding strength of the caseins to the iron is not so strong that the iron is not released or absorbed with the casein peptide (Ishikawa et al., 2007).

In this study various treatments of cow and goat milk were analysed for bioavailability using the Caco- 2 cells. Cow and goat milk was fortified with 5 and 10 mM of iron, with or without the addition of ascorbic acid. The addition of ascorbic acid was investigated as Davidsson et al., (1998) showed that this improved the absorption of iron. Ascorbic acid can act as a weak ligand and chelate the iron allowing the iron to remain soluble and keep it stable against oxidation and the formation of iron polymers (Jacobs & Miles, 1969; Zhu et al., 2006). Furthermore caseinates from cow and goat milk were analysed with 5 mM of iron as well as whole digests of cow and goat caseinate with the same iron content. Finally, calcium and iron precipitated cow and goat phosphopeptides were investigated to determine whether the different peptide compositions and the presence of ferric iron (from the iron precipitated peptides) had an effect on the iron absorption.

8.2 Results and Discussion

8.2.1 Oxidative stability of cow and goat skim milk and cow and goat phosphopeptides in a linoleic acid emulsion

Skim milk from cow and goat milk and phosphopeptides from the respective caseinates were analysed for their ability to protect lipids from iron which is a pro-oxidant (Yen et al., 1999). Linoleic acid was used as the lipid model as it is a polyunsaturated fat and in food such as infant formula it is critical that these remain un-oxidised. Oxidation is a major problem in food products with higher levels of polyunsaturated fatty acids (Waraho, McClements & Decker, 2011). Cow and goat skim milk and peptides derived from these were measured on an equal protein basis of 1 % (w/w) with the addition of 1 mM ferrous sulfate and compared with a blank containing no iron and a positive control containing the same amount of iron but with no protein (refer to section 3.7.8).

The effect of time on the response of the malondialdehyde (MDA) was dependent on the sample type, whether it was a control, milk or peptide ($p<0.0001$). Figure 8-1 shows the positive control was linear ($R^2 = 0.9859$) with respect to time; the 0 hour aliquot contained 0.25 ± 0.01 µg MDA/mL which increased to 3.09 ± 0.03 µg MDA/mL after 72 hours at 30 ^{o}C producing the greatest amount of MDA out of all the samples. It was evident that when iron was not bound to any protein that the oxidation of the linoleic acid occurred rapidly. Within 4 hours the MDA content was significantly greater than the other samples. The blank, containing no iron, began with an MDA content of 0.18 ± 0.03 µg MDA/mL which reached 1.25 ± 0.16 µg MDA/mL ($R^2 = 0.9698$). This indicates that there may have been some oxidation of the linoleic acid initially as the study by Sugiarto et al., (2010) had an absorbance reading of close to zero at the beginning of the trial. This may have caused an acceleration of auto-oxidation of the lipid throughout the experiment. Another potential source of oxidation may have occurred during the homogenisation step with the emulsifier as air was entrained into the solution and the increase of oxygen into the solution may have initiated more oxidation (Coupland & McClements, 1997). As all samples were prepared similarly, with the length of homogenisation timed, it would be expected that there would be little variation, which was confirmed with the low standard error in the results. Attempts were made to eliminate some potential oxidation by purchasing

freshly manufactured bio-reagent grade linoleic acid, however it appears that the fatty acid is highly unstable even when bought and used immediately.

The goat milk produced the lowest MDA content overall, including the blank, indicating that there is antioxidant capacity. The goat milk is able to scavenge the natural oxidation products resulting from linoleic acid oxidation. The initial MDA content was 0.25 ± 0.02 μg/ mL which increased to 0.46 ± 0.04 μg/mL after 72 hours. The goat peptide had a lower MDA response compared to the blank within the time range of 23 to 56 hours. At 48 hours the MDA content of the blank was 0.80 ± 0.08 μg MDA/ mL while at the same time the goat peptide had an MDA content of 0.74 ± 0.04 μg MDA/ mL ($p=0.4274$). By 56 hours the blank MDA content was 0.90 ± 0.04 μg MDA/ mL and goat contained 0.97 ± 0.03 μg MDA/ mL ($p=0.1967$); there was also no significant difference at 72 hours between the goat peptide and the blank ($p=0.4136$) indicting that the goat peptides prevented increased oxidation caused by iron. The cow milk and cow peptide had a higher content of MDA over the sampling time by 24 hours compared to both treatments of goat protein. The cow peptide had a lower MDA content compared to the cow milk for most of the holding time however became significantly greater by the 72 hour time period where the oxidation increased significantly to 1.98 ± 0.09 μg MDA/ mL versus 1.57 ± 0.06 μg MDA/ mL for cow milk ($p=0.0338$).

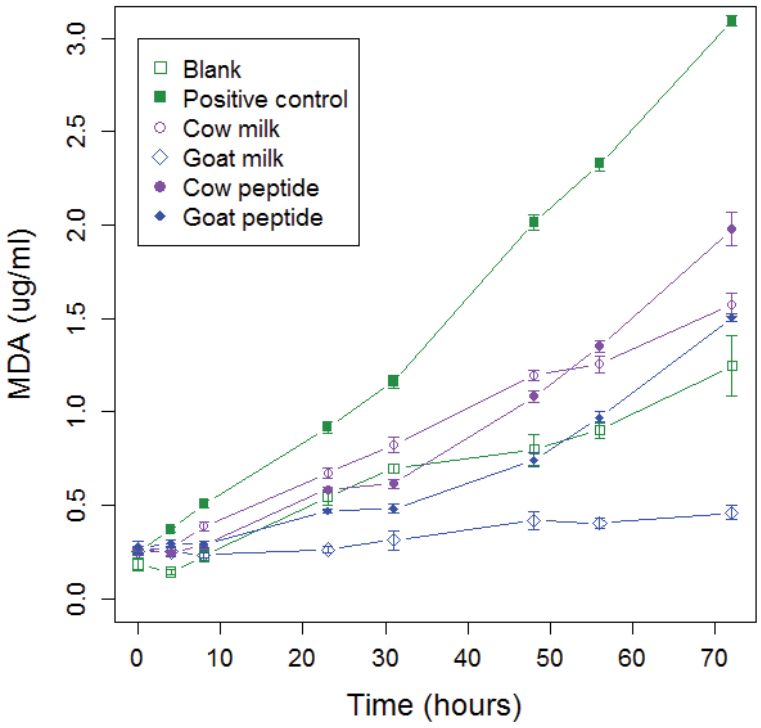


Figure 8-1: MDA content of blank, positive control, cow milk, goat milk, cow phosphopeptide and goat phosphopeptide over 72 hours at 30 ºC; error bars indicate standard error, n=3.

Mora-Gutierrez et al., (2010) compared cow casein with two samples of goat caseins: one with a low α_{s1}- casein content and one with a high α_{s1}- casein content. There was no significant difference between all samples in terms of hydroperoxide values however at 0.1 % protein low α_{s1}- casein content goat casein in 5 % oil reduced TBARS formation compared to cow casein and high α_{s1} content goat casein. The authors believed that as this protein mixture (low α_{s1}-casein) contained more α_{s2}-casein which is positively charged, and would therefore be expected to repel the positively charged iron ions and other divalent ions, keeping them away from the casein emulsified fat. They used NMR to show that the caprine caseins have a greater ability to adsorb onto and into the fat globules as there are more hydrophobic regions compared to cow caseins and therefore they reduced the interaction of iron and fat. It is unclear whether casein micelles would behave in the same way in the present trial as the proteins were not in the caseinate form however with the large difference between the cow and goat milk MDA values it would indicate that the goat caseins must structure the iron differently than cow or structure around the fat emulsion differently to protect the iron from the fat. Caseins can display antioxidant behaviour

due to their natively unfolded state allowing more interaction via the amino acid structure (Díaz et al., 2003; Mora-Gutierrez et al., 2010). Tyrosine, histidine, glutamic acid and aspartic acid have been reported to have some chelation and radical scavenging ability while methionine and proline are targets for free radical oxidation (Rival et al., 2001a). The amino acid analysis in section 6.2.8 found that goat casein contained more proline, threonine and valine compared to cow casein therefore, of the amino acids tested there is not much evidence that the amino acid composition was a factor for greater scavenging activity in goat milk. In spite of this, as the primary structure of the cow and goat caseins are different (Haenlein, 2004); there may be more accessibility to the radical scavenging amino acids in goat milk. The whey proteins may also have an effect of the antioxidant capacity as well as other serum components that are present in skim milk that vary in quantity. Goat milk contains more phosphorus, chloride, vitamin A, B1, B12 and vitamin C (Jandal, 1996) which may have the ability to bind iron to reduce potential interactions with fats. It is therefore likely that the antioxidant capacity of goat milk is greater than cow milk due to a combination of amino acid structure of the caseins and other components within the milk.

Díaz et al., (2003) showed that casein and phosphopeptides derived from cow milk behaved very similarly in terms of hexanal and hydroperoxide production over 8 days of testing when the protein content was adjusted to a 15 μM phosphorus basis. When the concentration was increased to 75 μM phosphorus the CPP performed worse than casein, producing more of the oxidation products. Casein hydrolysates produced lower amounts of hexanal and hydroperoxide than both casein and CPPs overall. This indicated that the phosphopeptide content of the protein is not the only factor that determined the antioxidant capacity of the solution. Similarly, Rival et al., (2001b) measured lipoxygenase activity of different forms and fractions of bovine casein. They measured the inhibition activity of the enzyme which consumes oxygen in the reaction as an indicator of oxidation with linoleic acid. They found that the highest inhibitory activity was from κ-casein, a whole tryptic digest of casein and whole casein while the dephosphorylation of casein did not significantly affect the activity compared to the other samples. However, the digestion of β-casein by trypsin improved the inhibition of activity with a reduction of 27 % compared to 17 % for the undigested β-casein. This may help to explain why there were differences seen between cow and goat milk

and peptides as the goat species milk contains more β-casein. The authors speculated that when the β-casein was digested the bioactive sequences of the protein were exposed while in the intact protein they were not as accessible. The only amino acid that was present at a higher level in the goat phosphopeptide compared to cow was histidine at 5.82 mg/ 100 mg compared to cow peptide of 0.67 mg/ 100 mg, in addition to a higher serine content of 22.49 mg/100 mg compared to cow peptide of 18.08 mg/100 mg. This was the only amino acid that had scavenging activity according to Díaz et al., (2003) and Mora-Gutierrez et al., (2010) in contrast to what Rival et al., (2001a) reported.

The whole digests and intact caseins may have a stronger inhibitory effect than CPPs due to the size and composition of the proteins or peptides. Intact caseins, compared to peptides, are long flexible strands with regions of hydrophobicity and hydrophilicity; refer to Figure 8-2 for a schematic of the proposed mechanism. The hydrophilic regions, with negative charges, are the regions that bind iron while the hydrophobic sections do not, but interact with fats. It is likely that the proteins are able to partition the iron away from the fat so that the iron cannot get in close enough proximity to act as a pro-oxidant. Similarly with the whole casein digests, there will be sections of proteins that were broken down into predominantly hydrophobic peptides. These will have a high affinity to the fat and may coat the globules so that the iron bound, hydrophilic peptides cannot interact. In purified CCPs these protective 'mechanisms' are no longer present as all peptides in the isolate have regions that bind iron, so while the iron is bound there is a higher chance that the fat and iron could interact.

Díaz et al., (2003) analysed casein phosphopeptides with calcium concentrations up to 100 mM in the presence of different concentrations of phosphopeptides and found that calcium did not have a significant effect on the rate of oxidation. Therefore while peptides contained about 7 % calcium, which is slightly greater than in the milks the different concentration of calcium, is unlikely to be a factor for increased oxidation.

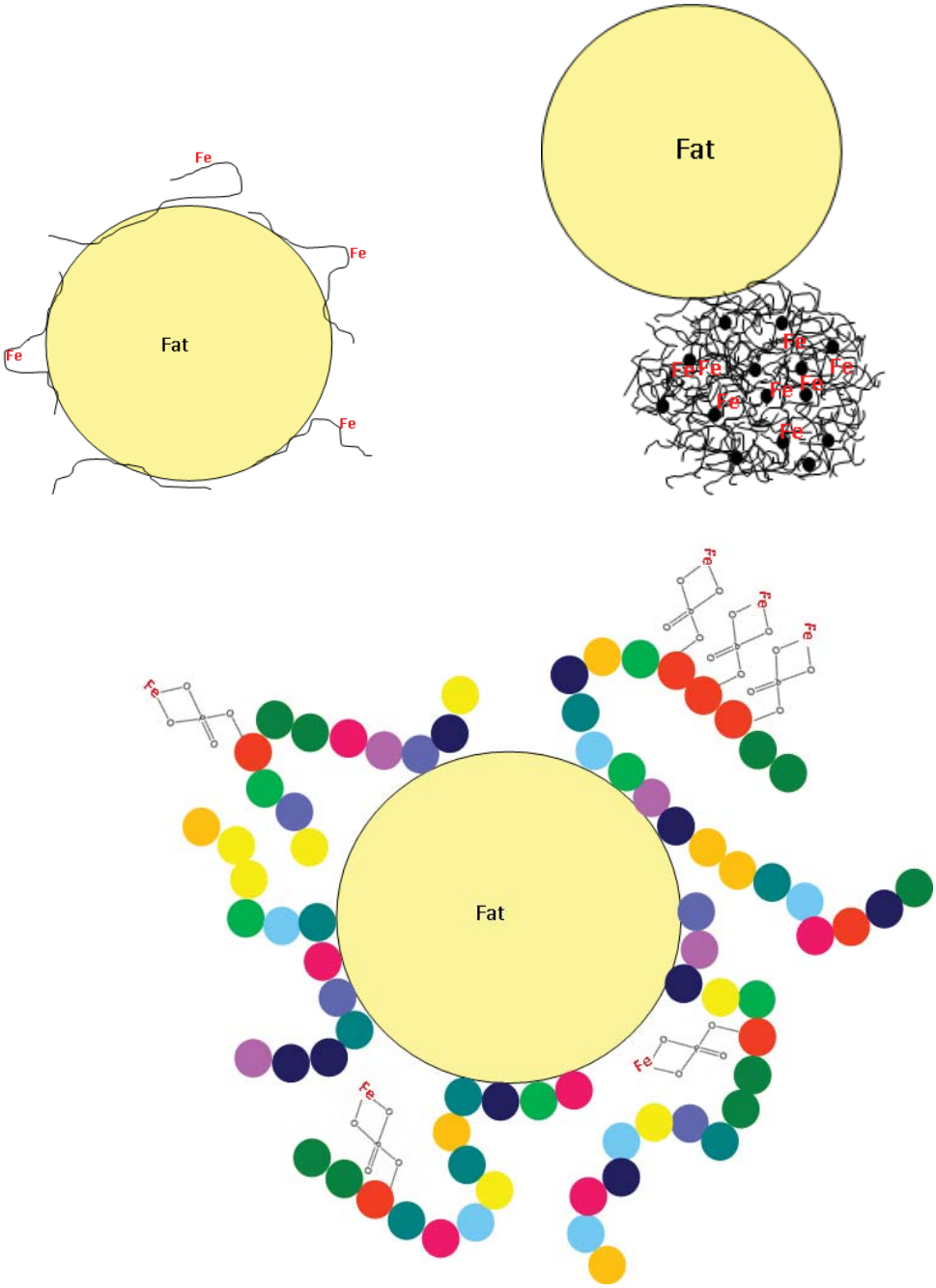


Figure 8-2: Schematic of intact casein proteins (top left) and micelles (top right) surrounding a fat globule with hydrophobic sections interacting while hydrophilic sections partition iron-bound sections away from the fat. Conversely, short hydrophilic peptides (bottom, amino acids represented by coloured circles) that contain a high concentration of iron allow a close proximity of iron to the fat increasing the chance of interaction over time.

8.2.2 Caco-2 cell line analysis of bioavailability and absorption of iron from cow and goat milk and their products

8.2.2.1 Cow and goat skim milk and the effect of the addition of ascorbic acid on ferritin response

Samples were prepared from wet blended cow and goat skim milk powder with the 5 mM and 10 mM fortification levels used (refer to section 3.7.9). Figures 8-3 and 8-4 show that without ascorbic acid, a ferritin response of 143.85 ± 12.42 ng ferritin/ mg cell protein for cow milk and 82.24 ± 5.15 ng ferritin/ mg cell protein for goat milk was obtained at 5 mM iron ($p=0.0006$). The response was significantly higher than the negative control of ferrous sulfate with no ascorbic acid of 11.96 ± 1.63 ng ferritin/ mg cell protein. Ascorbic acid had a significant effect at 5 mM on the iron uptake by the cells, producing a large ferritin response. The ascorbic acid was added at a ratio of 10:1 ascorbic acid: ferrous sulfate. The cow milk with ascorbic acid produced 526.89 ± 28.71 ng ferritin/ mg cell protein while the goat milk produced 693.89 ± 69.24 ng ferritin/ mg cell protein ($p<0.001$). Ascorbic acid has a strong positive effect on the uptake of iron. This indicates that the milk proteins do improve iron solubility for iron uptake however significantly less than with ascorbic acid added.

Authors have reported the effect of ascorbic acid on improving iron absorption or uptake of iron however with mixed results (Gillooly et al., 1984; Hunt, Gallagher & Johnson, 1994; Davidsson, et al., 1998; Hurrell & Egli, 2010). The mechanism for the improved absorption is attributed to the reducing power and chelating ability of ascorbic acid with iron (Teucher, Olivares & Cori, 2004). Ascorbic acid is able to reduce the ferric form of iron to ferrous which is able to be taken up by the divalent metal transporter (DMT-1), a membrane protein from the intestinal lumen to the enterocytes. Ascorbic acid can also act as a weak ligand and chelate the iron allowing the iron to remain soluble and keep it stable against oxidation and the formation of iron polymers (Zhu et al., 2006). It is known that when iron binds to casein proteins the ferrous iron is oxidised to the ferric form (Hegenauer et al., 1979) and therefore the iron must be reduced by the duodenal cytochrome b (Dcytb) enzyme prior to uptake by the divalent metal transporter 1 (DMT-1) protein. The introduction of ascorbic acid may therefore change the pathway in which some of the iron is transported. It seems likely that the ascorbic acid may bind any free iron not bound to the proteins preventing oxidation and allowing transport across the membrane. The

disadvantage of using ascorbic acid is that it is not stable under standard thermal processing conditions and would need to be dry blended in food (Teucher, Olivares & Cori, 2004).

When the iron concentration was increased to 10 mM there was no significant increase in iron uptake from the cow and goat milk without ascorbic acid compared to the lower fortification level for cow milk: 135.1 ± 16.63 ng ferritin/ mg cell protein (p=0.8095), and goat milk: 95.93 ± 8.57 ng ferritin/ mg cell protein (p=0.706). There was some evidence for a significant difference between species at 10 mM addition (p=0.0583). The addition of ascorbic acid did not improve the iron uptake, unlike in the 5 mM fortification level; there was no significant difference in the cow milk with ascorbic acid (155.44 ± 13.88 ng ferritin/ mg cell protein, p=0.5754) or goat milk (110.25 ± 13.77 ng ferritin/ mg cell protein, p=0.6934) with the same iron fortification level.

This data shows that the iron fortification level of milk is very important in the response of iron uptake and that more does not necessarily mean more iron will be absorbed. The addition of an uptake promoter of ascorbic acid does not improve the effect. It may be that the increased concentration allows for more interactions between iron ions and therefore there is more chance for oxidation. Glahn et al., (1998) added iron to infant formula at 10 µmol/L and 200 µmol/L to mimic the level of iron in cow and human milk, respectively. When the iron loading was increased the availability of the iron in the digest was 0.100 ± 0.021 % for 10 µmol/L and 0.056 ± 0.012 % at the higher loading while the availability of the iron in the bottom chamber after membrane filtration was 0.66 ± 0.12 % with 10 µmol/L and 0.47 ± 0.08 % with 200 µmol/L. The cell uptake of iron did increase with increasing iron loading; with 10 µmol/L the uptake was 10.0 ± 2.1 pmol/ 2hr and with 200 µmol/L the uptake was 112 ± 24 pmol/2 hr. In the present study, the 10 mM iron fortification level would be expected to increase however it may be different due to the high iron content. The level of iron fortification is significantly higher than fortification levels in milk, as shown by Glahn and worker's (1998) fortification levels. The increased iron load may have exceeded a saturation point after dilution causing poorer performance.

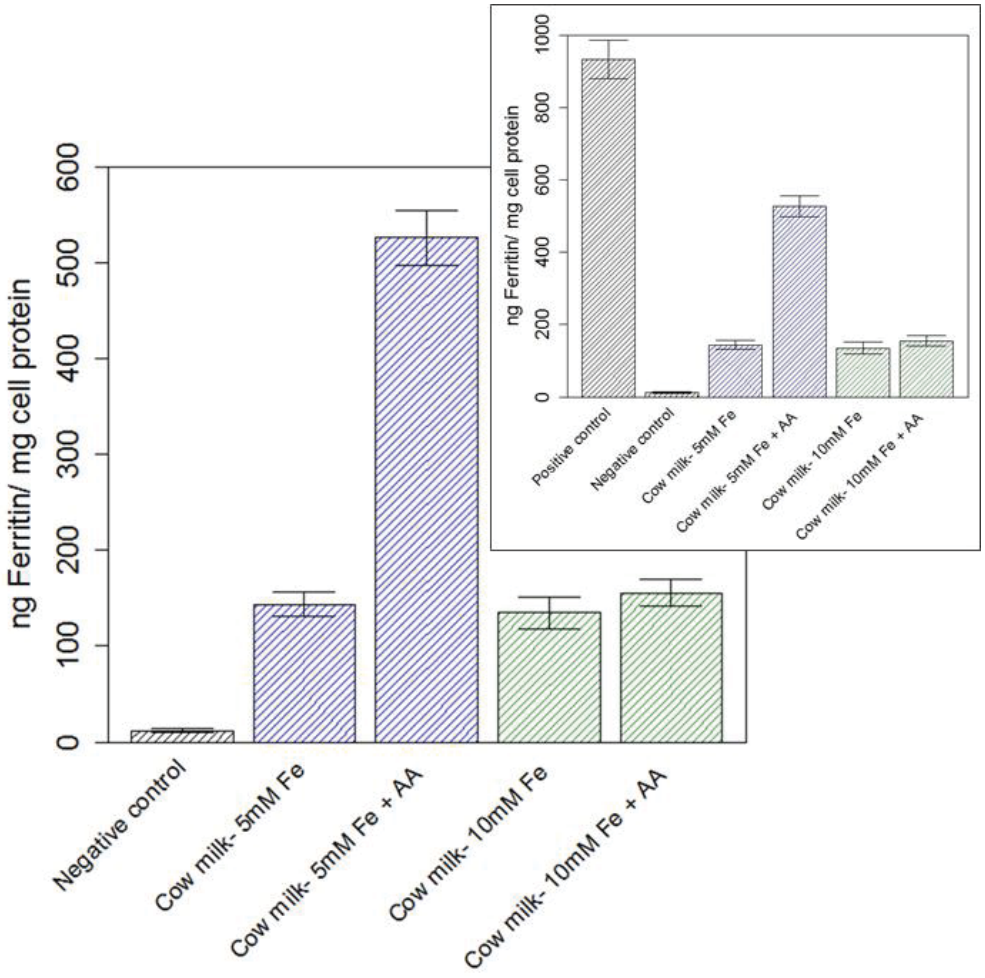


Figure 8-3: Ferritin response of cow milk treatments; insert shows the positive control for reference; positive control is iron with ascorbic acid; error bars indicate standard error, n=7.

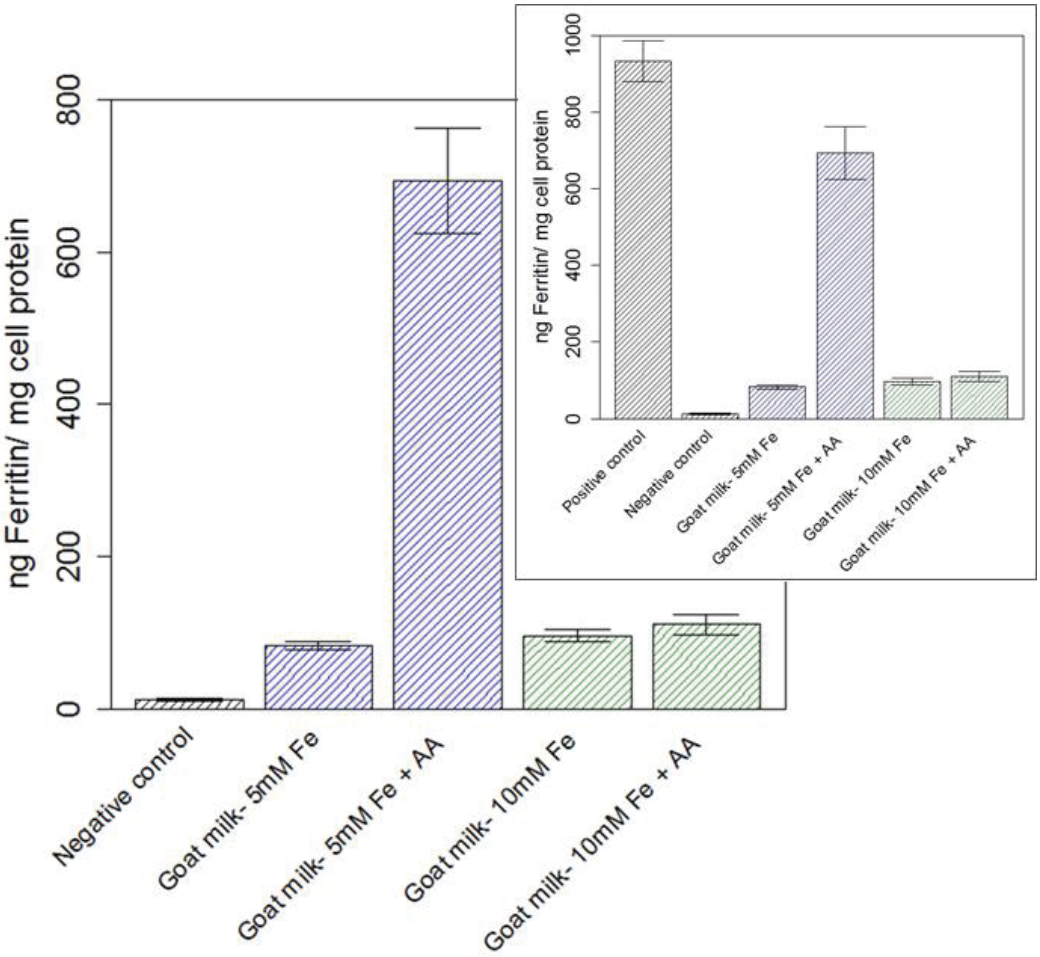


Figure 8-4: Ferritin response of goat milk treatments; insert shows the positive control for reference; error bars indicate standard error, n=7.

8.2.2.2 Cow and goat caseinate fortified with ferrous sulfate on ferritin response

Sodium caseinate precipitated from cow and goat milk was fortified with 5 mM ferrous sulfate for Caco-2 cell bioavailability analysis. The cow caseinate had a significantly lower response of 71.03 ± 5.18 ng ferritin/ mg cell protein compared to cow milk ($p=0.047$). With goat caseinate resulted in a higher mean ferritin production compared to goat milk (101.63 ± 12.01 ng ferritin/ mg cell protein compared to 82.24 ± 5.15 ng ferritin/ mg cell protein), although the difference was not statistically significant ($p=0.5935$) (refer to Figure 8-5). Additionally, there was no significant difference between the species origin of the caseinates ($p=0.400$). The addition of ascorbic acid was not tested.

It would be expected that the dissociation of the casein micelle into the caseinate monomers may allow an increased absorption due to a decrease in the overall protein size, compared to a whole casein micelle (Bouhallab et al., 2002). This did not occur as the cow caseinate performed worse while the goat caseinate was not different from the intact milk. A possible explanation may be that a digestion phase occurs to simulate the gastric and intestinal phase in the method prior to addition to the Caco-2 cells. This may have resulted in the peptides producing similar molecular weight products in both caseinate and milk as no pre-digestion had occurred. It may be that the individual caseinate protein size is still not small enough and reduces dialysability (Hurrell et al., 1989). The cow caseinate decreased the iron absorption which may have been due to the reduction in stability of the caseinates compared to milk as shown in section 5.2.2, as evidenced by the precipitation of the solution in the presence of iron. It is also likely that as the minerals and other proteins have been removed there is a stronger binding of the iron compared to the goat and therefore the iron is not released to the cells. This difference may be attributable to the composition of the caseinates as goat has more β- casein which has a lower binding strength to α- and therefore may be able to release iron more effectively at the DMT1 site.

8.2.2.3 Cow and goat caseinate hydrolysate fortified with ferrous sulfate on ferritin response

Cow and goat caseinate was digested using trypsin and the whole digest was fortified with 5 mM ferrous sulfate. The ferritin response for cow was 115.53 ± 20.63 ng ferritin/ mg cell protein and the response for goat was 143.94 ± 19.06 ng ferritin/ mg cell protein (p=0.3318) (refer to Figure 8-5). Both treatments improved the mean response compared to the intact caseinates with some evidence of significance (p= 0.076 for cow species, p=0.0848 for goat species). Compared to their respective intact milks there was no significant difference for cow (p= 0.2625) but the response was significantly greater for goat caseinate hydrolysate (p=0.0171).

The results of the goat hydrolysate followed the expected trend where the iron uptake improved as the protein size decreased. Hurrell et al., (1989) found that the dialysability of iron increased with increased hydrolysis of casein and as a result reduced the inhibitory effect that intact casein had on iron absorption in humans. Ait-Oukhatar et al., (1997) digested β-casein which compared to intact β-casein improved the iron uptake in rat models. In the current study, the cow caseinate hydrolysate

increased compared to the caseinate however produced a response lower than cow milk. This may be due to the different composition of the caseinate starting product, with a higher level of α_{s1} casein preventing release to the transporter protein. In contrast, the digestion of goat casein did improve the ferritin response.

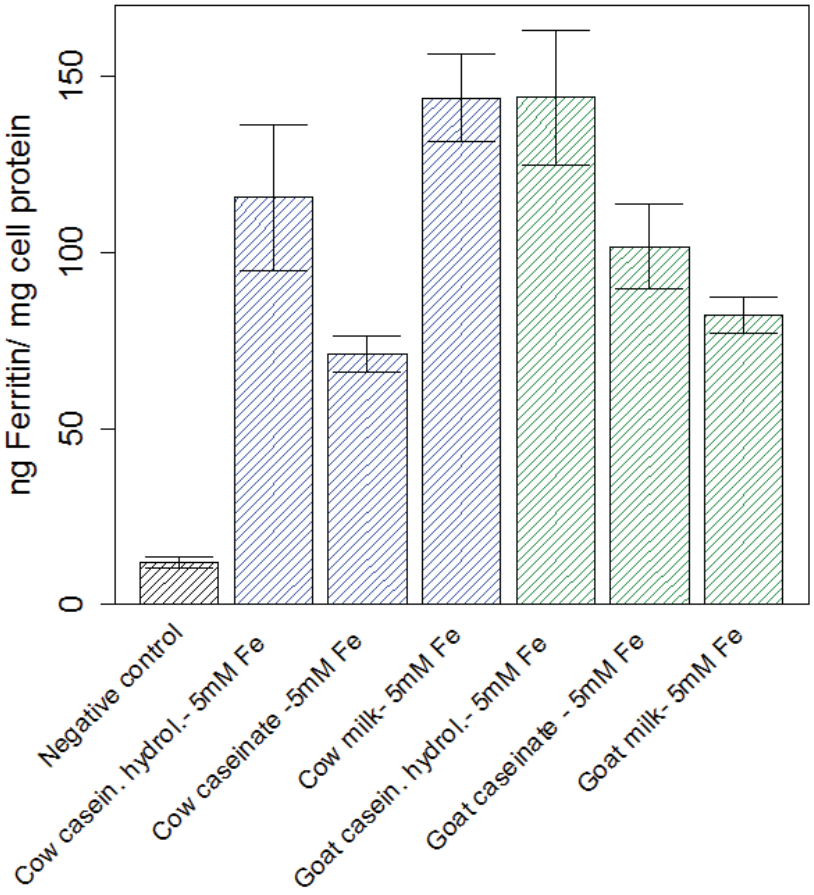


Figure 8-5: Ferritin response of cow and goat caseinate hydrolysate with 5 mM iron fortification in comparison to their respective milks. The positive control was omitted but is the same value as Figure 8-2; error bars indicate standard error, n=7.

8.2.2.4 Calcium and iron precipitated cow and goat phosphopeptides on ferritin response

There was no significant difference between the calcium and iron treatments between or within the species at a 95 % confidence level. The amount of ferritin produced for the treatments were 122.3 ± 12.7 ng/ mg cell protein for calcium precipitated cow peptides and 116.7 ± 11.3 ng/ mg cell protein for calcium precipitated goat peptides (refer to Figure 8-6 and 8-7). For the iron precipitated peptides the response was 105.0 ± 9.3 ng/mg cell protein for cow peptides and 138.1 ± 19.7 ng/ mg cell protein for goat

peptides. The responses were significantly greater than the negative control and significantly smaller than the positive control of ferrous sulfate and ascorbic acid. The use of calcium or iron to precipitate the phosphopeptides shows no difference in the amount of ferritin produced under the conditions used in Caco-2 cells ($p>0.10$).

The presence of calcium in the calcium precipitated peptides does not negatively affect the absorption of iron. However it cannot be stated with certainty that the removal of the calcium prior to the iron fortification would improve absorption as the amino acid analysis and RP-HPLC analysis showed the precipitation of peptides using calcium and iron yielded different compositions. The presence of calcium in a selectively precipitated digest of caseinate however does not decrease the production of ferritin compared to a digest selectively precipitated with iron with no calcium present. Previous studies in human models have found that calcium can negatively affect the absorption of iron. Hallberg et al., (1992) compared the iron absorption using cow and human milk and the absorption of iron when the milks were fortified with calcium. Cow milk caused lower iron absorption than human milk however they did not believe that human milk contained any 'iron enhancing' factors, rather that the differences in calcium content affected the absorption of iron. When human milk was fortified with calcium the iron absorption decreased. Cook, Dassenko & Whittaker (1991) found that calcium supplements inhibited iron absorption when taken with food and some forms decreased absorption when taken without food. Perales et al., (2006) also found that there was a negative correlation between the calcium content in milk and the iron that was dialyzable, this carried over to the amount that was able to be taken up by the Caco-2 cells. However, Ames et al., (1999) found that in children with low or high calcium diets the iron intake and iron incorporation into the red blood cells was not significantly different.

The non-significant difference between the phosphopeptides from cow or goat caseinate is likely due to the selective precipitation causing an isolation of low molecular weight peptides that have high affinity for divalent cations. They are therefore able to bind iron strongly and can pass through the 10 kDa membrane easily. The original composition of the cow or goat milk and their intrinsic differences are not a key factor in the functionality of the absorption, as any potential iron absorption inhibitors have been removed. At this level the primary amino acid composition is the main factor that would influence the transport of iron. López-Aliaga et al., (2009)

found that goat milk fortified with calcium did not affect the nutritive utilization of iron while cow milk reduced the apparent digestibility coefficient of iron. The reasons they stated for this difference was due to the variations in composition of the milks. There is a higher content of soluble whey proteins in goat milk compared to cow milk may allow them to be better iron carriers overall. The fat composition in goat milk consists mainly of medium chain fatty acids that can breakdown to become iron carriers. Additionally goat milk has higher levels of ascorbic acid and vitamin A which may have also caused the difference in iron utilisation. In terms of this study, all these factors have been removed and therefore cannot cause any potential increase in iron absorption in the Caco-2 cells. López-Aliaga et al., (2009) also noted that lysine may be able to reduce Fe^{3+} to Fe^{2+} that may improve absorption; the lysine content of the phosphopeptides was much lower in the calcium precipitated samples of 2.42 mg/100 mg and 2.05 mg/100 mg for cow and goat, respectively. In the iron precipitated samples the content was higher at 6.01 mg/100 mg and 6.31 mg/100 mg for cow and goat, respectively however these are both a decrease from the starting caseinate material of 7.78 mg/100 mg and 7.93 mg/100 mg for cow and goat, respectively. It is therefore unlikely that differences in the lysine content would be responsible for the improved absorption of the iron in the cell line.

Kibangou et al., (2005) compared two purified peptides from α_s- and β- casein using a rat model and Caco-2 cells. Both models gave similar results with the β (1-25) fragment allowing higher uptake and absorption than ferrous sulfate while α_{s1} (59-79) and α_{s2} (2-12) was less effective than ferrous gluconate which could have been due to the peptide binding the iron too tightly and not releasing iron at the intestine. There is a difference in conformation when these peptides bind calcium and therefore the binding conformation is likely to be different with iron also, therefore the peptide type is important.

As goat casein is higher in β-casein it would be expected that there would be a higher concentration of β-derived peptides and therefore a higher uptake and absorption, as shown here. It must be noted that the structure of the β-casein from goat milk is not the same to cow milk. According to SwissProt™, the phosphorylated sequence of cow β casein contains phosphorylation modifications at position 30, 32, 33, 34 and 50 (for most variants) while goat β casein contains the same modifications however there is also a phosphothreonine at position 27 that could change the electronegativity. This

could reduce the ability of the iron to be released in the same way as cow derived peptides. It is also likely that the differences in sequence particularly of the phosphoserine clusters of the α-caseins could change how the goat peptides bind and release the iron compared to cow peptides.

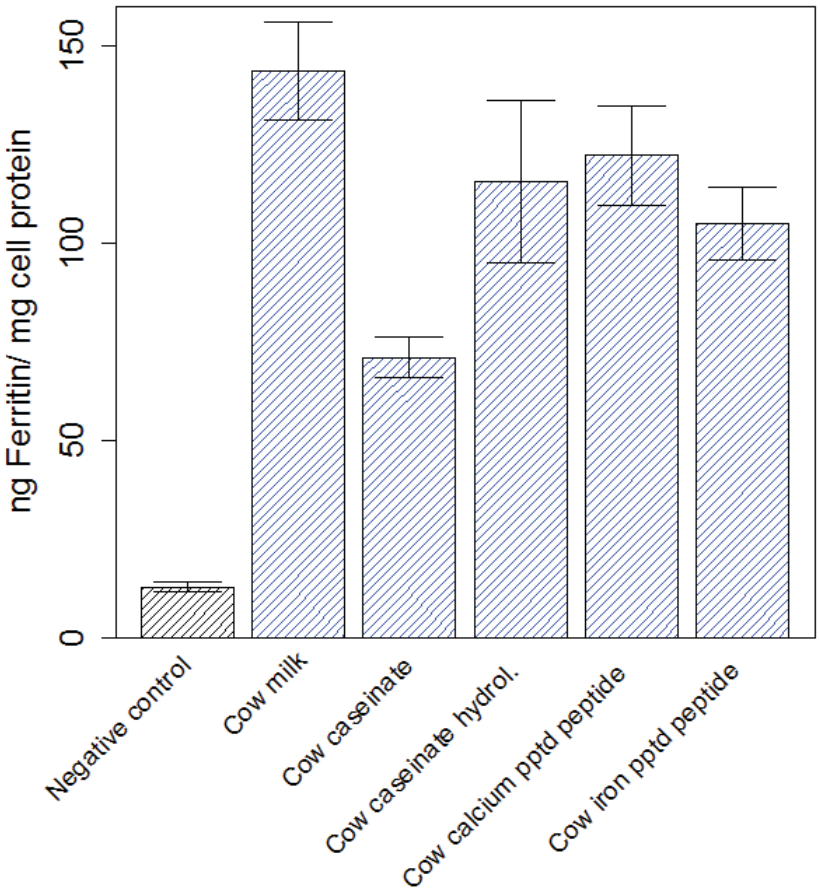


Figure 8-6: Ferritin response of cow phosphopeptides precipitated with calcium or iron with 5 mM iron fortification in comparison to their respective milks. The positive control was omitted but is the same value as Figure 8-2; error bars indicate standard error, n=7.

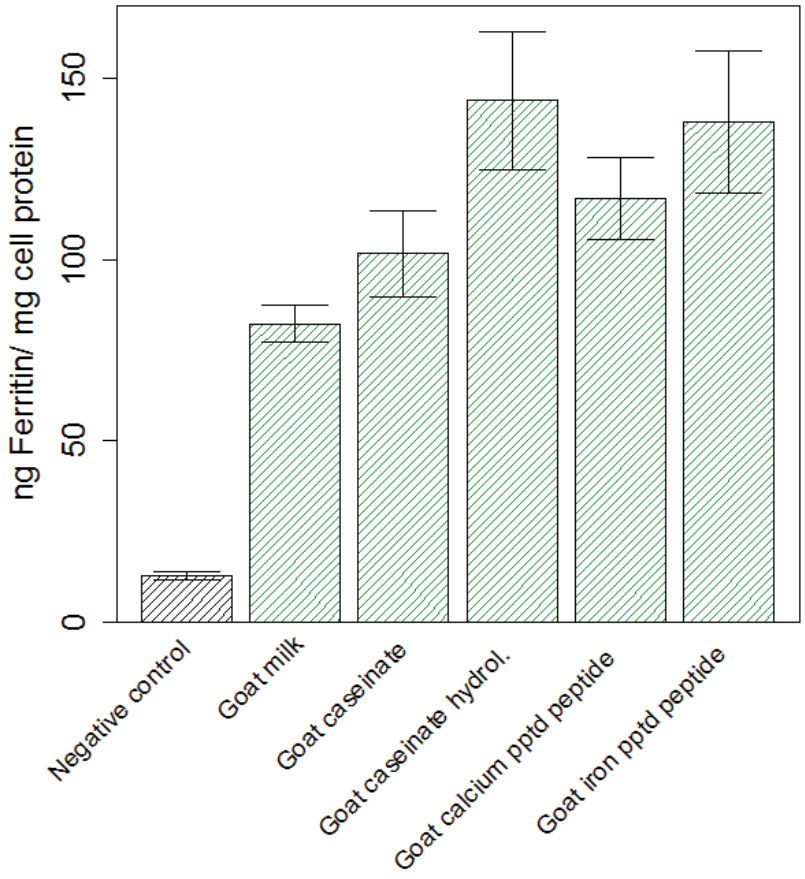


Figure 8-7: Ferritin response of goat phosphopeptides precipitated with calcium or iron with 5 mM iron fortification in comparison to their respective milks. The positive control was omitted but is the same value as Figure 8-2; error bars indicate standard error, n=7.

8.2.2.5 Comparison of cow and goat casein derived protein fractions on ferritin response on an equal iron filtrate basis

The method used to mimic the digestion system prior to applying the digest to the cell line involves a filtration step via a 10 kDa membrane. The solubility of each digest varied and different concentrations of iron were able to pass through the membrane. This section presents the data of ferritin produced on an equal filtrate iron concentration. This is particularly important for the phosphopeptides as the ratio of protein to iron is different from the other samples. This method of reporting gives an indication of how the protein delivers the iron to the cell revealing how well the iron is released by the protein source to be absorbed by the cell.

Of the cow milk derived fractions, cow milk fortified with 5 mM iron and ascorbic acid and cow caseinate hydrolysate with 5 mM iron were found to have the best response on an equal iron filtrate basis at 5.85 ± 0.32 ng ferritin/ nM iron and 5.15 ±

0.92 ng ferritin/nM iron (p=0.50) (refer to Figure 8-8). The cow milk fortified with 10 mM iron and ascorbic acid had the lowest response of 1.56 ± 0.14 ng ferritin/nM iron. This indicates that the addition of a high amount of iron and ascorbic acid does not improve transport into the cell. It would be more suitable to add iron to a hydrolysed caseinate product without the addition of high amounts of ascorbic acid as this would lead to good solubility of the iron however a similar ferritin response could be achieved with 5mM iron fortified cow milk with ascorbic acid.

Goat milk with 5 mM iron and ascorbic acid gave the highest response overall at 12.30 ± 1.23 ng ferritin/ nM iron followed by 10 mM goat milk with no ascorbic acid of 5.15 ± 0.46 ng ferritin/ nM iron ($p<0.001$). This was not significantly different from the other treatments of goat caseinate and caseinate hydrolysate (4.69 ± 0.55 ng ferritin/ nM iron and 4.83 ± 0.64 ng ferritin/ nM iron ($p>0.05$), respectively) (refer to Figure 8-9). Goat milk with 5 mM iron, without ascorbic acid, performed the worst out of all the goat protein treatments at 2.72 ± 0.17 ng ferritin/ nM iron. The literature (López-Aliaga et al., 2000; Barrionuevo et al., 2002; Alférez et al., 2006; Nestares et al., 2008) showed a much higher iron absorption of iron fortified goat's milk compared to cow or soy milk however in the absence of ascorbic acid this was not seen in the current study with the cow milk which had a greater response of 4.09 ± 0.35 ng ferritin/ nM iron at 5 mM iron ($p=0.004$). Goat milk with 5 mM iron and goat milk with 10 mM iron and ascorbic acid (2.49 ± 0.3 ng ferritin/ nM iron) performed similarly indicating that at 10 mM with ascorbic acid the performance is significantly inhibited which was also seen in the cow milk treatment and therefore the iron loading is a factor in the efficacy of absorption in Caco-2 cells.

The positive control (not shown) had a response of 37.37 ± 2.13 ng ferritin/ nM filtrate indicating that iron in the presence of ascorbic acid alone still performs better than the samples however the negative control containing no ascorbic acid had no response as there was no iron detected and therefore the samples do perform better than iron alone.

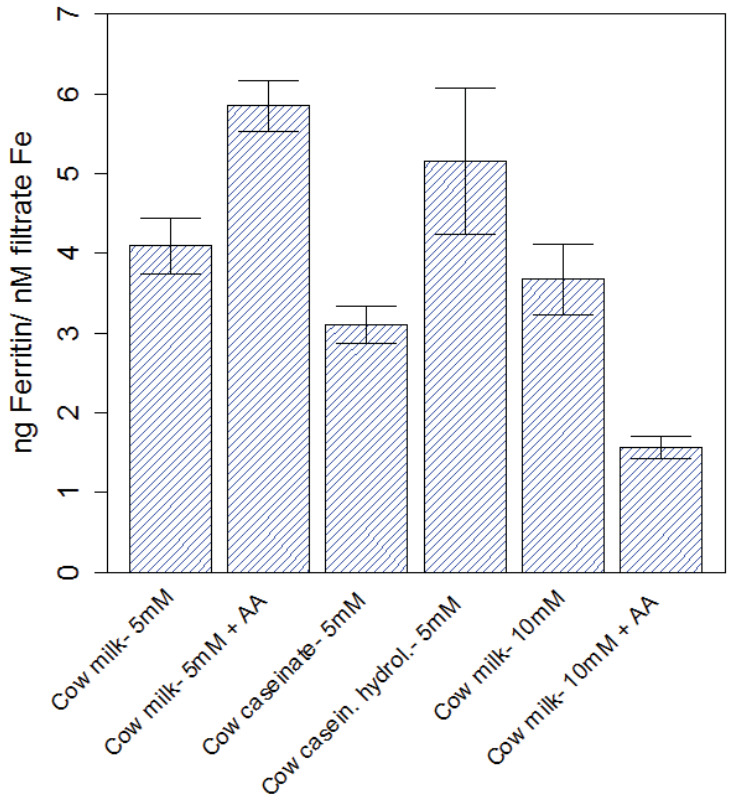


Figure 8-8: Ferritin response of all cow milk derived treatments on an equal iron filtrate basis of ng Ferritin/ nM iron; error bars indicate standard error, n=7.

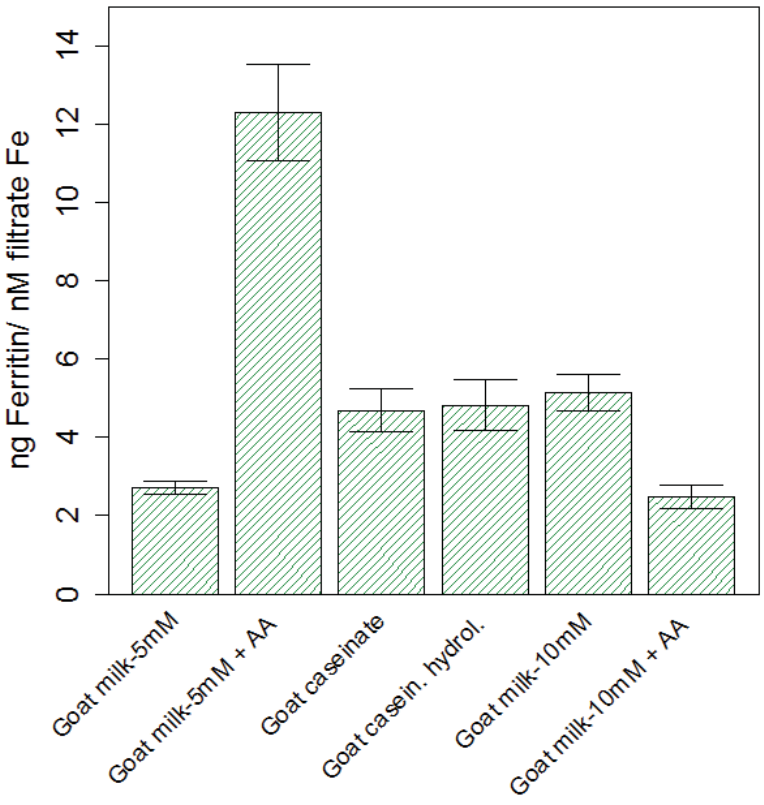


Figure 8-9: Ferritin response of all goat milk derived treatments on an equal iron filtrate basis of ng Ferritin/ nM iron; error bars indicate standard error, n=7.

8.2.2.6 Comparison of cow and goat phosphopeptides on an equal iron filtrate basis on ferritin response

The response of the cells with respect to the amount of soluble iron present in the filtrate revealed that the calcium precipitated cow phosphopeptides with iron added produced 6.54 ± 0.40 ng ferritin/ nM iron while peptides precipitated with iron produced a response of 7.56 ± 0.67 ng ferritin/ nM iron (p=0.2269), Figure 8-10. Goat peptides precipitated with calcium produced 9.64 ± 0.94 ng ferritin/ nM iron while with iron precipitation the response was 8.08 ± 0.78 ng ferritin/nM iron (p=0.2304). The use of calcium to precipitate the peptides did not appear to reduce iron binding, dialysability and delivery with the calcium still being present in solution. Calcium may either produce an isolate that gives a better peptide profile for iron delivery than the iron precipitate or it has no inhibitory effect on iron binding in its presence (Hegenauer et al., 1979b). From the iron chelation capacity studies (section 7.2) it was shown that the presence of calcium has only small effects on the binding of iron to the peptides selected under the same conditions. Therefore there is potential for the calcium precipitation method to be used in manufacturing, due to the higher stability of calcium with ethanol, without the need for calcium to be removed via ion exchange.

Comparing the results to the highest responses from all the treatments the calcium or iron precipitated cow peptides did not give a significantly different response from cow milk fortified with 5 mM iron with ascorbic acid of 5.85 ± 0.32 ng ferritin/ nM iron (p=0.1992). The iron precipitated peptides performed significantly better than iron fortified cow milk (p=0.0458). The calcium precipitated cow peptides response was significantly greater than cow milk with 10 mM and ascorbic acid and cow caseinate while for iron precipitated peptides the response was significantly greater than 10 mM cow milk, 10 mM + ascorbic acid cow milk, 5 mM cow milk and cow caseinate with 5 mM iron.

The goat phosphopeptides precipitated with calcium showed no significant difference to the highest responding treatment of goat milk fortified with 5 mM iron with ascorbic acid of 12.3 ± 1.23 ng ferritin/ nM iron (p=0.1046) while the iron precipitated peptides performed more poorly than the goat milk (p=0.0134). The goat phosphopeptides precipitated with calcium performed significantly better than 10 mM goat milk, 10 mM + ascorbic acid goat milk, 5 mM goat milk, 5 mM goat caseinate and 5 mM goat caseinate hydrolysate. The goat phosphopeptides precipitated with iron performed

significantly better than 10 mM + ascorbic acid goat milk, 5 mM goat milk, 5 mM goat caseinate and 5 mM goat caseinate hydrolysate with some evidence for 10 mM goat milk (p=0.0899).

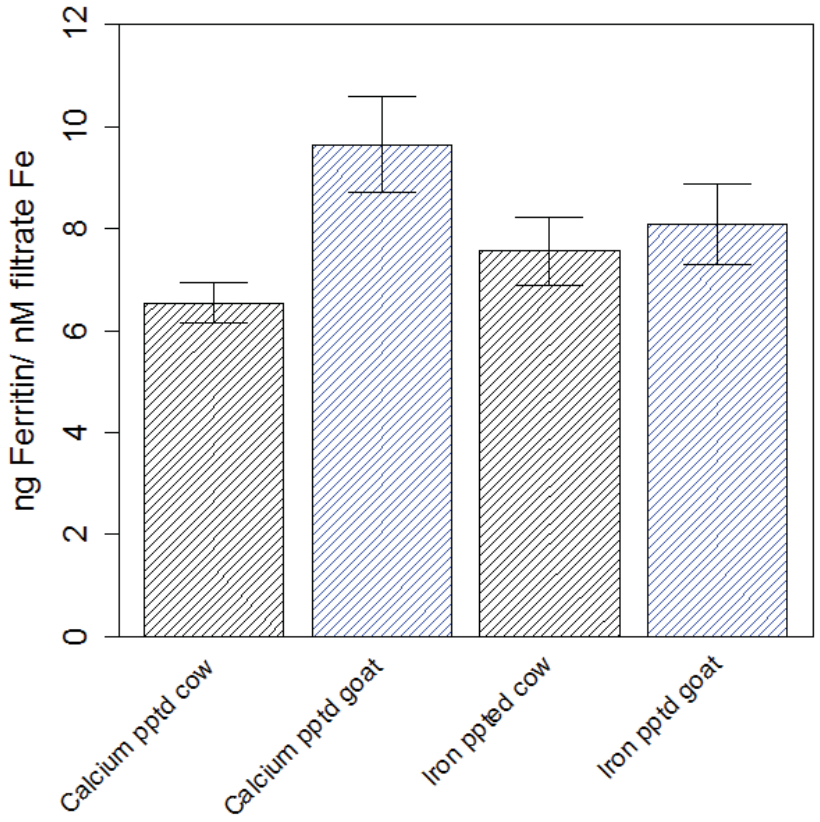


Figure 8-10: Comparison of calcium and iron precipitated cow and goat phosphopeptides on an equal iron filtrate basis; error bars indicate standard error, n=7.

8.3 Conclusion

The Caco-2 cell analysis has shown that ascorbic acid can improve iron absorption for both cow and goat milk, however this response is concentration dependent. At 10 mM there was no improvement in the amount of ferritin produced when ascorbic acid was added. When caseinate was isolated from cow and goat milk there was a different response from the species; the cow caseinate produced less ferritin compared to the cow milk while the goat caseinate did not produce a significantly different response to the goat milk. There may have not been a significant enough reduction in size to improve the dialyzability in addition to possible instability of the caseinate- iron complex. While the goat hydrolysate improved ferritin production comapred to goat milk, likely due to the reduction in protein size, the cow caseinate hydrolysate did not

improve against cow milk. This was attributed to the different casein composition, with cow casein containing more α_{s1}- casein that can hold iron tightly, prevent delivery to the cells. The phosphopeptides produced the same effect on ferritin production, regardless of the ion that was used to precipitate them and the species origin. When comparing to the previous treatments, the cow phosphopeptides did not improve the ferritin response to the cow milk and the goat phosphopeptides produced a non-signficantly difference to the goat caseinate hydrolysate, which had the greatest response. However, when comparing on an equal filtrate basis, the amonut of ferritin produced by the phosphopeptides was greater than all treatments apart from goat milk fortified with 5 mM Fe and ascorbic acid. This indicates that the phosphopeptides are able to deliver the iron to the cells more effectively after dialysis compared to most of the other treatments.

Based upon the literature it appears that the results for the cow milk and peptides have followed an expected trend where the milk and phosphopeptide produce similar levels of oxidation products. The goat peptides produced a lower MDA content than the cow samples while the goat milk was significantly better in terms of antioxidant capacity. The literature does not explain this strong antioxidant power however there is limited work on goat milk. It is likely that with the high β-casein content compared to cow milk this would have a positive effect and the differences in the amino acid composition may have added to this. It was also speculated that the structure of the casein micelle would have influenced the interaction of iron and fat. At this iron to protein ratio a significant amount of iron would be expected to bind to the micelle and it is likely most would be contained within the micelle structure therefore likely partitioned from the fat emulsion. The phosphopeptides are significantly smaller than the micelles and as no more than two phosphoserine residues are required to chelate an iron ion; this could be from a single peptide containing two residues or two peptides. In either case it is likely that the proximity of the iron to the fat would be a lot closer than with a micelle bound iron atom. Therefore while the peptides may be able to bind more iron they are more susceptible to becoming pro-oxidants toward the fat as there is a higher chance for interaction.

9 Conclusions and Recommendations

The bioavailability of iron in formulated foods is currently low due to iron being susceptible to oxidation and precipitation when added in a non-chelated form. The purpose of the thesis was to gain a fundamental understanding of the functionality of goat casein phosphopeptides as a chelator for iron allowing transportation to the intestinal wall for absorption. Cow casein was concurrently analysed due to the more extensive work performed on this species. The approach used was to assess how the complex would behave under industrial processing conditions.

The work presented in the thesis is novel as iron binding is characterised in goat milk, caseinate, caseinate hydrolysates and finally phosphopeptides and is compared with the products of cow milk. This gives an in depth analysis of the differences that the species type has on the interactions of milk proteins with iron. While there are significant differences in the casein micelles in terms of physicochemical properties these diminish as the proteins are purified. Differences in iron binding occurred with the sodium caseinates due to the different ratios of α_s- and β- caseins however after precipitation with cations the concentration of serine and phosphorus became similar as non-phosphorylated peptides were removed. Therefore the origin of the phosphorylated peptides did not appear to influence the measured properties significantly. The RP-HPLC chromatograms produced different peptide patterns and therefore the isolates between species varied but no significant difference in the iron binding properties resulted as measured by the ferrozine trials. In contrast, there were differences in the ability for the peptides to prevent iron mediated oxidation in a linoleic emulsion and therefore these subtle differences in the peptide extract may have an effect on the stabilising properties with iron.

This section reviews and summarises the research and identifies the main methods used and the implications they have on the study. The recommendations cover avenues of work that could be carried out to gain a more in depth understanding of the structure and functions of the peptides as well as different extraction methods.

9.1 Iron fortification of cow and goat milk

The main focus of literature in terms of iron fortification of goat milk concerns bioavailability studies in animal models, rather than the physicochemical characterisation that has been carried out extensively in cow milk. As goat milk is

becoming an increasingly popular dairy source a fundamental understanding of physicochemical responses with iron fortification is required. This was carried out in depth as this section of work provided a good foundation into how caseins and iron interact in a non-modified system.

The micelle size of goat milk was found to be smaller than cow milk in non-fortified milks. The result was expected as goat milk contains less α_{s1} casein which has been shown to reduce the ability for growth (Pierre et al., 1995).

Adding ferrous sulfate to reconstituted powdered milk or adding ferrous sulfate to liquid milk prior to spray drying, resulting in a dry blended and wet blended formulation, allowed a comparison of two potential processing techniques that could be used for iron fortified foods. The work gave an indication as to where in the processing line iron would best be added in the formulation and manufacturing process. Upon addition of increasing concentrations of iron (0- 20 mM) the micelle size did not change in dry blended cow milk while the size did increase, from 0 to 20 mM (the highest addition level) in goat milk however the goat milk micelle size remained significantly smaller than cow milk for all samples. The wet blend milks were affected by the addition of iron in terms of the micelle size distribution with the cow milk having a large variation in size; in contrast only with 5 mM iron addition to goat milk did the size become significantly larger than the control. This variation in size has not been reported in literature before as further processing of iron fortified milk has not been performed. It was unclear whether this was real variation or due to experimental error; a replicate was not manufactured due to time constraints.

There was a slight increase in the fraction of iron bound during wet blend processing compared to dry blending; on average from 0.73 (dry blend) to 0.80 (wet blend) for cow milk and 0.72 (dry blend) to 0.86 (wet blend) for goat milk. This could either be due to the high solids concentration of the milk prior to iron addition or some interaction during spray drying that forces more iron into the micelle or onto the surface of the micelle. The goat milk bound more iron on an equal protein basis, which is unexpected due to the lower content of α_{s1}- casein which contains more phosphoserine groups than β-casein. The elemental analysis of the wet blended milk showed that there was some movement of the minerals when iron was added, in particular the phosphate micellar concentration increased. This indicates that as the

iron concentration is increased, more phosphate moves into the micellar phase (or precipitates with iron) possibly to stabilise the iron.

The micellar moisture content for both cow and goat milk decreased with increasing iron regardless of the processing method. The wet blended milks had a slightly higher micellar moisture content than the dry blended milks at low iron addition but lost more moisture with increasing iron compared to the dry blended milk. This is due to the exchange of ions on the protein strands causing the expulsion of water. The increase of micellar phosphorus showed that there was a movement of ions therefore the micelle is becoming more mineralised adding to the loss of water. Overall, cow milk micelles were more hydrated than the goat milk which is related to the mineralisation and size of the micelles.

SAXS analysis of the dry and wet blended milks at various temperatures revealed that four features could be assigned to the casein micelles within the milk. These were the overall micelle size, incompressible (and compressible regions), the CCP particles and protein inhomogeneities. The feature that changed the most significantly with increasing iron was the CCP which provides strong evidence that the iron is interacting on this structural level rather than precipitating on the surface of the micelle. The iron is either precipitating around the CCP or forming a complex. The elemental analysis of the milk showed that more phosphorus was moving into the micellar phase so it may be that the iron and phosphorus are interacting together with the CCP to form stable mineral aggregates. The wet blended milks showed differences to the dry blended milks at this level also with the structure becoming more ordered such that the spacing between the CCP is more regular; the implications of this are unknown.

An interesting phenomenon occurred where the 5 mM wet blended cow milk had a greater micellar moisture content than the other samples which correlated with a significantly larger micelle size. SAXS of this sample at 37 ^{o}C also indicated that the incompressible region was qualitatively different to the other samples. This indicates that all these changes are related and in this particular treatment the micelles swelled causing the structure to become more open. This work has shown that iron addition not only has an effect on the CCP but additionally results in protein mediated structural changes in the micelle.

In determining where in the processing line the iron could be added, there was some variation in the amount of iron bound and how the casein micelle changes between the two methods. Adding iron prior to spray drying resulted in a higher iron binding capacity and caused structural changes in the CCP however at high iron concentrations the moisture loss is greater which could affect solubility. The concentrations of iron used in this study are significantly higher than what would be used in a milk product therefore this may not be a concerning factor. Only, if a small concentration of iron fortified milk was added to a formulation as an ingredient (where the milk was highly saturated with iron) could this be an issue. In the current process the iron was added as if the milk had already undergone evaporation. The manufacturing option of iron addition prior to evaporation was not examined. Potential problems with evaporation of iron fortified milk would be that the prolonged heating would accelerate oxidation of the iron. If the iron is not sufficiently bound to the caseins this may result in insoluble polymers of iron which could negatively impact colour, flavour and bioavailability. There was also more variation in the micelle size for cow milk which may indicate that there was stability issues when iron was spray dried.

Finally, calcium was removed from the micelles to determine how iron behaves in calcium depleted milk systems. The goat micelle appeared to be more resistant to micellar breakdown than cow micelles as relatively more sedimentation on ultra-centrifugation occurred after iron fortification. Based on the number distribution the cow milk had particle sizes in the range of 20 nm while goat had predominant peaks at 200 nm. The Z-average sizes also decreased with increasing iron concentration indicating the additional ionic strength causes a salting of the proteins reducing electrostatic repulsion within the particles. The higher β-casein content of goat milk may have maintained or reformed the structure of the micelle.

9.1.1 Recommendations

The iron fortified milks were tested approximately 2 hours after fortification (dry blend) or reconstitution (wet blend). It is unclear whether this time was adequate for mineral equilibration. This time has been shown to be adequate for unfortified milks, however the system had been altered therefore the equilibration time may have increased. It would therefore be interesting to measure the characteristics (pH, micelle size, fraction of iron bound to the micellar phase, moisture content) over several hours to see when the milk reached equilibrium.

SAXS revealed that there were structural differences between wet blended and dry blended milks and there were also differences in the amount of iron bound and micellar moisture content. Currently, it is unclear whether this is due to the spray drying process or due to the iron being added at high milk solids, mimicking the addition of iron after evaporation. Adding iron to a non-concentrated milk solution and then evaporating and spray drying could prove interesting to determine what factor is causing these changes. The effect of heating iron during evaporation to produce a concentrated solution would also indicate the stability of the bound iron, in terms of protein binding, oxidation and precipitation. A further variable that may impact structure on a commercial process is the practice of a high heat treatment. It is typical to use a high heat treatment (up to 90 ^{o}C), in manufacturing settings, immediately prior to spray drying to reduce viscosity and thus increase spray drier throughput via enhanced atomisation and higher feed total solids.

Further work is required on modelling the cow and goat milk SAXS data to understand the feature characteristics and the changes that occur as the iron loading is increased. This will allow a quantitative description of the data, rather than the current qualitative analysis. Due to the high complexity of the system modelling of the data still needs to be carried out in order to achieve this task. It would be very useful in understanding the internal structural changes that occur with iron fortification and various process and temperature effects.

The addition of iron to a ~70 % calcium depleted milk solution showed that cow and goat milk behave differently. The goat milk appeared to be more resistant to micelle dissociation and therefore could be more stable during industrial processing. This could have beneficial outcomes in manufacturing costs. Calcium depleted milk solutions cannot be increased to the typical solids content compared to skim milk solutions as viscosity becomes a limiting factor. However the goat micelles that have a more intact micelle structure may allow more evaporation of water and therefore will be cheaper to process. A study looking into the stability during spray drying could be carried out on the calcium depleted micelles to look at iron binding capacity, moisture content and protein stability and how it varies from cow milk.

9.2 Isoelectric precipitation of sodium caseinate

This chapter investigated the isolation of the casein from skim milk to produce the starting material for the casein phosphopeptides. The aim was to compare the behaviour of cow caseinate to goat caseinate following isoelectric precipitation. In addition, studies were performed on iron binding with the caseinates.

The process of isolating caseinate from cow's milk is a routine industrial process which can be replicated at pilot scale giving an essentially whey protein free product. The cow casein produces a solid precipitate that is easily handled. It was found under the same conditions at pilot scale as cow milk, the goat casein could not be readily isolated. This could be of significant technological concern if processes were to be employed in caseinate extraction. Goat casein does not appear to be as easy to handle due to the extremely soft and breakable curd. The goat casein was isolated via centrifugation. Soluble components at pH 4.2 were removed in the supernatant phase. The caseins were converted to caseinate after washing with the addition of alkali to pH 6.7.

The purity of the caseinates was checked using RP-HPLC and the cow caseinate was compared to a commercial preparation. As goat caseinate is not commercially produced this could not be compared however it was evident that the composition was different from cow with a much larger β-casein peak occurring.

Iron was added at 5, 11 and 22 mM to the caseinates at 3.5 % (w/w) protein. The iron addition showed that oxidation occurred rapidly after addition with all concentrations as the colour changed to orange. It is established that the oxidation state of ferrous iron changes to the ferric state upon binding however it is likely that there were unbound ions that were oxidising and polymerising in solution. The proteins were not stable over time and formed gelatinous solutions after approximately three hours. While the solubility results indicated that all the fortified caseinates were predominantly soluble however there was significant variation in the particle size indicating aggregation of the casein proteins. The high levels of iron in the ferric form would have caused rapid neutralisation of the negatively charged phosphoserine groups. There are no serum counter-ions present in the solution to balance the charges when iron is in excess in solution which could promote aggregation. The iron behaves differently to calcium due to the different binding mechanism; iron can bind three oxygen atoms in the ferric

state while calcium binds two via ionic binding which may explain the increased rate of aggregation.

9.2.1 Recommendations

The isoelectric precipitation of goat caseinate proved to be difficult with the precipitate being prone to breaking up with any mechanical manipulation. A systematic approach into finding a pilot scale methodology and ultimately a full scale technique for isolating goat caseinate should be carried out. It is likely that a centrifugal technique such as what was used at lab scale would be effective however micro-filtration could also be an option. Refinement of these techniques to optimise caseinate yield and reduce whey proteins would have to be carried out.

A more in depth investigation could be carried out into the stability and binding capacity of the caseinates with lower iron concentrations. There is already literature concerning cow caseinate and iron fortification so the focus should be on goat caseinate. While this may be of interest due to the initial findings of differences in stability, the focus of the iron binding for this thesis was more in terms of selected peptide fractions rather than intact caseinates hence the limited emphasis on this area.

9.3 Comparison of calcium, iron precipitated and IMAC eluted cow and goat phosphopeptides

The aim of this chapter was to characterise the iron binding capability of casein phosphopeptides, via several methods. The first step in this process was to develop a method to isolate the peptide extracts. Selective precipitation using calcium and iron were carried out according to methods from Reynolds and co-workers as well as the use of IMAC which was expected to produce a high purity fraction. It was hypothesised that these techniques would produce isolates with high concentrations of serine residues, indicating peptides rich in phosphopeptide clusters.

Cow and goat caseinate solutions were digested with trypsin at 1:50 or 1:200 E: S. Within the initial 2 hours the intact caseinates were broken down significantly with further changes in the chromatogram occurring as the hydrolysis time progressed at both tryptic ratios. Smaller peptides formed over time, with the 1:50 ratio giving a significantly higher degree of hydrolysis with about 20 % more cleavage sites hydrolysed. In terms of scale up conditions it would be expected that the 1:200 ratio

would be adequate in terms of concentration with a heat inactivation step, reducing the costs as well as achieving a hydrolysed product and reducing the risk of non-specific cleavage. There were differences in the peptide profiles between the cow and goat caseinates, although the importance of the profile difference is difficult to distinguish as a large number of peaks eluted from the digest. The number of peaks was in the expected range based on literature and predicted cutting profiles. While two ratios of trypsin were used on the caseinates producing different values of degree of hydrolysis, the peptide length was unknown which subsequently would affect the yield when precipitated with calcium or iron. The peptide length could have an effect on the functionality and iron binding capacity of the isolates. If the degree of hydrolysis is high then most peptides would be expected to be small which may reduce the yield as a high proportion of the precipitated peptides would contain serine however this may reduce the ability of the peptide to stabilise iron against other components in a formulation. The use of different enzymes may have also affected the peptide composition however these investigations were beyond the scope of this work.

The isolation of the phosphopeptides was conducted via calcium and ethanol precipitation according to methods from Adamson & Reynolds (1995) with some modifications. The isolates were analysed to check that phosphoserine containing peptide purification had occurred. For the calcium precipitated samples, there was little difference in amino acid profile and phosphorus content between species however there were some differences in the RP-HPLC chromatographic profile of the extract. Compared to the original digest there was an increase in serine content followed by glutamic acid and a large reduction in the number of peaks in the chromatogram, in the amino acid analysis and RP-HPLC data, respectively. On a molar ratio approximately 70 % of the serine groups isolated were phosphorylated. This was unexpected as according to the literature 100 % of serine residues are phosphorylated in both cow and goat casein (Ellegård et al., 1999). This may indicate that either the reaction may have caused some de-phosphorylation or a high concentration of natively de-phosphorylated serine was isolated.

The method used for the isolation of phosphopeptides was modified for the use of ferrous sulfate rather than calcium to determine whether an iron ion could produce an extract with similar results in terms of amino acid and RP-HPLC profile. A similar trend did occur for the iron precipitated peptides in terms of reduction of peaks and

phosphorus content however the amino acid analysis indicated that the isolation pathway was different to the calcium extraction as the increase in serine residues was lower while glutamic acid was higher. This is surprising due to extensive literature showing the high affinity of iron for phosphoserine groups however the mechanism for iron binding is different to calcium. The oxidation state of the iron changes to the trivalent form and binds via coordination bonds; in contrast, calcium remains in the same ionic state. The oxidation of iron may cause a shift in equilibrium allowing secondary binding with carboxylic acid groups, namely glutamic acid. This was seen in the caseinate bound iron where there was a greater instability of the caseinates due to the different binding mechanism indicating that less iron would be required to precipitate the phosphopeptides. Notably, there was an excess of iron in the sedimented peptide isolate during the fortification of the caseinate digests, indicating that some co-precipitation of unbound iron occurred. This would affect the functionality in formulations as the free iron would act as a pro-oxidant on fats. While the composition of the peptides differs this could provide a direct way to fortify peptides with iron for enhanced absorption however the serine content may limit the effectiveness of iron transport in the digestive system. On the basis of the higher concentration of serine in the calcium precipitated phosphopeptides along with the higher stability of the cation, this method was chosen for subsequent studies.

While there were significant differences between cow and goat milk in terms of physicochemical properties, the differences have appeared to reduce after selective precipitation. While the effect of calcium and iron caused significant differences in the composition, it was less so between the species.

Immobilised metal affinity chromatography is a technique used for the isolation of phosphoserine containing peptides due to the high selectivity of the iron chelated resin. While the IMAC appeared to separate the phosphopeptides from the non-binding peptides the serine isolation was not as high as the other methods, although the magnitude of overall selection was close to that of the iron precipitation. The limited isolation throughput the method allowed at laboratory scale made it difficult to conduct further testing on the phosphopeptide fractions. This technique would be more difficult to use in an industrial application without further refinement.

9.3.1 Recommendations

Mass spectrometry of the isolates would provide a much more detailed analysis of what peptide sequences from each method were obtained and allow a comparison in more detail than amino acid analysis alone. This could give an insight into the size, abundance and range of peptides that were selected, and confirm the presence of phosphoserine rich residues and clusters on the peptides. Attempts were made to use this technique however due to the complexity of the methodology to obtain logical results; no useful data could be presented.

As the iron precipitated peptides contained unbound iron, developing a method of isolating the iron-bound peptides could allow a more precise analysis of the functionality of the peptide isolates. Filtration could be used to separate the free iron however it may be difficult to isolate if the ferric iron has polymerised to a size range of the peptides.

9.4 Iron chelation capacity of phosphopeptides

Casein phosphopeptides from the calcium precipitated digests were investigated for their ability to bind iron and maintain stability under various processing conditions. This chapter first investigated how to remove calcium from the peptides which were highly saturated with the ion. Two methods were used: dialysis and ion exchange. It is likely that in an ingredient calcium would be present and therefore both types of peptides were investigated for their iron binding capacity/ stability to compare the effect of calcium. The effect of temperature, ionic strength, pH, holding time and iron loading were assessed as these changing environments could be present during industrial manufacture. Likewise, the peptides were added into milk to simulate the addition of the complex into a food formulation. This was carried out in two different orders: by adding the iron to a pre-mixed milk and peptide solution or mixing the iron and peptide first then adding it to milk. It was hypothesised that adding the pre-mixed iron and peptide solution first would improve binding due to the increased likelihood of the iron and peptide interacting within the three minute binding time.

9.4.1 Calcium removal techniques

Isolated peptides from the calcium and ethanol precipitation were used for iron binding analysis. Calcium removal from the phosphopeptides was required so that the potential maximum binding of iron could be studied. Although the binding strength of iron is

greater than calcium for the same phosphoserine binding site (Hegenauer, Saltman & Nace, 1979), it is ideal to reduce any competition between the calcium and iron so that optimal iron binding can be studied. Dialysis in a 40 mM sodium formate buffer at pH 3.0 was first trialled. This method was used as it was expected that the protein chains become protonated which would then unbind calcium, due to the ionic bonding type, that could then be dialysed out. However there was only a ~25 % decrease in calcium and lowering the pH further did not cause any more calcium to be removed. It was concluded that the dialysis in terms of buffer or membrane type may have inhibited the calcium from being removed from the dialysis tube.

The use of cation exchange resins has also been used to remove calcium from phosphopeptides. Ion exchange is commonly used to remove calcium and therefore it was concluded that this would be an acceptable method in the system. The solution can be decanted from the resin without concern of the calcium re-entering the solution. The amount of calcium remaining in the protein fraction was below the detectable limits of the titration used.

9.4.2 Iron binding capacity of cow and goat peptides under various processing conditons

There are several factors that occur in a factory line that could affect the availability of the iron: the pH of the solution, temperature changes during processing, holding times at various temperatures the formulation in which the ingredient is added in and the iron to protein ratio. These were addressed by measuring iron binding capacity under conditions expected in commercial use. These conditions included adding iron to peptide solution with different pH levels and ionic strength, heating or cooling the peptide solutions to look at the effect of temperature, altering holding times to determine whether binding can increase and mixing peptides with milk to examine any interactions between the peptides and intact milk proteins. The investigations were carried out in the presence and absence of calcium to examine the potential effects of calcium.

The pH and ionic strength of the buffer was first investigated as this would allow testing of a wide range of conditions to deduce the best buffer system to allow an optimisation of the binding of iron. A high (400 mM) and low (50 mM) ionic strength buffer was used to assess the effect of salt in solution. Higher concentrations of salt

did cause a reduction in binding presumably due to salt shielding of the negatively charged groups. At low pH the iron is freely soluble and little binding can occur due to the protonated protein. For optimal binding of iron the system must be close to neutral pH, which is within the expected range of food formulations. This is different to the literature; Gaucheron et al., (1996) found that when the pH was lowered from 6.7 to 3.7 the iron remained bound. This indicates that iron remains bound when the pH is lowered but will not bind at low pH.

Different types of buffer at pH 6.7 had an effect on the iron binding. Imidazole was initially selected as this has been used as a SMUF (Anema & Li, 2003) however HEPES has also been used for this pH range (Surigato, Ye & Singh, 2009) and water was used as a reference for no buffering. It was found that the imidazole was the best at maintaining binding of the protein to iron while HEPES could not maintain binding at high temperatures. The HEPES looses buffering function above 50 ^{o}C and possibly promotes oxidation of the iron.

The temperature is important in a factory setting, formulations will go through a range of temperature processes including pasteursation, evaporation and spray drying as well as selected mixing temperatures or cold storage. Temperatures were investigated from 4 ^{o}C to 72 ^{o}C, either with a short 3 minute holding time (based on literature of the method) or longer ramping or holding times. When increasing amounts of iron was added to the peptides with a three mintue binding time, at 37 ^{o}C the binding was optimised for both cow and goat peptides; increasing the temperature did not significantly improve the amount of iron that could be bound. Processes that involve higher temperatures may therefore not cause a significant effect on the iron binding capacity of iron when complexed to the phosphopeptides however when the holding time increased to 25 mintues it appeared that the iron binding decreased above 50 ^{o}C. At 4 ^{o}C it was found that the iron binding was poorer than at 20 ^{o}C and above. This was interesting as Raouche et al., (2009a) found iron binding in milk improved at cold temperatures however this may be due to the reduction of hydrophobic bonding, which could affect tertiary and quaternary protein structures. In contrast, phosphopeptides have a much shorter chain length and have minimal tertiary and quaternary structures and thus would not be impacted by cold temperatures in the same way.

The iron binding capacity of the peptides was signficantly higher than the amount of iron that intact milk proteins could bind. The maximum binding at 37 ºC was 50.16 ± 0.63 mg Fe/ g protein for goat peptides and 51.10 ± 0.07 mg Fe/ g protein for cow peptides in the absence of calcium, increasing the iron beyond this concentration resulted in a decrease in the amount of iron bound.

The peptides were added to diluted milk to determine whether the order of addition altered the iron binding properties. It was expected that adding the iron and peptide mix to the milk would increase the binding capacity of the protein. There was a slight improvement in the amount of iron that could be bound, with more binding when the peptide and iron were mixed first in the absence of calcium. It was speculated that the peptides act as a phosphate source to stabilise the iron in the casein micelles. This could have important implications when creating an ingredient in milk based formulations.

9.4.2.1 Comparison of cow and goat peptide structures for iron binding capacity

The iron binding of the goat derived peptides was usually lower than the cow peptides under the conditions tested. The yield of the peptides after calcium precipitation was fairly similar as was the phosphate, calcium content and the changes in the amino acid profile. It is known that the β- casein content is greater in goat however the phosphoserine clusters are more numerous in the α_s- caseins with 8-13 clusters versus 5 on the β-casein protein. The cow peptides may have more iron binding capacity as the α_s- casein peptides may be able to structure differently than to the β- clusters due to the neighbouring residues left on the peptides which may contribute to secondary binding. The similar binding is due to a nearly equal amount of phosphoseryl residues being present in the precipitated product but the slightly different peptide composition may have an overall effect on the binding.

9.4.3 Recommendations

An important consideration that was not tested in this study is the effect of spray drying. This process is very important in dairy industries as it allows for long term storage and lower cost transport. Spray drying involves vaporising atomised droplets of solution at very high temperatures with a short residence time. Based on the results of the study it appears that the iron would remain bound however this would be an

important process operation that would have to be analysed for further development of the product.

A scale up of these testing conditions may give a better estimation of how the peptides would behave in food formulations. The protein and iron concentrations used in the study were extremely dilute compared to food or milk solutions, at 0.2 mg/mL. The peptide extraction was not scaled up due to the cost of extraction, mainly due to the volumes of ethanol required; however this could be recycled in manufacturing. A scale up would allow pilot scale analysis of the stability of the iron-peptide complex as well as the development of an ingredient in a food.

9.5 Oxidative Stability and Caco-2 cell analysis of cow and goat milk products

Not only do the phosphopeptide isolates need to bind iron but they need to protect the iron from interacting with other components as well as delivering the iron in an absorbable form. A linoelic acid emulsion was used as the fat to be oxidised according to the method of Hegenauer et al., (1979a). Caco-2 cells were used as this technique gives an estimation of how iron absorbs in the intestinal tract without using animal models.

9.5.1 Anti-oxidant properties of cow and goat milk and phosphopeptides

The oxidation of the linoleic acid emulsion in the presence of iron showed that the rate of oxidation was linear for both the positive control (the emulsion and 1 mM iron in buffer) and the blank (the emulsion and buffer with no iron) reaching 3.09 ± 0.03 μg MDA/mL and 1.25 ± 0.16 μg MDA/mL after 3 days with an incubation temperature of 30 ^{o}C. Malondialdehyde (MDA) is a marker for oxidation products and therefore lower amounts produced indicate a greater resistance to oxidation. Goat milk only produced 0.46 ± 0.04 μg MDA/mL after the storage period. Goat milk contains more hydrophobic sections on the protein strands which can interact with the fat gloubles and therefore sterically prevent iron interaction with fat (Mora-Gutierrez et al., 2010). Rival et al., (2001a) determined that some amino acids (tyrosine, histidine, glutamic acid and aspartic acid) have antioxidant or radical scavenging activity however the amino acid analysis of the caseinate showed little difference in composition of residues between cow and goat casein. It may be that the goat micelles allow a different conformation of the hydrophobic residues so that other potential iron binding regions

(such as phosphoserine clusters and glutamic acid) are positioned to more readily interact with iron or radicals better. As the caseinate composition is fairly similar the serum proteins, salts or low molecular weight compounds may also have an effect on the oxidative stability.

The goat phosphoeptides produced less MDA than the cow milk and peptides. From the amino acid composition and the literature the only residue that was found to be signficantly more than in the cow peptide was histidine and to a lesser extent, serine which may explain the lower oxidation product content. The cow peptides produced more MDA than the goat peptides but produced less MDA than cow milk however between 48 and 56 hours there was a cross over and the oxidation products of the cow peptides exceeded that of cow milk.

Milk may therefore be more stable over extended periods of time. This may be due to the steric separation that they are able to maintain compared to peptides. As peptides are extremely small compared to micelles it is likely that there is a greater chance for the iron to still interact with fat even when bound to the peptides. The casein proteins that make up milk are long disordered strands (for example, β- casein can bind up to 7 cations (Baumy & Brule, 1988)). The ratio of iron ions to protein is therefore a lot lower than the phosphopeptides, in addition to this, the caseins still retain their hydrophobic sections which are more likely to interact with fat and therefore sterically partition the bound iron from the fat. This does not occur in the phosphopeptides as the hydrophobic regions would mostly be eliminated from the peptide isolate and therefore there is a higher chance that iron can get close to the fat and oxidise it. This may indicate that a mixture of peptides and milk such as in a formulation would work synergistically. The hydrophobic sections of the caseins would have a higher affinity to the fat and therefore prevent random interaction of the iron-bound peptides to the fat.

9.5.2 Bioavailability of iron chelates using Caco-2 cells

The iron absorption of the milk and the products were analysed using Caco-2 cells. Cow and goat milk with 5 mM iron both improved in bioavailability in the presence of ascorbic acid, however increasing the iron concentration to 10 mM or 10 mM with ascorbic acid did not improve the iron absorption. The use of caseinate produced a lower response to milk with cow caseinate while there was no significant difference

for goat, with no added ascorbic acid. This supports the findings that caseinate is not stable in the presence of iron even with the reduction in size from the breakdown of the micelle resulting in an equal or poorer bioavailabilty. The hydrolysis of the goat caseinate improved absorption compared to goat caseinate while the cow caseinate digest showed no improvement compared to cow milk with no added ascorbic acid. This may be due to the differences in α_{s1} casein (Kibangou et al., 2005) as it has been shown that α_s-casein peptides bind the iron too tightly and is therefore not release to the intestine. Goat casein has a lower α_s-casein content compared to cow casein and the higher β-casein may facilitate better delivery to the absoprtion site.

The isolation of the phosphopeptides showed that the use of calcium or iron as the precipitant made no difference in the response therefore calcium may not have an inhibitory effect as has been stated with iron absorption studies. When the results were presented on a nM Fe filtrate basis it was found that while the goat milk with 5 mM iron and ascorbic acid still delivered the greatest amount of iron, the phosphopeptides were the next best, indicating that the iron bound to the peptides is uptaken into the cells well despite the iron being in the ferric state initially.

Taking into consideration the oxidative stability and the bioavailability of the milk proteins, native goat milk may be the most stable and functional of all the treatments. In terms of the anti-oxidant capacity this performed signficantly better than the other treatments however the goat peptides were not significantly different from the blank control (containing no iron) and therefore still has anti-oxidant potential. The bioavailablity of the goat milk only performed well with the addition of ascorbic acid in which signficant amounts were added. The phosphopeptides may provide a more natural approach which performed better than the milks with no ascorbic acid on an iron filtrate basis. Adding the iron bound phosphopeptide to milk would likely be the best method of fortification as high iron binding can be achieved that is stable but will allow the milk proteins to protect the peptides against iron.

9.5.3 Recommendations

Investigations into the oxidative stability of the different casein forms could provide a better understanding of how they interact with fats. Tryptic digests of caseinate without any fractionation have been shown to have stronger antioxidant properties than isolates (Rival et al., 2001a; Rival et al 2001b) and therefore a whole tryptic digest

of the caseinates may give antioxidant properties. Adding the peptides in a milk solution could also help prove the theory that the large caseins would protect the peptides from interaction, while allowing a high iron binding capacity.

TEM or SEM could have been used to look at whether proteins or casein micelles were interacting with the lipid globules to validate the hypothesised mechanism in Figure 8-2.

In terms of measuring the iron bioavailablity of Caco-2 cells, a more realistic model may allow a better estimation of how iron is absorbed. In the current study the iron concentrations are signficantly higher than what would be used in food and based on the Caco-2 cell results more iron may not necessarily be better. Using milk and phosphopeptides fortified with iron at a RDI level could produce different and more relevant data. Similarly to the oxidation studies, combining the milk and phosphopeptides could provide good insight into a potential optimimum formulation for iron absorption.

9.6 Summary

The aim of the thesis was to investigate the feasibility of producing an ingredient that could bind iron, that is stable against processing conditions encountered during manufacturing, is stable in terms of causing minimal lipid oxidation and bioavailable allowing a more efficient uptake of the added iron, therefore reducing iron loading in the final product. The hypothesis was that isolating phosphopeptides which are rich in phosphorylated serine residues would increase the ratio of iron to protein and allow efficient and stable binding, meeting the criteria above. It was also hypothesised that goat milk derived ingredients would be superior over cow milk based ingredients based on several studies that found iron absorption was better facilitated by goat milk products. Figure 9-1 shows a summary of the different protein isolations performed on cow and goat skim milk and the analysis performed at each stage to characterise, determine iron binding and functionality. As little research has been conducted on the iron fortification of goat milk, an investigation into the comparison of cow and goat milk with iron fortification was also carried out giving a more complete picture of how iron interacts with milk proteins. It was proven that phosphopeptides are able to improve iron binding on an equal protein basis compared to intact milk proteins and can be stable in terms of iron binding under certain conditions. There were drawbacks

to using small peptides in the fat oxidation studies however it was shown there are potential synergistic effects between the peptides and intact milk proteins. In terms of goat milk derived peptides being a superior ingredient to cow peptides, this was not shown in the iron binding studies however intact goat milk does have beneficial properties with the interaction of iron compared to cow milk.

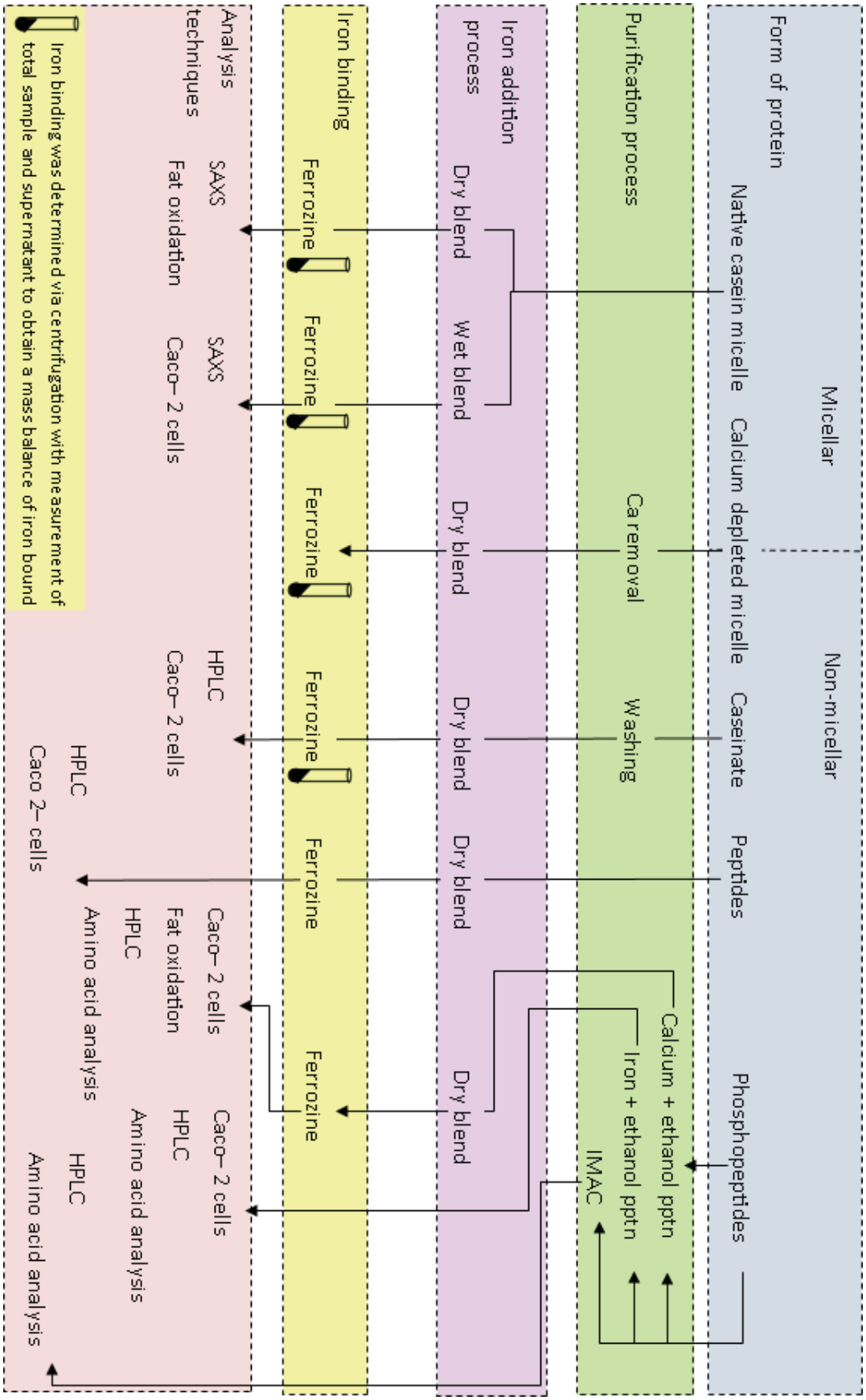


Figure 9-1: Summary of the protein fractions produced and analysed for iron binding and characterisation.

10 List of publications

Peer reviewed papers	Ingham, B., Erlangga, G. D., Smialowska, A., Kirby, N. M., Wang, C., Matia- Merino., L., Haverkamp, R. G. & Carr, A. J. (2015). Solving the mystery of the internal structure of casein micelles. Soft Matter, 11, 2723-2725. DOI: 10.1039/C5SM00153F Ingham, B., Smialowska, A., Erlangga, G. D., Matia-Merino., L., Kirby, N. M., Wang, C., Haverkamp, R. G. & Carr, A. J. (2016). Revisiting the interpretation of casein micelle SAXS data. Soft Matter, 12, 6937-6953. DOI:10.1039/c6sm01091a Smialowska, A., Matia-Merino, L. & Carr, A. J. (2017). Assessing the iron chelation capacity of goat casein digest isolates. Journal of Dairy Science, 100, 2553-2563. DOI: http://dx.doi.org/10.3168/jds.2016-12090 Smialowska, A., Matia-Merino, L., Ingham, B. & Carr, A. J. (2017). Effect of calcium on the aggregation behaviour of caseinate. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 522, 113-123. DOI:http://dx.doi.org/10.1016/j.colsurfa.2017.02.074 Smialowska, A., Matia-Merino, L. & Carr, A. J. (2017). Oxidative stability of iron fortified goat and cow milk and their peptide isolates. Food Chemistry, 237, 1021-1024. DOI: https://doi.org/10.1016/j.foodchem.2017.06.006
Conference presentation	Assessing the iron chelation capacity of caprine phosphopeptides at the Food Factor I 2016, Barcelona, Spain
Conference poster presentation	Assessing the iron chelation capacity of caprine phosphopeptides at the IDF World Dairy Summit 2016, Rotterdam, the Netherlands Insights into the aggregation behaviour of calcium caseinate studied by small angle X-ray scattering at the IDF World Dairy Summit 2016, Rotterdam, the Netherlands

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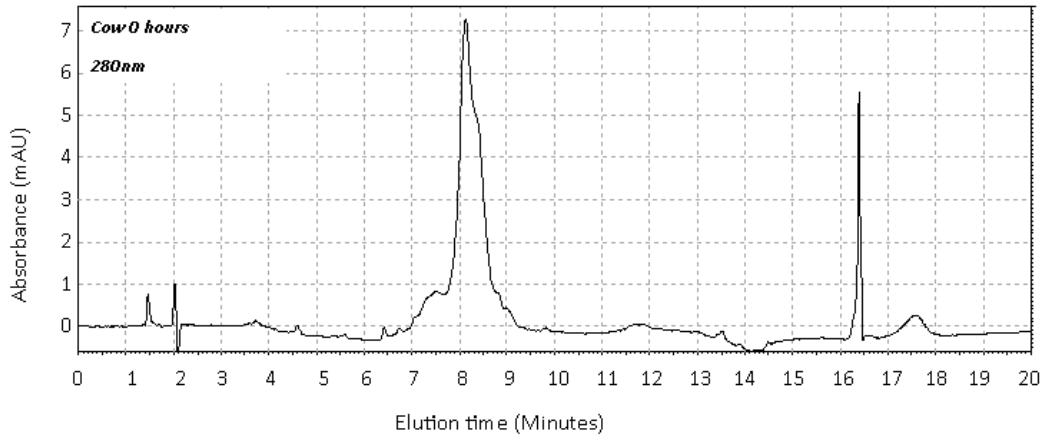
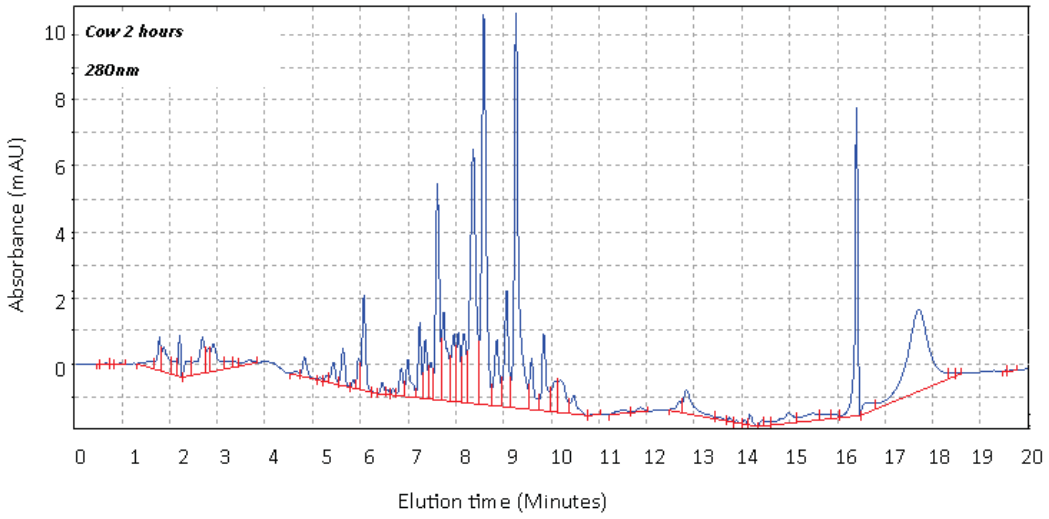
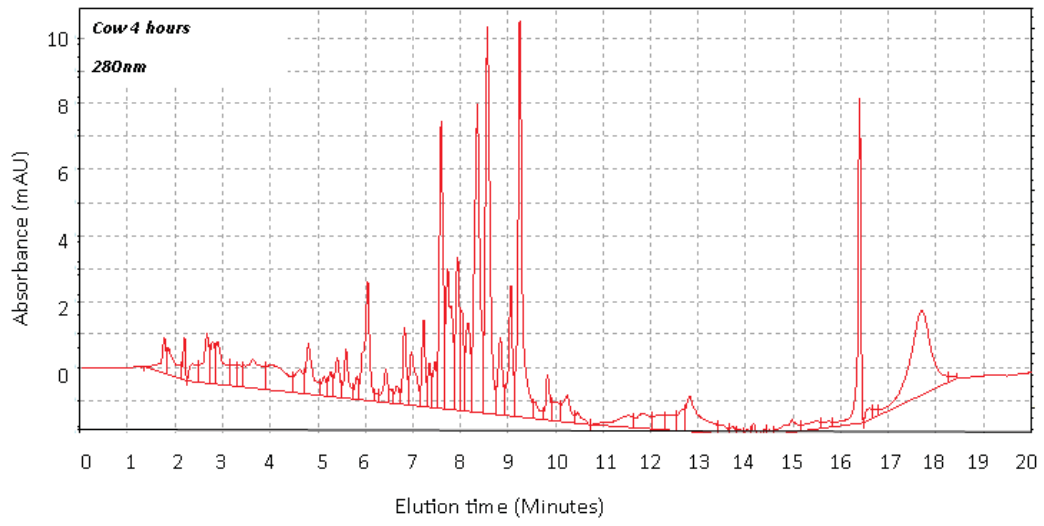
Appendix A

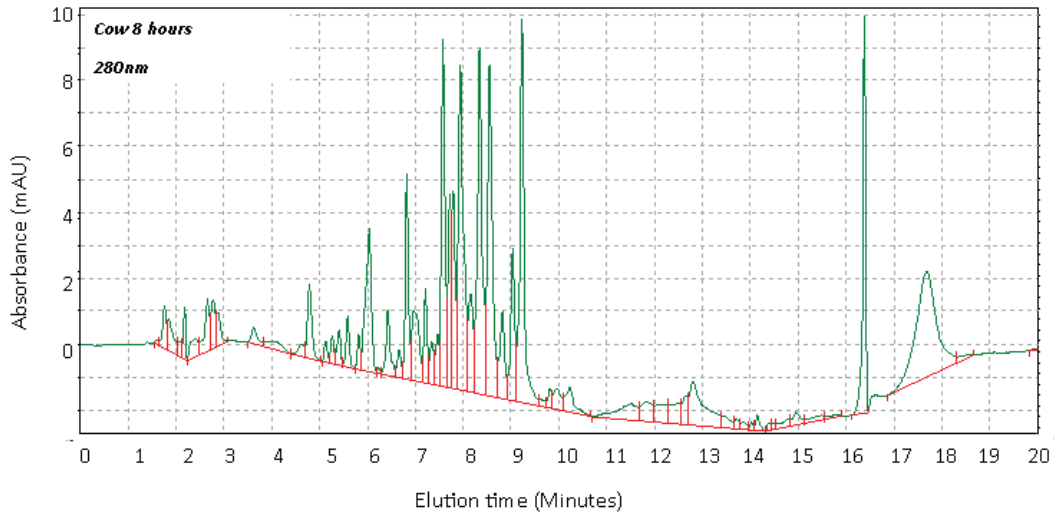
A Time series of cow and goat caseinate digestion

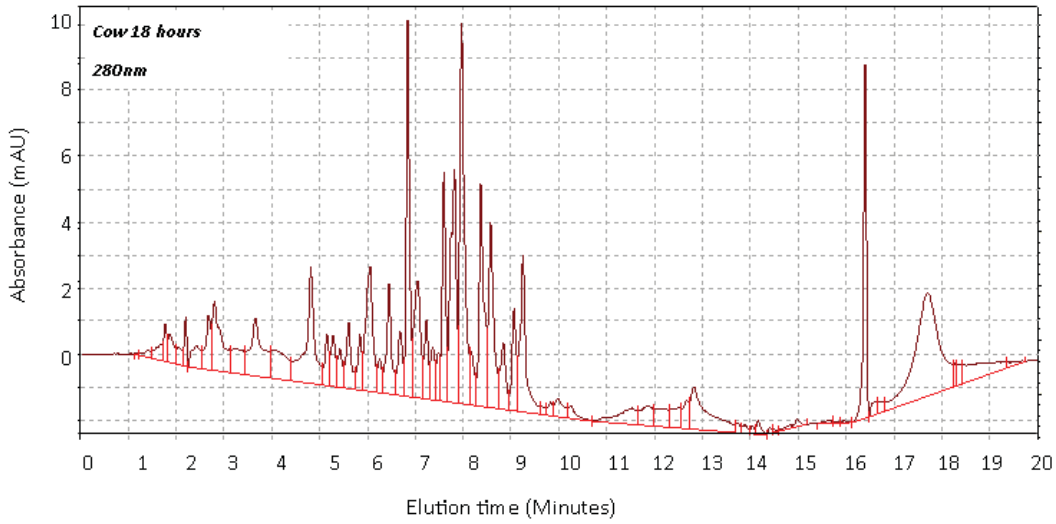
A.1 Chromatograms from section 6.2.2- 1: 200 E:S ratio

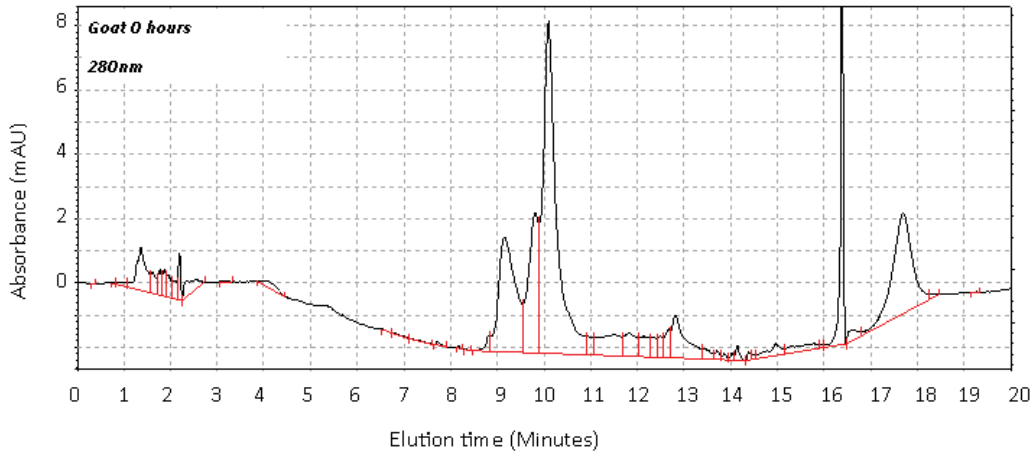


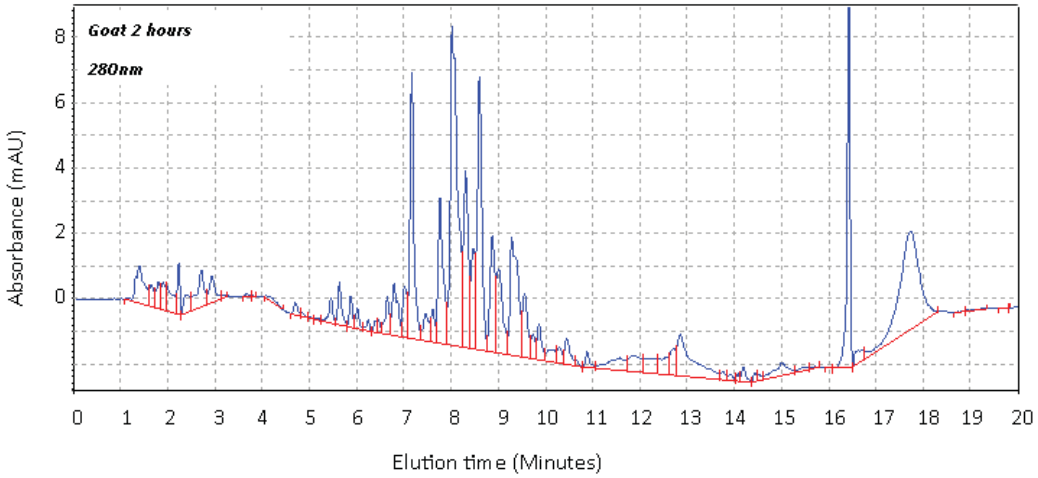


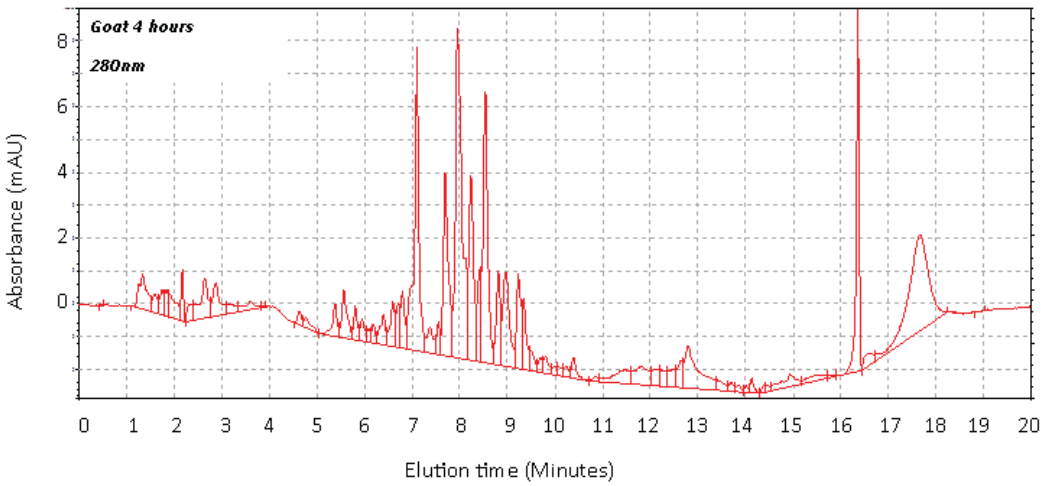


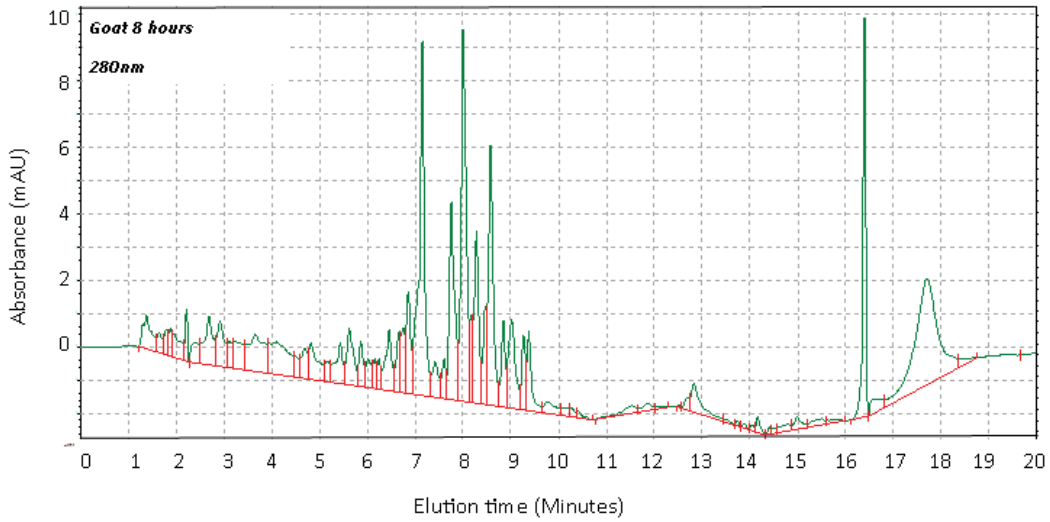

Cow 8 hours
280nm
Absorbance (mAU)
Elution time (Minutes)

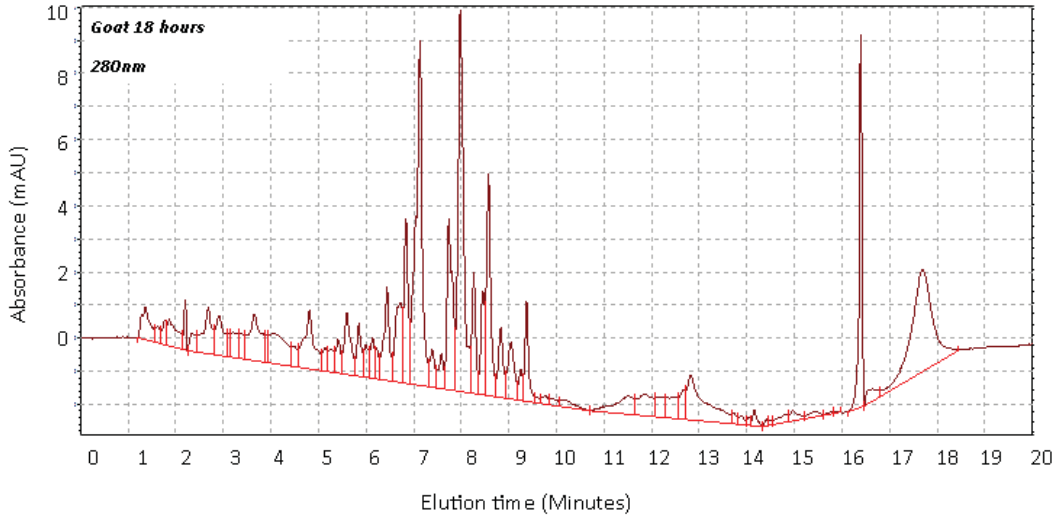

Cow 18 hours
280nm
Absorbance (mAU)
Elution time (Minutes)


Goat 0 hours
280nm
Absorbance (mAU)
Elution time (Minutes)

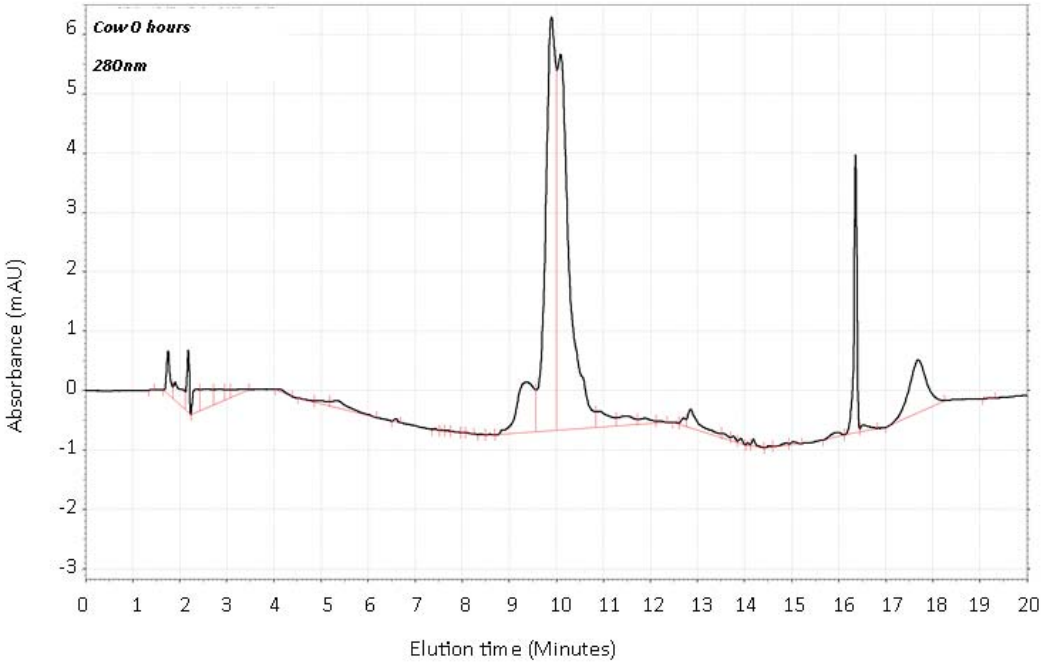

Goat 2 hours
280nm
Absorbance (mAU)
Elution time (Minutes)

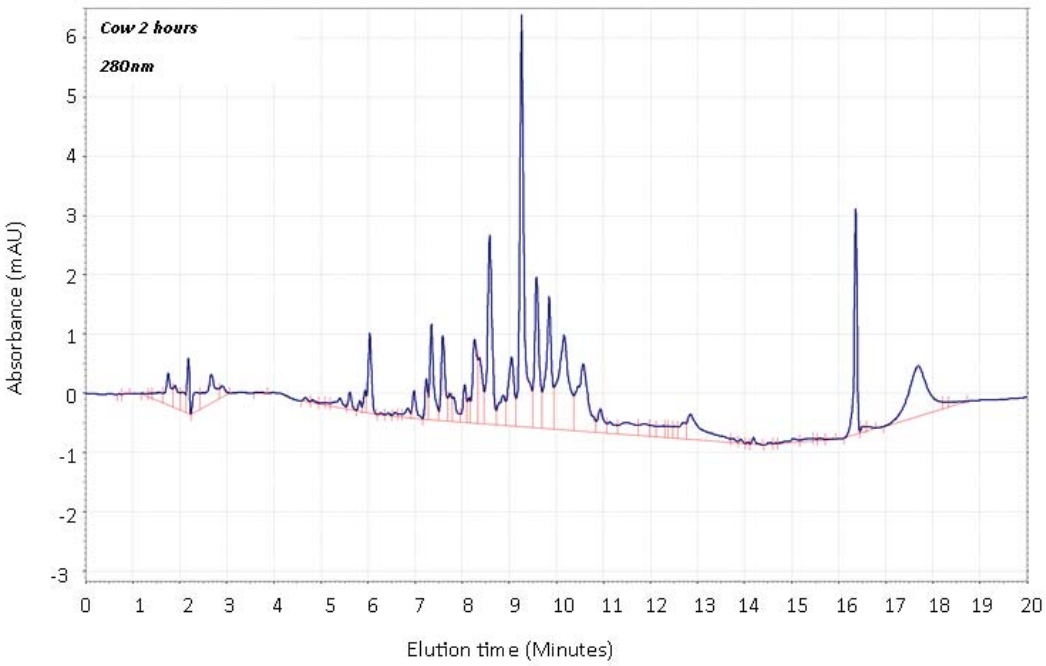

Goat 4 hours
280nm
Absorbance (mAU)
Elution time (Minutes)

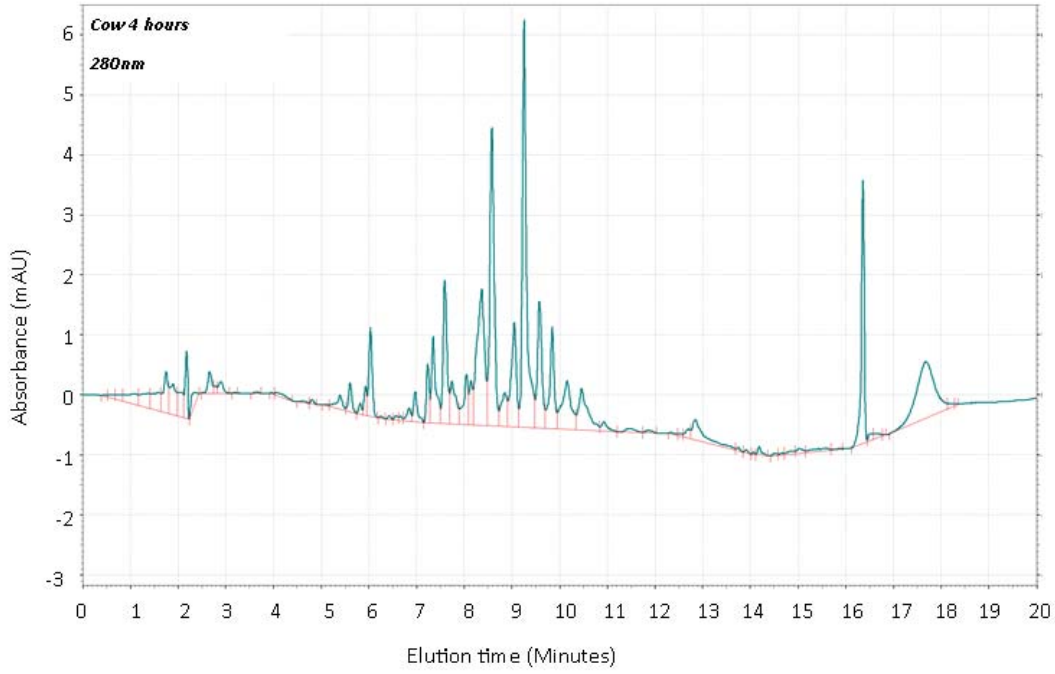

Goat 8 hours
280nm
Absorbance (mAU)
Elution time (Minutes)

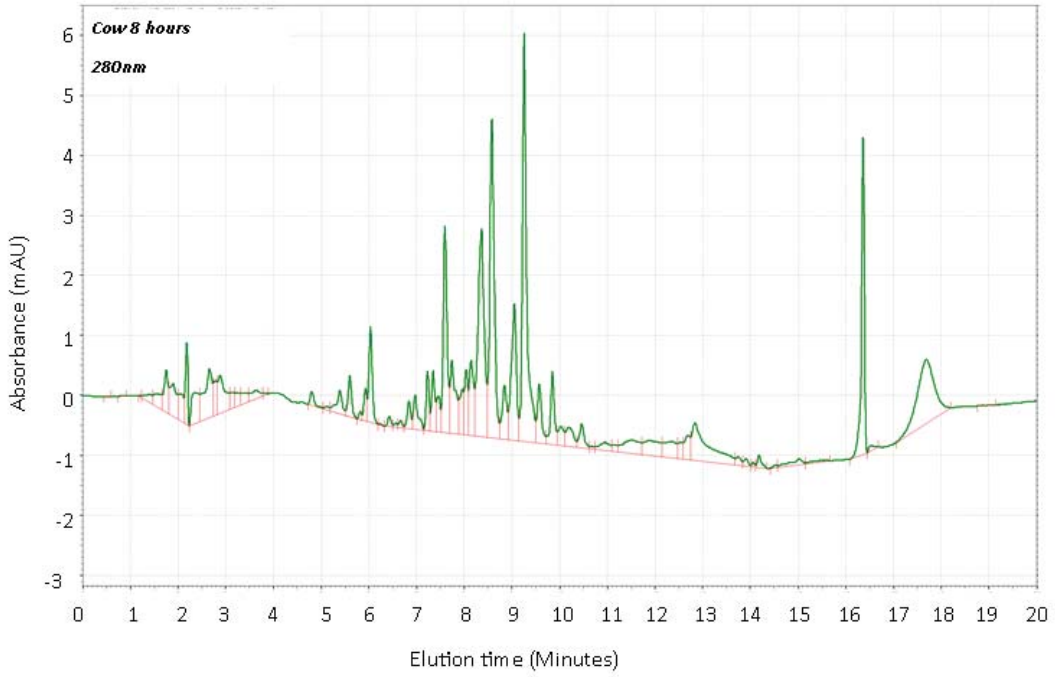

Goat 18 hours
280nm
Absorbance (mAU)
Elution time (Minutes)

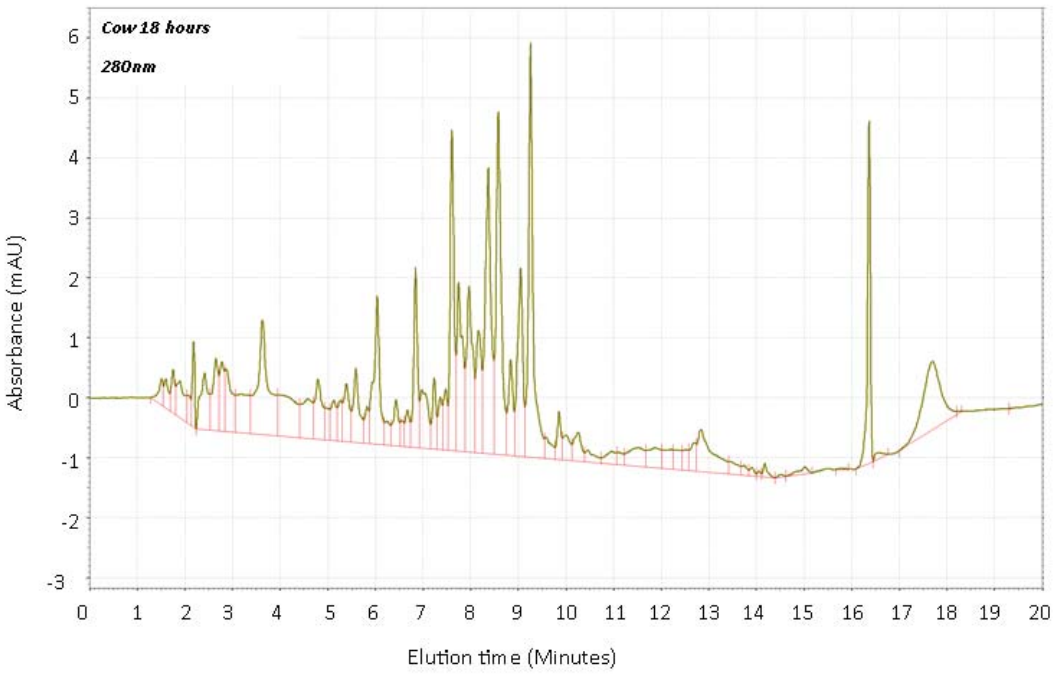
A.2 Chromatograms from section 6.2.2- 1: 200 E:S ratio

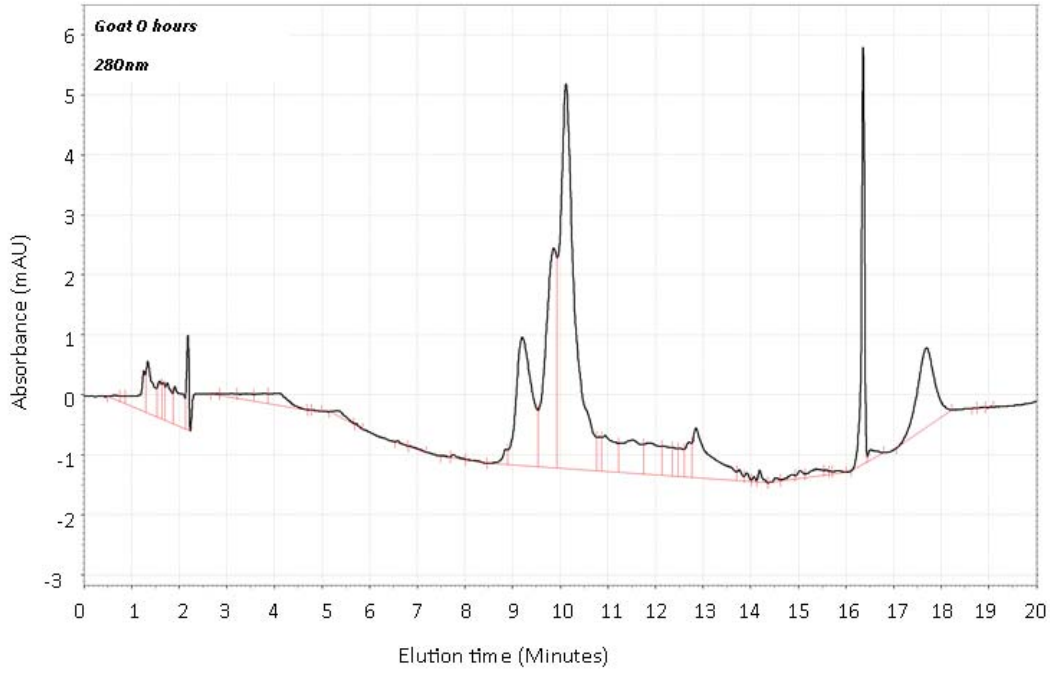


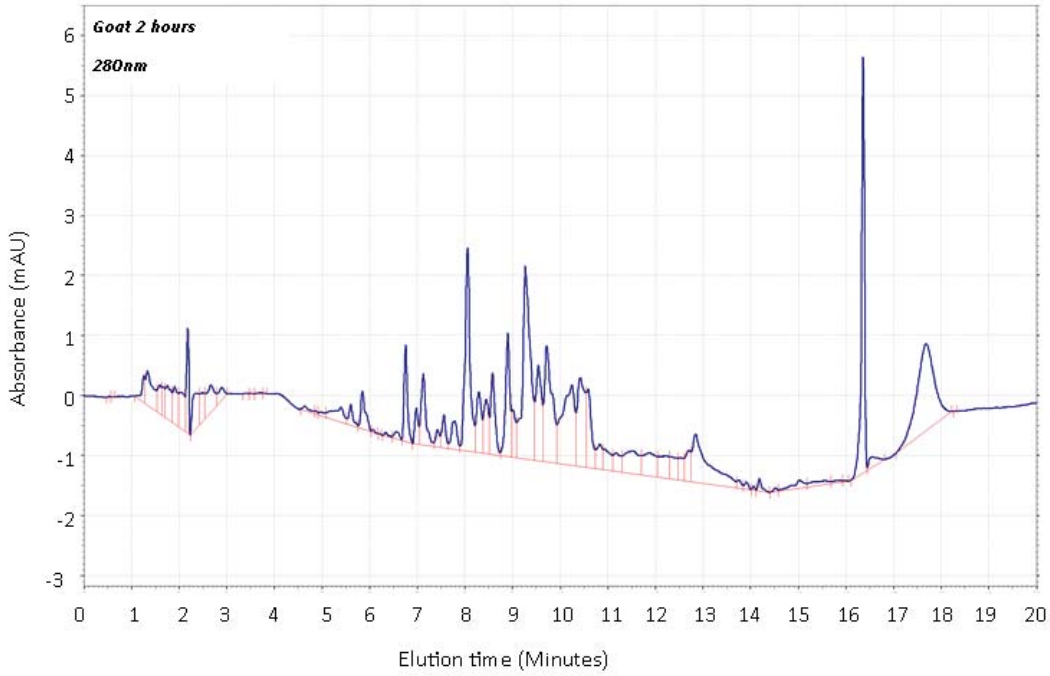


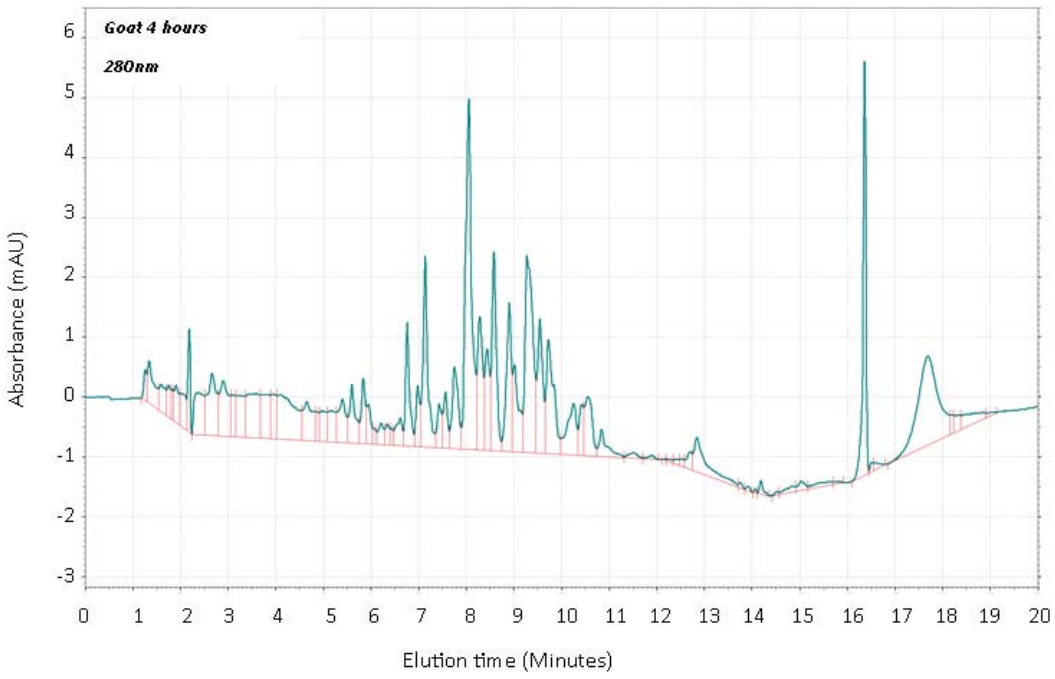

Cow 4 hours
280nm
Absorbance (mAU)
Elution time (Minutes)

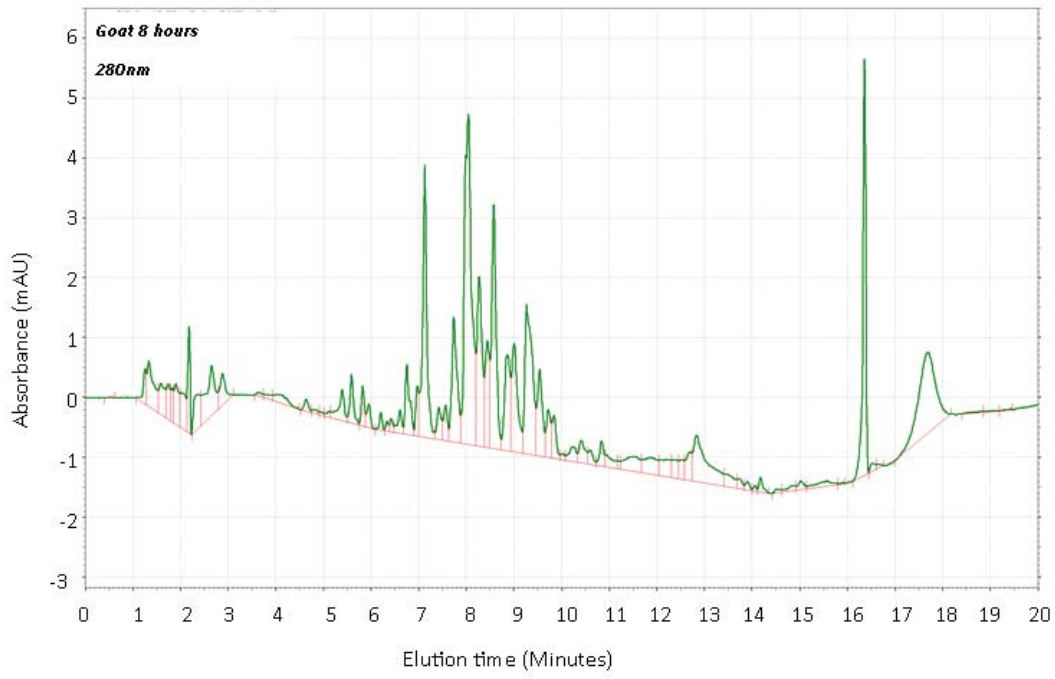

Cow 8 hours
280nm
Absorbance (mAU)
Elution time (Minutes)

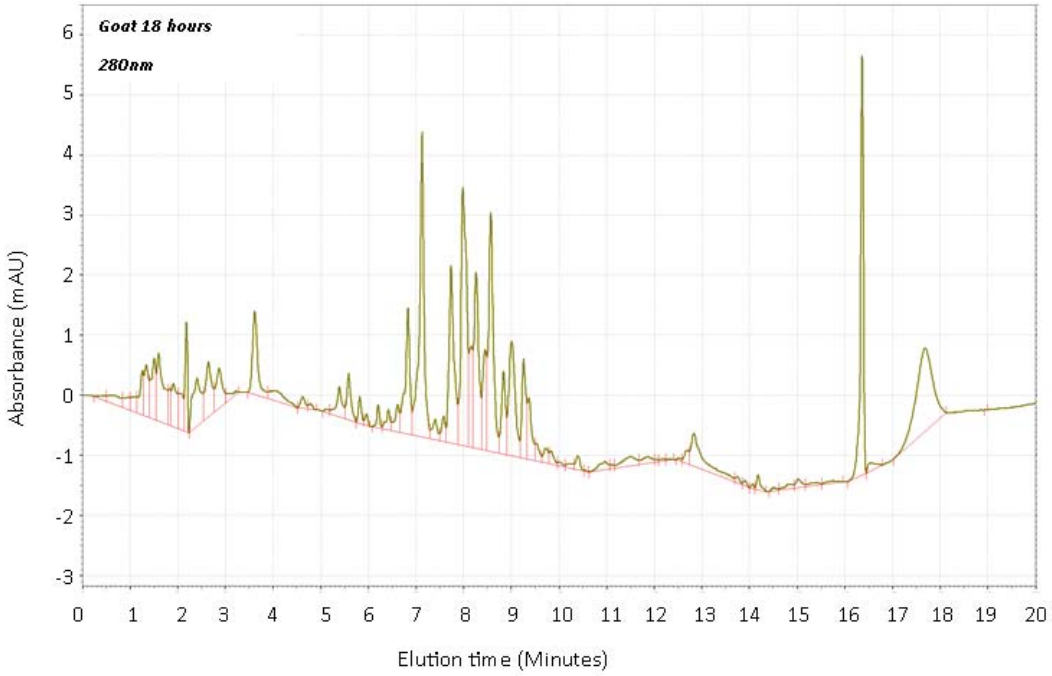

Cow 18 hours
280nm
Absorbance (mAU)
6
5
4
3
2
1
0
-1
-2
-3
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Elution time (Minutes)


Goat 0 hours
280nm
Absorbance (mAU)
Elution time (Minutes)


Goat 2 hours
280nm
Absorbance (mAU)
Elution time (Minutes)


Goat 4 hours
280nm
Absorbance (mAU)
Elution time (Minutes)


Goat 8 hours
280nm
Absorbance (mAU)
Elution time (Minutes)


Goat 18 hours
280nm
Absorbance (mAU)
6
5
4
3
2
1
0
-1
-2
-3
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Elution time (Minutes)

Appendix B

B Method development of IMAC: testing iron and buffers

B.1 Introduction

Determining the correct buffer for use in the IMAC column for washing and elution is important to ensure that as well as removing either non-binding or binding peptides at the correct point in elution, the iron leaching is minimised. Co-elution of iron could cause problems in the analysis as it interferes with protein concentration determination and mass sequencing. Upon discussion with Prof. FitzGerald (University of Limerick, Ireland) it was suggested to use water with the pH adjusted; a similar method was published by Guo et al., (2015). Trials were carried out to determine the acidic conditions required for the washing phase that reduces the level of iron leaching and alkaline conditions for elution. This was done by testing two pH levels and two ionic strengths with no sample, and then measuring any iron losses with the ferrozine reagent. Similarly, this was carried out in alkaline conditions to determine if iron will leach with the binding peptides.

B.1.1 Method

B.1.1.1 Buffer selection

A high and low ionic strength treatment was used in the acidic pH range; a low ionic strength buffer was only used in the alkaline pH buffers. The buffer contained 0.1 M added sodium chloride to produce the high ionic strength buffer. A high ionic strength buffer was tested as this has been reported to reduce electrostatic charges between the iron and resin however this may also increase the tendency for iron to be pulled off the resin. The low ionic strength buffer contained no added salt and contained only MilliQ water adjusted with acid or base.

The pH of the washing buffer is usually in the acidic range of pH 4-3 for iron charged columns (Andersson & Porath, 1986; Porath, 1992; Nuwaysir & Stults, 1993; Ueda et al., 2003; Tsai et al., 2008) and therefore buffers were made at pH 3.0 and 4.0, in the presence or absence of sodium chloride to 0.1 M.

B.1.1.2 Buffer preparation

The buffers were prepared by adding 1 M or 1 mM HCl to MilliQ water with constant stirring at room temperature, the experiments were performed at ambient temperature. The acid was added drop-wise and allowing the water to equilibrate for 10 minutes to ensure the correct pH was achieved. For the high ionic strength buffers, reagent grade sodium chloride was added prior to acidification and ensured the salt was fully dissolved. The buffers were de-gassed just before use and the pH checked again to ensure it was in close range to the target.

B.1.1.3 Column preparation

The column was extensively washed prior to iron charging by rinsing with water, 0.1 M EDTA and NaOH, pH 10 and lastly with water to equilibrate. A 20 mM $FeCl_3$ solution was applied and rinsed with water until negligible iron was detected using the ferrozine reagent.

B.1.1.4 Column elution

The buffers were applied to the column and the iron concentration of the eluent was tested at approximate 2 column volume (CV) intervals. The aim of the trial was to compare the initial iron content of the eluents by looking at the approximate first 10 CVs rather than determining the volume of buffer that has to be passed through to achieve no iron leaching. Because the column must be used in a semi continuous process, i.e. the resin must be hydrated at all times, there may be a dilution effect of the water already in the column with the added buffer and therefore in the final eluent. To reduce the tendency to measure the water in the first eluent fraction the first 5 mL of the run was excluded as this is 1mL short of the column volume- the first measurement may therefore be slightly diluted by water.

The eluted fractions were analysed for iron content using the Ferrozine method described in section 3.6.3.

B.1.2 Results

B.1.2.1 Treatments

B.1.2.1.1 Effect of low ionic strength at pH 4.0 on the iron leaching and pH

The first replicate (refer to Figure B-1) of the low ionic strength and at pH 4.0 are shown in black and grey for iron concentration and pH, respectively. It appears that as

the pH increases the iron concentration also increases, which is unexpected as ferric chloride is acidic. The replicate (a) followed a more expected trend whereby as the pH dropped the iron concentration increased or vice versa, as more ferric ions in solution would lower the pH. It is unclear whether the iron is causing the pH to drop due to the acidity of the iron or the pH is changing due to the possible dilution with water and therefore causing the iron to bind or unbind. The third replicate, shown in cyan showed that a small amount of iron came off in the first 2 CV with little change in iron concentration for the rest of the elution. Overall the elution shows that there is variability in the amount of iron that comes off the column indicating that the iron complexed to resin can be sensitive to experimental conditions such as loading, water washing, equilibration etc.

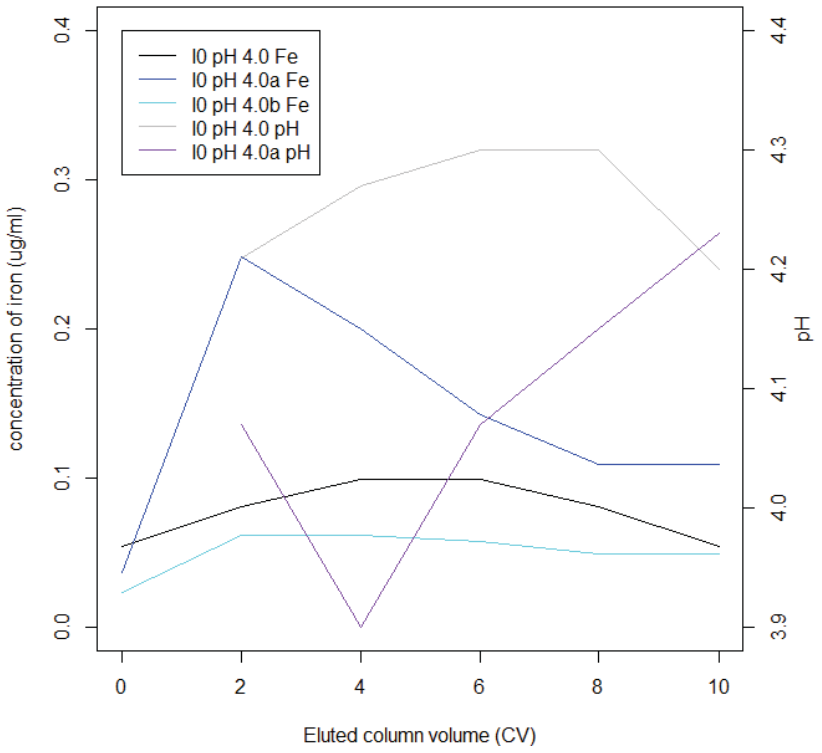


Figure B-1: Replicates of pH 4.0, I=0 buffers showing the iron concentration and pH of the eluents.

B.1.2.1.2 Effect of low ionic strength at pH 3.0 on the iron leaching and pH

The amount of iron that leached during elution at pH 3.0 buffer with no ionic strength increased in a curvilinear fashion over the CVs. The amount of iron that came off the column was greater by an order of magnitude compared to section B.1.2.1.1 where the

pH was 4.0 with no added salt. This may indicate the lower pH causes ionisation of the resin which causes iron ions to dissociate from the ligands. The overall trend of the pH after 10 CVs indicates that the increase in iron in the eluted fractions causes a decrease in pH.

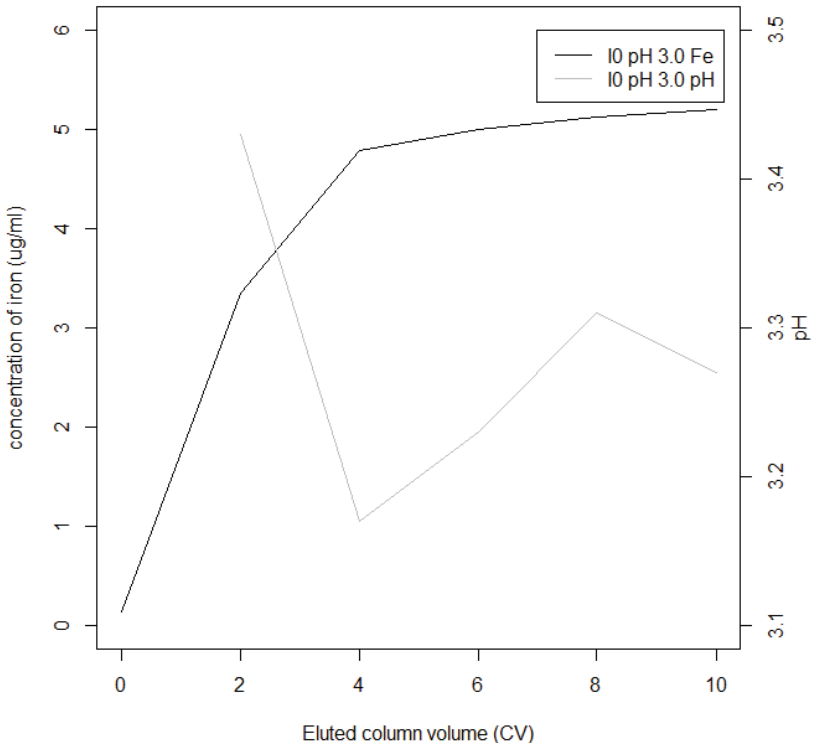


Figure B-2: Iron concentration and pH of eluents using buffer at low pH (3.0) and low ionic strength (I=0) sample.

B.1.2.1.3 Effect of high ionic strength at pH 3.0 and 4.0 on the iron leaching and pH

The concentration of iron that eluted at pH 4.0 (black line) was an order of magnitude greater than in section B.1.2.1.1 when there was no added ionic strength. Similarly, at pH 3.0 the amount of iron leached was around double than section B.1.2.1.2. The pH of the eluted fractions tends to reflect the amount of iron present in the eluent; with more iron present the pH is lower. This confirmed that using a buffer with added ionic strength can increase the amount of iron that unbinds into the eluent. This could cause significant problems when isolating phosphopeptides as these could co-elute with the iron in the washing phase.

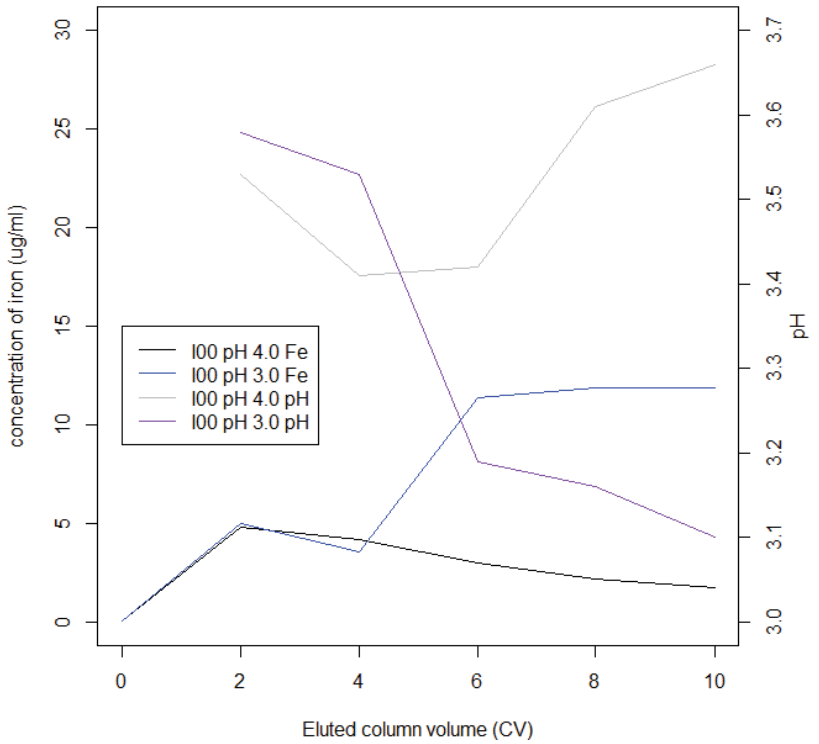


Figure B-3: Iron concentration and pH of eluents using buffer using high ionic strength samples (I=100) with buffer of pH 3.0 or 4.0.

B.1.2.1.4 Effect of high pH of pH 8.0 or 9.0 with low ionic strength (I=0) on iron leaching

An alkaline elution buffer is used to elute off phosphopeptides after the washing buffer phase has been completed. Iron leaching must be minimised to reduce potential interference with analysis. The pH 8.0 buffer resulted in a lower amount of iron eluting off in the fractions. However, at pH 9.0 there is only slightly more iron eluting with the buffer. In any case, the amount of iron leaching from the column in alkaline conditions is an order of magnitude lower than the acidic washing buffer. Therefore, in terms of iron leaching an alkaline buffer in the range of pH 8-9 with low ionic strength would be suitable.

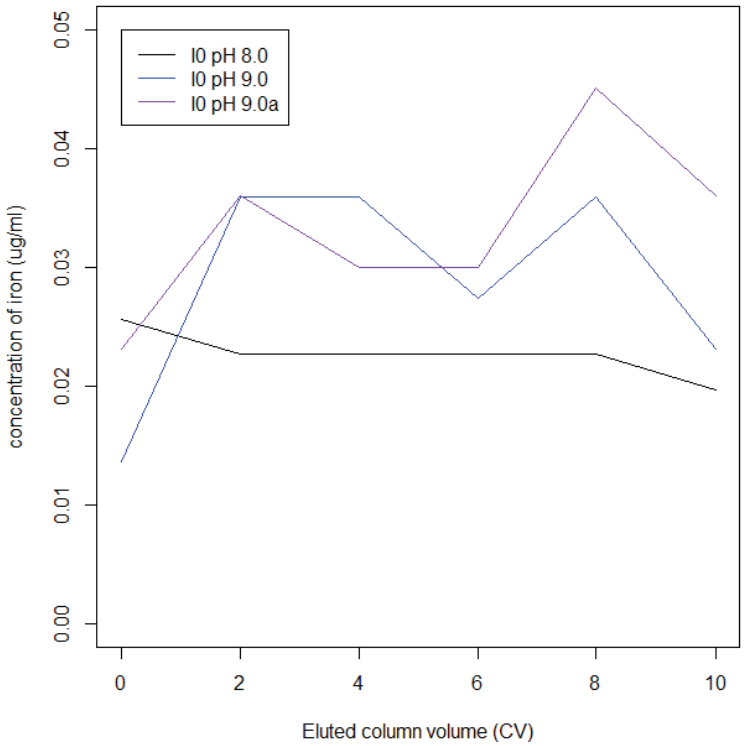


Figure B-4: Iron concentration of eluents using an alkaline eluent (buffer) (pH 8.0 or pH 9.0) with low ionic strength.

B.1.2.1.5 Summary of the conditions for washing and elution

The best conditions found for the column washing buffer and elution buffer were found to be solutions with low ionic strength. This is due to there being very little salt to compete for the binding sites with iron. The acidified buffer at pH 4.0 gave more variation in the eluted fractions however this could be caused by slight changes in ionic strength caused by the HCl and other variations in the method. The iron concentration decreases with column volumes applied which indicates that iron is not being released from the column by the buffer. Figure B-5 shows an averaged curve for the amount of iron that co-elutes after a water washing phase.

When looking in detail at the relationship between the eluted fraction iron concentration and pH producing trends in terms of iron leaching, there is compelling evidence to suggest that using a low ionic strength and higher pH reduces the iron leaching of the resin. A high ionic strength causes too much competition between the salts and the iron for the resin binding sites and ultimately causes the iron to lose its covalent bond with the IDA, instead electrostatically associating with the salts. At a lower pH it is likely the iron would favour associating with the hydronium ions for form aqua complexes or ligands as the iron becomes more soluble at lower pH. It is

concluded that the washing buffer cannot have a high ionic strength and a pH greater than 4.0 would reduce iron leaching.

The elution buffer in the alkaline range elutes less iron with water adjusted to pH 8.0 and 9.0 with NaOH. An alkaline buffer is required to elute the phosphopeptides, based on their *pI*. Using a buffer that is strongly alkaline, for example pH 12, in the column for prolonged periods of time can cause damage to the resin and increases the chance of ferric polymers forming, either preventing peptide elution or the iron complexing out of the column. Therefore a mildly alkaline solution was tested, between pH 8.0 and 9.0 as this is a commonly used range for the column. A more alkaline pH may allow the phosphopeptides to be removed quicker and therefore it would be more efficient to use a solution closer to pH 9.0.

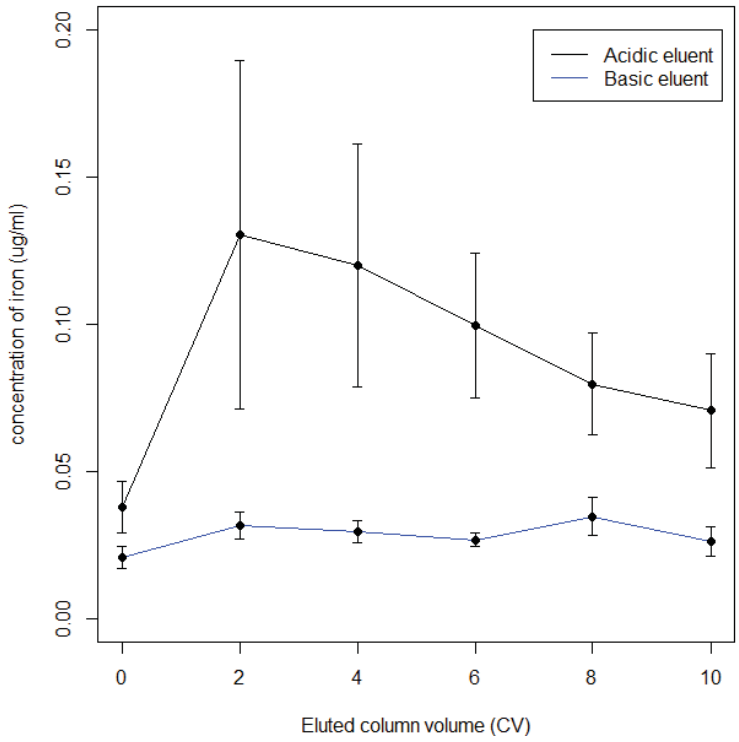
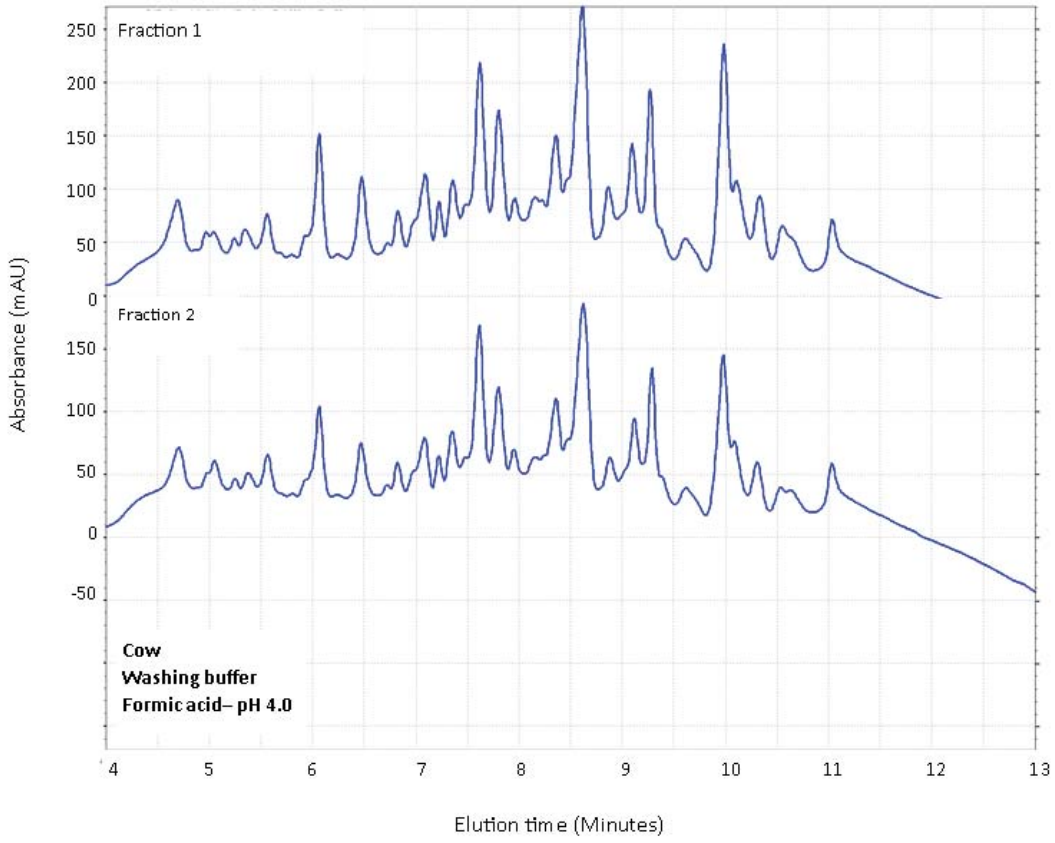
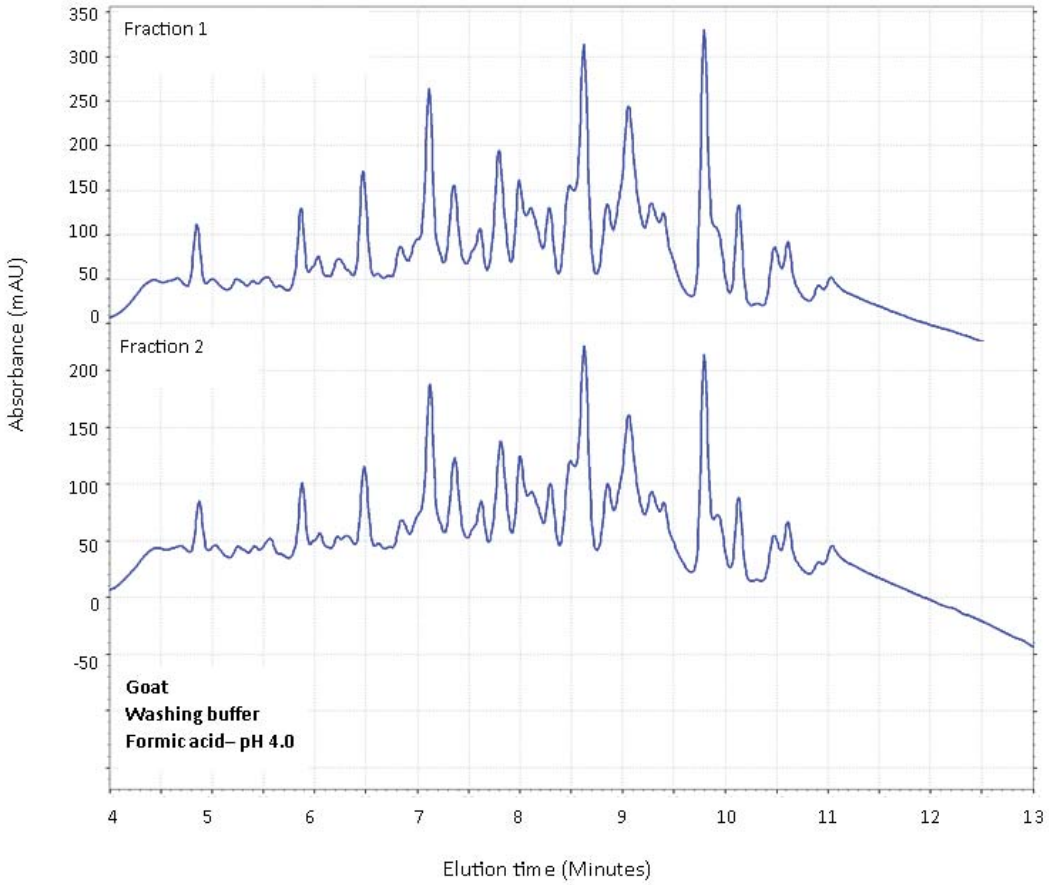
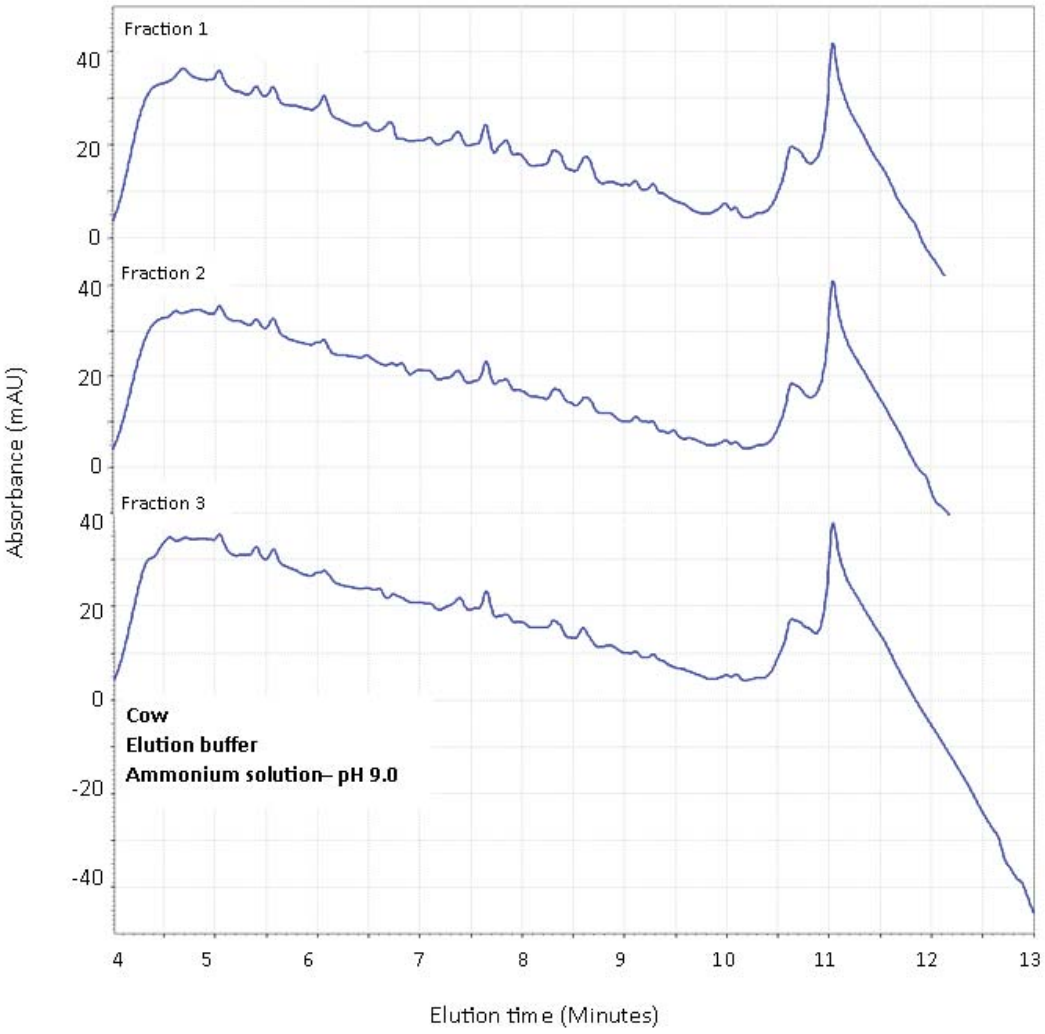


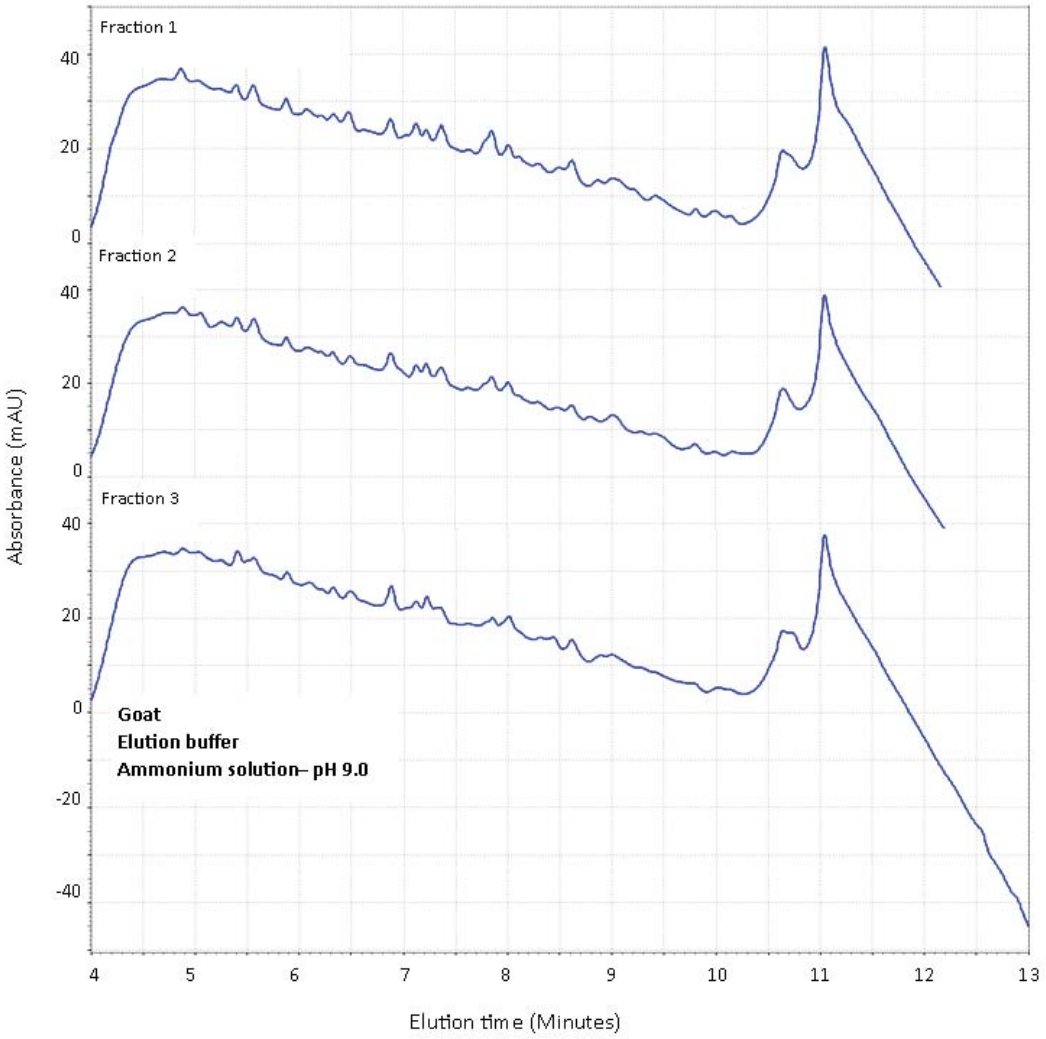
Figure B-5: Iron leaching of treatments that produced the lowest amount of leaching during washing and elution.

B.2 Washing and elution chromatographs of section 6.2.15




Fraction 1
Fraction 2
Goat
Washing buffer
Formic acid– pH 4.0
Absorbance (mAU)
Elution time (Minutes)
350
300
250
200
150
100
50
0
-50
4
5
6
7
8
9
10
11
12
13


Fraction 1
Fraction 2
Fraction 3
40
20
0
-20
-40
Absorbance (mAU)
Cow
Elution buffer
Ammonium solution– pH 9.0
4
5
6
7
8
9
10
11
12
13
Elution time (Minutes)



B.3 References

1. Guo, L., Harndey, P, A., O'Keeffe, M. B., Zhang, L., Li, B., Hou, H. & FitzGerald, R. J. (2015). Fractionation and identification of Alaska Pollock skim collagen-derived mineral chelating peptides. Food Chemistry, 173, 536-542.