Interactions of Iron, Protein and Orthophosphate in Milk Systems

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With the blessings of my parents

I dedicate this thesis to my

Wife (Ketki) and Son (Aayush)

Abstract

Although iron deficiency is the largest nutritional disorder affecting the human population, few food products in daily use are fortified with adequate (25% of daily requirements per serving) concentrations of iron. The interaction of iron with food components and the consequential deteriorative effects on colour and taste thwart its use as a fortificant of choice. Milk proteins, especially caseins, are a class of metallo-protein which chelate iron and prevent its interaction with food matrix. However, addition of high levels of iron to protein solutions causes precipitation.

As iron and calcium bind to similar sites on the proteins in milk, the effect of calcium depletion on the iron-binding properties of the milk proteins was examined. A weakly acidic cation-exchange resin was used to remove three different levels i.e. 18–22, 50–55 and 68–72% of calcium from milk, designated as low, medium and high CDM respectively. The depletion of calcium from milk by ion exchange affected its physico-chemical properties, with the extent being highly dependent on the level of calcium depletion. The integrity of the casein micelles was retained at up to ~ 20% calcium depletion from normal milk, but there was substantial disintegration of the casein micelles at calcium depletion levels of > 50%.

Five levels of iron (5, 10, 15, 20 and 25 mM) were added to each of these calcium-depleted milks (CDM) and the resultant milks were analysed for particle size, microstructure and the distribution of protein and minerals between the colloidal and soluble phases. The properties of the milks with different calcium contents were variably affected by the addition of iron. In both normal milk and the low CDM, the majority (> 90%) of the added iron bound to caseins within the casein micelles,

minimally affecting the hydrodynamic diameter, ζ-potential and protein distribution. Protein solubility was adversely affected in the medium CDM, whereas most of the protein and the added iron were associated with the non-sedimentable phase in the high CDM. The high concentration (~ 20 mM) of ferric iron in the non-sedimentable phase of the high CDM (~ 70% calcium depletion) presented a distinct advantage of the calcium depletion process over traditional processes for the iron fortification of milk systems. However, a reduction in aqueous phosphorus, in proportion to added iron, was observed in all milk systems. The addition of orthophosphate prevented aggregation and promoted the formation of small fibrous structure particles in calcium-depleted milk by reducing inter-particle iron bridging. In contrast, added orthophosphate promoted the formation larger unstable aggregates in calcium-depleted milk upon calcium re-addition. The differing effects of added orthophosphate on the iron and calcium-induced aggregation of proteins in calcium-depleted milk were related to the binding characteristic of respective cations. The inclusion of iron in the calcium-restored milk generated substantially smaller aggregates, which were promoted by the addition of orthophosphate. The presence of iron probably blocked the polymerisation pathway required for casein micelle structure formation but not the interaction of calcium with caseins.

The effect of orthophosphate addition on the iron-induced aggregation of caseins in sodium caseinate was examined. The binding of iron at greater than equimolar concentration of organic phosphorus on caseins resulted in the precipitation of both the iron and caseins. In the presence of orthophosphate, however, higher concentrations of iron could be added to sodium caseinate solution (~ 6.9% by weight of casein) with little sedimentation of protein. The presence of

orthophosphate prevented iron-induced casein precipitation in sodium caseinate solutions and also improved the solubility of iron. The formation of small aggregates upon iron addition to sodium caseinate solution containing orthophosphate was responsible for the high solubility of casein-iron complexes. The ^{31}P-NMR study, SDS-PAGE and size exclusion chromatography analysis of the soluble casein-iron complexes revealed that a cluster of inorganic ferric phosphate was stabilised by caseins. The concentration of iron bound by caseins in the soluble form was at least 5 to 10 times higher than in previous studies, thus creating an opportunity to develop an iron ingredient for food fortification.

The effect of exchanging the sequence of adding iron and orthophosphate to sodium caseinate solutions was examined. Surprisingly, the casein-iron precipitates (formed upon > 5 mM iron addition to sodium caseinate solutions) were redispersed upon orthophosphate addition. The added orthophosphate adsorbed onto the casein-iron precipitate. This adsorption displaced a part of the organic phosphorus (contributed by caseins) bound to iron and increased the surface negative charge on the casein-iron precipitate, which probably led to the redispersion of caseins and iron. The adsorption of orthophosphate occured on ferric hydroxide formed in the absence of casein, but does not solubilise iron, suggesting that the presence of caseins was critical for redispersion of the precipitate. Precipitate with higher protein content could redisperse greater concentrations of iron, while requiring lower orthophosphate content. Optimally four moles of iron could be solubilised by one mole of casein in these experiments. The adsorption of orthophosphate onto protein-iron complexes and its consequent solubilisation is a novel finding with a potential to create a new iron fortificant.

Overall, this research highlights the important role that orthophosphate plays in the binding of iron to milk proteins. The soluble protein–iron complexes created in this work could be used to fortify liquid food products. Moreover, two processes involving entirely different mechanisms for the formation of soluble protein–iron complexes have been proposed, which has opened up a number of avenues for further research. The products and processes as an outcome of this research are protected by a patent, namely “Micronutrient fortification process and its usage”, issued by the New Zealand Patent Office on 24 June 2014.

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Chapter 1. Introduction

Iron is the most abundant metal on earth and constitutes 5% of the earth's crust (Fredette et al., 2007). Ironically, the deficiency of iron is the largest nutritional disorder affecting world population. Iron is critical for regular metabolic functions and its deficiency affects almost every organ of the human body. The most important roles of iron in the human body involve oxygen transport, energy production and immunity (Carpenter et al., 1992). Overall, the deficiency of iron not only affects human health but also reduces a nation's productivity (Haas et al., 2001). Fortification of foods with iron has been recommended as a long term strategy for reducing iron deficiency. However, iron is a highly reactive metal and interacts with the food components, affecting the taste and colour of the fortified foods (Hegenauer et al., 1979a). Iron is thus recognised as the most difficult micronutrient for food fortification (FAO, 1997).

The non-heme sources of iron, e.g. inorganic salts, are generally used for food fortification. Iron salts are categorised into either insoluble, semi-soluble and soluble sources according to their solubility in water (Allen et al., 2006). Soluble sources of iron, like ferrous sulphate, are the most bioavailable but they are also the most reactive with the food matrix. Hence, insoluble sources of iron are preferred as a source for food fortification due their lower deteriorative effects. Unfortunately, the insoluble sources of iron could not be used for fortification of liquid food products, due to sedimentation problems. The chelated forms of iron, e.g. ferrous bis-glycinate, sodium feredetate, have now emerged as a choice of fortificant for liquid food products. The chelation of iron with other molecules reduces its interaction with food components. However, the high cost of these ingredients and instability during processing prevent their use in everyday food products.

Caseins in milk belong to the group of metal-chelating phosphoproteins, due to the presence of largely, clustered phosphorylated serine residues. These naturally occurring phosphoproteins act as multi-dentate ligands that bind metals strongly. Multivalent metals, such as calcium, copper, iron and magnesium, bind preferably to the phosphoserine residues compared to monovalent ions. Electrostatic interactions are involved in the binding of calcium and magnesium to caseins whereas coordination bonds are involved in the binding of iron and copper to caseins. Since coordination bonds are stronger than the electrostatic bonds, the binding constant for caseins to bind iron is higher than that for calcium. This property of caseins to bind iron has been exploited both commercially and scientifically to create ingredients for food fortification. While academic studies have successfully fortified milk and cheese with casein bound iron, commercial applications have largely attempted creating an ingredient for food fortification. The ferric form of iron has been used as a preferred source for iron, owing to its higher capacity and stability to bind caseins as compared to the ferrous form (Raouche et al., 2009a). Ferrous iron is easily oxidised to ferric form in the presence of oxygen commonly encountered in food processing operations (Emery, 1992).

Although the iron-casein complexes have been produced commercially, the highest concentration of iron added to casein in a dispersible format was 1% on w/w basis (Sher et al., 2006). Iron-casein complexes containing higher concentration of iron (> 1% w/w) have been made by freeze drying the casein precipitate generated upon iron addition (Zhang et al., 1989a). Consequently, these complexes could not be included in liquid food products. The precipitation of milk protein (especially caseins) at high levels of iron addition is a major drawback in the development of a commercially-viable ingredient.

The research undertaken to date on the binding of iron to proteins in milk, sodium caseinate and pure caseins has provided the following insights :

- Added iron binds mainly to proteins in milk. Caseins in milk have a higher capacity and ability to bind iron as compared to whey proteins.
- The higher iron binding capacity of caseins is due to the presence of clusters of phosphoserine residues. Among α_{s1}-, α_{s2}-, β- and κ-caseins, caseins with higher levels of phosphoserine residues bind higher amounts of iron.
- The binding of iron to casein is *via* coordination bonds with oxygen of the phosphoseryl residues. These coordination bonds formed remain unaffected by changes in ionic strength, pH and temperature.
- Precipitation of caseins occurs when charges on the caseins are neutralised upon iron addition. The precipitation of caseins in sodium caseinate solution (2.5% w/v protein) occurs upon addition of ≥ 2 mM ferric iron. However, when added to milk, up to 20 mM of iron could be added with minimal precipitation.
- The addition of ferric iron to milk results in a reduction of aqueous phosphorus content in a concentration-dependent manner. The formation of insoluble ferric phosphate has been confirmed inside the casein micelle upon high levels (20 mM) of iron addition.

One of the most notable observations in the above studies was the difference in the amount of iron that could be added to milk and sodium caseinate with minimal precipitation. Up to 10 times the concentration of iron could be added to milk with minimal protein precipitation as compared to sodium caseinate. The presence of minerals in milk was probably responsible for the higher amounts of iron that could be bound to proteins. However, milk contains ~32 mM calcium of which ~70% is bound to

the proteins. Both iron and calcium bind to phosphoserine residues on the caseins. Thus it would be interesting to further explore the binding of iron to milk proteins in the absence or presence of calcium in milk systems.

Another important area of research would be to understand the effect of phosphorus on the binding of iron to caseins. A reduction in the soluble phosphorus content has been observed in a number of studies (Hekmat et al., 1998; Raouche et al., 2009b) that have examined the effect of iron addition on the distribution of minerals in milk.

This thesis aims to further investigate the binding of iron to proteins in milk and in sodium caseinate by altering the mineral composition of these systems. In the case of milk, the effects of calcium depletion on the binding of iron to caseins have been examined. Furthermore, the effects of iron addition on the distribution of minerals in the milks with different levels of calcium depletion have been investigated. The information from this work helped design further experiments for characterising the effects of orthophosphate on the precipitation of caseins in sodium caseinate. Finally, based on the knowledge gained, new mechanisms have been put forward explaining the role of inorganic phosphorus in the binding of iron to caseins.

The research encompasses the following objectives:

1. To determine the effect of calcium-depletion on iron binding properties of milk.
2. To examine the influence of iron addition on the physico-chemical and microstructural properties of calcium-depleted milks.
3. To study the effect of calcium re-addition and exogenous orthophosphate inclusion on the properties of iron fortified calcium-depleted milk.

4. To elucidate the effect of exogenous orthophosphate inclusion on the iron-induced precipitation of caseins in sodium caseinate.
5. To examine the effect of orthophosphate addition on the physico-chemical properties of precipitated casein-iron complexes in sodium caseinate.

Chapter 2. Literature review

2.1. Introduction

Iron deficiency is a global nutritional problem. Almost 1.62 billion people around the world (24%) are estimated to be suffering from anaemia (McLean et al., 2009), an advanced stage of iron deficiency (Morón et al., 2009). Affected population is concentrated mainly in the developing nations, with the highest proportion (47-67%) being in the African continent and the largest number (315 million) in South East Asia. Iron deficiency affects people of all ages but predominantly pre-school children and pregnant women (McLean et al., 2009).

Iron is essential for optimal functioning of red blood cells which carry oxygen throughout the body. Lack of iron can result in a range of health issues, with the most severe being iron deficiency anaemia (IDA). Some of the symptoms associated with iron deficiency are fatigue and general weakness, shortness of breath, hair loss and restless leg syndrome. Statistics from the WHO global database show that IDA affects 47% of preschool age children. High rates of anaemia are also seen in pregnant women, with up to 42% being affected (Benoist et al., 2008). Children of school going age with iron deficiencies have shown decreased concentration spans and performance, in comparison to their counterparts (Falkingham et al., 2010). Overall it is obvious that iron deficiency has extensive global implications.

It is well known that non-haem iron from plants is not easily absorbed in the body. This is due partially to the presence of inhibitors or anti-nutritional factors present in plant based foods (Lynch, 2000). Phytic acid (myo-inositol hexaphosphate) has the ability to

chelate some micronutrients, such as iron, calcium and zinc, therefore making it insoluble and unavailable for absorption by the body. The level of phytate present in a range of foods was determined (Gibson et al., 2010) and phytate was found to be present in high amounts in unrefined grains and legumes.

The literature review covers the basic aspects of milk proteins relevant to the binding of iron. Furthermore, the effects of adding iron on the properties of milk and model systems have been reviewed. The manufacturing processes and applications of protein-iron complexes in food products undertaken by academia and industry have also been examined.

2.2. Distribution and absorption of iron

A normal person contains approximately 2.5 to 4 g of iron depending on gender, body weight and age. Of this, the majority (65 to 70%) of iron is present in the red blood cells as haemoglobin. At least 15% of iron is stored in muscles as myoglobin, with the remaining portion stored in the liver, spleen and bone marrow as ferritin. Most of the iron in the human body is recycled through the phagocytosis of red blood cells. Even though women have less iron (approx. 3 g) than men, they have a higher requirement for iron due to menstrual blood loss and increased requirement during pregnancy. Of the total amount of iron in human body, only 0.5 to 2 mg of iron enters and leaves human body on a daily basis (Andrews, 2000). Regular consumption of minor but important quantities of iron is required to maintain these small losses of iron on a daily basis and makeup for higher demands during pregnancy, growth and blood loss.

Absorption of iron in the human body is highly regulated with only 10 to 15% of the total ingested iron being absorbed (Lynch, 2000). The low bioavailability of non-haem

iron sources may be a blessing in disguise, as if it were more bioavailable it could lead to excess iron in the body, with serious repercussions to health. Absorption of iron in the human body is affected by multiple factors including: its form (haem or non-haem), oxidation state (+2 or +3), properties of the food matrix, inhibitory substances present in the food and iron status of individual and health of individual. Multiple dynamic equilibrium adjustments and regulation mechanisms determine the iron homeostasis (balance) in human body, which depends on the ability of the digestive and absorptive processes to extract iron from the ingested food (Lynch, 2000). Iron absorption occurs predominantly in the duodenum and upper jejunum (Conrad et al., 1999; Crichton, 1991). The absorption of iron rather than its excretion is considered the most important determinant of iron balance in human body (Finch, 1981).

Dietary iron is available either as a relatively easily absorbed haem iron or as non-haem iron which is considered less easily available. Haem iron is primarily available *via* a diet rich in meat, fish and poultry. This high bioavailability of haem iron is attributed mainly to its solubility in alkaline conditions present in the duodenum and jejunum (West et al., 2008). Non-haem sources of iron are available mainly from plant sources, as ferrous or ferric form. The low bioavailability of non-haem sources of iron is related to their low solubility at the intestinal pH. The ferrous form of iron can be easily oxidised to its ferric state in the presence of oxygen commonly encountered under normal food preparation conditions. The fact that ferric salts of iron are precipitated as ferric hydroxide above pH 3 makes them unavailable for absorption in the duodenum (Conrad et al., 2002). The availability of non-haem iron in the duodenum is governed by the ability of dietary and intestinal derived substances to chelate iron. The role of amino acids released during gastric digestion is to bind iron and deliver in a soluble form to the duodenum, therefore aiding in its absorption. Inhibition of iron absorption in the

presence of phytate, tannin, oxalate and polyphenols in the diet is due to their ability to precipitate and form insoluble complexes (Conrad et al., 2002).

Once iron is delivered to the duodenum in a soluble state, irrespective of its form (haem or non-haem), absorption of iron follows different pathways for entry into a common pool of iron. Two pathways demonstrated for haem iron absorption include the direct absorption of intact metalloporphyrin receptor and presence of haem transporter which carries haem from the small intestinal lumen directly into the cytoplasm (West et al., 2008). Whatever the mode of haem iron absorption, it is eventually cleaved by oxygenase to release iron into the common pool (Grasbeck et al., 1979). Non-haem iron that reaches the duodenum in a soluble state in the ferric form is first acted upon by ferric reductase which converts it to the ferrous form before it is transported across the membrane by divalent metal transporter 1 (DMT1) (Nancy, 1999).

2.3. Chemistry of iron

Iron is a highly reactive transition element with atomic number 26 and atomic weight 55.845. The electronic configuration of iron is written as:

$$1s^2, 2s^2, 2p^6, 3s^2, 3p^6, 4s^2, 3d^6$$

It has 26 protons and 30 neutrons in its nucleus and can occur in wide range of oxidation states (-2 to +6). It easily loses its valence electrons (two electrons from 4s orbitals and one electron from 3d orbital) to achieve a more stable configuration hence the +2 (Ferrous) and +3 (Ferric) states are most commonly encountered in aqueous solutions.

Acids are characterised by their preference for hard or weak bases. Hardness of acids increases with an increase in the oxidation state (Nicholls, 1974). According to Pearson (1963) acid base concept, Fe^{+3} is a hard acid, whereas, Fe^{+2} a borderline between hard and soft Lewis acid as indicated in Table 2.1. In general, hard acid forms strong bonds with strong base (oxygen) ligands and is not easily oxidized. On the other hand Fe^{+2} forms complexes with a wide variety of both hard and soft ligands, although nitrogen and sulphur ligands are preferred over oxygen ligands (Crichton, 2001).

***Table 2-1*:** *Lewis acids and bases*

	Hard	Borderline	Soft
Acids	H^+, li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Al^{3+}, Mn^{2+}. Fe^{3+}, Co^{3+}, La^{3+}	Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}	Cu^+, Ag^+, Au^+, Hg^{2+}, TI^+
Bases	NH_3, RNH_2, N_2H_4, OH^-, ROH, RO^-, CO_2^{-3}, F^-, Cl^-	N^{3-}, N_2, Br^-	H^-, R^-, CO, RSH

Adapted from (Crabb et al., 2010b)

Iron forms coordination complexes, wherein both electrons in the complex are provided by the ligands. Ferrous and ferric iron are found mostly with a coordination number 6 and frequently form octahedral complexes with ligands (Gerloch et al., 1994). The strength of these complexes is determined by the crystal field splitting energy (Δ) of ligands. A large Δ, which is due to the presence of strong ligand, results in the formation of low spin complexes, whereas the weak field ligands results in higher spin complexes and higher ionic radius. Low spin complexes are exchange inert and strongly bind field ligands, like OH^- (Petrucci, 2011). This theory explains why ferric ion which is a strong acid with low ionic radius, forms strong complex with oxygen bearing

ligands. Ferric iron binds strongly to major milk proteins and exerts less catalytic effect on oxidation than ferrous iron (Hegenauer et al., 1979a).

2.4. Iron fortification of foods

Fortification of foods is a common method used to deliver nutritionally-important minerals in required quantities to the consumer. Many technological problems can occur in fortified foods due to the chemical reactivity of minerals. These problems are reflected as changes in colour, flavour and functional properties of the food product. The solubility and chemical reactivity of the added mineral determine the kind and extent of reactions that may occur within a food system. The following factors are considered important when choosing a mineral for fortification of foods: (1) relative bioavailability of the mineral (2) reactivity of the mineral (3) stability of the mineral under processing and storage conditions (4) compatibility with other food components. One of the main conflicts that has arisen surrounding iron fortification is retaining its bioavailability while maintaining the product characteristics. The general consensus is that greater bioavailability is found in iron ingredients that have improved solubility at the duodenal pH. Table 2.2 lists the common iron fortificants and properties that are important for food applications. The most commonly used iron fortificants are ferrous sulphate, ferrous fumarate, NaFeETDA and elemental iron.

Table 2-2: *Characteristics and applications of common iron fortificants*

Fortificant	Characteristics	Iron%	Relative bio-availibility	Application
Ferrous sulphate.$7H_2O$	Bluish green crystals, water soluble, saline styptic taste, off flavour generation, pH -3.7, easily oxidised in moist air	20	100	Powdered milk, infant formula, wheat products
Ferrous gluconate	Yellowish grey to yellowish green powder, caramel odour, water soluble	12	89	Powdered milk, infant formula, wheat products
Ferrous lactate	Yellow to green powder, sweet taste, water soluble	19	106	Cereals, milk
Ferric ammonium citrate	Reddish brown granular powder, water soluble	16 –18	-	Powdered milk, infant formula
Ferrous fumarate	Reddish brown powder, slightly soluble in water, odourless and tasteless	33	100	Infant cereals, corn meal, wheat flour, salt, semolina bread flour
Ferric citrate	Brown granular powder, slowly soluble in water	16 – 18	-	Infant cereals, corn meal, wheat flour, salt
Ferric pyrophosphate	Yellowish to white, odourless, insoluble in water	24 – 26	21-74	Infant cereals, rice, salt
Carbonyl Iron	Grey coloured, water insoluble	> 98	5-20	Wheat flour
NaFeEDTA	Yellowish to brown crystalline powder,odourless,	12 – 13	> 100	Cereals,condiments, sugar, soy sauce, fish sauce

Nestel Penelope and Nalubola Ritu. Technical Brief on Iron Compounds for Fortification of Staple Foods.INACG, 2002. Hurrell Richard “Iron”, The Mineral Fortification of Foods. 1st ed. 1999: 54-93

Ferrous sulphate is water soluble, highly bioavailable and cheap, making it a popular choice with many food manufacturers. However, its high solubility means that it is

likely to interact with other ingredients in the food matrix leading to oxidation of fats, discolouration and deterioration of organoleptic properties over long-term storage (Edmonson et al., 1971; Wang et al., 1973). Less soluble iron fortificant can be incorporated into food products relatively easily and their reduced solubility means that there are fewer side effects experienced with the product (Hurrell, 2002). Ferrous fumarate has a similar bioavailability to ferrous sulphate and it works well in most food products; however, issues have arisen when acid conditions in food products caused complete dissolution of the fumarate affecting both product colour and taste (Hurrell, 2002). Chelated forms of iron are also a convenient choice with solubility occurring at high pH values which render it available for absorption within the body. As iron is bound to a ligand, it is prevented from interacting with various inhibitors present in the food matrix (Hegenauer et al., 1979b). The inclusion of these fortificants is dependent on product characteristics, such as pH. NaFeEDTA is an example of one such chelated iron product that is widely used in both the food and animal feed industries, at a higher cost to the producer and therefore to the consumer. Another example of an iron fortificant that is used frequently in the food industry is ferrous glycinate which is stable under most conditions but is not stable at low pH (Ding et al., 2011) .

As mentioned previously, many compounds present in or added to foods can act as anti-nutrients or inhibitors to iron absorption. On the other hand, there are also substances that enhance iron absorption in the body. Addition of substances, such as ascorbic acid, to a food product has been shown to improve absorption as they can (1) reduce ferric iron to ferrous form making it available for absorption (2) have the ability to chelate iron making it a soluble at the pH of the duodenum. An increase in iron absorption due to ascorbic acid has been documented in numerous *in vitro* and *in vivo* trials (Cook et al., 2001; Forbes et al., 1989; Kim et al., 2011; Siegenberg et al., 1991). Other

substances which may play a role in iron absorption are cysteine-containing amino acids (Taylor et al., 1986).

2.5. Aspects of milk proteins

Milk is Nature's vehicle for delivering essential nutrients (fat, protein, lactose, vitamin and minerals) from mother to its offspring. Caseins, the major proteins in milk (80% of total proteins), are synthesized in the mammary glands and serve as a carrier of calcium and phosphorus in a highly bioavailable form (Holt, 1992). Caseins are present in milk as colloidal particles called casein micelles (Fox et al., 2008). Whey proteins are present in the soluble state and constitute ~ 20% of the total proteins in milk. Whey proteins constitute a heterogeneous group of proteins which remain soluble in milk upon enzymatic or isoelectric precipitation of caseins (Hambraeus, 1982). The technological and sensory properties of most dairy products are largely governed by the properties of casein micelle. Recently, the role of milk proteins as carrier of essential minerals, vitamins and bioactives has been well documented (García-Nebot et al., 2010; Livney, 2010; Raouche et al., 2009a).

2.5.1. Caseins

Bovine milk is a major source of high quality dietary protein which also serves to regulate various physiological functions in human body. The presence of essential amino acids and unique digestibility features of different proteins give milk proteins an important dietary status. Several reviews provide an in-depth look at milk proteins (Dalgleish et al., 2004; Fox et al., 2008; Holt, 1992; Holt et al.; Horne, 1998; Horne, 2006; McMahon et al., 1984; McMahon et al., 2008). This section provides an overview

of the compositional and structural properties of milk proteins relevant to the topic of this thesis.

The composition and structural properties of major milk proteins are shown in Table 2.3. Casein is a general name given to a group of phosphoproteins (α_{s1}-, α_{s2}-, β- and κ-caseins) present in milk. Specificity of caseins to bind calcium as colloidal calcium phosphate prevents their precipitation. Synergistically, the colloidal calcium phosphate plays an important role in the stability and structure of caseins micelle.

Table 2-3*: *Composition and structural attributes of milk proteins

	Whey Proteins			**Caseins**			
	β-lg	α-lac	BSA	α_{S1}-	α_{S2}-	β-	κ-
Proteins (g/L)	3.0	0.7	0.1-0.4	12-15	3-4	9-11	2-4
Molecular Weight (Da)	18362	14194	65000	23612	25228	23980	19005
Total residues	162	123	581	199	207	209	169
Apolar residues [%]	34.6	36	28	36	40	33	33
Proline residues	8	2	28	17	10	35	20
Phosphoserine groups	0	0	0	8–9	10–13	5	1

Data obtained from Fox (2009) and Kinsella et al (1989)

Caseins are considered as class of 'intrinsically unstructured' proteins as they lack the cysteine residues, which form disulphide bonds and stabilize the tertiary structure of globular proteins (Farrell, 2011). Unlike whey proteins, all caseins contain phosphorylated serine residues in clusters of 2, 3 or 4, located unevenly along the polypeptide chain length (Figure 2.1). This gives α_{s1}-, β-, κ-casein a phosphorus content

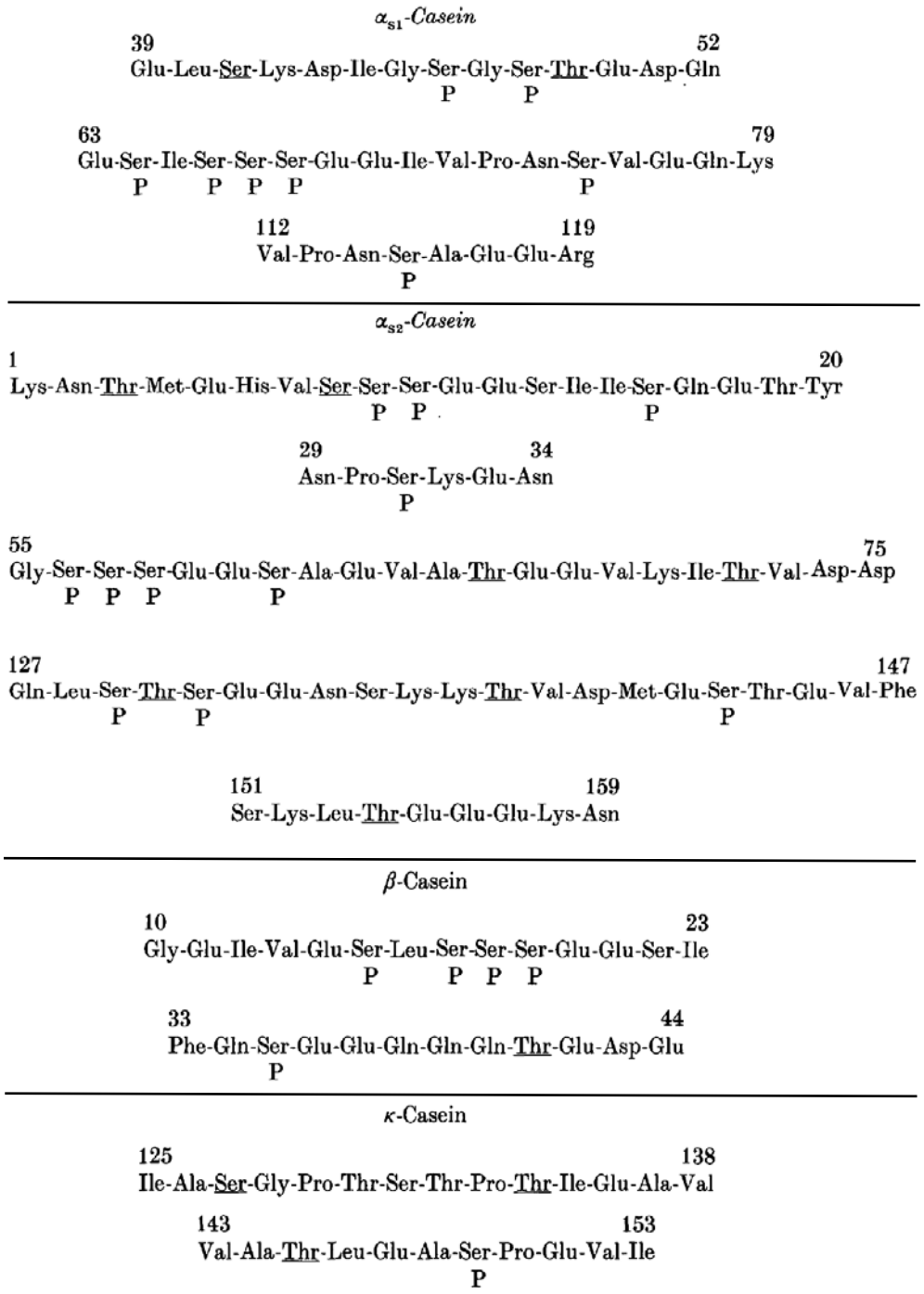
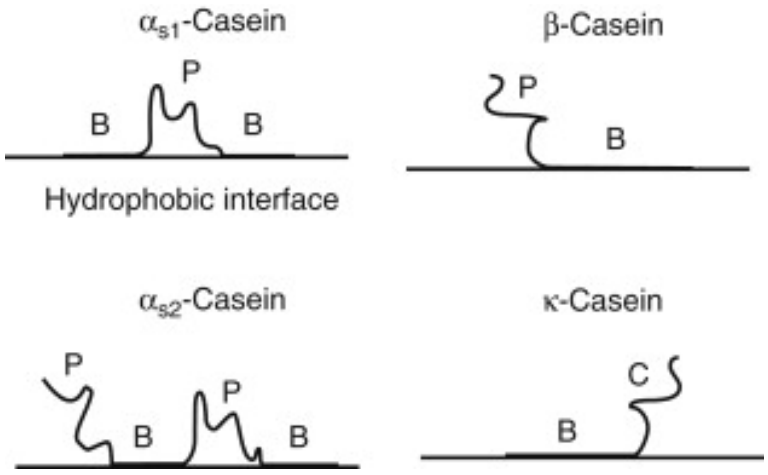


Figure 2-1*: *Phosphorylated amino acids on fractions of different caseins. Adopted from West (1986).

of 1%, 0.5% and 0.2% respectively (Dickson et al., 1971). The molecular weight of phosphoserine residue is 167 daltons (West, 1986). The clusters of phosphorylated serine residues act as multidentate ligands capable of binding to the calcium phosphate

nanoclusters in preference to single phosphate ions in the serum (Holt, 2004). This clustering of phosphoserine residues gives the caseins distinct hydrophilic and hydrophobic regions and hence their ampiphatic nature (Horne, 2006) as shown in Fig 2.2.

The ampiphatic nature is preserved across species for major α_{S1}- and β-caseins (Schmidt, 1970). Caseins lack a stable structure (α-helix and β sheet) owing to high level of uniformly distributed proline residues and have been termed as rheomorphic proteins (Holt et al., 1993).



B denotes hydrophobic regions and **P** hydrophilic regions

Adapted from Horne (2011)

Figure 2-2**:** *Depiction of the crosslinking of casein molecules via hydrophobic interaction sites.*

Phosphorylation of caseins is important for the structural integrity, nutritional quality and technological properties of milk (West, 1986). Those caseins (α_{s1}-, α_{s2}-, β-casein) with a high degree of phosphorylation are sensitive to Ca^{+}, whereas κ-casein which

contains only one phosphorylated residue stabilizes the former group of caseins against calcium-induced precipitation.

Most interesting characteristics of these individual caseins is their tendency to self-associate/polymerise *via* hydrophobic interactions as shown in Figure 2.3. α_{s1}-Casein self-associates into polymer-like chain with hydrophobic regions joined end to end (Thurn et al., 1987), whereas, β-casein associates into spherical particles in the absence of calcium (Swaisgood, 2003). However, this self-association of caseins (especially for α_{s1}- and α_{s2}-casein) is limited by electrostatic repulsive interactions, due to the available charge on amino acids, which is governed by pH and ionic strength (Horne, 1998). The self-association characteristics of β-casein are governed by the large hydrophobic region in its polypeptide chain and β-casein exists as a monomer at low temperature due to the absence of hydrophobic interactions (Morris, 2002; Swaisgood, 2003). The ability of α_{s1}- and α_{s2}- caseins to exist as oligomers and polymers at low temperatures points to the involvement of hydrogen bonds and secondary structures at the hydrophobic surface governing their association behaviours (Swaisgood, 2003). Both α_{s1}- and α_{s2}- casein show a pH and ionic strength-dependent association in a stepwise manner, i.e. monomer to dimer, dimer to trimer and so on (Rollema, 1992; Schmidt, 1970), as indicated below:

$$(\alpha_{s1}\text{-casein})_n + (\alpha_{s1}\text{-casein}) \longrightarrow (\alpha_{s1}\text{-casein})_{n+1}$$

Schmidt (1970) used light scattering to monitor the influence of ionic strength on the association behaviour of α_{s1}-casein (Table 2.4).

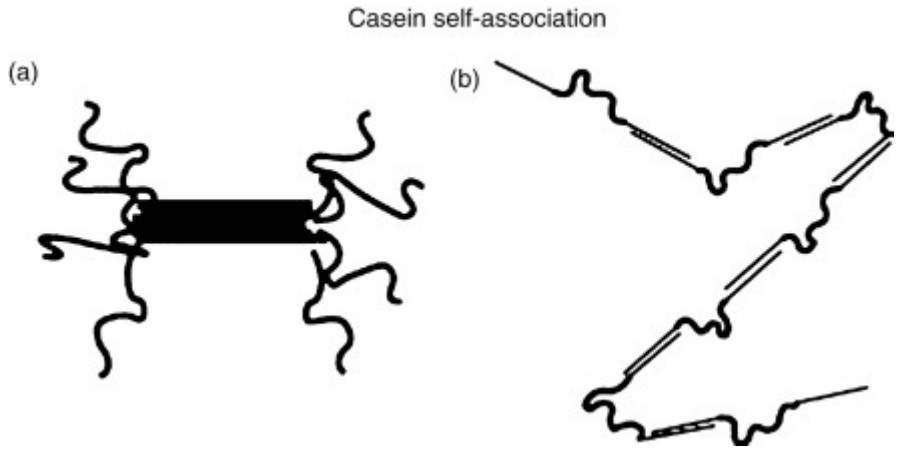


Figure 2-3**:** *Structures depicting the self-associating polymers of (A) β-casein and (B) α_{S1}-casein based on interactions of their hydrophobic regions (Horne, 1998).*

Table 2-4**:** *Influence of ionic strength on the association behaviour of α_{s1}- casein*

Ionic strength	**Existence of α_{s1}- casein**
<0.003	Monomers
0.01	Monomers and dimers
0.2	Dimers and tetramers

Increasing the ionic strength results in increased association due to shielding of charges on the amino acid residues, thereby reducing the electrostatic repulsions (Swaisgood, 2003). β-Casein self-associates into discrete polymers (Rollema, 1992), containing 15-60 monomers above a critical micelle concentration (0.5 mg/mL to 2 mg/mL) (Evans et al., 1979; Schmidt et al., 1972) at temperatures above 5^{o}C as indicated below:

$$n(\beta\text{-casein}) \longrightarrow (\beta\text{-casein})_n$$

κ-Casein self-association involves both hydrophobic attraction and intermolecular disulphide linkages produced by reaction of its cysteine residues (Horne, 2009). The self-association behaviour of individual caseins (α_{s1}-, β-) in the absence of Ca^{2+} is shown in Figure 2.3.

When present as mixtures, as in sodium caseinate, the caseins show different degree of de-mixing, resulting in mutual solubilisation of caseins (Farrer et al., 1999). The association behaviour of caseins is concentration-dependent; at low casein concentration, κ-casein dissociates and forms associated structures independently (Pepper et al., 1982). A submicellar structure with an average size of 20 nm for associated particles of casein solutions has been found, using dynamic light scattering and gel permeation chromatography (Chu et al., 1995; Pepper et al., 1982; Pitkowski et al., 2009). A trimodal distribution was observed by size exclusion chromatography for 2% sodium caseinate solutions; the different peaks consisted of mixtures of different caseins and contained monomers, casein complexes and aggregates (Lucey et al., 2000). Furthermore, the distribution was concentration-dependent; increasing the casein concentration resulted in an increase in the proportion of larger-sized aggregates, whereas the proportions of smaller aggregates decreased. Lucey et al., 2000) characterised commercial sodium caseinate samples, using multi angle laser light scattering (MALLS) technique and suggested that the rod-shaped self-associated structures were 169 nm in length and 1 nm in diameter, as calculated from the observed Rg (Z-average root mean-square radius) of 48.7 nm. Similar rod-like aggregate images were observed by Farrer and Lips (1999) using TEM. Thurn et al (1987) studied the association of α_{s1}- casein and reported the formation of extended rigid structures with persistence length of 130 nm. In the absence of calcium, caseins interact with each

other, with the interactions between κ-casein and α_{s1}- casein being stronger than α_{s1}- and β-caseins (Farrell, 2011).

The self-association of caseins follows different pathways in presence of calcium (Pitkowski et al., 2009). Calcium binds to the phosphoserine and carboxylic acid residues of individual caseins and causes their aggregation by shielding the net charge and lowering electrostatic repulsions (Byler et al., 1989). There are ~14 binding sites for calcium on caseins in sodium caseinate (Mekmene et al., 2011). The extent of aggregation in the absence of κ-casein depends on the concentration of calcium added and the number of phosphoserine residues available on the caseins. Due to the presence of just one phosphoseryl residue, κ-casein is affected little by the presence of calcium. This property of κ-casein limits the size of aggregates owing to their association with α_{s1}- and β-caseins in the presence of calcium (Dickson et al., 1971).

2.5.2. Casein micelles

Casein micelles differ from casein aggregates due to the presence of inorganic phosphate resulting in the formation of nanoclusters of calcium phosphate in their structure (Horne, 2011). Casein micelles exist mostly (95%) as roughly spherical colloidal particles of sizes ranging from 50-500 nm (Fox et al., 2008), with an average hydrodynamic diameter of 180-200 nm and containing approximately 2 X 10^4 casein molecules (Dalgleish, 2011; Gaucheron, 2005). They are highly hydrated (3.5 g water per g of protein) and occupy one tenth of the volume of milk (Dalgleish et al., 2012). Table 2.5. shows some characteristics of casein micelles (Fox et al., 2008).

Table 2-5: *Some characteristics of casein micelles*

Characteristics	**Value**
Diameter	120 nm (range: 50-500 nm)
Surface area	8 x 10 cm^2
Volume	2.1 x 10 cm^3
Density (hydrated)	1.0632 g cm^{-3}
Mass	2.2 x 10-15 g
Water content	63%
Hydration	3.7g H_2O g^{-1}protein
Voluminosity	44 cm^3 g^{-1}
Molecular mass (hydrated)	1.3 x 10^9 Da
Molecular Mass (dehydrated)	5 x 10^8 Da
No. of peptide chains	5 x 10^3
No. of particles per mL milk	10^{14} – 10^{16}
Surface of micelles per mL of milk	5 x 10^4 cm^3

Adapted from (Fox et al., 2008)

The casein micelle is presumed to have a sponge-like (Bouchoux et al., 2010) open structure, with the space within filled by milk serum. The organisation of caseins into casein micelles along with the presence of amorphous calcium phosphate provide them enhanced stability and conformational flexibility to interact with target molecules (Holt et al., 1996; Smyth et al., 2004). A typical casein micelle with a radius of ~100 nm contains 800-1100 nano-clusters with uniform composition, containing ~355 ± 20 positively charged amorphous calcium phosphate ($CaHPO_4$ x 2 H_2O) electrostatically bound by nearly 50 peptide chains *via* the negatively charged phosphoserine (Choi et

al., 2011; Cross et al., 2005; Holt et al., 1998; Little et al., 2004). Because, the physico-chemical properties of casein micelle affect the properties of both traditional and novel dairy products, enormous research has gone into the study of the surface and internal structures of casein micelles (Dalgleish, 1998; Dalgleish et al., 2004; de Kruif et al., 2012; Griffin et al., 1985; Hansen et al., 1996; Holt, 1992; Holt et al., 2003; McMahon et al., 1984; Payens, 1979; Schmidt, 1980; Tuinier et al., 2002; Waugh et al., 1965).

Various structural models of casein micelles have been presented after the discovery, isolation and characterisation of micelle stabilizing κ-casein by Waugh and Von Hippel in 1956 (Fox, 2009). Schmidt (1980) presented the first widely used 'submicelle' model based on protein interactions leading to formation of spherical aggregates (submicelles) held together by calcium-phosphate linkages (Qi, 2007). The following sequence of events proposed by Bauchheim and Welsch (1973) explains the submicelle model:

Casein monomers $\longrightarrow$ caseinate subunits + calcium phosphate $\longrightarrow$ casein micelles

A 'nanocluster' model considers the binding capacity of casein phosphopeptides to calcium phosphate which are present as a nanoclusters of 2-3 nm diameter surrounded by 50 phosphopeptide chains (Holt, 2004), homogeneously distributed throughout the casein micelle structure. This model could explain the internal structure and heterogeneity observed by transmission electron microscopy (TEM), but had limitations with respect to explanation for surface location of κ-casein and hence the termination of micellar growth (Horne, 2006). Surface hydrophobicity of caseins, their interactions leading to formation of phosphopeptide loop and bridging across calcium phosphate nanoclusters have been proposed as prerequisite for micelle growth and assembly in 'dual binding' model proposed by Horne (1998). 'Dual Binding' model encompasses both self-association behaviour and calcium bridging interactions of caseins to describe

the micellar integrity. A recent model put forward by de Kruif et al (2012) encompasses hydrophobic interactions, hydrogen bonding, ion bonding and weak electrostatic interactions all under the term 'weak interactions' responsible for self-association within the casein micelles along with bridging across the calcium phosphate. Although differing in various aspects, the above mentioned models agree with respect to the cementing role of calcium phosphate, the surface location and steric stabilization role of κ-casein (Fox, 2009).

Transmission and scanning electron microscopy have been used to decipher the internal and external structures of casein micelles but these techniques are limited by artifacts generated due to fixatives and metal staining used to accentuate the structures (Qi, 2007). Images of electron microscopy showing raspberry-like casein micelle structure supporting the 'submicelle' model of casein micelle turned out to be artifacts of sample preparation and treatment (McMahon et al., 1998). McMahon and Oommen (2008) proposed an interlocking lattice model based on TEM images using freeze-dried surface immobilized casein micelle without resin embedding. This model suggests that the electron dense region consisted of short linear and branched polymer chains of caseins interlocked by calcium phosphate nanoclusters. The tubular structures as proposed by McMahon and Oommen were also observed by Dalgleish et al (2004) using cold field-emission ultra high resolution scanning electron microscope (FESEM). Fibril-like structure in bovine casein micelles has also been reported by Lencki (2007) using Congo Red absorbance spectroscopy, Thioflavin T fluorescence spectroscopy and TEM imaging. He observed amyloid-like protofibrils of caseins 7 to 10 nm in length with 5 nm dense aggregate junction zones ascribed to interactions of these fibrils with calcium. Similarly, the existence of complex network of protein chains was confirmed by Marchin et al (2007) using small angle X-ray scattering analysis. Hence, the submicelle

model explaining spherical submicelles has now become obsolete with polymers/tubules/fibrils being used to demonstrate the latest models of casein micelle structure.

2.5.3. Casein micelle integrity

Casein micelles are stable to various technological treatments, such as heating, concentration, cooling and homogenization (Fox et al., 2008; Marchin et al., 2007). The role of calcium phosphate in maintaining the integrity of casein micelle is unanimously agreed in all casein micelle models till date. Casein micelle integrity is maintained even upon acidification up to pH 5.2, renneting by chymosin and upon chilling, indicating the role of various interaction energies in maintaining the structure. Casein micelles can be disintegrated by removing or binding calcium at the normal pH of milk (Griffin et al., 1988; Holt et al., 1986b; Lucey et al., 1997) and reformed into a micellar structure upon calcium re-addition (Grimley et al., 2010). The ability of casein micelle to maintain its structure upon acidification points to the importance of hydrophobic interactions in micelle integrity (Dalgleish et al., 1988; McGann et al., 1974). The role of hydrophobic interactions can also be envisaged by studying the effect of temperature on the binding of calcium to β-casein. β-Casein does not precipitate in presence of Ca^{2+} at low temperatures but at temperatures around 20^{o}C, aggregation occurs (Dalgleish et al., 1980) due to dominance of hydrophobic attractions (Horne, 1998). Dissociation of casein micelle into smaller particles by urea without disruption of calcium phosphate bridges also demonstrates the importance of hydrophobic and hydrogen bonding in casein micelle integrity (Aoki et al., 1986; McGann et al., 1960). Although both ‘interlocking lattice’ and ‘dual binding’ model presented by McMahon (2008) and Horne (1998) use hydrophobic interactions and bridging across the calcium phosphate

nano clusters to explain micellar integrity, the more elaborately discussed 'dual binding' model has been used within the scope of this thesis.

The 'dual binding' model groups both hydrogen bonding and hydrophobic attraction under the label of hydrophobic interactions (Horne, 2006). Self-association behaviours of caseins are well recognised and are driven by these interactions, but the associations are limited by electrostatic repulsions due to charge on amino acids involved. This model considers casein micelle as hydrophobic colloid, stabilized by delicate balance of hydrophobic attraction and electrostatic repulsion ((Horne, 1998):

Interaction energy = electrostatic repulsion + hydrophobic interaction

The positively charged calcium phosphate acts as neutralizing agent for negatively charged phosphoserine residues on caseins to the extent that enables the hydrophobic attraction to dominate and form the micellar structure (Horne, 1998). Electrostatic repulsions due to the presence of charged residues on amino acid clusters, are dependent on pH and ionic strength. Disruption of micellar structure which occurs at normal pH of milk due to any process (sequestration, dialysis, ion exchange etc.) of calcium removal is probably due to increase in the available charge and consequent shift of balance from hydrophobic attraction to electrostatic repulsion.

2.5.4. Dissociation of casein micelles

Dissociation of casein micelle can be achieved by any process which disrupts the balance between attractive and repulsive forces in favour of repulsion. Casein micelle contains approximately 3×10^3 nanogranules of calcium phosphate in an amorphous state (Gaucheron, 2005). Removal of substantial amount of calcium upon acidification without disruption of casein micelle can be achieved, possibly because of neutralization

of charged residues due to protonation upon acidification, leading to association of casein micelles (Dalgleish et al., 1989; Lucey et al., 1997). During sequestration by EDTA, protein charges remain unchanged and disruption of micelle is the result of the net decrease in the serum Ca^{+} activity and increased phosphate activity (Ward et al., 1997), whereas the reverse is true for micelle disintegration during acidification. Disintegration of casein micelle also occurs upon urea addition, due to disruption of the hydrophobic interactions. However, such caseins re-aggregate to form smaller-sized particles (McGann et al., 1974).

Lucey et al (1997) studied the effects of pH and concentrations of calcium and phosphate on the dissociation of casein micelles acidified to pH 5.3. They indicated that removal of calcium at normal pH of milk results into immediate disintegration of the casein micelle, probably due to the generation of charged residues and an increase in electrostatic repulsion (Lucey et al., 1997). Udabage et al (2000) reported that the maximum level of calcium solubilisation, without disruption of casein micelle structure, lies between 44 and 90% of calcium removal. Similar observations have been reported by Griffin et al (1988), who concluded that it was not possible to remove substantial amount of calcium without some micellar disaggregation. However, a critical concentration of calcium depletion is required to substantially disintegrate the casein micelle, below which the casein micelle integrity is maintained (Lin et al., 1972).

Holt et al (1986a) showed that dissociation of casein micelles could be achieved when free Ca^{2+} concentration in the serum was less than 1 mM, pointing to the importance of free Ca^{2+} concentration in the serum phase of milk. Furthermore, this dissociation could also be achieved upon removal of colloidal phosphate at pH 6.7 without reducing the free Ca^{+} ion concentration in the serum, indicating pivotal role of colloidal phosphate in

casein micelle integrity, although increasing Ca^{2+} could retard some dissociation of casein micelle. Importantly, irrespective of the mode of dissociation achieved, β-casein and κ-casein were preferentially dissociated as compared to α_{s1}- or α_{s2}-caseins, especially at lower temperatures (Holt, 1998).

Both phosphate and citrate salts have been used to reduce the free Ca^{2+} concentration in the serum phase (Tsioulpas et al., 2007). However, both these salts act differently; citrate reduces the free Ca^{2+} concentration by forming soluble calcium citrate complex, whereas phosphate addition results in precipitation of Ca^{+} as calcium phosphate (Tessier et al., 1958).

A detailed study tracking the casein micelle upon addition of polyphosphate and EDTA has put forward a hypothesis of "all or nothing" for the casein dissociation from the micelle (Pitkowski et al., 2007). They concluded that casein micelle remained intact until a critical concentration of calcium was depleted confirming earlier reports (Griffin et al., 1988; Marchin et al., 2007). Furthermore, the dissociation process followed a two-step kinetics, with a portion of micelle being rapidly dissociated upon chelation of calcium and remainder followed a slow process of dissociation in a concentration- and temperature-dependent manner. Cryo TEM images of 60% and 80% calcium-depleted milks indicated the presence of smaller-sized, less dense granular particles remaining in the suspension (Marchin et al., 2007). The temperature of milk during the calcium depletion process determines the composition of undissociated casein micelle. At low temperatures, higher dissociation of β-casein from the micelle was observed (Lin et al., 1972), whereas at room temperature the composition remained unchanged (Pitkowski et al., 2007).

Udabage et al (2000) investigated the reversibility of mineral-induced changes in casein micelle at a fixed pH. The soluble casein content was positively related to the concentration of calcium chelators, and that all the calcium chelators (citrate, Pi and EDTA) decreased the Ca^{+} activity to the same extent. The amount of soluble casein and associated minerals, which was reflected in the reduction in micelle diameter, was found to be directly proportional to the concentration of added EDTA and citrate. However, this dissociation was not completely reversible and only intermediate values for micellar caseins were obtained upon calcium addition. In the same experiment, addition of calcium chloride was accompanied by a reduction in serum phosphorus content, indicating the transfer of phosphorus along with added calcium to the micellar phase.

2.6. Minerals in milk

Minerals in milk constitute 0.7% by wt. of milk and make an important contribution to nutritional (bone growth, development and cellular functions), technological (structure and properties of milk products) and physico-chemical properties. Calcium, magnesium, phosphate and citrate are major salts in the colloidal phases associated with casein micelles and play an important role in micelle integrity and structure. Most of the anions, monovalent cations (Na^{+}, K^{+} and Mg^{+}) and trace minerals are present in the soluble phase of milk, along with whey proteins, either as free ions or complexes.

Although various techniques (ultracentrifugation, renneting and ultrafiltration) are used to study the partitioning of minerals between the soluble and colloidal phases, ultracentrifugation at 100,000 g for 1 h at 20^{o}C is considered to be most reliable owing to inclusion of minerals attached to whey proteins in its calculation (Gaucheron, 2005). Concentrations of major minerals in milk and their distributions are shown in Table 2.6.

Table 2-6: *Composition and distribution of minerals in milk*

Minerals	**Per kg milk**
Calcium (mg)	1043 – 1283
Colloidal calcium	65%
Calcium bound to Pser (mg)	300
Calcium bound as CCP (mg)	400***
Soluble calcium	35%
Total ionic calcium	5%
Total phosphorus (mg)	900-1000
Colloidal phosphorus	63.5%***
Organic phosphorus	18%***
Inorganic phosphorus (CCP)	82%
Soluble phosphorus	36.5%***
Bound to Ca and Mg	10%
Phosphate ion	90%
Total citrate (mg)	1300 – 2000
Colloidal citrate	11%
Soluble citrate	89%
Bound to Ca and Mg	85%
Ionic citrate	15%
Magnesium (mg)	100 – 150
Soluble magnesium	65%
Colloidal magnesium	35%
Sodium (mg)	400 – 600
Potassium (mg)	1200 – 1600

Compiled and calculated from Gaucheron (2005) and Tsioulpas et al (2007)

*** calculated values

CCP – Colloidal calcium phosphate

Pser - Phosphoserine

Approximately 40% of total buffering capacity of milk is due to soluble minerals and 20% is contributed by colloidal minerals (Françoise et al., 2005). Since minerals in

colloidal and soluble phase are in a dynamic equilibrium, the partitioning is affected by changes in temperature (Law, 1996; Pouliot et al., 1989; Rollema et al., 1989; Visser et al., 1986), pH (Ahmad et al., 2008; Dalgleish et al., 1988; Dalgleish et al., 1989; Visser et al., 1986), ionic strength (Jimenez-Lopez et al., 2011; Legraet et al., 1993) and/or addition (Gaucher et al., 2007; Griffin et al., 1988; Raouche et al., 2009a; Udabage et al., 2000) or removal (Ranjith et al., 1999; Ward et al., 1997) of minerals in milk and milk products. Phosphate, citrate, lactate, carbonate, acetate and propionate provide important contributions to the total buffering capacity of milk, especially in the range of pH 5.1–5.5 and 6.3–6.6 (Françoise et al., 2005). Addition of minerals and acids/bases to milk changes the ionic strength and the pKa values of the minerals. Consequently, the changes in ionic strength could influence the self-association behaviour of caseins and casein micelle integrity (Horne, 1998).

Among minerals, calcium is the most studied because of its influence on the stability of milk. The concentration of Ca^{2+} in the serum phase is important for stability of casein micelle (Holt, 1992) and reduction of Ca^{2+} results in disintegration of the casein micelle at the physiological pH of milk (Udabage et al., 2000). Ca^{2+} ion in the serum acts by influencing the surface negative charge of casein micelles thereby maintaining the balance of repulsive and attractive forces between casein micelles (Horne et al., 1982). However, the Ca^{2+} concentration is limited by its low solubility in the presence of inorganic phosphorus. Addition of exogenous orthophosphate results in the precipitation of calcium phosphate leading to a reduction of Ca^{2+} concentration (Udabage et al., 2000). In the presence of caseins, the calcium phosphate salts do not sediment independently and interactions with the soluble caseins occur (Horne et al., 1980). Almost all of calcium can be exchanged between the colloidal and soluble phases in less than one hour at elevated temperature (Pierre et al., 1981). The colloidal calcium

phosphate of milk solubilizes at lower temperature and precipitates upon heating (Holt, 1995).

The association of ions in the serum phase depends on the affinity constants of different ions at particular pH and the solubilities of the salts formed, depending on the temperature (Gaucheron, 2005). Citrate ions due to their higher affinity constants for Ca^{2+} and Mg^{+} ion act as chelators (Tsioulpas et al., 2007). Increasing the citrate content at normal pH of milk causes more binding of calcium to citrate and causes disintegration of casein micelles (Udabage et al., 2000).

In milk, the inorganic phosphorus (or orthophosphate) is distributed between the colloidal and soluble phase. In the colloidal phase, it forms the part of colloidal calcium phosphate, whereas in the soluble phase it is present mainly in the ionic form (Table 2.6). The inclusion of inorganic phosphorus is a pre-requisite for the formation of casein micelle (Aoki et al., 1987). The amorphous calcium phosphate nano-cluster consists of a calcium-rich and calcium-poor phases which have a Ca: P ratio of 2:1 and 1.5:1 respectively. The calcium-rich regions are responsible for binding of the nano-cluster to the organic phosphorus on caseins (Cross et al., 2005). The removal of this inorganic phosphorus from casein micelle, upon dialysis of milk, results in the disintegration of casein micelle (Aoki et al., 1988; Holt et al., 1986b). Furthermore, the crystalline structure of calcium phosphate observed in casein micelle under the TEM exists only in the presence of inorganic phosphorus (Guo et al., 2003).

2.7. Ion exchange of milk

One of the first example of ion exchange was mentioned in the old testament (Second book of Moses, Ex 15, 23 EU), wherein rotted cellulose contained in wood was used to de-bitter brackish water (Kammerer et al., 2010). Modern ion exchange resins are synthetically manufactured cross-linked polymer matrix with uniformly distributed fixed functional groups carrying oppositely charged ion which can be exchanged. Ion exchange (IE), as the name suggests, is a reversible and selective exchange of counter ions between an insoluble solid ion exchange resin and the interacting liquid medium. This exchange is *via* electrostatic interactions to achieve electro-neutrality within the resins (Kammerer et al., 2010). Ion exchange does not involve any chemical reaction but a rearrangement of exchanging ions in the solution and the resin to ensure maximum stability (Paterson, 1970b). The exchange rate is determined by the rate of diffusion of ions involved. A simple equation of the process may be described as below, where I^- represents co-ion and A^+ and B^+ the counter ions.

$$I^-A^+ \quad + \quad B^+ \quad \rightleftarrows \quad I^-B^+ \quad + \quad A^+$$

[IE resin] [Liquid] [IE resin] [Liquid]

Kinetics of ion exchange can be described as counter flow of two similar charges across the resin surface. The accumulation of charges at the surface of the ion exchange resins and the uncompensated charge on co-ion within resin creates an electrical potential across the exchanger-solution interface called the Donnan Potential (Paterson, 1970a). A negative Donnan potential forms at this interface for cationic resins which prevents the co-ion entry inside the ion exchange resin (Harland, 1994b). However, the principle of Donnan equilibrium is valid only for dilute solutions with counter ion concentration

less than 0.1 M (Fernandez et al., 1994). At higher concentrations, an anionic exchange along with cation exchange is observed which has been attributed to formation of ionic complexes (Fernandez et al., 1994). Although theoretical electroneutrality is envisaged throughout the ion exchange process, slight variations are observed during the actual process resulting from the difference in the diffusion rates of the counter ions creating diffusion potential across the resin surface (Kammerer et al., 2010).

Selectivity or affinity of ion exchange resins is affected by the characteristics of the ion exchange medium, resin and ions available for exchange. It is generally accepted that ion exchange affinity of ions is directly related to their charge and atomic number (DeSilva, 1999). This implies that K^+ or Na^+ form ion exchange resin will preferably exchange Ca^{2+} over Mg^{2+} and other monovalent ions present in the exchanging medium.

A resin with negatively charged functional group and an exchangeable cation attached is known as cation exchange resin, while in opposite positions make up an anionic exchange resin. Although many functional groups can be incorporated in the polymer matrix, most commonly employed cationic exchange groups are sulphonic ($R\text{-}SO_3H$) and carboxylic (R-COOH), representing a strong and weak acid cation exchange resin, respectively. Strong cation exchange resins are characterised by their ability to split neutral salts, whereas weakly acidic cation exchange resins are used for ion exchange of alkaline earth metals. Since, milk contains both proteins (neutral) and salts, weakly acidic cation exchange resin is preferred choice for calcium removal (Grimley et al., 2010; Lin et al., 2006; Ranjith et al., 1999), although earlier attempts to remove calcium has been done using mixed bed resins (Bishov et al., 1958).

Ion exchange has found applications in multidisciplinary fields for various processes, such as separation, decalcification, demineralisation and salt conversion catalysis. In the

food industry, the greatest applications are for water softening, waste water treatment, food processing (decolourisation, decaffeination, isolation of bionutrients etc.). Ion exchange has been used traditionally for manufacture of whey protein isolate, lactoferrin and demineralisation of whey by the dairy industry. Its use to remove calcium and take advantage of modified physico-chemical properties of milk has been commercially explored (Bhaskar et al., 2011; Lyman et al., 1933; Stahl. et al., 1984).

IE for removal of calcium from milk has been performed using column bed methods (Bishov et al., 1958; Coleman et al., 1954; Josephson et al., 1947) and batch methods (Grimley et al., 2010; Lin et al., 2006; Ranjith et al., 1999). The column bed method involves recirculation of the milk through a column saturated with IE resin at a constant flow rate, implying that the ratio of milk to resin is less than unity. In the batch method, different ratio of resin to milk volume has been attempted to remove varying amounts of calcium. Lin et al (2006) used highest ratio (1:1) of resin to milk to remove up to 80% calcium from permeate and skimmed milk, using sodium form of IE resin. Ranjith et al (1999) and Grimley et al (2010) used 10% and 3% v/v of resin in milk to remove 80% and 51% of total calcium from milk, respectively.

Removal of calcium from milk induces fundamental changes in the structural, physico-chemical and functional properties of milk, which have a direct influence on the technological aspects (rennet coagulation, heat stability, emulsifying capacity etc.) of milk and milk products.

As discussed earlier the calcium phosphate exists in dynamic equilibrium between the colloidal and soluble phases of milk (de la Fuente, 1998) and plays an important role in the structural integrity of casein micelles (Holt, 1992). Of the total calcium in milk, about 5 to 10% exists in the ionic form, which is bound by the cation exchange resin

during the ion exchange process (Grimley et al., 2010). However, the exchangeability of calcium between these two phases is very rapid (Pierre et al., 1983). As a result colloidal calcium is transferred to the soluble phase and is subsequently exchanged with ions on the resin.

The process of ion exchange for calcium depletion from milk is not new and was first reported by Lyman et al (1933). The following section describes the effects of ion exchange process on the physico-chemical, sensory and technological properties of milk and milk products.

2.7.1. Physico-chemical properties

The effects of calcium depletion on mineral content, sedimentation behaviour, ζ-potential, pH, colour and rennet coagulation have been assessed by a number of researchers (Coleman et al., 1954; Grimley et al., 2010; Haller et al., 1950; Ranjith et al., 1999). In an attempt to study the effect of different levels of calcium removal on physico-chemical properties of milk, Bishov et al (1958) used mixed bed anionic and cation exchange resin column in 3:2 ratio. They observed that the decrease in the quantity of sediment and opacity were directly proportional to the level of calcium depletion. Similar results with respect to a decrease in sedimentation of casein (Udabage et al., 2000) and opacity/turbidity were observed by other researchers (Burgess, 1982; Coleman et al., 1954; Faka et al., 2009; Grimley et al., 2010; Kulozik, 1998; Lin et al., 2006; Rialland et al., 1982) working on calcium removal from milk. Reduction in the opacity is caused by the disruption of the native casein micelle structure (Burgess, 1982), due to removal of calcium and breakage of linkages between the casein and inorganic constituents (Holt et al., 1986b). Similar reductions in the opacity have also

been observed by addition of calcium sequestrant, like EDTA (Griffin et al., 1988; Udabage et al., 2000).

The effects of calcium depletion on the pH of milk are contradictory. Lin et al (2006) and Grimley et al (2010) reported an increase in the pH of milk, but Ranjith et al (1999) reported a decrease in pH from 6.72 down to 6.38 upon 80% calcium removal using a similar weakly acidic cation exchange resin. Mixed bed anionic and cation exchange resin was found to cause a reduction in pH from 6.7 to 6.4 when 91% of calcium was removed from milk (Coleman et al., 1954).

Reduction of total calcium content is not exclusive with the ion-exchange method, but a substantial reduction in magnesium, potassium and phosphorus contents have been reported depending on ion with which the exchange takes place (Na^+ form or K^+ form). The affinity of weakly acidic cation exchange resin increases with atomic number and corresponding valences (Harland, 1994a). Bishov et al (1958), using mixed bed cation and anion exchange resin, have shown a reduction in all major minerals in milk, following the order: $Ca^{2+} > Mg^+ > K^+ > P > Na^+$. Calcium-depleted milk, produced using weakly acidic cation exchange resin, shows that almost 50% of magnesium and phosphorus were removed from milk when 60% of calcium was removed using K^+ form of resin (Ranjith et al., 1999). Although the reduction of phosphorus would not be envisaged according to the principles of ion exchange, this reduction was attributed to the precipitation of calcium phosphate at the resin surface in contact with milk. Similar reductions in phosphorus contents have been reported by Layman et al (1933) and Bishov et al (1958). Grimley (2010) reported reduction in potassium content of milk using sodium form of weakly acidic cation exchange resin.

An increase in negative ζ-potential from -24.4 to -30.6 has been reported by Grimley et al (2010) with reductions in total divalent cations concentration in skim milk diluted 30 times in deionised water. The ζ-potential was restored back to its original value upon calcium addition as calcium chloride.

2.7.2. Sensory properties of milk

Milk has a very delicate, bland, sweet taste which is characteristic to its components present; any change in salt balance is bound to affect the sensory attributes. It is evident that the changes in the flavour of calcium-depleted milk are related to the amount of calcium removed, the form of resin (Na^+, K^+) and their respective mixtures used. Haller et al (1950) reported the production of oxidised flavour in milk when 10 to 15% calcium was removed using Na^+ form of ion exchange resin. However, no change in the appearance or taste of milk was observed by Lyman et al (1933) upon 22% calcium depletion from milk. The effects of different forms (H^+ or Na^+) of ion exchange resins and their mixtures on the sensory quality of whole milk and reconstituted non-fat dry milk were investigated by Coleman et al (1954). They observed that development of acidity and perception of saltiness were the limiting factors when H^+ and Na^+ forms of resins were used. Although saltiness was considered as a limiting factor when mixed bed resin was used, it produced less flavour changes when 50% or 30% calcium was removed from whole milk and non-fat dry milk, respectively.

Ranjith et al (1999) observed the effects of Na^+ and K^+ form of resin individually and in mixtures on the flavour of raw skim milk treated to remove almost all divalent ions. A 'bicarbonate type' and 'soapy/salty' flavour was reported using K^+ and Na^+ form of resin. A 1:2 molar mixture of Na^+ and K^+ form resin was considered to be the best for overcoming off flavour problems.

Although flavour changes observed would be the limiting factor for extensive use in any food, these changes could be masked or minimized using various technologies. Moreover, the sensory attributes of the final product would be governed by the level of calcium-depleted milk used in the final formulation. Technological advantages of using calcium-depleted milk are immense and are discussed in the next section.

2.7.3. Effect on technological properties

All dairy products, unlike many other food products, originate from a single raw material. The technologies involved for the manufacture of these products involve many unit operations, such as heating, freezing, curdling and renneting. Since minerals play an important role in the stability of milk, ion exchange of milk affects several technological properties. The main objective of ion exchange in most applications in the dairy industry has been to improve productivity, lower production costs and provide innovative solutions.

Ion exchange (IE) of milk to obtain milks with softer curd formation suitable for infant nutrition is among the first published application of IE in the dairy industry (Lyman et al., 1933). It was observed that Zeolite-treated milk with 22% calcium removal failed to curdle by chymosin, resulting in a soft curd. Josephson and Reeves (1947) extended the principle of earlier experiment by Lyman to evaporated milks and found a stabilizing effect of ion exchange treatment on the stability of evaporated milk during sterilization. They used sodium form IE resin (Amberlite IR-100) to generate 60% calcium-depleted milk which was then added as a fluid, concentrate or powder to the evaporated milk. They showed that the need for stabilizing salts could be completely eliminated by using IE-treated milk at 0.5 to 2.0% of original milk.

The removal of calcium from milk improves various functional properties of milk, such as viscosity, water binding emulsification and foaming (Burgess, 1982). All these properties were put to use by Stahl (1984) during the manufacture of freeze thaw-stable icecream using more than 70% of calcium-depleted milk. The textural properties of set, stirred and drinking yoghurt were improved using 30% to 70% calcium-depleted milk and/or ingredient during product manufacture (Bhaskar et al., 2011). Most innovative and advanced use of this technique is for the production of modified milk protein concentrates and their subsequent use for manufacture of different cheeses and milk gels (Dybing et al., 2003).

Calcium depletion has not always been the aim of the ion exchange process, Wilcox and Valley (1959) utilized the ion exchange process to manufacture low sodium milk with over 90% reduction from the initial content in milk. Unlike the previous authors who used cation form of IE resin to replace sodium in milk, Stimpson (1955) used mixed bed hydrogen and hydroxyl form resin for deionisation of milk. The objective of this patent was to reduce the total ash content in milk to less than 30% of the initial amount present. Otting (1940) in his patent describes a method of converting finely divided casein in aqueous state to sodium or potassium caseinate, using respective form of IE resins. The ion exchange process was performed by a batch process at 38^{o}C. Rialland (1982) prepared an acidified milk with pH around 3.8 using acid form resin. Acidified milk was prevented from curdling by performing the ion exchange process at chilled temperatures (0-4^{o}C). This acidified milk was then used for the manufacture of casein, acid curds and lactoserum.

2.8. Iron binding in milk

Milk is a poor source of iron and contributes only 0.2-0.5 mg/L to the daily nutritional requirements of the human body (Flynn et al., 1997). Of this minor quantity of iron in cows' milk, ~ 14% is associated with the fat globule membrane, ~ 24% is bound to casein, ~ 29% to whey proteins and the rest (~ 32%) is associated with the low-molecular weight fraction (Brile et al., 1982).

When added externally, iron binds mainly to the proteins with less than 3% being associated with the fat fraction (King et al., 1959). Among the milk proteins, ~ 90% of iron added to milk binds to the caseins (Demott et al., 1976; Raouche et al., 2009a). Demott and Dincer (1976) studied the binding of iron to milk proteins and reported that added iron was bound to α_{s1}-, β- and κ-casein in the ratio 72:21:4 which was directly proportional to the concentration of phosphoserine residues available for binding of added iron. The small amount of iron in the serum phase of milk is either bound to whey protein, or in a iron-phosphate or iron-citrate complex, or could be bound to small diffusible molecules such as sulphur-containing compounds or ortotic acid (Bachran et al., 1980; Gaucheron et al., 1997a). Iron binds to caseins *via* coordination bonds with oxygen of the phosphoserine residues (Hegenauer et al., 1979c).

Hegenauer et al (1979b) showed that the caseins in the casein micelle can bind iron even when they are saturated with calcium. The higher equilibrium constant for iron-casein (10^{13} log) than calcium-casein complex is related to the increased ability of casein micelle to bind iron. The oxidation state of the added iron affects the efficiency of iron binding to casein micelle. Ferric iron binds more strongly to the casein micelle in skim milk in comparison to the ferrous iron (Raouche et al., 2009b).

Addition of iron to milk results in a drop in pH, change of colour, modification of salt balance between the soluble and colloidal phase and changes to casein conformation (Raouche et al., 2009b). Changes in flavour due to iron-induced oxidation of fat in milk are variable and related to the source of added iron. Soluble sources of iron, such as ferrous sulphate, cause substantial flavour deterioration due to fat oxidation in milk as compared to chelated forms of iron (Hegenauer et al., 1979b).

The high acidity of iron solution causes a reduction in the pH of milk; a maximum of 8 mM could be added to skim milk without protein precipitation (Gaucheron et al., 1997a). The binding of ferric iron to caseins also generates H^+ ions which contribute to the reduction in pH. Ferric iron reduces the pH more than ferrous form. Re-adjustment of pH and addition at low temperatures (~ 2^oC) permits the addition of up to 20 mM iron to skim milk without affecting its colloidal stability (Raouche et al., 2009a). This effect was attributed to the exchange of iron with the partially solubilised colloidal calcium at low temperatures. The iron bound to proteins in milk is stable even at low pH ~ 4.5, due to the involvement of coordination bonds in its binding to casein (Gaucheron et al., 1997a; Hekmat et al., 1998).

Addition of iron to milk, irrespective of its concentration and oxidation state, alters the distribution of minerals between the soluble and colloidal phase (Gaucheron et al., 1997a; Hekmat et al., 1998; Philippe et al., 2005; Raouche et al., 2009b). A reduction in soluble inorganic phosphorus content of milk as a function of added iron has been unanimously observed in these studies. Addition of 8 mM iron to milk permeate reduced its inorganic phosphorus content by ~ 92% which was attributed to the high stability constant (log 8.13 for HPO_4^{2-}) of iron-phosphate complex (Philippe et al., 2005). Raouche et al (2009b) showed the formation of insoluble ferric phosphate inside

the hydrophobic region of the casein micelle in skim milk using Mossbauer spectroscopy. On the contrary, even though a reduction of inorganic phosphorus in soluble phase was observed by Gaucheron et al (1997a), they suggested that colloidal phosphorus had no role to play in iron binding to caseins. This was based on the observation that colloidal phosphorus was released upon acidification of milk but iron was not released. However, it is important to note that the level of iron added was only 1.5 mM, whereas Rouche et al (2009b) added 20 mM iron to skim milk. Hekmat and McMahon (1998) observed that two-third of inorganic phosphorus was bound to the colloidal phase of milk upon iron addition (100 mg/L) at pH 6.7 and more phosphorus (~ 19%) was retained in iron-fortified milk compared to unfortified milk at pH 5.3, indicating the possible binding of phosphorus to the iron-casein complex.

Direct binding of iron to caseins is due to the higher stability constants of Fe-(serP-serP) ($10^{17.5}$) than Fe-$H_3P0^-_4$ ($10^{3.47}$) and Fe-$H_2P0^{2-}_4$ ($10^{8.30}$) (Hegenauer et al., 1979c). However, it was suggested that at higher concentrations of iron, the balance shifts towards the formation of colloidal mineral crystals (Raouche et al., 2009b).

2.9. Iron binding to milk proteins

The binding of iron to major milk proteins, i.e. caseins and whey proteins, has been investigated mostly in purified model systems, such as sodium caseinate, whey protein isolate and their purified fractions.

2.9.1. Caseins

Caseins belong to a class of metallo-proteins and bind iron more strongly than calcium. Coordination bonds with oxygen of the phosphoserine residues are primarily involved

in the binding of iron to caseins. The specificity of caseins for iron binding is principally related to the clustering of these phosphoserine residues. The binding of iron to caseins alters their conformation, as indicated by ultraviolet and fluorescence spectroscopy (Reddy et al., 1991), HPLC (Gaucheron et al., 1996), extrinsic fluorescence (Philippe et al., 2005) and absorption spectroscopy (Hegenauer et al., 1979b). The change observed in retention time and peak area patterns were higher for caseins with greater phosphoserine residues upon iron addition as indicated by HPLC (Gaucheron et al., 1996). A reduction in extrinsic/intrinsic fluorescence of sodium caseinate solutions upon iron addition has been attributed to a change in protein conformation, possibly due to the exposure of hydrophobic regions on caseins towards a more polar environment. Webb et al (1958) studied the effect of Fe^{+3} binding to phosphoseryl residues of egg yolk phosvitin and indicated that its binding reduces the electrostatic repulsion between phosphate groups which induces a structural change to β-structure.

The binding sites on caseins comprise of phosphoserine and also carboxylic acid residues (Bernos et al., 1997). Dephosphorylation of caseins has been shown to reduce, but not eliminate, their iron binding capacity (Bernos et al., 1997), indicating the binding of iron to other amino acids. Scatchard's plot analysis of β-casein suggests that it can bind at least 7 ferrous iron and the binding sites consist of 5 phosphoserine and two carboxylic acid residue on each molecule (Baumy et al., 1988b). Sugiarto et al (2009) showed the presence of 14 binding sites for ferrous iron on commercial sodium caseinate with a log K_{app} of 5.3.

Decreasing the pH reduces the ionisation of the negatively charged amino acid side chains, thus reducing their ability to bind cations by electrostatic interactions. The

binding of iron to caseins remains unaffected by a reduction in pH up to ~ 5.0 (Baumy et al., 1988b; Hegenauer et al., 1979c). Hegenauer et al (1979c), in their pioneering work on iron (III)-phosphoprotein chelates, demonstrated that the stoichiometric equilibrium constants of ferric iron and α_{s1}-casein did not significantly vary between pH 5.79 and pH 7.68, with an average value of 17.5 which signifies a strong complex. Reddy and Mahoney (1991) reported that the number of iron binding sites on α_{s1}-casein was independent of pH. Sugiarto et al (2009) have shown that the binding ability of sodium caseinate for ferrous iron (12 mg iron/g of protein) remained unchanged between pH 5.5 and 7.0. The ability of caseins to bind iron even at pH near their isoelectric point suggests an involvement of coordination bonds.

Increasing the ionic strength decreases the pKa of the second ionisation of amino acids thus reducing their affinity to bind iron. Iron (Fe^{+3}) bound to caseins was also stable to changes in ionic strength from 0-0.12 M (Gaucheron et al., 1997c). Similar observations have been reported for ferrous iron bound to β-casein by Baumy and Brule (1988b).

The sodium caseinate solutions with 1.5 mM added iron and heated at 90^{o}C for 15 min were stable and did not release the bound iron (Gaucheron et al., 1996; Gaucheron et al., 1997c). The iron bound to caseins did not release upon incubation with 20 mM sodium phosphate solution. However, in the presence of EDTA and citrate, a reversible transfer of iron from caseins to EDTA was observed (Gaucheron et al., 1996).

The addition of iron to casein above a certain concentration, when the binding sites are saturated, results in protein precipitation (Baumy et al., 1988b). The concentration at which precipitation occurs is governed by oxidation state of added iron. Ferrous iron precipitates casein at 4 mM level whereas only 2 mM of ferric iron caused precipitation in sodium caseinate solution containing 25 g/L of protein (Gaucheron et al., 1996). The

iron-induced precipitation of caseins has been attributed to neutralisation of negative charge on caseins and/or the formation of intermolecular iron adducts.

The binding of a ferrous ion to casein induces its oxidation to the ferric state (Emery, 1992; Hegenauer et al., 1979b; Manson et al., 1978). This oxidation reaction was related to the phosphoseryl residues on the casein as similar reactions were not observed upon dephosphorylation of caseins (Manson et al., 1972). Addition of ferric chloride to the reaction vessel containing O_2 and caseins (α_{s1}- and β-casein) instead of ferrous form did not cause any oxidation (Manson et al., 1978). The following reaction was proposed by Manson et al (1978):

$$O_2 + 4Fe^{2+} + 4H^{+} \longrightarrow 2H_2O + 4Fe^{3+}$$

2.9.2. Whey proteins

Approximately 10% of the iron added to skim milk binds to proteins in the supernatant, which has been shown to consist mainly of whey proteins (Gaucheron et al., 1997a; Hegenauer et al., 1979b; Raouche et al., 2009a). Among the major whey proteins, α-lactalbumin binds higher (6.0 mM) amounts of iron than β-lactoglobulin (3.2 mM) at pH 6.6 and ionic strength of 0.01 M (Baumy et al., 1988a). Decreasing the pH to 5.0 and increasing the ionic strength up to 1.0 M reduced the amount of iron bound to individual whey proteins i.e. α-lactalbumin and β-lactoglobulin. Sugiarto et al (2009) reported that the iron (ferrous) binding capacity of commercial whey protein isolate decreased from 8 mg iron/g of protein to 1 mg iron/g of protein upon reducing the pH from 7.0 to 5.5.

The probable sites for iron binding to whey proteins include the carboxylic acid residues of aspartic acid and glutamine acids. Sugiarto et al (2009) using Scatchard plots have

shown the presence of 8 binding sites with a K_{app} of 3.6. The absence of the phosphoserine residues, along with the compact structures of whey proteins are responsible for the lower ability of whey proteins to bind iron (Sugiarto et al., 2009). Table 2.7 provides a summary of the iron-binding studies undertaken over the years.

Table 2-7: *Summary of various iron binding studies*

Iron salts/chelates	Quantity	Protein source	Author
Ferrous chloride	6.64 mM	β-Casein	Baumy and Brule, 1988.
Ferric nitrilo-triacetate, ferric casein complex, ferric fructose	10-5 mg/kg	Casein and skim milk	Milkichael, et al, 1975.
Ferric chloride	10.6 mg/L	Milk	Demott and Dincer, 1976.
Ferric nitrilo-triacetate, ferric fructose, ferric lactobionate, ferrous sulphate, ferric polyphosphates	1 mM/L	Skim milk	Hegenauer, J., et al., 1979.
Ferrous sulphate, ferric pyrophosphate	0-20 mg/g protein	Casein	Potter and Nelson, 1979.
Ferric chloride, ferrous chloride	1.5 mM/L	Sodium caseinate and skim milk	Gaucheron et al., 1996, 1997.
Ferric chloride	100 mg/L	Acidified milk, yoghurt	Hekmat and McMahon.1998
Ferric chloride	0-8 mM/kg	Phospho-caseinate powder	Philippe et al . 2005.
Ferrous sulphate	0-20 mM/kg	Sodium caseinate, whey protein isolate	Sugiarto et al., 2009.
Ferrous chloride, ferric chloride	0-20 mM/kg	Skim milk	Raouche et al., 2009.

2.10. Formation and applications of protein-iron complexes

A protein-iron complex is formed when the iron salt donates an ion to casein or whey protein. This feature has been implemented knowingly or unknowingly during iron fortification experiments and dairy-based iron fortification programmes over many decades. The ability of a complex to prevent any detrimental change in a product depends on the type of salts (ferrous or ferric), the stability constant of the chelating ligand vis-a-vis protein, processing conditions and on the iron salt introduction step to the food matrix.

The development of an iron and whey protein complex as an ingredient for nutritional applications was first reported by Jones et al (1972). The complex was prepared by mixing soluble ferripolyphosphate with cottage cheese whey at pH 4.6. This procedure precipitated the whey proteins, which were then dialyzed against distilled water to remove lactose and as much mineral salts as possible. A white coloured fine powder was produced after lyophilisation of this complex that contained 10% iron, 13% phosphorus and 50% protein. The results showed that the bioavailability of this complex was superior to ferrous sulphate in weaning rats.

Zhang and Mahoney (1989b) prepared a iron-casein lyophilized complex by mixing different quantities of 0.2 M $FeCl_3$ into skimmed milk. These complexes were prepared by precipitation of casein at pH 4.6, followed by lyophilisation. A process for the preparation of saturated iron-lactoferrin by immobilizing and exchanging both lactoferrin and iron (ferrous sulphate) from milk (or milk systems) onto a cation exchange resin has been described in the patent by Palmano (2008). The invention claims that iron is loaded specifically into the iron-binding pockets of lactoferrin.

Several iron protein complexes have been developed and marketed by different companies, including Nestle, Unilever, Campina and Snow brand milk products. The iron chelation property of casein has been most commonly exploited. Sher et al (2006) described a patent in which ferric iron was bound to casein at pH between 5.8 and 6.2. The solution was then stirred for a maximum of 3 h and the pH adjusted to pH 6.8 – 7.0 before spray drying. The above procedure produced a ferric-casein complex with an optimum iron content of 1% by weight of casein. Ferric sulphate has been used as the iron fortificant by Hamashita et al (2000) for preparing an iron-casein complex with reduced astringency. Hydrogen carbonic acid or carbonic acid has been included in the iron-casein complex to reduce the astringency associated with the complex. Furthermore, the complex formed was hydrolysed using a protease to improve its heat stability. Marshman and Velikov (2009) used insoluble ferric pyrophosphate or orthophosphate along with gum arabic to produce iron nano-particles with particle size less than 1000 nm. Claims in this patent suggest it is suitable for multiple elements, including zinc, manganese and magnesium and that particle suspensions are stable to sedimentation.

The iron-whey protein preformed complex as explained earlier has been used to fortify pasteurized whole milk and found to be comparable to the control milk without added iron (Schmidt et al., 1972). A careful examination of published research shows that iron has been successfully incorporated in most of the dairy products where protein-iron complex is formed. The ferric form has almost always been found to be better than ferrous in this respect, owing to its resistance to oxidation and reduction reactions. The earliest example of ferrous oxidation by oxygen in milk leading to flavour and colour changes was reported for pasteurized whole milk, where de-aeration of milk was recommended before addition of ferrous sulphate at only 10 mg/L (Edmonson et al.,

1971). The importance of protein concentration in the binding of ferric compounds was observed during preparation of iron fortified non-fat milk powder. Addition of iron to concentrated milks, which contain more proteins, was recommended to prevent oxidized flavour (Kurtz et al., 1973). Cocoa and chocolate containing foods have been recommended for iron fortification owing to the presence of antioxidants, masking of colour, if any and greater acceptability with children (Douglas et al., 1981; Kinder et al., 1942). Among the nine different fortificants evaluated, ferric-polyphosphate-whey protein complex produced the least changes in organoleptic quality of prepared and srored samples.

Iron fortification of different cheeses has been achieved successfully without affecting their consumer acceptability with an upper level of 140 mg/kg being used for Harvati cheese (Jackson et al., 1992). It is interesting to note that higher retention (90%) was observed upon direct ferric chloride addition to milk than with microencapsulated ferric chloride (70%), thereby indicating the formation of iron-protein complexes and stability at the low pH. Cheddar and Mozzarella cheeses have been fortified with approximately 40 mg/kg of iron. Fortification of cheese milk was done with preformed protein-iron complexes (casein-iron, whey protein-iron) or by forming the complex upon addition of ferric chloride to milk (Rice et al., 1998; Zhang et al., 1990) without altering cheese quality. Formation of protein-iron complexes by adding ferric chloride to milk was considered a commercially viable option due to ease of incorporation during Mozzarella cheese manufacture. Better organoleptic qualities were observed for white soft cheese and Harvati cheese upon iron fortification at 80 and 140 mg/kg levels with ferric chloride. Hekmat and McMahon (1998) studied the distribution of casein-iron, iron-whey protein complex and ferric chloride in yoghurt after fortifying the milk at 100 mg Fe/L. They observed that iron added as iron-casein complex or ferric chloride was

preferentially retained in the casein micelles, whereas, iron-whey protein complex was distributed throughout the non-micellar portion.

The application of these complexes as ingredients has recently been envisaged by increasing the extent of iron binding to casein micelles (Raouche et al., 2009a). However, the suitability of such high iron bound milk based ingredients and their stability under processing conditions still remains to be explored.

2.11. The opportunities and gaps

The literature review demonstrates the vast amount of information available regarding the physico-chemical, application and mechanistic aspect of iron binding to milk proteins. The high affinity of milk proteins, especially caseins, to bind iron is evident. Hence, fortifying milk with iron is a viable option that could not only ensure good nutrition but also help alleviate iron deficiency. The most important question then arises - why has the iron binding ability of milk proteins not been exploited for food fortification?

The most important deterrent would probably be the fortification step itself. The slow addition of a minor quantity (~2.5 mg/serving) of iron to the large volume of milk processed in a dairy plant makes the fortification process unattractive. Secondly, the reactivity of iron with food constituents leading to problems such as oxidation and precipitation thwarts its prospect for fortification of milk and dairy products. Iron bound to milk proteins has shown potential as a fortificant for milk and dairy products, such as cheese and yoghurt in small scale studies. The binding of iron to milk proteins, especially caseins, prevents its interactions with food constituents thereby preventing the detrimental effects associated with fortification. Iron could be bound to proteins

both in milk or caseinate. Ferric iron binds more strongly to the caseins as compared to ferrous form. The addition of high ferric iron concentrations to milk would be the most attractive due to the simple preparation process. However, the high SNF and low protein content of skim milk restrict the concentration of iron achieved in the final ingredient.

An interesting alternative is binding of iron to protein solutions, such as sodium caseinate. Sodium caseinate has the advantage of having a high protein concentration and the presence of only caseins, which bind iron with very high affinity, providing an opportunity to prepare a commercially viable iron fortificant. However, precipitation of proteins was observed at > 4 mM level of ferric iron addition to sodium caseinate solutions containing similar protein as in milk. Hence, the use of pure protein solutions as a carrier for iron in a dispersible format is restrictive.

A thought provoking observation from the literature review above is the higher amounts of iron bind to the caseins in milk than in sodium caseinate. The difference could be due to the mineral composition of milk. Among the minerals available in milk, the distribution of inorganic phosphorus between the aqueous and colloidal phase is markedly affected upon iron addition. Hence, the role of inorganic phosphorus in the binding of iron to caseins in sodium caseinate needs to be studied.

Two hurdles discussed above show a clear opportunity and need for further research to enable higher iron binding to milk proteins especially in a dispersible format. Normal milk contains high concentration of calcium, which along with phosphorus (both organic and inorganic) aggregate caseins into a casein micelle structure. The added iron binds to the caseins already interacting with the colloidal calcium phosphate. Hence, the instability observed at high concentrations of iron addition might be due to the binding of both calcium and added iron to caseins in milk. Depleting a part of the original

calcium from milk should improve the processing stability of milk at higher concentrations of added iron. The Reference Dietary Intake (RDI) values for iron are ~1% of the daily calcium requirements. Hence, depleting a part of the calcium from milk to replace with iron becomes a sensible proposition.

Chapter 3. Materials and Methods

3.1. Materials

3.1.1. Chemicals

The list of chemicals used in the experiments and other relevant information are given in Table 3.1.

Table 3-1**:** *List of chemicals used*

Sr. No	Material	Specification	Manufacturer
1	Water	Mili Q, ISO 3696	Millipore Corporation, USA
2	Ferric chloride hexahydrate	> 99% pure	Sigma Aldrich, USA
3	Amberlite IRC 86 (Weakly acidic cation exchange resin)	H^+ form	Fluka analytical, France
4	Element standards (calcium, magnesium, iron, potassium, phosphorus and sodium)	1000 ± 4 mg/L	Fluka analytical, Switzerland
5	Sodium hydroxide and potassium hydroxide	Analytical grade	Merck Ltd, Germany
8	Sulphuric acid	98% pure	Sigma Aldrich, USA
9	Nitric acid	69% pure	Sigma Aldrich, USA
10	Potassium phosphate (monobasic and dibasic)	Analytical grade	Sigma Aldrich, USA
11	Lanthanum trichloride	Analytical grade	Sigma Aldrich, USA
12	Dipotassium hydrogen orthophosphate	General grade	Ajax Finechem, Australia
13	Iron (III) phosphate dihydrate	Laboratory grade	Sigma Aldrich, USA

3.1.2. Protein Sources

3.1.2.1. Skim milk powder (SMP)

Low heat skim milk powder was purchased from Fonterra Co-operative Ltd, New Zealand with the following specification.

Parameter	Limits
Protein (N x 6.38) (g/100g) as is	32.4
Moisture (g/100g)	4.0
Fat (g/100 g)	<1.25
Ash (g/100 g)	3.6
Titratable Acidity (% m/v)	0.15
Whey protein nitrogen index (mg/g)	> 6.0

3.1.2.2. Sodium caseinate

Edible grade sodium caseinate (Alanate 180, Fonterra, New Zealand) was purchased from Davies Trading Co., Palmerston North, New Zealand, with the following specification:

Parameter	Limits
Protein (N x 6.38)(g/100g) as is	92.7
Moisture (g/100 g)	4.3
Fat (g/100 g)	0.2
Ash (g/100 g)	3.6
Inhibitory Substances (IU/mL)	< 0.005

3.2. Methods

3.2.1. Studies on milk systems

3.2.1.1. Resin conversion

The weakly acidic cation exchange resin (Amberlite IRC86, Fluka analytical, France) available in H^+ form was converted to K^+ form at room temperature. The H^+ form resin was treated with three bed volumes of 1M potassium hydroxide solution for 45 min until the wet volume of the resin increased by 80%. The converted resin was washed with deionised water till a pH of ~7 was obtained for the wash water. A single batch was converted and used for a set of experiments.

Table 3-2*: *Properties of weakly acidic cation exchange resin

Functional group	-	Poly-acrylic co-polymer
Functional group	-	COO^-
Ionic form	-	H^+
Physical form	-	Amber coloured spherical beads
Total exchange capacity	-	$\geq$ 4.10 eq/L (H^+ form)
Moisture holding capacity	-	47 to 53% (H^+ form)
Specific gravity	-	1.17 to 1.195 (H^+ form)
Shipping weight	-	790 g/L
Harmonic mean size	-	0.580 – 0.780 mm

Weakly acidic cation exchange resin in K^+ form

3.2.1.2. Volume of resin

The following calculation shows the approximate volume of ion-exchange (IE) resin in K^+-form required for ~70% calcium depletion from 200 mL normal milk.

Total exchange capacity of resin is 4.1 meq/L. Thus, 1 L volume of resin can bind 4.1 M potassium ions (K^+) which is equivalent to 156.36 g of K^+. The average concentration of calcium in milk is ~ 30 mM i.e. 1.2 g calcium per L. Hence, the amount of calcium per 200 mL milk was 0.24 g calcium. Theoretically, 1 M of potassium on the resin will be exchanged for 1 M eq wt. of calcium from milk. Equivalent weight of calcium is 20.03. One litre of 4.1 meq weakly acidic cation exchange resin in K^+-form has the capacity to remove 82.14 g of calcium. Hence, the volume of resin theoretically required to remove 0.24 g of calcium from 200 mL milk would be 2.92 mL. However, the IE capacity of the resin is dependent on the temperature at which the IE process is performed. Preliminary experiments indicated that 3.75% of IE resin was sufficient to deplete ~ 70% calcium from milk at cold temperatures within 3 h.

3.2.1.3. Ion exchange process

Low heat SMP was reconstituted in deionised water (11% w/v) at 38-42^{o}C for 2 h and kept overnight at 5^{o}C for hydration. Sodium azide at 0.02% w/v was added to milk as a preservative. Milks with varying levels of calcium were obtained by ion-exchange in a batch process (5-10^{o}C). Different volumes of resin ranging from 0-3.75% were used to achieve different levels of calcium in milk. The volume of IE resin required was measured in a measuring cylinder. The excess water was drained by decantation and resin added directly to gently stirred milk. The depletion of calcium was monitored (in triplicate) using complexiметric titration as described in section 3.2.3.1.1. The pH of the

calcium-depleted milk samples was re-adjusted to 6.8, using 1 M NaOH solution and the final volume adjustment was done for all samples. The samples were stored overnight at ~ 5^{o}C until the iron addition step. Process flowchart for ion exchange treatment of milk is shown in Figure 3.1.

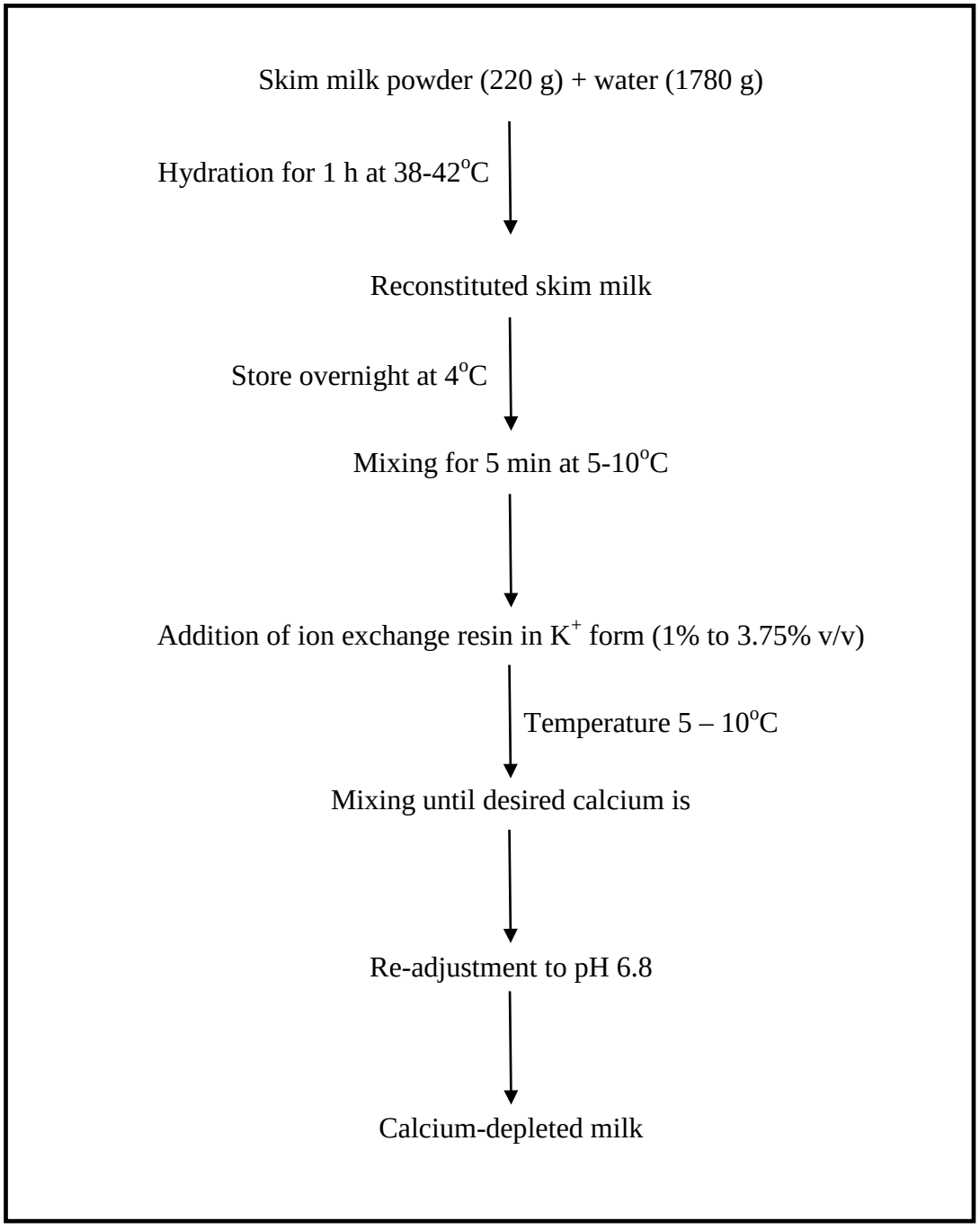


Figure 3-1: *Flowchart for ion exchange treatment of milk.*

3.2.1.4. Iron addition procedure

Iron addition to the normal milk and calcium-depleted milks (CDMs) was performed after overnight storage at ~ 5°C. Freshly prepared 0.5 M $FeCl_3$ solution was added dropwise to the vigorously stirred milks (~ 5°C) by maintaining the pH 6.8 ± 0.1 using 1 M NaOH (refer to Figure 3.2). Iron-added milks were then stirred for 30 min at 5°C.

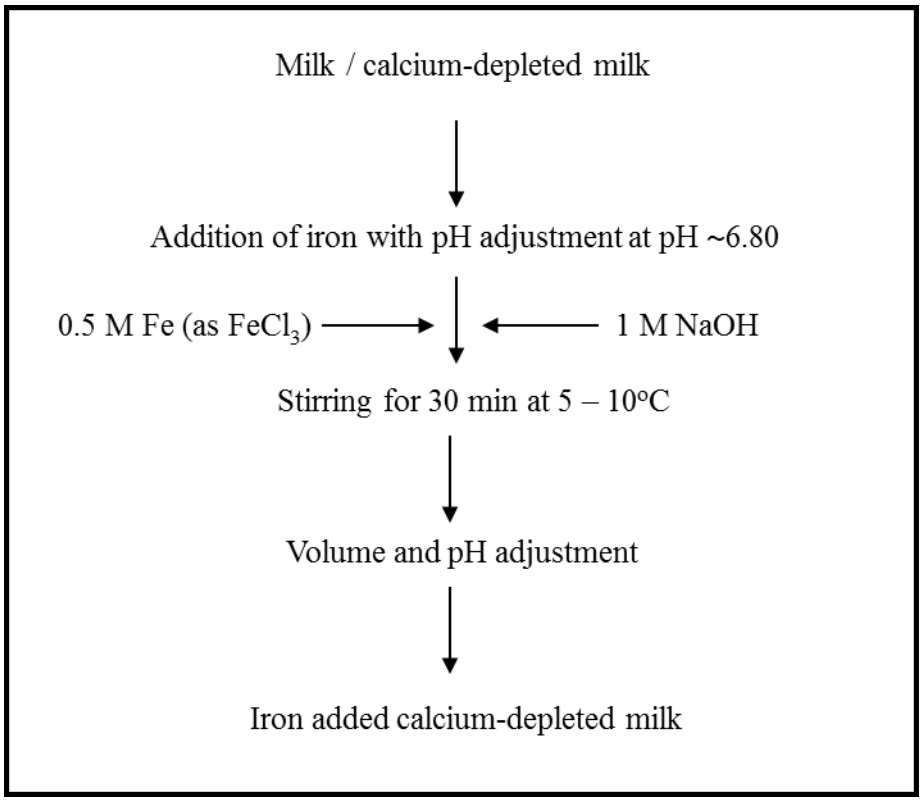


Figure 3-2*: *Flowchart for iron addition to calcium-depleted milk.

3.2.1.5. Addition of orthophosphate and calcium

In the experiments wherein exogenous orthophosphate was included, dipotassium hydrogen orthophosphate was added in the powder form to the calcium-depleted milk at ~ 5°C and stirred for 10 min. Iron was then added to the solution as described in step 3.2.1.4.

Calcium (1 M) was added to iron-fortified calcium-depleted milks at ~ 5°C and stirred at room temperature for 30 min. These samples were stored overnight at room temperature and pH readjusted to ~ 6.8. The final volumes of all the samples were adjusted before proceeding for mineral and protein partitioning. Process flowchart for iron and/or calcium addition to calcium-depleted milk is shown in Figure 3.3.

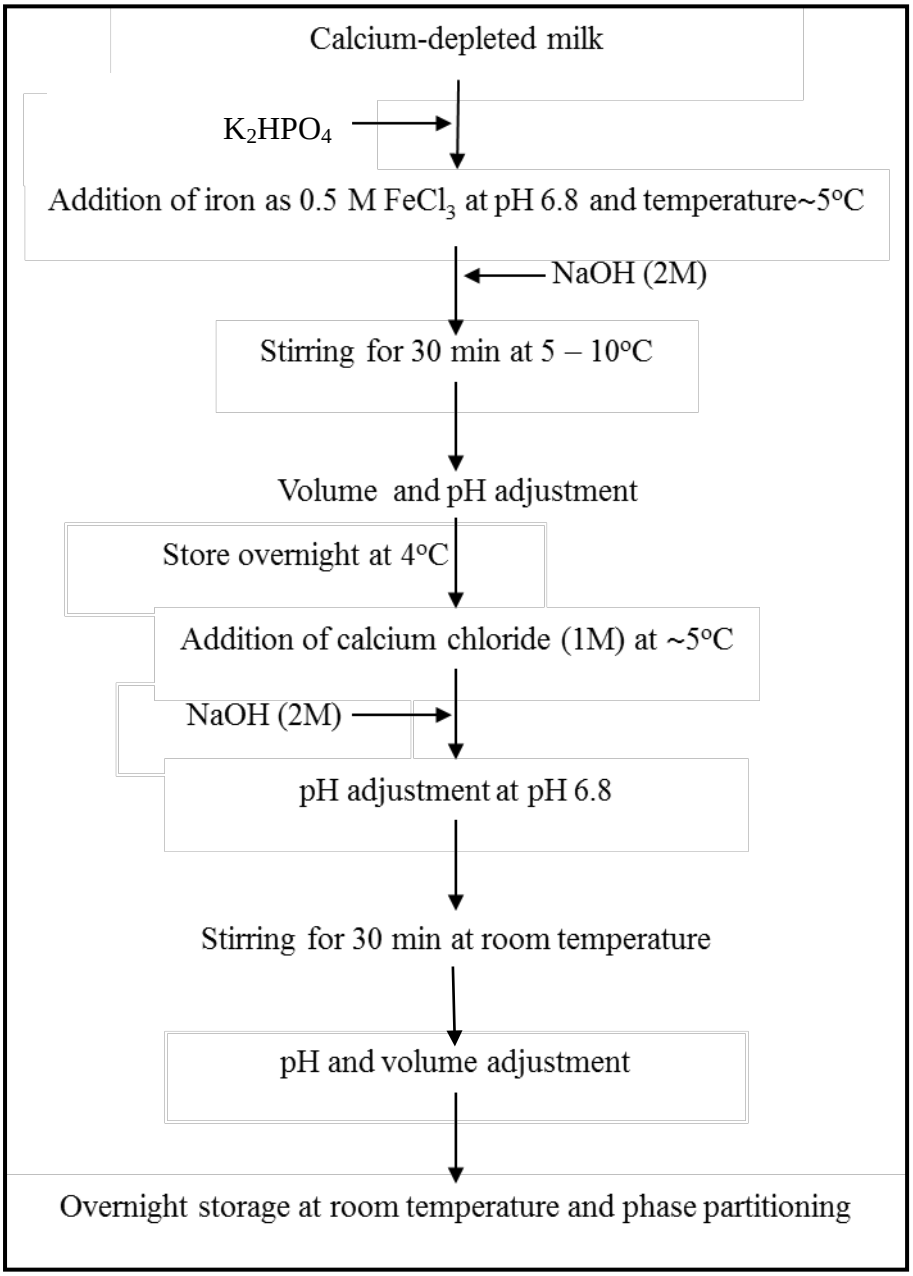


Figure 3-3: *Flowchart for iron and/or calcium addition to calcium-depleted milk.*

3.2.1.6. Phase partitioning

All milks were partitioned into sedimentable and non-sedimentable (soluble) phases by ultracentrifugation at 100,000 *g* for 1 h at 20°C (Figure 3.4). The supernatant was collected by inverting the tubes. Values for minerals in the non-sedimentable phase were corrected for the excluded volume effect, as proposed by Davies and White (1960).

Conversion Factor (CF) = Wt. of water in 100 g milk/Wt. of water in 100 g serum

Sub-samples of the supernatant were ultrafiltered using 10,000 MWCO (regenerated cellulose) membrane (Amicon Ultra, Millipore). A correction factor of 0.96 was applied for mineral estimation in the ultrafiltration permeate.

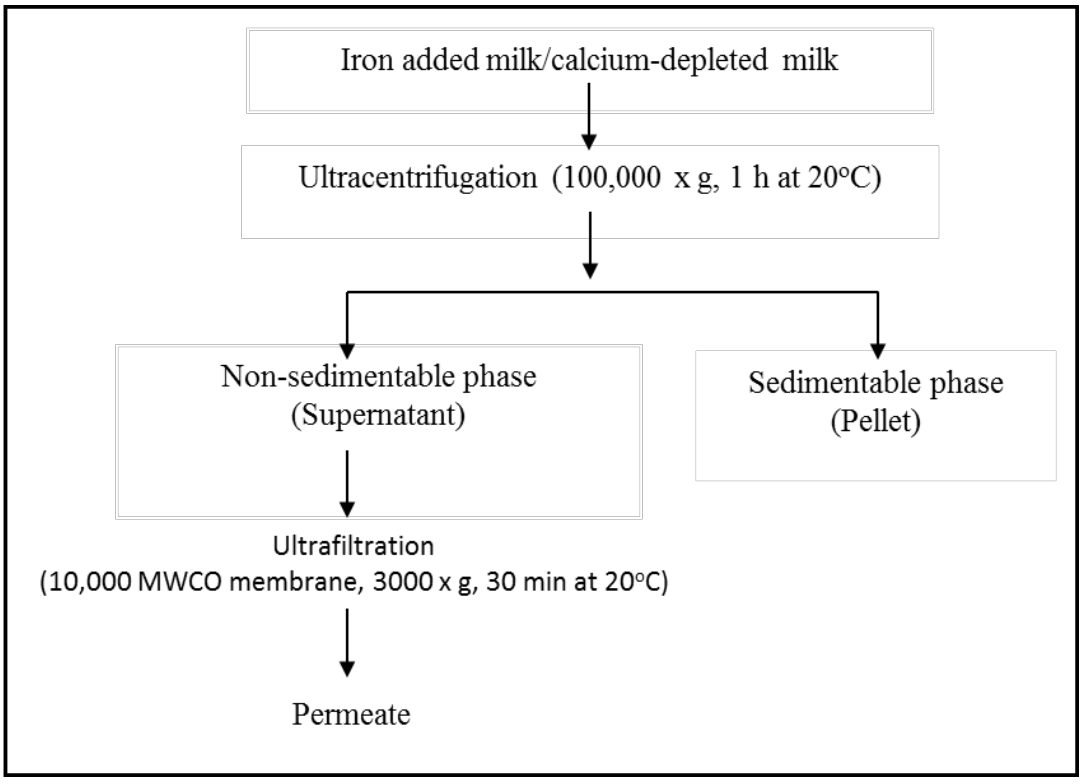


Figure 3-4**:** *Flowchart showing partitioning of phases in iron fortified calcium-depleted milk.*

3.2.2. Studies on sodium caseinate

3.2.2.1. Sample preparation

3.2.2.1.1. Iron addition to sodium caseinate solution containing orthophosphate

Sodium caseinate powder was dispersed in deionised water (Milli Q, Millipre Corp.) containing sodium azide at 50 ± 2^{o}C for 2 h and stored overnight at ~ 4^{o}C. In samples containing orthophosphate, powdered di-potassium hydrogen orthophosphate was added to the sodium caseinate solution and left stirring for 20 min at ~ 5^{o}C. Ferric chloride solution (0.5 M) was added dropwise to the vigorously stirred sodium caseinate solutions by maintaining the pH at 6.8 using 1 M NaOH or 1 M HCl. The final pH of the solutions was adjusted to pH 6.8 after 30 min stirring and volume of all samples was adjusted to achieve a protein content of 20 mg/mL (see figure 3.5).

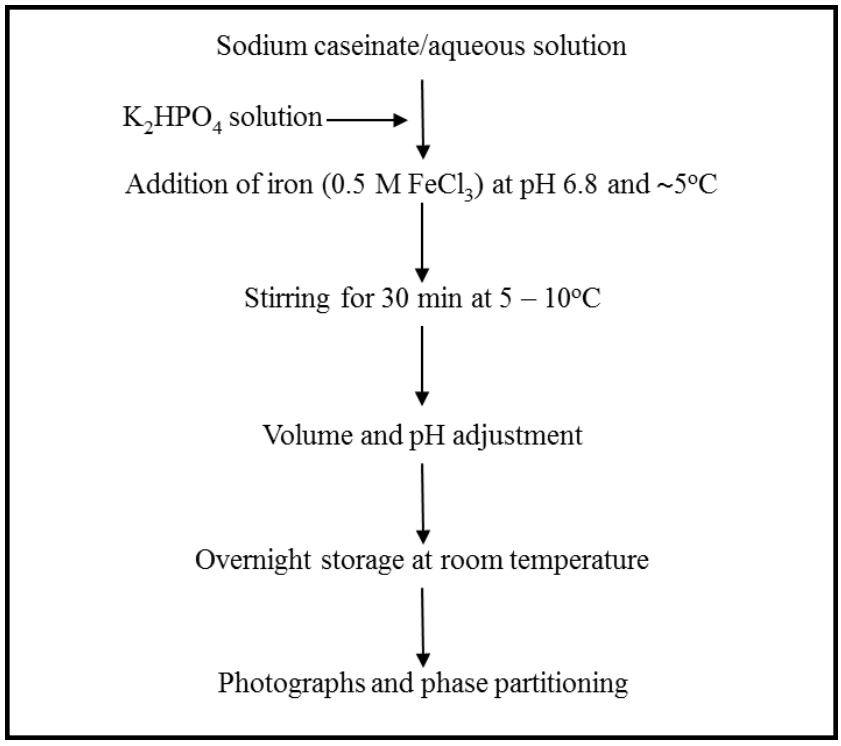


Figure 3-5*: *Flowchart for iron addition to sodium caseinate solution containing orthophosphate.

3.2.2.1.2. Orthophosphate addition to sodium caseinate solution containing iron

The iron solution (0.5 M $FeCl_3$) was added to a bulk sodium caseinate solution (800 g) at ~ 5°C, while maintaining the pH at ~ 6.8. This resulted in precipitation. The suspension was stirred for 30 min at ~ 5°C and then adjusted to a desired volume using Milli Q water. The iron added suspension was then divided equally into four portions (~103 g each) and the orthophosphate solution (5 mL) added thereafter. The pH of the iron added sodium caseinate solutions was readjusted to ~ 6.8 after phosphorus addition using 1.5 M HCl. The volumes of the samples were adjusted using Milli Q water to compensate for the differences in the amount of HCl required for pH adjustment and then allowed to stir at room temperature for another 30 min. The solutions were analysed after overnight storage at room temperature (See Figure 3.6).

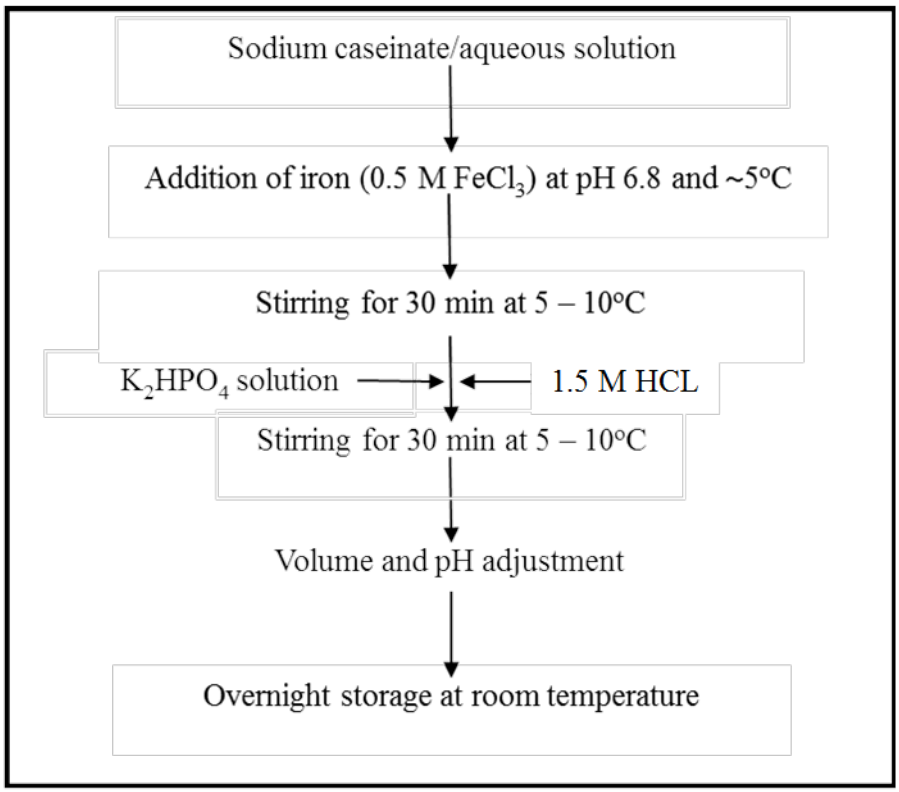


Figure 3-6*: *Flowchart for orthophosphate addition to sodium caseinate solution containing iron.

3.2.2.2. Ferric phosphate addition to caseins

A calculated amount of powdered ferric orthophosphate providing different concentrations of iron (0-20 mM) was added to sodium caseinate solutions and pH of the solutions adjusted to 6.8. The samples were stirred for 30 min at ~ 5°C and transferred to glass vials. The glass vial was sealed and placed on a rocking platform for 24 h at room temperature. The solutions were then centrifuged at 500 *g* for 20 min at 20°C and analysed for iron content in the supernatant.

3.2.2.3. Phosphorus adsorption experiments

These experiments were performed to ascertain the adsorption of orthophosphate onto ferric hydroxide precipitate prepared as described above. Ferric hydroxide solution was prepared by adding 15 mM iron as 0.5 M $FeCl_3$ to 96 mL de-ionised water at ~ 5°C. The pH of the solutions was adjusted to 6.8 using 2 M NaOH and the solution stirred for 30 min at ~ 5°C. Inorganic phosphorus (as K_2HPO_4) was added to the above solution at nine different concentrations, ranging from 0 to 2000 mg/kg and pH re-adjusted to 6.8. The volume of all the samples was then adjusted to 110 mL using Milli Q water. Since ferric hydroxide was insoluble in water, the solutions were allowed to stir for 24 h at room temperature and then centrifuged at 500 *g* for 20 min at 20°C. The supernatant was filtered through 0.45 micron syringe filter (Sartorius Stedium, Germany). The adsorption of phosphorus was determined by the difference in the phosphorus content of the original solution and the filtrate.

3.2.2.4. Phase partitioning

The samples were left overnight at room temperature and centrifuged at 500 *g* for 20 min at 20°C. The permeate was obtained from the supernatant after ultrafiltration using 10,000 MWCO (regenerated cellulose) membrane (Amicon Ultra, Millipore) in a centrifuge operating at 3000 *g* for 20 min. Flowchart indicating the partitioning of phases in iron-fortified sodium caseinate solutions is shown in Figure 3.7.

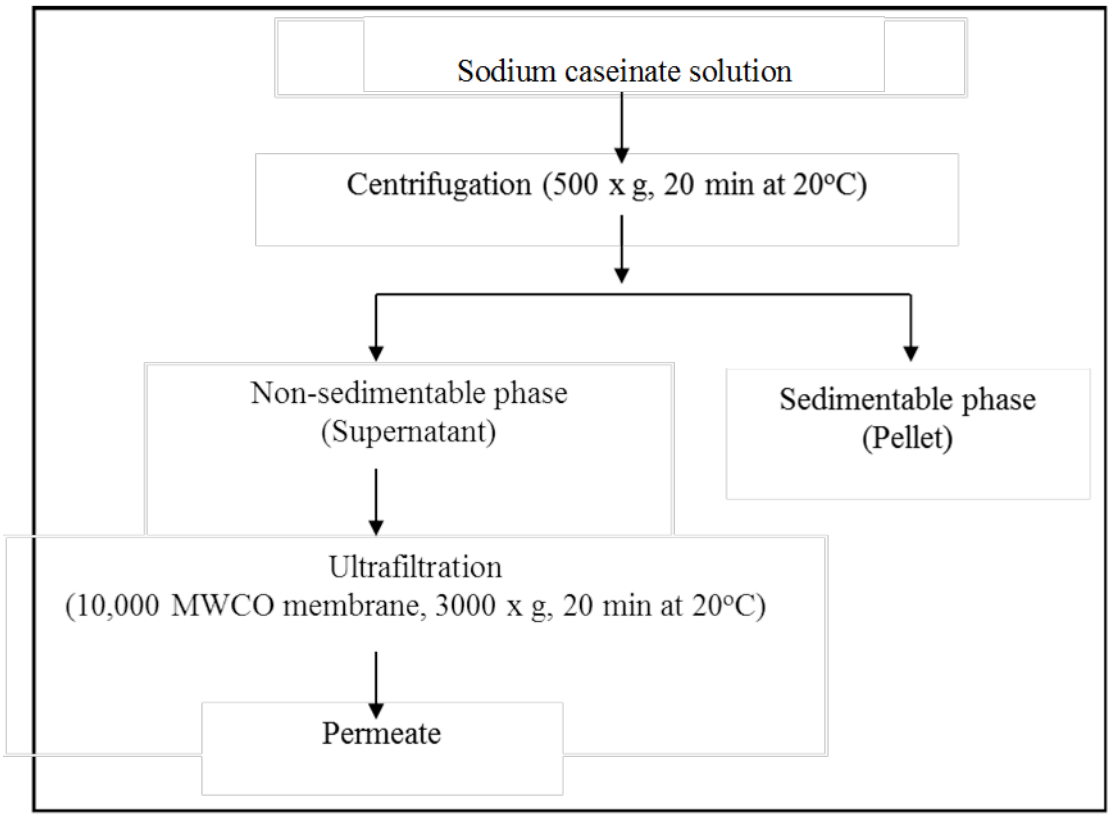


Figure 3-7*: *Flowchart showing partitioning of phases in iron added sodium caseinate solution.

3.2.3. Mineral and protein estimation

3.2.3.1. Determination of calcium by complexiметric titration

Complexiметric titration was used to monitor the concentration of calcium in milk. The method (Patton et al., 1956) relies on the ability of Patton Reeder's (PR) indicator to complex with calcium in milk. The complexed calcium when titrated with EDTA solution gives a blue colour change at the end of the titration process.

The reaction: $Ca\text{-}PR + EDTA^{4-} \longrightarrow PR + [Ca\text{-}EDTA]^{2-}$

The method involved the following steps:

1. Pipette 10 mL milk into a 250 mL conical flask.
2. Add 40 mL deionised water and 4 mL of 8 M sodium hydroxide solution.
3. Allow the solution to stand for 3-5 min with intermittent stirring.
4. Add 0.1 g of PR indicator and swirl the solution to dissolve the indicator.
5. Titrate the sample with 0.025 M EDTA solution.
6. Use the method ratio Ca^{2+}: EDTA = 1:1, calculate the concentration of calcium in the sample.

3.2.3.2. Mineral analysis by Atomic Absorption Spectroscopy (AAS)

Cations (iron, calcium, magnesium, potassium and sodium) were analysed using a single flame atomic absorption spectrophotometer (GBC Scientific Equipment, Hampshire, IL, USA) following wet digestion of the milks with concentrated sulphuric acid and nitric acid (Marshall, 2010). All cations, except calcium, were analysed using

the air-acetylene flame. Calcium was analysed using the oxidising nitrous oxide-acetylene flame; 5000 ppm of lanthanum trichloride was included as the releasing agent in milk samples.

3.2.3.2.1. Stock solution

For calcium, magnesium and iron analysis in normal milk and calcium-depleted milks, stock solutions containing calcium, magnesium and iron were prepared from 1000 ± 4 mg/kg standards as follows:

- 25 g of 1000 ppm Ca standard
- 5 g of 1000 ppm Mg standard
- 15 g of 1000 ppm Fe standard
- 5 mL of 25% concentrated nitric acid

The above quantities were weighed directly into a 100 mL volumetric flask and volume made up to 100 mL.

3.2.3.2.2. Working standards

Working standards containing different concentrations of individual elements based on the estimation limit of the AAS were prepared from the stock solution shown in Table 3.3. The final volume was made up with deionised water after adding lanthanum trichloride to all standard solutions (10% of the final volume). Working standard containing sodium and potassium were prepared directly using 1000 ± 4 mg/kg standards.

Table 3-3*: *Working standard concentrations of elements used for AAS analysis

Calcium	Iron	Magnesium	Sodium	Potassium
		ppm		
5	3	1	2	2
10	6	2	4	4
15	9	3	6	6
20	12	4	8	8
25	15	5	10	10

3.2.3.2.3. Sample preparation

The following procedure was followed for sample preparation which was adapted from Marshall (2010)

1. 2 g sample was weighed accurately in 125 mL Erlenmeyer flask.
2. Blank solution was prepared by mixing 3 mL of H_2SO_4 and 5 mL of HNO_3, then heated to remove HNO_3 and made up to 100 mL using deionised water.
3. The samples after addition of acids were heated at 200^oC (boiling), until brown-yellow fumes were observed.
4. Once the brown-yellow fumes ceased, 3-5 mL of HNO_3 added.
5. The flask was put on the hot plate to allow the HNO_3 boiling off.
6. The sample was then allowed to cool and then quantitatively transferred to 100 mL volumetric flask with sufficient rinsing of the Erlenmeyer flask.

7. Further dilutions were performed using Milli Q water, if required as shown in Table 3.4.

Table 3-4*: *Dilution of samples for respective element analysis

Sample	Calcium	Magnesium	Iron	Sodium	Potassium
Milk	100 X	50 X	100 X	50 X	100 X
CDM	50 – 100 X	50 X	50 – 100 X	50 X	200 X
Supernatant	50 – 100 X	50 X	50 – 100 X	50 X	100 – 200 X
Permeate	0	0	0	25 X	25 X

Table 3.5 and 3.6 show the parameters used to analyse the minerals in samples by AAS.

Table 3-5*: *Instrumental parameters for element analysis by AAS

Parameters	Elements				
	Calcium	Magnesium	Iron	Sodium	Potassium
Lamp Current (mA)	5.0	3.0	7.0	5.0	6.0
Wavelength (nm)	422.7	285.2	248.3	589.0	766.5
Slit Width (nm)	0.5	0.5	0.2	0.2	0.2

Mineral concentrations in the non-sedimentable, sedimentable phase and the permeate were determined as follows:

Non-sedimentable = CF x element concentration in non-sedimentable phase

Sedimentable = 100 – concentration in non-sedimentable phase

Permeate = 0.96 (correction factor) x element concentration in permeate

Table 3-6*: *Typical operational parameters of AAS

Sample measurement parameters	
Measurement mode	Integration
Sample introduction	Automatic
Replicates	3
Calibration parameters	
Conc. units	µg/mL
Conc. decimal places	3
Check sample conc	1.000 µg/mL
Flame type	Air-Acetylene
Fuel flow	2.00 l/min
Oxidant flow	10.0 l/min
Burner angle	10.0°
Workhead height	15.0 mm

3.2.3.3. Phosphorus

Phosphorus was determined as described in ISO 9874 (International Standards Organization, 2006). The proportionate distribution of phosphorus was determined by analysing the total phosphorus content of the sample, the non-sedimentable phase and the permeate. The procedure used is described briefly.

3.2.3.3.1.1. Reagent preparation

Reagent 1-dilute sulphuric acid:carefully add 27.8 mL of concentrated sulphuric acid to 72.2 mL of water.

Reagent 2 – sodium molybdate solution: weigh 2.5 g of sodium molybdate dehydrate into a 100 mL volumetric flask. Add sufficient volume of dilute sulphuric acid to dissolve the sodium molybdate dehydrate. Dilute to the mark with the same sulphuric acid and mix.

Reagent 3 - ascorbic acid solutions: weigh 0.5 g of ascorbic acid into a 10 mL glass test tube and make up to 10 mL with water (prepare fresh).

Molybdate/ascorbic acid solution (MAS): add 2.5 mL of sodium molybdate solution and 1 mL of ascorbic acid solution and make up to 10 mL.

3.2.3.3.1.2. Preparation of standards and test solution

1. Prepare 10 mg/L stock solution from 1000 mg per litre stock solution by diluting 1mL of concentrated stock solution up to 100 mL in volumetric flask.
2. Pipette 0, 0.1, 0.2, 0.3 and 0.5 mL, respectively of the above stock solution into 10 mL glass test tube with stopper.

3. Dilute the contents with 2.5 mL of water in all tubes.

4. Add 0.2 mL of MAS solution and make total volume to 5 mL. The resulting solution contains 0, 10, 20, 30, 50 microgram phosphorus per 50 mL.

5. Boil the contents in water bath for 15 min. give the same treatment to both sample and standards.

6. Cool the solution to room temperature with cold water and check the absorbance at 820 nm.

The volume of water, Molybdenum/ascorbic acid solution and sample or standard solutions is shown in Table 3.7.

Table 3-7*: *Volume of solutions for phosphorus analysis

Solutions	**Standards**					**Sample**
P std.	0	0.1	0.2	0.3	0.5	0.2
MAS	0.2	0.2	0.2	0.2	0.2	0.2
Water	4.8	4.7	4.6	4.5	4.3	4.6
Total	5	5	5	5	5	5

P std: phosphorus standard, MAS: Molybdenum/ascorbic acid solution

Formula:

$Wp = m1/200\ m_o$

Wp = Total phosphorus content in %

m_1 = Mass of phosphorus, read or calculated from calibration graph, in micrograms

m_o = Mass of the test portion in grams

The absorbance at 820 nm read from the UV-vis absorption spectrophotometer was entered as the value for y and the polynomial equation solved to obtain the value of x.

The value of x equates to m_1 in the formula for total phosphorus (w_p).

3.2.3.4. Protein

The protein content was determined by the semi-Kjeldahl method using a factor of 6.38 for converting total nitrogen content to protein content:

% Nitrogen = [(A x B) x 14 x 100] / (1000 x C)

A = mL of HCl used

B = exact molarity of HCl

C = weight in g of the original sample

3.2.4. Characterisation techniques

3.2.4.1. Intrinsic (tryptophan) fluorescence

Fluorescence spectrum to determine λ_{max} and to measure intensity was obtained on a spectroflourimeter (Jasco model 6200) using classical right-angle spectroscopy. Fixed wavelength emission intensity was measured at 343 nm for all samples with an excitation wavelength at 295 nm. The samples were diluted 150 times in pH 6.80, 50 mM potassium phosphate buffer. Five readings were taken for each sample in duplicates.

3.2.4.2. Z-average diameter

The hydrodynamic mean (Z-average) diameter was obtained by dynamic light scattering using Zetasizer Nano – ZS (Malvern Instruments, United Kingdom). Milk samples were diluted 100 times in the respective permeates. A 1.2 mL sample was poured into a

disposable cuvette with 1 cm^2 pathlength. The analysis was performed at a back scattering angle of 173^o for the incident 4 mV helium/neon laser, having wavelength of 633 nm. Each sample was analysed at least in duplicate and 3 readings were taken for each replicate. In order to achieve a good count rate across the samples in iron-added sodium caseinate solution, 70-fold dilution using deionised water was performed. The number average diameter was used to monitor the breakdown of casein micelle upon ion-exchange treatment.

Dynamic light scattering measures the size of particles based on their Brownian motion. Large particles have relatively slower Brownian motion as compared to smaller particles and the instrument relates the diffusion of these particles in the solvent into their size. The rate of diffusion is measured by analysing the fluctuation of the monochromatic light source i.e. helium/neon laser, onto an optical arrangement. Stokes equation is used to calculate the translational diffusion coefficient which gives the size of the particles as the hydrodynamic diameter:

$$d(H) = \frac{kT}{3\pi\eta D}$$

where:

d(H) = hydrodynamic diameter, *D* = translational diffusion coefficient, *k* = Boltzmann's constant, *T* = absolute temperature, η = viscosity

The hydrodynamic diameter (Z-average diameter) represents a hypothetical sphere with similar translational diffusion coefficient as the average of the particles measured. The Z-average diameter is derived from cumulants analysis via autocorrelation function.

3.2.4.3. Particle size distribution

The particle size distribution of iron and calcium added calcium-depleted milks were obtained using Mastersizer 2000 (Malvern Instruments, Worcestershire, UK). A common refractive index of 1.37 was chosen for all the samples. Mastersizer uses static light scattering measurement technique, where a series of photosensitive detectors measure the scattered light. The size distribution data is deduced from the angle of the light scattered by particles which is inversely proportional to the size of the particles.

3.2.4.4. Zeta-potential (ζ-potential)

The ζ-potential values were obtained using Zetasizer Nano-ZS (Malvern Instruments, United Kingdom). The samples were diluted 50 times in their respective permeates. Measurements were taken in a folded capillary cell (Model DTS 1060C, Malvern Instruments Ltd, Worcestershire, UK). The ζ-potential measurement cell was flushed three times with deionised water and rinsed with the sample solution before measurement. The ζ-potential of 30 mM iron added samples as shown in Figure 5.4 was performed after dilution in Milli Q water using the procedure as described by Grimley et al (2010).

ζ-potential measures the potential across the diffuse layer of the electrical double layer formed around the particles (Figure 3.8). The charge on the diffuse layer affects the electrophoretic mobility of the particles which is calculated by Henry equation:.

$$U_E = \frac{2\varepsilon\zeta}{3\eta}.f(Ka)$$

where U_E = electrophoretic mobility, z = zeta potential, ε = dielectric constant, η = viscosity, (κa) = Henry's function

The samples placed in micro-electrophoresis unit are subjected to electrical potential, thus mobilising the charged particles to their oppositely charged electrode placed at either end of the capillary cell. The optical arrangement measures the change in frequency of light fluctuations at 13^o angle. The electrophoretic mobility is converted using a combination of laser Doppler velocimetry and phase analysis light scattering (PALS) by M3-PALS technique by the Zetasizer Nano ZS.

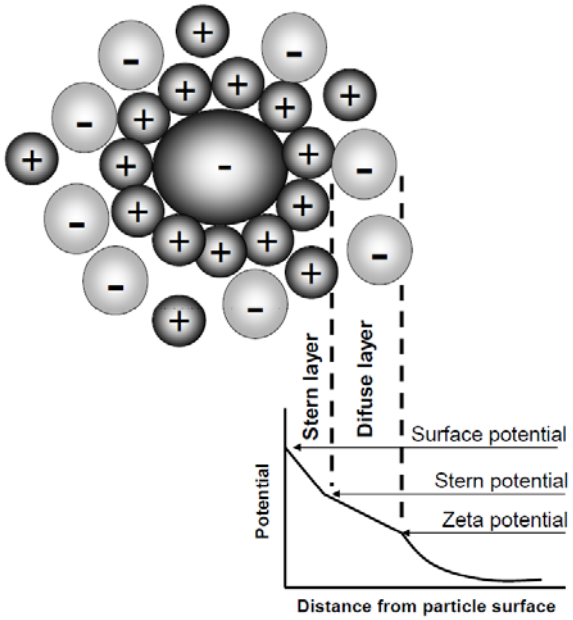


Figure 3-8*: *Schematic representation of zeta-potential.

3.2.4.5. Transmission electron microscopy (TEM)

The microstructures of (freshly prepared) milk samples were studied using TEM. Undiluted samples were injected and sealed in agarose tubes within 4 h of preparation. Primary fixation and secondary fixation were performed using 3% glutaraldehyde and

1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) respectively, with buffer washings between the two fixations, adapted from Karlsson et al (2007). Dehydration of the samples was achieved using an acetone series before infiltration and embedding in fresh resin (Procure 812). Ultra-thin sections (100 nm thick) were cut using a diamond knife and an ultra-microtome (Leica, Vienna, Austria). Samples were stained for 4 min with saturated uranyl acetate and lead citrate before being viewed in a transmission electron microscope (Philips EM 201C, Eindhoven, The Netherlands).

3.2.4.6. High Pressure Liquid Chromatography (HPLC) – Size Exclusion Chromatography (SEC)

The size exclusion chromatography of sodium caseinate solutions was performed based on the procedure described by Lucey et al (2000). Size-based separation of proteins was performed on a prepacked, cross-linked agarose based, gel filtration column (Superose® 6 HR 10/30) with 30 cm length. The column had an optimum separation range (based on globular proteins) between 5000 and 500,000 daltons and a bed volume of 24 mL. The SEC column was connected to a high pressure liquid chromatography instrument (Agilent technologies, Palo, Alto, CA, USA) for providing uniform flow of eluent through the gel filtration column. The outlet of the gel filtration column was connected to the UV detector which monitored the protein at 280 nm. The setup mentioned above is shown in Fig. 3.9.

The eluent buffer for SEC contained 20 mM immidazole and 50 mM sodium chloride and pH 7.0. The iron-added samples where gross aggregation of proteins was observed were omitted from the SEC study. All samples were filtered through 0.45 microns hydrophilic syringe filter (Sartorius Stedium) prior to injection through the SEC column. The column was equilibrated with the eluent for 1 h with buffer at flow rate of

0.8 mL/min. The column pressure at this flowrate was ~ 8 bar excluding the system back pressure. A 50 µl sample was injected to the eluent buffer at a nominal flow rate of 0.4 mL/min with a run time of at least 60 minutes. The column was washed, after every 10 sample runs, with 0.2 M sodium hydroxide solution at a flowrate of 0.5 mL/min and rinsed with eluent untill base line stabilisation. Each sample was analysed at least in duplicate.

The fractions of protein peaks obtained using SEC were integrated using the EZ chrom Elite graphical programming software. The fractions obtained by SEC of the samples were collected manually and subjected to particle size measurements, using the Zetasizer, as discussed earlier.

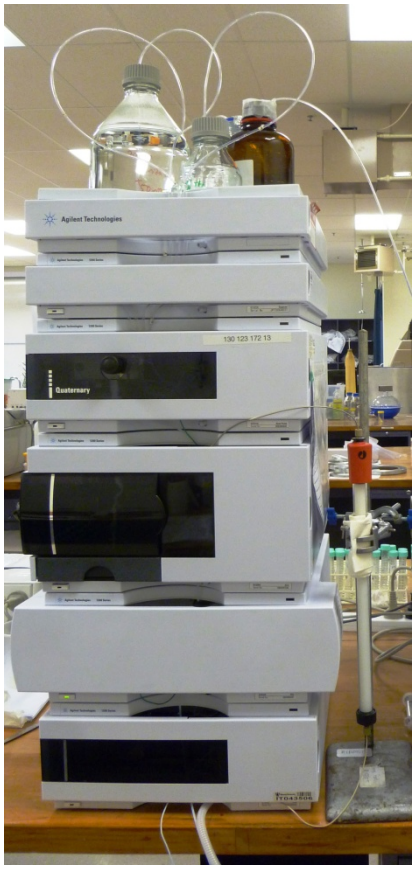


Figure 3-9*: *Photograph of HPLC-SEC system.

3.2.4.7. Turbidity measurements

Turbidity of the sodium caseinate samples was determined on a single cell UV-visible spectrophotometer (Genesys 10 UV, Thermo Scientific). Samples (3mL) were placed in 1 cm path length disposable cuvette (4 mL) and the absorbance measured at 650 nm in duplicate. Milli Q water was taken as the reference standard. For calcium-depleted milks, the samples were diluted 1:1 with deionised water before turbidity measurements.

3.2.4.8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed for the samples at least in duplicate. The fractions collected from the SEC column were freeze-dried and then run on SDS-PAGE. The SDS-PAGE technique was performed as described by Tercinier et al (2013) on Mini-Protean II dual slab cell system (Bio-Rad laboratories, Hercules CA, 94547, USA).

The lyophilised samples were dissolved in sample buffer which contained 2% SDS, 25% glycerol, 0.01% bromophenol blue, 13% 0.5 M Tris-HCl buffer pH 6.8 and β-mercaptoethanol as the reducing agents. Since the protein content of the collected fractions varied, SDS-PAGE was carried out initially to determine the optimal dilution required for each samples. The samples diluted in buffers were heated at 95^{o}C for 5 min and then cooled before loading onto the SDS-PAGE gels.

The SDS-PAGE gels consisted of a stacking and resolving gel. The resolving gel was prepared by mixing 2.5 mL Tris-HCl buffer (1.5 M), 100 mL 10% SDS, 5.3 mL of 30% acrylamide solution and volume made up to 25 mL after adjusting the pH to 8.8. The above mixture was degassed for 20 min under vacuum. The degassed mixture was

added with 5 µl TEMED (N,N,N'-tetramethylethylenediamine) and 50 µl 10% ammonium persulphate solution mixed gently and loaded onto the dual gel casting apparatus. Approximately 2 mL of deionised water was added on top of the resolving gel and the gel left for setting at room temperature. After a 45 min setting time, the water above the resolving gel was tipped and completely removed by absorbing with a blotting paper whip. The stacking gel which was prepared as described below was poured on top of the resolving gel.

Stacking gel was prepared by mixing 1.25 mL of 0.5 M Tris-HCl buffer, 50 µl 10% SDS, 650 µl acrylamide solution and deionised water. After degassing the above mixture for 20 min, 5 µl TEMED and 25 µl ammonium persulphate were added to the stock stacking gel solution and mixed gently. The above mixture was immediately added on top of the preset resolving gel and 0.75 mm plastic comb with 10 slots was inserted. The gel was allowed to set for further 45 min. The prepared gel was then set up on a vertical electrode buffer chamber.

The electrode buffer stock solution (5X) was made by mixing 9 g Tris base, 43.2 g glycine, 3 g SDS in deionised water which was finally made up to 1 litre after adjusting the pH to 8.6. The stock electrode buffer solution was diluted 1:4 ratio with deionised water before being added to the electrolysis chamber.

The electrode buffer was added to the electrolysis chamber till the precast gel was immersed. Approximately 10 µl of sample buffer was injected carefully into the sample well using a syringe. The electrolysis unit was connected to a power supply from Bio-Rad 1000/500 model and run at 180 mV voltage and 70 mA for 1 h. The gels were then transferred carefully to a flat base plastic container for staining and destaining operation.

The brilliant blue staining solution ~ 50 mL (3 g brilliant blue, 200 mL acetic acid glacial, 500 mL propanol in 2 l deionised water) was poured onto the gels and agitated slowly for approximately 1 h in the closed plastic container. The staining solution was slowly tipped into the sink and the gels with rinsed with deionised water. The gels were then destained for 1 h in 100 mL solution containing 10% propanol and 10% glacial acetic acid. The gels were further destained in a fresh destaining solution for 19 h with mild agitation. The gels were stored in deionised water after destaining. The gels were scanned using a flatbed scanner (Scanmaker i900, Microtek, Carson, CA, USA).

3.2.4.9. ^{31}P-Nuclear Magnetic Resonance (NMR)

NMR spectroscopy examines the magnetic resonance property of the nucleus of an atom placed in a static magnetic field and exposed to oscillating magnetic field (Hornak, 1997). Only those atoms with unpaired neutron or proton can be analysed by NMR as they have a property called spin. Spin is a property like charge and is assigned in numerals + and - (Hornak, 1997). Every unpaired proton or neutron has a spin ½. Two atoms have opposing spin i.e. -1/2 and +1/2 as seen in the case of charge will neutralise the effect of spin hence, NMR measures the unpaired nuclear spins (Hornak, 1997). In the absence of a magnetic field, the nuclei spin are randomly oriented (Yadav, 2005). However, in the presence of external magnetic field they orient either parallel or anti-parallel to the applied field (Figure 3.10). In the magnetic field, the nuclei with unpaired protons or neutron absorb photon of a radio wave and is excited to higher energy state (Hornak, 1997).

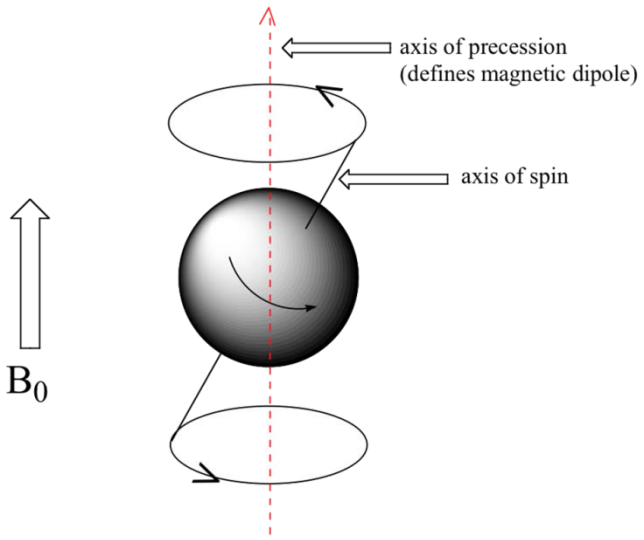


Figure 3-10**:** *Schematic representation of spinning nucleus and generated magnetic dipole.*

The presence of other nuclei and their respective bonding will alter the wavelength at which the photon is absorbed by the nuclei of interest occurs. The absorption of radio waves by the nuclei in different environment produces distinct signals which are recorded in an NMR spectra (Figure 3.11).

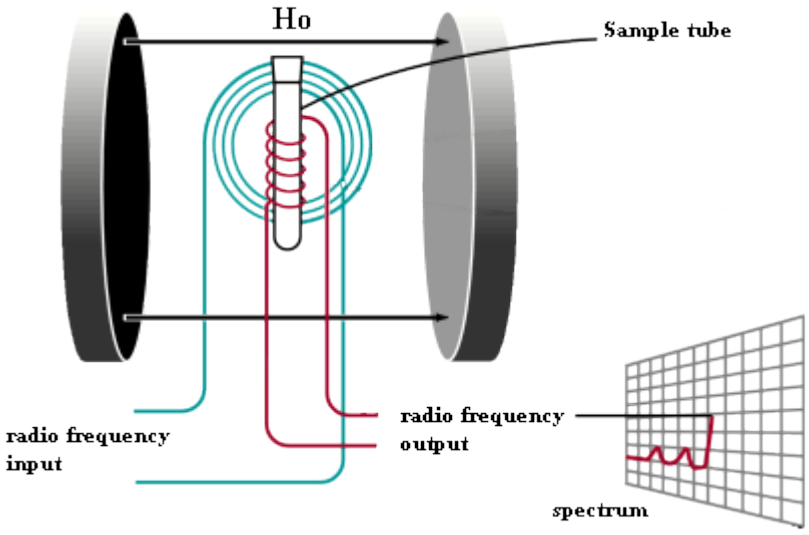


Figure 3-11**:** *Basic arrangement of Nuclear Magnetic Resonance (NMR) spectrometer (http://www.mhhe.com/physsci/chemistry/carey/student/olc/ch13nmr.html).*

Fresh samples were prepared for ^{31}P-NMR spectroscopy using sodium caseinate solutions (2% w/v protein) containing 0.02% w/v sodium azide and different concentrations of iron (0, 2, 4, 6, 8 and 10 mM). The phosphorus concentration was kept constant at 16 mM level.

^{31}P –NMR spectra were recorded using Bruker Avance 400 NMR spectrometer (Bruker Analytik, D-76287, Rheinstetten, Germany) operating at 161.97 MHz equipped with a 10 nm broad bandprobe. A 10 mL NMR tube was filled with 4.2 mL of sample and 0.42 mL of deuterium oxide (Cambridge Isotope Laboratories, Inc., USA). Triphenylphosphine $P(C_6H_5)_3$ dissolved in acetone at 20 mg/mL concentration contained in a concentric 3 mm NMR tube was used as an external standard. The peak for the standard was well separated from sample peaks. Each spectrum was recorded using inverse gated decoupling with a 30^o excitation pulse, spectral width of 64.9 kHz, 64k points and a recycle delay of 2 s.

Chapter 4. Influence of calcium-depletion on iron binding properties of milk

4.1. Introduction

Anaemia is a global epidemic, with over 1.6 billion people being affected worldwide (McLean et al., 2009). Nutritional iron deficiency is considered to be a contributory factor in at least 50% of these cases. The fortification of food with iron is considered to be a long term strategy for solving this problem. However, iron is also considered to be the most difficult micronutrient for food fortification, because of its reactivity with food components (Hurrell, 1997; Richard, 1999). The direct addition of iron salts into food products may result in rancidity because of lipid oxidation (Hurrell, 2002). Chelated forms of iron have thus emerged as a convenient choice for iron fortification, as these forms have reduced ability to interact with the food components. Early studies were aimed at fortifying milk with iron (Douglas et al., 1981), whereas subsequent work looked at the iron-binding ability of milk proteins (Demott et al., 1976; Gaucheron et al., 1997a; Hekmat et al., 1998).

The iron-binding ability of milk proteins, especially caseins, has been recognized for over 50 years. King et al. (1959) reported that the added iron bound mainly to the serum portion of the milk and later research demonstrated that it bound mainly to the caseins in skim milk (Carmichael et al., 1975; Raouche et al., 2009a). Caseins bind > 90% of the iron added to skim milk, with α_{s1}-casein, β-casein and κ-casein binding iron in the ratio 72:21:4 (Demott et al., 1976). The phosphorylated serine residues are the primary sites for iron binding and are frequently present in clusters of 2, 3 or 4 along the casein

chain length. This clustering of the phosphoserine residues, aided by the rheomorphic character of caseins, is responsible for the high metal-binding capacity of caseins. Thus, caseins act as multi-dentate ligands for binding iron *via* co-ordination bonds with oxygen of the phosphorylated residues (Bernos et al., 1997; Hegenauer et al., 1979c). The binding of iron at low concentration (~ 1.5 mM) to the proteins in skim milk is not affected by pH (4.8–6.7) and ionic strength, which has been demonstrated by the successful fortification of products such as yogurt and cheese (Hekmat et al., 1998; Zhang et al., 1989b). The addition of iron to milk alters the distribution of minerals between the soluble and colloidal phases, with a prominent reduction in inorganic phosphorus in the soluble phase (Gaucheron et al., 1997a; Raouche et al., 2009b).

At its normal pH, the calcium content of milk can be altered by exchanging the calcium ions with counter-ions on an ion-exchange resin. Ion exchange of multivalent cations (especially calcium) with monovalent ions (sodium, potassium) has been performed to improve the functionality and the stability of milk products (Bhaskar et al., 2011; Burgess, 1982; Lyman et al., 1933) and to manufacture novel products (Bhaskar et al., 2011). Up to 80% of the calcium in milk can be depleted using this technique (Ranjith et al., 1999). The depletion of calcium from milk induces changes to the structural and physico-chemical properties of the milk (Burgess, 1982; Grimley et al., 2010; Lin et al., 2006), but the effects on the iron-binding properties are not known.

The present work explores the effects of calcium depletion on the binding of iron to milk proteins. Furthermore, the distribution of minerals between the soluble and colloidal phases of milk is explored.

4.2. Results and discussion

4.2.1. Effect of calcium-depletion on the properties of milk

Three levels of calcium depletion were achieved using different volumes of ion exchange resin (0-3.75% by volume of milk). The reconstituted milk with three different levels of calcium depletion were designated as low (18-22%), medium (50-55%) and high (68-72%) calcium depleted milks (CDM) (Figure 4.1). The appearance of normal milk remained unaffected upon ~ 20% calcium depletion. However, a translucent greenish liquid was obtained for milks with > 50% calcium depletion, in agreement with the observations of Grimley et al (2010). The greenish colours of the CDMs were contributed by the presence of riboflavin. The high CDM was apparently less turbid than the medium CDM. The changes observed were due to reductions in the cation contents, especially the calcium content, due to the ion-exchange process.

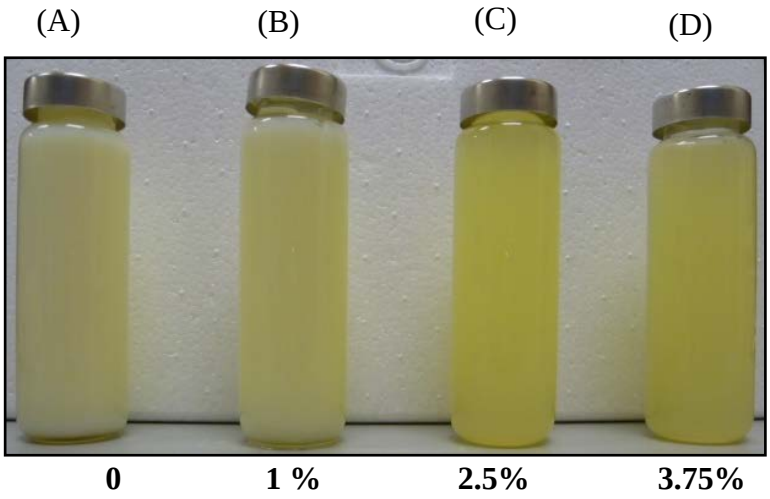


Figure 4-1*: *Photographs of glass vials containing (A) normal milk, (B) low CDM, (C) medium CDM and (D) high CDM. Volume of IE resin used for respective level of calcium depletion is also shown. CDM: calcium-depleted milk.

4.2.1.1. Effect of calcium-depletion on mineral composition of milk

The mineral compositions of normal milk (control) and the CDM are shown in Table 4.1. The calcium content of normal milk was 1097 mg kg^{-1}, which was reduced to 356 mg kg^{-1} by the ion-exchange treatment. As the potassium ions on the ion-exchange resin were exchanged for calcium ions in the milk, the proportionate potassium concentration increased from 1619 mg kg^{-1} in normal milk to 2514 mg kg^{-1} in the high CDM. The ion-exchange treatment also affected the magnesium content of the milk, which was reduced from 82 mg kg^{-1} in normal milk to 41 mg kg^{-1} in the high CDM, i.e., a 50% reduction. Ranjith et al. (1999) observed similar reductions in magnesium content when 60% of the calcium was depleted from milk. The affinity of resins for exchanging the counter-ions in solution is directly proportional to their charge and atomic number (DeSilva, 1999). This implies that a K^+ form of ion-exchange resin will preferably exchange Ca^{2+} over Mg^{2+} and other monovalent ions present in the exchanging medium. Moreover, as the atomic number of calcium (20) is higher than that of magnesium (12), it was preferentially exchanged during the ion-exchange process. The higher sodium contents in the CDM than in normal milk were due to the readjustment of the pH to 6.8 using sodium hydroxide solution.

Although a reduction in the anion concentrations is not envisaged in a cation-exchange process, reductions in total phosphorus content have been reported (Ranjith et al., 1999). In the present study, this decrease was ~ 15 to 20% in milk samples with calcium depletions of > 50% (Table 4.1). However, greater reductions in phosphorus content (up to 50%) were observed in earlier studies (Bishov et al., 1958; Lyman et al., 1933; Ranjith et al., 1999). Ranjith et al. (1999) proposed that the reduction was caused by the precipitation of calcium phosphate on to the surface of the ion-exchange resin.

Table 4-1: *Effect of calcium-depletion on concentrations of minerals in milk*

Milks	Calcium	Potassium	Magnesium	Sodium	Total phosphorus
	(mg/kg)				
Milk	1097.3 (±8.4)	1619.5 (±27.5)	81.7 (±3.8)	428.1 (±8.9)	1007.7 (±24.7)
Low CDM (18 to 22%)*	881.7 (±17.7)	1837.6 (±9.5)	72.5 (±0.3)	484.2 (±3.1)	957.4 (±0.6)
Medium CDM (50 to 55%)*	579.4 (±10.1)	2403 (±25.0)	46.5 (±1.5)	514.1 (±4.9)	855.6 (±18.6)
High CDM (68 to 72%)*	356.3 (±3.9)	2514 (±10.2)	40.7 (±1.0)	642.45 (±50.3)	932 (±4.4)

* Levels of calcium depletion

4.2.1.2. Physico-chemical properties

The effects of calcium depletion on selected characteristics of the milks are shown in Table 4.2. Calcium depletion by ion exchange was accompanied by a reduction in the pH of the milk, the extent being directly proportional to the amount of calcium depleted. The pH of the high CDM (~ 70% calcium depletion) was reduced to 6.28 after the ion-exchange treatment. A similar reduction in pH, from 6.72 to 6.38, was observed by (Ranjith et al., 1999), upon ~ 80% calcium depletion from milk using a comparable resin. All the treated milks were readjusted to pH 6.8 before further analysis.

The protein content in the non-sedimentable phase of normal milk (after ultracentrifugation at 100,000 *g* for 1 h at 20°C) was 0.83% (26% of the total) and

consisted mainly of whey proteins and some non-sedimentable casein particles (Hekmat et al., 1998). The non-sedimentable protein content was higher in the CDM; ~ 35, ~ 82 and ~ 94% of the total protein was found in the non-sedimentable phase of the low, medium and high CDM, respectively.

The Z-average diameters of the normal milk and the low CDM were ~ 260 nm, which suggested that depletion of ~ 20% calcium had little effect on the integrity of the casein micelles (Table 4.2). Similar trends were also reflected in the number average diameters observed for these milks, which were ~ 200 nm. In the medium CDM, the Z-average diameter reduced to ~ 128 nm, reflecting a decrease in the size of the casein micelles and the number average diameter showed an almost 5-fold decrease compared with normal milk. A further reduction in the calcium content (up to 70% in the high CDM) caused progressive disintegration of the remaining particles, as indicated by the decreasing value for the number average diameter. However, a slight increase in the Z-average diameter was observed in the high CDM, which was due to a small number of larger particles/structures in the system.

The average polydispersity index values were ~ 0.168 for both the normal milk and the low CDM, indicating a homogeneous distribution of particles. The polydispersity index increased 2-fold upon > 50% calcium depletion (Table 4.2), probably because of the simultaneous existence of micellar casein particles and variably associated caseins in the dispersions of the medium and high CDM.

All the ζ-potential values were in the range –16 ± 1 mV, irrespective of the calcium content in the milk. In normal milk and the low CDM, where the casein micelle structure was largely preserved, the negative ζ-potential could probably be attributed to

the polar residues of the κ-caseins on the surface of the casein micelles. However, as the casein micelle structure was substantially disintegrated in the medium and high CDM, the negative ζ-potential probably represented the surface charges on the polar residues on partially dissociated casein particles.

Table 4-2: *Effect of calcium-depletion on physico-chemical properties of milk*

Milks (calcium depletion)	**pH**	**Soluble protein (%)**	**Z-average diameter (nm)**	**Number average diameter (nm)**	**Polydispersity index**	**ζ-Potential (mV)**
Milk	6.80 (± 0.02)	0.83 (± 1.74)	261.4	200.5	0.17 (± 0.02)	-14.9 (± 0.4)
Low CDM (18 to 22%)*	6.58 (± 0.04)	1.10 (± 0.3)	258.0	198.9	0.16 (± 0.01)	-16.0 (± 0.3)
Medium CDM (50 to 55%)*	6.42 (± 0.04)	2.52 (± 0.36)	128.2	38.78	0.31 (± 0.03)	-15.1 (± 0.4)
High CDM (68 to 72%)*	6.28 (± 0.06)	2.99 (± 0.10)	158.0	20.14	0.45 (± 0.03)	-16.8 (± 0.9)

* Levels of calcium depletion

TEM showed that roughly spherical casein micelles of various diameters were present in normal milk (Figure 4.2A). However, the casein micelles were completely disrupted upon ~ 70% calcium removal from normal milk (Figure 4.2B). The low density of the residual casein micelle after substantial disruption makes it inappropriate for imaging using TEM.

The integrity of the casein micelles in milk is maintained by the balance of electrostatic repulsion forces and hydrophobic attraction forces, in which calcium phosphate nano-clusters play neutralizing and cementing roles (Horne, 1998). Disintegration of the casein micelles occurs when the balance is shifted towards electrostatic repulsion, caused by the charged residues on the caseins in the micelles.

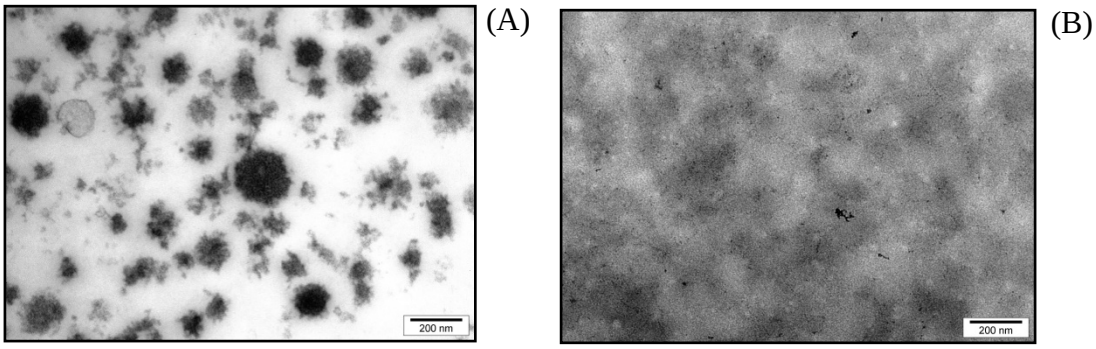


Figure 4-2*: *Microstructure of (A) normal milk and (B) 70% calcium-depleted milk (High CDM) (magnification – 64,000 X).

Furthermore, the colloidal calcium of the casein micelles is in dynamic equilibrium with the calcium in the serum phase of milk (Gaucheron, 2005). Rapid transfer of colloidal calcium to the soluble phase has been demonstrated (Pierre et al., 1981). The ion-exchange resin binds ionic calcium in the aqueous phase of milk (Grimley et al., 2010), resulting in a transfer of micellar calcium to the soluble phase. The reduction in the micellar calcium content of the casein micelle results in an increase in the negative charge on the caseins (Ward et al., 1997). Consequently, the balance shifts in favour of electrostatic repulsion, leading to disintegration (Horne, 1998; Udabage et al., 2000) of the casein micelles, as seen in the medium and high CDM. However, as the calcium content of the medium CDM was higher than that of the high CDM, larger amounts of calcium remained associated with the caseins in the former.

The caseins dissociated from the micelles in the serum phase of milk (Dalgleish, 2011; Pitkowski et al., 2007) may be further associated, because of hydrophobic interactions. Cryo TEM images of milks with 60 and 80% calcium depleted indicated the presence of smaller sized, less dense granular particles of the disintegrated casein micelles remaining in the suspension (Marchin et al., 2007). In this work, it was expected the milks with different calcium contents would have different iron-binding properties.

4.2.2. Effect of iron addition

Five different levels of ferric iron were added as ferric chloride (5, 10, 15, 20 and 25 mM) to the milks with different calcium content and the changes in the particle characteristics determined.

4.2.2.1. Intrinsic fluorescence

The binding of iron to milk proteins was confirmed by tryptophan fluorescence measurements which represent a change in the conformation of protein upon iron addition (Gaucheron et al., 1997a). Since the λ_{max} (wavelength of maximum fluorescence intensity) value for milk was observed at 343 nm, the fluorescence intensity was measured at this wavelength for all samples (Figure 4.3). The binding of iron to milk proteins was indicated by the lowering of the tryptophan fluorescence intensity with increasing iron concentration, as suggested by Gaucheron et al (1997a). A consistent non-linear decrease in the tryptophan fluorescence intensity was observed as a function of iron addition in all samples, in agreement with earlier reports (Gaucheron et al., 1997b). The decrease in tryptophan intensity indicates a change in the local environment in close proximity to tryptophan residues, mainly due to ligand binding and tryptophan-ligand energy transfer (Evans et al., 1975). Minimum values for

tryptophan fluorescence were observed for all samples at 25 mM iron addition. The intrinsic fluorescence measured at this level would be the fluorescence contributed mainly by tryptophan residues of whey proteins.

A similar decrease in intrinsic fluorescence from 230 to 170 was also observed by Gaucheron et al (1997a) upon 1.5 mM iron addition to skim milk. Evans et al (1975) studied the binding of different cations to ovotransferrin and observed that Fe^{2+}, Fe^{3+} and Cu^{2+} caused significant reductions in tryptophan fluorescence, due to tryptophan-ligand energy transfer.

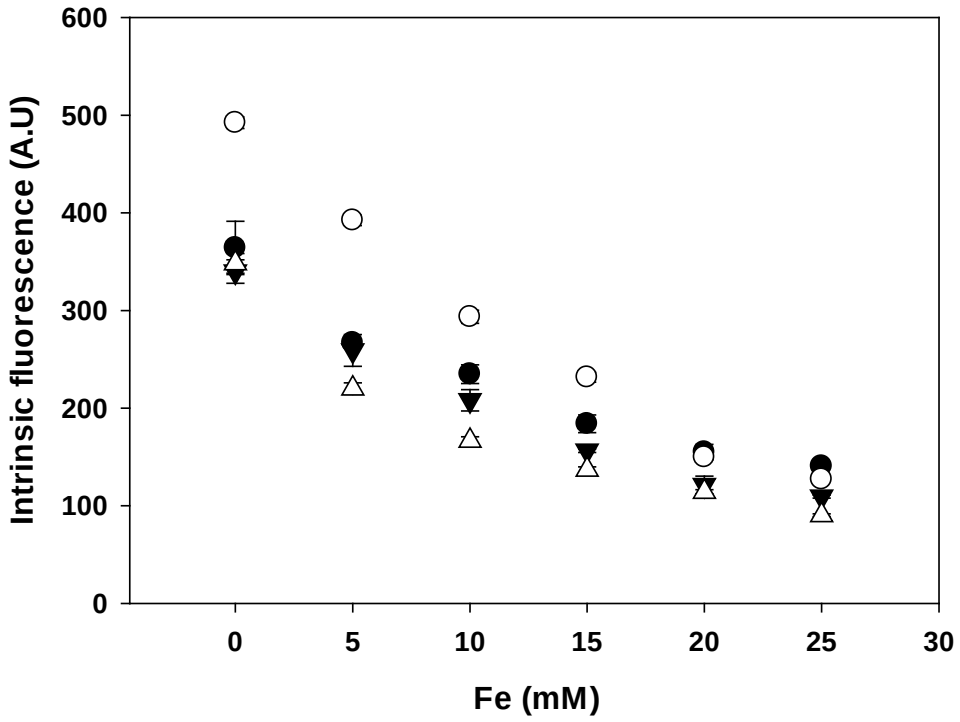


Figure 4-3**:** *The effect of iron addition on tryptophan (intrinsic) fluorescence intensity (at 343 nm) of normal milk (•), low CDM (o), medium CDM (▼) and high CDM (Δ). CDM: calcium-depleted milk.*

4.2.2.2. ζ-Potential

The negative ζ-potential values ranged from –14 to –17 mV for all milk samples, irrespective of their calcium content and these values remained stable upon iron addition to 25 mM (Figure 4.4). The results were in agreement with the trends observed by Raouche et al (2009a), who reported that the ζ-potential values were little affected on the addition of up to 20 mM ferric iron to skim milk. For the medium and high CDM, slightly higher negative ζ-potential values were observed for the latter, at all levels of iron addition.

Ferric iron is positively charged and its binding to the negatively charged phospho-serine residues on the caseins is expected to reduce the overall charge of the protein-iron complex formed. However, this was not observed in any of the milks, irrespective of their total calcium contents. It was probable that in normal milk and low CDM, where the casein micelle structure remained intact upon iron addition, the added iron bound in the interior of the micelle structure, leaving the surface negative charge contributed by κ-casein largely unaffected. The ζ-potential values for medium and high CDM suggested that the negative charge was also retained in the newly formed structures. This was interesting as the aggregation states of the caseins in these milks were markedly different.

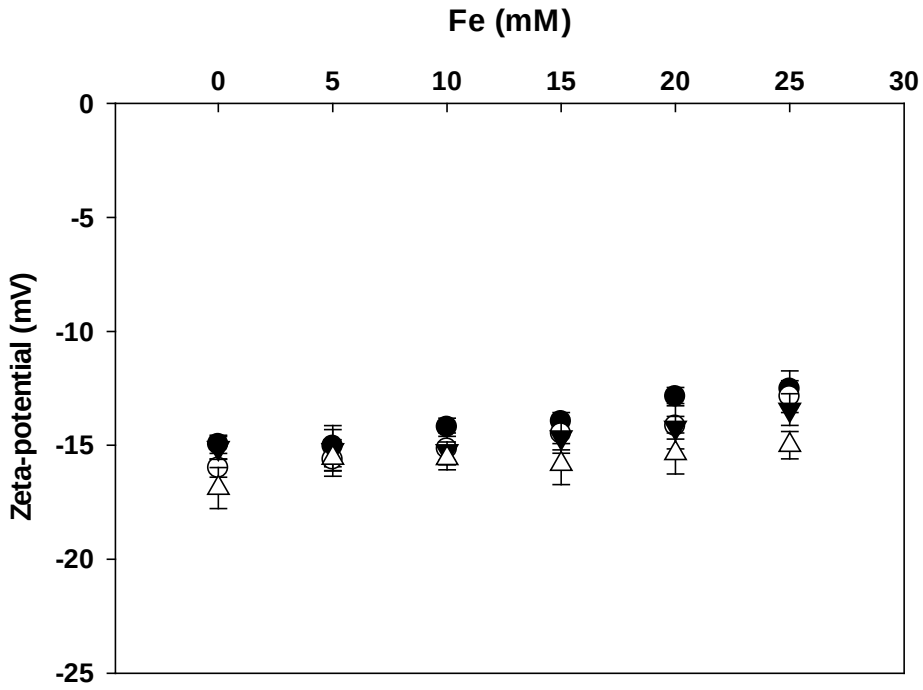


Figure 4-4*: *Effect of iron addition on the ζ-potential of normal milk (•), low CDM (o), medium CDM (▼) and high CDM (Δ). CDM: calcium-depleted milk.

4.2.2.3. Turbidity

The CDMs could be grouped into two distinct systems based on their turbidity values (Figure 4.5). Milks wherein the casein micelle integrity was maintained (as in normal milk and low CDM) had higher turbidity values, owing to the dense casein micelle structure, as compared to CDMs (medium and high CDM) with disintegrated casein micelle structure. The turbidity values of normal milk were unaffected by added iron. Depleting ~ 20% calcium from milk resulted in a lower turbidity value, although the casein micelle integrity was maintained as seen in the previous section. This decrease could be attributed to a decrease in the density of the casein micelle, caused by some dissociation of caseins and calcium. The turbidity remained fairly constant up to 10 mM iron addition but increased at higher iron concentrations, although the values remained

lower than that of normal milk. The turbidity values of medium and low CDM were substantially lower (< 0.6) at similar levels of iron addition. The turbidity values of both the medium and high CDM remained fairly constant up to 15 mM iron addition. An increase in the turbidity values was observed at 20 and 25 mM iron for medium and high CDM respectively.

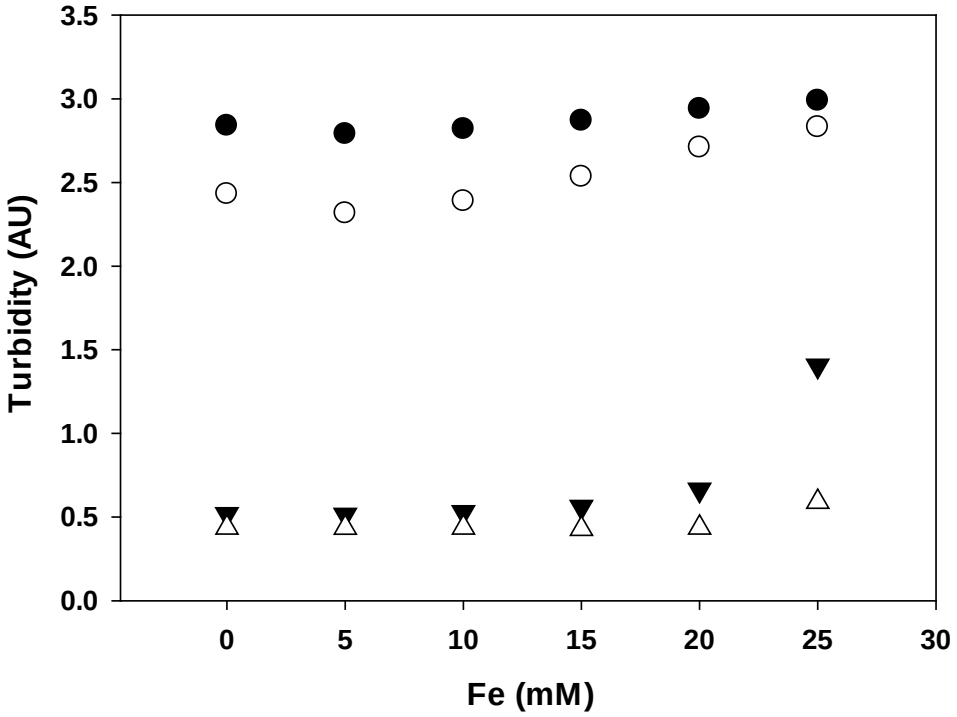


Figure 4-5*: *Effect of iron addition on the turbidity of normal milk (•), low CDM (o), medium CDM (▼) and high CDM (Δ). CDM: calcium-depleted milk.

4.2.2.4. Particle size

The Z-average diameters of normal milk and the low CDM remained fairly constant, regardless of the concentration of added iron (Figure 4.6). Similar results have been observed for Z-average diameter of normal milk upon addition of iron at lower concentration by Gaucheron et al (1997a). The Z-average diameters of the medium CDM were substantially lower and remained at ~ 127 nm up to 15 mM added iron. An

increase was observed at 25 mM added iron, although the value (184 nm) was lower than that of normal milk. In contrast, the Z-average diameter of the high CDM decreased gradually with increasing levels of iron addition.

The number average diameter values (data not shown) for normal milk and the low CDM were lower than the Z-average diameter values, but the trends were similar. For these milks, the diameter values remained fairly constant with iron addition, suggesting that the integrity of the casein micelles was preserved. However, for the medium and high CDM, the trends for the number average diameters were different from those for the Z-average diameters. The high and medium CDM had similar sized particles upon iron addition, although the values for the medium CDM were marginally higher than those for the high CDM. A slight increase in the number average diameter was observed for the medium CDM at 25 mM added iron, the extent of the change was substantially lower than that for the Z-average diameter. The Z-average diameter is based on the intensity variation observed for the particles under Brownian motion, which is biased towards the relatively larger particles/aggregates. The differences in the number average diameter and the Z-average diameter for the medium CDM at 25 mM added iron indicated that only a fraction of the particles were aggregated into relatively larger aggregates.

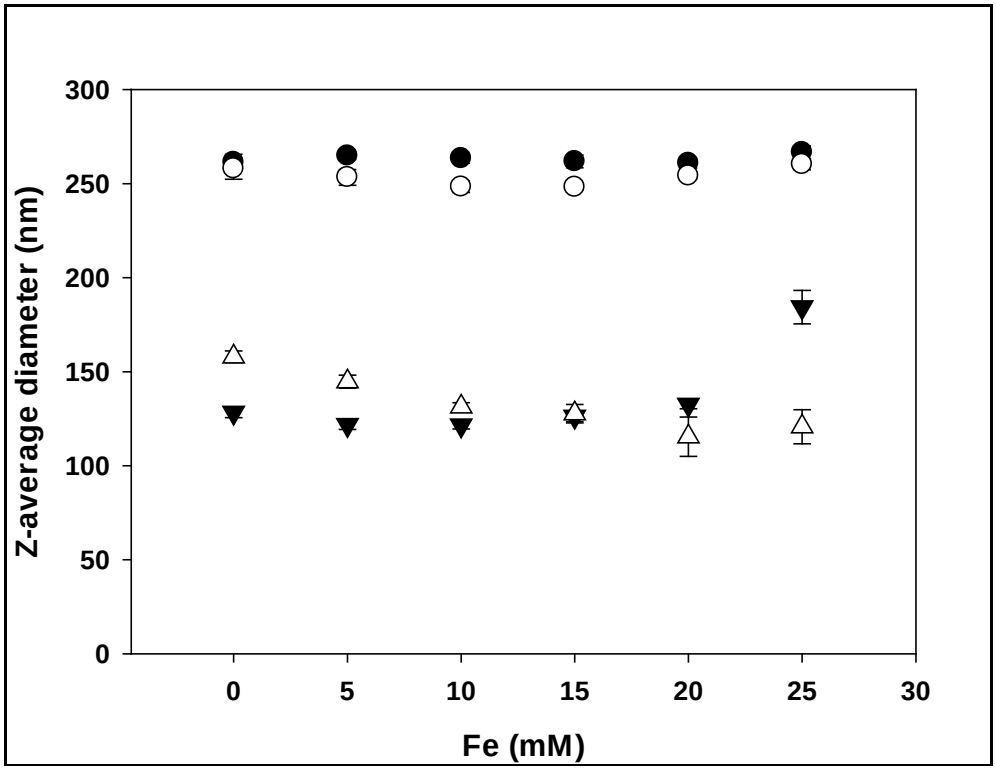


Figure 4-6*: *Effect of iron addition on the Z-average diameter (nm) of normal milk (•), low CDM (o), medium CDM (▼) and high CDM (⊿). CDM: calcium-depleted milk.

4.2.2.5. Microstructure

The microstructures of the iron-added high CDM samples were investigated using TEM (Fig. 4.7). The TEM image of high CDM did not show any dense structures, but dense fibrous structures were observed upon iron addition. The microstructures observed were similar in samples containing varying concentration of iron (5, 15 and 25 mM). Dense, minute circular particles with curved fibrous tail were observed. These structures existed independently and did not form into a larger network even at the highest level (25 mM) of added iron, which interestingly exceeded the level of calcium in normal milk.

It was clear from these observations that the roughly spherical structure of the "casein micelle" was specific to the presence of calcium in milk and could not be reformed by ferric iron under the conditions of this experiment. Although both ions bound to similar sites on casein, the differences in the aggregation were probably due to the type of bonds involved and/or their respective oxidation states. Caseins bind to calcium phosphate nano-clusters *via* electrostatic interactions in milk, whereas coordination bonds are involved in the binding of iron to casein. Furthermore, it has been reported that a cluster of at least three phosphoserine residues is required for the efficient binding of casein to colloidal calcium phosphate (Aoki et al., 1992). However, only two phosphoserine residues are needed to satisfy the coordination requirement for the binding of ferric iron to casein (Hegenauer et al., 1979c). The type of bonds and the number of phosphoserine residues required for interaction could be the reason for the different aggregation properties.

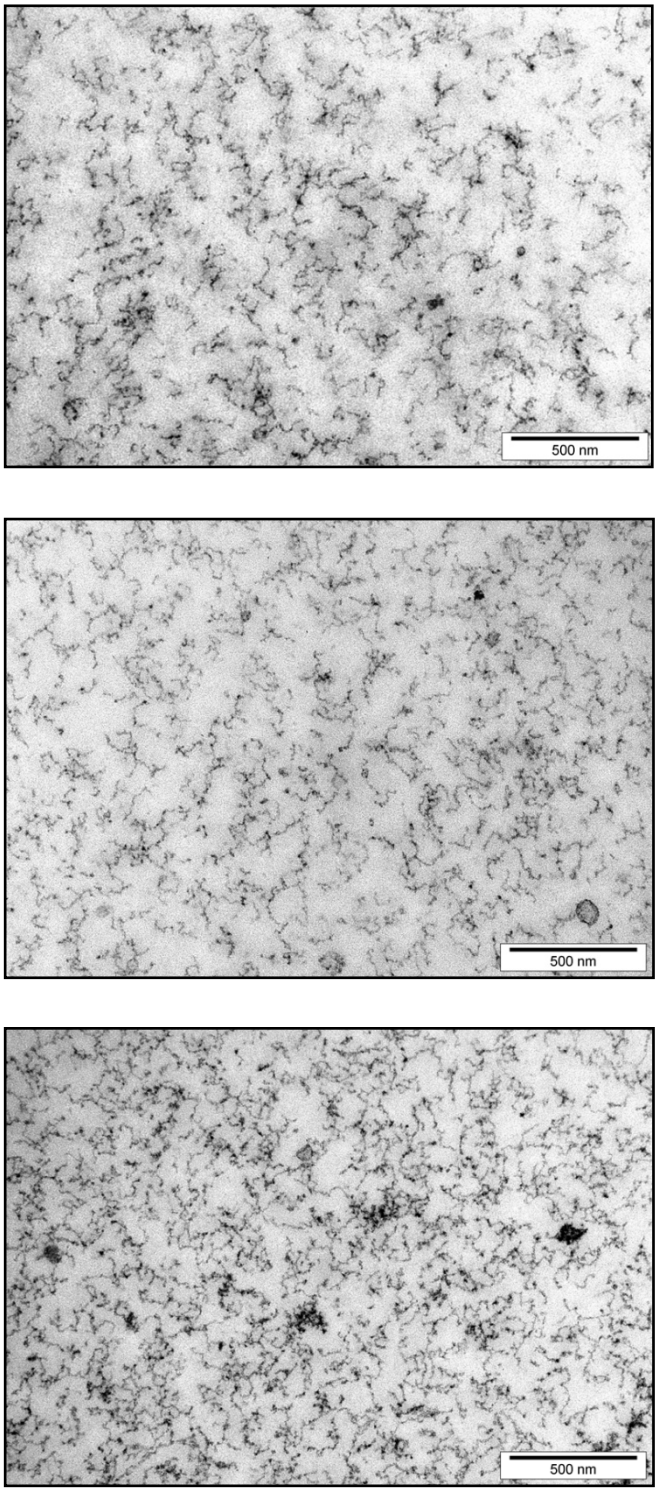


Figure 4-7*: *Microstructure of ~ 70% calcium-depleted milks containing (A) 5, (B) 15 and (C) 25 mM iron (Magnification 46000X).

4.2.2.6. Protein and minerals

The effects of iron addition on the distribution of non-sedimentable (soluble) iron, protein, calcium and phosphorus in normal milk, the low CDM, the medium CDM and the high CDM are shown in Figure 4.8. Overall, iron, protein and calcium followed similar trends in all milks, irrespective of their total calcium content. More importantly, a part of the added iron was always associated with the non-sedimentable phase of all samples.

Ferric iron is soluble under acid conditions (pH ~ 1.9) but at pH > 3.5 it forms an insoluble brick-red precipitate (Crabb et al., 2010a). The presence of iron in the non-sedimentable phase indicated that the solubility of ferric iron was altered by the presence of milk proteins. Among the proteins in milk (whey proteins and caseins), added iron binds mostly to caseins, irrespective of their state of aggregation. Caseins, especially α_s (α_{s1} + α_{s2})- and β-caseins, contain clusters of phosphoserine residues, which bind iron strongly (Horne, 1998), primarily *via* co-ordination links with oxygen (Gaucheron, 2000).

Table 4.3 shows the quantities of iron found in the ultrafiltration permeate of iron added milks. A very small proportion of the added iron was found in the permeate of all samples, irrespective of the added iron concentrations. Between 10 to 15 mg of iron per kg of milk was found in the ultrafiltration permeate of samples containing > 10 mM iron. This amount of iron represents approximately ~ 1% of the added iron. Since, ferric iron in the free form is insoluble at pH 6.8, low concentrations of iron in the permeate might constitute iron bound to low molecular weight fractions as suggested in earlier studies (Basch et al., 1974; Gaucheron et al., 1997b).

Table 4-3*: *Iron content in the permeate

Iron added (mM)	Iron in the permeate (mg/kg)			
	Milk	**Low CDM (18 – 22%)***	**Medium CDM (50 – 55%)***	**High CDM (68 – 72%)***
5	7.2 (±0.3)	8.4 (±0.3)	7.8 (±1.9)	6.8 (±0.7)
10	9.9 (±0.3)	13.1 (±1.5)	12.8 (±4.1)	8.1 (±0.8)
15	11.9 (±0.3)	14.3 (±0.9)	15.3 (±5.2)	13.7 (±2.3)
20	12.8 (±1.1)	17.1 (±1.2)	14.0 (±2.7)	9.4 (±0.6)
25	12.6 (±0.1)	16.5 (±0.1)	14.4 (±1.3)	11.8 (±1.1)

Corrections factor applied – 0.96, * Levels of calcium depletion

In normal milk, ~ 26% of the total protein and ~ 31% of the total calcium were associated with the non-sedimentable phase and were not significantly affected by iron addition (Figure 4.8A). Approximately 44% of the total phosphorus was present in the non-sedimentable phase of normal milk, which reduced to ~ 10% upon the addition of 25 mM iron. Less than 15% of the total iron was associated with the non-sedimentable phase at all levels of addition.

The depletion of ~ 20% calcium from normal milk resulted in ~ 10% increase in the non-sedimentable protein and phosphorus content in the low CDM (Figure 4.8B). The addition of iron to this system decreased the proportions of non-sedimentable protein and calcium, which reached similar proportions to those in normal milk. Phosphorus was most affected, with ~ 15% of the total phosphorus remaining in the non-sedimentable phase upon the addition of 25 mM iron. The proportion of iron in the non-sedimentable phase decreased at all levels of addition, with ~ 7% of the added iron remaining in the non-sedimentable phase at 25 mM added iron. The trends observed

showed that added iron bound mostly to the casein micelles, in agreement with previous work (Gaucheron et al., 1997b; Hegenauer et al., 1979b; Hekmat et al., 1998). The majority of the calcium present in normal milk and the low CDM was also associated with the non-sedimentable phase, irrespective of the iron content.

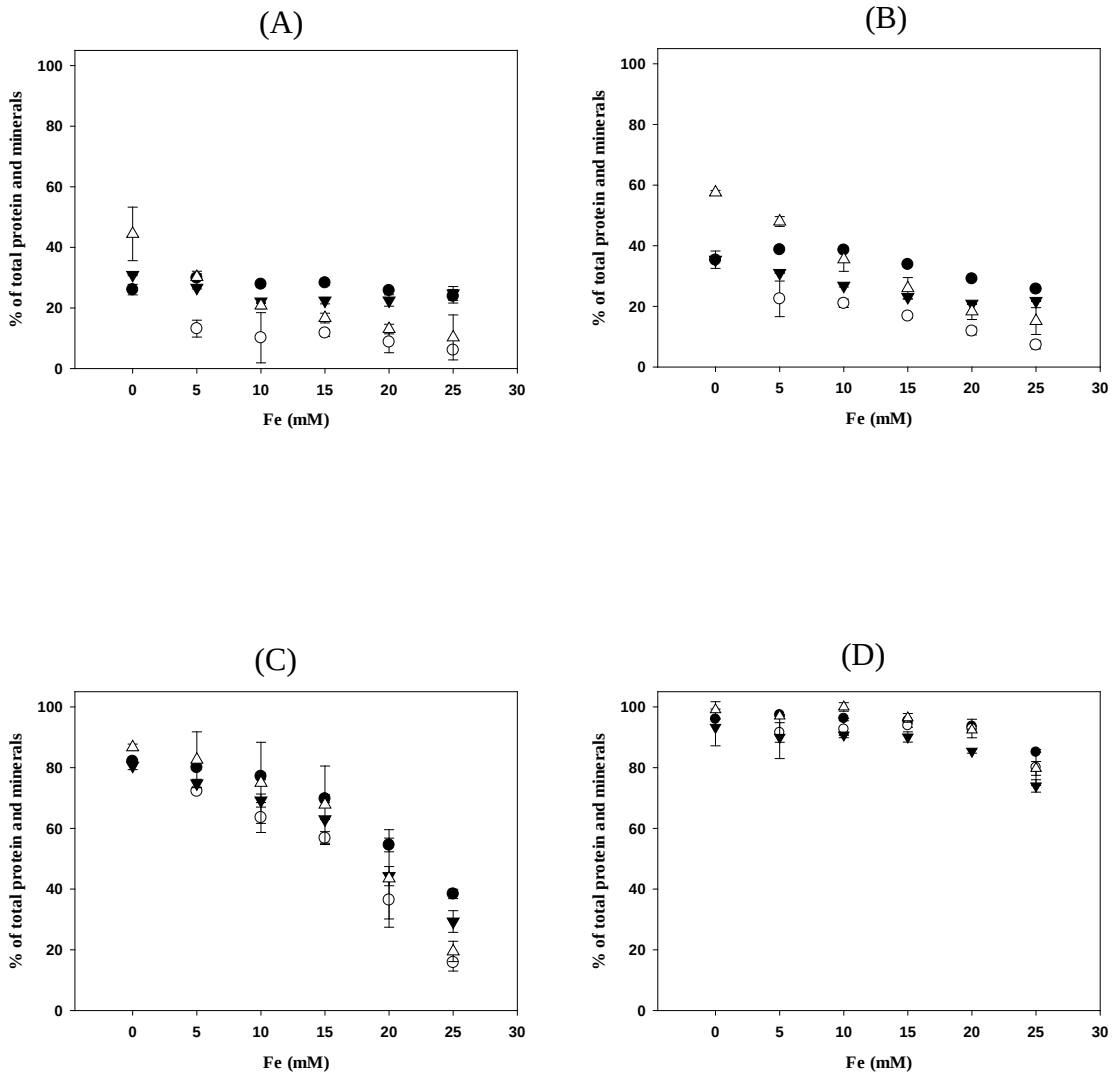


Figure 4-8**:** *Effect of iron addition (5, 10, 15, 20 and 25 mM) on proportional distribution of (•) protein, (o) iron, (▼) calcium and (Δ) phosphorus in the non-sedimentable phase (100,000 g for 1 h at 20°C) of (A) normal milk, (B) low CDM, (C) medium CDM and (D) high CDM. CDM: calcium-depleted milk.*

As the binding affinity of iron to casein is higher than that of calcium to casein, the exchange of iron with calcium in the casein micelle has been advocated (Hegenauer et al., 1979a). However, the present study found that calcium was not released into the non-sedimentable phase, even upon the addition of 20 mM iron to milk (Figure 4.8A). Therefore, it was probable that calcium, if displaced upon iron addition, remained associated with the casein micelle in some way. It is possible that the binding sites on the casein micelle were shared by calcium and added iron.

Substantially higher proportions of protein (> 80%) and phosphorus remained in the non-sedimentable phase of the medium and high CDM, which presumably consisted of solubilised caseins and the whey proteins. The addition of iron resulted in a concentration-dependent decrease in the proportions of non-sedimentable protein, calcium and phosphorus in the medium CDM, with considerable reductions above 10 mM iron addition. Similar trends were observed for the proportion of added iron in the non-sedimentable phase at all levels of addition. At the highest level of iron addition (25 mM), ~ 40, ~ 28, ~ 15% and ~ 19% of the total protein, calcium, iron and phosphorus was observed in the non-sedimentable phase. Positive linear correlations between the proportions of protein, iron, calcium and phosphorus in the non-sedimentable phase of the medium CDM were observed, suggesting that iron, calcium and phosphorus were bound to the sedimentable proteins in the medium CDM (Figure 4.9). Despite these reductions, higher proportions of all the above components were present in the non-sedimentable phase of the medium CDM, compared with normal milk and the low CDM.

Among all the milks, the high CDM had the lowest concentration of calcium (~ 30% of that in normal milk) and therefore the highest proportion of protein in the non-

sedimentable phase (Figure 4.8D). The non-sedimentable protein content of the high CDM was not affected up to 20 mM added iron, but a slight reduction at 25 mM added iron was observed. Approximately 80% of the total protein was still retained in the non-sedimentable phase of the high CDM, even after the addition of 25 mM iron. Both calcium and phosphorus were present mainly (> 90%) in the non-sedimentable phase of the high CDM up to 20 mM iron addition, but decreased slightly at 25 mM. The high CDM had > 90% of the added iron (up to 20 mM) in the non-sedimentable phase, with a minor reduction at 25 mM.

Figure 4.9 shows the SDS-PAGE on the supernatants of iron added milk samples. The presence of whey proteins along with non-sedimentable caseins was evident in the control milk. The addition of iron to milk decreased the concentration of non-sedimentable caseins, with no caseins present at 25 mM added iron (Figure 4.9 A). However, the whey proteins remained in the non-sedimentable phase of all iron added samples irrespective of its concentration. The higher proportion of iron in the non-sedimentable phase of CDM was due to the presence of caseins. Considering the protein in the non-sedimentable phase of 25 mM iron added milk consisted only of whey protein, the level of non-sedimentable caseins were calculated in all iron added CDM samples (Table 4.4). These results confirm that the increased proportion of iron in the non-sedimentable phase of CDM was due to the presence of caseins, as they have a higher affinity and capacity to bind iron. The presence of caseins in the non-sedimentable phase was observed in the supernatant of high CDM (Figure 4.9 B)

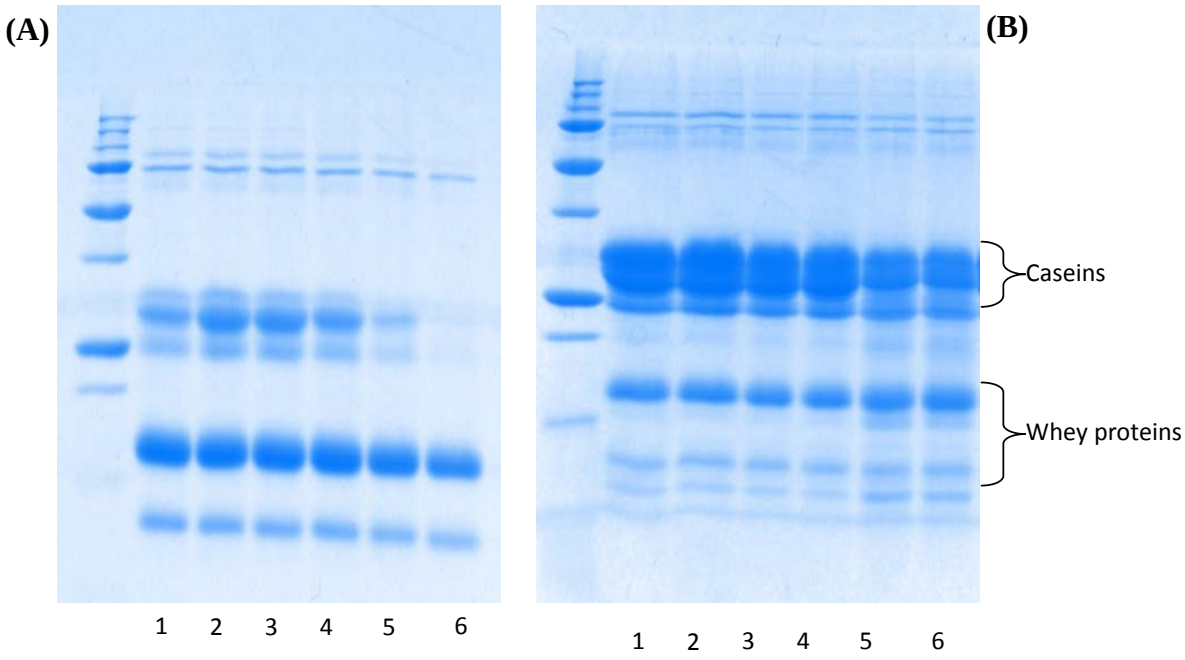


Figure 4-9**:** *SDS-PAGE of the non-sedimentable proteins (100,000 g, 1 h at 20^{o}C) in milk (Lane 1) and supernatants of samples containing different concentrations of iron i.e. Lane 2 – 5 mM, Lane 3 – 10 mM, Lane 4 – 15 mM, Lane 5 – 20 mM and Lane 6 – 25 mM of (A) normal milk and (B) ~70% calcium-depleted milk.*

Table 4-4: *Proportion of non-sedimentable caseins in iron added milks*

Iron added (mM)	**Non-sedimentable caseins (%)**			
	Milk	**Low CDM (18 – 22%)**	**Medium CDM (50 – 55%)**	**High CDM (68 – 72%)**
5	5.9	19.1	73.5	96.6
10	5.3	18.9	69.6	95.0
15	6.0	12.8	59.9	93.9
20	2.5	6.3	39.6	91.5
25	0.0	1.7	17.8	80.2

The non-sedimentable phase of 25 mM iron added milk contained 0.76% of protein and ~ 75 mg/kg of iron, indicating that whey proteins bound ~ 1% by weight of iron (10 mg iron/g of protein). Approximately ~ 41 mg of iron was bound per gram of protein in the non-sedimentable phase of high CDM at 25 mM iron addition, which corresponded to a 4-fold increase compared with normal milk. To our knowledge, this concentration of iron in the soluble phase of milk is the highest reported to date.

An interesting aspect of the above results was the difference in the proportionate distribution of iron in the medium CDM and the high CDM. The depletion of calcium above a critical concentration (> 50%) from milk resulted in substantial dissociation of the casein micelles, as seen earlier. Disintegration of the casein micelles results in the generation of different kinds of aggregated particles (Pitkowski et al., 2007). In the case of the medium CDM, the casein micelles were partly dissociated; however, the nature of these particles was uncertain. The results for calcium in the non-sedimentable phase of both the medium CDM and the high CDM suggested that a higher proportion of calcium was bound to caseins in the non-sedimentable phase of the former (Figure 4.8D). The higher tendency towards protein sedimentation in the medium CDM compared with the high CDM was probably due to the binding of iron to the caseins aggregated by calcium.

In contrast, relatively lower proportions of dissociated caseins in the non-sedimentable phase of the high CDM were associated with calcium. Hence, the majority of the iron added (> 83%) to the high CDM bound to these dissociated caseins and resulted in the formation of small non-sedimentable particles. Overall, these results suggested that the distribution of protein-iron complexes between the sedimentable phase and the non-sedimentable phase was governed by the calcium contents of the respective milks.

The association of > 90% of the added iron with the non-sedimentable phase in the high CDM upon 20 mM iron addition was notable. Protein precipitation has been observed in sodium caseinate solutions at < 5 mM iron addition (Gaucheron et al., 1996). The difference was probably related to the minerals present in the milk. A reduction in the concentration of soluble phosphorus in milk upon iron addition has been observed previously, with the formation of insoluble ferric phosphate (Raouche et al., 2009b). A decrease in this study was also observed, i.e., the proportion of non-sedimentable phosphorus reduced below the levels originally present in the milks, irrespective of their calcium contents. Hence, the role of phosphate in the solubility of iron–protein complexes is interesting and worth exploring.

4.2.2.7. Magnesium, sodium and potassium

Figure 4.10 shows the proportionate distribution of monovalent cations (sodium and potassium) and magnesium in the supernatants of normal milk and calcium-depleted milk. The monovalent cations in normal milk were almost entirely present in the non-sedimentable phase. The addition of iron to normal milk had little effect on the proportionate distribution of the monovalent cations. Similar observations were noted in calcium-depleted milks, where the proportionate distribution of sodium and potassium ions remained unaffected by iron addition. However, the proportionate distribution of magnesium decreased up to 10 mM iron addition level but remained fairly constant thereafter. The reduction observed in the distribution of magnesium was in contrast to the information provided by Raouche et al (2009b). They observed no increase in the colloidal magnesium contents upon iron addition. The difference might be due to the carbonation step used by these authors in the preparation of fortified milk. In the calcium-depleted milk, the trends for distribution of non-sedimentable magnesium were

similar to those observed for caseins. The proportion of magnesium associated with non-sedimentable phase increased with the level of calcium depletion. The addition of iron resulted in a reduction of the magnesium content in the low and the medium CDM, which indicated that at least a part of magnesium was bound to caseins. In the high CDM, a reduction in non-sedimentable magnesium content was observed only at 25 mM iron addition level, indicating that a part of the magnesium was bound to caseins.

Since, iron was also bound to the sedimentable casein fraction, the above results suggest that divalent magnesium was bound to the caseins and participated in the protein-iron complex formation. However, monovalent ions (sodium and potassium) did not play a role in the formation of protein-iron complexes.

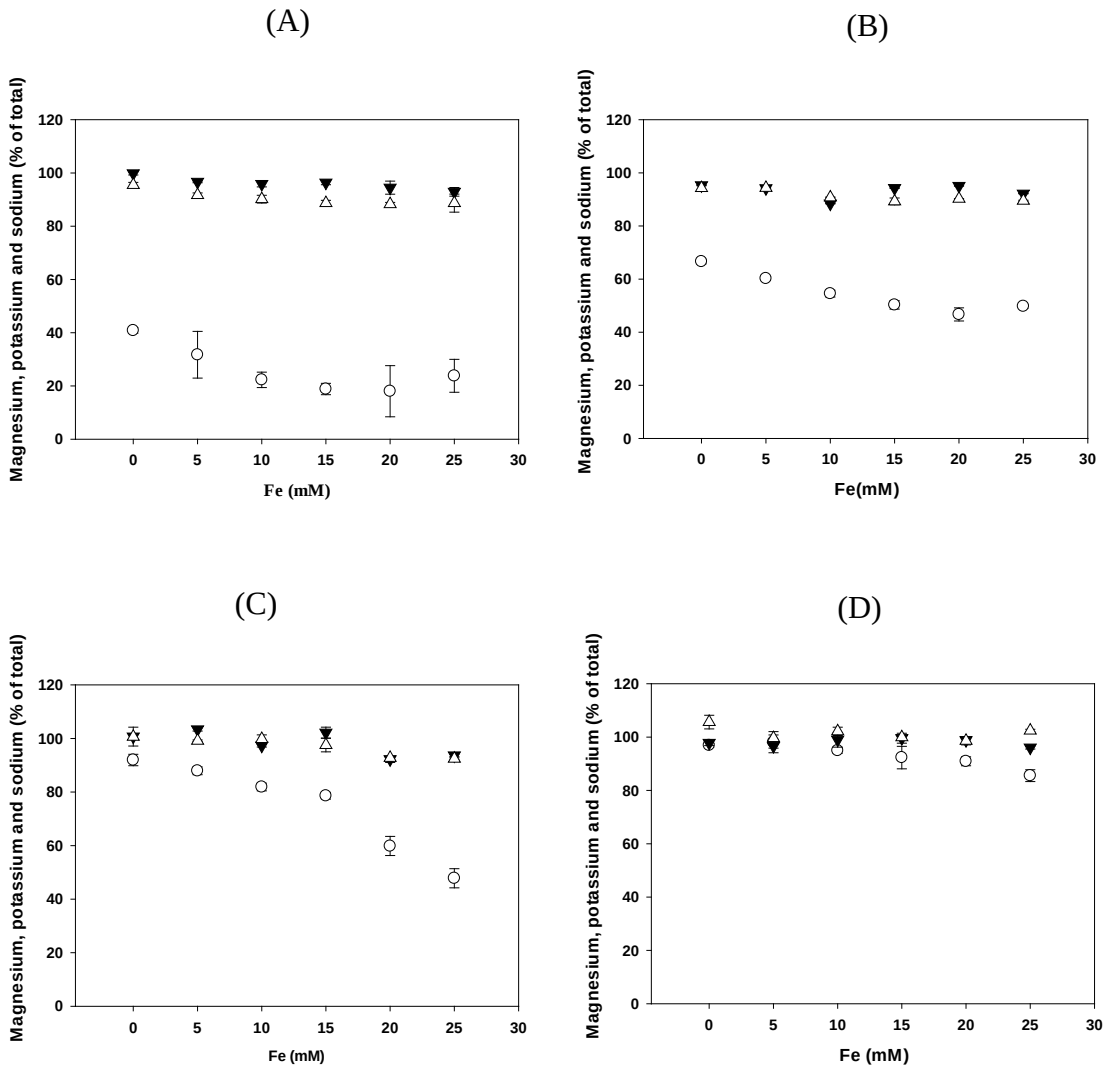


Figure 4-10**:** *Effect of iron addition (5, 10, 15, 20 and 25 mM) on proportional distribution of (Δ) potassium, (o) magnesium and (▼) sodium in the non-sedimentable phase (100,000 g for 1 h at 20°C) of normal milk (A), low CDM (B), medium CDM (C) and (D) high CDM. CDM: calcium-depleted milk.*

4.2.2.8. Phosphorus in permeate

The phosphorus content in the permeate of normal milk and the CDM constituted the phosphorus in "free" form (Figure 4.11). Approximately 34% of the total phosphorus was present in the permeate of normal milk and increased almost two-fold to ~ 70% in the high CDM. The increase in the permeate phosphorus content of the CDM suggested that a portion of the solubilised micellar phosphorus of the casein micelle existed in a diffusible form. An increase in the concentration of diffusible phosphorus upon disintegration of the casein micelle was also observed in an earlier study (Holt et al., 1986b). However, not all of the phosphorus that disintegrated from the casein micelle was diffusible, suggesting that a portion was associated with the protein in the non-sedimentable phase.

A decrease in the permeate phosphorus content was observed upon iron addition to all milks, irrespective of their total calcium content. The phosphorus content in the permeate of normal milk and the low CDM reduced almost nine-fold (to less than 6%) upon 25 mM iron addition. For the medium CDM, the permeate was completely depleted of aqueous phosphorus upon 20 mM iron addition. However, even upon 25 mM iron addition in the high CDM, ~ 8% of the total phosphorus still remained in the permeate. The decrease in permeate phosphorus indicated that phosphorus in free form (probably ionic) was interacting with the added iron. Furthermore, the interaction of this phosphorus with iron was not affected by the concentration of calcium present in the milk. It was probable that a salt of ferric phosphate was formed upon iron addition. However, ferric phosphate is insoluble and its formation inside the hydrophobic regions of the caseins micelle has been suggested using Mossbauer spectroscopy (Raouche et al., 2009b). The interaction of ionic phosphorus with iron, in the non-sedimentable

phase of calcium-depleted milks, indicates that salt of ferric phosphate was interacting with proteins.

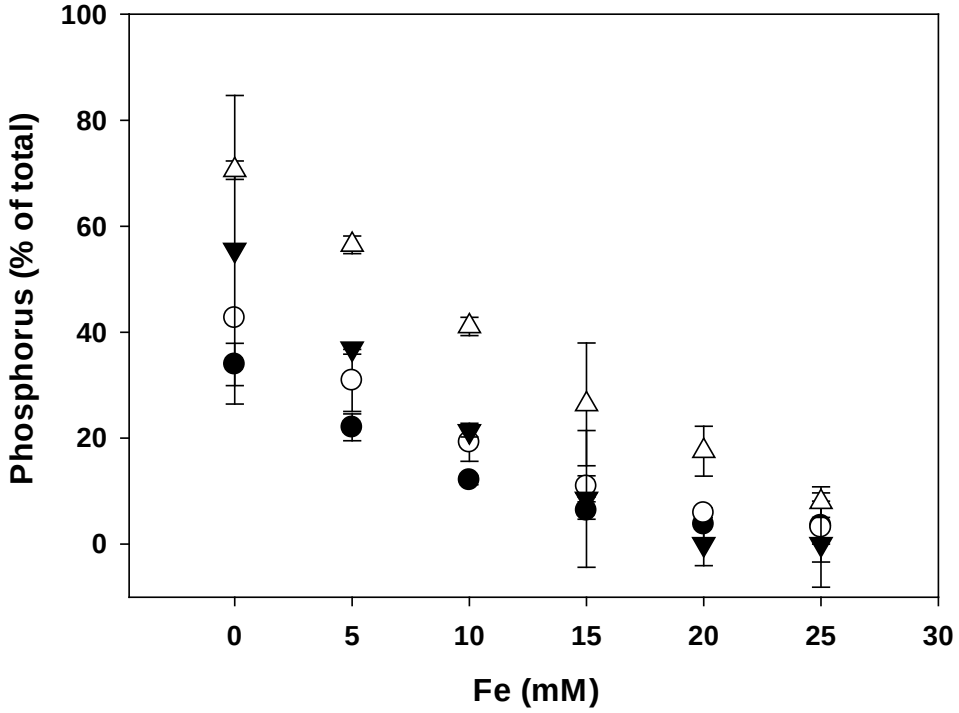


Figure 4-11*: *Effect of iron addition (5, 10, 15, 20 and 25 mM) on proportional distribution of phosphorus in the permeate of (•) normal milk, (o) low CDM, (▼) medium CDM and (Δ) high CDM. CDM: calcium-depleted milk.

4.3. Conclusions

The depletion of calcium from milk by ion exchange affected its physico-chemical properties, with the extent being highly dependent on the level of calcium depletion. The integrity of the casein micelles was retained at up to ~ 20% calcium depletion from normal milk, but there was substantial disintegration of the casein micelles at calcium depletion levels of > 50%. The properties of the milks with different calcium contents were variably affected by the addition of iron. In both normal milk and the low CDM, the majority (> 90%) of the added iron bound to caseins within the casein micelles, minimally affecting the hydrodynamic diameter, ζ-potential and protein distribution.

A reduction in aqueous phosphorus in proportion to added iron was also observed. Protein solubility was adversely affected in the medium CDM, whereas most of the protein and the added iron were associated with the non-sedimentable phase in the high CDM. The solubility of protein–iron complexes in the high CDM was related to the formation of small curved fibrous structures that resisted sedimentation upon ultracentrifugation. An interesting aspect of this study was the high concentration (~ 20 mM) of ferric iron in the non-sedimentable phase of the high CDM (~ 70% calcium depletion). This presents a distinct advantage of the calcium depletion process over traditional processes for the iron fortification of milk systems. Overall, this work suggests that the caseins in milk could form stable nano-complexes with ferric iron when ~ 70% of the calcium is depleted from milk, with potential applications in liquid food products. Finally, the study highlights that the binding of iron to caseins in milk with various calcium contents is different from than in model solutions (e.g., sodium caseinate or pure caseins) mainly due to the differences in their inorganic phosphorus content.

Chapter 5. Role of orthophosphate in iron- and/or calcium-induced aggregation of proteins in calcium-depleted milk

5.1. Introduction

Bovine milk is a rich source of calcium (~ 30 mM), which is distributed between the serum and colloidal phases (Gaucheron, 2005). Calcium, in the colloidal phase, is in part bound directly to caseins, as well as being associated with inorganic phosphorus as amorphous calcium phosphate (Holt, 1982). The calcium phosphate nanoclusters act as nucleation sites for the aggregation of the highly phosphorylated caseins (α_{s1}-, α_{s2}- and β-caseins), which, aided by hydrophobic interactions, lead to the formation of casein micelles (Fox et al., 2008; Holt, 1997; Horne, 2009). It has been proposed that, on average, a casein micelle with a radius of ~ 100 nm contains between 800 and 1100 nanoclusters (~ 2.3 nm), containing approximately 355 ± 20 calcium phosphates ($CaHPO_4$ x $2H_2O$) and surrounded by nearly 50 peptide chains bound by electrostatic interactions (Choi et al., 2011; Holt, 2004; Holt et al., 1998; Little et al., 2004). The clustering of phosphorylated serine residues on the caseins is considered to be a prerequisite for stabilization of the casein micelle structure (Aoki et al., 1992), although binding of colloidal calcium phosphate to carboxylic acid residues with similar affinity has been confirmed (Holt, 1997; Mekmene et al., 2011).

However, in the serum phase, only a limited amount of calcium is associated with phosphate because of the low solubility of calcium phosphate salts. Disintegration of the

casein micelle and solubilization of the caseins occur upon depletion of inorganic calcium or phosphorus or both from the casein micelle (Holt, 1982; Ranjith et al., 1999). The casein micelle can be re-formed, but with a lower hydrodynamic radius, upon re-addition of calcium to ion-exchanged milk (Grimley et al., 2010). The formation of casein-micelle-like structures has been reported upon calcium addition to sodium caseinate in the presence of orthophosphate (Aoki et al., 1987; Zhang et al., 1996). The dispersion stability of the particles formed upon calcium addition to caseins is governed by the concentration of phosphorus (Horne, 1982). The inclusion of exogenous orthophosphate in milk increases the sedimentation of calcium and decreases the level of ionic calcium (Mekmene et al., 2009; Udabage et al., 2000).

Caseins also bind other multivalent cations, such as zinc, magnesium, copper and iron, *via* the same phosphoseryl and carboxylic acid residues involved in the binding to the colloidal calcium phosphate (Baumy et al., 1988b; Philippe et al., 2005). However, the modes of binding are different. Magnesium and calcium bind electrostatically to the caseins whereas coordination bonds are involved in the binding of copper and iron. As the coordination bonds are stronger than electrostatic interactions, the affinity of binding is greater for iron and copper than for calcium and magnesium (Philippe et al., 2005). The binding of caseins to colloidal calcium phosphate nanoclusters is affected by ionic strength and temperature, but the iron remains bound to the caseins even at high ionic strengths, high temperature and pH between 4.0 and 7.8 (Baumy et al., 1988b; Gaucheron et al., 1996; Hekmat et al., 1998).

The present work aimed to examine the role of exogenous orthophosphate in the iron- and calcium-induced aggregation of proteins in ~ 70% calcium-depleted milk (CDM).

The CDM used in this study was prepared according to the process outlined in Chapter 4. Approximately 70% of the calcium was depleted from the reconstituted (11% w/v) skim milk, using a weakly acidic ion-exchange resin. Greater than 90% of the total caseins were present in the supernatant (100,000 *g* for 1 h at 20°C) of the CDM (~ 70% calcium depletion). Up to 20 mM iron could be added to the CDM with little protein sedimentation. The addition of iron to CDM resulted in a proportionate reduction in diffusible (ultrafiltration permeate) phosphorus in the CDM, which reduced from ~ 70 to ~ 8% upon 25 mM iron addition (Chapter 4). Although a reduction in the solubilities of protein and iron was observed at 25 mM iron addition, > 83% of these constituents were still present in the non-sedimentable phase.

5.2. Results and Discussion

5.2.1. The effect of exogenous orthophosphate on iron-induced aggregation of proteins in CDM

The effects of orthophosphate addition on the solubility of protein and iron were examined at 30 mM level of iron addition to CDM; under these conditions, a substantial protein sedimentation was observed. Di-potassium hydrogen orthophosphate at four different concentrations (8, 16, 24 and 32 mM) was added to the CDM prior to iron addition.

5.2.1.1. Particle size

The Z-average diameter of CDM with 30 mM added iron was relatively high (~ 500 nm) due to the presence of large protein aggregates (Figure 5.1). The inclusion of 8 mM orthophosphate in the CDM reduced the Z-average diameter to ~ 167 nm and it remained at ~ 123 nm in samples containing higher levels of exogenous orthophosphate. These results suggested that fortifying the CDM with orthophosphate promoted the formation of smaller aggregates. Consequently, the Polydispersity Index (PDI) value of 30 mM iron added CDM decreased considerably upon the inclusion of 32 mM exogenous orthophosphate, indicating the formation of relatively monodisperse particles.

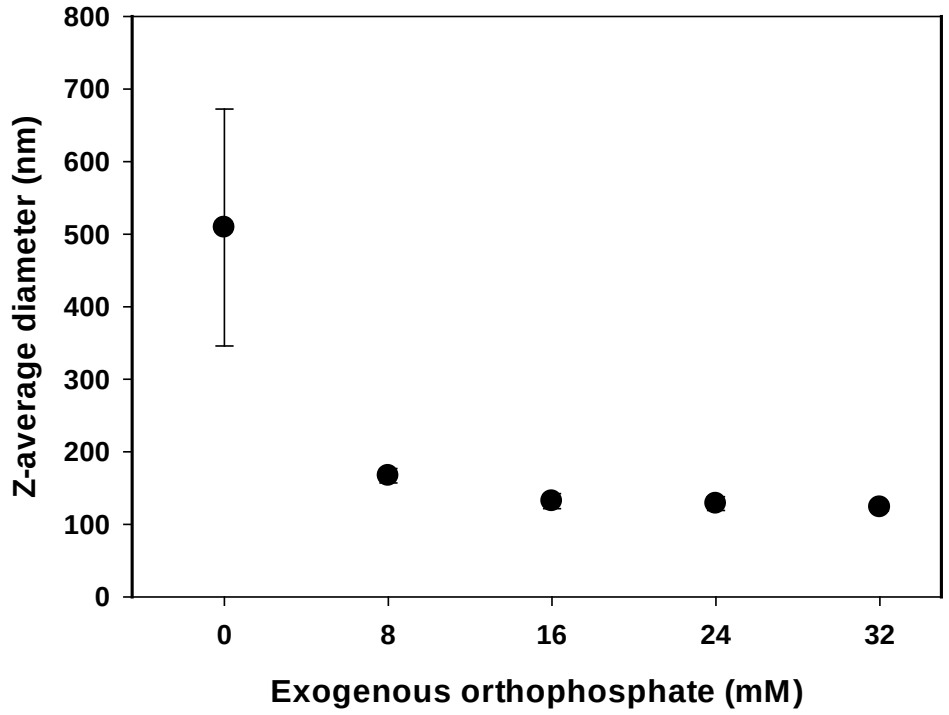


Figure 5-1*: *Effect of added orthophosphate on Z-average diameter (nm) of 30 mM iron added calcium-depleted milk (~ 70% calcium depletion).

5.2.1.2. Distribution of protein and minerals

Approximately ~ 36% of the total protein, ~ 11% of added iron and ~ 21% of the total phosphorus were present in the non-sedimentable phase upon 30 mM iron addition to CDM (Figure 5.2). The proportions of protein, iron and phosphorus in the non-sedimentable phase increased to ~ 88, ~ 73 and ~ 84%, respectively, when the CDM with 30 mM added iron contained 32 mM exogenous orthophosphate. A significant increase in the proportion of these constituents in the non-sedimentable phase even at the lowest concentration (8 mM) of added orthophosphate was observed. Quantitatively, ~ 38 mg of iron per gram of protein was associated with the non-sedimentable phase of CDM containing 30 mM iron and 32 mM exogenous orthophosphate. Overall, the

results indicated that increasing the inorganic phosphorus content of CDM improved the solubility of the protein-iron complex.

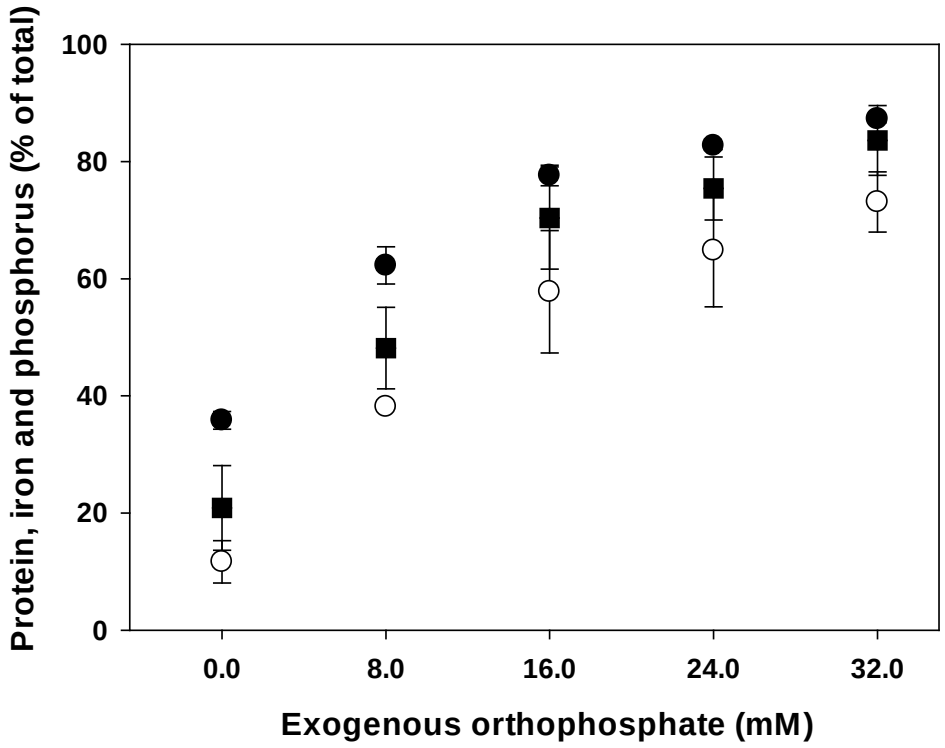


Figure 5-2**:** *Effect of orthophosphate addition to calcium-depleted milk (~ 70% calcium depletion) on non-sedimentable protein (●) iron (○) and phosphorus (■) upon 30 mM iron addition*

Figure 5.3 shows the proportion of phosphorus in the permeate after the addition of 30 mM iron to CDM containing different concentrations of exogenous orthophosphate. The phosphorus in the permeate represents the diffusible phosphorus, which exists mainly in the free ionic form. The addition of 30 mM iron to CDM reduced the permeate phosphorus content from ~ 21 to ~ 1 mM, suggesting an almost complete transfer of the diffusible inorganic phosphorus to the protein-iron complex (Figure 5.3). The inorganic phosphorus in the permeate increased, corresponding to the concentration of exogenous orthophosphate and constituted ~ 30% of the total (18 mM) for the sample to

which 64 mM had been added. However, the increase in the quantity of permeate phosphorus was only a portion of the added orthophosphate. Of the added orthophosphate, between 23 and 51% was present in the permeate of 30 mM iron containing CDM, indicating that part of the added orthophosphate was associated with the protein–iron complex. The concentration of orthophosphate that associated with the protein-iron complex was ~ 6, ~ 9.2, ~ 12.2 and ~ 13.5 mM for samples containing 8, 16, 24 and 32 mM added orthophosphate, respectively, indicating that a higher amount of diffusible phosphorus was associated with the protein-iron complexes in samples with greater orthophosphate content.

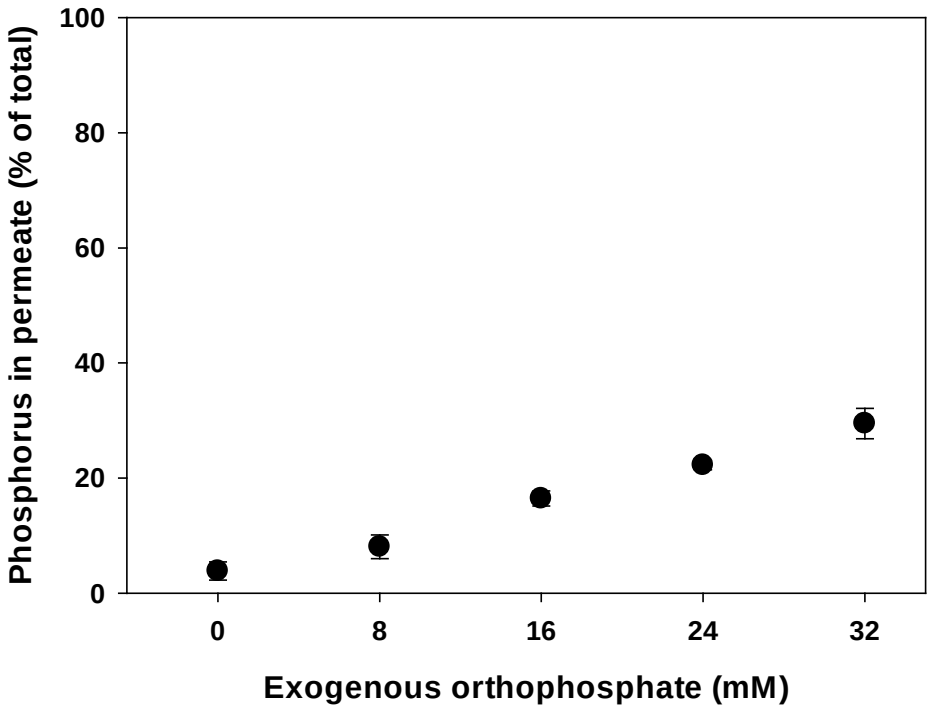


Figure 5-3*: *Effect of orthophosphate addition to calcium-depleted milk (~ 70% calcium depletion) on proportion of phosphorus in the permeate upon 30 mM iron addition.

5.2.1.3. ζ-potential

Figure 5.4 shows relative changes in the ζ-potential of 30 mM iron added CDM as a function of added orthophosphate concentration. A slightly higher negative ζ-potential was observed for samples containing exogenous orthophosphate suggesting a small increase in surface negative charge of the protein-iron complex. Thus it appears that the addition of exogenous orthophosphate reduced the influence of iron on screening the negative charge of the protein.

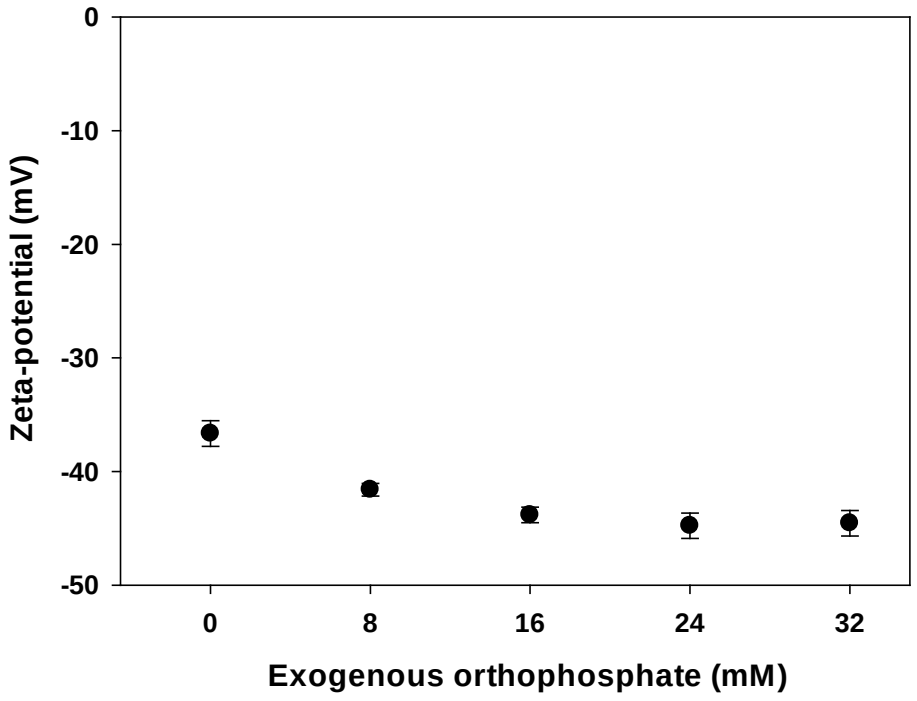


Figure 5-4*: *Effect of orthophosphate addition on ζ-potential (mV) of 30 mM iron added calcium-depleted milk (~ 70% calcium depletion)

5.2.1.4. Microstructure

CDM containing 30 mM iron showed considerable protein aggregation (Figure 5.5), with irregular aggregate structures. There were electron-dense regions in the centre of large aggregates and loose fibrous structures at the periphery. It appeared that the fibrous structures that formed upon the addition of iron to CDM were aggregated together into a larger randomly shaped network.

The microstructures of the CDM containing 30 mM iron and 16 or 32 mM exogenous orthophosphate consisted of small fibrous entities that existed largely independently (Figures 5.5B and C). This indicated that exogenous orthophosphate prevented the aggregation of fibrous structures. The proportional increase in the solubilization of the protein–iron complexes was probably due to the formation of these smaller fibrous structures (Figures 5.5B and C).

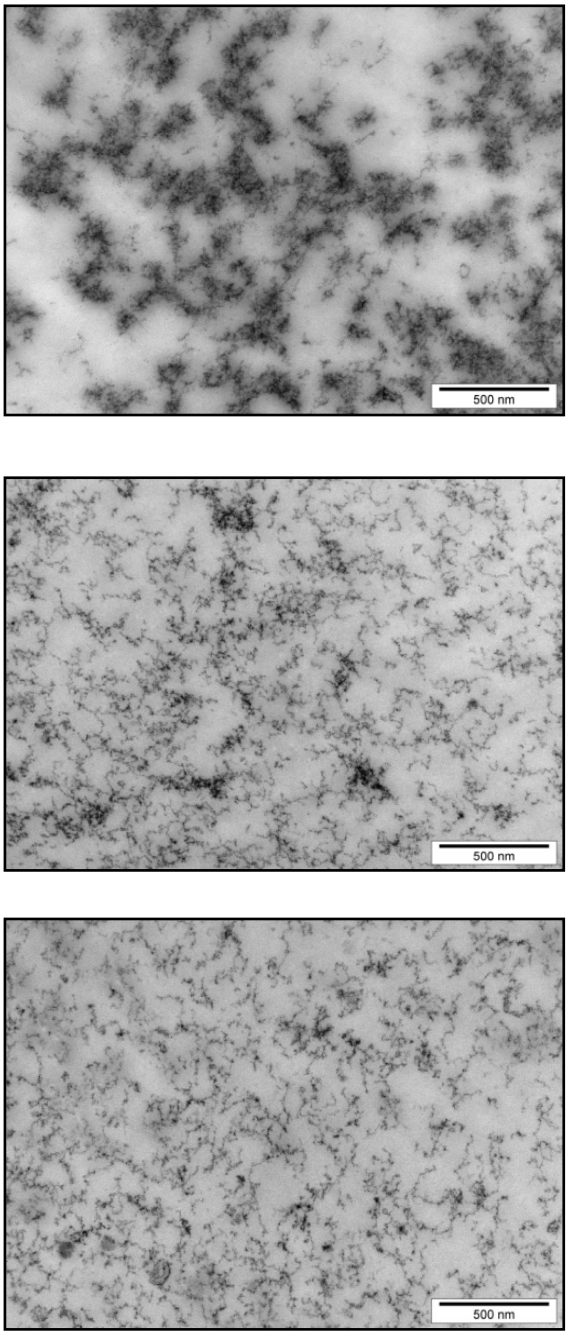


Figure 5-5*: *Microstructure of 30 mM iron added calcium-depleted milk (~ 70% calcium depletion) containing (A) 0, (B) 16 and (C) 32 mM of added orthophosphate (Magnification – 46,000 X).

Ferric iron is insoluble at the pH of these solutions and its presence in the non-sedimentable phase was due to its binding to caseins. The addition of minor quantities of iron (< 6 mM) to caseins (25 g/L) reduces their negative charge, leading to their

precipitation (Gaucheron et al., 1997c). The results of this experiment indicate that 30 mM iron could be added to CDM with added orthophosphate with minimal protein precipitation, which could be attributed to the involvement of diffusible phosphorus. Diffusible phosphorus exists in its ionised state as phosphate (PO_4^{n-}) in the aqueous phase (Corbridge, 1995) and can interact with iron, to form ferric phosphate salt. However, ferric phosphate salt is insoluble at the pH of these solutions (6.8) and it does not bind with caseins (as discussed in Chapter 6). The results of this experiment suggested that orthophosphate was possibly interacting with both protein and iron in the non-sedimentable phase. This interaction was responsible for maintaining the solubility of protein and iron. The phosphoserine residues on the caseins probably acted as nucleation sites for the formation and stabilization of the ferric phosphate (probably amorphous form) salt.

5.2.2. Effect of exogenous orthophosphate on aggregation of proteins in calcium-restored milk

The effect of adding various concentrations of calcium (10, 20 and 30 mM) on the Z-average diameter of CDM is shown in Figure 5.6. The Z-average diameter of the CDM containing up to 20 mM calcium was ~ 154 nm and increased more than 2-fold upon the addition of 30 mM calcium.

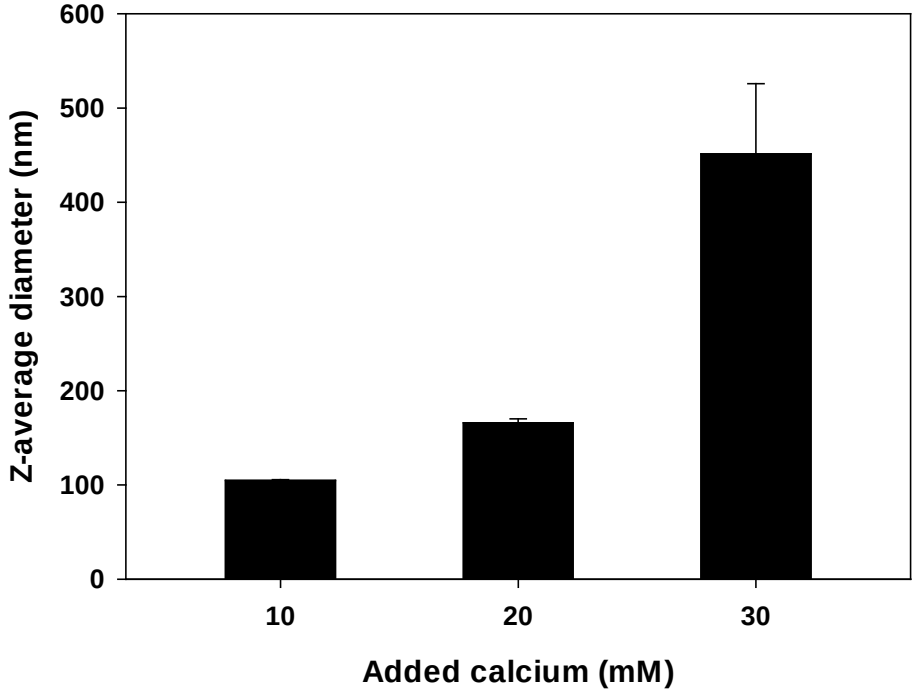


Figure 5-6**:** *Effect of calcium addition (0-30 mM) on the Z-average diameter (nm) of ~ 70% calcium-depleted milk*

The effect of exogenous orthophosphate on the aggregation of proteins in CDM were studied in CDMs added with 20 mM calcium which restored the calcium in CDM. Exogenous orthophosphate was added at two concentrations (6.5 and 16 mM) prior to

calcium addition (20 mM) because extensive precipitation and sedimentation was observed at higher levels.

5.2.2.1. Particle size

The Z-average diameter of CDM with 20 mM added calcium was ~ 154 nm and increased more than two-fold in samples containing 16 mM exogenous orthophosphate (Table 5.1) but the turbidity increased by only ~ 10%. The Z-average diameter is based on intensity weighted distribution of particles which is biased towards the presence of large aggregates. The difference in the increase of Z-average diameter and turbidity suggest that only a part of the proteins aggregated into substantially large aggregates in orthophosphate added calcium-restored milks. The results indicated that the addition of orthophosphate to CDM promoted the formation of some larger aggregates in the presence of calcium.

Table 5-1**:** *Effect of orthophosphate addition to CDM on the Z-average diameter and turbidity of calcium-restored milk (20 mM added calcium)*

Added orthophosphate (mM)	Z-average diameter (nm)	Turbidity (AU)
0	154.2 (±2.0)	1.82 (±0.1)
6.5	197.8 (±13.5)	1.92 (±0.02)
16	354.6 (±64.5)	1.95 (±0.06)

Interestingly, the aggregates in the calcium-restored milk were smaller than the casein micelles in the original milk. The re-formation of caseins into smaller aggregates compared with those in the original milk upon restoring the calcium in ion-exchanged skim milk using a similar ion-exchange resin has also been reported (Grimley et al., 2010). This was probably due to a change in the mineral composition of the normal milk upon ion-exchange treatment. Ion-exchange treatment reduced the total phosphorus content of normal milk by ~ 13%. Although the calcium content of CDM with 20 mM added calcium was similar to that of the original milk, the total phosphorus content was lower in the calcium-restored milk. Inorganic phosphorus is known as a kosmotrope (Collins, 1997), i.e., a small molecule with high negative charge. Kosmotropes bind adjacent water molecules strongly and promote hydrophobic interactions between micellar casein aggregates (Vaia et al., 2006). The reduced hydrophobic attraction forces have been linked to the re-formation of casein micelles into smaller aggregates in alkaline-disrupted milks (Huppertz et al., 2008). The reduced hydrophobic interactions, due to the reduction in total phosphorus content of CDM, might be a contributory factor in the formation of smaller aggregates in calcium-restored milk.

5.2.2.2. Distribution of protein and minerals

The changes observed in the particle size of calcium-restored milks with the addition of exogenous orthophosphate affected the distribution of protein, calcium and phosphorus between the centrifugal phases (Table 5.2). The non-sedimentable protein content of the CDM with added exogenous orthophosphate increased from ~ 38 to ~ 47% (Table 5.2) indicating that some of the caseins were present as small aggregates, which resisted sedimentation in these samples. An increase in the non-sedimentable protein content has

also been observed upon the addition of orthophosphate to skim milk (Jayani et al., 2010; Udabage et al., 2000). In contrast, the proportion of non-sedimentable calcium reduced by ~ 10% in samples containing 16 mM orthophosphate.

Table 5-2**:** *Effect of orthophosphate addition to CDM on non-sedimentable protein, calcium and phosphorus of calcium-restored milk (20 mM added calcium)*

Added orthophosphate (mM)	**Proportion in non-sedimentable phase (%)**		
	Protein	**Calcium**	**Phosphorus**
0	37.7 (±2.3)	44.1 (±1.7)	60.6 (±2.5)
6.5	41.3 (±2.6)	43.6 (±0.5)	66.5 (±0.7)
16	47.1 (±1.3)	40.5 (±0.5)	71.0 (±0.3)

Table 5-3: *Effect of orthophosphate addition on calcium and phosphorus in the permeate of calcium-restored milk (20 mM added calcium)*

Added orthophosphate (mM)	**Proportion in the permeate (%)**	
	Calcium	**Phosphorus**
0	25.3 (±0.6)	42.3 (±1.1)
6.5	21.8 (±0.7)	47.3 (±1.4)
16	15 (±0.6)	52.1 (±2.2)

The addition of 16 mM added orthophosphate reduced the proportion of calcium in the permeate of the calcium-restored milk (Table 5.3) from ~ 25 to ~ 15%. This indicated that the diffusible calcium content was governed by the concentration of orthophosphate in the respective samples. The proportion of phosphorus was higher in the permeate of the calcium-restored milks but represented only a part of the added orthophosphate (Table 5.3).

Greater than 70% of the total phosphorus in CDM existed in the permeate (~ 22.5 mM), as shown in Chapter 4. Higher proportions of permeate phosphorus were presumably present in CDM containing added orthophosphate. The addition of 20 mM calcium reduced the permeate phosphorus contents in all the samples. The difference in the permeate phosphorus contents of the CDM and calcium-restored milk indicated that higher amount of permeate phosphorus was reduced in samples with greater orthophosphate.

Approximately ~ 25% of the total calcium was present in the permeate of the calcium-restored milk and its concentration is dynamically controlled (Holt et al., 1981; Lewis, 2011). Phosphate has high affinity for calcium and has been used as a precipitant to reduce the ionic calcium in milk (Tessier et al., 1958). The decrease in the diffusible calcium of calcium-restored milks with added exogenous orthophosphate was due to its precipitation by the additional phosphate in these samples. An increase in calcium sedimentation at higher concentrations of added orthophosphate in milk was also reported by Udabage et al. (2000). Consequently, higher amounts of calcium phosphate were formed in calcium-restored milk with added orthophosphate. The formation of some large aggregates in calcium-restored milk with added orthophosphate was probably due to insufficient caseins being available to stabilize the additional calcium

phosphate salts formed. Horne (1982) has shown that relatively larger aggregates form when the orthophosphate content of the mixture is increased at fixed concentrations of casein and calcium.

5.2.2.3. Microstructure

The microstructure of calcium-restored milk containing 6.5 mM exogenous orthophosphate is shown in Figure 5.7. The addition of calcium aggregated the proteins into random shapes and sizes, in which some of the aggregates were roughly spherical, similar to the characteristic shape of the casein micelle. Notably, the protein aggregates formed had wide-ranging sizes and the electron-dense regions were uniformly distributed throughout the structure.

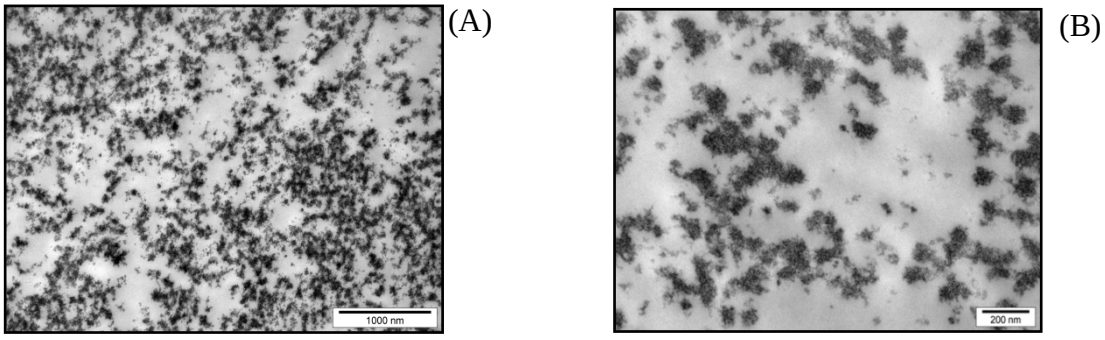


Figure 5-7*: *Microstructure of calcium-restored milk (~ 70% calcium depletion) containing 6.5 mM exogenous orthophosphate at two magnifications (A) 24000 X and (B) 64,000 X.

The effects of the addition of orthophosphate to CDM on the iron- and calcium-induced aggregation of the proteins were contrasting. The presence of orthophosphate prevented the aggregation of protein upon the addition of 30 mM iron to CDM, whereas it promoted the aggregation of protein in CDM where 20 mM calcium was added (calcium-restored milk).

The addition of iron to CDM resulted in the formation of small protein fibrous structures that remained in the non-sedimentable phase. Above a certain concentration of iron (> 25 mM), these fibrous structures aggregated and sedimented in the pellet (Figure 3A). Inter-particle iron bridging was probably responsible for this aggregation. The addition of orthophosphate increased the concentration of free ionic phosphorus in the permeate of CDM. The added iron probably interacted with the additional free ionic phosphorus available. This might reduce the inter-particle iron bridging, thereby promoting the formation of independent fibrous structures (Figure 3B). Thus, increasing the concentration of inorganic phosphorus in the sample promoted the formation of small, non-sedimentable aggregates upon iron addition.

The addition of 20 mM calcium to CDM possibly aggregated the proteins into a roughly spherical structure and reduced the non-sedimentable protein content to less than ~ 40% of the total. A higher proportion of protein in the non-sedimentable phase was reduced in the presence of calcium, compared with that in the presence of iron; this indicates that, compared with iron, calcium aggregated protein into larger aggregates in the CDM. At equimolar (8 mM) concentrations, Philippe et al. (2005) reported a greater reduction in non-sedimentable protein for calcium than for iron in a phospho-caseinate suspension.

The results of this work suggest that the nature of the cation added (i.e., iron or calcium) influenced the aggregation properties of the proteins in CDM. Although calcium and iron bind to similar sites on caseins, i.e., phosphoserine and carboxylic acid residues, they aggregate the proteins differently in CDM. A cluster of at least three phosphoserine residues is required for the efficient binding of caseins to calcium phosphate nanoclusters, whereas only two phosphoserine residues are required for the binding of

iron to caseins (Aoki et al., 1992; Hegenauer et al., 1979c). Iron is a transition metal whereas calcium is an alkaline earth metal, with different charge densities, ionic radii and coordination requirements. Furthermore, the binding of caseins to the calcium phosphate cluster involves electrostatic interactions (Gaucheron et al., 1997c) whereas iron binds *via* coordination bonds (Hegenauer et al., 1979c). Hence, the difference in the aggregation of iron and calcium might be related either to the number of phosphoserines required to stabilize the amorphous phosphate salts and their respective binding characteristics with ligands (amino acids on casein) or to the involvement of different casein fractions during the metal-induced aggregation. Thus, the different structures formed upon iron- and calcium-induced aggregation of caseins were due to their different binding characteristics.

5.2.3. Effect of iron addition to CDM on aggregation of proteins in calcium-restored milk

In these samples different concentrations of iron were added prior to restoring the calcium content of CDM.

5.2.3.1. Particle size

The changes in the Z-average diameter of calcium-restored milk upon iron addition to CDM are shown in Figure 5.8. The Z-average diameter of calcium-restored milk decreased upon 5 mM iron addition, irrespective of the orthophosphate content. Increasing the concentration of iron increased the Z-average diameter of all calcium-restored milks, but the extent varied. The increase in Z-average diameter was lower in the samples containing greater amounts of exogenous orthophosphate. The Z-average diameter for calcium-restored milk containing 6.5 mM added orthophosphate and 20 mM iron was found to be highest.

The effect of iron addition to CDM on particle size distribution (determined using Mastersizer) of calcium-restored milk is shown in Figure 5.8. Smaller-sized particles were formed in calcium-restored milk containing $\leq$ 10 mM iron, irrespective of the added orthophosphate concentration (Figure 5.8B, C and D). Increasing the iron content to 20 mM generated larger aggregates but only in the samples containing added orthophosphate (Figure 5.8C and D).

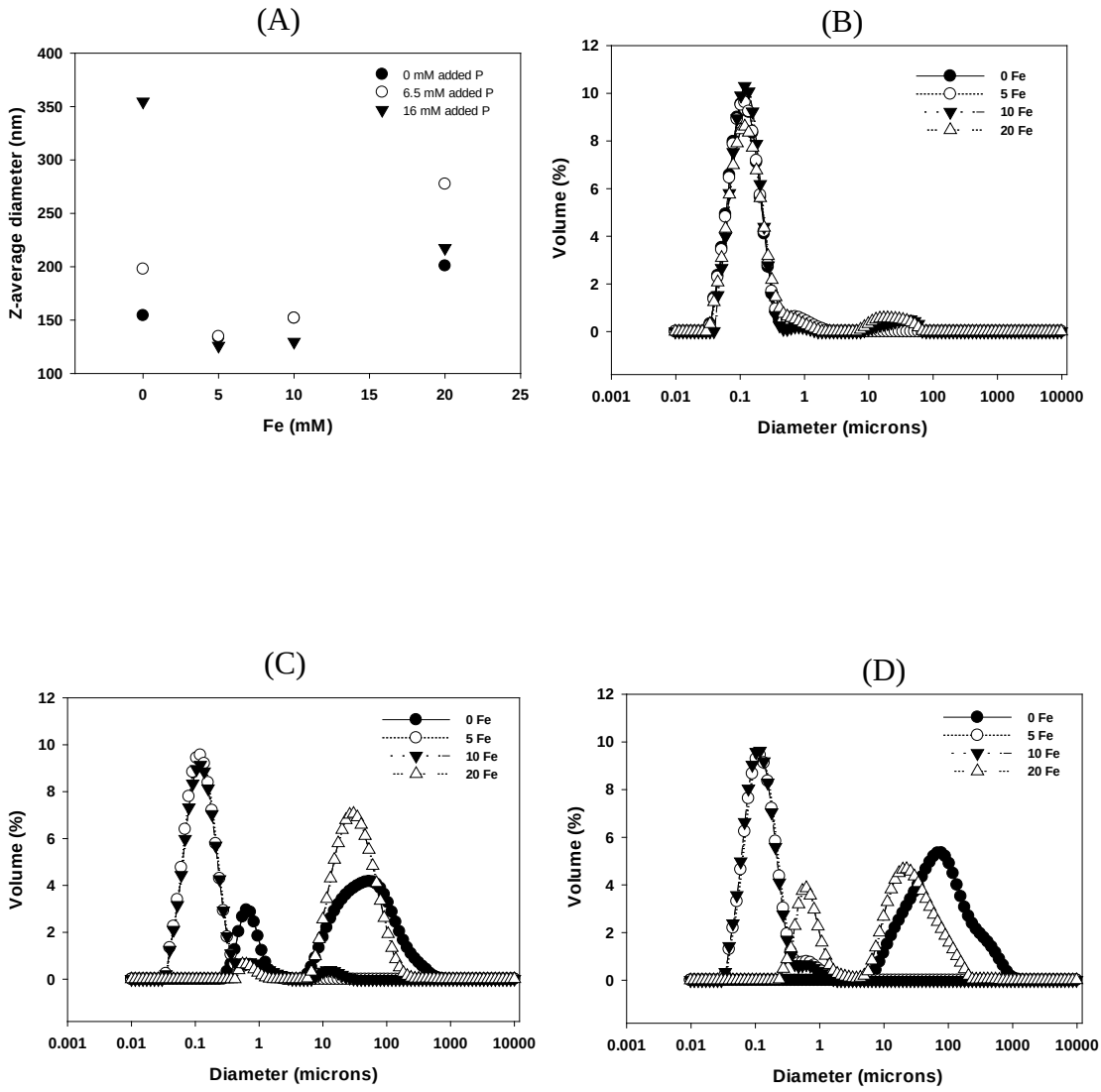


***Figure 5-8*:** *The effect of iron addition to calcium-depleted milk (~ 70% calcium depletion) on the (A) Z-average diameter (nm) and particle size distribution (determined by Mastersizer) of calcium-restored milk containing (B) 0, (C) 6.5 and (D) 16 mM exogenous orthophosphate.*

5.2.3.2. Distribution of protein and minerals

The effect of iron addition to CDM on the distribution of protein and minerals in calcium-restored milk containing varying exogenous orthophosphate contents is shown in Figure 5.9. Increasing the iron concentration decreased the amount of non-sedimentable protein, calcium and phosphorus in all calcium-restored milks, irrespective of their total orthophosphate content.

Less than 8% of the added iron was present in the non-sedimentable phase of calcium-restored milks containing 20 mM iron. The higher proportion of iron in the sediment was due to its binding to caseins which have a higher capacity to bind iron. Similar results have been reported by authors who studied the distribution of added iron in milk (Gaucheron et al., 1997a; Raouche et al., 2009b). Iron and calcium bind to similar sites on the caseins i.e. the phosphoserine and carboxylic acid residues. The affinity of caseins to bind iron is 13.5 log higher than that for calcium, presumably due to the coordination bonding involved as compared to the electrostatic interactions for the later (Hegenauer et al., 1979c). Although caseins bind iron more strongly than calcium, the addition of calcium did aggregate the caseins, thereby reducing the non-sedimentable protein and iron contents.

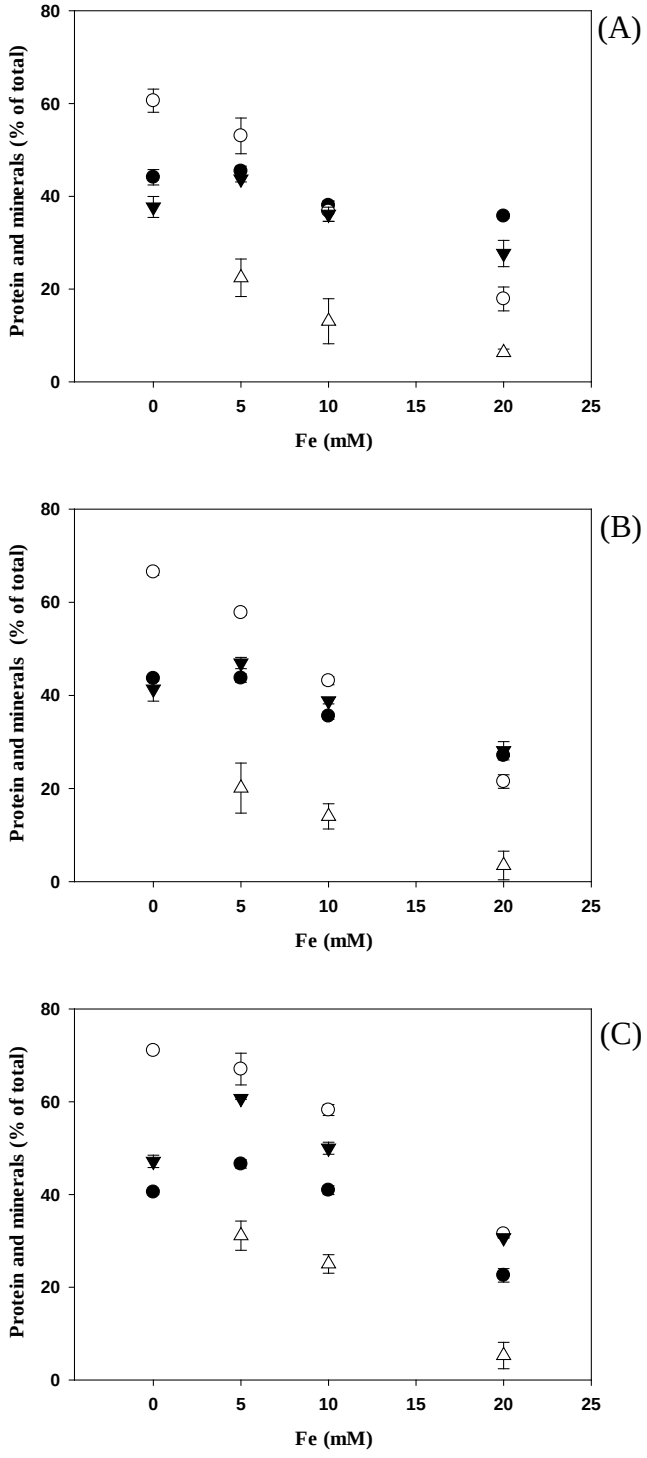


Figure 5-9**:** *The effect of iron inclusion (5, 10 and 20 mM) on the proportion of (●) calcium, (o) phosphorus, (▼) protein and (Δ) iron in the non-sedimentable phase (100,000 g for 1 h at 20°C) of calcium-restored milk containing (A) 0, (B) 6.5 and (C) 16 mM added orthophosphate.*

Figure 5.10A shows that the proportions of calcium in the permeate of calcium-restored milks were lower in the samples containing orthophosphate, irrespective of their iron concentration. The inclusion of iron did not increase the diffusible calcium content of calcium-restored milks, which indicates that the calcium was bound to proteins in CDM even upon iron addition. Thus, the proportions of calcium in the permeate were governed only by their orthophosphate content and not the presence of iron.

The phosphorus in the permeate reduced almost linearly in proportion to the concentration of iron in calcium-restored milks (Figure 5.10B). However, the extent of decrease corresponded to the orthophosphate content of the CDMs; less than 8% of the total phosphorus was diffusible in calcium-restored milk containing 20 mM iron, whereas ~ 25% of the total phosphorus was present in the permeate of sample containing 16 mM orthophosphate. The concentration of phosphorus in the permeate of these samples is shown in Table 5.4. The permeate phosphorus content decreased in calcium-restored milks with increasing amounts of added iron. The decrease in the amount of permeate phosphorus due to the addition of iron was calculated from the difference in the control (no added iron) and 20 mM iron containing calcium-restored milk. The result suggest that ~ 10.3, ~ 12.6 and ~ 14.3 mM of permeate phosphorus was additionally reduced upon the inclusion of 20 mM iron in calcium-restored milks containing 0, 6.5 and 16 mM of exogenous orthophosphate.

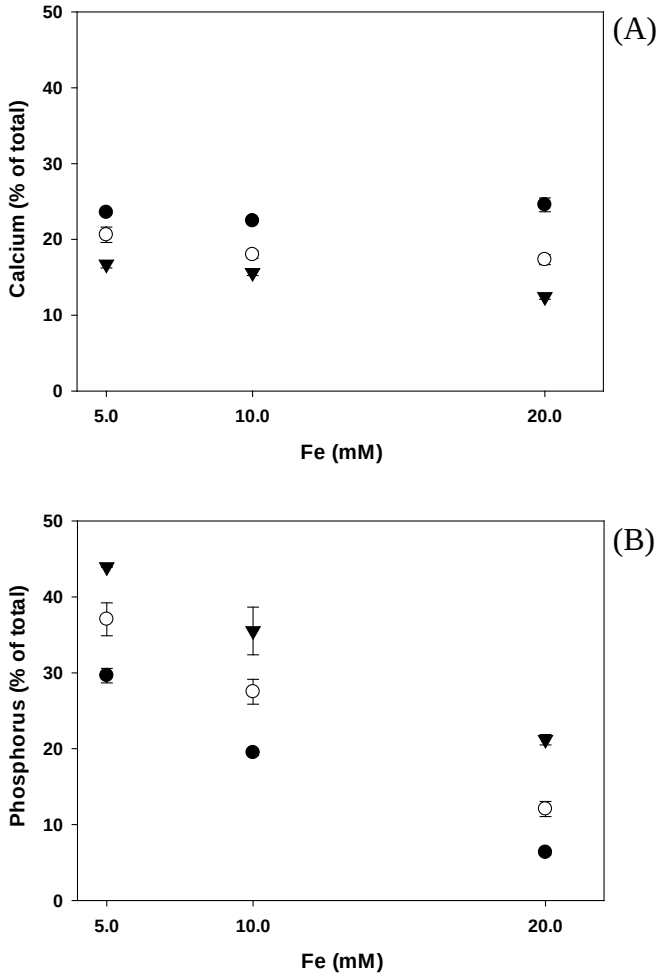


Figure 5-10**:** *The proportionate distribution of (A) calcium and (B) phosphorus in the permeate (MWCO - 10,000 daltons) of calcium-restored milk containing (●) 0 (o) 6.5 and (▼) 16 mM of added orthophosphate and varying iron concentrations (5, 10 and 20 mM).*

The addition of iron to CDM reduces the permeate phosphorus content as shown in Chapter 4. Hence, the amount of ionic phosphorus available to interact with calcium was presumably lower in calcium-restored milk containing iron. Consequently, lower amounts of calcium phosphate salts were probably formed in the calcium-restored milks containing higher concentrations of iron. The altered calcium phosphate content would be expected to affect the aggregation properties of caseins in calcium-restored milks.

Table 5-4*: *Phosphorus content of the permeate in calcium-restored milk containing various levels of iron

Fe (mM)	Added orthophosphate (mM)		
	0	**6.5**	**16**
5	8.83 (±0.3)	13.35 (± 0.8)	19.6 (±0.0)
10	5.75 (±0.1)	9.87 (±0.6)	15.64 (±1.4)
20	1.87 (±0.1)	4.29 (±0.3)	9.53 (±0.30)

5.2.3.3. Microstructure

The microstructure of 5 mM and 20 mM iron added calcium-restored milks containing 6.5 mM orthophosphate at two magnifications (34000 X and 64000 X) is shown in Figure 5.11. Clearly, the proteins were aggregated differently in these calcium-restored milks. Addition of 5 mM iron in calcium-restored milk resulted in the formation of relatively small independent structures (Figure 5.11A). At higher magnifications, the structures appeared to have electron dense region along with few protruding fibrous structures. The calcium-restored milk containing 20 mM iron showed entangled protein structures which associated into a network structure, with a little resemblance to the typical casein micelle structure (Figure 5.11B).

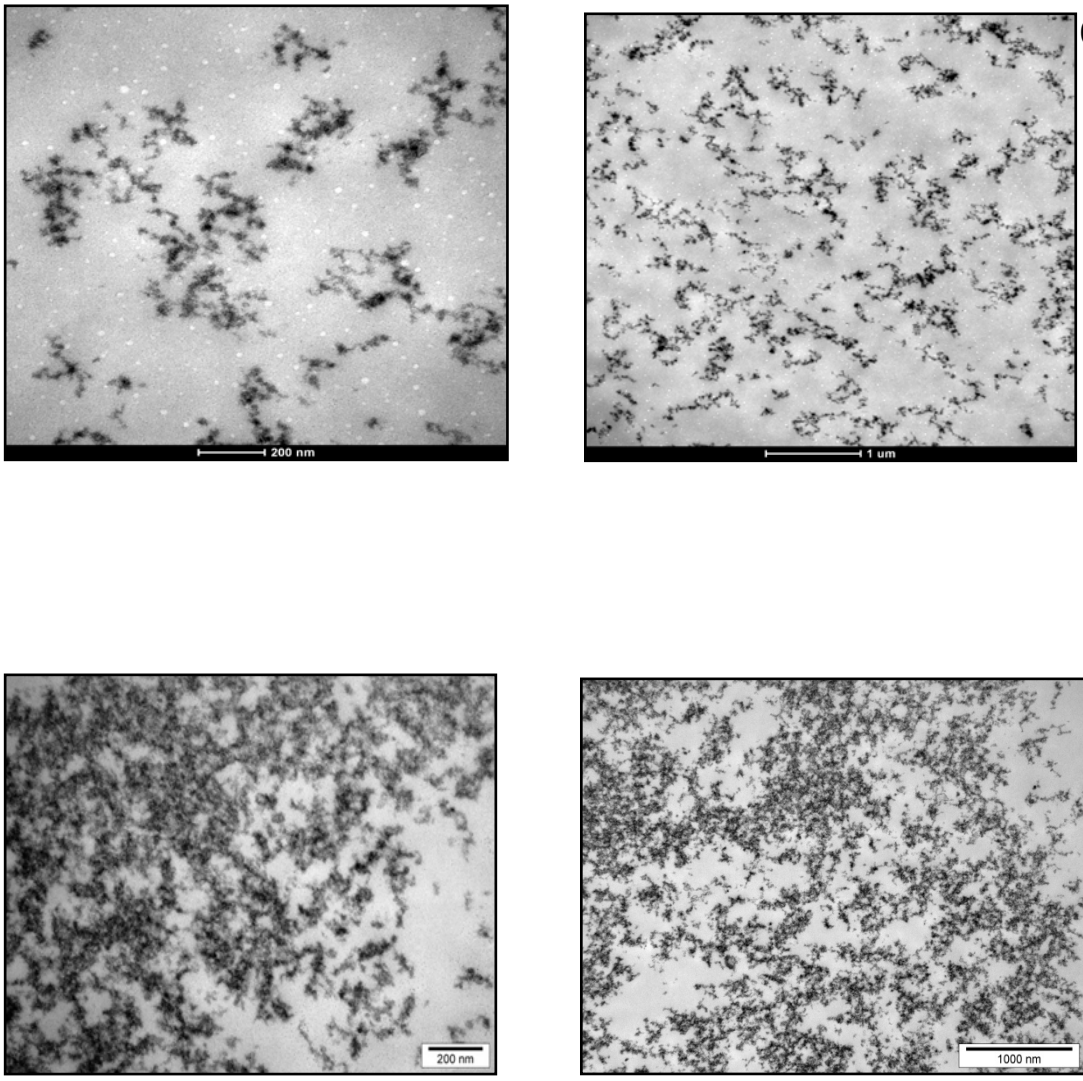


Figure 5-11**:** *Effect of iron, (A) 5 and (B) 20 mM, on the aggregation of proteins in calcium-restored milk containing 6.5 mM exogenous orthophosphate.*

Caseins associate into a roughly spherical structures upon calcium addition, similar to that of the characteristic casein micelle, but only in the presence of orthophosphate (Aoki et al., 1987; Schmidt, 1979). The mechanism and interactions involved in the association of caseins into the casein micelle structure have been discussed by Horne (2009). In brief, casein micelle structure formation involves a polymerisation pathway, where caseins aggregate *via* calcium phosphate nanoclusters and hydrophobic

interactions and the aggregation reaches completion upon the association of κ-casein on the surface of the micelle resulting in a roughly spherical structure. The binding of caseins to the calcium phosphate cluster involves electrostatic interactions with the phosphoserine and carboxylic acid residues available on caseins. It was highly probable that the presence of iron in these phosphoserine clusters disrupted the polymerisation pathway required for casein micelle formation, preventing the association of caseins into larger roughly spherical structures. This was evident from the lower Z-average diameters and microstructures of 5 mM iron added calcium-restored milk. The presence of a network structure in calcium-restored milks containing higher concentrations of iron could be due to inter-particle bridging caused by the high concentrations of total cations (iron and calcium). This confirms that the presence of iron alters only the aggregation of caseins, but not the interaction of calcium with the binding sites in caseins.

Figure 5.15 shows the effect of iron and/or calcium addition on the aggregation of proteins in calcium-depleted milk. The proteins aggregated into fibrous structure upon iron addition whereas roughly spherical aggregates of varying size formed in calcium-restored milk. The addition of calcium to iron added CDM aggregated the pre-formed fibrous structure into small independent structures.

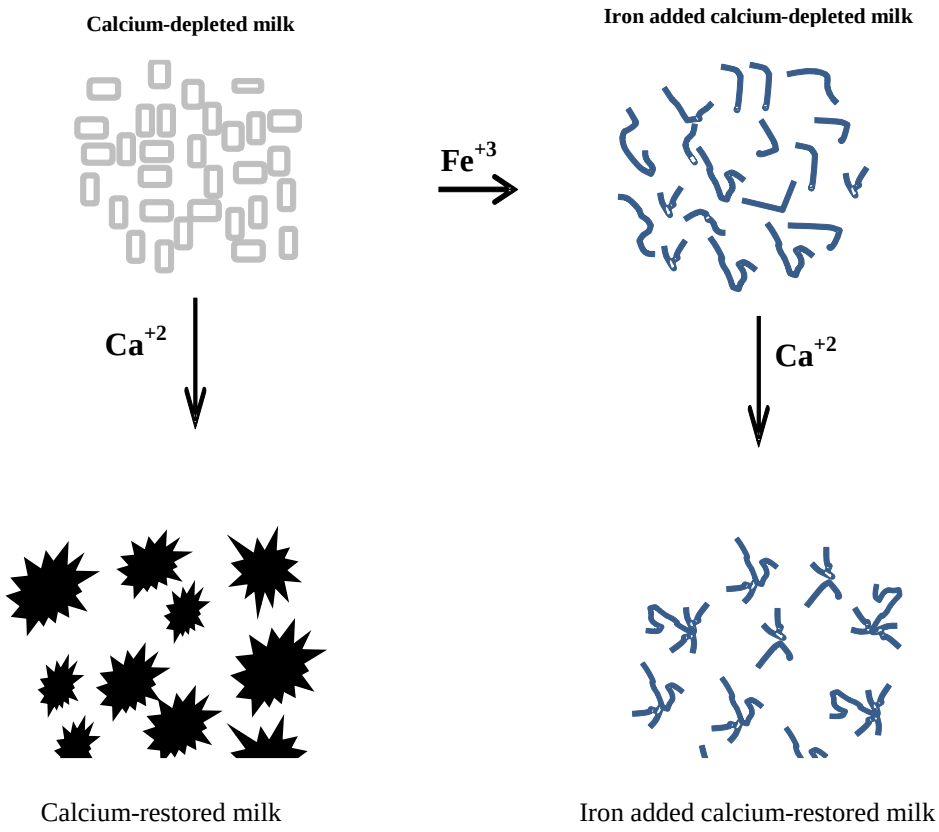


Figure 5-12*: *Schematic representing the effect of iron and/or calcium addition to CDM on the aggregation of proteins in calcium-restored milk

5.3. Conclusions

The addition of iron to CDM causes casein to aggregate into small fibrous particles whereas the addition of calcium aggregates caseins into roughly spherical particles. The addition of exogenous orthophosphate prevented aggregation and promoted the formation of smaller fibrous structures in CDM by reducing inter-particle iron bridging. In contrast, exogenous orthophosphate promoted the formation of larger aggregates in CDM upon the re-addition of calcium. The addition of exogenous orthophosphate reduced the diffusible calcium content in calcium-restored milk, thereby increasing the concentration of insoluble calcium phosphate. The caseins present in CDM were insufficient to stabilize the excess calcium phosphate formed, resulting in the formation

of some large protein aggregates. The different effects of exogenous orthophosphate on the iron- and calcium-induced aggregation of proteins in CDM were probably due to the type of bonds and the number of phosphoserines required to interact with these cations.

The inclusion of iron in calcium-restored milk generated substantially smaller aggregates, which were promoted by the addition of exogenous orthophosphate. The smaller protein aggregates affected the distribution of protein and minerals in calcium-restored milks containing iron. The presence of iron probably blocked the polymerisation pathway required for casein micelle structure formation but not the interaction of calcium with caseins.

Chapter 6. Iron binding in sodium caseinate in the presence of orthophosphate

6.1. Introduction

Casein, the principal protein in bovine milk, is extracted by acidifying skim milk to pH 4.6 at 20^{o}C. The precipitated casein fraction is separated from the whey, washed and dried. The acid-precipitated casein can be solubilized by reaction with alkali to produce caseinate. The major uses of casein and caseinate are as ingredients in foods to enhance their physical and functional properties, such as foaming, water binding, thickening and emulsification (Southward, 1989).

The casein fractions comprise four distinct proteins, i.e., α_{s1}-, α_{s2}-, β- and κ-casein (Fox, 2009; Singh, 2011). The level of phosphorylation in caseins is variable, with α_{s1}-, β- and κ-casein containing 1, 0.5 and 0.2% phosphorus, respectively (Dickson et al., 1971). Phosphorylation occurs at the serine residues by esterification with phosphate. The phosphoserine residues are clustered in groups of two, three, or four and strongly bind calcium and other metal ions (Gaucheron et al., 1997c). The binding of calcium to individual caseins has been studied extensively, with the binding capacity being found to be directly proportional to the number of phosphoserine residues. Various other cations (Mn^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+} and Cu^{2+}) have been shown to bind to caseins, with their binding ability being dependent on pH, ionic strength, temperature and the amount of phosphate in the solution (Baumy et al., 1988a; Gaucheron et al., 1997c; Philippe et al., 2005).

Among the multivalent ions, caseins bind iron more strongly than calcium. For instance, the binding constant of α_{s1}-casein for Fe^{3+} is more than threefold higher than that for calcium (Hegenauer et al., 1979c). Furthermore, the ferric form of iron binds more strongly to caseins than the ferrous form (Raouche et al., 2009a). Upon binding, ferrous iron is oxidized to the ferric state (Emery, 1992). The binding of iron results in precipitation of caseins, the concentration required for precipitation being dependent on the oxidation state (ferrous or ferric) of the iron, the phosphorylation of the caseins and the pH of the fortified solution. Ferric iron precipitates caseins at lower concentrations (2 mM) than ferrous iron (4 mM) (Gaucheron, 2000). Higher concentrations of ferrous iron could be added to sodium caseinate solutions when the pH was re-adjusted upon iron addition (Sugiarto et al., 2009). When ferric chloride was added to skim milk, it was found that 72% of added iron bound to α_s-casein, 21% bound to β-casein and 4% bound to κ-casein (Demott et al., 1976).

A number of studies have investigated the binding of iron to caseins in sodium caseinate solutions (Gaucheron et al., 1996; Gaucheron et al., 1997c; Hegenauer et al., 1979c; Sugiarto et al., 2009). Gaucheron et al (1997c) studied the effects of physico-chemical conditions (pH, ionic strength and temperature) on the binding capacities of the caseins for various cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+}). They found that the binding of iron and copper to caseins was unaffected by the physico-chemical conditions, which they attributed to the formation of strong coordination bonds with the phosphoserine residues. Hegenauer et al (1979c), in their pioneering work on the binding of Fe^{3+} to α_{s1}-casein, demonstrated that each iron bound to two phosphoserine residues with a stoichiometric equilibrium constant of 17.5, which indicates a strong complex. Sugiarto et al (2009) reported that there are 14 ferrous-iron-binding sites on the caseins in

sodium caseinate. Furthermore, the binding of iron to caseins is unaffected by changes in pH (5.7-7.0) and ionic strength (Baumy et al., 1988b; Sugiarto, 2004).

Studies on the binding of iron to caseins in the presence of orthophosphate are scarce. Hegenauer et al (1979c) briefly mentioned the formation of a spectroscopically distinct phosphoprotein-iron(III)-oxyanion complex with phosphate. Gaucheron et al (1996) incubated ferrous-iron-supplemented (1.5 mM) sodium caseinate in 20 mM disodium hydrogen orthophosphate solutions and observed no release of iron from the complex. However, a number of studies have highlighted the active role of orthophosphate in the calcium-induced precipitation of caseins (Alvarez et al., 2007; Aoki et al., 1996; Guo et al., 2003; Horne, 1982). Casein precipitation occurred at a lower concentration of calcium when orthophosphate was included in the reaction mixture. However, the size of the aggregate formed in the mixture of casein, calcium and phosphate was governed by their respective concentrations. Colloidally stable particles were formed at intermediate concentrations of orthophosphate, depending on the protein concentration and corresponded to the concentrations of calcium, phosphorus and casein in normal milk (Horne, 1982).

Although orthophosphate forms an important component of iron-fortified food products, few attempts have been made to understand its effect on the binding of iron to the caseins in sodium caseinate. This study examined how iron binding in sodium caseinate solutions is influenced by the presence of orthophosphate, using a range of techniques including ^{31}P-nuclear magnetic resonance (NMR) spectroscopy and size exclusion chromatography (SEC).

6.2. Results and discussion

6.2.1. Solubility of iron in aqueous solution

Ferric chloride formed a transparent orange-coloured aqueous solution with a pH of ~ 1.8. At pH 6.8, an insoluble brick-red precipitate was formed that sedimented immediately. The volume of sediment increased in proportion to the concentration of added iron (Figure 6.1A). Ferric iron hydrolyses at pH above 3.5 and forms insoluble ferric hydroxide precipitate *via* the following mechanism (Crabb et al., 2010a; Schwertmann et al., 2008):

$$Fe^{+3} + H_2O = [Fe\ (OH)]^{2+} + H^+$$

$$[Fe\ (OH)]^{2+} + H_2O = [Fe\ (OH)]^{+} + H^+$$

$$[Fe\ (OH)]^{+} + H_2O = Fe\ (OH)_3 + H^+$$

$$2Fe\ (OH)_3 = Fe_2O_3.3H_2O$$

The brick-red precipitate of ferric hydroxide did not form in orthophosphate containing solutions (Figure 6.1B and 6.1C). Instead, a fine yellow-coloured hydrous precipitate was formed immediately upon iron addition, and this precipitate sedimented slowly over a 24 h period. The volume of the precipitate formed increased with the concentration of added iron. This suggested that the precipitates had different ratios of Fe:P in the samples containing different iron contents (Fytianos et al., 1998; Suschka et al., 2001). Ferric iron is generally used a precipitant for orthophosphate in aqueous solutions and forms insoluble ferric phosphate (Fytianos et al., 1998). These observations suggest that ferric chloride would form insoluble precipitate at pH 6.8 in the presence or absence of orthophosphate, although the type of salts formed could be different.

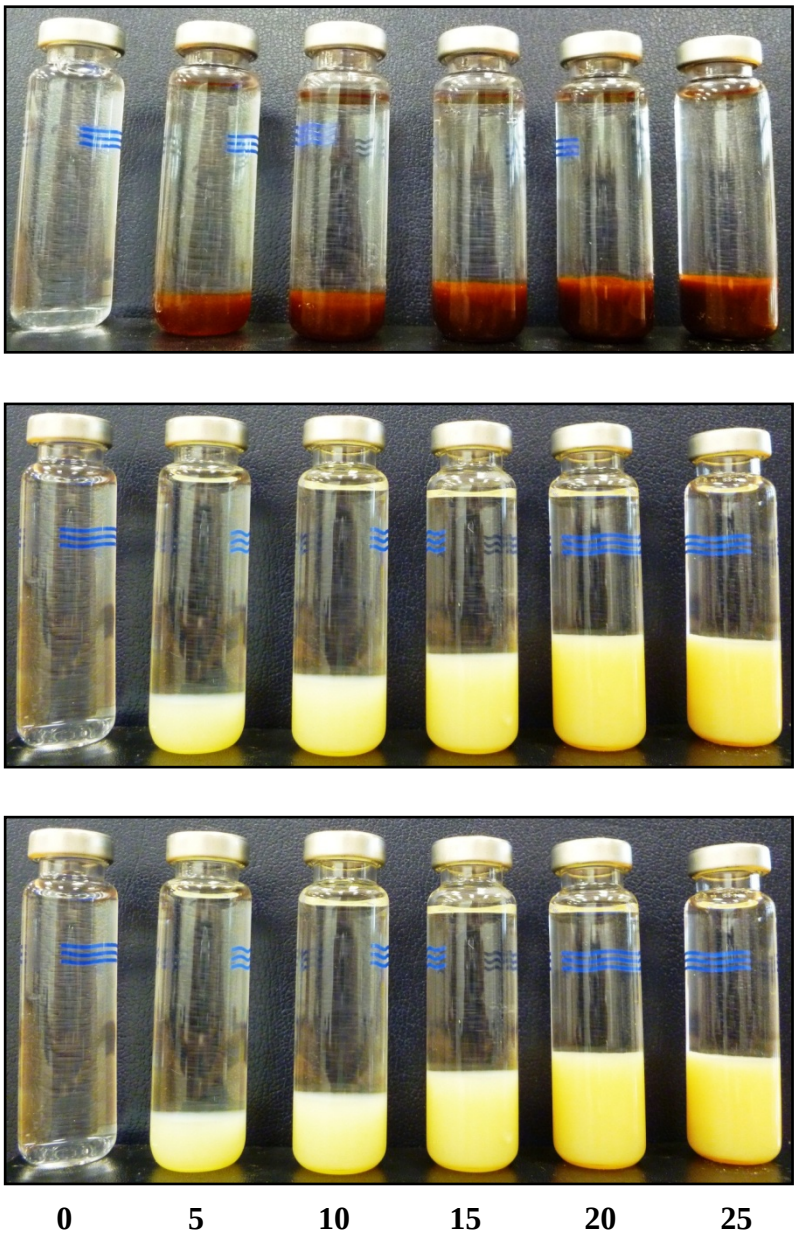


Figure 6-1*: *Solubility of ferric chloride solutions containing (A) 0, (B) 32 and (C) 64 mM of orthophosphate, at different iron concentrations (0, 5, 10, 15, 20 and 25 mM) after 24 h storage at room temperature and pH 6.8.

6.2.2. Effect of iron on the solubility of caseins

The effect of iron addition on the solubility of sodium caseinate solutions (1% to 3% w/v protein) is shown in Figure 6.2. The sodium caseinate solution appears transparent and remained apparently unaffected by protein concentration. Addition of 5 mM iron altered the transparency of solutions to yellowish-orange coloured dispersion. Increasing the iron content to 10 mM iron caused precipitation in all sodium caseinate solutions. The precipitation was observed as a floating mass at the top of the glass vial due to non-mixing over a 24 h period. The concentration of protein affected the level of iron at which gross precipitation occurred.

The proportions of soluble iron (obtained by centrifugation of the samples at 500 *g* for 20 min) in these sodium caseinate solutions are shown in Table 6.2. Around 36% of the added iron was soluble at 5 mM level in sodium caseinate solution containing 1% w/v protein. Increasing the protein content twofold increased the soluble iron content to ~ 93% at 5 mM added iron. However sodium caseinate solutions containing 3% w/v protein had less than 10% soluble iron at 5 mM added iron (Figure 6.2C). Above 5 mM added iron, the soluble iron content reduced substantially in all sodium caseinate solutions, irrespective of their protein content. The brick-red coloured precipitate of ferric hydroxide observed in aqueous solution at pH 6.8 did not form in the presence of sodium caseinate, indicating that conversion of ferric iron to ferric hydroxide was prevented by the presence of caseins.

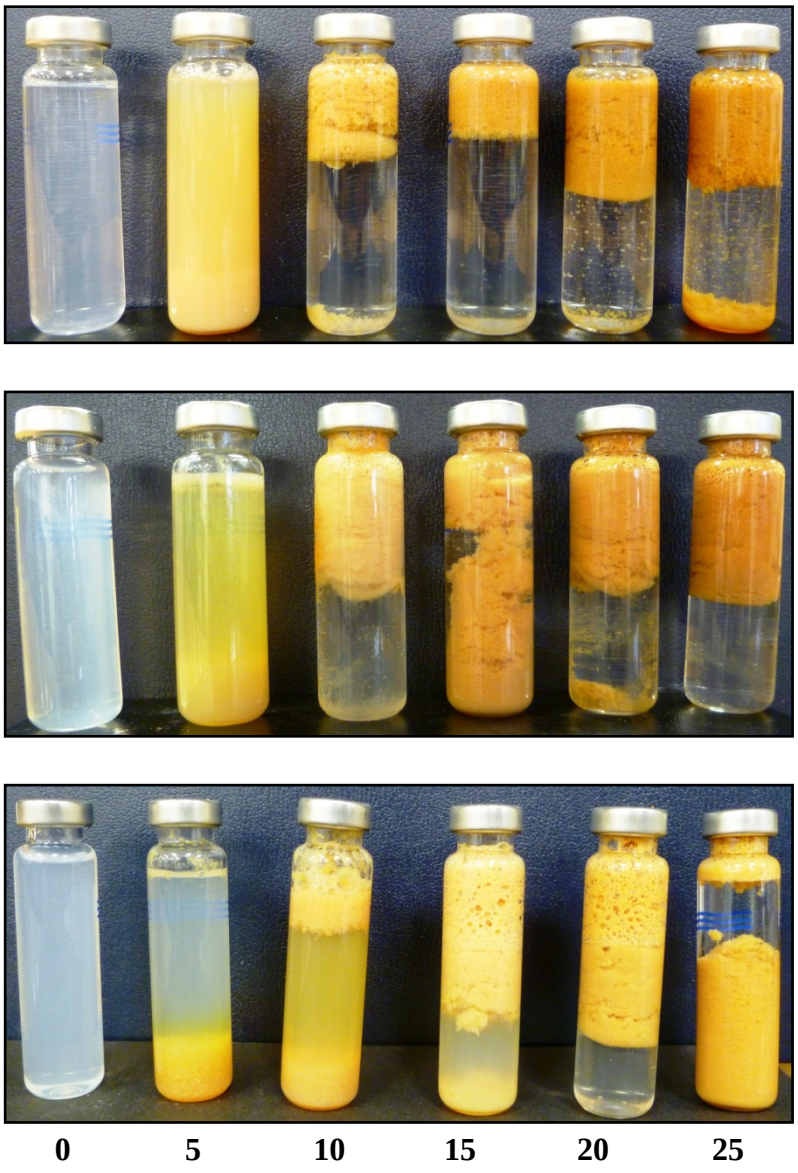


Figure 6-2*: *Solubility of sodium caseinate solutions containing (A) 1% w/v, (B) 2% w/v and (C) 3% w/v protein and different iron concentrations (0, 5, 10, 15, 20 and 25 mM) after 24 h storage at room temperature and pH 6.8.

Table 6-1**:** *Proportions of soluble iron in sodium caseinate solutions*

Protein (% w/v)	Added iron (mM)				
	5	**10**	**15**	**20**	**25**
1	36.3	0	0	0	0
2	93.3	0.7	0	0	0
3	6.3	11.3	3.4	0	0

6.2.3. Effect of orthophosphate on the solubility of iron and casein

The iron-induced precipitation of caseins in sodium caseinate solution (1% w/v protein) containing orthophosphate is shown in Figure 6.3. Dipotassium hydrogen orthophosphate (32 and 64 mM) was added to sodium caseinate solution and mixed for 20 min for complete dissolution prior to iron addition at ~ 5^{o}C.

The inclusion of orthophosphate in the mixture of iron and casein apparently improved the solubility of sodium caseinate solution (Figure 6.3). The solutions containing 32 mM orthophosphate formed a translucent yellowish coloured dispersion upon 5 mM iron addition, which was visibly less turbid as compared to the control. The apparent turbidity of orthophosphate added sodium caseinate solution increased with the concentration of iron and precipitation was observed at > 15 mM added iron. However, the casein precipitation was not extensive and a portion of protein was well dispersed

even upon 25 mM iron addition. Increasing the orthophosphate concentration to 64 mM prevented the extensive protein precipitation even upon 25 mM iron addition, but some precipitation still occured.

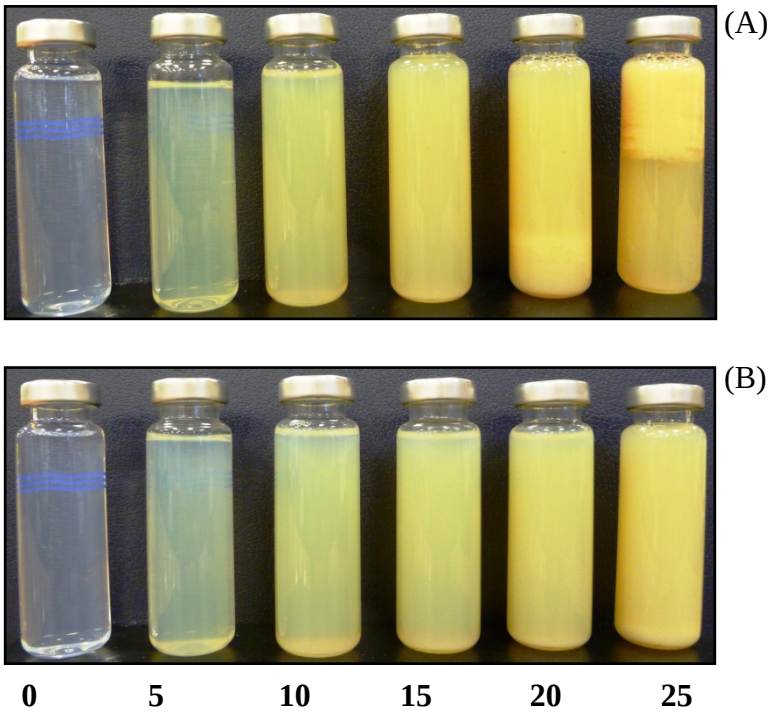


Figure 6-3*: *Solubility of sodium caseinate solutions (1% w/v protein) containing (A) 32 and (B) 64 mM of orthophosphate and different iron concentrations (0, 5, 10, 15, 20 and 25 mM) after 24 h storage at room temperature and pH 6.8.

A twofold increase in the protein content further improved the dispersion stability of iron added sodium caseinate solutions (Figure 6.4). Precipitation was observed only at 25 mM level in sodium caseinate solutions containing 32 mM orthophosphate. Increasing the orthophosphate content to 64 mM further improved the dispersion stability of sodium caseinate solutions at all levels of iron addition.

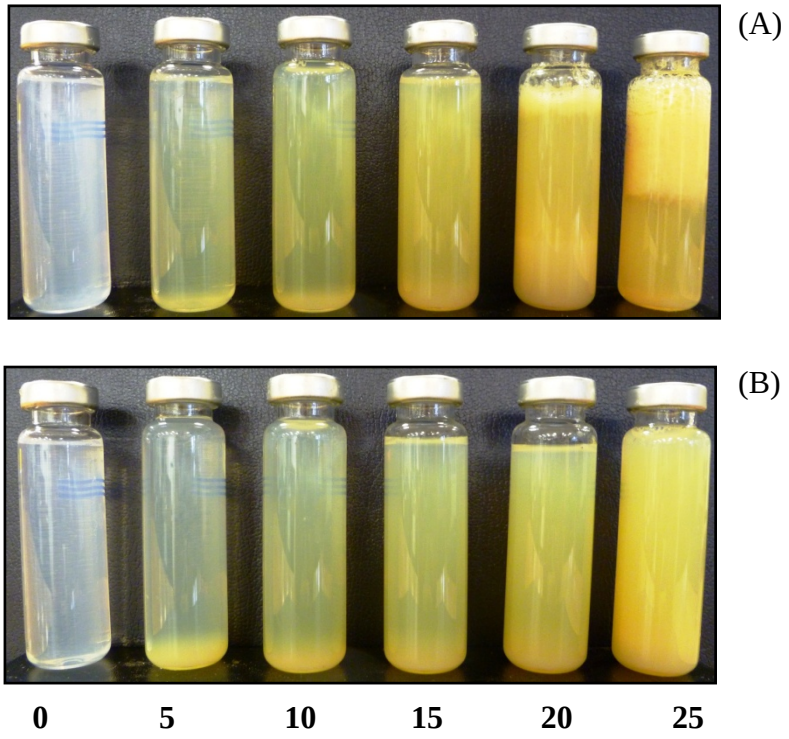


Figure 6-4**:** *Solubility of sodium caseinate solutions (2% w/v protein) containing (A) 32 and (B) 64 mM of orthophosphate and different iron concentrations (0, 5, 10, 15, 20 and 25 mM) after 24 h storage at room temperature and pH 6.8.*

The effect of orthophosphate on the solubility of sodium caseinate solution containing 3% w/v protein and iron was variable (Figure 6.5). Although gross precipitation of caseins was not observed in these solutions, yellow coloured sediment was observed, which was more visible in the samples containing 64 mM orthophosphate. The sediment was probably due to the localised protein sedimentation caused by the higher viscosity of these solutions containing greater protein content. This sediment did not form when these samples were stirred using high speed overhead stirrers. However under the stirring condition used, the amount of sediment seemed to decrease with increase in the concentration of iron added to sodium caseinate solutions containing orthophosphate.

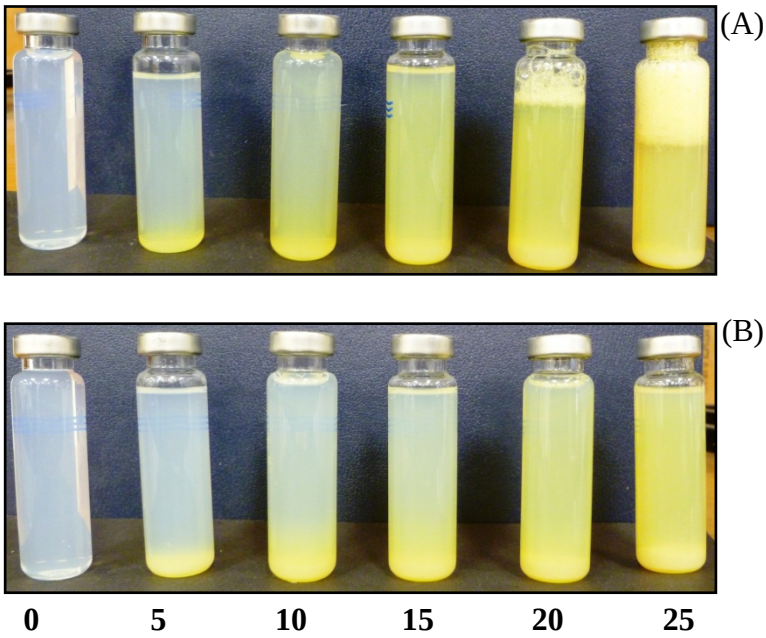


Figure 6-5*: *Solubility of sodium caseinate solutions (3% w/v protein) containing (A) 32 and (B) 64 mM of phosphorus as K_2HPO_4 and different iron concentrations (0, 5, 10, 15, 20 and 25 mM) after 24 h storage at room temperature and pH 6.8.

6.2.3.1. Distribution of protein, iron and phosphorus

The effect of iron addition on the precipitation of caseins in sodium caseinate solution (2% w/v protein) containing orthophosphate are reported and discussed herein. Samples that showed gross precipitation were not selected for this study.

The solubility of iron in 5 mM ferric chloride solution was markedly improved in the presence of sodium caseinate (2% w/v protein) (Figure 6.6A). However, substantial precipitation of iron occurred in solution containing 10 mM iron (Figure 6.6A), in agreement with previous work (Gaucheron et al., 1996; Sugiarto et al., 2010). At this concentration, sodium caseinate also showed extensive precipitation with > 90% of the total protein appearing in the sediment (Figure 6.6B).

The addition of orthophosphate (16, 32 or 64 mM) had a considerable effect on the solubility of both iron and protein in the sodium caseinate–ferric chloride mixtures (Figure 6.6). The inclusion of 16 mM orthophosphate markedly improved the solubility of both protein and iron in the solutions containing up to 15 mM ferric chloride. Increasing the concentration of orthophosphate to 64 mM extended the solubility range for both protein and iron to 25 mM. This suggested that the concentration of iron at which substantial protein precipitation occurred was governed by the amount of orthophosphate present in the sodium caseinate solution.

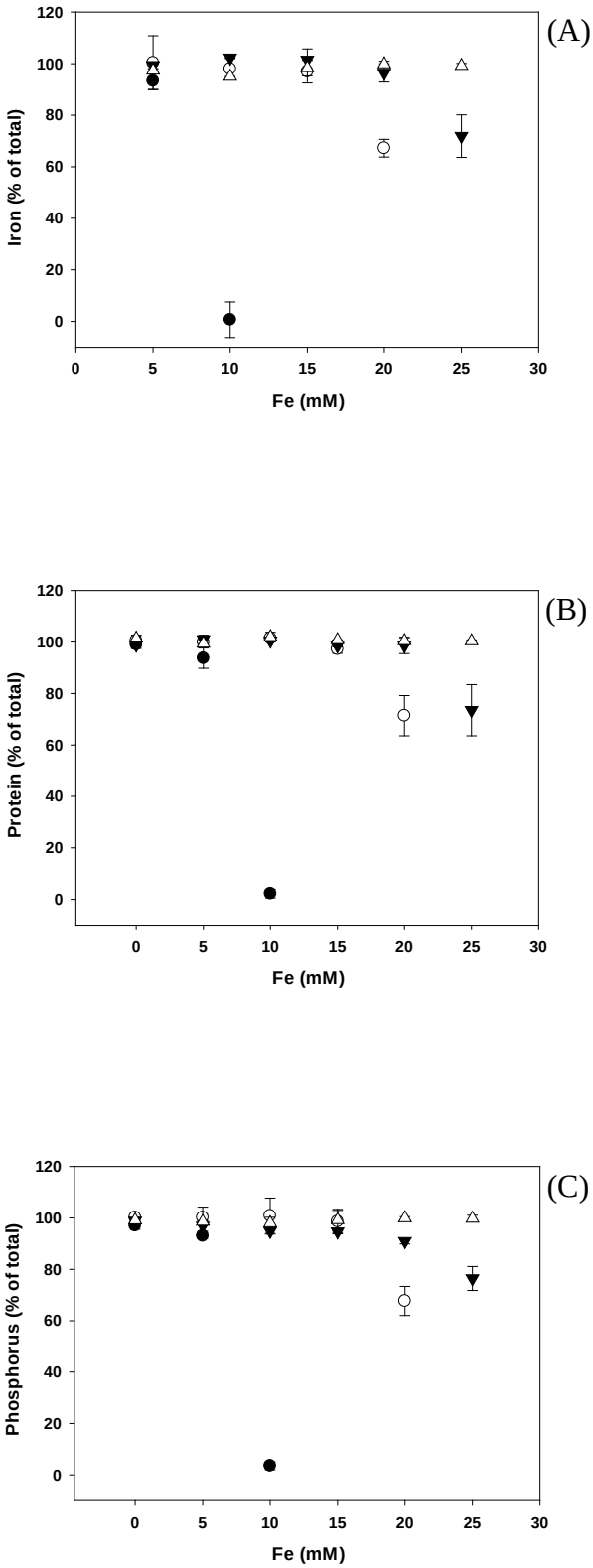


Figure 6-6*: *The proportion of soluble (500 g for 20 min at 20^{o}C) (A) iron, (B) protein and (C) phosphorus in sodium caseinate solution (2% w/v protein) containing (•) 0, (o) 16, (▼) 32 and (Δ) 64 mM orthophosphate.

The effect of iron on the solubility of phosphorus was examined in the supernatant, which contained both the organic phosphorus and the inorganic phosphorus (Figure 6.6C). The trends were similar and corresponded to the reductions observed for protein and iron.

Iron binds to caseins primarily *via* coordination bonds with the oxygen of the phosphoserine residues on the caseins, along with some carboxylic acid side chains (Philippe et al., 2005).These phosphoserine are located in clusters on the major caseins (α_s- and β-caseins) and act as multidentate ligands to bind the metal ions strongly. The solubility of ferric iron in the presence of caseins was maintained possibly because of its binding to these caseins, consequently preventing the formation of the insoluble ferric hydroxide precipitate. Sugiarto et al (2009) have shown, using ferrous sulfate, that there are 14 binding sites for iron on commercial sodium caseinate. The binding of positively charged iron to caseins reduces their negative charge, resulting in aggregation and subsequent sedimentation of the protein-iron complexes. Gaucheron et al (1996) proposed that the neutralization of the charges on the caseins and the formation of intra- and intermolecular bridging upon iron addition result in the precipitation of protein. They reported substantial precipitation of caseins in sodium caseinate solution (25 g/L) at above 4 mM (~ 6.7 mg iron/g protein) added iron (Gaucheron et al., 1997c).

The results showed that casein-iron mixtures containing 5 mM iron (~ 13 mg iron/g protein) formed a stable dispersion. The addition of ferric chloride to sodium caseinate solution reduced the pH, firstly because of the low pH of the ferric chloride solution and secondly because of the release of protons on the binding of iron to caseins. Gaucheron et al (1995) have shown that three protons are released when one iron atom binds to β-casein phosphopeptide. Re-adjustment of the pH to 6.8 after iron addition partly

overcame the reduction in negative charges on the amino acid side chain caused by the reduced pH of the solution, thereby preventing aggregation. Substantial precipitation of caseins occurred when the amount of added iron was above the equimolar concentration of organic phosphorus (5 mM). The inclusion of orthophosphate in sodium caseinate solutions prevented casein precipitation at > 5 mM iron addition. Remarkably, 16 mM orthophosphate increased the amount of iron that could be added to sodium caseinate solutions without substantial protein precipitation, by more than 300% to ~ 41 mg/g of protein.

Phosphorus in the permeate

Figure 6.9 shows the effect of iron addition on the proportion of phosphorus in the ultrafiltration permeates of sodium caseinate solutions containing various concentrations of orthophosphate (16, 32 and 64 mM). In the absence of iron, almost all the orthophosphate added to the sodium caseinate solutions was present in the permeate. This suggests that the orthophosphate was not interacting with the caseins and existed mainly in the free diffusible form.

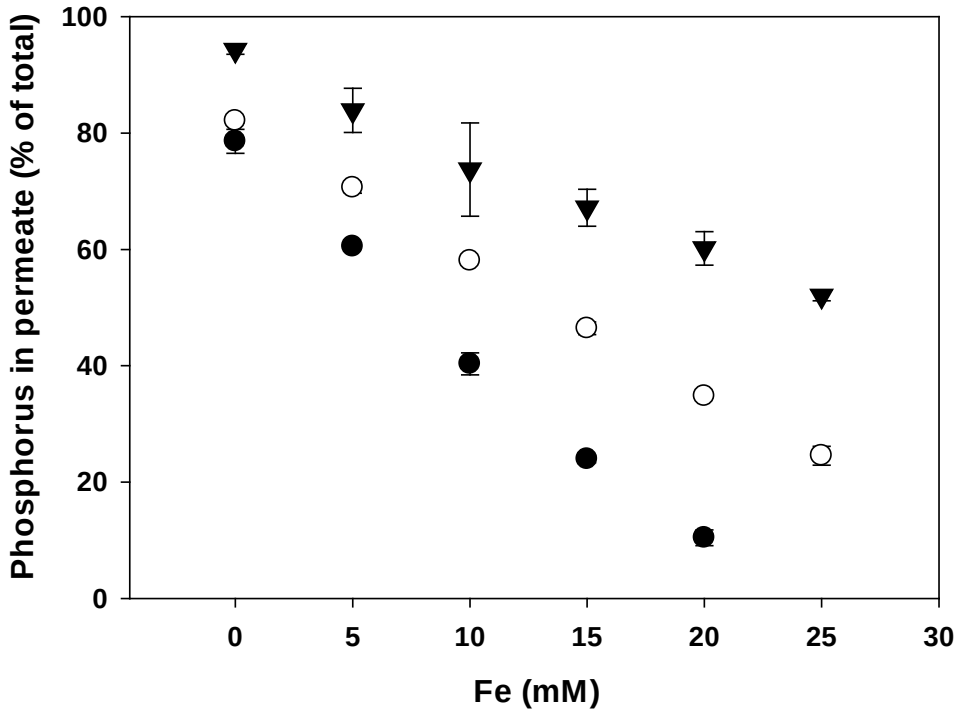


Figure 6-7*: *Effect of iron on the proportion of phosphorus in the permeate of sodium caseinate solution (2% w/v protein) containing (●) 16, (o) 32 and (▼) 64 mM orthophosphate.

An almost linear decrease in the phosphorus content of the permeate as a function of iron addition was observed (Figure 6.7). The phosphorus content of the permeate of the sodium caseinate solution containing 16 mM orthophosphate decreased to ~ 2.5 mM upon 20 mM iron addition. For samples with 32 and 64 mM orthophosphate, the addition of 25 mM iron reduced the permeate phosphorus content to ~ 9.6 and ~ 37 mM, respectively. It appeared that the reduction in the diffusible phosphorus content was associated with the formation of casein-iron complexes.

Table 6.2 gives estimates of the concentrations of phosphorus associated with the caseins on the addition of iron to sodium caseinate solutions containing orthophosphate.

The organic phosphorus concentrations of all sodium caseinate solutions were ~ 5 mM. The concentration of inorganic phosphorus bound to the casein-iron complex was calculated from the difference between the total and permeate phosphorus contents. The total amount of phosphorus bound to casein increased as the concentration of added iron in the sodium caseinate solutions increased, irrespective of the added orthophosphate concentration. Upon iron addition, greater amounts of phosphorus were associated with the caseins in sodium caseinate solutions containing greater orthophosphate concentrations. The increase in the concentration of orthophosphate associated with the caseins corresponded to the improved solubility of the caseins upon iron addition.

Table 6-2*: *Concentrations of total phosphorus bound to caseins in iron added sodium caseinate solutions

Iron (mM)	**Concentration of phosphorus bound to casein (organic phosphorus + inorganic phosphorus, mM)**		
	16	**32**	**64**
0	5.0	5.0	5.0
5	9.0	9.5	10.7
10	13.6	14.5	16.7
15	17.4	19.0	23.5
20	20.3	23.6	28.3
25	--	27.7	35.5

The nature of these interactions between inorganic phosphorus and the caseins was further investigated using ^{31}P-NMR spectroscopy.

6.2.3.2. ^{31}P-NMR spectroscopy

Figure 6.8 shows ^{31}P-NMR spectrum of iron-containing sodium caseinate solution (pH 6.8) in the presence of an external standard (triphenylphosphine). The peak for triphenylphosphine was separated from the organic and inorganic phosphorus peaks. The peak at 1 ppm represents inorganic phosphorus that existed as a contamination in the sodium caseinate solution but its concentration was negligible. The peaks between 1.2 and 3.2 ppm constitute multiple signals of organic phosphorus arising from the various casein fractions in sodium caseinate. These multiple resonance peaks have been extensively characterized in pure casein fractions by Sleigh et al (1983) and indicate that the organic phosphorus on the caseins is present in different environments. The spectrum is complex because of the presence of multiple caseins within the solution. However, it is well known that the phosphoserine residues on caseins are present in clusters of two, three, or four, thus creating regions of different environments for organic phosphorus (West, 1986).

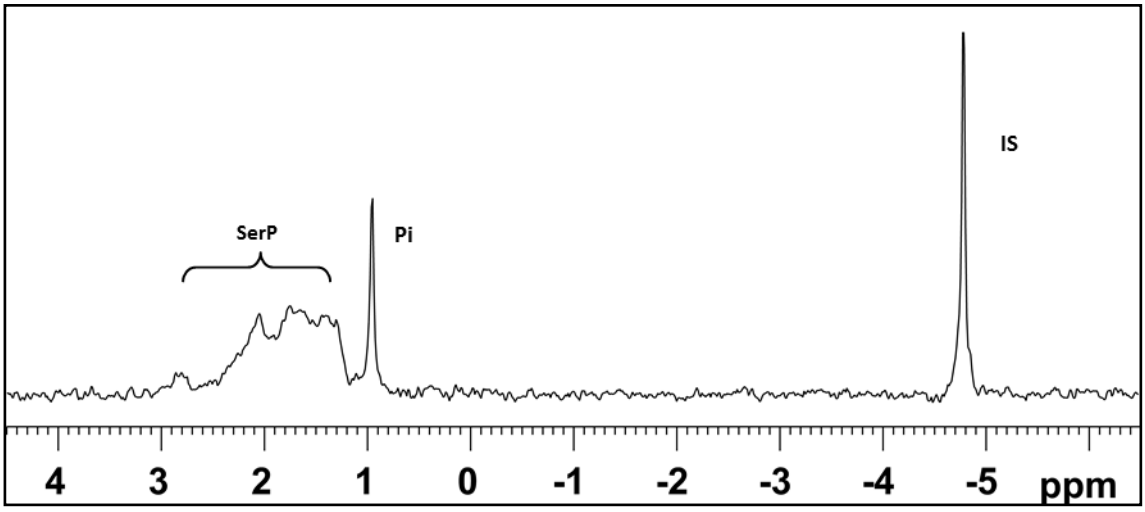


Figure 6-8: *^{31}P-NMR spectra of sodium caseinate solution (2% w/v protein) adjusted to pH 6.8. SerP, Pi and IS represent organic, inorganic and internal standard phosphorus resonance peaks respectively.*

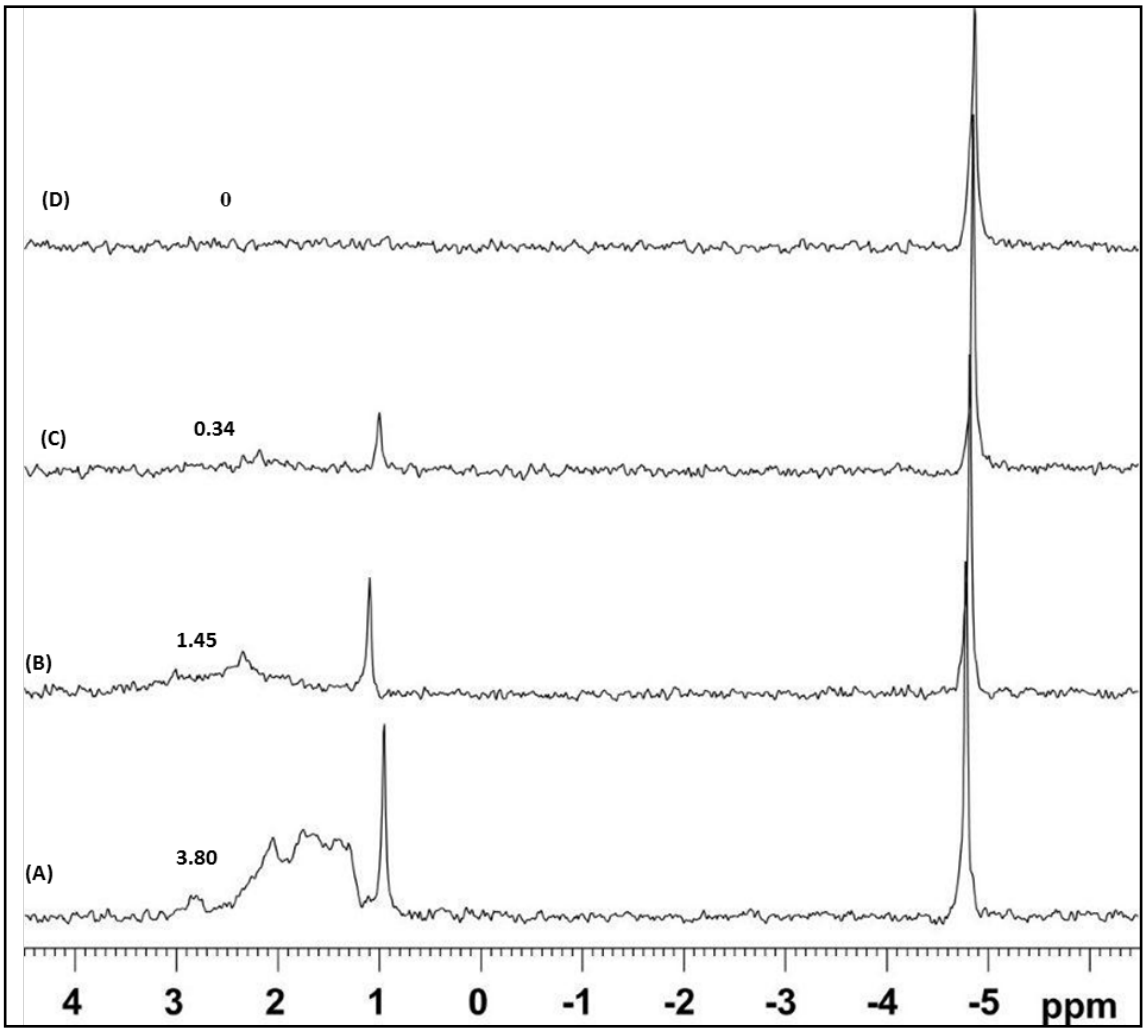


***Figure 6-9*: ^{31}*P-NMR spectra of sodium caseinate solution (2% w/v protein) containing (A) 2, (B) 4 and (C) 6 mM iron at pH 6.8. Integrals on specra represent area for organic phosphorus peaks.*

The ^{31}P-NMR spectra for the sodium caseinate solutions showed a significant reduction in the area of the organic phosphorus peaks upon iron addition (Figure 6.9). The resonance peaks for organic phosphorus were no longer visible upon 6 mM iron addition. Fe^{3+} is a paramagnetic ion and is expected to cause a broadening of ^{31}P signals. As the peak for the external standard were unaffected by the presence of iron, the reduction in the area of the organic phosphorus peak was probably due to an interaction with Fe^{3+}.

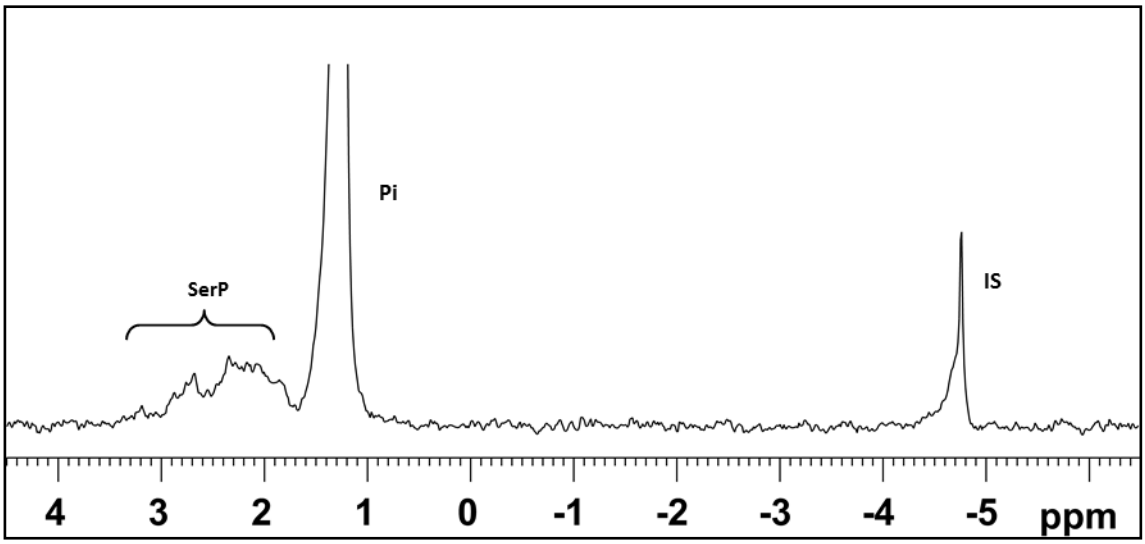


Figure 6-10**:** *^{31}P-NMR spectra of sodium caseinate solution (2% w/v protein) containing 16 mM orthophosphate (as K_2HPO_4) at pH 6.8. SerP, Pi and IS represent organic, inorganic and internal standard phosphorus resonance peaks respectively.*

The ^{31}P-NMR spectra for sodium caseinate solution containing orthophosphate showed a large inorganic phosphorus peak that was very close to the multiple peaks for organic phosphorus (Figure 6.10). The inorganic phosphorus peak would have masked a portion of organic peaks at higher concentrations; hence, the study was conducted using 16 mM orthophosphate. The resonance peak for inorganic phosphorus appeared as a sharp peak, indicating its presence in a free environment.

The addition of iron to the sodium caseinate solutions containing 16 mM orthophosphate resulted in a reduction in the organic phosphorus peaks, but the extent of the reduction was less than that in the control sodium caseinate solution (Figure 6.11). For example, resonance peaks were still evident in the ^{31}P-NMR spectra even upon 10 mM iron addition. In contrast, resonance peaks for organic phosphorus were

absent in the control sodium caseinate solution at above 4 mM iron. These differences in the organic phosphorus peaks suggest that the effect of iron on the peak-broadening phenomenon was reduced by the presence of orthophosphate. It was also observed that the area of the inorganic phosphorus peak (at 1 ppm) decreased as a function of added iron. Approximately 40% of the inorganic phosphorus remained in free form upon 10 mM iron addition (Table 6.3). This decrease corresponded to an equimolar reduction in inorganic phosphorus (10.2 mM) upon the addition of 10 mM iron. This suggested that the inorganic phosphorus interacted with the added iron in a concentration-dependent manner and possibly competed with the phosphoserine clusters on the caseins. The interaction between inorganic phosphorus and added iron appeared to prevent the precipitation of both casein and iron.

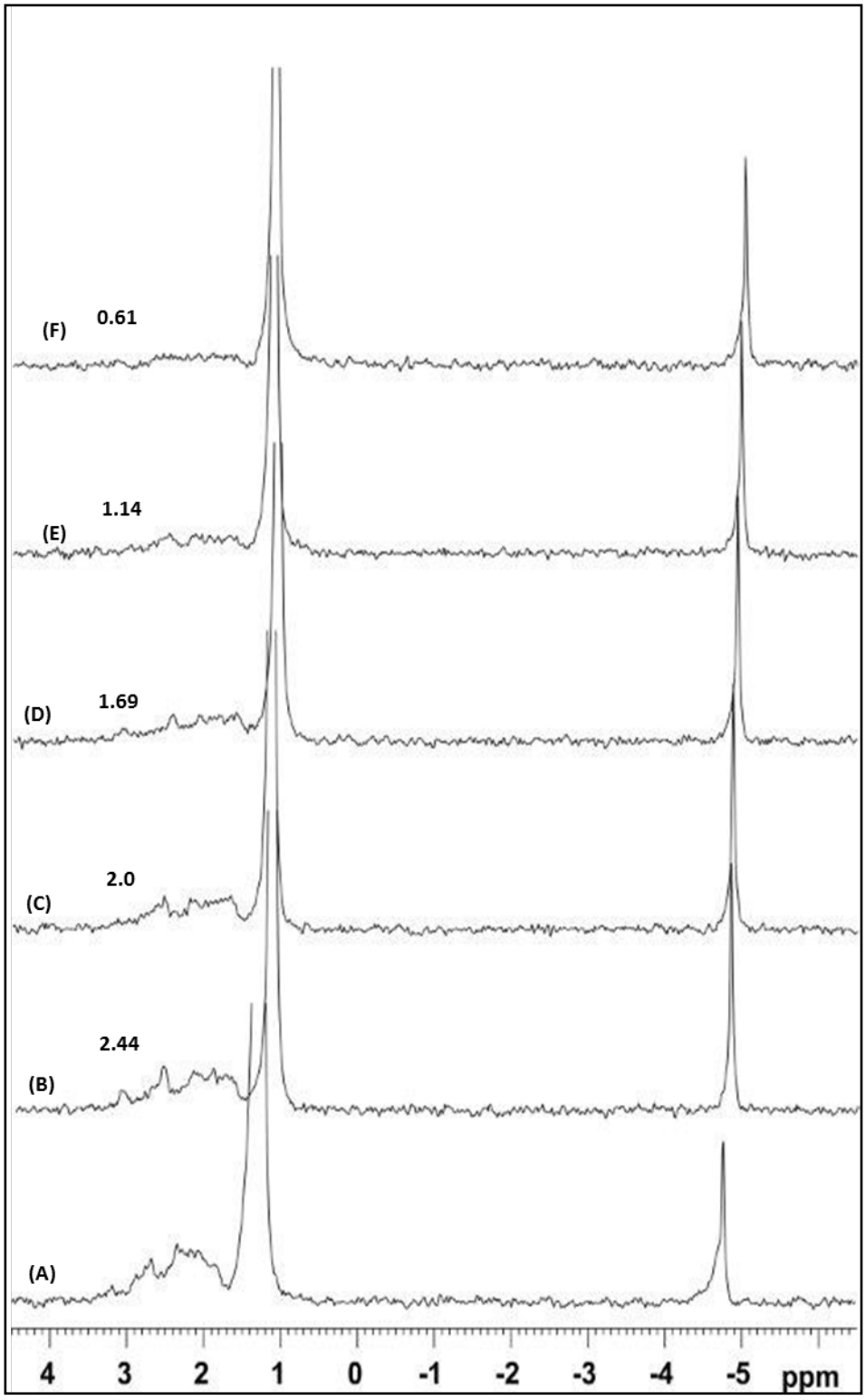


***Figure 6-11*: ^{31}*P-NMR spectra of sodium caseinate solution (2% w/v protein) containing 16 mM of orthophosphate upon (A) 0, (B) 2, (C) 4, (D) 6, (E) 8 and (F) 10 mM iron addition. Integrals on specra represent area for organic phosphorus peaks.*

Table 6-3**:** *Proportion of inorganic phosphorus (as estimated by ^{31}P-NMR spectroscopy) in iron added sodium caseinate solution containing 16 mM orthophosphate.*

	Fe (mM)					
	0	**2**	**4**	**6**	**8**	**10**
Proportion of inorganic phosphorus (%)	100	77.8	66.2	57.8	48.8	40.3

Commercial ferric phosphate which is a mixture of iron and inorganic phosphorus (phosphate salt) binds poorly to caseins in sodium caseinate solution (~ 10% w/v up to 20 mM level). Figure 6.12 compares the amount of iron bound to caseins as commercial ferric phosphate and in orthophosphate added sodium caseinate solution containing different concentrations of ferric chloride. The interaction product formed between iron and inorganic phosphorus in this experiment was soluble. The difference was probably related to the presence of organic phosphorus in the mixture of iron and inorganic phosphorus prepared using the procedure in this experiment. It was possible that organic phosphorus of the caseins was acting as a nucleation site for the growth of inorganic ferric phosphate cluster. Organic phosphorus associated with the caseins are soluble at pH 6.8, hence, the dispersibility of casein-iron-orthophosphate complex was due to the presence of caseins.

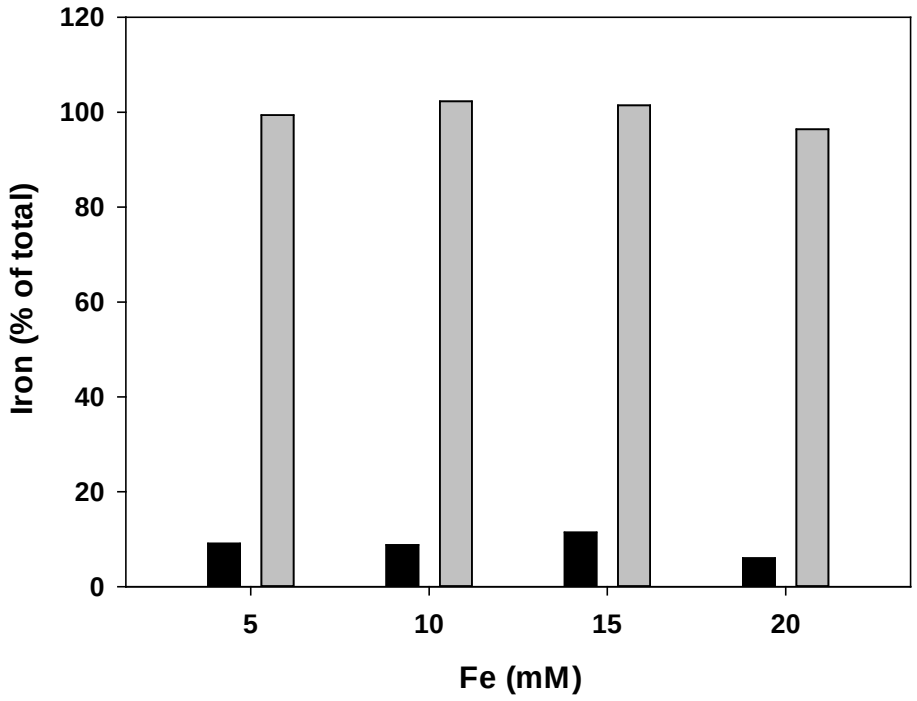


Figure 6-12**:** *Proportion of iron bound to caseins in the soluble phase of sodium caseinate solution when added as ferric phosphate dehydrate (black) and ferric chloride (grey) addition to orthophosphate (32 mM) added sodium caseinate solution*

6.2.3.3. Particle size characteristics

6.2.3.3.1. Turbidity and Z-average diameter

The turbidity of sodium caseinate solutions increased almost 7 fold upon 5 mM iron addition (Figure 6.13A). Substantial aggregation and sedimentation of proteins were observed at higher concentrations of iron addition to sodium caseinate solution. In the orthophosphate added samples, the increase in turbidity was minimal up to 15 mM level of iron addition. The turbidity increased substantially above 15 mM iron but only in the sample containing 16 mM orthophosphate. In the samples containing 32 and 64 mM orthophosphate, a significant increase in turbidity was observed at 20 and 25 mM iron respectively.

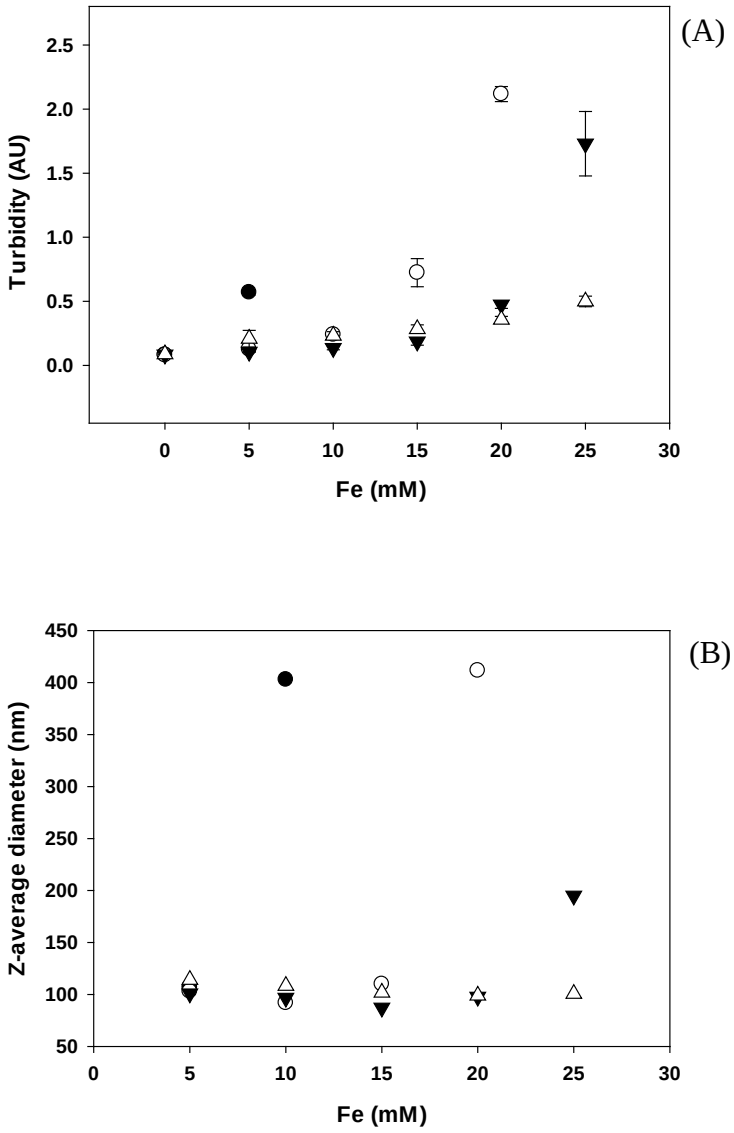


Figure 6-13**:** *Effect of iron addition on the (A) turbidity and (B) Z-average diameter (nm) of sodium caseinate solution (2% w/v protein) containing (•) 0, (o) 16, (▼) 32 and (Δ) 64 mM orthophosphate.*

With no added orthophosphate, the Z-average diameter of sodium caseinate solution increased markedly upon 10 mM iron addition (Figure 6.13B). Addition of orthophosphate stabilised the system and the increase in diameter occurred at higher levels of iron addition. No change in particle diameter was observed in the samples containing 64 mM orthophosphate up to 25 mM added iron.

A small but consistent rise in turbidity of these samples was observed where the Z-average diameter values remained fairly constant below the critical concentration. This suggests that relatively small aggregates increased in number in proportion to the added iron. This was further studied using size exclusion chromatography.

6.2.3.3.2. Size exclusion chromatography (SEC)

6.2.3.3.2.1. Sodium Caseinate

The typical elution profile of sodium caseinate solution (2% w/v protein) showed three different sizes of casein aggregate (Figure 6.14). Fraction 1 was clearly distinguishable and eluted close to the void volume; it was followed by two other fractions (fraction 2 and fraction 3) with larger volumes. The elution profile was similar to that reported by Lucey et al (2000). The total peak area percentages indicated that fractions 1, 2 and 3 represented ~ 4, ~ 18 and ~ 78% of the total, respectively. Thorough investigations of sodium caseinate solutions (2% w/v protein) by SEC have been reported in earlier studies (Lucey et al., 2000; Pepper et al., 1982) and indicate that fraction 2 and fraction 3 represented complexes and aggregates of various sizes and that caseins in the monomeric form (molecular weight between 25 and 30 kDa) eluted at the trailing edge of fraction 3. Larger casein aggregates eluted at the leading edge of fraction 2 and consisted mainly of κ-casein polymers and complexes of α_{s1}- and β-caseins with a molecular weight distribution between 200 and 45 kDa (Lucey et al., 2000). Presumably, in our experiments, fractions 1 and 2 consisted of larger casein aggregates whereas smaller casein aggregates and monomers were present in fraction 3. Samples that showed > 90% protein solubility upon iron and/or orthophosphate addition were characterized using SEC.

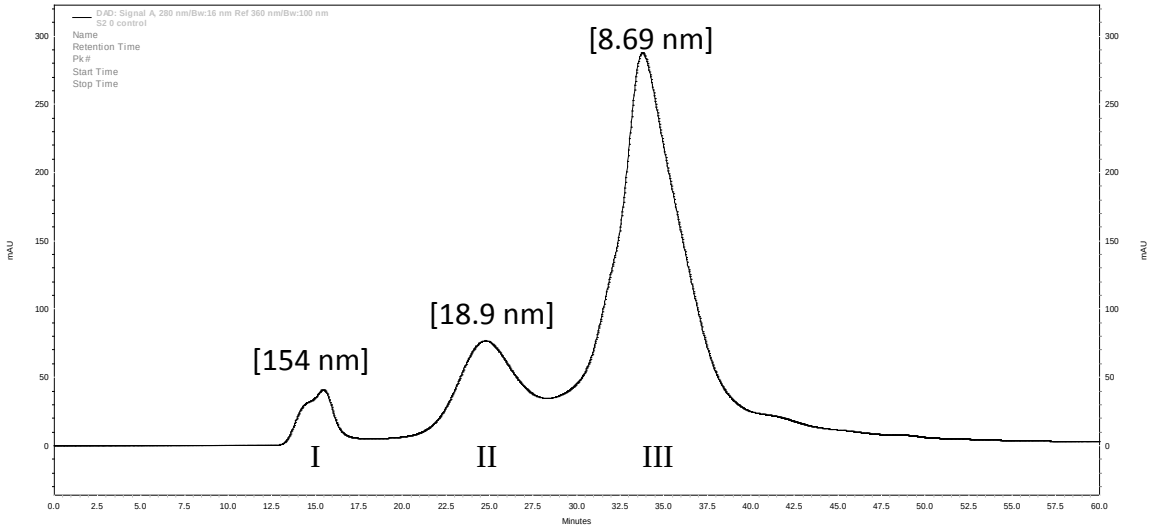


Figure 6-14**:** *Elution profiles of sodium caseinate solution (-) on Superose® 6 HR 10/30 column. Flow rate 0.4 mL/min, eluent – 20 mM imidazole, pH 7.0 and 50 mM NaCl. Values in brackets show volume average diameter of pooled peaks, determined by dynamic light scattering.*

Table 6.4 shows the volume average diameter of the fractions obtained by SEC as analysed by dynamic light scattering. The particles in fraction 1 had a volume average diameter of ~ 145 nm in all the sodium caseinate solutions irrelevant of orthophosphate concentration added. As shown by other researchers (Abdelkader et al., 2008; Lucey et al., 2000), a proportion of fraction 1 probably consisted of protein and fat complexes. The volume average diameter for fraction 2 obtained for all sodium caseinate solutions was at least seven-fold smaller than that of fraction 1. Fractions 2 contributed ~ 18% of the total protein and probably consisted of casein aggregates and κ-casein polymers. The volume average diameter of fraction 3 was ~ 8.5 nm for all the sodium caseinate solutions and represented the largest fraction (~ 78%) consisting probably of caseins monomers.

Table 6-4**:** *Volume average diameter of sodium caseinate fractions obtained by SEC*

Fractions	Orthophosphate (mM)			
	0	**16**	**32**	**64**
Fraction 1 (nm)	154.9 (±6.7)	141.1 (±4.2)	146.1 (±2.0)	135.4 (±8.7)
Fraction 2 (nm)	18.9 (±0.7)	20.8 (±0.4)	19.1 (±0.9)	18.6 (±0.5)
Fraction 3 (nm)	8.7 (±0.3)	8.6 (±0.1)	8.1 (0.1)	8.3 (±0.5)

The caseins in sodium caseinate solution associate by hydrophobic interactions mainly into aggregates of ~ 11 nm along with a small fraction of particles with radius ~ 80 nm (Chu et al., 1995; Lucey et al., 2000). The results suggested that most of the caseins eluted as monomers or small associated fractions in all sodium caseinate solutions irrespective of their orthophosphate content. The changes in diameters of the fractions were consistent with the principles of SEC, wherein the smallest size fractions elute at a later time.

6.2.3.3.2.2. Effect of iron addition

Among the iron added sodium caseinate samples, those having > 90% of soluble protein were characterised for particle size distributions using SEC.

The addition of 5 mM iron to the control sodium caseinate solution caused a marked change in its elution profile (Figure 6.15A). Fraction 3, which represented mainly casein monomers, was no longer evident in the elution profile and a significant reduction in area and a shift in fraction 2 to an earlier elution time were observed. These changes

followed the simultaneous appearance of a distinctly new fraction that eluted in the region of fraction 1.

The addition of 16 mM orthophosphate to the sodium caseinate solution considerably reduced the proportion of fraction 1 that formed upon 5 mM iron addition (Figure 6.15B). However, there was a substantial increase in the area of the fraction 2 peak. A substantial reduction in the fraction 3 peak was observed, but it was not diminished upon 5 mM iron addition. As the concentration of added iron was increased, the proportion of caseins in fraction 1 increased, whereas reductions in fraction 2 and fraction 3 were observed. Fraction 3 was diminished upon 15 mM iron additions. The changes in the elution profile of the sodium caseinate solution containing 32 mM orthophosphate upon iron addition were similar to that observed for 16 mM orthophosphate, although the concentration of iron required to diminish fraction 3 increased to 20 mM (Figure 6.15C).

In the sodium caseinate solution containing 64 mM orthophosphate, the increase in the area under fraction 1 upon 5 mM iron addition was markedly higher than in samples with lower orthophosphate concentrations. Moreover, the fraction 2 peak indicated a shift towards relatively larger particle diameters (Figure 6.15D). The increases in these fractions correlated with the decrease in fraction 3, although some caseins were still associated with fraction 3 at 25 mM iron additions. These results show that preferentially larger aggregates were formed upon iron addition to sodium caseinate solutions containing 64 mM orthophosphate. Overall, the results showed that the inclusion of orthophosphate in the mixture of iron and caseins reduced the size of the aggregates formed.

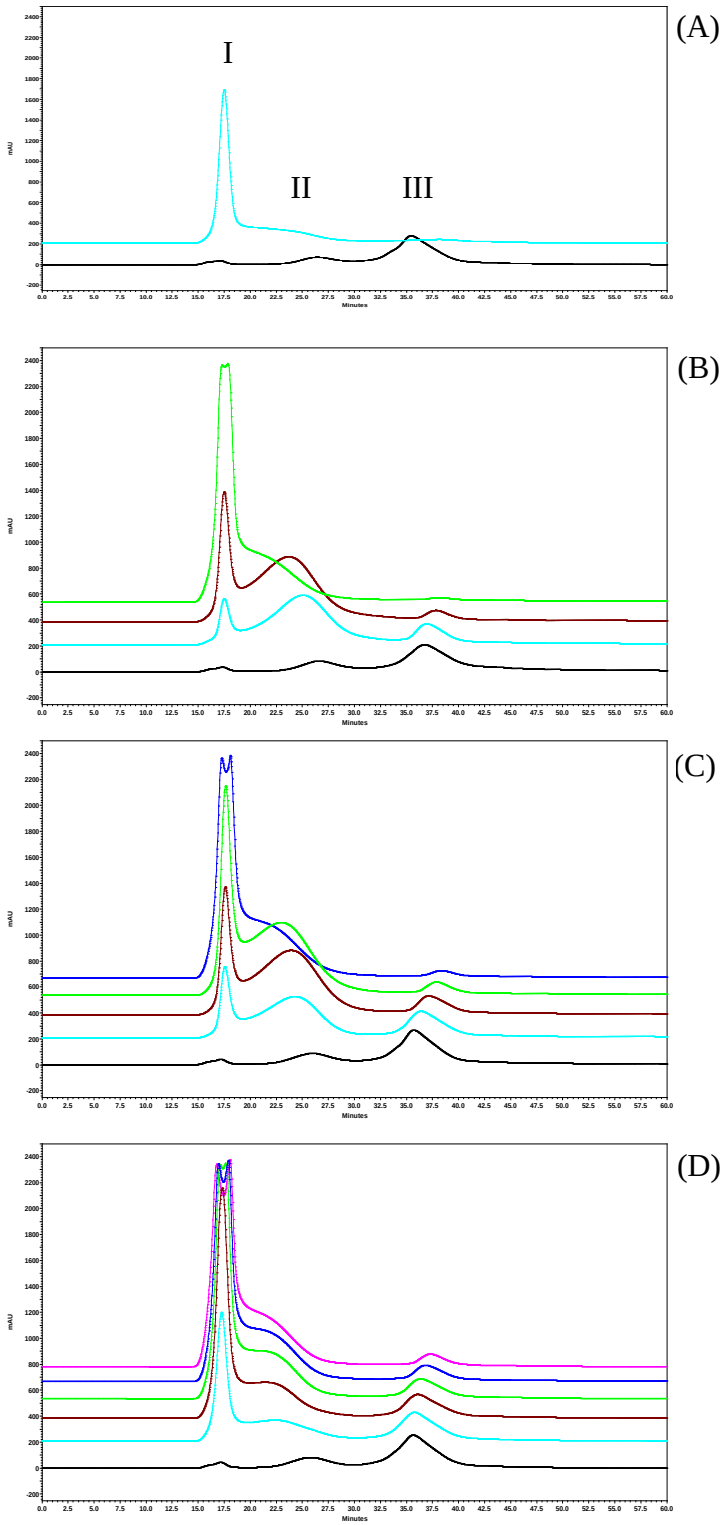


Figure 6-15**:** *Elution profiles of 0 mM (-), 5 mM (-), 10 mM (-), 15 mM (-), 20 mM (-) and 25 mM (-) iron added sodium caseinate solutions (2% w/v protein) containing 0 (A), 16 (B), 32 (C) and 64 (D) mM orthophosphate as K_2HPO4 on Superose® 6 HR 10/30 column. Flow rate 0.4 mL/min, eluent – 20 mM imidazole, pH 7.0 and 50 mM NaCl.*

The above elution profiles of iron added sodium caseinate solutions containing different concentrations of orthophosphate varied only in the proportions of 3 main fractions. However, unlike in the control sodium caseinate solution where most of the caseins were associated with fraction 1 upon 5 mM iron addition, substantial amounts of intermediate sized aggregates were formed in the samples containing orthophosphate.

The changes in the proportions of fractions upon iron addition are plotted in Figure 6.16. Addition of 5 mM iron to the control sodium caseinate solution caused a dramatic increase (from 4% to 70% of total) in the relative proportions of fraction 1, indicating that majority of the caseins were aggregated into relatively large particles (Figure 6.16A). In all the orthophosphate added sodium caseinate solutions, the increase in the proportion of fraction 1 upon 5 mM iron addition was lower than the control; however, the extent was variable. The increase in the proportion of fraction 1 for sodium caseinate solution containing 64 mM orthophosphate was more than two-fold higher as compared to those with lower levels of added orthophosphate. The highest proportion of caseins associated with fraction 1 was ~ 60% for 16, 32 and 64 mM orthophosphate added sodium caseinate solutions, but at different concentrations of added iron i.e. 15 mM, 20 mM and 25 mM respectively.

The trends for relative changes in fraction 2 were similar for sodium caseinate solution containing 16 and 32 mM orthophosphate (Figure 6.16B). An increase in fraction 2 up to 10 mM iron was observed, which reduced at a higher concentration in these samples. Approximately 30% of the caseins were associated with fraction 2 upon 15 and 20 mM iron addition to sodium caseinate solutions containing 16 and 32 mM orthophosphate, respectively. Interestingly for sodium caseinate solution containing 64 mM

orthophosphate, the proportion of fraction 2 increased slightly at all levels of iron addition and reached ~ 31% at 25 mM level.

A substantial decrease in fraction 3 was observed upon 5 mM iron addition to sodium caseinate solutions irrespective of their total orthophosphate content (Figure 6.16C). Approximately 78% of the caseins that existed as monomers in sodium caseinate solution containing 16, 32 and 64 mM orthophosphate, reduced to ~ 15, ~ 24 and ~ 30 percent, respectively upon 5 mM iron addition. At higher concentrations of iron (> 5 mM), fraction 3 decreased following the similar trends and reached ~ 4 ±1% of the total caseins, but at different iron concentrations depending on their respective orthophosphate content. These results suggest that the relatively larger aggregates formed in fraction 1 and fraction 2 upon iron addition were formed mainly at the expense of casein monomers.

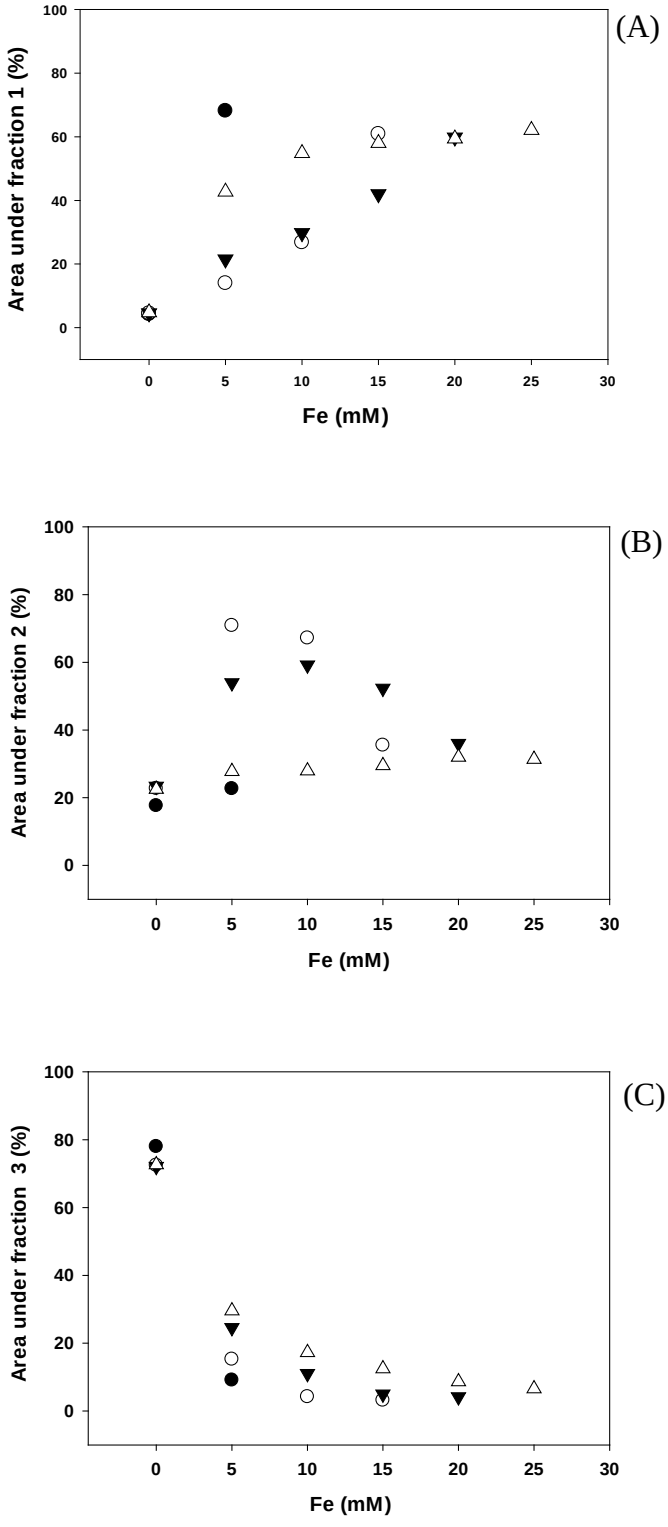


Figure 6-16*: *Effect of (•) 0, (o) 16, (▼) 32 and (⍙) 64 mM orthophosphate on changes in proportion of (A) fraction 1, (B) fraction 2 and (C) fraction 3 obtained from size exclusion chromatography of iron added sodium caseinate solutions.

A considerable decrease in fraction 3 was observed on the addition of 5 mM iron to the sodium caseinate solutions, irrespective of their total orthophosphate content. However, the concentration of iron at which the casein monomers (fraction 3) were almost entirely diminished was governed by the orthophosphate content of the sodium caseinate solutions. This suggests that a higher proportion of casein monomers was present in solutions containing greater orthophosphate concentrations at all levels of iron addition. This observation could be related to the area of the organic peaks in the ^{31}P-NMR spectra obtained for sodium caseinate solutions containing orthophosphate (16 mM) and up to 10 mM iron (Figure 6.11). The increased resonances for organic phosphorus upon iron addition to sodium caseinate solutions containing 16 mM orthophosphate were probably due to the free non-interacting caseins.

In samples with constant protein and orthophosphate concentrations, the sizes of the aggregates formed were governed by the concentration of added iron. At low levels of iron addition (≤ 10 mM), higher proportions of intermediate-sized aggregates (those associated with fraction 2) along with a small proportion of relatively larger aggregates (fraction 1) were formed. At higher iron concentrations (> 10 mM), the proportion of relatively larger aggregates (fraction 1) increased at the expense of both the intermediate-sized aggregates and casein monomers. Thus, the iron-induced aggregation of caseins in the samples with added orthophosphate was a sequential process. Firstly, casein monomers aggregated into sub-aggregates and, secondly, these sub-aggregates aggregated further and both steps occurred simultaneously (Figure 6.17). A similar aggregation of protein upon calcium addition to sodium caseinate containing 15 mM orthophosphate has been reported by Alvarez et al (2007).

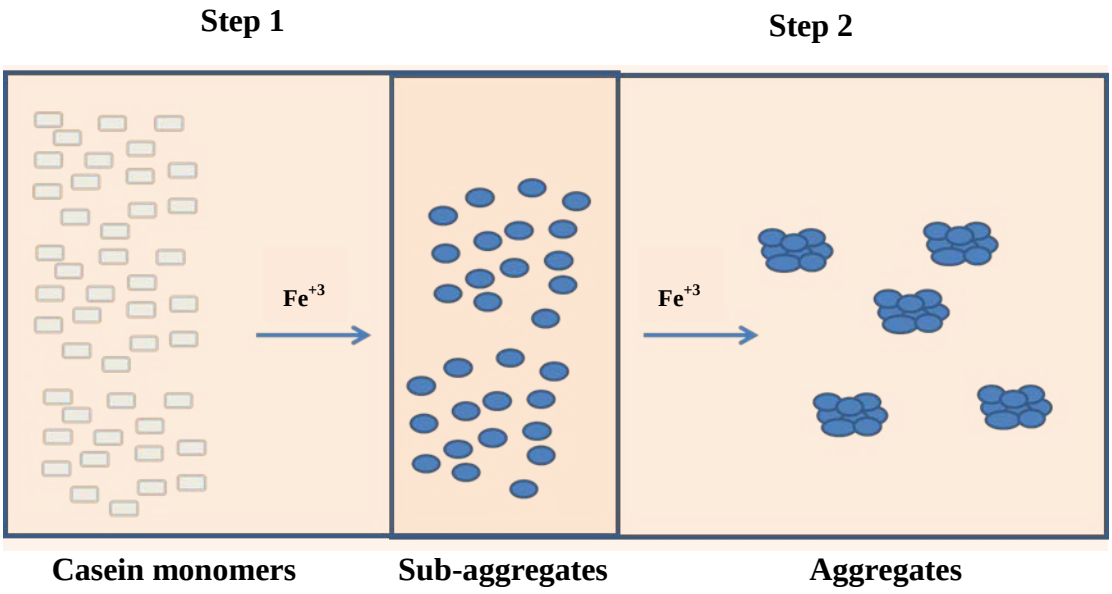


Figure 6-17*: *Schematic showing the iron-induced aggregation of caseins in orthophosphate added sodium caseinate solution.

6.2.3.4. SDS-PAGE

To ascertain the preferential involvement of caseins in iron binding, fraction 1 of sodium caseinate solutions containing 32 mM orthophosphate and different concentrations of added iron was analyzed by SDS-PAGE (Figure 6.18). The average proportions of α_{s1}-, α_{s2}-, β- and κ-caseins in the sodium caseinate solution were ~ 8.5, ~ 36.3, ~ 40.7 and ~ 14.5, respectively. All the major caseins (α_{s1}-, α_{s2}-, β- and κ-casein) were present in fraction 1 of the iron added sodium caseinate solution containing orthophosphate.

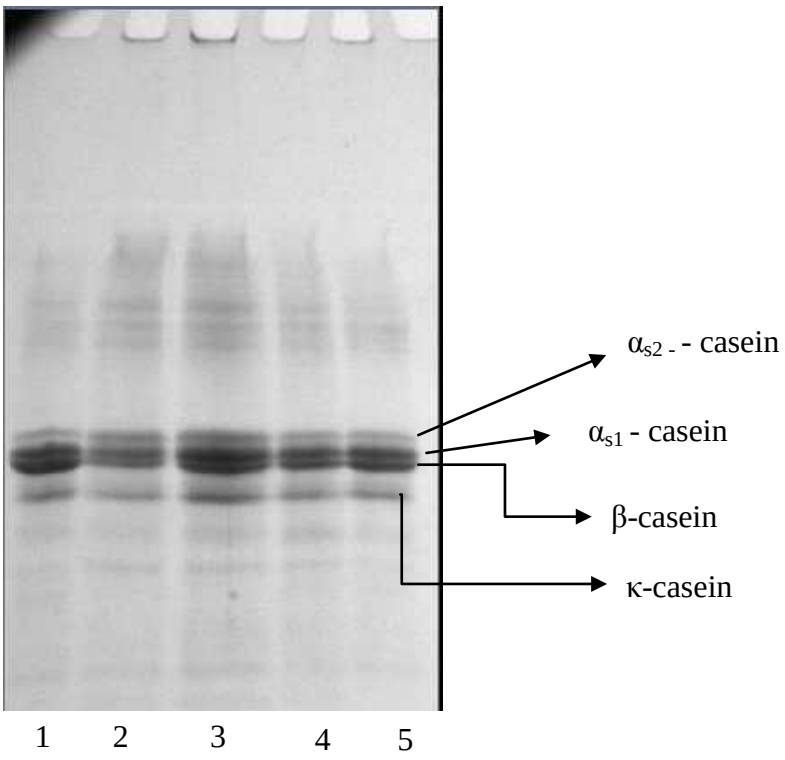


Figure 6-18**:** *SDS-PAGE gel of sodium caseinate (2% w/v protein) solution containing 32 mM of added orthophosphate (Lane 1) and fraction 1 collected from the size exclusion chromatography of solutions with different concentrations of iron, Lane 2 – 5: 5 mM, 10 mM, 15 mM, 20 mM added iron.*

Overall, the results show that the inclusion of orthophosphate in the mixture of iron and caseins raised the concentration of iron at which casein precipitation was induced. In the absence of orthophosphate, the added iron bound directly to the caseins, reducing charge and leading to its aggregation and precipitation.

Iron precipitates with orthophosphate in aqueous solutions and forms insoluble ferric phosphate salts with various Fe:P ratios (Fytianos et al., 1998). This precipitation also occurred in the sodium caseinate solutions containing orthophosphate, as indicated by a

proportionate reduction in the diffusible phosphorus content (Figure 6.7). However, it appeared that the precipitation of orthophosphate in the presence of caseins resulted in the solubilization of these precipitates. The interaction of iron with both the organic phosphorus and the inorganic phosphorus was shown using ^{31}P-NMR spectroscopy. Thus, the solubility of the iron-casein-orthophosphate complex was due to the presence of the caseins, which are soluble at pH 6.8. Furthermore, smaller aggregates were formed when iron was added to sodium caseinate in the presence of orthophosphate. Therefore it appears that the presence of orthophosphate performed two functions: (1) the interaction of orthophosphate with iron reduced the screening of the negative charge on the caseins; (2) this interaction resulted in the formation of smaller aggregates, which promoted dispersion stability. The extent of these influences was governed by the concentration of orthophosphate; an increase in its concentration increased the amount of iron that could be added to the sodium caseinate solution without substantial protein precipitation. The ^{31}P-NMR spectroscopy and SEC results suggest that relatively lower amounts of caseins interacted with iron in sodium caseinate solutions containing greater orthophosphate concentrations (Figure 6.15 and Figure 6.11). The interaction of iron with caseins was not selective and all casein fractions were involved in the aggregates formed. It was probable that colloidal structures of ferric phosphate were stabilized by caseins, as represented in Figure 6.19. The formation of ferric phosphate salts in the hydrophobic regions of the casein micelle of milk has already been established by Mossbauer spectroscopy (Raouche et al., 2009b).

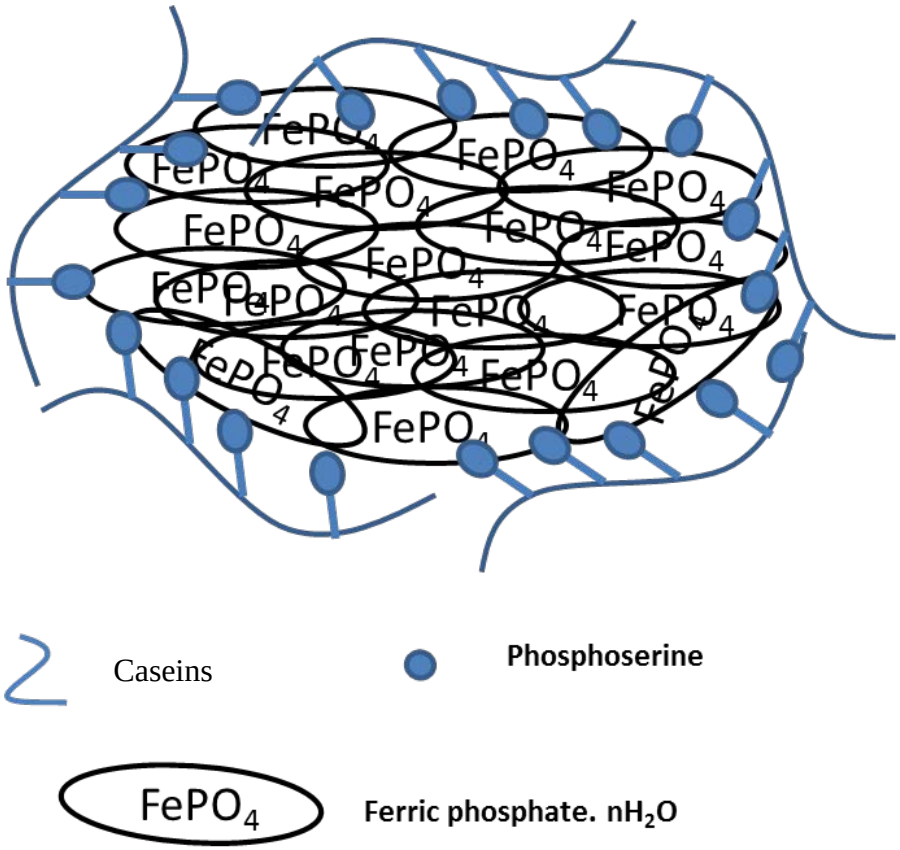


Figure 6-19*: *Schematic diagram representing the ferric phosphate stabilisation by caseins.

6.3. Conclusions

This study highlighted the role orthophosphate played in improving the solubility of iron and caseins. The binding of iron at greater than equimolar concentration of organic phosphorus on caseins results in the precipitation of both the iron and caseins. In the presence of orthophosphate, however higher concentrations of iron could be added to sodium caseinate solution (~ 6.9% by weight of casein) with little sedimentation of protein. Thus orthophosphate prevented iron-induced casein precipitation and also

improved the solubility of iron. The formation of small aggregates upon iron addition to sodium caseinate solution containing orthophosphate was responsible for the high solubility of casein-iron complexes. The ^{31}P-NMR study, SDS-PAGE and size exclusion chromatography analysis of the soluble casein-iron complexes propose that a cluster of inorganic ferric phosphate was stabilised by caseins. The concentration of iron bound by caseins in the soluble form was at least 5 to 10 times higher than in previous studies, thus creating an opportunity to develop a new ingredient for food fortification.

Chapter 7. The redispersion behaviour of casein-iron precipitate in the presence of orthophosphate

7.1. Introduction

The results presented in Chapter 6 showed that the presence of orthophosphate promoted the dispersion stability of casein-iron complex in two ways; firstly the interaction of orthophosphate with iron reduced the screening of negative charge on the caseins, and secondly this interaction resulted in the formation of smaller aggregates. Iron has a strong affinity for phosphate and generally forms an insoluble precipitate when added to aqueous solutions containing orthophosphate. Conversely, orthophosphate can interact with iron present in aqueous solutions as ferric hydroxide (Adegoke et al., 2013; Sparks, 2003). It is generally agreed that orthophosphate adsorbs on to the oxides of iron by displacing OH^- from the coordination sphere *via* the ligand exchange mechanisms (Lijklema, 1980). The adsorbed phosphate ion binds strongly to the ferric oxides and resist desorption (Barber, 2002). This adsorption phenomenon has been used to eleminate phosphate from natural and sewage waters.

Iron added to sodium caseinate solution binds to the caseins and forms insoluble precipitates at > 5 mM level at pH 6.8. Hence, the effect of orthophosphate addition on the physico-chemical properties of precipitated sodium caseinate solutions was examined in this chapter.

7.2. Results and discussion

7.2.1. The effect of iron on solubility of sodium caseinate solutions

Figure 7.1 shows the effect of adding 0 to 25 mM iron on the solubility of the sodium caseinate solutions containing 1 to 3% w/v protein after 24 h storage at room temperature. Addition of > 5 mM iron to sodium caseinate solutions resulted in protein precipitation, irrespective of the protein content. Addition of positively charged iron reduces the negative charge on the casein leading to aggregation and sedimentation, as shown in Chapter 6. The added iron binds primarily to the organic phosphorus associated with the phosphoserine residues on caseins. The total phosphorus contents of sodium caseinate solutions containing 1%, 2% and 3% w/v protein were ~ 2.5, ~ 5 and ~ 7.5 mM respectively (obtained by total phosphorus content analysis). At 25 mM iron addition, the ratio of iron:P was 10:1, 5:1 and 3.3:1 for sodium caseinate solution containing 1%, 2% and 3% w/v protein respectively. These observations suggest that iron added at greater than equimolar concentration of phosphorus (organic phosphorus) present in sodium caseinate solutions resulted in protein precipitation. Precipitation of caseins in sodium caseinate solution (2.5% w/v protein) containing > 4 mM addition of ferric chloride has also been observed by Gaucheron et al (1997c).

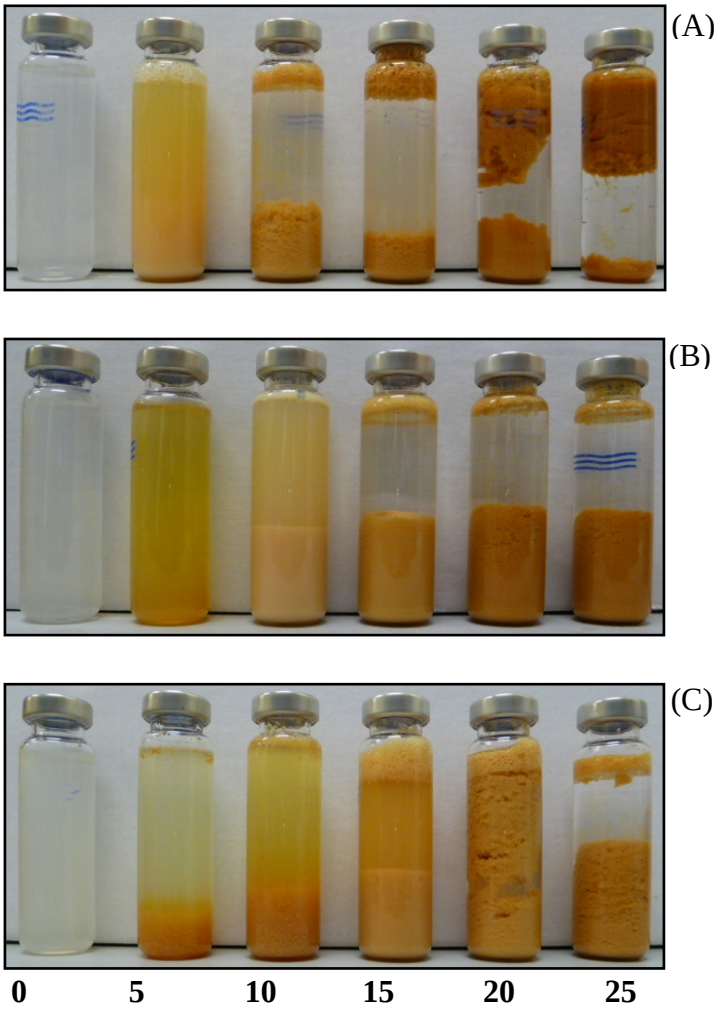


Figure 7-1**:** *The effect of iron addition (0, 5, 10, 15, 20 and 25 mM) on the solubility of sodium caseinate solutions containing (A) 1%, (B) 2% and (C) 3% w/v protein after 24 h storage at room temperature and pH 6.8.*

The effect of orthophosphate (i.e. 16, 32 and 64 mM) on the iron-induced precipitation in sodium caseinate was examined. The orthophosphate was added to the sodium caseinate solutions after 30 min of iron addition and stirring at ~ 5^{o}C. The pH of the orthophosphate added samples was adjusted to 6.8 and left to stirr at room temperature for further 30 min. The samples were then transferred to the glass vials and left to stand for 24 h (Figure 7.1).

The effect of adding 16 mM orthophosphate on the casein-iron precipitates obtained in solutions containing 1, 2 and 3% w/v protein is shown in Figure 7.2. Remarkably, the precipitation observed at > 5 mM iron addition level had disappeared, irrespective of the protein content. The redispersion was apparently more efficient in solutions containing higher protein content. Sedimentation was observed in the sodium caseinate sample containing 1% w/v protein when > 10 mM iron was added, but the extent was less than that in the control samples (no added orthophosphate).

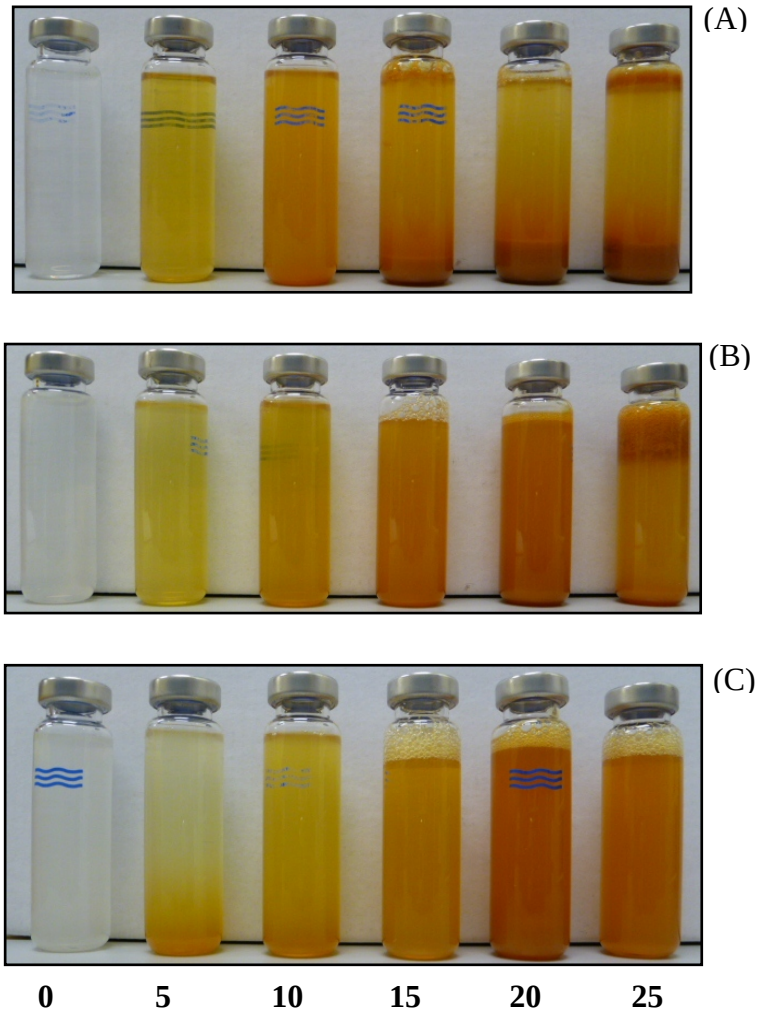


Figure 7-2**:** *The effect of 16 mM orthophosphate addition on the solubility of iron (0, 5, 10, 15, 20 and 25 mM) added sodium caseinate solutions containing (A) 1%, (B) 2% and (C) 3% w/v protein after 24 h storage at room temperature and pH 6.8.*

Further improvement was observed in the redispersion of casein-iron precipitate upon 32 mM orthophosphate addition in sodium caseinate solution with varying protein contents (1 to 3% w/v) (Figure 7.3). The redispersed solutions containing > 1% w/v protein were visibly less turbid. However, sedimentation was still observed in the sodium caseinate solution containing 1% w/v protein and > 10 mM iron.

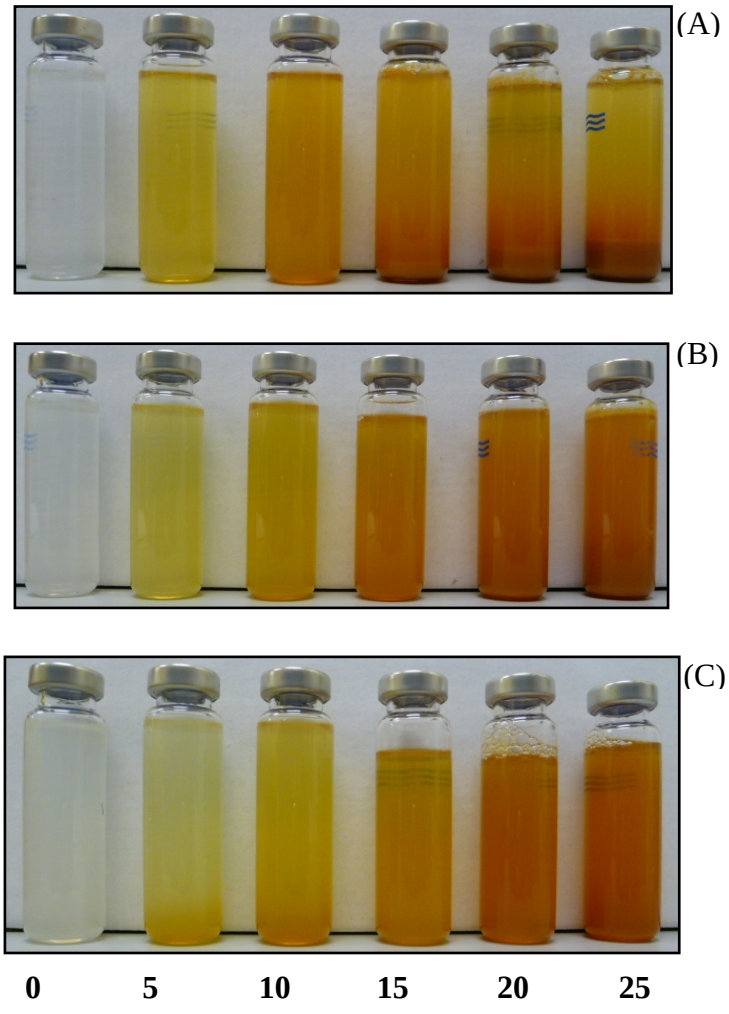


Figure 7-3**:** *The effect of 32 mM orthophosphate addition on the solubility of iron (0, 5, 10, 15, 20 and 25 mM) added sodium caseinate solutions containing (A) 1%, (B) 2% and (C) 3% w/v protein after 24 h storage at room temperature and pH 6.8.*

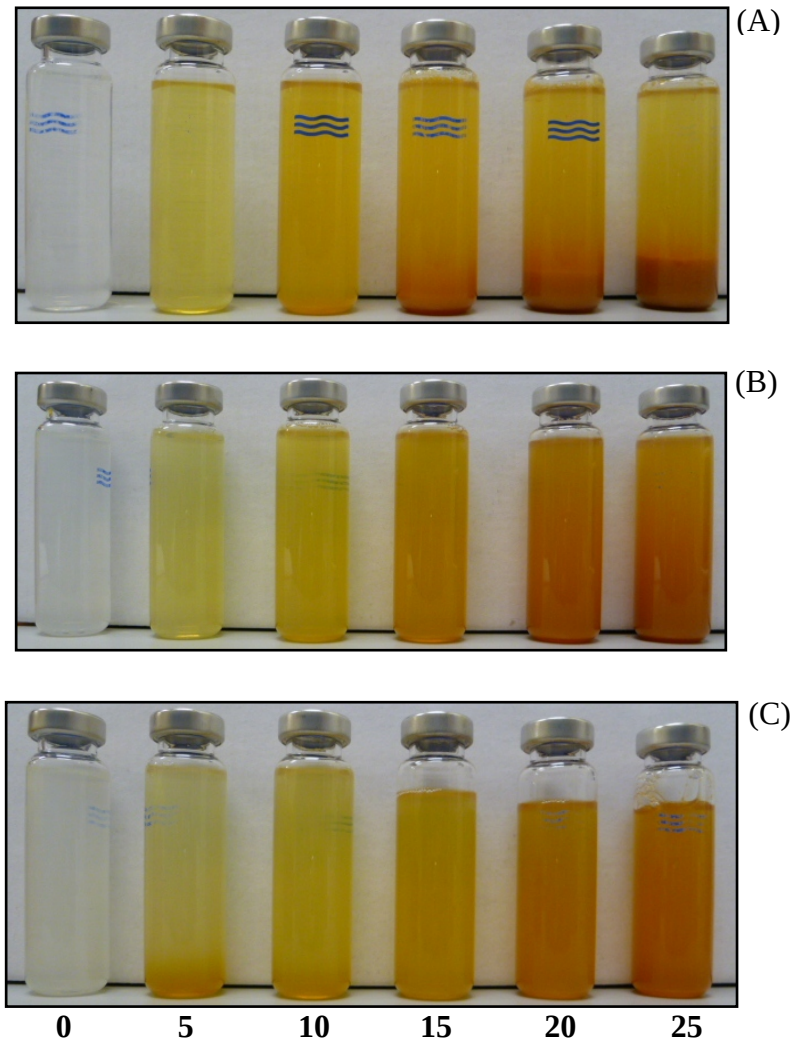


Figure 7-4**:** *The effect of 64 mM orthophosphate on the solubility of iron (0, 5, 10, 15, 20 and 25 mM) added sodium caseinate solutions containing (A) 1%, (B) 2% and (C) 3% w/v protein after 24 h storage at room temperature and pH 6.8.*

Irrespective of the protein and iron contents, samples containing 64 mM orthophosphate appeared slightly less turbid as compared to the samples with lower orthophosphate contents. However, substantial sedimentation was still observed in sodium caseinate solutions containing 1% w/v protein and > 10 mM iron.

A visual inspection of the photographs for iron-added sodium caseinate solutions suggests substantial redispersion of casein-iron precipitate even at the lowest concentration of orthophosphate addition. Further insights into the redispersion behaviour were gained by monitoring the turbidity and particle sizes which are reported and discussed in the next section.

7.2.1.1. Particle size characteristics

Sodium caseinate is a polydisperse system wherein the caseins (α_s-, β- and κ-caseins) are associated in a concentration-dependent manner into aggregates of ~ 10 nm diameter (Abdelkader et al., 2008; Chu et al., 1995). Addition of exogenous multivalent cation alters the above association and the aggregation of proteins. The particle size of the aggregates formed is important for determining the dispersion stability of the system.

Dynamic light scattering is generally used to study relative changes in the average particle size of the sodium caseinate solution (Abdelkader et al., 2008). The Z-average diameter is highly influenced by the presence of relatively small fraction of large aggregates in sodium caseinate which mask the smaller casein aggregates and monomers. It thus becomes imperative to use a more realistic method of volume particle size distribution, which is generated from fluctuations in the intensity data of Zetasizer. The particle size distribution provides a relatively clear picture of the changes in the volume of differently-sized aggregates formed in the redispersed of casein-iron complexes. The data could be used to observe the changes across sodium caseinate solutions containing different orthophosphate and iron content.

The effect of added orthophosphate concentration on the turbidity and volume particle size distribution of redispersed casein-iron precipitate in sodium caseinate solution

containing 1% w/v protein is shown in Figure 7.5. The turbidity values of sodium caseinate solutions, except at 5 mM added iron, were mostly above the detectable range of instrument, irrespective of the iron concentration. Addition of 16 mM orthophosphate to these iron-casein precipitates lowered the turbidity to within the detectable range of the instrument even at 25 mM iron addition. The turbidity values of all samples increased with increasing concentration of iron, irrespective of the orthophosphate content. At similar iron concentrations, the samples containing 64 mM orthophosphate had the lowest turbidity but the differences were small between the samples with varying orthophosphate contents. The results suggest that 16 mM orthophosphate addition was sufficient to substantially reduce the turbidity of iron added 1% w/v sodium caseinate solution.

Multiple populations of particles were observed in the volume particle size distribution of sodium caseinate solution (1% w/v protein) containing up to 25 mM iron and varying orthophosphate (0 - 64 mM) contents. The multiple populations could be segregated into three different diameter range: between 0 - 100 nm, 100 - 1000 and > 1000 nm. Significant proportions of the relatively small aggregates (0-100 nm) were observed in all the samples. The proportion of particles with diameter between 100 and 1000 nm increased in the samples with higher concentration of iron. Substantial increase in the proportions of relatively larger aggregates (> 1000 nm) was observed in samples that showed visible sedimentation (i.e. > 10 mM iron level).

Among the samples with varying orthophosphate concentrations, higher proportions of relatively smaller aggregates were observed in samples containing 32 mM orthophosphate compared with that containing 16 mM orthophosphate. However, further increase in orthophosphate concentration did not affect the volume particle size distribution.

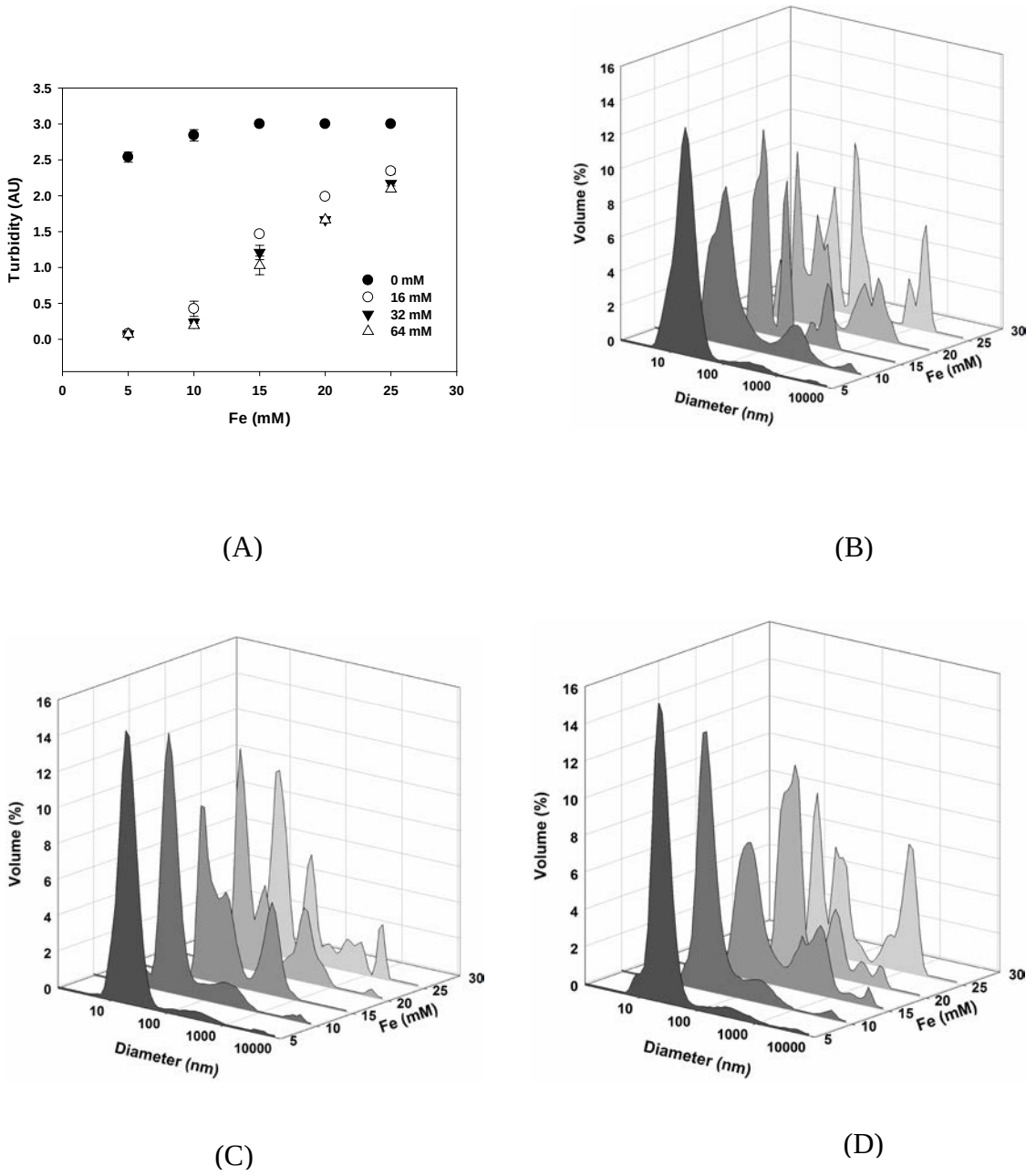
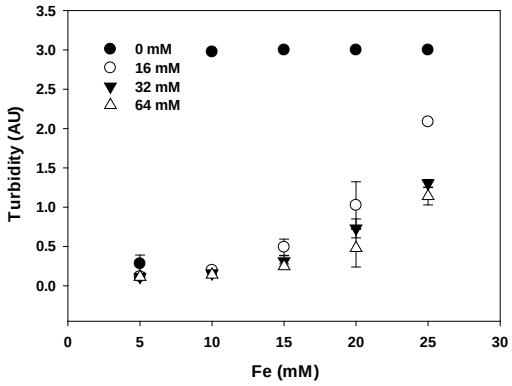
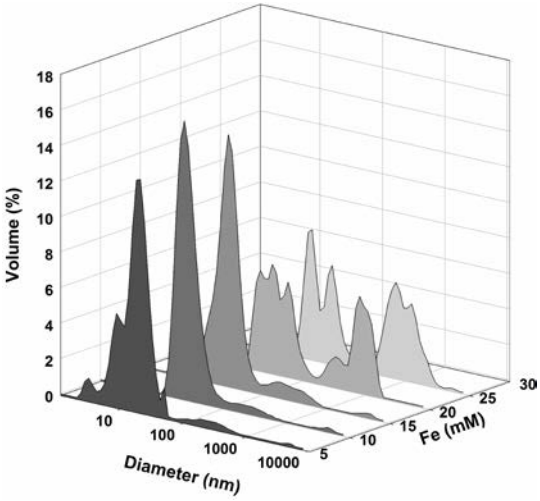


Figure 7-5**:** *Effect of iron addition (5, 10, 15, 20 and 25 mM) on the (A) turbidity and volume particle size distribution of sodium caseinate solution (1% w/v protein) containing (B) 16, (C) 32 and (D) 64 mM orthophosphate at pH 6.8.*

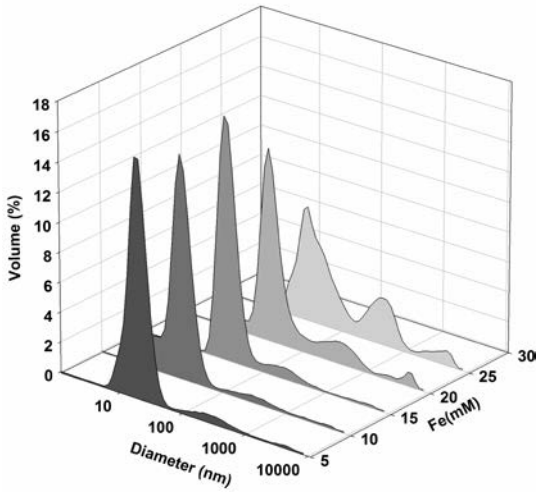
Overall, the samples with > 10 mM added iron showed substantial proportions of particles above 100 nm diameter. The turbidity and volume particle size distribution suggested that sodium caseinate solutions containing 1% w/v protein and 10 mM iron could be redispersed most efficiently upon 32 mM orthophosphate addition.



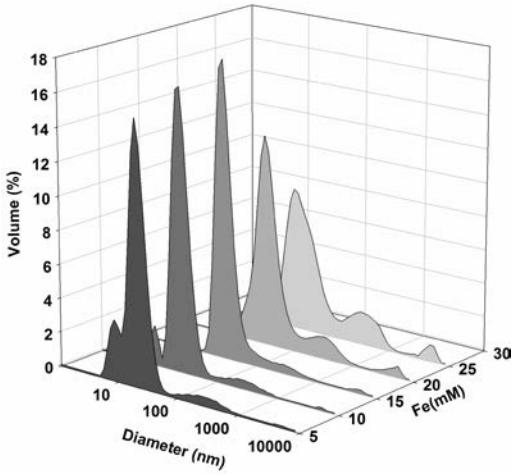
(A)



(B)



(C)



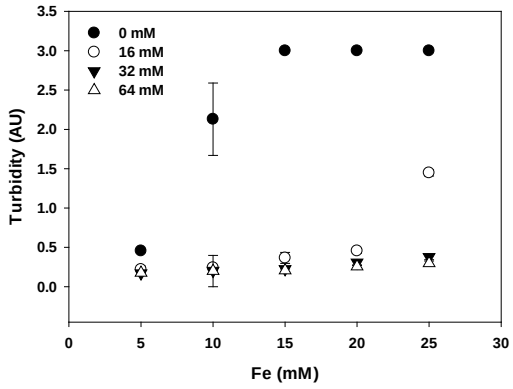
(D)

Figure 7-6*: *Effect of iron addition (5, 10, 15, 20 and 25 mM) on the (A) turbidity and volume particle size distribution of sodium caseinate solution (2% w/v protein) containing (B) 16, (C) 32 and (D) 64 mM orthophosphate at pH 6.8.

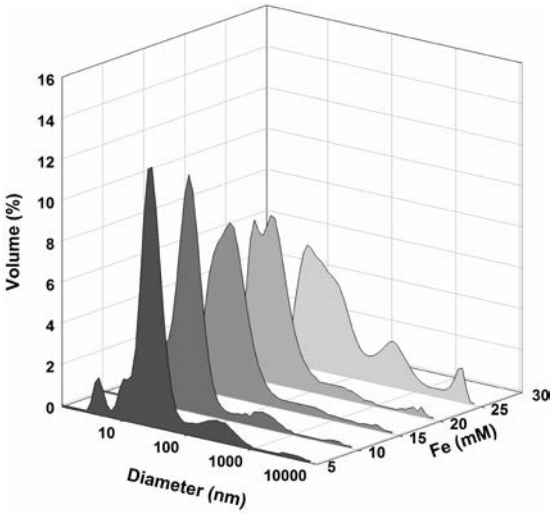
The concentration of iron at which substantial increase in turbidity occurred increased from 5 mM to 15 mM in sodium caseinate solution containing 2% w/v protein (Figure 7.6). A substantial reduction in turbidity as function of orthophosphate content was observed in sodium caseinate solution containing up to 25 mM iron. The turbidity values of the redispersed sodium caseinate solutions containing up to 15 mM level were similar and increased in the samples containing higher concentrations of iron.

Irrespective of the orthophosphate concentration, higher proportions of particles with less than 100 nm diameter were formed in sodium caseinate solution (2% w/v protein) containing up to 15 mM iron. Increasing the concentration of orthophosphate to > 16 mM raised the concentration of iron at which relatively larger particles (> 100 nm diameter) were formed to 20 mM. The particle size distributions were similar for iron-added sodium caseinate solutions containing 32 mM and 64 mM orthophosphate.

These results suggest that increasing the protein content of sodium caseinate to 2% w/v raised the threshold of iron at which relatively larger aggregates were formed. The optimum combination of protein, iron and orthophosphate for the formation of stable dispersion was 2% w/v protein, 15 mM and 32 mM respectively.


0 mM
16 mM
32 mM
64 mM
Turbidity (AU)
Fe (mM)

(A)


Volume (%)
Diameter (nm)
Fe (mM)

(B)

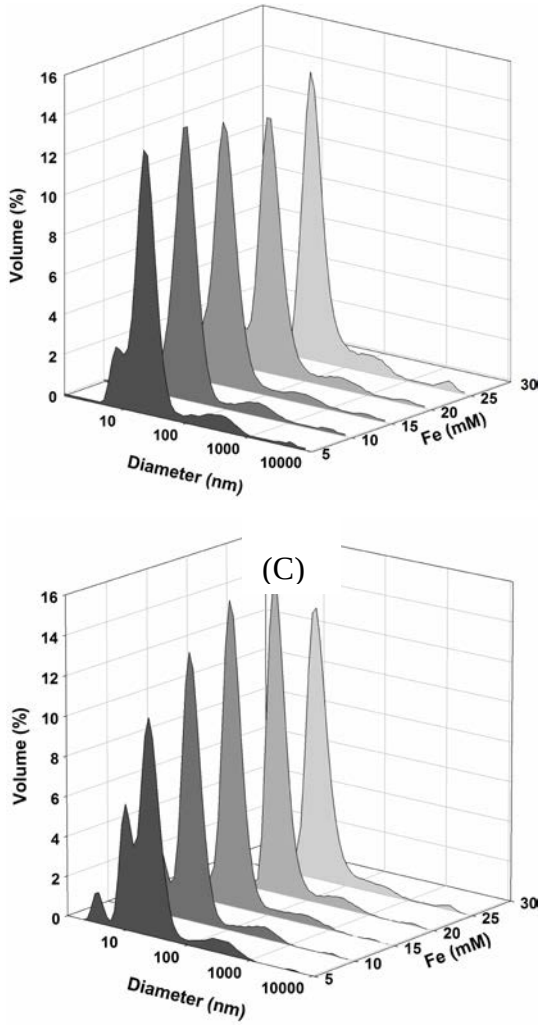


Figure 7-7**:** *Effect of iron addition (5, 10, 15, 20 and 25 mM) on the (A) turbidity and volume particle size distribution of sodium caseinate solution (3% w/v protein) containing (B) 16, (C) 32 and (D) 64 mM orthophosphate at pH 6.8.*

The turbidity of iron-added sodium caseinate solution containing 3% w/v protein was the lowest among the samples containing varying protein contents (Figure 7.7). Moreover with the exception of the sample containing 16 mM orthophosphate and 25 mM iron, the turbidity values of all other samples remained fairly constant.

The majority of the iron-added samples showed a monomodal distribution of particles with diameter < 100 nm, except the sample containing 16 mM orthophosphate and 25

mM iron. The samples containing 32 mM or 64 mM of phosphorus had similar particle size distributions at all levels of iron addition. Overall, the results suggest that increasing the protein content raised the concentration of iron at which stable dispersion could be formed upon addition of orthophosphate.

Protein	Iron (mM)					Orthophosphate (mM)
	5	10	15	20	25	
3%						64
						32
						16
2%						64
						32
						16
1%						64
						32
						16

Figure 7-8**:** *Diagram showing the concentrations of protein, iron and orthophosphate that form stable dispersion (Coloured green).*

Figure 7.8 shows the combination of iron and casein concentrations that could be redispersed by different concentrations of orthophosphate. By increasing the protein content of sodium caseinate solutions three-fold, more than double the amount of iron could be efficiently redispersed. The iron binding sites comprise mainly the oxygen of the organic phosphate esterified to serine residues on caseins (Hegenauer et al., 1979c). The organic phosphorus contents, as calculated from the total phosphorus content analysis, were ~ 2.5, ~ 5.0 mM and ~ 7.5 mM for sodium caseinate solution containing 1%, 2% and 3% w/v protein, respectively. The results of particle size

analysis show that a minimum of 10, 15 and 20 mM iron could be redispersed by sodium caseinate solution containing ~ 2.5, ~ 5.0 and ~ 7.5 mM organic phosphorus and different concentrations of orthophosphate. This suggests that one mole of organic phosphorus could bind three to four moles of iron in a stable dispersion by introducing varying concentrations of orthophosphate. Another interesting trend was that an increase in the amount of organic phosphorus (i.e. the protein content) reduced the amounts of orthophosphate required to redisperse higher concentrations of iron. For example, 16 mM orthophosphate could efficiently redisperse 10, 15 and 20 mM iron bound to caseins in sodium caseinate solution containing 1%, 2% and 3% w/v protein respectively. At similar protein contents, increasing the orthophosphate content from 16 to 64 mM raised the concentration of iron that could be efficiently redispersed by 5 mM. These observations suggest that the amount of protein played a critical role in the redispersion of the casein-iron precipitate by orthophosphate.

Phosphate ions belong to a family of kosmotrope i.e. a small molecule with high net negative charge (Collins, 1997). Kosmotropes increase the surface tension, decrease the solubility and aggregate proteins (Zhang et al., 2006), thus the redispersion of the casein-iron precipitate was unexpected. The iron-induced precipitation of casein occurs due to screening of the negative charge on caseins as well as the formation of intermolecular adducts (Gaucheron et al., 1996). Probably, an increase in the negative charge of the casein-iron precipitate upon orthophosphate addition was involved. Hence, further studies were planned to investigate the interactions between orthophosphate, casein and iron.

7.2.1.2. Effect of orthophosphate addition on the solubility of protein and iron in sodium caseinate solutions

The effect of orthophosphate addition (32 mM) on the solubility of protein and iron in sodium caseinate solution (2% w/v protein) was examined. Iron at different concentrations (5 to 25 mM) was added to sodium caseinate solution and pH adjusted to 6.8. The proteins in sodium caseinate solution were soluble at pH 6.8 but at > 5 mM iron addition, a small proportion of protein remained soluble. Since, added iron binds to caseins, it was probably associated with the precipitated caseins containing > 5 mM iron. Orthophosphate solution was added to these solutions and pH adjusted to 6.8 (see Figure 7.9).

As compared to the control (no added orthophosphate), the addition of 32 mM orthophosphate redispersed the majority of the precipitate, irrespective of the iron concentration added to sodium caseinate solution (Figure 7.9). A notable decrease in soluble protein and iron was observed only in the samples containing > 15 mM iron. As ferric iron is insoluble at pH 6.8, its presence in the supernatant suggests that it was associated with protein. The possibility of iron interaction with added orthophosphate independent of protein does not exist as ferric phosphate salts are insoluble at pH 6.8 (Fytianos et al., 1998). The results indicate that the redispersion of both the precipitated casein and iron was due to the interaction of added orthophosphate.

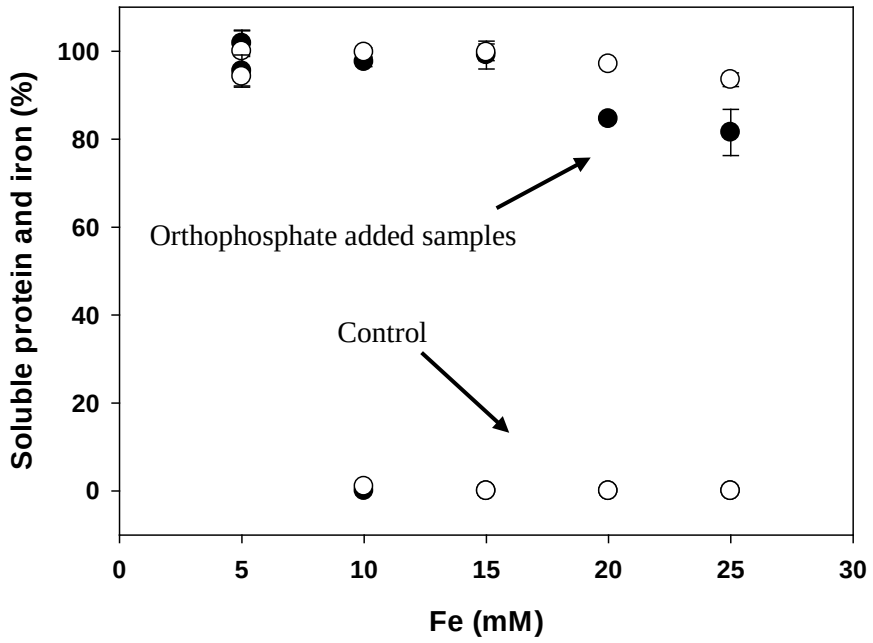


Figure 7-9*: *Proportion of soluble protein (○) and iron (●) in control (no added orthophosphate) and 32 mM orthophosphate-added sodium caseinate solution.

Since redispersion of the precipitate appears to be due to the addition of orthophosphate, its distribution in the supernatant and permeate was monitored (Figure 7.10). The phosphorus in the control sodium caseinate solution (no added orthophosphate) consists mainly of the organic phosphorus associated with caseins and it followed similar trend to the protein. The phosphorus in the supernatant of orthophosphate-added sodium caseinate solution containing 0 to 25 mM iron constitutes both the organic and inorganic phosphorus. Substantial redispersion of the casein-iron precipitate was observed upon orthophosphate addition, hence its concentration in the supernant remained unaffected by the presence of iron.

The proportion of phosphorus in the permeate of orthophosphate-added sodium caseinate solution with no added iron represent the phosphorus in “free” form. Almost 85% of the total phosphorus in this sample was present in the permeate, which was

equivalent to the amount of added orthophosphate, suggesting there was no interaction between caseins and added orthophate. However, in iron added samples the reduction in diffusible (permeate) phosphorus was observed and it correlated with the concentration of added iron. About ~ 40% of the total phosphorus was present in the permeate of sodium caseinate solution containing 25 mM iron. The decreases in the permeate phosphorus concentrations suggest that inorganic phosphorus was interacting with the casein-iron precipitate. This interaction was further investigated using ^{31}P-NMR spectroscopy.

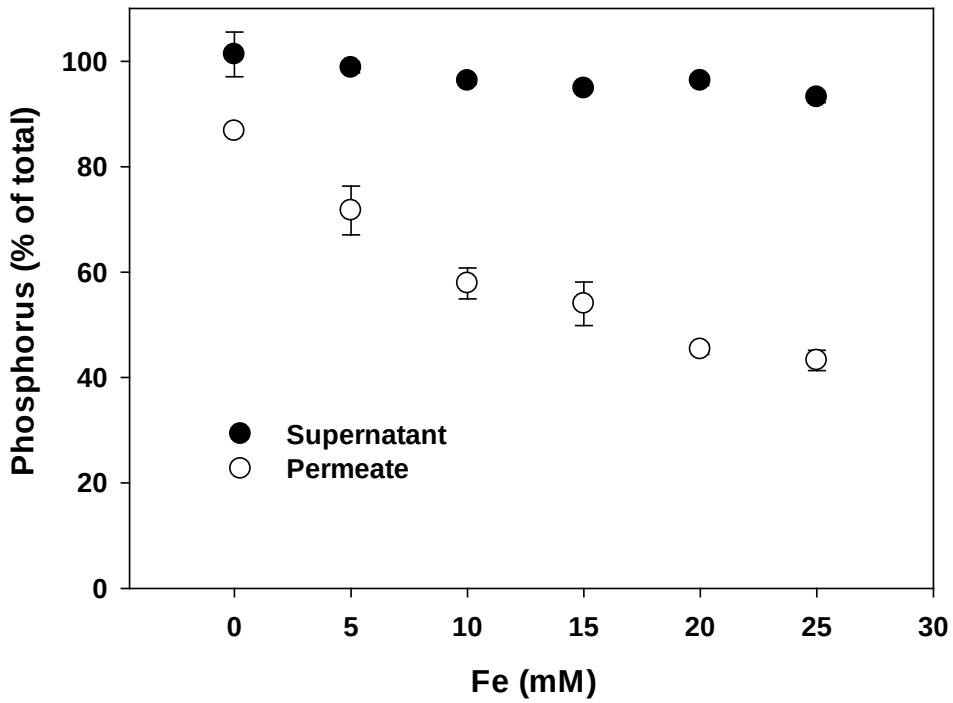


Figure 7-10**:** *Distribution of phosphorus in (•) supernatant and (○) permeate of 32 mM orthophosphate added sodium caseinate solution (2% w/v protein) containing 0 to 25 mM iron.*

7.2.1.3. ^{31}P-NMR spectroscopy

Iron is a paramagnetic atom and hinders the NMR signal (Gaucheron, 2000). Preliminary studies indicated that the ^{31}P-NMR signals for caseins were detectable up to 10 mM iron addition to orthophosphate containing solutions. Hence, the lower values for iron (0 – 10 mM) were selected for this study to observe the change in the ^{31}P-NMR spectra of organic phosphorus. The inorganic phosphorus appeared as a narrow peak close to the organic phosphorus peak and the two peaks overlaped at higher concentrations. Hence, 16 mM orthophosphate addition was chosen for this study which was sufficient to efficiently redisperse the casein-iron precipitate. The sodium caseinate solutions used for ^{31}P-NMR study contained 2% w/v protein, varying concentrations of iron (0, 2, 4, 6, 8, 10 mM iron) and 16 mM orthophosphate.

The ^{31}P-NMR spectra of sodium caseinate solutions showed distinct peaks for different clusters of organic phosphorus present on different caseins. The effect of iron addition on the broadening of the ^{31}P-NMR peaks for organic phosphorus has been discussed in Chapter 6. Figure 7.11 shows the effect iron had on the ^{31}P-NMR signals of organic phosphorus as a function of its concentration. A reduction in the area under organic phosphorus ^{31}P-NMR resonance peak was observed and these peaks were absent in sodium caseinate solution at > 4 mM iron. Substantial precipitation was observed at > 6 mM iron addition to sodium caseinate solutions.

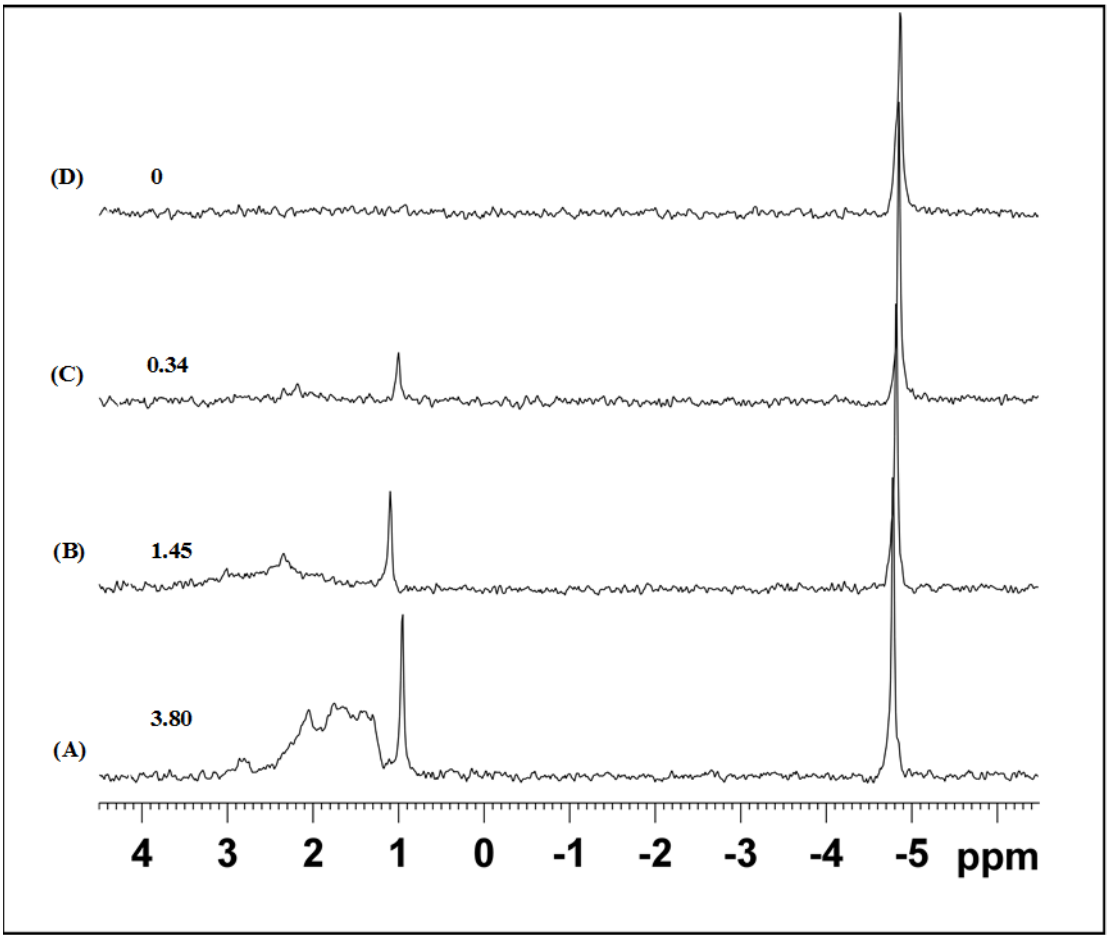


Figure 7-11**:** *^{31}P-NMR spectra of sodium caseinate solution (2% w/v protein) containing (A) 0, (B) 2, (C) 4 and (D) 6 mM iron after 24 h storage at room temperature. Numerals on spectra represent area for organic phosphorus peaks.*

The effect of orthophosphate addition on the ^{31}P-NMR signals of organic phosphorus in iron containing sodium caseinate solutions is shown in Figure 7.12. The orthophosphate was added to the iron containing sodium caseinate solutions and ^{31}P-NMR spectra recorded after 24 h storage at room temperature. A peak appeared at 1 ppm upon orthophosphate addition in all the samples and represented the inorganic phosphorus that existed in the free form.

The area under the ^{31}P-NMR organic phosphorus peaks was greater in orthophosphate added samples than the control (no added orthophosphate). Interestingly, the ^{31}P-NMR organic peaks were also observed in the samples containing > 4 mM iron. The results show that addition of orthophosphate to iron-added sodium caseinate solutions increased the concentration of organic phosphorus in the free environment. However, the area under organic phosphorus peaks decreased at higher concentrations of iron. The decrease in the area of organic phosphorus indicated the interaction of organic phosphorus with iron. The changes in the integrals of the organic phosphorus were accompanied by a reduction in the inorganic phosphorus contents, which was reduced by ~ 10 mM at equimolar concentrations of iron (Table 7.1). The greatest reduction in the free ionic phosphorus (4.84 mM) was observed in sodium caseinate solution containing 2 mM iron. The extent of free ionic phosphorus reduction decreased as iron concentration in the sodium caseinate solution increased.

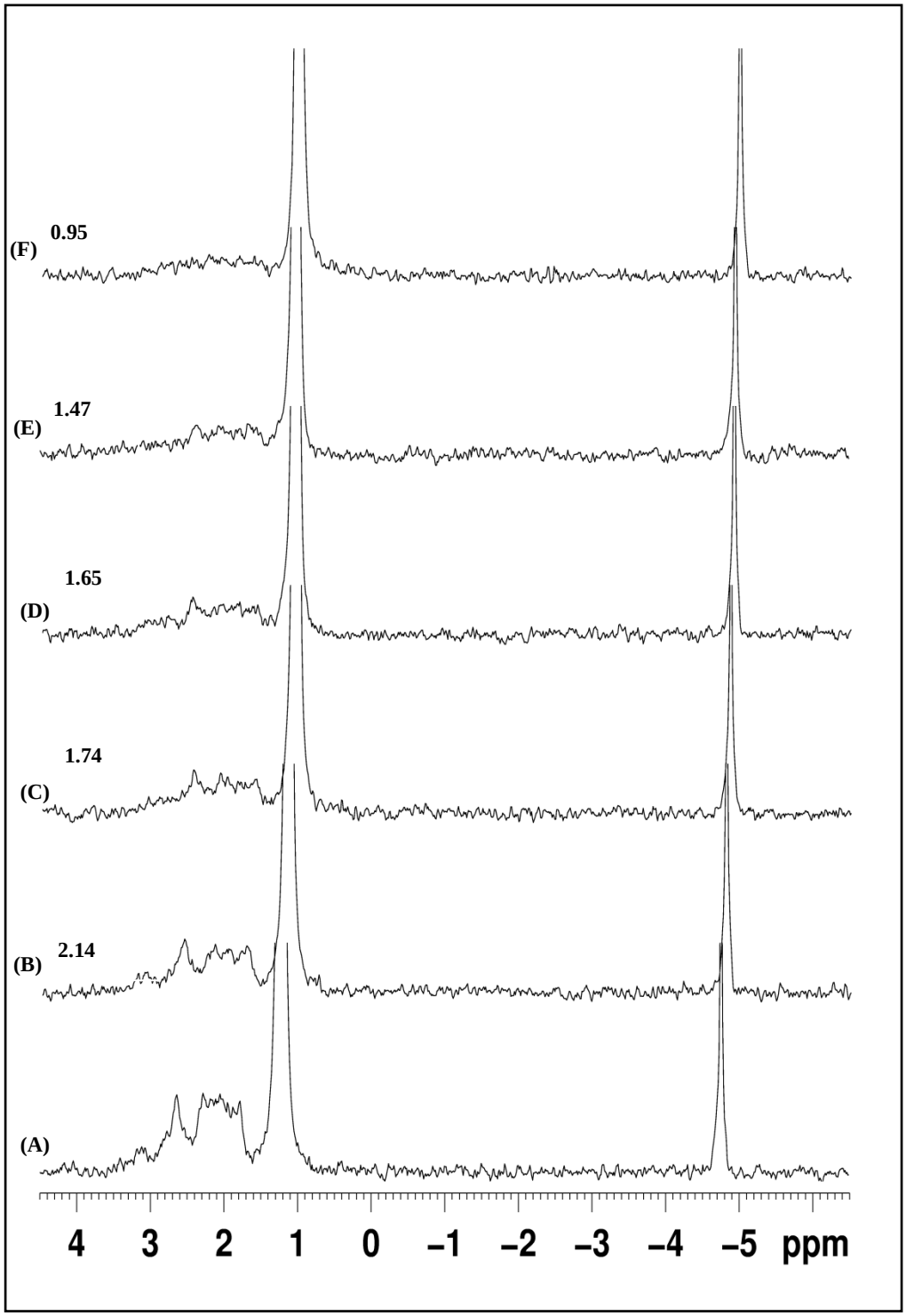


Figure 7-12*: *Effect of 16 mM orthophosphate on ^{31}P-NMR resonance spectra of sodium caseinate solutions (2% w/v protein) containing different iron concentrations i.e. (A) 0, (B) 2, (C) 4, (D) 6, (E) 8 and (F) 10 mM. Numerals on spectra represent area for organic phosphorus peaks.

Table 7-1**:** *Proportion of inorganic phosphorus (as estimated by ^{31}P-NMR spectroscopy) in 16 mM orthophosphate-added sodium caseinate solutions containing iron after 24 h storage at room temperature*

	Added iron (mM)					
	0	**2**	**4**	**6**	**8**	**10**
Proportion of inorganic phosphorus (%)	100	74.5	68.2	60	51.5	46.3

The results show that the relatively high area observed for the organic phosphorus peaks of sodium caseinate solutions containing iron was due to the addition of orthophosphate. However, the areas under the organic peaks were lower than that of sodium caseinate solutions with no added iron. This indicates that organic phosphorus on the caseins was interacting with iron in orthophosphate containing samples. However, the effect of iron on organic phosphorus was reduced upon orthophosphate addition. The increase in the area under organic phosphorus ^{31}P-NMR signals suggests that higher amounts of free phosphoserine residues were generated upon orthophosphate addition. Thus it appears that iron was interacting both with the organic and inorganic phosphorus. These results confirm two important aspects: firstly, the added orthophosphate was interacting in the region near where iron was bound to caseins and secondly, the iron was bound to caseins even in the presence of orthophosphate.

The precipitation of proteins occurs upon iron addition due to a reduction in the negative charge on caseins. The interaction between added orthophosphate and casein-iron precipitate was confirmed by ^{31}P-NMR investigation. The redispersion of the precipitate upon orthophosphate addition points to a possible increase in the surface

charge of the casein-iron precipitates. Phosphate ion is negatively charged and its interaction probably contributed to the increase in negative charge on the protein-iron complex (Stumm, 1995). These observations indicate that orthophosphate might be adsorbed onto the casein-iron complex. If this is the case, the extent of phosphate ion adsorption would be affected by its concentration. Hence, further experiments were conducted to compare the extent of permeate phosphorus reduction in sodium caseinate solutions with constant protein (2% w/v) and iron (15 mM) concentrations (Figure 7.13). Orthophosphate at three different concentrations i.e. 16, 32 and 64 mM was added to the casein-iron precipitate and the permeate analysed after 24 h storage at room temperature. The results showed that the phosphorus content of the permeate for samples with added 16, 32 and 64 mM orthophosphate were reduced by 49%, 28.5% and 26%, respectively.

Substantial redispersion of the casein-iron precipitate was observed upon 16 mM orthophosphate addition (Figure 7.14) where greater than 85% of the total protein and iron were present in the soluble phase. A slight further increase in the soluble protein and iron content was observed at 32 mM with no further change at 64 mM orthophosphate addition. The results show that the amounts of orthophosphate adsorbed onto the casein-iron complex was greater than actually required to redisperse the precipitate.

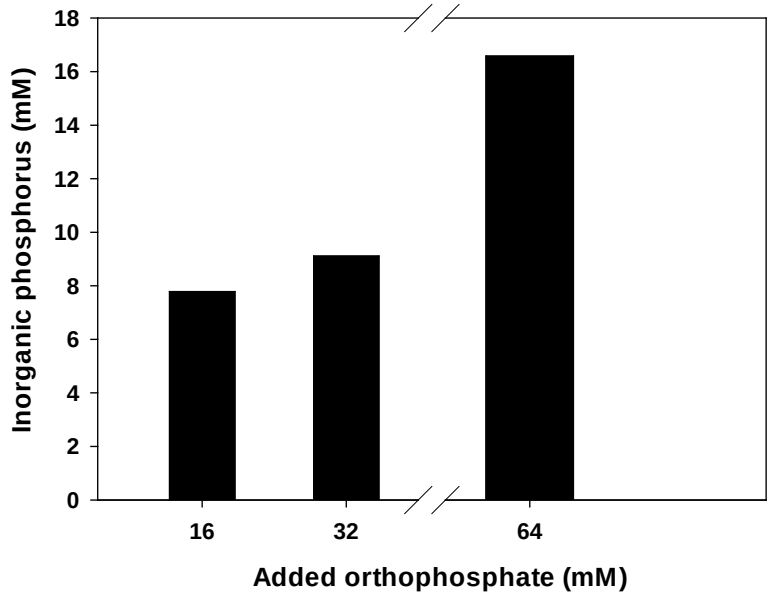


Figure 7-13**:** *Effect of added orthophosphate (16, 32 and 64 mM) on the the permeate phosphorus content of sodium caseinate solution (2% w/v protein) containing 15 mM iron.*

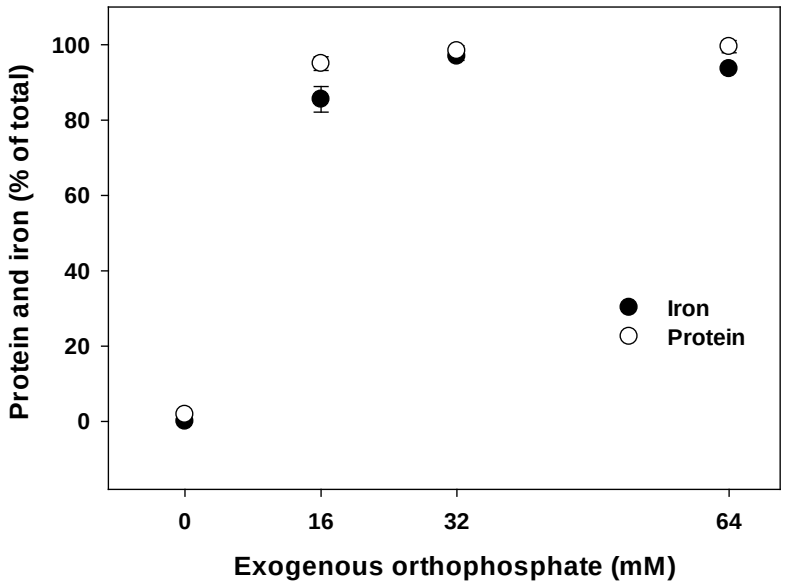


Figure 7-14**:** *Effect of orthophosphate (0, 16, 32 and 64 mM) addition on proportion of soluble protein and iron in sodium caseinate solution containing 15 mM iron.*

Orthophosphate added to sodium caseinate solution does not interact with the caseins in sodium caseinate solution and the presence of iron is critical for its interaction with casein-iron complex. Hence, it becomes essential to understand the interactions of iron and orthophosphate. Further experiments were conducted to observe the interaction of orthophosphate with iron added to aqueous solution maintained at pH 6.8 in the absence of caseins.

7.2.2. Interactions of orthophosphate with iron in the absence of casein

Ferric chloride hexahydrate is soluble in water and forms clear, orange coloured solution at pH ~ 1.8. However, when the pH of the solution is raised to 6.8, ferric iron hydrolyses rapidly to form insoluble ferric hydroxide (Crabb et al., 2010a; Schwertmann et al., 2008). The insoluble ferric hydroxide sedimented after 24 h storage at room temperature (Figure 7.15A). The addition of orthophosphate to ferric hydroxide in aqueous solution at pH 6.8 did not alter its colour or solubility (Figure 7.15B and C).

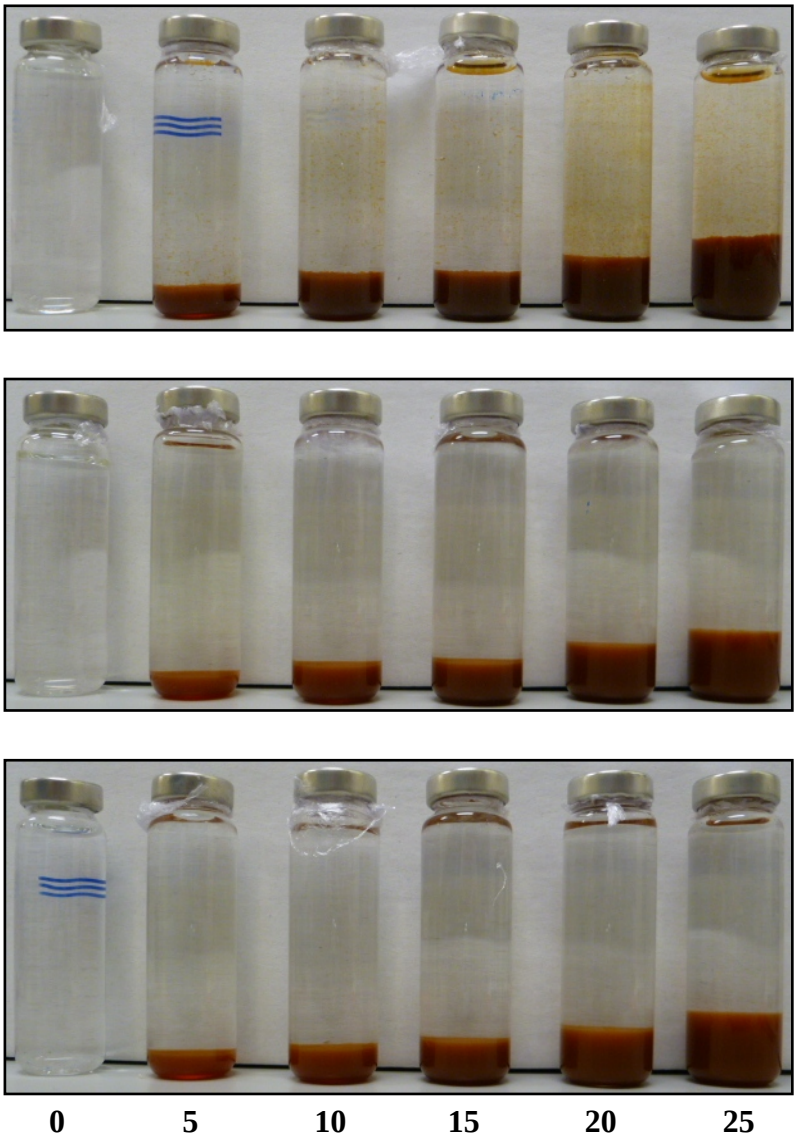


Figure 7-15**:** *The effect of orthophosphate (A) 0, (B) 32 and (C) 64 mM addition on the solubility of iron (0, 5, 10, 15, 20 and 25 mM) after 24 h storage at room temperature and pH 6.8.*

The interaction between orthophosphate and ferric hydroxide was assessed by adding varying concentrations of orthophosphate (0 to 64 mM) to aqueous solution containing 15 mM ferric chloride at pH 6.8 (Figure 7.16). The aqueous solutions maintained at pH 6.8 were stirred for 24 h at room temperature and the transfer of inorganic phosphorus to the ferric hydroxide/oxide complex quantified by the procedure described by Lijklema (1980) [see section 3.2.2.3]. Considerable adsorption of inorganic phosphorus

corresponding to the amount of orthophosphate added was observed onto the ferric hydroxide. The amount of phosphorus adsorbed increased from 4.5 mM to 9.5 mM upon raising the orthophosphate concentration from 8 to 64 mM. The increase in the amount of adsorbed phosphorus seemed to stabilize at 8 mM at > 32 mM orthophosphate addition. Similar trends in orthophosphate adsorption on freshly prepared ferric hydroxide solutions maintained at pH 7 have been reported by Lijklema (1980). This confirmed that the inorganic phosphorus would adsorb onto the iron in ferric hydroxide/oxide complex prepared under the conditions of this experiment. The adsorption of phosphorus is a well-established phenomenon and has been extensively studied in soil science (Adegoke et al., 2013; Barber, 2002; Crosby et al., 1984; Crosby et al., 1981).

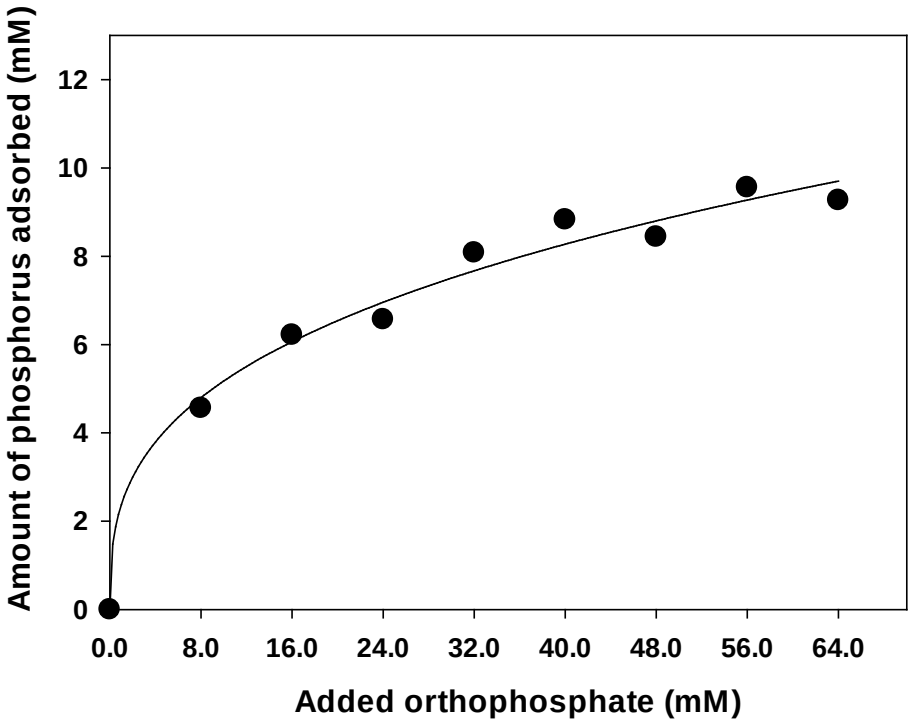
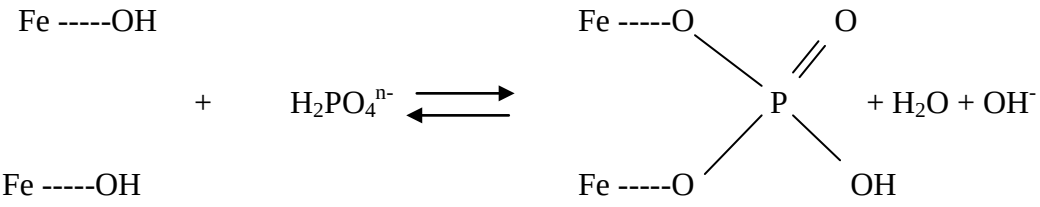


Figure 7-16**:** *The amount of inorganic phosphorus adsorbed onto ferric hydroxide (insoluble) at pH 6.8 after 24 h storage at room temperature.*

Inorganic phosphorus exists as phosphate ion in aqueous solution. Ferric iron is a lewis acid and has a strong affinity for strong base i.e. phosphate. The adsorption of phosphate onto ferric hydroxide is due to the high affinity between these cations (McBride, 1994) and is high in freshly prepared ferric hydroxide precipitate at pH 7.0 (Crosby et al., 1981). The adsorbed phosphate binds covalently to the ferric hydroxide (Stumm, 1995; Tan, 2011) leading to an increase in negative charge on the adsorbed surface. The most commonly proposed mechanism of covalent phosphate adsorption onto the ferric hydroxide is *via* ligand exchange. Ligand exchange entails a displacement and exchange of water molecule from the surface of the hydroxide metal complex by the adsorbing phosphate ion (Adegoke et al., 2013). Furthermore, the adsorption of phosphorus onto these complexes is enhanced by increasing ionic strength and temperature (Arai et al., 2001). Increasing ionic strength of the solution reduces the thickness of the electrical double layer around particle surface, thus making the surface more accessible to adsorption by surrounding ions. A simple equilibrium equation shows the mechanism of phosphorus adsorption:



The complexes formed by adsorption of phosphorus onto ferric hydroxide/oxide complexes are very stable and resist desorption (Barber, 2002; Parfitt et al., 1977). Although an increase in the negative charge is envisaged upon adsorption of orthophosphate onto the ferric hydroxide, it does not improve the solubility of ferric hydroxide-orthophosphate complex (see Figure 7.15).

7.2.3. Possible mechanisms

The precipitation of caseins at pH 6.8 occurs due to the neutralisation of charge on caseins by the binding of positively charged ferric iron (Gaucheron et al., 1996). Thus, the precipitated casein-iron complex had positively charged iron bound to its surface at pH 6.8. Iron has a very high affinity for phosphate ions and the adsorption of orthophosphate onto the casein-iron precipitates was clearly observed in this study. The adsorption of phosphate has been shown to affect the electrostatic properties of the surface and modify the flocculation and adhesion behaviour of the adsorbed surface (Stumm et al., 1992).

The iron-casein precipitate in these experiments was the surface onto which the adsorption of orthophosphate occurred. The adsorption of orthophosphate onto the casein-iron precipitate was comparable to its adsorption onto ferric hydroxide in this experiment, but there are some major differences, the most prominent being the presence of caseins. Figure 7.17 shows the structure of ferric hydroxide, which consists of four covalently-bonded units wherein oxygen atoms act as electron donors to the ferric ion. Notably, Fe-O bonds are formed in ferric hydroxide.

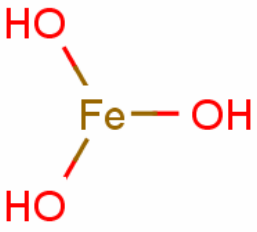


Figure 7-17*: *Molecular structure of ferric hydroxide.

The iron added to sodium caseinate solution binds primarily and preferentially to the phosphoserine residues on caseins. The molecular structure depicting the binding of iron to phosphoserine residues on caseins is shown in Figure 7.18.

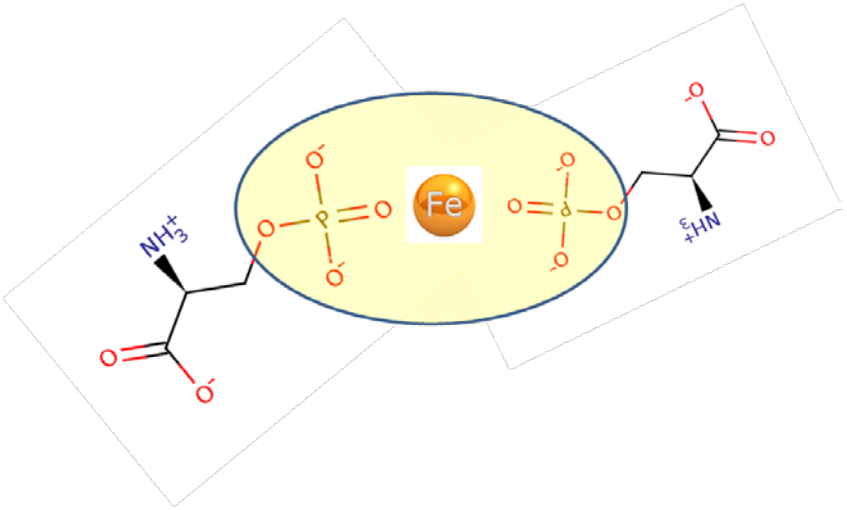


Figure 7-18**:** *Schematic representation of iron binding to phosphoserine on caseins as suggested by Hegenauer et al (1979c).*

Iron binds in octahedral coordination to at least two of these phosphoserine residues on caseins (Hegenauer et al., 1979c; Raouche et al., 2009b). As shown in Figure 7.18, the binding of iron to the oxygen of phosphoserine residues on caseins involves Fe-O bonds that are somewhat similar to those in ferric hydroxide. Thus, the primary Fe-O bonds required for orthophosphate adsorption exist on caseins-iron precipitate. Moreover, the coordination requirement of iron entails the presence of at least one water molecule in its coordination sphere during its binding to casein (Crabb et al., 2010b). The phenomenon of adsorption involving the displacement of a water molecule from the coordination sphere of iron by the adsorbed orthophosphate is well established (Adegoke et al., 2013; Crosby et al., 1984; Crosby et al., 1981). The ^{31}P-NMR investigation revealed that orthophosphate was indeed adsorbing on the region close to iron and organic phosphorus. Furthermore, the increase in the area of organic peaks

upon orthophosphate adsorption indicates the possibility that some organic phosphorus bound to iron was displaced by the adsorbed orthophosphate. Thus, the adsorption of orthophosphate onto the casein-iron precipitate could involve both the displacement of water molecule and/or displacement of organic phosphorus interacting with iron. The displacement of organic ligands bound to iron complexes by the adsorbate in soil has been demonstrated by Kudeyarova (2010). This adsorption of orthophosphate presumably increases the negative charge on the adsorbed surface by forming covalent bonds (Barber, 2002). The increase in the negative charge by the adsorbed orthophosphate was possibly enough to overcome the hydrophobic attraction forces leading to redispersion of the casein-iron complexes in the precipitate. The interaction of iron and orthophosphate with the phosphoserine cluster on caseins and the consequent effect on aggregation of proteins are shown in Figure 7.19.

The caseins in sodium caseinate contain clusters of phosphoserine residues with uncompensated negative charges. The binding of iron to these negatively charged phosphoserine residues above the equimolar concentration results in aggregation of caseins, probably due to charge neutralisation and iron bridging. The bonds between the casein and iron provide adsorption sites for added orthophosphate. The adsorption of orthophosphate probably displaces some of the organic phosphorus bound to iron and increases the negative charge on the casein-iron precipitates. The re-dispersion of the precipitated casein-iron complex is due to the increased repulsion owing to the increased negative charge.

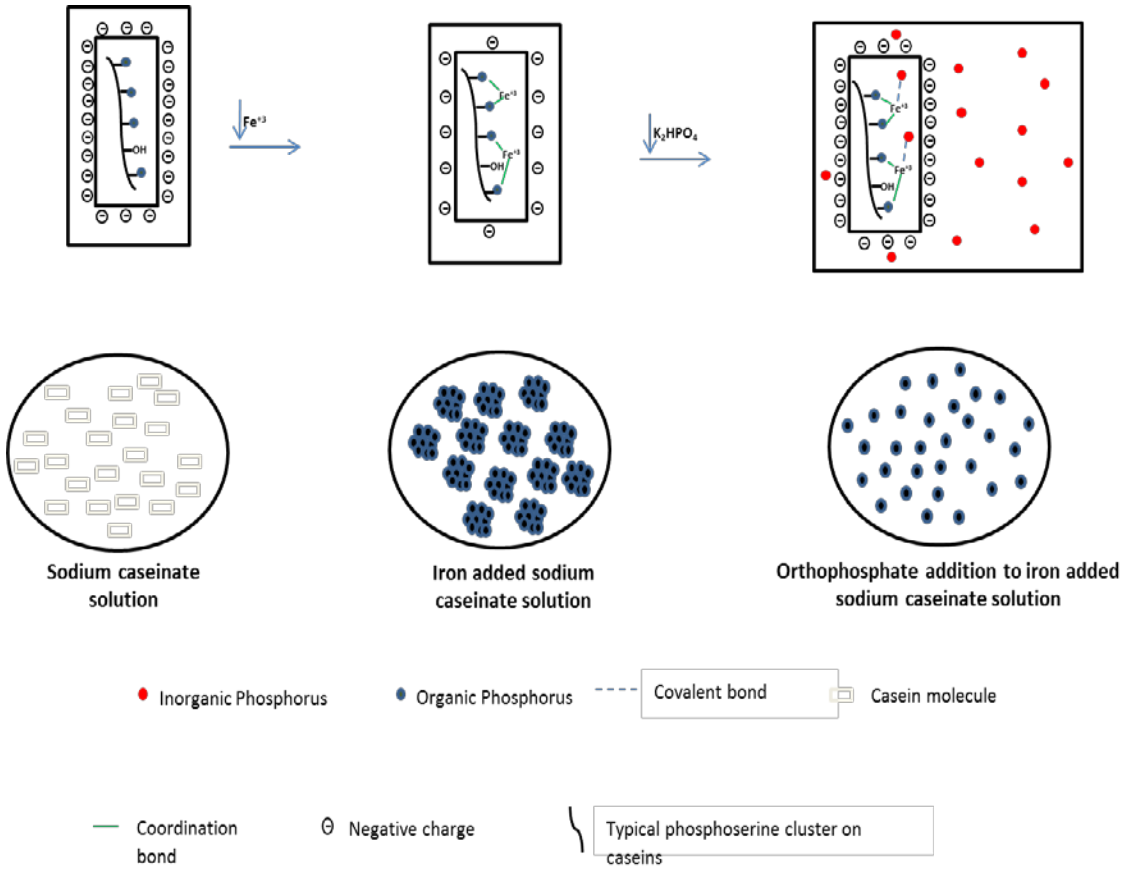


Figure 7-19*: *Schematic representing the changes in sodium caseinate solutions upon iron and orthophosphate addition at pH 6.8.

7.3. Conclusions

The casein-iron precipitates formed at > 5 mM iron addition to sodium caseinate solutions could be redispersed by the addition of orthophosphate. The added orthophosphate adsorbs onto the casein-iron precipitate. This adsorption displaces a part of the organic phosphorus (contributed by caseins) bound to iron and increased the surface negative charge on the casein-iron precipitate, which probably leads to the redispersion of caseins and iron. The adsorption of orthophosphate occurs on ferric hydroxide formed in the absence of casein, but does not solubilise iron, suggesting that the presence of caseins was critical for redispersion of the precipitate. The redispersion

of the precipitates by the addition of orthophosphate was affected by the concentration of iron and casein. The precipitate containing higher protein content could redisperse greater concentrations of iron while requiring lower orthophosphate content. Four moles of iron could be solubilised by a mole of caseins in these experiments. The adsorption of orthophosphate onto protein-iron complexes and its consequent solubilisation is a novel finding with a potential to create a new iron fortificant.

Chapter 8. Applications of research, overall conclusions and recommendations for further research

8.1. Applications of research

Milk proteins have proven to be good carriers for iron because of their ability to prevent its interaction with other food constituents, thereby increasing the shelf-life of iron-fortified products. Moreover, iron bound to caseins and whey proteins have good bioavailability when added to milk and cheese (Zhang et al., 1989a). Unfortunately, milk protein-iron complexes are not commonly recommended as a source of iron and there is no mention of such ingredients in World Health Organisation or Food Agriculture Organisation documents for iron fortificants (Allen et al., 2006; Nestel et al., 2002). Few commercial ingredients utilising iron-milk protein complexation are available for food fortification, because they have relatively low iron content. Table 8.1 lists the protein-iron complexes and their iron contents, cited in published literature and patents. Patents that propose modification of these basic complexes to lower the iron-associated astringency, improve their solubility and heat stability are also available.

Among these protein-iron complexes (Table 8.1), ferric iron bound to caseins is the most preferred combination, probably because it binds more strongly to caseins as compared to the ferrous form (Raouche et al., 2009a). Complexes containing > 1.25% of iron in the final products are generally insoluble and are prepared by iso-electric precipitation at pH 4.6, followed by subsequent lyophilisation of the protein-iron

complex. Hence, applications of these complexes, although containing higher iron content, are limited in liquid food products. Attempts to improve the solubility of these ingredients by enzymatic hydrolysis have been partly successful (Sakurai et al., 2000a).

Table 8-1**:** *The solubility characteristics of various milk protein-iron complexes.*

Ingredient/complex	**Iron (%)**	**Solubility**	**Author/inventor**
Ferri-succinylcasein	5.2	Insoluble	(Klotz et al., 2008)
Iron-casein complex	0.28 – 1.25	Soluble	(Sakurai et al., 2000b)
Iron-casein complex	1 (by wt. of casein)	Soluble	(Sher et al., 2006)
Ferric polyphosphate-whey protein	8 – 12	Insoluble	(Jones et al., 1975)
Casein-iron complex	2.3	Insoluble	(Zhang et al., 1989b)
Ferricplyphosphate-whey protein	4.2	Insoluble	(Zhang et al., 1989a)
Iron-whey protein	9.9	Insoluble	(Zhang et al., 1989b)
Iron-β-casein phosphopeptide	7 (of protein)	Soluble	(Bouhallab et al., 2002)
Iron-casein hydrolysate	5.6	Insoluble	(Chaud et al., 2002)

The soluble protein-iron complexes generally contain ~ 1% iron by weight of caseins which is insufficient to make these ingredients commercially attractive. The precipitation of caseins at higher levels of iron addition has restricted the amount of iron that can bind to caseins in a soluble format. Hence, increasing the iron content and

solubility are probably the most critical improvements required for mainstream applications of protein-iron complexes for iron fortification.

The soluble protein-iron complexes created in this work contained up to ~ 5.5% iron by weight of caseins thus substantially improving the iron loading onto caseins in a soluble form. These protein-iron complexes could be spray dried into powders with potential applications in a wide range of liquid food products. The protein-iron complexes are heat stable in aqueous solution for 30 min at 90^{o}C. The solubility and high heat stability ensure applications of these complexes in liquid products, especially heat treated (pasteurised, UHT etc.) products.

The process developed in this thesis of forming soluble protein-iron complexes and the spray dried ingredient thereof is protected by international patent namely "Micronutrient fortification process and its usage" issued by New Zealand Patent office on 20th June 2014. The detailed specification and the process employed in the manufacture of these complexes are available for viewing at:

http://www.iponz.govt.nz/app/Extra/IP/Mutual/Browse.aspx?sid=635425050244332707

The soluble iron fortificant has been named Ferri Pro^{TM} and represents the binding of ferric iron to milk proteins. Two variants of Ferri Pro^{TM} have been developed i.e. one prepared from skim milk and the other from sodium caseinate (Table 8.2). The total iron content in the final powdered ingredient differ between these variants but the amount of iron bound to caseins is almost similar (~ 4%).

The higher concentrations of iron in sodium caseinate-based Ferri Pro^{TM} is due to higher proportions of protein in the sodium caseinate base. These ingredients have good dispersibility and produce translucent aqueous solutions at 33% of Recommended

Dietary Allowances (RDA) concentrations for iron. In general 240 and 120 mg of powdered Ferri Pro™ (milk) and Ferri Pro™ (sodium caseinate) are required to fortify a serving of various standardised food products with 25% of the RDA of iron (Table 8.3).

Table 8-2**:** *Typical analysis of parameters for Ferri Pro™ powders*

Parameters	**Ferri Pro™ (Milk)**	**Ferri Pro™ (Sodium caseinate)**
Colour	Creamish	Yellowish cream
pH (1% solution)	~ 6.8 ± 0.1	~ 6.8 ± 0.1
Protein (g/100 g)	~ 29.3	~ 66.35
Sodium (g/100 g)	~ 1.23	~ 1.93
Iron (g/100 g)	~ 1.02	~ 2.40
Phosphorus (g/100 g)	~ 0.96	~ 3.9

Table 8-3: *Quantity of Ferri Pro™ required per serving to fortify standardised food products with iron*

Food	**Reference quantity**	**Maximum claim per reference quantity (% RDA)**	**Quantity of Ferri Pro™ powder to be added**	
			Ferri Pro (Milk, 1.25% Fe)	**Ferri Pro (Sodium Caseinate, 2.5% Fe)**
Biscuits containing not more than 200g/kg fat and 50g/kg sugar	35 g	3.0 mg (25%)	240 mg	120 mg
Cereal flours	35g	3.0 mg (25%)	240 mg	120 mg
Bread	50 g	3.0 mg (25%)	240 mg	120 mg
Pasta	35g uncooked	3.0 mg (25%)	240 mg	120 mg
Extracts of meat, vegetables or yeast	5g	1.8mg (15%)	144 mg	72 mg
Formulated beverages	600 mL	3.0 mg (25%)	240 mg	120 mg
Formulated meal replacements	One meal servings	4.8 mg (40%)	384 mg	192 mg
Formulated supplementary foods	One serving	6.0 mg (50%)	480 mg	240 mg

Advantages of Ferri Pro™:

1. The solubility of Ferri Pro™ makes it an ideal ingredient for iron fortification of liquid food products. Ferrous bis-glycinate and sodium feredetate are presently recommended as fortificants of liquid products (Martínez-Navarrete et al., 2002). However, these ingredients are expensive and problems with changes in colour and fat oxidation exist (Hurrell, 2002).
2. The high heat stability (stable to 90°C for 30 min) of Ferri Pro™ allows easy incorporation into heat treated liquid products.
3. Ferri Pro™ has a pH ~ 6.8 and could be easily added to milk products with neutral pH without affecting its overall quality.
4. Less than 150 mg of Ferri Pro™ is required to fortify a serving of food product with iron.
5. Ferri Pro™ could be manufactured with varying iron concentrations (0.5 – 2.8% iron in powdered product) suited to a specific final product application.
6. Ferri Pro™ powder has low bulk density and has a good flowability. The low bulk density allows it to be mixed properly (uniformly distributed) along with other bulk ingredients by geometrical mixing in a ribbon or double cone blenders.
7. The principal advantage is the ease of adapting Ferri Pro™ technology to commercial manufacturing. The protein-iron complex concentrate could be prepared in tanks using regular stirrers with variable speeds and pasteurised by batch or continuous methods common to dairy manufacturing plants.
8. Ferri Pro™ reduces the promotion of metal-induced fat oxidation and consequent changes in colour and flavour of iron-fortified food products.

The 15 mM iron added sodium caseinate solution as prepared by the process followed in Ferri Pro^{TM} constitutes ~ 450 servings (based on 2.5 mg per serve). Thus, the liquid Ferri Pro^{TM} solution could be easily mixed with the bulk product during the processing stage. Since the concentration of iron was higher in Ferri Pro^{TM} prepared from sodium caseinate, further sensory studies were conducted using this ingredient.

The strong interaction of iron with constituents of the food matrix is known to cause off-colour and flavour changes in fortified foods, which are accelerated at higher temperatures of processing and storage. The change in colour of food products is mainly due to the oxidation/reduction mechanisms between the food constituents (e.g. polyphenols) and iron (Mellican et al., 2003). A change in colour of food also affects the perceived flavour of the fortified foods (O'Donnell, 1997). The deterioration in the flavour of iron-fortified food products is mainly due to its interaction with fats (Hegenauer et al., 1979a).

Ferri Pro^{TM} was compared to iron-EDTA, ferrous gluconate, ferric ammonium citrate and ferrous sulphate in products such as milk, flavoured milks, yoghurts (plain and berry fruit), ice-cream, tea, chai latte and coffee. These products represent different conditions of pH, heating, presence of reducing/oxidising agents etc. The application trials were undertaken by the food product development specialists at the Riddet Institute.

Milk products were prepared using reconstituted whole milk (12% w/v) with the various iron sources added to a level of 25% of the RDA quantities recommended for respective products per serving. The iron-fortified milks were subjected to processing, such as pasteurisation, UHT, boiling, homogenisation and fermentation, as required for preparation of these products. The sensory analysis was performed with an average of 14 panellists using a 5-point hedonic scale.

The colour and flavour of pasteurised milk and UHT milk were similar and perceived acceptable after 24 refrigerated storage. The colour of pasteurised tea prepared using iron-fortified whole milk had variable levels of reduced lightness and showed an increase in red and yellow hues. Among the various iron fortificants used, tea containing Ferri Pro^{TM} had the least effect on colour, whereas a substantial darkening of sample containing iron-EDTA was observed. Similar observations in colour and flavour were seen for coffee and chai latte where the change in colour was least for samples containing pasteurised and UHT milks fortified with Ferri Pro^{TM}. Samples of coffee and chai latte containing commercial sources of iron chelated to EDTA were least acceptable in the overall acceptability category. Banana flavoured milk and milk containing mixed berry syrup were acceptable only for samples containing Ferri Pro^{TM}. A significant change in colour and flavour was observed for samples containing soluble sources of iron, such as ferrous sulphate, ferrous ammonium citrate, ferrous gluconate and iron-EDTA.

The deteriorative effect of fortifying chocolate milk with ferrous sulphate is well-known hence, only chelated forms of iron were compared for overall change in colour and flavour. The colour of UHT and pasteurised chocolate milk fortified with commercial iron-EDTA was darker than control and Ferri Pro^{TM}, but the flavour was deemed acceptable in all the samples by panellists suggesting chocolate milk could be fortified with chelated iron sources.

Three types of yoghurt i.e. natural, strawberry and blueberry, were fortified with Ferri Pro^{TM} and commercial iron-EDTA. The differences between the control and iron fortified yoghurt were minimal upon storage for 7 days under refrigerated conditions.

Based on these observations Ferri Pro™ was recommended as the iron fortificant of choice in the tested products.

Another major hurdle is in the fortification of cereal food products with iron. Cereal food products contain phytate, which bind strongly to ferric iron forming insoluble complexes and prevent it from being absorbed in the gastro-intestinal tract (Hurrell et al., 1992). Interestingly, the binding of iron to phytate also makes it a strong antioxidant when added to foods (Graf et al., 1987). The stability constant reported for phytate and ferric iron is between ~ 17.0 to 18.5 (Crea et al., 2008) which is similar to those observed for casein and ferric iron i.e. 17.5 (Hegenauer et al., 1979c). The solubility of Ferri Pro™ and the high affinity of casein-iron complex suggest that Ferri Pro™ could be used to fortify cereal products and yet maintain good bioavailability.

The trials for assessing the bioavailability of iron from Ferri Pro™ vis-a-vis ferrous sulphate in young women have been undertaken in collaboration with nutrition experts from Singapore (Professor Walczyk Thomas, Department of Chemistry, Faculty of Sciences, NUS) using a radio isotope method and the results are being analysed and will be published in due course.

8.2. Overall discussion and Conclusions

Iron added to milk binds strongly to proteins, especially caseins (Raouche et al., 2009a). Higher concentrations (~ 20 mM) of iron could be added to milk as compared to sodium caseinate or isolated caseins at the same casein concentration (< 5 mM, e.g. caseins; pure fractions or co-mixed) without any protein precipitation, an anomaly which has remained unexplored to date (Gaucheron et al., 1996; Raouche et al., 2009b). This thesis explored the reasons for these differences by determining interactions of

iron, phosphate and protein in milk and sodium caseinate solution. The range of iron concentrations (5 to 25 mM) used in this study were higher than in earlier studies. Since ferric iron binds more efficiently to milk proteins than ferrous form, it (ferric form) was chosen as a fortificant in this study.

Experiments on milk systems examined the effect of calcium-depletion and subsequent iron addition on the physico-chemical and microstructural properties of milk. Depleting ~ 70% calcium from milk enabled the binding of at least 20 mM iron to proteins in the soluble phase (100,000 *g* for 1 h at 20^{o}C). This study also highlighted the important role of diffusible inorganic phosphorus in the iron binding process. Hence, the physico-chemical effect of iron addition to sodium caseinate solutions containing orthophosphate was examined. The inclusion of orthophosphate enabled the addition of 20 mM iron to sodium caseinate solution (2% w/v protein) with little protein precipitation and this was at least 5 times higher than achieved in earlier studies.

Remarkably, the addition of orthophosphate to sodium caseinate solutions containing iron, redispersed the insoluble casein-iron precipitates (Chapter 7). The redispersion of precipitate involved the adsorption of orthophosphate onto the casein-iron precipitates. The high concentrations of soluble iron bound to caseins in these experiments could be used as a fortificant, suitable for, but not limited to milk and dairy products. The important results obtained during the entire study are discussed herein.

8.2.1. Influence of calcium-depletion on iron binding properties of milk

Iron added to normal milk binds mostly to the casein micelle, primarily due to the clusters of phosphoserine residues present on major caseins i.e. α_s- and β-casein . Up to 20 mM of ferric iron could be added to normal milk without affecting its colloidal

stability. The surface negative potential and structural integrity of the native casein micelle were retained, even at these relatively high levels of iron addition. As the native stabilisation mechanism of the casein micelle, i.e. steric stabilisation by the κ-casein, was not affected upon iron addition, it suggested that iron bound mainly to the interior of the micelle. Calcium and iron bind to similar sites on caseins in the casein micelle, but upon iron addition no displacement of colloidal calcium from the micelle into the serum phase was observed (Hegenauer et al., 1979c) . This indicates that the binding sites were shared by iron and calcium in the normal milk. The effect of calcium depletion on the physico-chemical properties of normal milk was investigated.

Calcium, at three different levels (~ 20%, ~ 50% and ~ 70%), was depleted from normal milk using a weakly acidic cation-exchange resin. The physico-chemical properties of ~ 20% calcium-depleted milk were similar to normal milk in terms of particle size and mineral distribution. The integrity of the casein micelle was maintained upon ~ 20% calcium depletion from normal milk depletion of > 50% calcium from normal milk resulted in substantial disintegration of the casein micelle and solubilisation of the colloidal constituents. The addition of iron to this calcium-depleted milk resulted in the formation fibrous structures, which seemed to increase with increasing the concentration of iron (Figure 8.1).

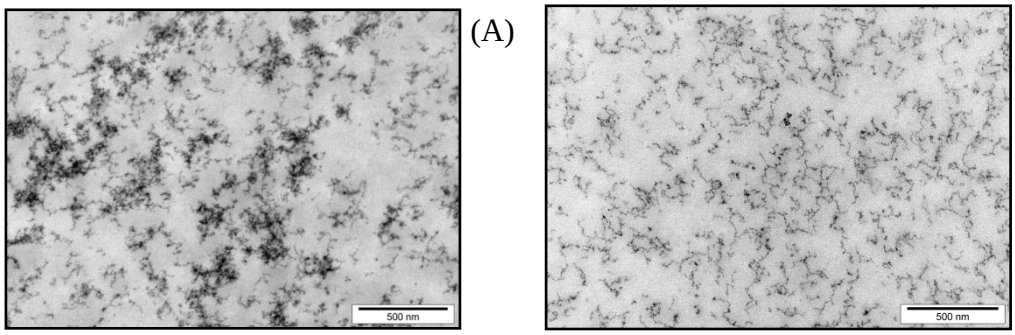


Figure 8-1**:** *Microstructure of 20 mM iron added (A) ~ 50% calcium-depleted milk and (B) ~ 70% calcium-depleted milk*

The distribution of these fibrous structures between the sedimentable and non-sedimentable phase was governed by the concentration of calcium in the respective calcium-depleted milk. In the calcium-depleted milk containing ~ 50% of the original calcium, majority of these fibrous structures were associated with the sediment at > 10 mM iron. However in ~ 70% calcium-depleted milk, these structures were present in the non-sedimentable phase even at > 20 mM added iron (Figure 8.1). Figure 8.2 shows the general aggregation behaviour of proteins upon iron addition to milks with varying levels of calcium depletion.

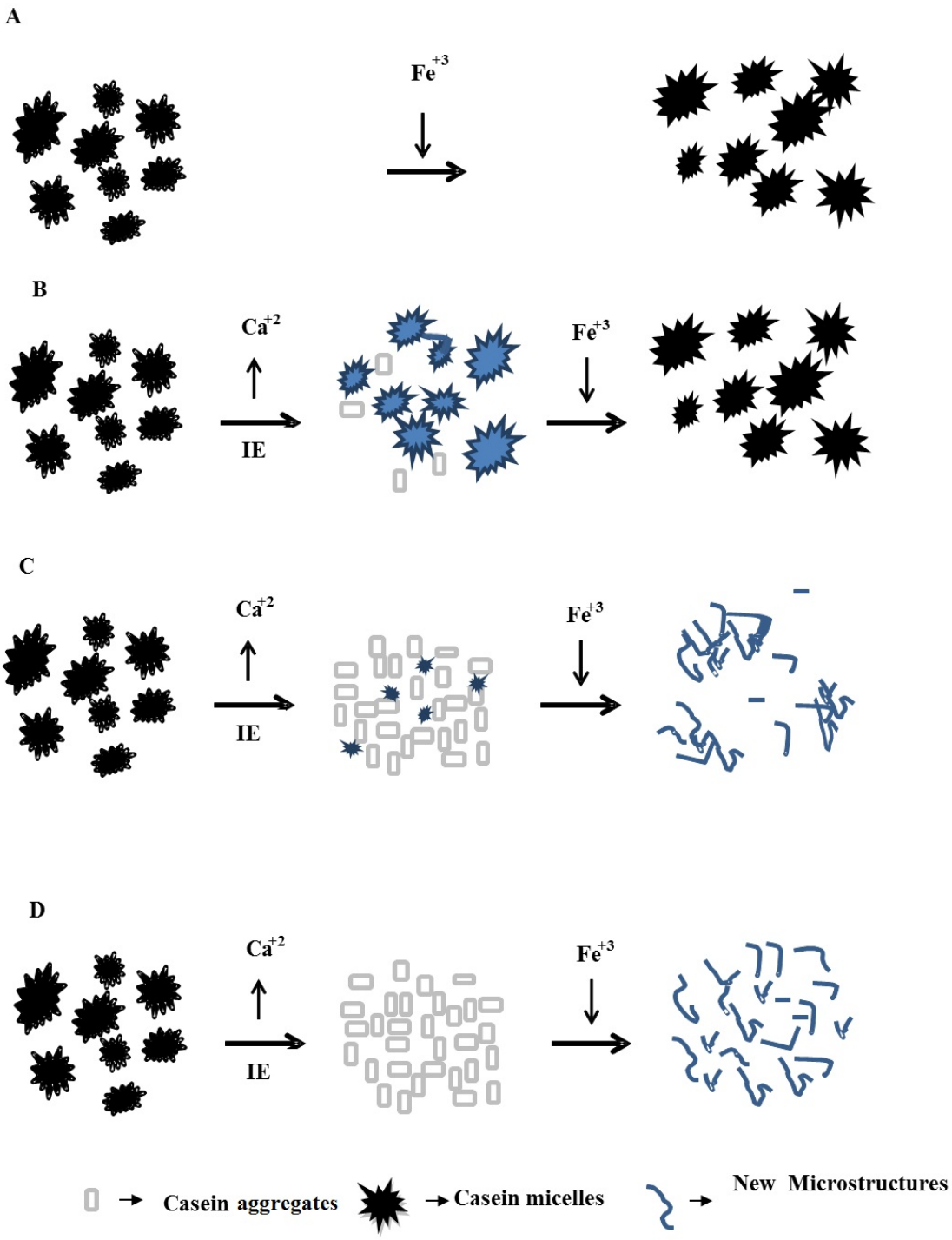


Figure 8-2*: *Diagram representing the effect of 20 mM iron addition on the micro-structure of (A) normal milk, (B) ~ 20%, (C) ~ 50% and (D) ~ 70% calcium-depleted milk.

The addition of positively charged ferric iron is expected to reduce the negative ζ-potential on caseins upon binding. However, relatively minor reductions (~ -1.5 mV) were observed upon iron addition to either milk or calcium-depleted milks. This was probably related to the re-distribution of minerals that occurs upon iron addition to milks, probably the diffusible inorganic phosphorus. A concentration-dependent transfer of aqueous phosphorus to the micellar phase was observed upon iron addition to milk and calcium-depleted milk. In calcium-depleted milk, iron was interacting with the diffusible phosphorus and the iron-phosphate complex formed was possibly stabilised by caseins. The interaction of iron with inorganic phosphate possibly prevented the screening of negative charges on caseins by the added iron.

Gross aggregation of protein-iron complexes in calcium-depleted milk (~ 70% calcium depletion) was observed at 30 mM level of iron (Figure 8.3A). The microstructure of these samples indicated aggregation of the fibrous entities at 30 mM iron addition to CDM. Fortifying the calcium-depleted milk with orthophosphate prevented this aggregation (Figure 8.3B) and promoted the formation of independent fibrous structures. It was probable that the interaction of iron with additional ionic phosphate in orthophosphate added CDM probably reduced the inter-particle iron bridging. In contrast, the additional ionic phosphate promoted aggregation and resulted in the formation of larger aggregates upon 20 mM calcium addition.

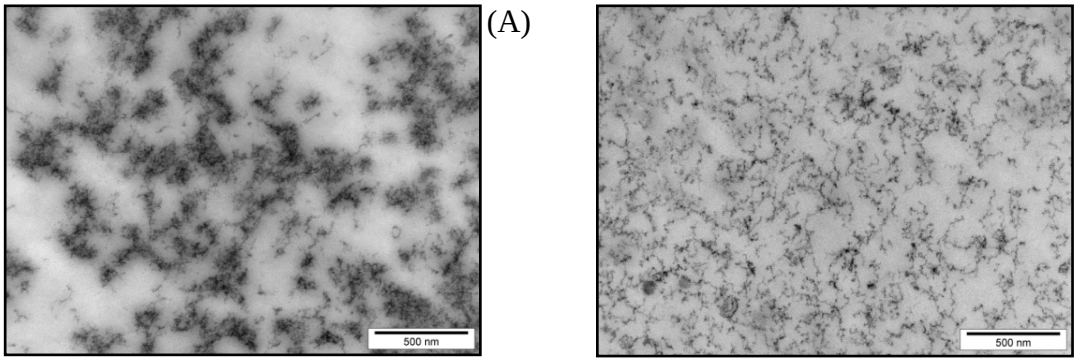


Figure 8-3*: *Microstructure of 30 mM iron added calcium-depleted milk containing (A) 0 and (B) 32 mM added orthophosphate.

Interestingly, the reduction in permeate phosphorus upon iron and calcium addition, to calcium-depleted milk was similar (16.4 ± 0.2 mM), yet they differed significantly in their protein aggregation properties. The contrasting effect was possibly related to the respective binding characteristics of iron and calcium. Iron binds *via* coordination bonds, whereas electrostatic interactions are involved in the binding of caseins to calcium phosphate. The comparison between calcium-and iron-induced aggregation of proteins has been discussed in Chapter 5.

The effect of iron inclusion on the aggregation properties of calcium-restored milk was examined. Interestingly the inclusion of up to 10 mM iron resulted in the formation of smaller particles even in samples containing added orthophosphate. It was highly likely that the presence of iron, bound to phosphoserine on caseins, disrupted the polymerisation pathway required for the formation of the casein micelle, thereby preventing the association of caseins into larger roughly spherical structures. However, the presence of iron on the caseins did not increase the concentration of calcium in the permeate. Since, both these ions binding to similar sites on caseins, it could be concluded that the binding sites were shared by the bound iron and calcium.

The experiments on milk systems highlight the important role of inorganic phosphorus in the binding and aggregation of proteins upon cation (iron and/or calcium) addition. Most importantly, it showed that the high concentrations of both calcium and iron that could be added to milk as opposed to caseinate were due to their interaction with inorganic phosphorus.

8.2.2. The stabilisation of ferric phosphate by caseins

The effect of orthophosphate inclusion in the mixture of iron and caseins was investigated in Chapter 6. Ferric iron forms insoluble ferric hydroxide in water at pH 6.8. In the presence of caseins, iron was solubilised up to 5 mM level. Increasing the iron concentration precipitated both casein and iron. However, the inclusion of orthophosphate in sodium caseinate solution prevented casein precipitation up to 25 mM level of iron addition. Ferric iron added to orthophosphate solution in the absence of caseins forms insoluble ferric phosphate at pH 6.8. Hence, the high concentrations of soluble iron in orthophosphate added sodium caseinate solutions was interesting.

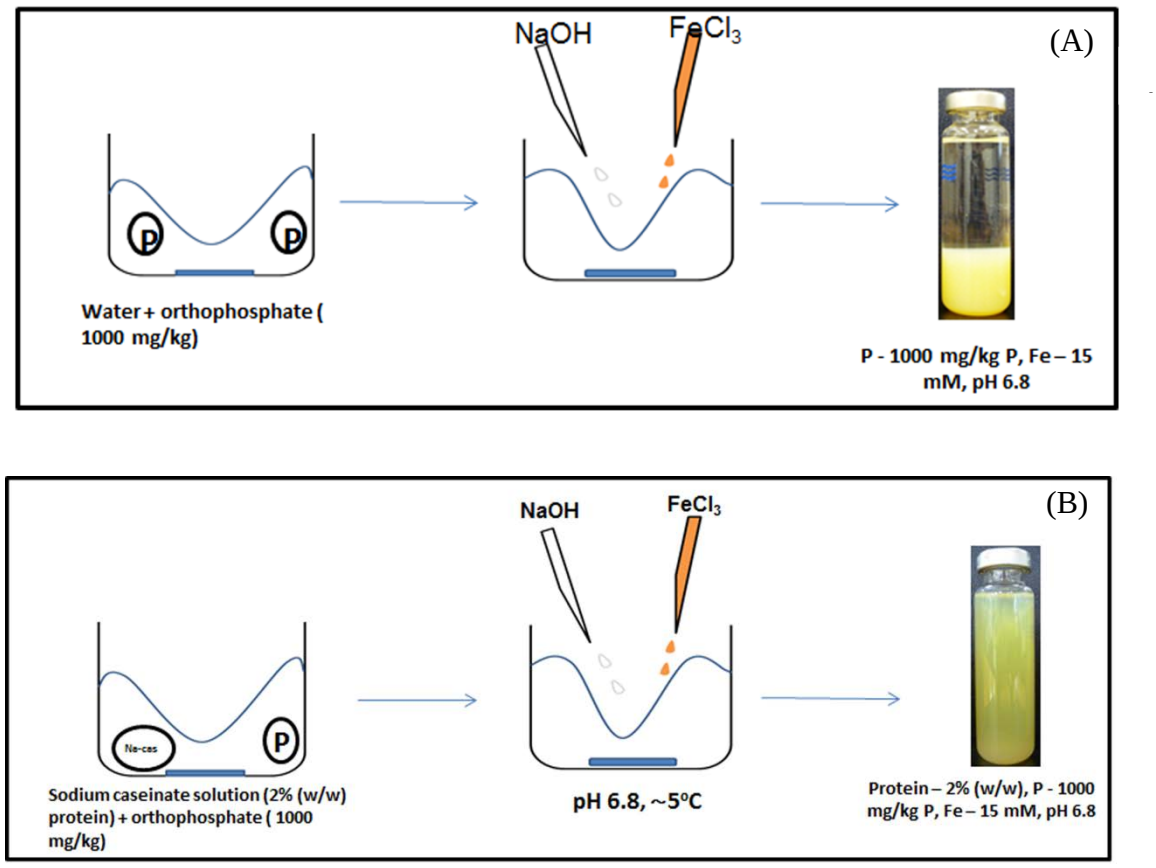


Figure 8-4*: *Effect of iron addition (15 mM) on solubility of solutions containing 32 mM orthophosphate in (A) water and (B) sodium caseinate (2% w/v protein) at pH 6.8.

A representative image depicting the process and effect of caseins on the dispersion stability of the complexes formed is shown in Figure 8.4. The solubility of the iron added to sodium caseinate solutions was governed by concentrations of protein (1 to 3% w/v) and orthophosphate (0 to 64 mM) present in solution. Upon increasing the orthophosphate concentration, higher amounts of iron could be solubilised by caseins in sodium caseinate and casein precipitation was also prevented. The effect of adding 15 mM iron to sodium caseinate solution in the presence and absence of orthophosphate is shown in Figure 8.5.

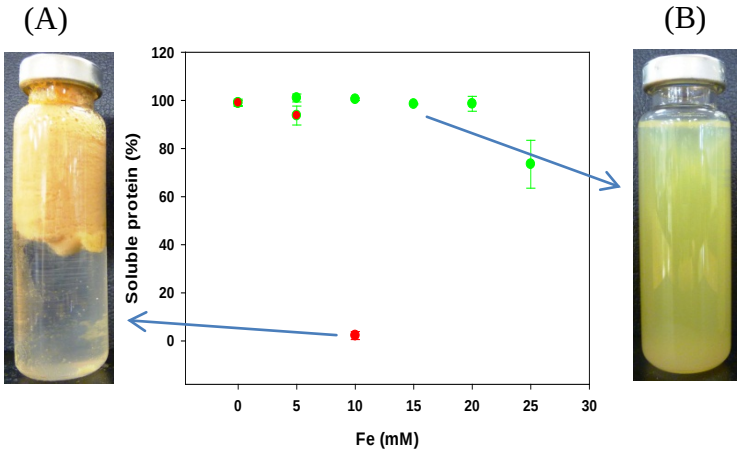


Figure 8-5*: *Effect of 15 mM iron addition to sodium caseinate solutions containing (A) 0 and (B) 32 mM orthophosphate.

Investigation into the role of orthophosphate indicated a concentration-dependent decrease in diffusible phosphorus upon iron addition (Figure 8.6). The reduction in permeate phosphorus correlated with an increase in iron solubilisation by caseins, confirming the important role of orthophosphate in the solubilisation of iron bound to caseins.

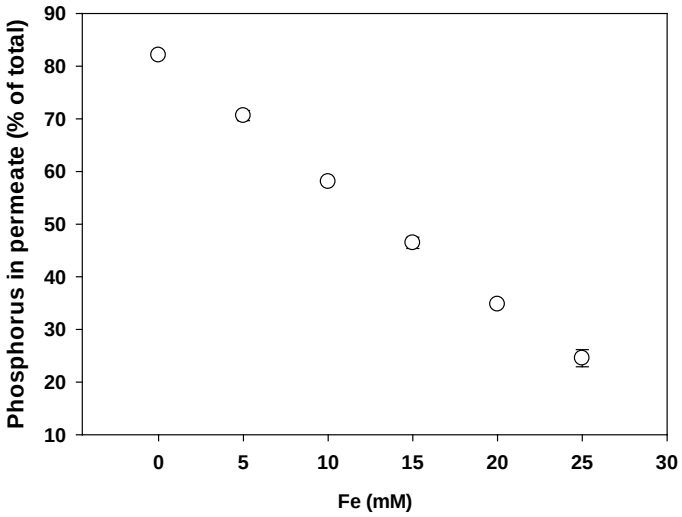


Figure 8-6*: *Effect of iron addition on proportion of phosphorus in permeate of sodium caseinate solution (2% w/v protein) containing 32 mM orthophosphate.

The ^{31}P-NMR investigation of iron added sodium caseinate solution containing orthophosphate revealed that iron was interacting with both the organic and inorganic phosphorus. Since the salts formed upon interaction of iron and orthophosphate are insoluble, it could be concluded that caseins were responsible for maintaining the solubility of the iron through the formation of a casein-iron-orthophosphate complex. It was probable that phosphoserine acted as the nucleation site for the growth of ferric phosphate salt. Size exclusion chromatography of the sodium caseinate solutions was performed to investigate the changes in size of the aggregates upon iron addition. The inclusion of orthophosphate resulted in the formation of smaller aggregates, which contributed to the solubility of iron-added sodium caseinate solutions.

Increasing the orthophosphate concentration also reduced the proportion of monomeric caseins involved in the aggregates formed upon iron addition. The composition of the aggregates formed upon iron addition to sodium caseinate solutions containing orthophosphate revealed involvement of all caseins fractions (i.e. α_{s1}-, α_{s2}-, β- and

κ-casein). Thus, the presence of orthophosphate reduced the screening of negative charge on caseins by the added iron and resulted in the formation of smaller aggregates which promoted solubility (Figure 8.7). It was hypothesised that a salt of ferric phosphate was possibly stabilised by caseins in iron-added sodium caseinate solutions containing orthophosphate.

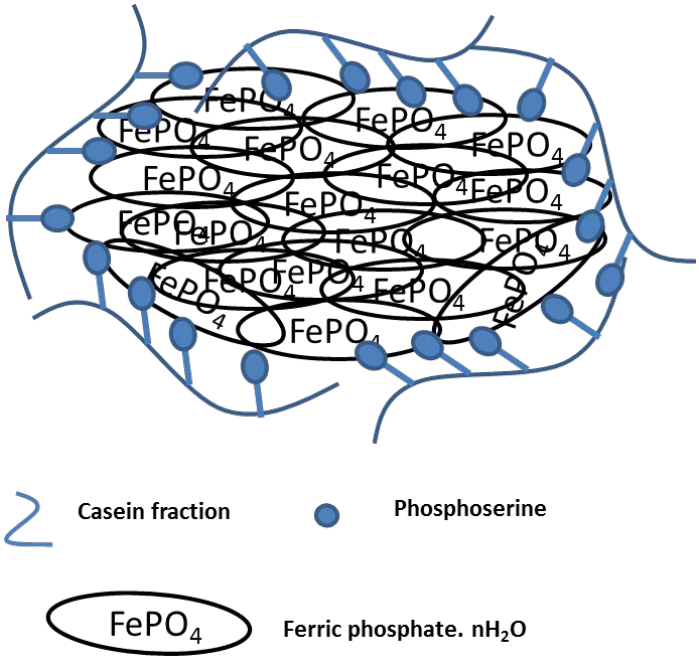


Figure 8-7**:** *Schematic showing the hypothesized stabilisation of ferric phosphate salt by caseins in sodium caseinate.*

8.2.3. The adsorption of phosphorus onto casein-iron complex

Interestingly, the addition of orthophosphate to iron-added sodium caseinate solutions redispersed the casein-iron precipitates. The particle size distribution analysis of the redispersed casein-iron precipitate containing varying concentrations of iron (0-25 mM) and protein (0-3% w/v) revealed that the ability of orthophosphate to redisperse the precipitate was governed by the concentration of caseins and bound iron. For example,

the addition of orthophosphate efficiently redispersed casein-iron precipitates in sodium caseinate solution with 1% w/v protein and 15 mM iron. However, if the protein concentration was increased to 3% w/v, casein-iron precipitate with 25 mM iron could be redispersed. Moreover, increasing the protein content reduced the amount of orthophosphate required for efficient redispersion of the casein-iron precipitate.

A reduction in permeate phosphorus content, which was positively related to the concentration of iron, was observed. The ^{31}P-NMR spectroscopy investigation revealed that addition of orthophosphate improved the resonance of organic phosphorus peaks, indicating an increased concentration of free phosphoserine residues. Additionally, the concentration of free inorganic phosphorus decreased with increase in the concentration of iron in sodium caseinate solution. The increased ^{31}P-NMR signals for organic phosphorus along with the reduction in inorganic phosphorus peaks suggested partial displacement of the former (organic phosphorus i.e. phosphoserine) by orthophosphate from the pre-existing casein-iron complex. However, a proportion of the organic phosphorus was still interacting with iron. These results hinted that orthophosphate was probably adsorbed onto the casein-iron precipitate. The phenomenon of orthophosphate adsorption is well-studied in soil science and commonly occurs for oxides of metals such as iron (Adegoke et al., 2013; Crosby et al., 1981). Adsorption of orthophosphate occurs on ferric hydroxide, mainly due to the high specificity of phosphate ions towards iron. Orthophosphate adsorbs covalently onto the ferric hydroxide mainly through ligand exchange phenomenon, which involves a displacement of H_2O/OH^- from the coordination sphere of iron (Goldberg et al., 1985). Substantial adsorption of orthophosphate was also observed onto ferric hydroxide complexes formed under the conditions of our experiments.

Iron binds to caseins by forming coordination bonds with oxygen of the phosphoserine and these iron-oxygen bonds possibly provide a site for orthophosphate adsorption. The presence of H_2O/OH^- bonds is expected on iron-casein complexes thus envisaging the possibility of orthophosphate adsorption through ligand exchange phenomenon. Moreover, ^{31}P-NMR study confirmed that inorganic phosphorus was indeed adsorbing in the region close to the iron that was bound to phosphoserine and displaced some of the organic phosphorus.

The adsorption of negatively charged orthophosphate onto the casein-iron precipitate probably increased the surface negative charge on casein-iron precipitate, resulting in inter-particle repulsion and consequent solubilisation. Ferric hydroxide remains insoluble upon orthophosphate adsorption whereas solubilisation of the casein-iron complex was observed. This was attributed to the presence of caseins, which are soluble at pH 6.8. Hence, it was hypothesized that the increase in surface negative charge caused by orthophosphate adsorption on the casein-iron precipitate leads to the redispersion.

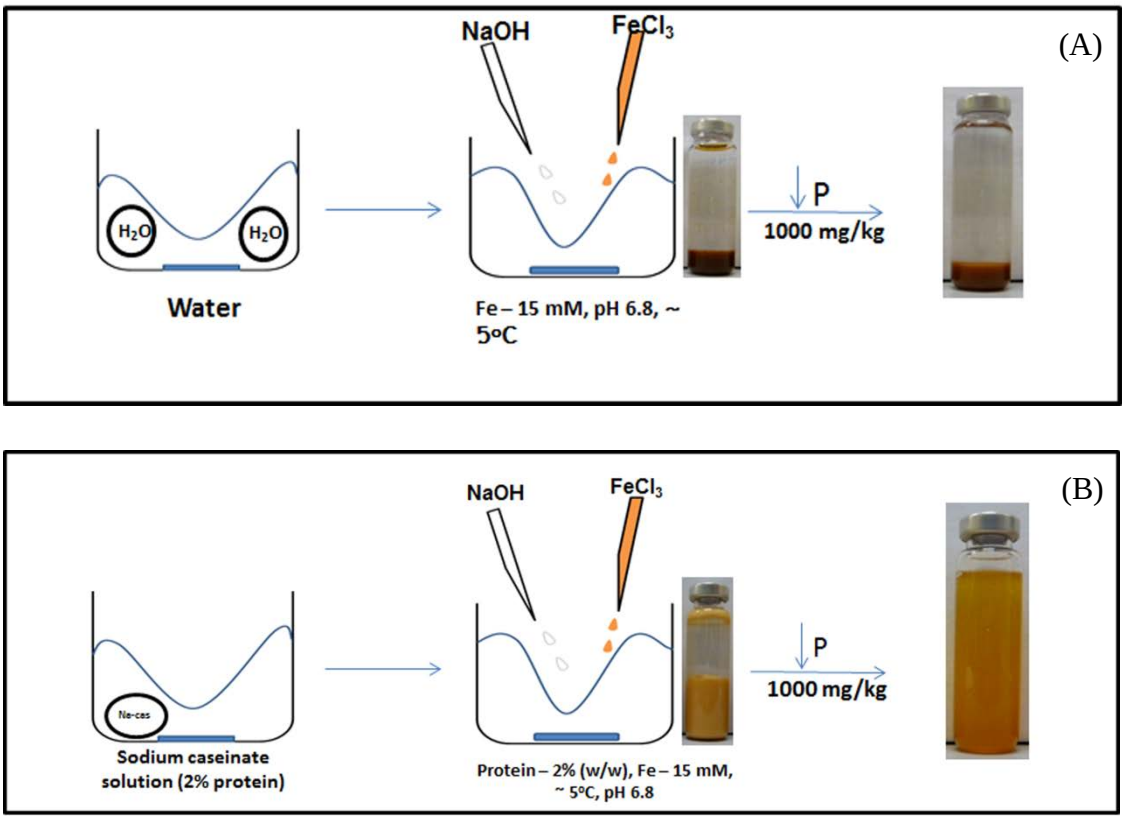


Figure 8-8**:** *Effect of orthophosphate (32 mM) on the dispersion stability of iron (15 mM) added to (A) water and (B) sodium caseinate solution (2% w/v protein) maintained at pH 6.8.*

The process and consequence of adding orthophosphate to freshly prepared ferric hydroxide or casein-iron precipitate is shown in Figure 8.8.

A visual observation of Figure 8.4 and Figure 8.8 suggest that exchanging the sequence of adding iron and phosphorus to sodium caseinate solutions results in different interactions between the same ingredients (iron, casein and orthophosphate). Moreover, the chemistry involved in both the cases is different i.e. precipitation of ferric orthophosphate and adsorption of orthophosphate onto casein-iron complexes.

The high affinity of iron towards orthophosphate ions was the governing factor in both these mechanisms. The addition of up to 20 mM iron to milk while still maintaining a good dispersion, as opposed to the precipitation of proteins observed at 5 mM levels in

sodium caseinate was related to the difference in their orthophosphate content. In the absence of milk proteins, orthophosphate interacts with iron either forming ferric phosphate or adsorbing onto ferric hydroxide. The outcomes of these experiments suggest that protein acted as the binder and contributed to the solubilisation of iron and its salts formed thereof.

8.3. Recommendations for further research

The strong affinity of caseins to bind iron has been known for more than 50 years. However, the precipitation of proteins at higher levels of addition (> 1% by weight of casein) circumvented its use as a potential carrier for iron. The use of caseins as a medium to bind high concentrations (> 5% by weight of protein) of iron yet maintaining good solubility characteristics was the main result of this study. This was achieved by manipulating the calcium content of milk and understanding its effect on the physico-chemical and microstructural properties of casein proteins upon iron addition. The ability of caseins to bind iron in a soluble form was improved by adding orthophosphate in the mixture. Hence, this study was focussed on investigating the role protein, iron and orthophosphate played in the formation of soluble protein-iron complexes. Most importantly, this work highlighted two different mechanisms by which soluble protein-iron complexes could be formed. These proposed mechanisms, i.e. the stabilisation of ferric orthophosphate by caseins and the adsorption of orthophosphate onto casein-iron precipitates have rarely been reported in food science. The work has therefore opened up new areas for further research.

Further research on iron binding to caseins in the presence of orthophosphate

This research proposed that the salt formed upon the interaction of iron with orthophosphate was dispersed in solution due to the presence of caseins. However, the exact mechanisms involved needs further investigation. Spectroscopic techniques, such as electron spin resonance (EPR), Mossbauer, IR, multiple-nuclear NMR and magnetic circular dichroism (CD) could help understand and identify the oxidation state of iron, bond length and atoms involved in the coordination sphere of the complex.

Further research on orthophosphate adsorption onto casein-iron complex

As mentioned previously, the phenomenon of orthophosphate adsorption onto the casein-iron complex is a relatively unexplored area in food science. However, this aspect has been studied extensively in soil science. A major difference between the effect of orthophosphate adsorption onto casein-iron complex and onto soil particles is that the casein-iron precipitate is solubilised upon this adsorption. The solubilisation of the precipitate opens up an interesting aspect for further research in food science. For example, a strong affinity and adsorption of orthophosphate has also been observed for calcium onto soil particles. Aspects that need further investigation include hysteresis of orthophosphate adsorption, the physico-chemical factors affecting the rate and capacity of adsorption and mechanistic understanding into the bonding of adsorbed orthophosphate onto casein-iron complex. A change in the NMR spectra of organic phosphorus was observed in this study upon the adsorption of orthophosphate, but further in-depth understanding of this bonding would be beneficial in food science.

Kinetic of the interactions involved

The analysis of the iron added solutions were conducted after 24 h storage in the entire study. A common observation has been a change in apparent turbidity during the 24 h period. Moreover, the changes observed were accelerated by an increase in temperature. Hence, further studies looking at the kinetics involved in the formation of soluble protein-iron complexes using spectroscopic techniques would provide greater insights into the changes occurring at molecular levels.

The effect of physico-chemical conditions

The pH of the iron added solutions in this study was maintained at 6.8. The release of iron bound to casein upon reducing the pH up to 3.8 has been extensively studied and was found to be stable up to pH 4.6 (Gaucheron et al., 1996). However, inorganic phosphorus was involved in the soluble protein-iron complexes formed in this study. Hence, it would be interesting to compare the release of bound iron and inorganic phosphorus from the protein-iron complexes upon reducing the pH. This should provide further evidence of the interactions (electrostatic or covalent) involved in the formation of complexes. The change in ionic strength of the sodium caseinate solutions would alter the thickness of the electrical double layer around the casein-iron aggregates formed and affect the adsorption of orthophosphate onto the complex. Hence, the effect of ionic strength on the adsorption of orthophosphate onto casein-iron precipitates needs further investigation.

The protein source

Whey proteins bind iron although to a lower extent than caseins. Preliminary experiments have suggested that inclusion of orthophosphate raised the concentration of

iron that could be added to whey protein isolate without precipitation and this necessitates a further study.

The effect of orthophosphate on other essential micronutrients (copper and zinc)

The mechanisms by which high concentrations of iron was bound to caseins could also be applied to other multivalent cations, especially calcium, copper and zinc. Preliminary experiments have shown that up to 1.5 mM of copper and 15 mM of zinc could be bound to caseins singly or combined in a dispersible format upon inclusion of 32 mM orthophosphate. These concentrations of copper and zinc correspond to their respective recommended Dietary Intake (RDI) values of ~ 900 mcg and ~ 11 mg respectively. Hence, it would be worthwhile to study the effect of single and multiple cation addition on the binding properties of caseins.

List of publications

Patent

- ✓ Mittal, V. A., Ellis, A., Das, S., Ye, A., & Singh, H. (2013). Mineral fortification process and its uses. Status - Granted

Peer reviewed papers

- ✓ Influence of calcium-depletion on iron binding properties of milk, Vikas Mittal; Ashling Ellis; Aiqian Ye; Harjinder Singh, *Journal of Dairy science* (Published online on 31st January, 2015).
- ✓ Iron binding to caseins in the presence of orthophosphate - *Food Chemistry* (submitted)

Text book chapter

- ✓ Ellis, A., Mittal, V., & Sugiarto, M. (2012). Innovation in iron fortification: is the future in iron-binding milk proteins? In D. Ghosh, S. Das, D. Bagchi & R. B. Smarta (Eds.), *Innovation in Healthy and Functional Foods* (pp. 249-268): CRC Press.

Conference presentation

- ✓ Formation of soluble protein-iron complexes in calcium-depleted milk at the IDF World dairy Summit 2013, Yokohama, Japan.

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