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Standardisation of encapsulation of iron fortified whey protein concentrate using alginate hydrogel

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Abstract
Iron deficiency anemia remains the most prevalent nutritional disorder globally, highlighting the urgent need for effective iron fortification strategies. Encapsulation of iron in food-grade matrices offers a promising approach to improve stability, bioavailability, and sensory acceptance. This study aimed to standardize the encapsulation of iron-fortified whey protein concentrate (WPC-Fe) using alginate hydrogels through ionotropic gelation. WPC-Fe complexes were prepared at a protein-to-iron ratio of 40:1 and incorporated into alginate solutions (1.0-2.0%, w/v), followed by cross-linking in calcium chloride solutions (3%, 5%, and 10%, w/v) at different pH levels (3, 5, and 7). The stability and bursting behavior of the beads were evaluated in 0.1 M phosphate buffer (pH 7). Results showed that no bead formation occurred at pH 3, while stable beads were formed at pH 5 and 7. Beads cross-linked with calcium chloride concentrations of 3% to 10% exhibited improved structural integrity. In phosphate buffer, most beads burst within 1 h, except for a few formulations prepared with 2% WPC-Fe/alginate and higher calcium chloride concentrations at pH 5 and 7. Comparable bursting behavior was observed for WPC and WPC-Fe beads, suggesting that protein-iron interactions did not enhance resistance against phosphate-induced destabilization. The findings demonstrate that pH and calcium chloride concentration are critical factors influencing bead formation and stability.

Keywords: Whey protein concentrate, iron fortification, alginate hydrogel, Ionotropic gelation, encapsulation

1. Introduction
Iron is a vital micronutrient required for immune function, energy metabolism, and oxygen transport. In humans, about two-thirds of body iron is bound to haemoglobin in red blood cells (Abbaspour *et al.*, 2014) [1]. It is also a vital component of metalloproteins such as ferritin, transferrin, and cytochromes, and serves as a cofactor for enzymes involved in redox reactions, electron transfer, and cellular energy production (Beard, 2001; ICMR-NIN, 2020) [2, 3]. Iron deficiency anaemia (IDA) is the most prevalent nutritional disorder worldwide, affecting approximately 30% of the global population (Murphy, 2002) [4]. This condition poses a significant public health challenge, particularly among vulnerable groups such as young children, menstruating adolescent girls, and pregnant women, with prevalence rates of 40%, 37%, and 30%, respectively. The major causes include inadequate dietary iron intake, impaired absorption, and increased losses due to factors such as heavy menstrual bleeding or gastrointestinal bleeding (Longo and Camaschella, 2015) [5].
According to the Recommended Dietary Allowances (RDAs), the daily iron requirement is 3 mg for infants aged 0-6 months, 19 mg for adult men, and 29 mg for adult women. During pregnancy, the requirement increases to 27 mg and remains at 23 mg during lactation, reflecting higher physiological demands (ICMR-NIN, 2020) [3]. Food fortification is a practical, cost-effective, and safe strategy to address iron deficiency, enabling controlled delivery of iron through common dietary staples. Ferrous sulfate is the most widely used fortificant due to its high bioavailability; however, its effectiveness is often limited by poor solubility and absorption under physiological conditions, as well as by the influence of dietary inhibitors and enhancers (Hurrell, 2002) [6]. Direct addition of iron can also impair sensory quality, reduce product stability, promote lipid oxidation, and cause gastrointestinal discomfort. Furthermore, its high reactivity may promote the formation of reactive oxygen species

(ROS), leading to lipid peroxidation, DNA damage, and enzyme inactivation (Rehman *et al.*, 2006) [7].

Encapsulation of iron within food-grade matrices offers a promising solution, protecting the fortificant from adverse interactions, enhancing bioavailability, and preserving the sensory attributes of fortified foods (Man *et al.*, 2022) [8]. Whey proteins are particularly effective carriers for iron fortification due to their high solubility, thermal stability, and ability to form gels, foams, and emulsions. Whey protein concentrate (WPC, 50-85% protein) and whey protein isolate (WPI, >90% protein) not only improve the bioavailability and stability of iron but also release amino acids during digestion that promote intestinal absorption (Mehra *et al.*, 2021) [9]. Encapsulation of iron within whey protein matrices further protects it from oxidation and degradation, while the high solubility of WPC supports efficient intestinal uptake.

Ionotropic gelation is a widely employed technique in food and pharmaceutical applications for encapsulating sensitive bioactive compounds. This process involves the interaction of polyelectrolytes, such as alginate, pectin, or carrageenan, with oppositely charged multivalent cations (e.g., Ca^{2+}), inducing a sol-gel transition and forming well-structured materials such as films, beads, hydrogels, and nanoparticles (Lakkis, 2007) [10]. In food systems, ionotropic gelation is particularly valuable for producing hydrogels, three-dimensional, water-swollen networks capable of encapsulating and protecting bioactives. These hydrogels can regulate the release of encapsulated compounds in response to environmental triggers such as pH, enzymes, or ionic strength, making them ideal for targeted delivery in functional foods and nutraceuticals (Li *et al.*, 2021) [11]. Ionic cross-linked hydrogels are typically formed at room temperature through electrostatic interactions between anionic polymer chains and metal cations, offering a mild, efficient, and biocompatible encapsulation method. Therefore, the present study aims to standardize the encapsulation of iron-fortified whey protein concentrate using alginate hydrogels.

2. Materials and Methods

Whey protein concentrate was obtained from Mahan Milk Proteins, Mumbai; sodium alginate from Meron Hydrocolloids, Kochi; and food-grade ferrous sulfate and sodium hydroxide from Annexe Saw Chemicals, Gujarat. The remaining reagents were all analytical grade.

2.1 Preparation of WPC-Iron complex

The 4% WPC solution on protein basis was subjected to heat treatment at 85 °C for 15 min and then cooled to 40 °C. After adjusting the pH to neutral, food-grade ferrous sulfate was added at a protein-to-iron ratio of 40:1. The mixture was kept in a shaking water bath at 40 °C for 2 h to facilitate binding. The solution was then subjected to freeze drying and stored at 7 °C (Lukose *et al.*, 2024) [12]. Figure 1 shows the flow chart for WPC-iron complex preparation.

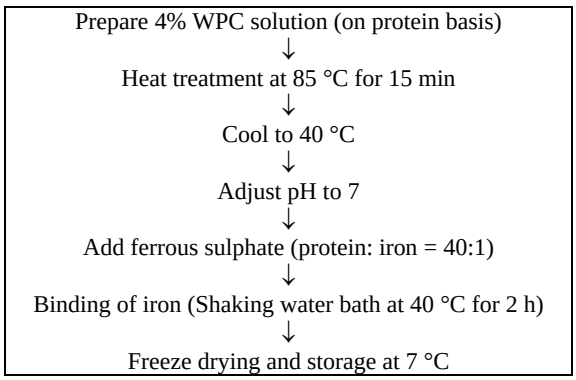


Fig 1: Preparation of WPC-Fe Complex

2.2 Preparation of WPC-Fe Hydrogel beads

Aqueous alginate solutions were prepared by dissolving the powdered ingredient in distilled water, followed by stirring at 60 °C for one hour and cooling to 35 °C. WPC-Fe solution was mixed with sodium alginate solution at a 1:1 (v/v) ratio with continuous stirring for one hour to obtain a uniform mixture. Before the preparation of beads, the pH of the mixed WPC-Fe/alginate solutions had been adjusted to 3, 5, or 7. Calcium chloride solutions (used for gelation) were also adjusted to the same pH values. WPC-Fe hydrogel beads were prepared by injecting the WPC-Fe/alginate solutions (pH 3, 5, or 7) into the corresponding calcium chloride solutions using a syringe. The distance between the syringe tip and the calcium chloride solution during bead formation was maintained at less than 1 cm. The beads were allowed to crosslink with Ca^{2+} at ambient temperature. After gelation, the hardened beads were collected by filtration and washed with distilled water to remove excess calcium ions from their surfaces (Zhang *et al.*, 2016) [13]. Preparation of the control WPC hydrogel followed the same method as that of the WPC-Fe sample. The flow chart of preparation of WPC-Fe hydrogel is shown in Figure 2.

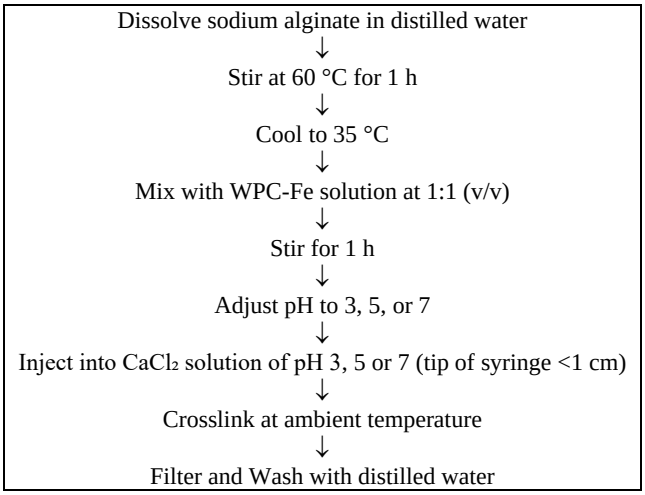


Fig 2: Preparation of WPC-Fe hydrogel

2.3 Preliminary Trial: Effect of Calcium Chloride Concentration on Bead Formation

The effect of calcium chloride concentration on bead formation was evaluated by keeping the WPC-Fe and sodium alginate concentrations constant at 2%. Calcium chloride concentrations ranging from 1% to 10% (w/v) were tested. The purpose of this preliminary trial was to determine the most appropriate calcium chloride concentrations for use in further experiments.

2.4 Experimental Design for Optimization

The selected calcium chloride concentrations (3%, 5%, and 10% w/v) were combined with three levels of sodium alginate and WPC-Fe concentrations (1.0%, 1.5%, and 2.0% w/v) at three pH levels (3, 5, and 7), resulting in 27 combinations. Bead formation was recorded as "Yes" or "No" for each combination. The same procedure was applied to the control (WPC hydrogel), giving a total of 54 combinations.

2.5 Bursting Behavior in Phosphate Buffer

A 0.25 g sample of dried beads obtained from each of the 54 combinations was accurately weighed and placed in 50 mL of phosphate buffer (pH 7.0) at room temperature. After 1 h of immersion, beads were visually examined, and the number of combinations in which beads remained intact was recorded (Menon and Sajeeth, 2013) [14].

3. Results and Discussions

3.1 Effect of Calcium Chloride Concentration on Bead Formation

At $CaCl_2$ concentrations of 1% and 2% (w/v), beads were formed upon contact with the gelling solution but quickly shrank and lost their shape due to insufficient calcium ions (Ca^{2+}) to form strong links between alginate chains. At $CaCl_2$ concentrations between 3% and 10% (w/v), well-formed beads were produced that retained their shape, indicating sufficient Ca^{2+} availability to create a dense and stable gel network. Higher calcium levels promoted more permanent inter-chain links within the alginate matrix, increasing bead firmness and rigidity. In the study of George and Abraham (2006) [15], increasing $CaCl_2$ concentration was shown to improve bead stability and hardness, while Li *et al.* (2016) [16] reported that within a moderate range (about 1-4%), $CaCl_2$ concentration may not significantly affect bead size or encapsulation efficiency once the minimum level for stable gelation is reached. Based on these results, 3%, 5%, and 10% (w/v) $CaCl_2$ were selected for subsequent experiments to represent varying crosslinking intensities. The morphological differences in beads formed at different calcium chloride concentrations are presented in Figure 3.

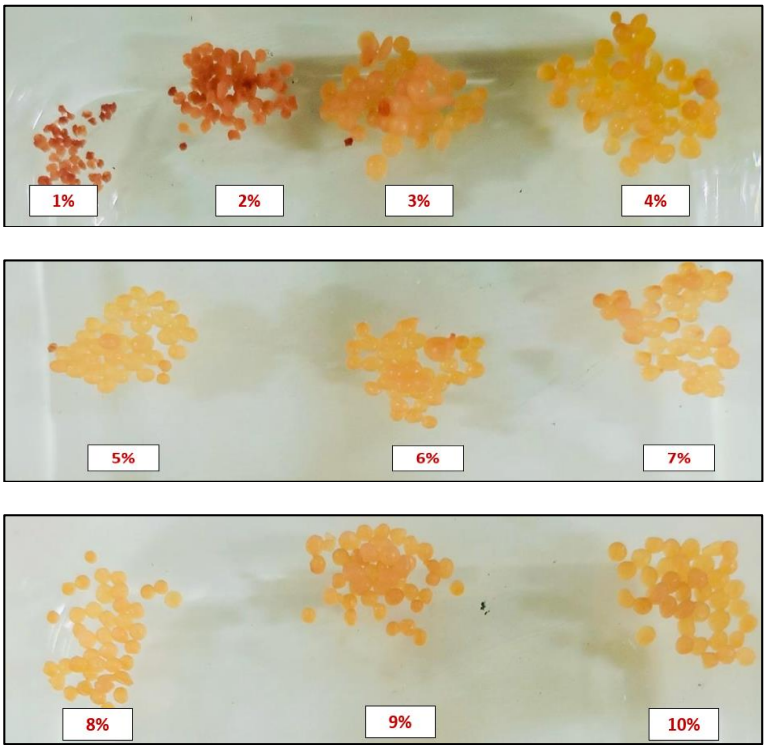


Fig 3: Morphological differences in beads formed at different calcium chloride concentrations

3.2 Effect of pH on bead formation

From Table 1, it can be conceived that no bead formation was observed at pH 3 in any tested combination, irrespective of WPC-Fe/sodium alginate concentration (1.0%, 1.5%, or 2.0% w/v) or $CaCl_2$ concentration (3%, 5%, or 10% w/v). The same outcome was recorded for the control WPC hydrogel. The initial pH of the WPC-sodium alginate mixture ranged from 6.25 to 6.60 for the control and 5.30 to 5.65 for the WPC-Fe/sodium alginate mixture, and was adjusted to pH 3.0 by the addition of 1 M citric acid. Acidification to pH 3 caused a marked decrease in viscosity, with the WPC-Fe/alginate solution becoming more fluid and noticeably lighter in color compared to pH 5 and pH 7 (Figure 4.).

This absence of bead formation at pH 3 can be attributed to the protonation of carboxylate groups ($-COO^-$) on guluronic acid residues, converting them to-COOH and thereby reducing their ability to electrostatically bind Ca^{2+} during gelation (Hu *et al.*, 2021) [17]. This disrupts the "egg-box" crosslinking structure essential for stable bead formation. Figure 5. shows beads formed at different pH levels.

Table 1: Bead formation at different pH levels (Yes/No).

No.	Sample concentration (WPC-Fe/alginate)	Calcium chloride concentration	pH	Bead formation (yes/no)
1	1%	3%	3	No
2			5	Yes
3			7	Yes
4		5%	3	No
5			5	Yes
6			7	Yes
7		10%	3	No
8			5	Yes
9			7	Yes
10	1.5%	3%	3	No
11			5	Yes
12			7	Yes
13		5%	3	No
14			5	Yes
15			7	Yes
16		10%	3	No
17			5	Yes
18			7	Yes
19	2%	3%	3	No
20			5	Yes
21			7	Yes
22		5%	3	No
23			5	Yes
24			7	Yes
25		10%	3	No
26			5	Yes
27			7	Yes

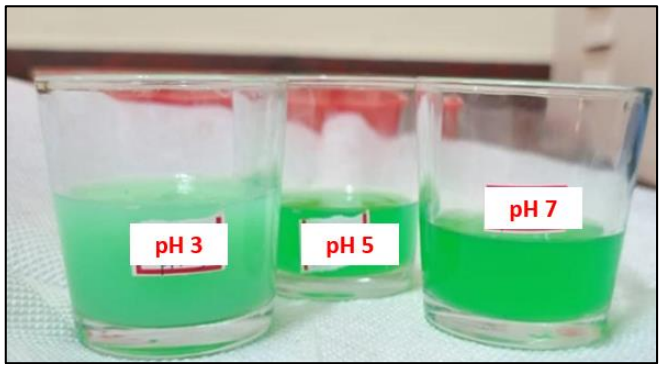
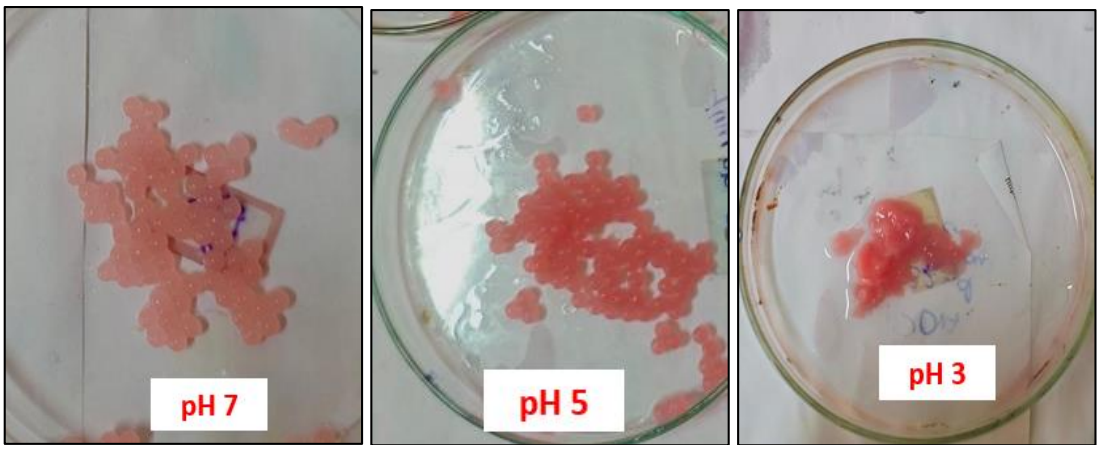


Fig 4: Color of the WPC-Fe/alginate solution after adjusting to the respective pH with 1 M citric acid



(a) (b) (C)

Fig 5: Bead formation at different pH levels, showing (a) no bead formation and (b, c) bead formation

3.3 Bursting behaviour of beads in phosphate buffer

The bursting behaviour of WPC-Fe hydrogel beads was evaluated in 0.1 M phosphate buffer (pH 7). From Table 2., it can be observed that after 1 h of incubation, only three combinations-2% WPC-Fe/alginate solution cross-linked with 5% $CaCl_2$ at pH 5, 2% WPC-Fe/alginate solution cross-

linked with 3% $CaCl_2$ at pH 7, and 2% WPC-Fe/alginate solution cross-linked with 5% $CaCl_2$ at pH 7 remained intact, while all other formulations exhibited bursting. A similar trend was observed for the control WPC beads.

Phosphate buffer was chosen for this study because it closely mimics physiological ionic strength and maintains a stable pH environment, making it a standard medium for evaluating the swelling and release behaviour of alginate-based hydrogels (George & Abraham, 2006; Anal & Stevens, 2005) [15, 18]. Moreover, phosphate ions are known to compete with alginate for calcium binding sites, leading to ion exchange between Ca^{2+} and phosphate anions. This exchange weakens the calcium-alginate cross-linked network, thereby promoting swelling and eventual bursting of beads in most formulations (Sriamornsak, 1998) [19]. The observation that only certain bead combinations resisted bursting highlights the role of both higher calcium chloride concentration and favorable pH conditions in forming a denser gel network, which provides greater structural integrity against phosphate-induced destabilization.

Table 2: Bursting behavior of WPC-Fe beads in 0.1 M phosphate buffer after 1 h.

No.	Sample concentration (WPC-Fe/alginate)	Calcium chloride concentration	pH	Bursting after 1 hour (yes/no)
1	1%	3%	3	Yes
2			5	Yes
3			7	Yes
4		5%	3	Yes
5			5	Yes
6			7	Yes
7		10%	3	Yes
8			5	Yes
9			7	Yes
10	1.5%	3%	3	Yes
11			5	Yes
12			7	Yes
13		5%	3	Yes
14			5	Yes
15			7	Yes
16		10%	3	Yes
17			5	Yes
18			7	Yes
19	2%	3%	3	Yes
20			5	Yes
21			7	No
22		5%	3	Yes
23			5	No
24			7	No
25		10%	3	Yes
26			5	Yes
27			7	Yes

(a) (b)

Fig 6: Bead stability in phosphate buffer: (a) beads that burst after 1 h; (b) beads that remained intact after 1 h

4. Conclusion

The present study demonstrated that bead formation and stability of WPC-Fe/alginate hydrogels were strongly influenced by calcium chloride concentration and pH. Beads did not form at pH 3, which may bed due to protonation of carboxylate groups, while well-structured beads were obtained at pH 5 and 7. In phosphate buffer (0.1 M, pH 7), most beads burst within 1 h, except for a few formulations with higher calcium chloride concentrations at favorable pH levels, indicating that denser cross-linking improved structural resistance. Interestingly, WPC-Fe/alginate beads exhibited similar bursting behavior to control WPC beads, suggesting that protein-iron interactions did not significantly

enhance bead stability against phosphate-induced destabilization.

Further studies are needed to evaluate additional bead characteristics, such as encapsulation efficiency, iron release profile, swelling kinetics, and *in vitro* bioaccessibility, in order to identify the most promising bead formulations for food fortification and targeted delivery applications.

5. Acknowledgment

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