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Phycocyanin Encapsulation in Carboxymethyl Chitosan and Whey Protein Isolate as a Strategy to Enhance Physicochemical Stability as a Food Coloring: A Review

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Abstract

Color additives, also known as food colorings, are substances added to food and beverages to enhance their visual appeal and consumer acceptance. The color of a food product serves as an indicator of attributes like flavor, safety, and nutritional value. Unlike other natural colors, blue-colored fruits and vegetables are rare, making blue associated with synthetic food coloring. Phycocyanin (PC), a natural blue pigment found in *Spirulina*, has garnered attention for its various biological properties, including antioxidant and anti-inflammatory activities. However, its industrial application limited by susceptibility to factors like high temperatures and pH variations during processing. Encapsulation, a technique involving the trapping of bioactive ingredients in carriers, can address these issues. In this study, chitosan derivatives, Carboxymethyl Chitosan (CMC)-Whey Protein Isolate (WPI) used as a coating for complexes in the encapsulation of phycocyanin. Encapsulation employed to improve pigment stability, and the study investigates the impact of ratios, pH, and $CaCl_2$ concentration addition as a crosslinking agent. The selection of the appropriate CMC-WPI ratio is crucial for achieving good physicochemical properties, high encapsulation efficiency, and survivability against gastrointestinal and storage conditions. The encapsulated phycocyanin demonstrates stability across various pH values, with the concentration of $CaCl_2$ influencing particle diameter and shape.

Keywords: color additive, phycocyanin, encapsulation, carboxymethyl chitosan, whey protein isolate, stability

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1. Introduction

A color additive or food coloring refers to any substance that, when added to food or beverages, imparts color. The incorporation of color is a crucial factor in enhancing the overall appeal and consumer acceptance of food and drinks. Consumers frequently use the color of a food product as an indicator of various attributes such as flavor, safety, and nutritional value [1-3]. The two types of coloring commonly used are natural coloring and synthetic coloring. To classify as natural food color additives, they must originate from natural sources and consist of raw materials derived from plants, animals, minerals, or microbes. Furthermore, it is crucial that the chromophore undergoes no chemical alterations during the processes of extraction or manufacturing [4]. Natural food colorings come with positive attributes like prevention of cardiovascular diseases, and activities that counteract obesity and diabetes, as well as anti-cancer [5], antioxidant, and anti-inflammatory effects [6-7].

Despite their color-enhancing qualities and health-promoting effects, natural food colorings encounter diverse issues, including instability and reduced bioavailability. The stability of natural food colorings is highly affected by factors such as pH, temperature, heat, and light, which can lead to color loss, resulting in fading and a decreased shelf life [8-9]. Food manufacturers increasingly favor synthetic food colors over natural ones to achieve specific properties like cost-effectiveness, improved appearance, intense color, stability, and uniformity [3]. Synthetic food colorings connected to

diverse health issues. Research has shown that these synthetic colors are a leading cause of food-related issues and can result in significant health problems, including hyperactivity in sensitive children, cancer, and allergies [1]. So, besides their instability, using natural colorings is a practical solution because they pose no negative effects when consumed and can be broken down, ensuring a high level of safety.

Phycocyanin (PC) is a natural blue pigment classified within the phycobiliprotein category, and it recognized as a primary pigment in *Arthrospira* (*Spirulina*). This pigment predominantly found in *cyanobacteria*, also known as blue-green algae (BGA), which considered one of the oldest life forms on Earth. The *Arthrospira* species, particularly *Arthrospira* (*Spirulina*) *plantesis* and *Arthrospira* (*Spirulina*) *maxima*, commonly utilized for the production of phycocyanin [10]. Notably, phycocyanin exhibits fluorescence ability [11], and it serves as a blue natural food colorant and a beneficial functional food ingredient, providing health benefits and acting as a natural colorant in various applications [12]. PC is attracting considerable attention due to its array of biological properties which present in Figure 1, such as antioxidant, hepato-protective, anti-inflammatory, neuro-protective, and antiproliferative activities [13-14].

The extraction of phycocyanin as a protein-pigment complex is quite straightforward due to its high solubility in water [15]. However, the industrial application of PC is constrained by its vulnerability to various industrial processes, such as elevated temperatures (e.g., pasteurization), acidic or alkaline pH conditions, or the use of oxidizing agents during extraction, purification, and product preparation. These processes can induce precipitation or dissociation phenomena, causing the loss of the blue color [16-17]. Encapsulation is a technique that involves trapping bioactive ingredients in suitable carriers to ensure protection and facilitate their precise delivery to the intended site through the creation of particles or capsules at micro- or nanometer scales. This process offers various advantages, such as heightened thermal and chemical stability, preservation or masking of flavor, taste, or aroma, controlled and targeted release, and improved bioavailability of pigments [17].

The choice of coating material is crucial in encapsulation, requiring non-reactivity with active compounds and essential protective properties like flexibility, strength, stability, and impermeability. In food applications, the coating must recognized as safe, inert to the active substance, biodegradable, and sufficiently robust to shield the encapsulated material from the environment [18]. Encapsulation efficiency is a measure of the successful entrapment of the core material within the capsules [19]. Therefore, differences in coatings can affect encapsulation efficiency, as they related to the amount of active material that can encapsulated. Encapsulated phycocyanin offers several advantages, including improved stability, protection from environmental conditions, and controlled release [20]. Various studies have conducted regarding coating materials for the phycocyanin encapsulation process. According to the study conducted by Dewi et al. (2017), alginate and κ-carrageenan show promise as coating materials for the phycocyanin encapsulation process.

This is attributed to the fact that the combination of maltodextrin-alginate and maltodextrin-κ-carrageenan results in a singular onset temperature, signifying the optimal dispersion of phycocyanin within the utilized coating materials [21]. Meanwhile, a study by Aprodu et al. (2019), indicates that encapsulation of anthocyanins with whey protein and casein shows high encapsulation efficiency of up to 99%. The pigment color and antioxidant content remain more stable during heating, and the addition of protein enhances the nutritional value of the product [22]. Furthermore, according to Zhang et al. (2020), the encapsulation efficiency of chlorophyll by 15% whey protein shows a value of 99.76%. Fluorescence and FTIR results indicate that whey protein successfully encapsulates chlorophyll [23]. The combination of chitosan derivative-WPI as encapsulating materials can used as microcapsule wall material because the amino and hydroxyl groups in chitosan can crosslink with protein, forming a stable complex. Several studies have utilized this combination of encapsulating materials, for example, garlic extract encapsulated with chitosan derivative-WPI can inhibit the degradation of phenolic compounds [24].

The same combination used for encapsulating curcumin, resulting in a delay in the release of curcumin [25]. According to the research conducted by Wang et al. (2021), the encapsulation of phycocyanin using chitosan-WPI encapsulating material results in a product with better temperature stability, stability in *in vitro* digestion simulation, maintains antioxidant activity stability, and imparts functional properties to coffee products [26]. Colorful food brings a sense of health and happiness to consumers [27]. Unlike other colors, blue-colored fruits and vegetables rarely found in nature. As a result, blue is not a part of the natural palette that stimulates appetite and tends to be associated with synthetic food coloring. The color blue considered 'unnatural' for food application [28]. Meanwhile, the use of blue coloring from phycocyanin considered to add functional properties to food [29]. Therefore, the addition of the blue pigment phycocyanin with an appropriate concentration of food coloring is necessary to achieve the desired characteristics of the product that consumers prefer.

2. Materials and Methods

2.1. Food Coloring

A color additive or food coloring is defined as any substance that, when introduced to food or beverages, provides color. The inclusion of color plays a pivotal role in elevating the overall attractiveness and acceptance of food and drinks by consumers. Often, consumers rely on the color of a food product as a signal for various attributes, including flavor, safety, and nutritional value [1-3-30]. The classification of colorants can become intricate due to variations in chemical structures, sources, and intended applications. An uncomplicated approach for their categorization relies on their origin, distinguishing between natural and synthetic types. Natural colorants can be derived from plants (such as curcumin, carotenoids, anthocyanins, betalains, and chlorophyll), animal cells (including carminic acid and kermesic acid), microorganism metabolism (like phycocyanin, carotenoids and chlorophylls), or minerals (such as titanium dioxide and calcium carbonate). Natural food colorings exhibit favorable qualities such as the prevention of cardiovascular disease, actions that mitigate obesity and diabetes, along with anti-cancer [5], antioxidant, and anti-inflammatory effects [6-7].

Despite their ability to enhance color and promote health, natural food colorings face various challenges, including instability and diminished bioavailability. The stability of natural food colorings is significantly impacted by variables like pH, temperature, heat, and light, potentially causing color loss, resulting in fading and a reduced shelf life [8-9]. Synthetic colorants are substances not naturally occurring and produced through chemical synthesis [31-35]. Food producers are increasingly opting for synthetic food colors over natural alternatives to attain distinct characteristics such as cost-effectiveness, enhanced visual appeal, vibrant color, stability, and consistency [3]. However, synthetic food colorings connected to diverse health issues. Research has shown that these synthetic colors are a leading cause of food-related issues and can result in significant health problems, including hyperactivity in sensitive children, cancer, and allergies [1]. Moreover, Chequer et al. (2015), conducted a study that evaluated the impact of this coloring agent on DNA, finding a dose-dependent genotoxic effect within the range of 0.5 to 20 μg mL^{-1} [36]. Some examples of synthetic colorants include Allura Red AC [37], Amaranth, Indigo Carmine, Erythrosine, Ponceau 4R [38], Tartrazine, Sunset Yellow FCF [39], Brilliant Blue, Black PN, and Red 2G [40]**.**

2.2. Phycocyanin

Phycocyanin (PC) is a naturally occurring blue pigment categorized as a phycobiliprotein, a category of light-harvesting apoproteins connected to open-chain tetrapyrroles as a prosthetic group, acknowledged as a primary pigment in *Arthrospira* (*Spirulina*). This pigment is primarily present in *cyanobacteria*, commonly known as blue-green algae (BGA), which recognized as one of the earliest life forms on Earth. *Arthrospira* species, especially *Arthrospira* (*Spirulina*) *plantesis* and *Arthrospira* (*Spirulina*) *maxima*, frequently employed for the synthesis of phycocyanin [10-41]. A study states that 47°C is the critical temperature point for the stability of phycocyanin [42]. Phycocyanin is noteworthy for its fluorescence capability [11], a physicochemical exchange of energy involves the absorption of shorter-wavelength photons by a molecule, which are subsequently re-emitted as photons with longer wavelengths , functioning as a natural blue coloring for food and a beneficial ingredient in functional foods [43]. It offers health advantages and serves as a natural colorant in various applications [12].

PC is attracting considerable attention due to its array of biological properties, such as antioxidant, hepato-protective, anti-inflammatory, neuro-protective, and antiproliferative activities [13-14]. A study conduct by Chittapun et al. (2020), reports that Phycocyanin is water-soluble and exhibits emission spectrum at 615-620 nm. Phycocyanin can inhibit antioxidants, trap alkoxyl, hydroxyl, and peroxyl radicals. Therefore, Phycocyanin is highly beneficial in preventing and slowing down the progression of various degenerative diseases [44]. The extraction of phycocyanin as a protein-pigment complex is quite straightforward due to its high solubility in water [15]. Achieving pure and stable C-PC (phycocyanin from *cyanobacteria*) requires careful selection of an extraction method, as the release of this pigment relies on the disintegration of the cell membrane. Numerous techniques for cell disruption have employed, with freezing and thawing cycles, bead milling, and mixing and homogenization being the most prevalent. Despite the relatively high purity achieved with the freezing and thawing method, this approach demands a lengthy processing time and presents challenges for scalability [45].

Conversely, bead milling is a rapid cell disruption method, but its application yields impure extracts with a high concentration of cell debris and other interfering compounds [46]. Mixing is a straightforward yet time-consuming method (typically hours) [47], while homogenization generally results in impure extracts. Consequently, the extraction of C-PC, aided by emerging technologies such as ultrasound, pulsed electric fields, and microwaves, has been explored as an alternative to overcome these limitations [48-49]. The industrial application of PC is constrained by its vulnerability to various industrial processes, such as presence of light, elevated temperatures (e.g., pasteurization), acidic or alkaline pH conditions, or the use of oxidizing agents during extraction, purification, and product preparation. These processes can induce precipitation or dissociation phenomena, causing the loss of the blue color [16-50].

2.3. Encapsulation

Encapsulation is a method involving the entrapment of bioactive ingredients in appropriate carriers to ensure protection and enable precise delivery to the intended site, achieved through the formation of particles or capsules at micro- or nanometer scales. This process provides numerous advantages, including increased thermal and chemical stability, preservation or masking of flavor, taste, or aroma, controlled and targeted release, and enhanced bioavailability of pigments. Bioactive compounds are easily volatile and highly sensitive to air, heat, light, and humidity. Therefore, encapsulation is a common method in the industry to protect bioactive compounds and preserve their taste and aroma [8-9-17]. The selection of coating material is critical in encapsulation, necessitating non-reactivity with active compounds and essential protective properties such as flexibility, strength, stability, and impermeability. In food applications, the coating must deemed safe, inert to the active substance, biodegradable, and sufficiently robust to shield the encapsulated material from the environment [18].

It is essential for the coating material to possess the capability to dissolve the active substance in the capsule under the desired release conditions. Achieving all the described properties with a single coating material is highly challenging. Therefore, it recommended utilizing combinations of various coating materials. Moreover, modified coating materials (such as modified cellulose) are preferable due to their improved physical and mechanical properties. In recent times, the most favored coating materials with film-forming ability include gums [51], proteins[52], natural and modified polysaccharides [53-54], synthetic polymers [55], gelatin [56], pectin [57], κ-carrageenan [58], agar [59], and whey [60-61]. The choice of encapsulation technique relies on the physical and chemical characteristics of both the core and the shell or coating material. The selection of the encapsulation method should be deliberate and not based on random attempts to find the correct process. The advantages obtained from encapsulation technology include achieving desired properties, enhancing product or process performance, and ensuring storage stability, among

other benefits [62]. Here are several techniques used in encapsulation as presented in Table 1.

Encapsulation usually carried out in micro or nano forms. Microencapsulation is the process of enclosing micron-sized materials within polymer shells. Hydrogels are one type of microencapsulation. Depending on their size, the formed capsules can referred to as macrocapsules, microcapsules, or nanocapsules, with diameters greater than 5000μm, ranging from approximately 1–5000μm, or less than 1μm, respectively [86]. Meanwhile, nanoencapsulation defined as the process of enclosing compounds in nano form within vesicular structures consisting of a polymer shell and usually a core serving as the active substance reservoir [87]. Nanocapsules can range from one to 1000 nm. Each nanocapsule can have different shapes, depending on the materials and methods used for preparation [88]. Encapsulation efficiency serves as a metric for the successful entrapment of the core material within the capsules [19]. The encapsulation efficiency (EE %) was determined using the formula:

$$EE\% = \left(\frac{Wt}{Wi}\right) \times 100\%$$

Here, Wt represents the total quantity of the incorporated material, and Wi is the total amount of the incorporated material added initially during the preparation process. The determination of Wt and Wi can be accomplished through spectroscopic or chromatographic methods. The efficiency of encapsulation can affected by several factors: How the target molecule distributes in the solvents during formulatio. The conditions employed in the encapsulation process, including temperature, pH, and mechanical stress. The variability in capsule sizes [89]. Numerous studies have conducted on encapsulation efficiency. Sittipummongkol et al. (2019), conducted a study by encapsulating neem seed oil with various coating materials (polyvinyl alcohol (PVA), gum arabic (GA), and whey protein isolate/maltodextrin (WPI/MD)) using a 1:2 ratio between neem seed oil and polymeric shell material. The encapsulation efficiencies obtained were 60.70 ± 2.73% (WPI/MD), 89.59-± 1.45% (GA), and 92.94 ± 3.87% (PVA), with the encapsulation method being spray-drying [72]. Another study conducted by Fadilah et al. (2023), where an encapsulation efficiency of 66.49% was achieved using κ-carrageenan as a coating material, along with the addition of 0.5 mL Tween 20 and 0.5 mL glutaraldehyde through the complex coacervation method [90].

Another study reported a maximum encapsulation efficiency of about 97% for electrosprayed κ-carrageenan nanoparticles [91]. In a different research, the encapsulation efficiency for curcumin, a natural bioactive compound, in pH-responsive hydrogel beads based on alginate, κ-carrageenan, and poloxamer found to be 95.74 ± 2.24% [92]. Encapsulation is a common practice in the industry, especially in the food sector, for preserving flavor compounds with specific taste and aroma. Taste and aroma are crucial active components in food. Controlled release of taste and aroma is a top priority in food processing. Various encapsulation methods yield different forms of encapsulation, such as pastes, powders, capsules, granules, and emulsion droplets. Most methods use flavor encapsulation to produce powder forms. The desired form depends on the end use of the flavor. Flavor powders used in the production of a wide range of food products, such as confectionery items (cakes, bread, biscuits, cookies, chocolate, and candies), powdered milk, instant desserts, instant beverages, baked goods, extruded snacks, and instant foods [93].

2.4. Chitosan

Chitosan derived from chitin by subjecting it to a deacetylation process, wherein chitin treated with a potent alkaline solution. The critical [94] factor for characterizing a chitosan sample is the degree of deacetylation. The degree of deacetylation plays a significant role in shaping the physical, biological, and chemical characteristics of the produced chitosan. This factor dictates the presence of exposed free amino groups resulting from the elimination of acetyl groups from the molecular chain of chitin, giving rise to the term deacetylation. It serves as a parameter for distinguishing between chitin and chitosan. The deacetylation process entails eliminating the acetyl group from the molecular chain of chitin, resulting in the presence of a complete amino group ($–NH_2$). The versatility of chitosan predominantly influenced by these highly reactive chemical amino groups. By adjusting factors such as reaction time, synthesis temperature, and the strength of the alkaline solution employed in the deacetylation process, chitosan with various chemical structures can be synthesized [95]. Chitosan is a linear polysaccharide composed of (β1 → 4) linkage of glucosamine and N-acetyl-glucosamine residues. Chitosan is soluble in aqueous media under acidic conditions through protonation of primary amines [96].

Crabs, shrimp, and several other crustacean shells contain chitin (β-(1 → 4)-N-acetyl-d-glucosamine), which, after reacting with the alkaline sodium hydroxide, produces a deacetylation product known as chitosan [96]. Chitosan possesses positively charged amino groups, creating a cationic biopolymer that is commercially available and water-soluble. The hydroxyl groups and cationicity within the chitosan structure offer numerous manipulation methods. Chitosan exhibits pH sensitivity due to D-glucosamine in its structure, making it soluble at acidic pH below 6 and insoluble at neutral pH [97]. Chitosan possesses high mucoadhesive and adsorption activities, antifungal capacity, film-forming capabilities, metabolic functions, and micro/nano formation. It exhibits biocompatibility, enhanced transfection, gel-forming properties, extremely low toxicity, waste inhibition characteristics, and hydrophilic traits, making it a suitable transport agent for delivering bioactive substances [98]. Microcapsules with chitosan layers utilized to protect active ingredients from external factors such as temperature and pH variations.

Various types of core materials, such as pharmaceuticals, food products, catalysts, oils, and pigments, can be microencapsulated using chitosan as the shell material. Chemically, chitosan contains free amine groups in both neutral and alkaline media, while protonated amines formed in acidic media. These groups are pH-sensitive, making chitosan-based compounds suitable for controlled release technology. Chitosan microcapsules containing drugs as active ingredients enable delayed release under specific conditions in targeted areas of the body. For example, lipophilic drugs encapsulated in chitosan for effective release in the human gastrointestinal tract [99]. Carboxymethyl Chitosan (CMC) is water-soluble derivatives of chitosan, each possessing strong positive and negative charges on their

surfaces, respectively. A new nanocomplex has developed using chitosan derivatives (CMC) to stabilize anthocyanins. Nanocomplexes form between anthocyanins and chitosan derivatives (CMC) through crosslinking interactions. At the optimal ratio, the resulting nanocomplexes exhibit high encapsulation efficiency, the desired particle size, and good stability [26-100].

2.5. Whey Protein Isolate (WPI)

Milk proteins fall into two primary groups: whey proteins and caseins [101]. Within whey proteins, whey protein isolate (WPI) stands out as a significant by-product derived from cheese, boasting a protein content exceeding 90%. WPI extensively utilized as carrier material for encapsulation due to its excellent emulsification and superior gelling properties. Additionally, WPI boasts high nutritional quality, offering excellent bioavailability with a concentrated presence of essential amino acids [102-103]. The key components of WPI include β-lactoglobulin (57%) and α-lactalbumin (19%), known for their outstanding abilities in forming protective films (wall system). In addition to β-lactoglobulin and α-lactalbumin, WPI also contains bovine serum albumin (7%), immunoglobulins (13%), and specific polypeptides (4%) [101-104-105]. Whey protein isolate (WPI) can also transformed into nanoparticles, serving as a promising material for microencapsulating various bioactive/hydrophobic compounds like vitamins, carotenoids, and flavor compounds [102]. This approach enhances stability and physiochemical properties [103-106-107]. The emulsifying properties of whey protein isolate can be improved through thermal treatment (heating above 60ºC), making it a useful encapsulating material with enhanced physicochemical characteristics [103-107].

In the study of the encapsulation of *Citrus reticulata* essential oil (CEO) conducted by Mahdi et al. (2022), it was concluded that the utilization of WPI in the formulation of wall materials, particularly with maltodextrin (MD) and gum Arabic (GA), enhances the physiochemical and stability properties of the nanoencapsulation of CEO. The nanoencapsulation of CEO using GA/MD/WPI improved the oxidative stability of the core materials, and the greatest enhancement observed when WPI incorporated into the wall materials. The active groups present in the amino acids of WPI can engage in intermolecular interactions with D-Limonene, suggesting that this interaction is likely responsible for the stabilization. In his study, he also noted that despite the poor flowability of the prepared powders, the formulations containing or utilizing their WPI composition showed enhanced flowability in comparison to the remaining preparations. An elevated proportion of WPI in the wall matrix led to enhanced encapsulation efficiency and reduced particle size in microcapsules containing wheat germ oil. This approach can also extended to encapsulate other essential oils [108].

3. Results and discussion

3.1. Encapsulation of Phycocyanin

3.1.1. Encapsulation of Phycocyanin with CMC-WPI Coating

Carboxymethyl Chitosan (CMC) is water-soluble chitosan derivatives with distinct negative charges. A novel nanocomplex (CMC) has developed to stabilize anthocyanins through cross-linking interactions. Whey protein isolate (WPI) is a valuable by-product of cheese production, containing over 90% protein. It serves as a preferred carrier for encapsulation, given its exceptional emulsification and gelling properties. WPI not only possesses high nutritional quality but also provides excellent bioavailability with a concentrated supply of essential amino acids [102-103]. Ionic gelation is a simple method that can used to encapsulate phycocyanin in CMC-WPI and $CaCl_2$ as crosslinking agent. CMC can bind to WPI to form a more stable polymer structure to entrap phycocyanin. The technical schematic shown in Figure 2. Baysan et al. (2021), investigated the effects of different combinations of coating material and stabilizer on the emulsion, physical, and chemical properties of propolis powder. The results showed that the use of WPI resulted in a higher yield of propolis powder compared to pure biopolymer [109].

On the other hand, Lee, J., and Duggan, E. (2022), explored the utilization of WPI microgels to encapsulate and shield vitamin D3 from environmental pressures. The study sought to evaluate the efficiency of WPI microgels in maintaining the stability of vitamin D3. WPI effectively preserved vitamin D3 at various temperatures over a four-week storage period, with <1% loss in the first week and >93% retention after four weeks. In contrast, unencapsulated vitamin D3 experienced significant losses, emphasizing the microgels' ability to protect and maintain vitamin D3 activity during long-term storage [110]. Encapsulation coating made of a mixture of (CHC/CMC)-WPI has used in various studies to enhance the stability and slow the release of anthocyanins in simulated digestion and prepared instant coffee. Wang et al. (2021), reported that the thermal stability of ACNs within the ACN-CHC/CMC-WPI nanocomplexes surpassed that of ACNs in an aqueous solution (ACN-AS). After heating, ACN-AS retained 48.3% of ACNs with a half-life of 291 min, while ACN-CHC/CMC-WPI nanocomplexes showed a higher retention rate of 60.7% and an extended half-life of 416 min.

The nanocomplex solution remained clear and transparent without precipitation post-heating. Additionally, the nanocomplexes exhibited superior stability and sustained release properties during simulated gastrointestinal digestion compared to ACN-AS. Moreover, the antioxidant activity of ACN-CHC/CMC-WPI nanocomplexes was higher (86.6%) than that of anthocyanin-loaded chitosan derivatives (ACN-CHC/CMC) (48.9%). Therefore, encapsulating phycocyanin using the CHC/CMC-WPI coating material is interesting for further study [26]. Freeze-drying used in phycocyanin encapsulation with CHC/CMC-WPI coating to improve the stability of the pigment. Phycocyanin is a water-soluble blue pigment extracted from microalgae that is sensitive to temperature, light, pH, and oxygen, which limits its applications in food and other products. Microencapsulation of phycocyanin using freeze-drying can improve its stability and protect it from degradation [111-112]. Many factors can influence encapsulation stability. Some of the factors that will explained in this paper are the effect of ratio, pH and concentration of $CaCl_2$.

3.1.2 The effect of ratio

The ratio of bioactive core content to coating material can significantly affect the encapsulation characteristics of the resulting product. Ge et al. (2018) conducted a study on the influence of the CHC: CMC ratio as

an encapsulation coating material at various ratios. As the CHC/CMC ratio decreased in the nanocomplex, the average particle size initially decreased and then increased. The smallest particle size was 178.1 nm, obtained from the nanocomplex with a CHC: CMC ratio of 1.2[91]:1 [100]. The phenomenon of larger particles in nanocomplexes with the lowest CHC/CMC ratio has reported previously [113]. Tirta et al. (2023) conducted a study on the influence of the WPI ratio on encapsulation coating material. In their study, it concluded that the effect of the WPI ratio on survivability during storage, yield, and moisture content was not apparent [114]. According to the results of the study conducted by Zhao et al. (2022), the higher the content of WPI in the coating material, the lower the encapsulation efficiency (EE) produced. In the WPI ratio of 2, an EE of 93.29 ± 0.41% was obtained, while in the ratio of 1, an EE of 97.8 ± 0.44% was obtained [115].

Makouie et al. (2020), in their study, added that the higher the content of whey protein isolate (WPI) in the coating material, the smaller the particle size produced. They also mentioned that raising the amount of whey protein isolate led to an elevation in the zeta potential of the microcapsules. This could be a result of the WPI having a net negative charge at pH levels higher than its isoelectric pH. Additionally, this effect can linked to an effective protein coating, causing an increase in the negative repulsive force on the particle surface, ultimately resulting in a more negative zeta potential. An increase for WPI can also reduce electrical conductivity in particles. This study confirms that selecting the appropriate wall material ratio is vital in phycocyanin encapsulation with good physicochemical properties, high encapsulation efficiency, and survivability against gastrointestinal and storage conditions [116].

3.1.3 The effect of pH

pH is an important variable in the phycocyanin encapsulation process. In Pradeep and Nayak (2019) study, the primary focus was on the stability of phycocyanin when subjected to encapsulation through the extrusion method. Existing literature indicates that phycocyanin experiences significant instability in its natural state beyond 50°C, leading to changes in its properties and denaturation. To evaluate the stability of microencapsulated PC, experiments conducted at varying pH levels (4.5, 5.5, 6.5, and 7.0) and two distinct temperatures (45°C and 55°C) [91].The degradation of PC quantified by assessing the relative concentration following incubation at the specified temperatures (45°C and 55°C).

Optimal stability observed at pH 6.5, while the least stability occurred at pH 7.0 and pH 4.5. The protein underwent degradation under both acidic and alkaline pH conditions, leading to reduced relative concentrations. Notably, minimal degradation noted at pH 6.5 under both 45°C and 55°C, suggesting that encapsulated PC exhibited instability at extreme pH values [91]. Zheng et al. (2014), reported similar findings, highlighting the instability of phycobiliprotein in highly acidic and alkaline environments. In contrast, encapsulated PC demonstrated stability across a wide range of pH values in their study [117].

3.1.4 The effect of $CaCl_2$ addition as crosslinker

$CaCl_2$ used as a crosslinker in the encapsulation of phycocyanin. It used to form a gel matrix with substances, which helps in the encapsulation of phycocyanin, providing stability and antioxidant activity. The use of $CaCl_2$ as a crosslinker is preferred due to the non-toxic nature of Ca^{2+} and its frequent use in crosslinking applications [91-118-119]. It observed that the utilization of calcium as a crosslinker had a notable impact on maintaining the integrity of the enclosed protein. The protein encapsulated within the matrix to shield it from organic solvents, and the matrix played a crucial role in safeguarding the structural integrity of the protein [91]. Hadiyanto et al. (2017), stated that the concentration of $CaCl_2$ did not markedly impact the encapsulation efficiency and phycocyanin load. This lack of influence can attributed to the substantial molecular weight of phycocyanin, which hinders its release from the gel [118].

Pan-utai and Iamtham (2019), conducted a study on the influence of various concentrations of $CaCl_2$ as a cross-linker on the particle diameter and shape in the encapsulation of phycocyanin. Different particle shapes and diameters observed at various $CaCl_2$ concentrations, where the ideal particle shape should be spherical. Based on the obtained data, the optimal concentration for encapsulation was found to be 2.5% (w/w), resulting in spherical particles with high encapsulation efficiency [112]. According to Zheng et al. (2014) in their research, a 3% $CaCl_2$ concentration can produce uniform microcapsules with a spherical shape. However, with concentrations of 1% and 5% $CaCl_2$, microcapsules of various sizes and non-spherical shapes can formed. Therefore, in their study, they utilized a 3% $CaCl_2$ concentration in the microcapsule preparation. This study affirms that the concentration of $CaCl_2$ plays a crucial role in the encapsulation process, influencing both the particle diameter and the shape of the encapsulated phycocyanin [117].

3.2. Discussion

3.1.2. Recent Progress and Future Perspective for Encapsulation of Phycocyanin with CMC-WPI Coating

The encapsulation of phycocyanin with CMC-WPI coating is a topic of recent research that holds promise for various applications. Phycocyanin, a natural blue pigment found in blue-green algae, has attracted attention due to its potential health benefits and uses in the food and pharmaceutical industries. Encapsulation technologies aim to improve the stability and bioavailability of phycocyanin, making it more suitable for use in different products. Recent studies have explored various encapsulation techniques, including spray drying, extrusion, electrospraying, emulsion, lipid encapsulation, and sol-gel method. These techniques offer different advantages in terms of the encapsulation efficiency, particle size, and stability of the resulting phycocyanin microcapsules.

In addition to the encapsulation techniques, researchers have also investigated different materials for the encapsulation of phycocyanin. Both natural polymers, such as alginate, chitosan, and gelatin, and synthetic polymers have studied for their suitability as wall materials for microencapsulated phycocyanin. Furthermore, studies have focused on the development of complex composites, such as alginate-agavins-polysaccharide beads, for the controlled release of phycocyanin in simulated gastrointestinal conditions [120]. The stability of encapsulated phycocyanin has been a key focus of research, with studies evaluating the effects of temperature, pH, and storage conditions on the stability of phycocyanin microcapsules.

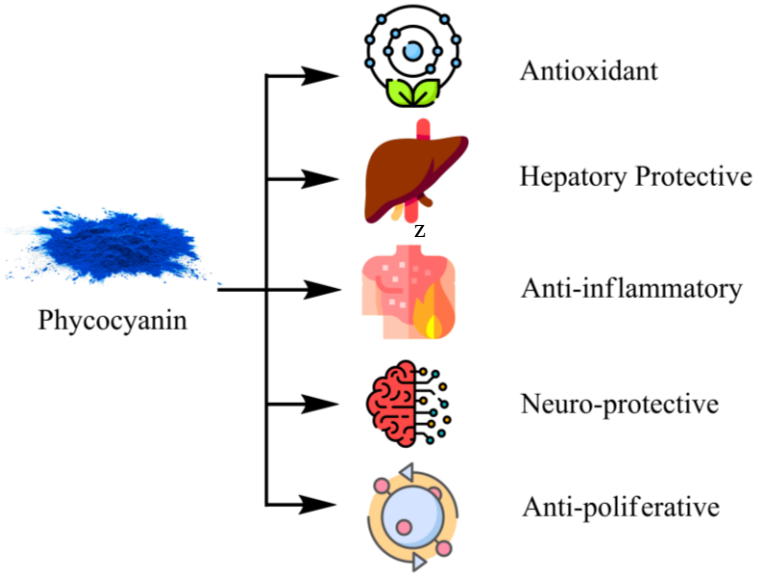

Figure 1. Some of phycocyanin beneficial

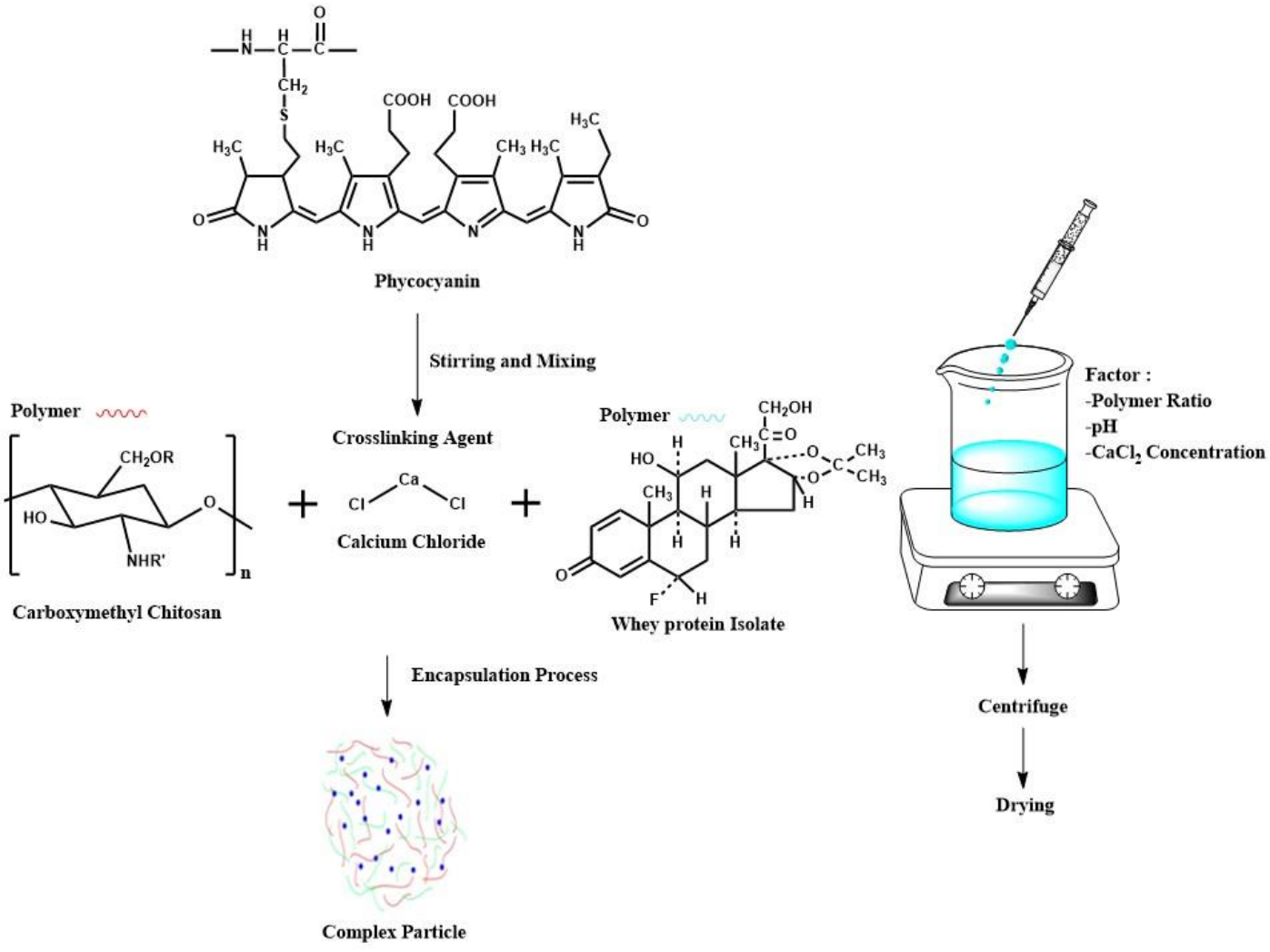

Figure 2. Schematic illustration of preparing CMC-WPI-PC complex

Table 1 Various techniques for encapsulation

Technique	Active Substance	Coating	Enhanced Property	References
Extrusion	Phage SL01	Alginate/ κ-carrageenan	Better survivability (pH, enzymes) and bioavailability	[63]
	Polyphenols from *Mesona chinensis* Benth extract	Alginate	Bioaccessibillity and bioavailability	[64]
	Polyphenols of Piper Betel leaves	Alginate	Taste, stability, and oxidation	[65]
Emulsion electro-spraying assisted by pressurized gas	Algae oil	Wheat gluten extract	Bioavailability, oxidation, controlled release, organoleptic properties	[66]
Emulsion	Curcumin	Sunflower oil, carboxymethylcellulose, lecithin	Less degradation, bioavailability, photochemical stability	[67]
Nanoemulsion	Thymne oil	Chitosan	Control release	[68]
	Vitexin	Medium-chain triglyceride	Bioavailability, antioxidant activity, storage stability and *in vitro* digestibility	[69]
Spray drying	Anthocyanins	Maltodextrin	Stability and shelf life	[70]
	Saccharomyces boulardii	Rice protein, maltodextrin	Prolonged storage, less degradation, effectiveness	[71]
	Neem seed oil	polyvinyl alcohol, gum arabic, and whey protein isolate/maltodextrin	Polyvinyl alcohol: water solubility, biodegradability, and film-forming ability Gum arabic: good emulsifier, has sufficient viscosity, can be easily solubilized in water, and is capable of forming polymeric films Maltodextrin: good film-forming ability and can be combined with protein or starch to produce shell material Whey protein isolate: used in combination with maltodextrin because of its good emulsification and antioxidant properties	[72]
Multiple step preparation including modified Störber sol-gel Process	Mn_3O_4	Hollow carbon sphere coated by graphene layer	Improving the efficiency of lithium-ion batteries	[73]
Freeze drying	Anthocyanins and phenolic acids from Blackthorn (Prunus spinosa L.) extract	Maltodextrin	Bioavailability, physico-chemical and biological degradation, shelf life.	[74]
	Propolis	Whey protein isolate	Taste, bioavailability, odor.	[75-76]
Synthesis of QD, growth of iron shell, and oxidation to form iron oxide shell	Quantum dots	Iron oxide	Optical properties	[77]
Sol-gel method	SiO_2	ZnO	Photoactivity properties	[78]
Pelletization process, coating processes	Calcium acetate/sodium carbonate (or composite of two), superabsorbent polymers, poly(ethylene glycol)	Epoxy resin and fine sand	Alkali resistance, durability, mineralization time, waterproof.	[79]
Vacuum facilitated infusion	Curcumin	*Geotrichum candidum arthrospores*	Chemical stability, water solubility, bioavailability.	[80]
Self-assembly of biopolymers	Anthocyanins	Whey protein isolate and pectin	Molecular instability	[81]
Internal phase separation	N-acetylcysteine	Ethylcellulose	Astringency, sulfur smell, bitter aftertaste.	[82]
Lipid encapsulation	Gamma-oryzanol	Stearic acid, sunflower oil/rice bran phospholipids, Tween 80	Water solubility and size	[83]
Electrospraying	D-limonene	κ-carrageenan	Biodegradable, great viscosity, and series of potential biomedical attributes	[84]
Ionic Gelation	Vitamin D3	Carboxymethyl chitosan-soy protein isolate	Higher encapsulation efficiency, exhibit release of vitamin D3 in simulated gastric fluid and intestinal	[85]

Understanding the factors that influence the stability of encapsulated phycocyanin is crucial for its successful application in various products. Overall, the research on the encapsulation of phycocyanin with CMC-WPI coating reflects a growing interest in the development of functional food and pharmaceutical products that harness the potential of this natural pigment. The combination of advanced encapsulation techniques and the use of diverse materials holds promise for the future application of phycocyanin in a range of products.

4. Conclusion

The encapsulation of phycocyanin using a CMC-WPI coating represents a promising approach to address the challenges associated with the stability of this natural blue pigment. The nanocomplexes formed through the interaction of carboxymethyl chitosan (CMC) and whey protein isolate (WPI) demonstrate high encapsulation efficiency and stability, with optimal results achieved at a specific ratio. The addition of whey protein isolate (WPI) further enhances the encapsulation process, providing stability against environmental pressures and improving the overall physicochemical properties of the encapsulated phycocyanin. The study emphasizes the importance of selecting the appropriate ratio of CMC and WPI for achieving desired encapsulation characteristics. The ratio significantly influences particle size, encapsulation efficiency, and zeta potential. Additionally, the findings underscore the role of pH in the encapsulation process, with optimal stability observed at specific pH levels.

The use of $CaCl_2$ as a crosslinker highlighted as a crucial factor, affecting both the integrity of the encapsulated protein and the characteristics of the resulting particles. Overall, the encapsulation of phycocyanin with CMC-WPI coating proves effective in improving the stability of the pigment, making it more resistant to factors such as temperature, light, pH variations, and oxygen. This encapsulation approach holds promise for applications in the food industry, where the stability of food colorings is crucial. Further research and exploration of this encapsulation technique may unveil its potential in enhancing the utilization of phycocyanin in various products, contributing to both visual appeal and potential health benefits associated with its antioxidant and anti-inflammatory properties.

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