



Development of a Whey Protein Hydrogel as an Alternative for the Microencapsulation of Calyx Extracts from *Hibiscus sabdariffa*[†]

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Abstract: In the present study, a whey protein-based hydrogel was developed as an alternative for the microencapsulation of *Hibiscus* extracts. The resulting hydrogels showed a high percentage of moisture and water activity, along with a partial degradation of anthocyanins during their production and storage. The hydrogels exhibited particle sizes between 8 and 15 µm and retained some of the characteristic color properties of extracts. The results obtained provide a novel alternative for the microencapsulation of bioactive compounds through the use of protein-based carrier systems, highlighting their potential for the development of innovative encapsulation systems.

Keywords: emulsion method; thermal gelation; carrier system; natural colorant



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1. Introduction

There is currently a growing trend in the production of natural foods due to the demand from consumers who are increasingly concerned about the quality of their diet and its impact on their health. For this reason, significant research has been conducted to explore natural food ingredients, such as pigments, that may offer potential health benefits for humans [1]. In nature, there is a wide variety of fruits and vegetables from which pigments can be obtained. One example is the dried calyxes of the *Hibiscus* flower (*Hibiscus sabdariffa*), which are a rich source of bioactive compounds that provide numerous benefits for human health [2], and have potential applications in both the food and pharmaceutical industries, where their medicinal properties have been extensively studied. In this regard, Galicia-Flores et al. [3] documented several positive effects of calyxes, such as diuretic and choleretic properties, reduction in blood pressure, and lower cholesterol levels. Additionally, it has been suggested that, due to their antioxidant properties, calyxes may play an important role in the prevention and treatment of certain types of cancer [4].

Their beneficial contributions are due to the presence of different functional compounds, such as phenolic compounds, organic acids, proanthocyanidins, flavonoids, and anthocyanins [3]. Anthocyanins are the most important compounds because they are responsible for their characteristic color; however, these molecules are susceptible to environmental factors such as increases in pH, temperature, and oxygen, which makes them relatively unstable [5]. This represents a problem, as the handling of natural pigments requires control and stability in order to adapt them to the conditions of use, processing, and storage. These techniques aim to protect sensitive compounds by forming microparticles with a porous polymeric membrane that encloses an active substance. This approach

offers several advantages, such as protecting the encapsulated compound from degradation caused by heat, oxygen, light, and moisture. Additionally, it allows for a gradual release of the compounds and improves the malleability of the materials [6]. There is a wide variety of potentially applicable microencapsulation technologies, ranging from well-established to relatively new methods. In recent years, studies have shown that the microencapsulation of phenols using protein hydrogels is a promising method for anthocyanin stabilization [7] and their incorporation into food systems.

In accordance with the above, the objectives of the present work were as follows: first, the preparation and physicochemical characterization of *Hibiscus* extracts; second, the microencapsulation of *Hibiscus* flower pigments through the development of a three-dimensional network structure based on whey protein; and finally, the physicochemical characterization of the generated hydrogels.

2. Methodology

2.1. Raw Material Procurement

The dried *Hibiscus* calyxes were obtained from a local market in Orizaba, Veracruz, Mexico, and whey protein was acquired from Amhfer Foods (CDMX, México).

2.2. Anthocyanin Extraction

Anthocyanin extraction was performed as reported by Parra-Campos & Ordóñez-Santos [8]. Briefly, extraction was carried out using a 60% ethanolic solution acidified to pH 1.5 with 1 M HCl, at a 1:10 (*w/v*) ratio. The mixture was shaken for 1 h, vacuum filtered, and concentrated at 45 °C under vacuum conditions in a rotary evaporator (Buchi, Flawil, Switzerland). Final soluble solids were adjusted to 5 °Brix, measured with a refractometer (Atago, Tokyo, Japan).

2.3. Determination of Total Monomeric Anthocyanin Content in Extracts

The content of total anthocyanins in the extract and hydrogel was determined by the differential pH method, according to the method described by Parra-Campos & Ordóñez-Santos [8], using two buffer solutions: potassium chloride (KCl), pH 1.0 (0.025 M), and sodium acetate (CH₃COONa), pH 4.5 (0.4 M). These were measured at absorbance values of 520 nm (maximum molar absorbance of anthocyanins) and 700 nm (correction factor) in a UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The results were expressed as total anthocyanins, following Equations (1) and (2).

$$A = (A_{520} - A_{700})_{\text{pH1}} - (A_{520} - A_{700})_{\text{pH4.5}} \quad (1)$$

$$\text{Anthocyanins} \frac{\text{mg}}{\text{L}} = \frac{A * \text{MW} * \text{DF} * 1000}{\epsilon * 1} \quad (2)$$

where *A* is the result of Equation (1), *MW* is the molar weight of cyanidin 3-glucoside (449.2 g·mol^{−1}) (C3G), *DF* is the dilution factor, ϵ is the molar extinction coefficient, and 1 is the beam path.

2.4. Whey Protein-Based Hydrogel Generation

Hydrogel generation was performed as reported by Bilek et al. [7]. A 15% (*w/v*) aqueous whey protein solution was prepared using deionized water. Subsequently, 33.33 mL of the milk protein solution was mixed with 5 g of the extract, SPSL-EJ. The SPSL-EJ solution was homogenized and acidified to pH 1.5 using 3 M HCl.

The SPSL-EJ solution was centrifuged at 5000 × *g* for 5 min at 20 °C using a microcentrifuge (DLAB, Beijing, China) to separate the insoluble fractions. The supernatant was then recovered and mixed with soybean oil at 50 °C in a 1:5 (*v/v*) ratio, supernatant/soybean oil, to carry out microencapsulation by the emulsification method. The mixture was then heated from 50 °C to 80 °C over a period of 6 min and kept at this temperature for 10 min to allow gelation. Once the time had elapsed, the suspension was allowed to cool to 20 °C and

centrifuged again under the same conditions. The oil was discarded, and the sedimented microcapsules were recovered and washed with NaCl at pH 1.5, acidified with 1 M HCl. Finally, the samples were centrifuged and stored at 4 °C in amber bottles until analysis.

2.5. Physicochemical Characterization of Hydrogels

2.5.1. Evaluation of Color Properties

The color of the hydrogels was evaluated using a CR400 colorimeter (Minolta, Osaka, Japan). The results were interpreted according to the CIE Lab scale, with its values used for the determination of Chroma, Hue angle, and total color difference (ΔE), as shown in Equations (3), (4), and (5), respectively [9].

$$\text{Chroma} = \sqrt{a^2 + b^2} \quad (3)$$

$$\text{Hue angle} = \tan^{-1} \left(\frac{b}{a} \right) \quad (4)$$

$$\Delta E = \sqrt{(L_0 - L_1)^2 + (a_0 - a_1)^2 + (b_0 - b_1)^2} \quad (5)$$

where the subscript 0 corresponds to the reference values.

2.5.2. Water Activity and Moisture Content

The moisture content of the hydrogel was determined through the gravimetric method using a drying oven. About 1 g of the hydrogel was weighed and then dried at 105 °C for 24 h. The results were calculated according to moisture percentage. The water activity (a_w) was measured using a water activity meter (Novasina, LabMaster, Lachen, Switzerland), placing 1 g of the sample. The determination was carried out at 25 °C, and the equipment was calibrated with standard supersaturated salt solutions before each measurement.

2.5.3. Particle Size

Particle size was determined by calculating the arithmetic mean of 300 hydrogel particles measured using a Motic BA310E optical microscope (NJ, USA) and Motic imaging software, Images Plus 2.0.

2.6. Statistical Analysis

All determinations were carried out in triplicate, and the results are presented as means with standard deviation. Analysis of variance (ANOVA) was performed using Minitab software v. 17 (PA, USA).

3. Results and Analysis

3.1. Characterization of Whey Protein-Based Extracts and Hydrogels

As mentioned above, *Hibiscus* calyces are a rich source of compounds such as organic acids and pigments such as anthocyanins. Therefore, they are considered a rich source of antioxidants and are used in a wide number of products marketed as natural and healthy products, such as jellies, teas, sauces, syrups, wines, and extracts [10], with special emphasis on anthocyanins. The concentration of total anthocyanins obtained for the concentrated extract was 81.37 ± 2.63 mg cyanidin-3-glucoside·L⁻¹, similar to that reported by Nhut-Pham et al. [11], who reported values ranging from 87.47 to 158.56 mg cyanidin-3-glucoside·L⁻¹ for extracts with different mass-to-solvent ratios.

Obtaining lower concentrations could be related to aspects specific to the crop, such as seasonal variation or genetic variability, since the influence of these factors on the nutritional characteristics and concentration of functional compounds has been well established [12]. The hydrogels exhibited a 38% reduction in the monomeric anthocyanin content, which could be related to the degradation of the molecule due to its exposure to relatively high temperatures during some of the steps involved in the emulsification technique. This is consistent with the findings reported by Bilek et al. [7], who evaluated the degradation of

black carrot anthocyanins microencapsulated in a whey protein-based hydrogel during each step of this technique. The authors reported a degradation range of 10.18% to 22.22%, with the degradation being more pronounced in those steps that involve the use of temperature.

Table 1 shows the color data. The parameter a , which corresponds to the red/green color, was 31.09 ± 0.37 ; that is, the concentrated extracts are located in red colors, which is reflected in the obtaining of a Hue angle of 6.87 ± 0.52 , corresponding to shades or nuances in this color, while the values obtained for the hydrogel were 16.91 ± 1.84 and 19.99 for the parameters a and Hue angle, respectively, i.e., a 45.60% reduction in the red color (a^* parameter) and the tendency of this toward green colors, which could be related to the degradation of anthocyanins. With respect to the Hue angle, it increased more than twice the initial value (in relation to the initial extract). Although this could be interpreted as an increase in the concentration of anthocyanins, the results obtained for total anthocyanins suggest that this is rather related to the loss of water and the incorporation of the pigment into the microstructured protein system, which makes it perceived differently. The behavior in chroma (color intensity or saturation) was similar to that of parameter a , showing that the extracts have a more intense red color.

Table 1. Characterization of concentrated extracts and whey protein hydrogels.

Parameter	Extract	Hydrogel
Moisture content (%)	81.01 ± 1.20	82.22 ± 1.003
Water Activity	-	0.93 ± 0.003
Color parameters		
L	33.53 ± 0.51	29.70 ± 1.59
a	31.09 ± 0.37	16.91 ± 1.84
b	3.75 ± 0.31	6.09 ± 1.03
Chroma	31.32 ± 0.39	18.02 ± 1.65
Hue angle	6.87 ± 0.52	19.99 ± 2.26
ΔE	0.58 ± 0.30	14.99 ± 1.78
Particle size (μm)	-	7.42 ± 3.81
Total anthocyanins (C3G)	81.37 ± 2.63	50.31 ± 0.85

Both a_w and moisture content parameters are frequently used to evaluate the stability of a food product. The water activity describes the degree of “binding” and, therefore, its capacity to participate in degradation reactions, as can be seen in Table 1. The a_w values in the hydrogels generated were 0.93 ± 0.003 , which means that a large fraction of the water contained in the gel is in free form. This behavior is expected in a product such as hydrogels, as their moisture content is high; these have gained great attention for that characteristic, as they have the ability to absorb a large amount of water within their structure (up to 100 times their weight) without dissolving [13], and they are able to release up to 95% of the adsorbed water to the surrounding environment. Similar results were obtained by Cassiani et al. [14], who reported values from ≈ 0.91 to 0.98 for different whey protein mixes.

The particle size in an encapsulating system is important as it determines the release mechanisms. Protein gelation for the manufacture of fragments can be classified according to particle size; those with a size $<100 \mu\text{m}$ are classified as micrometric (microhydrogels) and those with a size $<0.1 \mu\text{m}$ as nanohydrogels [15]. The values obtained in the present work ranged from 8.75 ± 1.70 to $15.15 \pm 2.19 \mu\text{m}$, so they can be considered as microhydrogels. These values were lower than those obtained by Habibi et al. [16], who reported a mean particle size of $\approx 63.5 \mu\text{m}$ for whey protein and monoacylglycerol bigels.

3.2. Stability of Color During Storage

Color is one of the most important parameters during processing, since it represents the first contact with the consumer. Therefore, it is necessary to control its stability during storage. At the end of storage, a tendency for the red colors to decrease was observed,

registering a reduction from 24.01 ± 4.10 to 16.10 ± 1.34 (on a^* value), is to say a loss of $32.94 \pm 3.72\%$ on this value. As with extraction, this could be related to a decrease in pigment concentration. As there was a smaller amount of encapsulated pigment, the appreciation of yellow tones, described by parameter b , showed a tendency to increase from 7.37 ± 0.31 to $9.37 \pm 1.35\%$, that is to say, an increment to 27.13% in relation to the initial value. These changes during storage were perceptible to the human eye, according to the ΔE scale, whose value was 8.43 ± 1.69 .

4. Conclusions

The results of the present study provide a novel alternative for the microencapsulation of bioactive compounds using protein-based carrier systems, which is relevant to the development of innovative encapsulation systems. The hydrogels generated exhibited a particle size that classifies them as microhydrogels, with high moisture content and water activity. Partial degradation of anthocyanins was observed due to the use of heat during processing, as well as a decrease in color properties during storage for three weeks. This highlights the need for further stability testing during storage.

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