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Research Article

# Microencapsulation of Black Carrot Anthocyanins for Enhanced Thermal Stability

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## Abstract

Pigments obtained from plants and algae are utilized as colour additives in food and pharmaceutical formulations due to the advantages of being non-toxic and possessing several biological activities. However, the low stability limits the utilization of natural pigments and therefore strategies such as chemical modification or encapsulation are required. This study aimed to improve the thermal stability of black carrot anthocyanins by microencapsulation. For this purpose, anthocyanin-rich black carrot extracts were obtained by ultrasound-assisted extraction and four different solvent systems were compared regarding extraction yield. The effect of parameters such as concentration and flow rate of alginate solution, stirring rate and temperature of $CaCl_2$ solution and needle diameter on the average size, polydispersity (PDI), and sphericity of alginate microparticles were examined. Optimum conditions were elicited as 2% concentration and 1 ml/min flow rate for alginate solution, 40 rpm stirring rate of $CaCl_2$ solution at 4°C and 0.45 mm of needle size resulting in 462.4 µm of particle size. Heat treatment was also applied and the retention efficiencies were determined as 96.92% and 75.82% for encapsulated and free anthocyanins, respectively. In addition, the half-life of anthocyanin-rich extract has been shown to increase from 7.5 h to 66.5 h by microencapsulation. These findings indicated the ability of alginate microparticles for the protection of black carrot anthocyanins from thermal degradation and improvement of storage stability.

*Keywords:* Alginate microparticles, Anthocyanin, Black carrot, Encapsulation, Thermal stability.

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## 1. Introduction

Colour additives or food colorants have been utilized as an indicator for the freshness of food as well as in non-food applications such as pharmaceutical formulations [1, 2]. These compounds are categorized based on various properties and one of the most popular classifications is the division as natural and synthetic colorants. Synthetic colorants have several advantages over their natural counterparts such as high resistance to degradation by light, heat or oxygen and low cost [3, 4]. However, the use of synthetic colorants as mixtures or high consumption may cause undesirable side effects such as hypersensitivity reactions [1]. Due to these limitations, increasing health concerns and consumer demand, natural colorants are becoming more popular.

The most common natural food colorants are chlorophyll for green, carotenoids for yellow and orange, anthocyanins for red and purple colour and these colorants are obtained from plants or algae by various extraction methods. While the main objective of previous applications was their colouring feature, they attract attention nowadays due to their possible health benefiting effects [5, 6].

Anthocyanins are water-soluble pigments which are responsible for the red, blue and purple colour of fruits and vegetables, and belong to the flavonoid class of phenolic compounds. They display a great number of biological activities and health promoting benefits such as radical scavenging, antimutagenic, anti-inflammatory, antihypertensive activities and reducing the risk of cancer, diabetes, cardiovascular and neurodegenerative disorders. Although anthocyanins possess significant potential as a food colorant, they are known to be instable under heat and light exposure and pH changes, which is a limitation that needs to be overcome [7–11].

The common methods for the stability enhancement of anthocyanins are chemical modification such as acylation and encapsulation [12]. Encapsulation is a viable approach to protect active compounds from harsh environmental conditions, to enable easier handling or to provide a controlled release behaviour [13, 14]. Emulsification, gelation, liposomal encapsulation and spray-drying are widely applied encapsulation strategies that mainly utilize polysaccharides, gums and proteins as encapsulating agents [15]. The ionotropic gelation or ionic cross-linking method is based on the crosslinking of polyelectrolyte polymers such as alginate, chitosan and pectin by counter ions [16]. In particular, sodium alginate is widely utilized in food, biomedical and pharmaceutical applications. It is a linear and anionic polysaccharide composed of α-1,4-l-guluronic acid and β-1,4-d-manurunic acid units and is commercially available from brown algae [17]. It is biodegradable, biocompatible and nontoxic and has the ability to form cross-linked gel structures in the presence of divalent and multivalent cations [18].

The objective of this study was to improve the thermal stability of black carrot anthocyanins by encapsulation. For this purpose, black carrot anthocyanins were obtained by ultrasound-assisted extraction by using water and ethanol at different concentrations. Alginate microparticles were prepared by ionotropic gelation method and the effects of parameters such as alginate concentration, flow rate of alginate solution, stirring rate and temperature of $CaCl_2$ solution and needle diameter were examined. Heat treatment was also applied to free and encapsulated anthocyanins to determine the influence of encapsulation on thermal stability. Thus, the effect of alginate based microencapsulation on the thermal stability of anthocyanin-rich black carrot extracts obtained by ultrasound-assisted extraction was determined.

## 2. Materials and Methods

### 2.1. Plant Material

Black carrots were purchased from a local market. They were washed with tap water, sliced into small pieces and stored at -20°C until extraction experiments. Sodium alginate was from Tito. Calcium chloride, potassium chloride, sodium acetate and citric acid were purchased from Merck. Ethanol and methanol were from Honeywell.

### 2.2. Ultrasound-assisted Extraction of Black Carrot Anthocyanins

Anthocyanin extraction was carried out by ultrasound-assisted extraction method. Ultrasound-assisted extraction is one of the modern extraction techniques which overcome the limitations of laborious and time-consuming conventional extraction approaches and is extensively used in food and pharmaceutical industries. In ultrasound-assisted extraction, mass transfer is enhanced by high shear forces and acoustic-induced cavitation in liquid medium [19, 20]. Consequently, enhanced yield and extract quality, lower energy and solvent consumption and reduced extraction time were obtained compared to conventional extraction techniques. In addition, ultrasound-assisted extraction is favourable for efficient extraction of thermally unstable compounds for which low or unsatisfactory yields are achieved by conventional methods [21–23].

In this study, sliced black carrots were weighed and mixed with extraction solvent (100% (v/v) water, 30% (v/v) ethanol, 70% (v/v) ethanol, 100% (v/v) ethanol) at a constant solid:liquid ratio of 1:20. An ultrasonic bath (Weightlab Instruments, Türkiye) with 40 kHz of frequency and 80 W of ultrasonic power was used and extraction experiments were conducted at 40°C for 40 min of extraction duration. After the extraction, samples were filtered through a filter paper. All experiments were done in triplicate.

### 2.3. Determination of Anthocyanin Content

The anthocyanin content of extracts was determined by the spectrophotometric pH differential method [24].This method is based on the reversible transformation of anthocyanin structure at different pH values. Anthocyanin molecules are pigmented at pH 1 while neutralized and become colorless at pH 4.5. The concentration of anthocyanin is proportional to the absorbance difference at $\lambda_{vis\text{-}max}$ (510 nm) and calculated by using the molar extinction coefficient and molecular weight of the major anthocyanin the sample [25, 26]. Briefly, two dilutions of samples were prepared by 0.025 M potassium chloride and 0.4 M sodium acetate solutions for the pH adjustment of samples to pH 1 and pH 4.5, respectively. The absorbance of obtained dilutions was measured at 510 and 700 nm wavelengths and, monomeric and total anthocyanin contents were calculated as cyanidin-3-glucoside equivalent by the following equations:

$$Monomeric\ anthocyanins\ \left(\frac{mg}{L}\right) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l} \quad (1)$$

$$A = (A_{510} - A_{700})_{pH=1} - (A_{510} - A_{700})_{pH=4.5} \quad (2)$$

$$Total\ anthocyanins\ \left(\frac{mg}{L}\right) = \frac{A' \times MW \times DF \times 1000}{\varepsilon \times l} \quad (3)$$

$$A' = (A_{510} - A_{700})_{pH=1} \quad (4)$$

where DF is dilution factor, MW is molecular weight (449.2 g/mol), l is optical path length and ε is extinction coefficient for cyanidin-3-glucoside which is equal to 26900 L $mol^{-1}$ $cm^{-1}$.

### 2.4. Preparation of Alginate Microparticles

Alginate microparticles were prepared by the ionotropic gelation method [27]. Briefly, alginate solutions at two different concentrations (1% and 2%) were prepared by dissolving sodium alginate in distilled water overnight at room temperature. The obtained alginate solution was pumped through a needle by a syringe pump (NE-300, New Era Instruments, USA) into 2% (w/v) $CaCl_2$ solution and prepared microparticles were kept in $CaCl_2$ solution for 45 min under continuous stirring. Then, alginate microparticles were removed by filtration, rinsed with distilled water and dried at room temperature for 72 h prior to size measurement. Experiments were carried out to determine the effect of parameters such as alginate concentration, flow rate of alginate solution, temperature and stirring speed of $CaCl_2$ solution and the needle size on the size, size distribution and sphericity of alginate particles as shown in Table 1. For all experiments, $CaCl_2$ concentration, dripping distance (5 cm) and volumetric ratio of alginate and $CaCl_2$ solutions (1:10) were kept constant.

**Table 1:** Experimental conditions for the preparation of alginate microparticles.

| Exp. No | Alginate solution | | $CaCl_2$ Solution | | Needle |
|---|---|---|---|---|---|
| | Concentration | Flow rate | Stirring rate | Temperature | Size |
| 1 | 1% | 1 ml/min | 40 rpm | 24ºC | 0.8 mm |
| 2 | 1% | 1 ml/min | 80 rpm | 24ºC | 0.8 mm |
| 3 | 2% | 1 ml/min | 40 rpm | 24ºC | 0.8 mm |
| 4 | 2% | 1 ml/min | 80 rpm | 24ºC | 0.8 mm |
| 5 | 1% | 3 ml/min | 40 rpm | 24ºC | 0.8 mm |
| 6 | 2% | 3 ml/min | 40 rpm | 24ºC | 0.8 mm |
| 7 | 1% | 3 ml/min | 40 rpm | 4ºC | 0.8 mm |
| 8 | 2% | 3 ml/min | 40 rpm | 4ºC | 0.8 mm |
| 9 | 2% | 1 ml/min | 40 rpm | 4ºC | 0.45 mm |
| 10 | 2% | 3 ml/min | 40 rpm | 4ºC | 0.45 mm |

### 2.5. Measurement of microparticle size, sphericity and polydispersity index

Randomly selected alginate microparticles were examined and images were taken by light microscopy. Average particle size was determined by ImageJ software [28] and polydispersity index (PDI) [29] was calculated by equation (5):

$$PDI = \left(\frac{\sigma}{d}\right)^2 \tag{5}$$

where σ is standard deviation and d is mean particle diameter.

The shape of produced microparticles was characterized by sphericity factor (SF) [30] and sphericity coefficient (SC) [31] as dimensionless shape indicators. The equations (6) and (7) were used to calculate SF and SC, respectively.

$$Sphericity\ factor = \frac{(d_{max} - d_{min})}{(d_{max} + d_{min})} \tag{6}$$

$$Sphericity\ coefficient = \frac{d_{min}}{d_{max}} \tag{7}$$

In equations (6) and (7), $d_{max}$ is the maximum (length) and $d_{min}$ is the minimum (width) diameters of particles. Microparticles with SF < 0.05 and SC close to 1 were considered as spheres.

### 2.6. Encapsulation of Black Carrot Anthocyanins in Alginate Microparticles

Encapsulation of black carrot extract was conducted by two different methods as in situ and post loading. In the first method, black carrot extract was mixed with alginate solution and the obtained mixture was added dropwise to the $CaCl_2$ solution under optimum conditions determined in the previous step. After the preparation, anthocyanin loaded alginate microparticles were removed by filtration and the pH differential method was applied to the remaining solution to determine the encapsulation efficiency. In the post loading method, encapsulation was carried out by absorption after the preparation of microparticles. Prepared alginate microparticles were immersed in an extract solution and left agitation for 3 h at room temperature. At the end of 3 h, the amount of unloaded anthocyanin was determined by the pH differential method. The following equation was used to calculate encapsulation efficiency [32].

$$Encapsulation\ efficiency\ (\%) = \frac{Initial\ anthocyanin\ amount - Unloaded\ anthocyanin\ amount}{Initial\ anthocyanin\ amount} \times 100 \quad (8)$$

### 2.7. Comparison of Thermal Stability of Free and Encapsulated Black Carrot Anthocyanins

Free and encapsulated black carrot extracts were immersed in a water bath at 70°C during 3 h and the anthocyanin content of samples was determined by the pH differential method. Free and encapsulated extracts stored at 4°C were used as controls. Thermal stability of black carrot extract was evaluated by calculation of retention efficiency as follows (Equation 9) [33].

$$Retention\ efficiency\ (\%) = \frac{Anthocyanin\ content\ \ after\ storage\ at\ 70°C}{Anthocyanin\ content\ after\ storage\ at\ 4°C} \times 100 \quad (9)$$

To determine the storage behavior, reaction constant (k) and half-life ($t_{1/2}$) were calculated for free and encapsulated black carrot anthocyanins by following equations (Equations 10 and 11) [34]:

$$\ln\left(\frac{C_t}{C_0}\right) = -kt \quad (10)$$

$$t_{1/2} = -\frac{\ln 0.5}{k} \quad (11)$$

where $C_0$ is the initial anthocyanin content, $C_t$ is the anthocyanin content at the specific time, t is the time and k is the reaction constant.

### 2.8. Statistical Analysis

The paired Student's t-test was used and the normality of the data was analyzed by the Kolmogorov-Smirnov test. In all tests, significant differences were considered when $p < 0.05$ [35].

## 3. Results and Discussion

### 3.1. Ultrasound-assisted Extraction of Black Carrot Anthocyanins

Ultrasound-assisted extraction was applied to black carrot and, anthocyanin content of extracts obtained by four different extraction solvents namely water, ethanol, 30% (v/v) EtOH and 70% (v/v) EtOH were determined by pH differential method. Extraction with 100% (v/v) water yielded the lowest anthocyanin content (46.5 mg/L) which was followed by 100% (v/v) ethanol (47.4 mg/L) with an insignificant difference. As shown in Figure 1, using an ethanol-water mixture as an extraction solvent significantly improved the amount of extracted anthocyanin compared to the experiments in which pure solvents were used. EtOH of 70% (v/v) that yields 112.7 mg/L of anthocyanin content has been chosen as an extraction solvent and black carrot extract obtained by 70% (v/v) EtOH has been used for further encapsulation and thermal stability experiments.

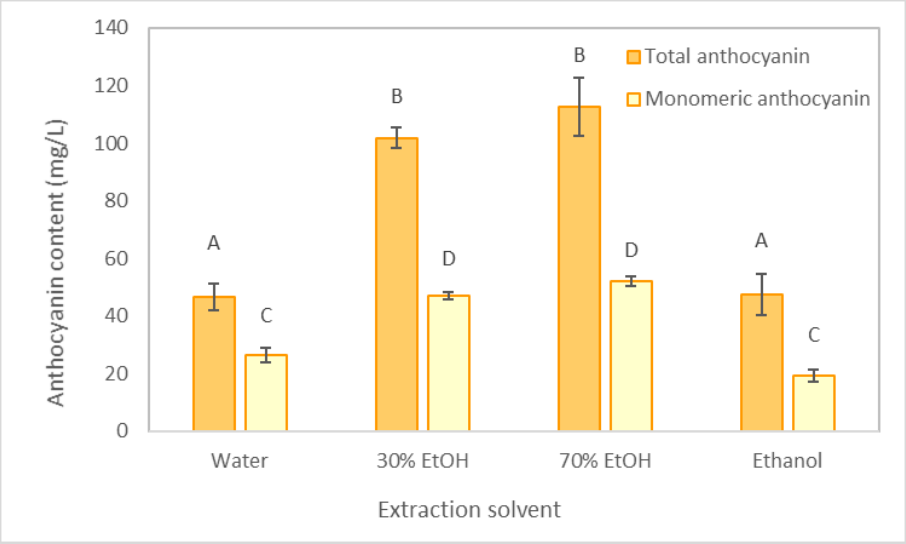


**Figure 1.** Anthocyanin content of black carrot extracts obtained by different solvents.

### 3.2. Preparation of Alginate Microparticles

Alginate microparticles were prepared by ionotropic gelation and calculated average microparticle sizes were present in Table 2.

**Table 2:** Average diameter, polydispersity index (PDI), sphericity factor (SF) and sphericity coefficients (SC) of prepared alginate microparticles.

| Exp. No | Diameter (μm) | PDI | SF | SC |
|---|---|---|---|---|
| 1 | 541.2 | 0.109 | 0.178 | 0.717 |
| 2 | 679.8 | 0.045 | 0.168 | 0.704 |
| 3 | 776.2 | 0.084 | 0.075 | 0.861 |
| 4 | 742.7 | 0.070 | 0.299 | 0.546 |
| 5 | 612.9 | 0.016 | 0.177 | 0.709 |
| 6 | 628.2 | 0.021 | 0.131 | 0.775 |
| 7 | 601.2 | 0.032 | 0.384 | 0.467 |
| 8 | 648.6 | 0.005 | 0.150 | 0.746 |
| 9 | 462.4 | 0.010 | 0.094 | 0.846 |
| 10 | 507.3 | 0.004 | 0.150 | 0.726 |

The effect of alginate concentration on average particle size, PDI value and sphericity was tested for 1% (w/v) and 2% (w/v) concentrations. For a needle diameter of 0.8 mm, increased alginate concentration resulted in the formation of larger particles probably due to the rise of viscosity by increased polymer concentration which may lead to the formation of larger particles [31]. Stirring of the gelation bath is applied to prevent the aggregation of beads and to obtain a more homogenous cross-linking reaction. However, increased stirring rate creates strong centrifugal forces and may lead to the deformation of particle shape [36]. In accordance with this, lower $CaCl_2$ stirring rate resulted in the production of microparticles with higher sphericity at constant alginate concentration (Figure 2A and 2B). Therefore, further experiments were conducted at 40 rpm stirring rate of the $CaCl_2$ bath. At a 40 rpm stirring rate, lower alginate concentration resulted in the production of microparticles with higher PDI value and lower sphericity that indicated the benefit of higher alginate concentration to produce spherical microparticles with narrow size distribution. When the alginate droplet enters the gelation solution, the drag forces acting on the droplet lead to shape deformation. It has been reported that alginate solution with low concentration and viscosity creates droplets less resistant to shape change while higher alginate concentration makes a huge contribution to retaining the spherical shape of the droplets [37]. The flow rate of alginate solution was also found to have a significant impact on sphericity and PDI value rather than average particle size. For 1% (w/v) alginate concentration, an increased flow rate mainly affected the PDI value of produced microparticles (decreasing from 0.109 to 0.016) while at 2% (w/v) alginate concentration, a higher flow rate tended to produce microparticles with less sphericity. To determine the effect of the temperature of the $CaCl_2$ solution, microparticle production was conducted at 4°C and 24°C. For 2% (w/v) alginate concentration, $CaCl_2$ solution at 4°C resulted in the production of particles with narrow size distribution while its effect on particle size and sphericity was insignificant. Smrdel et al., prepared alginate beads by ionotropic gelation and the temperature of gelation bath has been shown to be ineffective on particle size while the higher gelation temperature improved particle sphericity [38]. In addition, shape and size distribution of alginate microparticles tended to be irregular with increased stirring rate of $CaCl_2$ solution. By changing the needle diameter from 0.8 mm to 0.45 mm, a huge decrease was obtained for average particle size while the obtained particles were more spherical in shape and had a lower PDI value (Figure 2C). Since the average particle size, size distribution and sphericity have a significant impact on the release behavior, they are of prime importance for the application of particles. For instance, the reduction of particle size results in an increased surface area to volume ratio and leads to overcoming mass transfer limitations while tear shaped microparticles possess a strong burst release profile for the encapsulated agent. Furthermore, the mechanical and chemical features of microparticles are significantly affected by particle sphericity. Spherical beads have been shown to possess higher mechanical strength compared to non-spherical counterparts [29, 31, 39]. Consequentially, 2% (w/v) concentration and 1 ml/min flow rate for alginate solution, 40 rpm stirring rate of $CaCl_2$ solution at 4°C and 0.45 mm of needle size were found to be the most appropriate conditions for the production of alginate microparticles with lower size, narrow size distribution and higher sphericity.

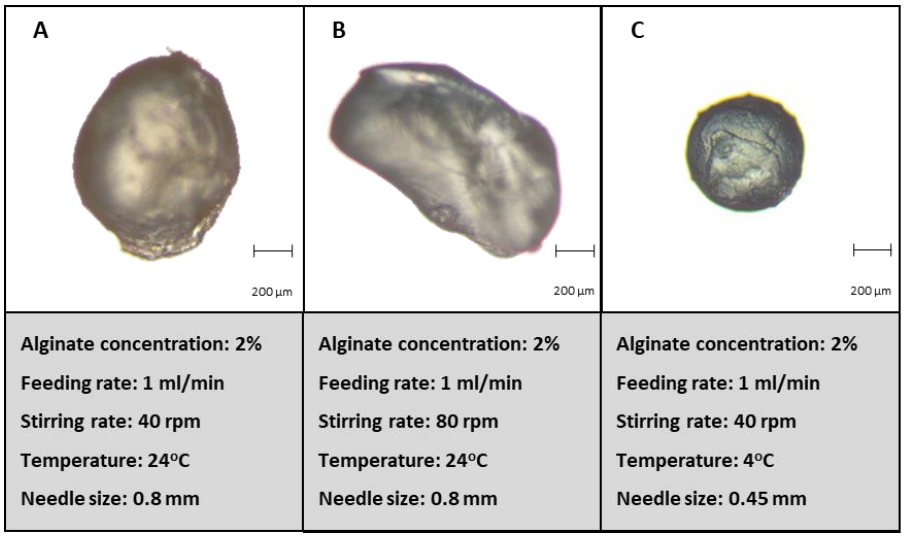


**Figure 2.** Optical microscope images of randomly selected alginates microparticles prepared at different conditions.

### 3.3. Encapsulation of Black Carrot Anthocyanins in Alginate Microparticles

Encapsulation of black carrot extract was conducted by two different methods as in situ and post loading, and both approaches were compared in terms of encapsulation efficiency. For the first method, 70.8% of initial anthocyanin was encapsulated during particle formation. However, absorption based encapsulation approach resulted in a significantly higher encapsulation efficiency as 87.7%. In previous studies, anthocyanin-rich extract from haskap berries was encapsulated in calcium-alginate microparticles with an encapsulation efficiency of 68.03% [40] while the encapsulation efficiencies of hibiscus anthocyanins by dripping extrusion were reported to vary between 67.9 and 88.1% [41] which are in agreement with the obtained encapsulation efficiencies in our study. A higher encapsulation efficiency was obtained by post loading method which is probable due the release of extract into the gelation bath during in situ loading. Therefore, post loading was chosen as the encapsulation approach of anthocyanins in alginate microparticles.

### 3.4. Comparison of Thermal Stability of Free and Encapsulated Black Carrot Anthocyanins

Free and encapsulated black carrot extracts were incubated at 4°C and 70°C and anthocyanin content of samples was compared to control groups to determine the thermal stability. After exposure to 70°C for 3 h, the retention efficiencies were determined as 96.92% and 75.82% and degradation constants were 0.092 $h^{-1}$ and 0.0104 $h^{-1}$ for free and encapsulated anthocyanins, respectively. In addition, the half-life of anthocyanin rich extract was improved from 7.5 h to 66.5 h by encapsulation. These results elicited the ability of alginate microparticles for the protection of black carrot anthocyanins from thermal degradation and improvement of storage stability.

## 4. Conclusion

The color and stability of anthocyanin change during processing and storage of foods and, various encapsulation approaches are applied for the protection of these compounds from harsh environmental conditions. In this study, black carrot anthocyanins were obtained by ultrasonic-assisted extraction and encapsulated to enhance thermal stability. For this purpose, alginate microparticles were produced at different conditions and characterized in terms of average particle size, particle sphericity and PDI values. Optimum conditions for the production of alginate

microparticles with lower size, higher sphericity and narrower size distribution were elicited as 2% (w/v) alginate concentration, 1 ml/min flow rate, 4ºC $CaCl_2$ temperature, 40 rpm stirring rate and 0.45 mm needle diameter resulting in particle size of 462.4 μm, sphericity factor of 0.094 and PDI of 0.01. Thermal stability tests were also conducted and the obtained results revealed that half-life of encapsulated anthocyanins was significantly higher than its free form. In conclusion, this study suggests that encapsulation of black carrot anthocyanins in alginate microparticles improved their thermal stability and possessed a high potential to be utilized as an ingredient for food applications.

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