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Research Article

Effect of Freeze-Thaw Pretreatment on Extraction Yield and Antioxidant Bioactivity of Corn Carotenoids (Lutein and Zeaxanthin)

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As a green and low-energy pretreatment method, the effect of freeze-thaw (FT) pretreatment on extraction yield and antioxidant bioactivity of carotenoids of the corn gluten meal (CGM) were evaluated in this study. The CGM particles ruptured in FT treatment due to the repeated damage caused by FT to CGM particles. The carotenoid compounds of pretreated CGM were lutein, zeaxanthin, β-carotene, and cryptoxanthin. Among them, the major carotenoids are lutein and zeaxanthin. The optimized FT pretreatment conditions included freezing temperature of −20°C, moisture content of 100%, and 2 cycles. An increase in the yield of lutein and zeaxanthin was observed in the range of 2.23–16.39 μg/g and 4.66–36.3 μg/g as a result of pretreatment as against 1.17 and 2.52 μg/g of the untreated sample, respectively. Moreover, the release of lutein and zeaxanthin from CGM was facilitated by FT pretreatment and increased the antioxidant activity of the carotenoids.

1. Introduction

Carotenoids, especially lutein and zeaxanthin, are functional pigments that are present in many kinds of plants, animals, and microorganisms [1]. It was reported that the intake of foods containing lutein and zeaxanthin, like vegetables and fruits, decreases the risk of cancer, angiocardiopathy, and other degenerative and chronic diseases [2]. Above all, lutein and zeaxanthin are regarded as macular pigment accumulated in the macula of the retina of the eye where they play a protective role against the development of age-related macular degeneration (AMD) and other ocular diseases [3, 4]. Unfortunately, since lutein and zeaxanthin are not produced in the body, their intake is primarily dependent on the consumption of foods rich in carotenoids [5].

Corn gluten meal (CGM), a primary residue of corn starch processing, contains 60–70% (w/w) of proteins and a small percentage of starch, fiber, lipid, and carotenoids [6]. CGM is known to exhibit poor functional properties in its original form. Since CGM cannot be easily digested and absorbed in the body, its wide application in the food industry is restricted, and it is chiefly used in the form of animal feed additive [7, 8]. However, CGM is considered as a very good and chief source of carotenoids, especially yellow carotenoids (α- and β-carotenes) and xanthophylls [9]. The lutein and zeaxanthin are the predominant carotenoids in CGM, which are very beneficial for healthy vision [10].

Unfortunately, lutein and zeaxanthin are not largely available from CGM, due to its high proportion of protein and dense microstructure. The chief protein fractions of CGM are zein and glutelin, which constitute approximately 65% (w/w) and 30% (w/w), respectively. Since it contains a relatively high proportion of hydrophobic amino acids, its solubility in aqueous systems is low. It shows stable structures [11], which can be a major obstacle for lutein and zeaxanthin extraction. For the above reasons, pretreatment methods are considered to be important for the recovery of carotenoid compounds [12], especially lutein and zeaxanthin. Therefore, the key step for the extraction of lutein and zeaxanthin is the pretreatment of CGM.

Due to thermolability and photodecomposition of lutein and zeaxanthin [13], CGM should be pretreated in low energy and at low temperatures, whereas the previous pretreatment technology in particular accounts for most of energy consumption, which can cause degradation and loss of activity of lutein and zeaxanthin [14]. Conventional pretreatment methods for CGM are mostly physical and chemical, such as milling, sonication, enzyme, microwave, and organic solvents treatment [15–17]. Unfortunately, these methods may consume high amount of energy or induce loss of biological activity of lutein and zeaxanthin. Therefore, developing cost-effective and environmentally friendly techniques for CGM pretreatment to enhance lutein and zeaxanthin extraction efficiency and maintaining their bioactivity is indeed a critical step toward the success of commercial production of carotenoids from CGM.

The freeze-thaw method is regarded as an efficient and inexpensive physical technique for disintegration of plant tissue and cell membrane. It has been shown that freeze-thawing of plant samples was able to induce the maximum degree of cell membrane permeabilization [18]. On the same principle, the freezing process may induce alterations in the corn protein tissue by formation and accretion of ice crystals, dehydration, and also by an increase in the solute concentration [19]. For this reason, freeze-thaw (FT) pretreatment can be used to disrupt rigid protein microstructure, resulting in loosening the structure of CGM. The release of bound carotenoid compounds is facilitated by the breakdown of CGM during FT pretreatment [20].

Thus, CGM FT pretreatment can prove to be an important method for increasing the amount of carotenoids and their antioxidant activity due to the low-temperature treatment, which can protect the bioactive components from degradation.

The objective of this study was to choose a suitable pretreatment process of CGM, which can be used as an alternate for the enhanced extraction of carotenoids. Furthermore, we aimed at studying how the methods for the FT pretreatment affect the structure of CGM and extraction efficiency of lutein and zeaxanthin. The relationship between the changes in main carotenoid components and antioxidant properties of the carotenoid compounds was also taken into account.

2. Materials and Methods

2.1. Materials. Corn gluten meal (CGM) was obtained from Cofco Biochemical Energy (Longjiang) Co. Ltd. (Heilongjiang, China). Lutein, zeaxanthin, β-carotene, β-cryptoxanthin, 1, 1-diphenyl-2-picrylhydrazyl radical, 2, 2-Diphenyl-1-(2, 4, 6-trinitrophenyl) hydrazyl (DPPH), and 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were from Sigma Co. (St. Louis, MO, USA). Methyl tert-butyl ether (MTBE) and methanol were purchased from Tedia Co., Inc. (Fairfield, USA). Hexane, anhydrous sodium sulfate, acetone, and ethanol were from Shanghai Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals and reagents were of analytical grade.

2.2. Freeze-Thaw (FT) Treatment. In order to investigate the effect of FT treatment on the extraction of the CGM carotenoids, the CGM was pretreated by means of FT experiments [21, 22]. The dried CGM was sieved to select a particle diameter of under 0.25 mm. 5 g of CGM was mixed with 1–6 mL distilled water and maintained for 12 h, and the samples were, respectively, placed at different freezes of −20°C, −40°C, −80°C, and −196°C (liquid nitrogen N_2 freezing) for 2 h. After freezing, the thawing process was initiated by placing the CGM samples in an incubator for 2 h at 20°C. Later, the samples were freeze-dried (LD-53, Millrock, USA) to remove moisture before extraction. The CGM samples were subjected to 1, 2, 3, 4, and 5 freeze-thaw cycles (FTC), respectively.

2.3. Carotenoids Extraction and Assay. Five grams of untreated or FT-treated CGM were added to 50 mL of ethanol. Carotenoids were extracted at 20°C for 12 h in dark, then the supernatant was filtered, and 2 mL of 40% methanolic potassium hydroxide was mixed with the supernatant and kept for saponification at 20°C in dark for 20 h. Then, 50 mL of hexane was added to partition carotenoids and mixed for 5 min; after that, 10% sodium sulfate solution was added and diluted. The mixture was kept at 20°C to form two clear separate phases. The carotenoids were collected from the upper hexane layer and evaporated completely. The samples were resolubilized in methanol (10 mL) and filtered using a 0.45-μm membrane filter for HPLC analysis and antioxidant assay. The whole separation process was performed in dark, and nitrogen was used to protect the carotenoids from isomerization or degradation.

The amount of carotenoids was, respectively, assayed by HPLC at 450 nm (Agilent Technologies, Palo Alto, CA, USA) as earlier reported in [23], (column: YMC-C_{30}, 250 × 4.6 mm, 5 μm particle size, Agilent Technologies, Germany). The mobile phase was methyl tert-butyl ether and methanol at a linear gradient from 0 min (5 : 95, v/v) to 30 min (30 : 70, v/v). The methanol was stabilized with triethylamine (TEA) to 0.1%, at a flow rate of 0.9 mL/min; the column temperature was maintained at 25°C, and 20 μL of sample was injected.

2.4. Observation of CGM Morphology by SEM. Morphology of both treated and untreated CGM samples was studied by scanning electron microscopy (SEM). A thin layer of dried CGM was placed on a copper sample-holder, using a double-sided carbon tape, and coated with gold (10 nm thickness) to make the sample conductive. Then, the sample was observed under a scanning electron microscope (S-3400N, Hitachi, Tokyo, Japan) at an accelerating voltage of 15 kV [24].

2.5. Antioxidant Activities

2.5.1. DPPH Radical Scavenging Activity Assay. Assay of DPPH radical scavenging activity of the carotenoids was determined by the method described by earlier researchers with a few modifications [25]. 2 mL of the carotenoid sample was mixed with 2 mL of 0.16 mM DPPH dissolved in ethanol. The mixed solution was mixed vigorously and kept at

25°C for 30 min in dark. The absorbance of the resulting solution A_{sample} was recorded at 517 nm. A blank (A_{blank}) was prepared in the same way except that ethanol was used as a substitute for samples. The DPPH scavenging activity was calculated as

$$\text{DPPH scavenging activity } (\%) = \left[\frac{\left(A_{blank} - A_{sample}\right)}{A_{blank}}\right] \times 100. \quad (1)$$

2.5.2. ABTS Radical Scavenging Activity. ABTS radical scavenging activity was evaluated by the method described in [26]. Stock solution of ABTS radical (7 mM ABTS in 2.45 mM potassium persulfate) was prepared and stored at 25°C for 16 h in dark. Working ABTS solution with an absorbance of 0.7 ± 0.02 at 734 nm was prepared by diluting the stock solution with distilled water. An aliquot (200 μL) of the carotenoid samples was mixed with 2 mL of the ABTS reagent and incubated for 10 min in dark. Absorbance was measured at 734 nm. The ABTS scavenging activity of the samples was calculated as follows:

$$\text{ABTS scavenging activity } (\%) = \left[\frac{\left(A_{blank} - A_{sample}\right)}{A_{blank}}\right] \times 100, \quad (2)$$

where A_{blank} is the absorbance without sample at 734 nm and A_{sample} is the absorbance of the sample solution.

2.5.3. Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity was evaluated by the method described in [27] with little changes. Briefly, 0.5 mL of the carotenoid sample was mixed with 1 mL of 6 mM $FeSO_4$, and 1 mL of 6 mM salicylic acid dissolved in ethanol. After the addition of 1 mL of 6 mmol/L H_2O_2, it was vigorously mixed and incubated at room temperature in dark for 30 min, and the resulting absorbance A_1 was read at 510 nm. In blank sample A_0, 1 mL distilled water was used instead of the sample. A_2 was prepared in the same manner except that 1 mL distilled water was used instead of $FeSO_4$. The hydroxyl radical scavenging activity was calculated as follows:

$$\text{Hydroxyl radical scavenging } (\%) = \left[1 - \frac{\left(A_1 - A_2\right)}{A_0}\right] \times 100. \quad (3)$$

2.6. Data Analysis by Statistical Methods. All experiments were performed in triplicates. The obtained experimental results were tested for significance ($p \leq 0.05$) for analysis of variance using SPSS software. All data were expressed as the mean ± standard deviation (SD).

3. Results and Discussion

3.1. Analysis of Major Carotenoid Compounds by HPLC. Analysis of the carotenoids obtained from CGM after the application of FT treatments was performed by HPLC. Figures 1(a) and 1(b) show chromatogram profiles detected at 450 nm for carotenoids obtained from an extraction of untreated and pretreated CGM. Carotenoids obtained from different pretreatment conditions resulted in similar chromatogram profiles. As shown in Figure 1, the major carotenoids identified in extracts of CGM, such as lutein, β-carotene, zeaxanthin, and cryptoxanthin, were influenced by FT pretreatment techniques.

The results showed that although the application of a FT treatment caused an increase in the amount of carotenoid compounds, the composition of the carotenoids was unaffected. The pretreatment caused an increase in the yield of lutein and zeaxanthin ranging from 2.23–16.39 μg/g and 4.66–36.3 μg/g as against 1.17 and 2.52 μg/g of the untreated sample, respectively, and the extraction yield of lutein and zeaxanthin was increased more than 14 folds by FT pretreatment. Ahmad-Qasem et al. have reported similar findings when they studied the application of FT for improving the extraction of other compounds from plant tissues [28].

3.2. Influence of Freeze-Thaw Temperature on Extraction Yield of Lutein and Zeaxanthin. FT treatments of different intensities at various temperatures on the extraction yield of lutein and zeaxanthin were analyzed. Figure 2 indicates the variation in the extraction yield of lutein and zeaxanthin with respect to FT temperature (−20°C, −40°C, −80°C, and −196°C). It could be seen that the control group (unpretreated CGM) displayed lower extraction yield of lutein and zeaxanthin. However, in pretreated CGM samples, the lutein and zeaxanthin extraction yield exhibited a gradual decrease as the FT treatment temperature drops. The optimum FT temperature for a higher yield of lutein and zeaxanthin was −20°C. This is because the CGM was frozen more slowly at −20°C, and the size of ice crystal in CGM was larger than that at other lower temperature (especially at −196°C), which alters the internal structure of CGM due to mechanical compression, causing structural damage during the thawing process [29]. The freezing temperatures (−20°C, −40°C, and −80°C) can be controlled in a manner which allows growth of relatively large ice crystals in the CGM particle, where the freezing rate is relatively slow; a small number of ice crystals but having large size can be formed, which could cause more damage to CGM. Therefore, the extraction yields of lutein and zeaxanthin were highest at −20°C FT pretreatment, where large ice crystals can be easily formed, causing damage to insoluble constituent that bounded carotenoids in the freezing process [30].

3.3. Effect of Moisture Content on Extraction Yield of Carotenoids. The optimal moisture content is very important for the efficient FT pretreatment of CGM. Thus, the influence of moisture content on the extraction yield of lutein and zeaxanthin was investigated, and the results are presented in Figure 3, respectively. Various moisture contents (20%, 40%, 60%, 80%, 100%, and 120%) of CGM were used for FT pretreatment. The extraction yields of lutein and zeaxanthin of the control group (without moisture) were

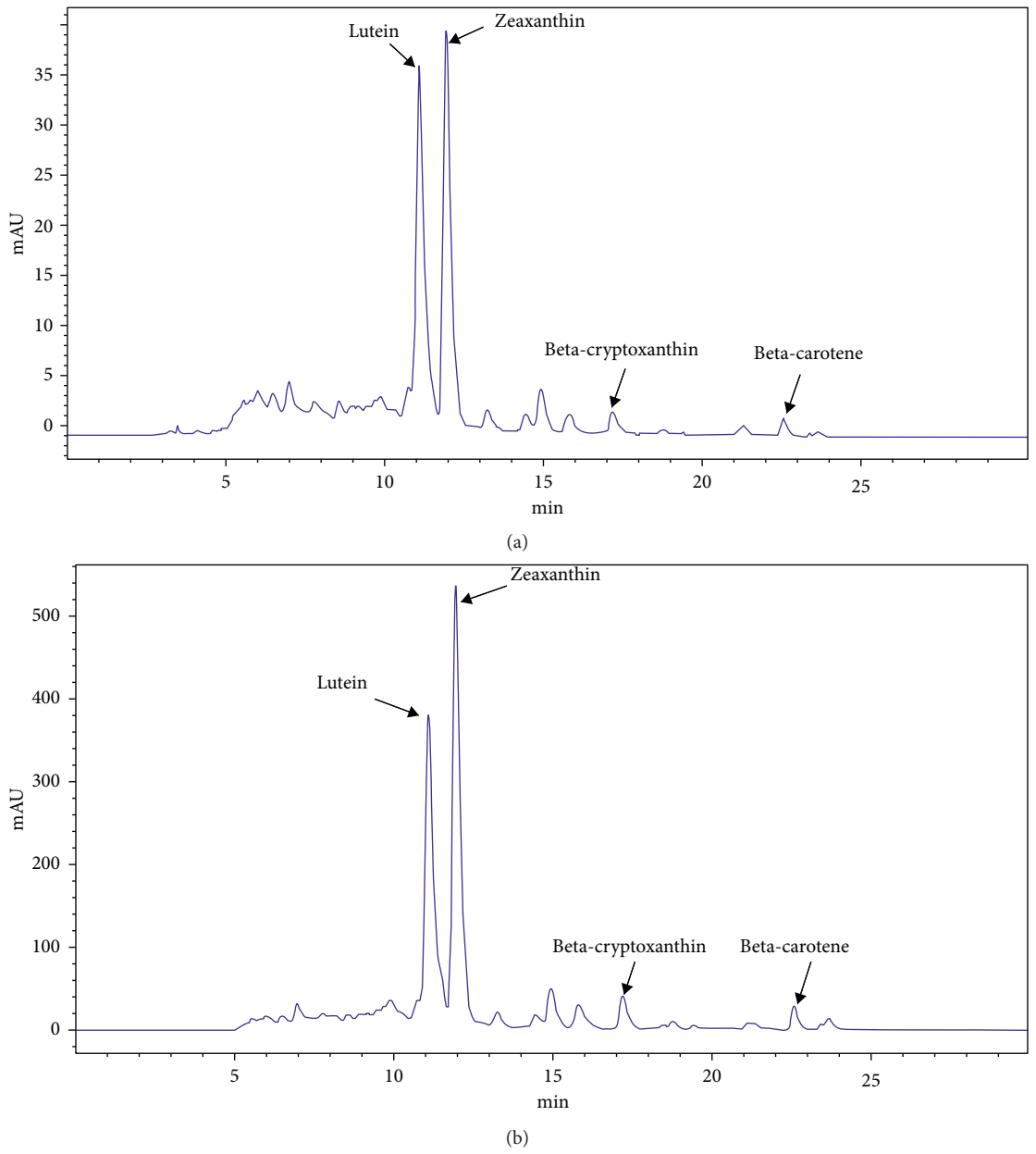


Figure 1: HPLC analysis of major carotenoid compounds of CGM. (a) No pretreatment; (b) freeze-thaw pretreatment.

low. When water was added into the CGM, it was seen that the extraction yield of carotenoids increased with an increase in the moisture content up to 100%; the highest yield of lutein and zeaxanthin (16.39 ± 0.752 and 36.30 ± 1.139 μg/g) resulted from the 100% moisture content of freeze-thaw pretreated CGM, while there was no corresponding increase seen for higher moisture content (100–120%). These results indicate that the optimal percentage of moisture content which is necessary to obtain a good extraction yield was 100% with respect to the dried CGM. The suitable moisture content of CGM is known to be the chief reason for the structural damage for FT pretreatment of CGM and lead to higher extraction yield of carotenoids. This is because moisture can permeate into CGM by adsorption, and they were frozen to form large ice crystals to breakdown the dense microstructure of CGM, and once the moisture in CGM was enough (100%), there would not be any further significant microstructure damage; the extraction yield of carotenoids cannot continue to increase. Therefore, the FT pretreatment of CGM with 100% moisture content was the most appropriate method to improve carotenoids extraction.

3.4. Effect of Number of Freeze-Thaw Cycles on Extraction Yield of Carotenoids. The extraction of carotenoids from the CGM subjected to five freeze-thaw cycles (FTC) pretreatment are shown in Figure 4. After just one FTC, the extraction yield of carotenoids was increased; with the increase of FTC, a significant increase in extraction yield of carotenoids was found after two FTC pretreatment and

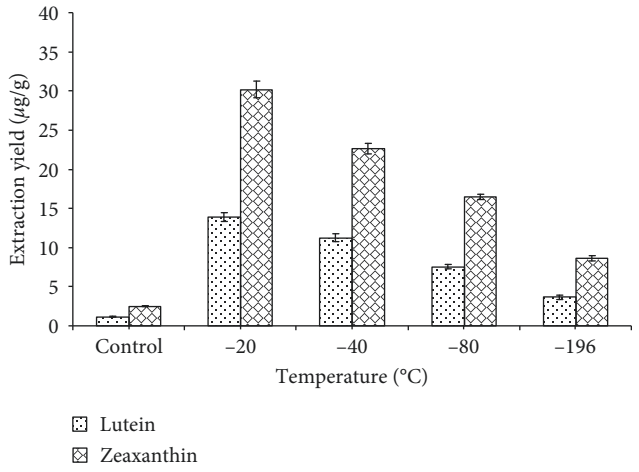


Figure 2: Effect of FT temperature on extraction yield of carotenoids.

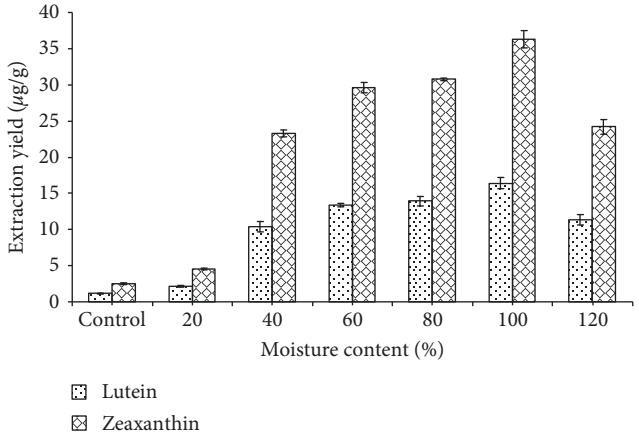


Figure 3: Effect of moisture content on extraction yields of carotenoids.

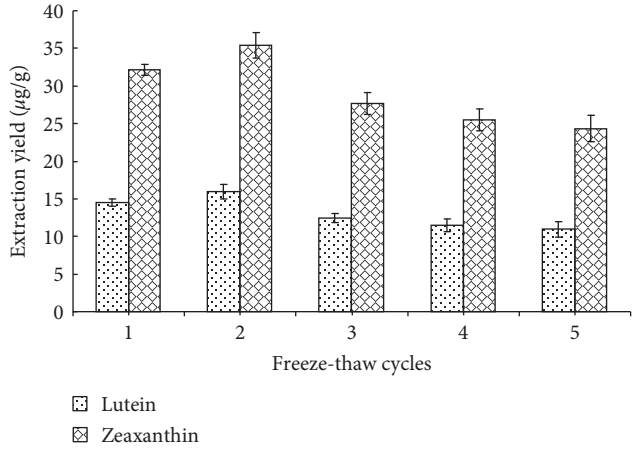


Figure 4: Effect of freeze-thaw cycles on extraction yield of carotenoids.

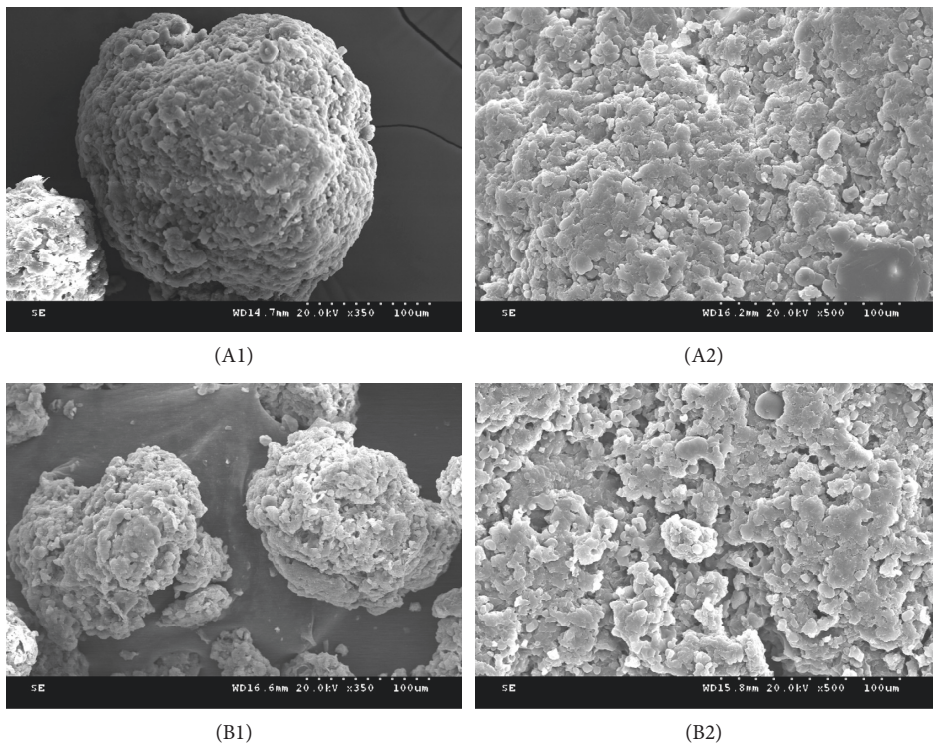
extraction yield decreased gradually more than two FTCs. These results suggest that a higher release of carotenoids possibly occurred due to the multiple freeze-thaw processes.

The result suggests that the repeated melting and reformation of ice crystals may cause further protein collapse [31], and the CGM may be damaged by repeated mechanical damages due to ice crystal expansion. More carotenoids could be extracted from damaged CGM particles after ice crystal thawing. More than two FTC pretreatments cannot lead to high extraction yield, which could be due to degradation and loss of lutein and zeaxanthin.

3.5. Microstructure of Freeze-Thaw Pretreated CGM. The total carotenoid contents of the extracts from CGM pretreated by FT were significantly higher than that of the extract from untreated CGM. These results might be explained by the scanning electron microscopy (SEM) photographs of CGM shown in Figure 5 (A and B). In the case of untreated CGM, since no FT pretreatment was applied, a regular spherical particle and clear intact surface were observed, and the total carotenoid contents of the extracts from the CGM was therefore significantly low. On the contrary, in the cases of CGM pretreated by FT technology, the compact globular microstructures were damaged, the gap between components became larger and formed a porous structure, samples became less dense, and their cross-linked structures were destroyed. The tightly bound corn starch converted into loose polysaccharides, and the long-chain zein transformed into the short-chain ones. Because the main components of CGM are zein and starch, carotenoids were bound with them, and therefore, the dissolution of carotenoids in organic solvent was promoted [32]. These mechanisms caused the damage of the structure by FT as can be seen in Figures 5(B1) and 5(B2). For the above reasons, the utilized FT pretreatments finally accelerated carotenoid release and enhanced the extraction efficiency [33]. This suggested that the FT could also be the crucial process for obtaining the ideal lutein and zeaxanthin.

3.6. Determination of Antioxidant Activity. Main carotenoid content and antioxidant capacity of extract from the CGM with and without FT pretreatment were compared, and the contribution of carotenoids to the total antioxidant activity can be analyzed by their correlation between the main component (lutein and zeaxanthin) and the antioxidant assays.

The DPPH, ABTS, and hydroxyl radical scavenging activity indicated higher contribution of lutein and zeaxanthin to the antioxidant properties of the CGM carotenoids (Table 1). These results are in agreement with previous studies which indicated that FT pretreatment could improve the lutein and zeaxanthin extraction of CGM. The content of lutein and zeaxanthin had a close correlation with the antioxidant property and was the major contributor to the antioxidant properties of CGM carotenoids. The DPPH, ABTS, and hydroxyl radical scavenging activities of lutein and zeaxanthin from the FT-pretreated CGM were significantly higher than that from the untreated group. One the contrary, low temperature could minimize the loss of carotenoids during the extraction process. Therefore, the



(A1) (A2)

(B1) (B2)

Figure 5: Transmission electron microscope (TEM) images of CGM. (A1, A2) no pretreatment; (B1, B2) freeze-thaw (−20°C, 100% moisture content, freeze-thaw twice) pretreatment.

Table 1: Lutein and zeaxanthin content and scavenging activity of the CGM with and without pretreatment. Data represent the mean value ± standard deviation ($n = 3$).

Pretreatment method	Lutein (μg/mL)	Zeaxanthin (μg/mL)	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)	Hydroxyl radical scavenging activity (%)
No pretreatment	0.70 ± 0.052	1.76 ± 0.046	8.36 ± 0.472	7.66 ± 0.38	4.76 ± 0.216
Freeze-thaw pretreatments	9.83 ± 0.451	21.78 ± 0.683	94.59 ± 0.569	75.42 ± 2.022	55.38 ± 1.538

FT-pretreated CGM provided carotenoid extracts with a higher antioxidant potential.

4. Conclusion

Freeze-thaw pretreatment was found to significantly affect the structure of the CGM, and satisfactory extraction yields for the lutein, zeaxanthin, β-carotene, and cryptoxanthin and other bioactive compounds were obtained through the FT treatment. The pretreatment of the CGM at a temperature of −20°C, with 100% moisture content and two freeze-thaw cycles, could increase the yields of lutein and zeaxanthin more than 14 folds and 14.4 folds, respectively, compared to the control (untreated CGM). Moisture content and FT pretreatment temperature of the CGM were found to significantly increase the levels of major carotenoid bioactive compounds of the CGM. Similarly, FT pretreatment was found to significantly increase the antioxidant activity of the CGM carotenoids as there was a close correlation between bioactive compounds and antioxidant activity. However, extremely low temperature and excessively multiple freeze-thaw cycles were found to decrease the levels of major carotenoid compounds, as well as the antioxidant activity.

These results suggest that FT pretreatment could significantly increase the levels of bioactive compounds and reduce the degradation and loss of the carotenoids during the extraction process. It is an efficient and novel pretreatment method that can improve the yield as well as antioxidant activity of carotenoid compounds in CGM. This may be useful for potential applications for development of active ingredients extraction. Future studies are recommended to comprehensively study the impact of a wide range of FT-assisted mechanical treatments on the different types of CGM bioactive compounds.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Additional Points

Practical Application. The application of freeze-thaw (FT) pretreatment has potential for increasing the extraction yield and antioxidant bioactivity of the carotenoids from corn gluten meal (CGM). CGM is an excellent and important source of carotenoids (lutein and zeaxanthin), while lutein and zeaxanthin are not largely available from CGM due to high proportion of lipophilic protein and dense microstructure. FT pretreatment could significantly increase the levels of carotenoids and reduce their degradation and bioactivity loss during the extraction process. The FT pretreatment is a green and low-energy technology, which can be beneficial in food industries for enhancing the key carotenoids extraction processes.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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