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Effect of Microfluidization on Bioaccessibility of Carotenoids from *Chlorella ellipsoidea* during Simulated Digestion

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ABSTRACT: This study focused on the bioaccessibility of carotenoids from *Chlorella ellipsoidea* using a simulated digestion model. To increase the bioaccessibility of carotenoids, *C. ellipsoidea* was pulverized by microfluidization at pressures up to 20000 psi. The microfluidization treatment significantly reduced mean microalga particle size from 2463 to 361 nm. The major carotenoid in *C. ellipsoidea* was zeaxanthin, with two minor carotenoids, antheraxanthin and *β*-carotene. After microfluidization, the zeaxanthin and *β*-carotene contents in *C. ellipsoidea* were not changed in comparison to the untreated group, whereas the antheraxanthin content was significantly reduced. The bioaccessibility of carotenoids in untreated *C. ellipsoidea* was very low (zeaxanthin, 2.60%; *β*-carotene, 1.69%). Approximately 95% of total *C. ellipsoidea* carotenoids could not be released and micellized by simulated digestion. The microfluidized microalga (at 20000 psi) was 10 times more effective for zeaxanthin and *β*-carotene micelle formation compared with untreated *C. ellipsoidea*, showing higher bioaccessibility of carotenoids (zeaxanthin, 32.60%; *β*-carotene, 18.19%). These results indicate that microfluidization may be useful for disrupting *C. ellipsoidea* cell walls and improving zeaxanthin and *β*-carotene bioaccessibility from *C. ellipsoidea* during the digestion process.

KEYWORDS: *Chlorella ellipsoidea, carotenoid, microfluidization, bioaccessibility, simulated digestion, zeaxanthin, β-carotene*

INTRODUCTION

Carotenoids are pigments that are synthesized by plants and microorganisms but not animals.[1,2] Many carotenoids act as potent free radical quenchers, singlet oxygen scavengers, lipid antioxidants, and photoprotectants under excess light.[3−5] Some carotenoids, such as lutein and zeaxanthin, may abate the risk of eye disorders, whereas *β*-carotene and lycopene may lessen the risk of cardiovascular diseases.[6−8]

Microalgae are generally recognized as a major natural source for novel functional compounds such as proteins, vitamins, minerals, and fatty acids.[9,10] *Chlorella ellipsoidea*, a green microalga, is widely used in Japan and Korea to feed rotifers and brine shrimp.[11,12] Interestingly, *C. ellipsoidea* is abundant in several types of carotenoids, especially zeaxanthin, which plays a role in reducing the risk of age-related macular degeneration in humans.

Recently, many studies have examined carotenoid bioavailability using simulated in vitro digestion models, which enable the mimicking of the human gastrointestinal environment and investigation of the influence of different factors on carotenoid absorption.[13,14] Carotenoid bioavailability is actually influenced by many factors, including the physicochemical properties of these compounds, the food matrix, processing, and interactions with other dietary compounds.[15] To utilize fully the functional bioactivity of carotenoids that are abundant in microalgae, they must be efficiently released from the microalgae matrix, transferred to lipid droplets, and then incorporated into mixed bile salt micelles during the simulated digestion process.[16]

Microfluidization, a sort of wet milling technique, is a nascent technology in food processing, which is widely used in the preparation of nanoemulsions and liposomes and the production of submicrometer pharmaceutical suspensions.[17,18] It is possible to create fine emulsions from large particles using high-pressure homogenization, because the specialized geometry of the chambers and the high pressure squeezes the liquid stream to reach extremely high velocities, resulting in particle size reduction by collision energy.

Our recent study demonstrated that microfluidization was effective in improving lutein bioaccessibility from *Chlorella vulgaris* during simulated digestion.[19] In this study, the bioaccessibility of several carotenoids in *C. ellipsoidea* was ascertained by simulated in vitro digestion. Microfluidization was then used to evaluate the influence of *C. ellipsoidea* pulverization on the bioaccessibility of their carotenoids.

MATERIALS AND METHODS

Samples and Chemicals. A culture broth of *C. ellipsoidea* was obtained from Aquanet Corp. (Tongyeong, Korea). Standard zeaxanthin and *β*-carotene were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pepsin, pancreatin, pancreatic lipase, and bile extract were also acquired from Sigma-Aldrich. HPLC grade *tert*-butyl methyl ether (TBME), ethanol, methanol, and water were purchased from Fisher Scientific (Springfield, NJ, USA). Unless indicated, all other chemicals were supplied by Sigma-Aldrich.

Microfluidization Procedures. The *C. ellipsoidea* culture broth was crushed using a Microfluidizer M-110EH (Microfluidics Co., Newton, MA, USA). The microalgae were microfluidized with a single treatment of either 5000, 10000, or 20000 psi, using a combination of a 200 *μ*m auxiliary chamber H30Z and a 100 *μ*m interaction chamber H10Z. Treated solutions were cooled under running water, and a portion of the microfluidized *C. ellipsoidea* was sampled for particle cell size analysis. The remaining microfluidized *C. ellipsoidea* solution and nonprocessed *C. ellipsoidea* solution were freeze-dried using a vacuum

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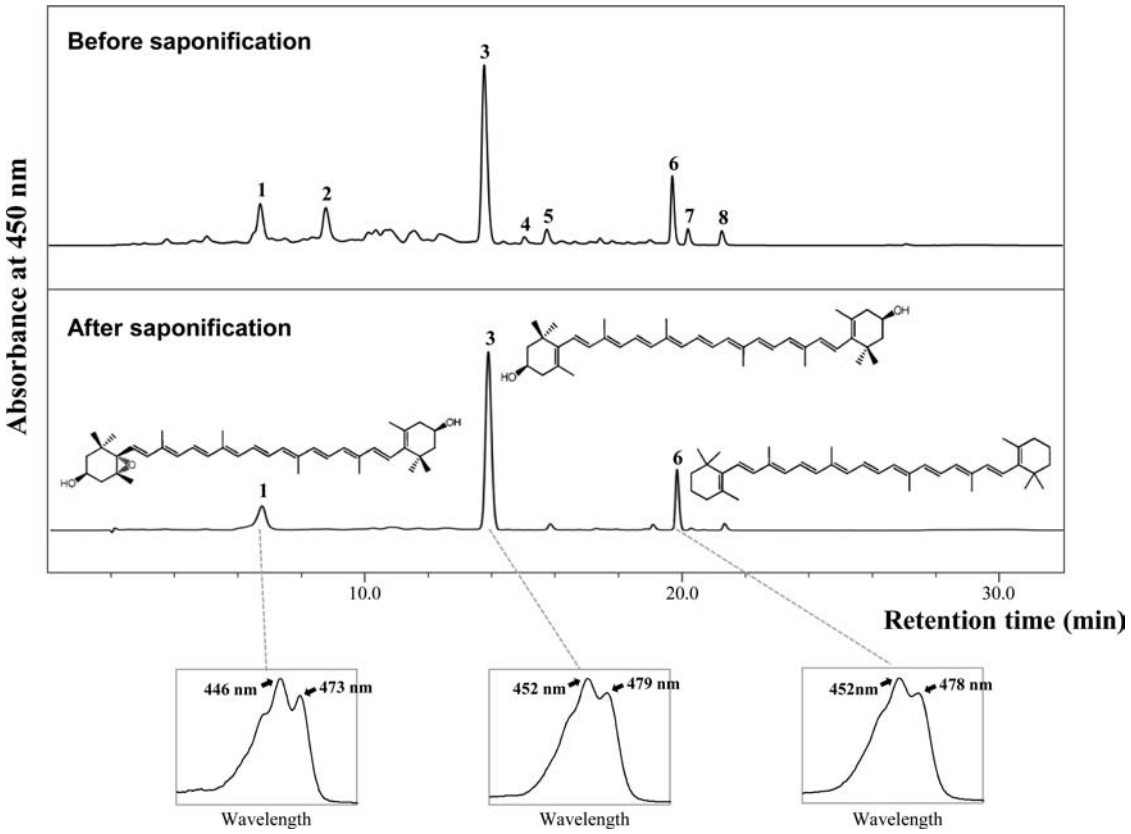

Figure 1. HPLC chromatogram before and after saponification of *C. ellipsoidea* extract and photodiode array spectra of the main carotenoids in *C. ellipsoidea*. Peaks: 1, antheraxanthin; 2, unidentified chlorophyll; 3, zeaxanthin; 4, unidentified chlorophyll; 5, unidentified carotenoid; 6, β-carotene; 7, unidentified chlorophyll; 8, unidentified carotenoid.

freeze-drier (Ilshin Laboratory, Korea) and stored at −80 °C before simulated digestion.

Measurement of Cell Size. Mean particle size and the polydispersity index of several *C. ellipsoidea* samples were analyzed by laser diffraction using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK), to ascertain the impact of microfluidization on cell wall breakdown. The scattering angle was fixed at 90°, and the temperature was maintained at 20 °C.

In Vitro Digestion. The in vitro digestion procedure was performed according to the method of Garrett et al. with minor modifications.[20] Briefly, *C. ellipsoidea* samples (200 mg) were mixed with 10 mL of saline solution containing NaCl, KCl, and $CaCl_2$ (120, 5, and 6 mM, respectively) and then slightly homogenized (Heidolph DIAX 900 Homogenizer, Berlin, Germany) for 10 s.

Three thousand units of α-amylase was added per gram of sample, and the pH was adjusted to 6.5, after which the mixture was incubated at 37 °C for 5 min in a shaking water bath (Lab companion, Jeio Tech, Korea) at 95 rpm for the simulated oral phase of digestion. To mimic the gastric phase of human digestion, the pH was acidified to 2.0 with HCl, and 1 mL of porcine pepsin solution (0.04 g/mL HCl) was then added, creating a final volume of 20 mL. The samples were overlaid with nitrogen gas and incubated at 37 °C for 1 h in a shaking water bath at 95 rpm. The intestinal phase involved increasing the pH to 5.3 with 0.9 M sodium bicarbonate, followed by the addition of 200 μL of the bile salts glycodeoxycholate (0.04 g in 1 mL of saline), taurodeoxycholate (0.025 g in 1 mL of saline), and taurocholate (0.04 g in 1 mL of saline), as well as 100 μL of pancreatic lipase (40 mg in 1 mL of 0.1 M sodium bicarbonate) and 100 μL of pancreatin (0.04 g in 500 μL of saline). The pH of each sample was increased to 6.5 with 1 N NaOH and overlaid with nitrogen. Samples were incubated for 2 h at 37 °C in a shaking water bath, to complete the intestinal phase of the in vitro digestion process. To isolate the aqueous micelles, the digestate was subject to ultracentrifugation for 95 min at 194270*g* using a Beckman Ultracentrifuge (Optima L-100 XP Ultracentrifuge, Beckman Coulter, Fullerton, CA, USA). The micellar fraction was prepared by filtration (0.2 μm; Millipore, Bedford, MA, USA) of the aqueous fraction to eliminate microcrystalline aggregates of carotenoids. The resulting micelles were stored at −80 °C before HPLC analysis. Bioaccessibility (%) was defined as the proportion of carotenoids present in the micelles compared with that contained in the nondigested *C. ellipsoidea*.

Carotenoid Extraction. Carotenoids were extracted from raw and microfluidized *C. ellipsoidea* using the method of Chitchumroonchokchai et al.[21] to determine major carotenoids before and after microfluidization. Fifty milligrams of freeze-dried powders from raw and microfluidized *C. ellipsoidea* was extracted by adding 3.0 mL of petroleum ether/acetone (2:1) containing 4.5 mM BHT, mixing on a vortex mixer for 1 min, and centrifuging at 3350*g* for 5 min to accelerate phase separation. The extraction procedure was repeated three times, and petroleum ether fractions were combined and dried at room temperature with nitrogen purging, followed by resolubilization in a methanol/TBME (90:10) solution that was used as a mobile phase. The extract was saponified (in an additional step to remove chlorophylls) with 30% methanolic potassium hydroxide at room temperature for 3 h. Following saponification, the alkali was eliminated completely by repeated washing, and the organic phase was evaporated to dryness using nitrogen purging. The residue was redissolved in a mobile phase solution.

Thawed samples (1−3 mL) of homogenized food and aqueous micellar fraction were extracted using the same method as the extraction procedure for freeze-dried *C. ellipsoidea* powders except for the omission of saponification.

HPLC Analysis. All extracts were analyzed using an analytic UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) equipped with an UltiMate 3000 autosampler column compartment, an HPG3200 binary pump, an UltiMate 3000 UV detector, and

Chromeleon software. A YMC C30 carotenoid column (3 μm particle size, 150 mm × 4.6 mm; Waters, Milford, MA, USA) was used to analyze carotenoids. The optimum mobile phase system comprised methanol/TBME (10:90) (solvent A) and water/methanol (5:95) (solvent B) with the following conditions: a linear gradient from 10 to 35% A for 20 min; a linear increase to 95% A for 5 min; isocratic at 95% A for 5 min; and a linear decrease to 10% A in 1 min. The column was equilibrated for 15 min at the starting conditions before each injection. The flow rate was 1.0 mL/min at 30 °C with an injection volume of 20 μL. Detection was conducted at 450 nm for antheraxanthin, zeaxanthin, and β-carotene. For quantitative analysis, the standard curves of zeaxanthin and β-carotene were calibrated using the linear least-squares regression equation derived from the peak area, whereas the concentration of antheraxanthin was tentatively calculated using the zeaxanthin standard curve.

Data Analysis. A minimum of three independent observations were made for each experimental group, and each experiment was repeated at least once to provide a minimum of $n = 3$ to determine significant differences in treatments. All data are expressed as the mean ± standard error of the mean (SEM). The Student−Newman−Keuls test was performed to separate treatment means when significant differences ($p < 0.05$) occurred. The statistical software used was GraphPad Prism 4.

RESULTS AND DISCUSSION

Identification of Major Carotenoids in *C. ellipsoidea*. *C. ellipsoidea* was used in this study to evaluate the influence of microfluidization on carotenoid bioaccessibility. The major carotenoids in *C. ellipsoidea* were identified using HPLC after solvent extraction. Figure 1 is a typical chromatogram of the main carotenoids in *C. ellipsoidea*. Because all chlorophylls should be removed by saponification, the chromatogram after saponification shows only carotenoid peaks. The result showed that peaks 2, 4, and 7 were chlorophyll pigments. Peaks 1, 3, and 6 were tentatively identified as antheraxanthin, zeaxanthin, and β-carotene, respectively. The major carotenoid peak from the *C. ellipsoidea* HPLC chromatogram was zeaxanthin, with two minor peaks, antheraxanthin and β-carotene. This result was in agreement with another study[22] that dealt with the extraction optimization of zeaxanthin from *C. ellipsoidea.* Peaks 5 and 8 might be considered the isoforms of zeaxanthin and β-carotene.

Violaxanthin, which has been reported in other studies[12,22] as one of the carotenoids in *C. ellipsoidea*, was not detected in this study. In fact, violaxanthin, antheraxanthin, and zeaxanthin are the key carotenoids in the xanthophyll cycle, which involves the enzymatic removal of epoxy groups from xanthophylls as a mechanism to reduce the amount of energy that reaches the photosynthetic reaction centers.[23] According to another study,[24] when green algae were suddenly exposed to high light, the diepoxide xanthophyll violaxanthin was rapidly transformed, via the intermediate antheraxanthin, to epoxide-free zeaxanthin through the function of the enzyme violaxanthin deepoxidase. It is considered that the lack of detection of violaxanthin in this study was caused by somewhat increased light conditions when *C. ellipsoidea* was cultured, which led to the de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin. Additionally, it was ascertained that the zeaxanthin in *C. ellipsoidea* was composed not of an ester form, but a free form. According to Chitchumroonchokchai's study,[25] intestinal Caco-2 cells took up free zeaxanthin more efficiently than they did zeaxanthin esters. Their result reveals a possibility that *C. ellipsoidea*, which contains the free form of zeaxanthin abundantly, might be a more bioavailable zeaxanthin source compared with the generally zeaxanthin ester-rich plant foods.

Effect of Microfluidization on the Size Distribution of *C. ellipsoidea*. Microfluidization was performed to increase the bioaccessibility of carotenoids. After the *C. ellipsoidea* culture solution was microfluidized at different pressures, the size of *C. ellipsoidea* was measured using a particle size analyzer to evaluate the effect of microfluidization on *C. ellipsoidea* cell wall pulverization. Figure 2 shows the particle size distribution of *C. ellipsoidea* before and after microfluidization at each pressure (5000, 10000, and 20000 psi). *C. ellipsoidea* without microfluidization exhibited the general feature of one singular group showing similar cell size. The size distribution graphs at 5000, 10000, and 20000 psi were shifted toward smaller sizes in proportion to the microfluidization pressure. In particular, *C. ellipsoidea* microfluidized at a pressure of 20000 psi had the most extensive distribution in the size range of 100−5000 μm and had the biggest distributional proportion in the range of 200−300 μm, indicating that microfluidization with high pressure effectively damaged the surface cell wall of *C. ellipsoidea*. According to Table 1, the particle size of *C. ellipsoidea* significantly diminished in proportion to pressure intensity. Interestingly, the polydispersity index, which was defined as the log-normal distribution width of the particle diameter, was not highest at 20000 psi but at 10000 psi. This

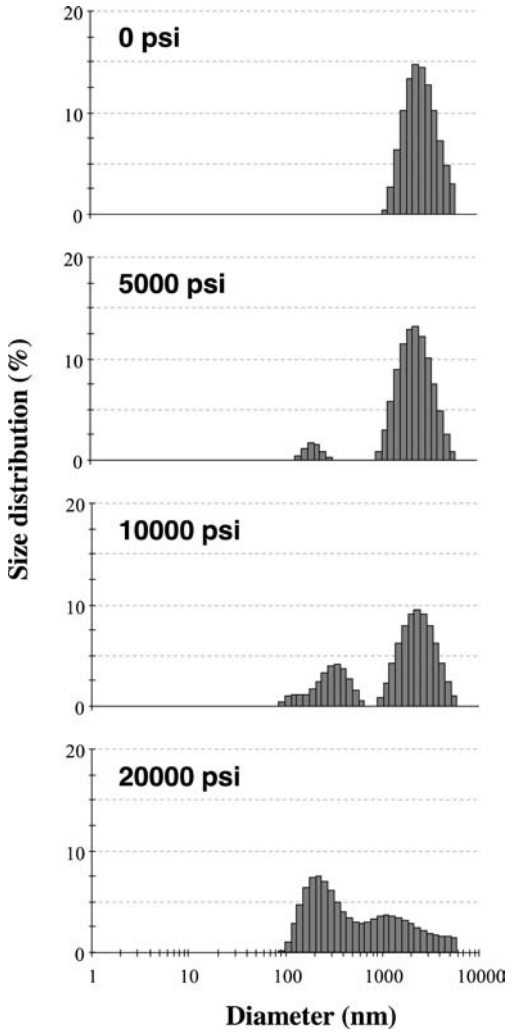

Figure 2. Particle size distribution of *C. ellipsoidea* before and after microfluidization at 5000, 10000, and 20000 psi.

Table 1. Influence of Microfluidization Pressure on Particle Size and the Polydispersity Index of *C. ellipsoidea*[a]

microfluidization pressure (psi)	mean particle size (nm)	polydispersity index
0	2463.67 ± 25.32 a	0.128 ± 0.020 a
5000	1782.67 ± 173.56b	0.321 ± 0.020 b
10000	821.57 ± 20.84 c	0.605 ± 0.028 c
20000	361.43 ± 8.19 d	0.455 ± 0.021 d

[a]Data represent the mean value ± SEM of three independent experiments. Different letters indicate significant differences ($P < 0.05$) between treatment means.

result reveals that the C. ellipsoidea microfluidized at 10000 psi contained various sizes of particles compared with 20000 psi-treated group and this might be due to the incomplete disruption of cell at 10000 psi.

Many types of mechanical and physical equipment, such as jet mills, dyno mills, and other homogenizers, have recently been applied in studies of microalgae cell rupture.[26,27] In our previous study,[19] microfluidization was used for the first time to pulverize the cell wall of *C. vulgaris*. The treatment of microfluidization at more than 10000 psi enabled the disintegration of the cell wall into a nanoscale size in the same manner as this study. These results indicate that microfluidization is a useful tool for microalgae cell disruption.

Carotenoid Analysis after Microfluidization and Simulated Digestion. Carotenoid content in *C. ellipsoidea* was measured using HPLC before and after microfluidization to assess the influence of microfluidization on carotenoid stability. Table 2 shows the carotenoid content for untreated *C. ellipsoidea* (UCE) and for *C. ellipsoidea* microfluidized at 5000, 10000, and 20000 psi (MCE5000, MCE10000, and MCE20000). Zeaxanthin was the most abundant carotenoid in *C. ellipsoidea* (1.85 mg/g *C. ellipsoidea*). About 70% of the total carotenoids in *C. ellipsoidea* was zeaxanthin, and the others were antheraxanthin and β-carotene (17 and 13%, respectively). In terms of zeaxanthin and β-carotene, there was no significant difference between UCE and MCE groups, showing that the content of zeaxanthin and β-carotene remained stable despite the application of microfluidization. However, the antheraxanthin content decreased with microfluidization. In particular, MCE10000 and MCE20000 exhibited significant content losses compared with UCE (30 and 66%, respectively), indicating that antheraxanthin was likely to break down in the process of microfluidization with high pressure.

Table 2. Carotenoid Content of Untreated *C. ellipsoidea* (UCE) and Microfluidized *C. ellipsoidea* (MCE5000, MCE10000, and MCE20000)[a]

	μg/g dry weight of chlorella		
	antheraxanthin	zeaxanthin	β-carotene
UCE	471 ± 32 a	1849 ± 200 a	352 ± 11 a
MCE5000	412 ± 70 ab	1830 ± 301 a	393 ± 32 a
MCE10000	328 ± 24 b	1999 ± 189 a	337 ± 44 a
MCE20000	160 ± 21 c	1875 ± 131 a	360 ± 24 a

[a]Data represent the mean value ± SEM of at least three independent experiments. Different letters indicate significant differences ($P < 0.05$) between treatment means.

Some studies have shown that antheraxanthin was likely to be adversely affected by food processing, such as drying and heating. According to Daood's study,[28] when pepper was dried at 90 °C, only 55% of antheraxanthin remained after the processing, compared with pepper dried at room temperature. In another study,[29] antheraxanthin content in orange juice notably diminished after microwave heating at 85 °C for 1 min, whereas no significant β-carotene decrease was observed. According to other studies,[30,31] the 5,6-epoxide groups of antheraxanthin and other similar carotenoids, such as violaxanthin, are transformed to the 5,8-furanoid derivatives that are catalyzed by acids. This transformation could be caused by the action of external acids during fermentation of food preparation or by contact with the organic acids that are generated from disrupting processing such as heating or grinding.[32] In this regard, antheraxanthin degradation in this study might be due to the increase of organic acids releases after microfluidization.

Simulated digestion was performed to evaluate the effect of microfluidization on increasing carotenoid bioaccessibility from *C. ellipsoidea* (Table 3). The bioaccessibility of carotenoids in UCE was extremely low compared to MCE groups. Approximately 95% of total carotenoids could not be released and micellized from the *C. ellipsoidea* cell matrix during simulated digestion, but remained in the insoluble digestate. Microfluidization remarkably improved the bioaccessibility of zeaxanthin and β-carotene, but not that of antheraxanthin in *C. ellipsoidea* during the digestion process. A significant increase appeared in the bioaccessibility of zeaxanthin and β-carotene when *C. ellipsoidea* was microfluidized at more than 10000 psi. In MCE20000, the contents of zeaxanthin and β-carotene were 12.5 and 10.8 times higher, respectively, than in UCE. Particularly, no antheraxanthin was detected after simulated digestion. The lack of detection of antheraxanthin might be because antheraxanthin could be transformed to the 5,8-furanoid derivative by acids, as discussed above, because the simulated digestion included the acidification procedure to mimic the gastric phase.

Table 3. Bioaccessibility of Carotenoids from UCE and Microfluidized *C. ellipsoidea* (MCE5000, MCE10000, and MCE20000) during Simulated Digestion[a]

	carotenoid bioaccessibility (%)		
	antheraxanthin	zeaxanthin	β-carotene
UCE	ND[b]	2.60 ± 0.08 a	1.69 ± 0.24 a
MCE5000	ND	7.81 ± 0.63 a	2.96 ± 0.88 a
MCE10000	ND	21.99 ± 3.72 b	10.13 ± 0.99 b
MCE20000	ND	32.60 ± 2.97 c	18.19 ± 3.44 c

[a]Bioaccessibility (%) is defined as the proportion of carotenoids present in the micelles, compared with that contained in the original *C. ellipsoidea*. Data represent the mean value ± SEM of at least three independent experiments. Different letters indicate significant differences ($P < 0.05$) between treatment means. [b]ND, not detected in micelles.

In this study, the bioaccessibility of zeaxanthin was greater than that of β-carotene in the same samples. This result was in accordance with the results from other studies that showed zeaxanthin was more bioavailable than β-carotene.[21,33] The chemical structures of carotenoid molecules seemed to be a key factor in their localization in the lipid bilayer of a food matrix.[34] β-Carotene, a pure hydrocarbon with no oxygen, is highly hydrophobic; thus, it was generally located in the emulsion droplet core, whereas zeaxanthin, a xanthophyll containing a

hydroxyl group, was preferentially distributed in the oil/water interface.[35] Interestingly, microfluidization enabled an upsurge in the bioaccessibility of both zeaxanthin and β-carotene in the same manner.

When *C. vulgaris* was treated with the same simulated digestion system as in our previous study,[19] the bioaccessibility of lutein was 26%, which was approximately 10 times higher than zeaxanthin bioaccessibility in *C. ellipsoidea*. This distinction between *C. vulgaris* and *C. ellipsoidea* may have originated from a difference in their culture system. It was reported that when green algae were cultured in a bioreactor without strong sunlight, as with *C. vulgaris*, the heterotrophic cultures induced enlarged cells with extended surface area and thinner cell walls than outdoor-cultured cells such as *C. ellipsoidea*.[36] Another study showed that the chlorella grown under autotrophic condition contained a higher amount of cellulose compared with heterotrophic cells.[37] In fact, the mean particle size of *C. ellipsoidea* (2.5 μm) was smaller than that of *C. vulgaris* (3.6 μm), which explains why microfluidization was more effective in the less-bioavailable *C. ellipsoidea*.

In conclusion, the bioaccessibility of carotenoids from *C. ellipsoidea* was investigated using simulated digestion, finding serious limitations in carotenoid bioaccessibility. Microfluidization effectively improved the poor bioaccessibility of zeaxanthin and β-carotene in *C. ellipsoidea*, whereas antheraxanthin showed a tendency to degrade when subject to microfluidization. Therefore, microfluidization is an effective processing tool for the increase of carotenoid bioaccessibility during microalgae digestion.

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Notes

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ABBREVIATIONS USED

TBME, *tert*-butyl methyl ether; BHT, butyl hydroxytoluene; UCE, untreated *C. ellipsoidea*; MCE5000, microfluidized *C. ellipsoidea* at a pressure of 5000 psi; MCE10000, microfluidized *C. ellipsoidea* at a pressure of 10000 psi; MCE20000, microfluidized *C. ellipsoidea* at a pressure of 20000 psi; MCE, microfluidized *C. ellipsoidea*; SEM, standard error of the mean.

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