Supercritical carbon dioxide anti-solvent precipitation of anti-oxidative zeaxanthin highly recovered by elution chromatography from Nannochloropsis oculata

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1. Introduction

Microalgae, full of carotenoids, have been considered a new kind of nutritional resources that cultivated from sea water. Commercial applications of microalgae in food industry are in different forms such as tablets, capsules and liquids. These microalgae were also widely investigated in the fields of food supplements and drug manufacture [1]. Carotenoids are essential nutrients in human diet. They mostly can be used as natural food colorants and additive in animal feed. Nowadays, carotenoids also have applications in cosmetics, especially in neutraceuticals. Lutein and zeaxanthin that are two major carotenoids in human act as strong antioxidants and filter high-energy blue light [2]. Moreover, zeaxanthin is found in the macular pigment in high concentrations (approximately twice than lutein) and plays a critical role in the pathogenesis of age-related macular degeneration (ARMD). In the eye disease dose-control study, a high dietary intake of zeaxanthin was related to a lower risk of ARMD and the improvement of human visual performance [3]. It is estimated that the ratio of lutein to zeaxanthin in the diet ranges from 4:1 to 7:1 [4]. In fact, humans are unable to synthesize zeaxanthin, and the intake of zeaxanthin is not in a substantial amount in common dietary life. Usually, the majority of these lutein and zeaxanthin was extracted from the Marigold flowers planted in Europe. Nevertheless, zeaxanthin could be existed in a few species of bacteria and microalgae, which are possibly grown and cultivated in island countries like Taiwan. The extraction of these anti-oxidative carotenoids from microalgae is important not to be highly relied on a huge amount of importing lutein and zeaxanthin from foreign countries in years.

Supercritical fluid anti-solvent (SAS) processes recently have been adopted as an alternative in purifying thermo-labile compounds from natural materials. It is possible to totally remove the anti-solvent (e.g., carbon dioxide) by reducing pressure to the gas phase. Moreover, the supercritical fluid molecule has high mass transfer coefficient in capable of penetrating easily into solution to lead a super-saturation of solutes and to make a precipitation rapidly. In common, particles down to nano size containing a large amount of bioactive compounds are not easily obtained when using conventional liquid anti-solvents or by other techniques such as the jet milling or the dry spraying [5]. Carotenoids become unstable in an environment of light and oxygen. Relatively low solubilities of most carotenoids of natural materials in supercritical CO2 allow suitable usage of CO2 as anti-solvent for the SAS precipitation of these thermo-labile compounds at...
nearly room temperature [6–8]. Hence, the SAS processes largely employed in the micronization of pharmaceutical ingredients have been recently reported [9,10]. Supercritical carbon dioxide is highly dissolved into a few organic solvents and makes enormously expansion ratio, especially in the critical point [11], leading a strong influence of the super-saturation ratio on the shape and size of crystals. Supercritical carbon dioxide was proved to be a very suitable anti-solvent for the production of micro-sized algal pigments [12]. Besides, several authors have also utilized the SAS for the separation of bioactive compounds from natural materials, including flavonoids, β-carotene, ginkgolides and lycopene [10,13,14]. However, experimental data on phase equilibrium between supercritical carbon dioxide and organic solvent is a key in understanding the SAS process as supercritical, superheated, liquid and supercritical carbon dioxide and organic solvent is a key in understanding the SAS process as supercritical, superheated, liquid and supercritical, respectively. The co-existing phases directly influence morphology, size and distribution of particles [15,16]. The SAS processes have been carried out in one-phase region while no interface exists between the solution and the anti-solvent fluid, thus precipitation was primarily caused by nucleation and small-size particles usually presented less agglomeration [17]. Pressure, temperature, solution concentration, feed flow rate and CO2 flow rate of the SAS precipitation were studied in accomplishing an optimal condition in obtaining small particles with narrow particle size distribution [18–21]. In order to form nano-particles containing high amount of carotenoids obtained from algal extracts, column fractionation coupled with precipitation process is necessary for the preparation of carotenoids-rich precipitates. This study examined ultrasonic solvents extraction of carotenoids from Nannochloropsis oculata coupled with column fractionation in obtaining zeaxanthin-rich elution, and then, submicron-sized bioactive precipitates of the purest zeaxanthin were successfully generated after the SAS precipitation process.

2. Materials and chemicals

2.1. Materials

N. oculata was cultivated by sea water in a few 5 ton polypropylene tanks and 500 g alga in dry basis were obtained from marine research center in Institute of Marine Biology of National Sun Yat-sen University (Kaohsiung, Taiwan) and from Genereach-Biotech Company (Central Science Park, Taichung, Taiwan). Microalgae (abbr. NCHU099) were ground for 5 s into powders using a blade-type grinder and then collected by sieving through a 100 mesh stainless steel screen under dimmed light. It was then freeze-dried and stored in a freezer at −80 °C before extraction.

Analytical grade solvents used for the extractions, the column chromatography and the SAS process, include 99% ethanol (Mallinckrodt, USA), 99.5% acetone (Mallinckrodt, USA), 99.5% dichloromethane (DCM) (Mallinckrodt, USA), HPLC grade solvents used for mobile phase in HPLC, include 99.5% methanol (Mallinckrodt, USA), 99.5% methyl tert-butyl ether (Mallinckrodt, USA), Ultra pure water (>18 MΩ) was obtained by using Ultrapure™ water purification system (Louton Co. Ltd., Taipei, Taiwan) and was filtered through a 0.45 μm membrane filter prior to use. The analytical grade of PS100 resin (Mitsubishi, Japan) were purchased and used without further purification. And 99.95% CO2 (Toyo gas, Taiwan) used for the SAS precipitation. The authentic standards of carotenoids included 90.0% lutein (Fluka, Switzerland), >95% zeaxanthin (Fluka, Switzerland), 95% β-cryptoxanthin (Sigma–Aldrich, USA), 95% trans-β-apo-8′-carotenal (Fluka, Switzerland), 95% mix isomers of α-carotene:β-carotene = 2:1 (Sigma–Aldrich, USA).

2.2. Solvent extraction

2.2.1. Soxhlet extraction

Freeze-dried algal powder (10.0 g) was extracted by using dichloromethane over 16 h in a 175 mL Soxhlet extractor. After extraction, solvents were removed under vacuum and then weighed. The extracts were stored in −80 °C refrigerator before column chromatography. The whole extractive and quantitative procedures were carried out under dimmed light.

2.2.2. Ultrasonic extraction

A stirred ultrasonic extractor similar to the work of Chen et al. [22] was employed without modification. 5 g of freeze-dried microalgae was extracted in 50 mL of the deionized water were obtained by using this apparatus equipped with frequency of 40 kHz and 300 W of power. The equipment performed at three 303 K and 323 K. The extraction time is 30 min. After extraction, the solution was filtered through a 0.45 μm syringe filter. Then, solvent was removed under vacuum and the residue was then weighed. The extracts were stored in −80 °C refrigerator before column chromatography fractionation. The whole extractive and quantitative procedures were carried out under dimmed light.

2.3. Column chromatography fractionation

The freeze-dried microalgae (3.00 ± 0.01 g) were exhaustively extracted in acetone with a 303 mL. The algal extracts were concentrated under vacuum to yield a loading sample (0.210 g) for the open-type column chromatography. The loading sample was dissolved in the pharmaceutical grade ethanol. The solution was subjected to a 3 cm (i.d.) × 30 cm (L) glass column which was packed with polyethylene based resin as the stationary phase. Isocratic elution was carried out using the mobile phase of EtOH and acetone (98:2). Finally, total 3 fractions were collected, and the solvent of each fraction was removed under vacuum and then weighed individually. The purified samples were stored in −80 °C refrigerator before the HPLC analysis and the SAS precipitation.

2.4. SAS precipitation process

A SC-CO2 precipitation setup similar to the work of Wu et al. [10] was employed with a minor modification. Fig. 1 presents the schematic flow diagram of the SAS precipitation. Liquid CO2 was compressed using a high-pressure pump (Spe-ed SFE, Applied Separations, USA) (4) into the first surge tank (75 mL, L/D = 30) (8). It entered a second surge tank (750 mL, L/D = 10) (11) at a constant flow rate after it was preheated using a heat exchanger (7). A coaxial flow nozzle of 0.0007 inch inside diameter was inserted into the entrance of the precipitator to act as the inlet of the solution feed. Then, CO2 flowed (54 g/min) through a metering valve (SS-31R54-A, Swagelok, USA) (6–3) into the visible precipitator (TST, Taiwan) (12) and various concentration (0.4, 0.8 mg/mL) of feed solutions were delivered into the precipitator at flow rates of 1–2 mL/min via a high-pressure liquid pump (L-6200A, Hitachi, Japan) (13). A stainless frit (37 μm) and an online filter (0.45 μm) were placed at the bottom of precipitator to prevent the penetration of particles. The operating pressure (200 bar) was regulated using a back-pressure regulator (26–1722, Tescom, USA) (9–3), and the operating temperature (313 K) was controlled using a water bath circulator (5–3). The consumption of CO2 was measured using a wet gas meter (TG20, Ritter, Germany) (16).

2.5. Quantification of carotenoids

High-performance liquid chromatography (HPLC) was performed using a Hitachi 2130 pump and 2400 UV series system
(Hitachi Ltd., Tokyo, Japan). The analysis was carried out with a reversed-phase YMC C-30 (5 μm, 250 mm × 4.6 mm i.d.) and a Phenomenex Luna security guard cartridge C-18 (5 μm, 4 mm × 2.0 mm i.d.). The microalgal extracts were eluted using mobile phases of water (A) methanol (B) and methyl tert-butyl ether (C). The eluent flow rate was maintained at 1 mL/min, the injection volume was 20 μL, and the detection wavelength and column temperature were set at 450 nm and 303 K. The elution gradient was as follows: 0 min, 10% A, 90% B, 0% C; 5 min, 4% A, 81% B, 15% C; 25 min, 4% A, 81% B, 15% C; 50 min, 4% A, 31% B, 65% C. The identity of zeaxanthin was confirmed by comparing their HPLC retention time with the analytical standards at the wavelength of 450 nm.

2.6. Analysis of particles size distribution and morphology

The mean particle size and particle size distribution (PSD) were determined using a light scattering particle size analyzer (Beckman Coulter, Counter F5, USA). The morphologies of the particles were examined using a field emission scanning electron microscope (FESEM) (JSM-7401F, JEOL, Japan). Before SEM examination, particles were sputtered with a layer of platinum film and the images were observed under a voltage of 2 kV.

2.7. Antioxidant assay of algal extracts

3.94 mg of DPPH powder was dissolved in the 50 mL methanol to form a 200 μM purple blue solution. 1 mL of the purple blue solution was mixed with 3 mL of the algal extract solution at the concentration ranging from 0.1 mg/mL to 2.5 mg/mL in a quartz tube. After reaching for 30 min and the absorption of this solution was measured at 517 nm by an UV-Vis spectrophotometer (Hitachi, U-3000, Japan). The DPPH scavenging ratio of the extract was calculated to determine a half-effective concentration of the extract (i.e., EC50 value), which is a measure of when 50% of the DPPH free radicals in the solution were being scavenged. The scavenging ratio value is defined by \[ \left( \frac{\text{ABS}_{\text{blank}} - \text{ABS}_{\text{sample}}}{\text{ABS}_{\text{blank}}} \right) \times 100\% \], where blank is the absorption without adding the purified microalgal sample and ABS_{\text{blank}} is the absorption of the purified microalgal sample.

2.8. Adult retinal pigment epithelium-19 (ARPE-19) cell assays

ARPE-19 cells were from mice epithelium cancer cells (BCRC 60383, Taiwan). After defrosting, ARPE-19 cells were cultured in the 1:1 mix medium of Dulbecco’s Modified Eagle Medium and Ham’s F-12 Nutrient Mixture (DMEM-F12) (HyClone Laboratories Inc., Logan, USA) complemented with 10% fetal bovine serum (FBS) (HyClone Laboratories Inc., Logan, USA) and 1% penicillin/streptomycin solution (Taiwan Veterans Pharmaceutical Co. Ltd., Taoyuan, Taiwan). In the beginning, cultures (DMEM-F12) with the cells were added in the 10 cm of tissue culture-treated dishes. The cells were grown at 310 K in a humidified 5% CO2 condition. After the cells plating for 48 h, the medium was removed and washed using a phosphate buffer solution (PBS) and Trypsin EDTA Glucose (TEG). The ARPE-19 cells were separately seeded (5 × 10^4 cell/well) into a 12-well plate in triplicates and then incubated 24 h for the cell attachment. The medium was then aspirated and the fresh medium containing 1 mL of various concentrations ranged from 0 to 15 μg/mL of the test sample was added to the cultures. The cells were again incubated in the presence of each sample at 310 K for 24 h under humidified air containing 5% CO2. Cell viability was individually evaluated by two treatments: (1) with the addition of H2O2 and (2) without the addition of H2O2 treatment (as a negative control). After the treatment with H2O2 (30%, 1 mL) for
the SAS anti-solvent precipitation study. Fig. 2 shows the elution from the feed. Finally, fraction 2 was dried and collected as the feed for the purification of zeaxanthin in the second fraction was fifteen-fold higher than that of the feed. The purity of zeaxanthin was enhanced from 19.18 mg/g in the acetone extract to 303.02 mg/g in the column fraction. Specially, only a small amount of zeaxanthin was lost during the whole partition process according to the duplicate experiments. The purity of zeaxanthin in the second fraction was fifteen-fold higher than that of the feed. Finally, fraction 2 was dried and collected as the feed for the SAS anti-solvent precipitation study. Fig. 2 shows the elution chromatography spectra of these three fractions analyzed by the HPLC-UV quantification at 450 nm. The HPLC spectra revealed that the amount of other carotenoids was decreased except zeaxanthin in the partition. Finally, the amount of zeaxanthin in three fractions is higher than those of other carotenoids. This stationary and mobile phase partition showed that these fractions are completely separated. The chromatogram of algal samples in the column partition was shown in Fig. 2b–d. It is a superior one-step column fractionation process in refining zeaxanthin efficiently from the algal solution. A few different kinds of cultivated algal materials were further tested in this column fractionation process. The recovery and purity of zeaxanthin are similar as mentioned. To reach the goal of concentrating zeaxanthin by this column fractionation, several process factors such as loading capacity of the column, pretreatment of the feed and kinds of mobile and stationary phases have also been preliminarily examined.

3. Results and discussion

3.1. Solvent extractions

Table 1 lists experimental data of Soxhlet solvent extractions which last 16 h and three ultrasonic extractions which last 0.5 h respectively. It is clear that the ultrasonic extractions display more efficiency by recovering zeaxanthin from N. oculata within 0.5 h. Although 16-h Soxhlet extractions recover total carotenoids (C_{cars}) with 100% recovery, zeaxanthin is a thermal sensitivity compound and is particularly degraded easily in long presence of oxygen and light [12]. Under long time Soxhlet extraction at elevated temperature, the zeaxanthin would not be fully recovered since it may be destroyed gradually with time up to 16 h. For this reason, ultrasonic acetone extraction is an acceptable method to recover zeaxanthin within half of an hour, as shown in Table 1. At the moderated extraction temperature (i.e., 303 K), the US-acetone 1 extract gives high content of zeaxanthin than high temperature (i.e., 323 K) because the oxidation of zeaxanthin may be involved at the 323 K extraction. Besides, the matrix of the ethanol extracts was more complex than those of the acetone extracts, resulting in low recovery of zeaxanthin.

3.2. Column chromatography fractionation

Based on the high recovery of zeaxanthin in the ultrasonic extraction better than that of Soxhlet extraction, a fixed amount of the ultrasonic acetone extract was dissolved in ethanol and loaded into an open-type column for the elution chromatography experiments at a fixed flow rate of 7 mL/min with a period of 8 h. Table 2 presents the concentration of zeaxanthin in each fraction. Fraction 2 effluent was identified as zeaxanthin-rich by the utilization of the HPLC analysis, which was enhanced from 19.18 mg/g in the acetone extract to 303.02 mg/g in the column fraction. Specially, only a small amount of zeaxanthin was lost during the whole partition process according to the duplicate experiments. The purity of zeaxanthin in the second fraction was fifteen-fold higher than that of the feed. Finally, fraction 2 was dried and collected as the feed for the SAS anti-solvent precipitation study. Fig. 2 shows the elution chromatography spectra of these three fractions analyzed by the HPLC-UV quantification at 450 nm. The HPLC spectra revealed that the amount of other carotenoids was decreased except zeaxanthin in the partition. Finally, the amount of zeaxanthin in three fractions is higher than those of other carotenoids. This stationary and mobile phase partition showed that these fractions are completely separated. The chromatogram of algal samples in the column partition was shown in Fig. 2b–d. It is a superior one-step column fractionation process in refining zeaxanthin efficiently from the algal solution. A few different kinds of cultivated algal materials were further tested in this column fractionation process. The recovery and purity of zeaxanthin are similar as mentioned. To reach the goal of concentrating zeaxanthin by this column fractionation, several process factors such as loading capacity of the column, pretreatment of the feed and kinds of mobile and stationary phases have also been preliminarily examined.

3.3. SC-CO2 anti-solvent precipitation

The algal extracts obtained after the column fractionation process of ultrasonic acetone extract were mixed to form the sample of zeaxanthin of 292.2 mg/g. The concentration of feed solution and the flow rate of the anti-solvent are two major parameters in affecting the particle size of the precipitates. The effect of the precipitates, reported by Wu et al. [10]. In this SAS study, feed concentrations of 0.4–0.8 mg/mL, solution flow rates of 1–2 mL/min and injection times of 1–10 min were chosen, the rest SAS operational conditions (pressure of 200 bar, temperature of 313 K and CO2 flow rate of 54 g/min) were kept constant in examination of how the amount of zeaxanthin and mean size of the precipitates changed with the injection time and the feed flow rate. According to experimental data of Chang and Randolph, the solubility of carotenoids in the supercritical CO2 phase is scarce (i.e., 10⁻⁵ mol fraction) [8]. The loss of carotenoids in the process period of the SAS process is negligible. Our experimental data showed that total amounts of zeaxanthin in the SAS precipitates were higher than those in the feeds. The run #A (one of a few SAS runs) showed that the content of zeaxanthin increased from 292 mg/g (29.2%) in the fractioned sample to 507.3 mg/g (50.7%) in the precipitate, shown in Table 3. The amount of zeaxanthin in the precipitates and the total yield were 507.3 mg/g and 40.6%, respectively shown in run #A of the 6 min injection time, while the amount of zeaxanthin and the total yield were changed to 368.3 mg/g and 49.7%, respectively shown in run #B of the 1 min injection time. The amount of zeaxanthin decreased at high feed flow rate and high solution concentration, shown by run #C and run #D. Fig. 3 indicates that morphology and size of the precipitate could be changed by varying the injection time and the feed flow rate. At constant CO2 flow rate of 54 g/min, small plate type particles could be obtained at low feed flow rate of 1 mL/min with 1 min of the injection time (run #B), even at the solution concentration of 0.8 mg/mL. It is displayed that the mean size of the precipitate becomes large and agglomerated at 2 mL/min when the injection time increased to 6 min (run #A). The mean sizes of precipitates were changed from 276 nm (run #A) down to 80 nm (run #B), measured by a Coulter counter in duplicate, respectively.

3.4. Antioxidant capacity

A few algal samples including the ultrasonic acetone extract, the partial purified fractions (fractions 1–3) were subjected to the assay of antioxidant capacity. Fig. 4 shows anti-oxidative capacities of the samples determined by the DPPH radical scavenging assay. Our experimental data showed that the EC50 values of scavenging the DPPH free radicals by α-tocopherol, the ultrasonic extract, the purified fraction (F-2) were 0.06, 1.00, and 0.46 mg/mL, respectively. It has been noted that α-tocopherol used as a positive control had the best effect over all the other samples. It is indicated that the C_zea value of the fraction 1 (F-1) was lowest (not detectable) presented the lowest effect on the DPPH scavenging ability; similar
Table 1
Solvent extraction of 5 g of *N. oculata*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TY (%)</th>
<th>C&lt;sub&gt;zea&lt;/sub&gt; (mg/g&lt;sub&gt;ext&lt;/sub&gt;)</th>
<th>C&lt;sub&gt;fuco&lt;/sub&gt; (mg/g&lt;sub&gt;ext&lt;/sub&gt;)</th>
<th>C&lt;sub&gt;/H9251&lt;/sub&gt;-car (mg/g&lt;sub&gt;ext&lt;/sub&gt;)</th>
<th>R&lt;sub&gt;zea&lt;/sub&gt; (%)</th>
<th>R&lt;sub&gt;fuco&lt;/sub&gt; (%)</th>
<th>R&lt;sub&gt;/H9251&lt;/sub&gt;-car (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soxhlet-CH&lt;sub&gt;2&lt;/sub&gt;Cl&lt;sub&gt;2&lt;/sub&gt; (16 h, 315 K)</td>
<td>8.81</td>
<td>12.20</td>
<td>0.74</td>
<td>1.36</td>
<td>84</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>US-EtOH (0.5 h, 303 K)</td>
<td>32.74</td>
<td>3.19</td>
<td>0.22</td>
<td>0.00</td>
<td>82</td>
<td>96</td>
<td>0</td>
</tr>
<tr>
<td>US-acetone 1 (0.5 h, 303 K)</td>
<td>4.77</td>
<td>23.68</td>
<td>0.61</td>
<td>0.63</td>
<td>89</td>
<td>43</td>
<td>29</td>
</tr>
<tr>
<td>US-acetone 2 (0.5 h, 323 K)</td>
<td>6.53</td>
<td>19.49</td>
<td>0.57</td>
<td>1.08</td>
<td>100</td>
<td>56</td>
<td>68</td>
</tr>
</tbody>
</table>

Soxhlet: Soxhlet extraction; US: stirred ultrasonic extraction (Solvent to solid ratio = 80); TY: total yield = (weight of extract/weight of feed) × 100%; C<sub>zea</sub>: concentration of zeaxanthin in extracts; C<sub>fuco</sub>: concentration of fucoxanthin in extracts; C<sub>/H9251</sub>-car: concentration of /H9251-carotene in extracts; R<sub>zea</sub>: recovery of zeaxanthin; R<sub>fuco</sub>: recovery of fucoxanthin; R<sub>/H9251</sub>-car: recovery of /H9251-carotene.

Table 2
Experimental data on column partition fractionation of algal extract.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>W&lt;sub&gt;e&lt;/sub&gt; (mg)</th>
<th>TY (%)</th>
<th>C&lt;sub&gt;fuco&lt;/sub&gt; (mg/g)</th>
<th>R&lt;sub&gt;fuco&lt;/sub&gt; (%)</th>
<th>C&lt;sub&gt;zea&lt;/sub&gt; (mg/g)</th>
<th>R&lt;sub&gt;zea&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-1</td>
<td>62.3</td>
<td>39.2</td>
<td>0.71</td>
<td>67.9</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>F-2</td>
<td>9.3</td>
<td>5.2</td>
<td>ND</td>
<td>0</td>
<td>303.02</td>
<td>92.4</td>
</tr>
<tr>
<td>F-3</td>
<td>14.1</td>
<td>8.9</td>
<td>ND</td>
<td>0</td>
<td>17.91</td>
<td>8.3</td>
</tr>
</tbody>
</table>

W<sub>feed</sub>: 159 mg; C<sub>zea,feed</sub>: 19.18 mg/g<sub>ext</sub>; C<sub>fuco,feed</sub>: 0.41 mg/g<sub>ext</sub>; eluent: EtOH/acetone = 98/2; W<sub>e</sub>: weight of each fraction; C<sub>zea</sub>: concentrations of zeaxanthin in fraction; R<sub>zea</sub>: recovery of zeaxanthin = (W<sub>e</sub> × C<sub>zea</sub>) / (W<sub>feed</sub> × C<sub>zea,feed</sub>) × 100%; ND: not detectable.

Table 3
Experimental data on SC-CO<sub>2</sub> antisolvent precipitation of algal solution at 200 bar and 313 K.

<table>
<thead>
<tr>
<th>Run</th>
<th>t (min)</th>
<th>Q&lt;sub&gt;feed&lt;/sub&gt; (mL/min)</th>
<th>C&lt;sub&gt;feed&lt;/sub&gt; (mg/mL)</th>
<th>TY (%)</th>
<th>C&lt;sub&gt;zea&lt;/sub&gt; (mg/g)</th>
<th>R&lt;sub&gt;zea&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>2</td>
<td>0.4</td>
<td>40.6</td>
<td>507.3</td>
<td>70.6</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>1</td>
<td>0.8</td>
<td>49.7</td>
<td>368.3</td>
<td>62.4</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>2</td>
<td>0.8</td>
<td>39.6</td>
<td>453.8</td>
<td>61.5</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>2</td>
<td>0.8</td>
<td>41.7</td>
<td>391.9</td>
<td>55.9</td>
</tr>
</tbody>
</table>

Run A: W<sub>feed</sub> = 4.8 mg at 6 min; B: W<sub>feed</sub> = 0.8 mg at 1 min; C: W<sub>feed</sub> = 1.6 mg at 1 min; D: W<sub>feed</sub> = 4.8 mg at 3 min; Q<sub>CO<sub>2</sub></sub>: flow rate of CO<sub>2</sub> = 54 g/min; Q<sub>feed</sub>: solution flow rate; C<sub>feed</sub>: concentration of solution; TY: total yield of precipitates = (W<sub>precipitates</sub>/W<sub>feed</sub>) × 100%; C<sub>zea</sub>: concentrations of zeaxanthin in precipitates; R<sub>zea</sub>: recovery of zeaxanthin = (W<sub>precipitates</sub> × TY × C<sub>zea</sub>) / (W<sub>feed</sub> × C<sub>zea,feed</sub>) × 100%; C<sub>zea,feed</sub> = average zeaxanthin concentration in feed = 292.2 mg/g.

Fig. 3. Morphology (SEM) and particle size distribution (PSD) of two algal precipitates at 200 bar and 313 K.
Table 4
Effect on proliferation of ARPE-19 cells by column fractions, SAS precipitates and 95% zeaxanthin.

<table>
<thead>
<tr>
<th>Samples</th>
<th>C (µg/mL)</th>
<th>Viabilityb (% seeded cells)</th>
<th>SDab</th>
<th>Viabilityc (% seeded cells)</th>
<th>SDac</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-2</td>
<td>0</td>
<td>114.13</td>
<td>13.26</td>
<td>34.57</td>
<td>4.14</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>102.88</td>
<td>28.07</td>
<td>71.88</td>
<td>13.09</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>106.17</td>
<td>30.83</td>
<td>85.60</td>
<td>8.50</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>102.88</td>
<td>14.79</td>
<td>75.17</td>
<td>15.72</td>
</tr>
<tr>
<td>SAS precipitates</td>
<td>0</td>
<td>230.99</td>
<td>10.75</td>
<td>25.56</td>
<td>3.31</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>183.63</td>
<td>28.12</td>
<td>46.00</td>
<td>1.35</td>
</tr>
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<td>6.62</td>
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<td>150.29</td>
<td>24.10</td>
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<td>95% Zeaxanthin</td>
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<td>16.39</td>
<td>43.01</td>
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<td>144.32</td>
<td>12.41</td>
<td>75.63</td>
<td>7.54</td>
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<td>147.67</td>
<td>10.21</td>
<td>93.55</td>
<td>11.44</td>
</tr>
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</table>

F-2: column fraction #2; C: dose concentration.

s Standard deviation (n = 3).
b ARPE-19 cell was not treated with H2O2.
c ARPE-19 cell was treated with 1 h H2O2.

4. Conclusions

This study demonstrated the purification of zeaxanthin of the ultrasonic acetone extracts obtained from N. oculata after the reverse phase column elution chromatography coupled with the supercritical anti-solvent (SAS) precipitation process. A polystyrene based resin was chosen as the absorbent in elution chromatography to yield a purest zeaxanthin effluent with recovery of 92.4%. Experimental results show that the amount of zeaxanthin in the second fraction was 15-fold higher than that in the extract. High amount of zeaxanthin in the SAS precipitates could be obtained after the selection of supercritical anti-solvent CO2 at a suitable injection time. At the same flow rate of anti-solvent, high flow rate of feed solution results in large particles at a long time injection due to agglomeration and accumulation. In vice versa, at low flow rate of feed solution did provide small particle at a short injection time evidenced that the SAS process is feasible to generate nano-sized particles. Supercritical carbon dioxide anti-solvent precipitations of the column fractionated algal solution do produce nano-scale particles containing high amount of zeaxanthin. Two kinds of chemical antioxidant capacities tests, including the DPPH free radical scavenging and the ARPE-19 cell proliferation assays were applied for the bioactivity assay of several algal samples.

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References