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Demonstration of continuous supercritical carbon dioxide anti-solvent purification and classification of nano/micro-sized precipitates of algal zeaxanthin from *Nannochloropsis oculata*

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ABSTRACT

This study examined time dependent continuous supercritical anti-solvent (SAS) recrystallization applied for purifying and generating zeaxanthin rich particulates from micro-algal *Nannochloropsis oculata*. Feed solutions varied from 0.4 to 1.2 mg/ml subjected into a 250 ml SAS crystallizer to yield a few classifications of nano- or micro-sized purified precipitates corresponding to different recrystallization time zones. The effect of operational conditions on amount, recovery of the zeaxanthin and mean size, morphology of the precipitates was obtained from experimentally designed SAS process. The mean size of particles falls within several hundreds of nanometers and the content of zeaxanthin in the particulates range from 65 to 71%, which relies highly upon the recrystallization time. This work demonstrated an environmental benign process in producing nano- or micro-sized particles containing rich-zeaxanthin from algal solution by using supercritical anti-solvent within minutes.

1. Introduction

Zeaxanthin ($C_{40}H_{56}O_2$) is the principal pigment obtained from yellow corn or marigold and possesses important physiological functions in the human body. Dietary xanthophylls such as lutein and zeaxanthin play important roles on against of the age-related macular degeneration (AMD) especially notably on the age-related eye disease (AED) (Krishnadev *et al.*, 2010; Moeller *et al.*, 2000; SanGiovanni *et al.*, 2007). Lutein and zeaxanthin have been found to be accumulated in high concentration within human retina (Bernstein *et al.*, 2001). Photoprotective mechanisms are important effects to prevent the harmful reactions in plants (marigold) or in algae. The harmful reactions were induced by the excess of photon absorbed from the sunlight irradiation. Carotenoids have been suggested as key molecules in the photoprotective mechanisms. For example, β-carotene can act as an effective quencher of singlet oxygen in the photosystem II reaction center (Telfer *et al.*, 1994). Another example, xanthophylls, especially zeaxanthin, is involved in the process of non-photochemical energy dissipation (Demmigadams, 1990; Gilmore *et al.*, 1994; Havaux and Niyogi, 1999; Horton and Ruban, 2005; Niyogi, 1999). The algae or marigold containing high zeaxanthin can be cultivated in the environment mentioned above. However, the traditional extraction of these bioactive carotenoids from these natural materials using organic solvents is not so comfortable and efficient due to low recovery of the carotenoids from vaporizing the solvents. According to experimental results of the Chen's work, traditional extraction method is not as efficient as those of supercritical extraction method (Chen *et al.*, 2009a). Supercritical anti-solvent precipitation of solutes from liquid phase solution has been extensively applied in pigment dispersion and pharmaceutical recrystallization to produce fine particles with high yield (Fages *et al.*, 2004; Liau *et al.*, 2010; Park *et al.*, 2010; Shen *et al.*, 2010). This approach has been also utilized in the separation of bioactive compounds from natural materials, including flavonoids, β-carotene, ginkgolides, lycopene, multi-phenolic acids derivative (Catchpole *et al.*, 2004; Chen *et al.*, 2005, 2009b; Cocero and Ferrero, 2002; Fages *et al.*, 2004; Miguel *et al.*, 2006; Wu *et al.*, 2009). Additionally, experimental data on the phase equilibrium between supercritical carbon dioxide (SC-CO_2) and organic solvent are important to understand the supercritical anti-solvent (SAS) process, as supercritical, superheated, liquid and co-existing phases directly influence the morphology, size and distribution of particles (Chang and Randolph, 1989). Super-saturation of solutes in a high-

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pressure solution is important to generate micronized particles in an SC-CO_2 anti-solvent precipitation process. However, the transient super-saturation of the solutes in such a high-pressure system that is mixed with SC-CO_2 is difficult to be determined (Chang *et al.*, 1991). There is no open literature reported regarding the nucleation of carotenoids from natural algal materials. The solubility parameter of zeaxanthin in SC-CO_2 has been calculated by Sajilata *et al.* (2010) and the SC-CO_2 extraction modified with methanol was also studied. In our work, the generation of nano- and micro-sized zeaxanthin rich particles obtained from the purified algal solutions based on experimentally designed SC-CO_2 precipitations was investigated. The effect of operational parameters (*i.e.* recrystallization time, feed concentration) on amount of zeaxanthin and particle size of the precipitates was examined.

2. Experimental

2.1. Materials and reagents

500 g of algae in dry basis were donated from Marine Research Center, National Sun Yat-Sen University (Kaohsiung, Taiwan) and Genereach Biotech. Company (Taichung Science Park, Taiwan). The species of *Nannochloropsis oculata* equivalent to The Culture Collection of Algae at The University of Texas at Austin (UTEX, LB-2164) was defrosted, grown in several open flasks equipped with in-cabinet fluorescent light by nutritional medium for a few days, and cultivated in two of 2 tons polypropylene tanks by sea water under open system for 20 days. The sea water was drawn from local seashore area. After harvested, the algae was aggregately precipitated, centrifuged by a high speed centrifuge, was freeze-dried, and then collected by sieving through a 100 mesh stainless steel screen under dimmed light. It was stored in a freezer at −80 °C before extraction.

Analytical grade solvents used for extractions, SAS processes, column chromatography and fractionations, including 99% ethanol (Mallinckrodt, USA), 99.5% acetone (Mallinckrodt, USA), 99.5% dichloromethane (DCM) (Mallinckrodt, USA). HPLC grade solvents used for mobile phase in HPLC, including 99.5% methanol (Mallinckrodt, USA), 99.5% methyl tert-butyl ether (Mallinckrodt, USA). Ultra pure water (>18 M) was obtained by using Ultrapure™ water purification system (Louton Co., Ltd., Taipei, Taiwan). The analytical grade of F_{254} Silica-gel 60 resin (Merck, Germany) was purchased and used without further purification. Carbon dioxide with a purity of 99.95% (Toyo gas, Taiwan) was used for SAS re-crystallization. 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), and 95% mix isomers of β-carotene: α-carotene = 2:1 (Sigma-Aldrich, USA).

2.2. Extraction and elution chromatography

The freeze-dried microalgae (50.0 g) were exhaustively extracted in dichloromethane with a 300 ml Soxhlet extractor, which was similar to the study of Liau *et al.* (2010). The algal extracts were concentrated under vacuum in yielding a load sample (3.6 g) for column chromatography. The loading sample was dissolved in the mixed solvents of ethyl acetate (EA) and *n*-hexane (1:3). The solution was subjected to a 10 cm (ID) × 30 cm (L) glass column which was packed with silica gel as the stationary phase. Isocratic elution was carried out using EA and *n*-hexane (1:3) at the flow rate of 7 ml/min. Finally, total 32 fractions were collected, and the solvent of each fraction was removed under vacuum and then weighed individually. The fractions from number 24 to 32 were collected as a zeaxanthin-rich portion as the feed of the SAS process. The properties of those fractions were studied in our previous work (Shen *et al.*, 2010). The purified samples were stored in −80 °C freezer before HPLC analysis and supercritical anti-solvent (SAS) precipitation.

2.3. Supercritical anti-solvent precipitation

Fig. 1 shows a conceptual flow diagram of the SAS precipitation that is similar to our previous work (Wu *et al.*, 2009). Liquid CO_2 was compressed using a high-pressure pump (*Spe-ed* SFE, Applied Separations, USA) and it was preheated using a heat exchanger. Then, CO_2 flowed (18, 36, 54 g/min) through a metering valve (SS-31RS4-A, Swagelok, USA) into a visible precipitator (TST, Taiwan),

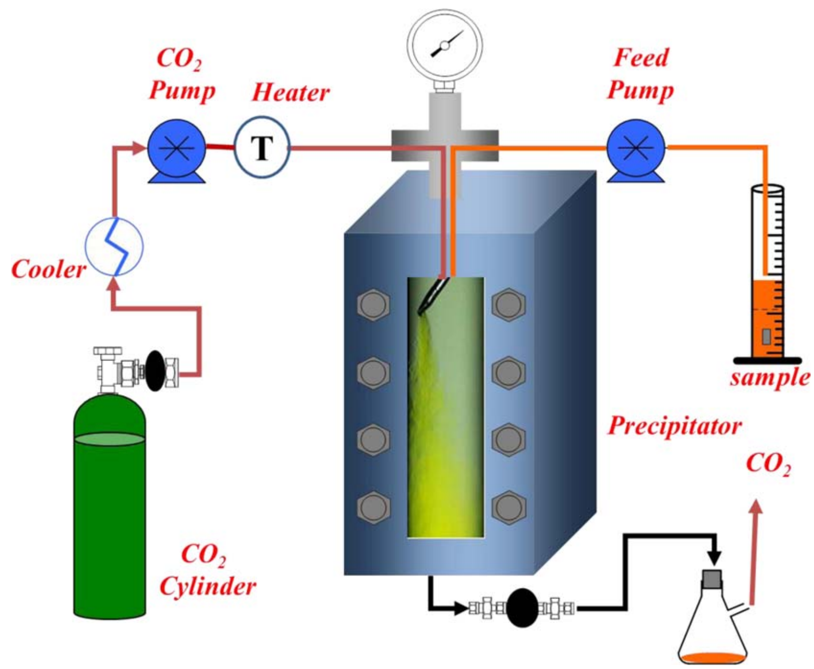

Fig. 1. Concenptual flow diagram of supercritical antisolvent precipitation of zeaxanthin-rich particulates.