Extraction of Astaxanthin from *Haematococcus pluvialis* using Supercritical CO$_2$ at High Pressure

Siti Machmudah$^1$, Artiwan Shotipruk$^2$, Motonobu Goto$^1$*, Mitsuru Sasaki$^1$, and Tsutomu Hirose$^1$

$^1$Department of Applied Chemistry and Biochemistry, Kumamoto University, Kumamoto, Japan

$^2$Chemical Engineering Department, Chulalongkron University, Bangkok, Thailand  

*Corresponding author: mgoto@kumamoto-u.ac.jp

ABSTRACT

In this study, astaxanthin from *Haematococcus pluvialis* has been extracted using supercritical CO$_2$ and modified ethanol to obtain high concentrated of astaxanthin. The antioxidant activity of extract has also been tested by diphenyl pycril hydroxyl (DPPH) and measured by UV-Vis Spectrophotometer. The effect of pressure, temperature, CO$_2$ flow rate and the existence of ethanol on total extraction yield, astaxanthin extracted and astaxanthin concentrated in the extract were studied. Extraction was carried out at 20 – 55 MPa of pressure, 313 – 353K of temperature, 2 – 4 ml/min of CO$_2$ flow rate, 1.67 – 7.5 % volume of ethanol:CO$_2$. The extract was analyzed by a Shimadzu Liquid Chromatograph LC-10AD, equipped with Diode Array Detector SPD-M10A and a 5C18-MS Waters of column. Maximum astaxanthin content in *H. pluvialis* sample was obtained by soxhlet extraction using dichloromethane as solvent. Maximum astaxanthin content was 3.43% weight. The amount of the total extract, astaxanthin extracted, and astaxanthin content in the extract increased with increasing temperature and pressure. Extraction yield increased with increasing CO$_2$ flow rate, while the amount of astaxanthin extracted and the astaxanthin concentrated almost did not change with the increase in CO$_2$ flow rate. The highest extraction yield, astaxanthin extracted and concentrated were obtained at high pressure and temperature. By using ethanol as an modifier, higher astaxanthin could be obtained at moderate pressure and high temperature. The addition of the modified ethanol could more than twice enhance the amount of astaxanthin extracted. The antioxidant activity of extract degraded with the existence of modified ethanol.

Keywords: Astaxanthin, *Haematococcus pluvialis*, supercritical carbon dioxide, modifier

Introduction

Astaxanthin (3,3'-dihydroxy-β,β'-carotene-4,4'-dione) is a ketocarotenoid oxidized from β-carotene and has been used as pigmentation source in the poultry and aquaculture industries. Due to its attractive red colour and higher antioxidant activity than α-carotene,
β-carotene, lutein, lycopene, cantaxanthin and vitamin E, astaxanthin can also be used as a food colourant and in medicine (Yuan, 1996; Guerin, 2000). The most important commercial application of astaxanthin, obtained either from synthetic origin or from natural sources, e.g. microalgae, yeast, or crustacean by products, is in the aquaculture industry where it is used in the formulation of feeds for farmed salmon to provide the typical muscle color, which is widely accepted by consumers throughout the world (Johson, 1991). Astaxanthin has several key biological functions in fish. It serves as a precursor of vitamin A, it is associated with reproduction and embryo development and also with protecting cells against oxidative damage. In human nutrition, astaxanthin has been gaining widespread popularity as a dietary supplement due to its powerful antioxidant properties. Currently, several astaxanthin products derived from microalgae are available in the marketplace, and being promoted as anticancer and anti-inflammatory agents as well as immunostimulants (Higuera-Ciapara, 2004).

Some microorganisms are rich in astaxanthin, but the Chlorophyte alga *Haematococcus pluvialis* is believed to accumulate the highest levels of astaxanthin in nature. Commercially grown *H. pluvialis* contains between 1.5-3.0% astaxanthin. The astaxanthin in *H. pluvialis* is approximately 70% monoesters, 25% diesters and 5% free, and all of the free astaxanthin and its monoesters and diesters have optically pure 3S, 3’S chirality (Figure 1) (Denery, 2004).

Supercritical fluids are now widely accepted for extraction, purification, recrystallization, and fractionation operations in many industries. The technology is used to process hundred of millions of pounds of coffee, tea, and hops annually, and it is increasingly becoming of common use in the pharmaceuticals industry for purification and nano-particle formation. Supercritical fluid processing is also gaining in the botanicals, vitamins, and supplements industries, where they are becoming synonymous with the highest purity and quality.

Supercritical fluid extraction (SFE) is far more efficient than traditional solvent separation methods. Supercritical fluid solvents are environmentally friendly and recyclable. Supercritical CO$_2$ is certainly a Green Solvent; by far the most common supercritical fluid is gaseous carbon dioxide. By adjusting the processing and temperature, the gas can act like liquid solvent, but with selective dissolving powers. In the supercritical fluid phase, extraction concentration is carried out simply with changes in pressure, which results in a pure product fraction and a clean CO$_2$ gas stream, which is completely recycled to the process. Moreover,
there are no hazardous waste streams, no harsh organic chemicals or residues, and the gaseous solvent is recyclable.

Organic solvents such as acetone and hexane have been used industrially for the extraction of astaxanthin from microalgae. The concentration of astaxanthin in these extracts, however, is limited because liquid solvents can not differentiate between the lipids and the carotenoid. By using supercritical fluid, much higher astaxanthin extract concentrations are achievable because of the ability to tailor their dissolving power (by tuning pressure). Lipids and carotenoids respond differently to supercritical CO$_2$ and can be selectively separated, resulting in an extract high in the carotenoids. Phasex Corporation has used supercritical CO$_2$ for the extraction of astaxanthin from microalgae at operating temperature and pressure less than 50°C and 30 MPa, respectively. Valderrama et al. (2003) conducted supercritical CO$_2$ extraction of astaxanthin from the microalgae at the operating temperature of 60°C and the pressure of 30 MPa and demonstrated the importance of algae cellular breaking and the use of ethanol modifier on extraction efficiency.

Furthermore, this study was designed to extract high concentrate astaxanthin from *Haematococcus pluvialis* using supercritical CO$_2$ with/without modified ethanol. The extraction process was optimized by varying the key extraction factors of pressure, temperature, ethanol-CO$_2$ ratio, and CO$_2$ flow rate.

**Material and Methods**

Dried samples of microalgae *H. pluvialis* were obtained from Cyanotech Corporation, Hawaii Ocean, Science Technology Park with 5% astaxanthin feed grade powder. Samples were stored in a refrigerator at 278K until use. Standard astaxanthin and the HPLC-grade solvents used for analysis were purchased from Wako Chemical Ind., Japan. CO$_2$ was obtained from Uchimura Co., Japan.

The schematic flow diagram of the extraction apparatus is illustrated in Figure 2. The apparatus includes chiller (Sibata, Coolman C-560, Japan), high pressure pump (Jasco PU-2080-100 MPa, Japan), modifier pump (Syringe pump Model 260D, ISCO, Japan), heating chamber (ST-110, ESPEC, Japan), extraction cell (Thar Tech, Inc. USA, 50 ml of volume), back pressure regulator (AKICO Co., Japan), collection vessel and wet gas meter (Sinagawa Co., Japan), using liquid CO$_2$ as a supercritical fluid, and ethanol as an modifier. Astaxanthin was extracted from *H. pluvialis* under various temperatures (313 – 353K), pressures (20 – 55 MPa), CO$_2$ flow rate (2-4 ml/min) and ratio of CO$_2$/ethanol (1.67-7.5%). In each experiment, approximately 7 grams of *H. pluvialis* was loaded in a 50 ml extraction cell and the remaining volume was filled with glass beads in the bottom and upper of cell. The cell was placed in the heating chamber to maintain the operating temperature. In each step, the extract was collected in collection vessel at every 30 to 60 min for 4 hours to prepare the sample solution for HPLC analysis. The total extract was weighted immediately after collection. To determine the percentage of astaxanthin extracted, the maximum astaxanthin content in microalgae *H. pluvialis* was determined via soxhlet extraction with dicipromethane.
for 6 hours. About 6 grams of *H. pluvialis* was extracted by 200 ml of solvent. Furthermore, 3 ml of methanol was added in 1 ml of extract for analyzing.

Astaxanthin in the extract was analyzed by a Shimadzu Liquid Chromatograph LC-10AD, equipped with Diode Array Detector SPD-M10A. Sample solution was injected through a 20 ml loop and separated on the 5C18-MS Waters of column (5µm; 4.6x150 mm) at ambient temperature. Isocratic elution was performed with methanol-acetonitrile-dichloromethane-water (85:5:5:5, v/v) mobile phase at a flow rate of 1.2 ml/min, and the detection wavelength was kept at 480 nm.

In addition, the effect of extraction condition on the antioxidant activity of extracts has also been investigated. In this study, the antioxidant activity is the ability of a compound to act as donor for hydrogen atoms or electron. Hydrogen donating ability of the aqueous extracts was examined in the presence of 1,1-diphenyl-2-picrylhydrazyl (DPPH) stable radical at 517 nm using UV-VIS spectrophotometer. DPPH solution was freshly prepared as a free radical source. 15 ml of methanol and 0.5 ml of DPPH were added in 0.5 ml of extract and incubated at room temperature (± 303K) for 30 min. As control 20% (v/v) of DPPH methanolic solution was prepared and treated as the samples. For characterization of the activity, the inhibition in percentage was expressed as follow.

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\text{Inhibition (\%)} = \left[ \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \right] \times 100\%
\]
Result and Discussion

Based on the soxhlet extraction, maximum astaxanthin content in *H. pluvialis* algae was 34.3 mg/g or 3.43% in weight. The amount of astaxanthin extracted was calculated based on this total amount.

The effect of temperature on extraction yield, astaxanthin extracted and concentrated were studied at pressure of 55 MPa and CO$_2$ flow rate of 3 ml/min. Figures 3, 4 and 5 show the effect of temperature on extraction yield, astaxanthin extracted and concentrated, respectively. The extraction yield is defined as weight of extract divided by weight of the original sample. Astaxanthin extracted was defined as mass of astaxanthin in the extract divided by maximum astaxanthin content in initial sample. Astaxanthin concentrated was expressed as mass of astaxanthin in the extract divided by mass of extract. Extraction yield, astaxanthin extracted, and astaxanthin concentrated increased with an increase in temperature. These results indicate that the total extract and the astaxanthin extraction are dependent on solute vapor pressure which increased with an increase in temperature. The degree in which extraction yield increased was however smaller than those of the astaxanthin extracted and the astaxanthin concentrated which increased more dramatically with increasing temperature. The increase in temperature from 343 K to 353K however does not increase the amount of astaxanthin extracted and concentrated. At 55 MPa, the highest extraction yield, the amount of astaxanthin extracted, and concentrated were 21.8%, 77.9%, and 12.3%, respectively, and were obtained at 343K.

![Fig. 3.](image1.png)  
![Fig. 4.](image2.png)

Fig. 3. The effect of temperature on extraction yield as function of time at 55 MPa and 3 ml/min.

Fig. 4. The effect of temperature on astaxanthin extracted as function of time at 55 MPa and 3 ml/min.

The effect of pressure on the extraction yield, astaxanthin extracted and astaxanthin concentrated using supercritical CO$_2$ at temperature of 343 K and CO$_2$ flow rate of 3 ml/min are shown in Figure 6, 7 and 8. Generally, extraction yield increased with increasing pressure. The total extract and the astaxanthin extracted increased gradually up to 50 MPa and the dramatic increase was observed at the pressure of 55 MPa. The dependency on the pressure
was expected as the CO$_2$ density increases at higher pressure, and therefore the solvent power to dissolve the substances increases. At 343 K, the highest amount of extraction yield and astaxanthin extracted were obtained at 55 MPa, and they were about 21.8% and 77.9%, respectively. The highest astaxanthin concentrated was also resulted at 55 MPa in which the value of about 12.3 % was obtained, suggesting that the selectivity of astaxanthin extraction was high at this pressure.

The effect of CO$_2$ flow rate on extraction yield, astaxanthin extracted and astaxanthin concentrated using supercritical CO$_2$ was studied at pressure of 50 MPa and temperature of 323K. Figure 9, 10 and 11 show the effect of CO$_2$ flow rate on extraction yield, astaxanthin extracted and astaxanthin concentrated, respectively, as function of CO$_2$ consumption. As seen in Figure 9 and 10, extraction yield and the astaxanthin extracted slightly increased with
increasing CO₂ flow rate, and tended toward the same value at higher CO₂ consumption. The increasing CO₂ flow rate generally causes the increasing number of molecule CO₂ per unit volume to enter the extractor, thus increasing inter-molecular interaction between CO₂ and solute, with the result that solute dissolved in CO₂ increase. Figure 11 however shows that CO₂ flow rate does not have a significant effect on the astaxanthin concentrated as the various flow rates yield almost the same values. The small effect of flow rate on the extraction process may be caused by the fact that at these rates, the CO₂ was not able to be distributed evenly throughout the extractor. Furthermore, the experiments were conducted at lower temperature, and the extraction may be highly influenced by the solubility limitation. Increasing the mass transfer by increasing the CO₂ flow rate may not enhance the extraction rate and the yield significantly. At this condition, the highest extraction yield, astaxanthin extracted and concentrated obtained were only 12.2%, 29% and 8.1%, respectively.

The effect of modifier concentration in CO₂ on astaxanthin extracted was studied at the pressure of 40 MPa and at the temperature of 343K, and the results are shown in Figure 12. Astaxanthin extracted increased with increasing ethanol concentration due to the additional ethanol in supercritical CO₂ cause the solvent power enhancement of supercritical CO₂. Modifier also can enhance separation factor or selectivity of astaxanthin. At this condition the astaxanthin extracted could reach more than 80%. The additional ethanol in supercritical CO₂ was effective at 5%. For larger modifier concentration the selectivity of astaxanthin becomes smaller due to other components in the feed were easily extracted. As the result, the other components in the extract become larger; on the other hand the selectivity of astaxanthin becomes smaller. As shown in Figure 12, for 7.5% of modifier concentration astaxanthin extracted is lower than for 5% of modifier concentration.

![Fig. 9. The effect of CO₂ flow rate on extraction yield as function of CO₂ consumption at 50 MPa and 323K.](image1)

![Fig. 10. The effect of CO₂ flow rate on astaxanthin extracted as function of CO₂ consumption at 50 MPa and 323K.](image2)
Fig. 11. The effect of CO$_2$ flow rate on astaxanthin concentration as function of CO$_2$ consumption at 50 MPa and 323K.

Fig. 12. The effect of modifier concentration in CO$_2$ on astaxanthin extracted as function of time at 40 MPa and 343K.

The comparison of the amount of astaxanthin extracted by SC-CO$_2$ extraction with and without ethanol as modifier is shown in Figure 13. The comparison was made for extractions under various pressures at the temperature of 313K and the CO$_2$ flow rate of 3 ml/min. The concentration of 1.67% ethanol in CO$_2$ was used and was found to enhance the amount of astaxanthin extracted by two folds. The addition ethanol enhances the solvent power of SC-CO$_2$ and causes the swelling of the matrix, thus increases the internal volume and the surface area for the contact with SC-CO$_2$ (Lim et al., 2002).

Fig. 13. Comparison of SC-CO$_2$ extraction without and with 1.67% modifier at various pressures, at 313K and 3 ml/min.

Fig. 14. The effect of pressure on the antioxidant activity of the extract without modifier at 313K.
The antioxidant activity of extract was expressed as the inhibition in percentage. The effect of extraction condition on antioxidant activity of the extract has been investigated. Figure 14 and 15 show the effect of pressure on the antioxidant activity of the extract without and with modifier, respectively. Even though it was small, the antioxidant activity of the extract increased with an increase in pressure. When ethanol was added in supercritical CO\(_2\), the antioxidant activity decreased dramatically. The percentage inhibition decreased about 30% of it without modifier.

![Figure 15. The effect of pressure on antioxidant activity of the extract with co-solvent (E/S=1.67%) at 313K of temperature.](image)

**Conclusion**

Extractions of astaxanthin from *Haematococcus pluvialis* using supercritical CO\(_2\) without and with ethanol as modifier have been conducted. For supercritical CO\(_2\) extraction without modified ethanol, generally, extraction yield, the amount of astaxanthin extracted, and the astaxanthin concentration increased with an increase in temperature and pressure. The amount of extraction yield and the amount of astaxanthin extracted slightly increased with increasing CO\(_2\) flow rate, while and the astaxanthin concentrated was not changed. The highest astaxanthin extracted and astaxanthin concentrated were 77.9% and 12.3%, respectively, obtained at 55 MPa, 343K, and at the CO\(_2\) flow rate of 3 ml/min. In other words, high astaxanthin extracted and astaxanthin content in the extract could generally be obtained at high pressure and temperature.

Extraction of astaxanthin using supercritical CO\(_2\) with modifier could twice enhance the amount of astaxanthin extracted and effective extraction was achieved at more moderate pressure (40 MPa). The amount of astaxanthin extracted was found to increase with increasing modifier concentration. In this work, the addition of modified ethanol in supercritical CO\(_2\) was the most effective at 5%. When the modifier was used, CO\(_2\) flow rate considerably affect the astaxanthin extracted and higher amount of astaxanthin was extracted as CO\(_2\) flow...
rate decreased. The antioxidant activity of extract degraded with the existence of modified ethanol.

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