CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Astaxanthin standard was obtained from Wako Chemical, USA. SC-CO_2 was carried out with high purity carbon dioxide. The *Haematococcus pluvialis* strain powder samples were the commercial algae powder (NatuRose®), manufactured by Cyanotech, USA; they were stored in an oxygen free package and kept in a refrigerator at 4°C until use.

3.2 Supercritical fluid extraction and astaxanthin analysis

In this study, a statistical experimental design was used in order to optimize the conditions for the SC-CO_2. The variables considered here were pressure, temperature, and extraction time. Each supercritical fluid extraction experiment was conducted in a SFX™ 220 supercritical fluid extraction system with a 10 ml extraction chamber, a restrictor, extractor and restrictor temperature controller as shown in Figure 3.1. For each experimental run, 0.5 g dried *Haematococcus pluvialis* algae were loaded in the extraction chamber. To distribute the algae sample throughout the extraction chamber, the chamber was filled with silica sand. The extract was trapped in acetone and analyzed using a spectrophotometer, Genesys 20 (Thermo spectronic, USA) at the wavelength 475 nm.

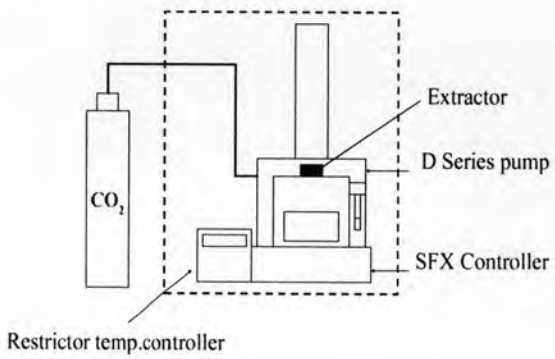

Figure 3.1 SFX 220 extraction system of supercritical carbon dioxide extraction

3.3 Soxhlet extraction

After *Haematococcus pluvialis* was extracted by supercritical carbon dioxide, some amount of astaxanthin still remained in the sample residue. Thus the soxhlet extraction with acetone solvent was used to determine the total mount of astaxanthin in the extract. The 0.5 g of *H. pluvialis* algae was extracted with 250 ml acetone using soxhlet apparatus as in Figure 3.2. Then the extract was measured for the astaxanthin concentration by using spectrophotometer, Genesys 20 (Thermo spectronic, USA) as in Figure 3.3.

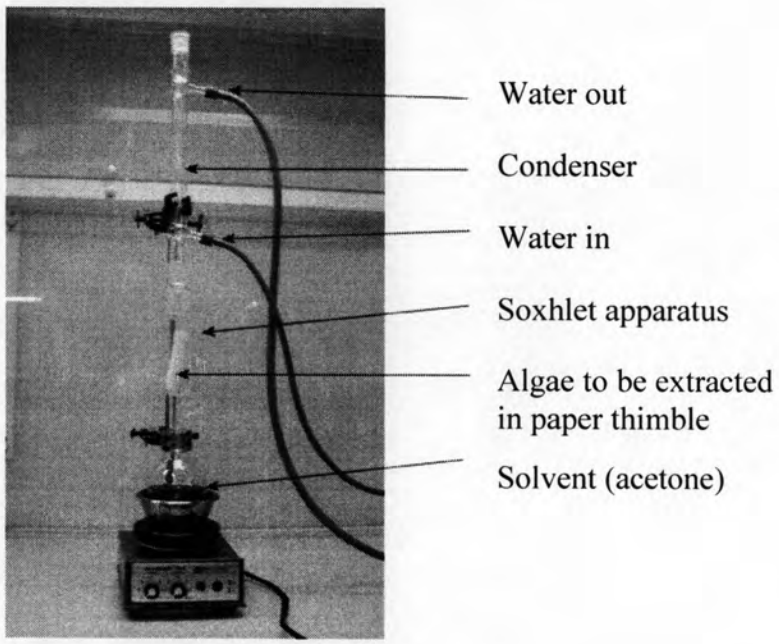

Figure 3.2 Soxhlet apparatus

Figure 3.3 Spectrophotometer, Genesys 20 (Thermo spectronic, USA)

3.4 Analysis of extract antioxidant activity

ABTS method is one of the spectrophotometric methods that use to determine the total antioxidant activity of concerned substances. In this method ABTS [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] have to be in radical forms which is called stable radical cation, $ABTS^{\bullet+}$. The improved technique for the generation of $ABTS^{\bullet+}$ is the reaction between ABTS and potassium persulfate that leads to direct production of the blue/green $ABTS^{\bullet+}$ chromopore, a stable and intense radical as shown in Figure 3.4. Its maximum absorption occurred at the wavelengths 645 nm, 734 nm and 815 nm as well as the more commonly used maximum at 415 nm as shown in Figure 3.5. In this experiment, the selected absorption of $ABTS^{\bullet+}$ was 734 nm because it was not overlap with astaxanthin absorption at 475 nm.

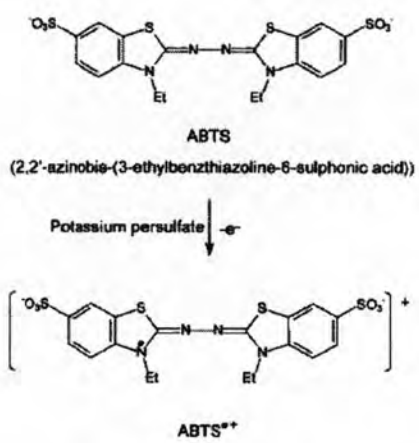

Figure 3.4 Formation of ABTS radical cation, $ABTS^{\bullet+}$

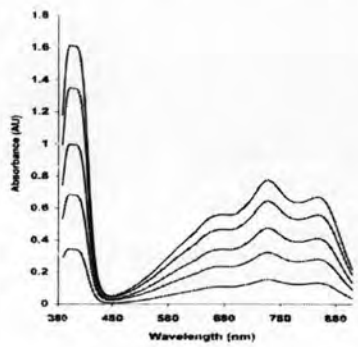

Figure 3.5 Absorption spectra of ABTS radical cation, $ABTS^{\bullet+}$

In this study, the antioxidant activity of the *Haematococcus pluvialis* extract was measured using a ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) method, modified from that described in previous research (*Re et al., 19999*). The extract was diluted in series by acetone and each diluted solutions were added into $ABTS^{\bullet+}$ solution (aqueous solution of 7mM ABTS and 2.45 mM potassium persulfate having absorbance of 0.70 ± 0.02 at 734 nm) with the volume ratio 1:2 (extract:ABTS solution). The solutions were mixed using a vortex and the mixture were incubated in the dark at room temperature for 10 min, after which the absorbance was measured at the wavelength of 734 nm using acetone to ABTS (1:2) as a reference.

For comparing the antioxidant activity in various extracts, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index. The value of IC_{50} can found from the plot of percent inhibition (PI) versus the corresponding concentration of astaxanthin, in which the values of PI can be calculated using the following equation:

$$PI\ (\%) = [1-(A_t / A_r)] \times 100 \qquad (3.1)$$

Where A_t and A_r are absorbance of test sample and absorbance of the reference, respectively.

3.5 Experimental design and statistical analysis

In this study the experimental design was used to evaluate the main and interaction effects of the factors: temperature (X_1), pressure (X_2), and extraction time (X_3) on astaxanthin yield as well as antioxidant activity of the extracts obtained from SC-CO_2 process. Seventeen experiments were performed with three experiments as the repeatability of the measurements at the center of the experimental domain. All factors and levels tested were reported in Table 3.1.

Table 3.1 Factors and levels tested for the designed experiment

Factor	Low level (-1)	Medium level (0)	High level (+1)
Temperature (X_1, °C)	40	60	80
Pressure (X_2, bar)	300	400	500
Extraction time (X_3, hour)	1	2.5	4

The statistical analysis of variance (ANOVA) of the experimental results was employed to determine the main effect and interaction of the factor effects using SPSS 9.0 program. The response surface equations were then proposed, from which the optimal conditions were determined. Detailed statistical data analysis and experimental design is described in Chapter 2.