



CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

2.1 *Haematococcus pluvialis*

Haematococcus pluvialis is an important green microalgae, a rich source of natural arietenoids pigments. The most important carotenoids found in this algae is astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) which is the most antioxidative of all the carotenoids. Naturally, *Haematococcus* occurs in continental and coastal rock pools and water holes. The algae can be classified as follows.

Phylum : Chlorophyta
Class : Chlorophyceae
Order : Volvocales
Family : Haematococcaceae
Genus : Haematococcus
Species : pluvialis

In general, there are 2 main stages of growth of *H. pluvialis* cell. During the first stage, *H. pluvialis* is in vegetative form when grown under optimal conditions. These vegetative cells are spherical or ellipsoid with 2 flagellas used for cell movement. The cells are enclosed by a cell wall which is separated from the protoplast by a watery jelly region. In the second stage, when the condition is not suitable for vegetative growth, such as under limited nutrients such as nitrogen and phosphate, or under light induction, the flagellated cells gradually transform into cyst cells or the aplanospores. These cyst cells are in the resting stage which allows them to survive for prolonged periods until the environment becomes more favorable. During this stage, the cells develop a distinct red color as it accumulate the valuable-product, astaxanthin, as can be seen in Figure 2.1.

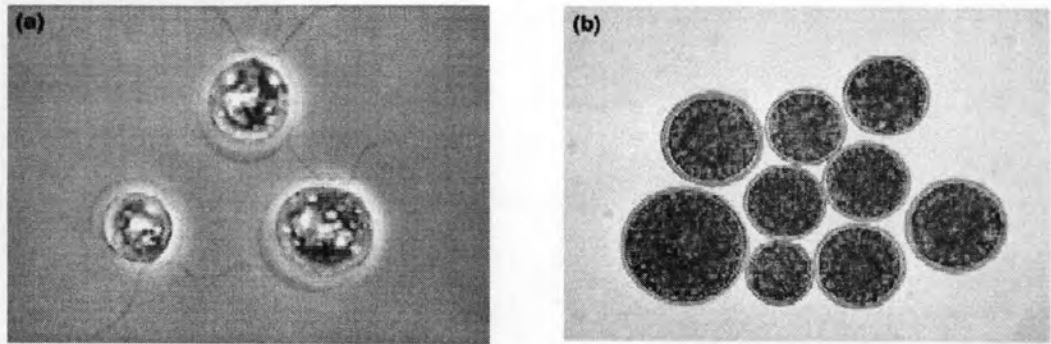


Figure 2.1 *H. pluvialis* (a) vegetative cells and (b) cyst cells.

2.2 Life Cycle of *Haematococcus pluvialis*

The life cycle of *H. pluvialis* can be classified into 4 stages as shown in Figure 2.2.

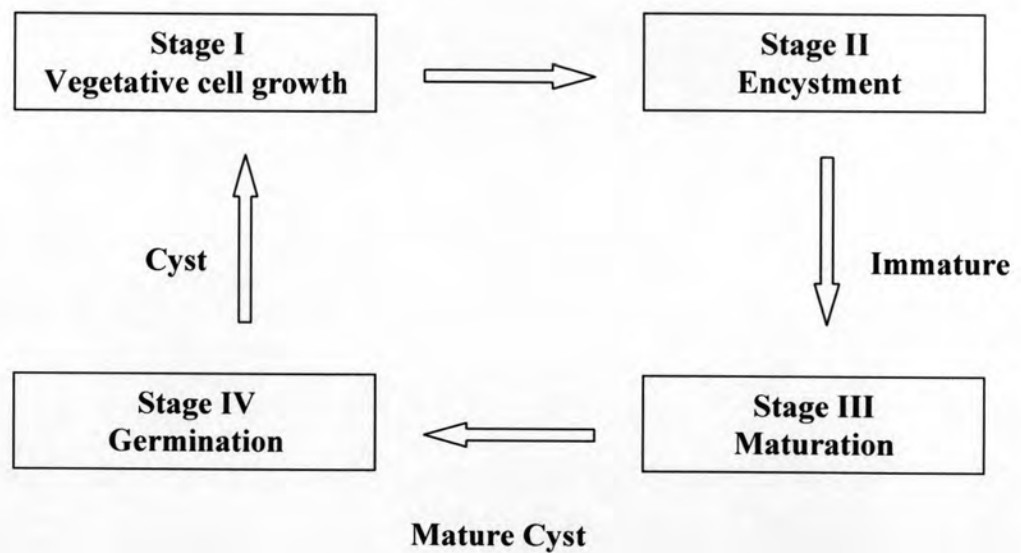


Figure 2.2 Life Cycle of *Haematococcus pluvialis*

Stage I: Vegetative cell growth

In this stage, *Haematococcus* takes the form of vegetative green cells in which accumulation of protein and chlorophyll pigment occurs. The cells can reproduce themselves by means of cell division.

Stage II: Encystment

Under favorable conditions, vegetative green cells transform into immature cyst cells by losing their flagella and developing thick cell walls. This is the most prominent form of the alga in non-synchronous cultures.

Stage III: Maturation

When the conditions become unfavorable, the immature cyst cells stop dividing. The cells accumulate astaxanthin and as a result, turn red and increase in size. At this stage, the cell walls thicken further as the cells mature.

Stage IV: Germination

When the environmental conditions become favorable, mature cyst cells return to typical vegetative cells.

2.3 Astaxanthin

Astaxanthin is the most commonly occurring red carotenoids in marine and aquatic animals, such as in shellfish lobsters and shrimps, and in fish eggs. The compound exhibits antioxidant properties superior to beta-carotene and vitamin C. Thus, the potential benefits of this powerful antioxidant to human health are being of high interest.

Chemical name of astaxanthin is 3,3'-dihydroxy- β,β -carotene-4,4'-dione. It is an oxycarotenoid, or more specifically, ketocarotenoid, whose molecular formulae is $C_{40}H_{52}O_4$. The molecular weight of the compound is 596.82 and the molecular structure is shown in Figure 2.3.

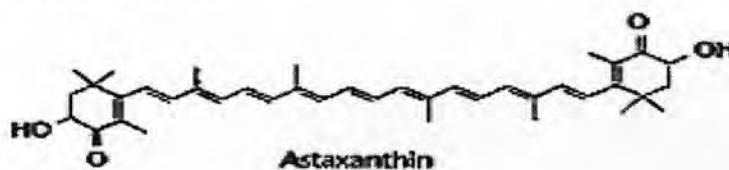


Figure 2.3 Astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione)

The molecular structure is similar to that of the familiar carotenoids, beta-carotene. However, the small differences in the structures of the molecules confer large differences in the chemical and biological properties. A comprehensive study

found astaxanthin to be twice as effective as beta-carotene, and about 80 times more effective than vitamin E, in quenching singlet oxygen in chemical solution (Di Mascio et al., 1991).

2.4 Biosynthesis pathway of astaxanthin

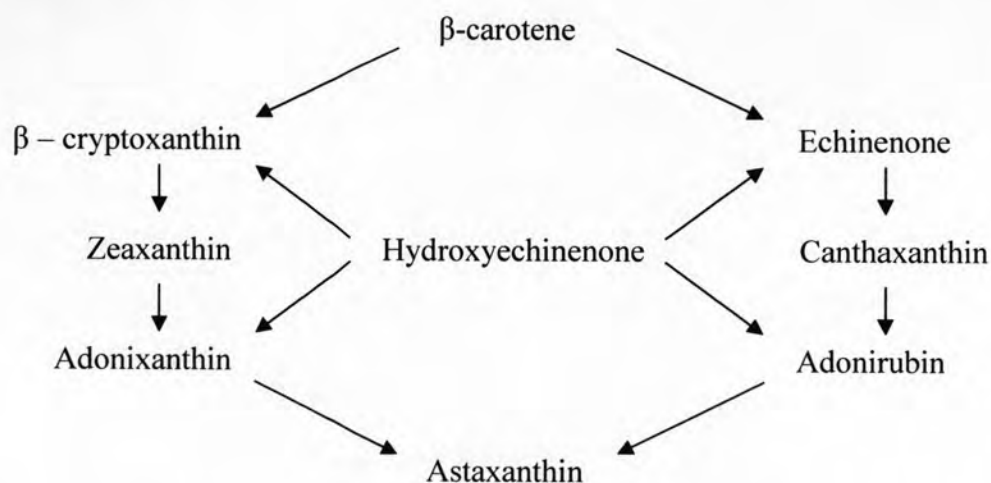


Figure 2.4 Biosynthesis pathway of astaxanthin

Biosynthesis pathway of astaxanthin occurs through the combined action of hydrolase and ketolase activities. It starts from β -carotene from which different carotenoids intermediates are derived during the process, and finally, astaxanthin is produced as shown in Figure 2.4.

2.5 Conventional extraction

Conventionally, carotenoids are extracted from natural materials by organic solvent extraction. The compounds are highly soluble in several organics solvents, such as methanol, ethanol, hexane, and acetone. But the solvent extraction of carotenoids from natural matrices is time consuming. They require multiple extraction steps and need large amounts of organic solvents, which are often expensive and potentially harmful. Additionally, the concentration of carotenoids in the extracts is limited because the liquid solvents cannot differentiate between the lipids and the

carotenoids. Supercritical fluid extraction is currently considered as a good candidate for extraction of plant material.

2.6 Supercritical fluid extraction

A supercritical fluid (SF) is a matter at a supercritical state (the temperature and pressure above its critical points). At this stage, the fluid is compressible and behaves like a gas. On the other hand, its density is closer to the typical density of a liquid, that is, between 0.1 to 1.0 g/ml. The supercritical fluid region can be drawn on a phase diagram as shown below.

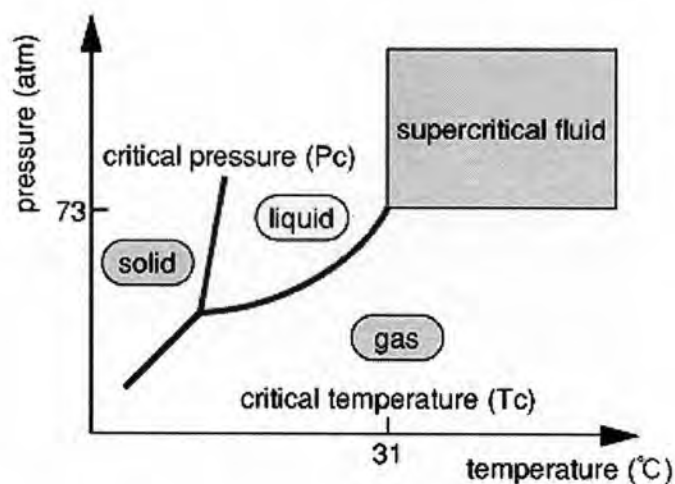


Figure 2.5 Phase diagram of supercritical carbon dioxide

The curves in the P-T diagram above indicate the coexistence of two phases e.g. solid-gaseous, solid-liquid, and liquid-gaseous. These are so called sublimation, melting, and vaporization equilibrium, respectively. The intersection of three curves, where the solid, liquid, and gaseous phases coexist in equilibrium, is called a triple point (TP). At liquid-gas equilibrium, the coexistence curve does not continue indefinitely. The curve suddenly breaks at a point which is designated by a critical temperature (T_c) and a critical pressure (P_c). This point is called a critical point (CP), which is a characteristic of each substance. The values for T_c and P_c for selected substances are shown in Table 2.1. Above this point, the liquid and the gas occur as a single phase, called supercritical fluid. At the supercritical state, the fluid process

interesting properties. Typical ranges of density, viscosity, and diffusion coefficients of supercritical fluids are summarized in Table 2.2.

Table 2.1 Features of various solvents at the critical point.

Solvents	Critical temperature (°C)	Critical pressure (bar)	Critical density (g/ml)
<u>Inorganic</u>			
CO ₂	31.1	72	0.47
N ₂ O	36.5	70.6	0.45
Ammonia	132.5	109.8	0.23
Water	374.2	214.8	0.32
Helium	-268	2.2	0.07
<u>Hydrocarbons</u>			
Methane	-82	46.0	0.169
Ethane	32.3	47.6	0.2
Propane	96.7	42.4	0.22
<u>Alcohols</u>			
Methanol	239	78.9	0.27
Ethanol	243.4	72	0.276

Table 2.2 Comparison of the properties of supercritical CO₂ and those of ordinary gases and liquids.

Phases	Density (g/cm ³)	Viscosity (g/cm·s)	Diffusion coefficient (cm ² /s)
Gases	$0.1 \times 10^{-3} - 2 \times 10^{-3}$	$1 \times 10^{-4} - 3 \times 10^{-4}$	0.1 - 0.4
SC-CO ₂ at T _c , P _c	0.47	3×10^{-4}	7×10^{-4}
SC-CO ₂ at T _c , 6P _c	1.0	1×10^{-3}	2×10^{-4}
Liquids	0.6 - 1.6	$0.2 \times 10^{-2} - 3 \times 10^{-2}$	$0.2 \times 10^{-5} - 2 \times 10^{-5}$

As shown in the table, the properties of supercritical fluid such as density, diffusivity, and viscosity lie between those of liquids and gases, which are summarized in the following section.

2.7 Properties of supercritical fluid

2.7.1 Density

The density of a supercritical fluid is dependent on its pressure and temperature. At a constant temperature, the density increases with increasing pressure and at a constant pressure; the density decreases with increasing temperature. In general, changing in pressure, temperature or both can alter the density of supercritical fluid over a wide range. The relationship between pressure and density of supercritical fluid at a given temperature can be explained by the ideal gas equation of state as expressed in equation (2.1)

$$PV = zRT \quad (2.1)$$

when $\rho = M/V \quad (2.2)$

the equation becomes $\rho = MP / zRT \quad (2.3)$

where V is the molar volume, z is the compressibility factor, R is the gas constant, ρ is the density, and M is the molecular weight.

2.7.2 Diffusivity

Normally, the diffusivity of a solute in supercritical fluid is between those in liquid and gases as shown in Table 2.2. The diffusivities of a solute in supercritical carbon dioxide and in ordinary liquids as a function of temperature and pressure are shown in Figure 2.6 and it can be concluded that

- 1) The diffusivity of a solute in a supercritical fluid is more than in a liquid solvent.
- 2) The diffusivity of a solute in a supercritical fluid decreases as pressure increases.

- 3) The diffusivity of a solute in a supercritical fluid increases with increasing temperature.

This interesting property of supercritical fluid leads to faster mass transfer than that of ordinary liquid solvent.

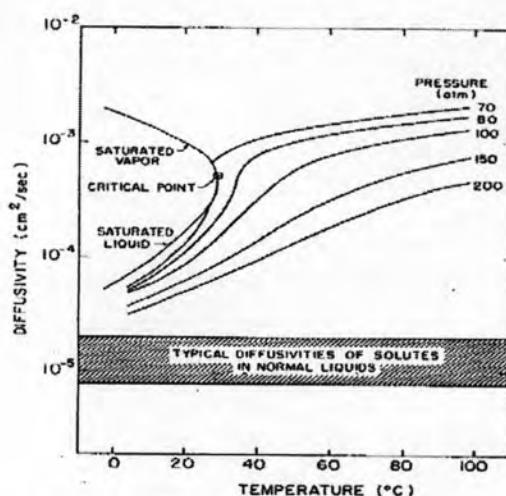


Figure 2.6 Diffusivities of solute in SC-CO₂ as a function of temperature and pressure

2.7.3 Viscosity

Generally, the viscosity of a fluid such as gas, liquid, or supercritical fluid depends on the temperature. But for the supercritical fluid, pressure markedly influences its viscosity despite it has little effect on that of liquid. At constant temperature, increase in pressure results in increased supercritical fluid viscosity and hence diminishing the solute diffusivity.

2.7.4 Polarity

A property that influences the solubility of fluid and can be altered to modify the selectivity of an extraction process is polarity. Normally, it occurs when the center of negative charge of a molecule does not coincide with that of its positive charge. This property can be represented by a parameter called a dipole moment. Dipole moments of various substances are shown in Table 2.3

Table 2.3 Permanent dipole moment of some supercritical fluids.

Fluid	Dipole moment (Debye)
CO ₂	0.0
SF ₆	0.0
Xe	0.0
Ethane	0.0
n-Butane	0.0
N ₂ O	0.2
Freon-12	0.2
Freon-11	0.5
Freon-22	1.4
NH ₃	1.5
CHF ₃	1.6
MeOH	1.7

According to the Table 2.3, supercritical fluids can be classified into polar, scarcely polar, and non-polar. For example, CO₂, which have zero dipole moment, is said to be non-polar.

2.7.5 Dielectric constant

Dielectric constant is one of the most important properties defining the solubility in fluids. It decreases as the temperature increases and as the pressure decreases. For example, at 25°C and 1 atm, water has a relatively high dielectric constant of 78.5. The value decreases to 12 at 1000°C. Because of its low dielectric constant at high temperatures, water shields the electron potential between ions weakly, so dissolved ions can freely form ion-pairs. Under these conditions, supercritical water behaves as a non-polar rather than a polar solvent. These properties partly account for its ability to dissolve non-polar organic compounds.

For supercritical carbon dioxide, the dielectric constant also increases with pressure as shown in Figure 2.7. Its dielectric constant in a dense state (200 bar, 40 °C) is about 1.5. As a result, it can be assimilated to a highly non-polar solvent which is appropriate for dissolving non-polar substances.

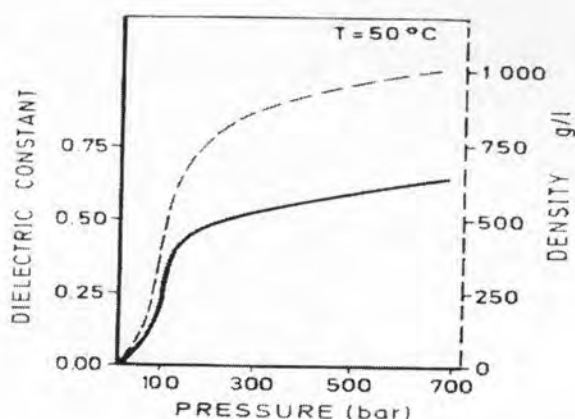


Figure 2.7 Influence of pressure on the dielectric constant and density of SC-CO₂ at a constant temperature.

2.8 Solubility in supercritical fluid

The solvating power of a supercritical fluid is highly dependent on its temperature and pressure, as can be summarized as follows.

- 1) The solubility increases with increasing pressure. The increase is very sharp near the critical point as a result of marked changes in solvent density.
- 2) The solubility increases, remains constant or decreases with increase in the temperature at a constant pressure, depending on the predominant factor, the solute vapor pressure or the solvent density. At low pressures, the solubility decreases somewhat with temperature and at high pressures, it increases markedly with temperature. The maximum solubility increases with increasing temperature at high pressures. The former effect arises from the decrease in density with temperature, whereas the latter, which involves the dense, less readily compressible region, results from the vapor pressure of the solid. At a given solvent density, its solubility increases with increasing temperature.
- 3) The solubility increases with increasing solvent density.

As an example, Figure 2.8 shows the solubility of naphthalene with pressure at two different temperatures in supercritical fluid, which corresponds with the conclusions above.

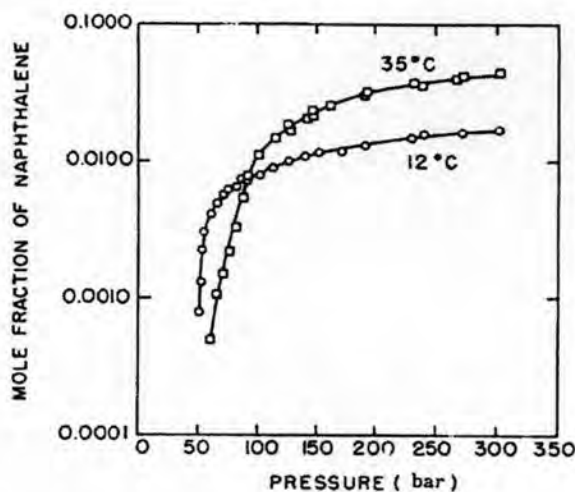


Figure 2.8 Variation of the solubility of naphthalene in supercritical fluid as a function of pressure and temperature

2.9 Experimental design and statistical data analysis

Most of previous studies about extractions optimized the process conditions by conducting one-factor-at-a-time experiments. The method assumes no interactions between the variables, and thus could lead to unbiased results. On the other hand, statistical experimental design is a powerful method for determining the factors effects and their interactions, which allow process optimization to be conducted effectively. So in this work, the experimental design was used to investigate the effects of the operating conditions on the extraction yields and their antioxidant activities. Several designs of experiments are used to allow the determination of factor effects and the effect of the factors interaction on the interested response. These include full factorial design, in which all n^k experiments are required for k factors each having n levels. This requires a large number of experiments, particularly when the number of factors and levels to be investigated is high. Alternatively, fractional factorial could be used in which only fractions of the full factorial experiments are needed to be conducted. Alternatively central composite design (CCD) analysis designs are other options that can be used to analyze the experimental data which

require even fewer number of experiments. Generally, these designs are useful for fitting a data containing curvature and different types of CCDs are such as Box-Behnken design, rotatable design, and face-centered central composite design. Detailed discussion on different CCDs can be found in Statistical Design and Analysis of Experiments (Mason, 1989). In this study, the face-centered central composite design was employed. This design consists of a 2^k factorial (coded to the usual ± 1 notation) augmented by $2k$ axial points $(\pm\alpha, 0, 0, \dots, 0)$, $(0, \pm\alpha, 0, \dots, 0)$, $(0, 0, \pm\alpha, \dots, 0)$, \dots , $(0, 0, 0, \dots, \pm\alpha)$ and n_0 center points $(0, 0, \dots, 0)$, where k is the number of factor and the α equals to 1 as in Figure 2.9. The face-centered central composite design has the advantage than other designs as it requires fewer experimental runs than the other designs. The experimental matrix for three factors faced-centered design is shown in Table 2.4.

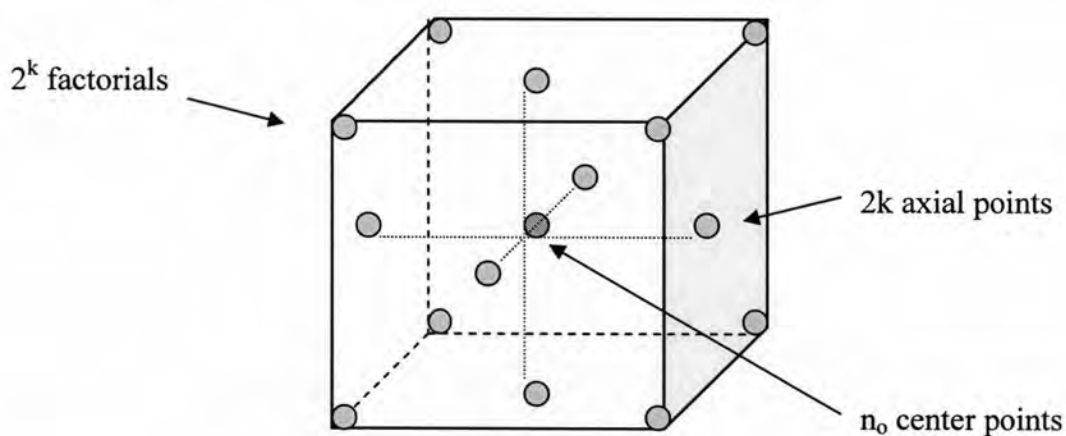


Figure 2.9 Face-centered central composite design

Table 2.4 Experimental matrix for the faced-centered design with 3 factors.

Run	Factor A	Factor B	Factor C
1	1	1	1
2	1	1	-1

3	1	-1	1
4	1	-1	-1
5	-1	1	1
6	-1	1	-1
7	-1	-1	1
8	-1	-1	-1
9	1	0	0
10	0	1	0
11	0	0	1
12	-1	0	0
13	0	-1	0
14	0	0	-1
15	0	0	0
16	0	0	0
17	0	0	0

To analyze the CCD data, analysis of variance (ANOVA) is used to determine the factors that have significant effects on the response. In addition, the analysis of central composite designs further proceeds by fitting to the data the general mathematical model (response equation). In this study, a quadratic equation was used which have the following form.

$$Y = \beta_0 + \beta_1 X_1 + \dots + \beta_k X_k + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \dots + \beta_{k-1} X_{k-1} X_k + \beta_{11} X_1^2 + \dots + \beta_{kk} X_k^2$$

where Y is the response, $X_1, X_2, X_3, \dots, X_k$ are the interested factors, β_0 is y -interception, $\beta_1, \beta_2, \beta_3, \dots, \beta_k$ are the partial regression coefficients. In other words, the model is fitted to the observed values of the dependent variable Y , that include (1) main effects for factors X_1, \dots, X_k , (2) their interactions ($X_1 X_2, X_1 X_3, \dots, X_{k-1} X_k$), and (3) their quadratic components (X_1^2, \dots, X_k^2).

In this study, the statistical performing and the model refining were evaluated by using SPSS 9.0, which allows the determination of factor effects and interaction

effects, the response equations, from which optimization of extraction conditions could be achieved.

2.10 Literature reviews on SC-CO₂ extraction

Due to many advantages SC-CO₂ offers, it has therefore been applied to extraction many valuable products from plant and marine materials. Examples of extraction of plant derived products are given in Table 2.5. These include extraction of spices and herbs for flavoring compounds and essential oils from plant materials (Novak et al., 1989, Roy et al., 1996) as well as from fermentation broth (Fabre et al., 1999). For SC-CO₂ extraction of carotenoids related compounds from natural materials including marine materials, literatures include the extraction of carotenoids from *Spirulina platensis* (Careri et al., 2001) and the extraction of carotenoids and other lipids from *Chlorella vulgaris* (Mendes et al., 1995). These studies demonstrated that the extraction yields and the carotenoids fraction in the oil increased with increasing pressure. The reviews of literature on SC-CO₂ extraction of carotenoids are given in Table 2.6. For extraction of astaxanthin, studies include extraction astaxanthin from various samples such as *Phaffia rhodozyma* (Matsuyama et al., 1998) and *H. pluvialis* (Majewski et al., 2000, Machmudah et al., 2004), and *Callinectes sapidus* and shell waste (Valenzuela et al., 2000).

Table 2.5 Review of previous investigation on supercritical fluid extraction of plant materials

References	Materials	Compounds	Methods	Conditions					Conclusions
				Temp. (°C)	Pressure (bar)	Sample loading (g)	Solvent flow rate (ml/min)	Extraction chamber (L)	
Novak and Robey, 1989	Spices and herbs	Flavor	SC-CO ₂	20-80	60-300	N/A	4550 (kg/h)	974	1) High yield and high quality extracts were resulted. 2) SC-CO ₂ allows controlled blending for product flavor and fragrance profile.

Table 2.5 Review of previous investigation on supercritical fluid extraction of plant materials (continue)

References	Materials	Compounds	Methods	Conditions					Conclusions
				Temp. (°C)	Pressure (bar)	Sample loading (g)	Solvent flow rate (ml/min)	Extraction chamber (ml)	
Roy et al., 1996	Peppermint leaves	Essential oils and cuticular wax	SC-CO ₂	40	100-300 optimal press. at 300 bar	23-24	0.028 - 0.066	~ 177	1) The extraction rate of cuticular waxes increased with increasing pressure, while that of essential oils was almost constant. 2) The concentration of cuticular waxes was close to the solubility, while that of essential oils was much smaller than the solubility.

Table 2.5 Review of previous investigation on supercritical fluid extraction of plant materials (continue)

References	Materials	Compounds	Methods	Conditions					Conclusions
				Temp. (°C)	Pressure (bar)	Sample loading (g)	Solvent flow rate (ml/min)	Extraction chamber (ml)	
Fabre et al., 1999	Fermentaion broth of yeast and L-phynylalanine	rose aroma	SC-CO ₂	32-42 suitable temp. 35 to 40°C	200	50 ml	N/A	350	The suitable operating conditions are 200 bar and 35-40 °C.
Palma and Taylor, 1999	Grape seed	Polyphenolic compounds	Near critical carbon dioxide	35-55 optimal temp. at 55°C	N/A	0.03	1	1	1) The best conditions were 0.95 g/ml CO ₂ density, 10% methanol, and 55 °C. 2) The recovery was 79%wt. with relative S.D. of 7.3%.

ภาควิชาเทคโนโลยีการเกษตร
 มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี

Table 2.5 Review of previous investigation on supercritical fluid extraction of plant materials (continue)

References	Materials	Compounds	Methods	Conditions					Conclusions
				Temp. (°C)	Pressure (bar)	Sample loading (g)	Solvent flow rate (ml/min)	Extraction chamber (ml)	
Tonthubthimthong et al., 2001	Neem seeds	Nimbin	SC-CO ₂	35-60 optimal temp.at 55 °C	100-260 optimal press. at 230 bar	2	0.24 - 1.24	10	The best experimental extraction conditions are 230 bar, 35 °C, and the solvent flow rate of 1.24 ml/min. (35 %wt.) Crude oil extracted increases with increasing pressure at constant temperature and with increasing temperature when pressure > 242 bar.
Wang et al., 2001	Ginseng root hair	Ginseng root hair oil and ginsenosides	SC-CO ₂ and EtOH as a co-solvent	35-60 optimal temp.at 60 °C	104-312 optimal press. at 312 bar	80	5	300	

Table 2.6 Review of previous investigation on supercritical fluid extraction of carotenoids related compounds from natural materials.

References	Materials	Compounds	Methods	Conditions					Conclusions
				Temp. (°C)	Pressure (bar)	Sample loading (g)	Solvent flow rate (ml/min)	Extraction chamber (ml)	
Majewski et al., 2000	Algae <i>H. pluvialis</i>	Astaxanthin	SC-CO ₂ and EtOH as a co-solvent	60	300	N/A	N/A	450	The extract yields were lower than 1 %wt. for crushed algae. The yield was 1.3 %wt. for twice grinded and 1.6 %wt. for twice-grinded algae with use of co-solvent.
Mendes et al., 1995	Algae <i>Chlorella vulgaris</i>	Carotenoids and other lipids	SC-CO ₂	40-55 optimal temp. at 55°C	200-350 optimal press. at 350 bar	5	400	N/A	Extraction yields increase with increasing press. at constant temp. or with temp. at constant press.

Table 2.6 Review of previous investigation on supercritical fluid extraction of carotenoids related compounds from natural materials. (continue)

References	Materials	Compounds	Methods	Conditions					Conclusions
				Temp. (°C)	Pressure (bar)	Sample loading (g)	Solvent flow rate (ml/min)	Extraction chamber (ml)	
Matsuyama et al., 1998	Yeast <i>Phaffia rhodozyma</i>	Astaxanthin	SC-CO ₂	~ 40	197	0.3	N/A	50	The astaxanthin and the carotenoids content of extracted oil were 86 %wt. and 92 %wt., respectively.
Valenzuela et al., 2000	Blue crab (<i>Callinectes sapidus</i>) shell waste	Astaxanthin	SC-CO ₂ and EtOH as a co-solvent	45-65 optimal temp. at 45°C	295-335 optimal press. at 340 bar	25	3400 - 4800	100	The maximum extraction conditions from the experiments occurred at 340 bar, 45 °C, and solid loading of 25 g

Table 2.6 Review of previous investigation on supercritical fluid extraction of carotenoids related compounds from natural materials. (continue)

References	Materials	Compounds	Methods	Conditions					Conclusions
				Temp. (°C)	Pressure (bar)	Sample loading (g)	Solvent flow rate (ml/min)	Extraction chamber (ml)	
Careri et al., 2001	Algae <i>Spirulina platensis</i>	Carotenoids	SC-CO ₂ and EtOH as a co-solvent	40-80 optimal temp. at 60, 76, 80°C for 3 carotenoids	150-350 optimal press. at 350 bar	0.5	2	7	1) The relationships between variables and responses are polynomial functions. 2) The significant effects for the extraction are pressure, modifier, extraction time, and interaction effects.

Table 2.6 Review of previous investigation on supercritical fluid extraction of carotenoids related compounds from natural materials. (continue)

References	Materials	Compounds	Methods	Conditions					Conclusions
				Temp. (°C)	Pressure (bar)	Sample loading (g)	Solvent flow rate (ml/min)	Extraction chamber (ml)	
Machmudah et al., 2004	Algae <i>H. pluvialis</i>	Astaxanthin	SC-CO ₂	40-80 (optimal temp. at 70°C)	200-550 (optimal press. at 550 bar)	7	2-4	50	1) The optimum conditions are 70°C, 550 bar, and flow rate 3 ml/min 2) The highest amount of astaxanthin extracts and astaxanthin content are 77.9 %wt. and 12.3 %wt.