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APPENDICES

APPENDIX A

DATA ANALYSIS

A-1 Standard calibration curve of astaxanthin

Table A-1 Standard calibration curve data

Concentration of std. astaxanthin (mg/l)	Absorbance at 475 nm.			
	Exp.1	Exp.2	Exp.3	Average
1	0.195	0.197	0.192	0.195
2	0.393	0.392	0.388	0.391
3	0.588	0.590	0.580	0.586
4	0.783	0.775	0.788	0.782
5	0.979	0.978	0.974	0.977

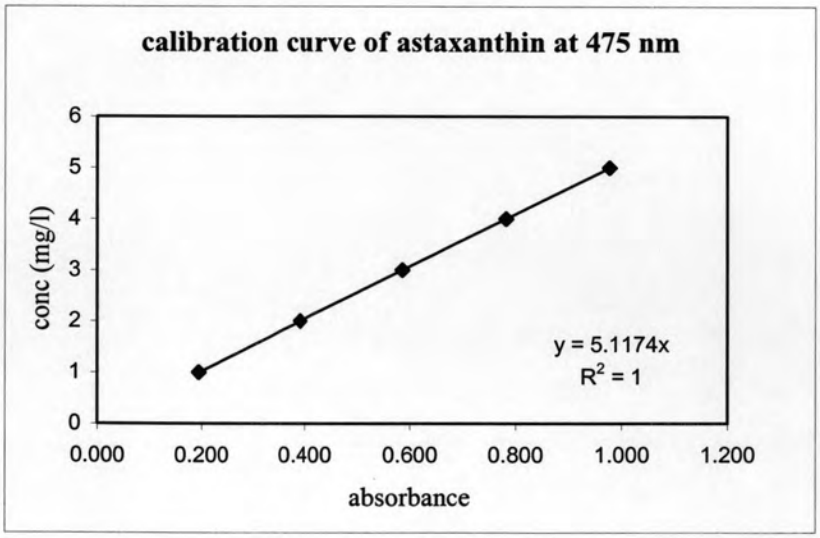

Figure A-1 Standard calibration curve of astaxanthin

A-2 Experimental data of astaxanthin extraction

Table A-2 Experimental data of total astaxanthin in *H. pluvialis* by soxhlet extraction

Experiment	Absorbance at 475 nm	Concentration of astaxanthin (mg/g)	% wt.
1	0.429	27.4	2.74
2	0.429	27.4	2.74
3	0.430	27.5	2.75
Average	0.429	27.5	2.75

Table A-3 Experimental data of astaxanthin yields (n=3)

Run	Temp. (°C)	Press. (bar)	Time (hour)	Astaxanthin yields (mg)				
				Exp.1	Exp.2	Exp.3	Avg.	% wt.
1	80	500	4	12.49	11.04	10.73	11.42	83.16
2	80	500	1	10.29	10.52	10.23	10.34	75.33
3	80	300	4	7.42	9.31	9.39	8.71	63.41
4	80	300	1	5.58	5.26	6.83	5.89	42.89
5	40	500	4	10.49	11.55	10.06	10.70	77.92
6	40	500	1	9.65	10.58	9.16	9.80	71.33
7	40	300	4	10.67	9.83	11.49	10.66	77.64
8	40	300	1	7.73	7.00	8.96	7.89	57.48
9	80	400	2.5	8.81	10.45	10.23	9.83	71.60
10	60	500	2.5	8.55	11.02	10.87	10.15	73.88
11	60	400	4	11.57	10.96	10.91	11.14	81.16
12	40	400	2.5	8.11	9.68	9.60	9.13	66.48
13	60	300	2.5	9.69	8.60	8.55	8.94	65.14
14	60	400	1	10.15	7.29	9.52	8.99	65.43
15	60	400	2.5	11.40	9.83	11.76	10.99	80.06
16	60	400	2.5	10.07	10.17	10.09	10.11	73.65
17	60	400	2.5	10.13	9.60	9.98	9.90	72.10

Table A-4 Experimental data of extract antioxidant activity (n=3)

Run	Temp. (°C)	Press. (bar)	Time (hour)	IC_{50} (mg/l)				
				Exp.1	Exp.2	Exp.3	Avg.	$1/IC_{50}$
1	80	500	4	3.29	2.05	1.77	2.37	0.422
2	80	500	1	3.16	2.52	2.55	2.74	0.365
3	80	300	4	2.73	1.68	1.65	2.02	0.496
4	80	300	1	1.97	1.41	1.94	1.77	0.564
5	40	500	4	1.93	2.06	1.47	1.82	0.550
6	40	500	1	3.46	2.61	2.06	2.71	0.369
7	40	300	4	3.94	1.94	1.79	2.56	0.391
8	40	300	1	4.99	1.99	2.03	3.01	0.333
9	80	400	2.5	1.90	2.23	2.21	2.12	0.473
10	60	500	2.5	1.90	2.42	2.38	2.23	0.448
11	60	400	4	2.71	2.89	3.31	2.97	0.337
12	40	400	2.5	1.92	2.26	2.24	2.14	0.468
13	60	300	2.5	2.88	1.96	1.33	2.06	0.486
14	60	400	1	3.30	1.81	2.38	2.50	0.401
15	60	400	2.5	2.53	2.00	2.31	2.28	0.439
16	60	400	2.5	3.34	2.31	2.23	2.63	0.380
17	60	400	2.5	3.60	2.22	2.40	2.74	0.365

APPENDIX B

STATISTICAL ANALYSIS

B-1 Astaxanthin yields

In this experiment, the operating conditions were varied to finalize the best condition for supercritical carbon dioxide extraction of astaxanthin from *Haematococcus pluvialis*. The results analysis can be determined by using the statistical testing program SPSS 9.0 and the results as shown in Table B-1.

Table B-1 ANOVA Table for astaxanthin yields

Dependent Variable: ASTAMGAV

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	28.969[a]	14	2.069	6.187	.148
Intercept	1149.519	1	1149.519	3437.214	.000
X1	1.045	2	.523	1.562	.390
X2	9.879	2	4.939	14.769	.063
X3	9.263	2	4.632	13.849	.067
X1 * X2	3.393	1	3.393	10.146	.086
X1 * X3	6.613E-03	1	6.613E-03	.020	.901
X2 * X3	1.629	1	1.629	4.871	.158
X1 * X2 * X3	2.113E-03	1	2.113E-03	.006	.944
Error	.669	2	.334		
Total	1623.160	17			
Corrected Total	29.638	16			

a. R Squared = .977 (Adjusted R Squared = .819)

Consider the hypotheses testing for the astaxanthin yields as below

1) Testing for the effect of each level of factor X_2 (operating pressure)

   H_0: No difference between each level of factor X_2 or the operating pressure cannot affect the astaxanthin yields

   H_1: At least one level different from another level of factor X_2 or the operating pressure can affect the astaxanthin yields

   Statistical testing: F = 4.939 / 0.334 = 14.769 or sig. = 0.063

Refuse H_0 if sig. < 0.10. In this case sig. = 0.063 < 0.10, thus refuse H_0 that means the operating pressure can affect the astaxanthin yields.

2) Testing for the effect of each level of factor X_3 (extraction time)

H_0: No difference between each level of factor X_3 or the extraction time cannot affect the astaxanthin yields

H_1: At least one level different from another level of factor X_3 or the extraction time can affect the astaxanthin yields

Statistical testing: F = 4.632 / 0.334 = 13.849 or sig. = 0.067

Refuse H_0 when sig. < 0.10 at confident interval 90%. In this case sig. = 0.067 < 0.10, thus refuse H_0 that means the extraction time can affect the astaxanthin yields.

3) Testing for the interaction effect of each level of factor X_1 and X_2 (interaction between operating temperature and pressure)

H_0: No difference between interaction level of factor X_1 and X_2 or the interaction between the operating temperature and pressure cannot affect the astaxanthin yields

H_1: At least one interaction level different from another interaction level of factor X_1 and X_2 or the interaction between the operating temperature and pressure can affect the astaxanthin yields

Statistical testing: F = 3.393 / 0.334 = 10.146 or sig. = 0.086

Refuse H_0 when sig. < 0.10 at confident interval 90%. In this case sig. = 0.086 < 0.10, thus refuse H_0 that means the interaction between the operating temperature and pressure can affect the astaxanthin yields

B-2 Antioxidant activity

Table B-2 ANOVA Table for extract antioxidant activity

Dependent Variable: INVEIC50

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	7.561E-02[a]	14	5.401E-03	3.529	.243
Intercept	2.410	1	2.410	1574.806	.001
X1	1.023E-02	2	5.113E-03	3.341	.230
X2	7.752E-03	2	3.876E-03	2.533	.283
X3	1.877E-03	2	9.383E-04	.613	.620
X1 * X2	2.738E-02	1	2.738E-02	17.890	.052
X1 * X3	7.813E-03	1	7.813E-03	5.105	.152
X2 * X3	7.688E-03	1	7.688E-03	5.024	.154
X1 * X2 * X3	5.000E-07	1	5.000E-07	.000	.987
Error	3.061E-03	2	1.530E-03		
Total	3.202	17			
Corrected Total	7.867E-02	16			

a. R Squared = .961 (Adjusted R Squared = .689)

Consider the hypotheses testing for the extract antioxidant activity as below

Testing for the interaction effect of each level of factor X_1 and X_2 (interaction between operating temperature and pressure)

H_0: No difference between interaction level of factor X_1 and X_2 or the interaction between the operating temperature and pressure cannot affect the extract antioxidant activity

H_1: At least one interaction level different from another interaction level of factor X_1 and X_2 or the interaction between the operating temperature and pressure can affect the extract antioxidant activity

Statistical testing: $F = 0.02738 / 0.001530 = 17.89$ or sig. = 0.052

Refuse H_0 when sig. < 0.10 at confident interval 90%. In this case sig. = 0.052 < 0.10, thus refuse H_0 that means the interaction between the operating temperature and pressure can affect the extract antioxidant activity.

B-3 Optimal condition for astaxanthin yields

Table B-3 ANOVA Table for astaxanthin yields

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	27.391	9	3.043	9.482	.004[a]
	Residual	2.247	7	.321		
	Total	29.638	16			

a. Predictors: (Constant), X2X3, X1X3, X1X2, X3X3, X3, X2, X1, X2X2, X1X1

b. Dependent Variable: ASTAMGAV

Table B-4 Coefficients for astaxanthin yields

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	10.107	.242		41.692	.000
	X1	-.199	.179	-.116	-1.111	.303
	X2	1.032	.179	.599	5.760	.001
	X3	.972	.179	.565	5.425	.001
	X1X1	-.458	.346	-.171	-1.323	.228
	X2X2	-.393	.346	-.146	-1.135	.294
	X3X3	.127	.346	.047	.367	.724
	X1X2	.651	.200	.338	3.251	.014
	X1X3	2.875E-02	.200	.015	.144	.890
	X2X3	-.451	.200	-.234	-2.253	.059

a. Dependent Variable: ASTAMGAV

From ANOVA Table that use for the hypotheses testing which are

H_0: $\beta_1 = \beta_2 = \beta_3 = 0$

H_1: at least one value of $\beta_i \neq 0$; i = 1, 2, 3

Statistical testing: F = 3.043 / 0.321 = 9.482 or sig. = 0.004

The result shows that sig = 0.004 < 0.05, thus refuse H_0 that means at least one factor has relation to the astaxanthin yields and have to determine whether factor that has relation to the astaxanthin yields by using t-test. From Table B-4, consider the hypotheses testing for the constant and the regression coefficients as below.

1) Testing for the constant (β_0)

H_0: $\beta_0 = 0$ vs. H_1: $\beta_0 \neq 0$

Statistical testing: t = 41.692 or sig = 0.000

The result shows that sig = 0.000 < 0.05, thus refuse H_0 that means the equation for this relation is not pass the origin point.

2) Testing for the regression coefficient (β_2)

H_0: The operating pressure does not has relation to the astaxanthin yields when another factors are constant

H_1: The operating pressure has relation to the astaxanthin yields when another factors are constant

Statistical testing: t = 5.760 or sig = 0.001

The result shows that sig = 0.001 < 0.05, thus refuse H_0 that means the operating pressure has relation to the astaxanthin yields when another factors are constant.

3) Testing for the constant (β_3)

H_0: The extraction time does not has relation to the astaxanthin yields when another factors are constant

H_1: The extraction time has relation to the astaxanthin yields when another factors are constant

Statistical testing: t = 5.425 or sig = 0.001

The result shows that sig = 0.001 < 0.05, thus refuse H_0 that means the extraction time has relation to the astaxanthin yields when another factors are constant.

4) Testing for the constant ($\beta_1\beta_2$)

H_0: The interaction between operating temperature and pressure does not has relation to the astaxanthin yields when another factors are constant

H_1: The interaction between operating temperature and pressure has relation to the astaxanthin yields when another factors are constant

Statistical testing: t = 3.251 or sig = 0.014

The result shows that sig = 0.014 < 0.05, thus refuse H_0 that means the interaction between operating temperature and pressure has relation to the astaxanthin yields when another factors are constant.

B-4 Optimal condition for extract antioxidant activity

Table B-5 ANOVA Table for extract antioxidant activity

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	6.656E-02	9	7.395E-03	4.273	.034[a]
	Residual	1.211E-02	7	1.731E-03		
	Total	7.867E-02	16			

a. Predictors: (Constant), X2X3, X1X3, X1X2, X3X3, X3, X2, X1, X2X2, X1X1

b. Dependent Variable: INVEIC50

Table B-6 Coefficients for extract antioxidant activity

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.413	.018		23.196	.000
	X1	2.090E-02	.013	.236	1.589	.156
	X2	-1.16E-02	.013	-.131	-.882	.407
	X3	1.640E-02	.013	.185	1.247	.253
	X1X1	4.392E-02	.025	.318	1.728	.128
	X2X2	4.042E-02	.025	.292	1.590	.156
	X3X3	-5.76E-02	.025	-.417	-2.265	.058
	X1X2	-5.85E-02	.015	-.590	-3.977	.005
	X1X3	-3.13E-02	.015	-.315	-2.125	.071
	X2X3	3.100E-02	.015	.313	2.108	.073

a. Dependent Variable: INVEIC50

From ANOVA Table that use for the hypotheses testing which are

H_0: $\beta_1 = \beta_2 = \beta_3 = 0$

H_1: at least one value of $\beta_i \neq 0$; i = 1, 2, 3

Statistical testing: F = 0.007395 / 0.001731 = 4.273

The result shows that sig = 0.034 < 0.05, thus refuse H_0 that means at least one factor has relation to the extract antioxidant activity and have to determine whether factor that has relation to the astaxanthin yields by using t-test. From Table 4.9, consider the hypotheses testing for the constant and the regression coefficients as below.

1) Testing for the constant (β_0)

   H_0: $\beta_0 = 0$ vs. H_1: $\beta_0 \neq 0$

   Statistical testing: t = 23.196 or sig = 0.000

   The result shows that sig = 0.000 < 0.05, thus refuse H_0 that means the equation for this relation is not pass the origin point.

2) Testing for the constant ($\beta_1\beta_2$)

   H_0: The interaction between operating temperature and pressure does not has relation to the extract antioxidant activity when another factors are constant

   H_1: The interaction between operating temperature and pressure has relation to the extract antioxidant activity when another factors are constant

   Statistical testing: t = -3.977 or sig = 0.005

   The result shows that sig = 0.005 < 0.05, thus refuse H_0 that means the interaction between operating temperature and pressure has relation to the extract antioxidant activity when another factors are constant.

APPENDIX C

LIST OF PUBLICATION

Praiya Thana, Siti Machmudah, Motonobu Goto, Mitsuru Sasaki, Artiwan Shotipruk, "Optimization of Supercritical Carbon Dioxide Extraction of Astaxanthin from *Haematococcus pluvialis*", Proceedings of The Thai Chemical Engineering and Applied Chemistry Conference 16th, Bangkok, Thailand, 26-27 October, 2006, Ref. No.BIO-008-P.

Optimization of Supercritical Carbon Dioxide Extraction of Astaxanthin from *Haematococcus pluvialis*

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Abstract

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a powerful biological antioxidant, produced in microalgae *Haematococcus pluvialis*. It exhibits antioxidant properties superior to beta-carotene and vitamin C, thus its potential benefits to human health are being of high interest. In general, astaxanthin products are highly sensitive to light, oxygen, and heat, supercritical carbon dioxide (SC-CO_2) is therefore used as an extraction solvent instead of conventional organic solvent to avoid the degradation due to extended exposure to oxidative stresses during the conventional process. In addition, the method causes no harmful organic solvents in the products. In determining the suitable extraction conditions, most of the previous studies employed one-variable-at-a-time experiments, in which the interactions between the process variables could not be determined. In this work, experimental design was used to investigate the effect of temperature (40-80 °C), operating pressure (300-500 bar), and extraction time (1-4 hours) of supercritical carbon dioxide (SC-CO_2) extraction on astaxanthin yields and antioxidant activity (IC_{50}). The results show that the pressure (X_2), the extraction time (X_3), and the interaction of temperature and pressure (X_1X_2) have significant effect to astaxanthin yields. However only the interaction of temperature and pressure (X_1X_2) have significant effect to extract antioxidant activity.

Keywords: Astaxanthin; *Haematococcus pluvialis*; Supercritical; Extraction; Carbon dioxide

1. Introduction

Haematococcus pluvialis is one of the most important microalgae producing many kinds of carotenoids such as beta-carotene, zeaxanthin, lutein, and astaxanthin. Amongst variety of carotenoids produced, astaxanthin is dominant product. Astaxanthin is a powerful biological antioxidant as it exhibits strong free radical scavenging activity and protects against lipid peroxidation and oxidative damage of LDL-cholesterol, cells and tissues, and cell membranes. For these reasons, there has been increasing interest to extract astaxanthin from *Haematococcus pluvialis*.

Supercritical fluid extraction (SFE) is a modern technology with increasing applications in the food and food processing industry. The principle of the process consists of utilizing a supercritical fluid whose physicochemical properties are between those of a liquid and a gas. Its temperature and pressure are above the critical values, which lead these solvents to possess special properties such as high diffusivity, low viscosity, and low surface tension. This allows the fluid to better diffuse through natural solid matrix, and thus better extract the natural compounds compared to the conventional liquid solvents. When the operating pressure and temperature are reduced, the loss of these special characteristics occurs. Thus, the solute can be extracted from the solvent at supercritical condition, and separated when pressure and temperature are reduced below its critical condition. The most frequently employed supercritical solvent in food and natural product processing is carbon dioxide (CO_2) due to its low toxicity, good safety, and low critical temperature.

Although supercritical carbon dioxide (SC-CO_2) is nowadays considered as a new alternative for benign extraction of natural compounds, the selection of the operating conditions for specific application is still an area of active research. For extraction of carotenoids from marine materials, many recent studies have been carried out to investigate the effect of operating conditions and to find optimal conditions for the process [1]. Studies on extraction of astaxanthin from *Haematococcus pluvialis* have also been reported [2, 3]. It was found in our previous investigation that the total amount of astaxanthin in the extract and its concentration

in the extract were influenced by the extraction pressure and temperature. Furthermore, using ethanol as a co-solvent could enhance the amount of astaxanthin, however the antioxidant activity was decreased due to the presence of large amount of other non-active compounds being extracted concurrently [3]. Despite the interesting information obtained from the previous studies, the process conditions have been optimized by merely conducting one-variable-at-a-time experiments. This can cause biased results obtaining suboptimality. Statistical experimental design has been demonstrated to be a powerful tool for determining the factors effects and their interactions, which allows process optimization to be conducted effectively. The techniques are used widely in various applications including in chemical industry. As an example related to this proposed study, the method has successfully applied it in order to investigate the effects of extraction parameters on supercritical fluid extraction of carotenoids in *Spirulina platensis* [1].

In this work, the experimental design was used to investigate the effects of operating pressure (300-500 bar), temperature (40-80 °C), and extraction time (1-4 hours) on the amount of astaxanthin as well as antioxidant activity of the extract.

2. Materials and Methods

2.1 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.

2.2 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 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. In addition, the soxhlet extraction 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.

2.3 Analysis of Antioxidant Activity

The antioxidant activity of astaxanthin extracts from *Haematococcus pluvialis* were measured using ABTS method. The concentration of the sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index for the purposes of comparing the antioxidant activity in various extracts. The extracts were diluted in series with acetone and each diluted extracts were added to $ABTS^{\bullet+}$ solution (produced by ABTS 7 mM and 2.45 mM potassium persulfate both in final concentration. Then adjusted an absorbance to 0.70 ± 0.02 at 734 nm) with the ratio of 1:10 (extract: $ABTS^{\bullet+}$solution). The mixtures were then incubated at room temperature in darkness for 10 min, after which the absorbance was measured at the wavelength of 734 nm.

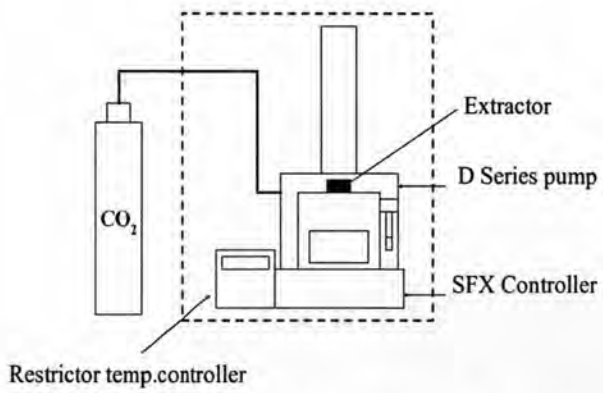

Figure 1 SFX 220 extraction system of supercritical carbon dioxide extraction

2.4 Experimental Design

In this study, the central composite 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 1.

Table 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, h)	1.0	2.5	4.0

The experimental data were fitted with second order response surface models, which have the following form:

$$\hat{Y} = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3}\sum_{j=1}^{3} b_{ij} X_i X_j \qquad (1)$$

where Y is the predicted response, X_i is the coded values of the factor, a polynomial coefficients; b_0 is the intercept term; b_i is the main effects for each variable; b_{ij} is the interaction effects. Finally, the response surfaces of the variables inside the experimental domain were analyzed by analysis of variance (ANOVA) using statistical package SPSS version 9.0 (SPSS Italia, Bologna, Italy). Further details in statistical data analysis and experimental design can be found in [4].

3. Results and Discussion

The three-level factorial design was used to evaluate the both main and interaction effects of the operating conditions and extraction time on the supercritical carbon dioxide extraction. The three interested factors are operating temperature (X_1) in the range of 40-80 °C, operating pressure (X_2) in the range of 300-500 bar, and extraction time (X_3) in the range of 1-4 hour. All data obtained are shown in Table 2.

Table 2 Experimental matrix and values of observed responses

Run	X_1	X_2	X_3	Astaxanthin (mg/0.5 g)	$1/IC_{50}$ (l/mg)
1	1	1	1	11.42	0.422
2	1	1	-1	10.34	0.365
3	1	-1	1	8.71	0.496
4	1	-1	-1	5.89	0.564
5	-1	1	1	10.70	0.550
6	-1	1	-1	9.80	0.369
7	-1	-1	1	10.66	0.391
8	-1	-1	-1	7.89	0.333
9	1	0	0	9.83	0.473
10	0	1	0	10.15	0.448
11	0	0	1	11.14	0.337
12	-1	0	0	9.13	0.468
13	0	-1	0	8.94	0.486
14	0	0	-1	8.99	0.401
15	0	0	0	10.99	0.439
16	0	0	0	10.11	0.380
17	0	0	0	9.90	0.365

From the soxhlet extraction, the total amount of astaxanthin in algae was 13.73 mg in 0.5 g dry algae or 2.75%wt. Thus on this basis, Table 2 indicated that the highest amount of astaxanthin yields was 11.42 mg in 0.5 g (or 83.05%wt.) and was obtained at 80 °C, 500 bar, after 4 hours. In addition, the results show that the extract obtained at 80 °C, 300 bar, and 1 hour had the highest antioxidant activity ($1/IC_{50}$ was 0.564 l/mg or IC_{50} was 1.77 mg/l). To investigate the effects of extraction condition, all experimental data were analyzed by using statistical analysis (SPSS 9.0). Enter method was used to calculate the estimated coefficients of the polynomial functions of response surfaces for both astaxanthin yields and extract antioxidant activity. Table 3, the ANOVA table for the two models of astaxanthin yields and extract antioxidant

activity in term of $1/IC_{50}$ which indicates that both models are considered to be statistically significant (sig. < 0.05) at 95% confidence. That means at least one of the interested factors has a significant correlation with the response. The calculated coefficients of all factors were shown in Table 4 and Table 5.

Table 3 ANOVA table for refined models

Model	Sum of Square	df	Mean Square	F	Sig.
Astaxanthin Yields					
Regression	27.391	9	3.043	9.482	0.004
Residual	2.247	7	0.321		
Total	29.638	16			
Antioxidant Activity (1/ IC_{50})					
Regression	$6.7x10^{-2}$	9	$7.4x10^{-3}$	4.273	0.034
Residual	$1.2x10^{-2}$	7	$1.7x10^{-3}$		
Total	$7.9x10^{-2}$	16			

Table 4 Regression coefficient of polynomial functions of response surfaces of astaxanthin content

	Coefficients	S.D.	t	Sig.
Astaxanthin Yields (mg)				
Constant	10.107	0.242	41.692	0.000
X_1	-0.199	0.179	-1.111	0.303
X_2	1.032	0.179	5.760	0.001
X_3	0.972	0.179	5.425	0.001

X_1^2	-0.458	0.346	-1.323	0.228
X_2^2	-0.393	0.346	-1.135	0.294
X_3^2	0.127	0.346	0.367	0.724
X_1X_2	0.651	0.200	3.251	0.014
X_1X_3	$2.875x10^{-2}$	0.200	0.144	0.890
X_2X_3	-0.451	0.200	-2.253	0.059
$R^2 = 0.924$				

Table 5 Regression coefficient of polynomial function of response surfaces of antioxidant activity

	Coefficients	S.D.	t	Sig.
1/ IC_{50} (l/mg)				
Constant	0.413	0.18	23.196	0.000
X_1	$2.090x10^{-2}$	0.13	1.589	0.156
X_2	$-1.16x10^{-2}$	0.13	-0.882	0.407
X_3	$1.640x10^{-2}$	0.13	1.247	0.253
X_1^2	$4.392x10^{-2}$	0.25	1.728	0.128
X_2^2	$4.042x10^{-2}$	0.25	1.590	0.156
X_3^2	$-5.76x10^{-2}$	0.25	-2.265	0.058
X_1X_2	$-5.85x10^{-2}$	0.15	-3.977	0.005
X_1X_3	$-3.13x10^{-2}$	0.15	-2.125	0.071
X_2X_3	$3.100x10^{-2}$	0.15	2.108	0.073
$R^2 = 0.846$				

3.1 Effect of Operating Temperature and Pressure

For supercritical fluid extraction, the solubility of solute depends on the supercritical fluid density and the solute vapor pressure, which are controlled by temperature and pressure of the supercritical solvent. In the predicted model, the temperature did not have important effect to the astaxanthin yields. But it had a

significant effect to the extract antioxidant activity in term of the interaction between the temperature and pressure, X_1X_2 (sig. 0.005 < 0.05).

However, the effect of pressure plays an important role for both astaxanthin yields and extract antioxidant activity (IC_{50}). The results show that the main effect of pressure, X_2 (sig. 0.001< 0.05) and the interaction effect between temperature and pressure, X_1X_2 (sig. 0.014 < 0.05) were significant to the astaxanthin yields. As a result, the extraction yields increased when the operating pressure increased. For the extract antioxidant activity, only the interaction effect between temperature and pressure, X_1X_2 (sig. 0.005 < 0.05) was significant. Figure 2 shows three-dimensional plot of the response surface for the astaxanthin yields for which the polynomial function is:

$$Y = 10.107 - 0.199X_1 + 1.032X_2 + 0.972X_3 - 0.458X_1^2 - 0.393X_2^2 + 0.127X_3^2 + 0.651X_1X_2 + 0.0288X_1X_3 - 0.451X_2X_3 \quad (2)$$

From the approximate model, the optimal condition for astaxanthin yields was at 90 °C, 640 bar, 2.9 hours and the astaxanthin extract was 22.66 mg/g or 82.40 % yield (IC_{50} 1.83 mg/l). Since this condition was out of the experimental range, the optimal condition within the experimental range was instead determined to be 70 °C, 500 bar, and 4 hours. At this condition, astaxanthin extract was 23.04 mg/g (or 83.78 %wt.) and IC_{50} 2.45 mg/l. Figure 3 shows three-dimensional plot of the response surface for the extract antioxidant activity ($1/IC_{50}$), for which the polynomial function is:

$$Y = 0.413 + 0.0209X_1 - 0.0116X_2 + 0.0164X_3 + 0.04392X_1^2 + 0.04042X_2^2 - 0.0576X_3^2 - 0.0585X_1X_2 - 0.0313X_1X_3 + 0.0310X_2X_3 \quad (3)$$

The experimental results obtained here did not suggest the optimal conditions in which the highest antioxidant activity was achieved, but instead the minimum point was obtained at 50 °C, 354 bar, and 2.68 hours of extraction, in which the $1/IC_{50}$ was 0.460 or IC_{50} was 2.18 mg/l).

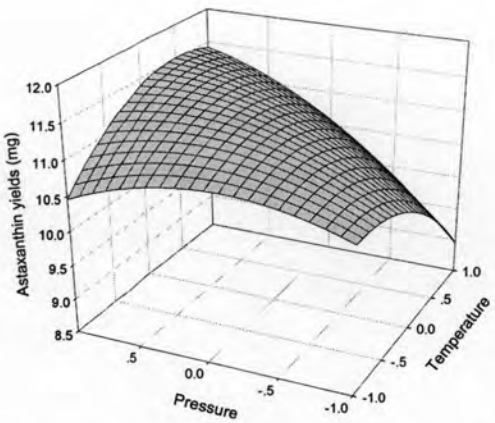

Figure 2 Three-dimensional plot of the response surface for the astaxanthin yields at 4 hour

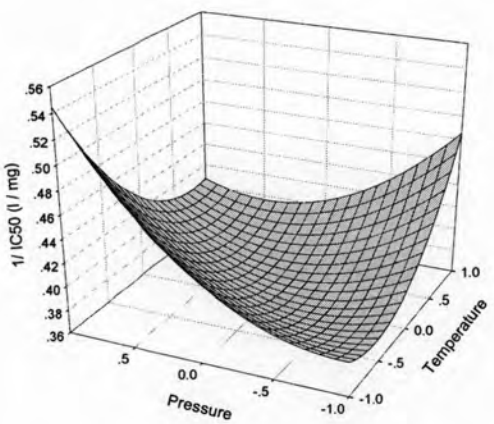

Figure 3 Three-dimensional plot of the response surface for the antioxidant activity ($1/ IC_{50}$) at 4 hour

3.2 Effect of Extraction Time

For the extraction time, the effects on both astaxanthin content and its antioxidant activity were investigated. Experimentally, the astaxanthin yields increased when the extraction time increased. According to the predicted model, the main effect of extraction time (X_3) was significant to the astaxanthin yields (sig. $0.001 < 0.05$) but the curvature effect and the interactions effects with other variables were not important. Additionally, all effects of the extraction time were not significant to antioxidant activity.

4. Conclusions

The experimental design approach allowed us to find the significant effects and polynomial functions for describing the effects of operating temperature, operating pressure and extraction time of the astaxanthin extraction from *Haematococcus pluvialis* using SC-CO_2 process.

In this study, the significant effects for the astaxanthin yields were the main effect of pressure, extraction time, and the interaction effect between temperature and pressure. The optimal condition for astaxanthin yields was at 70 °C, 500 bar, 4 hours and the extract was 23.04 mg/g or 83.78 % yield (IC_{50} 2.45 mg/l). For the extract antioxidant activity, only the interaction between temperature and pressure was significant effect. The minimum condition for antioxidant activity was found to be at 50 °C, 354 bar, 2.68 hours and at this condition, the IC_{50} value was 2.18 mg/l.

5. Acknowledgements

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6. References

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