CHAPTER IV

RESULTS AND DISCUSSION

In this study, the experiments were carried out to determine the effects of operation conditions of supercritical carbon dioxide extraction on astaxanthin yields from *Haematococcus pluvialis* and the antioxidant activity of the extract. Experimental design and analysis was employed to investigate the main effects and the interaction effects of the three interested factors, which were the operating temperature (X_1), the operating pressure (X_2), and the extraction time (X_3). Then the experimental results were fitted with response surface, from which the optimal conditions for extraction were obtained.

4.1 Supercritical carbon dioxide extraction (SC-CO_2) and design of experiment (DOE)

Supercritical carbon dioxide (SC-CO_2) extraction is interesting as alternative technique for natural extraction since it has advantages over the conventional extraction solvent such as being non-toxic and requiring mild operating conditions. SC-CO_2 is appropriate for extraction of astaxanthin, a very strong antioxidant, from *Haematococcus pluvialis*, and in this study, the experimental design was therefore used to evaluate the main and the interaction effects on the performance of SC-CO_2, measured in terms of astaxanthin yield and antioxidant activity of the extract. The investigated factors were the operating temperature (40-80 °C), pressure (300-500 bar), and extraction time (1-4 hours). Here, the astaxanthin yield was referred as percent yield which was defined as the percentage of the total amount of astaxanthin that was extracted. The antioxidant activity of the extract was here measured in terms of IC50, the concentration of the extract that gives a 50% reduction in the absorbance of the free radical, ABTS [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)]. Table 4.1 shows the factors and levels tested for the designed experiment while the experimental matrix and all experimental results are shown in Table 4.2. From the table, the maximum yield (83.16 %) was obtained at 80 °C, 500 bar, and after 4 hours of extraction. This was 22.84 mg/g of algae which was comparable to the amount of extract reported in previous study (Machmuda, 2006) in which the astaxanthin extracted was 26.72 mg/g, or equivalent to the recovery of 77.9 %, which were obtained at 70 °C, 550 bar, and 4 hours. For antioxidant activity, a reciprocal of the IC_{50} is alternatively used to indicate the antioxidant activity of the extract. The higher value of 1/IC50 indicates the higher antioxidant activity of the extract. From the experimental results, the highest activity ($1/IC_{50} = 0.564$) was found at 80 °C, 300 bar, and after 1 hour, and the lowest activity ($1/IC_{50} = 0.333$) was found at 40 °C, 300 bar, and after 1 hour of extraction.

Table 4.1 Factors and levels tested in the design of 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

Table 4.2 Experimental matrix and all experimental results for the design of experiment (n=3).

Run	Temp (X_1)	Press (X_2)	Time (X_3)	Astaxanthin (mg/0.5 g)	% yield	IC_{50} (mg/l)	$\frac{1}{IC50(mg/l)}$
1	1	1	1	11.42	83.16	2.37	0.422
2	1	1	-1	10.34	75.33	2.74	0.365
3	1	-1	1	8.71	63.41	2.02	0.496
4	1	-1	-1	5.89	42.89	1.77	0.564
5	-1	1	1	10.70	77.92	1.82	0.550

6	-1	1	-1	9.80	71.33	2.71	0.369
7	-1	-1	1	10.66	77.64	2.56	0.391
8	-1	-1	-1	7.89	57.48	3.01	0.333
9	1	0	0	9.83	71.60	2.12	0.473
10	0	1	0	10.15	73.88	2.23	0.448
11	0	0	1	11.14	81.16	2.97	0.337
12	-1	0	0	9.13	66.48	2.14	0.468
13	0	-1	0	8.94	65.14	2.06	0.486
14	0	0	-1	8.99	65.43	2.50	0.401
15	0	0	0	10.99	80.06	2.28	0.439
16	0	0	0	10.11	73.65	2.63	0.380
17	0	0	0	9.90	72.10	2.74	0.365

4.1.1 Statistical analysis of astaxanthin yields

From the experimental results, the statistical analysis, or in particular, the analysis of variance (ANOVA) was used to determine the factors that have important effects on the astaxanthin yield. The more details of this method were described in Chapter 2. The analysis results obtained using a statistical program, SPSS 9.0, are shown in Table 4.3.

Table 4.3 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)

From Table 4.3, it was found that at the confidence interval of 90% ($p<0.1$), the factors that have significant effects on the astaxanthin yields were the operating pressure, the extraction time, and the interaction between operating temperature and pressure. Each of these effects is described in more detail as follows.

4.1.1.1 Main effect of operating pressure to astaxanthin yields

Figure 4.1 shows the main effect of operating pressure to astaxanthin yields and the results show that the astaxanthin yields were higher when the pressure was operated in the range of 300-500 bar. This agrees generally with theory, which relates the dissolving power of SC-CO_2 in with its density, or the probability of interaction between supercritical solvent and solute.

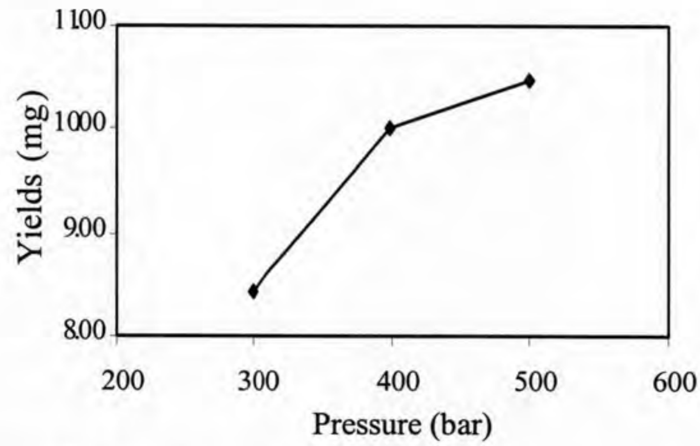

Figure 4.1 Main effect of operating pressure to astaxanthin yields

4.1.1.2 Main effect of extraction time to astaxanthin yields

The main effect of the extraction time to the astaxanthin yields is shown in Figure 4.2. The results show that the astaxanthin yields were higher with increasing extraction time between 1-4 hours. This is because increasing the time of extraction increases the contact time between the supercritical solvent and the solute.

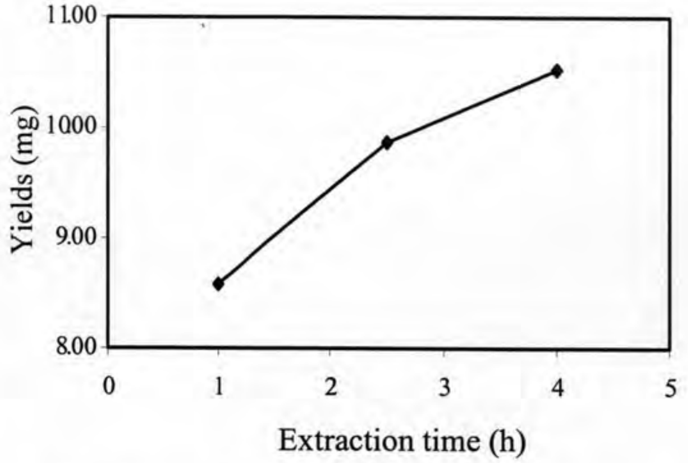

Figure 4.2 Main effect of extraction time to astaxanthin yields

4.1.1.3 Interaction effects of temperature and pressure on astaxanthin yield

The statistical analysis of the experimental results shows that the interaction between operating temperature and operating pressure affect significantly the astaxanthin yield. The interaction effect between the operating temperature and the operating pressure is plotted in Figure 4.3. The result shows that at pressure 300 bar, the extraction yields were lower when the temperature increased. While at the pressure of 400 and 500 bar, the extraction yields were slightly higher and sharply higher when the temperature increased. The reason of this observation is that the solubility of organic compound depends on a complex balance between supercritical fluid density and solute vapor pressure, which are both controlled by the fluid pressure and temperature.

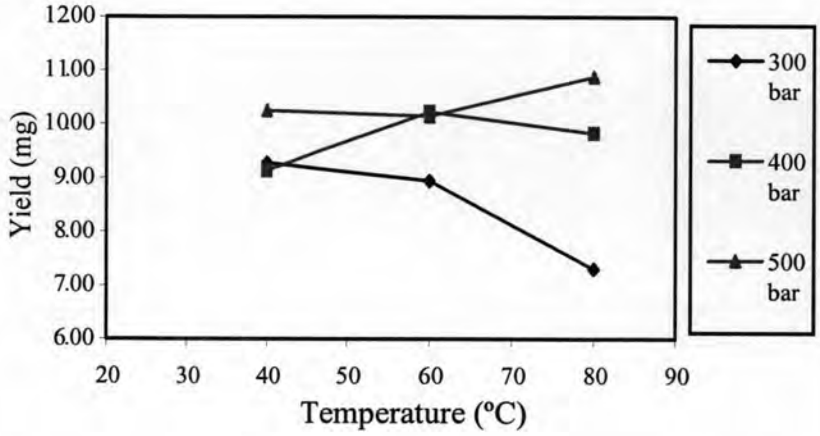

Figure 4.3 Interaction effect between temperature and pressure for astaxanthin yields

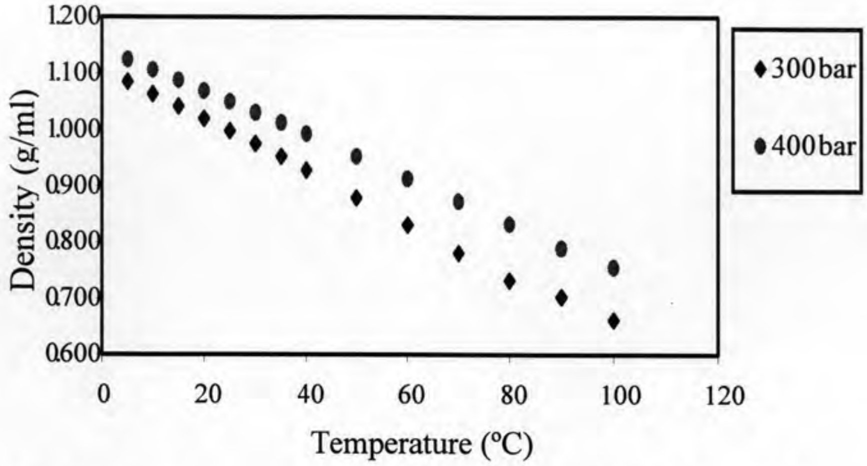

Figure 4.4 Density of pure carbon dioxide calculated by P-R EOS (Mamata, 2000)

The change in solvent density in response with the change in temperature and pressure can be seen in Figure 4.4. From this figure, it is seen that when the pressure increases, the supercritical fluid density increases. However, the temperature increase although causing a decrease in the fluid density, could be responsible for the increase in the solute vapor pressure. In other words, the solubility of the solute may increase, remain constant or decrease with increase in the temperature at a constant pressure, depending on whether the solute vapor pressure or the solvent density is the predominant factor. 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.

4.1.2 Statistical analysis of extract antioxidant activity

The experimental data of antioxidant activity of the extracts obtained at different SC-CO_2 operation conditions were analyzed using ANOVA and the results obtained using a statistical program SPSS 9.0 are shown in Table 4.4.

Table 4.4 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)

From the statistical analysis above, the factors that affects the extract antioxidant activity was the interaction between operating temperature and pressure. This effect can be explained as follows.

4.1.2.1 Interaction effects of operating condition to extract antioxidant activity

The statistical results from the data on antioxidant activity of the extracts show that the interaction effects between temperature and pressure was a significant factor. The interaction effect between the operating temperature and the operating pressure is shown in Figure 4.5. When temperature increased, the extracts antioxidant activities increased at lower pressure of pressure 300 bar, while the extract antioxidant activities decrease with increasing temperature at higher pressure of 500 bar. This result was opposite to what was observed for the astaxanthin yield in which higher yield was obtained at lower temperature when the pressure was low, while the yield decreased with increasing temperature when extraction pressure was high. The interaction between temperature and pressure of the supercritical fluid on the antioxidant activity of the extract is rather complex and from the findings of this study, no direct relationship between the amount of astaxanthin extracted and the antioxidant activity of the extract could be made.

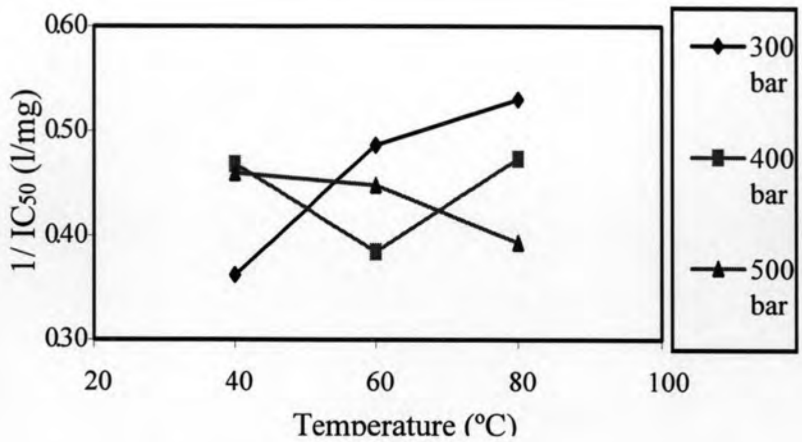

Figure 4.5 Interaction effect between temperature and pressure on antioxidant activity of the extract

4.2 Optimal condition for SC-CO_2 extraction of *H. pluvailis*

4.2.1 Optimal condition of astaxanthin yields

The relations between each factors and the extract yields were modeled with a response surface using a 2nd order polynomial model. Using the statistical program SPSS 9.0 and the analysis results obtained are summarized in Table 4.5, 4.6, and 4.7.

Table 4.5 Model summary for astaxanthin yields

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.961[a]	.924	.827	.5665

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

Table 4.6 ANOVA Table for optimal condition of 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

Table 4.7 Coefficients for response surface equation for astaxanthin yields.

Model		Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	t	Sig.
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

Therefore, the response surface equation obtained from the analysis is as follows.

$$Y = 10.107 - 0.199X_1 + 1.032X_2 + 0.972X_3 - 0.458X_1^2 - 0.393X_2^2 + 0.127X_3^2 + 0.651X_1X_2 + 0.0288X_1X_3 - 0.451X_2X_3 \quad (4.1)$$

where Y is the astaxanthin yields, X_1, X_2, and X_3 are the operating temperature, the operating pressure and the extraction time, respectively. The response surface of astaxanthin extract is shown in Figure 4.6 and the plot of observed value and predicted value is shown in Figure 4.7 which indicates that the model gives good prediction of the experimental results.

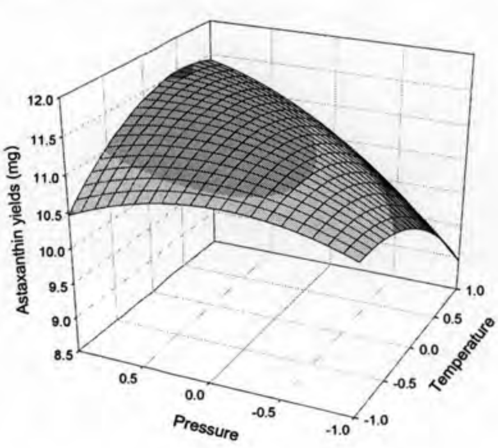

Figure 4.6 Response surface of astaxanthin yields

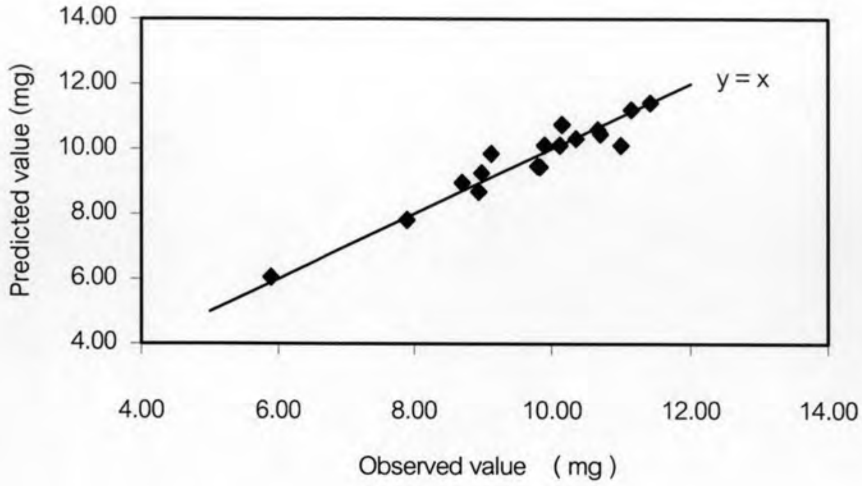

Figure 4.7 Observed value vs. predicted value of astaxanthin yields model

From the response surface equation for astaxanthin yields, the optimal condition of this extraction can be determined by taking the partial derivative of the equation with respect to each of the three variables, and set these partial derivatives equal to 0, and solve the equation. The resulting equations are:

$$dY / dX_1 = 0, \quad 0 = -0.199-0.458X_1 + 0.651X_2 + 0.0288X_3 \qquad (4.2)$$

$$dY / dX_2 = 0, \quad 0 = 1.032 + 0.651X_1 - 0.393X_2 - 0.451X_3 \qquad (4.3)$$

$$dY / dX_3 = 0, \quad 0 = 0.972 + 0.0288X1 - 0.451X_2 + 0.127X_3 \qquad (4.4)$$

The solutions of these equations yield the optimal condition which was 90 °C, 640 bar, 2.9 hours of extraction, which yields the highest amount of astaxanthin of 22.66 mg/g or 82.40 % yield. This condition was the extrapolated result and therefore would not be accurately predicted. Thus the optimal condition was proposed within the range of this experiment to be at the temperature of 70 °C, the pressure of 500 bar, and the extraction time of 4 hours, which yielded the amount of astaxanthin extract of 23.04 mg/g dry algae or 83.78 %wt.

4.2.2 Optimal condition of extract antioxidant activity

The analysis of the relationship between the factors and the extract antioxidant activity given by SPSS statistical program is summarized in Figure 4.8, 4.9, and 4.10

Table 4.8 Model summary for extract antioxidant activity

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.920[a]	.846	.648	4.16E-02

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

Table 4.9 ANOVA Table for optimal condition of 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 4.10 Coefficients for response surface equation for extract antioxidant activity

		Unstandardized Coefficients		Standardized Coefficients		
Model		B	Std. Error	Beta	t	Sig.
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 this analysis, the response surface equation is obtained as follows:

$$Y = 0.413 + 0.0209X_1 - 0.0116X_2 + 0.0164X_3 + 0.04392X_1^2 + 0.04042X_2^2 - 0.0576X_3^2 - 0.0585X_1X_2 - 0.0313X_1X_3 + 0.0310X_2X_3 \quad (4.5)$$

where Y is the extract antioxidant activity, expressed in term of $1/IC_{50}$, X_1, X_2, and X_3 are the operating temperature, the operating pressure and the extraction time, respectively. The response surface for antioxidant activity is shown in Figure 4.8 and the plot of the observed and the predicted value are shown in Figure 4.9.

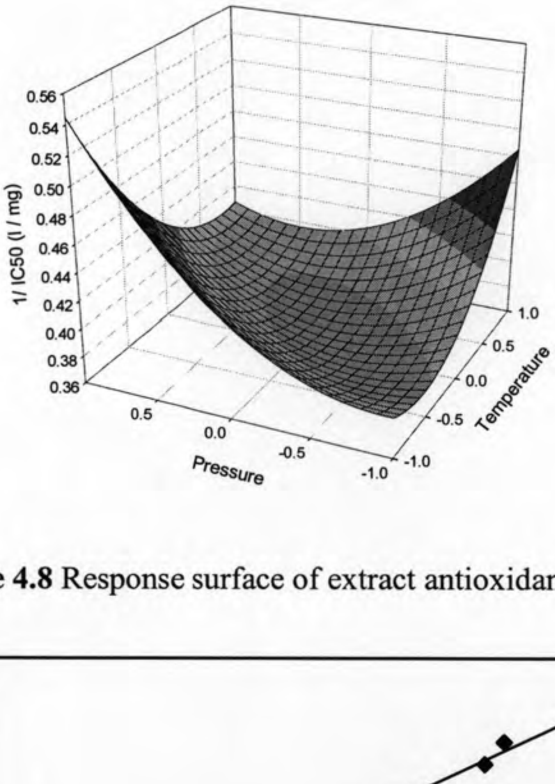

Figure 4.8 Response surface of extract antioxidant activity

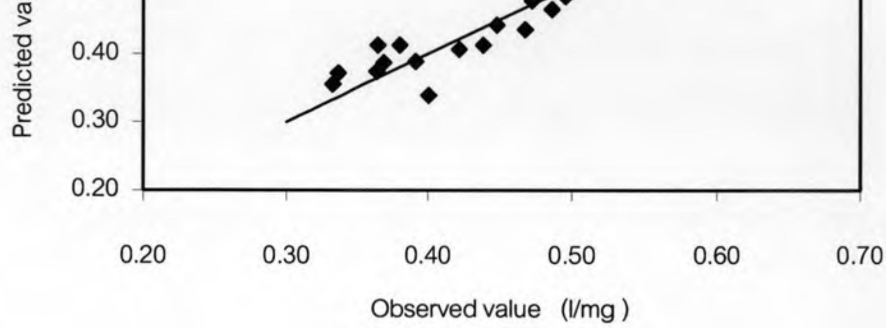

Figure 4.9 Observed value vs. predicted value of extract antioxidant activity model

In this case the solutions for the following equations gave a minimum point for antioxidant activity.

$$dY / dX_1 = 0, \quad 0 = 0.0209 + 0.04392X_1 - 0.0585X_2 - 0.0313X_3 \tag{4.6}$$

$$dY / dX_2 = 0, \quad 0 = - 0.0116 - 0.0585X_1 + 0.04042X_2 + 0.0310X_3 \tag{4.7}$$

$$dY / dX_3 = 0, \quad 0 = 0.0164 - 0.0313X1 + 0.0310X_2 - 0.0576X_3 \tag{4.8}$$

The minimum point of antioxidant activity, or $1/IC_{50}$, was found to be at 50 °C, 354 bar, and 2.68 hours of extraction. At this point, the IC_{50} value was 2.18 mg/l. The experimental results obtained here did not suggest the optimal conditions in which the highest antioxidant activity was achieved. In this regards, the optimal conditions for extraction would be recommended to be at the conditions for maximizing astaxanthin yield, which was found in the previous section. At this condition, the antioxidant activity was found based on the response surface equation to be 1.83 mg/l. Comparison of this value with those obtained in the experimental results, this value indicates that the extract has a reasonably high antioxidant activity.