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OPTIMISATION OF SUPERCRITICAL FLUID EXTRACTION OF ASTAXANTHIN FROM *PENAEUS MONODON* WASTE USING ETHANOL-MODIFIED CARBON DIOXIDE

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Abstract

Some studies demonstrated that astaxanthin surpasses the antioxidant benefits of beta-carotene, zeaxanthin, canthaxanthin, vitamin C, and vitamin E. *Penaeus monodon* (Tiger shrimp) is one of the most valuable traded crustacean products in which astaxanthin can be found in its by-products. The extraction of thermolabile compound like carotenoids at lower temperatures through supercritical carbon dioxide (SC-CO_2) can reduce the potential isomerization and degradation of the extraction product. In this study, astaxanthin had been extracted using SC-CO_2 with 15% (v/v) ethanol as an entrainer and the recovered astaxanthin was analyzed using High performance liquid chromatography (HPLC). A central composite design (CCD) was employed to study the effect of three SC-CO_2 parameters namely temperature (X_1) from 40 to 80°C, pressure (X_2) from 150 to 250 bar and extraction flow rate (X_3) from 1 to 3 ml/min on the astaxanthin complex yield, (Y_1) and free astaxanthin content, (Y_2). The nonlinear regression equations were significantly ($p<0.05$) fitted for both responses with high R^2 (>0.9261), which had no indication of lack of fit. The results indicated that a combined set of values of temperature (56.88°C), pressure (215.68 bar) and extraction flow rate (1.89 ml/min) was predicted to provide the optimum region in terms of astaxanthin complex yield, (58.50 ± 2.62 µg/g) and free astaxanthin content (12.20 ± 4.16 µg/g) studied.

Keywords: *Penaeus monodon*, astaxanthin, carotenoids, SFE, Optimization, HPLC.

1. Introduction

Astaxanthin, (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a ketocarotenoid present in seafood products, especially in shells of lobster and shrimp. Due to its attractive pink color and higher antioxidative activity than those of α-carotene, β-carotene, lutein, lycopene, canthaxanthin and vitamin E [1, 2], it was applied in pharmaceutical, cosmetic, food industries and as an important source of pigmentation in aquaculture industries [3, 4]. Several authors had reported that astaxanthin exert a protective effect against chronic diseases such as cancer [5, 6].

Nowadays, shrimp is the world's most valuable seafood product, accounting for about 15 percent of the total value global fisheries products in 2010 [7]. The increase in shrimp export was achieved mainly by a surge in production from aquaculture mostly contributed by the native shrimp species, the Tiger shrimp (*Penaeus monodon*) [8]. The global production of this species reached 600 000 tonnes/yr [8].*Penaeus monodon* is endemic and found in the waters of Malaysia [9]. In *Penaeus monodon*'s waste, astaxanthin is the major carotenoid and exists mainly as astaxanthin esters of various fatty acids [10, 11]. During processing of shrimps to human food, 30% of the total weight was wasted [12]. The recovery of astaxanthin from the waste would not only improve the economy for shrimp processors, but also help minimize the potential pollution of the shrimp waste. As a source of natural antioxidants, it is considered completely safe in comparison with synthetic antioxidants.

The wide scale use of organic solvents by a diverse range of global industries represents a serious threat to the environment. Supercritical fluid extraction (SFE) process is an eco-friendly alternative to the use of organic solvents in extraction. The emerging stricter environmental regulations concerning the use of common industrial solvents, most of which are harmful to human health, have stimulated the growth of the SFE technologies. Supercritical fluids are advantageous due to simplicity and less degradation of thermolabile compounds [13]. Compared to the conventional extraction, SFE requires less time due to higher mass transfer rates in supercritical fluids than in liquid solvents. However, one shortcoming of SC-CO_2 is that it often incapables of extracting slightly polar analytes from solid matrices due to its low solvating power and weak interaction with the matrices [14]. For extraction of astaxanthin, the use of polar extrainers was required to substantially improve the extraction efficiency of CO_2 by raising the solubility of the analytes and reducing their interaction with the matrix [15-17].

Other than that, it was found that reports regarding the extraction of astaxanthin from crustacean by-products using SFE were still very limited; in which most of the studies only focused on extraction of this carotenoid from the microalgae (*Haematoccocus pluvialis*) and the influences of temperature, pressure and extraction flow rate on the extraction yields have been vaguely studied. Therefore, through this study, we can update on some existing data, and also to produce new data on carotenoids content of the said crustacean waste from Malaysia. The objective of this study was to determine the effect of Supercritical Fluid Extraction with 15% (v/v) ethanol as co-solvent on the amount of astaxanthin extracted and astaxanthin content (free astaxanthin) and to optimize the processing procedure to extract astaxanthin from Tiger Shrimp waste. The variables studied were pressure, temperature and extraction flow rate.

2. Materials and Methods

2.1. Materials and reagents

Standard (3s, 3's) – Astaxanthin of 96.0% purity was purchased from Sigma-Aldrich Co (St. Louis, MO, USA). The ethanol of analytical grade, 100% acetonitrile and ethyl acetate of HPLC grade were obtained from MERCK, (Frankfurt, Darmstadt, Germany). Commercial grade liquefied carbon dioxide (purity, 99.7%), supplied in cylinder with dip tube was purchased from Gas Pantai Timur (Bangsar, Kuala Lumpur, Malaysia). The column used was a ZORBAX Eclipse XDB-C18 end capped 5 µm, 4.6 x 150mm reverse phase column (Agilent Technologies, USA).

2.2. Sample preparation

Sample of fresh tiger shrimps (*P. monodon*) (50-60 counts kg^{-1}) were obtained from Tanjung Bidara Beach, Malacca. The shrimps were transported in an expanded polystyrene box to the laboratory under iced condition (-4°C). The shrimp's waste was freeze-dried using a freeze dryer (FDU-2100, Tokyo Rikakikai Co., Ltd, Japan) for 72 hours and then grounded for approximately 15-20 minutes using a grinder (Multifunction disintegrator SY-04, Golden Bull). Later, the sample was kept at -80°C until further analysis.

2.3. Experimental design using response surface methodology (RSM)

The central composite design (CCD) was applied in the experimental design to study the responses, namely astaxanthin yield, Y_1 and free astaxanthin content, Y_2. The independent variables were X_1, X_2 and X_3 representing temperature, pressure, and extraction flow rate. The following were the settings for the independent variables (low/high values): temperature (40-80°C), pressure (150-250 bar) and extraction flow rate (1-3 ml/min). Each variable was coded at three levels: -1, 0, +1, giving them a range of 40-80°C, 150-250 bar and 1-3 ml/min, respectively, (Table 1). Sample was held in static extraction for 15 minutes, followed by a dynamic extraction for 120 minutes. Six replicate runs at the centre point (0, 0) of the design were considered in order to allow the estimation of the pure error. The experimental design was developed to (i) find a relationship between each response and three independent variables and (ii) to determine the optimum level of the independent variables which was our main goal. The experimental design and data analysis were carried out using response surface methodology with the Design Expert Software version 6.0.8 (Stat Ease, USA).

Table 1. Levels of independent variables established according to the CCD.

		Level		
Independent variables	Coded Factor	Low (-1)	Med (0)	High (1)
Temperature (° C)	X_1	40	60	80
Pressure (bar)	X_2	150	200	250
Extraction flow rate (ml/min)	X_3	1	2	3

2.4. Statistical analysis

For predicting both responses, namely Y_1 and Y_2, a quadratic polynomial regression model was assumed. Equation 1 has been proposed for each responses of Y.

$$Y = b_0 + \sum_{i=1}^{3} biXi + \sum_{i=1}^{3} \sum_{j=1}^{3} bijXiXj \quad (1)$$

where Y is the predicted response, X_i is the coded values of the factor, 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 model was evaluated using coefficient of determination (R^2) and the variables inside the experimental domain were analyzed by analysis of variance (ANOVA). The fitted full quadratic polynomial equations were obtained by holding one of the independent variables at a constant value and changing the level of the other variables to develop the contour plots [18].

2.5. Verification of model

The experiments for optimum point were done in triplicate and each set of yields were averaged. Then the results were tested by two sample *t*-test at the least significant difference of 95% confidence level for each yield and individual astaxanthin content using MINITAB release 16 software.

2.6. Supercritical fluid extraction

The SFE system includes a solvent pump (Series III, Lab Alliance, USA), a CO_2 pump (PU-2080, JASCO Corporation, Japan), a back-pressure regulator (BPR) (BP-1580-81, JASCO Corporation, Japan), an extractor vessel enclosed in an air-circulating oven (FX2-2, Sheldon Manufacturing Inc., USA), a pressure transmitter (682-8, Dwyer Instrument, Inc., USA) and a sample collector. In order to maintain its liquid state before it was pumped to the extractor, the CO_2 was chilled to -2°C using a chiller (Protech Electronic, Malaysia). The extractor consists of a high pressure stainless steel vessel that was filled with 5.00 ± 0.02 g of the freeze-dried sample. At both inlet and outlet of the vessel, glass wool was used to prevent any possible carryover of solid material. A static period of 15 min was used to allow contact between the sample and supercritical solvent, followed by a dynamic extraction for 120 minutes. The flow rate of the ethanol used as co-solvent was adjusted to desired concentration of 15% (v/v) ethanol to CO_2. With the purpose of sustaining the system pressure, a back-pressure regulator was employed while the needle valves controlled the flow of the supercritical fluid extraction process. The extraction product was collected together with ethanol at the end of the experiment through a sample collector in a previously weighed bottle, which was wrapped in aluminium foil and kept refrigerated (5°C) during and after extraction in order to avoid oxidation of the components extracted. Later, the collection bottle was kept under refrigeration (-20°C) in the dark prior to analysis.

2.7. Astaxanthin yield quantification

For the determination of the astaxanthin yield, the established method of Simpson and Haard [19] as explained in detailed by Sachindra et al. [12] has been employed in this study. The SFE extract was dried to constant weight under a gentle stream of oxygen-free nitrogen. Then the dry extract was diluted in a known amount of petroleum ether and the amount of astaxanthin was quantified by absorbance measured at 468 nm using Varian Cary 50 UV-Vis spectrophotometer. The carotenoid yield (CY) was calculated as astaxanthin using Eq. (2) [19]:

$$CY(\mu g \text{ astaxanthin}/g \text{ sample}) = \frac{(A_{468nm})(V_{extract})(\text{Dilution factor})}{0.2(W_{sample})} \quad (2)$$

where A is absorbance, V is volume of extract, 0.2 is the A468 of 1 l µg/ml of standard astaxanthin and W is weight of sample in gram.

2.8. Free astaxanthin identification and quantification

The carotenoid extracts obtained were passed through a filter of 0.45 µm pore size and injected in HPLC (Agilent model 1200 series) comprising of a binary pump with autosampler injector, micro vacuum degassers, thermostatted column compartment and a diode array detector in accordance to the established methods described in detail previously [20]. The column used was a ZORBAX Eclipse XDB-C18 end capped 5 µm, 4.6x150 mm reverse phase column (Agilent Technologies, USA). The solvents used are (A) acetonitrile: water (9:1 v/v) and (B) ethyl acetate. The solvent gradient used was developed as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 ml/min. The identification of carotenoids was done by comparing the retention times with the astaxanthin standard purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Astaxanthin standard with five different concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 µg/ml) was injected separately and detected to obtain the data for standard curve with $R^2 = 0.995$. The standard curve was positioned on a peak area-astaxanthin concentration matrix, in which the vertical axis was peak area (mAU/ second) while the horizontal axis was different standard (3s, 3's) – Astaxanthin concentration (µg/ml). The carotenoid concentration was calculated by comparing its relative proportion, as reflected by integrated HPLC peak areas, to total carotenoid content determined by spectrophotometry.

3. Results and Discussion

3.1. Model fitting

Table 2 shows the matrix of optimization. The experiments were performed in random order. The ranges of the parameter were selected based on the preliminary study of the extraction using SC-CO_2 and ethanol as co-solvent. Response surface analysis initiated the development of the polynomial regression relationship where each response variable (Y_i) was determined as a function of temperature (X_1), pressure (X_2) and extraction flow rate (X_3). The estimated regression coefficients of the response models together with the corresponding R^2 values and lack of fit tests were presented in Table 3.

There was a significant (p<0.05) regression relationship between the independent variables (temperature, pressure and extraction flow rate) and the responses (astaxanthin yield and free astaxanthin content). Table 3 shows high R^2 values ranging from 0.9261 to 0.9705 obtained by the response surface analysis. This indicated that at least 95% of the variation of the response variables could be precisely described by the regression models relating the independent variables and responses. This finding successfully demonstrated a satisfactory fitness of the response surface models applied for explaining the response variations as a function of three independent variables (temperature, pressure and extraction flow rate) (Table 3). The generated models significantly illustrated the actual relationships among the reaction parameters and sufficiently explained the data variation.

As shown in Table 4, the main effects of temperature and pressure gave significant ($p < 0.05$) effects on the astaxanthin yield and free astaxanthin content. The full quadratic regression model demonstrated the highest R^2 and p values for lack of fit test and the lowest p value for regression compared to other regression models. According to Table 4, temperature and pressure gave the most significant ($p < 0.05$) effect on the astaxanthin yield (Y_1) and free astaxanthin content (Y_2). Temperature and fluid pressure were found to play important role for extracting our targeted compounds. In this study, there was no interaction between independent variables on astaxanthin extraction. This is consistent with other work for supercritical extraction of astaxanthin from *Haematoccocus pluvialis* using ethanol-modified CO_2 conducted at operating temperature of 40-70°C and pressure of 30-55 MPa [21].

Table 2. Matrix of the CCD.

Run	Temperature (°C)	Pressure (bar)	Extraction flow rate (ml/min)	Astaxanthin yield (µg/g)	Astaxanthin content (µg/g)
1	40	150	1	30.933	6.518
2	80	150	1	16.941	1.709
3	40	250	1	36.805	8.322
4	80	250	1	23.971	4.791
5	40	150	3	27.951	7.392
6	80	150	3	10.007	2.000
7	40	250	3	34.290	8.119
8	80	250	3	23.864	3.802
9	40	200	2	33.900	7.501
10	80	200	2	19.133	5.899
11	60	150	2	43.090	9.106
12	60	250	2	50.073	11.220
13	60	200	1	53.918	10.006
14	60	200	3	59.952	11.009
15*	60	200	2	60.313	12.914
16*	60	200	2	58.904	12.882
17*	60	200	2	59.851	12.807
18*	60	200	2	60.422	12.866
19*	60	200	2	52.601	9.021
20*	60	200	2	60.779	12.611

*Center point for central composite design (CCD)

Table 3. Regression coefficients, R^2, adjusted R^2, probability values, and lack of fit for two dependent variable[a].

Regression coefficients	Astaxanthin yield (Y_1)	Astaxanthin content (Y_2)
b_0	56.89376	11.84486
b_1	-6.9963	-1.9651
b_2	4.0081	0.9529
b_3	-0.6504	0.0976
b_{1^2}	-27.5004	-4.63691
b_{2^2}	-7.43541	-1.17391
b_{3^2}	2.918091	-0.82941
b_{12}	1.0845	0.294125
b_{13}	-0.193	-0.17113
b_{23}	0.91175	-0.29463
R^2	0.970458	0.926123
R^2 (adj)	0.943869	0.859634
Regression (*P* value)	< 0.0001[b]	< 0.0002[b]
lack of fit (*P* value)	0.1805[c]	0.7590[c]

[a]Key: b_i the estimated regression coefficient for the main effects; b_{ii}, the estimated regression coefficient for the quadratic effects; b_{ij}, the estimated regression coefficient for the interaction effects; 1, temperature, 2, pressure and 3, extraction flow rate. [b]significant (p<0.05). [c]Not significant (p>0.05)

Table 4. *F* ratio and *p* value for each independent variable effects in the polynomial response surface models[a]

Variables		Main effects			Quadratic effects			Interaction effect		
		X_1	X_2	X_3	X_1^2	X_2^2	X_3^2	X_1X_2	X_1X_3	X_2X_3
Astaxanthin yield (Y_1)	*p*	< 0.000[b]	0.011[b]	0.623	< 0.000[b]	0.012[b]	0.260	0.466	0.895	0.539
	F	29.852	9.798	0.258	126.840	9.272	1.428	0.574	0.018	0.406
Astaxanthin content (Y_2)	*P*	< 0.001[b]	0.049[b]	0.824	< 0.000[b]	0.180	0.333	0.552	0.728	0.551
	F	21.156	4.975	0.052	32.393	2.076	1.036	0.379	0.128	0.380

[a]Key: X_1, X_2 and X_3 the main effects of pressure, temperature and extraction flow rate, respectively. X_1^2, X_2^2 and X_3^2 the quadratic effects of pressure, temperature and extraction flow rate, respectively. X_1X_2, X_1X_3 and X_2X_3 the interaction effects of pressure, temperature and extraction flow rate. [b]Significant at p<0.05.

3.2. Response analysis

3.2.1. Effect of operating temperature on astaxanthin yield and free astaxanthin content

The effects of temperature and pressure on astaxanthin yield and free astaxanthin content were presented in Figs. 1 and 2. Figure 1 shows the effect of temperature on astaxanthin yield at 40, 60 and 80°C and constant pressure of 150, 200 and 250 bar. Nobre et al. [22] reported that the solubility of the solutes is dependent upon two factors: the vapour pressure of the solutes (favoured by the temperature) and the

density of the solvent (favoured by the pressure, at contant temperature). The solubility was therefore controlled by the trade-off between these two factors.

According to Figs. 1 and 2, the amount of astaxanthin yield and free astaxanthin content increased steadily with an increase of temperature from 40-60°C. These findings illustrated that the astaxanthin yield and free astaxanthin content were affected by the solute pressure which rose with the increasing of temperature. According to Fuente et al. [23], the increase of vapour pressure with an increase in temperature will improve the solubility of astaxanthin in solvent. However, the increase in temperature may also lead to a reduction in SC-CO_2 density; which cause a decrease in solute solubility [23]. Apart from that, with the increase in temperature the mass transfer and the interaction between SC-CO_2 and the solute in the cellular matrix can be improved as this will enhance the diffusivity of the solvent.

Nevertheless, further increase in temperature to 80°C did not have significant effect on the amount of astaxanthin extracted and its free content in the extract. The extraction at this temperature seemed to degrade astaxanthin where the extraction amount went down. These result agreed well with those of other findings in the temperature range of (70-80°C) [16,24]. For an instance, Machmudah et al. [16] found that the rise of temperature from 70º C to 80°C did not increase the amount of astaxanthin extracted and its content in the microalga *Haematococcus pluvialis* extract. Through visual observation, the extraction at temperature higher than 70°C had led to excessive burnt of the sample [16].

3.2.2. Effect of operating pressure on astaxanthin yield and free astaxanthin content

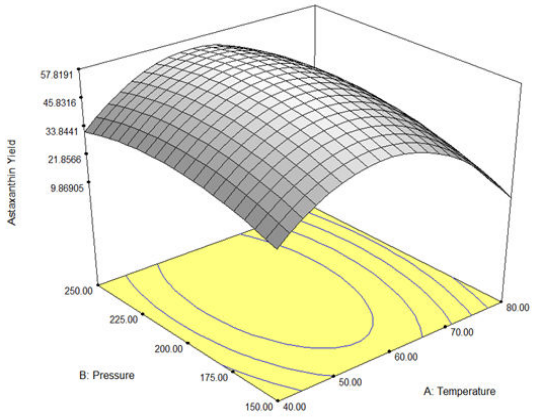
Figure 1 and Fig. 2 illustrate the effect of pressure on astaxanthin yield and free astaxanthin content. It is apparent from the results that the increase in pressure had positive effect in the amount of astaxanthin extracted and its free content for the rising of pressure from 150 to 200 bar. This is consistent with the existing literatures which reported this positive effect even at a more pronounced level [16,24,25]. The positive effect of pressure was caused by the increment in solvent power and density of CO_2 with pressure that enhanced the solubility of astaxanthin in CO_2.

Normally, because of the "cross-over" pressure, the effect of temperature in SC CO_2 extraction is a function of the extraction pressure. Generally, the cross-over pressure for natural compounds is slightly above the critical pressure of the CO_2 which is 7.3 Mpa. For carotenoids, the cross-over pressure is below 200 bar [23,26]. At near-critical pressure, where the CO_2 is highly compressible, an increase in temperature decreases the solubility of the solutes because of the decrease in density. Whereas, above the cross-over pressure, the solubility increases as temperature increases because of the more pronounced increment in solubility and volatility of the solute. In this study, the decrease in astaxanthin yield and free astaxanthin content when there was an increase in temperature from 60°C to 80°C at 250 Mpa did not agree with observations of others [15,27], who found temperature had positive effect at high pressures (even though not at low pressure) [27].

Nonetheless, this phenomenon was also observed previously in supercritical extraction of astaxanthin from *Haematoccocus pluvialis* using ethanol-modified CO_2 by Bustamante et al. [21], who obtained similar results and affirmed that the negative

effect of temperature reported may be due to the presence of oxygen in vials where the extract were kept during the experiment or in the CO_2 supplied that oxidize the astaxanthin [21]. Another factors deserving attention with respect to the oxidation of astaxanthin are the temperature and storage time [28]. When the temperature and storage time increase, the oxidation of astaxanthin increased. Armenta and Guerrero-Legarreta [28] found that 97% and 88% of natural and synthetic astaxanthin were oxidized under a combination of full light, air, and 45°C at 8 weeks of storage [28]. Astaxanthin is prone to isomerization and oxidative degradation because of the existence of long-chain conjugated double bonds [29,30]. Other than that, it was postulated that the ethanol-mediated isomerization of astaxanthin also lead to oxidation [30]. It could therefore be drawn from these results that even though temperature can increase the extraction of astaxanthin, it can also cause deterioration of the analyte during extractions, especially when using ethanol as modifier.

(a)



(b)

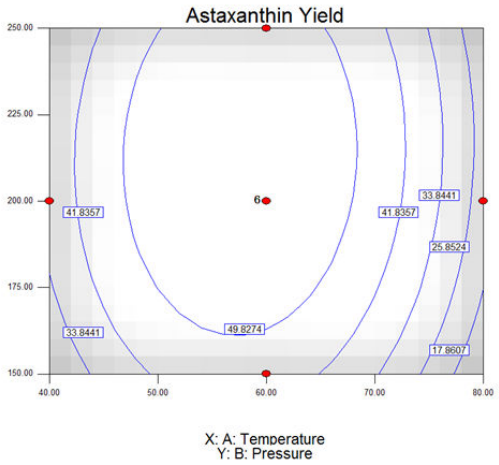
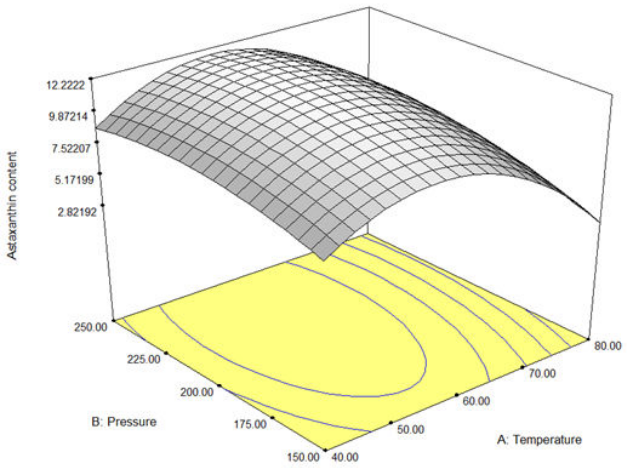


Fig. 1. (a) Response surfaces and (b) contour curves for the extraction yield of astaxanthin (µg astaxanthin/g (d.w.b)) as a function of extraction pressure (bar) and temperature (°C).

(a)



(b)

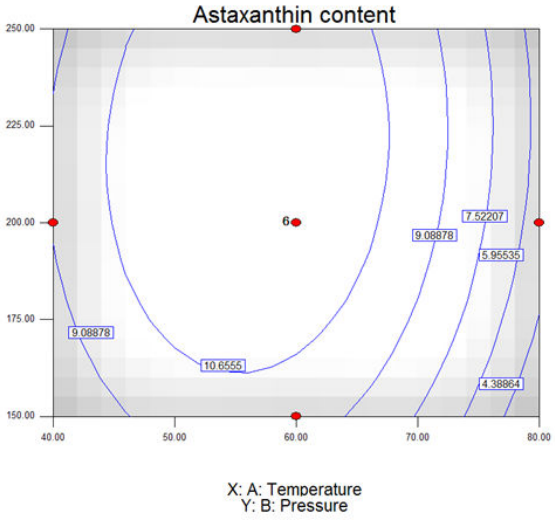


Fig. 2. (a) Response surfaces and (b) contour curves for the astaxanthin content (µg astaxanthin/g (d.w.b)) as a function of extraction pressure (bar) and temperature (°C).

3.2.3. Effect of extraction flow rate on astaxanthin yield and free astaxanthin content

Figure 5 shows the response optimization of the effects of 215.68 bar, 56.88°C and 1.89 ml/min on astaxanthin yield (µg astaxanthin/g (d.w.b)) and astaxanthin content (µg astaxanthin/g (d.w.b)). The figure also demonstrated the effect of extraction flow rate on astaxanthin yield and free astaxanthin content. For SC-CO_2 extraction, the effect of extraction flow rate depended largely on the balance between mass transfer and contact time. It would be expected that an increase in

the flow rate would increase the mass transfer. However when the flow rate increased, the contact time would be reduced. These findings indicate that the compensation between these two factors played a key role in affecting the final extraction rate of astaxanthin yield and its free content.

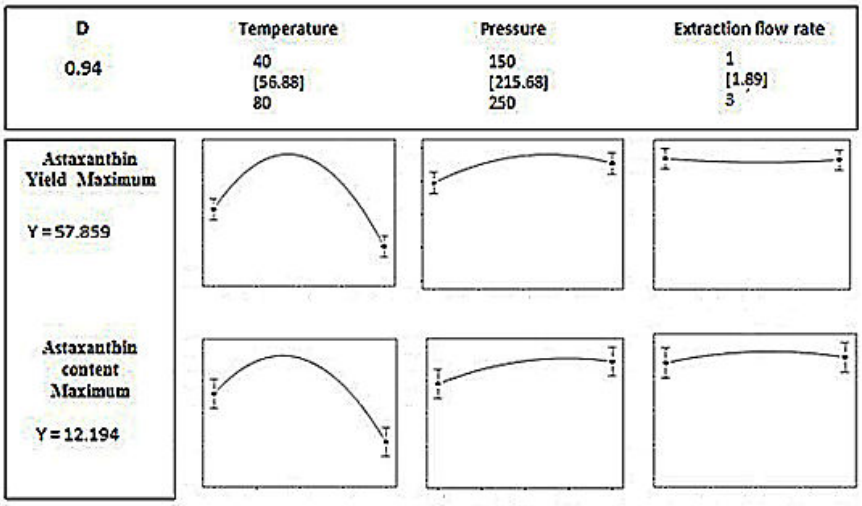


Fig. 3. Response optimization of the effects of 215.68 bar, 56.88°C and 1.89 ml/min on astaxanthin yield (µg astaxanthin/g (d.w.b)) and astaxanthin content (µg astaxanthin/g (d.w.b)).

The extraction flow rate of 1.89 ml/min gave the highest amount of astaxanthin being extracted. At flow rate of 1.89 ml/min, the residence time was sufficient to allow more effective contact between solvent and solute. In contrast, when the flow rate was increased to 3 ml/min, a shorter contacting time was involved. This lead to less viscous or pale orange astaxanthin extract was obtained due to incomplete extraction. Krichnavaruk et al. [24] showed the same trend when extracted astaxanthin from *Haematoccocus pluvialis* with 10% soybean oil as extrainer at a flow rate of 4 ml/min. Therefore, in this study it was shown that the total astaxanthin yield was negatively influenced by the extraction flow rate.

Other than that, it was also reported that normally at early stage of extraction, there was a rapid increase of solute amount in the extracted stream [24]. This could be due to the extraction of surface attached solute occurred during this stage. Meanwhile, the increase in the flow rate would slow down the external mass transfer resistance which gave rise to a faster initial rate of extraction [24]. Nevertheless at later stage, the extraction was manipulated mostly by the diffusion of solute from the cell matrix. At the same time, by increasing the flow rate, the rate of diffusion could not be controlled which resulted in almost similar rate of extraction at later stage of extraction.

3.3. Optimization condition

In order to determine an optimum set level of temperature, pressure and extraction flow rate for determining the desirable astaxanthin yield and free astaxanthin content,

the graphical and numerical optimization were performed in this study. Numerical optimization was carried out to find the exact value of multiple response optimization which leads towards the desirable goals. The overall optimum region was achieved at pressure of 215.68 bar, temperature of 56.88°C and extraction flow rate of 1.89 ml/min as shown in Fig. 3. At this optimum condition, the corresponding predicted response value for astaxanthin yield is 57.8587 µg/g and free astaxanthin content is 12.1944 µg/g respectively. The closeness between the predicted and experimental values confirmed the accuracy of the response surface models fitted to the experimental data.

3.4. Verification of the model

Table 5 illustrates the experimental and predicted values for both responses. The experimental response values were observed to be in good agreement with the predicted values. It was shown that there are no significant (p>0.05) difference between the experimental and predicted values. The corresponding experimental value for astaxanthin yield and free astaxanthin content were 58.50 ± 2.62µg/g and 12.20 ± 4.16µg/g.

The operating condition of extraction, including temperature, pressure and extraction flow rate for maximizing astaxanthin complex yield and free astaxanthin content were calculated by using the predictive equation simulated from RSM. After extraction at the optimum condition, the actual determination of astaxanthin complex yield and free astaxanthin content was carried out by UV-visible spectrophotometer and HPLC analysis. Later, the results were compared to the predicted value.

Table 5. Experimental and predicted values for the response variables[a].

Run	Astaxanthin yield[b] (µg/g)			Astaxanthin content[b] (µg/g)		
	Y_o	Y_i	Y_o-Y_i	Y_o	Y_i	Y_o-Y_i
1	30.933	30.318	0.615	6.518	5.948	0.570
2	16.941	14.542	2.399	1.709	1.771	-0.062
3	36.805	34.342	2.463	8.322	7.854	0.468
4	23.971	22.904	1.067	4.791	4.855	-0.064
5	27.951	27.580	0.371	7.392	7.074	0.318
6	10.007	11.032	-1.025	2.000	2.214	-0.214
7	34.290	35.250	-0.960	8.119	7.803	0.316
8	23.864	23.041	0.823	3.802	4.118	-0.316
9	33.900	36.390	-2.490	7.501	9.173	-1.672
10	19.133	22.397	-3.264	5.899	5.243	0.656
11	43.090	45.450	-2.360	9.106	9.718	-0.612
12	50.073	53.466	-3.393	11.22	11.624	-0.404
13	53.918	60.462	-6.544	10.006	10.918	-0.912
14	59.952	59.161	0.790	11.009	11.113	-0.104
15	60.313	56.894	3.419	12.914	11.845	1.069
16	58.904	56.894	2.010	12.882	11.845	1.037
17	59.851	56.894	2.957	12.807	11.845	0.962
18	60.422	56.894	3.528	12.866	11.845	1.021
19	52.601	56.894	-4.293	9.021	11.845	-2.824
20	60.779	56.894	3.885	12.611	11.845	0.766

[a]Y_o experimental value; Y_i predicted value; and Y_o-Y_i residue. [b]No significant (p>0.05) difference between experimental (Y_o) and predicted (Y_o) values.

4. Conclusions

These findings highlighted the potential of Supercritical Fluid Extraction (SFE) of astaxanthin from shrimp waste to be further developed for pilot scale production. Within the explored experimental region, the best extraction condition of SC-CO_2 with 15% (v/v) ethanol as an entrainer are 215.68 bar, 56.88°C and 1.89 ml/min, that allowed recovering of 58.50 ± 2.62 µg/g astaxanthin yield and 12.20 ± 4.16 µg/g free astaxanthin content for 120 min extraction time. The analysis of variance (ANOVA) demonstrated that temperature and pressure had a significant effect on the astaxanthin yield and free astaxanthin content. For commercialization purpose, results from this study can be employed as a guide for a pilot scale production of astaxanthin from shrimp by-products using SFE.

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