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Optimisation of ultrasound-assisted extraction of natural pigment from annatto seeds by response surface methodology (RSM)

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ABSTRACT

The present study reports on the extraction of natural pigment from annatto seeds. Response surface methodology (RSM) was used to investigate the effect of process variables on the ultrasound-assisted extraction (UAE). Four independent variables including temperature (20–80 °C), sonication time (2–10 min), duty cycle (0.2–0.8 s) and the ratio of seeds to the solvent (5–20%) were studied. According to the results, the optimal UAE condition was obtained with a temperature of 72.7 °C, extraction time of 7.25 min, the ratio of seed to solvent of 14% and duty cycle of 0.8 s. At these conditions, extraction yield determined as 6.35% and the absorbance value as 0.865%. The experimental values under optimal condition were in good consistent with the predicted values, which suggested UAE is more efficient process as compared to conventional extraction.

1. Introduction

Colour is the first notable characteristic of a food and often predetermines our expectation. Demand increased for more varieties and more-stable and vivid colours, as more and more processed foods fed our growing nation. Refrigeration, canning, dehydration, smoking, bottling, and exposure to light, air, moisture and temperature extremes all tend to alter the natural colour, making colour additives a hot commodity to restore expectations. Nowadays, public concern about the safety of synthetic colourants is increased; therefore, natural pigments attract more attention, which can be served as a functional component besides creating desirable colour in food products (Li, Zhang, & Liu, 2013). Natural colourants are considered GRAS because most of them are non-allergic, biodegradable, non-toxic and non-carcinogenic (Sinha, Chowdhury, Saha, & Datta, 2013; Sinha, Saha, & Datta, 2012).

Annatto extract refers to a series of colourant preparation consisting of Carotenoid-type pigments, extracted from outer coating of the seeds of the tropical bush *Bixa orellana* L. (Rajendran, 1990). Among all natural colourants, annatto ranks second in economic importance worldwide, and is widely used in the food and cosmetic industries (Lauro, 1991). The annatto seed extract (ASE) also has documented antioxidant and antimicrobial properties (Kurniawati, Soetjipto, & Limantara, 2007).

The carotenoid cis-bixin, also known as 9′cis-bixin or α-bixin (oil soluble) is the main component of the pericarp extract, comprising up to 80% of the weight and the remaining 20% includes norbixin (alkaline water soluble), several apo-carotenoids and volatile compounds (Galindo-Cuspinera, Westhoff, & Rankin, 2003; Lauro, 1991). ASE has been considered safe human consumption, since it has been used as a food colourant by peoples in Latin America for many centuries. Indeed, it has been reported that annatto extract does not exert any genotoxicity, subacute and chronic toxicity, reproductive toxicity or carcinogenicity and has been employed in the food industry as an important colourant, mainly in dairy products such as cheese and butter (Henry, 1996).

Traditional methods of colour extraction such as maceration are very time-consuming and require relatively large quantities of solvents. Nowadays, various modern extraction methods have been developed for the extraction of active components from plants, such as ultrasonic-assisted extraction (UAE), supercritical fluid extraction, enzymatic extraction, and dispersive liquid–liquid microextraction (Salar Bashi, Mortazavi, Rezaei, Rajaei, & Karimkhani, 2012; Wang & Weller, 2006).

Compared to some other extraction techniques such as microwave-assisted extraction and supercritical fluid extraction, the ultrasonic device is less expensive and much easier in practice (Ma, Chen, Liu, & Ye, 2009; Salar Bashi et al., 2012). In recent years, there have been several reports on the application of UAE in the extraction of different components (Ahmed, Akter, & Eun, 2011; Teng, Jo, & Choi, 2010; Zou, Jia, Li, Wang, & Wu, 2013) and many natural products (Da Porto, Porretto, & Decorti, 2013). The

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increment in extraction obtained by using ultrasound is mainly attributed to the effect of acoustic cavitations produced in the solvent as a result of ultrasound wave passage (Ghafoor, Choi, Jeon, & Jo, 2009; Zou et al., 2013). Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the tissue, increasing the contact surface area between the solid and liquid phase. As a result, the solute quickly diffuses from the solid phase to the solvent (Rostagno, Palma, & Barroso, 2003; Zou et al., 2013). However, it was unknown whether the extraction efficiency of ASE from annatto seeds can be improved by the UAE.

In order to optimise the extraction conditions when using ultrasound for the extraction of herbal components, including concentration of solvent, extraction time, duty cycle, and solvent-to-material ratio, response surface methodology (RSM) has been used. By establishing a mathematical model, RSM evaluates multiple parameters and their interactions using quantitative data, effectively optimising complex extraction procedures in a statistical way, thus reducing the number of experimental trials required (Salar Bashi et al., 2012; Zhang, Xu, Wang, Wu, & Chen, 2010).

The objectives of this study were to determine the optimal extraction conditions for the ASE using UAE and comparing extraction efficiency and antioxidant property of obtained colour with conventional method.

2. Materials and methods

2.1. Material

All chemicals were of analytical grade and obtained from Merck (Germany), Sigma–Aldrich (UK). Annatto seeds were purchased from Hyderabad, India.

2.2. Ultrasound-assisted extraction (UAE)

Annatto seeds were soaked in *n*-hexane for 6 h in order to remove oils and the defatted seeds used for dye extraction (Castello, Chandra, Phatak, & Madhuri, 2004). In our preliminary tests, chloroform showed the best colour extracting efficiency from annatto seeds among different solvents such as acetone, diethyl ether, ethyl acetate and ethanol. Therefore, we used the chloroform as a solvent for the extraction process. Samples were placed in capped glass tube mixed with chloroform and then immersed in a water bath. UAE process performed in XL2020 modele's of ultrasonic device (20 kHz, 550 W, Misonix, Germany). The working frequency and power was fixed at 20 kHz and 200 W, respectively. After ultrasonic extraction, the extracts were filtered through Whatman filter paper NO.1 and then vacuum-dried in the 1410D-2E vacuum oven (Shel Lab, USA) to produce dye powder.

2.3. Dye measurement (Y_1)

The powders in equal weight (2 mg) were dissolved in 20 ml chloroform and having been diluted to 10^{-2}, colouring strength was measured spectrophotometrically using UV-160A spectrophotometer (Shimadzu, Japan), at the 502 nm as maximum absorbance value for bixin in chloroform (Vasu, Palaniyappan, Kothandam, & Badami, 2010). The resultant absorbance was considered as response.

2.4. Determination of extraction yield (Y_2)

The obtained powder was weighed and the mass ratio of the powder to the weight of the seeds was taken into account as the extraction yield. Indeed, extraction yield was calculated according to following formula and considered as the second response.

$$\text{Extraction yield } \% = W_1 - W_2/W_1 \times 100$$

W_1 = weight of powderW_2 = weight of annatto seeds

2.5. DPPH assay

Determination of antioxidant activity performed according to the method of Panovska et al. 1 mL of ASE dissolved in acetone was added to 4 mL of 0.1 mM DPPH in a solution of 95% methanol. This solution was used as a blank. Absorbance at 517 nm was measured spectrophotometrically after 30 min incubation. The inhibition activity was calculated by the following formula (Kurniawati et al., 2007).

$$\% \text{ Inhibition} = [1 - (\text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}})] \times 100$$

$\text{Abs}_{\text{sample}}$: absorbance value of sample
$\text{Abs}_{\text{control}}$: absorbance value of control

2.6. Experimental design

A five level, four variables central composite rotatable design was employed for optimisation with respect to four important reaction variables the extraction temperature, sonication time, duty cycle and seed to solvent ratio. Off time for the ultrasound device considered to burst of the bubbles, so the duty cycle (on time/off time) and total time were 0.2–0.8 and 1 s, respectively. The independent variables and their levels are shown in Table 1. The factorial design consisted of sixteen factorial points, eight axial points and seven center points leading to sets of experiments in two of replication. Regression analysis was performed on the data of response variables such as the extraction yield and the absorbance values obtained.

2.7. Statistical analysis

The least square multiple regression methodology was used to enquire the relationship between the independent and dependent variables. The multiple regression equation was used to fit the second-order polynomial equation based on the experimental data as follows:

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 \\ & + \beta_{33} X_3 X_3 + \beta_{44} X_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 \\ & + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \end{aligned}$$

where Y represents the predicted response, β_0, is the model intercept, β_1, β_2, β_3, β_4, β_{11}, β_{22}, β_{33}, β_{44} and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} are linear quadratic and interaction coefficients respectively, and X_1, X_2, X_3 and X_4 are the independent.

The models were compared based on the coefficient of determination (R^2), adjusted coefficient of determination (R^2-adj) and predicted coefficient of determination (R^2-pred).The coefficient of determination (R^2) is defined as the regression of sum of squares

Table 1
Uncoded and coded levels of the independent variables of the extraction process.

Independent variables	Symbol	Coded levels				
		−2	−1	0	+1	+2
Temperature (°C)	X_1	20	35	50	65	80
Extraction time (min)	X_2	2	4	6	8	10
Duty cycle (s)	X_3	0.2	0.35	0.5	0.65	0.8
Seed to solvent ratio (%)	X_4	5	8.75	12.5	16.25	20

proportion to the total sum of squares which illustrates the adequacy of a model. R^2 ranges from 0 to 1. R^2 values closer to 1, means the model is more accurate. The high adjusted and predicted coefficient of determination also illustrate whether the model adequately fits the data (Badwaik, Prasad, & Deka, 2012). After selecting the most accurate model, the analysis of variance (ANOVA) was used to investigate the statistical significance of the regression coefficients by conducting the Fisher's *F*-test at 95% confidence level. The interactive effects of the factors were observed using surface plots, derived from the chosen model.

Finally, the entire process was optimised. The aim of the optimisation was to maximise the responses with the same weight ($w = 1$) and the credibility of the optimum conditions was diagnosed through the desirability values of the responses which range from 0 to 1. The closer values of desirability to 1 showed the more desirable and credible optimal conditions.

3. Results and discussion

3.1. Fitting the response surface models

According to the created design, 25 experiments were performed in duplicate and the obtained results are depicted in Table 2.

The values of R^2, R^2-adj and R^2-pred revealed that the full quadratic models were the more adequate than other models for the extraction efficiency and absorbance values. The values of R^2, R^2-adj and R^2-pred for the extraction efficiency were 94.31, 92.61 and 88.68, respectively; the values of R^2, R^2-adj and R^2-pred for the absorbance values were 89.94, 86.94 and 79.98, respectively.

Table 3 shows each coefficient values of extraction yield with respective *P* values. All of linear coefficients were significant except pulsation time. Among quadratic coefficients just pulsation time * pulsation time was insignificant and interactive coefficient of total time * seed to solvent ratio and temperature * seed to solvent ratio were found to be significant.

Table 4 shows each coefficient values of absorbance values with respective *P* values. All of linear coefficients were significant except pulsation time. Among quadratic coefficients just pulsation temperature * temperature was insignificant and all of interactive coefficient found to be significant.The two models are as follows:

$$\begin{aligned}\text{Extraction yield} = {} & -9.535 + 0.091\,X_1 + 0.635\,X_2 + 131.547\,X_4 \\ & - 0\,X_1^2 - 0.021\,X_2^2 - 337.587\,X_4^2 - 0.357\,X_1X_4 \\ & - 1.408\,X_2X_4.\end{aligned}$$

$$\begin{aligned}\text{Absorbance values} = {} & 0.0053\,X_1 + 0.0442\,X_2 + 6.6342\,X_4 \\ & - 0.0020\,X_2^2 + 0.4161\,X_3^2 - 16.0090\,X_4^2.\end{aligned}$$

The analysis of variance (ANOVA) was used to evaluate the significance of the quadratic polynomial models (Yuan, Gao, Mao, & Zhao, 2008). For each terms in the models, a large *F*-value and a small *P*-value would imply a more significant effect on the respective response variable (Quanhong & Caili, 2005). Therefore, the linear term of seed to the solvent ratio (X_4) and quadric term of seed to the solvent ratio (X_4^2) have the largest effect on the extraction yield. The linear term of temperature (X_1) showed a significant effect ($P < 0.05$) on the extraction yield; however, the other two linear terms (sonication time (X_2) and duty cycle (X_3)) did not show a significant effect ($P > 0.05$). The quadric term of extraction time also had a significant effect ($P < 0.05$) on the extraction yield, whereas, the effect of other three quadric terms were insignificant ($P > 0.05$).

The linear term of seed to the solvent ratio (X_4) and quadric term of seed to the solvent ratio (X_4^2) having the largest effects on the absorbance values ($P < 0.001$). The linear terms of temperature (X_1) and sonication time (X_2) also show a significant effect ($P < 0.05$); but, the effect of duty cycle was not significant ($P > 0.05$). The quadric terms of sonication time (X_2^2) and duty cycle (X_3^2) also had a significant effect on the absorbance values ($P < 0.05$).

In addition, none of the interactive terms had a significant effect on the absorbance values and on the extraction yield ($P > 0.05$).

The fitness of the model was investigated through lack-of-fit test ($P > 0.05$), which indicated suitability of models to accurately predict the variation (Quanhong & Caili, 2005).

Table 2
The CCD matrix and the experimental data for the responses.

Treatment NO.	Extraction temperature	Sonication time (min)	Seed to solvent ratio (%)	Duty cycle (s)	Extraction yield (%)	Absorbance values
1	35	8	8.75	0.65	3.98	0.741
2	50	10	12.5	0.50	6.09	0.730
3	35	4	16.25	0.65	5.41	0.799
4	50	6	12.5	0.50	5.55	0.783
5	35	8	8.75	0.35	3.92	0.691
6	35	4	8.75	0.65	3.59	0.657
7	65	8	16.25	0.65	5.85	0.868
8	50	6	20	0.50	4.39	0.807
9	35	4	8.75	0.35	3.38	0.642
10	65	4	8.75	0.35	3.91	0.711
11	65	8	8.75	0.65	5.19	0.758
12	65	4	16.25	0.65	5.71	0.849
13	35	4	16.25	0.35	5.32	0.790
14	35	8	16.25	0.35	5.36	0.817
15	50	6	12.5	0.80	5.72	0.819
16	65	4	8.75	0.65	5.12	0.728
17	80	6	12.5	0.50	6.00	0.722
18	50	2	12.5	0.50	4.28	0.719
19	20	6	12.5	0.50	4.40	0.741
20	50	6	12.5	0.20	5.65	0.769
21	65	4	16.25	0.35	5.06	0.831
22	65	8	8.75	0.35	5.63	0.728
23	50	6	5	0.50	2.85	0.527
24	35	8	16.25	0.65	5.63	0.824
25	65	8	16.25	0.35	5.72	0.857

Table 3
Estimated regression coefficients for extraction yield.

Term	Coefficient	SE coefficient	*P*
Constant	−9.535	1.2745	0.000
Temperature	0.091	0.0204	0.000
Total time	0.635	0.1494	0.000
Pulsation time (s)	2.160	2.0396	0.295
Seed to solvent ratio	131.547	8.1586	0.000
Temperature * Temperature	0.000	0.0001	0.009
Total time * Total time	−0.021	0.0076	0.008
Pulsation time (s) * Pulsation time (s)	0.067	1.3501	0.960
Seed to solvent ratio * Seed to solvent ratio	−337.587	21.6015	0.000
Temperature * Total time	0.000	0.0014	0.725
Temperature * Pulsation time (s)	0.025	0.0180	0.173
Temperature * Seed to solvent ratio	−0.357	0.0722	0.000
Total time * Pulsation time (s)	−0.204	0.1354	0.138
Total time * Seed to solvent ratio	−1.408	0.5415	0.012
Pulsation time (s) * Seed to solvent ratio	−11.556	7.2196	0.116

Table 4
Estimated regression coefficients for absorbance value.

Term	Coefficient	SE coefficient	*P*
Constant	0.0100	0.1386	0.943
Temperature	0.0053	0.0022	0.022
Total time	0.0442	0.0162	0.009
Pulsation time (s)	−0.2858	0.2218	0.204
Seed to solvent ratio	6.6342	0.8873	0.000
Temperature * Temperature	0.0000	0.0000	0.064
Total time * Total time	−0.0020	0.0008	0.017
Pulsation time (s) * Pulsation time (s)	0.4161	0.1468	0.007
Seed to solvent ratio * Seed to solvent ratio	−16.0090	2.3495	0.000
Temperature * Total time	−0.0002	0.0001	0.238
Temperature * Pulsation time (s)	−0.0003	0.0019	0.860
Temperature * Seed to solvent ratio	−0.0028	0.0078	0.720
Total time * Pulsation time (s)	0.0080	0.0147	0.588
Total time * Seed to solvent ratio	−0.0704	0.0588	0.238
Pulsation time (s) * Seed to solvent ratio	−0.7167	0.7852	0.366

3.2. Effects of extraction conditions on the extraction yield

Fig. 1(a) presents the interaction between the ratio of the seed to the solvent and sonication time. Initially, the extraction efficiency increased by increasing the ratio of the seed to the solvent but subsequently decreased, this is probably due to saturation of the solvent. As shown in Figs. 1(a) and (b), expected yield value increases with increasing sonication time. Maximum extraction yield was achieved by seed to the solvent ratio of 12.5% and sonication time of 10 min.

Fig. 1(b) describes interactive effects of temperature and seed to the solvent ratio on the extraction yield. The extraction efficiency was increased by increasing extraction temperature. This phenomenon most likely due to improvement in mass transfer rate at higher temperature, because at higher temperature the solubility of the pigments increased and the solvent viscosity reduced. However, further increase in seed to solvent ratio, a decline in total pigments extraction yield was observed. The phenomenon of solvent saturation is clearly demonstrated in Fig. 1(b). As shown in Fig. 1(b), it can be concluded that extraction yield was maximum when seed to the solvent ratio and extraction temperature were approximately 12% and 80 °C, respectively.

The interaction between temperature and sonication time on the extraction yield when the duty cycle and seed to solvent ratio were kept at 0.5 s and 12.5%, respectively; indicate that extraction yield increased by increasing temperature and sonication time.

3.3. Effects of extraction conditions on the absorbance values

The interactive effect of seed to the solvent ratio and sonication time on the absorbance values when the duty cycle and temperature were kept at 0.5 s and 50 °C, respectively; demonstrate that the absorbance values initially increased by increasing sonication time and seed to the solvent ratio, but decreased in the long extraction time. This can be due to the formation of sound chemical components in high extraction time. These components might have oxidative effects through producing free radicals and destroying conjugated double bond, so the absorbance values decreased. Zhang et al. (2010) in their study on UAE of ellagic acid from *Platycarya strobilacea* and Ghafoor and Choi (2009) in their work on UAE of phenolic compounds and antioxidants from grape peel obtained similar results in long sonication time.

Fig. 2(a) shows the interaction between temperature and sonication time on the absorbance values. As can be seen in Fig. 2(a), the absorbance values increased by increasing temperature and sonication time, but at longer time of extraction, the absorbance values decreased as temperature increased. Ghafoor and Choi (2009) also obtained the similar results. This could be attributed to the thermal decomposition of conjugated double bonds in higher temperature. Deleterious effect of long extraction time on the absorbance value is obvious in Fig. 2(a).

Fig. 2(b) demonstrates the effect of sonication time and duty cycle variations on the absorbance value of annatto extract.

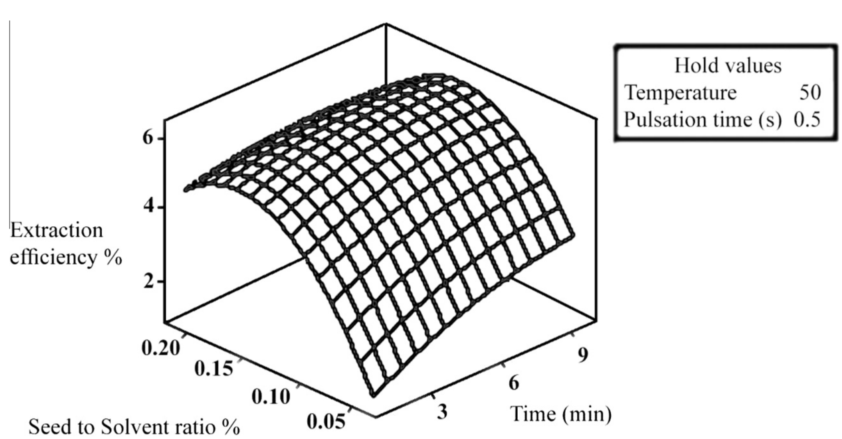

Fig. 1a. The interactive effect of seed to solvent ratio and sonication time on the extraction efficiency.

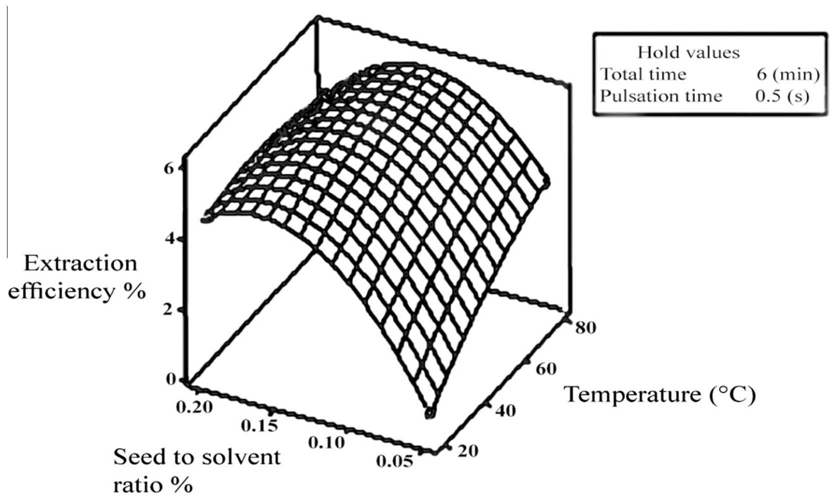

Fig. 1b. The interactive effect of seed to the solvent ratio and extraction temperature on the extraction efficiency.

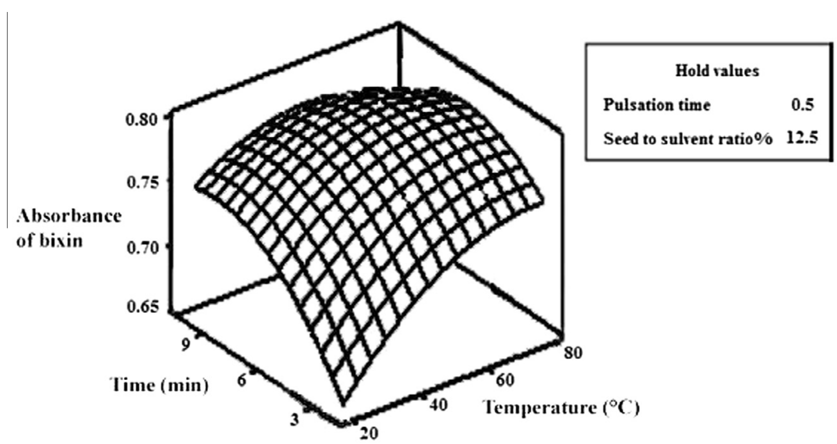

Fig. 2a. The interactive effect of extraction temperature and sonication time on the absorbance values.

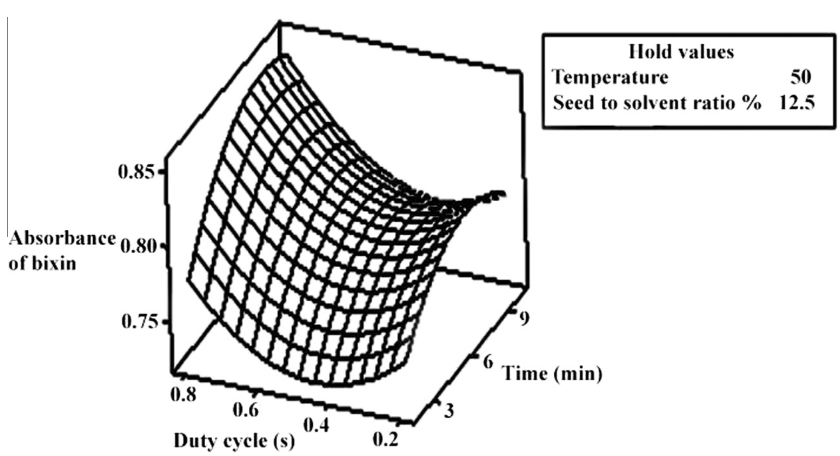

Fig. 2b. The interactive effect of extraction time and duty cycle on the absorbance values.

Initially, the absorbance value increased by increasing extraction time but subsequently it decreased. This could be due to the destruction of conjugated double bonds by sound chemical components produced in long extraction time. According to the Fig. 2(b), the deleterious effects of long extraction time on the conjugated double bonds decreased in high amounts of duty cycle, because the off time is decrease and there is no opportunity for sound chemical components to activate. Generally, the absorbance value has increased by increasing the duty cycle.

3.4. optimisation of the extraction process

The numerical optimisation technique used to optimise the UAE process, when weight and importance value for both responses were considered equal (Li et al., 2013). The extraction time of 7.25 min, the seed to the solvent ratio of 14%, temperature of 72.7 °C and duty cycle of 0.8, were found as the optimal conditions of the UAE process. The absorbance value of 0.865% and the extraction yield of 6.35% were acquired as the predicted results whose desirability values were equal to 1.000.

3.5. Comparison of UAE and conventional extraction

Response surface methodology was also used to evaluate the optimal extraction parameters for the conventional method. At temperature of 48.33 °C, extraction time of 2 h, the ratio of seed to the solvent of 12.89% and solvent concentration of 100% chloroform were found as the optimum conditions of the process under which the absorbance value determined as 0.597% and the extraction yield was 3.95% of annatto seed. However, for the UAE process extraction time of 7.25 min, the seed to the solvent of 14%, temperature of 72.7 °C and duty cycle 0.8 s, were found to be as the optimum conditions, the absorbance value of 0.865% and the extraction efficiency of 6.35% of annatto seeds were obtained; that both responses are higher from conventional method.

The results show that UAE method is more efficient than conventional method. Zhang et al. also reached the conclusion about the astaxanthin extraction from *Haematococcus pluvialis* (Zhang et al., 2010).

The result of DPPH assay showed that IC_{50} of ASE extracted by UAE was less than ASE extracted by conventional method. IC_{50} of the ASE extracted by UAE and the conventional extraction were 547.8 and 549.3 ppm, respectively (IC_{50} of β-Carotene was 552.1 ppm). In fact, the antioxidant activity of the ASE extracted by UAE is higher than the ASE extracted by the conventional method, because carotenoids were damaged in long extraction time of conventional extraction. Oancea, Grosu, Ketney, and Stoia (2013) observed that antioxidant activity of anthocyanin extracted from blackberry and sweet cherry cultivar by UAE was more than that of conventional extraction method.

4. Conclusion

The results revealed that ultrasound-assisted extraction is an effective technique for annatto colour extraction in comparison with conventional extraction method. The benefit of this this technique include lower extraction time, less carotenoid damage and more satisfactory colour obtained in both quantity and quality.

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