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ORIGINAL ARTICLE

Multivariate statistical analysis and optimization of ultrasound-assisted extraction of natural pigments from waste red beet stalks

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Abstract In this study, ultrasound-assisted extraction (UAE) of natural pigment extraction from waste red beet stalks were optimized under four factors (extraction temperature, ultrasonic power, extraction time and solid–liquid ratio) by using three level Box-Behnken response surface design. Extraction temperature, ultrasonic power and solid–liquid ratio were significantly influenced the extraction yield of pigments. Extraction temperature of 53 °C, ultrasonic power of 89 w, extraction time of 35 min and SL ratio of 1:19 g/ml was identified as the optimal condition. Under this condition, the actual yield of (betacyanin of 1.28±0.02 and betaxanthin of 5.31±0.09 mg/g) pigments was well correlated with predicted values (betacyanin was 1.29 mg/g and betaxanthin was 5.32 mg/g).

Keywords Natural pigment · Extraction · Box-Behnken design · Optimization

Introduction

Pigments extracted from natural sources have pinched much interest due to their functional properties and its harmfulness to the consumers. Moreover, it can be used not only in food and cosmetic industries but also in the development of functional foods. Most of the consumers were attracted by the color of the food materials. The interest in use of natural pigments in food products manufacturing was increased due to the toxicity of some synthetic colorants (Wang et al. 2011) and the recent development in food industries are replacing synthetic colors with natural colors obtained from natural resources (Es-Safi 2004; Wang et al. 2006; Zhang et al. 2011; Maran et al. 2015a, b). Therefore, finding new natural colorants from natural source especially from waste plant material is not only a way to reduce the environmental pollution but also important to produce value added healthier products in low cost.

Red beet (*Beta vulgaris* L.) is a root vegetable that grows primarily in the ground with a leafy top that grows aboveground. It is widely used for industrial production of juices, concentrates and powders, which find further applications in dairy products, fruit fillings, confectionary, meat substitutes and sausages (Gliszczynska-Swiglo et al. 2006; Kanner et al. 2001; Stinzing and Carle 2007). The pigment present in the red beet (betalain) exhibit good antiviral and antimicrobial activities, cancer preventive agents, and may be used as a source of essential dietary amino acids (Delgado-Vargas et al. 2000; Loginova et al. 2011). After harvesting of red beet, the stalks of red beet were discarded as a waste materials and it may pollute the environment. But the stalks contains considerable amount of pigments, considered not only to have good coloring potential but also possess positive physiological benefits for human health.

However, available literature shows that, no experimental work has been carried out to extract pigments from waste beet stalks using ultrasound-assisted extraction (UAE) method. In recent years, UAE method has received considerable attention and promising alternative to conventional extraction method (Ebringerová and Hromádková 2010; Rastogi 2011; Awad et al. 2012; Galanakis 2013; Moorthy et al. 2015; Maran et al. 2015a, b), owing to its advantageous such as shorten

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the extraction time, reduce the use of solvent, enhancement of extraction yield and improve the quality of extracts (Tao et al. 2014; Maran and Priya 2015a, b, c, d, e). Hence, the objectives of this present study are to extract natural pigments (betacyanin and betaxanthin) from waste red beet stalks by UAE method. Four factors (extraction temperature, ultrasonic power, extraction time and solid–liquid ratio) three level Box-Behnken response surface design was used in this study to examine and optimize the consequence of process variables on the extraction yield of pigments.

Materials and methods

Materials

Waste red beet stalks were acquired from local market near Chennai, TamilNadu, India and utilized as raw materials for this study. The stalks were cleaned in running tap water, dried at hot air oven in 40 °C until it attains constant weight, pulverized to powder form and stored at −20 °C prior to the experiments. Standard indicaxanthin and betanin was procured from Sigma-Aldrich chemicals, Mumbai and used to determine the betacyanin (BC) and betaxanthin (BX) content of the extracts (Prakash Maran and Manikandan 2012).

UAE of pigments

UAE of pigments from waste red beet stalks were carried out according to the method described by Maran and Priya (2015a). Ultrasonic probe was directly submerged in the mixture (10 g of leaf powder with appropriate volume of distilled water), desired power of probe and temperature was controlled and set with the help of an amplitude controller. At a particular extraction time,

Table 1 Experimental design matrix with observed results

Run order	Temperature (C, X_1)	Power (w, X_2)	Time (min, X_3)	SL ratio (g/ml, X_4)	Betacyanin (mg/g)	Betaxanthin (mg/g)
1	50	90	30	20	1.19	5.14
2	50	90	30	20	1.23	5.12
3	60	90	30	10	0.91	4.62
4	50	90	15	10	0.61	3.61
5	50	90	45	30	0.94	4.52
6	60	60	30	20	0.89	4.21
7	50	60	15	20	0.65	3.41
8	50	90	30	20	1.23	5.18
9	50	90	15	30	0.43	3.09
10	40	90	45	20	0.82	4.12
11	50	120	15	20	0.12	2.19
12	50	60	30	10	0.62	4.11
13	60	120	30	20	0.54	3.41
14	50	120	45	20	0.82	4.41
15	40	60	30	20	0.41	3.38
16	60	90	45	20	1.11	4.81
17	60	90	15	20	0.82	3.79
18	50	120	30	10	0.67	3.18
19	40	90	30	30	0.51	4.19
20	50	90	45	10	0.83	4.69
21	50	90	30	20	1.21	5.17
22	50	60	45	20	0.62	3.76
23	50	60	30	30	0.63	3.58
24	50	120	30	30	0.42	3.41
25	50	90	30	20	1.23	5.18
26	40	90	15	20	0.23	3.08
27	40	120	30	20	0.38	3.01
28	60	90	30	30	0.95	4.28
29	40	90	30	10	0.51	3.48

Table 2 Adequacy of the model tested for responses

Source	Sequential sum of squares p-value	Lack of fit p-value	R^2	Adjusted R^2	Predicted R^2	PRESS	Remarks
Betacyanin							
Linear	0.0284	<0.0001	0.353	0.245	0.190	2.222	
2FI	0.8536	<0.0001	0.433	0.118	0.045	2.619	
Quadratic	<0.0001	0.0157	0.986	0.972	0.922	0.213	Suggested
Cubic	0.0853	0.0322	0.997	0.988	0.692	0.846	Aliased
Betaxanthin							
Linear	0.0285	<0.0001	0.352	0.244	0.172	14.551	
2FI	0.8622	<0.0001	0.430	0.114	−0.040	18.289	
Quadratic	<0.0001	0.0019	0.985	0.971	0.917	1.459	Suggested
Cubic	0.0030	0.0416	0.999	0.996	0.908	1.622	Aliased

the mixture was taken, filtered, centrifuged at 792*g* for 15 min (Remi R-24 Centrifuge, India), supernatant liquid was collected and used for the examination of pigments. To minimize the variability in experimental data, experiments were carried out in a randomized order. According to the Table 1, the experiments were carried out in triplicates, average value was calculated and used for the determination of pigments (betacyanin and betaxanthin) (Table 1).

Determination of total betacyanin and betaxanthin content

The total betacyanin and betaxanthin content of the extracts were determined according to the method described by Maran et al. (2015a, b). The following equation was used to determine the betacyanin (BC) and betaxanthin (BX) content of the extracts as indicaxanthin and betanin equivalents (Prakash Maran et al. 2013)

$$\mathrm{BS(mg/g)} = \frac{\mathrm{A} \times \mathrm{DF} \times \mathrm{MW}}{\varepsilon \times 1} \quad (1)$$

where BS is betacyanin or betaxanthin, A is absorption value at 600 nm, DF is dilution factor, MW is molecular weight (indicaxanthin=308 g mol^{-1} and betanin=550 g mol^{-1}), is molar extinction coefficient (indicaxanthin=48, 000 L mol^{-1} cm^{-1} and betanin=60,000 L mol^{-1} cm^{-1}), and 1 is path length (1 cm) of the cuvette.

Experimental design

Under response surface methodology, Box-Behnken experimental design (BBD) with four factors three level was executed in this study to evaluate and optimize the influence of process variables such as extraction temperature (40–60 °C), ultrasonic power (60–120 w),

Table 3 ANOVA and significance of regression coefficients

Source	Sum of squares	DF	Mean square	*F*-value	*p*-value
Betacyanin					
Model	2.70	14	0.19	71.42	<0.0001
X_1	0.46	1	0.46	171.65	<0.0001
X_2	0.06	1	0.06	23.33	0.0003
X_3	0.43	1	0.43	160.21	<0.0001
X_4	0.01	1	0.01	2.25	0.1561
X_{12}	0.03	1	0.03	9.47	0.0082
X_{13}	0.02	1	0.02	8.32	0.0120
X_{14}	0.00	1	0.00	0.15	0.7063
X_{23}	0.13	1	0.13	49.27	<0.0001
X_{24}	0.02	1	0.02	6.25	0.0255
X_{34}	0.02	1	0.02	7.78	0.0145
X_1^2	0.38	1	0.38	140.88	<0.0001
X_2^2	1.07	1	1.07	395.59	<0.0001
X_3^2	0.41	1	0.41	152.74	<0.0001
X_4^2	0.40	1	0.40	148.24	<0.0001
Betaxanthin					
Model	17.32	14	1.24	67.86	<0.0001
X_1	1.24	1	1.24	68.08	<0.0001
X_2	0.67	1	0.67	36.86	<0.0001
X_3	4.25	1	4.25	232.95	<0.0001
X_4	0.03	1	0.03	1.76	0.2063
X_{12}	0.05	1	0.05	2.53	0.1337
X_{13}	0.00	1	0.00	0.01	0.9420
X_{14}	0.28	1	0.28	15.11	0.0016
X_{23}	0.87	1	0.87	47.94	<0.0001
X_{24}	0.14	1	0.14	7.92	0.0138
X_{34}	0.03	1	0.03	1.68	0.2160
X_1^2	1.93	1	1.93	105.91	<0.0001
X_2^2	7.65	1	7.65	419.23	<0.0001
X_3^2	2.81	1	2.81	154.08	<0.0001
X_4^2	1.61	1	1.61	88.27	<0.0001

extraction time (15–45 min) and solid-liquid (SL) ratio (10–30 g/ml) on the maximum extraction yield of pigments from waste red beet stalks. The experimental design consists of 29 experiments including five centre points (used to estimate experimental error) were ascertained and total number of experiments (N) were calculated from the following equation (Prakash Maran et al. 2015).

$$N = 2K(K-1) + C_0 \quad (2)$$

where, K is number of factors and C_0 is the number of central point.

The relationship between independent variables and responses were expressed by a second-order polynomial mathematical equation and the generalized form was given as follows

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i} \sum_{<j=2}^{k} \beta_{ij} X_i X_j \quad (3)$$

where, Y is the response; X_i and X_j are variables (i and j range from 1 to k); β_0 is the model intercept coefficient; β_j, β_{jj} and β_{ij} are interaction coefficients of linear, quadratic and the second-order terms, respectively; k is the number of independent parameters (k=4 in this study) (Maran 2015).

Statistical analysis

Pareto analysis of variance (ANOVA) and multiple regression analysis through least square method were employed in this study to analyze the experimental data. Stat-ease design expert software (Version 8.7.1) and Microsoft Excel® 2003 (Open database connectivity data source running under windows) were used to carry out the statistical analysis of experimental data.

Optimization and validation of optimized condition

Numerical optimization technique was employed in this study to optimize the process variables and regression equations developed in this study were utilized in order to locate the optimal extraction condition. Additional triplicate experiments were performed at the determined optimal condition and the average value was used to validate the optimal condition.

Results and discussions

Experimental data analysis and model fitting

The experimental data was analyzed using different models (linear, interactive (2FI), quadratic and cubic) and the results are shown in Table 2. From the results (Table 2), it was observed that, quadratic model incorporating linear, interactive and quadratic terms showed higher R^2, adjusted R^2, predicted R^2 and also low p-values, when compared with other models. Hence, quadratic model was adopted in this study to express the influence of process variables over the extraction yield of pigments. By employing multiple regression analysis on the experimental data, the final quadratic equations narrate the relationship between independent variables and responses in terms of coded factors is given below

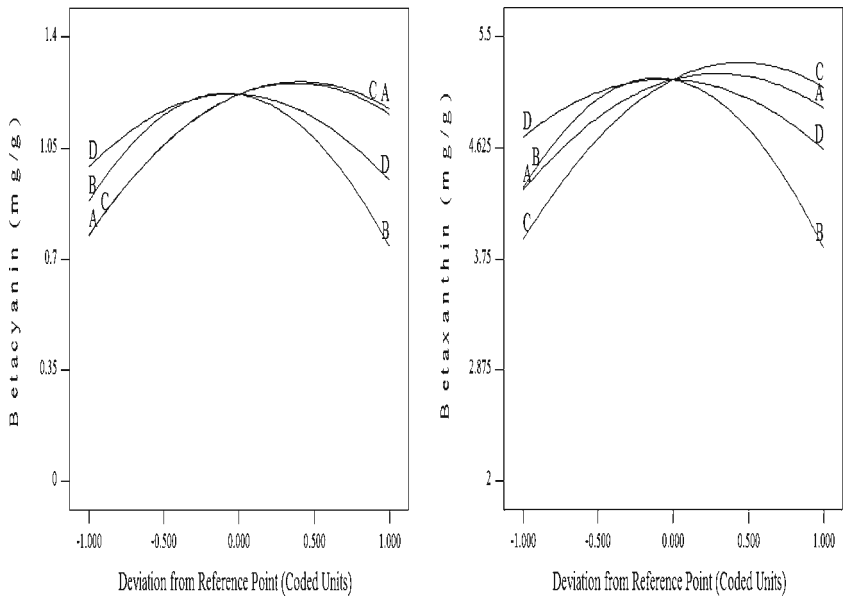

Fig. 1 Perturbation plots showing the effect of process variables on pigments yield

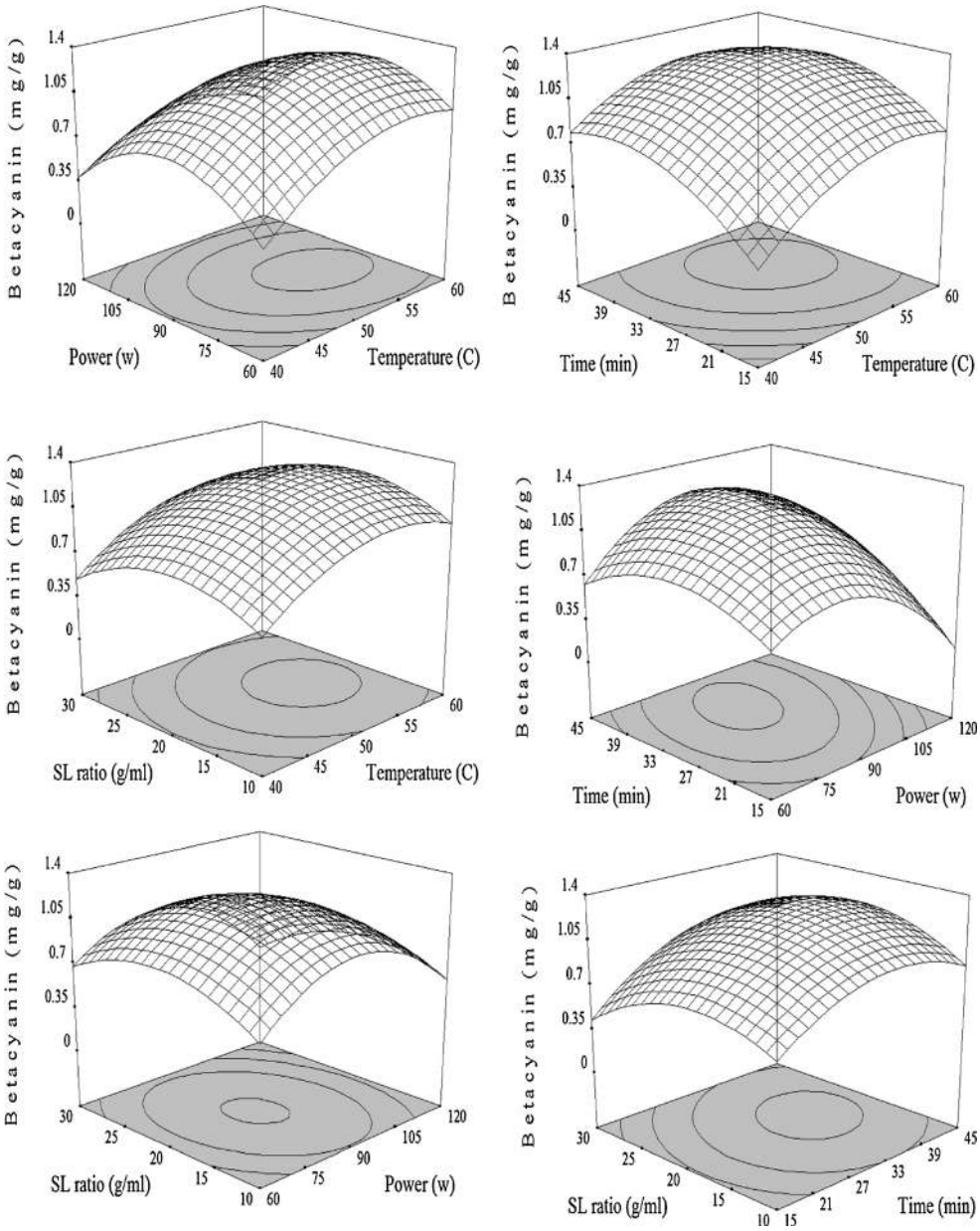

Fig. 2 Effect of process variables on betacyanin yield

$$BC = 1.22 + 0.19X_1 - 0.072X_2 + 0.18X_3 - 0.023X_4 - 0.08X_1X_2 - 0.069X_1X_3 - 0.009X_1X_4 + 0.16X_2X_3 - 0.059X_2X_4 + 0.073X_3X_4 - 0.18X_1^2 - 0.36X_2^2 - 0.19X_3^2 - 0.16X_4^2 \quad (4)$$

$$BX = 5.16 + 0.28X_1 - 0.19X_2 + 0.57X_3 - 0.032X_4 - 0.08X_1X_2 - 0.052X_1X_3 - 0.32X_1X_4 + 0.32X_2X_3 + 0.15X_2X_4 + 0.076X_3X_4 - 0.32X_1^2 - 0.089X_2^2 - 0.585X_3^2 - 0.63X_4^2 \quad (5)$$

Statistical analysis

Pareto analysis of variance (ANOVA) was used in this study to analyze the experimental data and the results are shown in Table 3. The results showed that, high model *F*-values (71.42 for BC and 67.86 for BX) with low probability value ($p<0.0001$) demonstrated the implication of developed regression models (Maran and Prakash 2015). The determination coefficient ($R^2=$ 0.986 for BC and 0.985 for BX) values indicated that

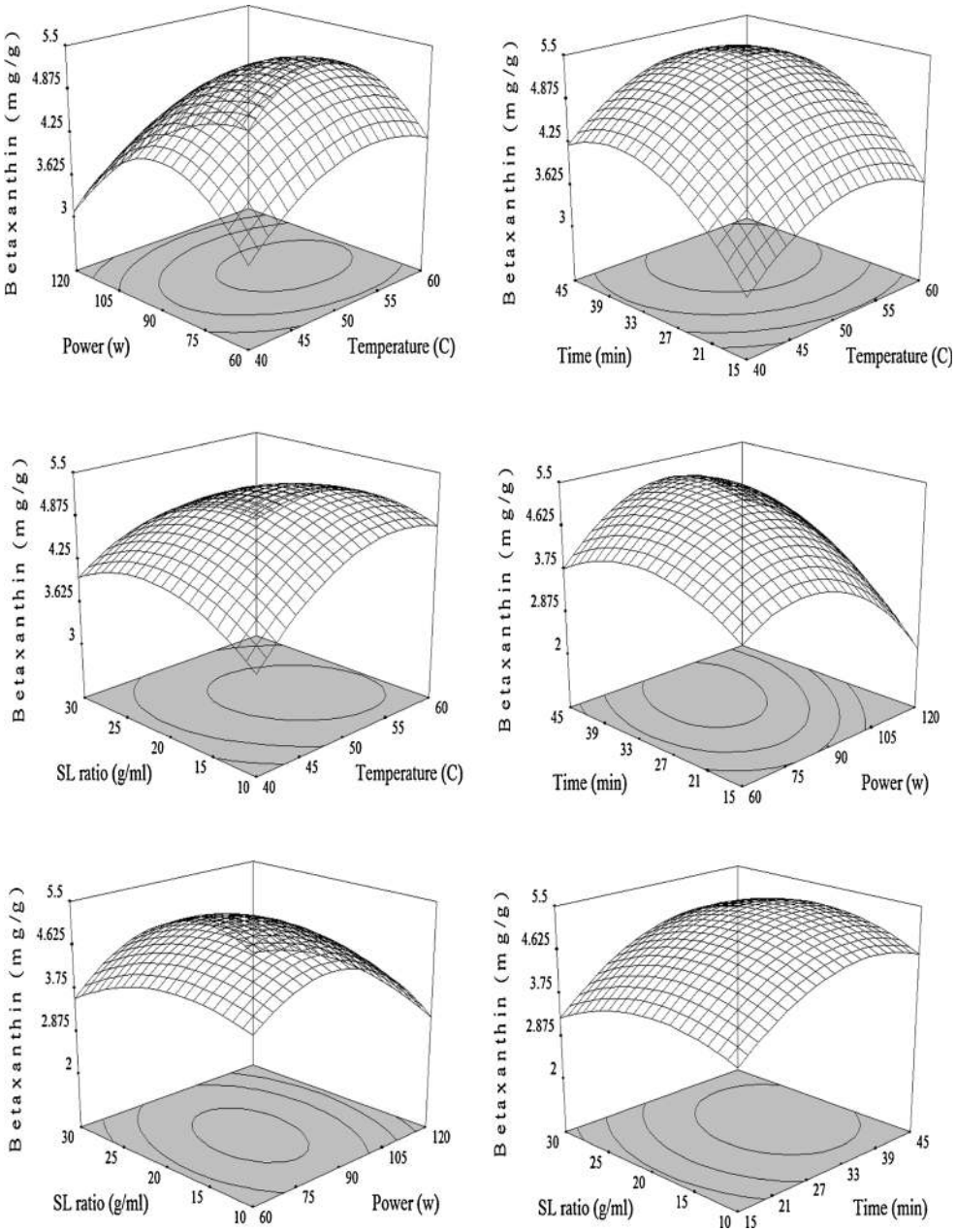

Fig. 3 Effect of process variables on betaxanthin yield

only 0.014 and 0.015 % of experimental data was not clarified by that equations (Maran et al. 2015a). Moreover, adjusted R^2 values (0.972 for BC and 0.971 for BX) are very close to R^2 and exhibited an excellent relationship between the experimental and predicted response values (Maran and Priya 2015b). The reliability of the conducted experiments was confirmed by low CV values (7.01 for BC and 3.37 for BX) of the responses. The high value of adequate precision (>30) for both response indicates an adequate signal and these models were significant for this extraction process. The statistical analysis of the experimental data confirmed that, the regression equations developed in this study were able to predict the experimental data in an accurate and precise manner.

Effect of process variables

Perturbation plot

Perturbation plot is one of the essential illustrative demonstration in order to estimate the effect of process variables in a single graphical illustration (Gupta and Ako 2005; Mishra et al. 2008). From the Fig. 1, it was found that, the responses were more sensitive to alteration in all factors and it was

confirmed by steep curvature all factors (extraction temperature (A), ultrasonic power (B), extraction time (C) and SL ratio (D)).

Response surface analysis

Three-dimensional (3D) response surface plots were generated from the developed models (Eqs. 4–5) in order to visualize and study the relationship between the response and process variables and it was shown in Figs. 2 and 3. Experiments were performed to study the effect of temperature (40–60 °C) over the extraction yield of pigments from waste red beet stalks. From the results, it was observed that, increase in temperature from 40–55 °C has the capability to create cavitation threshold, which can leads to form acoustic cavitation and cavitational nucleus. Collapse of caviation bubbles can dislocate the structure of plant matrix (Maran and Priya 2015c) and hasten the solubility and diffusivity of solvent in to the plant matrix and enhanced the extraction yield of pigments (Yang and Zhai 2010) (Fig. 2). Beyond the temperature of 55 °C, the characteristic of ultrasonic cavitation was altered, which decreases the intensity of mass transfer augmentation and diminished the extraction yield.

The effect of different ultrasonic power (60–120 w) on the extraction yield of pigments was studied and the results are shown in Fig. 2. As ultrasonic wave was passed through the liquid medium, violent shock wave and high-speed jet were generated by ultrasound provokes the swelling of the materials and enlargement of the pores in the materials (Vinatoru 2001; Luque-García and Luque de Castro 2003; Quan et al. 2009), which allows higher diffusivity across the plant materials and enhanced the extraction yield of pigments. However, beyond ultrasonic power of 100 w can increased the bubble numbers in the solvent during cavitation, which could reduce the efficiency of ultrasounic energy transmitted into the medium (Filgueiras et al. 2000) and weaken the extraction yield. Effect of time on the extraction yield of pigments from red beet stalks was investigated and the results are shown in Fig. 3. There was a steady increase in the yield of pigment up to 38 min and it could be explained that, swelling and hydration of plant material could be accelerated by cavitation effect of the ultrasonic waves during the earlier period of extraction (Wang et al. 2012). The asymmetric collapse of micro-bubbles near surfaces was also associated with microjets that could cause the disruption and good penetration of solvent (Vilkhu et al. 2008) into the matrix (Sun and Tomkinson 2002) through diffusion which improves the washing out of pigment content from plant material to surrounding solvents and enhanced the extraction performance. However, heating effect and exposure of ultrasound treatment for longer extraction time cause the structural destruction and decomposition of pigments, which reduced the extraction yield. Increase in SL ratio up to 1:25 g/ml enhanced the contact area between material and solvent, lower the concentration and viscosity of the extraction solvent which could lead to dissolve the pigments in the solvent and augmented the extraction yield of pigments. Since the formation of cavitation requires the negative pressure in the rarefaction region of wave function overcome the natural cohesive forces (Xu et al. 2014) and decreased the extraction yield (Fig. 3).

Determination and validation of optimized condition

Numerical optimization method was employed in this study to optimize the extraction process conditions. First and second order derivatives of regression models (Eqs. 4–5) were derived and second order derivatives showed negative values and it signifies the applicability of maximization (Maran and Priya 2015d). Algebraic solution gave the optimal condition in coded form was: $X_1=0.36$ °C, $X_2=-0.056$ w, $X_3=0.387$ min and $X_4=-0.073$ g/ml. The corresponding optimum extraction conditions in actual form was extraction temperature of 53 °C, ultrasonic power of 89 w, extraction time of 35 min and SL ratio of 19 g/ml and the maximum yield of betacyanin was 1.29 mg/g and betaxanthin was 5.32 mg/g respectively. Triplicate experiments were performed under the optimized conditions and the mean values (betacyanin of 1.28±0.02 mg/g and betaxanthin of 5.31±0.09 mg/g) obtained from real experiments, demonstrated the validation of the optimized conditions.

Conclusion

In this study, an efficient UAE method was successfully employed to extract natural pigments from waste red beet stalks. BBD was applied in this study to determine the optimal condition for maximum recovery of natural pigments. The maximum yield of pigments (betacyanin of 1.29 mg/g and betaxanthin of 5.32 mg/g) was obtained under the following optimal condition: extraction temperature of 53 °C, ultrasonic power of 89 w, extraction time of 35 min and SL ratio of 1:19 g/ml. Under this condition, the actual yield of pigments (betacyanin of 1.28±0.02 and betaxanthin of 5.31±0.09 mg/g) were well matched with the predicted yield. From the results, it can be concluded that, UAE method has a strong potential method for extraction of pigments from waste red beet stalks.

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