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Full Length Article

Optimization of Ultrasound-Assisted Extraction of Pigment from *Tremella sanguinea*

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Abstract

Tremella sanguinea Peng, one of the species in *Tremella* family, was an edible and medicinal fungus. Its fruit bodies were rich in natural yellow pigment with some nice features, such as thermo-stability, acid stability and water solubility. Thus, it had a wide prospect in business application; but till now, the standardized extraction process combining with ultrasound-assisted extraction (USAE) technique and the optimal parameters had not been reported. In our research, the pigment was isolated and analyzed by high performance liquid chromatography (HPLC). Plackett-Burman (PB) design, steepest ascent experiments and response surface methodology (RSM) design were applied to screen out the optimal parameter values of extraction with ultrasound assistance. The results showed that the maximum absorption wavelength of the pigment was 450 nm. Ultrasound power, extract temperature, solvent concentration and solvent sample ratio had significant influence on extraction values. When the input power, extract temperature, ethyl alcohol concentration and solvent sample ratio were 248.01 W, 87.37°C, 44.04% and 152.78, respectively, the pigment yield reached a maximum of 885.55 U/g. These findings contributed to the establishment of USAE procedure for *T. sanguinea* Peng pigment. © 2019 Friends Science Publishers

Keywords: Ultrasound-assisted extraction (USAE); Pigment; Response surface methodology (RSM); *Tremella sanguinea* Peng

Introduction

Natural and edible pigments had been widely used as coloring agents in food, pharmaceutics and cosmetics field in two decades (Xiao *et al.*, 2011). Most familiar natural pigments were flavonoids (Forkmann, 1991), carotenoids (Durante *et al.*, 2014; Wallace *et al.*, 2016), betalains (Gandía-Herrero and García-Carmona, 2013), Monascus pigments (Feng *et al.*, 2012), curcumin (Velíšek *et al.*, 2007) and phenolics (Li *et al.*, 2018; Muazzam and Farman, 2018). As green and natural ideas became a trend in food industrial development, more researches that focused on the exploration and application of edible pigments were fast developed.

Tremella sanguinea Peng, a new species identified in *Tremella* in 1990, had been regarded as an edible and medicinal fungus in China for their therapeutic effects (Peng, 1990; Wang *et al.*, 2016). The fruit body had been used for the treatment of dysentery, inflammatory disorder of the intestine and massive hemorrhage disease in traditional Chinese medicine (Chen, 1992). The fresh fruit body was in blood red and the dried product was in black color. The interesting thing was that the fruit body was rich in natural water-soluble yellow pigment. The pigment showed stability in low pH environment and high temperature tolerance which were suitable for food colorant in beverage industry and vinegar (Xia *et al.*, 2016). However, extraction methods of the pigment had not been investigated, which restricted further research and its application.

Extraction of natural pigments from fungi could be carried out in various ways, such as Soxhlet, maceration, heat reflux, supercritical extraction and microwave-assisted extraction (Esclapez *et al.*, 2011). Although these techniques had been demonstrated to be effective in compound extraction, the former three extraction techniques were laborious, time-consuming, high energy consumption and the latter two methods required expensive equipments (Esclapez *et al.*, 2011). Ultrasound-assisted extraction (USAE) had been demonstrated to improve the extraction efficiency and rate, reduced the temperature, saved the solvents consume (Chemat *et al.*, 2017). Nowadays, it was considered as a highly efficient, cheap and simple method suitable for industrial extraction (Wang and Weller, 2006; Khoddami *et al.*, 2013).

Industrial extraction of *T. sanguinea* required highly efficient optimal parameters. The parameters for optimization were the first step for transferring one research found to a practical application. Response surface methodology (RSM) was an important tool in parameters optimization. Response values and the independent variables could be determined by RSM design, which simulated a fully approximate real situation by the

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regression equation. In the case of low-frequency and high-power USAE, some variables were worth considering which would influence pigment yield, *e.g.*: input power, temperature, solvents, solvent-sample ratio and so on (Esclapez *et al.*, 2011). Therefore, the aims of this work were to explore for the influencing factors of USAE and optimize the extraction parameters by RSM.

Materials and Methods

T. sanguinea Peng used in this Study

The fruiting bodies of *T. sanguinea* Peng were collected from farmers of Baokang, Hubei province, China. In April of the last year, the mycelia of *T. sanguinea* Peng were inoculated in the caves on Logs of Fagaceae. Then these inoculated logs were stacked up in the woods. They were covered with branches, leaves and put up plastic sheds if necessary in the mycelia growth. The logs were leaned against the shelves prepared beforehand in the woods when the mycelia growth were finished. The fruiting bodies were cropped in September and October when temperature was at 12°C ~20°C. After then, they were quickly dried and collected for the experiment. These experiment materials were identified by Chenqiwu (a professor of Yangtze University) according to the document description (Peng, 1990), the botanical description (Table 1).

Experimental Setup

Ultrasonic extraction was performed by an ultrasonic washing machine (DL-820B, China). The fruiting bodies were crushed and sieved through 40 mesh before baking. The powder was kept away from light in a glass desiccator. Ethanol was purchased from Sinopharm Chemical Reagent Limited Corporation (Shanghai, China). Acetonitrile (TEDIA, U.S.A.) was chromatographic grade. Unless otherwise stated, all chemicals used were of analytical grade.

Pigment Extraction and Measurement of Color Values

Pigment extraction was conducted with an ultrasonic-assisted sonication water bath with a fixed frequency of 40 kHz. Roughly, 0.5 g of the *T. sanguinea* powder was added to a 100 mL conical flask containing 40% (v/v) ethanol. The conical flask was connected with a Liebig condenser and placed on the plate of the ultrasonic washing container. The ultrasonic wave power (0–800 W) and temperature (20–100°C) were set to different levels in experiments. Upon completion of the extraction process, the conical flask was cooled to room temperature. Pigment solution was obtained by centrifugation at 5000 RPM for 15 min. Pigment solution was measured of absorption value at 450 nm after dilution. The extraction efficiency could be defined as color value (450 nm, yellow) and calculated as formula (1):

$$\text{Color values}\left(\frac{\text{U}}{\text{g}}\right)=\frac{\text{Absorption values}\times\text{dilution ratio}}{\text{weight of sample (g)}} \quad (1)$$

Isolation Using HPLC

Further separation and purification of the pigment extraction was carried out by HPLC-DAD (Agilent Technologies, Santa Clara, C.A., U.S.A.). Chromatogram conditions: HPLC was equipped with a C18 reverse phase column (250 mm×4.6 mm×5 um) (Agilent Technologies, Santa Clara, C.A., U.S.A.). The column oven was set to 30°C, DAD detection wavelength was 400–600 nm and the injection volume was 20 μL. The mobile phases were water (mobile phase A) and acetonitrile (mobile phase B) and the working program was kept at 10% B at a flow rate of 1 mL/min.

Screening of Important Components

Primarily, the Plackett-Burman design experiment (Plackett and Burman, 1946) was carried out to investigate the most significant parameters which affected extract efficiency. Parameters included the input power of the ultrasound waves, extract temperature, extract time, solvent pH, ethanol concentration and the solvent-to-solid ratio were investigated in different experiments. The name, symbol codes, and levels of experimental variables were shown in Table 2 and 3.

Statistical Analysis

Results of the analysis of variance (ANOVA) for the data and the model coefficients computed by Design Expert software (version 8.0.6, Stat-Ease Inc.) were shown in Table 4.

Path of Steepest Ascent

Four key variables were confirmed based on the results of the PB design, the center values of which were starting points of the path of steepest ascent. The step-size was determined by the experimenter based on process knowledge and other practical considerations. The direction of steepest ascent was the direction in which the response increased the most. All factor levels were fixed, except that the known key factors identified using PB design. The path of speed ascent factors and their experimental trials were shown in Table 5.

Pigment Extraction by RSM

Based on the results of the PB test, the four independent variables (A_1, A_2, A_5 and A_6), chosen for pigment

Table 1: The botanical description of T. *sanguinea* Peng used in this study

Features	Contents
Taxonomic status	Eumycophyta, Basidiomycotina, Tremellales, Tremellaceae, *Tremella*
Distribution	In woods at an altitude of 700-1400 meters in southwest of China
Cropping season	September - October of every year
Shape features of fruiting bodies	Individual or in groups, hemisphere, 5~24 cm×5~20 cm×5~8 cm in volume.
Color	Dark red in fresh and black in dried products
Optimum growth temperature	15-20℃
Relative humidity	75%-90%
Light environment	Diffuse light

Table 2: Levels and codes of variables for Plackett–Burman design

Variables	Symbol codes	Coded levels	
		-1	1
Input power (W)	A_1	0	200
Temperature (°C)	A_2	60	80
Time (min)	A_3	10	30
pH	A_4	4	6
Ethanol concentration (%)	A_5	20	40
Solvent-to-solid ratio	A_6	50	100

Table 3: Results of Plackett-Burman design for pigments extraction by ultrasound wave

Trials	Variables						Color values
	A_1	A_2	A_3	A_4	A_5	A_6	(U/g)
1	-1	-1	-1	1	1	1	383
2	-1	-1	1	1	1	-1	375.7
3	-1	1	-1	-1	-1	1	475
4	1	-1	1	1	-1	1	456.3
5	1	1	1	-1	1	1	649.3
6	-1	1	1	1	-1	1	513
7	1	-1	1	-1	-1	-1	427
8	1	1	-1	1	-1	-1	504.3
9	1	1	-1	1	1	-1	586.7
10	-1	1	1	-1	1	-1	541.3
11	-1	-1	-1	-1	-1	-1	352.5
12	1	-1	-1	-1	1	1	519.3

Table 4: ANOVA for evaluation of the regression model in Plackett-Burman design

Variables	Coefficients	*F*-value	*P*
Input power (W)	41.87	45.57	0.001**
Temperature (°C)	62.98	103.13	<0.001**
Time (min)	11.82	3.63	0.115
pH	-12.12	3.82	0.108
Ethanol concentration (%)	27.27	19.33	0.007**
Solvent-to-solid ratio	17.37	7.84	0.038*

optimization using RSM design, were evaluated at three levels (-1, 0, +1) with 27 experimental runs including three repetitive central points in Table 6 by Design Expert software (version 8.0.6, Stat-Ease Inc.). The equation, used to generate surface graphs for RSM, was calculated by the formula (2):

Table 5: Experimental trials in path of steepest ascent

Trials	Factors				Color values
	A_1	A_2	A_5	A_6	(U/g)
1	120	70	30	75	538.5
2	160	75	35	100	641
3	200	80	40	125	752.125
4	240	85	45	150	873.73
5	280	90	50	175	855.875
6	320	95	55	200	768.9

Table 6: Levels and codes of variables for Box-Behnken design

Variables	Symbol Coded	Levels		
		-1	0	1
Input power (W)	A_1	200	240	280
Temperature (°C)	A_2	80	85	90
Ethanol concentration (%)	A_5	40	45	50
Solvent-to-solid ratio	A_6	125	150	175

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n} \beta_{ij} X_i X_j \qquad (2)$$

In the equation, Y was the predicted response value, β_0 was the intercept term, β_i was the linear term, β_{ii} was the squared term, β_{ij} was the interaction term and X_i and X_j were the coded levels of independent factors.

All experiments were performed in triplicate. Optimal levels of the variables which significantly affected extract efficiency were determined by regression equation and Response surface plots.

Results

HPLC, Visible Absorption Spectra of Pigment in *Tremella sanguinea* Peng

In this study, pigment in *T. sanguinea* Peng was separated by HPLC using an C18 reverse phase column with a SPD-1315A UV/Vis detector. Chromatogram of pigment derivatives were presented in Fig. 1. It showed that a peak with retention time of 2.47 min was separated successfully. The maximum absorption wavelength of this peak was 450 nm which was correspondent with the yellow color of the extract solution.

Screening of Significant Parameters

The results in Table 3 showed a wide variation in color values, which reflected the importance of design optimization. The ANOVA results of each variables were also presented in Table 4. Temperature, the input power of ultrasound, ethanol concentration and solvent-to-solid ratio showed a significant effect on the color values of pigments ($P < 0.01$). Subsequently, the four factors were chosen out for the following experiments.

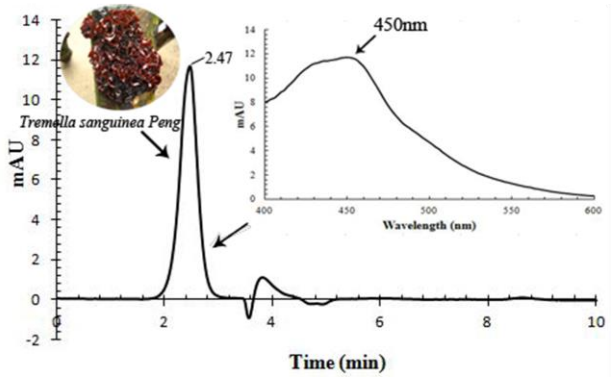

Fig. 1: Spectrograph and chromatogram analyse of pigment derivatives by HPLC

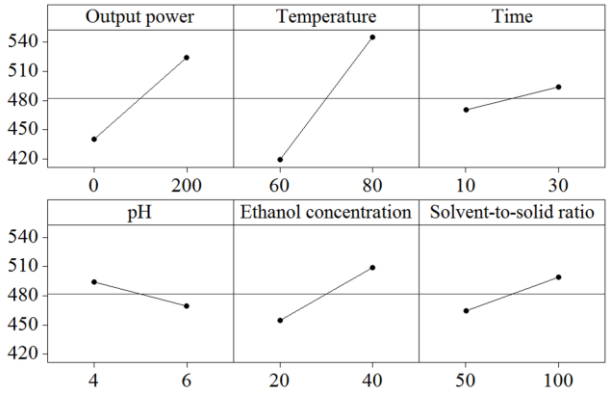

Fig. 2: Main effects of six variables on pigment extraction

Results of the Steepest Ascent Design

The PB results only helped us to determine rough levels of the selected variables. Thus, the steepest ascent design was employed in the following experiments, which would efficiently explore for regions near the best values. The main effects of variables were also revealed in Fig. 2, which represented the direction of steepest ascent. Ethanol concentration, temperature, input power and solvent-to-solid ratio were all significant positive effects. The steepest ascent approach design was designed to quickly the optimum and the results were shown in Table 5. The color value of Trial 4 was 873.73 U/g and a decreasing value appears in Trial 6. Therefore, Trial 4 was designed as the temporary center of RSM.

Optimization of Screened Variables

To increase pigment production further, the RSM design was applied to optimize pigment extraction; the factors and levels were shown in Table 6 and the corresponding color values were listed in Table 7. Fitting of the experimental data by regression analysis gave rise to a second-order polynomial equation model as follows:

$$Y=879.32+23.55A+30.46B-15.24AB-21.06AC-11.61BC49.16A2-32.43B2-23.97C2-20.44D2 \quad (3)$$

The ANOVA results of RSM were shown in Table 8. *p* values less than 0.01 indicated that the coefficient was most significant. The linear coefficients (A and B), interaction term coefficients (AB, AC and BC) and coefficients of the quadratic term (A^2, B^2, C^2 and D^2) were significant ($P < 0.01$). The *F*-value for this model was 52.13, implying that it was significant ($P < 0.01$). The *P*-value (0.6513) for lack of fit indicated that the proposed model fitted well with the experimental results.

According to the experimental results of RSM (Table 8), the input power and temperature showed significant effects on color values ($P < 0.05$). The results from the 3D surface plots in Fig. 3 indicated that the yields increased significantly with an increase in power up to 248–249 W and then began to decline with a further increase in input power (Fig. 3a and 3b). It was noteworthy that the highest yield was attained when the input power was 248.01 W. The same trend was also observed for the temperature range from 80°C to 90°C. The maximum yield was obtained at 87.85°C. Increasing temperature would result in an increase in color values and a decrease at the flex point.

However, no significant difference in pigment yield was found in the investigated solvent interval (40–50%) ($P > 0.05$). The solvent-to-solid ratio might one factor worth considering. Generally, high solvent-to-solid ratio was corresponded to a higher total amount of extraction compounds obtained within the limits, regardless of the solvent used. Although in Fig. 2, the solvent-to-solid ratio had shown a positive effect on the extraction results in PB design, no significant influence of pigment yield had been found in for solvent-to-solid ratios from 125:1 to 175:1 in the further optimal design ($P > 0.05$).

According to the results of Fig. 3, the optimal parameters were as follows: input power, 248.01 W; temperature, 87.37°C; solvent concentration, 44.04%; solvent-to-solid ratio, 152.78.

Verification of the Predictive Model

Although, the optimal values of extraction parameters could be determined by such an optimization programs (RSM), they still needed to be validated by verification experiments. The mean value of the pigment was 880.95±9.39 U/g, which was closed to the predicted value (885.55 U/g) of the model. The results showed that the RSM model was suitable for pigment extraction.

Discussion

Natural sources of pigments included plant, animals and microorganisms (Vendruscolo *et al.*, 2016). Filamentous fungi included the fungi species in Zygomycetes,

Table 7: Results of Box-Behnken design for pigment extraction by ultrasound wave

Trials	Factors				Color values (U/g)
	A_1	A_2	A_5	A_6	
1	-1	-1	0	0	724.35
2	1	-1	0	0	798.67
3	-1	1	0	0	828.98
4	1	1	0	0	842.35
5	0	0	-1	-1	832.56
6	0	0	1	-1	841.51
7	0	0	-1	1	839.33
8	0	0	1	1	829.68
9	-1	0	0	-1	782.95
10	1	0	0	-1	834.01
11	-1	0	0	1	795.03
12	1	0	0	1	832.24
13	0	-1	-1	0	780.45
14	0	1	-1	0	862.95
15	0	-1	1	0	808.77
16	0	1	1	0	844.84
17	-1	0	-1	0	755.15
18	1	0	-1	0	850.58
19	-1	0	1	0	799.55
20	1	0	1	0	810.74
21	0	-1	0	-1	804.11
22	0	1	0	-1	840.36
23	0	-1	0	1	795.08
24	0	1	0	1	857.49
25	0	0	0	0	876.25
26	0	0	0	0	873.85
27	0	0	0	0	887.85

Ascomycetes and Basidomycetes, in which some fungi were known to produce pigments, such as *Monascus* spp., *Neurospora* spp. (Gmoser *et al.*, 2017). Nowadays, pigments produced by filamentous fungi included melanin (Hou *et al.*, 2019), *Monascus* pigment (Silveira *et al.*, 2013) and carotenoids (Wang *et al.*, 2014). In comparison to the pigments from plant and animals, filamentous fungi were suitable for extensive production because the cultivation of which didn't compete farm land with agriculture production, no seasonal impediments, easy to harvest (Carvalho *et al.*, 2006; Gomes and Takahashi, 2016). These advantages determined filamentous fungi to apply as pigment producers in industry. To date, *Monascus* pigment, β-carotene and astaxanthin had been commercially produced by microbial fermentation (Gmoser *et al.*, 2017). As a species of filamentous fungi, *T. sanguinea* Peng produced yellow pigment with highly hydrophilic character, insensitivity to heat, acid stability (Xia *et al.*, 2016). In the experiment, we optimized the extraction parameters and obtained the optimum of the pigment yield from fruit bodies. We will try to produce the pigment by fermentation in the future.

USAE had been reported for the extraction of natural pigments from some plants (Chen *et al.*, 2018). Generally, USAE parameters included power, solvent concentration, extraction temperature (Kuo *et al.*, 2013). The extraction yield usually increased in line with the power intensity applied (Zou *et al.*, 2010). This was related to cavitation phenomena. Within a certain range, increasing the input power would increase the number of cavities in the media. The caivitation phenomena led to high shear forces in the media, which would disrupt cell walls and result in a fully contact between solvent and substrates (Chemat *et al.*, 2017). But the extraction field was rarely increased with the input power raised beyond a certain range. The sound intensity exceeded a certain point, excessively dense cavities were generated in the liquid near the ultrasonic transducer, resulting in the cushioning effect, which hindered the transmission of ultrasound, thus weakening the effect of input power (Keris-Sen *et al.*, 2014). Li *et al.* (2004) noted the similar tendency in the extraction of soybean oil in the difference ultrasound intensity, who observed that an increase in the oil yield up to 20.9 W/cm^2, but beyond which no further increase was found.

Temperature would strongly impact the properties of solvents. Usually, the increase in the extraction temperature led to the enhancement of the mass transfer, desorption and solubility (Esclapez *et al.*, 2011). In our results, temperature showed the positive effects on the extraction yield, this beneficial effect of temperature rose from 60°C~80°C. It could be explicated by the increase in the number of cavitation bubbles and in the solid-solvent contact area (Chemat *et al.*, 2017). This effect was weakened when the temperature was close to the boiling point of the solvents (Palma and Barroso, 2002). The optimal extraction temperature in the experiment was 87.37°C, which approached the boiling point of 45% ethanol aqueous solution (82.45°C). In addition, our previous work had shown that pigments extracted from *T. sanguinea* Peng were unstable beyond 90°C (Xia *et al.*, 2016).

Extraction solvents, especially ethanol and aqueous ethanol had been widely recognized as extraction solvents adequate for natural compound (including phenolic compounds, Monacolin K and anthocyanin) (Prasad *et al.*, 2011; Singgih *et al.*, 2014; Thao *et al.*, 2015). Moreover, aqueous ethanol was much safer and more suitable for the food industry than other solvents from a toxicological point of view (Huh *et al.*, 2004). The principle of extraction was usually based on the affinity of target compounds in the solvent and solute (Esclapez *et al.*, 2011). In the results of the Plackett-Burman design, ethanol aqueous showed a significant and positive effect on color values from 20% to 40%, This effect might be connected with the changes in the physical properties of the solvent, such as the density, viscosity and constant dielectric (Parra-Campos and Ordóñez-Santos, 2019), which increased the solubility of the compound and the driving force within the particles (Cacace and Mazza, 2003). But no difference was found in 40–50% aqueous ethanol, which suggested that the pigment was near saturation in such a solvent interval concentration.

Table 8: ANOVA for response surface quadratic model

Source	Sum of squares	df	Mean square	F-value	*P*-value
Model	35883.89	14	2563.13	52.13	<0.0001**
A-Input power	6654.29	1	6654.29	135.32	<0.0001**
B-Temperature	11134.96	1	11134.96	226.45	<0.0001**
C-Ethanol concentration	16.50	1	16.50	0.34	0.5732
D-Solvent-to-solid ratio	14.85	1	14.85	0.30	0.5927
AB	928.73	1	928.73	18.89	0.0010**
AC	1774.09	1	1774.09	36.08	<0.0001**
AD	47.96	1	47.96	0.98	0.3429
BC	538.94	1	538.94	10.96	0.0062*
BD	171.09	1	171.09	3.48	0.0868
CD	86.49	1	86.49	1.76	0.2095
A^2	12886.69	1	12886.69	262.07	<0.0001**
B^2	5609.24	1	5609.24	114.07	<0.0001**
C^2	3063.47	1	3063.47	62.30	<0.0001**
D^2	2227.51	1	2227.51	45.30	<0.0001**
Residual	590.07	12	49.17		
Lack of Fit	477.94	10	47.80	0.85	0.6513
Pure Error	112.11	2	56.05		

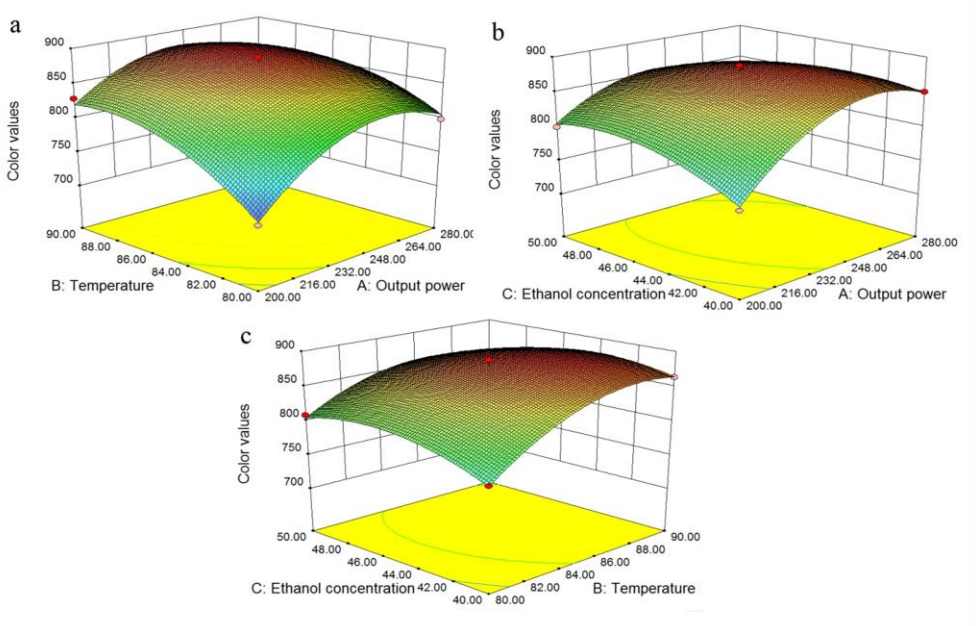

Fig. 3: 3D response surface and contour plots showing combined effect of extraction parameters on color values

Conclusion

The optimal value of pigment extraction (885.55 U/g**)** was obtained with the input power of 248.01 W, extract temperature of 87.37°C, ethyl alcohol concentration of 44.04% and solvent/solid ratio of 152.78.

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