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Microwave assisted extraction of anthocyanin from *Hibiscus rosa-sinensis*

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Abstract
This paper discusses about the optimization of process parameters for the microwave assisted extraction (MAE) of anthocyanin from *Hibiscus rosa-sinensis* using ethanol and water as solvent. The experimental design was formulated with the help of response surface methodology (RSM). The process parameters *viz. m*icrowave power, exposure time and solvent to sample ratio were optimized based on the yield of anthocynine content. The maximum yield of anthocyanin was obtained with the treatment combination of 400 W microwave powers, 180 s extraction time, 25 (v/w) solvent to sample ratio in water as a solvent, whereas with ethanol as a solvent a microwave power of 400 W, exposure time of 120 s with 25 (v/w) solvent to sample ratio reported highest anthocynine yield. Microwave assisted extraction of anthocyanin using acidified ethanol gave higher anthocyanin content (maximum 179.75 mgL^{-1}) compared to MAE assisted extraction of water (101.356 mgL^{-1}).

Keywords: Microwave power, extraction time, solvent to sample ratio

Introduction
Natural colourants are gaining importance in the food industry due to adverse human health effects associated with the use of synthetic colourants (Zarena and Sankar, 2012) [20]. The behavioral and neurological effects caused by the synthetic dyes used in the food industry may adversely affect human health (Arapitsas and Turner, 2010). Therefore, anthocyanins, can be considered as one of the best alternate for colouring due to their high potential in terms of low cost, high colourant power and high stability. Extraction of anthocyanins from a great variety of plants including cereals, flowers, fruits, and vegetables were practiced from the mid-1970s.
Hibiscus is popular flowering plants in the genus o*f Malvaceae* including in the mallow family. *Hibiscus rosa-sinensis* is colloquially known as China rose shoe black plant, Chinese hibiscus, and Hawaiian hibiscus. The hibiscus flowers are an excellent source of anthocyanin and widely available in India. Only very few studies are on extraction of anthocynine from *Hibiscus rosa-sinensis* are found reported.
A number of anthocyanin extraction methods have been suggested by researchers (Zarena and Sankar, 2012; Arapitsas and Turner, 2010; Welch, 2008, Wang and Weller, 2006; Gachovska *et al*., 2010; Wang *et al*., 2011) [20, 18, 17, 5, 16]. A number of pitfalls were reported for these methods such as extraction yield, high extraction time, degradation of anthocyanin due to high temperature (Khanh *et al.*, 2015).
Microwave radiations cause ionic conduction and rotation of permanent dipoles in liquid which results in molecular movements and an increase in temperature without altering their molecular structures (Kaufmann and Christen, 2002) [6]. Microwave extraction significantly reduces exposure timeand solvent utilization and improves the extraction efficiency (Eskilsson and Björklund, 2000). In this study microwave assisted extraction of anthocyanin using water and ethanol as solvents was carried out with the objective of optimizing the process parameters.

Materials and Methods
Plant material
Hibiscus flowers of the variety *rosa-sinensis* were collected locally. Petals were separated and dried at 60 °C for 2 h and powdered through hand crushing. The powders were kept in LDPE pouches for further analysis.

Microwave assisted extraction
Microwave assisted extraction of anthocyanins from *Hibiscus rosa-sinensis* was carried out by heating the solvent-sample mix in a microwave reactor (Microwave output 960 W, frequency

of 2450 MHz). *Hibiscus* powder of 1 g was measured and transferred into a beaker (250 mL) containing different volumes such as 20, 35, and 40 mL of solvent (deionized water /ethanol). Water and acidified ethanol (1% HCl in 95% ethanol) were used as solvent in the same proportion of solvent sample ratio explained above. Then the powder and solvent were thoroughly mixed using a stirrer for 5 min to provide sufficient diffusion of solvents in to *Hibiscus* powder. The solvent-sample mix were subjected to microwave treatment at various combination of process parameters *viz.* solvent/sample ratios (10, 25, 40 mL g^{-1}), microwave power (100, 400, 700 W), exposure times (60, 180 and 540 s in water, 60, 120, 180 s in case of acidified ethanol) (Table 1 and 2). After the treatment, the samples were allowed to cool down to room temperature followed by centrifugation at 5000 rpm for 10 min. The supernatants collected were filtered using Whatsman filter paper. A rotary evaporator was used to evaporate solvents (water/ethanol) retained in the sample material and the remaining extracts were collected for further studies.

Experimental design and statistical analysis

Response surface methodology (RSM) was employed to optimize the process conditions for the extraction of anthocyanin compounds from *Hibiscusrosa sinensis*. Design expert software version 7.0 was used for the experimental design and statistical analysis. One-way analysis of variance (ANOVA) was used to analyze variations between experimental data. The effect of all independent variables on the responses was studied using the analysis of the Box-Behnken method of three-level three-factor design. The independent variables *viz.* microwave irradiation power, exposure time and solvent/sample ratio were denoted as A, B and C, respectively. The lowest and highest values for exposure time were fixed as 1and 5 minutes for water and 1 and 3 for acidified ethanol. The sample to solvent ratio was set at 1:10 and 1:40. The microwave irradiation power was fixed between the power levels of 100 to 700W (Table1 & 2).The entire design includes 17 treatment combinations with five repetitions of the center point (Table 3&4), and the response function (Y) was divided into different components *viz*. linear, quadratic, and interactive.

$$Y=a_0+ a_1A+ a_2B + a_3C+ a_{12}AB+ a_{13}AC+ a_{23}BC+ a_{11}A^2+ a_{22}B^2+ a_{33}C^2 \text{…….(1)}$$

Where Y represents total anthocyanin yield; $a_0$stands for the model intercept; and the different coefficients such as coefficient of linear terms (a_1,a_2 and a_3), quadratic (a_{11} a_{22} a_{33}) and interactive terms(a_{12} a_{13} a_{23}) respectively; A, B and C are the coded independent variables. The effect and regression coefficients of individual linear, quadratic, and interactive terms were determined using the ANOVA tables. All terms in the polynomial were analyzed with F-value at probabilities of 0.001, or 0.05 and significant model terms were identified. The model was analyzed using R^2, predicted-R^2, adjusted $R^2$and PRESS (prediction error sum of squares). The regression coefficients were applied to conduct statistical calculations and response surface and contour plots were generated from the regression models (Myers and Montgomery 1995).

Table 1: Independent variables and their coded and actual values used for optimization (water).

Independent variables	Units	Code levels		
		-1	0	+1
Microwave irradiation power (A)	W	100	400	700
Microwave Exposure time (B)	s	60	180	300
Solvent to sample ratio (C)	mL g^{-1}	10	25	40

Table 2: Independent variables and their coded and actual values used for optimization (acidified ethanol).

Independent variables	Units	Code levels		
		-1	0	+1
Microwave irradiation power (A)	W	100	400	700
Microwave Exposure time (B)	s	60	120	180
Solvent to sample ratio(C)	mL g^{-1}	10	25	40

Determination of the Total Monomeric Anthocyanin (TMA)

The total anthocyanin yield can be calculated using a pH-differential assay as reported by Giusti and Wrolstad, (2001). The structural changes in the anthocyanin content during differences in pH values can be utilized for measurement of total monomeric anthocyanin. The extracted samples were diluted with distilled water to obtain adequate sample dilution. The buffer solutions with pH 1.0 (potassium chloride, 0.025M) and pH 4.5 (sodium acetate, 0.4M) were prepared. The maximum test portion added should be ≤10 mL (1-part test portion, 4 parts buffer). Absorbance of the samples was taken at both 520 and 700 nm for pH 1.0 buffer, and pH 4.5 buffers using a spectrophotometer. The absorbance of the samples was read versus a blank cell filled with distilled water. Calculated anthocyanin pigment concentration obtained is expressed as cyanidin-3-glucoside equivalents, as follows:

$$\text{TMAC (cyanidin-3-glucoside equivalents, mg/L)} = \frac{A \times MW \times DF \times 10^3}{eA} \text{ …….. (1)}$$

where A = (*A*520nm – *A* 700nm)pH 1.0 – (*A*520nm – *A*700nm) pH 4.5

Molecular weight of cyanidin-3-glucoside = 449.2 g/mol

DF = dilution factor; e = molar absorptivity ofcyanidin-3-glucoside.

Result and Discussion

Microwave assisted extraction of anthocyanin using water as a solvent

The experimental values obtained for various treatment combinations with the use of the Box-Behnken design were illustrated in Table 3. The lowest experimental value of 25.43 mg/L is achieved in run 16 (microwave power of 100 W within microwave exposure time of 60 s and solvent/sample ratio of 25 (mL g^{-1}). The highest value of 101.31 mg/L was observed in run 1 (microwave power of 400 W, microwave exposure time of 180 s and solvent/sample ratio of 25 mL g^{-1}).

Table 3: Box-Behnken experiment design matrix with observed values

Run	Microwave power(W)	Exposure time(s)	Solvent /sample ratio mL/g	Total anthocyanin content(mg/L)
2	100	300	25	49.44
3	700	60	25	76.432
4	400	300	10	83.923
5	700	300	25	89.432
6	400	180	25	98.345
7	100	180	40	29.45
8	400	300	40	60.367
9	400	180	25	100.995
10	400	60	10	72.167
11	700	180	40	83.453
12	100	180	10	53.765
13	400	180	25	96.543
14	400	180	25	99.567
15	400	60	40	54.456
16	100	60	25	25.432
17	700	180	10	87.456

The quadratic equation for the response anthocynin yield for regression model was obtained as follows.

anthocynin yield= 99.36+22.4A +6.83B -8.70C-2.75AB+5.08AC-1.46BC -21.69A^2-17.49B^2-14.14C^2…….(1)

Increase in microwave irradiation power,exposure time and decrease in solvent sample ratio may enhance the anthocyanin content.From the ANOVA table (3). Microwave power, exposure time and solvent material ratio were found to have significant effect on the total anthocyanin content($P \leq 0.05$).

The response surface optimization of total anthocynin yield indicated that the proposed model was adequate with nonsignificant lack of fit and satisfactory values of R^2. The R^2, Adj. R^2 and Pred. R^2 values for the total anthocynin yield was 0.9913, 0.9802and 0.8844 respectively. The reasonable agreement between pred. R^2 and Adj.R^2 depicts that model fits well. The coefficient of variation for the total anthocynin yield observed is 4.68. The probability (p) values of the regression model were more than 0.05 suggest that the model fits well for the experimental design.

The effects of microwave power (A) and exposure time (B) on the total anthocynine content are presented in Fig. 1.1. The increase in irradiation power (A) resulted in a positive change in the total anthocynine content. Similar trend was also observed for microwave exposure time (B) when the solvent sample ratio kept constant. The effects of microwave power and solvent sample ratio on the extraction of total anthocynin yield were depicted in Fig. 1.2, a linear increase in total anthocynin yield was observed with the increase of microwave power and solvent sample ratio at a fixed extraction time. Fig. 1.3 depicts that total anthocynin yield was affected by exposure time and solvent sample ratio, which denotes a clear increase in total anthocynin yield with the raise of the two variables.

The total anthocyanin content increased to a maximum of 101.356 mg/L at a power of 400W. Further increase in power from 400 to700 W reported a decrease in the anthocyanin content. This decrease at higher power could be due to higher boiling and evaporation rate of solvents in the microwave extraction (Desai and Parikh, 2012) [9]. At a low power density of 100 W the anthocyanin yield was found to be less. This trend might be due to the temperature being not enough to burst open the plant cells.

The increase in microwave power might have resulted in the cell wall rupture and hence improve the extraction yield because of ease of penetration of the solvent into the sample matrix (Mendes *et al.*, 2016). The rise in microwave power results in an increase in extract temperature. The higher temperatures resulted in rupture of cell walls and reduced diffusion resistance of anthocyanin due to intracellular pressure build up. On the other hand, the increase in temperature caused a reduced viscosity of solvent helped to increase the solubility of anthocyanin (Chan *et al.*, 2015; Xue *et al.*, 2018, Liazid *et al.*, 2011) [2, 8]

Total anthocyanin and C-3-G extraction showed an increase with the increase in solvent to sample ratio. A very high solvent sample ratio, a very slow increase in the TOTAL anthocynin yield was reported, because the solubility of samples would retard at low extract concentrations (Duan *et al.*, 2015) [3]. The exposure time rather than 180s with microwave power of 400 W in the current research might have accelerated the degradation rate of some thermo-sensitive bioactive compounds (Prommuak *et al.*, 2008; Trabelsi *et al.*, 2010) [15].

The plant cells require sufficient time to penetrate solvents into cells. On the other hand, medium microwave power is required to collapse the cell structures. The higher microwave power requires very short time for extraction of anthocyanin.

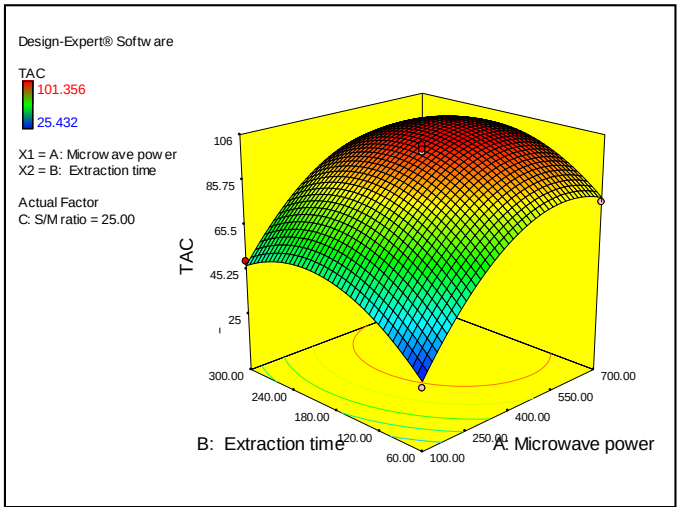

Fig 1.1: 3D surface plot of total anthocynin yield (mgL^{-1}) (Y) as a function of A (Microwave power (W), B (Exposure time (S).

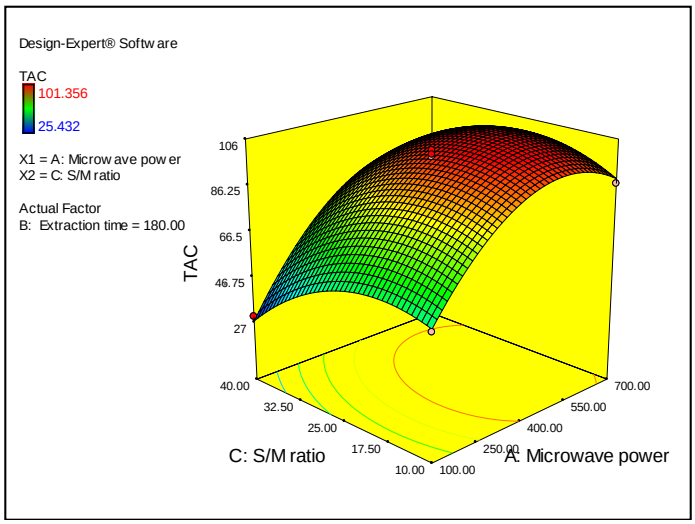

Fig 1.2: 3D surface plot of total anthocynin yield (mgL^{-1}) (Y) as a function of A (Microwave power (W), B (Solvent/sample ratio (mLg^{-1}).

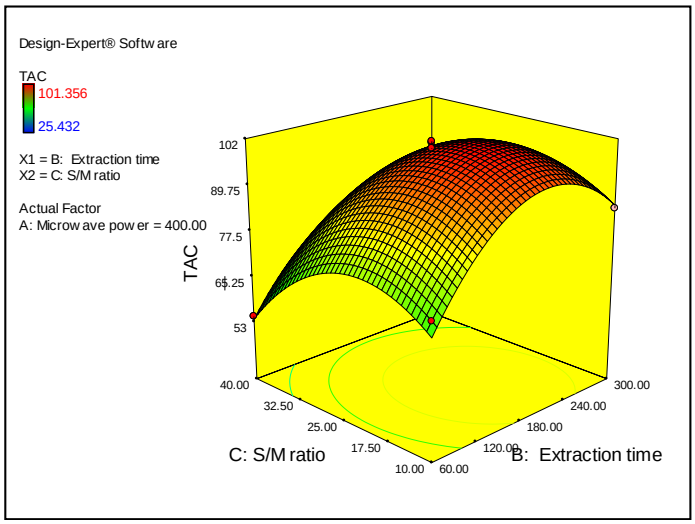

Fig 1.3: 3D surface plot of total anthocynin yield (mgL^{-1}) (Y) as a function of A (Exposure time (s), B Solvent/sample ratio (mLg^{-1}).

Microwave assisted extraction of anthocyanin using acidified ethanol as a solvent

The experimental values obtained for various treatment combinations with the use of the Box-Behnken design were illustrated in Table 5. The lowest experimental value of 103.95 mg/L is achieved in run 16 (microwave power of 100 W within microwave exposure time of 60 s and solvent/sample ratio of 25 (mL g^{-1}). The highest value of 179.754 mg/L was observed in run 1 (microwave power of 400 W, microwave exposure time of 180 s and solvent/sample ratio of 25 mL g^{-1}).

Table 4: Box-Behnken experiment design matrix with observed values (acidified ethanol as solvent)

Run	Microwave power (W)	Exposure Time (s)	Solvent/sample ratio (mL/g)	Total anthocyanin content (mg/L)
1	400	120	25	175.432
2	100	180	25	140.208
3	700	60	25	157.36
4	400	180	10	166.324
5	700	180	25	173.46
6	400	120	25	178.654
7	100	120	40	130.723
8	400	180	40	155.299
9	400	120	25	173.53
10	400	60	10	132.75
11	700	120	40	169.66
12	100	120	10	114.078
13	400	120	25	177.901
14	400	120	25	179.754
15	400	60	40	146.262
16	100	60	25	103.95
17	700	120	10	170.33

The quadratic equation for the response total anthocyanin content for regression model was obtained as follows.

$TAA= 177.05+23.48A +12.25B -1.93C-4.29AB -5.08AC-6.13BC-17.89A^2-4.67B^2-122C^2$…(2)

Following regression model was obtained to predict the total anthocyanin yield from *Hibiscus.* The independent variables such as microwave power, exposure time, solvent material ratio observed to have a significant effect on total anthocyanin content ($P \leq 0.05$).

The response surface optimization of extraction yield indicated that the proposed model was adequate with nonsignificant lack of fit and satisfactory values of R^2. The R^2, Adj. R^2 and Pred. R^2 values for the total anthocynin yield was 0.99, 0.98, and 0.91 respectively, which indicates good fitness of model due to the Pred. R^2 in reasonable agreement with Adj. R^2. The coefficients of variations for the total anthocynin yield observed 2.01. The probability (p) values of the regression model were more than 0.05 suggesting that the model fits well for the experimental design.

The highest extraction yield (179.754 mg (cyanidin-3-glucoside). L^{-1}) was observed at microwave power of 400 W, exposure time of 120 s and solvent/sample ration of 25 mLg^{-1},whereas, the lowest value of 103.95 (mg (cyanidin-3-glucoside) L^{-1}) was obtained in run 16 at microwave power of 100 W, exposure time of 60 s and solvent/sample ratio of to 25 mLg^{-1}. By multiple regression analysis on the experimental data, a mathematical model that is corresponding to the extraction of anthocynine could be obtained.

The effects of microwave power (A) and microwave exposure time (B) on the total anthocynin yield value keeping the solvent /sample ratio (C) constant are presented in Fig. 2.1. The rise in both irradiation power (A) and exposure time (B) increased the extraction yield gradually. The effects of microwave power and solvent sample ratio on the extraction of anthocynin were shown in Fig. 2.2. A linear increase in total anthocynin yield with the increase of microwave power and solvent sample ratio at a fixed exposure time was observed. The obtained response surface profiles Fig. 2.3 demonstrates the impacts of B (exposure time) versus C (solvent/sample ratio) at a constant level of microwave power (A). An increase in B from 60 up to about 156.43 s and C from 10 to about 25 (mL. g^{-1}) boosted total anthocynin yield value up to 185.112 (mg L^{-1}).

A similar trend of changes in process parameter as in water assisted MW extraction was observed in an acidified ethanol assisted extraction also. But when acidified ethanol was used as a solvent, it was observed that a reduced process time due to the low boiling point of ethanol which results in rapid boiling and evaporation of solvent.

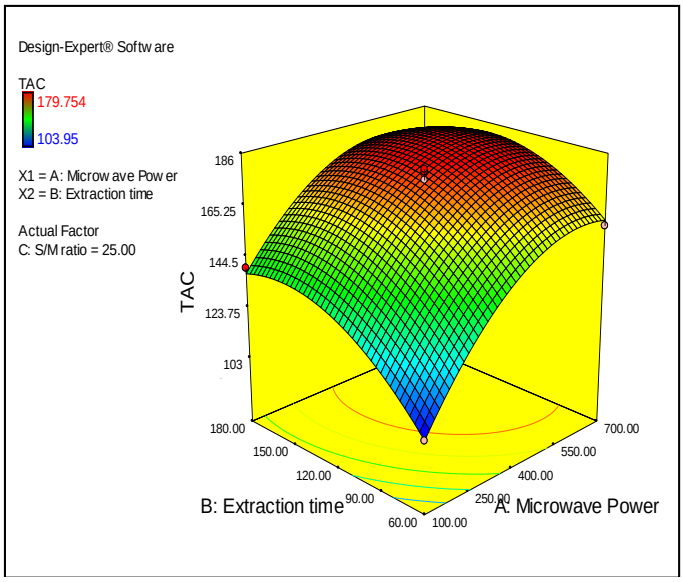

Fig 2.1: 3D surface plot of total anthocynin yield (mgL^{-1}) (Y) as a function of A (Microwave power (W), B (Exposure time (S).

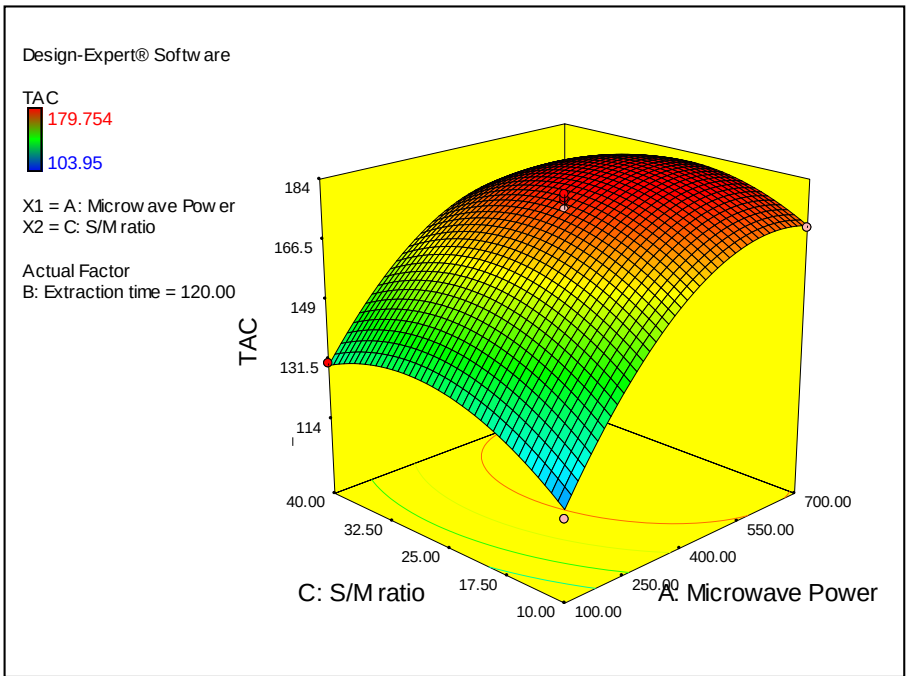

Fig 2.2: 3D surface plot of total anthocynin yield (mgL^{-1}) (Y) as a function of A (Microwave power (W), B (Solvent/sample ratio (mLg^{-1}).

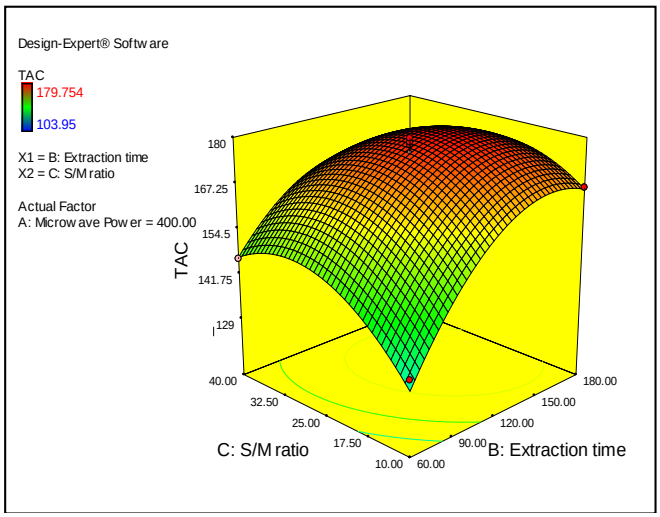

Fig 2.3: 3D surface plot of total anthocynin yield (mg L^{-1}) (Y) as a function of A (time (s), B (Solvent/sample ratio (mLg^{-1}).

Optimization of process variables

The optimal conditions for the microwave assisted extraction of anthocyanin for selected variables were obtained by solving the regression equation. The maximum desirability for total anthocynine yield was reported with microwave power of 497.62 W, exposure time of 151.18 s sand solvent to sample ratio of 24.20 mL g^{-1}. The corresponding response value for the predicted total anthocynine content with maximum desirability was 102.215 (mgL^{-1}) in water as a solvent. The optimized process conditions for anthocyanin extraction with acidified ethanol as a solvent were irradiation power of 587.81W, exposure time of 119.2 s and solvent-sample ratio of 22.67. The predicted total anthocyanin yield with maximum desirability from the optimized condition was 183.621 mg/L.

Conclusion

In the present study, the optimization of three major process variables such as irradiation power exposure time and solvent-sample ratio during microwave assisted extraction of anthocyanin from *Hibiscus Rosa-senesis* were carried out. The response surface analysis of process variables gave optimum process variables with maximum desirability for the best set of response properties such as exposure time of 180 s, a sample - solvent ratio of 1:25, and a microwave irradiation power of 400 W in case of water and exposure time of 120s, sample - solvent ratio of 1:25 and MW irradiation power of 400 W in case of acidified ethanol respectively. The study concludes that microwave assisted extraction of anthocyanin using acidified ethanol suggest higher optimum anthocyanin content (Maximum 179.75 mgL^{-1}) compared to MW assisted extraction using water as solvent (101.356 mgL^{-1}).

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