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Aqueous extraction of anthocyanin from hibiscus by coupling instantaneous pressure drop and microwave assisted process

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Abstract
This paper discusses about the optimization of process parameters for the extraction of anthocyanin from *Hibiscus rosa-sinensis* by instantaneous pressure drop pre- treatment and microwave assisted extraction process using water as solvent. The experimental design was formulated with the help of response surface methodology (RSM). The process parameters *viz.*, process pressure, treatment time, microwave power 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 1.12 kg/cm2 process pressure, 2 min treatment time, 960 W microwave powers, 27.5 solvent to sample ratio. Conventional aqueous extraction of anthocyanin resulted in less yield (Cold extraction- 85 mg/L, Hot extraction- 93.5 mg/L). The conventional method was found to be time consuming compared to coupled system which will take few minutes.

Keywords: Instantaneous pressure drop, microwave power, process pressure, treatment time, solvent-sample ratio

Introduction
As a result of the negative consequences that synthetic colourants have on human health, natural colourants are becoming more and more important in the food sector (Laleh *et al.*, 2006) [14]. Due to its tremendous potential in terms of low cost, strong colourant power, and high stability, anthocyanins are one of the best colouring alternatives. Hibiscus (*Hibiscus rosa sinensis*, Malvaceae) is a glabrous shrub widely grown in Kerala and has several forms with varying colours of flowers. However red coloured flower is preferred for medicinal uses. The flowers are observed to be promoters of hair growth and aid in healing ulcers and are effective in the treatment of arterial hypertension and to have significant antifertility effect (Jadhav *et al.*, 2009) [13]. Flowers are rich in bioactive compounds such as anthocyanin and other flavonoids which are responsible for its antioxidant, antibacterial, anti-inflammatory, hepatoprotective and anticholesterol activities. Calyx of Hibiscus contain cyanidin-3-sambubioside as major anthocyanin. Aside from their colouring potential, these compounds act as health promoting ingredients (Jabeur *et al.*, 2017) [12].
Researchers (Aryanti, 2017; Galvao *et al*., 2018; Welch, 2008; Wang and Weller, 2006; Chumsri *et al*., 2007; Wang *et al*., 2011) [3, 20, 19, 18] have proposed a number of anthocyanin extraction techniques. These methods were shown to have a variety of drawbacks, including low extraction yield, prolonged extraction times, and anthocyanin destruction at high temperatures (Cisse *et al*., 2012) [7].
A swelling operation using 'Instantaneous pressure drop' process prior to extraction could produce high quality anthocyanin by improving the hydration kinetics and capacity. The process is based on fundamental studies concerning thermodynamics of instantaneity (Allaf and Louka, 2004) [1]. Instantaneous pressure drop process (IPD) is a thermo- mechanical process that consists of subjecting a product to high pressure saturated dry steam for a short period of time followed by an abrupt pressure drop towards a vacuum. This abrupt pressure drops simultaneously triggers the autovaporization of the water, and swelling, with a possible rupture of cell walls and instant cooling of products, which stops thermal degradation. Subjecting the raw material to IPD process prior to anthocyanin extraction help the product get higher global diffusivity by improving the hydration kinetics leading to easy leach out of high quality anthocyanin through swelled cells (Allaf *et al.*, 2013) [2]. Microwave energy could be used effectively to mediate extraction of anthocyanin in place of conventional aqueous methods. During microwave heating of food materials, the internal heating of the in-situ water within the plant material by the microwaves leads to the rupture of the cells freeing the anthocyanin and bioactive compounds easily.

The use of microwaves for extraction of active components could result in enhanced performance in terms of quality and quantity such as high extraction and efficiency, less extraction time and yield. (Chan *et al.*, 2011) [5].

The application of an Instantaneous Pressure drop to the size reduced Hibiscus petals could result in increased structure swelling rate and higher diffusivity constant. This coupled with subsequent aqueous microwave assisted extraction could provide efficient extraction of high quality anthocyanin in less time, energy and cost. In this study Coupled IPD and microwave assisted extraction of anthocyanin from Hibiscus using water as solvent was carried out with the objective of optimizing the process parameters.

Materials and Methods Plant material

Red co;oured fresh Hibiscus flowers (*Hibiscus rosa sinensis*) were collected from nearby locality. Cleaned and petals were separated and dried in a hot air tray drier at 60°C for 2 h. The dried petals were crushed in a 900W power grinder and kept it in LDPE pouches at ambient temperature for further use.

Conventional extraction process

Cold extraction was done by soaking the dried flower sample in cold water for 24 h and then filtered off using sterile whatman filter paper. Hot extraction was done by soaking the sample at boiling temperature for 30 min, and then filtered through sterile filter paper. For ethanol extraction of anthocyanin, sample was placed in ethanol solvent in a conical flask and then kept in a rotary shaker at 250 rpm for 30 min and then filtered with the help of muslin cloth. The extracts was then collected in amber coloured bottles and stored under refrigerated condition.

IPD pre- treatment process

In this study an IPD system would be used for pre-treatment of Hibiscus towards the extraction of anthocyanin. The IPD system subjects the material to saturated steam, during a short time, followed by an instant pressure drop which will lead to auto evaporation of water, product texturing, and cooling. It induces the product to an abrupt transition from high steam pressure towards vacuum. This would enable the biomaterial to get higher global diffusivity and easy leach out of high quality anthocyanin through swelled cells.

IPD system consists of steam generation system, processing vessel, vacuum pump, vacuum tube and an IPD valve. The steam generator used was filled half with water, closed and heated by LPG. Steam control valve was closed during the steam generation. Two grams of dried Hibiscus powder was transferred into an open steel pan and placed inside the processing vessel and closed it. The vacuum pump was switched on and thereby an initial vacuum was created in vacuum tank. An initial vacuum was also established in the processing vessel as air is drawn out from the processing vessel via polyurethane hose by opening pneumatic valve. The initial vacuumisation is carried out to facilitate and mediate the close exchange between the incoming steam and the product surface. Then the pneumatic valve was closed.

Steam from steam generator regulated by the steam control valve, was allowed to pass to the processing vessel via silicon tube. Saturated steam is injected into the reactor at a fixed process pressure level as per the experimental design. Heat transfer is performed mainly by steam condensation leaving its latent heat which assures a very high coefficient of heat transfer. The steam control valve was then closed and the material was subjected to an instant controlled pressure drop towards vacuum by opening the IPD valve. The resulting auto vaporisation induces an instant cooling of the samples too. After a vacuum stage as per the treatment time as defined in experimental design, the pressure in the vessel is released by opening the pressure release valve and the sample was recovered from the processing vessel for the microwave assisted extraction process.

Microwave assisted extraction

The IPD pre- treated Hibiscus petals were collected into a beaker. In this study, aqueous extraction of anthocyanin from Hibiscus was carried out by adding measured quantity of distilled water to each sample as per the experimental plan. The microwave power level was set in the control panel of microwave reactor for various treatment conditions as per the experimental design. The beaker was placed inside the microwave reactor and subjected to microwaves at preset power level thereby leaching out the anthocyanin into the solvent matrix. 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 water retained in the sample material and the remaining extracts were collected for further studies (ashitha *et al*., 2020)

Conventional aqueous extraction of anthocyanin

Conventionally anthocyanin was extracted by cold extraction and hot extraction in aqueous medium. Cold extraction was done by soaking the dried flower sample in cold water for 24 h and then filtered off using sterile whatman filter paper. Hot extraction was done by soaking the sample at boiling temperature for 30 min, and then filtered through sterile filter paper. A rotary evaporator was used to evaporate water retained in the sample material. The extracts was then collected in amber coloured bottles and stored under refrigerated condition (Ruban and Gajalakshmi, 2012).

Experimental design and statistical analysis

To improve the process conditions for extracting anthocyanin compounds from Hibiscus rosa sinensis, response surface methodology (RSM) was used. Version 7.0 of Design Expert software was utilised for the statistical analysis and experimental planning. Analysis of variance (ANOVA), a one-way statistical method, was employed to examine differences between experimental data. The analysis of the Box-Behnken method of the three-level, three- factor design was used to examine the impact of all independent factors on the responses. The independent variables *viz.* process pressure, treatment time, solvent- sample ratio and microwave power were denoted as A, B, C and D respectively. The lowest and highest values for process pressure were fixed as 0.56 and 1.12 kg/cm2. The range for treatment time was 1 to 3 minutes and the solvent to sample ratio was set at 15 to 40 ml/g. The microwave irradiation power was fixed between the power levels of 480 to 960W (Table1). The response function (Y) was broken down into three components, *viz.* linear, quadratic, and interactive, and the overall design contains 29 treatment combinations with five repetitions of the centre point (Tables 2).

$$Y = b0 + b1 X1 + b2 X2 + b3 X3 + b4 X4 + b11 X12 + b22 X22 + b33 X32 + b44 X 2^4 X1 X3 + b14 X1 X4 + b23 X2 X3 + b24 X2 X4 + b34 X3 X4 \quad \text{……..(1)}$$

Where Y refers for total anthocyanin yield, b0 stands for the model intercept, and other coefficients such as coefficient of linear terms (b1, b2, b3 and b4), quadratic (b11, b22, b33 and b44) and interactive terms (b12, b13, b14, b23, b24 and b34) correspondingly. X1, X2, X3 and X4 are the
coded independent variables. Individual linear, quadratic, and interaction factors' effects and regression coefficients were calculated using ANOVA tables. With the help of an F-value analysis at probabilities of 0.001 or 0.05, all of the polynomial's terms were recognised and significant model terms were identified. Using R2, predicted-R2, adjusted-R2, and PRESS (prediction error sum of squares), the model was examined. The statistical calculations using the regression coefficients produced response surface and contour maps from the regression models (Montgomery 2001) [16].

Table 1: Independent variables and their coded and actual values used for optimization

Independent variables	Units	Coded levels		
		-1	0	+1
Process pressure (X1)	kg/cm2	0.56	0.84	1.12
Treatment time (X2)	min	1	2	3
Solvent- sample ratio (X3)	W	15	27.5	40
Microwave power (X4)	ml/g	480	720	960

Determination of the Total Monomeric Anthocyanin (TMA)

According to Giusti and Wrolstad (2001) [10], a pH-differential assay can be used to determine the total anthocyanin output. The total monomeric anthocyanin can be measured using the structural alterations in the anthocyanin content caused by variations in pH levels. To achieve an acceptable sample dilution, the extracted samples were diluted with distilled water. pH 1.0 (potassium chloride, 0.025M) and pH 4.5 (sodium acetate, 0.4M) buffer solutions were created. A maximum of 10 mL should be added as the test portion (1-part test portion, 4 parts buffer). Using a spectrophotometer, the samples' absorbance was measured at wavelengths of 520 and 700 nm for pH 1.0 buffer and pH 4.5 buffers. The samples' absorbance was measured in comparison to that of a blank cell filled with distilled water. The amount of calculated anthocyanin pigment is represented as cyanidin-3-glucoside equivalents.

TMAC (cyanidin-3-glucoside equivalents, mg/L) was determined by

Where A = (A530nm – A700nm) pH 1.0 – (A530nm - A700nm) pH 4.5 …… (3)

Where A is the absorbance of sample, MW is the molecular weight of cyanidin– 3-glucoside
(449.2 g/mol), DF is the dilution factor and E is the molar absorptivity of cyanidin-3-glucoside.

Results and Discussion

Coupled IPD and Microwave assisted extraction of anthocyanin

Table 2 provides an illustration of the experimental results for several treatment combinations using the Box-Behnken design**.** The anthocyanin yield varied between 82.05 and
126.88 mg/ L of the sample. The maximum anthocyanin yield was obtained at a process pressure of 1.12 kg/cm2, treatment time of 2 minutes, microwave power of 960 W and solvent-sample ratio of 27.5 ml/g. Minimum anthocyanin yield of 82.05 mg/L was observed in run 29 (process pressure of 0.56 kg/cm2, treatment time of 2 minutes, microwave power of 720 W and solvent- sample ratio of 15 ml/g).

Table 2: Box- Behnken experimental design matrix with observed values

Run	Process pressure Kg/cm2	Treatment time, min	Microwave power, W	Solid-solvent ratio, ml/g	Anthocyanin yield, mg/L
1	0.84	2	720	27.5	115.00
2	0.56	1	720	27.5	85.21
3	0.84	3	960	27.5	117.13
4	0.84	3	720	40	123.02
5	0.84	3	480	27.5	112.74
6	0.84	1	720	40	104.47
7	0.56	2	720	40	98.61
8	1.12	2	720	40	117.56
9	1.12	2	480	27.5	114.20
10	0.84	3	720	15	114.91
11	0.84	2	960	15	111.75
13	0.56	2	480	27.5	95.21
14	1.12	2	720	15	112.74
15	0.84	1	480	27.5	97.40
16	0.84	1	960	27.5	110.02
17	1.12	3	720	27.5	125.13
18	0.84	2	480	15	90.45
19	0.56	3	720	27.5	109.38
20	0.84	2	480	40	105.42
21	0.84	2	720	27.5	114.98
22	1.12	2	960	27.5	126.88
23	0.84	1	720	15	88.53
24	0.84	2	960	40	125.13
25	1.12	1	720	27.5	98.56
26	0.56	2	960	27.5	109.38
28	0.56	2	720	15	82.05

The following is the quadratic equation for the response anthocynin yield using the regression model:

Anthocyani yield = 115+ 12.72 A+ 8.46 B+ 4.98 C+ 3.52 D– 5.17 AB- 4.96 AC -4.38 AD -
4.22 C2- 6.89 D2 ……(4)

Where, A is the Process pressure, B is the Treatment, C is the Microwave power and D is solvent- sample ratio.

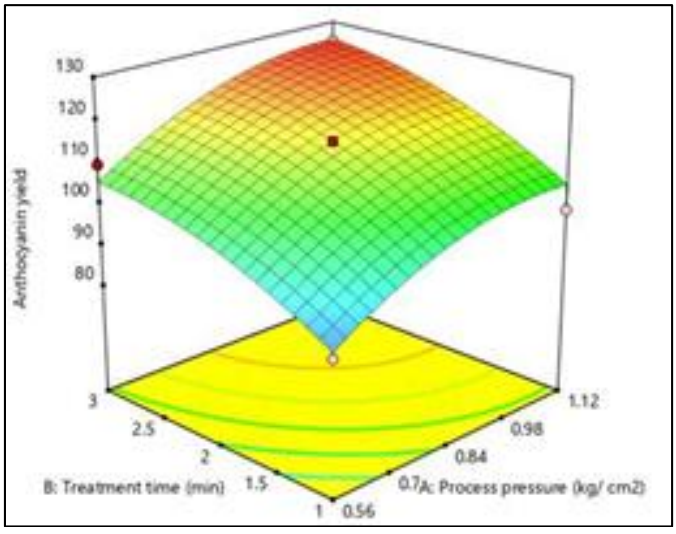

Fig 1: Effect of anthocyanin yield as a function of process pressure (kg/cm2) and treatment time (min)

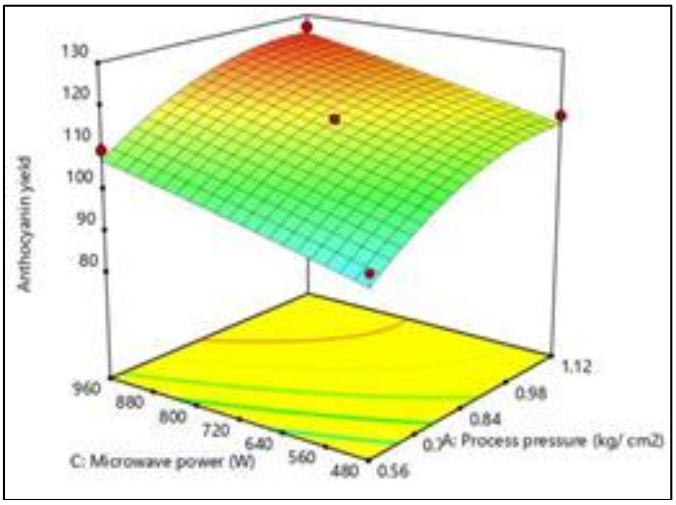

Fig 2: Effect of anthocyanin yield as a function of process pressure (kg/cm2) and microwave power (W)

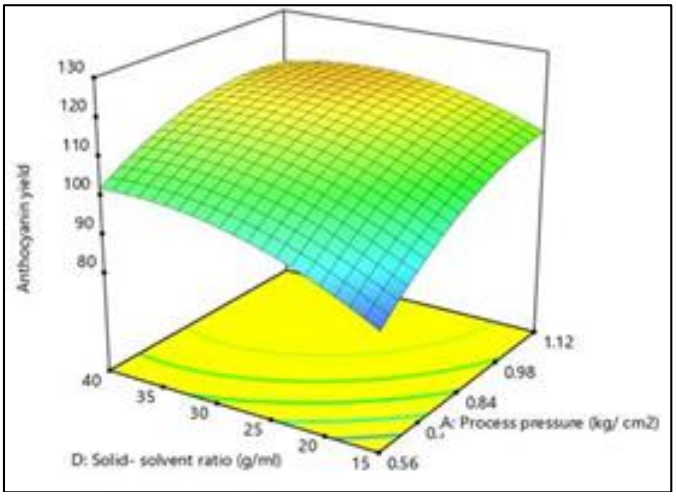

Fig 3: Effect of anthocyanin yield as a function of process pressure (kg/cm2) and solvent- sample ratio (ml/g)

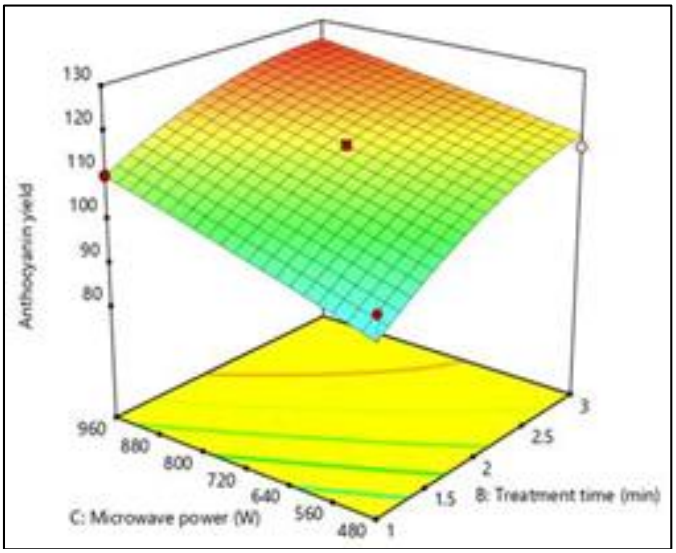

Fig 4: Effect of anthocyanin yield as a function of treatment time (min) and microwave power (W)

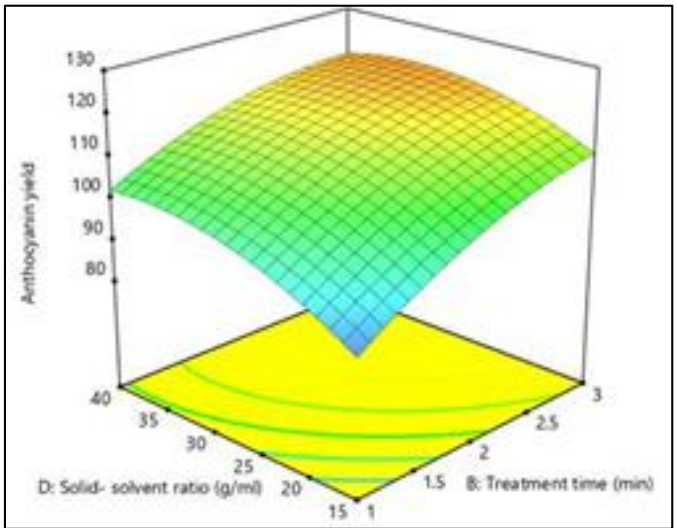

Fig 5: Effect of anthocyanin yield as a function of treatment time (min) and solvent- sample ratio (ml/g)

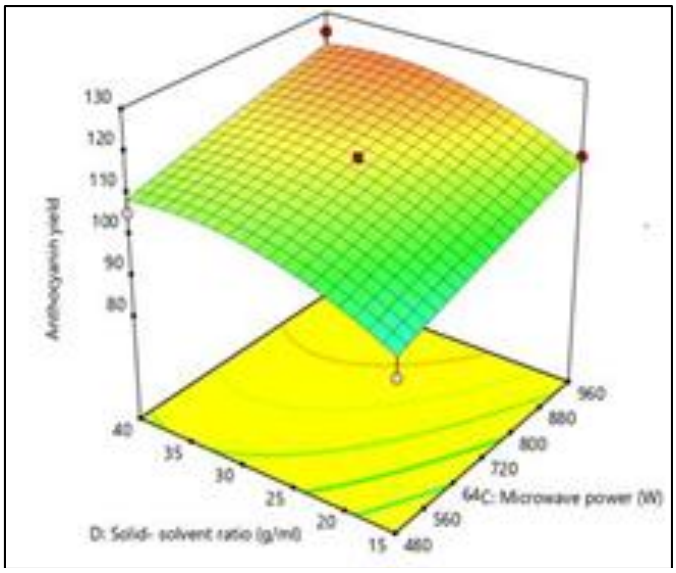

Fig 6: Effect of anthocyanin yield as a function of microwave power (W) and solvent- sample ratio (ml/g)

The ANOVA table (2) shows that the total anthocyanin concentration was significantly affected by microwave power, exposure period, and solvent material ratio ($P \leq 0.05$). The response surface optimization of otal anthocynin yield revealed that the suggested model was suitable, with nonsignificant lack of fit and acceptable R2 values. The values of R2, Adjusted R2 and Predicted R2 for the anthocyanin yield were 95.41, 90.82 and 73.57% respectively. The good fit of the model is indicated by the fair agreement between predicted R2 and Adjusted R2. The observed total anthocynin yield had a 4.68 coefficient of variation. The probability (p) values of regression model were more than 0.05, which indicates that the model fits the experimental design well. Lack of fit was insignificant and F-value suggested that the model was significant at one per cent and five per cent level of significance. Therefore, second order model was adequate in describing the anthocyanin yield.

It could be perceived from the Figure 4.1 (a), (b) and (c) that anthocyanin yield increased when process pressure was increased from 0.56 kg/cm2 to 1.12 kg/cm2. The saturated steam, during the compression step, heats the product and may increase slightly the product humidity. Due to the instant pressure drop, an abrupt auto evaporation of water cools the product and allowed the cells to swell up and easy leach out of anthocyanin was effected. The structure collapse, stickiness, and agglomeration were never observed in the temperature/water content domain below glass transition (Hamoud-Agha *et al*., 2019).

A maximum anthocyanin yield was observed when both the process pressure and solid- solvent ratio were the highest with the limits of the designed levels (Figure 4.1 c). The total anthocyanin content recorded a maximum of 126.88 mg/L at a microwave power level of 720W. Further increase in power from 720 to 960 W showed a decreasing anthocyanin content (Figure 4.1 d). This decrease at higher power could be due to higher boiling and evaporation rate of solvents in the microwave extraction (Desai and Parikh, 2012). At a low power of 480 W the anthocyanin yield was found to be less which might be due to the temperature being not enough to burst open the plant cells.

Due to the ease with which the solvent was able to penetrate the sample matrix due to the increase in microwave power, the extraction yield may have improved (Mendes *et al*., 2016). The extract temperature rises as a result of the increased microwave power. Due to an increase in intracellular pressure, higher temperatures caused cell walls to rupture and lowered anthocyanin diffusion resistance. On the other hand, the reduction in solvent viscosity brought about by the rise in temperature served to boost the solubility of anthocyanin. (Chan *et al.*, 2011) [5].

The extraction of C-3-G and total anthocyanin increased as the ratio of solvent to sample increased. Because the solubility of the materials would slow down at low extract concentrations, a very high solvent sample ratio was used, leading to a very sluggish increase in the TOTAL anthocynin yield. (Duan *et al*., 2015) [9]. The Figure confirms that higher solute to solvent ratio significantly yielded higher anthocyanin content from the Hibiscus extract. This is a general behaviour during extraction. By increasing the volume of solvent, the dissolved extracted materials would be higher thus resulting in higher extraction yield. In addition, the mass transfer parameter is also affected by the volume of solvent. The higher the solvent volume results in larger mass transfer and more accelerated diffusion into the medium (solvent) (Xu *et al*., 2016) [21]. Similar results were also found in extraction of anthocyanin by ultrasound assisted extraction from mulberry (Zhou *et al*., 2015) [22].

The plant cells need enough time for the solvents to enter the cells. On the other hand, to collapse the cell structures, medium microwave power is needed. The extraction of anthocyanin demands a greater microwave power and takes very little time.

Conventional extraction of anthocyanin

Conventional aqueous extraction methods resulted in less quality and lower yield of anthocyanin. Cold extraction of Hibiscus petals gave 85 mg/L of anthocyanin. Hot aqueous extraction resulted 93.5 mg/L of anthocyanin. The colour of hot extracted anthocyanin was reduced due to thermal degradation and also the conventional method was found to be time consuming when compared to coupled IPD and microwave assisted system.

Optimisation of process variables

By resolving the regression equation, the ideal circumstances for the coupled IPD and microwave aided extraction of anthocyanin for particular variables were discovered. From the analysis, a process pressure of 0.905 kg/cm2; treatment time of 2.272 minutes; microwave power of 707.302 W and solvent- sample ratio of 30.415 ml/g were found to be optimum values. The maximum desirability for total anthocynine yield was recorded as 0.936. The equivalent response value in water as a solvent was 119.858 (mg/L) for the corresponding total anthocynin content with highest desirability.

Conclusion

During the Instantaneous pressure drop pre- treatment and microwave-assisted extraction of anthocyanin from *Hibiscus Rosa-senesis*, the present work optimised four important process factors, including process pressure, treatment duration and solvent-sample ratio. The best set of response properties were determined by the response surface analysis of process variables. For example, process pressure of 0.905 kg/cm2; treatment time of 2.272 minutes; microwave power of 707.302 W and solvent- sample ratio of 30.415 ml/g resulted in anthocyanin yield of 119.858 mg/L. The study finds that, coupled instantaneous pressure drop pre- treatment and microwave assisted extraction of anthocyanin yielded higher compared to conventional aqueous extraction of anthocyanin.

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