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EXTRACTION, CHARACTERIZATION AND OPTIMIZATION OF BETALAINS FROM RED BEETROOT USING RESPONSE SURFACE METHODOLOGY

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The objective of the current research was to assess the optimal extraction conditions with respect to ethanol concentration and time to recover betalains from red beetroot (*Beta vulgaris* L.). Purposely, betalains were extracted from beetroot using water and ethanol based solvent modules at three different time intervals *i.e.,* 20, 40 and 60 min and the obtained extracts were analyzed for betacyanins, betaxanthins and total betalains. The obtained data was further analyzed using three level-two factor central composite design using response surface methodology. The data was fitted to second order polynomial equation by applying multiple regression analysis. The optimal conditions were obtained using maximum desirability function that found to be 31.03, 45.01 and 35.34% aqueous ethanol for betacyanins, betaxanthins and total betalains, respectively whilst, optimum extraction time was recorded to be around 43 min. The experimental values were close to the predicted values that proves the adequacy of the model employed. It was suggested that aqueous-ethanol is suitable for maximal recovery of beetroot betalains instead of ethanol or water as a sole solvent.

Keywords: Beetroot, functional food, phenolic acids, bioactive moieties, betacyanins, betaxanthins, extraction solvent.

INTRODUCTION

Red beetroot (*Beta vulgaris* L.) is a biennial herbaceous plant of greater food and medicinal value. Moreover, it resides amongst top 10 vegetables based on total phenolic density. It possesses various phenolic acids like p-coumaric, ferulic, vanillic, protocatechuic, syringic and p-hydroxybenzoic acids (Janiszewska, 2014). Red beetroot is an important functional food primarily containing diversity of coloring compounds with considerable biological activity (Esatbeyoglu *et al.*, 2015). These pigmented compounds are employed in food industries for value addition as additives. Various other industries such as pharmaceutics, cosmetics and livestock feed are also getting benefit from these compounds in a variety of ways (Boo *et al.*, 2012). The betalains in beetroot are considered as the dominant moieties (de Zwart *et al.*, 2003; Vali *et al.*, 2007; Dias *et al.*, 2009) especially betacyanins and betaxanthins (Esatbeyoglu *et al.*, 2015; Singh and Hathan, 2014).

The utilization of biologically active moieties from source materials to produce functional food ingredients, dietary supplements, nutraceutics and pharmaceutical & cosmetic applications involves series of steps decisive in final product quality. Amongst, extraction of the compound of interest is first and most crucial step that involves highly precise choice and control of allied unit operations vital to ensure optimal recovery of potentially active constituents (Abascal *et al.*, 2005). As the qualitative and quantitative analyses of phytochemicals rely on use of appropriate extraction procedure, hence the selection of extraction technique is important, and its allied requirements must be taken into consideration for targeted isolation of the compounds. Amongst various factors directly affecting the quality of plant extracts, properties of plant matrix, solvent being used, pressure, temperature of the extraction medium and time length are of immense contemplation (Hernandez *et al.*, 2009). Characteristically, compounds with biologic worth are present with other phytochemicals that may have conjugated structures. However, plant bioactives can be found and characterized in various plant parts like flesh, leave, flower, stem, seeds and fruits (Azmir *et al.*, 2013).

For extraction of bioactives, solvent extraction models are commonly employed owing to their easier use, applicability in various modules and efficiency. Generally, yield of an extract via chemical extraction procedure primarily depends upon the type of solvent and its polarity, time & temperature during the process, physical properties of plant material and sample to solvent ratio alongside its chemical composition. As the plant materials contain several simple to complex types of phenolic moieties, hence their solubility is governed by polarity of solvent being used and chemical nature of plant material. On the other hand, these bioactives may also be linked to other plant components like proteins and carbohydrates that may require certain additional steps to get purified extract. In this respect, no single method can extract all biologically active constituents from the sample.

Nevertheless, better control of all the variables can ensure good quality extract with least destruction of compounds to estimate their contents and activities. It has been noted that the rate of polyphenols extracted from a sample depends upon selection of right solvent. Commonly, ethanol, methanol, ethyl acetate and acetone solely or in various combinations to each other or some other solvents like water are used in extraction of bioactives from plant materials (Xu and Chang, 2007). In general, lower molecular weight phenolic compounds are efficiently extracted by using methanol. On the other hand, aqueous acetone performs better for extraction of flavanols with higher molecular weights (Dai and Mumper, 2010). However, ethanol is considered a good solvent with an additive property of being consumable and safer for human being (Shi *et al.*, 2005).

To isolate betalains from beetroot or other plants, the plant material is generally ground or macerated followed by extraction of beet pigments using water at cold or room temperature. While, in certain cases ethanol or methanol (20-50% v/v) is used along with water to achieve maximum extraction. Conversely, fermentation may also be needed to reduce the sugar levels in the extract ultimately increasing the betacyanins content in the final extract. Nonetheless, a slight acidification is required to precipitate betacyanins. Moreover, betanin degradation is quite rapid so it is necessary to avoid complex pigment disintegration. Purposely, prolonged cold water extraction under darkness may be employed to overcome this condition (Delgado-Vargas *et al.*, 2000).

In addition, extraction temperature and time also affect the efficiency of phenolic compounds recovery from plants which demonstrates divergent effects of oxidation of analytes and solubilization in solvent phase (Robards, 2003). A controlled increase in temperature during extraction enhances solubility of targeted compound by promoting mass transfer rate and solubility. However, surface tension and viscosity of the solvents decrease with increase in temperature aiding in penetration of solvent through plant matrices and promoting extraction rate. Nonetheless, higher temperatures along with longer extraction time may increase the risk of bioactives degradation ultimately lowering the qualitative and quantitative phenolic yields. To overcome these challenges, conventional methods are often coupled with recent technologies like microwaving, sonication, accelerated & pressurized solvent extractions that result in more specific and shorter time procurement of extracts. Hence, extraction procedure must be critically considered and designed in accordance to the specific needs of expected outcomes (Dai and Mumper, 2010). Hence, the present research was designed to estimate optimum ethanol concentration and time to extract red beetroot betalains.

MATERIALS AND METHODS

***Procurement and preparation of raw material*:** Fresh red beetroot was procured from local market and graded based on uniformity in size, color, shape and absence of any physical damage. The raw material was washed to remove dust, dirt or any allied impurities. Afterwards, peeling of the red beetroot was carried out. The prepared commodity was stored at refrigeration temperature (4-6ºC) until further use for experiments.

***Extraction of beetroot bioactive moieties*:** The bioactive moieties of red beetroot were extracted using solvents; water, ethanol and aqueous-ethanol (50% v/v). This selective solvent combination gives an ethanolic content variation of 0, 50 and 100%. Purposely, freshly chopped red beetroot was separately macerated in respective solvents acidified with 0.5% acetic acid by volume. The mass to solvent volume ratio was 1:5. The samples were ultrasonicated at 37 kHz for 15 minutes at 55°C using ElmaSonic (E 60H, Cousins, UK) followed by agitation at 320 rpm at 40°C. The extracts were recovered after three specified intervals *i.e.*, 20, 40 and 60 min followed by filtration through filter paper. Afterwards, filtered extracts were concentrated using Rotary Vacuum Evaporator (EYELA, N-N series, Japan) at 40°C. Further concentration was carried out by lyophilization process using laboratory freeze dryer (Martin Christ, Germany). The lyophilized extracts were used for betalains analyses.

***Characterization of betalains*:** The concentrated extracts were subjected to the determination of two classes of betalains namely betaxanthins and betacyanins (Ravichandran *et al.*, 2013). Accordingly, the extracts were diluted using 0.05M phosphate buffer of pH 6.5 to obtain the absorbance values between 0.8-1.0. Certainly, the absorbencies of the prepared extracts were then noted at 480 nm for betaxanthins and 538 nm for betacyanins. Betaxanthins and betacyanins were calculated using the following expressions;

$$\text{Betaxanthins (mg/L)} = \frac{\text{A} \times \text{DF} \times \text{MW} \times 1000}{\text{e} \times \text{l}}$$

$$\text{Betacyanins (mg/L)} = \frac{\text{A} \times \text{DF} \times \text{MW} \times 1000}{\text{e} \times \text{l}}$$

Where, A : Absorbance value at 480 nm for betaxanthins and 538 nm for betacyanins, DF : Dilution Factor, MW : Molecular weight 308 g/mol for betaxanthins and 550 g/mol for betacyanins, e : Extinction coefficient 48,000 L/mol.cm for betaxanthins and 60,000 L/mol.cm for betacyanins, l : Path length (1 cm)

The total betalains were calculated by adding the betacyanins and betaxanthins contents. The results were expressed as mg/g of the dried extracts obtained from red beetroot.

***Experimental design for model fitting and response surface analysis*:** Central Composite Design (CCD) was employed for evaluation of response surface profile based on data collected from experiments. The independent variables described as X_1 and X_2 for ethanol concentration (%) and time (min), respectively were coded at three levels as described in Table 1. The number of experiments (N) were calculated based on the expression $N = 2k(k-1) + C_0$ using 2 central points

that accounts for a total of 10 experiment runs as *k* represents number of factors.

Table 1. Independent variables of experimental design: coded and actual.

Variables	Factors (X)	Levels		
		-1	0	1
Ethanol Concentration (%)	X_1	0	50	100
Extraction Time (Min)	X_2	20	40	60

The association between the response and the variables was established as a function of factors [Y= $f(x_1, x_2) + e$] where e represents the error describing differentiation. The response surface behavior was evaluated based on response function of (Y_i) by employing a second-order polynomial equation.

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{i} \sum_{<j=2}^{k} \beta_{ij} x_i x_j + e_i$$

Where Y corresponds to the response whilst, x_i and x_j are independent variables ranging from 1 to k (number of independent variables) whilst the interaction coefficients are described by β_i, β_{jj} and β_{ij} for linear, quadratic and second order terms, respectively whereas β_0 represented the model intercept and e_i describes the error.

***Statistical analysis*:** All the data obtained regarding characterization of betalains were subjected to analysis of variance (ANOVA) with two factors factorial under completely randomized design followed by mean comparisons through Tukey's honest significant difference (HSD) test using Statistix 8.1 statistical package. For obtaining values of optimal extraction parameters, response surface methodology (RSM) was employed using SAS JMP (version 10) statistical software. Microsoft Excel (v2013) was used for data handling and generation of bar charts.

RESULTS AND DISCUSSION

***Characterization of betalains*:** The characterization of betalains involved the determination of betacyanins (red to violet), betaxanthins (yellow to orange) and total betalains in concentrated extracts obtained from red beetroot using different solvents and time combinations. In this regard, mean values regarding betalainic constituents of beetroot extracts are illustrated in Fig. 1 and 2 representing effects of solvent and time on different fractions of the pigments. The factorial analysis depicted highly significant (P<0.01) effect of both factors (solvent and time) on recovery of betalainic constituents whereas, their interactive effect was found to be non-significant. Amongst the solvents, betacyanins being water soluble moieties were efficiently extracted in aqueous extracts as depicted by mean value 54.32±2.74 mg/g of extract (Fig. 1). Whilst, the use of aqueous-ethanol resulted in highest extraction of betacyanins (60.21±2.39 mg/g). Conversely, ethanolic extracts exhibited least betacyanins *i.e.* 14.30±1.72 mg/g. As a function of time, maximum amount of betacyanins was obtained in extracts recovered at 40 min of extraction time (49.34±2.38 mg/g) followed by a depreciation in betacyanins at 60 min (43.05±2.34 mg/g). Also, lowest amount of this pigment was observed at 20 min (Fig. 2). These results depict a curvature response of pigment recovery as a function of time in which the betacyanins yield was increased while increasing the time and after 40 min it started decreasing. As an interactive effect of solvent and time, maximum betacyanins were recovered by using aqueous-ethanol combination at 40 min (69.69±2.36 mg/g).

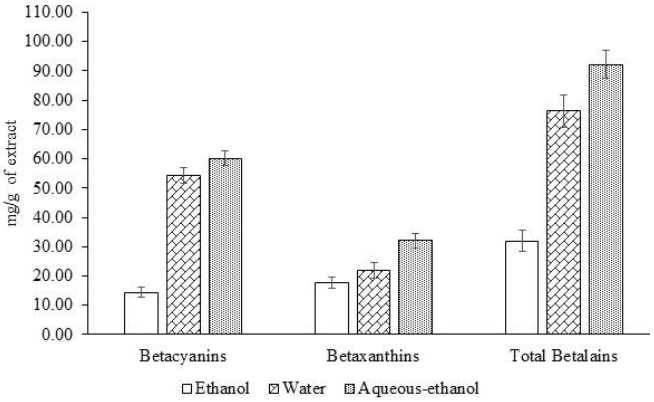

Figure 1. Effect of solvent on betalains content of red beetroot extracts.

Mean values pertaining to betaxanthins in beetroot extracts depicted highest concentrations in aqueous-ethanol solvent as 32.06±2.53 mg/g followed by water and ethanolic extracts 22.01±2.73 and 17.69±1.77 mg/g, respectively (Fig. 1). Over the time, similar pattern of increment in betaxanthins was observed as in case of betacyanins representing highest levels at 40 min extraction interval (28.66±2.33 mg/g) trailed by 60 min (23.56±2.58 mg/g) and 20 min (19.55±2.12 mg/g) as illustrated in Fig. 2. These results also depicted a curvature response with respect to time intervals *i.e.* increasing trend towards 40 min intervals whilst, decreasing pattern after 40 min of extraction time. As an interactive effect, maximum concentrations of betaxanthins (35.41±2.25 mg/g) were recorded in aqueous-ethanol based solvent model at 40 min extraction time.

The total betalains were the combined contents of betacyanins and betaxanthins. Regarding total betalains harvested through various solvents, highest values were documented for aqueous-ethanol combined extraction treatment as 92.27±4.92 mg/g followed by 76.34±5.47 mg/g in water extracts. However, least values were seen in ethanolic extracts as 31.99±3.49 mg/g total betalains (Fig. 1). This information provides enough evidence that theses pigments are primarily water soluble and the addition of ethanol to water increases the extraction capacities of the solvent mixture. Amongst the

treatments, highest betalains were exhibited by extracts obtained through aqueous-ethanol at 40 min as 105.10±4.61 mg/g followed by 94.50±5.51 mg/g at 60 min. Whereas, water extracts depicted 90.78±5.99 and 74.93±5.60 mg/g of betalains at 40 and 60 minutes intervals. In contrary, lowest amounts regarding betalains were reported for ethanolic extracts as 38.10±3.52, 30.39±3.65 and 27.47±3.30 mg/g at 40, 60 and 20 min, respectively.

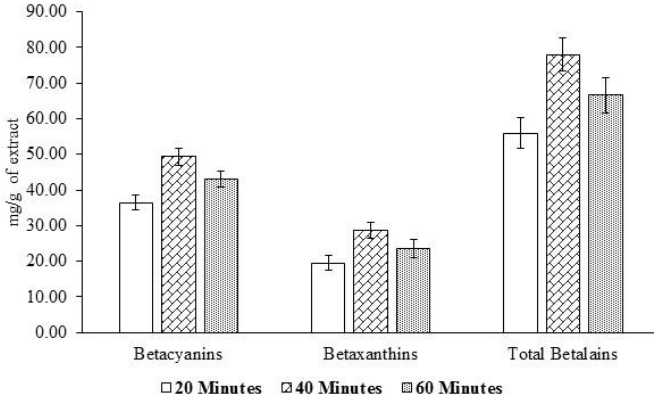

Figure 2. Effect of time on betalains content of red beetroot extracts.

The declining trend regarding betalains concentration with extension in time after 40 min can possibly be due to thermal degradation as a result of increased duration in extraction medium. Moreover, overall yield of the extract is also decreased with possible withdrawal of some debris material which can interfere with the analysis procedures. Although, solid impurities were filtered but there are chances that pigmented bodies may interact with soluble fractions of compounds/material present in the extract solution which can cause decreased detection of the compounds of interest.

The results of the instant investigation are in line with the previous findings of Jiratanan and Liu (2004) who studied antioxidant activities of table beet and green beans. They deduced that betacyanins in raw canned beets were 65.50±2.5 mg/100g beet. They further illustrated that thermal degradation of these compounds occur as a function of time when exposure to heat is extended from 15 to 45 min. They observed 24, 62.2 and 80.6% decrement in betacyanin contents than raw beet on treatment at 105, 115 and 125°C, respectively, for 30 min. Similar pattern of degradation was also observed in case of betaxanthins that showed linear decrease in betalainic profile of beet as a function of temperature and time. This implies that extraction time and temperature are cardinal factors in determining the final extract quality.

Recent investigation by Vulic *et al.* (2014) demonstrated that beetroot pomace obtained after extraction of juice from it also contains higher phytochemical densities in terms of betalainic constituents. They delineated that beetroot pomace contains 37.22 mg betanin/100g dry beet pomace. Furthermore, it has also been noticed that beet pomace possessed 17.95 mg isobetanin/100g on dry weight basis. These values are lesser than the current findings which is logical as majority of the beetroot betalainic constituent like betacyanins and betaxanthins have been removed in juice. However, whole beetroot sample was used for extraction of betalains in current study.

One of the peers Canadanović-Brunet *et al.* (2011) also evaluated antimicrobial and antioxidant potential of beetroot pomace extract. They observed that beetroot pomace extract contained 18.78-24.18 mg betacyanins/g of dry extract whilst, 11.19-22.90 mg betaxanthins/g of dry extract after solid phase extraction. The current investigation witnessed relatively higher contents with respect to betacyanins and betaxanthins than previously reported values of Canadanović-Brunet *et al.* (2011). This variation in concentration of compounds can be attributed to higher pigment densities in whole beetroot compared to its pomace which remained after extraction of juice from it. However, pomace can be a valuable source of pigmented bioactive moieties as described in various studies (Canadanovic-Brunet *et al.*, 2011; Vulic *et al.*, 2014).

In another investigation, Slavov *et al.* (2013) reported betalainic profile and antioxidant activities of beetroot juice and extracts in response to thermal and microwaving procedures. They recorded that extract possessed betacyanins as 44.4 mg/g fresh matter alongside betaxanthins as 16.6 mg/g fresh matter in untreated beetroot. Furthermore, they noticed that the quantities of betacyanins and betaxanthins were increased when beetroot was given microwaving pretreatment. They also reported that 1048 mg of betalains were obtained from 100 g of dry mass that corresponds to 124.7 mg betalains in terms of 100 g fresh weight of red beet. These findings support the results of our research as the results in most of the samples were in line with their study.

The trends observed in current research regarding extraction of pigments as a function of time is consistent with the findings of Cardoso-Ugarte *et al.* (2014). They reported that the concentration of betalains extracted from conventional and microwave assisted extraction procedures increased with increase in time. Nevertheless, the amount of pigment obtained after 40 min extraction time has been seen steady. Furthermore, pretreatment with microwaving improved extraction yields of betalains. They explicated that maximum extractable pigment possessed 364.83 mg/100g betanines whereas, betaxanthins were quantified as 195.84 mg/100g in red beet.

***Response surface analysis*:** Response surface methodology (RSM) is a useful empirical technique to assess the combined effect of multiple factors to obtain optimal response (Singh *et al.*, 2017). RSM allows multivariate evaluation of independent variables simultaneously to establish relationship between predicted and experimental results (Rožić *et al.*, 2010). Central composite design provides

enough flexibility to assess multivariate response surface effect. This design further allows to fit the models based on factorial layout with larger number of independent variables (Mason *et al.*, 2003). In the current research, 10 experiments with 2 center points were analyzed to find optimal conditions for recovery of betalainic constituents like betacyanins (BC), betaxanthins (BX) and Total Betalains (TB). The results for each experiment regarding betacyanins, betaxanthins and total betalains are summarized in Table 2 depicting enough correlation between the experimental and predicted values. The quadratic model adequacy is satisfactorily being met as the sum of squares represent very low *p*-values and their "adjusted *R*-squared" are sufficiently describing the data (Table 3). The data was fitted to second-order polynomial equation involving interactive terms. Since the adopted model can poorly describe the data and may lead to misinterpreted

Table 2. Experimental design matrix and responses.

Run	Ethanol Concentration (%)	Time (Min)	Betacyanins (mg/g)		Betaxanthins (mg/g)		Total Betalains (mg/g)	
	X_1	X_2	$Y_{Experimental}$	$Y_{Predicted}$	$Y_{Experimental}$	$Y_{Predicted}$	$Y_{Experimental}$	$Y_{Predicted}$
1	0	20	47.55	46.81	15.75	16.36	63.30	63.16
2	0	40	63.44	61.16	27.34	26.55	90.78	87.71
3	0	60	51.98	55.01	22.95	23.13	74.93	78.14
4	50	20	48.39	54.15	28.81	27.49	77.20	81.64
5	50	40	69.69	67.70	35.41	36.30	105.10	104.01
6	50	40	69.69	67.70	35.41	36.30	105.10	104.01
7	50	60	62.54	60.76	31.96	31.50	94.50	92.25
8	100	20	13.38	8.37	14.09	14.80	27.47	23.17
9	100	40	14.88	21.13	23.22	22.23	38.10	43.36
10	100	60	14.63	13.39	15.76	16.04	30.39	29.43

Table 3. Adequacy and parameter estimate for response surface model.

Betacyanins	**Estimates**	**Standard Error**	**DF**	**SS**	**MS**	**F-value**	***P*-value**
Model	67.70	3.34	5	4634.31	926.86	29.69	0.0029*
X_1	-20.01	2.28	1	2403.20	2403.20	76.99	0.0009*
X_2	3.31	2.28	1	65.54	65.54	2.10	0.2209
X_{12}	-0.795	2.79	1	2.53	2.53	0.08	0.7901
X_1^2	-26.56	3.66	1	1645.83	1645.83	52.73	0.0019*
X_2^2	-10.25	3.66	1	245.32	245.32	7.86	0.0486*
Residual error			4	124.85	31.21		
Lack of fit			3	124.85	41.62		
R^2	0.974						
Adjusted-R^2	0.941						
Betaxanthins							
Model	36.30	0.74	5	576.32	115.26	75.15	<0.0005*
X_1	-2.16	0.51	1	28.04	28.04	18.28	0.0129*
X_2	2.00	0.51	1	24.08	24.08	15.70	0.0166*
X_{12}	-1.38	0.62	1	7.65	7.65	4.98	0.0893
X_1^2	-11.91	0.81	1	408.47	408.47	266.32	<0.0001*
X_2^2	-6.81	0.81	1	108.10	108.10	70.48	0.0011*
Residual error			4	6.14	1.535		
Lack of fit			3	6.14	2.05		
R^2	0.989						
Adjusted-R^2	0.976						
Total Betalains							
Model	104.01	2.90	5	7913.82	1582.76	67.04	0.0006*
X_1	-22.18	1.98	1	2950.38	2950.38	125.64	0.0004*
X_2	5.31	1.98	1	169.07	169.07	7.20	0.0550
X_{12}	-2.18	2.42	1	18.97	18.97	0.81	0.4196
X_1^2	-38.47	3.17	1	3453.20	3453.20	147.05	0.0003*
X_2^2	-17.06	3.17	1	679.10	679.10	28.92	0.0058*
Residual error			4	93.93	23.48		
Lack of fit			3	93.93	31.31		
R^2	0.988						
Adjusted-R^2	0.974						

* Significant effect (P <0.05); DF = Degree of Freedom; SS = Sum of Squares; MS= Mean Sum of Squares; X_1 = Ethanol Concentration; X_2 = Time

exploration and optimization of response surface, hence it is necessary to check fitness of good model which was carried out by analysis of variance (ANOVA) and regression analysis (Singh *et al.*, 2017; Maran and Manikandan, 2012). The results from adequacy and ANOVA depicted that the model is adequately fitting the responses and representing the actual relationship between the response and independent variables (Table 3).

Due to the inclusion of interactive terms in the polynomial equation, the ANOVA is followed by F-test statistic that describes individual effect of each factor along with interactive terms (Singh *et al.*, 2017). The *p*-values from Table 3 depict that ethanol concentration and time were significantly influencing the response variable in all cases of BC, BX and TB. Whereas, the interactive terms can be seen to impart no significance value to the trait. The F values of influencing factors are larger enough ($P<0.05$) to represent significant model coefficients. The coefficients of determination (R^2) regarding BC, BX and TB were 0.974, 0.989 and 0.988 which corelate that the model is describing at least 95% of the data values sufficiently. It also suggests that the actual data values are in proximity of the linearity of

Table 4. Critical values, desirability and linearity of model (Experimental vs Predicted).

Response	Yield (mg/g)	Ethanol Concentration (%)	Time (min)	Desirability	R^2	Adjusted R^2	Root Mean Square Error
Betacyanins	71.79	31.03	43.52	0.993	0.974	0.970	3.89
Betaxanthins	36.57	45.01	43.15	0.950	0.989	0.988	0.87
Total Betalains	107.71	35.34	43.48	0.960	0.988	0.987	3.41

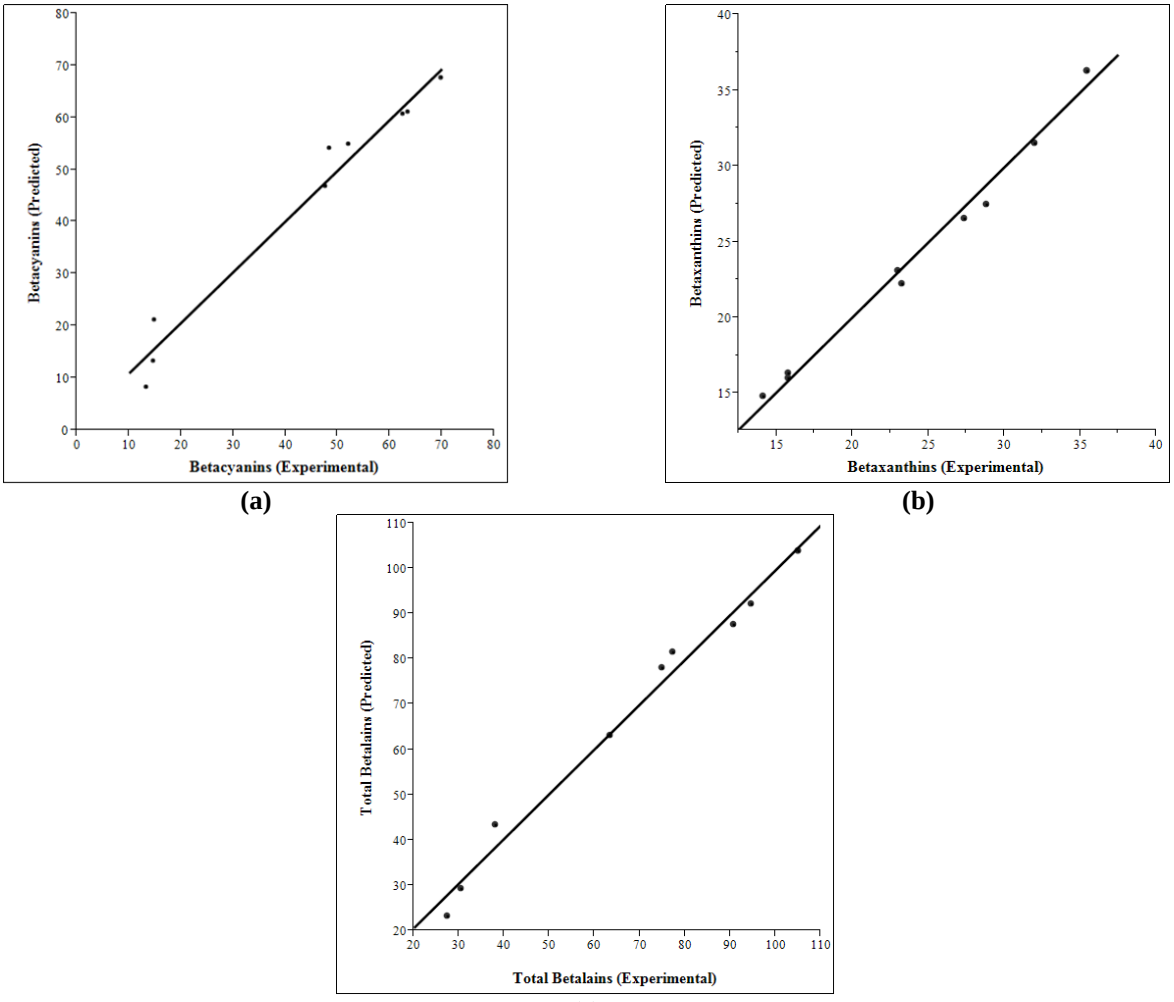

Figure 3. Comparative description of experimental and predicted values of betalainic constituents; (a) betacyanins (b) betaxanthins (c) total betalains.

equation. The promising correlation between the experimental and predicted data points are also being confirmed from the values of adjusted *R*-squared (Table 3). Very low values of P, goodness of fit and high coefficients of determination also advocate the sufficiency of the quadratic model and polynomial equation to predict actual relationship between the variables. It also emphasizes that the equation adequately forecast the data points closer to the observed values.

To get satisfactory results from the model, it is necessary to check that the predicted data points are closer in proximity to the experimental observations (Swamy *et al.*, 2014). A simple and quick way of analyzing this property is to fit the experimental values versus predicted values (Maran and Manikandan, 2012). In this respect, the observations collected from experiments were plotted against the predicted values and the linearity of the curve was evaluated by fitting a linear regression (Fig. 3). The results depicted high R^2 values *i.e.* 0.974, 0.989 and 0.988 for BC, BX and TB, respectively. Furthermore, low values of root mean square error also supported the linearity of the prediction formula (Table 4).

With respect to response surface plot (Fig. 4), the variation in levels of independent variables (ethanol concentration and time) showed significant effect on response variable (pigment yield). The independent variables showed a curvature effect on the response where the lower values of the ethanol concentration and time resulted in lowered yield of BC, BX and TB. From the response plot (Fig. 4) and parameter estimates (Table 3), it is evident that the quadratic terms are influencing the response negatively whilst, linear terms are

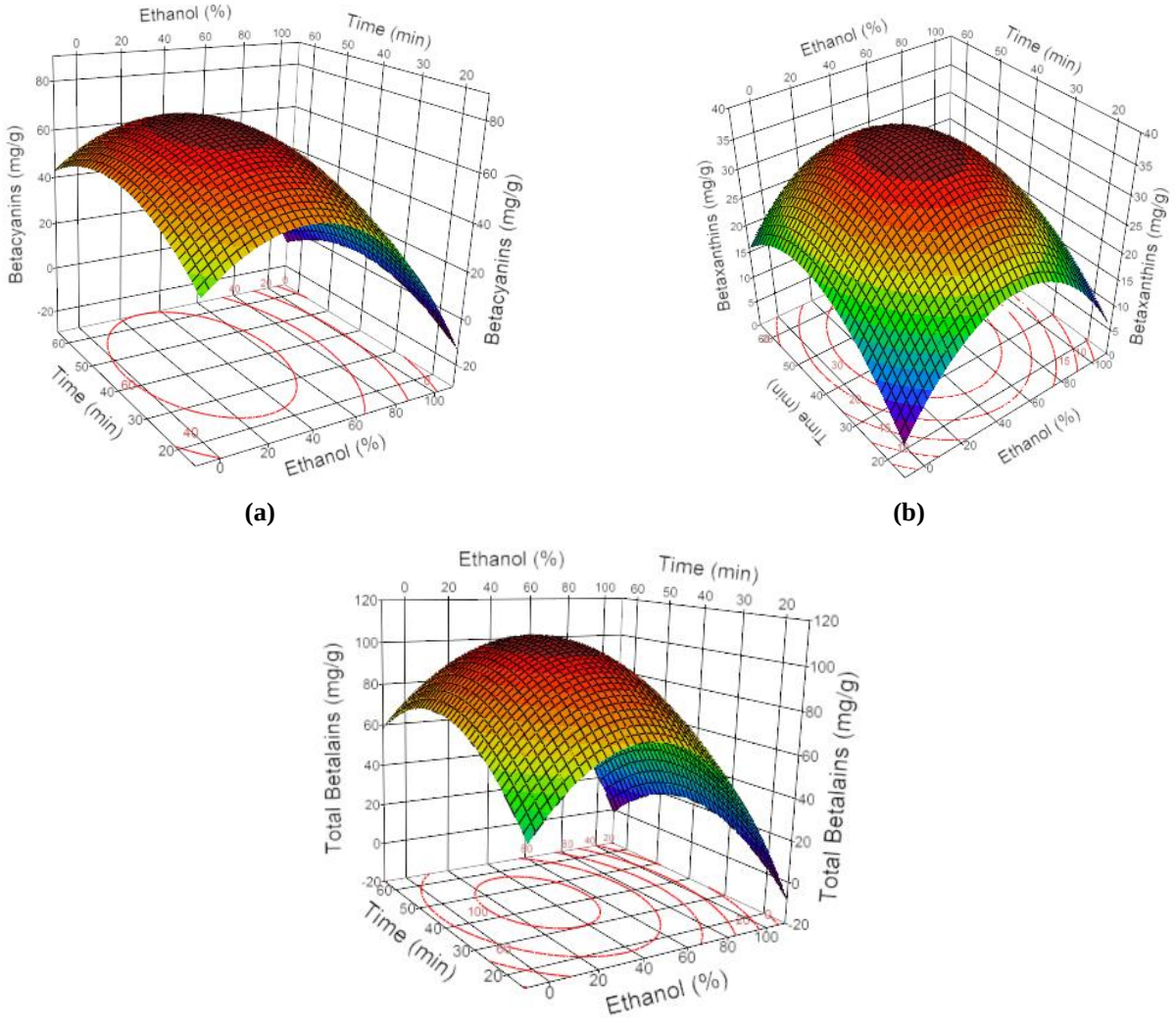

Figure 4. Response surface plots representing effects of solvent and time on betalainic constituents; (a) betacyanins (b) betaxanthins (c) total betalains.

positively correlated to the model except the ethanol concentration where the negative association is observed. This negative association can be attributed to hydrophilicity of the pigments as the red beet betalains are water soluble moieties as evident from the studies of Swamy *et al.* (2014). In case of BC and TB, it was noticed that the increasing ethanol concentration elevated the yield until the maximum yield is acquired at 31% and 35% of ethanol concentration, respectively at around 43 min extraction time. This implies a hydrophilic nature of betacyanins however depicted an increased yield by the addition of ethanol to solvent model. Whereas, maximal recovery of betaxanthins was obtained at comparatively higher levels of ethanol concentration (45%) whilst the time window remains around 43 min. Furthermore, the desirability function was applied to assess the optimal conditions for maximum yield of the betalains from red beetroot. In this regard, the desirability function in maximizing the responses for BC, BX and TB predicted 71.79, 36.57 and 107.71 mg/g, respectively in red beetroot extract. At these critical values, maximal factor values were recorded as 31.03, 45.01 and 35.34% for BC, BX and TB, respectively whilst, time factor was found to be around 43 min for all pigments (Table 4).

The positive relation between time with increased yield of BC and BX has also been observed in the study of Maran and Manikandan (2012). They extracted the betalains from prickly pear and applied Box-Behnken design for optimization of process parameters in the similar manner as adopted in the current study. They deduced that pigment yield increased with increased extraction time where maximum yield is obtained at 115 min. In another investigation by Singh *et al.* (2017), time was positively related to betanin yield in a microwave assisted extraction module analyzed using RSM. In another investigation, Swamy *et al.* (2014) reported positive relationship between process parameters and high pigment yields were obtained in aqueous extraction model. They also reported that temperature and mass of the plant material used for extraction also affect the pigment yields. However, all such parameters were controlled in current study that reduce the chance of error due to such factors. Nevertheless, the natural colorants like betalains are highly susceptible to process parameters and a slight change in the variables can cause significant change in final quality and quantity of the pigment. Azmir *et al.* (2013) provided enough scientific evidences for extraction of bioactives from plant materials. Nevertheless, the maximal extraction of colorants with high quality attributes is necessary especially from plant-based commodities. In this respect, the response surface methodology provides appropriate scientific approach to predict extraction conditions for optimal recovery of plant pigments.

***Conclusions*:** The present study showed that extract composition of red beetroot regarding betalains is highly influenced by time and composition of the solvent. In aqueous-ethanol based solvent module, the pigments were recovered with improved yield which represents that water and ethanol as sole solvent are not suitable for betalains extraction from red beetroot. Furthermore, extraction time also influences the pigment yield significantly and the RSM analysis revealed optimum time interval for betalains extraction around 43 min. Whereas, ethanol greatly influences the betalains yield in final extract but its only 30-45% concentration of ethanol shall yield higher pigment. Nevertheless, other variables like extraction temperature, solid-liquid ratio and sonication pulses etc. may be altered and further analyzed for more reliable parameter estimates to obtain betalains from red beetroot especially for food applications.

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