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A Comparative Study on Process Optimization of Betalain Pigments Extraction from Beta vulgaris subsp. vulgaris: RSM, ANN, and hybrid RMS-GA Methods

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Abstract

Beta vulgaris subsp. vulgaris (red Swiss chard) leaf stalks offer a rich source of betalains, natural pigments with promising applications in the food industry. This study employed response surface methodology (RSM) in conjunction with a 3-level Box-Behnken design to optimize independent extraction variables, including temperature, extraction time, and solid-to-liquid ratio, for maximizing betalains extraction from red Swiss chard. Betacyanins and betaxanthins, the key natural pigments, were targeted as response variables. Statistical analysis revealed the optimal conditions for extraction: 21.14 minutes of extraction time, 52.98°C temperature, and a solid-to-liquid ratio of 21.61 mg/mL, resulting in the maximum extraction of betacyanins (15.53 mg/100g) and betaxanthins (9.5 mg/100g). To enhance prediction accuracy, an artificial neural network (ANN) model was employed, outperforming RSM predictions. Moreover, incorporating a genetic algorithm (GA) into the RSM regression equation predicted even higher betalain contents, with betacyanins reaching 16.53 mg/100g and betaxanthins 10.52 mg/100g. Confirmation experiments conducted under RSM-GA predicted optimum conditions demonstrated mean betacyanin and betaxanthin contents of 16.54 mg/100g and 10.49 mg/100g, respectively. The superior predictive capabilities of the ANN model and the synergistic integration of GA with RSM highlight innovative

approaches for enhancing extraction efficiency. Furthermore, the characterized extract exhibit attributes such as aggregated morphology, amorphous nature and high thermal stability.

Keywords: waste valorization**;** Swiss chard; Betacyanin; Betaxanthin; Food colorant; Process optimization

1. Introduction

Food safety is always a major concern, especially in emerging nations where the demand for food additives continues to rise. The methods used in food production may significantly impact the finished goods' overall flavor and appearance [1]. In most cases, the degrading effects of food manufacturing procedures result in the loss of natural pigments in various food items, including cream cakes, ice creams, and snack foods. Therefore, the need for food colors is crucial, and they have a big impact on the characteristic of food quality [2]. From the consumer's perspective, one of processed food items' most important quality characteristics is their color. Adding colorants to restore colors is one strategy the food processing sectors can use. While it may not be possible to avoid food colors in food manufacturing sectors completely, it is important to ensure they are used safely and responsibly [3].

Synthetic food colorants can affect human health in various ways, including by causing allergic reactions, child hyperactivity, cancer, neurotoxicity, reproductive toxicity, and organ failure [4]. Additionally, several synthetic food colorings have been banned or restricted in several nations over safety issues. Red 2G, Red 40, Yellow 5, Sudan I-IV, and Brilliant Blue FCF are some colorants that are banned for use in food. It is significant to note that laws governing food additives can differ from nation to nation, and what may be restricted in one nation may be permitted in another [5]. For instance, despite being banned in many European countries, Brilliant Blue FCF is still used as a food ingredient in India [6]. Some food producers are now concentrating on using alternative methods to improve the appearance of their products due to the negative health effects of synthetic food colorants, such as natural food ingredients and cutting-edge food processing techniques. These techniques can produce high-quality, aesthetically pleasing food items while reducing the need for synthetic food colors [7].

Natural food colorants can produce a variety of hues, from vivid yellows and oranges to deep reds and purples, and they are color additives that come from natural sources like fruits, vegetables, and spices. Additionally, the possibility of using microalgal pigments like chlorophylls, carotenoids, and phycobiliproteins as food coloring is being extensively researched [8]. Even though natural food colorants can be more expensive and challenging to work with, many food manufacturers are now concentrating on using them to enhance the quality of their products and satisfy consumer demand for healthy foods. Historically, natural food colorants such as turmeric,

annatto, carrot juice, and beetroot juice have been used worldwide. The extracts from beets and related vegetables are one of the most commonly used natural food coloring ingredients in various foods. The primary component of the red colorant extracted from Beta vulgaris is betalain pigments [9].

Betalains are natural pigments that are not only nontoxic; they could be used as an alternative to supplement therapies in inflammation, oxidative stress, dyslipidemia-related diseases, and cancer [10]. Due to their low cost, toxicological safety, accessibility, excellent biodegradability, and possible positive effects on health, betalains can replace artificial food colors without posing any health risks. Recent studies show betalains have antiviral, antimicrobial, and antioxidant properties that may be used in various therapeutic procedures. Normally, betalains comprise betaxanthins and betacyanins, which may be used as culinary coloring agents. Red beets are frequently found to have high levels of betacyanins (300 to 600 mg / Kg of raw beet) and betaxanthins (320 to 420 mg/kg of raw beet) pigments [11].

Unfortunately, betalains have received little attention because they are limited distribution and even restricted occurrence in common edible plants. However, there has been a noticeable increase in interest in recent years, especially in the food processing industry [12]. Among the well-known natural food color sources, the leaf stalks of *Beta vulgaris* subsp. V*ulgaris* (red Swiss chard) is one of the most extensive betalain sources. Since Swiss chard is easy to cultivate, it is well familiarized in gardens worldwide [13]. It has been established that red Swiss chard leaf stalks contain significant betalain pigments. These water-soluble pigments can be used in the pharmacological, medical, and food sectors. Hence, red Swiss chard leaf stalks can be exploited as a potential source of natural pigments [14].

Traditional extraction procedures have various flaws that emphasize the importance of a statistical optimization approach. These procedures frequently lack selectivity, resulting in the extraction of undesirable components alongside the intended molecule. Furthermore, they can be inefficient and time-consuming, necessitating long equilibrium times and physical labor. The widespread use of organic solvents raises environmental and safety issues. Furthermore, scalability becomes an issue, preventing these approaches from being used in large-scale industrial operations [15]. Conventional extraction procedures may degrade samples or fail to extract particular types of chemicals efficiently. The lack of automation further limits the reproducibility and throughput of the extraction process. To overcome these limitations, a statistical optimization technique may be used to routinely analyze and optimize various extraction parameters. The extraction process may be fine-tuned using statistical models and experimental design approaches such as response surface methodology or factorial design to improve selectivity, efficiency, and yield while minimizing solvent use and sample degradation [16]. Statistical optimization provides a systematic and

effective method for overcoming traditional extraction approaches' drawbacks and improving the whole extraction process.

While much research has concentrated on extracting betalain pigments from beetroot, just a few have investigated the extraction of betalains from red Swiss chard [17]. Furthermore, research on optimizing the extraction of betalains from red Swiss chard using statistical methods is yet to be available. Hence, in the present study, the aqueous extraction of betalains from the leaf stalks of red Swiss chard was optimized using response surface methodology (RSM) through Box–Behnken design (BBD) and genetic algorithm (GA). Artificial neural networks (ANN) were also used to model the extraction process to make precise predictions about the quantity of betalain pigments that would be extracted under a particular process circumstance. The three independent process parameters, solid-liquid (SL) ratio, extraction temperature, and extraction time, were statistically optimized for maximized betacyanins and betaxanthins extraction. Finally, the obtained betalains product was characterized using analytical techniques such as scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), thermogravimetric analysis / differential scanning calorimetry (TGA/DSC), UV-Visible spectroscopy and high-performance liquid chromatography–mass spectrometry (HPLC - MS) [18].

2 Materials and methods

2.1 Materials and Chemicals

The leaf stalks of *Beta vulgaris* subsp. *Vulgaris* were collected from various home gardens across Addis Ababa, Ethiopia (Figure 1). Methanol (≥ 98.8 % purity) and acetonitrile solution (HPLC grade) were procured from Sigma–Aldrich chemicals, Addis Ababa. Ethiopia.

2.2 Extraction Process

2.2.1 Raw Material Preparation

Red Swiss chard stalks were meticulously separated and thrice cleaned with distilled water. After cleaning, the stalk samples were shade dried for 72 h at room temperature and then ground using a laboratory grinding mill. To eliminate larger-sized particles, the pulverized plant material was sieved using an ASTM sieve of 40 mesh size. Lastly, the sieved stalk powder samples were collected in dark bags and stored at -20 ^{0}C in a deep freezer for further experiments.

2.2.2 Aqueous Extraction of Pigments

The dried stalk powder was well-mixed with double distilled water at a predetermined amount (different SL ratios) in the conical flask as per the design of the experiments. During the

experiments, all the flasks were covered with an aluminum foil wrap to avoid solvent evaporation. Based on the predetermined BBD design of experiments (DOE), the flasks were maintained at different temperatures in the water bath. The samples were drawn from individual flasks at different time intervals based on the DOE combinations followed by centrifugation at 5000 RPM for 10 min in a laboratory centrifuge (H3-18K, KeCheng Centrifuge, China). The betaxanthins and betacyanins content of the supernatants from the centrifuged samples were quantified using a spectrophotometry-based assay. All the batch extraction experiments were executed in triplicates, and the average concentration of betaxanthins and betacyanins in the extract was taken as the response for RSM-based experiments [19]. To conduct characterization studies, the concentrated product was derived from the extract after removing the solvent using a rotary evaporator (SH-RE05L, SH Scientific, Korea).

2.3. Estimation of total betacyanins and betaxanthins Content

The extract's total betacyanins and betaxanthins Content was estimated using a spectrophotometric method described by Maran et al., 2013 [20]. Briefly, 10 mL of different aliquots of Swiss chard extract was added to the equal volume of 50% aqueous methanol solution in a conical flask, and the mixture was gently agitated for 30 mins at 200 RPM in a rotary shaker (BTL-45, Bio Techno Lab, India) maintained at 30 ^{0}C. After thorough mixing, the flask's content was centrifuged for 20 min at 5000 RPM (H3-18K, KeCheng Centrifuge, China). The supernatant was filtered using filter paper, and the filtrate obtained was further subjected to spectrophotometric analysis using a spectrophotometer (Model-525, LALCO, India). The betaxanthins and betacyanins are assessed in terms of betanin and indicaxanthin equivalents, respectively, using the following formula [21],

$$BA(mg/g) = \frac{D \times A \times MW}{\varepsilon \times L} \ldots\ldots\ldots (1)$$

Where BA refers to the total betaxanthins or betacyanins content, MW is the molecular weight of indicaxanthin (308 g/mol) or betanin (550 g/mol), A denotes the observed value of absorption at 600 nm, and ε represents the molar extinction coefficient of betanin (60,000 L/mol/cm) or indicaxanthin (48,000 L/mol/cm). On the denominator of equation 1, D represents the dilution factor, and L refers to the path length of the cuvette (1 cm).

2.4 Experimental Design

The core objective of this study is to maximize the extracting yield of betalain pigments by optimizing the important process variables. For that, the independent variables such as temperature, SL ratio, and extraction time were considered, and the limits within which the RSM-based

experiments were conducted include 40-70 ^{0}C of temperature, 10-30 mg/mL of S/L ratio, and 10-30 min of extraction time. The process was optimized to attain the highest yield of betacyanins and betaxanthins. Three factors with a three-level Box-Behnken design (BBD) were employed for the RSM approach [22]. The total number of experiments (L) was calculate using the following equation,

$$L = 2L(n-1) + a_0 \ldots\ldots (2)$$

Where n is the number of selected parameters, and a_0 refers to the number of central points. Based on equation 2, 17 experimental combinations (Table 1) with five replicates at the center points were estimated for betalains extraction from red Swiss chard. Using the experimental data, a second order polynomial equation was generated to express the correlation between the selected parameters and response variables (betacyanins and betaxanthins). In general, this polynomial equation can be represented by the following expression,

$$BA = \varphi_0 + \sum_{g=1}^{k} \varphi_h Y_h + \sum_{h=1}^{k} \varphi_{hh} Y_h^2 + \sum_g \sum_{h=2}^{k} \varphi_{hg} Y_g Y_h + \alpha_g \ldots\ldots\ldots (3)$$

Where BA refers to the response (betacyanins and betaxanthins content), Y_i and Y_j are the selected factors (g and h range from 1 to k), φ_o is the model coefficient for intercept, and φ_{hh}, φ_{gh}, and φ_h are the coefficient of interaction for second-order, quadratic, and linear terms respectively. The term 'k' denotes the number of independent parameters (here, k = 3). A statistical software, Design Expert 12.0.7.1 (Stat-Ease Inc., Minneapolis, USA), was used to generate the empirical model, response surface plots, and the statistical fitness of the model.

2.5 Statistical analysis, Optimization and Validation

The data acquired from the optimization experiments were subjected to multiple regression analysis using the least square method. In order to ensure statistical appropriateness, a Pareto analysis of variance was carried out for the significant regression coefficients of linear, quadratic, and interaction terms of the developed model equations [23]. The models were verified for statistical significance ($p \leq 0.05$) based on the student F-test. The acquired models were examined further using descriptive statistics like p-value, f-value, absolute average relative deviation, the mean sum of squares, coefficient of variation, determination coefficient, and correlation coefficient [24]. The condition for maximizing the targeted yield of betalain pigments (betaxanthins and betacyanins) from red Swiss chard was ascertained from the regression models, and the optimal condition was validated by comparing the predicted yield with the experimental yield.

2.6. Artificial Neural Networks

To validate the RSM results, an artificial neural network (ANN) based prediction system was used. ANN functions similarly to the brain's neurons, and their primary goal is to predict the optimum response by analyzing the experimental parameters and results obtained from BBD experiments. ANN neurons are typically linked by their synaptic weights. Neurons are trained according to their assigned function (tansig or purelin) to produce an optimal response. The connections between neurons are modified during training until a sufficiently optimal output is obtained [25]. The architecture of the ANN comprises three layers: input, output, and hidden layer. The input layer comprises three layers, each representing one of the independent variables used in this study (Time, Temperature, and solid solvent ratio). The output layer comprises two layers that depict the responses obtained from the extraction experiments (Quantity of betacyanins and betaxanthins obtained per 100g of stalk). The number of neurons in the hidden layer was optimized by training the network with hidden layer neurons in the range of 1 to 25. The number of hidden layer neurons that lead to minimum mean square error was selected to train the network. The input and target datasets used for ANN training are adapted from the RSM study's process conditions. The experimental data were separated into three groups – training (70%), validation (15%), and testing (15%) dataset. Prior to training, the data points of independent variables and their corresponding response values were normalized using the following equation:

$$\text{Normalized data} = \frac{2*(X_A - X_{min})}{X_{max} - X_{min}} - 1 \quad \ldots\ldots\ldots (4)$$

X_A is the actual value, X_{min} is the lowest value, and X_{max} is the highest value of the factors used in the RSM and responses of each experiment. Normalization confines data to a value range of -1 to 1, allowing for rapid data convergence. De-normalization (reverse of normalization) was used to convert the normalized output into actual output after the input was processed into an appropriate output. Also, six extraction experiments were conducted separately from BBD trials under varying experimental conditions to validate the predictive capacity of trained networks. To measure the performance of ANN, metrics such as root mean square error (RMSE), correlation coefficient (R2), and absolute average deviation (AAD) were determined using the following expressions:

$$\text{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(t_i - o_i)^2} \quad \ldots\ldots\ldots (5)$$

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(t_i - o_i)^2}{\sum_{i=1}^{n}(o_i)^2} \quad \ldots\ldots\ldots (6)$$

$$\text{AAD (\%)} = \left[\frac{1}{n}\sum_{i=1}^{n}(t_i - o_i)/o_i\right] * 100 \quad \ldots\ldots\ldots (7)$$

Where t_i is the data predicted by RSM and ANN, o_i is the actual response, and n is the total number of experimental runs performed [26]. The nntool (Neural Network Toolbox) of MATLAB 9.12 (R2022a, Mathworks Inc., MA, USA) was used to conduct ANN modeling.

2.7 Genetic Algorithm

The genetic algorithm is a type of nonlinear search algorithm that simulates the process of evolution inspired by Darwin's theory of natural selection. GA is frequently used to solve constrained and unconstrained models related to optimization. In this technique, the random number function generates different data points representing each iteration's population. Only optimized points that lead to a global solution will be used for future iterations. Because our study focuses on two different responses, we used a multi-objective optimization procedure in GA known as the non-dominated sorting genetic algorithm II (NSGA-II algorithm) to determine the optimal conditions required to maximize the extraction of betacyanins and betaxanthins. In NSGA-II, population fitness was assessed using regression equations derived from RSM as the fitness function. The GA-based optimization was carried out with the help of the MATLAB 8.1 optimization toolbox (R2022a, Mathworks Inc., MA, USA). The goal of GA-based optimization is to increase the extraction of both betacyanins and betaxanthins. The NSGA-II algorithm was limited by the upper and lower limits of the independent variables used in RSM-based experiments [27]. The parameters used to run the NSGA-II algorithm include population size = 60, cross-over rate = 0.8, mutation rate = 1.0, and maximum iteration time = 120 minutes.

2.8 Characterization of betalain pigments

The betalain pigments extracted from the leaf stalks of red Swiss chard were characterized using different characterization techniques such as SEM, FTIR, XRD, TGA/DSC, and HPLC-MS. Using a scanning electron microscope (EVO18, Carl Zeiss, Germany), the morphological features of the powdered extract were assessed. The Fourier-transform infrared spectroscopy (FTIR) analysis was carried out to determine the functional group present in the extract using an FTIR spectrometer (IRAffinity-1S, Shimadzu, Japan). In addition, the crystallographic and thermal characteristics of the Swiss chard extract were determined using an X-ray diffractometer (3rd Generation Empyrean, Malvern Pananalytics, UK) and simultaneous thermal analyzer (STA449 F3 Jupiter, Netzsch, Germany), respectively. The optical properties of the extract were characterized using a double-beam UV-Visible spectrophotometer (Model-525, LALCO, India). Finally, the chemical constituents of the Swiss chard extract were identified using liquid chromatography integrated with a photodiode array detector and mass spectrometer (410 Prostar Binary LC with 500 MS IT PDA Detectors, Varian Inc, USA).

3. Results and Discussion

3.1 Analysis of Experimental Data and Fitting of Model

Different model equations (quadratic, interactive (2FI), cubic, and linear) were developed using the obtained experimental data. Table 2 shows the experimental outcome and predicted values for each combination of runs which was predetermined by the design of the experiment. Table 3 presents the significance of the developed model for betacyanins and betaxanthins extraction. The statistical data were analyzed based on R2 (adjusted), R2 (predicted), and p-values (sequential and lack of fit). Based on the statistical appropriateness, a quadratic model was selected to study the influence of selected independent variables for pigment extraction [28].

Further, the multiple regression analysis and the second order polynomial model were developed from the experimental data. The generated equation can express the correlation between the selected independent variables (extraction time, temperature, and SL ratio) and the response variables (total betacyanins and betaxanthins content). The developed second order polynomial equations are given below,

$$Betacyaninyield(mg/g) = -74.3083 + 2.15(Extractiontime) + 1.91(Temperature) + 1.62(SLratio) - 0.014(Extractiontime)(Temperature) + 0.0002(Extractiontime)(SLratio) - 0.001(Temperature)(SLratio) - 0.0328 \ldots\ldots \quad (8)$$

$$Betaxanthinyield = -15.696 + 0.497(Extractiontime) + 0.591(Temperature) + 0.491(SLratio) - 0.0009(Extractiontime)(Temperature) + 0.0008(Extractiontime)(SLratio) - 0.007(Temperature)(SLratio) - 0.0109 \ldots\ldots \quad (9)$$

3.2 Analysis of the statistical significance for model development

In this study, the Pareto analysis of variance (ANOVA) was undertaken to test the suitability and significance of the generated model for predicting the yield of betacyanins and betaxanthins from the leaf stalk of Swiss chard. The software tool suggested and proved that the second polynomial equation had the best adequacy. Hence, the fitness of the polynomial equations was studied using the ANOVA test. The results from ANOVA for both betacyanins and betaxanthins extraction models are given in Table 4 [29]. Based on the statistics, at a probability value of < 0.0001, the developed models were observed to fit well with the experimental data. The high Fisher's value for the models indicates that the models are interpreted adequately. In the present study, Fisher's value was 89.32 for betacyanins and 13.15 for betaxanthins yield. From the results, the lack of Fit value (F-value of 3.43 and 3.95 for betacyanins and betaxanthins, respectively) and

p-value (0.1326 for betacyanins and 0.1088 for betaxanthins) indicated the appropriateness of the selected experimental data to construct statistical models for predicting the yield of betacyanins and betaxanthins [30]. The goodness of the fitting of experimental data to the models was ascertained by the coefficient of determination (R^2). In this study, R^2-values for betacyanins and betaxanthins extraction were found to be 0.9914 and 0.9442, respectively, which indicate that only 0.0086% and 0.0558% of the deviations were identified to be unexplained [31].

3.3. Effect of process parameters on betalains extraction

A perturbation plot can be useful to investigate the effect of all chosen individual parameters over the selected ranges and to identify the most effecting parameter. In this study, using the Design-Expert software, perturbation plots were generated to examine the individual effect of extraction time, temperature, and SL ratio. Figure 2 shows the perturbation plot for the influence of individual variables on the yield of betacyanins (Figure 2a) and betaxanthins (Figure 2b) yield. From the plot, it was observed that significant curvature with steep slopes was seen for all the selected parameters, which indicates a good influence of process parameters on the pigment yield.

3.4 Observed versus RSM-predicted response analysis

Residuals represent the discrepancy between a dependent variable's observed or actual values and the predicted values estimated by a statistical model. The predicted versus actual responses and normal residual plots are shown in Figure 3. Figures 3a and 3b depict the alignment of predicted responses over actual or observed ones, demonstrating that the model fittings used to predict how betacyanins and betaxanthins concentration will respond to changes in independent variables are suitable [32].

Residual plots are graphical representations that display the relationship between the residuals and the independent variables or predicted values. These plots allow for observing the model's validity by examining the patterns or trends in the residuals. Figures 3c and 3d show that the data displayed a predominantly linear pattern along the equality line, indicating a well-fitted plot. Also, The residuals were distributed in an S-shaped pattern, often associated with an ideal plot. Moreover, the linear relationship observed in the error definition suggested a normal distribution of errors [33]. Both models exhibited no signs of experimental aberrations, reinforcing the reliability of the results.

3.5 Interpretation of response surface plots

3D response surface plots are used (generated using model equations 8 and 9) to illustrate the graphical correlation between the selected factors and responses [34]. The 3D response surface

plots depicting the yield of betacyanins and betaxanthins are shown in Figure 4. Among the selected factors, temperature is one of the most important parameters that significantly affect the extraction of betalain pigments. From the observations, temperature showed both a negative and positive influence on the extraction of betalains. An increase in temperature from 40 to 55 ^{0}C positively influenced the extraction process and increased the pigment yield. After 55 ^{0}C, a constant decline in the concentration of betalains pigment in the extraction mixture was recorded. The increasing betalains yield over increasing temperatures upto 55 ^{0}C is attributed to the fact that the diffusivity and solubility of chemical constituents of plant materials increase linearly with increasing temperature [35]. However, a rise in the temperature decreases the solvent's viscosity and surface tension, which may further modify the mass transfer potential. Thus, in this study, the increase in the temperature beyond 55 ^{0}C led to a constant reduction in the pigment yield. In addition, the degradation of betalain pigments at elevated temperatures may cause a decrease in the pigment yield [36]. In the case of extraction time, an increase in pigment yield was recorded till 20 min. Beyond that, a decreasing trend in pigment extraction was observed. During the initial 20 min, hydration and swelling of the plant substances were getting enhanced because of the effect of cavitation, which normally increases the plant matrix's diffusion [37]. Thus, the results showed an increasing profile at this period, and such occurrence is well-explicated by Fick's 2nd law of diffusion. Based on this law, beyond a certain time of extraction, the solute (pigment) and solvent (water) reach a final equilibrium [38]. Also, the exposure of the extracted pigment under the elevated temperature beyond the optimal time led to the degradation of pigments, resulting in the decreasing yield of betalain pigments.

Next to extraction time, the SL ratio was observed to influence the extraction yield of betalain pigments significantly. It was found that an increase in SL ratios from 10 to 20 mg/ml has greatly increased the pigments yield, and it is due to the enhancement in the dissolving potential and penetration of the betalain pigments at higher plant material concentration in the solvent. However, if the SL ratio is further increased beyond 20 mg/mL, the yield of pigment got reduced because of high solvent viscosity. The solvent's inherent cohesive force increases when the solvent's viscosity peaks, preventing cavitation and reducing betalains yield [39].

3.6 ANN based modelling of the extraction processed

Table 2 shows the RSM, and ANN predicted responses to 17 BBD experiments. The best ANN architecture has three input, seven hidden, and two output layers (Figure 5a). A log-sigmoid transfer function (logsig) activates the hidden layers, and a linear transfer function activates the output layer (purelin). Feed-forward backpropagation is the network type used to run ANN. Levenberg-(Trainlm) and Marquardt's learning rule are used as training functions. The maximum

epoch is 5000, and the acceptable mean-squared error (MSE) is 0 [40].

During ANN modeling, the root mean square deviation decreased significantly as the correlation coefficient approached one. The maximum observed R2-value (overall) was 0.9934. The regression plots for ANN training, validation, testing, and overall are shown in Figure 5b. The R2-value is close to one, indicating that the ANN-predicted and observed data are well matched. As a result, ANN was adopted in this investigation to model the process of natural food colorant extraction from the leaf stalks of red Swiss chard. The weights and bias values associated with each of the layers in the best architecture of ANN are listed below,

iw{1,1} = [-4.0072 -1.7324 1.42;
2.2045 -2.2011 1.2303;
2.5659 0.37426 0.54945;
-1.8266 1.3968 -1.8082;
-2.5055 0.72496 -0.17289;
2.5286 -0.32052 -2.4057;
-0.87323 2.791 0.96917;
2.3213 -0.012148 -2.3841;
2.216 -1.6087 -1.5717;
-1.5977 -1.6989 -2.863]

iw{2,1} = [0.88875 -0.028272 -0.34436 -0.15312 0.058546 0.49538 -0.56527 0.97864 -0.7916 -1.3096;
1.2364 -0.38984 0.70527 0.1446 -0.27042 -0.62108 -0.70872 0.81402 0.25756 -1.0398]

b{1}= [1.7412;
-2.2821;
-2.2176;
1.3695;
1.9379;
1.504;
-1.0121;
1.7833;
2.2084;
-2.0887]]

b{2}= [-1.9181;
-1.3506]

3.7. RSM vs ANN models

Response Surface Methodology (RSM) and Artificial Neural Network (ANN) models are utilized for system analysis and optimization, albeit with different approaches and characteristics. RSM focuses on statistical modeling and optimization of response variables by fitting mathematical models to experimental data, enabling the identification of optimal input values. On the other hand, ANN models leverage the interconnectedness of artificial neurons to learn complex non-linear relationships between input and output variables from large datasets. In this study, both RSM and

ANN were used to model the process of extraction of betalain pigments from Swiss chard. Compared to RSM-predicted betacyanins and betaxanthins concentration, ANN-predicted values substantially better fit the experimental data (Table 2). Nevertheless, both models performed well in terms of predicting the responses with a good level of accuracy. The residual plots depicted in figure 6 show that many residual values deduced from ANN-predicted responses are near zero, indicating a high level of similarity between the experimental data and ANN-predicted data. Furthermore, ANN performance indicators like RMSE, R^2, and AAD were discovered to be more significant than RSM performance (Table 5). Hence, the ANN was successfully used to model the extraction of food colorants from redSwiss chard.

3.8 Optimum condition predicted by RSM and RSM-GA

The current study used second-order polynomial models to determine response parameters and optimal conditions. The desirability function method was used to optimize the various responses simultaneously. Numerical optimization aids one in selecting the best values for independent and dependent variables. It involves selecting from several optimization choices, such as range, maximum, minimum, goal, or none, to obtain the optimal output value under given conditions. Specific ranges were allocated to the input variables in this study to achieve a maximum value for the response variable [41]. The optimization ramp and the optimization threshold for betacyanins and betaxanthins concentration are shown in figure 7 and table 6, respectively.

The desirability function values measure how desired or optimum a certain combination of input factors is when utilizing Response Surface Methodology (RSM). The desirability function aggregates many responses or objectives into a single value, allowing the evaluation and comparison of various experimental circumstances. The desirability function values typically range from 0 to 1, 1 representing the most desirable or optimal outcome. A desirability value of 0 indicates that the combination of input factors does not meet the desired objectives or falls outside the specified range [42]. In this study, the desirability function value obtained was 0.968, indicating a better balance or compromise between the responses.

Using the desirability function of RSM, the optimum conditions predicted to yield the maximum quantity of betacyanins and betaxanthins are 21.14 min of extraction time, 52.98 ^{0}C of temperature, and 21.61mg/mL of S/L ratio. The total betacyanins and betaxanthins content that could be extracted at optimal conditions were predicted to be 15.53 mg/100g, and 9.5 mg/100g, respectively. Because the desirability function lacks a pareto front, the single optimal solution depicted above is deceptive. When using the RSM-GA hybrid, 16 pareto solutions were obtained (Table 7). Among these solutions, solution number 5 shows the best response for both betacyanins

and betaxanthins yield. A confirmatory experiment over the optimum condition showed mean betacyanins and betaxanthins content of 16.54 mg/100g and 10.49 mg/100g, respectively. Therefore, the results predicted by the RSM-GA hybrid are more robust than the RSM desirability function.

According to Kugler et al. [43], the total concentration of betalain pigments extracted from a purple-red variety of Swiss chard using methanol as a solvent is 50.6 mg/kg of fresh weight of the stalk (betaxanthins = 20.3 mg/kg and betacyanins = 30.3 mg/kg). In this study, using aqueous extraction, the total concentration of betalains obtained is 27.03 mg/100g of the stalk's dry weight. Extraction from dry material has several advantages over extraction from wet material, including increased stability and preservation of the extracted substances' integrity and activity, ease of handling and reduced processing complexity, increased concentration of desired compounds, reduced risk of microbial contamination, potential cost savings through the elimination of energy-intensive drying processes, and greater versatility in application and storage [44]. These benefits make dry material extraction favorable regarding quality control, efficiency, cost-effectiveness, and adaptability in various sectors, including food, pharmaceuticals, and research.

3.9 Characterization of extracted betalain pigments

3.9.1 SEM analysis

The scanning electron microscopy images of the food colorant extracted from the leaf stalks of Swiss chard at different magnification is depicted in Figure 8. The SEM images show that the food colorant extracted from Swiss chard formed aggregates of irregular shape with some porous structures. The size of the aggregates varied from 12 µm to 305 µm with an average size of 115 µm. The observed morphology of the food colorant extracted from Swiss chard showed many similarities with the sugar beet extract [45]. SEM analysis enables the visualization and characterization of particles within the extract, offering valuable insights into their size, shape, and surface features. This information plays a crucial role in understanding the physical properties of the extract and detecting specific components or contaminants. As this study focuses on utilizing the extract as a food colorant, SEM analysis is particularly relevant for assessing its dispersive properties. Examining the particle size and shape helps determine if they are suitable for achieving an even distribution within a food matrix or if issues like clumping or sedimentation may arise [46]. The SEM analysis revealed that the extract forms irregularly shaped aggregates with porous structures, which could hinder the uniform dispersion of the food colorant within a food matrix. Consequently, implementing additional processing steps to transform the extract into a food colorant can address these dispersion challenges.

3.9.2 UV-Visible spectra analysis

The UV-Visible spectra of the extract were recorded between 200 – 800 nm and illustrated in figure 8e. Betalain pigments are generally classified into betaxanthins (yellowish orange) and betacyanins (reddish purple). Betalain pigments' maximum absorption (λ_{max}) varies from 450 to 600 nm of wavelength [52]. The extract obtained from red Swiss chard showed two characteristic peaks at 482 and 535 nm corresponding to the λ_{max} of betaxanthin and betacyanin pigments, respectively.

3.9.3 XRD analysis

The crystallographic characteristics of the extracted food colorant were studied using the XRD analysis. Figure 8f shows the X-ray diffraction pattern of the Swiss chard leaf stalk extract. The broad peaks and a large amount of noise in the XRD pattern of the extract prove that the extracted colorant lacks crystallinity and hence, is amorphous. The amorphous materials are hygroscopic and can readily form aggregates [47]. The hygroscopic characteristic of the extract might result in the aggregated morphology of the obtained extract.

3.9.4 FTIR analysis

The presence of various functional groups on the extracted food colorant was determined through FTIR analysis. The FTIR spectrum of the Swiss chard leaf stalk extract is illustrated in figure 8g. A broad peak at 3362 cm-1 is assigned to the extract's hydroxyl O-H stretching of the carboxylic acids [48]. The sharp peaks at 1624 cm-1 and 1420 cm-1 in the FTIR spectrum are due to the presence of asymmetric and symmetric stretching of C=O group, respectively [49]. The peak at 831 cm-1 corresponds to the alkane out of plane =CH group. All these functional groups indicate the diverse phytochemical constitution of the extracted food colorant. The betanin compound extracted from beetroot showed FTIR spectrum similar to that obtained from Swiss chard [50].

3.9.5 TGA/DSC analysis

TGA/DSC measurements were used to study the thermal properties of the isolated betalain pigments from red Swiss chard at temperatures ranging from 25 to 600 ^{0}C. Figure 9 depicts the TGA/DSC plots of the extracted betalain pigments, and the thermal analysis results show distinct weight loss phases across the examined temperature range. No weight loss was detected in the TGA plot until 100 oC, followed by the first weight loss stage at 130 ^{0}C. The 8% weight loss reported during this stage was caused by the evaporation of residual moisture in the sample. The second weight loss stage (56.75 % weight loss) was observed between 210 and 400 ^{0}C, and it is attributed to the decomposition of saccharide compounds in the extract [51]. Any rise in temperature over 400

^{0}C resulted in a progressive decrease in extract weight, with a residual mass of 26.09% remaining at the end of the analysis (at 598.1 ^{0}C). The extract is expected to degrade completely beyond the temperature of 600 ^{0}C. In DSC analysis, an endothermic peak was observed at 205.2 ^{0}C, and it is apparent that the peak indicated the availability of the water molecule even at high temperatures. Overall, the thermal study revealed that the pigments isolated from Swiss chard were thermally stable, implying that the derived food colorant can tolerate heat processing in the food sector.

3.9.6 HPLC-MS analysis

The HPLC chromatogram of the aqueous extract of betalain pigments from red Swiss chard was taken at two different wavelengths (480 nm for betaxanthins and 538 nm for betacyanins) and illustrated in figure 10. The MS analysis over the eluents showed the presence of five betaxanthins pigments such as Glutamine-bx (Vulgaxanthin I), Proline-isobx (Isoindicaxanthin), Valine-isobx, Isoleucine-bx (Isovulgaxanthin IV) and Isoleucine-isobx. In addition, four betacyanins pigments, such as Betanin, Isobetanin, Isoprebetanin, and Betanidin, were identified from the extract. The observed betalain compounds in the leaf stalk extract of red Swiss chard showed many similarities to the compounds found in the aqueous extract of various beetroot cultivars [53]. Therefore, the extraction parameters optimized in this study gave a good yield of various betalain pigments from red Swiss chard.

4. Conclusion

Red Swiss chard (Beta vulgaris subsp. vulgaris) is a cost-effective source for extracting betalain pigments, which have potential applications as food colorants. In this study, the aqueous extraction of betacyanins and betaxanthins from the leaf stalks of Red Swiss chard was modeled using Response Surface Methodology (RSM) and Artificial Neural Network (ANN). The findings can be summarized as follows:

- The RSM model showed a strong correlation (high coefficient of determination, R2) between the predicted and experimental data for betacyanins and betaxanthins extraction, indicating a good fit of the regression model.

- According to the RSM model, the optimal conditions predicted for betacyanins and betaxanthins extraction were 15.53 mg/100g and 9.5 mg/100g, respectively. The analysis of variance (ANOVA) indicated that the solid-liquid ratio had the most significant influence on the extraction process.

- The ANN modeling of the Box-Behnken design (BBD) experiments demonstrated superior prediction capabilities compared to RSM.

- The hybrid RSM-Genetic Algorithm (GA) approach predicted the highest amounts of extractable betacyanins and betaxanthins from Red Swiss chard leaf stalks as 16.53 mg/100g and 10.52 mg/100g, respectively.
- Characterization studies revealed that the extracted pigments were in an aggregated state with an amorphous nature, exhibited high thermal stability, and consisted of four betacyanin and five betaxanthin compounds.

Overall, this study highlights the effectiveness of RSM and ANN in modeling the extraction of betalain pigments from Red Swiss chard, with ANN showing superior predictive performance. The extracted pigments demonstrated desirable characteristics for potential food colorant applications.

Declarations

Ethical approval: Not applicable.

Competing interest: Not applicable.

Conflict of interest: The authors declare that they have no conflict of interests.

Consent for publication: All authors agree to the publishing of the paper.

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Reference

1. Liguori J, Trübswasser U, Pradeilles R, et al (2022) How do food safety concerns affect consumer behaviors and diets in low-and middle-income countries? A systematic review. Glob Food Sec 32:100606.

2. Jurić S, Jurić M, Król-Kilińska Ż, et al (2022) Sources, stability, encapsulation and application of natural pigments in foods. Food Rev Int 38:1735–1790.
3. Benucci I, Lombardelli C, Mazzocchi C, Esti M (2022) Natural colorants from vegetable food waste: Recovery, regulatory aspects, and stability—A review. Compr Rev Food Sci Food Saf 21:2715-2737.
4. Dey S, Nagababu BH (2022) Applications of food colour and bio-preservatives in the food and its effect on the human health. Food Chemistry Advances 1:100019.
5. Oplatowska-Stachowiak M, Elliott CT (2017) Food colors: Existing and emerging food safety concerns. Crit Rev Food Sci Nutr 57:524-548.
6. Varghese R, Ramamoorthy S (2023) Status of food colorants in India: conflicts and prospects. Journal of Consumer Protection and Food Safety 1-12.
7. Neves MIL, Silva EK, Meireles MAA (2021) Natural blue food colorants: Consumer acceptance, current alternatives, trends, challenges, and future strategies. Trends Food Sci Technol 112:163-173.
8. Bhatia L, Bachheti RK, Garlapati VK, Chandel AK (2020) Third-generation biorefineries: a sustainable platform for food, clean energy, and nutraceuticals production. Biomass Convers Biorefin 1-16.
9. de Oliveira SPA, do Nascimento HMA, Sampaio KB, de Souza EL (2021) A review on bioactive compounds of beet (*Beta vulgaris* L. subsp. *vulgaris*) with special emphasis on their beneficial effects on gut microbiota and gastrointestinal health. Crit Rev Food Sci Nutr 61:2022-2033.
10. Rahimi P, Abedimanesh S, Mesbah-Namin SA, Ostadrahimi A (2019) Betalains, the nature-inspired pigments, in health and diseases. Crit Rev Food Sci Nutr 59: 2949-2978.
11. Butera D, Tesoriere L, Di Gaudio F, et al (2002). Antioxidant activities of Sicilian prickly pear (Opuntia ficus indica) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin. J Agric Food Chem 50:6895-6901.
12. Prajapati RA, Jadeja GC (2023) Optimization of ultrasound-assisted deep eutectic solvent extraction of betanin and its application in chitosan-based biofilm. Biomass Convers Biorefin 1-13.
13. Stintzing FC, Herbach KM, Mosshammer MR, Kugler F, Carle R (2008) Betalain pigments and color quality. In: Culver A (ed) Colour quality of fresh and processed food, 1 st edn. ACS, Washington, DC, pp 82-101.
14. Gamba M, Raguindin PF, Asllanaj E, et al (2021) Bioactive compounds and nutritional composition of Swiss chard (Beta vulgaris L. var. cicla and flavescens): a systematic review. Crit Rev Food Sci Nutr 61: 3465-3480.
15. Bitwell C, Sen IS, Luke C, Kakoma MK (2023) A review of modern and conventional extraction techniques and their applications for extracting phytochemicals from plants. Sci Afr 19:e01585.

16. Gupta GK, Mondal MK (2023) Mechanism of Cr (VI) uptake onto sagwan sawdust derived biochar and statistical optimization via response surface methodology. Biomass Convers Biorefin13:709-725.
17. Gamba M, Raguindin PF, Asllanaj E, Merlo F, Glisic M, Minder B, Bussler W, Metzger B, Kern H, Muka T (2021) Bioactive compounds and nutritional composition of Swiss chard (Beta vulgaris L. var. cicla and flavescens): a systematic review. Crit Rev Food Sci Nutr 60:3465-3480.
18. Carreón-Hidalgo JP, Román-Guerrero A, Navarro-Ocaña A et al, (2023) Chemical characterization of yellow-orange and purple varieties of Opuntia ficus-indica fruits and thermal stability of their betalains. J Food Sci. 88:161-174.
19. Chaari M, Akermi S, Elhadef K, et al (2022) From modeling and optimizing extraction of peels beetroot (Beta vulgaris L.) betalains to in silico probing of their antibacterial multitarget mechanisms. Biomass Convers Biorefin 1-24.
20. Maran JP, Manikandan S, Mekala V (2013) Modeling and optimization of betalain extraction from Opuntia ficus-indica using Box–Behnken design with desirability function. Ind Crops Prod 49:304-311.
21. Moßhammer MR, Maier C, Stintzing FC, Carle R (2006) Impact of Thermal Treatment and Storage on Color of Yellow-Orange Cactus Pear (*Opuntia ficus-indica* [L.] Mill. cv.'Gialla') Juices. J Food Sci 71: C400-C406.
22. Ferreira SC, Bruns RE, Ferreira HS, et al (2007). Box-Behnken design: An alternative for the optimization of analytical methods. Anal Chim Acta 597:179-186.
23. Candioti LV, De Zan MM, Cámara MS, Goicoechea HC (2014) Experimental design and multiple response optimization. Using the desirability function in analytical methods development. Talanta 124:123-138.
24. Rosa ME, Ferreira AM, Neves CM, et al (2023) Valorisation of red beet waste: one-step extraction and separation of betalains and chlorophylls using thermoreversible aqueous biphasic systems. Green Chem 25:1852-1864.
25. Borjan D, Šeregelj, Andrejč DC (2022) Green techniques for preparation of red beetroot extracts with enhanced biological potential. Antioxidants 11:805.
26. Gebremariam EC, Malede YC, Prabhu SV, Varadharajan V, Manivannan S, Jayakumar M, Gurunathan B (2023) Development of bio-based adhesive using tannery shaving dust: Process optimization using statistical and artificial intelligence techniques. Bioresour Technol Rep 101413.
27. Kong Y, Zhang Z, Peng Y (2022) Multi-objective optimization of ultrasonic algae removal technology by using response surface method and non-dominated sorting genetic algorithm-II. Ecotoxicol Environ Saf 230:113151.

28. Varadharajan V, Shanmugam S, Ramaswamy A (2017) Model generation and process optimization of microwave-assisted aqueous extraction of anthocyanins from grape juice waste. J Food Process Eng 40:e12486.
29. Thirugnanasambandham K, Sivakumar V (2017) Microwave assisted extraction process of betalain from dragon fruit and its antioxidant activities. J Saudi Soc Agric Sci 16:41-48.
30. Greenland S, Senn SJ, Rothman KJ, Carlin JB, Poole C, Goodman SN, Altman DG (2016) Statistical tests, P values, confidence intervals, and power: a guide to misinterpretations. Eur J Epidemiol 31:337-350.
31. Bonan S, Fedrizzi G, Menotta S, Elisabetta C (2013) Simultaneous determination of synthetic dyes in foodstuffs and beverages by high-performance liquid chromatography coupled with diode-array detector. Dyes Pigm 99:36-40.
32. Adam AA, Soleimani H, Shukur MFBA, et al (2022) A new approach to understanding the interaction effect of salt and plasticizer on solid polymer electrolytes using statistical model and artificial intelligence algorithm. J Non Cryst Solids 587:121597.
33. Myers RH, Montgomery DC, Anderson-Cook CM (2016) Response surface methodology: process and product optimization using designed experiments. John Wiley & Sons, New Jersey
34. Tang DS, Yin GM, He YZ, et al (2009) Recovery of protein from brewer's spent grain by ultrafiltration. Biochem Eng J 48:1-5.
35. Jun X (2013) High-pressure processing as emergent technology for the extraction of bioactive ingredients from plant materials. Crit Rev Food Sci Nutr 53:837-852.
36. Basavaraja T, Joshi A, Sethi S, Kaur C, Tomar BS, Varghese E, Dahuja A (2022) Stability enhancement of beetroot betalains through copigmentation. J Sci Food Agric 102:5561-5567.
37. Rahman MM, Lamsal BP (2021) Ultrasound-assisted extraction and modification of plant-based proteins: Impact on physicochemical, functional, and nutritional properties. Compr Rev Food Sci Food Saf 20:1457-1480.
38. Kumar S, Rawson A, Kumar A, et al (2023) Lycopene extraction from industrial tomato processing waste using emerging technologies, and its application in enriched beverage development. Int J Food Sci Technol 58:2141-2150.
39. Hu S, Gil-Ramírez A, Martín-Trueba M, Benítez V, Aguilera Y, Martín-Cabrejas MA (2023) Valorization of coffee pulp as bioactive food ingredient by sustainable extraction methodologies. Current Research in Food Science 100475.
40. Hilali S, Wils L, Chevalley A, Clément-Larosière B, Boudesocque-Delaye L (2022) Glycerol-based NaDES as green solvents for ultrasound-assisted extraction of phycocyanin from Arthrospira platensis—RSM optimization and ANN modelling. Biomass Convers Biorefin 12(Suppl 1):157-170.

41. Mourabet M, El Rhilassi A, El Boujaad, H, Bennani-Ziatni M, Taitai A (2017) Use of response surface methodology for optimization of fluoride adsorption in an aqueous solution by Brushite. Arab J Chem 10*:*S3292-S3302.
42. Labidi A, Tebassi H, Belhadi S, Khettabi R, Yallese MA (2018) Cutting conditions modeling and optimization in hard turning using RSM, ANN and desirability function. J Fail Anal Prev 18:1017-1033.
43. Kugler F, Stintzing FC, Carle R (2004) Identification of betalains from petioles of differently colored Swiss chard (Beta vulgaris L. ssp. cicla [L.] Alef. cv. Bright Lights) by high-performance liquid chromatography− Electrospray ionization mass spectrometry. J Agric Food Chem *52:*2975-2981.
44. Stéphane FF, Jules BK, Batiha GE, Ali I, Bruno LN (2021) Extraction of bioactive compounds from medicinal plants and herbs. In: El-Shemy H (ed) Natural Medicinal Plants. IntechOpen, London.
45. Ermis E, Özkan M (2021) Sugar beet powder production using different drying methods, characterization and influence on sensory quality of cocoa-hazelnut cream. J Food Sci Technol 58:2068-2077.
46. Tatiana P, Barroso S, Mendes S, et al (2020) Optimization of phycobiliprotein pigments extraction from red algae Gracilaria gracilis for substitution of synthetic food colorants. Food chem 321:126688.
47. Rivas JC, Cabral LMC, Rocha-Leão MHMD (2021) Microencapsulation of guava pulp using prebiotic wall material. Braz J Food Technol 24.
48. Kushwaha R, Kumar V, Vyas G, Kaur J (2018) Optimization of different variable for eco-friendly extraction of betalains and phytochemicals from beetroot pomace. Waste and Biomass Valorization 9:1485-1494.
49. Sengupta D, Mondal B, Mukherjee K (2015) Visible light absorption and photo-sensitizing properties of spinach leaves and beetroot extracted natural dyes. Spectrochim Acta A Mol Biomol Spectrosc 148:85-92.
50. Devadiga D, Ahipa TN (2020) Betanin: A Red-Violet Pigment-Chemistry and Applications. In: Samantha AK (ed) Chemistry and Technology of Natural and Synthetic Dyes and Pigments, 1 st edn. IntechOpen, London, UK, pp 88939.
51. Arya OP, Adhikari P, Pandey A, Bhatt ID, Mohanty K (2022) Health-promoting bioactive phenolic compounds in different solvent extracts of *Curcuma caesia Roxb.* rhizome from North-East India. J Food Process Preserv 46:e16805.
52. Łata E, Fulczyk A, Kowalska T, Sajewicz M (2020) Development of a Novel Thin-Layer Chromatographic Method of Screening the Red Beet (Beta vulgaris L.) Pigments in Alimentary Products. J Chromatogr Sci *58:*5-15.

53. Slatnar A, Stampar F, Veberic R, Jakopic J (2015) HPLC-MSn identification of betalain profile of different beetroot (*Beta vulgaris* L. ssp. *vulgaris*) parts and cultivars. J Food Sci 80:C1952-C1958.