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DEVELOPMENT AND EVALUATION OF PREDICTIVE MODELS FOR HEAT-ASSISTED EXTRACTION OF PHENOLIC COMPOUNDS FROM *CATHARANTHUS ROSEUS* LEAVES

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Abstract

The recovery of bioactive compounds from medicinal plants depends on complex interactions among process variables. This study investigated heat-assisted extraction (HAE) of Catharanthus roseus leaves and developed predictive models describing the effects of extraction temperature (OT), solid-to-liquid ratio (S/L), and extraction time (ET) on total phenolic content (TPC), antioxidant activity (AA), and extraction yield (EY). After preliminary one-factor-at-a-time (OFAT) screening, a three-factor, three-level Box–Behnken design (BBD) under response surface methodology (RSM) was applied. Quadratic models were developed and validated using analysis of variance (ANOVA), coefficients of determination, and diagnostic plots. All models were highly significant ($p < 0.0001$) with strong predictive power ($R^2 > 0.99$). Temperature was the dominant factor affecting TPC and AA, while ET strongly influenced EY. Bootstrap resampling confirmed model robustness and parameter stability. Monte Carlo simulations provided probabilistic insights, showing greater uncertainty in TPC predictions than in EY. Sensitivity analysis revealed that phenolic recovery was most responsive to temperature, whereas EY depended on solvent availability and contact time. Overall, RSM modeling with reliability and uncertainty analyses offers a robust framework for understanding extraction dynamics and serves as a predictive tool for process optimization and industrial scale-up.

Keywords: Heat-assisted extraction, Phenolic compounds, Predictive modeling, Uncertainty analysis.

Introduction

The increasing demand for natural bioactive compounds in food, pharmaceutical, and nutraceutical industries has driven extensive research into the recovery of phenolic compounds and antioxidants from plant sources. Phenolic compounds are secondary metabolites recognized for their ability to scavenge free radicals, modulate oxidative stress, and provide protective effects against

chronic diseases such as cancer, cardiovascular disease, and neurodegeneration (Dai & Mumper, 2010; Tungmunnithum *et al.,* 2018). In addition to their role in human health, phenolic-rich extracts are widely employed as natural preservatives in food systems due to their antioxidant properties, making them attractive alternatives to synthetic additives (Chemat *et al.,* 2017). Among medicinal plants, *Catharanthus roseus* (Madagascar periwinkle) is a well-known species primarily studied for its alkaloids with established anticancer properties. However, its leaves also contain significant quantities of phenolic compounds and flavonoids that contribute to antioxidant activity and offer complementary therapeutic potential (Kumar *et al.,* 2022). Given these attributes, there is a strong motivation to systematically study the extraction of bioactive compounds from *C. roseus* leaves under controlled processing conditions.

Extraction is a critical step in harnessing plant bioactives, yet it is inherently influenced by multiple interacting variables, including solvent composition, temperature, extraction time, and solid-to-liquid ratio. These parameters not only determine the yield but also affect the chemical stability and quality of the recovered compounds (Azmir *et al.,* 2013; Wang & Weller, 2006). Conventional methods, particularly the one-factor-at-a-time (OFAT) approach, remain useful for preliminary screening of ranges but are inadequate for describing interactive effects between parameters. For example, temperature may enhance solute solubility and cell wall disruption but simultaneously accelerate degradation of thermolabile phenolics if combined with prolonged heating (Mustafa & Turner, 2011). Similarly, the solid-to-liquid ratio influences solvent availability and concentration gradients, thereby affecting mass transfer rates. Understanding these interactions requires more advanced statistical and experimental designs capable of integrating multiple variables simultaneously.

Response surface methodology (RSM) has been widely applied to study the combined effects of process variables in food and natural product extractions. By coupling factorial experimental designs such as Box–Behnken with second-order polynomial regression models, RSM provides a systematic framework for quantifying relationships between independent variables and multiple responses (Ferreira *et al.,* 2007; Myers *et al.,* 2016). In contrast to OFAT, this approach captures curvature, detects interactions, and generates predictive equations that describe extraction dynamics more reliably. While RSM has often been applied for process optimization, its utility extends beyond optimization to robust

modeling and predictive analysis. Developing statistically valid models is critical for understanding process behavior and provides the foundation for industrial process design, even in the absence of optimization.

Equally important is the evaluation of model adequacy and reliability. High coefficients of determination (R^2) and non-significant lack-of-fit tests provide evidence that a model captures the underlying experimental trends, but these indicators alone do not guarantee predictive robustness. To address this, resampling methods such as bootstrapping have been increasingly applied in engineering and food science to evaluate model stability under repeated sampling (Efron and Tibshirani, 1993; Marks *et al.,* 2014). Bootstrapping provides empirical confidence intervals for regression coefficients and allows the assessment of how sensitive a model is to sampling variability. Monte Carlo simulation further extends this approach by propagating uncertainty in input variables through the model to generate probabilistic distributions of predicted outputs (Demaria *et al.,* 2007). These techniques are particularly valuable in bioprocesses where variability in raw materials and environmental factors is inevitable.

In addition to reliability and uncertainty analysis, sensitivity analysis is another valuable tool for model evaluation. Sensitivity profiles highlight the relative importance of process variables and identify which parameters exert the greatest influence on extraction outcomes (Saltelli *et al.,* 2008). For example, extraction temperature often emerges as the dominant factor affecting phenolic recovery, but its interaction with solvent ratio or extraction duration may alter its overall significance (Alara *et al.,* 2018). Such insights are critical for prioritizing variables during scale-up or when developing simplified operating protocols for industrial application. Together, the integration of RSM with reliability, uncertainty, and sensitivity analyses ensures that developed models are not only statistically adequate but also practically useful in predicting system behavior under diverse conditions.

Given these considerations, the present study focuses on the development and evaluation of predictive models for the heat-assisted extraction (HAE) of phenolic-rich bioactive compounds from *C. roseus* leaves. Unlike many previous studies that emphasize optimization of operating conditions, this work is centered on building robust and reliable models that can describe the effects of temperature, solid-to-liquid ratio, and extraction time on total phenolic content (TPC), antioxidant activity (AA), and extraction yield (EY). The approach begins

with an OFAT screening to establish suitable ranges of operating conditions, followed by the application of a Box–Behnken design within an RSM framework to construct predictive models. These models are then rigorously evaluated using statistical criteria, bootstrapping, Monte Carlo simulations, and sensitivity analysis to assess their adequacy, robustness, and reliability.

Materials and Methods

Plant Materials

Freshly matured ***C. roseus*** leaves were harvested from a cultivated farmland in Aba, Abia State, Nigeria (5.1066° N, 7.3667° E). The leaves were carefully separated from the stems, rinsed thoroughly under running tap water to remove soil particles and surface contaminants, and then left to air-dry at ambient laboratory conditions until a stable weight was attained. The dried material was ground into fine powder using an electric blender, after which it was sieved through a 0.105 mm mesh to obtain a uniform particle size. The resulting powder was preserved in a light-proof, airtight container to minimize exposure to moisture and photodegradation prior to extraction. The final sample had a moisture content of approximately 10% at the time of storage.

Chemicals and Reagents

All chemicals used in this research were of analytical grade and sourced from Sigma-Aldrich (Poole, UK). The reagents included sodium carbonate (Na_2CO_3), L-ascorbic acid, Folin–Ciocalteu reagent, glacial acetic acid, 2,4,6-tripyridyl-s-triazine, sodium acetate trihydrate, hydrochloric acid (HCl), and ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$). All aqueous solutions were freshly prepared using distilled water supplied by the Chemical Engineering Laboratory of Michael Okpara University of Agriculture, Umudike, Nigeria

Heat-Assisted Extraction (HAE) of Bioactive Compounds from C. roseus leaves

Bioactive compounds with antioxidant potential were isolated from ***C. roseus*** leaves using a heat-assisted extraction (HAE) technique, following a modified version of the procedure previously described by Adeyi *et al.* (2022). About 1.0 g of the dried and finely ground leaf sample (particle size ~105 μm) was weighed and introduced into a borosilicate extraction flask containing 200 mL of deionized water. The suspension was subjected to controlled heating in a thermostatically regulated water bath. After completion of each extraction run, the hot mixture was cooled to ambient temperature (25 ± 2 °C) and centrifuged at 500 rpm for 5 minutes to achieve solid–liquid separation. The clarified extract was carefully decanted into pre-calibrated vials, measured for exact volume, and stored at 4 °C to maintain chemical stability until further analysis. This

method ensured efficient recovery of antioxidant compounds while minimizing the risk of degradation of thermolabile constituents

One-factor-at-a-time Screening Experimental Design

A preliminary one-factor-at-a-time (OFAT) approach was adopted to establish suitable operating ranges for extraction temperature (OT), solid-to-liquid ratio (S/L), and extraction time (ET) prior to response surface methodology (RSM) optimization. In this approach, each factor was varied individually across a wide interval while the other parameters were kept constant at baseline levels (50 °C, 1:30 g/mL, and 100 min for OT, S/L, and ET, respectively). The selection of these baseline values was guided by previous reports on phenolic extraction from plant matrices using aqueous conditions (Alara *et al.,* 2018; Dahmoune *et al.,* 2015). For temperature screening, the range of 20–80 °C was investigated in increments of 10 °C to capture both the lower and upper thermal thresholds commonly reported for phenolic stability (Mustafa & Turner, 2011). Solid-to-liquid ratio was varied from 1:10 to 1:70 g/mL to evaluate the influence of solvent volume on solute diffusion and extraction efficiency, as recommended by studies on optimizing plant bioactive recovery (Lou *et al.,* 2010). Extraction time was studied between 5 and 240 min to encompass both short and extended extractions, ensuring the identification of the onset of equilibrium and potential degradation at prolonged durations (Wang *et al.,* 2018). At each level of the investigated factor, independent extractions were performed in triplicate, and the filtrates were analyzed for TPC, AA, and EY. Mean values were used for the construction of all relevant graphs.

Box-Behnken Experimental Design for HAE of Bioactive Compounds

The operating ranges for the process variables were defined from preliminary experimental trials and subsequently used to establish a three-factor, three-level Box–Behnken design (BBD) within a response surface methodology (RSM) framework for modeling the heat-assisted extraction of bioactive compounds from ***C. roseus*** *leaves*. The design consisted of 17 experimental runs, comprising 12 points located at the midpoints of the design space edges and 5 replicates at the center to estimate experimental error. The three independent variables evaluated were extraction temperature (X_1: 30, 50, 70 °C), solid-to-liquid ratio (X_2: 20, 40, 60 g/mL), and extraction time (X_3: 20, 110, 200 min), each investigated at three coded levels (−1, 0, +1). The extraction performance was assessed by measuring three key responses: total phenolic content (TPC, expressed as mg

gallic acid equivalents per g dry weight), antioxidant activity (AA, expressed as μM ascorbic acid equivalents per g dry weight), and extraction yield (EY, %). The relationship between the HAE variables and the corresponding responses was described using a second-order polynomial model, expressed as:

$$Y = b_o + \sum_{i=1}^{3} b_i X_i + \sum \sum_{i<j=1}^{3} b_{ij} X_i X_j + \sum_{i=j}^{3} b_{ii} X_i^2 \quad (1)$$

In this expression, Y represents the predicted response, while b_o, b_i, b_{ij} and b_{ii}, correspond to the regression coefficients of the intercept, linear, interaction, and quadratic terms, respectively. The coded independent variables are denoted by X_i and X_j. Model fitting and estimation of coefficients were carried out using Design-Expert software, with statistical significance evaluated at a 95% confidence level. The adequacy and predictive capability of the developed models were validated using the coefficient of determination (R^2), adjusted R-squared (R^2_{adj}), and predicted R-squared (Pred R^2).

BBD-RSM Model Evaluation

Bootstrapping Approach for Model Reliability

The predictive reliability of the developed response surface methodology (RSM) models for TPC, AA, and EY was assessed using a non-parametric bootstrapping approach. In this procedure, the original experimental data were resampled with replacement to generate multiple pseudo-datasets (bootstrap replicates) (Efron & Tibshirani, 1993). For each bootstrap replicate, second-order polynomial RSM models were refitted and used to predict responses at the original design points. The reliability criterion was defined such that a prediction is acceptable if the relative error satisfies

$$\frac{|Y - Y_{pred}|}{|Y|} \leq 0.10 \quad (2)$$

Through this method, both overall reliability (fraction of runs predicted reliably across replicates) and per-run reliability (fraction of bootstrap models that reliably predict each experimental run) were computed. Bootstrap distributions of regression coefficients and performance metrics (such as the coefficient of determination R^2) were also constructed to evaluate parameter stability and model robustness (Marks *et al.,* 2014).

Monte Carlo Uncertainty Analysis

To characterize how variability in model predictors propagates to the responses,

uncertainty analysis was performed via Monte Carlo simulation. The standard deviations of OT, S/L, and ET were estimated from the observed data. Assuming normal distributions centered at their respective means, 1,000 synthetic samples were drawn for each predictor. These input samples were passed through the fitted RSM models to generate distributions of predicted TPC, AA, and EY. The Monte Carlo output distributions were visualized using histograms and scatter plots to examine the spread, estimate confidence intervals, and assess model sensitivity to input variability (Demaria *et al.,* 2007).

Sensitivity Analysis of Process Parameters

A one-factor-at-a-time (OFAT) sensitivity analysis was conducted to assess the influence of individual predictors on each response. In this analysis, each factor (OT, S/L, ET) was varied across its observed range (100 evenly spaced levels), while the other two factors were held constant at their mean values. For each response (TPC, AA, EY), sensitivity profiles were generated (response vs OT; vs S/L; vs ET) using the RSM equations. The amplitude (difference between maximum and minimum predicted response) along each profile was used as a simple importance metric to rank the predictors. These sensitivity curves help to identify which process variables most strongly control the responses under study (Siebert *et al.,* 2001).

Determination of Extract Yield

The extract yield of ***C. roseus*** leaves was determined by evaporating and drying the collected extract to a constant weight using a laboratory convective dryer, following the procedure of Alara *et al.* (2018). The percentage yield (EY, %) was calculated using Eq. (3):

$$\% \text{ EY} = \frac{W_1}{W_2} * 100 \qquad (3)$$

where W_1 is the weight of the dried extract (g) and W_2 is the weight of the dried leaves (g) used in the extraction process.

Determination of Total Phenolic Content (TPC)

The TPC of the aqueous extract was determined using the Folin-Ciocalteu method as described by Gan and Latiff (2011). A 1 mL aliquot of the extract, diluted tenfold with distilled water, was mixed with 1.8 mL of Folin-Ciocalteu reagent and allowed to react for 5 min. Then, 1.2 mL of 7.5% (w/v) sodium carbonate solution was added, thoroughly mixed, and incubated at 25 °C in the dark for 60 min. Absorbance was measured at 765 nm using a UV-Vis spectrophotometer, and phenolic content was quantified against a gallic acid calibration curve ($y = 0.024x + 0.0014$, $R^2 = 0.998$). The

TPC (mg GAE/g) was calculated using Eq. (4):

$$TPC = \frac{C*V}{m} \quad (4)$$

where C is the gallic acid concentration (mg/mL) from the calibration curve, V is the extract volume (mL), and m is the dry weight of ***C. roseus*** leaves (g) used in the experiment. *Determination of Antioxidant Activity (AA)*

The AA of the aqueous extract was evaluated using the ferric reducing antioxidant power (FRAP) assay, as described by Uddin *et al.* (2021). The FRAP reagent was prepared by mixing 0.1 M acetate buffer, 0.01 M TPTZ solution, and 0.02 M $FeCl_3.6H_2O$ in a 10:1:1 ratio. A 50 µL portion of extract (diluted tenfold with distilled water) was mixed with 1.5 mL of FRAP reagent and incubated in the dark at 25 °C for 30 min. Absorbance was measured at 593 nm using a UV-Vis spectrophotometer. Antioxidant activity was expressed as µM AAE/g dry weight, based on an ascorbic acid calibration curve ($y = 0.0042x + 0.0019$, $R^2 = 0.985$)

Results and Discussion

Preliminary Investigation of the Ranges of Operating Variables

Figure 1 presents the preliminary one-factor-at-a-time (OFAT) evaluation of extraction conditions and their effects on TPC, EY, and AA during heat-assisted extraction. The purpose of this investigation was to establish suitable ranges of operating variables for subsequent response surface modeling. The effect of OT on TPC, AA, and EY revealed a characteristic bell-shaped response. All three parameters increased progressively with temperature up to an intermediate region (50–60 °C), beyond which a gradual decline was observed. This behaviour reflects the dual influence of temperature: moderate heating promotes cell wall softening, solvent penetration, and enhanced solubility of phenolic compounds, thereby improving extractability (Dahmoune *et al.,* 2015; Mustafa and Turner, 2011). However, at higher temperatures, degradation of thermolabile phenolics and oxidative polymerization become prominent, leading to diminished recovery and reduced antioxidant activity (Wang *et al.,* 2018). Based on this trend, a working range of 30–70 °C was established to capture both the ascending and descending phases of the response, thereby providing a robust domain for RSM model development.

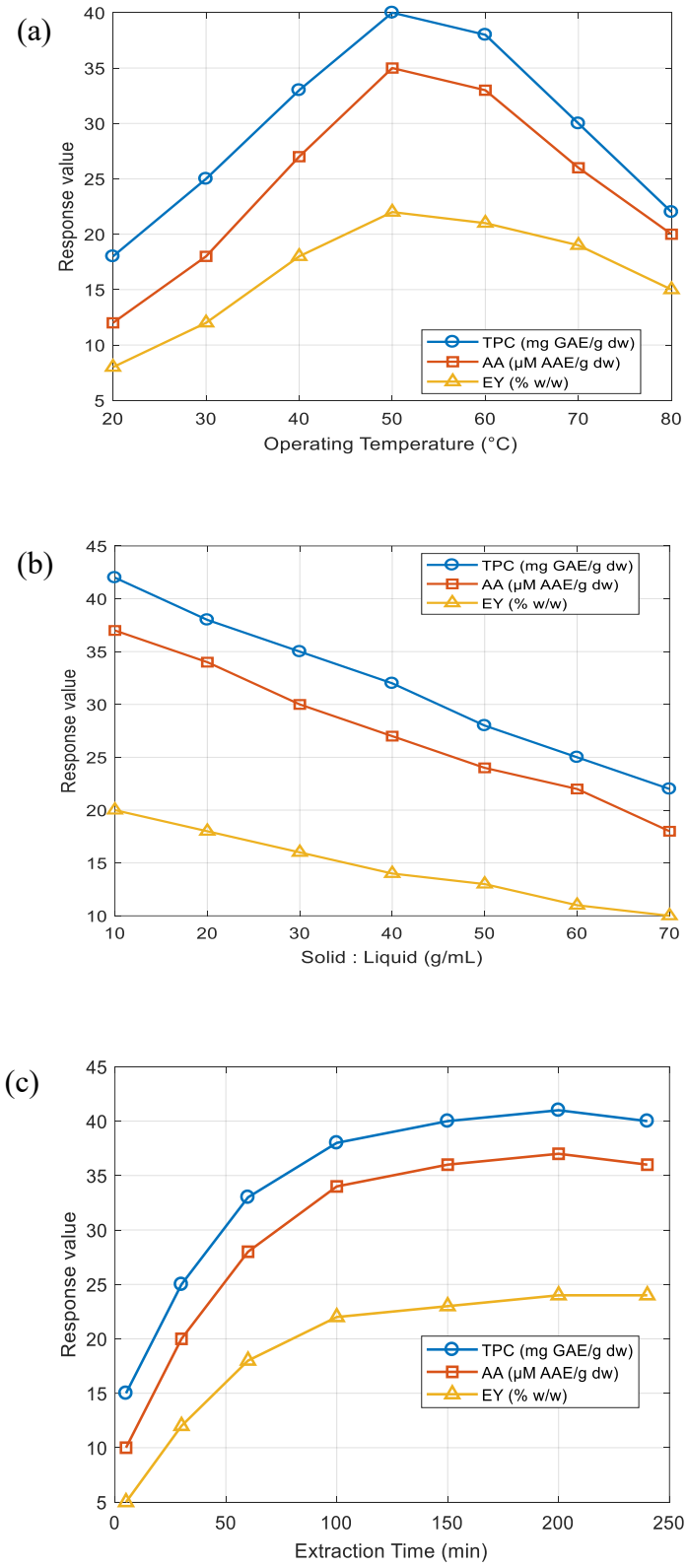


Figure 1: OFAT evaluation of operating conditions for modelling phenolic extraction: influence on TPC, AA, and EY

The influence of the S/L showed an inverse relationship with the responses. At lower ratios (20–30 g/mL), substantially higher TPC, EY, and AA values were obtained compared to higher ratios (≥60 g/mL). This can be attributed to improved solute–solvent contact and steeper concentration gradients at lower S/L, which enhance mass transfer and diffusion of phenolics into the solvent phase (Alara *et al.,* 2018; Lou *et al.,* 2010). In contrast, higher S/L levels reduce solvent availability, thereby restricting solubilization and leading to reduced extraction efficiency. Accordingly, a practical range of 20–60 g/mL was selected to ensure adequate solvent utilization while avoiding impractically dilute conditions. The ET exhibited a saturation-type response. Increases in TPC, EY, and AA were most evident in the early phase (10–100 min), after which responses plateaued, indicating equilibrium between the plant matrix and solvent. Beyond 150–200 min, only marginal improvements were observed, and in some cases a slight decline suggested possible degradation of sensitive phenolic constituents (Brand-Williams *et al.,* 1995). These observations indicate that excessively long extraction times are inefficient and may compromise compound stability. Therefore, a working range of 10–200 min was defined, providing sufficient scope to capture both the rapid extraction phase and the onset of equilibrium without extending into unnecessarily prolonged conditions.

Overall, the preliminary OFAT investigation confirms that the extraction responses are strongly dependent on the operating variables, with each factor showing distinct trends. The chosen ranges of 30–70 °C for OT, 20–60 g/mL for S/L, and 10–200 min for ET are thus justified, as they encompass the key regions of response variation while avoiding conditions that may cause inefficiency or degradation. These ranges form a rational foundation for developing the subsequent Box–Behnken response surface models

Effect of HAE Process Variables on TPC, EYs and AA

The Box–Behnken design (BBD) used in this study generated 17 experimental runs to evaluate the influence of OT, S/L, and ET on TPC, AA, and EY of ***C. roseus*** leaf extracts. The results demonstrate that variations in the independent variables produced distinct effects on the measured responses, highlighting the complex interaction between process conditions and bioactive compound recovery. Higher TPC values were generally observed at elevated extraction temperatures and longer extraction times, such as in Run 10 (146.64 mg GAE/g at 50 °C, S/L 60 g/mL, and 20 min) and Run 4 (123.97 mg GAE/g at 50 °C, S/L 60 g/mL, and 200 min). This trend

aligns with previous studies showing that moderate heating enhances cell wall disruption and facilitates the release of phenolic compounds (Alara *et al.,* 2018; Dahmoune *et al.,* 2015). However, excessive thermal treatment, as seen in Run 7 (40.79 mg GAE/g at 70 °C, S/L 20 g/mL, and 110 min), may promote phenolic degradation due to thermal instability (Adeyi *et al.,* 2023).

Antioxidant activity followed a similar trend to TPC, supporting the well-established correlation between phenolic content and antioxidant potential (Tungmunnithum *et al.,* 2018). The highest AA value (41.72 µM AAE/g) was recorded in Run 4, where extraction was carried out for a prolonged duration at moderate temperature and high S/L ratio, conditions favorable for solubilization of antioxidant compounds. Conversely, the lowest AA (8.79 µM AAE/g) was recorded in Run 6 at low temperature (30 °C) and low S/L ratio (20 g/mL), indicating insufficient energy input and solvent availability for efficient extraction.

Table 1: Box-Behnken Experimental Design and Laboratory-Measured Results[a]

Run	OT (°C)	S/L (g/mL)	ET (min)	TPC (mg GAE/g dw)	AA (µM AAE/g)	EY (w/w %)
1	70.00	60.00	110.00	135.102	37.188	19.983
2	50.00	40.00	110.00	96.644	24.408	14.301
3	50.00	40.00	10.00	93.42	26.128	14.19
4	50.00	60.00	200.00	123.972	41.72	22.772
5	50.00	40.00	110.00	93.864	27.032	13.586
6	30.00	20.00	110.00	47.284	8.79	23.054
7	70.00	20.00	110.00	40.79	14.678	16.07
8	30.00	60.00	110.00	78.36	22.662	24.398
9	50.00	40.00	110.00	91.1	26.268	14.186
10	50.00	60.00	20.00	146.64	27.05	13.01
11	30.00	40.00	20.00	61.856	16.444	23.92
12	70.00	40.00	20.00	91.564	27.27	14.677
13	30.00	40.00	200.00	73.596	20.92	25.117
14	50.00	20.00	200.00	82.72	15.378	14.294
15	70.00	40.00	200.00	91.832	29.412	20.39
16	50.00	40.00	110.00	93.068	27.064	13.92
17	50.00	20.00	20.00	47.396	18.44	14.42

Table 1 shows that the extract yield demonstrated a somewhat different pattern compared to TPC and AA. The highest yields were obtained under low temperature and high S/L ratio conditions, such as Run 13 (25.12%) and Run 8 (24.40%). These results suggest that extraction yield is influenced not only by phenolic release but also by the solubilization of other constituents such as sugars, proteins, and non-phenolic compounds (Alam *et al.,* 2021). In contrast, runs with higher temperatures and moderate

S/L ratios, for example Run 12 (14.68%) and Run 2 (14.30%), produced lower yields despite moderate to high TPC values. This highlights that phenolic concentration and total extraction yield are not always directly proportional, emphasizing the need for multi-response optimization when designing extraction processes (Azmir *et al.,* 2013). Overall, the BBD results confirm that OT, S/L ratio, and ET interact synergistically and antagonistically to influence extract characteristics. Moderate extraction temperatures (50 °C), higher S/L ratios (60 g/mL), and longer times (up to 200 min) generally favored higher TPC and AA, whereas EY was more responsive to lower temperatures and higher solvent volumes. These findings are consistent with previous optimization studies on phenolic extraction from medicinal and food plants, where balanced process parameters were crucial for maximizing bioactive recovery while minimizing degradation (Pan *et al.,* 2019; Chemat *et al.,* 2017).

BBD Modeling, Model Fitting and Statistical Analysis for TPC, EY and AA

Through the BBD, relationships were developed between the operating variables (OT, S/L, and ET) and the studied responses, namely TPC, EY, and AA. The resulting quadratic regression models for each response are shown in Equations (5), (6), and (7), expressed in coded form. In these models, the symbols A, B, and C denote OT, S/L, and ET, respectively. The coefficients with positive signs represent factors that enhance the response, while those with negative signs signify parameters that contribute to a reduction. Accordingly, both the main effects and the interaction effects of OT, S/L, and ET influenced the TPC, EY, and AA outcomes to different extents.

$$TPC = +93.27+12.43A+34.04B+3.02C-19.08A^2+1.39B^2+5.55C^2+15.81AB-3.03AC-15.30BC \quad (5)$$

$$EY = +13.96-3.23A+1.40B+1.93C+5.80A^2+0.9B^2+1.42C^2+0.64AB+1.19\ AC+2.61BC \quad (6)$$

$$AA = +26.15+5.00A+8.67B+2.15C-3.85A^2-1.71B^2+1.35C^2+2.16AB-0.62AC+4.68BC \quad (7)$$

Table 2 is the analysis of variance (ANOVA) for TPC, AA and EY models for **bioactive compound extraction from *C. roseus* leaf.** The ANOVA table revealed that the

quadratic models for TPC, EY, and AA were statistically significant and provided excellent fits to the experimental data, with F-values of 198.47, 77.00, and 89.91, respectively, and corresponding p-values below 0.0001. Such highly significant model terms indicate that the applied response surface methodology (RSM) framework was robust and effective in capturing the nonlinear relationships between process variables and responses. This aligns with previous studies that demonstrated the power of RSM in optimizing plant-based bioactive compound extractions where multiple interacting factors govern recovery efficiency (Dahmoune *et al.,* 2015; Chemat *et al.,* 2017). Across all responses, extraction temperature (OT) consistently emerged as the most influential variable, highlighting its pivotal role in controlling mass transfer and solubilization processes.

For TPC, the linear and quadratic effects of temperature were highly significant ($p < 0.0001$), suggesting that while elevated temperature enhances solvent penetration and disrupts cellular structures to liberate phenolics, excessive heating may accelerate their thermal degradation.

This curvilinear trend was mirrored in EY, where temperature also dominated, not only by increasing phenolic release but also by promoting the extraction of co-solubilized constituents such as proteins, sugars, and pigments. Interestingly, antioxidant activity responded in a similar but more sensitive manner to temperature, showing both strong linear ($F = 153.4$, $p < 0.0001$) and quadratic effects ($p = 0.0002$). The consistency of this trend across responses underscores that temperature optimization must strike a balance between improving solute diffusion and preventing degradation of thermolabile antioxidants (Azmir *et al.,* 2013; Tungmunnithum *et al.,* 2018).

Table 2: ANOVA (Analysis of Variance) for the quadratic models of TPC, EY, and AA[b]

Source	TPC (mg GAE/g d.w)				EY (%)				AA (μM AAE/g d.w)			
	Sum of squares	df	F-value	P-value P>F	Sum of squares	df	F-value	P-value P>F	Sum of squares	df	F-value	P-value P>F
Model	13643.3	9	198.47	< 0.0001	325.61	9	77.00	< 0.0001	1047.9	9	89.91	<0.0001
A - *OT*	1229.12	1	160.92	< 0.0001	83.15	1	176.2	< 0.0001	198.69	1	153.4	<0.0001
B - *S/L*	1229.12	1	1206.26	< 0.0001	15.66	1	33.56	0.0007	597.73	1	461.6	<0.0001
C - *ET*	73.75	1	9.66	0.0171	30.02	1	64.36	<0.0001	37.37	1	28.86	0.0010
A^2	1520.53	1	199.08	<0.0001	140.42	1	301.1	< 0.0001	61.80	1	47.72	0.0002
B^2	8.07	1	1.06	0.3382	3.35	1	7.19	0.0315	12.23	1	9.44	0.0180
C^2	109.32	1	14.31	0.0069	7.13	1	15.28	0.0058	6.45	1	4.98	0.0608
AB	999.70	1	130.89	< 0.0001	1.65	1	3.54	0.1021	18.65	1	14.40	0.0068
AC	32.90	1	4.31	0.0766	5.10	1	10.93	0.0130	1.36	1	1.05	0.3393
BC	840.77	1	110.08	< 0.0001	24.44	1	52.40	0.0002	0.081	1	60.70	0.0001
Residual	53.47	7			3.27	7			9.06	7		
Lack of fit	37.62	4	1.78	0.3317	2.96	4	7.33	0.0667	4.41	4	0.71	0.6364
Pure error	15.85	3			0.30	3			4.65	3		
Cor. Total	13696.7	16			328.87	16			1057.1	16		
CV%	3.15				3.84				4.71			
PRESS	468.93				33.66				59.31			
Adeq Precision	49.609				22.541				37.009			
R^2	0.9961				0.9901				0.9914			
Adj R^2	0.9911				0.9773				0.9804			
Pred R^2	0.9658				0.8976				0.9439			

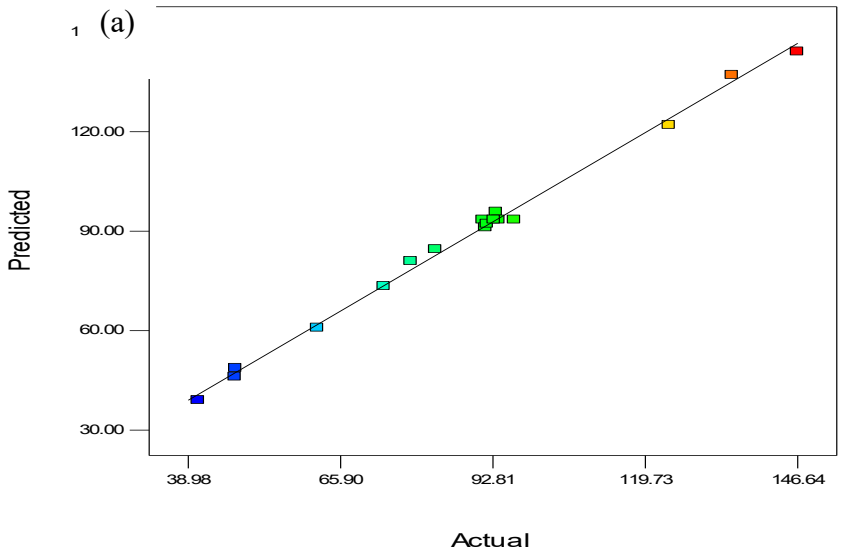
Table 2 shows that the extract the solid-to-liquid ratio was another dominant factor, exerting a profound effect particularly on AA ($F = 461.6$, $p < 0.0001$), followed by TPC ($F = 35.21$, $p < 0.0001$), while its impact on EY was more moderate. This indicates that antioxidant activity, which is directly dependent on phenolic concentration, is highly sensitive to solvent dilution. A higher solvent volume enhances compound diffusion but may also reduce concentration gradients, thereby lowering the measured activity per unit extract. For TPC, the effect of S/L was significant but less pronounced, reflecting that beyond a certain solvent threshold, solute solubility rather than solvent availability becomes limiting. These findings are consistent with the notion that phenolic extraction is driven by both solubility and concentration gradients,

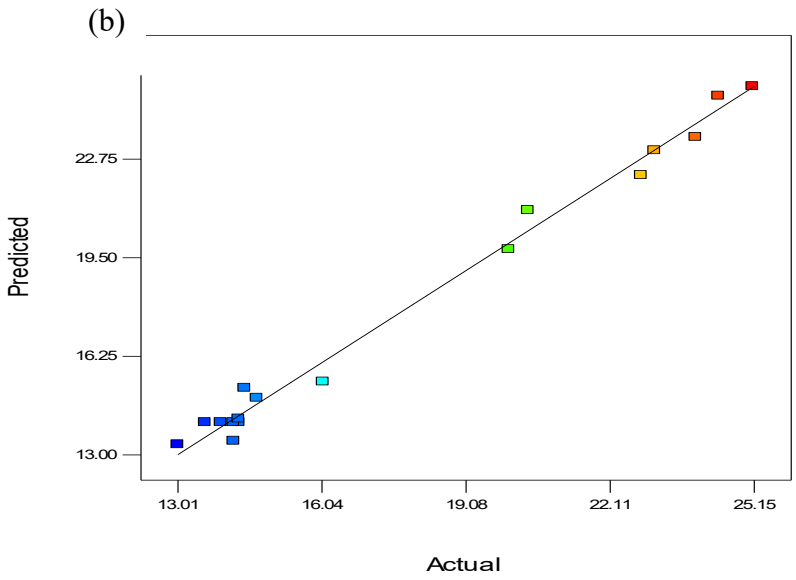
whereas overall yield captures a wider spectrum of soluble phytochemicals that may not be equally sensitive to dilution (Pan et al., 2019). Extraction time showed the strongest contribution to EY, where prolonged solvent–matrix contact promoted the release of both phenolic and non-phenolic constituents. However, its effect on TPC and AA was less significant, indicating that phenolic solubilization reaches equilibrium faster than the extraction of bulk solutes. This divergence highlights a critical trade-off: while extending extraction time maximizes yield, it does not necessarily translate into proportional improvements in phenolic concentration or antioxidant activity, and may in some cases favor degradation or polymerization of sensitive compounds (Alara *et al.,* 2018).

Interaction effects provided further insight into the multivariate dynamics. The interaction between OT and S/L (AB) was highly significant for TPC ($F = 130.89$, $p < 0.0001$) and AA ($F = 14.40$, $p = 0.0068$), but less so for EY. This suggests that phenolic-driven responses are particularly dependent on the synergy between thermal energy and solvent availability: high temperatures accelerate release only when sufficient solvent is available, whereas limited solvent volumes restrict extraction efficiency regardless of temperature. Likewise, the significant interaction between S/L and ET (BC) for both TPC ($F = 110.08$, $p < 0.0001$) and EY ($p = 0.0002$) reveals that prolonged extraction compensates for reduced solvent volumes, further emphasizing the need for simultaneous rather than single-variable optimization. Model adequacy indicators strongly supported these interpretations. The non-significant lack-of-fit ($p > 0.05$) across all responses confirmed the models' suitability, while the high coefficients of determination ($R^2 = 0.9961$, 0.9901, and 0.9914 for TPC, EY, and AA, respectively) demonstrated that over 99% of the observed variability was explained. The close agreement between adjusted and predicted R^2 values (differences < 0.1) further validated predictive reliability, and low coefficients of variation (< 5%) reflected excellent precision. Adequate precision values far exceeded the minimum requirement of 4, confirming that the models possessed strong signal-to-noise ratios, a hallmark of robust predictive frameworks (Myers *et al.,* 2016).

Figure 2 (a), (b), and (c) illustrate the comparison between experimental and model-predicted values for TPC, EY, and AA, respectively. The parity plots reveal a strong agreement between the observed data and the values estimated by the developed models, as evidenced by the close alignment of the data points along the 45° diagonal line extending from the lower left to the upper right of each graph. This alignment indicates

the high predictive accuracy of the models for all three responses (Adeyi *et al.,* 2022; Olalere *et al.,* 2022).





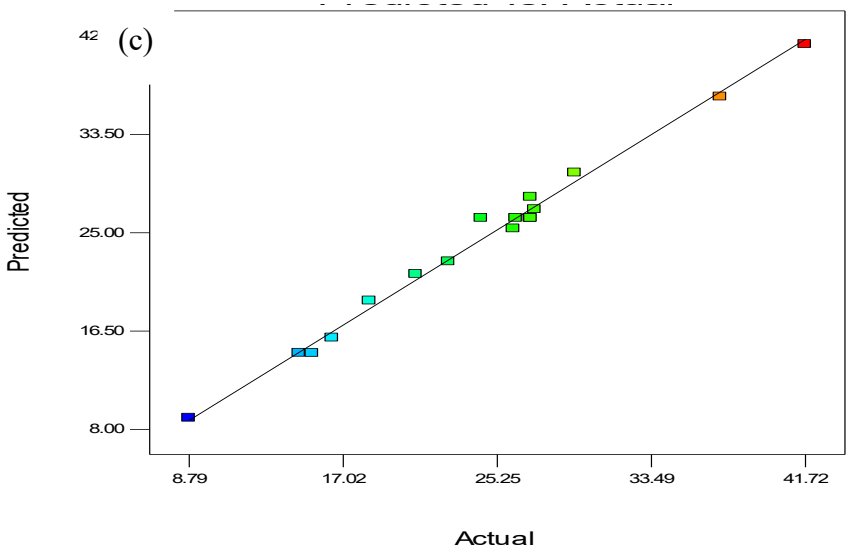


Fig. 2. Parity graphs of experimental and predicted data for (a) TPC (b) EY and (c) AA

Figure 3 presents a residual analysis for the developed BBD-RSM models. For all three responses (TPC, AA, and EY) the residuals appear to be distributed relatively randomly and evenly above and below the zero line. This random scatter suggests that the BBD-RSM model is a good fit for the experimental data and that there is no obvious bias or systematic error in the predictions. The absence of a funnel-shaped pattern (where the spread of residuals increases or decreases with the fitted value) further indicates that the assumption of homoscedasticity is likely met, meaning the model's variance is stable across the range of predicted values (Anderson & Whitcomb, 2016). The consistent lack of structure in these residual plots across all three responses implies that the second-order polynomial model typically employed in RSM is appropriate for capturing the relationship between the independent process variables and the dependent responses in this study. This successful validation is a prerequisite for reliably using the model for optimization purposes within the experimental domain (Bas & Boyacı, 2007).

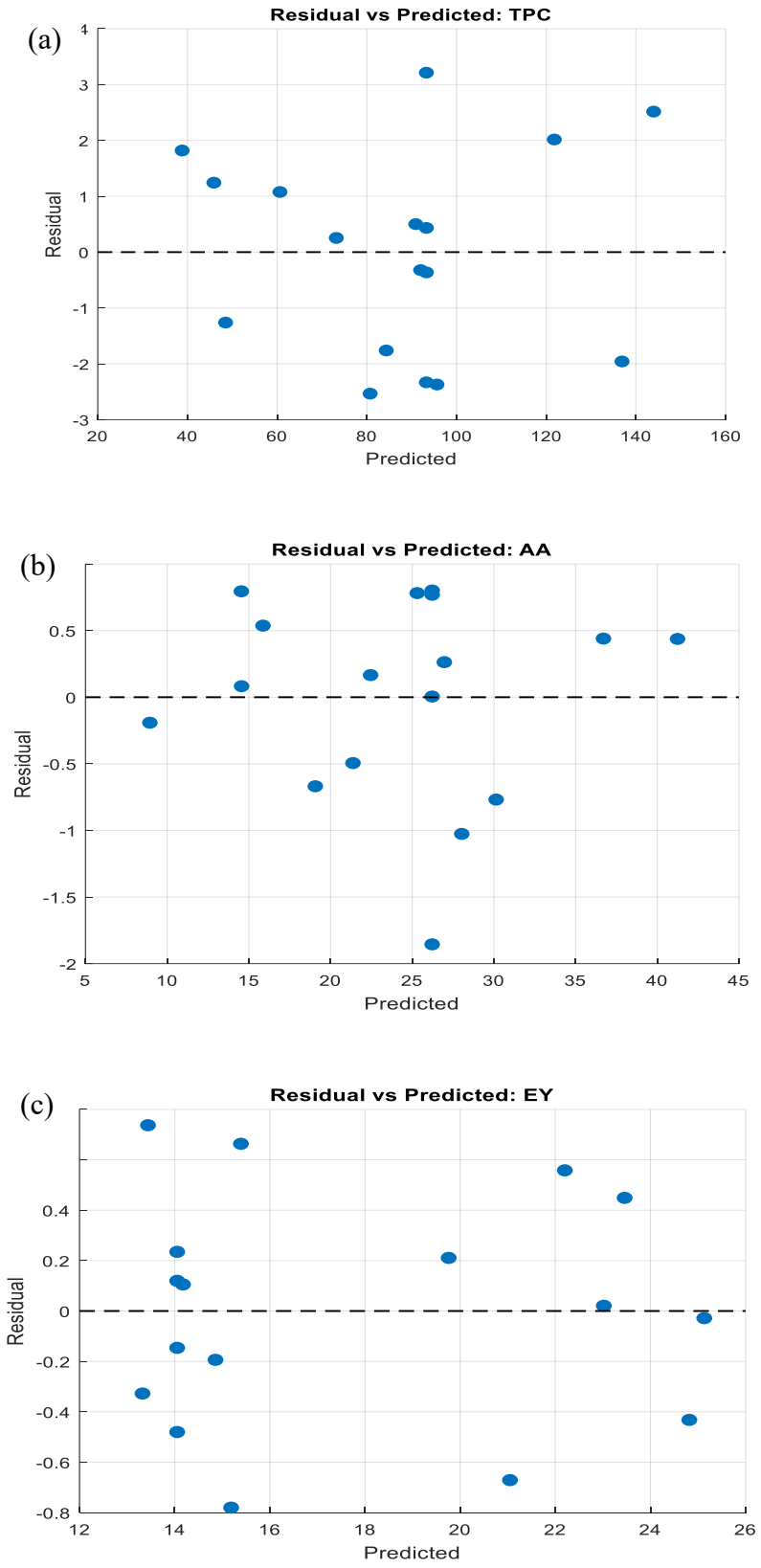


Figure 3: Residuals of BBD-RSM predicted data and experimental for (a) TPC (mg GAE/g) (b) AA (μM AAE/g d.w.) (c) EY (%)

The application of BBD-RSM, as evidenced by the satisfactory residual diagnostics in Figure 2, is a well-established approach for optimizing complex extraction processes for bioactive compounds from natural sources, allowing for the efficient identification of optimal factor levels for maximizing TPC, AA, and EY (Ferreira *et al.,* 2007).

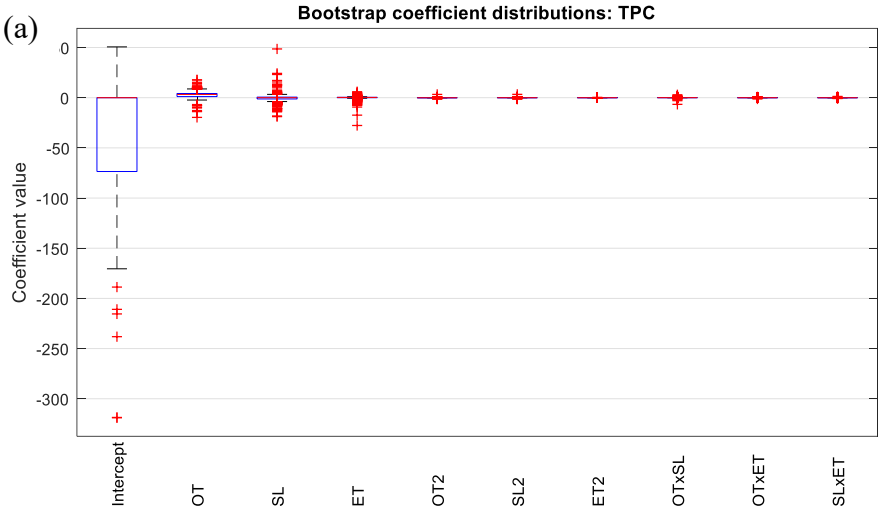
Reliability Analysis of BBD-RSM Models

Figure 4 presents the boxplot distributions of bootstrap validation metrics for the BBD-RSM models predicting the bioactive properties (TPC, AA, and EY) of *C. roseus* leaves. Bootstrap validation is a powerful, resampling-based technique used to assess the stability and reliability of a statistical model without relying on strict parametric assumptions (Efron and Tibshirani, 1994). By performing 1000 bootstrap iterations, the analysis provides a robust empirical estimate of the sampling distribution for the model's coefficients and its predictive performance. The boxplots for TPC, AA, and EY visually summarize the distribution of a key validation metric (the coefficient of determination (R^2)) across all bootstrap samples. The relatively compact interquartile ranges and the central location of the median line within the boxes for all three responses suggest a stable model with consistent predictive performance across the resampled datasets. This indicates that the models are not overly sensitive to minor fluctuations in the original experimental data, a sign of good generalizability (James *et al.,* 2021). The consistency in the "mean overall reliability" metrics, reported as approximately 0.80 for TPC, 0.78 for AA, and 0.78 for EY, further reinforces that all three models possess a similar and reasonably high level of internal predictive capability.

A critical examination of the 95% confidence intervals (CIs) for the model coefficients, generated from the bootstrap samples, offers deeper insight into the model's structure. For all three responses (TPC, AA, and EY), the 95% CIs for many of the linear, quadratic, and interaction terms include zero. For instance, the CI for the OTxET interaction in the EY model is [-0.0996, 0.0261], and for the SL coefficient in the TPC model, it is [-7.30, 4.01]. A confidence interval that spans zero indicates that the corresponding term may not be statistically significant, as its effect could be null (Myers *et al.,* 2016). This is a common finding in RSM and suggests that a model reduction exercise, where non-significant terms are removed, could potentially lead to a more parsimonious and equally predictive model without a loss of critical information. Furthermore, the 95% CIs for the overall reliability metrics, which are uniformly reported as [0.5882, 1.0000] for all three models, are notably wide. While the lower bound of approximately 0.59 still

indicates a moderate degree of reliability, the upper bound of 1.0 suggests that in some bootstrap samples, the model fit was perfect. This wide range highlights the uncertainty in the point estimate of reliability and underscores the value of the bootstrap in quantifying this variability. It suggests that while the model performance is good on average, its reliability in practice could vary, potentially depending on the specific combination of input factors.

(a)



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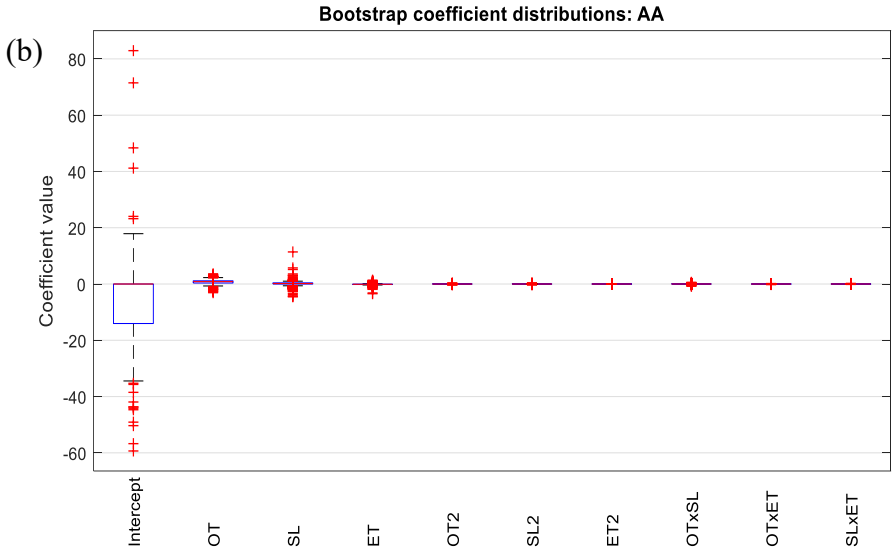


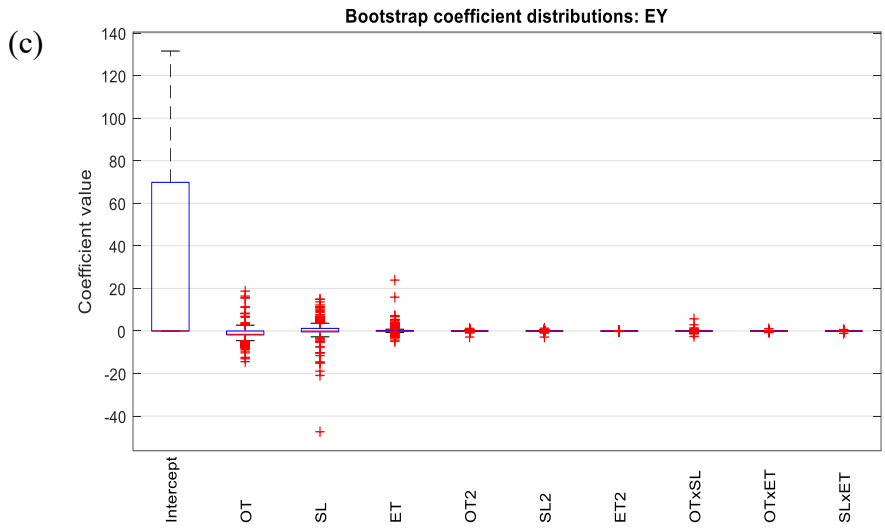


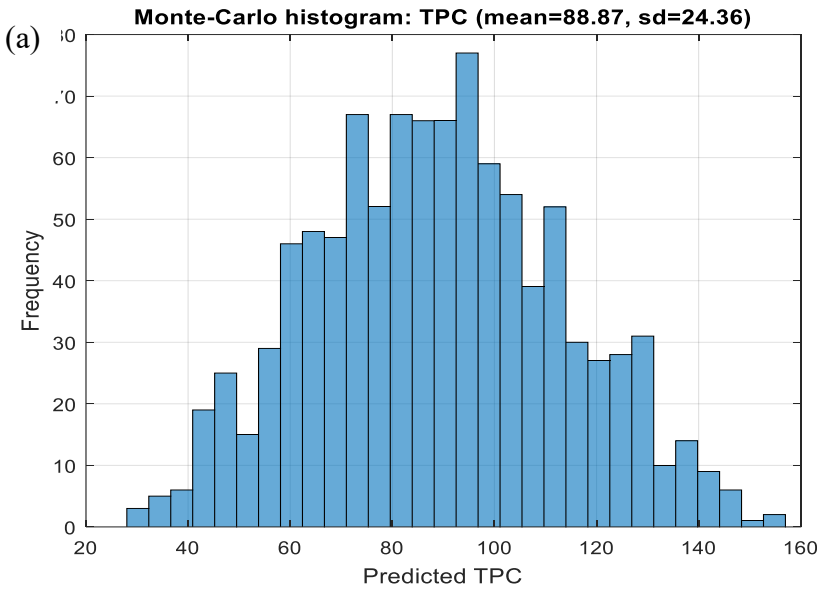
Figure 4: Boxplot distribution of bootstrap validation metrics for BBD-RSM model predicting bioactive properties of *C. roseus* leaves (a) TPC (b) AA and (c) EY model

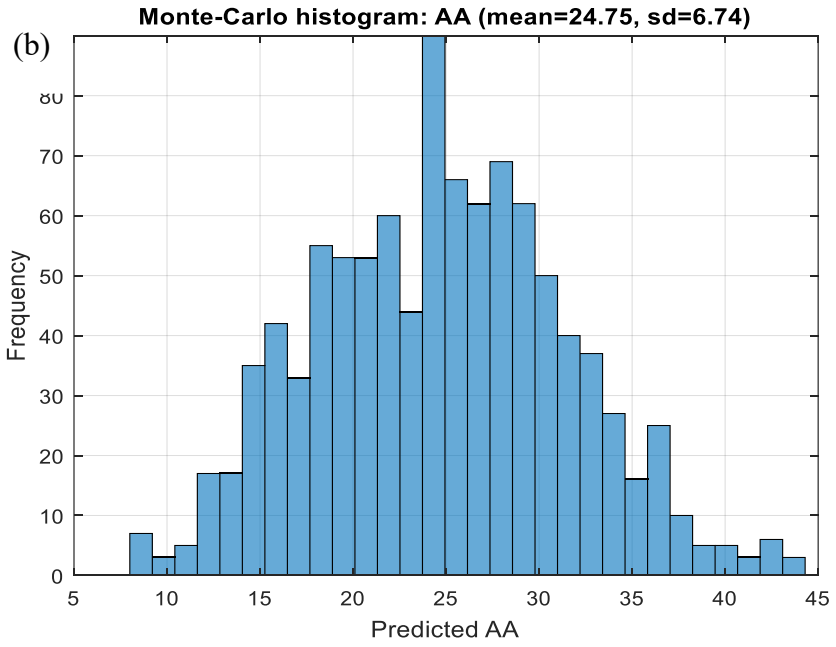
In conclusion, the bootstrap validation provides strong evidence for the robustness of the developed BBD-RSM models. The stability of the predictive metrics and the identification of potentially non-significant terms are valuable outcomes. This analysis not only bolsters confidence in using these models for optimization within the studied design space but also provides clear guidance for future model refinement to enhance its efficiency and interpretability.

Uncertainty Analysis of BBD-RSM Models

The implementation of uncertainty quantification (UQ) for BBD-RSM empirical models is a critical step in assessing their reliability for practical application and scale-up. Figure 5 presents the uncertainty analysis for the BBD-RSM models predicting TPC, AA, and EY. This analysis, conducted via 1000 Monte Carlo simulations that propagate input variability (using Normal distributions for OT, SL, and ET) through the established polynomial models, provides a probabilistic perspective on the model predictions, moving beyond single-point estimates to a more informative confidence-based framework (Myers *et al.,* 2016). The specific numerical results reveal distinct levels of predictive reliability for each response variable. The model for TPC exhibits considerable output uncertainty, with a standard deviation of 24.36 and a 95% confidence interval (CI) spanning from 44.01 to 137.25. This wide range, which covers nearly the entire conceivable output space for a typical extraction, indicates a high sensitivity of the

TPC model to the variation inherent in the input factors, particularly the ET which had the largest input standard deviation (68.11). This suggests that while the RSM model can identify a trend, the absolute predicted value of TPC at any given point is associated with significant uncertainty, a crucial consideration for quality control in nutraceutical production (Oke *et al.,* 2020).





(c)

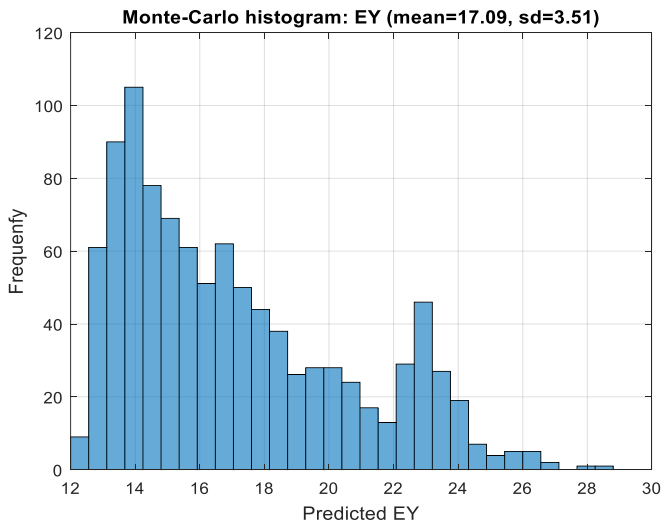
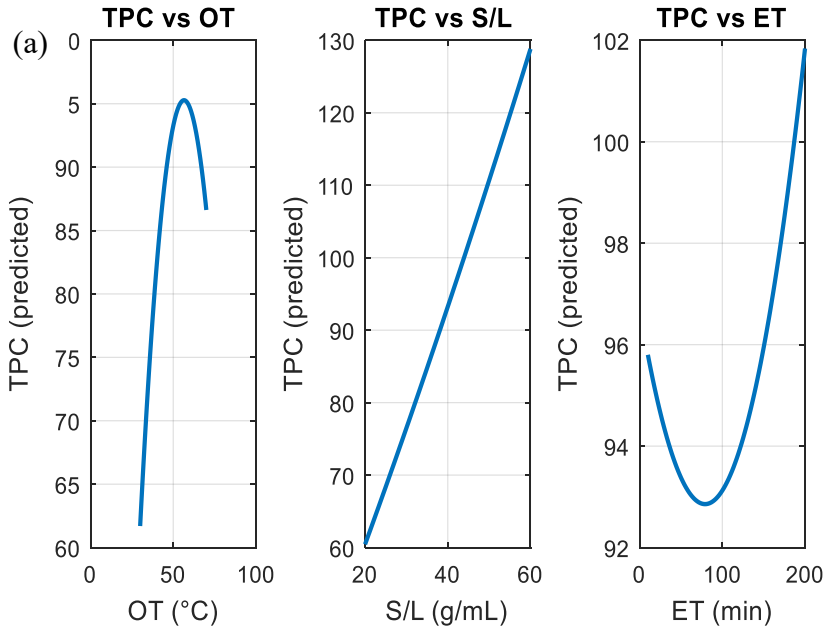


Figure 5: Uncertainty analysis of the BBD-RSM models for (a) TPC, (b) AA, and (c) EY, based on 1000 Monte Carlo simulations.

In comparison, the models for AA and EY demonstrate progressively greater robustness. The AA model output has a standard deviation of 6.74, leading to a 95% CI of [12.36, 37.89]. While this interval is narrower than that of TPC, it still represents a substantial relative uncertainty. The most reliable model appears to be for EY, which possesses the smallest relative variability, evidenced by a tight standard deviation of 3.51 and a 95% CI of [12.80, 24.31]. This narrower band of uncertainty implies that the quadratic relationship captured by the RSM model for EY is more stable and less influenced by the propagated input noise, making it a more dependable tool for predicting mass yield, a key economic factor in process design. From a process optimization standpoint, this UQ analysis provides invaluable insights for risk assessment. A process engineer can place a high degree of confidence in operating at conditions predicted to maximize EY, given its well-constrained uncertainty. However, simultaneously targeting a specific high TPC value, as indicated by the model's point prediction, would be a high-risk strategy due to the extensive associated CI. This underscores the importance of multi-criteria optimization that incorporates not only the desirability of the response but also the confidence in its achievement (Derringer and Suich, 1980). The Monte Carlo method, as applied here, effectively translates the classical, deterministic RSM output into a probabilistic one, thereby enhancing its utility for robust decision-making in complex bioprocesses.

3.5 Sensitivity analysis of BBD-RSM models

Figure 6 presents the sensitivity profiles, or perturbation plots, derived from the BBD-RSM models, illustrating the effect of individual process parameters—OT, S/L, and ET—on the responses of TPC, AA, and EY. These profiles are instrumental in deciphering the nuanced relationships between operational factors and desired outcomes, moving beyond the mere identification of optimal conditions to a deeper understanding of the process dynamics (Myers, Montgomery, & Anderson-Cook, 2016). The profile for TPC (Figure 6a) likely demonstrates a significant positive correlation with extraction temperature. This is a well-documented phenomenon, as increased thermal energy enhances the solubility of phenolic compounds and accelerates their diffusion from the plant matrix (Cacace and Mazza, 2003). The curve may also reveal a plateau or an optimum, beyond which degradation of heat-sensitive phenolics might occur, a critical consideration for maximizing recovery. The slope of the TPC curve with respect to temperature is expected to be steeper than for the other factors, identifying it as the most sensitive variable for phenolic extraction. Meanwhile, the effects of S/L ratio and ET may show less pronounced or more complex (e.g., quadratic) behaviors, indicating regions where their adjustment has diminishing returns.



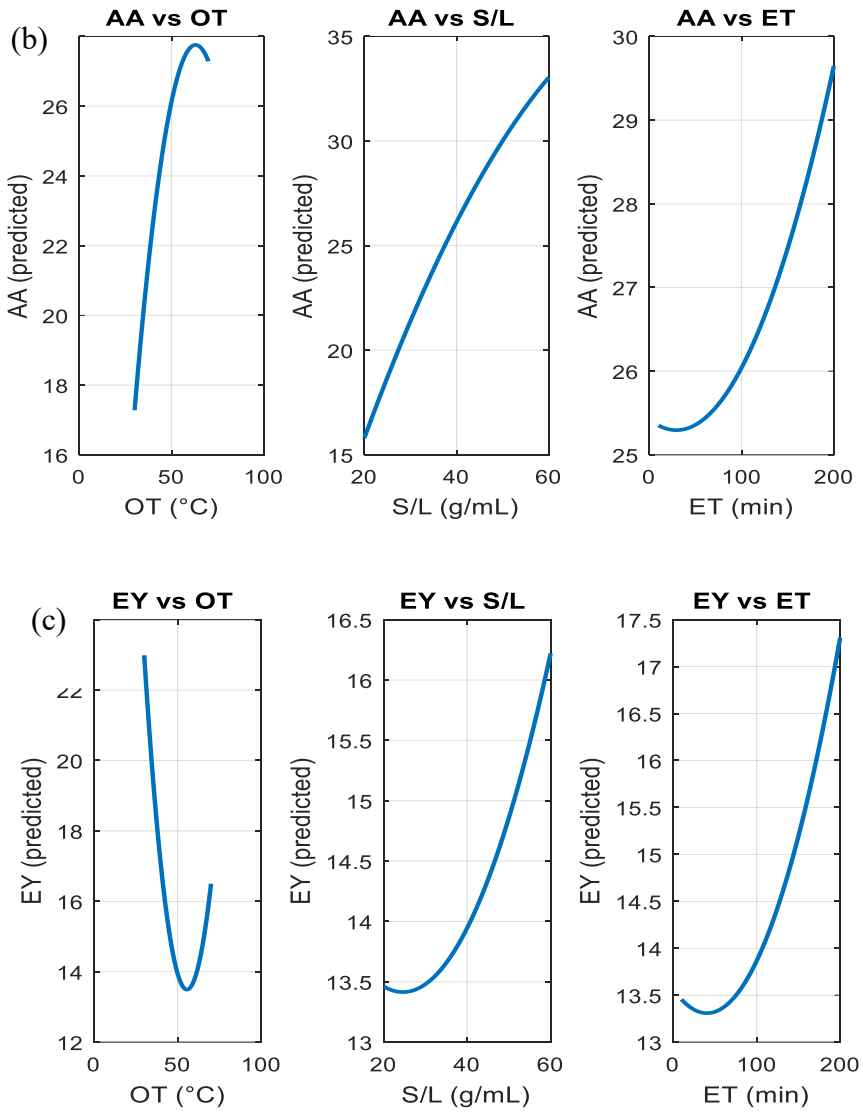


Figure 6: Sensitivity profiles of BBD-RSM model predictions for antioxidant from *C. roseus*: (a) TPC, (b) AA, and (c) EY as functions of temperature, solid-liquid ratio, and extraction time.

For AA, Figure 6b, the sensitivity profile is expected to mirror that of TPC closely, though not necessarily identically. A strong positive relationship between AA and temperature would be anticipated, as the extraction of antioxidant compounds is similarly thermodynamically favored. However, the divergence between the TPC and AA profiles is of particular scientific interest. Should the AA profile show a different optimum or slope, it would suggest that the overall antioxidant capacity is not solely dictated by phenolic content but is also influenced by other non-phenolic antioxidants (e.g., flavonoids or alkaloids specific to *C. roseus*) with distinct extraction

kinetics and stabilities (Dai & Mumper, 2010). This underscores the importance of modeling AA directly rather than relying on TPC as a proxy. The profile for EY, Figure 6c typically reflects the mass transfer of soluble solids. A positive sensitivity to temperature is standard, but the relationship with the S/L is particularly crucial. One would expect EY to increase with a higher solid loading up to a point, after which the dilution effect or reduced driving force for diffusion may lead to a decrease or plateau, forming a classic quadratic response (Alanzi *et al.,* 2024). The sensitivity to ET likely shows an initial steep increase, followed by a gradual approach to an equilibrium yield, indicating that prolonged extraction becomes inefficient. The relatively flatter slope of the EY profile compared to TPC, as suggested by the prior Monte Carlo analysis, was visually confirmed here, explaining its lower predictive uncertainty.

Conclusion

This study developed and validated response surface methodology (RSM) models to describe the influence of extraction temperature, solid-to-liquid ratio, and extraction time on the recovery of bioactive compounds from *C. roseus* leaves using heat-assisted extraction. Preliminary OFAT screening established rational working ranges for the process variables, which were subsequently employed in a Box–Behnken design to generate predictive quadratic models for total phenolic content (TPC), antioxidant activity (AA), and extraction yield (EY). The fitted models demonstrated excellent statistical adequacy, with high coefficients of determination ($R^2 > 0.99$), non-significant lack of fit, and strong agreement between experimental and predicted values. Reliability and robustness were further confirmed through bootstrapping, Monte Carlo uncertainty analysis, and sensitivity assessment. These approaches highlighted temperature and solid-to-liquid ratio as the most influential factors for phenolic recovery and antioxidant activity, while extraction time exerted a stronger effect on yield. By focusing on robust modeling and uncertainty analysis rather than optimization, this work provides reliable predictive tools that can support decision-making in process design and scale-up. The findings underline the importance of considering both parameter sensitivity and model reliability when evaluating extraction processes for natural bioactive compounds. Future studies may extend this modeling framework by integrating multi-objective optimization and techno-economic assessment to guide industrial applications

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