Optimization of Ultrasound-assisted Extraction Using Response Surface Methodology for Total Anthocyanin Content, Total Phenolic Content, and Antioxidant Activities of Roselle (*Hibiscus sabdariffa* L.) Calyces and Comparison with Conventional Soxhlet Extraction

Tanvir Ahmed[1*], Shakhawat Ullah[2], Md Suzauddula[3], Sadia Haque Khan[4]

[1]Department of Food Engineering and Tea Technology, Shahjalal University of Science and Technology, Sylhet-3114, Bangladesh. tanvirsust2011@gmail.com

[2]Farm Technology, Irrigation, and Water Resources Management Division, Rural Development Academy, Bogura, Bangladesh. shakhawat64@gmail.com

[3]Department of Nutrition and Food Engineering, Daffodil International University, Dhaka 1341, Bangladesh. mdsuzauddula@gmail.com

[4]Department of Microbiology, University of Dhaka, Dhaka, 1000, Bangladesh. sadiahaquek@gmail.com

*Corresponding author: tanvirsust2011@gmail.com

Abstract

Response surface methodology (RSM) and a Box-Behnken design (BBD) were used to determine optimum conditions for ultrasound-assisted extraction of *Hibiscus sabdariffa* L. calyces. The current study applied BBD to explore the effects of X_1: ultrasonic temperature (30-80 °C), X_2: ultrasonic time (20-50 min.), and X_3: solid-to-solvent ratio (1:10 – 1:60) on total anthocyanin content (TAC), total phenolic content (TPC), and antioxidant activities (DPPH and FRAP assays). ANOVA results revealed that TAC, TPC, DPPH, and FRAP all had R^2 values of 0.98, 0.97, 0.98, and 0.98, respectively, indicating that models designed with second-order polynomials were capable of reliably analyzing interactions between parameters (response and independent variables) satisfactorily. It was determined from RSM study that 80 °C ultrasound temperature, 48 min. ultrasound time, and 1:60 solid-to-solvent ratio were the optimum extraction parameters for maximizing TAC, TPC, DPPH and FRAP. The experimental values for TAC, TPC, DPPH, and FRAP were 311 ± 5 mg CGE/100 g, 572 ± 7 mg GAE/100 g, 974 ± 3 µmolTE/100 mL, and 2332 ± 3 µmolTE/100 mL, respectively, under the optimal conditions. Also, a good agreement was found between experimental and predicted values, with a residual standard error of less than 5%. Compared to the yield of Soxhlet extraction for TAC (176 ± 4 mg CGE/100 g), TPC (210 ± 3 mg GAE/100 g), DPPH (534 ± 2 µmolTE/100 mL), FRAP (1732 ± 3 µmolTE/100 mL), the extraction efficacy of the UAE process under optimized conditions demonstrated to be more effective. Therefore, based on the needs of the industry and sustainable development, the UAE process might be an economical alternative to traditional extraction methods.

Keywords: *Hibiscus sabdariffa* L., Calyces, Antioxidant activity, Response Surface Methodology, Ultrasound-assisted extraction, Soxhlet extraction

1. Introduction

There has been a significant uptick in demand for natural foods in the last decade, especially those high in or mostly comprised of substances derived from plants. Many of plants derived foods are promoted and researched for their potential health benefits, which has led to increased interest in them. Foods with high concentrations of active biological components, such as anthocyanins, flavonoids, polyphenolic acids, and vitamins, are also referred to as functional foods. Besides being a source of nutrients, these foods could provide specific health benefits to humans [1]. Researchers have been looking for biologically active compounds in foods that can promote health and well-being, and perhaps prevent certain diseases. Consequently, there is an increasing interest in Roselle calyces and their extracts for their potential as functional foods.

The Roselle, *Hibiscus sabdariffa* L., belongs to the Malvaceae Family and is a bushy, erect annual herbaceous shrub with an upright, branched stem. The most essential part of roselle is calyces. The calyces of roselle have been used for centuries in a variety of medicinal and culinary applications, including refreshing drinks, jams, syrup, wines, and natural food colorants. As a rich source of phenolic compounds and anthocyanins, roselle calyces have the potential to be used in a variety of ways [2]. These compounds may be a naturally occurring source of antioxidants and a crucial factor in determining the antioxidant potential of foods. According to research conducted by Prasongwatana et al. [3], the uricosuric impact of Roselle calyces extract makes it a viable therapeutic agent for kidney stone disease. Since ancient times, the calyces of Roselle have been used to treat patients with hypertension, high fever, and gastrointestinal disease because of their high antioxidant content. In the meantime, studies conducted by Olvera-Garcia et al. [4] showed that calyces extract possesses numerous therapeutic properties in *in vitro* and *in vivo* studies, such as anti-diabetic, anti-inflammatory, anti-neurodegenerative, and anticancer capabilities. As the calyces of the *Hibiscus sabdariffa* L. plant have demonstrated therapeutic promise and include phenolic compounds and anthocyanins, it is essential to investigate the antioxidant activities of calyces.

Different technologies can be used for the extraction of anthocyanins and phenolic compounds from solid materials, including conventional methods (Soxhlet, agitation, maceration, hydrodistillation) and novel methods (supercritical fluids, ultrasound-assisted, microwave, enzyme)[5]. Conventional procedures have drawbacks, such as the high cost of a solvent, time, and energy usage, which motivate the search for alternate methods that are more effective, economical, and safe. In this context, there has been extensive research into green extraction alternatives, such as those based on alternative solvent usage (other than petroleum), renewable plant resources, and emerging technologies that may permit decreasing cost, energy, and time inputs [6]. Recent years have seen a surge in interest in ultrasound-assisted extraction (UAE) technology because of its many attractive features, such as high repeatability, minimal solvent use, high extraction efficiency, low cost, easy

operation, and low pollution to the environment. Moreover, UAE processes have been found to enhance higher extraction yields because cavitation occurs when air bubbles rapidly form and collapse in ultrasound-treated fluids, causing local pressure and temperature increases as well as large amounts of energy. As a result, increased diffusion across the cell wall or its breakdown could increase cell viability, which in turn may enable cell contents to be liberated [7].

The extraction efficiency varies based on different factors such as solid-to-solvent ratio, solvent type, extraction temperature, time, and pressure. Several innovative technologies have the potential to improve extraction efficiency over conventional techniques like Soxhlet by reducing the amount of solvent required, extraction temperature and time, temperature, and/or enhancing extract yield and quality under optimal conditions [8]. Consequently, increasing the contents of anthocyanins, phenolic compounds, and antioxidant activities requires optimizing the process parameters. The response-surface methodology (RSM) and classical single-factor experiments are the two most common approaches to optimizing a system [9]. One-factor-at-a-time methods involve varying one factor at a time while keeping all others constant; although these methods are popular, they are time-consuming and provide limited information about how the factors interact [10]. Besides, RSM can be applied to investigate the effect of multiple variables. RSM not only defines the effect of independent variables but also their interactions. Moreover, RSM can be used to analyze, improve, and optimize the independent factors that influence experiment outcomes. It has been found that the central composite design (CCD) and Box-Behnken design (BBD), which have been frequently used in RSM experiments, have been utilized in many studies. As the focus of most RSM studies is second-order models, BBD is specifically developed to fit such models. Besides, a second-order regression model fits with the BBD using only three levels of each factor, whereas the CCD requires five levels. Having said that, the BBD also requires fewer experimental runs than other methods. [11].

Although several separate studies on the extraction of total anthocyanin content (TAC), total phenolic content (TPC), and antioxidant activity from *Hibiscus sabdariffa* L. calyces can be found in the literature, there are currently few reports describing the optimized conditions for their extraction. Due to the compositional variability of TAC and TPC's natural sources (e.g., fruits, flowers, leaves, stems, and roots), extraction conditions cannot be extrapolated directly from previously investigated sources. Thus, it is essential to undertake independent studies to optimize the extraction of TAC, and TPC from *Hibiscus sabdariffa* L. by selecting the crucial factors for each extraction technique. In our investigation, no UAE study has yet been reported that optimizes the UAE extraction temperature, time, and solid-to-solvent ratio for *Hibiscus sabdariffa* L. calyces to determine the levels of anthocyanins, phenolics, and antioxidant activity. Thus, the present study aimed to optimize the UAE extraction time, temperature, and solid-to-solvent ratio using RSM for extraction of TAC, TPC and determine the antioxidant activity (DPPH, FRAP) of *Hibiscus sabdariffa* L. calyces. As a prototype

of conventional methods, Soxhlet extraction (SE) was carried out and its results were compared with UAE.

2. Materials and methods

2.1. *Materials and Chemicals*

The fresh *Hibiscus sabdariffa* L. was collected from Horticulture & Nursery Unit, Rural Development Academy, Bogura, Bangladesh (27°59′ latitude and 58°29′ longitude, elevation 1845 m). First, the samples were cleaned with distilled water, and then the calyces were removed. Before freeze drying, the samples were frozen at -90 °C. Samples were freeze-dried for five days at -50 °C to -54 °C using a freeze dryer (model: BK-FD505, BIOBASE, China) followed by grinding into powder (mesh size 30) using a commercial blender (model: QB1004, Ninja, USA). In the airtight jar, the powdered sample was stored in the -80 °C freezer until further use. For TAC, TPC, and antioxidant assays (DPPH, FRAP), all solvents and standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Extraction methods and design of experiments

The UAE method was used for extraction and optimization under three independent variables with three different levels. In addition, the current study also conducted SE for comparison with UAE. The present study conducted a preliminary screening of the literature and determined the appropriate ranges of independent process variables for the UAE, namely X_1 (temperature, °C), X_2 (time, min.), and X_3 (solid-to-solvent ratio, g/mL) (Table 1). Independent variables are selected based on their influence on the TAC, TPC, and antioxidant assays (DPPH and FRAP). The extractions were performed using a BBD with three factors and three levels. The variables were coded to three levels, namely −1, 0, and +1, based on the following equation:

$$y = a_0 + a_1x_1 + a_2x_2 + a_{11}x_1^2 + a_{22}x_2^2 + a_{12}x_1x_2 \ldots\ldots\ldots\ldots\ldots\ldots$$

----------Equation 1

Where y is the measured response variable, x_1 and x_2 represent the levels of independent variables. a_o is a constant (predicted response at the centre), a_1, a_2; a_{11}, a_{22}; and a_{12} are the linear, quadratic, and two-factor interaction coefficients of the model, respectively. ANOVA was performed using the Design-Expert® statistical software (version 12.0.3) to determine the effects of significant interactions in the model ($p < 0.01$ or $p < 0.05$). Model F-value, lack of fitness, and coefficient of determination (R^2) were calculated to assess the fitness of the model.

2.3. Ultrasound-assisted extraction (UAE) from Hibiscus sabdariffa L. calyces

An ultrasonic bath (Model: Power-Sonic 405, Hwashin Technology Co., Korea) with a capacity of 6 L, 200 W, and 40 kHz was used to extract by following the method described by Borges et al. [12] with minor modifications. Each experiment was conducted in a 250 mL Erlenmeyer flask using calyces' dry powder (1 g) and 50% (v/v) of ethanol using the BBD experimental design (Table 2) following a three-level three-factor full factorial design with center points. Following sonication, the mixture was poured into a flask and placed well in the ultrasonic bath. Then, the mixtures were centrifuged (6000 rpm for 10 min at room temperature). The samples were filtered after extraction with Whatman filter papers (number 4). In order to analyze TAC, TPC, DPPH, and FRAP, samples were stored at 4 °C in an amber flask.

2.4. Soxhlet extraction (SE) from Hibiscus sabdariffa L. calyces

A comparison of UAE and conventional solvent extraction was made by using SE following the Kothari et al. [13] method after optimizing the UAE process. An amount of dried calyces (6 g) was filled with cellulose thimble and 50% (v/v) of ethanol was used for extraction. During SE, the operating conditions were identical to the UAE-derived optimal conditions, such as extraction temperature, time, and the ratio of solid-to-solvent. In the following steps, the extracts were combined and reduced to 25 mL by using a rotary evaporator (model: Buchi R-210; Flawil, Switzerland), filtered by injection disk filter (0.45 μm), and diluted in ethanol with 25 mL.

2.5. Total anthocyanins content (TAC)

The content of monomeric anthocyanins was determined using the official AOAC method 2005.02.[14]. Based on the color change of anthocyanin with pH, this method uses pH differentials to determine the color: at pH 4.5, colorless hemiketal is predominant, whereas, at pH 1.0, colored oxonium ions occur. Concentration determines the absorbance variation of pigments at 530 nm. The samples were diluted properly in sodium acetate buffer (.4 M) and potassium chloride buffer (pH 1.0, 0.025 M), and the absorbance at 530 and 710 nm was determined. The TAC concentration was calculated in terms of mg of cyanidin-3-glucoside equivalents/100g (mg CGE/100g) of extract using Equation 2:

$$\text{TAC (mg CGE/100g)} = \frac{[(X520 - X700)pH\ 1.0\ - (X520 - X700)pH\ 4.5) \times MW\ \times DF}{\varepsilon\ \times\ l}$$

----------Equation 2

where l is standard path length (cm), ε = the molar extinction coefficient for cyanidin-3-glucoside (26,900 L/mol/cm), DF is the dilution factor, MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), and X is the absorbance.

2.6. Total phenolic content (TPC)

In the current investigation, following the procedure demonstrated by Silva et al.[15], *Hibiscus sabdariffa* L. calyces were extracted with ethanol and their TPC determined. The TPC was calculated using the Folin–Ciocalteau method. 0.6 mL of the ethanol extract was mixed with 6 mL of Folin-Ciocalteau reagent in a 1 mL sodium carbonate solution at 4% concentration. The solution was left to incubate for 2h at room temperature in the dark. UV-1800 Spectrophotometric (Shimadzu Scientific Instruments, Japan) measurements were performed at 765 nm. Per 100 mg of extract, TPC was measured in mg gallic acid equivalents (mg GAE/100 mg).

2.7. Determination of antioxidant activities (DPPH and FRAP assays)

The FRAP and DPPH assays were performed on *Hibiscus sabdariffa* L. calyces to evaluate their antioxidant activities. The current investigation followed the protocol reported by Silva et al. [16] and Thaipong et al.[17], consequently, to determine DPPH and FRAP. Measurements of the absorbance of DPPH and FRAP were performed with a UV-1900 spectrophotometer (Shimadzu Scientific Instruments, Japan) at 517 nm and 593 nm, respectively. The unit of DPPH and FRAP was expressed as micromoles of Trolox equivalent (TE) per 100 mL of the sample (μmolTE/100 mL).

2.8. Model validation

A comparison of adjusted R^2 and predicted R^2 was conducted in order to validate the model. The present study validated the model derived from RSM using triplicate tests under optimal conditions. For comparisons between predicted values and experimental results, residual standard error (RSE) percentages were used (Equation 3).

$$\text{Residual standard error (RSE) (\%)} = \frac{Experimental\ value - Predicted\ value}{Predicted\ value} \times 100 \text{ ----------Equation 3}$$

3. Results and discussion

In the current investigation, solid-to-solvent ratio, ultrasonic temperature, and ultrasonic time, optimization was carried out to maximize TPC, TAC, and antioxidant activity (DPPH, FRAP) of *Hibiscus sabdariffa* L. calyces' extracts. The first step of the current investigation was to identify the variables in the UAE process that influence the levels of TAC, TPC, and antioxidant activities. Multiple studies were conducted using single factor testing to analyze how different processing factors affected the UAE process. Most of the previous studies addressed the effects of different variables on the efficiency of the extraction process such as solid-to-solvent ratio, solvent content, ultrasound time, ultrasound temperature, and frequency [7]. Several discrepancies have also been observed in UAE treatment, with the extraction of TAC, TPC, and antioxidant activity varying based on pretreatment of the residue sample, cultivar zone, and species [18]. It is possible that few sonication factors, such as the frequency of ultrasonic waves, and ultrasonic power, could adversely affect the TAC, TPC, and antioxidant capacity. For instance, a frequency of between 30 and 40 kHz

was used in grape by-products for anthocyanins extraction, and it was reported to be most effective at 35 kHz [19]. Similarly, according to the RSM study of Gonzalez-Centeno et al. [20], 40 kHz was optimal for extracting phenolics from grape pomace compared to 82 kHz, and 122 kHz frequencies. Research on plant materials used in the UAE reveals that most authors only specify the ultrasonic power and frequency used by their respective systems [21–23]. Consequently, 40 kHz was selected in the present investigation as the frequency for UAE optimization. There may be some explanation for why low frequencies are preferred, such as being conducive to a limited number of larger cavitation bubbles and a larger cavitation effect. This cavitation effect diminishes with increased ultrasonic frequency [24]. Cavitation bubbles must be compressed and rarefied for a minimum amount of time in order to form and grow. Insufficient time in the cycle prevents the bubble from forming and expanding. Besides, in response to increases in power or power density, UAE extraction yields initially rise, but then decline after reaching a peak. Using coffee grounds waste as a sample, Al-Dhabi et al. [25] investigated how power, temperature, and sonication time interact. They found that the TPC yield increased between 100 and 200 W, while yield declined at power levels above 250 W. Conversely, Gonzalez-Centeno et al. [20] found that total antioxidant yields, phenolic, and flavonoid content were linearly related. A further explanation for the higher yield is the increased contact area between solvent and solid caused by ultrasonic vibrations. Hydrodynamic force, which causes tissue disruption, is also boosted when power is increased [26]. A high level of power, however, produces a greater number of bubbles. Thus, the cavitation effect is reduced when the bubbles volume concentration increases. Bubble implosion is reduced when more bubbles are formed, because there are more collisions, deformations, and collapses of non-spherical bubbles [27]. In addition, a coating of cavitation bubbles surrounding the probe tip reduces yield when energy cannot be transmitted into the extraction medium as a result of the saturation effect. Similarly, in the UAE, a range of solvents have also been employed to extract various chemicals, including water, acetone, alcohols, ethanol, etc. Plant sources of phenolic chemicals are commonly extracted using alcohol or acetone mixed with water. It has been shown in numerous studies that ethanol is the most effective solvent for removing phenolic compounds from composted vegetable and fruit scraps due to its strong affinity. Due to its low price and renewable source (sugar cane), ethanol is preferred as a solvent for its GRAS status (generally recognized as safe) [28]. Up to a certain point, ethanol concentration increases boost phenolic component yields, but further increases have detrimental effects. A similar pattern has been observed when extracting cyanidin-3-O-glucoside from jabuticaba peel [28] and TPC from *Stevia rebaudiana* leaf [29]. The dielectric constant of the solvent falls as the ethanol content increases, increasing the phenolic compound's solubility and diffusivity. Having high ethanol concentrations (i.e., very pure ethanol solvent) can lead to dehydration of plant tissue and protein denaturation, resulting in lower yields. So, the present study selected 50% (v/v) ethanol for the UAE and SE process. For the optimization study of solid-to-solvent ratio, ultrasonic temperature, and ultrasonic time in the UAE process, The range of low and high values (Table 1)

were established based on prior research into the levels of TAC, TPC, and antioxidant activity in different plant materials [30–34].

3.1. Fitting the response surface models (RSM) of the UAE

As shown in Table 2, a BBD was developed with regard to optimizing the UAE variables (solid-to-solvent ratio, ultrasound time, and temperature) in terms of maximizing TAC, TPC, and investigating the antioxidant activities (DPPH and FRAP) from *Hibiscus sabdariffa* L. calyces' extracts. Table 2 also presents the experimental values of TPC, TAC, and antioxidant activities (DPPH and FRAP) of *Hibiscus sabdariffa* L. calyces extracts under various experimental conditions. The experimental values for TAC (213 ± 2 - 321 ± 2 mg CGE/100 g), TPC (371 ± 4 - 531 ± 3 mg GAE/100 g), DPPH (509 ± 5 - 948 ± 3 μmolTE/100 mL) and FRAP (1739 ± 10 - 2262 ± 11 μmolTE/100 mL) demonstrating the significant influence of process factors on the evaluated responses. Data was analyzed using multiple regression methods to estimate regression coefficients for a second-order polynomial model. Using the experimental data for each of the responses, the RSM models are derived based on the following equations:

$$\text{TAC (mg CGE/100 g)} = 177.69 - 1.47X_1 + 3.47X_2 + 1.62X_3 + 0.03X_1X_2 + 0.02X_1X_3 - 0.04X_2X_3 + 0.01X_1^2 - 0.05X_2^2 - 0.02X_3^2 \quad \text{----------Equation 4}$$

$$\text{TPC (mg GAE/100 g)} = 374.97 - 3.07X_1 + 1.16X_2 + 0.39X_3 - 0.03X_1X_2 - 0.03X_1X_3 + 0.03X_2X_3 + 0.06X_1^2 + 0.03X_2^2 + 0.03X_3^2 \quad \text{----------Equation 5}$$

$$\text{DPPH (μmolTE/100 mL)} = 1016.73 - 12.12X_1 - 4.58X_2 - 9.87X_3 + 0.03X_1X_2 + 0.07X_1X_3 - 0.03X_2X_3 + 0.14X_1^2 + 0.06X_2^2 + 0.08X_3^2 \quad \text{----------Equation 6}$$

$$\text{FRAP (μmolTE/100 mL)} = 1257.13 + 4.39X_1 + 18.95X_2 + 5.26X_3 - 0.07X_1X_2 + 0.20X_1X_3 + 0.07X_2X_3 - 0.006X_1^2 - 0.03X_2^2 - 0.23X_3^2 \quad \text{----------Equation 7}$$

As depicted in Table 2, X_1: ultrasound temperature, X_3: solid-to-solvent ratio, and X_1X_3: interaction of ultrasound temperature and solid-to-solvent ratio had significant ($p<0.01$ or $p< 0.05$) effects on most of the responses. Table 3 summarizes the data of the adequacy of models, goodness of fit, and analysis of variance (ANOVA). The regression models for the TAC, TPC, DPPH, and FRAP with satisfactory coefficients of multiple determinations (TAC (Predicted R^2: 0.82, Adjusted R^2: 0.96, R^2: 0.98), TPC (Predicted R^2: 0.81, Adjusted R^2: 0.94, R^2: 0.97), DPPH (Predicted R^2: 0.83, Adjusted R^2: 0.96, R^2: 0.98), and FRAP (Predicted R^2: 0.86, Adjusted R^2: 0.95, R^2: 0.98)) suggests the fitness of the RSM used in this study. The factor selected for the model contributed greatly to the variance shared among samples, indicated by a high R^2. F-critical values for ultrasound temperature, ultrasound time, DPPH, and FRAP were 42.36, 24.34, 50.31, and 30.37, respectively. Those results demonstrate that RSM techniques are capable of accurately modeling the extraction process due to their low Prob > F-value ($p < 0.01$) and high F-value. It is also evident from the data that the lack of fit was not significant when compared with the pure error. Lack of fit values are statistically

insignificant, thus indicating the model's accuracy in prediction [35]. Furthermore, a lower coefficient of variation (C.V.) generally indicates a more credible and accurate experiment [36]. The C.V. value ranged from 2.34 – 3.80%, satisfying the consistency and precision of the BBD experiment.

3.2. Effect of UAE parameters on total anthocyanin content (TAC) based on RSM

The main effect plot and 3D surface plot of the solid-to-liquid ratio, ultrasound temperature, and ultrasonic time on the TAC are shown in Figure 1A and Figure 2(A, B, C) respectively. It is clear from the Figures that increased TAC concentrations are associated with temperatures between 30 and 80 °C. Despite prior research showing anthocyanin stability decreasing above 60°C [37,38], at increased extraction temperatures, the anthocyanin content is likely to be higher because of enhanced solvent properties, denaturation of the plant matrix, and improved diffusion. As a result of ultrasound treatment combined with heating, localized microfractures can be created in the cell walls, thus facilitating anthocyanin release [39,40], which is primarily localized in the hypodermic epidermal cells. Additionally, Hu et al.[41] hypothesized that even though ultrasound cavitation intensities are weaker at high temperatures, the damaging effects are also reduced. Anthocyanin extraction by ultrasound is therefore not necessarily affected by temperature changes under certain conditions and might even be increased. A study by Wang et al.[41] reported that increasing the temperature of UAE process from 60 to 70 °C and 80 °C led to an enhance in the yield of blueberry anthocyanins in MeOH extracted. Moreover, after 30 min. of ultrasonic treatment, TAC levels peaked, gradually increasing with increasing ultrasonic treatment time. In contrast, longer ultrasonic treatment times decreased anthocyanin recovery. It may be possible to intensify the extraction of solutes from the plant matrix with an appropriate ultrasonic treatment because of the cavitation effect [42]. Anthocyanins may undergo chemical decomposition by prolonged ultrasonic treatment at a higher temperature, as confirmed by other studies [43,44]. Similarly, with increasing the ratio of solid-to-solvent from 1:10 to 1:40, TAC gradually increased, but further ratio increases resulted in lower content. It is possible that a high solid-to-solvent ratio would increase the contact among plant materials and solvents, hence enhancing anthocyanin content. In contrast, a further increased solid-to-solvent ratio might cause some anthocyanins in the extract to be oxidized, which decreases anthocyanin content [45]. Similar trending results were also recorded by Wang et al.[46] for extracting TAC from purple sweat potato using UAE.

3.3. Effect of UAE parameters on total phenolic content (TPC) based on RSM

Ultrasonication and high extraction temperatures can alter the diffusion coefficients of phenolic compounds, assisting them in migrating from the plant matrix into a solvent. The application of ultrasound is useful because it can be used to grind up the vegetal matrix, allowing the cell contents to spill out into the extraction medium. Figure 1B and Figure 3 (A, B, C) shows the main effect plot

and 3D surface plot, respectively, of the solid-to-liquid ratio, ultrasound time, and ultrasound temperature on the TPC. It is apparent from the figures that extraction at higher temperatures, specifically from 50 to 80 °C, resulted in a gradual rise in TPC. UAE operations at higher temperatures have not been fully explained, although several hypotheses exist [7]. One theory proposed that when cavitation bubbles are generated during the rarefaction cycle, elevated solvent vapor pressure fills them and reduces the pressure difference between their interiors and exteriors. Cavitation bubbles are more numerous and more widespread at higher temperatures, but their weaker implosion causes less sparing to the cell, reducing the yield. Second, it was hypothesized that the cavitation bubbles formed at high temperatures caused an increase in shear stress, which in turn led to deterioration of the phenolic component. Thirdly, increasing the temperature of a solvent increases its surface tension; as a result, the intensity of collapsing cavitation bubbles reduces the mass transfer of the component. Researchers have found that raising the temperature increases yields without lowering further. For instance, a peak in yield was observed at the highest measured temperature, when the TPC of grape seed rose with increasing temperature, according to the study of Ghafoor et al.,[47]. Correspondingly, Ramic et al.[43] and Rodrigues et al.[48] both observed the same trend with TPC in black chokeberry waste and coconut (*Cocos nucifera*) shell powder. A biomolecule like polyphenols also has a highly time-dependent extraction yield. Extraction yield increases with longer extraction times in the current study (Figure 1 B, Figure 4a, Figure 4c). Ultrasonic parameters impacted the profile of the TPC extraction curve similarly as previous studies reported that longer extraction times and higher temperatures are generally required for olive leaf extracts [49] and seed cake extracts [50]. There are two possible mechanism might be involved in the process: (1) solubilization of phenolics from surface cells of plant materials; and (2) diffusion and osmosis-based processes (slow extraction) release deep-buried chemicals [51]. The results (Figure 1B, Figure 3B, Figure 3C) also demonstrated that the UAE process solid-to-solvent ratio influenced the TPC in the same way as they were influenced by ultrasound temperature and time. Based on these findings, mass transfer between solids and solvents may be driven primarily by concentration gradients between solids and solvents. Since low solid-to-liquid solutions have high viscosities, cavitation is more difficult because the rarefaction cycle must resist a stronger cohesive force. Cavitation increases when the solids-to-liquids ratio increases, resulting in decreased viscosity and concentration of the extraction medium. With a higher concentration difference, the solute could diffuse more readily and dissolve more readily in the solvent, thus enhancing the extraction process. More matrix fragmentation, erosion, and pore development in the by-product are all associated with a higher solids-to-liquids ratio, all of which contribute to a higher TPC [52]. A study on *Aquilaria crassna* showed that an increases in the solid-to-solvent ratio such as 1:10 to 1:20 dramatically increased the TPC, but a ratio of 1:160 did not significantly improve phenolic compound extraction efficiency from *Aquilaria crassna* [53]. Rodriguez et al.[54] reported that coconut shell powder contains more phenolic compounds in the solid-to-liquid ratio between 1:20 and 1:50.

3.4. Effect of UAE parameters on antioxidant activities (DPPH, FRAP) based on RSM

The efficiency of antioxidant assays can affect the antioxidant activity of a sample, so the present study used DPPH and FRAP antioxidant assays to investigate the impact of ultrasound time, ultrasound temperature, and the ratio of solid-to-liquid on *Hibiscus sabdariffa* L. calyces' antioxidant capacity is depicted in Figure 1C as the main effect plot, and in Figure 4(A-C) as 3D response surface plots. Figure 4(A-C) exhibits that the interaction between the ratio of solid-to-solvent and ultrasound temperature significantly affects the DPPH activity more than ultrasound time. It is also evident from Figure 1C that the DPPH activity of *Hibiscus sabdariffa* L. calyces as the solid-to-solvent ratio between 10 – 60 g/mL and the ultrasound temperature increased between 30 °C – 80 °C. It is believed that higher temperature decreases viscosity and increases solubility in solvents of substances. Furthermore, higher temperatures improve the dispersion and diffusion of the extracted solvent, which speeds up extraction [55]. A similar range of temperature (30 – 70 °C) was also used to investigate the DPPH activity of *Curcuma caesia* rhizome using UAE, where the best DPPH activity was recorded at 60 °C [56]. Similar results were also reported on *Chenopodium quinoa* seeds extracts [57], *Citrus unshiu* extracts [58]. The degradation of antioxidant compounds and diluting of the solution led to a decline in DPPH scavenging activity. Similar trending results of DPPH were also recorded by Gogoi et al.[59] for *Pleurotus citrinopileatus* mushroom. There was no discernible correlation between extraction time and DPPH activity, suggesting that phenolics influencing DPPH assay values may be recovered in less time than optimal [60].

Similarly, Figure 1D and Figure 4 (D-F) show how the ratio of solid-to-liquid, ultrasound time, as well as temperature, impact the FRAP activity of *Hibiscus sabdariffa* L. calyces. From the results, it is evident that the ratio of solid-to-solvent and ultrasound temperature plays the most important role of the three independent variables. Increasing the ultrasound temperature led to a rapid increase in FRAP activity. Studies have demonstrated a temperature-dependent pattern of FRAP activity, possibly because solvent characteristics as well as the decomposition of extracts vary with temperature. The boost in FRAP activity from 60 to 80 °C could be explained by the fact that a greater number of chemicals are extracted at higher temperatures due to the relaxation of the food matrix and enhanced medium diffusivity throughout the extraction process. In Burmese grape pulp, the optimum temperature for FRAP activity was found to be 80 °C, as reported by Hossain and Hossain [61]. Previous research conducted in the UAE by Hani et al. [62] and Deng et al. [63] found that 61 °C and 69 °C, respectively, are the optimum temperatures for bitter gourd and sugar apples. In contrast, a rise from 35 to 50 min. of extraction time resulted in a decline in FRAP values. Long-term exposure to environmental factors including oxygen and light may cause phenolic molecules to oxidize, leading to a decrease in FRAP action [64]. According to Fick, the solute concentrations in the bulk solution (extraction solvent) and the solid matrix (plant sample) approach equilibrium after a specific time (which is known as the second rule of diffusion) [65]. Based on the findings of Chen

et al. [66], and Kashyap et al. [67], the fruits from *Lycium ruthenicum* Murr. and Meghalayan cherry had the highest FRAP activity when extracted for 30 and 31 min., respectively. Moreover, various solid-to-solvent ratios (1:10 to 1:60) were examined in order to determine the FRAP activity of *Hibiscus sabdariffa* L. calyces. From 1:10 to 1:40, the solid-to-solvent ratio increased gradually, as shown in Figure 1D and Figure 4 (E, F), while a downward trend also was observed after the solid-to-solvent ratio of 1:40. Kumar et al. [7] explained that it is more challenging to achieve a cavitation effect when the solid-to-solvent ratio is larger. Since there is more solvent in a system with a lower solid-to-solvent ratio, the solute is more likely to dissolve in the solvent due to the increase in the contact area, and the ultrasonic intensity operating on the solid material may have a greater influence on fragmentation and erosion.

3.5. Determination of Optimal conditions and model validation

The goal of this research was to find out the optimal UAE process variables for maximally extracting the TAC, TPC, and measuring DPPH as well as FRAP from *Hibiscus sabdariffa* L. calyces. The optimal level of independent variables for maximum extraction efficiency was calculated by a numerical optimization procedure that relied on experimental and statistical analysis. Derringer and Suich [68] developed a method for optimizing two responses simultaneously. The method involves converting each predicting response into a range of 0 to 1, which is defined as a dimensionless partial desirability function. An overall desirability response is then created by combining the partial desirability functions. A single optimal extraction condition was obtained for all responses: solid-to-solvent ratio of 1:60, ultrasound time of 48 °C, and ultrasound temperature of 80 °C. Based on the desirability function, D = 0.95 (Figure 5), predicted values and optimal conditions were determined. It can be inferred that all responses are concurrently located within a desirable range if D is positive (> 0). Response values around 1 stipulate that the combined effect of the various criteria is a global maximum, and therefore that the actual values are quite close to the desired ones [69]. Estimated optimal conditions were used for experimental confirmation of the results. Predicted and experimental values are presented in Table 4. The experimental values under the optimal conditions were as TAC: 311 ± 5 mg CGE/100 g, TPC: 572 ± 7 mg GAE/100 g, DPPH: 974 ± 3 µmolTE/100 mL, and FRAP: 2332 ± 3 µmolTE/100 mL. The model predicted values of TAC (301 mg CGE/100 g), TPC (553 mg GAE/100 g), DPPH (948 µmolTE/100 mL), and FRAP (2229 µmolTE/100 mL) all agreed with the current. Residual standard error (RSE) percentages were determined for analysis of discrepancies between predicted and experimental values. Values of RSE < ± 0.5 were regarded to be consistent with the prediction in the current investigation. The RSE values (Table 4) in the current investigation indicated that there are no consequential discrepancies between predicted and experimental values found for *Hibiscus sabdariffa* L. calyces extracts. According to the BBD-derived model, the ratio of solid-to-solvent, extraction temperature, and time can be predicted with a high degree of accuracy in the UAE.

3.6. Comparison of UAE and Soxhlet extraction method (SE)

Effectiveness in extracting TAC and TPC and measuring antioxidant activity (DPPH and FRAP) was investigated by comparing the UAE technique to the SE. It is clear from the data that UAE has a higher extraction capacity for TAC (311 ± 5 mg CGE/100 g), TPC (572 ± 7 mg GAE/100 g), DPPH (974 ± 3 µmolTE/100 mL), and FRAP (2332 ± 3 µmolTE/100 mL) as compared to SE (TAC: 176 ± 4 mg CGE/100 g, TPC: 210 ± 3 mg GAE/100 g, DPPH: 534 ± 2 µmolTE/100 mL, FRAP: 1732 ± 3 µmolTE/100 mL). Several variables, including chemical reactions, gas-liquid phase transition, and gas diffusion, may account for the increased extraction yield of TAC, TPC, DPPH, and FRAP from UAE [69]. Additionally, the optimized UAE process in the present study exhibited higher TAC, TPC, DPPH, and FRAP for *Hibiscus sabdariffa* L. calyces extracts compared to other studies investigated by Duy et al. [70] for solvent-based extraction method (TPC: 763 mg GAE/L, DPPH: 870-928 µmol TE/L, FRAP: 3494–3460 µmol TE/L), Jafarian et al. [71] for aqueous and alcoholic extracts of calyces (TPC: 24.36 to 44.34 mg GAE/100 g, DPPH: 43.85% to 91.77%), Sirag et al.[72] for calyces ethanolic extract (TPC: 41.07 mg GAE/g, DPPH: 80%), Salmerón-Ruiz et al.[73] for water extract of *Hibiscus sabdariffa* L. calyces (TAC: 132.7 ± 7.8 mg CGE/100 mL, DPPH: 800.6 ± 69.9 µmolTE/100 mL), Chikhoune et al.[74] for microwave-assisted extraction (TAC: 0.279 ± 0.010 mg CGE/100 g, TPC: 3.734 ± 0.180 mg GAE/100 g), Purbowati and Maksum [75] for microwave-assisted extraction (TAC: 13.8381 mg/100g).

4. Conclusion

The findings from the current study suggested that the RSM model optimized the extraction conditions effectively based on the experimental results, indicating that the model was appropriate. An ultrasound temperature of 80 °C, a time of 48 min., and a solid-to-solvent ratio of 1:60 were the optimal UAE process conditions. From an ecological perspective, the proposed method might be acceptable since it uses solvents not harmful to food, pharmaceuticals, and other industries. It is also possible to reduce the disposal issues associated with industrial waste by quantifying TAC, TPC, and antioxidant activity by using RSM-based approaches. In industry, ultrasound batch reactors are widely used to extract antioxidants, so the developed optimized conditions can be scaled up. It is also worth noting that the ultrasound power, duty cycle, and frequency may also affect the TAC, TPC, and antioxidant activity of *Hibiscus sabdariffa* L. calyces. Further research is therefore needed to improve and optimize extraction efficiency based on the variables mentioned above for industrial purposes. In addition, it would be beneficial to focus future research on the extraction, isolation, and purification of the anthocyanins, and phenolic components of *Hibiscus sabdariffa* L. calyces extract for use as food supplements and other industrial applications. Thus, the present study could be considered the first step toward further research into *Hibiscus sabdariffa* L.as a health food, including extraction, separation, and nutraceutical analysis of its antioxidative compounds.

Ethics declarations

Competing interests

The authors declare no competing interests.

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

Tanvir Ahmed: Formal data analysis, Data calculation, Formal data analysis, Writing- Original Draft

Shakhawat Ullah: Investigation**,** Data curation**,** Formal data analysis, Data calculation, Formal data analysis, Writing- Review & Editing

Md Suzauddula: Methodology, Formal data analysis, Data calculation, Writing- Review and Editing

Sadia Haque Khan: Methodology, Formal data analysis, Data calculation, Writing- Review and Editing

Funding

Not applicable

Availability of data and materials

Not applicable

References

[1] GalanakisCM. Functionality of food components and emerging technologies. Foods 2021;10:128.

[2] SalemMA, ZayedA, BeshayME, Abdel MesihMM, BenKhayalRF, GeorgeFA, et al. Hibiscus sabdariffa L.: phytoconstituents, nutritive, and pharmacological applications. Adv Tradit Med 2021:1–11.

[3] PrasongwatanaV, WoottisinS, SriboonlueP, KukongviriyapanV. Uricosuric effect of Roselle (Hibiscus sabdariffa) in normal and renal-stone former subjects. J Ethnopharmacol 2008;117:491–5.

[4] Olvera-Garc\'\iaV, Castaño-TostadoE, Rezendiz-LopezRI, Reynoso-CamachoR, deMej\'\iaE, ElizondoG, et al. Hibiscus sabdariffa L. extracts inhibit the mutagenicity in microsuspension assay and the proliferation of HeLa cells. J Food Sci 2008;73:T75--T81.

[5] Wong-PazJE, Muñiz-MárquezDB, Aguilar-ZárateP, Ascacio-ValdésJA, CruzK, Reyes-LunaC, et al. Extraction of bioactive phenolic compounds by alternative technologies. Ingredients Extr. by Physicochem. methods food, Elsevier; 2017, p. 229–52.

[6] Picot-AllainC, MahomoodallyMF, AkG, ZenginG. Conventional versus green extraction techniques—A comparative perspective. Curr Opin Food Sci 2021;40:144–56.

[7] KumarK, SrivastavS, SharanagatVS. Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review. Ultrason Sonochem 2021;70:105325.

[8] UsmanI, HussainM, ImranA, AfzaalM, SaeedF, JavedM, et al. Traditional and innovative approaches for the extraction of bioactive compounds. Int J Food Prop 2022;25:1215–33. https://doi.org/10.1080/10942912.2022.2074030.

[9] Ou-AniO, OucheikhL, DabbousA, ZniniM, CostaJ, MajidiL. Optimization of hydrodistillation extraction using response surface methodology and Chemical composition of essential oil from Moroccan endemic medicinal plant Ballota hirsuta 2022.

[10] BreigSJM, LutiKJK. Response surface methodology: A review on its applications and challenges in microbial cultures. Mater Today Proc 2021;42:2277–84.

[11] Tanvir Ahmed, Md Rahmatuzzaman Rana, Mahjabin Rahman MaishaASM, Sayem, Mizanur RahmanRA. Optimization of ultrasound-assisted extraction of phenolic content & antioxidant activity of hog plum (Spondias pinnata L. f. kurz) pulp by response surface methodology. Heliyon 2022:131932. https://doi.org/10.1016/j.heliyon.2022.e11109.

[12] BorgesGDSC, VieiraFGK, CopettiC, GonzagaLV, FettR. Optimization of the extraction of flavanols and anthocyanins from the fruit pulp of Euterpe edulis using the response surface methodology. Food Res Int 2011;44:708–15. https://doi.org/10.1016/j.foodres.2010.12.025.

[13] KothariV, GuptaA, NaraniwalM. Comparative study of various methods for extraction of antioxidant and antibacterial compounds from plant seeds. J Nat Remedies 2012;12:162–73.

[14] LeeJ, DurstR, WrolstadR. AOAC official method 2005.02: total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method. Off Methods Anal AOAC Int 2005;2.

[15] SILVATLL, SILVAEPda, AsquieriER, VIEIRAECS, SilvaJS, SILVAFAda, et al. Physicochemical characterization and behavior of biocompounds of caja-manga fruit (Spondias mombin L.). Food Sci Technol 2018;38:399–406. https://doi.org/10.1590/fst.03717.

[16] SilvaEM, SouzaJNS, RogezH, ReesJ-F, LarondelleY. Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian region. Food Chem 2007;101:1012–8. https://doi.org/10.1016/j.foodchem.2006.02.055.

[17] ThaipongK, BoonprakobU, CrosbyK, Cisneros-ZevallosL, ByrneDH. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J Food Compos Anal 2006;19:669–75. https://doi.org/10.1016/j.jfca.2006.01.003.

[18] Borrás-Enr\'\iquezAJ, Reyes-VenturaE, Villanueva-Rodr\'\iguezSJ, Moreno-ViletL. Effect of ultrasound-assisted extraction parameters on total polyphenols and its antioxidant activity from mango residues (Mangifera indica L. var. Manililla). Separations 2021;8:94.

[19] CorralesM, ToepflS, ButzP, KnorrD, TauscherB. Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: A comparison. Innov Food Sci \& Emerg Technol 2008;9:85–91.

[20] González-CentenoMR, KnoerzerK, SabarezH, SimalS, Rosselló C, FemeniaA. Effect of acoustic frequency and power density on the aqueous ultrasonic-assisted extraction of grape pomace (Vitis vinifera L.)--A response surface approach. Ultrason Sonochem 2014;21:2176–84.

[21] XuY, PanS. Effects of various factors of ultrasonic treatment on the extraction yield of all-trans-lycopene from red grapefruit (Citrus paradise Macf.). Ultrason Sonochem 2013;20:1026–32. https://doi.org/10.1016/j.ultsonch.2013.01.006.

[22] DahmouneF, BoulekbacheL, MoussiK, AounO, SpignoG, MadaniK. Valorization of Citrus limon residues for the recovery of antioxidants: Evaluation and optimization of microwave and ultrasound application to solvent extraction. Ind Crops Prod 2013;50:77–87. https://doi.org/10.1016/j.indcrop.2013.07.013.

[23] CarreraC, Ruiz-RodriguezA, PalmaM, BarrosoCG. Ultrasound assisted extraction of phenolic compounds from grapes. Anal Chim Acta 2012;732:100–4.

https://doi.org/10.1016/j.aca.2011.11.032.

[24] LeongT, AshokkumarM, KentishS. The fundamentals of power ultrasound—a review. Acoust Aust 2011;39:54–63.

[25] Al-DhabiNA, PonmuruganK, JeganathanPM. Development and validation of ultrasound-assisted solid-liquid extraction of phenolic compounds from waste spent coffee grounds. Ultrason Sonochem 2017;34:206–13. https://doi.org/10.1016/j.ultsonch.2016.05.005.

[26] SamaramS, MirhosseiniH, TanCP, GhazaliHM, BordbarS, SerjouieA. Optimisation of ultrasound-assisted extraction of oil from papaya seed by response surface methodology: Oil recovery, radical scavenging antioxidant activity, and oxidation stability. Food Chem 2015;172:7–17.

[27] DezhkunovNV, FrancescuttoA, CiutiP, SturmanF. Temperature Dependence of Cavitation Activity at Different Ultrasound Intensity. XVI Sess. Russ. Acoust. Soc., 2005, p. 74–7.

[28] RodriguesS, FernandesFAN, deBritoES, SousaAD, NarainN. Ultrasound extraction of phenolics and anthocyanins from jabuticaba peel. Ind Crops Prod 2015;69:400–7. https://doi.org/10.1016/j.indcrop.2015.02.059.

[29] RaspeDT, CiottaSR, ZorzenonMRT, DacomeAS, daSilvaC, MilaniPG, et al. Ultrasound-assisted extraction of compounds from Stevia leaf pretreated with ethanol. Ind Crops Prod 2021;172:114035. https://doi.org/10.1016/j.indcrop.2021.114035.

[30] PinelaJ, PrietoMA, PereiraE, JabeurI, BarreiroMF, BarrosL, et al. Optimization of heat-and ultrasound-assisted extraction of anthocyanins from Hibiscus sabdariffa calyces for natural food colorants. Food Chem 2019;275:309–21.

[31] Vázquez-SánchezAY, Aguilar-ZárateP, Muñiz-MárquezDB, Wong-PazJE, RojasR, Ascacio-ValdésJA, et al. Effect of ultrasound treatment on the extraction of antioxidants from Ardisia compressa Kunth fruits and identification of phytochemicals by HPLC-ESI-MS. Heliyon 2019;5:e03058. https://doi.org/10.1016/j.heliyon.2019.e03058.

[32] IzadiyanP, HemmateenejadB. Multi-response optimization of factors affecting ultrasonic assisted extraction from Iranian basil using central composite design. Food Chem 2016;190:864–70. https://doi.org/10.1016/j.foodchem.2015.06.036.

[33] LiuY, SheX-R, HuangJ-B, LiuM-C, ZhanM-E. Ultrasonic-extraction of phenolic compounds from Phyllanthus urinaria: optimization model and antioxidant activity. Food Sci Technol 2017;38:286–93. https://doi.org/10.1590/1678-457X.21617.

[34] XuJ, WangW, LiangH, ZhangQ, LiQ. Optimization of ionic liquid based ultrasonic assisted extraction of antioxidant compounds from Curcuma longa L. using response surface methodology. Ind Crops Prod 2015;76:487–93.

[35] HossainMA, AhmedT, HossainMS, DeyP, AhmedS, HossainMM. Optimization of the factors affecting BT-2 black tea fermentation by observing their combined effects on the quality parameters of made tea using Response Surface Methodology (RSM). Heliyon 2022;8:e08948. https://doi.org/10.1016/j.heliyon.2022.e08948.

[36] HimelM, RafiA, AhmedT, HossainMA, MoazzemM, others. Response surface optimization to extract antioxidants from freeze-dried seeds and peel of pomegranate (Punica granatum L.). Biomass Convers Biorefinery 2022:1–16.

[37] KechinskiCP, GuimarãesPVR, NoreñaCPZ, TessaroIC, MarczakLDF. Degradation kinetics of anthocyanin in blueberry juice during thermal treatment. J Food Sci 2010;75:C173--C176.

[38] BuckowR, KastellA, TerefeNS, VersteegC. Pressure and temperature effects on degradation kinetics and storage stability of total anthocyanins in blueberry juice. J Agric Food Chem 2010;58:10076–84.

[39] BarbaFJ, BrianceauS, TurkM, BoussettaN, VorobievE. Effect of alternative physical treatments (ultrasounds, pulsed electric fields, and high-voltage electrical discharges) on selective recovery of bio-compounds from fermented grape pomace. Food Bioprocess Technol 2015;8:1139–48.

[40] BarbaFJ, GalanakisCM, EsteveMJ, FrigolaA, VorobievE. Potential use of pulsed electric technologies and ultrasounds to improve the recovery of high-added value compounds from blackberries. J Food Eng 2015;167:38–44.

[41] WangW, JungJ, TomasinoE, ZhaoY. Optimization of solvent and ultrasound-assisted extraction for different anthocyanin rich fruit and their effects on anthocyanin compositions. LWT-Food Sci Technol 2016;72:229–38.

[42] ZhuZ, GuanQ, GuoY, HeJ, LiuG, LiS, et al. Green ultrasound-assisted extraction of anthocyanin and phenolic compounds from purple sweet potato using response surface methodology. Int Agrophysics 2016;30.

[43] RamićM, VidovićS, ZekovićZ, VladićJ, CvejinA, PavlićB. Modeling and optimization of ultrasound-assisted extraction of polyphenolic compounds from Aronia melanocarpa by-products from filter-tea factory. Ultrason Sonochem 2015;23:360–8.

628 [44] TiwariBK, PatrasA, BruntonN, CullenPJ, O'donnellCP. Effect of ultrasound processing on
629 anthocyanins and color of red grape juice. Ultrason Sonochem 2010;17:598–604.

630 [45] GolmakaniM-T, MoayyediM. Comparison of microwave-assisted hydrodistillation and
631 solvent-less microwave extraction of essential oil from dry and fresh Citruslimon (Eureka
632 variety) peel. J Essent Oil Res 2016;28:272–82.

633 [46] WangF, ZhangS, DengG, XuK, XuH, LiuJ. Extracting Total Anthocyanin from Purple
634 Sweet Potato Using an Effective Ultrasound-Assisted Compound Enzymatic Extraction
635 Technology. Molecules 2022;27:4344.

636 [47] GhafoorK, ChoiYH, JeonJY, JoIH. Optimization of ultrasound-assisted extraction of
637 phenolic compounds, antioxidants, and anthocyanins from grape (Vitis vinifera) seeds. J
638 Agric Food Chem 2009;57:4988–94.

639 [48] RodriguesS, PintoGAS. Ultrasound extraction of phenolic compounds from coconut (Cocos
640 nucifera) shell powder. J Food Eng 2007;80:869–72.

641 [49] \cSahinS, \cSaml\iR. Optimization of olive leaf extract obtained by ultrasound-assisted
642 extraction with response surface methodology. Ultrason Sonochem 2013;20:595–602.

643 [50] TehS-S, BirchEJ. Effect of ultrasonic treatment on the polyphenol content and antioxidant
644 capacity of extract from defatted hemp, flax and canola seed cakes. Ultrason Sonochem
645 2014;21:346–53.

646 [51] VuongQV, GoldsmithCD, DangTT, NguyenVT, BhuyanDJ, SadeqzadehE, et al.
647 Optimisation of ultrasound-assisted extraction conditions for phenolic content and
648 antioxidant capacity from Euphorbia tirucalli using response surface methodology.
649 Antioxidants 2014;3:604–17.

650 [52] PredescuNC, PapucC, NicorescuV, GajailaI, GoranGV, PetcuCD, et al. The influence of
651 solid-to-solvent ratio and extraction method on total phenolic content, flavonoid content and
652 antioxidant properties of some ethanolic plant extracts. Rev Chim 2016;67:1922–7.

653 [53] StamatopoulosK, ChatzilazarouA, KatsoyannosE. Optimization of multistage extraction of
654 olive leaves for recovery of phenolic compounds at moderated temperatures and short
655 extraction times. Foods 2013;3:66–81.

656 [54] RodriguesS, PintoGAS, FernandesFAN. Optimization of ultrasound extraction of phenolic
657 compounds from coconut (Cocos nucifera) shell powder by response surface methodology.
658 Ultrason Sonochem 2008;15:95–100.

[55] SridharA, PonnuchamyM, KumarPS, KapoorA, VoD-VN, PrabhakarS. Techniques and modeling of polyphenol extraction from food: a review. Environ Chem Lett 2021;19:3409–43.

[56] JemainSFP, JamalP, RausAR, AmidA, JaswirI. Effects of process conditions on the ultrasonic extraction of phenolics scavenger from Curcuma caesia rhizome. Int Food Res J 2017;24.

[57] CarciochiRA, ManriqueGD, DimitrovK. Optimization of antioxidant phenolic compounds extraction from quinoa (Chenopodium quinoa) seeds. J Food Sci Technol 2015;52:4396–404.

[58] JeongS-M, KimS-Y, KimD-R, JoS-C, NamKC, AhnDU, et al. Effect of heat treatment on the antioxidant activity of extracts from citrus peels. J Agric Food Chem 2004;52:3389–93.

[59] GogoiP, ChutiaP, SinghP, MahantaCL. Effect of optimized ultrasound-assisted aqueous and ethanolic extraction of Pleurotus citrinopileatus mushroom on total phenol, flavonoids and antioxidant properties. J Food Process Eng 2019;42:e13172.

[60] TzimaK, BruntonNP, LyngJG, FrontutoD, RaiDK. The effect of Pulsed Electric Field as a pre-treatment step in Ultrasound Assisted Extraction of phenolic compounds from fresh rosemary and thyme by-products. Innov Food Sci \& Emerg Technol 2021;69:102644.

[61] HossainMA, HossainMS. Optimization of Antioxidant Extraction from Freeze-dried Pulp, Peel, and Seed of Burmese grape (Baccaurea ramiflora Lour.) by Response Surface Methodology. Biomass Convers Biorefinery 2021. https://doi.org/10.21203/rs.3.rs-347432/v1.

[62] HaniNM, TorkamaniAE, AbidinSZ, MahmoodWAK, JulianoP. The effects of ultrasound assisted extraction on antioxidative activity of polyphenolics obtained from Momordica charantia fruit using response surface approach. Food Biosci 2017;17:7–16. https://doi.org/10.1016/j.fbio.2016.11.002.

[63] DengG-F, XuD-P, LiS, LiH-B. Optimization of ultrasound-assisted extraction of natural antioxidants from sugar apple (Annona squamosa L.) peel using response surface methodology. Molecules 2015;20:20448–59. https://doi.org/10.3390/molecules201119708.

[64] ChanSW, LeeCY, YapCF, Wan AidaWM, HoCW. Optimisation of extraction conditions for phenolic compounds from limau purut (Citrus hystrix) peels. Int Food Res J 2009;16.

[65] VickersNJ. Animal communication: when i'm calling you, will you answer too? Curr Biol

2017;27:R713--R715. https://doi.org/10.1016/j.cub.2017.05.064.

[66] ChenS, ZengZ, HuNA, BaiBO, WangH, SuoY. Simultaneous optimization of the ultrasound-assisted extraction for phenolic compounds content and antioxidant activity of Lycium ruthenicum Murr. fruit using response surface methodology. Food Chem 2018;242:1–8. https://doi.org/10.1016/j.foodchem.2017.08.105.

[67] KashyapP, RiarCS, JindalN. Optimization of ultrasound assisted extraction of polyphenols from Meghalayan cherry fruit (Prunus nepalensis) using response surface methodology (RSM) and artificial neural network (ANN) approach. J Food Meas Charact 2021;15:119–33. https://doi.org/10.1007/s11694-020-00611-0.

[68] DerringerG, SuichR. Simultaneous optimization of several response variables. J Qual Technol 1980;12:214–9.

[69] CiricA, KrajncB, HeathD, OgrincN. Response surface methodology and artificial neural network approach for the optimization of ultrasound-assisted extraction of polyphenols from garlic. Food Chem Toxicol 2020;135:110976.

[70] DuyNQ, ThoaiH, LamT. Effects of Different Extraction Solvent Systems on Total Phenolic, Total Flavonoid, Total Anthocyanin Contents and Antioxidant Activities of Roselle (Hibiscus sabdariffa L.) Extracts. ASIAN J Chem 2019;31:2517–21.

[71] JafarianS, MortazaviA, KenariRS, RadAHE. Total phenolic content \& antioxidant activity of roselle (Hibiscus sabdariffa L.) calyces extracts. 2014.

[72] SiragN, ElhadiMM, AlgailiAM, HassanHM, OhajM. Determination of total phenolic content and antioxidant activity of roselle (Hibiscus sabdariffa L.) calyx ethanolic extract. Stand Res J Pharm Pharmacol 2014;1:34–9.

[73] Salmerón-RuizML, Dom\'\inguez-AvilaJA, Ayala-ZavalaJF, Alvarez-ParrillaE, Villegas-OchoaMA, Sáyago-AyerdiSG, et al. Optimization of total anthocyanin content and antioxidant activity of a Hibiscus sabdariffa infusion using response surface methodology. Biotecnia 2019;21:114–22.

[74] ChikhouneA, GagaouaM, NanemaKD, SouleymaneAS, HafidK, AlianeK, et al. Antioxidant activity of Hibiscus sabdariffa extracts incorporated in an emulsion system containing whey proteins: Oxidative stability and polyphenol--whey proteins interactions. Arab J Sci Eng 2017;42:2247–60.

[75] PurbowatiISM, MaksumA. The antioxidant activity of Roselle (Hibiscus sabdariffa Linii)

phenolic compounds in different variations microwave-assisted extraction time and power. IOP Conf. Ser. Earth Environ. Sci., vol. 406, 2019, p. 12005.

List of Tables

Table 1. Independent variable levels in experimental design for response surface analysis

Independent variables	Symbols	Coded level		
		-1	0	1
Temperature (°C)	X_1	30	55	80
Time (min.)	X_2	20	35	50
Solid to solvent ratio (g/mL)	X_3	10	35	60

764 Table 2. Box-Behnken design of three variables with their measured values of responses

Run	Temperature (°C)	Time (min.)	Solid to solvent ratio (g/mL)	Total Anthocyanin Content (TAC) (mg CGE/100 g)	Total Phenolic content (mg GAE/100 g)	Antioxidant activity	
						FRAP (µmolTE/100 mL)	DPPH (µmolTE/100 mL)
1	30	50	35	213 ± 2	482 ± 8	1751 ± 10	523 ± 7
2	55	20	60	269 ± 6	439 ± 4	1881 ± 15	645 ± 3
3	55	50	60	221 ± 3	531 ± 3	1889 ± 12	598 ± 5
4	55	35	35	265 ± 3	421 ± 5	2040 ± 5	612 ± 8
5	80	20	35	289 ± 5	462 ± 3	2262 ± 11	865 ± 3
6	55	35	35	265 ± 2	427 ± 7	1997 ± 8	596 ± 3
7	80	50	35	315 ± 7	495 ± 5	2146 ± 12	913 ± 4
8	30	20	35	234 ± 4	394 ± 8	1765 ± 8	523 ± 9
9	80	35	10	292 ± 3	481 ± 4	1886 ± 15	887 ± 5
10	30	35	60	215 ± 2	498 ± 7	1655 ± 9	509 ± 5
11	30	35	10	234 ± 4	375 ± 5	1739 ± 10	632 ± 7
12	55	20	10	237 ± 5	371 ± 4	1843 ± 12	715 ± 11
13	80	35	60	321 ± 2	525 ± 3	2311 ± 7	948 ± 3
14	55	35	35	273 ± 2	398 ± 3	2107 ± 11	607 ± 5

15	55	50	10	256 ± 2	413 ± 3	1740 ± 9	715 ± 5

765

766 Table 3. Analysis of variance (ANOVA) of the quadratic models for TAC, TPC, and antioxidant activity

767

Source	TAC	TPC	DPPH	FRAP
X_1	<0.0001**	0.002**	<0.0001**	<0.0001**
X_2	0.252	0.001**	0.989	0.139
X_3	0.721	0.0002**	0.019*	0.009**
X_1X_2	0.016*	0.092	0.399	0.311
X_1X_3	0.015*	0.031*	0.016*	0.003**
X_2X_3	0.004**	0.117	0.409	0.275
X_1^2	0.082	0.003**	0.001**	0.880
X_2^2	0.015*	0.435	0.395	0.044*
X_3^2	0.038*	0.053	0.014*	0.002**
R^2	0.98	0.97	0.98	0.98
Adjusted R^2	0.96	0.94	0.96	0.95
Predicted R^2	0.82	0.81	0.83	0.86
F value	42.36	24.34	50.31	30.37
Prob > F	0.0003**	0.001**	0.0002**	0.0008**
C.V. (%)	2.52	2.95	3.80	2.34

Lack of fit	2.68	0.58	16.23	0.45

768 Data are significant at $^{**}p < 0.01$, $^{*}p < 0.05$; TAC: total anthocyanin content; TPC: total phenolic content; DPPH: 2,2-diphenyl1 picrylhydrazyl radical
769 scavenging ability; FRAP: Ferric reducing antioxidant power.

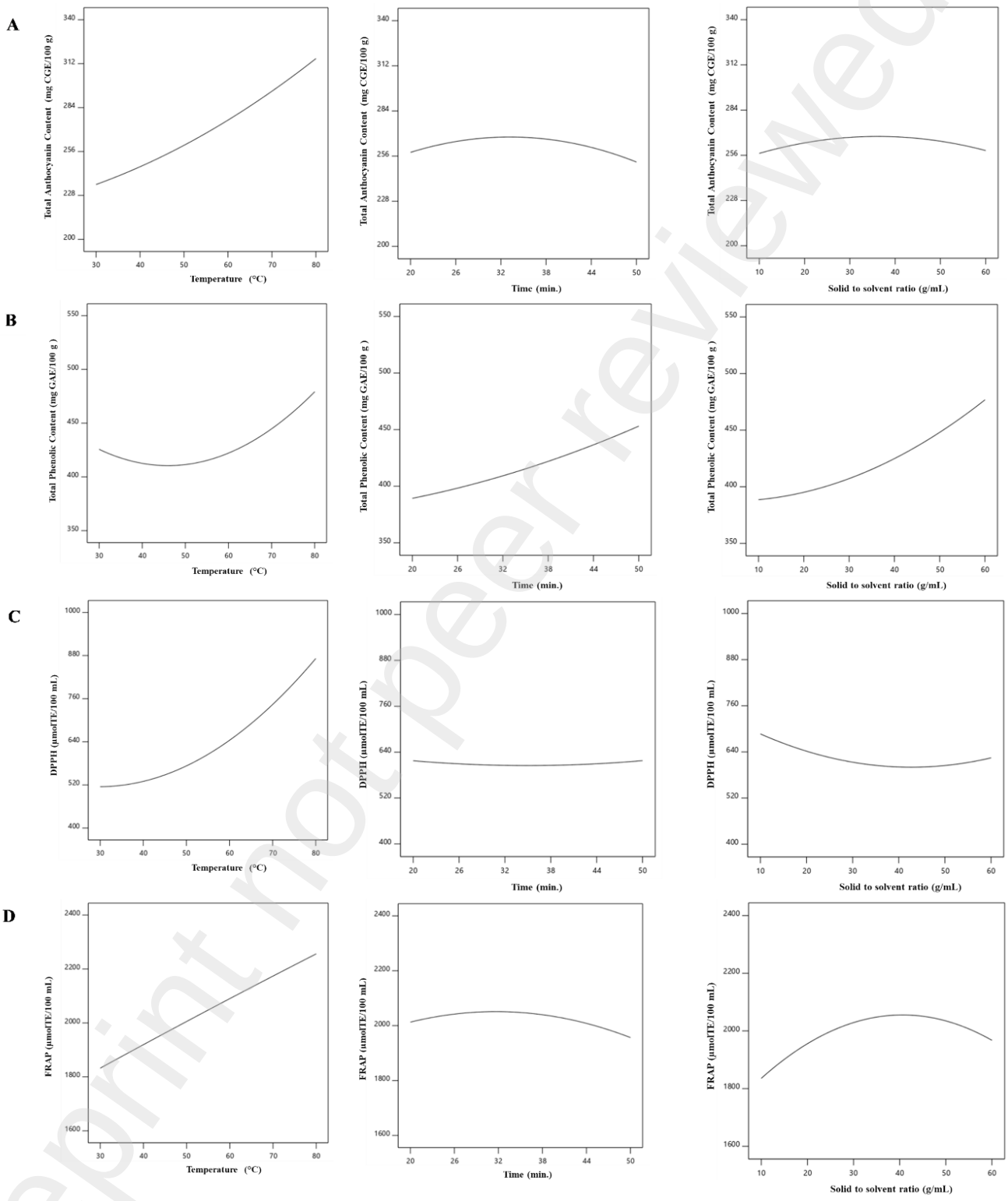
770 Table 4. Experimental values of UAE of TAC, TPC, DPPH, and FRAP under the optimized conditions and comparison to Soxhlet extraction

771

Optimized conditions	Temperature (°C)	80				
	Time (min.)	48				
	Solid to solvent ratio (g/mL)	60				
Response variables		Target	Predicted value	Experimental value	RSE (%)	Soxhlet extraction
	TAC (mg CGE/100 g)	maximum	301	311 ± 5	3.32	176 ± 4
	TPC (mg GAE/100 g)	maximum	553	572 ± 7	3.44	210 ± 3
	DPPH (µmolTE/100 mL)	maximum	948	974 ± 3	2.74	534 ± 2
	FRAP (µmolTE/100 mL)	maximum	2229	2332 ± 3	4.62	1732 ± 3

772 RSE: Residual Standard Error; Experimental values expressed as mean ± standard deviation of the mean (n = 3)

773 **List of Figures**



774

775 Figure 1. Main effects plot of ultrasound temperature, ultrasound time, and solid-to-solvent ratio on
776 (A) total anthocyanin content (B) total phenolic content (C) DPPH: 2,2-diphenyl1-picrylhydrazyl
777 radical scavenging activity; (D) FRAP: Ferric reducing antioxidant power.

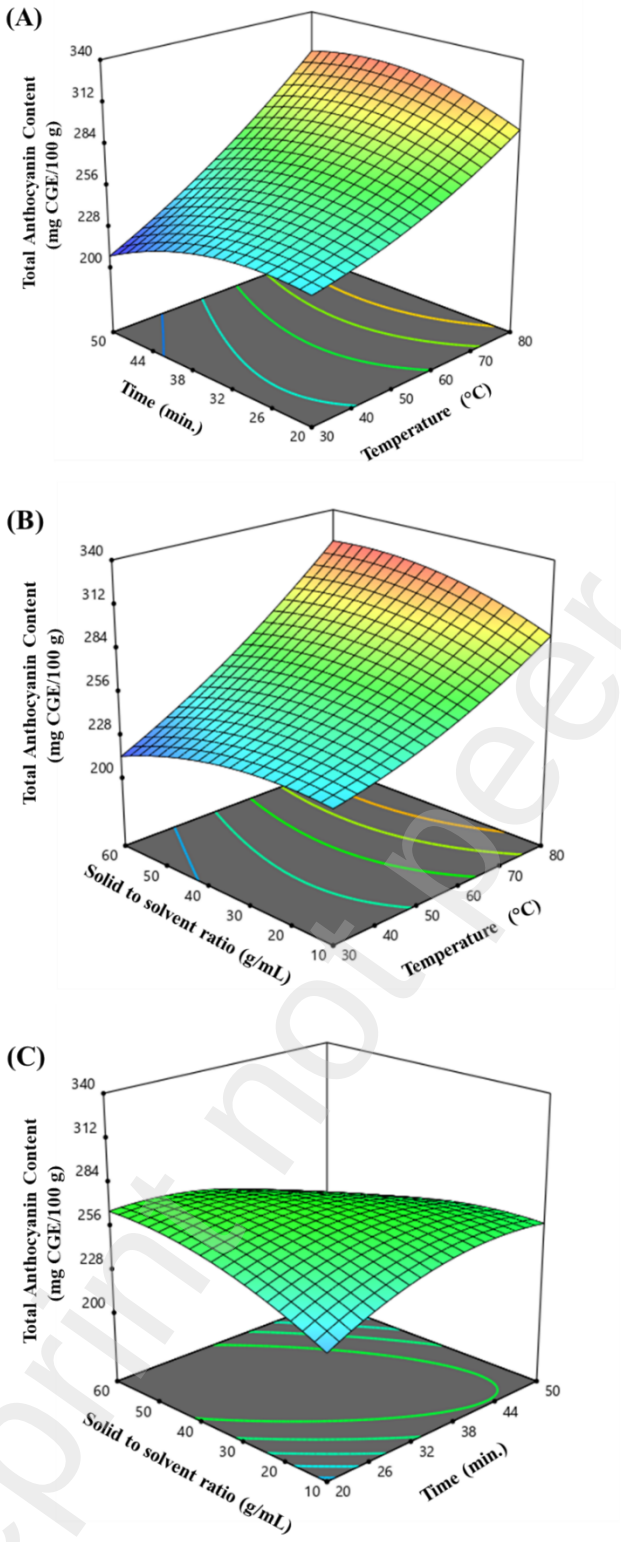


Figure 2. Response surface plots for interaction effects of ultrasound temperature, ultrasound time, and solid-to-solvent ratio on the total anthocyanin content of *Hibiscus sabdariffa* L. calyces.

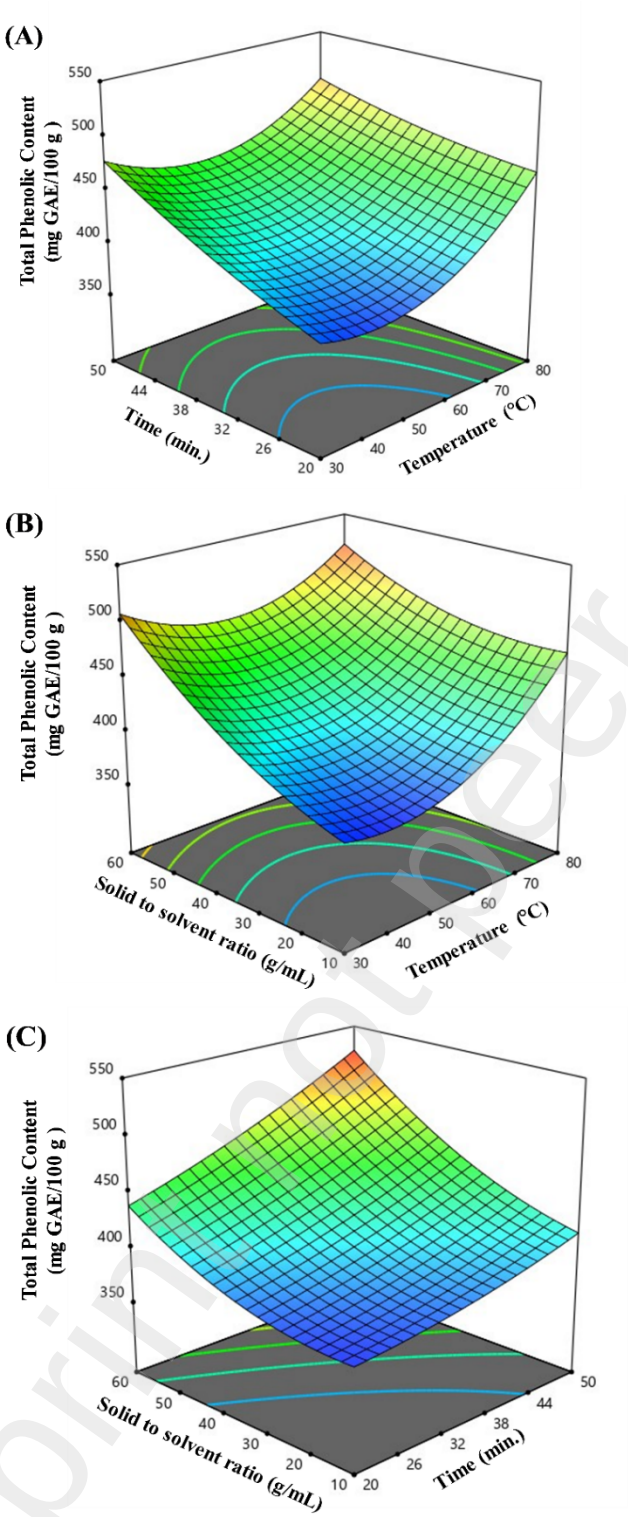


Figure 3. Response surface plots for interaction effects of ultrasound temperature, ultrasound time, and solid-to-solvent ratio on the total phenolic content of *Hibiscus sabdariffa* L. calyces.

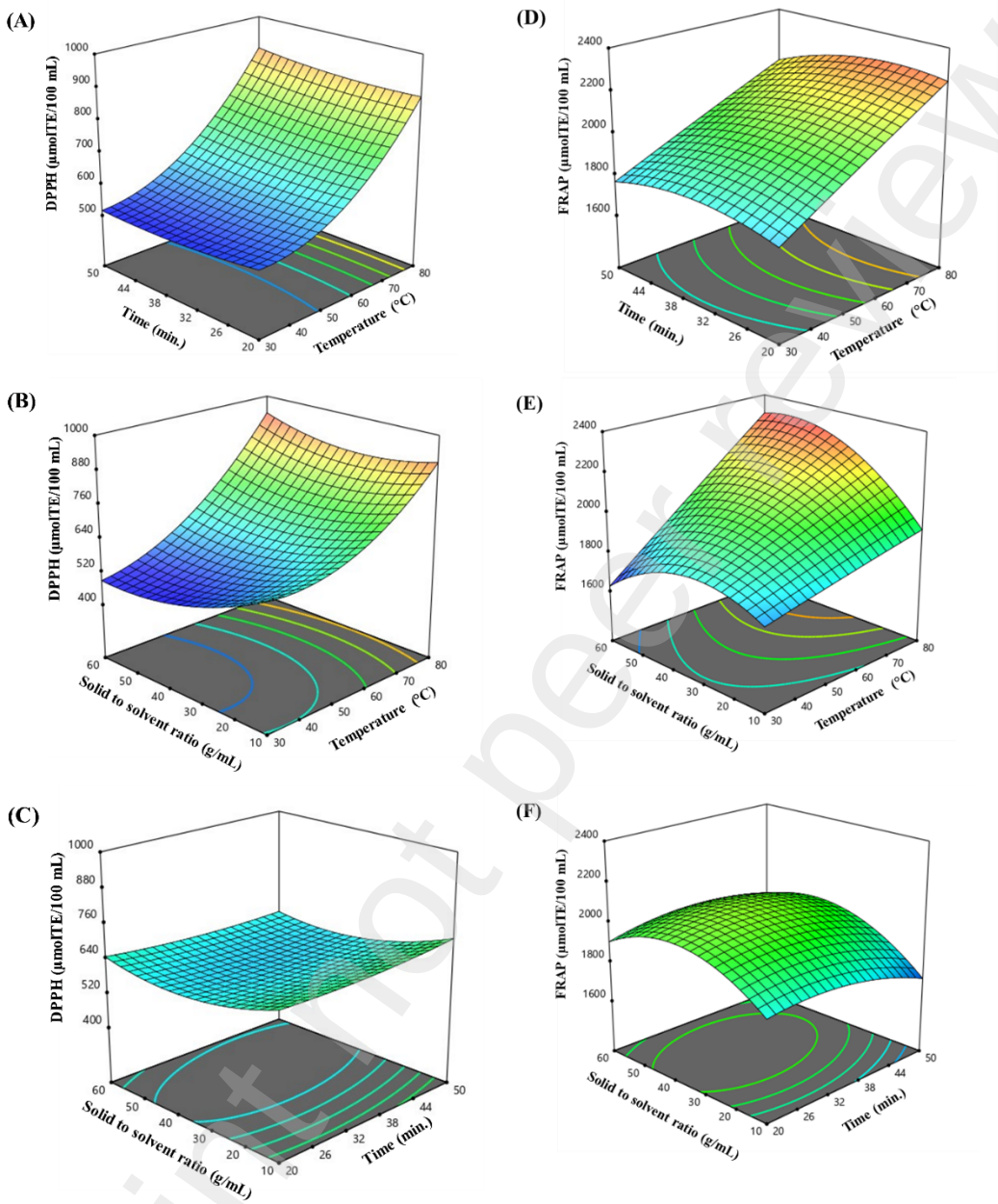


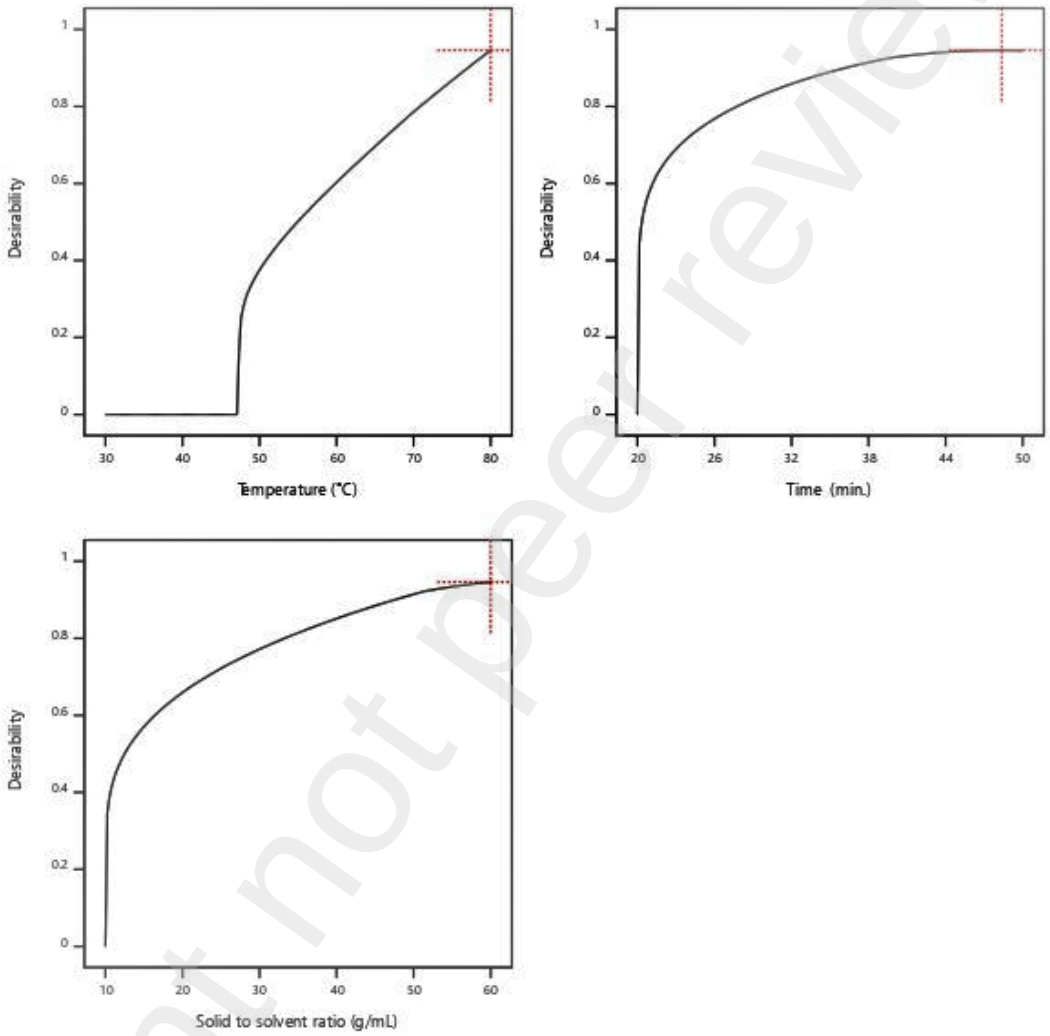
Figure 4. Response surface plots for interaction effects of ultrasound temperature, ultrasound time, and solid-to-solvent ratio on the DPPH (A, B, C) and FRAP (D, E, F) assays of *Hibiscus sabdariffa* L. calyces.

797

798

799

800



801

802 Figure 5. Desirability function plot of ultrasound temperature, ultrasound time, and solid-to-solvent
803 ratio