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Extraction Optimization of Phenolics from *Beta Vulgaris* Stems by High-Intensity Ultrasound with Response Surface Methodology Approach

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Abstract

High-intensity ultrasound-assisted extraction was used to extract total soluble phenols, flavonoids, and anthocyanins from *Beta vulgaris* stems, and to evaluate their antioxidant activities (DPPH, ABTS, and FRAP). The effect of extraction time (X_1: 2, 4, and 6 min), ultrasound power (X_2: 80%, 90% and 100%), and liquid-to-solid ratio (X_3: 10:1, 15:1, and 20:1 mL/g) was investigated using response surface methodology. The high-intensity ultrasound-assisted extraction models for all responses were adjusted to a 2nd-order polynomial equation (R^2 = 0.94 - 0.99, lack of fit > 0.05). Optimal high-intensity ultrasound-assisted extraction conditions differ for each response, X_1: 3.08 min, X_2: 100%, and X_3: 17.32:1 mL/g for total soluble phenols, X_1: 5.99 min, X_2: 95.04% and X_3: 17.28:1 mL/g for total flavonoids, and X_1: 6 min, X_2: 92.24%, and X_3: 12.97:1 mL/g for total anthocyanins. Moreover, all evaluated conditions exhibited antioxidant properties by DPPH, ABTS, and FRAP. Furthermore, the validated high-intensity ultrasound-assisted extraction conditions (3.08 min for extraction time, 100% power ultrasound, and 17.32 mL/g of liquid-to-solid ratio) yielded 2.35 times higher soluble phenols content than conventional extraction method (magnetic stirring at 400 rpm for 60 min), with higher ($p < 0.05$) flavonoids, ABTS, and FRAP values, and similar values ($p > 0.05$) for anthocyanins and DPPH. Furthermore, shikimic, protocatechuic, 4-hydroxybenzoic, gallic, chlorogenic, neochlorogenic, and trans-ferulic acids were higher under high-intensity ultrasound-assisted extraction than conventional extraction according to the HPLC analysis. It demonstrated that high-intensity ultrasound-assisted extraction is an efficacious technology for extracting bioactive molecules. In addition, future research could focus on isolating and purifying the phenolic compounds extracted from *B. vulgaris* stem powder, which have potential applications in food and non-food industries.

Keywords: Beetroot stalk, Box-Behnken design, Soluble phenols, Flavonoids, Anthocyanins, Antioxidant activity, Green extraction

Introduction

Approximately 30% of the food intended for human consumption is either wasted or lost during the process of production and consumption. This inefficiency results in social, economic, food security, and environmental problems [1]. Consequently, in 2015, global leaders created a strategy with 17 sustainable development goals (SDG) to eliminate poverty, safeguard the environment, and ensure universal

prosperity as part of the 2030 Agenda. Within this framework, SDG 12 aims to establish a circular economy, focusing on responsible production and consumption, and emphasizing sustainable resource use. Target 12.3 of SDG 12 aims to halve food waste, including postharvest and food processing losses [2]. Plant-based sources, mainly fruits and vegetables, account for 40% - 50% of all food waste [1]. In this context, these agri-food waste raw materials and their by-products, such as peels, seeds, and stems, contain bioactive compounds that can be extracted and utilized in higher-value products for food, pharmaceutical, and cosmetic applications [3,4].

Beta vulgaris (beetroot) is a tuber from the *Chenopodiaceae* family. It ranks among the top ten vegetables with superior antioxidant properties and is commonly consumed raw or cooked in various forms, such as juice, jams, pickles, salads, and soups [5]. In 2021, 42 million tons were produced worldwide [6]. The primary parts of this tuber include the root, bulb, stem, and leaves [7], which generate food residues following beetroot bulb processing [8]. In this context, *B. vulgaris* stems constitute 35% of the total beetroot weight (approximately 14 million tons of BVS were produced in 2021), which are typically discarded as food waste or utilized for compost or animal feed [9-11]. Conversely, BVS represents a substantial underutilized agri-food waste with significant potential for industrial applications. For instance, it has been documented that BVS can be consumed after steaming or stir-frying [7]. Furthermore, BVS powder contains protein, dietary fiber, lipids, iron, and potassium [8,9]. Recent investigations have explored the use of BVS powder as a functional colorant for stirred yogurt [10] and BVS juice for fortifying orange juice [12]. Furthermore, microfiltration and ultrafiltration produce a clarified *B. vulgaris* stem extract with potential industrial uses [13]. *B. vulgaris* stem is a potential source of phytochemicals, such as phenolic acids, flavonoids (mainly anthocyanins), non-flavonoids, and betalains (betacyanins if red and betaxanthins if yellow) with antimicrobial, anti-inflammatory, antiviral, antidiabetic, and antioxidant properties [7,8,14,15]. Given *B. vulgaris* stem potential for recovering bioactive compounds, various studies have investigated diverse extraction techniques for their valorization [16].

Several conventional and advanced methodologies are available for extracting bioactive compounds from plant materials [5]. The recovery of bioactive compounds from *B. vulgaris* stem is typically performed using conventional methods. For instance, the use of magnetic stirring for 60 min at 25 °C, followed by overnight refrigeration, using a 70% methanol:water solution, has been investigated [15]. Koubaier *et al.* [14] macerated *B. vulgaris* stem powder in water for 72 h at room temperature, while other studies have macerated *B. vulgaris* stem powder up to 96 h [16]. Additionally, Soxhlet extraction has been utilized for 480 min using hexane to extract phenolics from *B. vulgaris* stem [16]. However, these methods are expensive (consumption of large amounts of solvent and energy), tedious (long extraction times), have low selectivity and recovery yields, and potentially compromise compound stability (degradation of compounds due to oxidation, hydrolysis, and ionization) [5]. In contrast, advanced extraction technologies have emerged as efficacious and eco-friendly alternatives for extracting bioactive compounds from vegetables and their by-products. These technologies are categorized as green methods due to their typically low cost (reduced solvent and energy consumption) and rapid processing (low extraction times), resulting in superior recovery yields [17]. Among them, pressurized liquid [18], supercritical CO_2 [16], and ultrasound [11] have been investigated for extracting bioactive molecules from *B. vulgaris* stem.

High-intensity ultrasound-assisted extraction is an effective technique characterized by simplicity, low cost, effectiveness, and efficiency for extracting bioactive compounds from *B. vulgaris* waste [19]. Like most extraction techniques, high-intensity ultrasound-assisted extraction is predicated on a solid-liquid leaching process involving mass and energy transfer [20], with acoustic cavitation (formation, growth, and implosion of air bubbles) being the primary physical effect [11,16]. This process generates various shear and mechanical forces that break down the plant cell wall, enabling and accelerating the liberation of biomolecules, and enhancing the recovery yield [17,20]. High-intensity ultrasound-assisted extraction has been investigated for extracting bioactive molecules from *B. vulgaris* leaves [4,16,21-23], dried pulp [19,20,24], roots [17,25] and stems [16,11]. Nevertheless, optimizing the high-intensity ultrasound-assisted

extraction process is an imperative step required to achieve higher recovery yields, as multiple factors (temperature, extraction time, solvent, frequency, pulse cycle, liquid-to-solid ratio, and ultrasound power) influence the extraction procedure. In this context, response surface methodology and statistical designs such as Box-Behnken are utilized to optimize complex extraction processes. These statistical techniques are based on mathematical models that evaluate the effects and interactions between factors and responses, determine the optimal high-intensity ultrasound-assisted extraction experimental conditions, and maximize the recovery yield [26]. Recently, the high-intensity ultrasound-assisted extraction of betalains from *B. vulgaris* stem waste has been optimized by response surface methodology, in which power intensity (48 - 80 W/cm^2), solid:liquid ratio (10 - 30 g/mL), and sonication time (20–30 min) were investigated through a Box-Behnken design [11]. However, no information in the literature was found on optimizing the extraction of total soluble phenols, flavonoids, and their antioxidant properties from *B. vulgaris* stems by high-intensity ultrasound-assisted extraction.

Accordingly, this work aims to evaluate the effect of extraction time (2, 4 and 6 min), ultrasound power (80%, 90%, and 100%), and liquid-to-solid ratio (10:1, 15:1 and 20:1 mL/g), on the ultrasound-assisted extraction of total soluble phenols, total flavonoids, total anthocyanins, and antioxidant properties of *Beta vulgaris* stem waste. Additionally, the optimized ultrasound extraction conditions were compared with magnetic stirring.

Materials and methods

This work was conducted in 2 stages. The initial stage aimed to determine the highest content of total soluble phenols, total flavonoids, and total anthocyanins, and to evaluate the antioxidant capacities (measured by DPPH, ABTS, and FRAP assays) of *B. vulgaris* stem powder subjected to high-intensity ultrasound-assisted extraction. Additionally, the optimal high-intensity ultrasound-assisted extraction conditions were identified using the response surface method. The following stage focused on assessing the effect of independent variables on responses during high-intensity ultrasound-assisted extraction under optimal conditions and their comparison with those of a conventional extraction method.

Materials and reagents

Beta vulgaris stems (**Figure 1(A)**) were donated from a local market at Tepatitlan, Jalisco, México. *B. vulgaris* stems were cleaned with potable water and dehydrated in a convective drying oven (Memmert GmbH, Schwabach, Germany) at 40 °C for 24 h (**Figure 1(B)**) [11]. Subsequently, the *B. vulgaris* stems were ground using a Nutribullet® food processor and sieved through a 35-mesh screen (Humboldt, AASHTO M92, IL, USA) to produce a fine powder (< 500 µm), as shown in **Figure 1(C)**. The *B. vulgaris* stem powder was stored under refrigeration (4 °C) away from light exposure for further analysis. All chemicals and reagents employed in this investigation were of analytical and HPLC grade.

Figure 1 Fresh (A), dried (B), and powder (C) of *Beta vulgaris* stem.

First stage

Experimental design

The experimental design was established employing STATISTICA software v. 10 (Statsoft, Tulsa, OK, USA). A Box-Behnken design was implemented for 3 factors (X_1, X_2, and X_3), 3 levels (–1, 0, +1), and 3 central points, with 15 experimental runs (in a random sequence to reduce systematic errors) to determine the optimal high-intensity ultrasound-assisted extraction conditions. The 3 independent variables were extraction time (X_1, 2, 4, and 6 min), ultrasound power (X_2, 80%, 90% and 100%), and liquid-to-solid ratio (X_3, 10:1, 15:1, and 20:1 mL/g). The responses investigated were soluble phenols (Y_1, mg of gallic acid equivalents per gram, GAE/g), flavonoids (Y_2, mg of cathechin equivalents per gram, CE/g), anthocyanins (Y_3, mg of cyanidin-3- *O*-glycoside per gram, C3G/g), DPPH (Y_4, mmol of Trolox equivalent per gram, TE/g), ABTS (Y_5, mmol TE/g), and FRAP (Y_6, mmol TE/g).

High-intensity ultrasound-assisted extraction

For the extraction of soluble phenols, flavonoids, and anthocyanins from the *B. vulgaris* stem powder, a PZ-550LI high-intensity ultrasonic processor with 550 W output power and 20 kHz frequency (XMSJ, Zhengzhou City, China) equipped with an ultrasonic probe (6 mm diameter) was employed. A constant 3:1 s on/off pulse cycle was applied [27]. The process involved mixing 1 g of *B. vulgaris* stem powder with an acidified methanol:water (80:20 v/v with 2% v/v HCl at 2 M) solution [28]. An ice bath maintained a constant high-intensity ultrasound-assisted extraction temperature of 25 ± 2 °C. Following sonication under specific experimental conditions, cold centrifugation at 4 °C (Hermle Z32HK, Wehingen, Germany) for 10 min (8,000×*g*) was performed. Supernatants were collected and preserved at −20 °C for future examination.

Quantification of total soluble phenols

In a 2 mL tube, 12 µL of the sample, 12 µL of Folin–Ciocalteu reagent (Sigma-Aldrich, USA), 116 µL of 7.5% w/v Na_2CO_3, and 164 µL of distilled water were mixed and incubated in darkness for 15 min. After this period, 200 µL of the mixture was placed in a 96-well plate, and the absorbance was read in a plate reader at 750 nm (ACCURIS Instruments, SmartReader MR-9600, Nankín, China) [29]. A calibration curve (R^2 = 0.998) of gallic acid (Sigma-Aldrich, USA) was constructed, and the results were expressed as mg equivalents of gallic acid per g of dry sample (mg GAE/g).

Quantification of total flavonoids

In a 2 mL tube, 100 µL of extract and 430 µL of a 5% sodium nitrite solution (Sigma-Aldrich, USA) were mixed and incubated for 5 min. Lately, 30 µL of 10% aluminum chloride (Golden-Bell, Mexico) was added and incubated for 1 min. Then, 440 µL of sodium hydroxide (NaOH 1 M) was added and homogenized in a vortex, and 200 µL of the mixture was placed in a 96-well plate and read at a wavelength of 490 nm in a microplate reader [30]. A calibration curve (R^2 = 0.999) was constructed with catechin (Sigma-Aldrich, USA), and the results were expressed as mg equivalents of catechin per gram of dry extract (mg QE/g).

Quantification of total anthocyanins

The total anthocyanin content was estimated by the pH differential method. The process involved diluting the extract (1:9) into 2 separate buffers (buffer of sodium acetate 0.4 M at pH 4.5 and buffer of potassium chloride 0.025 M at pH 1), and incubation for 30 min. Following this, 200 µL of each mixture was placed in a 96-well plate. Then, a microplate reader recorded the absorbance at 700 and 520 nm. The TAs were expressed as cyanidin-3-*O*-glucoside per gram (C3G/g), based on C3G molecular weight (449.2 g mol^{-1}) and molar extinction coefficient (26,900 L/cm mg), as recommended [31].

Antioxidant activity by DPPH, ABTS, and FRAP assays

To assess the scavenging activity of the DPPH• radical, a 96-well microplate was used, in which 260 µL of DPPH solution (Sigma Aldrich, USA) at 190 µM was mixed with 40 µL of extract and incubated under agitation (200 rpm) for 30 min in darkness. The absorbance was measured at 517 nm using a microplate reader. A calibration curve (R^2 = 0.992) was constructed with the Trolox standard (Sigma-Aldrich, USA), and the results were expressed as millimoles of Trolox equivalent per gram (mmol TE/g) [32]. The DPPH

reagent was mixed with methanol and prepared 15 min before use.

To determine ABTS + radical scavenging activity, in a 96-well microplate, 265 µL of ABTS + solution at 7 mM was mixed with 35 µL of extract and incubated under agitation (200 rpm) for 10 min in dark conditions. Then, the absorbance was read at 734 nm in a microplate reader. A calibration curve ($R^2 = 0.999$) was constructed with the Trolox standard (Sigma-Aldrich, USA), and the results were expressed as millimoles Trolox equivalent per gram (mmol TE/g) [33]. The ABTS + solution was made by mixing the ABTS reagent (38.4 mg), potassium persultate (6.62 mg), and 10 mL of phosphate buffer (0.1 M, pH 7.4) under magnetic stirring for 16 h in darkness; then, the working solution was spectrophometrically adjusted to 7 mM, using sodium persulfate solution (2.45 mM).

To conduct the FRAP assay, 36 µL of the extract sample, 264 µL of FRAP solution, and 9 µL of distilled water were combined in a 96-well plate and stirred (200 rpm) in dark conditions (30 min). Then the absorbance (595 nm) was measured in a microplate reader. A calibration curve ($R^2 = 0.997$) was constructed with the Trolox standard (Sigma-Aldrich, USA), and the results were expressed as millimoles Trolox equivalent per gram (mmol TE/g). The FRAP working solution was prepared by combining 2.5 mL of 20 mM ferric chloride ($FeCl_3$), 2.5 mL of 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40 mM hydrochloric acid (HCl), and 25 mL of 0.3 mM sodium acetate buffer, and adjusted to pH 3.6. Then, the mixture was placed at 37 °C for 3 h, in a water bath [34].

Response surface methodology analysis

Following the quantification of all dependent variables, the response surface method was employed to identify the optimal high-intensity ultrasound-assisted extraction conditions for extracting soluble phenols, flavonoids, and anthocyanins from the *B. vulgaris* stem powder. Therefore, a polynomial model (2^{nd} order) incorporating all the terms (linear, quadratic, and interaction) was applied to forecast the response (Eq. (1)).

$$Y=b_0+\sum_{i=1}^{n}(b_i x_i)+\sum_{i=1}^{n}(b_i x_{ii}^2)+\sum_{j=1\neq i}^{n}(b_{ij}x_i x_j)+\varepsilon \quad (1)$$

Y: is the projected response (soluble phenols, flavonoids, and anthocyanins), b_0 is model constant, b_i are model coefficients in its linear form, b_{ii} are model coefficients in its quadratic form, b_{ij} is the model interaction coefficient, *xi* and *xj* stands for the coded levels of the independent variable (extraction time, ultrasound power, and liquid-to-solid ratio), and ε is the experimental error.

The F-ratio was utilized to evaluate model suitability. A lack-of-fit test was employed to assess the adequacy of the fitted model. Concurrently, the R-square and R-adjusted values were examined at a 95% confidence interval to evaluate model performance.

Second stage

Experimental validation of optimal high-intensity ultrasound-assisted extraction

From the optimal high-intensity ultrasound-assisted extraction conditions obtained through response surface methodology analysis for soluble phenols, flavonoids, and anthocyanins, theoretical optimal high-intensity ultrasound-assisted extraction conditions for soluble phenols were selected for experimental validation of the model's accuracy, comparing predicted and experimental values. The conventional extraction method was also compared to the optimal high-intensity ultrasound-assisted extraction conditions for soluble phenols in a 1-factorial experimental design. Additionally, soluble phenols, flavonoids, anthocyanins, DPPH, ABTS, and FRAP (as described in previous sections) were evaluated, along with their effectiveness. The efficacy of high-intensity ultrasound-assisted extraction was assessed by applying Eq. (2) [35].

$$\text{Effectiveness (n-times)} = \frac{\text{Total soluble phenols content by UAE}}{\text{Total soluble phenols content by conventional extraction}} \quad (2)$$

Conventional extraction

For magnetic stirring extraction, 1 g of *B. vulgaris* stem powder was combined with acidified methanol:water (80:20 v/v with 2% v/v HCl at 2 M) solution in amber glass bottles and agitated at room temperature (25 °C) at 400 rpm for 60 min using a magnetic stirrer, followed by cold centrifugation at 4 °C for 10 min (8,000×*g*) [28]. Supernatants were collected and preserved at −20 °C for future examination. The

amount of solvent used in magnetic extraction was considered based on the optimal high-intensity ultrasound-assisted extraction conditions obtained in the 1st phase for soluble phenols.

High-performance liquid chromatography (HPLC) analysis

To identify phenolic compounds in *B. vulgaris* stem extracts obtained through optimal high-intensity ultrasound-assisted extraction conditions and conventional extraction, HPLC analysis was accurately conducted using the method established by Aguilar-Hernández *et al.* [36]. The methanolic extracts were evaporated to dryness at room temperature and redissolved in 1 mL of acidified water containing 2% acetic acid (v/v). Then, it was filtered through 0.22 µm membrane filters, and injected (30 µL) into an HPLC system (Agilent Technologies 1260 Infinity, Waldbronn, Germanyequipp) equipped with a photodiode array detector and a C18 reverse-phase column (250 mm long, 4.6 mm in diameter, 5 µm particle size; Thermo Scientific, Sunnyvale, CA, USA). The mobile phase was composed of acidified water containing 2% acetic acid, as eluent A, and a mixture of acidified water (0.5% acetic acid) and methanol as eluent B. The samples and standards underwent analysis through a gradient program starting 0% B (0 - 35 min), following to 35% B (35 - 55 min), and increasing to 75% B (55 - 60 min), and 100% B (60 - 70 min), and returning to 0% B, all at a flow rate of 0.4 mL/min. The peak areas were detected at 280 and 320 nm. Quantification of phenolic compounds was performed using a calibration curve of standards ranging from 0.5 to 300 µg/mL, and the results were expressed in mg/100 g.

Statistical analysis

In the 1st stage, data was analyzed by RSM. In the 2nd phase, the data were examined using analysis of variance (ANOVA, $p < 0.05$), while Tukey's test ($\alpha = 0.05$) was applied to compare the extraction methods ($p < 0.05$). Data was presented as means ± standard deviation (n = 3). In the second stage, the statistical comparison between optimal high-intensity ultrasound-assisted extraction conditions and the conventional extraction method was performed by the Student T test ($p < 0.05$). Statistical analysis of the results was conducted using STATISTICA software v. 10 (Statsoft, Tulsa, OK, USA). All experiments (extractions and measurements) were performed in triplicate.

Results and discussion

Table 1 lists the experimental and predicted values of soluble phenols (TSPs), flavonoids (FLAs), and anthocyanins (TAs) from *B. vulgaris* stem powder by high-intensity ultrasound-assisted extraction, along with the residual standard error. Statistical differences were observed among high-intensity ultrasound-assisted extraction runs for TSPs, FLAs, and TAs, depending on the experimental conditions ($p < 0.05$). The maximum TSPs and FLAs (309.14 mg GAE/g and 38.38 mg CE/g) were observed under identical high-intensity ultrasound-assisted extraction conditions (6 min extraction time, 100% ultrasound power, and 15:1 mL/g liquid-to-solid ratio). In contrast, the minimum TSPs content (161.54 mg GAE/g) was observed at 6 min of extraction time, 90% of ultrasound power, and 20:1 mL/g liquid-to-solid ratio, while the lowest FLAs content (23.17 mg CE/g) was found at 4 min of extraction time, 80% of ultrasound power, and 10:1 mL/g liquid-to-solid ratio. Regarding TAs, the highest content (0.447 mg C3G/g) was achieved with an extraction time of 2 min, 80% ultrasound power, and a liquid-to-solid ratio of 15:1 mL/g. Conversely, the lowest TAs content (0.180 mg C3G/g) was observed at an extraction time of 4 min, 80% ultrasound power, and a liquid-to-solid ratio of 10:1 mL/g. The observed values exceed those reported in a water bath at 50 °C (3.65 mg GAE/g) [9], magnetic stirring (15 mg GAE/g) [14], maceration (16 to 31 mg GAE/g), Soxhlet (11 to 55 mg GAE/g), supercritical CO_2 (98 mg GAE/g), pressurized liquid (14 to 16 mg GAE/g) and ultrasound bath (14 to 33 mg GAE/g) [16,18] extractions of total phenols from *B. vulgaris* stem waste. Similar trends were observed in total flavonoid content compared to those reported in magnetic stirring overnight or vortexing for 1 min, with a flavonoid content ranging from 5.46 to 31.17 mg CE/g from *B. vulgaris* stem waste [10,15]. Conversely, the anthocyanin content obtained in this study (0.19 - 0.38 mg C3G/g) was lower than that reported by Abdel-Aziz *et al.* [15], who reported an anthocyanin content of 0.56 mg of malvidin-3-glucoside equivalents per gram. However, they quantified the total anthocyanin content from the stem-leaf extracts. The efficacy of high-intensity ultrasound-assisted extraction is attributed to

the cavitation effect (micro-bubbles and micro-jets), which enhances the extraction and recovery process of phenolic compounds by disrupting the plant cell wall and facilitating solvent infiltration into the plant material [22,26]. Other studies have demonstrated that ultrasound-assisted extraction can effectively and efficiently extract bioactive molecules from *B. vulgaris* byproducts [4,16,17,19-25].

Table 1 Box-Behnken experimental design, experimental and predicted values of the soluble phenols, flavonoids, anthocyanins contents, and residual standard error after high-intensity ultrasound extraction from *Beta vulgaris* stem powder.

Run	Predictors[1]			Response variables		RSE (%)	Response variables		RSE (%)	Response variables		RSE (%)
	X_1 (min)	X_2 (%)	X_3 (mL/g)	Experimental TSPs[1]	Predicted TSPs[4]		Experimental FLAs[2]	Predicted FLAs[4]		Experimental TAs[3]	Predicted TAs[4]	
1	2	100	15:1	272.86 ± 2.50^{c}	289.49	–5.74	36.18 ± 0.29^{b}	38.48	–0.25	0.276 ± 0.003^{f}	0.276	0.0
2	6	100	15:1	309.14 ± 1.65^{a}	285.63	8.23	38.38 ± 0.09^{a}	38.07	0.26	0.275 ± 0.01f^{g}	0.275	0.0
3	4	80	10:1	189.87± 2.47^{j}	195.49	–2.88	23.17 ± 0.04^{k}	23.38	–0.93	0.180 ± 0.01^{j}	0.180	0.0
4	2	90	20:1	237.31 ± 0.83^{f}	231.12	2.68	29.21 ± 0.27^{g}	29.83	0.33	0.264 ±0.001^{g}	0.264	0.0
5	6	90	20:1	161.54 ± 1.65^{k}	193.41	–16.48	36.32 ± 0.07^{b}	36.42	–0.27	0.294 ± 0.002^{e}	0.294	0.0
6	6	80	15:1	195.37 ± 3.30^{i}	175.74	11.17	29.21 ± 0.40^{h}	29.11	0.34	0.198 ± 0.001^{i}	0.198	0.0
7	4	90	15:1	263.41 ± 4.38^{d}	234.38	12.39	31.80 ± 0.33^{e}	33.39	–4.77	0.381 ± 0.005^{c}	0.386	–1.3
8	4	90	15:1	213.47 ± 3.32^{h}	234.38	–8.92	34.02 ± 0.53^{d}	33.39	1.87	0.395 ± 0.001^{b}	0.386	2.3
9	2	80	15:1	188.90 ±2.38^{j}	219.52	–13.91	26.86 ± 0.13^{i}	26.95	–0.36	0.447 ± 0.001^{a}	0.447	0.0
10	4	100	20:1	299.39 ±3.80^{b}	288.38	3.82	32.35 ± 0.30^{e}	32.57	–0.67	0.283 ± 0.01^{ef}	0.283	0.0
11	6	90	10:1	192.70 ± 3.13^{ij}	205.19	–6.09	30.62 ± 0.22^{f}	30.71	–0.32	0.441 ± 0.002^{a}	0.441	0.0
12	4	100	10:1	242.31 ± 1.65^{f}	260.19	–6.87	30.81 ± 0.40^{f}	30.59	0.71	0.241 ± 0.006^{h}	0.241	0.0
13	4	80	20:1	189.75 ± 1.73^{j}	173.21	9.55	25.58 ± 0.03^{j}	25.36	0.86	0.189 ± 0.001^{ij}	0.189	0.0
14	2	90	10:1	257.94 ± 6.3^{e}	219.32	17.61	35.65 ± 0.18^{c}	35.55	0.28	0.251 ± 0.025^{h}	0.251	0.0
15	4	90	15:1	225.83 ± 1.54^{g}	234.38	–3.65	32.03 ± 1.12^{e}	33.39	–4.09	0.367 ± 0.001^{d}	0.386	–4.9

All values are means ± standard deviation (n = 3). Different letters on each line indicate statistically significant differences between treatments by Tukey´s test (α = 0.05). X_1: Extraction time; X_2: Ultrasonic power; X_3: Ratio Liquid-to-solid ratio; TSPs: Total soluble phenols (mg GAE/g dry basis), FLAs: Total flavonoids (mg CE/g dry basis), TAs: Total anthocyanins (mg C3G/g dry basis). RSE: residual standard error. [1]Gallic acid equivalents (mg GAE/g dry basis), [2]Catechin equivalent (mg CE/g dry basis), [3]Cyanidin-3-*O*-Glucoside (mg C3G/g dry basis); [4]Values were predicted using a 2nd-order polynomic equations, R^2 = 0.9275, R^2 = 0.9910, R^2 = 0.9934, respectively.

Effect of high-intensity ultrasound-assisted extraction on antioxidant activity of *Beta vulgaris* stem powder

It is well-established that the high-intensity ultrasound-assisted extraction conditions influenced the recovery yield of bioactive compounds (including soluble phenols. Flavonoids, and anthocyanins) from plant materials, subsequently impacting the resulting extract's antioxidant properties [26]. **Table 2** lists the effect of high-intensity ultrasound-assisted extraction on the antioxidant activity of *B. vulgaris* stem powder by DPPH, ABTS, and FRAP assays. Statistically significant differences were observed among high-intensity ultrasound-assisted extraction treatments for DPPH, ABTS, and FRAP activities, depending on the experimental conditions ($p < 0.05$). The maximum DPPH (189.10 mmol TE/g) and ABTS (243.31 mmol TE/g) activities were at 6 min of extraction time, 100% ultrasound power, and 15:1 mL/g liquid-to-solid ratio. These results agree with treatments with higher soluble phenols and flavonoids contents (**Table 1**). Similar trends were previously reported in DPPH and ABTS activities after ultrasound-assisted extraction of bioactive compounds from coffee pulp [37]. DPPH and

ABTS free radicals are inhibited by antioxidant compounds, including phenolic acids and flavonoids. These compounds donate electrons and hydrogen atoms, neutralizing these free radicals [38]. Furthermore, the maximum FRAP activity (317.37 mmol TE/g) was observed at 2 min of extraction time, 80% ultrasound power, and 15:1 mL/g liquid-to-solid ratio; these conditions correspond to the treatment with higher anthocyanin content (**Table 1**). It has been reported that anthocyanins can reduce F^{3+}-TPTZ complex to Fe^{2+}-TPTZ by abstracting an electron from a reductant [39]. Other studies have demonstrated that *B. vulgaris* stem extracts exhibit antioxidant properties by DPPH and ABTS [10]. Battistella-Lasta *et al.* [18] reported DPPH EC_{50} values of 394 µg/mL, while values of 15.7 µmol TE/g for ABTS and 18 µmol TE/g for FRAP were found for *B. vulgaris* stem powder using pressurized liquid extraction. The antioxidant values obtained in this work are higher than those reported in beetroot pulp extract obtained by ultrasound for DPPH (0.19 mmol TE/g), ABTS (0.15 mmol TE/g), and FRAP (4.88 mmol TE/g), evidencing that *B. vulgaris* stem powder could be used as a source of antioxidant compounds [19].

Table 2 Antioxidant activity of *Beta vulgaris* stem powder after high-intensity ultrasound-assisted extraction.

Run	Predictors			Response variables (mmol TE/g)		
	X_1 (min)	X_2 (%)	X_3 (mL/g)	DPPH	ABTS	FRAP
1	2	100	15:1	162.58 ± 0.01^{b}	211.51 ± 0.12^{b}	110.47 ± 9.20^{f}
2	6	100	15:1	189.10 ± 2.13^{c}	243.31 ± 1.18^{a}	292.09 ± 21.75^{b}
3	4	80	10:1	80.80 ± 0.01^{l}	103.78 ± 0.24^{f}	208.34 ± 5.83^{d}
4	2	90	20:1	121.79 ± 1.18^{gh}	156.73 ± 0.43^{e}	131.67 ± 10.32^{f}
5	6	90	20:1	120.02 ± 0.22^{i}	157.58 ± 0.74^{e}	126.86 ± 0.84^{f}
6	6	80	15:1	84.30 ± 0.58^{k}	103.09 ± 0.45^{f}	126.02 ± 0.93^{f}
7	4	90	15:1	136.04 ± 0.73^{e}	160.56 ± 0.36^{d}	235.81 ± 11.44^{c}
8	4	90	15:1	123.44 ± 0.32^{f}	157.56 ± 5.03^{e}	246.25 ± 42.61^{c}
9	2	80	15:1	89.63 ± 0.14^{j}	104.49 ± 0.74^{f}	317.37 ± 10.43^{a}
10	4	100	20:1	169.30 ± 0.01^{c}	211.10 ± 0.12^{a}	119.36 ± 7.60^{f}
11	6	90	10:1	80.40 ± 0.14^{l}	103.39 ± 0.01^{f}	237.28 ± 3.11^{c}
12	4	100	10:1	123.34 ± 2.49^{fg}	157.97 ± 0.09^{e}	127.22 ± 3.64^{f}
13	4	80	20:1	156.27 ± 0.50^{d}	167.12 ± 0.68^{c}	129.68 ± 0.87^{c}
14	2	90	10:1	163.14 ± 0.14^{c}	213.10 ± 0.01^{b}	115.53 ± 7.64^{f}
15	4	90	15:1	120.40 ± 0.01^{hi}	158.07 ± 0.09^{e}	168.12 ± 18.65^{e}

All values are means ± standard deviation (n = 3). Different letters on each line indicate statistically significant differences between treatments by Tukey´s test (α = 0.05). X_1: Extraction time; X_2: Ultrasonic power; X_3: Ratio Liquid-to-solid ratio.

Fitting the response surface models of the high-intensity ultrasound-assisted extraction for total soluble phenols, total flavonoids, and total anthocyanins from *Beta vulgaris* stem powder

As shown before, there are significant influences ($p < 0.05$) of extraction factors on the evaluated responses (soluble phenols, flavonoids, and anthocyanins). Data was analyzed using multiple regression coefficients for a 2nd-order polynomial model. Most of the regression coefficients were significant ($p < 0.05$) for soluble phenols, except for X_1^2 and X_1^2*X_2 ($p > 0.05$). For flavonoids, the non-significant ($p > 0.05$) terms were X_1*X_2^2 and X_2*X_3. Furthermore, all linear and interactive factors were significant ($p < 0.05$) for anthocyanins. The mathematical model for the soluble phenols, flavonoids, and anthocyanins was derived, excluding statistically non-significant coefficients ($p > 0.05$) to increase the predictive capability of the models [40]. It has been reported that during the optimization of high-intensity ultrasound-assisted extraction of phytochemicals from plant materials, specific terms of the analysis of variance model may be non-significant without affecting the

model's prediction ability [27,41]. The response surface models are described as follows:

$$\text{Soluble phenols (mg GAE/g)} = -6175.53 + 1819.82X_1 + 144.35X_2 - 0.79X_2^2 - 23.71X_3 - 0.66X_3^2 - 42.73X_1 * X_2 + 0.23X_1 * X_2^2 + 10.61X_1 * X_3 - 1.36X_1^2 * X_3 + 0.29X_2 * X_3 \quad (3)$$

$$\text{Flavonoids (mg CE/g)} = -310.57 + 23.12X_1 - 2.05X_1^2 + 6.72X_2 - 0.031X_2^2 + 1.92X_3 - 0.109X_3^2 - 0.511X_1 * X_2 + 0.038X_1^2 * X_2 + 0.682X_1 * X_3 - 0.50X_1^2 * X_3 \quad (4)$$

$$\text{Anthocyanins (mg C3G/g)} = 15.01 - 6.34X_1 + 0.17X_1^2 - 0.30X_2 + 0.001X_2^2 + 0.056X_3 - 0.002X_3^2 + 0.12X_1 * X_2 - 0.0005X_1 * X_2^2 - 0.001X_1^2 * X_2 + 0.014X_1 * X_3 - 0.002X_1^2 * X_3 + 0.0001X_1 * X_3 \quad (5)$$

Table 3 summarizes the analysis of variance, model adequacy, and fit accuracy. The regression models for soluble phenols (R^2: 0.9275, R-adjust: 0.9138, R^2-predict: 0.9476), flavonoids (R^2: 0.9910, R-adjust: 0.9884, R^2-predict: 0.9920), and anthocyanins (R^2: 0.9934, R-adjust: 0.9914, R^2-predict: 0.9939), exhibited satisfactory coefficients of multiple determinations, indicating a favorable correlation among the experimental and predicted data for the quadratic model, as demonstrated in diverse studies (R^2 values from 0.96 to 0.99) during ultrasound-assisted extraction of bioactive compounds from plant materials [28,36,42]. Furthermore, the lack of fit of all responses was deemed non-significant ($p > 0.05$) in the analysis of variance model analysis, suggesting the accuracy of the model [43]. Furthermore, all high-intensity ultrasound-assisted extraction conditions exhibited comparable values between experimental and predicted data for soluble phenols, flavonoids, and anthocyanins, exhibiting residual standard error values ranging from < 1 to 17% (**Table 1**). These results were consistent with other studies that optimized high-intensity ultrasound-assisted extraction processes using a Box-Behnken design and response surface methodologies [28,36,42].

Table 3 Summary of analysis of variance of the quadratic response model for ultrasound-assisted extraction of total soluble phenol, total flavonoid, and total anthocyanin from *Beta vulgaris* stem powder.

Source	Soluble phenols			Flavonoids			Anthocyanins		
	SS	F-value	*p*-value	SS	F-value	*p*-value	SS	F-value	*p*-value
X_1	465.26	3.43	0.073	4.75	22.79	< 0.001	0.0117	188.16	< 0.001
X_1^2	311.50	2.29	0.139	66.81	319.81	< 0.001	0.0001	0.249	0.621
X_2	46599.47	344.03	< 0.001	460.04	2202.22	< 0.001	0.0001	2.79	0.104
X_2^2	1773.32	13.09	0.001	80.63	385.99	< 0.001	0.085	1374.79	< 0.001
X_3	326.09	2.40	0.130	2.31	11.10	0.002	0.007	113.27	< 0.001
X_3^2	3038.55	22.43	< 0.001	82.01	392.59	< 0.001	0.061	989.23	< 0.001
X_1*X_2	669.95	4.94	0.033	4.93	23.61	< 0.001	0.046	742.39	< 0.001
X_1*X_2^2	12649.59	93.39	< 0.001	0.22	1.09	0.302	0.082	1331.04	< 0.001
X_1^2*X_2	474.38	3.50	0.070	13.79	66.05	< 0.001	0.023	376.93	< 0.001
X_1*X_3	83.20	0.61	0.438	97.85	468.42	< 0.001	0.019	310.02	< 0.001
X_1^2*X_3	4435.19	32.74	< 0.001	5.90	28.25	< 0.001	0.012	205.40	< 0.001
X_2*X_3	2453.18	18.11	0.001	0.57	2.73	0.107	0.0007	12.74	< 0.001
Error	4334.36			6.68			0.001		
R^2	0.9275			0.9910			0.9934		

Source	Soluble phenols			Flavonoids			Anthocyanins		
	SS	F-value	*p*-value	SS	F-value	*p*-value	SS	F-value	*p*-value
R^2-adj	0.9138			0.9884			0.9940		
R^2-pred	0.9476			0.9920			0.9939		
Lack of fit	0.0545			0.1633			0.2335		

Effect of high-intensity ultrasound-assisted extraction parameters on total soluble phenols, total flavonoids, and total anthocyanins based on response surface method

The 3D surface plots and main effects plots of the extraction time, ultrasound power, and liquid-to-solid ratio on the soluble phenols, flavonoids, and anthocyanins contents from *B. vulgaris* stem powder are shown in **Figures 2** and **3**, respectively. Moreover, the optimal high-intensity ultrasound-assisted extraction conditions were identified using a desirability function that ranges from 0 to 1 (where 1 is an entirely desirable response) to maximize the values of dependent variables during high-intensity ultrasound-assisted extraction [44]. Regarding soluble phenols (**Figures 2(a)** to **2(c)**), they tend to increase as the liquid-to-solid ratio increases; moreover, this variable is dependent on the power ultrasound and extraction time, where the highest soluble phenols content is extracted after 3 min at 100% of power ultrasound and 17:1 mL/g of liquid-to-solid ratio, with a maximum soluble phenols content of 295 mg GAE/g and a desirability of 0.90 (**Figure 3(a)**). Similar trends were observed for flavonoids (**Figures 2(e)** to **2(g)**), with higher results (38 mg CE/g) obtained at 17:1 mL/g liquid-to-solid ratio, higher ultrasound power (95%), and extraction time (6 min), with a desirability of 1.0 (**Figure 3(b)**). In contrast to soluble phenols, flavonoids require higher extraction time during high-intensity ultrasound-assisted extraction, associated with the sensitivity of these molecules, as reported by Sanou *et al.* [45]. These results agree with the principles of mass transfer phenomena between solvents and solids, which are predominantly influenced by concentration gradients. When the liquid-to-solid ratio is low, the solution's viscosity increases, making cavitation more difficult because the rarefaction phase must overcome a stronger cohesive force. Conversely, as the liquid-to-solid ratio rises, cavitation becomes more pronounced due to decreased viscosity and a lower concentration of dissolved solids. This enhances the extraction process by promoting greater matrix fragmentation, erosion, and pore development in the plant cell, all of which contribute to a higher TSPs content [26]. Moreover, the Pareto Chart (**Figures 2(d)** and **2(h)**) showed that the ultrasound power (X_2) exhibited the most significant effect (positive effect) that influenced the high-intensity ultrasound-assisted extraction of soluble phenols and flavonoids, respectively. It has been reported that the ultrasound-assisted extraction yield of soluble phenols from *Phyllanthus emblica* plant showed a significant increase when the liquid-solid ratio was changed from 10:1 to 20:1 [46]. Alves *et al.* [44] reported that increasing the ultrasound-assisted extraction volume from 10 to 20 mL/g and the nominal ultrasound power from 20% to 80% significantly enhanced the recovery of bioactive compounds from *Monteverdia aquifolia* leaves. Chen *et al.* [47] reported that phenolic content increases when the liquid-to-solid ratio increases in ultrasound-assisted extraction from coffee leaves and noted that larger volumes of solvent could more effectively recover phenolic compounds by increasing the concentration gradient. However, using excessive amounts of solvent volume led to waste. Thus, determining the optimal solvent volume is essential for maximizing the efficiency of the extraction process [47]. Conversely, the best high-intensity ultrasound-assisted extraction conditions for anthocyanins are 12.97:1 mL/g of liquid-to-solid ratio, an extraction time of 6 min, and 92% ultrasound power (**Figures 2(i)** to **2(k)**), with an anthocyanins content of 0.43 mg C3G/g and a desirability of 0.96 (**Figure 3(c)**), being ultrasound power the most significant factor that influences the extraction of anthocyanins (**Figure 2(l)**). By increasing the proportion of solids in the solvent, the interaction between them can be enhanced, leading to a higher

recovery yield of anthocyanins. However, if this proportion becomes excessively high, it may cause some anthocyanins in the extract to oxidize, thereby decreasing their overall content [48]. It has been documented that increasing ultrasound power typically enhances the physical and chemical effects of cavitation during high-intensity ultrasound-assisted extraction, thereby increasing the recovery yield of bioactive compounds through enhanced mass transfer. However, after reaching the maximum peak, the yield may decrease, mainly at longer extraction times, due to the degradation of compounds [26]. Diverse authors have reported desirability values from 0.85 to 1.0 during the ultrasound-assisted extraction of bioactive compounds from plant materials, suggesting significant acceptability [27,49].

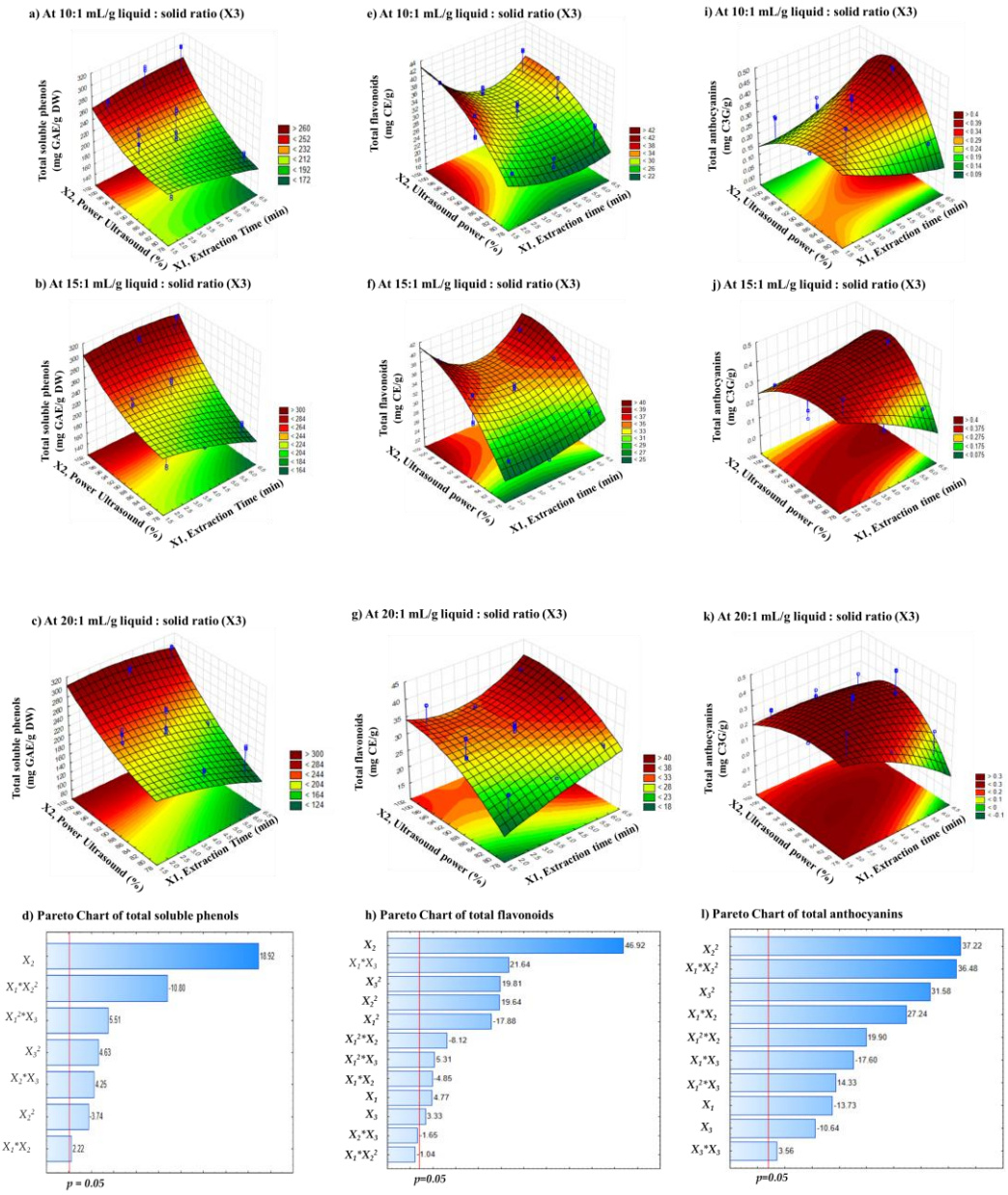

Figure 2 Response surface plots and Pareto Chart for interaction effects of extraction time, ultrasound power, and liquid-to-solid ratio on the soluble phenols (a - d), flavonoids (e - h), and anthocyanins (i - l) from *Beta vulgaris* stem powder.

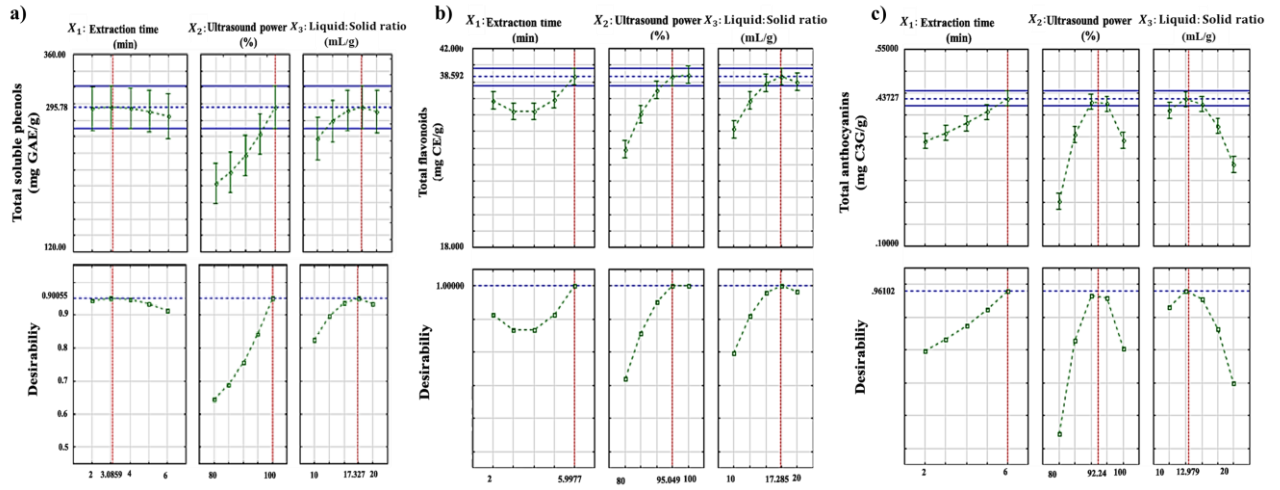

Figure 3 Main effects plot of extraction time, ultrasound power, and liquid-to-solid ratio on soluble phenols (a), flavonoids (b), and anthocyanins (c) from *Beta vulgaris* stem powder.

Verification of total soluble phenols predictive model and comparison of ultrasound-assisted extraction with a conventional extraction method

Among all estimated optimal high-intensity ultrasound-assisted extraction conditions (soluble phenols, flavonoids, and anthocyanins), the high-intensity ultrasound-assisted extraction conditions for soluble phenols (extraction time: 3.08 min, ultrasound power: 100%, and liquid-to-solid ratio: 17.32 mL/g) were selected for experimental model reliability confirmation. Under these conditions, the predicted soluble phenols values (295.78 - 321.27 mg GAE/g) aligned with the experimental soluble phenols results (296.79 - 306.59 mg GAE/g, **Table 4**), confirming the adequacy and reliability of the soluble phenols fitted model. As a result, effective extraction of soluble phenols from *B. vulgaris* stem powder using high-intensity ultrasound-assisted extraction was possible, aligning with findings reported in another research [50].

The effectiveness of extracting soluble phenols from *B. vulgaris* stem powder was investigated by comparing the high-intensity ultrasound-assisted extraction conditions with a conventional extraction method; moreover, flavonoids, anthocyanins, and antioxidant activity was also determined using DPPH, ABTS, and FRAP assays (**Table 4**). The soluble phenols yield from *B. vulgaris* stem powder when high-intensity ultrasound-assisted extraction was applied (301.66 mg GAE/g) was 2.35 times higher than that obtained from the conventional extraction method (128.26 mg GAE/g) ($p < 0.05$). Additionally, the extraction time was reduced by 98% in the high-intensity ultrasound-assisted extraction compared to the evaluated conventional extraction method. Furthermore, other parameters such as flavonoids (34.83 mg CE/g), ABTS (192.87 mmol TE/g), and FRAP (199.12 mmol TE/g), exhibited significantly ($p < 0.05$) higher values when high-intensity ultrasound-assisted extraction was applied compared to those of conventional extraction method (30.90 mg CE/g, 0.29 C3G/g, 141.95 mmol TE/g, and 50.32 mmol TE/g, respectively); moreover, no differences ($p > 0.05$) were detected in anthocyanins (0.27 and 0.29 mg C3G/g) and DPPH (147.59 and 146.09 mmol TE/g) values for the high-intensity ultrasound-assisted extraction and conventional extraction methods. These results align with earlier studies, which demonstrated that high-intensity ultrasound-assisted extraction leads to a more efficient extraction of bioactive compounds compared to traditional methods, in a shorter time frame. The chemical and physical effects of ultrasound cavitation promote the breakdown of the cell wall and assist in releasing phenolic compounds [35,36]. Singh *et al.* [11] reported an increase in phenolic compounds in *B. vulgaris* stem extracts when high-intensity ultrasound-assisted extraction was applied (70 mg GAE/g) compared to the Soxhlet (50 mg GAE/g) and maceration (60 mg GAE/g).

Table 4 Comparison of experimentally optimal high-intensity ultrasound-assisted extraction with a conventional extraction method.

Parameter	[1]HIUAE	[2]Conventional extraction
Total soluble phenols (mg GAE/g)	301.66 ± 4.87[a]	128.26 ± 9.73[b]
Total flavonoids (mg CE/g)	34.83 ± 0.39[a]	30.90 ± 0.39[b]
Total anthocyanins (mg C3G/g)	0.27 ± 0.003[a]	0.29 ± 0.002[a]
DPPH (mmol TE/g)	147.59 ± 1.53[a]	146.09 ± 0.35[a]
ABTS (mmol TE/g)	192.87 ± 3.56[a]	141.95 ± 0.52[b]
FRAP (mmol TE/g)	199.12 ± 11.30[a]	50.32 ± 5.84[b]
Effectiveness (n-times)	2.35	

All values are means ± standard deviation (n = 3). Different letters on each line indicate statistically significant differences in extraction methods by Student T test ($\alpha = 0.05$). [1]High-intensity ultrasound-assisted extraction conditions (HIUAE): 3.08 min for extraction time, 100% power ultrasound, and 17.32 mL/g of liquid-to-solid ratio. [2]Magnetic stirring at 400 rpm for 60 min.

HPLC analysis of *Beta vulgaris* stem powder

The HPLC analysis of extracts obtained from *Beta vulgaris* stem powder using the optimal high-intensity ultrasound-assisted extraction conditions and conventional extraction showed the presence of shikimic acid (**Figure 4**). The shikimic acid content in high-intensity ultrasound-assisted extraction (10,880 mg/100 g, **Figure 4(a)**) was significantly higher than that obtained by conventional extraction (422 mg/100 g, **Figure 4(b)**). The shikimic acid pathway is a common approach for the biosynthesis of lignin, aromatic amino acids (tryptophan, tyrosine, and phenylalanine), and other secondary metabolites in plants such as phenolic compounds (i.e., gallic acid, chlorogenic acid, pyrogallol, and catechol) and alkaloids. Due to its structure, which comprises 6-membered carbocyclic rings and 3 asymmetric centers, shikimic acid is widely used in synthetic biology to obtain a wide range of compounds with pharmaceutical properties, including anti-inflammatory, analgesic, and antiviral agents (Tamiflu®) [51]. It has been reported that shikimic acid can accumulate in various plant organs, including bark and stem [52]. Therefore, the ultrasound cavitation can break the cell wall of *B. vulgaris* stem, facilitating the release of shikimic acid compared to the conventional extraction method [17,20]. In this context, *B. vulgaris* stem could be a source of shikimic acid for potential pharmacological applications.

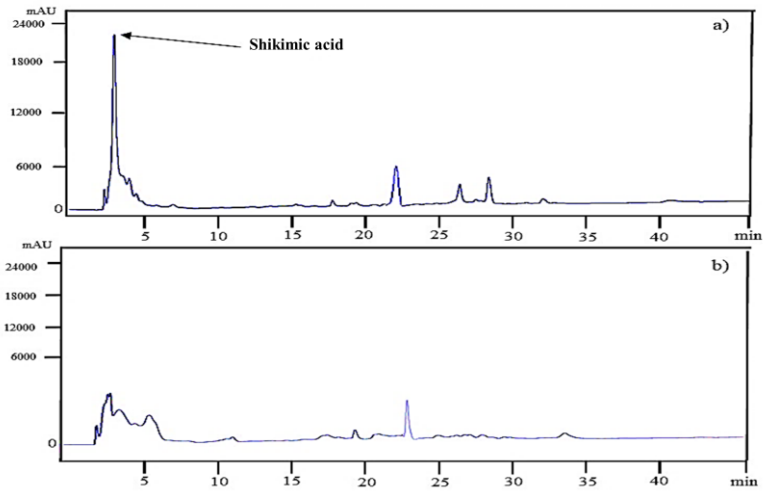

Figure 4 Chromatograms of a) high-intensity ultrasound-assisted extraction, and b) conventional extraction of *Beta vulgaris* stem powder.

Additionally, 7 phenolic compounds were identified in the extract obtained under high-intensity ultrasound-assisted extraction, including protocatechuic, 4-Hydroxybenzoic, gallic, caffeic, chlorogenic, neochlorogenic, and trans-ferulic acids. On the other hand, with the conventional extraction, only 4 phenolic compounds were identified, including protocatechuic, chlorogenic, neochlorogenic, and trans-ferulic acids (**Table 5**). High-intensity ultrasound-assisted extraction demonstrated a significantly higher phenolic content ($p < 0.05$) compared to the traditional extraction. Within high-intensity ultrasound-assisted extraction, the most abundant phenolic compounds were trans-ferulic acid (9.63 mg/100 g), caffeic acid (3.11 mg/100 g), and neochlorogenic acid (2.38 mg/100 g). Conversely, the conventional extraction yielded lower concentrations of trans-ferulic acid (7.42 mg/100 g), neochlorogenic acid (2.08 mg/100 g), and protocatechuic acid (0.88 mg/100 g). It has been reported that traditional extraction techniques often result in low recovery rates and inferior quality due to potential degradation. High-intensity ultrasound-assisted extraction offers a technological alternative for extracting phenolic compounds from *B. vulgaris* stem powder [24,26].

Some studies have reported the presence of a variety of phenolic compounds from *Beta vulgaris* stem, using diverse solvents and extraction methods. Chlorogenic, gallic, ferulic, syringic, caffeic, and coumaric acids, methyl gallate, catechin, and rutin were identified in aqueous extract from *B. vulgaris* stem [10]. Koubaier *et al.* [14] characterized the phytochemical profile of an acetonitrile fraction of *B. vulgaris* stem and reported the presence of gallic, ferulic, chlorogenic, caffeic, vanillic, syringic, and ellagic acids, quercetin, myricetin, and kaempferol. Abdo *et al.* [8] informed the presence of cinnamic, ferulic, caffeic, syringic, ellagic, and coumaric acids, vanillin, rutin, naringenin, quercetin, and catechin in a methanol-ethanol-water extract from *B. vulgaris* stem; however, gallic and chlorogenic acids were not detected. Batistella-Lasta *et al.* [18] informed that the type and content of phytochemicals depended on the extraction method and solvent used. They used ethanol and reported the presence of 3 - 4 dihydroxybenzoic, caffeic, chlorogenic, ferulic, gallic, syringic, and sinapic acids by pressurized liquid extraction, but this phenolic profile differs from those obtained by Soxhlet and ultrasound-assisted extraction, where most of these compounds were not detected, proving the effect of the extraction technique on the phytochemical profile of *Beta vulgaris* stem powder extracts.

Table 5 Comparison of the phenolic profile of *Beta vulgaris* stem powder using high-intensity ultrasound-assisted extraction and conventional extraction.

Compounds	[1]HIUAE (mg/100 g)	[2]Conventional extraction (mg/100 g)
Hydroxybenzoic acids		
Protocatechuic acid	1.21 ± 0.05[a]	0.88 ± 0.06[b]
4-Hydroxybenzoic acid	1.74 ± 0.06[a]	nd
Gallic acid	1.21 ± 0.01[a]	nd
Hydroxycinnamic acids		
Caffeic acid	3.11 ± 0.06[a]	nd
Chlorogenic acid	1.74 ± 0.05[a]	0.67 ± 0.01[b]
Neochlorogenic acid	2.38 ± 0.01[a]	2.08 ± 0.04[b]
Trans-ferulic acid	9.63 ± 0.01[a]	7.42 ± 0.18[b]

All values are means ± standard deviation of 3 determinations (n = 3). nd: not detected. Different letters on each line indicate statistically significant differences in extraction methods by Student's t-test (α = 0.05). [1]High-intensity ultrasound-assisted extraction (HIUAE) conditions: 3.08 min for extraction time, 100% power ultrasound, and 17.32 mL/g of liquid-to-solid ratio. [2]Magnetic stirring at 400 rpm for 60 min.

Conclusions

Variations in high-intensity ultrasonic-assisted extraction parameters, including extraction time, ultrasound power, and liquid-to-solid ratio, showed a positive effect on soluble phenols, flavonoids, anthocyanins, and antioxidant capacities (DPPH, ABTS, and FRAP) of *Beta vulgaris* stem powder. Although the optimal high-intensity ultrasound-assisted extraction conditions differed for each response, all responses were successfully fitted to a 2nd-order polynomial equation. Furthermore, high-intensity ultrasound-assisted extraction (extraction time of 3.08 min, ultrasound power of 100%, and liquid-to-solid ratio of 17.32 mL/g) proved more effective than the conventional extraction method for soluble phenols, flavonoids, ABTS, and FRAP, while yielding comparable values for anthocyanins and DPPH. The high-intensity ultrasound-assisted extraction of *B. vulgaris* stem powder identified many hydroxybenzoic and hydroxycinnamic acids, including protocatechuic, 4-hydroxybenzoic, gallic, caffeic, chlorogenic, neochlorogenic, and trans-ferulic acids. The concentration of these compounds was greater in high-intensity ultrasound-assisted extraction compared to the conventional extraction method. This research emphasized that the stems of *B. vulgaris*, an often-overlooked agricultural byproduct, could serve as a significant source of phenolic compounds with antioxidant benefits. Additionally, it demonstrated that high-intensity ultrasound-assisted extraction is an efficacious technology for extracting bioactive molecules, which have potential applications in food and non-food industries. Future research should explore the use of natural deep eutectic solvents to enhance the recovery yield of phenolic compounds from *B. vulagirs* stem powder through greener extraction methods. Furthermore, studies on the isolation and purification of the phenolic compounds in *B. vulgaris* stem powder should be performed, as well as on evaluating its stability, bioavailability, and bioaccessibility for use in various pharmaceutical, cosmetic, nutraceutical, and other industrial applications. In this study, a laboratory-scale ultrasound was employed; however, further research should focus on pilot-scale or industry-scale up extraction.

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Declaration of generative AI in scientific writing

No generative AI tools were used in the writing of this manuscript.

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