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Microwave-Assisted Extraction of Phenolic Compounds from Broccoli (*Brassica oleracea*) Stems, Leaves, and Florets: Optimization, Characterization, and Comparison with Maceration Extraction

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Academic Editor: Jennifer Keogh

Special Issue: Valorisation of By-products as Opportunity to Innovation Functional Foods

Recent Progress in Nutrition
2022, volume 2, issue 2
doi:10.21926/rpn.2202011

Received: February 17, 2022
Accepted: March 25, 2022
Published: April 01, 2022

Abstract

Microwave-assisted extraction (MAE) to obtain phenolics from vegetable wastes has been of recent interest. Broccoli is one of the most globally produced vegetables, and around 43% of the harvest is considered waste. Thus, given the significant quantity of broccoli waste generated, the objective of this work was to optimize the MAE, to maximize the total phenolic content (TPC) from broccoli by-products (leaves and stems) and broccoli florets. The Response Surface Analysis was used in the optimization model to evaluate the impacts of methanol concentration, time, and temperature, and their interactions on the TPC of the broccoli extracts. The optimal MAE conditions were found to be 74.54% (methanol), 15.9 min, and 74.45 °C for broccoli stems; 80% (methanol), 10 min, and 73.27 °C for broccoli leaves; and 80% (methanol), 18.9 min, and 75 °C for broccoli florets. Under these conditions, the broccoli leaves exhibited the highest TPC (1940.35 ± 0.794 µg GAE/g DW), followed by the florets (657.062 ± 0.771 µg GAE/g DW) and stems (225.273 ± 0.897 µg GAE/g DW). The antioxidant activity of the broccoli extracts was evaluated under the optimal conditions by DPPH and ABTS assays, and the same behavior was observed in both studies, the broccoli leaves exhibited the

highest antioxidant activity, among florets and stems. In addition, vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids in the broccoli extracts were identified and quantified using HPLC. Furthermore, MAE was found to increase the phenolic yield up to 45.70% for broccoli leaves, 133.57% for broccoli florets, and 65.30% for broccoli stems, in less time compared with maceration extraction. MAE proved to be an efficient and sustainable technique to obtain phenolics from broccoli by-products, which can constitute a viable solution for valorizing broccoli wastes.

Keywords
Broccoli by-products; antioxidant activity; bioactive compounds; phenolic acids; green extraction; revalorization

1. Introduction

Food waste is a major worldwide concern in terms of environmental sustainability, food safety, and the need to feed the growing global population [1]. Among the food wastes, fruits and vegetables represent around 33% of the total food waste from the post-harvest to the distribution level [2]. Broccoli (*Brassica oleracea L.* var. Parthenon) ranks in the top 5 most-produced vegetables in the world, with 24.17 million metric tons (MMT), the main global producers are China, India, USA, and Mexico [2, 3]. However, it is estimated that approximately 43% of the total broccoli harvest is considered waste, such as leaves and stems [4]. The large amounts of broccoli waste have a negative effect on the agricultural environment and food security. Some studies have revealed that significant amounts of bioactive compounds and essential nutrients are present in the broccoli by-products, such as phenolic compounds, glucosinolates, flavonoids, carotenoids, and sterols [5-8]. The bioactive compounds are extra-nutritional components with the capacity of modulating metabolic processes, and some of them hold antioxidant, anti-microbial, or anti-inflammatory properties [9, 10]. Among the bioactive compounds, phenolic acids have been of recent interest, due to their antioxidant and antimicrobial properties, which make them have a commercial value in different industries, including cosmetics, pharmaceuticals, and food [1, 11, 12]. The enormous range of health advantages and industrial applications of phenolic acids has prompted scientists to enhance extraction and purification strategies for these naturally given compounds [11]. Furthermore, the use of phenolic acids in food enhancement has sparked a lot of interest, since they can be used to retard lipid oxidation and can be added to fresh or processed meats to improve color stability, retard the appearance of off-flavors, and prevent oxidative rancidity [1, 13]. The evaluation of phenolic acids properties, such as antioxidant activity demonstrates the high potential and added value of these compounds for further industrial applications.

The main phenolic acids found in broccoli are caffeic acid, chlorogenic acid, neochlorogenic acid, gallic acid, ferulic acid, and sinapic acid [8, 14]. The phenolics can be obtained from broccoli by-products, using different extraction techniques: conventional (Soxhlet, maceration, hydro-distillation), or non-conventional (microwave-assisted, ultrasound-assisted, supercritical fluid). However, recent trends in extraction techniques are focused on finding solutions that minimize the

time, and use of solvents for phenolic extraction and still maintain high-quality extracts; this can be accomplished using green extraction or non-conventional techniques [5, 15-17].

Green extraction techniques are also known as non-conventional techniques since they employ organic solvents, take less time to extract, and use less energy, all of which have a positive influence on the environment [17]. The microwave-assisted extraction (MAE) is one of the most used, due to its several advantages such as the efficient cell wall disruption in less time, less use of solvent, high selectivity, cost-effective in comparison with maceration, and the mature and developed process both in laboratory and industry level; thus, the microwaves provide dielectric heating and solute dissolution [1, 3]. The comparison between conventional and non-conventional methods of extraction, becomes crucial to enrich and propose a solid base to choose the most appropriate extraction method to extract phenolics from broccoli waste. Generally, methanol/water, and methanol/ethanol/water mixtures are commonly used for extracting phenolics from broccoli by MAE [5]. However, there are few examples and experimentation on MAE of broccoli by-products; more experimentation needs to be carried out to establish the proper parameters for phenolic extraction by MAE from broccoli wastes. The successful phenolic green extraction from broccoli by-products may represent an alternative option to reuse and valorize the vegetable wastes, optimizing the resources and offering a sustainable solution for waste utilization.

Some comparisons between the total phenolic content from different broccoli parts have demonstrated that the leaves and stems have similar content and antioxidant activity to the edible parts of the broccoli (florets) [8, 18]. To our knowledge, as far as broccoli by-products extraction of phenolics, [5, 8, 19-23] have done similar research; however, those studies are not optimizing MAE methodologies, nor comparing the key desirable attributes between the edible broccoli parts (florets), broccoli by-products (leaves, and stems), and conventional extraction methods.

According to the above, the aim of this research is to perform the optimization of the microwave-assisted extraction of broccoli by-products: leaves and stems, and broccoli edibles: florets, to maximize the total phenolic content; identify the main phenolic acids, evaluate their antioxidant activity (with DPPH and ABTS radical scavenging activity analyses), and compare against conventional extraction methods, in this case: maceration. Response Surface Analysis (RSA) is employed in the optimization model to evaluate the impacts of solvent concentration, time, and temperature, and their interactions on the total phenolic content of the broccoli extracts. It is expected that the broccoli leaves, and stems exhibit a similar or higher amount of total phenolic content, and antioxidant activity compared with the broccoli florets. Furthermore, the MAE should have a higher phenolic extraction yield compared to maceration extraction in less time. This study is focused on phenolic acid identification through High-Performance Liquid Chromatography (HPLC) due to the properties and commercial value of these specific compounds.

2. Materials and Methods

2.1 Sample Preparation

The broccoli (*Brassica oleracea*) florets, stems, and leaves were collected from a local market: Chez Robin in Montreal, Quebec. The broccoli by-products were cut into small pieces (3 to 4 cm) and separated from each other, 500 g of each category (florets, leaves, and stems). Then the materials were lyophilized in a Freeze-Dryer (Labconco Catalog No. 7670520, Serial No. 091017338G, USA), and they were ground to a fine powder with the help of a commercial blender

(Retsch, Knife Mill Grindomix GM 200) at 5000 rpm for 1 min. Finally, the materials were stored at -20 °C until further analysis. The sample preparation was based on previous research on broccoli samples [19, 22, 24].

2.2 Maceration Extraction

2.5 g of each of the previously treated broccoli samples, consisting of stems, leaves, and florets were extracted with 4 different solvents: 50 mL methanol (80% v/v), 50 mL methanol (40% v/v), 50 mL methanol (60% v/v), and 50 mL of distilled water (methanol free), in 100 mL closed flasks. The maceration was carried out at room temperature for 24 h, with constant agitation at 250 rpm. The maceration extraction process was an improvement based on [19, 22, 24] previous studies. Then, the mixture was centrifuged (Centrifuge, Sorvall Legend X1R- Thermo Scientific) for 20 min at 10350 rpm, and 4 °C. Finally, the supernatant was filtered through a 0.20 µm PTFE syringe-filter (Fisher Scientific), then the aqueous phase was stored at -20 °C until further analysis.

2.3 Microwave-assisted Extraction (MAE) and Optimization

The MAE of all the broccoli by-product samples was carried out using a Mini WAVE Digestion Module (SCP Science Canada) that operates at a frequency of 2.45 GHz using 6 cylindrical quartz reactor vessels of 50 mL. In the MAE experiments, 2.5 g of each of the previously treated broccoli samples were extracted in 50 mL of solvent, with a liquid solid ratio of 20:1. Table 1 shows the variables and the levels proposed in the experimental designs. The solvent selected for the MAE was methanol (at different concentrations, see Table 1), which has been proved to be one of the best polar solvents for phenolic extractions [20, 22]. The parameters such as time and temperature were established, monitored, and controlled in the Mini Wave Digestion module set up. After each extraction, the mixture was centrifuged (Centrifuge, Sorvall Legend X1R- Thermo-Scientific) for 20 min at 10350 rpm, and 4 °C. Finally, the supernatant was filtered through a 0.20 µm PTFE syringe-filter (Fisher Scientific), then the aqueous phase was stored at -20 °C until further analysis.

Table 1 Experimental variables and levels used in Central Composite Rotatable Design.

Variables	Variable	Levels				
		-α	**-1**	**0**	**+1**	**+α**
Solvent concentration (methanol% v/v)	A	26.36	40	60	80	93.63
Time (min)	B	6.59	10	15	20	23.40
Temperature (°C)	C	48.18	55	65	75	81.81

In total 3 MAE optimizations were done, one for each category: broccoli stems, leaves, and florets. Response Surface Analysis (RSA) modeling technique with Central Composite Rotatable Design (CCRD) was selected for the experiment design of the Microwave-assisted extraction (MAE) to evaluate the effect of temperature (°C), time (min), and solvent concentration (% v/v) in the total phenolic content (TPC) of the broccoli by-products.

The experimental design was based on the CCRD with 20 experimental runs for each optimization, including 6 central points, 8 factorial runs, and 6 axial points. The choice of the axial

runs (α) gives the design the rotatable aspect, where α represents the extreme values (low and high). For the three variables (temperature, time, and solvent concentration), the value of α is 1.682. The CCRD design uses least-squares regression to fit the experimental data to a quadratic model.

Rotatability was the criteria for choosing RSA, due to two factors: the TPC optimization purpose, and the position of the optimum values which was unknown before the experiments; it was reasonable to select a design that allowed for equal precision and reasonable distribution of the data points [25]. Furthermore, the modeling technique used offers the advantage of reducing time and expenses [26]. In all the experiments, the three independent variables (methanol concentration, time, and temperature) were correlated in order to maximize TPC response of the broccoli extracts.

The RSA of the data, and optimization of the models were performed using Design Expert software (version 13.0 Stat-Ease Inc. Minneapolis, MN, USA). The ANOVA (Analysis of variance), the variables (methanol concentration, time, and temperature), and the responses (TPC) under the optimized conditions were validated using the same software.

2.4 Determination of Total Phenolic Content (TPC)

To quantify the TPC from the different extracts, the Folin-Ciocalteu method was followed, with minor adaptations from [27-30]. A mixture consisting of 100 µL of the extract, 475 µL of distilled water and 100 µL of Folin-Ciocalteu reagent, was stored in the dark for 5 min at room temperature, then 1325 µL of 75g/L sodium carbonate (Na_2CO_3) were added into the mixture, homogenized, and incubated at room temperature in darkness for 2 h. The absorbance was measured at 765 nm. The same procedure was used using gallic acid (0 to 100 ppm) as standard compound.

The sample concentration (µg gallic acid equivalent (GAE)/mL) was calculated based on the standard gallic acid calibration curve. The TPC results are expressed in µg gallic acid equivalent (GAE)/g dry weight (DW).

2.5 Phenolic Acids Characterization

The phenolic acids were characterized for the samples under the optimum MAE conditions, for each category: broccoli stems, leaves, and florets. A high-performance liquid chromatography (HPLC) method was used to characterize the phenolic acids, with some changes from [27, 28, 31]. The calibration solutions were made with standards of vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids, in aliquots, in concentrations between 0 to 100 ppm diluted in 0.1% formic acid and distilled water 99.9% (v/v). Before the HPLC method, all the samples, including the standards, were filtered through a 0.20 µm PTFE syringe-filter (Fisher Scientific).

The HPLC (Agilent 1100 Series) used a C18 column (Gemini, 5µ 150 × 4.60 mm), a mobile phase A: formic acid 0.1% + 99.9% water (v/v), and a mobile phase B: formic acid 0.1% + acetonitrile 99.9% (v/v). The flow rate was 0.4 mL/min, with an injection volume of 5 µL, and 40 °C. The gradient was as follows: 99% A, 1% B for 10 min; 50% A, 50% B, for 20 min; and then 99% A, 1% B, for 10 min. The absorbance was set at 330 nm. The identification and quantification of the phenolic compounds were performed by comparing the retention time of pure standards solutions.

2.6 Determination of Antioxidant Activity (AA)

2.6.1 DPPH (2,2-Diphenyl-1-Picrylhydrazyl Hydrate) Radical Scavenging Method

The AA was determined through an DPPH assay, based on [27, 30, 32] experimentations for the samples under the optimum MAE conditions, for each category: broccoli stems, leaves, and florets. A methanolic-DPPH stock solution was prepared (0.048 mg/mL), and 5 aliquots of diluted extracts to facilitate the quantification.

Then a mixture consisting in 500 µL of extract and 500 µL of the DPPH-methanolic solution was vortexed and incubated in the dark for 30 min at room temperature. Finally, the absorbance was measured at 517 nm. The control sample consisted in 500 µL of methanol and 500 µL of the DPPH-methanolic solution. To quantify the percentage of inhibition in the samples, the equation (Eq. 1) was used, which is the mean inhibitory concentration, that is, the concentration of antioxidant compounds that can inhibit 50% of the DPPH radical.

$$\%\text{Inhibition} = \frac{\mathrm{A_c} - \mathrm{A_s}}{\mathrm{A_c}} \times 100\% \qquad (Eq.1)$$

Where A_c is the control absorbance and A_s the sample absorbance. Finally, a standard commercial Trolox calibration curve was developed (0 to 100 ppm) to quantify the AA in the sample, and the same procedure of the DPPH assay was followed. The AA in the samples is expressed in µg of Trolox Equivalents (TE)/g of dry weight (DW) of the sample. The analyses were made in triplicates for each broccoli sample.

2.6.2 ABTS (2,2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid)) Radical Scavenging Method

The antioxidant activity was also determined by an ABTS assay, based on [18, 33-35] experimental investigation on the samples under the optimum MAE conditions, for each category: broccoli stems, leaves, and florets. A mixture of 7 mmol/L ABTS and 140 mmol/L potassium persulfate ($K_2S_2O_8$) was stored in the dark at 25 °C for 14 h. Then, the ABTS radical solution was diluted to the absorbance level of 0.70 ± 0.02 at 734 nm, using an aqueous methanol solution (67% v/v).

Then a mixture consisting of 20 µL of the broccoli extracts (the extracts were diluted to facilitate the analysis), and 2 mL of the ABTS radical solution was stored in the dark for 6 min at room temperature. Finally, the absorbance was measured at 734 nm. A mixture of 2 mL of the ABTS radical solution and 20 µL of methanol was used as control.

Finally, a standard commercial Trolox calibration curve was developed (0 to 100 ppm) to quantify the AA in the sample, and the same procedure of the ABTS assay was followed. The AA in the samples is expressed in µg of Trolox Equivalents (TE)/g of dry weight (DW) of the sample. The analyses were made in triplicates for each broccoli sample.

3. Results

3.1 Optimization: Microwave-assisted Extraction (MAE) of Broccoli Samples: Stems, Leaves and Florets

3.1.1 Analysis of Responses Models: Response Surface Analysis (RSA)

RSA with CCRD was used to evaluate the effect of three variables: methanol concentration (v/v%), extraction time (min), and temperature of the extraction(°C), on the total phenolic content (TPC) of the broccoli samples. In all the experiments, the three independent variables were correlated to maximize TPC response. The surface response model obtained for the TPC was of second order for the three analyses (broccoli stems, leaves and florets), the results of the analysis are shown in Table 2. For all the models, the results were significant at *p*-values <0.05, and the models were focused on maximizing the adjusted R^2 and the predicted R^2, in general a greater R^2 suggests a better fit for the model.

Table 2 Reduced mathematical models for the total phenolic content (TPC) response and its evaluation parameters based on Fit Summary and Model Summary Statistics of the broccoli samples.

Broccoli Samples	Model[a]	Model[b]	R^2	Adjusted R^2	Predicted R^2	Std. Dv.	Sequential[c] *p*-value	Lack of fit[c] *p*-value
Stems	$Y = -2.81A^2 - 2.75BC + 23.58A + 5.60B + 12.17C + 191.81$	$Y = -0.007029A^2 - 0.054935BC + 2.02245A + 4.69122B + 2.04101C - 53.70021$	0.9907	0.9823	0.9748	3.11	*	ns
Leaves	$Y = -8.12A^2 - 1.90B^2 + 35.11A + 7.75B + 16.79C + 1908.51$	$Y = -0.020293A^2 - 0.076083B^2 + 4.19045A + 3.83297B + 1.67921C + 1580.61459$	0.9950	0.9905	0.9829	3.37	***	ns

Florets	$Y = -2.50B^2 - 2.50C^2 + 30.26A + 6.67B + 15.07C + 609.28$	$Y = -0.100193B^2 - 0{,}025048C^2 + 1.51319A + 4.33981B + 4.76289C + 272.17951$	0.9912	0.9833	0.9429	3.82	*	ns

[a] Final Equation in Terms of Coded Factors; [b] Final Equation in Terms of Actual Factors; [c] the *p*-value results are indicated as follows: ns: $p > 0.05$; *: $0.05 < p < 0.01$; **: $0.01 < p < 0.001$; ***: $p < 0.001$; Y: total phenolic content (TPC) expressed in μg gallic acid equivalent (GAE)/g dry weight (DW); A: Methanol concentration (%); B: Time (min); C: Temperature (°C).

In the case of the broccoli stems model, only the methanol concentration was significant for the quadratic terms (Table 2), the reduction of the whole quadratic model was carried out since there were some insignificant terms. Furthermore, in the Analysis of variance (ANOVA) for the reduction of quadratic model, the F-value was 184.52, and *p*-value was <0.0001 which provide evidence that the model is significant. Figure 1 shows the RSA for the TPC in the broccoli stems model. The graph corresponds to the interaction between the most significant variable, methanol concentration, with the other two variables: time and temperature. In Figure 1A, the interaction between methanol concentration and time during the MAE is shown. The experimental runs showed that the maximum TPC (224.174 μg GAE/g DW) was obtained using the highest values of methanol concentration (80% and 93.63% v/v) for 20 min. In Figure 1B, the interaction between methanol concentration and temperature is observed. Although in the model, the temperature is not significant in quadratic terms, it affects the TPC; at higher temperatures (65 °C, and 75 °C) the TPC increases, compared to the lower temperatures.

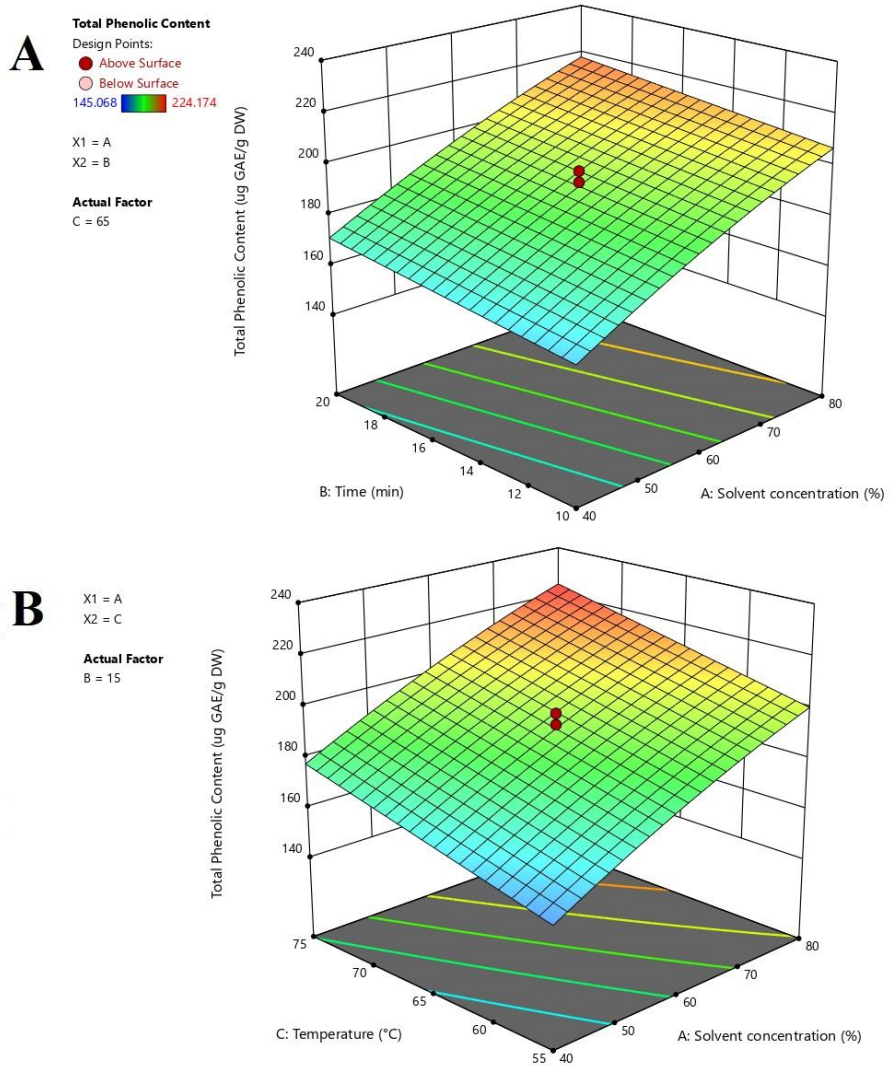

Figure 1 3D Broccoli stems Response Surface plot of total phenolic content (TPC). (A) Interaction AB, Solvent concentration (methanol%), and time (min). (B) Interaction AC, Solvent concentration (methanol%), and temperature (°C).

The RSA for the broccoli leaves samples showed that the methanol concentration and time were significant in quadratic terms (Table 2). The temperature affected the quadratic model in terms of lineal behavior. Due to the presence of several insignificant terms, the quadratic model was reduced. The ANOVA for the reduced quadratic model showed a Model F-value of 396.21, and *p*-value was <0.0001 which implied that the model was significant; the Lack of Fit was not significant, so that the model fits. Figure 2 shows the RSA for the TPC in the broccoli leaves model. Figure 2A indicates the interaction between methanol concentration, and time during the MAE. The experimental runs exhibited that the maximum TPC (1960.12 µg GAE/g DW) was obtained at a methanol concentration of 80% (v/v), for 20 min; the behavior is similar to the broccoli stems model (Figure 1A). In general, the broccoli leaves presented higher amounts of TPC compared with the broccoli stems, which agrees with the established values by [5, 14, 19, 23]. Figure 2B shows the interaction between methanol concentration, and temperature. In the experiments, the 75 °C temperature exhibited the highest amount of TPC at 80% (v/v) methanol concentration. In Figure

2C, the interaction between time and temperature is shown; between 15 min and 20 min at 75 °C, the TPC increases, compared to the lower times.

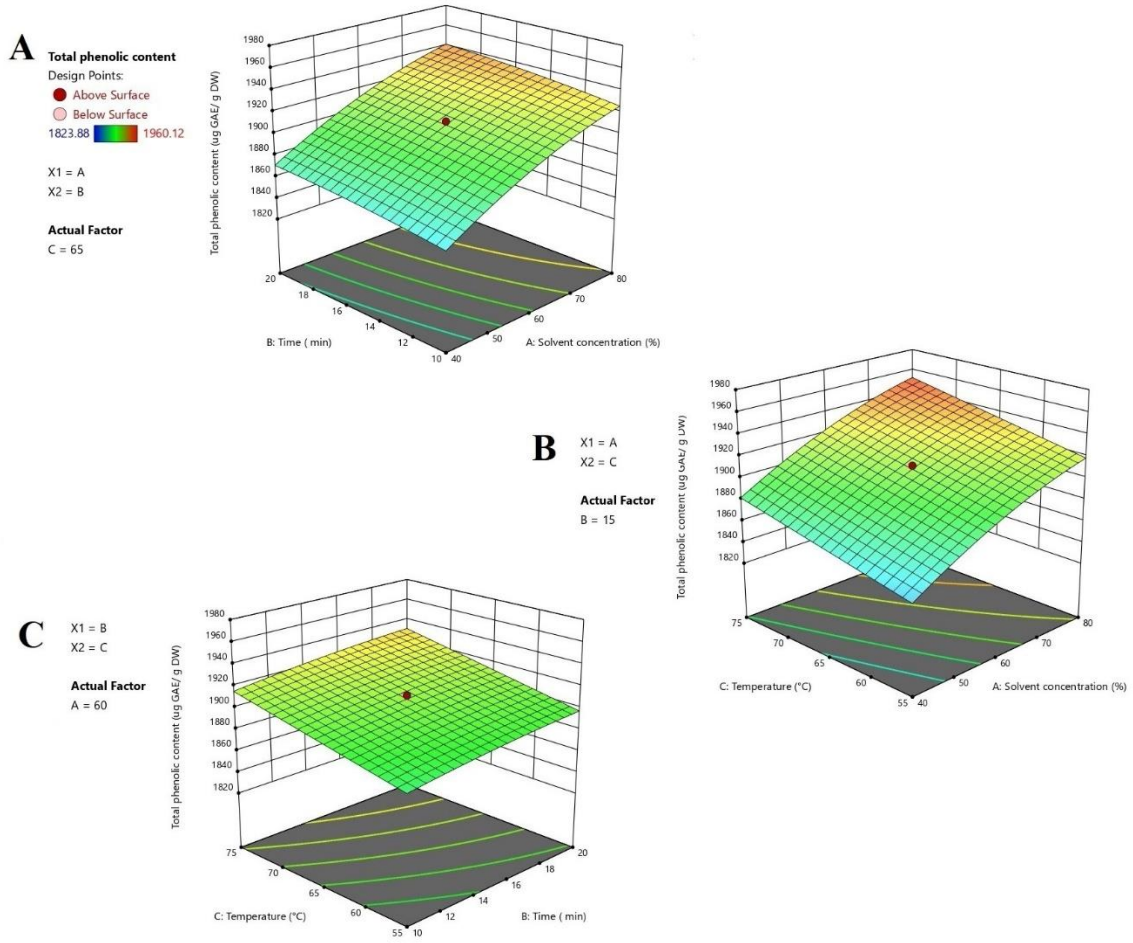

Figure 2 3D Broccoli leaves Response Surface plot of total phenolic content (TPC). (A) Interaction AB, Solvent concentration (methanol%), and time (min). (B) Interaction AC, Solvent concentration (methanol%), and temperature (°C). (C) Interaction CB, Time of extraction (min), and temperature (°C).

In the broccoli florets model, the time and temperature of extraction were significant in quadratic terms (Table 2). However, the methanol concentration shows a linear behavior in the quadratic model. The model was reduced, due to the presence of insignificant terms. The ANOVA for the reduced quadratic model exhibited a Model F-value of 193.07, and a *p*-value <0.0001, providing support for the model to be significant. Figure 3 presents the RSA for the TPC in the broccoli florets model. Figure 3A indicates the interaction between time, and temperature during the MAE. The experimental runs showed that the highest TPC (668.049 µg GAE/g DW) was obtained at 65 °C for 15 min. Figure 3B exhibits the interaction between time, and methanol concentration; in the experiments, the highest TPC was obtained at 93.63% (v/v) of methanol concentration. Figure 3C shows the interaction of temperature, and methanol concentration; at higher temperatures and concentrations the TPC increases. The broccoli stems, leaves, and florets models exhibited similar behaviors; in the experiments for the three models, the highest amounts of TPC were found in

methanol concentrations ≥80% (v/v), between 15 min to 20 min, and temperatures between 65 °C to 75 °C, which agrees with the established by [5, 14, 20].

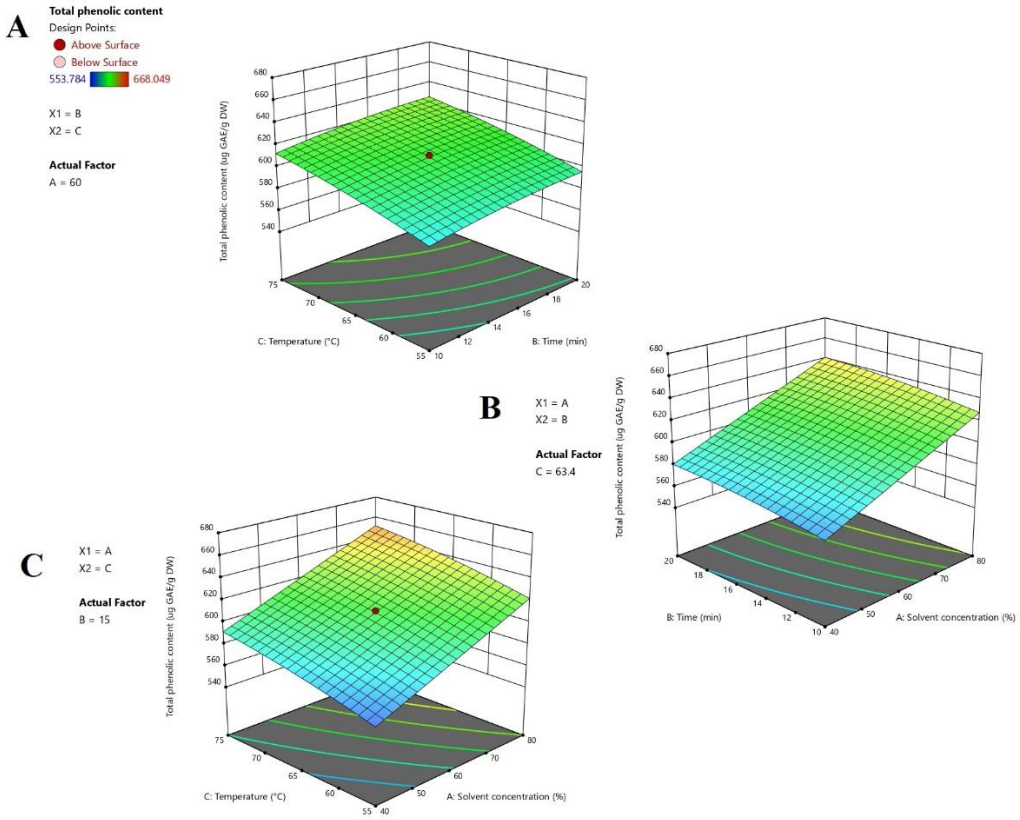

Figure 3 3D Broccoli florets Response Surface plot of total phenolic content (TPC). (A) Interaction BC, Time (min) and temperature (°C) of the extraction. (B) Interaction AB, Solvent concentration (methanol%), and time (min). (C) Interaction AC, Solvent concentration (methanol%), and temperature (°C).

3.1.2 Optimization and Validation of the Total Phenolic Content (TPC) Responses

The model optimization and validation of the TPC responses for the broccoli stems, leaves and florets were made to streamline the MAE process and propose the optimal and most efficient parameters (methanol concentration, time, and temperature) all in the range to maximize the TPC, through RSA. The optimal values calculated for the independent parameters using Design Expert 13 software are presented in Table 3. Those values were estimated using the mathematical models shown in Table 2. For the validation of the adequacy of the model, triplicate experiments were carried out under optimized MAE conditions and the observed values of TPC responses were obtained. The values of TPC in the broccoli stems, leaves, and florets are very close to those estimated with the model (see Table 3). As a result, the model proved its capacity for prediction. According to [25] the lower the Relative Standard Deviation (RSD) value, the more precise the data collection is.

Table 3 Optimization and validation values of the total phenolic content (TPC) responses for the broccoli samples*.

Broccoli Sample	Optimized conditions			Predicted TPC (µg GAE/g DW)	Observed TPC (µg GAE/g DW) **	RME (%)	RSD (%)
	Methanol concentration (% v/v)	Time (min)	Temperature (°C)				
Stems	79.54	15.9	74.45	224.206	225.273 ± 0.897	0.48	0.48
Leaves	80.00	10.0	73.27	1939.73	1940.350 ± 0.794	0.03	0.11
Florets	80.00	18.9	75.00	655.82	657.062 ± 0.771	0.18	0.33

TPC: total phenolic content; GAE: gallic acid equivalent; DW: dry weight. RME: Relative Mean Error; RSD: Relative Standard Deviation *: all the predicted solutions presented a Desirability of 1.0; **: Each value was expressed by mean ± SD.

3.2 Antioxidant Activity (AA) Evaluation by DPPH and ABTS Assays of the Validated Broccoli Samples: Stems, Leaves, and Florets

The DPPH and ABTS radical scavenging methods were used to measure the AA of the validated broccoli extracts (the samples under the optimal MAE conditions). The results of the mean total AA evaluation are shown in Table 4; the AA in the samples is expressed in µg of Trolox Equivalents (TE)/g of dry weight (DW) of the sample.

Table 4 ABTS and DPPH radical scavenging activities (AA values) for the validated broccoli samples.

Broccoli Sample	TPC (µg GAE/g DW)	AA in DPPH assay (µg TE/g DW)	AA in ABTS assay (µg TE/g DW)
Stems	225.273 ± 0.897^{a}	193.110 ± 0.415^{c}	212.118 ± 0.213^{a}
Leaves	1940.350 ± 0.794^{b}	632.057 ± 0.087^{a}	1034.220 ± 0.324^{b}
Florets	657.062 ± 0.771^{c}	290.973 ± 0.669^{b}	452.169 ± 0.093^{c}

TPC: total phenolic content; GAE: gallic acid equivalent; DW: dry weight; AA: antioxidant activity; TE: Trolox equivalents; DPPH: 2,2-diphenyl-1-picrylhydrazyl hydrate; ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); Each value was expressed by mean ± SD; Same letters in the same column refer to means not statistically different ($p > 0.05$).

In the broccoli extracts the TPC was significantly correlated with DPPH assay ($p < 0.001$, r = 0.897). The AA with the ABTS assay was also correlated with the TPC ($p < 0.001$, r = 0.858). Other authors [19, 23] have reported similar correlations between the TPC and AA for broccoli extracts. Broccoli by-products such as leaves, and stems contain high total phenolics and show high and similar activities compared with broccoli florets. The AA of all the broccoli extracts was higher in the ABTS assay compared with the DPPH assay, among DPPH and ABTS analyses, the broccoli leaves extracts had the highest AA and TPC followed by florets, and stems (see Table 4).

3.3 Phenolic Acids Characterization: Application of HPLC Method to the Validated Broccoli Samples: Stems, Leaves, and Florets

The identification and quantification of phenolic acids in all the validated broccoli extracts were based on calibration curves of external standards (vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids). The analyses were made in triplicates of each sample. The results of the phenolic acid characterization are shown in Table 5. Among the individual phenolic acids in the extracts, chlorogenic, neochlorogenic and ferulic acids were quantifiable in sufficient amounts in the three broccoli samples (stems, leaves, and florets). Caffeic and p-coumaric acids were quantifiable in the broccoli leaves extracts. Gallic acid and vanillic acid were quantifiable in the broccoli stems and florets extracts, while sinapic acid was only quantifiable in the broccoli leaves and florets extracts.

Table 5 Phenolic acids identification and quantification for the validated broccoli samples.

Phenolic acid	Retention time (min)	Broccoli sample concentration (µg/mL)		
		Stems	Leaves	Florets
Caffeic	27.333	ns	1.959 ± 0.042	ns
Chlorogenic	26.051	0.869 ± 0.011	2.153 ± 0.005	1.001 ± 0.004
Ferulic	30.224	21.920 ± 0.004	23.845 ± 0.021	21.954 ± 0.084
Gallic	21.127	17.127 ± 0.023	ns	21.736 ± 0.014
Neochlorogenic	24.958	8.789 ± 0.031	12.148 ± 0.008	9.020 ± 0.032
p-coumaric	29.680	ns	5.502 ± 0.063	ns
Sinapic	29.935	ns	9.149 ± 0.045	2.065 ± 0.055
Vanillic	27.339	17.582 ± 0.011	ns	29.171 ± 0.067

y: Area (mAU*s) milli-Absorbance Units; x: Concentration of the phenolic compound (µg/mL); ns: not significant, low concentrations unable to quantify; Each value was expressed by mean ± SD.

Figure 4 shows the phenolic acid chromatograms of the broccoli extracts. Figure 4A represents the chromatogram for the broccoli stem extract, in this chromatogram 5 phenolic acids were identified in sufficient amounts; ferulic acid was found to be in highest amount, followed by vanillic acid, gallic acid, neochlorogenic acid, and chlorogenic acid (see Table 5). Figure 4B shows the phenolic acids chromatogram of the broccoli leaves extract, where 6 phenolic acids were identified: ferulic acid being in the highest concentration, followed by neochlorogenic acid, sinapic acid, p-coumaric acid, chlorogenic acid, and caffeic acid.

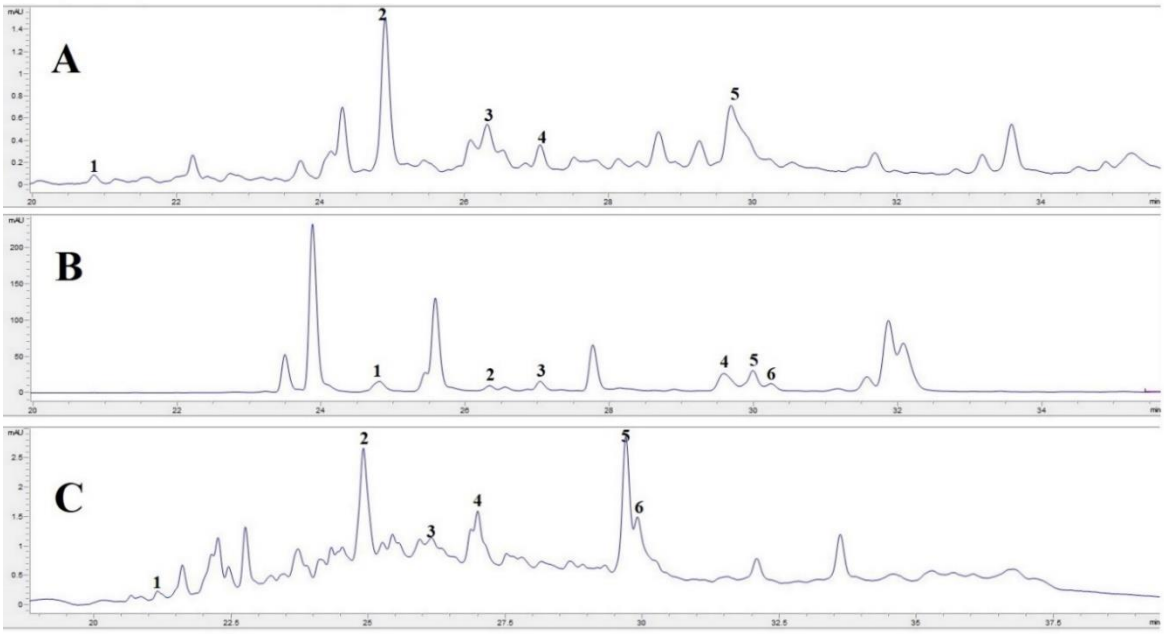

Figure 4 Phenolic acids chromatograms of the validated broccoli extracts (stems, leaves, and florets). (A) Chromatogram of the broccoli stems extract: 1- gallic acid, 2- neochlorogenic acid. 3- chlorogenic acid, 4-vanillic acid, 5- ferulic acid. (B) Chromatogram of the broccoli leaves extract: 1- neochlorogenic acid, 2-chlorogenic acid, 3- caffeic acid, 4- p-coumaric acid, 5- sinapic acid, 6- ferulic acid. (C) Chromatogram of the broccoli florets extract: 1- gallic acid, 2- neochlorogenic acid, 3- chlorogenic acid, 4- vanillic acid, 5- sinapic acid, 6-ferulic acid.

The phenolic acid chromatogram of the broccoli florets extract is shown in Figure 4C. In the broccoli florets extract, 6 phenolic acids were identified; vanillic acid (Table 5) was found in the highest amounts, followed by ferulic acid, gallic acid, neochlorogenic acid, sinapic acid, and chlorogenic acid.

3.4 Comparison between the MAE Validated Broccoli Sample Extracts and Maceration Broccoli Sample Extracts

The total concentration of phenolic compounds extracted from the broccoli stems, leaves, and florets, with methanol concentrations of 80% (v/v), 60% (v/v), 40% (v/v), and methanol free (distilled water) were compared after 24 h of extraction. The comparison of the TPC between a common maceration extraction and the MAE from the validated broccoli samples, is shown in Table 6. The TPC is expressed in μg gallic acid equivalent (GAE)/g dry weight (DW). The optimized MAE process increased the total phenolic yield in the broccoli stems by 0.49%, 9.04%, 19.18%, and 65.30% in comparison to the maceration extraction with methanol concentrations of 80%, 60%, 40%, and distilled water as solvent, respectively. In the case of broccoli leaves, the total phenolic yield increased by 13.20%, 13.49%, 25.07%, and 45.70% using MAE in comparison with maceration extraction, under the previously mentioned methanol concentrations. Finally, the MAE process increased the total phenolic yield in the broccoli florets by 89.23%, 99.32%, 107.62%, and 133.57%, in comparison with maceration extraction with the same methanol concentrations.

Table 6 Comparison of total phenolic content (TPC) of broccoli samples (stems, leaves, and florets) with maceration method and the validated broccoli extracts with MAE.

Extraction method	Methanol concentration (% v/v)	Time	Temperature (°C)	TPC of broccoli samples (μg GAE/g DW)		
				Stems	Leaves	Florets
Maceration	80.00	24 h	24.00	224.174 ± 0.922	1714.011 ± 1.223	347.228 ± 0.956
	60.00			206.595 ± 0.721	1709.616 ± 0.946	329.649 ± 1.567
	40.00			189.016 ± 1.188	1551.404 ± 0.792	316.465 ± 1.683
	0.00*			136.278 ± 1.034	1331.664 ± 1.834	281.306 ± 0.871
MAE	79.54	15.9 min	74.45	225.273 ± 0.897	-	-
	80.00	10 min	73.27	-	1940.350 ± 0.794	-
	80.00	18.9 min	75.00	-	-	657.062 ± 0.771

* Methanol free, distilled water used as solvent; TPC: total phenolic content; GAE: gallic acid equivalent; DW: dry weight; MAE: Microwave-assisted extraction; Each value was expressed by mean ± SD.

In terms of extraction time, the highest TPC results in the broccoli extracts were also observed in MAE with the optimal conditions of 10 min to 18.9 min, compared with 24 h in maceration extraction. MAE has been demonstrated to be a rapid extraction technique in comparison to a 24 h maceration extraction for obtaining phenolics from the broccoli samples in a short period of time. The results (Table 6) showed the highest TPC at the highest methanol concentration (80% v/v) in maceration for the three broccoli categories, which supports the results obtained in MAE.

4. Discussion

4.1 Optimization: Microwave-assisted Extraction (MAE) of Broccoli Extracts

The MAE process begins with the solvent penetration (in this case methanol) into the broccoli samples, then the components break down with the help of electromagnetic waves, the solubilized compounds are moved from the insoluble matrix to the bulk solution, and the liquid and residual solid phase are separated [1, 36]. The microwave radiation absorption in the extraction system enhanced the heat buildup of the extraction solution, resulting in the dissolution of phenolics into the solution for 15 to 20 minutes (see Table 3), the same behavior was observed by [15, 37] in MAE for different plant samples. In the experiments, the increase in temperature, and solvent concentration enhanced the TPC extraction of all the broccoli by-products. The same behavior was presented in the study of [20] for lyophilized broccoli samples (by-products not specified), with optimal MAE conditions of 71.51 °C, for 17 min, methanol concentration of 72.06% v/v, and 160 W. However, it is demonstrated that prolonged exposure times (more than 20 min) at high temperatures degrade the phenolic compounds, reducing the extraction yield in the microwave field [15].

According to Table 3 results, the broccoli leaves exhibited the highest amount of TPC (1940.35 ± 0.794 μg GAE/g DW) under the optimal conditions, followed by the broccoli florets (657.06 ± 0.771 μg GAE/g DW), and finally the broccoli stems (225.27 ± 0.897 μg GAE/g DW). Other authors reported similar results with different extraction methods on broccoli samples; [38] reported 317 μg GAE/mL

of TPC in broccoli stems using Microwave hydro diffusion and gravity assisted extraction. [22] presented a TPC of 5.4 mg GAE/g DW, for a mixture of broccoli stems and leaves. [14] reported a TPC of 4.14 mg GAE/g DW for broccoli leaves, 2.51 mg GAE/g DW for broccoli florets, and 1.41 mg GAE/g DW for broccoli stems, using solid liquid extraction. [23] presented a TPC of 1310 mg GAE/100 g DW for broccoli leaves, 215.6 mg GAE/100 g DW for broccoli stems, and 528.9 mg GAE/100 g DW for broccoli florets, using maceration.

Some authors reported higher amounts of TPC in the broccoli extracts, compared with the results of this study, this can be explained since the conditions of the extraction vary in several factors, such as the type of extraction (the present study focuses on MAE, compared with convention methods, such as solid-liquid extraction and maceration), the solvent concentration (this experiment used aqueous methanol in different concentrations, compared with the use of absolute methanol in [14] experiments), time (the conventional extraction methods require more extraction time compared with green extraction such as MAE), and temperature. At higher solvent concentrations the TPC increases [1, 20]; However, the purpose of MAE is to reduce the use of solvent in less time to obtain bioactive compounds from the plant samples.

Although the values mentioned vary depending on experimental conditions and extraction methods, in general the broccoli leaves exhibited higher amount of TPC, followed by the florets and stems. It has been observed that in general the peels and outer parts of fruits and vegetables (in this case the leaves), present a higher amount of polyphenol content, since this part of the plants are exposed to an aggressive or stressful environment, secondary metabolism is induced, resulting in increased phenolic compound production [39]. Moreover, MAE has demonstrated to be a good extraction method to obtain phenolics in less time, and at lower temperatures compared to the other extraction methods, such as maceration, Soxhlet extraction, and ultrasound-assisted extraction [3, 33, 40].

4.2 Antioxidant Activity (AA) Evaluation and Phenolic Acids Identification of the Validated Broccoli Extracts

4.2.1 DPPH and ABTS Radical Scavenging Methods

Any compound that delays or inhibits oxidative damage to a target molecule is considered as an antioxidant. Antioxidant molecules such as phenolic acids scavenge free radicals, inhibiting the oxidative pathways that contribute to degenerative diseases [41]. In the case of the DPPH assay, the DPPH radical is reduced in the presence of antioxidants, which causes the solution to fade. The methanolic solutions acquire a violet color characterized at 517 nm [33, 42]. On the other hand, the ABTS test compares antioxidants to a Trolox standard in terms of their capacity to scavenge the ABTS produced in aqueous phase [43].

Among DPPH and ABTS analyses (see Table 4), the broccoli leaves extract had the highest AA (632.057 ± 0.087 DPPH μg TE/g DW; 1034.220 ± 0.324 ABTS μg TE/g DW) and TPC followed by florets (290.973 ± 0.669 DPPH μg TE/g DW; 452.169 ± 0.093 ABTS μg TE/g DW), and stems (193.110 ± 0.415 DPPH μg TE/g DW; 212.118 ± 0.213 ABTS μg TE/g DW). The activities of the broccoli by-products in terms of DPPH and ABTS radical scavenging were considerably different. However, the AA was higher in the ABTS assay in all the broccoli extracts compared with the DPPH assay. It is demonstrated that the DPPH decolorizing process was not promoted by the components of the

extracts, and so had limited activity, compared with ABTS assay, which agrees with the established method by [44].

According to [45] the outer regions of most fruits and vegetables exhibit higher AA, since antioxidants play a protective role in them. In this study the TPC was significantly correlated with the AA with the DPHH and ABTS assays. The phenolic content is directly related to the AA [27], so it is expected to have a higher AA in the broccoli leaves compared to the florets and stems. Broccoli extracts using MAE showed a direct correlation between the TPC and AA responses, the higher the TPC, the higher the AA; moreover, it is reported that MAE exhibits higher AA compared to the other extraction methods, such as ultrasound assisted extraction, and Soxhlet extraction [33]. However, in the literature there are contrasting statements about positive or negative correlations between the TPC and AA [23, 44]. This could be explained by the different kinetic profiles of phenolic compounds against the DPPH and ABTS radicals, such as, the need for a longer reaction time, other unspecified reactions between the phenolic compounds, as well as other radical reagent parameters like pH, temperature, and solvent choice [33, 44].

A study made by [24] showed an AA of 77.84 mg TE/g DW, for a mix of broccoli leaves and stems, and 51.06 mg TE/g DW for broccoli florets. Another study by [46] showed an AA of 67.32 mg TE/g DW for broccoli florets. [23] studied different broccoli cultivars and found that the AA of the leaves was the highest and that of the stems was the lowest. The mentioned studies exhibited the same behavior of AA as this study since leaves presented the highest AA. All the validated broccoli samples presented antioxidant activity; the antioxidant potential of the broccoli extracts might be attributed to the vegetable natural antioxidants [19, 23].

Broccoli by-products (leaves and stems) have a similar profile to their edible counterparts (florets); the experiments not only revealed that they include phenolics, but they also exhibited higher AA (leaves), which increase their chances of being employed for extraction of bioactive chemicals, particularly those linked to key health benefits. [32] stated that samples extracted with acidic methanol at 70 °C had greater AA, which corresponded to those with higher polyphenol concentration, which agrees with the results of this experiment, since the optimal conditions for MAE extraction exhibited that at 80% v/v methanol concentration, 74.45 °C (stems), 73.27 °C (leaves), and 75 °C (florets) the TPC and AA were the highest.

4.2.2 Phenolic Acids Identification

Vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids were identified in the validated broccoli extracts of leaves, stems, and florets (see Table 5). [24] also identified neochlorogenic acid (7.2 mg/g DW), chlorogenic acid (0.2 mg/g DW), and caffeic acid (trace level) in a mix of broccoli leaves and stems extract. Another study in broccoli by-products: leaves and stems, [32] showed that the predominant phenolics in the broccoli extracts were neochlorogenic acid (124.7 μg/g DW), chlorogenic acid (128.0 μg/g DW), sinapic acid (80.8 μg/g DW), and ferulic acid (88.2 μg/g DW). [19] found chlorogenic acid (112.44 mg/g DW), and sinapic acid (9.85 mg/g DW) in different cultivars of broccoli leaves, and chlorogenic acid (8.63 mg/g DW) in broccoli stems.

[47] studied dried broccoli florets ethanol extracts and found that gallic acid had the highest concentration (3884.59 μg/g DW) followed by chlorogenic acid (140.60 μg/g DW); other phenolic acids detected were ellagic acid, cinnamic acid, and syringic acid. [48] studied fresh broccoli florets

and found that the predominant phenolic acids in the samples were gallic acid (1.80 mg/100 g Fresh Weight (FW)), chlorogenic acid (1.38 mg/100 g FW), and sinapic acid (1.25 mg/100 g FW). The phenolic acid concentrations vary due to several factors, such as the extraction conditions, types of extraction, genetic, agronomic, and environmental factors, that enhance the final concentration [49].

4.3 Comparison between MAE and Maceration of the Broccoli Extracts

In both extraction techniques (MAE and maceration), the broccoli leaves exhibited the highest amount of TPC followed by the florets, and stems (see Table 6). The extraction conditions for obtaining the greatest amount of TPC in maceration were the same for all the broccoli samples: 80% (v/v) methanol concentration, 24 h, and room temperature; under these conditions, the broccoli leaves exhibited 1714.011 ± 1.223 µg GAE/g DW of TPC, followed by florets 347.228 ± 0.956 µg GAE/g DW, and stems 224.174 ± 0.922 µg GAE/g DW.

The solvent concentration is similar to the optimal conditions found in MAE, which range from 79.54 to 80% (v/v) of methanol. The highest amounts of TPC were found in the highest solvents concentrations, which agrees with other studies [20]. However, in MAE the time varies between 10 to 18.9 min, against the 24 h maceration extraction, remarking the time efficiency of the MAE process. Temperature is another important factor in the extractions, in the optimized MAE temperatures between 73.26 and 75 °C were employed, which was at room temperature in maceration; due to the long period of extraction in maceration, it is not recommended to employ high temperatures, since prolonged exposure to high temperatures reduces the phenolic extraction yield due to the breakdown of the chemical active structures of phenolic compounds [50].

Overall, there is a significant increase in the total phenolic yield using MAE as a method to extract phenolics, compared to the maceration extraction, other authors reported the same behavior comparing both techniques [51-54]. MAE was found to increase the phenolic yield up to 45.70% for broccoli leaves, 133.57% for broccoli florets, and 65.30% for broccoli stems. Moreover, higher TPC was obtained by the MAE method compared to the maceration approach, further confirming its high efficiency. Similar results were also reported in comparing MAE with conventional extraction techniques in extracting polyphenols from other plant samples [51, 54, 55]. Among other advantages of MAE, some studies have demonstrated that MAE exhibit better quality extracts, high selectivity, and cost-effectiveness in comparison with maceration extraction [3, 40].

5. Conclusions

The effect of three variables: methanol concentration, temperature, and time, in MAE to extract phenolic acids from Broccoli stems, leaves, and florets, was evaluated and then optimized through RSA methodology, with CCRD as an upgrading technique to maximize the TPC. A second-order polynomial regression model with high reliability was obtained for the three broccoli samples, the optimal extraction conditions were: 74.54% (methanol concentration), 15.9 min, and 74.45 °C for broccoli stems; 80% (methanol concentration), 10 min, and 73.27 °C for broccoli leaves; and 80% (methanol concentration), 18.9 min, and 75 °C for broccoli florets. The TPC values obtained under the optimal MAE conditions were: 225.273 ± 0.897 µg GAE/g DW, 1940.35 ± 0.794 µg GAE/g DW, and 657.062 ± 0.771 µg GAE/g DW, for the broccoli stems, leaves, and florets respectively. The results showed that the broccoli by-products (leaves and stems) contain significant amounts of

phenolic compounds. The broccoli leaves not only exhibited higher amounts of phenolic content compared to the florets, but also higher antioxidant activity: 632.057 ± 0.087 DPPH μg TE/g DW; 1034.220 ± 0.324 ABTS μg TE/g DW (broccoli leaves), 290.973 ± 0.669 DPPH μg TE/g DW; 452.169 ± 0.093 ABTS μg TE/g DW (broccoli florets), and 193.110 ± 0.415 DPPH μg TE/g DW; 212.118 ± 0.213 ABTS μg TE/g DW (broccoli stems).Therefore, the broccoli by-products can constitute a viable solution for repurposing and valorizing broccoli wastes. Moreover, MAE remarkably increased the TPC, and the phenolic yield values of the broccoli extracts compared to the maceration extraction in a shorter period. MAE proved to be an efficient green extraction technique to obtain phenolics. Furthermore, several phenolic acids were identified in the broccoli by-products, with HPLC method, such as vanillic, sinapic, caffeic, chlorogenic, ferulic, gallic, neochlorogenic, and p-coumaric acids. Both the food and cosmetic industries are increasingly interested in using phenolic extracts as antioxidants, and broccoli by-products might be one of them. However, further extensive investigations of prospective industrial uses, as well as economic considerations, should be done in the future for the industrial application of broccoli by-product extracts.

Acknowledgments

All the authors thank McGill University, the National Council for Science and Technology (CONACyT), and the Institute of Financing and Information for Education (EDUCAFIN) for supporting the research.

Author Contributions

Sheila Lucía Rodríguez García were responsible for the study design, statistical analysis, experimental design, testing, data collection, and manuscript preparation. All authors assisted with manuscript editing.

Funding

Funding for this work was provided by the National Council for Science and Technology (CONACyT), and the Institute of Financing and Information for Education (EDUCAFIN).

Competing Interests

No potential conflict of interest is reported by the authors.

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