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Seeking Optimal Extraction Method for Augmenting *Hibiscus sabdariffa* Bioactive Compounds and Antioxidant Activity

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Abstract: The dried flowers of *Hibiscus sabdariffa* (HS), available worldwide, have various applications in both non-medicinal and medicinal fields. The growing global interest in the health benefits of HS is linked to its potential prevention or management of non-communicable diseases. The aim of this research was to find the optimal extraction method that ensures the maximum yield of multiple beneficial bioactive components, such as polyphenols, anthocyanins, vitamin C, β-carotene, antioxidant activity, free radical scavenging activity DPPH and ferric reducing antioxidant power (FRAP). To this end, stirring, pulsed electric field, and ultrasound-assisted extraction were evaluated, either alone or in combination. Under optimized extraction conditions, the obtained extract exhibited an elevated total polyphenol content (37.82 mg of gallic acid equivalents/g dry weight (dw)), total anthocyanin content (610.42 μg of cyanidin equivalents/g dw), total carotenoids content (921.84 μg of β-carotene equivalents/g dw), and ascorbic acid content (507.44 mg/100 g dw). Remarkably, the extracts exhibited strong antioxidant properties (487.51 μmol of ascorbic acid equivalents (AAE)/g dw and 243.42 μmol AAE/g dw as evidenced by FRAP and DPPH assays, respectively). This research advances the parameters that should be employed to produce the optimal and nutritionally enhanced HS flower extracts, that can be used in the commercial sector.

Keywords: hibiscus; extraction; ultrasonication; pulsed electric field; antioxidants; polyphenols; anthocyanins; response surface methodology; principal component analysis; partial least squares analysis

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1. Introduction

The *Hibiscus sabdariffa* (HS), a member of the Malvaceae family, is commonly found in tropical and subtropical regions, including China, Thailand, Indonesia, Egypt, Sudan, Saudi Arabia, Taiwan, Vietnam, Nigeria, and Mexico [1]. This annual plant is well-known for its production of red flowers and is recognized by various names such as roselle, Jamaica, Red Sorrel, Indian Sorrel, wonjo, and karkade [2]. In addition to its ornamental appeal, the HS is valued for its diverse bioactive components, including phenolic acids, flavonoids, anthocyanins, and organic acids [3]. These compounds contribute to its various therapeutic applications, as evidenced by its use in traditional medicine, suggesting management of type 2 diabetes [4,5], as well as beneficial effects over nephropathy [6,7], iron-deficiency anemia [8], hypertension [9], and cardiovascular diseases [10]. Furthermore, the intense red color of HS and its extracts makes it a sought-after natural dye in the cosmetic and food industries [11]. Moreover, dehydrated HS flowers are extensively utilized in the formulation of an array of consumables, encompassing beverages, tea, jellies, jams, sauces, wines, syrups, ice cream, and chutneys [12]. This versatility underscores the relevance of HS in both botanical and commercial domains.

The extracts from the HS flowers have garnered significant attention from researchers due to their potent antioxidant activity [13]. To maximize the isolation of bioactive compounds and antioxidant potential of these extracts, various studies have been undertaken, exploring various extraction parameters. For instance, water-based extractions have been conducted employing substance-to-solvent ratios ranging from 1:10 to 1:100, temperatures spanning 25 to 100 °C, and extraction durations ranging from 5 to 600 min [14–16]. Additionally, extractions utilizing methanol at concentrations of 100% and 80% have been performed at both elevated (64 °C) and ambient (25 °C) temperatures, respectively [17,18]. Notably, alongside conventional extraction with stirring, environmentally sustainable extraction approaches have been investigated, including microwave-assisted extraction, employing 70% ethanol as solvent, with a substance-to-solvent ratio of 1:10, and an extraction duration of 2 min [19]. However, a comprehensive extraction method encompassing all critical factors (temperature, solvent, and extraction time) for the preparation of HS extracts with maximal content in bioactive compounds and antioxidant capacity, remains elusive.

A modern research area is the development of green extraction methods, which aim at improving the performance and environmental sustainability of extracting natural products. Green extraction methods are characterized by low energy consumption, high selectivity and efficiency, minimal use of harmful solvents, and safe and high-quality outcomes [20]. Advantages include minimized solvent consumption, absence of hazardous substances, shortened extraction durations, and low energy consumption [21–23]. Some of the most widely used green extraction techniques include pulsed electric field (PEF) extraction [24], ultrasound-assisted extraction (UAE) [25], pressurized liquid extraction (PLE) [26], microwave-assisted extraction (MAE) [27], supercritical fluid extraction (SFE) [28], and enzyme-assisted extraction (EAE) [29]. These techniques have been successfully applied to various aromatic and medicinal plants for various purposes. Examples include the use of MAE for extracting mint (*Mentha piperita* L.) [30], PLE for extracting two species of eucalyptus (*Eucalyptus marginata* and *Eucalyptus pauciflora*) [31], and the extraction of *Clitoria ternatea* through the use of UAE [32].

The aim of this study was to develop an optimized procedure for the preparation of extracts from HS flowers, rich in total polyphenol content (TPC). As such, pivotal extraction parameters, such as the composition of the solvent, as well as the extraction time and temperature were studied. Moreover, alongside the conventional extraction process with stirring (ST), the use of PEF or ultrasound (US) as pretreatment steps was examined, either alone or in combination. To aid the above study, a response surface methodology (RSM) was employed. The extract obtained under the optimum conditions is expected to contain a high amount of TPC and as such, high antioxidant activity.

2. Materials and Methods

2.1. Chemicals and Reagents

The details about all the chemicals and reagents that were used to accomplish this experimental survey are presented in the Supplementary Material.

2.2. Flower Collection and Preparation

The flowers of HS were gathered in late September of 2023, from a field in the Larissa region of Thessaly, central Greece (39°32′43.0″ N 22°25′46.0″ E, as per Google Earth coordinates). The flowers were manually harvested at their mature stage. Subsequently, the flowers were washed with tap water, rinsed with deionized water, and dried using a paper towel before being subjected to lyophilization in a Biobase BK-FD10P lyophilizer (Jinan, China) for 24 h at 7 Pa and temperature around −54 °C. The lyophilized flowers were finely ground to a fine powder and sieved using an Analysette 3 PRO (Fritsch GmbH, Oberstein, Germany). Particles with an average diameter of 106 μm were obtained and used for further experiments.

2.3. Extraction Procedure

One gram of powdered HS flowers was introduced into a 25 mL glass bottle, and 20 mL of an extraction solvent was added. Table 1 presents the composition of the extraction solvent, which includes varying concentrations of ethanol—C_{EtOH} (%, *v*/*v*)—ranging from 0 to 100% *v*/*v*. The convention extraction process was stirring (ST) at 500 rpm using a magnetic stirrer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at different temperatures (T) and durations (t), as outlined in Table 1. Some samples underwent PEF and US treatments, noting four different extraction techniques, as indicated in Table 1. The coded variable level 5 is concerned only C_{EtOH}, t and T. In all cases, the sample underwent hydration by immersing the dry powder in the different C_{EtOH} solvent for a t of 10 min. The extraction steps, for means of simplicity, are depicted in Figure 1.

Table 1. The actual and coded levels of the independent variables were used to optimize the process.

Independent Variables	Code Units	Coded Variable Level				
		1	**2**	**3**	**4**	**5**
Technique	X_1	ST	PEF + ST	US + ST	PEF + US + ST	–
C_{EtOH} (%, *v*/*v*)	X_2	0	25	50	75	100
t (min)	X_3	30	60	90	120	150
T (°C)	X_4	20	35	50	65	80

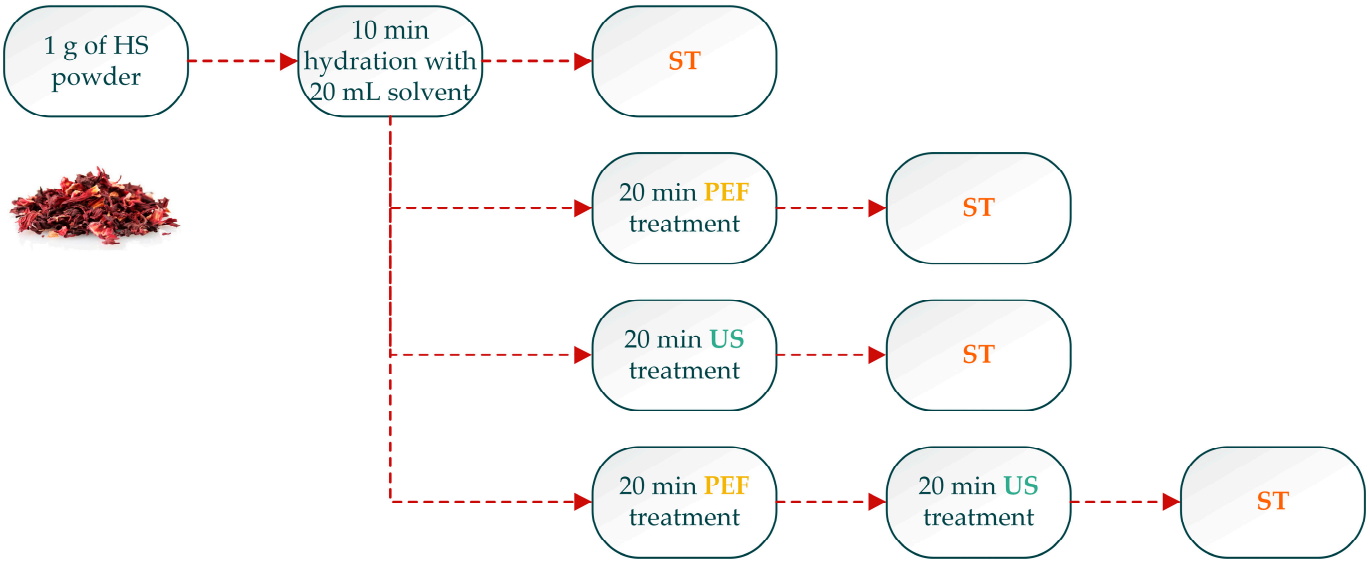

Figure 1. The extraction process of HS (*Hibiscus sabdariffa*) dry flowers using different techniques (ST: stirring, PEF: pulsed electric field, US: ultrasound).

To process the samples using PEF, two custom stainless-steel chambers (Val-Electronic, Athens, Greece), a mode/arbitrary waveform generator (UPG100, ELV Elektronik AG, Leer, Germany), a digital oscilloscope (Rigol DS1052E, Beaverton, OR, USA), and a high-voltage power generator (Leybold, LDDidactic GmbH, Huerth, Germany) were employed [33]. The electric field density was set at 1.0 kV/cm, with a pulse period of 1 ms (frequency: 1 kHz) and a pulse length of 10 µs. The US treatment was carried out by placing the Duran bottle in an Elmasonic P unit (Elma Schmidbauer GmbH, Singen, Germany), the temperature was maintained at 30 °C, and the frequency was set to 37 kHz and 180 W.

In all cases, after the extraction step was completed, the sample underwent centrifugation (Remi Elektrotechnik Ltd., Palghar, India) for 10 min at 3600× g. Subsequently, the supernatant was collected and preserved at −40 °C until further analysis.

2.4. Response Surface Methodology (RSM) Optimization of Extraction and Experiment Design

The Supplementary Material contains comprehensive details on how Response Surface Methodology (RSM) was utilized to enhance the sample's effectiveness in bioactive compounds.

2.5. HPLC-Based Analysis of the Pelargonin Chloride Content and the Other Polyphenolic Compounds

The detailed procedure is given in the Supplementary Material.

2.6. Analyses of Extracts

Detailed information on the analyses of the extracts, including TPC, TAC, antioxidant activity through the DPPH and the FRAP assays, TCC, and AAC, can be found in the Supplementary Material [34–38]. All results are expressed to the dry weight (dw) of the flower.

2.7. Statistical Analysis

The detailed presentation of the statistical analysis program used for this study is included in the Supplementary Material.

3. Results and Discussion

All the collected and studied flowers exhibited red calyces with five large petals interconnected by the corolla. The flowers had a length ranging from 5.10 to 5.15 cm, empirically and scientifically suitable for full bloom [39]. To prepare extracts from the HS flowers with a high concentration of bioactive compounds an RSM approach was applied. This method facilitated the systematic examination of various factors influencing the extraction process, including conventional extraction method (ST), green extraction methods (US and PEF), and their combinations. In addition, more extraction parameters such as extraction t, T, and C_{EtOH} (%, *v/v*) solvent mixtures were taken into consideration. In accordance with earlier investigations and preliminary experiments, the most suitable ratio of solid-to-C_{EtOH} (%, *v/v*) solvent ratio was determined to be 1:20 [34].

3.1. Extraction Optimization

Employing the RSM approach, the influence of each factor was evaluated and adjusted to attain optimal extraction efficiency. The recorded outcomes for each extract are outlined in Table 2. It can be seen that the design points 5, 15, and 20 are not particularly promising. On the contrary, design point 13 exhibited the most promising results. This signifies that the treatment with PEF and US constitutes the most optimal combination. Also, avoidance of excessively high T (<65 °C) coupled with a one-hour extraction t creates the ideal extraction conditions, yielding a highly enriched extract, as exemplified by design point 13.

Table 2. Experimental results for the four explored independent variables and the corresponding responses of the dependent variable.

Design Point	Independent Variables				Responses					
	X_1	X_2	X_3	X_4	TPC [1]	TAC [2]	FRAP [3]	DPPH [4]	TCC [5]	AAC [6]
1	3	1	3	4	25.30	341.08	295.60	164.82	710.02	381.32
2	3	2	1	3	29.62	604.39	477.68	243.42	763.65	480.75
3	2	3	4	3	21.29	543.96	226.20	119.58	921.84	441.42
4	2	4	5	4	12.27	347.47	149.64	106.69	708.51	327.26
5	3	5	4	2	3.90	135.53	82.72	66.59	256.92	267.87
6	4	1	4	5	32.33	285.56	354.95	105.94	578.57	448.03
7	4	2	3	1	34.43	465.42	358.60	108.00	690.92	401.23
8	1	3	3	2	29.42	578.07	274.41	81.21	847.53	484.04

Table 2. *Cont.*

Design Point	Independent Variables				Responses					
	X_1	X_2	X_3	X_4	TPC [1]	TAC [2]	FRAP [3]	DPPH [4]	TCC [5]	AAC [6]
9	1	4	4	1	23.38	398.22	301.91	82.18	689.48	386.66
10	1	5	1	4	12.75	162.97	216.52	32.14	231.75	303.81
11	1	1	2	3	32.26	468.95	386.91	119.12	545.52	461.45
12	1	2	5	5	35.97	430.21	487.51	123.12	594.45	487.20
13	4	3	2	4	37.82	539.70	467.69	135.80	768.40	507.19
14	3	4	2	5	24.51	479.91	472.39	109.09	450.20	507.44
15	2	5	3	5	6.60	80.51	100.24	25.01	215.26	239.33
16	2	1	1	1	14.78	444.93	155.15	102.84	428.31	351.04
17	2	2	2	2	23.50	610.42	269.15	137.85	769.89	480.28
18	3	3	5	1	22.39	599.57	474.49	125.81	750.33	481.11
19	4	4	1	2	25.11	361.03	265.08	76.82	427.64	393.68
20	4	5	5	3	8.66	145.46	111.72	1.44	176.06	298.24

[1] Total polyphenol content (TPC) in mg gallic acid equivalents (GAE)/g dw; [2] Total anthocyanin content (TAC) in µg cyanidin equivalents (CyE)/g dw; [3] Ferric reducing antioxidant power (FRAP) in µmol ascorbic acid equivalents (AAE)/g dw; [4] 2,2-Diphenyl-1-picrylhydrazyl (DPPH) in µmol AAE/g dw; [5] Total carotenoid content (TCC) in µg β-carotene equivalents (CtE)/g dw; [6] Ascorbic acid content (AAC) in mg/100 g dw.

Figure 2 depicts an HPLC-DAD chromatogram at 280 nm and 320 nm for an HS extract. As can be seen, various polyphenolic compounds such as 3-hydroxytyrosol, pelargonin chloride, rutin, and hesperidin were identified at 280 nm [40–43] as well as chlorogenic acid and luteolin at 320 nm [44].

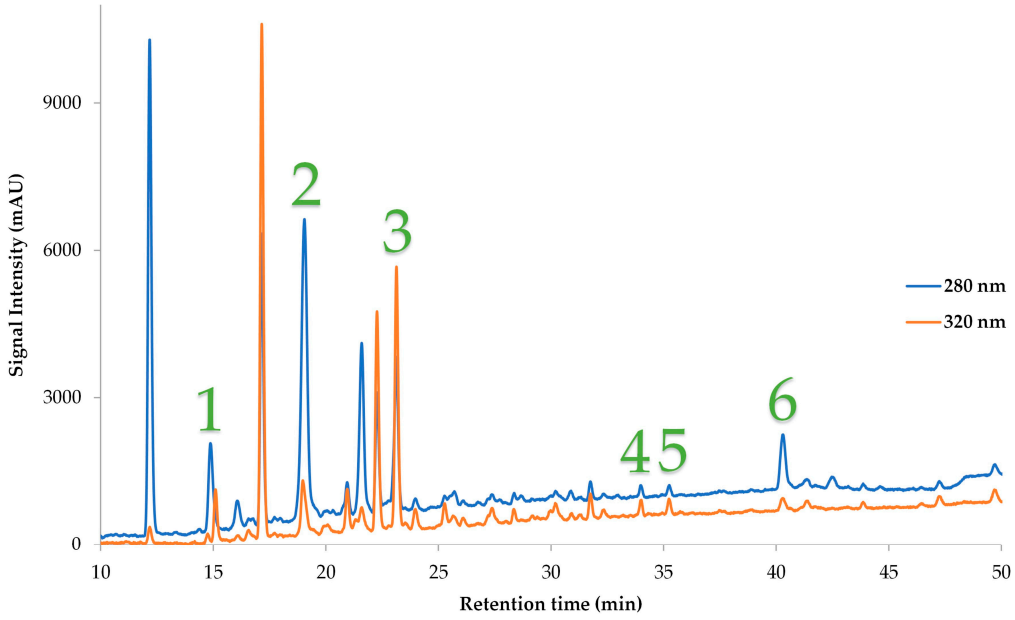

Figure 2. Representative HPLC chromatogram at 280 nm and 320 nm of an HS extract showcasing the identified polyphenolic compounds. 1: 3-Hydroxytyrosol; 2: Pelargonin chloride; 3: Chlorogenic acid; 4: Rutin; 5: Luteolin-7-glucoside; 6: Hesperidin.

Optimizing extraction parameters was important, as it resulted in achieving the highest extraction efficiency while minimizing the utilization of energy, *t*, and solvents. Enhancing these parameters is crucial for minimizing the environmental footprint of the entire extraction process [45]. C_{EtOH} selection significantly affects compound extraction [46]. For example, bioactive compounds characterized by medium polarity, such as the polyphenols abundantly present in the present samples, are not efficiently extracted with water owing to their lower polarity [47]. As a result, ethanol has been extensively utilized to enhance

extraction efficiency [48]. This outcome was further corroborated in the present study, as regarding the solvent parameter, the presence of ethanol was deemed indispensable.

Table 3 presents the statistical parameters, second-order polynomial equations (models), and coefficients (with values exceeding 0.96) for each model. These findings indicate a strong alignment between the developed models and the data. Accompanying this, Figures S1–S6 illustrate plots comparing the actual response against the predicted response for each examined parameter, along with desirability functions. For a comprehensive understanding of the TPC, three-dimensional response plots are presented in Figure 3, while corresponding three-dimensional response plots for the remaining parameters can be found in Figures S7–S11.

According to the Pareto plots, the positive or negative influence of each extraction factor on the isolation efficiency of the respective bioactive element is presented in Figure 4. The prominently negative correlation observed in the C_{EtOH} (%, *v*/*v*) across all bioactivities (TPC, TAC, TCC, and AAC), as well as antioxidant capacity, regardless of the determination method, is intriguing. This implies that neither 100% water nor 100% ethanol as solvents assist in achieving maximal values of the aforementioned bioactivities, confirming results presented in Table 2, where the highest bioactivity values were achieved with ethanol–water mixtures. In contrast, the extraction method appears to positively influence both the isolation of TPC and TAC, suggesting a potential association between these methods. However, this result conflicts with the DPPH antioxidant method, which is negatively affected by the extraction method factor. Notably, a positive correlation is observed with the extraction *T* for the FRAP antioxidant method, whereas *T*, in combination with C_{EtOH} (%, *v*/*v*) and extraction *t*, exhibits a negative correlation for the values of DPPH and FRAP, respectively.

Table 3. Mathematical models developed through RSM were employed to optimize the extraction of HS. These models included only significant terms.

Responses	Second-Order Polynomial Equations (Models)	R^2 Predicted	R^2 Adjusted	*p*-Value	Equation
TPC [1]	$Y = 17.33 - 18.64X_1 + 15.67X_2 + 1.46X_3 + 6.2X_4 + 4.43{X_1}^2 - 3.31{X_2}^2 - 0.53{X_3}^2 + 1.0{X_4}^2 + 0.09X_1X_2 - 0.06X_1X_3 - 1.12X_1X_4 + 1.01X_2X_3 - 1.22X_2X_4 - 1.1X_3X_4$	0.9738	0.9004	0.0050	(2)
TAC [2]	$Y = 294.78 - 66.1X_1 + 466.69X_2 - 128.22X_3 + 14.9X_4 - 16.9{X_1}^2 - 93.75{X_2}^2 + 2.13{X_3}^2 + 8.85{X_4}^2 + 15.6X_1X_2 + 28.93X_1X_3 - 0.13X_1X_4 + 11.21X_2X_3 - 12.1X_2X_4 - 8.57X_3X_4$	0.9795	0.9221	0.0027	(3)
FRAP [3]	$Y = 71.14 - 120.69X_1 + 316.19X_2 - 151.96X_3 + 120.44X_4 + 26.76{X_1}^2 - 60.63{X_2}^2 + 13.76{X_3}^2 + 44.87{X_4}^2 + 8.92X_1X_2 + 15.58X_1X_3 - 31.57X_1X_4 + 32.22X_2X_3 - 40.81X_2X_4 - 41.59X_3X_4$	0.9652	0.8676	0.0097	(4)
DPPH [4]	$Y = -192.21 + 141.65X_1 + 107.58X_2 - 79.76X_3 + 107.46X_4 - 14.19{X_1}^2 - 18.06{X_2}^2 + 3.26{X_3}^2 + 3.36{X_4}^2 - 10.9X_1X_2 - 0.22X_1X_3 - 13.91X_1X_4 + 20.1X_2X_3 - 17.83X_2X_4 - 7.57X_3X_4$	0.9626	0.8580	0.0114	(5)
TCC [5]	$Y = -458.87 + 164.06X_1 + 344.69X_2 + 251.8X_3 + 215.7X_4 - 10.31{X_1}^2 - 67.16{X_2}^2 - 6.52{X_3}^2 - 55.52{X_4}^2 - 35.63X_1X_2 - 31.84X_1X_3 + 26.19X_1X_4 - 10.7X_2X_3 + 24.33X_2X_4 - 13.78X_3X_4$	0.9636	0.8618	0.0107	(6)
AAC [6]	$Y = 376.88 - 137.83X_1 + 313.01X_2 - 95.12X_3 + 4.76X_4 + 0.03{X_1}^2 - 54.98{X_2}^2 - 1.87{X_3}^2 + 18.3{X_4}^2 + 17.99X_1X_2 + 28.4X_1X_3 - 2.75X_1X_4 + 5.54X_2X_3 - 22.93X_2X_4 - 4.29X_3X_4$	0.9717	0.8925	0.0059	(7)

[1] TPC: Total polyphenol content; [2] TAC: Total anthocyanin content; [3] FRAP: Ferric reducing antioxidant power; [4] DPPH: 2,2-Diphenyl-1-picrylhydrazyl; [5] TCC: Total carotenoid content; [6] AAC: Ascorbic acid content.

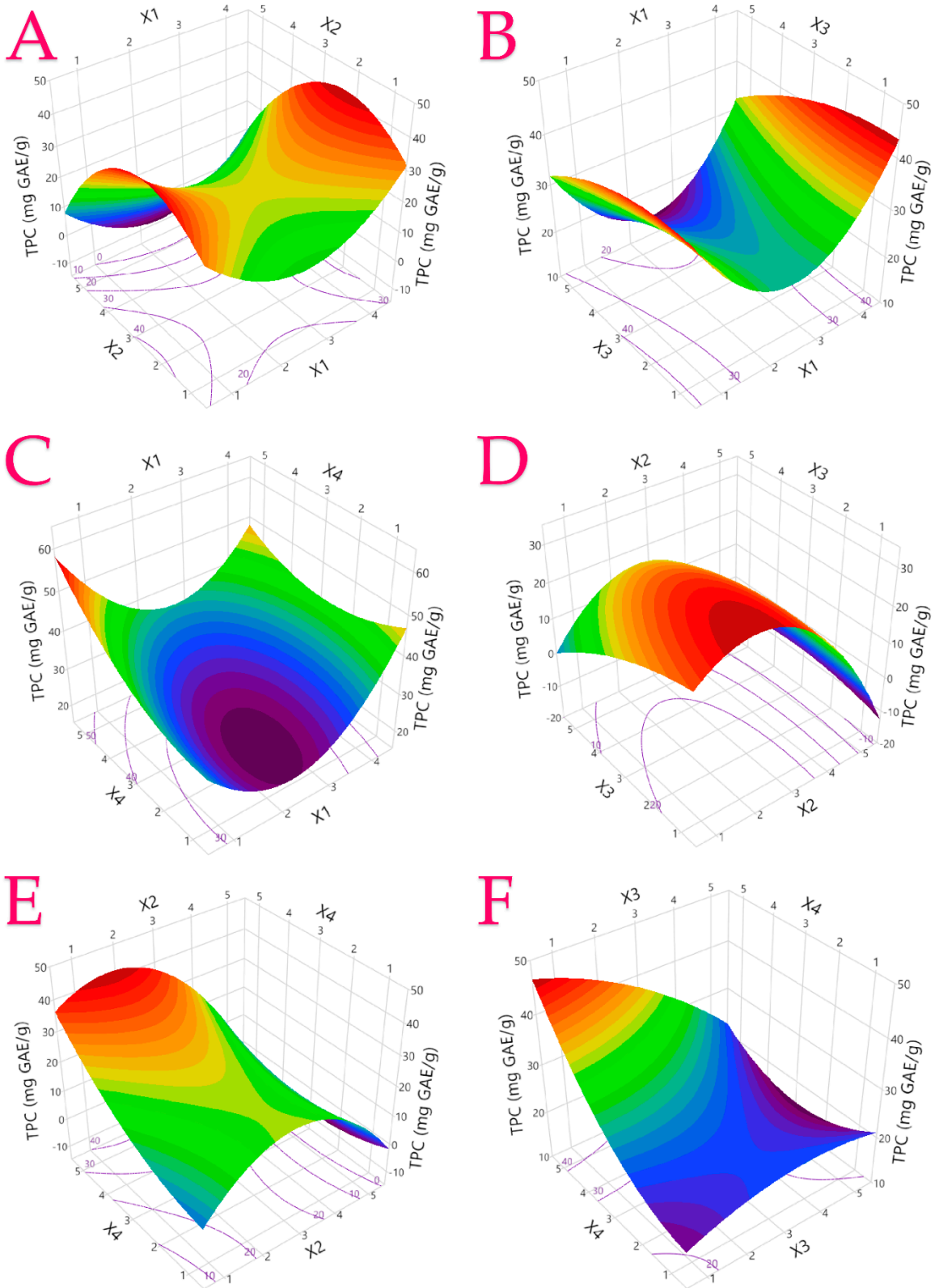

Figure 3. The optimized extraction of HS extracts is depicted in 3D graphs illustrating the influence of the process variables on the response (Total polyphenol content—TPC, mg GAE/g). Plot (**A**) shows the interaction between X_1 and X_2; plot (**B**) demonstrates the interaction between X_1 and X_3; plot (**C**) illustrates the interaction between X_1 and X_4; plot (**D**) showcases the interaction between X_2 and X_3; plot (**E**) displays the interaction between X_2 and X_4; plot (**F**) represents the interaction between X_3 and X_4.

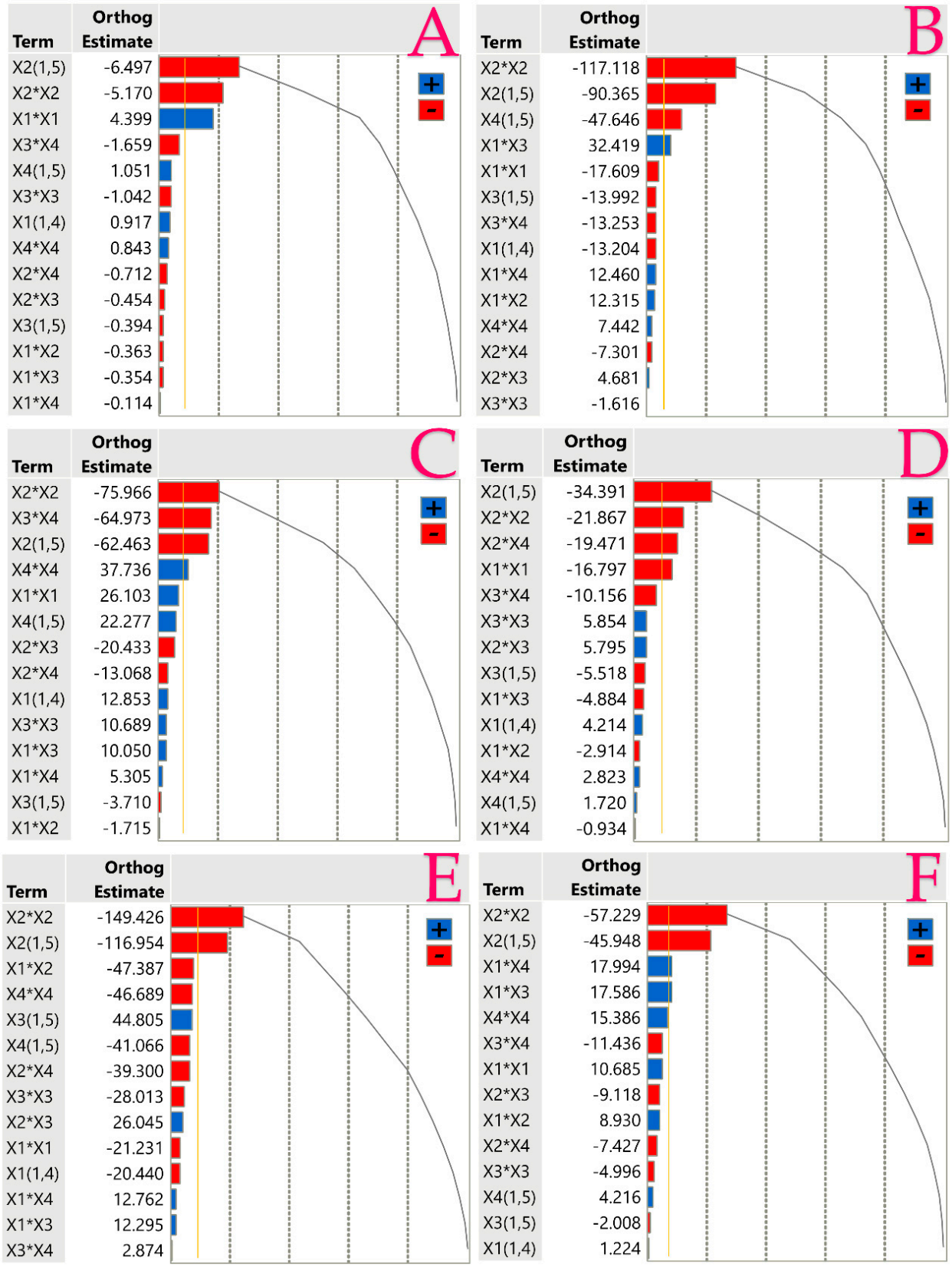

Figure 4. Pareto plots of transformed estimates for TPC (**A**), TAC (**B**), FRAP (**C**), DPPH (**D**), TCC (**E**), and AAC (**F**) assays. A reference gold line is drawn on the plot to indicate the level of significance ($p < 0.05$).

3.2. Analysis of the Extracts

3.2.1. TPC and TAC of the Extracts

The adopted extraction techniques and parameters notably affected the TPC and TAC in the extracts, as outlined in Table 2. The TPC ranged from 3.90 mg gallic acid equivalents (GAE)/g dw to 37.82 mg GAE/g dw, thus the quantity could increase by up to 869.83%. The highest extraction yields of phenolics were attained through the utilization of a 25% C_{EtOH} coupled with a short extraction *t*, as outlined in Table 4. Notably, the increase in *T*, along with the integration of both the green extraction processings prior to stirring, results in a further enhancement of the TPC content. Comparing the results with recent studies that employed conventional extraction methods, either with boiling water or ethanol solutions at 23 °C, notable differences were observed. Specifically, in the former case, a value of 14.24 mg GAE/g dw of the flower was recorded [49], while in the latter, using a 50% ethanolic solution, the value was 35.71 mg GAE/g dw. However, when employing a 75% ethanolic solution, the value decreased to 35.30 mg GAE/g dw [50]. By comparing all the results, it is once again evident that ethanol is indeed a suitable solvent for extracting bioactive compounds of HS, and complementary techniques preceding conventional extraction can increase the TPC to very significant levels.

Table 4. Maximum predicted responses and optimum extraction conditions for the dependent variables.

Responses	Optimal Conditions				
	Maximum Predicted Response	**Technique (X_1)**	***C* (%, *v*/*v*) (X_2)**	***t* (min) (X_3)**	***T* (°C) (X_4)**
TPC (mg GAE/g dw)	41.17 ± 7.73	PEF + US + ST (4)	25 (2)	60 (2)	65 (4)
TAC (μg CyE/g dw)	655.67 ± 113.56	US + ST (3)	50 (3)	30 (1)	65 (4)
FRAP (μmol AAE/g dw)	603.11 ± 121.21	US + ST (3)	50 (3)	30 (1)	65 (4)
DPPH (μmol AAE/g dw)	243.42 ± 46.76	US + ST (3)	25 (2)	30 (1)	50 (3)
TCC (μg CtE/g dw)	944.36 ± 194.3	ST (1)	50 (3)	120 (4)	50 (3)
AAC (mg/100 g dw)	561.11 ± 67.68	US + ST (3)	50 (3)	30 (1)	65 (4)

Concerning TAC, the quantities exhibited a range of values from 80.51 to 610.42 μg cyanidin equivalents (CyE)/g dw, contingent upon the employed extraction method. It was demonstrated that the optimum C_{EtOH} (%, *v*/*v*) was a 50% solution, with a minimal extraction *t*. The inclusion of US prior to the conventional stirring was also deemed imperative. In a previous investigation examining the enhancement of TAC levels in HS flowers through green extraction techniques such as maceration and ultrasonic turbolization, acidified water emerged as the preferred solvent. Maximum quantities for each method were documented as 96.30 and 87.30 μg CyE/g dw, respectively [51]. These recorded values were notably 533.87% and 599.22% lower compared to the levels achievable by following the recommended extraction parameters for obtaining an anthocyanin-rich extract from HS. Moreover, in a previous study where an ethanolic extract of HS flowers was studied, the amount of TAC was recorded at 359.3 mg CyE/100 g dw (i.e., 3593 μg CyE/g dw) [52]. Despite the impressive recorded amount, the content could be enhanced by 41.14% if the appropriate extraction conditions were applied.

As indicated in Table 4, the predicted values obtained by Partial Least Squares (PLS) for the optimal extraction stand at 41.17 mg GAE/g dw for TPC and 655.67 μg CyE/g dw for TAC. Nevertheless, considering the maximum values outlined in Table 5, the predicted responses, following the same extraction method and parameters for the preparation of a single optimal extract, contained 36.56 mg GAE/g dw for TPC and 681.96 μg CyE/g dw for TAC. These findings underscore the significance of meticulously choosing extraction techniques and C_{EtOH} (%, *v*/*v*), as they can substantially augment the efficiency and yield of bioactive compounds. This approach not only proves to be more energy-efficient but also enhances overall effectiveness in the extraction process.

Table 5. Highest desirability for all variables using the partial least squares (PLS) prediction profiler under the optimal extraction conditions (X_1:3, X_2:2, X_3:1, X_4:5).

Variables	PLS Model Values	Experimental Values
TPC (mg GAE/g dw)	36.56	40.49 ± 2.67
TAC (μg CyE/g dw)	681.96	642.18 ± 34.68
FRAP (μmol AAE/g dw)	690.18	697.1 ± 23.7
DPPH (μmol AAE/g dw)	227.12	216.14 ± 14.91
TCC (μg CtE/g dw)	781.14	676.86 ± 41.29
AAC (mg/100 g dw)	595.96	536.05 ± 15.55

3.2.2. Polyphenolic Compounds of the Optimum Extract

Pelargonin emerged as the principal polyphenolic compound within the optimal extract, as presented in Table 6, comprising approximately 87.80% of the total polyphenolic compounds. Pelargonin is classified as an anthocyanin, commonly present in red fruits and vegetables, functioning as a natural colorant [53]. Identified mainly in dates, pelargonin has been investigated for its anti-allergic properties [54]. Moreover, it is reported to exhibit oxygen radical absorbance capacity [55]. Pelargonin chloride has been previously identified in HS flowers from a variety in Mexico in minimal quantities. More specifically, it was found in amounts of 0.00217 mg/g dw [56], which is much lower than what can be obtained by applying ideal extraction procedures. According to previous studies, the leaves of HS are rich in chlorogenic acid [57], known for their pharmacological activities, encompassing anticancer, antidiabetic, antiviral, and antipyretic properties [58,59]. Besides the leaves of the HS, it is evident (Table 6) that the flowers also contain a considerable amount of chlorogenic acid. Additionally, it is apparent that hesperidin is present in the flowers, which has neuroprotective effects observed across various models of central nervous system disorders [60]. In a preceding investigation, the calyces of HS exhibited a hesperidin content of 0.14 mg/g dw, resulting in a 600% reduced content compared to the optimal extract of HS presented herein. This means that the enriched extract, besides excelling in polyphenolic compounds, may also possess all the beneficial properties of these compounds. Figure 5 shows the chemical structures of the polyphenolic compounds that were identified in the extracts of HS.

Table 6. Polyphenolic compounds extracted under optimal conditions (X_1:3, X_2:2, X_3:1, X_4:5).

Polyphenolic Compound	Optimal Extract (mg/g dw)
3-Hydroxytyrosol	1.33 ± 0.05
Pelargonin chloride	34.63 ± 1.94
Chlorogenic acid	1.27 ± 0.09
Rutin	0.68 ± 0.03
Luteolin-7-glucoside	0.54 ± 0.03
Hesperidin	0.98 ± 0.06
Total identified	39.44 ± 2.20

3.2.3. Antioxidant Properties of the Extracts

The antioxidant properties of the HS extracts acquired in this study were assessed through two distinct methodologies, namely, FRAP and DPPH. Optimal extraction methods for both approaches necessitate ultrasonic treatment prior to agitation, a relatively elevated T ranging from 50 to 65 °C, a C_{EtOH} of up to 50%, and a short t, as depicted in Table 2.

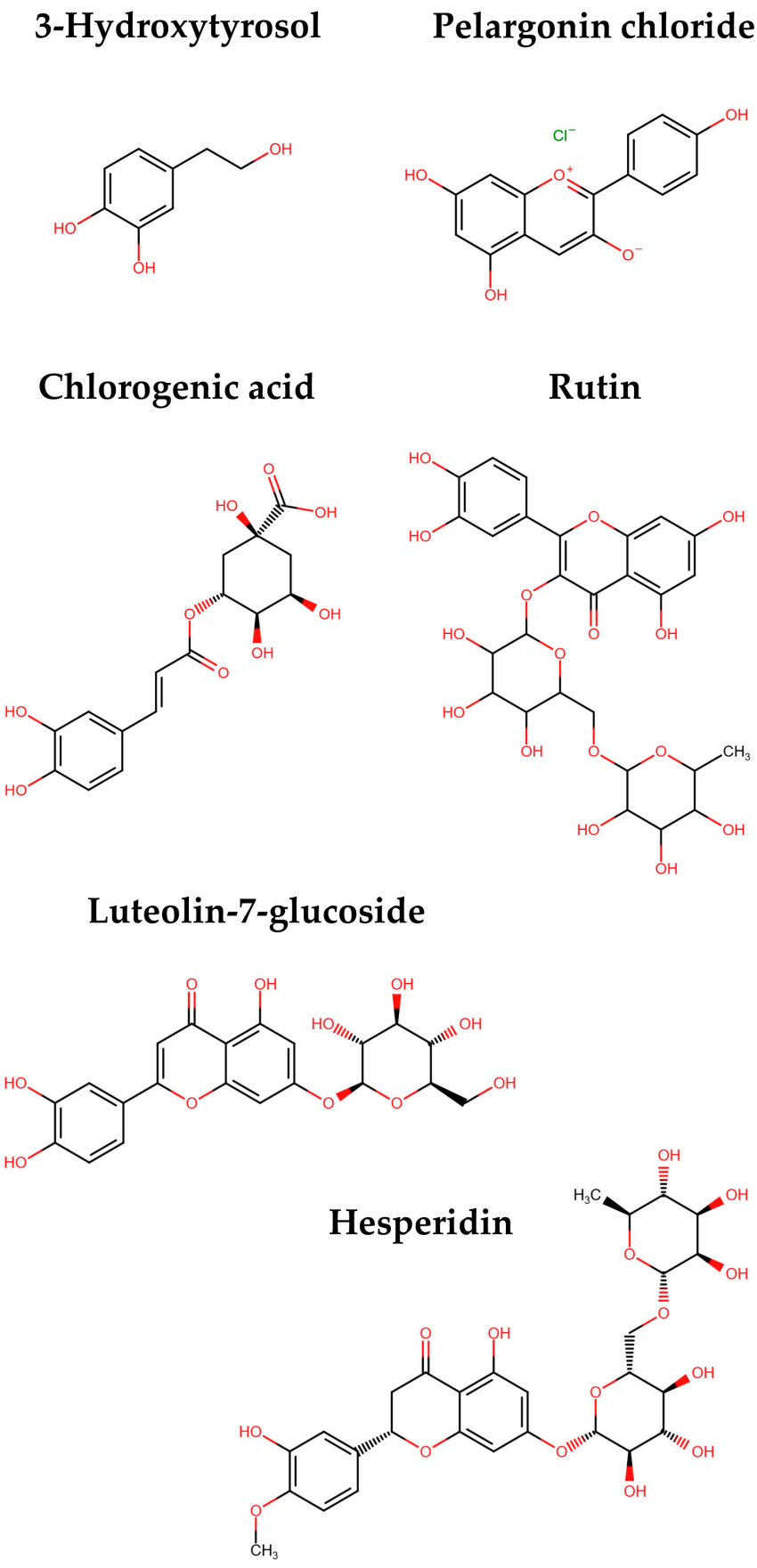

Figure 5. Chemical structures of all the polyphenolic compounds identified in HS extracts.

Regarding the antioxidant capacity via both methods, it can be seen that the samples exhibit significant variability in their capacity depending on the method employed. Specifically, the values range from 82.72 to 487.51 μmol ascorbic acid equivalents (AAE)/g dw according to Table 2, indicating that the antioxidant capacity can increase by up to 489.32%, in the FRAP method. Simultaneously, in the DPPH method the free radical scavenging capability increased by up to 16,800%. The experimental value of 216.14 ± 14.91 μmol AAE/g dw for the DPPH assay corresponds to an ascorbic acid concentration (AAC) of 10,826 ± 670 μmol/L. The IC_{50} value of the antioxidant ascorbic acid is 854.24 μmol/L, while the HS optimal extract has an IC_{50} value of 8545.32 μmol/L. The lower the IC_{50} value, the higher the antioxidant capacity. In a previous method, an attempt to optimize the antioxidant capacity of HS leaves using various solvents (methanol, 80% alcohol, hot and cold water) and drying methods (room, sun, oven, microwave, cross flow, infra-red, and freeze-dryer) was made. It was found that with 80% alcoholic solvent on freeze-dried leaves, the antioxidant capacity reached 202.93 μmol AAE/g dw for the FRAP method. Conversely, the highest total antioxidant activity was exhibited from the sun-dried leaf cold water extract at 394.39 μmol AAE/g dw with the room-dried leaf 80% alcohol extract following closely at 369.12 μmol AAE/g dw [61].

As per Table 4, the highest predicted values for each antioxidant capacity measurement method were 603.11 ± 121.21 μmol AAE/g dw for FRAP, whereas for DPPH, the corresponding value was 243.42 ± 46.76 μmol AAE/g dw, with desirability of 0.86 and 0.87, respectively, compared to the maximum value of 1. Comparing the results, it is evident that the HS extract can achieve and surpass the antioxidant capacity of the leaves using suitable methods, creating an extract with pronounced multifaceted antioxidant capability.

3.2.4. TCC and AAC of the Extracts

Carotenoids offer a plethora of health benefits and aesthetic therapeutic implications, including immune system strengthening, resilience against serious maladies such as cancer, and noteworthy anti-aging attributes [62,63]. Additionally, ascorbic acid, a natural antioxidant [64], not only serves as an antioxidant but also improves the absorption of non-heme iron [65]. A noteworthy outcome is that the extract of HS flowers is quite rich in TCC and AAC, with values ranging from 176.06 to 921.84 μg β-carotene equivalents (CtE)/g dw (a 423.59% increase) and 239.33 to 507.44 mg/100 g dw (indicating a 112.03% increase) after various processing conditions. This finding aligns with prior investigations where the abundant presence of both TCC and AAC in HS flowers has been emphasized [66,67]. In another study examining the nutrient content of HS flowers, the quantities recorded in TCC and AAC were 0.53 ± 0.27 mg/100 g dw (530 μg/100 g dw) and 141.09 mg/100 g dw, respectively. Therefore, once again, the importance of finding suitable extraction conditions for all plant extracts becomes evident [68]. Of equal significance is that, according to Table 4, achieving the maximum TCC quantity (944.36 μg CtE/g dw) requires simple stirring and a 50% ethanol concentration, while obtaining the maximum AA quantity (561.11 mg/100 g dw) necessitates pre-processing with US, the same C_{EtOH} (%, *v/v*), and minimal extraction *t*. The implementation of these extraction methodologies grants the formulation of an exceptionally enriched HS extract, characterized by heightened levels of AA and TC suitable for immediate and beneficial applications in the fields of medical science, cosmetic therapeutics, and nutritional sciences. The chemical structure of β-carotene and vitamin C are shown in Figure 6.

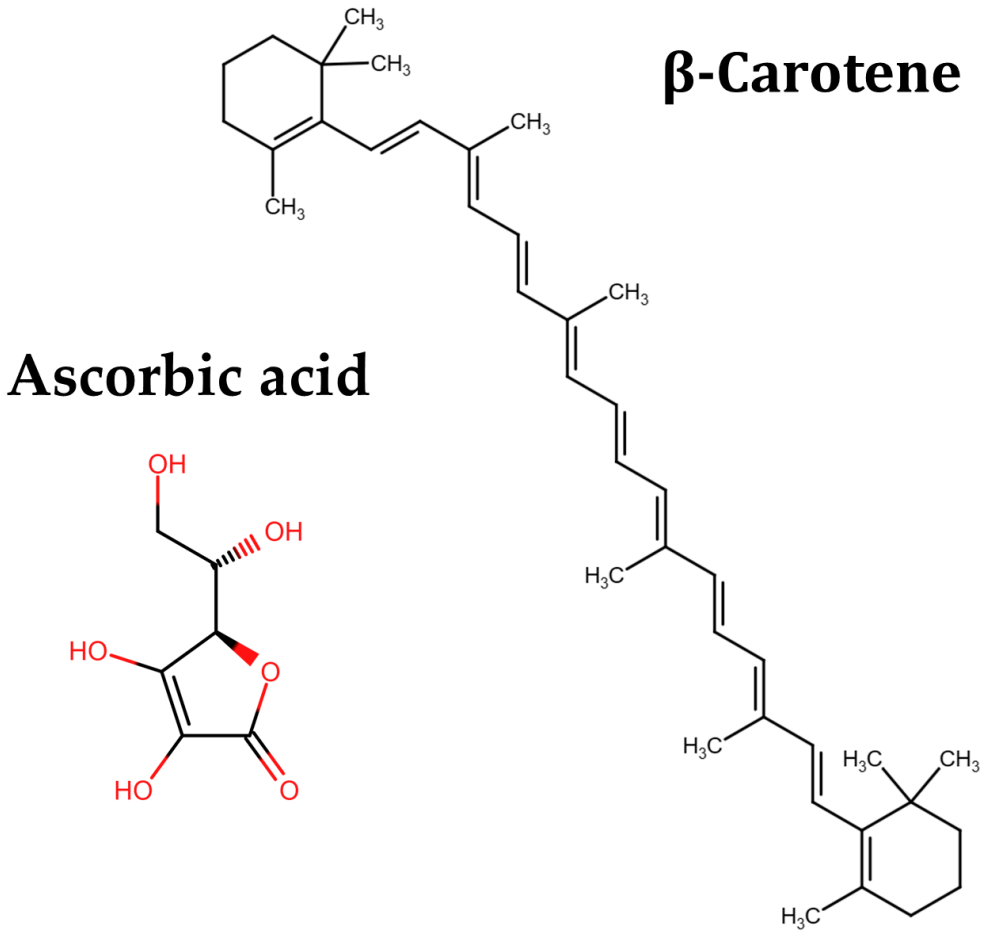

Figure 6. Chemical structure of β-carotene and ascorbic acid.

3.3. Principal Component Analysis (PCA) and Multivariate Correlation Analysis (MCA)

In Figures 7 and 8, the correlation of values among various bioactive compounds is depicted, revealing noteworthy outcomes. In Figure 7 it can be seen that component 1 explained 76.9% of the variability, presenting a positive correlation with all of the examined variables TPC, TAC, FRAP, DPPH, TCC, and AAC. Furthermore, it is discernible that among the extraction factors, the one that predominantly influences the increase in various bioactive compounds is X_2. It is extensively noted that ethanol plays a significant role in enhancing the isolation of bioactive compounds. Regarding Figure 8, it is worth noting that in numerous studies, the correlation among variables is often low or even negative. However, in the current research, not only a negative correlation is not observed, but the lowest correlation is 0.6, with a maximum value of 1, and this is observed in only one variable, that of antioxidant capacity through the scavenging of free radicals. Most bioactive substances exhibit a maximum correlation coefficient of 1. Specifically, the variables TPC, FRAP, and AAC recorded the highest correlation, demonstrating that both TP and AA exhibit strong antioxidant activity. Additionally, a robust correlation was observed between the TAC variable and both AAC and TCC. These results are highly encouraging, as it is rare to observe such a high degree of correlation in multivariate systems with multiple sensitive variables.

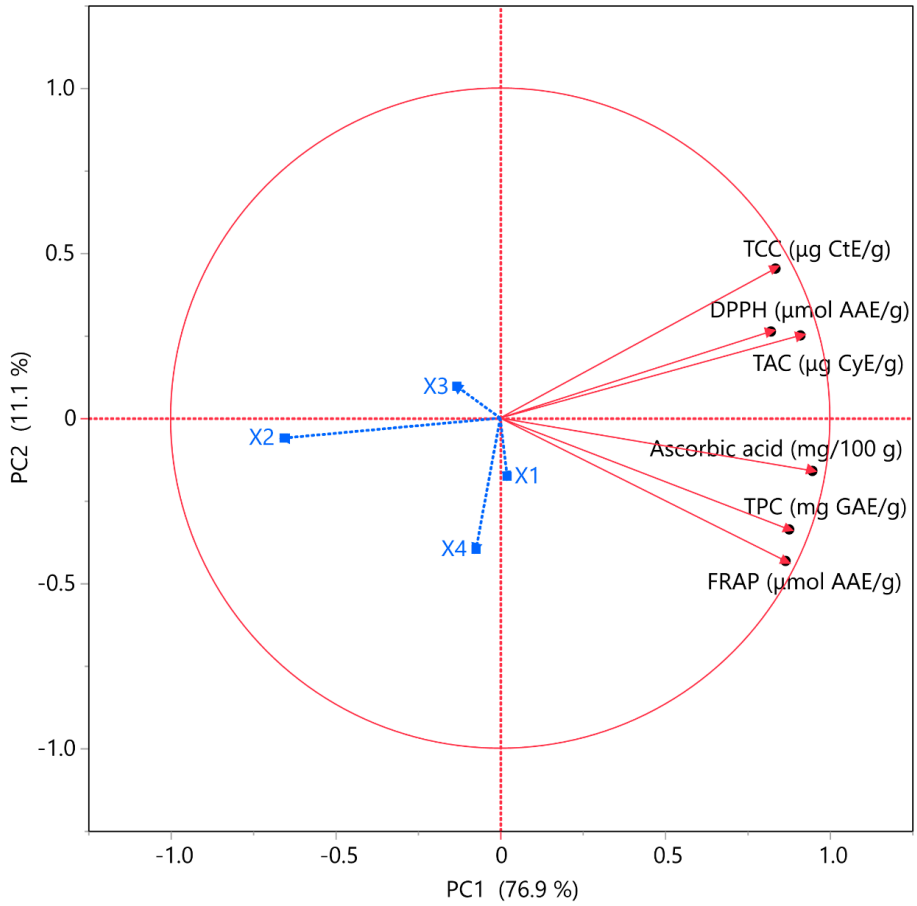

Figure 7. Principal component analysis (PCA) for the measured variables. Each *X* variable is presented with a blue color.

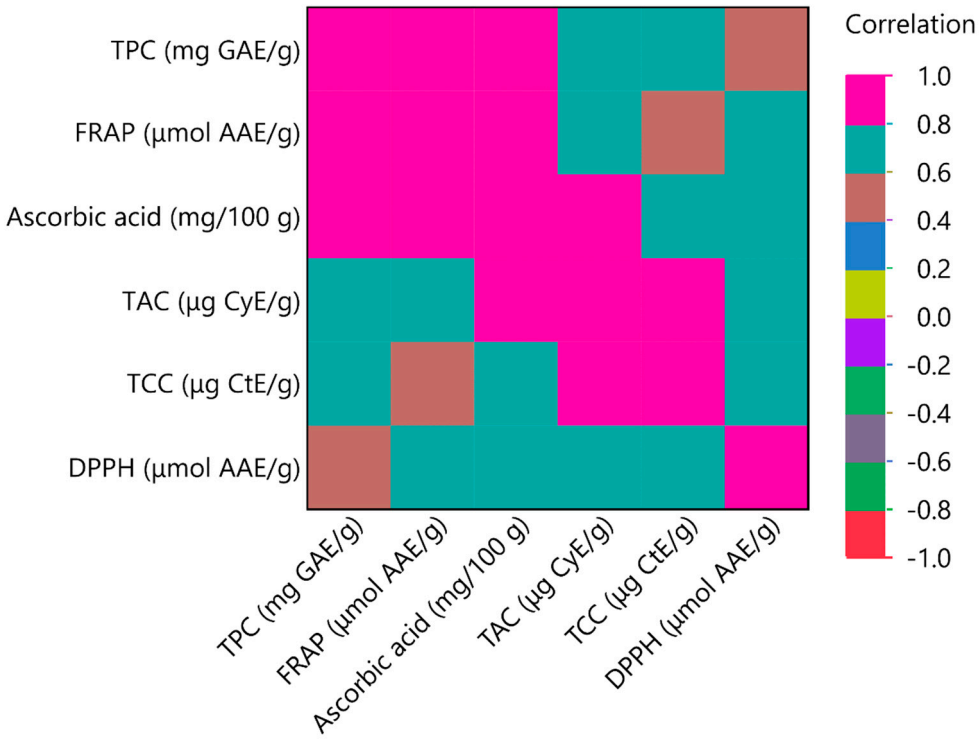

Figure 8. Multivariate correlation analysis of measured variables.

3.4. Partial Least Squares (PLS) Analysis

A PLS analysis, as depicted in Figure 9, was carried out to identify the key factors among the investigated extraction variables (X_1, X_2, X_3, and X_4). It is crucial to emphasize that PLS offers valuable insights for optimizing extraction conditions and contributes to comprehending how different factors interact to influence the yields of bioactive compounds. Figure 9 illustrates the quantities of all potentially extractable bioactive compounds by following the suggested extraction conditions based on the PLS analysis. The desirability of the results is notably high, registering a value of approximately 0.8930. The result, as indicated in Table 4, is logical and it is demonstrated that for almost all bioactive elements and antioxidant capacity, the maximum values are ensured through the application of the same parameters.

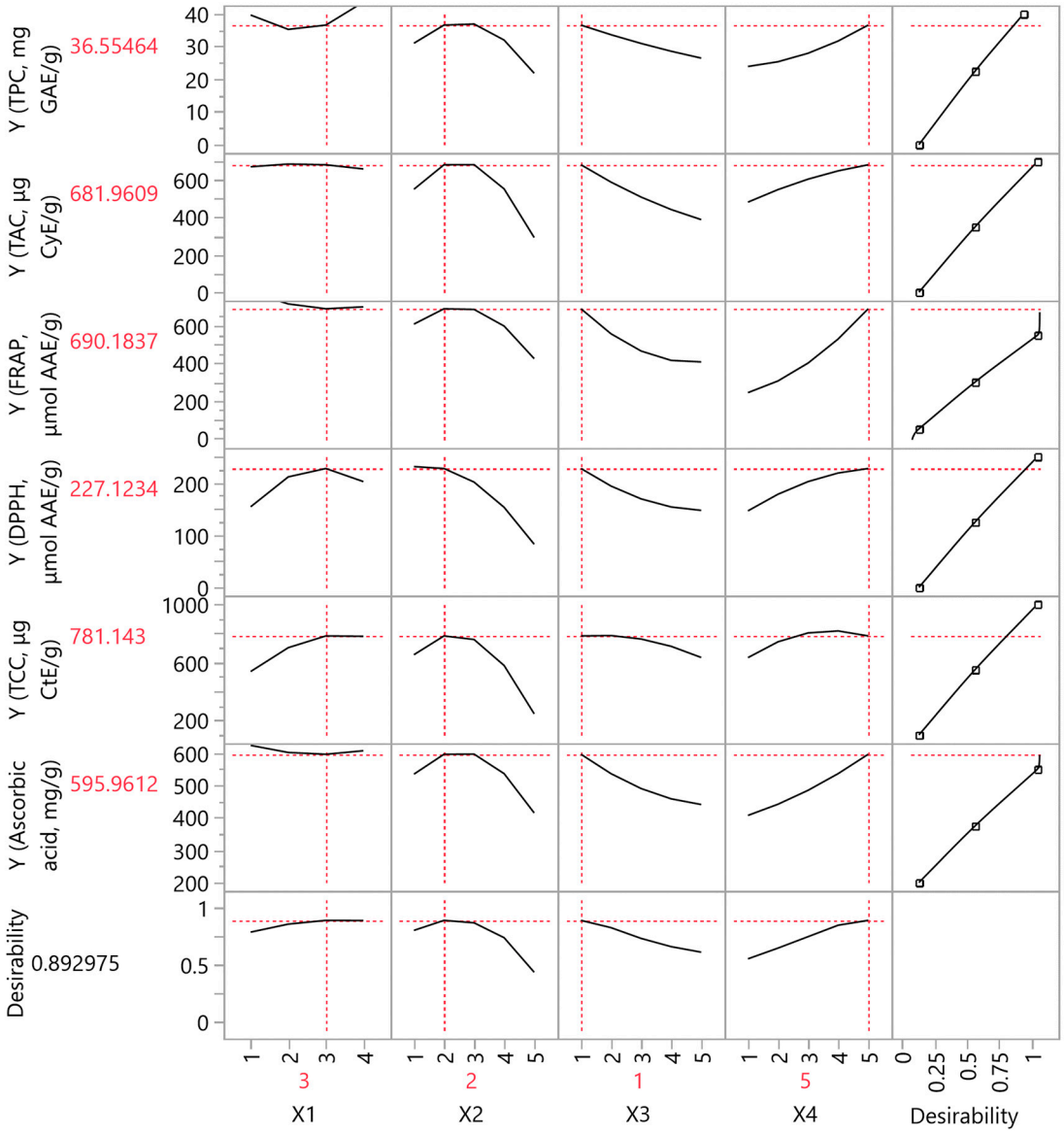

Figure 9. Partial least squares (PLS) prediction profiler of each variable and desirability function with extrapolation control for the optimization of HS extracts.

Upon comparing the values provided by the PLS model with those obtained through experimental analysis, a correlation of 0.9888 was observed, indicating no deviations, with a *p*-value of <0.0001.

4. Conclusions

The dried flowers of HS are highlighted as a versatile source with extensive applications in both the non-pharmaceutical and pharmaceutical sectors. The global interest in HS revolves around perceived health benefits, particularly in preventing or managing non-communicable diseases. This study, acknowledging the diverse bioactive compounds responsible for the myriad properties of HS, delves into three distinct extraction methods, including conventional ST and two environmentally friendly approaches, US and PEF, both individually and in combination. Through stochastic optimization, this research aims to produce the most nutrient-rich HS extract using more advanced and eco-friendly methods. Indeed, according to our results, the use of US before ST (at least for t 30 min) along with 25% C_{EtOH}, at 80 °C T was deemed the most suitable for the abundance of bioactive substances (36.56 mg GAE/g dw for TPC, 681.96 µg CyE/g dw for TAC, 781.14 µg CtE/g dw for TCC, 595.96 mg/100 g dw for AAC) and antioxidant capacity (690.18 µmol AAE/g dw and 227.12 µmol AAE/g dw for FRAP and DPPH method, respectively). Simultaneously, the application of the aforementioned extraction parameters offered a remarkable increase in TPC, TAC, TCC, and AAC reaching 837.46, 747.02, 343.67, and 149.02%, respectively. This study not only provides optimized extraction processes to maximize the overall nutritional content or individual nutrients of HS flower extracts but also underscores the diverse applications of this extract.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12030581/s1, Supplementary Material File—Materials and Methods. Figures S1–S6 comprise plots that illustrate the comparison between the actual response and the predicted response for each parameter under examination, accompanied by the desirability functions. Figures S7–S11 present three-dimensional response plots for the remaining responses.

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