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Enhancing Antioxidant Properties of *Prunus spinosa* Fruit Extracts via Extraction Optimization

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Abstract: *Prunus spinosa* fruit, commonly known as blackthorn or sloe, possesses a wide range of health-promoting properties, including antioxidant and antibacterial activities. In this study, we investigated the effects of various extraction parameters, such as temperature, time, and solvent composition, on the extraction of bioactive compounds from *P. spinosa* fruit. Response surface methodology was employed to optimize these parameters and evaluate their impact on the antioxidant properties of the extracts. Furthermore, techniques such as ultrasound (US) and pulsed electric field (PEF) were applied, both individually and in combination, to explore their potential effects on the extraction process. The optimal extraction technique proved to be the combination of PEF and US, followed by stirring. The most suitable solvent was 75% ethanol, whereas the optimum extraction time and temperature were 30 min and 80 °C, respectively. Our findings revealed that under the optimum extraction parameters, a significant improvement in the extraction efficiency of bioactive compounds from *P. spinosa* fruit was achieved. More specifically, the optimal conditions, according to partial least squares (PLS) analysis, were a combination of all three extraction modes (PEF-US-ST), the shorter extraction time of the present study (30 min), and the corresponding higher temperature (80 °C). As expected, the presence of ethanol was considered necessary, even in an amount of 25%. The total polyphenol content was found to be 30.74 mg gallic acid equivalent (GAE)/g, the total flavonoids content 3.23 mg rutin equivalents (RtE)/g and the total anthocyanins 125.2 μg cyanidin-3-*O*-glucoside equivalents (CyE)/g. HPLC-DAD analysis showed that neochlorogenic acid was the polyphenol with the highest concentration (4.13 mg GAE/g) in *P. spinosa* fruit. The antioxidant activity of the optimized, according to PLS analysis, extract was evaluated and found to be 146.09 μmol ascorbic acid equivalent (AAE)/g determined by ferric reducing antioxidant power (FRAP) assay, and by the radical scavenging activity (DPPH) assay was 18.56 μmol AAE/g. Additionally, the ascorbic acid was determined to be 119.4 mg/100 g. Overall, this study contributes valuable insights into the extraction optimization process and the potential applications of *P. spinosa* fruit in the development of functional foods and pharmaceutical products.

Keywords: *Prunus spinosa*; extraction; antioxidant activity; pulsed electric field; ultrasound-assisted extraction; response surface methodology; multivariate correlation analysis; partial least squares

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1. Introduction

Nowadays, there is growing recognition of the importance of plants, particularly wild plants, as significant sources of natural bioactive compounds in functional products. Polyphenols are a large group of naturally occurring organic compounds found in plants. They are characterized by having multiple phenolic (hydroxyl) groups in their chemical

structure. Flavonoids are one major subclass of polyphenols, consisting of compounds with two aromatic rings, connected by a three-carbon chain. Anthocyanins are a subclass of flavonoids and, by extension, a subgroup of polyphenols. Compounds belonging to the abovementioned groups/sub-groups have shown potential in the prevention and complementary therapy of various chronic diseases, including cardiovascular diseases, which are a leading cause of global fatalities [1]. Given the essential role of antioxidants in human health, efforts have been undertaken to develop food products with high antioxidant content [1,2]. Ethnobotanical knowledge serves as a valuable guide in the search for plant species that can be used to retrieve antioxidant compounds [3]. In this context, the *Prunus spinosa* fruit emerges as a promising candidate, necessitating further investigation.

Prunus spinosa L., classified under the *Prunus* genus, is a tree or shrub belonging to the Rosaceae family [4]. Commonly known as "blackthorn", "sloe", or "wild plum", it thrives in the Mediterranean, Europe, West Asia, and West Africa [5,6]. This species grows to four meters and produces little circular drupes 10–15 mm in diameter. The outer part of the fruit is bluish black and has green astringent flesh [7,8]. *P. spinosa* blooms in April–May [8]. Usually, bitter raw fruit is used to make jams, composts, and liqueurs [9]. These composts made from this fruit can raise body resistance, build blood, and relieve rheumatic pain [10]. In fact, blackthorn has a long history as a therapeutic agent [5]. This fruit contains several bioactive compounds, making it worthy of further investigation, despite its inedibility and little industrial use.

The fruit of *P. spinosa* exhibits significant antioxidant activity due to its high content of polyphenols and vitamin C [7,11]. Due to the content of antioxidants, blackthorns can reduce oxidative stress, which causes aging and tumor formation [12]. Polyphenols may prevent degeneration, cardiovascular disease, and cancer [13]. Moreover, they are rich in anthocyanins, imparting their distinctive color [14,15]. The potential anti-inflammatory properties of the fruit is intriguing for further research, as well as their antibacterial properties. Due to the rise in antibiotic resistance among pathogens, the antimicrobial properties of *P. spinosa* have gained attention, particularly in addressing microbial resistance [16–18]. Last but not least, ripe *P. spinosa* fruit has demonstrated antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella abony* NCTC 6017, and some ATCC strains of *Staphylococcus aureus* [19]. *P. spinosa* is an overlooked resource that can be used as a raw material for generating compounds in the food industry [20]. Consequently, research has focused on identifying its phenolic components and examining its biological effects [9].

Despite the nutritional properties of *P. spinosa* fruit, its in-depth exploration has been limited, possibly due to its seasonal nature and late ripening [5]. This study aims to comprehensively investigate the bioactive compounds and antioxidant activities present in the fruit of *P. spinosa*. By conducting extractions and analyses of polyphenolic and flavonoid content, as well as ascorbic acid (vitamin C), at the optimum sample, we aim to gain a deeper understanding of the nutritional properties of this fruit. Response surface methodology (RSM) was employed to optimize the extraction process, taking into account parameters such as temperature, solvent composition, and time, and to evaluate their impact on the antioxidant activity of the extracts. Additionally, the effect of US and PEF techniques as pretreatment steps to enhance the extraction yield of bioactive compounds was examined. By providing specific insights into the bioactive constituents and antioxidant potential of *P. spinosa* fruit, our research aims to contribute to a better understanding of their nutritional significance. The optimization of extraction conditions and the evaluation of innovative pretreatment methods will promote the potential applications of this fruit in various food and health-related industries.

2. Materials and Methods

2.1. Chemicals and Reagents

All information about chemicals and reagents is given in the Supplementary Materials.

2.2. Plant Collection and Preparation

Fruit of *P. spinosa* L. was collected in October of this year from wild plants found in a hilly area near Spathades, Kalambaka, central Greece, according to Google Earth version 7.3.2.5776 Latitude: 39.7082 and Longitude: 21.7135. To facilitate the analysis for this study, a collection of fruit was obtained from multiple trees located within the boundaries of the same orchard. The fruit was manually collected at the mature stage. Fruit was thoroughly washed with tap water and dried with a paper towel. The fruit flesh and peel were meticulously separated from the kernel using a knife, sliced into smaller pieces, and placed in a Biobase BK-FD10P (Jinan, China) lyophilizer. The lyophilized fruit was ground to fine powder at Analysette 3 PRO (Fritsch GmbH, Oberstein, Germany), and particles with an average diameter of 106 μm were used for further study.

2.3. Extraction Procedure

One gram of powdered fruit was placed in a 25 mL glass bottle with 20 mL of the extraction solvent. In Table 1, the composition of the extraction solvent composition (ethanol at various concentrations ranging from 0 to 100% *v/v*) is displayed. Extraction was carried out by stirring (ST) at 500 rpm in a magnetic stirrer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at various temperatures and durations of time, as illustrated in Table 1. As shown in Table 1, the samples were also subjected to US and PEF treatments. First, the sample was subjected to hydration by immersing the dry powder in the solvent for 10 min. Then, PEF treatment was applied for 20 min, followed by US treatment for another 20 min for the combined method. Finally, the samples were subjected to ST extraction, as previously stated. The procedure followed is illustrated in Figure 1. In the case of single technique pre-treatment, the same procedure was followed, while either the PEF or US treatment steps were omitted.

Table 1. Process optimization utilizing both the actual and coded levels of the independent variables.

Independent Variables	Code Units	Coded Variable Level				
		1	2	3	4	5
Technique	X_1	ST [1]	PEF [2] + ST	US [3] + ST	PEF + US + ST	–
C (%, *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

[1] ST: stirring, [2] PEF: pulsed electric field, [3] US: ultrasound.

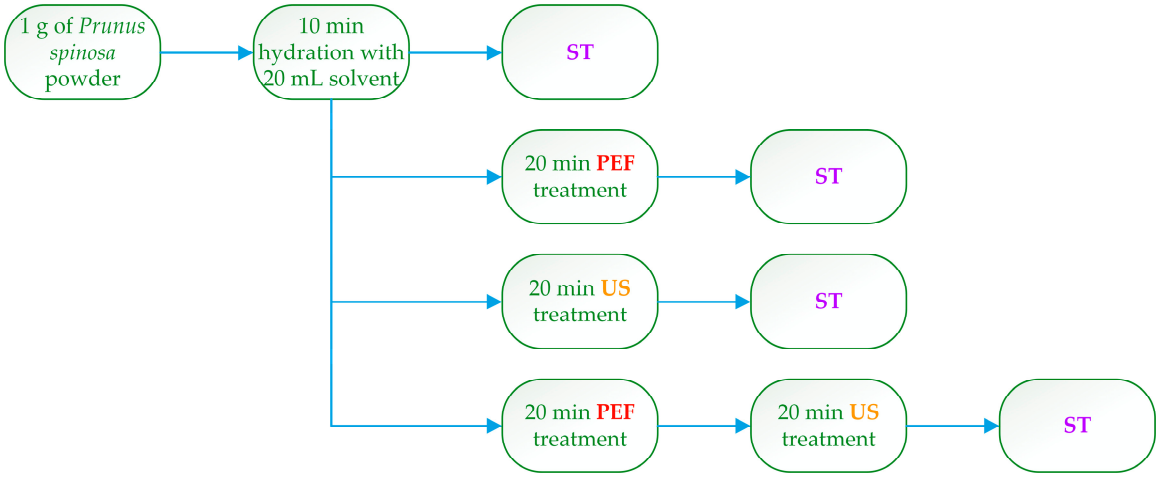

Figure 1. The extraction process of *P. spinosa* fruit powder using different techniques (ST: stirring, PEF: pulsed electric field, US: ultrasound).

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, LD Didactic GmbH, Huerth, Germany) were used for PEF processing of the samples [20,21]. The electric field density was set to 1.0 kV/cm, the pulse period was 1 ms (frequency: 1 kHz), and the pulse length was 10 µs. The temperature was kept at 30 °C in the Elmasonic P machine (Elma Schmidbauer GmbH, Singen, Germany) for US treatment, which ran at 37 kHz.

In all cases, the sample was centrifuged (Remi Elektrotechnik Ltd., Palghar, India). for 10 min at 3600 × *g* following extraction, and the supernatant was collected and stored at −40 °C until further analysis.

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

Using an RSM technique, polyphenols, primarily neochlorogenic acid, total polyphenol content (TPC), and total anthocyanins were extracted with the best possible yield. As a result, the purpose of the design was to increase neochlorogenic acid content as well as TPC, and anthocyanin pigment values. This was accomplished by adjusting the extraction process, solvent concentration (ethanol, EtOH) (*C*, % *v/v*), extraction duration (*t*, min), and extraction temperature (*T*, °C). An experiment with a main effect screening design and twenty design points served as the basis for optimization. According to the experimental design, the process variables were established in five levels. Table 1 shows the coded and actually occurring levels. Analysis of variance (ANOVA) and summary-of-fit tests were used to determine the overall model significance (R^2, *p*) and the significance of the model (equations) coefficients at a minimum level of 95%.

A second-order polynomial model, shown in the following Equation (1), was also used to predict the response variable as a function of the examined independent factors:

$$Y_k = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y_k is the predicted response variable; X_i and X_j are the independent variables; β_0, β_i, β_{ii}, and β_{ij} are the intercept, regression coefficients of the linear, quadratic, and interaction terms of the model, respectively.

2.5. HPLC-Based Determination of the Neochlorogenic Acid Content and Other Polyphenolic Compounds

A Shimadzu CBM-20A liquid chromatograph and a Shimadzu SPD-M20A diode array detector (both supplied by Shimadzu Europa GmbH, Duisburg, Germany) were used for the analysis. The compounds were separated using a Phenomenex Luna C18(2) column from Phenomenex Inc. in Torrance, CA, USA, maintained at 40 °C (100 Å, 5 µm, 4.6 × 250 mm). The mobile phase consisted of 0.5% aqueous formic acid (A) and a mixture of 0.5% formic acid in acetonitrile/water (6:4) (B). The gradient program used was as follows: 0% B to 40% B, then to 50% B in 10 min, to 70% B in another 10 min, and then held constant for 10 min. The flow rate of the mobile phase was 1 mL/min. The retention time and absorbance spectrum were compared to those of pure chemical standards to identify the compounds and then quantified using calibration curves (0–50 µg/mL).

2.6. Analyses of Extracts

All information regarding the analyses of the extracts for their TPC, total flavonoid content (TFC), total anthocyanins (TA), ascorbic acid content, and their antioxidant activity (assessed using the DPPH free radical scavenging assay and the FRAP assay) and color analysis are given in detail in Supplementary Materials [21–24].

2.7. Statistical Analysis

JMP® Pro 16 software (SAS, Cary, NC, USA) was used for the experimental design as well as the statistical analysis related to the response surface methodology (RSM), distribution analysis, multivariate correlation analysis (MCA), and partial least squares (PLS) analysis. The extraction processes were repeated three times, and the quantitative analysis was performed in triplicate, providing a total of three measurements for each sample. The results are reported as average values with standard deviations.

3. Results and Discussion

The extraction and utilization of bioactive compounds from *P. spinosa* has gained considerable interest in recent times [25]. Given the abundance of bioactive compounds in *P. spinosa*, such as polyphenols and anthocyanins, the use of these compounds into value-added products holds significant importance. Therefore, the maximum amount of these bioactive compounds from *P. spinosa* was extracted by investigating the key factors influencing the extraction process. The parameters under study included solvent composition (water, ethanol, and their 25, 50, and 75% *v/v* mixtures), extraction duration (ranging between 15 and 180 min), and extraction temperature (ranging between 20 and 80 °C). Additionally, the liquid-to-solid ratio, a crucial factor in extraction processes, was optimized, with 20 mL of solvent per 1 g of *P. spinosa* fruit powder yielding the best results based on preliminary studies. Lower solvent concentrations resulted in inadequate extraction, while higher concentrations did not show improvements, making this liquid-to-solid ratio the standard for all extracts prepared. To enhance the extraction process, the potential of US and PEF as pretreatment steps, both separately and in combination, were examined. These energy-efficient approaches offer scalability and were assessed for their ability to improve the extraction yield of bioactive compounds on a larger scale. PEF was employed prior to US, and the sequential use of both techniques was also examined.

The optimization of extraction parameters is crucial for maximizing effectiveness while minimizing resource consumption and ensuring a more environmentally friendly process [23,25]. Furthermore, the solvent composition plays a vital role, as the properties of the solvent significantly influence compound extraction [26]. For instance, polar solvents, such as water, are inefficient in extracting moderately polar molecules, such as polyphenols. As a result, ethanol and other organic solvents are commonly used to enhance the extraction process [21,22,27,28]. According to the results of the present study (vide infra), a high ethanol concentration in the extraction solvent was necessary to achieve the most effective polyphenol extraction. Combining green extraction methods such as PEF and US with traditional techniques has been shown to improve efficiency [21–23,25–27]. These methods disrupt cellular membranes, making desirable compounds easier to extract. Previous studies have demonstrated that employing PEF or US, or a combination of both, prior to the main extraction process results in enhanced effectiveness [23,25].

3.1. Optimization of the Extraction

In order to investigate the effects of each extraction parameter and optimize the extraction technique, an RSM approach was employed. The responses measured included TPC, neochlorogenic acid, and TA. Furthermore, HPLC-DAD was employed to identify and quantify several polyphenolic substances, including gallic acid, procatechuic acid, catechin, cyanidin 3-*O*-glucoside, delphinidin 3,5-di-*O*-galactoside, rutin, neochlorogenic acid, chlorogenic acid, delphinidin 3,5-di-*O*-glucoside, cyanidin 3-*O*-(6″-malonylglucoside), quercetin 3-*O*-galactoside, quercetin 3-β-D-glucoside, kaempferol 3-*O*-β-rutinoside, and kaempferol 3-glucoside. The experimental parameters for extract preparation and the measured responses are displayed in Table 2, while the concentrations of the polyphenols before optimization are shown in Table S1. As shown in Table 2, the TPC values ranged from 6.12 to 24.20 mg GAE/g, marking a statistically significant difference of 74.71% in their TPC. Neochlorogenic acid values were between 1.63 and 4.57 mg/g, a difference of 63.02% in the content of this compound in the extracts. The TA values measured ranged

between 11.5 and 149.2 µg CyE/g, resulting in another statistically significant difference in the TA content among the extracts. Notably, some polyphenols were recovered in low quantities, with catechin values ranging from 0.15 to 0.70 mg/g, neochlorogenic acid from 1.63 to 4.57 mg/g, cyanidin-3-*O*-glucoside from 0.15 to 1.04 mg/g, chlorogenic acid from 0.14 to 0.40 mg/g, delphinidin 3,5-di-*O*-galactoside from 0.19 to 0.63 mg/g, cyanidin 3-*O*-(6″-malonylglucoside) at from 0.11 to 1.25 mg/g, and rutin from 0.09 to 0.22 mg/g.

Table 2. Results from the experiments on the dependent variable's responses and the four independent variables under examination.

Design Point	Independent Variables				Responses					
	X_1	X_2	X_3	X_4	Total Polyphenols (mg GAE/g)		Neochlorogenic Acid (mg/g)		Total Anthocyanins (µg CyE/g)	
					Measured	Predicted	Measured	Predicted	Measured	Predicted
1	3	1	3	4	8.20	7.97	3.04	2.87	38.8	38.6
2	3	2	1	3	12.56	12.61	3.77	3.56	101.5	99.2
3	2	3	4	3	14.62	13.24	2.92	2.72	140.5	137.0
4	2	4	5	4	13.42	15.11	2.62	2.98	123.8	130.2
5	3	5	4	2	4.83	5.13	1.83	1.93	73.2	71.6
6	4	1	4	5	7.89	8.40	3.55	3.63	30.8	31.5
7	4	2	3	1	12.15	12.30	2.66	2.69	59.2	61.1
8	1	3	3	2	11.82	13.27	2.86	2.61	129.8	126.6
9	1	4	4	1	24.20	23.43	2.42	2.43	125.0	126.8
10	1	5	1	4	5.60	5.19	1.95	1.97	89.4	89.2
11	1	1	2	3	4.61	5.57	2.35	2.60	38.4	42.0
12	1	2	5	5	23.73	22.87	4.57	4.46	134.6	132.0
13	4	3	2	4	22.49	21.59	2.84	2.96	129.9	126.6
14	3	4	2	5	13.59	14.15	2.64	2.65	149.0	152.3
15	2	5	3	5	6.32	6.23	1.78	1.70	90.8	88.5
16	2	1	1	1	3.08	2.73	1.69	1.60	11.5	8.7
17	2	2	2	2	6.12	5.13	2.33	2.58	89.2	93.2
18	3	3	5	1	19.50	19.94	2.60	2.63	149.2	148.2
19	4	4	1	2	22.82	23.50	2.02	2.06	98.8	101.0
20	4	5	5	3	7.22	6.41	2.06	1.86	65.6	64.7

Table 3 presents the statistical parameters, second-order polynomial equations (models), and coefficients for each model, with coefficients exceeding 0.95, indicating excellent fitting of the produced models. Notably, the total anthocyanins model exhibited an exceptional coefficient value of 0.9954, highlighting a strong correlation ($p < 0.0001$). Visual representations of the responses, along with the desirability functions, can be found in Figures S1–S3. Additionally, three-dimensional response plots for TPC, neochlorogenic acid, and TA are depicted in Figures S4–S6. For a comprehensive view of the identified polyphenols in *P. spinosa* samples, the results are given in Figure 2, which displays a representative chromatogram of the detected polyphenols.

Table 3. *Prunus spinosa* extraction from hydroethanolic solutions was optimized utilizing a variety of methods and mathematical models developed with RSM.

Responses	Second-Order Polynomial Equations (Models)	R^2	p	Equation
Total polyphenols	$Y = -3.2 - 2.98X_1 - 3.59X_2 + 12.2X_3 - 0.55X_4 + 2.27{X_1}^2 + 0.26{X_2}^2 + 1.6{X_3}^2 - 0.4{X_4}^2 + 0.35X_1X_2 - 4.27X_1X_3 + 1.39X_1X_4 - 1.25X_2X_3 + 1.26X_2X_4 - 1.68X_3X_4$	0.9857	0.0011	(2)
Neochlorogenic acid	$Y = -0.69 + 0.29X_1 + 2.86X_2 - 1.7X_3 + 1.07X_4 + 0.05{X_1}^2 - 0.4{X_2}^2 + 0.01{X_3}^2 + 0.17{X_4}^2 - 0.14X_1X_2 + 0.19X_1X_3 - 0.27X_1X_4 + 0.29X_2X_3 - 0.37X_2X_4 + 0.03X_3X_4$	0.9461	0.0267	(3)
Total anthocyanins	$Y = -110.55 + 4.07X_1 + 126.57X_2 + 6.67X_3 + 11.21X_4 - 4.63{X_1}^2 - 19.43{X_2}^2 + 2.55{X_3}^2 + 2.21{X_4}^2 + 3.54X_1X_2 - 0.61X_1X_3 + 0.89X_1X_4 - 1.64X_2X_3 - 1.49X_2X_4 - 4.71X_3X_4$	0.9954	<0.0001	(4)

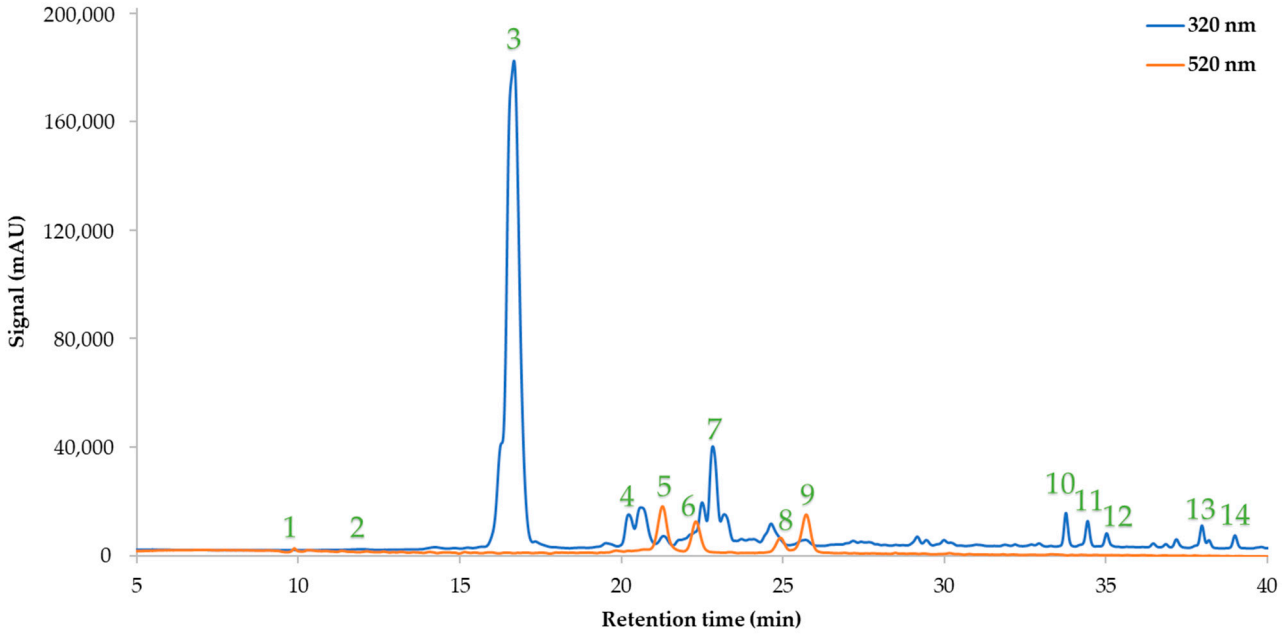

Figure 2. Exemplary HPLC chromatogram at 320 nm of a *P. spinosa* extract demonstrating the phenolic compounds that were identified. 1: Gallic acid; 2: Procatechuic acid; 3: Neochlorogenic acid; 4: Catechin; 5: Cyanidin 3-*O*-glucoside; 6: Delphinidin 3,5-di-*O*-galactoside; 7: Chlorogenic acid; 8: Delphinidin 3,5-di-*O*-glucoside; 9: Cyanidin 3-*O*-(6″-malonylglucoside); 10: Rutin; 11: Quercetin 3-*O*-galactoside; 12: Quercetin 3-β-D-glucoside; 13: Kaempferol 3-*O*-β-rutinoside; 14: Kaempferol 3-glucoside.

3.2. Multivariate Correlation Analysis (MCA)

Following the determination of the parameters *L**, *a**, and *b** for color (Table 4), an attempt was made to establish the compatibility of the parameters with TPC, neochlorogenic acid, and TA. The color of a fruit or its extract is often attributed to the high or low amounts of these phenolic compounds. The color of each sample varied according to the pretreatment and extraction temperature applied to it. At low temperatures, the samples exhibited a yellow hue, whereas pretreatment only with US produced a green hue. At high temperatures, the samples were red in color. In Figure 3, it can be observed that both lightness and yellowness show a negative correlation with the above-mentioned polyphenolic substances. On the contrary, redness shows a high correlation up to a value of 0.8, with a maximum of 1. This result is expected since the fruit is intensely red and appears to be attributable to the polyphenolic compounds contained in the fruit. A further important result is a high correlation value between these polyphenolic compounds, which is also to be expected since neochlorogenic acid and anthocyanins are part of the total polyphenols [29].

During the extraction process carried out at various temperatures and the pretreatment process, particularly when using US or PEF, both methods can generate localized heating. This could influence the extraction kinetics of phenolic compounds, including neochlorogenic acid and anthocyanins, which might, in turn, affect the color of the samples. Higher temperatures generally increase the diffusion rate of phenolic compounds from the sample matrix to the solvent, leading to higher extraction yields. However, prolonged exposure to high temperatures might also result in the degradation of phenolic compounds, affecting the overall color profile. Moreover, temperature can affect the solubility of pigments, especially in the case of anthocyanins. Higher temperatures may enhance pigment solubility, leading to more intense colors, while lower temperatures might limit pigment extraction and result in reduced color intensity.

Table 4. Color analysis results from the experiments and the four independent variables under examination.

Design Point	Independent Variables				Color Coordinates		
	X_1	X_2	X_3	X_4	L^*	a^*	b^*
1	3	1	3	4	47.5	24.2	20.0
2	3	2	1	3	35.3	26.0	16.6
3	2	3	4	3	35.0	26.0	11.7
4	2	4	5	4	43.4	16.6	20.0
5	3	5	4	2	52.2	15.6	31.5
6	4	1	4	5	41.7	25.2	16.4
7	4	2	3	1	40.4	24.4	16.9
8	1	3	3	2	34.0	22.1	12.7
9	1	4	4	1	37.2	20.3	21.2
10	1	5	1	4	49.3	17.1	32.0
11	1	1	2	3	46.4	22.6	21.3
12	1	2	5	5	34.9	26.0	11.7
13	4	3	2	4	34.8	21.5	12.4
14	3	4	2	5	38.9	20.5	16.1
15	2	5	3	5	51.3	13.7	31.0
16	2	1	1	1	52.9	17.9	23.7
17	2	2	2	2	42.6	23.4	22.1
18	3	3	5	1	33.5	22.6	11.1
19	4	4	1	2	41.6	18.9	19.5
20	4	5	5	3	40.8	24.4	20.8

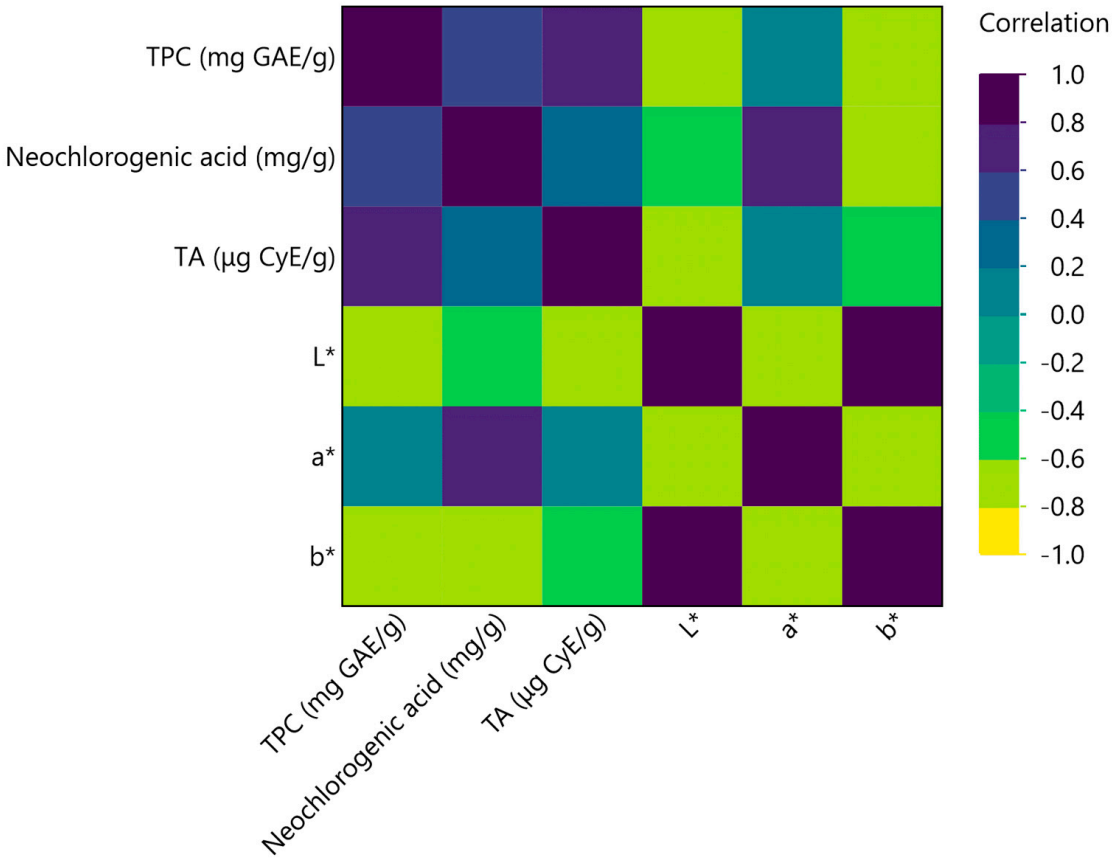

Figure 3. Multivariate correlation analysis of measured variables (total polyphenol content-TPC, neochlorogenic acid content, total anthocyanins-TA, and L^*, a^*, b^* color coordinates).

3.3. Analysis of the Optimum Extract

To determine the maximum predicted values for TPC, neochlorogenic acid, and TA, the desirability function was utilized, and the results are presented in Table 5. Under these conditions, the highest quantity of total polyphenols was extracted from *P. spinosa* fruit, achieved by a short extraction time (30 min) and low temperature (35 °C), using a 75% ethanol solution combined with pretreatment steps of PEF and US before the standard extraction (ST). Neochlorogenic acid exhibited its maximum extraction at a moderately high temperature (80 °C), with a dilute ethanolic solution (25% *v/v*) and ST lasting for 150 min. For TA, the most effective extraction was accomplished using a 50% ethanol solution, at an intermediate temperature (65 °C), with pretreatment of US before ST, and ST lasting for 30 min. Notably, according to the RSM, the combination of PEF, US, and ST proved to be the most efficient extraction technique. This results from the PLS analysis. As can be seen in Table 5, the variable X_1, which is the technique, combined with the rest of the data, concludes that this combination of techniques is the one that will lead to the optimal extraction conditions. Compared to other studies, Athanasiadis et al. [25] utilized PLS analysis to find the optimal conditions for extracting bioactive compounds from orange peel waste, and concluded that the combination of PEF and US raised the amount of bioactive compounds extracted. In another study, Athanasiadis et al. [23] applied a PLS analysis in order to determine the optimal conditions for the extraction of bioactive compounds from quince peels, and found that the best extraction technique is just ST at 65 °C for more than 120 min. This means that each fruit needs a specific combination of extraction conditions, and not a specific one is followed.

Table 5. Maximum predicted responses and optimum extraction conditions for the dependent variables using hydroethanolic solutions.

Responses	Optimal Conditions				
	Maximum Predicted Response	**Technique (X_1)**	***C* (%, *v/v*) (X_2)**	***t* (min) (X_3)**	***T* (°C) (X_4)**
Total polyphenols (mg GAE/g)	23.5 ± 3.98	PEF + US + ST (4)	75 (4)	30 (1)	35 (2)
Neochlorogenic acid (mg/g)	4.46 ± 0.8	ST (1)	25 (2)	150 (5)	80 (5)
Total anthocyanins (μg CyE/g)	153.26 ± 13.5	US + ST (3)	50 (3)	30 (1)	65 (4)

After optimization, a significant increase in the concentrations of total polyphenols, ranging from 9 to 70%, was recorded. The optimized recovery yields for the polyphenols are listed in Tables 6 and 7. Previous studies reported lower levels of polyphenols extracted from *P. spinosa* fruit, such as 2.52 mg GAE/g dw by Opriş et al. [30], 71.23% lower than our findings. Similarly, studies on *P. spinosa* fruit by González-de-Peredo et al. [31] and Ozzengin et al. [6] resulted in TPC values of 5.56 ± 0.18 mg/g and 4.41 ± 2.47 mg/g, respectively, which were 36.53 and 49.66% lower than our findings. In the first case, the *P. spinosa* fruit underwent ultrasound-assisted extraction, operating at 200 W and a working frequency of 24 kHz. In the second case, a total of 10 g of plum pulp was mixed with 100 mL of distilled water and subjected to a 12 h extraction process in a refrigerated environment. Flavonoids were also found in the *P. spinosa* fruit extract, with a maximum TFC of 3.23 ± 0.17 mg RtE/g dw achieved under optimal extraction conditions. The combination of PEF, US, and ST, similar to TPC extraction, resulted in the highest TFC. Ethanol mixed with water at a ratio of 75% was found to be the most suitable solvent for TFC extraction, further supported by comparisons with other studies using methanol or recovering lower TFC levels from *P. spinosa* fruit. This is strengthened by the comparison of our results with a previous study in *P. spinosa* fruit, from Magiera et al. [32] where 1.43 ± 0.09 mg/g TFC was recovered, after using 75% methanol as a solvent and 500.1 g of dried plum pulp, and the solution was subjected to sequential reflux for a duration of 20 min for each reflux cycle. Moreover, Prvulovic et al. [33] examined the TFC in *Prunus avium* fruit by sonicating 5 g of plant material in 70% acetone for 20 min, and obtained

0.70 mg/g. Another study was conducted by the same research team [34] in *P. avium* petioles, following the same extraction procedure, but this time, 1.94 mg/g dw of TF were found. These quantities were 55.73, 73.33, and 39.94% less than the TFC determined in *P. spinosa* in this study. Regarding TA, after optimization, the maximum quantity was secured. The maximum TA value of the extract is presented in Table 6. In further research by Damar and Yilmaz [35], 97.76 µg/g TA from *P. spinosa* fruit were reported, the experimental setup of which consisted of a GM 4200 generator, an ultrasonic converter UW 200, an SH 200 G booster horn, and a titanium probe TS 109 with a diameter of 9 mm. A 1:1 dilution of blackthorn extract with distilled water was prepared by homogenizing the mixture using a Waring blender. The resulting mixture, weighing 12.5 g, was transferred into a double-walled extraction tube. Subsequently, 112.5 g of distilled water was added to the tube. This study reported a TA value of 22.05% lower than our findings. Comparing our results to studies on other *Prunus* species, specifically *Prunus domestica*, Ozzengin et al. [6] obtained 106.2 µg/g TA, and as such, *P. spinosa* fruit was found to contain 15.18% higher TA concentrations than *P. domestica*.

Table 6. Using the partial least squares (PLS) prediction profiler under optimal extraction conditions, the maximum desirability for all variables is obtained. (X_1:4, X_2:2, X_3:1, X_4:5).

Variables	PLS Model Values	Experimental Values
Total polyphenols (mg GAE/g)	31.54	30.74 ± 1.23
Neochlorogenic acid (mg/g)	4.62	4.13 ± 0.08
Total anthocyanins (µg CyE/g)	129.4	125.2 ± 5.8

Table 7. Quality parameters, color analysis, and polyphenolic compounds under optimal extraction conditions.

Quality Parameters	Optimal Extract
Total flavonoids (mg RtE/g)	3.23 ± 0.17
FRAP (µmol AAE/g)	146.09 ± 3.2
DPPH (µmol DPPH/g)	200.15 ± 6.36
A_{AHP} (µmol AAE/g)	18.65 ± 1.58
Ascorbic acid (mg/100 g)	119.4 ± 4.63
Color analysis	
L^*	33.4 ± 1.3
a^*	23.1 ± 0.9
b^*	11.9 ± 0.5
Polyphenolic compounds (mg/g)	
Gallic acid	0.05 ± 0
Procatechuic acid	0.03 ± 0
Neochlorogenic acid	4.13 ± 0.08
Catechin	0.39 ± 0.01
Cyanidin 3-*O*-glucoside	1.16 ± 0.08
Delphinidin 3,5-di-*O*-galactoside	0.75 ± 0.02
Chlorogenic acid	0.3 ± 0.02
Delphinidin 3,5-di-*O*-glucoside	0.36 ± 0.01
Cyanidin 3-*O*-(6″-malonylglucoside)	1.04 ± 0.06
Rutin	0.19 ± 0.01
Quercetin 3-*O*-galactoside	0.08 ± 0.01
Quercetin 3-β-D-glucoside	0.08 ± 0
Kaempferol 3-*O*-β-rutinoside	0.13 ± 0
Kaempferol 3-glucoside	0.07 ± 0
SUM	8.76 ± 0.31

The most abundant polyphenol recovered from *P. spinosa* fruit was neochlorogenic acid, which is consistent with previous studies [36,37]. Neochlorogenic acid offers beneficial

effects both for fruit and human health, including anti-inflammatory and antioxidant properties [38,39], particularly against pneumonia, at a concentration of 100 μM [38]. Additionally, it potentially contributes to the formation of anthocyanins [40]. The maximum amount recovered for neochlorogenic acid was 13.44% of the total polyphenol content. Levels of neochlorogenic acid in related *Prunus* species have been studied thoroughly, with Tomić et al. [41] recovering 3.95 ± 0.47 mg/g fresh weight from *Prunus domestica* L. fruit and Yilmaz et al. [42] recovering 0.22 ± 0.01 mg/g dw from *Prunus cerasus* L. pomace. The concentration of neochlorogenic acid in *P. spinosa* fruit was remarkably higher, showing a 1777% difference compared to the latter study. Likewise, cyanidin-3-*O*-glucoside was found by Crupi et al. [43] in *P. avium* fruit in an amount 72.89% lower than the present results. Celik et al. [44] reported chlorogenic acid and rutin 56.67 and 99.75% times less, respectively, in *P. spinosa* fruit. This indicates that the extraction conditions need to be optimized to recover all possible individual phenolic compounds.

Antioxidant activity was assessed using FRAP and DPPH assays, with the best results obtained in extracts containing 75% ethanol as the extraction solvent. Extraction duration played a vital role, as longer extraction periods yielded higher antioxidant activity. The ideal temperature, however, appears to be a midpoint of 65 °C. Interestingly, different extraction methods were applied depending on the antioxidant test used, with the FRAP method benefitting from PEF and US prior to ST, while the DPPH assay required PEF before ST to enhance DPPH free radical scavenging. After optimization, the DPPH value of the extract was 200.15 ± 6.36 μmol DPPH/g, indicating a considerable improvement in scavenging ability.

Remarkably, high levels of ascorbic acid were recovered from *P. spinosa* fruit under optimal conditions, with the most effective extraction achieved using PEF, US, and ST, and a 75% ethanol solvent for 30 min at 80 °C. The results indicated that temperature and extraction time were essential variables in maximizing the extraction yield. Di Mateo et al. [45] extracted 2 g of fresh fruit tissue in 30 mL methanol solution, and recovered 105.24 ± 1.60 mg/100 g fresh weight (fw) from *P. avium* fruit, an amount similar to our finding. Usenik et al. [14] also studied *P. avium* fruit, where 10 g of fruit pulp was dissolved in 50 mL of twice-distilled water and extracted for 30 min at ambient temperature, and they recovered 17.2 mg/100 g fw, 85.59% lower than our finding.

Fruit color is a key characteristic, as it may be a guide to consumer acceptance and preference or rejection [6]. Therefore, it can also be concluded that the color of the product in which the fruit or its extract will be used is of utmost importance. Regarding *Prunus spinosa*, color has been studied both in the fruit pulp and skin and in foods where its extract has been used as a natural food colorant. In the first case, values of lightness (L^*), redness (a^*), and yellowness (b^*) were 15.5 to 27.9, from 2.6 to 7.1, and from 1.8 to 6.6, respectively, in the skin and 19.3 to 31.3, from 0.9 to 5.3, and from 4.9 to 14.6, respectively, in the pulp are reported [6]. Regarding foodstuffs, two dairy confectionery products, doughnut icings and "beijinho" pastry, were prepared. After direct measurement of color, L^*, a^*, and b^* values for doughnut icings and "beijinho" were obtained, 46 ± 1, 24 ± 2, 2.6 ± 0.4, and 60 ± 6, 17 ± 3, 9 ± 5, respectively [46]. In the present study, among the 20 different samples, the values of L^*, a^*, and b^* varied as follows 33.5 to 52.9, 13.7 to 26, and 11.1 to 32, respectively, and are presented in Table 4. For the sample obtained after extraction under the optimum conditions (Table 7), the values were for L^* 33.4 ± 1.3, for a^* 23.1 ± 0.9, and for b^* 11.9 ± 0.5, leading to the conclusion that extracts of specific parameters intensify the color.

To identify the most crucial extraction parameters (X_1, X_2, X_3, X_4), partial least squares (PLS) analysis was conducted (Figures 4 and 5). Figure 4 shows the PLS analysis used to generate a graph (correlation loading plot) displaying *P. spinosa* extraction procedures and hydroethanolic solutions. Figure 5 depicts the PLS prediction profiler of each variable and desirability function with extrapolation control for *P. spinosa* extract optimization utilizing various extraction procedures and hydroethanolic solutions. The concentration of the solvent ($X_2 \times X_2$) emerged as more important than other factors in the extraction of bioactive compounds. A high ethanol content (at least 75%) in the solvent produced

the best results for most compounds. Factors $X_3 \times X_4$ (time and temperature) also showed a significant impact, while factor X_1 (extraction technique) had relatively less influence. The optimized extraction conditions for *P. spinosa* fruit are summarized in Table 6, and the experimental values were found to correlate closely with those predicted by the PLS model. Finally, Table 7 provides the quality parameters and polyphenolic compounds under optimal extraction conditions.

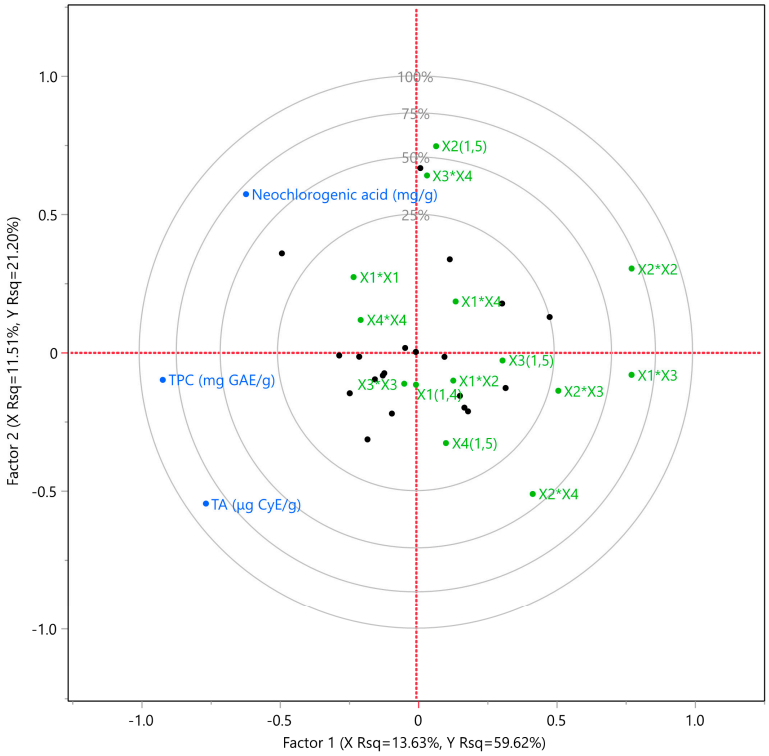

Figure 4. PLS (partial least squares) analysis was used to generate a graph (correlation loading plot) displaying *Prunus spinosa* extraction procedures and hydroethanolic solutions.

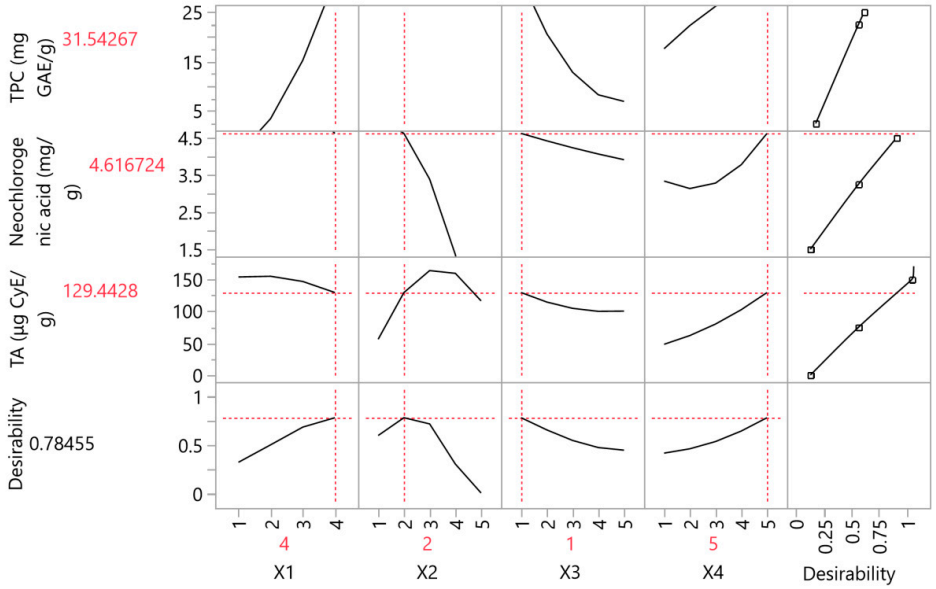

Figure 5. Using several extraction techniques and hydroethanolic solutions, *Prunus spinosa* extracts were optimized using a partial least squares (PLS) prediction profiler for each variable and desirability function with extrapolation control.

The suggested extraction conditions for *P. spinosa* fruit are shown in Table 6, combined with the results of the various variables that were examined throughout the experiment. Upon comparison of the values given by the PLS model with those obtained after experimental analysis, the correlation among them is found to be 0.999, and they show no deviations, with the *p*-value being 0.0027.

4. Conclusions

In this study, we conducted a comprehensive exploration and optimization of various extraction procedures and factors to obtain the most efficient scheme for extracting bioactive components from whole *P. spinosa* fruit. The results revealed that a combination of PEF, US, and ST, a straightforward and cost-effective approach, proved to be the most suitable extraction method. This approach, carried out at a slightly elevated temperature (80 °C) for a short duration of 30 min, demonstrated exceptional efficacy in extracting valuable bioactive compounds from the fruit. These conditions were determined through RSM, ANOVA, MCA, and PLS analyses. One critical finding was the significant impact of the extraction solvent on the overall efficiency of the process. Ethanol, when used as a solvent with a proportion of 25% deionized water, led to superior extraction efficiencies. This highlights the importance of carefully selecting the solvent composition to maximize the yield of bioactive compounds from *P. spinosa*. The relevance of antioxidants in promoting human health cannot be overstated, and there is a growing demand for food products enriched with high antioxidant content. Our research offers valuable insights into the potential of *P. spinosa* as a promising source of bioactive compounds. The optimized extraction conditions can be utilized to produce extracts with enhanced antioxidant properties, contributing to the development of functional foods and nutraceuticals that offer valuable health benefits. Our findings encourage further research and exploration of the bioactive compounds present in *P. spinosa* fruit. By delving deeper into the fruit's chemical composition and potential applications, new opportunities may arise.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9080942/s1, Figure S1: Plots A, B, and C display the actual response versus the predicted response (Total polyphenol content—TPC, mg GAE/g) for the optimization of *Prunus spinosa* extracts carried out with hydroethanolic solutions and different extraction methods, as well as the studentized residuals and the desirability function, respectively. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model. Figure S2: Plots A, B, and C display the actual response versus the predicted response (Neochlorogenic acid, mg/g) for the optimization of *Prunus spinosa* extracts carried out with hydroethanolic solutions and different extraction methods, as well as the studentized residuals and the desirability function, respectively. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model. Figure S3: Plots A, B, and C display the actual response versus the predicted response (Total anthocyanins—TA, μg CyE/g) for the optimization of *Prunus spinosa* extracts carried out with hydroethanolic solutions and different extraction methods, as well as the studentized residuals and the desirability function, respectively. Asterisks and colored values denote statistically significant values, while inset tables include statistics relevant to the evaluation of the resulting model. Figure S4: The optimal extraction of *Prunus spinosa* extracts using different extraction methods and hydroethanolic solutions is shown in 3D graphs that show the impact of the process variables considered in the response (Total polyphenol content—TPC, mg GAE/g). Plot (A), covariation of X_1 and X_2; plot (B), covariation of X_1 and X_3; plot (C), covariation of X_1 and X_4; plot (D), covariation of X_2 and X_3; plot (E), covariation of X_2 and X_4; plot (F), covariation of X_3 and X_4. Figure S5: The optimal extraction of *Prunus spinosa* extracts using different extraction methods and hydroethanolic solutions is shown in 3D graphs that show the impact of the process variables considered in the response (Neochlorogenic acid, mg/g). Plot (A), covariation of X_1 and X_2; plot (B), covariation of X_1 and X_3; plot (C), covariation of X_1 and X_4; plot (D), covariation of X_2 and X_3; plot (E), covariation of X_2 and X_4; plot (F), covariation of X_3 and X_4. Figure S6: The optimal extraction of *Prunus spinosa* extracts using different extraction methods and hydroethanolic solutions is shown in 3D graphs that show the impact of the process variables considered in the response (Total anthocyanins—TA, μg

CyE/g). Plot (A), covariation of X_1 and X_2; plot (B), covariation of X_1 and X_3; plot (C), covariation of X_1 and X_4; plot (D), covariation of X_2 and X_3; plot (E), covariation of X_2 and X_4; plot (F), covariation of X_3 and X_4. Table S1: Design points under investigation and the actual concentration of polyphenolic compounds, represented in mg/g dw. Reference [47] is cited in the Supplementary Materials.

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