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Capsicum chinense Polyphenols Extraction by Supercritical Fluids Using Response Surface Methodology (RSM)

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Abstract: *Capsicum chinense*, commonly known as the habanero pepper, is renowned for its culinary and medicinal value due to a great abundance of polyphenolic compounds. The pursuit of eco-friendly methods for extracting these metabolites, which produce high-purity extracts applicable to the food and pharmaceutical sectors, has led to the adoption of green technologies such as supercritical fluid extraction (SFE). In this methodology, by manipulating factors like temperature, pressure, and extraction time, the goal of producing extracts with elevated phenolic content from plant materials can be achieved. In this study, a central compound design (CCD) was conducted with the response surface methodology (RSM) to optimize the extraction of polyphenols from *Capsicum chinense* using supercritical fluids. The optimal conditions for total polyphenol extraction were determined as 63.1 °C, 1161.82 psi, and an extraction time of 132 min, with a total polyphenol content (TPC) of 1870 mg of gallic acid equivalent (GAE)/100 g extract. Additionally, concentration of several individual polyphenols were optimized, including catechin (236.27 mg/100 g extract, 62.8 °C, 1150 psi, and 132 min), chlorogenic acid (447.08 mg/100 g extract, 63.1 °C, 1150 psi, and 131.9 min), vanillic acid (136.38 mg/100 g extract, 41.9 °C, 1150 psi, and 132 min), diosmin + hesperidin (92.80 mg/100 g extract, 63 °C, 3200 psi, and 132 min), rutin (40 mg/100 g extract, 63.03 °C, 3200 psi, and 132 min), among others. These findings highlight the potential of supercritical fluid extraction for obtaining high yields of polyphenols from *Capsicum chinense*. The use of SFE-RSM also may optimize the extraction of specific phenolic compounds, and at the same time, it provides valuable insights for the development of extracts with enhanced bioactive properties for various applications in the food and pharmaceutical industries.

Keywords: *Capsicum chinense*; supercritical fluid extraction; polyphenols; response surface methodology

Citation: Avilés-Betanzos, K.A.; Scampicchio, M.; Ferrentino, G.; Ramírez-Sucre, M.O.; Rodríguez-Buenfil, I.M. *Capsicum chinense* Polyphenols Extraction by Supercritical Fluids Using Response Surface Methodology (RSM). *Processes* **2023**, *11*, 2055. https://doi.org/10.3390/pr11072055

Academic Editor: Maria Angela A. Meireles

Received: 19 June 2023
Revised: 4 July 2023
Accepted: 6 July 2023
Published: 10 July 2023

1. Introduction

Habanero pepper (HP) is a highly significant crop for the Yucatan Peninsula, in terms of both its culinary value and economic impact, due to its distinctive organoleptic and globally recognized bioactive properties. These properties stem from a range of secondary metabolites found within the habanero pepper, where capsaicin is the most recognized metabolite responsible for its characteristic pungency, although the HP also contains other notable bioactive compounds, such as phenolic compounds. These compounds are generated through the activation of metabolic pathways, including the shikimic acid and phenylpropanoid pathways, as a response to various biotic and abiotic factors like weather, humidity, type of soil, UV-light exposure, among others, specific to the southeastern region of Mexico [1–3].

The habanero pepper contains a diverse range of phenolic compounds, which can be categorized as polyphenols and further classified into subgroups such as hydroxycinnamic

acids, hydroxybenzoic acids, flavan-3-ols, flavones, flavanones, flavonols, and flavonoids, among others [4,5].

In general, phenolic compounds exhibit diverse bioactive properties such as antioxidant, anti-inflammatory, anticancer, and anti-obesogenic, to name a few. As a result, there is a significant interest in exploiting the potential of these phenolic compounds derived from habanero pepper, developing applications including food, cosmetics, and pharmaceutical fields [6,7]. For example, catechin is classified as flavan-3-ols, a characteristic phenolic compound of the habanero pepper [4,5]. It exhibits bioactive properties extensively studied, such as UV protection, antimicrobial, anticancer, and antiviral activities, with applications in cosmetics and pharmaceuticals [8].

Chlorogenic acid, classified as a hydroxycinnamic acid [5], is also an important phenolic compound to mention. It is considered the second most abundant phenolic compound in habanero peppers, as reported by Troconis-Torres et al. [4]. Chlorogenic acid has properties that can be exploited for the benefit of human health, including antioxidant, anti-inflammatory, and antibacterial activities, and hepatoprotective capacity through the regulation of cellular apoptosis. Consequently, several applications have been developed, primarily in the field of food, such as colorants, food packaging, and prebiotics, with the aim to capitalize on its bioactive properties [9]. Other metabolites of interest include diosmin and hesperidin, classified as flavanones, which are used in the pharmaceutical industry for the treatment of circulatory problems such as varicose veins or hemorrhoids. However, they have also been reported to develop other properties such as antidiabetic, hepatoprotective, and neuroprotective, among others [10].

Obtaining phenolic-rich extracts from food matrices can be a challenging task in the context of the current emphasis on environmental sustainability through green chemistry [11,12]. This approach arises mainly from the need to avoid the utilization of organic solvents, including methanol, acetone, hexane, petroleum ether, and others, due to their potential toxicity and the requirement of multiple unit operations for solvent recovery and disposal [13].

These factors contribute to increasing costs in both, the extraction process and the final product. Even when solvents are employed in conjunction with recognized green extraction technologies, such as ultrasound-assisted extraction or microwave-assisted extraction, the inherent challenges associated with solvent usage persist [11].

A viable option is the use of supercritical fluid extraction, also recognized as a green technology [14]; this technology presents an advantage over traditional extraction methods such as maceration, Soxhlet, and even more over recent ones like ultrasound, in terms of using a non-toxic solvent for both those performing the extractions and final consumers. It also involves lesser usage of organic solvents, recovery and reuse of the supercritical solvent (mainly CO_2), and non-polluting waste [15].

This green technology is based on reaching liquid–gas behavior of a solvent, mainly CO_2, by increasing temperature and pressure to reach the critical point. The main objective is to enhance the solvent capacity, penetration, diffusion, and density, and reduce viscosity, leading to an increase in the porosity of the food matrix due to a rapid expansion of the cell wall. This process enables the extraction of various bioactive compounds with high purity (>99%), primarily non-polar metabolites, due to the non-polar nature of CO_2 [15,16]. One way to enhance the extraction of phenolic compounds is by modifying the polarity of the extraction through the addition of co-solvents such as ethanol or water [17].

Some authors, such as de Aguiar et al. [18], report a supercritical fluid extraction of phenolic compounds from Malagueta peppers (*Capsicum frutescens*) where a concentration of 3600 $\pm$ 200 mg GAE/100 g extract was achieved under conditions of temperature (Tp) of 40 °C, pressure (Ps) of 2175.75 psi, and an extraction time (Et) of 300 min. Moreover, Deka et al. [19] reported the extraction of phenolic compounds from Bhut Jolokia chili (*Capsicum assamicum*), obtaining a concentration of phenolic compounds of 4250 $\pm$ 2.26 mg GAE/100 g extract under conditions of Tp of 60 °C, Ps of 3002.28 psi, and Et of 73 min. Additionally, Grande-Villanueva et al. [20] reported the extraction of phenolic compounds

using supercritical fluid technology under conditions of Tp of 40 °C, Ps of 2900.75 psi, and Et of 240 min, achieving a concentration of 3700 ± 30 mg GAE/100 g extract. However, this concentration did not show a statistically significant difference ($p > 0.05$) when utilizing higher Tp (60 °C) and Ps (3625.94 psi) with the same Et (240 min) reaching a concentration of 3600 ± 10 mg GAE/100 g extract.

Although supercritical fluid extraction has shown to be a viable option for obtaining phenolic compounds from *Capsicum* spp., the available information primarily focuses on the extraction of capsaicin. Consequently, there is a lack of information regarding the optimization of extraction conditions for total polyphenol content (TPC) and even less for individual phenolic compounds [18,21–24]. The above is due to the non-polar nature of CO_2 and the recent interest in studying the phenolic compounds of habanero pepper, particularly those from the Yucatan Peninsula [3,15]. Thus, the use of mathematical and statistical tools such as response surface methodology (RSM) would be suitable for finding the optimal extraction conditions of phenolic compounds from habanero pepper fruit (*Capsicum chinense*).

RSM offers advantages such as reduced resources (fewer experimental trials) and less time consumed compared to other experimental designs. Additionally, it generates a mathematical model that predicts response optimization by analyzing the independent variables and their interactions [25]; this behavior is important to understand to maximize the concentration of phenolic compounds in the extract.

The objective of this study was to obtain a habanero pepper (*Capsicum chinense*) extract with the highest concentration of phenolic compounds by optimizing the conditions of supercritical fluid extraction using response surface methodology.

2. Materials and Methods

2.1. Raw Materials

In the community of Chicxulub pueblo, Yucatán, Mexico, habanero pepper plants (*Capsicum chinense* Jacq.) were cultivated under controlled greenhouse conditions. The specific geographic coordinates were 21°08′50.5″ N and 89°29′42.8″ W.

The cultivation took place in lithic leptosol soil, already classified according to the World Reference Base for Soil Resources (WRB) classification. In the common Mayan language, this soil is known as Tzek'el lu'um.

The fruits of the habanero pepper were harvested on 11 December 2019, three months after the initial planting. At the time of harvest, the peppers were still in an immature state, characterized by their green color.

2.2. Habanero Pepper Drying and Sieved Process

Following harvest, the freshly picked habanero peppers in an immature green state were transported to the CIATEJ facilities at the southeast campus. At this location, the peppers underwent a product classification process, segregating the immature green fruits from those displaying color changes to discard, such as green-orange and orange. Additionally, other plant residues such as leaves, stems, and peduncles were separated from the fruits.

Once the green habanero pepper fruits were collected and classified, they were placed in aluminum trays and subjected to drying using a FELISA oven (Barcelona, España, model FE-292) at a temperature of 65 °C for a duration of 72 h [26]. Subsequently, the dried habanero pepper fruits were pulverized using an Oster® blender (Mexico City, Mexico) and passed through a #35 sieve with a particle size of 500 µm. Finally, the resulting habanero pepper powder was stored in plastic bags lined with aluminum foil at room temperature until further use.

2.3. Habanero Pepper Polyphenols Extraction

2.3.1. Experimental Design

A central composite design 2^3 (CCD)) was implemented to optimize temperature (X_1), pressure (X_2), and extraction time (X_3) as extraction conditions for the phenolic compounds extraction from a habanero pepper.

For each factor, two levels were taken into consideration. For temperature (Tp), the lower level was set at 45 °C (−1), while the higher level was established at 60 °C (1). Regarding pressure (Ps), the experimental conditions consisted of a low level at 1450 psi (−1) and a high level at 2900 psi (1). The extraction time (Et) implemented was 60 min (−1) and 120 min (1), as low and high levels, respectively. Three central points (0) were also implemented: Tp of 52.5 °C, Ps of 2175 psi, and an Et of 90 min. Finally, according to the response surface methodology, the star points (second experimental design) were added once the data (first 12 experiments) fit a second-order mathematical model, where the values for Tp were 41.9 °C (−1.414) and 63.1 °C (1.414), for Ps 1150 psi (−1.414) and 3200 psi (1.414), and Et 48 min (−1.414) and 132 min (1.414), were established.

With the assistance of the statistical software, a canonical analysis was conducted on the complete experimental design (Table 1) in order to determine the ideal conditions for achieving a habanero pepper extract with the maximum concentration of total polyphenols.

Table 1. Central composite design (CCD) 3^2 to evaluate the extraction conditions of phenolic compounds from habanero pepper by supercritical fluids.

Exp	Factors						Variable Response	
	Coded Values			Real Values			TPC (mg GAE/100 g Ext)	Individual Polyphenols * (mg/100 g Xt)
	X_1	X_2	X_3	Tp (°C)	Ps (psi)	Et (min)		
1	−1	−1	−1	45	1450	60	Y_1	Z_1
2	1	−1	−1	60	1450	60	Y_2	Z_2
3	−1	1	−1	45	2900	60	Y_3	Z_3
4	1	1	−1	60	2900	60	Y_4	Z_4
5	−1	−1	1	45	1450	120	Y_5	Z_5
6	1	−1	1	60	1450	120	Y_6	Z_6
7	−1	1	1	45	2900	120	Y_7	Z_7
8	1	1	1	60	2900	120	Y_8	Z_8
9	0	0	0	52.5	2175	90	Y_9	Z_9
10	0	0	0	52.5	2175	90	Y_{10}	Z_{10}
11	0	0	0	52.5	2175	90	Y_{11}	Z_{11}
12	−1.414	0	0	41.9	2175	90	Y_{12}	Z_{12}
13	1.414	0	0	63.1	2175	90	Y_{13}	Z_{13}
14	0	−1.414	0	52.5	1150	90	Y_{14}	Z_{14}
15	0	1.414	0	52.5	3200	90	Y_{15}	Z_{15}
16	0	0	−1.414	52.5	2175	48	Y_{16}	Z_{16}
17	0	0	1.414	52.5	2175	132	Y_{17}	Z_{17}

Note: Tp = temperature; Ps = pressure; Et = extraction time; TPC = total polyphenol content; GAE = gallic acid equivalent; Xt = extract; * each polyphenol was reported individually.

The response variables measured in this study were the total polyphenol content (TPC) and the concentration of individual polyphenols in the habanero pepper extract.

2.3.2. Extraction of Polyphenols by Supercritical Fluids

The process was conducted according to the procedure conducted by Santos et al. [22] with some modifications; the extraction of polyphenols began by weighing a sample of 40 g of habanero pepper powder (previously sieved, #35, particle size $\leq$ 500 µm). Subsequently, 20% ethanol (8 g) was added to change the polarity of the extraction process. To prevent pepper particles from clogging the equipment's outlet pipes during the extraction process, the habanero pepper powder was packed using filter paper. The packed habanero pepper powder was placed inside the extraction vessel (500 mL) of supercritical fluid extraction equipment (SFT-150, Supercritical Fluid Technologies, Inc., Newark, DE, USA).

The extractions were performed using the static mode, with the equipment stabilized at the defined pressure and temperature for the specific extraction time according to the

experimental design. At the end of the extraction time, the habanero pepper extract (Xt) was collected, weighed, and stored under refrigeration until further use.

2.4. Determination of Total Polyphenol Content in Habanero Pepper Extract

The Folin–Ciocalteu methodology, as described by Singleton et al. [27], was used to evaluate the extracts. However, some modifications were made to the procedure. In this modified approach, 25 µL of the extracted sample was mixed with 25 µL of distilled water. Then, 3 mL of distilled water and 250 µL of Folin's reagent were added to the mixture, which was allowed to stand for 5 min. Subsequently, 750 µL of 20% sodium carbonate ($NaCO_3$) and 950 µL of distilled water were added, and the solution was incubated for 30 min. Finally, the absorbance of the samples was measured at 765 nm using a UV-Vis spectrophotometer (JENWAY®, model 6700, Vernon Hills, lL, USA). The results were expressed as milligrams of gallic acid equivalent per 100 g of extract (GAE/100 g Ext) based on the calibration curve.

2.5. Determination of Individual Polyphenol in Habanero Pepper Extract

The individual polyphenol determination was conducted using a UPLC Acquity H-class system (Waters, Milford, MA, USA) equipped with a diode array detector (DAD). An Acquity UPLC HSS C18 column was utilized for the analysis. To establish a calibration curve, 19 polyphenol standards (Sigma-Aldrich®) were employed. The calibration curve was developed by preparing a stock solution with a concentration of 1 mg/mL containing the following polyphenols: gallic acid, protocatechuic acid, chlorogenic acid, ferulic acid, coumaric acid, cinnamic acid, catechin, rutin, kamepferol, quercetin, luteolin, vanillin, ellagic acid, diosmin, hesperidin, neohesperidin, naringenin, apigenin, and diosmetin.

Both the calibration curve and the extracts determinations underwent analysis with the following specific conditions. A column temperature of 45 °C and an injection volume of 2 µL were used for both samples. A wavelength of 280 nm was selected for detection. Solvent A, containing 0.2% acetic acid, and solvent B, consisting of acetonitrile with 0.1% acetic acid, were utilized for the mobile phase. The elution gradient followed a predetermined pattern: from 0 to 10 min, the mobile phase composition transitioned from 99% A to 70% A; from 10 min to 12 min, the mobile phase composition remained at 70% A; and from 12 to 15 min, the composition returned to 99% A. Each injection took approximately 15 min to be completed [28]. The chromatograms resulting from the calibration curve are shown in Figure S1.

2.6. Statistical Analysis

The experiments were conducted using a randomized experimental factorial design 3^2. The data presented are expressed as means ± standard deviations. Linear correlation analysis was performed to examine the relationship between the concentrations of total polyphenols and the individual polyphenols in the extracts. This analysis utilized Pearson's correlation coefficient (r) and was supplemented with a principal component analysis (PCA). Data analysis was performed using the statistical software Statgraphics Centurion XVII.II-X64 (Statgraphics Technologies Inc. version 16.1.03, Virgin, UT, USA), Excel (version 2108, Microsoft Corporation, Redmond, WA, USA), and R 4.0.3 (The R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Total Polyphenol Content in Habanero Pepper Extract

The highest concentration of total polyphenols (1656.42 ± 5.29 mg GAE/100 g Xt) was obtained using supercritical CO_2 and ethanol as co-solvent (20%) under the conditions of 52.5 °C, a pressure of 1150 psi, and an extraction time of 90 min. On the other hand, the lowest concentration of polyphenols (62.21 ± 0.39 mg GAE/100 g Xt) was achieved with a temperature, pressure, and extraction time of 52.5 °C, 2175 psi, and 90 min, respectively. These last conditions correspond to the central points of the experimental design (Table 2).

Table 2. Factorial design 3^2 for the extraction conditions evaluation of phenolic compounds from habanero pepper using supercritical fluids.

Exp	Factors						Variable Response
	Coded Values			Real Values			TPC (mg GAE/100 g Xt)
	X_1	X_2	X_3	Tp (°C)	Ps (psi)	Et (min)	
1	−1	−1	−1	45	1450	60	736.62 ± 1.46 ^{k}
2	1	−1	−1	60	1450	60	600.74 ± 1.47 ^{j}
3	−1	1	−1	45	2900	60	361.76 ± 2.4 ^{f}
4	1	1	−1	60	2900	60	1075.00 ± 1.68 ^{m}
5	−1	−1	1	45	1450	120	322.060 ± 1.69 ^{e}
6	1	−1	1	60	1450	120	758.24 ± 1.76 ^{l}
7	−1	1	1	45	2900	120	67.93 ± 0.20 ^{b}
8	1	1	1	60	2900	120	403.68 ± 2.81 ^{h}
9	0	0	0	52.5	2175	90	62.74 ± 0.44 ^{a}
10	0	0	0	52.5	2175	90	62.90 ± 0.38 ^{a}
11	0	0	0	52.5	2175	90	62.21 ± 0.39 ^{a}
12	−1.414	0	0	41.9	2175	90	115.50 ± 0.40 ^{d}
13	1.414	0	0	63.1	2175	90	736.81 ± 3.6 ^{k}
14	0	−1.414	0	52.5	1150	90	1656.42 ± 5.29 ^{n}
15	0	1.414	0	52.5	3200	90	377.51 ± 1.28 ^{g}
16	0	0	−1.414	52.5	2175	48	93.49 ± 0.57 ^{c}
17	0	0	1.414	52.5	2175	132	569.37 ± 3.60 ^{i}

Note: Tp = temperature; Ps = pressure; Et = extraction time; TPC = total polyphenol content; GAE = gallic acid equivalent; Xt = extract. Different lowercase letters on each column show a statistically significant difference; values are means ± SD (n = 4).

Total Polyphenol Content Response Surface Modelling

In the second-order analysis of the completed experimental design (seventeen experiments: eleven experiments of the first experimental design plus star points) a *p*-value < 0.0001 and an R^2 = 72.80 were obtained for total polyphenol content (TPC), indicating an adjustment of the TPC values to a second-order model. The analysis involved achieving multiple regression coefficients (Table S1), which were used to design the following predicted Equation (1) for the TPC from the habanero pepper (*Capsicum chinense* Jacq.):

$$Y = 15559.1 - 293.297X_1 - 7.219X_2 - 84.728X_3 + 1.236{X_1}^2 + 0.081X_1X_2 + 1.667X_1X_3 + 0.0006{X_2}^2 + 0.0334X_2X_3 + 0.0236{X_3}^2 - 0.0007X_1X_2X_3 \quad (1)$$

Y = TPC (mg GAE/100 g Xt)
X_1 = temperature (°C)
X_2 = pressure (psi)
X_3 = extraction time (min).

According to the mathematical model, in order to achieve the optimal total polyphenol content (TPC) of 1870 mg GAE/100 g extract (Xt) from habanero pepper powder, the following parameters should be applied for supercritical fluid extraction: a temperature (Tp) of 63.1 °C, a pressure (Ps) of 1161.82 psi, and an extraction time (Et) of 132 min. Figure 1 shows the response surface plots (A–C) and contour plots (a–c) obtained through canonical analysis of the total polyphenol content (TPC) values from the experimental design. Figure 1A displays the response surface obtained from the interaction of Ps and Et factors, while Tp was fixed at its optimal value (63.1 °C). The interaction of Tp and Et factors, with a fixed Ps value (1161.82 psi) is depicted in Figure 1B. Both Figure 1A,B exhibit a plateau of maxima and minima, where two zones of maximum response or TPC concentration (red color) and two areas of minimum response or low TPC concentration (blue color) can be observed.

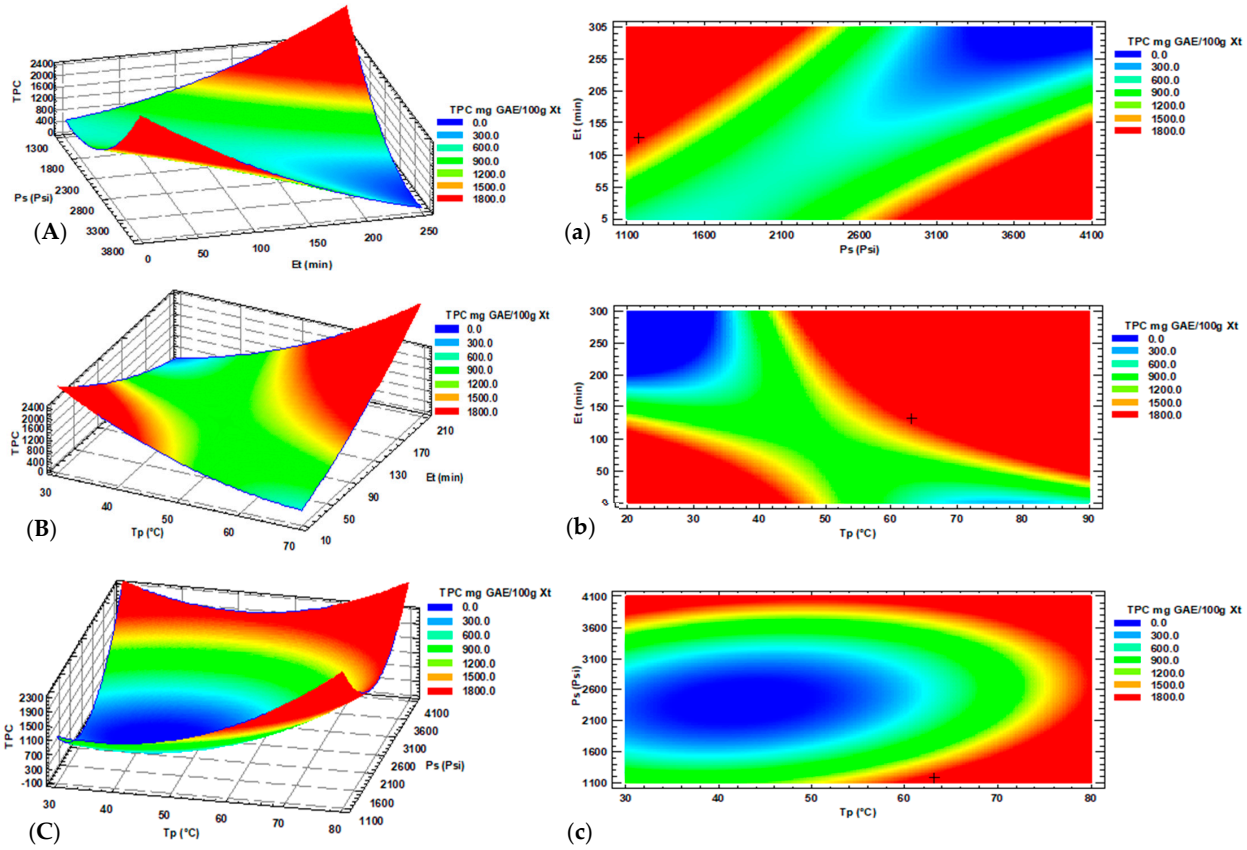

Figure 1. Response surface of total polyphenol content of (**a**) interaction between Ps = pressure and Et = extraction time; (**b**) interaction between Tp = temperature and Et = extraction time; (**c**) interaction between Tp = temperature and Ps = pressure; and contour plots of (**A**) interaction between Ps = pressure and Et = extraction time; (**B**) interaction between Tp = temperature and Et = extraction time; (**C**) interaction between Tp = temperature and Ps = pressure; + indicates the response variable optimal value.

Figure 1C illustrates the response surface obtained from the interaction of Tp and Ps, with a fixed extraction time of 132 min. Each contour plot displays the maximum TPC predicted by the mathematical model, indicated by the symbol "+".

Results of ANOVA for the experimental design showed that the main factors Tp ($p < 0.0001$) and Ps ($p < 0.0001$), the binary interactions of Tp-Ps ($p = 0.0258$), Ps-Et ($p < 0.0452$), the ternary interaction Tp-Ps-Et ($p = 0.0074$), and the quadratic term of the Ps factor ($p < 0.0001$), all exhibited a significant effect on the TPC concentration in the extract obtained from habanero pepper using supercritical fluids (Table S2).

3.2. Individual Polyphenols from Habanero Pepper Extract

From CCD 2^3, it was observed that, under the extraction conditions of Tp = 45 °C, Ps = 1450 psi, and Et = 120 min (experiment #5), the highest concentrations of protocatechuic acid (21.76 ± 0.32 mg/100 g Xt), vanillic acid (96.02 ± 0.15 mg/100 g Xt), ellagic acid (9.78 ± 0.02 mg/100 g Xt), and diosmetin (14.77 ± 0.13 mg/100 g Xt) were obtained. However, protocatechuic acid was not detected in experiments #14, #15, #16, and #17 (Table 3), while ellagic acid was not detected in experiments #16 and #17 and diosmetina was not detected in experiments #14 and #15.

Table 3. Individual polyphenols from the habanero pepper extract obtained by supercritical fluid extraction using a central composite design 2^3.

Exp	Factors			Variables Response *							
	Tp (°C)	Ps (psi)	Et (min)	Protocatechuic Acid	Catechin	Chlorogenic Acid	Coumaric Acid	Cinnamic Acid	Rutin	Quercetin + Luteolin	Kaempferol
1	45	1450	60	12.45 ± 0.03 ^{g}	**143.63 ± 0.12** ^{o}	31.17 ± 0.08 ^{cde}	1.92 ± 0.16 ^{d}	7.39 ± 0.04 ^{j}	7.73 ± 0.09 ^{d}	1.30 ± 0.00 ^{a}	13.84 ± 0.02 ^{cd}
2	60	1450	60	3.76 ± 0.06 ^{c}	4.58 ± 0.02 ^{b}	4.42 ± 0.02 ^{a}	1.65 ± 0.00 ^{b}	2.09 ± 0.01 ^{e}	2.46 ± 0.04 ^{b}	4.91 ± 0.26 ^{b}	3.47 ± 0.14 ^{a}
3	45	2900	60	19.27 ± 0.31 ^{k}	25.95 ± 0.06 ^{i}	81.67 ± 0.24 ^{g}	**3.16 ± 0.29** ^{f}	2.92 ± 0.03 ^{f}	4.40 ± 0.08 ^{c}	24.30 ± 0.21 ^{f}	8.38 ± 2.21 ^{b}
4	60	2900	60	1.42 ± 0.10 ^{b}	3.20 ± 0.63 ^{a}	1.87 ± 0.20 ^{a}	1.60 ± 0.01 ^{b}	1.66 ± 0.00 ^{d}	2.94 ± 0.13 ^{b}	**425.69 ± 3.25** ^{g}	2.33 ± 0.14 ^{a}
5	45	1450	120	**21.76 ± 0.32** ^{l}	15.55 ± 0.02 ^{e}	21.23 ± 0.13 ^{bc}	1.80 ± 0.08 ^{bc}	2.78 ± 0.00 ^{f}	4.44 ± 0.01 ^{c}	1.23 ± 0.35 ^{a}	3.61 ± 0.21 ^{a}
6	60	1450	120	15.44 ± 0.06 ^{h}	137.33 ± 0.12 ^{n}	**288.59 ± 0.76** ^{k}	0.00 ^{a}	**10.35 ± 0.02** ^{k}	0.00 ^{a}	11.71 ± 0.28 ^{de}	67.57 ± 0.08 ^{j}
7	45	2900	120	12.67 ± 0.01 ^{g}	88.92 ± 0.58 ^{m}	164.79 ± 2.47 ^{j}	0.00 ^{a}	0.94 ± 0.00 ^{c}	0.00 ^{a}	9.20 ± 0.09 ^{cd}	39.06 ± 0.92 ^{g}
8	60	2900	120	17.43 ± 0.22 ^{j}	29.39 ± 0.07 ^{k}	27.98 ± 1.34 ^{bcd}	2.53 ± 0.07 ^{e}	6.24 ± 0.31 ^{i}	**21.95 ± 1.14** ^{e}	2.58 ± 1.52 ^{ab}	11.08 ± 0.16 ^{bc}
9	52.5	2175	90	9.74 ± 0.04 ^{e}	24.71 ± 0.06 ^{h}	129.91 ± 1.58 ^{i}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	8.91 ± 0.02 ^{c}	24.56 ± 0.18 ^{f}
10	52.5	2175	90	9.00 ± 0.12 ^{d}	23.11 ± 0.18 ^{fg}	121.24 ± 0.16 ^{i}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	15.04 ± 0.33 ^{e}
11	52.5	2175	90	8.78 ± 0.18 ^{d}	23.05 ± 0.20 ^{f}	110.38 ± 0.97 ^{h}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	9.52 ± 0.03 ^{cd}	22.41 ± 0.10 ^{f}
12	41.9	2175	90	11.94 ± 0.02 ^{f}	27.48 ± 0.25 ^{j}	27.83 ± 0.35 ^{bcd}	0.00 ^{a}	5.15 ± 0.01 ^{h}	0.00 ^{a}	10.46 ± 0.03 ^{cde}	69.52 ± 0.95 ^{j}
13	63.1	2175	90	16.39 ± 0.07 ^{i}	32.88 ± 0.08 ^{l}	41.44 ± 3.42 ^{e}	0.00 ^{a}	0.00	0.00 ^{a}	10.29 ± 0.09 ^{cde}	52.61 ± 0.35 ^{h}
14	52.5	1150	90	0.00 ^{a}	27.65 ± 0.29 ^{j}	30.78 ± 0.52 ^{cd}	0.00 ^{a}	3.87 ± 0.04 ^{g}	0.00 ^{a}	9.14 ± 0.09 ^{cd}	**90.81 ± 3.85** ^{k}
15	52.5	3200	90	0.00 ^{a}	24.27 ± 0.23 ^{gh}	19.27 ± 0.20 ^{b}	0.00 ^{a}	0.59 ± 0.02 ^{b}	0.00 ^{a}	9.84 ± 0.09 ^{cde}	53.50 ± 2.33 ^{h}
16	52.5	2175	48	0.00 ^{a}	9.14 ± 0.06 ^{c}	33.43 ± 0.36 ^{de}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	10.52 ± 0.12 ^{cde}	14.61 ± 0.07 ^{cd}
17	52.5	2175	132	0.00 ^{a}	11.87 ± 1.30 ^{d}	53.73 ± 0.52 ^{f}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	12.44 ± 0.55 ^{e}	62.70 ± 0.25 ^{i}

Exp	Factors			Variables Response *							
	Tp (°C)	Ps (psi)	Et (min)	Vanillic Acid	Ferulic Acid	Ellagic Acid	Diosmin + Hesperidin	Neohesperidin	Naringenin	Apigenin	Diosmetin
1	45	1450	60	10.15 ± 0.06 ^{d}	2.47 ± 0.35 ^{f}	2.30 ± 0.03 ^{c}	1.98 ± 0.01 ^{a}	3.62 ± 0.01 ^{bc}	8.43 ± 0.04 ^{c}	6.30 ± 0.04 ^{c}	4.23 ± 0.01 ^{c}
2	60	1450	60	1.57 ± 0.30 ^{ab}	0.36 ± 0.02 ^{ab}	1.82 ± 0.24 ^{b}	2.20 ± 0.39 ^{a}	1.48 ± 0.49 ^{ab}	0.00	2.55 ± 0.23 ^{b}	2.47 ± 0.17 ^{b}
3	45	2900	60	51.98 ± 0.07 ^{i}	0.86 ± 0.00 ^{bc}	7.71 ± 0.54 ^{i}	0.00	10.99 ± 0.94 ^{d}	**11.42 ± 2.05** ^{d}	**7.49 ± 0.80** ^{d}	9.04 ± 0.11 ^{e}
4	60	2900	60	1.34 ± 0.44 ^{a}	0.00 ^{a}	0.00	0.00	0.00	0.00	0.00	0.00
5	45	1450	120	**96.02 ± 0.15** ^{l}	1.27 ± 0.62 ^{cd}	**9.78 ± 0.02** ^{j}	4.31 ± 0.09 ^{a}	4.44 ± 0.09 ^{c}	7.90 ± 0.11 ^{c}	6.03 ± 0.06 ^{c}	**14.77 ± 0.13** ^{f}
6	60	1450	120	3.67 ± 0.04 ^{bc}	**2.68 ± 0.27** ^{f}	4.27 ± 0.27 ^{e}	0.00	0.00	9.29 ± 0.06 ^{c}	0.00	3.55 ± 0.07 ^{bc}
7	45	2900	120	3.23 ± 0.07 ^{ab}	1.45 ± 0.01 ^{d}	2.98 ± 0.01 ^{d}	0.00	0.00	0.00	0.00	0.00
8	60	2900	120	54.35 ± 0.18 ^{j}	0.25 ± 0.04	6.21 ± 0.00 ^{h}	**54.51 ± 9.59** ^{b}	**16.52 ± 3.31** ^{f}	1.53 ± 0.14 ^{b}	3.01 ± 0.01 ^{b}	9.58 ± 0.19 ^{e}
9	52.5	2175	90	2.28 ± 0.12 ^{ab}	1.27 ± 0.03 ^{cd}	2.93 ± 0.02 ^{d}	0.00	0.00	0.00	0.00	0.00
10	52.5	2175	90	42.18 ± 0.00 ^{h}	0.97 ± 0.00 ^{cd}	2.74 ± 0.02 ^{d}	0.00	0.00	0.00	0.00	0.00
11	52.5	2175	90	35.25 ± 0.17 ^{g}	0.00 ^{a}	2.81 ± 0.01 ^{d}	0.00	0.00	0.00	0.00	0.00
12	41.9	2175	90	36.04 ± 0.07 ^{g}	0.00 ^{a}	5.44 ± 0.10 ^{g}	0.00	0.00	0.00	0.00	0.00
13	63.1	2175	90	66.40 ± 0.18 ^{k}	0.84 ± 0.04 ^{bc}	7.47 ± 0.27 ^{i}	1.67 ± 0.04 ^{a}	0.00	0.00	0.00	0.00
14	52.5	1150	90	25.91 ± 0.19 ^{e}	0.82 ± 0.02 ^{bc}	4.99 ± 0.00 ^{f}	0.00	0.00	0.00	0.00	0.00
15	52.5	3200	90	30.28 ± 0.10 ^{f}	0.00 ^{a}	2.88 ± 0.02 ^{d}	0.00	0.00	0.00	0.00	0.00
16	52.5	2175	48	30.93 ± 2.88 ^{f}	0.86 ± 0.00 ^{bc}	0.00	0.00	0.00	0.00	0.00	6.40 ± 1.02 ^{d}
17	52.5	2175	132	5.49 ± 0.12 ^{c}	1.17 ± 0.02 ^{cd}	0.00	0.00	0.00	0.00	0.00	6.32 ± 1.18 ^{d}

Note: Tp = temperature; Ps = pressure; Et = extraction time; * all response variables were reported as mg/100 g of extract. Different lowercase letters on each column show a statistically significant difference; values are means ± SD (n = 3). Bold shows the highest values.

3.2.1. Catechin

Under supercritical extraction conditions of 45 °C, 1450 psi, and 60 min of extraction (experiment #1), the highest concentration of catechin (143.63 ± 0.12 mg/100 g Xt) was obtained, while the lowest concentration of this metabolite was achieved under 60 °C, 2900 psi, and a 60 min (experiment #4).

3.2.2. Chlorogenic, Cinnamic, and Ferulic Acid

The highest concentrations of chlorogenic acid (288.59 ± 0.76 mg/100 g Xt), cinnamic acid (10.35 ± 0.02 mg/100 g Xt), and ferulic acid (2.68 ± 0.27 mg/100 g Xt) were achieved by supercritical fluid extraction from habanero pepper powder at a temperature of 60 °C, a pressure of 1450 psi, and an extraction time of 120 min. Regarding chlorogenic acid, the lowest concentration (1.87 ± 0.20 mg/100 g Xt) was detected when extraction was performed at 60 °C, with Ps = 2900 psi and Et = 60 min. Cinnamic acid (#9, #10, #11, #13, #16, and #17) and ferulic acid (#4, #11, #12, and #15) were not detected in the extracts obtained from several experiments of the central composite design (CCD) 2^3.

3.2.3. Coumaric Acid, Naringenin, and Apigenin

Other phenolic compounds that presented a high concentration in habanero pepper extracts were coumaric acid (3.16 ± 0.29 mg/100 g Xt), naringenin (11.42 ± 2.05 mg/100 g Xt), and apigenin (7.49 ± 0.80 mg/100 g Xt) under the conditions of experiment #3, at 45 °C in combination with 1450 psi and 60 min. In several extracts obtained from different experiments (#6, #7 and #9 to #17), the aforementioned metabolites were not detected.

3.2.4. Rutin, Diosmin, Hesperidin, and Neohesperidin

Finally, in experiment #8 (Tp = 60 °C, Ps = 2900 psi, and Et = 120 min), high concentrations of rutin (21.95 ± 1.14 mg/100 g Xt), diosmin + hesperidin (54.51 ± 9.59 mg/100 g Xt), and neohesperidin (16.52 ± 3.31 mg/100 g Xt) were obtained. These metabolites were not detected under the conditions of the central points (#9, #10, and #11) and star points (#12 to #17) of the CCD 2^3.

3.2.5. Individual Polyphenol Response Surface Modeling

Table 4 presents the *p*-values, R^2 values, and the prediction equation for the second-order model obtained from the statistical analysis of each individual polyphenol value derived from the 2^3 central composite design. The analysis was conducted to optimize the conditions of supercritical fluid extraction for phenolic compounds from habanero peppers.

According to the analysis of the results, all individual polyphenols were adjusted ($p < 0.05$) to a second-order mathematical model. However, protocatechuic acid and kaempferol exhibited a coefficient of determination (R^2) below 0.7, suggesting that the model, as well as the response surface and contour plot (Figures S2 and S3) may not be suitable for predicting their concentrations in habanero pepper extracts obtained by supercritical fluids under varying conditions of temperature, pressure, and extraction time.

The optimal conditions, as well as the optimal predicted values for each individual polyphenol obtained from the canonical analysis, are shown in Table 5.

Based on the data presented in Table 5, five distinct groups of individual polyphenols exhibited comparable optimal extraction conditions. One group comprises protocatechuic acid, cinnamic acid, quercetin + luteolin, ferulic acid, and naringenin, which share similar extraction conditions involving temperature (41.9 °C), pressure (3180–3200 psi), and extraction time (48–53.5 min). Another group consists of catechin, chlorogenic acid, cinnamic acid, and kaempferol, which shared a pressure of 1150 psi, an extraction time of 132 min with a temperature of 62.8 °C to 63.1 °C. Moreover, rutin, diosmin + hesperidin, and neohesperidin exhibited optimal conditions for supercritical fluid extraction at 63 °C, 3200 psi, and an extraction time of 132 min. A fourth group was found with an optimal temperature of 41.9 °C, pressure of 1150–1186 psi, and extraction time of 131.43–132 min and consisted of vanillic acid, ellagic acid, and diosmetin.

Table 4. Multiple linear regression results for the values of individual polyphenols obtained from the 2^3 central composite design.

IP	R^2	Prediction Equation (Y)
PtAc	65.5	$190.68 - 9.90X_1 + 0.085\,X_2 + 1.47\,X_3 + 0.1081X_1^2 - 0.0013\,X_1X_2 - 0.02\,X_1X_3 - 0.0000019\,X_2^2 - 0.00088\,X_2X_3 - 0.0010\,X_3^2 + 0.000015X_1X_2X_3$
Ctn	79.2	$3574.97 - 73.95X_1 - 1.13\,X_2 - 33.61\,X_3 + 0.1936\,X_1^2 + 0.02\,X_1X_2 + 0.62\,X_1X_3 + 0.000016X_2^2 + 0.0124\,X_2X_3 + 0.0013\,X_3^2 - 0.0002\,X_1X_2X_3$
ChAc	77	$1573.03 - 29.89X_1 - 0.58\,X_2 - 34.73\,X_3 - 0.1154\,X_1^2 + 0.01\,X_1X_2 + 0.73\,X_1X_3 - 0.000021\,X_2^2 + 0.0136\,X_2X_3 - 0.0019\,X_3^2 - 0.0002\,X_1X_2X_3$
CuAc	72.9	$6.07 - 0.433\,X_1 + 0.01\,X_2 + 0.30\,X_3 + 0.0093\,X_1^2 - 0.0003\,X_1X_2 - 0.008\,X_1X_3 + 0.00000099\,X_2^2 - 0.0002\,X_2X_3 + 0.0006\,X_3^2 + 0.0000043\,X_1X_2X_3$
CnAc	82.4	$217.45 - 5.46\,X_1 - 0.04\,X_2 - 1.20\,X_3 + 0.0328\,X_1^2 + 0.0004\,X_1\,X_2 + 0.02\,X_1\,X_3 + 0.0000031\,X_2^2 + 0.00024\,X_2\,X_3 + 0.00064\,X_3^2 - 0.0000048\,X_1\,X_2\,X_3$
Rt	85.4	$96.99 - 2.61\,X_1 + 0.02\,X_2 + 0.66\,X_3 + 0.0323\,X_1^2 - 0.0009\,X_1X_2 - 0.02\,X_1X_3 + 0.0000034\,X_2^2 - 0.00079\,X_2X_3 + 0.002\,X_3^2 + 0.000017\,X_1X_2X_3$
Q + L	88.7	$-201.156 + 4.13\,X_1 + 0.11\,X_2 + 0.48\,X_3 - 0.0179X_1^2 - 0.002\,X_1X_2 - 0.003\,X_1X_3 - 0.0000027\,X_2^2 - 0.00049\,X_2X_3 - 0.00053\,X_3^2 + 0.0000071\,X_1X_2X_3$
Kpf	49.4	$732.83 - 16.18\,X_1 - 0.31\,X_2 - 7.36\,X_3 + 0.0329\,X_1^2 + 0.005\,X_1X_2 + 0.19\,X_1X_3 + 0.000014\,X_2^2 + 0.0037\,X_2X_3 - 0.0108\,X_3^2 - 0.000073\,X_1X_2X_3$
VaAc	76.3	$-1057.69 + 10.58\,X_1 + 0.64\,X_2 + 19.12\,X_3 + 0.1012\,X_1^2 - 0.01\,X_1X_2 - 0.30\,X_1X_3 - 0.000011\,X_2^2 - 0.0079\,X_2X_3 - 0.0122\,X_3^2 + 0.00014\,X_1X_2X_3$
FeAc	86.1	$-30.61 - 1.70\,X_1 + 0.07\,X_2 + 1.78\,X_3 + 0.0385X_1^2 - 0.0013\,X_1X_2 - 0.03\,X_1X_3 + 0.0000017\,X_2^2 - 0.00082\,X_2X_3 - 0.0011\,X_3^2 + 0.000014\,X_1X_2X_3$
EgAc	79.3	$2.952 - 2.06\,X_1 + 0.03\,X_2 + 1.42\,X_3 + 0.0341\,X_1^2 - 0.0007\,X_1X_2 - 0.02\,X_1X_3 + 0.0000012\,X_2^2 - 0.0005\,X_2X_3 - 0.0014\,X_3^2 + 0.0000099\,X_1X_2X_3$
D + H	82.9	$10.23 - 1.38\,X_1 + 0.10\,X_2 + 2.67\,X_3 + 0.0513\,X_1^2 - 0.003\,X_1X_2 - 0.07\,X_1X_3 + 0.0000046\,X_2^2 - 0.002\,X_2X_3 + 0.0028\,X_3^2 + 0.000045\,X_1X_2X_3$
NeHe	82.4	$-32.62 - 0.27\,X_1 + 0.08\,X_2 + 1.49\,X_3 + 0.0273\,X_1^2 - 0.002\,X_1X_2 - 0.035\,X_1X_3 + 0.0000029\,X_2^2 - 0.0011\,X_2X_3 + 0.0017\,X_3^2 + 0.000022\,X_1X_2X_3$
Ngn	76.8	$126.47 - 3.70\,X_1 + 0.01\,X_2 - 0.49\,X_3 + 0.0284\,X_1^2 - 0.0003\,X_1X_2 + 0.007\,X_1X_3 + 0.0000030\,X_2^2 - 0.00023\,X_2X_3 + 0.0018\,X_3^2 + 0.0000023\,X_1X_2X_3$
Agn	73.5	$14.26 - 0.88\,X_1 + 0.03\,X_2 + 0.66\,X_3 + 0.0187\,X_1^2 - 0.0007\,X_1X_2 - 0.02\,X_1X_3 + 0.0000019\,X_2^2 - 0.00052\,X_2X_3 + 0.0012\,X_3^2 + 0.00000979\,X_1X_2X_3$
Dmt	91.7	$-79.59 + 1.36\,X_1 + 0.08\,X_2 + 1.45\,X_3 + 0.0121\,X_1^2 - 0.0015\,X_1X_2 - 0.04\,X_1X_3 + 0.0000013\,X_2^2 - 0.0011\,X_2\,X_3 + 0.0043\,X_3^2 + 0.00002\,X_1X_2\,X_3$

Note: IP = individual polyphenol; PtAc = protocatechuic acid; Ctn = catechin; ChAc = chlorogenic acid; CuAc = coumaric acid; CnAc = cinnamic acid; Rt = rutin; Q+L = quercetin + luteolin; Kpf = kaempferol; VaAc = vanillic acid; FeAc = ferulic acid; EgAc = ellagic acid; D + H = diosmetin + hesperidin; NeHe = neohesperidin; Ngn = naringenin; Agn = apigenin; Dmt = diosmetin; X_1 = temperature (°C); X_2 = pressure (psi); X_3 = extraction time (min); psi = pound per square inch; min = minutes; Y = individual polyphenol of interest.

Table 5. Optimal conditions and predicted values of individual polyphenols extracted from habanero pepper by supercritical fluid extraction.

Individual Polyphenol	Optimal Conditions			Optimal Value (mg/100 g Xt)
	Tp (°C)	Ps (psi)	Et (min)	
protocatechuic acid	41.9	3200	48	29.16
catechin	62.8	1150	132	236.27
chlorogenic acid	63.1	1150	131.9	447.08
coumaric acid	41.9	3200	48.3	5.54
cinnamic acid	63.1	1150	132	21.96
rutin	63.03	3200	132	40.05
quercetin + luteolin	41.9	3200	53.53	32.88
kaempferol	62.9	1150	132	130.31
vanillic acid	41.9	1150	132	136.38
ferulic acid	41.9	3199	48.22	18.60
ellagic acid	41.9	1150	131.43	14.63
diosmin + hesperidin	63.03	3200	132	92.80
neohesperidin	63.1	3200	131.99	31.98
naringenin	41.9	3189	48	21.11
apigenin	43.3	3200	48	11.74
diosmetin	41.9	1186	132	26.65

Note: Tp = temperature; Ps = pressure; Et = extraction time; psi = pound per square inch; min = minutes; Xt = habanero pepper extract.

Figure 2 displays the response surfaces and contour plots illustrating the Tp-Ps interactions while maintaining Et fixed at its optimal value (according to individual polyphenols, Table 5). The selection criteria for these plots was a majority polyphenol with a coefficient of determination greater than 0.75.

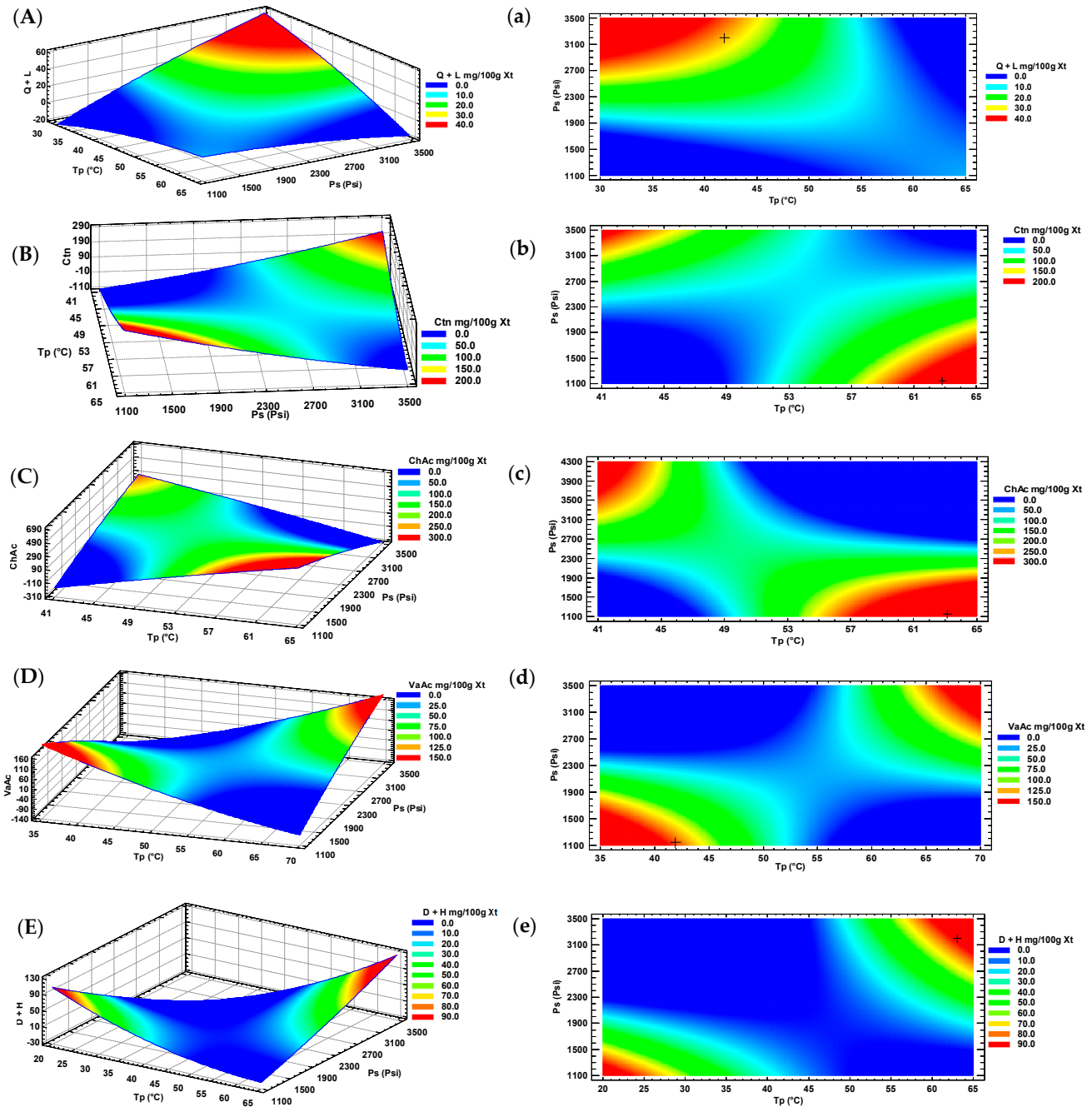

Figure 2. Temperature (Tp) and pressure (Ps) interaction. Response surface (capital letters) and contour plots (lowercase letter) of individual polyphenols; (**A**), (**a**) quercetin + luteolin; (**B**), (**b**) chlorogenic acid; (**C**), (**c**) catechin; (**D**), (**d**) vanillic acid; (**E**), (**e**) diosmetin + hesperidin; Xt = habanero pepper extract; "+" indicates the response variable optimal value.

All the predominant individual polyphenols exhibited behavior that corresponds to a saddle-shaped plateau of maxima and minima, where two areas of maximum response (red color) can be observed, separated by a wide area of blue color where temperature intersects pressure, resulting in a minimum concentration of the metabolite in the extract.

While the majority of polyphenols exhibit similar behavior when extracted by supercritical fluids under conditions of temperature, pressure, and extraction time, are affected differently by those factors. Table 6 presents the results of the ANOVA for each individual polyphenol and the significant effect for each factor from the CCD 2^3.

Table 6. Individual polyphenols *p*-value in the CCD 2^3.

Source	Individual Polyphenol *p*-Value													
	Ctn	**ChAc**	**CuAc**	**Rtn**	**Q + L**	**VaAc**	**FeAc**	**EgAc**	**D + H**	**NeHe**	**Ngn**	**Apg**	**Dmt**	**CnAc**
A	0.1064	0.7692	0.5033	0.0770	0.0117	0.1508	0.0037	0.2016	0.0020	0.8514	0.0052	0.0009	0.0011	<0.0001
B	0.0079	0.3333	0.2405	0.0190	0.0024	0.8847	0.6914	0.0490	0.0059	0.0034	0.0305	0.2583	0.1661	<0.0001
C	0.0863	0.0005	0.0194	0.1405	0.4243	0.1559	0.0159	0.0004	0.0014	0.3868	0.8405	0.0640	0.0014	0.0077
AA	0.0634	0.5317	0.0037	0.0054	0.1239	0.2007	<0.0001	<0.0001	0.0762	0.0120	0.0090	0.0116	0.0799	0.0000
AB	0.1593	<0.0001	0.0289	<0.0001	<0.0001	0.0041	0.6030	0.1270	0.0001	0.0130	0.5335	0.0988	0.0001	0.3248
AC	<0.0001	0.0114	0.0612	<0.0001	0.0001	0.5742	0.0030	0.3208	0.0005	<0.0001	<0.0001	0.0133	0.0021	<0.0001
BB	0.1302	0.2798	0.0037	0.0054	0.0315	0.1875	0.0120	0.0756	0.1264	0.0120	0.0091	0.0117	0.0801	<0.0001
BC	0.0711	0.0388	0.7343	0.0002	0.0002	0.0148	0.0003	0.0690	0.0002	0.1832	0.0004	0.5946	0.0004	0.5573
CC	0.8293	0.8682	0.0032	0.0047	0.4614	0.0195	0.0040	0.0012	0.1194	0.0106	0.0079	0.0103	<0.0001	0.0158
ABC	<0.0001	0.0002	0.0002	<0.0001	0.0560	<0.0001	<0.0001	0.0001	0.0001	<0.0001	0.4944	0.0003	<0.0001	0.0015

Note: A = temperature; B = pressure; C = extraction time; Ctn = catechin; ChAc = chlorogenic acid; CuAc = coumaric acid; Rt = rutin; Q + L = quercetin + luteolin; VaAc = vanillic acid; FeAc = ferulic acid; EgAc = ellagic acid; D + H = diosmin + hesperidin; NeHe = neohesperidin; Ngn = naringenin; Apg = apigenin; Dtm = diosmetin; CnAc = cinnamic acid; values under 0.05 indicate a significant effect on the concentration of the metabolite in the habanero pepper extract. The factors that show an effect on the individual polyphenol are in red color.

The triple interaction (Tp, Ps, and Et) presented a significant effect on the concentration of the individual polyphenols, while did not show a significant effect on the concentration of quercetin + luteolin (Q + L) and naringenin. However, the double interactions of the factors temperature–pressure ($p < 0.0001$), temperature–extraction time ($p = 0.0001$), and pressure–extraction time ($p = 0.0002$) presented an effect on Q + L, while only the double interaction of temperature with extraction time showed an effect ($p < 0.0001$) on naringenin.

Individually, pressure was the main factor that affected (Table 6) a greater number of individual polyphenols (Ctn, Rtn, Q + L, EgAc, D + H, NeHe, Ngn, and CnAc), followed by temperature (Q + L, FeAc, D + H, Ngn, Apg, Dmt, and CnAc) and extraction time (ChAc, CuAc, FeAc, EgAc, D + H, Dmt, and CnAc).

3.3. Principal Component Analysis (PCA)

The PCA allowed us to identify the experimental conditions that mostly influence the concentration of phenolic compounds extracted from habanero pepper, as well as to determine the interaction between variables. According to this analysis (Figure 3), the individually extracted polyphenols showed an association with the extraction conditions of experiment #1 (A), temperature of 45 °C, pressure of 1450 psi, and extraction time of 60 min (Figure 3a). Also, it can be observed in Figure 3b that cluster #1 formed by the individual polyphenols and CTP shows similarities with cluster #2 formed by experiments #2 (B), #9-#11 (I), #12 (J), #13 (K), #14 (L), #15 (M), #16 (N), and #17 (O).

On the other hand, in Figure 3b, cluster #3 consisting of experiments #3 (C), #5 (E), and #8 (H), does not exhibit similar characteristics to the other experiments, or any association with the phenolic compounds extracted from the habanero pepper leaf. Similar behavior is observed with the last cluster (#4), which only registered experiment #6 (F) under the extraction conditions of temperature 60 °C, pressure 1450 psi, and extraction time 120 min.

Finally, it can be observed that chlorogenic acid (4), quercetin + luteolin (8), and kaempferol exhibit a positive association with CTP (1), whereas rutin (7), vanillic acid (10), and diosmin + hesperidin (13) show a negative association with the total polyphenol content.

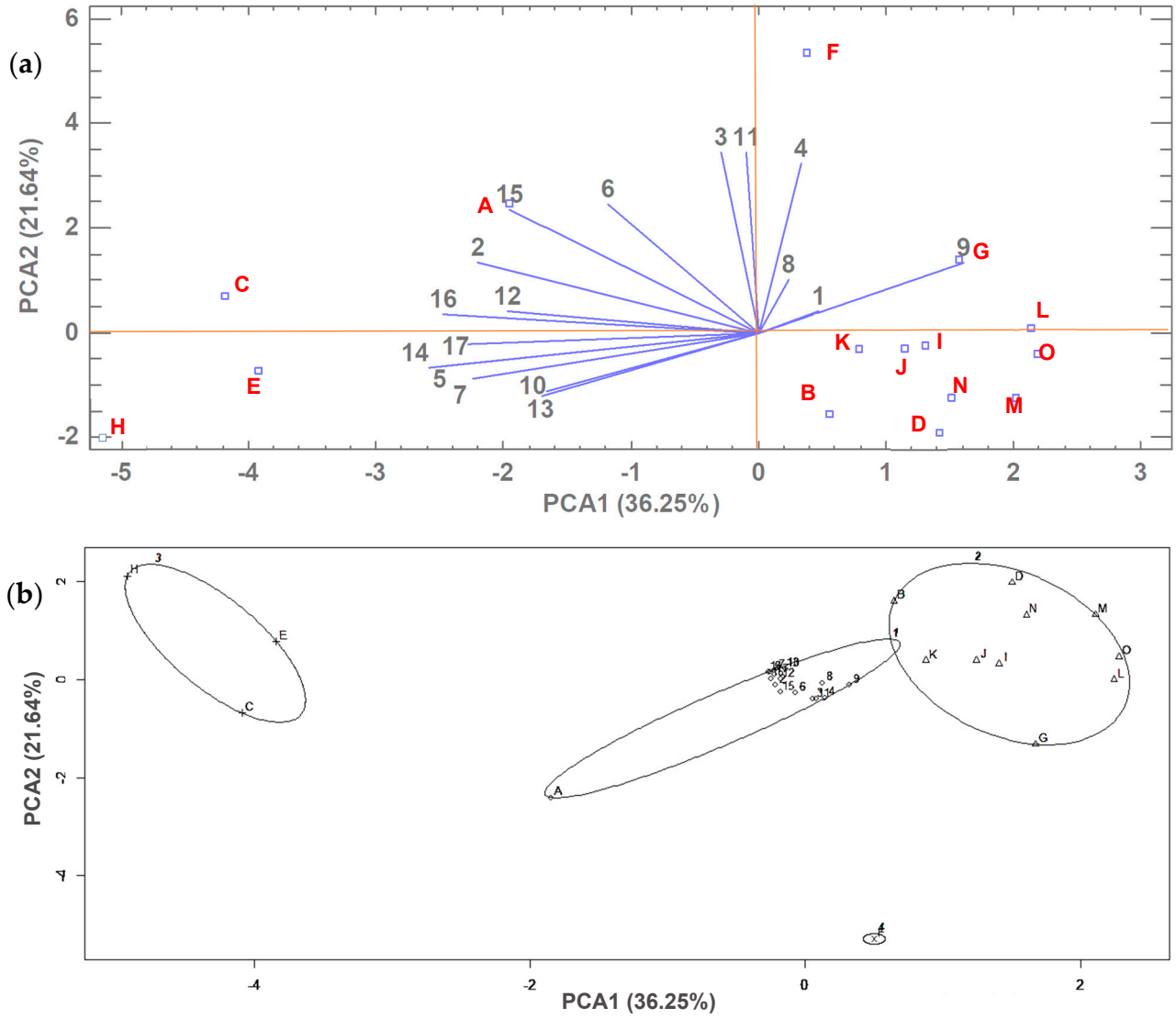

Figure 3. Principal component analysis (**a**) and cluster of k means (**b**) depending on experiments from CCD 2^3 for the evaluation of the factors temperature (Tp), pressure (Ps), and extraction time (Et). Numerations and capital letters: 1 = total polyphenol content (TPC); 2 = protocatechuic acid; 3 = catechin; 4 = chlorogenic acid; 5 = coumaric acid; 6 = cinnamic acid; 7 = rutin; 8 = quercetin + luteolin; 9 = kaempferol; 10 = vanillic acid; 11 = ferulic acid; 12 = ellagic acid; 13 = diosmin + hesperidin; 14 = neohesperidin; 15 = naringenin; 16 = apigenin; 17 = diosmetin; A = Tp (45 °C), Ps (1450 psi), Et (60 min); B = Tp (60 °C), Ps (1450 psi), Et (60 min); C = Tp (45 °C), Ps (2900 psi), Et (60 min); D = Tp (60 °C), Ps (2900 psi), Et (60 min); = E = Tp (45 °C), Ps (1450 psi), Et (120 min); F = Tp (60 °C), Ps (1450 psi), Et (120 min); G = Tp (45 °C), Ps (2900 psi), Et (120 min); H = Tp (60 °C), Ps (2900 psi), Et (120 min); I = Tp (52.5 °C), Ps (2175 psi), Et (90 min); J = Tp (41.9 °C), Ps (2175 psi), Et (90 min); K = Tp (63.1 °C), Ps (2175 psi), Et (90 min); L = Tp (52.5 °C), Ps (1150 psi), Et (90 min); M = Tp (52.5 °C), Ps (3200 psi), Et (90 min); N = Tp (52.5 °C), Ps (2175 psi), Et (648 min); O = Tp (52.5 °C), Ps (2175 psi), Et (132 min).

4. Discussion

According to canonical analysis, the optimal conditions for obtaining a maximum extracted concentration of total polyphenols (1870 mg GAE/100 g Xt) from habanero pepper were a temperature (Tp) of 63.1 °C, a pressure (Ps) of 1161.82 psi, and an extraction time (Et) of 132 min. The temperature chosen aligns with previous findings in the literature regarding the extraction of phenolic compounds from various sources. For instance, Gelmez et al. [29] optimized the supercritical fluid extraction conditions for phenolic compounds

from raw wheat germ, exploring different temperature ranges (44–60 °C) and determining an optimal temperature of 59 °C to achieve a concentration of 955 mg GAE/100 g extract. Consistent with our study, it was observed that temperature, as well as the interaction with pressure, significantly affected ($p < 0.05$) the total polyphenol concentration of the extract. Grande-Villanueva et al. [20] also reported a higher concentration of phenolic compounds (367 mg GAE/100 g extract) extracted from *Capsicum annuum* at a temperature of 60 °C and a pressure of 4351.13 psi. They conducted the analysis of different extracts obtained at temperatures of 40 °C and 60 °C, using a range of pressures from 2175 psi to 5076.32 psi. As stated by Wu et al. [30], increasing the temperature during supercritical fluid extraction of phenolic compounds improves the solubility of these metabolites in CO_2. Additionally, this temperature change promotes volatilization, leading to an increase in extraction efficiency. However, it is important to note that higher temperatures can result in a decrease in CO_2 density, reducing the solvent capacity and potentially yielding an extract with lower phenolic compound content. Therefore, it is crucial to simultaneously increase both temperature and pressure, which explains the interaction effect of these two factors. In the present study, the optimal pressure (1161.82 psi) required to obtain a polyphenol-rich extract from habanero pepper was lower than reported in the literature. This can be attributed to the use of ethanol (20% v/w) during the extractions, which maintains a high solvent density throughout the process and induces swelling of the food matrix particles. Consequently, this reduces the pressure needed to achieve a high concentration of the target metabolites [31], leading to lower operational requirements of the equipment (electricity consumption) and reducing production costs [30].

Temperature and pressure also play a crucial role in optimizing the extraction conditions to obtain individual phenolic compounds. Catechin, a distinctive phenolic compound found in habanero pepper, has been observed to present a high concentration within extracts obtained using supercritical fluids. According to the mathematical model obtained in this study, the maximum concentration of catechin was achieved using conditions of Tp = 62.8 °C, Ps = 1150 psi, and Et = 132 min. These results differ from those reported by Sökmen et al. [32], where they evaluated pressure, temperature, and extraction time factors using ethanol as a co-solvent for the extraction of catechin from green tea. The best extraction performance was obtained with a temperature of 60 °C, a pressure of 3625.94 psi, and an extraction time of 3 h. This difference could be attributed to the extraction method of the present study, which used a static mode instead of a dynamic mode [32]. The static mode allows for a longer contact time between the solvent (CO_2) and the co-solvent (ethanol) with the sample; this permits 1) higher catechin extraction at lower temperatures and pressures (due to a higher density of the co-solvent) and 2) to facilitate saturation of the metabolite (catechin) in the solvent by enlarging extraction time, as predicted by the mathematical model [33–35]. Another major compound in the extracts obtained by SFE was chlorogenic acid, which exhibited optimal extraction conditions similar to catechin, at a temperature of 63.1 °C, a pressure of 1150 psi, and an extraction time of 131.9 min. Daraee et al. [36] reported optimal extraction conditions for chlorogenic acid from sunflower seeds (*Helianthus annus*) at 40 °C, with a pressure of 2451.14 psi, and an extraction time of 104.6 min. These conditions are consistent with the predicted conditions of the mathematical model in the current study. An important effect of temperature and pressure on the concentration of chlorogenic acid in the extract was also reported. The increase in temperature raises the vapor pressure of the metabolite, resulting in better yields at low pressures. Conversely, an increase in pressure promotes an increase in solvent and co-solvent density, leading to better yields at low temperatures. Pellicanò et al. [37] reported that phenolic compounds such as catechin, chlorogenic acid, quercetin, naringenin, and luteolin are positively affected (higher concentration) by increasing pressure during supercritical fluid extraction of tomato peel (*Solanum lycopersicum*). Quercetin, another phenolic compound obtained in high concentration in habanero pepper extracts, was optimized under the conditions of 41.9 °C and a pressure of 3200 psi, with a predicted concentration of 32 mg/100 g Xt. Similar conditions were reported by Ekinci [38], who

aimed to optimize quercetin extraction from sumac fruit (*Rhus coriaria* L.) using SFE. In this study, different temperatures (40–70 °C), pressures (2175.57–3625.94 psi), and ethanol percentages as a co-solvent (2–6% *w*/*w*) were evaluated. The optimization revealed that the optimal conditions were a temperature of 40 °C, a pressure of 3625.94 psi, and 6% ethanol content, resulting in concentrations of 2196 µg/100 g of sumac fruit, which were 16% higher compared to other extraction conditions. Unlike the presented study, the interaction of temperature and pressure was significant ($p > 0.05$), which could be attributed to the low ethanol concentration (6%) and the polar nature of quercetin [39].

Vanillic acid from habanero pepper was also optimized under SFE conditions of Tp = 41.9 °C, Ps = 1150 psi, and Et = 132 min. It was observed that, similar to other optimized polyphenols, the double interaction of pressure with temperature, as well as the triple interaction of the extraction factors, had a significant effect ($p < 0.05$). According to Farías-Campomanes et al. [40], the best extraction conditions for vanillic acid by SFE involve a temperature close to 40 °C (313 K), a pressure of 2900.75 psi, and an extraction time of 120 min. These conditions were established based on the extraction of phenolic compounds from *Vitis vinifera* grape residues.

The extracts obtained from habanero pepper by SFE exhibited high concentrations of different individual polyphenols considered of interest due to their bioactive properties. For instance, catechin develops anti-inflammatory, antioxidant, and antiobesogenic properties, the latter attributed to its ability to improve lipid metabolism and microbiota [41]. Chlorogenic acid has also been extensively studied for its bioactive properties, particularly for its capacity to mitigate and prevent the development of metabolic syndrome [42]. Additionally, several clinical studies have demonstrated its positive effects in the treatment of obesity [43], diabetes [44], and hypertension [45].

On the other hand, quercetin and luteolin exhibit different bioactive properties compared to the aforementioned metabolites. Quercetin can prevent the onset of hyperuricemia by inhibiting various enzymes (adenosine deaminase, xanthine oxidase, and ketohexokinase) that promote urate production. Additionally, it can be employed for the prevention of COVID-19 by targeting the S-receptor-binding protein's domain of SARS-CoV-2, as demonstrated by Shabir et al. [46]. In contrast, luteolin demonstrates potent effects in modulating proinflammatory mechanisms. It has the capacity to hinder the NF-kB transcription factor, thereby impeding the inflammatory response resulting from the synthesis and formation of the NLRP3 protein complex together with the proinflammatory cytokine pro-IL-1B. This particular attribute of luteolin supports potential therapeutic benefits for conditions determined by imbalanced inflammatory responses, such as arthritis, as proposed by Caporali et al. [47]. Finally, it is important to optimize the vanillic acid of SFE extraction. The vanillic acid found in extracts of habanero pepper has been recently studied for its promising neuroprotective capacity against inflammation induced by bacterial lipopolysaccharides (LPS) that trigger neuroinflammation [48], which is associated with the onset of diseases such as Alzheimer's [49] and Parkinson's [50].

This study presents innovative evidence that enables the targeted extraction of specific phenolic compounds from Habanero pepper using supercritical fluid extraction (SFE), resulting in extracts with high phenolic concentration. Furthermore, it is demonstrated that the extraction of a phenolic-rich extract is suitable for utilization in the pharmaceutical or food industry due to the bioactive properties of its components. However, given the high purity achieved through SFE and the sensitivity that phenolic compounds exhibit to temperature, ultraviolet light, and moisture, among others, it is recommended to explore options that preserve the bioactive characteristics, such as microencapsulation and various techniques like spray drying, spray cooling, freeze drying, and coacervation [51,52].

5. Conclusions

It has been demonstrated that supercritical fluid extraction technology is a favorable and sustainable option for extracting phenolic compounds from habanero peppers. The results have shown that by modifying the factors of temperature, pressure, and extraction

time, it is possible to direct the extraction process toward specific phenolic compounds. Through the optimization of extraction conditions, extracts with high concentrations of phenolic compounds were obtained, with temperature and pressure being the most influential factors in the extraction of individual polyphenols. It was generally observed that the 19 identified polyphenols in the extracts exhibited an association with the extraction conditions of 45 °C, 1450 psi, and 60 min.

Finally, the extracts obtained from habanero peppers through supercritical fluid extraction technology can be considered bioactive ingredients to develop pharmaceuticals, nutraceuticals, and functional foods for the prevention and treatment of diseases such as arthritis, diabetes, and obesity, among others. This study suggested conducting future studies to evaluate different preservation techniques for preserving the bioactive properties of the Habanero pepper leaf extract. Some encapsulation techniques, such as spray drying, freeze-drying, or coacervation, could be useful in increasing their shelf life.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr11072055/s1, Table S1: multiple regression coefficients for TPC; Table S2: ANOVA of complete experimental design of TPC; Figure S1: chromatogram corresponding to calibration curve of individual polyphenols; (a) polyphenol calibration curve Mix 1; (b) polyphenol calibration curve Mix 2; (c) polyphenol calibration curve Mix 3. Numeration: 1 = gallic acid; 2 = protocatechuic acid; 3 = catechin; 4 = chlorogenic acid; 5 = ferulic acid; 6 = cumaric acid; 7 = cinnamic acid; 8 = rutin; 9 = quercetin + luteolin; 10 = kamepferol; 11 = vanillin; 12 = ellagic acid; 13 = diosmin + hesperidin; 14 = neohesperidin; 15 = naringenin; 16 = apigenin; 17 = diosmetin; Figure S2: Response surface plots (capital letters): A) Temperature (Tp) interaction with extraction time (Et) of protocatechuic acid (PtAc); B) Pressure (Ps) interaction with extraction time (Et) of PtAc; C) Temperature (Tp) interaction with pressure (Ps) of PtAc; D) Temperature (Tp) interaction with extraction time (Et) of protocatechuic acid (PtAc); E) Pressure (Ps) interaction with extraction time (Et) of Kfp; F) Temperature (Tp) interaction with pressure (Ps) of Kfp; and contour plots (lowercase): a) Tp interaction with Et of PtAc; b) Ps interaction with Et of PtAc; c) Tp interaction with Ps of PtAc; d) Tp interaction with Et of Kfp; e) Ps interaction with Et of Kfp; f) Tp interaction with Ps of Kfp. Abbreviations: Xt = Habanero pepper extract. Figure S3: Response surface plots (capital letters): A) Temperature (Tp) interaction with extraction time (Et) of Kaempferol (Kpf); B) Pressure (Ps) interaction with extraction time (Et) of PtAc; C) Temperature (Tp) interaction with pressure (Ps) of PtAc; D) Temperature (Tp) interaction with extraction time (Et) of kaempferol (Kfp); E) Pressure (Ps) interaction with extraction time (Et) of Kfp; F) Temperature (Tp) interaction with pressure (Ps) of Kfp; and contour plots (lowercase): a) Tp interaction with Et of PtAc; b) Ps interaction with Et of PtAc; c) Tp interaction with Ps of PtAc; d) Tp interaction with Et of Kfp; e) Ps interaction with Et of Kfp; f) Tp interaction with Ps of Kfp. Abbreviations: Xt = Habanero pepper extract.

Author Contributions: Conceptualization, I.M.R.-B. and K.A.A.-B.; methodology, K.A.A.-B.; software, K.A.A.-B. and I.M.R.-B.; validation, I.M.R.-B., M.O.R.-S., M.S. and G.F.; formal analysis, I.M.R.-B., M.O.R.-S., and M.S.; investigation, K.A.A.-B. and I.M.R.-B.; resources, I.M.R.-B.; data curation, I.M.R.-B. and M.S.; writing—original draft preparation, K.A.A.-B.; writing—review and editing, I.M.R.-B., M.O.R.-S., and M.S.; visualization, I.M.R.-B.; supervision, I.M.R.-B.; project administration, I.M.R.-B.; funding acquisition, I.M.R.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Council of Science and Technology of México (CONACYT), which financed project No. 257588, and scholarship 661099 for Kevin Alejandro Avilés-Betanzos.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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