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Antioxidant-Rich Extracts from Lemon Verbena (*Aloysia citrodora* L.) Leaves through Response Surface Methodology

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Abstract: A member of the Verbenaceae family, *Aloysia citrodora*, or lemon verbena, is a medicinal herb with antioxidant compounds. The aim of this study was to develop a green, optimized method for the bioactive compound (carotenoids, ascorbic acid, and polyphenols) extraction from lemon verbena leaves through response surface methodology (RSM). The bioactive compound recovery was shown to be significantly affected by the extraction technique (both with pulsed electric field and ultrasound-assisted extraction), along with an extraction solvent, based on partial least squares analysis. Consequently, the maximum polyphenol yield required a double-assisted extraction with a relatively low extraction duration (60 min) at a high temperature (80 °C), with a moderate-polarity extraction solvent (50% *v*/*v* ethanol). With the optimized method, the total polyphenol content (TPC) was measured at 175.03 mg gallic acid equivalents/g, whereas chromatographic analysis revealed that verbascoside was the most prevalent polyphenol (132.61 mg/g). The optimum extract provided a high antioxidant capacity through the measurements of FRAP (1462.17 μmol ascorbic acid equivalents (AAE)/g), DPPH (1108.91 μmol AAE/g), and H_2O_2 (1662.93 μmol AAE/g). Total carotenoids were measured at 499.61 μg/g, with ascorbic acid at 8.36 μg/g. Correlation analyses revealed a negative correlation of the latter compound with color coordinates. This study highlights the potential of lemon verbena leaves to be used in pharmaceutical and food industries.

Keywords: *A. citrodora*; extraction; pulsed electric field; ultrasonication; Box–Behnken design; antioxidant activity; polyphenols; verbascoside; principal component analysis; partial least squares analysis

Citation: Athanasiadis, V.; Chatzimitakos, T.; Makrygiannis, I.; Kalompatsios, D.; Bozinou, E.; Lalas, S.I. Antioxidant-Rich Extracts from Lemon Verbena (*Aloysia citrodora* L.) Leaves through Response Surface Methodology. *Oxygen* **2024**, *4*, 1–19. https://doi.org/10.3390/oxygen4010001

Academic Editor: Márcio Carocho

Received: 23 December 2023
Revised: 17 January 2024
Accepted: 18 January 2024
Published: 22 January 2024

1. Introduction

Medicinal plants are a significant source of compounds that exhibit bioactivities, rendering them valuable molecules for therapeutic, cosmetic, and food-related products [1]. Phenolic compounds are major bioactive compounds found in plants [2]. They offer notable therapeutic properties and function as natural antioxidants and preservatives [3]. The efficacy of plant extracts, in terms of a specific activity, is dependent on their chemical composition, which can exhibit variations due to environmental factors [4,5] (such as temperature, altitude, soil type, etc.) under which the plant was grown [6], as well as due to variations between different plant species [7]. As such, the efficacy of plant extracts rich in polyphenols can vary, due to the factors mentioned above [8]. Considering their beneficial health effects and antioxidant activity, polyphenols are among the most heavily researched classes of bioactive molecules [9]. They may promote human health when consumed daily, significantly including inhibitory activity against a variety of serious chronic diseases, including cancer, diabetes, and others [10,11]. Owing to the fact that polyphenols are molecules that stand out due to their antioxidant properties, much effort is being placed to produce polyphenol-rich extracts from various plants. This is necessitated by the high demand for natural antioxidants that can be used to substitute their synthesized analogues

in various products [12]. Furthermore, extensive research has been conducted on the antimicrobial properties of polyphenols found in medicinal plants, targeting a diverse array of microorganisms. Particular attention has been drawn to tannins and flavonols as a result of their broad antimicrobial activity compared to other polyphenols. The majority of these compounds interfere with a variety of microbial virulence factors including the inhibition of biofilm formation and neutralization of bacterial toxins, whereas they demonstrate synergistic effects when combined with antibiotics [13]. The food industry is willing to use these compounds as natural preservatives since they increase the shelf life of numerous products [14]. For instance, antioxidant activity in cottage cheese has been boosted by *Rosmarinus officinalis* extract, whereas polyphenol and flavonoid concentration in extruded corn snack and bacterial content have all been improved by herbal mixtures containing *Laurus nobilis*, and *Curcuma longa* [15].

The Verbenaceae family includes approximately 2000 species and more than 100 genera, grown over a broad geographic range that covers tropical, subtropical, and temperate regions across the globe [16]. Since ancient times, aromatic species in this family have been utilized as flavorings in foods and beverages [17]. Moreover, they have been used in traditional medicinal remedies. Lemon verbena (*Aloysia citrodora*) is a member of the Verbenaceae family that originated in South America but, nowadays, grows throughout North Africa, Southern Europe, and parts of Iran. Lemon verbena is a well-known aromatic species that is rich in antioxidant compounds with high antioxidant activity, such as polyphenols, ascorbic acid, and carotenoids [18–20]. Its extracts are widely applicable in the fields of aromatherapy and perfumery. In traditional medicine, its extracts have been employed to treat several digestive issues including flatulence, indigestion, and acidity [21]. Antibacterial, antifungal, anti-inflammatory, and antioxidant properties have been attributed to lemon verbena water infusions [22]. The high content of verbascoside, also known as acteoside, in lemon verbena leaf extract is well acknowledged. It has substantial biological properties such as anxiolytic, antioxidant, anticancer, neuroprotective, antimicrobial, anesthetic, and sedative effects [19,23]. It is also reported that lemon verbena leaf extract contains a high concentration of other polyphenols, tannins, and flavonoids [24].

Green extraction techniques could be used either as pretreatment or as sole extraction techniques for the sustainable recovery of several bioactive compounds from plant sources [25]. These are environmentally friendly techniques as they lack the use of organic solvents and demand lower extraction time and energy consumption [26]. Despite the several human benefits associated with the intake of *A. citrodora*, there is a scarcity of research related to the green manufacturing of extracts abundant in antioxidant polyphenols. The object of this study was to optimize a green extraction process to prepare extracts, rich in antioxidant compounds (i.e., polyphenols, ascorbic acid, and carotenoids), that could potentially be used by food and pharmaceutical industries. To do so, a response surface methodology (RSM) was employed to optimize the extraction procedure. More specifically, green solvent mixtures consisting of water and ethanol, along with the temperature and the duration of the extraction process, were studied. To further enhance this process, green sample pretreatment techniques (i.e., pulsed electric field (PEF) and ultrasonication (US)) were implemented to assist conventional extraction (stirring). The optimum conditions were determined with a partial least squares (PLS) model.

2. Materials and Methods

2.1. Chemicals and Reagents

Hydrochloric acid, methanol, L-ascorbic acid, phosphate buffer solution, aluminum chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH) 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), trichloroacetic acid, β-carotene analytical standard, and all chemical standards for the HPLC determination of polyphenols were obtained from Sigma-Aldrich (Darmstadt, Germany). Ethanol, gallic acid, and the Folin–Ciocalteu reagent were bought from Panreac Co. (Barcelona, Spain). From Merck (Darmstadt, Germany), iron (III) chloride was purchased. Anhydrous sodium carbonate was purchased from Penta (Prague, Czech Republic). Hy-

drogen peroxide (35% v/v) was purchased from Chemco (Malsch, Germany). Deionized water was used for all conducted experiments.

2.2. Lemon Verbena Leaves Material

For all experiments, lemon verbena leaves were gathered from a local market in Karditsa, Greece. The leaves were rinsed extensively with distilled water and dried with paper towels. The sample underwent freeze-drying using a Biobase BK-FD10P freeze-dryer (Jinan, China). The dried lemon verbena plant was then ground to a fine powder (<400 μm diameter) using a blender. Finally, until further analysis, the powder was preserved at −40 °C.

2.3. Extraction Process

To identify the optimal conditions for the recovery of antioxidant compounds from lemon verbena, different combinations of extraction with other pre-treatment techniques were used. In all cases, 1 g of dried powder was mixed with 20 mL of solvent. The solvents employed were 0–100% v/v ethanol in water. PEF and US techniques were used to further assist the conventional extraction (stirring) process. In the case that these techniques were employed, the leaves powder was hydrated by adding the corresponding solvent and left for 10 min. After that, the sample was treated either with PEF or with US for 20 min, whereas when the two techniques were combined, a 20 min PEF treatment was followed by a 20 min treatment with US. Finally, all samples underwent an extraction step under stirring. During the PEF process of the samples, a digital oscilloscope (Rigol DS1052E, Beaverton, OR, USA), two custom stainless-steel chambers (Val-Electronic, Athens, Greece), a mode/arbitrary waveform generator (UPG100, ELV Elektronik AG, Leer, Germany), and a high-voltage power generator (Leybold, LD Didactic GmbH, Huerth, Germany) were used. An electric field strength of 1.0 kV/cm was chosen, with a pulse period of 1 ms (frequency: 1 kHz) and a pulse length of 10 μs. An Elmasonic P machine (Elma Schmidbauer GmbH, Singen, Germany) was used for US treatment and maintained a temperature of 30 °C while operating at 37 kHz.

For the stirring process, the powder–solvent mixtures were transferred to screw-capped glass bottles and heated at 20–80 °C for 30–150 min, under continuous stirring at 500 rpm. After the extraction was completed, the samples were centrifuged at 10,000× g in a NEYA 16R centrifuge (Remi Elektrotechnik Ltd., Palghar, India) for 10 min. Finally, the supernatants were collected and stored at −40 °C until further analysis. The extraction took place in various combinations of the examined parameters with the coded levels shown in Table 1.

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

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

2.4. Optimization with Response Surface Methodology (RSM) and Experimental Design

To assess the antioxidant activity of lemon verbena extracts and to isolate bioactive compounds as efficiently as possible, the RSM technique was used. Thus, efficiently maximizing the levels of these values was the primary goal of the design. This was accomplished by initially optimizing the extraction technique, along with the solvent concentration (C, % v/v of ethanol), extraction time (t, min), and extraction temperature (T,

°C). Based on an experiment that used a Box–Behnken design with a main impact screening arrangement, the optimization of the extraction process was determined. On the basis of optimization, an experiment was conducted with a main effect screening design and 20 design points. The experimental design required setting up the process variables at five different levels. Table 1 displays the coded and observed levels. The overall significance of the model (R^2, p) and the significance of the model coefficients (equations) were determined using analysis of variance (ANOVA) and summary-of-fit tests, with a minimum level of 95%. In addition, the response variable was predicted as a function of the examined independent factors using a quadratic (second-order) polynomial model, as illustrated in Equation (1):

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

where X_i and X_j represent the independent variables, and Y_k defines the predicted response variable. The model linear, quadratic, and interaction terms are represented by the intercept and regression coefficients, β_0, β_i, β_{ii}, and β_{ij}, respectively.

The RSM was used to identify the largest peak area and evaluate the impact of a significant independent variable on the response. To visually depict the model equation, the creation of three-dimensional surface response graphs occurred.

2.5. Bioactive Compounds Determination

2.5.1. Total Polyphenol Content (TPC)

A previously established methodology [27] was applied to determine TPC. Briefly, 200 µL of the sample was mixed with 200 µL of Folin–Ciocalteu reagent, and after 2 min, 1600 µL of 5% w/v aqueous sodium carbonate solution was added in a 2 mL Eppendorf tube. The mixture was incubated at 40 °C for 20 min, and the absorbance was recorded at 740 nm in a Shimadzu UV-1700 PharmaSpec Spectrophotometer (Kyoto, Japan). The total polyphenol concentration (C_{TP}) was calculated from a gallic acid calibration curve from 10–100 mg gallic acid/L. The total polyphenol yield (Y_{TP}) was determined as mg gallic acid equivalents (GAE) per g of dry weight (dw), using the following Equation (2):

$$\text{TPC (mg GAE/g dw)} = \frac{C_{TP} \times V}{w} \quad (2)$$

where the volume of the extraction medium is indicated with V (expressed in L) and the dry weight of the sample as w (expressed in g).

2.5.2. HPLC Quantification of Polyphenolic Compounds

High-Performance Liquid Chromatography (HPLC) was used to detect and quantify individual polyphenols from the sample extracts, as established in our previous research [27]. A Shimadzu CBM-20A liquid chromatograph and a Shimadzu SPD-M20A diode array detector (DAD) (both purchased from Shimadzu Europa GmbH, Duisburg, Germany) were employed for the analysis of lemon verbena extracts. A volume of 20 µL of properly diluted extracts was injected into the system. The compounds were separated into a Phenomenex Luna C18(2) column from Phenomenex Inc. in Torrance, CA, USA, and kept at 40 °C (100 Å, 5 µm, 4.6 mm × 250 mm). The mobile phase included 0.5% aqueous formic acid (A) and 0.5% formic acid in acetonitrile/water (6:4) (B). The gradient program was as follows: from 0 to 40%, B for 40 min; then to 50%, B for 10 min; and to 70%, B for another 10 min and then constant for 10 min. The total chromatographic analysis lasted 70 min. The flow rate of the mobile phase was set at 1 mL/min. Scanning was conducted in the wavelength range of 190–800 nm. The compounds were identified by comparing the absorbance spectrum and retention time to those of pure standards and then quantified through calibration curves (0–50 µg/mL). The results were given in mg/g dw.

2.5.3. Ascorbic Acid (AA) Content

Ascorbic acid (AA) concentration was evaluated using a previously established method [28]. A quantity of 100 µL sample extract along with 500 µL of 10% (*v*/*v*) Folin–Ciocalteu reagent was mixed with 900 µL of 10% (*w*/*v*) trichloroacetic acid in an Eppendorf tube. The absorbance was measured at 760 nm after 10 min. Ascorbic acid was used as the calibration standard.

2.5.4. Total Carotenoids (TC) Determination

A slightly modified method introduced by Ayour et al. [29] was employed to determine the total carotenoid (TC) content of the extracts. Briefly, a ten-fold dilution was used in the samples during their preparation, and therefore, the absorbance was recorded at 450 nm. The TC content was expressed as mg of β-carotene equivalents per gram of dried weight, using a calibration curve based on β-carotene.

2.6. Antioxidant Capacity of the Extracts

2.6.1. Ferric-Reducing Antioxidant Power (FRAP) Assay

An established technique by Shehata et al. [30] was used for the evaluation of FRAP. In a 2 mL Eppendorf tube, 100 µL of properly diluted sample was mixed with 100 µL of $FeCl_3$ solution (4 mM in 0.05 M HCl). The mixture was incubated at 37 °C for 30 min, with 1800 µL of TPTZ solution (1 mM in 0.05 M HCl) being immediately added right after, and the absorbance was measured after 5 min at 620 nm. The ferric-reducing power (P_R) was calculated using an ascorbic acid calibration curve (C_{AA}) in 0.05 M HCl with ranging values (0.05–0.5 mM). The P_R was calculated as mmol of ascorbic acid equivalents (AAE) per kilogram of fw, using Equation (3):

$$P_R\ (\mu\text{mol AAE/g fw}) = \frac{C_{AA} \times V}{w} \quad (3)$$

where V is represented (in L) as the entire volume of the extraction medium, and w (in g) represents the dried weight of the material.

2.6.2. DPPH• Antiradical Activity Assay

The extracted polyphenols from the dried material were evaluated for their antiradical activity (A_{AR}) using a slightly modified DPPH• method, as previously established by Shehata et al. [30]. In brief, 50 µL of the sample was mixed with a quantity of 1950 µL of a 100 µM DPPH• solution in methanol, with the solution being kept at room temperature for 30 min in the dark right after. Following that, the absorbance was measured at 515 nm. Moreover, a blank sample was used instead of the sample, including DPPH• solution and methanol, with the absorbance immediately being measured. To calculate the percentage of scavenging, Equation (4) was employed:

$$\%\ \text{Scavenging} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (4)$$

An ascorbic acid calibration curve in Equation (5) was used to evaluate antiradical activity (A_{AR}), which was expressed as µmol AAE per gram of dw:

$$A_{AR}\ (\mu\text{mol AAE/g fw}) = \frac{C_{AA} \times V}{w} \quad (5)$$

2.6.3. Hydrogen Peroxide (H_2O_2) Scavenging Assay

A previously mentioned method [28] was applied for the H_2O_2 scavenging assay. A quantity of 600 µL of a H_2O_2 solution (40 mM, made in phosphate buffer, pH 7.4) was

mixed with 400 µL of the extract in an Eppendorf tube. The absorbance was then measured after 10 min at 230 nm. Equation (6) describes the scavenging capacity of the H_2O_2:

$$\% \text{ Scavenging of } H_2O_2 = \frac{A_o - (A - A_A)}{A_o} \times 100 \quad (6)$$

where the absorbances of the blank solution, the extract solution in the absence of hydrogen peroxide, and the sample are denoted by A_o, A_c, and A, respectively.

The concentration of ascorbic acid ranged in the calibration curve (C_{AA}, 50–500 µmol/L in 0.05 M HCl), and the following Equation (7) was used to determine the anti-hydrogen peroxide activity (A_{AHP}) as µmol AAE per g of dw:

$$A_{AHP} \text{ (µmol AAE/g fw)} = \frac{C_{AA} \times V}{w} \quad (7)$$

where V denotes the volume of the extraction medium (in L), and w is the dry weight of the sample.

2.7. Color Determination of the Extracts

The CIELAB color of the lemon verbena extracts was measured by using a previously established methodology [31] using a colorimeter (Lovibond CAM-System 500, The Tintometer Ltd., Amesbury, UK), where the CIELAB parameters (L^*, a^*, and b^*) measured the aqueous extracts. Three parameters were fundamental to measure the color of the extracts: the L^* value denotes the lightness of a color, ranging from 0 (representing black) to 100 (representing white); the a^* value specifies the degree of redness (negative values) or greenness (positive values) in a color; similarly, the b^* value measures the extent of yellowness (negative values) or blueness (positive values) in a color. The measure of color intensity is denoted by C_{ab}^* or C^* (chroma, saturation). The hue angle (h_{ab} or H) and psychological coordinate chroma were measured using the following equations:

$$C_{ab}^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (8)$$

$$h_{ab}^o = \arctan\left(\frac{b^*}{a^*}\right) \quad (9)$$

2.8. Statistical Analysis

The statistical analysis related to response surface methodology and distribution analysis, which were applicable through JMP® Pro 16.2 software (SAS, Cary, NC, USA). The quantitative analysis was performed in triplicate, and the extraction procedures were repeated at least twice for each batch of lemon verbena extract. The results were represented in the form of means and standard deviations. Principal component analysis (PCA), multivariate correlation analysis (MCA), and partial least squares (PLS) analysis were conducted through JMP® Pro 16.2 software (SAS, Cary, NC, USA).

3. Results and Discussion

To maximize effectiveness and guarantee a more environmentally friendly extraction process, extraction parameter optimization was essential [32,33]. In this context, the composition of the solvent is crucial, since the solvent characteristics have a significant effect on compound extraction [34]. As an example, moderately polar molecules like polyphenols are difficult to extract with highly polar solvents like water. Consequently, organic solvents are commonly employed to enhance the extraction procedure. Specifically, ethanol could be combined with water to produce an extraction solvent suitable for use in the food industry [35]. The main purpose of this study was to enhance the extraction of polyphenols from lemon verbena. RSM was used to investigate the aforementioned conditions and extraction technique combinations to determine the most efficient model for producing

extracts rich in bioactive compounds (i.e., polyphenols, ascorbic acid) as well as extracts with high antioxidant activity. For the most effective extraction of polyphenols, a moderate concentration of ethanol in the extraction solvent was required, according to the findings of the present study (*vide infra*). It has also been demonstrated that the efficiency could be increased by combining the two green extraction methods (PEF and US) with conventional extraction (stirring). The desired compounds can be more easily extracted using these techniques, which induce cellular membrane disruption [36]. Prior research has also proved that utilizing PEF and US with the extraction procedure could increase its efficacy [37].

3.1. Total Polyphenol Content and Antioxidant Activity of the Extracts

The results obtained from the FRAP, DPPH, and H_2O_2 assays, in addition to the measured and predicted responses for TPC, are provided in Table 2. It appeared that the outcomes for TPC varied considerably between the samples. Specifically, TPC varied between 40.66 and 163.71 mg GAE/g, with design point samples 10 and 12 representing the lowest and highest values, respectively, with the samples exhibiting a four-fold difference in the TPC. In a study conducted by Rashid et al. [38], lemon verbena extracts were analyzed using different solvents, recording comparable results. TPC recorded ~120 mg GAE/g when ethanol was the extraction solvent, whereas half the value was measured when water was the extraction solvent. In our case, water recorded ~90–113 mg GAE/g, and pure ethanol yielded ~40–100 mg GAE/g of polyphenols.

Regarding the antioxidant assays, the data revealed considerable variance as well. For example, the range for the FRAP assay was 576.19–1326.45 μmol AAE/g, the range for the DPPH assay was 438.94–958.98 μmol AAE/g, which corresponds to a two-fold increase, and the range for the H_2O_2 assay was 350.44–1579.52 μmol AAE/g, which represents a nearly five-fold increase. A previous study suggested that the antioxidant capacity of an extract is influenced by both the quantity of polyphenols present and the characteristics of the antioxidant compounds [39]. The implementation of ethanol led to hydroethanolic extracts that were not only potent scavengers against the DPPH radical but also in the other two antioxidant capacity assays. The reason for this could lie in the moderate-polarity polyphenols yielded compared to pure water [40]. As demonstrated on TPC, it was found that these extracts contained a higher concentration of polyphenols.

Table 2. Experimental findings for the three independent variables under investigation and the dependent variable's responses.

Design Point	Independent Variables				Responses							
					TPC (mg GAE/g)		FRAP (μmol AAE/g)		DPPH (μmol AAE/g)		Hydrogen Peroxide (μmol AAE/g)	
	X_1	X_2	X_3	X_4	Actual	Predicted	Actual	Predicted	Actual	Predicted	Actual	Predicted
1	3	1	3	4	90.79	93.72	1006.24	979.75	644.16	614.36	464.60	455.58
2	3	2	1	3	104.69	96.84	1145.26	1134.95	818.78	796.75	537.46	560.86
3	2	3	4	3	137.66	128.44	1145.77	1158.92	876.98	872.69	1110.67	1079.76
4	2	4	5	4	112.12	111.65	1102.59	1082.75	811.47	821.07	814.02	785.12
5	3	5	4	2	52.95	56.58	576.19	616.29	438.94	467.04	441.47	395.83
6	4	1	4	5	113.29	109.05	1062.39	1064.93	797.22	804.01	819.28	829.54
7	4	2	3	1	145.38	141.77	1131.38	1114.51	823.26	814.26	1425.10	1398.20
8	1	3	3	2	146.63	140.00	1103.47	1095.68	916.73	883.51	1445.65	1425.62
9	1	4	4	1	132.12	132.55	989.12	970.74	747.02	741.83	1103.30	1119.77
10	1	5	1	4	40.66	44.28	630.85	639.32	477.72	484.86	411.12	392.76
11	1	1	2	3	104.86	99.95	886.66	881.56	533.61	547.39	718.37	717.05
12	1	2	5	5	163.71	168.85	1326.45	1342.51	958.98	962.97	1579.52	1581.80
13	4	3	2	4	139.61	155.73	1265.94	1332.61	896.30	948.87	1418.19	1416.07
14	3	4	2	5	150.96	143.15	1272.40	1234.30	937.16	910.02	1320.31	1261.63
15	2	5	3	5	100.25	99.85	834.32	843.55	617.24	620.13	755.40	828.63
16	2	1	1	1	97.19	98.06	864.21	886.87	577.51	582.23	350.44	311.01
17	2	2	2	2	98.45	114.71	1068.68	1063.70	790.31	817.87	841.65	930.57
18	3	3	5	1	126.37	128.41	1121.96	1136.56	770.65	781.03	837.94	864.99
19	4	4	1	2	138.85	133.65	1161.42	1138.35	767.30	755.60	1140.69	1158.81
20	4	5	5	3	66.76	66.03	712.20	689.67	530.45	505.30	459.21	480.83

Table 3 displays the concentrations of the main polyphenols that have been identified using HPLC-DAD, whereas a representative chromatogram is illustrated in Figure 1. Phenolic acids (vanillic acid, *p*-coumaric acid, and ferulic acid) cumulated to form a combined 0.5–8.94 mg/g, whereas flavonoids (rutin, quercetin 3-D-galactoside, verbascoside, narirutin, and kaempferol 3-glucoside) had a total sum of 22.77–163.38 mg/g. The beneficial properties of these polyphenols should be highlighted. Firstly, *p*-coumaric was found to reduce basal oxidative stress more efficiently than vitamin E in animal models. It also possesses anti-inflammatory, antimicrobial, and chemopreventive properties [41,42]. In addition, the hydrophilic biomolecule verbascoside, which was found to be the major polyphenol, exhibits antitumor, antidepressant, neuroprotective, anti-inflammatory, and antioxidant properties [43]. Naturally occurring flavanone narirutin possesses several health-promoting properties, such as antioxidant, anti-inflammatory, and anticancer activities. As previously stated, the combination of techniques observed in sample 12 appeared to be advantageous in terms of extracting a substantial amount of verbascoside (147.21 mg/g), marking ~91% of the quantified polyphenols from the specific sample. However, variance in the concentration of polyphenolic compounds extracted using different techniques was noticeable. Therefore, it is evident that an extraction model incorporating the aforementioned parameters was imperative for the optimal extraction of polyphenols.

Table 3. Coded values of the four independent variables under investigation, and the actual concentration of polyphenolic compounds, represented in mg/g dw.

Design Point	Independent Variables				Responses							
	X_1	X_2	X_3	X_4	VA	*p*-CA	FA	RT	Q3G	VB	NRT	KG
1	3	1	3	4	0.53	5.65	1.86	1.64	1.90	36.03	6.36	1.84
2	3	2	1	3	0.53	5.90	0.48	1.43	1.82	59.22	3.05	1.72
3	2	3	4	3	0.22	5.25	1.79	1.43	1.77	115.78	3.28	1.79
4	2	4	5	4	0.17	0.07	0.53	1.46	1.76	88.67	3.12	2.89
5	3	5	4	2	0.01	0.01	0.50	1.43	0.01	21.77	0.58	1.86
6	4	1	4	5	0.24	5.56	1.83	1.99	2.07	98.21	0.91	2.59
7	4	2	3	1	0.21	6.25	1.76	3.25	1.78	122.68	0.23	2.10
8	1	3	3	2	0.20	5.75	1.79	3.41	1.79	130.98	1.01	1.81
9	1	4	4	1	0.14	1.00	0.92	1.44	1.75	119.80	1.90	3.48
10	1	5	1	4	0.01	0.07	0.51	0.01	1.76	21.02	0.68	1.87
11	1	1	2	3	0.28	5.13	1.90	1.48	1.76	60.13	0.88	1.92
12	1	2	5	5	0.27	5.45	2.13	1.95	2.17	147.41	0.52	2.23
13	4	3	2	4	0.44	5.15	1.86	1.37	1.79	125.21	0.31	1.81
14	3	4	2	5	0.33	1.95	0.72	1.44	1.80	125.14	0.43	2.12
15	2	5	3	5	0.01	0.02	0.52	1.46	1.77	58.09	1.31	1.95
16	2	1	1	1	0.23	5.12	1.81	1.41	2.36	84.58	0.29	1.90
17	2	2	2	2	0.25	5.44	1.77	1.43	2.72	81.25	0.01	1.90
18	3	3	5	1	0.19	5.51	1.84	1.45	1.78	85.42	0.21	1.83
19	4	4	1	2	0.56	1.28	1.04	1.25	1.78	128.25	0.05	2.11
20	4	5	5	3	0.05	0.01	0.52	0.01	0.01	29.15	0.01	1.91

VA: Vanillic acid; *p*-CA: *p*-Coumaric acid; FA: Ferulic acid; RT: Rutin; Q3G: Quercetin 3-D-galactoside; VB: Verbascoside; NRT: Narirutin; KG: Kaempferol 3-glucoside.

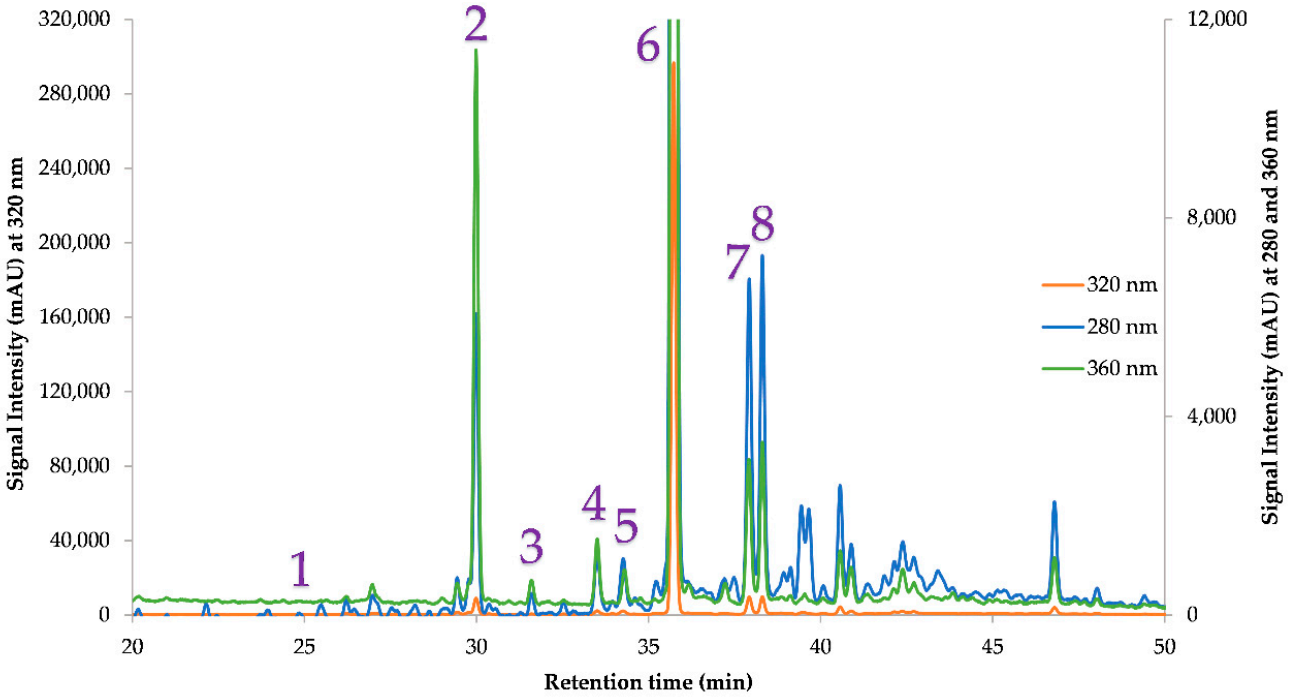

Figure 1. Exemplary HPLC chromatogram at 280, 320, and 360 nm of lemon verbena extract demonstrating polyphenolic compounds that were identified. 1: Vanillic acid; 2: *p*-Coumaric acid; 3: Ferulic acid; 4: Rutin; 5: Quercetin 3-D-galactoside; 6: Verbascoside; 7: Narirutin; 8: Kaempferol 3-glucoside.

3.2. Other Bioactive Compounds, Biological and Physicochemical Determination of Extracts

There is a robust relationship between color characteristics and carotenoids, which are pigments present in many plant species. Since they affect the product appearance, carotenoids quantification is an important quality indicator [44,45]. Table 4 shows the results of the analysis of the parameters affecting the color of the samples (L^*, C^*, H, and carotenoids) as well as their ascorbic acid content. TC concentration ranged from 287.89 to 602.56 μg CtE/g. Since carotenoids are known lipophilic compounds, the highest concentrations of total carotenoids appear to be in samples containing ethanol which lowers the polarity of the extraction solvent, such as design points 4, 6, 12, and 14. Regarding color analysis, the L^* coordinate had little variance (32.6–47.6) compared to the C^* coordinate (4–29.2). An even greater variance was observed for H (14.5–124.5) which marked an approximately nine-fold difference between samples 11 and 15. It could be concluded that the implementation of ethanol as a solvent resulted in a brighter and more vivid color, as observed in design points 5 and 10, compared to water which led to darker and blurry extracts (i.e., design points 1 and 11). A small variance was observed in AA concentration, as a two-fold difference was recorded between design points 10 and 12 (4.26–9.47 mg/g).

Finally, in Table 5, the statistical parameters, quadratic (second-order) polynomial equations (models), and coefficients (coefficients > 0.95) obtained for each model are presented, suggesting a good fit for the developed models. Plots of the actual response versus the predicted response for each examined parameter as well as the desirability functions are given in Figures S1–S4. More statistic details are given in the Supplementary Material. Three-dimensional response plots for TPC are given in Figure 2. In Figure 2A, it can be concluded that TPC is highly affected by X_1 (US + ST) and X_2 (25% v/v ethanol as solvent), whereas in Figure 2B, it seems that a high extraction time (i.e., X_3 variable was optimum for >120 min of extraction) is required for the optimum polyphenol yield. In Figure 2E, a positive correlation is revealed with polyphenol recovery and variable X_4, which demands a high extraction temperature (>50 °C). Three-dimensional response plots for the rest of the responses are found in Figures S5–S7, following a similar rationale.

Table 4. Coded values of the four independent variables under investigation, and the actual concentration of total carotenoids, ascorbic acid content, and coordinates of color analysis.

Design Point	Independent Variables				Responses				
	X_1	X_2	X_3	X_4	Carotenoids (μg CtE/g)	Ascorbic Acid (mg/g)	L^*	C^*	Hue
1	3	1	3	4	599.33	7.41	34.9	4.0	34.6
2	3	2	1	3	379.60	7.30	40.6	19.3	48.8
3	2	3	4	3	506.48	7.01	35.8	9.7	43.7
4	2	4	5	4	540.31	5.92	36.1	10.9	124.4
5	3	5	4	2	451.57	4.52	44.6	29.2	100.1
6	4	1	4	5	557.95	9.41	35.6	7.9	54.6
7	4	2	3	1	327.25	7.32	39.5	17.7	36.4
8	1	3	3	2	316.52	7.33	39.3	13.9	43.6
9	1	4	4	1	403.37	5.85	41.3	20.5	102.1
10	1	5	1	4	383.59	4.26	47.6	26.5	121.2
11	1	1	2	3	472.51	6.85	32.6	9.9	14.5
12	1	2	5	5	593.25	9.47	34.4	9.4	50.7
13	4	3	2	4	443.83	6.24	37.7	12.7	52.5
14	3	4	2	5	602.56	7.68	37.2	8.9	116.9
15	2	5	3	5	473.67	4.95	41.0	23.3	124.5
16	2	1	1	1	287.89	6.43	44.6	20.4	53.2
17	2	2	2	2	334.44	7.45	43.4	23.3	40.9
18	3	3	5	1	543.60	6.96	33.6	13.0	28.4
19	4	4	1	2	452.69	6.71	40.9	16.9	104.7
20	4	5	5	3	441.58	4.54	45.1	23.0	123.4

Table 5. Mathematical models created using RSM were used to optimize the extraction of lemon verbena. The models contained only significant terms.

Responses	Second-Order Polynomial Equations (Models)	R^2 Predicted	R^2 Adjusted	p-Value	Eq.
TPC	$Y = 115.38 - 37.27X_1 + 22.92X_2 + 57.33X_3 - 48.54X_4 + 7.14{X_1}^2 - 8.47{X_2}^2 - 2.11{X_3}^2 + 3.58{X_4}^2 + 5.88X_1X_2 - 8.87X_1X_3 + 4.46X_1X_4 - 4.67X_2X_3 + 6.25X_2X_4 - 0.59X_3X_4$	0.9558	0.8322	0.0169	(10)
FRAP	$Y = 555.9 - 3.4X_1 + 347.79X_2 + 127.21X_3 - 106.27X_4 + 9.4{X_1}^2 - 77.88{X_2}^2 + 7.66{X_3}^2 + 10.63{X_4}^2 + 16.88X_1X_2 - 36.84X_1X_3 + 16.43X_1X_4 - 16.72X_2X_3 + 19.82X_2X_4 - 5.13X_3X_4$	0.9858	0.9460	0.0011	(11)
DPPH	$Y = 300.72 - 54.29X_1 + 628.91X_2 - 76.72X_3 - 131.07X_4 + 1.98{X_1}^2 - 103.17{X_2}^2 - 11.73{X_3}^2 + 24.91{X_4}^2 - 1.41X_1X_2 + 18.3X_1X_3 + 0.29X_1X_4 + 7.88X_2X_3 - 12.61X_2X_4 + 21.45X_3X_4$	0.9815	0.9296	0.0022	(12)
Hydrogen Peroxide	$Y = 321.24 - 776.65X_1 + 1041.24X_2 + 479.02X_3 - 397.71X_4 + 136.8{X_1}^2 - 184.79{X_2}^2 - 89.51{X_3}^2 + 69.27{X_4}^2 + 54.16X_1X_2 + 0.57X_1X_3 - 23.9X_1X_4 - 15.65X_2X_3 - 0.58X_2X_4 + 33.12X_3X_4$	0.9912	0.9665	0.0004	(13)

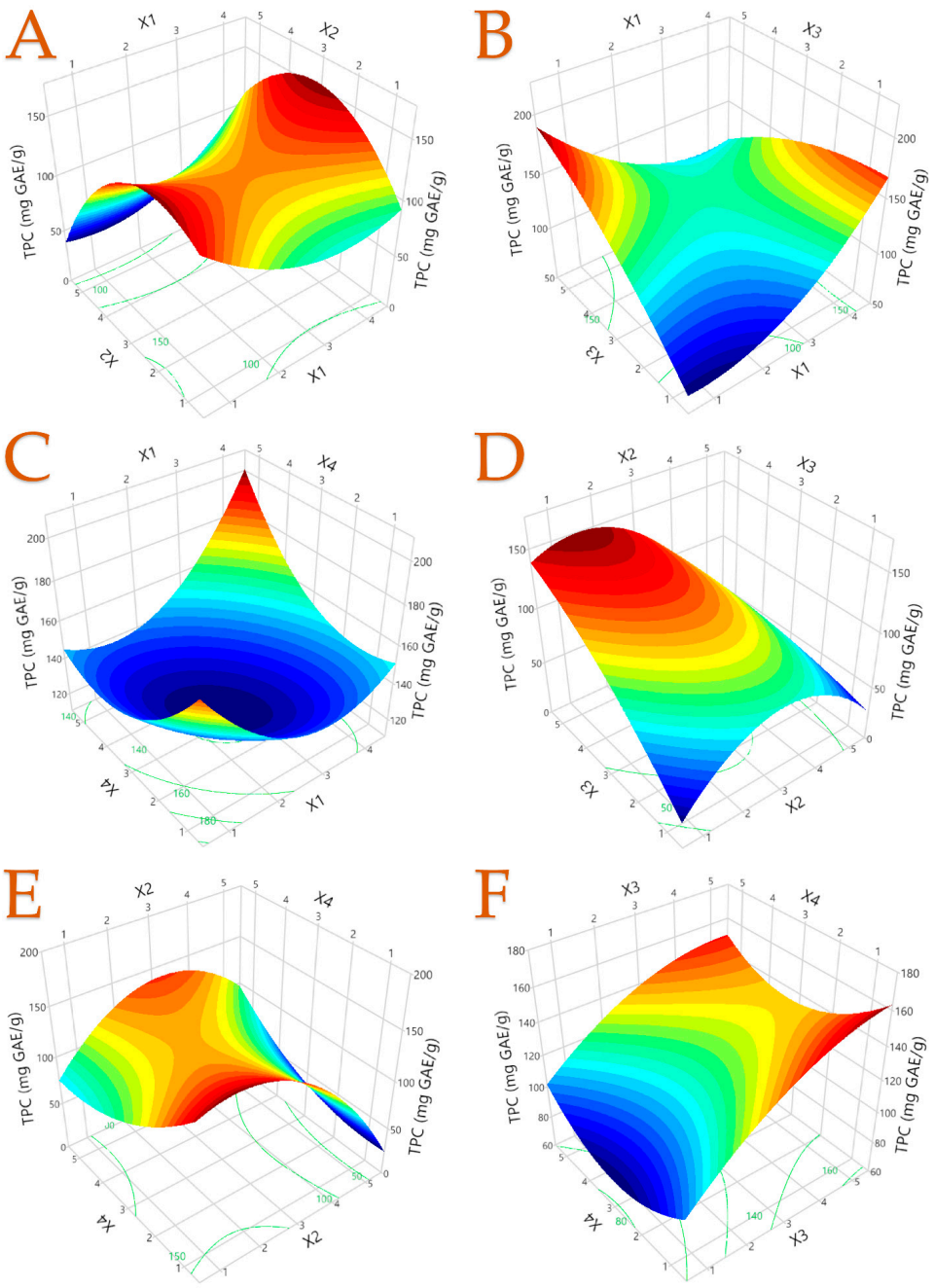

Figure 2. The optimal extraction of lemon verbena extracts is shown in 3D graphs that show the impact of the process variables considered in the response (total polyphenol content—TPC, mg GAE/g). Plot (**A**), covariation of X_1 and X_2; plot (**B**), covariation of X_1 and X_3; plot (**C**), covariation 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.

3.3. Optimal Extraction Conditions

To increase efficiency, the extraction parameters must be optimized. Diverse bioactive compound structures may present complications for the extraction process due to solubility and polarity fluctuations [46]. Furthermore, the extraction method and various processing parameters have a major impact on the yield and antioxidant capacity of the extract. Therefore, it becomes critical to optimize this process [47]. In recent years, significant progress has been made toward developing extraction techniques that minimize the use of hazardous and toxic solvents, safeguard human health, and consume little energy. The

utilization of an eco-friendly solvent is crucial for the effective implementation of this technique [48]. Water, for instance, is the most affordable and environmentally friendly solvent. It is a highly effective solvent for the extraction of polar molecules. However, to extract less polar compounds, organic solvents like ethanol or methanol could be implanted to solubilize bioactive components such as polyphenols in a more effective way than water [49]. To that end, binary solvents may yield greater efficiency, and more specifically, ethanol and water could be a unique combination due to their food-grade capability [50]. To minimize energy consumption during the extraction process, it is crucial to further optimize the extraction duration and temperature. Since earlier research has proven the efficacy of both short [51] and long [52] extraction durations, a comprehensive evaluation is required to identify the effect of time on extraction. High temperatures are also known to improve extraction processes by making solutes more soluble and diffusion coefficients stronger. Nevertheless, since polyphenols are thermolabile compounds, it is essential to consider that they could decompose beyond a certain point [53]. Typically, the optimal temperature range for traditional extraction methods to achieve the highest polyphenol recovery is 50–80 °C [54,55]. It is worth mentioning that the matrix composition is also one of key factors that inevitably affect the efficiency of the extraction process. The diverse structures of bioactive components, along with their varying polarities and solubilities, can make the extraction process more complicated.

To this end, the desirability function was applied to determine the highest expected levels of antioxidant activity (as measured by FRAP, DPPH, and H_2O_2) and TPC. The maximum values of the assays were obtained by employing various extraction conditions. To effectively measure TPC from lemon verbena at a predicted value of 168.85 mg GAE/g dw, a 150 min extraction with 25% v/v was demanded at 80 °C using a conventional stirring technique. The same conditions were also required for the maximum predicted response of 1342 µmol AAE/g for the FRAP assay. Table 6 provides additional details regarding the optimal conditions for extraction.

Table 6. Maximum predicted responses and optimum extraction conditions for the dependent variables; coded variable levels are given in parentheses.

Responses	Optimal Conditions				
	Maximum Predicted Response	**Technique (X_1)**	**C (%, v/v) (X_2)**	**t (min) (X_3)**	**T (°C) (X_4)**
TPC (mg GAE/g)	168.85 ± 33.33	ST (1)	25 (2)	150 (5)	80 (5)
FRAP (µmol AAE/g)	1342.51 ± 119.04	ST (1)	25 (2)	150 (5)	80 (5)
DPPH (µmol AAE/g)	996.11 ± 83.47	PEF + US + ST (4)	50 (3)	90 (3)	65 (4)
Hydrogen Peroxide (µmol AAE/g)	1581.8 ± 177.18	ST (1)	25 (2)	150 (5)	80 (5)

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

To obtain further details from the variables and perform a more comprehensive data analysis, PCA was utilized. The objective of this analysis was to determine whether TPC and antioxidant compounds (specifically, ascorbic acid and individual polyphenols), antioxidant assays (including FRAP, DPPH, and H_2O_2), color coordinates, and carotenoids exhibited any correlation between them. In order to select the two principal components depicted in Figure 3, eigenvalues >1 were considered. These components accounted for a combined 61.6% of the variance. The results revealed a positive or negative correlation between the parameters. For example, a much-anticipated result was the positive correlation between antioxidant assays with TPC and verbascoside. As a result, it can be noticed that they are discriminated and properly grouped. On the other hand, discrimination was also observed between color coordinates, as they were influenced by the same parameters, such as solvent concentration.

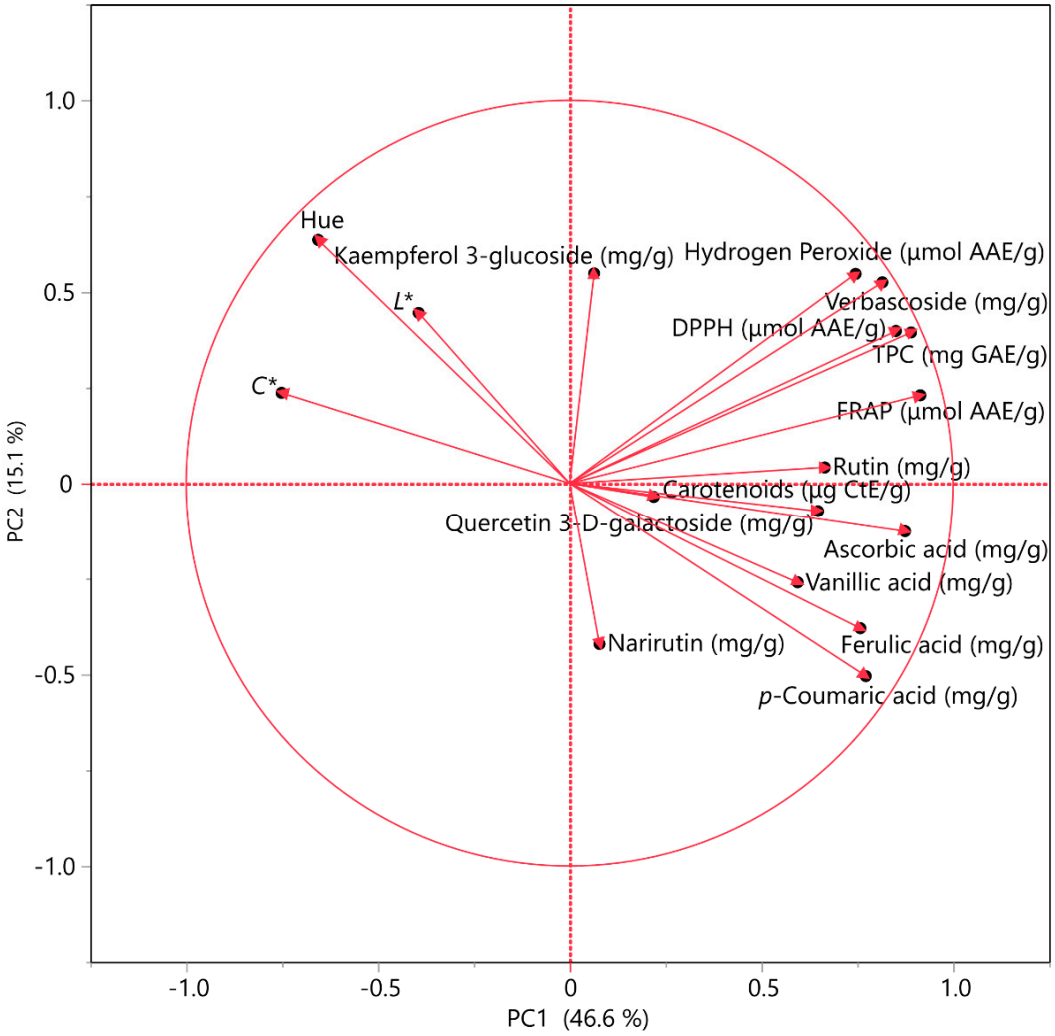

Figure 3. Principal component analysis (PCA) for the measured variables.

Additionally, an MCA was conducted to provide additional insight into the correlation between the variables under investigation. One significant benefit of this analysis in comparison to the previous one is its ability to quantify the degree of positive or negative correlation between the variables. The color scale utilized in this particular context represents correlation values between −1 and 1, as explained in the corresponding caption. The outcomes of this analysis are illustrated in Figure 4. AA was positively correlated with the majority of individual polyphenols (>0.6), but it could be observed that it was negatively correlated with color coordinates. Specifically, this correlation was found strong (>0.6), which would be caused by the different extraction solvent. It was previously revealed that the incorporation of ethanol as an extraction solvent led to brighter and more colorful extracts. However, it was additionally shown that the higher the presence of ethanol, the more decreased the concentration of AA.

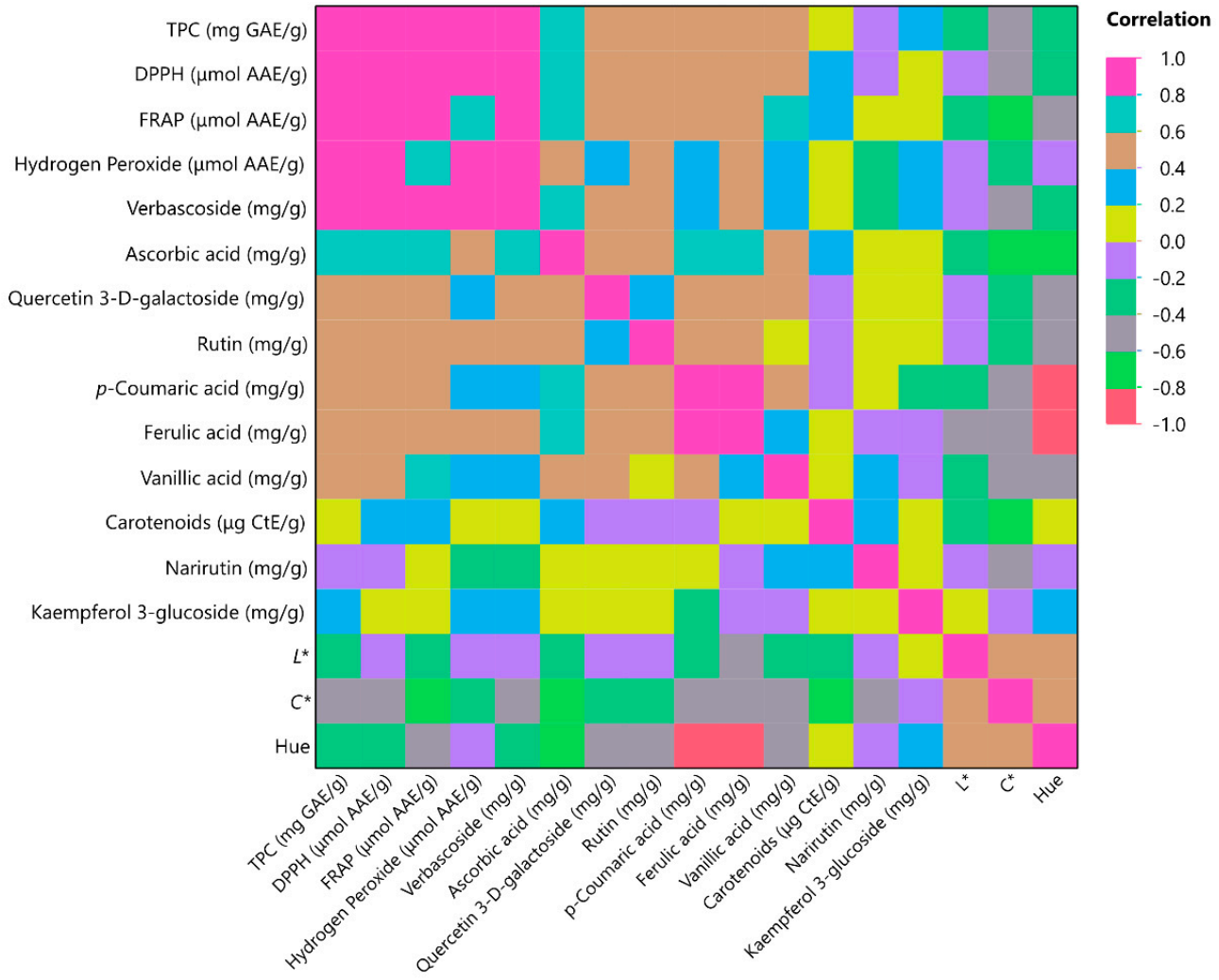

Figure 4. Multivariate correlation analysis of measured variables.

3.5. Partial Least Squares (PLS) Analysis

A PLS model was used to assess the significance of the extraction parameters (X_1, X_2, X_3, and X_4). Figure 5 depicts the application of the PLS model to create a correlation loading plot, which visually displays the impact of extraction conditions of lemon verbena. A higher projection factor, especially over 0.8, indicates a bigger contribution from this variable. It can be concluded that in the X_1 variable, solely stirring was not enough to yield maximum polyphenols; however, this was possible through the PEF-assisted, US-assisted, or combined PEF- and US-assisted extractions. In the X_2 parameter, it was observed that a moderate-polarity solvent (i.e., 50% v/v) was found to be optimum in all assays. Extraction duration (i.e., parameter X_3) did not have a significant impact, though it was noticed that 60 min of extraction was the most beneficial. Finally, in all cases, it was observed that a high temperature (X_4 parameter at 80 °C) was required for the most favorable outcomes.

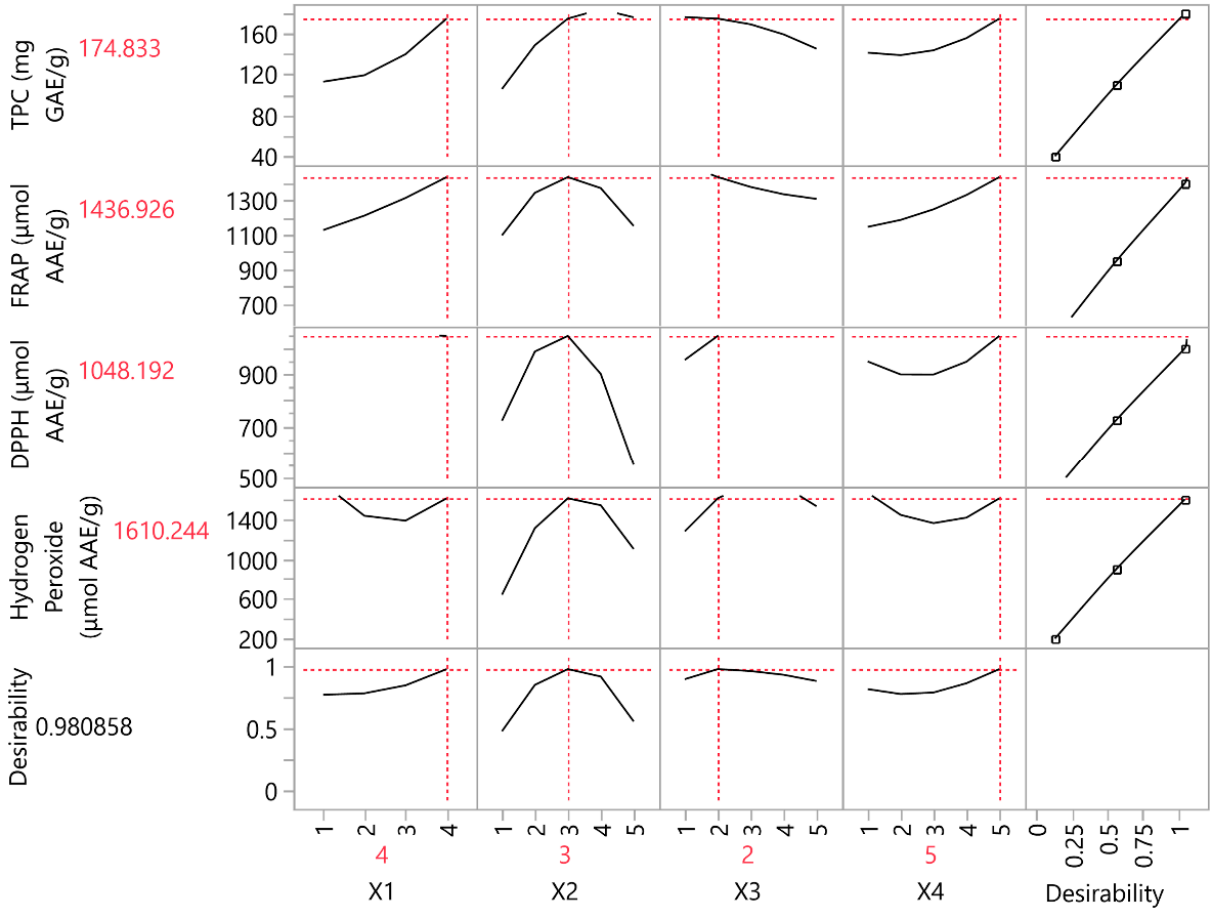

Figure 5. Partial least squares (PLS) prediction profiler of each variable and desirability function with extrapolation control for the optimization of lemon verbena extracts.

When the values from the experimental analysis are compared with those from the PLS model, a correlation of 0.9991 can be observed, yet there are no deviations between the two sets of data (p = 0.0005). Table 7 represents the PLS-predicted values with the corresponding experimental values of TPC and antioxidant assays, in which the optimum technique was found to be PEF + US + ST, requiring extraction with 50% v/v of ethanol for 60 min at 80 °C. Table 8 shows the values of several individual antioxidant compounds and color properties in these optimum extraction conditions.

Table 7. Maximum desirability for all variables using the partial least squares (PLS) prediction profiler under the optimal extraction conditions (X_1:4, X_2:3, X_3:2, X_4:5).

Variables	PLS Model Values	Experimental Values
TPC (mg GAE/g)	174.83	175.03 ± 11.9
FRAP (µmol AAE/g)	1436.93	1462.17 ± 30.71
DPPH (µmol AAE/g)	1048.19	1108.91 ± 42.14
Hydrogen Peroxide (µmol AAE/g)	1610.24	1662.93 ± 68.18

Following optimization, TPC showed a slight increase from the initial RSM results, leading to 175.03 mg GAE/g, whereas major polyphenols, verbascoside, narirutin, and *p*-coumaric acid were measured at 132.61, 5.27, and 1.49 mg/g, respectively. The total sum of the measured polyphenols was 148.42 mg/g, a value not far from the TPC. In a study by Polumackanycz et al. [56], the chemical composition of lemon verbena extracts was evaluated with infusion, decoction, and hydromethanolic extraction. Rutin concentration ranged from 3.32 to 7.79 mg/g dw, leading to comparable results with our study. However,

p-coumaric acid was found significantly lower than our optimal sample by up to ten-fold. Regarding other bioactive compounds, TC was measured at 499.61 μg CtE/g, whereas AA was quantified at 8.36 mg/g. Guimarães et al. [57] explored the antioxidant properties of four medicinal plants, including lemon verbena. They used decoction and infusion extraction techniques, in which TPC was measured at 221.90 and 445.04 mg GAE/g, respectively. The ascorbic acid concentration was comparable to our extracts as it was quantified at 6.80 and 8.05 mg/g, respectively. Antioxidant assays were also further increased from their corresponding RSM values from 5 to 16%, verifying the importance of the PLS model. Regarding the DPPH assay, our optimum sample reached 1108.91 μmol AAE/g, indicating a vast antioxidant capacity. For comparison, Portmann et al. [58] investigated the bioactive compounds and antioxidant capacity from two species of aqueous lemon verbena extracts through infusion and decoction techniques. They found that DPPH ranged from 57 to 232 μmol AAE/g dw, indicating a significant difference from our sample.

Table 8. Different parameters and polyphenolic compounds analysis under optimal extraction conditions (X_1:4, X_2:3, X_3:2, X_4:5).

Parameters	Optimal Extract
Carotenoids (μg CtE/g)	499.61 ± 32.47
Ascorbic acid (mg/g)	8.36 ± 0.28
L^*	36.67 ± 0.2
C^*	12.9 ± 0.6
Hue	55.1 ± 2.6
Polyphenolic compounds (mg/g)	
Vanillic acid	0.37 ± 0.02
p-Coumaric acid	5.27 ± 0.11
Ferulic acid	1.88 ± 0.04
Rutin	2.27 ± 0.13
Quercetin 3-D-galactoside	2.23 ± 0.07
Verbascoside	132.61 ± 9.81
Narirutin	1.49 ± 0.03
Kaempferol 3-glucoside	2.3 ± 0.12

4. Conclusions

Through the extensive investigation and optimization of different conditions, this study aimed to determine the optimal extraction method for bioactive components recovered from lemon verbena. The experiments were conducted using water and ethanol mixtures and eco-friendly and food-grade solvents with adjustable polarity. While the PLS model revealed which parameters were most important for extraction, RSM allowed for the fine-tuning of those parameters. It was revealed that the implementation of PEF and US in conventional extraction was of high importance. However, it should be noted that further green techniques or extraction conditions could have been investigated for the effective recovery of bioactive compounds. In addition, by using a moderate-polarity mixture (50% *v*/*v* ethanol), increasing the extraction temperature (80 °C) within a moderate extraction duration (60 min) improved the effectiveness of the extraction of bioactive compounds. The application of MCA and PCA revealed a strong negative correlation between AA and color coordinates, which would be primarily due to solvent polarity. The considerably high TPC which was measured (175.03 mg GAE/g dw), along with other compounds such as AA and TC render lemon verbena leave extracts capable of providing pharmaceutical and food industries with plenty of health-promoting bioactive compounds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/oxygen4010001/s1, Figures S1–S4 comprise plots that illustrate the comparison between the actual response and the predicted response for each parameter under

examination, accompanied by the desirability functions. Figures S5–S7 present three-dimensional response plots for the remaining responses.

Author Contributions: Conceptualization, V.A., T.C. and S.I.L.; methodology, V.A., T.C. and S.I.L.; software, V.A. and T.C.; validation, V.A., T.C., D.K., I.M. and E.B.; formal analysis, V.A. and T.C.; investigation, V.A. and T.C.; resources, S.I.L.; data curation, V.A., T.C. and S.I.L.; writing—original draft preparation, V.A. and D.K.; writing—review and editing, V.A., T.C., D.K., I.M., E.B. and S.I.L.; visualization, V.A. and T.C.; supervision, S.I.L.; project administration, S.I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All related data and methods are presented in this paper. Additional inquiries should be addressed to the corresponding author.

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

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