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Enhancement of the Green Extraction of Bioactive Molecules from *Olea europaea* Leaves

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Abstract: Olive leaves are a rich source of polyphenols that have beneficial antihypertensive, hypocholesterolemic, cardioprotective, and anti-inflammatory effects. The aim of this study was to compare the efficiency of conventional extraction (CE), microwave-assisted extraction (MWE), and microwave–ultrasound-assisted extraction (MWUE) for the extraction of bioactive molecules from olive leaves using water as a solvent and to define the optimal extraction conditions for all three methods used. CE conditions (temperature, time, magnetic stirrer rotational rate and particle diameter) and MWE extraction and MWUE conditions (microwave power, time, particle diameter, and temperature) were optimized using response surface methodology (RSM) based on the Box–Behnken experimental design. The total polyphenol content and antioxidant activity of all prepared extracts was analyzed and compared. The results showed that MWUE provided the highest amount of total polyphenols (Total Polyphenolic Content (TPC) = 273.779 ± 4.968 mg_{GAE} $g_{d.m.}{}^{-1}$) and the highest antioxidant activity, which was about 3.1 times higher than CE. Optimal extraction conditions were determined to be 80 °C, 15 min, 200 µm, and 750 min-1 for CE, 700 W, 7.5 min, 300 µm, and 80 °C for MWE, and 800 W, 5 min, 100 µm, and 60 °C for MWUE. Considering the maximum amount of total polyphenols extracted, the results suggest that MWUE is the most effective green extraction process that extracted the highest amount of polyphenols and could be used by the food industry for commercial exploitation of currently unprofitable plant bioactive sources.

Keywords: olive leaves; microwave-assisted extraction; microwave–ultrasound-assisted extraction; response surface optimization

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1. Introduction

The olive tree is a plant that grows in abundance in the Mediterranean region. The most common species is *Olea europaea* L., which is extensively dispersed along a coast of Southern Europe, Western Asia and North Africa [1,2]. As previously stated by Ozturk et al. [2], *Olea europaea* grows in a form of short, dense tree up to 10 m tall, and the most essential portion of this tree is its fruit. Moreover, 10% of the whole mass of olives is composed of olive leaves [3]. According to Proietti et al. [4], leaves are gathered during the olive tree trimming and mostly disposed of as waste, and it is currently not profitable. Many nations utilize olive leaves as animal fodder, burning surplus branches following trimming [5]. Olive leaves are accessible as a low-cost natural resource and can been used in a large-scale industrial production of functional foods [6]. Olive leaves are a rich and cheap source of polyphenols that could be extracted and valorized for development of health-promoting products [7]. According to Vogel et al. [8], a large number of studies reported beneficial anti-hypertensive, hypocholesterolemic, cardioprotective and anti-inflammatory

effects of the polyphenolic components found in olive leaves. Due to that, there is an increasing interest in the food industry for production of functional foods fortified with olive extracts containing biologically active compounds [9].

The variables impacting the extraction procedure have to be explored to maximize the yield of bioactive molecules that are extracted from the plant materials [10,11]. In the case of extraction of bioactive molecules, solvent extraction is a favorable procedure because thermal delicate materials are recoverable at low temperatures [10]. As stated by Bosiljkov et al. [12], extraction techniques of bioactive molecules must be based on green and feasible methods and green extraction principles. Taking into account the principles of green extraction, water can be employed as the solvent. According to Goldsmith et al. [5], water is a low-cost polar solvent that has been demonstrated to successfully extract a wide range of phenolic molecules with significant antioxidant properties from a variety of plant sources. Taking into consideration the green extraction principles, which include sustainable energy utilization and the use of innovatory applied science [13], microwave-assisted extraction and ultrasound-assisted extraction are considered to be highly effective [14,15]. Pinto et al. [16] described microwave-assisted extraction as a green approach that uses microwaves that are capable of entering the sample and reacting with polar components. In comparison to traditional extraction procedures, the heat and pressure generated during the microwave-assisted extraction process enhance mass transfer and aid in removing plant components in less time with a higher yield [16–20]. However, microwave radiation may generate extreme heating on certain areas of the treated sample, which can have a negative effect on the heat sensitive bioactive molecules [21]. This problem can be minimized by combing the microwaves with ultrasound. By creating impact forces, ultrasonic waves may cause solid particles to rupture as a result of the cavity effect's instantaneous high temperature and pressure [22]. Reports reveal efficient application of microwave–ultrasound-assisted extraction for extraction of phenolic compounds, flavonoids, triterpenoids and vitamin C from *Clinacanthus nutans* [23,24], proteins from barley [21], polysaccharides from *Camptotheca acuminate* [22] and flavonoids from *Lotus plumule* [25].

To analyze the effects of all variables on the extraction process and to estimate optimal extraction conditions, statistical and mathematical modeling methods have been successfully applied over the years [26,27]. The traditional approach (one-factor-at-a-time) is deemed less trustworthy since it does not account for interacting effects among components and it is time-consuming and costly [28]. To address this issue, the response surface methodology (RSM) was developed and is now commonly used to optimize extraction conditions. RSM is a sophisticated mathematical approach that is frequently utilized in numerous sectors to optimize particular experimental settings. Furthermore, it concurrently assesses the effects of many factors and their interactions on one or more response variables, resulting in a smaller number of experiments required to assess the extraction process [19,20,28,29].

The objectives of this study were to evaluate the optimum process conditions of microwave-assisted (MWE) and microwave–ultrasound-assisted extraction (MWUE) for extraction of bioactive molecules from olive leaves using RSM based on Box–Behnken design of experiment. Moreover, the effectiveness of the selected green extraction technologies was compared to the conventional extraction (CE). To the best of our knowledge, this is the first report of comparison and optimization of green extraction processes for the extraction of bioactive molecules from olive leaves. This study also aimed to select the most efficient method for bioactive molecule extraction from olive leaves that can be used in the food industry for commercial application of a currently non-profitable plant source.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Materials

Dried olive leaves (*Olea europaea* L.) were bought at a specialized herb shop (Suban d.o.o., Zagreb, Croatia). Olive leaves were gathered in Croatia's southernmost region at the

time period of 2015. Dehydrated leaves were wrapped in paper pack and kept in a dark and dry environment (T = 20 °C and RH = 50%). Dry matter of the dried olive leaves was measured gravimetrically using a standard AOAC method [30].

2.1.2. Chemical

Folin-Ciocalteu's reagent and sodium carbonate were from Kemika (Zagreb, Croatia). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and potassium peroxodisulphate from Fluka (Buchs, Switzerland). ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), DPPH (1,1-diphenyl-2-picrylhydrazyl), TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) and gallic acid (3,4,5-trihydroxybenzoic acid), iron(II) sulfate heptahydrate and acetic acid from Sigma-Aldrich Chemie (Steinheim, Germany). Methanol and sodium acetate trihydrate J. T. Baker (Deventer, The Netherlands), ethanol from Carlo Erba Reagents (Milano, Italy), and iron (III) chloride hexahydrate from Gram Mol (Zagreb, Croatia).

2.2. Methods

A short description of the methodology used in this work is presented in Figure 1.

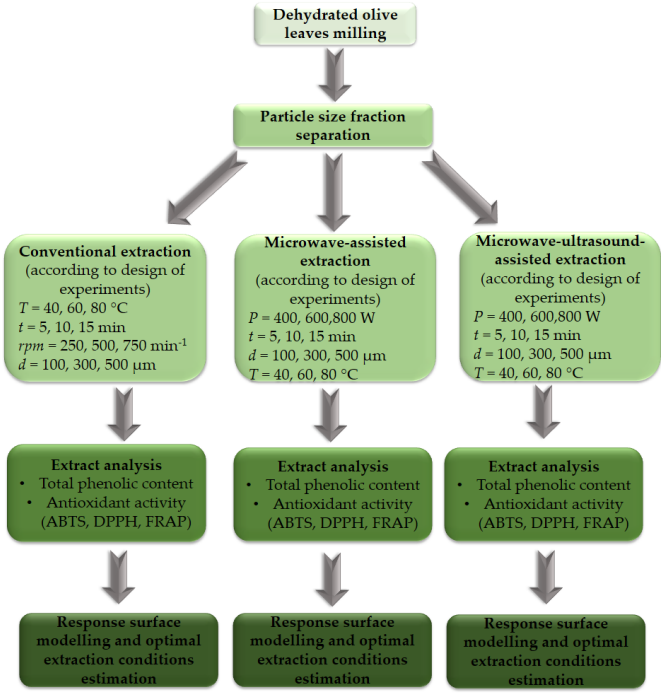

Figure 1. Flow diagram of the methodology used in this research.

2.2.1. Milling and Separation of Particle Size Fractions

Dehydrated olive leaves were ground using the IKA Tube control batch mill (IKA-Werke, Staufen, Germany). Milling process settings were as follows: 15,000 rpm with adjustable milling time (10–40 s). Standardized DIN sieves (Fritsch, Idar-Oberstein, Germany) with pores diameter d = 100, 250, 355, 500, 800 and 1000 µm were used to separate particle size fractions of ground olive leaves. Seven distinct particle size fractions were obtained (<100, 100, 250, 355, 500, 800 and 1000 µm) by a subsequent sieving of the milled herbal material. Three fractions (d = 100, 355, 500 µm) were used for further experiments.

2.2.2. Conventional Extraction (CE) Procedure

One gram of ground olive leaves was extracted with 50 mL of deionized water in 200 mL glass at a selected temperature (40, 60, 80 °C ± 0.5 °C) in the Ika HBR4 digital oil-bath (IKA-Werk GmbH & Co. KG, Staufen, Germany). Following the completion of the

extraction procedure, samples were filtered using a cellulose paper filter (LLG Labware, Meckenheim, Germany) and stored at 4 °C until analysis.

2.2.3. Microwave-Assisted Extraction (MWE) and Microwave–Ultrasound-Assisted Extraction (MWUE) Procedure

MWE and MWUE were performed in the MW-ER-02 extractor (Lab Kits, Miami, USA). Impact of the MW and simultaneous effect of the MV and US (p = 50 W, f = 40 kHz) on the extraction productivity was examined. One gram of dry olive lives was extracted with 50 mL of deionized water in a 250 mL Erlenmeyer flask. Following the completion of the extraction procedure, samples were filtered using 100 percent cellulose paper filter (LLG Labware, Meckenheim, Germany) and stored at 4 °C until analysis.

2.2.4. Total Polyphenolic Content (TPC) and Antioxidant Activities (AA) Measurement

TPC was measured spectrophotometrically according to Pinelo et al. [31]. AA was measured using the ABTS free radical method [32], DPPH method [33] and FRAP method [34]. All results were expressed per gram of dry matter (DM) of olive leaves. All measurements were conducted in triplicate and results expressed as the average value ± standard deviation.

2.2.5. Design of the Experiments and Response Surface Modeling

The effect of process variables was evaluated using Box–Behnken experimental design (BB DOE) at three levels (−1, 0, 1). For each extraction type, 27 experiments were conducted randomly. In the case of CE process variables of interest were: (i) extraction temperature, X_1 (T = 40, 60, and 80 °C), (ii) extraction time, X_2 (t = 5, 10, and 15 min), (iii) magnetic stirrer rotational rate, X_3 (rpm = 250, 500, and 750 min^{-1}) and (iv) particle diameter, X_4 (d = 100, 300, and 500 μm). In the case of the MWE and MWUE processes, the effects of (i) microwave power, Z_1 (p = 400, 600, and 800 W), (ii) extraction time, Z_2 (t = 5, 10, and 15 min), (iii) particle diameter, Z_3 (d = 100, 300, and 500 μm) and (iv) extraction temperature, Z_4 (T = 40, 60, and 80 °C) were analyzed. Experimental measurement data were fit to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i \cdot X_i + \sum_{i=1}^{3} \beta_{ii} \cdot X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} \cdot X_i \cdot X_j$$

where Y is the predicted response, β_0, β_{i}, β_{ii} and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction phrases, respectively, and X_i and X_j are the independent variables [19]. Response surface analysis was carried out employing Statistica v.14 software package (Tibco Software Inc., Palo Alto, CA, USA). Simultaneous optimization of all four (TPC, ABTS, DPPH, FRAP) output variables was performed.

2.2.6. Statistical Analysis

The descriptive statistical analysis (mean values, standard deviations, and data distribution normality test) in this work was conducted in the Statistica v.14 software package (Tibco Software Inc., Palo Alto, CA, USA). Correlations between extraction process variables and output variables were analyzed based on the Pearson correlation coefficients using the Statistica v.14 software package.

3. Results

In this work, biologically active compounds were extracted from olive leaves by CE, MWE and MWUE process. To define the process conditions that ensure optimum concentrations of polyphenols and antioxidant activity, the experimental plan was developed and analyzed using the RSM methodology.

3.1. Response Surface Analysis of the Total Phenolic Content and Antioxidant Activity of the Olive Leaves Aqueous Extracts Produced by Conventional Extraction

In the case of the classical extraction process, the influence of four process variables (T, t, rpm and d) was analyzed at three levels. Experimentally measured data (TPC and AA determined by ABTS, DPPH and FRAP method) of 27 experiments are given in Table 1.

The results showed that the highest amount of total polyphenols (TPC = 88.305 ± 3.271 mg_{GAE} $g_{d.m.}{}^{-1}$) was extracted in Experiment No. 4 (80 °C, 15 min, 500 min^{-1}, 300 µm), which is approximately 1.5 fold higher in comparison to Experiment No. 2 (40 °C, 15 min, 500 min^{-1}, 300 µm) where TPC value was 57.725 ± 6.277 mg_{GAE} $g_{d.m.}{}^{-1}$. Similar results for polyphenolic concentration in olive leaves infusions were presented by Medina et al. [35] where TPC was in a range of 44.79–108.27 g kg^{-1} and by Feng et al. [36] for methanol–water olive leaves extract, where TPC was in the range of 17.58–51.07 mg_{GAE} $g_{d.m.}{}^{-1}$. Furthermore, Lins et al. [37] presented 131.7 mg_{GAE} $g_{d.m.}{}^{-1}$ of TCP in n-hexane extract of olive leaves, while Topuz and Bayram [38] obtained TPC in a range of 35.10–49.81 mg_{GAE} $g_{d.m.}{}^{-1}$ for extraction in methanol. Nicolí et al. [39] presented a phenolic profile and AA for ethanol extracts (60% ethanol solution) of olive leaves of 15 Italian Olea europaea L. cultivars, where TCP was in a range of 11–49 mg_{GAE} $g_{d.m.}{}^{-1}$ depending on the cultivar. Presented experimental conditions (No. 4 and No. 2) differed only in extraction temperature, demonstrating that the extraction temperature had an important effect on the extraction efficiency. The obtained results were consistent with the correlation coefficients shown in Table S1. The correlation coefficient showed a significant positive influence of temperature on the TPC values (r = 0.677), indicating that higher amounts of TPC are extracted at higher temperatures. Alternatively, correlation coefficients showed negative correlations between extraction time and TPC, ABTS, DPPH and FRAP. The extraction time for this experiment was selected based on literature data [40], where the dynamic extraction experiments of Lamiaceae family plant were performed and it was shown that prolongation of the extraction time over 15 min had no influence on extraction performance. Peršurić et al. [41] also showed that an extraction time of longer than 15 min had no influence of the extraction yield of the polyphenols from olive lives. Furthermore, the extraction temperature had a positive effect on the extraction yield [41].

The greatest AA values of the extracts analyzed by the FRAP and the DPPH method were also observed for Experiment No. 4 (80 °C, 15 min, 500 min^{-1}, 300 µm), with FRAP = 1.049 ± 0.010 $mmol_{FeSO_4 \cdot 7H_2O}$ $g_{d.m.}{}^{-1}$ and DPPH = 0.401 ± 0.014 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$, respectively. Similar results were presented by Lins et al. [37], where AA measured by FRAP method was 1.0837 $mmol_{FeSO_4 \cdot 7H_2O}$ $g_{d.m.}{}^{-1}$ for n-hexane extract of olive leaves prepared by maceration process. Conversely, Nicolí et al. [39] presented results of AA measured by DPPH of olive leaves of 15 Italian Olea europaea L. cultivars in range of 0.867–2.989 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$. The higher AA presented by Nicolí et al. [39] can be explained by the use of 60% ethanol solution as the extraction solvent. However, according to Martiny et al. [42], water is the most suitable extraction solvent (non-toxic, no need for solvent removal) when the extract is further used in pharmaceutical and food industry.

In this work, the highest value of AA measured by the ABTS method (ABTS = 0.457 ± 0.061 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$) was obtained for Experiment No. 14 (60 °C, 5 min, 500 min^{-1}, 500 µm) while the lowest value (ABTS = 0.245 ± 0.047 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$) was acquired for the extract prepared under Experiment No. 27 (60 °C, 10 min, 500 min^{-1}, 300 µm). This can be compared with results presented by Sánchez-Gutiérrez et al. [43] where AA expressed by ABTS was in a range from 0.253 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$ for water extract to 0.301 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$ for 50% ethanol extract. These results are consistent with the correlation coefficients (Table 2). The correlation coefficients show a significant positive correlation between TPC and DPPH (r = 0.506) and TPC and FRAP (r = 0.454), and positive correlation between TPC and ABTS (r = 0.156). As stated by Piluzza and Billitta [44] this finding indicates that phenolic content might be utilized to predict AA properties. Al-Marazeeq et al. [45] conducted a study on the influence of solvent type (80% methanol, 80% ethanol, acetone and distilled water) and extraction conditions on the proportion of total polyphenolic content in olive leaves extract. The total polyphenolic content ranged from TPC = 158–392 mg_{GAE} $g_{d.m.}{}^{-1}$, which was consistent

with the data presented in this research. Second order polynomial equations were applied to fit the experiment to the model data (Table 2, Figure 2a[1]–d[1]).

In the case of the TPC, the RSM model showed that temperature (X_1), particle size (X_3), and rotational rate (X_4) had positive effects on the TPC, while time (X_2) had a negative influence on the TPC (Table 2). As presented at Figure 2a[1], the effect of extraction temperature and extraction time on TPC was analyzed by keeping particle size and rotational rate constant by increasing extraction temperature from 40 to 80°C, while the concentration of extracted TCP increased from 68.551 to 88.305 $mg_{GAE}\ g_{d.m.}{}^{-1}$. As stated by Anne and Nithyanandam [46], an increase in extraction temperature reduces the viscosity of the solvent which improves the mass transfer. The present results agree with Santos et al. [47], Vieira et al. [48] Saifullah et al. [49] and Monteleone et al. [50], who all reported positive effects of the extraction temperature on polyphenol content in plant extracts prepared by conventional extraction. The negative effect of the extraction time can be explained by Fick's second law: by prolonging the extraction time solvent will be statured by the phenolic compounds and the extraction rate will decrease. The same effect of input variables was observed for AA measured by FRAP method (Table 2, Figure 2d[1]). In the case of AA measured by the ABTS method, the model demonstrated that extraction temperature, extraction time and particle diameter had a negative effect, while magnetic stirrer rotational rate had a positive effect on the ABTS (Table 2, Figure 2b[1]). Alternatively, in the case of AA determined by the DPPH method, the developed model demonstrated that only temperature had a positive influence on the analyzed variable (Table 2, Figure 2c[1]).

The highest fit of experimental and model predicted data was detected for the CE process in the case of AA measured by DPPH method (r^2 = 0.871). As stated by Le Man et al. [51], a model is considered acceptable for $r^2 > 0.75$. As a result, it is possible to deduce that all models developed for characterization of the CE process in this work adequately describe the process. However, considering that only the high R^2 value does not necessarily ensure that the model fits the data adequately, residual analysis was used to confirm the model's goodness-of-fit (Figure 3).

The residuals are defined as the differences between experimental values and model predicted values of specific variable. The residuals for the TCP, ABTS, DPPH and FRAP models were determined to be normally distributed (Figure 3a[1]–a[4]). Furthermore, the normality criterion was fulfilled because the residual plots were gathered nearly along a straight line. The normal distribution of the residuals was further confirmed by the bell-shaped histograms that displayed the measurement distribution (Figure 3c[1]–c[4]). Residual versus predicted value graphs (Figure 2b[2]–b[4]) show that there is no pattern in the residuals, showing that the models match the experimental data well. Furthermore, the residuals were found to vary around the center value (Figure 3d[1]–d[4]) with no clear outliers.

Additionally, the ANOVA demonstrated that the developed quadratic models were significant (for $p < 0.05$) with p values in the range from <0.001 to 0.020 (Table S2). As stated by Teng et al. [52], greater regression coefficients and lower p value for each component in the model imply a more significant influence of the studied variable. The statistical study also revealed that the lack of fit values of the models were not significant ($p > 0.05$) and that F values were large (in the range from 1.360 for FRAP to 4.690 for TPC). The collected findings show that the proposed models are reliable within the range of the analyzed variables.

Table 1. Box–Behnken design of experiment for CE process based on three process variables with their observed responses. Factor levels are shown in brackets.

Run	*T*/°C	*t*/min	*rpm*/min^{-1}	*d*/μm	TPC/ $mg_{GAE}\ g_{d.m.}{}^{-1}$	ABTS/ $mmol_{Trolox}\ g_{d.m.}{}^{-1}$	DPPH/ $mmol_{Trolox}\ g_{d.m.}{}^{-1}$	FRAP/ $mmol_{FeSO_4 \cdot 7H_2O}\ g_{d.m.}{}^{-1}$
1	40 (−1)	5 (−1)	500 (0)	300 (0)	69.551 ± 7.417	0.371 ± 0.059	0.337 ± 0.056	0.741 ± 0.005
2	40 (−1)	15 (1)	500 (0)	300 (0)	57.725 ± 6.277	0.312 ± 0.039	0.302 ± 0.002	0.618 ± 0.004
3	80 (1)	5 (−1)	500 (0)	300 (0)	73.172 ± 5.876	0.361 ± 0.043	0.307 ± 0.009	0.812 ± 0.041
4	80 (1)	15 (1)	500 (0)	300 (0)	88.305 ± 3.271	0.353 ± 0.012	0.401 ± 0.014	1.049 ± 0.010
5	60 (0)	10 (0)	250 (−1)	100 (−1)	66.131 ± 10.256	0.376 ± 0.104	0.293 ± 0.002	0.718 ± 0.076
6	60 (0)	10 (0)	750 (1)	100 (−1)	69.115 ± 4.069	0.377 ± 0.023	0.306 ± 0.026	0.743 ± 0.041
7	60 (0)	10 (0)	250 (−1)	500 (1)	68.832 ± 1.358	0.384 ± 0.048	0.278 ± 0.017	0.717 ± 0.004
8	60 (0)	10 (0)	750 (1)	500 (1)	69.178 ± 5.755	0.329 ± 0.038	0.242 ± 0.097	0.726 ± 0.288
9	60 (0)	10 (0)	500 (0)	300 (0)	64.598 ± 2.119	0.352 ± 0.002	0.305 ± 0.009	0.819 ± 0.133
10	60(0)	5 (−1)	500 (0)	100 (−1)	69.753 ± 4.095	0.355 ± 0.024	0.319 ± 0.038	0.637 ± 0.105
11	60 (0)	15 (1)	500 (0)	100 (−1)	61.326 ± 1.930	0.362 ± 0.084	0.281 ± 0.002	0.647 ± 0.099
12	60 (0)	5 (−1)	500 (0)	500 (1)	70.063 ± 4.095	0.457 ± 0.061	0.343 ± 0.031	0.654 ± 0.117
13	60 (0)	15 (1)	500 (0)	500 (1)	60.499 ± 4.748	0.368 ± 0.067	0.265 ± 0.019	0.622 ± 0.006
14	40 (−1)	10 (0)	250 (−1)	300 (0)	63.366 ± 13.121	0.405 ± 0.085	0.317 ± 0.048	0.699 ± 0.063
15	80 (1)	10 (0)	250 (−1)	300 (0)	75.649 ± 1.047	0.313 ± 0.067	0.334 ± 0.015	0.676 ± 0.150
16	40 (−1)	10 (0)	750 (1)	300 (0)	68.543 ± 0.332	0.351 ± 0.012	0.294 ± 0.024	0.821 ± 0.100
17	80 (1)	10 (0)	750 (1)	300 (0)	72.827 ± 1.388	0.396 ± 0.126	0.346 ± 0.039	0.778 ± 0.017
18	60 (0)	10 (0)	500 (0)	300 (0)	66.460 ± 4.331	0.282 ± 0.009	0.301 ± 0.029	0.795 ± 0.003
19	60 (0)	5 (−1)	250 (−1)	300 (0)	70.615 ± 3.014	0.338 ± 0.016	0.396 ± 0.007	0.858 ± 0.074
20	60 (0)	15 (1)	250 (−1)	300 (0)	66.189 ± 7.616	0.358 ± 0.086	0.337 ± 0.004	0.845 ± 0.045
21	60 (0)	5 (−1)	750 (1)	300 (0)	69.644 ± 6.191	0.312 ± 0.040	0.338 ± 0.005	0.745 ± 0.002
22	60 (0)	15 (1)	750 (1)	300 (0)	67.193 ± 1.988	0.331 ± 0.037	0.338 ± 0.004	0.762 ± 0.033
23	40 (−1)	10 (0)	500 (0)	100 (−1)	61.944 ± 2.955	0.351 ± 0.019	0.341 ± 0.002	0.815 ± 0.002
24	80 (1)	10 (0)	500 (0)	100 (−1)	69.271 ± 4.108	0.357 ± 0.102	0.328 ± 0.022	0.820 ± 0.106
25	40 (−1)	10 (0)	500 (0)	500 (1)	67.634 ± 8.389	0.358 ± 0.012	0.311 ± 0.017	0.913 ± 0.086
26	80 (1)	10 (0)	500 (0)	500 (1)	73.958 ± 3.064	0.296 ± 0.026	0.304 ± 0.013	0.813 ± 0.044
27	60 (0)	10 (0)	500 (0)	300 (0)	76.379 ± 3.121	0.245 ± 0.047	0.313 ± 0.038	0.965 ± 0.095

Table 2. RSM models for description of extraction of TPC and AA (ABTS, DPPH and FRAP) from olive leaves by CE, MWE and MWUE processes. Statistically significant coefficients are marked bold.

	Variable	Model Equation	R^2
conventional extraction	*TPC*	$Y = \mathbf{68.175} + \mathbf{11.197}\cdot X_1 - \mathbf{3.720}\cdot X_2 + 0.848\cdot X_3 + 2.093\cdot X_4 - 1.203\cdot {X_1}^2 + 1.036\cdot {X_2}^2 + 0.733\cdot {X_3}^2 + 3.180\cdot {X_4}^2 + \mathbf{12.097}\cdot X_1X_2 - 3.999\cdot X_1X_3 - 0.503\cdot X_1X_4 + 0.987\cdot X_2X_3 - 0.567\cdot X_2X_4 - 1.633\cdot X_3X_4$	0.827
	ABTS	$Y = \mathbf{0.385} - 0.0148\cdot X_1 - 0.018\cdot X_2 + \mathbf{0.003}\cdot X_3 - 0.013\cdot X_4 - 0.028\cdot {X_1}^2 - 0.032\cdot {X_2}^2 - \mathbf{0.037}\cdot {X_3}^2 - \mathbf{0.052}\cdot {X_4}^2 + 0.026\cdot X_1X_2 + 0.069\cdot X_1X_3 - 0.043\cdot X_1X_4 - 0.001\cdot X_2X_3 - 0.048\cdot X_2X_4 - 0.031\cdot X_3X_4$	0.778
	DPPH	$Y = \mathbf{0.3341} + \mathbf{0.020}\cdot X_1 - \mathbf{0.019}\cdot X_2 - 0.014\cdot X_3 - 0.010\cdot X_4 - 0.026\cdot {X_1}^2 - \mathbf{0.032}\cdot {X_2}^2 - 0.021\cdot {X_3}^2 - 0.005\cdot {X_4}^2 + 0.064\cdot X_1X_2 + 0.017\cdot X_1X_3 - 0.003\cdot X_1X_4 + 0.030\cdot X_2X_3 - 0.019\cdot X_2X_4 - 0.020\cdot X_3X_4$	0.871
	FRAP	$Y = \mathbf{0.731} + \mathbf{0.033}\cdot X_1 - \mathbf{0.005}\cdot X_2 + 0.002\cdot X_3 + 0.014\cdot X_4 - \mathbf{0.007}\cdot {X_1}^2 + 0.061\cdot {X_2}^2 - 0.029\cdot {X_3}^2 - 0.064\cdot {X_4}^2 + 0.111\cdot X_1X_2 - 0.009\cdot X_1X_3 - 0.049\cdot X_1X_4 + 0.084\cdot X_2X_3 - 0.021\cdot X_2X_4 - 0.026\cdot X_3X_4$	0.754
microwave-assisted extraction	*TPC*	$Y = \mathbf{124.059} + \mathbf{13.197}\cdot Z_1 - 10.988\cdot Z_2 + 6.355\cdot Z_3 + \mathbf{25.868}\cdot Z_4 - \mathbf{11.958}\cdot {Z_1}^2 + 8.607\cdot {Z_2}^2 + \mathbf{10.672}\cdot {Z_3}^2 + 0.668\cdot {Z_4}^2 + 16.824\cdot Z_1Z_2 - 0.108\cdot Z_1Z_3 - 11.263\cdot Z_1Z_4 + 0.297\cdot Z_2Z_3 + 8.179\cdot Z_2Z_4 - 4.045\cdot Z_3Z_4$	0.768
	ABTS	$Y = \mathbf{0.593} - \mathbf{0.017}\cdot Z_1 + 0.050\cdot Z_2 + 0.081\cdot Z_3 - \mathbf{0.151}\cdot Z_4 + \mathbf{0.086}\cdot {Z_1}^2 + \mathbf{0.101}\cdot {Z_2}^2 + 0.067\cdot {Z_3}^2 + 0.046\cdot {Z_4}^2 + 0.028\cdot Z_1Z_2 + 0.157\cdot Z_1Z_3 + 0.095\cdot Z_1Z_4 - 0.029\cdot Z_2Z_3 - 0.118\cdot Z_2Z_4 - 0.107\cdot Z_3Z_4$	0.849
	DPPH	$Y = \mathbf{0.408} + 0.009\cdot Z_1 - \mathbf{0.018}\cdot Z_2 + 0.023\cdot Z_3 + \mathbf{0.067}\cdot Z_4 + 0.005\cdot {Z_1}^2 + \mathbf{0.031}\cdot {Z_2}^2 + 0.025\cdot {Z_3}^2 + 0.001\cdot {Z_4}^2 - 0.031\cdot Z_1Z_2 + 0.022\cdot Z_1Z_3 + 0.001\cdot Z_1Z_4 + 0.017\cdot Z_2Z_3 + 0.024\cdot Z_2Z_4 + 0.012\cdot Z_3Z_4$	0.858
	FRAP	$Y = \mathbf{0.977} - 0.025\cdot Z_1 - \mathbf{0.401}\cdot Z_2 - 0.075\cdot Z_3 + \mathbf{0.140}\cdot Z_4 + 0.050\cdot {Z_1}^2 - 0.074\cdot {Z_2}^2 + 0.074\cdot {Z_3}^2 - 0.028\cdot {Z_4}^2 - 0.209\cdot Z_1Z_2 + 0.346\cdot Z_1Z_3 + \mathbf{0.284}\cdot Z_1Z_4 + 0.384\cdot Z_2Z_3 - 0.189\cdot Z_2Z_4 + 0.087\cdot Z_3Z_4$	0.817
ultrasound-microwave-assisted extraction	*TPC*	$Y = \mathbf{159.091} + \mathbf{11.247}\cdot Z_1 - \mathbf{39.523}\cdot Z_2 - 10.311\cdot Z_3 + 26.713\cdot Z_4 - 2.106\cdot {Z_1}^2 - 23.453\cdot {Z_2}^2 - 7.442\cdot {Z_3}^2 + 11.836\cdot {Z_4}^2 - \mathbf{64.399}\cdot Z_1Z_2 - 14.828\cdot Z_1Z_3 + 19.174\cdot Z_1Z_4 + 48.693\cdot Z_2Z_3 - 4.796\cdot Z_2Z_4 - 2.249\cdot Z_3Z_4$	0.875
	ABTS	$Y = \mathbf{0.579} + \mathbf{0.107}\cdot Z_1 - \mathbf{0.249}\cdot Z_2 - 0.045\cdot Z_3 - 0.132\cdot Z_4 - \mathbf{0.0370}\cdot {Z_1}^2 - 0.034\cdot {Z_2}^2 - 0.057\cdot {Z_3}^2 + 0.030\cdot {Z_4}^2 - 0.213\cdot Z_1Z_2 - 0.133\cdot Z_1Z_3 - 0.143\cdot Z_1Z_4 - 0.272\cdot Z_2Z_3 + 0.006\cdot Z_2Z_4 + 0.234\cdot Z_3Z_4$	0.898
	DPPH	$Y = \mathbf{0.472} + 0.015\cdot Z_1 - \mathbf{0.126}\cdot Z_2 + 0.053\cdot Z_3 + \mathbf{0.021}\cdot Z_4 - 0.052\cdot {Z_1}^2 - 0.077\cdot {Z_2}^2 + 0.054\cdot {Z_3}^2 + 0.054\cdot {Z_4}^2 - 0.247\cdot Z_1Z_2 - 0.064\cdot Z_1Z_3 + 0.002\cdot Z_1Z_4 + 0.032\cdot Z_2Z_3 - \mathbf{0.059}\cdot Z_2Z_4 - 0.208\cdot Z_3Z_4$	0.799
	FRAP	$Y = \mathbf{1.067}\text{-}\ 0.052\cdot Z_1 - \mathbf{0.084}\cdot Z_2 + 0.052\cdot Z_3 + \mathbf{0.145}\cdot Z_4 + 0.095\cdot {Z_1}^2 + 0.168\cdot {Z_2}^2 - 0.007\cdot {Z_3}^2 - \mathbf{0.061}\cdot {Z_4}^2 - \mathbf{0.489}\cdot Z_1Z_2 - 0.049\cdot Z_1Z_3 - 0.009\cdot Z_1Z_4 - 0.011\cdot Z_2Z_3 + 0.009\cdot Z_2Z_4 + 0.023\cdot Z_3Z_4$	0.863

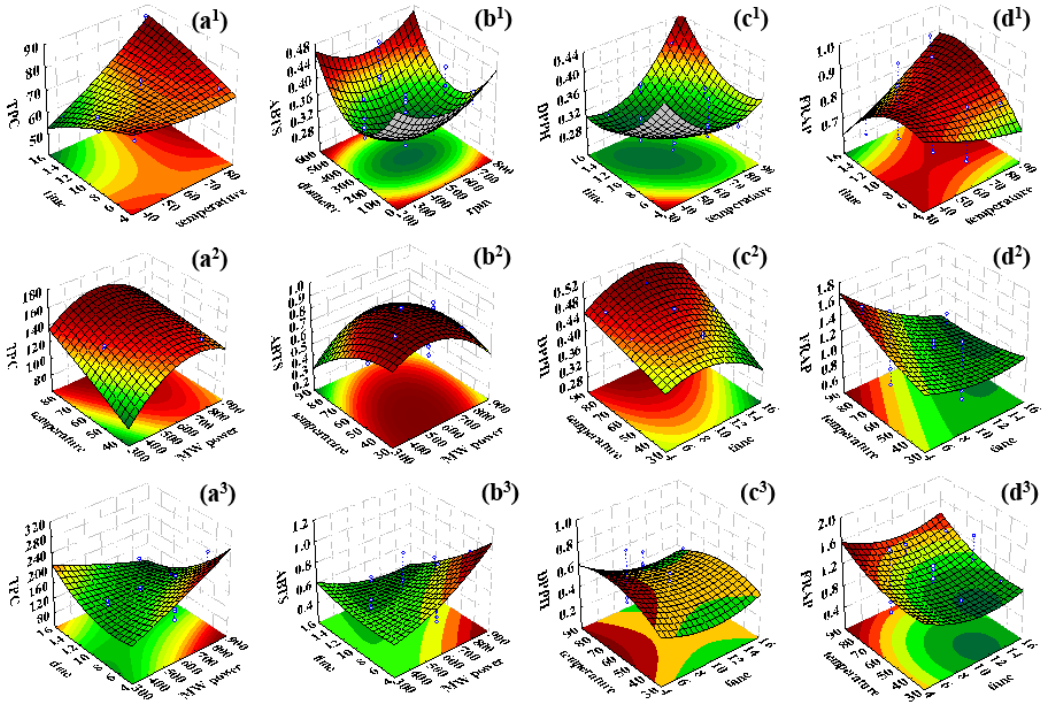

Figure 2. Three dimensional response surface plots of the significant variables on the extraction process outputs: (**a**) TPC, (**b**) ABTS, (**c**) DPPH, and (**d**) FRAP. (1) CE, (2) MWE, and (3) MWUE.

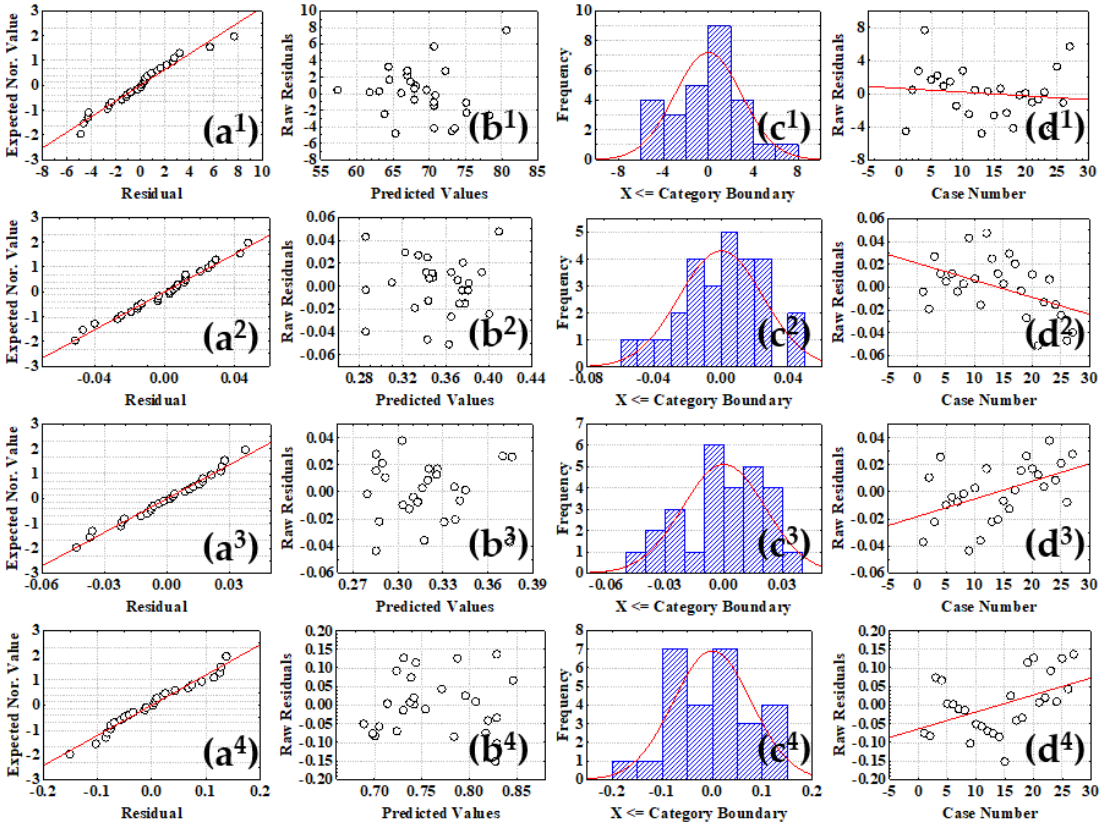

Figure 3. Residual analysis for the RSM models for CE: (**a**) normal probability plot of the residuals, (**b**) residuals versus predicted values, (**c**) histogram of residuals, and (**d**) residuals versus case number. (1) TPC, (2) ABTS, (3) DPPH, and (4) FRAP.

3.2. Response Surface Analysis of the Total Phenolic Content and Antioxidant Activity of the Olive Leaves Aqueous Extracts Produced by Microwave-Assisted Extraction and Microwave–Ultrasound-Assisted Extraction

Recently, a fast and reliable extraction method utilizing microwaves has been developed. Microwave-assisted extraction is often used for analysis of organic compound traces in solid samples [53]. It is also applied for extraction of natural molecules such as flavonoids and caffeine, as well as polyphenolic compounds from tea [54], grape seeds [55], lettuce (Lettuce sativa) [56], Punica granatum peel [57], coffee (*Coffea robusta* L. Linden), bee pollen [58] and many others. In this work aqueous extracts from olive leaves were also prepared by microwave–ultrasound-assisted extraction process. Microwaves and ultrasound effects (p = 50 W) were analyzed simultaneously on the effectiveness of extraction of biologically active substances from olive leaves. This method connects the ultrasonic vibration cavitation with MW and enhances the extraction at low temperature and air pressure [23]. The influence of four variables (T, t, d, p) on the extraction efficacy of MWE and MWUE processes was analyzed (Table 3).

Outcomes show that, for the microwave-assisted extraction, the greatest total polyphenolic concentration (TPC = 157.523 ± 2.336 mg_{GAE} $g_{d.m.}{}^{-1}$) was measured in the extract prepared according to the process conditions of Experiment No. 26 (600 W, 15 min, 300 µm, 80 °C). Da Rosa et al. [59] showed an 82% increase in TCP extraction yield for MWE at 86 °C (TPC = 105.05 mg_{GAE} $g_{d.m.}{}^{-1}$) using water as solvent in comparison to maceration at room temperature (TPC = 57.28 mg_{GAE} $g_{d.m.}{}^{-1}$). Chanioti et al. [60] obtained TPC of 34.53 mg_{GAE} $g_{d.m.}{}^{-1}$ for conventional extraction at 60°C for 1 h in buffer media and TPC of 28.00 mg_{GAE} $g_{d.m.}{}^{-1}$ for MWE at the same temperate for 30 min, while Kirbaşlar and Şahin [61] obtained only 6 mg_{GAE} $g_{d.m.}{}^{-1}$ during MWE extraction. For microwave-assisted extraction obtained correlation coefficients (Table S1) demonstrated a significant positive relationship among TPC and extraction temperature (r = 0.576) and a positive relationship among microwave power and TPC (r = 0.294). Obtained correlation coefficients also showed that there was a significant negative correlation between extraction time and TPC (r = −0.401), a positive correlation between microwave power and TPC (r = 0.113) and a positive relationship between extraction temperature and TPC (r = 0.271) for MWUE. For MWE, the greatest AA by the DPPH method (DPPH = 0.461 ± 0.009 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$) was determined in the extract prepared under the process conditions of Experiment No. 8 (600 W, 10 min, 500 µm, 80 °C), which was higher than the AA presented by Chanioti et al. [60] (DPPH = 0.091 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$) and Sánchez-Gutiérrez et al. [43] (DPPH = 0.158 $mmol_{Trolox}$ $g_{d.m}.{}^{-1}$). The greatest AA by the FRAP method in the extract prepared under the process conditions of Experiment No. 25 (600 W, 5 min, 300 µm, 80 °C) was FRAP = 1.569 ± 0.572 $mmol_{FeSO_4 \cdot 7H_2O}$ $g_{d.m.}{}^{-1}$. Both antioxidant activities measured by DPPH and FRAP methods after microwave-assisted extraction showed approximately 1.6-fold higher values compared to those obtained by the CE.

For the extracts produced by MWUE, the maximum TPC of 273.779±4.968 mg_{GAE} $g_{d.m.}{}^{-1}$ was measured in the extract prepared according to Experiment No. 2 (800 W, 5 min, 300 µm, 60 °C). The highest measured antioxidant activities were also obtained for the extract prepared according to Experiment No. 2 (800 W, 5 min, 300 µm, 60 °C); ABTS = 1.014± 0.041 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$, FRAP = 1.837 ± 0.026 $mmol_{FeSO_4 \cdot 7H_2O}$ $g_{d.m.}{}^{-1}$, and DPPH = 0.933 ± 0.042 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$. The maximum amount of extracted polyphenols in the case of MWUE was 1.7 times gather than MWE, and 3.1 times greater than CE. According to previous research, it is proposed that microwaves have a membrane breaking effect on the cells, which enhances the extraction yield. Conversely, ultrasound creates microscopic bubbles in the plant material, and the collapse of these bubbles creates high-shear gradients that cause microstreaming that disrupts the cell walls [27]. It is proposed that the combination of these two effects improved the extraction efficiency in this research.

Table 3. Box–Behnken design of experiment for MWE and MWUE processes established with three process variables and their observed responses. Factor levels are shown in brackets.

					Microwave-Assisted Extraction				Microwave–Ultrasound-Assisted Extraction Process			
Run	***P*/W**	***t*/min**	***d*/μm**	***T*/°C**	***TPC***	***ABTS***	***DPPH***	***FRAP***	***TPC***	***ABTS***	***DPPH***	***FRAP***
1	400 (−1)	5 (−1)	300 (0)	60 (0)	133.279 ± 3.223	0.503 ± 0.021	0.389 ± 0.022	0.829 ± 0.083	146.858 ± 2.417	0.558 ± 0.143	0.469 ± 0.009	0.770 ± 0.168
2	800 (1)	5 (−1)	300 (0)	60 (0)	140.329 ± 7.659	0.552 ± 0.044	0.438 ± 0.017	1.181 ± 0.517	273.779 ± 4.968	1.014 ± 0.041	0.933 ± 0.042	1.837 ± 0.026
3	400 (−1)	15 (1)	300 (0)	60 (0)	90.209 ± 10.542	0.619 ± 0.005	0.420 ± 0.031	0.815 ± 0.075	147.805 ± 3.628	0.386 ± 0.047	0.456 ± 0.005	1.452 ± 0.874
4	800 (1)	15 (1)	300 (0)	60 (0)	130.909 ± 1.674	0.724 ± 0.005	0.407 ± 0.022	0.748 ± 0.088	145.928 ± 11.687	0.416 ± 0.017	0.425 ± 0.013	0.314 ± 0.791
5	600 (0)	10 (0)	100 (−1)	40 (−1)	114.747 ± 2.412	0.668 ± 0.012	0.374 ± 0.017	0.741 ± 0.071	139.686 ± 1.229	0.934 ± 0.005	0.422 ± 0.030	0.836 ± 0.029
6	600 (0)	10 (0)	500 (1)	40 (−1)	122.573 ± 3.673	0.779 ± 0.067	0.374 ± 0.032	0.637 ± 0.002	138.947 ± 4.328	0.458 ± 0.012	0.404 ± 0.007	0.772 ± 0.011
7	600 (0)	10 (0)	100 (−1)	80 (1)	147.286 ± 11.975	0.669 ± 0.098	0.461 ± 0.009	0.919 ± 0.134	160.582 ± 0.560	0.409 ± 0.061	0.474 ± 0.014	1.070 ± 0.018
8	600 (0)	10 (0)	500 (1)	80 (1)	147.023 ± 2.903	0.577 ± 0.019	0.486 ± 0.002	0.990 ± 0.128	155.346 ± 2.296	0.400 ± 0.009	0.039 ± 0.031	1.099 ± 0.023
9	600 (0)	10 (0)	300 (0)	60 (0)	144.724 ± 10.899	0.742 ± 0.078	0.433 ± 0.005	0.933 ± 0.226	191.953 ± 1.044	0.871 ± 0.013	0.656 ± 0.011	0.466 ± 0.028
10	400 (−1)	10 (0)	300 (0)	40 (−1)	103.274 ± 1.872	0.873 ± 0.011	0.442 ± 0.027	1.358 ± 0.967	136.212 ± 1.675	0.491 ± 0.019	0.441 ± 0.012	0.958 ± 0.044
11	800 (1)	10 (0)	300 (0)	40 (−1)	134.746 ± 0.718	0.692 ± 0.164	0.451 ± 0.018	0.879 ± 0.007	98.924 ± 7.168	0.609 ± 0.016	0.323 ± 0.002	0.659 ± 0.033
12	400 (−1)	10 (0)	300 (0)	80 (1)	135.748 ± 9.539	0.499 ± 0.063	0.458 ± 0.000	0.915 ± 0.037	154.739 ± 6.687	0.615 ± 0.205	0.601 ± 0.007	1.299 ± 0.191
13	800 (1)	10 (0)	300 (0)	80 (1)	144.695 ± 10.278	0.510 ± 0.039	0.469 ± 0.013	1.005 ± 0.035	155.800 ± 10.381	0.446 ± 0.095	0.486 ± 0.002	0.962 ± 0.048
14	600 (0)	5 (−1)	100 (−1)	60 (0)	126.139 ± 7.506	0.570 ± 0.013	0.416 ± 0.009	1.735 ± 1.252	248.462 ± 4.706	0.521 ± 0.002	0.544 ± 0.011	0.949 ± 0.055
15	600 (0)	15 (1)	100 (−1)	60 (0)	120.603 ± 2.890	0.497 ± 0.012	0.386 ± 0.008	0.710 ± 0.110	147.407 ± 6.897	0.499 ± 0.024	0.438 ± 0.016	0.876 ± 0.059
16	600 (0)	5 (−1)	500 (1)	60 (0)	125.021 ± 0.533	0.748 ± 0.045	0.405 ± 0.020	1.030 ± 0.352	157.989 ± 8.071	1.024 ± 0.370	0.506 ± 0.012	1.096 ± 0.059
17	600 (0)	15 (1)	500 (1)	60 (0)	120.082 ± 2.415	0.616 ± 0.177	0.409 ± 0.037	0.774 ± 0.093	154.320 ± 2.694	0.457 ± 0.011	0.464 ± 0.018	0.978 ± 0.032
18	600 (0)	10 (0)	300 (0)	60 (0)	145.619 ± 6.261	0.803 ± 0.012	0.467 ± 0.019	0.945 ± 0.103	192.185 ± 4.097	0.875 ± 0.269	0.691 ± 0.013	0.907 ± 0.019
19	400 (−1)	10 (0)	100 (−1)	60 (0)	121.011 ± 9.311	0.722 ± 0.119	0.394 ± 0.044	1.049 ± 0.248	136.310 ± 5.320	0.524 ± 0.018	0.425 ± 0.025	0.885 ± 0.016
20	800 (1)	10 (0)	100 (-1)	60 (0)	116.626 ± 2.051	0.522 ± 0.150	0.374 ± 0.006	0.681 ± 0.092	140.470 ± 1.098	0.759 ± 0.282	0.435 ± 0.002	0.894 ± 0.026
21	400 (−1)	10 (0)	500 (1)	60 (0)	137.222 ± 6.833	0.651 ± 0.226	0.422 ± 0.027	0.815 ± 0.081	164.974 ± 13.254	0.534 ± 0.049	0.563 ± 0.003	1.178 ± 0.041
22	800 (1)	10 (0)	500 (1)	60 (0)	132.621 ± 12.457	0.764 ± 0.032	0.446 ± 0.010	1.139 ± 0.018	139.479 ± 10.821	0.504 ± 0.028	0.445 ± 0.016	1.007 ± 0.002
23	600 (0)	5 (−1)	300 (0)	40 (−1)	131.107 ± 0.689	0.595 ± 0.039	0.398 ± 0.002	1.066 ± 0.372	134.462 ± 7.030	0.571 ± 0.068	0.455 ± 0.002	1.061 ± 0.004
24	600 (0)	15 (1)	300 (0)	40 (−1)	121.445 ± 4.840	0.822 ± 0.019	0.333 ± 0.024	0.916 ± 0.094	136.503 ± 0.349	0.495 ± 0.039	0.469 ± 0.045	0.935 ± 0.013
25	600 (0)	5 (−1)	300 (0)	80 (1)	150.826 ± 0.152	0.639 ± 0.062	0.460 ± 0.007	1.569 ± 0.572	163.048 ± 0.160	0.479 ± 0.002	0.574 ± 0.009	1.315 ± 0.028
26	600 (0)	15 (1)	300 (0)	80 (1)	157.523 ± 2.336	0.632 ± 0.006	0.441 ± 0.003	1.040 ± 0.035	155.496 ± 1.004	0.415 ± 0.048	0.469 ± 0.025	1.222 ± 0.013
27	600 (0)	10 (0)	300 (0)	60(0)	144.644 ± 8.239	0.837 ± 0.005	0.448 ± 0.002	1.098 ± 0.089	190.806 ± 8.900	0.897 ± 0.069	0.629 ± 0.024	1.233 ± 0.004

In the cases of TPC and DPPH, RSM models showed that microwave power (Z_1), particle diameter (Z_3), and temperature (Z_4) had a significant positive effect on the analyzed variable (Table S2), while extraction time (Z_2) had a negative effect (Table 2, Figure 2a^2,c^2). Furthermore, the RMS model showed positive effects of microwave power (Z1) and extraction temperature (Z4) on ABTS (Table 2, Figure 2b^2) and a positive effect of extraction temperature (Z4) on FRAP (Table 2, Figure 2d^2). Similarly, research by Saifullah et al. [62] and Chowdhury et al. [63] showed a positive effect of microwave power and extraction temperature on the TPC of the prepared extract, while Kumar et al. [20] presented positive effects of microwave power and extraction time on TPC. Furthermore, Kumar et al. [19] showed a negative effect of microwave power on FRAP, while Kumar et al. [20], Chowdhury et al. [63], Nguyen et al. [64] and Saifullah et al. [62] presented a positive effect of microwave power on FRAP.

For microwave–ultrasound-assisted extraction, the RSM model showed that the microwave power (Z_1) ($p < 0.001$) and the extraction temperature (Z_4) ($p = 0.006$) had a significantly positive effect on TPC (Table 2, Figure 1a^3, Table S2). For the same extraction process, RSM models showed that only microwave power (Z_1) had a positive effect on ABTS (Table 2, Figure 1b^3, Table S2), microwave power (Z_1), particle dimeter (Z_3) and extraction temperature on DPPH (Table 2, Figure 1c^3, Supplementary Table S2) and particle dimeter (Z_3) and extraction temperature on FRAP (Table 2, Figure 1d^3, Table S2). At the moment, there are no available data on MWUE of bioactive compounds from olive leaves, but there are some examples of MWUE application. Yu et al. [23] presented efficient application of MWUE for extraction of polyphenols from Clinacanthus nutans where the solid-to-liquid ratio was noted to be the most significant variable for the TCP extraction yield based on RSM model, while Chamutpong et al. [24] concluded that there is a positive effect of ultrasonic power and microwave power on extraction of phenolic compounds for the same plant material. Furthermore, Li et al. [25] noted ultrasonic power and extraction time to be significant for MWUE of flavonoids from Lotus plume, while Liu et al. 2019 [65] presented extraction temperature and extraction time to be the most important variables for the extraction yield of flavonoids from sweet potato leaves.

For the microwave-assisted extraction, the highest fit of experimental data and the developed RSM model was found for DPPH ($r^2 = 0.858$) (Table 2), while for the microwave–ultrasound-assisted extraction process for ABTS ($r^2 = 0.898$). The applicably of the RSM model describing MWE and MWUE was evaluated based on residual analysis (Figure 4).

The findings revealed that, for all developed RMS models, residuals were normally distributed (Figure 4a1*–a4*,a1**–a4**) following bell-shaped histograms (Figure 4c1*–c4*,c1**–c4**). The residual vs. predicted value plots (Figure 4b1*–b4*,b1**–b4**) indicate that there is no pattern in the residuals and that residuals are distributed around a central value (Figure 4c1*–c4*,c1**–c4**) without obvious outliers demonstrating that the models closely match the observed data.

3.3. Optimization and Model Validation for CE, MWE and MWUE

On the basis of the desirability profiles produced from the RSM projected values, optimization was performed simultaneously for all the evaluated chemical characteristics of the extracts. The desirability scale of 0 (undesirable) to 1 (extremely desirable) was employed, and the optimization matrix design revealed that the optimum process conditions for conventional extraction were T = 80 °C, t = 15 min, d = 200 µm and rpm = 750 min^{-1} (Table 4).

The experimental values under optimal process conditions were: TPC = 79.547 ± 2.680 mg_{GAE} $g_{d.m.}{}^{-1}$, ABTS = 0.431 ± 0.072 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$, DPPH = 0.416 ± 0.014 $mmol_{Trolox}$ $g_{d.m.}{}^{-1}$ and FRAP = 0.742 ± 0.119 $mmol_{FeSO_4 \cdot 7H_2O}$ $g_{d.m.}{}^{-1}$, which is almost identical to the model-predicted values. The optimum extraction settings for MWE were p = 700 W, t = 15 min, d = 200 µm and T = 80 °C and for the MWUE p = 800 W, t = 5 min, d = 100 µm and T = 60 °C. As presented in Table 4, experimental data obtained for the MWE and MWUE were consistent with the predicted values. For all three extraction procedures, experimental data and model predicted data were shown to be insignificantly different at

$p > 0.05$ based on a paired t-test (Table 4). Based on the presented results, extraction settings estimated based on by RSM may be treated correctly and predictably [33,34].

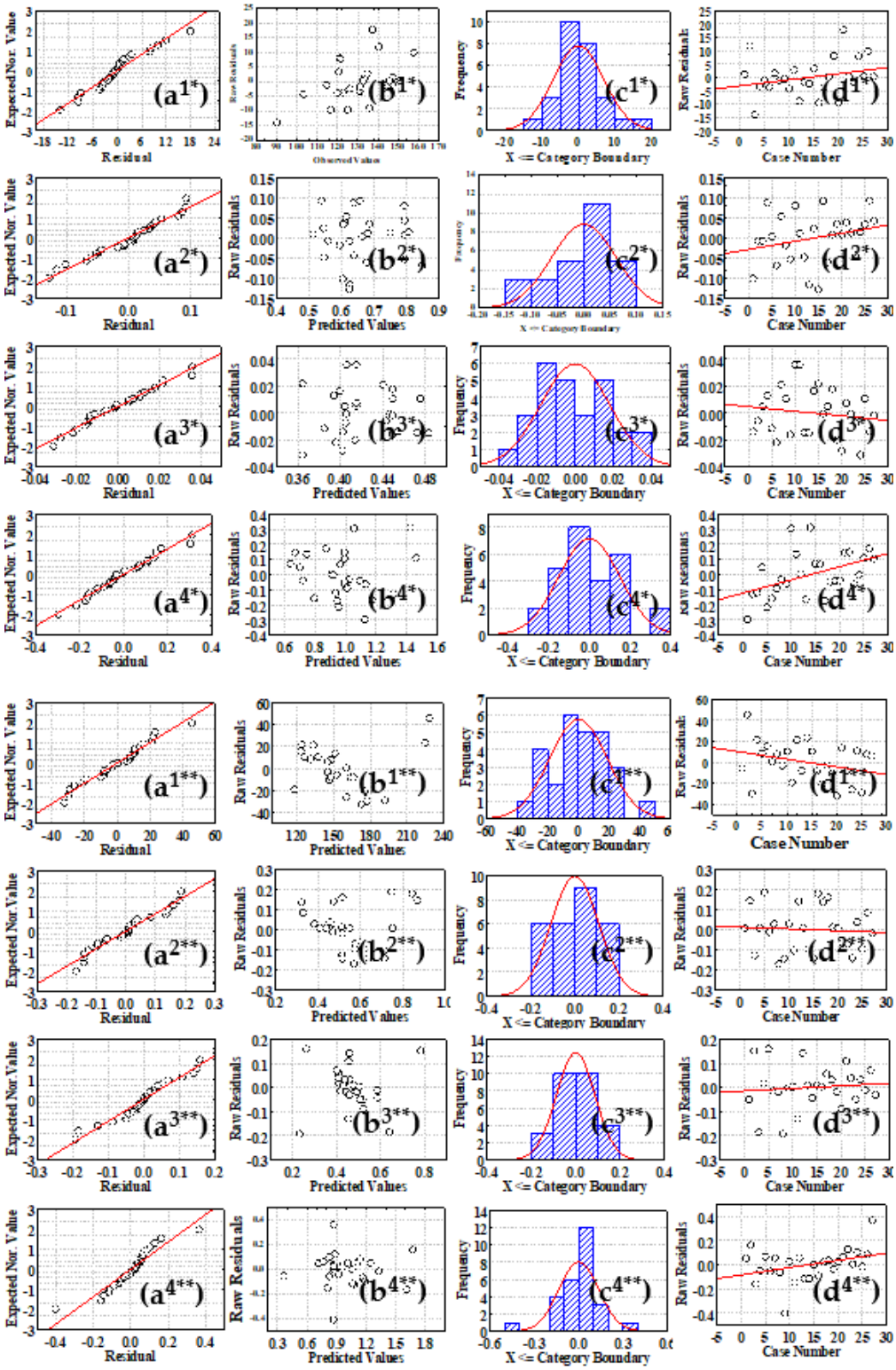

Figure 4. Residual analysis for the response surface regression models MW and MWU extraction. (a) Normal probability plot of the residuals, (b) residuals versus the predicted values, (c) histogram of the residuals, and (d) residuals versus the case number. (1) TPC, (2) ABTS, (3) DPPH, and (4) FRAP. * microwave extraction, ** microwave–ultrasound-assisted extraction.

Table 4. Predicted and experimental values of response variables tested at optimal CE, MWE and MWUE conditions.

Response Variable	Optimum CE Conditions				Optimum Value	
	Temperature/°C	Time/min	Magnetic Stirrer Rotational Rate/min^{-1}	Particle Diameter/μm	Predicted	Experimental
TPC	80	15	750	200	78.170	79.547 ± 2.680
ABTS					0.443	0.431 ± 0.072
DPPH					0.425	0.416 ± 0.014
FRAP					0.777	0.742 ± 0.119
Response Variable	**Optimum MWE Conditions**				**Optimum Value**	
	Microwave Power/W	**Time/min**	**Particle Diameter/μm**	**Temperature/°C**	**Predicted**	**Experimental**
TPC	700	7.5	300	80	151.540	152.405 ± 7.237
ABTS					0.659	0.651 ± 0.026
DPPH					0.479	0.451 ± 0.014
FRAP					1.335	1.424 ± 0.283
Response Variable	**Optimum MWUE Conditions**				**Optimum Value**	
	Microwave Power/W	**Time/min**	**Particle Diameter/μm**	**Temperature/°C**	**Predicted**	**Experimental**
TPC	800	5	100	60	272.480	273.129 ± 5.339
ABTS					0.879	0.881 ± 0.014
DPPH					0.803	0.778 ± 0.023
FRAP					1.659	1.598 ± 0.308

4. Conclusions

The current study found that microwave–ultrasound-assisted extraction can be used as an efficient method for extracting bioactive molecules from olive leaves. MWUE provided higher polyphenol content and antioxidant activity in comparison to microwave-assisted extraction and the conventional extraction method. The results suggest that the use of water as a green solvent can ensure effective extraction of bioactives. Response surface modeling (RSM) was used to evaluate the effects of extraction conditions on the TPC and AA of the aqueous olive leaf extracts prepared by the CE, MWE and MWUE methods. The RSM models described the experimentally obtained data well. The optimal conditions for the conventional extraction process were estimated to be 80 °C, 15 min, 200 μm and 750 min^{-1} for CE, 700 W, 7.5 min, 300 μm and 80 °C for MWE and 800 W, 5 min, 100 μm and 60 °C for MWUE. It can be concluded that MWUE can be efficiently used for commercial exploitation of bioactives from various plant sources through green extraction principles used in the food industry.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9020033/s1, Table S1: Correlation matrix for the (a) conventional extraction process and (b) microwave-assisted extraction process and ultrasound-microwave-assisted extraction process. Table S2: Analysis of variance (ANOVA) for the response surface polynomial model for (a) conventional extraction process, (b) microwave-assisted extraction process, and (c) ultrasound–microwave-assisted extraction process.

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