

Article

Ultrasound-Assisted Production of Virgin Olive Oil: Effects on Bioactive Compounds, Oxidative Stability, and Antioxidant Capacity

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Abstract

This study investigated the effects of ultrasonic treatment of olive paste prior to malaxation on oil yield (Y), enzyme activity and virgin olive oil (VOO) quality in four Croatian olive varieties: Istarska Bjelica, Rosulja, Oblica and Levantinka. The oils were extracted using the Abencor system according to a central composite experiment design, with treatment durations of 3–17 min and power levels of 256–640 W. The parameters analyzed included Y, oxidative stability index (OSI), antioxidant capacity (AC), phenolic and α -tocopherol content, volatile compounds, fatty acid profile, and the activity of lipoxygenase, β -glucosidase, polyphenol oxidase, and peroxidase. Olive variety was the most influential factor in all variables. The response surface methodology showed that ultrasonic treatment at low-to-medium intensity improved several quality attributes. For example, Y increased by 4% in Oblica, phenolic content increased by up to 17% in Istarska Bjelica, and OSI and AC increased by 13–15% in Istarska Bjelica and Levantinka. In contrast, longer treatment and higher ultrasound power had a negative effect. No significant differences were found in other parameters examined. Overall, the application of ultrasound led to measurable, though moderate, improvements in Y and VOO quality, with results strongly dependent on olive variety and treatment conditions. These results underline the need for further optimization tailored to each variety.

Keywords: ultrasound; virgin olive oil; response surface methodology; oil yield; oxidative stability; antioxidant capacity; phenolic composition; α -tocopherol; volatile compounds; lipoxygenase; β -glucosidase; polyphenol oxidase; peroxidase



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1. Introduction

Virgin olive oil (VOO) is highly valued as a cornerstone of the Mediterranean diet due to its distinctive sensory properties and well-documented health benefits [1]. In the olive fruit, the oil is stored in the cytoplasm in specialized leucoplasts called elaioplasts. During the crushing phase of VOO production, the structure of the fruit is broken by

mechanical forces, releasing oil from the broken cells. However, some cells remain intact and the oil released from the broken cells often forms emulsions with the aqueous phase, hindering separation [2]. Consequently, conventional VOO production is characterized by a relatively low oil yield (Y), which is improved by malaxation—a controlled mixing of the olive paste at controlled temperatures (≤ 28 °C) for up to 60 min. This process also influences the overall quality of VOO [3]. During malaxation, the oil droplets coalesce through hydrophobic interactions, while compounds such as triacylglycerols, fatty acids, pigments and phenols released during crushing are distributed between the oil and water phases and transformed by endogenous enzymes (EE) of the olive fruit [4]. Enzymes of the lipoxygenase (LOX) pathway (acyl hydrolase, lipoxygenase, hydroperoxide lyase, alcohol dehydrogenase and alcohol acyl transferase) produce the desirable C5 and C6 volatiles that shape the positive sensory properties of VOO [5]. The phenolic composition is strongly influenced by β -glucosidase (β -GLU), which hydrolyzes phenolic glycosides to release sugars and phenolic aglycones, increasing both sensory and nutritional value. Conversely, the oxidoreductases polyphenol oxidase (PPO) and peroxidase (POX) degrade phenols. POX oxidizes phenols by using hydroperoxides, while PPO catalyzes the hydroxylation to catechins and the oxidation to o-quinones, which lead to melanin formation via enzymatic browning reactions [6]. To improve nutritional and sensory quality, it would be ideal to increase LOX and β -GLU activity while reducing POX and PPO activity.

Although it is essential for improving Y and VOO quality, malaxation is also a limiting factor. It interrupts the continuity of processing and significantly increases costs due to the energy required to maintain the paste temperature [7]. For this reason, strategies such as the optimization of conventional parameters (time and temperature) or the introduction of innovative technologies—including flash thermal treatment, ultrasound and pulsed electric fields—have been investigated as alternatives or complements to malaxation [8].

High-power ultrasound induces both thermal and mechanical effects in VOO production [9]. The thermal effect results from the conversion of ultrasound energy into heat, which reduces the viscosity of the paste and promotes coalescence of the oil droplets. The mechanical effect is primarily caused by acoustic cavitation, in which gas bubbles form, grow and collapse, leading to a local increase in temperature and pressure [4]. Cavitation disrupts cell structures and membranes, improves mass transfer and can increase both Y and the concentration of bioactive compounds [3,9,10]. Ultrasound also offers sustainability benefits as it improves energy efficiency and reduces production costs by shortening or even replacing conventional malaxation [11]. In addition, the by-products resulting from ultrasound treatment contain lower concentrations of phenolic compounds, which are one of the main causes of ecotoxicity in pomace and wastewater. In addition, ultrasonic treatment can reduce antinutritional factors in olive pomace. Olive pomace contains about 0.12% phytic acid [12] and 7.8–9.5% saponins [13]. Previous studies have shown that ultrasound is effective in reducing the content of antinutrients in other plant matrices such as soybean and millet [14,15]. These results suggest that similar effects may occur in olive pomace; however, targeted studies are needed to confirm this and to elucidate the underlying mechanisms.

Despite these advantages, ultrasound can generate free radicals during sonication, which can impair product quality. Careful selection of ultrasonic parameters and temperature monitoring are therefore crucial as they influence the extent of radiation-mediated reactions that affect the oxidation of bioactive compounds [16]. This effect can be observed in the stability of phenolic and volatile compounds. In several studies [9,17,18], a slight decrease in volatile compounds was observed, which was attributed to the increase in temperature and the inactivation of enzymes of the LOX pathway due to cavitation. Similarly, a slight decrease in phenolic content was observed in the study by Gila et al. [18], while a

greater decrease was observed in the studies by Iqdam et al. [19] and Jiménez et al. [20], suggesting that prolonged or intense ultrasound exposure may cause phenol degradation.

To date, the use of ultrasound in VOO production has been investigated in Spanish, Italian, Turkish and Tunisian olive varieties [9,10,17–19,21–25], but not in Croatian autochthonous varieties. Although Croatia is a small VOO producer by European and world scale, with a production of about 3500 tons in the last two years—corresponding to 1.6%, 1.4% and 0.3% of the total production of Italy, Greece and Spain, respectively [26]—its VOOs are of exceptional quality, as evidenced by numerous international awards [27]. Production is concentrated on the Adriatic coast (Istria, Kvarner and Dalmatia), which accounts for 15% of the country's total area [28]. The use of new technologies, such as ultrasound, has the potential to increase oil production while maintaining quality. The leading Croatian olive varieties are Oblica, Istarska Bjelica, Rosulja and Levantinka. Oblica is the predominant variety, accounting for around 75% of olive groves nationwide. In Istria, Istarska Bjelica is the most commonly cultivated variety, followed by Rosulja, while Levantinka is mainly cultivated in central and southern Dalmatia [29,30]. These varieties differ genetically and chemically, but remain underexplored with respect to novel processing technologies. Investigating their responses to ultrasound provides information on cultivar-specific responses and offers practical guidance for optimized VOO production strategies tailored to individual varieties. Therefore, the aim of this study was to investigate the effects of ultrasound application prior to malaxation on Y, OSI, AC and the composition of phenols, tocopherols, volatiles and fatty acids in VOOs from Istarska Bjelica, Rosulja, Oblica and Levantinka.

2. Materials and Methods

2.1. Chemicals

The chemicals used for the analyses were obtained from various manufacturers:

- Sigma-Aldrich (St. Louis, MO, USA): benzamide, butylhydroxytoluene (BHT), L-dithiothreitol, ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), triton X-100, polyvinylpyrrolidone (PVP), guaiacol, catechol, coomassie brilliant blue G 250, linoleic acid, sodium acetate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), diethyl ether, ethyl acetate, acetonitrile, standards for phenolic compounds (hydroxytyrosol, tyrosol, p-coumaric acid, hydroxytyrosol acetate, oleacein, methyl hemiacetal of oleocanthal, oleuropein aglycone, ligstroside aglycone and oleocanthal), standards for oleuropein, 4-methyl-2-pentanol and methyl pentadecanoate.
- Kemika (Zagreb, Croatia): boric acid, anhydrous disodium hydrogen phosphate, potassium chloride, potassium phosphate, sodium dihydrogen phosphate-2-hydrate, isopropanol, isooctane, acetic acid, potassium hydroxide, sodium chloride, sodium thiosulfate, anhydrous sodium hydrogen sulphate and acetone.
- Alfa Aesar (Haverhill, MA, USA): D-norleucine.
- Lach-Ner (Neratovice, Czech Republic): ethanol and potassium iodide.
- Fluka (Buchs, Switzerland): tween-40 and orthophosphoric acid.
- T.T.T. (Sveta Nedjelja, Croatia): hydrogen peroxide and formic acid.
- Honeywell (Offenbach, Germany): methanol, hexane and heptane.
- Thermo Fischer Scientific (Waltham, MA, USA): starch and hydrochloric acid.
- Santa Cruz Biotechnology (Dallas, TX, USA): bovine serum albumin.
- LGC (Teddington, UK): α -tocopherol standard.
- Millipore (Burlington, MA, USA): β -, γ - and δ -tocopherol standards.
- Larodan (Solna, Sweden): standards for hydroperoxyoctadecatrienoic acid (HPOT).

2.2. Plant Material

Olive fruits (*Olea europaea* L.) of four autochthonous Croatian varieties (Oblica, Levantinka, Rosulja and Istarska Bjelica) were used. Oblica and Levantinka were obtained from the orchard of the Institute for Adriatic Crops and Karst Reclamation (Split, Dalmatia), and Rosulja and Istarska Bjelica from an agricultural holding in Bale (Istria). The harvest was carried out in mid-October 2022 for Oblica, Rosulja and Istarska Bjelica and in early November 2022 for Levantinka. The ripening index was determined based on the degree of pigmentation of the skin and pulp. It was 1.75 for Oblica, 2.01 for Levantinka, 2.19 for Rosulja and 1.32 for Istarska Bjelica. Istarska Bjelica and Rosulja were characterized by higher moisture content (59.3% and 64.5%) and lower oil content (16.5% and 10.3%), while Oblica and Levantinka had lower moisture content (46.4% and 41.0%) and higher oil content (20.4% and 22.7%). The ripening index, moisture and oil content of the olive fruits were determined according to the methods of the International Olive Council [31].

2.3. Virgin Olive Oil Production

The olives were processed no later than 24 h after harvesting. VOO was produced on a laboratory scale using an Abencor MC2 system (Ingenierías y Sistemas, Seville, Spain), which simulates the industrial extraction. After cleaning and washing, ~800 g of olives was crushed into a paste using a metal hammer mill (MM-100) equipped with a 4.5 mm sieve and operated at 3000 rpm. The paste was subjected to ultrasound pretreatment following a central composite design with treatment duration and power as variables (Table 1). The ultrasonic treatment was performed using a Sonorex Digiplus ultrasonic bath (BANDELIN electronic, Berlin, Germany; 640 W, 120–240 V, 20 kHz) at an initial water temperature of 25 °C. During the treatment, the paste was continuously stirred to ensure uniform exposure and the temperature was measured before and after the treatment (Table 1).

Table 1. Central composite experimental design for the production of virgin olive oil and corresponding changes in the temperature of the olive paste during ultrasound treatment.

Sample	Treatment Duration (min)	Ultrasonic Bath Power (W)	ΔT During the Treatment (°C)
1-control	0	0	28.3 ± 1.7 *
2	10	256	−1.2
3	5	320	−1.4
4	15	320	1.9
5	3	448	−1.4
6-central	10	448	1.4
7	17	448	3.9
8	5	576	−0.3
9	15	576	4.4
10	10	640	2.4

* Temperature of the olive paste after crushing (average for all varieties $\pm$ standard deviation).

Malaxation was carried out at 27 °C for 40 min in a thermomixer (TM-100). The oil was separated using a CF-100 vertical centrifuge (90 s, 3500 rpm), followed by clarification in a Rotina 320R centrifuge (Hettich, Tuttlingen, Germany; 4 min, 5000 rpm, 18 °C). The oils were preserved in dark glass containers under a nitrogen atmosphere at temperatures below 20 °C. The analytical evaluations were performed shortly after production. First, the basic quality parameters, phenolic compounds, AC, OSI and tocopherols were determined, followed by volatile compounds and the fatty acid composition and finally the enzyme activity.

2.4. Determination of Oil Yield

Y (%) was determined using Equation (1) according to the method of Peres et al. [32]:

$$Y = \frac{V(\text{VOO}) \times \rho}{m} \times 100, \quad (1)$$

where V(VOO) is the volume of VOO produced (mL), ρ is the density of VOO (0.915 g/mL) and m is the mass of olive paste used for oil extraction (g).

2.5. Basic Quality Parameters

The peroxide value, free acidity and specific absorption in the ultraviolet (K232, K268 and ΔK) of the oils produced were measured by standard methods of ISO and International Olive Council [33–35].

2.6. Determination of Fatty Acid Composition

Gas chromatography (GC) was used to analyze the fatty acid composition according to a slightly modified ISO method [36]. Methyl pentadecanoate in isooctane (C15 methyl ester, $\gamma = 1.25 \mu\text{g/mL}$) was used as an internal standard and replaced pure isooctane in the sample preparation protocol. 1 μL of the prepared fatty acid methyl esters (FAMES) was injected into the instrument in split mode (50:1). They were separated on a DB-23 capillary column (60 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA, USA) with a stationary phase of 50% cyanopropylmethylpolysiloxane. The carrier gas was helium at a constant flow rate of 1.5 mL/min. The injector and the flame ionisation detector (FID) were both kept at 250 $^{\circ}\text{C}$. The column temperature was at the beginning 60 $^{\circ}\text{C}$, then increased to 220 $^{\circ}\text{C}$ at a rate of 7 $^{\circ}\text{C}/\text{min}$ and held at this temperature for 17 min. The fatty acids were identified by comparing their retention times with those of a certified standard mixture (FAME 37, Supelco, Bellefonte, PA, USA). The internal standard method was used to quantify the individual fatty acids.

2.7. Enzyme Activity Assay

2.7.1. Olive Paste and Acetone Powders

Olive paste was used to isolate and determine the activity of LOX, while acetone powder was prepared for the extraction of β -GLU, PPO and POX. After malaxation, 30 g of the olive paste was sealed in plastic bags, frozen in liquid nitrogen and preserved at -20°C until analysis. Another 90 g of the paste was processed into acetone powder according to a modified protocol by Romero-Segura et al. [37]. In brief, 1200 mL of cold acetone (-20°C) was added to the paste and homogenized for 2 min in a GLH 850 homogenizer (Omni International, Kennesaw, GA, USA) at 15,000 rpm. The homogenate was vacuum filtered and the residue was subjected to two further homogenizations with 250 mL of cold acetone each. The final residue was rinsed with 50 mL of diethyl ether, frozen in liquid nitrogen, sealed in plastic bags and stored in a freezer until further use.

2.7.2. Lipoxygenase Extraction and Activity Assay

LOX was isolated according to the method of Luaces et al. [38] with slight alterations. 5 g of olive paste was mixed with 20 mL of extraction buffer (100 mM phosphate buffer, pH 6.7) using a GLH 850 homogenizer at 11,000 rpm for two 1 min intervals. The homogenate was vacuum filtered through a double layer of Miracloth and centrifuged at $27,000 \times g$ for 30 min at 4 $^{\circ}\text{C}$. The collected supernatant was used as a crude enzyme extract.

The activity of LOX was determined according to the protocol of Soldo et al. [39]. Linoleic acid (25 mM) was used as substrate. The reaction mixture consisted of 4 mL of 0.1 M sodium phosphate buffer (pH 6.0), 500 μL of linoleic acid and 500 μL of enzyme

extract. The reaction was performed at 25 °C with constant magnetic stirring for 30 min. The reaction was terminated by lowering the pH to 2.0 with 3 M HCl and then 1 mL of 0.25 mM BHT was added as an internal standard.

The hydroperoxides formed in the reaction were extracted by three successive extractions with 10 mL of hexane–isopropanol (95:5, *v/v*). The final extraction was followed by centrifugation at 5000 rpm for 10 min. The combined hexane phases were evaporated until dry under reduced pressure (<200 mbar) at 40 °C using a rotary evaporator (Rotavapor, Heidolph Instruments GmbH & Co., Ltd., Schwabach, Germany). The dried residue was reconstituted in 1.5 mL of acetonitrile–water (67:33, *v/v*) and sonicated for 10 s in an ultrasonic bath. 10 µL of the prepared hydroperoxide extract was injected into the HPLC (Agilent 1200 Series, Agilent Technologies, Santa Clara, CA, USA) with a diode array detector (DAD). HPOTs were separated on a reverse-phase C18 column (Luna, 250 mm × 4.6 mm, 5 µm particle size, 100 Å pore size; Phenomenex, Torrance, CA, USA) at 35 °C. The mobile phases were A-0.25% acetic acid in water and B-acetonitrile. They flowed through the system at a constant rate of 1.0 mL/min. The gradient program was: 63% B at 0 min, held until 17 min; increased to 80% B at 20 min; held at 80% B until 32 min; returned to initial conditions at 35 min; followed by a 10 min equilibration. The detection was performed at 234 nm.

HPOTs were identified by comparing their retention times and UV spectra with those of 13(S)-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid commercial standard. Quantification was performed using the internal standard method based on the response factor ratio between HPOTs and BHT. LOX activity was expressed as the amount of HPOT (µmol) synthesized per mg of protein per minute at 25 °C according to Equation (2):

$$EA = \frac{\sum A \times RRF_{BHT/HPOT} \times n(BHT)}{A(BHT) \times \gamma(\text{proteins}) \times V \times t'} \quad (2)$$

where EA is the enzyme activity (µmol/mg·min), $\sum A$ is the sum of the HPOTs peak areas, RRF is the ratio between the response factors of BHT and HPOT, $n(BHT)$ is the amount of BHT added to the reaction mixture (µmol), $A(BHT)$ is the BHT peak area, $\gamma(\text{proteins})$ is the protein concentration in the enzyme extract (mg/mL), $V(E)$ is the volume of enzyme extract used for the reaction (µL) and t is the reaction time (min). The RRF was calculated according to Equation (3):

$$RRF_{BHT/HPOT} = \frac{\frac{\text{peak area BHT}}{\mu\text{mol BHT injected}}}{\frac{\text{peak area HPOT}}{\mu\text{mol HPOT injected}}} \quad (3)$$

2.7.3. β-Glucosidase Extraction and Activity Assay

β-GLU was isolated and its activity was measured according to a modified method by Romero-Segura et al. [37]. The enzyme extracts were prepared by suspending 3 g of acetone powder in 12 mL of extraction buffer (100 mM borate buffer, pH 9.0, containing 5 mM EDTA, 1 mM PMSF and 0.25% (*w/v*) dithiothreitol). The mixture was stirred in an ice bath on a magnetic stirrer for 1 h and then centrifuged at 27,000× *g* at 4 °C for 30 min. The collected clear supernatant was used as crude enzyme extract to determine the enzyme activity. β-GLU activity was determined in a reaction medium consisting of 1.5 mL of oleuropein solution (5 mM in 50 mM sodium acetate buffer, pH 5.5) and 500 µL of enzyme extract. The reaction was carried out at 45 °C for 30 min and terminated by the addition of 1.5 mL of methanol. 20 µL of the reaction mixture was then injected into the HPLC-DAD instrument (Agilent 1200 Series, Agilent Technologies, Santa Clara, CA, USA). A reversed-phase C18 column (Luna, 250 mm × 4.6 mm, 5 µm particle size, 100 Å pore size; Phenomenex, Torrance, CA, USA) was used for separation under isocratic conditions. The mobile phase was 0.1% formic acid in water/methanol (1:1, *v/v*) at a flow

rate of 1.0 mL/min. The chromatograms were recorded at 280 nm and the oleuropein concentrations before and after the reaction were calculated using Equation (4):

$$c(\text{OLE}) = \frac{A(\text{OLE}) + 9.5302}{32.092}, \quad (4)$$

where $c(\text{OLE})$ is the concentration of oleuropein ($\mu\text{mol/mL}$) and $A(\text{OLE})$ is the area under the oleuropein peak in the chromatogram ($\text{mAU}\cdot\text{s}$). This external calibration curve was generated for the range of 0.2 to 1.2 mM oleuropein with a linearity range of 0.4 to 0.8 and $R^2 = 0.82$. The enzymatic activity of β -GLU was expressed as the amount of residual oleuropein (μmol) reduced per mg of protein per minute according to Equation (5):

$$\text{EA} = \frac{\Delta c(\text{OLE}) \times V(\text{RM})}{\gamma(\text{proteins}) \times V(\text{E}) \times t'} \quad (5)$$

where EA is the enzyme activity ($\mu\text{mol}/\text{mg}\cdot\text{min}$), $\Delta c(\text{OLE})$ is the concentration of residual oleuropein after reduction by the 30 min reaction ($\mu\text{mol/mL}$), $V(\text{RM})$ is the volume of the reaction mixture (mL), $\gamma(\text{proteins})$ is the concentration of proteins in the enzyme extract (mg/mL), $V(\text{E})$ is the volume of enzyme extract (μL) used in the reaction mixture, and t is the reaction time (min).

2.7.4. Polyphenol Oxidase Extraction and Activity Assay

The enzyme extracts were prepared according to the modified method of Peres et al. [40]. For this purpose, 3 g of acetone powder was mixed with 2.5% polyvinylpyrrolidone (PVP) and 12 mL of extraction buffer (0.05 M potassium phosphate, 1 M potassium chloride, min pH 6.2). The mix was stirred for 1 h in an ice bath with a magnetic stirrer and then centrifuged at $27,000 \times g$ for 30 min at 4°C . The collected supernatant was used as crude enzyme extract to determine the enzyme activity. PPO activity was determined in a reaction medium containing 2.5 mL of catechol solution (30 mM) and 500 μL of enzyme extract. The reaction was performed at room temperature and the increase in absorbance at 420 nm, corresponding to catechol oxidation, was monitored spectrophotometrically after 1 min according to the method of Caponio et al. [41]. PPO activity was described as the amount of o-quinone (μmol) formed per mg of protein per minute, calculated with a molar extinction coefficient (ϵ) of $1623 \text{ M}^{-1}\cdot\text{cm}^{-1}$, according to Equation (6):

$$\text{EA} = \frac{\Delta A \times V(\text{RM}) \times 1000}{\epsilon \times d \times \gamma(\text{proteins}) \times V(\text{E}) \times t} \quad (6)$$

where EA is the enzyme activity ($\mu\text{mol}/\text{mg}\cdot\text{min}$), ΔA is the difference in absorbance between the sample with the enzyme extract and the blank samples, $V(\text{RM})$ is the volume of the reaction mixture (mL), ϵ is the extinction coefficient of the product, d is the length of the cuvette (1 cm), $\gamma(\text{proteins})$ is the concentration of proteins in the enzyme extract (mg/mL), $V(\text{E})$ is the volume of the enzyme extract (μL) and t is the reaction time (min).

2.7.5. Peroxidase Extraction and Activity Assay

The enzyme extracts for POX activity were prepared as described in paragraph 2.7.4. POX activity was determined spectrophotometrically based on the increase in absorbance at 470 nm due to the formation of tetraguaiacol ($\epsilon = 26,600 \text{ M}^{-1}\cdot\text{cm}^{-1}$) from the peroxidation of guaiacol according to the modified method of Caponio et al. [41]. The reaction mix contained 2 mL guaiacol (30 mM) and 1 mL H_2O_2 (30%, 4 mM), which were added simultaneously. The absorbance was measured after 10 min of reaction at room temperature. POX activity was defined as the amount of tetraguaiacol (μmol) formed per mg of protein per minute. Enzyme activity was calculated using the same equation used for PPO activity.

2.7.6. Total Protein Content

The amount of total proteins in enzyme extracts was quantified using the Bradford reagent with crystalline bovine serum albumin (BSA) as the standard protein [42]. The protein concentration was calculated according to Equation (7):

$$\gamma \text{ (proteins)} = \frac{A + 0.0436}{13.7292}, \quad (7)$$

where γ (proteins) is the concentration of proteins in the enzyme extract (mg/mL) and A is the absorbance at 595 nm. 300 μ L of the enzyme extract was added to 1.2 mL of Bradford reagent and the absorbance was measured against a blank at 595 nm after a 5 min reaction at room temperature.

2.8. Determination of Volatile Compounds

Gas chromatography–mass spectrometry (GC/MS) was used to determine the composition and concentration of the volatile compounds. For sample preparation, 10 g of VOO were added to 20 mL glass vials to which 0.015 g of internal standard (0.15% 4-methyl-2-pentanol in refined sunflower oil) was added. The vials were sealed with silicone septa and placed in a heating block with magnetic stirrer (Pierce Reacti-Therm Heating/Stirring Module, Artisan, Champaign, IL, USA) at 40 °C. After 10 min of conditioning, a 2 cm divinylbenzene/carboxene/polydimethylsiloxane SPME fiber (Supelco, Bellefonte, PA, USA) was placed in the headspace for 30 min and desorbed in the injector of a 6890N Network GC system (Agilent Technologies, Santa Clara, CA, USA) in split-less mode at 260 °C for 1 min. An HP-5 capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m; Agilent Technologies) with 5% phenylmethylpolysiloxane as the stationary phase and helium as the carrier gas at a flow rate of 1.5 mL/min was used to separate the components. The oven temperature was as follows: initially 30 °C (held for 3 min), then increased to 150 °C at 5 °C/min and to 250 °C at 20 °C/min and held at 250 °C for 5 min. An inert mass-selective detector 5973 operated in electron ionization mode and scanned m/z 50–550. The temperatures of the ion source and the transfer line were 250 °C and 260 °C, respectively. A mix of n-alkanes (C8–C20) in dichloromethane was analyzed under identical conditions to calculate Kovats retention indices. The volatile compounds were identified by comparing their mass spectra with those of the NIST mass spectral library and by comparing the Kovats indices with literature values. Quantification was performed using the response of the internal standard and its known concentration.

2.9. Determination of Phenolic Compounds

The phenolic composition was determined using the RP-HPLC method. The phenolic compounds were extracted according to the standard method of the International Olive Council [43] with syringic acid as internal standard. The prepared methanolic extracts (20 μ L) were analyzed by HPLC according to the method of Škevin et al. [44]. The concentration of total and individual phenols was expressed in mg tyrosol/kg oil.

2.10. Determination of Tocopherols

The tocopherols were analyzed according to the ISO method [45]. The oil sample (0.1 g) was weighed into a 10 mL volumetric flask and diluted with n-heptane. 20 μ L of the prepared solution was injected into a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, SAD). The tocopherols were separated on a LiChroCART Silica 60 column (250 mm $\times$ 4.6 mm $\times$ 5 μ m; Merck, Darmstadt, Germany) at room temperature by isocratic normal phase chromatography. The mobile phase was 0.7% 2-propanol in n-hexane at a flow rate of 0.9 mL/min. The tocopherols were detected with a fluorescence detector

($\lambda_{\text{excitation}} = 295 \text{ nm}$, $\lambda_{\text{emission}} = 330 \text{ nm}$) with a total run time of 25 min. The individual tocopherols were identified by comparison with the retention times of the commercial standards. Quantification was performed by expressing the concentration of each tocopherol detected as equivalents of α -tocopherol (mg/kg).

2.11. Determination of Oxidative Stability

Oxidative stability was assessed by measuring the induction time on a differential scanning calorimeter (DSC 214 Polyma, NETZSCH-Gerätebau GmbH, Selb, Germany) according to a modified protocol by Tan et al. [46]. VOO ($4.0 \pm 0.3 \text{ mg}$) was weighed into an aluminum pan. It was then hermetically sealed with a lid perforated in the center and placed in the sample chamber of the device. Calibration was performed with high-purity indium and the baseline was established with an empty pan. The isothermal method was used at 140°C . The sample was heated at a rate of $20^\circ\text{C}/\text{min}$ under a constant flow of nitrogen ($40 \text{ mL}/\text{min}$). After a 5 min equilibrium period at 140°C , the nitrogen atmosphere was replaced with purified oxygen (99.95%) at a flow rate of $100 \text{ mL}/\text{min}$. Nitrogen was supplied as a protective gas at $60 \text{ mL}/\text{min}$ throughout the analysis. The DSC curves were analysed with the NETZSCH Proteus Thermal Analysis software (version 8.0.1). The induction time was determined as the intersection between the extrapolated baseline and the tangent of the exothermic part of the recorded exotherm.

2.12. Determination of Antioxidative Capacity

The AC of VOO was determined by measuring the reduction of the DPPH radical at room temperature using electron paramagnetic resonance (EPR) on a Magnettech MS-5000 spectrometer (Freiberg Instruments GmbH, Freiberg, Germany). The parameters used were: frequency of magnetic field modulation-100 kHz, magnetic field strength-331–343 mT, field sweep range-12 mT, sweep time-30 s, microwave power-10 mW and modulation amplitude-0.2 mT. $20 \mu\text{L}$ of VOO was added to $980 \mu\text{L}$ of a 0.15 mM DPPH solution in ethyl acetate and then mixed vigorously. The reaction lasted exactly 30 min in the dark before the EPR spectra were recorded. The blank sample contained $20 \mu\text{L}$ of ethyl acetate instead of VOO. The amplitudes of the EPR signals of the DPPH radical were calculated by integrating the EPR spectra with EW (EPRWare) Scientific Software Service. The reduction of the DPPH radical signal, which indicates antioxidant activity, was calculated using the following Equation (8):

$$\text{DPPH radical reduction (\%)} = \frac{A_0 - A_{30}}{A_0} \times 100, \quad (8)$$

where A_0 is the amplitude of the blank sample and A_{30} is the amplitude of the EPR signal of DPPH radical in the VOO solution, measured after 30 min of reaction time.

2.13. Statistical Analysis

The VOO samples were produced with the application of ultrasound pretreatment according to the central composite experiment design with two independent variables: treatment duration (min) and ultrasonic bath power (W). The experimental matrix shown in Table 1, paragraph 2.3., consisted of 10 experimental conditions, including a control with no ultrasound application (0 min, 0 W) and a central point that was repeated five times to allow estimation of experimental errors and to evaluate the reproducibility of the system. The treatment duration ranged from 3 to 17 min, while the ultrasound power varied between 256 and 640 W (axial points). A one-way analysis of variance (ANOVA) was carried out to analyze the influence of variety. Significant differences between the control samples of the different olive varieties were determined using the (two-tailed) Tukey's post hoc test at $p \leq 0.05$.

Response surface methodology (RSM) was used to understand the influence of ultrasonic pretreatment on the main quality and composition parameters of VOO. A two-factor interaction model (2FI) was developed to describe the influence of ultrasound power and treatment time on Y, OSI, AC, phenolic and tocopherol content and linoleic acid concentration for different olive varieties. The empirical equation (first order multiple regression equation) was developed (9):

$$\text{Response} = \beta_0 + \beta_1 \times A + \beta_2 \times B + \beta_3 \times C_1 + \beta_4 \times C_2 + \beta_5 \times C_3 + \beta_6 \times C_4 + \beta_7 \times A \times B + \beta_8 \times A \times C_1 + \beta_9 \times A \times C_2 + \beta_{10} \times A \times C_3 + \beta_{11} \times A \times C_4 + \beta_{12} \times B \times C_1 + \beta_{13} \times B \times C_2 + \beta_{14} \times B \times C_3 + \beta_{15} \times B \times C_4 \quad (9)$$

where A = time, B = power, C₁ = Istarska Bjelica variety, C₂ = Rosulja, C₃ = Oblica and C₄ = Levantinka. The predicted response was modelled with a series of regression coefficients (β), including the intercept (β_0), linear (β_1 – β_6) and interaction coefficients (β_7 – β_{15}). Data processing, statistical and graphical design, including one-way ANOVA, Tukey's post hoc test and RSM, was carried out using Excel (version LTSC Professional Plus 2021; Microsoft Office, Redmond, WA, USA) and XLSTAT statistical software (version 2024.3.0. Premium; Lumivero, Denver, CO, USA). The results are shown as mean $\pm$ standard deviation. All analyses were conducted with a minimum of two replicates.

3. Results and Discussion

The application of ultrasound in VOO production is attracting increasing interest as a technology that can improve the release of intracellular components and the functionality of the oil [17]. In this study, the effects of ultrasound pretreatment on VOO production were investigated under laboratory conditions in four Croatian olive varieties, focusing on both compositional changes and biochemical responses. All samples, regardless of treatment, met the official criteria for extra VOO according to the EU regulation [47]. The peroxide values ranged from 1.0 to 3.0 meq O₂/kg, the free fatty acids from 0.19 to 0.42% oleic acid, and the UV absorption indices (K232, K268, ΔK) remained within acceptable limits. In agreement with previous studies conducted at both industrial and laboratory scale [9,10,17,18,22], no significant differences were found between control and ultrasound-treated samples for these primary quality parameters. Therefore, they are not discussed further in this paper so that the focus can be placed on more sensitive indicators of oil quality and technological response. Since the olive variety has a strong influence on the chemical composition, phenolic content and antioxidant capacity (AC) of VOO-and consequently on the response to ultrasound-assisted extraction [48]-it is important to first investigate the intrinsic differences between the varieties studied.

3.1. Influence of Olive Variety

The differences between Istarska Bjelica, Rosulja, Oblica and Levantinka were significant in almost all measured chemical and biochemical parameters, confirming previous studies emphasizing the importance of genotype on oil quality outcomes [29,49].

3.1.1. Oil Yield

As shown in Figure 1 Levantinka exhibited the highest Y, while Rosulja showed the lowest. Similar trends have been described in the literature, with Levantinka showing a Y-value of around 20% and Rosulja of 5–8% [30,50,51]. In contrast, laboratory-scale Y values of 18–21% have been reported for Oblica and about 21% for Istarska Bjelica [29,30], which corresponds to values 1.5–2 times higher than those observed in this study. However, some previous studies have documented lower Y values that are comparable to our results [50,52]. The reduced Y in this study is likely attributable to the pronounced drought in 2022 during pit hardening and intensive fruit growth, as well as differences in ripening indices, with

Istarska Bjelica and Oblica exhibiting slightly lower ripening indices than Levantinka and Rosulja [30,53]. In addition, excessive rainfall shortly before harvest in Istria may have increased fruit moisture, diluting Y during extraction [54]. It is known that Y is influenced by several factors, including the ripeness of the fruit, climatic conditions, agronomic practices and technological parameters applied during processing [55,56].

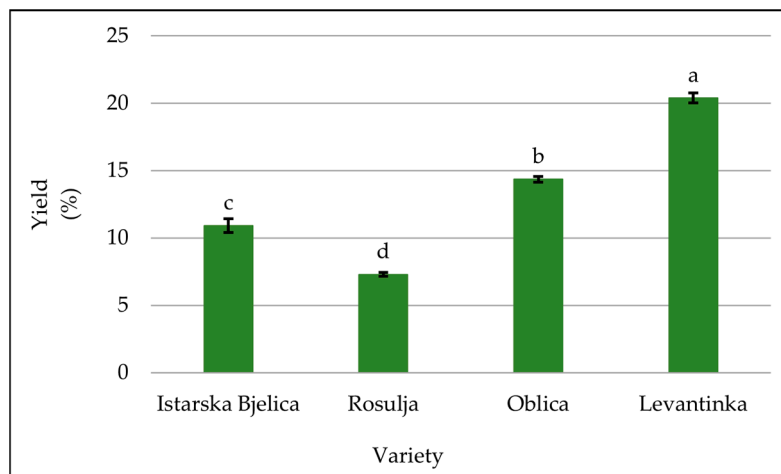


Figure 1. Oil yield (Y, %) of VOO control samples from different autochthonous Croatian olive varieties. Different lowercase letters above the bars indicate significant differences between varieties as determined by Tukey’s post hoc test ($p < 0.05$). The one-way ANOVA showed a highly significant effect of variety on oil yield ($p < 0.001$).

3.1.2. Fatty Acid Composition

The fatty acid composition of VOO is strongly influenced by the olive variety, the degree of ripeness of the fruit and the geographical origin, in particular the latitude and climatic conditions [57]. Table 2 shows the major fatty acids in the VOO samples and their classification into fatty acid groups: saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

Table 2. Major fatty acids and fatty acid groups—saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA)—as a percentage of total fatty acids in VOO control samples of autochthonous Croatian olive varieties.

Fatty Acids/Fatty Acid Groups (mg/g)	<i>p</i> -Value	Variety			
		Istarska Bjelica	Rosulja	Oblica	Levantinka
C16:0	<0.0001	13.4 ± 0.2 b	14.1 ± 0.1 a	13.8 ± 0.2 a	11.5 ± 0.1 c
C18:0	<0.0001	3.1 ± 0.1 b	2.3 ± 0.0 c	2.4 ± 0.1 c	4.1 ± 0.1 a
C18:1	<0.0001	70.1 ± 0.2 c	72.5 ± 0.2 b	63.7 ± 0.5 d	73.6 ± 0.6 a
C18:2	<0.0001	8.6 ± 0.2 b	6.6 ± 0.0 c	16.1 ± 0.4 a	6.9 ± 0.3 c
SFA	<0.0001	17.2 ± 0.3 a	17.0 ± 0.0 ab	16.7 ± 0.1 b	16.3 ± 0.2 c
MUFA	<0.0001	71.6 ± 0.2 b	74.3 ± 0.2 a	64.9 ± 0.4 c	74.7 ± 0.6 a
PUFA	<0.0001	9.3 ± 0.3 b	7.4 ± 0.0 c	16.9 ± 0.4 a	7.5 ± 0.4 c
MUFA/PUFA	<0.0001	7.7 ± 0.2 b	10.0 ± 0.0 a	3.8 ± 0.1 c	10.0 ± 0.5 a

The *p*-values indicate the influence of the olive variety on the measured parameters. If significant ($p \leq 0.05$), Tukey’s post hoc test was performed, and different letters within each row indicate significant differences.

The fatty acid composition of the VOOs produced in this study was consistent with previous reports [29,52,58]. MUFAs predominated, accounting for 64–75% of the total fatty acids, with oleic acid constituting about 98% of the MUFA fraction. The high oleic acid content supports the health claim “high unsaturated fat”, indicating a contribution to the maintenance of normal blood cholesterol levels [59]. Detailed fatty acid profiles

of different olive varieties illustrate the variability of oil composition and its impact on nutritional quality and oxidative stability. The MUFA/PUFA ratio is a key indicator of oxidative stability, reflecting the oil's resistance to oxidative degradation and consequently its shelf life under appropriate storage conditions [29]. In this study, Levantinka and Rosulja exhibited a MUFA/PUFA ratio of about 10, Istarska Bjelica had a ratio of around 8 and Oblica had the lowest ratio of about 4, indicating greater susceptibility to oxidation. The lower ratio in Oblica is attributable to the reduced oleic acid content combined with the highest linoleic acid content of the varieties studied. Comparisons with the literature show some variability: in the study by Žanetić et al. [29], the MUFA/PUFA ratio for Oblica was about twice as high as that observed here, while the ratio for Levantinka is consistent with our results. Conversely, the study by Jukić Špika et al. [58] reported a MUFA/PUFA ratio for Oblica that is consistent with the present results. These differences likely reflect the variations in growing conditions in the different harvest years.

3.1.3. Endogenous Enzymes

Olive paste was used for the isolation and determination of LOX activity, while the enzymes β -GLU, POX and PPO were first concentrated in acetone powder prior to extraction (Table 3). The endogenous enzymes (EE) play a crucial role in VOO production as they largely determine the quality and nutritional value of the oil. During the crushing phase, the plant cell structures are broken down, releasing chemical components such as triacylglycerols, fatty acids, pigments and phenolic compounds, which serve as substrates for the EE. In the subsequent malaxation phase, these enzymes are activated and catalyze the conversion of the substrates into volatile and phenolic compounds that contribute to the nutritional, sensory and antioxidant properties as well as the oxidative stability of the VOOs produced [4].

Table 3. The activity of endogenous enzymes ($\mu\text{mol}/\text{mg min}$) in VOO control samples of different autochthonous Croatian olive varieties.

Activity of Endogenous Enzymes ($\mu\text{mol}/\text{mg min}$)	<i>p</i> -Value	Variety			
		Istarska Bjelica	Rosulja	Oblica	Levantinka
LOX	<0.0001	0.120 ± 0.012 b	1.638 ± 0.072 a	0.132 ± 0.004 b	0.190 ± 0.016 b
β -GLU	<0.0001	<LOD *b	0.031 ± 0.001 a	<LOD *b	<LOD *b
PPO	<0.0001	1.593 ± 0.112 a	<LOD *c	0.016 ± 0.021 c	0.459 ± 0.026 b
POX	<0.0001	<LOD *b	<LOD *b	<LOD *b	0.007 ± 0.002 a

Results marked with * are below LOD (limit of detection) of the method, LOD (β -GLU) = $0.001 \mu\text{mol}/\text{mg min}$, LOD (PPO) = $0.007 \mu\text{mol}/\text{mg min}$; LOD (POX) = $0.001 \mu\text{mol}/\text{mg min}$; the *p*-values indicate the influence of the olive variety on the measured parameters. If significant ($p \leq 0.05$), Tukey's post hoc test was performed, and different letters within each row indicate significant differences.

The olive variety exerted a significant influence on the overall activity of the enzymes. Among the control samples, Rosulja exhibited the highest LOX activity compared to the other three varieties, which corresponds to the highest maturity level (2.19). This observation is consistent with previous reports [60], which indicate that LOX activity increases significantly as the olive fruit ripens. Compared to the results of Soldo et al. [60], LOX activity in the present study was about two to three times higher in Oblica and Levantinka, which is related to their higher ripening indexes. β -GLU activity was detected exclusively in Rosulja, the variety with the highest maturity index. This is in agreement with the study by Susamci et al. [61], who reported that β -GLU activity generally increases with fruit ripening, although the response can vary greatly between cultivars. In this study, β -GLU activity was measured in acetone powders following the protocol of Romero-Segura et al. [37]. While acetone precipitation effectively concentrates proteins, it can also partially

inactivate the enzymes due to the sensitivity of the enzyme to organic solvents [62,63], which likely explains the absence of detectable β -GLU activity in the other varieties and may lead to an underestimation of β -GLU activity. For a more accurate assessment, future studies should consider measuring β -GLU activity directly in the olive paste, as is common in LOX analysis. No PPO activity was detected in Rosulja, while Istarska Bjelica exhibited the highest PPO activity, demonstrating a contrasting pattern to that of LOX. This observation is consistent with literature reports indicating that PPO and POX activities generally decrease during fruit ripening [6]. POX activity was only detected in Levantinka, although at very low levels, in agreement with previous reports [40,41].

3.1.4. Volatile Compounds

The volatile compounds in VOO can be divided into products of the lipoxygenase pathway (Σ LOX), oxidation products (Σ OX) and products of microbial activity (Σ MBA) [64]. The composition of the volatile compounds in the VOOs produced is summarized in Table 4. Among the detected compounds, 2-methylbutanal was identified as the only product of microbial activity responsible for the musty deficiency. 2,4-Hexadienal, 4-oxohex-2-enal, pentanal and nonanal were classified as oxidation products (Σ OX), while all other detected compounds originated from the lipoxygenase pathway (Σ LOX). The compounds derived from the LOX pathway contribute to the characteristic green and fruity aroma of VOO, while the oxidation products are associated with undesirable rancid off-flavors. VOO exhibits a high resistance to non-enzymatic oxidation due to its oleic acid content and natural antioxidants. Oxidation occurs mainly by autoxidation, a radical-mediated chain reaction that consumes antioxidants and generates toxic free radicals and hydroperoxides [64]. The content and composition of volatile compounds are strongly influenced by the olive variety, the degree of ripeness of the fruit, the geographical origin, the agronomic practices and the processing methods [60].

In the control samples, aldehydes dominated the volatile profile of most olive varieties, with the exception of the Levantinka variety, in which aldehydes and alcohols were present in approximately equal amounts. The varieties from the Istria region exhibited a higher aldehyde content, while the Dalmatian varieties contained a higher level of alcohols. The ketone content was highest in Istarska Bjelica, while the ester content was highest in Levantinka. In addition, the Istrian varieties had the highest levels of LOX-derived compounds and oxidation products, while MBA-derived compounds were most abundant in Levantinka and Istarska Bjelica. A comparison with the literature reveals variability across studies. Rosulja in previous reports [65] showed a fivefold lower total aldehyde content than in this study, while other volatile groups were similar. In contrast, the study by Žanetić et al. [29] reported that Oblica had a threefold higher aldehyde content, a twofold higher alcohol content and a slightly higher ester content, while Levantinka had a fivefold higher aldehyde content, a similar alcohol content and a fourfold lower ester content.

To assess the actual contribution of volatile compounds to the flavor of VOOs, their concentration should be considered in relation to the established flavor thresholds. Detailed flavor thresholds for volatile compounds of VOOs were reported by Cecchi et al. [5]. In accordance with this study, several OX and MBA products in the control samples exceeded their taste thresholds. In particular, 2-methylbutanal, pentanal and 2,4-hexadienal exceeded the thresholds in all varieties except Levantinka, while nonanal exceeded its threshold in all varieties except Oblica (when the lowest threshold of 0.15 mg/kg was applied). These compounds contribute to a complex array of flavors, including cheese, malty, cocoa, almond, sweet, ripe, wood, bitter, oil, pungent, strawberry, fruit, tomato, green, floral, cut grass, fresh, fat, solvent, citric, rancid, wax, soap and tallow. Among the LOX products, several compounds were present above their flavor thresholds. These included 2-hexenal,

(Z)-2-penten-1-ol, 1-penten-3-ol and 1-penten-3-one in all varieties except Levantinka; hexenyl acetate in Levantinka only; 1-hexanol in Oblica and Levantinka; 3-hexenyl acetate in Rosulja and Levantinka (when a lower threshold of 0.2 mg/kg was applied); (Z)-3-hexen-1-ol in all varieties (when lower thresholds of 1.1 and 1.5 mg/kg were applied); and (E)-2-penten-1-ol only in Levantinka (when the lower threshold of 0.25 mg/kg was applied). These compounds contribute to various sensory attributes including bitter, almond, green, cut grass, leaf, fruity, apple, sharp, sweet, astringent, fat, plastic, butter, rubber, pungent, banana, olive, walnut husk, fresh, nut, wet earth, lawn, hay, herb, floral, green, fish, metallic, train oil, mustard, spicy, strawberry, resin, aromatic, alcoholic, rough, soft, tomato, olive paste and mushroom.

Table 4. Volatile compounds and groups of volatile compounds (mg/kg) in VOO control samples of different autochthonous Croatian olive varieties.

Groups of Volatile Compounds (mg/kg)	<i>p</i> -Value	Variety			
		Istarska Bjelica	Rosulja	Oblica	Levantinka
2-Methylbutanal	0.126	0.22 ± 0.04	0.04 ± 0.03	0.08 ± 0.02	0.38 ± 0.33
Pentanal	0.154	0.29 ± 0.30	0.35 ± 0.30	<LOD *	<LOD *
2-Pentenal	0.834	0.24 ± 0.03	0.16 ± 0.11	0.18 ± 0.03	0.19 ± 0.19
2-Hexenal	<0.0001	15.27 ± 1.31 b	37.18 ± 4.21 a	9.42 ± 0.95 b	10.92 ± 5.43 b
2,4-Hexadienal	0.005	2.92 ± 0.23 a	3.28 ± 0.26 a	2.97 ± 0.27 a	1.54 ± 0.76 b
4-Oxohex-2-enal	<0.0001	3.54 ± 0.40 a	2.27 ± 0.25 b	2.10 ± 0.24 b	0.52 ± 0.38 c
Nonanal	<0.0001	0.32 ± 0.05 a	0.36 ± 0.06 a	0.12 ± 0.00 b	0.21 ± 0.03 b
3-Hexenal + 2-Methyl-4-pentenal	<0.0001	14.31 ± 0.77 a	7.20 ± 0.89 c	10.32 ± 0.94 b	1.81 ± 0.98 d
Total aldehydes	<0.0001	37.11 ± 1.90 b	50.84 ± 4.77 a	25.19 ± 2.43 bc	15.57 ± 7.42 c
1-Penten-3-ol	0.111	0.39 ± 0.41	1.49 ± 1.46	2.51 ± 2.51	5.42 ± 3.42
(E)-2-Penten-1-ol	0.560	0.16 ± 0.01	0.05 ± 0.06	0.04 ± 0.04	0.50 ± 0.86
(Z)-2-Penten-1-ol	0.192	1.93 ± 0.06	1.32 ± 0.08	0.91 ± 0.19	1.19 ± 1.03
(Z)-3-Hexen-1-ol	0.338	2.20 ± 0.26	1.17 ± 2.02	3.39 ± 0.10	2.13 ± 1.86
2-Hexen-1-ol	0.483	<LOD *	0.10 ± 0.02	0.05 ± 0.05	0.90 ± 1.56
1-Hexanol	0.512	0.10 ± 0.02	<LOD *	0.57 ± 0.11	1.09 ± 1.89
Total alcohols	0.006	4.77 ± 0.62 b	4.13 ± 1.66 b	7.48 ± 2.62 ab	11.23 ± 1.92 a
1-Penten-3-one	0.003	6.11 ± 0.23 a	1.93 ± 1.67 b	2.02 ± 1.95 b	<LOD *b
Total ketones	0.003	6.11 ± 0.23 a	1.93 ± 1.67 b	2.02 ± 1.95 b	<LOD *b
3-Hexenyl acetate	<0.0001	<LOD *b	0.44 ± 0.25 b	<LOD *b	2.69 ± 0.42 a
Hexyl acetate	<0.0001	<LOD *b	<LOD *b	<LOD *b	1.62 ± 0.10 a
Total esters	<0.0001	<LOD *b	0.44 ± 0.25 b	<LOD *b	4.31 ± 0.46 a
ΣLOX §	<0.0001	40.75 ± 2.44 b	51.05 ± 2.86 a	29.42 ± 2.58 c	28.45 ± 4.86 c
ΣOX ¥	<0.0001	7.07 ± 0.48 a	6.26 ± 0.42 ab	5.19 ± 0.50 b	2.27 ± 1.13 c
ΣMBA £	0.126	0.22 ± 0.04	0.04 ± 0.03	0.08 ± 0.02	0.38 ± 0.33

Results marked with * are below LOD (limit of detection) of the method, LOD = 0.003 mg/kg; the *p*-values indicate the influence of the olive variety on the measured parameters. If significant ($p \leq 0.05$), Tukey's post hoc test was performed, and different letters within each row indicate significant differences; § ΣLOX accounts for the sum of 2-pentenal, 2-hexenal, 3-hexenal + 2-methyl-4-pentenal, 1-penten-3-ol, (E)-2-penten-1-ol, (Z)-2-penten-1-ol, (Z)-3-hexen-1-ol, 2-hexen-1-ol, 1-hexanol, 1-penten-3-one, 3-hexenyl acetate and hexyl acetate; ¥ ΣOX accounts for the sum of 2,4-hexadienal, 4-oxohex-2-enal, pentanal and nonanal; £ ΣMBA accounts for 2-methylbutanal.

3.1.5. Phenolic Compounds and Tocopherols

Several phenolic compounds were detected in the VOOs, including phenolic alcohols (hydroxytyrosol and tyrosol), *p*-coumaric acid, hydroxytyrosol acetate, oleacein, methyl hemiacetal of oleocanthal, oleocanthal, and the aglycones of oleuropein and ligstroside, as summarized in Table 5. The phenolic profile is characteristic of the olive variety, but is also influenced by several factors, including fruit maturity, geographical origin, climate, and agronomic and technological practices [66]. Among the control samples of the four varieties studied, Istarska Bjelica exhibited the highest total phenolic content (TPC), together with the highest concentrations of oleocanthal and ligstroside aglycones. Levantinka showed the

highest content of phenolic alcohols, while Rosulja contained the highest concentrations of methyl hemiacetal of oleocanthal, oleuropein aglycones and oleacein. Oblica displayed the lowest content of TPC, oleuropein aglycones, methyl hemiacetal of oleocanthal, phenolic alcohols and oleacein, while presenting the highest concentration of *p*-coumaric acid. Comparisons with the literature highlight variability of the phenolic composition. The study by Šarolić et al. [67] reported that Levantinka exhibited approximately half of the TPC determined in the present study, while Oblica showed twice the TPC. In the study by Franić et al. [52], a lower TPC was reported for Levantinka (279 mg/kg), which is similar to the values determined here for Oblica and Rosulja, while Istarska Bjelica exhibited a TPC about twice as high as in the present study (582 mg/kg). According to the EU Commission Regulation [59], olive oil may bear the health claim “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress” if it contains at least 5 mg of hydroxytyrosol and its derivatives per 20 g of oil. Of the varieties examined in this study, only Rosulja VOO fulfills this criterion.

Table 5. The concentration of phenolic compounds and α -tocopherol (mg/kg) in VOO control samples of different autochthonous Croatian olive varieties.

Phenolic Compounds (mg/kg)	<i>p</i> -Value	Variety			
		Istarska Bjelica	Rosulja	Oblica	Levantinka
Hydroxytyrosol	0.157	2 ± 0	7 ± 2	<LOD *	14 ± 22
Tyrosol	0.112	4 ± 0	<LOD *	4 ± 0	11 ± 15
<i>p</i> -Coumaric acid	<0.0001	3 ± 0 b	<LOD *c	7 ± 1 a	3 ± 0 b
Hydroxytyrosol acetate	0.089	<LOD *	<LOD *	<LOD *	1 ± 1
Oleacein	<0.0001	136 ± 23 a	145 ± 4 a	38 ± 8 c	71 ± 18 b
Methyl hemiacetal of oleocanthal	<0.0001	19 ± 1 b	32 ± 2 a	7 ± 4 c	15 ± 3 b
Oleuropein aglycone	<0.0001	85 ± 6 b	110 ± 5 a	24 ± 11 d	46 ± 6 c
Ligstroside aglycone	<0.0001	78 ± 12 a	32 ± 1 b	35 ± 3 b	41 ± 3 b
Oleocanthal	<0.0001	94 ± 2 a	42 ± 1 d	46 ± 1 c	60 ± 4 b
Total	<0.0001	420 ± 12 a	368 ± 9 b	161 ± 26 d	263 ± 30 c
α -tocopherol (mg/kg)	<0.0001	332 ± 52 a	272 ± 10 b	273 ± 22 b	367 ± 18 a

Results marked with * are below LOD (limit of detection) of the method, LOD = 0.2 mg/kg; the *p*-values indicate the influence of the olive variety on the measured parameters. If significant ($p \leq 0.05$), Tukey's post hoc test was performed, and different letters within each row indicate significant differences.

The concentration of α -tocopherol, the predominant tocopherol in the VOOs, is also shown in Table 5. The olive variety exerted a strong influence on the α -tocopherol content. In the control samples, Levantinka exhibited the highest α -tocopherol content, while Oblica and Rosulja contained the lowest values. These results are in agreement with the results of the study by Jukić Špika et al. [68], who found similar α -tocopherol concentrations in Oblica (263–284 mg/kg). For the other varieties, the literature values are generally lower than the values observed in this study. In the study by Šarolić et al. [67], lower α -tocopherol levels were found in Oblica (213 mg/kg) and Levantinka (222 mg/kg), which can be attributed to higher ripening indices. This is in agreement with the results of Kafkaletou et al. [69] showing that α -tocopherol concentrations decrease with fruit ripening. Similarly, the study by Koprivnjak et al. [70] reported lower α -tocopherol content in Istrian varieties, with Istarska Bjelica showing values about three times lower than those observed here. According to the EU Regulation on nutrition and health claims [71,72], foods may bear the claim “Vitamin E contributes to the protection of cells from oxidative stress” if they contain at least 1.8 mg of vitamin E per 100 g. All VOOs analyzed in this study meet this requirement.

3.1.6. Oxidative Stability and Antioxidant Capacity

The oxidative stability of VOOs was evaluated by induction time, defined as the duration (in minutes) during which the oil resists oxidation at elevated temperatures (140 °C). Longer induction times indicate higher concentrations of antioxidants, which confer greater resistance to oxidative deterioration and improve storage stability. The most important factors contributing to oxidative stability include phenolic compounds, tocopherols and the fatty acid profile [73]. Among the control samples (Table 6), Rosulja exhibited the highest oxidative stability, while Oblica showed the lowest. This trend corresponds to the lowest total phenolic content of Oblica and the highest content of polyunsaturated fatty acids, especially linoleic acid. Similar observations were made in the study by Bilušić et al. [74], where Oblica also showed the shortest induction time among the Croatian varieties studied.

Table 6. Oxidative stability index (OSI, min) and antioxidant capacity (AC, % DPPH radical reduction) in VOO control samples of different autochthonous Croatian olive varieties.

Parameter	<i>p</i> -Value	Variety			
		Istarska Bjelica	Rosulja	Oblica	Levantinka
OSI (min)	<0.0001	155.2 ± 4.6 b	206.3 ± 0.6 a	55.1 ± 2.7 d	138.8 ± 4.0 c
AC (% DPPH radical reduction)	<0.0001	58.33 ± 1.89 b	73.28 ± 2.12 a	39.09 ± 3.35 c	56.61 ± 4.28 b

The *p*-values indicate the influence of the olive variety on the measured parameters. If significant ($p \leq 0.05$), Tukey's post hoc test was performed, and different letters within each row indicate significant differences.

The antioxidant capacity (AC) of VOO reflects its ability to counteract oxidative reactions associated with oxidative stress and the development of various diseases. Phenolic compounds and tocopherols contribute the most to the AC of VOO [23]. In this study, AC was determined in vitro using the DPPH assay and expressed as a percentage of DPPH radical reduction. A higher percentage indicates a higher concentration of antioxidants, giving the oil greater stability, slowing degradation during storage and offering potential health benefits to the consumer [75]. As shown in Table 6, Rosulja exhibited the highest AC among the control samples, while Oblica showed the lowest, reflecting the pattern observed for oxidative stability. The AC of Rosulja is consistent with the values from the study of Koprivnjak et al. [51], while Istarska Bjelica showed a slightly higher AC (78%) in their study compared to the present results.

3.2. Influence of Ultrasound

Previous studies have demonstrated that ultrasound can affect oil yield (Y), oxidative stability index (OSI), antioxidant capacity (AC) and phenolic and tocopherol content [9,10,17–20,22,76,77]. In contrast, the effect of ultrasound on volatile compounds was generally described as negligible [5,18,22,24,77] or resulted in a slight decrease [9,17,18], which was attributed to temperature increases and inactivation of lipoxygenase (LOX) by acoustic cavitation. In the study by Yahyaoui et al. [25], no significant effect of ultrasound on LOX activity was observed, while β -glucosidase (β -GLU) exhibited a slight decrease. On the other hand, polyphenol oxidase (PPO) and peroxidase (POX) activities were more affected [3,4]. Mild or short ultrasound exposure stimulated enzyme activity through heat and micro-mixing effects, while higher power levels resulted in inhibition of the enzymes or reduced activity. These results emphasize the complex and parameter-dependent effects of ultrasound on both the Y and the quality of VOO.

To better understand the effects of ultrasound treatment on the main quality and compositional parameters of VOOs from Croatian olive varieties, response surface methodology

(RSM) was used. This statistical approach allows the simultaneous assessment of multiple variables and their interactions and provides an efficient framework for modeling the complex processes involved in olive paste treatment. In this study, two-factor interaction (2FI) models were developed to describe the effects of ultrasonic power (256–640 W) and treatment time (3–17 min). The model parameters for Y, OSI, AC, phenolic and tocopherol content, and linoleic acid concentration are shown in Table 7. Models were also created for other parameters investigated, but these were not statistically significant and are therefore not discussed further.

Table 7. The parameters of the two-factor interaction model (regression coefficients, *p*-value, coefficient of determination (R^2) and lack of fit) for oil yield (Y), oxidative stability index (OSI), antioxidant capacity (AC), linoleic acid content, total phenolic compounds and α -tocopherol content as a function of the duration of ultrasonic treatment (min), power (W) and olive variety.

Model Parameter	Y (%)	OSI (min)	AC (% of DPPH Radical Reduction)	Linoleic Acid (mg/g)	TPC (mg/kg)	α -Tocopherol (mg/kg)
Intercept	12.923	130.94	53.037	93.6	296.586	287.106
<i>p</i> -value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Time	0.159	−3.908	−2.012	−0.166	−12.653	−2.557
<i>p</i> -value	0.093	0.004	<0.0001	0.603	0.000	0.375
Power	0.105	−0.879	−0.453	−0.649	−3.305	−0.228
<i>p</i> -value	0.247	0.477	0.272	0.037	0.275	0.935
Variety-Istarska Bjelica	−2.167	24.252	8.156	−9.462	147.029	13.317
<i>p</i> -value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.001
Variety-Levantinka	7.05	7.46	−3.413	−27.823	−41.035	35.01
<i>p</i> -value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Variety-Oblica	0.9	−92.602	−22.275	68.004	−179.161	−33.683
<i>p</i> -value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Variety-Rosulja	−5.783	60.89	17.533	−30.719	73.168	−14.644
<i>p</i> -value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0
Time * Power	−0.184	−1.325	0.4	1.066	−3.992	16.688
<i>p</i> -value	0.164	0.462	0.505	0.019	0.365	<0.0001
Time * Variety-Istarska Bjelica	0.217	−1.066	−0.403	0.053	12.657	−6.509
<i>p</i> -value	0.182	0.63	0.585	0.923	0.021	0.193
Time * Variety-Levantinka	0.064	−1.879	−1.322	0.191	−8.421	−5.966
<i>p</i> -value	0.691	0.398	0.076	0.729	0.122	0.233
Time * Variety-Oblica	−0.131	1.542	−0.204	−1.087	−0.853	1.509
<i>p</i> -value	0.419	0.487	0.783	0.051	0.875	0.762
Time * Variety-Rosulja	−0.151	1.403	1.929	0.842	−3.382	10.966
<i>p</i> -value	0.352	0.527	0.01	0.129	0.532	0.03
Power * Variety-Istarska Bjelica	−0.276	−7.309	−2.812	0.493	−15.387	0.169
<i>p</i> -value	0.082	0.001	0	0.356	0.004	0.972
Power * Variety-Levantinka	0.115	5.803	1.782	1.131	8.789	−11.743
<i>p</i> -value	0.461	0.009	0.014	0.036	0.095	0.016
Power * Variety-Oblica	0.363	3.574	2.376	−3.343	9.826	−1.184
<i>p</i> -value	0.024	0.1	0.001	<0.0001	0.062	0.806
Power * Variety-Rosulja	−0.202	−2.068	−1.347	1.719	−3.229	12.757
<i>p</i> -value	0.199	0.336	0.061	0.002	0.537	0.009
R^2	0.991	0.988	0.958	0.997	0.966	0.653
R^2 adjusted	0.988	0.985	0.952	0.996	0.962	0.607

As previously noted, Y was mainly determined by the olive variety. While a slight increase in Y was observed with longer ultrasound pretreatment times, neither treatment time ($p = 0.093$) nor ultrasound power ($p = 0.247$) exerted a statistically significant main effect on Y (Table 7, Figure 2). This indicates that ultrasound treatment alone did not consistently improve oil recovery under all conditions. However, a significant interaction between ultrasound power and Oblica variety suggests that ultrasound can moderately improve oil recovery in certain varieties, likely by promoting cell wall disruption and facilitating oil release. Specifically, application of 640 W for 10 min increased Y in Oblica by approximately 4% compared to the control. These results are consistent with previous studies emphasizing that varietal differences in cell structure, moisture content and oil distribution play an

important role in extractability [78]. Similar improvements in Y were observed in other varieties subjected to ultrasonic pretreatment prior to malaxation under industrial conditions, including an increase of 3.4% in Ogliarola Garganica (maturity index 2.82) [17], 4.4% and 4.6% in Nocellara and Coratina, respectively [22], and an increase of 4.3% in Chemlali after a 10 min ultrasonic treatment [76]. Recent studies have also investigated the use of ultrasound in VOO production, including energy cost–benefit analyses demonstrating the potential profitability of the process. For example, in the study by Grillo et al. [79], an 18% increase in Y and a 48% reduction in energy consumption (from 0.964 kW/kg to 0.500 kW/kg) was observed, while in the study by Boffa et al. [80], a 43% increase in Y and a ~35% reduction in energy consumption per kilogram of oil (from ~555 kWh to ~381 kWh) was observed.

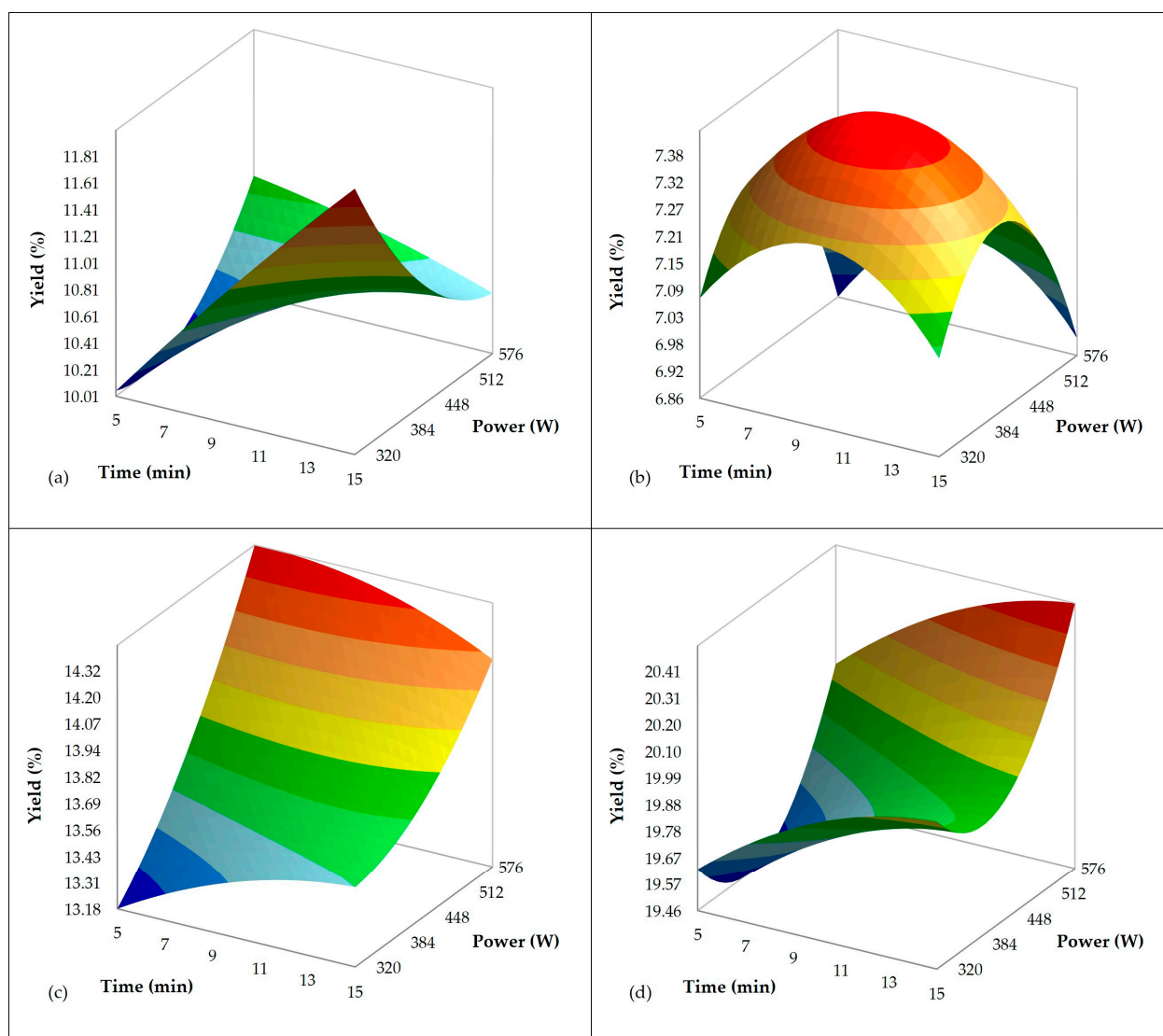


Figure 2. Three-dimensional response surface plots illustrating the effect of ultrasound pretreatment parameters—treatment time (min) and ultrasound power (W)—on oil yield in autochthonous Croatian olive varieties: (a) Istarska Bjelica, (b) Rosulja, (c) Oblica and (d) Levantinka.

The OSI is a key parameter reflecting the resistance of VOO to lipid oxidation and serves as a reliable predictor of shelf life. The model developed for OSI (Table 7, Figure 3) showed high predictive accuracy ($R^2 = 0.988$; adjusted $R^2 = 0.985$), indicating that the combined effects of ultrasound parameters and olive variety explained almost all of the observed variability in OSI. Among the ultrasound factors, treatment duration had a statis-

tically significant negative effect on OSI ($p = 0.004$), suggesting that prolonged sonication promotes oxidative degradation. This is consistent with previous reports showing that acoustic cavitation during ultrasound treatment can generate localized heating and reactive oxygen species, thereby accelerating oxidative reactions [81]. In contrast, ultrasound power alone had no significant effect ($p = 0.477$), emphasizing that duration of exposure is a more critical determinant of oxidative damage than power intensity. These results are consistent with the observations in the research of Iqdiam et al. [19], who reported a significant decrease in OSI after 8 and 10 min of direct and indirect high-power ultrasound treatments, while shorter exposure times (0–6 min) had no significant effect ($p > 0.05$). Notably, 10 min of direct high-power ultrasound was reported to reduce induction time by approximately 8%. This suggests that ultrasound only begins to compromise oxidative stability beyond a critical exposure threshold.

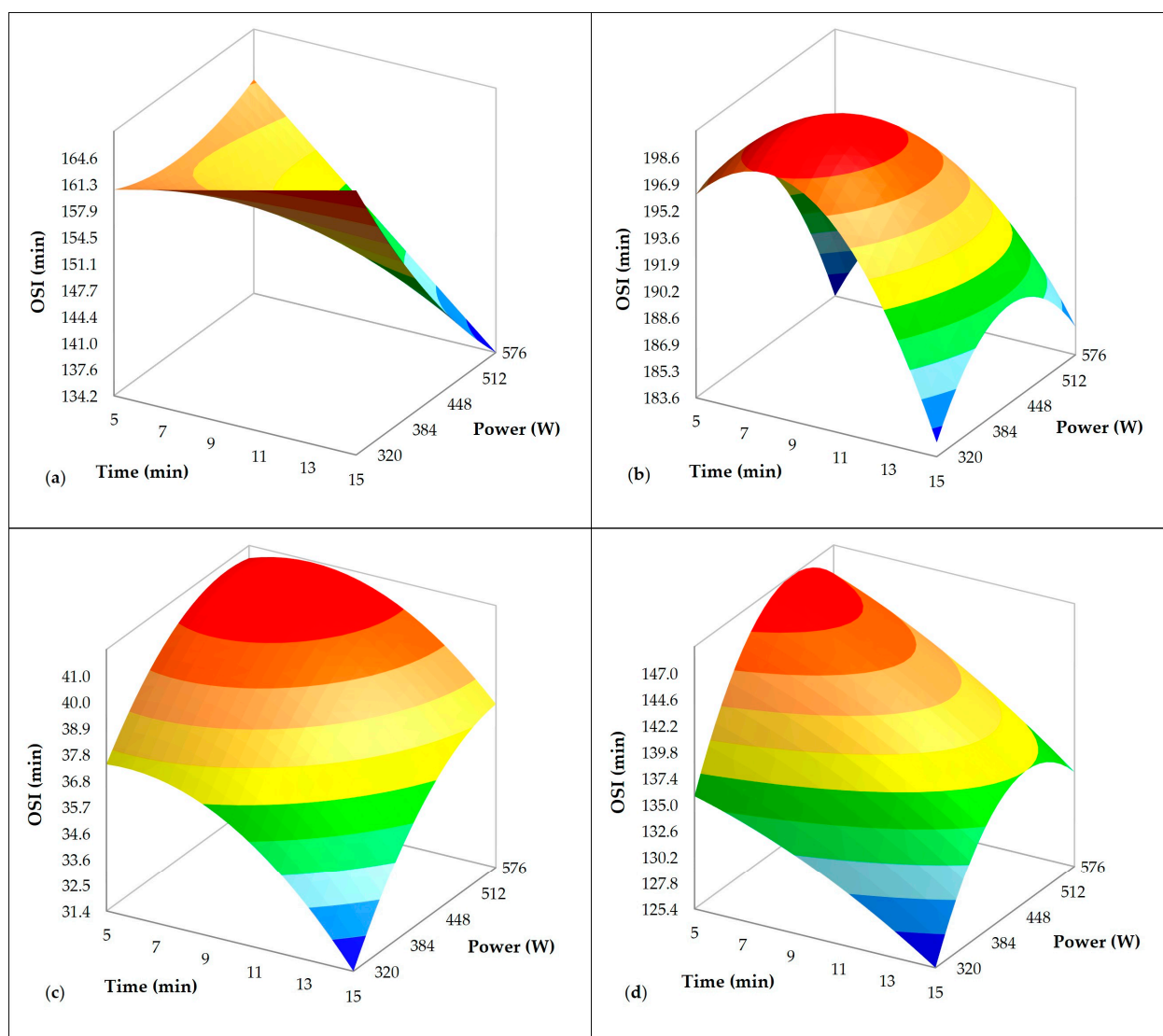


Figure 3. Three-dimensional response surface plots illustrating the effect of ultrasound pretreatment parameters—treatment time (min) and ultrasound power (W)—on oxidative stability index (OSI, min) in autochthonous Croatian olive varieties: (a) Istarska Bjelica, (b) Rosulja, (c) Oblica, and (d) Levantinka.

The negative interaction of ultrasound power observed in Istarska Bjelica (Figure 3a) indicates that even phenolic-rich cultivars [82] can be susceptible to oxidation when exposed to excessive ultrasound energy, illustrating the fine balance between beneficial extraction

and detrimental degradation. Accordingly, Istarska Bjelica showed the highest increase compared to the control sample (15%) and among all varieties at the lowest ultrasound power. In contrast, the interaction of ultrasound power in Levantinka (Figure 3d) was significant and positive, suggesting that moderate ultrasound energy can promote the release of antioxidants without inducing significant oxidative stress in this variety. These observations are in agreement with the results of Servili et al. [22], who reported that ultrasound under optimized conditions—such as elevated pressure—can selectively increase the extraction of antioxidants and improve oil quality.

Overall, these results underline the importance of variety-specific optimization of ultrasound parameters. Short to moderate treatment durations may provide benefits by promoting the release of bioactive compounds, while prolonged sonication may compromise oxidative stability, especially in varieties that are more sensitive to oxidative degradation.

The regression model developed for linoleic acid content (Table 7, Figure 4) demonstrated excellent predictive accuracy ($R^2 = 0.997$; adjusted $R^2 = 0.996$). The models were created using mass fractions in mg/g of oil rather than percentages, allowing small changes in concentration to be detected and providing higher resolution and precision. Ultrasound power was found to be a significant negative factor ($p = 0.037$), indicating that higher sonication intensity decreases linoleic acid content, likely due to oxidative degradation by radicals generated during cavitation. In contrast, treatment duration had no statistically significant main effect ($p = 0.603$), suggesting that prolonged exposure alone may not significantly alter linoleic acid content unless combined with high power. These results are consistent with the observed decrease in OSI and support the assumption that ultrasound-induced oxidation primarily targets unsaturated lipids such as linoleic acid.

In the Oblica variety (Figure 4c), high-power ultrasound treatment ($p < 0.001$) led to a significant reduction in the linoleic acid content. This result is in line with expectations, as Oblica contains at least twice the amount of linoleic acid compared to the other varieties studied (Table 2). Linoleic acid is particularly susceptible to oxidation, and high-power ultrasound (20 kHz, 150 W) has been shown to accelerate oxidative processes in oils. For example, the research by Chemat et al. [83] reported that sonication of refined sunflower oil for only 2 min increased peroxide values from 5.38 to 6.33 meq O_2 /kg, indicating the onset of oxidation. While the immediate changes in fatty acid composition were minimal, the profile of volatile compounds revealed the formation of hexanal and limonene as a result of sonication.

The regression model for TPC (Table 7, Figure 5) exhibited a strong fit ($R^2 = 0.966$; adjusted $R^2 = 0.962$), confirming that, in addition to variety, ultrasound treatment also influences the phenolic profile of VOO. Among the ultrasound parameters, treatment duration had a highly significant negative effect (-12.653 mg/kg per minute, $p < 0.001$), while ultrasound power alone had no significant effect. Notably, the interaction between treatment duration and Istarska Bjelica variety was significant and positive ($p = 0.021$), indicating that this variety is less susceptible to phenolic losses with prolonged sonication. Accordingly, the highest TPC increase (17%) was recorded at a medium ultrasound duration. Previous studies confirm the variety- and condition-dependent effects of ultrasound on phenolic composition. In the study by Tamborrino et al. [9], an increase of 41% in TPC and 68% in oleacein was observed in the Peranzana variety, while oleocanthal increased only slightly (~10%) and phenolic alcohols decreased. Taticchi et al. [17] observed a 10% increase in TPC in Ogliarola Garganica oils, primarily due to oleuropein aglycone species. In the study by Servili et al. [22], a 28% increase in TPC was observed in the Nocellara variety, with a significant increase in oleocanthal content (39% in Nocellara, 19% in Coratina) and a considerable increase in ligstroside and tyrosol content. In contrast, Gila et al. [18] found that ultrasound had little effect on phenolic content and even caused a slight

decrease in Arbequina oils, which they attributed to a moderate increase in temperature during treatment.

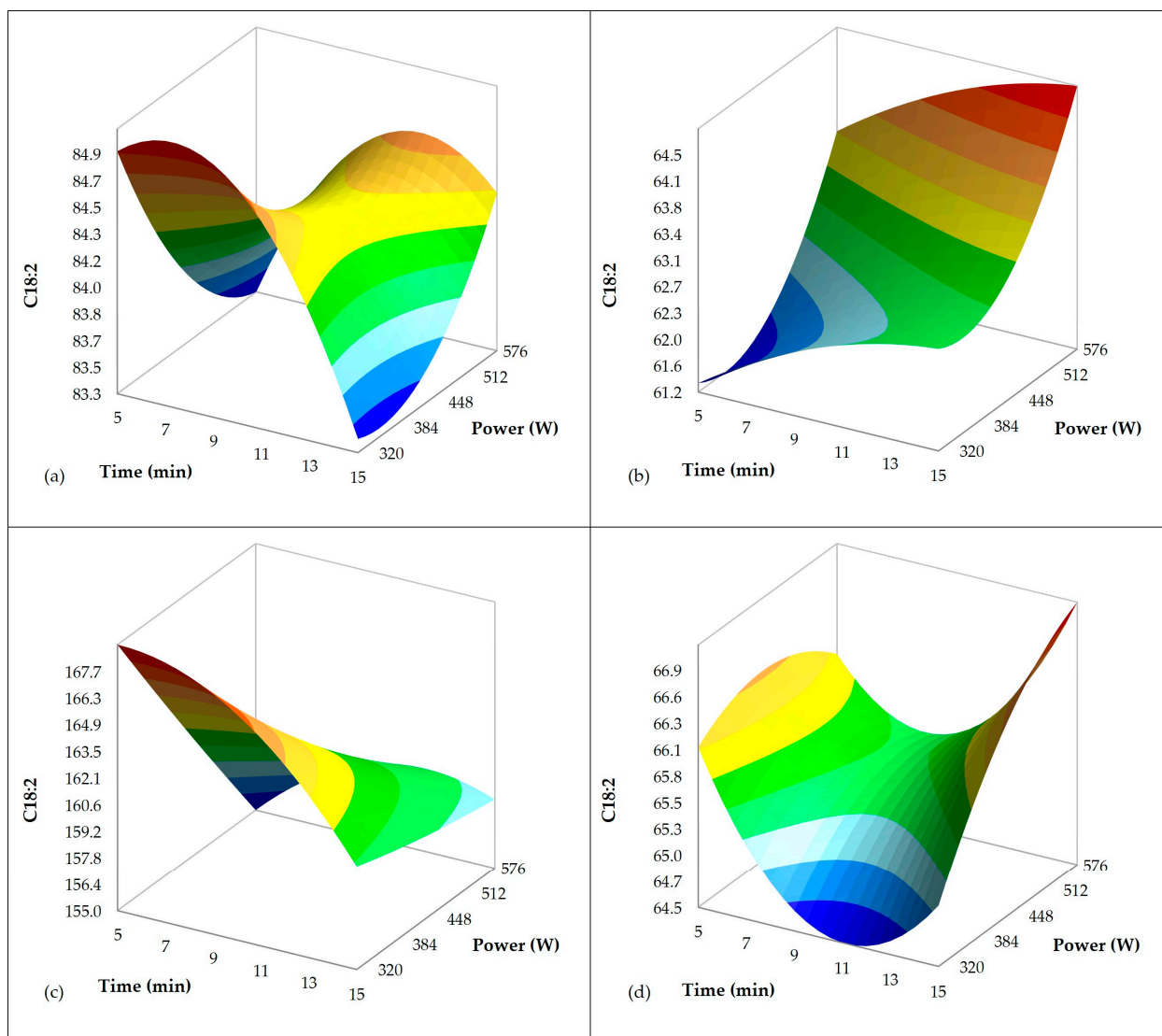


Figure 4. Three-dimensional response surface plots illustrating the effect of ultrasound pretreatment parameters—treatment time (min) and ultrasound power (W)—on linoleic acid content (mg/g) in autochthonous Croatian olive varieties: (a) Istarska Bjelica, (b) Rosulja, (c) Oblica, and (d) Levantinka.

On the other hand, the study by Iqdiam et al. [19] reported that a 10 min direct high-power ultrasound treatment resulted in a significant reduction in TPC by approximately 19% in Arbequina and 22% in Frantoio, indicating a significant loss of antioxidants with prolonged sonication. These results are in agreement with those of Jiménez et al. [20], who also observed a degradation of phenols due to ultrasound exposure. These contrasting results can be explained by the dual role of ultrasound: enhancement of phenolic extraction by disrupting cell structures and facilitating the release of bound compounds [3], while at the same time promoting oxidative reactions that consume phenols as primary antioxidants to neutralize free radicals generated during sonication [16]. This mechanism is supported by the observed decrease in OSI and linoleic acid content, suggesting a coupled degradation pathway in which phenols are consumed to counteract oxidation, accelerating the degradation of the unsaturated fatty acids and reducing the overall stability of the oil. The results for Istarska Bjelica show that short ultrasonic treatments can increase phenolic

content without inducing significant oxidation losses, highlighting the critical importance of optimizing sonication duration to balance these competing effects.

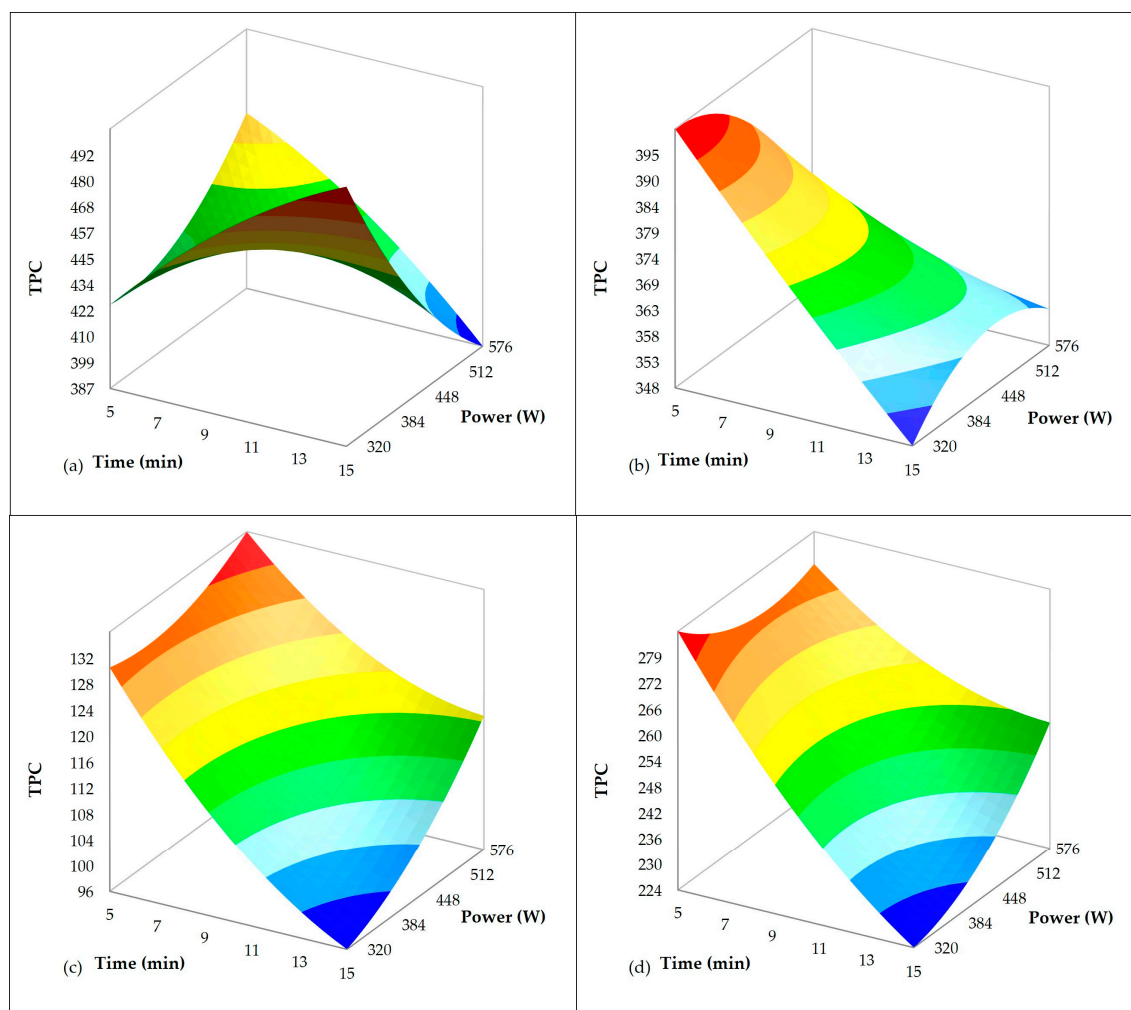


Figure 5. Three-dimensional response surface plots illustrating the effect of ultrasound pretreatment parameters—treatment time (min) and ultrasound power (W)—on total phenolic content (TPC) in autochthonous Croatian olive varieties: (a) Istarska Bjelica, (b) Rosulja, (c) Oblica, and (d) Levantinka.

Phenolic compounds also play a key role in the sensory profile of VOO, contributing to bitterness and pungency [1]. Consequently, ultrasound-induced changes in phenolic content can modulate sensory quality. An increase in phenol concentration due to ultrasonic treatment generally resulted in a stronger perception of bitterness, pungency and spiciness compared to control samples [5,11,23,80]. Conversely, a reduction in phenolic content was associated with a decrease in bitter and pungent notes and an overall improvement in sensory acceptance of VOOs subjected to ultrasound pretreatment [20,84].

The regression model for α -tocopherol content (Table 7, Figure 6) showed moderate explanatory power ($R^2 = 0.653$; adjusted $R^2 = 0.607$), reflecting the complex behavior of tocopherols during ultrasound treatment. Individually, neither treatment time nor ultrasound power had a statistically significant effect; however, their interaction was highly significant ($p < 0.0001$), indicating that the combined application of higher ultrasound intensity and longer exposure may significantly affect tocopherol content, probably by promoting release from the cell matrix. This effect was particularly evident in Rosulja, which showed a 24% increase in α -tocopherol. Similar trends were observed in the studies by Taticchi et al. [17] and Pagano et al. [10], who found an 11% increase in α -tocopherol content

in Ogliarola Garganica, while no significant effect of ultrasound on tocopherol content was observed in the study by Gila et al. [18]. In the present study, α -tocopherol content decreased in all varieties except Rosulja (Figure 6), emphasizing the variety-dependent response to sonication.

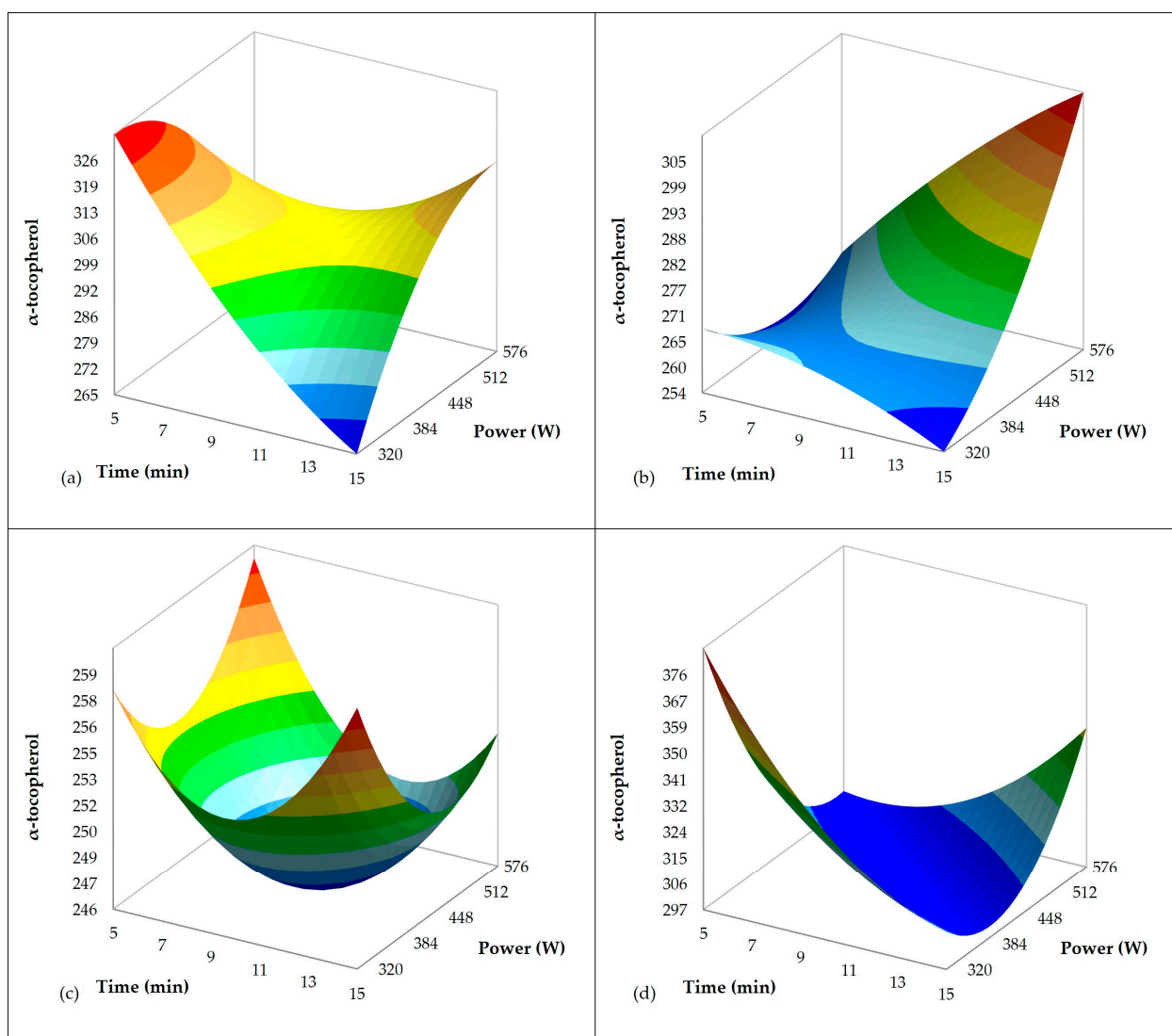


Figure 6. Three-dimensional response surface plots illustrating the effect of ultrasound pretreatment parameters—treatment time (min) and ultrasound power (W)—on α -tocopherol content in autochthonous Croatian olive varieties: (a) Istarska Bjelica, (b) Rosulja, (c) Oblica, and (d) Levantinka.

The regression model for AC, expressed as percentage of DPPH radical reduction (Table 7, Figure 7), demonstrated strong predictive power ($R^2 = 0.958$; adjusted $R^2 = 0.952$). Treatment duration had a highly significant negative effect ($p < 0.0001$), indicating that prolonged ultrasound exposure reduces AC, likely due to thermal degradation of antioxidant compounds [18]. In agreement with this, the highest increase in AC compared to the control sample and among all varieties was observed in Levantinka (13%) with shorter ultrasound duration. Ultrasound power alone had no significant effect on AC ($p = 0.272$). However, the interaction between power and Istarska Bjelica variety was significantly negative ($p < 0.001$), indicating that higher ultrasound power decreases AC in this variety. These results are consistent with the effects observed for TPC, the primary antioxidants in VOO. In Rosulja, however, the interaction between treatment duration and ultrasound

power showed a significant positive effect on AC, which is likely related to the observed increase in α -tocopherol content under extended sonication.

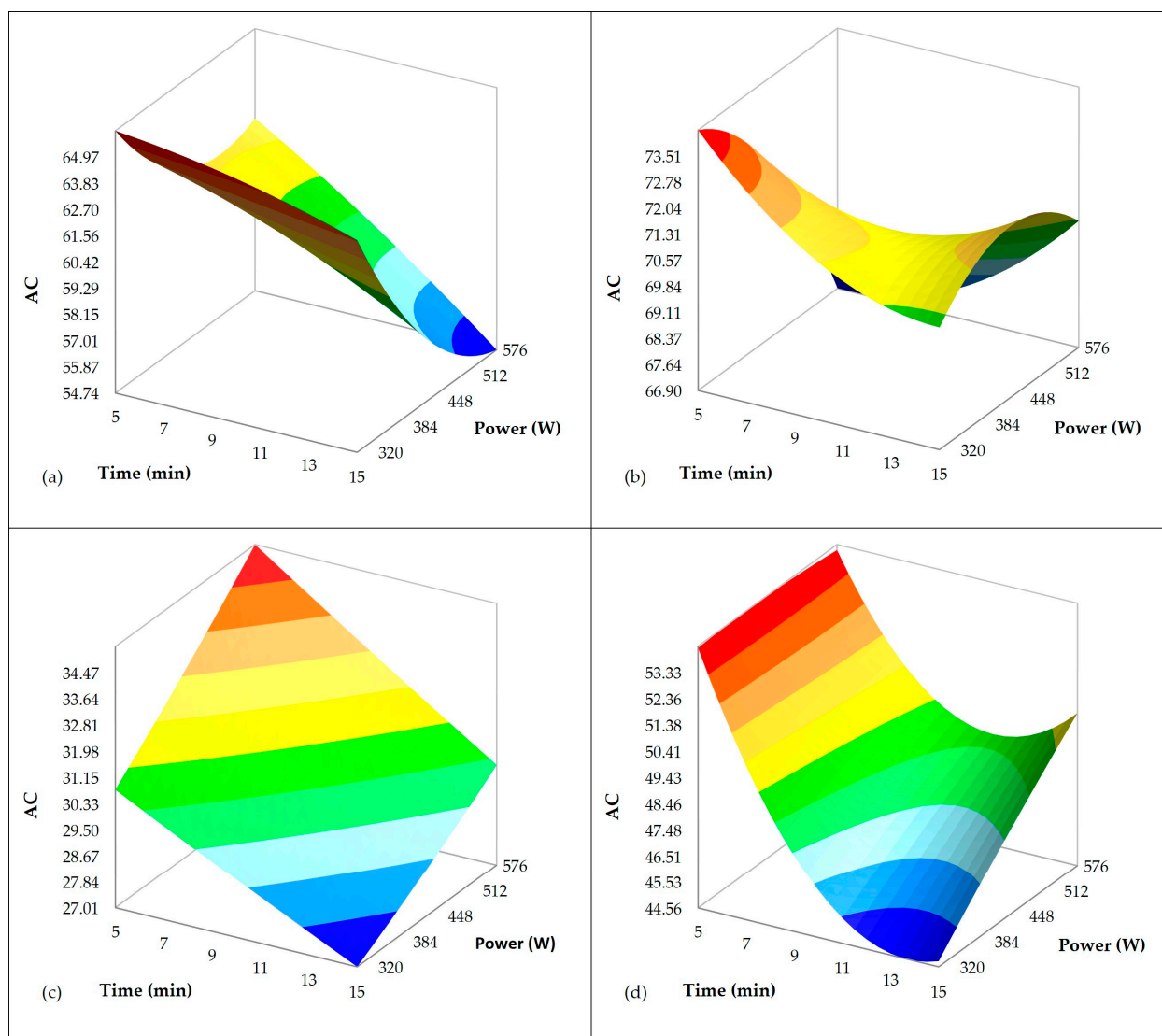


Figure 7. Three-dimensional response surface plots illustrating the effect of ultrasound pretreatment parameters—treatment time (min) and ultrasound power (W)—on antioxidant capacity (AC, % DPPH radical reduction) in autochthonous Croatian olive varieties: (a) Istarska Bjelica, (b) Rosulja, (c) Oblica, and (d) Leventinka.

4. Conclusions

Previous research has demonstrated that ultrasound can enhance oil yield (Y) while maintaining product quality. Although Croatia is recognized for producing exceptional quality VOOs, production is geographically restricted and relatively limited in scale. The application of ultrasound could therefore be a promising strategy to increase production efficiency. However, Croatian autochthonous olive varieties have not yet been systematically evaluated with respect to novel processing technologies. To address this gap, the present study investigated the effects of ultrasound pretreatment on Y, endogenous enzyme activity and quality characteristics of VOOs from four autochthonous Croatian olive varieties: Istarska Bjelica, Rosulja, Oblica and Leventinka. In this experimental setup, the olive paste was subjected to ultrasound treatment before malaxation, with the duration of the treatment ranging from 3 to 17 min and the power between 256 and 640 W.

The effect of ultrasound was strongly dependent on the variety. The treatment duration generally had a stronger influence than the power. A moderate increase in Y (~4%) was only observed in Oblica, while other varieties showed no significant improvement. Prolonged ultrasound exposure resulted in a decrease in total phenolic content (TPC), oxidative stability index (OSI) and antioxidant capacity (AC), except in Rosulja where AC increased. High ultrasound power was associated with lower linoleic acid content, indicating increased oxidative degradation and contributing to the observed decrease in AC in Istarska Bjelica. The α -tocopherol content was generally negatively affected by ultrasound, except in Rosulja, where extreme ultrasound conditions resulted in a significant increase, likely contributing to the increase in AC. Basic quality parameters, volatile compounds and the activity of enzymes remained largely unaffected by the ultrasound treatment.

These results suggest that excessive sonication may compromise bioactive compounds and oxidative stability, highlighting the need for careful optimization. Short-to-moderate ultrasonic durations at low-to-moderate power are recommended to achieve a balance between improved extraction and preservation of VOO quality. The following optimal conditions are suggested for the studied varieties: 256 W for 10 min for Istarska Bjelica, 448 W for 3 min for Rosulja and Levantinka and 320 W for 5 min for Oblica.

Although ultrasound is already applied in the industry, our findings and previous studies indicate that a universal approach is not suitable for all olive varieties. Instead, the parameters of the ultrasound treatment should be tailored to the specific variety. It is therefore recommended to conduct further experiments to optimize the conditions for each variety, with the aim of maximizing Y and maintaining quality while minimizing the risk of oxidative degradation. In this way, ultrasound technology can be more effectively integrated into industrial VOO production.

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Abbreviations

The following abbreviations are used in this manuscript:

Y	Yield
VOO	Virgin olive oil
OSI	Oxidative stability index
AC	Antioxidant capacity
EE	Endogenous enzymes of the olive fruit
LOX	Lipoxygenase

B-GLU	β-Glucosidase
PPO	Polyphenol oxidase
POX	Peroxidase
BHT	Butylhydroxytoluene
EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenylmethylsulfonylfluoride
PVP	Polyvinylpyrrolidone
DPPH	2,2-diphenyl-1-picrylhydrazyl
HPOT	Hydroperoxyoctadecatrienoic acid
GC	Gas chromatography
FID	Flame ionization detector
FAME	Fatty acid methyl ester
DAD	Diode array detector
DSC	Differential scanning calorimeter
EPR	Electron paramagnetic resonance
ANOVA	Analysis of variance
RSM	Response surface methodology
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
MBA	Microbiological activity
OX	Oxidation
TPC	Total phenolic content

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