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Innovative Approaches to Enhance Activity of Endogenous Olive Enzymes—A Model System Experiment: Part I—Thermal Techniques

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Abstract: The aim of this study was to investigate the influence of thermal treatment and ultrasound on the activity of β-glucosidase and lipoxygenase, enzymes that determine the phenolic composition and sensory profile of virgin olive oil. Enzyme activity was determined spectrophotometrically in model systems consisting of commercial enzymes and their substrates. Thermal treatment was performed by tempering the enzymes and substrates at temperatures between 15 and 40 °C. Enzyme activity was measured 1 min after reaction and again after the additional incubation of the reaction mixture at 25 °C for 30 min to simulate the behavior of the enzymes during the malaxation process. Ultrasonic treatment of the model solutions was performed at 128, 320, and 640 W of the ultrasonic bath power for 1, 5, and 12 min. Enzyme activity was determined immediately after treatment and again after incubation at 25 °C for 30 min. The higher temperatures during thermal treatment increased the activity of both enzymes. During ultrasound treatment, the activity of both enzymes was positively affected by its duration. The higher power of ultrasound was a better choice for β-glucosidase and the lower one for lipoxygenase. The stimulation of enzyme activity by the studied techniques resulted in an acceleration of enzymatic reactions during the additional incubation, suggesting that the malaxation process could be shortened in virgin olive oil production.

Keywords: enzymatic activity; β-glucosidase; lipoxygenase; model system; virgin olive oil production; thermal treatment; ultrasound treatment; malaxation

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1. Introduction

Due to its exceptional sensory properties and beneficial effects on human health, virgin olive oil (VOO) is enjoying increasing popularity worldwide, beyond its traditional status as a staple food in the Mediterranean. The particular composition of fatty acids, especially oleic acid, and the high phenolic content contribute to the fact that VOO can prevent a number of diseases including cardiovascular and metabolic disorders [1,2].

VOO is obtained from olive fruit purely through mechanical processes, which include crushing and malaxation as the key production steps [3]. Crushing breaks the cellular structure of the olive fruit and releases the oil droplets contained in the cell vacuoles and cytoplasm of the fruit, while at the same time initiating a cascade of chemical and biochemical reactions [4]. These processes continue during malaxation, where the olive paste is kneaded at a defined temperature, which in turn facilitates the fusion of dispersed oil droplets into larger drops that can be separated by mechanical processes. At the same time, pectolytic, cellulolytic, and hemicellulolytic enzymes are activated, which break down the cell walls and alter the rheological properties, resulting in a lower viscosity of the olive paste [5]. The chemical and enzymatic reactions that occur cause the transformation of

phenolic compounds and lead to the formation of volatile compounds that are crucial for the sensory and nutritional profile of VOO. The changes in the phenolic profile of olive fruit are mainly caused by β-glucosidase (β-GLU), which hydrolyzes oleuropein, demethyloleuropein, and ligstroside, the major hydrophilic secoiridoids in olive fruit, releasing their aglycones [6]. Along with β-GLU, esterases also contribute to the formation of various aglycones including oleacein and oleocanthal [7]. These products are more lipophilic and partition into the oil phase, increasing the proportion of phenolic compounds in VOO. On the other hand, the activity of peroxidases and polyphenol oxidases during malaxation can lead to the oxidation and degradation of phenolic compounds [8]. The study by Miho et al. [9] found that the duration of malaxation had a negative effect on most secoiridoid aglycones, with the exception of oleacein and oleocanthal, whose concentration increased during the 10 to 30 min malaxation. An increase in malaxation temperature leads to an increase in β-GLU activity, solubilization, and distribution of phenolic compounds, but may also cause an increase in phenol degradation due to polyphenol oxidase and peroxidase activity [8]. Therefore, the net effect of temperature on phenolic content depends on which mechanism predominates [10]. The time and temperature of malaxation also directly affect the formation of volatile compounds via the lipoxygenase pathway [11]. Lipoxygenases (LOX) are the first enzymes in this chain reaction, converting linoleic and linolenic acids formed from triacylglycerides and phospholipids by acyl hydrolases into their hydroperoxides. The hydroperoxides are then converted to C6 aldehydes, alcohols, and esters. This process produces the aromatic compounds of VOO such as hexanal, hexan-1-ol, trans-2-hexenal, and trans-2-hexen-1-ol [12]. Lower temperatures during malaxation favor the formation of aldehydes, while higher temperatures promote the conversion of aldehydes to alcohols [13]. The duration of malaxation also has an influence, but to a lesser extent, than temperature. Cevik et al. [14] recommended a malaxation duration of 40 min to achieve an optimum balance between the desired and undesired volatile compounds.

In conventional virgin olive oil production, limited production systems result in low oil recovery, with 10–20% of the oil remaining in the pomace [15]. Therefore, in recent years, innovative technologies have been developed aiming to increase the oil yield, improve the shelf life, and reduce the production costs while maintaining the sensory quality and increasing bioactive compound content [16]. Among the most promising technologies for VOO production are thermal processes such as flash thermal treatment (FTT) and ultrasound (US) as well as non-thermal processes such as pulsed electric field [17–19]. The use of heat exchange in VOO production technology has been explored by several authors, initially with the aim of heating the olive paste before malaxation by tubular heat exchangers [20–22]. Depending on the temperature applied, FTT modification has the potential to shorten the malaxation time, increase the content of phenols, and modify the profile of volatile compounds. In addition, US treatment of olive paste after crushing and before malaxation has been shown to increase the production yield due to rapid heating of the olive paste and the destruction of cell walls due to cavitation and implosion, which lead to the release of additional oil [23]. Studies by Jiménez et al. [24] and Clodoveo et al. [25] have shown that US treatment can alter the profile of volatile compounds, increase the content of tocopherols, chlorophyll, and carotenoids, but decrease the content of polyphenols, bitterness, and pungency. Almeida et al. [26] found that US treatment of the paste after mixing increased the content of polyphenols, C5 and C6 volatiles, bitterness, pungency, fruitiness, and utilization of DMU extraction, but decreased the tocopherol content. Since it has been shown that temperature and duration of malaxation can cause changes in enzyme activity, the conditions of the thermal processes may be critical in determining the nutritional and sensory profile of VOO. In addition, US treatment can lead to structural changes in the enzymes and alter their activity by changing the substrate affinity and stability [27].

Although several studies have investigated the effects of FTT and US on virgin olive oil production, the studies focused mainly on the products of complex reactions rather than determining the activity of individual enzymes. Therefore, this study endeavored

to determine the direct effect of these thermal techniques on the activity of β-GLU and LOX as the key enzymes affecting the quality of VOO. The enzyme activity was assessed in model systems consisting of enzymes and their substrates.

2. Materials and Methods

2.1. Materials

The enzymes used in this experiment, β-glucosidase (β-GLU) from almond (Prunus dul-cis) and lipoxygenase (LOX) from soybean (Glicine max) and their substrates, 4-nitrophenyl-β-D-glucopyranoside (*p*-NPG) and linolenic acid (ALA), respectively, were purchased from Sigma Aldrich (St. Louis, MO, USA), as were Coomassie Brilliant Blue G-250 and *p*-nitrophenol (*p*-NP). The bovine serum albumin standard was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Tween 40 from Fluka Chemie GmbH (Buchs, Switzerland), and ethanol from Gram-mol (Zagreb, Croatia). Sodium acetate, sodium hydroxide, disodium hydrogen phosphate, and sodium dihydrogen phosphate dihydrate were purchased from Kemika (Zagreb, Croatia). Acetic and hydrochloric acids were purchased from Lach-Ner (Neratovice, Czech Republic), and *o*-phosphoric acid from Carlo Erba Reagents GmbH (Emmendingen, Germany).

2.2. Model Systems

The model solution for the determination of β-glucosidase (β-GLU) activity contained 1.5 mL of the *p*-NPG solution (15 mM) in 0.05 M sodium acetate buffer (pH 5.5) and 20 µL of the enzyme solution (5 mg/mL) diluted in the same buffer.

To determine the activity of lipoxygenase (LOX), a model solution was prepared with 2.5 mL of 0.1 M phosphate buffer (pH 6.0), 50 µL of enzyme solution (5 mg/mL) in the same buffer, and 50 µL of ALA (10 mM). ALA was prepared according to the method described by Axelrod et al. [28]. The 25 mM solution of ALA was prepared by dissolving the appropriate amount of the fatty acid in water containing 1% Tween-40 and 25 mM NaOH. To remove dissolved oxygen, the water was previously treated with nitrogen. The prepared concentrated solution was divided into aliquots, stored at −80 °C and diluted to the desired concentration with oxygen-free water before each use.

2.3. Quantification of Proteins

The exact protein concentration in the enzyme solutions was determined by the Bradford method [29]. Enzyme solutions were diluted 30-fold to achieve a reliable absorbance range between 0.1 and 1. To perform the measurements, 300 µL of the diluted enzyme solution was added to 1.2 mL of Bradford reagent (1% solution of Coomassie Brilliant Blue G-250 in water containing 5% (*v*/*v*/) ethanol and 10% (*v*/*v*) *o*-phosphoric acid) and allowed to react for 5 min. Absorbance was measured at 595 nm using a UV–Vis spectrophotometer (UviLine 9400, SECO-MAM, Alès, France). Bovine serum albumin (concentration from 0.5 to 5 mg/mL) was used as a standard for the calibration curve. Protein determinations were performed three times for each enzyme solution.

2.4. Simulation of Malaxation Process

To simulate the behavior of enzymes in olive paste during the malaxation process, model systems of enzymes and their corresponding substrates, prepared as described in Section 2.2, were incubated at 25 °C for up to 60 min. Enzyme activity was measured after 1 min and then every 10 min.

2.5. Thermal Treatment

The components of each model solution (prepared as described in Section 2.2) were separately tempered in a water bath to 15, 20, 25, 30, 35, and 40 °C before mixing them to determine the enzyme activity at the temperature used to temper the enzyme and substrate. After preparation, the model solutions were left to react at the indicated temperatures for

1 min, and then the enzyme activity was measured. After additional incubation of the reaction mixture at 25 °C for 30 min, the activity was determined again.

2.6. Ultrasound Treatment

A Bandelin sonorex digiplus ultrasonic bath (Bandelin electronic, Berlin, Germany) with a declared maximum power of 640 W was used for ultrasonic treatment (US). The enzyme solution and substrate were tempered separately to 25 °C prior to treatment; this was also the starting temperature of the ultrasonic bath. Treatments of each model solution (prepared as described in Section 2.2) were performed at a power of 128, 320, and 640 W (20, 50, and 100% of the maximum power of the ultrasonic bath—640 W), and the treatment duration was 1, 5, and 12 min.

Enzyme activity was determined immediately after US treatment and after additional incubation of the treated reaction mixture at 25 °C for 30 min.

2.7. Enzyme Activity

The β-GLU activity was determined by monitoring the increase in absorbance at 405 nm related to the increasing amount of *p*-NP released from the *p*-NPG [30]. Measurements were performed using two blank samples: a solution of *p*-NPG with 20 µL of acetate buffer in place of the enzyme and an enzyme solution with 1.5 mL of acetate buffer in place of the *p*-NPG solution. These solutions were thermally or ultrasonically treated like the model solutions to exclude any increase in absorbance not caused by the enzyme itself. *p*-NP (concentration from 0.15 to 15 mM) was used to construct a calibration curve for the quantification of the hydrolytic reaction.

LOX activity was determined according to the method published by Luaces et al. [31]. The method is based on the spectrophotometric measurement of formed linolenic acid hydroperoxides (HPOT) at 234 nm. Similar to the determination of β-GLU activity, the absorbance of the model solution was corrected by the absorbance of two blanks: a solution of ALA and an enzyme solution. The quantification of HPOT was performed using a calculated molar extinction coefficient of 2.5×10^4 M^{-1} cm^{-1}.

Enzyme activity was defined as the amount of product (*p*-NP or HPOT) released by 1 mg of enzyme.

2.8. Statistical Evaluation

Each set of experimental conditions was tested in three replicates. The statistical significance threshold was set at $p = 0.05$ for all relevant statistical tests. In the thermal treatment study, the effect of temperature (15, 20, 25, 30, 35, and 40 °C) as an independent factor on β-GLU and LOX activity was examined. Results were analyzed by one-way analysis of variance (ANOVA), and the Tukey multiple comparison test was used to determine the differences between means. The study of US treatment on the activity of β-GLU and LOX activity immediately after and after additional incubation was based on a full-factorial design that included two independent factors: (i) time (1, 2, and 5 min) and (ii) power (128, 320, and 640 W), both tested at 3 levels with a total of 9 experiments conducted. To estimate the effects of the studied factors and their interaction on the analyzed parameters, two-way ANOVA was used in combination with Tukey's multiple comparison tests. Nonlinear regression was used to model the behavior of β-GLU and LOX activity during additional incubation and as a function of temperature reached by direct (TT) or indirect (US) heat input. Exponential equations (one phase association, exponential growth), second-order equations, and growth equations (Gompertz and beta-growth) were evaluated, and the best-fitting models were selected to predict the behavior of enzymatic activity. All statistical analyses were performed using the XLSTAT software solution [32].

3. Results and Discussion

3.1. Enzyme Activity during Simulated Malaxation Process

Olive fruit endogenous enzymes play a crucial role in the nutritional quality and sensory properties of virgin olive oil. Although their activity is mainly determined by agronomic factors (variety and ripeness), enzyme activity can be influenced by regulating production parameters such as temperature and/or time of the malaxation process [4,33,34]. Figure 1 shows the enzyme activity of β-GLU (1a) and LOX (1b) over a period of 60 min at 25 °C, the temperature most commonly recommended for VOO production in the malaxation process, which can take up to 1 h (usually 30 to 45 min) [35].

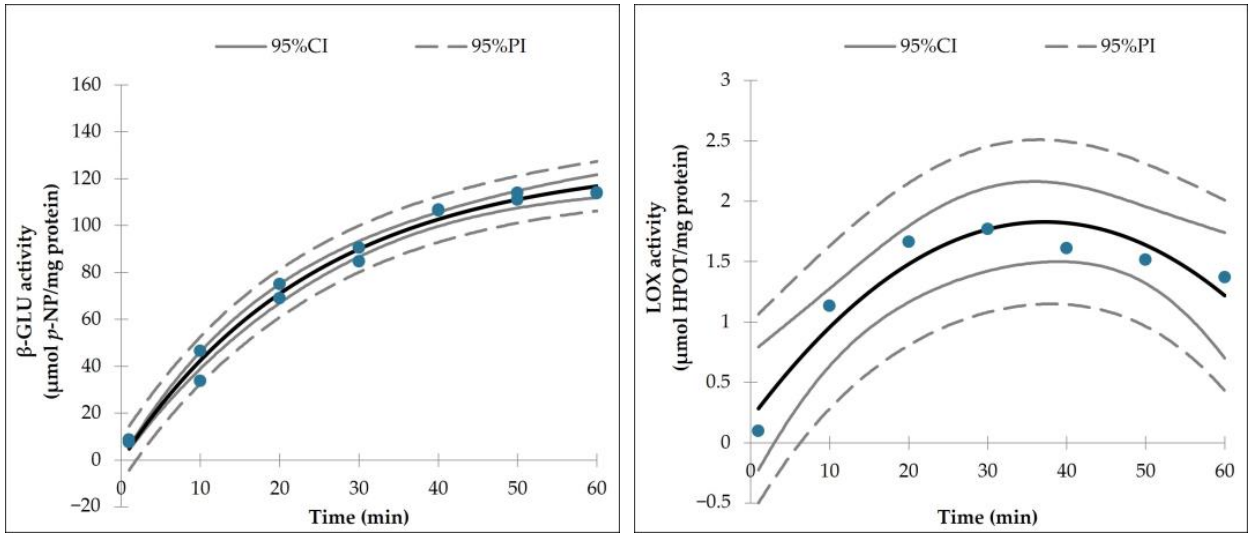

Figure 1. Nonlinear regression models for enzyme activity over time at 25 °C: (**a**) β-GLU—one phase association exponential function; (**b**) LOX—second-order polynomial function. The black line represents the model curve, the gray solid line (95% CI) represents the 95% confidence interval, and the gray dashed line (95% PI) represents the 95% prediction interval. The circles represent plot-level data points.

The concentration of *p*-NP, a product of β-GLU, increased continuously with time. The highest activity occurred in the first 20 min of the reaction, reaching 63% of the final concentration of *p*-NP. In the last 20 min of the reaction, a significant decrease in the activity rate was observed, probably due to exhaustion of the substrate for the reaction. During this period, only 6.5% of product was formed. The best-fitting model for β-GLU activity was determined to be an exponential function of the one-phase association presented by Equation (1):

$$\beta - \text{GLU activity } (\mu\text{mol } p - \text{NP/mg protein}) = 128.064 \times \left(1 - e^{-0.0404 \times \text{Time(min)}}\right) \quad (1)$$

with a coefficient of determination R^2 = 0.989. During the first 20 min of the reaction, LOX activity (Figure 1b) followed a pattern similar to β-GLU. Comparable results were reported by Fauconnier and Marlier [36], who found that almost all of the linolenic fatty acid (more than 96%) from LOX was consumed within the first 20 min of the reaction when the substrate concentrations in the model solutions were lower than 1 mmol/L. The results shown in Figure 1b indicate that after these first 20 min, the increase in the HPOT concentration was minimal during the next 10 min and then decreased until the end of the simulated malaxation process. An explanation for this behavior was found in the spontaneous decomposition of the formed HPOT [37]. The concentration of HPOT in

the reaction mixture depends on the reaction rate of the formation of HPOT versus the reaction rate of its decomposition. The results of the present study show that the HPOT formation and decomposition rates were similar from the 20th to the 30th minute. After that, the decomposition rate increased, which explained the decrease in HPOT concentration. Due to the decreasing concentration at the end of the simulated malaxation process, the activity of LOX did not follow an exponential curve like β-GLU, but could be described by the second-order polynomial function of Equation (2) with a determination coefficient $R^2 = 0.907$.

$$\text{LOX activity } (\mu\text{mol HPOT/mg protein}) = 0.1975 + 0.0876 \times \text{Time(min)} - 0.0012 \times \text{Time(min)}^2 \quad (2)$$

3.2. Influence of Thermal Treatment on Enzyme Activity

As mentioned earlier, temperature is one of the most important factors affecting the activity of endogenous enzymes, along with the duration of the malaxation process [4]. Higher temperatures during the malaxation process favor the phenolic content of the produced VOO due to increased β-GLU activity and inactivation of other endogenous enzymes such as polyphenol oxidase and peroxidase, which have a negative effect on polyphenols [38]. However, increasing the temperature during malaxation may have a negative effect on the volatiles of VOO due to the inactivation of LOX and the formation of volatiles that may impart an unpleasant odor to the oil [35]. Therefore, lower malaxation temperatures (<30 °C) are recommended to obtain good VOO quality [6]. It has already been reported that the use of heat exchangers to rapidly heat or cool the paste before the malaxation phase can have an impact on the nutritional value and sensory quality of the oil [18,39]. To investigate how thermal pretreatment affects the activity of the enzymes, β-GLU and LOX and their substrates were tempered to temperatures between 15 and 40 °C. Activity was measured after 1 min of reaction at the indicated temperature and again after the reaction mixtures were allowed to react at 25 °C for 30 min, simulating the conditions during the malaxation process. The results are shown in Table 1.

Table 1. Enzyme activity of thermally treated (TT) β-glucosidase (β-GLU) and lipoxygenase (LOX) and after the additional incubation of the model systems at 25 °C for 30 min.

Source of Variation	β-GLU Activity (μmol *p*-NP/mg Protein)		LOX Activity (μmol HPOT/mg Protein)	
	TT Treatment	TT Treatment and Incubation	TT Treatment	TT Treatment and Incubation
Temperature (°C)	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.002$
15	0.00 ± 0.00 d	66.11 ± 1.34 d	0.10 ± 0.01 d	1.13 ± 0.02 a
20	0.00 ± 0.00 d	81.02 ± 2.12 bc	0.08 ± 0.02 d	1.08 ± 0.02 a
25	0.43 ± 0.60 cd	74.61 ± 0.52 c	0.14 ± 0.01 d	1.06 ± 0.01 ab
30	2.54 ± 0.56 bc	89.09 ± 2.17 ab	0.26 ± 0.00 c	1.11 ± 0.00 a
35	13.00 ± 2.60 ab	86.12 ± 3.51 abc	0.33 ± 0.01 b	1.12 ± 0.04 a
40	23.22 ± 1.35 a	91.58 ± 0.56 a	0.52 ± 0.02 a	0.98 ± 0.00 b

Values with different letters in each column are statistically different ($p \leq 0.05$) according to Tukey's multiple comparison tests.

The β-GLU showed no activity after 1 min of reaction at temperatures below 20 °C, but at higher temperatures, the activity increased exponentially. Romero-Segura et al. [30] reported a similar trend for purified olive β-GLU with the highest activity at 45 °C. After an additional incubation of the β-GLU model system, there was a significant increase in the concentration of *p*-NP in all samples. However, temperature had a significant effect on the final *p*-NP concentration ($p < 0.001$). The Tukey test showed that the activity of β-GLU pretreated at 15 °C and then incubated at 25 °C for 30 min was significantly lower than in the other samples during the simulated malaxation process. As expected, the highest activity was measured in the reaction mixtures pretreated at the highest temperature, but

these samples were not different from those treated at 30 and 35 °C. Therefore, taking into account the lower energy consumption when heating the medium to 30 °C compared to 40 °C, thermal treatment at 30 °C before malaxation can be considered as optimal for β-GLU activity.

Regarding the effect of temperature on the activity of LOX, it was previously reported that 30 °C is the optimal temperature for LOX. Moreover, the results showed an inhibition of HPOT synthesis at higher temperatures. At 50 °C, LOX was inactivated up to 70% [4]. The results presented in Table 1 show that the activity of LOX increased with temperature and the increase was particularly pronounced at higher temperatures (≥30 °C). The highest activity of LOX was measured at 40 °C. The discrepancy with previous reports is due to the fact that soybean LOX was used in this experiment, which has a slightly different thermal stability than olive LOX [40]. Simulating the malaxation process by incubating the pretreated solutions at 25 °C for 30 min significantly increased the HPOT concentration in the model solutions, and the temperature of the thermal treatment itself had a significant effect on the final HPOT concentration ($p \leq 0.05$). Samples that were tempered at 40 °C had a significantly lower HPOT concentration after the additional incubation than other samples (Tukey's test). This could be explained by the fact that the enzyme activity measured after 1 min at 40 °C according to Tukey's test was significantly higher compared with the other samples, and we can assume that the accelerated production of HPOT continued during the first few minutes of the simulated malaxation process simply because of the higher enzyme activity at the elevated temperature, as the mixture had to cool down to 25 °C for a longer time. The maximum HPOT concentration in these samples was probably reached sometime during the 30 min period, and because of the spontaneous decomposition of the HPOT mentioned earlier, we probably did not measure their concentration at its peak.

3.3. Influence of Ultrasound on Enzyme Activity

The effect of ultrasound treatment (US) itself and its use as a pretreatment of the malaxation process on the activity of β-GLU and LOX is shown in Table 2. Results are presented as the least squares means (LS means) ± standard error by US parameters (time and power). According to preliminary experiments, 12 min was chosen as the longest US treatment because the solutions treated with the max power reached a final temperature of 40 °C during this time (maximum temperature in the TT experiment). Both β-GLU and LOX activity were positively affected by the duration of US treatment. This was expected, and the results are consistent with those in Figure 1. The power of US treatment also had a significant effect on the activity of both enzymes ($p \leq 0.05$), but in the completely opposite manner. While β-GLU responded best to the highest power applied (640 W), lower ultrasound intensity was better for LOX. A power-dependent increase in β-GLU activity [41] and a decrease in LOX activity [42] were also previously reported. The interaction of time and power during US treatment significantly affected the activity of β-GLU ($p < 0.001$). The highest activity of β-GLU was obtained after 12 min of treatment at 640 W. The concentration of *p*-NP in the solution after this treatment was 47% higher than in samples treated with 50% less power during the same time. For LOX, the interaction of time and power was not significant, but the highest activity was obtained after 12 min of treatment at 128 and 320 W of power. The difference in the behavior of these two enzymes was due to their thermal stability, as mentioned earlier. Although US treatment is considered as a non-thermal technique in food processing, it does generate heat that is proportional to the time and power applied [23].

The concentration of *p*-NP formed increased significantly upon additional incubation of the β-GLU model solution at 25 °C for 30 min. This indicates that US treatment did not cause inactivation of β-GLU. On the contrary, similar activity values were obtained for all samples, which could mean that the US treatment increased the activity of the enzyme and its curve reached a plateau somewhere during this 30 min incubation. Statistical analysis showed that the time of pretreatment had no effect on the concentration of *p*-NP at the end of the experiment ($p > 0.05$), while the power and the interaction of time and power were

found to be significant ($p \leq 0.05$). LOX showed a similar behavior, except that both factors studied, the time and power, and their interaction, were found to be significant ($p \leq 0.05$). It has also been shown that a lower power during the US treatment was a better choice to obtain a higher concentration of HPOT after the additional incubation of the model solutions. Interestingly, the 12 min US treatment resulted in a higher HPOT concentration right after the treatment than at the end of the experiment, regardless of the applied power. This could probably be explained by the spontaneous degradation of HPOT. We observed that the HPOT concentration decreased after 30 min of incubation at 25 °C (Figure 1b). Due to the heat generated during the 12 min US treatment and the increase in activity of LOX with temperature (Table 1), it can be assumed that the HPOT concentration reached its maximum before the end of the 30 min incubation process and we measured it when it started to decrease.

Table 2. Enzyme activity of β-glucosidase (β-GLU) and lipoxygenase (LOX) after ultrasound (US) treatment and after additional incubation of the model systems at 25 °C for 30 min as an effect of time and power, expressed as the least square mean ± standard error.

Source of Variation	β-GLU Activity (μmol *p*-NP/mg Protein)		LOX Activity (μmol HPOT/mg Protein)	
	US Treatment	US Treatment and Incubation	US Treatment	US Treatment and Incubation
Time (min) *	$p < 0.001$	$p = 0.319$	$p < 0.001$	$p = 0.006$
1	19.36 ± 0.62 c	99.09 ± 1.31 a	0.24 ± 0.01 c	1.40 ± 0.02 ab
5	39.77 ± 0.49 b	101.85 ± 2.34 a	0.80 ± 0.08 b	1.28 ± 0.03 b
12	73.15 ± 2.48 a	99.80 ± 1.52 a	1.78 ± 0.13 a	1.61 ± 0.12 a
Power (W) **	$p < 0.001$	$p = 0.016$	$p = 0.014$	$p < 0.001$
128	24.85 ± 0.67 c	97.16 ± 1.77 b	1.11 ± 0.03 a	1.76 ± 0.03 a
320	42.00 ± 0.41 b	99.88 ± 2.24 ab	0.94 ± 0.13 ab	1.29 ± 0.11 b
640	65.43 ± 2.49 a	103.70 ± 1.14 a	0.77 ± 0.08 b	1.24 ± 0.05 b
Time (min) × Power (W)	$p < 0.001$	$p = 0.033$	$p = 0.086$	$p = 0.005$
1 × 128	8.79 ± 1.22 g	98.05 ± 2.60 ab	0.27 ± 0.01 d	1.84 ± 0.06 a
1 × 320	15.08 ± 1.22 g	101.92 ± 2.78 ab	0.17 ± 0.03 d	1.04 ± 0.03 b
1 × 640	34.20 ± 0.69 f	97.32 ± 0.95 b	0.27 ± 0.01 d	1.33 ± 0.01 ab
5 × 128	14.81 ± 1.44 g	99.64 ± 4.51 ab	1.11 ± 0.06 bc	1.77 ± 0.01 a
5 × 320	42.67 ± 0.00 e	97.62 ± 5.29 ab	0.71 ± 0.04 cd	1.06 ± 0.02 b
5 × 640	61.85 ± 0.30 c	108.30 ± 0.87 a	0.58 ± 0.23 cd	1.02 ± 0.10 b
12 × 128	50.95 ± 0.69 d	93.79 ± 1.08 b	1.95 ± 0.07 a	1.68 ± 0.07 a
12 × 320	68.26 ± 0.22 b	100.12 ± 3.09 ab	1.93 ± 0.38 a	1.77 ± 0.34 a
12 × 640	100.26 ± 7.42 a	105.5 ± 3.17 ab	1.46 ± 0.10 ab	1.37 ± 0.12 ab

Values with different letters in each column subsection are statistically different ($p \leq 0.05$) according to Tukey's multiple comparison tests; * Results represent LS mean ± standard error of all powers applied at a given time; ** Results represent LS mean ± standard error of all tree treatment times at the indicated power of ultrasound.

3.4. Effect of Heating Source on Enzyme Activity

US can affect enzyme activity by increasing the temperature of the reaction medium, but also, more directly, by affecting the structure of the enzymes [10]. The aim of this part of the experiment was to distinguish between these two effects of US treatment. The activity of the enzymes is presented as a function of temperature, for TT as a function of the temperature used in the experiment and for US as the final temperature reached during the treatment.

Figure 2 shows the activity of β-GLU after TT and US (Figure 2a,b), and the activity after additional incubation of the treated solutions at 25 °C for 30 min (Figure 2c,d). The behavior of β-GLU was described and predicted by exponential curves for all the treatments studied; the parameters of the models are listed in Table 3 along with their coefficients of determination. Comparing the results presented in Figure 2a,b, a significantly higher

enzyme activity was obtained with US than with TT at the same temperatures. One of the reasons for this is certainly the time required to reach the desired temperature with US (up to 12 min to reach 40 °C), whereas the reaction time of TT was always 1 min. However, the activity of β-GLU was significantly higher, even when comparing the 1 min treatments. At the lowest power (128 W), the temperature increase was minimal, only 0.2 °C, but the activity of the enzyme was 10-fold higher. This increase was even greater at higher US power. At 640 W US for 1 min, the temperature increased by 2 °C, but the activity of β-GLU was almost 13 times higher than the activity after TT at 27 °C (calculated from the model for TT shown in Table 3).

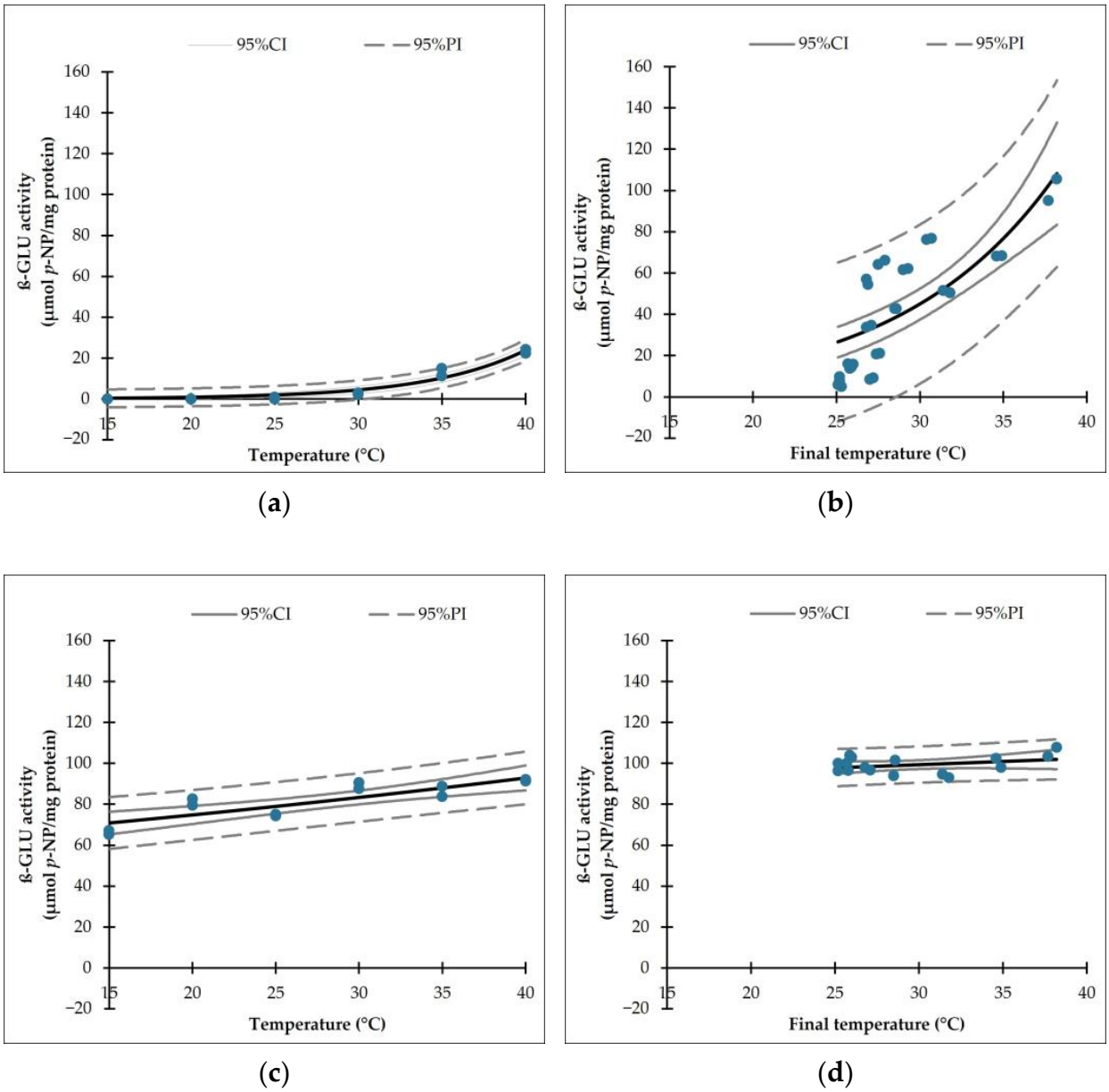

Figure 2. Nonlinear exponential curve function for β-glucosidase (β-GLU) activity as a function of temperature for: (**a**) thermal treatment (TT); (**b**) ultrasonic treatment (US); (**c**) TT and incubation for 30 min at 25 °C; (**d**) US and incubation for 30 min at 25 °C. The black line represents the model curve, the gray solid line (95% CI) represents the 95% confidence interval, and the gray dashed line (95% PI) represents the 95% prediction interval. The circles represent plot-level data points.

Table 3. Parameter estimates of the exponential curve for β-glucosidase (β-GLU) activity as a function of temperature.

	β-GLU Activity (μmol *p*-NP/mg Protein)			
	TT Treatment	**US Treatment**	**TT Treatment and Incubation**	**US Treatment and Incubation**
	Figure 2a	**Figure 2b**	**Figure 2c**	**Figure 2d**
Model *	β-GLU activity = a × $e^{(k \times T)}$			
a	0.0277	0.8650	60.1699	90.6092
k	0.1690	0.1258	0.0108	0.0031
R^2	0.963	0.929	0.727	0.113

* T in the model represents the temperature (°C).

The increased activity after US compared to TT at all temperatures was also evident after additional incubation of the model solutions (Figure 2c,d). Fan et al. [41] investigated the effects of US on the properties and activity of β-GLU. Similar to our results, an increase in β-GLU activity was observed with US, which was directly correlated with changes in the structure of the enzyme. These changes also affected the active site of the enzyme, making it more available to the substrate and accelerating the reaction and shortening the time for hydrolysis of the phenolic compounds.

Selected exponential models have been shown to fit the TT and US treatments well and have very high coefficients of determination ($R^2 > 0.9$). On the other hand, this type of model is not the best fit for the results of β-GLU activity after additional incubation, especially for US ($R^2 = 0.113$), because there was not much difference in the enzyme activity between the analyzed samples. The reason for this, as explained in the previous subsection, was the increased activity of the enzyme, which resulted in all of the substrate being consumed during the additional incubation. This could mean that the US pretreatment of the olive paste could shorten the malaxation time to obtain VOO with the same concentration of phenolic compounds. This reduction in malaxation time should be further investigated, first by monitoring enzyme activity during additional incubation on model solutions, but then also on real olive samples, to gain a comprehensive perspective.

The activity of LOX (Figure 3) was affected by US in a similar manner to the activity of β-GLU. The concentration of HPOT formed during 1 min of TT at 25 °C doubled with US without a significant increase in the final temperature of the model system. Interestingly, the highest HPOT concentration in this whole experiment was reached after 12 min of US at lower applied powers and without additional incubation (Figure 3b). This suggests that US increases the activity of LOX to the extent that no additional incubation is required, which could mean that the malaxation process is not essential for VOO production after US pretreatment of the olive paste. However, such conclusions can only be drawn after additional experiments on the influence of US parameters on other enzymes involved in the LOX pathway and after extensive experiments on real olive samples, which would include the determination of the enzyme activity, volatiles, and sensory properties of such oils.

The activity of LOX as a function of temperature, as shown in Figure 3, was described for all treatments with a second-order polynomial function. The parameters of the models are shown in Table 4. This model was the best choice for TT and US prior to additional incubation, and these two models had a good coefficient of determination (0.980 and 0.707, respectively). However, predicting the activity of LOX after incubation proved to be difficult. The coefficient of determination was 0.352 for TT after incubation and only 0.006 for US and incubation. Similar to the activity of β-GLU, the plateau of HPOT concentration was reached sometime during the 30 min incubation at 25 °C of the reaction mixture, but beyond that, spontaneous decomposition of HPOT was also present. This is why the results of the activity of LOX after US and incubation are so inconsistent, and the model we chose, or any of the models we used, did not fit the results.

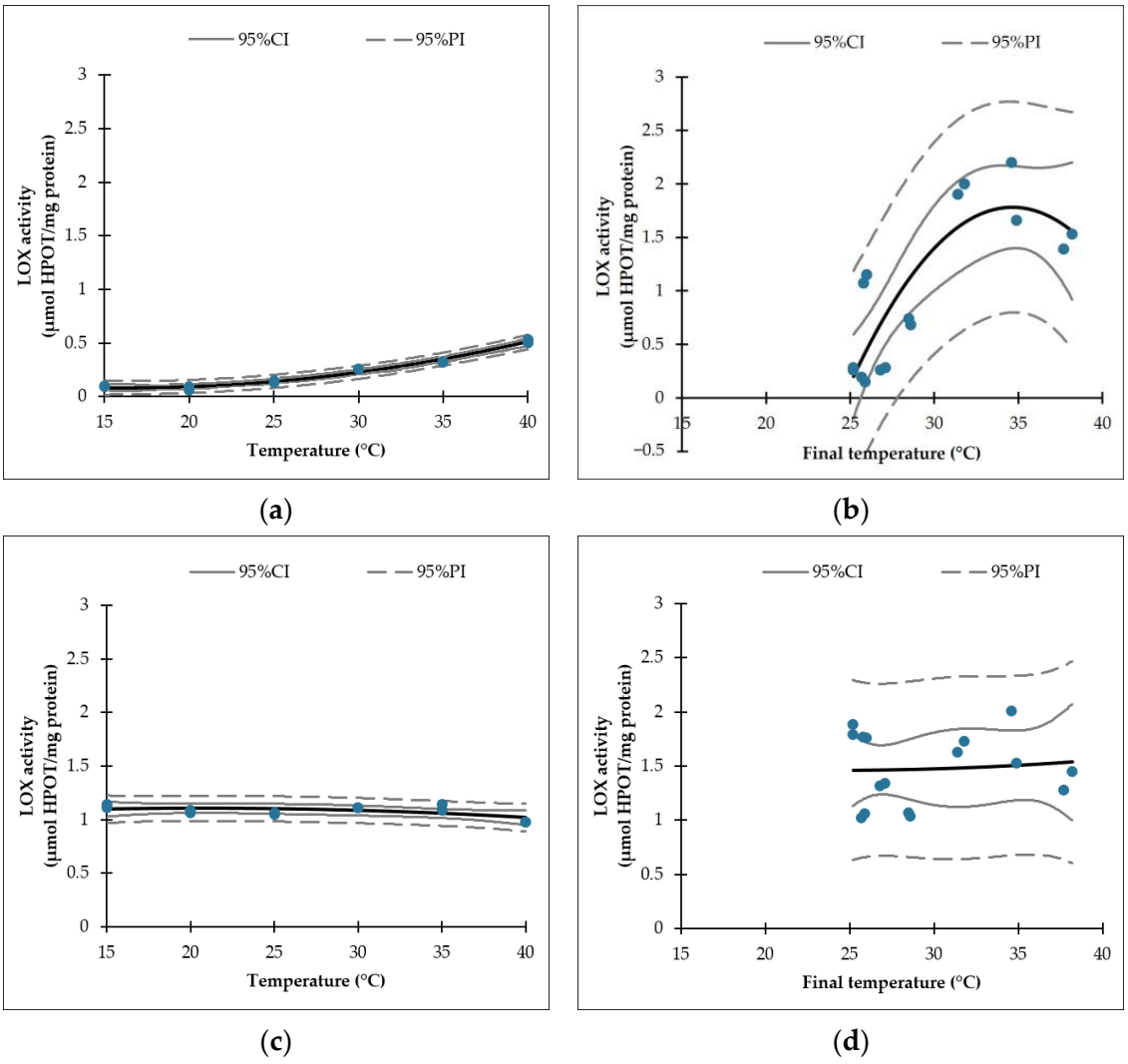

Figure 3. Second-order nonlinear function for LOX activity as a function of temperature for: (**a**) thermal treatment (TT), (**b**) ultrasonic treatment (US), (**c**) TT and incubation for 30 min at 25 °C, and (**d**) US and incubation for 30 min at 25 °C. The black line represents the model curve, the gray solid line (95% CI) represents the 95% confidence interval, and the gray dashed line (95% PI) represents the 95% prediction interval. The circles represent the plot-level data points.

Table 4. Parameter estimates of the exponential curve for lipoxygenase (LOX) activity as a function of temperature.

	LOX Activity (µmol HPOT/mg Protein)			
	TT Treatment	**US Treatment**	**TT Treatment and Incubation**	**US Treatment and Incubation**
	Figure 3a	**Figure 3b**	**Figure 3c**	**Figure 3d**
Model *	LOX activity = a + b × T + c × T^2			
a	0.2808	−19.4915	1.0045	1.6760
b	−0.0243	1.2277	0.0096	−0.0178
c	0.0008	−0.0177	−0.0002	0.0004
R^2	0.980	0.707	0.352	0.006

* T in the model represents the temperature (°C).

4. Conclusions

New technologies are being studied to improve the production of high-quality virgin olive oil. Understanding their effects on the activity of the enzymes responsible for the quality of the produced oil, β-GLU and LOX, is essential for proper control of the production process. The thermal techniques investigated in this study, thermal treatment and ultrasound, significantly affected the activity of endogenous enzymes. Higher temperatures used for thermal treatment increased the activity of both enzymes studied. Moreover, the activity of both enzymes correlated positively with the duration of ultrasound treatment, with β-GLU responding better to higher power, whereas LOX responded much better to lower power. The results clearly indicate that the temperature released during ultrasound treatment is only one of the reasons for the increase in enzyme activity, and that other reasons could be due to the structural changes in the enzymes caused by ultrasound.

Stimulation of enzyme activity by thermal treatment, especially by ultrasound, resulted in the acceleration of enzymatic reactions during the additional incubation of reaction mixtures for 30 min at 25 °C, which was performed to simulate the malaxation process. These results suggest that the introduction of either of these techniques in the production of virgin olive oil could shorten or even eliminate the malaxation process. However, these results should be confirmed on real olive samples, where not only the enzyme activity but also the nutritional value, sensory properties, oxidative stability, and extractability of such oils would be determined.

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