

Article

Innovative Approaches to Enhance Activity of Endogenous Olive Enzymes—A Model System Experiment: Part II—Non-Thermal Technique

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Abstract: The aim of this study was to determine the optimal parameters of pulsed electric field treatment to increase the activity of β -glucosidase and lipoxygenase, crucial enzymes that improve the nutritional quality and sensory properties of virgin olive oils. The activity of the enzymes was determined spectrophotometrically immediately after treatment of model systems (commercial enzymes and their substrates) with pulsed electric fields and after simulation of the malaxation process for 30 min at 25 °C. Pulsed electric field treatments were performed with electric field strengths of 2.67 and 13.33 kV/cm at frequencies of 25 and 125 Hz for 1, 2, and 5 min. The results showed a positive correlation of β -glucosidase activity with the electric field strength and time, with the first of the two factors having the greatest influence. Lipoxygenase activity was affected only by the duration of treatment: positively immediately after treatment and negatively after simulation of the malaxation process. Based on these results, pretreatment with a moderate pulsed electric field for 2 min before the malaxation process could be proposed to increase the activity of β -glucosidase and lipoxygenase, e.g., for the production of virgin olive oils with higher concentrations of polyphenols and desirable volatile compounds.

Keywords: pulsed electric field; enzymatic activity; β -glucosidase; lipoxygenase; model system



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1. Introduction

Innovative technologies are an effective tool for solving existing problems in food production. Their application can increase value and sustainability in the food industry. Some of the technologies, such as microwave treatment, ultrasound, high-pressure processing, pulsed electric fields, and supercritical fluid extraction, have found their industrial application in the production of certain foods, mainly for the purpose of preservation and extraction [1]. Numerous new opportunities for the use of these innovative technologies and their optimization continue to be the focus of recent food technology research [2–5].

In recent years, the possibility of applying a pulsed electric field (PEF) in food production has attracted great scientific interest, and virgin olive oil (VOO) production is no exception. The introduction of a PEF can contribute to an optimal conditioning of the olive paste in the phases of crushing and malaxation, which is very important for oil extractability. The objective of olive fruit crushing is to break the oily cells. About 76% of the oil in olive fruit is found in the vacuoles of the cells of the mesocarp and about 24% in the cytoplasm, which is distributed in small droplets bound to colloids [6]. The oil from vacuoles can be obtained by crushing, but the oil distributed in the cytoplasm is quite difficult to extract and is generally lost with the pomace or vegetation water. Moreover, despite modern mills, it is possible that up to 20–25% of the undestroyed cells of the mesocarp are present in the

pomace after oil extraction [7]. To improve the extractability of the oil, the olive paste is subjected to malaxation in conventional production. Malaxation is carried out for 30 to 45 min at lower temperatures (<30 °C) to allow coalescence of the small oil droplets and to ensure optimal activity of the endogenous olive enzymes, resulting in a favorable chemical composition of the oil [8]. However, the current technology of VOO production hardly solves the problem of distribution of hydrophilic bioactive compounds (such as phenols) between the oil and water phases. The result is the production of VOO with a relatively low concentration of such compounds, i.e., with low nutritional value and insufficiently pronounced sensory properties.

In most of the PEF studies on VOO production, PEF treatment was investigated as a pretreatment of malaxation with the aim of reducing the malaxation time and lowering the temperature to which the olive paste was exposed. The first one was performed by Guderjan et al. [9], who found an increase in the oil extraction yield of 6.5–7.4% depending on the conditions applied. Other authors also noted an increase in processing yield ranging from 13.3% to 18% [6,10,11]. In addition, the results of some previous studies have shown that PEF treatment significantly improves the transition of phenolic compounds into the oil phase without negatively affecting oil quality parameters and tocopherol content. As for the sensory quality, the sensory characteristics of PEF–VOO either slightly improved or did not change, and most importantly, neither off-flavors nor defects were detected in the sensory evaluation [10–13]. However, it seems that the application of the PEF process does not always have the same effects on the properties of VOO. In contrast to the aforementioned studies, Abenoza et al. [6] found a decrease in total phenols and higher peroxide levels in Arbequina VOO produced with PEF pretreatment. These oils also showed a decrease in chlorophylls and carotenoids. Regarding the sensory profile, PEF did not cause any deficiencies. However, it increased fruitiness but also decreased bitterness and pungency.

Several mechanisms have been proposed for the effect of PEF on increasing the extraction yield and changing the chemical composition of VOO. PEF is based on the use of short electric pulses that lead to the electroporation of cellular structures. The short-term exposure to high-voltage electric pulses causes porosity of the cell wall and destabilizes its lipid–protein interlayer, making the cell permeable to lower-molecular-weight molecules that cause swelling and subsequent destruction of the cell structure. During the PEF treatment, these short electric pulses (1–100 μ s) with an electric field of 5 to 80 kV/cm are applied to a food product placed in a chamber between two electrodes. Except for causing the electroporation of the cell structures, the electric field facilitates the coalescence process, contributing to a more effective separation of oil and water [14]. Due to the increased porosity of the cell membrane, intracellular components escape from the cell, and mass transfer increases. Enzymes and substrates previously located in different cellular compartments come into contact, and numerous biochemical reactions crucial for the formation of valuable bioactive VOO compounds begin [12]. In addition, PEF leads to changes in the secondary and tertiary structure of enzymes, thus altering their activity. PEF technology is widely used in the food industry for inactivation of endogenous enzymes, and the effects on enzyme activity depend on PEF conditions but also on the enzyme itself. High-voltage pulses (10–50 kV/cm) are used to inactivate enzymes [15]. However, some studies have shown that the use of a lower voltage reduces inactivation or even increases the activity of endogenous enzymes. The results of previous studies indicate that among the different types of enzymes responsible for the nutritional and sensory quality of VOO, peroxidase, polyphenol oxidase, and hydroperoxide lyase are susceptible to PEF treatment, while lipoxygenase and β -glucosidase show relatively high PEF resistance [16,17].

All of the above processes occur simultaneously in real olive systems, and it is almost impossible to distinguish their influence on oil quality individually. However, it is crucial to understand each process individually in order to optimize PEF parameters, as they can often have opposite effects on the quality of the final product. Since the electroporation of cells caused by PEF treatment is well described, the real challenge is to choose the right PEF parameters to modify enzyme activity and ultimately improve the nutritional and sensory

quality of VOO. Therefore, in the present study, we investigated the effect of low-voltage PEF treatment on the activity of the pure endogenous olive enzymes β -glucosidase and lipoxygenase. We used model systems to determine the direct effects of this non-thermal technique on the activity of these two enzymes, with the aim of defining the optimal PEF parameters to enhance the activity of β -glucosidase and lipoxygenase.

2. Materials and Methods

2.1. Materials

The commercial enzymes β -glucosidase (β -GLU) from almond (*Prunus dulcis*) and lipoxygenase (LOX) from soybean (*Glicine max*), 4-nitrophenyl- β -D-glucopyranoside (*p*-NPG), linoleic acid (LA), *p*-nitrophenol (*p*-NP), and Coomassie Brilliant Blue G-250 were all purchased from Sigma Aldrich (St. Louis, MO, USA). 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 dehydrate were purchased from Kemika (Zagreb, Croatia). Acetic and hydrochloric acids were purchased from Lach-Ner (Neratovice, Czech Republic), while *o*-phosphoric acid was purchased from Carlo Erba Reagents GmbH (Emmendingen, Germany).

2.2. Model Systems

The model solution for the determination of β -glucosidase (β -GLU) activity contained 15 mL of *p*-NPG solution (15 mM) in 50 mM of sodium acetate buffer (pH 5.5) and 50 μ L of enzyme solution (5 mg/mL) diluted in the same buffer. β -GLU activity was defined as the amount of product (*p*-nitrophenol-*p*-NP) released by 1 mg of enzyme measured at 405 nm.

To determine the activity of lipoxygenase (LOX), defined as the amount of hydroperoxides of linoleic acid-hydroperoxyl-octadecadienoic acid (HPOD) released by 1 mg of enzyme, a model solution was prepared with 15 mL of 0.1 M phosphate buffer (pH 6.0), 50 μ L of enzyme solution ($\gamma = 5$ mg/mL) in the same buffer, and 300 μ L of LA (10 mM). LA was prepared in the same way as linolenic acid (ALA) in the first part of this study [18]. LOX activity was measured at 234 nm.

2.3. Quantification of Proteins

Protein concentration was determined using the Bradford method [19] described in our previous work [18].

2.4. Simulation of Malaxation Process

To simulate the behavior of the enzymes in the olive paste during the malaxation process, the model systems 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 at regular intervals (every 10 min during the 60 min) following the methods for determination of enzyme activity described in our previous research [18].

2.5. PEF Treatment and Determination of Enzyme Activity

The high-intensity pulsed electric field device used in this study was the HVG60/1 PEF (Impel d.o.o., Zagreb, Croatia). PEF treatments were performed by adding the reaction mixture of enzyme and substrate (prepared as described in Section 2.2), both tempered to 25 °C, to the treatment chamber. The treatment chamber consisted of two stainless steel electrodes with a diameter of 50 mm and a distance of 7.5 mm (Figure 1). Treatments were performed with electric field strengths of 2.67 and 13.33 kV/cm at frequencies of 25 and 125 Hz. The duration of the pulses was 2 μ s, and the total treatment time was 1, 2, and 5 min.

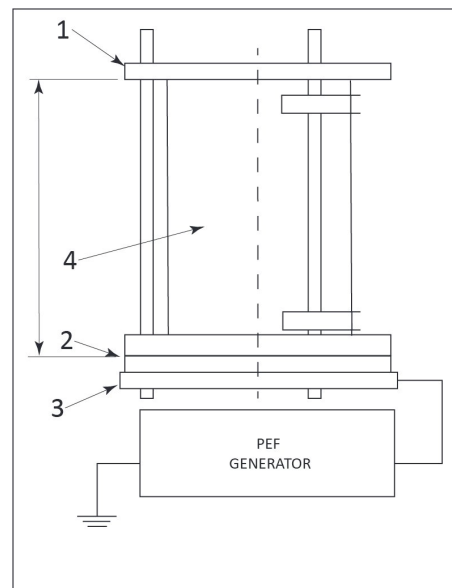


Figure 1. Experimental setup for PEF treatment (1—high voltage electrode (movable); 2—ground electrode; 3—isolated bottom base; 4—reactor chamber).

The enzyme was determined immediately after PEF treatment and after additional incubation of the treated reaction mixture at 25 °C for 30 min according to the methods described in our research on thermal techniques [18].

2.6. Statistical Analysis

The activity of β -GLU and LOX during a 60 min incubation at 25 °C was modeled using nonlinear regression by evaluating exponential equations (one phase association and exponential growth), second-order equations, and growth equations (Gompertz and beta growth). The models with the best fit were selected and used to predict the enzyme activity. The effect of the PEF treatment was determined based on a full factorial design with 3 independent factors: (i) electric field strength (2.67 and 13.33 kV/cm; coded as -1 and $+1$), (ii) frequency (25 and 125 Hz; coded as -1 and $+1$), and time (1, 2, and 5 min; coded as -1 , 0, and $+1$), with a total of 12 experiments. Three replicates were performed for each factor combination, and the results were used to calculate the average value and standard deviation. The obtained results were analyzed using one-way analysis of variance (ANOVA) followed by a pairwise post hoc Tukey's comparison test used to determine differences between means. For all relevant statistical analysis methods, the confidence level threshold was set at 95%.

Response Surface Methodology (RSM) was used to determine the influence of independent factors on β -GLU and LOX activity immediately after PEF treatment and after an additional 30 min incubation at 25 °C. The results for the observed dependent variables based on full factorial design were used to build regression models that included linear and interaction coefficients because none of the quadratic terms were considered significant. Models were evaluated based on lack of fit and coefficients of determination (R^2). Factorial ANOVA was used to determine the influence of PEF parameters on the obtained results. Nonlinear modeling and one-way ANOVA were performed in XLSTAT 2023 software (Lumivero, Denver, CO, USA), while Design Expert 10 (Stat-Ease, Inc., Minneapolis, MN, USA) was used for RSM.

3. Results and Discussion

3.1. Simulation of Malaxation Process

Olives contain a complex system of endogenous enzymes, the amount of which depends on the variety and ripeness of the fruit. The endogenous enzymes play a crucial role in both the quantity and quality of the VOO produced. During the crushing of the

fruit in the VOO production, the enzymes come into contact with their substrates, but the activity of the enzymes is determined by the processing parameters (time and temperature) during the malaxation of the paste [20]. The activity of β -GLU and LOX at 25 °C over a period of 60 min is presented in Figure 2a,b, respectively.

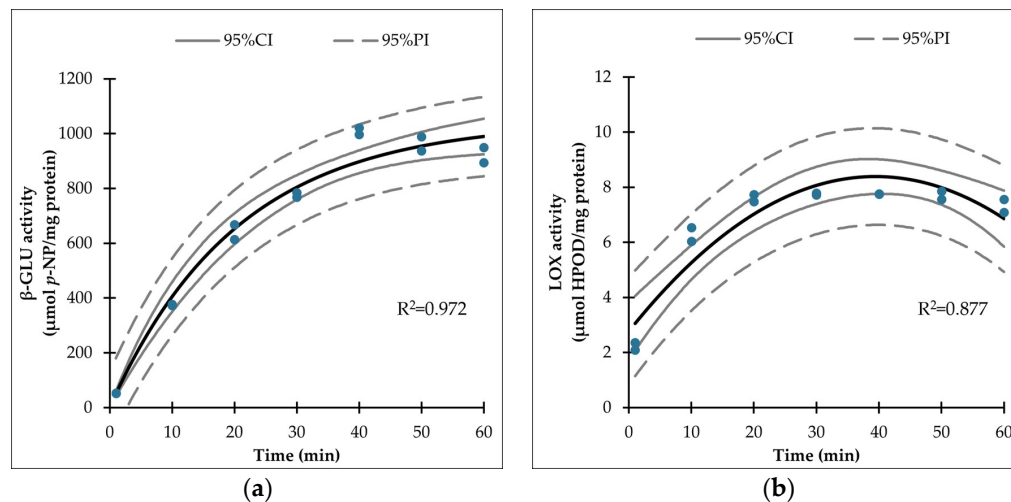


Figure 2. Nonlinear regression models for the activity of enzymes over time at 25 °C: (a) β -GLU—one phase association exponential function; (b) LOX—polynomial second-order function. The black line represents the model curve, the gray full line represents the 95% confidence interval, and the gray dashed line represents the 95% prediction interval. The circles represent plot-level data points.

The β -GLU activity can best be described by the exponential function of the one-phase association presented in Equation (1):

$$\beta - \text{GLU activity } (\mu\text{mol } p - \text{NP/mg protein}) = 1044.78 \times \left(1 - e^{-0.04893 \times \text{Time}(\text{min})}\right) \quad (1)$$

with a coefficient of determination $R^2 = 0.972$. The production rate of p -NP was highest in the first 20 min of the reaction, after which the reaction gradually slowed and the curve approached the plateau, which in this case was 1044.78 $\mu\text{mol } p\text{-NP/mg protein}$. The dynamics of product formation were similar to the results we obtained in the first part of this research [18]. However, in the present research, the plateau toward which the curve moves was 8 times higher, and the association rate constant was 20% higher. These results were expected due to the 4-times-higher substrate-to-enzyme ratio (900 $\mu\text{mol } p\text{-NPG/mg protein}$) in this study compared to that in the aforementioned study (225 $\mu\text{mol } p\text{-NPG/mg protein}$).

The activity of LOX, i.e., the HPOD concentration, during the simulation of the malaxation process is best described by the second-order polynomial function (Figure 2b) given in Equation (2):

$$\text{LOX activity } (\mu\text{mol HPOD/mg protein}) = 2.7827 + 0.2845 \times \text{Time (min)} - 0.0036 \times \text{Time (min)}^2 \quad (2)$$

with an R^2 of 0.877. The second-order polynomial function was also the best fit for the LOX activity during the simulated malaxation process in our previous research [1]. In the aforementioned study, we used linolenic acid (ALA) as the LOX substrate, but the dynamics of formation and decomposition of the corresponding hydroperoxide (hydroperoxyl octadecatrienoic acid (HPOT)) were similar. In both experiments, the highest rate of hydroperoxide formation occurred in the first 20 min of the reaction, after which the reaction slowed, reaching the maximum concentration after 39.5 min in the present study and after 36.5 min in the previous study [18]. After reaching the maximum, a decrease in the hydroperoxide concentration was observed in both experiments. This indicates that the hydroperoxides, both HPOD and HPOT, were stable for about 20 min, after which

their accelerated degradation began. However, according to the obtained models, the maximum HPOT concentration in the present study was 4.7 times higher than in the last study (8.404 μmol HPOD/mg protein compared to 1.796 μmol HPOT/mg protein). The significantly higher concentrations of HPOD in the present study can be explained by the 6-fold higher substrate-to-enzyme ratio used for the model system. In addition, it has been previously published that the type 1 LOX, which is both soybean LOX and olive LOX, shows a slight preference for LA over ALA [21,22].

3.2. Influence of PEF Treatment on Enzyme Activity

The PEF as a non-thermal technology is being widely studied for its application in food processing. Research has shown that PEF affects cell structure, more specifically the cell membrane, via electroporation without extensive heating [23]. This phenomenon is one of the reasons for the incorporation of PEF in VOO production. It is being investigated as a possible tool to assist the malaxation process in order to improve the oil extraction. In addition, a PEF has also been shown to affect the quality of the oil by triggering the release of micro-components and endogenous enzymes through pores of different sizes and shapes formed by high-voltage impulses that facilitate their contact [12]. It has also been reported that a PEF can modulate the activity of enzymes by affecting their structure. Although most studies have investigated the inactivation of endogenous enzymes (alkaline phosphatase (ALP), pectin methyl esterase (PME), polygalacturonase (PG), peroxidase (POD), polyphenol oxidase (PPO), LOX, hydroperoxide lyase (HPL), and β -GLU) involved in determining the quality of dairy, fruit, and vegetable products by PEF processing, the increase in activity under different PEF conditions has been reported. Apart from the electric field strength and treatment time, the pulse width, frequency, pulse polarity, and even the shape of the pulse wave affect the degree of enzyme inactivation (or activation) by a PEF [15,24].

In this study, we investigated the effect of PEF technology on the activity of β -GLU and LOX, enzymes that can improve the nutritional quality and sensory properties of VOOs. Luo et al. [16] reported that a lower electric field strength (8 kV/cm) favored the activity of LOX and that more than 90% of the activity was retained after a PEF treatment of 1000 μs . Increasing the electric field strength significantly decreased the activity of LOX. On the other hand, Aguiló-Aguayo et al. [17] reported that β -GLU activity can be significantly increased by PEF treatment, but they used a much higher field strength (35 kV/cm). Therefore, we investigated the effects of lower electric field strengths (2.67 and 13.33 kV/cm) on the activity of the above enzymes. However, we extended the treatment time enormously (up to 5 min) to compensate for the low power of the electric field. The effects of the PEF on the activity of β -GLU and LOX are shown in Table 1.

Table 1. Enzyme activity of β -glucosidase (β -GLU) and lipoxygenase (LOX) after pulsed electric field (PEF) treatment and after additional incubation of the model systems at 25 °C for 30 min as an effect of electric field strength, frequency, and time.

Electric Field Strength (kV/cm)	Frequency (Hz)	Time (min)	Final Temperature (°C)	β -GLU Activity after PEF Treatment (μmol <i>p</i> -NP/mg protein) *	β -GLU Activity after PEF Treatment and Incubation (μmol <i>p</i> -NP/mg protein) *	LOX Activity after PEF Treatment (μmol HPOD/mg protein) *	LOX Activity after PEF Treatment and Incubation (μmol HPOD/mg protein) *
Control		1	25.0 \pm 0.2	2.74 \pm 0.23 i	522.90 \pm 1.63 f	1.87 \pm 0.16 e	8.51 \pm 0.13 b
Control		2	25.0 \pm 0.2	158.03 \pm 0.48 c	809.23 \pm 12.07 de	3.77 \pm 0.17 d	8.62 \pm 0.14 b
Control		5	25.0 \pm 0.2	254.65 \pm 11.78 a	849.27 \pm 18.43 cd	5.68 \pm 0.09 b	8.40 \pm 0.25 b
2.67	25	1	23.7 \pm 0.9	21.86 \pm 1.63 ghi	594.76 \pm 3.96 f	3.73 \pm 0.20 d	10.51 \pm 0.17 a
2.67	125	1	23.7 \pm 0.8	28.56 \pm 6.14 ghi	652.45 \pm 62.70 ef	3.89 \pm 0.09 d	10.57 \pm 0.08 a
2.67	25	2	22.9 \pm 0.8	32.90 \pm 6.06 fghi	544.66 \pm 16.09 f	4.81 \pm 0.10 c	9.73 \pm 0.38 a
2.67	125	2	23.3 \pm 0.4	35.21 \pm 8.86 fgh	546.80 \pm 15.39 f	4.57 \pm 0.12 c	10.68 \pm 0.43 a
2.67	25	5	22.6 \pm 0.2	90.42 \pm 0.23 de	574.00 \pm 37.06 f	6.08 \pm 0.04 ab	9.78 \pm 0.50 a
2.67	125	5	22.6 \pm 0.3	105.09 \pm 11.19 d	599.05 \pm 30.07 f	6.27 \pm 0.13 a	9.81 \pm 0.05 a

Table 1. Cont.

Electric Field Strength (kV/cm)	Frequency (Hz)	Time (min)	Final Temperature (°C)	β -GLU Activity after PEF Treatment ($\mu\text{mol } p\text{-NP/mg protein}$) *	β -GLU Activity after PEF Treatment and Incubation ($\mu\text{mol } p\text{-NP/mg protein}$) *	LOX Activity after PEF Treatment ($\mu\text{mol HPOD/mg protein}$) *	LOX Activity after PEF Treatment and Incubation ($\mu\text{mol HPOD/mg protein}$) *
13.33	25	1	22.2 \pm 1.1	15.55 \pm 3.59 ghi	997.98 \pm 81.87 abc	3.34 \pm 0.29 d	10.35 \pm 0.27 a
13.33	125	1	22.2 \pm 0.8	13.01 \pm 2.39 hi	986.58 \pm 97.40 bcd	3.82 \pm 0.27 d	9.98 \pm 0.13 a
13.33	25	2	21.7 \pm 0.4	46.40 \pm 2.99 fg	1004.75 \pm 16.13 abc	4.73 \pm 0.06 c	9.65 \pm 0.18 a
13.33	125	2	22.4 \pm 0.7	62.24 \pm 9.26 ef	1126.73 \pm 2.79 a	4.66 \pm 0.05 c	10.03 \pm 0.25 a
13.33	25	5	22.1 \pm 0.1	170.20 \pm 0.00 bc	986.37 \pm 13.44 bcd	5.98 \pm 0.03 ab	9.92 \pm 0.33 a
13.33	125	5	22.0 \pm 0.1	193.87 \pm 14.94 b	1044.89 \pm 36.45 ab	5.82 \pm 0.01 ab	9.84 \pm 0.43 a

* PEF treatment significantly affected enzyme activity according to one-way ANOVA ($p \leq 0.05$); values with different letters in each column are statistically different ($p \leq 0.05$) according to Tukey's multiple comparison test.

3.2.1. β -Glucosidase

The activity of the enzyme was measured immediately after PEF treatment and after additional incubation of the model solutions for 30 min at 25 °C to simulate the malaxation process (Table 1). The results for β -GLU showed that a 1 min treatment did not significantly alter activity regardless of the field strength or frequency applied. In fact, the concentration of p -NP in samples treated for 1 min was not significantly different from the control after 1 min of reaction time, as shown by Tukey's multiple comparison test. The results for β -GLU activity after 2 min of treatment seemingly increased with increasing electric field strength, although Tukey's test showed that there was no significant difference between these samples. However, these differences were evident and significant at the longest treatment duration of 5 min. A stronger electric field resulted in a significant increase in p -NP concentration, but frequency had no effect. These results are consistent with those of Lu et al. [25], who found a significant increase in β -GLU activity when the electric strength was increased from 5 to 15 kV/cm. However, in samples treated longer (2 and 5 min), the concentration of p -NP was significantly lower than in controls. These results, although unexpected, could be explained by a significant drop in temperature during treatment. Due to the small volume of the reaction mixture (15 mL), the temperature of the model systems dropped as the solution came into contact with the metal electrodes. The temperature dropped from 25 °C (control) to an average of 22.6 °C after 2 min of treatment and to 22.3 °C after 5 min. These results are consistent with our previous study [1], in which we found that β -GLU activity could not be detected in the temperatures below 25 °C after a minute of reaction time.

After additional incubation of the treated and untreated model systems, the concentration of p -NP increased significantly in all samples. However, the samples treated with a low electric field strength (2.67 kV/cm) did not differ significantly from each other regardless of the duration of treatment or the frequency used. The activity of these samples was comparable to that of the 1 min control. The other controls (2 and 5 min) showed significantly higher β -GLU activity compared with the samples treated for the same time with a low electric field strength. This could probably be explained by the lower initial temperature of these samples at the beginning of the incubation period and the consequent lower activity of the enzyme. In contrast, a higher field strength (13.33 kV/cm) significantly increased the β -GLU activity. According to Tukey's test, the activities of the treated samples were significantly higher than those of the corresponding controls. Some previous research has shown that moderate electric field strength can increase the activity of β -GLU by altering the secondary and tertiary structure of the enzyme, thereby creating more active sites or increasing the size of the existing ones [17,25]. The highest β -GLU activity was obtained after a 2 min incubation of the sample treated in an electric field of 13.33 kV/cm at 125 Hz. The measured activity was higher than the plateau defined in the model for β -GLU activity at 25 °C for 60 min (Figure 2a), implying that olive paste pretreated with a PEF could yield oils with a higher phenolic concentration as a direct result of higher β -GLU activity.

The measured activities of the enzyme after PEF treatment and additional incubation of the model solutions were used to construct three-factor regression models. The model parameters of the obtained models are listed in Table 2. The best-fitting model for β -GLU activity after PEF treatment proved to be a model containing linear and factor interaction components with a good coefficient of determination ($R^2 = 0.986$) and a non-significant lack of fit. All three independent factors, as well as the interactions of electric field strength and time and frequency and time, had a significant effect on enzyme activity. Since the regression coefficients are presented in terms of coded factors, their effects on enzyme activity can be compared. Therefore, it can be concluded that the time of PEF treatment affects the β -GLU activity three times more than the field strength or the interaction of field strength and time. Frequency and its interactions have a much smaller effect on β -GLU activity. Figure 3 shows the response surface plots of two factor interactions with a third factor set to the mean. The results clearly show a positive influence of all three factors on enzyme activity, with time and electric field strength being the most important.

Table 2. Model parameters—regression coefficients in terms of coded factors (A—electric field strength, B—frequency, and C—time), p -values, coefficients of determination (R^2), and lack of fit for β -glucosidase (β -GLU) and lipoxygenase (LOX) activity after PEF treatment and after additional incubation for 30 min at 25 °C.

Source of Variation	β -GLU Activity after PEF Treatment	β -GLU Activity after PEF Treatment and Incubation	LOX Activity after PEF Treatment	LOX Activity after PEF Treatment and Incubation
Intercept	77.42	804.36	4.99	10.03
p -Value	<0.001	<0.001	<0.001	0.038
A	20.15	219.63	−0.08	−0.11
p -Value	<0.001	<0.001	0.157	0.155
B	5.06	21.16	0.03	0.08
p -Value	0.009	0.072	0.612	0.293
C	61.90	−3.36	1.11	−0.23
p -Value	<0.001	0.800	<0.001	0.016
AB	1.94	-	-	-
p -Value	0.268			
AC	22.27	-	-	-
p -Value	<0.001			
BC	5.02	-	-	-
p -Value	0.022			
Lack of fit	0.818	0.117	0.001	0.122
R^2	0.986	0.952	0.933	0.338
R^2 adjusted	0.981	0.944	0.906	0.239

The activity of β -GLU after additional incubation can also be described by a linear three-factor model without factor interactions. The model parameters listed in Table 2 show that, of the three parameters studied, only electric field strength had a significant effect on the final concentration of p -NP. These results are consistent with the results of Tukey's test (Table 1) and can be explained by the fact that conformational changes on the enzyme caused by a stronger electric field (13.33 kV/cm) increased the activity of the enzyme. The maximum product concentration in these samples was reached sometime during the 30 min incubation period, making the time of the PEF treatment insignificant. The effect of electric field strength, as the only significant factor, on β -GLU activity after additional incubation of the model solutions is shown in Figure 4.

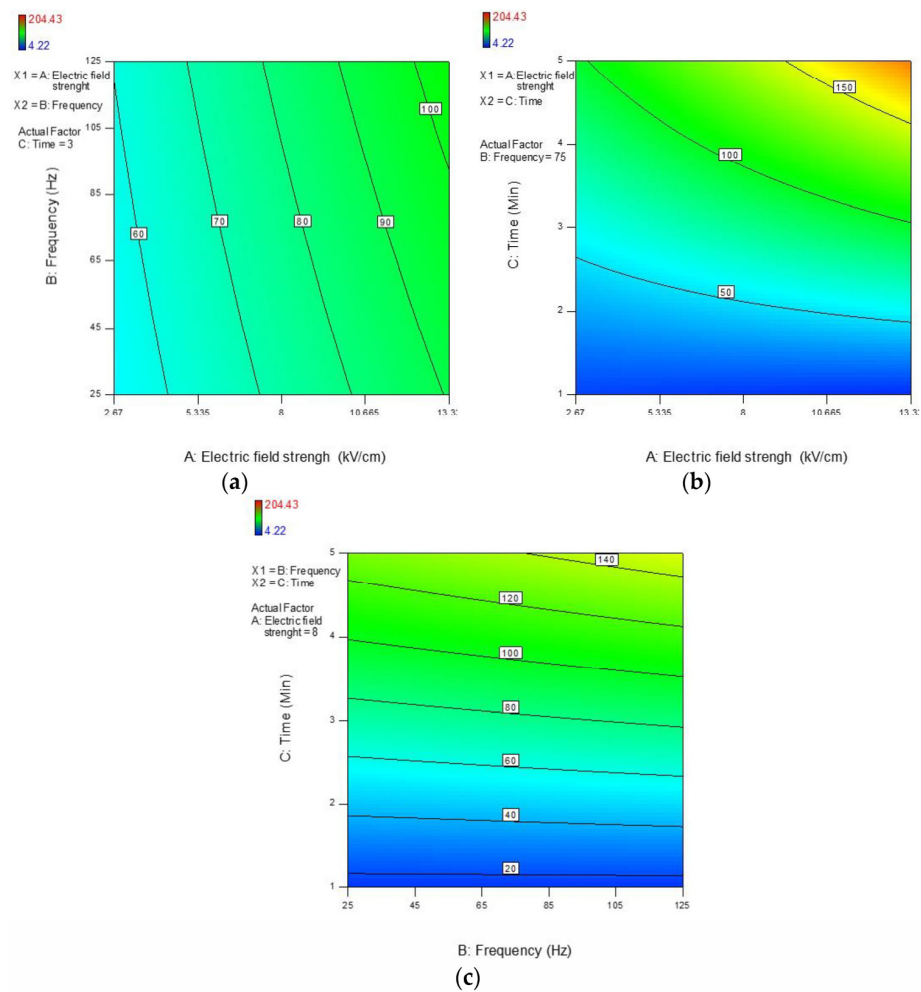


Figure 3. Response surface plots for β -glucosidase (β -GLU) activity after PEF treatment as affected by (a) electric field strength and frequency with the time set to 3 min, (b) electric field strength and time with the frequency set to 75 Hz, and (c) frequency and time with the electric field strength set to 8 kV/cm.

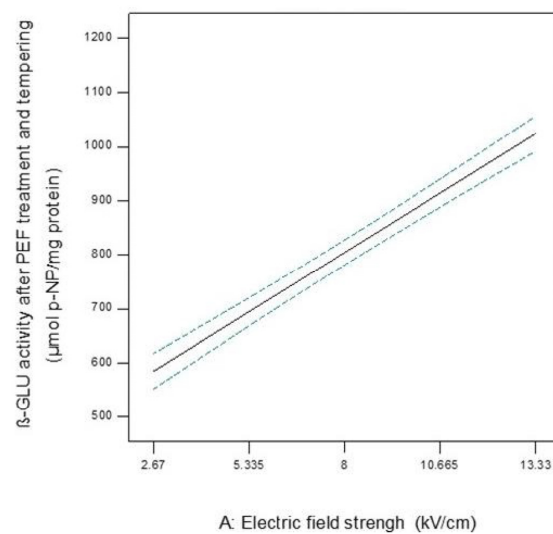


Figure 4. β -glucosidase (β -GLU) activity after PEF treatment and additional incubation for 30 min at 25 °C as a function of the electric field strength. The black line represents the model curve and the blue dashed line represents the 95% confidence interval.

3.2.2. Lipoxygenase

The results of LOX activity affected by PEF treatment immediately after treatment and after stimulation of the malaxation process are shown in Table 1. All treated samples had higher LOX activity than the control samples. The activity of LOX was significantly affected by the time of PEF treatment. In Tukey's test, PEF-treated samples were grouped by time. Interestingly, 1 min of PEF treatment doubled the activity of LOX at both field strengths. According to Tukey's test, there was no significant difference between the samples treated for 1 min and the 2 min control. However, in the samples treated for 2 min, the activity of LOX increased on average by 25% compared to the control and by 6% after 5 min of treatment. The parameters of the linear regression model (listed in Table 2) showed a good R^2 and an adjusted R^2 , but the model had a significant lack of fit (0.001). The model confirmed what was evident from the results of Tukey's test, namely that only the duration of PEF treatment had a significant effect on the activity of LOX.

The results of the activity of LOX after additional incubation of the model solution showed that there was no difference between the treated samples. However, all treated samples had significantly higher HPOD concentrations than the controls. Since the controls reached a plateau comparable to the plateau defined by the malaxation model for the activity of LOX (Figure 2b), and all PEF-treated samples extended this plateau, it can be concluded that treatment of LOX with a weak or mild electric field can increase its activity. According to the model parameters (Table 2), the linear model is not best suited to describe the activity of LOX ($R^2 = 0.338$), with only time having a significant influence among the three factors studied.

The influence of time, the only significant factor in the activity of LOX both immediately after PEF treatment and after additional incubation of the samples, is shown in Figure 5. Treatment time had a positive effect on the activity of LOX immediately after PEF treatment (Figure 5a). However, after the incubation period, the opposite effect was observed (Figure 5b). This is probably due to the spontaneous decomposition of the formed hydroperoxides, which was more pronounced with time [26].

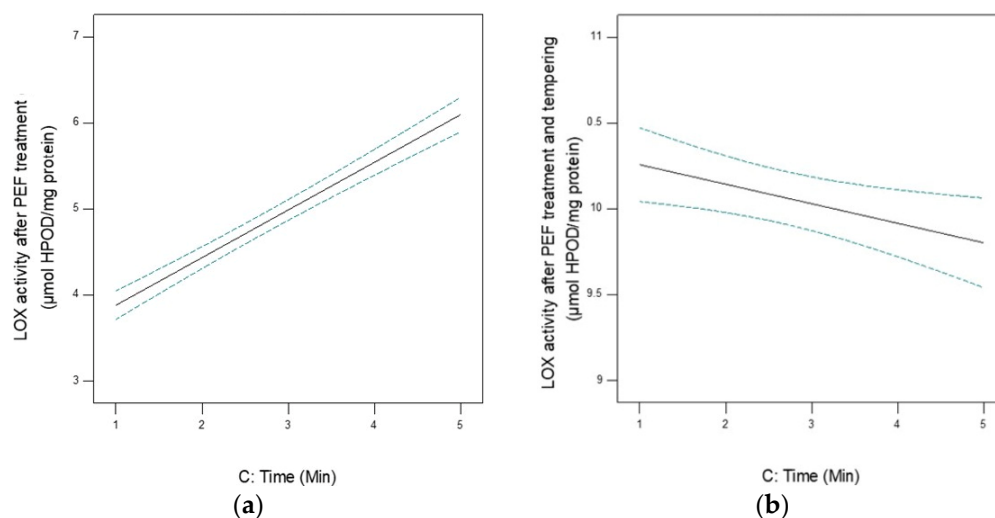


Figure 5. Lipoxygenase (LOX) activity as a function of time after (a) pulsed electric field (PEF) treatment and (b) PEF treatment and additional incubation for 30 min at 25 °C. The black line represents the model curve and the blue dashed line represents the 95% confidence interval.

4. Conclusions

PEF treatment increases the activity of β -GLU and LOX in model systems, but the increase in the concentration of enzyme products depends on the parameters used for the treatment. A low electric field strength (2.67 kV/cm) had no significant effect on β -GLU activity, whereas a moderate strength electric field (13.33 kV/cm) significantly increased its activity. In contrast, LOX activity increased slightly at both electric field strengths but was

mainly dependent on the duration of treatment. These results suggest that the application of a moderate electric field strength for 2 min prior to malaxation of olive paste can increase the activity of β -GLU and LOX, resulting in oils with higher concentrations of polyphenols and desirable volatile compounds. However, in order to draw such conclusions, the effects of selected PEF parameters on other enzymes that determine the phenolic and sensory profile of the oil (e.g., peroxidase, polyphenol oxidase, and hydroperoxide lyase) need to be investigated. Experiments with model systems using pure enzymes are important for a better understanding of the changes they undergo during PEF treatment, and the results obtained can serve as a starting point for optimizing the PEF treatment of the real system, i.e., olive paste, in order to produce VOO with higher nutritional value and better sensory quality.

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