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Optimization of Enzymatic Assisted Extraction of Bioactive Compounds from *Olea europaea* Leaves

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Abstract: Nowadays, the circular economy trend drives researchers in the recovery of various bioactive compounds from agri-food by-products. Enzyme-assisted extraction (EAE) has been shown to be an innovative green technology for the effective extraction of various phytochemicals from agri-food section by-products; therefore, this study aimed to evaluate the application of EAE as green technology to obtain extracts from olive leaves (*Olea europaea*) for potential industrial production. The used enzymes were Celluclast, Pectinex XXL and Viscozyme L. EAE was conducted under various enzyme dose combinations and an incubation time of 120 min. Obtained extracts were characterized in terms of total polyphenols (TP) and total antioxidant activity (AA). Firstly, the enzyme synergistic effect in the enzymatic extraction of polyphenols was evaluated. TP optimal extraction conditions (468.19 mg GAE (gallic acid equivalent)/L of extract) were achieved after EAE using Pectinex and Viscozyme enzymes (50–50 *v*/*v*) and for AA (69.85 AA%). According to the above results, a second experiment investigated the effect of incubation time (min.) and enzyme dose (mL) on the optimal extraction conditions of olive leaves. The final results after optimization were 75% higher than the control sample for the TP content (605.55 mg GAE/L) and 8% higher for the AA (70.14 AA%). These results indicated that EAE is an excellent choice for the green extraction of polyphenols from the olive leaves.

Keywords: plant by-product; antioxidants; green technology; enzyme preparation; polyphenols

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1. Introduction

Olives are of the most widely produced crops, with 65%, 16%, and 15% of the world's output grown in Europe, Asia, and Africa, respectively [1]. Moreover, Proietti et al. [2] estimated that an olive tree produces approximately 11,777 leaves, most of which are thrown away as waste. However, the leaves can be used as a profitable raw material for continuous large-scale industrial production for a considerable amount of time [3]. Furthermore, since their import is likely to include waste management services for olive oil producers, the business that processes leaves stands a very high chance of increasing its profit margin.

Along with macronutrients and micronutrients, a typical diet also includes specific chemical compounds, mostly found in fruits and vegetables, such as phenolic acids, flavonoids, anthocyanins, catechins, quercetin, tannins, stilbenes, etc. [4], which have been shown in several studies to have a significant biological impact. These substances are referred to as bioactive substances and have a variety of functions for human health. Bioactive chemicals are regarded as secondary metabolites of plants and are crucial for pest and disease resistance, as well as species preservation. Each year's action and promotion of health advantages pique their attention [5]. The olive (*Olea europaea* L.), one of the most significant fruit plants in the Mediterranean region, is prized for its nutritional and

health benefits all over the world. Olive oil is made from the fruits of the olive using mechanical methods. The phenolic compounds' antioxidant activity is responsible for their health-promoting properties, and their pharmacological actions have been documented in the literature [6]. Because of their potent antioxidant activity, by-products from the olive oil industry provide a prospective source of phenolic compounds [5]. An increasing number of people have been interested in giving these goods more value in recent years, for both nutritional and environmental reasons. Numerous investigations have been conducted in this context to comprehend the function of the numerous natural chemicals found in these products. Based only on the quantity of phenol subunits present, phenols can be divided into simple phenols and polyphenols. Therefore, simple phenols, phenolic acids, coumarins, flavonoids, and stilbenes, as well as hydrolyzed and condensed tannins, lignans, and lignins, are all included in the phrase "plant phenolics". In vitro biological actions of phenols include the scavenging of free radicals, the regulation of enzyme activity, and the prevention of cell proliferation. According to Cherng et al. [7], they have anti-inflammatory, anti-ulcer, anti-allergic, and antibiotic properties. They also show antibacterial action.

The biological cycle of the olive affects significant variations in phenolic chemicals, both quantitatively and qualitatively [8]. Phenolic compounds are present in both fruits and leaves in substantial amounts, and they are transported from the fruit to the olive oil during processing. These compounds determine the taste and antioxidant activity of olive oil, which is why they are crucial to its quality. The class of molecules known as phenolics includes over 8000 naturally occurring substances. These compounds always have an aromatic ring with at least one hydroxyl substituent, or a phenol [5]. According to Abaza et al. [9], olive leaves contain flavonoid and phenolic compounds that exhibit a range of biological activities. These chemicals may also be accountable for the pharmacological effects of olive leaves, or at the very least, for the synergistic increase in these effects.

Oleuropein and its derivatives are the principal constituents of olive leaves, Which include hydroxytyrosol, other polyphenols, triterpenes, which comprise flavonoids (rutin and diosmin) and oleanolic acid. These elements give the tree, as well as its fruits and foliage, resilience against disease and insect damage [5]. Oleuropein, which was initially discovered in 1908, is thought to be the cause of many of the medicinal benefits of extracts from olive leaves and the cause of the bitter flavor of olive oil and olives as well [10]. Strong antioxidant and free radical scavenging properties, as well as antimicrobial, hypoglycemic, anti-toxoplasmosis, antiviral, antifungal, anti-aggregation, and platelet hypolipidemic properties have all been reported for oleuropein [11]. According to Rahmanian et al. [12], six major polyphenolic compounds were found to be present in olive leaf extract, following a qualitative and quantitative compositional analysis using high performance liquid chromatography (HPLC) in conjunction with photo diode array detection (DAD). These compounds include oleuropein (24.5%), verbascoside (1.1%), luteolin-7-Oglucoside (1.4%), apigenin-7-Oglucoside, hydroxytyrosol (1.5%), and tyrosol (0.7%). Lutein, sesamol, ellagic acid, and apigenin-7-O-glucoside (1.4%) were also obtained from the leaves.

The amount found in the leaf is significantly higher than in other parts of the tree, while being present in the olive fruit and oil [13]. The chemical composition of olive leaves is influenced by several elements, including olive variety, temperature, extraction methods, wood ratio, tree age, genetics, and cultivation methods [14]. Since the concentration of phenolic components varies among plant materials, it is essential to determine the best extraction conditions and characterize the extract's antioxidant activity and composition.

Thus, it is advised to use whole leaf extracts rather than only their individual components, such as oleuropein, when making nutraceuticals, functional foods, and food additives [15]. Evaluating various (new) technologies that support polyphenolic stability during extractions is crucial. At the same time, it should be more effective, efficient, and environmentally friendly [16].

Compared to conventional methods, extraction by EAE offers several advantages. These include mild reaction conditions, which involve short reaction times and low temperatures, the ability to extract large amounts of bioactive compounds (by bioaccessing

even defined molecules within cellular organelles like vacuoles and plant cell walls that would otherwise be inaccessible), high bioavailability, high quality, and low residue levels. Additionally, EAE can reduce production costs by eliminating the need for the multiple installations required for classical extraction processes [17].

The aims of the study were as follows: (i) to develop a green enzymatic extraction process for the recovery of polyphenols from the olive leaves, (ii) to evaluate the synergistic effects of three (3) commercial enzymes in the enzymatic extraction process recovery of polyphenols from the olive leaves, and (iii) by using the optimal enzyme mixture found in step (ii), to evaluate the optimal extraction conditions for the recovery of polyphenols from the olive leaves.

To the best of our knowledge, step (ii) and step (iii) of the current study are, for first time, reported for olive leaves and are the innovative points of this study.

2. Materials and Methods

2.1. Plant Material

Olive (*Olea europaea*) leaves, Koroneiki variety harvested year 2023, were obtained from a producer in the region of Agrinio, Greece. The leaves were dried at 50 °C for 5 h [18]. The residual humidity was determined using a moisture analyzer, AXIS AS-60 (AXIS Sp. z o.o. ul. Kartuska 375b, 80-125 Gdańsk, Poland), and was found to be 5.93%. Dried olive leaves were stored in a metal container at room temperature until used.

2.2. Chemicals and Reagents

For analytical purposes, the following reagents were used: 2,2-Diphenyl-1-picrylhydrazyl (DPPH·) (Sigma-Aldrich, Darmstadt, Germany); absolute methanol (CH_3OH), sodium carbonate (Na_2CO_3), and acetate buffer ($CH_3COONa \times 3H_2O$) (Merck Darmstadt, Germany); Folin–Ciocalteu reagent from Sigma-Aldrich; and gallic acid (3,4,5-trihydrobenzoic acid) 99% isolated from *Rhus chinensis* Mill (JNK Tech. Co., Seongnam, Republic of Korea). All the other reagents and solvents used were of analytical grade.

2.3. Enzyme Preparations

The following commercial enzyme preparations were used: pectinolytic preparation Pectinex XXL, cellulolytic preparation Celluclast and Viscozyme L. (Cellulase, Hemicellulase, Xylanase) (all from Novozymes A/S, Bagsvaerd, Denmark).

2.4. Enzyme-Assisted Extraction

Finely grounded (particle size < 700 μm) olive leaves (Figure 1) were mixed with water (10:1, *v*/*w*), acidified (pH 4.0) with HCl, and left for 1 h for rehydration at 25 °C. After pH adjustment (pH 4.0), the suspension (100.0 g) was placed in a 50 °C water bath (Memmert Schutzart DIN 40,050-IP 20, Germany) for 20 min before enzymes were added. After incubation at 50 °C, the sample was placed in a boiling water bath for 10 min to inactivate the enzymes, then immediately cooled and finally filtered through a paper filter under vacuum and weighed to determine the extract yield [19]. The same process was followed for the control sample and, instead of enzymes, distilled water was used at the same amount as the enzymes in its sample.

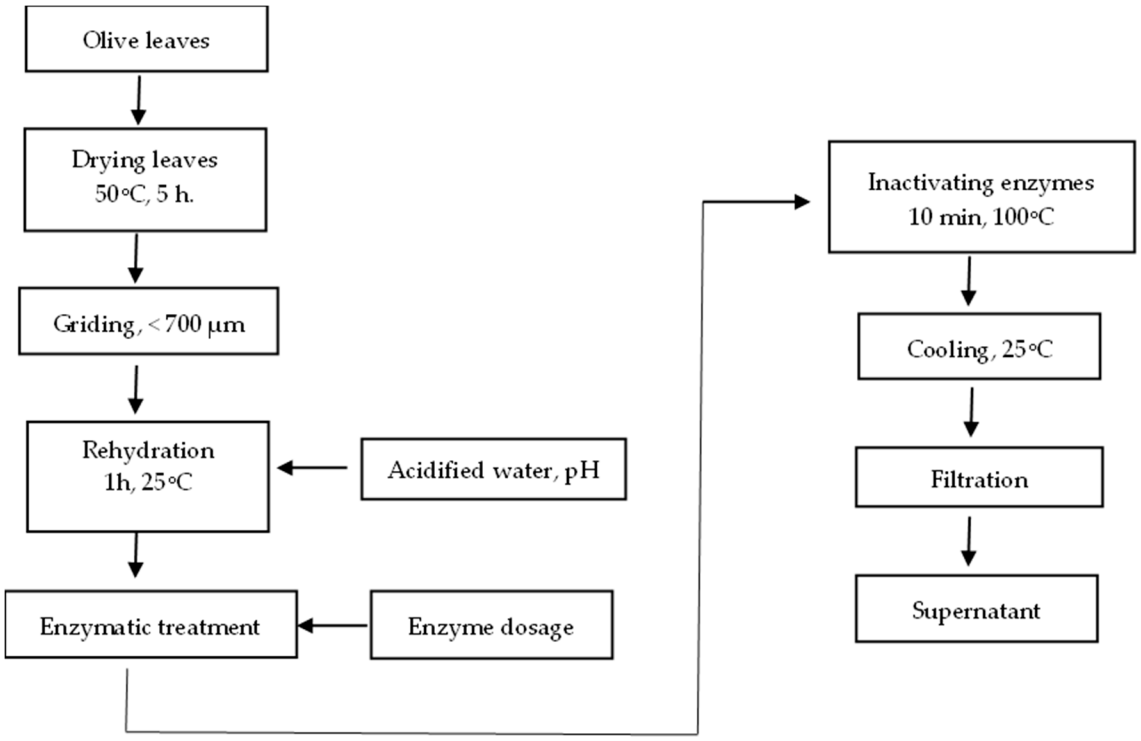


Figure 1. Process flowchart for extraction of olive leaves (*Olea europaea*).

2.5. Phytochemical Analyses

All measurements were performed with a SHIMADJU UV/VIS spectrophotometer (UV-1900, Kyoto, Japan) using 1 cm pathlength cuvettes. The content of total polyphenols (TPP) was determined using the method of Karabagias et al. [20], with the following modifications, at room temperature: in a 5 mL volumetric flask, 0.20 mL of the ethanolic extracts of grape origin, followed by 2.50 mL of distilled water and 0.25 mL Folin–Ciocalteu reagent, were added. After 3 min, 0.50 mL of saturated sodium carbonate (Na_2CO_3, 30% *w*/*v*) was also added into the mixture. Finally, the obtained solution was increased to a volume of 5 mL using distilled water. This solution was left for 2 h in the dark at room temperature and the absorbance was measured at λ = 760 nm. For the total polyphenols test, distilled water was used as the blank sample. The results were presented as equivalents of gallic acid (GAE). Each sample was analyzed in triplicate (n = 3).

The total antioxidant capacity was determined using the DPPH assay (measuring free radical scavenging activity). The DPPH assay was based on the method of Karabagias et al. [20], with the following small modifications, at room temperature: 1.9 mL of absolute ethanol solution of DPPH·(1.34×10^{-4} mol/L) and 1 mL of acetate buffer 100 mmol/L (100 Mm) (pH = 7.10 ± 0.01) were placed in a cuvette, and the absorbance of the DPPH· was measured at t = 0 (A_0). Subsequently, 0.1 mL of each extract studied was added to the above medium and the absorbance was measured at regular time periods, until the absorbance value reached a plateau (steady state, At). The reaction in all cases was completed in 15 min and the absorbance was measured at λmax = 517 nm. Each sample was measured in triplicate (n = 3). For this antioxidant test, ethanol and acetate buffer (2:1, *v*/*v*) were used as the blank sample.

2.6. Experimental Design

According to a recent publication [19], a simplex-centroid design for a mixture with three components was applied (Figure 2). Enzyme preparations (single or mix) were used as 1.2% (*v*/*v*) solution and the incubation time was 120 min.

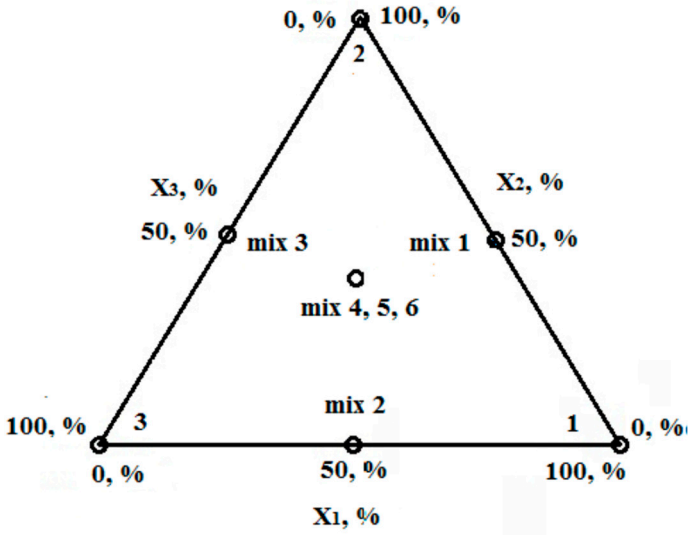


Figure 2. Ternary diagram for the simplex-centroid design: 1—100% Celluclast (X_1), 2—100% Pectinex XXL (X_2), 3—100% Viscozyme L (X_3), mix 1—X_1/X_2 = 1:1; mix 2—X_1/X_3 = 1:1; mix 3—X_2/X_3 = 1:1; mix 4, 5, 6—$X_1/X_2/X_3$ = 1:1:1.

An optimal central composite design (OCCD) of type 2n + 2n + n0 was applied. The influence of the independent variables was determined by means of the response surface methodology [19]. Table 1 shows the levels of the two independent variables—enzyme dose (0.02–0.18%) and reaction time (30–210 min). The enzyme used was a 1:1 mixture of the Viscozyme L. and pectinolytic (Pectinex XXL) preparations. The experimental data were fitted to a second-degree regression equation, as follows:

$$y = b_0 \sum_{i=1}^{n} b_i x_i \ + \sum_{i=1}^{n} b_{ii} \ x_i^2 + \sum_{i=1}^{n} \sum_{j=1}^{n} b_{ij} \ x_i x_j \quad (1)$$

where y is the dependent variable (response), b_0 is the model intercept, b_i, b_{ii}, and b_{ij} are the linear, quadratic, and interaction regression coefficients, respectively, x_i and x_j are the independent variables, and n is equal to the number of the tested factors (n = 2 in this study).

Table 1. Independent variable values and corresponding levels.

Factor	Minima	Centre Point	Maxima	Axial Point. a
Enzyme dose (%E/S [a])—X_1	0.02	0.1	0.18	−a = −1 +a = +1
Time (min.)—X_2	30	120	210	−a = −1 +a = +1

[a] mL enzyme preparation per 100 g substrate.

2.7. Statistical Analysis

The results reported in the present study are the mean values of at least three analytical determinations and the coefficients of variation expressed as the percentage ratios between the standard deviations and the mean values were found to be <5% in all cases. The means were compared using one-way ANOVA and Tukey's test at a 95% confidence level.

3. Results

3.1. Selection of the Mixture of Enzyme Preparations

Table 2 shows the results after the extractions, evaluating the synergy of the enzymes in order to find the most effective enzyme combination for polyphenol extraction from the olive leaves.

Table 2. Treatment variants and results [a] for the experimental design.

	Yield (%)	TPP [b] (mg GAE/L)	DPPH [c] (AA %)
Control (no enzyme)	64.00 ± 3.20 a	442.13 ± 22.11 a	64.76 ± 3.24 a
X_1	62.36 ± 3.12 ab	434.90 ± 21.74 a	66.51 ± 3.33 a
X_2	63.18 ± 3.16 ac	387.53 ± 19.38 b	68.36 ± 3.42 a
X_3	66.82 ± 3.34 a	377.63 ± 18.88 b	70.48 ± 3.52 a
Mix 1 (X_1/X_2)	64.17 ± 3.21 a	447.64 ± 22.38 a	69.05 ± 3.45 a
Mix 2 (X_1/X_3)	56.90 ± 2.84 b	465.91 ± 23.30 a	70.32 ± 3.52 a
Mix 3 (X_2/X_3)	61.68 ± 3.08 ab	468.19 ± 23.41 a	69.85 ± 3.49 a
Mix 4, 5, 6 ($X_1/X_2/X_3$)	58.19 ± 2.91 bc	464.64 ± 23.23 a	70.08 ± 3.50 a

[a] Means ± standard deviation (n = 3). [b] Results are expressed as milligram gallic acid equivalents (GAE) per 1 L. [c] Results are expressed as antioxidant activity (%). Different lowercase letters within a column indicate significant differences (Tukey's test. $p < 0.05$).

Significant increases in the recovery rates of total polyphenols and antioxidants were observed due to the enzymatic treatments (see Table 2, Figure 3a,b). The binary combination containing Viscozyme and pectinolytic preparations (X_2/X_3 = 1:1) resulted in the highest yield of total polyphenols, reaching a 5.9% higher value than the control sample (without enzymatic treatment).

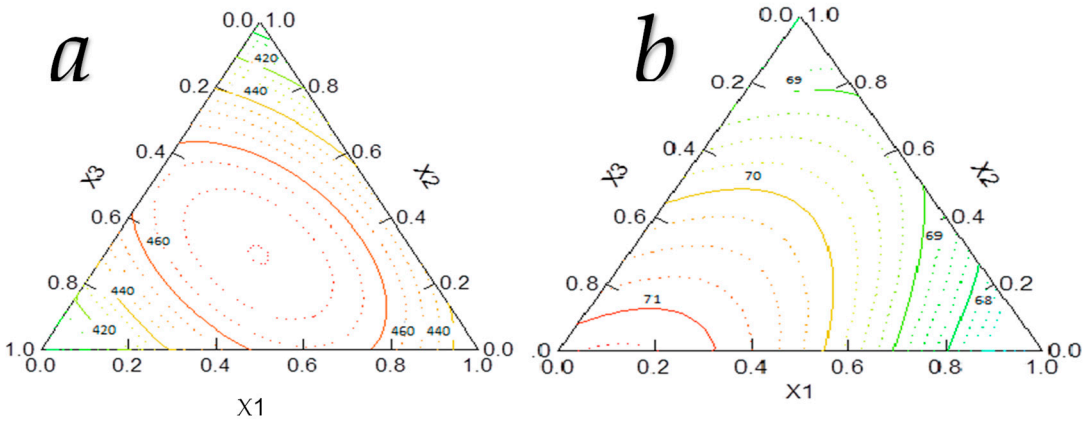


Figure 3. Simplex contour plots for: (**a**) TPP, (**b**) DPPH.

This value (468.19 mg GAE/L) is higher than that for microwave-assisted enzymatic extraction (34.53 mg GAE/g) [21] but lower than that (54.92 mg GAE/g) reported for 80% ethanolic extraction [22]. However, possible differences in the polyphenolic content of the raw materials should be taken into account.

Interestingly, similar effects concerning the total polyphenols and antioxidants were observed for the Viscozyme and cellulolytic preparations mixture (X_1/X_3 = 1:1) and for all enzyme mixtures ($X_1/X_2/X_3$ = 1:1:1), which is probably due to the secondary xylanase activity of commercial pectinase [23].

3.2. Optimization of the Process Parameters

Table 3 shows the total polyphenols and the antioxidant capacity of olive leaves after extraction with the most effective combination of enzymes (Pectinase and Viscozyme, Mix 3, X_2/X_3) as shown in Table 2, modifying the dose and the incubation time to evaluate the most optimum extraction conditions using enzymes. The code values in Table 3 mean minima "−", · centre point "0" and maxima "+" for enzyme dose and incubation time, as described in Table 1.

Table 3. Experimental design matrix and results for the optimal central composite design.

No	Coded Values		Enzyme Dose (%E/S [a])	Time (min)	TPP [b] (mg GAE/L)	DPPH [c] (AA%)	Yield [d], (%)
			X_1	X_2	Y_1	Y_2	Y_3
1	−	−	0.02	30	553.99 a	55.23 a	57.13 ad
2	+	−	0.18	30	495.11 b	57.22 a	64.98 bcg
3	−	+	0.02	210	602.88 cd	58.61 a	57.73 ad
4	+	+	0.18	210	572.06 ac	58.33 a	55.54 ad
5	−	0	0.02	120	530.78 ab	56.85 a	61.05 dce
6	+	0	0.18	120	582.61 ac	57.78 a	58.22 adf
7	0	−	0.1	30	605.55 c	57.31 a	70.14 g
8	0	+	0.1	210	510.42 ab	58.82 a	62.42 bef
9	0	0	0.1	120	556.85 ad	58.21 a	65.49 bef
10	0	0	0.1	120	557.44 ad	58.22 a	65.72 bef
11	0	0	0.1	120	556.95 ad	57.98 a	66.12 bef

[a] mL enzyme preparation per 100 g substrate; [b] results are presented as mg gallic acid equivalents (GAE) per L; [c] results are presented as antioxidant activity (%); [d] results are presented as % per 100 g. Different lowercase letters within a column indicate significant differences (Tukey's test. $p < 0.05$).

Significant variations in the yields of total polyphenols and antioxidants were observed in response to the different enzymatic treatments (see Table 3), increasing the TP by 37% and AA by 8%, compared to the control sample. In contrast with the findings of other researchers [19,24], an increase in the enzyme dose did not affect the recovery rate of total polyphenols; however, in some cases, an increase in enzyme dose decreased the total polyphenols content. The negative effects of the higher enzyme dose showed that Viscozyme, which activates rutinase, with the action of synergistic pectinase, significantly reduced total phenolic substances due to the loss of rutin [25], one of the most abundant flavonoids in olive leaves [22], but not the antioxidant activity, which remained stable.

The same results were obtained for the incubation time on the total polyphenol yield; an increasing incubation time decreased the total polyphenol yield due to thermal degradation, which were the same results as found by other researchers [26,27].

The experimental data in Table 3 were used to determine the coefficient of two second-order polynomial equations, as follows:

$$Y_1 = 553.87 + 9028.4X_1 - 6.27X_2 - 100{,}211.1{X_1}^2 + 0.06{X_2}^2 - 8.44X_1X_2 + 278{,}661.1{X_1}^3 - 0.00015{X_2}^3 - 0.075X_1{X_2}^2 + 137.2{X_1}^2X_2, \text{(mg GAE/L)} \quad (2)$$

$$R^2 = 0.99$$

$$Y_2 = 53.999 + 40.0077X_1 + 0.021X_2 - 125.25{X_1}^2 - 0.079X_1X_2 - 0.00000636777{X_2}^2, \text{(AA\%)} \quad (3)$$

$$R^2 = 0.97$$

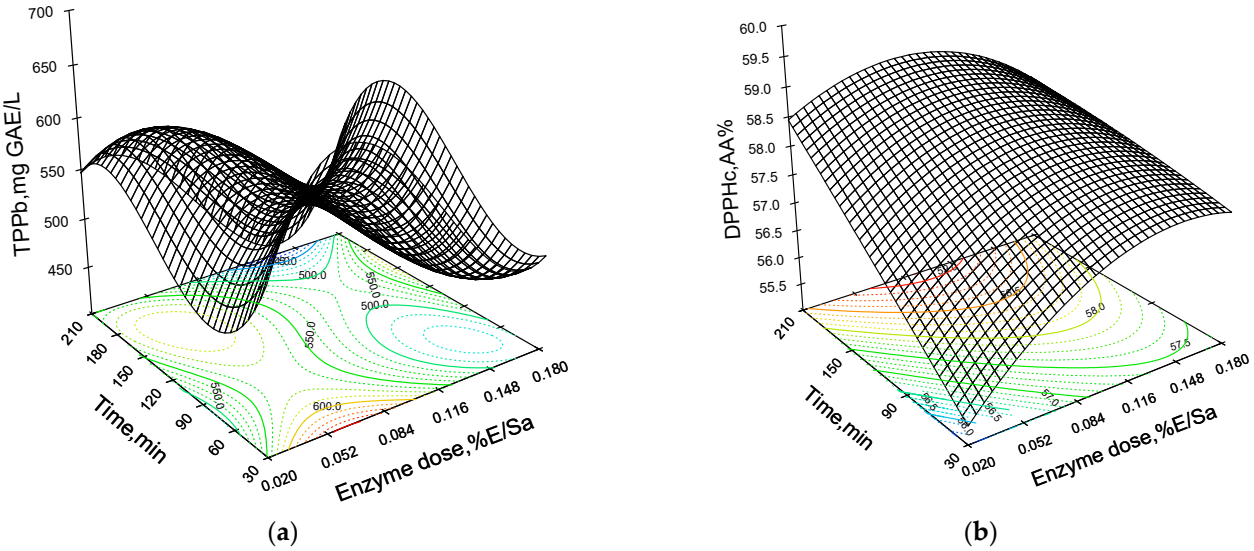
where Y_1 and Y_2 are the predicted responses for TPP and DPPH, respectively, X_1 is the enzyme dose, and X_2 is the incubation time.

After the optimization process of enzyme dose and extraction time (Table 3), the final results under the optimal conditions (605.55 mL GAE/L) are still higher than the results mentioned above. The final results are also higher than the total polyphenols content that was obtained using cyclodextrins and glycerin as co-solvents (54.33 mg GAE/g) [28] but lower than the microwave and ultrasound extractions (104.22 mg GAE/g and 80.52 mg GAE/g), respectively [29].

All of the R^2 (coefficient of determination) values were greater than 0.95, implying that the models accurately represent the experimental data [30].

Both the incubation time and enzyme dose produced positive linear and negative quadratic effects on total polyphenols. This means that the yield of total polyphenols (Figure 4a) increases when the incubation time or enzyme dose increases up to a certain point, after which they begin to decrease. Positive linear and negative quadratic effects of

incubation time were also reported for total polyphenols in extracts from rose petals and saffron tepals [30,31].



(**a**) (**b**)

Figure 4. Response surfaces showing the effects of enzyme mixture dose, %E/Sa—grams of enzyme mixture per 100 g substrate, and incubation time, min, on (**a**) TPP and (**b**) DPPH.

Positive linear effects of incubation time and negative quadratic effects of enzyme dose were obtained for the total antioxidant capacity values (Figure 4b), suggesting similar changes to those observed for the total polyphenols content.

A graphical optimization of the extraction conditions was carried out in order to maximize the yields of total polyphenols and antioxidants. Figure 5 shows the overlapping region, defining the intervals of variation in the enzyme mixture dose (0.06–0.15%) and treatment time (30–120 min) that satisfy the optimization criterion.

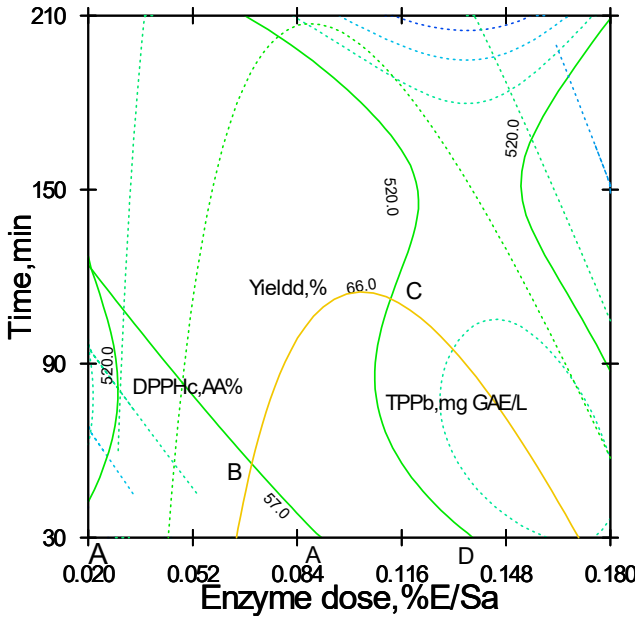


Figure 5. Graphical optimization of the extraction conditions—enzyme mixture dose, %E/Sa—grams of enzyme mixture per 100 g substrate, and incubation time, min.

4. Discussion

This work presents a novel method for more effectively extracting polyphenols from olive leaves, leading to environmentally friendly extracts and procedures. The results show that, by using green extraction techniques, it is possible to limit the usage of organic solvents by developing easy and cheap methods for extracting bioactive plant polyphenols. Table 4 summarizes the results of other studies on extracting polyphenols from olive leaves, in comparison with the current method. As can be seen, after using the optimal combination of enzymes and incubation time, the total polyphenols obtained using enzymatic-assisted extraction are higher than in all other methods, except the results from microwave-assisted extraction and ultrasound-assisted extraction, which are higher.

Table 4. Total phenolic content of olive leaves in different extraction methods.

Extraction Method	Total Polyphenol Content	Reference
Enzyme-assisted extraction	605.55 mg GAE/L	Current study
Microwave-assisted enzymatic extraction	34.53 mg GAE/g	[21]
Ethanol 80%	54.92 mg GAE/g	[22]
Cyclodextrins and glycerin co-solvents	54.33 mg GAE/g	[28]
Microwave-assisted extraction	104.22 mg GAE/g	[29]
Ultrasound-assisted extraction	80.52 mg GAE/g	[29]

The results of this study indicate that the recovery of polyphenolic antioxidants from olive leaves is improved by enzyme-assisted extraction, particularly when a binary enzyme combination consisting of Viscozyme L. and pectinase preparations (1:1) is used. The optimum range to obtain extracts with a high concentration of total polyphenols and antioxidants is defined by the variable intervals of the enzyme mixture dose (0.06–0.15%) and incubation time (30–120 min). This novel method provides an environmentally friendly replacement for conventional extraction methods, rendering it a green technology.

5. Conclusions

The obtained results of this study clearly show that the recovery of polyphenols from olive leaves is enhanced by enzyme-assisted extraction, particularly when ternary enzyme combinations, including pectinolytic and Viscozyme preparation, are used. The reason that these two enzymes are more effective is based on the construction of the olive leaf cell wall, which contains cellulose, pectin, and hemicellulose [32]. Enzymatic and organic solvent-free extraction methods have an environmentally friendly substitute. The innovative and very promising results of this work motivate us to undertake further research, which should be carried out based on the polyphenolic profile of the olive leaves and the olive tree varieties to achieve the maximum quantity and the highest quality of polyphenolic yields. Specifically, our future research plans are as follows: (i) to analyze the effects of different olive tree varieties and different collecting periods on the number of secondary metabolites in the leaves, for example, after a long heat wave, (ii) the scale-up of the enzymatic-assisted extraction of bioactive compounds from *Olea europaea* leaves to an industrial scale, and (iii) the application of extracted bioactive compounds to active food packaging.

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Conflicts of Interest: Author Alexios Vardakas was employed by the company GAEA Products S.M. S.A. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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