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Comparison between different flavored olive oil production techniques: healthy value and process efficiency

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Abstract:	We have compared three different production methods, which may be suitable to obtain flavored olive oil. The comparison was made employing two different herbs, thyme and oregano. The traditional and widespread method consist in the infusion of herbs into the oil. A second scarcely diffused method is based on the addition of herbs to the crushed olives before the malaxation step during the extraction process. The third innovative method is the implementation of the ultrasound before the olive paste malaxation. The main objective of the study is to verify the effect of the treatments on the quality of the product, assessed by means the chemical characteristics, total phenol content and radical scavenging activity of the resulting oils. Simultaneously, considerations on the efficiency, time consumption and labor, were made to identify the more suitable method for the large scale production, in order to enhance product quality and improve the process efficiency. The less favorable method was the addition

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	of herbs directly to the oil. A positive effect was achieved by the addition of herbs to the olive paste and other advantages were attained by the employment of ultrasound. These last two methods allow to produce oils "ready to sell", instead the infused oils need to be filtered. Moreover, the flavoring methods applied during the extraction process determine a significant increment of phenolic content and radical scavenging activity of olive oils. The increments were higher when oregano is used instead of thyme. Ultrasound inhibited the olive polyphenoloxidase, the endogenous enzyme responsible for olive oil phenol oxidation. This treatment of olive paste mixed with herbs before malaxation was revealed as the most favorable method due the best efficiency, reduced time consumption and minor labor, enhancing product quality of flavored olive oil.
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ABSTRACT

Three different flavoring methods of olive oil were tested employing two different herbs, thyme and oregano. The traditional method consist in the infusion of herbs into the oil. A second scarcely diffused method is based on the addition of herbs to the crushed olives before the malaxation step during the extraction process. The third innovative method is the implementation of the ultrasound before the olive paste malaxation. The objective of the study is to verify the effect of the treatments on the quality of the product, assessed by means the chemical characteristics, the phenol composition and the radical scavenging activity of the resulting oils. The less favorable method was the addition of herbs directly to the oil. A positive effect was achieved by the addition of herbs to the olive paste and other advantages were attained by the employment of ultrasound. These last two methods allow to produce oils “ready to sell”, instead the infused oils need to be filtered. Moreover, the flavoring methods applied during the extraction process determine a significant increment of phenolic content and radical scavenging activity of olive oils. The increments were higher when oregano is used instead of thyme. Ultrasound inhibited the olive polyphenoloxidase, the endogenous enzyme responsible for olive oil phenol oxidation. This treatment of olive paste mixed with herbs before malaxation was revealed as the most favorable method due the best efficiency, reduced time consumption and minor labor, enhancing product quality of flavored olive oil.

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Abstract Three different flavoring methods of olive oil were tested employing two different herbs, thyme and oregano. The traditional method consist in the infusion of herbs into the oil. A second scarcely diffused method is based on the addition of herbs to the crushed olives before the malaxation step during the extraction process. The third innovative method is the implementation of the ultrasound before the olive paste malaxation. The objective of the study is to verify the effect of the treatments on the quality of the product, assessed by means of the chemical characteristics, the phenol composition and the radical scavenging activity of the resulting oils. The less favorable method was the addition of herbs directly to the oil. A positive effect was achieved by the addition of herbs to the olive paste and other advantages were attained by the employment of ultrasound. These last two methods allow to produce oils "ready to sell", instead the infused oils need to be filtered. Moreover, the flavoring methods applied during the extraction process determine a significant increment of phenolic content and radical scavenging activity of olive oils. The increments were higher when oregano is used instead of thyme. Ultrasound inhibited the olive polyphenoloxidase, the endogenous enzyme responsible for olive oil phenol oxidation. This treatment of olive paste mixed with herbs before malaxation was revealed as the most favorable method due to the best efficiency, reduced time consumption and minor labor, enhancing the product quality of flavored olive oil.

Keywords *Origanum vulgare* L.*; Thymus vulgaris* L.; *Olea europaea* L.; total phenol content; radical scavenging activity.

Introduction

Flavored olive oils are common on the marketplace [1]. Producers usually add to virgin olive oils other ingredients such as vegetables, herbs, spices or other fruit with the aim to improve the nutritional value, enrich the sensory characteristics, and improve the shelf life of the product [2,3]. Aromatic plants can be added with the aim to enrich the virgin olive oil with their content of essential oils and other compounds characterized by antimicrobial and antioxidant properties [4]. Moreover, flavored oils are appreciated by consumers for their rich taste and their gastronomy and culinary use [5,6]. There is an increasing attention for consumers about the nutritional effects of herb and spices because they are considered

sources of natural antioxidants. Oregano and thyme are, among the commercially available herbs, the ingredients with the highest total antioxidant capacity and antimicrobial activity. [7]. Moreover they show a significant reduction of pharmacological dosage of commercially available antibiotics when co-administered [4, 8-13]. From the technological point of view, herbs and spices have been used as natural antioxidants for stabilizing the monounsaturated oils, such as olive oil [14]. Different flavoring systems are available and new ones can be developed to optimize the process. Infusion is a well-known aromatization method: herbs and spices are finely ground and mixed with virgin olive oil. The mixture is left at room temperature for a definite time and with periodic shaking. Then the mixture is filtered to remove the solid parts and becomes clear and "ready to use". This technique is time-consuming but widespread.

Considering the industrial need to speed up the processes, and the strong interest among consumers in buying and eating healthy foods and seasonings, the most traditional flavoring system was compared with other ones. Two different herbs, oregano (*Origanum vulgare* L.) and thyme (*Thymus vulgaris* L.) were added to the olive paste, during the olive oil extraction process, immediately after crushing, and then the malaxation was conducted with or without the application of ultrasound (US). US is considered an emerging technology able to meet the innovation demand of the virgin olive oil (VOO) industry [15-17]. US is a form of energy generated by sound waves of frequencies above 16 kHz . US can be useful to optimize the aromatization process of the olive oil due to the mechanical effect [15-16] generated by the cavitation phenomena [18]. Cavitation can facilitate the extraction and the dissolution of herbal essential oils into the EVOO[16].

The main objective of the study was to compare the three flavoring methods in order to verify the effect of the treatments on the healthy value of the product, assessed by means of the chemical characteristics, total phenol content and radical scavenging activity of the resulting oils [19]. Simultaneously, considerations on the efficiency, time consumption and labor, were made to identify the more suitable method for the large scale production, in order to enhance product quality, extend the shelf life and improve the process efficiency.

Materials and Methods

Reagents. All chemicals and reagents were of analytical grade and solutions were freshly prepared before use. The complete list is given in the Electronic Supplementary Material (ESM).

Plant material. Olive fruits (*Olea europaea* L.) of Coratina variety were harvested in olive groves of the same area near Andria (Apulia–Italy) in the 2013/2014 crop season. The olives were randomly picked by hand, using rakes and then were put into 30 kg boxes and immediately brought to the pilot plant [20, 21]. Fine cut herbs, oregano and thyme (certified AIAB-Associazione Italiana per l'Agricoltura Biologica), dried and under vacuum, were kindly provided by Sandemetrio Opificio Erboristico (S.da Prov.le 374, Km 5 - 73040 Specchia (LE) Italy).

EVOO extraction system. The pilot plant is described by [22]. The flow chart of the experimental plan is shown in Figure S1 (ESM). Three samples were collected as control, without any flavoring (C); three samples were incorporated with thyme and oregano fine cut leaves (10 g/L of olive oil); the herbs were macerated for 15 days into the olive oils at room temperature and then filtered through Whatman no. 4 paper. Three samples were extracted after the addition of herbs to the olive paste (10 g/kg) before the malaxation. Three samples was extracted after the addition of herbs to the olive paste (10 g/kg) and sonicated for 6 minutes before the malaxation.

Efficiency evaluation in terms of time-consumption of flavoring process. The three different flavoring methods were evaluated in terms of time-consumption (h) requested to complete the flavoring phase: (i) the infusion of herbs into the oil; namely, infused with oregano (OI) and infused with thyme (TI); (ii) the addition of herbs to the crushed olives before the malaxation step during the extraction process; namely, malaxed with oregano (OM) and malaxed with thyme (TM); (iii) the US treatment of the olive paste mixed with herbs before the malaxation step during the extraction process; namely, sonicates with oregano (OS) and sonicated with thyme (TS).

Olive oils quality indices. Free acidity, coefficients of specific extinction at 232 and 270 nm (K_{232} and K_{270}), and peroxide value (PV) of EVOO samples obtained from the assay were determined in analytical duplicate according to regulation (EU) No 1348/2013.

Extraction and analyses of polyphenols from EVOO samples and aromatic herbs. Polar extracts of extra virgin olive oil and aromatic herbs (oregano and thyme) were obtained by a liquid/liquid extraction method according to previously published procedure [23 - 25]. The obtained polyphenols were determined by colorimetric methods using Folin-Ciocalteu reagent [26] and HPLC-UV-MS^n analysis [27 - 29], as reported in detail in ESM.

Radical Scavenging Activity (RSA). Radical Scavenging Activity (RSA) of polar fraction extracted from olive oil samples and the presence of hydrogen donors in polar fraction extracted from olive oil samples were examined by reduction of DPPH in methanol according to [30].

Enzyme PPO, extraction and assay. The PPO extraction procedure was as reported by Ortega-Garcia et al. [31] with some modifications, and analyzed by Bradford assay [32] (see ESM).

Statistical analysis. Olive oil extraction experiments were performed in triplicate and chemical analyses of the oil obtained were conducted in duplicate. The results were expressed as mean value ± Standard Deviation (SD). Statistical analysis was carried out using Microsoft Excel software. Significant differences between treatments were determined using one-way analysis of variance (ANOVA) followed by ''t- test''. Regard to the final results of RSA analysis were expressed as mg of gallic acid for Kg of oil (Sigmoidal dose-response (variable slope), $R^2 = 0.996$) [33]. Regard to the extraction and assay of enzyme PPO as well as the HPLC polyphenol quantification, one-way analysis of variance (ANOVA), using the Tukey's honestly significant differences (HSD) post hoc test, with the SPSS Base 11.5 software (SPSS Inc., Chicago, IL, USA) was performed. Statistical significance for the tests was set at $p < 0.05$.

Results and Discussion

Influence of the ultrasound treatment on quality parameters of olive oil. All samples showed very low values of acidity, peroxide value, K_{232} and K_{270} (Table 1). Considering these chemical parameters, no significant differences were found attributable to the flavoring method ($P < 0.05$). Recently a very interesting paper reported that the infusion of different herbs and spices can affect virgin olive oils quality indices [33]. The discordance in the results can be probably due to the difference in (i) the olive

cultivar, (ii) the presence in the oil of traces of water and enzymes (unfiltered oil may and not dried spices contain water and enzymes that can favor the increment of free acidity and oxidative product), (iii) the system employed to dry the herbs and spices (oven- drying or sun-drying) that can affect the storability of the infused oil during the maceration [34].

Total polyphenols content and HPLC-UV-MS^n determination. Total polyphenols content (TPC) of the different EVOO extracts were reported in Table 1; moreover, their characterization was performed by HPLC-UV-MS^n analyses which allowed to identify 35 phenolic compounds (present in EVOO or derived by the processing with oregano and thyme) on the basis of their deprotonated molecular ions $[M-H]^-$, MS/MS fragment ions and elution order (Table 2) [27 - 29]. The incorporation of herbs positively affected the olive oils' phenolic composition and the consequent expected bioactivity, particularly if herbs were added to the crushed olives before the malaxation (Table 3). In infused oils, the TPC showed a slightly but significant increment only when oregano (OI) was added instead of thyme (TI) respect to the control sample (C) (Table 1). In opposition, the oil obtained by means of the addition of herbs to the olive paste during the extraction process, before the malaxation, showed a hard increase of TPC. In fact, when the olive paste was malaxed with thyme and oregano, the resulting oils (TM and OM) showed an almost three folds improvement of TPC respect to C and the infused TI and OI samples (Table 1). This interesting increment can be attributed to the presence of vegetation water into the olive paste, which can act as solvent for the improvement of herbs polar compounds' extraction, as proved by the increment of the concentrations of organic acids (coumaric, caffeic, ferulic, and rosmarinic acids) as well as flavones and flavonols (kaempferol, quercetin, naringenin, and apigenin) (Table 3), especially when dried material is used. In fact, solvent extraction from dried material is a two stage process involving: (i) steeping vegetal materials in solvent to facilitate swelling and hydration processes; (ii) the mass transfer of soluble constituent from the material to solvent by osmotic and diffusive processes [35]. Moreover, the continuous mixing of olive paste with herbs probably promote the releasing of oregano and thyme phenols into the oily phase speeding the process respect to the infusion methods, and the herb glycosidic phenols can be easily hydrolysable by olive endogenous ß-

glucosidase, active during malaxation. Evidently, the lack of vegetation water is instead a limit for the diffusion of polar herb phenols in infused oils, as confirmed by the absence of glicosidic derivatives, such as rutin or kaempferol glicosides (Table 3).

Ultrasound treatment, disrupting the biological cell walls, can facilitate the previous described phenomena allowing a higher extraction of polyphenols from herbs but also from olive paste, as revealed by the higher concentrations of tyrosol, hydroxytyrosol, and oleuropein derivatives in OS and TS than the other samples (Table 3). Overall, the positive effect of ultrasound was a further increment of TPC; indeed, comparing TM and OM with the oil obtained sonicating the olive paste mixed with the herbs (TS and OS), the increment of TPC was equal to 13.3 and 13.5%, respectively (Table 1).

The significant increment of polyphenols in the sonicated oils can be also attributed to the effect of ultrasound on polyphenoloxidase (PPO) activity. Figure 1 shows the ultrasound inhibition effect of olive PPO. The EVOO quality is intimately affected by its content in phenolic compounds. PPO is responsible for oxidative losses of phenolics during olive paste malaxation. EVOO phenols play a key role in the shelf life of the product due to their activity delaying oxidation processes. They act as chain breakers by donating radical hydrogen to alkylperoxyl radicals, produced by lipid oxidation and contribute to the formation of stable derivatives.

Radical scavenging activity. The increment of TPC determined an increase of the radical scavenging activity (RSA) of the product. A significant correlation was found between TPC and RSA (R^2 = 0.9192), confirming that the incorporation of herbs, particularly before the malaxation, had a positive effect also on the olive oils' radical scavenging activities (Table 1). In fact, in infused oils, the RSA showed a significant increment when thyme (TI) and oregano (OI) were added respect to the control sample (C) (untreated). The increment was equal to 60 and 33% for TI and OI respectively. The FOO infused with thyme (TI) was characterized by a lower TPC and a higher RSA than the oil infused with oregano (OI). Following the trend of TPC, the oil obtained adding the herbs to the olive paste during the extraction process, before the malaxation, showed a hard increase of RSA. In fact, when the olive paste was malaxed with thyme and oregano, the resulting oils (TM and OM) showed an improvement of RSA

respect to the infused ones (TI and OI) equal to about 2 and 4 times respectively. The effect of ultrasound on RSA of FOOs was significantly positive only when thyme was added to the olive paste. Comparing the TM with the oil obtained sonicating the olive paste mixed with the thyme (TS), the increment of RSA was equal to 34%. No significant differences were registered when the FOOs were extracted from sonicated olive pastes mixed with oregano respect to the malaxed ones with the same herb.

Process efficiency evaluation. The process efficiency evaluation in terms of time-consumption for the flavoring process revealed that the less favorable method was the addition of herbs directly to the oil. In fact, this method required time and labor without advantages regarding to the phenolic content and radical scavenging activity of the FOOs. The addition of herbs to the crushed olives before the malaxation required few minutes and, after the extraction, FOOs were immediately clear. The time of contact between the herbs (60 min) 30 min of "pre-heating time" (time needed to warm up the olive paste until 30 °C) and 30 min of "effective malaxing phase". When the third innovative method, consisting in the implementation of the ultrasound before the olive paste malaxing, was applied, a significant reduction (equal to the 33%) of the "pre-heating time" was attained due to the thermal effects of ultrasound (10 min vs. 30 min needed by traditional convective system) due to the thermal effect of ultrasound. The reduction of the time necessary for the flavoring phase is accompanied by the high product quality and extended shelf life determined by the higher phenol content and radical scavenging activity, more evident in the sonicated samples.

Conclusions and Perspectives. The addition of thyme and oregano to the crushed olives before the malaxation step allowed to obtain a clear product ready-to-sell a in a short period a clear product ready-to-sell, characterized by a higher TPC and RSA. The implementation of new and emerging technologies, such as ultrasound, improved the efficiency of the production process, and the mechanical effects due to cavitation increased the nutritional value of the product. The increments were higher when oregano was used instead of thyme. US treatment of olive paste mixed with herbs before malaxation was revealed as the most favorable method due the best efficiency, reduced time

consumption and minor labor, enhancing product quality of FOOs. To our knowledge, before this study, the infusion system for aromatizing olive oils had not been compared with the addition of herbs to the olive paste before the malaxation, during the extraction process. Moreover, before the present study, there had been no published data on the innovative US treatment of olive paste mixed with herbs, and the effects of US on endogenous polyphenol oxidase. However, further research will be necessary to determine if these results can be extended to other vegetables, herbs, spices or fruit, characterized by healthy properties and available to flavoring VOO.

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Table 1. Qualitative indices of olive oils: Free Fatty Acids (FFA), Peroxide Value (PV), Coefficients of specific ex
Content (TPC) and Radical Scavenging Activity (RSA), obtained with three different flavoring methods (values
replicates). 1 (C): Control (EVOO); 2 (TI): Infused with Thyme (EVOO + Thyme – 10 g/L); 3 (OI): Infused with
g/L); 4 (TM): Malaxed with Thyme (OP + Thyme – 10 g/kg); 5 (OM): Malaxed with Oregano (OP + Oregano –
Thyme (OP + Thyme – 10 g/kg); 7 (OS): Sonicated with Oregano (OP + Oregano – 10 g/kg). The tests were cond
the same column with different letters differ significantly ($p < 0.05$).

Sample	FFA (% oleic acid)	PV ($meqO_2/kg$)	K_{232}	K_{270}	TPC (mg of gallic acid/kg of oil)	(DPPH: r
C	0.27±0.04 a	7.7±0.3 b	1.63±0.03	0.11±0.01	125±8 a	
TI	0.32±0.03 ab	8.1±0.3 c	1.63±0.03	0.11±0.01	129±10 a	
OI	0.29±0.03 ab	8.4±0.2 c	1.69±0.02	0.11±0.01	175±7 b	
TM	0.29±0.05 ab	6.9±0.2 a	1.62±0.03	0.11±0.01	345±7 c	
OM	0.30±0.03 ab	7.2±0.3 ab	1.65±0.03	0.11±0.01	348±10 c	
TS	0.34±0.02 b	7.0±0.4 a	1.66±0.02	0.11±0.01	391±3 d	
OS	0.29±0.03 a	7.1±0.2 a	1.69±0.03	0.11±0.01	395±3 d	

Table 2. List of polyphenols identified in aromatic herbs and extra virgin olive oil.

COMPOUND	RT (min)	$[M-H]^-$	MS/MS fragments	Detected in
Gallic acid	3.20	169		O
Vanillin	4.22	151	123	EVOO
Syringic acid	4.02	197	182, 167	O, T
Hydroxytyrosol	4.27	153	123	EVOO
Protocatechuic acid	4.56	153	109	O, T
Caffeic acid-O-hexoside 1	5.43	341	179, 135	O, T
Vanillic acid	5.72	167	152, 108	EVOO
Caffeic acid-O-hexoside 2	6.38	341	179, 135	O, T
***p*-Hydroxybenzoic acid**	6.76	137	93	O, T
Caffeic acid-O-hexoside 3	7.30	341	179, 135	O, T
***m*-Hydroxybenzoic acid**	7.45	137	93	O
Tyrosol	7.76	137		EVOO
Homovanillic acid	8.63	181	137	O, T, EVOO
Caffeic acid	8.68	179	135	O, T, EVOO
Apigenin-C-hexoside-C-hexoside	10.08	593	503, 473	O, T
Ferulic acid-O-hexoside	10.32	355	193	O, T
Coumaric acid	11.54	163	119	O, T, EVOO
Hydroxytyrosol acetate	11.98	195		EVOO
Rutin	12.94	609	301	O, T
Kaempferol-3O-rutinoside	13.10	593	285	T
Quercetin-3O-glucoside	13.64	463	301, 300	T
Kaempferol-3O-glucoside	13.93	447	285	O, T
Apigenin-7O-glucoside	15.34	431	269	T
Ferulic acid	16.31	193	134	O, T, EVOO
Rosmarinic acid	16.55	359	197, 161	O, T
DAFOA (decarboxymethylated)	19.49	319	199, 111	EVOO
Kaempferol	20.04	285	151	O, T
Luteolin	20.06	285		EVOO
Acetoxy-pinoresinol	20.42	415		EVOO
Quercetin	20.79	301	151	O
Naringenin	21.79	271	151	O, T, EVOO
Apigenin	21.85	269	151	O, T, EVOO
Methoxyluteolin	22.23	299	227, 199	EVOO
AFOA	22.71	377	307, 275	EVOO
AFLA	24.70	361	315, 291, 259	EVOO
Carnosol	27.00	329	285	O, T

RT = retention time; $[M-H]^-$ = deprotonated molecular ion; O = oregano; T = thyme; EVOO = extra virgin olive oil; DAFOA = dialdehydic form of oleuropein aglycone; AFOA = aldehydic form of oleuropein aglycone; AFLA = aldehydic form of lingstroside aglycon.

Table 3. Identification and quantification of individual polyphenols (mean ± SD) of aromatized extra-virgin oli

Different letters in the rows represent statistically significant differences ($p < 0.05$).

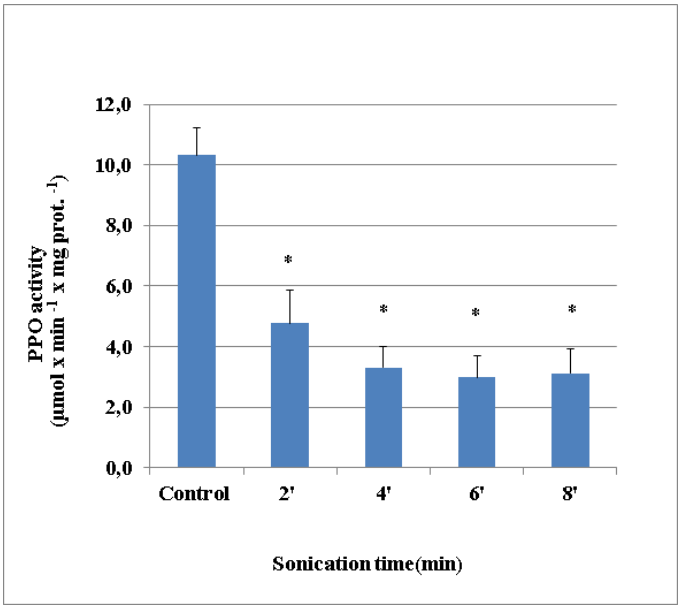
COMPOUND	C	TI	OI	TM	OM
Gallic acid	n.d.	n.d.	tr	n.d.	tr
Vanillin	0.125±0.017a	0.10±0.03a	0.105±0.011a	0.12±0.03a	0.12±0.02a
Syringic acid	n.d.	0.080±0.011bc	0.126±0.19d	0.029±0.012a	0.06±0.02b
Hydroxytyrosol	44±5a	46±8a	47±9a	48±6a	48±4a
Protocatechuic acid	n.d.	0.20±0.02a	0.27±0.02a	9.9±1.6b	13.0±1.7c
Caffeic acid-O-hexoside 1	n.d.	n.d.	n.d.	0.30±0.04a	tr
Vanillic acid	0.13±0.03	0.10±0.02	0.11±0.03	0.123±0.017	0.12±0.03
Caffeic acid-O-hexoside 2	n.d.	n.d.	n.d.	0.41±0.11a	0.9±0.5b
***p*-Hydroxybenzoic acid**	n.d.	tr	0.24±0.04a	0.65±0.11a	0.97±0.18ab
Caffeic acid-O-hexoside 3	n.d.	tr	n.d.	0.24±0.04a	0.56±0.12b
***m*-Hydroxybenzoic acid**	n.d.	n.d.	n.d.	n.d.	tr
Tyrosol	10.5±0.5a	11.0±0.9a	10.7±0.4a	10.7±0.8a	11.3±0.9a
Homovanillic acid	0.006±0.002a	0.07±0.02b	0.09±0.02bc	0.12±0.05c	0.16±0.02de
Caffeic acid	0.026±0.005a	0.49±0.005a	0.75±0.03a	17.6±1.0b	19.7±0.4c
Apigenin-C-hexoside-C-hexoside	n.d.	n.d.	tr	0.025±0.005b	0.083±0.007d
Ferulic acid-O-hexoside	n.d.	n.d.	n.d.	0.034±0.003b	0.020±0.004a
Coumaric acid	0.240±0.015a	1.59±0.06b	2.70±0.06c	5.1±0.3d	5.8±0.3d
Hydroxytyrosol acetate	3.9±0.4a	3.7±0.5a	3.5±0.7a	3.4±0.4a	3.4±0.5a
Rutin	n.d.	n.d.	n.d.	0.14±0.06a	0.18±0.06a
Kaempferol-3O-rutinoside	n.d.	n.d.	n.d.	0.18±0.04a	n.d.
Quercetin-3O-glucoside	n.d.	n.d.	n.d.	0.12±0.02a	n.d.
Kaempferol-3O-glucoside	n.d.	tr	n.d.	1.2±0.7b	0.33±0.02a
Apigenin-7O-glucoside	n.d.	n.d.	n.d.	0.84±0.13a	n.d.
Ferulic acid	0.014±0.002a	0.036±0.005a	0.77±0.07b	2.27±0.14c	3.47±0.13d
Rosmarinic acid	n.d.	tr	tr	6.0±0.4a	7.5±0.3b
DAFOA (decarboxymethylated)	10.9±1.5a	10±3a	11±3a	11±3a	12±2a
Kaempferol + Luteolin	0.63±0.04a	0.91±0.07ab	1.08±0.04b	2.99±0.12c	3.00±0.17c
Acetoxy-pinoresinol	2.7±0.3b	2.8±0.2b	3.0±0.2b	1.99±0.15a	2.6±0.2ab

Quercetin	n.d.	n.d.	n.d.	n.d.	0.79±0.14a
Naringenin	0.026±0.004a	0.23±0.02ab	0.331±0.019b	0.74±0.07c	0.99±0.09d
Apigenin	0.53±0.03a	1.10±0.09c	0.92±0.08bc	0.84±0.15ab	1.35±0.15c
Methoxyluteolin	0.39±0.05ab	0.43±0.02b	0.47±0.07b	0.28±0.04a	0.42±0.02ab
AFOA	8.7±0.6a	10±2a	8.3±0.8a	7.7±0.6a	8.2±0.7a
AFLA	5.2±0.3ab	5.0±0.7ab	4.5±0.4a	4.6±0.4a	4.3±0.5a
Carnosol	n.d.	5.3±0.3b	3.2±0.5a	12.4±1.0c	15.8±1.7d

C = control; OI = infused with oregano; TI = infused with thyme; OM = malaxed with oregano; TM = malaxed
oregano; TS = sonicated with thyme; DAFOA = dialdehydic form of oleuropein aglycone; AFOA = aldehydic form
aldehydic form of lingstroside aglycone.

Figure 1. Ultrasound inhibition effect of olive PPO. Olive paste was sonicated for 2, 4, 6, and 8 minutes before mala

significance vs control untreated (Anova test, $p < 0.05$).



ELECTRONIC SUPPLEMENTARY MATERIAL OF THE ARTICLE:

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THE EXTENDED AND DETAILED VERSION OF MATERIALS AND METHODS

Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, sodium carbonate, standard compounds as tyrosol and gallic acid, HPLC solvents and other reagents were purchased from Sigma (Sigma-Aldrich Chemical Company, Dorset, UK). Coumaric acid, caffeic acid, gallic acid, apigenin, luteolin, rutin, and hydroxytyrosol were purchased from Extrasynthese (Genay – France). All other chemicals and reagents were of analytical grade and solutions were freshly prepared before use.

Extraction of polyphenols from EVOO samples and aromatic herbs.

1 g of olive oil sample was diluted in 1 ml of n-hexane. Diluted samples were extracted by two 5 mL portions of methanol:water 70:30 (v:v) solvent. After separation from the lipidic fraction by centrifugation (6000 rpm for 10 min), the two hydrophilic extracts were combined.

Determination of total polyphenols.

The total phenols content in the polar fraction extracted from olive oil samples was measured by colorimetric methods using Folin-Ciocalteu reagent. For this purpose, 100 µL of phenolic extract or standard were pipetted in a 10 mL test tube and mixed with 100 µL 2 N Folin–Ciocalteu reagent and, after 5 min, with 800 µL of Na_2CO_3 5%. The mixture was held at 40 °C for 20 min and then, at room temperature for 15 min. The total phenol content was determined colorimetrically at 750 nm using Lambda Bio 20 UV/Vis Spectrometer Perkin Elmer. The results were expressed as mg of gallic acid for kg of oil. A calibration curve of gallic acid was acquired in the concentration range of 10-100 µg/mL ($R^2 = 0.949$).

HPLC-UV-MS^n analysis of polyphenols

Separation and identification of polyphenols were carried out by means of an HPLC-UV-ESI-MS^n system consisting of a 600-MS multi solvent delivery pump (Waters, Milford, MA, USA), a single wavelength UV-Vis detector (1100 series Agilent Technologies, Palo Alto, CA, U.S.A.) and an LCQ ion trap mass spectrometer (Thermo Electron Co., San Jose, CA, USA).

Briefly, after filtration through 0.45 μm pore size regenerated cellulose filters (VWR International, USA) EVOO and aromatic herbs exctracts were injected onto a reversed stationary phase column, Luna C18 (150 x 2 mm i.d., particle size 3 μm, Phenomenex, USA) protected by a C18 Guard Cartridge (4.0 x 2.0 mm i.d., Phenomenex). HPLC separation was accomplished using a binary mobile phase composed of (solvent A) acetonitrile and (solvent B) water containing 0.1% (v/v) formic acid. The following gradient was adopted: 0 min, 90% B; 1 min, 90% B; 15 min, 70% B; 22 min, 50% B; 28 min, 0%; 34 min, 0% B; 36 min, 90% B, followed by washing and re-equilibrating the column. The column temperature was controlled at 25 °C and the flow was maintained at 0.4 mL/min. UV-Vis detection wavelength was set at 280 and 320 nm. HPLC-ESI-MS data were acquired under negative ion mode using Xcalibur (Thermoquest) software. The optimized electrospray/ion optics parameters were as follows: spray voltage, 4.0 kV; sheath gas (nitrogen), 9 L/min; nebulizer, 40 psi, capillary voltage, 35.0 V; capillary temperature, 200 °C; tube lens offset voltage, 15.0 V.

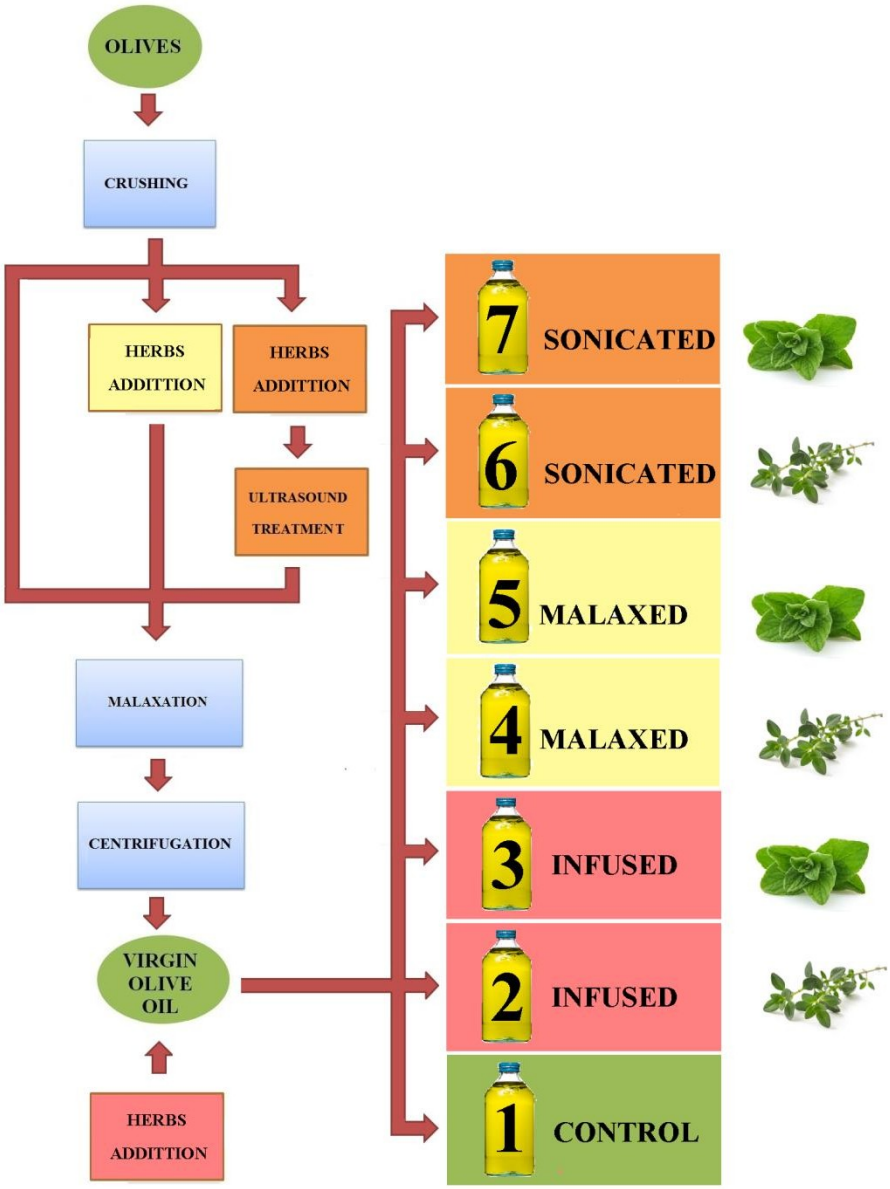
Typically, three runs were performed during HPLC-ESI-MS analysis of each sample. First, an MS full-scan acquisition (*m/z* range 100 – 1000) was performed to obtain preliminary information on the predominant *m/z* ratios observed during the elution. Subsequently, MS^2 spectra were acquired by fragmenting some precursor ions selected in the MS spectra. Tentative compound identification was achieved by combining different

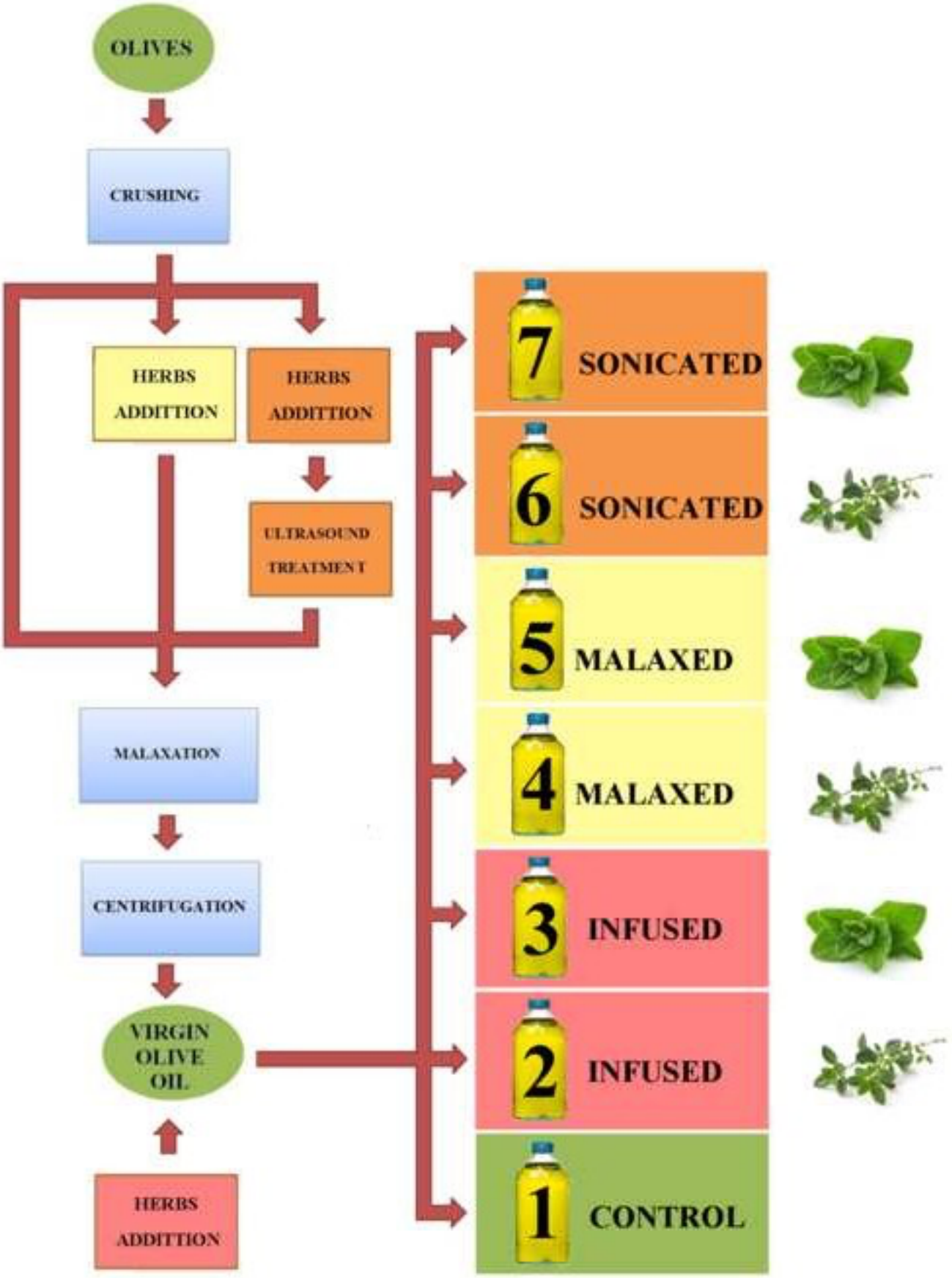
information: UV absorption, retention times (RT), and mass spectra (MS and MS^2) which were compared with those from pure standards, when available, and/or interpreted with the help of structural models already hypothesized in the literature. Polyphenols were quantified at 280 nm as apigenin (R^2 = 0.99903) and hydroxytyrosol (R^2 = 0.99918) or at 320 nm as caffeic acid (R^2 = 0.99914) and coumaric acid (R^2 = 0.99899) equivalents.

Enzyme PPO: extraction and assay

Fruit samples were frozen in liquid nitrogen and pulverized with a pestle and mortar. One to five grams of frozen sample were used to obtain a dried acetone powder and stored a -20 °C. Immediately before each PPO assay, 10 mg of acetone powder was resuspended in the proportion of 1:60 (w/v) in 0.1 M phosphate buffer, pH 6.2 with 0.3 mg/mL of type-II trypsin inhibitor,stirred for 1 h at 4°C, centrifuged at 5,000 *g* for 10 min at 4°C and filtered through glass wool. The crude extract was used for protein quantification and PPO assays. Protein concentration was determined by the Bradford method. Polyphenol oxidase activity based on an initial rate of increase in absorbance at 410 nm was determined spectrophotometrically using ultraviolet-visible spectrophotometer Ultrospec 7000 (GE, Company, UK). The assays were performed at 30°C in a medium containing 0.1M sodium phosphate buffer, pH 6.2, 40 mM catechol, 10 µL of fruit enzyme extract in a total volume of 1 mL. Specific activity was expressed as µmol/min x mg/protein.

Figure S1. Flow chart of the experimental plan. 1 (C): CONTROL (EVOO); 2 (TI): Infused with Thyme (EVOO + Thyme – 10 g/L); 3 (OI): Infused with Oregano (EVOO + Oregano – 10 g/L); 4 (TM): Malaxed with Thyme (OP + Thyme – 10 g/kg); 5 (OM): Malaxed with Oregano (OP + Oregano – 10 g/kg); 6 (TS): Sonicated with Thyme (OP + Thyme – 10 g/kg); 7 (OS): Sonicated with Oregano (OP + Oregano – 10 g/kg). The tests were conducted in triplicate.

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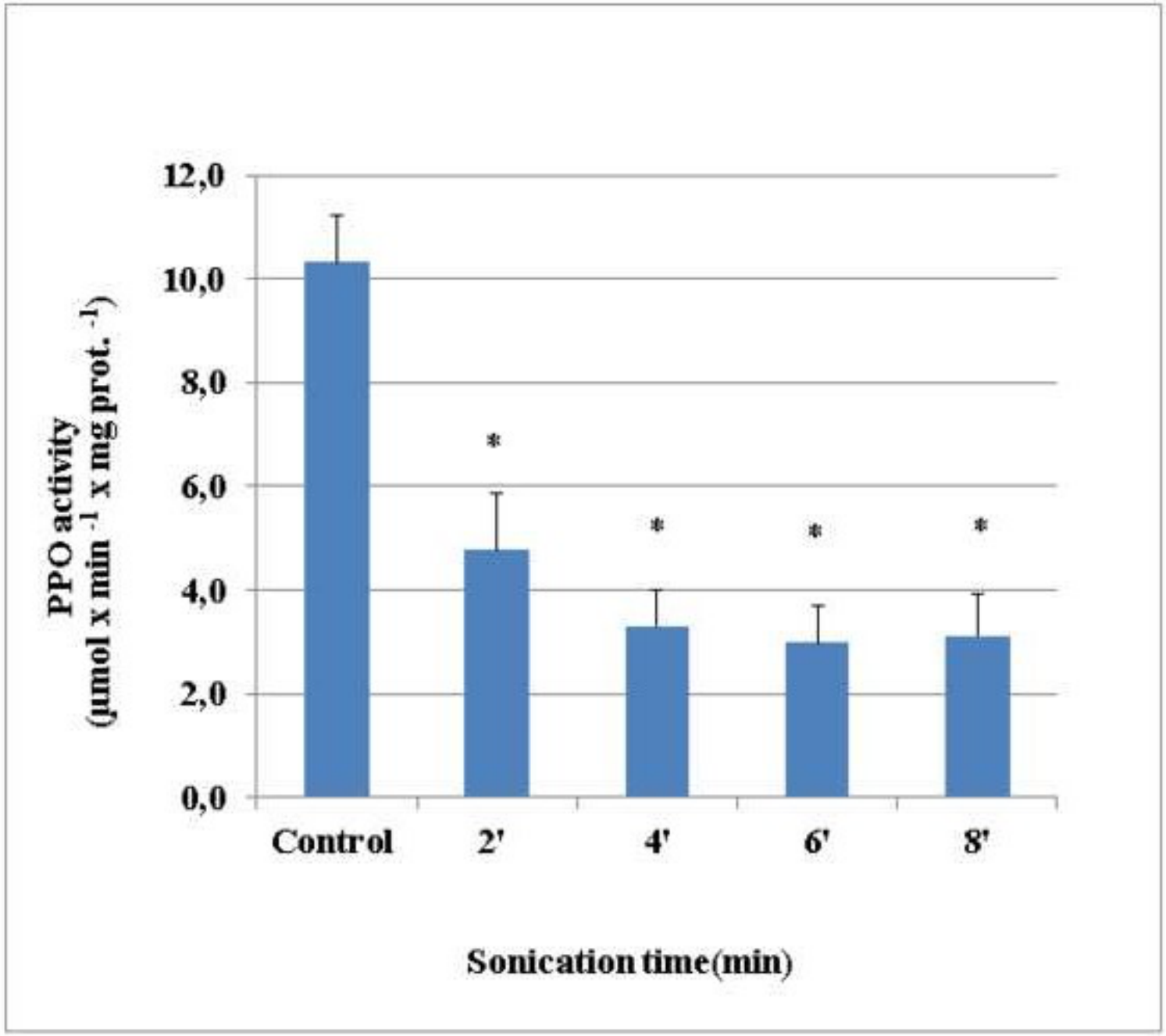
1 CONTROL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