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Short note

Effects of ozonation on the phenolic fraction of olive oil mill wastewater (OOMW): a study case

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Abstract: Olive oil mill wastewater (OOMW) is considered the most polluting fraction of olive processing residues, due to its high content in polyphenols. In this study it has been examined the possibility of using an ozone source as a strong oxidant agent, to lower the phenolic fraction of OOMW, allowing its use in agriculture. The OOMW, coming from a continuous 3-phase olive oil mill located in the province of Arezzo (Italy) was submitted to ozonation for 1, 3, and 8 hours, using an ozone generator. Total polyphenols, antioxidant activity, and the amount of tyrosol and hydroxytyrosol were determined on the derived samples. To measure the biotoxicity of the treated OOMW the germination test of radish seeds was used. The results of the chemical determinations highlighted the effect of dephenolyzation performed by ozonation of OOMW, with a significant decrease of antioxidant activity. Hydroxytyrosol was significantly lowered, depending on the duration of treatment, while tyrosol resulted less affected. The germination test showed that, with a 50% dilution of OOMW, the biotoxicity decreases as the ozone treatment increases.

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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1. Introduction

The production of olive oil in the world since 2010 has exceeded 3 million of tons; in the last decades olive growing has expanded beyond its traditional cultivation areas, characterized by warm and arid climate (Marone and Fiorino, 2012), thanks to the development of continuous oil separation techniques during the 1960s and 1970s, determining a qualitative improvement and a faster processing of the product (Kapellakis *et al.*, 2008), the harvesting mechanization (Fiorino *et al.*, 2010), the improvement of cultivation techniques, as well as new training systems and canopy management (Tous *et al.*, 2010). As processing by-product, important amounts of pomace (solid residues) and olive oil mill wastewater

(OOMW) are obtained (Table 1) (Di Giovacchino and Preziuso, 2006), both considered environmental pollutants.

In the past, the olive pomace obtained from pressure and continuous three-phase mills was used only to produce oil by means of chemical solvents (Di Giovacchino and Preziuso, 2006), whereas with the evolution of agriculture practices, and the search for new renewable sources of energy, it was employed as raw material for biogas production and, more recently, it started to be considered a dietary component of dairy cattle in order to improve the characteristics of both milk (Castellani *et al.*, 2017) and derived products (Castellani *et al.*, 2018).

The major problem related to the widespread use of pomace in agriculture depends on the high presence of phenolic compounds, with strong antimicrobial activity that determine a remarkable biotoxicity. Phenols show antioxidant properties but also phytotoxic actions in soil and are credited to reduce microbial growth in both anaerobic and aerobic digester. This aspect was explained by Girardi *et al.* (2014), who investigated different oxidative chemical treatments able to reduce both the phenolic content and the triglyceride fraction in various olive solid residues obtained through different extraction processes. In this way it was possible to obtain combined phenol-free compounds and reduced triglyceride, for a semi-finished product appropriated for agricultural purposes, using Hydrogen peroxide (H_2O_2) alone or combined with Fe^{2+} (the Fenton system).

The OOMW is probably the most polluting and biotoxic by-products of the olive processing because of their remarkable antioxidant capacity deriving from the phenolic compounds (De Marco *et al.*, 2007), even if it represents a promising resource for agriculture, especially considering their contribution in terms of water intake, and the high quantities of nutritive minerals directly deriving from the fruit juices.

An INCO-MED research project entitled ‘‘New Technologies for Olive Mill Waste Water Detoxification and Product Recovery’’ (NewTech OMW, Contract ICA3-CT-2002-10033) has been granted by the European Commission to valorize the OOMW. Started in 2003 and involving four Mediterranean countries, it deals with the treatment of OOMW with the aim to develop low-cost, low technology, and environment-friendly treatments to recover high added-value products for different agricultural uses. Among the explored solutions can be listed the addiction of fungal cells, soil minerals or pure oxidation catalysts (Gianfreda *et al.*, 2006).

Recently, Kerasioti *et al.* (2017) presented a work focused on the investigation of the effects of livestock feed supplemented with OMW on the enzymatic activity and protein expression of antioxidants enzymes, in liver and spleen tissue of sheeps. Therefore, in this case was presented a solution for the development of a low-cost intervention for pathological conditions associated with oxidative stress, while at the same reducing the potential risk of environmental pollution.

Among the oxidant agents commonly distributed in nature, the strongest is certainly represented by ozone (O_3), a component of the atmosphere, generated by the absorption of UV radiation by oxygen. It is considered a strong oxidant which guarantees a broad spectrum of bactericidal effects both in the gaseous and aqueous states. High reactivity, penetrability, and spontaneous decomposition without leaving any harmful by-product, justify the numerous potential uses and make it preferable compared to other oxidizing agents, such as sodium hypochlorite and hydrogen peroxide (Brodowska *et al.*, 2017). In food industry, the ozone application is mostly related to decontamination of water and detoxification of product surface, such as the elimination of mycotoxins and pesticide residues from some agricultural products (Jin-Gab *et al.*, 1999).

Because of its high oxidizing potential and the minimal environmental impact, this compound has been used for the first time in this work for the detoxification of OOMW through the control of the phenolic fraction in order to induce a decrease of the antioxidant activity. The aim of this study was therefore the development of a simple, cheap and low environmental impact methodology, transferable on

Table 1 - Quantity of olive pomace and olive oil mill wastewater (OOMW) obtained in olive processing by different mechanical systems

	Pressure	Decanter 3-phase	Decanter 2-phase and half	Decanter 2-phase
Olive pomace (kg t^{-1})	250-350	450-550	550-650	800-850
Olive oil mill wastewater (L t^{-1})	400-500	600-800	150-300	-

From Di Giovacchino and Preziuso, 2006.

an industrial scale, for oxidative detoxification of OOMW through ozonation.

2. Materials and Methods

Reagents and standards

Hydroxytyrosol, tyrosol, gallic acid, ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), TPTZ (2,4,6-tripyridyl-s-triazine), Trolox [(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid], Folin-Ciocalteu's reagent, sodium carbonate, potassium persulfate, $FeCl_3 \cdot 6H_2O$, methanol and HPLC-grade ethanol were purchased from Sigma-Aldrich (Milan, Italy), while ethyl acetate was obtained from Carlo Erba (Milan, Italy). All chemicals and reagent grade used in the present research were used without further purification steps.

Olive oil mill wastewater (OOMW) collection

The used OOMW from season 2017 (from October to December) was obtained in march of the present year from a decanting tank of a continuous three-phase olive oil extraction system located in the province of Arezzo (Italy), where the main cultivars are, in order, Moraiolo, Leccino and Frantoio.

Ozonation of OOMW

The OOMW was treated with ozone for 1, 3 and 8 h. For each condition, 500 mL of crude OOMW were placed in a glass beaker with a total capacity of 800 mL (base diameter: 10 cm; height: 13.5 cm), and ozone was insufflated at room temperature by using a OZOsteril generator (P.M.G. Depurazione, Vercelli, Italy) with a production capacity of 250 mg/h and an emission flow equal to 62±0.3 mL/min (Power: 30 W max). Samples were then aliquoted and stored at -20°C until use.

Seeds germination test

To evaluate the effect of ozonation on the biotoxicity of OOMW, a germination test was performed using radish seeds (*Raphanus sativus* L.), according to the methodology used for assessing compost maturity (Warman, 1999). Comparison was made among the germination of seeds in three different substrates: distilled water (W), untreated OOMW (C), and OOMW treated with ozone for 1, 3, and 8 hours (without dilution and after a 50% dilution (v/ v) with distilled water).

For each testing condition were prepared 10 Petri dishes (diameter 9.0 cm, height 1.5 cm) with a double layer of filter paper (Whatman® 41) soaked in 10 mL of each substrate, each dish containing 10 radish seeds appropriately arranged (Fig. 1).

The dishes were placed in a climatic chamber in the dark and at a temperature of 25±1°C. The surveys on germination were carried out after 1 day (T1), 3 days (T2) and 6 days (T3), counting the seeds for which it was possible to observe the appearance of the *plumula*. The obtained data were submitted to one way and multiway analysis of variance (ANOVA). Separation of means was performed by the Fisher's LSD test ($p<0.05$). Computations were performed by Statgraphics Centurion XV v. 15.0.04.

Extraction of phenolic compounds from OOMW

The recovery of OOMW phenolic compounds was performed according to the procedure reported by Dammak *et al.* (2016) with slight modifications. Ethyl acetate was added to OOMW samples (1:1; v/v) and the resulting mixture was stirred for 30 min at room temperature. After centrifugation at 4000 rpm for 5 min, the organic phase was separated, filtered with paper filters (Whatman® 41) and concentrated in a rotary vacuum evaporator (40°C). The residual was dissolved in methanol and filtered through a syringe filter (0.45 µm) before subsequent analysis.

Determination of total phenolic content

The total polyphenol content in samples of treated OOMW was estimated by the Folin-Ciocalteu colorimetric method, according to the procedure of Singleton and Rossi (1965). 10 µL of each sample were mixed with 90 µL of distilled water and 500 µL of freshly prepared 0.2 N Folin-Ciocalteu's reagent (1:10 v/v with water). After 10 min, 400 µL of satured

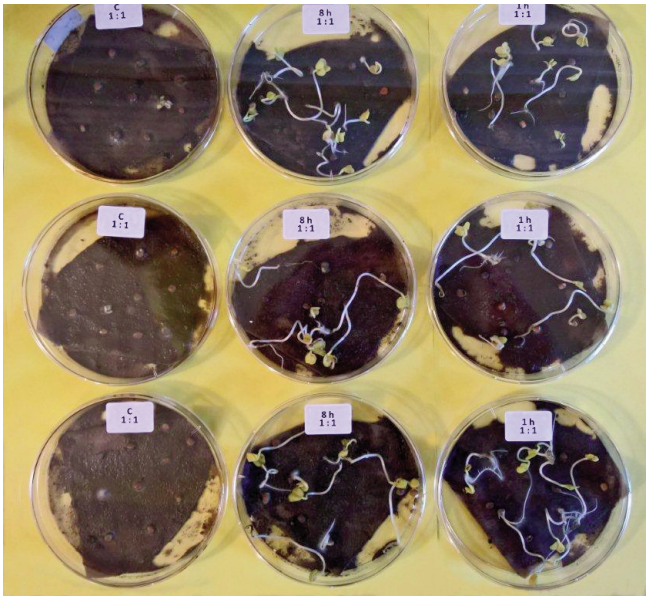

Fig. 1 - Radish seeds germination at T3; on the left, Petri dishes with untreated OOMW; in the middle, OOMW 8h; on the right, OOMW 1h (diluted 1:1 v/v).

sodium carbonate (75 g/L) were added. After incubation at 23°C (room temperature) for 1.5 h, the absorbance of the resulting blue coloured solution was measured at 765 nm with JENWAY 6305 UV/vis spectrophotometer. Quantitative evaluations were performed by using a standard calibration curve of six points (R^2 = 0.9944) ranging from 0 to 50 µg/mL of gallic acid in 80% methanol. The total phenolic content was expressed as gallic acid equivalents (GAE), in milligrammes per milliliter of sample.

Evaluation of total antioxidant capacity

The estimation of total antioxidant capacity (TAC) in OOMW extracts was carried by comparing two different approaches: the ABTS method and the ferric reducing antioxidant power (FRAP). With regard to the ABTS was used a modified methodology previously reported by Ozgen *et al.* (2006). ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) 7 mM was incubated at room temperature for 12-14 h with an oxidant (2.45 mM potassium persulfate) to obtain a stable, dark blue-green radical solution. The solution was than diluted with 80% HPLC-grade ethanol to an absorbance of 0.70±0.2 at 734 nm to form the test reagent. Reaction mixtures containing 10 µL of sample and 1.990 µL of reagent were incubated in the dark at room temperature for 6 min, and the reduction of color deriving from the antioxidants action was measured at 734 nm with JENWAY 6305 UV/vis spectrophotometer. The absorbance was compared to that of the calibrated Trolox standard (range 0-16 µM). Additional dilution was needed if the absorbance values were over the linear range of the standard curve. Results were expressed in terms of Trolox Equivalent Antioxydant Capacity (TEAC; mmol/mL).

The FRAP assay was done according to Thaipong *et al.* (2006). The fresh working solution was prepared by mixing in a 10:1:1 ratio 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution, and $FeCl_3 \cdot 6H_2O$ solution. 10 µL of each OOMW extract were allowed to react with 1.990 µL of the FRAP solution for 30 min in dark condition. The formation of colored product (ferrous tripyridyltriazine complex) was recorded at 593 nm. Results are expressed in mM TEAC per ml of OOMW extract.

HPLC analysis

The evaluation of phenolic profile was performed by using a HPLC method; attention was focused on the relative amounts of hydroxytyrosol and tyrosol before and after the oxidative treatments. The HPLC instrument was a Varian system coupled to a UV/Vis detector set at 280 nm and equipped with a SUPELCOSIL LC-18 column (5 µm particle size, 25 cm x 4.6 mm; Sigma Aldrich, Milan, Italy) maintained at 40°C in an oven. The eluent flow was fixed at 1 mL/min and the used HPLC grade solvents were water and methanol. The elution gradient starts with 80% of water and 20% of methanol; after 30 minutes the water starts to be excluded in favor of the methanol, until reaching the 100% of methanol after 50 minutes from the beginning of the analysis. This condition is then kept constant for further 20 minutes. At the end of the analysis (70 min) the ratio between the solvents was turned back to the initial 80:20 and kept for other 10 min (equilibration of the instrument). The biophenols of interest were identified by comparing the elution times of chromatograms obtained for OOMW extracts with those of standards.

3. Results

Chemical effects of OOMW ozonation

Following the ozonation of OOMW, a simple and efficient extraction of phenolic component from each sample was obtained by using ethyl acetate, allowing to analyze in an accurate way the total phenolic content, the antioxidant activity and relative quantities of two biophenols of particular interest: hydroxytyrosol and tyrosol.

The first noteworthy finding concerns the fact that the OOMW used in this study is characterized by a higher phenolic content than that reported in other studies (Ochando-Pulido *et al.*, 2015; Dammak *et al.*, 2016).

After treatment with ozone, a marked and time-dependent reduction of the phenolic content was evidenced. As reported in Table 2, the reduction of TPC is significant after 3 hours of ozonation (from 54.60 mg/mL to 35.34 mg/mL; $P<0.01$), and reaches the minimum value (16.19 mg/mL; $P<0.01$) after 8 hours of treatment.

These results correlates with the TAC which was evaluated in each sample through two different approaches; both the ABTS and the FRAP assay showed the lowest antioxidant potential after 8 hours of ozonation ($P<0.01$), that is precisely in the samples poorest in terms of phenolic compounds.

The HPLC analysis was performed with the aim of determining the influence of the ozone oxidation

Table 2 - Analytical parameters collected after oxidative treatments of OOMW with ozone for 1, 3 and 8 hours

	C	1h	3h	8h
Total phenolic compounds (GAE mg/mL)	54.60±2.98 a	51.10±2.59 a	35.34±4.68 b	16.19±0.42 c
Total antioxidant capacity				
ABTS (TEAC µmol/mL)	6.26±0.41 a	5.16±0.63 a	4.52±0.49 b	1.75±0.35 c
FRAP (TEAC µmol/mL)	3.72±0.12 a	3.03±0.06 b	2.13±0.02 c	1.62±0.14 d
Hydroxytyrosol*,§	741 a	639 b (81.0)	471 c (61.4)	490 c (66.1)
Tyrosol*,§	630 a	595 a (88.1)	511 b (78.0)	518 b (78.8)
pH	4.52±0.03 a	4.49±0.02 a	4.51±0.04 a	4.48±0.03 a

Values in the same row followed by different letters differ significantly (significance was set at P<0.05).
* Arbitrary unit.
§ % residual inside the round brackets.

treatments on the relative concentrations of three biophenols. In particular the attention was focused on hydroxytyrosol and tyrosol, two compounds commonly found in the by-products of the olive oil production industry, to which a high antioxidant activity is associated (De Marco *et al.*, 2007). The analysis showed only a partial reduction of hydroxytirosol and tyrosol content; as reported in Table 2, hydroxytyrosol showed a minimum residual of 61% after 3 h of ozonation, while tyrosol did not fall below the 78% even after 8 hours of treatment. This datum is not in full agreement with the marked reduction of the TPC previously reported.

Unexpected is the data concerning pH (Table 2), which does not seem to undergo significant changes.

Seed germination test

Table 3 shows the germination ability of the radish seeds, placed on different germination substrates: solutions of untreated OOMW (C) and OOMW derived from different ozonation times (1h, 3h, and 8h, respectively) diluted to 50%; as control, distilled water (W) was used. For each test, the number of seeds germinated after 1, 3 and 6 days (T1, T2, and T3, respectively), was counted. The trial was carried out also on the undiluted OOMW, but in this case no germination was obtained: therefore these data have not been reported.

From Table 3, it is clear that the radish has a high germination ability and, after 1 day, all the seeds placed in distilled water (W) germinated. In the 50% diluted OOMW (C) an inhibition is still measurable, and only after 6 days of staying on the substrate it was possible to observe the beginning of germination (on average 6.0%). It is also evident the "time effect" of ozonation, which allowed a germination percentage of 30% already on the first day for OOMW treated for 8 hours (T1) and 17% for OOMW treated for 3 hours (T1). In the case of the best result (8 hours of ozonation, T3), the percentage of germination is statistically equal to that obtained by using distilled water as a substrate (Fig. 2).

Table 3 - Average number of seed germinated per Petri dish (ten seeds) with different treatments (W, C, OOMW 1h, 3h, 8h) after 1 (T1), 3 (T2), and 6 days (T3), respectively

	Ozone treatment				
	W	C	1h	3h	8h
T1	10.0±0.0 d	0.0±0.0 a	1.0±0.0 b	1.7±1.49 b	3.0±1.15 c
T2	10.0±0.0 c	0.0±0.0 a	6.7±1.57 b	7.0±2.83 b	7.7±0.67 b
T3	10.0±0.0 c	0.6±0.52 a	7.0±1.49 b	7.3±2.83 b	8.7±0.82 c

Values in the same row followed by different letters differ significantly (significance was set at P<0.05).

The effect of the different ozonation times on the germination is highlighted in figure 2, that clearly shows as in OOMW (C) germination starts only after the third day of incubation, while the seeds placed on ozonated substrates arrange in a sequence (OOMW 1h, OOMW 3h, and OOMW 8h) which reflects the increases in the ozonation. The number of seeds that germinate continues to increase after the third day practically only in the OOMW 8h test, and on the sixth day (T3) 87% of the incubated seeds germinated.

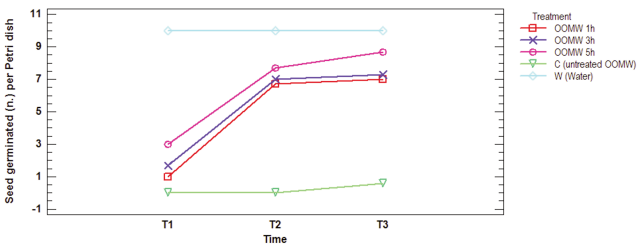

Fig. 2 - Interaction plot (treatment x time) from multiway ANOVA.

4. Discussion and Conclusions

Ozonation has proven to be a valuable tool for lowering the phenolic fraction in the OOMW. In the present study, using a laboratory instrument, after 8 hours of ozonation the phenolic fraction decreased to less than one third of the initial amount, with an almost equivalent lowering of the antioxidant activity, according to previous works; hydroxytyrosol showed to be reduced in a more marked way with respect to the tyrosol. The findings concerning the germination essay evidenced the detoxifying effect of ozonation; after 8 hours of ozonation the percentage of germinated seeds is statistically equal to that obtained using only distilled water as a substrate, while in the untreated solution the seeds germination is still inhibited after 6 days. These results are also confirmed by comparing the succession of the final germination percentages obtained using different ozonation times: 6% for untreated OOMW, 70% after 1, 73% after 3, and 87% after 8 hours of ozonation, respectively.

Further investigations should be performed to deeply investigate the biochemical effects of ozonation on OOMW, but the treatment is really promising for an effective dephenolyzation of the OOMW, even for oil mill industrial plants allowing to transform this by-product, currently considered highly polluting, in a crop product suitable for energy production in bioreactors, as natural fertilizer, or foodstuff supplement.

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