

## Olive Mill Wastewater Anaerobically Digested: Phenolic Compounds with Antiradical Activity

Francesco La Cara<sup>\*a</sup>, Elena Ionata<sup>a</sup>, Giovanni Del Monaco<sup>a</sup>, Loredana Marcolongo<sup>a</sup>, Marta R. Gonçalves<sup>b,c</sup>, Isabel Paula Marques<sup>b</sup>

<sup>a</sup>Istituto di Biochimica delle Proteine, CNR, Via Pietro Castellino 111, 80131 Napoli, Italia

<sup>b</sup>LNEG, Estrada Paço do Lumiar, 1649-038 Lisboa, Portugal

<sup>c</sup>IBB, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[f.lacara@ibp.cnr.it](mailto:f.lacara@ibp.cnr.it)

The recovery of phenolic compounds, present in the olive fruits and its by-products, has been intensively studied by the antioxidant properties. Olive mill wastewater (OMW) is a phenolic-rich industrial effluent that can be advantageously valorized by the anaerobic digestion to the methane and agricultural fertilizer productions. The objective of this work was to evaluate the antiradical activity of OMW after anaerobic digestion in order to maximize the valorization of this type of effluents. The digested flow was obtained from an anaerobic hybrid reactor treating OMW at different organic loading rates (OLR), from 3.3 to 7.1 kg COD m<sup>3</sup> d<sup>-1</sup>. OLR rise was applied by increasing progressively the OMW volume fraction from 8 % to 83 % in the feed mixture. The input and output streams, obtained at different OMW volume fractions, were characterized in terms of antiradical activity and phenolic compounds identification and quantification. Despite of the fraction decrease on total phenolic compounds provided by OMW anaerobic digestion, the antiradical activity is still significantly high (EC50 = 3.24) in the digested effluent. Oleuropein was the main phenolic compound present in the substrate before and after anaerobic digestion (about 15 % of the initial value). Others phenolic compounds present are: gallic acid, hydroxytyrosol, tyrosol, and quercetin. These data confirmed that, after the OMW anaerobic treatment to produce biomethane, the remaining flow yet contain useful compounds with antiradical activity.

### 1. Introduction

The Mediterranean region is world's leading olive growing area, and olive processing has been an important and traditional industry for its countries since ancient times. Worldwide olive oil production is reported to be about 3,024,000 t for the years 2009/2010 and the highest amount is in European Union (EU) with 74 % of the total (IOOC, 2010). The production of olive oil, depending on the process used, usually yields next to 20 % olive oil, 30 % semi-solid waste and 50 % aqueous liquor. The liquid effluent of olive oil process, the olive mill wastewater (OMW), amounts to 0.5–1.5 m<sup>3</sup> per 1000 kg of olives. OMW is characterized by intensive violet-dark brown up to black color, strong specific olive oil smell, high degree of organic pollution (chemical oxygen demand; 40-220 g L<sup>-1</sup> and biochemical oxygen demand; 35-110 g·L<sup>-1</sup>), pH between 3 and 6, total organic compound of 25-45 g·L<sup>-1</sup>, high electrical conductivity, high content of polyphenols (0.5–24 g·L<sup>-1</sup>), reducing sugars up to 60 % of the dry substance, high content of solid matter, and potassium as the predominant inorganic material (~4 g·L<sup>-1</sup>) (Niaounakis and Halvadakis, 2006).

The amount of such pollutant compounds prohibit OMW, generated during the oil extraction processes, to be directly discharged into water or onto land. Several treatment procedures including physical, chemical, biological or combined technologies have been tested to reduce undesirable properties of OMW. Among the diverse biological procedures studied, the utilization of the anaerobic digestion was reported as one of the most promising technologies for the disposal of OMW (Marques, 2000; Marques, 2001; Sampaio et al., 2011; Gonçalves et al., 2012).

OMW represents a severe environmental problem due to its highly polluting organic fluid also arising from polyphenols content. In spite of its low biodegradability, since it contains dozens of phenolic compounds, OMW is also regarded as a potent and cheap source of natural antioxidants (Bertin et al., 2011). Consequently, during recent years, several studies have been undertaken to elucidate the potentiality of these compounds present in the OMW (Takaç and Karakaya, 2009; He J. et al., 2011).

The aim of the work is to maximize the OMW valorization through the assessment of its ability to provide compounds of industrial interest after the energetic valorization step (anaerobic digestion). So, the goal of this study is to evaluate antiradical activity of OMW digested anaerobically in a hybrid digester, working under different operational conditions.

## 2. Materials and Methods

### 2.1 Chemicals

Chemical reagents and solvents for HPLC analysis are all of analytical grade and were from SIGMA-Aldrich (St. Louis, MO, USA).

### 2.2 Experimental set-up

The anaerobic digestion experiments were performed in an up-flow anaerobic hybrid digester. The unit (Figure 1) was built out of polyvinyl chloride (PVC) pipe with a total volume of about 2 dm<sup>3</sup>. A packed bed, selected in previous studies (Marques, 2001), was used to fill only 1/3 of reactor's height.

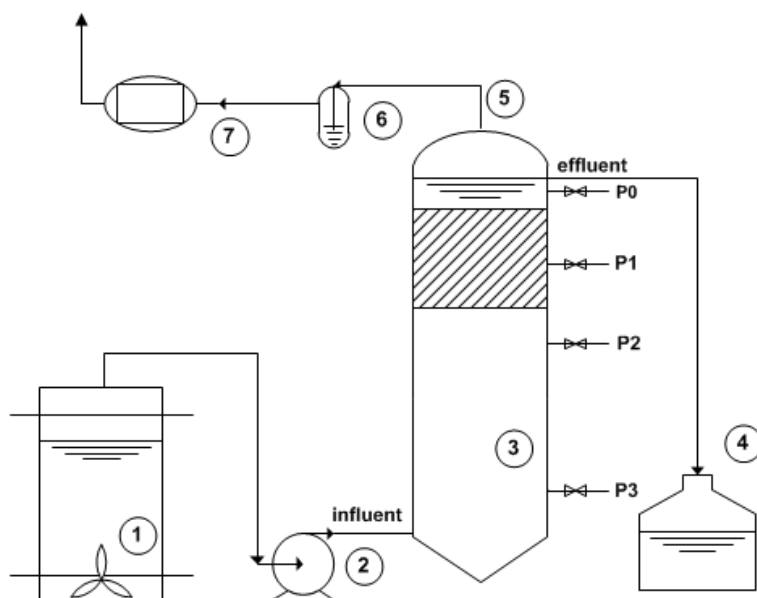


Figure 1: Experimental set of the hybrid digester. (1) feeding tank; (2) peristaltic pump; (3) hybrid digester; (4) treated effluent; (5) Biogas exit; (6) liquid trap; (7) gas counter. Sampling zones: P0 – 56 cm, P1 – 43 cm, P2 – 31 cm and P3 – 7 cm

No device separator of solid/liquid/gas was installed and no substrate recycle was provided. The digester was semi-continuously fed by a time controlled peristaltic pump and maintained at  $37 \pm 1$  °C using a water jacket. The feed tank temperature was kept at about 4 °C. The gas production was measured by a wet gas meter and corrected to standard conditions for temperature and pressure (0 °C, 100 kPa). Four ports were located along the digester length to access different zones: sludge bed (P3; 7 cm), immediately below filter zone (P2; 31 cm), in the middle of the filter zone (P1; 43 cm) and in the liquid top layer (P0; 56 cm).

### 2.3 Substrates and samples

The effluent generated in the olive oil campaign of 2007/2008 was collected in an olive oil mill (three-phase continuous extraction) in Rio Maior (Portugal) and stored in the olive mill underground tanks. The OMW complementary substrate, piggery effluent, was provided by a pig fattening installation also in Rio Maior. The effluent was collected in plastic containers, characterized at the time of arrival at the laboratory and then stored in the dark at 4 °C.

The anaerobically digested effluents were obtained from an anaerobic hybrid reactor (Figure 1) treating OMW at different organic loading rates ( $3.3 - 7.1$  Kg COD  $m^3 \cdot d^{-1}$ ). The reactor started to be fed with an OMW volume fraction of 8 % in the feed mixture. The volume fraction of raw OMW was gradually increased in the feed mixture from 8 % to 83 % under a HRT (hydraulic retention time) of about 6 days. Piggery effluent was used to add up to the total volume of the mixture. The digester was operated for about 300 days (Gonçalves et al., 2012). During all the operation time, no nutrients were added; no chemical correction of pH and no dilution with tap water were performed.

### 2.4 Antiradical activity

The antiradical activity of the digested OMW was determined by a modification of the method described by von Gadow et al. (1997). Briefly, one milliliter of a  $6 \times 10^{-5}$  M methanolic solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was added to 10 mL of a methanolic solution of the OMW samples mixed and placed in 1-cm glass cuvettes. The decrease in absorbance at 515 nm was determined continuously with data acquisition at 2-s intervals with a spectrophotometer Varian, Cary 50 for 16 min (until the absorbance stabilized).

The inhibition percentage (IP) of the DPPH radical by the phenolic compounds of the OMW extracts was calculated according to the formula

$$IP = [(A_{C(0)} - A_{C(t)}) / A_{C(0)}] \times 100 \quad (1)$$

where  $A_{C(0)}$  is the absorbance of the control at  $t = 0$  min and  $A_{C(t)}$  is the absorbance of the reaction solution at  $t = 16$  min (Cruz et al., 2001).

### 2.5 Total polyphenols analysis

Total phenolic compounds concentrations, were expressed as mg of caffeic acid equivalent (CAE) and were determined via a modified Folin–Ciocalteu method (Singleton and Rossi, 1965).

### 2.6 HPLC analysis of phenolic compounds

Aliquots of 0.1 ml of each sample were diluted in 5 ml of acid methanol (70:29:1; methanol:water:HCl) and incubated at 37 °C for 30 min in a rotary shaker. The suspension was centrifuged for 15 min at 3500 rpm and the supernatant was recovered and used for polyphenols assay.

HPLC/UV analysis of single phenolic compounds was performed utilizing a  $250 \times 4.6$  mm (5  $\mu$ m)  $C_{18}$  Hypersil column (Thermo Electron Corporation, Bellefonte, PA, USA) used with a Securityguard precolumn (Phenomenex, United Kingdom) with a  $C_{18}$  cartridge in combination with a Thermo-Finnigan Surveyor HPLC system (solvent degasser, quaternary pump, thermostatically controlled column oven set at 25 °C, a photodiode array detector set to collect overall data from 200-600 nm, and selected wavelengths of 230, 254 and 280 nm).

Peak identifications were confirmed from retention times, UV spectroscopic data, and direct comparison to pure standards. The solvent flow rate was 0,9 ml/min and the mobile phase was a four-step linear solvent gradient system (0–30 min, 10% B; 30–35 min, 55% B; 35–40 min, 100% B; 40–45 min, 100 % B) using 2 % acetic acid in water as solvent A and 0.5 % acetic acid in 50 % acetonitrile as

solvent B. Identification of phenolic compounds in the OMW extracts was performed by HPLC-UV, comparing the relative retention times and UV spectra with those of standard solutions.

### 3. Results and discussion

OMW was treated by anaerobic digestion without any pretreatment. Biogas productivities up to  $3.2 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$  were obtained (Gonçalves et al., 2012). After the anaerobic treatment, the effluent remained with a dark-brown colour and with a significant fraction of phenolic compounds (50-60 %), independently of the OMW volume fraction in the digester feed (Gonçalves et al., 2012).

The antioxidant activity and total phenolic compounds of the effluents were evaluated before and after the treatment. The highest antiradical activity values (6.7 and 8.5 EC50) corresponded to the maximum polyphenols contents ( $1.1$  and  $1.3 \text{ g}\cdot\text{L}^{-1}$ ) and the highest volume fraction of OMW in the digester feed (69 and 83 % OMW) (Table 1).

Table 1: Total phenolic compounds and antiradical activity in OMW

Reactor operation period		Total polyphenols mg CAE mL <sup>-1</sup>	% of DPPH inhibition	Antiradical activity EC50*
8	IN	0.25	53.8	2.32
8	OUT	0.18	48.9	1.84
12	IN	0.27	53.3	2.54
12	OUT	0.12	50.4	1.19
69	IN	1.07	80.4	6.65
69	OUT	0.30	55.0	2.73
83	IN	1.32	77.4	8.53
83	OUT	0.38	52.5	3.62
Piggery effluent	-	0.20	49.6	2.02

\*The antiradical activity was defined as the amount of antioxidant (expressed as  $\mu\text{g}$  of total polyphenols) necessary to decrease the initial DPPH concentration by 50 % (EC50 = Efficient Concentration)

The antiradical activity of effluent vs influent did not decreased very much at 8 % v/v OMW. On other hand, the decrease of antiradical activity was almost constant (41-47 %) by increasing the OMW amount in influent (12 to 83 % OMW).

During the anaerobic process a decrease in polyphenol concentrations and in antiradical activity occurred but the reduction of the former was higher than the antiradical activity. This data suggests that anaerobic treatment is a process able to remove/convert phenolic compounds but it does not eliminate the antiradical power of the digested flows. A similar removal data were obtained comparing the IN and the OUT of the digester operating with the lower volumes fractions of OMW (8 and 12 %). On the other hand, the amount of polyphenol and antiradical activity of such substrates were similar of the piggery effluent content (Table 1).

The HPLC sample analyses showed several peaks corresponding to different phenols among which some compounds were identified: phenyl acids (gallic, caffeic and ferulic acid), phenyl alcohols (hydroxytyrosol, tyrosol), catechin, rutin, quercetin and oleuropein (Table 2).

Oleuropein was the main phenolic compound present in the substrate before and after anaerobic digestion. The concentration of such compound increased from values of about 100 to  $1100 \mu\text{g mL}^{-1}$  as the amount of OMW in influent was implemented. Except for the operational period using 8 v/v % OMW in digester influent, the oleuropein removal capacity of the system was increased with the OMW volume implementation in the feed (76-91%). This may results from the positive evolution of biomass in terms of acclimation to the toxic phenolic compounds of the influent.

Table 2: HPLC analysis of phenolic compounds ( $\mu\text{g}\cdot\text{mL}^{-1}$ )

OMW (v/v, %)	GA	HT	T	C	CA	FA	R	O	Q
8	2.30	nd	38.48	38.50	13.85	1.38	0.19	179.5	3.63
8	0.75	nd	nd	nd	nd	nd	nd	194.0	23.63
12	nd	nd	nd	13.0	7.63	9.00	0.30	93.55	21.15
12	4.56	nd	8.05	23.80	1.33	nd	nd	22.50	6.95
69	25.02	87.50	35.95	13.0	23.58	55.98	nd	300.0	4.88
69	30.65	nd	nd	nd	nd	nd	nd	27.20	10.50
83	5.85	52.50	55.15	16.25	8.65	21.25	0.14	1125	4.05
83	1.38	nd	26.38	nd	3.18	nd	nd	115.8	23.70
Piggery effluent	1.38	8.13	21.05	16.08	5.55	14.10	nd	8.18	nd

GA = gallic acid; HT = hydroxyl-tyrosol; T = tyrosol; C = catechin; CA = caffeic acid; FA = ferulic acid; R = rutin; O = oleuropein; Q = quercetin; nd = not detected

After the anaerobic treatment, a significant fraction of phenolic compounds can be present. In particular, gallic acid, hydroxytyrosol, tyrosol, and quercetin were identified and quantified.

A general decrease in concentration of the identified phenolic compounds was observed. The exception is related to the quercetin whose concentration was increased during anaerobic process in almost operational situations (Table 2). The quercetin is a flavonoid widely distributed in nature with antioxidants properties that act as a scavenger substance against the free radical formation in the human body (Formica and Regelson, 1995).

In conclusion these data confirmed that, after the OMW treatment by anaerobic digestion to produce biomethane, the remaining flow yet contain useful compounds with antiradical activity.

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