Innovative method for recovery and valorization of hydroxytyrosol from olive mill wastewaters

A. Bonetti, S. Venturini, A. Ena and C. Faraloni

ABSTRACT

The nutritional properties of olive oil can be attributed to its oleic acid and phenolic compounds content, acting as natural oxidants to prevent human diseases. In particular, hydroxytyrosol has an anti-inflammatory action similar to omega 3 fatty acids from fish oil. The olive oil production was conducted by two extraction procedures: first, a two-phase extraction giving extra-virgin olive oil and humid pomace, second, a three-phase working process of humid pomace, obtaining another minimum quantity of extra-virgin olive oil, 'dry' pomace devoid of polyphenols, and mill wastewaters rich in anti-oxidant compounds. The aim of this processing was to employ water to extract the highest concentration of polyphenols from humid pomace and convey them in oil mill wastewaters for extraction. Processed olives were 37,200 kg, pomace deprived of polyphenols was equal to 20,400 kg and processing was performed with 500 kg of olives per hour. This method offers advantages of using cheap equipment and technical simplicity.

Key words | hydroxytyrosol, olive oil mill wastewater, olive oil mill wastewater treatment, polyphenols

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INTRODUCTION

Mediterranean people have been growing olive trees for thousands of years, acquiring strong competence in methods for olive oil extraction. Today, 98% of the 900 million olive trees covering 10 million hectares worldwide are located in the Mediterranean Basin (Sesli & Yegenoglu 2009). The leading olive oil producers are Spain, Italy, Greece and Portugal. Olive oil has excellent nutritional properties, and its consumption, traditionally restricted to the Mediterranean area (77% of the worldwide output), is now increasing all over the world (Dermeche *et al.* 2013). The success of olive oil can be explained by the benefits of the Mediterranean diet, which is associated with lower incidences of atherosclerosis, some types of cancers, and cardiovascular and neurodegenerative diseases (Owen *et al.* 2000; Perona *et al.* 2006). These apparent health benefits have been partially ascribed to the dietary consumption of virgin olive oil by Mediterranean populations. Moreover, the rising popularity of this food is predominantly attributed to its oleic acid and phenolic compounds content, which may, respectively, affect plasma lipid/lipoprotein profiles (Fki *et al.* 2005a, 2005b) and act as natural oxidants to prevent human diseases (Owen *et al.* 2000). In addition, phenolic compounds have anti-inflammatory, antiproliferative and anti-atherogenic properties (Atmani *et al.* 2009; Cicerale *et al.* 2010; Loizzo *et al.* 2011).

Olive fruits contain characteristic polyphenolic antioxidant compounds. In plant cultures, polyphenols, together with flavonoids, play a key role in protecting tissues from UV, which causes lesions, and herbivore attack (Caldwell *et al.* 1983). Moreover, these compounds protect plant cell metabolism from oxidative damage (Yamasaki *et al.* 1997; Visioli *et al.* 2002). Among them, hydroxytyrosol, which is synthesized by olive plants to protect them from adverse weather conditions, has been demonstrated to have biological activities, including inhibition of cancer cells and protection against oxidative effects.

Hydroxytyrosol from olives exhibits an ORAC activity (ability to neutralize free radicals) 10–15-fold higher than that of vitamin C, quercetin, or vitamin E.

Hydroxytyrosol is a very small molecule, able to penetrate tissue very quickly, and it has been confirmed as one of the most interesting biophenols present in olive fruits. Many studies have confirmed the importance for human health of this compound: in Sweden, patients with

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rheumatoid arthritis exhibited a marked diminution of pain and inflammation after three months of a Mediterranean diet employing olive oil. The conclusion was that polyphenols or hydroxytyrosol in olive oil possess an anti-inflammatory action similar to omega 3 fatty acids from fish oil. Considering the high antioxidant activity of polyphenols, adding these substances to food not only increases nutraceutical value for this type of food, but preserves the food itself from degradation (Sköldstam *et al.* 2003).

Despite these interesting properties, hydroxytyrosol is not commercially available in high amounts yet. Several methods have been proposed for hydroxytyrosol production by means of chemical (Tuck *et al.* 2000) or enzymatic synthesis (Espin *et al.* 2001), but protocols are usually slow and expensive, resulting in a small number of commercially available products containing pure hydroxytyrosol. In an *in vitro* model of neuroinflammation, hydroxytyrosol was shown to inhibit production of pro-inflammatory cytokines, suggesting that the anti-inflammatory activity of hydroxytyrosol may impact neuroinflammation in Parkinson's and other neurodegenerative diseases (Bitler *et al.* 2012).

Hydroxytyrosol in olive fruit is synthesized through oleuropein (phenolic compound present in olive fruits and leaves) hydrolysis. Studies on rats have demonstrated that oleuropein (most representative polyphenol in extra-virgin olive oil, which is hydrolysed to hydroxytyrosol and elenolic acid during milling) possesses effects on spontaneous tumours: they regress after treatment with oleuropein at 1% in water after 9–12 days (Puel *et al.* 2006)

When olives are milled to produce extra-virgin olive oil, these important phenolic compounds are quite completely conveyed in olive oil mill waste waters. More than 99% of active polyphenols in olive fruits are present from 100 to 300-fold more concentrated in olive oil mill waste water. It has been demonstrated that polyphenols from olive oil mill wastewater increase antioxidant capacity of blood plasma in rats, evidencing a role in heart protection.

Olive oil is defined as the oil obtained from olive fruit, and its production process includes different phases. The extraction of olive oil is achieved through discontinuous (pressing) or continuous (centrifuging) processes. Water is used in some of these steps to squeeze out most of the oil from the olive. Before milling, olive fruit is pitted and then processed in mills. Once the olive fruit has been crushed, the resulting paste is mixed and malaxed to increase the percentage of available oil and help small droplets to coalesce and agglomerate, facilitating the separation of the oil from water phases. The discontinuous pressing process is the oldest and most widespread method for processing olive fruit to obtain olive oil. After grinding, olive paste is pressed to compact the solid phase of the olive paste and to percolate the liquid phases (oil and vegetation water). This two-phase process permits obtaining extra-virgin olive oil and olive pomace (olive cake). This mixed by-product, with an emulsion containing the olive oil, is separated by decantation from the remaining oil mill wastewaters (OMWW).

This method offers advantages, as only cheap equipment and technical simplicity are required (Niaounakis & Halvadakis 2006). By contrast, disadvantages consist of process discontinuity and production of OMWW with a higher chemical oxygen demand compared with OMWW produced by the other process (Di Giovacchino *et al.* 2002). In major olive oil-producing countries such as Italy and Portugal, 5,000–6,000 and 1,000 mills, respectively, are operating using the traditional discontinuous process.

Unfortunately, olive oil extraction generates great amounts of two by-products, consisting of a solid residue and an effluent known as olive OMWW. Both of them, and especially OMWW, are considered harmful to the environment, as several studies have demonstrated their negative effects on soil microbial populations (Paredes *et al.* 1987; Della Greca *et al.* 2001; Rana *et al.* 2003). This is due to the fact that olive phenols are major contributors to this pollution due to their toxicity and anti-microbial activity. Estimates put the annual worldwide production of OMWW at between 7 and over 30 million m^3 (Aragon & Palancar 2000; Niaounakis & Halvadakis 2006).

Italy is the only olive-oil producing country in the world where legislation for disposal of olive mill wastes exists. Italian law allows the spreading of OMWW on fields to help the olive oil industry, which otherwise will be compromised (Law n. 574 1996). The disposal is up to 50 and 80 m^3 $ha^{-1}y^{-1}$, for traditional and modern plants, respectively. However, the law's application is not practically feasible due to the hilly landscape of Italian olive-growing regions, the difficulty of spreading during the winter season for lack of accessible soil, and the high energy cost required for transport and spreading of OMWW (Altieri & Esposito 2008).

Several techniques have been used to recover phenolic compounds from olive by-products, including enzymatic preparation, solvent extraction, membrane separation, centrifugation and chromatographic procedures (Dermeche *et al.* 2013). Solvent extraction is the most commonly employed technique to extract phenolic compounds, and ethyl acetate is the most effective solvent for the treatment of OMWW under acidic conditions (Allouche *et al.* 2004), even if the disadvantages of employing solvents have been

overcome by other techniques such as employment of supercritical CO_2 and ultrasound-assisted extraction (Usaquen-Castro *et al.* 2006). Finally, Garcia-Castello *et al.* (2010) recovered and concentrated OMWW phenolic compounds with an integrated membrane system based on membrane techniques.

This paper presents an innovative solution to exploit OMWW with an immediate economic valorization of humid pomace. A mill prototype has been projected with an initial discontinuous two-phase process to produce extra-virgin olive oil and humid pomace.

In the subsequent three-phase process, the pomace will be processed by using different water/solvent quantities. The application of this technology allowed us to produce: (a) dephenolized pomace to be used in biodigestors; (b) clean water to be reused in the mill; (c) heavy quantities of OMWW containing polyphenols; and (d) kernels to produce thermal energy.

METHODS

Prototype construction

A mini-mill prototype has been assembled by Dr Ena's group at CNR-ISE Florence (Figure 1). The prototype has been built in such a way as to perform each phase of olive oil production, olive oil production, de-phenolization of pomace and polyphenol enrichment of mill wastewaters in a structured sequence, so as to better study the functioning of each production phase. Two working phases are present: the first is a two-phase extraction giving extra-virgin olive oil and humid pomace; the second phase is a three-phase working process of humid pomace, so as to obtain another minimum quantity of extra-virgin olive oil, 'dry' pomace devoid of polyphenols, and mill wastewaters rich in anti-oxidant compounds. The aim of working with a two-phase process followed by a three-phase process permits addition

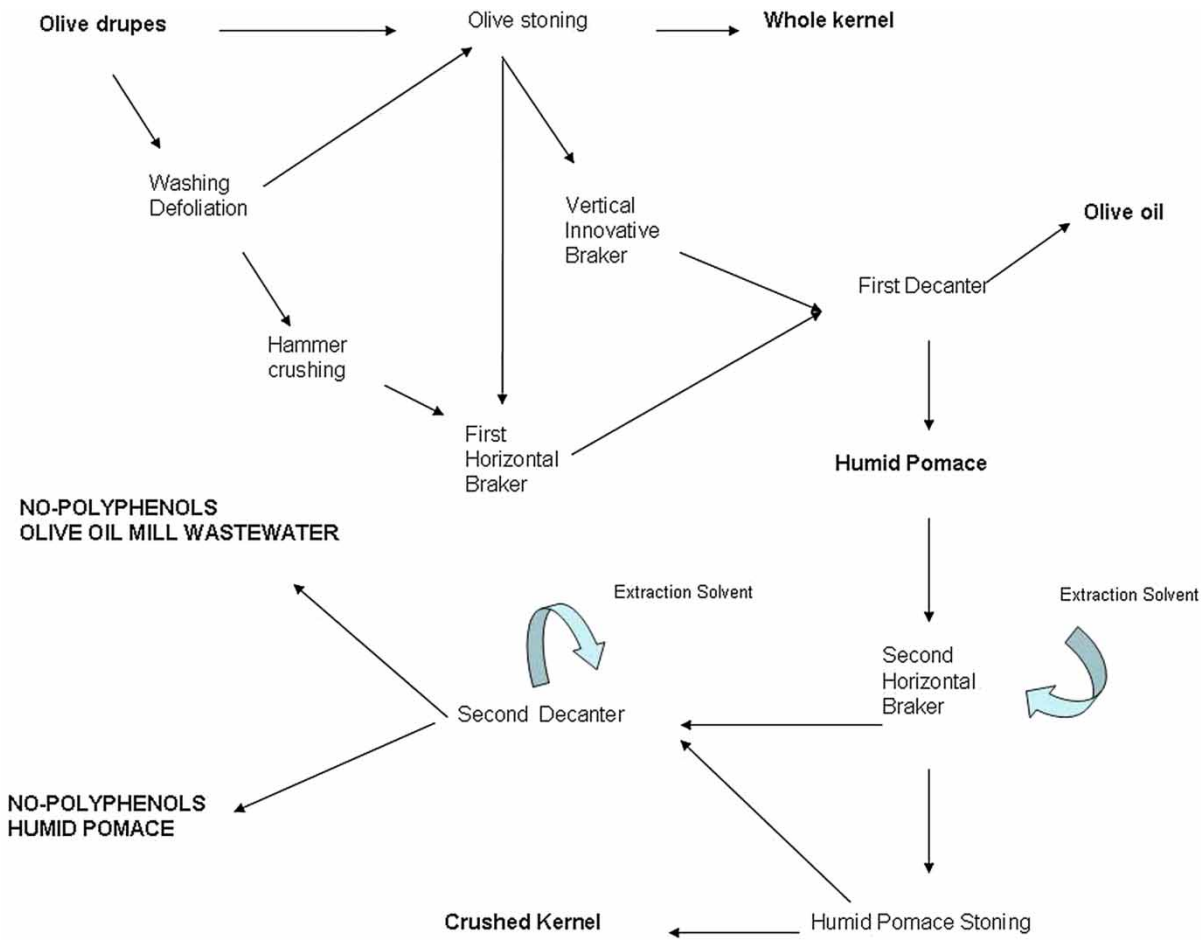

Figure 1 | Scheme of the prototype.

of water to extract the highest concentration of polyphenols from humid pomace, convey them in OMWW and then extract them. Processed olives were equal to 37,200 kg, pomace devoid of polyphenols was equal to 20,400 kg, and processing was performed with 500 kg/h of olives.

Polyphenol extraction from humid pomace

Tests were performed to assess the optimal water quantity to obtain the highest concentration of polyphenols in mill wastewater. Humid pomace was inserted in a horizontal press connected to a three-phase decanter and processed according to three sets of six experiments each, according to the scheme in Table 1. The concentration of polyphenols in humid pomace was estimated to be 8.74 g/kg as determined by the Folin–Ciocalteu test (Folin & Ciocalteu 1927).

Polyphenol concentration and desorption

OMWW obtained after the milling process was adsorbed on active carbon resins (Ena *et al.* 2009) and concentrated until polyphenol concentration on activated carbon (AC) plant was 5–10 g/L. To this end, two concentrators were employed: a solid–liquid and an ultrasound extractor, and the following parameters were determined: optimal time for desorption, optimal solvent for polyphenol extraction and optimal extraction temperature.

Ultrasound extraction

In the first set of experiments, an ultrasound extractor was employed with a power of 9 Hz. For desorption, 3.5 L of a food-grade solvent given by isopropanol and ethanol at pH 3.2 was employed together with all active carbon (equal to 335 g of dry AC). A power of 9 Hz and times of 20 and 30 min were chosen for the analysis. Two more experiments were subsequently performed, the first one with a total extraction time of 60 min with a intermediate polyphenol desorption after 30 min, and the second one with a total extraction time of 90 min with two intermediate desorptions at 30 and 60 min.

Solid–liquid extraction

A second set of experiments was performed to further improve the extracting method by employing a solid–liquid extractor with different compressing and decompressing cycles. The first set of experiments was effected with 14 cycles of extraction for a total of 126 min by employing the same solvent in ultrasound extraction. The second set of experiments was effected with a total time of 45 min. Finally, the last set of experiments was performed with seven cycles, with a total time of 63 min.

Polyphenol quantification

Polyphenol concentration was determined according to the Folin–Ciocalteu test, modified. Briefly, in a 50 mL flask, 1 mL of sample was made to react with 5 mL of Folin–Ciocalteu reagent. After 5 min, 10 mL of a sodium carbonate solution was added to stop the reaction. The volume was adjusted to 50 mL, and after 3 h, samples were read at 730 nm and absorbance registered. Polyphenol concentration was calculated in g/kg (Folin & Ciocalteu 1927).

Extra-virgin olive oil and OMWW analysis

Olive oil obtained from this milling procedures was analysed according to EC Regulation n. 2568/91 (1991). All concentrated samples from OMWW were analysed for the

Table 1 | The three sets of experiments with different water quantities in decanter and press

	Set A		Set B		Set C	
	II Decanter	II Press	II Decanter	II Press	II Decanter	II Press
Exp. 1	200 L/h	No water	100 L/h	No water	100 L/h	250 L/h
Exp. 2	500 L/h	No water	200 L/h	No water	200 L/h	250 L/h
Exp. 3	800 L/h	No water	350 L/h	No water	350 L/h	250 L/h
Exp. 4	200 L/h	250 L/h	500 L/h	No water	500 L/h	250 L/h
Exp. 5	500 L/h	250 L/h	650 L/h	No water	650 L/h	250 L/h
Exp. 6	800 L/h	250 L/h	800 L/h	No water	800 L/h	250 L/h

content of main ions and pesticides according to standard procedures.

Standards and chemicals

Authentic standards of hydroxytyrosol were purchased from Carlo Erba. Methanol, acetic acid and all solvents used for high pressure liquid chromatography (HPLC) were of analytical or HPLC grade from Carlo Erba, Milan, Italy. Ethanol and isopropyl alcohol employed for the extraction of polyphenols from AC resins were of food-grade and purchased from Carlo Erba, Milan, Italy.

Standard solutions, calibration curves and calculation of hydroxytyrosol content

Hydroxytyrosol stock solutions were prepared by dissolving the crystalline standard first in 1,000 ppm stock solutions. Subsequently, stock solutions were diluted to 5 ppm with 80% methanol (v/v). Calibration curves were obtained for each standard with high linearity ($r > 0.996$) by plotting the standard concentrations as a function of the peak area obtained from HPLC analysis with 25 μL injections. For this purpose, the stock solutions of the standards were diluted with 80% methanol to five different concentrations ranging from 1 to 20 mg/L. Each concentration was analysed by triplicate injections.

Preparative HPLC

The purification of hydroxytyrosol for nuclear magnetic resonance (NMR) analysis was achieved on a Phenomenex Kinetex Phenyl-Hexyl 100 A 150×4.6 mm reverse-phase C18 using a Varian multisolvent pump ProStar 210, and photodiode array detector Varian ProStar 335. The solvent system was (A) H_2O/CH_3COOH (99.9:0.1) and (B) ACN/H_2O/CH_3COOH (95:4.9:0.1). Separations were done in isocratic mode with a flow rate of 2 mL/min. The detection was at 260 nm. Each injection volume was 1.5 mL. If the peak collected was not pure, it was re-chromatographed under the above conditions. All chromatograms were analysed using Workstation Galaxie LC software.

HPLC–diode array detection (DAD) analysis

Analyses of hydroxytyrosol were carried out using a Varian multisolvent pump ProStar 210 and a photodiode array detector Varian ProStar 335. For the separations, a Phenomenex Kinetex Phenyl-Hexyl 100 A 150×4.6 mm reverse-phase C18 column with identical pre-column operating at 25 °C was employed. The eluent was composed of (A) H_2O/CH_3COOH (99.9:0.1) and (B) methanol/H_2O/CH_3COOH (95:4.9:0.1). A three-step linear solvent gradient system was used starting from 5 to 99% of solution B for a 69-min period at a flow rate of 1.0 mL/min. The percentage of solution B reached 25% from 2 to 22 min, then 99% from 23 to 55 min, then 5% from 55 to 69 min. The injection volume was 25 μL. UV–Vis spectra were recorded in the 278–325 nm range and the chromatograms were recorded at 278 nm.

HPLC–mass spectrometry (MS) analysis

HPLC–MS analyses were carried out with a Finnigan Surveyor LC System linked to a TSQ Quantum-Finnigan mass spectrometer with an electro-spray interface (Finnigan). The separations were performed using a Phenomenex Kinetex Phenyl-Hexyl 100 A 150×4.6 mm reverse-phase C18 column operating under the same chromatographic conditions as previously reported.

Spectra were recorded in negative (Fragmentor 80) and positive (Fragmentor 120) ion mode. The mass spectrometer operating conditions were: capillary temperature 300 °C, nebulizer pressure 45 psi, spray voltage 3,200 V, mass range of 50–1,000 amu, quadrupole temperature 40 °C.

NMR analysis

NMR analysis of the purified hydroxytyrosol was carried out at 600 MHz for 1H spectra (Varian Inova 600 MHz spectrometer, equipped with an indirect detection probe). A standard sample of authentic hydroxytyrosol was prepared by dissolving 1.0 mg of the compound in 0.7 mL of CD3OD. An analytical sample of purified hydroxytyrosol was prepared from 1.0 mg of compound dissolved in 0.7 mL of CD3OD. Direct comparison between the proton spectra of the two samples confirmed the structural identification of hydroxytyrosol.

RESULTS AND DISCUSSION

Prototype construction

Prototype functioning is reported in Figure 1. This multiphase olive processing permitted the employment of water as solvent during the pomace processing, increasing polyphenol concentration in mill wastewater rather than in pomace. 'Pitting' could be effected during either the two-phase extraction or the three-phase extraction. In both

cases, the resulting product could be employed for production of thermal energy. Moreover, kernel elimination reduces the activity of polyphenol-oxidase, which degrades phenolic anti-oxidant compounds during mechanical extraction of olive oil (Di Giovacchino *et al.* 2002). The machine is an innovative product: its innovation consists in the sequential setting up of its components: each single unit is present on the market, but they have never been employed for both olive oil and pomace processing at the same time. The chemical composition of the produced olive oil was analysed, and the results are shown in Table 2. As shown, this could be considered a first-class extra-virgin olive oil. In particular, the content of oleic acid, giving the highest nutritive value to the oil, resulted at high concentration, whereas the content of palmitic acid, the major saturated fat, was quite low. Linolenic acid was also found at low concentration (<1%). Although considered beneficial from a nutritional point of view, it is a polyunsaturated acid, and it cannot exceed 1%, as it is particularly unstable and susceptible to oxidation, conferring rancidity to the oil.

Polyphenol extraction from humid pomace

Results on polyphenol content of the three different sets of experiments are reported in Table 3. Results showed that, by increasing water quantity in the decanter, polyphenol recovery increased (Set A in comparison with Set B). For all tests, recovery percentage was around 100%. However, polyphenol concentration in OMWW in Set B tests was equal to 0.75 g/kg (Set B, experiment 6), a value too high for anaerobic fermentation in digestors, so a last group of experiments (Set C) was designed to decrease polyphenol concentration in humid pomace. Results in Table 3 reported that humid pomace exhibited a polyphenol concentration of 0.22 g/kg (Set C, experiment 6), a value acceptable in digestors, as methane-producing bacteria tolerate it. We hypothesized that the higher recovery values observed in

Table 2 | Complete chemical analysis of the extra-virgin olive oil produced with the prototype

Parameter	Measure unit	Result	Uncertainty	EC limits
Acidity	% M/M Oleic Acid	0.41	± 0.02	< 0.6
Peroxides number	meq.O_2/Kg	5.15	± 0.26	< 16
K232	K	1.53	± 0.03	< 2.50
K270	K	0.109	±0.011	< 0.22
Delta K	ΔK	0.003	±	< 0.01
Tocopherols	mg/Kg	337	±57	> 40
Polyphenols	mg/Kg	383	±0.38	> 60
Refractive Index at 25 °		1.4669	±0.0003	1.4665–1.4679
Myristic Acid	% M/M Tot. Fatty Acids	0.01	±0.01	<0.05
Palmitic Acid	% M/M Tot. Fatty Acids	11.97	±1.56	8–14
Palmitoleic Acid	% M/M Tot. Fatty Acids	1.15	±0.30	0.5–1.5
Heptadecanoic Acid	% M/M Tot. Fatty Acids	0.10	±0.05	
% M/M Tot. Fatty Acid	% M/M Tot. Fatty Acids	0.21	±0.10	
Stearic Acid	% M/M Tot. Fatty Acids	2.09	±0.29	1.1–3
Oleic Acid	% M/M Tot. Fatty Acids	74.94	±3.75	73–83
Linoleic Acid	% M/M Tot. Fatty Acids	7.73	±1.93	<9
Linolenic Acid	% M/M Tot. Fatty Acids	0.93	±0.10	<0.9
Arachidonic Acid	% M/M Tot. Fatty Acids	0.39	±0.02	<0.6
Eicosenoic Acid	% M/M Tot. Fatty Acids	0.32	±0.04	<0.4
Behenic Acid	% M/M Tot. Fatty Acids	0.13	±0.01	<0.2
Lignoceric Acid	% M/M Tot. Fatty Acids	0.05	±0.03	<0.2
Trans-oleic Isomers	% M/M Tot. Fatty Acids	0.01	±0.00	<0.05
Trans Linoleic + Linolenic Isomers	% M/M Tot. Fatty Acids	<0.01	±0.00	<0.05

Table 3 | Polyphenol concentrations in samples obtained from the three sets of experiments

	Polyphenols in humid pomace (g/kg)	Polyphenols in dephenolized pomace (g/kg)	Polyphenols in OMWW (g/L)	Total polyphenols (g/kg)	Recovery (%)
Set A					
1	6.24	1.31	4.91	6.22	100
2	4.37	0.75	3.52	4.27	98
3	3.36	0.58	2.77	3.35	100
4	4.60	0.75	3.79	4.54	99
5	3.50	0.47	2.97	3.44	98
6	2.82	0.36	2.45	2.86	100
Set B					
1	7.28	1.81	6.32	8.13	112
2	6.24	1.54	5.78	7.32	117
3	5.83	1.23	5.15	6.39	110
4	4.37	0.92	4.25	5.17	118
5	3.80	0.83	3.61	4.44	117
6	3.36	0.75	3.2	3.89	117
Set C					
1	5.14	0.93	4.80	5.73	111
2	4.60	0.80	4.12	4.92	107
3	3.91	0.63	3.22	3.85	98
4	3.50	0.44	2.80	3.24	93
5	3.12	0.38	2.67	3.05	98
6	2.82	0.22	2.58	2.86	99

all Set B experiments and in experiment 2 of Set C could probably be due to a hydrolysis reaction of polyphenols, with a consequent higher polyphenol concentration detected by Folin–Ciocalteu reagent. The subsequent step involved concentration of OMWW and desorption on AC resins. Considering an activity of the mini-mill of 8 h, 24 h are necessary to recover distilled water from OMWW to be employed for the mill's necessities.

Polyphenol concentration and desorption

Polyphenols contained in OMWW from Set C experiments, adsorbed on AC plant according to the method developed by Dr Ena's group at ISE-CNR in Florence (Ena *et al.* 2009), were desorbed by employing an ultrasound concentrator (Table 4). The initial concentration of polyphenols was comparable to the one obtained in literature (Fki *et al.* 2005a, 2005b). Adsorption from OMWW was performed only one time, polyphenols in OMWW being equal to 5.14 g/L and 0.22 g/L before and after AC plant treatment, respectively (99% of recovery) (Table 3, Set C). The desorption results of the four experiments are shown in Table 4. The results evidenced that the best time of extraction with this extractor was 30 min. At 60 and 90 min, no significant differences in desorption were observed.

To improve the polyphenol desorption from the AC plant, a solid–liquid extractor was utilized, with an extraction time similar to those employed for ultrasound extraction, corresponding to 14 extraction cycles of 45 min. The results are reported in Table 4. It can be observed that no differences in recovery were found, with respect to ultrasound extractions, the recovery being from 41.8 to 49.9%. In another set of experiments, the recovery of polyphenols exhibited no significant differences by employing a reduced cycle number, seven cycles, with a total time of 63 min (Table 4), with a strong energy saving. Desorption experiments performed with ultrasound (US) or solid–liquid extractor exhibit polyphenol concentrations ranging from 1.440 g/L to 2.45 g/L in US extraction and from 1.24 to 3.22 g/L in the solid–liquid extractor. The phenolic concentrations reported in this paper are higher than that reported by Garcia-Castello *et al.* (2010). Nevertheless, the recovery here is around 44%

Table 4 | Polyphenol concentrations obtained from OMWWs processed by ultrasound and solid–liquid concentrators

Ultrasound extractor	Time (min)	Total adsorbed polyphenols (g/L)	Polyphenols desorbed (g/L)	Recovery (%)
Exp. 1	20	5.16	1.47	28.48
Exp. 2	30	5.19	1.440	27.74
Exp. 3	30	4.06	1.268	31.23
	60	4.38	1.370	31.27
Exp. 4	30	5.19	2.455	47.30
	60	5.19	2.39	46.05
	90	5.19	2.43	46.82
Solid–liquid extractor	Cycles/Min			
Exp. 1	14/126	6.32	2.97	46.99
Exp. 2	14/126	3.95	1.74	44.05
Exp. 3	14/126	5.92	2.79	47.13
Exp. 4	14/45	2.87	1.28	44.59
Exp. 5	14/45	5.48	2.45	44.70
Exp. 6	14/45	7.22	3.22	44.59
Exp. 7	7/63	2.77	1.24	44.76
Exp. 8	7/63	5.18	2.32	44.78
Exp. 9	7/63	6.96	3.11	44.68

as compared with 78% reported by Garcia-Castello *et al.* (2010); concentrated polyphenols are higher than those reported by these authors (0.50 g/L).

Concentrated OMWW extracts obtained by Set C experiments have been analysed, and results are displayed in Table 5. Total polyphenols were equal to 50.41 g/L in US experiments, with a content of hydroxytyrosol equal to 17.87 g/L, with a percentage of 35.4% of total polyphenols. Solid–liquid extractions exhibited a total polyphenol content of 50.58 and 51.31 g/L for 7 and 14 cycles, respectively. We hypothesize that the similar polyphenol concentration obtained with 7 and 14 cycles could be due to solvent saturation. Hydroxytyrosol recovery was equal to 44.44 and 31.34 g/L, values higher than those reported by Garcia-Castello *et al.* (2010). Chemical oxygen demand (COD) values of these extracts are equal to 11,640 mgO_2/L, with a reduction of 78% with respect to initial COD value of OMWW of 52,500 mgO_2/L (data not shown). These values are similar to those obtained through microbial treatments of olive oil by products with *Aspergillus niger* (Aissam *et al.* 2007).

Essential minerals such as iron (Fe), zinc (Zn) and copper (Cu) were present, as were some microelements. Heavy metals such as Pb, Hg, Cd and Sn were present at very low concentrations with respect to legal limits (EC regulation 1881/2006 2006). Moreover, the results showed very low concentrations of phosphorus pesticides and chlorine pesticides below legal limits, permitting this product to be defined as 'biological'. In addition, bacterial count was null.

HPLC and MS analysis

All extracts from Set C experiments have been analysed for a quali-quantitative evaluation of polyphenols, in particular hydroxytyrosol. Results are shown in Figure 2(a), where hydroxytyrosol exhibited a retention time of 12 min. Preparative HPLC is shown in Figure 2(b), where peaks A, B and C have been analysed by MS and NMR and resulted to be hydroxytyrosol (peak A), δ-glutarolactone (peak B) and pinoresinol (peak C) (Figures 3–5, respectively). HPLC analyses referring to the last experiment of Set C (Table 3) of concentrated extracts and de-phenolized pomace are shown in Figure 4(a) and 4(b). Results obtained from humid pomace, OMWW and de-phenolized humid pomace confirmed the presence of bioactive compounds such as polyphenols from sub-products of olive oil milling; in particular, analyses demonstrated the presence of high concentrations of a high biological value compound in these matrices, hydroxytyrosol. Figure 5(a) and 5(b) show polyphenols concentration in centrifuged humid pomace, and hydroxytyrosol percentage in each humid pomace fraction. More than half of extracted polyphenols by percentage was hydroxytyrosol. Analyses of de-phenolized humid

Table 5 | Chemical analysis of OMWW concentrated extracts

		US	Timatic 14 cycles	Timatic 7 cycles
Total polyphenols	g/L	50.41	50.58	51.31
Hydroxytyrosol	g/L	17.87	44.44	31.38
COD	mgO_2/kg	11,640	11,540	11,440
pH	–	3.29	3.18	3.17
Cd	mg/L	0.012	0.11	0.014
Cr	mg/L	3.571	2.597	1.007
Cr VI	mg/L	<0.1	<0.1	<0.1
Fe	mg/L	16.16	8.369	5.254
Mn	mg/L	4.494	3.86	1.921
Hg	mg/L	0.0087	0.0103	0.0084
Ni	mg/L	6.712	3.32	3.359
Pb	mg/L	0.553	1.654	0.629
Cu	mg/L	6.706	19.58	4.264
Zn	mg/L	64.51	30.37	30.71
Ca	mg/L	772.6	739.4	428.7
Mg	mg/L	449.5	465	333.9
K	mg/L	26,680	24,030	22,390
Sulphate	mg/L	757.3	632.7	693
Chlorine	mg/L	1,465.1	1,429.5	1,527.5
Phosphorus (Orthophosphate)	mg/L P	519.6	555.5	545.3
Phosphorus (Total)	mg/L P	18.01	1,942	1,820
Peroxides	meqO_2/kg	1.08	1.1	0.96
Tocopherol	mg/L	<10	<10	<10
Ammonia N	mg/L	21.1	23.4	14.1
Nitrous N	mg/L	<0.025	<0.025	<0.025
Nitric N	mg/L	<0.1	<0.1	<0.1
Anionic surfactants	mg/L	<0.025	<0.025	<0.025
Cationic surfactants	mg/L	<0.2	0.2	<0.2
Non-ionic surfactants	mg/L	<0.03	<0.03	<0.03
Total surfactants	mg/L	<0.3	<0.3	<0.3
Phosphorus pesticides	mg/L	<0.01	<0.01	<0.01
Total pesticides (Phosp. Pest. excluded)	mg/L	<0.005	<0.005	<0.005
Aldrin	mg/L	<0.001	<0.001	<0.001
Dieldrin	mg/L	<0.0001	<0.0001	<0.0001
Endrin	mg/L	<0.001	<0.001	<0.001
Isodrin	mg/L	<0.001	<0.001	<0.001
Total bacterial count at 22 °C	UFC/mL	0	0	0
Total bacterial count at 36 °C	UFC/mL	0	0	30

UFC, units for colony.

pomace, obtained at the end of experiment 6, Set C (Figure 5(c)) and Figure 5(d), exhibited a very low content of polyphenols, with hydroxytyrosol percentages from 3.2 to 0%. These data confirmed how the mini-mill prototype was able to produce a humid pomace completely devoid of polyphenolic compounds.

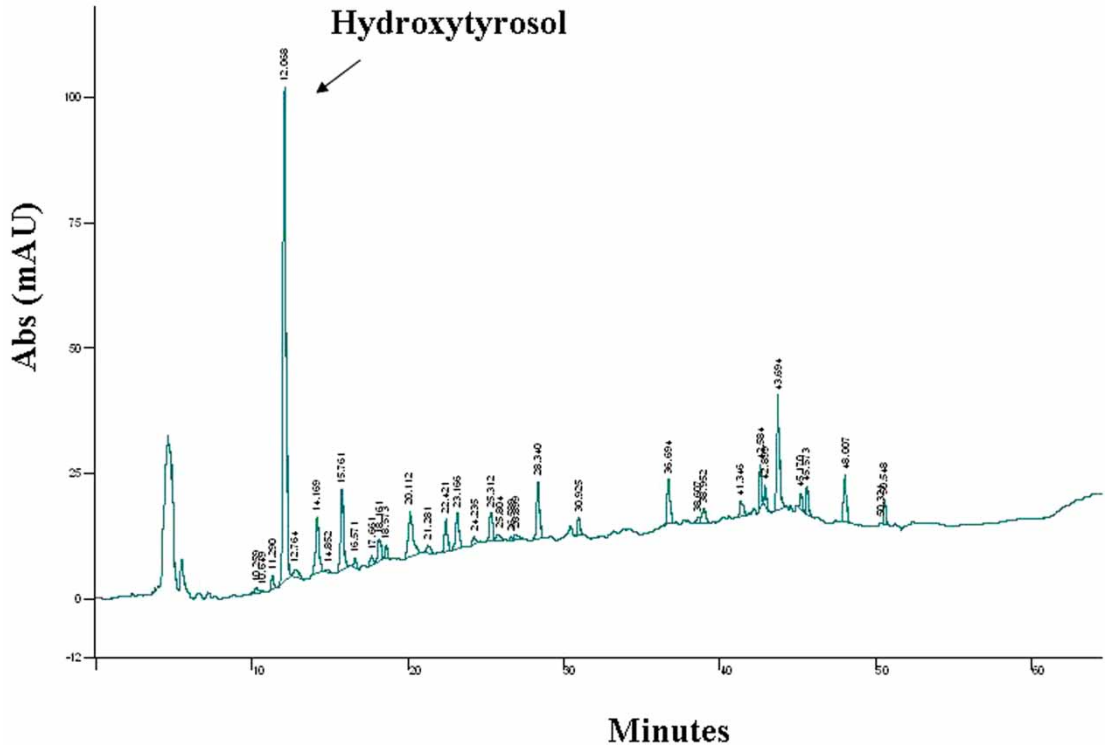

Figure 2 | Chromatogram of an OMWW concentrated extract.

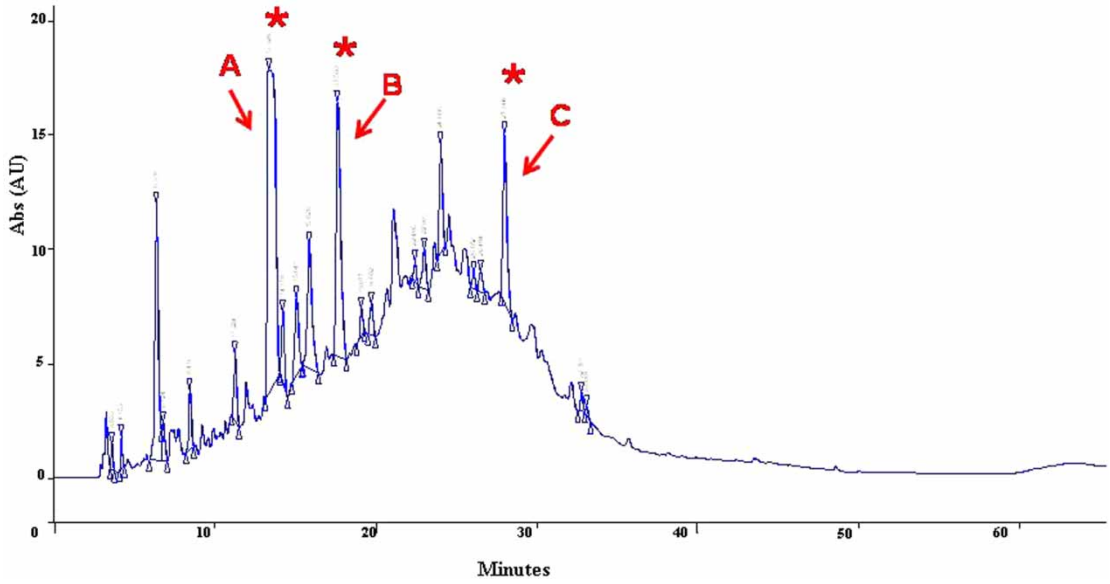

Figure 3 | HPLC semi-preparative analysis of OMWW concentrated extracts. A = hydroxytyrosol, B = δ-glutarolactone, C = pinoresinol. Asterisks added to emphasize peaks.

Employment of de-phenolized humid pomace in biodigestor

De-phenolized humid pomace was employed for methane production. In particular, humid pomace was inserted gradually into the biodigestor, mixed at different percentages with ensilage, to avoid a biological shock to bacteria present in the biodigestor. By starting with 3,000 kg/d of humid pomace, after 20 days humid pomace contribution was 14,000 kg. Considering that biomass consumption in a

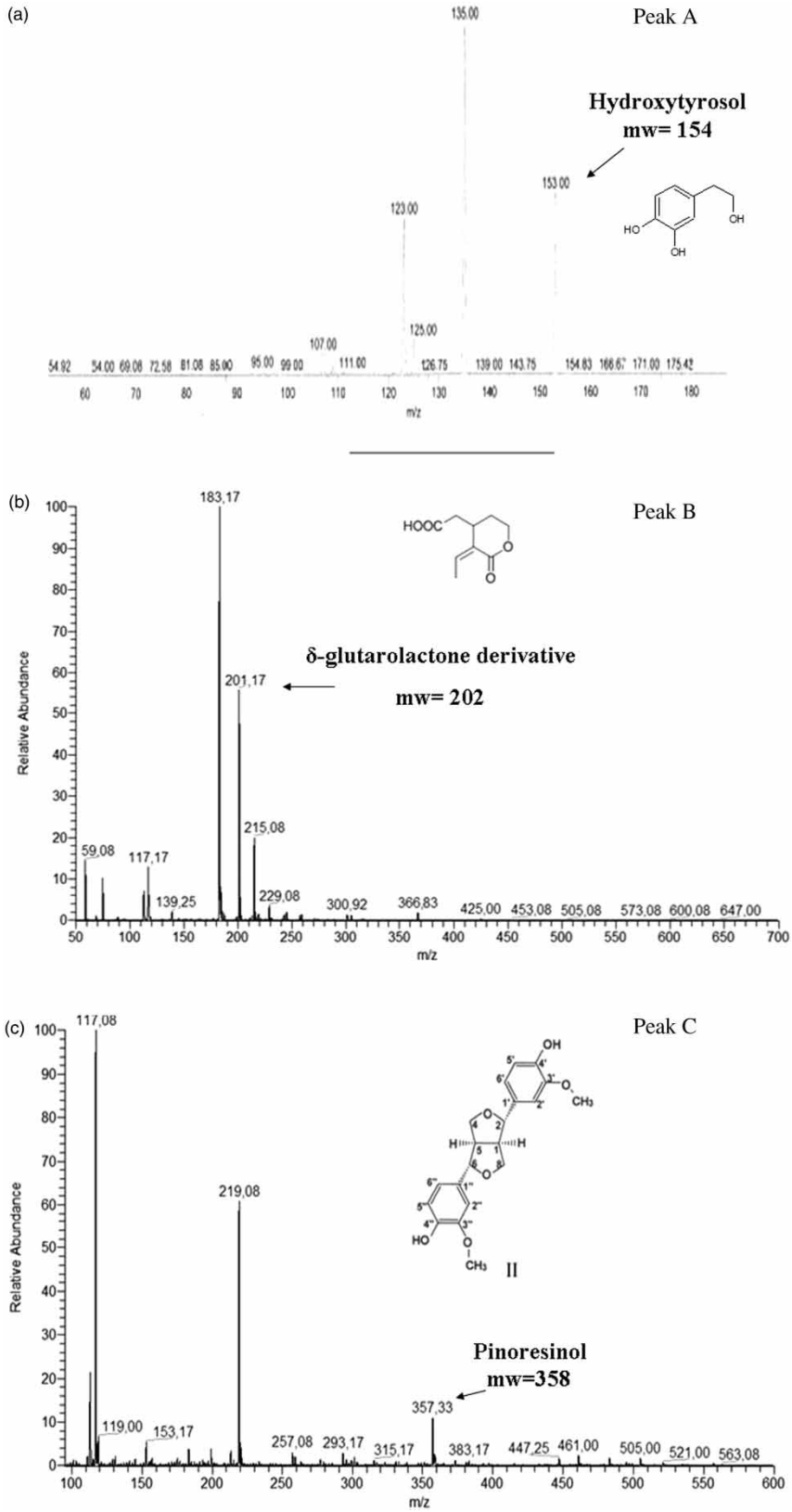

Figure 4 | (a) MS spectra of peak A of HPLC semi-preparative analysis identified as hydroxytyrosol; (b) MS spectra of peak B of HPLC semi-preparative analysis identified as δ-glutarolactone; (c) MS spectra of peak C of HPLC semi-preparative analysis identified as pinoresinol.

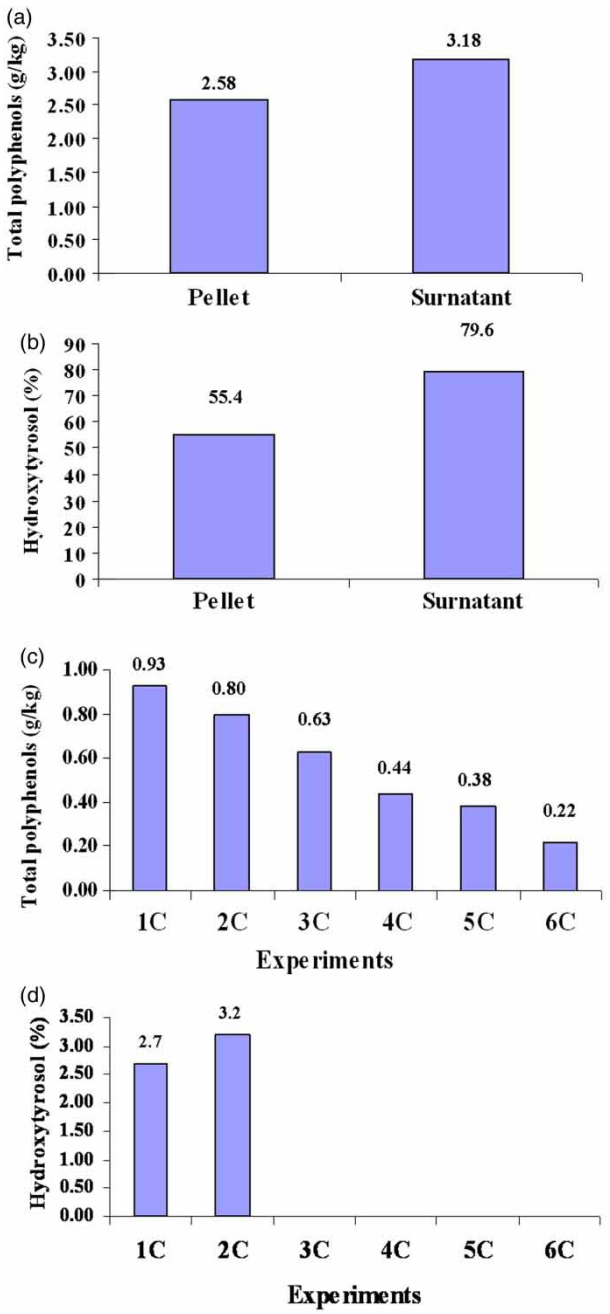

Figure 5 | (a) Total polyphenols concentration in an OMWW concentrated extract and (b) relative percentages of hydroxytyrosol in these extracts. (c) Total polyphenols concentration in de-phenolized humid pomace samples of experiment 6, Set C. (d) Relative percentages of hydroxytyrosol in (c) samples.

biodigestor is 45,000 kg, it can be concluded that 30% of ensilage was humid pomace.

It has been observed that kernel removal from pomace was an important step, as this procedure avoids problems at the solid–liquid separation phase in the digestor and material stratification. Moreover, employment of humid pomace increased methane percentage from 50 to 54% and was employed for electricity production. Final residues from digestor activity have been successfully employed as fertilizer.

CONCLUSIONS

The idea of recovery and valorization of olive oil mill sub-products enabled the introduction of many innovations which could transmit a turning point in olive oil chain production. By exploiting existing machineries in an olive oil mill in a combined (2 + 3) working process, we were able to obtain:

(a) high quality extra-virgin olive oil;
(b) de-phenolized humid pomace for employment in biodigestors;
(c) polyphenol-rich OMWW to be extracted;
(d) recovery of nuts, to be employed in thermal energy production;
(e) clean water to be re-used in mill;
(f) polyphenol-enriched extracts to be exploited in the food and cosmetic market.

Innovations consist in: (i) use of a two-phase process in which extra-virgin olive oil and humid pomace were produced, followed by a second three-phase process handling humid pomace to de-phenolized pomace to be employed in biodigestors; (ii) production of a concentrated OMWW enriched in polyphenols; (iii) recovery of clean water to be re-used in mill. In particular, this process recovers 97% of the water used in the three-phase process. In fact, considering that during the three-phase process we employed 8,400 L of water and that 20,400 kg of humid pomace produced during olive milling contained 8,160 L of water, we could recover 96.90% of clean water. This non-conventional technology enabled us to extract from OMWW a polyphenols concentration higher than that reported in literature (Garcia-Castello *et al.* 2010), with a hydroxytyrosol recovery of 79.6%. These values are similar to those reported in the literature (Fernandez-Bolanos *et al.* 2002).

Simple, innovative, non-conventional technologies together with long-used processes permit obtaining a 'no-residue' working process with a total recovery of products without any waste. The presented solution combines the extraction of valuable phytochemical compounds with beneficial properties for the pharmaceutical, cosmetics and food industry, with bioconversion of olive mill by-products without environmental impact.

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