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Sustainability, Innovation and Green Chemistry in the Production and Valorization of Phenolic Extracts from *Olea europaea* L.

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Abstract: A *circular economy* process applied to the agro-industrial sector of the olive oil production, based on environmentally and economically sustainable procedures, was described. *Olea europaea* L. tissues and by-products (leaves, and pitted olive pulp) represent a renewable and low-cost source of polyphenols, in particular hydroxytyrosol (HTyr), a natural occurring compound well known for its biological properties. *Olea europaea* L. green leaves (GL), dried leaves (DL) and pitted olive pulp were extracted by water in a pneumatic extractor to obtain polyphenolic fractions on an industrial scale. After the extraction step, an environmentally and economically sustainable separation process, based on membrane technology and the following concentration step, produced three fractions, named *Soft Extract Olea GL*, *Soft Extract Olea DL* and *Soft Extract Olea HTyr*, which find industrial applications in food, nutraceutical, pharmaceutical, feed and agronomic fields. Novel functionalized extracts containing hydroxytyrosol methyl carbonate (HTyr-MC) were obtained from HTyr-enriched fractions through green chemistry procedures, which appear to be a promising tool to increase the applications of the polyphenolic extracts.

Keywords: *Olea europaea* L. by-products, sustainable extraction, membrane separation technology, hydroxytyrosol, hydroxytyrosol methyl carbonate, functionalized extracts, circular economy, green chemistry

1. Introduction

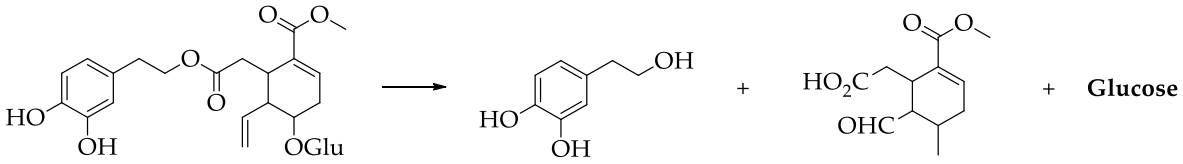
Sustainability and innovation are some of the keywords of a novel concept of economy, named *circular economy*, based on legislative proposals suggested and adopted by the European Community to increase the global competitiveness, the economic growth and create new jobs saving resources and energy [1]. The aim of the *circular economy* is "closing the loop" of product lifecycles through greater recycling and re-use, and bring benefits for both the environment and the economy [2].

This approach can be applied in almost all manufacturing. For example, the agro-industrial field offers a good opportunity when considering the large amounts of wastes and by-products produced every year during fruit and vegetables processing. In this area, a *circular economy* process can be

achieved through efficient small and industrial scale bioenergy plants, biorefineries and environmentally friendly processes to obtain bioactive compounds that can be used as active ingredients for agronomic, cosmetic, food, feed and pharmaceutical formulations [3, 4]. Among these, polyphenols are excellent candidates, which have been demonstrated to have many therapeutic properties. In fact, they have shown effectiveness as antioxidants in processed foods, wine, beverages, even at concentrations below 100 ppm; they promote health by reducing the onset of inflammation, cardiovascular illnesses, arthritis, and other free radicals mediated diseases including several kinds of cancer [5]. The main sources of polyphenols are vegetables and fruits, including extra-virgin olive oil, an essential component of the Mediterranean diet [6-7]. Unfortunately, blood bioavailability of these compounds - the fraction absorbed by the human body - is low. In the last years, food, pharmaceutical and cosmetic industries have introduced functionalized products e.g. products containing vegetal secondary metabolites such as polyphenols, to increase prevention against the above-mentioned diseases. This is also a response to the increasing demand related to the "natural lifestyle choices" of consumers. As a result, the polyphenols market has grown rapidly over recent years and is expected to increase at the annual rate, expressed as Compound Annual Growth Rate (CAGR), of 9% by 2020. At the same time, it is estimated that polyphenols consumption will increase from 12,200 tons to 25,000 tons reaching a market worth € 900 million, affecting mainly three geographic areas (Asia Pacific, North America and Europe).

A strong contribution in terms of the source of polyphenols is provided by olive tree cultivation particularly widespread in the Mediterranean countries (Spain, Italy, Greece and Portugal) and in the Northern African countries (Syria, Turkey, Morocco and Tunisia). The process of olive oil production creates a large amount of waste, including olive mill wastewaters, olive pulp and leaves. Olive mill wastewaters are the main waste produced from three-phase olive processing while olive pulp is the main waste deriving from two-stage olive oil processing. This last technique, promoted from the European Community, was recently applied from Spain and Italy to eliminate the production of olive mill wastewaters characterized by high level of toxicity and costs for disposal [8]. Olive leaves can be considered a waste because derive from both olives processing and pruning practices. These raw materials are a precious source of bioactive compounds including low-molecular weight phenols [9]. In olive leaves, the main constituent is oleuropein (Scheme 1), a phenolic secoiridoid glycoside which by enzymatic or chemical hydrolysis, produces hydroxytyrosol (HTyr), elenolic acid, and glucose [10]. Among these, HTyr is a biologically outstanding compound for its properties, in particular for the strong antioxidant activity [11]. These peculiar properties and the absence of genotoxicity [12], make HTyr a good candidate for use as a preservative, thus potentially replacing synthetic food and cosmetic additives such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). It is worth mentioning that these additives have recently raised concerns about their possible mutagenic and carcinogenic effects. At the same time, HTyr plays an important role in pharmaceutical applications for its health-beneficial properties such as anticancer activity [13].

Scheme 1. Products of hydrolysis of oleuropein.



Oleuropein **HTyr** **Elenolic acid**

In recent years, international scientific research has proved several biological effects of polyphenols present in olive leaves and by-products. Numerous studies have shown that they exhibit a wide number of properties including antiradical activity [14] and antimicrobial activity against microorganisms related to human health, food processing and agriculture pest control. For example, their effectiveness was tested against *Helicobacter pylori*, the agent responsible for peptic ulcers and some types of gastric cancer as well as against several foodborne pathogens such as *Salmonella enteritidis* and *Staphylococcus aureus* [15, 16].

Taking into account the wide applicability, several extractive procedures have been optimized to recover low molecular weight polyphenols from *Olea* tissues and olive oil by-products [17, 18]. Among them, membrane separation technology has been recently developed to fractionate olive mill wastewaters [19-21]. This technology offers several advantages over the conventional ones, mainly in terms of low energy consumption, no additive requirements and no phase change. However, in most cases it can be applied only on a laboratory scale.

The case-study here described represents an original example of a *circular economy* process applied to the agricultural system of the olive oil processing on an industrial scale. It concerns the valorization of *Olea europaea* L. leaves and pitted olive pulp as a source of bioactive polyphenols to produce standardized extracts to be used in food, nutraceutical, pharmaceutical, feed and agronomic fields. An example of green chemistry was reported in order to obtain novel functionalized extracts from HTyr-enriched fractions.

2. Materials and Methods

2.1 Reagents, plant material and instruments

Reagents and solvents were supplied by Sigma-Aldrich (Milan, Italy) and used without further purification. Hydroxytyrosol was synthesized as we already described using a patented procedure [22, 23]. Silica gel (200-300 mesh) and silica gel F254 plates were furnished by Merck (Milan, Italy).

Olea leaves and pitted olive oil (Frantoio cultivar) were collected in Tuscany (Siena, Italy), Latium (Rieti, Italy) and Apulia (Foggia, Italy) during the year 2015.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a spectrometer 400 MHz Bruker using chloroform-d_3, acetone-d_6 and methanol-d as solvents. All chemical shifts are expressed in parts per million (δ scale) and coupling constants are reported in Hertz (Hz).

The HPLC/DAD analyses were performed with an HP 1100L liquid chromatograph equipped with a diode array detector (Agilent Technologies, Palo Alto, CA), and an analytical column LiChrosorb RP18 250 4.60 mm, 5µm (LichroCART, Merck Darmstadt, Germany) maintained at 26 °C. The eluents were H_2O adjusted to pH= 3.2 with HCOOH (solvent A) and CH_3CN (solvent B). A four-step linear solvent gradient was used starting from 100% of solvent A up to 100% of solvent B, for 88 minutes at a flow rate of 0.8 ml min^{-1}, in accordance with a previous paper [24].

2.2 Extraction of low-molecular phenolic compounds

Olea europaea L. extracts were obtained according to a patented eco-friendly and economically sustainable process [25, 26]. The extractions were carried out in a Pneumatic Extractor Timatic series (Tecnolab S.r.l., Spello, Perugia, Italy) using water as extractive solvent at industrial scale. In detail, 30 kg of GL or 30 kg of DL or 50 kg of pitted olive pulp required 200 L of solvent. The process was carried out in a stainless steel basket at ~~a~~ 70 °C in water for 30 or 60 min. The working cycle was fully automatic and alternated between a dynamic phase, obtained with a set pressure (7-9 bar), and a static phase necessary for transferring the substance into the extraction solvent. Forced percolation was generated during the stationary phase, which, thanks to the programmable recirculation, ensured a continuous flow of solvent within the plant matrix. This avoided the over-saturation and the formation of preferential channels, thus ensuring the total extraction of polyphenols from the plant materials. In the pretreatment phase, to avoid the polyphenol oxidation, the pH was lowered from the original value of 5.7 to 3.5, in order to inactivate the polyphenol oxidase enzyme present in the aqueous raw extracts, and to create the optimal conditions for the subsequently addition of the pectinase enzyme when the processed sample was "pitted olive pulp". The pH was lowered by adding concentrated HCl and citric acid (1% w/V).

2.3 Fractionating of the phenolic extracts

The fractionating of the extracts was performed by the membrane technology, in particular by Microfiltration (MF), Ultrafiltration (UF), Nanofiltration (NF) and Reverse Osmosis (RO). The membranes were characterized by different molecular weights with cut-off and filtration degrees. During the manufacturing process, the MF stage was carried out with tubular ceramic membranes in titanium oxide while the UF, NF and RO stages were conducted with spiral wound module membranes in polyethersulfone (PES) [26]. At the end of each working cycle, the membrane modules were automatically washed to remove the eventual sediments occurring on the membranes and then reduce the membrane fouling. In particular, MF membrane was washed with tap water for 5 minutes, a basic solution for 30 min, then an acidic medium until neutral pH and finally with distilled water; NF and RO were simply washed firstly with tap water and then with distilled water [25, 26].

2.4 Concentration of the fractions deriving from MF, UF and RO

Each fraction deriving from MF, UF and RO stages was concentrated using a scraper evaporator series provided with a heat-pump (C&G Depurazione Industriale Company, Firenze, Italy). Three concentrates were obtained: *Soft Extract Olea GL* from GL; *Soft Extract Olea DL* from DL and *Soft Extract Olea HTyr* from pitted olive pulp.

Figure 1. Separation system based on membrane technology: Microfiltration (MF), Ultrafiltration (UF) and Reverse Osmosis (RO).

2.5 Functionalization of the HTyr-enriched fraction (Soft Extract Olea HTyr)

Methanol or ethanol (5 mL) was added to 100 mg of *Soft Extract Olea HTyr*. After the removal of the precipitate represented by inorganic salts, the filtered solution containing standardized HTyr was recovered. The alcoholic solvent was removed under reduced pressure; then, the residue was treated with dimethyl carbonate (DMC) at the reflux temperature in the presence of a catalyst (1,8-diazabicyclo [5.4.0] undec-7-ene, DBU, or sulfuric acid) for 3 or 7 h [27].

3. Results and discussion

Olea europaea green leaves (GL) and dried leaves (DL) of Frantoio cultivar were the first matrices utilized to recover low-molecular phenolic compounds. Table 1 reports the experimental conditions of the extractions performed from GL (*entries 1 and 2*) and DL obtained by two different drying processes: room temperature for 15 days (*entries 3 and 4*) or ventilated stove at 40 °C for 3 days (*entries 5 and 6*). The extractions were performed on 15% w/V on fresh weight and dry weight for 30 and 60 minutes in a pneumatic extractor, as described in the Experimental Section. As reported in the last column of Table 1, the percentage of the total polyphenols and oleuropein extracted from leaves depends on the starting material status, namely green leaves (GL) or dried leaves (DL). Prolonging the extraction time from 30 to 60 minutes, an increase of total polyphenols and oleuropein was observed in all cases (compare *entry 1* to *entry 2*; *entry 3* to *entry 4*; *entry 5* to *entry 6*) but these results may not justify the production of high extraction volumes in the light of the raw material low cost.

Table 1. Experimental data of the extraction of *Olea europaea* GL and DL (Frantoio cultivar).

	Samples	Extracted leaves (%)[1]	Extraction time (min)	Total polyphenols, oleuropein (%)[2]
entry 1	GL	15	30	13.6 (38.5)
entry 2	GL	15	60	17.8 (43.9)
entry 3	DL, rt, 15 days	15	30	12.3 (19.5)
entry 4	DL, rt, 15 days	15	60	16.1 (22.0)
entry 5	DL, ventilated stove, 40 °C, 3 days	15	30	11.4 (26.0)
entry 6	DL, ventilated stove, 40 °C, 3 days	15	60	16.8 (41.0)

[1] mg g^{-1} fresh weight for GL; mg g^{-1} dry weight for DL. [2] Oleuropein (in brackets).

As previously said, pitted olive pulp was also used as raw material, being a consistent olive oil by-product deriving from a two-stage olives processing. The industrial process to obtain standardized phenolic extracts included three steps: 1) extraction of low-molecular phenolic compounds from raw materials; 2) fractionation of the phenolic extracts; 3) concentration of the fractions. The first step was carried out in the previously described Pneumatic Extractor Timatic series, and was followed by an enzymatic pretreatment. In particular, to reduce the effect of membrane clogging by the solids present in the pitted olive pulp, the commercial enzyme complex Pectinex SMASH XXL (Novo Nordisk, Franklinton, N.C.) extracted from *Aspergillus niger* was employed. The preliminary enzymatic phase allowed both the releasing of the bioactive compounds from the Olea stuff and the optimized recovery of the hydroxytyrosol. The fractionation of the phenolic extracts was then performed by an innovative separation system based on membrane technology defined as BAT (Best Available Technology) and recognized by the EPA (Environmental

Protection Agency) [25, 26]. This separation system consisted in Microfiltration (MF), Nanofiltration (NF) and Reverse Osmosis (RO) stages. The third and final step was carried out in order to increase the title of bioactive compounds in the final extracts. It was performed in a heat-pump evaporator using the fractions deriving from MF, NF and RO. Three concentrates extracts were obtained: *Soft Extract Olea GL* from GL; *Soft Extract Olea DL* from DL and *Soft Extract Olea HTyr* from pitted olive pulp. For each of them, the total polyphenols and the content of hydroxytyrosol, secoiridoid, elenoic acid derivatives, flavonoids, verbascoside and lignans was quantified by HPLC/DAD/ESI-MS analysis (Table 2). As showed, the content in total polyphenols of the fractions obtained from green leaves (*Soft Extract Olea GL*) and pitted olive pulp (*Soft Extract Olea HTyr*) was very similar (24 and 29% *w*/*w*, respectively) whereas that coming from the dried leaves (*Soft Extract Olea DL*) was significantly lower (about 6% *w*/*w)* [28]. In particular, *Soft Extract Olea HTyr* was rich in HTyr and derivatives (96.5% of the total polyphenols); *Soft Extract Olea GL* contained secoiridoids as the main components and among them, oleuropein accounted for 67.2% of the total polyphenols. As expected, *Soft Extract Olea DL* contained secoiridoids in only 19.2% of the total polyphenols being a great loss of oleuropein during the drying process.

Table 2. Content of polyphenols in the fractions obtained by the industrial plant.

	Compound	Soft Extract Olea GL *	Soft Extract Olea DL *	Soft Extract Olea HTyr *
entry 1	Hydroxytyrosol derivatives	24.69 ± 3.47	25.21 ± 1.56	279.89 ± 18.24
entry 2	Secoiridoid derivatives	164.19 ± 1.47	11.09 ± 0.45	nd
entry 3	Elenolic acid derivatives	28.34 ± 0.43	7.54 ± 0.40	0.51 ± 0.04
entry 4	Flavonoids	1.42 ± 0.06	4.30 ± 0.31	7.83 ± 0.25
entry 5	Verbascoside	1.27 ± 0.01	1.00 ± 0.41	1.69 ± 0.17
entry 6	Lignans	6.76 ± 0.10	5.85 ± 1.05	nd
entry 7	Total Polyphenols	244.15 ± 5.54	57.63 ± 4.42	289.93 ± 18.70

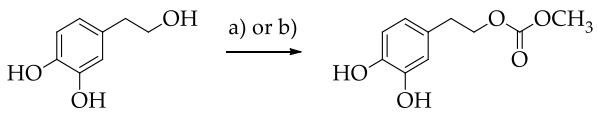
* Data expressed in mg g^{-1}; nd= not detected.

An economic estimate of the described industrial platform was calculated considering 79,300 kg of processed olives which produced 66,600 kg of pitted olive pulp. After extraction, membrane separation and concentration processes, a total of 2,940 kg of extracts were obtained (yield: 4.4%). The production costs and market prices of the different extracts increased proportionally to the HTyr percentage. In particular, the production cost varied from 11.34 to 16.34 €/Kg and the market price from 30 to 100 €/Kg. The net income per year estimated for the platform was about € 70,000.

All extracts have industrial applications. *Soft Extract Olea GL* and *Soft Extract Olea DL* can be employed in the food industry to preserve meat and bakery products [29, 30] whereas *Soft Extract Olea HTyr*, rich in HTyr, can be used in pharmaceutical, nutraceutical and cosmetic applications also in combination with chestnut tannin extracts [31]. In this paper, we described an original approach about the utilization of HTyr-enriched extracts. In particular, *Soft Extract Olea HTyr* was functionalized to obtain novel HTyr-derived compounds with the aim to increase the industrial applications of the natural extracts [32]. In this context, green chemistry procedures are an important tool to guarantee the sustainability of the process [33]. As an example of a HTyr-derived compound obtained from *Soft Extract Olea HTyr*, we reported the functionalization of HTyr present into extracts to produce hydroxytyrosol methyl carbonate (HTyr-MC), which has been obtained up to now in our

laboratory by treating pure HTyr in dimethyl carbonate (DMC) in the presence of a catalyst [27]. As it is well known, DMC is an ecofriendly chemical able to act both as solvent [34] and reagent [35] depending on the experimental conditions. The selective introduction of the methyl carbonate moiety into HTyr reduced its hydrophilicity while the catecholic moiety was responsible for the antioxidant activity of the final product. In fact, antioxidant tests confirmed the efficiency of HTyr-MC as a radical scavenger [27] and the lack of cytotoxicity make it possible to be used as a preservative for food applications [36]. Recently, HTyr-MC was added into the poly(vinyl alcohol) (PVA) matrix to obtain novel PVA-based binary films useful for packaging applications showing an antioxidant effect on food and then preserving food susceptible to the oxidation [37]. Anticancer assays performed on melanoma (M14), pulmonary (H125), colon (WiDr) and promyelocytic leukaemia (HL60) cell lines revealed that HTyr-MC was more effective than HTyr in cell growth inhibition and apoptosis induction [38]. Moreover, HTyr-MC was used as a synthetic precursor of a large panel of novel hydroxytyrosol derivatives [39-42]. Due to these various applications, obtaining functionalized extracts containing HTyr-MC from *Olea europaea* by-products appeared to be a target for increasing the potentiality of the application of these extracts as an integrated step of the *circular economy* process applied to olive processing. As detailed in the Experimental Section and depicted in Scheme 2, *Soft Extract Olea HTyr* was converted into the corresponding HTyr-MC-enriched fraction in satisfactory yield after a preliminary filtration of the plant material with an alcoholic solvent in order to eliminate the insoluble salts and the following treatment of the residue with DMC/DBU or DMC/H_2SO_4.

Scheme 2. Obtaining of functionalized *Soft Extract Olea HTyr*.

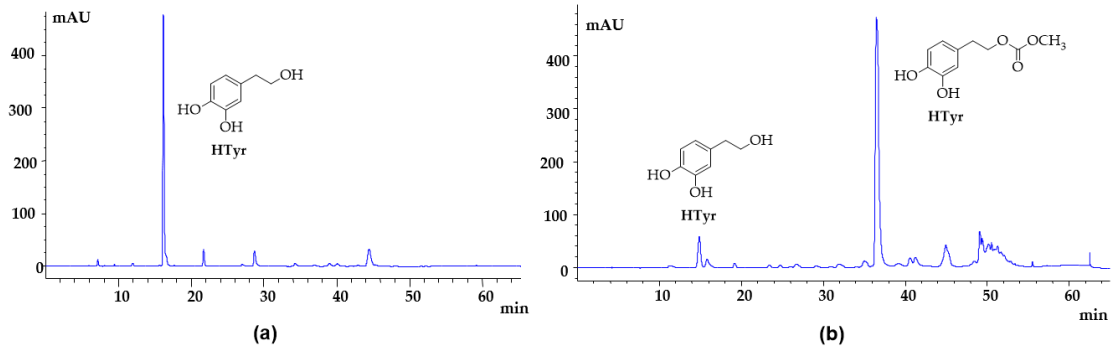


Soft Extract Olea HTyr **Novel functionalized extracts**

a) DMC/DBU, reflux, 3 h; b) DMC/H_2SO_4, reflux, 7 h

The functionalization of *Soft Extract Olea HTyr* was monitored by HPLC analysis with a UV-Vis detector selected at 280 nm (Figure 2). Depending on the catalyst (DBU or sulfuric acid), after the work-up of the mixture of reaction, HTyr-MC were obtained in 90 and 92% yield, respectively. The satisfactory results obtained from *Soft Extract Olea Htyr* gave favor to using the HTyr-enriched phenolic fraction as starting material for novel bioactive compounds.

Figure 2. HPLC/DAD chromatograms of *Soft Extract Olea HTyr* **(a)**; after the green chemistry derivatization **(b)**.



5. Conclusions

Olive oil by-products and olive leaves coming from pruning practices represent an attractive source of biologically active compounds and green chemistry procedures can play an effective role to increase their potentiality of application. In this research, *Olea europaea* green leaves, dried leaves and pitted olive pulp were used to obtain different polyphenolic extracts useful for industrial applications and the production of novel molecules. The technology optimized to prepare these extracts is environmentally and economically sustainable and applicable on an industrial scale. The extracts could be employed to design and market a great number of stabilized or enhanced products, such as baked foods, cosmetics and supplements for human health. Novel functionalized extracts were prepared applying green chemistry semi-synthesis techniques from HTyr-enriched fractions, offering additional potentialities of use. The results described in this paper highlight the possibility of the creation of a multifunctional platform based on the reuse of agricultural waste and agro-industrial by-products by sustainable processes according to the *circular economy* concept in order to "close the loop" of product lifecycles.

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Author Contributions: Annalisa Romani designed the industrial plant for the optimization of polyphenol high-scale extraction and separation via membrane-technology. Patrizia Pinelli performed the analytical characterization of polyphenols of the extracts. Francesca Ieri, expert of the industrial plant, estimated the economic impact. Roberta Bernini planned, designed and performed the experiments about the preparation and characterization of HTyr-MC into phenolic extracts. All authors have contributed to writing the paper. Roberta Bernini and Annalisa Romani have done the critical revision and editing.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

HTyr: Hydroxytyrosol

HTyr-MC: Hydroxytyrosol methyl carbonate
GL: Green leaves
DL: Dried leaves
Soft Extract Olea GL: *Olea europaea* fraction deriving from green leaves
Soft Extract Olea DL: *Olea europaea* fraction deriving from dried leaves
Soft Extract Olea HTyr: *Olea europaea* fraction deriving from pitted olive pulp
HPLC/DAD: High Performance Liquid Chromatography/Diode Array Detector

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