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ABSTRACT

The polyphenols from Olea europaea L. matrices (leaves, olives, oil and by-products) are well known for the biomedical effects related mainly to the antioxidant activity. In this study, a sustainable extractive technology followed by membrane separation methods was described to obtain different commercial extracts for applications in the functional food industry, pharmaceutical and cosmetic fields. In particular, the fractions enriched with hydroxytyrosol (OH-Tyr) can be used in pharmaceutical, nutraceutical and cosmetic applications, whereas those with lower phenol concentrations can be employed in the food industry to preserve meat and bakery products. Finally, an example of green chemistry procedure applied to OH-Tyr enriched fractions was described as a sustainable tool to increase the potentiality of application of these extracts.

Keywords: polyphenolic antioxidants, hydroxytyrosol, sustainable extraction, membrane separation technology, HPLC/DAD, antioxidant activity, green economy, green chemistry, lipophilization

INTRODUCTION

The sustainable technology presented in this paper consists of a water extraction of *Olea europaea* L. matrices (leaves, olives, oil and by-products) following by different steps of fractionation via membrane technologies. The resulting fractions, characterized and quantified by HPLC/DAD-ESI/MS, have an economic value for applications related to their antioxidant properties. Despite the fact that by-products of plant food processing represent a major disposal problem for the relative industry, they are also promising sources of compounds, which can be exploited for their favorable technological or nutritional properties.
The aim of this paper is to describe a sustainable extractive technology followed by membrane separation methods applied on by-products of the processing of olive oil, in order to obtain different commercial extracts, which are useful for different applications thanks to their antioxidant and antiradical properties.
Among bioactive phenolic compounds, hydroxytyrosol (OH-Tyr) plays an important role as nutritional supplement or ingredient of functional foods (Bernini *et al.*, 2013). As a matter of facts, OH-Tyr is a non-genotoxic and non-mutagenic antioxidant at concentrations that far exceed those attainable after oral intake (Aunon-Calles *et al.*, 2013) and has granted Generally Recognized As Safe (GRAS) authorization by the FDA. However, OH-Tyr is a strongly hydrophilic molecule, and its poor solubility in lipid media is a limiting factor

for the incorporation into oils and fats. Therefore, the search for novel lipophilic derivatives is of interest in both the pharmaceutical and food industries to extend the potentiality of application of OH-Tyr in lipid media and increase its bioavailability (Bernini *et al.*, 2015).

BACKGROUND

The polyphenols from *Olea europaea* L. matrices (olive oil by-products, leaves and olive pulp) are known for their good antioxidant properties and protective biological and biomedical effects. The chemical characterization and quantitative evaluation of these minor polar compounds can be useful to obtain active principles with important applications in pharmaceutical, cosmetic and functional food products. The main constituent of olive leaf is a phenolic secoiridoid glycoside, oleuropein, which can be broken down into elenolic acid, a powerful anti-bacterial molecule (Covas, 2007) and hydroxytyrosol (OH-Tyr), a small molecule well known for its strong antioxidant activity (Saija *et al.*, 1998). In vitro tests have shown that flavonoids in olive leaf extracts have recognized antiradical features (Benavente-García *et al.*, 2000). Moreover, in vivo studies have demonstrated the effectiveness of olive leaf extract in lowering blood pressure and this effect seems to be mainly ascribed to oleuropein and hydroxytyrosol (Franconi *et al.*, 2006). The useful antioxidant properties of hydroxytyrosol may be important in the search for "natural" replacements for "synthetic" antioxidant food additives such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which in recent time have raised concerns about their possible mutagenic and carcinogenic effects. A previous investigation (De Leonardis *et al*., 2008) demonstrated that olive leaf phenol extract is a good antioxidant for food lipids, even at lower doses than 100 mg kg^{-1} (as hydroxytyrosol), and it has neither cytotoxic effects nor inhibits the growth of lactic acid bacteria. For these reasons, and in view of their recognized nutraceutical activities, olive leaf phenol extracts can be used as a foodstuff ingredient. Moreover, the antimicrobial properties of phenol compounds coming from olive products have recently been investigated against *Helicobacter pylori* as well as several food-borne pathogens (Medina *et al.*, 2013), confirming important applications of these extracts, not only for food processing control and preservation during storage, but also for counteracting microorganisms harmful to human health. The potentiality of application of phenolic compounds can be extended increasing their solubility in lipid media, as a consequence of their structural modifications. In this context, green chemistry procedures represent an important tool for safety concerns.

MAIN FOCUS OF THE PAPER

Aim, Issues, Controversies, Problems

The polyphenols from *Olea europaea* L. matrices (leaves, olives, oil and by-products) are well known for the biomedical effects related mainly to the antioxidant activity. Different commercial extracts, for applications in the functional food industry, pharmaceutical and cosmetic fields, can be obtained from this sustainable extractive technology. Moreover, the innovative process of separation used allowed for obtaining individual extracts that can be modified in order to have specific biological properties and different applications in pharmaceutical, cosmetic and food industries (innovative natural additives and functional food). The research limitations concern the standardization of the process technologies, and the quality of the product, with particular regard to the titer in active principles. OH-Tyr enriched fractions can be exploited as new food ingredients and, for the first time in this research, as starting materials for the synthesis of the corresponding hydroxytyrosol fatty acid esters by an eco-friendly procedure. The aim of this paper was then to obtain OH-Tyr lipophilic derivatives, showing an amphiphilic nature due to the presence of the catecholic moiety on one side of the molecule and the alkyl chain on the other, to increase their solubility in lipid media without decrease the biological activity.

Method or Approach

The green-extraction was performed in a Rapid Extractor Timatic series (from Tecnolab S.r.l., Perugia, Italy) using a solid-liquid extraction technology. The extraction was performed with water, in a stainless steel basket

at a temperature of 60°C. The working cycle is fully automatic and alternates 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 is generated during the stationary phase, which, thanks to the programmable recirculation, ensures a continuous flow of solvent to the interior of the plant matrix. This avoids over-saturation and the formation of preferential channels, thus ensuring total extraction of the active principles from the vegetal matrix.

This innovative separation process performed with physical technologies (PCT/IT/2009/09425529 by Phenofarm S.r.l.) is defined as BAT (Best Available Technology) and recognized by the EPA (Environmental Protection Agency) (Romani *et al.*, 2010). The technology studied consists of an integrated system of all the filtration stages: Micro (MF), Ultra (UF), Nano (NF) and Reverse Osmosis (RO). The different filtration stages are characterized by different molecular weights with cut-off and filtration degrees. During the manufacturing process, the MF stage is carried out with tubular ceramic membranes in titanium oxide and the UF, NF, and RO stages are conducted with spiral wound module membranes in polyethersulfone (PES). This design maximizes the surface area in a minimum amount of space. Less expensive but more sensitive to pollution, this ecofriendly system consists of consecutive layers of large membranes and supporting material in an envelope-type design rolled up around a perforated steel tube (Pizzichini *et al.*, 2009).

This sustainable technology consists of the water extraction of the aforementioned vegetal material and the steps of fractionation by different membranes. These fractions, characterized and quantified by HPLC/DAD-ESI/MS, have an economic value for the applications related to their antioxidant properties.

The lipophilization reactions of HTyr enriched fractions were performed according to an efficient and sustainable procedure (Bernini *et al.* 2007, 2008).

RESULTS AND IMPLICATIONS

The extraction and separation on different membranes permitted to have three fractions of Olea. In particular, concentrates from microfiltration (CMF), nanofiltration (CNF) and, finally, from reverse osmosis (CRO) were obtained using green olive leaves and dried leaves, as starting materials. The HPLC/DAD/ESI-MS quali-quantitative analyses were performed for each sample obtained from the industrial plant. Hydroxytyrosol and derivatives, secoiridoids, hydroxycinnamic acids, verbascoside, flavonoids and trace amounts of others phenolic compounds were characterized and quantified. The content in total polyphenols of the extracts obtained from green leaves and pitted olive pulp are very similar (24.4 and 28.9% p/p), whereas that coming from the dried leaves is 5.7% p/p. The quantitative data of each polyphenol in the raw fractions of the industrial plant are reported in **Tables 1a** and **1b**.

	Plant Fractions ($g\ l^{-1}$)			
	GL Olea CMF	**GL Olea CNF**	**GL Olea CRO**	**DL Olea CRO**
Hydroxytyrosol derivatives	0.29 ± 0.10	4.69 ± 0.67	6.18 ± 0.58	3.63 ± 0.64
Secoiridoid der.	2.74 ± 1.75	25.13 ± 8.88	26.62 ± 8.14	2.44 ± 1.74
Elenolic acid der.	0.82 ± 0.28	4.05 ± 1.33	4.15 ± 0.45	1.05 ± 0.37
Hydroxycinnamic derivatives	0.03 ± 0.02	0.24 ± 0.13	0.30 ± 0.67	0.21 ± 0.12
Flavonoids	0.15 ± 0.09	0.56 ± 0.18	0.83 ± 0.13	0.29 ± 0.21
Verbascoside	0.09 ± 0.03	0.99 ± 0.31	0.83 ± 0.23	0.71 ± 0.49
Lignans	nd	3.18 ± 1.16	nd	nd
Total Polyphenols	4.12 ± 2.12	38.84 ± 10.31	38.91 ± 8.24	8.33 ± 2.51

	Concentrated Fractions (mg g^{-1})		
	Soft Extract Olea OH-Tyr	**Soft Extract Olea GL**	**Soft Extract Olea DL**
Hydroxytyrosol derivatives	279.89 ± 18.24	24.69 ± 3.47	25.21 ± 1.56
Secoiridoid der.	nd	164.19 ± 1.47	11.09 ± 0.45
Elenolic acid der.	0.51 ± 0.04	28.34 ± 0.43	7.54 ± 0.40
Hydroxycinnamic derivatives	7.83 ± 0.25	1.42 ± 0.06	4.30 ± 0.31
Flavonoids	nd	1.27 ± 0.01	1.00 ± 0.41
Verbascoside	1.69 ± 0.17	6.76 ± 0.10	5.85 ± 1.05
Lignans	nd	17.48 ± 0.01	2.65 ± 0.23
Total Polyphenols	289.93 ± 18.70	244.15 ± 5.54	57.63 ± 4.42

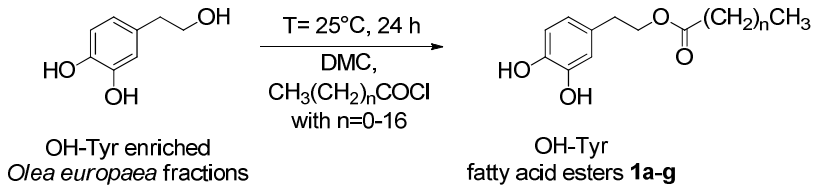
Tables 1a and **1b**. *HPLC/DAD quantitative data of each polyphenol in the raw and concentrated fractions of the industrial plant. CMF = Concentrate of Microfiltration; CNF = Concentrate of Nanofiltration; CRO= Concentrate of Reverse Osmosis; GL= Green Leaves; DL = Dried Leaves; nd = not detected.*

The GL Olea CMF fraction is more diluted than the others (CNF and CRO) because of the higher cut-off of the membrane, allowing for the passage of the main part of the molecules, and then concentrated in the following nanofiltration and reverse osmosis steps. Even so, this fraction can still be used for animal feed and agriculture applications. The concentrations of CNF and CRO from GL are very similar, with a titer in total polyphenols of 3.9% *p*/*V*. The main phenolic compounds in all the fractions, the secoiridoids, show the lowest quantity in the CRO fraction obtained from dried leaves due to the decomposition of oleuropein, one of the main secoiridoid compounds, during the drying process itself. Table 1 also illustrates the concentrated fractions obtained by using a heat pump evaporator, as the initial extracting materials are not only GL and DL, but also pitted olive pulp. This last fraction was called Olea OH-Tyr since the main compounds are hydroxytyrosol (OH-Tyr) and its derivatives. The low concentration of secoiridoids is still evident in the concentrated fractions or soft extracts from olive leaves after their drying process. The titer in total polyphenols of the soft extracts obtained from green leaves and pitted olive pulp are very similar, 24.4 - 28.9% *p*/*p*, whereas that coming from the dried leaves is 5.7% *p*/*p*.

With these fractions, functionalized oils and bakery products naturally stabilized were obtained. In particular, the fractions enriched with OH-Tyr can be used in pharmaceutical, nutraceutical and cosmetic applications, whereas those with lower phenol concentrations can be employed in the food industry, to preserve meat and bakery products (Bargiacchi *et al*., 2014; Forte *et al*., 2013). Due to acting as blood-pressure regulators and modulators of triglyceride and cholesterol levels, the Olea fractions could be used in new formulations of nutraceutical beverages and powders for food supplements (Romani *et al*., 2014).

Finally, an example of green chemistry procedure was reported in order to obtain lipophilic hydroxytyrosol fatty acid esters for the first time directly from natural extracts. The OH-Tyr enriched fractions were employed as substrate and treated with acyl chlorides showing different length (C2-C18, even number) in dimethyl carbonate for 24 h at room temperature (**Scheme 1**). The esterification reactions proceeded in 22-58% yields (calculated by HPLC/DAD/MS) without any catalyst. In consideration of the presence of other compounds into extracts, the sustainability and accessibility of the procedure, these results resulted interesting and promising for similar structural modifications.

Scheme 1. Lipophilization of the OH-Tyr enriched fraction.



FUTURE RESEARCH DIRECTIONS

Future perspective of the developing of innovative process of separation used allowed for obtaining individual extracts that can be modified in order to have specific biological properties and different applications in pharmaceutical, cosmetic and food industries (innovative natural additives and functional food). This new technology prioritizes the prevention and reduction of waste, then its reuse and recycling and, lastly, the optimization of its final disposal. The economic benefits are the getting increased value from co-products and waste streams in the competitive field of Beverage and Food Industries, where the development of sustainable business models are increasing (Donnelly et al., 2015; Romani et al., 2015). Pure HTyr esters and the corresponding esterified *Olea* fractions will be tested on tumoral cell lines for biomedical applications. Further tests will be carried out to investigate their potential utilization in crop protection, and as food/feed supplements.

CONCLUSION

This communication evidenced that by-products of plant food processing represent a promising sources of biologically active compounds and green chemistry is a useful tool to increase their potentiality of application. The processes based on the circular economy and the applications of green technologies are important firstly for their sustainability and secondly because they may be able to create communication among different industrial areas, from food to pharmaceutical, to agronomy up to the energy sector.

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