ChemistrySelect

Research Article
doi.org/10.1002/slct.202403476

Chemistry Europe
European Chemical Societies Publishing

www.chemistryselect.org

Sustainable Extraction of Hydroxytyrosol from Olive Leaves Based on a Nature-Inspired Deep Eutectic Solvent (NADES)

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Within the circular economy framework, the valorisation of olive leaves as an effective source of organic compounds rather than an agricultural waste, represents a great opportunity. Olive leaves contain bioactive polyphenols, such as the health-enhancing catechol hydroxytyrosol (HT), derived primarily from oleuropein. This study establishes a novel and sustainable approach for the extraction of HT from olive leaves, utilizing eco-friendly deep eutectic solvents derived from natural sources (NADES). By optimizing the extraction process, the study highlights the efficacy of the eutectic mixture cholinium/glycerol (1:2 mol/mol) in achieving HT-rich extracts from olive leaves (0.12 ± 0.03 g HT/g of dry extract). Indeed, the method demonstrates a remarkable yield of HT, reaching 12.3 ± 0.9 g/kg of leaf powder, under optimized conditions. The antioxidant properties of the extract are thoroughly assessed, suggesting its potential health benefits. Significantly, the study underscores the scalability of the extraction method, showcasing its viability for large-scale production while maintaining excellent outcomes. Furthermore, the sustainability of the extraction method is boosted by the reuse of NADES across multiple extraction cycles, enhancing resource efficiency and reducing environmental impact. This research unlocks the potential of olive leaves as a valuable source of bioactive compounds and outlines a sustainable extraction approach aligning with the principles of the circular economy.

1. Introduction

Agri-food processing by industries involves the generation of a significant amount of waste and by-products, detrimental for the environment. Within the framework of sustainable development, the correct and efficient management of food wastes represents nowadays one of the main concerns for industries.[1,2]

Therefore, in the present worldwide climatic and energetic emergencies, the valorization of agri-food residues to obtain precious molecules contained in various waste sources represents a great opportunity to achieve the transition toward a sustainable and circular economy.[3–5]

Among agri-food wastes, in recent years, those resulting from the production of olive oil have been arousing great interest.[6] The olive oil agro-industry is a key economic sector for the producing countries, mainly in the Mediterranean region. Spain, Italy, and Greece are the main European producers of olive oil. Spain is currently the leading country in Europe and the world, with around 60% of the EU's olive oil production and 45% of the world's.[7,8]

Olea europaea L. leaves represent a copious waste resulting from olive cultivation, generated mainly during the pruning and harvesting of olive trees. It should be noted that, out of the total pruning residues (leaves, twigs, and branches), the leaves constitute 25% of the dry weight.[6,9] Moreover, when the olives arrive at the mill, they are subjected to a precleaning process in which a significant quantity of olive leaves are separated. It is estimated that for every 100 kg of olives processed, approximately 8 kg of leaves remain as waste and require adequate disposal. Other studies report that for each liter of olive oil produced, 6.23 kg of pruning residues (branches and leaves) are produced.[6,9]

This waste biomass is mainly intended to be burned, composted or to be used for animal feed.[9,10] Therefore, it is desirable to implement alternative strategies aimed at valorising olive leaves rather than considering them merely as waste.

Olea europaea L. leaves constitute a bioresource of active polyphenolic metabolites with recognized benefits for the human health. In *Olea europaea* trees, polyphenolic compounds are the secondary plant metabolites that have been demonstrated to exhibit biologically relevant activities, such as anti-inflammatory and antioxidant properties, as well as long-term human health-promoting properties related to cardioprotective and neuroprotective effects.[11,12]

The most abundant phenol of *O. europaea*, present both in olive leaves and fruits, is oleuropein **1**, a bioactive glycosylated secoiridoid consisting of three components: glucose, elenolic acid, and hydroxytyrosol (HT).[13] Specifically, elenolic acid represents the central core of oleuropein, bound to the catechol HT by an ester bond, and linked to a molecule of glucose by a glycosidic bond (Figure 1).

HT is an *ortho*-biphenolic compound recognized as the molecule responsible for the bioactivity of the oleuropein. The

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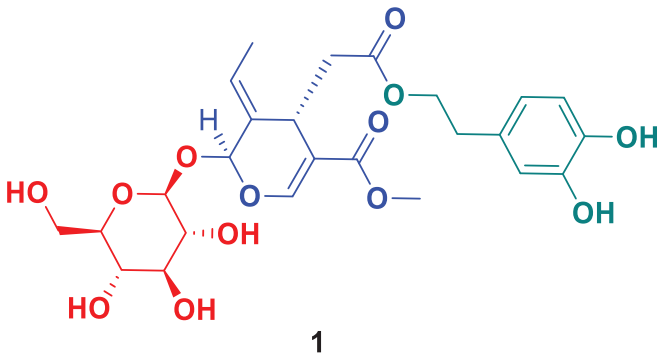


Figure 1. Oleuropein **1** and its components: glucose (red), elenolic acid (blue), and hydroxytyrosol (green).

catechol HT itself has been hypothesized to exert a wide range of biological properties including cardioprotective, anticancer, neuroprotective, and antimicrobial effects. HT bioactivity has primarily been linked to its potent antioxidant properties, stemming from the *o*-dihydroxyphenyl moiety, which has a propensity to convert into the HTox quinone form, thus enabling it to function as a free radical scavenger (Scheme 1).[14]

HT is rarely in free form in nature, but its production requires a process of cleavage of oleuropein by enzymatic or chemical hydrolysis. The enzymatic hydrolysis of the oleuropein, which occurs naturally in olive tree, involves specific enzymes such as β-glycosidase and esterase. On the other hand, acid hydrolysis of oleuropein represents the most used method in the laboratory and in industrial processes to generate free HT (Scheme 2).[15]

Nowadays, several HT formulations are commercially available.[16,17] Therefore, due to its human health-beneficial properties, in recent years HT is arousing growing interest from researchers, trying to find procedures that aim to obtain large quantities of HT, through enzymatic or chemical synthetic processes,[18–20] or using mill waste.

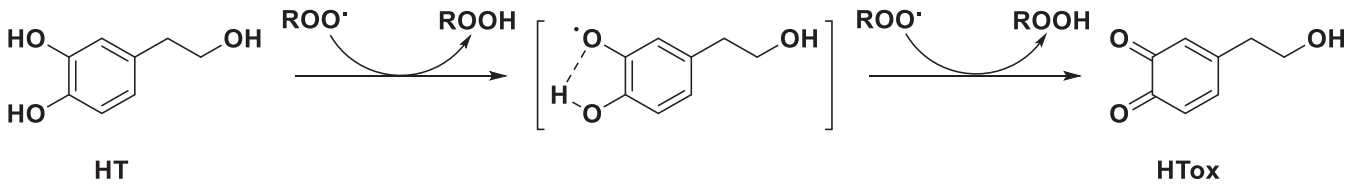
Among the mill waste, several studies have focused on the extraction of HT from olive oil mill wastewaters,[21,22] being rich in this polyphenol. However, the extraction of HT from olive leaves has received much less consideration.[15,23,24]

Usually, recovery of bioactive polyphenols from olive leaves involves conventional extraction methodologies based on the use of ethanol,[25] methanol,[26] dimethyl sulfoxide,[27] and hexane[28] as solvents. Most of these conventional volatile organic solvents are often toxic to humans and the environment,[29] and should be replaced in the rational design of future chemical processes, fully respecting humans and the environment.

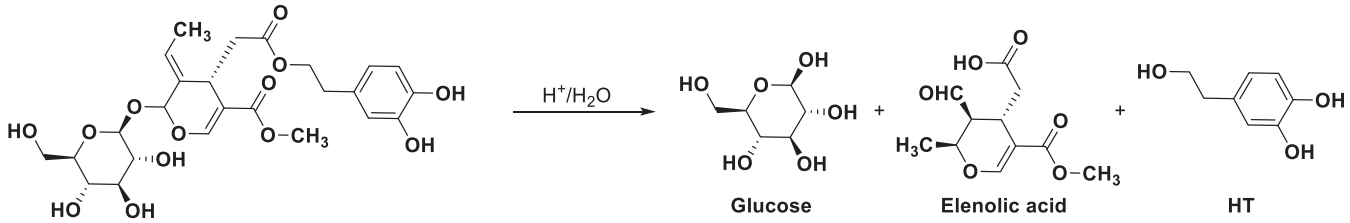
Due to ecological and toxicological issues and in the search for more sustainable alternatives, natural deep eutectic solvents (NADESs) are emerging as a promising new class of unconventional and environmentally friendly ionic solvents for use in the polyphenol extraction field.[30]

NADESs are combinations of at least two safe and cost-effective components. They are usually composed of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), which can form a eutectic mixture, with a melting point much lower than that of either of the individual components. Compared with conventional organic solvents, NADESs exhibit low volatility, high thermal stability, and nonflammability. Typical components of NADESs are cholinium chloride (ChCl), glycerol, urea, natural carboxylic acids, carbohydrates, polyalcohols, etc.; these components are nature-inspired molecules derived renewable sources; thus, their biodegradability is extraordinarily high, and their toxicity is very low or nonexistent. Moreover, NADESs display high tunable solvent properties by simply changing the molar ratio or the nature of the components.[31,32]

There is a growing interest regarding the extraction of the bioactive polyphenols from olive leaves using NADESs as solvents, especially in the last few years. For example, Batista et al. showed that the mixture ChCl:acetic acid, among the carboxylic acid-based DESs tested, extracted the largest amount of phenolic compounds from olive leaves.[33] L-Lactic acid combined with glycerol or glycine has also been demonstrated to be a suitable solvent for obtaining oleuropein-rich extracts.[34,35] However, only a few studies have been reported regarding the extraction of the antioxidant catechol HT from olive leaves employing NADESs. For instance, in 2020 it was described that the eutectic mixture citric acid:glycine:water combined with an ultrasound-assisted



Scheme 1. Mechanism of free radical scavenger exerted by HT.



Scheme 2. Products of acid-catalyzed hydrolysis of oleuropein.

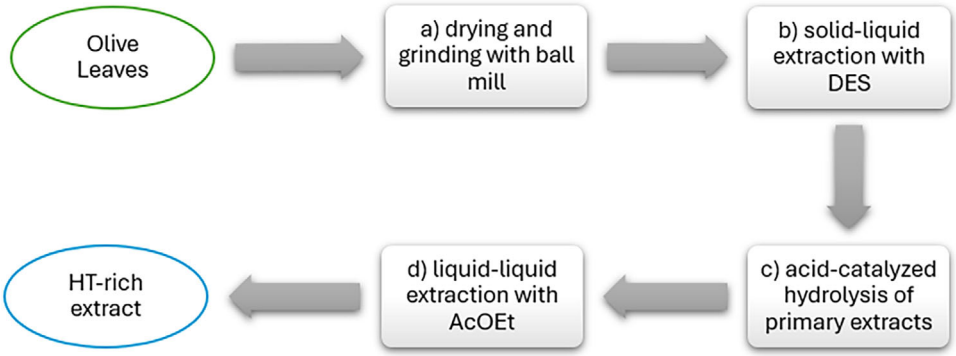


Figure 2. Schematic diagram of the production of HT-rich extracts from olive leaves.

extraction method had the highest extraction of HT from olive leaves.[36]

The aim of the present study is the development of a new, simple, and sustainable method to obtain an olive leaf extract with a high HT content. In particular, the bioactive catechol HT was recovered from olive leaves through an innovative, eco-friendly, and high-performance extraction methodology involving the use of the NADES cholinium chloride/glycerol (1:2 mol/mol) as green and reusable extraction medium, instead of common volatile and toxic organic solvents deriving from petroleum.

2. Results and Discussion

In this paper we described an efficient method for the extraction of the biologically valuable HT from olive leaves as natural source. The process is characterized by high efficiency and sustainability since it allows the extraction of HT up to 12.3 ± 0.9 g from each kilogram of dry olive leaves. The method is based on the use of a nature-inspired, nontoxic, and biodegradable deep eutectic solvent (DES), ChCl:glycerol 1:2 mol/mol, easily reused for up to six consecutive extraction cycles. Our attention has been focused on the optimization of the HT recovery, due to its high biological and commercial value as a powerful antioxidant compound. Furthermore, the method allowed the extraction of a small amount of the phenolic compound tyrosol (T) that was also quantified.

2.1. Optimization of HT Recovery from Olive Leaves

Our investigation has been focused on the optimization of HT recovery from olive leaves starting from the application of four primary steps (Figure 2): (a) drying of olive leaves to constant weight and grinding with ball mill technique to increase the surface area of the solid material, (b) solid–liquid extraction using an appropriate DES, (c) acidic hydrolysis of extracts to obtain HT from oleuropein, and (d) liquid-liquid extraction of the hydrolysate, by employing eco-friendly ethyl acetate to obtain organic extracts with very high concentration in HT.

Table 1. Comparison of olive leaves pretreatment methods before the solid–liquid extraction.[a)]

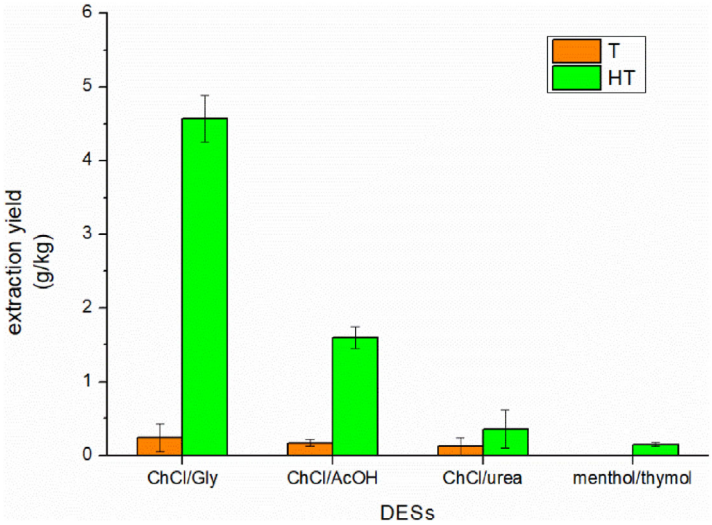
Entry[a)]	Pretreatment Method	T (g/kg)	HT (g/kg)
1	Freezing and cutting	N.R.[b)]	N.R.
2	Ventilated oven and milling	N.R.	0.3 ± 0.1
3	Air-desiccator and milling	0.2 ± 0.2	4.6 ± 0.3

[a)] HT and T amounts (g/kg) refer to the grams of phenol derivatives recovered for each kilogram of leaves powder. Experimental conditions: 1 g of leaves powder was suspended in 10 mL of a 70:30 v/v mixture of DES/water (DES: ChCl/glycerol 1:2 mol/mol) and was subjected to ultrasonication at 80 °C for 1 h. After this time, the extracts were separated from the olive leaf powder by centrifugation (1500 rpm, 10 min) and were then subjected to acidic hydrolysis (HCl 1.5 M) under reflux for 45 min. The reaction mixture was then extracted with AcOEt (3 × 15 mL) to recover polyphenols. The combined organic phases were evaporated under vacuum to obtain a brown oil. A sample of this oil was subsequently collected and analyzed by HPLC.
[b)] Not revealed.

2.1.1. Drying and Grinding of Olive Leaves

The olive leaves (*Olea europaea*, *Leccino cultivar*) used in this study were harvested in November 2022. To understand the influence of pretreatment of the leaves, three different techniques were tested: (a) Initially, the fresh leaves were frozen at −20 °C and then shredded using a cutter into pieces ranging in size from 1 to 2 mm. (b) The second methodology involved the use of a ventilated oven. The fresh leaves were dried at a temperature of 60 °C for 24 h and then finely grounded using a steel ball mill with an oscillation frequency of 30 Hz for 30 s, producing a powder with particle sizes ranging from 100 to 300 µm. (c) For the third method, the olive leaves drying process was conducted in a dryer for 12 days at 30 °C and a relative humidity ranging between 17% and 20%. The dried leaves were subsequently grounded using a steel ball mill with an oscillation frequency of 30 Hz for 30 s, resulting in a powder with particle sizes ranging from 100 to 300 µm. The powders resulting from the three techniques were stored in a freezer at −20 °C until use. Subsequently, the samples were extracted using the methodology reported in Table 1. The analysis of the extracts and the quantification of HT and T content in the samples were performed by High-Performance Liquid Chromatography

Table 2. Effect of the DES composition on the HT and T content in the extracts after hydrolysis.[a)]



Entry[a)]	Deep Eutectic Solvent	T (g/kg)	HT (g/kg)
1	ChCl/gly	0.2 ± 0.2	4.6 ± 0.4
2	ChCl/AcOH	0.17 ± 0.05	1.6 ± 0.2
3	ChCl/urea	0.1 ± 0.1	0.4 ± 0.3
4	menthol/tymol[b)]	N.R.[c)]	0.15 ± 0.03

[a)] HT and T amounts (g/kg) refer to the grams of phenol derivatives recovered for each kilogram of leaves powder. Experimental conditions: 1 g of leaves powder was suspended in 10 mL of a 70:30 v/v mixture of DES/water and subjected to ultrasonication at 80 °C for 1 h. After this time, the extracts were then separated from the olive leaf powder by centrifugation (1500 rpm, 10 min) and were then subjected to acidic hydrolysis (HCl 1.5 M) under reflux for 45 min. The reaction mixture was then extracted with AcOEt (3 × 15 mL) to recover polyphenols. The combined organic phases were evaporated under vacuum to obtain a brown oil. A sample of this oil was subsequently collected and analyzed by HPLC.
[b)] In the case of the hydrophobic eutectic mixture menthol/tymol, no water was added.
[c)] Not revealed.

(HPLC) (see the Experimental Section for further details). As reported in Table 1, the comparison between olive leaves pre-treatment methods shows how the third method appears to be the best in terms of HT extraction. Therefore, this method was used in the subsequent experiments described in the text.

2.1.2. Solid–Liquid Extraction: The Nature of DES Components

The second experimental parameter subjected to the optimization has been the nature of DES employed to extract the organic compounds HT and T. We chose four natural DESs (NADESs) with complementary features, namely, cholinium chloride (ChCl)/glycerol (gly) (1/2 mol/mol) and ChCl/urea (1/2 mol/mol) as representatives of neutral hydrophilic DESs, ChCl/acetic acid (AcOH) (1/2 mol/mol) as acidic hydrophilic DES and finally, the lipophilic DES composed by an equimolar mixture of menthol and thymol (menthol/thymol, 1/1 mol/mol).

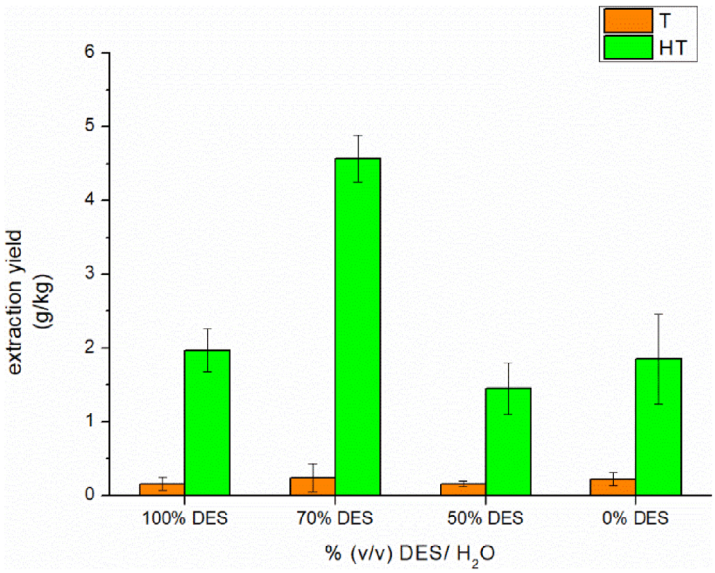
In all the extraction experiments, hydrophilic DESs were mixed with water (DES/H_2O 70:30 v/v) to decrease the viscosity of the medium and improve the mass transfers.

The HT and T contents in the extracts have been expressed in g/kg and it refers to the grams of HT or T extracted per kilogram of leaves powder. All extractions were carried out by sonication for 60 min at 80 °C with a powder/solvent ratio of 1:10 w/v. After this time, the acidic hydrolysis of the extract was performed, under reflux for 45 min. To maximize the recovery of HT from oleuropein, we opted for the acidic hydrolysis instead of a faster hydrolysis in basic media. This choice was deliberate as HT is prone to instability in basic conditions, where it undergoes a rapid polymerization. Hence, we favored an approach that mitigates the potential for HT degradation and guarantees a superior yield from the extracts.[37]

From the data reported in Table 2, it is clear that the hydrophilicity of DESs is a key factor in the extraction of HT and T, and in fact the lipophilic mixture menthol/thymol is practically ineffective. This result was somewhat expected due to the high solubility of HT in water, namely 50 g/L.

Among hydrophilic DESs, those composed by hydroxylated H-bond donors are more successful in the extraction of HT and T with a pronounced preference for the ChCl/gly eutectic mixture, able to extract 4.6 ± 0.4 g of HT for a kilogram of olive leaves. It is noteworthy that in a very recent paper related to the extraction of antioxidant compounds from olive leaves based on the NADES ChCl/gly, the extraction performed in similar conditions (ChCl:gly 1:5 with 30% water, 80 °C as extraction temperature, and 2 h process time under magnetic stirring, resulted in a 0.128 g of HT per kilogram of fresh olive leaves.[39]

Table 3. Influence of water in the extraction of HT and T. [a)]



Entry[a)]	Extraction Solvent	T (g/kg)	HT (g/kg)
1	ChCl/gly	0.16 ± 0.09	2.0 ± 0.3
2	ChCl/gly 70% v/v	0.2 ± 0.2	4.5 ± 0.3
3	ChCl/gly 50% v/v	0.16 ± 0.04	1.5 ± 0.4
4	H_2O	0.22 ± 0.09	1.9 ± 0.6

[a)] HT and T amounts (g/kg) refer to the grams of phenol derivatives recovered for each kilogram of leaves powder. Experimental conditions: 1 g of leaves powder was suspended in 10 mL of a solvent composed by DES (DES: ChCl/glycerol 1:2 mol/mol) and water in the range 0/100–100/0. The mixture was subjected to ultrasonication at 80 °C for 1 h. After this time, the extracts were separated from the olive leaf powder by centrifugation (1500 rpm, 10 min) and were then subjected to acidic hydrolysis (HCl 1.5 M) under reflux for 45 min. The reaction mixture was then extracted with AcOEt (3 × 15 mL) to recover polyphenols. The combined organic phases were evaporated under vacuum to obtain a brown oil. A sample of this oil was subsequently collected and analyzed by HPLC.

2.1.3. Solid–Liquid Extraction: The Role of Water

Based on our prior experience regarding the dramatic impact of water content on the physicochemical properties of hydrophilic DESs,[38,40] we investigated the influence of water on the extraction process.

Therefore, four extractions were performed using ChCl/gly with increasing water content in the range of 0%–70% v/v.

As shown in Table 3, the best solvent was found to be the DES with a water content of 30% v/v, which exhibited an HT amount of 4.5 ± 0.3 g/kg. In all other cases, the HT content was reduced by more than half and remained essentially constant regardless the amount of water (Table 3). The observed results could be rationalized by considering that the mixture DES/H_2O 70:30 v/v represents the best compromise between several key features related to the extraction medium such as lipophilicity, viscosity, and ability to wet the solid. In particular, the addition of 30% of water to DES represents the optimal in term of the solubility of polar molecules, such as HT, in the extraction medium. Moreover, the addition of the appropriate amount of water to the medium, compared to pure DES, decreases its viscosity, as reported in the literature,[41] and consequently increases the mass transfer processes and the wettability of solids, two fundamental parameters in the extraction processes. On the other hand, an excess of water in the medium effects negatively the extraction of HT.

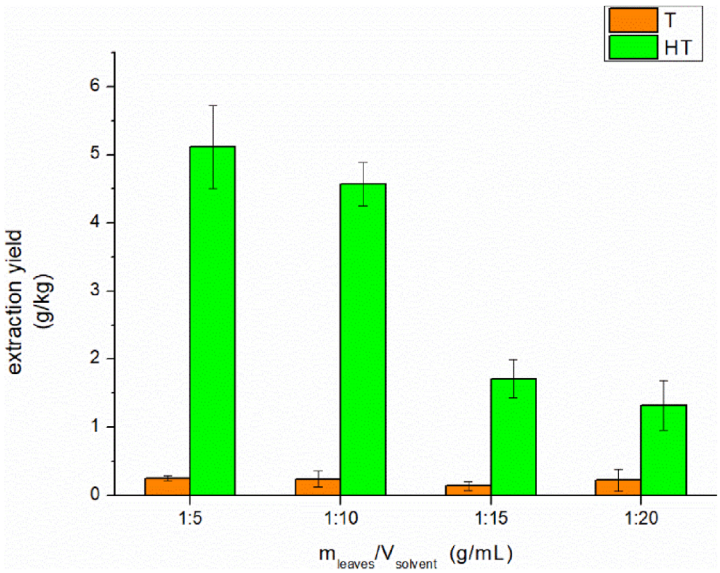
2.1.4. Solid–Liquid Extraction: The Influence of Leaves Powder/DES ratio

Regarding the ratio between the mass of leaves powder and the volume of extraction medium, a brief investigation was conducted with the aim to minimize the amount of DES to be used; the results are summarized in Table 4.

Experiments suggest that when the solid–liquid ratio is in the range from 1:5 to 1:10 g/mL, the content of HT and T in the extract is optimal and essentially similar within the experimental error. However, when the liquid phase increases, a significant decrease in the extraction of HT was experienced, reaching the minimum level of 1.3 ± 0.4 g/kg of HT when a 1:20 solid/liquid ratio was employed.

This apparently counterintuitive behavior can be explained by considering that the solvent used during the solid–liquid extraction process is mainly composed by DES (ChCl/gly) which cannot be removed by simple evaporation, due to its negligible

Table 4. Influence of leaves powder/solvent ratio (in g/mL) used in the solid/liquid extraction.[a)]



Entry[a)]	Leaves (g)/Solvent (mL) Ratio	T (g/kg)	HT (g/kg)
1	1:5	0.25 ± 0.04	5.1 ± 0.6
2	1:10	0.2 ± 0.1	4.6 ± 0.3
3	1:15	0.14 ± 0.06	1.7 ± 0.3
4	1:20	0.2 ± 0.2	1.3 ± 0.4

[a)] HT and T amounts (g/kg) refer to the grams of phenol derivatives recovered for each kilogram of leaves powder. Experimental conditions: 1 g of leaves powder was suspended in appropriate volume of a solvent composed by DES (DES: ChCl/glycerol 1:2 mol/mol) and water in 70:30 v/v ratio. The mixture was subjected to ultrasonication at 80 °C for 1 h. After this time, the extracts were separated from the olive leaf powder by centrifugation (1500 rpm, 10 min) and then subjected to acidic hydrolysis (HCl 1.5 M) under reflux for 45 min. The reaction mixture was then extracted with AcOEt (3 × 15 mL) to recover polyphenols. The combined organic phases were evaporated under vacuum to obtain a brown oil. A sample of this oil was subsequently collected and analyzed by HPLC.

volatility, and, considering its hydrophilicity, it remains in the aqueous phase during the subsequent liquid–liquid extraction.

Consequently, the presence of larger amounts of DES in the aqueous layer during the liquid–liquid extraction with AcOEt, could enhance the solubility of HT and T in the water phase, thus limiting the transfer of phenolic compounds into the organic layer.

2.1.5. Solid–Liquid Extraction: Use of Magnetic Stirring versus Ultrasounds

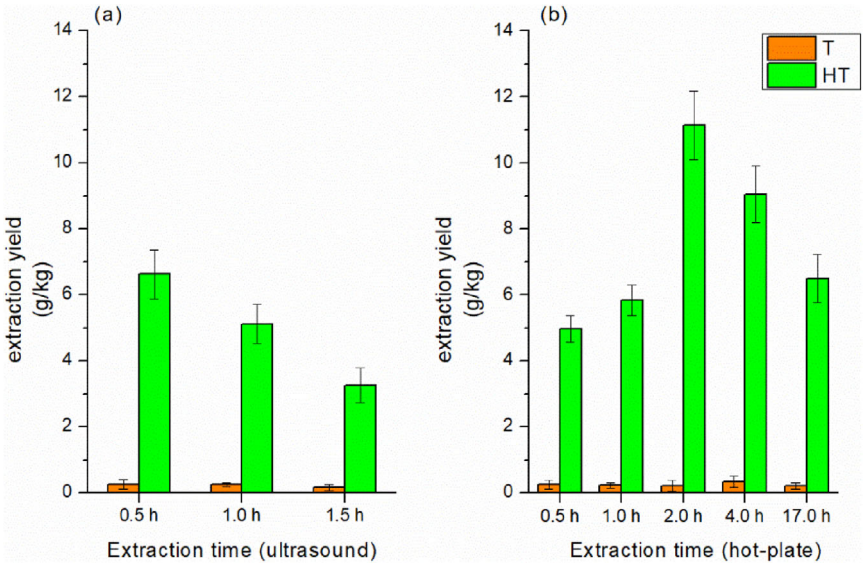
Finally, two experimental techniques for the solid–liquid extraction were screened: the conventional magnetic stirring on a hot plate and the ultrasound-assisted extraction, that were performed at different times (Table 5). The experimental conditions were the same for all tests performed: extraction solvent ChCl/gly with 30% v/v of water, leaves/solvent ratio 1:5 (g/mL), and extraction temperature 80 °C.

Extractions under sonication were carried out at different times: 30, 60, and 90 min. As can be seen from the Table 5a, longer extraction times negatively influences the efficiency of the process. In particular, extraction at 30 min proved to be the most efficient with an amount of 6.6 ± 0.7 g of HT per kilogram of leaves. Surprisingly, the amount of HT in the extracts dramatically decreased to 5.1 ± 0.6 g/kg and 3.3 ± 0.5 g/kg at 60 and 90 min, respectively. We hypothesized that the decrease in HT content in the extracts with increasing time is not due to a lower efficiency in solid–liquid extraction, but rather to the increase in degradative phenomena particularly accelerated by sonication, as observed also for other natural polyphenols such as caffeic acid, rutin, and cyanidin-3-glycoside.[42]

On the contrary, the conventional extraction performed under magnetic stirring on a hot plate gave the best results after 2 h at 80 °C: in this case, 11.1 ± 1.0 g/kg of HT were found in the extracts (Table 5, entry 6). The increase of the extraction time caused, also in this case, a degradation of polyphenolic compounds.

Therefore, based on this experimental screening, it can be concluded that the highest recovery of HT, from powdered olive leaves, is achieved by suspending 1.0 g of leaf powder in 5 mL of a 70:30 v/v mixture of DES/water (DES: ChCl/glycerol 1:2 mol/mol) for 2 h at 80 °C under vigorous magnetic stirring. After this first process, the extracts undergo acidic hydrolysis under reflux

Table 5. Influence of different experimental techniques used in the solid/liquid extraction.[a)]



Entry[a)]	Technique	Time (h)	T (g/kg)	HT (g/kg)
1	Ultrasound	0.5 h	0.3 ± 0.1	6.6 ± 0.7
2	Ultrasound	1 h	0.25 ± 0.05	5.1 ± 0.6
3	Ultrasound	1.5 h	0.16 ± 0.09	3.3 ± 0.5
4	Hot plate	0.5 h	0.3 ± 0.1	5.0 ± 0.4
5	Hot plate	1 h	0.23 ± 0.08	5.9 ± 0.5
6	Hot plate	2 h	0.2 ± 0.2	11.1 ± 1.0
7	Hot plate	4 h	0.3 ± 0.2	9.1 ± 0.9
8	Hot plate	17 h	0.22 ± 0.09	6.5 ± 0.7

[a)] HT and T content in the extract obtained under ultrasound (graph a) or magnetic stirring on a hot plate (graph b). HT and T amounts (g/kg) refer to the grams of phenol derivatives recovered for each kilogram of leaves powder. Experimental conditions: 1 g of leaves powder was suspended in 5 mL of a 70:30 v/v mixture of DES/water (DES: ChCl/glycerol 1:2 mol/mol). The mixture was subjected to ultrasonication or magnetic stirring on a hot plate at 80 °C for different extraction times. After this time, the extracts were separated from the olive leaf powder by centrifugation (1500 rpm, 10 min) and were then subjected to acidic hydrolysis (HCl 1.5 M) under reflux for 45 min. The reaction mixture was then extracted with AcOEt (3 × 15 mL) to recover polyphenols. The combined organic phases were evaporated under vacuum to obtain a brown oil. A sample of this oil was subsequently collected and analyzed by HPLC.

for 45 min. With this optimized two-step process we recovered 11.1 ± 1.0 g/kg of HT (Table 5, entry 6).

2.1.6. Solid–Liquid Extraction: The Optimized One-Pot Procedure

To streamline the entire methodology, we considered that a one-pot procedure, combining both the extraction and the hydrolysis steps (Figure 3), could be advantageous within the context of sustainable chemistry.

Hence, four experiments were conducted, wherein 1.0 g of powdered olive leaves was suspended in 5 mL of a 70:30 v/v mixture of DES/HCl_{aq} (DES: ChCl/glycerol 1:2 mol/mol, HCl_{aq}: 1.5 M) and stirred at 80 °C for different times. The results are reported in Table 6.

We were pleased to discover that after stirring the suspension for just 60 min at 80 °C, it was possible to recover an even greater amount of HT (12.3 ± 0.9 g/kg, Table 6, entry 2) compared to the best result obtained with the two-step methodology (11.1 ± 1.0 g/kg, Table 5, entry 6).

This one-pot procedure, with a recovery rate of HT from powdered olive leaves up to 12.3 ± 0.9 g $kg^{-1}h^{-1}$, offers a quicker approach to HT recovery, while improving sustainability by reducing process duration, energy consumption, and waste generation.

2.1.7. Solid–Liquid Extraction: The Reuse of DES

To further increase the sustainability of the HT polyphenol extraction process, the reuse of the eutectic mixture was tested for multiple consecutive extractions. Using the optimized one-pot procedure, an extraction was carried out starting from 1.0 g of olive leaves powder. Then, 5 mL of the extraction solvent consisting of a 70:30 v/v mixture of DES/HCl_{aq} (DES: ChCl/glycerol 1:2 mol/mol, HC_{laq}: 1.5 M) was added. The extraction mixture was placed on a preheated oil bath at 80 °C, under magnetic stirring for 60 min. Subsequently, the powder was separated from the extraction solvent by centrifugation at 1500 rpm for 15 min. The supernatant, consisting of the organic compounds in a mix-

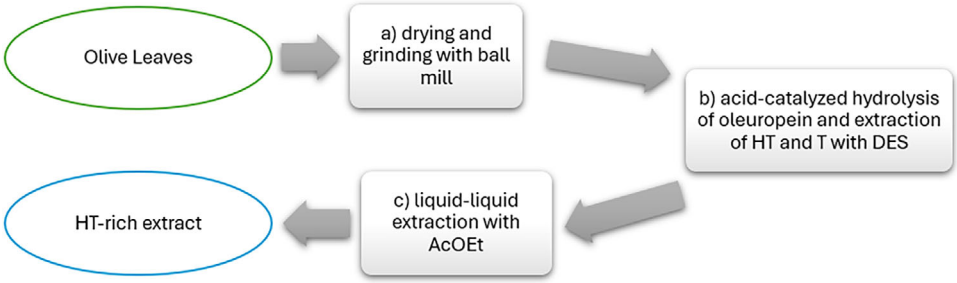
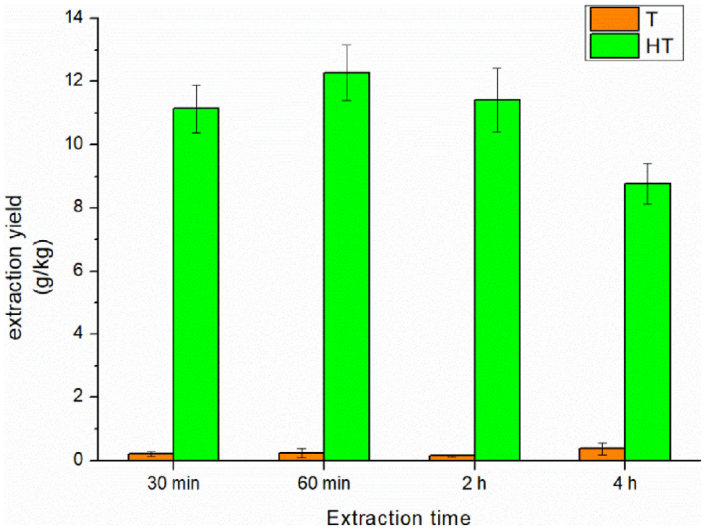


Figure 3. Schematic diagram of the optimized production of HT-rich extracts from olive leaves with a one-pot process for the hydrolysis of oleuropein and DES-mediated extraction of polyphenols.

Table 6. One-pot extraction–hydrolysis process at different times.[a)]



Entry[a)]	Time (h)	T (g/kg)	HT (g/kg)
1	0.5	0.21 ± 0.07	11.1 ± 0.8
2	1	0.2 ± 0.2	12.3 ± 0.9
3	2	0.16 ± 0.04	11.4 ± 1.0
4	4	0.4 ± 0.2	8.8 ± 0.6

[a)] HT and T amounts (g/kg) refer to the grams of phenol derivatives recovered for each kilogram of leaves powder. Experimental conditions: 1.0 g of leaves powder was suspended in 5 mL of a 70:30 v/v mixture of DES/HCl_{aq} (DES: ChCl/glycerol 1:2 mol/mol, HCl_{aq}: 1.5 M). The suspension was subjected to magnetic stirring on a hot plate at 80 °C for different times (see the Experimental Section for further details).

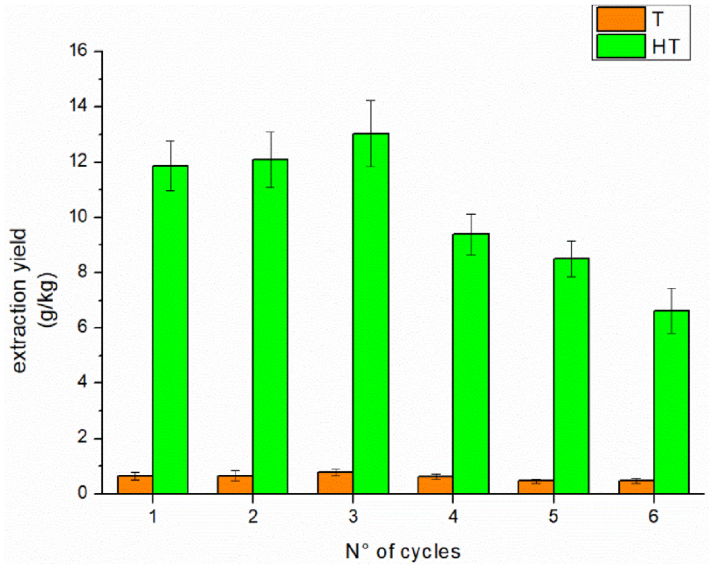
ture of ChCl/gly and aqueous HCl, was extracted with AcOEt (3 × 15 mL) to recover the polyphenols. The remaining aqueous layer was concentrated under vacuum to remove water and HCl until reobtaining the DES mixture, which could be reused for further extractions (see the Experimental Section for more details). The procedure proved to be very effective, maintaining high extraction recovery of HT for up to three consecutive runs (Table 7).

The recovery power of HT showed a decline starting from the fourth cycle. This could be due to the accumulation of polar organic substances in the eutectic mixture during the initial three cycles, potentially impeding the extraction of phenolic compounds in the following runs.

2.1.8. HT Extraction from Olive Leaves in Multigram Scale

The optimized one-pot extraction process was also tested on a multigram scale. Specifically, 30 g of powdered olive leaves were suspended in a solvent mixture consisting of a 70:30 v/v mixture of ChCl/gly and aqueous HCl (1.5 M), in a 500 mL round bottomed flask equipped with a mechanical stirrer (see the Experimental Section for more details). This extraction/hydrolysis step was carried out for 1 h at 80 °C yielding 3.39 g of dry extract. We were pleased to find that the amount of HT present in the extract was very similar to that obtained in the microscale process, ranging in a value of 12.3 ± 1.0 g of HT per kilogram of leaves.

Table 7. Sequential extractions to produce HT-rich extracts by DES reuse.



Run[a)]	T (g/kg)	HT (g/kg)
1	0.6 ± 0.2	11.9 ± 0. 9
2	0.7 ± 0.2	12.1 ± 1.0
3	0.8 ± 0.1	13.0 ± 1.2
4	0.62 ± 0.09	9.4 ± 0.8
5	0.46 ± 0.06	8.5 ± 0.6
6	0.48 ± 0.08	6.6 ± 0.8

[a)] HT and T amounts (g/kg) refer to the grams of phenol derivatives recovered for each kilogram of leaves powder. Experimental conditions for the first cycle: 1 g of leaves powder was suspended in 5 mL of a 70:30 v/v mixture of DES/HClaq (DES: ChCl/glycerol 1:2 mol/mol, HClaq: 1.5 M) and stirred at 80 °C for 1 h. After a centrifugation process to remove solids, extraction with AcOEt to recover phenolic compounds, and concentration under vacuum to evaporate water and HCl, the same DES was reused up to five consecutive extractions (see the Experimental Section for further details).

2.1.9. Antioxidant Activity of HT-Rich Extracts

Finally, the antioxidant activity and the total polyphenolic content of olive leaf dry extracts, both expressed as HT equivalents, were assessed to determine their biological significance. Antioxidant capacity of olive tree leaf extracts was determined to be 0.29 ± 0.03 g HT/g of dry extract, while the total polyphenol content was determined to be 0.18 ± 0.03 g HT/g of dry extract (see the Experimental Section). The last data is in accordance with the amount of HT determined by HPLC and found to be 0.12 ± 0.03 g HT/g of dry extract. This scenario suggests that HT is the predominant polyphenolic compound present in the extracts, and that the higher antioxidant capacity found can be attributed to additional non-polyphenolic molecules.

3. Conclusions

In this work, we disclosed a safe, fast, and efficient chemical process for the recovery of HT from olive leaves. If compared with similar methodologies, the procedure has an improved degree of sustainability since it is based on the use of a nonflammable, nontoxic, bio-based DES, namely the mixture cholinium chloride/glycerol 1:2 mol:mol, used in combination with a 1.5 M aqueous solution of HCl. The methodology, consisting in a one-pot two-step procedure, permitted the extraction of the oleuropein from powdered olive leaves and its contextual hydrolysis to afford a solution of HT in DES/water. The final liquid–liquid extraction with AcOEt, and the subsequent solvent evaporation, allowed the production of a solid extract with a content in HT up to 12.3 ± 0.9 g for a kilogram of leaves and containing 0.12 ± 0.03 g HT /g extract while the extraction solvent could be reused up to three times while maintaining a high extraction yield. Finally, the process remains effective when scaled up to 30 g.

4. Experimental Section

4.1. Chemicals and Reagents

Reagents and solvents, unless otherwise specified, were purchased from Sigma-Aldrich (Sigma-Aldrich) and TCI (Tokyo Chemical Industry) and used without any further purification. NADESs used in this study are as follows: cholinium chloride (ChCl)/glycerol (gly) (1/2 mol/mol), ChCl/urea (1/2 mol/mol), ChCl/acetic acid (AcOH) (1/2 mol/mol), and the DES composed by an equimolar mixture of

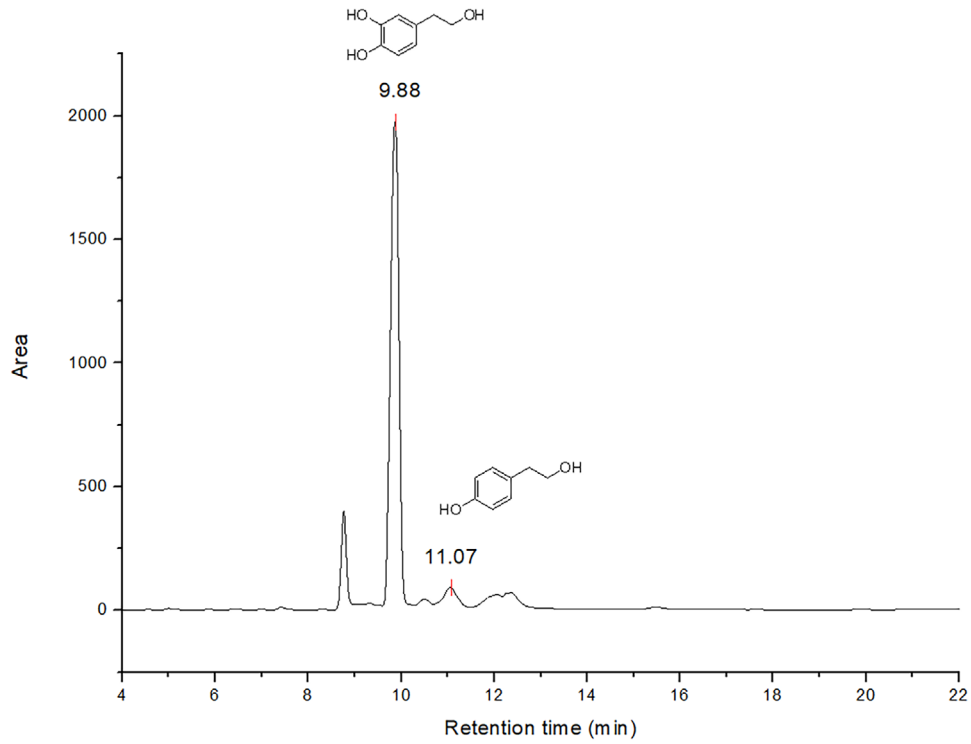


Figure 4. A typical HPLC chromatogram of olive leaves extract.

menthol and tymol (1/1 mol/mol). All the NADESs used in this study were prepared by heating the corresponding individual components under stirring at 60–80 °C for 10–30 min until a clear eutectic mixture was obtained.

4.2. Pretreatment of Olive Leaves

The olive leaves used in this study were harvested in November 2022 from the "Leccino" cultivar in Lecce, Apulia, Italy. The olive leaves were washed with distilled water to remove solids, dirt, and any pesticide residue. The drying process of olive leaves was conducted in a vertical desiccator cabinet with electronic dehumidification system and air circulation for 12 days at 30 °C to remove as much humidity as possible. After this period the relative humidity was found to range between 17% and 20%. The dried leaves were subsequently pulverized using a steel ball mill with an oscillation frequency of 30 Hz for 30 s, resulting in an olive leaf powder with particle sizes ranging from 100 to 300 µm. The powder was stored at −20 °C until further use.

4.3. Quantification of Hydroxytyrosol (HT) and Tyrosol (T) in Olive Leaf Extracts

The analysis of the extracts and the quantification of hydroxytyrosol content in the samples were performed using an HPLC system (Agilent model, series 1260) equipped with a binary pump (G1312B), an autosampler (G1367E), a thermostated column compartment (G1316C), and a UV DAD detector (G1315C), controlled by Agilent ChemStation software. The analysis was conducted using a C-18 HPLC column (LiChrospher RP18 5 µm, 250 × 4.6 mm), with a mobile phase flow rate of 1.00 mL min^{-1}, employing a 10 µL injection volume, with UV detection at 280 nm for HT and 275 for T at 25 °C. The mobile phase consisted of H_2O (HPLC grade, Sigma Aldrich) with 1% H_3PO_4 (A) and MeOH/MeCN 1:1 v/v (B, HPLC grade, Sigma Aldrich). The chromatographic method is based on a gradient elution: A/B 93:7 from 0 to 4 min, A/B 86.5:13.5 from 5 to 17 min, A/B 84:16 until 18 min, followed by reaching 100% B at minute 22. Elution continues for another 5 min with 100% B. Subsequently, from minute 27 to minute 30, the elution gradient returns to initial conditions (A/B 93:7), and the column is stabilized for an additional 5 min. Analytical grade hydroxytyrosol and tyrosol standards (purity ≥98.0%) were used to construct their respective calibration curves reporting peak area versus concentration. Each analysis was repeated three times, and the mean value was recorded. Linearity was assessed using the coefficient of determination R^2, which was found to be 0.9992 for HT and 0.9996 for T. For each experiment carried out in this study, the extraction of powdered olive leaves was performed in triplicate, and the error bars shown in the histograms correspond to the standard deviation of the three measurements. A typical HPLC chromatogram of the extract is presented in the following Figure 4. HT and T eluted at 9.88 min and 11.07 min, respectively.

4.4. Optimized One-pot Procedure for the Extraction of HT from Olive Leaves

In a 20 mL vial, 1.0 g of olive leaf powder was added, followed by the addition of the extraction solvent (5 mL), which consisted of a 70:30 v/v mixture of ChCl/gly and aqueous HCl (1.5 M). The extraction mixture was then heated in an oil bath at 80 °C, while being stirred magnetically for 1 h. Once the extraction was complete, the mixture was centrifuged at 1500 rpm for 15 min to separate the liquid (supernatant) from the plant material (pellet). The pellet was washed twice with H_2O (5 mL each time) through centrifugation (1500 rpm, 10 min). The collected supernatant was then extracted with AcOEt (3 × 15 mL) to recover polyphenols. The combined organic phases

were evaporated under vacuum to obtain a brown oil. A sample of this oil was subsequently solubilized in AcOEt and analyzed by HPLC.

4.5. Procedure for Sequential Extractions of HT from Olive Leaves by DES Re-using

In a 20 mL vial, 1.0 g of olive leaf powder was added, followed by the addition of the extraction solvent (5 mL), which consisted of a 70:30 v/v mixture of ChCl/gly and aqueous HCl (1.5 M). The extraction mixture was then heated in an oil bath at 80 °C, while being stirred magnetically for 1 h. Once the extraction was complete, the mixture was centrifuged at 1500 rpm for 15 min to separate the liquid (supernatant) from the plant material (pellet). The pellet was washed twice with H_2O (5 mL each time) through centrifugation (1500 rpm, 10 min). The aqueous supernatant collected was subsequently extracted with AcOEt (3 × 15 mL) to recover polyphenols. The remaining aqueous layer was concentrated under vacuum to remove water and HCl until the DES mixture was obtained again, which could be reused for a further extraction starting from 1.0 g of fresh olive leaf powder.

4.6. Procedure for the HT and T Extraction from Olive Leaves in Multigram Scale

In a 500 mL round bottomed flask, equipped with a mechanical stirrer, 30 g of olive leaf powder was added, followed by the addition of the extraction solvent (150 mL), which consisted of a 70:30 v/v mixture of ChCl/gly and aqueous HCl (1.5 M). The extraction mixture was then heated in an oil bath at 80 °C, while stirred for 1 h at 150 rpm. Once the extraction was complete, the mixture was centrifuged at 8000 rpm for 10 min at 12 °C to separate the liquid (supernatant) from the plant material (pellet). The pellet was washed twice with H_2O (100 mL each time) through centrifugation (8000 rpm, 5 min, 12 °C). The collected supernatant was then extracted with AcOEt (3 × 250 mL) to recover polyphenols. The combined organic phases were evaporated under vacuum to obtain a brown oil. A sample of this oil was subsequently taken and analyzed by HPLC.

4.7. Measurement of Antioxidant Capacity of Olive Leaf Extracts

Antioxidant capacity of olive tree leaf extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) decoloration assay. Briefly, a 60 μM solution of DPPH in ethanol was prepared, with absorbance approximately 1.0 at 518 nm. Then, 950 μL of DPPH was mixed with 50 μL of extract dissolved in ethanol (in triplicate) or pure ethanol, and the absorbances (A_{518} and $A_{0,\ 518}$ respectively) were read after 20 min of incubation, respectively. The resulting values A_{0518}–A_{518} were converted into HT equivalent antioxidant capacity by using a calibration curve constructed with a series of HT solutions in ethanol in the range 10–80 μg/mL and reported the values of A_{0518}–A_{518} as a function of the HT concentration.

Total polyphenolic content of olive tree leaf extracts was determined using the Folin assay. Specifically, 250 μL of extract was mixed with 250 μL of Folin reagent (diluted 1:5 v/v in water) and 500 μL of NaOH 0.35 M. After 5 min of incubation, the absorbance (A_{720}) was read at 720 nm. This value was converted into HT equivalent concentration using a calibration curve constructed with a series of HT solutions in water in the concentration range 4–40 μg/mL and reporting the values of A_{720} as a function of the HT concentration.

Acknowledgements

Francesco Messa acknowledges Regione Puglia for funding "assegni di RIcerca per riPARTire con le Imprese (RIPARTI)" – Project "Solventi Ionici Naturali per la Valorizzazione di Composti Organici d'Interesse da Scarti della Filiera Olivicola-Olearia", project no. e94b807b, in the framework of POC PUGLIA FESR-FSE 2014/2020 projects. All authors acknowledge A.C.L.I. RACALE – Società Agricola Cooperativa for their collaboration and for providing the natural raw materials.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Deep eutectic solvents · Hydroxytyrosol · Natural antioxidants · Olive leaves · Tyrosol

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Manuscript received: July 24, 2024