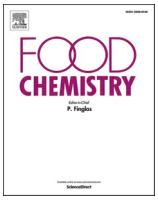
Food Chemistry 447 (2024) 139038

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Novel procedures for olive leaves extracts processing: Selective isolation of oleuropein and elenolic acid

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ARTICLE INFO

Keywords:
Elenolic acid
Lamellar solids
Oleuropein
Olive leaves
Silica sulphuric acid
Solid phase adsorption

ABSTRACT

Several processes have been developed in the past to selectively extract oleuropein and its aglycones from olive derived materials. In the present manuscript, we outline a novel approach for processing olive leaves aqueous extracts. This allowed first to select microwave irradiation as the methodology able to provide a large enrichment in oleuropein. Subsequently, the use of lamellar solids led to the selective and high yield concentration of the same. Adsorption on solids also largely contributed to the long term chemical stability of oleuropein. Finally, an eco-friendly, readily available, and reusable catalyst like H_2SO_4 supported on silica was applied for the hydrolysis of oleuropein into hydroxytyrosol and elenolic acid. This latter was in turn selectively isolated by an acid-base work-up providing its monoaldehydic dihydropyran form (7.8 % extractive yield), that was unequivocally characterized by GC–MS. The isolation of elenolic acid in pure form is described herein for the first time.

1. Introduction

Olea europea L. (olive, Fam. Oleaceae) is among the most important crops in countries of the Mediterranean area. A large number of preparations having in general beneficial effects for human health have been obtained, tested, and commercialized over the last years. In this context, quite recently, much attention has been devoted to the valorization of by-products deriving from olive processing, like leaves. Indeed, these represent the most abundant waste of the olive oil and olive mills industry (>10 % of the total weight of harvested fruits) and agronomy practices, like pruning (around 30 Kg per olive tree) (Calvano & Tamborrino, 2022). Nevertheless, the use of olive leaves as remedies for diseases has been known since ancient times. The first report about their use to this concern dates to the Greek and Roman ages. Nowadays, several products having olive leaf extracts as the main ingredients are marketed and claimed to prevent and ameliorate symptoms of a wide array of diseases. These include those caused by bacteria, viruses, and fungi, hypertension, arrhythmia, intestinal spasms, cancer, neurological disorders, and others (Selim et al., 2022). These effects are typically ascribed to the presence of the secoiridoid glycoside oleuropein (**1**), and of its aglycones hydroxytyrosol (**2**) and elenolic acid (**3**) (Fig. 1), the latter very little studied until now, and of other reported polyphenols and phenylpropanoids in general like dimethyloleuropein, ligstroside, verbascoside, oleuroside, tyrosol, apigenin, quercetin, kaempferol, hesperidin, luteolin, caffeic acid, ferulic acid, chlorogenic acid, and *p*-coumaric acid. Oleuropein is among the most abundant secondary metabolite isolable from a natural source accounting, on average, for more than 9 % of the dry weight of leaves. Nevertheless, the content of this compound strongly depends on many endogenous and exogenous factors, like the botanical variety and existence of different cultivars, climate, stage of crop cycle, type of the extraction process, temperature, pH of the extraction media, number of extractions steps, and others (Monteleone et al., 2021). This can deeply influence the overall quality, efficacy, and commercial value of olive leaf-based phytopreparations (Safarzadeh Markhali et al., 2020). Thus, the search for methodologies with the aim of optimizing and standardizing the content of oleuropein and other relevant secondary metabolites found in olive leaves still represents a challenging and intriguing field of research. This is of pivotal importance, not only for quality control purposes of the whole olive leaf processing, but also for manufacturing in the future phytotherapeutic and nutraceutical entities approaching the market (Lafka et al., 2013). To this regard, an innovative and green chemical

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https://doi.org/10.1016/j.foodchem.2024.139038
Received 22 December 2023; Received in revised form 5 March 2024; Accepted 11 March 2024
Available online 19 March 2024

procedure, based on solid phase adsorption / desorption, to selectively isolate oleuropein in very good yield from aqueous olive leaf extracts has been developed and reported herein. In the meantime, we also focused our attention in trying to obtain from the same extracts elenolic acid (**3**) in pure form. Until now, the isolation of this compound has been very difficult due to a series of reasons, like chemical stability (Antoniadi et al., 2022) and co-occurrence of isoforms deriving from keto-enol tautomeric equilibria (Abbattista et al., 2019). Pharmacological investigations on elenolic acid are also of great importance. This secondary metabolite exhibited a promising preventive and therapeutic potential. Indeed, it has been reported to display *in vitro* and *in vivo* effects as an anti-diabetic (Wang et al., 2022), hepatoprotective (Habibi et al., 2021), and anti-influenza virus (Salamanca et al., 2021) agent. Based on previous findings on the selective isolation of whole classes of secondary metabolites of plant origin, we thus hypothesized that the extraction in heterogenous media by differently structured solid supports could also be applied to the isolation of oleuropein and elenolic acid.

2. Materials and methods

2.1. Chemical reagents and standards

H_2O HPLC-grade (>18.2 MΩ/cm resistivity) used to extract plant materials and HPLC analyses was obtained by using an Elix 3 Milli-Q water deionization and purification system (Millipore, Bedford, MA, USA). MeOH Chromasolv® (purity grade ≥ 99.9 %), EtOAc (purity grade ≥ 99.5 %), HCl 37 %, HCOOH 98 %, 3-hydroxytyrosol (CAS No.10597–60-1, analytical standard), and oleuropein (CAS No. 32619–42-4, analytical standard) were purchased from Merck Sigma-Aldrich (Darmstadt, Germany). CH_3CN (purity grade ≥ 99.9 %) was obtained from Fisher Scientific Italia (Rodano MI, Italy). $NaHCO_3$ was purchased from Carlo Erba reagents (Cornaredo, Milan, Italy). For GC analyses, standard solutions as well as all samples have been derivatized using BSTFA reagent (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1 % of TMCS, trimethylchlorosilane, LiChropur™) obtained from Supelco® (a Sigma-Aldrich Corporation subsidiary), using the already recently reported procedure (Fiorito et al., 2022a). Silica sulfuric acid (SiO_2-OSO_3H), used as the catalyst for the hydrolysis of oleuropein, was prepared *in house* by the reaction between silica gel (high-purity grade), purchased from Honeywell Research Chemicals (Charlotte, NC, USA) and H_2SO_4 96 %, obtained from Carlo Erba reagents (Cornaredo, Milan, Italy). All solid sorbents tested for oleuropein adsorption from aqueous olive leaf extracts were all commercial products and were kindly donated by Prolabin & Tefarm Srl (Perugia, Italy). Their characterization was accomplished by this latter company and chemico-physical properties matched those already reported for the same materials (Genovese et al., 2020). All solids used in the present study are listed in Table 1.

2.2. Plant material and sample treatment

O. europaea (cultivar "Gentile") leaves (geographical origin, Abruzzo region, Italy) were collected from olive trees during olive harvesting and the taxonomic identificationof the plant source has been accomplished by Authors from Chieti. Voucher specimens (OEL-1–2023) have been stored in the deposit of the laboratory of Chemistry of Natural Products and Phytochemistry of the Department of Pharmacy of the University "Gabriele d'Annunzio" of Chieti-Pescara. Leaf samples have been stored in our laboratory and air-dried. Subsequently, they were grounded with an electric spice mill for approximately 2 min. to achieve the finest particle size possible. The obtained powder was stored at 4 °C in the dark before carrying out next experiments. For the extraction procedures, triturated leaves (20 g) have been suspended in the extractive solvent (H_2O, 300 mL) and subjected to microwave (MW) irradiation for 1 min. at 200 W (equivalent to a temperature of 80 °C). The same apparatus, as previously reported, namely a Monowave Edu (Anton Paar GmbH, Graz, Austria) one, was employed to this aim (Fiorito et al., 2015). After filtration under vacuum, the filtrates have been analyzed by HPLC to determine the original content in oleuropein. Subsequently, each solid support listed in Table 1 (100 mg) was added to 5 mL of the original extractive aqueous solution to evaluate their adsorption and preconcentration capacities for oleuropein. The resulting mixtures were kept under vigorous magnetic stirring overnight at room temperature,

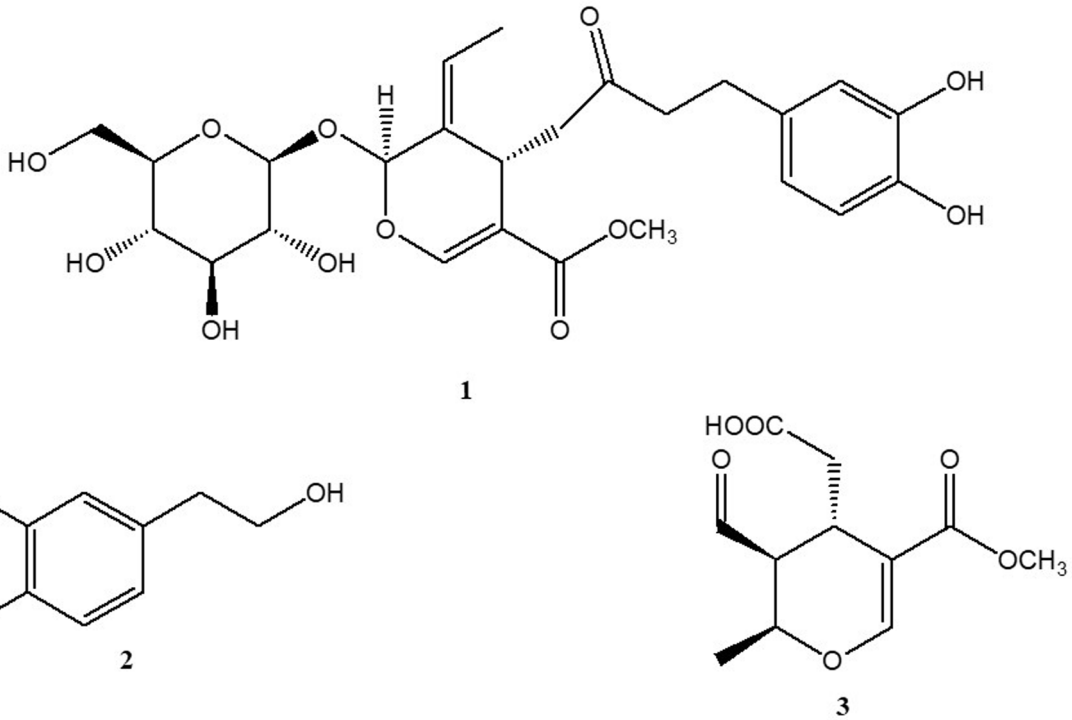

Fig. 1. Chemical structure of oleuropein (**1**), hydroxytyrosol (**2**), and elenolic acid (**3**).

Table 1
List of inorganic and inorganic / organic solids studied herein for the selective extraction of oleuropein from olive leaves aqueous extracts.

	Entry
Layered double hydroxides	
Zinc aluminum oleate	**A**
Zinc aluminum nitrate	**B**
Zinc aluminum chloride	**C**
Magnesium aluminum nitrate	**D**
Magnesium aluminum azelate	**E**
Magnesium aluminum hydroxide chloride	**F**
Magnesium aluminum hydroxide acetate	**G**
Magnesium aluminum hydroxide carbonate	**H**
Magnesium aluminum acetate	**I**
Zinc hydroxy chloride	**L**
Lamellar solids	
Zirconium phosphate (type B)	**M**
Zirconium phosphate (type B) + octadecylamine	**N**
Oxides / Hydroxides	
Magnesium oxide	**O**
Magnesium hydroxide	**P**
Phyllosylicates	
Bentonite	**Q**
Talc	**R**
Mica L	**S**
Mica F	**T**
Mica SFG 20	**U**
Magnesium aluminum D. BenzenSolfonate SDS 01 H8L	**V**
Zinc aluminum D. BenzenSolfonate SDS 02 H8L	**Z**
Florisil®	**AZ**
Alumina	**BZ**

centrifuged (5000*g*, 5 min., room temperature), and filtered under vacuum. The content of oleuropein retained by the solids have been estimated by HPLC analyses, double-checking its quantity in the filtrate and the one obtained after desorption by extensive washing of solids on filters with MeOH (5 x 3 mL). SiO_2-OSO_3H has been prepared from silica and H_2SO_4 96 % following an already reported procedure (Manna et al., 2015). Briefly, 15 g of SiO_2 gel have been dispersed in Et_2O (70 mL). This mixture has been initially stirred for 10 min., then added dropwise H_2SO_4 conc. (3 mL) dissolved in Et_2O (5 mL). After 1 h the solvent has been evaporated to complete dryness in a hot air oven at 60 °C followed by heating for 12 h at 120 °C. A light brown powder for direct use was obtained. Thus, SiO_2-OSO_3H (1.0 g) was suspended into 10 mL of double distilled H_2O and oleuropein (200 mg), purified by the solid phase adsorption procedure outlined above, was added. The resulting mixture was vigorously magnetically stirred for 2 h at room temperature. The suspension was filtered under vacuum and the filtrate analyzed by HPLC showing the total absence of oleuropein. Consequently, the filtrate was basified to pH 8.5 with a 9 % solution of $NaHCO_3$ and extracted with EtOAc (3 x 5 mL). The aqueous phases were recovered, acidified to pH = 3 with a 10 % solution of HCl, and extracted with EtOAc (2 x 5 mL). The collected organic phases were dried over anhydrous $MgSO_4$, and the solvent evaporated to complete dryness under vacuum to provide a raw solid (45 mg). This was analyzed by GC–MS after derivatization, following the same protocol described above, to assess the presence of elenolic acid. This material was further purified by semipreparative HPLC, providing 7 mg of a white waxy solid that was further characterized by ^1H and ^{13}C NMR spectroscopy, following the same general route as already reported (Bruyere et al., 2011).

2.3. HPLC analyses and method validation

The same apparatus equipped with a fluorescence detector (mod. G1321A, Agilent, Santa Clara, CA, USA), as already described, was used to perform HPLC analyses (Fiorito et al., 2022b). The separation was achieved employing a Phenomenex® RP C18 (4.6 mm ∅ x 250 mm, 5 μm particle size) column. The mobile phase consisted of a H_2O / HCOOH 99.6 / 0.4 (solvent A) and CH_3CN / HCOOH 99.6 % / 0.4 % (solvent B) mixtures. A gradient mode at a flow rate of 1.0 mL / min. was applied as the following: 0.0——10.0 min. from 5 % to 20 % B, 10.01——15.0 min. from 20 % to 30 % B, 15.01——18.0 min. 30 % B, 18.01——25.0 min. from 30 % to 50 % B, and finally 25.01——30.0 min. from 50 % to 5 % B. The column temperature was kept fixed at 25 °C and the injection volume was 20 μL. The wavelength for both qualitative and quantitative analyses by DAD was set at 280 nm. The fluorescence detector signal for the qualitative analysis was monitored at 520 nm and 460 nm, both with excitation at 240 nm. All solutions were filtered through a 0.22 μm pore size Durapore® membrane (Merck Sigma-Aldrich, Milan, Italy) before performing HPLC runs. Statistical analyses and data were managed by Open Labs software (Agilent Technologies, Santa Clara, CA, USA). Calibration curves of oleuropein were drawn by injecting pure standard solutions at twelve concentrations values, namely 0.50 μg/mL, 1 μg/mL, 2.5 μg/mL, 5.0 μg/mL, 10.0 μg/mL, 20.0 μg/mL, 25.0 μg/mL, 50.0 μg/mL, 75.0 μg/mL, 150.0 μg/mL, 300.0 μg/mL, 600.0 μg/mL. The retention time recorded for oleuropein was 17.5 min. with an r^2 value of 0.9993. The HPLC method was validated according to the ICH guidelines in terms of the following parameters: precision, accuracy, linearity, limits of detection (LOD), and limits of quantification (LOQ). These and other relevant data have been summarized in Table 2.

The intra-day and inter-day precisions were determined following the already reported procedure (Fiorito et al., 2022b). Precision was measured at three concentration levels for quality control (QC) samples, namely QC_{Low} = 1.0 μg/mL, QC_{Medium} = 75.0 μg/mL, and QC_{High} = 600.0 μg/mL. Accuracy was calculated by spiking samples deriving from extraction of olive leaves with three concentrations of the oleuropein pure chemical standard (low, medium, and high spikes). LOD and LOQ values were determined by the injection of serial dilutions of the corresponding standard solutions, featured by a signal-to-noise (S/N) ratio of 3.3 and 10.0 as the reference, respectively. All calculations were done in triplicate.

2.4. GC–MS qualitative analyses

Gas chromatography-mass spectrometry (GC–MS) analyses have been accomplished using a 8860 GC with 5977B GC/MSD Agilent

Table 2
HPLC-DAD method validation parameters for oleuropein.

Slope (S)	6402
Intercept	−766
SD_s (slope)	971
SD_i (intercept)	47
LOD (μg/mL) [3.3 x SD_s / S]	0.50
LOQ (μg/mL) [10 x SD_s / S]	1.51
Precision	
Intra-day	**% RSD (n = 3)**
QC_{low}	100.1 / 0.997
QC_{medium}	100.2 / 0.996
QC_{high}	100.1 / 0.999
Inter-day	**% RSD(n = 3)**
QC_{low}	100.2 / 0.998
QC_{medium}	100.2 / 0.999
QC_{high}	100.3 / 0.999
Accuracy	
Intra-day	**% Recovery (n = 3)**
QC_{low}	100.8 / 0.995
QC_{medium}	101.0 / 0.996
QC_{high}	100.9 / 0.998
Inter-day	**% Recovery (n = 3)**
QC_{low}	100.1 / 0.998
QC_{medium}	100.6 / 0.996
QC_{high}	101.2 / 0.996
Capacity factor	1.05 ± 0.02
Resolution	1.10 ± 0.02
Peak symmetry	1.01 ± 0.01
Tailing	0.98 ± 0.02

apparatus. Separation of analytes of interest have been obtained by a J&W DB-5 ms Ultra Inert GC Agilent Technologies Column (30 m length, ∅ 0.25 mm capillary column coated with a 1 μm film thickness stationary phase). The sample volume of 0.2 μL was injected into the above-mentioned instruments using an AOC-20i + s autoinjector. The temperature of the injection port has been set at 240 °C in split mode 25:1 ratio. The GC oven temperature has been programmed as the following: 5 min. at 140 °C, increased by 4 °C / min. to the final value of 310 °C (with 2.5 min hold at 300 °C). Solvent delay was set to 7 min. The ion source temperature in the MS has been set at 230 °C and the Interface at 280 °C. Total Ion Chromatogram (TIC) has been created for *m/z* range 50——600. GC peaks have been identified by comparing their mass spectra to the database of the National Institute of Standards and Technology (NIST 11, Mass Spectral Library 2011/EPA/NIH, version updated on June 18th, 2021). Data have been analyzed by using commercial software Origin Pro 2018 (OriginLab Corporation, Northampton, MA) and Jamovi (Version 1.6). All experiments were done in triplicate.

2.5. Purification and quantitative determination of elenolic acid

Semipreparative-scale HPLC purification of elenolic acid was carried out on a Shimadzu system equipped with a SIL-20A autosampler, a LC-20AD pump, a CTO-10AS column oven, a SPD-20A UV–VIS detector, and a FRC-10A fraction collector. The separations were performed on a Phenomenex C18(2) Luna column (10.0 mm ∅ x 250 mm, 5 μm particle size, 100 Å pore size) (Phenomenex, Torrance, CA, USA), with the oven temperature set at 40 °C. The mobile phase consisted of a H_2O / CH_3CN / HCOOH (94.9 % / 0.5 % / 0.1 %) (solvent A) and CH_3CN / H_2O / HCOOH (94.9 % / 0.5 % / 0.1 %) (solvent B) mixture working in a gradient mode at a flow rate of 4.0 mL/min. The gradient was changed over time as the following: 0.0——10.0 min, from 5 % to 20 % B, 10.01——15.0 min. from 20 % to 30 % B, 15.01——18.0 min. 30 % B, 18.01 – 25.0 min. from 30 % to 50 % B, 25.01——30.0 min. from 50 % to 5 % B. Finally, the initial experimental conditions were maintained for 5 min. The peak corresponding to elenolic acid, having a retention time of 7.8 min, was collected manually from injections of 90 μL of the crude extract originally made at a concentration of 45 mg/mL.

2.6. Statistical analysis

Statistical analyses were elaborated following the same general procedure, namely Student's *t* test, as already reported in the literature (Fiorito et al., 2022b).

3. Results and discussion

The first step of our study consisted in the extraction of finely powdered olive leaves. Several attempts to this concern were accomplished (e.g. maceration, ultrasound- and MW-assisted, and Soxhlet extractions). Conditions were optimized by applying MW irradiation to an aqueous suspension rigorously for 1 min. at 200 W of potency, equivalent to a temperature of 80 °C. Other processes, employing the same extractive solvent or a mixture of H_2O / MeOH 8:2, indicated several times in the literature as the most performing means (Malik & Bradford, 2008), led to poorer yields in oleuropein (e.g. maceration) even after prolonged times (up to 48 h), as revealed by HPLC analyses, or to its extensive chemical disruption (e.g ultrasounds assisted and Soxhlet extractions). Although the application of heat, as occurs during MW extraction, has been described to provide a rapid thermal degradation of oleuropein (Martínez-Navarro et al., 2021), the indicated time (1 min.) represented an excellent compromise. In fact, lower time resulted in lower yields, while higher ones provide a deep darkening of extractive solutions probably due to a partial but extensive oxidation of polyphenols, including the desired secoiridoid glycoside. As revealed by HPLC analyses of the filtrates, a comparison between all extraction processes revealed that the MW-assisted one led to obtain a quantity of oleuropein 2.3 times higher than the other ones, as briefly reported in Table 3.

In very recent years, we oriented many efforts to the optimization of solid phase adsorption procedures based on the use of layered biocompatible materials to absorb and/or intercalate different types of secondary metabolites from chemically complex plant-derived matrices. In this context, we investigated the performance of the panel of solid sorbents listed in Table 1. These comprised layered double hydroxides (clays), zirconium phosphate-based solids, magnesium oxide and magnesium hydroxide, and phyllosilicates. All were assayed for their performance in the selective adsorption of oleuropein from the olive leaf aqueous extracts, obtained as described above. To this aim, 5 mL of each aqueous suspension were added 100 mg of each sorbent, and the resulting mixtures were magnetically stirred at room temperature for 24 h. Each mixture has been transferred in an Eppendorf tube, centrifuged (5000g, room temperature, 5 min.), and the supernatant discarded. Oleuropein retained on each solid was desorbed by extensive washing with MeOH and the corresponding filtrates analyzed by HPLC. Data so obtained represented the results of the solid phase adsorption experiments and are illustrated in Table 4. For all experiments, *t*-test values [95 % confidence level ($\nu = 2$)] are lower than the theoretical ones (data not shown).

Data reported in Table 4 show that all solid sorbents were able to absorb oleuropein in good to excellent yields. 5 out of 23 supports provided nearly quantitative yield. The good yields obtained in general could be due to the fact that oleuropein, through the many OH functions present in the sugar and polyphenolic portions, is able to effectively interact through complexation with the metal cations that characterize the surface and inner structures of the sorbent materials. In this context, it is noteworthy to highlight how the best results in terms of adsorption yields were recorded for those materials containing organic anions as part of the crystal structures (e.g. intercalation), namely Zn Al oleate (entry A), Mg Al azelate (entry E), Mg Al hydroxide acetate (entry G), Mg Al D. BenzenSolfonate SDS 01 H8L (entry V), and Zinc Al D. BenzenSolfonate SDS 02 H8L (entry Z). This may indicate how the adsorption of oleuropein is greatly favored by additional hydrophobic and Van der Waals interactions with such organic moieties respect to purely inorganic materials. We also studied the chemical stability of oleuropein retained by each solid by desorption every 48 h in the same way as described above. We found that this secoiridoid remained virtually chemically unaltered during a period of investigation of 60 days with a decrease < 1.0 %. Thus, it is worth emphasizing that the described treatment may be a strategy to simultaneously improve the isolation and the stability of oleuropein for subsequent treatments. Values reported in Table 4 were the results of the optimization of the solid phase adsorption step considering the influence of key parameters like sorbent loading and times. Increases of loading of solid supports up to 500 mg and of times to 72 h provided no substantial improvements of the adsorption yields. Any increase of above 35 °C resulted in the temperature- and time-dependent chemical degradation of oleuropein.

By the procedure described above, we realized that we could easily handle quantities of pure oleuropein sufficient for further chemical

Table 3
Comparison of extractive yieldsof oleuropein from olive leaves under optimized conditions for each of the methodologies employed. Values are expressed as mg/gand as mean ± SD (n = 3).

Method	Extractive yield (mg/g)
Maceration*	44.3 ± 0.16
Soxhlet**	27.9 ± 0.08
Ultrasound***	29.9 ± 0.24
Microwave****	81.3 ± 0.34

*24 h, room temperature; **2h; ***10 min, 60 °C; ****_Vide supra_ for optimized experimental conditions.

Table 4
Quantitative determination of oleuropeinadsorbed onto sorbents (A-Y) from olive leaves aqueous extracts HPLC-DAD. Values are expressed as mg/g, percentage, and as mean ± SD (n = 3).

Sample entry	Oleuropein	
	mg/g	%
Raw extract*	81.3 ± 0.34	–
A	79.4 ± 0.21	97.6 ± 0.02
B	65.0 ± 0.31	79.9 ± 0.01
C	64.8 ± 0.24	79.7 ± 0.02
D	66.2 ± 0.22	81.4 ± 0.02
E	81.2 ± 0.12	99.9 ± 0.01
F	67.4 ± 0.32	82.9 ± 0.03
G	76.2 ± 0.18	93.7 ± 0.02
H	62.4 ± 0.23	76.7 ± 0.01
I	72.6 ± 0.17	89.2 ± 0.02
L	68.8 ± 0.26	84.6 ± 0.01
M	49.1 ± 0.19	60.4 ± 0.02
N	52.2 ± 0.25	64.2 ± 0.02
O	41.6 ± 0.19	51.1 ± 0.01
P	40.1 ± 0.20	49.3 ± 0.03
Q	55.2 ± 0.21	67.9 ± 0.02
R	57.3 ± 0.20	70.4 ± 0.01
S	61.8 ± 0.18	76.0 ± 0.02
T	64.4 ± 0.17	79.2 ± 0.01
U	62.8 ± 0.17	77.2 ± 0.01
V	80.4 ± 0.13	99.1 ± 0.03
Z	80.4 ± 0.12	99.1 ± 0.02
AZ	65.9 ± 0.22	81.0 ± 0.03
BZ	52.8 ± 0.24	64.9 ± 0.01

* MW extraction

processing. Among these, we thought worthy of interest attempting the isolation in pure form of elenolic acid. Indeed, the isolation of such an active principle did not have been subject of extensive research. This may be probably because it did not have been claimed until now to exert valuable and powerful biological activities like oleuropein and hydroxytyrosol. However, quite recently, elenolic acid was seen to have appreciable antiviral and human metabolism modulatory properties (Wang et al., 2022; Salamanca et al., 2021). Considering that this terpenoid moiety is directly linked to the sugar core of oleuropein by a glycosidic linkage, we decided to perform acid hydrolysis of this latter and hopefully of the ester residue of the hydroxytyrosol portion. Unfortunately, any attempts to operate in the homogeneous phase with either inorganic (e.g. sulphuric, hydrochloric) or organic (acetic, lactic, citric, *p*-toluensulfonic) acids, at different concentrations and pH values, and at temperature up to 40 °C in the time range 10 min. – 24 h, resulted in an extensive chemical degradation of the starting material and / or in obtaining by-products resulting from a partial hydrolysis (e.g. oleacein) (Shimamoto et al., 2023) or only hydroxytyrosol. In recent years, SiO_2-SO_3H has been recognized as a valuable and powerful alternative to mineral acids to accomplish numerous inorganic and organic reactions (Pramanik & Bhar, 2021). The advantages of –SO_3H functionalized silica are attributed to its thermal stability, easy recovery, and efficient recyclability, without a significant loss of catalytic activity. Furthermore, this heterogeneous solid support allows to perform reactions under milder conditions and in most cases without affecting chemically labile groups (Kaur et al., 2015). Thus, after the optimization of the experimental conditions, and having determined a 1:5 oleuropein / SiO_2-SO_3H ratio as the best promoter, we made the secoiridoid glycoside to react with the solid support in a heterogeneous reaction medium. The analysis of the filtrate by HPLC showed the complete disappearance of peaks related oleuropein and the formation of new unresolved peaks centered around an R_t of 6.84 min. These were attributed to overlapping peaks of hydroxytyrosol and elenolic acid, having similar polarity. To verify and validate such a hypothesis, we submitted the same sample to GC–MS analyses after derivatization as TMS ethers. As a result, two distinct peaks having R_t of 24.44 min. and 26.59 min. clearly appeared in the chromatogram and these were attributed to derivatized hydroxytyrosol and elenolic acid. To further confirm this finding, we separated by acid-base work-up the two products obtained after the hydrolysis with SiO_2-SO_3H. Thus, the filtrate was basified to pH 8.5 – 9 with a solution of $NaHCO_3$ and the resulting solution extracted with EtOAc. After having combined and dried, the organic phases were discarded, while the aqueous ones were acidified to pH 3 with diluted HCl and extracted with EtOAc. The two organic solutions so obtained were separately analyzed by GC–MS after derivatization as previously reported. Each of the two chromatograms showed only one peak with R_t values of 24.42 min. and 26.57 min., that can be unambiguously attributed (by comparison of the MS fragmentation with that reported in the NIST database) to hydrotyrosol and more interestingly to elenolic acid as illustrated in Fig. 2.

After purification we could get elenolic acid in pure form in 7.8 % overall yield from oleuropein, employing the hydrolytic procedure promoted by SiO_2-SO_3Hin H_2O. Other than by its MS spectrum fragmentation, the structure of the product we obtained was confirmed to be elenolic acid also by comparison of its ^1H and ^{13}C NMR data with those reported in the literature for the same compound (Christophoridou et al., 2005; Rigakou et al., 2019).

Performing the study depicted in the present paper, we succeeded in setting up three different methodologies to obtain enriched phytopreparations and / or to isolate individual compounds from raw olive leaf materials. These represent valid alternatives to what has been reported so far in the literature on the same topic. In the first step of our investigation, we have shown how the extraction of the vegetable matrix promoted by MW for a very short period provided an appreciable enrichment in oleuropein of the related aqueous extracts. In the recent past, several other processes for the convenient extraction of this secoiridoid from the same source have been developed. These included conventional and modern techniques employing different solvents. All have been very recently excellently reviewed by Khalil and coworkers highlighting advantages and drawbacks of each process (Khalil et al., 2023). To this concern, it is evident how the application of MW for the extraction of oleuropein from olive leaves is not strictly “new”, as some authors already applied such a method for the same purpose adopting slightly different experimental conditions (Martiny et al., 2022; Sánchez-Gutiérrez et al., 2021; Şahin et al., 2017; Japón-Luján et al., 2006). However, data we recorded herein are a confirmation of the effectiveness of MW and H_2O as a mean for the extraction of oleuropein, especially in terms of yields and purity. The second step of our study comprised the assessment of the efficiency of a panel of solid sorbents belonging to different chemical classes (e.g. layered double hydroxides, zirconium-based lamellar solid, Mg oxide and hydroxides, and phyllosilicates) for the adsorption of oleuropein from aqueous olive leaf extracts. To the best of our knowledge, such a process, that employs olive leaves as the starting material, is reported herein for the first time in the literature. Indeed, the solid phase adsorption of this secoiridoid is a field of research that received relatively little attention, in particular when compared to the whole number of investigations reported and devoted to oleuropein extraction. The few articles appeared in the literature during the last two decades were all focused on the use of solid sorbents not belonging to layered double hydroxides and / or lamellar solids in general. Thus, Ozcan and Demirli in, 2013 reported the use of molecularly imprinted polymers for the same purpose, but obtained low extractive yields in the desired secondary metabolites (2.4 %). Other materials employed comprised the combination of ionic liquids and polymer networks (Liu et al., 2015), porous materials with carboxyl-modified multi-walled carbon nanotubes (Liu et al., 2015), macroporous resins (e.g. Amberlites XAD 2, XAD 4, XAD 7HP, and XAD 16) (Şahin & Belgin, 2017; Johnson and Mitchell, 2019; Liu et al., 2020), and boric acid affinity resins (Liu et al., 2021).These methodologies, although effective and representing easy to handle procedures, have some disadvantages like partial selectivity in the adsorption of oleuropein, low extractive yields, use of toxic and polluting solvents either for adsorption and / or desorption, and some difficulties for the synthesis of

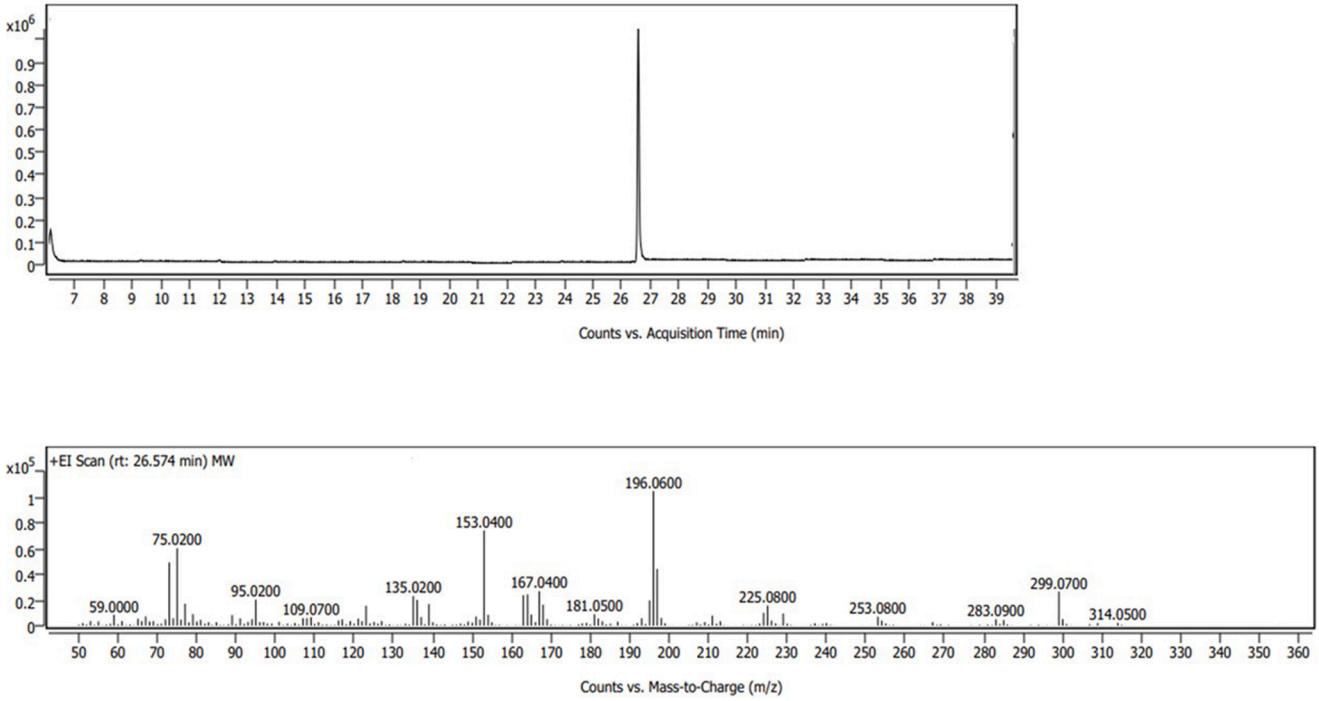

Fig. 2. GC chromatogram MS-fragmentation spectrumof pure elenolic acid.

sorbents. Thus, the one we proposed herein represents, in our opinion, a valid alternative to the exisiting methodologies for the solid phase adsorption of oleuropein from olive leaf aqueous extracts. The use of lamellar solids favourably compares to the already reported processes mainly in terms of extractive yields, selectivity, easy work-up, and low cost and ready accessibility of sorbent materials. Furthermore, it is worthy to underline how the retention of oleuropein by the listed solids largely contribute to its chemical stability over a long period of time. It is intriguing the fact that the same phenomenon was experienced in the case of another very easily oxidizable phytochemical, like crocetin from saffron aqueous extracts (Fiorito et al., 2022c). It may be hypothesized to this concern that clays may preserve the chemical integrity of such active principles by a sort of "encapsulation" of molecules into the inner surface featuring their multilammelar structure. So the use of clays to enhance oleuropein chemical stability over time is a valid alternative to the few methods for the same purpose reported in the literature until now. The most explicative examples to this aim include its chemical transformation (e.g. peracetylation) (Bonacci et al., 2018), use of surfactants (Stamatopoulos et al., 2014), and inclusion in liposomes (González-Ortega et al., 2021). In the third and last step of the experimental route outlined herein, we set up a method to selectively obtain elenolic acid by hydrolisis of oleuropein. Again, few methodologies to obtain this terpenoid have been reported in the literature. Indeed, most of the hydrolitic reactions using oleuropein as the starting material have been devoted to get pure hydroxytyrosol. The reported processes typically employed isolated enzymes (Chatzikonstantinou et al., 2022) and mineral acids (Romero et al., 2020). It i snoteworthy to highlight that, under the experimental conditions used to obtain hydroxytyrosol, the terpenoid structure of oleuropein underwent extensive chemical degradation, so that elenolic acid could not practically be recovered. Indeed, the enrichment of phytopreparations with this terpenoid can be to date effectively managed only by enzymatic hydrolysis (Ramirez et al., 2016; Yuan et al., 2015). Nevertheless, even by this method, elenolic acid did not have been so far obtained in pure form. As an explicative example to this concern, Isenolic®, the most marketed product in the world containing compound (**3**), used as an immun adjuvant and an anti-influenza virus agent, is an enriched extract containing elenolic acid in the concentration range 4 % – 8 %. Furthermore, all purification methodologies reported to date in the literature did not aim to isolate this compound, but only to its qualitative and quantitative determination in chemically complex matrices (Przybylska et al., 2023). Some chemical synthesis of elenolic acid are actually available (e.g. in principal in order to provide a pure chemical compound for pharmacological assays and / or pure analytical standard), but all are practically useless due to the high number of synthetic steps (>10 in most cases), to the lack of diasteroselectivity, to the very low overall yields, and finally to the highly toxic and polluting reagents and solvents employed to accomplish the whole synthetic routes. Thus, to the best of our knowledge, the ones we reported herein is the first and to date only route to easily obtain elenolic acid in pure form by chemical hydrolisis.

4. Conclusions

In this manuscript a novel methodology to process olive leaves has been described. It was demonstrated, as one of the few examples reported in the literature, that the selective removal of oleuropein by clays modified with organic anions can occur. This finding in our opinion represents a concrete address to develop novel and alternative methodologies towards the separation of and / or enrichment with oleuropein of olive-leaf based phytopreparations. Furthermore, the one reported herein is the first example, to the best of our knowledge, of the isolation of elenolic acid, that has nowadays a well recognized therapeutic potential for several acute and chronic diseases affecting humans and animals. As a final consideration, data reported in the present manuscript, largely contribute to enforce the concept of olive leaves, so far thought as waste material from drupe harvest, as high value commercial products. Indeed, we have shown how, by application of simple and easy to handle experimental procedures, and the use of low cost materials and equipments, the same matrix is a unique source of powerful biologically active phytochemicals.

CRediT authorship contribution statement

Serena Fiorito: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Chiara Collevecchio:** Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Roberto Spogli:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Francesco Epifano:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Salvatore Genovese:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

Authors wish to acknowledge University “G. D’Annunzio” of Chieti-Pescara for financial support to this research (FAR 2021).

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