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Technical Report

Supercritical fluid extraction of bioactive compounds from *Olea europaea* pruning wastes

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Abstract:

The aim of this report is to describe a single step supercritical fluid extraction (SFE) that enables the simultaneous isolation of phenolic compounds, carotenoids, and tocopherols from olive (*Olea europaea*) leaves. Utilizing CO_2 and bio-ethanol as extraction solvents, the optimized conditions achieved recoveries of approximately 76 % for oleuropein, 84 % for α-tocopherol, 88 % for lutein, and 90 % for β-carotene. Although the recoveries obtained were slightly lower than those achieved through ultrasound-assisted extractions, the SFE approach offered significant advantages, including automation, the use of safer solvents, and improved environmental compatibility.

Keywords: Supercritical fluid extraction, olive leaves, bioactive compounds

1. Introduction

Pruning residues from olive trees (*Olea europaea*) are increasingly recognized as a rich source of bioactive molecules. Such materials, traditionally discarded or burned for energy purposes, are now being reconsidered for their ability to add value to agricultural biomass and support circular economy strategies.

A single SFE step was optimized to recover three different classes of bioactive compounds, namely tocopherols, carotenoids, and phenols, from Olea europaea leaves, which have different octanol-water partition coefficients (log *P*). In order to investigate the extraction recovery, the same matrices were initially extracted using three different validated ultrasound-assisted extraction (UAE) procedures [1,2,3], each selective for the molecular classes of interest.

2. Experimental

2.1 Chemicals and reagents

Water (H_2O), acetonitrile (ACN), methanol (MeOH) (UHPLC-MS grade, purity ≥ 99.9 %), acetone (Ace), isopropanol (IPA), hexane (Hex), bio-ethanol (bio-EtOH), methyl tert-butyl ether (MTBE), dichloromethane (DCM) (gradient grade for HPLC, purity ≥ 99.9 %), formic acid (FA), and all the standards were purchased from Merck KGaA (Darmstadt, Germany).

2.2 Samples and sample preparation

Olea europaea leaves were harvested in February 2025 in Messina, Italy. After harvesting, leaves were washed with distilled water and dried at ambient temperature until a constant weight was achieved. The dried samples were ground using a laboratory mill and sieved to obtain a particle size of less than 0.3 mm.

2.3 Instrumentation

The supercritical fluid extraction (SFE) was carried out using an SFE pretreatment system (Shimadzu Nexera UC) equipped with; a CBM-40 controller, an SFE-30A module, an LC-30AD$_{SF}$ CO_2 pump, an LC-40DXR modifier pump, an LC-40D make-up pump, an SFC-30A back pressure regulator, a DGU-405 degasser, an FRC-40 fraction collector.

The obtained extracts were analyzed using high-performance liquid chromatography (HPLC) coupled to different detectors according to the compounds class. The characterization of tocopherols was conducted utilizing a fluorometric detector (FLD: RF-20AXS, Shimadzu). A photodiode array detector (PDA: SPD-M30A, Shimadzu), coupled with a single quadrupole mass spectrometer (LCMS-2020, Shimadzu) equipped with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) interfaces, was used for the determination of carotenoids and phenols, respectively. All the employed analytical conditions are reported in Table 1.

Table 1: HPLC conditions used for bioactive compounds characterization.

Compound class	Column	Mobile phase		Pump time program	Flow rate (mL/min)	Injection volume (µL)	Oven temp. (°C)	Detection
		A	B					
Phenols	Ascentis Express C18 (150 mm × 4.6 mm I.D., 2.7 µm)	0.1 % FA in H_2O	0.1 % FA in ACN	Gradient: 0 min, 10 % B; 4 min, 35 % B; 12 min, 47 % B; 12.5 min, 60 % B; 16 min, 75 % B; 21 min, 100 % B	1	5	25	PDA: 280 nm; MS Interface: ESI; scan: 100-800 *m/z*
Carotenoids	Ascentis Express C30 (250 mm × 4.6 mm I.D., 2.7 µm)	MeOH/MTBE/ H_2O (90:8:2, *v/v/v*)	MeOH/MTBE/ H_2O (8:90:2, *v/v/v*)	Gradient: 0 min, 0 % B; 20 min, 20 % B; 60 min, 50 % B; 65 min, 100 % B; 70 min, 100 % B; 75 min, 0 % B	0.8	20	30	PDA: 450 nm; MS Interface: APCI; scan: 100-1000 *m/z*
Tocopherols	Ascentis Si (250 mm × 4.6 mm I.D., 5 µm)	Hex	IPA	Isocratic: 99:1, *v/v*	1.7	5	25	FLD: Ex. 290 nm, Em. 333 nm

2.4 UAE procedures

For the UAE procedures, 100 mg of dry leaf powder was used as starting material. Phenolic compounds were extracted using 1 mL of bio-EtOH/H_2O (8:2, *v/v*) for 10 min, 4 times until exhaustion. Carotenoids were extracted using 1 mL of Ace/MeOH (1:1, *v/v*) for 10 min (3 times until exhaustion). Tocopherols were extracted using 1 mL of Hex for 10 min (3 times until exhaustion). All the extractions were centrifuged at 4500 rpm for 5 min and filtered prior to HPLC analysis.

2.5 SFE optimization

The 0.2 mL stainless steel extraction vessels were directly filled with 100 mg of olive leaf powder.

In order to develop a multi-class SFE and obtain the best recovery for all the compound classes, several parameters were evaluated (static and dynamic extraction time, extraction temperature, extraction pressure, modifier percentage, and flow rate). All the tested methods are reported in Table 2. The flow rate was 1 mL/min except for MET.12, which was 2 mL/min. The extraction temperature was set at 40 °C for all methods, except for MET. 9 and MET. 10 performed at 50 and 60 °C, respectively.

3. Results and discussion

The target compounds span a very broad polarity range, with log *P* values varying from –2.3 (for oleoside) to 13.5 (for β-carotene). Considering this chemical diversity, the SFE method was optimized to produce a single step extract containing all the target compound classes while minimizing manual operations.

To simplify the discussion about the optimization workflow, only the extraction recovery of the most representative and abundant compound of each class was evaluated. Specifically, oleuropein was selected for polyphenols, α-tocopherol for tocopherols, lutein and β-carotene for carotenoids were selected.

Initial trials were conducted employing pure CO_2 as the extraction solvent. However, CO_2 alone was able to extract mainly non-polar compounds such as tocopherols and β-carotene. Consequently, the use of a polar modifier was mandatory to ensure the extraction of phenolic compounds and polar pigments. A 2 min static extraction step using 100 % CO_2 was applied in all tests to ensure complete filling of the 0.2 mL extraction vessel. This was followed by two dynamic steps: a 5 min dynamic extraction with 100 % CO_2 to recover non-polar compounds, and a subsequent 15 min dynamic extraction with 10 % bio-ethanol as the modifier.

Table 2: SFE methods tested. For all methods, 2 min of static extraction and 5 min of dynamic extraction at 100% CO_2 (Step1) were performed at the beginning of SFE.

Method	Dynamic extraction			Flow rate (mL/min)	BPR (MPa)	Temperature (˚C)
	Step	Time (min)	Modifier: bio-EtOH (%)			
MET. 1	2	15	10	1	25	40
MET. 2	2	15	30	1	25	40
MET. 3	2	5	10	1	25	40
	3	15	30			
MET. 4	2	5	10	1	25	40
	3	25	30			
MET. 5	2	5	10	1	15	40
	3	25	30			
MET. 6	2	5	10	1	35	40
	3	25	30			
MET. 7	2	5	10	1	35	50
	3	25	30			
MET. 8	2	5	10	1	35	60
	3	25	30			
MET. 9	2	5	10	1	35	40
	3	25	40			
MET. 10	2	5	10	2	35	40
	3	25	30			
MET. 11	2	15	10	1	35	40
	3	35	30			

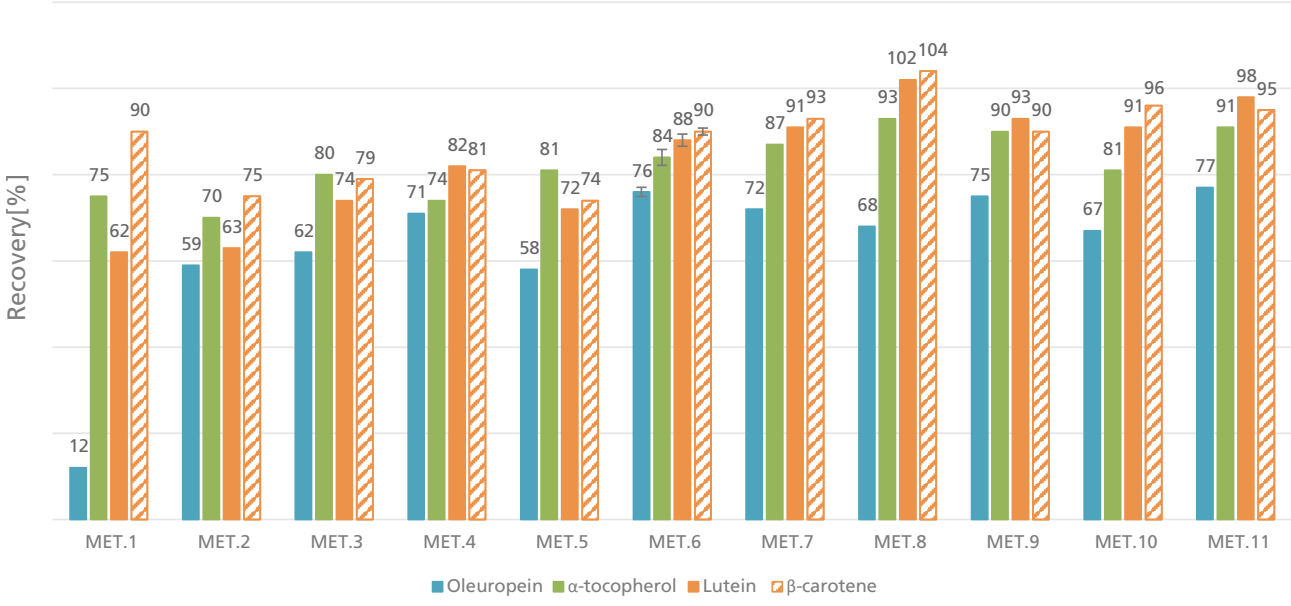

Figure 1: Bar graphs illustrating the recoveries of oleuropein, α-tocopherol, lutein, and β-carotene obtained for each of the SFE methods tested (UAE results were used as references) (refer to Table 2 for comprehensive details regarding the methods employed).

Under these conditions, with a pressure of 25 MPa, a flow rate of 1 mL/min, and a temperature of 40 ˚C , recoveries were observed to be 12 % for oleuropein, 75 % for α-tocopherol, 62% for lutein, and 90 % for β-carotene (MET. 1). Given that oleuropein, which is one of the most polar and abundant compounds, exhibited the lowest extraction efficiency, the amount of modifier was increased from 10 % to 30 % (MET. 2). As anticipated, the recovery of oleuropein significantly improved to 59 %, while the extraction of β-carotene decreased from 90 % to 75 %.

To address this issue, in MET. 3, the dynamic extraction was further subdivided into three steps: 5 min with 100 % CO_2, 5 minutes with 10 % bio-ethanol, and 15 min with 30 % bio-ethanol. This strategy resulted in a recovery of 62 % for oleuropein, along with recoveries of 80 %, 74 %, and 79 % for α-tocopherol, lutein, and β-carotene, respectively.

To further enhance oleuropein extraction, the dynamic extraction time at 30 % modifier concentration was extended from 15 to 25 minutes, resulting in an oleuropein recovery of up to 71 % (MET. 4).

The effects of pressure and temperature on extraction efficiency were also investigated. A reduction in pressure from 25 to 15 MPa (MET. 5) resulted in decreased recoveries for all target compounds. Conversely, increasing the pressure to 35 MPa (MET. 6) significantly improved recoveries, achieving 76% for oleuropein, 84% for α-tocopherol, 88% for lutein, and 90% for β-carotene. Additionally, raising the temperature from 40 ˚C to 50 ˚C (MET. 7) and then to 60 ˚C (MET. 8) enhanced the recovery of most compounds, with the exception of oleuropein, which exhibited lower recovery at higher temperatures. Therefore, 40 ˚C was selected as the optimal extraction temperature.

Additional adjustments were investigated to reach the best compromise among the compound classes. Raising the modifier level to 40 % for 25 min of dynamic extraction (MET. 9) produced minimal gains, as it negatively affected CO_2 supercritical properties at high modifier concentrations. Increasing the flow rate to 2 mL/min (MET. 10) mainly reduced oleuropein recovery by roughly 10 %, likely due to shorter contact time between solvent and matrix. Additionally, the higher modifier consumption also made it unattractive, and the flow rate was reset to 1 mL/min.

Finally, the dynamic stages using 10 % and 30 % of bio-EtOH were extended, from 5 and 25, to 15 min and 35 min, respectively (MET. 11). However, the modest improvement did not justify extending the total extraction duration by an additional 20 min.

Therefore, the optimized conditions identified as the best compromise among all the target compound classes were as follows (MET. 6):

- **2 min static extraction with 100 % CO_2;**
- **5 min dynamic extraction with 100 % CO_2;**
- **5 min dynamic extraction with 10 % bio-EtOH;**
- **25 min dynamic extraction with 30 % bio-EtOH.**

The operating parameters were: 1 mL/min flow rate, 35 MPa, and 40 ˚C. Under these optimized conditions, the following recoveries were achieved 76 % for oleuropein, 84 % for α-tocopherol, 88 % for lutein, and 90 % for β-carotene. The obtained recovery values are reported in Fig. 1. The chromatograms and quantitative results obtained from SFE are shown in Figure 2 and Table 3, respectively.

Conclusion

An SFE protocol was developed to produce a single step extract enriched in phenolic compounds, carotenoids, and tocopherols from olive pruning residues. The approach proved suitable for sustainable and large-scale processing, using only CO_2 and bio-EtOH and reducing both solvent risks and manual handling.

The single SFE procedure was completed in around 40 minutes, while the three separate UAE techniques required about 3 hours overall. Beyond its shorter runtime, SFE demonstrated superior performance over UAE in terms of extraction speed, automation, sample processing capacity, material sustainability, and operator safety, indicating its strong potential for facilitating the production of bioactive compounds and natural food additives under a circular-economy model.

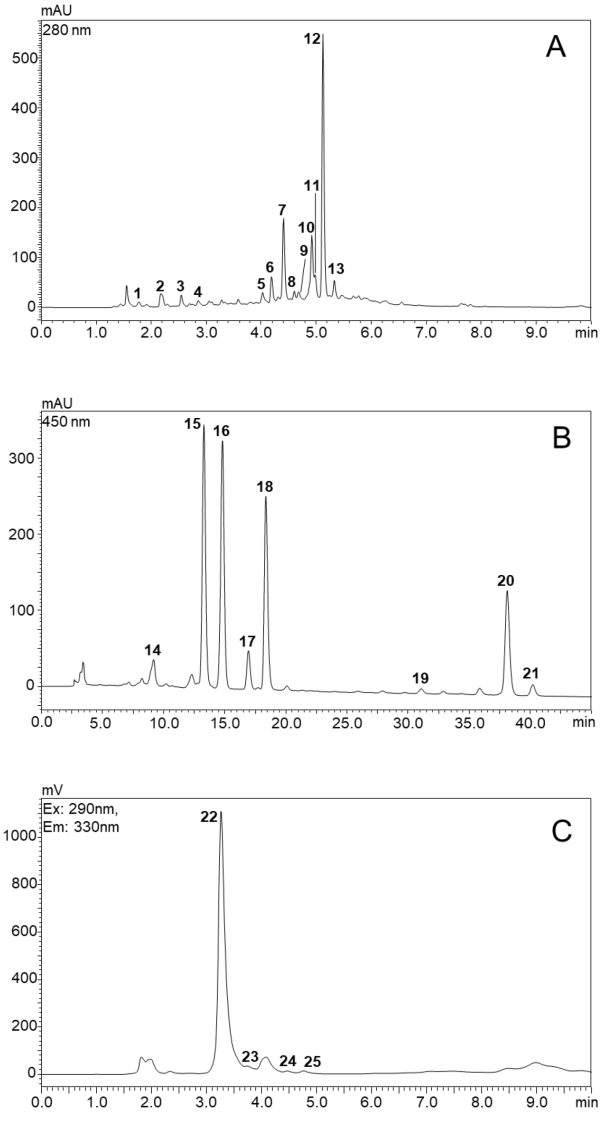

Figure 2: Chromatograms of phenolic compounds (A : PDA 280nm), carotenoids (B: PDA 450 nm), and tocopherols (FLD: Ex. 290 nm, Em. 330 nm). Compound names and quantitative results are reported in Table 3.

Table 3: Concentration (mg/kg ± standard deviation) of the bioactive compounds quantified in the SFE extracts.

NO	Compound	Quantified value
1	Gallic acid	92 ± 3
2	Hydroxytyrosol glucoside	942 ± 9
3	Hydroxytyrosol	535 ± 7
4	Oleoside	1337 ± 11
5	Hydroxyoleuropein	3675± 13
6	Verbascoside	2298 ± 13
7	Luteolin 7-O-glucoside	1953 ± 8
8	Oleuropein glucoside	2109 ± 10
9	Apigenin rutinoside	153 ± 3
10	Apigenin glucoside	1650 ± 13
11	Luteolin 4-O-glucoside	529 ± 8
12	Oleuropein	64519 ± 25
13	Lucidumoside C	1725 ± 15
14	Neoxanthin	97 ± 5
15	Chlorophyll b	276 ± 12
16	Lutein	865 ± 11
17	Zeaxanthin	107 ± 5
18	Chlorophyll a	338 ± 6
19	15/13 cis-β-carotene	8 ± 1
20	Trans-β-carotene	217 ± 4
21	9-cis-β-carotene	26 ± 2
22	α-tocopherol	171 ± 5
23	α-tocotrienol	14 ± 2
24	β-tocopherol	5 ± 1
25	γ-tocopherol	5 ± 1

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