

## Article

# Comparison of Different Extraction Methods for the Recovery of Olive Leaves Polyphenols

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**Abstract:** In the present study, advanced extraction techniques, microwave (MAE), ultrasound (UAE), and high pressure (HPAE)-assisted extraction, were applied to improve extraction efficiency of olive (*Olea europaea* L.) leaves polyphenols. The effect of sample mass (1.5 and 3 g), MAE—time (2, 8.5, and 15 min) and temperature (45 and 80 °C), UAE—time (7, 14, and 21 min) and amplitude (50 and 100%) and HPAE—time (1, 5.5, and 10 min) and pressure (300 and 500 MPa) on the concentration of each analyzed polyphenol compound was examined. Identified polyphenols were oleuropein, hydroxytyrosol, chlorogenic acid, caffeic acid, verbascoside, and rutin. All three advanced extraction techniques yielded higher content of total polyphenols when compared to the conventional heat-reflux extraction (CE) along with a significant reduction of extraction time from 60 (CE) to 2, 21, and 5.5 min in MAE, UAE, and HPAE, respectively. The most intensive values of tested parameters in each technique were the ones that promoted cell wall disruption, e.g., temperature of 80 °C in MAE, 100% amplitude in UAE and 500 MPa in HPAE. MAE and UAE were more efficient in total polyphenols' recovery than HPAE.

**Keywords:** olive leaves; polyphenols; oleuropein; microwave-assisted extraction; ultrasound-assisted extraction; high pressure-assisted extraction; heat-reflux extraction; extraction efficiency

## 1. Introduction

Olive (*Olea europaea* L.) is one of the most important crops in the Mediterranean region primarily used for oil production, where during pruning of the olive tree a significant amount of by-products (leaves and branches) are accumulated. Olive leaves are considered as an inexpensive, renewable, and abundant source of polyphenols [1] and it has been found that olive leaves extract (OLE) has antioxidative, anti-inflammatory, and antimicrobial properties against bacteria and fungi, and also shows anti-viral properties against several different viruses [2]. Phenolic compounds, including phenolic acids, phenolic alcohols, flavonoids and secoiridoids, are the ones responsible for these various positive properties. Several studies have reported the individual phenolic profile of olive leaves showing hydroxytyrosol, rutin, verbascoside, luteolin-7-glucoside, luteolin-4'-glucoside, oleuropein, oleuropein aglycone, and ligstroside aglycone being the most abundant among the large number of diverse phenolic compounds identified [3–8].

OLE polyphenolic content highly depends upon a plant's geographical origin and cultivar, as well as the efficiency of the applied extraction technique. The conventional extraction techniques such as Soxhlet and heat-reflux extraction are usually performed at high temperature, their duration is long and the requirement for sample and solvent, as well as human work, is large, therefore, causing high costs and having a negative impact on the environment and human health [9]. With an intention to solve

these limitations, some alternative techniques have been developed such as microwave, ultrasound and high hydrostatic pressure-assisted extraction. Each of these techniques has a unique mechanism to improve extraction efficiency. In microwave-assisted extraction (MAE), extraction occurs as the result of changes in the cell structure caused by electromagnetic waves [10], while in ultrasound-assisted extraction (UAE) the enhancement in extraction is mainly attributed to the effect of acoustic cavitation produced in the solvent by the passage of an ultrasound wave [11]. In high-pressure-assisted extraction (HPAE), high pressure disrupts the cell wall and compounds are released from the cytoplasm [12]. Furthermore, the extraction process itself can be affected by the specific extraction parameters such as the sample to solvent ratio, time, temperature, pressure, or ultrasound power. By prolonging the contact of the sample with a solvent or disrupting the cell walls so that more compounds can diffuse to the solvent, more intense extraction conditions (longer extraction time, higher temperature, pressure, or ultrasound power) could possibly improve the extraction yield. However, if applied in excess they could cause the degradation of some polyphenols. All these facts lead to inevitability and necessity for the optimization of the extraction process in order to achieve the maximum effectiveness in isolation of target compounds [1,4,13].

So far HPAE has not been used for the extraction of olive leaves polyphenols, while various studies have dealt with MAE and UAE optimization for olive leaves polyphenols' recovery [1,4,13–15]. However, the optimization of the polyphenols' extraction in the above-mentioned studies was based on the total phenolic content or antioxidant activity of the obtained extracts, without examining the influence of the extraction parameters of these techniques on individual phenolic compounds. Their general conclusion was that in comparison with conventional techniques MAE and UAE are able to provide OLE with similar content of bioactive compounds, but with a remarkable shortening of the extraction time. To the best of our knowledge, there are no studies dealing with the recovery of olive leaves polyphenols involving MAE, UAE, and HPAE simultaneously, as well as their effect on olive leaves individual polyphenols. Therefore, this study aimed to evaluate the effect of various MAE, UAE, and HPAE parameters on the concentration of individual OLE polyphenols as well as to compare these novel extraction techniques mutually and with conventional heat-reflux technique.

## 2. Materials and Methods

### 2.1. Chemicals

Ethanol (96%) was purchased from Gram-mol d.o.o. (Zagreb, Croatia). HPLC standards of oleuropein (OL), verbascoside (VB), rutin (RT), caffeic acid (CF), and chlorogenic acid (CA) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), while hydroxytyrosol (HT) was purchased from Extrasyntheses (Genay, France). All the solvents used for HPLC analysis were HPLC grade.

### 2.2. Plant Material

Olive leaves, cv. Oblica, were collected on Hvar, Croatia. Leaves were air-dried and stored in the dark at ambient temperature. Prior to extraction, leaves were ground with an electric grinder (CM3260, Grunding, Neu-Isenburg, Germany) and the obtained powder was immediately used for the extraction.

### 2.3. Extraction Conditions

The extraction conditions for MAE, UAE, and HPAE were selected based on the previous studies reporting the range of extraction parameters as the most successful for olive polyphenols' extraction [1,4,7,15,16] and, therefore, were taken as the framework for the experimental design applied in this study. For all extraction methods, various sample masses (1.5 and 3 g) were treated with an aqueous solution of ethanol (50%, *v/v*) used as an extraction solvent.

MAE was performed on Start S Microwave Labstation for Synthesis (Milestone, Bergamo, Italy). Extraction parameters were irradiation time (2, 8.5, and 15 min) and temperature (45 and 80 °C).

Microwave power was kept constant at 300 W until the target temperature was achieved. General extraction parameters were: time to achieve extraction temperature (2 min), mixing (50%), and ventilation after extraction (1 min).

An ultrasonic processor (UP) 400 S (Dr. Hielscher GmbH, Teltow, Germany) equipped with ultrasonic probe (surface 3.8 cm<sup>2</sup>) immersed 1 cm into the solution was used for UAE. The maximal nominal output power of the device was 400 W and the ultrasonic frequency was 24 kHz. Extraction time was 7, 14, and 21 min with an amplitude of 50 and 100%. The temperature was measured with an infrared thermometer and it did not exceed 60 °C.

HPAE was conducted in the FPG7100.100 system (Stansted Fluid Power Ltd., Harlow, UK), where the sample–solvent mixture was vacuum packaged and extracted at different pressures (300 and 500 MPa) and extraction times (1, 5.5, and 10 min) with glycol solution used as a pressure-transmitting media.

The temperature was varied as an extraction parameter only in the MAE technique since MAE offers the possibility of its full control during the process. In UAE, it can be controlled only partially in a temperature range by external cooling during the extraction process (it was measured by an infrared thermometer and kept below 60 °C), while HPAE was conducted at ambient temperature, where the increase of temperature as a function of pressure did not promote the degradation of analyzed compounds [17]. Therefore, in the UAE and HPAE the temperature was not taken as a variable.

In order to compare the efficiency of extraction methods listed above with a conventional extraction technique, heat-reflux extraction (CE) was also conducted under previously defined optimal conditions (data not shown): 3 g of the sample was placed into the flask containing 40 mL of the extraction solvent, extracted for 30 min, and decanted. The extraction of residue was then repeated under the same conditions and extracts from each cycle were combined.

After each extraction, obtained extracts were made up to 100 mL with the extraction solvent, centrifugated at 5000 rpm/10 min and the resulting supernatants were stored at −18 °C until further analysis. Each treatment was done in two replicates ( $n = 2$ ).

#### 2.4. HPLC Analysis

An HPLC system (Agilent Technologies HPLC 1200 Series, Santa Clara, CA, USA) equipped with a binary pump, autosampler, UV/Vis-Photo Diode Array Detector (DAD) and a Luna 5 µm C18 column (250 mm × 4.6 mm, 100 Å) (Phenomenex, Torrance, CA, USA) was used for the separation, identification, and quantification of polyphenols according to the method described by Richard et al. (2011) [18] with some modifications. Mobile phases were 0.1% formic acid in water ( $v/v$ ; solvent A) and 0.1% formic acid in methanol ( $v/v$ ; solvent B) and the following gradient was used: 0–3 min, 10% B; 3–30 min, 50% B; 30–40 min, 60% B; 40–45 min, 60% B; 45–50 min, 100% B; 50–60 min, 10% B. Operating conditions were: constant flow rate 1 mL min<sup>−1</sup>, column temperature 30 °C, injection volume 20 µL and UV/Vis-Photo Diode Array detection at 280 nm. Prior to automatic injection into the HPLC system, standards and samples were filtered using 0.45 µm nylon membrane.

Identification of polyphenols was carried out by comparing retention times and spectral data of the separated peaks with those of authentic standards. The quantification of polyphenols was performed by the external standard method. All standards were prepared as stock solutions in methanol. Stock solution was diluted to yield five increasing concentrations and five-point calibration curves were used to calculate the amount of each compound. All measurements were performed in duplicate ( $n = 4$ ) and results were expressed as mean value ± SD of mg g<sup>−1</sup>.

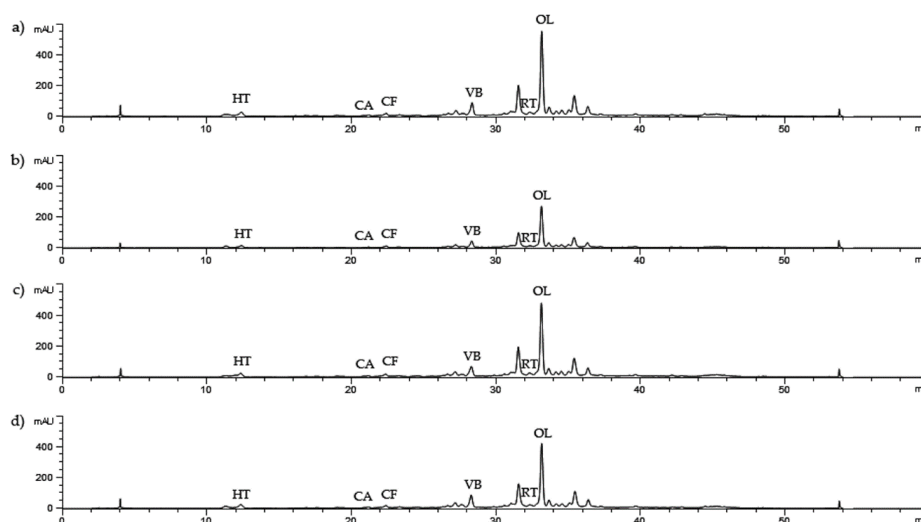
#### 2.5. Statistical Analysis

Statistical analysis was carried out using Statistica ver. 8.0 software (Statsoft Inc., Tulsa, OK, USA). Full factorial randomized experimental design was applied, and descriptive statistics were employed for the basic data evaluation. All continuous variables were analyzed by multivariate analysis of variance (MANOVA) and marginal mean values were compared with Tukey's HSD test. Principal

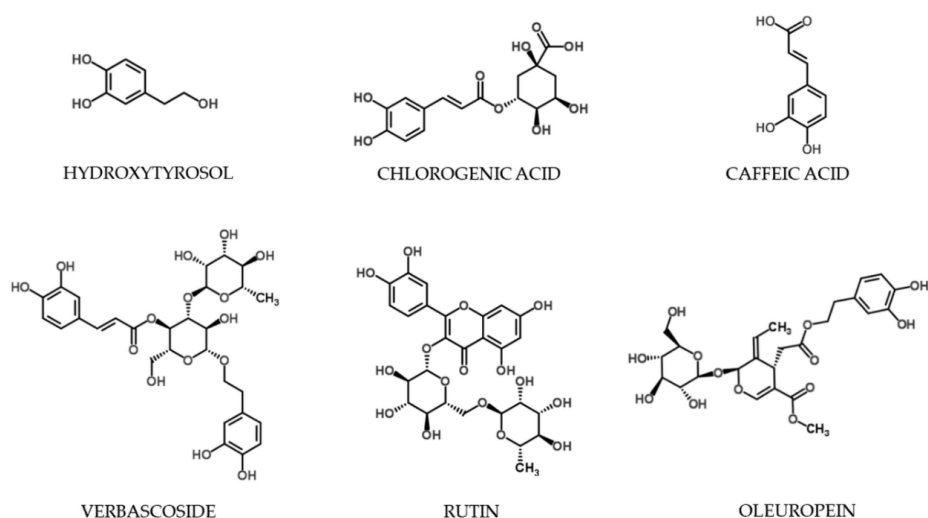
component analysis (PCA) was conducted in order to examine possible grouping of the samples. All tests were evaluated at the significance level  $p \leq 0.05$ .

### 3. Results and Discussion

In the present study, different conditions of MAE, UAE, and HPAE were applied for the polyphenols' recovery from olive leaves. Extracts were analyzed by HPLC and identified polyphenols, as well as their concentrations, are shown in Table 1. Results present the measurement values of individual phenols in extracts obtained by conducted extraction techniques in which specific extraction parameters were varied. HPLC analysis showed that composition of identified polyphenols remained unchanged for all experimental trials, containing six polyphenols listed according to their retention time: HT, CA, CF, VB, RT, and OL (Figures 1 and 2).



**Figure 1.** HPLC chromatograms at 280 nm of olive leaves extracts obtained by (a) microwave-assisted extraction (80 °C, 2 min, 3 g sample); (b) ultrasound-assisted extraction (50%, 21 min, 1.5 g sample); (c) high pressure-assisted extraction (300 MPa, 10 min, 3 g sample); (d) conventional heat-reflux extraction (2 × 30 min, 3 g sample) (HT = hydroxytyrosol, CA = chlorogenic acid, CF = caffeic acid, VB = verbascoside, RT = rutin, OL = oleuropein).



**Figure 2.** Chemical structure of polyphenols identified in olive leaves extracts.

**Table 1.** Olive leaves individual phenols (mg g<sup>−1</sup>) extracted by various extraction techniques.

Extraction Technique	Extraction Parameters			OL	HT	CA	CF	VB	RT	Total Sum
MAE	Time (min)	Temperature (°C)	Mass (g)							
	2	45	1.5	74.11 ± 0.00	1.10 ± 0.42	0.44 ± 0.00	0.26 ± 0.00	0.50 ± 0.00	2.57 ± 0.00	78.98 ± 0.42
	2	45	3.0	69.51 ± 0.43	0.83 ± 0.00	0.45 ± 0.01	0.28 ± 0.00	0.35 ± 0.01	2.23 ± 0.01	73.65 ± 0.43
	2	80	1.5	83.41 ± 0.06	0.86 ± 0.00	0.42 ± 0.00	0.24 ± 0.01	0.55 ± 0.01	2.70 ± 0.02	88.19 ± 0.09
	2	80	3.0	87.75 ± 0.11	0.95 ± 0.03	0.46 ± 0.01	0.32 ± 0.01	0.41 ± 0.00	2.30 ± 0.01	92.18 ± 0.11
	8.5	45	1.5	52.14 ± 0.04	0.61 ± 0.01	0.44 ± 0.00	0.20 ± 0.00	0.31 ± 0.00	8.68 ± 0.06	62.38 ± 0.02
	8.5	45	3.0	65.93 ± 0.10	0.80 ± 0.01	0.41 ± 0.00	0.26 ± 0.01	0.33 ± 0.00	2.15 ± 0.07	69.89 ± 0.20
	8.5	80	1.5	84.82 ± 0.21	0.94 ± 0.00	0.45 ± 0.00	0.29 ± 0.01	0.55 ± 0.00	2.43 ± 0.01	89.49 ± 0.24
	8.5	80	3.0	82.44 ± 0.57	0.93 ± 0.03	0.41 ± 0.02	0.29 ± 0.00	0.40 ± 0.00	2.14 ± 0.27	86.61 ± 0.89
	15	45	1.5	64.03 ± 0.05	0.82 ± 0.01	0.44 ± 0.01	0.24 ± 0.01	0.47 ± 0.00	2.76 ± 0.01	68.75 ± 0.05
	15	45	3.0	70.88 ± 0.05	0.87 ± 0.01	0.48 ± 0.03	0.33 ± 0.04	0.34 ± 0.00	2.46 ± 0.00	75.36 ± 0.12
	15	80	1.5	80.66 ± 0.25	0.92 ± 0.00	0.43 ± 0.03	0.26 ± 0.01	0.54 ± 0.00	2.42 ± 0.03	85.23 ± 0.25
	15	80	3.0	82.01 ± 0.18	0.82 ± 0.17	0.44 ± 0.00	0.30 ± 0.01	0.42 ± 0.04	2.42 ± 0.00	86.41 ± 0.04
UAE	Time (min)	Amplitude (%)	Mass (g)							
	7	50	1.5	79.28 ± 0.26	3.10 ± 1.11	0.46 ± 0.00	0.33 ± 0.00	0.50 ± 0.00	2.54 ± 0.01	86.20 ± 0.86
	7	50	3.0	76.42 ± 0.05	2.11 ± 0.00	0.53 ± 0.04	0.55 ± 0.04	0.39 ± 0.01	2.09 ± 0.04	82.07 ± 0.06
	7	100	1.5	67.30 ± 0.28	1.96 ± 0.21	0.37 ± 0.01	0.27 ± 0.01	0.49 ± 0.01	2.17 ± 0.00	72.54 ± 0.08
	7	100	3.0	84.62 ± 0.61	1.69 ± 0.04	0.59 ± 0.09	0.51 ± 0.16	0.42 ± 0.00	2.06 ± 0.04	89.88 ± 0.86
	14	50	1.5	60.33 ± 0.52	1.83 ± 0.06	0.35 ± 0.01	0.24 ± 0.01	0.47 ± 0.00	2.04 ± 0.00	65.24 ± 0.58
	14	50	3.0	56.84 ± 0.08	1.44 ± 0.01	0.39 ± 0.01	0.25 ± 0.01	0.37 ± 0.01	1.81 ± 0.00	61.09 ± 0.04
	14	100	1.5	66.01 ± 0.66	2.21 ± 0.02	0.42 ± 0.00	0.29 ± 0.01	0.52 ± 0.00	2.37 ± 0.01	71.81 ± 0.63
	14	100	3.0	81.38 ± 0.23	2.59 ± 0.02	0.53 ± 0.00	0.55 ± 0.00	0.44 ± 0.01	2.22 ± 0.02	87.69 ± 0.28
	21	50	1.5	85.90 ± 0.04	2.61 ± 0.01	0.50 ± 0.01	0.39 ± 0.01	0.56 ± 0.01	2.54 ± 0.01	92.49 ± 0.04
	21	50	3.0	74.35 ± 0.77	2.32 ± 0.04	0.48 ± 0.00	0.37 ± 0.01	0.39 ± 0.00	2.00 ± 0.00	79.90 ± 0.82
	21	100	1.5	77.91 ± 0.23	2.52 ± 0.02	0.48 ± 0.01	0.37 ± 0.01	0.54 ± 0.01	2.43 ± 0.03	84.23 ± 0.23
	21	100	3.0	80.91 ± 0.92	1.95 ± 0.00	0.54 ± 0.08	0.63 ± 0.00	0.42 ± 0.00	2.11 ± 0.02	86.55 ± 1.02
HPAE	Time (min)	Pressure (MPa)	Mass (g)							
	1	300	1.5	69.76 ± 0.42	1.58 ± 1.03	0.48 ± 0.01	0.27 ± 0.00	0.47 ± 0.00	2.80 ± 0.06	75.35 ± 0.55
	1	300	3.0	67.76 ± 0.49	0.82 ± 0.00	0.39 ± 0.01	0.22 ± 0.01	0.50 ± 0.00	2.51 ± 0.06	72.19 ± 0.44
	1	500	1.5	72.19 ± 0.18	0.85 ± 0.00	0.43 ± 0.01	0.25 ± 0.01	0.51 ± 0.00	2.61 ± 0.05	76.82 ± 0.14
	1	500	3.0	72.26 ± 0.33	0.85 ± 0.01	0.42 ± 0.01	0.25 ± 0.01	0.52 ± 0.00	2.51 ± 0.01	76.80 ± 0.32
	5.5	300	1.5	73.00 ± 0.83	0.85 ± 0.00	0.45 ± 0.00	0.25 ± 0.04	0.50 ± 0.00	2.60 ± 0.15	77.64 ± 1.02
	5.5	300	3.0	72.74 ± 0.25	0.86 ± 0.01	0.42 ± 0.03	0.25 ± 0.01	0.52 ± 0.00	2.54 ± 0.02	77.32 ± 0.30
	5.5	500	1.5	66.64 ± 1.20	0.79 ± 0.20	0.33 ± 0.16	0.35 ± 0.07	0.35 ± 0.00	2.14 ± 0.03	70.60 ± 1.51
	5.5	500	3.0	71.47 ± 0.21	0.85 ± 0.23	0.22 ± 0.04	0.32 ± 0.01	0.38 ± 0.00	2.27 ± 0.01	75.50 ± 0.45
	10	300	1.5	66.29 ± 0.49	0.95 ± 0.01	0.43 ± 0.01	0.30 ± 0.01	0.36 ± 0.00	2.26 ± 0.01	70.58 ± 0.50
	10	300	3.0	74.56 ± 1.98	1.04 ± 0.00	0.34 ± 0.13	0.30 ± 0.00	0.39 ± 0.01	2.25 ± 0.05	78.87 ± 2.05
	10	500	1.5	60.48 ± 0.95	0.77 ± 0.19	0.39 ± 0.00	0.26 ± 0.00	0.33 ± 0.01	2.04 ± 0.03	64.26 ± 0.73
	10	500	3.0	63.63 ± 0.18	0.65 ± 0.01	0.38 ± 0.01	0.26 ± 0.00	0.35 ± 0.00	1.93 ± 0.00	67.19 ± 0.20

MAE = microwave-assisted extraction, UAE = ultrasound-assisted extraction, HPAE = high pressure-assisted extraction, OL = oleuropein, HT = hydroxytyrosol, CA = chlorogenic acid, CF = caffeic acid, VB = verbascoside, RT = rutin. Results are expressed as mean ± SD.

The total sum of extracted polyphenols ranged from 61.09 to 92.49 mg g<sup>-1</sup> (Table 1), which is higher than 52.10 mg GAE g<sup>-1</sup> reported by Talhaoui et al. (2014) [3] but within the range of values reported by Ahmad–Qasem et al. (2013) [13] (66 mg g<sup>-1</sup>), Hannachi et al. (2019) [14] (81.96 mg GAE g<sup>-1</sup>), and Rafiee et al. (2011) [15] (69.03 mg TAE g<sup>-1</sup>). OL was the most abundant polyphenol, composing between 83.59% and 95.20% of all identified polyphenols, followed by RT (2.29–13.92%) and HT (0.95–3.58%). Present polyphenols, especially OL, which is typical for the *Oleaceae* family [19], have major biological and health properties [20–22]. The ratio between obtained compounds was also not influenced by the extraction method. Ahmad–Qasem et al. (2013) [13], Mkaouar et al. (2016) [5], Bouaziz and Sayadi (2005) [7], Mitsopoulos et al. (2011) [8], and Dekanski et al. (2009) [23] also confirmed that OL was the major compound in OLE. Other polyphenols identified in this research were also previously identified in OLE by other authors: VB [5,13], HT [5,8], RT [7,8], CA [8], and CF [23].

Based on the results from Table 1, statistical analysis was conducted in order to examine the influence of tested extraction parameters on yield of individual polyphenols, as well as their sum in order to provide information on at which of the tested conditions the highest yields of analyzed compounds were obtained, therefore, allowing an insight into each of the extraction techniques' efficiency. Statistical data are provided in Table 2.

### 3.1. Microwave-Assisted Extraction (MAE)

In order to establish the optimal MAE conditions for OLE polyphenols' extraction, time (2, 8.5, and 15 min), temperature (45 and 80 °C) and sample mass (1.5 and 3 g) were varied and their influence on the concentration of individual OLE polyphenols is shown in Table 2. Time had a significant influence ( $p \leq 0.05$ ) on all polyphenols except HT. Extraction of only 2 min resulted in the highest OL concentration and sum of all identified polyphenols. The lowest yields of OL, VB, CF, and CA were obtained after 8.5 min, except for RT which yielded the highest concentration at that extraction time (Table 2). Similarly to our results, Hannachi et al. (2019) [14] reported an optimal time of 1.81 min for the MAE of polyphenols from olive leaves, while Taamalli et al. (2012) [4], Japón-Luján et al. (2006) [24] and Rafiee et al. (2011) [15] reported a longer time of 6, 8, and 15 min, respectively. Differences in reported results could be attributed to the different extraction conditions used for the extraction [4,14,15,24], especially when MAE duration strongly depends on the applied temperature and/or microwave power. Accordingly, there are two general approaches established regarding the MAE conditions: the first is to apply short, intensive treatment and the second is to apply a prolonged, but moderate one [25].

Concentrations of all identified polyphenols, except HT and CA, were also affected by the temperature. Murakami et al. (2004) [26] also confirmed that temperature does not affect CA concentration since it was stable during heating at 180 °C for 180 min. OL, VB, and CF concentrations were significantly higher at 80 °C, while RT concentration was higher at 45 °C. Differences in optimal temperature could be explained by the fact that different polyphenols show different sensitivity to the heat treatment depending on their structures [27]. In general, flavonoids (e.g., RT) are more sensitive to thermal degradation than phenolic acids [28]. The elevated temperature usually improves the extraction yield and it shortens the extraction time, but at the same time, intense temperature or prolonged exposure to high temperature can cause a degradation of thermally sensitive compounds and therefore could result in a poor extraction yield [10]. This finding was confirmed by Taamalli et al. (2012) [4] who reported that the extraction efficiency of most of the OLE polyphenols, including OL and RT, increased with the temperature elevation up to 80 °C, while with further temperature increase the extraction efficiency decreased.

**Table 2.** Influence of extraction parameters on olive leaves individual phenols (mg g<sup>-1</sup>).

Extraction Technique	Source of Variation	OL	HT	CA	CF	VB	RT	Total Sum
MAE	Time (min)	$p < 0.01 *$	$p = 0.26$ ns	$p = 0.05 *$	$p = 0.03 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$
	2	78.69 ± 0.08 c	0.93 ± 0.05 a	0.44 ± 0.00 ab	0.27 ± 0.00 ab	0.45 ± 0.00 b	2.45 ± 0.03 a	83.25 ± 0.12 c
	8.5	71.33 ± 0.08 a	0.82 ± 0.05 a	0.43 ± 0.00 a	0.26 ± 0.00 a	0.40 ± 0.00 a	3.85 ± 0.03 b	77.09 ± 0.12 a
	15	74.40 ± 0.08 b	0.86 ± 0.05 a	0.45 ± 0.00 c	0.28 ± 0.00 c	0.44 ± 0.00 b	2.51 ± 0.03 a	78.94 ± 0.12 b
	Temperature (°C)	$p < 0.01 *$	$p = 0.25$ ns	$p = 0.21$ ns	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$
	45	66.10 ± 0.07 a	66.10 ± 0.04 a	0.44 ± 0.00 a	0.26 ± 0.00 a	0.38 ± 0.00 a	3.47 ± 0.02 b	71.50 ± 0.10 a
	80	83.51 ± 0.07 b	83.51 ± 0.04 a	0.44 ± 0.00 a	0.28 ± 0.00 b	0.48 ± 0.00 b	2.40 ± 0.02 a	88.02 ± 0.10 b
	Mass (g)	$p < 0.01 *$	$p = 0.89$ ns	$p = 0.53$ ns	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$
UAE	1.5	73.19 ± 0.07 a	0.88 ± 0.04 a	0.44 ± 0.00 a	0.25 ± 0.00 a	0.49 ± 0.00 b	3.59 ± 0.02 b	78.83 ± 0.10 a
	3.0	76.42 ± 0.07 b	0.87 ± 0.04 a	0.44 ± 0.00 a	0.30 ± 0.00 b	0.37 ± 0.00 a	2.28 ± 0.02 a	80.68 ± 0.10 b
	Time (min)	$p < 0.01 *$	$p = 0.16$ ns	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$
	7	76.90 ± 0.17 b	2.21 ± 0.12 a	0.48 ± 0.01 b	0.41 ± 0.02 b	0.45 ± 0.00 a	2.21 ± 0.01 b	82.67 ± 0.21 b
	14	66.14 ± 0.17 a	2.01 ± 0.12 a	0.42 ± 0.01 a	0.33 ± 0.02 a	0.45 ± 0.00 a	2.11 ± 0.01 a	71.46 ± 0.21 a
	21	79.77 ± 0.17 c	2.35 ± 0.12 a	0.50 ± 0.01 b	0.44 ± 0.02 b	0.47 ± 0.00 b	2.27 ± 0.01 c	85.79 ± 0.21 c
	Amplitude (%)	$p < 0.01 *$	$p = 0.55$ ns	$p = 0.03 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$
	50	72.18 ± 0.14 a	2.23 ± 0.09 a	0.45 ± 0.01 a	0.35 ± 0.01 a	0.45 ± 0.00 a	2.17 ± 0.01 a	77.83 ± 0.17 a
HPAE	100	76.35 ± 0.14 b	2.15 ± 0.09 a	0.48 ± 0.01 b	0.43 ± 0.01 b	0.47 ± 0.00 b	2.22 ± 0.01 b	82.12 ± 0.17 b
	Mass (g)	$p < 0.01 *$	$p = 0.02 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$
	1.5	72.79 ± 0.14 a	2.37 ± 0.09 b	0.43 ± 0.01 a	0.31 ± 0.01 a	0.51 ± 0.00 b	2.35 ± 0.01 b	78.75 ± 0.17 a
	3.0	75.75 ± 0.14 b	2.02 ± 0.09 a	0.51 ± 0.01 b	0.47 ± 0.01 b	0.40 ± 0.00 a	2.05 ± 0.01 a	81.20 ± 0.17 b
	Time (min)	$p < 0.01 *$	$p = 0.37$ ns	$p = 0.16$ ns	$p = 0.04 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$
	1	68.91 ± 0.29 b	1.01 ± 0.11 a	0.35 ± 0.02 a	0.29 ± 0.01 c	0.42 ± 0.00 a	2.43 ± 0.02 b	73.41 ± 0.31 b
	5.5	71.32 ± 0.29 c	0.92 ± 0.11 a	0.40 ± 0.02 a	0.27 ± 0.01 ab	0.44 ± 0.00 b	2.40 ± 0.02 b	75.76 ± 0.31 c
	10	67.46 ± 0.29 a	0.78 ± 0.11 a	0.41 ± 0.02 a	0.25 ± 0.01 a	0.42 ± 0.00 a	2.27 ± 0.02 a	71.60 ± 0.31 a
HPAE	Pressure (MPa)	$p < 0.01 *$	$p = 0.37$ ns	$p = 0.04 *$	$p = 0.19$ ns	$p < 0.01 *$	$p = 0.01 *$	$p < 0.01 *$
	300	68.06 ± 0.23 a	0.96 ± 0.09 a	0.42 ± 0.02 b	0.28 ± 0.01 a	0.42 ± 0.00 a	2.41 ± 0.02 b	72.54 ± 0.25 a
	500	70.40 ± 0.23 b	0.84 ± 0.09 a	0.36 ± 0.02 a	0.26 ± 0.01 a	0.44 ± 0.00 b	2.33 ± 0.02 a	74.64 ± 0.25 b
	Mass (g)	$p < 0.01 *$	$p = 0.34$ ns	$p = 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$	$p < 0.01 *$
	1.5	71.28 ± 0.23 b	0.97 ± 0.09 a	0.43 ± 0.02 b	0.25 ± 0.01 a	0.50 ± 0.00 b	2.59 ± 0.02 b	76.02 ± 0.25 b
	3.0	67.18 ± 0.23 a	0.84 ± 0.09 a	0.35 ± 0.02 a	0.30 ± 0.01 b	0.36 ± 0.00 a	2.15 ± 0.02 a	71.16 ± 0.25 a
	300	68.06 ± 0.23 a	0.96 ± 0.09 a	0.42 ± 0.02 b	0.28 ± 0.01 a	0.42 ± 0.00 a	2.41 ± 0.02 b	72.54 ± 0.25 a

MAE = microwave-assisted extraction, UAE = ultrasound-assisted extraction, HPAE = high pressure-assisted extraction, CE = conventional heat-reflux extraction, OL = oleuropein, HT = hydroxytyrosol, CA = chlorogenic acid, CF = caffeic acid, VB = verbascoside, RT = rutin. \*  $p \leq 0.05$ , ns = not significant ( $p > 0.05$ ). Results are expressed as mean ± SE. Values with different letters within column are statistically different at  $p \leq 0.05$ .



Sample mass also had a significant ( $p \leq 0.05$ ) influence on all identified polyphenols, except HT and CA. According to Spigno and De Faveri (2009) [16], if the solvent volume is maintained constant (changing only solids content), the temperature of the MAE system is constant which can result in a higher extraction yield at lower solid/solvent ratios. Indeed, higher VB and RT concentrations were achieved at lower solid/solvent ratios (1.5 g of sample). However, OL and CF concentrations were higher when using 3 g of the sample, which can be considered as a positive environmental effect since the consumption of solvent is lower.

### 3.2. Ultrasound-Assisted Extraction (UAE)

Extraction time (7, 14, and 21 min), amplitude (50 and 100%), as well as sample mass (1.5 and 3 g) were varied during UAE of olive leaves polyphenols. Obtained results were statistically analyzed and are given in Table 2. Time had a significant ( $p \leq 0.05$ ) influence on all polyphenols except HT and CF. The highest total polyphenols, OL, VB, and RT concentrations were obtained after 21 min, while the lowest OL, RT, and CA concentrations were obtained after 14 min. By applying a longer extraction time, the sample is in longer contact with the solvent, which facilitates a higher diffusion of the target compounds [11]. However, the overexposure to the ultrasound treatment can cause the heating effect, which leads to the degradation of some polyphenols [29]. Some authors [30,31] reported a positive effect of UAE time on the extraction yield in the blackthorn flower (*Prunus spinosa* L.) and sage (*Salvia officinalis* L.) extracts, while others reported that longer UAE time led to the lower extraction yield in banana peel [32]. Composition and stability of polyphenols vary in different types of material, which also explains these differences in the reported results. The temperature difference between MAE (80 °C) and UAE (60 °C) could explain why RT showed high sensitivity to elevated temperatures during longer extraction times in MAE, while the highest RT concentration in UAE was obtained even after 21 min (Table 2). In general, the optimal extraction time for the majority of OLE polyphenols was much shorter in MAE (2 min) than UAE (21 min), similarly to Hannachi et al. (2019) [14] who noted that optimal MAE and UAE time for olive leaves polyphenols was 1.81 and 28.69 min, respectively.

Amplitude also had an important role in the extraction of all identified polyphenols except HT and CF. Using 100% amplitude resulted in higher concentrations of all polyphenols influenced by this parameter, since more cell walls are damaged by applying higher ultrasound amplitude, therefore, causing the release of more polyphenols to the solvent [11].

Considering the sample mass, all identified polyphenols were significantly affected ( $p \leq 0.05$ ) by the amount of sample. Higher OL, CF, and CA concentrations were achieved by using 3 g of the sample, while higher RT, VB, and HT concentrations were achieved with lower sample mass (1.5 g). This difference could be linked with the specificity of an individual compound, although the increase of RT, VB, and HT yields with the solvent/sample ratio increase is consistent with the mass transfer principles, where the concentration gradient, which is the driving force, is higher when a lower solid/solvent ratio is used, thus leading to higher diffusion [1]. Sample mass in UAE had the same effect on OLE polyphenols as in MAE, where 3 g of the sample yielded the highest total sum of all polyphenols indicating a lower solvent consumption. Contrarily, Şahin and Şamlı (2013) [1] achieved higher olive leaves phenolic content in UAE extract by using a lower solid/solvent ratio.

### 3.3. High Pressure-Assisted Extraction (HPAE)

Table 2 provides the results of HPAE time (1, 5.5, and 10 min), pressure (300 and 500 MPa) and sample mass (1.5 and 3 g) effect on the OLE polyphenols. Pressure holding time had a significant ( $p \leq 0.05$ ) influence on all polyphenols, except HT and CA. Its main function is to form the equilibrium of solvent concentration between the inner and outer part of the cells and to get in full contact with the bioactive compounds and solvent [12]. Pressure holding time showed a similar influence on OL and VB concentration—with the time increase, from 1 to 5.5 min, concentrations of these compounds increased, but with a further increase (10 min) they decreased. Similar findings were previously reported by Chen et al. (2009) [33] in HPAE of ginsenosides from roots of ginseng. By increasing the extraction



time from 1 to 5 min, the yield of extracted saponins increased, but when extractions were longer than 5 min the yield was kept constant. According to Pascal's theory [12], during the high hydrostatic pressure (HHP) treatment, the pressure uniformly and instantly transfers to the whole material, which makes the extraction process fast, easy and effective. In comparison with MAE, where the optimal extraction time for total polyphenols was 2 min, HPAE optimal extraction time was slightly higher (5.5 min), whereas it was much shorter than the 21 min required for optimal UAE (Table 2). However, total polyphenols content extracted by HPAE in the optimal time was much lower than those obtained in the optimal MAE and UAE time (Table 2).

Furthermore, from Table 2 it can be observed that OL and VB concentrations were significantly higher at 500 MPa, while RT and CA concentrations were significantly higher at 300 MPa. Diverse data on optimal pressure were also previously reported [33–35] and Corrales et al. (2009) [36] described that due to the similar degree of cell membrane disruption, the content of anthocyanins extracted from grape skins did not significantly differ ( $p > 0.05$ ) upon different pressure intensities. HHP can enhance polyphenols' extraction by causing several different effects, where it will disrupt the cell wall and release the cytoplasm, which contains a high concentration of target material [12]. By applying HHP more solvent can permeate into the cells and as a consequence, more compounds can diffuse into the solvent [12]. Furthermore, the solubility of extracts is improved as the pressure increases [12]. A common trend that can be observed between MAE, UAE, and HPAE is that when a parameter that enhances a cell wall disruption (temperature in MAE (influenced by microwave power); amplitude in UAE; pressure in HPAE) is elevated, the total sum of the extracted polyphenols is higher (Table 2).

Sample mass showed to have an important role in the extraction of all identified compounds except HT (Table 2). It can be observed that OL, RT, VB, and CA concentrations were significantly higher when using 1.5 g of the sample, where an evidently lower sample mass/solvent ratio enhanced the possibility for bioactive compounds to get into contact with the extraction solvent. Since the dissolving process of bioactive compounds into the solvent is a physical process, it leads to higher leaching-out rates [12,35]. In general, using a higher ratio of solvent when compared to raw material gives a more dilute effect on the solvent side. This gives a larger concentration difference between the interior of the plant cells and the external solvent and, therefore, a faster extraction rate can be achieved [33]. While 1.5 g of the sample was the optimal sample mass in HPAE, the opposite result was obtained in MAE and UAE where a mass of 3 g resulted with higher total polyphenols extraction (Table 2).

In summary, based on the statistical analysis, results showed that optimal values of parameters that promote the highest total polyphenols extraction efficiency were 2 min/80 °C/3 g in MAE, 21 min/100%/3 g in UAE, and 5.5 min/500 MPa/1.5 g in HPAE.

### 3.4. Mutual Comparison of Advanced Extraction Techniques and with Conventional Heat-Reflux

In order to provide a better insight into the efficiency of tested extraction techniques, all techniques were mutually compared, as well as with the conventional heat-reflux. The comparison of grand mean values obtained for olive leaves individual polyphenols and their sum isolated by various advanced extraction techniques and the mean values of analyzed compounds and their sum for heat-reflux is presented in Table 3. When compared mutually, it can be seen that almost all polyphenols' yields were the highest when extracted by MAE and UAE, especially OL as the most abundant, while the ones obtained by HPAE were slightly lower. Moreover, the comparison of polyphenols' total sum clearly shows that the highest levels of polyphenols were achieved in MAE and UAE extracts (Table 3).

When compared to the CE, the total sum of all identified polyphenols, as well as OL and RT concentrations were the lowest when CE was used (Table 3). Similarly, Hannachi et al. (2019) [14] noted that when compared to the CE methods, MAE and UAE were more efficient for the extraction of polyphenols from olive leaves. What is often described as a highlight of advanced techniques is the lower level of biomaterial's heating in comparison with the CE. Since many organic compounds,

including polyphenols, are heat-sensitive, by applying a high temperature-extraction technique for a longer time they will be denatured, lose biologic activity, or change into another compound [37].

**Table 3.** Comparison of olive leaves individual phenols ( $\text{mg g}^{-1}$ ) extracted with various extraction techniques.

Extraction Technique	OL	HT	CA	CF	VB	RT	Total Sum
MAE	$74.81 \pm 2.12$	$0.87 \pm 0.03$	$0.44 \pm 0.00$	$0.27 \pm 0.01$	$0.43 \pm 0.02$	$2.94 \pm 0.36$	$79.76 \pm 1.92$
UAE	$74.27 \pm 1.90$	$2.19 \pm 0.10$	$0.47 \pm 0.02$	$0.39 \pm 0.03$	$0.46 \pm 0.01$	$2.20 \pm 0.05$	$79.97 \pm 2.01$
HPAE	$69.23 \pm 0.87$	$0.90 \pm 0.07$	$0.39 \pm 0.02$	$0.27 \pm 0.01$	$0.43 \pm 0.02$	$2.37 \pm 0.05$	$73.59 \pm 0.93$
CE	$67.05 \pm 1.21$	$0.89 \pm 0.07$	$0.41 \pm 0.01$	$0.27 \pm 0.01$	$0.47 \pm 0.02$	$2.01 \pm 0.06$	$71.11 \pm 1.30$

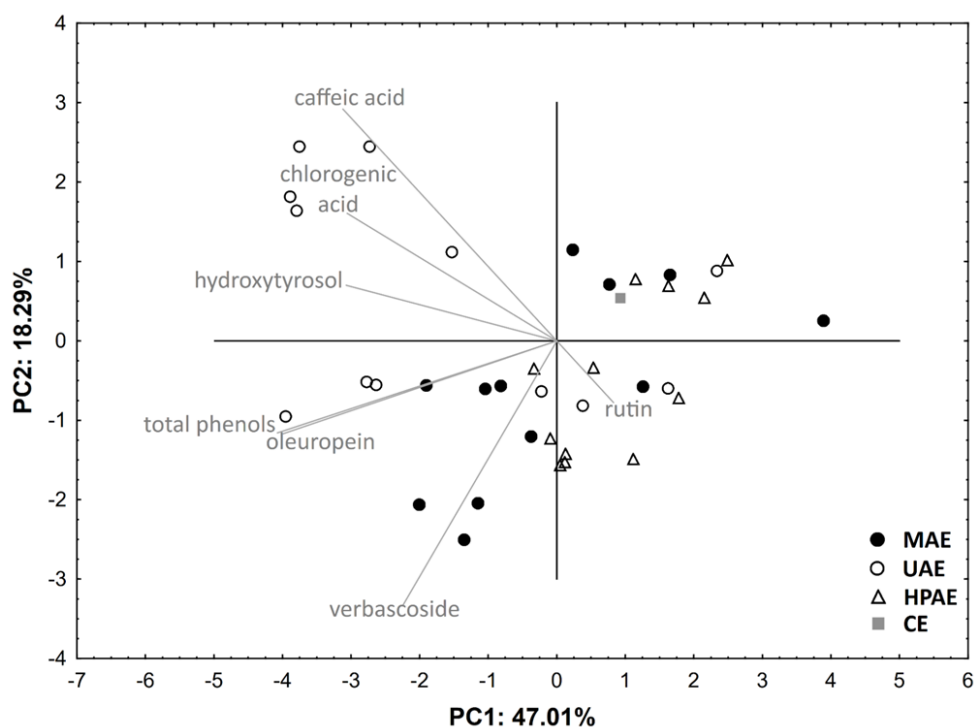
MAE = microwave-assisted extraction, UAE = ultrasound-assisted extraction, HPAE = high pressure-assisted extraction, CE = conventional heat-reflux extraction, OL = oleuropein, HT = hydroxytyrosol, CA = chlorogenic acid, CF = caffeic acid, VB = verbascoside, RT = rutin. For MAE, UAE, and HPAE results are expressed as mean  $\pm$  SE, for CE results are expressed as mean  $\pm$  SD.

In addition to the lowest total polyphenols' yield obtained by CE (Table 3), the crucial difference between CE and all other advanced extraction techniques applied in this study was a significant reduction of time. A time of 60 min in CE (two cycles of 30 min), as its optimal extraction condition, was required to extract the maximum yield of polyphenols, which was lower when compared to the concentrations of polyphenols extracted in the optimal time of 2, 21, or 5.5 min in MAE, UAE, and HPAE (Table 2), respectively.

Comparing the results in Table 3, much higher concentrations of all identified compounds were achieved with MAE ( $79.76 \text{ mg g}^{-1}$ ) and UAE ( $79.97 \text{ mg g}^{-1}$ ) than with HPAE ( $73.59 \text{ mg g}^{-1}$ ) and CE ( $71.11 \text{ mg g}^{-1}$ ). Therefore, similar polyphenols' extraction was achieved at a lower temperature ( $60^\circ\text{C}$ ) applied for a longer time (21 min) in UAE and higher temperature ( $80^\circ\text{C}$ ) applied for a shorter time (2 min) in MAE. In research by Hannachi et al. (2019) [14], a higher olive leaves total polyphenols' content was achieved with MAE when compared to UAE.

Regarding the effect of different extraction processes on individual polyphenols, an interesting observation was that HT concentration was not influenced by any of the applied extraction parameters. The reason could be that HT is an OL and VB moiety, and can be obtained by their degradation. OL degradation occurs by the opening of the oleanolic acid ring with a final rearrangement into HT [38]. Further, results of Briante et al. (2000) [39] indicate high stability of HT, since it was not qualitatively degraded when heated at  $70^\circ\text{C}$  for 3 h, while OL aglycon disappeared after 30 min at  $70^\circ\text{C}$ . Contrarily, RT seems to be the least stable compound throughout all of the examined extraction techniques. Furthermore, HT concentration was more than two-fold higher by UAE when compared to the other extraction techniques. Likewise, Xie et al. (2019) [40] considered UAE the most effective method for HT extraction from olive pomace.

Additionally, the possible grouping of OLE samples regarding the applied extraction technique was tested using PCA. Obtained results are presented in Figure 3, which provides the PCA biplot based on the MANOVA results (Table 2) giving a better visual perception of the extracts grouping by the applied extraction techniques. The first two components (PC1 and PC2) explained 65.30% of the total variance. As it can be seen, PCA showed a certain grouping of OLE, where most of the samples obtained using MAE and UAE were situated at negative values of PC1, while HPAE and CE samples as well, were situated at PC1 positive values of the biplot. All identified polyphenols, except RT, were considered as the most discriminating variables due to the strong/very strong correlation ( $r = 0.65\text{--}0.90$ ) with both principal components, which is in accordance with previously discussed data (Tables 1–3).



**Figure 3.** Distribution of olive leaves extracts in a two-dimensional coordinate system defined by the first two principal components (PC1 and PC2) according to the applied extraction technique (MAE = microwave-assisted extraction, UAE = ultrasound-assisted extraction, HPAE = high pressure-assisted extraction, CE = conventional heat-reflux extraction).

#### 4. Conclusions

Olive leaves extracts obtained using MAE, UAE, and HPAE consisted of HT, CA, CF, VB, RT and OL. The most abundant polyphenol in all extracts was OL (83.59–95.20%) with an average concentration of 74.81 (MAE), 74.27 (UAE), and 69.23 mg g<sup>-1</sup> (HPAE). Except for HT which was not influenced by any of the extraction parameters and RT which showed high sensitivity, statistical analysis showed that optimal values of parameters that resulted with the highest efficiency of olive leaves polyphenols' extraction were 2 min/80 °C/3 g in MAE, 21 min/100%/3 g in UAE, and 5.5 min/500 MPa/1.5 g in HPAE. Considering the average total polyphenols' content, MAE (79.76 mg g<sup>-1</sup>) and UAE (79.97 mg g<sup>-1</sup>) were more efficient in comparison with HPAE (73.59 mg g<sup>-1</sup>). Furthermore, all three alternative extraction techniques yielded a higher content of total polyphenols when compared to the CE (71.11 mg g<sup>-1</sup>) along with a significant reduction in time from 60 min in CE to 2, 21, and 5.5 min in MAE, UAE, and HPAE, respectively. Combining all obtained results, MAE and UAE could have a slight advantage over HPAE and could be successfully used for the recovery of olive leaves polyphenols with MAE being less time-consuming.

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