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Efficiency of Microwave and Ultrasound-Assisted Extraction as a Green Tool for Polyphenolic Isolation from Monofloral Honeys

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Abstract: Monofloral honey is obtained from the nectar of single-source plants and has a higher market value due to its polyphenol content associated with its biological properties, especially its antioxidant capacity. In this work, advanced extraction techniques using microwave- (MAE) and ultrasound-assisted (UAE) extraction of phenolic compounds in monofloral honey were optimized and compared. Optimal parameters for MAE were an irradiation time of 15 min, a temperature of 60 °C, and a microwave power of 300 W, and for UAE, a sonication time of 10 min, a temperature of 35 °C, and an ultrasound amplitude of 60%. The extraction solvent used was 70% ethanol. In the extracts of different monofloral honey samples (mint, fennel, raspberry, lavender, sage, buckwheat, maroon, heaven) obtained at optimal MAE and UAE conditions, polyphenolic compounds were determined using UPLC-ESI/MS^2 analysis and antioxidant capacity using ORAC, ABTS, and DPPH assays. The results showed that UAE was the more efficient technique for the extraction of total flavanones, flavones, hydroxycinnamic acids, and total phenols, and MAE for total flavonols and hydroxybenzoic acids. The antioxidant ORAC and DPPH capacity was higher for the extracts obtained with MAE, while the ABTS capacity was higher for those obtained with UAE.

Keywords: monofloral honey; polyphenolics; microwave and ultrasound assisted extraction; antioxidant capacity

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1. Introduction

Honey is a sweetening food obtained by bees (*Apis mellifere*) from the nectar of flowering plants. The chemical composition of honey is closely linked to its botanical origin, processing, seasons, and environmental conditions. In terms of its botanical origin, honey can be classified, as multifloral and monofloral honey, which is obtained from multiple plant species or from the nectar of a single plant source. Monofloral honey has a higher market value due to its biochemical composition, which is influenced by the floral source [1,2].

Apart from its high nutritional value due to the content of sugars, minerals, proteins, vitamins, and organic acids, monofloral honey contains a variety of bioactive compounds, and has significant antioxidant capacity due to its polyphenolic profile, as well as antimicrobial, antidiabetic, and anticancer activity [1,3]. In the last decade, the polyphenolic composition

of monofloral honeys and their relationships with biological activities have attracted great interest from researchers [1,4]. The antioxidant properties of honey are mainly attributed to the presence of flavonoids such as chrysin, pinocembrin, quercetin, galangin, kaempferol and hydroxycinnamic and hydroxybenzoic acids such as caffeic acid, chlorogenic acid, ferulic acid, gallic acid, ellagic acid, protocatechuic acid, and 4-hydroxybenzoic acid [5].

Isolation of polyphenolic compounds from honey is an important step in determining the beneficial effects of honey on human health, and a suitable extraction procedure is essential to obtain the highest possible yield of polyphenols. Conventional techniques for extracting polyphenols are usually time-, energy-, and solvent-consuming, and polyphenols can be degraded as heat-sensitive compounds. As a result, advanced extraction techniques, microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) have become increasingly popular in recent years as more efficient, faster, economical, and environmentally friendly methods for extracting bioactive compounds from various sample matrices [6]. MAE is a non-contact heating technique that helps to reduce the thermal gradient and accelerate energy transfer caused by electromagnetic waves. Microwave energy is used to heat solvents in contact with samples and disperse compounds of interest from the sample into an extractant. The main advantage of MAE is its ability to rapidly heat the sample solvent mixture, leading to its broad applicability for rapid extraction of analytes, including thermally unstable compounds, reduction of extraction time and solvent consumption, and simultaneous extraction of multiple samples. The efficiency of MAE depends on several factors, including solvent properties, sample material, and compounds to be extracted especially their dielectric constants. UAE uses sound waves (20–100 MHz), which can cause the cavitation process and improve the heat and mass transfer of the process. These waves consist of a series of compression and rarefaction cycles that propagate using a solid, liquid, or gaseous medium, causing displacement of molecules from their original position. Ultrasonic waves produce bubbles in the solvent, and ultrasonic vibrations compress these bubbles. Cavitation phenomena and mechanical mixing effects are the main mechanisms in UAE, increasing extraction efficiency and shortening extraction time. In addition, ultrasound avoids the thermal decomposition of heat-sensitive compounds because it is a non-thermal process [6,7]. MAE and UAE of phenolic compounds and antioxidants from different samples were studied by various researchers. Biesaga and Pyrzynska [7] studied the stability of polyphenols from honey during different extraction techniques, and the results showed higher stability of phenolic acids and flavonoids in samples during MAE and UAE treatments using acidified water (HCl, pH 2) or 40% methanol/acidified water (v/v) as extraction solvent. UAE conditions gave higher and/or similar phenolic acid yields compared to the commonly used conventional extraction techniques (solvent shaking), and UAE also reduced the time required for sample preparation.

Since extraction parameters such as temperature, time, type of solvent, power of microwave, frequency, and amplitude of ultrasound waves affect the yield of extract and bioactive compounds, they should be optimized with regard to the sample properties and the targeted bioactive compounds [8]. The polar solvents and their aqueous mixtures are usually used for the extraction of polyphenols from diverse matrices; many studies have shown that ethanol is a good solvent for the extraction of polyphenols and is also safe for human consumption [9].

The efficiency of the extraction procedures is mainly evaluated by the determination of total phenols using spectrophotometric methods and liquid chromatography combined with a mass spectrometry detector for the detailed chemical characterization of phenolic compounds in complex matrices [10]. The ability of phenolic compounds to act as antioxidants is their most important property, which is essential for scavenging radicals [11]. Numerous assays can be used to measure antioxidant capacity using different reaction mechanisms, such as the transfer of hydrogen atoms (ORAC) and single electrons (FRAP) or their combination (DPPH, ABTS) [12,13]. As extracts are increasingly used in industry and bee products are widely consumed and used as food or supplements, it is important to know how green extraction affects the phenolic content and antioxidant capacity of this matrix. The higher extraction yield of phenolics with high antioxidant potential is the focus

of many studies, but a higher content of phenols in the extract does not ensure a higher antioxidant capacity [11]. According to our knowledge, microwaves, and ultrasound have been used for the extraction of various types of raw materials, but data on the polyphenolic content and profile of honey produced using MAE and UAE are limited.

Therefore, the objective of our study was to determine the optimal conditions for MAE (irradiation time (5, 10, 15 min), temperature (60, 80, 100 °C), and microwave power (300, 450, 600 W)) and for UAE (sonication time (10, 20, 30 min), temperature (35, 50, 65 °C), and ultrasound amplitude (30, 60, 90%) of polyphenols from monofloral honey and to compare the extraction efficiency in terms of polyphenol content and antioxidant capacity. The polyphenol content of the monofloral honey extracts obtained at optimal MAE and UAE conditions was determined using UPLC ESI/MS^2 analysis, and the antioxidant capacity was determined using ORAC, ABTS, and DPPH assays, respectively.

2. Materials and Methods

2.1. Chemicals and Reagents

The Milli-Q water purification system (Millipore, Bedford, MA, USA) was used for the distilled water. Ethanol (96%) was obtained from Lachner (Neratovice, Czech Republic), formic acid (98–100%) from T.T.T. d.o.o. (Sveta Nedjelja, Croatia), and acetonitrile (HPLC grade) from J.T. Baker Chemicals (Deventer, The Netherlands). Folin–Ciocalteu reagent was purchased from Merck (Darmstadt, Germany), sodium phosphate (96%) and anhydrous sodium carbonate (99.5%) from Kemika (Zagreb, Croatia), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Acros Organics (Thermo Fisher Scientific, Geel, Belgium), fluorescein sodium salt from Honeywell Riedel-de-Haën (Bucharest, Romania) and 2,20-Azobis (2-amidinopropane) hydrochloride from Sigma-Aldrich (Steinheim, Germany). DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt stock solution, potassium persulfate ($K_2S_2O_8$) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). TPTZ (2,4,6-Tris-(2′-Pyridyl)-s-Triazine) was provided by Acros Organics B.V.B.A. (Thermo Fisher Scientific, Geel, Belgium). Authentic standards purchased from Sigma-Aldrich (St. Louis, MO, USA) were quercetin-3-glucoside, galangin, myricetin, naringenin, pinocembrin, chrysin, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, gallic acid, and vanillic acid, and from Extrasynthese (Genay, France) kaempferol-3-glucoside, rutin, apigenin, and luteolin. The apigenin was dissolved in ethanol containing 0.5% (*v*/*v*) dimethyl sulphoxide, while other standards were prepared as methanol stock solutions. To produce calibration curves, five working standard solutions were prepared by diluting the stock solutions.

2.2. Honey Samples

Different monofloral honey samples of mint (*Mentha* spp.)—samples 1, 2, 3, 4; fennel (*Foeniculum vulgare*)—sample 5; raspberry (*Rubus idaeus*)—sample 6; lavender (*Lavandula* sp.)—samples 7 and 8; ailanthus (*Ailanthus altissima*)—sample 9; Lovran maroon (*Castanea sativa* Mill.)—sample 10; sage (*Salvia officinalis* L.)—sample 11; buckwheat (*Fagopyrum esculentum* Moench)—sample 12; and maroon (*Castanea sativa* Mill.)—sample 13 were collected in year 2020 and 2021 from different geographical regions of Croatia (Osekovo, Bjelovar, Popovača, Zagreb, Županja, Vinkovci, Gorski Kotar, Lovran, Novi Vinodolski, Gorski Kotar, Ludbreg, Ičići, Omišalj) (Table 1).

Table 1. Monofloral honey samples.

No.	Monofloral Honey Samples	Collection Region	Collection Year
1	Mint (*Mentha* spp.)	Osekovo	2020
2	Mint (*Mentha* spp.)	Bjelovar	2020
3	Mint (*Mentha* spp.)	Popovača	2021
4	Mint (*Mentha* spp.)	Zagreb	2021

Table 1. *Cont.*

No.	Monofloral Honey Samples	Collection Region	Collection Year
5	Fennel (*Foeniculum vulgare*)	Županja	2021
6	Raspberry (*Rubus idaeus*)	Vinkovci	2021
7	Lavender (*Lavandula* sp.)	Gorski kotar	2021
8	Lavender (Lavandula sp.)	Novi vinodolski	2021
9	Ailanthus (*Ailanthus altissima*)	Ičići	2021
10	Lovran Marron (*Castanea sativa* Mill.)	Lovran	2021
11	Sage (*Salvia officinalis* L.)	Omišalj	2021
12	Buckwheat (*Fagopyrum esculentum* Moench	Ludberg	2021
13	Marron (*Castanea sativa* Mill.)	Gorski kotar	2021

2.3. Extraction of Polyphenols

2.3.1. MAE

To obtain the optimal MAE conditions for polyphenolic isolation from monofloral honey samples, the optimization was performed on mint honey according to the experimental design shown in Table 2. Constant extraction parameters were the time required to achieve extraction temperature (4 min), stirring (50%), and ventilation after extraction (2 min). A honey sample (1 ± 0.001 g) and 25 mL of aqueous ethanol solution (70%, *v*/*v*) were mixed in the extraction vessel with the addition of a magnetic stir bar and the extraction was carried out in the microwave reactor (Ethos Easy, Milestone, Sorisole, Italy) varying the irradiation time (5, 10, 15 min), temperature (60, 80, 100 °C) and microwave power (300, 450, 600 W). Temperature was set as a constant and controllable parameter during the extraction, thereby applying the selected microwave power only in short increments of time needed to achieve and maintain the constant temperature. After cooling at room temperature, the extracts were filtered (no. 40 filter paper, Whatman), made up to 25 mL in volumetric flasks with extraction solvent, and stored in plastic tubes at −18 °C in an inert gas atmosphere until analysis. All extractions were performed in duplicate (n = 2).

Table 2. TPC of mint honey extracts obtained using different MAE and UAE parameters.

MAE				UAE			
t **(min)**	**T (°C)**	**Power (W)**	**TPC (mg GAE/100 g)**	***t*** **(min)**	**T (°C)**	**Ultrasound Amplitude (%)**	**TPC (mg GAE/100 g)**
5	60	300	75.00 ± 2.09	10	35	30	55.86 ± 3.39
5	60	450	78.10 ± 3.79	10	35	60	71.27 ± 1.57
5	60	600	68.39 ± 2.27	10	35	90	61.35 ± 1.23
5	80	300	62.58 ± 3.07	10	60	30	63.61 ± 3.00
5	80	450	59.68 ± 3.40	10	60	60	62.46 ± 2.36
5	80	600	65.88 ± 3.05	10	60	90	74.21 ± 2.42
5	100	300	47.10 ± 1.97	10	90	30	71.12 ± 0.91
5	100	450	61.10 ± 1.81	10	90	60	74.12 ± 2.74
5	100	600	67.97 ± 3.18	10	90	90	58.19 ± 3.34
10	60	300	55.99 ± 3.56	20	35	30	61.40 ± 2.34
10	60	450	61.81 ± 1.50	20	35	60	74.41 ± 2.73
10	60	600	65.50 ± 3.54	20	35	90	76.35 ± 3.73
10	80	300	65.28 ± 0.78	20	60	30	70.04 ± 2.71
10	80	450	58.53 ± 2.41	20	60	60	72.38 ± 3.11

Table 2. *Cont.*

MAE				UAE			
***t* (min)**	**T (°C)**	**Power (W)**	**TPC (mg GAE/100 g)**	***t* (min)**	**T (°C)**	**Ultrasound Amplitude (%)**	**TPC (mg GAE/100 g)**
10	80	600	64.66 ± 2.65	20	60	90	59.68 ± 3.54
10	100	300	76.97 ± 2.42	20	90	30	61.79 ± 3.58
10	100	450	64.22 ± 2.11	20	90	60	68.71 ± 4.23
10	100	600	82.74 ± 1.81	30	90	90	88.95 ± 3.49
15	60	300	78.55 ± 4.15	30	35	30	65.52 ± 4.30
15	60	450	84.19 ± 0.57	30	35	60	69.21 ± 3.29
15	60	600	71.56 ± 1.53	30	35	90	67.49 ± 2.50
15	80	300	74.41 ± 2.74	30	60	30	70.10 ± 0.55
15	80	450	71.84 ± 1.44	30	60	60	67.56 ± 2.46
15	80	600	68.79 ± 2.92	30	60	90	63.78 ± 1.51
15	100	300	79.09 ± 3.21	30	90	30	66.87 ± 3.68
15	100	450	70.61 ± 1.27	30	90	60	76.67 ± 3.53
15	100	600	83.16 ± 0.96	30	90	90	87.86 ± 3.03

TPC = total phenolic content, MAE = microwave-assisted extraction, UAE = ultrasound-assisted extraction. Results are expressed as mean ± SD.

2.3.2. UAE

To obtain the optimal UAE conditions for polyphenolic isolation from monofloral honey samples, the optimization was performed on mint honey according to the experimental design shown in Table 2. A mass of honey sample (1 ± 0.001 g) was mixed with 25 mL of aqueous ethanol solution (70%, *v*/*v*) and stirred in a magnetic stirrer for 15 min. Then, extraction was carried out in an ultrasonic bath (Elmasonic P, Elma Schmidbauer GmbH and Co., Singen, Germany) varying sonication time (10, 20, 30 min), temperature (35, 50, 65 °C), and ultrasound amplitude (30, 60, 90%). The extracts were filtered (no. 40 filter paper, Whatman) and made up to 25 mL in volumetric flasks with extraction solvent. Extracts were stored in plastic tubes at −18 °C in an inert gas atmosphere until analysis. All extractions were performed in duplicate (n = 2).

2.4. Total Phenolic Content

To determine the total phenolic content (TPC) of the honey samples, the Folin–Ciocalteu method was used [14]. After 20 min of incubation, the absorbance of the reaction mixture was determined at 765 nm with a spectrophotometer (UV-VIS UviLine 9400, Secomam, France). Using gallic acid as a standard, the TPC was calculated and expressed as mg gallic acid equivalents (mg GAE)/100 g of honey. All samples were measured in duplicate (n = 2).

2.5. UPLC/ESI-MS^2 Determination of Phenolic Compounds

The phenolic characterization of the honey extracts obtained under optimal UAE and MAE conditions was performed using a triple quadrupole mass spectrometer (6430 QqQ) connected to a 1290 RRLC instrument (Agilent, Santa Clara, CA, USA) with a binary pump, autosampler, and thermostated column compartment, according to the method described by Elez Garofulic et al. [15]. Prior to analysis, honey samples were filtered (0.45 µm PTFE membrane filters), and the phenolic compounds were separated at 35 °C on an Agilent Zorbax Eclipse Plus C18 column (100 × 2.1 mm). The flow rate was at 0.35 mL/min, and the injection volume was 2.5 µL. For peak identification, electrospray ionization was performed in negative and positive ion modes (m/z 100 to 1000), and data were collected in multiple

reaction monitoring mode (MRM). The operating conditions of the ionization source were: positive/negative capillary voltage = 4000/3500 V, drying gas temperature = 300 °C, flow rate of 11 L h^{-1}, and nebulizer pressure = 40 psi. High-purity nitrogen was used as both inducing cone and collision gas. Agilent MassHunter software was used for qualitative and quantitative data analysis. The phenolic compounds were identified by comparing mass spectral fragmentation patterns of authentic standards and previously reported data. All standards were qualified and quantified in MRM mode using the optimized parameters. For the quantitative analysis, an external calibration was used, and in the absence of authentic standards, quantification was performed using calibration curves of standards from the corresponding phenol group. The isorhamnetin and quercetin are expressed as an equivalent of quercetin-3-glucoside and kaempferol as an equivalent of kaempferol-3-glucoside and 2,5-dihydrobenzoic acid, 3,4-dihydrobenzoic acid, and *p*-hydroxybenzoic acid as an equivalent of vanillic acid. A signal-to-noise ratio (S/N) of 3 and 10 was used to estimate the limits of detection (LOD) and quantification (LOQ) [15]. Concentrations of phenolic compounds were expressed as mg per 100 g of honey (mg/100 g) (n = 2).

2.6. Antioxidant Capacity Determination

2.6.1. Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was performed according to a previously described procedure using an automated plate reader (BMG LABTECH, Offenburg, Germany) [15]. A 96-well microplate with 150 µL of fluorescein was filled with the Trolox standard, or the sample, or sodium phosphate buffer (pH 7.4, 75 mM) as a blank. The reaction was started by adding 25 µL AAPH (240 mM) to the plate after incubation at 37 °C for 30 min. Fluorescence measurements (excitation λ = 485 nm/emission λ = 538 nm) were performed at 37 °C every 90 s for 120 min. ORAC values were expressed as µmol Trolox equivalent per 100 g of honey (µmol TE/100 g, n = 2).

2.6.2. ABTS (2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic Acid) Assay

The antioxidant capacity of ABTS was measured according to a modified method of Miller and Rice-Evans [16]. The 40 µL of the diluted sample is mixed with 4 mL of 1% ABTS●+, and the absorbance at 734 nm (UV-VIS UviLine 9400, Secomam, France) is measured after 1 min. Using Trolox as a standard, the ABTS values were expressed as µmol TE/100 g (n = 2).

2.6.3. DPPH (2,2-Diphenyl-1-picrylhydrazyl Radical) Assay

In a reaction with the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH●), the antioxidant capacity of the samples was evaluated [17]. The reaction mixture was incubated at room temperature for 20 min, and the absorbance was measured at 517 nm (UV-VIS UviLine 9400, Secomam, France). Using Trolox as a standard, the DPPH values were expressed as µmol TE/100 g (n = 2).

2.7. Statistical Analysis

Statistica ver. 10.0 software (Statsoft Inc., Tulsa, OK, USA) was used for statistical analysis. All extractions and analyses were performed in duplicate. A three-level full factorial design with 27 experimental trials was used to assess the effect of three independent variables, namely time, temperature, and power or amplitude during MAE and UAE, on the TPC in mint honey. The Shapiro–Wilk's test and Levene's test were used to test the normality and homoscedasticity of the data, respectively. Multifactorial analysis of variance (ANOVA) was used to assess normally distributed data (related to MAE extraction), and Tukey's HSD, a multiple comparison test, was used to compare marginal means between groups. The Kruskal–Wallis test was used to evaluate nonparametric data (related to the UAE extraction). For the mutual comparison of the honey samples as well as the efficiency of UAE and MAE in terms of phenolic compound content and antioxidant capacity, the mean values were compared using one-way ANOVA and Tukey's HSD test.

The significance level for all tests was $p \leq 0.05$, and the statistical data are reported as mean ± standard error (SE).

3. Results and Discussion

The influence of different MAE and UAE extraction parameters on the content of polyphenols in mint honey extracts was studied. Using the Folin–Ciocalteu method, the TPC of the obtained extracts was evaluated (Table 2), and using statistical analysis, the optimal MAE and UAE conditions were determined. The UPLC-ESI/MS^2 method was used to characterize and quantify the polyphenols in different monofloral honey extracts obtained under optimal extraction conditions, and their antioxidant capacity was determined using the ORAC, ABTS, and DPPH assays.

The TPC of the mint honey extracts obtained by MAE ranged from 47.10 to 84.19 mg GAE/100 g and by UAE from 55.85 to 87.86 mg GAE/100 g. According to our knowledge, the results of TPC obtained in MAE and UAE honey extracts are scarce. Our results are in agreement with the study of Chaikham and Prangthip [18], who treated longan flower honey with ultrasound, and with those of Pavlešić et al. [19] for mint honey conventionally extracted with water (76.7–90.1 mg GAE/100 g). The TPC of mint honey (23.7 mg GAE/100 g) conventionally extracted with 40% methanol/acidified water (*v*/*v*, pH = 2, HCl) [20] was considerably lower than in this study. In the study by Piljac-Žegarac [21], the mean TPC of different monofloral Croatian floral honeys conventionally extracted with water was 42.24 mg/100 g, which was lower than in this study. TPC values determined for Polish honeys ranged from 9.64 to 34.19 mg/100 g, with dark honeys having higher phenolic compound content [22]. The lower TPC was also found in Turkish wild mint (34.37 ± 0.44 mg GAE/100 g) [2] and in mint honey extract from Romania (50.82 mg GAE/100 g) [23].

3.1. MAE Optimization

The irradiation time (5, 10, 15 min), extraction temperature (60, 80, 100 °C), and microwave power (300, 450, 600 W) were varied to determine the optimal MAE conditions for the extraction of mint honey polyphenols, and their influence on TPC is shown in Table 3. In this study, the effect of irradiation time on the TPC of mint honey extracts was significant ($p \leq 0.01$), while extraction temperature and microwave power had no significant effect. Considering the results of statistical analysis, it was found that extraction at 60 °C and 300 W after 15 min of irradiation were the optimal MAE conditions to obtain a higher content of polyphenols. In general, increasing extraction time increases extraction yield since extraction is a mass transfer process. However, it is also known that excessive heat exposure can cause oxidation of phenolic compounds [24]. The duration of MAE is highly dependent on the temperature and/or microwave power applied [25]. The efficiency of extraction can be increased by raising the extraction temperature, as this can reduce the viscosity of the solvent and improve its solubility and mobility. However, excessive temperature can also affect the polyphenol content and cause oxidation of the polyphenols [24]. In this study, increasing the temperature from 60–100 °C resulted in no significant change (slightly lower) of the TPC of the honey extracts obtained. Wong Paz et al. [26] reported a similar trend for TPC from semiarid plants where the optimal temperature for MAE was 60 °C, as no significant change was observed when the temperature increased. For different sample matrices, other authors have reported that a higher extraction temperature results in a higher TPC. For example, Rodriguez-Gonzalez et al. [27] reported that higher TPC yields of pollen were at higher extraction temperatures because the extraction of phenolic compounds from pollen is enhanced due to the breaking of the cell wall and the ability of the solvent to transport more compounds. In this study, an increase in extraction time from 5 to 15 min significantly increased TPC, whereas the TPC of the extracts was slightly higher when 600 W was applied compared to 300 W. The intensity of the microwave power affects the interactions and the equilibrium rate and regulates the distribution of analytes between sample and solvent; increasing the microwave power can also increase the extraction yield. However, when the extraction time was longer, a decrease in extraction yield was

observed when the microwave power increased because high microwave power could increase the product temperature and decrease the extraction yield due to the degradation of phenolic compounds [28]. Therefore, a longer extraction time of 15 min with a lower microwave power of 300 W (due to energy savings and high TPC) was selected as optimal for the extraction of mint honey polyphenols. The highest TPC (13.73 mg GAE/g dw) was obtained in 60% ethanol extracts from coffee bee pollen when the MAE conditions were 314 W and 7 min [29] and in the study of Hayek [30] after melting the honey using microwaves with powers of 270, 450, and 900 W, the concentration of phenolic compounds decreased on average by 31.1–35.5%.

Table 3. Influence of MAE parameters on TPC of mint honey extracts.

MAE Parameters	TPC (mg GAE/100 g)
Time (min)	$p < 0.01$ *
5	65.09 ± 2.14 [a]
10	66.19 ± 1.99 [a]
15	75.80 ± 1.35 [b]
Temperature (°C)	$p = 0.17$
60	71.01 ± 2.11 [a]
80	65.74 ± 1.29 [a]
100	70.33 ± 2.72 [a]
Microwave Power (W)	$p = 0.55$
300	68.33 ± 2.63 [a]
450	67.79 ± 2.09 [a]
600	70.96 ± 1.68 [a]

* Statistically significant variable at $p \leq 0.05$. Results are expressed as mean ± SE. Means with the same letter within the column are not significantly different at $p \leq 0.05$. TPC = total phenolic content, MAE = microwave-assisted extraction.

3.2. UAE Optimization

The sonication time (10, 20, 30 min), temperature (35, 50, 65 °C), and ultrasound amplitude (30, 60, 90%) were varied to determine the optimal UAE conditions for the extraction of mint honey polyphenols, and their influence on TPC is shown in Table 4. In this study, the effect of ultrasound amplitude on the TPC of mint honey extracts was significant ($p = 0.03$), while extraction temperature and sonication time had no significant effect. Considering the results of the statistical analysis, extraction at 35 °C after 10 min of sonication at an ultrasound amplitude of 60% proved to be the optimal UAE conditions to obtain a higher content of polyphenols. Although an increase in ultrasound amplitude of 30–60% increased TPC, an amplitude of 60% was chosen because a further increase from 60 to 90% had no significant effect on TPC. The amplitude represents the height of the waves and the sonication intensity. High ultrasound amplitude generally increases extraction efficiency as it increases the number of compression and rarefaction cycles of ultrasound waves, improves mass transfer from the matrix to the solvent [31,32], and can reduce extraction time and energy consumption [33]. As ultrasound triggers cavitation and the development of small bubbles that are subjected to rapid adiabatic compression and expansion, this leads to an increase in temperature and pressure. Singh et al. [34] reported that the yield decreased when the amplitude was increased over a certain value, probably because high amplitude causes bubbles to develop, which prevents pressure waves from propagating. When longan flower honey was exposed to different ultrasound amplitudes (20–60%) and times (5–20 min), the increase in TPC was more pronounced in honey treated when ultrasound amplitude and time were increased (60%/20 min) [18]. According to Oroian et al. [31], TPC in propolis extract increased by 17.5% when the amplitude was increased from 20 to 100%. Furthermore, it was found that TPC

increased after 10 min of extraction, but increasing the extraction time from 20 to 30 min did not significantly increase the TPC of the obtained honey extracts. The explanation could be based on Fick's second law of diffusion, which says that the sample matrix and the extraction solvent reach a final equilibrium after a certain time. A longer extraction time may also lead to the oxidation of polyphenols and increase the loss of solvent by evaporation, which affects the solvent-to-solid ratio [32]. Therefore, 10 min was chosen as the optimal extraction time for extraction of honey polyphenols because a shorter extraction time can save time and costs. In the study by Peláez-Acero et al. [35], significant differences were found in extracts of six Mexican honey samples with respect to the ultrasonic treatment applied (10, 20, and 30 min), and the polyphenols reached a maximum concentration with 20 min of ultrasound. According to Borrás-Enríquez et al. [36], an increase in the concentration of polyphenolic compounds from mango manilla residues was observed between 10 and 20 min, while a decrease in concentration was observed for a period longer than 20 min. Similar results for UAE from green soybean pods were reported by Leksawasdi et al. [37], where the highest TPC was obtained at an extraction time of 10 min with 50% amplitude. The highest TPC for UAE from a sage was obtained at a sonication time of 11 min and a device output power of 100 W [38]. In this study, an increase in extraction temperature from 35–65 °C slightly increased the TPC of honey extracts. Many studies have shown that temperature has a positive effect on the extraction of polyphenols and that different temperature ranges can be used for various matrices [31,39], and some studies show no significant difference in TPC [40]. Oroian et al. [41] reported that the highest UAE extraction yield from Romanian propolis was at 58 °C (30 min/100 W). Higher temperature may have a positive effect on the solubility of polyphenols and decreases viscosity and surface tension [33,39]; however, heat degradation may occur at high temperatures. Specifically for honey treatments, higher losses of phenolic compounds (48.5%) were observed when honey was melted using ultrasonic treatment at 45 °C, 60 W, and 90 min [30]. The optimum UAE conditions for Chinese propolis were 40 °C, ultrasound time 20 min, and ultrasound power 135 W [42]. Chaikham et al. [18] subjected honey samples to various ultrasound treatments (20–60% amplitude/20 kHz/5–20 min), and samples heated at 50 and 70 °C for 5 min showed the highest content of phenolic compounds probably due to ultrasound stimulation and the disintegration of pollen. Probably, the higher temperature in this study did not significantly increase TPC due to the high solubility of honey polyphenols in an aqueous mixture of ethanol. Therefore, 35 °C was selected as the optimal extraction temperature for honey polyphenol extraction.

Table 4. Influence of UAE parameters on TPC of mint honey extracts.

UAE Parameters	TPC (mg GAE/100 g)
Time (min)	$p = 0.26$
10	65.80 ± 1.65 [a]
20	70.41 ± 2.17 [a]
30	70.56 ± 1.77 [a]
Temperature (°C)	$p = 0.18$
35	66.99 ± 1.61 [a]
50	67.09 ± 1.21 [a]
65	72.70 ± 2.48 [a]
Ultrasound amplitude (%)	$p = 0.03$ *
30	65.15 ± 1.25 [a]
60	70.76 ± 1.11 [b]
90	70.87 ± 2.72 [b]

* Statistically significant variable at $p \leq 0.05$. Results are expressed as mean ± SE. Means with the same letter within the column are not significantly different at $p \leq 0.05$. TPC = total phenolic content, UAE = ultrasound-assisted extraction.

3.3. Polyphenolic Characterization of Different Monofloral Honey Extracts

Individual polyphenols of monofloral honey extracts obtained at optimal MAE and UAE conditions were identified and quantified using UPLC-ESI/MS^2 analysis. A total of 21 polyphenolic compounds, consisting of flavonols, flavanones, flavones, and phenolic acids, were identified in honey extracts (Table 5, Supplementary Table S1). Fifteen compounds were identified based on the accurate mass data and retention times of authentic standards, while the structural characterization of the other compounds was based on the accurate mass, registered fragmentation patterns of mass spectra, and literature data.

Table 5. Mass spectrometric data and identification of phenolic compounds in different monofloral honey extracts obtained using optimized extraction conditions.

Compound	Rt (min)	Precursor Ion (*m*/*z*)	Product Ion (*m*/*z*)	Collision Energy (V)	Cone (V)	Mode	Concentration Range (mg/100 g)
Kaempferol-3-glucoside **	1.387	449	287	5	100	ESI (+)	0.029–0.191
Quercetin-3-glucoside **	1.713	465	303.1	5	100	ESI (+)	0.052–0.347
Isorhamnetin	2.085	317	257	10	100	ESI (+)	0.035–0.554
Galangin **	2.248	269	168.9	24	170	ESI (-)	0.034–0.554
Rutin **	6.811	611	303	15	120	ESI (+)	0.023–0.226
Kaemferol	9.600	287	165	10	100	ESI (+)	0.052–0.429
Myricetin **	10.594	316.8	151	22	150	ESI (-)	0.016–0.912
Quercetin	12.069	303	153	5	100	ESI (+)	0.071–0.815
TOTAL FLAVONOLS							0.672–1.870
Naringenin **	1.560	270.9	151	12	140	ESI (-)	0.015–0.078
Pinocembrin **	11.677	257	257	5	100	ESI (+)	0.142–0.407
TOTAL FLAVANONES							0.176–0.422
Apigenin **	1.577	271	153	30	80	ESI (+)	0.192–0.764
Chrysin **	1.805	253	253	5	140	ESI (-)	0.165–0.981
Apigenin-7-*O*-glucoside	8.300	433	271	24	200	ESI (+)	0.027–0.183
Luteolin **	10.965	287	153	25	140	ESI (+)	0.014–0.097
TOTAL * FLAVONES							0.653–1.787
Chlorogenic acid **	2.517	353	191	10	80	ESI (-)	0.035–0.380
Caffeic acid **	9.110	179	135	10	80	ESI (-)	0.110–0.250
p-coumaric acid **	9.481	163	119	10	80	ESI (-)	0.212–1.991
Ferulic acid **	9.777	193	149	6	100	ESI (-)	0.512–1.618
TOTAL HCA							1.108–4.009
3,4-Dihydroxybenzoic acid	1.665	152.9	108	20	90	ESI (-)	0.058–0.475
2,5- Dihydroxybenzoic acid	6.426	152.8	81.8	16	80	ESI (-)	0.041–0.428
Vanillic acid **	10.152	166.9	122.9	6	90	ESI (-)	0.143–0.582
p-hydroxybenzoic acid	11.568	137	93	5	80	ESI (-)	0.018–3.102
TOTAL HBA							0.407–3.831

** identification confirmed using authentic standards; HCA = hydroxycinnamic acids, HBA = hydroxybenzoic acids.

Several studies on European honeys have revealed that the phenolic profile of honeys is rich in flavonoid aglycones, benzoic acids, and cinnamic acids [43]. In this study, the identification of flavonols kaempferol-3-glucoside, quercetin-3-glucoside, galangin, rutin, and myricetin were performed using comparison with authentic standards, whereas the aglycones were determined in positive mode as isorhamnetin, quercetin, and kaempferol due to characteristic molecular ion at *m*/*z* 317, *m*/*z* 303, and *m*/*z* 287 [44]. Rutin was not detected in MAE honey samples of raspberry and ailanthus. Total flavonols in analyzed honey samples ranged from 0.672–1.870 mg/100 g, and the most abundant flavonols were myricetin (0.016–0.912 mg/100 g) and quercetin (0.017–0.815 mg/100 g). All of the detected flavonols were previously found in different monofloral honeys in varying contents. According to Lavag et al. [5], myricetin, quercetin, kaempferol, and isorhamnetin are the

most abundant flavonols in monofloral honeys. In general, quercetin was the predominant flavonoid in Argentine monofloral honeys, and total flavonol content ranged from 0.67 to 1.71 mg/100 g [45] which is consistent with the results of this study. Lower contents of quercetin (from 0.0580 to 0.15 mg/100 g) and myricetin (from 0.0004 to 0.0058 mg/100 g) were determined in different Croatian mint honeys [19]. In Polish buckwheat and wild raspberry honey, quercetin content was 0.057 and 0.010 mg/100 g, respectively, and higher content of galangin (0.070 and 0.054 mg/100 g) and kaempferol (0.035 and 0.099 mg/100 g) were also found [46]. In this study, galangin content ranged from 0.034 (ailanthus honey—MAE) to 0.554 mg/100 g (Lovran marron honey-UAE) and kaempferol from 0.052 (lavender honey-UAE) to 0.429 mg/100 g (ailanthus honey-UAE). The total content of flavanones in the analyzed honey extracts from UAE and MAE ranged from 0.176 (mint honey-UAE) to 0.422 mg/100 g (mint honey-MAE). The flavanones naringenin and pinocembrin were identified using comparison with authentic standards, and the content of pinocembrin (0.142–0.407 mg/100 g) was higher in analyzed samples than that of naringenin (0.015–0.078 mg/100 g). In Polish buckwheat and wild raspberry honeys, pinocembrin content (0.059 and 0.033 mg/100 g) was lower than in this study [46]. According to Pavlešić et al. [19], naringenin content in Croatian mint honey ranged from 0.01–0.0314 mg/100 g, which was also lower than in this study. Apigenin, chrysin, and luteolin were identified among the flavones by comparison with authentic standards, while apigenin-7-*O*-glucoside was identified on the basis of a characteristic fragment ion at m/z 271 and the loss of sugar moiety glucose (−162 amu). Total flavones ranged from 0.653 (marron honey-UAE) to 1.787 mg/100 g (lavender honey-UAE) in the honey extracts analyzed, with chrysin being the most abundant flavone (0.165–0.981 mg/100 g). In Polish buckwheat and wild raspberry honey, chrysin content (0.079 and 0.059 mg/100 g) was also higher than that of apigenin and luteolin but lower than in this study [46]. Lower contents of chrysin (0.112 to 0.51 mg/100 g) and apigenin (0.1065 to 0.64 mg/100 g) were also determined in Croatian mint honeys when compared to this study [19]. Furthermore, mint honey extracts obtained using UAE had higher chrysin (0.508–0.786 mg/100 g) and apigenin (0.207–0.764 mg/100 g) content than the extracts obtained using MAE (0.389–0.635 and 0.207–0.350 mg/100 g, respectively) (Supplementary Table S1). Among the hydroxycinnamic acids (HCA), the identification of chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and vanillic acid was carried out by comparison with authentic standards. The total content of HCA in the analyzed honey extracts obtained using UAE and MAE ranged from 1.108 to 4.009 mg/100 g and comprised the highest mass fraction in TPC. The most abundant HCA were *p*-coumaric (0.212–1.991 mg/100 g) and ferulic acid (0.512–1.618 mg/100 g). Halagarda et al. [46] in Polish buckwheat and wild raspberry honeys determined *p*-coumaric (0.427 and 0.276 mg/100 g), ferulic acid (0.047–0.087 mg/100 g) and caffeic acid (0.072 and 0.042 mg/100 g) in lower content. In this study, raspberry honey extracts obtained using UAE had higher *p*-coumaric (1.645 mg/100 g) and ferulic acid content (0.748 mg/100 g) than ones obtained using MAE (1.493 and 0.617 mg/100 g), while buckwheat MAE samples had higher *p*-coumaric (0.481 mg/100 g) and lower ferulic acid content (1.460 mg/100 g), (Supplementary Table S1). Pavlešić et al. [19] determined *p*-coumaric acid in the range of 0.34–0.8 mg/100 g and caffeic acid in the range of 0.13–0.272 mg/100 g in Croatian mint honeys, which was more or less similar to the results for *p*-coumaric acid (0.254–1.277 mg/100 g) and caffeic acid (0.120–0.250 mg/100 g) of mint extracts in this study, respectively. The total content of hydroxybenzoic acids (HBA) as the sum of vanillic, 2,5-dihydrobenzoic, 3,4-dihydrobenzoic, and *p*-hydroxybenzoic acids in the analyzed UAE and MAE honey extracts ranged from 0.407–3.831 mg/100 g. The most abundant HBA in analyzed samples were *p*-hydroxybenzoic acid ranging from 0.018 (lavender honey—UAE) to 3.102 mg/100 g (lavender honey—UAE) and vanillic acid ranging from 0.143 (lavender honey—UAE) to 0.582 mg/100 g (mint honey—MAE). In this study, *p*-hydroxybenzoic acid was determined to have a higher content in buckwheat honey extracts obtained using UAE (1.416 mg/100 g) and MAE (1.727 mg/100 g) than in Chinese buckwheat honey (0.7967 mg/100 g) [47]. The content of vanillic acid was also determined

in higher content in buckwheat honey extracts obtained using UAE (0.435 mg/100 g) and MAE (0.373 mg/100 g) than in Nordic buckwheat honey (0.025 mg/100 g) [48]. The different composition and content of polyphenols in honey are due to various factors, such as the flower and geographical origin and seasonal processing, as well as the packaging, storage, and extraction conditions [1]. In this study, 10 min, 35 °C, and 60% of amplitude were used as optimized conditions for UAE and 15 min, 60 °C, and 300 W for MAE. Tables 6 and 7 present the differences in the content of individual phenolic groups in honey samples influenced by the technique applied for the extraction. A statistically significant difference between UAE and MAE was found for different phenolic groups. The comparison of the mean values of UAE and MAE in terms of individual phenolic groups and TPC showed that the UAE showed higher efficacy, except for total flavonols and total HBA. This could indicate that these compounds are more stable in the presence of microwaves. Various studies reported that ultrasound treatments allow better preservation of flavonoids compared to conventional and some non-conventional extraction techniques. The increase in phenolic content is probably due to the decomposition of pollen stimulated by ultrasound, as honey does not contain intact cells [18]. When comparing individual honey samples within each extraction technique, sample 11 (sage honey) had the significantly lowest content of total flavonols and total flavanones compared to the other honey samples obtained using both UAE and MAE. Regardless of the extraction technique, the significantly highest content of total flavones was found in sample 8 (lavender honey) and the lowest content of total HBA. Moreover, the highest content of total HCA was found in sample 10 (Lovran maroon honey), while the lowest total polyphenols (as a sum of all determined phenolic groups) were found in sample 13 (maroon honey). In the study by Biesaga [7], the UAE also gave higher recoveries of phenolic compounds in comparison to MAE due to lower temperature, which is beneficial for preventing oxidation and degradation of phenolic compounds. Additionally, it was observed that the stability of polyphenols during extraction depends on their structure. The benzoic acid derivatives also showed higher stability during MAE, while flavonols showed significant degradation. When comparing the polyphenol yield from propolis, UAE was also a more efficient technique than MAE [41,49]. The benefits of UAE are mainly based on the mechanical effects of acoustic cavitation and are performed with less heat than MAE [49], but longer exposure to ultrasound can also decrease the concentration of polyphenols [35]. Quintero-Lira et al. [50] reported that ultrasound time between 10 and 15 min increased the concentration of various flavonoids. Some studies reported that UAE at 40 kHz promotes the generation of unstable bubbles and temporary cavitation and creates hydroxyl radicals and hydrogen atoms, which reduces the content of bioactive compounds with antioxidant activity [51,52]. In this study, 70% ethanol was used as the extraction solvent, and Kenari et al. [53] observed that extraction with an aqueous ethanol solution increased the yield of TPC from sesame cake because the intensity of ultrasonic cavitation increases in the presence of water due to the decrease in vapor pressure and viscosity of the mixture. However, Yildirim et al. [54] reported a higher content of total polyphenols and total flavanols in the propolis extracts obtained using MAE in comparison with UAE. MAE is based on the rapid heating of the solvent and adhesion of the extractable material to the solvent, which can shorten the extraction time, resulting in a higher content of extracted bioactive compounds, but its disadvantage is inhomogeneous heating [11]. It has also been reported that longer irradiation times during MAE can result in a decreased content of extracted bioactive compounds, possibly due to their degradation [49], and phenolic structures with more hydroxyl groups are less stable under different MAE conditions [55]. In general, the literature data confirm that the application of microwaves and ultrasound increases the phenolic content of the extracts, but the degradation of certain phenolic groups shows that optimization of the extraction conditions is necessary.

Table 6. Total flavonols, flavanones and flavones content of honey extracts obtained using UAE and MAE.

Sample		Total Flavonols (mg/100 g)			Total Flavanones (mg/100 g)			Total Flavones (mg/100 g)	
		UAE	MAE		UAE	MAE		UAE	MAE
		$p < 0.001$ *	$p < 0.001$ *		$p < 0.001$ *	$p < 0.001$ *		$p < 0.001$ *	$p < 0.001$ *
1	$p < 0.001$ *	$1.22 \pm 0.23^{f,A}$	$1.28 \pm 0.02^{f,B}$	$p = 0.001$ *	$0.25 \pm 0.06^{d,B}$	$0.21 \pm 0.01^{c,A}$	$p = 0.003$ *	$1.20 \pm 0.14^{f,B}$	$1.18 \pm 0.02^{e,A}$
2	$p = 0.002$ *	$1.67 \pm 0.26^{k,B}$	$1.61 \pm 0.02^{k,A}$	$p = 0.001$ *	$0.18 \pm 0.07^{a,A}$	$0.27 \pm 0.05^{f,B}$	$p < 0.001$ *	$1.06 \pm 0.11^{e,A}$	$1.29 \pm 0.03^{i,B}$
3	$p = 0.001$ *	$1.35 \pm 0.21^{h,A}$	$1.42 \pm 0.07^{i,B}$	$p < 0.001$ *	$0.32 \pm 0.04^{f,A}$	$0.42 \pm 0.04^{k,B}$	$p = 0.001$ *	$1.53 \pm 0.14^{l,B}$	$1.51 \pm 0.03^{l,A}$
4	$p < 0.001$ *	$1.48 \pm 0.08^{j,A}$	$1.58 \pm 0.03^{j,B}$	$p < 0.001$ *	$0.36 \pm 0.04^{g,B}$	$0.23 \pm 0.02^{de,A}$	$p < 0.001$ *	$1.41 \pm 0.02^{i,B}$	$0.85 \pm 0.02^{c,A}$
5	$p < 0.001$ *	$0.75 \pm 0.09^{b,A}$	$1.28 \pm 0.04^{f,B}$	$p = 0.001$ *	$0.22 \pm 0.01^{c,B}$	$0.20 \pm 0.01^{b,A}$	$p < 0.001$ *	$0.80 \pm 0.03^{b,B}$	$0.52 \pm 0.02^{b,A}$
6	$p < 0.001$ *	$1.03 \pm 0.09^{d,B}$	$0.94 \pm 0.03^{c,A}$	$p = 0.003$ *	$0.33 \pm 0.02^{f,A}$	$0.34 \pm 0.02^{i,B}$	$p < 0.001$ *	$1.46 \pm 0.06^{j,B}$	$1.27 \pm 0.04^{h,A}$
7	$p < 0.001$ *	$0.83 \pm 0.06^{c,A}$	$1.13 \pm 0.07^{d,B}$	$p < 0.001$ *	$0.20 \pm 0.04^{b,A}$	$0.38 \pm 0.06^{j,B}$	$p < 0.001$ *	$1.03 \pm 0.06^{c,A}$	$1.32 \pm 0.03^{j,B}$
8	$p < 0.001$ *	$1.03 \pm 0.16^{d,A}$	$1.15 \pm 0.09^{e,B}$	$p < 0.001$ *	$0.39 \pm 0.07^{i,B}$	$0.25 \pm 0.06^{e,A}$	$p < 0.001$ *	$1.79 \pm 0.05^{m,B}$	$1.62 \pm 0.01^{m,A}$
9	$p < 0.001$ *	$1.45 \pm 0.05^{i,B}$	$1.35 \pm 0.08^{h,A}$	$p < 0.001$ *	$0.38 \pm 0.01^{h,B}$	$0.31 \pm 0.05^{h,A}$	$p < 0.001$ *	$1.38 \pm 0.04^{h,B}$	$0.30 \pm 0.05^{a,A}$
10	$p = 0.028$ *	$1.34 \pm 0.21^{g,B}$	$1.32 \pm 0.05^{g,A}$	$p = 0.002$ *	$0.30 \pm 0.03^{e,B}$	$0.28 \pm 0.05^{g,A}$	$p < 0.001$ *	$1.05 \pm 0.10^{d,B}$	$0.27 \pm 0.03^{a,A}$
11	$p < 0.001$ *	$0.67 \pm 0.09^{a,A}$	$0.79 \pm 0.06^{a,B}$	$p = 0.032$ *	$0.18 \pm 0.04^{a,A}$	$0.19 \pm 0.04^{a,B}$	$p < 0.001$ *	$1.48 \pm 0.06^{k,B}$	$1.35 \pm 0.07^{k,A}$
12	$p = 0.004$ *	$1.87 \pm 0.07^{l,B}$	$1.85 \pm 0.04^{l,A}$	$p < 0.001$ *	$0.36 \pm 0.01^{g,B}$	$0.24 \pm 0.02^{d,A}$	$p = 0.002$ *	$1.29 \pm 0.07^{g,B}$	$1.26 \pm 0.04^{g,A}$
13	$p < 0.001$ *	$1.04 \pm 0.06^{e,B}$	$0.81 \pm 0.04^{b,A}$	$p < 0.001$ *	$0.41 \pm 0.02^{j,B}$	$0.27 \pm 0.03^{f,A}$	$p = 0.001$ *	$0.65 \pm 0.03^{a,A}$	$0.67 \pm 0.07^{b,B}$
Mean		1.21	1.27		0.30	0.28		1.24	1.03

* $p \leq 0.05$ Results are expressed as mean ± standard deviation. Different lowercase letters indicate statistically significant differences ($p \leq 0.05$) between honey samples within each extraction technique (column), while different uppercase letters indicate statistically significant differences ($p \leq 0.05$) between extraction techniques for individual honey samples within each group of phenolic compounds (row). UAE = ultrasound-assisted extraction, MAE = microwave-assisted extraction.

Table 7. Total HCA, total HBA and total phenols content of honey extracts obtained using UAE and MAE.

Sample		Total HCA (mg/100 g)			Total HBA (mg/100 g)			Total Phenols (mg/100 g)	
		UAE	MAE		UAE	MAE		UAE	MAE
		$p < 0.001$ *	$p < 0.001$ *		$p < 0.001$ *	$p < 0.001$ *		$p < 0.001$ *	$p < 0.001$ *
1	$p < 0.001$ *	$2.53 \pm 0.01^{g,B}$	$2.16 \pm 0.08^{e,A}$	$p < 0.001$ *	$2.60 \pm 0.04^{l,A}$	$2.85 \pm 0.01^{k,B}$	$p = 0.001$ *	$7.81 \pm 0.47^{i,B}$	$7.69 \pm 0.03^{j,A}$
2	$p < 0.001$ *	$2.99 \pm 0.17^{k,B}$	$2.58 \pm 0.04^{h,A}$	$p < 0.001$ *	$1.99 \pm 0.02^{h,B}$	$1.87 \pm 0.04^{e,A}$	$p < 0.001$ *	$7.90 \pm 0.62^{j,B}$	$7.61 \pm 0.07^{i,A}$
3	$p < 0.001$ *	$1.81 \pm 0.03^{c,A}$	$2.04 \pm 0.08^{d,B}$	$p = 0.138$	$0.70 \pm 0.06^{b,A}$	$0.68 \pm 0.05^{b,A}$	$p < 0.001$ *	$5.71 \pm 0.48^{b,A}$	$6.08 \pm 0.03^{d,B}$
4	$p < 0.001$ *	$2.40 \pm 0.10^{f,A}$	$2.76 \pm 0.07^{k,B}$	$p < 0.001$ *	$1.96 \pm 0.04^{g,B}$	$1.83 \pm 0.04^{d,A}$	$p < 0.001$ *	$7.61 \pm 0.04^{h,B}$	$7.23 \pm 0.03^{h,A}$
5	$p < 0.001$ *	$2.97 \pm 0.40^{j,B}$	$2.71 \pm 0.05^{j,A}$	$p < 0.001$ *	$1.91 \pm 0.03^{f,A}$	$2.21 \pm 0.04^{i,B}$	$p < 0.001$ *	$6.65 \pm 0.53^{e,A}$	$6.92 \pm 0.06^{f,B}$
6	$p < 0.001$ *	$2.72 \pm 0.22^{h,B}$	$2.60 \pm 0.06^{i,A}$	$p < 0.001$ *	$1.82 \pm 0.06^{d,A}$	$1.90 \pm 0.02^{f,B}$	$p < 0.001$ *	$7.35 \pm 0.02^{g,B}$	$7.05 \pm 0.09^{g,A}$
7	$p < 0.001$ *	$2.90 \pm 0.08^{i,B}$	$2.48 \pm 0.06^{g,A}$	$p < 0.001$ *	$3.83 \pm 0.20^{m,B}$	$3.49 \pm 0.04^{l,A}$	$p = 0.017$ *	$8.78 \pm 0.33^{l,A}$	$8.80 \pm 0.05^{l,B}$
8	$p < 0.001$ *	$3.10 \pm 0.03^{l,B}$	$2.87 \pm 0.04^{l,A}$	$p < 0.001$ *	$0.41 \pm 0.05^{a,A}$	$0.45 \pm 0.02^{a,B}$	$p < 0.001$ *	$6.72 \pm 0.35^{f,B}$	$6.34 \pm 0.22^{e,A}$
9	$p < 0.001$ *	$1.26 \pm 0.02^{b,B}$	$1.11 \pm 0.07^{a,A}$	$p < 0.001$ *	$2.10 \pm 0.08^{i,B}$	$2.05 \pm 0.07^{g,A}$	$p < 0.001$ *	$6.57 \pm 0.06^{d,B}$	$6.01 \pm 0.03^{c,A}$
10	$p < 0.001$ *	$4.01 \pm 0.05^{m,B}$	$3.84 \pm 0.07^{m,A}$	$p < 0.001$ *	$2.15 \pm 0.07^{j,B}$	$2.05 \pm 0.06^{g,A}$	$p < 0.001$ *	$8.85 \pm 0.46^{m,B}$	$7.76 \pm 0.07^{j,A}$
11	$p < 0.001$ *	$1.94 \pm 0.03^{d,B}$	$1.70 \pm 0.02^{c,A}$	$p < 0.001$ *	$1.65 \pm 0.02^{c,A}$	$1.80 \pm 0.05^{c,B}$	$p < 0.001$ *	$5.92 \pm 0.25^{c,B}$	$5.83 \pm 0.04^{b,A}$
12	$p < 0.001$ *	$2.27 \pm 0.03^{e,A}$	$2.40 \pm 0.04^{f,B}$	$p < 0.001$ *	$2.17 \pm 0.02^{k,A}$	$2.31 \pm 0.05^{j,B}$	$p < 0.001$ *	$7.96 \pm 0.13^{k,A}$	$8.07 \pm 0.16^{k,B}$
13	$p < 0.001$ *	$1.04 \pm 0.08^{a,A}$	$1.29 \pm 0.02^{b,B}$	$p < 0.001$ *	$1.89 \pm 0.04^{e,A}$	$2.18 \pm 0.07^{h,B}$	$p < 0.001$ *	$5.04 \pm 0.15^{a,A}$	$5.22 \pm 0.13^{a,B}$
Mean		2.46	2.35		1.94	1.97		7.14	6.97

* $p \leq 0.05$ Results are expressed as mean ± standard deviation. Different lowercase letters indicate statistically significant differences ($p \leq 0.05$) between honey samples within each extraction technique (column), while different uppercase letters indicate statistically significant differences ($p \leq 0.05$) between extraction techniques for individual honey samples within each group of phenolic compounds (row). HCA = hydroxycinnamic acids, HBA = hydroxybenzoic acids, UAE = ultrasound-assisted extraction, MAE = microwave-assisted extraction.

3.4. Antioxidant Capacity of Honey Extracts

Antioxidant capacity was determined in monofloral honey extracts obtained using optimized UAE and MAE using ORAC, ABTS, and DPPH assays (Table 8). To correctly describe the antioxidant capacity of honey in vitro, different assays related to reaction mechanisms, oxidizing species, and reaction conditions were used. ORAC is a hydrogen transfer assay that measures the substrate's ability to donate hydrogen, while ABTS and DPPH are the electron transfer assays that measure the substrate's reducing ability. All the samples showed considerable antioxidant capacity using all three assays. The honey extracts obtained using UAE had ORAC antioxidant capacity ranging from 381.83 to 597.93 µmol TE/100 g, ABTS in a range from 587.80 to 666.19 µmol TE/100 g and DPPH antioxidant capacity ranged from 190.40 to 243.99 µmol TE/100 g. The antioxidant capacity in MAE honey extracts obtained using ORAC ranged from 380.09 to 615.00 µmol TE/100 g, ABTS from 580.32 to 654.99 µmol TE/100 g, and DPPH from 186.83 to 243.26 µmol TE/100 g. The higher ABTS than DPPH capacity was also observed in Sicilian monofloral honeys [56]. Regardless of the extraction technique, the lowest and highest antioxidant ABTS capacity was determined in mint and raspberry honey extracts and DPPH capacity in sage and mint honey extracts, respectively. The lowest ORAC capacity was determined in sage honey extracts regardless of the extraction technique, while the highest ORAC value was determined in UAE mint honey and MAE Lovran maroon honey samples (Table 8). Previous studies reported that phenolic compounds are responsible for the antioxidant capacity of honey, and dark-colored honeys have higher antioxidant capacities [46,57]. The honeys with the highest phenolic contents (as a sum of individual phenolic groups) in this study were Lovran maroon honey (8.855 mg/100 g) obtained using UAE and lavender honey (8.799 mg/100 g) obtained using MAE. Depending on the flower source, geographical origin, and climatic conditions, the composition of polyphenolic compounds varies, but *p*-coumaric acid, ferulic acid, caffeic acid, pinocembrin, chrysin, quercetin, apigenin, and naringin are mainly present [46]. Due to the large differences in honey composition, the biological activities of the honey samples also varied [58]. Numerous free radical scavengers in honey can reduce the imbalance between the content of antioxidants and the production of free radicals. According to Chua et al. [58], certain side reactions, such as metal ion chelation and enzyme inhibition, may have no effect on DPPH free radicals. The range of DPPH capacity of Serbian honeys ranged from 45 to 275 µmol TE/100 g, with lavender honey (74 µmol TE/100 g) having a lower antioxidant capacity than in this study [59]. Sicilian monofloral honeys showed DPPH antioxidant capacity ranging from 8.5 to 238.4 µmol TE/100 g and ABTS from 19.2 to 270.3 µmol TE/100 g [56], which was also lower than in this study. The ORAC antioxidant capacity of honeys from different floral sources in North America ranged from 231 to 799 µmol TE/100 g [43], which was similar to the results of this study. Different solvents and extraction techniques can also lead to a different composition of phenolic compounds in the extracts due to the different solubility of the individual compounds, and the lower content of phenolic compounds with more hydrophobic properties than those with hydrophilic properties can affect the bioactivity of an extract [60]. For the comparison of the honey samples and the efficiency of UAE and MAE in terms of antioxidant capacity, the mean values were compared (Table 8). A statistically significant difference was found between the UAE and MAE techniques for individual honey samples within the ABTS, DPPH, and ORAC (except for samples 2 and 11) assays for antioxidant capacity determination. The higher ORAC and DPPH mean values were determined in honey extracts obtained using MAE and lower using the ABTS assay. Apart from the different mechanisms for measuring antioxidant content, antioxidant assays are affected by different structural properties of the extracted antioxidants, and the antioxidant behavior depends on the number and position of hydroxyl groups and other substituents in a molecule [61]. This study suggests that the higher content of total flavonols and HBA in the extracts obtained using MAE is probably responsible for the higher ORAC and DPPH capacity. In the study of Rodriguez-Gonzalez et al. [27], the antioxidant properties of bee pollen extracts measured using ABTS were also higher in

the extracts obtained using UAE than by MAE, probably due to the degradation of some non-phenolic compounds. The higher ABTS capacity of the UAE extracts compared to the DPPH capacity could be due to the ABTS radical having a stronger tendency to donate electrons than the DPPH radical. Other studies have also examined the effects of these techniques on antioxidant capacity and have come to different conclusions. Polyphenolic structural features, reaction mechanisms, and conditions of the assays used have been shown to influence antioxidant capacity. In general, comparable antioxidant capacities were found between honey extracts obtained using both techniques, although there are differences in the reaction mechanism between UAE and MAE. Fuente-Ballesteros et al. [62] reported that MAE is a faster technique but extracts less bioactive compound compared to UAE, possibly due to degradation processes that favor UAE over MAE as a more efficient extraction technique. In the study by Yildrim [54], the highest DPPH antioxidant capacity of propolis extracts was obtained using UAE compared to MAE and maceration. Quintero-Lira et al. [50] reported that the higher antioxidant ABTS capacity was determined in honey when UAE was applied for longer time (5, 10, and 15 min) due to the increased extraction and availability of phenolic acids and flavonoids. The ABTS values of Turkish honey samples extracted under different ultrasonic time (5, 10, 15, and 20 min) and temperature conditions (30, 45, 60, and 80 °C) increased with the increase in ultrasound time compared to the untreated samples, but the ABTS values were mainly affected by thermal treatment and to a lesser extent by ultrasound [63]. Regarding the honey variety, sample 11 (sage honey) had the significantly lowest ORAC and DPPH values compared to the other honey samples, regardless of the extraction technique used. Within the ABTS method, sample 1 (mint honey) obtained using UAE and MAE extraction had the lowest antioxidant capacity compared to the other honey samples while, at the same time, the highest DPPH capacity. Ruiz-Navajas et al. [64] reported different DPPH values of Mexican honey samples due to the content of phenolic acids and flavonoids and their ability to scavenge free radicals, donate hydrogen, chelate metal ions, or even serve as a substrate for superoxide or hydroxyl radicals. Apart from the polyphenol content, a variety of honey constituents, such as peptides, organic acids, enzymes, Maillard reaction products, and other minor compounds, provide the antioxidant effect of honey [43].

Table 8. Antioxidant capacity of honey extracts obtained using UAE and MAE.

Sample		ORAC (μmol TE/100 g)			ABTS (μmol TE/100 g)			DPPH (μmol TE/100 g)	
		UAE	MAE		UAE	MAE		UAE	MAE
		$p < 0.001$ *	$p < 0.001$ *		$p < 0.001$ *	$p < 0.001$ *		$p < 0.001$ *	$p < 0.001$ *
1	$p < 0.001$ *	$553.9 \pm 0.1^{d,A}$	$590.3 \pm 0.9^{fg,B}$	$p < 0.001$ *	$587.80 \pm 0.2^{a,B}$	$580.32 \pm 0.4^{a,A}$	$p = 0.002$ *	$243.99 \pm 0.1^{m,B}$	$243.26 \pm 0.4^{m,A}$
2	$p = 0.903$	$575.7 \pm 0.7^{h,A}$	$578.2 \pm 4.8^{ef,A}$	$p < 0.001$ *	$635.39 \pm 0.3^{c,B}$	$593.39 \pm 0.2^{c,A}$	$p < 0.001$ *	$237.35 \pm 0.6^{k,A}$	$240..3 \pm 0.3^{l,B}$
3	$p = 0.009$ *	$603.4 \pm 0.8^{k,B}$	$597.4 \pm 0.3^{fg,A}$	$p < 0.001$ *	$642.85 \pm 0.3^{d,B}$	$606.44 \pm 0.4^{d,A}$	$p < 0.001$ *	$225.23 \pm 0.3^{i,A}$	$235.23 \pm 0.3^{j,B}$
4	$p = 0.016$ *	$597.9 \pm 0.9^{j,B}$	$592.3 \pm 0.5^{fg,A}$	$p < 0.001$ *	$645.64 \pm 0.4^{d,A}$	$653.11 \pm 0.4^{l,B}$	$p < 0.001$ *	$228.63 \pm 0.2^{j,A}$	$230.75 \pm 0.5^{i,B}$
5	$p = 0.006$ *	$565.1 \pm 0.3^{ef,B}$	$561.5 \pm 0.3^{de,A}$	$p < 0.001$ *	$631.62 \pm 0.7^{c,A}$	$636.32 \pm 0.3^{g,B}$	$p < 0.001$ *	$204.33 \pm 0.3^{e,A}$	$211.49 \pm 0.2^{e,B}$
6	$p < 0.001$ *	$473.0 \pm 0.3^{b,A}$	$510.2 \pm 0.4^{b,B}$	$p < 0.001$ *	$666.19 \pm 0.1^{e,B}$	$654.99 \pm 0.2^{m,A}$	$p < 0.001$ *	$200.41 \pm 0.2^{b,A}$	$210.23 \pm 0.3^{d,B}$
7	$p < 0.001$ *	$562.9 \pm 0.2^{e,A}$	$574.0 \pm 0.1^{def,B}$	$p < 0.001$ *	$646.58 \pm 0.3^{d,A}$	$651.25 \pm 0.3^{k,B}$	$p < 0.001$ *	$211.49 \pm 0.1^{g,A}$	$215.76 \pm 0.4^{f,B}$
8	$p = 0.002$ *	$568.2 \pm 1.0^{g,B}$	$549.8 \pm 0.6^{cd,A}$	$p < 0.001$ *	$643.79 \pm 0.2^{d,A}$	$645.64 \pm 0.4^{i,B}$	$p < 0.001$ *	$209.15 \pm 0.4^{f,A}$	$217.29 \pm 1.1^{g,B}$
9	$p = 0.002$ *	$581.9 \pm 0.3^{i,B}$	$575.8 \pm 0.2^{def,A}$	$p < 0.001$ *	$647.51 \pm 0.3^{d,B}$	$637.24 \pm 0.4^{h,A}$	$p < 0.001$ *	$211.66 \pm 0.3^{h,A}$	$219.70 \pm 0.3^{h,B}$
10	$p < 0.001$ *	$583.3 \pm 0.5^{i,B}$	$527.5 \pm 0.4^{bc,A}$	$p < 0.001$ *	$636.31 \pm 0.3^{c,A}$	$647.52 \pm 0.3^{j,B}$	$p < 0.001$ *	$203.99 \pm 0.2^{d,A}$	$206.30 \pm 0.2^{c,B}$
11	$p = 0.078$	$381.8 \pm 0.5^{a,A}$	$380.1 \pm 0.6^{a,A}$	$p < 0.001$ *	$664.31 \pm 0.4^{e,B}$	$627.00 \pm 0.1^{f,A}$	$p < 0.001$ *	$190.40 \pm 0.4^{a,B}$	$186.83 \pm 0.4^{a,A}$
12	$p = 0.003$ *	$566.5 \pm 0.5^{fg,B}$	$558.9 \pm 0.3^{de,A}$	$p < 0.001$ *	$615.78 \pm 0.4^{d,B}$	$585.93 \pm 0.3^{b,A}$	$p = 0.002$ *	$240.94 \pm 0.4^{l,B}$	$240.05 \pm 0.3^{k,A}$
13	$p < 0.001$ *	$525.0 \pm 0.2^{c,A}$	$615.0 \pm 0.2^{g,B}$	$p = 0.012$ *	$645.46 \pm 0.3^{b,B}$	$613.92 \pm 0.3^{e,A}$	$p < 0.001$ *	$201.30 \pm 0.3^{c,A}$	$204.51 \pm 0.3^{b,B}$
Mean		549.1	554.7		639.17	625.62		216.07	220.13

* $p \leq 0.05$ Results are expressed as mean ± standard deviation. Different lowercase letters indicate statistically significant differences ($p \leq 0.05$) between honey samples within each extraction technique (column), while different uppercase letters indicate statistically significant differences ($p \leq 0.05$) between extraction techniques for individual honey samples within each antioxidant capacity assay (row). UAE = ultrasound-assisted extraction, MAE = microwave-assisted extraction, TE = Trolox equivalents.

4. Conclusions

In this study, UAE and MAE were optimized and compared for polyphenol extraction of monofloral honey. The optimal extraction conditions for UAE were 10 min, 35 °C, and 60 W, and for MAE, 15 min, 60 °C, and 300 W. The extraction technique had a significant effect on the polyphenol content and antioxidant capacity of the honey samples. Total phenolic acids and flavonols were the most abundant and the extracts had higher individual contents of *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid and vanillic acid, myricetin, quercetin, pinocembrin, naringenin and chrysin. UAE showed higher yields for total flavanones, flavones, HCA, and total phenols, and MAE for total flavonols and total HBA. The antioxidant capacity measured using ORAC and DPPH assays was higher in the extracts obtained by microwave, while the ABTS capacity was higher in those obtained using ultrasound extraction. In conclusion, monofloral honey extracts obtained using MAE and UAE are rich in polyphenols with high antioxidant capacity, but the influence of these extraction techniques on the bioactive compounds of honey and their degradation processes needs further investigation. Future research should investigate whether the combination of these techniques can further improve extraction yields in terms of total and individual phenolic content, as well as effects on bioavailability and bioactivity related to potential health benefits, as honey extracts could find a great application in the food and dietary supplement industry.

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