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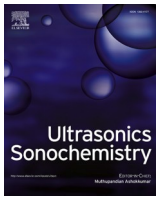

Do ultrasonic field effects upon the polyphenolics profile of propolis extracts improve their antioxidant and antimicrobial activity?

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ABSTRACT

Ultrasound-assisted extraction (UAE) was applied for polyphenols extraction from Romanian propolis, followed by comparison with previous maceration work. The effects consisted not only in time reduction and extraction yield increase, but also in polyphenolics profile modification in terms of flavonoids / polyphenolic acids ratio. The operating parameters were ultrasounds (US) field exposure time (10–100 min), solvent composition (water, 25 % and 50 % ethanolic solutions, w/w), and liquid:solid ratio (2:1, 4:1 and 6:1, w:w), while keeping temperature constant. 24 polyphenolic derivatives were quantified by UHPLC-HRMS. UAE favored the extraction of pinocembrin, isorhamnetin and chrysin in water and 25 % ethanol, leading to different profiles than maceration, and further influences upon the antioxidant and antimicrobial activity. All extracts demonstrated increased antibacterial and antifungal activity compared to maceration, particularly the 50 % ethanolic extracts, which presented a three-times larger antioxidant capacity. Chemometric methods (Principal Component Analysis – PCA and Partial Least Squares Regression – PLS) and a saturation type model were used to correlate the polyphenolics profiles and antioxidant capacity. Experimental and modelling results concluded that 50 % ethanolic solutions and UAE represent the favorable operating conditions in terms of yield and extracts quality.

1. Introduction

Numerous studies revealed the amazing and complex mechanism of the propolis action to preserve/improve human health through the activity of the polyphenolic derivatives [1–6]. Propolis compounds also inhibit some proteins responsible for cancer cells proliferation (e.g., cyclin B, Claudin-2) or activate others involved in the apoptosis process stopping cell division and enhancing the action of cancer drugs [7]. The antimicrobial efficacy of propolis and some of its individual components has been studied against a wide variety of microorganisms: bacteria [8–12], fungi [13–17], viruses [18–19] and parasites [20]. Previous studies on the antibacterial potential of propolis have shown that it has been more effective against Gram-positive bacteria compared to Gram-negative ones [8]. Propolis extracts, through the content of phenolic acids and flavonoids, present a fungistatic effect up to 12 h after administration, by reducing the ability to grow, germinate, and hyphae formation of *Candida* strains. Also, these extracts caused membrane and cell wall damage with intracellular content extravasation for fungal cells, but without mutagenic effects [15].

Over the years, researchers have worked on the isolation and quantification of flavonoids and other bioactive compounds from various natural matrices, prior to including in cosmetics, food and medicinal products [21] by classical extraction processes (e.g., maceration, Soxhlet) [22] and later focusing on the modern non-conventional methods such as UAE [23,24], microwave-assisted extraction (MAE) [25], or simultaneous UAE/MAE [26], supercritical fluid extraction [27] and ionic liquid extraction [23]. It has been reported that the highest flavonoid content and the biggest antioxidant capacity of propolis extracts were obtained for supercritical CO_2 extraction in 15 % ethanol, while MAE, followed by Soxhlet gave the highest total polyphenols content [28].

The advantages of using US in the extraction of polyphenolic compounds from biological matrices are discussed in various studies [21,29,30] and refer to simplicity, reduced extraction time, the

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possibility of using small amounts of solvent, increased efficiency for extracting thermolabile compounds thanks to the possibility of maintaining a low temperature in the ultrasonic bath and its ability to be easily implemented in various food or drug industries. When a liquid: solid mixture is subjected to an US field, cavitation bubbles begin to form, expanding and shrinking following the liquid compression and rarefaction, till those arrived at the critical dimension implode, thus producing liquid micro-jets at the liquid–solid interface, able to penetrate the matrix, shattering its walls, and enabling the migration of substances of interest in the liquid [31].

While most investigations report on results obtained using hydro-alcoholic mixtures in the 70–99 % range [2–4,22,23,31], larger proportions of water were also tested [32,33], demonstrating that water-based solvents tend to extract more phenolic acids than flavonoids. Extraction yield peaks around 40–60 % (v/v) ethanol, when a plateau is reached. Kara *et al.* [32] concluded that 5:1 and 10:1 represent the best liquid:solid ratios in terms of efficiency (costs and total phenolic content).

The study carried out by Aboulghazi *et al.* [33] varied ethanol content (40–80 %), liquid:solid ratio (10:1–30:1, v:w) and time (15 – 45 min) while using a 50 kHz sonic bath at 120 W and 35°C; total phenolics, total flavonoids, antioxidant activity, and extraction yield were the targeted outputs. Simultaneous optimization provided the optimal processing conditions: 15 min, for 30:1 liquid:solid ratio, using 58 % ethanol. The most abundant compound in the optimized extract was *epi*-catechin, (0.193 mg/g) followed by *p*-coumaric acid (0.053 mg/g).

Aiming to replace the volatile organic solvents, dos Santos *et al.* [34] used UAE to test the transfer of bioactive compounds from red Brazilian propolis into a mixture of ionic liquids with 10 % eutectic co-solvents. A 20 kHz ultrasonic horn was used for 5 min at 400 W, to homogenize the sample of propolis either with 70 and 95 % ethanol, or with 1-hexyl-3-methylimidazolium chloride in 10 % water, in three successive extractions. The aqueous extract presented twice the extractive potential of 95 % ethanol, an increase attributed to the presence of the ionic liquid, as its higher hydrophobicity leads to the transfer of hydrophobic components from the biomass based on π–π, n–π, and hydrophobic interactions. The eutectic solvents proposed by Trusheva *et al.* [35] involved citric acid or choline chloride mixed with alcohols. Temperature has seldom exceeded 60°C, to prevent degradation of the bioactive compounds [2,35,36]. Golmahi *et al.* [37] emphasized the influence of concentration, time, and temperature upon the total phenolic content and antioxidant activity of propolis ethanolic extracts.

The present work aims to find optimal conditions for polyphenolic compounds extraction from propolis, using sonication as intensifying technique, under isothermal conditions. The operating parameters were the US exposure time, liquid:solid ratio, and ethanolic solvent concentration. Another goal was to identify if the US field and exposure time influence the propolis standard profile of extracted polyphenolic derivatives using a UHPLC-HRMS method. If US exposure modifies the profile of extracted components, as well as their concentration, then both the antioxidant and the antimicrobial activities could be affected, and the present study aims to find out how much.

This study represents a continuation of a previously published investigation dealing with polyphenolics profile obtained by maceration [38], which will be compared to the UAE obtained profiles, being focused on US contribution to the process intensification, the way the new profiles influence the antioxidant and antimicrobial activities, and the quality of propolis extracts.

2. Materials and methods

2.1. Raw material – Propolis

The propolis used in this study was obtained from dr. Roxana Spulber, Institute for Research and Development for Beekeeping. It was harvested from Bihor County (Oradea Municipality), Romania, being produced by *Apis meliffera carpatica*. Propolis was finely grinded (0.1 mm) with a Retsch 200 mill (Haan, Germany) and stored at –20°C until subjected to the UAE.

2.2. Reagents

2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) – $ABTS^{\bullet+}$, (98 %), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid – Trolox, (95 %), $K_2S_2O_8$ (99 %), and ethanol (99.8 %) were purchased from Sigma-Aldrich (Steinheim, Germany), being used as shipped. Analytical standards for flavonoids (apigenin, (+)–catechin, chrysin, (–)–*epi*–catechin, galangin, hesperidin, isorhamnetin, kaempferol, pinocembrin, quercetin, rutin) and phenolic acids (caffeic, 3,4-dihydroxybenzoic, *t*-ferulic, gallic, 4-hydroxybenzoic acid, *p*-coumaric, syringic, and vanillic acids), phenolic acids derivatives (CAPE, ellagic, and chlorogenic acids), stilbenes (*t*-resveratrol) acid, vanillic acid) and terpenes (abscisic acid) from Sigma-Aldrich (Steinheim, Germany) were used to prepare 500 mg/L methanolic stocks. A mixed working standard in methanol was prepared by diluting individual stocks to 10 mg/L. Pipettes and class A volumetric glassware were used in all quantification experiments. Gradient grade methanol for liquid chromatography (99.9 %), and formic acid (98–100 %) were purchased from Merck (Darmstadt, Germany). Aqueous solutions were prepared with deionized water produced by a Milli-Q Millipore system (Bedford, USA). All chemicals for antimicrobial activity were microbiologically pure. Nutrient agar (NA) was purchased from Sigma–Aldrich (Steinheim, Germany), and yeast extract peptone dextrose (YPD), from Carl Roth (Karlsruhe, Germany).

2.3. UAE procedure

Based upon our previous findings [38], showing that using 70 % (w/w) alcoholic solution didn't improve the extraction of active species in an economic way, only three types of solvents (water, 25 % (w/w) ethanol, and 50 % (w/w) ethanol), three liquid:solid ratios (2:1; 4:1; 6:1, w:w) and five extractions times (10, 20, 30, 40 and 100 min) were envisaged. Although time of extraction is not a real independent parameter, it was considered as such because of the side effects of cavitation phenomena (characteristic to an intense US field) could have upon the polyphenolics profile, changing it due to the chemical reactions between the active species generated during the collapse of the cavitation bubbles and some or all the polyphenolics compounds [24]. The time of extraction with the highest antimicrobial and antioxidant performances could, thus, be found. For the sake of brevity, the ethanol concentrations and liquid:solid ratios were reported without w/w indication, in the entire paper. 5 g of propolis were added to 43 glass jars over which 10 g solvent were added for the ratio of 2:1, 20 g of solvent for the ratio of 4:1, and 30 g for the last ratio. The jars were closed tightly with lids and all samples were placed in pairs in the ultrasonic bath for extraction, always at the same height above the transducers. The jars for 100 min and 10 min exposure times were placed first for each liquid: solid ratio, the last being replaced, in due time, with the jars for 20-, 30-, and 40-min extraction time. Samples were filtered through filter paper in sterile plastic containers with lids and stored in the freezer (-20°C) until the analyzes were performed. The ultrasonic bath frequency was 40 kHz (Elma Transsonic, Germany), ultrasonic power was set to 110 W (100 %) and the water temperature (the coupling fluid) was maintained around the room ambient temperature, replacing it every other 10 min of sonication. The volume of water in the ultrasonic bath was always the same, 1300 mL.

2.4. Polyphenolic derivatives analysis by UHPLC-HRMS

Quantification was performed with an UltiMate 3000 UHPLC System (Thermo Fisher Scientific), coupled with a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ mass spectrometer equipped with Heated

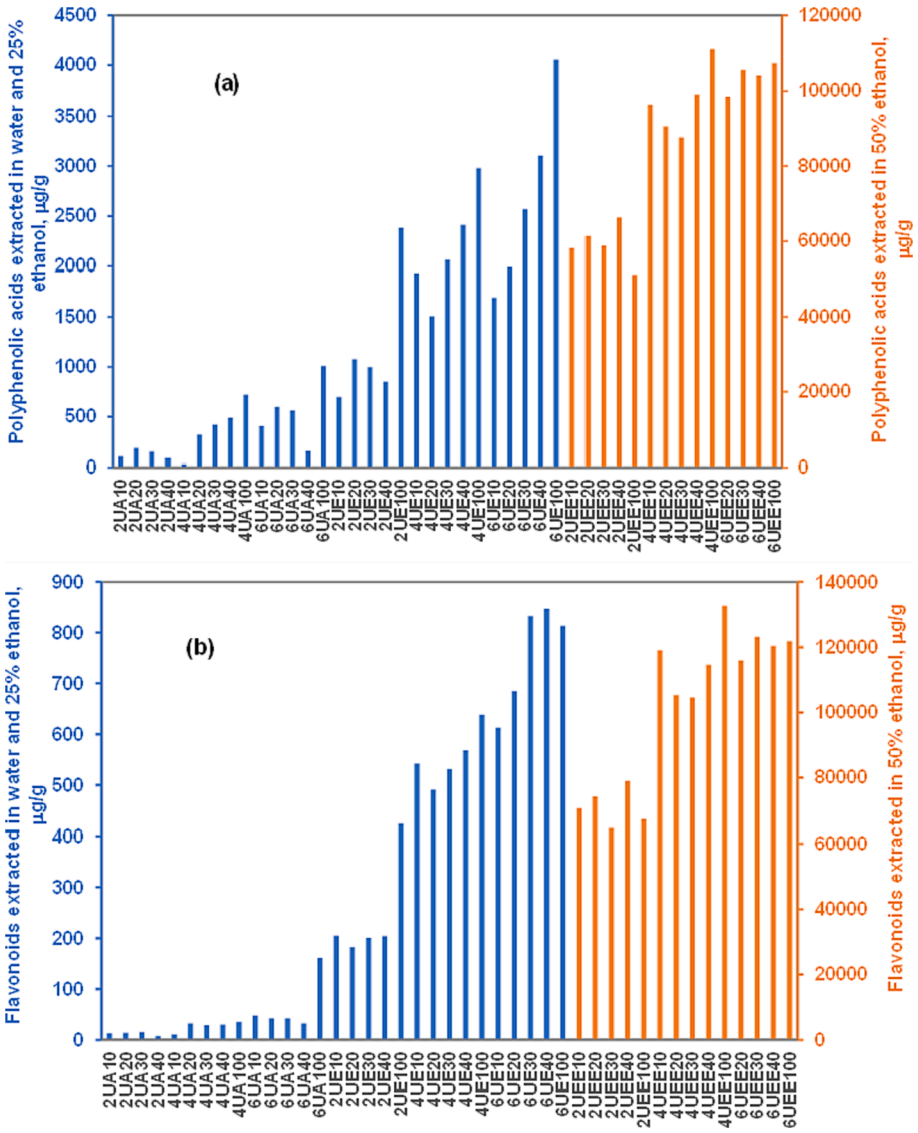

Fig. 1. Polyphenolic acids (a) and flavonoids (b) quantified in 0 – 50 % hydroalcoholic extractants, subjected to a 40 kHz and 110 W US field (Elma Transsonic bath), at room temperature.

Electrospray Ionisation (HESI) probe (Thermo Fisher Scientific). Chromatographic separation used a Kinetex® C18 column (100 × 2.1 mm, 1.7 µm particle diameter) at 30°C. Gradient elution at a 0.3–0.4 mL/min flow rate employed solvent A (water with 0.1 % formic acid) and solvent B (methanol with 0.1 % formic acid), as in the previous work [38]. The applied voltage was 2.5 kV, and the capillary temperature was 320 °C. The energy of the collision cell was set at 30 eV. Full scan in negative mode covered the 75–1000 *m/z* range; data were acquired at a resolving power of 70,000 FWHM at 200 *m/z*, while variable data-independent analysis MS2 (vDIA) was performed at 35,000 resolution. The isolation windows and scan ranges were set to 75–205, 195–305, 295–405, 395–505, and 495–1000 *m/z*. Nitrogen was used as collision and auxiliary gas, at flow rates of 11 and 48 arbitrary units, respectively. Calibrations solutions were prepared in the 50–1750 µg/L concentration range for each compound of interest, by serial dilution with methanol of the 10 mg/L standard mixture and the linear calibration curves for each compound were forced through origin. Data were processed with the Xcalibur software package (Version 4.1). The mass tolerance window was set to 5 ppm. Calibration parameters for the 24 polyphenolic derivatives are available in **Table S1 – Supplementary information (SI)**, online. Propolis extracts were filtered through a 0.45 µm polytetrafluoroethylene membrane and diluted before injection into the UHPLC-MS system. A typical chromatogram is presented in **Fig. S1 – SI**. Polyphenolic acids and flavonoids levels were reported as either µg/mL extract or µg/g propolis.

2.5. TEAC radical scavenging assay

The antioxidant capacity of the extracts was evaluated by their ability to scavenge $ABTS^{\bullet+}$, a long-lived free radical, as to engage in action both lipophilic and hydrophilic antioxidants [39]. The assay was carried out at 734 nm, against a reagent blank in water, using Trolox as model compound [38]. $ABTS^{\bullet+}$ assay parameters were 7.38 ± 0.03 µg/mL slope, −0.48 ± 0.17 µg/mL intercept, 0.9997 coefficient of determination, and 0.17 µg/mL standard error of response. Bias did not exceed 3.8 %, with 94.6 – 102.1 % recovery for the investigated concentration range.

2.6. Antimicrobial activity

A slightly modified disc diffusion method was used for the study of antimicrobial activity, as described in our previous research [38]. The

bacteriostatic activity of the solvent was always subtracted from the measured extracts inhibition zone diameter.

2.7. Data processing

The PCA, PLS, and nonlinear regression over the experimental data to evaluate the saturation model parameters were carried out using an inhouse software written in Matlab® R2022a (MathWorks, Natick, MA, USA) programming environment. US field topology was computed using Acoustic physics from COMSOL Multiphysics® 5.2a (COMSOL, Inc., Burlington, MA, USA).

3. Results and discussion

3.1. Composition of extracts

Extractions used 2:1, 4:1 and 6:1 liquid:solid ratios to obtain rather concentrated solutions of polyphenolic derivatives and provide a basis for evaluating the mass transfer intensification brought around by the US field. Apart from water, only 25 % and 50 % ethanol were used as extracting agents, as previous maceration research [38] demonstrated that the extracts in 50 % ethanol provided the best antimicrobial activity and the highest number of extracted compounds; the antioxidant capacity tends to level at higher alcohol concentrations. The extracted polyphenolic acids and flavonoids quantified by UHPLC-HRMS with relative standard deviations below 5 % are presented in Fig. 1, with samples differentiated by liquid:solid ratio (2U, 4U, 6U), solvent type (A – water, E – 25 % ethanol, EE – 50 % ethanol) and US field duration (10, 20, 30, 40, 100 min). 24 compounds were quantified in the 43 extracts, revealing significant differences between their average concentration levels: there were compounds extracted at average concentrations lower than 0.1 mg/g, between 0.1 and 1 mg/g, and higher than 1 mg/g.

A closer look to the extract profile with the liquid:solid ratio reveals caffeic, ferulic, and *p*−coumaric acids as best extracted compounds in water, at room temperature (**Fig. S2 - SI**). Despite the low average extraction yield recorded (0.04 %), the three polyphenolic acids represent 80 % of the quantified compounds. Kara *et al.* [32] reported the same three polyphenolic acids as main compounds extracted in water, after applying a routine consisting of 30 min US bath-stage combined with 24 h-maceration shaking at 200 rpm and room temperature.

Surprisingly, the US treatment allows flavonoids to solubilize even in aqueous extracts. Pinocembrin was leading, at a 14.6 μg/g average, accompanied by isorhamnetin, 9.6 μg/g, and small amounts of chrysin, 4.6 μg/g (6UA10 sample in **Fig. S2a - SI**). Longer exposure to US increased the amounts extracted, all remaining in the low values domain (**Fig. S2b - SI**). Working with 25 % ethanol as extraction solvent gave flavonoids the chance to increase to approximately 22 – 25 % of the quantified compounds, at the expense of the polyphenolic acids, which dropped to as much as 74 %. Caffeic, ferulic, and *p*-coumaric acids were still the main polyphenolic acids (**Fig. S2c - SI**), at levels higher than those obtained after 5 day-maceration [38]. The significant flavonoids present were isorhamnetin and pinocembrin. Their levels increased with liquid:solid ratio to a maximum of 298.8 μg/g. Pinocembrin was half of the isorhamnetin at all liquid:solid ratios.

Flavonoids exceed by 1.1 – 1.2 times the polyphenolic acids present in the 50 % ethanolic extracts, with US intensification (Fig. S2d - SI). Pinocembrin, followed by *epi*-catechin, and catechin are the leading flavonoids. Isorhamnetin maintained the level noticed in 25 % ethanol, and quercetin stayed around 6 mg/g. All other flavonoids did not exceed 3 mg/g; chrysin varied around 0.2 mg/g.

US field promoted the extraction of an average of 39.9 mg/g pinocembrin, like the 35.9 mg/g level reported by Woźniak *et al.* [40] for the 70 % ethanolic extract from Polish poplar propolis after 5 day-contact, using 10:1 liquid:solid ratio.

Ellagic acid was the top acid extracted in 50 % ethanol, 38.6 mg/g. Resveratrol and rutin were extracted at approximately 18 mg/g. Caffeic acid did not exceed the values extracted in 25 % ethanol (0.8 mg/g), but its phenyl-ester derivative, CAPE, increased significantly (24 mg/g in 6UEE100). *p*-Coumaric acid raised to approximately 7.2 mg/g, but lost its leading place in the polyphenolic acids group. Ferulic acid dropped below 0.2 mg/g.

Table 1
Process efficiency for different extraction techniques applied to propolis.

Solvent	Extraction yield, %					
	5 days maceration [38]			US 100 min		
	2:1	4:1	6:1	2:1	4:1	6:1
water	0.206	0.306	0.485	0.010	0.076	0.117
25 % ethanol	0.228	0.273	0.416	0.382	0.362	0.487
50 % ethanol	4.25	6.01	8.20	12.38	24.67	23.20

Hydroalcoholic solutions presented variable profiles for individual compounds concentration at different liquid:solid ratios and contact times. After 40 min sonication, increased levels were noticed for larger liquid:solid ratios (4:1 and 6:1), while longer sonication times showed either similar levels or higher ones in the 2:1 liquid:solid samples.

Did the US exposure bring around the expected results in terms of shortening the processing period and boosting the extraction yield compared to maceration? Data in Table 1 demonstrate that even the longest extraction time (100 min) spent in an ultrasonic bath has not brought around the much-sought enhancement of aqueous extractions compared to maceration. The main gain, apart from reducing the operating time, was the presence of around 10 % flavonoids in a solvent which, normally, solubilizes very slowly such structures. The process is intensified, but, still, the exposure time to US field was rather small, therefore not enough compounds were transferred to reach the thermodynamic equilibrium (Table 1, maceration). At the current average extraction rate (0.01 mg/g, 100 min) and if it keeps steady, the US intensified process would reach 0.206 mg/g after around one and half days. As the liquid:solid ratio increases, this time is slowly decreasing. Increasing the ethanol concentration to 25 %, the extraction yields were a little bit higher than those of maceration, because of the ethanol, which can form complex molecular association with the polyphenolic compounds, preventing them to reach the saturation levels. This way, the mass driving force is sufficiently high to ensure high mass fluxes from the solid phase into the liquid phase, responsible for reaching the reported yields after 100 min sonication.

The solution with 50 % ethanol offered the much-sought boost in the UAE compared to maceration (Table 1).

Again, this was a result of the synergistic effects of the US field, which intensify the mass transfer rate, acting upon, on one hand, the overall mass transfer rate and, in the other, damaging the matrix cells' walls, due to the collapse of the cavity bubbles in their vicinity, which form micro-jets towards these walls, and of the increased availability of ethanol molecules, which can arrest much more molecules of polyphenolic compounds in complexes, keeping, for a longer time, high mass driving forces. Both the increase in the overall mass transfer rates and quasi-steady mass transfer driving force permitted much higher mass fluxes from the solid phase into the liquid phase, thus ensuring the levels reported in Table 1.

Since Romanian propolis contains around 250 – 300 mg/g polyphenolic derivatives [41], 50 % ethanol used in the present study allowed the extraction of 45 to 84 % of the bioactive principles available at room temperature, as liquid:solid ratio changed from 2:1 to 6:1. Bankova *et al.* [23] reported a higher percentage of flavonoids in the extracts obtained using UAE, compared to maceration. Even if their statement was based on experiments carried out with higher alcohol containing solvents (60 – 70 %), the increase in flavonoids for UAE, in the present study, is noticeable, as well, for all solvents and extraction conditions used.

It must be mentioned that there was another contribution to getting different polyphenolics profiles for different concentrations of

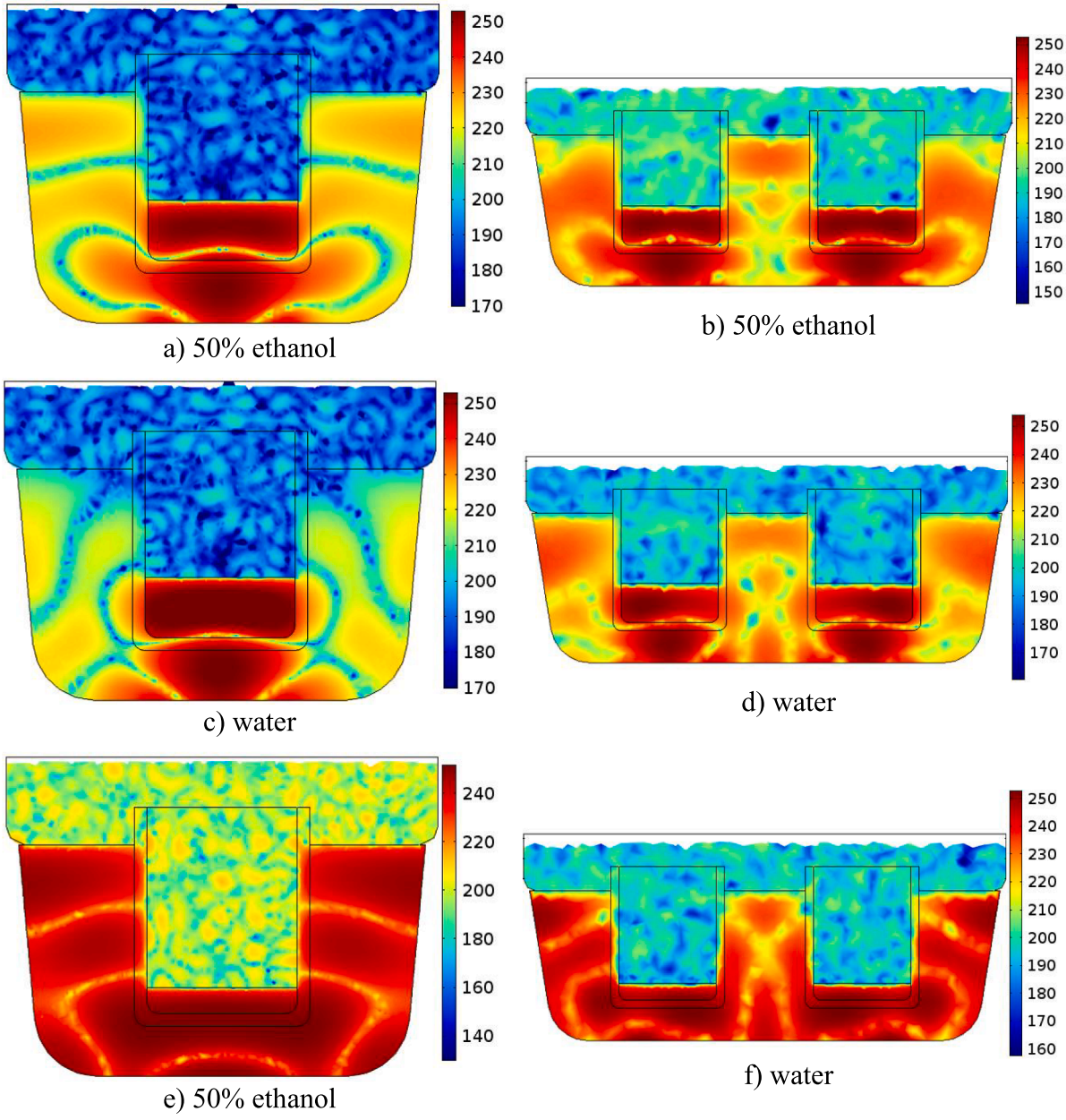

Fig. 2. US field distribution in cross sections YZ, passing through the center of one of the transducers (**a**, **c,** and **e**) and XZ, passing through the center of the US bath (**b**, **d,** and **f**). 50% ethanol (**a** and **b**) and water (**c** and **d**) are in beakers, for the 6:1 ratio case, while 50 % ethanol (**e**) and water (**f**) are in beakers, for the 2:1 ratio case, water being the coupling liquid. The intensity of the US field is given by the sound pressure level (dB, the speed of sound in water being the reference).

hydroalcoholic solution, namely, the US field topology, which was heavily influenced by both the physical properties of the fluid in which the former developed and its level in the vessel. For the same characteristics of the US parameters (amplitude of the transducer and the input power), the knots and venters distribution will be different for different fluids subjected to the US in the same ultrasonic bath, keeping the coupling liquid the same (Fig. 2).

The density (for the energy needed to periodically move the liquid mass), the viscosity (for the internal heat dissipation of the mechanical energy and the cavitation phenomena) and the interfacial tension (for the cavitation phenomena) are the main properties which will dictate the topology of knots and venters, together with the field distribution in between.

Two fluids were used to illustrate this, 50 % ethanol (density 900 kg/m^3, viscosity 1.05 mPa·s, interfacial tension 21.8 mN/m, Fig. 2**a, b,** and **e**) and water (density 1000 kg/m^3, viscosity 1 mPa·s, interfacial tension 72 mN/m, Fig. 2**c, d,** and **f**), while water was chosen as coupling liquid in the ultrasonic bath. The US field distribution was computed for the aforementioned intensity and frequency, the beakers being filled with the amounts corresponding to the highest ratio, 6:1, and to the lowest one, 2:1. The computations were done using Acoustic physics from COMSOL Multiphysics® 5.2a (the chosen fluid model was linear elastic), the ultrasonic bath geometry being implemented using COMSOL geometry primitives. The plane YZ passes through the center of one of the two transducers, while the plane XZ crosses the center of both transducers. Air is placed above the liquid phase. Physical properties of the extractants and air were computed using COMSOL's built-in properties from the provided materials library.

Fig. 2**a-d** clearly shows that the computed distribution field is completely different for the two fluids present in the beaker, which must

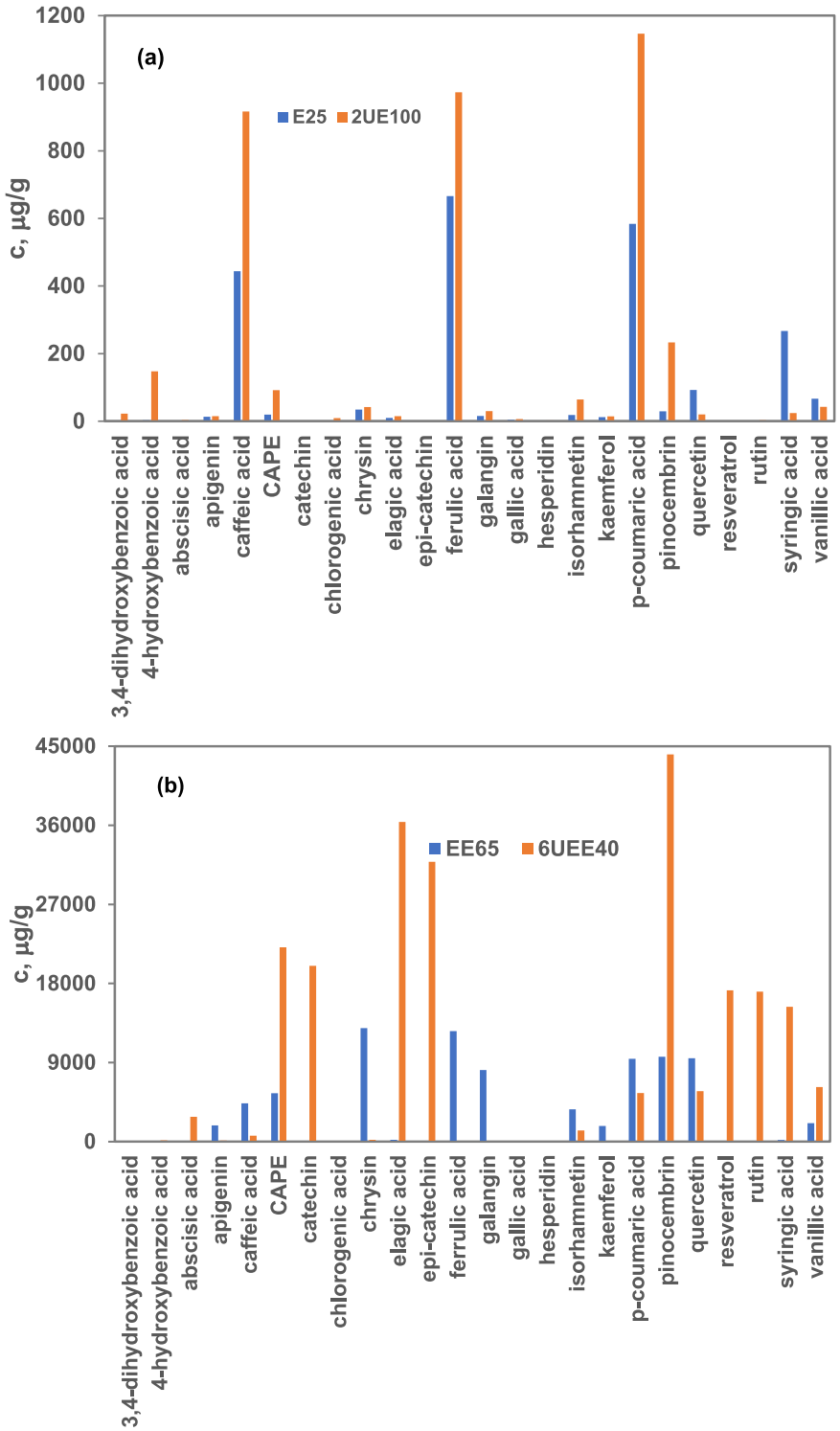

Fig. 3. Polyphenolics profile extracts variation with ethanol level and liquid:solid ratio for: **(a)** 25 % ethanol, 2:1 liquid:solid ratio, 5 day-maceration (E25) and 100 min in ultrasonic bath (2UE100); **(b)** 50 % ethanol extracts, 6:1 liquid:solid ratio 5 day-maceration (EE65) and 40 min in ultrasonic bath (6UEE40).

reflect in the interactions between the US field and the solid phase – therefore, when explaining the differences between the performance of different solvents subjected to an US field, keeping the operating conditions the same, the field distribution should be accounted for, as a hidden, but powerful cause. Unfortunately, the US field topology will change, also, when the level of the extractants in the beaker (or any other kind of vessels) changes, even if the height above the transducer is kept the same (Fig. 2**e** and **f**, against Fig. 2**a** and **d**). This supplemental change makes even more difficult to predict the relationship between the extraction performance and the US field topology.

The comparison of each polyphenolic compound extraction in maceration and UAE showed variable behavior, depending also on the ethanolic content in the extractant. UAE in 25 % ethanol leads, after 100 min, to larger amounts of compounds than the 5 day-maceration, the most important contributions coming from caffeic, ferulic, and *p*-coumaric acids, isorhamnetin, and pinocembrin (Fig. 3**a**). The largest

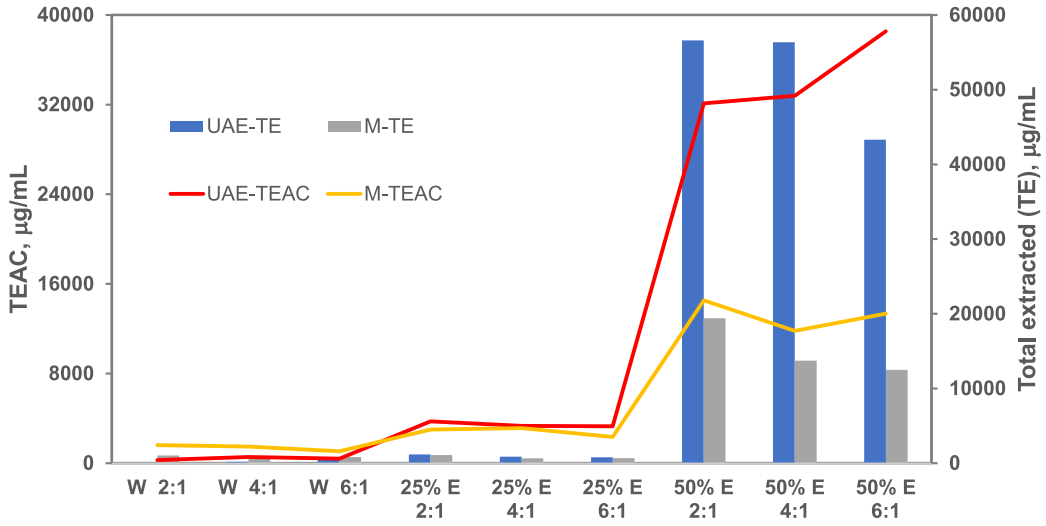

Fig. 4. Variation of antioxidant capacity and total extracted compounds with the experimental conditions in UAE for 100 min and 5−day maceration at similar liquid:solid ratios (2:1, 4:1, 6:1, w:w) in water (W), 25 % and 50 % ethanol as solvents. Maceration data were previously reported [38].

enhancement in the total extracted compounds was seen for the 2:1 liquid:solid ratio in US. Maceration went poorly for 2:1 liquid:solid, but US increased by 68 % the total extracted amount (2.278 to 3.822 mg/g), for the same ratio and the longest US exposure. The considerable US enhancement for the 50 % ethanol extractant came with a change in the extracted compounds profile. Resveratrol, rutin, and syringic acid were present in similar levels (15 mg/g), accompanied by vanillic acid, 6 mg/g (Fig. 3**b**). While absent in maceration, catechin and *epi*-catechin now exceed 32 mg/g. Resveratrol level was 3 orders of magnitude higher than the values reported by Duca *et al.* [42]. Unexpectedly, ferulic acid, isorhamnetin, kaempferol, and chrysin were less extracted when US field was applied. This might be due to the different US field influence with the increase of ethanol concentration.

Oroian *et al.* [22] found *p*-coumaric acid as the most abundant polyphenol in the Suceava county propolis (220 mg/g), but rather small levels of pinocembrin (13 mg/g). This difference in behavior might be related to the operating conditions applied: 70 % ethanol, 50:1 liquid:solid ratio, 20 kHz US probe horn for 2 × 15 min, while the temperature increased to 60°C. Still, the flavonoids to polyphenolic acids ratio was 1.16, like that obtained for the 50 % ethanolic extracts in the present study. The four factors, three levels Box-Behnken design study conducted by the same group [43] using a 25 kHz, 100 W ultrasonic bath identified the optimal conditions for the highest efficiency: 70 % ethanol, 58°C, and 30 min sonication time. The optimum extract polyphenolics profile missed, quite surprisingly, *p*-coumaric acid and had high levels of kaempferol (228 mg/g).

Pobiega *et al.* [13] reported a 11.86 % yield for Polish samples processed with 70 % ethanol (using 10:1 liquid:solid ratio, after 30 min sonication at 210 W, 20 kHz horn). They also concluded that solvent:propolis ratio did not affect the process efficacy, contrary to the present results. This could be the result of the different way of generating the US field, with a horn, which has a limited cone-like penetration depth, instead of a transducer. Thus, the longer sonication duration and the different ultrasonic provider (100 min, ultrasonic bath, 110 W, 40 kHz) used in the present study might explain the larger yields obtained.

Ramanauskiene *et al.* [44] carried out UAE using 2.5 – 10 % aqueous solutions of propolis and concluded that the polyphenolic content increased in time, the best level, 68 mg/mL, being recorded after 30 min. *p*-Coumaric acid represented approximately 35 % of polyphenols in the extracts analyzed, like the 31 % value found in the present study.

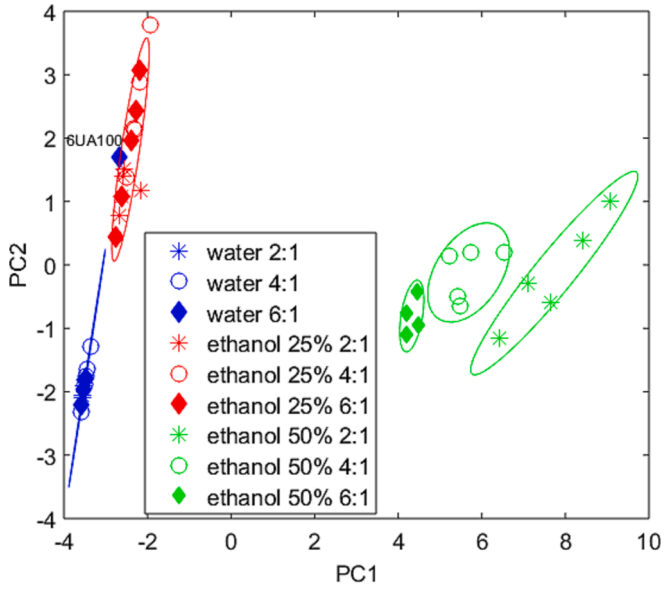

Fig. 5. Projections in PC1-PC2 coordinates of samples obtained in UAE.

3.2. Antioxidant capacity

The average antioxidant capacity of the extracts varied from 304 to 2928, and finally 40752 µg TEAC/mL, as ethanol percentage in the solvent increased. It followed the trend of total extracted compounds, as in the maceration research [38]. Antioxidant capacity of aqueous US extracts represented approximately 30 % of the values obtained after 5-day maceration at any liquid:solid ratio. 25 % ethanolic extracts presented rather similar antioxidant capacities, either by maceration or by 100 min US field exposure (Fig. 4). The extraction time reduction was the major plus. The three-times increase in the extracted antioxidants was closely followed by the TEAC values of the 50 % ethanolic extracts. Rather unexpectedly, extract dilution was accompanied by an increase in the antioxidant capacity, drawing attention to possible changes in the distribution profile of those 24 polyphenolics quantified (**Fig. S3 – SI**).

The average antioxidant capacity of 997 µmol TEAC/g propolis value of 50 % ethanol extracts in US is comparable to the 1219 µmol TEAC/g propolis reported by Cavalaro *et al.* [4] for a 20:1 (v:w) extract of green Brazilian propolis subjected to sonication with 49.5 % ethanol. Since the 35:1 (v:w) liquid:solid ratio led to an extract having 2417 µmol TEAC/g propolis, it is reasonable to explain these punctual differences by the

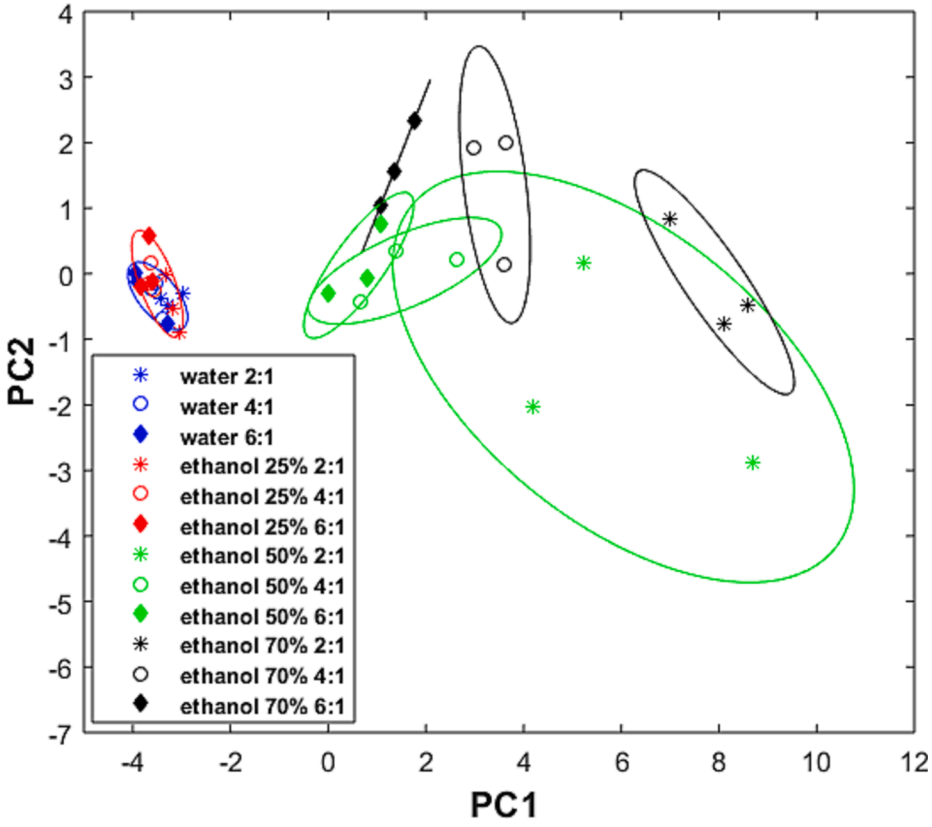

Fig. 6. Projections in PC1-PC2 coordinates of samples obtained in maceration (based on data obtained in the previous maceration study [38]).

operation conditions (more liquid phase, 28 W/L ultrasonic bath), source of propolis and US field topology. Ding *et al.* [45] determined a range of variation from 36.8 to 106.7 μmol TEAC/g of extract, different from the present study, 2.2 – 1030 μmol TEAC/g propolis. The difference is not unexpected considering the source of propolis (Chinese against Romanian), solvent (70 % ethanol against 0, 25 % and 50 % ethanol), and US bath characteristics (220 W, 40 kHz, 30 min against 110 W, 40 kHz, 10 – 100 min). Hegazi *et al.* [14] and lately Duca *et al.* [42] have long concluded that the antioxidant capacity of European propolis is very much determined by the flavonoids present, in good agreement with the present study.

3.3. Data processing

PCA analysis results (for centered and normalized data) proved that the first three PCs account for more than 90 % of samples variability

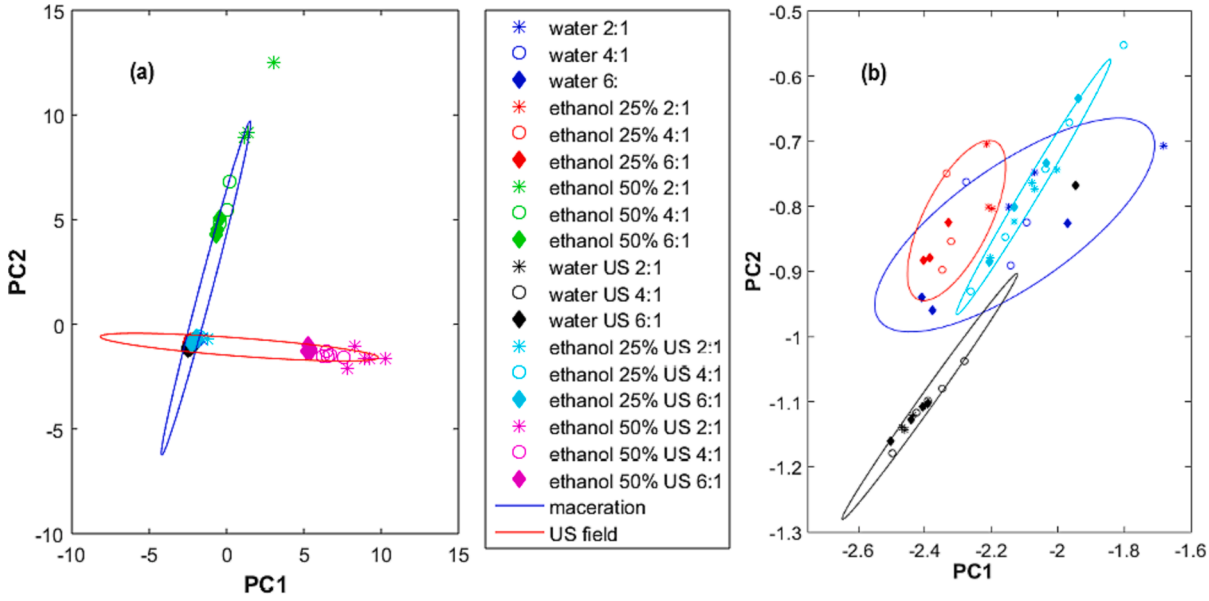

Fig. 7. Projections in PC1-PC2 coordinates of samples obtained using both maceration [38] and US extraction results: (a) grouping along maceration vs US field; (b) close-up for the projections in PC1-PC2 coordinates of water and 25 % ethanol samples.

Table 2
Components with significant contribution to the antioxidant capacity.

Water extract	25 % ethanolic extract	50 % ethanolic extract
ferulic acid, caffeic acid, *p*-coumaric acid, isorhamnetin, pinocembrin	caffeic acid, *p*-coumaric acid, ferulic acid, vanillic acid, isorhamnetin	*p*-coumaric acid, ferulic acid, abscisic acid, vanillic acid, catechin, *epi*-catechin, isorhamnetin, syringic acid, resveratrol, rutin

(78.9 % PC1, 11.7 % PC2, 3.3 % PC3). The UAE samples representation in PC1-PC2 coordinates revealed a clear grouping tendency according to the solvent used (Fig. 5). Samples did not differentiate by liquid:solid ratio and UAE time for water extracts. Separation is slightly higher for 25 % ethanolic extractant, but, still, all samples part the same ellipsis. Surprisingly, the water extract obtained at 6:1 liquid:solid ratio and US maximum time (6UA100) overlaps with the 25 % ethanol samples, mainly with 2:1 liquid:solid ratio (2UE100), suggesting a similar polyphenolics profile. This might stamp 6UA100 sample as outlier.

The polyphenolics profiles for 50 % ethanolic extracts differentiate according to the liquid:solid ratios, because of the US field influencing the liquid:solid interface processes, generating reactive species during the collapse of cavity bubbles; these can chemically react with polyphenolic compounds, thus affecting their composition. Also, the liquid:solid ratio changes the extractant height in the beaker which, by itself, induces changes in the US field (Fig. 2), synergistically with the higher ethanol concentration. The Yildirim studies [46], focused on polyphenolics extraction from propolis by maceration, US, and microwaves with variable ethanol concentrations, emphasized samples grouping according to solvent nature, but not based on operating conditions. PCA study of Oroian *et al.* [43] reported sample grouping by harvest year and, for some cases, by extraction time.

As for the contribution of each polyphenolic compound in the PCs, **Fig. S4 – SI** proves that there are components with high loadings in PC1 or in both PC1and PC2. Ferulic, *p*-coumaric, vanillic, syringic, and 3,4-dihydroxybenzoic acids and most of the flavonoids (catechin, *epi*-catechin, kaempferol, pinocembrin, resveratrol, apigenin) are representative for data variability. These results agree well with Duca *et al.* [42], where polyphenolic compounds were extracted by maceration at 20:1 liquid:solid ratio in 60 % ethanol.

PCA method was applied to the maceration obtained data [38] as well, leading to quite similar contributions (PC1 79.4 %, PC2 4.7 %, PC3 4.4 %) as for UAE. The aqueous and 25 % ethanolic samples are practically overlapping, which was not the case for UAE samples. Still, they are clearly differentiated from the 50 % and 70 % ethanol extracts (Fig. 6). An incipient sample grouping starts to discern, according to liquid:solid ratios, for 50 % ethanol, despite some overlapping. A completely different situation is the 70 % ethanol case, where a clear differentiation for the three ratios appears (Fig. 6), like for 50 % ethanol, in the UAE (Fig. 5). This might arise from the sonication side effects, given by the chemical interactions between the reactive species formed by cavitation and the polyphenolics, the former being differentiated according to the liquid:solid ratio, with consequences upon the profiles.

PCA applied for both maceration and UAE samples (PC1 59.2 %, PC2 33.2 %) lead to a clear separation of 50 % ethanolic samples obtained in US field and maceration (Fig. 7**a).** Water and 25 % ethanol extracts practically overlap (Fig. 7**b**), proving that US field did not essentially change the polyphenolics profile. Finally, PCA demonstrates that the solvent nature is the major factor differentiating the polyphenolic profile, while the US field and liquid:solid ratio play an important role for 50 % ethanol, where polyphenolic derivatives with complex structure (mainly flavonoids) are extracted.

PLS models (**Fig. S6 – SI**) demonstrated a good correlation between the 24 polyphenolic compounds profiles and the corresponding antioxidant capacity, given the high determination coefficient values for all cases, $R^2 > 0.96$. The VIP based selection of polyphenolic compounds

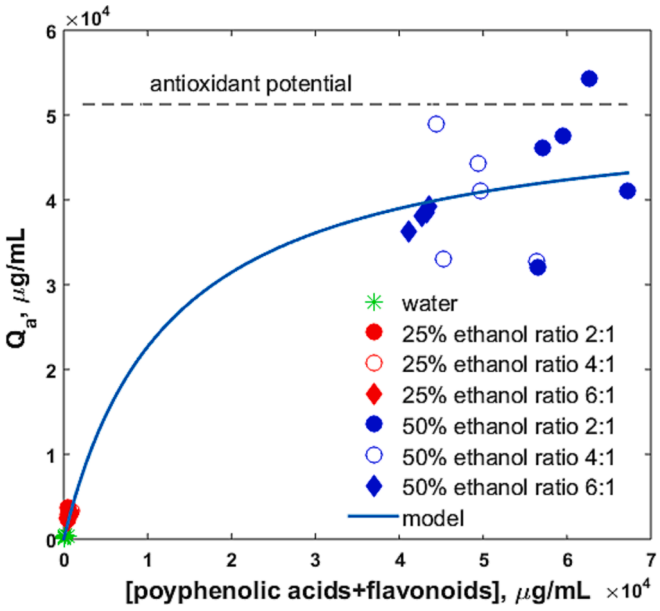

Fig. 8. Antioxidant capacity variation with total polyphenolics concentration.

with the largest contribution in the correlation proved that the solvent nature has a high influence (Table 2 and **Fig. S7 – SI**).

The PLS analysis showed that in sonicated water, the phenolic acids, which are mainly extracted, contribute to the build-up of antioxidant capacity. As pinocembrin and isorhamnetin are also present in comparable levels to the phenolic acids, they have also a high VIP score in the regression model. The high contribution components to the antioxidant capacity of 25 % ethanol extracts are, practically, the same as in water, in line with the extracted compounds profile. In more concentrated ethanolic solutions, the polyphenolics profile is complex, and both phenolic acids and flavonoids are important for the antioxidant capacity.

The parameters of a saturation model (1) as previously proposed [38] were determined by non-linear regression, to capture the influence of total concentration of polyphenolic components upon the capacity of the antioxidant activity:

$$Q_a = \frac{K_{\max} \cdot c_p}{K_c + c_p} \quad (1)$$

Q_a is the antioxidant capacity (μg TEAC/mL) and c_p is the polyphenolic compounds concentration (μg/mL). The model parameters are K_{max} (μg TEAC/mL), which can be interpreted as the extract antioxidant potential at theoretically very high polyphenolic concentration ($c_p \to \infty$), and K_c (μg/mL), standing for the critical concentration (c_p for which Q_a is half K_{max}). The model parameters were identified minimizing the objective function (2), n being the number of samples considered:

$$F = \sum_{i=1}^{n} \left(Q_{a,\text{exp}} - Q_{a,\text{model}}\right)^2 \quad (2)$$

The built-in function *ga* encoding the genetic algorithms in Matlab® was used to minimize the objective function (2). The model fits well the experimental data (Fig. 8), reflected in the value of the determination coefficient, $R^2 = 0.96$, for $K_{max} = 50761$ μg TEAC/mL extract, and $K_c = 11880$ μg polyphenolic derivatives/mL extract.

The maximum antioxidant capacity predicted by the model is quite close to the experimental data obtained for 50 % ethanolic extracts. Generally, data fit well the proposed model, but there is an unexpected pattern, better noticed for 50 % ethanol samples, at 2:1 and 4:1 liquid:solid ratios (Fig. 8): there are samples with higher polyphenolics content having similar or lower antioxidant capacity. At 6:1 liquid:solid ratio the data follow the general rule of increasing antioxidant capacity with

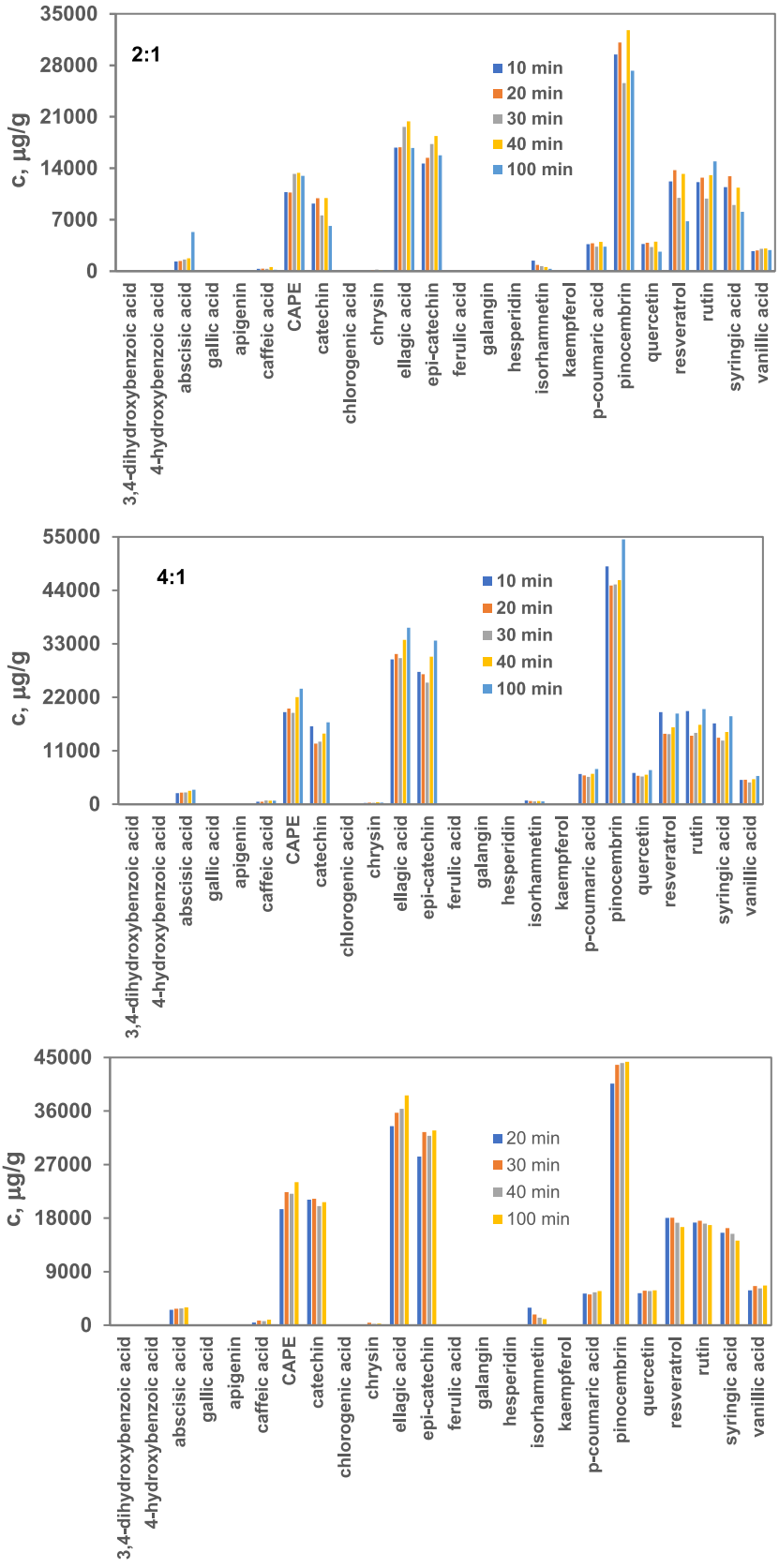

Fig. 9. Polyphenolic derivatives profiles for 2:1, 4:1, and 6:1 liquid:solid ratio in 50 % ethanol.

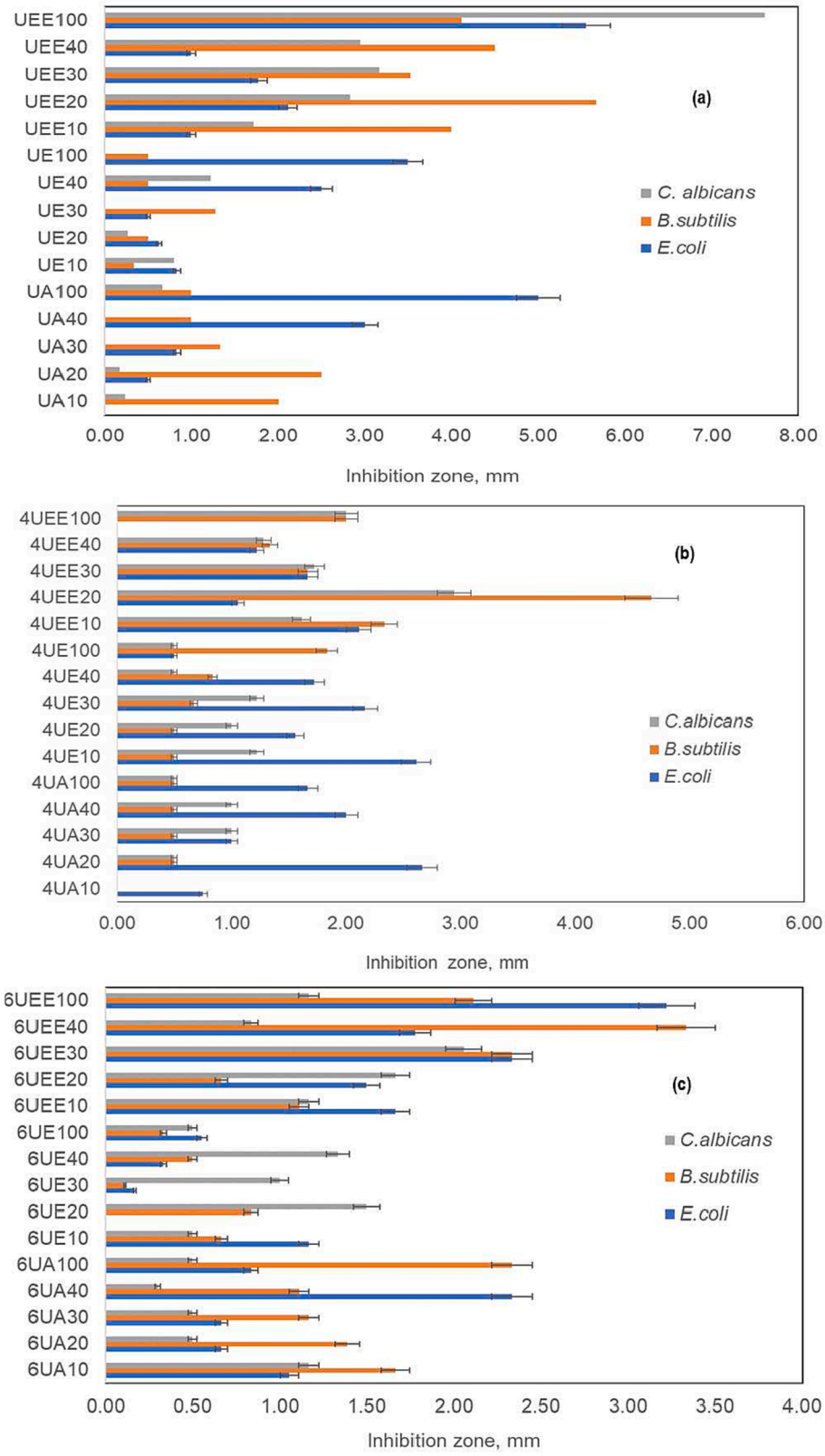

Fig. 10. The antimicrobial activity recorded after 24 h in UAE extracts obtained at different liquid:solid ratios: (a) 2:1, (b) 4:1 and (c) 6:1.

increased polyphenolics concentration. A possible explanation starts from the liquid–solid interface cavitations, generating free radicals, especially $HO^{-\bullet}$; their concentration is maximum at 2:1 ratio, because the liquid volume is minimum. Free radicals interact with extracted compounds, generating the experimentally noticed profile differences. The analysis of the experimental concentration profile supports this hypothesis (Fig. 9). The 2:1 and 4:1 liquid:solid ratios profiles vary with the US field duration, showing that the concentration of certain polyphenolics is not steadily increasing, possibly due to chemical reactions caused by free radicals. The 6:1 liquid:solid ratio concentrations profile

in Fig. 9 is almost constant in time.

4. Antimicrobial activity

The antimicrobial activity of propolis extracts in different solvents and at different exposure times to US is presented in Fig. 10**a-c**, by the measured size of the cell growth inhibition zone. The measurements accuracy depends upon the concentration profile of antimicrobial substances diffusing into the culture medium and determining the microbial growth inhibition. The measured inhibition zone appears as a hallow or clear circular zone around the disk impregnated in the propolis extract sample analyzed in triplicate.

The antibacterial activity against *E. coli* increased with the liquid: solid ratio, irrespective of the sonication time for aqueous extracts. The US exposure time influence upon the antibacterial activity against *E. coli* increased for 2:1 ratio, while for 4:1 and 6:1, higher value for inhibition zones were recorded for 10 and 40 min, respectively. These results may be a combination of both the cavitation phenomena, cumulating their effects in time, and the change in the US field topology, modifying the distribution of the cavitation bubbles, due to the change in the height of the solid–liquid mixture (Fig. 2), as the ratio increased (the beaker depth in the coupling liquid was, always, the same). According to the results, aqueous extract 2UA100 had the maximum inhibitory effect upon Gram-negative bacteria *E. coli*. Aqueous extracts had rather weak antimicrobial activity, confirmed by the results reported by Campos *et al.* [9] and Biria *et al.* [10]. They contain mainly phenolic compounds and a lower percentage of flavonoids, but when the latter are increased, a higher inhibitory effect upon Gram-negative bacteria is emphasized [47].

Gram-positive bacteria (*B. subtilis*) were most inhibited by the 2:1 liquid:solid ratio and moderate exposure time to US extract (2UA20). The second-best inhibitory effects had 6UA100 extract, thus showing that the change in the US field topology was beneficial for the longest extraction time. Quite interesting, the US exposure time had no effect upon the inhibitory capacity of 4:1 liquid:solid ratio extracts, which showed the same low value. Again, this could be the consequence of the US field topology change, induced by the liquid–solid mixture height. To increase the inhibition of Gram-positive and fungal bacteria, the extracts should have higher flavonoids concentrations, with profiles adapted to the specific characteristics of the cell wall morphology [48].

Maximum inhibition for unicellular fungi (*C. albicans*) was recorded for 6UA10 extract. Further increase of the US exposure time had deleterious effects upon the concentration of the compounds responsible for the *C. albicans* inhibition. In the case of 4:1 liquid:solid ratio, the highest inhibitory effects manifested after 30- and 40-min US exposure time, although they are a little lower than for the previous ratio. The 2:1 ratio extracts manifested the lowest inhibitory effects, on average, irrespective of the US exposure time.

Most antimicrobial activity studies showed that the presence of ethanol in various concentrations in a solvent stimulates the extraction of polar and non-polar compounds, and, also, the synergistic effect of the ethanol presence and the US exposure, stimulating the antimicrobial activity [44].

The antimicrobial activity for propolis extracts with 25 % ethanol is moderate. The most efficient extract for *E. coli* is 4UE10. *B. subtilis* is affected by extracts subjected to medium to long US exposure times for low liquid:solid ratios, while short US exposure times are needed for high extraction ratios to achieve inhibitory properties. Most efficient extract for *B. subtilis* is 4UE100 sample. Yeast (*Candida*) inhibition manifested for all extracts, due to presence of *p*-coumaric acid, regardless the extractive conditions, but most efficient extract is 6UE20, having the maximum inhibition zone.

Propolis extracts in 50 % ethanol in the presence of US are the most effective in terms of antimicrobial activity, due to the high concentration levels of pinocembrin, *epi*-catechin, rutin, resveratrol, and syringic acid. *E. coli* is susceptible to polyphenolics extracted during long US exposure times, regardless the liquid:solid extraction ratio. Most efficient extract inhibiting *E. coli* is 2UEE100, while 2UEE20 is the most efficient for *B. subtilis*. Yeasts are susceptible to compounds extracted in solutions with low liquid:solid ratios, and medium US exposure time. The maximum inhibition was provided by 2UEE30 extract against *C. albicans*.

In US aqueous extracts, the antimicrobial activity is due to *p*-coumaric, caffeic, and ferulic acids, higher than in maceration. The concentration of all compounds with antimicrobial activity increased in the 25 % ethanol extracts, compared to maceration, therefore their antimicrobial activity was higher. The 50 % ethanolic extracts are richest in substances with antimicrobial activity, which can act synergistically – the changes in their distribution increased, significantly, the antimicrobial activity.

For prokaryotes, small liquid:solid ratios are necessary for good inhibitory effects, regardless of the solvent nature, while for eukaryotes, the liquid:solid ratio should be maximum (6:1) for aqueous or weak alcoholic extracts, and 2:1 for richer ethanol extracts. The exposure times required to obtain extracts with good inhibitory effects are antagonistic for Gram-positive and Gram-negative bacteria, taking values at the extremities of the studied time interval, while for yeast, the exposure times should be low to moderate.

5. Conclusions

The 50 % ethanolic extract subjected to US offered the boost in both quantity and polyphenolics derivatives profile, compared to maceration, which significantly increased the extraction yield. Statistical data analysis demonstrated that the solvent nature is the most important operating parameter that determined the polyphenolic compounds profile change, while the US field and liquid:solid ratios played an important role. The results demonstrated that 50 % ethanol becomes profitable if associated with the US field, rendering unnecessary the usage of higher ethanol concentration extractants. So, the ultrasonic field effects upon the polyphenolics profile of propolis extracts really improve their antioxidant and antimicrobial activity.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2022.106274.

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