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Exploring the potential of microwaves and ultrasounds in the green extraction of bioactive compounds from *Humulus lupulus* for the food and pharmaceutical industry

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ABSTRACT

Currently, there is a continuous growth in demand for medicinal and aromatic plant derived molecules obtained in a sustainable and eco-friendly way. Among the medicinal plants that have a plethora of health-related properties, there is definitely hops. In the present study, microwaves (MW) and ultrasounds (US) were used to increase the extraction efficiency of bioactive compounds from hop cones using water, ethanol and their mixture as food-grade solvents. A multidisciplinary approach was used for the in-depth characterization of green extracts. The bioactive components were determined by chemical methods, using both spectrophotometric assays as well as high-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis of individual phenols, bitter acids and xanthohumol, and results were compared with those from a control extract, obtained using aqueous methanol (80 %) acidified with HCl (0.1 %). Moreover, we applied proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy to reveal insights on the different composition in terms of primary and secondary metabolites of the green extracts analyzed. Generally, microwave-assisted extraction (MAE) of hop cones with ethanol showed the strongest extraction power for almost all the bioactive compounds investigated, as well as the shortest extraction time (1 min) and the highest antiradical capacity. ^{1}H-NMR analyses showed an overall greater influence of solvent polarity, rather than extraction technique, on the average composition of the final extract. ^{1}H-NMR fingerprinting of hop aqueous extracts highlighted as main components sugars and amino acids, as well as other metabolites including trigonelline, an alkaloid with interesting biological properties. However, the two extraction techniques significantly affected the contribution of signals in the terpene or aromatic regions when ethanol and ethanol:water were used as solvents, proving a better extraction efficiency for these compounds of MW than US. Regardless the extraction technique and solvent used, the most abundant polyphenol compound in the green extracts analyzed was catechin, ranging from 2989 ± 10 to 14009 ± 248 μg g^{-1} (for UAE_{ETOH} and MAE_{ETOH}, respectively), followed by rutin and chlorogenic acid. 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) radical scavenging methods were used to evaluate the biological properties of green hop extracts, showing a superior free-radical scavenging capacity in MW irradiated ethanol samples compared to other samples tested. The interrelationships between the parameters analyzed and the different extraction conditions, as well as the relationships among variables, were investigated by principal component analysis.

1. Introduction

Hop cones, the inflorescences of the female plant of *Humulus lupulus* L., have an important role as flavoring and bittering agent in the brewing industry, which utilizes about the 98 % of the world hop's production (Moir, 2000). Currently, 91,881 ha of hops are grown in the world, for a total production of 148,603 tonnes (FAOSTAT, 2020). United States is the top world producer, followed by Ethiopia and China. Europe, with its leading countries (Germany, Czech Republic and Poland), covers the 34.3 % of the world hop's production (FAOSTAT, 2020). Although known to most as a key ingredient in beer, hop has been used since ancient times as a medicinal plant in the treatment of mental illnesses

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such as anxiety, restlessness, sleep disorders and insomnia (Wang et al., 2014).

For these purposes, hop extracts were combined with valerian one (*Valeriana officinalis* L). Until recently, this preparation represented the most frequently administered form of plant-based sleeping agents and sedatives (European Medicines Agency, 2020). The phytotherapeutic properties of hop are due to its secondary metabolites, which have been reported to exert a plethora of health-related beneficial effects (Olsovska et al., 2016). Among pharmaceutically important compounds from hops are polyphenols, having proven anticarcinogenic, antioxidant, antimicrobial, anti-inflammatory and estrogenic effects (Gerhäuser, 2005; Bocquet et al., 2018). Previous studies highlighted the nutraceutical potential of hop polyphenols, which has been reported to be similar to those of tea and grapes (Wang et al., 2014).

Among polyphenols, hop cones are characterized by the presence of prenylated phenolic compounds, mainly acylphloroglucinols (bitter acids), which are potential sources of treatment or prevention of many diseases, including cancer, diabetes, osteoporosis, cardiovascular diseases, inflammatory and metabolic disorders. In addition, the antimicrobial effects of β-acids against different microorganisms are significant (Van Cleemput et al., 2009). Xanthohumol, a prenylated chalcone, is one of the major prenylflavonoids present in hops, which has gained a lot of interest in recent years due to its high biological activity (Magalhães et al., 2010; Venturelli et al., 2016). Recently, several authors highlighted neuroprotective effects linked to this valuable compound (Rancán et al., 2017; Jiao et al., 2020; Wang et al., 2020), as well as a high anticancerogenic potential, both alone and in combination with other bioactive compounds (Gieroba et al., 2020; Krajka-Kuźniak et al., 2020).

Currently, for both brewing and herbal applications, hop extracts are increasingly used instead of whole flowers to preserve the most valuable compounds from oxidation and to allow a better dosage. However, the development of an efficient and sustainable extraction technology that guarantees the quality of the herbal preparation is mandatory (Chemat et al., 2012; Heng et al., 2013). The most widely used extraction system for the preparation of hop extracts is the one based on the use of supercritical carbon dioxide (Rój et al., 2015). Supercritical fluid extraction (SFE) is recognized as an eco-friendly process, requiring low energy input (Chemat et al., 2012). Unfortunately, SFE-based methods have some shortcomings such as the high establishment cost, the need for highly specialized technical personnel, no scalability and the selective solvent nature of CO_2, which is not suitable for the extraction of polar phenolics without the use of co-solvents (Ameer et al., 2017; Khaw et al., 2017).

Recently, other non-conventional and green extraction techniques, including UAE and MAE, have been developed to recover bioactive compounds from plants in order to obtain acceptable results in terms of both yields and environmental sustainability of the applied process (Ameer et al., 2017). In particular, MAE) provides fast extractions (few min) without the degradation of thermolabile compounds, with considerable savings in time and energy (Pérez-Serradilla and De Castro, 2011). This technique is quite adaptable on a small or large volume, meaning technology transfer from laboratory to industry scale can be done easily (Gallo et al., 2010). MAE also produces high extraction rates and good results with low costs (Gallo et al., 2010). The benefit of using US as an inexpensive green technique in plant extraction has already been demonstrated for a number of compounds of interest to both the pharmacology and food industries (Vilkhu et al., 2008; Vinatoru et al., 2017). Ultrasound-assisted extraction has a number of noticeable features like eco-friendliness, cost-effectiveness, simplicity and versatility (Ameer et al., 2017). However, the effectiveness of both extraction techniques (UAE and MAE) are found to be dependent on the solvent employed. The choice of the organic solvent used in plant extraction is generally quite restricted and, normally, solvents are used with boiling points not exceeding 70–75 °C, as if above this temperature the degradation or modification (i.e. through possible covalent interactions with other constituents of the plant matrix) of polyphenols may occur (Shi et al., 2002). The major solvents used for the extraction of phytochemicals include methanol and ethanol (Nour et al., 2014), while for the extraction of specific compounds (i.e. anthocyanins), methanol with HCl is one of the most common solvent mixture (Giusti et al., 2005). However, for food grade applications, ethanol is preferred.

At present, there are only few literature data about the effect of extraction conditions on the polyphenol content and antioxidative activities of hops and its products (Sanz et al., 2019). Moreover, to the best of our knowledge, there is a lack of literature data on the extraction of primary and secondary metabolites from hop by using MW and US. Starting from these considerations, the present research aimed to evaluate the potential of these green techniques for the simultaneous recovery of the main phytochemical compounds in hops and to investigate the influence of different green solvents on it. An in-depth characterization of the obtained green extracts was then carried out through a multidisciplinary analytical approach. Hop extracts were characterized by spectrophotometric and chromatographic analyses. In order to fully characterize the green extracts and the influence of both solvent and extraction technique on their phytochemical composition, ^{1}H-NMR metabolite profiling of hop extracts was carried out. Finally, data were applied to the principal component analysis (PCA) to gain an overview of the effects of the solvent in extraction method.

2. Materials and methods

2.1. Chemicals

All used reagents were of analytical spectrophotometric grade. The ethanol used for the US and MW extraction tests was of analytical grade (Sigma–Aldrich; Milan, Italy). The methanol used for the control extraction tests was of analytical grade (VWR; Milan, Italy). Folin Ciocalteau reagent, sodium carbonate, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,20-azinobis [3-ethylbenzothiazoline-6-sulfonate] (ABTS) and potassium persulfate were purchased from Sigma–Aldrich (Milan, Italy). Bitter acids mixture standard (International calibration extract, ICE-3) was from Labor Veritas Co. (Zürich, Switzerland). ICE-3 was reported to contain 13.88 % cohumulone and 30.76 % of *n*-humulone + adhumulone (α-acids), and 13.44 % of colupulone and 10.84 % of *n*-lupulone + adlupulone (β-acids). Phenolic standards used for identification and quantification purposes with high-performance liquid chromatography (HPLC) were purchased from Extrasynthese (Genay, France). Organic solvents used for chromatography were of HPLC ultragradient grade (Sigma-Aldrich, Milan, Italy), while the water employed was previously purified in a Milli-Q system (Millipore, Milan, Italy). Polytetrafluoroethylene (PTFE) membrane filters (0.45-μm pore size) from Pall (Pall Corporation, MI, USA) were used for filtration of both mobile phases and samples.

2.2. Plant material

Wild seedless hop cones were obtained in dried form (containing about 8 % by weight moisture) from random samples of the same accession present in the germoplasm collection field of the Lucanian agency for the development and innovation in agriculture (ALSIA; Potenza, Italy). Prior to be extracted, hop cones were grinded with a laboratory mill to a fine powder (sieve 0.5 mm) and kept protected from light and humidity until analysis.

2.3. Extraction processes

All extractions of dried hop cones were carried out using a solid: liquid ratio of 1:15 (w/v), previously optimized (data not shown), with the following methods:

2.3.1. Ultrasound-assisted extraction

Ultrasound-assisted extraction was performed in a temperature-controlled sonication bath (UTA-200, Falc, Italy), operating at 40 kHz. The operating conditions were chosen on the basis of our previous studies (data not shown). Hop samples (1 g) were first mixed with different solvents (ethanol, water, ethanol:water = 50:50 v/v) in test tubes with screw caps on a magnetic shaker (300 rpm; Heidolph Mr. 2002, Kelheim, Germany) in the dark and at room temperature (25 °C). Then, the mixture underwent to an UAE for 30 min at 25 °C under US irradiation. The resulting extracts were then centrifuged at 6792 *g*, for 15 min at 4 °C. Pellets were extracted once again in the same manner. Then, the supernatants were collected together and immediately analyzed.

2.3.2. Microwave-assisted extraction

Microwave-assisted extraction was performed in a closed vessel. The extraction was carried out in a CEM MARS 5 professional multimode oven operating at 2.45 GHz (CEM, Matthews, NC, USA). The extraction reactor cells were made by a closed pressure-resisting system that contains an inner vessel of pyrex ("H4500") with a volume of 100 ml and allows maximum pressure and temperature of $2.4*10^6$ Pa and 210 °C. The inner temperature of the sample vessel was measured and controlled with a MW-inert optic fiber temperature probe, while the pressure inside the MW system was measured by a gauge probe. Based on preliminary trials (data not shown), hop samples (1 g) were extracted with investigated solvents at 75 °C under MW irradiation (400 W). In details, the protocol was set for a ramp from room temperature to 75 °C in 5 min. The temperature probe was set to maintain the vessel temperature at 75 °C for another 1 min. During this time, the MW power will be injected intermittently to ensure the vessel remains at 75 °C. Then, extracted samples were immediately cooled down to room temperature, centrifuged at 6792 g, for 15 min at 4 °C and the supernatants collected and immediately analyzed.

2.3.3. Reference extraction process

Taking into consideration previous studies of the authors, regarding the biological activity and composition of plant extracts (data not shown), hydroalcoholic extracts (methanol/water = 80:20, v/v; where not differently specified, acidified with 0.1 % HCl (v/v)) were obtained from 1 g of dried hop cones by dynamic maceration (30 min at 25 °C, 300 rpm and in the dark) followed by ultrasonication (for 30 min at 25 °C) in an US bath, operating at 40 kHz. The resulting extracts (control samples, CTRL) were then centrifuged at 6792 *g*, for 15 min at 4 °C. Pellets were extracted once again in the same manner. Then, the supernatants were collected together and immediately analyzed.

Obtained crude extracts were analyzed without further purification. All extractions were performed in triplicate.

2.4. Determination of total polyphenols (TPs) and bitter acids in hop extracts

The content of total polyphenols (TPs) in the extracts was determined using the Folin–Ciocalteau assay (Carbone et al., 2011). The calibration curve was made with standard solutions of gallic acid in the range 0–100 ppm and measures were carried out at 765 nm using a UV–vis spectrophotometer (model 6300 PC, VWR, Milan, Italy). All analyses were performed in triplicate. TP yield was expressed as milligrams of gallic acid equivalents (GAE) per gram of dried sample

The bitter acid content of hop samples was determined on 2.5 g of ground hop cones, using toluene as extraction solvent, according to the official ASBC Hops-6 method (American Society of Brewing Chemists (ASBC, 2012). α- and β-acid content was determined spectrophometrically at 275, 325 and 355 nm; results were the average of three independent measurements and data were expressed as percentage of bitter acid on dry basis (db).

2.5. Determination of individual phenols and xanthohumol by HPLC

Hop polyphenols were separated and identified by an analytical HPLC system (Agilent 1100 series, Agilent, Italy) equipped with a photodiode array detector (DAD; Agilent Technologies, Italy). The DAD was fixed simultaneously at 280 nm (hydroxybenzoic acids (HB) and flavan-3-ols), 320 nm (hydroxycinnamic acids, HA) and 370 nm (flavonols). In addition, UV–vis spectra were recorded in the range 200−700 nm. The separation of phenolic compounds was made according to the protocol reported by Carbone and Mencarelli (2015), without modifications. The separation was performed on a Luna C18 column (Phenomenex, 4.6 × 250 mm; 5 μm particle size, set at 30 °C). The injection volume was 20 μL and the samples were membrane filtered (Millipore PTFE 0.45 mm, Milan, Italy) prior to the HPLC analysis. The different phenolic compounds were identified by their retention time, spectral data against individual standards and according to literature data and by the method of standard addition to the samples when necessary. Analytical data were evaluated using a chromatographic data management software system (Chemstation 32.1, Agilent Technologies). Ten-point calibration curves based on external standard solutions (0–100 ppm) were obtained for quantification. All analyses were performed in triplicate. Results were expressed as μg g^{-1} of dried sample.

Xanthohumol (XAN) was quantified according to Magalhães et al. (2010), with some modifications. Briefly, XAN was separated and quantified by an analytical HPLC system (Agilent 1100 series, Agilent, Italy) equipped with a photodiode array detector (DAD; Agilent Technologies, Italy) set at 370 nm. The separation was carried on a Synergi C18 column (Phenomenex, 4.6 × 150 mm; 4 μm particle size, set at 25 °C) used in combination with the following binary gradient: 40–99 % B for 15 min, 99 % B for 5 min. The time of post-run for reconditioning was 5 min. The injection volume was 25 μL and the samples were membrane filtered (Millipore PTFE 0.45 mm, Milan, Italy) prior to the HPLC analysis. The following mobile phase was used (flow rate 0.8 ml min^{-1}): solvent A, water with 0.1 % (v/v) formic acid (FA); solvent B, acetonitrile with 0.1 % FA. XAN was identified by its retention time, spectral data and by the method of standard addition to the samples when necessary. Analytical data were evaluated using a chromatographic data management software system (Chemstation 32.1, Agilent Technologies). Six-point calibration curve based on external standard solutions (0–100 ppm) were obtained for quantification. All analyses were performed in triplicate. Results were expressed as mg g^{-1} of dried sample.

2.6. Determination of individual bitter acids by HPLC

Different hop bitter acids were separated and identified by an analytical HPLC system (Agilent 1100 series, Agilent, Italy) equipped with a photodiode array detector (DAD; Agilent Technologies, Italy), according to the ASBC Standard Methods of Analysis (Hops-14 method; American Society of Brewing Chemists (ASBC, 2012).

The injection volume was 50 μL and the samples were membrane filtered (Millipore PTFE 0.45 μm, Milan, Italy) prior to the HPLC analysis. The separation was performed on a Synergi C18 column (Phenomenex, 4.6 × 150 mm; 4 μm particle size, set at 40 °C). The isocratic mobile phase was distilled water, methanol and FA in a ratio of 17:85:0.25 (v/v/v). Chromatograms were acquired at 326 nm. All analyses were performed in triplicate. For the quantification of α- and β-acids, a calibration curve was obtained from dilutions of ICE-3 standard. The results were expressed as % w/w.

2.7. H-NMR analysis of hop extracts

Different extracts were resuspended in 600 μL of deuterated solvents: water, methanol, a mix of methanol and water (1:1) respectively, containing 0.5 mM of trimethylsilyl propionate-d_4 (TSP) as internal standard. After centrifuging, the supernatant was transferred in a 5 mm thin-walled glass NMR tube for subsequent NMR spectral analysis.

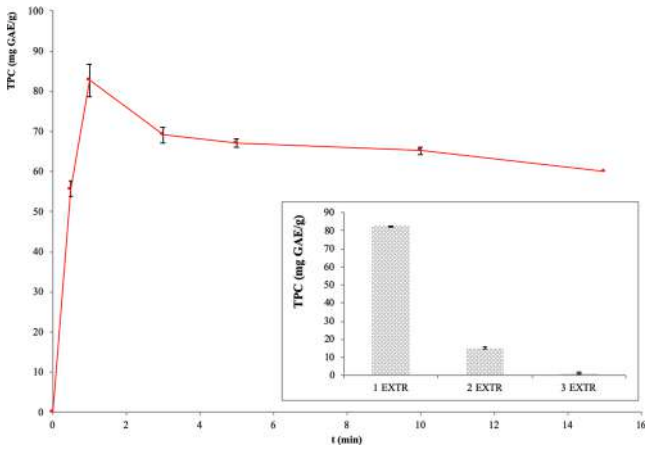


Fig. 1. Influence of time on microwave assisted extraction of hop polyphenols. TPC: total polyphenol content; GAE: gallic acid equivalents; EXTR: extraction.

NMR spectra were collected using a 700 MHz for ^{1}H, equipped with a 5 mm inverse TXI probe and z-axis gradient. ^{1}H-NMR spectra were acquired at 25 °C, with a spectral window of 15 ppm (carrier frequency at 4.7 ppm), 64 transients and 2 steady-state scans, using 3 s of acquisition time and 2 s of relaxation delay. ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) experiments were acquired with a spectral window of 15 ppm x 100 ppm (carrier frequencies at 4.85 and 46.5 ppm) using 2048 × 600 data points and 36 transients for the aliphatic region.

Quantification of hop metabolites in aqueous extracts was achieved using Chenomx NMRSuite v 8.5 (Chenomx Inc.), using as internal standard TSP 0.5 mM. The collected spectra were processed using 0.5 Hz of line-broadening followed by manual phase and baseline correction. All analyses were performed in triplicate.

2.8. Biological properties of hop green extracts: in vitro antiradical capacity (AC)

The radical scavenging power of the analysed samples was assessed by measuring their ability to scavenge synthetic radicals (e.g., $DPPH^{\bullet}$ and $ABTS^{\bullet+}$). ABTS radical cation decolourization assay was performed in 2.5 ml plastic cuvettes by pipetting 20 µL of extract (opportunely diluted) into 980 µL of $ABTS^{\bullet+}$ radical solution and the absorbance was measured after 60 min at 734 nm. Results were expressed as µg of hop sample on dry weight required to obtain 50 % $ABTS^{\bullet+}$ radical scavenging (half maximal effective concentration, EC_{50}) according to Carbone et al. (2011).

The $DPPH^{\bullet}$ quenching capacity of extracts was determined spectrophotometrically as reported by Carbone et al. (2011). Results were expressed as µg of sample (on dry weight basis) required to obtain 50 % DPPH scavenging (EC_{50}). All determinations were performed in triplicate.

2.9. Statistical analysis

Statistical analysis was performed with SPSS 20.0 software (SPSS, Inc., Chicago, Illinois). Data were reported as means ± standard deviation (SD) of three independent experiments with three replicates. Prior to chemometric applications, all variables were auto scaled (transformation into z-scores) to standardize the statistical importance of all responses. An exploratory data analysis was made to check the data normal distribution (Kolmogorov-Smirnov test) and the homogeneity of variance (Levene's test). Data were then subjected to two-way multivariate analysis of variance (two-way MANOVA) employing the Pillai's trace to assess significant effects of solvent and extraction technique on selected dependent variables ($p < 0.01$). MANOVA was followed up using univariate ANOVAs (REGWQ post-hoc test), while Pearson's correlation coefficient (r) was used to determine the correlation among variables; $p < 0.05$ and $p < 0.01$ were taken as significant and highly significant level, respectively. Principal component analysis (PCA) was used to establish the relationships among all variables under study.

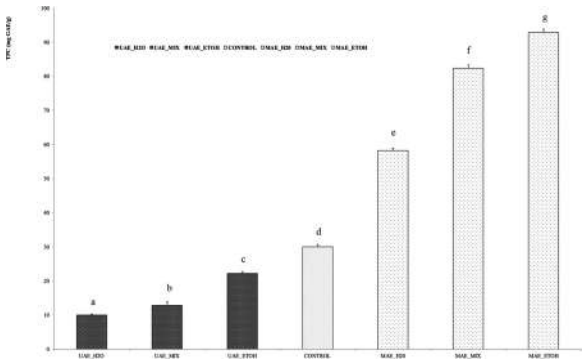


Fig. 2. Total polyphenol content (TPC) of hop green extracts (mean ± SD). Different letters indicate significant differences in the mean ($p < 0.05$). GAE: gallic acid equivalents; UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; ETOH: ethanol; MIX: ethanol:water mixture (50 %, v/v).

3. Results

A preliminary set of experiments with different extraction times (from 1 to 15 min) was carried out for MAE, in order to evaluate the effect of this parameter on the polyphenol extraction using ethanol as a model solvent (Fig. 1). The extraction of polyphenols was time-dependent, reaching maximum yield after 1 min of reaction, which was therefore chosen as the extraction time for all the following experiments with MAE. Similar results were reported by other authors (Alupului et al., 2012). Besides, in order to check the complete extraction of the phytochemicals from the matrix, three irradiation cycles of the sample of 1 min each were carried out (Fig. 1, inset). As it can be noticed, the first extraction led to a TP yield of about 82 %, with the third extraction able to recover only 1 % of the remaining TPC. Therefore, it was decided to proceed to a single extraction in all MAE experiments, in order to avoid the possible degradation of valuable compounds through prolonged MW irradiation.

3.1. Total polyphenol content of hop extracts

Fig. 2 shows the TP content of different hop extracts analyzed. TPC ranged from 8.7 ± 0.3 to 93.4 ± 0.2 mg GAE g^{-1} (for UAE_{H20} and MAE_{ETOH}, respectively). Multivariate analysis highlighted a significant effect of both solvent (S) and extraction technique (E), as well as their interaction (SxE), on this parameter ($p < 0.001$). With regard to the food grade solvents used in the present study, a linear increase in polyphenol content was observed as the concentration of organic solvent increased and higher yields of TPs were detected in ethanol samples regardless of the extraction technique used (20.3 ± 0.2 and 93.4 ± 0.2 mg GAE g^{-1} for UAE and MAE, respectively). Kowalczyk et al. (2013) showed that, under the same extraction conditions and hop variety analyzed, the replacement of 50 % water with methanol or ethanol in the solvent mixture resulted in a two-fold increase of TPC yield. Under our extraction conditions, the water replacement with 50 % ethanol resulted in a more limited increase than reported by these authors (~ + 30 % and + 41 % TPC for UAE and MAE, respectively), while replacing water with 80 % acidified methanol gave a three-fold increase in TPC. It is known that hydroalcoholic mixtures show a synergistic effect between water and alcohol, increasing the extraction performance of phenolic compounds from plant matrices. Besides, when water is used in combination with US, hydroxyl radicals can be produced and can react with the more easily oxidizable compounds present in the plant matrix and thus

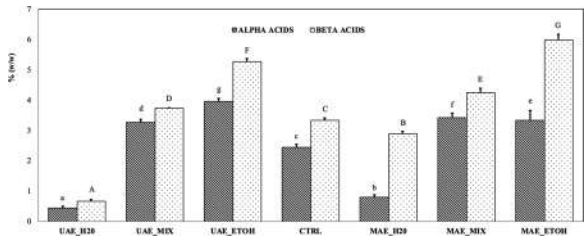


Fig. 3. Bitter acid content of hop green extracts (mean ± SD). Different letters indicate significant differences in the mean ($p < 0.05$). UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; CTRL: control; ETOH: ethanol; MIX: ethanol:water mixture (50 %, v/v).

decrease the reducing potential of the extract. This effect can be contained by adding organic solvents and acidifying (Soria and Villamiel, 2010; Annegowda et al., 2012).

Comparing the results of extraction efficiencies among techniques (Fig. 2), MAE gave significantly higher values ($p < 0.01$) than UAE, regardless the solvent used, in agreement with previous studies (Routray and Orsat, 2014). The combined use of ethanol with MAE allowed the recovery, in just one minute, of about five times more polyphenols than the combined use of ethanol and US. Finally, the combination of 80 % aqueous methanol with US gave higher TP values compared to those reported by Önder et al. (2013) for the extraction of a Turkish wild hop accession; however, the authors provided no details about the UAE employed for a correct comparison with our data.

3.2. Total bitter acid content of hop extracts

Fig. 3 shows the total bitter acid content of hop extracts analyzed. α-acid content (AA) ranged from 0.39 ± 0.06 to 4.0 ± 0.1 % (w/w; for UAE_{H2O} and UAE_{ETOH}, respectively); while β-acid content (BA) ranged from 0.64 ± 0.04 to 6.3 ± 0.3 % (w/w; for UAE_{H2O} and MAE_{ETOH}, respectively). Multivariate analysis highlighted a significant effect of both S and SxE on bitter acid content ($p < 0.001$). However, no significant influence of E was found for AA of sample analyzed, while for BA the influence of E was significant ($p < 0.001$). As far as α-acids are concerned, US extraction efficiency increased significantly with increasing the percentage of organic solvent ($p < 0.01$), in line with the low solubility of these acids in water. It is interesting to note that the combination of US with ethanol has led to an α-acid yield of about 62 % higher than conventional solid-liquid extraction with toluene (CTRL sample in Fig. 3). Aniol et al. (2008) showed a strictly dependence between the percentage of AA removed form spent hops and the increasing polarity of the solvent used under US extraction of the samples.

In the present study, MW irradiation of hop samples showed an increasing yield of AA inversely correlated with the polarity of the solvent used, whereas ethanol appeared significantly less efficient than ethanol:water in the extraction of α-acids ($p < 0.01$), even if the difference was minimal (about – 2 %). To the best of our knowledge, there are no previous studies on the influence of the solvent and these unconventional techniques on the extraction efficiency of bitter acids from hops.

As can be seen from Fig. 3, ethanol showed a better extraction efficiency for β-acids than α-acids, regardless the extraction technique (5.28 ± 0.06 and 6.3 ± 0.3 % w/w for UAE and MAE, respectively). These findings agree with the more hydrophobic character of β-acids compared to α- ones (Olsovska et al., 2016). Moreover, compared to the reference extraction method (solid-liquid extraction of hop cones with toluene according to ASBC Hops-6 method), US coupled with ethanol extracted about + 58 % of hop's β-acids, while using the same solvent in combination with MAE the yield was + 79 %. This is a very important finding in light of the recognized high antimicrobial and anti-infective power of hop's β-acids (Van Cleemput et al., 2009; Čermák et al., 2015). Among the state-of-the-art methods used for extracting bitter compounds from hops, the most widespread is supercritical fluid extraction with carbon dioxide, mainly focusing on α-acids recovery due to their brewing value (Daoudand and Kusinski, 1992). As far as we know, there are no literature data on the extraction efficiency of β-acids from *Humulus lupus* L as a function of solvent or extraction technique, despite the very high phytotherapeutic potential of these compounds.

Table 1
Phenolic composition (μg g^{-1}) of hop polyphenol extract determined by high-performance liquid chromatography (mean ± SD).

Compound	UAE[1]_H_20	UAE_MIX[4]	UAE_ETOH[2]	CTRL[3]	MAE[5]_H_20	MAE_MIX	MAE_ETOH
Hydroxybenzoic acids (HBA)							
p-hydroxybenzoic acid	714 ± 24a	n.d.	n.d.	1117 ± 44c	879 ± 10b	n.d.	n.d.
Gallic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Syringic acid	474 ± 16b	769 ± 12e	277.9 ± 0.4a	582 ± 23d	476 ± 6b	522 ± 12c	1182 ± 21f
Protocatechuic acid	928 ± 34e	861 ± 14d	n.d.	n.d.	751 ± 12c	590 ± 14b	554 ± 10a
Total HBA	2116 ± 74	1630 ± 26	277.9 ± 0.4	1699 ± 67	2106 ± 28	1112 ± 26	1736 ± 31
Hydroxycinnamic acids (HCA)							
p-Coumaric acid	389 ± 5b	491 ± 11c	142 ± 22a	615 ± 7e	516 ± 24d	372 ± 11b	830 ± 10f
Caffeic acid	46 ± 10a	n.d.	n.d.	n.d.	102 ± 35b	n.d.	133 ± 2c
Chlorogenic acid	989 ± 33f	904 ± 15e	355.6 ± 0.6a	725 ± 29c	1382 ± 18	640 ± 15b	769 ± 14d
Ferulic acid	143 ± 16e	23 ± 8a	n.d.	130 ± 14d	62 ± 12c	39 ± 12b	242 ± 4f
Total HCA	1567 ± 64	1418 ± 34	497.6 ± 22.6	1470 ± 50	2062 ± 89	1051 ± 38	1974 ± 30
Flavan-3-ols							
Catechin	6655 ± 220c	6541 ± 98c	2989 ± 10a	8058 ± 325d	8399 ± 187e	5919 ± 143b	14009 ± 248f
Epicatechin	414 ± 14b	610 ± 10e	286.8 ± 0.6a	730 ± 29f	508 ± 7d	480 ± 12c	1185 ± 21 g
Total flavans	7069 ± 234	7151 ± 108	3275.8 ± 10.6	8788 ± 354	8907 ± 194	6399 ± 155	15194 ± 269
Flavanols							
Rutin	1092 ± 1 g	633 ± 1e	295 ± 2a	866 ± 1f	487.8 ± 0.6c	528.8 ± 0.7d	437.7 ± 0.8b

n.d.: not detectable. Different letters in a row indicate the significant differences in the mean ($p < 0.05$).
[1] UAE: ultrasound-assisted extraction.
[2] ETOH: ethanol.
[3] CTRL: control.
[4] MIX: ethanol:water mixture (50 %, v/v).
[5] MAE: microwave-assisted extraction.

3.3. HPLC analysis of polyphenols

Hop polyphenols are not only an important group of compounds related to the pro-health potential of hop cone extracts, but they also act as key compounds in beer flavor quality and stability (Jaskula-Goiris et al., 2014). In the present study, three classes of phenolic compounds were analyzed: phenolic acids (HB and HA), flavan-3-ols and flavonols (Table 1). Multivariate analysis highlighted a significant effect of both S and E, as well as their interaction (SxE), on phenolic content ($p < 0.001$). The highest recovery of polyphenols was obtained using MW irradiation in combination with ethanol (19342 μg g^{-1} d.b.), following by MAE_{H2O} > CTRL > UAE_{H2O} > UAE_{MIX} > MAE_{MIX} > UAE_{ETOH}. The higher extraction efficiency of MAE compared to US extraction can be explained by reaching higher temperatures in a shorter period of time in the case of MW application, leading to a higher rate of release of phytocompounds into the solvent, also in view of the higher dissociation of polyphenols linked with the membrane, caused by MW. Besides, the lower yield of phenolic compounds obtained with UAE can be explained by a lower rate of rupture of cellular structure as compared to MW (Routray and Orsat, 2014), as well as to the occurrence of degradative mechanisms of active compounds due to oxidative pyrolysis caused by hydroxyl radicals during cavitation phenomenon (Ameer et al., 2017). As regards the effect of solvent on MAE extraction efficiency, ethanol, despite having a dielectric constant lower than water, has a dissipation factor 1.67 times higher than water, which is reflected in a higher heating rate and better extraction efficiency (Hemwimon et al., 2007; Routray and Orsat, 2014). Besides, with regard to samples extracted by US, the total polyphenol content decreased significantly in ethanol samples compared to the water ones (−63 %), as previously described (Ghitescu et al., 2015). In this case, we can hypothesize that one of the reasons of the observed trend may be that the vapor pressure (VP) of the solvents used is very different, as the vapor pressure has a great influence on the onset and intensity of acoustic cavitation (Tao et al., 2014). At 25 °C, the VP of water is 23.8 torr while that of ethanol is more than the double (58.9 torr). When the vapor pressure is higher, more bubbles are created, which collapse with less intensity due to a smaller pressure difference between inside and outside of bubbles. Another reason may be due to the higher swelling properties of water than that of ethanol, enhancing the mass transfer of metabolites, especially if it is considered that the starting matrix is not a fresh but dried sample (dried hop cones), resulting in different viscosities of the suspensions, which may affect the ultrasound action. In this regard, while water could enhance swelling of plant material, ethanol may weaken the interactions between the solutes and plant matrix. As a consequence, a synergistic effect in the extraction of bioactive compounds could be achieved with adding of water to ethanol, increasing the permeability of plant tissues while enabling better mass-transfer by diffusion. This could be explained the observed trend within the same technique: UAE_H_2O > UAE_MIX > UAE_EtOH. However, a direct comparison of the results obtained with literature data is particularly difficult because, to the best of our knowledge, there are no data available on the effect of different solvents in the ultrasonic extraction of specific phenolic compounds from hop cones. Finally, another important factor to be taken into consideration trying to elucidate the mechanisms underlying the US extraction is the difference in the polarity of the solvents used, which affects their solubilization capability of individual polyphenols within the same technique and it could partly explain the higher phenolic acid yield in the UAE_H_2O samples compared to UAE_EtOH ones. On the other hand, Zhang et al. (2015) studied the degradation of gallic acid mediated by US in a model extraction solution, pointed out that the degradation rate of the phenolic acid increased with further increasing ethanol concentration above 60 % v/v. However, the authors pointed out that the factors responsible for the phenol degradation are not only attributable to the acoustic cavitation mechanism, but also depend on the physical properties of the solvent used (e.g. surface tension, vapour pressure, etc.) in the US experiments. Regardless the extraction technique and solvent used, the most abundant polyphenol compound was catechin, ranging from 2989 ± 10 to 14009 ± 248 μg g^{-1} (for UAE_{ETOH} and MAE_{ETOH}, respectively), followed by rutin and chlorogenic acid. These values are higher than those found in other plant materials (Albuquerque et al., 2017), including green tea leaves, which are considered as the main source of catechins (Gadkari and Balaraman, 2015). Among HB investigated, no trace of gallic acid was found in any of the samples analyzed. This is probably due to genetic traits of wild hop accession used in the present study (Jelínek et al., 2010). The most abundant phenolic acid in this fraction was syringic acid, which was found in all extracts analyzed, ranging from 277.9 ± 0.4 to 1182 ± 21 μg g^{-1} (for UAE_{ETOH} and MAE_{ETOH}, respectively). Besides, *p*-hydroxybenzoic acid proved to be unstable, both under MAE and UAE, with increasing organic solvent concentration, underlying the role of the solvent on the recoveries of phenolic acids (Li et al., 2004). Moreover, *p*-hydroxybenzoic acid undergoes easily to decarboxylation, which can occur due to high temperature and also to US decomposition (Qiao et al., 2013; Castada et al., 2020).

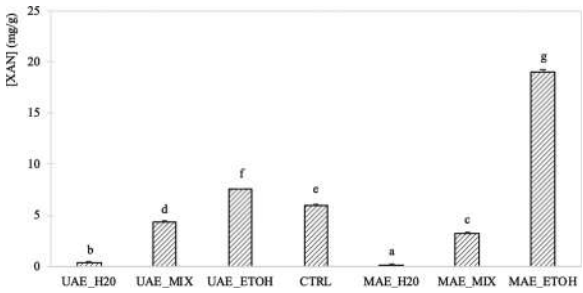


Fig. 4. Xanthohumol (XAN) content of hop green extracts (mean ± SD). Different letters indicate significant differences in the mean ($p < 0.05$). UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; CTRL: control; ETOH: ethanol; MIX: ethanol:water mixture (50 %, v/v).

As far as HA are concerned, the most abundant was chlorogenic acid, followed by *p*-coumaric one. Both acids were found in all the extracts tested, unlike the caffeic acid, which was found only in MAE_{H2O} and MAE_{ETOH} samples. Qiao et al. (2013) reported a stronger chemical degradative effect of US on caffeic acid in ethanol (80 %) rather than in water. Besides, the authors found chlorogenic acid to be stable in the seven solvents tested under US irradiation in agreement with our results, with the exception of pure ethanol, which gave a significant reduction of this compound (more than 60 %) if compared with other solvents used in UAE of hop cones. Our findings are similar to those reported by other authors (Kowalczyk et al., 2013), highlighting the significant influence of solvent type, as well as the extraction technique, on the concentration of particular phenolics in different extracts. As far as flavans are concerned, MW irradiation showed a better extraction efficiency than US, regardless the solvent used, for both catechin and epicatechin. Moreover, the highest yields were reached using MW in combination with ethanol for both compounds tested (14009 ± 248 and 1185 ± 21 μg g^{-1} for catechin and epicatechin, respectively), while the lowest ones when ethanol was used in combination with US (2989 ± 10 and 286.8 ± 0.6 μg g^{-1} for catechin and epicatechin, respectively). Previous studies showed that catechin could undergo epimerization, hydrolysis, and oxidation/condensation reactions during US irradiation (Zhu et al., 2018). UAE showed the best recovery capacity of hop rutin, whereas a great impact of the solvent was also observed (Biesaga, 2011).

3.4. HPLC quantification of xanthohumol in different green extracts

Xanthohumol is the most abundant prenylated chalcone that occurs only in hop plant, accumulating mainly in cones and, to a lower extent, in leaves (Čeh et al., 2007). The growing interest of researchers for this compound is due to its proven ability to inhibit the growth of different types of human cancer cells (i.e. breast, colon, hepatocellular, ovarian,

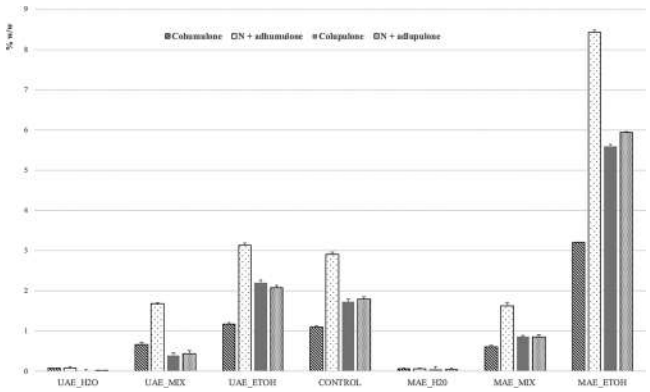


Fig. 5. HPLC profiles of different bitter acids in hop green extracts (mean ± SD). UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; ETOH: ethanol; MIX: ethanol:water mixture (50 %, v/v).

pancreatic and prostate cancer cells as well as leukemia cells), to induce both caspase-dependent and caspase-independent apoptosis and to inhibit invasion and angiogenesis (Miranda et al., 1999; Dorn et al., 2010; Saito et al., 2018).

Fig. 4 shows XAN content of hop extracts analyzed. As previously observed for the phenolic content of green extracts, multivariate analysis highlighted a significant effect of both S and E, as well as their interaction (SxE), on XAN content ($p < 0.001$). The maximum extraction yield was obtained using MW irradiation in combination with ethanol (19.1 ± 0.2 mg g^{-1}), following by $UAE_{ETOH} > CTRL > UAE_{MIX} > MAE_{MIX} > UAE_{H20} > MAE_{H20}$. Using ethanol as a solvent, MAE produced 2.5 times more xanthohumol than US in only 1 min of extraction ($p < 0.01$). According to Mongelli et al. (2015) the average XAN content of Italian wild hop accession is 0.407 mg 100 g^{-1} on dry basis. The authors extracted all samples under dynamic maceration for 3 h in 100 % methanol. Herein, MAE produced an increase of more than 4.6 times compared to this average value. Moreover, in the present study, MAE_{ETOH} sample showed a XAN content three times higher than that of the CTRL one extracted with 80 % methanolic solution. Hop XAN content, as well as the other secondary metabolites, is strictly dependent on genotype and environmental factors. However, when these parameters are set, the extraction method, as shown by the results reported in this paper, has a significant influence on the final content of extractable bioactive compounds. To the best of our knowledge, no literature data are available on the effect of these green extraction technologies on the hop XAN recovery.

3.5. HPLC analysis of hop bitter acids

Hop bitter acids, both α- and β- ones, are a mixture of several similar compounds each. The α-bitter acids are a mixture of six humulone analogues, while β-bitter acids consist of several analogues: lupulone, colupulone, adlupulone, prelupulone, and postlupulone, which are of minor importance in the brewing process, but exerting a plethora of biological activities (Van Cleemput et al., 2009; Karabín et al., 2016). Besides, co-humulone, the main component of α-acid fraction, is of interest from the brewing point of view. In fact, it leads to a lower foam and storage stability in beer, which means that a low percentage of this compound in hop extracts is of advantage. Fig. 5 shows the extraction efficiency of proposed methods in terms of different individual hop bitter compounds. Multivariate analysis highlighted a significant effect of both S and E, as well as their interaction (SxE), on different bitter compounds analyzed ($p < 0.001$). Higher yields of both total α- (4.326 ± 0.005 and 11.64 ± 0.01 % w/w, for UAE and MAE, respectively) and β-acids (4.052 ± 0.003 and 11.54 ± 0.02 % w/w, for UAE and MAE, respectively) were detected in ethanol samples, regardless of the extraction technique used. In this regard, it is noteworthy that all

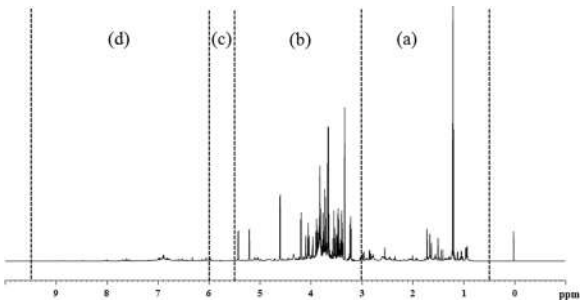


Fig. 6. A typical ^{1}H-NMR spectrum of hop extract. The main spectral regions are reported: aliphatic region from 0.5 to 3.0 ppm (a); sugars from 3.0 to 5.5 ppm (b); terpenes from 5.5 to 6.0 ppm (c) and aromatics from 6.0 to 9.5 ppm (c).

ethanolic extracts obtained by green technologies are characterized by higher bitter acid values than those obtained by classical extraction with toluene (CTRL). Moreover, on average, MAE showed better extraction efficiency for all bitter acids tested, regardless the solvent used. Among the compounds analyzed, the most representative ones were N + adhumulone (including humulone), ranging from 0.07 to 8.4 % w/w (for MAE_{H2O} and MAE_{ETOH}, respectively). As far as co-humulone content is concerned, the highest value was obtained in MAE_{ETOH} samples (3.21 ± 0.01 % w/w), while the lowest one was obtained with MW extraction using water as solvent (0.232 ± 0.001 % w/w); $p < 0.01$). As far as β-acids are concerned, the most representative ones were N + adlupulone (including lupulone), ranging from 0.0045 to 5.94 % w/w (for UAE_{MIX} and MAE_{ETOH}, respectively).

To the best of our knowledge, no literature data are available regarding the effect of these green extraction technologies on the individual hop bitter acids recovery.

3.6. ^{1}H-NMR analysis of green extracts

Most published studies focused on secondary metabolites of plant extracts, while little has been done on primary metabolites such as sugars, amino acids, and carboxylic acids, which have, at least, some nutritional value. In this study, we applied ^{1}H-NMR analysis to investigate all of the detectable metabolites in different hop extracts and the effects of the different extraction solvents and techniques on the hop metabolite fingerprinting. Fig. 6 shows a representative ^{1}H-NMR spectrum of the extracts analyzed in this paper. To make a quantitative analysis of the composition, we divided the spectrum into four regions, each marked with a letter: (a) which mostly contains signals from amino acids (when using H_2O as a solvent) or bitter acids (when using EtOH as a solvent), (b) which mostly contains sugary resonances, (c) which shows signals from terpenes and (d) which covers signals from aromatic compounds, mainly polyphenols (when using EtOH as a solvent). These four regions were integrated, and each value divided by the total integral of the spectrum to obtain the corresponding relative composition of each extract in terms of these regions. Fig. 7 shows the average composition, resulting from three independent experiments, for each of the classes of compounds identified (i.e. sugars, amino acids, terpenes and aromatics), according to the extraction technique and solvent used. Regardless the extraction technique used, sugars represented the most abundant compounds in H_2O and MIX samples (80 and 60 %, respectively). EtOH extracts, on the other hand, showed a 60 % of the total signals coming from the aliphatic region, predominantly from bitter acids. The relative contributions of the aliphatic or sugar regions to the total spectral intensity were very similar when using MAE or UAE. Interesting results were obtained when comparing the two extraction techniques for the contribution of signals in the terpene or aromatic regions. There was a significant higher contribution ($p < 0.05$) to the total integral of terpenes of the MAE_{EtOH} and MAE_{MIX} samples than that

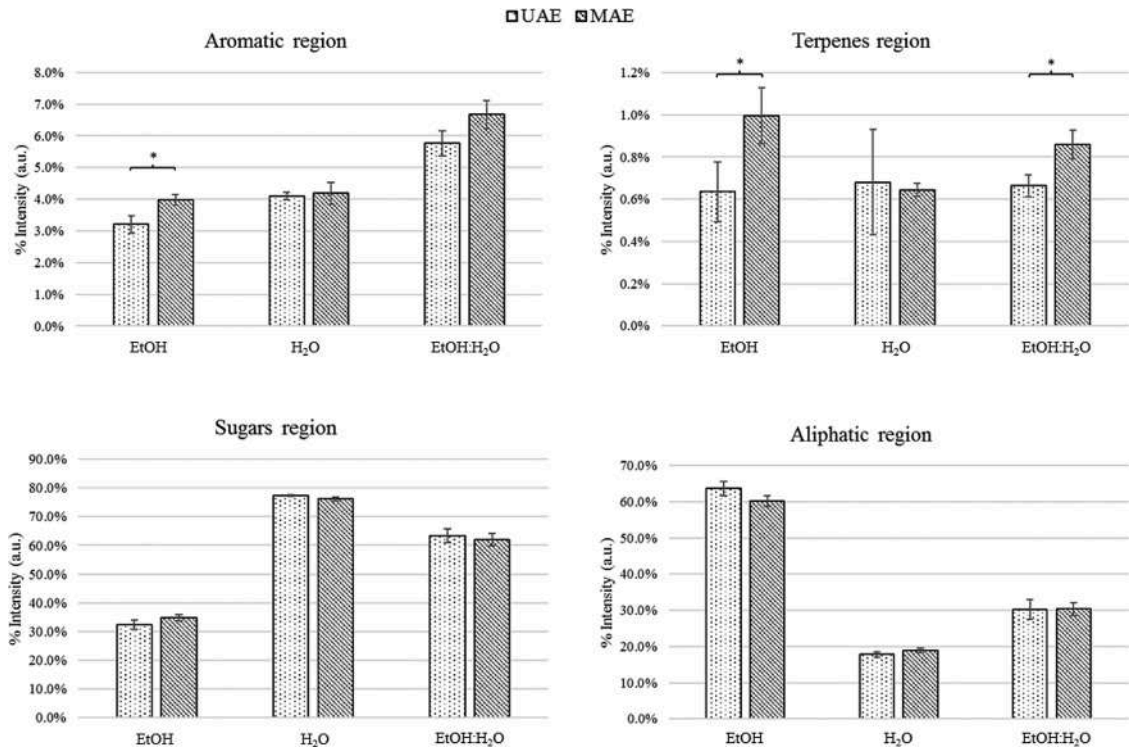


Fig. 7. Relative intensity of each spectral region calculated for different hop green extracts expressed as a fraction of the total spectral integral. ETOH: ethanol.

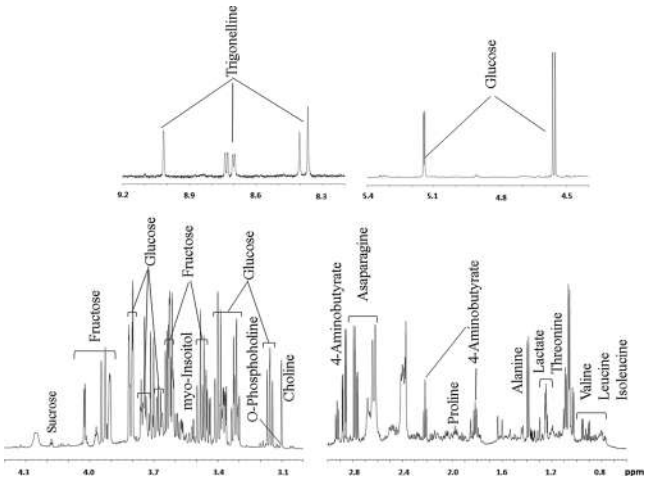


Fig. 8. A representative ^{1}H-NMR spectrum of aqueous hop extracts.

of the corresponding UAE extracts. MAE_{EtOH} samples also showed a higher enrichment in polyphenols than UAE ones, in line with the results described in the previous sections for this class of compounds.

In order to provide the identification and quantification of the most concentrated primary metabolites in hop cones, mainly amino acids and sugars, the analysis of the composition of aqueous extracts using spectral deconvolution was carried on (Fig. 8). A few other metabolites were identified, including myo-inositol and lactate (carbohydrate metabolism), choline, *o*-phosphocholine and ethanolamine (lipid metabolism) and two signalling molecules: 4-aminobutyrate and trigonelline. The former, also known as γ-aminobutyric acid (GABA), acts as an endogenous signalling molecule regulating plant growth and development among many other different functions (Carillo, 2018). Trigonelline is also a hormone involved in plant cell cycle regulation, nodulation and oxidative stress, and in helping plant survival and growth (Gupta, 2016). Trigonelline was found initially in *Trigonella foenum-graecum* L. (fenugreek) (Moorthy et al., 1989) and in high concentrations in Arabica coffee (Stennert and Maier, 1994), but in hop it was only recently identified (Benson, 2014). After the brewing process, trigonelline is found also in beer, with around 50 % carryover (Spevacek et al., 2016). Its presence in hop and beer is particularly interesting because of the many biological activities found for this alkaloid. In particular, it can be used as an anti-diabetic agent, improving glucose tolerance in obese diabetics (Yoshinari and Igarashi, 2010). In addition, trigonelline may be involved in various contexts as antibacterial, antiviral, sedative, anti-migraine, and anticancer (Zhou et al., 2012).

The concentrations of all identified metabolites were measured by

Table 2
Main metabolites (% w/w) identified in hop aqueous extracts (mean ± SD).

	Compound	UAE[1]	MAE[2]
Amino acids	Asparagine	75.2 ± 0.7	75.4 ± 0.7
	Proline	9.0 ± 0.4	6.7 ± 0.9
	Alanine	8.1 ± 0.5	8.6 ± 0.2
	Valine	2.7 ± 0.4	2.9 ± 0.2
	Isoleucine	1.8 ± 0.1	1.9 ± 0.4
	Threonine	1.7 ± 1.5	2.9 ± 1.4
	Leucine	1.6 ± 0.2	1.5 ± 0.1
Sugars	Glucose	58.5 ± 2.7	52.8 ± 13.4
	Fructose	32.3 ± 3.6	27.5 ± 8.1
	Sucrose	4.5 ± 2.8	14.0 ± 20.3
	Maltose	2.7 ± 0.8	3.6 ± 0.7
	Galactose	1.0 ± 0.1	1.0 ± 0.1
	Xylose	0.5 ± 0.1	0.6 ± 0.1
	Mannose	0.5 ± 0.1	0.5 ± 0.1
Other Metabolites	myo-Inositol	46.6 ± 4	45.8 ± 0.9
	Choline	20.1 ± 1.2	18.7 ± 1.9
	4-Aminobutyrate	16.9 ± 2.1	17.2 ± 0.8
	Trigonelline	4.8 ± 0.5	4.6 ± 0.3
	Lactate	4.8 ± 2.9	6.2 ± 1.4
	Ethanolamine	3.8 ± 0.4	3.8 ± 0.6
	o-Phosphocholine	3.0 ± 1.1	3.7 ± 1.8

[1] UAE: ultrasound-assisted extraction.
[2] MAE: microwave-assisted extraction.

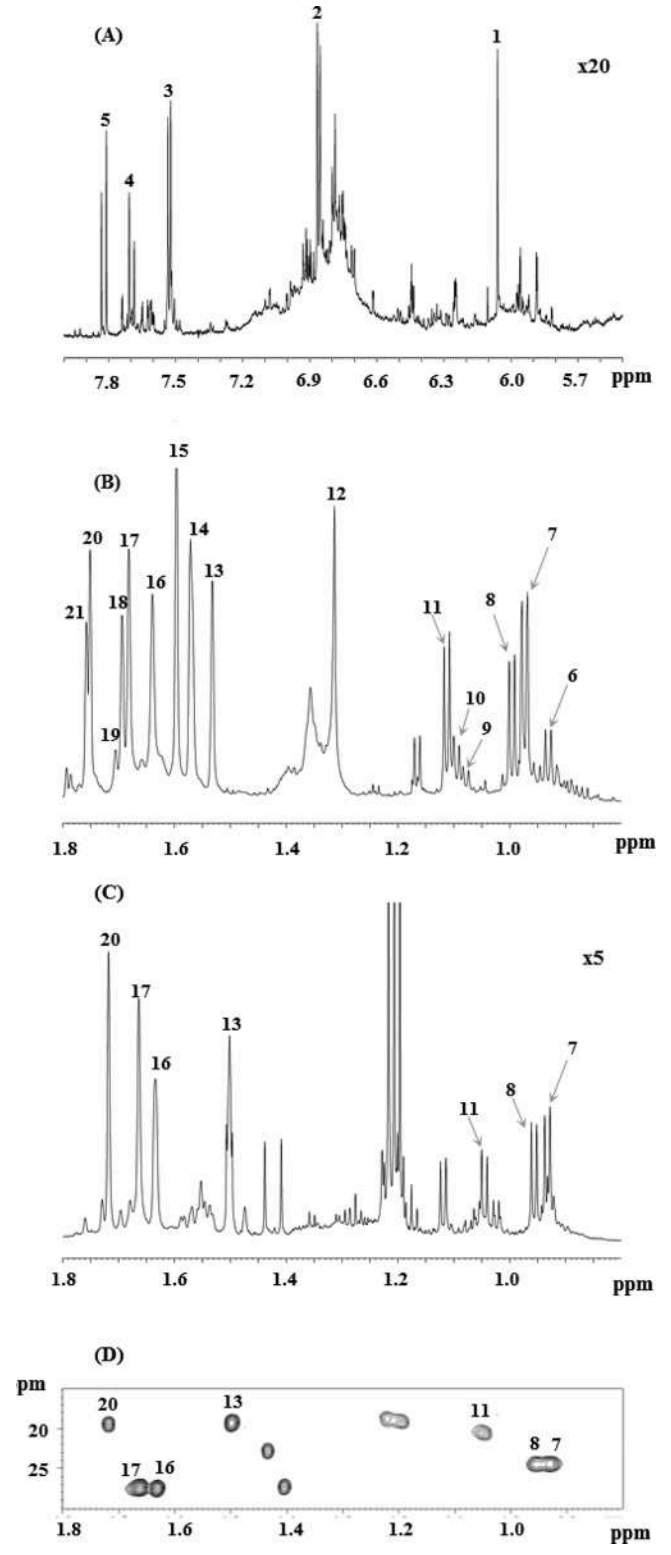


Fig. 9. Expanded aromatic region from 5.5 to 7.5 ppm (A) and bitter acids region from 0.8 to 1.8 ppm of the ^{1}H-NMR spectra of MAE_ETOH (B) and MAE_MIX samples (C), respectively; ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectrum for bitter acids region of MAE_MIX extract (D). MAE: microwave-assisted extraction; ETOH: ethanol; MIX: ethanol:water mixture (50 %, v/v).

Table 3
Chemical shifts used for the assignment of bitter acids and xanthohumol measured in the ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) experiments.

Compound	Assignment	^{1}H [ppm]	^{13}C [ppm]
Humulones & Lupulones	CH_3-11	1.661	26.1
	CH_3-10	1.736; 1.731	18.0
	CH_2-7 a,b	3.076	21.8
Humulones	CH_3-16	1.509	18.0
	CH_3-15	1.616	26.1
	CH_2-12	2.488	42.9
Lupulones	CH_3-15,20	1.545	26.1
	CH_3-16,21	1.573	18.2
Humulone & Lupulone	CH_3-4',5'	0.953	23.1
	CH_2-2'	2.844	50.1
Humulone	CH_2-2'a	2.704	48.8
Adhumulone	CH_3-4'	0.944	12.2
	CH_3-5'	1.058	16.7
Adlupulone	CH_3-4'	0.885	12.4
	CH_3-5'	0.907	14.5
Cohumulone & Colupulone	CH_3-3',4'	1.091	19.3
Cohumulone	CH-2′	3.802	35.9
Colupulone	CH-2′	3.991	37.0
Xanthohumol	CH_3-4",5"	1.711	26.0
	CH_2-1"	3.241	53.9
	OCH_3	3.903	56.4
	CH-5′	6.030	91.7

spectral deconvolution using a dataset of simulated spectra within the Chenomx software, and their w/w contributions to each compound family calculated (Table 2). No significant difference in the composition between UAE and MAE extracts was observed. Asparagine shows an outstanding prevalence among amino acids, representing a 75 % w/w of the total amino acids detected. This non-essential amino acid is critical for the production of the body's proteins, enzymes and muscle tissue. Supplements of this amino acid are claimed to balance nervous system function.

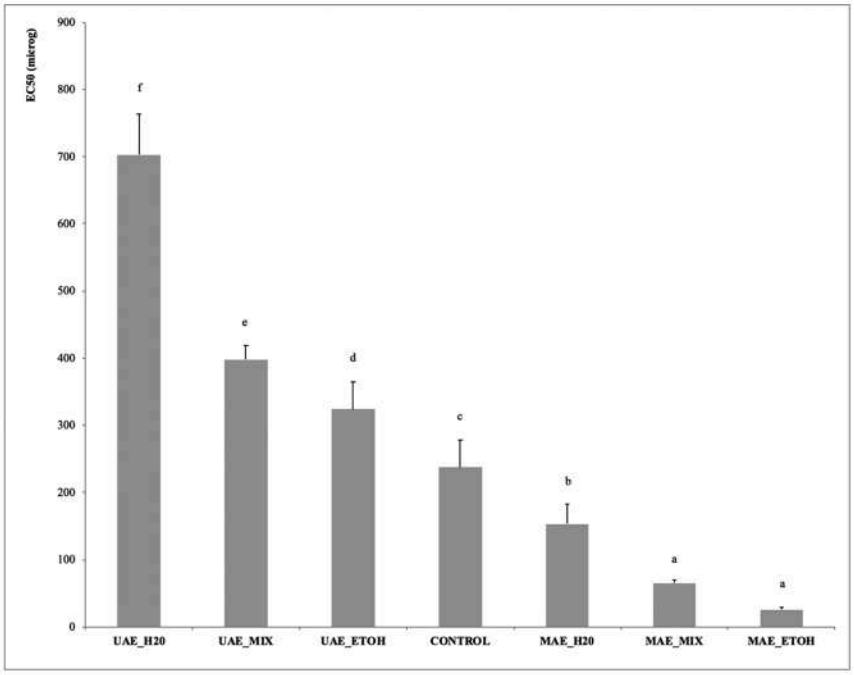
A distribution of amino acid concentrations very similar to that observed for hops was found in pea nodules, which contain an order of magnitude higher concentration of asparagine than each of the other major amino acids. (Gln, Ala, Asp and Glu) (Scharff et al., 2003).

Carbohydrate composition of extracts analyzed showed a large presence of glucose and fructose, constituting together almost 90 % w/w of all sugars extracted (Table 2). Sucrose, maltose, galactose, xylose and mannose completed the identified compounds of this family. It is worth noting, however, that carbohydrates do not represent a major constituent of hop, as different varieties show a sum of fructose, glucose and sucrose representing around 1 % of the dry hop material (MacWilliam, 1953). However, they represented the largest fraction of water-soluble compounds in H_2O extracts from hop cones in the present study, regardless the extraction technique used.

Secondary metabolites, including bitter acids and the prenylated chalconoid xanthohumol, were readily identified in EtOH extracts (Fig. 9A, B). The aliphatic region showed most of the signals belonging to methyl groups of α- and β-acids, whereas the aromatic region showed several resonances that can be attributed to xanthohumol. Assignment of these signals was confirmed by an ^{1}H-^{13}C HSQC experiment, leading to chemical shifts in agreement to those already assigned (Table 3; Farag et al., 2012). These compounds (i.e. bitter acids) were not detected by NMR in H_2O extracts, in line with their very low percentage estimated using a more sensitive technique (see Section 3.5). Moreover, we found some signals in the aliphatic region of the MAE_{MIX} samples (Fig. 9C) that could be assigned to bitter acids using a ^{1}H-^{13}C HSQC experiment (Fig. 9 D).

Comparison of the observed chemical shifts with those of Table 3 led to the conclusion that EtOH:H_2O mixture extracted mainly α-acids. This observation is in line with the fact that MW irradiation increases the yield of α-acids when the solvent polarity is increased (see Section 3.2).

a)



b)

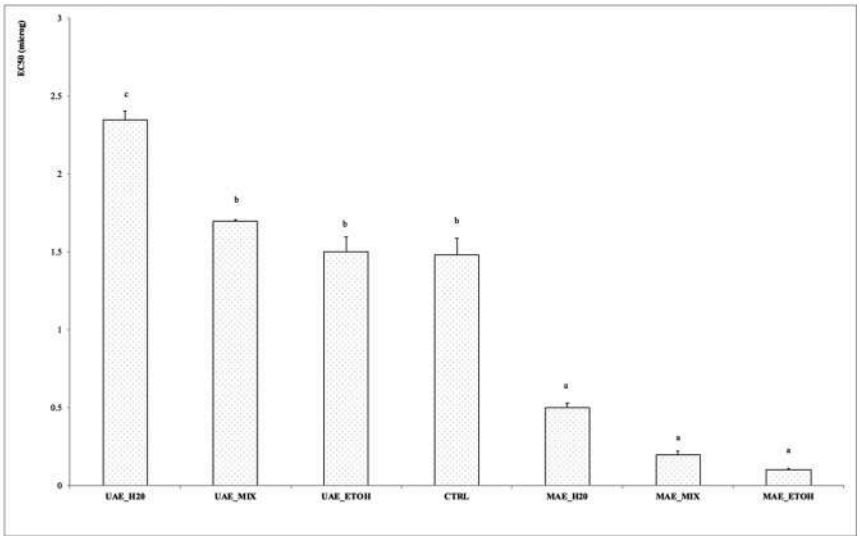


Fig. 10. Antiradical capacity of hop green extracts (mean ± SD) measured against a) 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$), b) 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS$^{\bullet+}$). Different letters indicate significant differences in the mean ($p < 0.05$). UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; ETOH: ethanol; CTRL: control; MIX: ethanol:water mixture (50 %, v/v); EC_{50}: half maximal effective concentration.

The results obtained with the NMR analysis suggests that this is true particularly for α-acids, while the extraction of β-acids decreased significantly using solvents of higher polarity, like the mixture EtOH: H_2O.

3.7. Biological properties of green hop extracts: in vitro antioxidant capacity

Bioactive compounds isolated from hop cones are reported to exert a plethora of healthy related activities, most of them related to their antioxidant potential. In order to investigate the radical scavenging properties of different green extracts analyzed, two different in vitro antiradical assays were performed, based on hydrogen and electron transfer. Fig. 10 shows the antiradical potential of hop extracts towards the synthetic chromogenic radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, expressed in terms of EC_{50}: the lower this value the higher the antiradical capacity of the extract. In both *in vitro* tests, the highest antiradical capacity values were recorded for the ethanol samples, even though all green extracts exhibited a radical scavenging capacity against DPPH$^{\bullet}$ at concentrations higher than those required for ABTS$^{\bullet+}$ quenching (EC_{50}: 26 and 0.1 μg db for DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, respectively), in agreement with the results reported by Di Sotto et al. (2018). These findings could be reasonably explained by the chemical structure of the bioactive components present in the analyzed green extracts, considering that steric accessibility is one of the major determinants of the DPPH$^{\bullet}$ quenching reaction (Prior et al., 2005).

MW irradiation of hop cones led to the higher antiradical capacity of MAE samples compared to UAE ones, regardless of the solvent and the *in*

Table 4
Correlation analysis.

	TPC	AA	BA	AC_{ABTS}	AC_{DPPH}	CoH	AdH	CoL	AdL	SA	*p*CA	CHLA	CAT	EPIC	RUT	XAN
TPC	1	.231	.541**	−.892**	−.865**	.557**	.553**	.585**	.599**	.510**	.510**	.027	.621**	.573**	−.475*	.499**
AA		1	.848**	-.286	−.469*	.504**	.550**	.491**	.465*	.224	−.097	−.799**	−.112	.188	−.642**	.578**
BA			1	−.561**	−.735*	.749**	.777**	.754**	.739**	.420*	.185	−.571**	.243	.449*	−.839**	.783**
AC_{ABTS}				1	.899**	-.332	-.336	-.359	−.366	−.289	−.305	−.071	−.376**	−.339	.642**	−.291
AC_{DPPH}					1	−.493**	−.508**	−.541**	−.540**	−.396*	−.426*	.109	−.449**	−.506**	.677**	−.498**
CoH						1	.998**	.989**	.982**	.774**	.567**	−.358	.685**	.799**	−.438*	.988**
AdH							1	.989**	.980**	.764**	.549**	−.400*	.658**	.790**	−.456*	.993**
CoL								1	.998**	.722**	.568**	−.382*	.682**	.794**	−.436*	.985**
AdL									1	.695**	.552**	−.380*	.676**	.775**	−.432*	.974**
SA										1	.873**	.096	.883**	.948**	−.083	.752**
*p*CA											1	.358	.956**	.940**	.117	.556**
CHLA												1	.344	.067	.308	−.438**
CAT													1	.938**	.005	.639**
EPIC														1	−.078	.797**
RUT															1	−.431**
XAN																1

TPC: total phenolic content; AA: alpha acid content; BA: beta acid content; AC_{ABTS}: antiradical capacity (ABTS in vitro test); AC_{DPPH}: antiradical capacity (DPPH in vitro test); CoH: cohumulone; AdH: N + adhumulone; CoL: colupolone; AdL: N + adlupulone; SA: syringic acid; *pCA*: *para*-coumaric acid; CHLA: chlorogenic acid; CAT: catechin; EPIC: epicatechin; RUT: rutin; XAN: xanthohumol. **: Correlation is significant at the 0.01 level. *: Correlation is significant at the 0.05 level.

Table 5
Loadings of the significant measured variables on the three first principal components (PCs).

Variables	Components		
	1	2	3
CoL	0.881		
CoHL	0.885		
AdH	0.899		
AdL	0.873		
XAN	0.916		
EPIC	0.526	0.817	
CAT		0.918	
SA	0.500	0.795	
*p*CA		0.947	
CHLA	−0.707	0.583	
TPC			0.825
AC_{DPPH}			−0.900
AC_{ABTS}			−0.965
RUT			−0.748
Eigenvalues	5.42	4.34	3.41
% of variance	62.22	17.17	14.70

Extraction method: principal component analysis. Rotation method: Varimax with Kaiser normalization. Component loadings with absolute values less than 0.400 have been left out of the table for ease of comparison.
CoL: colupolone; CoHL: cohumulone; AdH: N + adhumulone; AdL: N + adlupulone; XAN: xanthohumol; EPIC: epicatechin; SA: syringic acid; CAT: catechin; TPC: total polyphenol content; *p*CA: *para*-Coumaric acid; AC_{DPPH}: antiradical capacity measured by $DPPH^{\bullet}$; RUT: rutin; CHLA: chlorogenic acid; AC_{ABTS}: antiradical capacity measured by $ABTS^{\bullet+}$.

vitro test used ($p < 0.01$), in agreement with their TPC (Fig. 2). Multivariate analysis highlighted no significant differences among solvents used within the same extraction technique when $ABTS^{\bullet+}$ was tested. These results were in contrast with those reported by Kowalczyk et al. (2013), who found a significant difference in EC_{50} values among aqueous and different hydroalcoholic extracts of *H. lupulus* cones. However, the authors did not find any significant difference when different hydroalcoholic extracts were tested.

3.8. Correlation analysis

It is well recognized that the antiradical potential of *H. lupulus* can be ascribed to its pool of bioactive compounds, mainly phenolic ones (Krofta et al., 2008; Kowalczyk et al., 2013; Di Sotto et al., 2018). Table 4 shows the Pearson's correlation coefficients among antiradical capacity and bioactive constituents of *H. lupulus* extracts. Similarly, to Kowalczyk et al. (2013), the antiradical potential of green extracts correlated highly with their TPC ($r = -0.892$ and -0.865, for $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, respectively; $p < 0.01$).

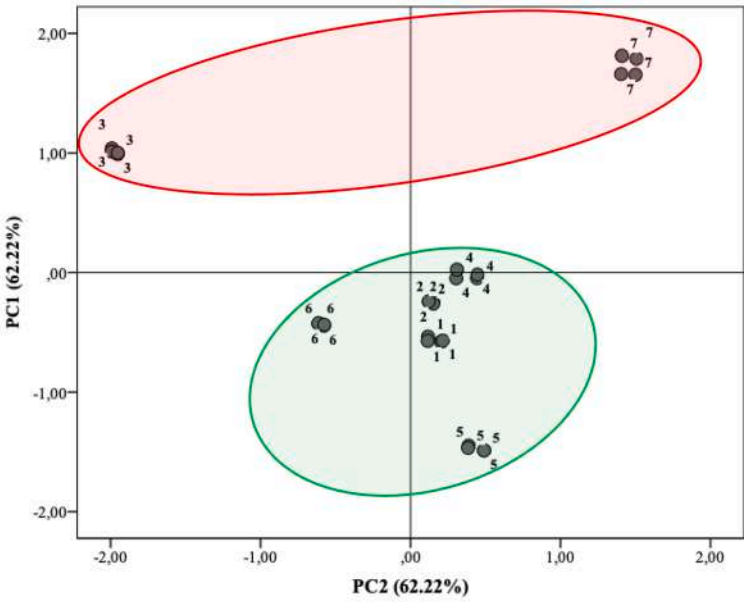
Correlation analysis also pointed out no statistically significant relationships between both TPC and AC_{ABTS} and AA, as also observed by other authors (Elrod et al., 2019), while only a weak negative correlation ($p < 0.05$) was observed between total α-acids and AC_{DPPH}, in contrast with the results reported Karabín et al. (2016). Besides, both antioxidant tests revealed a good and highly significant ($p < 0.01$) relationship with rutin, highlighting its role in the direct quenching of free radicals.

Interestingly, only weak but highly significant ($p < 0.01$) correlations were found among AC_{DPPH}, different bitter acids quantified by HPLC and xanthohumol, while no correlations were found among AC_{ABTS} and the same compounds, supporting the findings of other authors that these compounds contrast the oxidative stress not by directly scavenging the ROS but rather stimulating the endogenous defense mechanism of the cells (Di Sotto et al., 2018).

3.9. Principal component analysis

To better understand the interrelations amongst all the parameters

a)



b)

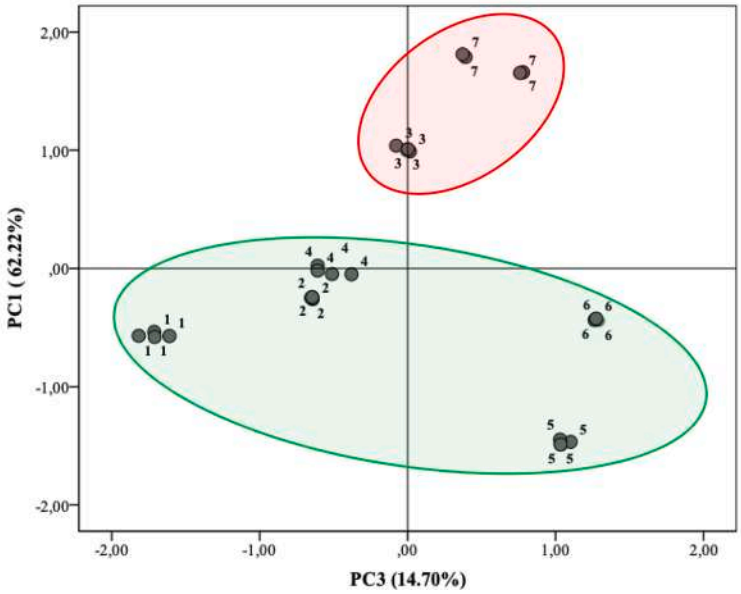


Fig. 11. Score plot of the principal components (PCs): a) PC1 *vs* PC2; b) PC1 *vs* PC3; c) PC2 *vs* PC3. The different extracts are indicated with the following numbers: 1) UAE_{H2O}; 2) UAE_{MIX}; 3) UAE_{ETOH}; 4) CTRL; 5) MAE_{H2O}; 6) MAE_{MIX}; 7) MAE_{ETOH}. UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; ETOH: ethanol; CTRL: control; MIX: ethanol:water mixture (50 %, v/v).

analyzed and technological treatments applied, the raw dataset was subjected to principal component analysis (PCA). PCA was conducted on selected variables, chosen on the basis of the analysis of the correlation and anti-image correlation matrices obtained from the standardized z-scores (Field, 2009), with orthogonal rotation (Varimax model). The Kaiser–Meyer– Olkin measure verified the sampling adequacy for the analysis (KMO = 0.71; Field, 2009). Bartlett's test of sphericity ($p < 0.001$) indicated that correlations between the considered items were sufficiently large for PCA. On the basis of eigenvalues > 1 (Kaiser's criterion) and of the scree plot (not shown), three principal components (PCs) accounting for 94.1 % of the total variance were considered significant. Component loadings after rotation were reported in Table 5, showing the major variables differentiating the green extracts. As we can see, phloroglucinol derivates and xanthohumol loaded highly on factor 1, explaining most of its variance (62 %; rotated solution). Conversely, flavans and phenolic acids loaded highly on factor 2 (17.2 %; rotated

solution), while the rest of the variance seemed related to different aspects of the antiradical capacity of green extracts (14.70 %; rotated solution).

Fig. 11 depicts the scores for samples analyzed in a two-dimensional plots (a–c). Ethanolic extracts had a high score in PC1, being related to high content of xanthohumol and bitter acids (Fig. 11b). Besides, PC2 was able to discriminate between UAE_{ETOH} and MAE_{ETOH} based on their phenolic profiles (Fig. 11a). Finally, MAE and UAE samples were mainly separated each other on PC3 on account of their overall antiradical properties (Fig. 11c).

4. Conclusion

The phytotherapeutic properties of hop cones are mainly due to its secondary metabolites, which have been reported to exert different health-related beneficial effects. The experimental results indicate that both green techniques, UAE and MAE, are effective to a different extent in recovering of hop secondary metabolites, while solvent polarity is a discriminating factor in the recovery of primary metabolites. As a consequence, our study has shown that, depending on the technologies and extraction conditions adopted, it is possible to obtain a different composition of bioactive compounds in the final product from the same matrix and that suitable combinations of solvents/green techniques can be tailored for specific purposes.

Moreover, the combined use of MW with ethanol to obtain hop bioactive extracts could be an important contribution to the development of new therapeutic phytoextracts with low toxicity, thanks to their high content of valuable phenolics, mainly xanthohumol. These findings appear relevant, considering that the use of ultrasonic and MW extraction of plant matrices is a very promising sector for the production of antioxidants on an industrial scale, also, in view of the low energy impact and, especially in the case of MW, the shorter extraction times used to achieve high yields of this high added value compounds.

Credit authorship contribution statement

KC: designed, supervised and revised the study; project administration, funding acquisition. **GP** and **DC**: performed the ^{1}H-NMR experiments and related data analysis; writing - review & editing. **VM**: investigation; writing - review & editing. **VM** and **KC**: analyzed the data and drafted this manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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