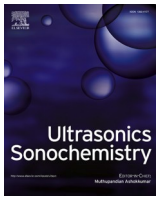
Ultrasonics Sonochemistry 116 (2025) 107322

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson



Evaluation of drying methods and green extraction techniques to enhance the recovery of bioactive compounds from hop leaves: A sustainable approach for the valorisation of agricultural by-products

Katya Carbone *, Valentina Macchioni

CREA Research Centre for Olive, Fruit and Citrus Crops, Via di Fioranello 52, 00134 Rome, Italy

ARTICLE INFO

Keywords:
Green extraction techniques
Hop leaves
Humulus lupulus L
Polyphenols
Xanthohumol, drying techniques

ABSTRACT

This study investigated the effects of different drying techniques and green extraction methods on bioactive compounds in hop leaves, typically considered as waste material. Freeze-drying (FD) and oven-drying (OD) were compared for drying the leaves of five hop varieties, while the study focused on the use of microwave (MAE) and ultrasound (UAE) as innovative extraction techniques. The influence of these factors was then evaluated on several bioactive compounds, including polyphenols, flavonoids, pigments, and xanthohumol, as well as the antioxidant capacity and α-glucosidase inhibition of the extracts obtained. MAE yielded higher total polyphenol and flavan content (TPC and FLC, respectively) values than UAE. Similarly, FD samples showed higher TPC and FLC values than OD ones, whereas chlorophyll *b* was consistently more abundant than chlorophyll *a* in all samples. HPLC analysis identified catechin, epigallocatechin gallate, and *p*-hydroxybenzoic acid as the predominant phenolic compounds. Xanthohumol concentrations ranged from 0.04 ± 0.00 to 1.12 ± 0.03 mg g^{-1}, with MAE yielding higher levels than UAE.

Multivariate analysis revealed that the drying process accounted for the largest proportion of variation in the phytochemical profile (37.1 %), followed by the extraction technique (27.3 %) and hop variety (14.3 %). The hop leaf extracts showed α-glucosidase inhibitory activity, with FD samples showing greater inhibition than OD ones. PCA highlighted the significant influence of the extraction method and drying process on the phytochemical composition of hop leaf extracts.

This research highlights the potential of hop leaves as a sustainable source of phytochemicals for the food, pharmaceutical, and nutraceutical sectors, and emphasises the importance of optimizing extraction and drying techniques.

1. Introduction

Hops (*Humulus lupulus* L.) are widely recognized for their applications in beer production, with research primarily focusing on the bioactive compounds in hop cones [1,2]. However, substantial volumes of hop leaves, stems, and unharvested cones are generated as waste during harvest, representing a significant opportunity for sustainable valorization [3]. The fresh biomass, generated when the plant is harvested, is estimated at about 10–15 tonnes *per* hectare, corresponding to about 2.6 kg *per* plant on average [4]. About hop leaves, it can be stated that they represent approximately 25 % of the plant, calculated on a dry basis (dw), depending on the variety under consideration [5,6].

Previous studies have highlighted the antioxidant, anti-inflammatory, and antibacterial properties of hop leaf extracts [1,3,7], yet the impact of different drying and extraction methods on the bioactive compound profile, particularly across hop varieties, has not been adequately addressed. In a recent study, Macchioni et al. evaluated the efficacy of two drying methods, namely oven drying (OD at 45 °C) and freeze-drying (FD), on hop leaves. FD was identified as the most appropriate method for maintaining the overall nutraceutical profile, whereas OD was more effective in preserving the carotenoids. However, the authors observed a strong interaction between the drying treatment applied and the genotype considered, emphasizing the need to tailor drying methods to specific hop varieties for optimal quality [8]. Furthermore, the selection of an appropriate drying method for hop leaves must consider the energy consumption involved. Based on

* Correspondence author.
E-mail address: katya.carbone@crea.gov.it (K. Carbone).

https://doi.org/10.1016/j.ultsonch.2025.107322
Received 13 January 2025; Received in revised form 7 March 2025; Accepted 21 March 2025
Available online 23 March 2025

estimates, the energy consumption for OD is approximately 6 % less than that for FD [9]. While this may result in some compromise to the phytochemical profile of the plant matrix, it would still allow the use of existing kilns on hop farms. As with other plant matrices, optimal recovery of bioactive compounds from leaves to produce high value-added hop extracts requires the selection of suitable solvents and extraction processes. In recent years, there has been a notable increase in scientific interest in the development of efficient and sustainable extraction technologies [10]. These technologies enable the formulation of phytoextracts that adhere to the principles of green chemistry principles. Currently, environmentally sustainable and unconventional extraction methods, such as ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), have emerged for the recovery of bioactive compounds from plant sources [11]. These techniques have been designed with the dual objective of optimizing yield and maintaining the environmental sustainability of the extraction process [8,12]. Although recent studies have begun to explore green extraction techniques for hop cones, few studies have focused on hop leaves, particularly comparing the efficiency of MAE and UAE in preserving and extracting their bioactive compounds. In this regard, it has been shown that these techniques are effective to in the recovery of secondary metabolites from hop cones [13,14]. While, to the best of our knowledge, there are no literature studies on the use of microwaves for the extraction of bioactive compounds from hop leaves, Muzykiewicz et al. evaluated and compared the antioxidant activity of alcoholic extracts of fresh hop leaves obtained by the use of ultrasound [15], while Macchioni et al. used UAE to recover phytochemicals from five different hop leaf varieties [8]. Despite the known bioactive potential of hop leaves, a comprehensive comparison of the effectiveness of green extraction techniques, specifically MAE and UAE, in preserving and extracting these compounds across different hop genotypes and drying processes, remains lacking. This gap limits the development of optimized valorization processes for hop leaf biomass.

Therefore, this study aimed to fill this gap by evaluating the efficiency of MAE and UAE in recovering bioactive compounds from hop leaves, considering the influence of hop variety and drying method. It focused on analyzing the content and profiles of key phytochemicals, including polyphenols and xanthohumol, and assess their biological properties in vitro, to establish a foundation for the sustainable valorization of hop leaves.

2. Materials and methods

2.1. Chemicals

Folin-Ciocalteau reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), vanillin, 2,2'-azinobis-(3-ethylbenzothiazolin-6-sulphonic acid) (ABTS$^{\bullet+}$), potassium persulphate and ethanol were purchased from Sigma-Aldrich (Milan, Italy). The standards used for identification and quantification by high-performance liquid chromatography (HPLC) were purchased from Extrasynthese (Genay, France). All other solvents and reagents used were of analytical grade. Prior to analysis, all the green extracts obtained were filtered through membrane filters (cellulose acetate) with a pore size of 0.45 μm purchased from Pall (Pall Corporation, Ann Arbor, MI, USA).

2.2. Plant material

At the time of harvesting, hop leaves from five varieties (Table 1) were sampled at an organic farm near Rome, Italy (41°63′46″N-12°87′18″E). The crop was harvested in 2023. A portion of leaves (300 g) from each genotype was oven dried (OD) at 45 °C using an air speed of 0.6 m/s, a relative humidity of less than 0.5 % and a system power of 1.4 kW/h (model 600, Memmert GmbH + Co. KG, Schwabach, Germany). This drying method and temperature were chosen to suit the kilns commonly used on hop farms. The remaining 300 g of leaves was freeze dried (FD) at −54 °C under a pressure of 0.075 mbar (model Modulyo 4 K, Edwards, UK) [8]. Dehydration of the samples by all the above methods was continued until a final moisture content of approximately 8–10 % was reached. At the end of each drying treatment, samples were finely ground (0.5 mm sieve), stored under vacuum and protected from light and moisture until analysis. Four replicates were made for each treatment.

2.3. Extraction processes

The powdered leaf samples were extracted with ethanol using at a solid-to-liquid ratio of 1:30, using the following methods.

2.3.1. Ultrasound-assisted extraction of hop leaves

Leaf samples were subjected to ultrasound extraction as outlined by Macchioni et al. [8], employing ethanol (96 %) as a food-grade solvent to obtain green extracts ready for use in various sectors, including phytotherapy and cosmetics. Briefly, samples (1.0 g) were first mixed with 15 mL of ethanol (96 %) in test tubes with screw caps on a magnetic stirrer (300 rpm; Heidolph Mr. 2002, Kelheim, Germany) in the dark and at room temperature (25 °C). Subsequently, the mixture was subjected to the extraction process for 30 min at 25 °C, under ultrasound irradiation in a temperature-controlled sonication bath (UTA-200, Falc, Italy), operating at 40 kHz. The resulting extracts were then centrifuged at 6792 × g for 15 min at 4 ∘C. Pellets were extracted once again in the same manner. The extracts obtained were stored at −80 °C, under vacuum until analysis.

2.3.2. Microwave-assisted extraction of hop leaves

Leaf samples were subjected to microwave extraction according to Carbone et al. [13], using ethanol (96 %) as solvent. Briefly, the extraction was carried out in a closed vessel, in a CEM MARS 5 professional multimode oven operating at 2.45 GHz (CEM, Matthews, NC, USA). The inner temperature of the sample vessel was measured and controlled with a microwave-inert optic fiber temperature probe, while the pressure inside the microwave system was measured by a gauge probe. Hop leaf samples (1.0 g) were extracted 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. The extracts obtained were stored at −80 °C, under vacuum until analysis.

Table 1
Sample codes.

Hop's purpose	Hops variety	Drying methods[a]	Code	Hops variety	Drying methods	Code
Dual	Chinook	FD	V1	Chinook	OD	V6
Dual	Centennial	FD	V2	Centennial	OD	V7
Bittering	Comet	FD	V3	Comet	OD	V8
Dual	Columbus	FD	V4	Columbus	OD	V9
Dual	Cascade	FD	V5	Cascade	OD	V10

[a] OD: oven-drying, FD: freeze-drying.

2.4. Phytochemical analysis of hop leaves

The total polyphenol content (TPC) of the samples was determined as described by Carbone et al., without modifications [13] at 765 nm using a UV–vis spectrophotometer (model 6300 PC, VWR, Milan, Italy). TPC was expressed as milligrams of gallic acid equivalents *per* gram of dried sample (mg GAe g^{-1}). Total flavan content (FLC) was determined using the vanillin assay method, as described by Carbone et al. [13]. The results were expressed as milligrams of catechin equivalents *per* gram of dried sample (mg CAE g^{-1}). The non-phenolic pigment content (i.e. chlorophyll *a* (Chla) and b (Chlb), total carotenoid content (TCC)) of the analysed samples was determined according to Lichtenthaler & Buschmann [16]. Results were given in μg g^{-1} of the dry sample. All analyses were performed in triplicate.

2.5. Determination of individual phenols and xanthohumol by HPLC

The separation and identification of hop leaf polyphenols were performed using an HPLC system (Agilent 1100 series, Agilent, Italy) with a photodiode array detector (DAD; Agilent Technologies, Italy), following the method outlined by Carbone and Mencarelli [17], without modifications. The different phenolic compounds were identified using a combination of three methods: first, by their retention time; second, by spectral data against individual standards; and third, by reference to data in the literature. The standard addition method was also used to confirm the identity of the compounds in the samples. The results were expressed as μg g^{-1} of dried hop leaves.

Xanthohumol (XN) was quantified according to Carbone et al. [13], without modifications, using an analytical HPLC − DAD system (Agilent 1100 series, Agilent, Italy), set at 370 nm. XN was identified by analyzing its retention time, spectral data, and the application of the standard addition method to the samples. The results were expressed as mg g^{-1} of dried sample.

2.6. Antiradical capacity (AC) assays

The antiradical capacity of hop leaf extracts was evaluated by measuring their ability to scavenge.

synthetic radicals (e.g., $DPPH^{\bullet}$ and $ABTS^{\bullet+}$), according to Carbone et al. [13], without modifications. The results were expressed as EC_{50}. All measurements were performed in triplicate.

2.7. Glucosidase inhibition assay

The α-glucosidase activity of analysed samples was evaluated according to the Sigma-Aldrich protocol for the enzymatic assay of α-glucosidase [18], using *p*-nitrophenyl-D-glucoside as substrate. Briefly, hop extracts were evaporated under vacuum using a rotary evaporator (50 °C, 60 rpm) (Büchi Rotavapor® R-300; Büchi, Milan, Italy), followed by nitrogen evaporation. Dried extracts were then dissolved in DMSO at a concentration of 1 mg mL^{-1} and then diluted using potassium phosphate buffer (pH 6.8; 67 mM) to achieve final test concentrations, ensuring that the final concentration of DMSO did not exceed 1 % in the assay to avoid enzyme inhibition. The α-glucosidase activity was spectrophotometrically determined, in the absence (negative control) or presence of leaf extracts (sample), by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-D-glucopyranoside at 400 nm.

Inhibition of the enzyme activity was expressed as percentage inhibition and calculated as follows:

$$\alpha\ glucosidase\ inhibition(\%) = \left[1 - \left(Abs_{sample} - Abs_{blank}\right) / \left(Abs_{negative\ control}\right)\right] \times 100$$

All determinations were performed in triplicate.

2.8. Statistical analysis

Statistical analysis was performed with SPSS 25.0 software (SPSS, Inc., Chicago, Illinois) and using the R software version 4.3.1 [52].

Data were reported as mean ± standard deviation (SD) of four independent experiments with three replicates. The interquartile range (IQR) method was utilised to detect any potential outliers and then the log transformation was applied to the entire dataset with a view to reducing skewness and thereby mitigating the impact of any outliers that were detected. An exploratory analysis of the data was carried out to check the normal distribution (Shapiro-Wilkinson test) and equality of variances (Levene's test). The results of these tests showed a non-normal distribution (all Shapiro-Wilk p-values < 0.05) and for some variables even heteroscedasticity after log-transformation. Therefore, non-parametric inferential analysis was used to analyze the data. The effect of genotype (V), drying method (T), extraction techniques (E) and their interactions on the variables considered in the study were analysed with a permutational multivariate analysis of variance (PERMANOVA; n. permutations: 999) using the adonis2 function from the vegan R-package [19].

PERMANOVA was then followed-up by Kruskal-Wallis tests for individual variables, using Dunn's test for post-hoc pairwise comparisons. Moreover, a correlation heatmap was computed using Kendall's Tau Coefficient, considering the limited sample size.

Finally, Principal Component Analysis (PCA) with varimax rotation was employed as an exploratory chemometric method to examine the data structure and to identify similarities and underlying patterns within the analysed samples.

3. Results and discussion

3.1. Phytochemical composition of hop leaf extracts

The present study analysed the overall phytochemical content of leaf extracts of five different hop varieties (Table 1). Irrespective of genotype, drying method and extraction technique employed, TPC ranged from 2.3 ± 0.5 to 46 ± 1 mg GAE g^{-1} (for the samples V10 U and V1 M, respectively), while FLC ranged from 0.44 ± 0.04 to 11.71 ± 0.06 mg CAE g^{-1} (for the samples V9 U and V2 M, respectively). These results are in line with the previous ones, with small differences in absolute values probably due to the different crop year considered [8] and, on average, higher than those reported by Čeh et al. for leaves of different hop varieties oven-dried at 45 °C [5].

As far as non-phenolic pigments are concerned, Chla ranged from 101 ± 2 to 723 ± 7 μg g^{-1} (for V6 UAE and V4 MAE, respectively), whereas Chlb ranged from 283 ± 4 to 1860 ± 19 μg g^{-1} (for V5 UAE and V9 MAE, respectively). According to literature studies shaded plants have a higher proportion of Chlb than Chla [20]. Finally, TCC ranged from 90 ± 4 to 291 ± 3 μg g^{-1} (for the samples V6 UAE and V10 MAE, respectively). These results confirm the high content of non-phenolic pigments in hop leaves, comparable and even higher than that reported for olive leaves [21], thus presenting an opportunity for their valorisation through the utilisation of this waste biomass. Emerging evidence, in fact, suggests that carotenoids and chlorophylls possess diverse biological functions beyond their fundamental antioxidant properties [22].

3.2. Influence of extraction method and drying technique on the phytochemical profile of hop leaf green extracts

Fig. 1a shows the effects of adopted extraction methods on the polyphenol content of hop leaf extracts as a function of drying technique and variety. The TPC-reducing effect of oven-drying has been observed in the literature for several plant matrices (e.g. spearmint and Carica papaya leaves) and was confirmed by Macchioni et al. for hop leaves [8,23,24]. This reduction may be attributed to the degradation of

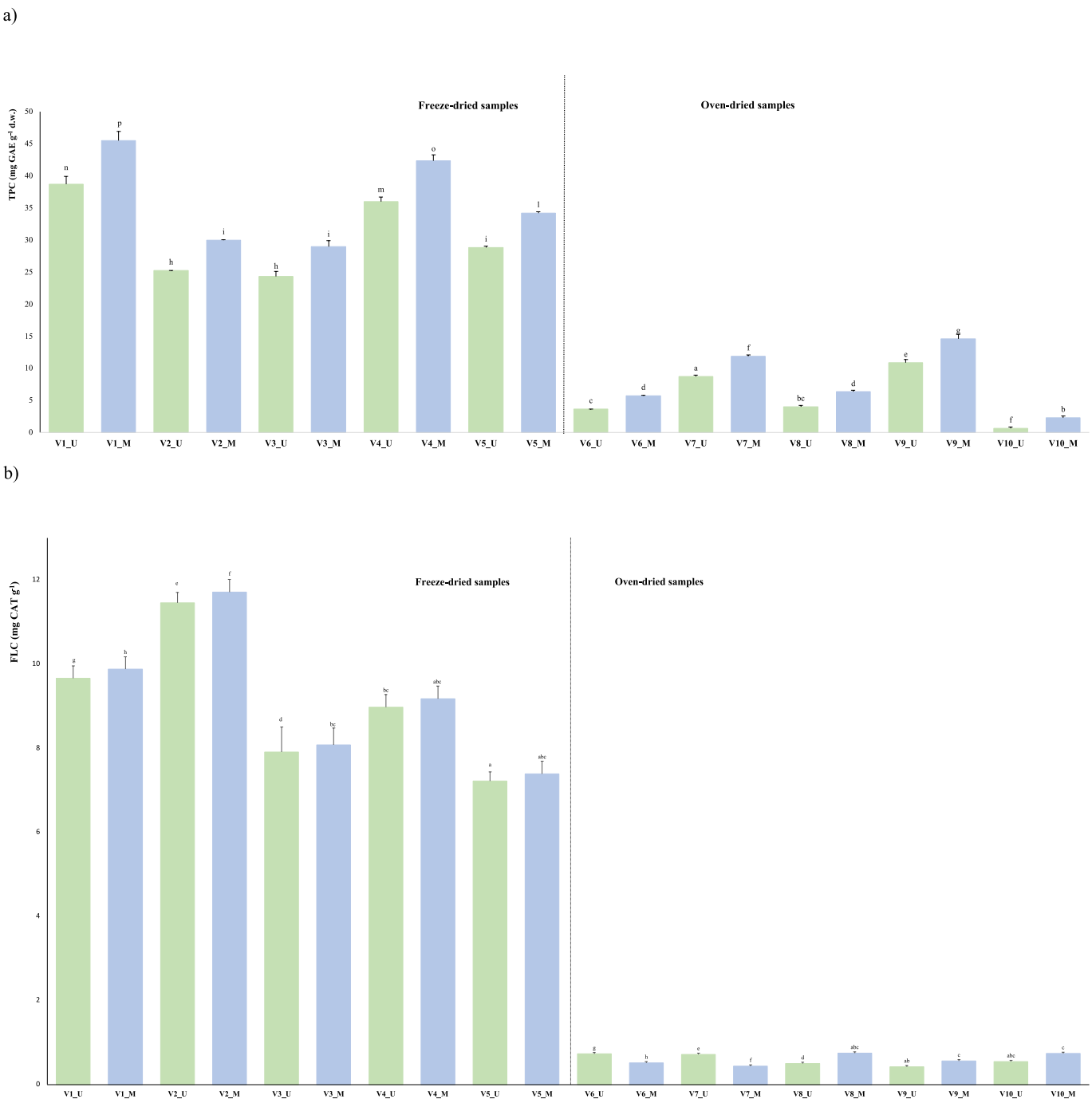


Fig. 1. a) Total polyphenol content of hop green extracts (mean ± s.d.). TPC: total polyphenol content; GAE: gallic acid equivalent; U: ultrasound- assisted extraction; M: microwave-assisted extraction. Different letters indicate significant differences in the mean ($p < 0.05$). b) Total flavan content of hop green extracts (mean ± s.d.). FLC: total flavan content; CAT: catechin equivalent. U: ultrasound- assisted extraction; M: microwave-assisted extraction. Different letters indicate significant differences in the mean ($p < 0.05$).

antioxidant compounds following exposure to thermal processing [25]. In contrast, the FD method involves the formation of minute ice crystals within the cell, which are expelled by sublimation during the freezing process, forming highly porous microstructures. Sublimation of water crystals facilitates the preservation of cellular structures, thereby enabling the retention of phenolic compounds and enhancing solvent access and extraction [26].

As far as extraction techniques are concerned, the highest TPC content was recorded, within each hop variety, for samples extracted by microwave. In addition, the average increase in TPC observed for the MAE samples was around 10 % compared to the UAE samples, regardless of the hop variety considered.

This finding is particularly significant considering that the extraction time of the MAE is substantially shorter than that of the UAE. In the present study, high-intensity ultrasound (40 kHz and 60 W cm^{-2}) was applied to the plant matrix, which was capable of producing a high cavitation effect by generating a strong local temperature rise that might have degraded and/or oxidised the polyphenol fraction, reducing its content in the extracts [27].

About the flavan-3-ols, the drying method applied exerted a highly significant influence on their content ($p < 0.001$), with a reduction of FLC in the OD samples of approximately 93 % compared to that of the FD

samples, irrespective of the hop variety and extraction technique under consideration (Fig. 1b). These findings are in agreement with the literature on different plant matrices [28]. Samoticha et al. demonstrated that the preservation of bioactive compounds is optimised by low temperatures and low oxygen levels, such as those encountered during freeze-drying [29]. The highest FLC was observed for Centennial among the FD samples ($p < 0.05$), whereas Comet and Cascade exhibited the highest FLC among the OD samples ($p < 0.05$), for both extraction techniques utilized.

The total leaf pigments, including chlorophyll-a, chlorophyll-b, and carotenoids, are essential for photosynthesis. The concentration of these pigments varies across different plant species. Their diversity and interrelationships can be attributed to a combination of internal factors and environmental conditions [30]. In the present study, three classes of hop leaf pigments were analysed: Chla, Chlb, and TCC (Fig. 2). The median values indicate that for all three variables considered, the MAE group had higher median values than the UAE one, which were statistically significant ($p < 0.01$), except for TCC, for which variety had the greatest effect ($p < 0.001$). On average, and without considering the variety or the drying process applied, the use of ultrasound resulted in a reduction of approximately 43 % in Chla and 64 % in Chlb in comparison to MAE. Furthermore, the concentration of Chlb was consistently higher than that of Chla in all the samples analysed ($p < 0.01$) on average. This difference has also been observed in the sunlight of some other leaf types, as well as when a single species has been grown under different light intensities [31]. With regard to TCC, higher values were recorded in the OD samples than in the FD ones for the Centennial, Comet and Cascade varieties ($p < 0.01$), irrespective of the extraction technique used.

3.3. *Influence of extraction method and drying technique on the antiradical capacity of hop leaf green extracts*

Research has indicated that phytochemicals extracted from hop leaves may offer a range of health benefits, as demonstrated by in vitro tests, with the majority of these benefits relating to their antioxidant properties [3,5,32,33]. The antiradical capacities of the hop extracts are shown in Fig. 3. Regarding the drying method, in general, highly statistically significant differences ($p < 0.001$) for both in vitro assays were observed between the FD and OD samples.

Moreover, the highest AC values were recorded for samples extracted by microwaves, although all green extracts showed the ability to quench

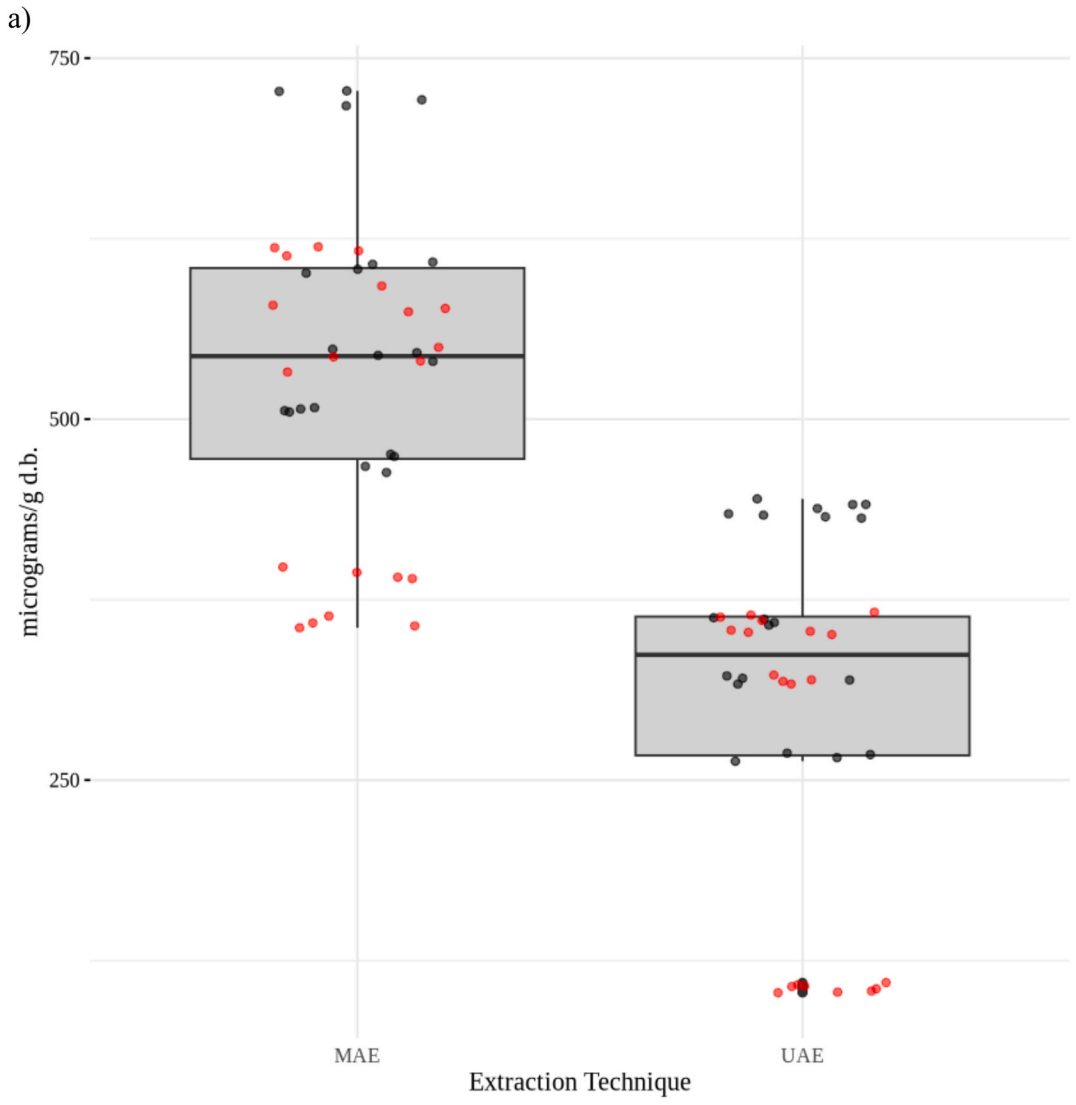


Fig. 2. Box-plot of total leaf content of hop green extracts. Box plot explanation: right edge of the box, 75th centile; left edge, 25th centile; vertical bar within box, median; right vertical bar outside box, maximum value; left vertical outside box, minimum value. Points outside the box are outliers or suspected outliers; a) Chlorophyll *a* content; b) Chlorophyll *b* content; c) TCC: total carotenoid content. UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction. In the graph, the red dots refer to oven-dried samples and the black dots to freeze-dried samples.

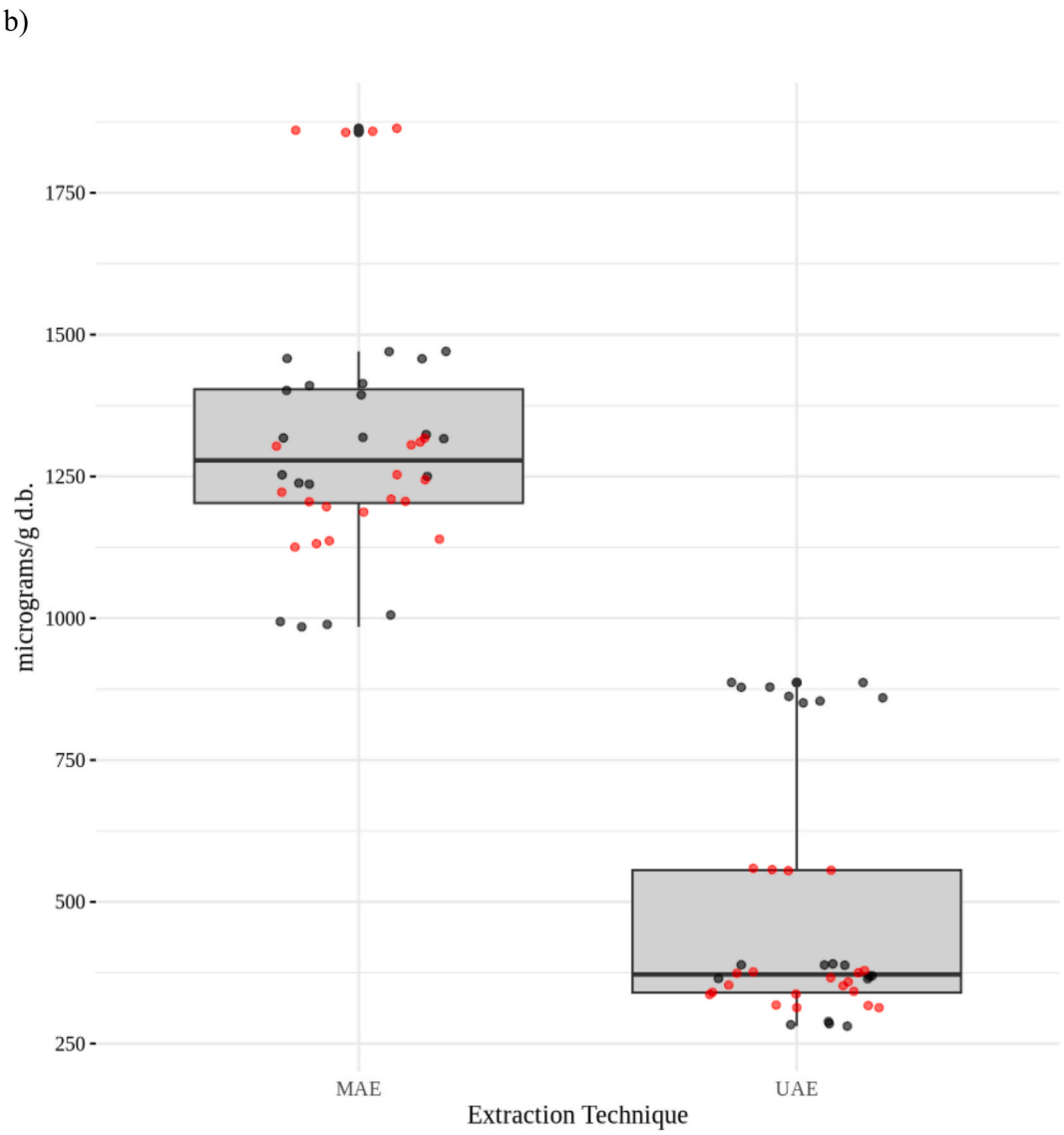


Fig. 2. (*continued*).

DPPH$^{\bullet}$ at concentrations higher than those required to quench ABTS$^{\bullet+}$. These results can be plausibly attributed to the chemical structure of the bioactive molecules in the analyzed green extracts, as steric accessibility is a key factor in the DPPH$^{\bullet}$ quenching reaction, and by their more or less lipophilic character [34]. Microwave-assisted extraction produced hop leaf extracts with stronger antioxidant properties than ultrasound-assisted extraction, and this was true across different drying process applied and in vitro assay used ($p < 0.01$). This effect was statistically significant and consistent with the higher phenolic content in MAE extracts.

3.4. HPLC analysis of polyphenols

In the present study, four classes of phenolic compounds were analysed, based on the available analytical standards: phenolic acids (hydroxybenzoic and hydroxycinnamic), flavan-3-ols and flavonols (Table 2). Regardless of variety and type of treatment, the most abundant compound present in hop leaf extracts was catechin, followed by epigallocatechingallate (EGCG) and *p*-hydroxybenzoic acid. The catechin content of hop leaf extracts ranged from 296 ± 4 to 2740 ± 211 μg g^{-1}, with the V9 UAE and V1 MAE samples displaying the lowest and highest concentrations, respectively. Conversely, the EGCG content varied from 111 ± 1 to 1986 ± 4 μg g^{-1} for the V7 UAE and V4 MAE samples, respectively. These findings align with the flavan monomer content observed in dual-purpose hop cones by Chenot et al. (427–4196 mg kg^{-1}) [35], indicating that hop leaves constitute a viable and cost-effective source of catechins. To the best of our knowledge, there are few studies in the literature on the quantification of the different polyphenols in hop leaves [36,37]. Keskin et al. [37] analysed the different polyphenol content in hop cones and leaves, without specifying the variety used. In the study, the most abundant compound in the leaves was epicatechin (825.57 ± 0.04 μg g^{-1}), while catechin was not detected. Recently, Calvert at al. [36] analysed the leaf polyphenol profiles of three different hop varieties (Cascade, Calypso and Contessa) at three different development stages (flower, middle and harvest stage), revealing that catechin was the most prevalent polyphenolic compound in hop leaves. However, the mean concentration was markedly lower than that observed in our findings, particularly in the case of the Cascade variety (average catechin content at harvest stage: 2.81 mg 100 g^{-1} db), probably due to the different extraction method adopted by the Authors. This highlights the pivotal role of the extraction process in tuning the amount and type of bioactive compounds that can be extracted from the plant matrices.

To the best of our knowledge, this study is the first to identify and

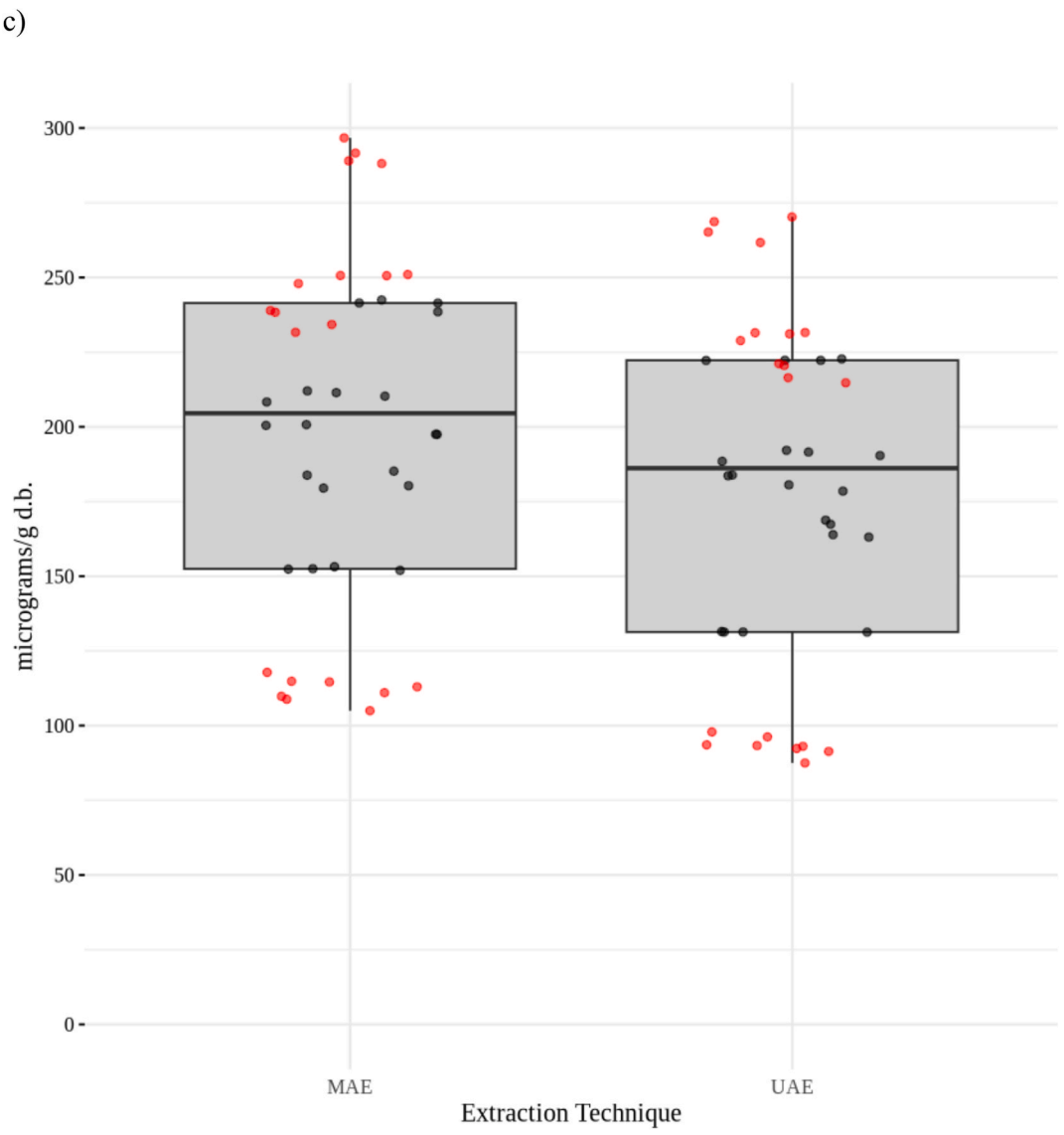


Fig. 2. (*continued*).

quantify ECGC in hop leaves. The beneficial effects of tea on human health are primarily attributed to this compound, which is considered the most significant bioactive constituent found within the beverage [38]. Herein, the EGCG content found in the analysed hop leaf samples was comparable to the values reported by Lee & Lee for green and black tea extracts obtained by the dipping method and using ethanol as a solvent, and even higher in the case of microwave extracts [39]. These findings suggest the potential for the use of hop leaves in the development of nutraceutical products and functional beverages, as well as to extend the shelf life and to improve the flavor stability.

As far as the extraction technique is concerned, microwave irradiation enhanced the extraction of both catechin and EGCG ($p < 0.05$ and $p < 0.001$, respectively), regardless the drying treatment applied to the plant matrix. It has been reported that catechins are susceptible to degradation when subjected to ultrasonic treatment [40]. Ultrasound waves create microscopic cavitation bubbles in the solvent, which grow during the rarefaction phase and collapse violently during the compression phase, leading to localized high temperatures (up to 5000 K) and pressures (up to 1000 atm) [41]. The collapse of these bubbles has been shown to result in the homolytic cleavage of solvent molecules, producing hydroxyl radicals (·OH) and hydrogen radicals (·H), leading to the oxidation of the hydroxyl groups on the aromatic rings. This results in structural modifications and the formation of quinone-like structures. Horžić et al. reported that, among the flavan-3-ol catechins present in yellow tea, catechin and EGCG were the most sensitive to degradation when subjected to ultrasound [41].

The results also indicated a substantial impact of the drying process on the flavan content of hop leaf extracts ($p < 0.001$), revealing a more substantial decrease in EGCG concentration than in catechin. While freeze-drying is a low-temperature process, during the oven-drying process, the high temperature and structural changes in the plant matrix can lead to the activation of oxidative enzymes, such as polyphenol oxidase (PPO) and peroxidase, and the release of hydrolytic enzymes, which can lead to the degradation of these compounds [42].

Regarding hydroxycinnamic acids (HCAs), only *p*-coumaric acid was identified in all hop varieties subjected to analysis. Its concentration was highest in the V3 MAE samples (743 ± 11 μg g^{-1}; $p < 0.05$). These findings are consistent with those previously documented in scientific literature, although the values reported here are, on average, higher, particularly for samples of freeze-dried leaves extracted using microwave technology [36,37]. Interestingly, cinnamic acid was detected only in V5 MAE samples. Of the factors analysed, the drying process had the greatest impact on the overall HCA concentration in the hop leaf extracts ($p < 0.01$). Madrau et al. reported a similar degradation trend

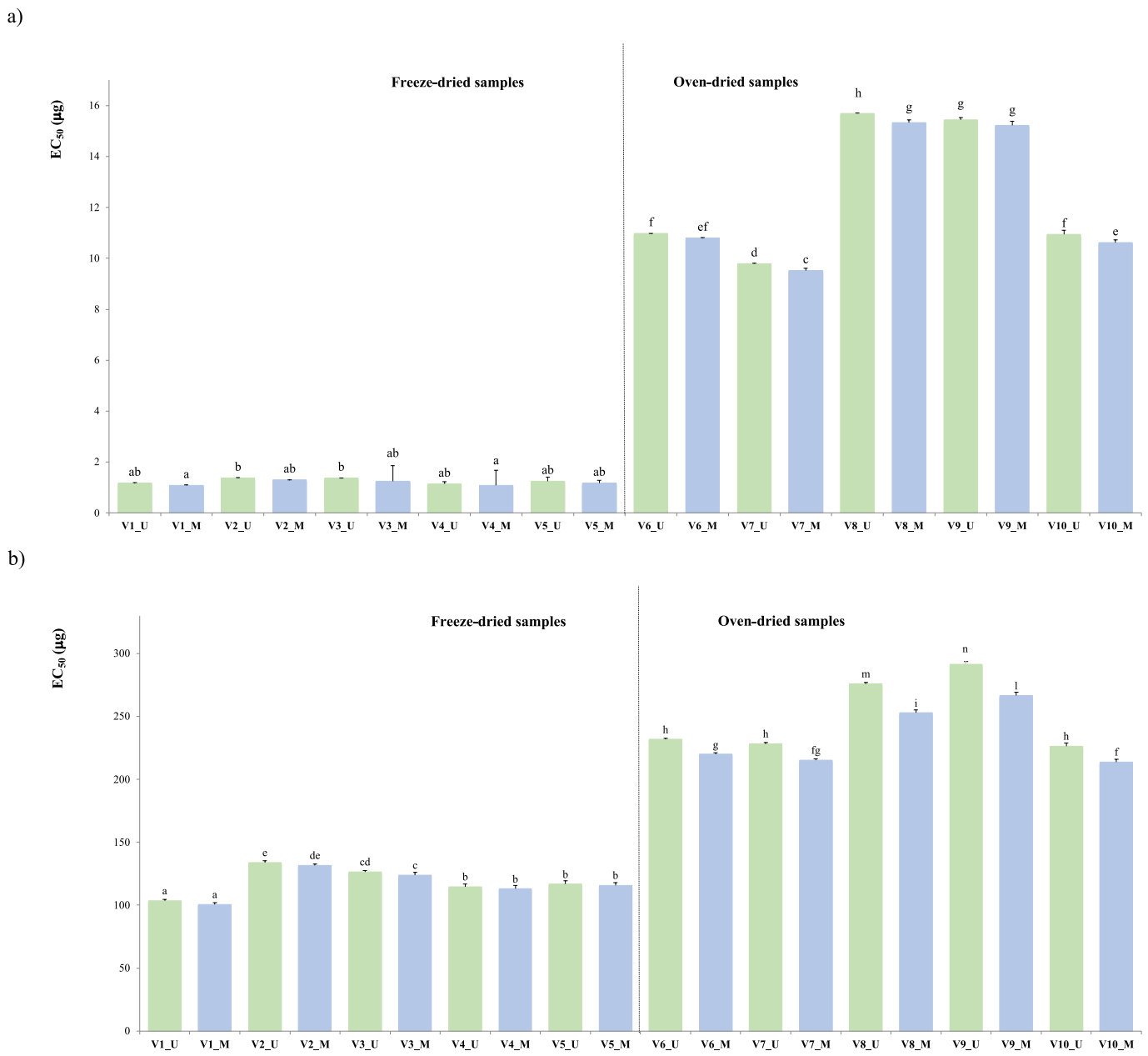


Fig. 3. Antiradical capacity of hop leaf extracts (mean ± s.d.). a) Results from $ABTS^{\bullet+}$ in vitro assay; b) Results from $DPPH^{\bullet}$ in vitro assay. All data are expressed in terms of EC_{50} as µg of dried sample. Different letters indicate significant differences in the mean ($p < 0.05$).

for both catechins and hydroxycinnamic acids. In their study on the effect of drying temperature on apricot polyphenols, the authors attributed this tendency to PPO activation at drying temperatures of 55 °C [43]. This temperature closely corresponds to the oven-drying temperature that was used in the present study (45 °C). Zhang reported that polyphenol oxidases (PPOs) in different plant species have different optimal temperatures, with the majority in the 30–50 °C range [44].

Regarding the hydroxybenzoic acids (HBAs), the most abundant acid was *p*-hydroxy benzoic one. This was followed by ellagic acid and syringic acid, irrespective of the factors analysed. It is noteworthy that syringic acid was only identified in the freeze-dried hop leaves of the Chinook variety (V1), regardless of the extraction method employed. To the best of our knowledge, this study represents the first report on the presence of this phenolic acid in hop leaf extracts, with a concentration exceeding that found in hop cones [45] and comparable to and in many cases higher than that reported for several food products, except grape seeds [46]. This observation is significant, because the high biological potential of this compound, which can also be utilized for industrial purposes, is well established [46].

The drying process had a substantial impact on the different factors analyzed ($p < 0.001$). Moreover, the microwave-irradiated OD samples exhibited a pronounced decrease in the mean concentration of these acids compared with the UAE OD samples. To the best of our knowledge, there are no literature studies on the combined effect of ultrasound/microwaves with different drying processes on the content and profile of HBAs. However, it can be assumed that the observed results were the result of a combination of effects. First, during oven drying, the compounds undergo oxidative degradation due to the PPOs present in the plant matrix, which are active at the process temperatures. Then, the application of ultrasound leads to structural deformation of the matrix itself due to the associated sono-physical effects, causing the cell walls to break down and releasing the phenolic acids bound to them. Conversely, microwaves preserve the cellular structures and inherently cause the

Table 2
Polyphenol profiles of hop leaf green extracts expressed as μg g^{-1} on dry basis (mean ± SD)[1].

Sample codes	*p*-hydroxybenzoic acid		Syringic acid		Ellagic acid		*p*-Coumaric acid		Caffeic acid		Ferulic acid		Cinnamic acid		Catechin		Epigallocatechingallate		Rutin	
Extraction	UAE	MAE	UAE	MAE	UAE	MAE	UAE	MAE	UAE	MAE	UAE	MAE	UAE	MAE	UAE	MAE	UAE	MAE	UAE	MAE
V1	690 ± 18o	630 ± 16*n*	257 ± 10a	237 ± 6a	105 ± 1f	nd	55 ± 3 g	168 ± 4i	312 ± 3o	185 ± 1*n*	921 ± 13p	251 ± 2 *m*	nd	nd	816 ± 3d	2740 ± 21 l	724 ± 13 cd	630 ± 24bc	nd	nd
V2	706 ± 19o	718 ± 1o	nd	nd	160 ± 4i	135 ± 2 g	105 ± 2 h	109 ± 3 h	63 ± 1f	109 ± 2 g	nd	57 ± 3ed	nd	nd	783 ± 6d	2063 ± 13i	199 ± 5a	1026 ± 5de	321 ± 3b	133 ± 2a
V3	718 ± 20o	503 ± 30lim	nd	nd	161 ± 6i	143 ± 6 h	66 ± 4 g	743 ± 11 l	nd	165 ± 2il	459 ± 5o	272 ± 5*n*	nd	nd	1808 ± 12 *g*	1195 ± 8 h	756 ± 3 cd	836 ± 4d	nd	nd
V4	518 ± 2 ml	478 ± 1ihl	nd	nd	nd	nd	63 ± 21 g	40 ± 2e	174 ± 5 ml	48 ± 5e	nd	36 ± 1bc	nd	nd	1089 ± 15f	807 ± 7d	811 ± 8d	1986 ± 4f	nd	nd
V5	1686 ± 5p	339 ± 7 g	nd	nd	212 ± 21	nd	105 ± 5 h	51 ± 2 fg	113 ± 4 g	169 ± 8li	129 ± 7gh	139 ± 3hgil	nd	26 ± 1	997 ± 7e	2032 ± 6i	560 ± 9b	1189 ± 6e	nd	nd
V6	nd	35 ± 1a	nd	nd	nd	nd	nd	17 ± 2ab	nd	31 ± 2b	nd	46 ± 2dbce	nd	nd	410 ± 2b	426 ± 8b	267 ± 8ab	410 ± 7b	nd	nd
V7	nd	54 ± 1bac	nd	nd	45 ± 2d	nd	18 ± 1cb	26 ± 1bac	39 ± 1c	47 ± 2e	nd	70 ± 2f	nd	nd	395 ± 4b	646 ± 16	111 ± 1a	622 ± 4bc	nd	nd
V8	283 ± 1f	33 ± 2a	nd	nd	37 ± 6c	nd	31 ± 1cb	14 ± 1ab	nd	51 ± 5e	144 ± 3llh	41 ± 2cb	nd	nd	560 ± 5c	540 ± 8c	287 ± 5a	470 ± 17b	nd	nd
V9	239 ± 13e	76 ± 10cb	nd	nd	28 ± 2b	4 ± 1a	21 ± 11cb	nd	48 ± 3e	21 ± 4a	23 ± 1a	nd	nd	nd	296 ± 4a	391 ± 14b	275 ± 3a	664 ± 11bc	nd	nd
V10	453 ± 1hi	172 ± 3d	nd	nd	57 ± 1e	nd	nd	37 ± 3e	nd	146 ± 7 h	nd	142 ± 5ihl	nd	nd	805 ± 8d	1113 ± 17f	319 ± 7ab	1006 ± 14de	nd	nd

[a]Different lower case letters in the same column indicate significant differences among samples ($p < 0.05$); nd: not determined.

rapid and intense heating of polar substances, which can potentially degrade the most sensitive molecules [26,47].

In general, regarding the influence of the drying method applied, the total phenolic acid content (PAC) quantified in the present study ranged from 256 to 1372 μg g^{-1} for FD and OD, respectively, and was independent of the genotype and extraction technique considered. Regarding the extraction technique, PAC ranged from 701 to 928 μg g^{-1} for MAE and UAE, respectively and independently of the genotype and drying method considered. In this case, the PERMANOVA showed a significant effect of factor E only for caffeic acid ($p < 0.05$) and ellagic acid ($p < 0.001$). Finally, regarding genotype, PAC ranged from 455 to 1051 μg g^{-1} for Columbus and Cascade respectively independently of the E and T factors, resulting significant only for ellagic acid ($p < 0.01$) and ferulic acid ($p < 0.001$). Nevertheless, the range of PAC reported herein was higher than that observed recently by Calvert et al. in hop leaf extracts for different genotypes [36].

3.5. HPLC quantification of xanthohumol in different green extracts

Xanthohumol (XN), a prenylated chalcone found in hops, plays important roles in aging, diabetes, inflammation, microbial infection, and cancer [1]. Fig. 4 shows the XN content of the analyzed hop extracts, which ranged from 1.12 ± 0.03 to 0.04 ± 0.00 mg g^{-1}, for V1 MAE and V9 MAE, respectively. In general, the mean XN content in the samples extracted via ultrasound was approximately half of that obtained through microwave extraction, with the exception of the Centennial and Columbus hop leaf samples, for which ultrasound-assisted extraction yielded extracts characterised by higher XN values than the corresponding MAE samples. Interestingly, the XN content of the UAE Cascade samples (V5 and V10) was consistent with the findings of Calvert et al. [36], whereas it was higher when referring to MAE samples (19.19 and 66.46 mg 100 g^{-1}, for V10 and V5 samples, respectively).

3.6. Multivariate analysis findings

The PERMANOVA analysis was conducted on the entire dataset according to the 2.8 subheading. The results showed significant effects ($p < 0.001$) on all the main factors and their interactions (Table 3).

Regarding the main effects, the non-parametric multivariate analysis pointed out that hop variety (V) explained 14.3 % of variation, whereas the drying process (T) showed the strongest effect, explaining 37.1 % of variation, while the extraction technique (E) explaining 27.3 % of variation. Regarding factor interactions, the strongest interaction was found for the two-way interactions VxT (8.9 % of variation explained) and VxE (7.1 % of variation explained).

The Kruskal-Wallis tests for each response variable against each factor were then conducted, and significant results for each factor were identified (Table S1). Then, Dunn's test for post-hoc comparisons on these significant variables for each factor was performed, highlighting as key findings that as far as genotype was concerned, TCC showed highly significant differences ($p < 0.001$) between Cascade *vs* Chinook, Cascade *vs* Columbus, Columbus *vs* Comet, and Centennial *vs* Columbus. Regarding the extraction technique, there were highly significant differences ($p < 0.001$) between MAE and UAE for Chla, Chlb, EGCG and ellagic acid. Finally, regarding the drying process, all significant variables, except for non-phenolic pigments and rutin, showed highly significant differences between FD and OD ($p < 0.001$).

Finally, Fig. 5 shows the relationships among the variables analyzed, using the Kendall's Tau correlation coefficients. The results indicated some good positive correlations (AC_{ABTS} and AC_{DPPH}: $\tau \approx 0.81$), whereas TPC and FLC were negatively correlated with both AC_{ABTS} and AC_{DPPH} (TPC with AC_{ABTS} and AC_{DPPH}: $\tau \approx -0.64$; FLC with AC_{ABTS} and AC_{DPPH}: $\tau \approx -0.54$). The results also indicated some significant moderate correlations: positive correlations between TPC and caffeic acid (CA) ($\tau \approx 0.48$) and FLC and *p*-coumaric acid ($\tau \approx 0.50$), and negative correlations between AC_{ABTS} and AC_{DPPH} and catechin and caffeic acid ($\tau \approx -0.55$).

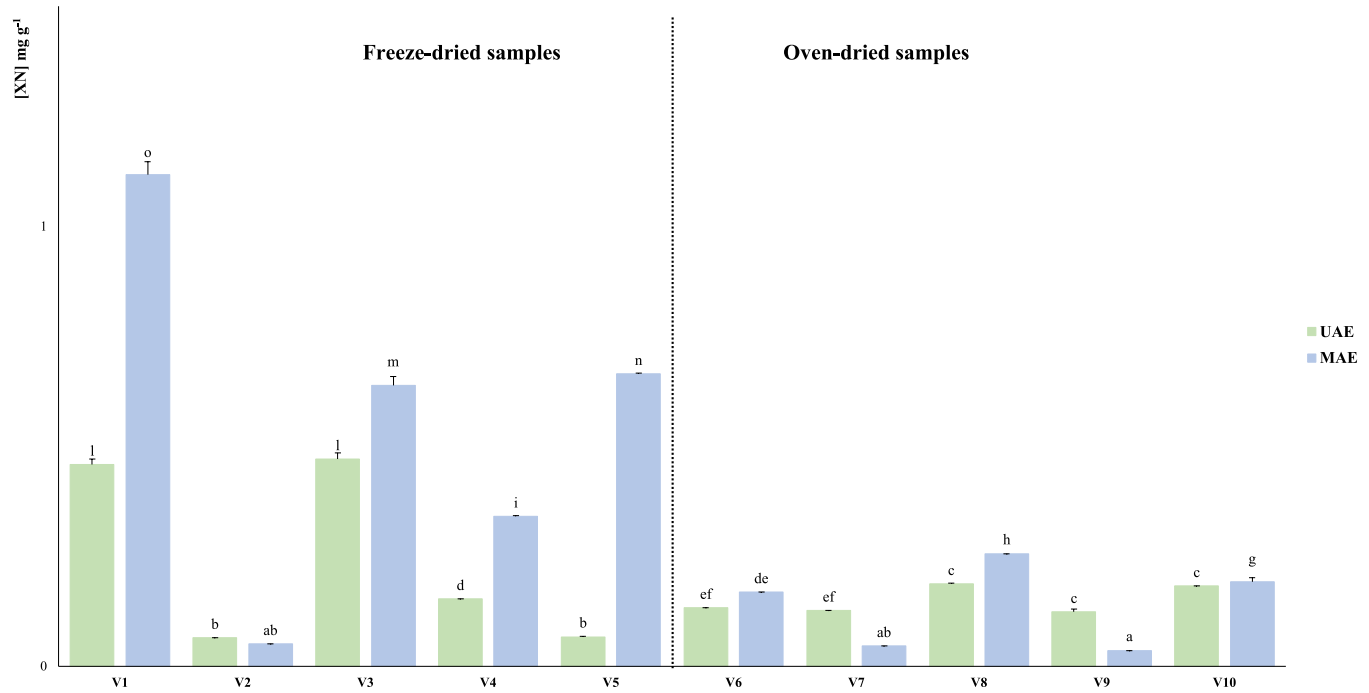


Fig. 4. Xanthohumol content of hop green extracts (mean ± s.d.). XN: Xanthohumol; UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction. Different letters indicate significant differences in the mean ($p < 0.05$).

Table 3
Permutational multivariate analysis (PERMANOVA) results as a function of hop variety (V), extraction technique (E), drying process (T), and their interactions.

Factor	Df	Sums of squares	F	R^2	p-value
Variety (V)	4	0.86401947	882.179853	0.14271127	0.001
Extraction technique (E)	1	1.65336067	6752.44722	0.27308784	0.001
Drying process (T)	1	2.2442292	9165.598	0.3706824	0.001
VxE	4	0.42829609	437.298224	0.07074225	0.001
VxT	4	0.5370773	548.365853	0.08870979	0.001
ExT	1	0.07584767	309.767509	0.01252786	0.001
VxExT	4	0.23679663	241.773737	0.03911202	0.001
Residuals	60	0.01469121		0.00242657	
Total	79	6.05431826		1	

3.7. Hypoglycemic potential of different hop leaf extracts

Type 2 diabetes is a metabolic disorder characterised by elevated blood glucose levels, insulin resistance, and a relative insulin deficiency. It is responsible for more than 90 % of all cases of diabetes on a global scale. A significant strategy employed in the management of post-prandial hyperglycaemia is the reduction or inhibition of carbohydrate absorption, achieved by the suppression of digestive enzymes such as α-amylase and α-glucosidase [48]. A novel therapeutic approach for managing this condition involves the use of natural inhibitors, such as polyphenols, which modulate starch breakdown and help maintain glucose levels in the bloodstream [49]. To investigate the hypoglycaemic potential of hop green leaf extracts, the samples were tested for their inhibitory activity against α-glucosidase in the range of 0–120 μg mL^{-1} (Fig. 6). The same trend was observed for all the samples analysed, regardless of the extraction method used.

The results of the non-parametric statistical analysis show significant differences between the groups of samples, as determined by the Kruskal-Wallis test, with a p-value of 0.0017, indicating that the different green hop leaf extracts had different effects on the different samples. The influence of the drying process proved to be a discriminating factor in the inhibitory activity of the extracts, showing that the OD samples were not able to exceed 30 % inhibition of the enzyme at the highest concentration tested (no significant differences in % inhibition were recorded between 100 and 120 mg mL^{-1} extract for any of the samples tested; $p > 0.05$). In terms of genotype, V1, V2 and V4 showed the highest inhibitory potential, regardless of the extraction method used, with V2 samples able to inhibit in vitro more than 70 % of the alpha glucosidase activity tested (72 ± 1 % and 71.3 ± 0.9 %, for V2_M and V2_U, respectively; $p > 0.05$). The IC_{50} (the sample concentration required to inhibit 50 % of the enzyme) values were calculated for all the samples that showed an inhibitory effect equal to or greater than 60 % (sample from V1 to V5). The lowest IC_{50} values were recorded for V2 MAE, followed by V1 MAE and V2 UAE (21.3 ± 0.6, 25.2 ± 0.8; 28.5 ± 0.6 μg mL^{-1}, respectively). To the best of our knowledge, this is the first study to investigate the inhibitory capacity of green hop leaf extracts on alpha-glucosidase activity. The IC_{50} values reported are in agreement with those reported by Papoutsis et al. for plant matrices characterised by strong inhibitory activity against the enzyme [50]. Moreover, V1 MAE samples showed a IC_{50} slightly lower than that reported for a methanolic extract of Chinook hop cones (35.34 μg mL^{-1}) by Do Nascimento et al. [51]. However, it should be noted that no positive control, such as acarbose, was utilised in this study. Consequently, the results presented herein represent a preliminary screening of the potential alpha glucosidase inhibitory activity of the different hop leaf green extracts for their exploitation in different areas.

3.8. Principal component analysis

To gain a deeper understanding of the interrelationships among the analysed parameters and the applied technological treatments, the raw dataset was subjected to principal component analysis (PCA). The PCA was performed on selected variables, which were chosen based on the correlation and anti-image matrices derived from the standardized z-scores, utilising the orthogonal rotation (Varimax model). The adequacy of the sampling for analysis was confirmed by the Kaiser-Meyer-Olkin measure (KMO = 0.72). Furthermore, the results of Bartlett's test of sphericity ($p < 0.001$) indicated that the correlations among the

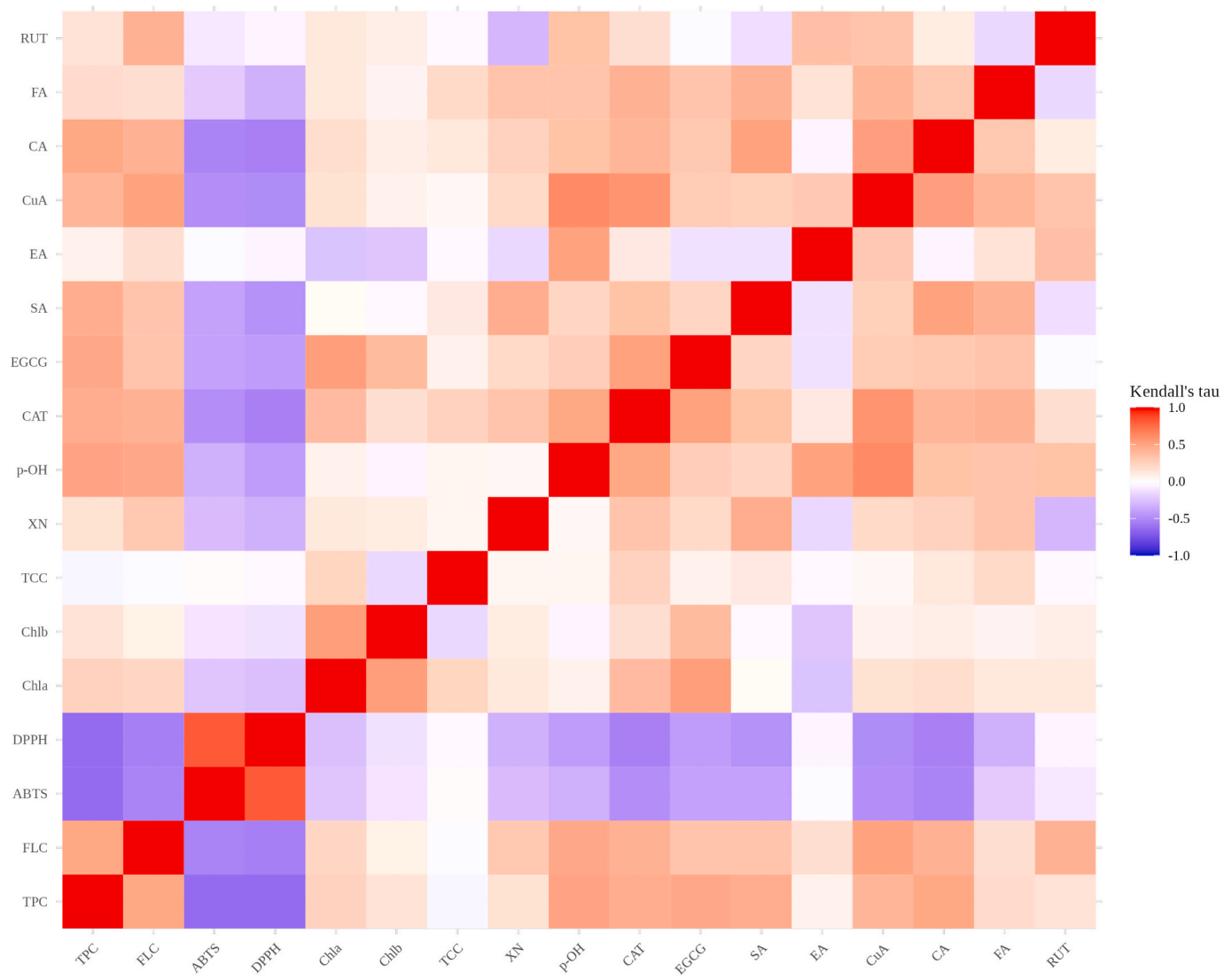


Fig. 5. Correlation matrix calculated from Kendall's tau coefficients of the hop leaf variables under investigation

variables were sufficiently strong to justify the use of PCA. The scree plot (not shown) and the eigenvalues > 1 (Kaiser's criterion) were used to determine the number of principal components (PCs) to be considered significant. It was determined that three PCs were significant, representing 81 % of the total variance. The PCA score plots (Fig. 7) showed clear differences between the samples from the different treatments, indicating that the final profile of the hop leaf extract was strongly influenced mainly by both the extraction technique and the drying method used.

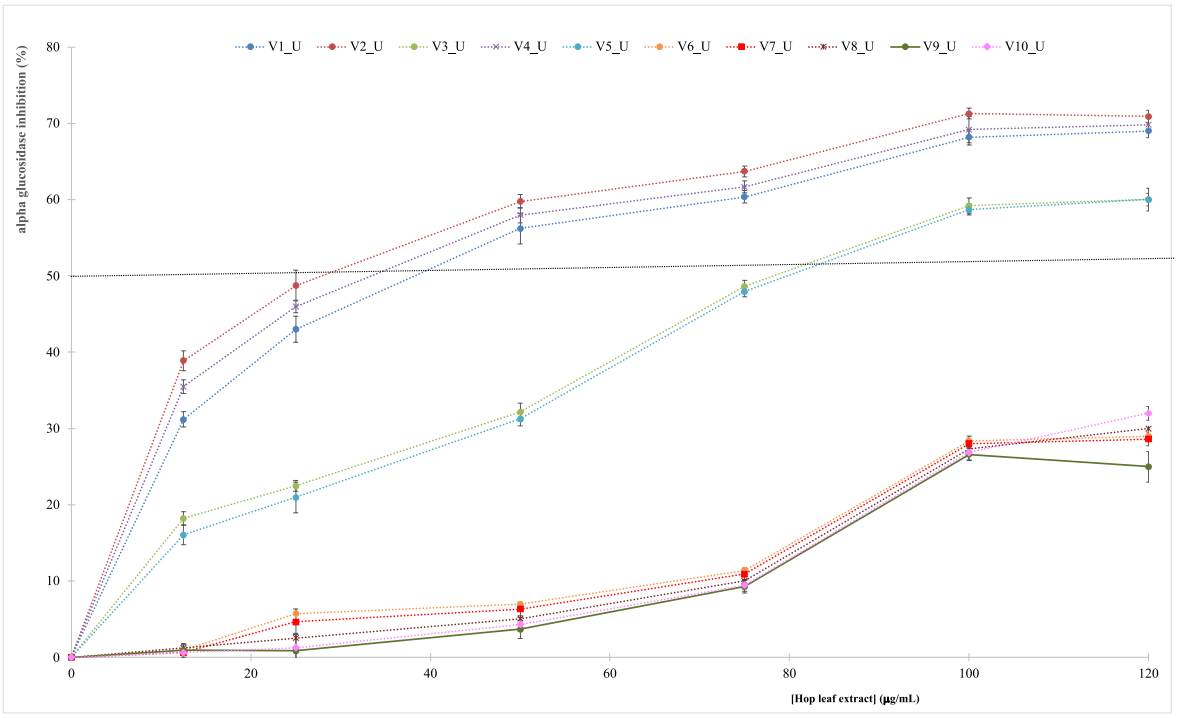
With regard to the first component, which accounted for 54.15 % of the total variance, the hop leaf extracts were separated mainly on the basis of the different drying processes to which the starting plant matrix (hop leaves) was subjected (Fig. 7a). On PC3 (13.10 % variance explained) the samples were grouped into two main clusters according to the extraction method used, each of which contained two subpopulations according to the drying method used (separated by PC1; Fig. 7b). Finally, on PC2 (13.75 % variance explained), genotype appeared to be the factor clustering the samples (Fig. 7c).

The differences observed in the PCA can be explained by several key metabolites highlighted in the loading plots of the three PCs (Fig. 8). PC loadings are a means of estimating the contribution of each independent variable to each PC in a PCA model. The magnitude of a variable's loading is directly proportional to its contribution to a particular component. By plotting the loadings of each PC, it is possible to identify the variables responsible for the groupings observed in the PCA score plot. The loadings plot indicates that PC1 was strongly associated with the total phytochemical profile of the extracts and with *p*-hydroxybenzoic acid. As expected, a strong negative correlation was also found between the sample AC and TPC and FLC, confirming what the correlation analysis showed (Fig. 5). The negative correlation is justified by the fact that in our study the AC was expressed as a function of the EC_{50} (the amount of antioxidant necessary to decrease the initial radical concentration by 50 %), where the lower this value the higher the antiradical potential of the matrix. PC2 was mainly associated with the content of phenolic acids, which, among the bioactive compounds analysed in this work, seem to be those most influenced by the drying process of the initial plant matrix and, to a lesser extent, to the genotype. Interestingly, PC2 also showed a strong positive correlation with the XN content in the extracts analysed. Finally, based on the Chlb and EGCG content, the samples were grouped on PC3.

4. Conclusions

One of the major challenges facing modern agriculture is the ability to implement sustainable supply chain management models. This is particularly important for crops such as hops, which produce more

a)



b)

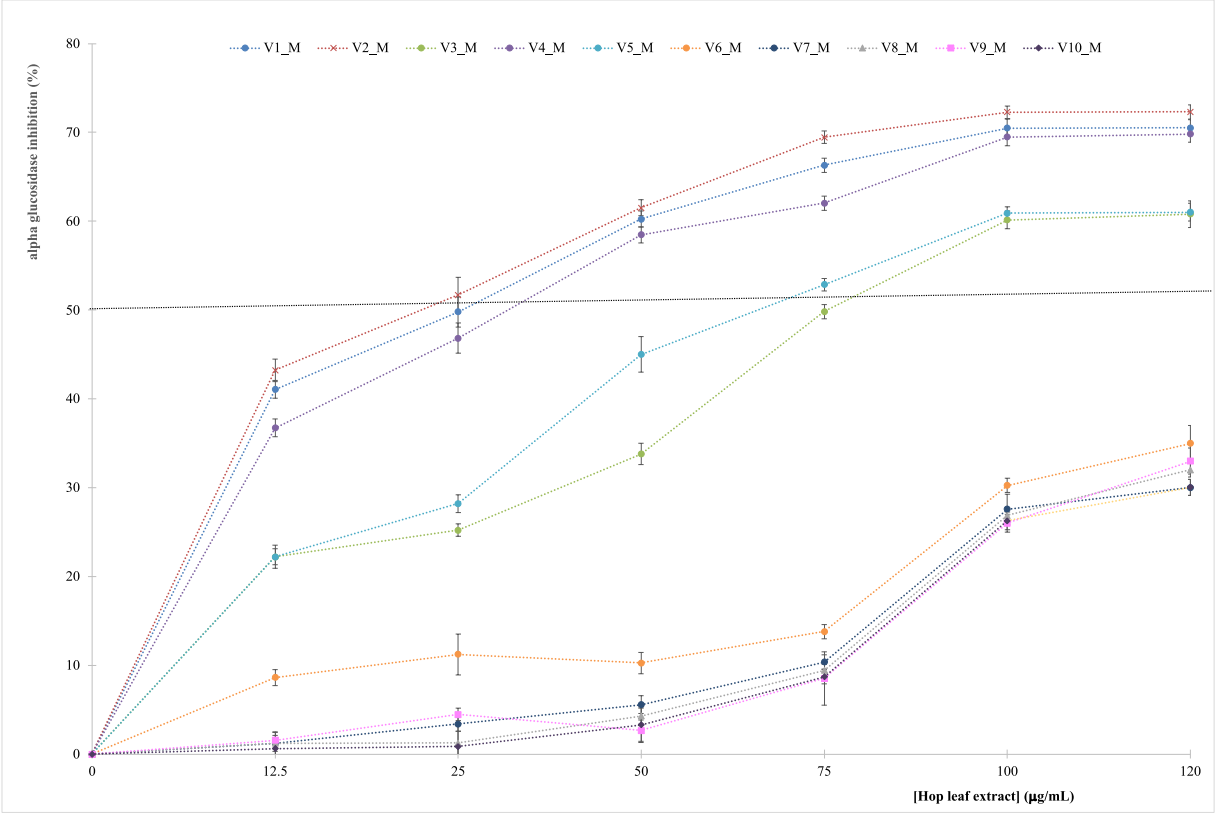


Fig. 6. Effects of green hop leaf extracts on alpha glucosidase activity. U: ultrasound-assisted extraction; M: microwave-assisted extraction.

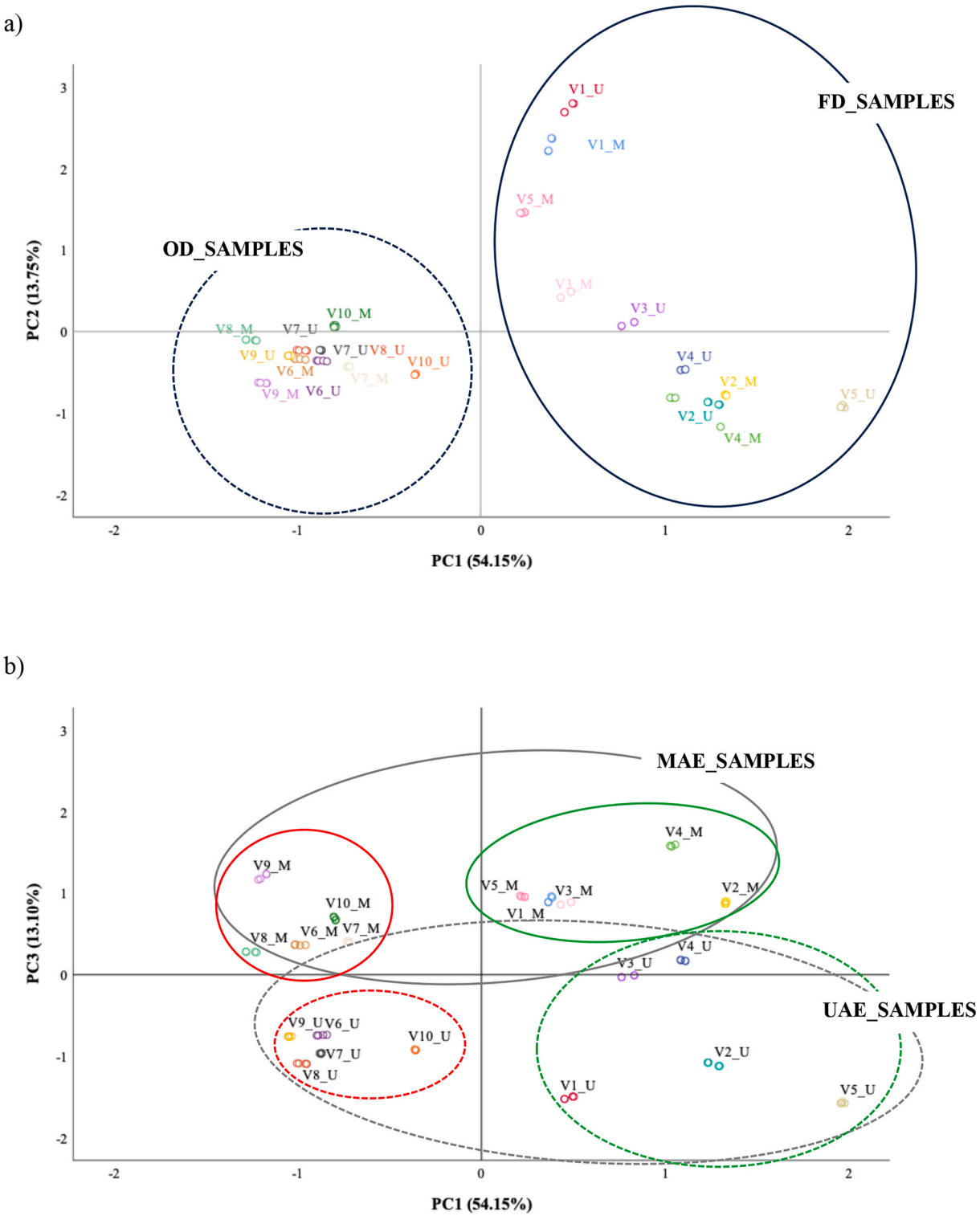


Fig. 7. PCA score plots UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; OD: oven-dried; FD: freeze-dried.

biomass than cones when harvested. Extracting valuable compounds from this biomass using environmentally friendly and energy-efficient extraction processes, which are more sustainable than traditional methods, and using existing on-farm drying kilns to stabilise the biomass may offer interesting prospects for farmers interested in this crop. In fact, the extracts obtained could be used both in beer production and in other sectors such as phytotherapy. The results reported herein indicate that the drying techniques significantly affect the final composition of hop leaf extracts. Freeze-drying generally preserved more bioactive compounds than oven-drying, but the genotype had a pivotal role in determining the final phytochemical profile of the extracts. This confirmed that the dehydration technique should be tuned in relation to the compounds of greatest interest or value for each genotype. In addition, microwave-assisted extraction was more efficient than

c)

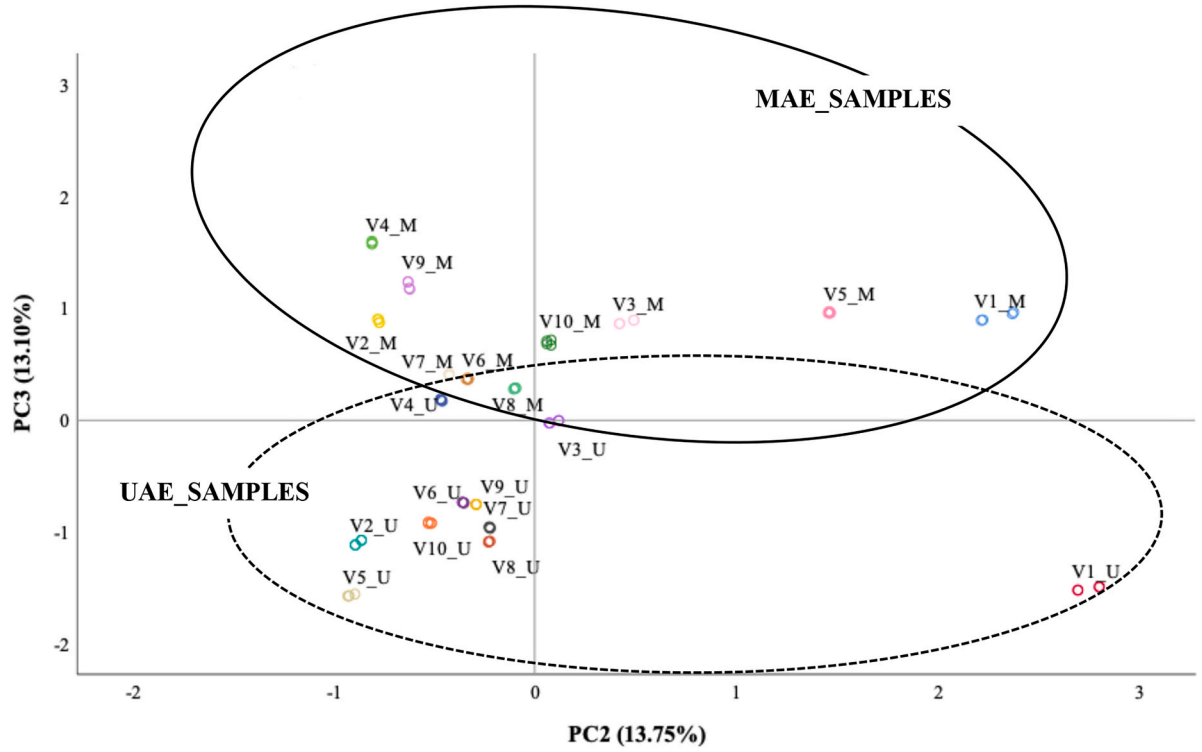


Fig. 7. (*continued*).

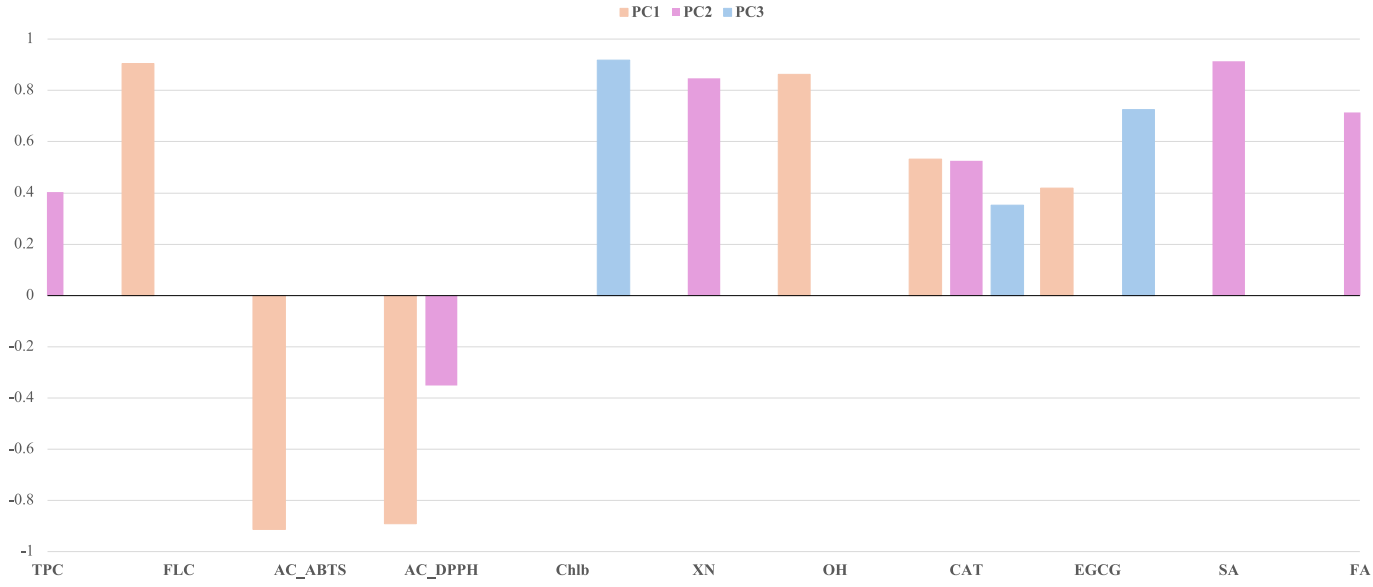


Fig. 8. PCA loading plots TPC: total polyphenol content; FLC: total flavan content; AC_ABTS: antiradical capacity (ABTS test); AC_DPPH: antiradical capacity (DPPH test); Chlb: chlorophyll *b*; XN: xanthohumol; OH: *p*-hydroxybenzoic acid; CAT: catechin; EGCG: epigallocatechingallate; SA: syringic acid; FA: ferulic acid.

ultrasonic extraction for most bioactive compounds and significantly reduced extraction times. Hop leaves, typically considered waste, have shown potential as a valuable source of bioactive compounds, including xanthohumol and some hop leaf extracts have shown promising α-glucosidase inhibitory activity, suggesting potential applications in managing type 2 diabetes. The study highlights the potential of hop leaves as a sustainable source of bioactive compounds for food, pharmaceutical, and nutraceutical applications, demonstrating the importance of optimizing extraction and drying techniques to maximize the recovery of valuable compounds from this biomass. However, the scope of the study was limited to certain phytochemicals and bioactivities and further research is needed to explore the full range of health benefits of hop leaf extracts. Similarly, in an era characterised by strong climatic changes, future research and applications of the results of this study cannot disregard how soil and climate conditions or agricultural practices might influence the potential variability of bioactive

compound content in hop leaves.

CRediT authorship contribution statement

Katya Carbone: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Valentina Macchioni:** Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation.

Funding

The research was carried out under the project LOB.IT with financial support from the Italian Ministry of Agriculture, Food Sovereignty and Forestry (D.M. n. 667550; 12.30.2022).

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2025.107322.

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