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Conventional and innovative extraction technologies to produce food-grade hop extracts: Influence on bitter acids content and volatile organic compounds profile

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Abstract: Hop extracts represent a natural alternative to synthetic food additives because of their high content of bitter acids and volatile organic compounds (VOCs) with bittering, flavoring, and antimicrobial properties. However, broader uses of hop extracts as natural techno-functional ingredients rely on the identification of sustainable and affordable extraction technologies allowing to diversify the processes and produce extracts characterized by different compositions and, consequently, qualitative properties.

Thus, this study is aimed to evaluate and compare the effect of innovative and conventional extraction methods on the bitter acids content and VOCs pattern of food-grade ethanolic hop extracts for food applications.

Innovative extractions were carried out by using two ultrasound systems (a laboratory bath [US] and a high-power ultrasound bath [HPUS]), and a high-pressure industrial process (high hydrostatic pressure [HHP]). Conventional extractions (CONV) were performed under dynamic maceration at 25 and 60°C; for ultrasound and conventional methods, the effect of the extraction time was also investigated.

Among the extracts, the highest and lowest content of bitter acids was found in CONV 60°C extracts, and HHP and CONV 25°C extracts, respectively. Of the 34 VOCs identified in dry hops, ~24 compounds were found in US, HPUS and CONV extracts, while only 18 were found in HHP. CONV extractions showed higher selectivity for sesquiterpenes, while US and HPUS showed higher selectivity for esters and monoterpenes. Hierarchical cluster analysis (HCA) and partial least squares-discriminant analysis (PLS-DA) allowed classifying hop extracts based on the extraction methods and also allowed highlighting the technological conditions to produce hop extracts with specific techno-functional and flavoring properties.

KEYWORDS
bitter acids, flavoring properties, hop extracts, *Humulus lupulus* L., SPME GC–MS, volatile compounds

Practical Application: The study showed that different extraction methods can lead to hop products with varying sensory and functional properties. By selecting the right extraction method, companies can produce hop extracts with specific compositions that meet their needs for clean label and sustainable food products, as well as new edible packaging or coatings.

1 | INTRODUCTION

Hop cones, the female inflorescences of *Humulus lupulus* L., are known worldwide as the main ingredient in the production of beer, which confer the essential character of bitterness and the overall flavor including the typical "spicy" or "hoppy" aroma (De Keukeleire et al., 2003; Krofta et al., 2018).

The bitterness properties of hops' cones are due to the presence and amount of bitter acids secreted by lupulin glands, consisting of two related series of homologs, that is, α-acids or humulones, which represent the larger portion, and β-acids or lupulones present in minor amount. The α-acids consist of a mixture of six humulone analogs among which the major ones are humulone (35%–70% of total α-bitter acids), cohumulone (20%–65% of total α-bitter acids), and adhumulone (10%–15% of total α-bitter acids), while β-bitter acids include lupulone (30%–55% of total β-bitter acids), colupulone (20%–55% of total β-bitter acids), adlupulone (5%–10% of total β-bitter acids), prelupulone, and postlupulone (Carbone et al., 2020; Hrnčič et al., 2019).

Besides α– and β-acids, the flavoring properties of hops depend on the presence and content of volatile compounds, synthesized by the plastids of glandular trichomes (G. Wang et al., 2008), which belong mainly to three main classes: (I) hydrocarbons (monoterpenes, sesquiterpenes, and aliphatic hydrocarbons), (II) oxygenated compounds (i.e., terpene alcohols, sesquiterpene alcohols), and (III) organosulfur compounds (i.e., thioesters, sulfides). The most abundant volatiles of the first class (50%–80%) are monoterpenes (α- and β-pinene, myrcene, and limonene) and sesquiterpenes (α-humulene, β-farnesene, β-caryophyllene, α- and β-selinene and γ-muurolene). The second class (up to 30%) is formed during the ripening, processing, and storage of hops and includes linalool, geraniol, caryophyllene oxide, and farnesol. The third class represents a minor part of the volatile fraction (<1%) and comprises compounds (e.g., hydrogen sulfide, dimethyl sulfide with a very low odor threshold) (Karabín et al., 2016; Krofta et al., 2018; Pistelli et al., 2018).

In the last decade, some studies have evidenced that hops' bitter acids, and terpenes (i.e., myrcene, α-muurolene, β-pinene, limonene, α-caryophyllene and β-humulene) besides their sensory effects also exert some antimicrobial activity and property of interest for food preservation purposes. In particular, terpenes showed moderate antimicrobial effects against Gram-negative bacteria (e.g., *Proteus vulgaris*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp.), some yeasts (e.g., *Candida albicans*), and Gram-positive bacteria (e.g., *E. faecalis and Streptococcus aureus*) (Bocquet et al., 2018). Likewise, bitter acids showed inhibitory activity toward Gram-positive species, such as *Lactobacillus, Streptococcus, Staphylococcus, Micrococcus*, and *Bacillus*, and certain fungi, such as *Penicillium* and *Aspergillus* species (Bocquet et al., 2018; Macchioni et al., 2021; Van Cleemput et al., 2009).

With the aim to respond to the growing interest of consumers toward "free-from" additives products and to help industries in replacing synthetic additives with natural alternatives, hop extracts have been proposed and tested with promising results as natural preservatives against foodborne pathogens in food products such as bread (Nionelli et al., 2018), meat (Kramer et al., 2015), ham (L. Wang et al., 2016), fruit, and vegetables (Rodrigues et al., 2021), as well as for the development of edible coatings for fresh-cut fruits (Carbone et al., 2021). However, the broader use of hop extracts as natural ingredients for producing innovative and clean-label products relies on the investigation and identification of extraction conditions allowing the production of extracts characterized by different volatile organic compounds (VOCs) and bitter acids composition and, consequently, with diversified techno-functional and sensory properties.

Extraction technologies applied for the production of plant extracts include several conventional methods (e.g., percolation and static or dynamic maceration) commonly used because of their easy and wide-ranging applicability; however, these methods present various drawbacks associated with the high consumption of solvents, prolonged extraction times, and degradation of thermosensitive biomolecules (Hrnčič et al., 2019).

In recent times, to overcome these disadvantages, innovative extraction techniques such as ultrasound-assisted extraction (UAE) and high hydrostatic pressure (HHP) extraction (Astray et al., 2020; Giacometti et al., 2018; Santarelli et al., 2022) have been explored. These methods offer highly selective and efficient recovery of compounds

of interest from different plants (e.g., grape and green tea leaves), shorter extraction time, and lower losses and degradation of volatile and thermolabile compounds (Corrales et al., 2009; Ma et al., 2009). Furthermore, these techniques are sustainable, and with respect to other innovative extraction methods (EMs), that is, supercritical fluid extractions, promise a great potential for industrial upscaling (Chemat, Rombaut, Meullemiestre, et al., 2017; Vincekovićet al., 2017), reduce establishment costs, and require much less specialized technical personnel (Ameer et al., 2017).

To the authors' knowledge, despite a wide literature focused on the characterization of the volatile profile and/or determination of bitter acids in hop cones of different varieties, cultivars, origin, and maturity grade (Eyres et al., 2007; Forteschi et al., 2019; Liu et al., 2018; Rodolfi et al., 2019; Shellie et al., 2009; Su & Yin, 2021; Van Opstaele et al., 2012), fewer are the studies focused on hop extracts and almost all were aimed to evaluate the extraction efficiency and selectivity of supercritical fluid EMs (Rodrigues et al., 2021; Sanz et al., 2019). Conversely, very scarce (Carbone et al., 2020) or even absent are the studies on the effect of ultrasound- and HHP-assisted extraction processes and related parameters on the volatile pattern and bitter acids content of food-grade hop extracts for food applications.

Thus, to fill this knowledge gap, this study was aimed at investigating the effect of nonconventional extraction techniques (UAE and HHP) on the extraction of these compounds. For comparison purposes, the effect of conventional extractions, that is, dynamic macerations, was also evaluated. For the UAEs and dynamic macerations, the effect of process parameters such as the extraction temperature and/or time was determined. To avoid purification processes and to produce food-grade extracts, ethanol was used as extraction solvent. The single and total bitter acids content of the extracts as well as the comprehensive profiling of VOCs was determined.

Finally, to discriminate the hop extracts based on the different extraction conditions, the interrelationships between extraction techniques and the corresponding parameters considered as well as the relationships among variables were investigated through a chemometric approach including both hierarchical cluster analysis (HCA) and partial least squares-discriminant analysis (PLS-DA). The results of this study could be of main importance to diversify the use of hops and hop extracts in food formulations and products.

2 | MATERIALS AND METHODS

2.1 | Hop raw material

A single batch of dried (moisture content <12% by weight) hop cones (*H. lupulus* L.; var Cascade) grown in Abruzzo (Italy) was provided by a local producer. Upon arrival, the product was vacuum-packed in high barrier (PA/PE) plastic bags, stored at −40°C, and kept protected from light and humidity until use.

2.2 | Chemicals

The bitter acids standard mixture International Calibration Extract ICE-3 containing 13.88% of cohumulone, 30.76% of n-humulone + adhumulone (α-acids), 13.44% of colupulone, and 10.84% of n-lupulone + adlupulone (β-acids) was purchased from Labor Veritas Co. (Zürich, Switzerland). Solid-phase microextraction (SPME) fiber was acquired from Supelco (Bellefonte, PA, USA). All the other chemicals were obtained from Sigma–Aldrich (Milan, Italy).

2.3 | Production of hop-food grade extracts by conventional and nonconventional EMs

Before extraction, an aliquot of 5 g of dry hops was ground three times in a coffee mill (De'Longhi KG210; De' Longhi Appliances Srl, Treviso, Italy) for 10 s (Inui et al., 2017), and then packed in high barrier (PA/PE/PE) plastic bags and stored at −40°C until extraction.

Conventional and nonconventional extractions were carried out as described in Santarelli et al. (2022). All extractions were carried out by using an ethanolic solution (ethanol: water = 50:50 v/v) as extraction solvent, and a matrix-solvent ratio of 1:50 (w/v). These conditions were applied to allow the extraction of phytochemicals with different polarities (Carbone et al., 2020; Santarelli et al., 2022).

For the conventional extractions, the hop powder and the extraction solvent were inserted within 50 mL glass vial. Before closure, the vial was conditioned under nitrogen. Thus, dynamic maceration was performed by stirring (300 rpm) at 25°C (CONV 25°C) and 60°C (CONV 60°C) for 15, 30, 60, and 120 min.

Nonconventional extractions were performed by using two ultrasonic baths and an HHP equipment. Before extraction, the hop powder and the extraction solvent were packed into plastic bags (PA/PE/PE, thickness: 50 μm) in the absence of air.

UAEs were performed at 25 ± 1°C for 15, 30, 60, and 120 min using thermally controlled ultrasonic tools: (i) a low power (100 W, 50 kHz) ultrasound (US) bath (Labsonic LBS1 -3; Falc, Bergamo, Italy) and (ii) a high power (800 W) ultrasound (HPUS) bath (Waveco®, Next Cooking Generation, Milano, Italy).

HHP extraction was performed using industrial scale equipment (Avure HPP AV-10; JBT, Chicago, USA). The bags were placed into a hydrostatic pressure vessel, and the pressure was raised to 600 MPa. The pressure holding time was 5 min, while the overall treatment duration was ca. 8 min. Water was used as a filling medium in the HHP vessel with an initial temperature of 3°C. Considering an adiabatic temperature increase of 3°C/100 MPa, the vessel water temperature at the fixed process pressure (600 MPa) was estimated to be ≤21°C.

All extractions were performed in triplicate.

After extractions, all differently processed hops-solvent mixtures were centrifuged at 4000 rpm (2470 × *g*) for 10 min at 4°C; the supernatants (i.e., the extracts) were, then, filtered with a nylon filter (0.45 μm) and stored at –40°C until analysis.

2.4 | Reference extraction process for bitter acids

To compare the *α*- and *β*-acids content of food-grade extracts with that obtained by the official EM, a reference extract was prepared using toluene as solvent according to the official American Society of Brewing Chemists (ASBC) Hops-6 method and used as a reference sample (CNTR).

All extractions were performed in triplicate.

2.5 | Determination of single bitter acids by high-pressure liquid chromatography

The bitter acids content was determined on the CNTR sample and on both conventional and nonconventional extracts (see Section 2.4).

Bitter acids analysis was performed by an analytical high-performance liquid chromatography (HPLC) system (Agilent 1100 series, Agilent, Italy) equipped with a diode array detector (DAD; Agilent Technologies, Italy). Before HPLC analysis, each sample was filtered by using a PTFE membrane (Millipore 0.45 mm, Milan, Italy).

Identification and quantification of *α*-acids (AA) and *β*-acids (BA) were carried out according to the official analytical method ASBC Standard Methods of Analysis (Hops-14 method; ASBC, 2012; Carbone et al., 2020). The sample (50 μL) was injected into a C18 column Synergi 4U Fusion RP (4.6 × 150 mm; 4 μm particle size, Phenomenex, Bologna, Italy), set at 40°C. The isocratic mobile phase was distilled water, methanol, and formic acid in a ratio of 17:85:0.25 (v/v/v). Chromatograms were acquired at 326 nm. For the quantification of *α*- and *β*-acids, a calibration curve was obtained from the dilution of the ICE-3 standard. The results are expressed as% w/w.

HPLC analyses were performed in triplicate on conventional and nonconventional extracts.

2.6 | Volatile organic compounds analysis

2.6.1 | SPME procedure

VOCs were evaluated in triplicate on both hop extracts and dry hop powder.

To obtain high VOCs' recovery from the samples, preliminary experiments were carried out to optimize the SPME conditions including fiber coating Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) versus Polydimethylsiloxane/Divinylbenzene (PDMS/DVB), sample amount (2.0, 1.0, 0.5, and 0.25 mL of hop extract; 2.0, 1.0, 0.5, and 0.25 g of dry hop), extraction and equilibration time (30, 20, 10, and 5 min), desorption times (15, 10, and 5 min), and extraction temperature (60, 40, and 30°C) (data not shown).

Results were used to identify the best analytical conditions, which are summarized as follows: PDMS/DVB (65 μm) was chosen as fiber (Hamm et al., 2003; Mariano et al., 2022); dry hop powder (0.025 g) or hop extract (0.250 mL) were inserted into a 20 mL-vial, hermetically closed with an aluminum crimp cap and a silicone/PTFE septum (VWR Internationals Srl, Milano, Italy). Quantities higher than those optimized caused GC-column saturation. To improve the extraction efficiency, before closure the ionic strength of the hops hydroalcoholic extracts was increased with the addition of 5 mg of NaCl. The extraction was carried out at 40°C in a water bath. Each sample was heated for 10 min at 40°C under constant agitation (300 rpm) in the pre-equilibration step then the fiber was exposed for 10 min.

2.6.2 | GC–MS analysis

GC analyses were conducted in triplicate with Perkin Elmer Gas Chromatograph Clarus® 580 coupled to a

Perkin Elmer mass spectrometer Clarus® SQ 8 S (Perkin Elmer, Boston, MA, USA). The chromatographic separation was performed on a Zebron capillary column (Phenomenex) (30 m ×, 0.25 mm ×, 0.25 μm film 82 thickness, 5% phenyl and 95% dimethylpolysiloxane stationary phase). Helium was used as a carrier gas at a flow rate of 1.0 mL min^{-1}; a split injection ratio of 1:10 was used. After the SPME extraction, the compounds were thermally desorbed at 240°C for 10 min. The oven temperature program was set as follows: 50°C for 1 min, from 50 to 180°C at a rate of 8°C min^{-1}, 180°C for 1 min, from 180 to 240°C at a rate of 10°C min^{-1}, and 240°C for 2 min (De Flaviis et al., 2022). For hop extracts solvent delay, of 1 min was applied. MS was operated in electron ionization mode (EI+) at 70 eV. The scan speed was 5.1 scans/s, and mass spectra were recorded in the range of 33−600 m/z.

For some terpenes that can elute very close to each other (*β*-pinene, limonene, *β*-myrcene), the identification was performed by the comparison of the retention time with those of the pure reference compound (Sigma–Aldrich, St. Louis, MO, USA).

The identification of the unknown VOCs was carried out by comparison with mass spectra of a library database (NIST14 Mass Spectral Library, National Institute of Standards and Technology, Gaithersburg, MD, USA), considering a minimum of 85% of similarity, and by comparing retention index (RI) with those reported in the literature (Benelli et al., 2018; Fiorini et al., 2019; Mancuso et al., 2019; Villa-Ruano et al., 2018). A mixture of C6–C40 n-alkanes (RESTEK, PA, USA) was injected using the analytical conditions reported above in order to determine the temperature-programmed RI according to the Van den Dool and Kratz (1963) formula. Identified peaks were elaborated with the TurboMass 6.1.0 software.

To determine the volatile profile of the extracts, for each identified compound, the relative peak area (%) was computed as follows (Equation 1):

$$\% \text{ RPA } = \frac{Aa}{\sum At} \times 100,$$

where Aa is the area of the selected peak and ΣAt is the sum of all the peak's areas in the chromatogram (Ligor et al., 2014).

2.7 | Statistical analysis

Data were reported as mean and standard deviation and analyzed by one-way analysis of variance (ANOVA) using XLSTAT 2021 (Addinsoft, Paris, France). Significant differences between means were calculated by HSD Tuckey's post hoc test at a level of $p < 0.05$.

Data collected on the CONV and UAE extracts were additionally processed by multivariate ANOVA to highlight, for the formers, the single and combined effects of the extraction temperature (T) and time (t), and for the latter the single and combined effects of the EM and time (t). Effective hypothesis decomposition was further computed. The sigma-restricted coding of effects was used, and for each effect, its sum of squares is the difference of the model sums of squares for all other effects from the whole model sums of squares. As such, the effective hypothesis decomposition sums of squares provide an unambiguous estimate of the variability of predicted values for the outcome uniquely attributable to each effect. Data were processed using STATISTICA for Windows (StatSoftTM, Tulsa, OK, USA) software.

To study the data structure, investigating similarities and hidden patterns among the extracts analyzed, HCA and PLS-DA were computed. These methods were applied to a normalized dataset to retrieve all relevant information systematically, using XLSTAT 2021 (Addinsoft). Clusters were computed by Ward's method based on Euclidean distance, and the significant differences among clusters were investigated by multivariate analysis followed by HSD Tuckey's post hoc test ($p \leq 0.05$). In PLS-DA, cross-validation was conducted through the general Jackknife method using five groups that one by one were removed to recompute the model. Qi^2 criterion was used to determine if the contribution of latent variables (LV) to all dependent variables were significant.

3 | RESULTS AND DISCUSSION

3.1 | Identification and quantification of *α*- and *β*-acids

In Table 1, the single and total content of AA (cohumulone and adhumulone) and of BA (colupulone and n-adlupulone) of hop extracts obtained by the different EMs is reported. The relative cohumulone content (%) has also been calculated and reported since this compound is responsible for the sour and unpleasant bitterness of beer, and a low relative quantity is usually appreciated (De Keukeleire, 2000; Forteschi et al., 2019).

In general, the CONV 60°C EM led to the highest extraction efficiency of AA content followed by UAE, HHP, and CONV 25°C. Conventional extractions at 60°C also showed the highest ability in extracting BA, followed by the other EMs with minor differences and a main effect of the extraction time.

By analyzing the extracts' composition in terms of individual hop bitter compounds, it can be observed that the most representative *α*-acid was the n-adhumulone,

TABLE 1 Bitter acids composition (% w/w) of different hop extracts determined by high-performance liquid chromatography analysis.

	% w/w															
Extraction method	**Extraction time (min)**	**Cohumulone**	**SD**	**n-Adhumulone**	**SD**	**Total α-acid**	**SD**	**Colupolone**	**SD**	**n-Adlu-pulone**	**SD**	**Total β−acids**	**SD**	**Relative cohumulone (%)[a]**	**SD**
CNTR	–	0.93h	0.04	2.43g	0.09	3.4i	0.1	1.27m	0.03	1.51g	0.05	2.78j	0.08	27.64c	0.07
CONV 25°C	15	3.4f	0.1	8.8ef	0.5	12.3gh	0.6	2.0hil	0.2	1.3gh	0.2	3.3hij	0.4	27.8bc	0.6
	30	3.4fg	0.04	8.9ef	0.1	12.3h	0.2	2.13hi	0.02	1.42gh	0.01	3.50hi	0.02	27.16c	0.03
	60	3.2g	0.2	8.5f	0.5	11.7gh	0.6	1.8l	0.2	1.2hi	0.10	3.0j	0.3	27.7c	0.3
	120	3.43ef	0.02	8.8ef	0.1	12.3gh	0.2	2.50fg	0.03	1.77f	0.01	4.3g	0.04	27.9bc	0.2
CONV 60°C	15	4.30a	0.06	11.4a	0.3	15.7a	0.4	4.8a	0.1	3.1ab	0.1	8.0a	0.2	27.5c	0.2
	30	4.1b	0.1	10.9abc	0.3	14.9abc	0.4	4.77a	0.05	3.14ab	0.07	7.9ab	0.1	27.11c	0.02
	60	3.88cd	0.04	10.33cd	0.07	14.2d	0.1	4.4b	0.2	3.0bc	0.1	7.4bc	0.3	27.29c	0.09
	120	4.07b	0.07	11.00ab	0.06	15.1abc	0.1	4.7ab	0.1	3.3a	0.1	8.0a	0.2	27.0c	0.3
HPUS	15	4.36a	0.04	10.8bc	0.9	15.1ab	1.0	3.7de	0.6	2.8cd	0.6	6.4de	1.2	28.9a	1.7
	30	3.8d	0.2	9.4e	0.4	13.2ef	0.2	2.79f	0.05	2.1e	0.1	4.9f	0.1	28.8ab	1.6
	60	3.57e	0.05	9.32e	0.08	12.90fg	0.03	2.3gh	0.2	1.6fg	0.1	3.8gh	0.3	27.7c	0.5
	120	4.0bc	0.3	10.5bcd	0.8	14.5bcd	1.1	3.5e	0.2	2.61d	0.02	6.1e	0.2	27.64c	0.07
US	15	3.84d	0.02	10.0d	0.5	13.9de	0.5	1.7l	0.1	1.07il	0.04	2.8j	0.1	27.7c	0.1
	30	3.9bcd	0.2	10.4bcd	0.6	14.4 cd	0.8	4.1c	0.4	2.8cd	0.3	6.9cd	0.6	27.4c	0.4
	60	3.49ef	0.04	9.3e	0.06	12.81fg	0.04	1.8il	0.2	1.4gh	0.0	3.2ij	0.3	27.2c	0.3
	120	3.9cd	0.1	10.0d	0.5	13.9de	0.6	3.9cd	0.1	2.7cd	0.2	6.6de	0.3	27.9bc	0.4
HHP	5	3.45ef	0.04	9.2e	0.2	12.6fg	0.3	1.33m	0.02	0.88l	0.02	2.2k	0.03	27.2c	0.3

Note: Data in columns with different letters were statistically different at p level < 0.05.

Abbreviations: CNTR, control extraction; CONV 25°C, conventional extraction at 25°C; CONV 60°C, conventional extraction at 60°C; HPUS, high-power ultrasound; US, ultrasound; HHP, high hydrostatic pressure; SD, standard deviation.

[a]Relative cohumulone is expressed as cohumulone to total alpha-acids ratio (%) (Forteschi et al., 2019).

with concentration ranging from 8.5% w/w (CONV 25°C 60 min) to 11.4% w/w (CONV 60°C 15 min), followed by the cohumulone with values ranging from 3.2% (CONV 25°C 60 min) to 4.4% w/w (HPUS 15 min). Regarding single BA, the colupulone concentration ranged from 1.3% w/w (HHP) to 4.8% (CONV 60°C 15 and 30 min), while n-adlupulone varied from 0.88% w/w (HHP) to 3.3 w/w (CONV 60°C 120 min).

Concerning the relative cohumulone, the highest content was found in the HPUS extracts, 15 and 30 min, while no significant differences were observed among all the remaining extracts under study.

It is interesting to note that in general, the samples extracted in ethanol 50% (v/v) showed, regardless of the EM and time, a similar or even greater content of bitter acids than the CNTR sample extracted with toluene, and this highlights the high extraction capability of the selected food-grade solvent toward this class of compounds.

Differences in hop raw material (i.e., pellets), varieties/cultivar/origin, and/or different EMs (i.e., supercritical fluid extraction, pulsed electric field, microwave) and solvents (i.e., methanol) (Maliar et al., 2017) make difficult the comparison of the results obtained in this study with current literature data. However, as concerns the CNTR sample, the concentration of AA and BA resulted similar to that found by Carbone et al. (2020) on wild hop extracts obtained with the same method and extraction solvent. In the same study, Carbone et al. (2020) also produced hop extracts using ethanol 50% (v/v) in combination with ultrasounds but found a content of AA and BA lower than that determined in this study in the US and HPUS ethanolic extracts. Finally, the relative cohumulone percentage found in the extracts agrees with those reported by Forteschi et al. (2019) in hop extracts obtained by constant stirring at room temperature using methanol diethyl ether and 0.1 M hydrochloric acid as solvents, and by Krofta (2003) in extracts of different hop varieties produced using methanol diethyl ether.

Multifactorial ANOVA analysis (Table 2) highlighted a significant ($p < 0.001$) effect of the individual T factor, with CONV 60°C > CONV 25°C, and of the t factor; on the other hand, their interaction ($t \times T$) showed a significant effect ($p < 0.5$) only for the extraction of cohumulone, colupulone, and total β-acids ($p < 0.001$).

As concerns the UAE extractions (US and HPUS), the EM factor significantly ($p < 0.05$) influenced the extraction of cohumulone, n-adlupulone, total β-acids, and relative cohumulone, while the factor t and the interactive effect EM $\times$ t affected ($p < 0.05$) all single AA and BA, and consequently, the total content of bitter compounds.

3.2 | HS-SPME analysis of VOCs

In Table 3, the volatile profile of hop extracts obtained by conventional and nonconventional technologies is reported. To evaluate the selectivity of the extraction solvent and the EMs for hop VOCs, the volatile pattern of dried hop powder is reported.

GC–MS analysis of dried hop cones allowed to detect and identify 34 compounds characteristic of the hop flavor (Forteschi et al., 2019; Liu et al., 2018; Rodolfi et al., 2019; Su & Yin, 2021; Van Opstaele et al., 2012) (see Table S1 for odor, flavor, and taste description of the identified VOCs). In particular, two main classes of aromatic compounds were found, that is, the terpenoids (i.e., monoterpene hydrocarbons, monoterpene alcohols, sesquiterpene hydrocarbons, sesquiterpene epoxides, sesquiterpene alcohols) and the esters. Among the former, humulene and β-myrcene were the most abundant followed by β-caryophyllene and β-farnesene, and this result was in accordance with data reported by Forteschi et al. (2019) and Rodolfi et al. (2019) for the *Cascade* variety. These compounds are the key characters that contribute to the unique hop aroma. In particular, humulene has a notable scent of woody; β-myrcene is the main hop component responsible for the pungent smell of fresh hops and shows geranium-like, lemon, and woody odor characteristics; β-caryophyllene presents a typical spicy, clove-like, and woody flavor and taste characteristics, while β-farnesene exhibits herbal, citrus-like, and woody taste and odor characteristics (Krofta, 2003; Liu et al., 2018).

Despite the aromatic profile of dried cones being similar to those reported in the literature for the same hop variety (Forteschi et al., 2019; Rodolfi et al., 2019), some differences in composition that could be ascribed to the different samples' harvest time and growing area were found. In particular, it is known that the harvesting time strongly influences the composition of terpenes since the biosynthesis of α-humulene, β-caryophyllene, and β-farnesene takes place at the beginning of growth, while β-myrcene, linalool, β-pinene, and D-limonene are produced in later times (Forteschi et al., 2019). Conversely, the content of α-pinene, limonene, β-caryophyllene, β-farnesene, muurolene, β-selinene, α-selinene, and geraniol is strictly related to the hop origin (Rodolfi et al., 2019).

As concerns the volatile aroma pattern of the hop extracts, regardless of the EM and time, it was characterized by the presence of α-humulene (~40%) and β-myrcene (~30%), followed by β-caryophyllene (~15%), β-farnesene (~5%), and by other volatile compounds, mostly esters, that although present in traces (<1%) may contribute to provid-

TABLE 2 Multifactorial analysis of variance (ANOVA) of the individual and interactive effects of extraction temperature (T) and time (t) for conventional extractions (25°C; 60°C) and of extraction method (EM) and time (t) for ultrasound-assisted extractions (US; HPUS) on single bitter acids content of hop extracts.

			Cohumulone	n-Adhumulone	Total α-acids	Colupolone	n-Adlupulone	Total β-acids	Relative cohumulone
F	CONV	t	15.05***	8.31***	9.84***	22.16**	22.12***	21.65***	4.7**
F		T	508.96***	404.15***	442.81***	3524.24**	1708.02***	2670.56***	18.3***
F		$t \times T$	3.46*	ns	ns	11.33***	ns	6.09***	ns
F	UEA	EM	8.54**	ns	ns	ns	9.42**	5.43***	4.41*
F		t	22.69***	5.61**	9.36***	49.76***	38.38***	46.27***	ns
F		EM × t	7.31**	3.66*	4.73***	42.21***	33.76***	39.51***	ns

Abbreviations: CONV, conventional extraction; ns, not significant; UAE, ultrasound-assisted extraction.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

ing additional notes to the flavor with "fruity", "green," and "floral" scents (see Table S1).

Moreover, volatile compounds found in traces (< 0.6%) in dry hops such as some esters (2-methylbutyl propanoate, methyl heptanoate, methyl (4E) -4-nonenoate, methyl 8-methyl-nonanoate), monoterpenes (cis-β-ocimene, 3-carene), and ketone (2-undecanone) were not detected in the hop extract (Table 3).

In general, by comparing the volatile composition of hop extracts obtained by conventional and nonconventional EMs, it is possible to note that irrespective of the extraction time and temperature, dynamic maceration (CONV 25°C and CONV 60°C) showed, in general, a higher selectivity toward most of the sesquiterpenes, that is, β-caryophyllene, cis-β-farnesene, α-selinene, γ-gurjunene, cis-muurola-3,5-diene, β-cadinene, and caryophyllene oxide, and allowed to recover also α-muurolene, and α-calacorene that were not detected in the other extracts.

The use of ultrasounds (US and HPUS) allowed extracting primarily some monoterpenes such as β-myrcene, β-pinene, and myrtenol, and a greater number of saturated esters. The higher extraction selectivity of ultrasounds toward esters, compared to conventional extraction at high temperature, was also found by Guimarães et al. (2019) in a prebiotic soursop whey beverage. The higher release of volatile compounds by UAE may be due to the enhancement of mass transfer rates enabling more interaction between solvent and glands and cellular materials and/or due to the low level of degradation of thermal compounds (Chemat, Rombaut, Sicaire, et al., 2017). In addition, differences in the composition among CONV and UAE extracts could be related to the transformations of unstable chemical compounds during the ultrasound application. Another hypothesis is that the different extraction of other nonvolatile compounds (Santarelli et al., 2022) may have influenced the interactions between solvent and VOCs and, in turn, their liquid-vapor partitioning (Dalla Rosa et al., 1994).

HHP extraction, compared with all other techniques, allowed to extract a limited number of VOCs (18 out of 34 compounds). Since other authors (Krofta et al., 2018) investigating the volatile profile of fresh hops homogenates subjected to high hydrostatic pressure processes did not find any loss of VOCs, this result highlights the lower capability of HHP at the conditions applied in this study to extract these volatile secondary metabolites.

Specific sensory analysis of the differently obtained samples was out of the scope of this study. However, by considering the odor and flavor descriptor of each volatile compound (Table S1) and the volatile pattern of the different hop extracts, it can be estimated that extracts obtained by dynamic maceration and HHP are characterized mostly by woody notes, while UAE extracts by fruity, green, and floral nuances.

In order to analyze the single and combined effect of the extraction temperature (T) and time (t) on every single aromatic compound of hop extracts obtained by conventional extractions, data were processed by multifactorial ANOVA (Table 4).

Extraction temperature (T) influenced ($p < 0.05$) the relative peak area (RPA) of all volatile compounds except that of β-pinene, β-myrcene, α-copaene, cis-β-farnesene, humulene, α-calacorene, methyl 4-decenoate, and methyl geranate. The effective hypothesis decomposition analysis for the T factor highlighted a higher RPA for α-pinene, limonene, α-phellandrene, myrtenol, β-caryophyllene, 2-methylbutyl 2-methylpropanoate, and methyl 6-methyl heptanoate in CONV 60°C extracts, while the remaining compounds were higher in the CONV 25°C ones (data not shown).

The extraction time (t) influenced ($p < 0.05$) the extraction of all detected compounds except for

TABLE 3 Percentage composition of volatiles (VOCs) in different food-grade hop extracts.

			Percentage peak area (PA %)								
	Average Rt (min)	RI	Dry hop	HPUS 15′	HPUS 30′	HPUS 60′	HPUS 120′	US 15′	US 30′	US 60′	US 120′
Terpenes											
Monoterpenes											
α-Pinene	6.39	949	0.022^{4fg}	0.070^{bc}	0.058^{bcde}	0.082^{b}	0.061^{bcd}	nd	nd	nd	0.195^{a}
β-Pinene	7.25	998	0.516^{def}	0.750^{ab}	0.571^{bcde}	0.738^{abc}	0.564^{cde}	0.480^{ef}	0.752^{ab}	0.667^{abcd}	0.562^{cde}
β-Myrcene	7.42	1006	35.6^{bcd}	38.5^{ab}	27.6^{ef}	43.4^{ab}	30.3^{cde}	30.2^{cde}	40.6^{ab}	37.6^{abc}	44.60^{a}
Limonene	8.22	1049	0.310^{a}	0.115^{b}	0.090^{cde}	0.115^{b}	0.116^{b}	0.269^{a}	0.094^{b}	nd	nd
α-Phellandrene	8.26	1051	0.278^{a}	0.090^{bc}	0.052^{bcd}	0.096^{b}	0.054^{bcd}	nd	0.088^{bc}	nd	nd
Myrtenol	9.36	1109	0.156^{cdef}	0.151^{cdef}	0.158^{cdef}	0.194^{cd}	0.186^{cde}	0.532^{b}	0.528^{b}	0.719^{a}	0.785^{a}
3-Carene	9.53	1119	0.550^{a}	nd	nd	nd	nd	nd	nd	nd	nd
cis-β-Ocimene	8.39	1064	0.081^{a}	nd	nd	nd	nd	nd	nd	nd	nd
Sesquiterpenes											
α-Ylangene	14.38	1395	0.599^{a}	0.159^{c}	0.187^{c}	0.149^{c}	0.199^{c}	0.436^{b}	0.521^{ab}	0.580^{ab}	0.623^{a}
α-Copaene	14.48	1402	0.463^{ab}	0.20^{cd}	0.217^{d}	0.193^{d}	0.200^{d}	0.362^{b}	0.495^{ab}	0.358^{bc}	0.422^{b}
β-Caryophyllene	15.25	1448	14.7^{abcde}	13.4^{cdef}	14.6^{abcde}	12.9^{def}	15.2^{abcde}	12.3^{ef}	10.9^{fg}	10.3^{fg}	8.22^{g}
cis-β-Farnesene	15.62	1535	8.89^{a}	3.94^{cd}	4.91^{c}	2.54^{f}	2.75^{de}	3.45^{de}	2.74^{de}	2.60^{e}	2.30^{e}
Humulene	15.83	1483	25.7^{h}	35.7^{efg}	41.8^{bc}	34.0^{fg}	42.8^{b}	43.0^{b}	36.1^{defg}	38.9^{bcde}	32.3^{g}
γ-Muurolene	16.08	1498	1.37^{defg}	1.08^{gh}	1.34^{efg}	0.922^{hi}	1.09^{fgh}	1.12^{hi}	0.912^{g}	0.893^{hi}	0.733^{i}
α-Selinene	16.47	1515	2.48^{bcde}	1.43^{f}	1.97^{cdef}	1.04^{f}	1.32^{f}	1.45^{ef}	1.04^{f}	0.99^{f}	1.43^{f}
γ-Gurjunene	16.69	1521	3.10^{cde}	2.17^{fg}	2.85^{cdef}	1.66^{g}	2.12^{fg}	2.35^{efg}	1.94^{fg}	2.02^{fg}	2.39^{defg}
cis-Muurola-3.5-diene	16.79	1535	0.585^{cd}	0.384^{de}	0.578^{cd}	0.270^{e}	0.352^{e}	0.3712^{e}	0.243^{e}	0.199^{e}	0.387^{de}
β-Cadinene	16.84	1538	1.25^{cde}	0.912^{efg}	1.33^{cd}	0.702^{fg}	0.901^{fg}	0.852^{fg}	0.623^{g}	0.650^{g}	0.682^{fg}
L-Calamenene	16.93	1543	0.312^{d}	nd	0.595^{ab}	0.310^{d}	0.423^{cd}	0.536^{abc}	0.504^{bc}	0.505^{bc}	0.688^{a}
α-Muurolene	17.15	1556	0.037^{cd}	nd	nd	nd	nd	nd	nd	nd	nd
α-Calacorene	17.23	1561	0.031^{cd}	nd	nd	nd	nd	nd	nd	nd	nd
Caryophyllene oxide	17.93	1603	0.096^{b}	0.041^{b}	0.091^{b}	nd	0.087^{b}	0.150^{b}	0.155^{b}	nd	0.208^{b}
cis-Z-α-Bisabolene epoxide	18.41	1629	0.277^{de}	0.247^{de}	0.434^{d}	0.214^{de}	0.335^{de}	0.923^{bc}	0.836^{bc}	1.13^{ab}	1.179^{ab}
Esters											
Saturated esters											
2-Methylbutyl propanoate	7.04	985	0.102^{a}	nd	nd	nd	nd	nd	nd	nd	nd
2-Methylbutyl 2-methylpropanoate	7.88	1031	0.314^{a}	0.062^{bcd}	0.038^{cd}	0.065^{bcd}	0.085^{abcd}	0.224^{abcd}	0.177^{abcd}	0.158^{abcd}	0.295^{ab}
Methyl heptanoate	8.38	1039	0.348^{a}	nd	nd	nd	nd	nd	nd	nd	nd
Methyl 6-methyl heptanoate	9.24	1103	0.147^{bcd}	0.044^{de}	0.055^{cde}	0.060^{cde}	0.083^{cde}	0.146^{bcd}	0.149^{bc}	0.272^{a}	0.243^{ab}
Methyl octanoate	9.95	1141	0.193^{bc}	0.066^{d}	0.077^{d}	0.055^{d}	0.073^{d}	0.144^{c}	nd	0.259^{ab}	0.297^{a}
Methyl nonanoate	11.79	1242	0.160^{ab}	0.231^{a}	0.130^{abc}	0.019^{bc}	0.242^{a}	nd	nd	nd	nd
Methyl (4E) -4-nonenoate	11.57	1230	0.098^{a}	nd	nd	nd	nd	nd	nd	nd	nd
Methyl 8-methyl-nonanoate	12.92	1307	0.031^{a}	nd	nd	nd	nd	nd	nd	nd	nd
Methyl 4-decenoate	13.3	1328	0.470^{bc}	0.170^{e}	0.186^{e}	0.135^{ef}	0.249^{de}	0.337^{cd}	0.448^{bc}	0.585^{b}	0.747^{a}
Methyl geranate	13.54	1342	0.690^{ab}	0.135^{d}	0.150^{cd}	0.099^{d}	0.195^{cd}	0.418^{bc}	0.488^{ab}	0.676^{ab}	0.718^{a}

(Continues)

TABLE 3 (Continued)

	Average Rt (min)	RI	Percentage peak area (PA %) Dry hop	HPUS 15′	HPUS 30′	HPUS 60′	HPUS 120′	US 15′	US 30′	US 60′	US 120′
Ketones											
2-Undecanone	13.04	1313	0.070^{a}	nd	nd	nd	nd	nd	nd	nd	nd
Total absolute area (10^9)			6.03^{a}	1.17^{bc}	1.11 ^{bc}	1.05^{bc}	1.06^{bc}	1.16^{bc}	1.18^{bc}	1.35^{bc}	1.36^{bc}

	Average Rt (min)	RI	Percentage peak area (PA %) CONV 25°C 15′	CONV 25°C 30′	CONV 25°C 60′	CONV 25°C 120′	CONV 60°C 15′	CONV 60°C 30′	CONV 60°C 60′	CONV 60°C 120′	HHP
Terpenes											
Monoterpenes											
α-Pinene	7.25	949	0.038^{cdef}	0.037^{def}	0.030^{efg}	0.033^{def}	0.056^{bcdef}	0.032^{def}	0.077^{b}	nd	nd
β-Pinene	6.39	998	0.455^{ef}	0.430^{ef}	0.401^{ef}	0.368^{f}	0.394^{ef}	0.414^{ef}	0.424^{def}	0.396^{ef}	0.800^{a}
β- Myrcene	7.42	1006	28.3^{def}	26.5^{def}	21.7^{fgh}	19.8^{gh}	24.7^{efg}	26.6^{efg}	27.9^{def}	23.2^{efgh}	16.17^{i}
Limonene	8.22	1049	0.092^{b}	0.086^{b}	0.075^{b}	0.067^{b}	0.079^{b}	0.093^{b}	0.102^{b}	0.088^{b}	nd
α-Phellandrene	8.26	1051	0.056^{bcd}	0.058^{bcd}	0.045^{bcd}	0.034^{cd}	0.055^{bcd}	0.060^{bcd}	0.065^{bc}	0.049^{bcd}	nd
Myrtenol	9.36	1109	0.098^{f}	0.103^{ef}	0.12^{cdef}	0.114^{def}	0.094^{f}	0.107^{ef}	0.093^{f}	0.202^{c}	0.515^{b}
3-Carene	9.53	1119	nd	nd	nd	nd	nd	nd	nd	nd	nd
cis-β-Ocimene	8.39	1064	nd	nd	nd	nd	nd	nd	nd	nd	nd
Sesquiterpenes											
α-Ylangene	14.38	1395	0.216^{c}	0.196^{c}	0.241^{c}	0.235^{c}	0.179^{c}	0.154^{c}	0.181^{c}	0.196^{c}	0.600^{a}
α-Copaene	14.48	1402	0.421^{b}	0.416^{b}	0.443^{b}	0.474^{ab}	0.393^{b}	0.385^{b}	0.421^{b}	0.437^{b}	0.594^{a}
β-Caryophyllene	15.25	1448	15.6^{abcd}	15.8^{abcd}	16.3^{abc}	16.8^{ab}	17.3^{a}	16.5^{ab}	16.2^{abc}	17.1^{a}	13.8^{bcde}
cis-β-Farnesene	15.62	1535	6.56^{b}	6.94^{b}	7.40^{b}	7.27^{b}	6.94^{b}	6.57^{b}	6.41^{b}	7.03^{bc}	2.62^{f}
Humulene	15.83	1483	37.0^{def}	37.9^{cdef}	39.3^{bcde}	40.5^{bcd}	38.7^{bcde}	38.4^{bcdef}	37.6^{cdef}	39.6^{bcde}	54.5^{a}
γ-Muurolene	16.08	1498	1.59^{cde}	1.68^{bcd}	1.91^{ab}	2.00^{a}	1.66^{bcd}	1.48^{cde}	1.57^{cde}	1.72^{abc}	1.40^{def}
α-Selinene	16.47	1515	2.71^{abcd}	2.83^{abcd}	3.32^{ab}	3.52^{a}	2.73^{abcd}	2.61^{abcd}	2.49^{bcd}	2.85^{abc}	1.82^{def}
γ-Gurjunene	16.69	1521	3.54^{abc}	3.74^{abc}	4.12^{ab}	4.37^{a}	3.56^{abc}	3.30^{bcd}	3.34^{bc}	3.73^{abc}	3.12^{cde}
cis-Muurola-3.5-diene	16.79	1535	0.665^{bc}	0.664^{bc}	0.826^{ab}	0.871^{a}	0.665^{bc}	0.630^{bc}	0.633^{bc}	0.733^{abc}	0.392^{de}
β-Cadinene	16.84	1538	1.43^{bc}	1.50^{abc}	1.71^{ab}	1.79^{a}	1.40^{bc}	1.46^{abc}	1.40^{bc}	1.53^{abc}	1.01^{def}
L-Calamenene	16.93	1543	0.502^{bc}	0.439^{bcd}	0.523^{bc}	0.525^{bc}	0.493^{bc}	0.430^{cd}	0.447^{bcd}	0.498^{bc}	nd
α-Muurolene	17.15	1556	0.086^{abc}	0.097^{abc}	0.128^{a}	0.133^{a}	0.096^{abc}	0.086^{abc}	0.052^{bcd}	0.112^{ab}	nd
α-Calacorene	17.23	1561	nd	nd	0.129^{a}	0.087^{ab}	0.067^{bc}	0.070^{bc}	0.038^{bcd}	0.057^{bc}	nd
Caryophyllene oxide	17.93	1603	0.151^{b}	0.044^{b}	0.652^{a}	0.603^{a}	0.051^{b}	0.068^{cb}	0.060^{b}	0.036^{b}	0.229^{b}
cis-Z-α-Bisabolene epoxide	18.41	1629	0.234^{de}	0.255^{de}	0.296^{de}	0.278^{de}	0.158^{de}	0.174^{e}	0.177^{de}	0.186^{de}	1.251^{a}
Esters											
Saturated esters											
2-Methylbutyl propanoate	7.04	985	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-Methylbutyl 2-methylpropanoate	7.88	1031	0.025^{cd}	0.034^{cd}	0.030^{cd}	0.020^{d}	0.033^{cd}	0.032^{cd}	0.044^{cd}	0.030^{cd}	0.268^{abc}
Methyl heptanoate	8.38	1039	nd	nd	nd	nd	nd	nd	nd	nd	nd
Methyl 6-methyl heptanoate	9.24	1103	nd	nd	nd	nd	nd	0.061^{cde}	nd	nd	0.192^{ab}
Methyl octanoate	9.95	1141	nd	nd	nd	nd	nd	nd	nd	nd	nd
Methyl nonanoate	11.79	1242	nd	nd	nd	nd	nd	nd	nd	nd	nd

(Continues)

TABLE 3 (Continued)

			Percentage peak area (PA %)								
	Average Rt (min)	RI	CONV 25°C 15′	CONV 25°C 30′	CONV 25°C 60′	CONV 25°C 120′	CONV 60°C 15′	CONV 60°C 30′	CONV 60°C 60′	CONV 60°C 120′	HHP
Methyl (4E) -4-nonenoate	11.57	1230	nd	nd	nd	nd	nd	nd	nd	nd	nd
Methyl 8-methyl-nonanoate	12.92	1307	nd	nd	nd	nd	nd	nd	nd	nd	nd
Methyl 4-decenoate	13.3	1328	0.187[e]	0.182[ef]	0.152[e]	0.161[e]	0.164[e]	0.143[e]	0.163[e]	0.156[e]	nd
Methyl geranate	13.54	1342	0.108[d]	0.107[d]	0.102[d]	0.122[d]	0.010[d]	0.093[d]	0.159[cd]	0.094[d]	0.733[a]
Ketones											
2-Undecanone	13.04	1313	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total absolute area (10^9)			1.43[bc]	1.41[bc]	1.50[bc]	1.57[bc]	1.72[b]	1.60[bc]	1.41[bc]	1.48[bc]	1.00[c]

Note: Data in rows with different letters were statistically different at p level < 0.05.
Abbreviations: CONV 25°C, conventional extraction at 25°C; CONV 60°C, conventional extraction at 60°C; HPUS, high-power ultrasound; US, ultrasound; HHP, high hydrostatic pressure; nd, not detected; RI, van den Dool & Kratz retention index referred to C6–C40 n-alkane mixture standard; Rt, retention time in minutes.

β-pinene, limonene, α-ylangene, α-copaene, cis-β-farnesene, α-selinene, α-muurolene, cis-Z-α-bisabolene epoxide, and methyl 4-decenoate. The combined effect of $T \times t$ significantly affected the extraction of all the detected aromatic compounds except for β-pinene, α-ylangene, α-copaene, cis-β-farnesene, α-selinene, γ-gurjunene, β-cadinene, L-calamenene, α-muurolene, cis-Z-α bisabolene epoxide, and methyl 4-decenoate.

Data of the UAE extracts were also processed by multifactorial ANOVA to investigate the single and combined effect of the two ultrasound EMs and of the extraction time (t) on the single aromatic compounds of hop extracts (Table 4). The results highlight that EM significantly ($p < 0.05$) affected the extraction of all the detected compounds apart from β-pinene, humulene, and γ-gurjunene. The effective hypothesis decomposition analysis for the EM factor indicated a higher RPA for β-myrcene, myrtenol, α-ylangene, α-copaene, L-calamenene, cis-Z-α-bisabolene epoxide, 2-methylbutyl 2-methylpropanoate, methyl 6-methyl heptanoate, methyl octanoate, methyl 4-decenoate, and methyl geranate in the US extracts, while the remaining compounds were higher in the HPUS extracts (data not shown). Moreover, conversely to the US, HPUS allowed also to extract a small amount of methyl nonanoate.

In both US- and HPUS-assisted extractions, the extraction time (t) influenced ($p < 0.05$) the aromatic compounds pattern of the hop extracts, except for α-ylangene, β-caryophyllene, humulene, 2-methylbutyl 2-methylpropanoate, and methyl nonanoate. In addition, the combined effect of EM $\times$ t significantly influenced the extraction of VOCs except for α-ylangene, α-copaene, caryophyllene oxide, 2-methylbutyl 2-methylpropanoate, and methyl nonanoate.

As already highlighted, there is limited information in the scientific literature on the effect of ultrasound or extraction temperature on the aroma composition of the related hop extracts. However, this study shows that the method of extraction (either conventional or non-conventional) may influence the chemical composition of hop extract and thus its sensory and techno-functional properties.

3.3 | Multivariate statistical analysis

To better understand the interrelations among all the variables analyzed and extractions parameters, the dataset was subjected to HCA and PLS-DA. HCA was used to explore the organization of samples analyzed in groups, and among groups depicting a hierarchy, considering all the normalized dataset comprising AA and BA, and all the identified volatile compounds. The resulting dendrogram is shown in Figure 1. At an average Euclidean distance between 100 and 120, an overall separation in two main clusters was found between the EMs and, in particular, between the conventional and nonconventional techniques.

Moreover, at an average distance ranging from 60 to 80, three clusters were observed. Interestingly, all the extractions carried out by conventional methods, independently of extraction temperature, were clustered together, and the extractions carried out by using the ultrasound technique were separated in two different clusters. Finally, it is possible to note that the HHP method was clustered with the US extraction.

HCA was then followed by ANOVA to highlight any significant difference among clusters linked to the

TABLE 4 Multifactorial analysis of variance (ANOVA) of the individual and interactive effects of extraction temperature (*T*) and time (*t*) for conventional extractions (25°C; 60°C), and of extraction method (EM) and time (*t*) for ultrasound-assisted extractions (US; HPUS), on relative composition of volatile organic compounds (VOCs) of hop extracts.

	F					
	CONV			**UEA**		
Compound	*t*	*T*	*t* × *T*	EM	*t*	EM × *T*
α-Pinene	123.6***	20.3***	136.4***	11.3***	68.9***	82.1***
β-Pinene	ns	ns	ns	ns	4.5*	10.8**
β-Myrcene	10.8**	ns	8.5**	7.9*	6.7*	26.3***
Limonene	ns	9.6*	7.2*	7.7*	94.3***	95.9***
α-Phellandrene	13.8**	19.2**	5.8*	446.9***	53.1***	158.5***
Myrtenol	24.4***	6.4*	20.4***	1091.6***	28.1***	15.6*
α-Ylangene	ns	19.2**	ns	187.4***	ns	ns
α-Copaene	ns	ns	ns	171.9***	5.3*	ns
β-Caryophyllene	4.1*	13.4**	5.3*	144.3***	ns	16.7***
cis-ß-Farnesene	ns	ns	ns	26.4***	22.3***	10.8**
Humulene	9.4**	ns	5.7*	ns	ns	29.3***
γ-Muurolene	10.8**	23.6**	5.8*	77.6***	27.6***	26.9***
α-Selinene	ns	7.2*	ns	8.9*	9.6**	11.4**
γ-Gurjunene	7.4*	26.0***	ns	ns	4.4*	6.8*
cis-Muurola-3,5-diene	10.0**	17.0**	4.1*	32.1***	21.1***	23.9***
β-Cadinene	6.9*	15.9**	ns	242.8***	60.1***	87.5***
L-Calamenene	9.2**	7.5*	ns	73.4***	26.8***	23.9***
α-Muurolene	ns	ns	ns	nd	nd	nd
α-Calacorene	7.1*	ns	16.8***	nd	nd	nd
Caryophyllene oxide	254.9***	1057***	279.0***	28.3***	21.9***	ns
cis-Z-α-Bisabolene epoxide	ns	39.5***	ns	786***	8.3*	20.2***
2-Methylbutyl 2-methylpropanoate	17.8***	35.4***	7.6***	14.5**	ns	ns
Methyl 6-methyl heptanoate	323.6***	323.6***	323.6***	152.6***	11.1**	5.8*
Methyl octanoate	nd	nd	nd	124.1***	43.7***	51.8***
Methyl nonanoate	nd	nd	nd	34.3***	ns	ns
Methyl 4-decenoate	ns	ns	ns	243.3***	21.9***	12.4*
Methyl geranate	4.5*	ns	8.5**	466.2***	15.7*	12.7*

Abbreviations: CONV, conventional extraction; nd, not detected; ns, not significant; UAE, ultrasound-assisted extraction.
*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.

different variables analyzed. Where significant *F*-values occur, HSD Tukey's post hoc comparisons were conducted to determine the simple effects between clusters. Significant differences ($p < 0.05$) were observed among the three clusters (Table 5). Based on the post hoc analysis, it was possible to group the hop extracts into three clusters: Cluster 1 (HPUS) mainly characterized by the presence of methyl nonanoate, Cluster 2 (US and HHP) grouping hop extracts with a high percentage composition of esters, and Cluster 3 (CONV 25°C and CONV 60°) grouping extracts with a high percentage composition of terpenes.

In order to test if different extraction conditions allow discriminating hop extracts and thus, to confirm the results of HCA, the data matrix was processed by PLS-DA. The three different clusters were used as predictor variables, and all aromatic compounds were used as explanatory variables.

The PLS-DA model depicted three significant LVs and explained the 91% and 73% of Y and X variance, respectively, with $Qi^2 = 0.844$. The model permitted to correctly classify all hop extracts with a classification rate of 100%.

Figures 2a,b and 3a,b show the score and correlation plots along the first two LV of the PLS-DA model. The discrimination among three clusters and conventional and nonconventional extractions was mostly due to the first

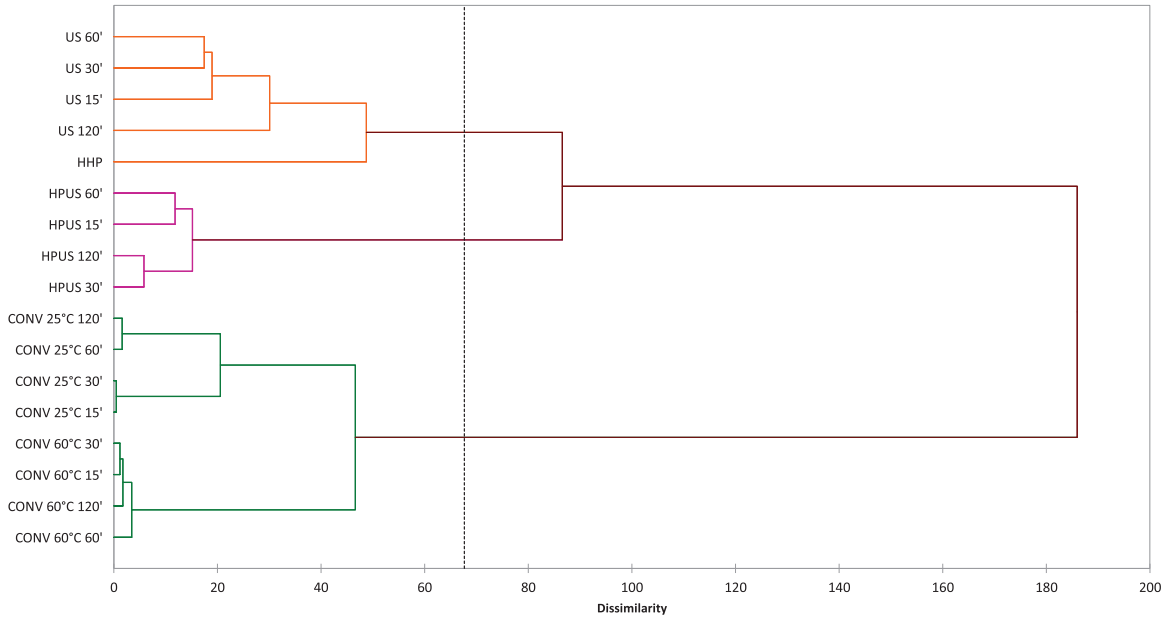

FIGURE 1 Hierarchical cluster analysis (HCA) of the functional traits of hop extracts. CONV 25°C: conventional extraction at 25°C; CONV 60°C conventional extraction at 60°C; HPUS: high-power ultrasounds; US, low power ultrasounds; HHP, high hydrostatic pressure; 15′, 30′, 60′, 120′: extraction times in minutes.

TABLE 5 Multivariate analysis of aromatic compounds of hop extracts.

	Cluster 1		Cluster 2		Cluster 3	
Variable	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
β-Pinene	0.7[a]	0.1	0.7[a]	0.1	0.41[b]	0.27
β-Myrcene	34.9[a]	6.9	33.8[a]	10.7	24.80[b]	3.2
2-Methylbutyl 2-methylpropanoate	0.06[b]	0.02	0.22[a]	0.06	0.031[b]	0.007
α-Phellandrene	0.07[a]	0.02	0.02[b]	0.04	0.05[ab]	0.010
Methyl 6-methyl heptanoate	0.06[b]	0.02	0.20[a]	0.06	0.008[c]	0.022
Myrtenol	0.017[a]	0.02	0.06[b]	0.1	0.12[a]	0.04
Methyl octanoate	0.07[ab]	0.01	0.1[a]	0.1	-	-
Methyl nonanoate	0.2[a]	0.1	-	-	-	-
Methyl 4-decenoate	0.18[b]	0.05	0.4[a]	0.3	0.16[b]	0.01
Methyl geranate	0.14[b]	0.04	0.61[a]	0.14	0.11[b]	0.02
α-Ylangene	0.17[b]	0.02	0.55[a]	0.08	0.20[b]	0.03
α-Copaene	0.2[b]	0.01	0.4[a]	0.100	0.42[a]	0.03
β-Caryophyllene	14[c]	1	11[b]	2	16.5[a]	0.4
cis-β-Farnesene	3.5[b]	1.1	2.7[c]	0.4	6.9[a]	0.4
γ-Muurolene	1.1[b]	0.2	1.0[b]	0.3	1.7[a]	0.2
α-Selinene	1.4[b]	0.4	1.3[b]	0.3	2.9[a]	0.4
γ-Gurjunene	2.2[b]	0.5	2.4[b]	0.5	3.7[a]	0.4
cis-Muurola-3.5-diene	0.4[b]	0.1	0.32[b]	0.09	0.71[a]	0.09
β-Cadinene	1.0[b]	0.2	0.8[b]	0.2	1.5[a]	0.1
α-Muurolene	-	-	-	-	0.1[a]	0.03
α-Calacorene	-	-	-	-	0.06[a]	0.04
cis-Z-α-Bisabolene epoxide	0.31[b]	0.09	1.1[a]	0.18	0.22[b]	0.05

Note: Different lowercase letters in a row indicate significant differences according to post hoc HSD Tukey's test ($p < 0.05$). Aromatic compounds are expressed as abundance relative (%).

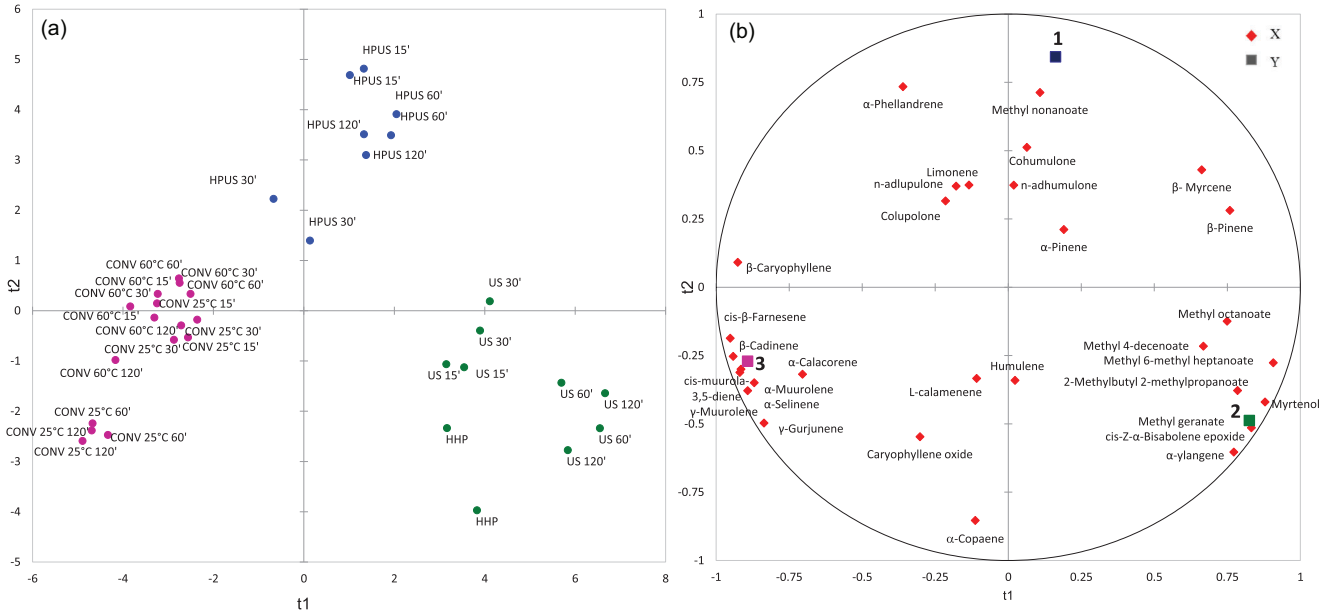

FIGURE 2 Scores (a) and correlation plot (b) of the partial least squares-discriminant analysis (PLS-DA) model along the first and second latent variables (LVs). CONV 25°C, conventional extraction at 25°C; CONV 60°C, conventional extraction at 60°C; HPUS, high-power ultrasounds; US, low power ultrasounds; HHP, high hydrostatic pressure; 15′, 30′, 60′, 120′: extraction times in minutes; 1: Cluster 1; 2: Cluster 2; 3: Cluster 3.

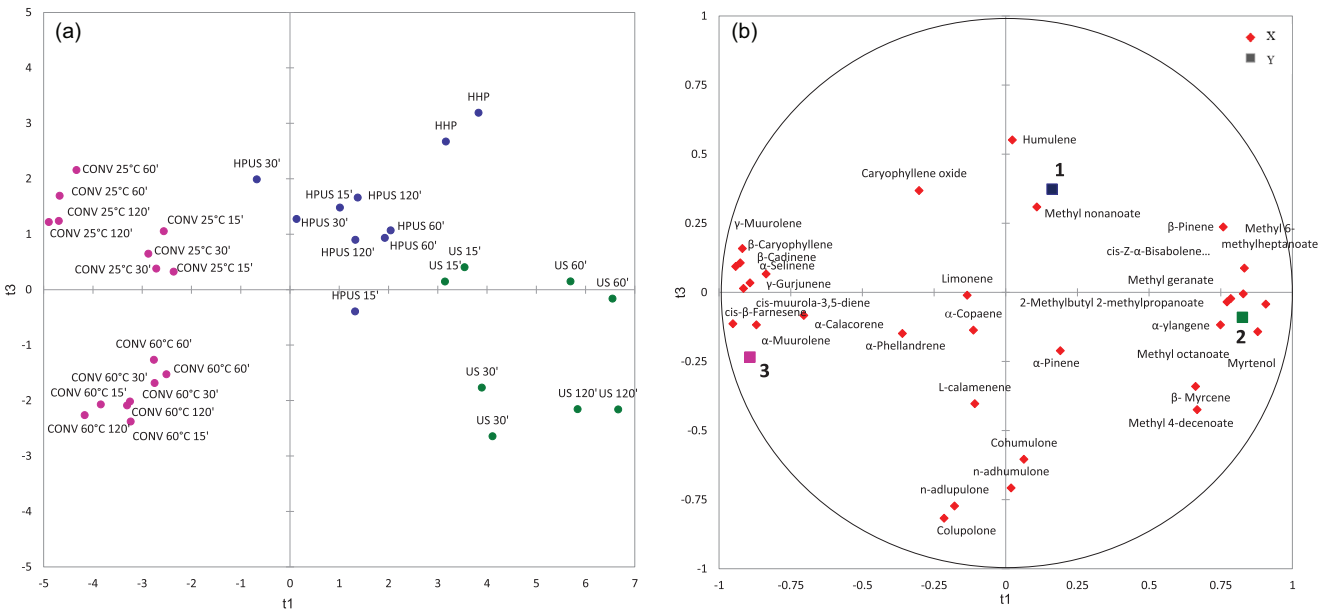

FIGURE 3 Scores (a) and correlation plot (b) of the partial least squares-discriminant analysis (PLS-DA) model along the first and third latent variables (LVs). CONV 25°C, conventional extraction at 25°C; CONV 60°C, conventional extraction at 60°C; HPUS, high-power ultrasounds; US, low power ultrasounds; HHP, high hydrostatic pressure; 15′, 30′, 60′, 120′: extraction times in minutes; 1: Cluster 1; 2: Cluster 2; 3: Cluster 3.

(Y 50%, X 44%) and second (Y 34%, X 18%) latent variables (Figure 2a), while the third (Y 7%, X 29%) latent variable (Figure 3a) mainly contributed to the discrimination of conventional extraction based on extraction temperature.

The VOCs with variable importance in projection (VIP) values greater than 1.0 on the first LV (t1) which characterize (w1 positive) US and HHP extractions (Cluster 2) were 2-methylbutyl 2-methylpropanoate, cis-Z-α-bisabolene epoxide, methyl 6-methyl heptanoate, methyl

geranate, methyl nonanoate, myrtenol, α-ylangene, and β-pinene. VIP values that characterize (w1 negative) conventional extraction (Cluster 3) were α-copaene, cis-muurola-3,5-diene, cis-β-farnesene, α-muurolene, α-phellandrene, α-selinene, β-cadinene, β-caryophyllene, γ-gurjunene, and γ-muurolene. The volatile compounds with VIP values greater than 1.0 on the second LV (t2) which characterize (w2 positive) HPUS extracts were methyl nonanoate, α-phellandrene, and β-caryophyllene.

From the results of the statistical analysis, it is interesting to highlight how despite the different statistical approaches it is possible to obtain the same results and discriminate the different hop extracts based on the different extraction conditions.

4 | CONCLUSION

The results of this study highlight that different extraction technologies and process conditions may deeply affect the composition of hop extracts leading to hop products with different sensory and techno-functional properties. Overall, the use of an aqueous ethanolic solution (50% v/v) in combination with UAEs and conventional extraction at 60°C allowed the production of hop extracts rich in bitter acids and characterized by a wide range of VOCs. In particular, among all the EMs under investigation, dynamic maceration at 60°C determined the highest extraction of bitter acids and sesquiterpenes with woody, citrus, herbal and spicy notes such as β-caryophyllene and cis-β-farnesene, while the use of ultrasounds (US and HPUS) allowed extracting, in general, more esters and monoterpenes with minty, woody, balsamic, and fruity notes such as β-myrcene, β-pinene and myrtenol. Compared to the other EMs, HHP highlighted a low capability to extract both bitter acids and VOCs.

Chemometric techniques, that is, HCA and PLS-DA effectively showed the optimal technological conditions to produce hop extracts with VOCs and bitter acid composition responding to specific technological, qualitative, and sensory needs for food applications.

This study deepened the knowledge of the effect of extraction technologies on the composition of hop extracts that could contribute to the development of new natural flavoring and/or preservative hop products to be used, beyond the craft and industrial brewery productions, for the development of sustainable and clean label foods (meat, bakery, minimally processed products, and non -alcoholic beverages) and new edible packaging or coatings.

AUTHOR CONTRIBUTIONS

Veronica Santarelli: Conceptualization; Methodology; Investigation; Formal analysis; Writing – original draft; Writing – review & editing. **Lilia Neri**: Conceptualization; Methodology; Formal analysis; Writing – original draft; Writing – review & editing; Investigation. **Katya Carbone**: Formal analysis; Writing – review & editing; Supervision; Visualization. **Valentina Macchioni**: Investigation. **Marco Faieta**: Visualization. **Paola Pittia**: Conceptualization; Writing – review & editing; Supervision; Project administration; Funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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