






# Comparative analysis of four hop cultivars grown in Brazil and the USA by GC-MS-based metabolomics

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## Abstract

**Why was the work done:** Although the third largest beer producer in the world, Brazil currently imports the majority of its hops. A recent development is the cultivation of hops (*Humulus lupulus* L.) in Brazil. In addition to genetic factors, the chemical composition of hops can exhibit variations due to conditions of cultivation. Accordingly, it is of value to characterise and differentiate hop cultivars grown in Brazil with the same cultivars grown in a long established location such as the United States of America.

**How was the work done:** Centennial, Chinook, Columbus, and Nugget cultivars grown in Brazil or in the USA were compared by metabolomic analyses of the chemical profiles using gas chromatography coupled to mass spectrometry. Principal component analysis showed sample grouping according to where the hops were grown. Partial Least Squares Discriminant Analysis allowed the characterisation of the main metabolites that discriminated hop samples from the two countries. A total of 31 metabolites were putatively identified, including monoterpenes, sesquiterpenes, oxygenated mono- and sesquiterpenes, esters, alcohols, and ketones.

**What are the main findings:** There were clear metabolic differences between the same hop varieties grown in Brazil or the USA. The metabolites with the greatest discriminating power for Brazilian hops were *trans*- $\alpha$ -bergamotene, 2-decanone, and *l*-gurjunene, while American hops presented  $\beta$ -copaene, humuladienone, and isopentyl isobutyrate. Notably, *trans*- $\alpha$ -bergamotene was present in Brazilian hops but absent from American hops.

**Why is the work important:** This study sheds light on the differences in the chemical composition of hops cultivated in Brazil compared those cultivated in the USA. This knowledge may stimulate new producers and contribute to the development of hop cultivation in Brazil.

## Keywords

chemical profile, metabolic fingerprinting, *Humulus lupulus* L, cultivation, geographical origin, secondary metabolite, variability, hops

## Introduction

The genus *Humulus* is part of the Cannabaceae family and comprises three main species: *Humulus lupulus* L. *Humulus japonicus*, and *Humulus yunnanens* (Neve 1991). Of them, *H. lupulus* produces oils and resins that are used in the production of beer. Approximately 98% of the world hop cultivation is used for beer with 2% used in food, medicine and cosmetics (Chadwick et al. 2006; Zanoli and Zavatti 2008). As hops are native to Central Europe, Asia, and North America it was thought that hops could not be cultivated in countries with a tropical climate due to lack of cold winters and long days required for hop flowering and production of lupulin. However, hops are now being cultivated in Australia, New Zealand, Argentina and Brazil (Jastrombek et al. 2022).

Brazil is the third largest beer producer in the world, producing about 14 billion litres of beer per year. Most of the hops used in Brazilian beer production are imported from Germany and the United States of America (USA) ([http://www.cervbrasil.org.br/novo\\_site/dados-do-setor/](http://www.cervbrasil.org.br/novo_site/dados-do-setor/)). Indeed, in 2022, Brazil spent US\$ 70 million to import 4,300 tons of hops and hop extracts (<http://comexstat.mdic.gov.br/pt/geral/15604>). Although the cultivation of hops in Brazil is a recent development, it has potential with 50 hop cultivars registered (<https://sistemas.agricultura.gov.br/snpc/cultivarweb/>) and some 50 hectares cultivated with an estimated production of 24 tons (<https://aprolupulo.com/>). According to the Ministry of Agriculture, Livestock and Supply, the varieties Chinook, Columbus, Nugget and Centennial exhibit good adaptation to the growing conditions in Brazil and are the most cultivated (<https://www.gov.br/agricultura/pt-br/assuntos/inspecao/produtos-vegetal/publicacoes/anuario-da-cerveja-2021.pdf>).

Hops contribute bitterness, aroma, flavour, body, foam formation and retention, aroma and flavour stability, together with antimicrobial and antioxidant activity to beer (Hieronymus 2012; Almaguer et al. 2014) The cones (inflorescence) of female plants have lupulin glands (glandular trichomes) where essential oils, prenylflavonoids, soft resins ( $\alpha$ - and  $\beta$ -acids) and hard resins are formed and stored (Hieronymus 2012; Mosher and Trantham 2017).

The  $\alpha$ -acids, after isomerisation to iso  $\alpha$ -acids during wort boiling, are the primary source of bitterness in beers (Eßlinger 2009; Mosher and Trantham 2017). The essential oils influence aromatic characteristics of hops, providing aroma and flavour to beer (Briggs et al. 2004; Eyres and Dufour 2008; Eßlinger 2009; Hieronymus 2012; Mosher and Trantham 2017). The monoterpene  $\beta$ -myrcene and the sesquiterpenes  $\alpha$ -humulene and  $\beta$ -caryophyllene are the major essential oils, and vary in the composition of terpenes, esters, ketones, alcohols, carboxylic acids, aldehydes, aliphatic hydrocarbons, phenols, furans, and sulphur compounds (Fix 1999; Hieronymus 2012; Dresel et al. 2015; Mosher and Trantham 2017; Roland et al. 2017; Machado et al. 2019).

The chemical composition of hops varies due to genetic and environmental factors (Abram et al. 2015; Sharp et al. 2017; Rettberg et al. 2018; Su and Yin 2021). According to Matsui et al (2016), the environmental factors that affect cultivation are divided into three classes, (i) 'natural origin' factors, associated with soil types and plant age; (ii) 'controlled by culture' factors related to management during and after cultivation, such as application of fertiliser, irrigation, pruning, harvesting and post-harvest processing; and (iii) 'climate-based' factors, such as temperature, rain and sunlight during the growing period. Of these, the 'controlled by culture' factor can be altered, with for example, the use of artificial lighting to increase productivity (Bauerle 2019). In countries where hop cultivation is well established, such as the USA, the production chain is consolidated through specialised cooperatives and processing units. Accordingly, a comparison of hop processing in Brazil and the USA reveals differences in harvesting and post-harvesting procedures, including conditions of harvesting in the field, removal of the cones from the vines (timing, peeling and cleaning), drying, pelletising, fractioning, storage, and distribution.

The metabolic variability resulting from environmental, cultural, and epigenetic factors of hop growing in different regions has been the focus of research. Rodolfi et al (2019), characterised Cascade hops grown in Italy and compared it with Cascade grown in Germany, the United States, and Slovenia. The results showed differences in the composition of monoterpenes and sesquiterpenes

from different locations (Rodolfi et al. 2019). Accordingly, the characterisation of hops by their cultivation location can provide valuable information for brewers and hop growers.

Brazil is at the beginning of its journey as a hop producer. Therefore, analysis of the chemical composition of hops cultivated in Brazil and comparison with hops grown in long established locations will provide useful insight. In this study, the chemical profiles of hops cultivated in Brazil and the USA were analysed by gas chromatography coupled to mass spectrometry (GC-MS) using metabolomic tools. The aim of this work was to compare hops cultivated in Brazil and the USA and to identify the main discriminating metabolites.

## Materials and methods

### Plant materials and sample preparation

Hop pellets of cultivars Chinook, Columbus, Nugget and Centennial (2020 harvest) were purchased from Yakima Chief hops in the USA. Hops cultivated in Brazil were obtained from Brava Terra (Columbus and Nugget) and from Dalcin (Centennial, Chinook and Nugget).

Extracts were prepared from 30 mg of pelleted, powdered (using mortar and pestle) hops and 1 mL of dichloromethane (Riedel de Haën, Germany), with vortex agitation (AV-2, Gehaka, 5 min at room temperature) and filtration through cotton wool. The solvent was evaporated, and the extract resolubilised in dichloromethane at a concentration of 10 mg/mL. For each sample, extractions were performed in triplicate.

### Metabolic profile acquisition using GC-MS

Analyses was performed using a gas chromatograph coupled to a quadrupole mass spectrometer (QP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) using an RTx-5MS column (30m x 25mm x 25 µm).

The following conditions were employed: linear temperature gradient of 4°C/min from 60°C to 300°C and held at 300°C for 10 minutes; carrier gas, Helium; column oven temperature, 60°C;

injection temperature, 260°C; injection mode, split; injection volume, 1.0 µL; flow control mode, linear velocity; pressure, 86.7 kPa; total flow, 11.4 mL/min; column flow, 1.40 mL/min; linear velocity, 43.2 cm/s; purge flow, 3.0 mL/min and split ratio, 5. The mass spectra were acquired in the scan mode between 35 and 600 *m/z*, with an ion source temperature of 250°C and an EI voltage of 70 eV.

Chromatograms and mass spectra were visualised using GC Solutions software (version 4.20 for Windows, Shimadzu Corporation, Kyoto, Japan). A blank of dichloromethane and quality control samples (C<sub>8</sub>-C<sub>40</sub> alkanes) were injected for every ten analysed samples.

### Data Processing

The data obtained from the GC-MS analyses, was converted to the \*.mzXML file format using the GC Solutions software, and processed using MzMine™ software (version 2.53 for Windows, BMC Bioinformatics, UK)

The following parameters were used: mass detection, mass detector-centroid (noise level, 5.0 x 10<sup>2</sup>); ADAP chromatogram builder (min group size in # of scans, 5; group intensity threshold, 5 x 10<sup>2</sup>; min highest intensity, 1 x 10<sup>3</sup>; *m/z* tolerance, 0.5 *m/z* or 0 mg/L); chromatogram deconvolution, algorithm-wavelets (ADAP) (S/N threshold, 10; S/N estimator, intensity window SN; min feature height, 1 x 10<sup>3</sup>; coefficient/area threshold, 100; peak duration range, 0.02-2.0; RT wavelet range, 0.01-0.20), *m/z* centre calculation – median; hierarchical clustering (min cluster distance (min), 0.01; min cluster size, 3; min cluster intensity, 1 x 10<sup>3</sup>; min edge-to-height ratio, 0.3; min delta-to-height ratio, 0.2; min sharpness, 100; shape -similarity tolerance, 80; choice of model peak based on, *m/z* value); alignment, ADAP aligner (GC) (min confidence, 0.01; retention time tolerance, 0.3 min (absolute); *m/z* tolerance, 0.5 *m/z* or 0 mg/L; score threshold, 0.7 and retention time similarity, retention time difference, 0.4).

After processing, the data was exported as a spreadsheet in \*.csv format and transformed using Log<sub>10</sub>.

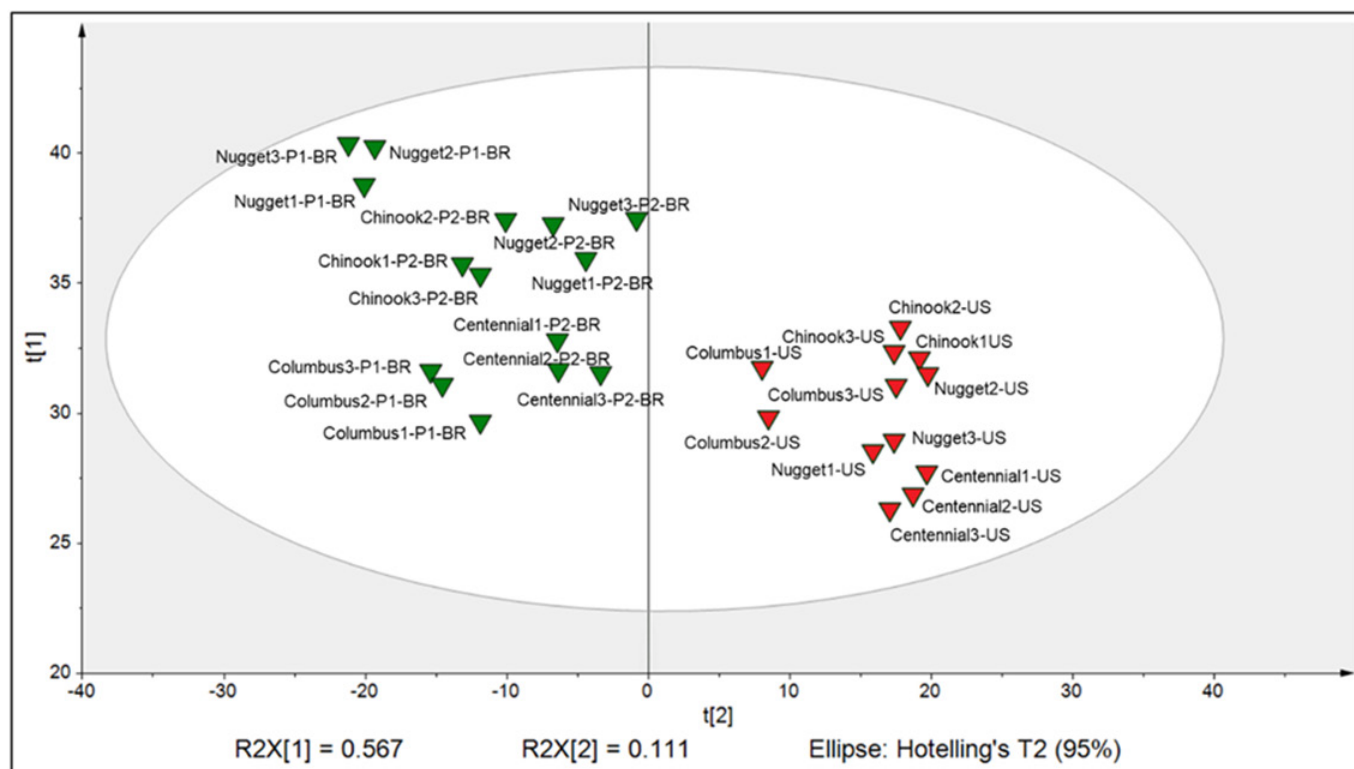
## Statistical analysis

Multivariate statistical analyses were performed using SIMCA software (version 13.0.3.0 for Windows, Umetrics, Umeå, Sweden). Data were first subjected to an unsupervised statistical analysis using principal component analysis (PCA). Then, a supervised statistical analysis using partial least squares discriminant analysis (PLS-DA) was performed to determine the discriminant metabolites for each cultivation site.

Discriminant metabolites of each group were selected using the variable importance in projection (VIP) plot from the PLS-DA, assuming that variables with VIP values greater than 1 were important for separating the groups. Metabolites detected by GC-MS were identified by comparing their mass spectra with the libraries of NIST (National Institute of Standards and Technology), Wiley, and FFNSC (Flavours and Fragrances of Natural and Synthetic Compounds) together with the retention indices from the literature with those calculated according to the equation of Van den Dool and Kratz (1963).

**Figure 1.**

**PCA score plot of hops cultivated in Brazil (green) and the USA (red) as a function of the first and second components ( $R^2_X = 0.783$ ;  $Q^2 = 0.629$ ).** The labels indicate each cultivar and the corresponding replicate (1, 2 and 3). For the hops cultivated in Brazil, P1 and P2 refer to those from Brava Terra and Dalcin.



## Results and discussion

The GC-MS chemical profiles of cultivars Chinook, Columbus, Nugget and Centennial from Brazil and the USA were initially explored by unsupervised statistical analysis. PCA (Figure 1) showed a tendency for samples to cluster according to where they were grown. The first and the second components explained 56.7% and 11.1% of the variance, with the second component (on the horizontal axis) responsible for separating the samples into two groups: hops cultivated in Brazil (left hemisphere) and the USA (right hemisphere). The blank and quality control samples were grouped, confirming the reproducibility of the GC-MS analyses and the efficacy of data processing (Supplementary Information Figure S1).

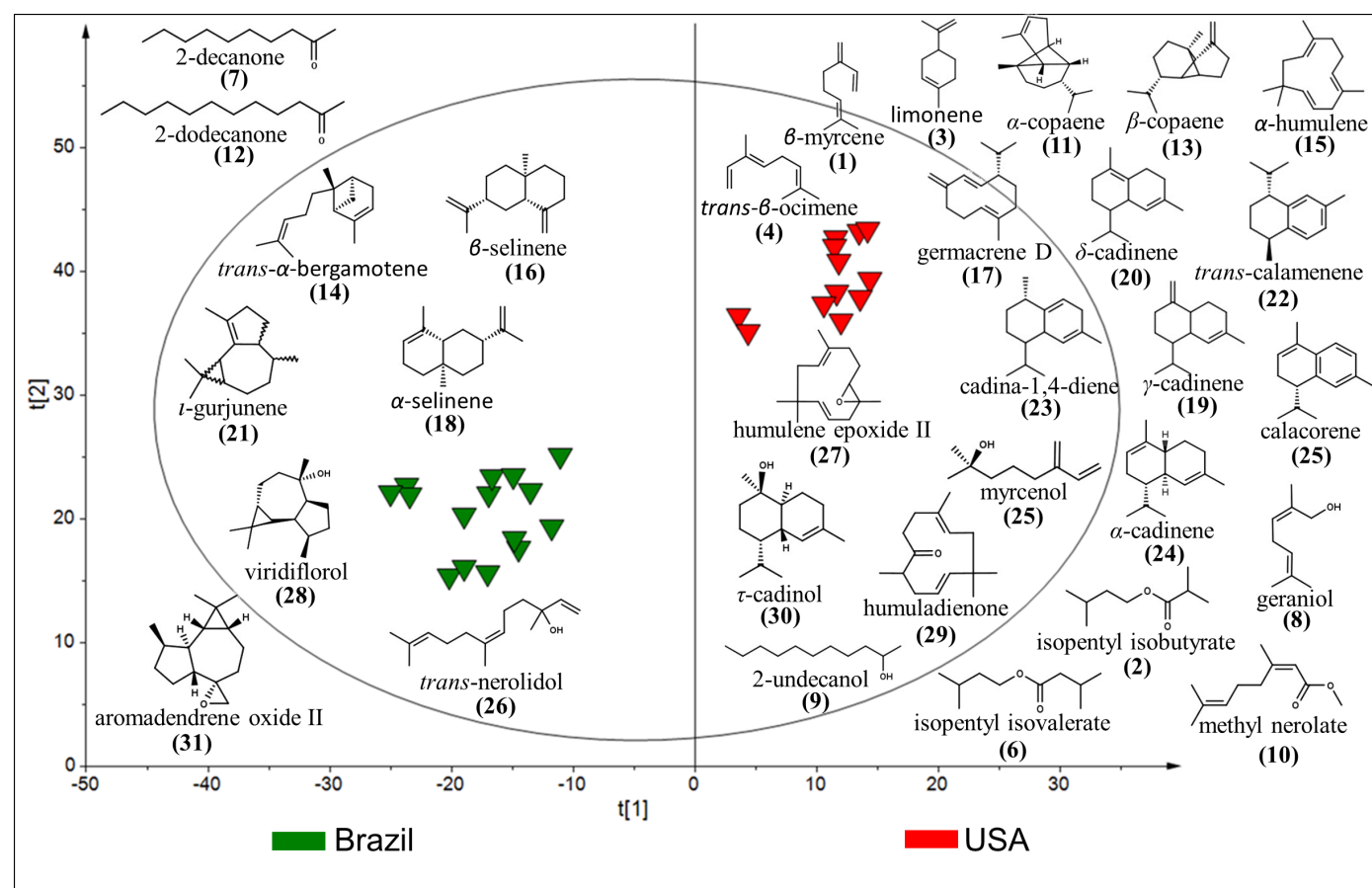
To determine the metabolites responsible for the differentiation of hops cultivated in Brazil or in the USA, supervised statistical analysis was performed. Partial least squares discriminant analysis (PLS-DA) confirmed the separation between hops cultivated in different locations (Figure 2). A total of 31

metabolites were putatively identified (Supplementary Information Table S1) according to level 2 for metabolite identification proposed by Sumner et al (2007). These included monoterpenes, sesquiterpenes, oxygenated mono- and sesquiterpenes, esters, alcohols, and ketones. The discriminant metabolites identified for each growing region and their respective chemical classes are reported in Table 1.

Samples of hops cultivated in the USA exhibited 22 discriminant metabolites (Figure 2), corresponding to three monoterpenes (1, 3, and 4), two oxygenated monoterpenes (5 and 8), 10 sesquiterpenes (11, 13, 15, 17, 19, 20, 22, 23, 24 and 25), three oxygenated sesquiterpenes (27, 29 and 30), three esters (2, 6 and 10) and an alcohol (9). With hops cultivated in Brazil, nine discriminant metabolites were putatively identified (Figure 2), including four sesquiterpenes (14, 16, 18, and 21), three oxygenated sesquiterpenes (26, 28, and 31) and two ketones (7 and 12).

Figure 2.

PLS score plot of hops cultivated in Brazil (green) and the USA (red), analysed by GC-MS ( $R_2Y = 0.989$ ;  $Q^2 = 0.946$ ). The name and structures of the discriminating metabolites for each location are presented in the plot.



In a study involving the autoxidation of constituents of hops, Dieckmann and Palamand (1974) observed  $\beta$ -myrcene as the precursor of substances contributing aroma such as limonene,  $\alpha$ - and  $\beta$ -pinene, linalool, geraniol, citral, and nerol. This is the result of four classes of reactions associated with myrcene autoxidation, namely cyclisation, oxidation, disproportionation, and polymerisation reactions.

Cyclisation reactions can generate the cyclic monoterpene limonene (3), the main metabolite present in citrus essential oils and responsible for the citrus notes in some hop cultivars. In addition to influencing aromatic characteristics, limonene can be the precursor of bicyclic monoterpenes such as  $\alpha$ - and  $\beta$ -pinene and camphene. It can also disproportionate into p-cymene and menthane (Dieckmann and Palamand 1974; Briggs et al. 2004).

**Table 1.**

**Discriminating metabolites and their chemical classes for Chinook, Columbus, Nugget and Centennial hops cultivated in Brazil and in the USA.** ID = metabolite identification; VIP = Variable Importance in Projection value.

Country	ID	VIP	Compound	Class	
USA	1	1.53	$\beta$ -myrcene	monoterpene	
	2	3.09	isopentyl isobutyrate	ester	
	3	2.30	limonene	monoterpene	
	4	1.35	<i>trans</i> - $\beta$ -ocimene	monoterpene	
	5	1.20	myrcenol	oxygenated monoterpene	
	6	1.94	isopentyl isovalerate	ester	
	8	1.95	geraniol	oxygenated monoterpene	
	9	1.46	2-undecanol	alcohol	
	10	1.81	methyl nerolate	ester	
	11	2.14	$\alpha$ -copaene	sesquiterpene	
	13	2.42	$\beta$ -copaene	sesquiterpene	
	15	1.07	$\alpha$ -humulene	sesquiterpene	
	17	1.53	germacrene D	sesquiterpene	
	19	1.77	$\gamma$ -cadinene	sesquiterpene	
	20	1.57	$\delta$ -cadinene	sesquiterpene	
	22	1.47	<i>trans</i> -calamenene	sesquiterpene	
	23	1.26	cadina-1,4-diene	sesquiterpene	
	24	1.80	$\alpha$ -cadinene	sesquiterpene	
	25	1.71	calacorene	sesquiterpene	
	27	1.66	humulene epoxide II	oxygenated sesquiterpene	
	29	2.63	humuladienone	oxygenated sesquiterpene	
	30	2.09	$\tau$ -cadinol	oxygenated sesquiterpene	
	Brazil	7	2.81	2-decanone	ketone
		12	2.21	2-dodecanone	ketone
		14	3.35	<i>trans</i> - $\alpha$ -bergamotene	sesquiterpene
		16	1.95	$\beta$ -selinene	sesquiterpene
		18	1.38	$\alpha$ -selinene	sesquiterpene
		21	2.42	<i>l</i> -gurjunene	sesquiterpene
		26	1.66	<i>trans</i> -nerolidol	oxygenated sesquiterpene
		28	1.92	viridiflorol	oxygenated sesquiterpene
31		1.20	aromadendrene oxide II	oxygenated sesquiterpene	

The oxygenated monoterpenes myrcenol (5) and geraniol (8) are also potential products of myrcene autoxidation generated from oxidation reactions differentiated American from Brazilian hops. Although geraniol is described as a specific metabolite of hops, the amount can vary considerably reflecting the geographic site, seasonal conditions, and agricultural practices (Liu et al. 2018; Su and Yin 2021). Furthermore, geraniol can be converted into  $\beta$ -citronellol during fermentation, transitioning from an aroma of roses to a citrus and floral aroma (King and Dickinson 2003; Takoi et al. 2010; Praet et al. 2012; Karabin et al. 2014). In general, oxygenated monoterpenes exhibit low odour thresholds and contribute to floral and fruity notes in hopped beers reflecting their high solubility (Kishimoto et al. 2006; Inui et al. 2013; van Opstaele et al. 2013; Lafontaine et al. 2019).

The esters isopentyl isobutyrate (2), isopentyl isovalerate (6), and methyl nerolate (10), and the aliphatic alcohol 2-undecanol (9) were identified as discriminant metabolites for hops cultivated in the USA. The branched chain esters isopentyl isobutyrate (2) and isopentyl isovalerate (6) are derived from amino acids, while methyl nerolate (10) is a terpene alcohol ester (Briggs et al. 2004). Esters in hop oil contribute to characteristic floral and fruity aromas, which are considered important indicators of hop quality (Sharpe and Laws 1981; Briggs et al. 2004). The aliphatic alcohol 2-undecanol (9) is derived from the ketone 2-undecanone, a methyl ketone considered a marker for hop cultivars, which contributes organoleptically to beer, with a flavour threshold of 70  $\mu\text{g/L}$  (Perpète et al. 1998).

Several sesquiterpenes differentiated hops cultivated in the USA from those from Brazil. The sesquiterpene  $\alpha$ -humulene (15) was the first metabolite identified in hop oil and is the most abundant sesquiterpene found in commercial cultivars (Sharpe and Laws 1981; Moir 2000; Almaguer et al. 2014). Furthermore, the ratio between the amount of  $\alpha$ -humulene and  $\beta$ -caryophyllene tends to be constant and specific to each cultivar and is useful for differentiating hops (Briggs et al. 2004). The sesquiterpene germacrene D (17) is considered a possible precursor in the enzymatic biosynthesis of several other sesquiterpenes, such as cadinenes (19, 20 and 24), cadine-1,4-diene (23), and

copaenes (11 and 13). Epoxidation reactions involving the sesquiterpene  $\gamma$ -cadinene (19) lead to the formation of the oxygenated sesquiterpene  $\tau$ -cadinol (30), also reported as a discriminant metabolite in American hops (Naya and Kotake 1972; Bülow and König 2000).

Oxygenated compounds found in hops can be divided into two groups: (i) formed by oxidised volatiles and derivatives from the decomposition of bitter acids, and (ii) formed by monoterpene alcohols, methyl ketones, methyl esters and esters of fatty acids, during cone maturation (Naya and Kotake 1972). The oxygenated sesquiterpenes humulene epoxide II (27) and humuladienone (29) are obtained through oxidative reactions of sesquiterpene  $\alpha$ -humulene as products of cone deterioration. Usually, oxidative reactions occur so quickly that the products are found in fresh hop oils (van Opstaele et al. 2013). Indeed, oxidation through incorrect processing or storage can result in desirable aromatic notes including herbal, floral, spicy, mouldy, or cedar (Sharpe and Laws 1981; van Opstaele et al. 2013; Rettberg et al. 2018).

### Discriminating metabolites in hops cultivated in Brazil

The ketones 2-decanone (7) and 2-dodecanone (12) were discriminants for hops cultivated in Brazil. Although ketones are minor metabolites in the volatile fraction of hop extracts, they contribute to floral, fruity, and citrus aroma in beers and are also indicators in distinguishing hops (Sharpe and Laws 1981; Perpète et al. 1998; van Opstaele et al. 2013; Yan et al. 2019).

In addition to ketones, several sesquiterpenes and oxygenated sesquiterpene derivatives were putatively identified as discriminants for Brazilian hops: *trans*- $\alpha$ -bergamotene (14),  $\alpha$ -selinene (18),  $\beta$ -selinene (16),  $\iota$ -gurjunene (21), *trans*-nerolidol (26), viridiflorol (28), and aromadendrene oxide II (31). The generation of the sesquiterpene  $\iota$ -gurjunene and the oxygenated sesquiterpenes viridiflorol and aromadendrene oxide II occur through arrangements of the precursor bicyclogermacrene, while *trans*-nerolidol is formed as a product of the acid hydrolysis of the oxygenated sesquiterpene farnesol, found in hop oil (Briggs et al. 2004).

The oxygenated sesquiterpene *trans*-nerolidol has pharmacological and biological properties, such as antimicrobial and antioxidant activity (Chan et al. 2016). Qualitative and quantitative differences in oxygenated terpene derivatives may occur in different hop cultivars. This is due to genetic factors but can also reflect cultivation conditions (geography, soil, agricultural practices, and post-harvest processing), suggesting their use in the characterisation and regional differentiation of hops (Likens and Nickerson 1967; Verzele and de Keukeleire 1991; Lafontaine and Shellhammer 2019; Machado et al. 2019)

In the study by Almeida et al (2020) of the antioxidant activity of essential oil from Cascade hops, differences in the chemical profile were associated with the site of cultivation. The sesquiterpenes  $\alpha$ -selinene,  $\beta$ -selinene, and *trans*- $\beta$ -farnesene were the main metabolites identified in the oil of Cascade hops cultivated in Brazil. However, the same cultivar grown in the USA showed high levels of  $\beta$ -myrcene,  $\alpha$ -humulene, and  $\beta$ -caryophyllene. Interestingly,  $\alpha$ -selinene and  $\beta$ -selinene were identified as discriminants for Brazilian hops.

In a study of the genetic diversity of European and North American wild hops, Patzak et al (2010) found that European hops contain high levels of selinene. Using a multidisciplinary approach, Dubbous-Wach et al (2021) evaluated wild hops and German cultivars grown in Corsica as well as in their countries of origin. The results indicated higher levels of  $\alpha$ -selinene in wild hops, which were linked to the environmental conditions of the area. Mongelli et al (2016) evaluated the suitability of growing Italian hop cultivars and ecotypes using a phytochemical approach. Selinene isomers were found in abundance in the volatile fraction of the ecotypes. These findings support the idea that selinenes can be used as chemical markers for hop cultivars (Paguet et al. 2022) and hops grown in Brazil.

The sesquiterpene *trans*- $\alpha$ -bergamotene was found in the Brazilian hops, but was not detected in American hops. This metabolite had the highest 'Variable Importance in Projection' value (Table 1) in the group of discriminating metabolites from Brazilian hops. Commonly, *trans*- $\alpha$ -bergamotene occurs in small amounts in hop oil, yet shows great

aromatic potential with a sweet, floral, and citrus aroma (Su and Yin 2021). Metabolic differences as a function of cultivation geography can contribute different aromatic characteristics to genetically identical hops, which may be of interest in differentiating beers.

## Conclusions

The results reported here demonstrate chemical differences between the same hop varieties grown in different geographies. Hops grown in Brazil contained ketones, sesquiterpenes, and oxygenated sesquiterpenes as discriminants. The sesquiterpenes *trans*- $\alpha$ -bergamotene and  $\iota$ -gurjunene, and ketone 2-decanone, contributed the highest discriminating power to the Brazilian hops. In addition, *trans*- $\alpha$ -bergamotene was present in all samples of Brazilian hops but absent in the American samples. Hops grown in the USA contained monoterpenes, oxygenated mono- and sesquiterpenes and esters as discriminants, primarily characterised by the sesquiterpene  $\beta$ -copaene, the oxygenated sesquiterpene humuladienone, and the ester isopentyl isobutyrate.

This study, unprecedented for hops cultivated in Brazil, enabled the characterisation of hops growing in different locations and provides insight as to the main differences in fragrant medium- and non-polar hop metabolites in Brazilian hops. This information about this raw material in beer may stimulate new producers and contribute to the development of hop cultivation in Brazil.

## Author contributions

**Guilherme Silva Dias:** resources, methodology, formal analysis, data curation, visualisation, writing (original draft).

**Marilia Elias Gallon:** investigation, conceptualisation, methodology, writing (review and editing), visualisation.

**Leonardo Gobbo-Neto:** conceptualisation, investigation, supervision, writing (review and editing), project administration, funding acquisition.



## Acknowledgements

G. S. Dias thanks the Coordination for the Improvement of Higher Education Personnel - CAPES (Process 88887.705690/2022-00) for the scholarship. L. Gobbo-Neto and G. S. Dias thank São Paulo Research Foundation (FAPESP) for research grant (Process 19/08651-7). L. Gobbo-Neto thanks the National Council for Scientific and Technological Development (CNPq) for the research grant (Process 305061/2021-6).

## Conflict of interest

No conflict of interest was reported by the authors.

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