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Metabolomic and Proteomic Profile of Dried Hop Inflorescences (*Humulus lupulus* L. cv. Chinook and cv. Cascade) by SPME-GC-MS and UPLC-MS-MS

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Abstract: Hop (*Humulus lupulus* L.) is grown mainly for the production of beer. The flowers of the female plant give it the bitter taste and pungent aroma. There are a large number of hop varieties differing in their α-acid content, essential oil levels and odor profiles. Aside from their use in brewing, more recently, hops have been used for the pharmacological properties of its derivatives that are of great importance to the pharmaceutical industry. Hop is known to have a fairly complex chemistry characterized by the presence of a variety of sesquiterpenoids, diterpenoids and triterpenoids, phytoestrogens and flavonoids. Additionally, considering the countless applications in the pharmacological sector in recent years, a chemical characterization of the different cultivars is essential to better identify the source of specific secondary metabolites. For this purpose, the dried inflorescences of two hop cultivars, Chinook and Cascade, were investigated using Solid-Phase Microextraction-Gas Chromatography-Mass Spectrometry and Liquid Chromatography-Tandem Mass Spectrometry (SPME-GC-MS and LC-MS-MS) to describe their metabolomic and proteomic profile. Furthermore, thanks to an in-depth statistical survey, it was possible to carry out a comparative study highlighting interesting implications deriving from this investigative study.

Keywords: proteins; volatile and non-volatile compounds; chromatographic analyses; multivariate statistical analysis

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1. Introduction

Humulus lupulus L., the common hop, is one of the 102 accepted species of the Cannabaceae family [1] and one of the two belonging to the *Humulus* genus present in Italy [2]. *H. lupulus* is a European Caucasian, dioecious, perennial plant with a climbing habit, 1 to 6 m high, widespread on the edges of humid woods, ditches, uncultivated areas and hedges, from the plain up to 1200 m asl. It has woody, striated, rough and branched stems with deflexed hairs, which, unable to stand alone, twist with the small-hooked thorns at any nearby support. The leaves are opposite, petiolate, broadly ovate-cordate, usually deeply three- to five-lobed and coarsely dentate (Figure 1A). The male flowers, white-yellow, 5 mm in diameter, are collected in a panicle inflorescence of 15–20 mm (Figure 1B). The female flowers gather in a pendent, glandular, cone-like, light green inflorescence formed by persistent ovate and acute bracts (Figure 1C). Even the infructescence (30 mm) has a conical shape. The flowering period is from May to August [2,3].

Figure 1. (**A**) *H. lupulus* leaves are deeply 3- to 5-lobed and coarsely dentate; (**B**) male flowers; (**C**) female inflorescence.

The natural distribution of *H. lupulus* is now obscured since inflorescences are widely used in brewing and the plant is consequently cultivated and often naturalized [3].

Cascade and Chinook are two of the many hop cultivars. The first one is a hop culti-var developed in the USDA breeding program in the 1950s, released as an American aromatic variety in 1972 and first used commercially in 1975 [4]. It was named after the Cascade Mountain range stretching along the west coast between the United States and Canada. The plant is characterized by elongated dark green cones with moderate to fairly high amounts of α-acids compared to many other types of hops. The resulting aroma is of medium intensity and very distinct. It has a pleasant, floral and spicy flavor, citrusy with a slight grapefruit characteristic [5]. As with the Cascade hop, Chinook hop, taking its name from an indigenous Native American tribe in the region around Washington state, is a USDA breeding program product with high α-acids and good storage properties released in 1985 [5]. It is a very distinctive dual-purpose hop used in beers as both bittering and aroma additions. Chinook hop has mainly spicy and pine characteristics with clear notes of grapefruit that accentuate its bitterness [6]. Both hops can be used to make any American and Indian Pale ales, but they are also suitable for seasonal ales, barley wine and some porters and stouts.

Inspired by our previous study on the chemical characterization of different cultivars of *C. sativa* L. inflorescences [7], in this work, we investigate the dried inflorescences of two hop cultivars, Chinook (Figure 2A) and Cascade (Figure 2B), using different techniques such as SPM-GC-MS and HPLC-MS -MS in order to obtain an exhaustive characterization of both volatile and non-volatile profile for comparative purposes. In addition, for the first time, we also report their protein content.

Figure 2. (**A**) *H. lupulus* L. "Chinook"; (**B**) *H. lupulus* L. "Cascade".

2. Materials and Methods

2.1. Materials

Formic acid, acetonitrile, ethanol, sodium dodecyl sulfate (SDS), urea, dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate and water were purchased from Sigma–Aldrich (Milano, Italy) and they were all LC-MS grade.

2.2. Plant Material

The two hop cultivars investigated in this study, Chinook and Cascade, both of American origin were grown in San Nicandro Garganico (Foggia, Italy) by the Azienda Agricola Vocino in 2021.

These two cultivars were chosen for their medium–high content of soft resins in particular of α- and β-acids, which have an antiseptic function in the production of beer during the boiling phase but also of defense against biotic and abiotic stresses in full field. Of the two cultivars, Cascade is the one that has been the most affected by the strong heat (abiotic stress), thus inducing an early pre-flowering at the expense of a lower yield of about 50% on average for each plant. Chinook, on the other hand, had a higher yield per plant, a low fungal attack on the flower and larger cones.

The irrigation of the plants, positioned at about 1 m from each other and with a distance between the rows of about 3 m, was carried out by drop without resorting to any treatment or chemical fertilization, while the soil was mainly worked manually. The manual harvesting of the plants was conducted at the end of August and the drying of the hop cones took place in a natural way, inside a dark and ventilated room with a constant humidity level (about 50% RH). After about 5 days, the hops were vacuum-packed and stored in a cool, dry place.

2.3. SPME Sampling

SPME technique was used to sample the volatile fraction. About 2 g of inflorescences from the two hop cultivars were placed inside a 15 mL glass vial with PTFE-coated silicone septum. The extraction of components was obtained using a SPME device (Supelco Inc., Belfont, PA, USA). The chosen fiber was coated with 50/30 µm DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane). The operative conditions following Cicaloni et al. [7] with minor modifications. For the desorption of the collected compound phase, the SPME fiber was inserted in the GC injector maintained at 250 °C in split mode.

2.4. GC-MS Analysis

A Clarus 500 model Perkin Elmer (Perkin Elmer Inc., Waltham, MA, USA) gas chromatograph coupled with a mass spectrometer equipped with an FID (flame detector

ionization) was used to perform the analyses of the headspace hop inflorescences. For the separation of compounds, a Restek Stabilwax polar capillary column and a Varian (VF-1ms) apolar column were used. For most of the compounds, with the non-polar column, higher relative amounts were obtained and are therefore reported.

The GC and MS parameters were assessed following Iannone et al. [8]. Briefly, the oven-programmed temperature was set initially at 55 °C and then increased to 220 °C at 6 °/min and finally held for 15 min. Helium was used as carrier gas at a constant rate of 1 mL/min. MS detection was performed with electron ionization (EI) at 70 eV operating in the full-scan acquisition mode in the m/z range of 40–500 amu. To identify the volatile compounds, we compared the mass spectra with those of pure components stored in the Wiley 2.2 and Nist 02 libraries database. The Linear Retention Indices (LRIs) were also calculated using a series of alkane standards (C_8–C_{25} *n*-alkanes), injected into both columns, and then compared with those in the literature. The relative amounts of the components were expressed as a percent peak area relative to the total peak area without the use of an internal standard and any factor correction. All analyses were carried out in triplicate.

2.5. UPLC-MS-MS Analysis

To investigate the non-volatile profile of the dried and powdered hop inflorescences, we used an Ultimate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA) that was controlled with Thermo Xcalibur software (Thermo Fisher Scientific, Waltham, MA, USA).

The samples were prepared by following this method: A total of 100 mg of powder was ultrasonicated for 20 min with 5 mL of 70% ethanol, followed by centrifugation (13,000 rpm, 4 °C) for 10 min. The resulting supernatant of the samples was injected into the UPLC-Q-Exactive plus system. The samples were separated using a column Acquity UPLC BEH C18 (2.1 mm × 15 cm, 1.7 µm, Waters, Waltham, MA, USA). The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). The gradient started with 2% of B, which was maintained constant for 1 min. Then, the organic phase was increased up to 100% in 50 min. The phase B was maintained at 100% for other 2 min and then returned to the initial condition. The flow rate was maintained at 0.2 mL/min and the injection volume of the sample was 10 µL. Additionally, the column temperature was kept at 35 °C. A Q-Exactive Plus™ quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to perform mass spectrometry analyses in the negative and positive ion modes, with a scan mass range set at m/z 200–2000.

HR-MS spectra were recorded in the positive and negative ion modes using the following parameters: spray voltage 3.5 kV (positive) and 3.0 kV (negative), sheath gas 20 (arbitrary units), auxiliary gas 5.0 (arbitrary units), capillary temperature 320 °C and resolution 35,000. MS/MS spectra were obtained by a Higher Energy Collision Dissociation (HCD) of 30 (arbitrary units). The accuracy error threshold was fixed at 5 ppm. The final annotated metabolome dataset was generated by Compound Discoverer 3.3 (Thermo Fisher Scientific, Waltham, MA, USA). The retention time (RT) was set to 0.2 min, with mass equal to 10 ppm, and other parameters were selected as the default values for peak extraction and peak alignment. All analyses were carried out in triplicate.

2.6. Proteomic Analysis

To describe the proteomic profile of the samples, we operated the procedure as follows: A total of 1 g frozen samples was homogenized (TissueRuptor homogenizer, Qiagen, Garstligweg, Switzerland) in 10 mL of 2% sodium Dodecyl Sulfate (SDS), dissolved in distilled water and centrifuged at 13,800× *g* rpm for 15 min. BCA assay by using bovine serum albumin (BSA) as standard was employed to quantify the protein concentration.

Filter-Aided Sample Preparation (FASP) method was conducted for protein digestion [9]. In particular, 200 µg of proteins were combined with 8 M urea to a final volume of 400 µL, uploaded in centrifugal ultrafiltration units with 30 kDa nominal molecular

weight cutoff (Microcon ®-30, Sigma-Aldrich, St. Louis, MO, USA). Successively, in order to reduce protein disulfide bridges, samples were incubated at RT for 30 min with 40 µL of 100 mM dithiothreitol (DTT) and then centrifuged at 13,800 rpm for 30 min. Once the flowthrough was discarded, the filter was then washed twice using 400 µL of 8 M urea by 30 min centrifugation at 13,800× *g* rpm. After that, the samples were incubated for 30 min in the dark to alkylate-free thiol groups with 100 µL of 100 mM iodoacetamide (IAA) in 8 M urea. Filter units were washed firstly with 400 µL of 8 M urea and successively with 400 µL of 50 mM ammonium bicarbonate, by centrifuging at 13,800× *g* rpm for 30 min and 20 min, respectively. Proteins digestion was carried out at 37 °C overnight using 1:50 dilution of Trypsin Gold-Mass Spec Grade (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate. After that, a centrifugation at 13,800× *g* rpm for 10 min was carried out to collect peptides, followed by two washes with 100 µL of 0.1% FA in distilled water. The digested samples were then desalted by using OASIS cartridges (Waters, Milford, MA, USA), brought to dryness, and reconstituted in formic acid (0.1%) in water to have a final concentration of 1 µg/mL.

Q Exactive™ HF-X hybrid quadrupole-Orbitrap™ mass spectrometer (Thermo Fischer Scientific, Waltham, MA, USA) was used to perform LC-MS/MS analyses. The peptide separation was carried out at 35 °C using an PepMap ™ RSLC C18 column, 75 µm × 150 mm, 2 µm, 100 Å (Thermo Fisher Scientific, Waltham, MA, USA) at a flow rate of 300 nL/min. The mobile phases A and B used for the analysis were 0.1% formic acid in water and 0.1% formic acid in 80% acetonitrile, respectively. The gradient started with 5% of B, that was maintained constant for 5 min. Then, the organic phase was increased up to 90% in 97 min and kept constant for 9 min and then returned to the initial conditions. These experiments were performed using a data-dependent acquisition (DDA) setting to select the "top twelve" most-abundant ions for MS/MS analysis.

Protein identification was performed using Proteome Discover 2.5 (Thermo Fisher Scientific, Waltham, MA, USA) and Sequest algorithm. The reference database was *Humulus* (Taxonomy ID: 3484) and the number of total proteins was 34.193, downloaded in June 2022.

2.7. Statistical Analysis

The resulting data matrix was imported into MetaboAnalyst 5.0 online platform [10] and graphically displayed by using several R packages ("ComplexHeatmap" version 2.11.1, "Circlize" version 0.4.13, and "ColorRamps" version 2.3).

The obtained data were normalized by sum. An heatmap was plotted to visualize the variations in potential markers and to separate metabolites into different groups by a hierarchical cluster analysis (HCA) based on Euclidean distance. Principal component analysis (PCA) was applied to provide an exploratory data analysis. An unsupervised PCA analysis on MetaboAnalyst 5.0 was carried out to determine how metabolites differ from each other, and which compounds contribute the most to this difference. Lastly, the differences in the metabolites were detected using PLS-DA. The corresponding VIP values were calculated using the PLS-DA model. The VIP value represents the difference between the considered variables. A VIP value above 1.5 indicated components that play an important role in differentiating between samples. Only components with VIP > 1.5 and $p < 0.05$ were selected as potential markers.

3. Results

3.1. GC-FID Chemical Composition

Using SPME-GC-MS technique, a total of thirty-five volatile compounds, listed in Table 1, was identified. In general, the sesquiterpene content exceeded the monoterpene content by about 50% in both varieties. β-Caryophyllene and humulene were the most abundant and with comparable relative abundances between Chinook (13.0 and 29.0%) and Cascade (12.9 and 30.0%). Among the monoterpenes, β-myrcene was the main one, but with a higher relative percentage in Cascade (27.1%) rather than in Chinook (19.4%). Quantitative similarities between the two cultivars regarding the percentage content of some compounds

were found. On the other hand, the qualitative differences were significant. In fact, the number of compounds detected in Chinook (31) was significantly higher than that in Cascade (18). In particular, compounds such as, α-pinene, terpinolene, *p*-cymen-8-ol, 4-decenoic acid, methyl ester, Z-, α-selinene, γ-cadinene and selina-3,7(11)-diene, ranging between 1.1 and 4.6%, were present only in Chinook and were missing in Cascade, in addition to other minor compounds with relative percentages lower than 1.0%. The relative chromatograms are reported in Figure 3.

Table 1. Chemical volatile composition (percentage mean value ± standard deviation) of the hop inflorescences.

N°	COMPONENT [1]	LRI [2]	LRI [3]	LRI [4]	Chinook	Cascade
1	3-penten-2-ol	768	774	1170	-	3.4 ± 0.03
2	α-thujene	819	823	1025	-	0.5 ± 0.03
3	butanoic acid, 3-methyl-	830	834	1639	-	0.7 ± 0.04
4	α-pinene	941	942	1018	4.6 ± 0.03	-
5	1-butanol, 2-methyl-, propanoate	970	968.4	1186	0.7 ± 0.03	-
6	β-myrcene	988	987	1171	19.4 ± 0.04	27.1 ± 0.05
7	propanoic acid, 2-methyl-, 2-methylbutyl ester	990	989	1190	0.7 ± 0.02	0.7 ± 0.03
8	heptanoic acid, methyl ester	1024	1026	1288	0.2 ± 0.02	0.5 ± 0.02
9	limonene	1031	1030	1201	2.3 ± 0.02	1.8 ± 0.02
10	β-terpinene	1033	1036	1190	0.3 ± 0.02	1.8 ± 0.03
11	α-ocimene	1045	1042	1210	0.7 ± 0.02	-
12	methyl, 6-methyl heptanoate	1072	1068	1310	0.3 ± 0.02	-
13	terpinolene	1083	1080	1270	3.0 ± 0.06	-
14	*p*-cymenene	1089	1091	1431	0.4 ± 0.03	-
15	perillen	1106	1102.1	1437	-	0.7 ± 0.03
16	octanoic acid, methyl ester	1125	1122	1381	0.1 ± 0.01	-
17	*p*-cymen-8-ol	1193	1189	1855	1.4 ± 0.02	-
18	nonanoic acid, methyl ester	1227	1224	1492	0.2 ± 0.02	-
19	2-undecanone	1295	1298	1596	0.4 ± 0.02	-
20	decanoic acid, methyl ester	1311	1309	1611	0.1 ± 0.02	-
21	4-decenoic acid, methyl ester, Z-	1315	*	1632	1.2 ± 0.02	-
22	methyl geranate	1328	1323	1673	0.6 ± 0.03	1.6 ± 0.02
23	α-cubebene	1352	1350	1462	2.2 ± 0.03	1.5 ± 0.02
24	ylangene	1381	1376	1493	0.5 ± 0.02	-
25	β-caryophyllene	1440	1440	1610	13.0 ± 0.05	12.9 ± 0.02
26	humulene	1477	1473	1665	29.0 ± 0.04	30.0 ± 0.05
27	β-eudesmene	1483	1481	1722	3.4 ± 0.03	3.1 ± 0.02
28	γ-muurolene	1490	1486	1681	4.0 ± 0.03	3.4 ± 0.03
29	α-selinene	1493	1489	1720	1.7 ± 0.02	-
30	γ-cadinene	1511	1509	1778	2.2 ± 0.02	-
31	δ-cadinene	1522	*	1750	4.4 ± 0.02	4.3 ± 0.02
32	α-muurolene	1525	*	1693	0.6 ± 0.02	5.3 ± 0.03
33	selina-3,7(11)-diene	1533	1530	1762	1.1 ± 0.02	-
34	caryophyllene oxide	1587	1585	1935	0.4 ± 0.02	-
35	humulene epoxide II	1610	1606	1988	0.9 ± 0.02	0.6 ± 0.03
	SUM				100.0	99.9
	Monoterpenoids				32.7	37.6
	Sesquiterpenoids				63.4	61.1
	Others				3.9	1.2

[1] The components are reported according to their elution order on the apolar column; [2] Linear Retention Indices measured on the apolar column; [3] Linear Retention Indices measured on the polar column; [4] Linear Retention indices from the literature; * LRI not available; - Not detected.

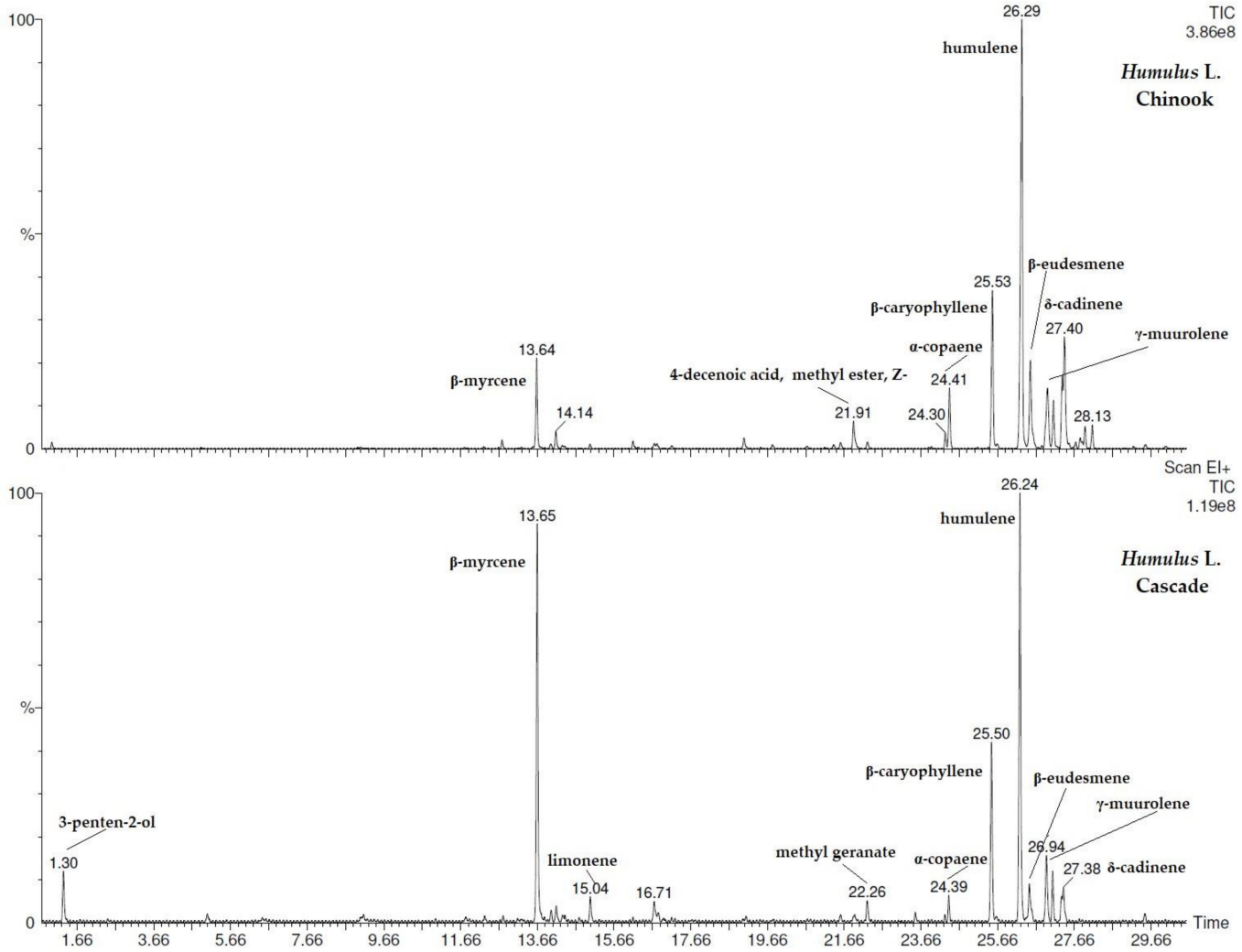

Figure 3. Chromatograms of the Chinook and Cascade hop dried inflorescences.

3.2. UPLC Chemical Composition

By UPLC analyses, fifty-six non-volatile compounds were detected and identified, forty-six in Chinook and forty-five in Cascade (Table 2). A series of flavonoids, α- and β-acids and other classes of compounds were found. Colupox A (25.0 and 32.3%), belonging to the class of organic compounds known as benzofurans, and adlupone (21.6 and 19.7%) belonging to that of *m*-benzoquinones, were the principal compounds in Chinook and Cascade, respectively, followed by gibberellin A12 (10.1 and 8.8%), lupulone F (6.4 and 6.5%) and bis(3-methyl-2-butenyl)phlorisovalerophenone (6.0 and 6.2%).

Morin (0.1%), manghaslin (0.1%), lupulone C (0.1%), quercetin 4-O-glucoside (0.2%), β-selinene epoxide (0.1%), gibberellin A17 (0.1%), trans-caffeic acid (0.1%) and quercetin 3-7-diglucoside (0.2%) were found only in Chinook. On the contrary, colupulone (0.1%), chlorogenic acid (0.1%), diprenylgenistein (0.1%), dihydroxanthohumol (0.1%) and secoisolariciresinol (0.1%) were detected only in Cascade.

Table 2. Chemical non-volatile composition (percentage mean value ± standard deviation) of *Humulus* L. inflorescences.

N°	COMPONENT [1]	Chinook	Cascade
1	hulupinic acid	0.1 ± 0.01	0.1 ± 0.01
2	isohumulone A	0.2 ± 0.03	0.1 ± 0.02
3	gibberellin A19	0.2 ± 0.04	0.1 ± 0.02
4	bis(3-methyl-2-butenyl)phlorisovalerophenone	6.0 ± 0.42	6.2 ± 0.40
5	postlupulone	3.2 ± 0.45	3.6 ± 0.85
6	adhumulone	0.1 ± 0.00	0.1 ± 0.01
7	morin	0.1 ± 0.01	-
8	manghaslin	0.1 ± 0.01	-
9	lupulone C	0.1 ± 0.01	-
10	quercetin 4-O-glucoside	0.2 ± 0.03	-
11	lupulone E	0.7 ± 0.05	1.1 ± 0.27
12	quercetin	3.5 ± 0.49	0.4 ± 0.03
13	colupox A	25.0 ± 0.42	32.3 ± 2.28
14	6,8-diprenylnaringenin	0.6 ± 4.42	1.3 ± 0.13
15	protocatechuic acid	1.0 ± 0.04	0.2 ± 0.05
16	β-selinene epoxide	0.1 ± 0.14	-
17	epicatechin	0.5 ± 0.01	0.1 ± 0.02
18	lupulone D	0.3 ± 0.07	0.3 ± 0.03
19	dl-phenylalanine	0.1 ± 0.05	0.1 ± 0.01
20	pterostilbene	0.2 ± 0.00	0.3 ± 0.03
21	gibberellin A12	10.1 ± 0.33	8.8 ± 0.62
22	gibberellin A17	0.1 ± 0.71	-
23	gibberellin A24	0.6 ± 0.01	1.0 ± 0.23
24	gibberellin A34 methyl ester	0.1 ± 0.11	0.1 ± 0.01
25	prelupulone	1.0 ± 0.00	0.6 ± 0.14
26	isoquercetin	3.2 ± 0.14	0.9 ± 0.07
27	colupulone	-	0.1 ± 0.01
28	lupulone B	0.1 ± 0.01	0.1 ± 0.01
29	kaempferol 3-neohesperidoside	1.5 ± 0.10	0.4 ± 0.03
30	oxyresveratrol	0.5 ± 0.07	0.1 ± 0.02
31	lupulone F	6.4 ± 0.45	6.5 ± 1.54
32	adlupulone	21.6 ± 3.05	19.7 ± 1.99
33	quercitrin	3.9 ± 0.68	1.6 ± 0.37
34	rutin	3.7 ± 0.26	0.5 ± 0.04
35	xanthohumol D	0.2 ± 0.03	0.1 ± 0.00
36	tretinoin glucuronide	0.2 ± 0.02	0.2 ± 0.01
37	chlorogenic acid	-	0.1 ± 0.01
38	gibberellin A53	-	-
39	kaempferol	0.1 ± 0.00	0.1 ± 0.03
40	diprenylgenistein	-	0.1 ± 0.01
41	dihydroxanthohumol	-	0.1 ± 0.00
42	tricyclodehydroisohumulone	0.8 ± 0.11	2.8 ± 0.28
43	8-prenylnaringenin	0.7 ± 0.13	8.8 ± 0.69
44	secoisolariciresinol	-	0.1 ± 0.01
45	trimethoxycinnamic acid	0.1 ± 0.01	0.3 ± 0.03
46	4,4-dihydroxy-dimethoxychalcone	0.8 ± 0.06	-
47	luteolin	1.2 ± 0.17	0.1 ± 0.03
48	trans-caffeic acid	0.1 ± 0.01	-
49	quercetin 3-7-diglucoside	0.2 ± 0.01	-
50	lupulone A	tr	tr
51	desmethylxanthohumol	-	tr
52	isoxanthohumol	-	-
53	pre-humulone	-	tr
54	post-humulone	tr	tr
55	xanthohumol	tr	tr
56	xanthohumol E	-	tr
	SUM	99.5	99.5

[1] The listed components were identified by UPLC/MS analyses; tr: traces (mean value < 0.1%); - Not detected.

3.3. Proteomic Content

By LC/MS-MS analyses, numerous molecules belonging to different protein classes were identified (Table 3). Of these, all were found in the Chinook cultivar, while five, namely humulone synthase 2, probable CoA ligase CCL13 (CCL13), 2-C-methyl-D-erythritol 2, 2-C-methyl-D-erythritol 2, mitochondrial branched-chain aminotransferase 1 and naringenin-chalcone synthase, were absent in Cascade.

Table 3. Proteomic list in the hop inflorescences.

Description	Protein Class	Peptides	AAs	Chinook	Cascade
ATP synthase CF1 β-subunit	ATP synthase	27	498	Y	Y
ribulose 1,5-bisphosphate carboxylase/oxygenase	other	23	475	Y	Y
ATP synthase CF1 α-subunit	ATP synthase	15	507	Y	Y
ATPase subunit 1	ATP synthase	17	509	Y	Y
photosystem II CP47 chlorophyll apoprotein	photosystem	14	508	Y	Y
Chalcone isomerase-like protein 2	flavonoid pathway	8	209	Y	Y
photosystem II CP43 chlorophyll apoprotein	photosystem	9	473	Y	Y
humulone synthase 1	bitter acid pathway	13	454	Y	Y
photosystem II protein D1	photosystem	7	353	Y	Y
photosystem I P700 apoprotein A2	photosystem	8	734	Y	Y
photosystem II protein D2	photosystem	5	353	Y	Y
chalcone isomerase-like protein 1	flavonoid pathway	11	214	Y	Y
humulone synthase 2	bitter acid pathway	8	454	Y	N
CCL2	flavonoid pathway	11	573	Y	Y
Phloroisovalerophenone synthase	bitter acid pathway	9	394	Y	Y
cytochrome f	photosystem	9	320	Y	Y
sucrose synthase, partial	sugar	7	309	Y	Y
A Chain A, Hop1	other	4	101	Y	Y
CCL13	flavonoid pathway	10	573	Y	N
peroxidase 52	oxidoreductase	7	327	Y	Y
Myrcene synthase, chloroplastic	terpenoid pathway	6	613	Y	Y
isopentenyl-diphosphate isomerase	terpenoid pathway	8	321	Y	Y
polyubiquitin, partial	ubiquitination	4	76	Y	Y
cytochrome b6	photosystem	3	232	Y	Y
ATP synthase CF1 epsilon subunit	ATP synthase	4	133	Y	Y
germin 3	oxidoreductase	3	214	Y	Y
farnesyl pyrophophate synthase	terpenoid pathway	5	342	Y	Y
ATP synthase CF0 subunit I	ATP synthase	5	191	Y	Y
chalcone synthase	flavonoid pathway	5	394	Y	Y
allene oxide cyclase C4	oxidoreductase	3	254	Y	Y
ATPase subunit 4	ATP synthase	3	198	Y	Y
ribosomal protein S7	ribosomal	4	155	Y	Y
photosystem II cytochrome b559 α-subunit	photosystem	3	83	Y	Y
NADH dehydrogenase subunit 9	ATP synthase	2	190	Y	Y
peroxiredoxin-2F	oxidoreductase	4	199	Y	Y
photosystem I subunit VII	photosystem	2	81	Y	Y
SKP1 component-like 1	ubiquitination	3	157	Y	Y
plastid allene oxide cyclase	oxidoreductase	2	255	Y	Y
ribosomal protein L14	ribosomal	2	122	Y	Y
protein phosphatase 2A 65 kDa subunit	other	3	334	Y	Y
2-C-methyl-D-erythritol 2	terpenoid pathway	2	245	Y	N
TMV resistance protein N-like protein	other	2	130	Y	Y
ATPase subunit 8	ATP synthase	2	159	Y	Y
Aromatic prenyltransferase PT1L	bitter acid pathway	3	414	Y	Y
mitochodrial branched-chain aminotransferase 1	bitter acid pathway	3	393	Y	N
naringenin-chalcone synthase	flavonoid pathway	2	389	Y	N

Y: Present; N: Not present; peptides: number of identified peptides; AAs: number of total protein amino acids; protein class: biological process/pathway to which the proteins belong.

A pie chart representing the percentage distribution of the different protein classes found in the inflorescences was reported (Figure 4). By observing the graph, it is evident that, among the recognized protein classes, the one relating to photosystems (19%) is the largest followed by that relating to ATP-Synthase (17%) and flavonoid pathways (13%). The same portion (11%) is attributed to the bitter acid pathway and oxidoreductases. Lastly, the terpenoid pathway class (9%) was also found.

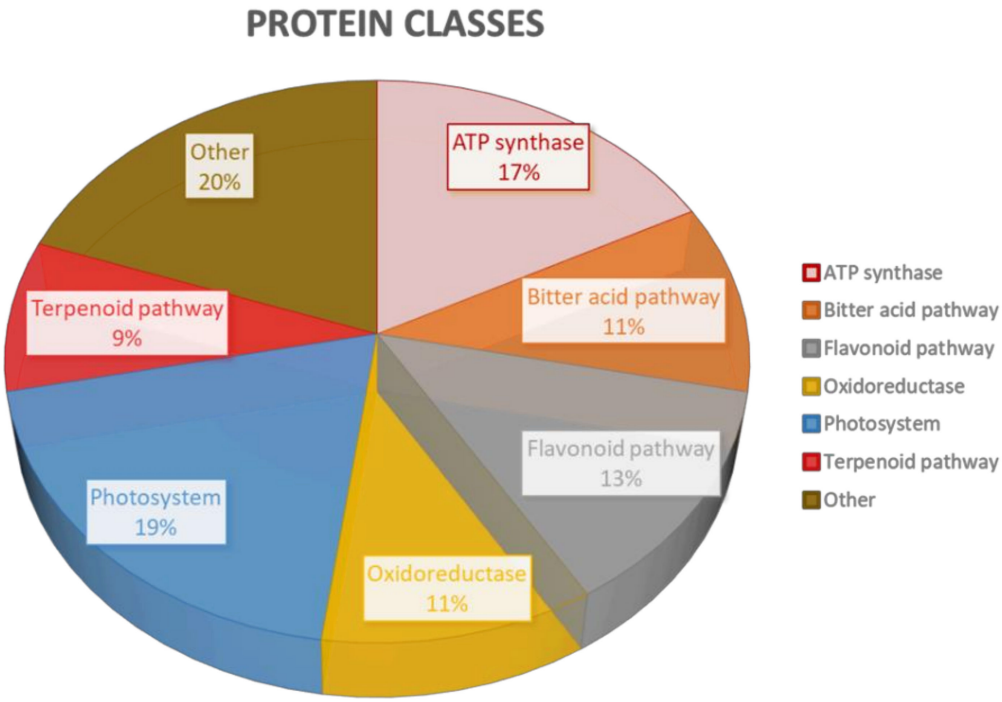

Figure 4. Percentages of protein classes.

These differentially expressed proteins essentially belong to two main pathways: the bitter acids pathway and xanthumol pathway. Specifically, mitochondrial branched-chain aminotransferase 1 and humulone synthase 2 are, respectively, the first and the last proteins involved in bitter acid pathway [11]. In hop, the bitter acids are particularly interesting for the pleasant bitter flavoring and for the overall microbial stabilization [11]. Conversely, in the xanthumol pathway, we identified CCL13 and naringenin-chalcone synthase, two proteins involved in xanthumol synthesis [11].

3.4. Multivariate Data Analysis

The metabolite profiling of the two inflorescences from hop cultivars revealed a large number of volatile and non-volatile compounds. These data were subjected to statistical analysis to better investigate the samples and their main differences in metabolite composition.

The complete list of volatile and non-volatile metabolites, normalized by sum, was reported in a heatmap (Figure 5). As far as the volatile components are concerned, the most relevant compounds were β-myrcene, β-caryophyllene and humulene, identified in both the cultivars. Similarly, the most relevant non-volatile metabolites were represented by colupox A, adlupulone and gibberellin A12.

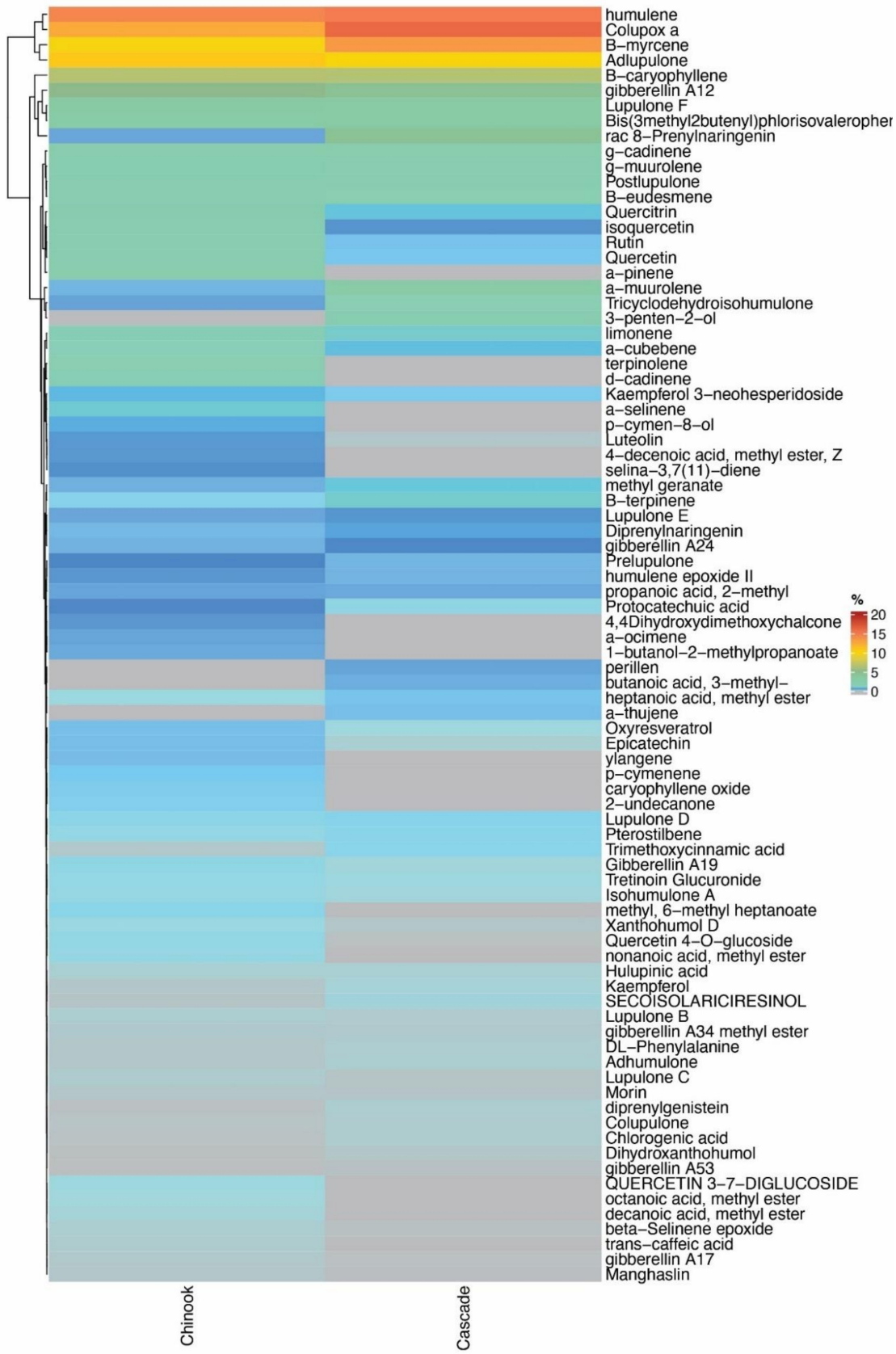

Figure 5. Heatmap of metabolite percentage normalized by sum in the inflorescence varieties of the two cultivars. Each cultivar is indicated in the column, and every row indicates a compound. Red indicates high abundance, whereas compounds under the detection threshold are in gray.

Next to the heatmap, a hierarchical clustering (HCA) technique was used to analyze the similarities of metabolite trends in the two samples. The resulting dendrogram was calculated using the Euclidean distance method. As shown in Figure 4, two main clusters can be observed. The first one is represented by the four metabolites showing the most significant composition percentage (humulene, colupox A, β-myrcene and adlupulone), and the second one is represented by the other fifty-two metabolites, in turn divided into two subclusters, including β-caryophyllene, gibberellin A12, lupulone F, bis(3-methyl-2-butenyl)phlorisovalerophenone and 8-prenylnaringenin in the first subcluster and all other compounds characterized by a lower percentage in the second one. Compounds under the detection threshold are represented in grey.

After a preliminary samples' examination, a better investigation of the hop-obtained data was performed. The principal component analysis (PCA, Figure 6) and partial least squares discriminant analysis (PLS-DA, Figure 7) segregated the samples on the basis of metabolite levels in each sample. Specifically, PCA unsupervised algorithm is an orthogonal linear transformation of possibly correlated variables into a smaller number of uncorrelated variables called principal components (PCs). In this case, the performed PCA was based on the first two principal PCs scores: PC1 explained 97.7% and PC2 explained 2.3%. As described above, hop cultivars were characterized by a different volatile and non-volatile metabolic composition. To find out which metabolites are responsible for such variation, a biplot was generated from PCA model. Additionally, supervised forms of discriminant analysis, such as PLS-DA [12], which rely on the class membership of each observation, were also commonly applied in metabolic fingerprinting experiments [12,13]. To identify the most important metabolites allowing discrimination between samples, we performed a supervised PLS-DA based on the variable importance in projection values (VIP), analyzing ten metabolites with a VIP score between 1.5 and 4.5. A metabolite with a VIP > 1.5 is regarded as significantly discriminant. In the biplot, it is observable that Chinook was mainly characterized by humulene, β-cariophyllene in the volatile fraction and by adlupulone, gibberellin A12 and lupulone F in the non-volatile profile. On the contrary, Cascade was mainly characterized by β-myrcene in the volatile fraction and by colupox A and 8-prenylnaringenin in the non-volatile profile. These three compounds are also mainly represented in VIP plot (Figure 7) with a score up to 3.5, followed by α-murolene and 3-penten-2-ol mostly identified in Cascade and α-pinene, rutin, quercetin, terpinolene and isoquercetin with a high score in Chinook.

Additionally, a fold change analysis (Figure 8) with the aim to compare value changes between the two varieties was performed. The result is plotted in log2 scale, so that same fold change (up/downregulated) has the same distance to the zero baseline. As shown in Figure 7, the metabolites upregulated in Cascade were 19, of which 8 are volatile and 11 non-volatile compounds. In contrast, the downregulated compounds in Cascade were 34, of which the flavonoid component is particularly represented with 11 metabolites (quercetin-4-O-glucoside, manghaslin, quercetin, epicatechin, isoquercetin, kaempferol-3-neohesperidoside, quercitrin, rutin, xanthohumol D, luteolin and quercetin-3-7-diglucoside).

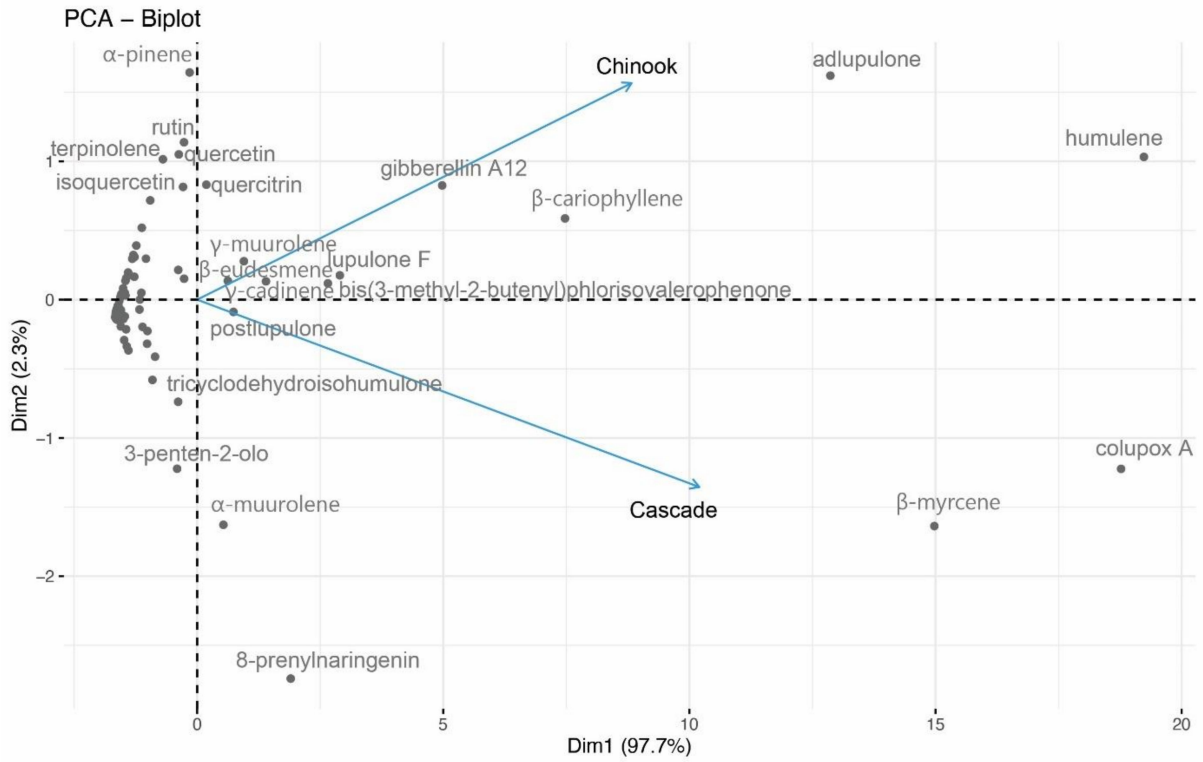

Figure 6. Principal component analysis (PCA) biplot. A biplot provides information on both metabolites and samples of a data matrix to be displayed graphically. Arrows represent the two samples Cascade and Chinoook; points represent all metabolites dataset. Compounds responsible for the divergence between the two inflorescences from hop cultivars were adlupulone, humulene and *β*-cariophyllene for Chinook and *β*-myrcene and colupox A for Cascade. Metabolites grouped at the origin of the graph do not contribute to the samples' variability.

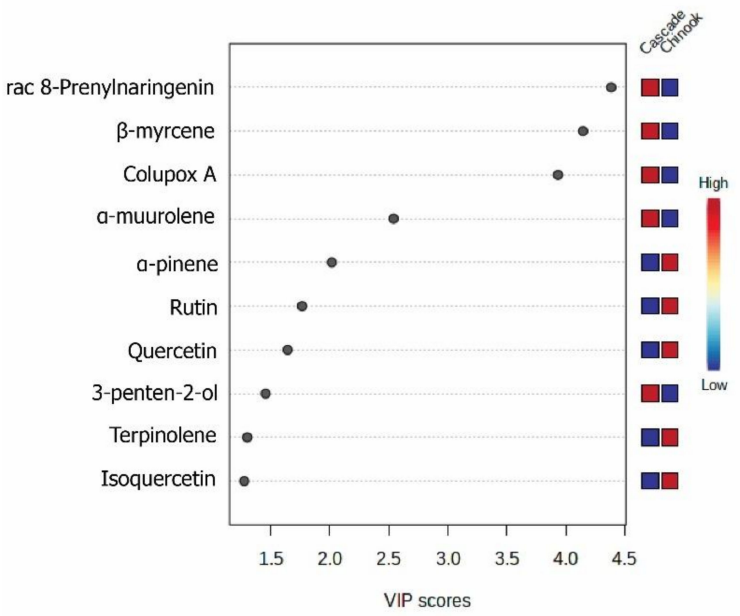

Figure 7. PLS-DA and variable importance in projection (VIP) plot. It displays the top 10 most important metabolite features identified by PLS-DA. Colored boxes on the right indicate the relative mean percentage of the corresponding metabolite for H. lupulus inflorescences. VIP is a weighted sum of squares of the PLS-DA loadings considering the amount of explained Y-variable in each dimension.

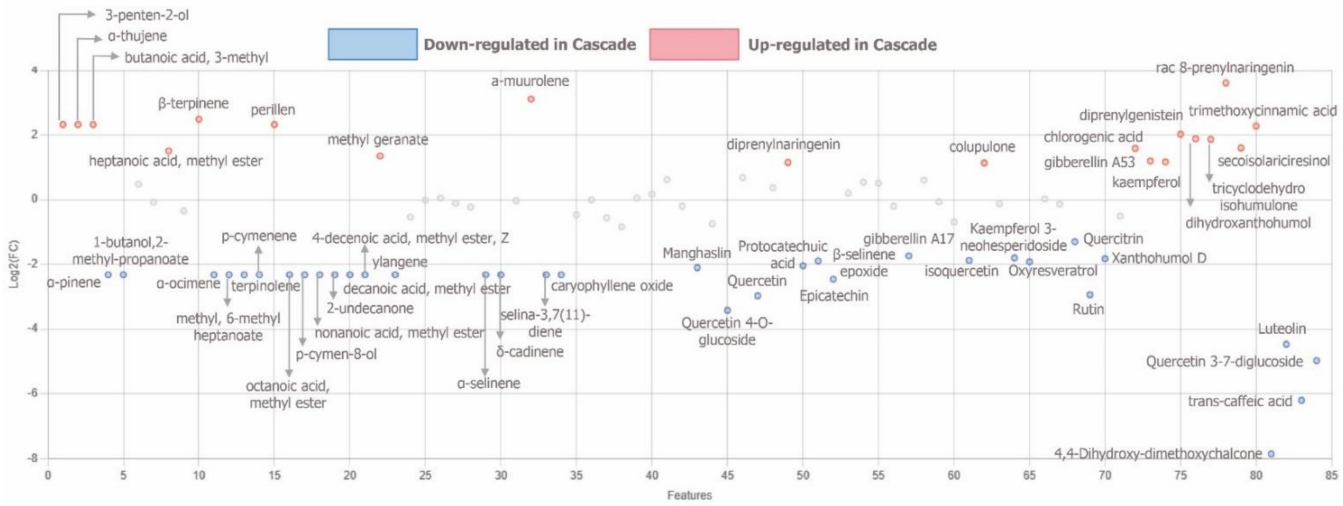

Figure 8. Important features selected by the fold-change analysis with a threshold of 2.5. The red circles represent features above the threshold upregulated in Cascade, and the blue circles represent the metabolites downregulated in Cascade. Metabolites that were not significantly changed are shown by gray dots. In the *y*-axis are reported values based on log2 scale, so that both upregulated and downregulated features can be plotted in a symmetrical way; in the *x*-axis are reported the feature values. The metabolites upregulated in Cascade were 19, whereas downregulated in Cascade, there were 34.

4. Discussion

In this work, for the first time, the dried inflorescences of two specific hop cultivars, Chinook and Cascade grown in southern Italy, were the subject of investigation in order to describe their volatile and non-volatile profile and their protein content. In recent years, the interest in natural substances has gradually increased with the aim of finding the greatest number of bioactive molecules. The female inflorescence of hop, particularly rich in endogenous substances, is traditionally used for the production of beer due to the aromatic characteristics it gives it. Thanks to the fact that some of the phytochemicals found in hops have a potential pharmacological value, they could be exploited as potential alternatives to synthetic products in antimicrobial and/or anticancer treatments.

Considering that there are hundreds of cultivated hop cultivars and other types being tested, their chemical characterization is essential to identify the source of specific secondary metabolites. To the best of our knowledge, there is no document in the literature that demonstrates the metabolomic characterization of the inflorescences of Chinook and Cascade, whose cultivation in Italy has only recently developed, nor their proteomic profile.

A recent paper reports a chemical composition study on extracts obtained from hops using different extraction techniques. *β*-Myrcene (from 7.1 to 10.6%), *α*-humulene (from 3.1 to 14.7%) and (*E*)-*β*-farnesene (from 0.9 to 14.1%) were the main detected volatile compounds. The percentage content of *α*-acids, *β*-acids, iso-*α*-acids and xanthohumol achieved by HPLC in the investigated hops extracts was also determined. [14].

A survey of 30 unique wild hop populations in the Maritimes region of eastern Canada was conducted to measure the phytochemical diversity of prenylcalcone, soft resins (*α*- and *β*-acids) and flavonol components [15].

In some works, the hop cones, that is, the immature inflorescences, have been the subject of investigation. Three different cultivars (Nugget, Saaz and Perle) were analyzed showing a profile rich in myrcene, *β*-caryophyllene and humulene, but with a predominance of *β*-farnesene in Saaz [16]. Topaz, Pacific jade, Cascade, CryoHop, Pacifica, Styrian Goldings, Hallertau, Hersbruker and Hallertau Blanc were other cultivars whose volatile composition has been described, highlighting *β*-myrcene as the major component (from 31.8% to 71.4%), but also with some differences in the qualitative composition [8].

Our results, obtained from the volatile fraction analysis of the dried inflorescences, highlight a profile rich in sesquiterpenes both in Chinook (63.4%) and Cascade (61.1%). In fact, for both cultivars humulene, the sesquiterpene compound was the most abundant molecule, followed by the monoterpene β-myrcene. In general, the number of identified constituents in Chinook was higher than that in the Cascade cultivar.

A somewhat more conspicuous number of papers discuss the chemical composition of essential oils (EOs) obtained from different hop cultivars [17–19]. Particular attention has been paid to EOs obtained from Cascade whose cultivation is constantly expanding, especially in Italy. Rodolfi et al. [20] characterized the EOs from Cascade hops grown in nine Italian regions, demonstrating how the cultivation area has a substantial effect on plant growth as well as crop years [21] by influencing their secondary metabolism. The chemical composition of EOs from Cascade cultivars cultivated in Brazil and in the USA has been also documented, showing qualitatively different volatile and phenolic contents [22]. Cascade EOs from the Mediterranean area (Sardinia) were also investigated. Their composition was in line with literature data, i.e., β-myrcene and α-humulene were the two mayor constituents. Furthermore, the percentage content of α- and β-acids was measured and varied between 5.0 and 9.0% [23].

In our study, the data obtained from the proteomic analysis showed more protein classes, some of which belong to the flavonoid (13%) and bitter acid (11%) pathways. Bitter acids, α-acids and β-acids are bioactive molecules endowed with pharmacological activities, such as antibacterial [24], anti-inflammatory [25] and anticancer [26]. Generally, their amounts depend largely on the hop types and growth conditions [27]. In our investigation, the percentage content of bitter acids was 34.7% in Chinook and 32.3% in Cascade and, among all, adlupulone was the one who achieved the highest percentages (21.6% and 19.7%, respectively). Further, traces of minor bitter acids, such as pre- and post-humulone, were found in both cultivars.

8-Prenylnaringenin (8-PN) and 6,8-diprenylnaringenin (6,8-DPN) were the prenylflavonoids more abundantly found in the two hops. In detail, 6,8-DPN reached percentage values higher in Cascade (8.8%, 1.3%) than in Chinook (0.7%, 0.6%). These compounds, in addition to the known anticarcinogenic, antibacterial and anti-inflammatory activities, also endow a powerful estrogenic activity [28]. Traces of other prenylflavonoids, such as, xanthohumol, isoxanthohumol (IXN), dihydroxanthumol (DXN), xanthohumol E and desmethylxanthoumol, were also detected. Among these, xanthohumol (XN) is the best studied cancer chemopreventive phytochemical isolated from hops. It has been shown to strongly inhibit the cDNA-expressed human cytochrome P450 enzymes that mediate the metabolic activation of many chemical carcinogens [29]. In recent years, IXN has received much attention for its biological effects. In fact, it showed an antiproliferative activity against human cell lines of breast cancer (MCF-7), ovarian cancer (A-2780), prostate cancer (DU145 and PC-3) and colon cancer (HT-29 and SW620) [30,31]. Furthermore, this compound inhibited the production of prostate membrane antigen (PSA) and exhibited an antiviral activity towards herpes virus (HSV1and HSV2) [32,33]. DXN has also been shown to possess cytotoxic activity, especially towards two colon (HCT116 and HT29) and two hepatocellular (HepG2 and Huh7) carcinoma cell lines [34].

Gibberellins (GAs) are phytohormones connecting environmental changes with plant development [35]. They have been found in samples of female plants of hop cv Nugget growing in Spain [36]. We detected the presence of Gibberellin A12 as the most abundant (10.1% and 8.8%) in Chinook and Cascade, followed by gibberellin A17, gibberellin A24 and gibberellin A34 methyl ester (from 0.1 to 1.0%).

Five phloroglucinol derivatives, whose percentage values range from 0.1 to 0.7%, such as lupulone B, lupulone D lupulone E, hulupinic acid and colupox A were present in both hop cultivars, while lupulone C were found only in Chinook. Noteworthy, colupox A reached high percentages (25.0% and 32.3%) in the two cultivars. These compounds were isolated from the hexane and methanol extracts of the female inflorescence pellet [25].

Finally, in our work, SPME-GC-MS and HPLC-MS-MS techniques have been shown to be suitable for the characterization of the volatile and non-volatile content of dried hop inflorescences. In fact, some previous works report chemical composition studies on hemp inflorescences [37], aerial parts of *Rosmarinus eriocalyx* [38], *Thymus munbyanus* subsp. *coloratus* [39] and *Peganum harmala* L. [40], using the same or similar investigation techniques.

5. Conclusions

The investigation techniques applied in this study were found to be suitable for the characterization of the two hop cultivars grown in Italy, thus proving suitable for the fingerprinting of this vegetable matrix. The obtained results highlight a composition rich in bioactive molecules belonging to different chemical classes with known biological activities; therefore, both Cascade and Chinook hops appear to be a high-quality raw material with great potential for future industrial and pharmaceutical applications. As the brewing industry has grown exponentially in recent years, interest in hop production has increased as well as the development of new cultivars. Therefore, their chemical characterization is fundamental to describe their metabolite profiling and identify bioactive molecules.

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Conflicts of Interest: The authors declare no conflict of interest.

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