Humulus lupulus L. cv. Cascade grown in Northern Italy: morphological and phytochemical characterization

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Abstract

Several aroma hops (*Humulus lupulus* L.) were recently introduced in Northern Italy as a small-scale production of excellence. In this preliminary study, the American cv. Cascade, was investigated in a combined morphological and phytochemical survey. Morphological investigation on trichome structure, density and distribution by scanning electron microscope (SEM) and light microscope was performed. Essential oil composition, α/β-acid and polyphenol profiles over three years were determined by GC-MS and HPLC analyses.

Two types of non-glandular (simple and cystolithic) and glandular (peltate and bulbous) trichomes were observed on leaves and female inflorescences. The peltate trichomes resulted as the main sites of terpene production and accumulation.

The essential oil profiles showed myrcene, β-caryophyllene, (*E*)-β-farnesene and humulene epoxide II as the dominant compounds over the three collection times, although with diverse relative abundances. The presence of two exclusive compounds, γ-muurolene and *trans*-γ-cadinene, characterized the investigated samples, potentially enhancing herbal, woody and spicy aroma traits of the Cascade hop cultivated in Northern Italy.

Bitter acid composition shows quantitative values consistent with literature data only at the second and third monitoring year. Qualitative differences in polyphenol content were also recorded, for the presence of quercetin-3-O-malonylglucoside and kaempferol-3-O-rutinoside, which may characterize this peculiar Italian cultivation.

Keywords: Hop, *Humulus lupulus* cv. Cascade, trichomes, essential oil, α/β-acids, polyphenols.

1. Introduction

Humulus lupulus L. (hop) is a climbing, perennial, dioecious plant belonging to the family Cannabaceae. It is native to Eurasia and, at present, it is widespread in the temperate zones of all continents, both in the wild and under cultivation (Pignatti 1982; Small 1980).

In Europe there is evidence on the use of *H. lupulus* since prehistoric times (Behre 1999). The ancient Romans, as mentioned by Pliny the Elder, employed leaves and inflorescences in some food preparations, as well as in the textile and cosmetic field (Grieve 1971; Lawless 1995; Barnes et al. 2002). Afterwards, the use of hop rapidly increased in the Middle Age, presumably because of its developed utilization in the brewing process. Cultivation of hop began in the mid-ninth century AC in Germany, then spreading throughout central Europe.

Nowadays, about 54% of the world production of hop for the brewing process still occurs in Central Europe, especially in Hallertau (Germany) and Zatec (Saaz, Czech Republic) regions. The USA and China account for about 36% and 6% of the world production, respectively (https://www.statista.com/statistics/757722/hop-production-global-by-country/, 2016).

The female inflorescences, usually called *hop cones* (Shephard et al. 2000), are the plant part of main interest, due to the presence of glandular trichomes responsible for the typical hop aroma. *Lupulin*, the mixture of trichomes obtained from the sieved cones, is listed in the European Pharmacopoeia (Eu. Ph.) for the sedative, antimicrobial and proestrogenic properties (Zanoli and Zavatti 2008; Van Cleemput et al. 2009).

In the wide panorama of *H. lupulus* varieties, the cultivar Cascade is an aroma hop selected in 1972 for brewing at the Oregon State University (Oregon, USA) from cv. Fuggle, cv. Serebrianker (a Russian variety) and an unknown American cultivar (Oliver 2012). Its name descends from the Cascade mountain range, extending through Washington and Oregon States. The popularity of the Cascade hop, especially in the USA craft brewery industry, is mainly due to the combination of high

66 production yield, resistance to downy mildew infections (Moir 2000) and to the characteristic floral,
67 fruity, particularly citrusy, aroma with little earthy or spicy notes (Kishimoto et al. 2006; Nance and
68 Setzer 2011).

69 Although bitter acids composition is usually used as quality parameter for hop, literature proposes
70 only a few contributions on the phytochemistry of cv. Cascade. The essential oil (EO) composition
71 was analyzed by GC-O, GCxGC (Eyres et al. 2007; Steinhaus et al. 2007) and GC-MS (Nance and
72 Setzer 2011; Mongelli et al. 2016). In particular, Nance and Setzer (2011) identified myrcene, α-
73 humulene, (E)-caryophyllene, and (E)-β-farnesene as the EO main components.

74 Polyphenolic components were characterized via HPLC-DAD by Magalhaes *et al.* (2010), Kavalier
75 et al. (2011) and De Cooman et al. (1998) applying diverse extraction methods and leading to the
76 identification of catechins, procyanidins, quercetin and kaempferol glucosides as principal
77 components.

78 Concerning the *indumentum* micromorphology, only few works exist on the ontogeny,
79 histochemistry and ultrastructure of glandular trichomes in different hop varieties (Oliveira and Pais
80 1988, 1990; Hirosawa et al. 1995; Saito et al. 1995; Kim and Mahlberg 2000; Kavalier et al. 2011).

81 In Italy, industrial beer production represents a minor economic sector; recently, however, a high-
82 quality production of craft beer is gradually spreading on a small-scale: up to 850 Italian micro-
83 breweries are now operating (AssoBirra 2016). They primarily import hops from abroad, however
84 several attempts have been made to improve production with local or regional raw materials.

85 The present research arises in this contest. We combined, for the first time, a study on the
86 morphological and phytochemical characterization of Cascade hop cultivated in Northern Italy. We
87 specifically analyzed: (i) trichome distribution pattern and histochemistry on young leaves and female
88 inflorescences (cones); (ii) the essential oils obtained from the cones across three consecutive years
89 and (iii) the composition of bitter acids and polyphenols, to assess the variability among the profiles.

2. Materials and Methods

2.1 Plant treatment

Cascade hop plantlets were purchased at Garten Eickelmann (Geisenfeld, Germany) and cultivated for 2 years in an experimental site (Farm La Morosina, Abbiategrasso, Milan, Italy), before the beginning of the monitoring campaign, in 2012. Plants were grown under a permanent trellis approximately 3 m tall, with spacing of 1 x 4.25 m between plants and rows, respectively; plants were irrigated by sprinklers.

Samples for the micromorphological investigation were collected in September 2012. Samplings of cones for the phytochemical investigation were performed at the end of September 2012 (S12), 2013 (S13) and 2014 (S14): the cones were collected at maturity and dried at 40°C in a thermostatic room, protected from light.

2.2 Micromorphological investigation

2.2.1 Scanning Electron Microscopy (SEM)

Fresh leaves, bracts, bracteoles and ovaries were collected from female plants and fixed overnight at 4°C in 4% (v/v) glutaraldehyde in deionized water. Fixed tissues were washed with deionized water and post-fixed with aqueous 2% osmium tetroxide for 2 hours. Samples were washed several times with deionized water and dehydrated using the following ethanol concentrations: 25, 50, 70, 80, 95 and 100% twice for 15 min. Samples were then critical point dried with liquid CO_2, mounted on aluminum stubs and sputtered with gold under vacuum (Nanotech sputter coater). Specimens were examined using a LEO 1430 Scanning Electron Microscope.

Three replicates for each plant part were analyzed to assess morphological variability.

2.2.2 Light Microscopy (LM)

LM investigation on historesin-fixed samples (leaves, bracts, bracteoles and ovaries) was performed to describe in detail the structure of the glandular trichomes. Histochemical tests were employed on fresh samples to evidence the main chemical classes of metabolites in the secretory products of the peltate trichomes of leaves and cones, with special emphasis on terpenes. Hand-made sections (40–50 μm thick) and semi-thin sections (20–25 μm thick) obtained by means of a cryostat, were stained with the following dyes: Sudan III/IV (Johansen 1940) and Fluoral Yellow-088 (Brundrett et al. 1991) for total lipids; Nadi reagent for terpenes (David and Carde 1964); Ruthenium Red and Alcian Blue for polysaccharides other than cellulose (Jensen1962); ferric trichloride for polyphenols (Gahan, 1984). Matchings for all the histochemical stains were performed with control procedures. At least five samples of each plant part were examined for each histochemical staining to assess the consistency of the results.

Observations were performed under a Leitz DM-RB Fluo Optic microscope equipped with a digital camera Nikon DS-L1.

2.3 Phytochemical investigation

2.3.1. Preparation and analysis of essential oils

Dried cones (50 g) were submitted to hydrodistillation for 2 hours using a Clevenger-type apparatus (2 L rounds bottom flask containing 1 L of water), and the obtained essential oil, dissolved in *n*-hexane (HPLC-grade, 5% solution), was immediately submitted to GC-MS analysis. The GC analyses were performed on a HP-5890 Series II instrument equipped with DB-WAX and DB-5 capillary columns (30 m x 0.25 mm, 0.25 μm film thickness) applying a linear temperature gradient from 60°C to 240°C at 3°C min^{-1}; injector and detector temperatures were 220°C; carrier gas helium (2 mL min^{-1}); detector dual FID; splitless injection. The identification of the components was performed, for both the columns, by comparison of their retention times with those of pure authentic samples and by their linear retention indices (lri) relative to the series of *n*-hydrocarbons.

GC-EIMS analyses were achieved with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Injector and transfer line temperatures were respectively kept at 250°C and 240°C; oven temperature programmed from 60°C to 240°C at 3°C min^{-1}; carrier gas helium at 1 mL min^{-1}; splitless injection. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their lri relative to the series of *n*-hydrocarbons, and on computer matching against commercial (NIST 2000 and ADAMS) and laboratory-made mass spectra library built up from pure substances and components of known essential oils and MS literature data (Stenhagen et al. 1974; Massada 1976; Jennings and Shibimoto 1980; Swigar and Silverstein 1981; Davies 1990; Adams 1995).

2.3.2 Extraction and sample preparation of α/β-acids and polyphenols

Dried cones were formerly ground to fine powder with an electric grinder. Then, a small amount of powder (250 mg) was subjected to extraction with a sequence of three solvents (petroleum ether 40-60, dichloromethane and methanol). Extraction was performed four times for each solvent with equal volumes and timing (10 mL, 30 min), leading to three organic fractions containing respectively terpenophenolics, pigments and polyphenols. This procedure was repeated several times on equal amounts of hop samples and gets yield ranges of 19.4-23.7%, 3.0-4.2% and 7.8-12.9% for extraction of *α*/*β*-acids, pigments and polyphenols, respectively, with total yields in extraction variable between 30.0% and 39.6%.

2.3.3 HPLC analysis of α/β-acids

The dry sample obtained with petroleum ether during the extraction procedure described above was redissolved in the same solvent, diluted in acetonitrile and then injected. HPLC analyses were performed at room temperature on a Varian Prostar HPLC equipped with Varian Prostar 335 PDA detector and Lichrocart® RP-18 column (250 x 4.6 mm, 3 µm, Merck KGaA, Darmstadt, Germany).

Eluent composition was varied between 0.1% formic acid in water (A) and pure acetonitrile (B) according to the following program: 0-5 min B 20-50%, 5-7 min B 50-100%, 7-19 min B 100%; the flow rate was 0.6 mL $min^{-1.}$ Resulting peak areas were quantified according to ASBC/EBC procedure by comparison with ICE-3 standard (IHSC 2010). Percentages of α/β-acids refers to the weight of starting samples.

2.3.4 HPLC analysis of polyphenols

HPLC-DAD analyses of samples obtained from methanol extraction were performed on the same system used for α/β-acids determination, using a binary mixture composed of 0.1% formic acid in water (A) and pure acetonitrile (B) and applying the following gradient program:0-10 min B 10-15%, 10-45 min B 15-40%, 45-52 min B 40-100%, 52-58 min B 100%, at a flow rate of 0.6 mL min^{-1}. LC-MS analyses were performed on a Thermo Finnigan LC-MS system, equipped with a PDA detector and a LCQ Advantage mass spectrometer, using the same column and gradient conditions, as reported previously (Araneo et al. 2013). Results are reported as integrated areas.

3. Results and Discussion

3.1 Micromorphological investigation

The young leaves and cones of *H. lupulus* cv. Cascade are characterized by a high number of non-glandular and glandular trichomes (Fig. 1). Both categories can be divided into different types according to their size, shape and localization.

Two types of non-glandular trichomes were identified: simple and cystolithic trichomes (Fig. 1). The former are medium-long, with an acute apex and a smooth surface (Fig. 1a, arrow); the cystolithic ones are shorter, enlarged at the base and ending with a pointed tip and exhibit calcium carbonate deposits on the surface (Fig. 1b).

184 Two main types of glandular trichomes were observed: peltate (Fig. 1c,d) and bulbous (Fig. 1e), both
185 consisting of a stalk and a multicellular secretory head. LM observation, in particular, allowed to
186 accurately characterize their structure and morphology (Fig. 1f-h).
187 The peltate ones consist of 2-4 basal epidermal cells, 2-4 stalk cells and of a very high number of
188 glandular cells arranged in a single layer (Fig. 1c,f,g); the glandular head is surrounded by a wide
189 subcuticular space in which the secretory material is stored. Two subtypes, differing in shape, size and
190 distribution pattern, were recognized: flattened, mainly located on leaves, with a head diameter in the
191 range 100-120 μm at maturity (Fig. 1c,f), and biconical, typical of cones, with a head diameter in the
192 range 150-180 μm (Fig. 1d,g).
193 Bulbous trichomes exhibit 2 basal epidermal cells, 2 stalk cells and 4 secreting cells (25-40 μm in
194 diameter) with a thin subcuticular space (Fig. 1e,h).

195 Figure 2 (a-f) shows in detail the trichome distribution pattern. Cystolithic and bulbous trichomes (Fig.
196 2a, arrow) are present on the adaxial leaf epidermis; the peltate trichomes are scattered on the whole
197 abaxial lamina and simple non-glandular hairs are exclusively located at the midrib (Fig. 2b, arrow).
198 Non-glandular and bulbous trichomes are densely distributed on the abaxial and adaxial surfaces of
199 bracts and bracteoles (Fig. 2c, d, e); peltate trichomes are present only on the abaxial surface and
200 appear much crowded at the basal region (Fig. 2c, e). The perianth is covered by high-density peltate
201 trichomes only (Fig. 2f).

202 The results of the histochemical investigation are showed in Figure 3. We focused attention on the
203 peltates, due to their greater density compared to bulbous trichomes. Regardless their distribution on
204 leaves and cones, these trichomes displayed consistent responses to all the employed histochemical
205 dyes.
206 The substances accumulated in the large subcuticular spaces are visible in the form of variable-sized
207 droplets, also in the stainless samples (Fig. 3a). These secretory products were intensely evidenced by
208 the total lipid-specific dyes, Sudan III/IV and Fluoral Yellow 088 (Fig. 3d,e). In particular, the

response to Nadi reagent gave clear positive responses, indicating the presence of terpenes (Fig. 3f). The employed hydrophilic tests invariably displayed negative results (Fig. 3b,c).

The micromorphological features of the *indumentum* of leaves and female inflorescences of *H. lupulus* cv. Cascade are consistent to those proposed in literature for other cultivars, in particular for the glandular trichomes (Oliveira and Pais 1988; Kim and Mahlberg 2000; Kavalier et al. 2011). Two types of glandular hairs were observed: peltate, which are large and contain up to 100-200 cells (Oliveira and Pais 1988, 1990) and bulbous glands, which are much smaller. For the latter, literature refers to the presence of 8 secreting cells at maturity (Oliveira and Pais 1988), whereas we detected 4 head cells in all the examined samples as in Sugiyama et al. (2006).

3.2 Phytochemical investigation

3.2.1 Essential oils

The overall composition of the essential oils of *H. lupulus* cv. Cascade obtained in the three collection times is shown in Table 1.

A total of 45 compounds were identified. The profiles obtained in September 2012 (S12), September 2013 (S13) and September 2014 (S14) are characterized by the presence of 34 (96.5%), 31 (97.8%) and 32 (98.85%) compounds, respectively.

Regarding the most represented chemical classes, monoterpenes were detected in slightly higher percentages than sesquiterpenes in S12 (49.6% and 41.1%, respectively). In S13, the sesquiterpenes prevail (57.6%), followed by the monoterpenes (39.1%). S14 is characterized by the clear prevalence of the monoterpenic fraction (78.7%) compared to the sesquiterpenic one (17.2%). Overall, the non-oxygenated terpenes increased from the 2012 to the 2014 samples, whilst the opposite behaviour was evidenced for the oxygenated ones.

Concerning the most abundant compounds, the investigated EO profiles show myrcene (*4*) as the main compound across the 3 years, with relative percentages of 41.6% in S12, 35.5% in S13 and 72.3% in S14. The sesquiterpenes α-humulene (*26*) (15.9% in S12, 26.8% in S13, 7.3% in S14), β-caryophyllene (*23*) (5.8% in S12, 12.4% in S13, 3.3% in S14), (*E*)-β-farnesene (*27*) (2.5% in S12, 5.1% in S13 and 2.8% in S14) and humulene epoxide II (*41*) (4.9% in S12, 1.4% in S13, 0.2 % in S14) followed.

The common compounds are 18. The exclusive compounds are 3 in S12 (*1, 19, 33*), 3 in S13 (*17, 35, 42*), 5 in S14 (*10, 14, 18, 28, 32*). These compounds are present in relative percentages always lower than 1.5%.

Noteworthy, the essential oil contains linalool (*11*) among its constituents, particularly the S12 sample (1.1%). Peacock and Deinzer report that most of the floral aroma of beers produced using Cascade hop are due to linalool and geraniol. The latter compound is not present in our samples but, according to the same authors, it may depend on the hop age because its amount increases during storage.

Literature about hop essential oil is quite rich, particularly for the "aroma hop" varieties, and the composition is very dependent on the variety, with some differences within the same ones, according to the geographical origin or cultivation/processing techniques.

If we restrict the comparison of our samples to the profile of the other investigated Italian cultivation of the Cascade hop (Mongelli et al. 2016), myrcene results about halved, while (*E*)-β-farnesene is present in higher percentages; some other differences emerge concerning the minor compounds are evident, probably depending on the diverse environmental factors, and cultivation conditions as well.

A relevant question is if the concentration changes of some terpenes, are more related to the age of the plant or to environmental factors. A recognized cause of terpene synthesis bursts is the presence of herbivores that are able to activate the production of terpenes as a defensive response (Pichersky and Gershenzon 2002).

Moreover, the comparison with the same cultivar grown in Oregon and Washington States, despite the differences in the analytical methodologies, showed a general consistency of the qualitative profiles (Nance and Setzer 2011; Lam et al. 1986), except for the presence of two exclusive compounds, γ-

muurolene and *trans*-γ-cadinene, in our samples. These two compounds may intensify some peculiar aromatic features of hop, such as the herbal, woody and spicy notes (Goncalves et al. 2014).

Recently, Lafontaine et al. (2019), even if the study was performed in Washington State, evidenced that the highest yield of essential oil for this cultivar was obtained from samples collected in September, the same period of harvesting of our samples. Furthermore, the same authors observed that during brewing earlier harvesting were useful for bittering whilst, if used for aroma, Cascade hop should be collected in September. All these results were consistent over three years.

3.2.2. α/β-acid composition

HPLC analysis revealed the presence of 4 peaks at Rt = 14.30, 14.80, 16.10 and 16.90 min (Fig. 4) attributed, respectively, to α-acids cohumulone (a), *n*-humulone + adhumulone (b), and to *β*-acids colupulone (c), *n*-lupulone + adlupulone (d) by ESI-MS spectra acquired for each peak.

Our samples showed variable values of *α*- and *β*-acids between S12 and the other two collection times, S13 and S14; in fact, the total *α*-acids content (which correspond to the sum of cohumulone, adhumulone and *n*-humulone percentages) moves from 2.19%(w/w) in S12 to 4.93% and 5.01% in S13 and S14, respectively. *β*-acids moves from 6.73% in S12 up to 7.56% in S13 and to 7.66% in S14.

Therefore, there is no qualitative variability among the *α*- and *β*-acid compositions over the three years, with the presence of the six principal derivatives of phloroglucinol (*n*-, co-, adhumulone and *n*-, co- and ad-lupulone) usually reported for hop. On the contrary, if we consider the quantitative distribution of each class of the above-mentioned compounds, it clearly comes out that S12 displays considerable differences in comparison to literature data. S12 profile shows a lower content of *α*-acids (2.19%) compared with literature (4.5-7.0%), while *β*-acids and cohumulone/*α*-acids percentages (6.73% and 30%, respectively) attest to comparable values (Nance and Setzer 2011; Goncalves et al. 2012). S13 and S14 profiles showed percentages in line with literature data.

3.2.3 Polyphenol content

Polyphenol analysis revealed the presence of ten main peaks (Fig. 5). Nine out of ten were identified by LC-PDA-MS analysis, five corresponding to flavonol glycosides. Polyphenols were: procyanidin B (P2), chlorogenic acid (P3), proanthocyanidins (P4, P5), quercetin-3-*O*-rutinoside (rutin, P6), quercetin-3-*O*-hexoside (P7), quercetin-3-*O*-malonylglucoside coeluted with kaempferol-3-*O*-rutinoside (P8), kaempferol-3-*O*-hexoside (P9) and kaempferol-3-*O*-malonylglucoside (P10) (Magalhaes et al. 2010; Li and Deinzer 2007). For peaks P7, P9, it was not possible to define the type of condensed hexoside (glucoside or galactoside) from data obtained by mass spectrometry.

Among flavonol glycosides, the polyphenolic composition of our samples was characterized by the presence of compounds already reported in literature for this cultivar (Magalhaes et al. 2010; Kavalier et al. 2011; De Cooman et al. 1998), except for quercetin-3-*O*-malonylglucoside and kaempferol-3-*O*-malonylglucoside, identified for the first time in Cascade hop, but present in some other hop cultivars (Aron 2011).

4. Conclusions

This study combined for the first time a morphological and phytochemical surveys on the Cascade hop cultivated in Northern Italy for commercial use.

The detailed micromorphological observation by light and scanning electron microscopy allowed to describe the non-glandular and glandular trichomes. The *indumentum* features proved consistent to literature information, with peltate trichomes resulting as the main sites of terpene production and accumulation.

The essential oil profiles showed changes in their composition over the three collection times, although the dominant compounds were consistently represented by myrcene, *β*- caryophyllene, (*E*)-*β*-farnesene and humulene epoxide II, in accordance with literature data. However, qualitative differences were

307 recorded, mainly due to the presence of the exclusive compounds, *γ*-muurolene and trans-*γ*-cadinene.
308 These two molecules may enhance some typical organoleptic traits of the Cascade hop cultivated in
309 Northern Italy, such as the herbal, woody and spicy aroma descriptors.
310 For what concerns bitter acid composition, the quantitative values resulted consistent to literature data
311 only at the second and third monitoring year. This may be ascribed to the adaptation to the new
312 environment. Qualitative differences in polyphenol content were also recorded, with particular
313 reference to the presence of quercetin-3-*O*-malonylglucoside and kaempferol-3-*O*-rutinoside, that may
314 characterize this peculiar Italian cultivation.
315 This preliminary information will be confirmed by further analyses over successive years.

316

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321

322 **Conflicts of Interest**

323 The authors declare no conflict of interest.

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417

418 **Table 1.** Constituents of essential oils obtained from the cones of *Humulus lupulus* L. cv. Cascade in
419 September 2012, 2013 and 2014. The compounds common to all the three profiles are evidenced in
420 grey.

	l.r.i.	Compounds	Relative abundance (%)			Identification
			September 2012 (S12)	September 2013 (S13)	September 2014 (S14)	
1	898	propyl butanoate	0.3	-	-	St
2	941	α-pinene	0.4	-	0.2	St
3	982	β-pinene	1.8	0.9	1.6	St
4	993	myrcene	41.6	35.5	72.3	St
5	1008	pentyl propanoate	1.0	-	0.2	St
6	1019	2-methylbutyl isobutyrate	-	0.2	0.4	RI, MS
7	1027	methyl heptanoate	0.6	-	0.4	St
8	1032	limonene	0.9	0.7	1.2	St
9	1052	(*E*)-β-ocimene	-	0.4	0.2	Stmix
10	1087	methyl 6-methylheptanoate	-	-	0.3	RI, MS
11	1101	linalool	1.1	0.4	0.6	St
12	1104	nonanal	0.4	0.3	0.2	St
13	1128	methyl octanoate	0.3	-	0.2	St
14	1210	methyl 4-nonenoate	-	-	0.2	RI, MS
15	1228	methyl nonanoate	0.3	-	0.2	St
16	1293	2-undecanone	0.4	0.2	0.1	St
17	1309	methyl (*E*)-2-decenoate	-	0.4	-	RI, MS
18	1311	methyl 4-decenoate	-	-	0.8	RI, MS
19	1316	(*E,E*)-2,4-decadienal	1.2	-	-	St
20	1325	methyl geranate	0.6	0.6	0.7	RI, MS
21	1377	α-copaene	0.5	0.4	-	St
22	1383	geranyl acetate	2.3	0.6	1.2	St
23	1419	β-caryophyllene	5.8	12.4	3.3	St
24	1430	β-copaene	0.2	0.5	0.1	RI, MS
25	1437	*trans*-α-bergamotene	0.4	0.3	0.1	RI, MS
26	1456	α-humulene	15.9	26.8	7.3	St
27	1459	(*E*)-β-farnesene	2.5	5.1	2.8	Stmix
28	1475	*trans*-cadina-1(6),4-diene	-	-	0.1	RI, MS
29	1479	γ-muurolene	1.7	1.3	0.7	RI, MS
30	1487	β-selinene	1.3	1.3	0.7	RI, MS
31	1495	α-selinene	1.4	1.5	-	RI, MS
32	1495	viridiflorene	-	-	0.9	RI, MS
33	1497	2-tridecanone	1.3	-	-	St
34	1500	α-muurolene	0.4	0.4	-	RI, MS
35	1508	(*E,E*)-α-farnesene	-	0.3	-	RI, MS
36	1514	trans-γ-cadinene	0.8	1.3	0.2	RI, MS
37	1516	geranyl isobutyrate	0.9	-	0.7	RI, MS
38	1524	δ-cadinene	1.3	2.1	0.6	RI, MS
39	1538	α-cadinene	-	0.2	0.2	RI, MS
40	1582	caryophyllene oxide	1.9	0.6	-	St
41	1607	humulene epoxide II	4.9	1.4	0.2	RI, MS
42	1628	1-*epi*-cubenol	-	0.2	-	RI, MS
43	1637	caryophylla-4(14),8(15)-dien-5-ol	1.4	0.8	-	RI, MS
44	1642	*epi*-α-cadinol	0.3	0.4	-	RI, MS
45	1654	α-cadinol	0.4	0.3	-	RI, MS
		Monoterpene hydrocarbons	**44.7**	**37.5**	**75.5**	
		Oxygenated monoterpenes	**4.9**	**1.6**	**3.2**	
		Sesquiterpene hydrocarbons	**32.2**	**53.9**	**17.0**	
		Oxygenated sesquiterpenes	**8.9**	**3.7**	**0.2**	
		Non-terpene derivatives	**5.8**	**1.1**	**3.0**	
		Total identified	**96.5**	**97.8**	**98.9**	

St: standard compound; Stmix: standard compound isomers mixture; RI: retention index; MS: mass spectrum

Figure Captions

Figure 1. SEM micrographs showing non-glandular (a-b) and glandular trichomes (c-e) of *H. lupulus* cv. Cascade: (a) simple trichomes; (b) cystolithic trichome with calcium carbonate deposits; (c) flattened peltate trichomes on leaf epidermis; (d) biconical peltate trichomes on inflorescences; (e) bulbous trichome. LM micrographs showing glandular trichome (f-h) of *H. lupulus* cv. Cascade: f) flattened peltate trichome; (g) biconical peltate trichome; (h) bulbous trichome. *Scale bars: a, f, g = 40 μm; b, h = 20 μm; c = 25 μm; d = 50 μm; e = 10 μm.*

Figure 2. SEM micrographs of *H. lupulus* cv. Cascade: (a) leaf adaxial epidermis with cystolithic hairs and bulbous trichomes (arrow); (b) leaf abaxial epidermis with peltate trichome on the interveinal areas and simple non-glandular trichomes on the midrib (arrow); (c) bract abaxial surface subtending a pair of female flowers; (d) bract adaxial surface; (e) abaxial basal part of a bracteole enclosing a single female flower; (f) ovary and perianth (enclosed within bracteole). *Scale bars: a = 100 μm; b, d-f = 200 μm; c = 1mm.*

Figure 3. LM micrographs showing the results of the histochemical investigation on peltate trichomes: (a) stainless peltate trichome; (b) Ruthenium Red; (c) Alcian Blue; (d) Sudan III/IV; (e) Fluoral Yellow 088; (f) Nadi reagent. *Scale bars = 40 μm.*

Figure 4. Chromatogram of bitter acids extracted by petroleum ether from H. lupulus cv. Cascade cones. The peaks correspond to (a) cohumulone, (b) adhumulone + n-humulone, (c) colupulone and (d) adlupulone + n-lupulone.

Figure 5. Chromatogram of methanolic extract from *H. lupulus* cv. Cascade plants.