

1 ***Humulus lupulus* L. cv. Cascade grown in northern Italy: morphological and**  
2 **phytochemical characterization**

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18 ***Humulus lupulus* L. cv. Cascade grown in northern Italy: morphological and**  
19 **phytochemical characterization**

20 **Abstract**

21 Several aroma hops (*Humulus lupulus* L.) were recently introduced in northern Italy as a small-  
22 scale production of excellence. In this preliminary study, the American cultivar Cascade, was  
23 investigated in a combined morphological and phytochemical survey. Morphological  
24 investigation on trichome structure, density and distribution was performed by scanning electron  
25 microscopy (SEM) and light microscopy (LM). Essential oil composition,  $\alpha/\beta$ -acid and  
26 polyphenol profiles over three years were determined by GC-MS and HPLC analyses.

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

30 The essential oil profiles showed myrcene,  $\beta$ -caryophyllene, (*E*)- $\beta$ -farnesene and humulene  
31 epoxide II as the dominant compounds over the three collection times, although with different  
32 relative abundances. The presence of two exclusive compounds,  $\gamma$ -muurolene and *trans*- $\gamma$ -  
33 cadinene, characterized the investigated cv. Cascade, potentially enhancing herbal, woody and  
34 spicy aroma traits of this cultivation in Northern Italy.

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

39

40 **Keywords:** Hop, *Humulus lupulus* cv. Cascade, trichomes, essential oil,  $\alpha/\beta$ -acids, polyphenols.

41

## 42 1. Introduction

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

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

51 In Europe there is evidence on the use of *H. lupulus* since prehistoric times (Behre 1999). The  
52 ancient Romans, as mentioned by Pliny the Elder, employed its leaves and inflorescences in some food  
53 preparations, as well as in textiles and cosmetic products (Grieve 1971; Lawless 1995; Barnes *et al.*  
54 2002). Afterwards, the use of hop rapidly increased in the Middle Age, presumably because of its  
55 developed utilization in the brewing process. Cultivation of hop began in the mid-ninth century AC in  
56 Germany, then spreading throughout central Europe.

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

61 In the wide panorama of *H. lupulus* varieties, the cultivar Cascade is an aroma hop selected in 1972  
62 for brewing at the Oregon State University (Oregon, USA) from cv. Fuggle, cv. Serebrianker (a  
63 Russian variety) and an unknown American cultivar (Oliver 2012). Its name descends from the  
64 Cascade mountain range, extending through Washington and Oregon States. The popularity of the  
65 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, there is little  
70 information regarding the phytochemistry of cv. Cascade. The essential oil (EO) composition was  
71 analyzed by GC-O, GCxGC (Eyres *et al.* 2007; Steinhaus *et al.* 2007) and GC-MS (Nance and Setzer  
72 2011; Mongelli *et al.* 2016). Nance and Setzer (2011) identified myrcene,  $\alpha$ -humulene, (*E*)-  
73 caryophyllene, and (*E*)- $\beta$ -farnesene as the EO main components.

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

78 Concerning the *indumentum* micromorphology, there are only few reports on the ontogeny,  
79 histochemistry and ultrastructure of glandular trichomes in different hop varieties (Oliveira and Pais  
80 1988, 1990; Hirokawa *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 EOs obtained from the cones across three consecutive years and (iii) the  
89 composition of bitter acids and polyphenols, to assess the variability among the profiles.

90

## 91 **2. Materials and Methods**

### 92 *2.1 Plant treatment*

93 Cascade hop plantlets were purchased at Garten Eickelmann (Geisenfeld, Germany) and cultivated  
94 for 2 years in an experimental site (Farm La Morosina, Abbiategrasso, Milan, Italy), before the  
95 beginning of the monitoring campaign, in 2012. Plants were grown under a permanent trellis  
96 approximately 3 m tall, with spacing of 1 x 4.25 m between plants and rows, respectively; plants were  
97 irrigated by sprinklers. No chemical field treatments were applied during plant growth, to evaluate  
98 spontaneous response of the plants to the environment.

99 Samples for the micromorphological investigation were collected in September 2012. Samplings  
100 of cones for the phytochemical investigation were performed in the second half of September 2012  
101 (S12), 2013 (S13) and 2014 (S14): the cones were collected at maturity and dried at 40°C in a  
102 thermostatic room, protected from light, to obtain 80% water loss (evaluated as sample weight loss).

### 103 *2.2 Micromorphological investigation*

#### 104 *2.2.1 Scanning Electron Microscopy (SEM)*

105 Fresh leaves, bracts, bracteoles and ovaries were collected from female plants and fixed overnight  
106 at 4°C in 4% (v/v) glutaraldehyde in deionized water. Fixed tissues were washed with deionized water  
107 and post-fixed with aqueous 2% osmium tetroxide for 2 hours. Samples were washed several times  
108 with deionized water and dehydrated using the following ethanol concentrations: 25, 50, 70, 80, 95 and  
109 100% twice for 15 min. Samples were then critical point dried with liquid CO<sub>2</sub>, mounted on aluminum  
110 stubs and sputtered with gold under vacuum (Nanotech sputter coater). Specimens were examined  
111 using a LEO 1430 Scanning Electron Microscope.

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

#### 113 *2.2.2 Light Microscopy (LM)*

114 LM investigation on historesin-fixed samples (leaves, bracts, bracteoles and ovaries) was  
115 performed to describe in detail the structure of the glandular trichomes. Histochemical tests were  
116 employed on fresh samples to evidence the main chemical classes of metabolites in the secretory  
117 products of the peltate trichomes of leaves and cones, with special emphasis on terpenes. Hand-made  
118 sections (40–50  $\mu\text{m}$  thick) and semi-thin sections (20–25  $\mu\text{m}$  thick) obtained by means of a cryostat,  
119 were stained with the following dyes: Sudan III/IV (Johansen 1940) and Fluoral Yellow-088  
120 (Brundrett *et al.* 1991) for total lipids; Nadi reagent for terpenes (David and Carde 1964); Ruthenium  
121 Red and Alcian Blue for polysaccharides other than cellulose (Jensen 1962); ferric trichloride for  
122 polyphenols (Gahan, 1984). Matchings for all the histochemical stains were performed with control  
123 procedures. At least five samples of each plant part were examined for each histochemical staining to  
124 assess the consistency of the results.

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

### 127 2.3 *Phytochemical investigation*

#### 128 2.3.1. *Preparation and analysis of essential oils*

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

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

### 148 2.3.2 Extraction and sample preparation of $\alpha/\beta$ -acids and polyphenols

149 Dried cones were formerly ground to fine powder with an electric grinder. Then, a small amount  
150 of powder (250 mg) was sequentially extracted with three solvents (petroleum ether 40-60°C,  
151 dichloromethane and methanol). Extraction was performed four times for each solvent with equal  
152 volumes and timing (10 mL, 30 min), leading to three organic fractions containing, among others,  
153 terpenophenolics, pigments and polyphenols, respectively. This procedure was repeated several times  
154 on equal amounts of hop samples, resulting in yield ranges of 19.4-23.7%, 3.0-4.2% and 7.8-12.9% for  
155 extraction of  $\alpha/\beta$ -acids, pigments and polyphenols, respectively, with total yields in extraction varying  
156 between 30.0% and 39.6%.

### 157 2.3.3 HPLC analysis of $\alpha/\beta$ -acids

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

162 Eluent composition was varied between 0.1% formic acid in water (A) and pure acetonitrile (B)  
163 according to the following program: 0-5 min B 20-50%, 5-7 min B 50-100%, 7-19 min B 100%; the  
164 flow rate was 0.6 mL min<sup>-1</sup>. Resulting peak areas were quantified according to ASBC/EBC procedure  
165 by comparison with ICE-3 standard (IHSC 2010). Percentages of  $\alpha/\beta$ -acids refers to the weight of  
166 starting samples. To obtain a clear identification of constituents, analyses were repeated on a Thermo  
167 Finnigan LC-MS system, equipped with PDA detector and LCQ Advantage mass spectrometer, using  
168 the same column and binary eluent program used for the quantification by HPLC-PDA, above  
169 reported.

#### 170 2.3.4 HPLC analysis of polyphenols

171 LC-MS analyses of samples obtained from methanol extraction were performed on the same  
172 Thermo Finnigan LC-MS system used for peak identification in  $\alpha/\beta$ -acids analysis. Eluent was a  
173 binary mixture composed of 0.1% formic acid in water (A) and pure acetonitrile (B), which was varied  
174 according to the following gradient program: 0-10 min B 10-15%, 10-45 min B 15-40%, 45-52 min B  
175 40-100%, 52-58 min B 100%, at a flow rate of 0.6 mL min<sup>-1</sup> (Araneo *et al.* 2013). Identification of the  
176 constituents was based on computer matching against commercial (NIST 2000) and laboratory-made  
177 mass spectra library built up from pure substances and MS literature data.

178

### 179 3. Results and Discussion

#### 180 3.1 Micromorphological investigation

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

184 Two types of non-glandular trichomes were identified: simple and cystolithic trichomes (Fig. 1). The  
185 former are medium-long, with an acute apex and a smooth surface (Fig. 1a, arrow); the cystolithic ones



186 are shorter, enlarged at the base and ending with a pointed tip and exhibit calcium carbonate deposits  
187 on the surface (Fig. 1b).

188 Two main types of glandular trichomes were observed: peltate (Fig. 1c,d) and bulbous (Fig. 1e), both  
189 consisting of a stalk and a multicellular secretory head. LM observation allowed to accurately  
190 characterize their structure and morphology (Fig. 1f-h).

191 The peltate ones consist of 2-4 basal epidermal cells, 2-4 stalk cells and of a very high number of  
192 glandular cells arranged in a single layer (Fig. 1c, f, g); the glandular head is surrounded by a wide  
193 subcuticular space in which the secretory material is stored. Two subtypes, differing in shape, size and  
194 distribution pattern, were recognized: flattened, mainly located on leaves, with a head diameter in the  
195 range 100-120  $\mu\text{m}$  at maturity (Fig. 1c, f), and biconical, typical of cones, with a head diameter in the  
196 range 150-180  $\mu\text{m}$  (Fig. 1d, g).

197 Bulbous trichomes exhibit 2 basal epidermal cells, 2 stalk cells and 4 secreting cells (25-40  $\mu\text{m}$  in  
198 diameter) with a thin subcuticular space (Fig. 1e, h).

199 Figure 2 (a-f) shows in detail the trichome distribution pattern. Cystolithic and bulbous trichomes (Fig.  
200 2a, arrow) are present on the adaxial leaf epidermis; the peltate trichomes are scattered overall abaxial  
201 lamina and simple non-glandular hairs are exclusively located at the midrib (Fig. 2b, arrow).

202 Non-glandular and bulbous trichomes are densely distributed on the abaxial and adaxial surfaces of  
203 bracts and bracteoles (Fig. 2c, d, e); peltate trichomes are present only on the abaxial surface and  
204 appear much crowded at the basal region (Fig. 2c, e). The perianth is covered by high-density peltate  
205 trichomes only (Fig. 2f).

206 The results of the histochemical investigation are shown in Figure 3. We focused attention on the  
207 peltates, due to their greater density compared to bulbous trichomes. Regardless of their distribution on  
208 leaves and cones, these trichomes displayed consistent responses to all the employed histochemical  
209 dyes.

210 The substances accumulated in the large subcuticular spaces are visible in the form of variable-sized  
211 droplets, also in the stainless samples (Fig. 3a). These secretory products were intensely evidenced by  
212 the total lipid-specific dyes, Sudan III/IV and Fluoral Yellow 088 (Fig. 3d,e). In particular, the  
213 response to Nadi reagent gave clear positive responses, indicating the presence of terpenes (Fig. 3f).  
214 The employed tests for polysaccharides and polyphenols invariably displayed negative results (Fig.  
215 3b,c).

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

223 The histochemical dyes we employed on the peltate trichomes revealed that EOs, in particular  
224 terpenes, are massively produced and released by these structures. This evidence agrees with the  
225 results by Oliveira and Pais (1988), who, however, documented the synthesis of essential oils also in  
226 the bulbous trichomes. Therefore, EOs appear to be synthesized by different types of secretory  
227 structures in hop cones. As regards to the other most important hop components responsible for the  
228 aroma and taste properties of beer, *i.e.* bitter acids and tannic acids, the same authors suggested that the  
229 former are produced exclusively by the peltate trichomes, whereas the latter are produced in laticifers  
230 (Oliveira and Pais, 1988).

231

## 232 3.2 Phytochemical investigation

### 233 3.2.1 Essential oils

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

236 A total of 45 compounds were identified. The profiles obtained in September 2012 (S12), September  
237 2013 (S13) and September 2014 (S14) are characterized by the presence of 34 (96.5%), 31 (97.8%)  
238 and 32 (98.85%) compounds, respectively.

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

245 Concerning the most abundant compounds, the investigated EO profiles show myrcene (4) as the main  
246 compound across the three years, with relative percentages of 41.6% in S12, 35.5% in S13 and 72.3%  
247 in S14. The sesquiterpenes  $\alpha$ -humulene (26) (15.9% in S12, 26.8% in S13, 7.3% in S14),  $\beta$ -  
248 caryophyllene (23) (5.8% in S12, 12.4% in S13, 3.3% in S14), (*E*)- $\beta$ -farnesene (27) (2.5% in S12,  
249 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)  
250 followed.

251 The most common compounds are 18. The exclusive compounds are three in S12 (1, 19, 33), three in  
252 S13 (17, 35, 42), five in S14 (10, 14, 18, 28, 32). These compounds are present in relative percentages  
253 always lower than 1.5%.

254 It is noteworthy that the EO contains linalool (11) among its constituents, particularly the S12 sample  
255 (1.1%). Peacock and Deinzer (1981) reported that most of the floral aroma of beers produced using cv.  
256 Cascade are due to linalool and geraniol. The latter compound is not present in our samples but,  
257 according to the same authors, it may depend on the hop age because its amount increases during the  
258 storage process.

259 Literature about hop EO is quite rich, particularly for the "aroma hop" varieties. The composition is  
260 very variable depending on the different cultivars, with some differences within the same variety,  
261 according to the geographical origin, the cultivation area or the cultivation/processing techniques.

262 If we compare our samples to the profile of the other investigated Cascade hop cultivated in Italy  
263 (Mongelli *et al.* 2016), myrcene was almost halved, while (*E*)- $\beta$ -farnesene was present in higher  
264 percentages; other differences emerged concerning the presence of several exclusive minor compounds  
265 in our samples (5,9,13,15,22), not identified by Mongelli *et al.* 2016. This variability could be related  
266 to the diverse environmental factors, cultivation conditions and harvesting period as well.

267 Moreover, the comparison with the same cultivar grown in Oregon and Washington, despite the  
268 differences in the analytical methodologies, showed a general consistency of the qualitative profiles  
269 (Lam *et al.* 1986; Nance and Setzer 2011), except for the presence of two exclusive compounds,  $\gamma$ -  
270 muurolene and *trans*- $\gamma$ -cadinene, in our samples. These two compounds may intensify some peculiar  
271 aromatic features of the Cascade hop cultivated in Italy, such as the herbal, woody and spicy notes  
272 (Goncalves *et al.* 2014).

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

279

280 3.2.2.  *$\alpha/\beta$ -acid composition*

281 HPLC analysis revealed the presence of 4 peaks at  $R_t = 14.30, 14.80, 16.10$  and  $16.90$  min (Fig. 4)  
282 attributed, respectively, to  $\alpha$ -acids cohumulone (a),  $n$ -humulone + adhumulone (b), and to  $\beta$ -acids  
283 colupulone (c),  $n$ -lupulone + adlupulone (d) by ESI-MS spectra acquired for each peak.

284 Our samples showed variable values of  $\alpha$ - and  $\beta$ -acids between S12 and the other two collection  
285 times, S13 and S14; in fact, the total  $\alpha$ -acids content (which correspond to the sum of cohumulone,  
286 adhumulone and  $n$ -humulone percentages) moves from 2.19%(w/w) in S12 to 4.93% and 5.01% in  
287 S13 and S14, respectively.  $\beta$ -acids moves from 6.73% in S12 up to 7.56% in S13 and to 7.66% in S14.  
288 Therefore, there is no qualitative variability among the  $\alpha$ - and  $\beta$ -acid compositions over the three  
289 years, with the presence of the six principal derivatives of phloroglucinol ( $n$ -, co-, adhumulone and  $n$ -,  
290 co- and ad-lupulone) usually reported for hop. On the contrary, if we consider the quantitative  
291 distribution of each class of the above-mentioned compounds, it clearly comes out that S12 displays  
292 considerable differences in comparison to literature data. S12 profile shows a lower content of  $\alpha$ -acids  
293 (2.19%) compared with literature (4.5-7.0%), while  $\beta$ -acids and cohumulone/ $\alpha$ -acids percentages  
294 (6.73% and 30%, respectively) attest to comparable values (Nance and Setzer 2011; Goncalves *et al.*  
295 2012). S13 and S14 profiles showed percentages in line with literature data.

296

### 297 3.2.3 Polyphenol content

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

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

310

#### 311 **4. Conclusions**

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

314 The detailed micromorphological observation by light and scanning electron microscopy allowed to  
315 describe the non-glandular and glandular trichomes. The *indumentum* features were consistent to  
316 literature information, with peltate trichomes as the main sites of terpene production and accumulation.  
317 The phytochemical data are generally in agreement with literature, though they showed quantitative  
318 differences in essential oil and bitter acid composition during the three monitoring years. This may be  
319 ascribed to the adaptation to the new environment.

320 Moreover, qualitative differences were recorded in essential oil composition and polyphenol content,  
321 mainly due to the presence of the exclusive compounds,  $\gamma$ -muurolene and *trans*- $\gamma$ -cadinene in EO and  
322 quercetin-3-*O*-malonylglucoside and kaempferol-3-*O*-rutinoside among polyphenols, that may  
323 characterize this peculiar Italian cultivation of the Cascade hop.

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#### 328 **Conflicts of Interest**

329 The authors declare no conflict of interest.

330 **REFERENCES**

- 331 Adams RP. 1995. Identification of essential oil components by gas chromatography/mass  
332 spectroscopy. Carol Stream: Allured. ISBN 0931710855
- 333 Araneo S, Santagostini L, Flamini, G, Iriti M, Cerasa F, Fico G. 2013. Luppolo coltivato in Italia:  
334 indagine fitochimica dal campo al boccale di birra (Hop cultivated in Italy: phytochemical study  
335 from field to beer) Acts of XIII Congress of the Italian Society of Phytochemistry. Gargnano, Italy,  
336 19-21 September, 34-35.
- 337 Aron P. 2011. The effect of hopping technology on lager beer flavor and flavor stability and the impact  
338 of polyphenols on lager beer flavor and physical stability, PhD Dissertation, Oregon University.
- 339 AssoBirra. Annual Report 2016.
- 340 Barnes J, Anderson L, Philipson J 2002. Herbal medicines: A Guide for Health Care Professionals,  
341 Pharmaceutical Press, London.
- 342 Behre K.E. 2002. The history of beer additives in Europe- a review, *Vegetation History and*  
343 *Archeobotany*, 8: 35-48
- 344 Brundrett MC, Kendrick B, Peterson CA. 1991. Efficient lipid staining in plant material with Sudan  
345 Red 7B or Fluoral Yellow 088 in polyethyleneglycol-glycerol. *Biotech Histochem.* 66: 111–116.
- 346 David, R, Carde JP. 1964. Coloration différentielle des inclusions lipidiques etterpéniques des  
347 pseudophylles du Pin maritime au moyen du reactif NADI. *C R Biol.* 258: 1338–1340.
- 348 Davies NW, 1990. Gas chromatographic retention indexes of monoterpenes and sesqui-terpenes on  
349 methyl silicone and Carbowax 20M phases. *J Chrom.* 503: 1-24. 10.1016/S0021-9673(01)81487-4
- 350 De Cooman L, Everaert E, De Keukeleire D. 1998. Quantitative analysis of hop acids, essential oils  
351 and flavonoids as a clue to the identification of hop varieties. *Phytochem Anal* 9: 145-150.



- 352 Eyres GT, Marriott PJ, Dufour J-P. 2007. Comparison of odor-active compounds in the spicy fraction  
353 of hop (*Humulus lupulus* L.) essential oil from four different varieties. J Agric Food Chem 55:6252-  
354 6261.
- 355 Gahan PB. 1984. Plant histochemistry and cytochemistry: an introduction. Academic Press, London.
- 356 Gonçalves J, Figueira J, Rodrigues F, Camara JS. 2012. Headspace solid-phase microextraction  
357 combined with mass spectrometry as a powerful analytical tool for profiling the terpenoid  
358 metabolomic pattern of hop-essential oil derived from Saaz variety. J Sep Sci 35: 2282-2296.
- 359 Gonçalves JL, Figueira JA, Rodrigues FP, Ornelas LP, Branco RN, Silva CL, Câmara JS. 2014. A  
360 powerful methodological approach combining headspace solid phase microextraction, mass  
361 spectrometry and multivariate analysis for profiling the volatile metabolomic pattern of beer starting  
362 raw materials, Food Chem. 160:266-280.
- 363 Grieve M 1971. A Modern Herbal, Dover Publications, New York.
- 364 Hirosawa T, Saito T, Tanaka T, Matsushima H, 1995. SEM observation and HPLC analysis of the  
365 accumulation of alpha-and beta-acids in the fresh developing hop (*Humulus lupulus* L.) peltate  
366 glandular trichomes. Microscopy. 44(3): 145-147.
- 367 International Hop Standards Committee (IHSC), 2010. J Inst Brew. 116: 197-198.
- 368 Jennings W, Shibamoto T. 1980. Qualitative analysis of flavor and fragrance volatiles by glass  
369 capillary chromatography. Academic Press. New York.
- 370 Jensen WA. 1962. Botanical histochemistry: principles and practice. WH Freeman & Co., San  
371 Francisco.
- 372 Johansen DA. 1940. Plant Microtechnique. McGraw-Hill Book Company, New York, London.

373 Kavalier AR, Litt A, Ma C, Pitra NJ, Coles MC, Kennelly EJ, Matthews PD. 2011. Phytochemical and  
374 morphological characterization of hop (*Humulus lupulus* L.) cones over five developmental stages  
375 using high performance liquid chromatography coupled to time-of-flight mass spectrometry,  
376 ultrahigh performance liquid chromatography photodiode array detection, and light microscopy  
377 techniques. *J Agric Food Chem.* 59: 4783-4793.

378 Kim ES, Mahlberg PG. 2000. Early development of the secretory cavity of peltate glands in *Humulus*  
379 *lupulus* L. (Cannabaceae). *Molec Cells.* 10: 487-492.

380 Kishimoto T, Wanikawa K, Kono K, Shibata K. 2006. Comparison of the odor-active compounds in  
381 unhopped beer and beers hopped with different hop varieties. *J Agric Food Chem.* 54: 8855-8861.

382 Lafontaine S, Varnum S, Roland A, Delpech S, Dagan L, Vollmer D, Kishimoto T, Shellhammer T.  
383 2019. Impact of harvest maturity on the aroma characteristics and chemistry of Cascade hops used  
384 for dry-hopping. *Food Chem.* 278: 228-239.

385 Lawless J 1995. *The Illustrated Encyclopedia of Essential Oils: The Complete Guide to the Use of*  
386 *Oils in Aromatherapy and Herbalism*, Element Books, Dorset, UK.

387 Li, H-J.; Deinzer, M.L. 2007. Tandem mass spectrometry for sequencing proanthocyanidins. *Anal.*  
388 *Chem.* 79: 1739-1748

389 Lam KC, Nickerson GB. Deinzer ML. 1986. A rapid solvent extraction method for hop essential oils. *J*  
390 *Agric Food Chem.* 34: 63-66.

391 Magalhaes PJ, Vieira JS, Goncalves LM, Pacheco JG, Guido LF, Barros AA. 2010. Isolation of  
392 phenolic compounds from hop extracts using polyvinylpyrrolidone: characterization by high-  
393 performance liquid chromatography-diode array detection-electrospray tandem mass spectrometry.  
394 *J Chrom A.* 1217: 3258-3268.

395 Massada Y, 1976. *Analysis of essential oils by gas chromatography and mass spectrometry.* J Wiley  
396 and Sons. New York.

- 397 Moir M 2000. Hops- A millennium review, J Am Soc Brew Chem 58: 131-146.
- 398 Mongelli A, Rodolfi M, Ganino T, Marieschi M, Caligiani A, Dall'Asta C, Bruni R. 2016. Are  
399 *Humulus lupulus* L. ecotypes and cultivars suitable for the cultivation of aromatic hop in Italy? A  
400 phytochemical approach. Ind Crop Prod. 83: 693-700.
- 401 Nance MR, Setzer WN. 2011. Volatile components of aroma hops (*Humulus lupulus* L.) commonly  
402 used in beer brewing. J Brew Dist. 2: 16-22.
- 403 Oliveira MM, Salomk M, Pais MS. 1988. Glandular trichomes of *Humulus lupulus* var. Brewer's  
404 Gold: ontogeny and histochemical characterization of the secretion. Nord J Bot – Sect Struct Bot. 8:  
405 349-359.
- 406 Oliveira MM, Pais MS. 1990. Glandular trichomes of *Humulus lupulus* var. Brewer's gold (hops):  
407 ultrastructural aspects of peltate trichomes. J Submicrosc Cytol Pathol. 22, 241–248
- 408 Oliver G. 2012. The Oxford Companion to Beer; Oxford University Press, London, UK.
- 409 Peacock VE, Deinzer ML. 1981. Chemistry of Hop Aroma in Beer. J. Am. Soc. Brew. Chem. 39: 136–  
410 141.
- 411 Pignatti S. 1982. Flora d'Italia. EdAgricole, Bologna, Italy.
- 412 Saito T, Hirose T, Horiuchi S, Murakami A, Matsushima H. 1995. A study of SEM examination on  
413 fresh hop (*Humulus lupulus* L.) peltate glandular trichomes. Microscopy. 44(1), 39-44.
- 414 Shephard HL, Parker JS, Darby P, Ainsworth CC. 2000. Sexual development and sex chromosomes in  
415 hop. New Phytol. 148: 397-411.
- 416 Small E. 1980. The relationships of hop cultivars and wild variants of *Humulus lupulus*. Can J Bot. 58:  
417 676–686.

- 418 Steinhaus M, Wilhelm W, Schieberle P. 2007. Comparison of the most odour-active volatiles in  
419 different hop varieties by application of a comparative aroma extract dilution analysis. Eur Food  
420 Res Technol. 226: 45–55.
- 421 Stenhagen E, Abrahamsson S, McLafferty FW. 1974. Registry of Mass spectral data. J. Wiley and  
422 Sons. New York.
- 423 Sugiyama R, Oda H, Kurosaki F. 2006. Two distinct phases of glandular trichome development in hop  
424 (*Humulus lupulus* L.). Plant Biotechnol J. 23: 493-496.
- 425 Swigar AA, Silverstein RM. 1981. Monoterpenes. Aldrich Chem. Comp. Milwaukee.
- 426 Van Cleemput M, Cattoor K, De Bosscher K, Haegeman G, De Keukeleire D, Heyerick A. 2009. Hop  
427 (*Humulus lupulus*) – derived bitter acids as multipotent bioactive compounds. J Nat Prod. 72, 1220-  
428 1230.
- 429 Zanolli P, Zavatti M. 2008. Pharmacognostic and pharmacological profile of *Humulus lupulus* L. J  
430 Ethnopharm. 116: 383-396.
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- 432

433 **Figure Captions**

434

435 **Figure 1.** SEM micrographs showing non-glandular (a-b) and glandular trichomes (c-e) of *H.*  
436 *lupulus* cv. Cascade: (a) simple trichomes; (b) cystolithic trichome with calcium carbonate  
437 deposits; (c) flattened peltate trichomes on leaf epidermis; (d) biconical peltate trichomes on  
438 inflorescences; (e) bulbous trichome. LM micrographs showing glandular trichome (f-h) of *H.*  
439 *lupulus* cv. Cascade: f) flattened peltate trichome; (g) biconical peltate trichome; (h) bulbous  
440 trichome. *Scale bars:* a, f, g = 40  $\mu\text{m}$ ; b, h = 20  $\mu\text{m}$ ; c = 25  $\mu\text{m}$ ; d = 50  $\mu\text{m}$ ; e = 10  $\mu\text{m}$ .

441

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

448

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

452

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

456

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

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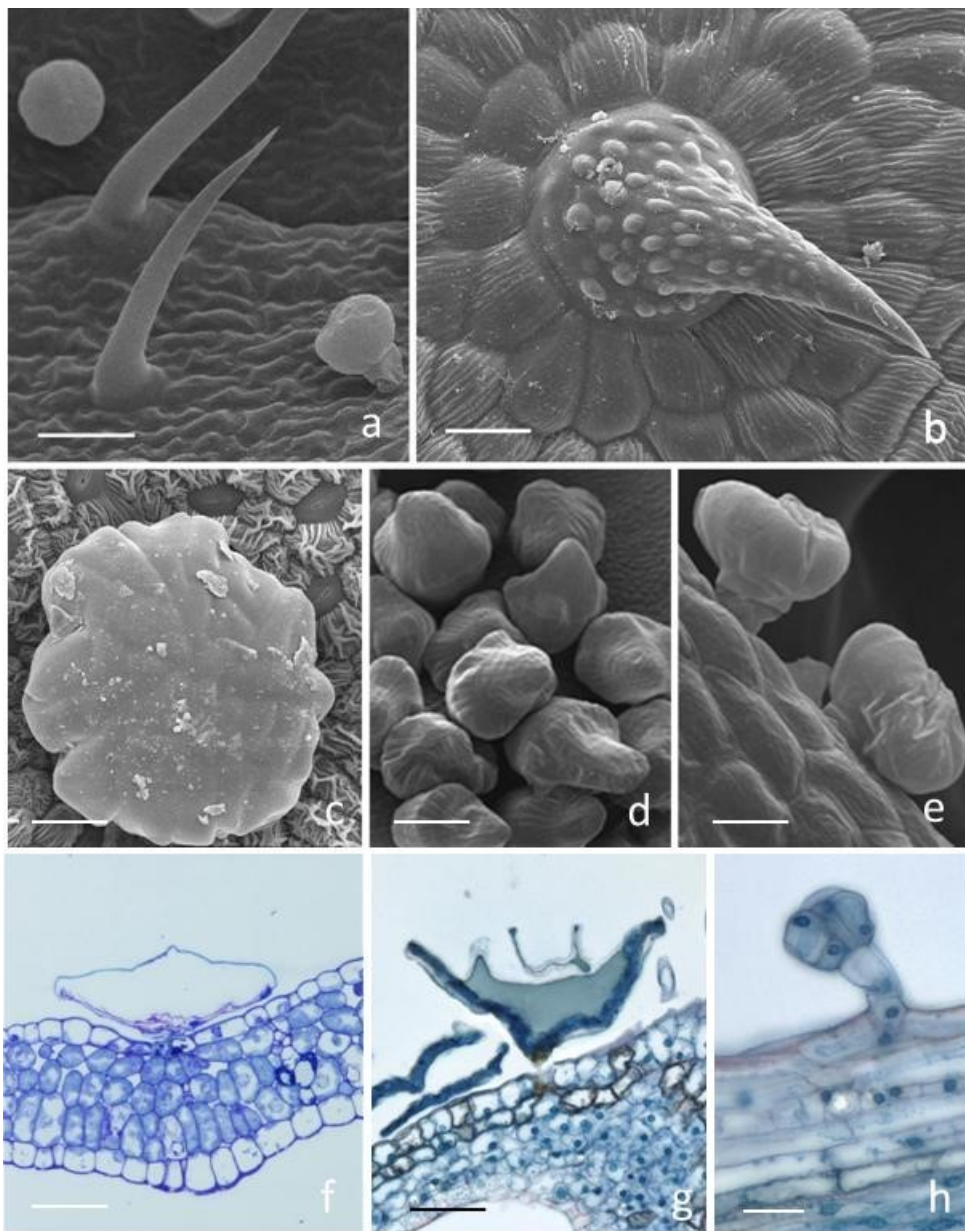
461 **Table 1.** Constituents of essential oils obtained from the cones of *Humulus lupulus* L. cv. Cascade in  
 462 September 2012, 2013 and 2014. The compounds common to all the three profiles are evidenced in  
 463 grey.

	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	$\alpha$ -pinene	0.4	-	0.2	St
3	982	$\beta$ -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	( <i>E</i> )- $\beta$ -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 ( <i>E</i> )-2-decenoate	-	0.4	-	RI, MS
18	1311	methyl 4-decenoate	-	-	0.8	RI, MS
19	1316	( <i>E,E</i> )-2,4-decadienal	1.2	-	-	St
20	1325	methyl geranate	0.6	0.6	0.7	RI, MS
21	1377	$\alpha$ -copaene	0.5	0.4	-	St
22	1383	geranyl acetate	2.3	0.6	1.2	St
23	1419	$\beta$ -caryophyllene	5.8	12.4	3.3	St
24	1430	$\beta$ -copaene	0.2	0.5	0.1	RI, MS
25	1437	<i>trans</i> - $\alpha$ -bergamotene	0.4	0.3	0.1	RI, MS
26	1456	$\alpha$ -humulene	15.9	26.8	7.3	St
27	1459	( <i>E</i> )- $\beta$ -farnesene	2.5	5.1	2.8	Stmix
28	1475	<i>trans</i> -cadin-1(6),4-diene	-	-	0.1	RI, MS
29	1479	$\gamma$ -muurolene	1.7	1.3	0.7	RI, MS
30	1487	$\beta$ -selinene	1.3	1.3	0.7	RI, MS
31	1495	$\alpha$ -selinene	1.4	1.5	-	RI, MS
32	1495	viridiflorene	-	-	0.9	RI, MS
33	1497	2-tridecanone	1.3	-	-	St
34	1500	$\alpha$ -muurolene	0.4	0.4	-	RI, MS
35	1508	( <i>E,E</i> )- $\alpha$ -farnesene	-	0.3	-	RI, MS
36	1514	<i>trans</i> - $\gamma$ -cadinene	0.8	1.3	0.2	RI, MS
37	1516	geranyl isobutyrate	0.9	-	0.7	RI, MS
38	1524	$\delta$ -cadinene	1.3	2.1	0.6	RI, MS
39	1538	$\alpha$ -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- <i>epi</i> -cubenol	-	0.2	-	RI, MS
43	1637	caryophylla-4(14),8(15)-dien-5-ol	1.4	0.8	-	RI, MS
44	1642	<i>epi</i> - $\alpha$ -cadinol	0.3	0.4	-	RI, MS
45	1654	$\alpha$ -cadinol	0.4	0.3	-	RI, MS
		<b>Monoterpene hydrocarbons</b>	<b>44.7</b>	<b>37.5</b>	<b>75.5</b>	
		<b>Oxygenated monoterpenes</b>	<b>4.9</b>	<b>1.6</b>	<b>3.2</b>	
		<b>Sesquiterpene hydrocarbons</b>	<b>32.2</b>	<b>53.9</b>	<b>17.0</b>	
		<b>Oxygenated sesquiterpenes</b>	<b>8.9</b>	<b>3.7</b>	<b>0.2</b>	
		<b>Non-terpene derivatives</b>	<b>5.8</b>	<b>1.1</b>	<b>3.0</b>	
		<b>Total identified</b>	<b>96.5</b>	<b>97.8</b>	<b>98.9</b>	

464 St: standard compound; Stmix: standard compound isomers mixture; RI: retention index; MS: mass  
465 spectrum  
466

467 Figure 1

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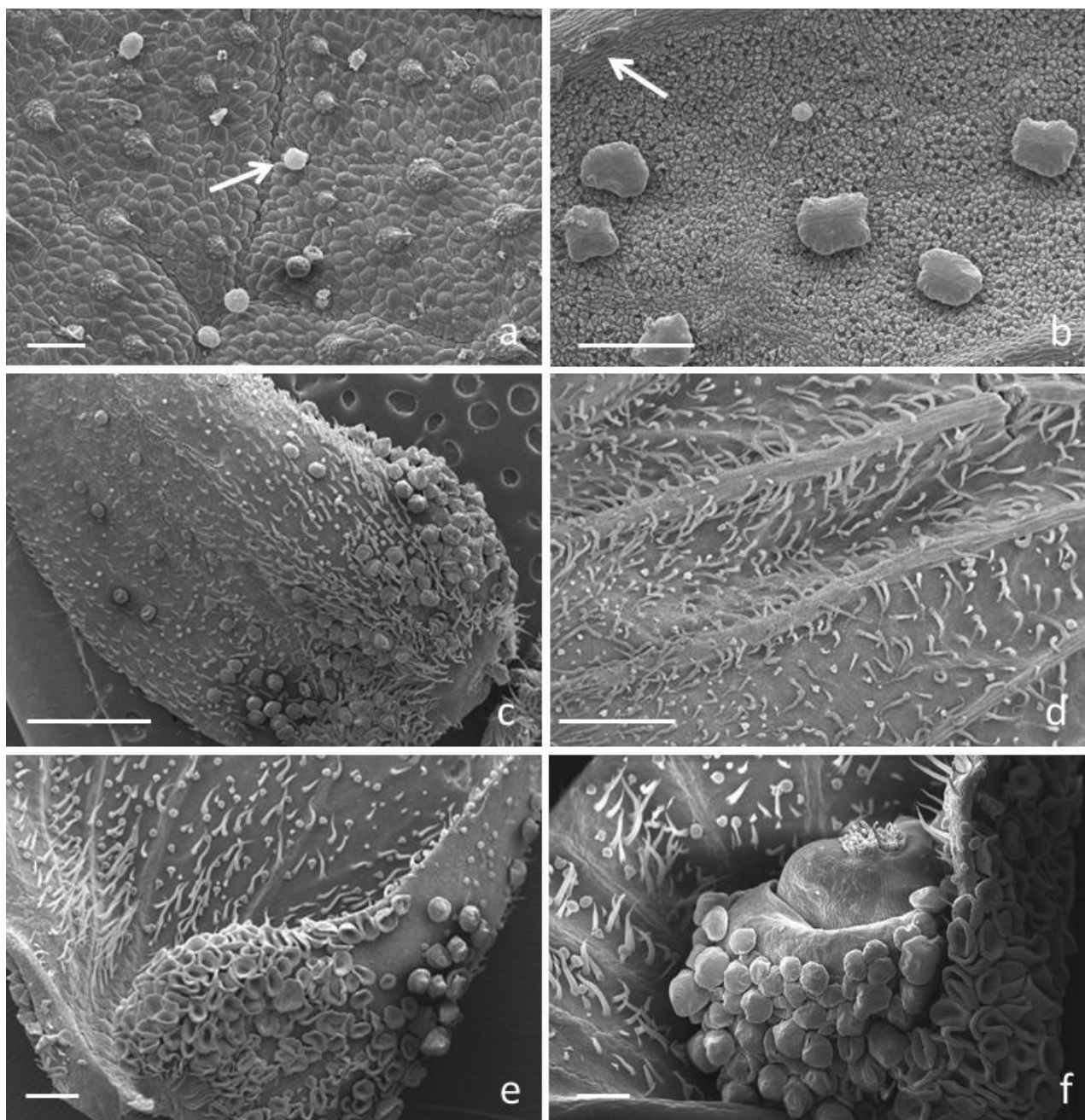
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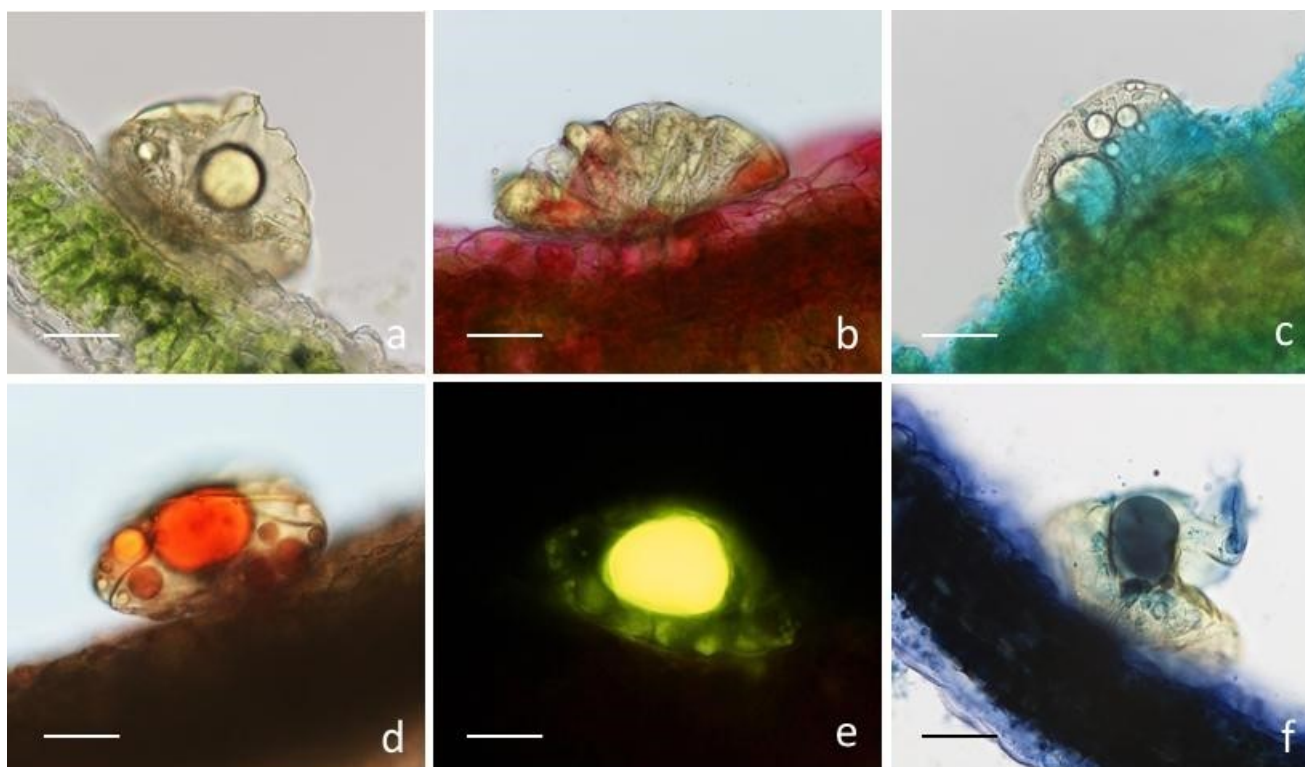
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478 Figure 3

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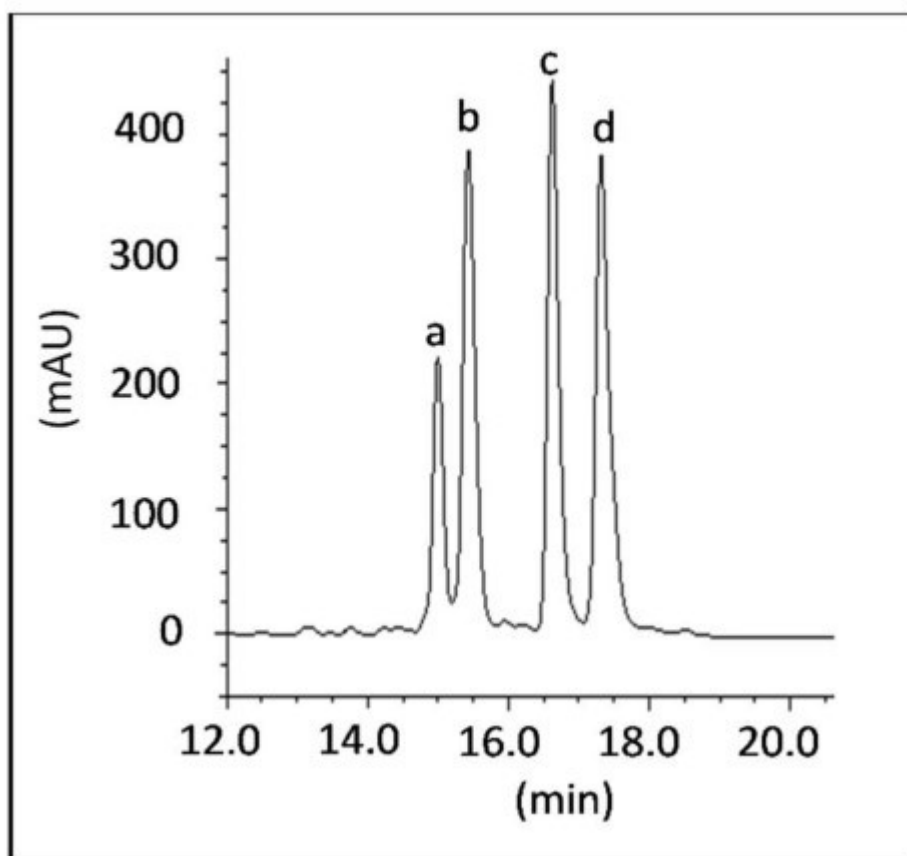


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482 Figure 4

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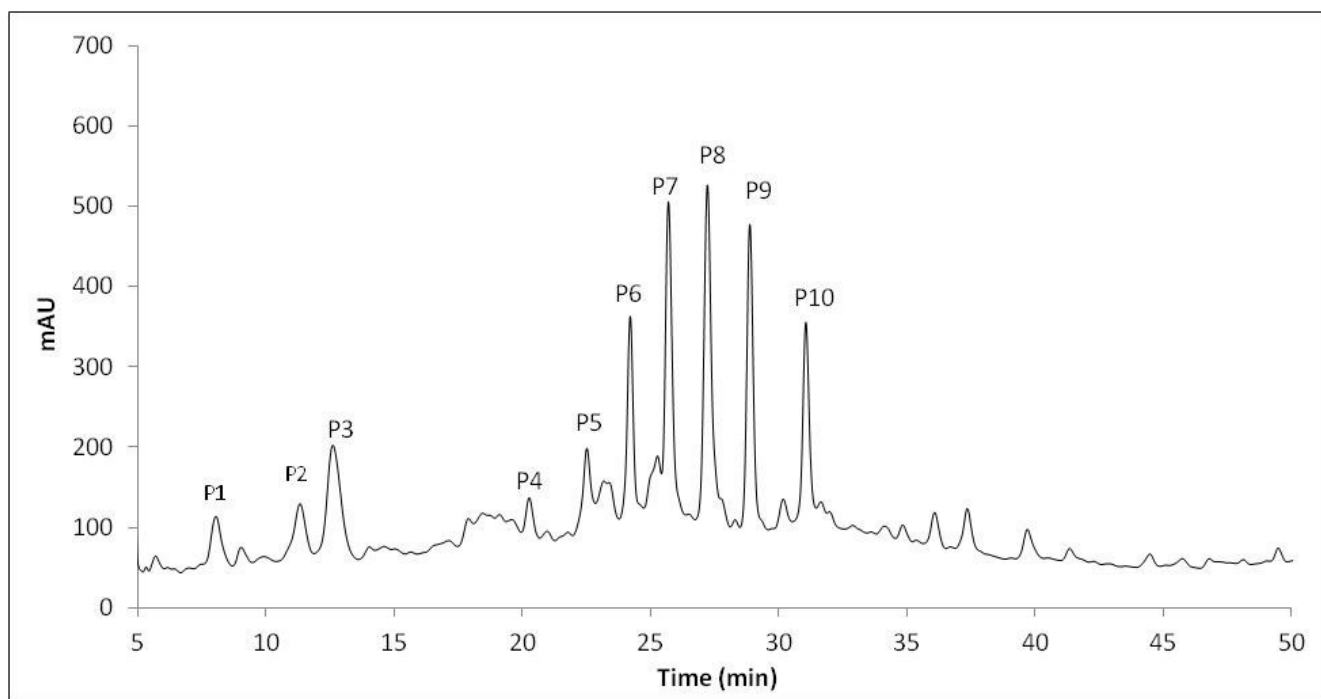
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489 Figure 5

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