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 cultivar Cascade, was investigated in a combined morphological and phytochemical survey. Morphological investigation on trichome structure, density and distribution was performed by scanning electron microscopy (SEM) and light microscopy (LM). 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 different relative abundances. The presence of two exclusive compounds, γ-muurolene and *trans*-γ-cadinene, characterized the investigated cv. Cascade, potentially enhancing herbal, woody and spicy aroma traits of this cultivation in Northern Italy.

The bitter acid composition showed quantitative values consistent with literature data only for 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, nowadays, it is widespread in the temperate zones of all continents, both in the wild and under cultivation (Small 1980; Pignatti 1982).

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 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 its leaves and inflorescences in some food preparations, as well as in textiles and cosmetic products (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).

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

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

Although bitter acids composition is usually used as quality parameter for hop, there is little information regarding the phytochemistry of cv. Cascade. The essential oil (EO) composition was analyzed by GC-O, GCxGC (Eyres *et al*. 2007; Steinhaus *et al*. 2007) and GC-MS (Nance and Setzer 2011; Mongelli *et al*. 2016). Nance and Setzer (2011) identified myrcene, α-humulene, (*E*)-caryophyllene, and (*E*)-β-farnesene as the EO main components.

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

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

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

The present research arises in this contest. We combined, for the first time, a study on the morphological and phytochemical characterization of Cascade hop cultivated in northern Italy. We specifically analyzed: (i) trichome distribution pattern and histochemistry on young leaves and female inflorescences (cones); (ii) the EOs obtained from the cones across three consecutive years 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. No chemical field treatments were applied during plant growth, to evaluate spontaneous response of the plants to the environment.

Samples for the micromorphological investigation were collected in September 2012. Samplings of cones for the phytochemical investigation were performed in the second half 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, to obtain 80% water loss (evaluated as sample weight loss).

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 subjected to hydrodistillation for 2 hours using a Clevenger-type apparatus (2 L round-bottom flask containing 1 L of water), and the obtained EO, 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 EOs 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 sequentially extracted with three solvents (petroleum ether 40-60°C, 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, among others, terpenophenolics, pigments and polyphenols, respectively. This procedure was repeated several times 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 extraction of *α*/*β*-acids, pigments and polyphenols, respectively, with total yields in extraction varying 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. To obtain a clear identification of constituents, analyses were repeated on a Thermo Finnigan LC-MS system, equipped with PDA detector and LCQ Advantage mass spectrometer, using the same column and binary eluent program used for the quantification by HPLC-PDA, above reported.

2.3.4 HPLC analysis of polyphenols

LC-MS analyses of samples obtained from methanol extraction were performed on the same Thermo Finnigan LC-MS system used for peak identification in *α*/*β*-acids analysis. Eluent was a binary mixture composed of 0.1% formic acid in water (A) and pure acetonitrile (B), which was varied according to 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} (Araneo *et al*. 2013). Identification of the constituents was based on computer matching against commercial (NIST 2000) and laboratory-made mass spectra library built up from pure substances and MS literature data.

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

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 μm at maturity (Fig. 1c, f), and biconical, typical of cones, with a head diameter in the
196 range 150-180 μm (Fig. 1d, g).
197 Bulbous trichomes exhibit 2 basal epidermal cells, 2 stalk cells and 4 secreting cells (25-40 μm 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 monoterpenic fraction (78.7%) compared to the sesquiterpenic 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 α-humulene (*26*) (15.9% in S12, 26.8% in S13, 7.3% in S14), β-
248 caryophyllene (*23*) (5.8% in S12, 12.4% in S13, 3.3% in S14), (*E*)-β-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*)-β-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, γ-
270 muurolene and *trans*-γ-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. α/β-acid composition*

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

284 Our samples showed variable values of *α*- and *β*-acids between S12 and the other two collection
285 times, S13 and S14; in fact, the total *α*-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. *β*-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 *α*- and *β*-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 *α*-acids
293 (2.19%) compared with literature (4.5-7.0%), while *β*-acids and cohumulone/*α*-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.

Among flavonol glycosides, the polyphenolic composition of our samples was characterized by the presence of compounds already reported in literature for this cultivar (De Cooman *et al*. 1998; Magalhaes *et al*. 2010; Kavalier *et al*. 2011), 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 were consistent to literature information, with peltate trichomes as the main sites of terpene production and accumulation.

The phytochemical data are generally in agreement with literature, though they showed quantitative differences in essential oil and bitter acid composition during the three monitoring years. This may be ascribed to the adaptation to the new environment.

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

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Conflicts of Interest

The authors declare no conflict of interest.

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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 μm; b, h = 20 μm; c = 25 μm; d = 50 μm; e = 10 μm.*

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 μm; b, d-f = 200 μm; 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 μm.*

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.

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

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

467 Figure 1

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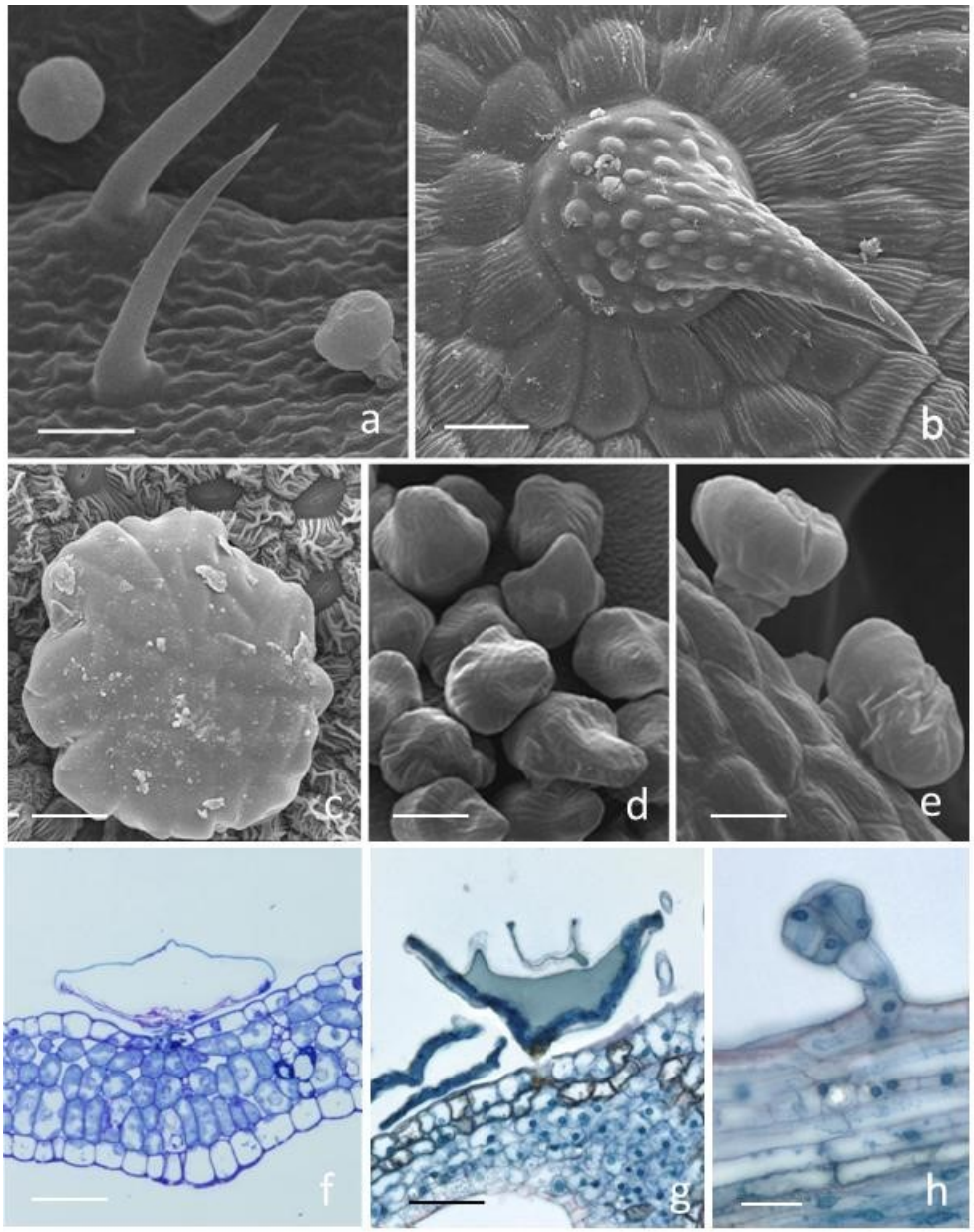

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473 Figure 2

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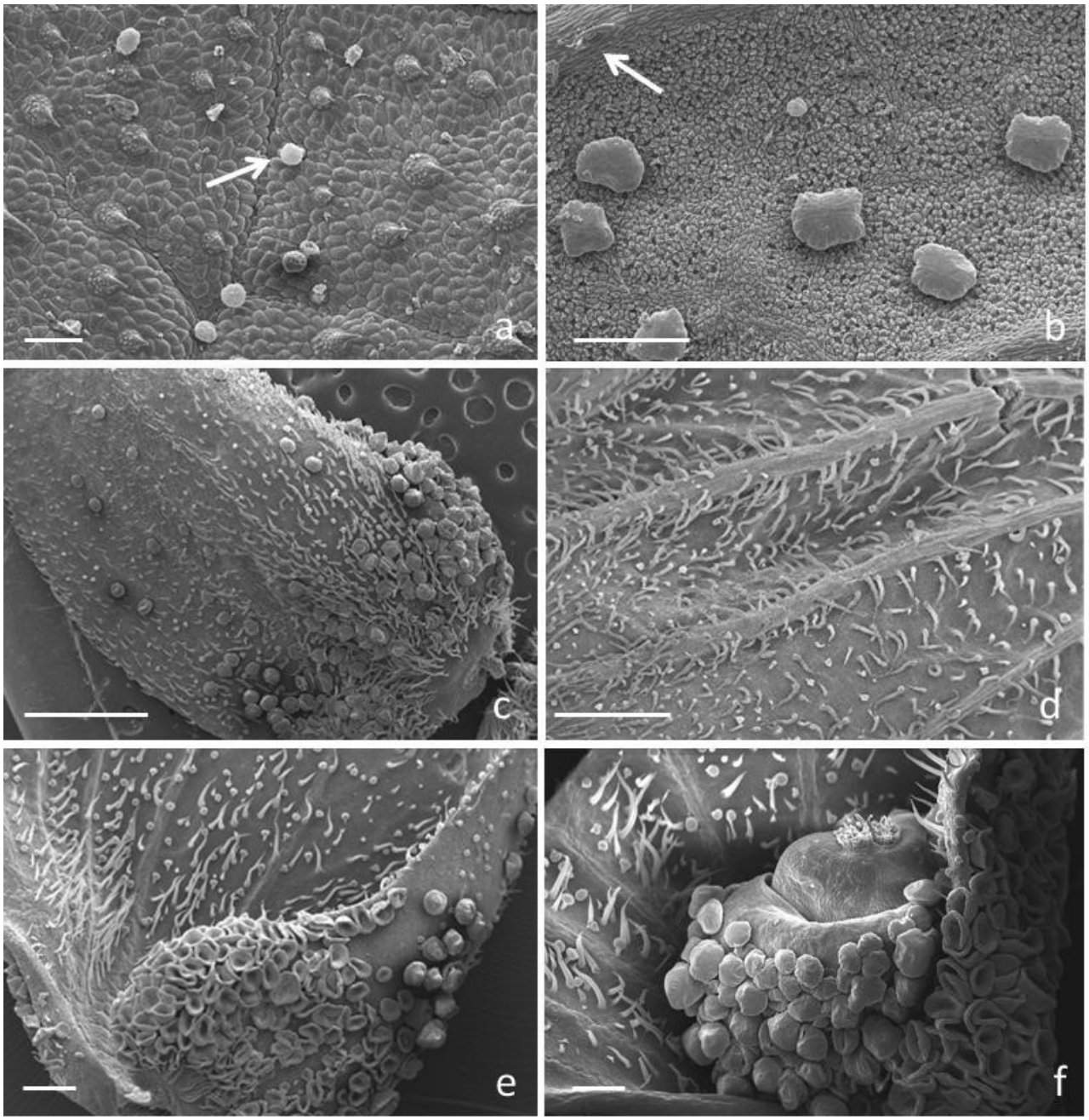

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478 Figure 3

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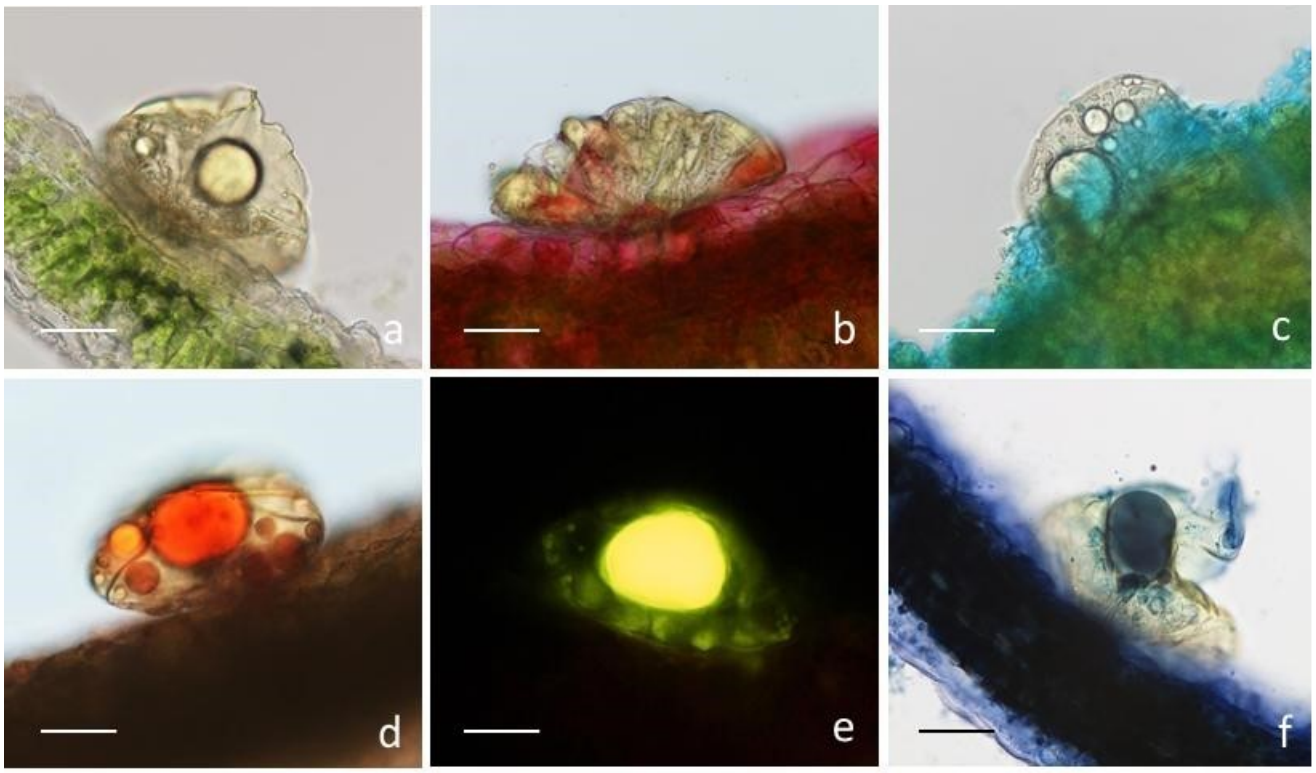

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482 Figure 4

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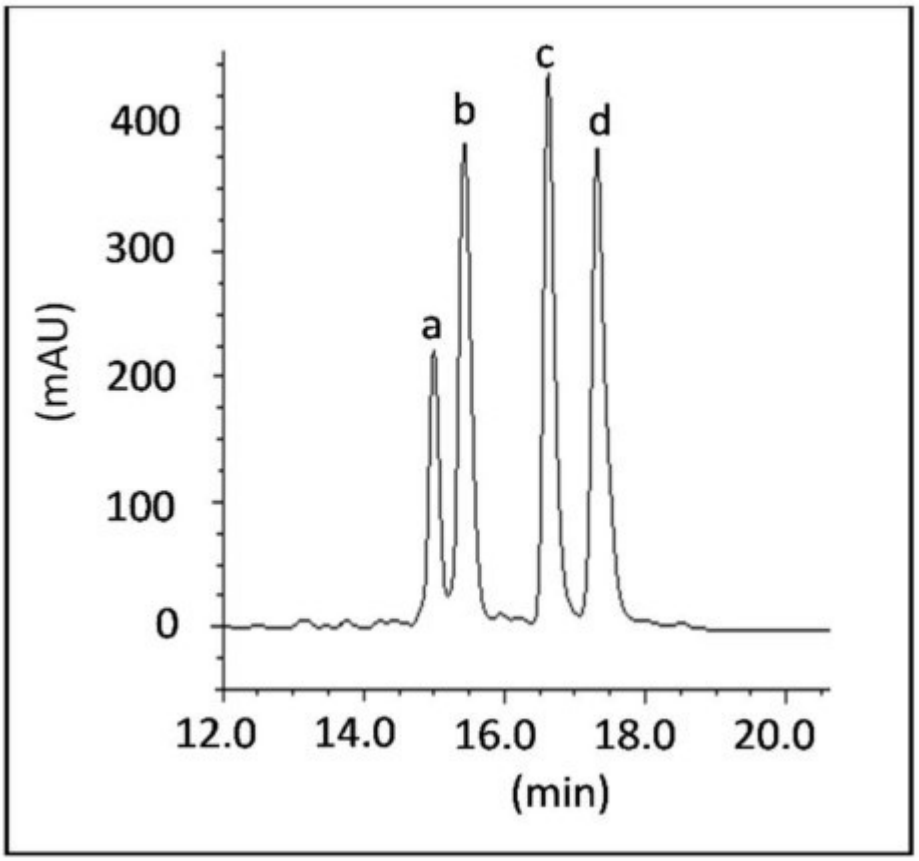

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489 Figure 5

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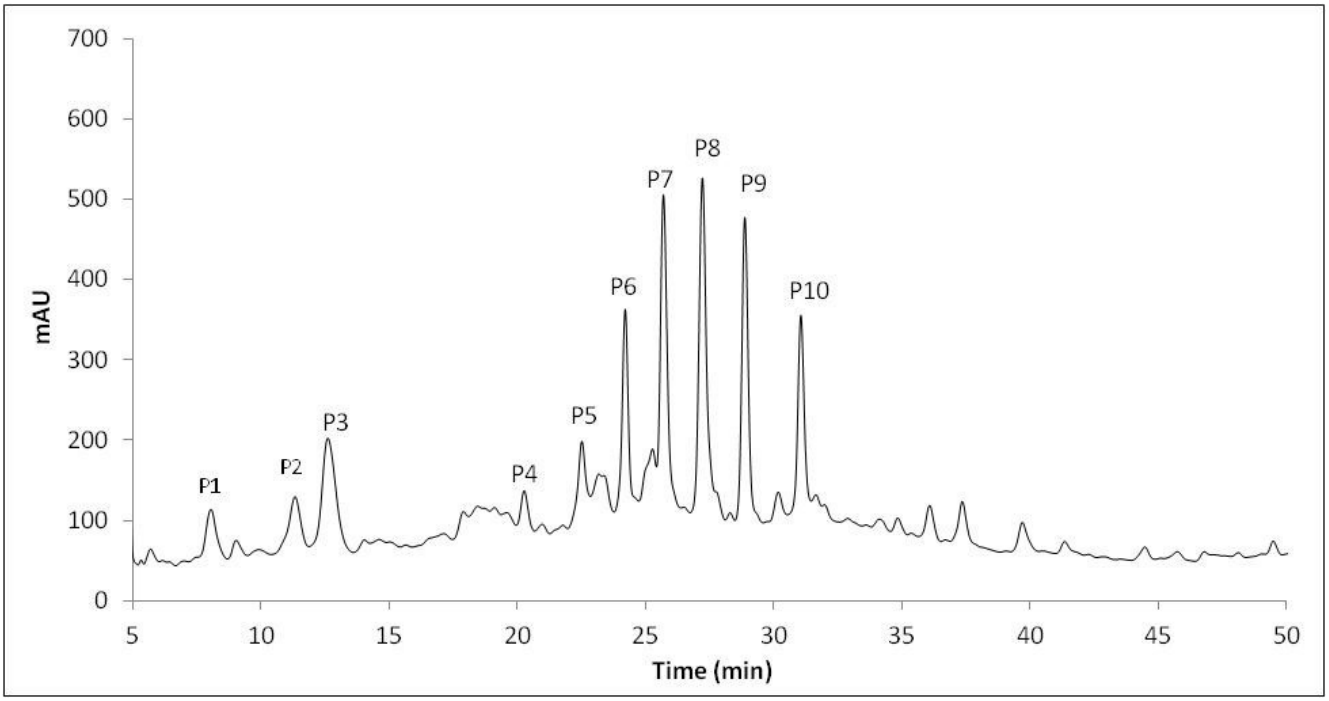

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