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Authors: Annalisa Maietti, Virginia Brighenti, Gianpiero Bonetti, Paola Tedeschi, Francesco Pio Prencipe, Stefania Benvenuti, Vincenzo Brandolini, Federica Pellati

1 **Highlights**

2 • A new HPLC method for the analysis of flavonols in young hop shoots was developed

3 • Quercetin and kaempferol glycosides were the main phenolics in hop shoots

4 • The content of flavonols is related to the *in vitro* antioxidant activity

5 • Hop shoots represent a new source of bioactive antioxidant compounds

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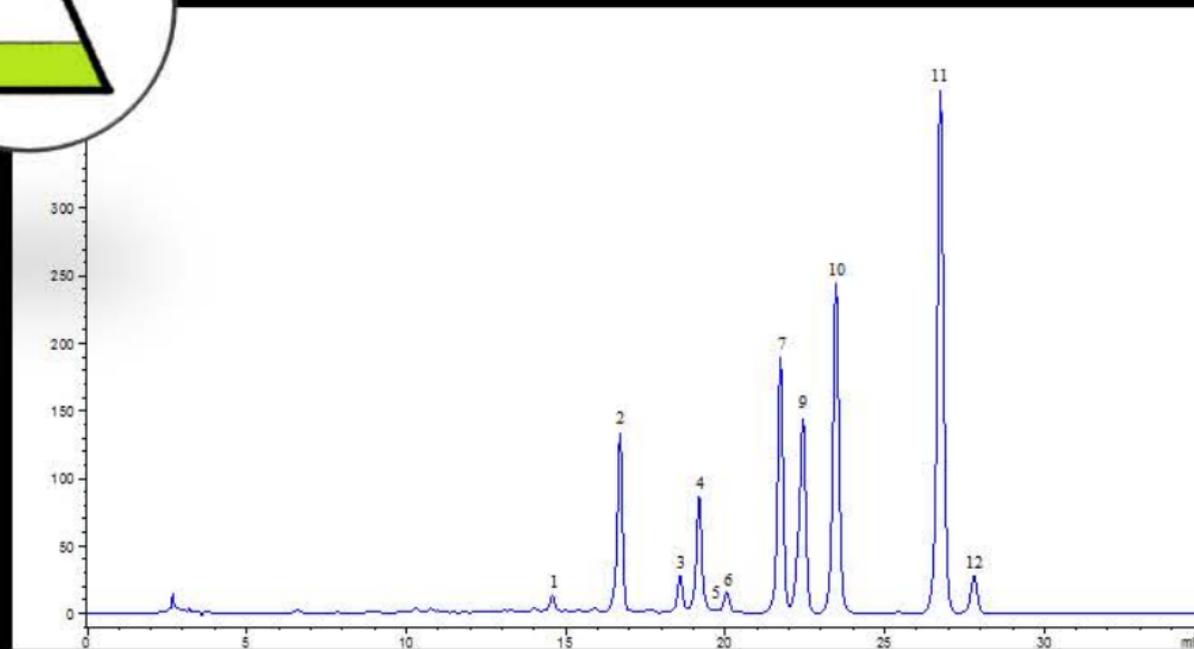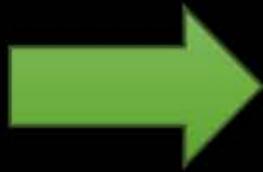
**Hop shoots** → **Extraction and HPLC analysis** → ***In vitro* antioxidant activity**

# Metabolite profiling of flavonols and *in vitro* antioxidant activity of young shoots of wild *Humulus lupulus* L. (hop)

Annalisa Maietti[a], Virginia Brighenti[b], Gianpiero Bonetti[a], Paola Tedeschi[a], Francesco Pio Prencipe[b], Stefania Benvenuti[b], Vincenzo Brandolini[a], Federica Pellati[b]*

[a]*Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17, 44121 Ferrara, Italy.*

[b]*Department of Life Sciences, University of Modena and Reggio Emilia, Via G. Campi 103, 41125 Modena, Italy*

*Corresponding author:*

Dr. Federica Pellati

Department of Life Sciences

University of Modena and Reggio Emilia

Via G. Campi 103, 41125 Modena, Italy

Phone: +39 059 2058565

E-mail: federica.pellati@unimore.it

Abbreviations: reactive oxygen species, ROS; diphenylpicrylhydrazyl, DPPH•; formic acid, HCOOH; methanol, MeOH; acetonitrile, ACN; water, $H_2O$; limit of detection (LOD); limit of quantification (LOQ); photochemiluminescence (PCL).

2

**Abstract**

*Humulus lupulus* L., commonly named hop, is well-known for its sedative and estrogenic activity. While hop cones are widely characterized, only few works have been carried out on the young shoots of this plant. In the light of this, the aim of this study was to identify for the first time the flavonoids present in young hop shoots and to compare the composition of samples harvested from different locations in Northern Italy with their antioxidant activity. The samples were extracted by means of dynamic maceration with methanol. The HPLC-UV/DAD, HPLC-ESI-MS and MS$^2$ analysis were carried out by using an Ascentis $C_{18}$ column (250 × 4.6 mm I.D., 5 μm), with a mobile phase composed of 0.1 M formic acid in both water and acetonitrile, under gradient elution. Quercetin and kaempferol glycosides were the main compounds identified and quantified in hop shoot extracts. Total flavonols ranged from 2698 ± 185 to 517± 47.5 μg/g (fresh weight).

The antioxidant activity was determined by means of the radical scavenging activity assay against diphenylpicrylhydrazyl (DPPH$^{\bullet}$) and by using a photochemiluscence assay with a Photochem$^{\circledR}$ apparatus. The results showed that hop shoots represent a new source of flavonols; therefore, they can be useful for a possible incorporation in the diet as a functional food or applied in the nutraceutical ambit.

**Keywords:** *Humulus lupulus*; hop shoots; flavonols; HPLC; MS; antioxidant activity.

## 1. Introduction

The harvesting and consumption of edible wild plants is an ancient custom for many people. Although their use has been limited in the last century, the interest for their healthy properties has never lessened. Today, the increase of cardiovascular, cancer and neurodegenerative diseases in industrialized countries has stimulated new interest in edible wild plants. Indeed, edible wild plants have a high nutritional value and they are a rich source of bioactive compounds [1], such as vitamins, carotenoids and polyphenols, which have been found to possess a great variety of biological properties, including the antioxidant activity. Several scientific studies have shown free radicals and, in particular, reactive oxygen species (ROS) as the main cause of aging and tissue damage. The overproduction of ROS, most frequently caused either by an excessive stimulation of NAD(P)H by cytokines or by the mitochondrial electron transport chain and xanthine oxidase, results in oxidative stress. Oxidative stress is a deleterious process that can be an important mediator of damage to cell structures and, consequently, cardiovascular disease, cancer, neurological disorders, including Alzheimer and Parkinson, and ageing [2]. Dietary antioxidants and other nutrients play an important role in preventing cells from radical-induced cytotoxicity [3].

*Humulus lupulus* L., commonly named hop, is a dioecious perennial plant belonging to the *Cannabaceae* family. Although hop is a spontaneous plant in central Europe, today it is widely cultivated in all temperate regions. Hop female flowers are used in the brewing process of beer, providing bitterness, flavour and aroma. The characteristic bitter grade and aroma that define a particular beer are influenced by many factors, including the hop cultivar employed.

In ancient times, hop has been used for its sedative action effect. The sedation, pre-anesthetic and anti-anxiety properties of hop extracts have been recently demonstrated in rats [4]. The frequent menstrual disturbances observed in female hop-pickers has suggested a potential hormonal activity of hop extracts; this estrogenic effect of hop has been attributed to 8-prenylnaringenin [5]. Other hop prenylflavonoids, including xanthohumol and other prenylchalcones, do not have any estrogenic activity, but they are considered to be possible cancer chemopreventive compounds [5,6].

Hop cones are widely characterized for their phenolics [7], stilbenes [8], prenylflavonoids and prenylphloroglucinols (bitter acids) [9,10]. Hop young shoots have been widely used as vegetables by the Romans. Today, hop young shoots, together with those from *Asparagus acutifolius* L. (wild asparagus), *Bryonia dioica* Jacq. (white bryony) and *Tamus communis* L. (black bryony)*,* are hand-picked in fresh and fertile lands at the edge of woods and ditches. They have been traditionally

107 consumed boiled as wild asparagus or to cook risotto and omelet. In Italy, the harvest of hop young

108 shoots is typical in the North and Central regions and it is made on the banks of rivers or in hill

109 areas, where there is a high humidity. As regards the composition, only few studies have been

110 carried out on hop young shoots for their content of carotenoids [11], organic acids [12] and

111 tocopherol [13].

112 In the light of all the above, the aim of this study was to identify for the first time the flavonoid

113 composition of hop young shoots by means of a new method based on HPLC-UV/DAD, HPLC-

114 ESI-MS and MS$^2$ and to compare these data with the antioxidant activity of the plant material

115 harvested from four different locations in Northern Italy.

116

117 **2.  Materials and methods**

118

119 *2.1. Chemicals and solvents*

120

121 Quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside were purchased

122 from Extrasynthese (Genay, France). Kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside and

123 2,2-diphenyl-1-picrylhydrazyl (DPPH$^•$) were from Sigma-Aldrich (Milan, Italy). All reference

124 compounds were of chromatographic grade. Formic acid (HCOOH), HPLC-grade methanol

125 (MeOH) and acetonitrile (ACN) were from Sigma-Aldrich (Milan, Italy). Water (H$_2$O) was purified

126 by using a Milli-Q Plus185 system from Millipore (Milford, MA, USA).

127

128 *2.2. Plant material*

129

130 Hop young shoots were hand-picked in April-May 2014 in three different locations of the Po river

131 banks, including Castelmassa (Rovigo), Cologna (Rovigo) and Santa Maria in Punta (Ferrara), and

132 in a hill area of Tuscany, named Vicchio (Florence). Each sample was packed in a plastic bag,

133 frozen at −20 °C on the same day and preserved until analysis.

134

135 *2.3. Extraction of secondary metabolites from hop shoots*

136

137 Dynamic maceration was chosen for the extraction of flavonoids present in hop shoots and MeOH

138 was used as the extraction solvent. In particular, the extraction procedure was performed on 2.0 g of

139 fresh sample with 10 mL of solvent at room temperature for 30 min under magnetic stirring. The

140 mixture obtained from the extraction was then centrifuged at 4000 rpm for 5 min and the

141 supernatant solution was filtered under vacuum into a volumetric flask. The residue of the first

142 extraction was re-extracted as previously described. Finally, the filtrates of the two extractions were

143 combined and brought to 25 mL in a volumetric flask. An aliquot of 5 mL of the extract was

144 concentrated under vacuum at 35 °C and then brought to the final volume of 1 mL with MeOH in a

145 volumetric flask. The concentrated extract was subsequently filtered by using a 0.45 μm PTFE filter

146 into a HPLC vial prior to the injection into the HPLC system.

147 The extraction procedure was carried out in duplicate for each sample.

148

149 *2.4 .HPLC-UV/DAD analysis*

150

151 HPLC-UV/DAD analyses were performed on an Agilent Technologies (Waldbronn, Germany)

152 modular model 1100 system, consisting of a vacuum degasser, a quaternary pump, an autosampler,

153 a thermostatted column compartment and a diode array detector (DAD). Chromatograms were

154 recorded by using an Agilent Chemstation for LC and LC-MS systems (Rev. B.01.03).

155 The HPLC analyses were carried out on an Ascentis $C_{18}$ column (250 × 4.6 mm I.D., 5 μm,

156 Supelco, Bellefonte, PA, USA). The mobile phase was composed of (A) 0.1 M HCOOH in $H_2O$ and

157 (B) ACN. The gradient elution was modified as follows: 0-15 min from 10% to 20% B, 15-35 min

158 from 20% to 30% B, 35-40 min from 30% to 40% B, 40-45 min from 40% to 50% B. The post-

159 running time was 5 min. The flow rate was 1.0 mL/min. The column temperature was set at 25 °C.

160 The sample injection volume was 10 μL. The UV/DAD acquisitions were carried out in the range

161 190-550 nm and chromatograms were integrated at 352 nm. Three injections were performed for

162 each sample.

163

164 *2.5. HPLC-ESI-MS and MS[2] analysis*

165

166 HPLC-ESI-MS and MS[2] analyses were carried out by using an Agilent Technologies modular 1200

167 system, equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a

168 thermostatted column compartment and a 6310A ion trap mass analyzer with an ESI ion source.

169 The HPLC column and the applied chromatographic conditions were the same as reported for the

170 HPLC-UV/DAD system. The flow rate was split 5:1 before the ESI source.

171 The HPLC-ESI-MS system was operated both in the positive and in the negative ion modes. For the

172 positive ion mode, the experimental parameters were set as follows: the capillary voltage was 3.5

6

173    kV, the nebulizer ($N_2$) pressure was 32 psi, the drying gas temperature was 350 °C, the drying gas

174    flow was 10 L/min and the skimmer voltage was 40 V. For the negative ion mode, the conditions

175    were set as follows: the capillary voltage was 4.0 kV, the nebulizer ($N_2$) pressure was 35 psi, the

176    drying gas temperature was 350 °C, the drying gas flow was 11 L/min and the skimmer voltage was

177    40 V.

178    Data were acquired by Agilent 6300 Series Ion Trap LC/MS system software (version 6.2). The

179    mass spectrometer was operated in the full-scan mode in the *m/z* range 100-1000. $MS^2$ spectra were

180    automatically performed with helium as the collision gas in the *m/z* range 50-1000 with the

181    SmartFrag function.

182

183    *2.6. HPLC-UV/DAD method validation*

184

185    The validation of the HPLC-UV/DAD method was performed in agreement with the international

186    guidelines for analytical techniques for the quality control of pharmaceuticals (ICH guidelines)

187    [14].

188    The stock standard solution of each compound (quercetin-3-*O*-galactoside, quercetin-3-*O*-

189    rutinoside, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside) was prepared as follows: an

190    accurately weighed amount of pure compound (2.1-5.0 mg) was placed into a 10 mL volumetric

191    flask; then, MeOH was added and the solution was diluted to volume with the same solvent. The

192    external standard calibration curve was generated by using six data points, covering the

193    concentration ranges: 7.8-313.0 µg/mL for quercetin-3-*O*-rutinoside (**3**); 5.3-213.0 µg/mL for

194    quercetin-3-*O*-galactoside (**5**); 7.8-312.0 µg/mL for kaempferol-3-*O*-rutinoside (**7**); 6.3-505.0

195    µg/mL for kaempferol-3-*O*-glucoside (**10**). Ten µL aliquots of each standard solution were used for

196    HPLC analysis. Injections were performed in triplicate for each concentration level. The calibration

197    curve was obtained by plotting the peak area of the compound at each level versus the concentration

198    of the sample. The quantification of compounds **3**, **5**, **7** and **10** was performed by using their

199    calibration curves. The amount of the other flavonols found in hop shoots was determined by using

200    the calibration curves of the reference compounds with the same chromophore. In particular, the

201    calibration curve of quercetin-3-*O*-rutinoside was used for compound **1**, that of quercetin-3-*O*-

202    galactoside for compound **6**, that of kaempferol-3-*O*-glucoside for compounds **11** and **12** and that of

203    kaempferol-3-*O*-rutinoside for compounds **2**, **4**, and **9**.

204    For reference compounds, the limit of detection (LOD) and the limit of quantification (LOQ) were

205    experimentally determined by HPLC analysis of serial dilutions of a standard solution to reach a

7

206 signal-to-noise ($S/N$) ratio of 3 and 10, respectively.

207 The accuracy of the analytical method was evaluated by means of the recovery test. This involved

208 the addition of a known quantity of standard compound to half the Vicchio sample weight to reach

209 100% of the test concentration. The fortified samples were then extracted and analysed with the

210 proposed method.

211 The precision of the extraction technique was validated by repeating six times the extraction

212 procedure of the same sample (Vicchio). An aliquot of each extract was then injected and

213 quantified. The precision of the chromatographic system was tested by performing intra- and inter-

214 day multiple injections of one extract from sample Vicchio and then checking the %RSD of

215 retention times and peak areas. Six injections were performed each day for three consecutive days.

216

217 *2.7. Determination of the DPPH• radical-scavenging activity*

218

219 This assay was performed according to Molyneux *et al*. [15]. Fifty µL of sample solution were

220 added to 1450 µL of a 0.06 mM DPPH• methanolic solution. The mixture was left to stand for 15

221 min in the dark. The reduction of the DPPH• radical was determined by measuring the absorption at

222 515 nm. The radical scavenging activity was calculated as a percentage of DPPH• discoloration, by

223 using the equation:

224 $$\% \text{ inhibition} = [(A_{T0} - A_{T15})/A_{T0}] \times 100$$

225 where, $A_{T15}$ is the absorbance of the solution after 15 min of incubation and $A_{T0}$ is the absorbance

226 of the DPPH• solution. Trolox was used for the standard calibration curve from 0.05 to 4 mM.

227

228 *2.8. Determination of the antioxidant activity by photochemiluminescence assay*

229

230 The photochemiluminescence (PCL) assay, based on the methodology of Popov *et al*. [16], was

231 used to measure the antioxidant activity of hop young shoot extracts with a Photochem® apparatus

232 (Analytic Jena, Jena, Germany) against superoxide anion radicals generated from luminol.

233 In particular, the antioxidant activity of the extracts was assessed by means of the ACL kit

234 (Analytic Jena, Jena, Germany). For the ACL assay, 2.3 mL of reagent 1 (solvent and dilution

235 reagent, MeOH), 0.2 mL of reagent 2 (buffer solution), 25 µL of reagent 3 (photosensitizer, luminol

236 1 mmol/L) and 10 µL of standard or sample solution were mixed and measured. Luminol is used as

237 a photosensitiser when exposed to UV light at a $\lambda_{max}$ of 351 nm, and as a detecting substance for

238 free radicals. Trolox was used for the standard calibration curve from 0.25 to 2 nM.

239 In the PCL-ACL assay, the photochemical generation of free radicals is combined with a sensitive

240 detection obtained by using chemiluminescence. In ACL studies, the kinetic light emission curve

241 was monitored for 3 min and expressed as mg/g Trolox equivalents. The areas under the curves

242 were calculated by using the PCL soft control and analysis software. Trolox or antioxidants from

243 the samples reduce the magnitude of the PCL signal, and hence, the area calculated from the

244 integral. The observed inhibition of the signal was plotted against the concentration of Trolox added

245 to the assay medium. The concentration of the added sample was such that the generated

246 luminescence during the 3 min sampling interval fell within the limits of the standard curve.

247

248 *2.9. Statistical analysis*

249

250 All statistical analyses were performed by using Excel software Office 2013. Concentration, means

251 and standard deviation data were calculated with Excel. Statistica 6.1 (Statsoft) was used for the

252 analysis of variances (ANOVA) and a Tukey's adjustment for multiple comparisons to test for

253 significant differences between the means. *P*-values under the significance level ($\alpha$) of 0.05 were

254 considered statistically significant.

255

256 **3. Results and discussion**

257

258 3.1. *Method development and identification of flavonols in hop shoot extracts*

259

260 In this study, the identification of flavonols in hop shoot extracts was carried out for the first time

261 on the basis of their UV/Vis spectra, together with MS and MS$^2$ data, which were compared with

262 those of reference standards, when commercially available, and with the literature. The flavonols

263 identified in the methanolic extracts of hop shoots are shown in Table 1.

264 Table 1

265 Since there are no studies focused on the phenolic composition of hop shoots, the characterization

266 of this plant material was an interesting topic to be properly investigated. Firstly, the research was

267 focused on the identification of the characteristic bioactive compounds present in well-known hop

268 cone extracts, including prenylflavonoids and prenylphloroglucinols [10,17,18]. To do this, the

269 extraction and HPLC analysis of hop shoot samples was initially performed under the same

270 conditions previously described in the literature [10]. However, the chromatograms recorded did not

271 reveal the presence of both prenylflavonoids and bitter acids in the hop shoot extracts analysed in

272 this study. This is probably due to the fact that these samples are composed of very young tissues,

273 where the biosynthesis of both prenylflavonoids and bitter acids does not take place. In the light of

274 all the above, the extraction and analytical conditions were completely modified and optimized, in

275 order to have a good recovery of bioactive compounds from hop shoots and a satisfactory

276 separation of the peaks observed in the HPLC chromatograms.

277 The chromatographic peaks were preliminarily assigned to a chemical class according to their

278 UV/Vis spectra. Indeed, all polyphenols have a characteristic UV/Vis spectrum with different $\lambda_{max}$:

279 in particular, flavonoids exhibit a first maximum in the 240-285 nm range (band II) and a second

280 one in the 300-550 nm range (band I) [19]. By combining this information with that obtained from

281 the MS experiments, a preliminary identification was carried out, and then it was confirmed by the

282 HPLC analysis of the reference standards commercially available under the same chromatographic

283 conditions. As regards HPLC-ESI-MS and $MS^2$ analyses, both positive and negative ion modes

284 were applied for the structural characterization of hop shoot constituents [19,20]. On the basis of

285 this approach, a total of 12 flavonol glycosides were identified (Table 1).

286 In particular, quercetin and kaempferol glycosides were firstly distinguished due to their different

287 UV/Vis behavior and $\lambda_{max}$ in the band I range: in fact, quercetin glycosides showed a $\lambda_{max}$ in the

288 355-360 nm range, while kaempferol glycosides in the 348-352 nm range.

289 As regards mass spectrometry, in the $MS^2$ spectra of these compounds recorded in the positive ion

290 mode, the cleavage of the glycosidic bond led to the elimination of the sugar residue, resulting in a

291 strong fragment at $m/z$ 303 and 287, corresponding to the aglycones, quercetin and kaempferol,

292 respectively. In the negative ion mode, most of the identified constituents generated the

293 corresponding aglycone at $m/z$ 300 (homolytic cleavage) and 301 (heterolytic cleavage) for

294 quercetin glycosides and at $m/z$ 284 (homolytic cleavage) and 285 (heterolytic cleavage) for

295 kaempferol glycosides, suggesting that the glycosylation site was located at the 3-position [21,22].

296 As regards the sugar moiety identification, MS methods can be used to obtain information on the

297 carbohydrate type and sequence. Even if glucose is the most common monosaccharide in flavonoid

298 glycosides, galactose along with rhamnose, xylose and arabinose are not uncommon [22].

299 Disaccharides are also often found in glycosylated flavonoids, the more common ones being

300 rutinose (rhamnosyl-(α1→6)-glucose) and neohesperidose (rhamnosyl-(α1→2)-glucose) [22]. The

301 cleavage at the glycosidic O-linkages with a concomitant H-rearrangement leads to the elimination

302 of monosaccharide residues, such as the loss of 162 u (hexose), 146 u (deoxyhexose), 132 u

10

303    (pentose) or 176 u (uronic acid), thus allowing the determination of the carbohydrate sequence [22].

304    In addition, the analysis of the product ion spectra of $[M + Na]^+$ adduct ions can provide additional

305    information on the size and pattern of glycoside substitutions on flavonols [23].

306    The $MS^2$ experiments carried out on the $[M + Na]^+$ adduct ion of compound **1** generated a base peak

307    at *m/z* 477, corresponding to $[rhamnose\text{-}glucose\text{-}rhamnose + Na]^+$, thus indicating the presence of a

308    trisaccharidic moiety [23]. This compound, showing a $[M + H]^+$ ion at *m/z* 757, according to its UV

309    spectrum and $MS^2$ fragmentation in the positive ion mode with the subsequent loss of a rhamnose

310    (*m/z* 611) and a rhamnose-glucose disaccharide unit (*m/z* 303), was identified as quercetin-3-*O*-(2-

311    rhamnosyl)-rutinoside [24]. The presence of the rutinoside moiety was deduced by the absence of

312    the $[M − H − 120]^-$ fragment in the negative ion mode, which is typically due to the presence of a

313    neohesperidoside disaccharide [24]. The fragmentation pattern of this constituent in the negative ion

314    mode was also found to be in agreement with other previous studies [25,26]. By following the same

315    strategy, compound **2** was identified as kaempferol-3-*O*-(2-rhamnosyl)-rutinoside on the basis of

316    the good agreement of its MS and $MS^2$ data with the literature [25,26].

317    Flavonol glycosides having a rhamnosyl-glucose as the sugar moiety, such as compounds **3**, **4** and

318    **7**, showed a base peak at *m/z* 331, corresponding to $[rhamnose\text{-}glucose\text{-} + Na]^+$; this suggested that

319    these compounds possess a disaccharide unit and not two sugars linked at different positions.

320    Compounds **3** and **7** were confirmed as rutinosides by the analysis of reference standards. As

321    regards compound **4**, it was identified as kaempferol-3-*O*-neohesperidoside, due to the product ion

322    $[M − H − 120]^-$ at *m/z* 473 in the negative ion mode, which revealed the presence of a (1→2)

323    interglycosidic linkage between the two monosaccharides [24]. In addition, the two product ions at

324    *m/z* 447 and 285, which correspond to the loss of rhamnose (−146 u) and rhamnosyl-glucose (−308

325    u), respectively, have relative abundances strikingly different, with a 1→2 linkage between the

326    monosaccharides which favours the elimination of the disaccharide residue to yield a deprotonated

327    aglycone ion [27]. Finally, the $MS^2$ data of compound **4** were found to be in good agreement with

328    the literature [25,26,28].

329    The fragmentation pathway observed for the $[M + H]^+$ ions of compounds **8**, **9**, **11** and **12** was based

330    on the release of the malonyl-glucose moiety (− 248 u) [22]. As regards the negative ion mode, the

331    major product ion in the $MS^2$ spectra of these compounds corresponds to $[M − H − CO_2]^-$,

332    originating from the decarboxylation of a malonic acid moiety [23]. The exact location of the

333    malonyl group on the glycosidic part is difficult to be defined on the basis of MS data, but it is

334    known to be predominantly located at the 6-position of a hexose moiety [22]. On the basis of the

335    good agreement of the MS and $MS^2$ data of compounds **8**, **11** and **12** both in the positive [27] and in

336 the negative ion mode [29,30], they were identified as quercetin-3-$O$-(6''-$O$-malonyl)-hexoside (**8**)

337 and kaempferol-3-$O$-(6''-$O$-malonyl)-hexoside (**11** and **12**); indeed, both glucose and galactose are

338 possible as the sugar moiety for these compounds [29]. Compound **9** was identified as kaempferol-

339 3-$O$-(6''-$O$-malonyl)-neohesperidoside, due to the good match of its MS and MS$^2$ data with the

340 literature [28]. In particular, the [M + H]$^+$ ion of **9** was 86 mass units larger than that of compound

341 **4**, indicating that compound **9** contains a malonyl group; this was further supported by the presence

342 of the product ion at $m/z$ 593 in the MS$^2$ spectrum acquired in the negative ion mode of compound

343 **9**, attributable to [M − H − malonyl]$^-$. The specific biosynthetic pathway for this natural compound

344 has been described by Kogawa *et al*. [31], as a malonylated derivative of its precursor kaempferol-

345 3-$O$-neohesperidoside (**4**).

346

347 *3.2. Method validation*

348

349 Over the concentration range tested, the method showed good linearity ($r^2 \geq 0.9995$) for the

350 reference standards chosen in this study.

351 The LOD values had a range from 1.8 to 2.5 µg/mL, while the LOQ range was from 5.3 to 7.8

352 µg/mL, which indicate that the method is sensitive.

353 The accuracy of the analytical procedure was evaluated by using the recovery test. The percentage

354 recovery values, obtained by comparing the results from samples and fortified samples, were found

355 to be higher than 80% and they can be considered satisfactory.

356 The low intra- and inter-day %RSD for retention times ($\leq 0.1$) and peak area ($\leq 3.0$) relative to the

357 target compounds and their low intra- and inter-day SD ($\leq 76$ µg/g) values for content indicate the

358 high precision of both the chromatographic system and the extraction procedure.

359 By taking into account all the information described above, it can be concluded that this method is a

360 reliable tool for the analysis of flavonols in hop shoots, conforming to the ICH guidelines.

361

362 3.3. *Quantitative analysis of flavonols in hop shoot samples*

363

364 Figure 1 shows a representative HPLC-UV/DAD chromatogram of a hop shoot extract obtained

365 with the method developed in this study (sample Vicchio).

366 <center>Figure 1</center>

367 The HPLC-UV/DAD method was applied to the quantitative analysis of flavonol glycosides in hop

368 shoot samples. Quantitative data, expressed as µg/g fresh weight, are shown in Table 2.

369                        Table 2

370    A noteworthy difference in the amount of total flavonols was observed between hop shoots of

371    different origin, with the sample Vicchio being the richest one (2698 ± 185 µg/g) and the sample

372    Castelmassa the poorest (517 ± 48 µg/g). In general, the most abundant compounds found in hop

373    shoot samples were kaempferol derivatives, such as kaempferol-3-*O*-(6"-*O*-malonyl)-glucoside (or

374    galactoside) (770-226 µg/g), kaempferol-3-*O*-glucoside (491-65 µg/g) and kaempferol-3-*O*-(6"-*O*-

375    malonyl)-neohesperidoside (401-54 µg/g).

376    Since the present work is the first one focused on the quali- and quantitative analysis of hop shoot

377    flavonoids, there are no comparative data in the literature. However, a good agreement was found

378    for the total content of flavonoids in hop shoots with the values previously described for edible

379    vernal early shoots from Portugal, including both white (2410 ± 124 µg/g) and black bryony (2010

380    ± 60.4 µg/g) [32]. In the case of young shoots from wild asparagus, the total flavonoid content

381    described in the literature was lower (301 ± 19.2 µg/g) [32].

382

383    *3.4. Antioxidant activity of hop shoot extracts*

384

385    The antioxidant activity of hop young shoot extracts was evaluated with two methods, i.e. by means

386    of the DDPH$^\bullet$ radical scavenging activity (Figure 2) and by the PCL-ACL assay (Figure 3). In both

387    cases, the results are expressed as mg of Trolox equivalents per g of shoots (fresh weight). As

388    shown in Figures 2 and 3, the antioxidant activity plots had the same trend for both methods.

389    However, the data obtained with the DPPH$^\bullet$ test were lower than those provided by the PCL-ACL

390    assay. This can be due to the fact that the DPPH$^\bullet$ radical has a higher molecular weight and steric

391    hindrance if compared with the superoxide anion radical ($O_2^{\bullet-}$), that may preclude the interaction with

392    some substrate molecules present in the extracts.

393                       Figures 2 and 3

394    By focusing on the results obtained with the PCL-ACL test (Figure 3), the Vicchio hop shoot

395    sample had the greatest antioxidant activity (1.07 ± 0.08 mg/g), followed by Santa Maria in Punta

396    and Cologna; Castelmassa presented the lowest antioxidant activity (0.68 ± 0.04 mg/g). It is

397    interesting to note that the antioxidant activity data of the PCL-ACL assay have a good correlation

398    with the total flavonol content, with an $r^2$ value of 0.9577.

399    In general, the phenolic composition and the antioxidant activity of vegetables are known to vary as

400    a function of the cultivar, the environmental conditions (including temperature, humidity and UV

401    irradiation) at the site of collection and the growing stage [33]. As regards the samples analysed in

this work, it should be pointed out that Vicchio came from Tuscany hills located at 203 m a.s.l., while Cologna, Santa Maria in Punta and Castelmassa samples were harvested from different points of the Po river banks. In particular, the Santa Maria in Punta and Cologna samples, collected near the Po river delta, showed a very similar antioxidant activity.

Additional samples collected from different areas are necessary to assess the influence of both abiotic and biotic factors on the bioactive constituents of this plant material and also on the antioxidant activity of the extracts.

## 4. Conclusions

The flavonol composition of hop young shoots was studied in this work for the first time by means of a validated method based on HPLC-UV/DAD, HPLC-ESI-MS and $MS^2$. Quercetin and kaempferol glycosides were found to be the main compounds in the extracts obtained from this plant material. These components were quantified and their content was found to be related to the antioxidant activity of the extracts, determined *in vitro* by means of the DPPH$^\bullet$ and the PCL-ACL assays.

These results highlight the importance of hop shoots as a potential source of flavonols, which can be useful for their role against biological radicals, such as the superoxide radical $O_2^{\bullet-}$. Therefore, hop young shoots should be considered as a new source of bioactive compounds to be used as a functional food or in the nutraceutical field. The difference in the antioxidant activity among samples of different origin suggests an influence of the environmental conditions on the biosynthesis of flavonol compounds.

## References

[1] D. Martins, L. Barros, A.M. Carvalho, I.C.F.R. Ferreira, Nutritional and *in vitro* antioxidant properties of edible wild greens in Iberian Peninsula traditional diet, Food Chem., 125 (2011) 488–494.

[2] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell Biol., 39 (2007) 44–84.

[3] Y.Z. Fang, S. Yang, G. Wu, Free radicals, antioxidants, and nutrition, Nutrition, 18 (2002) 872–879.

[4] R. Shishehgar, A. Rezaie, M. Nazeri, Study of sedation, pre-anensthetic and anti-anxiety effects of hop (*Humulus lupulus*) extract compared with diazepam in rats, J. Anim. Vet. Adv., 11 (2012) 2570–2575.

[5] P. Zanoli, M. Zavatti, Pharmacognostic and pharmacological profile of *Humulus lupulus* L., J. Ethnopharmacol., 116 (2008) 383–396.

[6] J.F. Stevens, J.E. Page, Xanthohumol and related prenylflavonoids from hops and beer: to your good health!, Phytochemistry, 65 (2004) 1317–1330.

[7] F.C. Önder, M. Ay, S.D. Sarker, Comparative study of antioxidant properties and total phenolic content of the extracts of *Humulus lupulus* L. and quantification of bioactive components by LC−MS/MS and GC−MS, J. Agric. Food Chem. 61 (2013) 10498−10506.

[8] V. Jerkovic, D. Callemien, S. Collin, Determination of stilbenes in hop pellets from different cultivars, J. Agric. Food Chem. 53 (2005) 4202−4206.

[9] J.F. Stevens, A.W. Taylora, G.B. Nickersonb, M. Ivancic, J. Henning, A. Haunold, M.L. Deinzera, Prenylfavonoid variation in *Humulus lupulus*: distribution and taxonomic significance of xanthogalenol and 4'-O- methylxanthohumol, Phytochemistry, 53 (2000) 759−775.

[10] F.P. Prencipe, V. Brighenti, M. Rodolfi, A. Mongelli, C. Dall'Asta, T. Ganino, R. Bruni, F. Pellati, Development of a new high-performance liquid chromatographymethod with diode array and electrospray ionization-massspectrometry detection for the metabolite fingerprinting of bioactivecompounds in *Humulus lupulus* L., J. Chromatogr. A, 1349 (2014) 50−59.

[11] P. Garcıa-Herrera, M.C. Sanchez –Mat, M. Camara, J. Tardıob, B. Olmedilla-Alonsoc, Carotenoid content of wild edible young shoots traditionally consumed in Spain (*Asparagus acutifolius* L., *Humulus lupulus* L., *Bryonia dioica* Jacq. and *Tamus communis* L.), J. Sci. Food Agric., 93 (2013) 1692–1698.

[12] M.C. Sanchez-Mata, R.D. Cabrera Loera, P. Morales, V. Fernandez-Ruiz, M. Camara, C. Dıez Marques, M. Pardo-de-Santayana, J. Tardıo, Wild vegetables of the Mediterranean area as valuable sources of bioactive compounds, Genet. Resour. Crop Evol., 59 (2012) 431–443.

[13] P. Morales, A.M. Carvalho, M.C. Sanchez-Mata, M. Camara, M. Molina, I.C.F.R. Ferreira, Tocopherol composition and antioxidant activity of Spanish wild vegetables, Genet. Resour. Crop. Evol., 59 (2012) 851–863.

[14] International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH), Guideline Q2(R1)-Validation of Analytical Procedures: Text and Methodology, ICH Secretariat,c/o IFPMA, Geneva, 2005, pp. 1.

466 [15] Molyneux P, The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating
467 antioxidant activity, Songklanakarin J. Sci. Technol., 26 (2004) 211–219.

468 [16] I. Popov, G. Lewin, Photochemiluminescent detection of antiradical activity. VI. Antioxidant
469 characteristics of human blood plasma, low density lipoprotein, serum albumin and amino acids
470 during in vitro oxidation, Luminescence, 14 (1999) 169–174.

471 [17] P.J. Magalhães, L.F. Guido, J.M. Cruz, A.A. Barros, Analysis of xanthohumol and
472 isoxanyhohumol in different hop products by liquid chromatography-diode array detection-
473 electrospray ionization tandem mass spectrometry, J. Chromatogr. A, 1150 (2007) 295–301.

474 [18] L. Dhooge, T. Naessens, A. Heyerick, D. De Keukeleire, A.J. Vlietinck, L. Pieters, S. Apers,
475 Quantification of xanthohumol, 8-prenylnaringenin and 6-prenylnaringenin in hop extracts and
476 derived capsules using secondary standards, Talanta, 83 (2010) 448–456.

477 [19] E. De Rijke, P. Out, W.M.A. Niessen, F. Ariese, C. Gooijer, U.A.T. Brinkman, Analytical
478 separation and detection methods for flavonoids, J. Chromatogr. A, 1112 (2006) 31–63.

479 [20] X. Qiao, W. Yang, D. Guo, M. Ye, Extraction, separation, detection and structural analysis of
480 flavonoids, Curr. Org. Chem., 15 (2011) 2541–2566.

481 [21] E. Hvattum, D. Ekeberg, Study of the collision-induced radical cleavage of flavonoid
482 glycosides using negative electrospray ionization tandem quadrupole mass spectrometry, J. Mass
483 Spectrom., 38 (2003) 43–49.

484 [22] F. Cuyckens, M. Claeys, Mass spectrometry in the structural analysis of flavonoids, J. Mass
485 Spectrom., 39 (2004) 1–15.

486 [23] P. Kachlicki, J. Einhorn, D. Muth, L. Kerhoas, M. Stobiecki,. Evaluation of glycosylation and
487 malonylation patterns in flavonoid glycosides during LC/MS/MS metabolite profiling, J.  Mass
488 Spectrom., 42 (2008) 572–586.

489 [24] D. Barreca, G. Gattuso, G. Laganà, U. Leuzzi, E. Bellocco, *C*- and *O*-glycosyl flavonoids in
490 Sanguinello and Tarocco blood orange (*Citrus sinensis* (L.) Osbeck) juice: identification and
491 influence on antioxidant properties and acetylcholinesterase activity, Food Chem., 196 (2016) 619–
492 627.

493 [25] W. Tao, N. Yang, J.-a. Duan, D. Wu, J.M. Guo, Y. Tang, D. Qian, Z. Zhu, Simultaneous
494 determination of eleven major flavonoids in the pollen of *Typha angustifolia* by HPLC-PDA-MS,
495 Phytochem. Anal., Phytochem. Anal., 22 (2011) 455–461.

497 [26] Y. Chen, H. Yu, H. Wu, Y. Pan, K. Wang, Y. Jin, C. Zhang, Characterization and
498 quantification by LC-MS/MS of the chemical components of the heating products of the flavonoids
499 extract in Pollen Typhae for transformation rule exploration, Molecules, 20 (2015) 18352–18366.

[27] C. Carazzone, D. Mascherpa, G. Gazzani, A. Papetti, Identification of phenolic constituentsin red chicory salad (*Cichorium intybus*) by high-performance liquid chromatography with diode array and electrospray ionization tandem mass spectrometry, Food Chem., 138 (2013) 1062–1071.

[28] K. Kazuma, N. Noda, M. Suzuki, Malonylated flavonol glycosides from the petals of *Clitoria ternatea*, Phytochemistry, 62 (2003) 229–237.

[29] H. An, H. Wang, Y. Lan, Y. Hashi, S. Chen, Simultaneous qualitative and quantitative analysis of phenolic acids and flavonoids for the quality control of *Apocynum venetum* L. leaves by HPLC–DAD-ESI-IT-TOF-MS and HPLC-DAD, J. Pharm. Biomed. Anal., 85 (2013) 295–304.

[30] M.N. Vieira, P. Winterhalter, G. Jerz, Flavonoids from the flowers of *Impatiens glandulifera* Royle isolated by high performance countercurrent chromatography, Phytochem. Anal., 27 (2016) 116–125.

[31] K. Kogawa, K. Kazuma, N. Kato, N. Noda, M. Suzuki, Biosynthesis of malonylated flavonoid glycosides on the basis of malonyltransferase activity in the petals of *Clitoria ternatea*, J. Plant Physiol., 164 (2007) 886–894.

[32] L. Barros, M. Dueñas, I.C.F.R. Ferreira, A.M. Carvalho, C. Santos-Buelga, Use of HPLC–DAD–ESI/MS to profile phenolic compounds in edible wild greens from Portugal, Food Chem., 127 (2011) 169–173.

[32] R. Ksouri, W. Megdiche, A. Debez, H. Falleh, C. Grignon, C. Abdelly, Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte Cakile maritime, Plant Physiol. Biochem., 45 (2007) 244–249.

**Figure captions**

**Figure 1**: Representative chromatogram obtained by HPLC-UV/DAD of an extract of hop shoots (sample Vicchio) at 352 nm.

17

530    **Figure 2**: DPPH$^{\bullet}$ radical scavenging activity of hop young shoot extracts of different origin. Data

531    are expressed as mg of Trolox equivalents for g of shoots (mean $\pm$ SD, $n = 6$). Mean values marked

532    with the same letter are not significantly different from each other ($p > 0.05$).

533

534    **Figure 3**: Antioxidant activity by PCL-ACL assay of hop young shoot extracts of different origin.

535    Data are expressed as mg of Trolox equivalents for g of shoots (mean $\pm$ SD, $n = 6$). Mean values

536    marked with the same letter are not significantly different from each other ($p > 0.05$).

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**Table 1**
Flavonols identified in extracts of hop shoots by HPLC-UV/DAD, HPLC-ESI-MS and MS[2].

| Peak number | Compound | $t_R$ (min) | UV $\lambda_{max}$ (nm) | MS ($m/z$) | MS[2] ($m/z$) |
|---|---|---|---|---|---|
| 1 | Quercetin-3-*O*-(2-rhamnosyl)-rutinoside[a] | 14.5 | 257, 355 | 757 [M + H]⁺ | 611 (76), 465 (36), 449 (24), 303 (100) |
| | | | | 779 [M + Na]⁺ | 633 (9), 477 (100), 331 (6) |
| | | | | 755 [M – H]⁻ | 609 (96), 591 (100), 581 (33), 573 (57), 489 (30), 445 (28), 301 (47), 300 (69) |
| 2 | Kaempferol-3-*O*-(2-rhamnosyl)-rutinoside[a] | 16.7 | 266, 348 | 741 [M + H]⁺ | 595 (52), 449 (26), 433 (19), 287 (100) |
| | | | | 763 [M + Na]⁺ | 617 (6), 477 (100), 331 (10) |
| | | | | 739 [M – H]⁻ | 593 (20), 575 (100), 393 (34), 285 (22), 284 (18) |
| 3 | Quercetin-3-*O*-rutinoside[b] | 18.5 | 256, 356 | 611 [M + H]⁺ | 465 (26), 303 (100) |
| | | | | 633 [M + Na]⁺ | 487 (74), 459 (20), 331 (100) |
| | | | | 609 [M – H]⁻ | 301 (100), 300 (30) |
| 4 | Kaempferol-3-*O*-neohesperidoside[a] | 19.1 | 266, 349 | 595 [M + H]⁺ | 449 (33), 287 (100) |
| | | | | 617 [M + Na]⁺ | 471 (38), 331 (100) |
| | | | | 593 [M – H]⁻ | 473 (18), 447 (15), 429 (26), 327 (15), 285 (54), 284 (100), 255 (35) |
| 5 | Quercetin-3-*O*-galactoside[b] | 19.5 | 256, 360 | 465 [M + H]⁺ | 303 (100) |
| | | | | 487 [M + Na]⁺ | 325 (100), 185 (54) |
| | | | | 463 [M – H]⁻ | 301 (100), 300 (66) |
| 6 | Quercetin-3-*O*-glucoside[b] | 20.0 | 255, 359 | 465 [M + H]⁺ | 303 (100) |
| | | | | 487 [M + Na]⁺ | 325 (100), 185 (28) |
| | | | | 463 [M – H]⁻ | 301 (100), 300 (60) |
| 7 | Kaempferol-3-*O*-rutinoside[b] | 21.7 | 265, 348 | 595 [M + H]⁺ | 449 (24), 287 (100) |
| | | | | 617 [M + Na]⁺ | 471 (67), 331 (100) |
| | | | | 593 [M – H]⁻ | 285 (100), 284 (7) |
| 8 | Quercetin-3-*O*-(6"-*O*-malonyl)-glucoside or galactoside[a] | 22.2 | - | 551 [M + H]⁺ | 303 (100) |
| | | | | 573 [M + Na]⁺ | 529 (100), 487 (13), 325 (28) |
| | | | | 549 [M – H]⁻ | 505 (100) |
| | | | | 505 [M – H – CO$_2$]⁻ | 463 (28), 301 (100), 300 (60) |
| 9 | Kaempferol-3-*O*-(6"-*O*-malonyl)-neohesperidoside[a] | 22.4 | 266, 350 | 681 [M + H]⁺ | 535 (100), 287 (43) |
| | | | | 703 [M + Na]⁺ | 659 (100), 617 (16), 513 (24), 417 (24), 373 (30) |
| | | | | 679 [M – H]⁻ | 635 (100) |
| | | | | 635 [M – H – CO$_2$]⁻ | 593 (64), 575 (33), 284 (100) |
| 10 | Kaempferol-3-*O*-glucoside[b] | 23.4 | 266, 348 | 449 [M + H]⁺ | 287 (100) |
| | | | | 471 [M + Na]⁺ | 309 (100), 185 (37) |

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| | | | | | |
|---|---|---|---|---|---|
| | | | | 447 [M − H]$^-$ | 285 (100), 284 (58) |
| **11** | Kaempferol-3-$O$-(6''-$O$-malonyl)-glucoside or galactoside[a] | 26.7 | 265, 348 | 535 [M + H]$^+$ | 287 (100) |
| | | | | 557 [M + Na]$^+$ | 513 (100), 471 (25), 309 (23) |
| | | | | 533 [M − H]$^-$ | 489 (100) |
| | | | | 489 [M − H − CO$_2$]$^-$ | 285 (100), 284 (7) |
| **12** | Kaempferol-3-$O$-(6''-$O$-malonyl)-glucoside or galactoside[a] | 27.8 | 266, 352 | 535 [M + H]$^+$ | 287 (100) |
| | | | | 557 [M + Na]$^+$ | 513 (100) |
| | | | | 533 [M − H]$^-$ | 489 (100) |
| | | | | 489 [M − H− CO$_2$]$^-$ | 285 (100), 284 (26) |

Experimental conditions as in Sections 2.4 and 2.5.

[a] Identified on the basis of MS and MS$^2$ data in comparison with the literature.
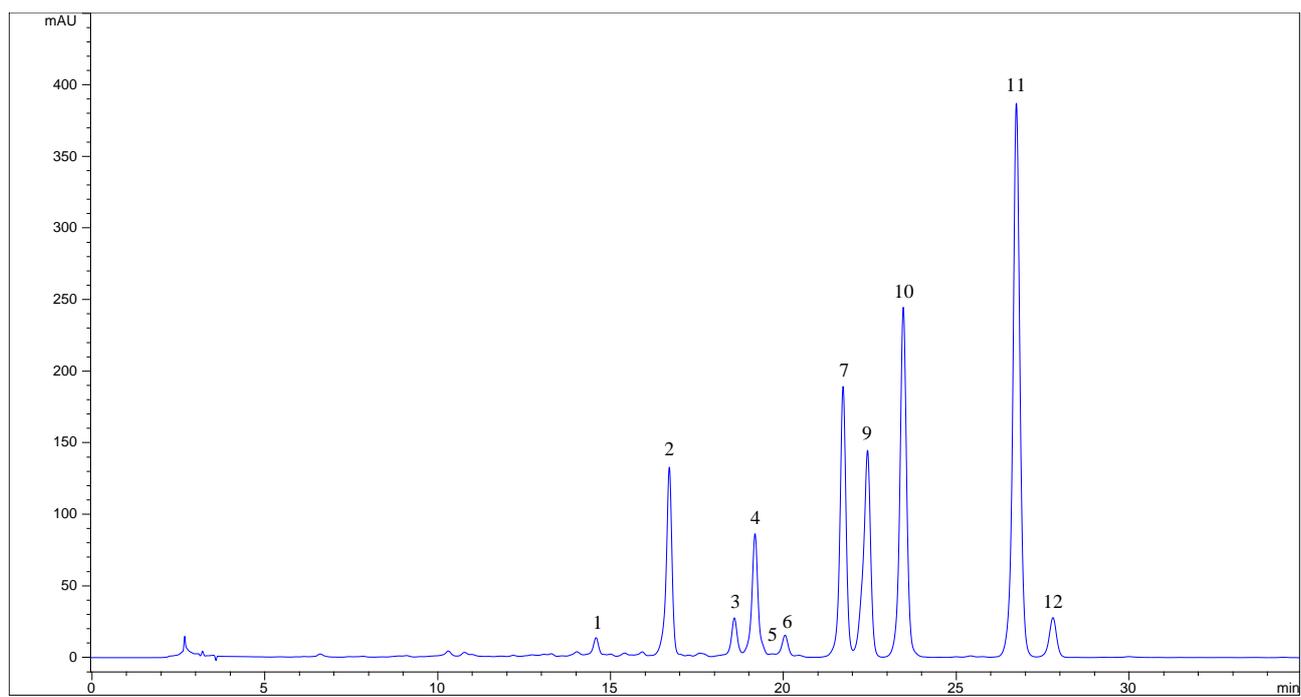
[b] Confirmed by using a reference standard .

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**Table 2**

Content of flavonol compounds in hop shoot extracts of different origin by HPLC-UV/DAD analysis (data are expressed as µg/g, fresh weight).

| Compound | Vicchio | Castelmassa | Cologna | Santa Maria in Punta |
|---|---|---|---|---|
| Quercetin-3-*O*-(2-rhamnosyl)-rutinoside (**1**) | 37.2 ± 2.4 | <LOD | <LOQ | 21.3 ± 3.6 |
| Kaempferol-3-*O*-(2-rhamnosyl)-rutinoside (**2**) | 311.9 ± 30.0 | 41.1 ± 3.2 | 96.1 ± 9.9 | 147.1 ± 16.9 |
| Quercetin-3-*O*-rutinoside (**3**) | 63.2 ± 7.3 | <LOQ | 18.0 ± 2.2 | 27.3 ± 4.8 |
| Kaempferol-3-*O*-neohesperidoside (**4**) | 161.2 ± 15.7 | 14.9 ± 1.2 | 36.7 ± 1.2 | 55.1 ± 7.3 |
| Quercetin-3-*O*-galactoside (**5**) | 10.7 ± 1.9 | <LOD | <LOQ | <LOQ |
| Quercetin-3-*O*-glucoside (**6**) | 22.0 ± 2.0 | <LOQ | <LOQ | <LOQ |
| Kaempferol-3-*O*-rutinoside (**7**) | 369.7 ± 31.8 | 92.4 ± 7.4 | 101.7 ± 9.3 | 145.2 ± 10.1 |
| Kaempferol-3-*O*-(6"-*O*-malonyl)-neohesperidoside (**9**) | 401.4 ± 31.7 | 53.7 ± 4.2 | 117.3 ± 19.1 | 146.4.4 ± 20.4 |
| Kaempferol-3-*O*-glucoside (**10**) | 490.7 ± 41.1 | 65.2 ± 9.8 | 110.9 ± 36.9 | 88.9 ± 1.8 |
| Kaempferol-3-*O*-(6"-*O*-malonyl)-glucoside or galactoside (**11**) | 769.5 ± 75.9 | 231 ± 18.5 | 226.0 ± 32.0 | 232.3 ± 25.5 |
| Kaempferol-3-*O*-(6"-*O*-malonyl)-glucoside or galactoside (**12**) | 60.6 ± 5.2 | 19.0 ± 1.3 | 19.3 ± 2.6 | 20.0 ± 2.2 |
| | | | | |
| Total flavonols | 2698 ± 185[a] | 517 ± 48[c] | 726 ± 137[b] | 884 ± 113[b] |

Experimental conditions as in Section 2.4.

Data are expressed as mean ($n = 6$) ± SD.

Mean values marked with the same letter are not significantly different from each other ($p > 0.05$).
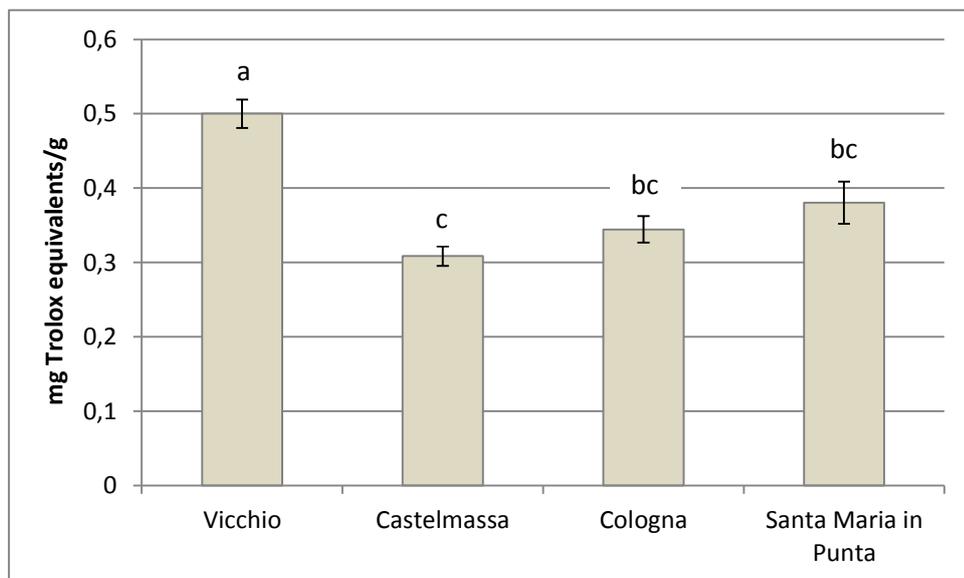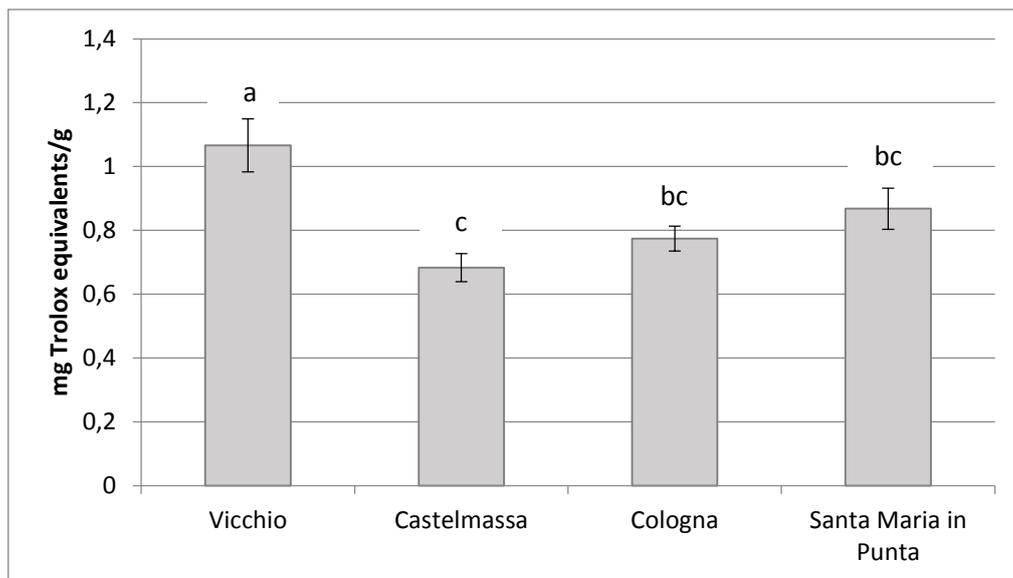
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**Figure 1**

**Figure 2**

**Figure 3**