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

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PII: S0731-7085(17)30475-2
DOI: http://dx.doi.org/doi:10.1016/j.jpba.2017.04.043
Reference: PBA 11241

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

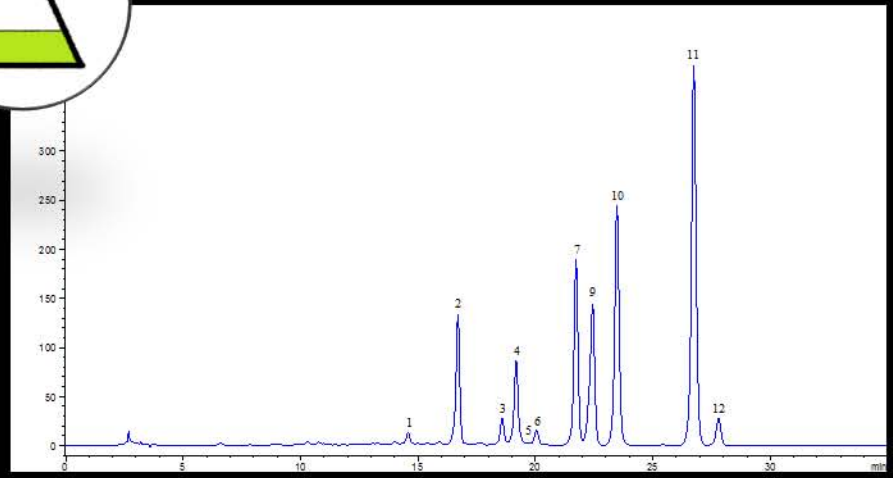
Received date: 24-2-2017
Revised date: 21-4-2017
Accepted date: 24-4-2017

Please cite this article as: Annalisa Maietti, Virginia Brighenti, Gianpiero Bonetti, Paola Tedeschi, Francesco Pio Prencipe, Stefania Benvenuti, Vincenzo Brandolini, Federica Pellati, Metabolite profiling of flavonols and in vitro antioxidant activity of young shoots of wild Humulus lupulus L.(hop), Journal of Pharmaceutical and Biomedical Analysishttp://dx.doi.org/10.1016/j.jpba.2017.04.043

Highlights

- A new HPLC method for the analysis of flavonols in young hop shoots was developed
- Quercetin and kaempferol glycosides were the main phenolics in hop shoots
- The content of flavonols is related to the *in vitro* antioxidant activity
- Hop shoots represent a new source of bioactive antioxidant compounds

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)

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

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® 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

consumed boiled as wild asparagus or to cook risotto and omelet. In Italy, the harvest of hop young shoots is typical in the North and Central regions and it is made on the banks of rivers or in hill areas, where there is a high humidity. As regards the composition, only few studies have been carried out on hop young shoots for their content of carotenoids [11], organic acids [12] and tocopherol [13].

In the light of all the above, the aim of this study was to identify for the first time the flavonoid composition of hop young shoots by means of a new method based on HPLC-UV/DAD, HPLC-ESI-MS and MS^2 and to compare these data with the antioxidant activity of the plant material harvested from four different locations in Northern Italy.

2. Materials and methods

2.1. Chemicals and solvents

Quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside were purchased from Extrasynthese (Genay, France). Kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) were from Sigma-Aldrich (Milan, Italy). All reference compounds were of chromatographic grade. Formic acid (HCOOH), HPLC-grade methanol (MeOH) and acetonitrile (ACN) were from Sigma-Aldrich (Milan, Italy). Water (H_2O) was purified by using a Milli-Q Plus185 system from Millipore (Milford, MA, USA).

2.2. Plant material

Hop young shoots were hand-picked in April-May 2014 in three different locations of the Po river banks, including Castelmassa (Rovigo), Cologna (Rovigo) and Santa Maria in Punta (Ferrara), and in a hill area of Tuscany, named Vicchio (Florence). Each sample was packed in a plastic bag, frozen at −20 °C on the same day and preserved until analysis.

2.3. Extraction of secondary metabolites from hop shoots

Dynamic maceration was chosen for the extraction of flavonoids present in hop shoots and MeOH was used as the extraction solvent. In particular, the extraction procedure was performed on 2.0 g of fresh sample with 10 mL of solvent at room temperature for 30 min under magnetic stirring. The

mixture obtained from the extraction was then centrifuged at 4000 rpm for 5 min and the supernatant solution was filtered under vacuum into a volumetric flask. The residue of the first extraction was re-extracted as previously described. Finally, the filtrates of the two extractions were combined and brought to 25 mL in a volumetric flask. An aliquot of 5 mL of the extract was concentrated under vacuum at 35 °C and then brought to the final volume of 1 mL with MeOH in a volumetric flask. The concentrated extract was subsequently filtered by using a 0.45 µm PTFE filter into a HPLC vial prior to the injection into the HPLC system.

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

2.4 .HPLC-UV/DAD analysis

HPLC-UV/DAD analyses were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1100 system, consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment and a diode array detector (DAD). Chromatograms were recorded by using an Agilent Chemstation for LC and LC-MS systems (Rev. B.01.03).

The HPLC analyses were carried out on an Ascentis C_{18} column (250 × 4.6 mm I.D., 5 µm, Supelco, Bellefonte, PA, USA). The mobile phase was composed of (A) 0.1 M HCOOH in H_2O and (B) ACN. The gradient elution was modified as follows: 0-15 min from 10% to 20% B, 15-35 min from 20% to 30% B, 35-40 min from 30% to 40% B, 40-45 min from 40% to 50% B. The post-running time was 5 min. The flow rate was 1.0 mL/min. The column temperature was set at 25 °C. The sample injection volume was 10 µL. The UV/DAD acquisitions were carried out in the range 190-550 nm and chromatograms were integrated at 352 nm. Three injections were performed for each sample.

2.5. HPLC-ESI-MS and MS^2 analysis

HPLC-ESI-MS and MS^2 analyses were carried out by using an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a thermostatted column compartment and a 6310A ion trap mass analyzer with an ESI ion source. The HPLC column and the applied chromatographic conditions were the same as reported for the HPLC-UV/DAD system. The flow rate was split 5:1 before the ESI source.

The HPLC-ESI-MS system was operated both in the positive and in the negative ion modes. For the positive ion mode, the experimental parameters were set as follows: the capillary voltage was 3.5

kV, the nebulizer (N_2) pressure was 32 psi, the drying gas temperature was 350 °C, the drying gas flow was 10 L/min and the skimmer voltage was 40 V. For the negative ion mode, the conditions were set as follows: the capillary voltage was 4.0 kV, the nebulizer (N_2) pressure was 35 psi, the drying gas temperature was 350 °C, the drying gas flow was 11 L/min and the skimmer voltage was 40 V.

Data were acquired by Agilent 6300 Series Ion Trap LC/MS system software (version 6.2). The mass spectrometer was operated in the full-scan mode in the *m/z* range 100-1000. MS^2 spectra were automatically performed with helium as the collision gas in the *m/z* range 50-1000 with the SmartFrag function.

2.6. HPLC-UV/DAD method validation

The validation of the HPLC-UV/DAD method was performed in agreement with the international guidelines for analytical techniques for the quality control of pharmaceuticals (ICH guidelines) [14].

The stock standard solution of each compound (quercetin-3-*O*-galactoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside) was prepared as follows: an accurately weighed amount of pure compound (2.1-5.0 mg) was placed into a 10 mL volumetric flask; then, MeOH was added and the solution was diluted to volume with the same solvent. The external standard calibration curve was generated by using six data points, covering the concentration ranges: 7.8-313.0 µg/mL for quercetin-3-*O*-rutinoside (**3**); 5.3-213.0 µg/mL for quercetin-3-*O*-galactoside (**5**); 7.8-312.0 µg/mL for kaempferol-3-*O*-rutinoside (**7**); 6.3-505.0 µg/mL for kaempferol-3-*O*-glucoside (**10**). Ten µL aliquots of each standard solution were used for HPLC analysis. Injections were performed in triplicate for each concentration level. The calibration curve was obtained by plotting the peak area of the compound at each level versus the concentration of the sample. The quantification of compounds **3**, **5**, **7** and **10** was performed by using their calibration curves. The amount of the other flavonols found in hop shoots was determined by using the calibration curves of the reference compounds with the same chromophore. In particular, the calibration curve of quercetin-3-*O*-rutinoside was used for compound **1**, that of quercetin-3-*O*-galactoside for compound **6**, that of kaempferol-3-*O*-glucoside for compounds **11** and **12** and that of kaempferol-3-*O*-rutinoside for compounds **2**, **4**, and **9**.

For reference compounds, the limit of detection (LOD) and the limit of quantification (LOQ) were experimentally determined by HPLC analysis of serial dilutions of a standard solution to reach a

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

The accuracy of the analytical method was evaluated by means of the recovery test. This involved the addition of a known quantity of standard compound to half the Vicchio sample weight to reach 100% of the test concentration. The fortified samples were then extracted and analysed with the proposed method.

The precision of the extraction technique was validated by repeating six times the extraction procedure of the same sample (Vicchio). An aliquot of each extract was then injected and quantified. The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of one extract from sample Vicchio and then checking the %RSD of retention times and peak areas. Six injections were performed each day for three consecutive days.

2.7. Determination of the DPPH$^{\bullet}$ radical-scavenging activity

This assay was performed according to Molyneux *et al*. [15]. Fifty μL of sample solution were added to 1450 μL of a 0.06 mM DPPH$^{\bullet}$ methanolic solution. The mixture was left to stand for 15 min in the dark. The reduction of the DPPH$^{\bullet}$ radical was determined by measuring the absorption at 515 nm. The radical scavenging activity was calculated as a percentage of DPPH$^{\bullet}$ discoloration, by using the equation:

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

where, A_{T15} is the absorbance of the solution after 15 min of incubation and A_{T0} is the absorbance of the DPPH$^{\bullet}$ solution. Trolox was used for the standard calibration curve from 0.05 to 4 mM.

2.8. Determination of the antioxidant activity by photochemiluminescence assay

The photochemiluminescence (PCL) assay, based on the methodology of Popov *et al*. [16], was used to measure the antioxidant activity of hop young shoot extracts with a Photochem® apparatus (Analytic Jena, Jena, Germany) against superoxide anion radicals generated from luminol.

In particular, the antioxidant activity of the extracts was assessed by means of the ACL kit (Analytic Jena, Jena, Germany). For the ACL assay, 2.3 mL of reagent 1 (solvent and dilution reagent, MeOH), 0.2 mL of reagent 2 (buffer solution), 25 μL of reagent 3 (photosensitizer, luminol 1 mmol/L) and 10 μL of standard or sample solution were mixed and measured. Luminol is used as

a photosensitiser when exposed to UV light at a λ_{max} of 351 nm, and as a detecting substance for free radicals. Trolox was used for the standard calibration curve from 0.25 to 2 nM.

In the PCL-ACL assay, the photochemical generation of free radicals is combined with a sensitive detection obtained by using chemiluminescence. In ACL studies, the kinetic light emission curve was monitored for 3 min and expressed as mg/g Trolox equivalents. The areas under the curves were calculated by using the PCL soft control and analysis software. Trolox or antioxidants from the samples reduce the magnitude of the PCL signal, and hence, the area calculated from the integral. The observed inhibition of the signal was plotted against the concentration of Trolox added to the assay medium. The concentration of the added sample was such that the generated luminescence during the 3 min sampling interval fell within the limits of the standard curve.

2.9. Statistical analysis

All statistical analyses were performed by using Excel software Office 2013. Concentration, means and standard deviation data were calculated with Excel. Statistica 6.1 (Statsoft) was used for the analysis of variances (ANOVA) and a Tukey's adjustment for multiple comparisons to test for significant differences between the means. *P*-values under the significance level (α) of 0.05 were considered statistically significant.

3. Results and discussion

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

In this study, the identification of flavonols in hop shoot extracts was carried out for the first time on the basis of their UV/Vis spectra, together with MS and MS^2 data, which were compared with those of reference standards, when commercially available, and with the literature. The flavonols identified in the methanolic extracts of hop shoots are shown in Table 1.

Table 1

Since there are no studies focused on the phenolic composition of hop shoots, the characterization of this plant material was an interesting topic to be properly investigated. Firstly, the research was focused on the identification of the characteristic bioactive compounds present in well-known hop cone extracts, including prenylflavonoids and prenylphloroglucinols [10,17,18]. To do this, the extraction and HPLC analysis of hop shoot samples was initially performed under the same

conditions previously described in the literature [10]. However, the chromatograms recorded did not reveal the presence of both prenylflavonoids and bitter acids in the hop shoot extracts analysed in this study. This is probably due to the fact that these samples are composed of very young tissues, where the biosynthesis of both prenylflavonoids and bitter acids does not take place. In the light of all the above, the extraction and analytical conditions were completely modified and optimized, in order to have a good recovery of bioactive compounds from hop shoots and a satisfactory separation of the peaks observed in the HPLC chromatograms.

The chromatographic peaks were preliminarily assigned to a chemical class according to their UV/Vis spectra. Indeed, all polyphenols have a characteristic UV/Vis spectrum with different λ_{max}: in particular, flavonoids exhibit a first maximum in the 240-285 nm range (band II) and a second one in the 300-550 nm range (band I) [19]. By combining this information with that obtained from the MS experiments, a preliminary identification was carried out, and then it was confirmed by the HPLC analysis of the reference standards commercially available under the same chromatographic conditions. As regards HPLC-ESI-MS and MS^2 analyses, both positive and negative ion modes were applied for the structural characterization of hop shoot constituents [19,20]. On the basis of this approach, a total of 12 flavonol glycosides were identified (Table 1).

In particular, quercetin and kaempferol glycosides were firstly distinguished due to their different UV/Vis behavior and λ_{max} in the band I range: in fact, quercetin glycosides showed a λ_{max} in the 355-360 nm range, while kaempferol glycosides in the 348-352 nm range.

As regards mass spectrometry, in the MS^2 spectra of these compounds recorded in the positive ion mode, the cleavage of the glycosidic bond led to the elimination of the sugar residue, resulting in a strong fragment at *m/z* 303 and 287, corresponding to the aglycones, quercetin and kaempferol, respectively. In the negative ion mode, most of the identified constituents generated the corresponding aglycone at *m/z* 300 (homolytic cleavage) and 301 (heterolytic cleavage) for quercetin glycosides and at *m/z* 284 (homolytic cleavage) and 285 (heterolytic cleavage) for kaempferol glycosides, suggesting that the glycosylation site was located at the 3-position [21,22].

As regards the sugar moiety identification, MS methods can be used to obtain information on the carbohydrate type and sequence. Even if glucose is the most common monosaccharide in flavonoid glycosides, galactose along with rhamnose, xylose and arabinose are not uncommon [22]. Disaccharides are also often found in glycosylated flavonoids, the more common ones being rutinose (rhamnosyl-(α1→6)-glucose) and neohesperidose (rhamnosyl-(α1→2)-glucose) [22]. The cleavage at the glycosidic *O*-linkages with a concomitant H-rearrangement leads to the elimination of monosaccaride residues, such as the loss of 162 u (hexose), 146 u (deoxyhexose), 132 u

(pentose) or 176 u (uronic acid), thus allowing the determination of the carbohydrate sequence [22]. In addition, the analysis of the product ion spectra of $[M + Na]^+$ adduct ions can provide additional information on the size and pattern of glycoside substitutions on flavonols [23].

The MS^2 experiments carried out on the $[M + Na]^+$ adduct ion of compound **1** generated a base peak at *m*/*z* 477, corresponding to [rhamnose-glucose-rhamnose+Na]$^+$, thus indicating the presence of a trisaccharidic moiety [23]. This compound, showing a $[M + H]^+$ ion at *m/z* 757, according to its UV spectrum and MS^2 fragmentation in the positive ion mode with the subsequent loss of a rhamnose (*m*/*z* 611) and a rhamnose-glucose disaccharide unit (*m*/*z* 303), was identified as quercetin-3-*O*-(2-rhamnosyl)-rutinoside [24]. The presence of the rutinoside moiety was deduced by the absence of the $[M - H - 120]^-$ fragment in the negative ion mode, which is typically due to the presence of a neohesperidoside disaccharide [24]. The fragmentation pattern of this constituent in the negative ion mode was also found to be in agreement with other previous studies [25,26]. By following the same strategy, compound **2** was identified as kaempferol-3-*O*-(2-rhamnosyl)-rutinoside on the basis of the good agreement of its MS and MS^2 data with the literature [25,26].

Flavonol glycosides having a rhamnosyl-glucose as the sugar moiety, such as compounds **3**, **4** and **7**, showed a base peak at *m/z* 331, corresponding to [rhamnose-glucose-+Na]$^+$; this suggested that these compounds possess a disaccharide unit and not two sugars linked at different positions. Compounds **3** and **7** were confirmed as rutinosides by the analysis of reference standards. As regards compound **4**, it was identified as kaempferol-3-*O*-neohesperidoside, due to the product ion $[M - H - 120]^-$ at *m*/*z* 473 in the negative ion mode, which revealed the presence of a (1→2) interglycosidic linkage between the two monosaccharides [24]. In addition, the two product ions at *m*/*z* 447 and 285, which correspond to the loss of rhamnose (−146 u) and rhamnosyl-glucose (−308 u), respectively, have relative abundances strikingly different, with a 1→2 linkage between the monosaccharides which favours the elimination of the disaccharide residue to yield a deprotonated aglycone ion [27]. Finally, the MS^2 data of compound **4** were found to be in good agreement with the literature [25,26,28].

The fragmentation pathway observed for the $[M + H]^+$ ions of compounds **8**, **9**, **11** and **12** was based on the release of the malonyl-glucose moiety (– 248 u) [22]. As regards the negative ion mode, the major product ion in the MS^2 spectra of these compounds corresponds to $[M - H - CO_2]^-$, originating from the decarboxylation of a malonic acid moiety [23]. The exact location of the malonyl group on the glycosidic part is difficult to be defined on the basis of MS data, but it is known to be predominantly located at the 6-position of a hexose moiety [22]. On the basis of the good agreement of the MS and MS^2 data of compounds **8**, **11** and **12** both in the positive [27] and in

the negative ion mode [29,30], they were identified as quercetin-3-*O*-(6"-*O*-malonyl)-hexoside (**8**) and kaempferol-3-*O*-(6"-*O*-malonyl)-hexoside (**11** and **12**); indeed, both glucose and galactose are possible as the sugar moiety for these compounds [29]. Compound **9** was identified as kaempferol-3-*O*-(6"-*O*-malonyl)-neohesperidoside, due to the good match of its MS and MS^2 data with the literature [28]. In particular, the $[M + H]^+$ ion of **9** was 86 mass units larger than that of compound **4**, indicating that compound **9** contains a malonyl group; this was further supported by the presence of the product ion at *m*/*z* 593 in the MS^2 spectrum acquired in the negative ion mode of compound **9**, attributable to $[M – H – malonyl]^-$. The specific biosynthetic pathway for this natural compound has been described by Kogawa *et al*. [31], as a malonylated derivative of its precursor kaempferol-3-*O*-neohesperidoside (**4**).

3.2. Method validation

Over the concentration range tested, the method showed good linearity ($r^2 \geq 0.9995$) for the reference standards chosen in this study.

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

The accuracy of the analytical procedure was evaluated by using the recovery test. The percentage recovery values, obtained by comparing the results from samples and fortified samples, were found to be higher than 80% and they can be considered satisfactory.

The low intra- and inter-day %RSD for retention times (≤ 0.1) and peak area (≤ 3.0) relative to the target compounds and their low intra- and inter-day SD (≤ 76 µg/g) values for content indicate the high precision of both the chromatographic system and the extraction procedure.

By taking into account all the information described above, it can be concluded that this method is a reliable tool for the analysis of flavonols in hop shoots, conforming to the ICH guidelines.

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

Figure 1 shows a representative HPLC-UV/DAD chromatogram of a hop shoot extract obtained with the method developed in this study (sample Vicchio).

Figure 1

The HPLC-UV/DAD method was applied to the quantitative analysis of flavonol glycosides in hop shoot samples. Quantitative data, expressed as µg/g fresh weight, are shown in Table 2.

Table 2

A noteworthy difference in the amount of total flavonols was observed between hop shoots of different origin, with the sample Vicchio being the richest one (2698 ± 185 µg/g) and the sample Castelmassa the poorest (517 ± 48 µg/g). In general, the most abundant compounds found in hop shoot samples were kaempferol derivatives, such as kaempferol-3-*O*-(6"-*O*-malonyl)-glucoside (or galactoside) (770-226 µg/g), kaempferol-3-*O*-glucoside (491-65 µg/g) and kaempferol-3-*O*-(6"-*O*-malonyl)-neohesperidoside (401-54 µg/g).

Since the present work is the first one focused on the quali- and quantitative analysis of hop shoot flavonoids, there are no comparative data in the literature. However, a good agreement was found for the total content of flavonoids in hop shoots with the values previously described for edible vernal early shoots from Portugal, including both white (2410 ± 124 µg/g) and black bryony (2010 ± 60.4 µg/g) [32]. In the case of young shoots from wild asparagus, the total flavonoid content described in the literature was lower (301 ± 19.2 µg/g) [32].

3.4. Antioxidant activity of hop shoot extracts

The antioxidant activity of hop young shoot extracts was evaluated with two methods, i.e. by means of the $DDPH^{\bullet}$ radical scavenging activity (Figure 2) and by the PCL-ACL assay (Figure 3). In both cases, the results are expressed as mg of Trolox equivalents per g of shoots (fresh weight). As shown in Figures 2 and 3, the antioxidant activity plots had the same trend for both methods. However, the data obtained with the $DPPH^{\bullet}$ test were lower than those provided by the PCL-ACL assay. This can be due to the fact that the $DPPH^{\bullet}$ radical has a higher molecular weight and steric hindrance if compared with the superoxide anion radical ($O_2^{\bullet-}$), that may preclude the interaction with some substrate molecules present in the extracts.

Figures 2 and 3

By focusing on the results obtained with the PCL-ACL test (Figure 3), the Vicchio hop shoot sample had the greatest antioxidant activity (1.07 ± 0.08 mg/g), followed by Santa Maria in Punta and Cologna; Castelmassa presented the lowest antioxidant activity (0.68 ± 0.04 mg/g). It is interesting to note that the antioxidant activity data of the PCL-ACL assay have a good correlation with the total flavonol content, with an r^2 value of 0.9577.

In general, the phenolic composition and the antioxidant activity of vegetables are known to vary as a function of the cultivar, the environmental conditions (including temperature, humidity and UV 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.

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

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

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

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

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

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

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

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

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

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

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

[26] Y. Chen, H. Yu, H. Wu, Y. Pan, K. Wang, Y. Jin, C. Zhang, Characterization and quantification by LC-MS/MS of the chemical components of the heating products of the flavonoids 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.

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 ± 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 ± SD, n = 6). Mean values
536 marked with the same letter are not significantly different from each other ($p > 0.05$).
537

539

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 λ_{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)

				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 .

543

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).

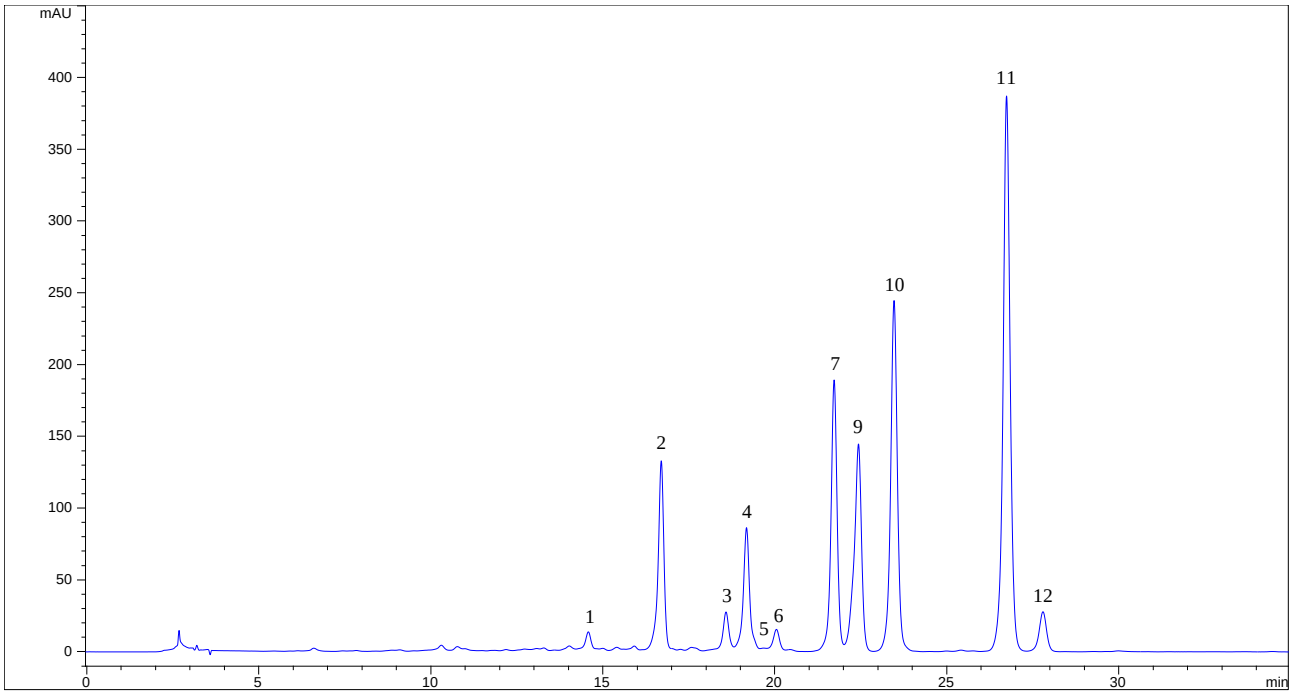

Figure 1

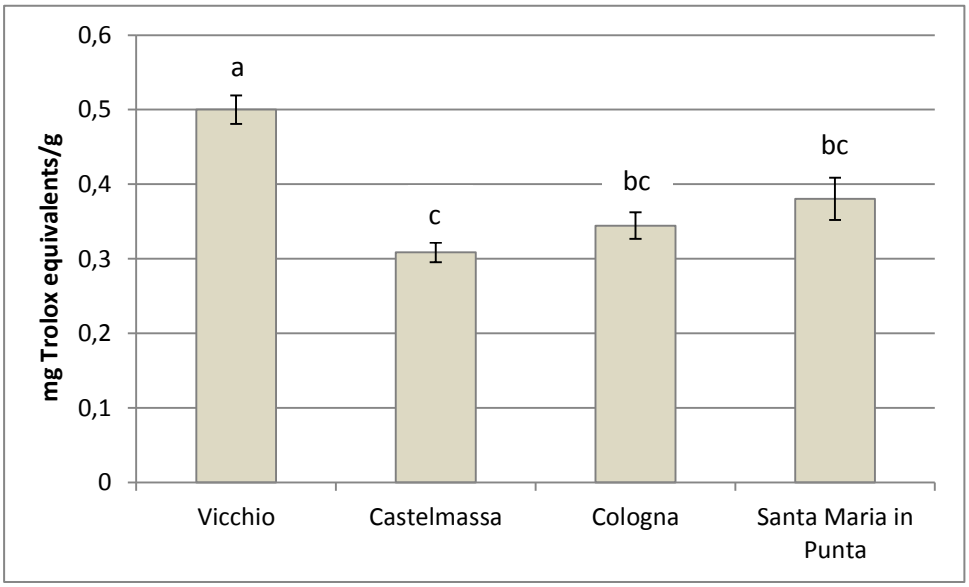

Figure 2

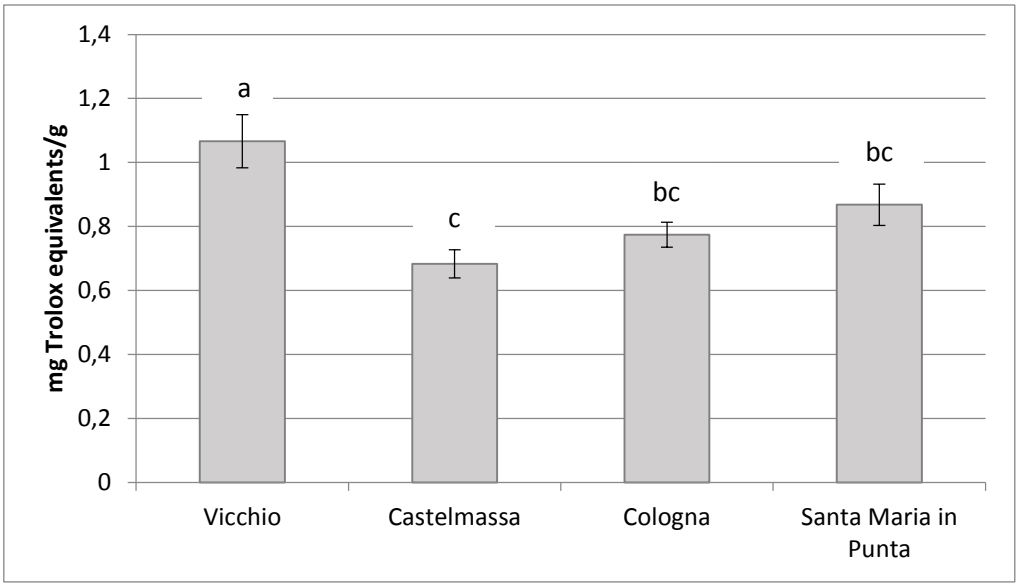

Figure 3