Highlights

1. Hemp inflorescences represent added-value co-products after seed harvest
2. Site (S) and harvest time (H) affected inflorescence yield; SxH influenced seed production
3. The EO composition changed more with harvest time, rather than the site
4. EOs were rich in sesquiterpenes, but monoterpene hydrocarbons were also relevant
5. Cannabinoids (mainly cannabidiol) were higher in the lowland early harvest EO

Valorisation of hemp inflorescence after seed harvest: cultivation site and harvest time influence agronomic characteristics and essential oil yield and composition

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Abstract

Cannabis sativa L. is a multipurpose crop, whose industrial varieties, complying with the 0.2% threshold set by the EU legislation, can be cultivated without restrictions by farmers. Other than its traditional use as a source of bast fibres from the stems, the fixed oil extracted from its seeds represents a valuable nutritional product. Its inflorescences are also a further exploitable threshing residue originating from seed harvest, as they can be used for the extraction of the essential oil (EO), a high-value added product.

This study aims at contributing to the re-evaluation of the industrial hemp cultivation as an agro-environmentally sustainable crop for the diversification of Mediterranean cropping systems, by exploring the possibility to recover the EO from the inflorescences after seed harvest.

The influence of the cultivation site (lowland and hillyland of Pisa province, Tuscany, central Italy) and the harvest time (August and September) have been investigated on the 'Fedora 17' monoecious hemp cultivar: the main agronomic traits in term of stem, seed and inflorescences production, as well as essential oil yield and composition have been evaluated.

The crops harvested in September exhibited higher total dry yield as well as higher inflorescence and stem yields, whilst neither the site nor the harvesting period influenced the seed production, which was significantly influenced by harvest time x cultivation site interaction. Both seed fixed oil and crude protein content were affected by the cultivation site only, but with an opposite trend: the highest seed oil content was reached in the hilly area, while the plants grown in the plain area exhibited the major seed protein content. All the extracted EOs were mainly rich in sesquiterpenes (mostly β-caryophyllene and its oxidized derivatives, and α-humulene), but monoterpene hydrocarbons were significantly represented as well (mainly α- and β-pinene, and myrcene). The EOs extraction yields were slightly higher in the earlier harvest for both sites.

Keywords: *Cannabis sativa* L.; Fedora 17; hemp by-products; threshing residue.

1. Introduction

Cannabis sativa L. is a species native to Central and North-eastern Asia, where evidences of its cultivation date back to over 5000 years ago (Li, 1973). Traditionally considered a multi-purpose crop, industrial hemp has been widely cultivated and used throughout history for its fibre, nutritional and medicinal properties (House et al., 2010; Kriese et al., 2004; Tang et al., 2006; Vera and Hanks, 2004). Hemp is an annual high-yielding crop with low environmental impact due to its low susceptibility to pests and diseases, making it a suitable crop to be used in both conventional and organic cropping systems (Angelini et al., 2016; Kreuger et al., 2011; Van der Werf et al., 1996). Compared with other crops, hemp requires a low level of irrigation and fertilizers after its establishment (Amaducci et al., 2008; Gandolfi et al., 2013). The stems provide fibres and hurds, while seeds are used for food, feed and pharmaceutical applications.

The hemp bast fibre is one of the most ecologically friendly, as well as the oldest of all natural fibres (Shahzad, 2012). Besides the traditional utilisation of the fibres, their use as reinforcement in biocomposites (mainly automotive), insulation materials and other non-woven applications

(technical textiles), has increased in recent years, as a response to the increasing demand for developing biodegradable, sustainable, and recyclable materials (Carus, 2017). Furthermore, the biomedical relevance of hemp is well documented, thanks to the wealth of its secondary metabolites identified so far (over 500) and their biological activity (Appendino et al., 2008; ElSohly and Slade, 2005; Pertwee, 2009; Turner et al., 1980). Hemp grain is generally composed of 25-35% of oil, 20-25% protein, 20-30% carbohydrates and 10–15% insoluble fibre, along with a rich array of minerals (Deferne and Pate, 1996; Oomah et al., 2002; Pate, 1999; Vonapartis et al., 2015). Moreover, hemp seed oil has positive health benefits, including lowering cholesterol and blood pressure (Callaway, 2004; Jones, 1995). The highly polyunsaturated oil of hemp seed is currently used for personal care products such as lotions, moisturizers, shampoos, and lip balms. The versatility of the hemp seed lends itself to the development of numerous products for the food, cosmetic, therapeutic, functional food, and nutraceutical industries (Oomah et al., 2002). Hemp seed production and its properties vary widely, depending mainly on the harvest date and on the agro-climatic and geographical conditions in which it is grown (Anwar et al., 2006; Campiglia et al., 2017; Tang et al., 2016). In recent years, the essential oil (EO) extracted from the inflorescences of *C. sativa* L. has gained interest, including it among the various products obtained from this plant (Bertoli et al., 2010). Hemp EO is synthesized in the glandular hairs, mainly present in the female flowers bracts and floral leaves, where the cannabinoids synthesis takes place as well (Kim and Mahlberg, 1991). It is a high value product, mainly composed of terpenes, of which monoterpenes are the major responsible for the fragrance profiles of different hemp EOs (Bertoli et al., 2010). Different cultivars, indeed, produce EOs with very diverse compositions, leading to various aroma profiles (Bertoli et al., 2010; Mediavilla and Steinemann, 1997; Nissen et al., 2010). Genetic improvement and selection may have contributed to the differentiation in the terpenoid content within the various selections and crossings. Besides the genetic variability, several agronomic and environmental factors are involved in both the yield and the composition of the produced essential oil. Among them, the sowing density can affect inflorescence and, consequently EO yield. The modulation of

the flowering time (Faux et al., 2013) exerted by the climatic and photoperiodic conditions may affect inflorescence development and consequently, EO production (Meier and Mediavilla, 1998), as well. In particular, weather seems to play a major role on the EO yield, as a quite dry period occurring between the beginning of female flowering and seed maturity is desirable. The production of essential oil increases in humid areas, as it is also the case for resin, the density of glandular trichomes and the content of cannabinoids. It seems that strong rains can destroy glandular trichomes and cause product decreases (Meier and Mediavilla, 1998). At the same time, the harvesting period plays an important role on EO yield: the flowers should be harvested when about 50% of the seeds are matured, shortly before their full maturity, which occurs when 75% of them are matured. The prevention of the pollination and the use of manual harvesting also increase the yields (Meier and Mediavilla, 1998).

The essence can be obtained from both fresh and dried hemp inflorescences, although it is generally preferable to hydrodistill fresh material. The drying process decreases the amount of obtainable oil but, if performed correctly and with a short storage period, it does not affect the quality of the extracted EO. The EO yield of different hemp varieties ranges from 0.11 to 0.25% (w/w) and generally shows a significant content of α-pinene (3-20%), β-pinene (1-8%), (*E*)-β-ocimene (1-10%), mycene (8-45%), terpinolene (0.12-22%) limonene (0.3-6.4%), β-caryophyllene (7.3-28.0 %) and α-humulene (3.2-12.6%) (Bertoli et al., 2010).

C. sativa L. essential oil extracted from industrial hemp varieties, cultivated for seed oil production, can represent an interesting and promising added-value by-product. These varieties (complying with the 0.2% threshold set by the EU legislation No. 2860/2000), can be cultivated without restrictions by farmers and EO extraction could be realized on the threshing residues, recovered during seed harvest and seed cleaning procedures.

Considering that there is a growing interest in hemp for seed production, this study aims at evaluating the possibility to obtain good agronomic performances in terms of seed yield, fixed oil and protein content, together with high quality EO from hemp inflorescences residues. For this

purpose, the effects of cultivation site and time of harvest on agronomic characteristics (plant height, density, stem and seeds yield, seed oil and protein content) and inflorescences essential oil yield and compositions were evaluated. The monoecious French cultivar 'Fedora 17' has been used and compared in two sites (hillyland and lowland) of central Italy, carrying out two distinct harvests (ripening phase in August and senescence phase in September). At the same time, the compositions of the EOs were compared to that of inflorescences coming from spontaneously reborn plants (volunteer plants), grown in the next year on the lowland site and harvested in November 2016.

2. Materials and Methods

2.1. Experimental conditions and plant material

Two experimental fields were established in 2015 in farms located in two contrasting sites: in the hilly (Santa Luce, latitude 43°28'N, longitude 10°34'E, and altitude 200 m a.s.l.) and plain area (Cascina, latitude 43°40'N, longitude 10°30'E, altitude 8 m a.s.l.) of Pisa Province (Tuscany region, central Italy). The soil of the hilly area is classified as *Vertisoil*, and it was characterized by clay-loam texture (35.8±4.3; 37.3±2.8, and 26.9±4.5 g 100 g^{-1} soil, of clay, silt and sand, respectively in the 0-30 cm soil layer), with a low content of both soil organic matter (1.29±0.42 mg g^{-1} soil) and available phosphorus (6.07±0.72 mg P_2O_5 kg^{-1}soil), a medium level of total nitrogen (1.11±0.07 mg g^{-1} soil), and a good content of exchangeable potassium (137.0±8.2 mg K_2O kg^{-1} soil). In the plain area, the soil was a typically alluvial clay loam soil (20.1±7.1; 42.4±2.7; and 37.5±6.7 g 100 g^{-1} soil, of clay, silt and sand, respectively in the 0-30 cm soil layer), with a good content of both organic matter (1.82±0.46 mg g^{-1} soil) and total nitrogen (1.31±0.21 mg g^{-1} soil), rich in exchangeable potassium (197.0±10.2 mg K_2O kg^{-1} soil) and poor of available phosphorus (13.42±2.41 mg P_2O_5 kg^{-1} soil). The two sites have a Mediterranean climate, with total rainfall during the 2015 growing season (from March to August) of 420 mm and 422 mm for Santa Luce and Cascina (long-term mean: 390 mm in the same period), with an average temperature of 21°C and 20.3°C mm (long-term mean: 19.3°C in the same period). Very high temperatures, 3.1°C above

128 the long-term average, have been reached in July in both sites, which has been recorded as the
129 hottest July since 1995.
130 Crop techniques and mechanization methods were defined in relation to the specific characteristics
131 of the area, and according to low input management practices applied by local farmers. In both
132 environments, tillage (medium-depth ploughing at 35 cm depth) was conducted in the autumn of
133 2014, while the seedbed was prepared in the following spring, immediately before planting, by a
134 pass with a double-disking harrow and a pass with a field cultivator. Two weeks before hemp
135 sowing, the fields were fertilized with 70 kg $ha^{-1}$of P_2O_5 as triple superphosphate. K fertilizer was
136 not applied due to the high levels present in the soil. Nitrogen fertilization was applied at a ratio of
137 50 kg N ha^{-1} as ammonium nitrate applied 21 days after crop emergence. Sowing took place on 8
138 and 10 April, at Santa Luce and Cascina respectively, by a pneumatic drill adopting a seed rate of
139 35-40 kg ha^{-1}. Row distance was set at 0.20 m (Cascina) and 0.13 m (Santa Luce). In both sites, the
140 crop was protected against weeds before inter row closure, by mechanical weeding and no
141 herbicides, neither pesticides were applied during the growing season. The cultivation was carried
142 out under rainfed conditions. Two distinct harvests were carried out during seed ripening: the first
143 one was accomplished in early August, with a seed moisture around 20%; the second harvest
144 occurred in September, when seed moisture decreased to 11%.
145 The monoecious variety used in this study was Fedora 17, a French cultivar, containing less than
146 0.2% w/w of Δ9-tetrahydrocannabinol (Δ9-THC) (Regulation EC No. 1124/2008, Annex XII),
147 commonly used for fibre production, and characterized by an early onset of the flowering phase.
148 The seeds were obtained from the "Coopérative Centrale des Producteurs de Chanvre" of Le Mans,
149 France.
150 In each site the field layout was a completely randomized block design to compare the two harvest
151 times. For each harvest time (August and September 2015), four randomized sample areas of 1 m^2
152 (1 x 1m) within each experimental field, for each cultivation site, were collected, in order to assess
153 the main biometric and productive characteristics. The plants were manually cut at the base of the

stem. The main biometric and productive characteristics were evaluated on a sub-sample of 20 representative plants. The different plant organs and components (stems, leaves, seeds, inflorescences) were separated and dried into a ventilated oven (35/40°C) until constant weight for dry weight determinations and for further processing and quality evaluations. Seeds were separated from the inflorescence by using a small table threshing machine and then dried (at 30°C) and cleaned. The empty seeds were removed and weighed for seed yield determination. The resulting inflorescences (threshing residues) were then evaluated for EO yield and composition. Seed yields were also evaluated for the entire experimental plot using a thresher plot-machine.

Besides the observations on 2015 hemp cultivation carried out in Santa Luce and Cascina, further investigations were carried out on volunteer plants grown from seeds that were dropped into the soil from the previous hemp crop in Cascina. Hemp regulations require that all volunteer plants be eliminated in the field/crop following hemp production. However, we wanted to evaluate if the essential oil characteristics differed between cultivated and volunteer hemp plants. Therefore, the volunteer plants were sampled from Cascina site in November 2016. The plants, irregularly reborn on the soil, were short (<1m stem height) and in the vegetative stage. The aerial parts were air-dried at 30°C and then analysed for EO yield and composition.

2.2. Seed oil and protein content

Four representative sub samples of grain were used for oil and protein determination. Seed protein content was measured according to the Kjeldahl method (Bremner and Mulvaney, 1982) (Kjeldahl N × 6.25). Samples were analysed for oil content according to the Association of Official Analytical Chemists (AOAC) methods (AOAC, 2000). Ether extract system was used for oil extraction from powdered samples, by an ANKOM model XT10 extractor (ANKOM Technology, Macedon, NY, USA). In a typical extraction process, 1 g powdered samples were immersed in boiling petroleum ether for 60 min to dissolve most of the soluble material. Total lipids were extracted by means of a chloroform/methanol solution (2:1, v/v), according to Rodriguez-Estrada et al. (1997).

180

181 *2.3. Essential oil hydrodistillation*

182 The hydrodistillations were carried out in a standard Clevenger apparatus for 2 hours. The
183 extractions were performed on representative sub samples of dried aerial parts: the EO yields,
184 calculated on a dry weight basis and expressed as g of essential oil per 100 g of inflorescences, are
185 reported in Table 1. For each Cascina and Santa Luce 2015 sample, hydrodistillation was carried
186 out in triplicates, whilst, due to the low amount of plant material, only one hydrodistillation was
187 performed for the Cascina November 2016 sample.

188

189 *2.4. GC – MS Analysis*

190 The hydrodistilled essential oils were diluted to 5% in *n*-hexane HPLC grade and then injected into
191 a GC – MS apparatus. Gas chromatography–electron impact mass spectrometry (GC–EIMS)
192 analyses were performed with a Varian CP-3800 gas chromatograph equipped with a DB-5
193 capillary column (30m×0.25 mm; coating thickness 0.25μm) and a Varian Saturn 2000 ion trap
194 mass detector. Analytical conditions were as follows: injector and transfer line temperatures 220
195 and 240 °C, respectively; oven temperature programmed from 60 to 240 °C at 3 °C/min; carrier gas
196 helium at 1 ml/min; injection of 0.2 μl (5% *n*-hexane HPLC grade solution); split ratio 1:30.
197 Identification of the constituents was based on a comparison of the retention times with those of the
198 authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons.
199 Computer matching was also used against commercial (NIST 14 and ADAMS) and laboratory-
200 developed library mass spectra built up from pure substances and components of known oils and
201 MS literature data (Adams, 1995; Davies, 1990; Jennings and Shibamoto, 1982; Masada, 1976;
202 Stenhagen et al., 1974).

203

204 *2.5. Statistical Analyses*

For the essential oil compositions, the statistical analyses were carried out using the JMP software package (SAS Institute, Cary, NC, USA). The correlation data matrix was a 99 × 9 matrix (99 compounds × 9 samples including replicates = 891 data). The principal component analysis (PCA) was performed selecting the two highest principal components (PCs) obtained by the linear regressions operated on mean-centred, unscaled data; as an unsupervised method, this analysis aimed at reducing the dimensionality of the multivariate data of the matrix, whilst preserving most of the variance (Choi et al., 2004). The Principal Component Analysis (PCA) was performed selecting the two highest PCs obtained by the linear regressions: the chosen PC1 and PC2 cover 46.49% and 26.97% of the variance, respectively, for a total explained variance of 73.46%. The Hierarchical Cluster Analysis (HCA) was performed using Ward's method, with squared Euclidian distances as a measure of similarity. Both the HCA and the PCA methods can be applied to observe groups of samples even when there are no reference samples that can be used as a training set to establish the model.

Regarding to biometric, productive and qualitative parameters, a two-way ANOVA with four replications (Gomez et al., 1984) was carried out in order to estimate the variance components of harvest time (H; August and September), cultivation site (S; Cascina and Santa Luce) and their reciprocal interaction (HxS). Means were separated on the basis of post-hoc LSD test with a significance level of 5%.

3. Results and discussion

3.1. Biometric and productive characteristics

The main biometric and productive traits, as well as the main qualitative parameters of the seeds (oil and protein content), are reported in Table 1. Significant differences for plant density and plant height were observed between the two cultivation sites, with more dense crops in Santa Luce and taller plants in Cascina. On the other hand, no effect of the harvest time and HxS interaction was

230 detected for these two traits. A significant increase of total plant and inflorescence dry yields was
231 observed from the 1st to the 2nd harvest time. Furthermore, higher total dry biomass as well as
232 inflorescence and stem yields were observed in Santa Luce in comparison with Cascina, probably
233 due to the greater plant density registered in this hilly site. In such conditions the plants resulted
234 shorter and reached the reproductive stage earlier than the plants cultivated in the plain area of
235 Cascina. However, the same trend was not observed for the seed yield, which remained almost
236 stable during the two harvests and between the two sites. In this case, a significant effect of HxS
237 interaction was detected with the main differences between Santa Luce and Cascina for the seed
238 yield obtained in August, that was higher in the hilly site. The tendentially lower yields obtained at
239 Cascina can be attributable to seed losses due to shattering. This phenomenon was responsible, in
240 turn, for spontaneously reborn plants (namely volunteer plants) from seeds naturally dropped into
241 the soil.
242 The seed production and the stem and inflorescence yields obtained in this trial were similar to
243 those reported in the literature. In particular, Tang et al. (2016) reported seed yield ranging from 0.3
244 Mg ha^{-1} to 2.4 Mg ha^{-1}in trials carried out in Italy, France and Czech Republic, for Fedora 17, which
245 proved to be a particularly suitable variety for seed production. At the same time, similarly to our
246 findings, a threshing residue up to 2 Mg ha^{-1} has been reported. Similarly, Campiglia et al. (2017),
247 in a field trial carried out on sandy loam soil in Viterbo (central Italy), reported, for Fedora 17, a
248 seed yield ranging from 1.04 to 2.02 Mg ha^{-1} and inflorescences yield from 1.48 to 2.63 Mg ha^{-1}. In
249 such conditions the best results were achieved at the greater plant density (120 plants m^{-2}) and the
250 higher nitrogen fertilization (100 kg N ha^{-1}).
251 The fixed oil content was not affected by the harvest time or HxS interaction, but significantly
252 varied depending on the cultivation site. The oil content ranged from 261 to 278 g kg^{-1} and rose
253 from Cascina to Santa Luce as plant density increased. Likewise, the crude protein content was
254 similar between the two harvests and varied from 178 to 204 g kg^{-1} with the lowest values in the
255 hilly site of Santa Luce, showing an opposite behaviour to that observed for the oil content. The

values of seed oil and crude protein content here observed, fall within the range reported previously (Campiglia et al., 2017; Vera and Hanks, 2004; Vonapartis et al., 2015).

3.2. Essential oil compositions and yields

The complete compositions of the extracted essential oils (EOs), the yields of extraction and the legend of the samples are reported in Table 2. The essential oil content and composition was assessed, not only on the inflorescences resulting from seed threshing (see Table 1), but also on small Fedora 17 plants reborn from seed shattering (volunteer plants) from the previous crop in Cascina, which were collected manually in November 2016. The yields of extraction were, for both sites, higher for the samples harvested in August, while the inflorescences from the volunteer plants showed the lowest yield (0.01% w/w).

Sesquiterpenes were the most abundant chemical class of compounds for all the samples, as they account for over 60% of all the EOs, with the hydrocarbon ones ranging from 40.1 to 51.6%. Their relative abundances were quite stable in samples from the same cultivation site. Monoterpene hydrocarbons follow as the second most abundant class of compounds in all the samples: they showed a decrement from August to September in the plants grown at Cascina, whilst the opposite was found for the plants from Santa Luce. In the volunteer sample, their relative abundance incremented up to 32.8%. The oxygenated monoterpenes increased in the late harvest, where their relative abundances reached up to 3-4%. The detected cannabinoids (cannabichromene and cannabidiol) ranged from a minimum of 0.5 to a maximum of 3.1% in SL2 and C1, respectively. Cannabinoids decreased from the August to the September specimens of the same area; they were not detected in the EO from volunteer plants.

β-caryophyllene was the most abundant compound in all the samples: its relative abundance ranged between 17.4 and 23.4%. Its oxidized derivatives were also detected in relevant relative amounts: caryophyllene oxide and caryophylla-4(14),8(15)-dien-5-ol accounted for 7.0 and 2.8% on average, respectively. The latter was less represented in the C3 sample, where it only reached 1.4%. 9-*Epi*-

(E)-caryophyllene, which was not detected in the C3 sample, ranged between 1.5 and 3.0% in the other EOs. Among the sesquiterpene hydrocarbons, α- and β-selinene exhibited a relevant presence, as they accounted for 2.5 and 3.5% on average, respectively; they were not detected in the volunteer plants. Valencene and viridiflorene, instead, were only found in the latter, where they represented 2.9 and 2.0%, respectively. α-Humulene, a typical constituent of *C. sativa* essential oil, showed a quite stable relevance in all the samples; it was slightly more abundant in the Santa Luce EOs from both the harvests.

The C3 composition was more variable compared to the other samples in terms of monoterpene hydrocarbons relative abundances. α-Pinene was far less represented (4.3%) in the C3 sample compared to the other ones (11.4-14.1%). β-Pinene showed a more stable behaviour: it slightly decreased in the C3 sample (2.3%), compared to C1 (3.3%) and C2 (3.6%). α- and β-pinene relevant presence is typical of the monoecious varieties of *C. sativa* (Bertoli et al., 2010). Myrcene, instead, showed an opposite behaviour, as it was almost three times more abundant in the C3 sample (9.7%) compared to all the other EOs (2.4-3.9%). The increment of myrcene in the essential oil of a plant belonging to the same family, *Humulus lupulus* L., has been linked to a later harvesting date (Matsui et al., 2016; Schnaitter et al., 2016). It is also reported as a result of drought conditions in *Ocimum basilicum* L. (Abdollahi Mandoulakani et al., 2017), as well as a response to thermogenic stress in *Macrozamia* cycad cones (Terry et al., 2016). A moderate to severe water stress-induced increment in the monoterpene hydrocarbons fraction was also reported in EOs extracted from *Helichrysum petiolare* Hilliard & B.L.Burtt (Caser et al., 2016). The results of the present study are in accordance with these findings: the volunteer plants have been harvested later compared to the other samples. Moreover, as they were not regularly watered, they might have suffered both thermal and water stress.

(*E*)-β-Ocimene reached up to 8.0% in the volunteer sample EO, whilst it ranged between 1.2 and 1.8% in the other EOs. Terpinolene is also more relevant in the C3 sample EO, where it was detected in higher percentages (4.2%) compared to its contribution in the other samples (1.3-1.9%).

Whilst cannabichromene was found exclusively in the SL1 sample as low as 0.1%, cannabidiol was found in all the EOs, with the exception of C3. The CBD content showed a decrement from the first harvest of August to the later one in both areas: it dropped from 3.1 to 1.3% in the Cascina samples and from 1.6 to 0.5% in the Santa Luce ones.

As expected, the differences in the essential oil compositions were more relevant between the inflorescence (threshing residue) of cultivated plants and the inflorescences collected from volunteer plants (spontaneously reborn from seeds fallen from the previous crop), rather than between the different cultivation sites. On the contrary, the extraction yields were slightly higher for the inflorescence obtained, after seed removal, from plants grown in the hilly area and harvested in August; the inflorescences obtained, in the same area, from the 2nd harvest (SL2), though, showed a higher yield reduction compared to C2.

The detected EOs compositions are, overall, very different from those reported by Nissen et al. (2010) for three other industrial cultivars: Carmagnola and Fibranova, which are dioecious cultivars of Italian origin, and Futura, a monoecious of French origin. Monoterpenes dominate the compositions, accounting for up to 60-70% of the total, with myrcene and α-pinene as the most abundant compounds in the oil; sesquiterpenes, instead, reach up to 20-30% (Nissen et al., 2010). In Novak et al. (2001) the myrcene predominance over β-caryophyllene is shown by all the EOs extracted from the five analyzed industrial cultivars (Felina 34, Fedrina 74, SwissMix, Kompolti and Secuemi) of *C. sativa* L. (Novak et al., 2001). The same behaviour is shown by other fibre hemp EOs are reported by Bertoli et al. (2010): Pop 2 (harvested in 2005), Carmagnola (harvested in 2005 and 2006), Red Petiole (harvested in 2005), which are all dioecious varieties, and the monoecious Felina 34 (harvested in 2005 and 2006). In the same study, the dioecious varieties Pop 2 (harvested in 2006), Pop 4 (harvested in 2006) and Pop 5 (harvested in 2005), and the dioecious Red Petiole (harvested in 2006) EOs showed a composition more similar to the ones in the present study, with a predominance of β-caryophyllene, and sesquiterpenes in general, over monoterpenes (Bertoli et al., 2010).

334

335 *3.3. Multivariate statistical analysis*

336 The hierarchical cluster analysis (HCA) dendrogram (Fig. 1) grouped samples based on the
337 cultivation site for the main crops, whilst the C3 volunteer sample was clustered separately: this
338 confirms its compositional difference compared to all the other EOs. In the other macro-cluster, the
339 Cascina and the Santa Luce samples were grouped in two different sub clusters: the compositions of
340 the EOs are influenced by the site of cultivation in a recognizable pattern, even at different
341 harvesting times.

342 The principal component analysis (PCA) plot (Fig. 2) confirmed the significant differences in terms
343 of composition of the EOs extracted from the volunteer sample: only C3 was plotted in the left
344 quadrants of the PCA plot, due to its larger relative abundances of myrcene, terpinolene, (*E*)-β-
345 ocimene, valencene and viridiflorene. Moreover, the score plot evidenced the influence of the
346 harvesting time on the quality of the extracted EO. The earlier harvested samples (C1 and SL1),
347 indeed, were plotted in the lower right quadrant of the PCA plot: their caryophylla-4(14),8(15)-
348 dien-5-ol, 9-*epi*-(E)- caryophyllene and cannabidiol relative abundances separated them in this
349 quadrant from the later harvested ones. C2 and SL2 were positioned in the upper right quadrant:
350 selina-3,7(11)-diene, caryophyllene oxide, santolina triene and sabinene played a major role in their
351 plotting.

352 The PCA result is not in disagreement with the HCA: they are to be interpreted as different aspects
353 of the compositional behaviour. The HCA, which does not take into account the covariance among
354 the samples, showed an overall qualitative proximity of the EOs compositions from the same site.
355 The PCA, instead, evidenced the quantitative influence of all the compounds in the EO, taking into
356 account the two most relevant linear regressions explaining most of the analysed covariance,
357 differentiating the samples according to their harvesting time.

358

4. Conclusion

The findings obtained in this study highlighted that the exploitation of the whole biomass of industrial hemp is of pivotal importance in order to develop a series of specific products and co-products (stems, seeds and inflorescences), in a modern biorefinery approach.

From an agronomic point of view, the cultivation of industrial hemp as multipurpose crop for fixed oil production and inflorescences for high quality EO still requires optimized agro-techniques, incorporating appropriate technical advances in agronomic practices in order to maximize both the seed production and the quantity and quality of the EO produced by the residual inflorescences. In particular, this study confirms the importance of genotype x environment interaction and harvest time of the seeds in defining the crop performances in term of productivity and quality of the final products. Certainly, the cultivation of industrial hemp for seed and both fixed and essential oil production could represent a valid alternative in order to increase the agro-environmental sustainability and diversification of Mediterranean cereal-based cropping, as well to contribute to differentiate and raise the income deriving from agricultural activity. In fact, the present study demonstrated the possibility to effectively exploit the inflorescences as added-value co-products after seed harvest, contributing to the overall economy of hemp cultivation. *C. sativa* L. essential oil can represent a high-value product, whose yield and composition are greatly influenced by the selected cultivar. 'Fedora 17' EO, investigated in the inflorescences during seed ripening, is mainly rich in sesquiterpene compounds, both oxygenated and hydrocarbon: the most represented ones are β-caryophyllene and its oxidized derivatives. Its monoterpene hydrocarbons fraction, though, is particularly relevant, even in the volunteer plants, where they are almost as represented as the sesquiterpene hydrocarbons. The obtained data have shown that the majority of the compositional variability was mainly due to the origin of the raw material and to the harvest time, rather than to the cultivation site. In fact, the volunteer plants showed a significantly higher relative abundance of monoterpene hydrocarbons, with a different profile of sesquiterpene hydrocarbons, as well. Finally, the inflorescences obtained from August harvest showed higher yields of extraction compared to the

385 later-harvested ones.

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388 **5. References**

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Tables

Table 1. Effect of harvest time, cultivation site and their reciprocal interaction on the main biometric, productive and qualitative characteristics of *Cannabis sativa* (var. Fedora 17). Values are expressed as mean ± standard deviation (n=4).

		Cultivation Site			
	Harvest time	Cascina (plain area)	Santa Luce (plain area)	*Mean Harvest*	*Significance*
Plant density (plants m^{-2})	August	72.86 ±12.45	132.05 ± 26.39	102.46	S = ***; H = n.s.; SxH = n.s.
	September	88.33 ± 7.64	142.31 ± 16.01	115.32	
	Mean Site	80.60 B	137.18 A		
Plant height (cm)	August	211.80 ± 22.63	172.00 ± 16.99	191.90	S = ***; H = n.s.; SxH = n.s.
	September	205.66 ± 29.64	159.56 ± 13.60	182.61	
	Mean Site	208.73 A	165.78 B		
Total dry yield (Mg ha^{-1})	August	15.55 ± 1.23	23.60 ± 2.57	19.58 B	S = ***; H = ***; SxH = n.s.
	September	18.56 ± 1.76	30.52 ± 2.88	24.54 A	
	Mean Site	17.06 B	27.06 A		
Inflorescence dry yield (Mg ha^{-1})	August	1.66 ± 0.25	2.17 ± 0.20	1.92 B	S = ***; H = ***; SxH = n.s.
	September	2.62 ± 0.24	3.35 ± 0.33	2.99 A	
	Mean Site	2.14 B	2.76 A		
Stem dry yield (Mg ha^{-1})	August	13.05 ± 0.98 c	20.34 ± 1.73 b	16.70 B	S = ***; H = ***; SxH = *
	September	14.94± 1.02 c	26.30 ± 1.89 a	20.62 A	
	Mean Site	14.00 B	23.32 A		
Seed yield (Mg ha^{-1})	August	0.84 ± 0.13 b	1.09 ± 0.16 a	0.97	S = n.s.; H = n.s.; SxH = *
	September	1.00 ± 0.03 ab	0.97 ± 0.09 ab	0.99	
	Mean Site	0.92	1.03		
Oil content (g kg^{-1})	August	265.80 ± 14.20	278.40 ± 16.47	272.10	S = *; H = n.s.; SxH = n.s.
	September	260.61 ± 9.18	275.60 ± 9.11	268.11	
	Mean Site	263.21 B	277.00 A		
Protein content (g kg^{-1})	August	200.70 ± 12.10	178.0 ± 13.47	189.35	S = **; H = n.s.;

	September	204.20 ± 15.31	180.24 ± 13.89	192.22	SxH = n.s.
	Mean Site	202.45 A	179.12 B		

Means followed by different letters are significantly different according to 2-way ANOVA with harvest time (H) and cultivation site (S) as variability factors. $LSD_{0.05}$ test has been used as *post-hoc* comparison. Lower-case letters indicate HxS interaction, upper-case letters indicate effect of harvest time and cultivation site. Significance is indicated as follows: ns, not significant; *, significant at $p < 0.05$ level; **, significant at $p < 0.01$ level; ***, significant at $p < 0.001$ level.

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Table 2. Complete compositions and extraction yields (%w/w dry weight) of the extracted essential oils.

Constituents	l.r.i.[a]	Relative abundance (%)				
		Cascina			Santa Luce	
		August 2015 (C1)	September 2015 (C2)	November 2016 (C3)	August 2015 (SL1)	September 2015 (SL2)
(*E*)-2-hexenal	856	-[b]	-	0.4	-	-
heptanal	901	0.1±0.1	0.5±0.3	0.5	0.1±0.1	0.2±0.0
santolina triene	909	0.1±0.1	0.4±0.2	0.2	0.1±0.1	0.5±0.1
α-pinene	939	14.1±7.3	12.9±9.4	4.3	12.6±5.9	11.4±2.0
camphene	954	0.4±0.2	0.5±0.3	-	0.4±0.2	0.5±0.1
sabinene	976	-	0.1±0.1	-	-	0.1±0.1
β-pinene	980	3.3±1.6	3.6±2.2	2.3	2.5±1.1	2.8±0.6
myrcene	992	3.9±1.4	3.9±2.1	9.7	2.4±0.8	3.2±0.5
α-phellandrene	1005	0.3±0.7	0.1±0.1	0.2	0.1±0.1	0.1±0.1
δ-3-carene	1011	1.3±1.8	0.4±0.2	1.2	0.1±0.1	0.2±0.0
α-terpinene	1018	-	0.1±0.1	0.2	0.0±0.1	0.1±0.0
p-cymene	1027	-	0.1±0.0	-	0.0±0.1	0.2±0.0
limonene	1031	1.7±0.7	1.4±0.3	1.1	0.7±0.2	0.8±0.1
1,8-cineole	1035	0.1±0.2	0.3±0.4	0.3	0.4±0.3	0.5±0.0
(*Z*)-β-ocimene	1041	0.3±0.2	0.2±0.1	1.1	0.2±0.1	0.3±0.0
(*E*)-β-ocimene	1051	1.4±0.7	1.5±0.5	8.0	1.2±0.5	1.8±0.3
γ-terpinene	1062	0.1±0.1	0.2±0.1	0.3	0.0±0.1	0.2±0.0

cis-sabinene hydrate	1070	-	0.2±0.0	0.2	0.0±0.1	0.2±0.0
terpinolene	1089	1.4±0.7	1.8±0.6	4.2	1.3±0.7	1.9±0.2
trans-sabinene hydrate	1099	-	0.1±0.2	-	-	0.1±0.1
linalool	1101	-	0.1±0.1	0.2	-	-
nonanal	1103	0.2±0.1	0.4±0.1	1	0.1±0.1	0.2±0.0
cis-*p*-menth-2-en-1-ol	1123	-	-	0.1	-	0.1±0.1
trans-pinene hydrate	1124	-	0.1±0.1	-	-	-
trans-pinocarveol	1140	0.1±0.1	0.6±0.0	0.1	0.4±0.1	0.7±0.1
trans-sabinol	1141	-	0.2±0.0	-	-	0.2±0.2
geijerene	1143	-	-	-	-	0.2±0.2
*iso*pulegol	1146	-	0.1±0.1	-	-	-
camphene hydrate	1150	-	0.1±0.1	-	0.1±0.1	0.1±0.2
β-pinene oxide	1158	-	-	0.2	-	-
(*E*)-2-nonenal	1163	-	-	0.2	-	-
pinocarvone	1164	-	0.2±0.0	-	0.1±0.1	0.3±0.0
borneol	1167	-	0.1±0.1	-	0.1±0.1	0.1±0.1
pinocampheol	1170	0.1±0.1	0.9±0.1	-	0.5±0.1	1.0±0.0
p-cymen-8-ol	1183	-	0.1±0.1	0.1	-	0.1±0.1
α-terpineol	1190	0.1±0.1	0.2±0.0	-	0.1±0.0	0.2±0.0
myrtenol	1192	-	0.1±0.1	-	-	-
hexyl butyrate	1193	-	-	0.2	-	-
cyclosativene	1368	-	0.1±0.1	-	-	-
α-ylangene	1372	-	-	-	-	-

1-hexyl-1-hexanoate	1385	-	-	0.3	-	-
sativene	1395	-	0.1±0.2	-	-	0.1±0.1
*iso*caryophyllene	1405	0.4±0.0	0.6±0.2	0.4	0.4±0.1	0.6±0.1
α-gurjunene	1409	-	-	-	0.0±0.1	-
cis-α-bergamotene	1416	0.1±0.1	0.2±0.0	-	0.2±0.1	0.2±0.0
β-caryophyllene	1418	20.5±3.1	17.4±0.4	20.9	23.4±4.4	22.4±1.6
trans-α-bergamotene	1438	1.1±0.3	1.3±0.3	1.8	1.9±0.4	1.9±0.1
α-humulene	1455	7.0±0.9	5.9±0.9	6.5	8.5±1.3	8.1±0.3
(*E*)-β-farnesene	1460	-	-	3.3	-	-
*allo*aromadendrene	1461	-	2.1±2.9	-	1.5±2.1	2.2±3.1
9-*epi*-(*E*)-caryophyllene	1467	3.0±0.6	1.5±2.1	-	3.0±2.8	2.2±3.1
β-chamigrene	1475	-	-	-	0.1±0.1	-
γ-muurolene	1477	0.2±0.1	0.4±0.1	-	0.3±0.2	0.4±0.1
β-selinene	1485	3.0±1.7	3.3±1.2	-	4.0±0.9	4.3±0.2
valencene	1492	-	-	2.9	-	-
viridiflorene	1493	-	-	2.0	-	-
α-selinene	1494	2.4±1.2	2.5±0.7	-	2.8±0.5	3.0±0.2
α-bulnesene	1505	-	-	-	0.2±0.2	-
(*E*,*E*)-α-farnesene	1507	-	-	0.5	-	-
β-bisabolene	1509	0.2±0.2	0.3±0.1	-	0.3±0.1	0.3±0.0
trans-γ-cadinene	1513	0.1±0.2	0.2±0.2	-	0.4±0.2	0.2±0.3
7-*epi*-α-selinene	1518	0.2±0.3	-	-	0.2±0.2	0.3±0.0
δ-cadinene	1523	0.3±0.1	0.8±0.5	-	0.5±0.2	0.5±0.0

β-sesquiphellandrene	1524	-	-	0.3	-	-
zonarene	1530	-	-	-	-	0.2±0.2
(*E*)-γ-bisabolene	1535	-	-	0.2	-	-
selina-3,7(11)-diene	1542	1.4±0.8	5.4±1.5	2.4	3.9±2.1	4.5±0.2
germacrene B	1556	-	0.2±0.3	-	-	-
cis-longipinanol	1557	-	-	-	-	0.3±0.4
longicamphenylone	1559	-	-	-	0.0±0.1	-
dimethyl ionone	1563	0.7±0.2	0.4±0.1	-	0.5±0.2	0.3±0.4
(*E*)-nerolidol	1565	0.6±0.4	1.1±0.2	0.4	0.6±0.3	0.6±0.0
caryophyllene alcohol	1569	0.3±0.3	-	-	0.5±0.2	0.4±0.0
caryophyllene oxide	1581	6.6±1.3	8.5±2.4	7.3	6±1.6	6.9±0.8
globulol	1583	-	0.3±0.4	-	-	-
carotol	1594	-	-	0.5	-	-
5-*epi*-7-*epi*-α-eudesmol	1606	0.4±0.4	0.1±0.2	-	0.3±0.1	0.1±0.1
humulene oxide II	1607	2.2±0.5	2.4±1.0	2.9	1.9±0.5	2.0±0.2
10-*epi*-γ-eudesmol	1623	1.3±0.6	0.7±0.7	1.9	0.4±0.6	0.3±0.5
1-*epi*-cubenol	1628	-	-	1.2	-	-
eremoligenol	1632	-	-	-	0.3±0.4	-
caryophylla-4(14),8(15)-dien-5-ol	1636	4.0±2.2	2.2±0.2	1.4	3.6±1.5	2.8±0.1
epoxy-*allo*aromadendrene	1641	0.5±0.4	1.1±0.1	1.2	0.8±0.4	0.4±0.6
cubenol	1643	-	-	-	-	0.2±0.3
selina-3,11 dien-6-alpha ol	1644	0.2±0.3	0.8±1.2	-	0.3±0.4	1.5±0.9
α-eudesmol	1652	-	0.2±0.4	-	0.3±0.6	-

selin-11-en-4-α-ol	1653	0.6±0.8	0.2±0.2	-	0.2±0.3	-
7-*epi*-α-eudesmol	1655	-	-	0.8	-	-
intermedeol	1667	-	0.3±0.4	-	-	-
14-hydroxy-9-*epi*-(*E*)-caryophyllene	1668	1.9±1.1	1.0±0.4	-	1.7±0.7	-
(*Z*)-α-santalool	1677	1.3±1.1	1.1±0.3	-	1.4±0.7	-
elemol acetate	1681	-	-	-	-	0.5±0.7
epi-α-bisabool	1685	-	0.4±0.5	-	0.1±0.2	0.6±0.3
juniper camphor	1692	-	0.3±0.4	0.3	-	0.1±0.2
acorenone B	1698	-	-	-	-	0.3±0.4
caryophyllene acetate	1701	0.8±0.5	0.1±0.2	-	0.4±0.4	-
hexahydrofarnesyl acetone	1845	0.3±0.2	0.2±0.1	1.0	0.3±0.3	0.2±0.0
cannabichromene	2427	-	-	-	0.1±0.1	-
cannabidiol	2431	3.1±1.9	1.3±0.4	-	1.6±1.9	0.5±0.3
Monoterpene hydrocarbons		28.2±12.4	27.1±16.1	32.8	21.7±8.8	24.1±3.8
Oxygenated monoterpenes		0.4±0.2	3.2±0.0	1.2	1.7±0.5	3.7±0.3
Sesquiterpene hydrocarbons		40.1±6.4	42.3±7.1	41.2	51.6±5.1	51.2±3.6
Oxygenated sesquiterpenes		20.8±7.9	21.0±7.6	17.9	18.8±5.6	17±1.5
Apocarotenoids		1.0±0.3	0.6±0.2	1.0	0.8±0.4	0.4±0.4
Cannabinoids		3.1±2.0	1.3±0.4	-	1.7±2.0	0.5±0.3
Non-terpene aldehydes		0.3±0.2	0.8±0.4	2.1	0.2±0.2	0.4±0.0
Non-terpene esters		-	-	0.5	-	-
Total identified (%)		94.7±3.2	96.3±1.0	96.2	96.4±1.6	97.4±0.9

Extraction yield (% w/w)	0.06±0.02	0.04±0.01	0.01	0.12±0.03	0.02±0.00

[a] Linear retention indices on a DB5 column; [b] Not detected.

Figure captions

Figure 1. Hierarchical cluster analysis (HCA) dendrogram for the complete compositions of the essential oils extracted from all the samples.

Figure 2. Principal component analysis (PCA) score plot for the complete compositions of the essential oils extracted from all the samples.

Figure 1

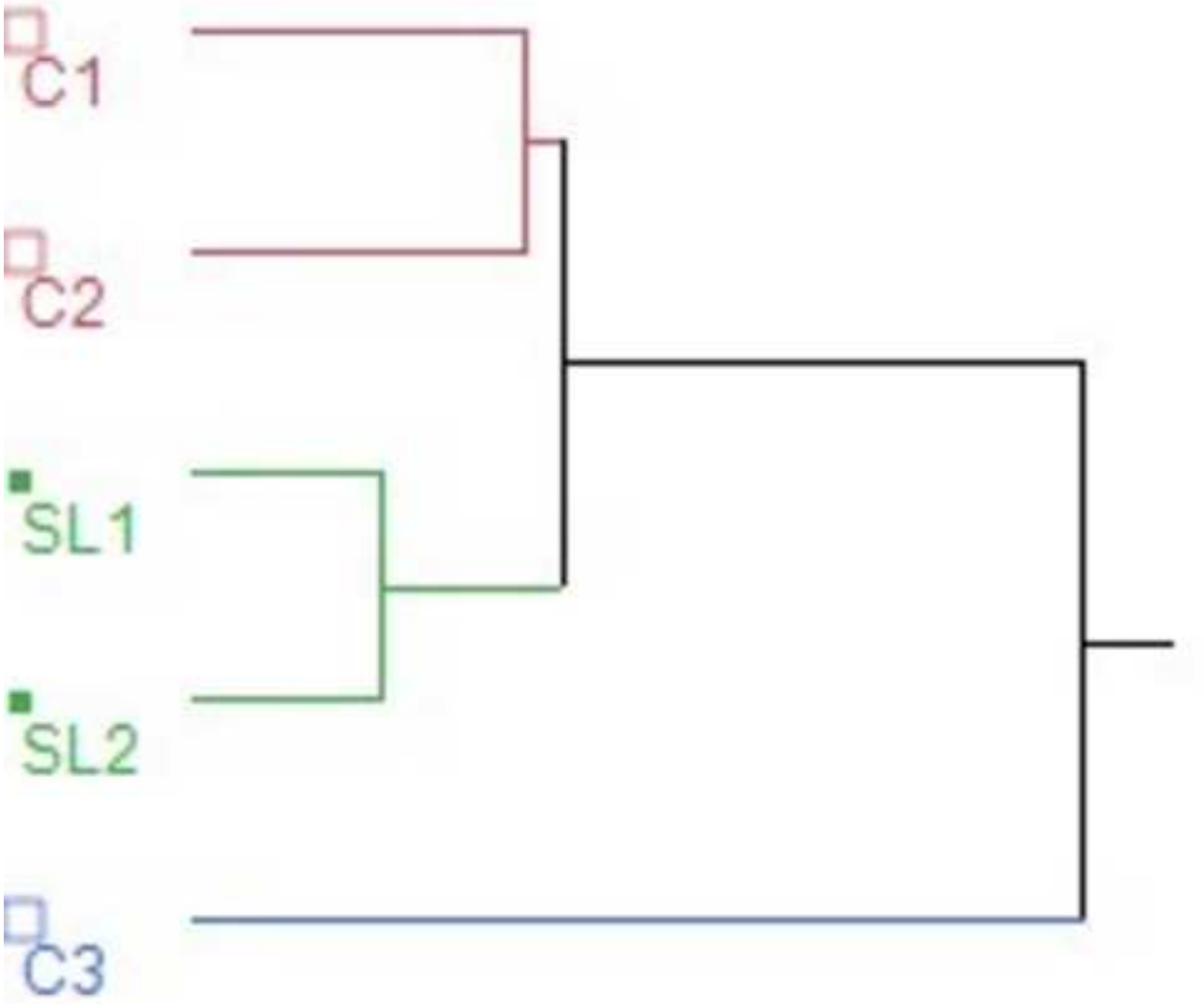

Figure 2