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Phenolic compounds profile and antioxidant properties of six sweet cherry (*Prunus avium*) cultivars

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1 **Abstract**

2 Sweet cherry (*Prunus avium*) fruits are a nutritionally important food rich in dietary phenolic
3 compounds. The aim of this study was to investigate the phenolic profile and chemometric
4 discrimination of fruits from six cherry cultivars using a quantitative metabolomics approach, which
5 combine non-targeted mass spectrometry and chemometric analysis. The assessment of the phenolic
6 fingerprint of cherries allowed the tentative identification of 86 compounds. A total of 40
7 chlorogenic acids were identified in cherry fruit, which pointed out hydroxycinnamic acid
8 derivatives as the main class of phenolics by number of compounds. Among the compounds
9 detected, 40 have been reported for the first time in sweet cherry fruit. Hydroxycinnamic acids are
10 also the quantitatively most represented class of phenolic compounds in the cherry cultivars with
11 the exception of Lapins and Durone della Marca where the most representative class of phenolic
12 compounds were anthocyanins and flavan-3-ols, respectively. This non-targeted approach allowed
13 the tentative identification of the cultivar-compound relationships of these six cherry cultivars.
14 Both anthocyanins and colorless phenolic compounds profile appeared to be cultivar-dependent. In
15 detail, anthocyanins and flavonols patterns have the potential to be used for the determination of a
16 varietal assignment of cherries.

17 **Keywords:** cherries, mass spectrometry, polyphenols fingerprint, metabolomics, principal
18 components analysis

19 **1. Introduction**

20 Dietary habits are thought to be pivotal in the prevention of chronic and degenerative diseases such
21 as cancer, cardiovascular disease and metabolic syndrome-related disorders (Del Rio et al., 2013).

22 In this context, the daily intake of total polyphenols has been inversely associated with the risk of
23 cardiovascular diseases, all-cause mortality in subjects at high cardiovascular risk and cancer
24 (Tresserra-Rimbau et al., 2014a and 2014b). Not only total polyphenols, but also the intake of single
25 classes of phenolic compounds may be positive for human health. Some human randomized
26 intervention studies evidenced that the intake of flavan-3-ols-rich food (such as cocoa),
27 anthocyanins-rich food (such as berries) and flavanone rich food (such as citrus fruit) may have
28 beneficial effects on clinically significant risk factors associated with cardiovascular diseases (Del
29 Rio, et al., 2013).

30 Sweet cherries (*Prunus avium*) have been described as a rich source of dietary phenolic compounds
31 with plenty of health benefits and playing an important role in preventing several chronic diseases
32 (Ferretti, Bacchetti, Belleggia, & Neri, 2010; Mc Cune, Kubota, Stendell-Hollis, & Thomson,
33 2011). Extracts of sweet cherries have shown antioxidant properties both in cell-free and cell-based
34 assays and *in vitro* anti-proliferative activity against human cancer cells from colon (HT-29 and
35 HCT-15) and stomach (MKN45) (Bastos et al., 2015; Serra, Duarte, Bronze, & Duarte, 2011).

36 Consumption of sweet cherry fruit or juice (Bing cultivar) results in a significant reduction in
37 systolic and diastolic blood pressure and heart-rate in hypertensive subjects (Kent, Charlton, Jenner,
38 & Roodenrys, 2016) as well as several biomarkers associated with inflammatory diseases in healthy
39 humans (Kelley et al., 2013).

40 Some of the phenolic compounds reported to be present in sweet cherries include hydroxycinnamic
41 acids, anthocyanins, flavan-3-ols and flavonols (Ballistreri et al., 2013; de Pascual-Teresa, Santos-
42 Buelga, & Rivas-Gonzalo, 2000; Gao, & Mazza, 1995; Girelli, De Pascali, Del Coco, & Fanizzi,
43 2016; Picariello, De Vito, Ferranti, Paolucci, & Volpe, 2016). Sweet cherries have an anthocyanin

44 composition of 3-glucoside and 3-rutinoside of cyanidin as the major anthocyanins and the 3-
45 rutinoside of peonidin and pelargonidin as the minor anthocyanins (Ballistreri et al., 2013; Gao, &
46 Mazza, 1995). Caffeoylquinic and coumaroylquinic acids have been described as the major
47 hydroxycinnamic acids in sweet cherries (Ballistreri et al., 2013; Gao, & Mazza, 1995). Among
48 flavan-3-ols and flavonols, epicatechin and quercetin-3-rutinoside have been detected as the main
49 compounds belonging to these classes present in sweet cherries (de Pascual-Teresa et al., 2000;
50 Pacifico et al., 2014). The amount of individual identified phenolic compounds in sweet cherries is
51 inconstant, depending strongly on the cultivars (Picariello et al., 2016).

52 Therefore, some efforts aiming at the identification of phenolic compounds in sweet cherry fruit
53 have been done. Nevertheless, the comprehensive characterization of this phenolic-rich matrix is
54 still lacking. Most of the studies carried out so far identified and quantified sweet cherry phenolics
55 by using high performance liquid chromatography coupled with photodiode array detector (HPLC-
56 DAD) (Ballistreri et al., 2013; de Pascual-Teresa, et al., 2000; Gao, & Mazza, 1995), but very few
57 studies applied mass spectrometry detection for the qualitative analysis and characterization of
58 sweet cherry fruit (Bastos et al., 2015; Pacifico et al., 2014; Picariello, et al., 2016). To the best of
59 our knowledge, the most detailed study carried out in order to estimate the phenolic composition of
60 sweet cherries allowed the identification of 21 compounds belonging to different phenolic classes
61 (Picariello, et al., 2016).

62 This work, mainly aimed at HPLC-mass spectrometry identification and quantification of phenolic
63 compounds in six Italian cherry cultivars. Nowadays, there are two different approaches processing
64 and explaining metabolomic data (Wishart, 2008). In one version, known as “chemometric
65 approach”, chemical compounds are not initially identified. The complex data recorded by mass
66 spectrometry are directly used for global multivariate statistical analysis such as principal
67 component analysis (PCA). After identifying significant differences, the most informative peaks in
68 the spectra are characterized and quantified. In the second approach, known as “quantitative

69 approach” most of the compounds in the sample are identified and quantified using mass
70 spectrometry. This information is then used to perform multivariate statistical analysis allowing to
71 establish the most important differences between samples (Wishart, 2008). We utilized thea
72 “quantitative metabolomics” approach, which combine non-targeted mass spectrometry and
73 chemometric analysis ~~(Wishart, 2008)~~. Non-targeted procedure has been recently utilized to
74 investigate the phenolic fingerprint of different vegetable materials overcoming the difficulties and
75 disadvantages of traditional targeted approaches (Calani et al., 2013; Mena et al., 2016). The
76 applied non-targeted procedure integrates a fast separation with the possibility of analyzing a large
77 amount of data. The chemometric ~~approach~~ analysis allowed the identification of the intra- and
78 inter-specific variability in cherry polyphenols and which factors contribute most to this variability.
79

80 **2. Materials and methods**

81 **2.1. Materials**

82 Cyanidin 3-O-glucoside, quercetin 3-O-glucoside, 5-O-caffeoylquinic acid, 5-O-feruloylquinic acid,
83 catechin, epicatechin, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox),
84 2,4,6-tripyridyl-S-triazine (TPTZ), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)
85 and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (Milan, Italy). Acetonitrile,
86 methanol, formic acid were obtained from Carlo Erba (Milan, Italy). Solid phase extraction (SPE)
87 cartridge (C18, 50 µm, 60 Å, 500 mg) were supplied by Waters (Milan, Italy). The absorbance was
88 read using a Jasco V-550 UV/Vis spectrophotometer.

89

90 **2.2. Cherry**

91 Samples of six sweet cherry (*Prunus avium*) varieties (Della Marca, Celeste, Bigarreau, Durone
92 Nero, Lapins and Moretta) were harvested at commercial maturity in Vignola (Modena province,
93 Italy) during spring (Celeste, Bigarreau, Durone Nero and Moretta cultivars) or summer (Lapins
94 and Della Marca cultivars) 2015. For each variety, about 2 kg of cherries were randomly sampled
95 from several trees and processed immediately or frozen within 1 h after harvest and stored at -80°C
96 until used. The cherry cultivars were selected according to the different skin and flesh colors, from
97 pale yellow flesh and slightly reddish skin to dark red cultivars (**see supplementary Figure S1**).

98

99 **2.3. Extraction of phenolic compounds**

100 Sweet cherry fruits (30 g) were pitted and homogenized with 50 mL of water/methanol/formic acid
101 (28:70:2, v/v/v) with an Ultra-Turrax homogenizer for 2 min. The suspension was then stirred for
102 120 min at 30°C, centrifuged (3000g, 30 min, 4°C) and the supernatant filtered on paper. The
103 extracts (1 mL) were then passed through a SPE cartridge preconditioned with 4 mL of acidified
104 methanol (containing 0.1% of formic acid), followed by 5 mL of acidified water (containing 0.1%

105 of formic acid). Elution was carried out with acidified water (6 mL) to eliminate the unbound
106 material and phenolic compounds were then desorbed by elution with 3 mL of acidified methanol.
107 The obtained polyphenol-rich extracts were then used for the subsequent analysis. Each sample was
108 extracted in triplicate.

109

110 ***2.4. Identification and quantification of phenolic compounds by liquid chromatography***

111 ***electrospray ionization ion trap mass spectrometer (LC-ESI-IT-MS)***

112 Sweet cherry polyphenol-rich extracts were analyzed on a HPLC Agilent 1200 Series system
113 equipped with a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 µm particle size,
114 Hamilton company, Reno, Nevada, USA). The mobile phase consisted of (A) H₂O/formic acid
115 (99:1, v/v) and (B) acetonitrile/formic acid (99:1, v/v). The gradient started at 4% B for 0.5 min
116 then linearly ramped up to 30% B in 60 min. The mobile phase composition was raised up to 100%
117 B in 1 min and maintained for 5 min in order to wash the column before returning to the initial
118 condition. The flow rate was set at 1 mL/min. After passing through the column, the eluate was
119 split, and 0.3 mL/min was directed to an Agilent 6300 ion trap mass spectrometer. Two MS
120 experiments were performed, one in ESI negative ion mode and one using positive ESI ionization
121 (for anthocyanins), under the same chromatographic conditions. ESI-MS parameters were as
122 follows: potential of the ESI source, 4 kV; capillary temperature, 400°C (Del Rio et al., 2004).
123 Identification of phenolic compounds in all samples was carried out using full scan, data-dependent
124 MS² scanning from *m/z* 100 to 800 and selected reaction monitoring.

125 Anthocyanins in sweet cherry extracts were quantified in cyanidin 3-O-glucoside equivalents.

126 Chlorogenic acids were quantified as 5-O-caffeoylquinic acid equivalents. Flavan-3-ols and
127 flavonols were quantified as epicatechin and quercetin-3-glucoside equivalents, respectively.

128 Hydroxybenzoic acids were quantified as gallic acid equivalents. Quantitative results were

129 expressed as mg of compounds per 100 g of fresh weight fruit.

130 The limits of detection (LOD) were 0.1 ng for anthocyanins, 0.27 ng for hydroxybenzoic acids, 0.14
131 ng for chlorogenic acids, 1.04 ng for flavan-3-ols, 0.19 ng for flavonols and other flavonoids. The
132 limits of quantification (LOQ), defined as the lowest quantifiable concentration, were 0.6 ng for
133 anthocyanins, 1.9 ng for hydroxybenzoic acids, 1.3 ng for chlorogenic acids, 2.4 ng for flavan-3-ols,
134 2.1 ng for flavonols and other flavonoids.

135

136 **2.5. Antioxidant capacity analysis**

137 The total antioxidant capacity was performed by using four different assays.

138 The ABTS assay was carried out according to Re et al. (1999). The ABTS scavenging capacity was
139 expressed as μmol of trolox equivalent per 100 g of fresh weight fruit, by means of a calibration
140 curve obtained with trolox (50 to 500 $\mu\text{mol/L}$), in the same assay conditions.

141 For the determination of the reducing ability of samples, a protocol based on the ferric
142 reducing/antioxidant power (FRAP) assay was utilized (Benzie & Strain, 1999). FRAP values were
143 referred to a linear regression curve plotting absorbance versus trolox concentration in the range of
144 50-1000 $\mu\text{mol/L}$, and expressed as μmoles of trolox equivalent per 100 g of fresh weight fruit.

145 The capacity to scavenge hydroxyl radicals was evaluated according to the method reported by
146 Tagliazucchi, Helal, Verzelloni, & Conte (2016). The hydroxyl radical scavenging capacity was
147 expressed as μmol trolox per 100 fresh weight fruit. Calibration curve was created by using trolox
148 standard solution at concentrations ranging between 350 and 1500 $\mu\text{mol/L}$.

149 The superoxide anion radical scavenging activity was determined by the method of Bamdad &
150 Chen (2013). The superoxide anion scavenging capacity was expressed as μmol trolox per 100 fresh
151 weight fruit, by means of a calibration curve obtained with trolox (1000 to 10000 $\mu\text{mol/L}$), in the
152 same assay conditions.

153

154 **2.6. Statistics**

155 All data are presented as mean \pm SD for three replicates for each prepared sample. Univariate
156 analysis of variance (ANOVA) with Tukey's post-hoc test was applied using Graph Pad prism 6.0
157 (GraphPad Software, San Diego, CA, U.S.A.) when multiple comparisons were performed. The
158 differences were considered significant with $P < 0.05$. Principal component analysis was carried out
159 using the analytical data as variables and utilizing the software Solo (Eigenvector Research Inc.,
160 Manson, WA, U.S.A).

161 **3. Result and discussion**

162

163 **3.1. Identification of the major phenolic compounds in the six cherry cultivars**

164 In this study, the fruits of six sweet cherry cultivars (*Prunus avium*) were compared for their
165 phenolic profile and content. The phytochemical composition of these fruits, focusing on the
166 phenolic fraction, was investigated using a non-targeted procedure through LC-ESI-MS/MS
167 experiments. This approach allowed the full characterization of the phenolic fraction of cherries and
168 the tentative identification of 86 compounds (**Table 1**).

169 Six compounds were identified by comparison with reference standards, while the remaining 80
170 compounds were tentatively identified based on the interpretation of their fragmentation patterns
171 obtained from mass spectra (MS² experiments) and by comparison with the literature. The mass
172 spectrum data along with peak assignments for the identified phenolic compounds are described in
173 **Table 1** and in supplementary **Figures S2-S7**.

174

175 **3.1.1. Chlorogenic acids**

176 Six signals (peaks 1-6) at m/z 353 ($[M-H]^{-1}$) were observed that eluted between 14 and 27 min
177 (**Figure S2**). These components were easily identified as caffeoylquinic acids (CQAs) using the
178 hierarchical keys previously developed by Clifford, Johnston, Knight, & Kuhnert (2003). The peaks
179 1 and 2 both showed in the MS² spectra a base peak at m/z 191 and an intense secondary peak at m/z
180 179 (**Table 1**), consistent with the fragmentation pattern of 3-CQA (Clifford et al., 2003). Using the
181 same chromatographic conditions, Clifford, Kirkpatrick, Kuhnert, Roozendaal, & Rodrigues
182 Salgado (2008) found that *cis*-3-CQA eluted earlier than *trans*-3-CQA. Peaks 3 and 5 had a MS²
183 base peak at m/z 173 which is diagnostic of 4-CQA. On the basis of the elution order peak 3 was
184 identified as *cis*-4-CQA and peak 5 as *trans*-4-CQA. Peaks 4 and 6 had a fragmentation MS²
185 spectra consistent with that of an authentic 5-CQA standard. Based on the elution order peak 4 was

186 identified as *trans*-5-CQA and peak 6 as *cis*-5-CQA (Clifford et al., 2008). Peaks 7-12 had a ([M-
187 H]⁻¹) at *m/z* 337 (**Figure S2**), which on the basis of their MS² spectra (**Table 1**) and in comparison
188 with the scheme proposed by Clifford et al. (2003) were identified as coumaroylquinic acids
189 (CoQAs). In particular, peaks 7 and 8 were assigned to the isomers *cis*- and *trans*-3-CoQA, peaks 9
190 and 10 to the isomers *cis*- and *trans*-4-CoQA and peaks 11 and 12 to the isomers *trans*- and *cis*-5-
191 CoQA (Clifford et al., 2008). Five feruloylquinic acids (FQAs) with a negative charged molecular
192 ion at *m/z* 367 ([M-H]⁻¹) were identified in sweet cherries. Following the hierarchical scheme and
193 the order of elution they were identified as *cis*-3-FQA (peak 13), *trans*-3-FQA (peak 14), *cis*-4-
194 FQA (peak 15), *trans*-5-FQA (peak 16 also co-eluted with an authentic standard) and *cis*-5-FQA
195 (peak 17) (**Table 1** and **Figure S2**). Compounds corresponding to the peaks 1-17 had already been
196 described in sweet cherry cultivars (Moeller, & Herrmann, 1983). The following minor
197 hydroxycinnamic acids have been described in sweet cherries for the first time in this study. Seven
198 signals (peaks 18-24) at *m/z* 515 were observed. Five compounds (peaks 18-22), eluting between 13
199 and 25 min, produced at MS² *m/z* 353, 341 and 179. According to Clifford, Wu, Kirkpatrick, &
200 Kuhnert (2007), they can be classified as caffeoylquinic acid-glycosides with the caffeic acid
201 moiety (origin of *m/z* 179) attached to both the quinic acid (origin of *m/z* 353) and the hexose
202 (origin of *m/z* 341). The other two compounds with *m/z* 515 were identified as 3,5-dicaffeoylquinic
203 acid (peak 23) and 4,5-dicaffeoylquinic acid (peak 24) by their fragmentation pattern (Clifford,
204 Knight, & Kuhnert, 2005). Peaks 25-27 had a negative charged molecular ion at *m/z* 353 ([M-H]⁻¹)
205 which is characteristic of both caffeoylquinic acid lactones (CQLs) and caffeoylshikimic acids
206 (CSAs). Based on previously published fragmentation spectra (Jaiswal, Matei, Subedi, & Kuhnert,
207 2014), peaks 25 and 27 were identified as CSAs, whereas peak 26 contained both 3- and 4-CQL.
208 Two additional lactones, 3-coumaroylquinic acid lactone (3-CoQL) and 4-CoQL were identified as
209 compounds in peaks 28 and 29 (Jaiswal et al., 2014). Peaks 30 and 31 exhibited the same molecular
210 ion at *m/z* 499. However, they differed in their MS² fragment ion spectra (**Table 1**). According to

211 Clifford, Mark, Knight, & Kuhnert (2006a) they were tentatively identified as 3-*p*-coumaroyl-5-
212 caffeoylquinic acid and 3-caffeoyl-4-*p*-coumaroylquinic acid, respectively. Several
213 hydroxycinnamic acid hexoses were identified in cherries, for the first time. In particular, peak 32
214 was identified as coumaroyl hexose (m/z 325), peaks 33 and 34 as caffeoyl hexose (m/z 341), peak
215 38 as feruloyl hexose (m/z 355) and peak 39 sinapoyl hexose (m/z 385), by its fragmentation spectra
216 (Dall'Asta et al., 2012; Clifford et al., 2007). Two additional signals were found at m/z 341 (peaks
217 35 and 36). They were always characterized by a loss of 162 Da (hexoside moiety) with the
218 appearance of a daughter ion at m/z 179 consistent with the presence of a caffeic acid residue. In
219 keeping with published data, these compounds were tentatively identified as caffeic acid-glycosides
220 (Clifford et al., 2007). Similarly, peak 40, which was characterized by a negative molecular ion at
221 m/z 385, was identified as sinapic acid-glycoside. Peak 37 had a $[M-H]^-$ at m/z 327, which upon
222 MS^2 fragmentation yielded a daughter ion at m/z 165, consistent with a loss of 162 Da (hexoside
223 moiety). A comparison with previous finding indicated that peak 36 is probably caffeoyl alcohol-
224 hexoside (Vanholme et al., 2012).

225 A total of 40 chlorogenic acids were identified in cherry fruit, which indicated hydroxycinnamic
226 acid derivatives as the main class of phenolics by number of compounds.

227

228 **3.1.2. Flavan-3-ols**

229 Four monomeric flavan-3-ols already described in cherries were found (**Figure S3**) (de Pascual-
230 Teresa et al., 2000; Bastos et al., 2015). Catechin and epicatechin (peaks 41 and 42; m/z 289) were
231 identified by comparison of retention time and fragmentation spectra with authentic standards.
232 Epicatechin-3-gallate and (epi)catechin-glucoside (peaks 43 and 44; m/z 441 and 451, respectively)
233 were identified based on their fragmentation patterns (MS^2 experiments) and by comparison with
234 the literature (Bastos et al., 2015; Del Rio et al., 2004).

235 Five type-B procyanidin dimers ((epi)catechin-(epi)catechin) were identified at m/z 577 (peaks 47-
236 51) (**Figure S3**). The fragmentation pattern reported in **Table 1** is consistent with previously
237 reported data (Gu et al., 2003). De Pascual-Teresa et al. (2000) identified in sweet cherries six type-
238 B procyanidin dimers and one procyanidin trimer. We were not able to identify B-type trimer in our
239 study but we reported for the first time the presence of two B-type procyanidin tetramers, one B-
240 type procyanidin pentamer and one propelargonidin dimer. The doubly charged ion, $[M-2H]^{2-}$ 576,
241 was present as two peaks (45 and 46) and gave MS^2 fragments at m/z 289 ((epi)catechin), 425, and
242 407, a fragmentation pattern consistent with B-type tetrameric procyanidin (Wollgast, Pallaroni,
243 Agazzi, & Anklam, 2001). Peak 53 had a negative double charged ion at m/z 720 (molecular weight
244 of 1440 Da consistent with B-type procyanidin pentamer) which gave product ions in the MS^2
245 spectra at m/z 289 ((epi)catechin), 577 (procyanidin dimer), 405 and 407, and was tentatively
246 identified as B-type procyanidin pentamer (Wollgast et al., 2001). Finally, peak 52 presented a $[M-$
247 $H]^-$ at m/z 561 whose fragmentation pattern (**Table 1**) was identical to that of the propelargonidin
248 dimer (epi)afzelechin-(epi)catechin, previously describe in strawberries (Gu et al., 2003).

249

250 **3.1.3. Flavonols and other minor colorless phenolic compounds**

251 Among the 7 flavonol derivatives (**Table 1** and **Figure S4**) detected, 5 (compounds 54, 55, 57, 58
252 and 59) had been previously identified in sweet cherries (Chaovanalikit, & Wrolstad, 2004; Bastos
253 et al., 2015; Picariello et al., 2016), while compounds 56 and 60 have been described in sweet
254 cherry for the first time. Compound 60 was identified by comparison with previously reported data
255 (Mena et al., 2016). Compound 56 had the same negative molecular ion (m/z 463) as compound 54,
256 which was identified as quercetin-3-*O*-glucoside by comparison with an authentic standard. The
257 analysis of MS^2 spectra revealed the loss of 162 Da (hexose group) to produce an m/z 301
258 (quercetin) daughter ion. This compound was therefore tentatively identified as quercetin-hexoside.

259 Compounds 61 and 62 presented an identical pseudomolecular ion $[M-H]^-$ at m/z 433 releasing a
260 fragment ion at m/z 271 (loss of a hexose group), which might be coherent with naringenin-
261 hexoside (**Table 1** and **Figure S5**) (Bastos et al., 2015). Compounds 63 and 64 showed the same
262 negative molecular ion (m/z 611) which gave product ions in the MS^2 spectra at m/z 285 and 303
263 characteristic of taxifolin aglycone (Bastos et al., 2015). The loss of 308 Da is typical of a rutinose
264 moiety, and therefore these compounds were tentatively identified as isomers of taxifolin-rutinoside
265 (Bastos et al., 2015). On the other hand, compounds 65 and 66 presented the same negative
266 molecular ion (m/z 465) and a fragmentation pattern typical of taxifolin-hexoside (Bastos et al.,
267 2015).

268 Three hydroxybenzoic acid-glycosides and two hydroxybenzoyl acid hexoses have been described
269 for the first time in sweet cherries in this study (**Table 1** and **Figure S6**). Four signals (peaks 67-70)
270 at m/z 315 were observed. All of the compounds in the MS^2 experiments gave a base peak at m/z
271 153, corresponding to protocatechuic acid aglycone that correspond0s to a loss of 162 Da (hexose
272 moiety). The MS^2 spectra of the compounds 69 and 70 were also characterized by fragment ions at
273 m/z 195 $[M-H-(CH_2O)_4]^-$, 267 $[M-H-(CH_2O)-H_2O]^-$, 207 $[M-H-(CH_2O)_4-H_2O]^-$ and 177 $[M-H-$
274 $(CH_2O)_3-H_2O]^-$ which could be produced by the loss of a $-CHOH$ unit. This behavior is indicative of
275 sugar fragmentation and, is essentially identical to that of caffeoyl hexose (Clifford et al., 2007).

276 We tentatively assigned these to isomeric protocatechuoyl hexose. Whereas, compounds 67 and 68
277 did not show any evidence of sugar fragmentation and were, therefore, tentatively identified as
278 protocatechuic acid-glycoside. Protocatechuic acid (m/z 153) was also found as aglycone (peak 71).

279 Two signals (peaks 72 and 73) at m/z 299 gave a base peak in the fragmentation spectra at m/z 137,
280 which is indicative of the presence of a hydroxybenzoic acid residue. This fragment originated by
281 the loss of 162 Da suggesting the presence of a hexoside moiety. Peak 73 showed evidence of sugar
282 fragmentation (signals at m/z 269, 239, 209 and 179 characteristic of the loss of $-CHOH$ units)
283 whereas peak 72 did not show any evidence of sugar fragmentation. The two compounds were,

284 therefore, tentatively identified as hydroxybenzoyl-glycoside (peak 72) and hydroxybenzoic acid
285 hexose (peak 73). Peak 74 showed a molecular negative ion at m/z 329, which fragmented in the
286 MS² experiments giving a base peak at m/z 167, suggesting the presence of a vanillic acid residue.
287 The loss of 162 Da and the absence of sugar fragmentation evidence prompt us to tentatively
288 identify this compound as vanillic acid-glycoside.

289

290 **3.1.4. Anthocyanins**

291 A total of 12 anthocyanins were identified in sweet cherries (**Table 1** and **Figure S7**). Compounds
292 75-80 had been already identified in sweet cherries, while compounds 81-86 have been described in
293 sweet cherries for the first time (Chaovanalikit, & Wrolstad, 2004; Gao, & Mazza, 1995).

294 Compound 81 had a positive charged molecular ion at m/z 757, yielding MS² ions at m/z 595, 449
295 and 287. The fragments at m/z 595, 449 and 287 correspond to the loss of a hexose moiety (-162
296 Da), a coumaroyl moiety (-146 Da) and a further hexose moiety (-162 Da), respectively. The ion at
297 m/z 287 suggested that the aglycone is cyanidin and the compound was therefore tentatively
298 identified as cyanidin-3-(6-O-p-coumaroyl)-5-O-diglucoside (Flamini, 2013).

299 Compound 82 had a positive charged molecular ion at m/z 581, yielding a MS² ion at m/z 287,
300 which suggests the presence of cyanidin as aglycone. This compound was identified, on the basis of
301 published data, as cyanidin-3-sambubioside (Giusti, Rodríguez-Saona, Griffin, Wrolstad, 1999).

302 Compounds 83-86 were tentatively identified by comparison with previously reported data
303 (Flamini, 2013; Pereira-Caro, Cros, Yokota, & Crozier, 2013).

304

305 **3.2. Profile of phenolic compounds in the six cherry cultivars**

306 **Table 2** and **Figure 1** provide information about the amount of the 86 tentatively identified
307 phenolic compounds in the six cherry cultivars.

308

309 3.2.1. Chlorogenic acids

310 Caffeoylquinic and coumaroylquinic acids were the main chlorogenic acids found in the studied
311 cherry cultivars (in average 42.85% of total identified phenolic compounds). Among them, 3-
312 coumaroylquinic acid was the major compound detected, with the exception of the cultivar Lapins
313 where 3-caffeoylquinic acid was present at a higher amount. Interestingly, both the caffeoylquinic
314 and coumaroylquinic acids were found in the cherries as *trans* and *cis* isomers. It is known that,
315 naturally, plants synthesize the *trans*-isomers over the *cis*-isomers (Clifford, Jaganath, & Clifford,
316 2006b). The latter *cis* isomers have been reported to be formed in tissue or extracts previously
317 exposed to UV light. It has been hypothesized that chlorogenic acids present in the plant tissue
318 exposed to natural UV light (such as fruits) undergo *trans-cis* isomerization, whereas in the
319 unexposed tissue, such as coffee seeds, they remain stable (Clifford et al., 2005, 2006b, 2008).
320 Isomerization can also take place during MS experiments with electrospray ionization (Xie et al.,
321 2011). However, *trans-cis* isomerization was not observed when a pure standard of *trans* 5-
322 caffeoylquinic acid was injected into the mass spectrometer at the same conditions of the extract
323 excluding the possibility of an artefact due to the electric field during MS experiments. To the best
324 of our knowledge, this is the first demonstration of the presence in high amounts of the *cis* isomers
325 of chlorogenic acids in cherries. The amount of the *trans* isomer of 3-coumaroylquinic acid varied
326 from 53.42 mg/100g (cultivar Della Marca) to 452.52 mg/100g (cultivar Durone Nero), whereas the
327 quantity of the *cis* isomer ranged between 15.15 mg/100g (cultivar Della Marca) and 220.54
328 mg/100g (cultivar Moretta). Previous studies found that the fruit of the sweet cherry cultivar Sam
329 was that with the highest amount (131.5 mg/100g) of *trans* 3-coumaroylquinic acid (Gao, & Mazza,
330 1995), which is lower than the amount found in this study in the fruit of the cultivars Durone Nero,
331 Bigarreau, Moretta and Celeste. Among the other coumaroylquinic acids, 4-coumaroylquinic acid
332 was always present at a higher concentration than 5-coumaroylquinic acids. The cultivar with the
333 highest concentration of coumaroylquinic acids was Durone Nero where they accounted for 32.79%

334 of the total phenolic compounds, with the *trans* isomers of 3-coumaroylquinic acids accounting for
335 23.83% and the *cis* isomer for 7.16% of the total phenolic compounds. The cultivar with the highest
336 content of caffeoylquinic acids was Lapins, which contained 230.20 mg/100g of total
337 caffeoylquinic acids, representing the 18.69% of total phenolic compounds. The amount of *trans* 3-
338 caffeoylquinic acids found in the tested cultivars is in keeping with previous studies (Moeller, &
339 Herrmann, 1983; Gao, & Mazza, 1995). Additional minor hydroxycinnamic acid derivatives were
340 found in sweet cherry cultivars, with *trans*-3-feruloylquinic acid and caffeic acid-glycoside being
341 the most representative.

342

343 **3.2.2. Flavan-3-ols**

344 Among the identified flavan-3-ols, epicatechin was the predominant ranging in concentration
345 between 136.61 mg/100g (cultivar Lapins) and 397.19 mg/100g (cultivar Durone Nero). The
346 amount of epicatechin was from 5 to 40 times higher than that previously reported in sweet cherry
347 cultivars (Arts, van de Putte, & Hollman, 2000; de Pascual-Teresa et al., 2000). Catechin, instead,
348 was always present at lower concentration. The other two identified flavan-3-ol monomer,
349 epicatechin-3-gallate and catechin-glucoside were present at low concentration in all the analyzed
350 cultivars, with the exception of the cultivars Durone Nero and Bigarreau which contained catechin-
351 glucoside in appreciable amount (7.77 and 10.16 mg/100g, respectively).

352 The total procyanidin content in the cherry cultivars ranged from 13.39 to 41.69 mg/100g in the
353 cultivars Lapins and Durone Nero, respectively. The total levels of procyanidins in sweet cherries
354 are in line with those reported by Chaovanalikit, & Wrolstad (2004), who found that the sweet
355 cherry cultivars Royal Ann and Rainier contained 20.2 and 7.2 mg/100g of total procyanidins,
356 respectively.

357 The cultivar with the highest concentration of flavan-3-ols (monomers + oligomers) was Durone
358 Nero (515.64 mg/100g) where they accounted for 27.16% of the total phenolic compounds. In the

359 cultivar Della Marca, the amount of total flavan-3-ols account for about the 56% of total phenolic
360 compounds.

361

362 ***3.2.3. Flavonols and other minor colorless phenolic compounds***

363 The sweet cherry cultivars analyzed in this study contained seven flavonols, with their
364 concentration ranging from 11.39 to 85.64 mg/100g in the cultivars Della Marca and Bigarreau,
365 respectively. In all the cultivars, quercetin-3-rutinoside was the main flavonol detected in amounts
366 comprised between 5.13 and 51.97 mg/100g. Previous studies reported quercetin-3-rutinoside
367 quantities in sweet cherry cultivars between 7.8 and 34.2 mg/100g (Serra et al., 2011).

368 Four dihydroflavonols were identified in some of the cherry cultivars, in amounts that exceeded
369 those of flavonols. In the cultivar Lapins, the two isomers of taxifolin-rutinoside accounted for
370 9.94% of total phenolic compounds, representing the third most concentrated colorless phenolic
371 compound after 3-caffeoylquinic acid and epicatechin. Taxifolin glycosides have been recently
372 reported in sweet cherry fruits in amounts comparable with that found in this study (Bastos et al.,
373 2015).

374 Isomers of the flavanone naringenin-hexoside and of hydroxybenzoic acids glycoside were found in
375 the different cultivars at low concentrations, representing less than 1% of total phenolic compounds.

376

377 ***3.2.4. Anthocyanins***

378 Cyanidin-3-rutinoside and cyanidin-3-glucoside were the main anthocyanin detected in the four
379 anthocyanin-rich cherry cultivars (Moretta, Durone Nero, Bigarreau and Lapins) representing the
380 87.11-97.57% of total anthocyanins. The remaining anthocyanins consisted of ten minor
381 compounds with peonidin-3-rutinoside and pelargonidin-3-rutinoside being the most representative.
382 Significant differences in the concentration of total and individual anthocyanins in sweet cherry
383 cultivars have been previously reported (Esti, Cinquanta, Sinesio, Moneta, & Di Matteo, 2002; Gao,

384 & Mazza, 1995; Mozetič, Trebše, Simčič, & Hribar, 2004). The data obtained in the present study is
385 in keeping with these studies.

386 The highest amount of cyanidin-3-rutinoside was found in the cultivar Lapins (389.90 mg/100g)
387 where it represented the 31.65% of total phenolic compounds and the 84.25% of total anthocyanins.

388

389 **3.3. Antioxidant activity analysis**

390 To fully characterize the properties of the sweet cherry cultivars the ability to scavenge some
391 physiologically relevant radicals (superoxide anion and hydroxyl radical), organic nitro-radical
392 ABTS as well as the reducing power were also evaluated. In this study, the cultivar Lapins and
393 Moretta showed a significantly higher ABTS radical scavenging activity as well as a significantly
394 higher reducing power in comparison to other cultivars (**Figure 2A** and **2B**). The antioxidant
395 capabilities of the sweet cherry extracts determined with the FRAP and ABTS assays provided
396 values between 533.1 and 3153.6 $\mu\text{mol trolox}/100\text{g}$ fresh weight and between 1323.6 and 6784.9
397 $\mu\text{mol trolox}/100\text{g}$ fresh weight, respectively, being within the order of magnitude already reported
398 for cherries (Mc Cune et al., 2011; Picariello et al., 2016). However, when the scavenger ability
399 against physiologically relevant radicals were considered, the cultivar Durone Nero showed the
400 highest scavenger ability (**Figure 2C** and **2D**).

401

402 **3.4. Chemometric approach to evaluate the relationships among the results**

403 To achieve a better understanding of the characteristics of the different cherry cultivars and to
404 identify a potential relational network between cherry cultivars and phenolic compounds, principal
405 component analysis (PCA) was applied (**Figure 3**). Three principal components explained 80.53%
406 of the total variation. The bi-plot PC1 vs PC2 showed a clear splitting of the cultivars: the lightest
407 cherries negatively linked to the PC1 whereas the darkest cultivars had positive scores on the same
408 component (**Figure 3A** and **B**). The first group, Della Marca e Celeste, had negative scores on PC1

409 and were characterized by the presence of glycosides of hydroxycinnamic acids and caffeic acid
410 derivatives, and a low amount of anthocyanins. Otherwise, Bigarreau, Durone Nero, Lapins and
411 Moretta constituted the second group, positively linked to PC1, characterized by a high content in
412 anthocyanins. This clusterization obtained by PCA clearly reflected the visible differences due to
413 the cultivar and the type of cherries themselves (**Figure 3A and B**). PC2, mainly associated with
414 hydroxybenzoic and hydroxycinnamic acids, had positive loadings for protocatechuic acid and
415 glycosides of hydroxybenzoic acid and protocatechuic acid-glycoside, hydroxybenzoyl hexose,
416 vanillic acid-glycoside, caffeic acid derivatives and caffeoylquinic acid-glycoside (**Figure 3A and**
417 **C**).

418 Focusing on PC2 it is possible to notice how anthocyanins split themselves depending on cultivars:
419 peonidins, malvidins and derivatives had positive PC2 scores; differently cyanidins and
420 pelargonidins had no remarkable connection to the second component. The discrimination among
421 the darkest cultivars, which were split into two groups, was highlighted on PC2. The first group
422 composed of Durone Nero and Bigarreau showed a high content of flavan-3-ols, such as catechin
423 and epicatechin, and flavonols and derivatives, kaempferol-3-glucoside and kaempferol-3-
424 rutinoside. Moretta and Lapins characterized the second group and showed a positive correlation to
425 the second component and a high amount of hydroxybenzoic and hydroxycinnamic acids and
426 derivatives of both classes (**Figure 3A and C**). The third component explained about 19% of the
427 total variation and the bi-plot showed a lower data scattering between the axes. A clear
428 discrimination among darkest cultivars was also shown on PC3: Moretta and Bigarreau had positive
429 scores on PC3, otherwise Durone Nero and Lapins had a negative score on the same component
430 (**Figure 3B and C**). PC3 had positive loadings for tetramer and dimer B type of procyanidins and
431 was negatively correlated to the *cis* isomer of 4- and 5-feruloylquinic acid. It can also be noted that
432 a large amount of flavonols and derivatives were negatively linked to PC3, such as kaempferol-3-
433 rutinoside, kaempferol-3-glucoside, quercetin-7-O-glucoside-rutinoside and quercetin-3-O-

434 rutinoid

435 It should be noted that Celeste showed the most balanced phenolic profile among the cultivars
436 tested, exhibiting medium contents for all the compounds identified. Celeste showed constant
437 medium-low values for PC1, PC2 and PC3.

438

439 ***4. Conclusion***

440 The quantitative metabolomics approach allowed the tentative identification of 86 individual
441 phenolic compounds in cherry cultivars. Among the detected compounds, 40 have been reported for
442 the first time in cherry fruits. This non-targeted approach investigating the phenolic fingerprinting
443 and chemometric discrimination of the six cherry cultivars allowed the tentative identification of the
444 cultivar-compound relationships of these six cherry cultivars. Results reported in this study showed
445 that both cherry colorless phenolic compounds and anthocyanins vary, depending on the cultivar. In
446 detail, the anthocyanins and flavonols patterns have the potential to be used for the determination of
447 a varietal assignment of cherries. This is of paramount importance considering that most of the
448 produced sweet cherries are processed in semi-transformed products in which the original cultivar is
449 lost. The definition of easy-to-identify markers and the application of fast and reproducible
450 metabolomics approach is of preeminent importance for the identification of the cultivar used for
451 the production of processed foods. However, further studies are necessary to better understand how
452 the agro-climatic factors (such as growing, harvesting time, seasonal variability) may influence the
453 phenolic composition of the different cherry cultivars.

454

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Figure captions

Figure 1. Global percentage of hydroxycinnamic acids, flavan-3-ols, flavonols, hydroxybenzoic acids, anthocyanins and other flavonoids in the six sweet cherry cultivars.

Figure 2. Antioxidant capacity (expressed as $\mu\text{mol trolox}/100\text{g}$ of fresh weight), measured by ABTS (A), FRAP (B), superoxide anion (C) and hydroxyl scavenging (D) assays, of the polyphenols-rich extracts of six cherry cultivars (y-axes). Each sample was run in triplicate and results are reported as mean values \pm SD. Values in the same graph with different lowercase letter are significantly different ($P < 0.05$).

Figure 3. Principal component analysis of cherry cultivars. (A) Loading plot of PC1 versus PC2. (B) Loading plot of PC1 versus PC3. (C) Loading plot of PC2 versus PC3. Code number of compounds is reported in **Table 1**. The symbol ● identified cherry cultivars whereas the symbol ▲ identified the compounds.