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

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Abstract

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

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

1. Introduction

Dietary habits are thought to be pivotal in the prevention of chronic and degenerative diseases such as cancer, cardiovascular disease and metabolic syndrome-related disorders (Del Rio et al., 2013). In this context, the daily intake of total polyphenols has been inversely associated with the risk of cardiovascular diseases, all-cause mortality in subjects at high cardiovascular risk and cancer (Tresserra-Rimbau et al., 2014a and 2014b). Not only total polyphenols, but also the intake of single classes of phenolic compounds may be positive for human health. Some human randomized intervention studies evidenced that the intake of flavan-3-ols-rich food (such as cocoa), anthocyanins-rich food (such as berries) and flavanone rich food (such as citrus fruit) may have beneficial effects on clinically significant risk factors associated with cardiovascular diseases (Del Rio, et al., 2013).

Sweet cherries (*Prunus avium*) have been described as a rich source of dietary phenolic compounds with plenty of health benefits and playing an important role in preventing several chronic diseases (Ferretti, Bacchetti, Belleggia, & Neri, 2010; Mc Cune, Kubota, Stendell-Hollis, & Thomson, 2011). Extracts of sweet cherries have shown antioxidant properties both in cell-free and cell-based assays and *in vitro* anti-proliferative activity against human cancer cells from colon (HT-29 and HCT-15) and stomach (MKN45) (Bastos et al., 2015; Serra, Duarte, Bronze, & Duarte, 2011). Consumption of sweet cherry fruit or juice (Bing cultivar) results in a significant reduction in systolic and diastolic blood pressure and heart-rate in hypertensive subjects (Kent, Charlton, Jenner, & Roodenrys, 2016) as well as several biomarkers associated with inflammatory diseases in healthy humans (Kelley et al., 2013).

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Cherry

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

2.3. Extraction of phenolic compounds

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

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

2.4. Identification and quantification of phenolic compounds by liquid chromatography electrospray ionization ion trap mass spectrometer (LC-ESI-IT-MS)

Sweet cherry polyphenol-rich extracts were analyzed on a HPLC Agilent 1200 Series system equipped with a C18 column (HxSil C18 Reversed phase, 250×4.6 mm, 5 μm particle size, Hamilton company, Reno, Nevada, USA). The mobile phase consisted of (A) H_2O/formic acid (99:1, v/v) and (B) acetonitrile/formic acid (99:1, v/v). The gradient started at 4% B for 0.5 min then linearly ramped up to 30% B in 60 min. The mobile phase composition was raised up to 100% B in 1 min and maintained for 5 min in order to wash the column before returning to the initial condition. The flow rate was set at 1 mL/min. After passing through the column, the eluate was split, and 0.3 mL/min was directed to an Agilent 6300 ion trap mass spectrometer. Two MS experiments were performed, one in ESI negative ion mode and one using positive ESI ionization (for anthocyanins), under the same chromatographic conditions. ESI-MS parameters were as follows: potential of the ESI source, 4 kV; capillary temperature, 400°C (Del Rio et al., 2004). Identification of phenolic compounds in all samples was carried out using full scan, data-dependent MS^2 scanning from *m/z* 100 to 800 and selected reaction monitoring.

Anthocyanins in sweet cherry extracts were quantified in cyanidin 3-O-glucoside equivalents. Chlorogenic acids were quantified as 5-O-caffeoylquinic acid equivalents. Flavan-3-ols and flavonols were quantified as epicatechin and quercetin-3-glucoside equivalents, respectively. Hydroxybenzoic acids were quantified as gallic acid equivalents. Quantitative results were expressed as mg of compounds per 100 g of fresh weight fruit.

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

2.5. Antioxidant capacity analysis

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

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

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

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

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

2.6. Statistics

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

3. Result and discussion

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

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

Six compounds were identified by comparison with reference standards, while the remaining 80 compounds were tentatively identified based on the interpretation of their fragmentation patterns obtained from mass spectra (MS^2 experiments) and by comparison with the literature. The mass spectrum data along with peak assignments for the identified phenolic compounds are described in **Table 1** and in supplementary **Figures S2-S7**.

3.1.1. Chlorogenic acids

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

identified as *trans*-5-CQA and peak 6 as *cis*-5-CQA (Clifford et al., 2008). Peaks 7-12 had a ([M-H]$^{-1}$) at *m/z* 337 (**Figure S2**), which on the basis of their MS^2 spectra (**Table 1**) and in comparison with the scheme proposed by Clifford et al. (2003) were identified as coumaroylquinic acids (CoQAs). In particular, peaks 7 and 8 were assigned to the isomers *cis*- and *trans*-3-CoQA, peaks 9 and 10 to the isomers *cis*- and *trans*-4-CoQA and peaks 11 and 12 to the isomers *trans*- and *cis*-5-CoQA (Clifford et al., 2008). Five feruloylquinic acids (FQAs) with a negative charged molecular ion at *m/z* 367 ([M-H]$^{-1}$) were identified in sweet cherries. Following the hierarchical scheme and the order of elution they were identified as *cis*-3-FQA (peak 13), *trans*-3-FQA (peak 14), *cis*-4-FQA (peak 15), *trans*-5-FQA (peak 16 also co-eluted with an authentic standard) and *cis*-5-FQA (peak 17) (**Table 1** and **Figure S2**). Compounds corresponding to the peaks 1-17 had already been described in sweet cherry cultivars (Moeller, & Herrmann, 1983). The following minor hydroxycinnamic acids have been described in sweet cherries for the first time in this study. Seven signals (peaks 18-24) at *m/z* 515 were observed. Five compounds (peaks 18-22), eluting between 13 and 25 min, produced at MS^2 *m/z* 353, 341 and 179. According to Clifford, Wu, Kirkpatrick, & Kuhnert (2007), they can be classified as caffeoylquinic acid-glycosides with the caffeic acid moiety (origin of *m/z* 179) attached to both the quinic acid (origin of *m/z* 353) and the hexose (origin of *m/z* 341). The other two compounds with *m/z* 515 were identified as 3,5-dicaffeoylquinic acid (peak 23) and 4,5-dicaffeoylquinic acid (peak 24) by their fragmentation pattern (Clifford, Knight, & Kuhnert, 2005). Peaks 25-27 had a negative charged molecular ion at *m/z* 353 ([M-H]$^{-1}$) which is characteristic of both caffeoylquinic acid lactones (CQLs) and caffeoylshikimic acids (CSAs). Based on previously published fragmentation spectra (Jaiswal, Matei, Subedi, & Kuhnert, 2014), peaks 25 and 27 were identified as CSAs, whereas peak 26 contained both 3- and 4-CQL. Two additional lactones, 3-coumaroylquinic acid lactone (3-CoQL) and 4-CoQL were identified as compounds in peaks 28 and 29 (Jaiswal et al., 2014). Peaks 30 and 31 exhibited the same molecular ion at *m/z* 499. However, they differed in their MS^2 fragment ion spectra (**Table 1**). According to

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

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

3.1.2. Flavan-3-ols

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

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

3.1.3. Flavonols and other minor colorless phenolic compounds

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

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

Three hydroxybenzoic acid-glycosides and two hydroxybenzoyl acid hexoses have been described for the first time in sweet cherries in this study (**Table 1** and **Figure S6**). Four signals (peaks 67-70) at *m/z* 315 were observed. All of the compounds in the MS^2 experiments gave a base peak at *m/z* 153, corresponding to protocatechuic acid aglycone that correspond0s to a loss of 162 Da (hexose moiety). The MS^2 spectra of the compounds 69 and 70 were also characterized by fragment ions at *m/z* 195 $[M-H-(CH_2O)_4]^-$, 267 $[M-H-(CH_2O)-H_20]^-$, 207 $[M-H-(CH_2O)_4-H_20]^-$ and 177 $[M-H-(CH_2O)_3-H_20]^-$ which could be produced by the loss of a –CHOH unit. This behavior is indicative of sugar fragmentation and, is essentially identical to that of caffeoyl hexose (Clifford et al., 2007). We tentatively assigned these to isomeric protocatechuoyl hexose. Whereas, compounds 67 and 68 did not show any evidence of sugar fragmentation and were, therefore, tentatively identified as protocatechuic acid-glycoside. Protocatechuic acid (*m/z* 153) was also found as aglycone (peak 71). Two signals (peaks 72 and 73) at *m/z* 299 gave a base peak in the fragmentation spectra at *m/z* 137, which is indicative of the presence of a hydroxybenzoic acid residue. This fragment originated by the loss of 162 Da suggesting the presence of a hexoside moiety. Peak 73 showed evidence of sugar fragmentation (signals at *m/z* 269, 239, 209 and 179 characteristic of the loss of –CHOH units) whereas peak 72 did not show any evidence of sugar fragmentation. The two compounds were,

therefore, tentatively identified as hydroxybenzoyl-glycoside (peak 72) and hydroxybenzoic acid hexose (peak 73). Peak 74 showed a molecular negative ion at *m/z* 329, which fragmented in the MS^2 experiments giving a base peak at *m/z* 167, suggesting the presence of a vanillic acid residue. The loss of 162 Da and the absence of sugar fragmentation evidence prompt us to tentatively identify this compound as vanillic acid-glycoside.

3.1.4. Anthocyanins

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

Compound 81 had a positive charged molecular ion at *m/z* 757, yielding MS^2 ions at *m/z* 595, 449 and 287. The fragments at *m/z* 595, 449 and 287 correspond to the loss of a hexose moiety (-162 Da), a coumaroyl moiety (-146 Da) and a further hexose moiety (-162 Da), respectively. The ion at *m/z* 287 suggested that the aglycone is cyanidin and the compound was therefore tentatively identified as cyanidin-3-(6-O-p-coumaroyl)-5-O-diglucoside (Flamini, 2013).

Compound 82 had a positive charged molecular ion at *m/z* 581, yielding a MS^2 ion at *m/z* 287, which suggests the presence of cyanidin as aglycone. This compound was identified, on the basis of published data, as cyanidin-3-sambubioside (Giusti, Rodríguez-Saona, Griffin, Wrolstad, 1999).

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

3.2. Profile of phenolic compounds in the six cherry cultivars

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

3.2.1. Chlorogenic acids

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

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

3.2.2. Flavan-3-ols

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

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

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

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

3.2.3. Flavonols and other minor colorless phenolic compounds

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

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

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

3.2.4. Anthocyanins

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

384 & Mazza, 1995; Mozetiĉ, Trebŝe, Simĉiĉ, & 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 μmol trolox/100g fresh weight and between 1323.6 and 6784.9
397 μmol trolox/100g 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

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

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

rutinoside

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

4. Conclusion

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

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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 μmol trolox/100g 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 ± 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.