PHENOLIC MATURITY IN cv. SANGIOVESE: EVOLUTION OF THE CHARACTERISTICS OF ANTHOCYANINS AND TANNINS IN SKIN AND SEEDS

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Introduction

Grape skin and seed flavonoids are important contributors to the sensory attributes and aging potential of red wines (Río Segade et al., 2008). The knowledge of flavonoid evolution and extractability during ripening is crucial for winemaking decisions and wine grade allocations (Mercurio et al., 2010). In red grape varieties, the berry flavonoids are mainly represented by anthocyanins, flavan-3-ol monomers and proanthocyanidins, along with lower amounts of flavonols (Cheynier et al.,2006).

Anthocyanins accumulate in the skin, and they determine berry and wine color. They include non-acylated glucoside, acetylglucoside, coumaroylglucoside and caffeoylglucoside derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin (Fournand et al., 2006). These compounds are synthesized from veraison and their quantity is determined by factors such as vine genotype, vigor and environmental parameters such as temperature, light incidence, water availability and agronomic practices (Filippetti et al., 2013; Pastore et al., 2013).

Catechin, epicatechin, epigallocatechin and epicatechin-gallate are present as free monomers and as subunits of polymeric compounds (proanthocyanidins) and constitute the class of flavanols, also known as tannins (Downey et al., 2006). They are localized in the skin hypodermal layers and also in the soft parenchyma of the seed (Adams, 2006) and contribute to wine bitterness, astringency, body, mouthfeel and color stability (Gawel, 2006). Tannins accumulate in the berry skin and seeds from fruit-set to veraison and after this stage their concentration can decline (Downey et al., 2003) or remain unchanged (Kennedy et al., 2001a). The quantity of tannins in the skin is usually lower than in the seeds (Ristic et al., 2010) or in some cases similar (Harbertson et al., 2002).

The extractability of berry skin and seed flavonoids is defined as the percentage of total anthocyanins and tannins extracted during vinification. This is a key determinant of the phenolic content of wines. In Tempranillo grapes, anthocyanin extractability peaks in over-ripe berries even though the total anthocyanin content has already declined (Hernández-Hierro et al., 2012). In contrast, no changes in anthocyanin extractability were observed from veraison to harvest in Shiraz berries (Fournand et al., 2006). Differences in anthocyanin extractability among varieties could be correlated with the polysaccharide content of the skin (Romero-Cascales et al., 2005). Extractability increases when there are low concentrations of pectin, cellulose, arabinoxylan, arabinogalactan and xyloglucan (Ortega-Regules et al., 2006).

The extractability of skin and seed tannins during berry development is different between varieties: tannins become easier to extract during the ripening of Tempranillo berries (Canals et al., 2005) but harder to extract during the ripening of Monastrell berries (Bautista-Ortín et al., 2012). Other studies

have shown no relationship between ripening and tannin extractability in Pinot Noir and Monastrell berries (Pastor del Rio et al., 2006; Fournand et al., 2006).

Once grape is harvested, fermentation management also affects the phenolic content of wines. Anthocyanin extraction mostly occurs during early fermentation (Nagel et al., 1979), and non-acylated anthocyanins (particularly malvidin) are the most abundant derivatives in wine (Mayen et al., 1994). Skin tannins are also extracted predominantly during the early stages of fermentation, whereas seed tannins take longer to be released and are therefore most abundant in wines with long maceration times (Peyrot de Gachons and Kennedy, 2003). The grape variety also affects the relative contribution of skin and seed tannins, e.g. Uva di Troia berries release a higher proportion of skin tannins compared to Aglianico berries, which release more seed tannins under the same conditions (Gambuti et al., 2009).

In this survey we investigated the evolution of anthocyanin and tannin extractability from post-veraison to harvest in *Vitis vinifera* L. cv. Sangiovese. This red berry variety is the most-widely cultivated in Italy, and is used for the production of premium Tuscan red wines, but it is characterized by high susceptibility to bunch rot and low level of anthocyanins.

In the last few years, elevated temperatures during ripening affected negatively the acidity content and the synthesis of anthocyanins in Sangiovese berries (Filippetti et al., 2011), while sugar accumulation rate was reported higher and wines resulted unbalanced with excessive levels of alcohol, poor color and high astringency. This situation increased the interest on understanding the evolution and extractability of flavonoids during ripening.

With this aim, we investigated the impact of ripening stage on the concentration and extractability of anthocyanins and tannins in Sangiovese berries. The extractability was measured after prolonged maceration in a model hydroalcoholic solution to simulate the conditions of red wine vinification and thus provide data that is useful to viticulturists and winemakers.

Materials and methods

Plant material, leaf area and yield components

The study was conducted in the 2013 season in a 7-year-old irrigated vineyard of *Vitis vinifera* L. cv. Sangiovese (clone 12T grafted onto SO4 rootstock), located in Bologna, Italy (latitude 44°25'N; longitude 11°28'W). Vines were spaced 1 m within the row and 2.8 m between the rows and were trained to a vertically shoot positioned (VSP) spur pruned cordon. Each vine was winter-pruned leaving six nodes of two buds (12 buds per vine). During the growing season, the number of shoots and clusters was kept uniform by shoot and cluster thinning. Shoots were trimmed twice, in June and July, and pest management was carried out according to Emilia-Romagna region standard practices.

Berry sampling

Four replicates, consisting of four vines each, were established in the vineyard. Every 10 days, from one week after full veraison (19 August) to harvest (2 October), a random sample of 120 berries was collected from each replicate (480 berries total) by cutting with scissors through the pedicel. Each sample was then divided into four subsamples which were used to determine: a) must biochemical parameters (40 berries); b) extractable anthocyanins and tannins (40 berries); c) total anthocyanins (20 berries); d) total tannins (20 berries). The berries for the determination of must biochemical

parameters and for extractable anthocyanins and tannins were immediately processed, while the remaining subsamples were frozen and stored at –80°C. Harvest was delayed so long as rot infection appeared on bunches in order to increase the concentration of soluble solids and to investigate the behavior of phenolic compounds over a broad period. The vegetative behavior and productivity of the vines, was typical for Sangiovese cultivated in fertile soils, confirming that the plants were healthy and well balanced.

Biochemical analysis of musts

Must parameter subsamples (40 berries each) were analyzed to determine the soluble solids concentration using a temperature-compensating Maselli R50 refractometer (Maselli Misure, Parma, Italy). The must pH and titratable acidity were measured using a Crison Titrator (Crison Instruments, Barcelona, Spain).

Extraction of anthocyanins and tannins using a model hydroalcoholic solution

Whole (not ground) skins and seeds from 40 berries were soaked separately and shaken daily, in different tubes containing 80 mL of a hydroalcoholic solution for 15 days at 28°C. The duration and the temperature imposed to the extractions were chosen to simulate the winemaking conditions and so to determine the concentration of extractable anthocyanins and tannins. The hydroalcoholic solution comprised 6 g/L tartaric acid, 40 mL/L 1 N NaOH, 100 mg/L potassium metabisulphite and a proportion of ethanol that raised from 0 to 13% in the first 12 days of extraction. This concentration was reached by adding every two days 2 mL of ethanol absolute (12 mL total) to simulate alcoholic fermentation. The extracts were centrifuged (15 minutes, 13000 rpm) and an aliquots of the supernatant (400 µL) were dried under vacuum at 20°C. Pellets were stored at -20°C.

Exhaustive extraction of anthocyanins and tannins

Total anthocyanins were extracted from the skins of 20 berries by soaking the peeled skins in 100 mL methanol for 24 h, then storing the extracts at –20 °C (Mattivi et al., 2006). Total tannins were extracted from the skins and seeds of 20 berries grounded separately to a fine powder before extracting 1 mg of the sample in 1 mL 70% (v/v) acetone in water, for 24 hours in dark room (Downey et al., 2003). Skin and seed extracts were then centrifuged (15 minutes, 13000 rpm) and two 400 µL aliquots of the supernatant were dried under vacuum at 20 °C. Pellets were stored at -20°C.

Anthocyanin determinations

Total and extractable anthocyanins were separated by HPLC as described by Mattivi and co-workers (Mattivi et al., 2006), using a Waters 1525 instrument equipped with a diode array detector (DAD) and a reversed-phase column (RP18 250 x 4 mm, 5 µM) with a pre-column (Phenomenex, Castel Maggiore, BO, Italy). The concentration was determined by measuring absorbance at 520 nm. A calibration curve was established using a malvidin-3-glucoside standard (Sigma-Aldrich, ST. Louis, MO, USA).

Tannin determinations

Total and extractable tannins were measured by HPLC with the equipment described above. The tannin content was determined by acid-catalyzed cleavage in the presence of excess phloroglucinol (Kennedy and Jones, 2001 b). Individual reversed-phase HPLC separations were used to determine the abundance of free monomers and cleaved proanthocyanidins by measuring absorbance at 280 nm (Downey et al., 2003). The concentrations of free monomers and hydrolyzed terminal subunits

were determined from standard curves prepared with commercial standards of catechin, epicatechin, epicatechin-gallate and epigallocatechin (Extrasynthese, France). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction coefficients (Kennedy et al., 2001b).

The seed tannin content was assigned to free monomers, terminal subunits and extension subunits, whereas the skin tannin content was assigned to terminal subunits and extension subunits. The mean degree of polymerization (mDP) was calculated by summing terminal and extension subunits and dividing by terminal subunits (Downey et al., 2003). The phenolic concentration was expressed as mg per kg of berries (mg kg^{-1}), in order to take into account variations in berry weight that modify the concentration of these compounds during ripening.

Statistical analysis

Data were analyzed by longitudinal data analysis using the mixed procedure available in SAS v9.0 (SAS Institute, Inc., Cary, NC, USA), with compound symmetric (cs) as covariance structure.

Results

Berry development and composition

The accumulation of soluble solids and the evolution of berry weight during ripening is shown in Table 1. Sugar concentration grew in a near linear manner until harvest whereas berries stopped growing 28 days after full veraison. The skin weight increased slowly until two weeks before harvest, while the seed weight remained constant during the examined ripening period.

Table 1. Mean weight of berry, skin and seed, and soluble solids concentration during ripening.

Days after full veraison	Soluble solids (°Brix)	Berry weight (g)	Skin weight (g/berry)	Seed weight (g/berry)
7	14.9 d	2.42 a	0.250 a	0.108
18	17.5 c	2.68 b	0.264 ab	0.095
28	19.2 b	2.82 c	0.287 b	0.092
38	19.8 b	2.79 c	0.319 c	0.099
51	21.7 a	2.86 c	0.315 c	0.099
Significance	*	***	**	ns

Different letters within a column indicate significant differences.
Asterisks indicate significance at: * P < 0.05; ** P < 0.01; *** P < 0.001; ns not significant.

Anthocyanin and skin tannin analysis

The levels of both total and extractable anthocyanins increased until harvest, as shown in Table 2. The total anthocyanin concentration almost doubled during this period whereas the extractable anthocyanin more than tripled. The concentration of total skin tannin terminal and extension subunits dropped after the first sampling date. In contrast, the concentration of the extractable portion of the same subunits increased: terminal subunits raised only after the first sampling date, while extension subunits increased until two weeks before harvest.

Table 2. Total and extractable anthocyanins and skin tannins subunits (mg kg $^{-1}$) during ripening.

Days after full veraison	Total			Extractable		
	anthocyanins	skin tannin terminal subunits	skin tannin extension subunits	anthocyanins	skin tannin terminal subunits	skin tannin extension subunits
7	342.51 a	77.44 b	1574.98 b	26.65 a	26.75 a	283.58 a
18	458.68 b	60.88 a	1257.82 a	50.79 b	43.47 b	444.42 b
28	540.05 bc	61.45 a	1277.24 a	64.56 c	42.40 b	466.34 b
38	552.80 bc	58.09 a	1261.58 a	73.12 cd	47.28 b	579.26 c
51	595.14 c	56.67 a	1223.49 a	81.99 d	42.45 b	564.55 c
Significance	**	***	**	***	**	*

Different letters within a column indicate significant differences.
Asterisks indicate significance at: * P < 0.05; ** P < 0.01; *** P < 0.001; ns not significant.

Seed tannin analysis

The concentration of total and extractable seed tannin monomers declined throughout ripening, mainly in the 28 days following full veraison (Table 3), although the decline in total monomers was sharper than that of the extractable portion. The total concentration of seed tannin terminal and extension subunits declined in the 28 days following full veraison, remaining stable thereafter. The evolution of extractable terminal and extension subunits showed a decline after the first sampling date, followed by a plateau until harvest.

Table 3. Total and extractable seed tannins subunits (mg kg $^{-1}$) during ripening.

Days after full veraison	Total			Extractable		
	monomer subunits	terminal subunits	extension subunits	monomer subunits	terminal subunits	extension subunits
7	539.77 c	457.39 c	1523.09 c	270.81 c	279.32 b	604.72 b
18	303.40 b	303.60 b	1174.10 b	174.05 b	132.72 a	282.92 a
28	174.00 a	224.49 a	949.86 a	148.87 a	114.79 a	335.19 a
38	171.23 a	241.05 a	1021.84 a	116.29 a	143.65 a	375.90 a
51	138.49 a	226.33 a	953.06 a	128.00 a	153.49 a	342.12 a
Significance	***	***	**	***	***	***

Different letters within a column indicate significant differences.
Asterisks indicate significance at: * P < 0.05; ** P < 0.01; *** P < 0.001; ns not significant.

mDP

Both total and extractable skin tannins mDP increases during ripening, but for total skin tannins this increase was only 6%, whereas for the extractable portion was 21% (Table 4). The mDP of total seed tannins increased in the 28 days following full veraison, while no substantial change was observed for that of the extractable portion. The mDP of seed tannins resulted lower than that of skin tannins, in both fractions.

Table 4. Skin and seed tannins mDP during ripening.

Days after full veraison	Total		Extractable	
	skin tannin mDP	seed tannin mDP	skin tannin mDP	seed tannin mDP
7	21.31 a	4.37 a	11.67 a	3.18
18	21.67 ab	4.85 b	11.24 a	3.16
28	21.82 ab	5.24 c	11.86 a	4.42
38	22.77 b	5.24 c	13.37 ab	3.63
51	22.63 b	5.21 c	14.19 b	3.22
Significance	*	*	**	ns

Different letters within a column indicate significant differences.
Asterisks indicate significance at: * P < 0.05; ** P < 0.01; *** P < 0.001; ns not significant.

Extractability

The extractability of anthocyanins, skin tannins and seed tannins was calculated as a percentage relative to the total amount. Both anthocyanins and skin tannins became more extractable during ripening (Table 5). The increase in anthocyanin extractability was linear from post-veraison to the sampling date before harvest, whereas in the same time-period, the extractability of skin tannins increased in a biphasic manner. The extractability of seed tannins did not show a specific developmental trend.

Table 5. Extractability (%) of anthocyanins, skin tannins and seed tannins during ripening.

Days after full veraison	Anthocyanins exctractability	Skin tannin extractability	Seed tannin extractability
7	7.78 a	18.81 a	45.82
18	11.07 b	37.21 b	33.11
28	11.96 bc	37.87 b	44.41
38	13.23 c	47.48 c	44.34
51	13.78 c	47.42 c	47.32
Significance	**	**	Ns

Different letters within a column indicate significant differences.
Asterisks indicate significance at: * P < 0.05; ** P < 0.01; *** P < 0.001; ns not significant.

Discussion

The concentration of skin and seed flavonoids in Sangiovese berries were analyzed using different extraction methods: exhaustive extractions with methanol and acetone to measure the total content of anthocyanins and tannins respectively, and a model hydroalcoholic solution (13.5% ethanol) to measure the extractable fraction obtained during a typical vinification process. This approach also allowed the analysis of differences in the amount of each phenolic compound, so we could measure their extractability during ripening.

The accumulation of extractable anthocyanins was faster than the accumulation of total anthocyanins from one week after full veraison to the point of harvest. These metabolites therefore appear to become more extractable during ripening, correlating with the degradation of cell walls in the skin, as previously reported by Rio Segade et al (2008). The extractability of skin tannins in our Sangiovese berries increased dramatically from one week to 38 days after full veraison, reflecting a sharp increase in the abundance of extractable tannins and a simultaneous decline in the concentration of total tannins. This decrease may be caused in part by dilution effects representing berry growth and, as reported in Shiraz (Downey et al., 2003), by stable associations between tannins and other cellular components such as cell wall polysaccharides, lignins and proteins. Anyway, the literature contains conflicting reports concerning the fate of total skin tannins during berry ripening. For example, only minor fluctuations were observed in Cabernet Sauvignon, Shiraz and Pinot Noir berries (Harbertson et al., 2002), while it was found an increase of skin tannins in Cabernet Sauvignon berries, during the last phases of ripening (Bindon et al., 2014). These diverse profiles may reflect varietal differences and changes in the environmental conditions, including vintage-specific effects (Cadot et al., 2011). The increase in extractable skin tannins we observed is consistent with those reported for Shiraz berries (Canals et al., 2005) and for Cabernet Sauvignon berries (Bindon et al., 2014), in the latter case based on extraction in a solution containing 10% ethanol. The same authors described also the role of cell wall pores in sequestrating skin tannin that results in a limitation of their extractability in the last phases of ripening. This could explains why also the extractability of Sangiovese skin tannins did not increase anymore in the last two weeks before harvest.

The mDP of total skin tannins was similar to other red berry varieties during ripening (Downey et al., 2003; Kennedy et al., 2001b; Cohen et al., 2012; Hanlin and Downey, 2009), although the mDP in the extractable fraction was lower than the total fraction, as reported also by Mattivi and co-workers. (Mattivi et al., 2009) This may reflect the greater extraction efficiency of acetone compared to hydroalcoholic solutions (Pastor del Rio and Knnedy, 2006; Downey and Hanlin, 2010). Bindon and Kennedy (2011) reported that the affinity of skin cell wall material for high-molecular-mass skin tannins decreases during ripening, making the longer polymers more extractable later in the ripening process. This explains the steep increase we observed in the mDP of extractable skin tannins compared to the slower increase in the mDP of total skin tannins.

Each component of the Sangiovese total and extractable seed tannins (monomer, terminal and extension subunits) declined sharply after full veraison, followed by small fluctuations until the point of harvest, as reported in other red grape varieties (Harbertson et al., 2002; Pastor del Rio and Kennedy, 2006; Ristic and Iland 2005). This decline could reflect the oxidative cross-linking of polymers (Cadot et al., 2006) and the formation of branched polymers that are more resistant to hydrolysis (Downey et al., 2003), which could reduce the efficiency of extraction even with strong solvents such as acetone.

In our study the mDP of total seed tannins was lower than that of total skin tannins, but there was no clear trend during ripening as reported for Shiraz berries (Downey et al., 2003). Furthermore, it was

reported that the mDP of seed tannins extracted with acetone was higher than the mDP of the same compounds extracted using a hydroalcoholic solution (Bautista-Ortín et al., 2012).
Overall, the extractable portion of phenolic compounds in the skin of Sangiovese berries increased during ripening, with anthocyanins peaking at harvest and tannins peaking at the previous sampling date. In contrast, the seed tannin concentration declined sharply within 20 days after full veraison and no substantial changes were observed during the remaining ripening period.

Conclusions

This study showed the flavonoid concentration of Sangiovese berries during ripening and the extractability of these compounds after simulated maceration. Our approach confirmed that ripening stage had a strong impact on the increase of extractable anthocyanins and skin tannins. This reflected a rise in the extractability of these compounds independent of the accumulation of their total amount, probably due to modification in skin cell wall structure. In contrast, ripening stage seems to have no effects on the extractability of seed tannins as both total and extractable portions declined from post-veraison to harvest.
The knowledge of dynamic flavonoid in berry and wine-like solution during ripening, can be used by viticulturists and winemakers to improve Sangiovese harvest management, although further researches are needed to understand in more detail the effects of phenolic maturity on wine sensory attributes.

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