Extraction kinetics of anthocyanins from skin to pulp during carbonic maceration of winegrape berries with different ripeness levels

Extraction kinetics of anthocyanins from skin to pulp during carbonic maceration of winegrape berries with different ripeness levels

Caterina Pace†, Simone Giacosa†, Fabrizio Torchio, Susana Río Segade, Enzo Cagnasso, Luca Rolle*

Università degli Studi di Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari. Via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy.

*Corresponding author: luca.rolle@unito.it; Tel.: +39 011 6708558; Fax: +39 011 6708549
†These authors contributed equally to the study.

Running title: Extraction kinetics of anthocyanins during carbonic maceration

ABSTRACT

The evolution of the content and profile of anthocyanins was studied in the skin and pulp of Gamay winegrapes during twelve days of carbonic maceration. The ripening effect was also investigated using berries belonging to two density classes (A=1094-1100 kg/m^3 and B=1107-1115 kg/m^3). The ripest berries showed a higher extraction yield, even though the differences among density classes tended to decline towards the end of the process, and few significant differences were found in the anthocyanin profile. The maceration time influenced strongly not only the content and extraction yield, but also the qualitative composition of anthocyanins towards the predominance of malvidin derivatives. Finally, the extraction yield of anthocyanins was positively related with the ethanol formed and the skin mechanical properties using linear regression models, which showed that the skin hardness is likely to be an important variable in modeling daily anthocyanin extraction during carbonic maceration, particularly from the sixth day.

Keywords: carbonic maceration; anthocyanin; extraction kinetics; red winegrapes; ripeness; skin hardness

1. Introduction

Carbonic maceration (CM) is a winemaking process exploiting the adaptability of intact grape berries to a medium saturated with carbon dioxide (Tesniere & Flanzy, 2011). Under this anaerobic atmosphere, a series of transformations inside the whole berry are induced by the activity of endogenous enzymes present in the grapes. During the CM process, the berries undergo intracellular fermentation without yeasts intervention causing the transformation of a small amount of sugars into alcohol (1.5-2% v/v alcohol), the reduction of the malic acid content, and the extraction and formation of secondary metabolites, in particular phenolic and volatile compounds (Ducruet, 1984; Gómez-Míguez & Heredia, 2004; Spranger et al., 2004; Tesniere & Flanzy, 2011).

In practice, in winery, the intact grape clusters are put into a closed tank and kept under CO_2 atmosphere for seven or more days depending on temperature, grape ripeness and cultivar (Chinnici, Sonni, Natali, Galassi, & Riponi, 2009). Inside the tank, the grapes respiring also consume oxygen and the anaerobic metabolism occurs whenever the oxygen concentration is low in gaseous or liquid environment, but the intensity of the phenomena decreases in liquid environment (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2000). For this reason, some importance was attributed to the physico-mechanical properties of the berries to resist the compression in carbonic maceration vinification (Giacosa et al., 2013). After CM, the grapes are pressed and the juice completes alcoholic fermentation without skin maceration (Tesniere & Flanzy, 2011). Therefore, this type of winemaking technology can significantly affect the phenolic composition of the wine. Many studies reported in the scientific literature showed the differences in the phenolic profile of CM wines with respect to other conventional young wines produced using different vinification techniques (Spranger et al., 2004; Sun, Spranger, Roque-do-Vale, Leandro, & Belchior, 2001). A low content of tannins is generally typical of the ‘young’ wines produced by CM (Pellegrini, Simonetti,

Gardana, Brenna, Brighenti, & Pietta, 2000; Sacchi, Bisson, & Adams, 2005). However, a correct extraction of anthocyanins from the skin into the pulp should be guaranteed during CM in order to assure positive chromatic characteristics because these are strongly related to consumer preference of these wines (Parpinello, Versari, Chinnici, & Galassi, 2009). Specific studies showed that CM wines are characterized by high contents of B-type vitisins and ethyl-bridged anthocyanin-flavanol adducts resulting from the high concentration of acetaldehyde produced by the anaerobic metabolism (Chinnici et al., 2009), but low contents of total and monomeric anthocyanins were observed (Castillo-Sánchez, Mejuto, Garrido, & García-Falcón, 2006; Gómez-Míguez & Heredia, 2004).

The content and profile of anthocyanins in winegrapes are of considerable importance in evaluating their oenological potentiality because these compounds are directly associated with the wine color. The extraction of anthocyanins from the grape into the wine depends on the tendency of the berry skin to yield them (González-Neves et al., 2004). Their extractability generally increases throughout grape ripening as a consequence of the degradation of the skin cell wall by pectolytic enzymes, and the changes in the polysaccharide composition, cellulose content and degree of methylation of pectins (Hernández-Hierro et al., 2014; Ortega-Regules, Ros-García, Bautista-Ortín, López-Roca, & Gómez-Plaza, 2008; Ribéreau-Gayon et al., 2000). However, while the anthocyanin extraction into the must-wine during a classical maceration process was extensively investigated (Canals, Llaudy, Valls, Canals, & Zamora, 2005; González-Neves, Gil, & Barreiro, 2008; Romero-Cascales, Fernández-Fernández, López-Roca, & Gómez-Plaza, 2005; Rolle, Torchio, Ferrandino, & Guidoni, 2012b), to our knowledge very scarce data were reported in the scientific literature about the release of anthocyanins from the berry skin to the pulp during CM.

Therefore, in this work, we investigated the evolution of anthocyanins in the skin and pulp of berries undergoing CM with the aims of: *i*) to determine the extraction kinetics of

these compounds during the process; *ii*) to evaluate the impact of two different levels of berry maturity on the content and composition of anthocyanins released; and *iii*) to relate the anthocyanin extraction with the physico-mechanical properties of the berry skin.

2. Material and methods

2.1. Grapes

Vitis vinifera L. cv. Gamay grapes were collected at Semptember 30th, 2012 at technological maturity from a vineyard (45°7′28.29″N 6°58′59.51″E) located at Chiomonte (TO) in the Susa Valley (Piedmont, northwest Italy). A 10-kg set of Gamay grape berries were randomly picked in the vineyard with attached pedicels from different parts of the bunch, transported into boxes to the laboratory and immediately processed. The study was carried out separately on the berries belonging to two different ripening stages (i.e. density classes; Singleton, Ough, & Nelson, 1966) that were: A=1094-1100 kg/m^3 and B=1107-1115 kg/m^3. Density separation was carried out by flotation in different saline solutions (from 100 to 190 g/L sodium chloride) as described by Rolle et al. (2011a) in order to obtain a homogeneous sample of berries. The floating berries were washed with water, visually inspected before the CM process and those with damaged skins were discarded.

2.2. Carbonic maceration process

Three groups of 120 intact whole berries for each ripening stage were placed into a 500 mL glass jar (Bormioli S.p.A., Fidenza, Italy), which was previously saturated with food grade carbon dioxide (Rivoira S.p.A., Milano, Italy) and hermetically closed. The jars were stored in a chamber at controlled temperature of 28 °C for 12 days. Approximately every 12 h, the jars were saturated with carbon dioxide. The CM process was chemically monitored by sampling at 0 (fresh grape), 3, 6, 9 and 12 days.

2.3. Chemical analysis

2.3.1. Reagents and standards. Solvents of HPLC-gradient grade and all other chemicals of analytical-reagent grade were purchased from Sigma (Milan, Italy). The solutions were prepared in deionized water produced by a Purelab Classic system (Elga Labwater, Marlow, United Kingdom). Anthocyanin standards (delphinidin-3-O-glucoside chloride, malvidin-3-O-glucoside chloride, petunidin chloride, peonidin-3-O-glucoside chloride and cyanidin-3-O-glucoside chloride) were supplied from Extrasynthèse (Genay, France).

2.3.2. Anthocyanin extraction and determination. Ten berries for both trial replicates and sampling point were weighed before anthocyanin extraction. The berry skins, removed manually from the pulp, were quickly immersed in 25 mL of a buffer solution containing 12% v/v ethanol, 600 mg/L $Na_2S_2O_5$, 5 g/L tartaric acid and adjusted to pH 3.20 by the addition of 1M NaOH (Torchio, Cagnasso, Gerbi, & Rolle, 2010). Afterwards, the skins were homogenized at 8000 rpm for 1 min with an Ultraturrax T25 high-speed homogenizer (IKA Labortechnik, Staufen, Germany) and centrifuged for 15 min at 3000 × g at 20 °C. The pulp was collected in a beaker containing $Na_2S_2O_5$ (100 mg) and subsequently diluted (9:1, m/m) with 5 mol/L sulphuric acid (Di Stefano & Cravero, 1991). Afterwards, the pulp was homogenized at 9500 rpm for 30 s with an Ultraturrax T10 high-speed

homogenizer (IKA Labortechnik, Staufen, Germany) and centrifuged in a PK 131 centrifuge (ALC International, MI, Italy) for 15 min at 3000 × g at 20 °C. The supernatant was then used for analysis.

The content of total anthocyanins in skin and pulp samples was determined by a spectrophotometric method using a UV-1601PC spectrophotometer (Shimazdu Scientific Instruments Inc., Columbia, MD, USA) (Di Stefano & Cravero, 1991; Torchio et al., 2010). The total anthocyanins index (TAI) was expressed as malvidin-3-glucoside chloride. The relative standard deviation of TAI, based on repeated analyses (n=20) of ten sample extracts, was 1.14% (Torchio et al., 2010).

The determination of individual anthocyanins was performed after concentration of the berry skins or pulp extract using a SEP-PAK C18 cartridge (Waters Corporation, Milford, MA, USA) with methanol as eluent. The chromatographic system employed was a P100 pump equipped with an AS3000 autosampler (Spectra Physics Analytical, Inc., San Jose, CA, USA), a 20-µL Reodyne sample loop, a LiChroCART column (25 cm x 0.4 cm i.d.) purchased from Merck (Darmstadt, Germany) and packed with LiChrospher 100 RP-18 (5-µm) particles supplied by Alltech (Deerfield, IL, USA), and a Spectra Focus Diode Array Detector (DAD, Spectra Physics Analytical, Inc., San Jose, CA, USA) operating at 520 nm. The mobile phases consisted of formic acid/water (10:90, v/v) (mobile phase A) and a mixture formic acid/methanol/water (10:50:40, v/v) (mobile phase B). The two mobile phases were filtered through a 0.20 µm PTFE membrane filter (Whatman International Ltd., Maidstone, Kent, UK). The mobile phase flow-rate was 1 mL/min. A linear gradient was used for the separation of anthocyanins starting at 72% A, and decreasing to 55% A in 15 min, to 30% A in 20 min, to 10% A in 10 min and to 1% A in 5 min, and then back to 72% A in 3 min. The column was then equilibrated for 10 min prior to each analysis. The data treatment was carried out using the ChromQuest chromatography data system (ThermoQuest, Inc., San Jose, CA, USA). The identification of the free forms of anthocyanins was achieved by comparing their retention times with those of pure standards. The acylated forms of anthocyanins were identified by matching the DAD spectrum and retention time of each chromatographic peak, and by comparing these data with those available in the literature (Pomar, Novo, & Masa, 2005; Rolle & Guidoni, 2007). Individual anthocyanins were determined by comparing the area of the appropriate peak with the total peak area and were expressed in percentages. All of the analyses were performed in duplicate and then averaged.

2.3.3. Technological parameters of pulp. Other ten berries for both trial replicates and sampling point were manually crushed, and the must obtained was centrifuged for 10 min at 3000 x *g* and analyzed. Organic acids, reducing sugars and ethanol were quantified by HPLC using a 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD set to 210 nm and a refractive index detector (RI). The analyses were performed isocratically at 0.8 mL/min flow-rate and 65 °C column temperature with an 300 × 7.8 mm i.d. Aminex HPX-87H cation exchange column and a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA). The mobile phase was 0.0065 mol/L H_2SO_4 (Giordano, Rolle, Zeppa, & Gerbi, 2009). The data analysis was carried out using the ChemStation software (Agilent Technologies, Santa Clara, CA, USA).

2.4. Instrumental texture analysis

Skin physico-mechanical properties. Before the anthocyanin analysis, the skin hardness was assessed on the whole berries by a non-destructive puncture test. The texture test was performed using a Universal Testing Machine (UTM) TA.XTplus texture analyzer (Stable Micro Systems - SMS, Godalming, Surrey, UK) equipped with a HDP/90 platform, a SMS P/2N needle probe and a 5 kg load cell. The test speed was 1 mm/s and the compression applied was 3 mm. Each berry was individually

placed on the metal plate of the UTM with the pedicel in a horizontal plane in order to be consistently punctured in the lateral side. The berry skin hardness was defined by the break energy (W_{sk}), expressed in mJ (Rolle et al., 2012b). The use of a needle probe allows separate estimation of this skin mechanical characteristic, minimizing the possible interferences caused by the pulp firmness on the results. All data acquisitions were made at 500 points per second, and the W_{sk} parameter was calculated from force-distance curves using the Texture Exponent software package (Stable Micro Systems).

2.5. Statistical analysis

Statistical analyses were performed using the SPSS statistics software package (version 19.0; IBM Corporation, Armonk, NY, USA).

3. Results and discussion

As expected, the fresh berries of cv. Gamay belonging to the two ripening stages considered were characterized by different reducing sugar and organic acid contents, as these primary metabolites are being directly influenced by the grape berry density (Rolle, Torchio, Giacosa, Río Segade, Cagnasso, & Gerbi, 2012a). The total reducing sugar contents in the berry must were 219 g/L (i.e. 12.5% v/v as potential alcohol) and 252 g/L (i.e. 14.5% v/v as potential alcohol) for the ripening stage A and B, respectively. Tartaric acid and malic acid amounts were 7.2 g/L and 2.6 g/L, respectively, in the berry must for the ripening stage A, and 6.4 g/L and 2.2 g/L, respectively, for the ripening stage B.

Table 1 shows the anthocyanin contents and profiles of the berry skin and pulp for the ripening stage A and B. Because of the density classification of the berries by flotation, a high homogeneity in the data can be observed. The manual operation of berry peeling was made with extreme care. Therefore, it is possible to consider that the small difference (9 mg/kg grape) in the release of anthocyanins from the skin to the pulp was a result of the ripening stage of the berries. Although the total content of anthocyanins in the pulp was very low (< 27 mg/kg grape), as expected, the value was higher in the ripest berries because of the structural properties and composition of the cell-walls of the skin that may facilitate the anthocyanin release already before harvest (Hernández-Hierro et al., 2014; Ortega-Regules, Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2006a).

In the skin, a high difference in the total content of anthocyanins (200 mg/kg grape) was detected among the berries belonging to the two density classes considered. Therefore, for Gamay grapes, the level of sugars was particularly important as a criterion for the selection of the harvest date because it is strongly related to the anthocyanin accumulation. This behavior was different in respect to other varieties such as Shiraz (Fournand, Vicens, Sidhoum, Souquet, Moutounet, & Cheynier, 2006), Carménère and Cabernet Sauvignon (Obreque-Slier, Peña-Neira, López-Solís, Zamora-Marín, Ricardo-Da Silva, & Laureano, 2010), and Nebbiolo (Rolle et al., 2012a), where the anthocyanin content was not significantly different for sugar contents higher than 220 g/L. However, in Barbera grapes, Torchio et al. (2010) found a significant increase in the richness in these compounds in most of the growing zones studied for sugar contents comprised between 235 and 269 g/L.

Gamay grapes were characterized by an anthocyanin profile made up mainly of molecules tri-substituted in the B-ring with a prevalence of malvidin 3-glucoside. Among di-substituted anthocyanins, peonidin-3-glucoside was the most abundant red pigment. The percentage of acetyl-glucoside derivatives was less than 1.5%. The impact of the ripening stage of the grapes on the anthocyanin profile was not significant. In fact, very few differences were detected in both the anthocyanin profile of the skin and pulp among berries belonging to the two density classes considered. Respect to the literature, some small differences in the ΣPeonidin derivatives / ΣMalvidin derivatives ratio were observed because of a higher percentage found (about 10%) of peonidin derivatives in our grapes (Jin, He, Bi, Cui, & Duan, 2009; Rolle & Guidoni, 2007). However, although the anthocyanin fingerprint seems to be closely related to genetic characteristics, differences in the profile of these pigments can be found as a result of agroecological factors (Ortega-Regules, Romero-Cascales, López-Roca, Ros-García, & Gómez-Plaza, 2006b).

The values of the skin break energy (W_{sk}) for the berries at the two different ripeness stages A and B (0.448±0.069 mJ and 0.471±0.082 mJ, respectively) did not show significant differences, confirming that the skin hardness cannot be used as a ripeness predictor (Río Segade, Orriols, Giacosa, & Rolle, 2011). However, within the same variety, growing locations and annual bioclimatic indices affect this skin mechanical characteristic (Maury, Madieta, Le Moigne, Mehinagic, Siret, & Jourjon, 2009). On average, in comparison with other varieties, Gamay grapes showed low W_{sk} values (Giacosa et al., 2013). It is important to consider these mechanical aspects because a relationship between the wine-grape skin hardness and the kinetics of anthocyanin extraction was found (Rolle et al., 2012b).

The release of anthocyanins from the skin to the pulp, evaluated as the percentage and concentration of those remaining in the skin, for all trials performed was shown in Figure 1. For each sample, about 96±1% of the amounts initially present in the skins were recovered in residual solid parts, pulp juice and natural juice escaped from the berry in the glass jar. This percentage of recovery agreed with that reported by Fournand et al. (2006) in a previous study.

A significant progressive diffusion of these red colored pigments from skins was detected for all twelve days of the carbonic maceration (CM) process. In particular, the berries of the ripening stage B showed a high release of anthocyanins in the first three days (> 300 mg/kg, about 36%), and it experienced a slowdown from the ninth day from which only 6% was released. On the contrary, the berries belonging to density class A showed a slow anthocyanin release in the first three days of the process (< 100 mg/kg, about 15%), and a constant and regular anthocyanin extraction during the following days until the twelfth day. In each sampling point, the berries of density class B showed higher extraction yield, although the differences tended to decline towards the end of the process. Analyzing the final percentage of non-extracted anthocyanins, the berries of density class B achieved a minimal average value (9.3%), which was not significantly lower than the one corresponding to class A (11.5%). When compared with the results of other studies on the phenols extraction with different models (Fournand et al., 2006; Guidoni & Hunter, 2012; Rolle et al., 2012b), it is interesting to highlight that this vinification technique seems to allow anyway very good extraction yields. However, as a consequence of the results obtained for both grape total anthocyanin content and extractability, in the seasons where the berries are harvested with lower sugars levels, the CM process cannot be stopped before the tenth day, in order not to penalize the color of the wines produced.

To acquire wider information it becomes necessary to extend the study to check possible correlations between data obtained on the anthocyanin extraction and chemical-physical parameters of the grapes during the CM process. Therefore, two important variables that several authors reported in the scientific literature as factors affecting the anthocyanin extractability were monitored: the ethanol content of the medium (Canals et al., 2005), which is formed during CM by enzymatic activity inside the berry (Flanzy, Flanzy, & Benard, 1989), and the skin hardness (Rolle et al., 2012b). Figure 2 shows the values of the skin break energy and the ethanol content for Gamay

berries at two different levels of ripeness during CM winemaking. The behavior of these two parameters was similar for the berries belonging to the two different density classes considered (A and B) during the whole CM process. For each sampling point, no significant differences were found among the berries with different richness in sugars. Therefore, no impact of the ripening stage of the berries on the anthocyanin extraction can be directly attributable to these two parameters. The final content of ethanol was 2.3% v/v and 3.1% v/v for the berries of density classes A and B, respectively. A progressive and regular formation of ethanol was detected already in the first phases of the CM process, with an average daily production of about 0.3% v/v. The ethanol concentration obtained at the end of the CM process was in agreement with those reported by Flanzy et al. (1989) in a previous work on the Carignan variety.

While the formation of alcohol during CM vinification was already known, the changes in the physico-mechanical parameters of the skin were not still investigated. Despite a certain dispersion of W_{sk} data among the berries, a clear trend of this parameter was observed during the CM process. After an initial phase comprising the first six days in which the W_{sk} values remained practically constant, a general hardening of the skin was detected with an increase of about 0.1 mJ (corresponding to about 20%) at the twelfth day respect to the fresh grape. In traditional maceration process using a wine-like solution, hard skins presented a higher extractive capacity for anthocyanins when compared with the corresponding soft skins (Rolle et al., 2012b). According to Canals et al. (2005), total content of anthocyanins increases even when different alcohol content is present in the medium during the first three days of traditional maceration, but stabilizes subsequently. Therefore, the results obtained highlight that the increase in the extraction of anthocyanin compounds (Figure 1) from the sixth day of the CM process is mainly associated with the skin hardening.

The effects of independent variables such as W_{sk} (mJ) and ethanol content (% v/v) on the percentage of total anthocyanins yet present in the skin (TA, %, dependent variable) during CM winemaking, for the berries belonging to the ripening stages A and B, were individually described by the following equations:

$$TA\ (\%) = 153.21 - 159.01\ W_{sk}\ (mJ)\ (R = 0.682,\ p < 0.001,\ SEC = 13.21)$$

$$TA\ (\%) = 100.31 - 16.53\ Ethanol\ (\%\ v/v)\ (R = 0.914,\ p < 0.001,\ SEC = 7.34)$$

where R = correlation factor of calibration; SEC = standard error of calibration.

According to the coefficients of the two independent variables, a increase either in the value of W_{sk} or in the ethanol content contributes significantly to decrease the percentage of anthocyanins present in the skin. This confirmed that an increase in the two parameters results in a higher extraction yield of anthocyanins. Although the coefficient of the W_{sk} parameter was higher than that obtained for the ethanol content, the correlation factor of calibration was lower and the standard error of calibration was higher for the first. In fact, univariate linear regression using the W_{sk} parameter as independent variable explained 44.4% of the total variation observed in TA, whereas the one corresponding to the ethanol content explained 82.8%. This difference can be due to the fact that the effect of the W_{sk} value is significant only from the sixth day of maceration. When the effects of independent variables were simultaneously evaluated, the following equation was obtained:

$$TA\ (\%) = 112.38 - 29.49\ W_{sk}\ (mJ) - 15.00\ Ethanol\ (\%\ v/v)\ (R = 0.918,\ p < 0.001,\ SEC = 7.29)$$

In this case, the correlation factor of calibration increased and the standard error of calibration decreased using multivariate linear regression, when compared with univariate regression, which permitted to explain 83.1% of the total variation observed in TA. Although the weight of the W_{sk} parameter decreased strongly, it is still higher than that associated with the ethanol content. Anyway, the two independent variables contribute favorably to release

anthocyanins from the skin. Therefore, the skin hardness is likely to be a new variable that should be considered in modeling daily anthocyanin extraction during CM winemaking, particularly from the sixth day.

Table 2 shows the profile of anthocyanins yet present in the skin and of those released into the pulp during the CM process for Gamay grapes at the two ripening stages considered. The ripening effect on the anthocyanin profile was small. Although lower percentages of delphinidin, cyanidin and petunidin derivatives, in favor of higher ones of peonidin and malvidin derivatives, were generally found in the skin and pulp of the riper berries at each sampling point, the differences among berries of density classes A and B were not usually significant. Particularly in the skin, the significant differences in the anthocyanin profile among berries belonging to the two density classes were detected until the ninth day, excepting for delphinidin derivatives also with differences in subsequent days. In the pulp, only the percentages of cyanidin derivatives were significantly different among berries of density classes A and B during the first three days of maceration.

The maceration time is important for the color of the resulting wine because the anthocyanin profile of the skin and pulp was different depending on the time at which the process was interrupted. As maceration progressed, the percentage of malvidin derivatives remaining in the skin increased significantly at the expense mainly of delphinidin, petunidin and peonidin derivatives, particularly from the sixth day. After twelve days of CM, the percentage of malvidin derivatives yet present in the skin was higher than 80%, with a variation with respect to fresh grapes of +15.0 and +18.9% as function of the ripening stage. These results agreed with those reported by other authors (García-Beneytez, Revilla, & Cabello, 2002; Guidoni & Hunter, 2012), who found higher proportions of malvidin derivatives remaining in the skin at the end of traditional red winemaking. Therefore, malvidin derivatives were the least extracted anthocyanin compounds from the skin during CM winemaking. On contrary, delphinidin derivatives were released almost completely at the end of the CM process with a variation of about -5% with respect to fresh grapes. Regarding cyanidin derivatives, about 30% of those initially present in the skin were extracted. Petunidin and peonidin derivatives showed different behavior as function of the ripening stage of the berries. About 45% of both petunidin and peonidin derivatives were released from the fresh skin for the berries belonging to density class A, whereas 65% of petunidin derivatives and 25% of peonidin derivatives were extracted for riper berries. The percentages obtained for peonidin forms of anthocyanins yet present in the skin were in agreement with those previously published for different winegrape varieties after traditional red winemaking (García-Beneytez et al., 2002).

In the pulp, the relative amount of delphinidin and petunidin derivatives remained practically invariable during the CM process. However, the percentage of malvidin derivatives increased significantly (with a variation of +14.9-20.9% at the twelfth day with respect to fresh grapes as function of the ripening stage) in detriment of peonidin (-13.0-21.2%) and cyanidin (-0.9-1.5%) derivatives. This trend agreed with that occurred in the skin and with that described by other researchers from the fresh grape to the wine (Cagnasso, Rolle, Caudana, & Gerbi, 2008; García-Beneytez et al., 2002; Romero-Cascales et al., 2005) or from the grape must to the wine during the first days of traditional maceration (González-Neves et al., 2008; Guidoni & Hunter, 2012). The remarkable loss of di-substituted anthocyanins is probably due to the complex processes of combination, oxidation and insolubilization that characterize anthocyanin-like substances during winemaking (Cheynier, Souquet, Kontek, & Moutounet, 1994). These losses may be a likely reason for the strong influence of malvidin derivatives released from the skin on the anthocyanin profile of the pulp. At the end of the CM process, the anthocyanin profile of the pulp consisted mainly of

malvidin derivatives (about 82%), and therefore they are primarily responsible for the red color of the resulting wines, with a relatively small incidence of peonidin derivatives (9.4-12.1% as function of the ripening stage). This aspect may be interesting from a technological point of view, because the anthocyanin profile of the wine could be more or less modified towards a higher prevalence of tri-substituted anthocyanins (more stable), such as malvidin derivatives, on di-substituted forms (more prone to oxidative degradation), such as cyanidin and peonidin derivatives, depending on the CM time. Furthermore, the percentage of unacylated and acetylated glucosides in the pulp increased significantly in detriment of cinnamoylated glucosides during CM winemaking. The decrease of these last compounds in the pulp can be due to reduced extraction rates of anthocyanins acylated with p-coumaric acid (García-Beneytez et al., 2002; Guidoni & Hunter, 2012) or, once extracted, to their low stability (Dallas, Ricardo-Da-Silva, & Laureano, 1995) and easy interaction with components of the surrounding matrix (Guidoni & Hunter, 2012).

4. Conclusions

This work was focused on the extraction kinetics of anthocyanins from the skin to the pulp during carbonic maceration winemaking of Gamay berries at two ripening stages. The ripening effect was studied in density sorted berries and it was particularly evident for total content of anthocyanins, whereas few significant differences were found in the anthocyanin profile. Higher total contents of anthocyanins were released in the riper berries but, as maceration progressed, the differences in the extraction yield among berries of different density classes became smaller. Regarding the anthocyanin profile, after twelve days, only the percentage of delphinidin derivatives in the skin was significantly different among berries belonging to different density classes. The maceration time was a very influential factor on the content, extraction yield and profile of anthocyanins and, therefore, could play a key role in the color stability of the wines produced. In fact, tri-substituted anthocyanins extracted in the pulp were more abundant with larger maceration times, achieving percentages of malvidin derivatives higher than 80% at the twelfth day. The increasing trend of the anthocyanin extraction yield with the maceration time was explained and modeled on the basis of an increase in the ethanol content formed in the pulp during the winemaking process, but also of an increase in the skin hardness particularly from the sixth day. Therefore, the skin mechanical properties could be proposed as factors controlling the release of anthocyanins from the skin into the pulp during carbonic maceration, which determines the qualitative and quantitative composition of these red pigments in the resulting wine.

References

Cagnasso, E., Rolle, L., Caudana, A., & Gerbi, V. (2008). Relationship between grape phenolic maturity and red wine phenolic composition. *Italian Journal of Food Science, 20*, 365–380.

Canals, R., Llaudy, M. C., Valls, J., Canals, J. M., & Zamora, F. (2005). Influence of ethanol concentration on the extraction of color and phenolic compounds from the skin and seeds of Tempranillo grapes at different stages of ripening. *Journal of Agricultural and Food Chemistry, 53*, 4019–4025.

Castillo-Sánchez, J. J., Mejuto, J. C., Garrido, J., & García-Falcón, S. (2006). Influence of wine-making protocol and fining agents on the evolution of the anthocyanin content, colour and general organoleptic quality of Vinhao wines. *Food Chemistry, 97*, 130–136.

Cheynier, V., Souquet, J. M., Kontek, A., & Moutounet, M. (1994). Anthocyanin degradation in oxidising grape musts. *Journal of the Science of Food and Agriculture, 66*, 283–288.

Chinnici, F., Sonni, F., Natali, N., Galassi, S., & Riponi, C. (2009). Colour features and pigment composition of Italian carbonic macerated red wines. *Food Chemistry, 113*, 651–657.

Dallas, C., Ricardo-Da-Silva, J. M., & Laureano, O. (1995). Degradation of oligomeric procyanidins and anthocyanins in a Tinta Roriz red wine during maturation. *Vitis, 34*, 51–56.

Di Stefano, R., & Cravero, M. C. (1991). Metodi per lo studio dei polifenoli dell'uva. *Rivista di Viticoltura e di Enologia, 44*, 37–45.

Ducruet, V. (1984). Comparison of the headspace volatiles of carbonic maceration and traditional wine. *Lebensmittel-Wissenschaft and Technologie, 17*, 217–221.

Flanzy, C., Flanzy, M., & Benard, P. (1989). *Tecnologia dei vini novelli*. Brescia, Italy: Edizioni AEB SpA.

Fournand, D., Vicens, A., Sidhoum, L., Souquet, J. M., Moutounet, M., & Cheynier, V. (2006). Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages. *Journal of Agricultural and Food Chemistry*, *54*, 7331–7338.

García-Beneytez, E., Revilla, E., & Cabello, F. (2002). Anthocyanin pattern of several red grape cultivars and wines made from them. *European Food Research and Technology, 215*, 32–37.

Giacosa, S., Torchio, F., Río Segade, S., Gaiotti, F., Tomasi, D., Lovat, L., Vincenzi, S., & Rolle, L. (2013). Physico-mechanical evaluation of the aptitude of berries of red wine grape varieties to resist the compression in carbonic maceration vinification. *International Journal of Food Science and Technology, 48*, 817–825.

Giordano, M., Rolle, L., Zeppa, G., & Gerbi, V. (2009). Chemical and volatile composition of three Italian sweet white passito wines. *Journal International des Sciences de la Vigne et du Vin*, *43*, 159–170.

Gómez-Míguez, M., & Heredia, F. J. (2004). Effect of the maceration technique on the relationships between anthocyanin composition and objective color of Syrah wines. *Journal of Agricultural and Food Chemistry, 52*, 5117-5123.

González-Neves, G., Charamelo, D., Balado, J., Barreiro, L., Bochicchio, R., Gatto, G., Gil, G., Tessore, A., Carbonneau, A., & Moutounet, M. (2004). Phenolic potential of Tannat, Cabernet-Sauvignon and Merlot grapes and their correspondence with wine composition. *Analytica Chimica Acta, 513*, 191–196.

González-Neves, G., Gil, G., & Barreiro, L. (2008). Influence of grape variety on the extraction of anthocyanins during the fermentation on skins. *European Food Research and Technology, 226*, 1349−1355.

Guidoni, S., & Hunter, J. J. (2012). Anthocyanin profile in berry skins and fermenting must/wine, as affected by grape ripeness level of *Vitis vinifera* cv. Shiraz/R99. *European Food Research and Technology, 235*, 397–408.

Hernández-Hierro, J. M., Quijada-Morín, N., Martínez-Lapuente, L., Guadalupe, Z., Ayestarán, B., Rivas-Gonzalo, J. C., & Escribano-Bailón, M. T. (2014). Relationship between skin cell wall composition and anthocyanin extractability of *Vitis vinifera* L. cv. Tempranillo at different grape ripeness degree. *Food Chemistry, 146*, 41–47.

Jin, Z.-M., He, J.-J., Bi, H.-Q., Cui, X.-Y., & Duan, C.-Q. (2009). Phenolic compound profiles in berry skins from nine red wine grape cultivars in Northwest China. *Molecules, 14*, 4922–4935.

Maury, C., Madieta, E., Le Moigne, M., Mehinagic, E., Siret, R., & Jourjon, F. (2009). Development of a mechanical texture test to evaluate the ripening process of Cabernet Franc grapes. *Journal of Texture Studies*, *40*, 511–535.

Obreque-Slier, E., Peña-Neira, A., López-Solís, R., Zamora-Marín, F., Ricardo-Da Silva, J. M., & Laureano, O. (2010). Comparative study of the phenolic composition of seeds and skins from

Carménère and Cabernet Sauvignon grape varieties (*Vitis vinifera* L.) during ripening. *Journal of Agricultural and Food Chemistry*, *58*, 3591–3599.

Ortega-Regules, A., Romero-Cascales, I., López-Roca, J. M., Ros-García, J. M., & Gómez-Plaza, E. (2006b). Anthocyanin fingerprint of grapes: environmental and genetic variations. *Journal of the Science of Food and Agriculture, 86*, 1460–1467.

Ortega-Regules, A., Romero-Cascales, I., Ros-García, J. M., López-Roca, J. M., & Gómez-Plaza, E. (2006a). A first approach towards the relationship between grape skin cell-wall composition and anthocyanin extractability. *Analytica Chimica Acta, 563*, 26−32.

Ortega-Regules, A., Ros-García, J. M., Bautista-Ortín, A. B., López-Roca, J. M., & Gómez-Plaza, E. (2008). Changes in skin cell wall composition during the maturation of four premium wine grape varieties. *Journal of the Science of Food and Agriculture, 88*, 420–428.

Parpinello, G. P., Versari, A., Chinnici, F., & Galassi, S. (2009). Relationship among sensory descriptors, consumer preference and color parameters of Italian Novello red wines. *Food Research International, 42*, 1389–1395.

Pellegrini, N., Simonetti, P., Gardana, C., Brenna, O., Brighenti, F., & Pietta, P. (2000). Polyphenol content and total antioxidant activity of *vini novelli* (young red wines). *Journal of Agricultural and Food Chemistry, 48*, 732–735.

Pomar, F., Novo, M., & Masa, A. (2005). Varietal differences among the anthocyanin profiles of 50 red table grape cultivars studied by high performance liquid chromatography. *Journal of Chromatography A, 1094*, 34−41.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. J., & Lonvaud, A. (2000). Handbook of Enology. Volume 1. *The microbiology of wine and vinification*. Chichester, UK: John Wiley & Sons Ltd.

Río Segade, S., Orriols, I., Giacosa, S., & Rolle, L. (2011). Instrumental texture analysis parameters as winegrapes varietal markers and ripeness predictors. *International Journal of Food Prop*erties, *14*, 1318−1329.

Rolle, L., & Guidoni, S. (2007). Color and anthocyanin evaluation of red winegrapes by CIE L*, a*, b* parameters. *Journal International des Sciences de la Vigne et du Vin, 41*, 4, 193–201.

Rolle, L., Río Segade, S., Torchio, F., Giacosa, S., Cagnasso, E., Marengo, F., & Gerbi, V. (2011a). Influence of grape density and harvest date on changes in phenolic composition, phenol extractability indices, and instrumental texture properties during ripening. *Journal of Agricultural and Food Chemistry, 59*, 8796–8805.

Rolle, L., Torchio, F., Ferrandino, A., & Guidoni, S. (2012b). Influence of wine-grape skin hardness on the kinetics of anthocyanin extraction. *International Journal of Food Properties, 15*, 249–261.

Rolle, L., Torchio, F., Giacosa, S., Río Segade, S., Cagnasso, E., & Gerbi, V. (2012a). Assessment of physicochemical differences in Nebbiolo grape berries from different production areas and sorted by flotation. *American Journal of Enology and Viticulture, 63*, 195–204.

Romero-Cascales, I., Fernández-Fernández, J. I., López-Roca, J. M., & Gómez-Plaza, E. (2005). The maceration process during winemaking extraction of anthocyanins from grape skins into wine. *European Food Research and Technology, 221*, 163–167.

Sacchi, K. L., Bisson, L. F., & Adams, D. O. (2005). A review of the effect of winemaking techniques on phenolic extraction in red wines. *American Journal of Enology and Viticulture, 56*, 197–206.

Singleton, V. L., Ough, C. S., & Nelson, K. E. (1966). Density separations of wine grape berries and ripeness distribution. *American Journal of Enology and Viticulture, 17*, 95–105.

Spranger, M. I., Clímaco, M. C., Sun, B., Eiriz, N., Fortunato, C., Nunes, A., Leandro, M. C., Avelar, M. L., & Belchior, A. P. (2004). Differentiation of red winemaking technologies by phenolic and volatile composition. *Analytica Chimica Acta, 513*, 151–161.

Sun, B., Spranger, I., Roque-do-Vale, F., Leandro, C., & Belchior, P. (2001). Effect of different winemaking technologies on phenolic composition in Tinta Miùda red wines. *Journal of Agricultural and Food Chemistry, 49*, 5809–5816.

Tesniere, C., & Flanzy, C. (2011). Carbonic maceration wines: characteristics and winemaking process. *Advances in Food and Nutrition Research, 63*, 1–15.

Torchio, F., Cagnasso, E., Gerbi, V., & Rolle, L. (2010). Mechanical properties, phenolic composition and extractability indices of Barbera grapes of different soluble solids contents from several growing areas. *Analytica Chimica Acta, 660*, 183–189.

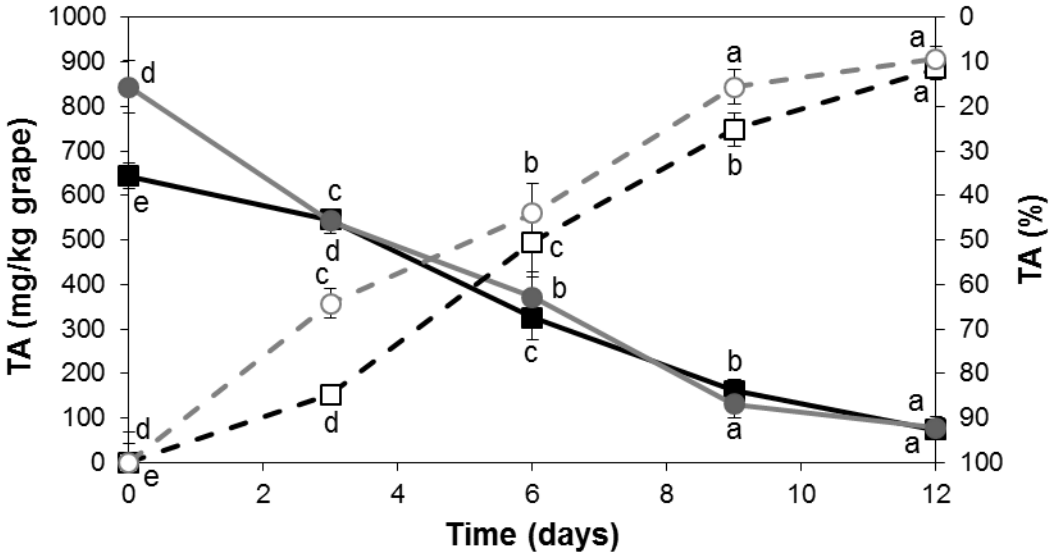

Fig. 1. Total anthocyanins remaining in the skin for density class A (■ , □) and B (● , ○), expressed as concentration (■, ●) and percentage (□, ○), for Gamay grape berries during carbonic maceration winemaking. Different letters indicate significant differences among days of winemaking (Tukey-b test; $p < 0.05$). Density class: A= 1094-1100 kg/m^3, B= 1107-1115 kg/m^3.

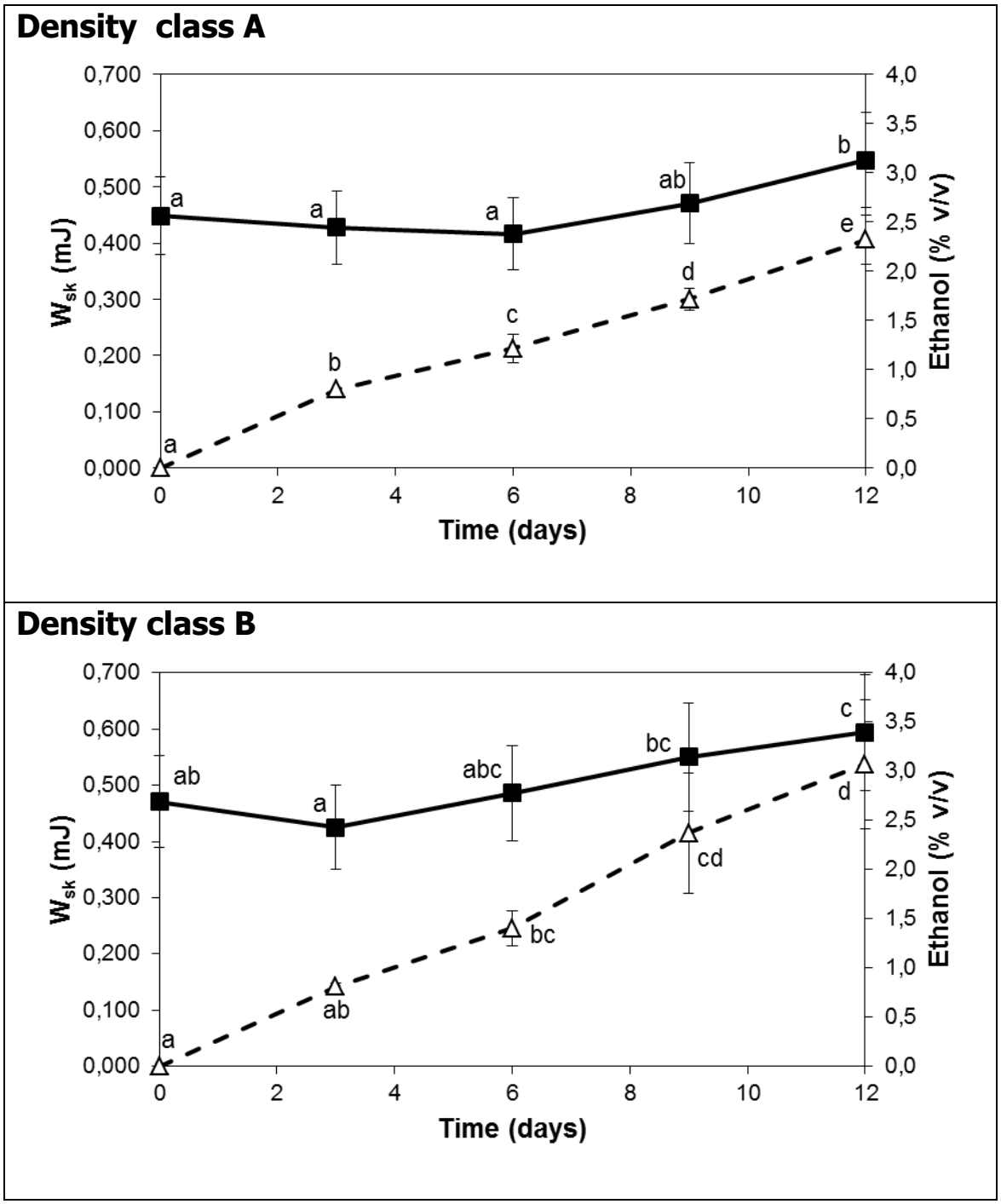

Fig. 2. Skin break energy (W_{sk}, ■) and ethanol content of berries (△) for Gamay grapes at two different levels of ripeness during carbonic maceration winemaking. Different letters indicate significant differences among days of winemaking (Tukey-b test; $p < 0.05$). Density class: A= 1094-1100 kg/m^3, B= 1107-1115 kg/m^3.

Table 1
Anthocyanin composition of the skin and pulp for fresh Gamay grape berries at two different levels of ripeness.

Compound	A		B		Sign[a]	Sign[b]
	Skin	Pulp	Skin	Pulp		
Total anthocyanins (mg/kg grape)	643.54±28.13	13.98±2.12	843.63±57.93	22.74±3.69	**	*
ΣDelphinidin derivatives (%)	7.56±0.42	2.45±0.29	6.22±1.22	2.01±0.35	ns	ns
ΣCyanidin derivatives (%)	2.32±0.28	3.47±0.29	1.85±0.29	2.64±0.37	ns	*
ΣPetunidin derivatives (%)	7.99±0.35	2.94±0.29	7.44±1.01	3.01±0.35	ns	ns
ΣPeonidin derivatives (%)	20.64±2.98	30.60±3.86	17.93±2.17	25.13±2.56	ns	ns
ΣMalvidin derivatives (%)	61.49±3.76	60.54±3.64	66.56±3.96	67.21±3.13	ns	ns
ΣSimple glucosides (%)	91.62±0.53	89.86±2.31	92.57±0.22	90.32±2.06	*	ns
ΣAcetyl glucosides (%)	1.34±0.24	0.00±0.00	1.35±0.10	0.00±0.00	ns	ns
ΣCinnamoyl glucosides (%)	7.04±0.55	10.14±2.31	6.08±0.12	9.68±2.06	*	ns

All data are expressed as average value ± standard deviation (n= 3). ([a]) and ([b]) indicate significant differences among the two ripeness levels for the skin and pulp, respectively, at the same winemaking day. Sign: *,** and ns indicate significance at $p < 0.05$, 0.01 and not significant, respectively. Density class: A= 1094-1100 kg/m^3, B= 1107-1115 kg/m^3.

Table 2
Anthocyanin profile of the skin and pulp for Gamay grape berries at two different levels of ripeness during carbonic maceration winemaking.

Compound (%)	Day	A		B		Sign[a]	Sign[b]
		Skin	Pulp	Skin	Pulp		
ΣDelphinidin derivatives	0	7.56±0.42cd	2.45±0.29	6.22±1.22c	2.01±0.35	ns	ns
	3	10.35±1.06d	3.41±0.93	4.36±1.02b	2.20±0.34	**	ns
	6	5.28±2.41bc	3.12±1.82	3.17±0.53b	2.38±0.54	ns	ns
	9	4.01±1.23ab	3.96±2.59	0.86±0.19a	2.21±1.65	*	ns
	12	1.95±0.46a	3.06±0.85	0.80±0.51a	1.64±0.59	*	ns
Sign[c]		***	ns	***	ns		
ΣCyanidin derivatives	0	2.32±0.28ab	3.47±0.29b	1.85±0.29b	2.64±0.37b	ns	*
	3	2.54±0.25b	2.25±0.28a	1.62±0.17ab	1.63±0.14a	**	*
	6	2.02±0.47ab	1.55±0.57a	1.44±0.16ab	1.61±0.11a	ns	ns
	9	1.68±0.14a	1.95±0.45a	1.05±0.26a	1.70±0.28a	*	ns
	12	1.62±0.32a	1.98±0.24a	1.31±0.26ab	1.73±0.42a	ns	ns
Sign[c]		*	**	*	**		
ΣPetunidin derivatives	0	7.99±0.35bc	2.94±0.29	7.44±1.01b	3.01±0.35	ns	ns
	3	9.85±0.88c	4.48±1.47	6.10±0.89b	3.62±0.26	**	ns
	6	6.85±1.96ab	4.21±1.89	5.44±0.71b	3.95±0.71	ns	ns
	9	6.03±1.17ab	5.25±2.56	2.60±0.27a	3.20±1.65	**	ns
	12	4.36±0.70a	4.10±1.46	2.50±1.31a	2.42±1.60	ns	ns
Sign[c]		**	ns	***	ns		
ΣPeonidin derivatives	0	20.64±2.98c	30.60±3.86c	17.93±2.17	25.13±2.56c	ns	ns
	3	17.25±1.80bc	17.73±3.30b	18.10±0.90	18.62±0.73b	ns	ns
	6	18.12±1.61bc	14.86±1.89ab	17.03±1.66	18.37±2.32b	ns	ns

	9	13.58±1.32ab	14.11±0.50ab	13.77±1.70	15.26±1.19ab	ns	ns
	12	11.64±2.35a	9.38±1.22a	13.79±4.09	12.10±3.93a	ns	ns
Sign[c]		**	***	ns	***		
ΣMalvidin derivatives	0	61.49±3.76a	60.54±3.64a	66.56±3.96a	67.21±3.13a	ns	ns
	3	60.02±1.95a	72.12±4.24b	69.82±1.94a	73.93±0.48ab	**	ns
	6	67.73±4.94ab	76.26±5.50b	72.92±0.62a	73.69±1.20ab	ns	ns
	9	74.71±2.63bc	74.73±6.00b	81.73±2.12b	77.62±3.17bc	*	ns
	12	80.43±3.18c	81.48±3.02b	81.60±4.28b	82.11±5.14c	ns	ns
Sign[c]		***	**	***	**		
ΣSimple glucosides	0	91.62±0.53ab	89.86±2.31a	92.57±0.22ab	90.32±2.06a	*	ns
	3	93.56±0.84b	93.91±0.99b	93.43±0.77b	95.59±1.40b	ns	ns
	6	92.82±0.81ab	94.94±0.18b	93.88±0.42b	95.80±0.19b	ns	**
	9	92.90±0.67ab	96.36±0.16b	92.31±0.57ab	96.82±0.37b	ns	ns
	12	91.06±0.93a	95.46±0.51b	91.01±1.47a	96.06±0.51b	ns	ns
Sign[c]		*	***	*	***		
ΣAcetyl glucosides	0	1.34±0.24a	0.00±0.00a	1.35±0.10a	0.00±0.00a	ns	ns
	3	1.23±0.08a	0.28±0.20ab	1.25±0.08a	0.18±0.03a	ns	ns
	6	1.27±0.13a	0.37±0.08bc	1.11±0.09a	0.42±0.10b	ns	ns
	9	1.06±0.19a	0.64±0.05c	1.26±0.18a	0.62±0.07b	ns	ns
	12	1.86±0.15b	1.69±0.19d	1.86±0.26b	1.43±0.17c	ns	ns
Sign[c]		**	***	**	***		
ΣCinnamoyl glucosides	0	7.04±0.55	10.14±2.31c	6.08±0.12ab	9.68±2.06b	*	ns
	3	5.21±0.76	5.81±0.79b	5.32±0.69ab	4.24±1.37a	ns	ns

	6	5.91±0.80	4.70±0.24ab	5.01±0.34a	3.79±0.09a	ns	**
	9	6.03±0.50	3.00±0.11a	6.43±0.51ab	2.56±0.37a	ns	ns
	12	7.08±0.84	2.85±0.38a	7.13±1.28b	2.51±0.36a	ns	ns
Sign[c]		ns	***	*	***		

All data are expressed as average value ± standard deviation (n= 3). ([a]) and ([b]) indicate significant differences among the two ripeness levels for the skin and pulp, respectively, at the same winemaking day. Different letters within the same column ([c]) indicate significant differences among days of winemaking at the same ripeness level for the skin and pulp (Tukey-b test; $p < 0.05$). Sign: *,**,*** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively. Density class: A= 1094-1100 kg/m^3, B= 1107-1115 kg/m^3.