Impact of maceration enzymes on skin softening and relationship with anthocyanin extraction in winegrapes with different anthocyanin profiles.

IMPACT OF MACERATION ENZYMES ON SKIN SOFTENING AND RELATIONSHIP WITH ANTHOCYANIN EXTRACTION IN WINEGRAPES WITH DIFFERENT ANTHOCYANIN PROFILES

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ABSTRACT

The impact of an enzyme preparation on the extraction of grape skin anthocyanins into a wine-like solution was evaluated during maceration (25 °C, 8 days). The study was performed on *Vitis vinifera* L. cv. Cabernet Sauvignon and Nebbiolo because of their different anthocyanin profiles, which are mainly composed of tri- and di-substituted forms, respectively. Maceration enzymes enhanced the skin releasing capacity for anthocyanins between 8% and 15% more depending on the enzyme dose and variety, and shortened the time required to reach the maximum extraction yield by about 40 h, when compared to the control samples. The effect of enzymes on the profile and total content of anthocyanins during maceration was significant only for Cabernet Sauvignon. Therefore, a variety effect was found. Particularly, the relative amount of malvidin glucosides increased up to 9% more whereas that of delphinidin, cyanidin and peonidin glucosides decreased up to 5, 2 and 3% more, respectively, with the addition of the enzyme preparation. The results also showed lower anthocyanin degradation at maceration times longer than 72 h with the use of enzymes. Furthermore, the relationship between this enzyme effect and the instrumental skin hardness was assessed for the first time, as no previous study deals with the variation in the mechanical properties of the berry skin after using maceration enzymes. Significant positive relationships were found between the skin softening and the anthocyanin extraction yield ($R>0.69$, $p<0.01$), which confirmed that the skin degradation by enzymes facilitates the anthocyanin release.

Keywords: maceration enzymes; skin mechanical properties; extraction; anthocyanins; winegrapes

1. Introduction

Color is one of the most important attributes in the initial assessment of the red wine. High colored wines are usually associated with high perceived quality scores (Parpinello, Versari, Chinnici, & Galassi, 2009). Anthocyanins contribute strongly to the sensory quality of red wines because these compounds and their interactions with other phenolic compounds are responsible for the color and its stability during ageing (Boulton, 2001; Fulcrand, Dueñas, Salas, & Cheynier, 2006; Monagas, Martín-Álvarez, Bartolomé, & Gómez-Cordovés, 2006).

Anthocyanins are mainly located inside the skin cell vacuoles and are partially extracted from the berry skin into the must/wine during winemaking (González-Neves, Gil, & Barreiro, 2008). The anthocyanin content and composition of red wines depend on the amount of pigments in the berry skin at harvest and on the easiness of their extraction. Although the qualitative and quantitative composition of anthocyanins in the wine is directly related to the winegrape variety, ripening stage, culture practices, growing season and environmental conditions, the oenological practices also play an important role (González-Neves et al., 2008; González-Neves, Gil, Favre, & Ferrer, 2012; Sacchi, Bisson, & Adams, 2005).

Exogenous enzymes are widely used in red winemaking, attempting to accelerate the extraction of anthocyanins from the berry skin and thus increasing the color intensity of the resulting wine (Bautista-Ortín, Martínez-Cutillas, Ros-García, López-Roca, & Gómez-Plaza, 2005; Gil-Muñoz et al., 2009; Ortega-Heras, Pérez-Magariño, & González-Sanjosé, 2012; Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2012; Soto Vázquez, Río Segade, & Orriols Fernández, 2010). The commercial enzyme preparations mainly show pectolytic (polygalacturonase, pectin methyl esterase, pectin lyase), cellulase, hemicellulase and acid protease activities (Romero-Cascales, Fernández-Fernández, Ros-García, López-Roca, & Gómez-Plaza, 2008). Maceration enzymes degrade the berry skin pecto-cellulosic cell walls by partial hydrolysis of structural polysaccharides. Therefore, the permeability of the cell wall is increased facilitating the diffusion process of anthocyanins from the vacuoles into the must during fermentation (Romero-Cascales et al., 2008, 2012).

Contradictory results have been reported about the impact of maceration enzymes on the anthocyanin content and color intensity in red wines (Sacchi et al., 2005). The discrepancies are probably due to the different nature and activities of the commercial enzyme preparations (Bautista-Ortín et al., 2005; Romero-Cascales et al., 2008), as well as to varietal and vintage effects on the grape anthocyanin content and composition or on the skin cell wall

morphology and composition (Bautista-Ortín, Fernández-Fernández, López-Roca, & Gómez-Plaza, 2007; Ducasse et al., 2010; Ortega-Heras et al., 2012).

The differences in the mechanical properties of the berry skin are also linked to variations in the chemical composition of the cell walls, which determines the resistance of the skin to the anthocyanin release (Hernández-Hierro et al., 2014). In fact, the berry skin hardness and berry skin thickness influence the rate and extent of the anthocyanin extractability (Río Segade, Giacosa, Gerbi, & Rolle, 2011; Rolle, Torchio, Ferrandino, & Guidoni, 2012). Although it is well known that the degradation of the cell walls causes the skin softening (Ortega-Regules, Ros-García, Bautista-Ortín, López-Roca, & Gómez-Plaza, 2008), the effect of maceration enzymes on the skin mechanical properties has not been quantified to date.

The maceration time also affects the anthocyanin release, chromatic characteristics and color stability in the red wine. A longer maceration time usually contributes to a greater anthocyanin extraction from the skins and improves the color stability of the wine (González-Neves et al., 2008; Kelebek, Canbas, & Selli, 2009; Romero-Cascales, Fernández-Fernández, López-Roca, & Gómez-Plaza, 2005; Romero-Cascales et al., 2012; Sacchi et al., 2005). Nevertheless, this relationship may be affected by the participation of extracted anthocyanins in oxidation and polymerization reactions occurring during the maceration process, their partial adsorption by the yeasts, and their fixation onto the grape solid parts (Bautista-Ortín et al., 2007; González-Neves et al., 2008; Romero-Cascales et al., 2005, 2012).

Given that the production of high quality red wines demands the exploitation of the intrinsic chromatic characteristics of the grapes and their preservation in the final product, the aim of this work was to evaluate the effect of an enzyme preparation used at two different doses on the kinetics and extent of the anthocyanin extraction during maceration, and to relate this effect with the skin softening of two red winegrape cultivars. In particular, *Vitis vinifera* L. cv. Cabernet Sauvignon and Nebbiolo were selected on the basis of their different anthocyanin content and profile mainly composed of tri- and di-substituted anthocyanins, respectively. The two varieties are widely used to produce high quality red wines that are commercialized in worldwide.

2. Materials and methods

2.1. Grape samples

In this study, whole bunches of red grape *Vitis vinifera* L. cv. Cabernet Sauvignon and Nebbiolo were harvested from various vines in commercial vineyards located in the same

growing zone (Piedmont, Cuneo province, north-west Italy) in 2013. Once in the laboratory, for each variety, a subsample consisting of approximately 1.5 kg of grapes (1000-1200 berries) was randomly selected by picking berries with attached short pedicels from different positions in the cluster (shoulders, middle and bottom). For each subsample, the berries were sorted according to their density by flotation in saline solutions of different concentrations (from 100 to 190 g/L sodium chloride, corresponding to densities comprised between 1069 and 1125 kg/m^3) as described by Rolle et al. (2012a). The presence of the pedicel prevents saline solution entry into the berry during flotation. This densimetric sorting allows obtaining more homogeneous samples and minimizing the possible ripening effect among berries. The study was carried out on the berries belonging to the most representative class with a density of 1094 kg/m^3 for Cabernet Sauvignon and 1100 kg/m^3 for Nebbiolo, and a relative weight of about 60% w/w. The sorted berries were washed with water and visually inspected; those with damaged skins were discarded. For each variety studied, one set of 30 sorted berries (three replicates of ten berries) was randomly selected for each experiment, and each replicate was accurately weighed. Finally, the remaining berries, subdivided into two replicates, were used for determining the technological ripeness parameters in the grape juice obtained by manual crushing and centrifugation.

2.2. Instrumental texture analysis

A Universal Testing Machine (UTM) TA.XTplus texture analyzer (Stable Micro Systems, Godalming, Surrey, UK), equipped with a HDP/90 platform and a 5 kg load cell, was used for skin texture analysis. The berry skins were manually removed from the pulp using a laboratory spatula. The skin hardness was assessed before and after maceration by a puncture test using a SMS P/2N needle probe (Stable Micro Systems) and a test speed of 1 mm/s as described by Giacosa, Marengo, Guidoni, Rolle, & Hunter (2015). Each skin was individually punctured, and two parameters were measured: skin break force (N, as F_{sk}) and skin break energy (mJ, as W_{sk}). The first variable corresponds to the skin resistance to the needle probe penetration and the second variable is represented by the area under the force-time curve, which is limited between 0 and F_{sk} (Letaief et al., 2008). All data acquisitions were made at 500 points per second, and the skin mechanical properties were calculated from force-distance curves using the Texture Exponent software package (Stable Micro Systems).

2.3. Chemical analysis

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, UK). 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).

Technological ripeness parameters. pH was determined by potentiometry using an InoLab 730 pHmeter (WTW, Weilheim, DE), and titratable acidity (g/L tartaric acid) was estimated using the OIV method (OIV, 2008). Organic acids (citric acid, tartaric acid and malic acid) and reducing sugars (glucose and fructose) (g/L) were determined using a HPLC system equipped with a diode array detector (DAD) set to 210 nm and a refractive index detector, respectively (Giordano, Rolle, Zeppa, & Gerbi, 2009).

Extraction and determination of anthocyanins. To evaluate the effect of the use of an enzyme preparation during the maceration process, a total of three experimental tests were performed. Once the skins were punctured, they were quickly immersed into 20 mL of a hydroalcoholic buffer at pH 3.2 containing 5 g/L tartaric acid, 100 mg/L sodium metabisulphite and 12% v/v ethanol (wine-like solution) for the control samples, while doses of 20 and 50 mg/kg (2 and 5 g/100 kg of grapes, respectively) of an enzyme preparation (AEB, France) containing 70.3% pectin lyase, 22.2% polygalacturonase and 7.5% cellulase activities were added to the hydroalcoholic buffer in other samples (ED20 and ED50, respectively). Solution aliquots were taken at different maceration times and were used for determining extracted skin anthocyanins during the maceration step. After remaining 192 h at 25 ºC, the residual berry skins were quickly immersed into 20 mL of a new hydroalcoholic buffer containing 2 g/L sodium metabisulphite. 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 supernatant was then used for determining non-extracted skin anthocyanins in order to estimate the extraction yield (%) as the extracted anthocyanins/extracted+non-extracted anthocyanins ratio (Río Segade et al., 2011). Total contents of anthocyanins (expressed as mg malvidin-3-O-glucoside chloride/L) were determined by a spectrophotometric method (Torchio, Cagnasso, Gerbi, & Rolle, 2010; Torchio, Río Segade, Gerbi, Cagnasso, & Rolle, 2011).

The determination of the anthocyanin profile was performed after the berry skin extract had been submitted to reverse-phase solid-phase extraction (RP-SPE) using a 1 g Sep-Pak C-18 cartridge (Waters Corporation, Milford, MA, USA) with methanol as the eluent.

The HPLC-DAD system, chromatographic conditions and peak identification were previously reported in the literature (Rolle et al., 2012a). A LiChroCART analytical column (25 cm × 0.4 cm i.d.) purchased from Merck (Darmstadt, Germany), which was packed with LiChrospher 100 RP-18 (5 μm) particles supplied by Alltech (Deerfield, IL, USA), was used. The mobile phases were: A = formic acid/water (10:90, v/v); B = formic acid/methanol/water (10:50:40, v/v). Individual anthocyanins were expressed in percentages. All of the analyses were performed in duplicate.

2.4. Statistical analysis

Statistical analyses were carried out using the SPSS Statistics software package version 19.0 (IBM Corporation, Armonk, NY, USA).

3. Results and discussion

3.1. Technological ripeness parameters

As a consequence of the selection of the berries belonging to the most representative density class for each winegrape variety, Cabernet Sauvignon (1094 kg/m^3) and Nebbiolo (1100 kg/m^3) berries showed different technological ripeness parameters. Sugars and organic acids are primary metabolites directly influenced by the grape berry density (Rolle et al., 2012a). Nebbiolo grapes were richer in reducing sugars (257 g/L) than Cabernet Sauvignon (230 g/L). However, the parameters related to the acidity were quite similar for the two varieties (pH 3.10, 8.96 g/L tartaric acid as titratable acidity, 3.5 g/L malic acid, 6.9 g/L tartaric acid, 0.3 g/L citric acid for Cabernet Sauvignon, and pH 2.96, 8.81 g/L tartaric acid as titratable acidity, 2.5 g/L malic acid, 7.4 g/L tartaric acid, 0.30 g/L citric acid for Nebbiolo).

3.2. Anthocyanin extraction

Figure 1 shows the differences among Cabernet Sauvignon and Nebbiolo varieties in the extraction kinetics and completeness of anthocyanins from the skins into a wine-like solution during maceration without the addition of enzymes (control samples), and with the addition of 20 and 50 mg/kg of an enzyme preparation (ED20 and ED50 samples, respectively). The extraction percentages of anthocyanins were significantly higher for Nebbiolo skins at any maceration time when no enzyme was used, or a dose of 20 mg/kg of the enzyme preparation was added (71% and 79%, respectively, for Nebbiolo; 59% and 69%, respectively, for Cabernet Sauvignon). However, these differences decreased with increasing the added amount of the enzyme preparation. At the end of maceration (time of 192 h),

differences of 10.9, 8.5 and 4.4% were observed in the extraction percentage of anthocyanins among Cabernet Sauvignon and Nebbiolo varieties for control, ED20 and ED50 samples, respectively. Using a dose of 50 mg/kg of the enzyme preparation, the percentages of extracted anthocyanins for the two winegrape varieties studied were not significantly different after 192 h of maceration (81% for Nebbiolo and 76% for Cabernet Sauvignon). Furthermore, the extraction yield of anthocyanins increased significantly with the addition of the enzyme preparation at any maceration time, and even with increasing the enzyme dose at any time for Cabernet Sauvignon skins but from 144 h for Nebbiolo. The maximum releasing capacity was reached after 48 h of maceration, after which the extraction percentages of anthocyanins were kept practically constant in most cases, independently on the variety and enzyme dose. Other works reported that an extraction time of 48 h was sufficient to achieve a plateau for anthocyanins (Río Segade et al., 2011; Rolle et al., 2012b). On the other hand, maceration enzymes may be important tools for shortening the times needed to release most of extractable anthocyanins from the skins. The use of this enzyme preparation speeded up the maximum extraction yield of anthocyanins by about 40 h, compared to the control samples.

3.3. Anthocyanin profile and content

The qualitative and quantitative compositions of skin anthocyanins released into the wine-like solution during the maceration process with and without the addition of the enzyme preparation are shown in Tables 1 and 2 for Cabernet Sauvignon and Nebbiolo winegrapes, respectively. Unacylated anthocyanins predominated in the two varieties studied throughout the maceration process. The Cabernet Sauvignon variety is rich in trisubstituted anthocyanins (ranging from 62% to 78%) with a profile mainly constituted of malvidin derivatives (Table 1). The relative amount of malvidin-3-glucoside varied during maceration between 43% and 58% as a function of the addition of the enzyme preparation and the maceration time. Higher percentages of this anthocyanin compound corresponded to maceration times longer than 24 h with some exceptions. Delphinidin-3-glucoside was the second most abundant anthocyanin form, particularly at the beginning of maceration (about 11-14%). However, its relative abundance decreased during maceration, this reduction being more significant when an enzyme preparation dose of 50 mg/kg was used. At the end of maceration in the presence of 50 mg/kg enzyme preparation, the percentage of petunidin-3-glucoside was higher than that of delphinidin-3-glucoside. The proportions of peonidin-3-glucoside and cyanidin-3-glucoside also decreased during the maceration process, although this decrease was not significant for peonidin-3-glucoside with the addition of 20 mg/kg enzyme preparation probably due to the

higher variability in the percentages at maceration times longer than 72 h. As a result, the malvidin-3-glucoside/peonidin-3-glucoside ratios or the trisubstituted anthocyanins/disubstituted anthocyanins ratios increased significantly during the maceration process from about 6 to 8, 10 and 20 for control, ED20 and ED50 samples, respectively. This effect of the maceration time on the anthocyanin profile agreed with that observed in musts made from winegrapes rich in trisubstituted anthocyanins (González-Neves et al., 2008), particularly for cyanidin, peonidin and malvidin forms during the first days of maceration. At the end of maceration (time of 192 h), the anthocyanin profile of Cabernet Sauvignon agreed with that published for the wines at the end of alcoholic fermentation (Gil-Muñoz et al., 2009).

For the Nebbiolo variety, peonidin-3-glucoside was the predominant anthocyanin compound with percentages comprised between 35% and 45%, and the richness in disubstituted anthocyanin forms ranged from 43% to 59% (Table 2). The anthocyanin profile was slightly different as a function of the maceration time (Table 2). In fact, the relative abundances of peonidin-3-glucoside and cyanidin-3-glucoside decreased constantly and significantly during the maceration process, with the exception of peonidin-3-glucoside in the control samples where the time effect was not significant. This decrease caused a constant and significant increase in the proportions of petunidin-3-glucoside and malvidin-3-glucoside, with the exception of the latter when an enzyme preparation dose of 50 mg/kg was used probably due to the greater variability in the results obtained at times longer than 120 h. The variation in the percentage of delphinidin-3-glucoside as maceration progressed was not significant. Although peonidin-3-glucoside predominated over malvidin-3-glucoside in the first days, their proportions were increasingly similar while maceration occurred. The malvidin-3-glucoside/peonidin-3-glucoside ratios higher than 1 were achieved at maceration times longer than 144 h when an enzyme preparation dose of 50 mg/kg was used. Consequently, the trisubstituted anthocyanins/disubstituted anthocyanins ratios higher than 1 were obtained at maceration times longer than 120 h for control, ED20 and ED50 samples. In varieties characterized by an important presence of disubstituted anthocyanins, such as Nebbiolo, a remarkable loss of these anthocyanin compounds has been also noticed during winemaking by oxidation, polymerization and insolubilization processes (Cagnasso, Rolle, Caudana, & Gerbi, 2008; Cheynier, Souquet, Kontek, & Moutounet, 1994). Therefore, the prevalence of malvidin-3-glucoside over peonidin-3-glucoside is possible in Nebbiolo wines (Cagnasso et al., 2008). As already mentioned, the loss of disubstituted anthocyanins during maceration was also found in the Cabernet Sauvignon variety, although in a lesser extent than

Nebbiolo. Some authors have reported that the diffusion of disubstituted anthocyanins is faster than the one corresponding to trisubstituted anthocyanins in winegrape varieties with very different ratios between the two types of anthocyanins, but the percentage of trisubstituted anthocyanins increases during maceration (Río Segade et al., 2014).

On the other hand, the relative abundance of malvidin-3-glucoside increased with the enzyme addition for the Cabernet Sauvignon variety (Table 1), particularly at a dose of 50 mg/kg of preparation, although this enzyme effect was significant only at some maceration times. Malvidin derivatives are the most stable forms of anthocyanins, and their higher diffusion from the skin during the first hours of maceration may contribute favorably to the color stability of the resulting wine. The inverse significant effect was observed with the addition of the increasing dose of the enzyme preparation on the percentages of delphinidin-3-glucoside and peonidin-3-glucoside at times longer than 120 h, and even on that of cyanidin-3-glucoside at any maceration time. Particularly, 3´-hydroxylated molecules (delphinidin, cyanidin and petunidin) are more prone to oxidation (Cheynier et al., 1994), and therefore the wines with higher proportions of these molecules are more sensitive to the color degradation. Anthocyanins with ortho-hydroxylated groups cannot interact with flavanols and ethanal to stabilize red pigments. In this sense, the use of the enzyme preparation at a dose of 50 mg/kg may favor the color stability. However, for the Nebbiolo variety, the anthocyanin profile was not significantly influenced by the addition of enzymes at any maceration time (Table 2).

Acylated anthocyanins are very important because they participate in intramolecular copigmentation processes, protecting the flavylium cation (Gil-Muñoz et al., 2009). Although the percentages of these compounds for the Cabernet Sauvignon variety are relatively high (Table 1), the relative abundance of acetylated derivatives of anthocyanins (ranging from 9% to 26%) did not vary consistently during the maceration process in control, ED20 and ED50 samples. Nevertheless, a significant increase was found in the proportion of cinnamoylated derivatives with increasing the maceration time, particularly when the enzyme preparation was added at a dose of 50 mg/kg. At each maceration time, the effect of the addition of the enzyme preparation on the relative amount of cinnamoylated derivatives was not significant (Table 1). For the Nebbiolo variety, acylated anthocyanins accounted for small percentages (less than 8%, Table 2). The percentage of acetylated derivatives showed a little increase during maceration up to the last step (times of 96 h, 72 h and 144 h for control, ED20 and ED50 samples, respectively), from which the percentage began to decrease. Instead, the relative abundance of cinnamoylated forms increased significantly with increasing the

maceration time up to 48 h but a decrease was then detected. The addition of enzymes was not a significant factor in the anthocyanin profile at any maceration time (Table 2).

For the Cabernet Sauvignon variety, the content of total anthocyanins increased significantly during the first 48 h of maceration independently on the presence of enzymes, but from this maceration time the anthocyanin content decreased significantly without the addition of the enzyme preparation (Table 1). When the anthocyanin content at each maceration time was compared among control, ED20 and ED50 samples, the addition of the enzyme preparation at doses of 20 and 50 mg/kg did not facilitate the anthocyanin release into the wine-like solution, but it prevented better the loss of anthocyanins released. Other researchers have reported no effect of maceration enzymes on the anthocyanin content in Cabernet Sauvignon wines during alcoholic fermentation (Puértolas, Saldaña, Condón, Álvarez, & Raso, 2009). The significantly higher concentrations of total anthocyanins found in enzyme-treated samples after 72 h of maceration may be linked to the enhanced release of skin phenolics acting as copigments, and consequently protecting anthocyanins against oxidation (Bautista-Ortín et al., 2005; Boulton, 2001; Ducasse et al., 2010; Ortega-Heras et al., 2012). In fact, Hernández-Hierro et al. (2014) suggested the existence of copigmentation processes between polyphenols present in the cell wall material and anthocyanins while the extraction is taking place. The content of total anthocyanins in Nebbiolo increased significantly during the first 48 h of maceration, but the anthocyanin content decreased then significantly (Table 2). However, the total content of anthocyanins in the Nebbiolo variety was not significantly affected by the presence and dose of the enzyme preparation at any maceration time (Table 2).

The effect of the addition of enzymes on the anthocyanin content and profile has been studied on different varieties, although discrepant results were obtained. Kelebek et al. (2009) observed that the anthocyanin profiles of control and enzyme-treated wines made from Kalecik Karasi grapes were similar, and the enzyme treatments during maceration did not have any selective effect on individual anthocyanins. Gil-Muñoz et al. (2009) found no qualitative change at the end of alcoholic fermentation in Cabernet Sauvignon and Syrah wines. Nevertheless, the content of individual anthocyanins increased with the addition of enzymes. On the contrary, Borazan and Bozan (2013) showed that the enzyme-treated wines made from Okuzgozu grapes contained almost two times less individual anthocyanins than the control wines after skin fermentation, this decrease being considerable after 3 days of maceration. As a consequence of variety differences, the content of individual and total anthocyanins was differently affected by the use of maceration enzymes. Some authors found

that the enzymatic treatment increased faster the content of total anthocyanins compared to the control sample during the first maceration days for Monastrell and Kalecik Karasi wines (Bautista-Ortín et al., 2005; Kelebek et al., 2009; Romero-Cascales et al., 2012). However, this content decreased gradually after reaching the maximum value probably due to the fixation of anthocyanins onto the yeasts or grape solid parts, and to oxidation and polymerization reactions occurring during maceration simultaneously to the extraction (Kelebek et al., 2009; Romero-Cascales et al., 2012). In contrast, Borazan and Bozan (2013) reported that the content of total anthocyanins in Okuzgozu wines after skin fermentation did not increase with the enzyme addition, instead it decreased. Other researchers found that maceration enzymes did not facilitate the anthocyanin diffusion from the skins for Monastrell and Cabernet Sauvignon varieties (Bautista-Ortín et al., 2007; Busse-Valverde, Gómez-Plaza, López-Roca, Gil-Muñoz, & Bautista-Ortín, 2011; Puértolas et al., 2009). This variability in the results obtained has been already attributed to variety differences, to the different nature and activities of enzymes used in the commercial preparations, and to the presence of some side activities such as β-glucosidase (Bautista-Ortín et al., 2005; Romero-Cascales et al., 2008, 2012). In fact, Romero-Cascales et al. (2008) characterized the main enzymatic activities present in six commercial maceration enzymes, and they found that high pectin and pectate lyase activities, medium polygalacturonase and pectin methyl esterase activities and no cellulase and β-glucosidase activities had the highest positive effect on the extracted content of anthocyanins during maceration of Monastrell skins. The absence of β-glucosidase activity in the enzyme preparation used in the present work could have prevented the release of the aglicone and the spontaneous transformation to colourless forms.

3.4. Skin mechanical properties

Instrumental texture parameters of the skin for Cabernet Sauvignon and Nebbiolo winegrapes were determined before and after the maceration process in order to evaluate if they can justify the differences found in the releasing capacity for anthocyanins (Table 3). The initial values (time 0) of F_{sk} and W_{sk} were comprised in the usual range for these varieties, particularly from Piedmont growing zone (Río Segade et al., 2014; Rolle, Gerbi, Schneider, Spanna, & Río Segade, 2011; Rolle et al., 2012a). The berry skin break force can be considered a good mechanical parameter to estimate anthocyanin extraction kinetics with adequate reliability. The toughest skins show greater capacities for the anthocyanin release at maceration times longer than 4 h (Rolle et al., 2012b). In this work, at the initial time (time 0), the differences found in the mechanical properties that define the skin hardness (F_{sk} and W_{sk})

were not significant among Cabernet Sauvignon and Nebbiolo varieties or among the skins of each winegrape variety used for each treatment (control, ED20 and ED50). Therefore, the differences found in the anthocyanin release during maceration cannot be attributed to variations in the initial skin hardness. The relationships between the skin mechanical properties and the anthocyanin extraction yield are variety dependent (Río Segade et al., 2014). The chemical composition of grape skin cell walls may determine the mechanical resistance of the berry skin to the anthocyanin release (Hernández-Hierro et al., 2014). Nevertheless, at the end of maceration (time of 192 h), Nebbiolo skins were significantly harder than Cabernet Sauvignon skins according to the skin break force when no enzymatic treatment was applied. In the absence of exogenous enzymes, the magnitude of the degradation of the skin cell walls during maceration was different for the two varieties studied. It is well known that maceration enzymes promote this degradation (Bautista-Ortín et al., 2005), and therefore the variety differences in the skin hardness were avoided. The enzymes act preferentially on the pectin fraction of the skin cell wall, where they produce the greatest degradation (Romero-Cascales et al., 2012). Some authors have linked this degradation to the softening of the fruit (Rosli, Civello, & Martínez, 2004).

The degradation of the skin cell walls during maceration favors the anthocyanin release because they act as a barrier against the diffusion of phenolic compounds from the vacuoles (Bautista-Ortín et al., 2005). However, the instrumental measurement of the skin mechanical degradation promoted by the use of maceration enzymes was not previously performed. In the present work, according to the significant reduction of the values of F_{sk} and W_{sk} after maceration, a softening effect was observed in the skin during maceration, this effect being particularly important when the enzyme preparation was added. Significant correlations (correlation factor $R>0.5$, $p<0.05$) were found for the two varieties between the variation (Δ) in the values of F_{sk} and W_{sk} from the initial to final time (times of 0 and 192 h) and the corresponding anthocyanin extraction yield (n=18, 2 varieties x 3 treatments x 3 replicates of 10 berry skins). So, the percentage of extracted anthocyanins was stronger correlated with ΔF_{sk} ($R=0.797$, $p<0.001$) than with ΔW_{sk} ($R=0.695$, $p<0.01$).

Rolle et al. (2012b) reported that the effect of the skin hardness on the diffusion of the different anthocyanin compounds into the hydroalcoholic buffer solution is only significant when macerating for times less than 10 min. Therefore, in the present work, the different behavior of Cabernet Sauvignon and Nebbiolo to the presence of maceration enzymes, in relation to the relative amounts of individual anthocyanins, was not due to the initial

mechanical properties of the skin but probably to the different anthocyanin profile of each variety.

4. Conclusions

The enzyme preparation used in this work influenced the mechanical properties of the berry skin, increasing the softening that naturally occurs during maceration as a result of the degradation process. This effect of enzymes on the skin hardness was instrumentally quantified for the first time, and the mechanical properties of the skin may be considered predictors of the extraction yield of anthocyanins during maceration. Furthermore, the use of enzymes permitted to increase the extraction yield of anthocyanins, to short the maceration time, and to prevent the loss of the anthocyanins released during maceration. However, the anthocyanin profiles of Cabernet Sauvignon and Nebbiolo varieties during maceration were differently affected by the presence of enzymes, showing a variety effect. The influence of the variety was independent on the skin hardness, but the anthocyanin composition of winegrapes may affect.

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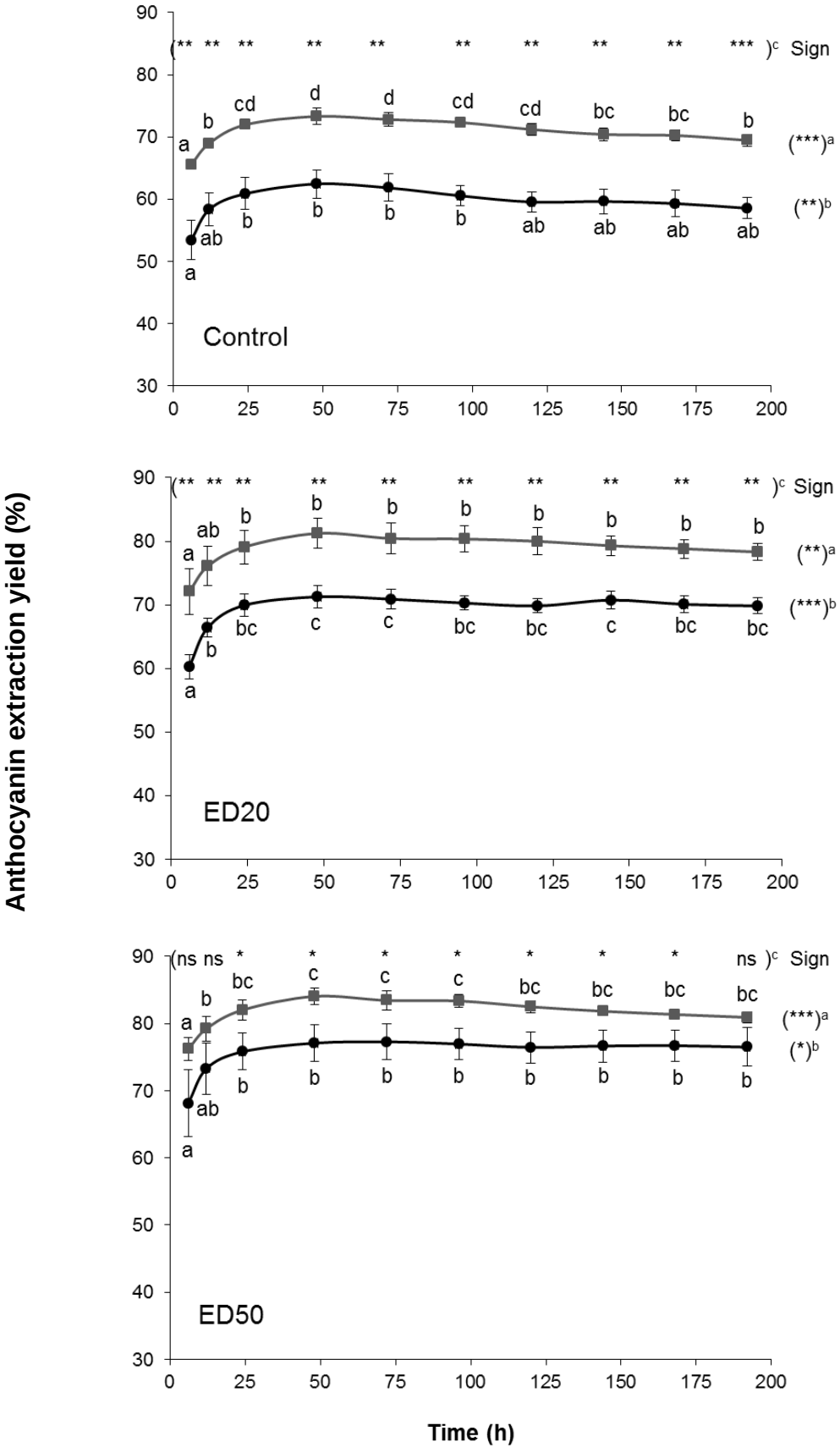
Anthocyanin extraction yield (%)
Time (h)
Control
ED20
ED50
Sign
(***)a
(**)b
(**)a
(***)b
(***)a
(*)b

Figure 1. Effect of enzymatic treatment on the anthocyanin extraction during maceration for Nebbiolo (■) and Cabernet Sauvignon (●) winegrapes. All data are expressed as average value ± standard deviation (n=3). [a,b,c]Sign: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively, for the differences among maceration times for each variety ([a,b]) and among varieties for each maceration time ([c]). Different Latin letters within the same line indicate significant differences among maceration times for Nebbiolo ([a]) and Cabernet Sauvignon ([b]) varieties (Tukey-b test, $p < 0.05$). ED20= enzyme dose of 20 mg/kg, ED50= enzyme dose of 50 mg/kg.

Table 1. Anthocyanin profile and content of Cabernet Sauvignon winegrapes during the maceration process with and without enzymatic treatment.

Treatment	Maceration time (h)	Dp-3-G (%)	Cy-3-G (%)	Pt-3-G (%)	Pn-3-G (%)	Mv-3-G (%)	∑Acetyl (%)	∑Cinnamoyl (%)	Total (mg/L)
Control	6	12.56±0.82c	3.31±0.17c,β	7.18±0.47ab	7.94±0.27d	45.11±2.86ab	19.72±4.07b	4.18±0.20a,β	422±33a
	12	12.00±0.70bc	3.00±0.14bc,β	6.89±0.39a	7.22±0.06bc	42.78±0.39a,α	23.17±1.36b,β	4.95±0.28ab	515±33b
	24	11.84±0.81abc	2.94±0.15bc,β	7.32±0.39ab	7.31±0.12cd	46.61±0.68abc,α	18.96±0.39b,β	5.02±0.39ab	510±28b
	48	12.66±0.71c	2.99±0.14bc,β	8.26±0.26b	7.36±0.18cd	50.47±2.16cd	13.44±1.54a	4.82±0.30ab	611±30d
	72	12.24±0.70bc	2.84±0.12b,γ	8.50±0.44b	7.49±0.29cd,β	54.21±0.97d,β	10.32±1.04a,α	4.41±0.40ab	595±27cd
	120	10.30±1.26abc,αβ	2.38±0.24a,β	7.40±0.76ab	6.43±0.23a	46.63±1.53abc,α	21.79±3.36b	5.08±0.26b	540±14bc,α
	144	11.02±1.06abc,β	2.44±0.15a,β	8.19±0.42ab	6.69±0.19ab,β	53.26±2.55d	13.80±1.27a	4.59±0.20ab	543±33bc,α
	168	10.01±0.77ab,β	2.20±0.18a,β	7.46±0.48ab	6.27±0.40a,β	48.04±2.48bc	21.15±2.89b	4.86±0.35ab	535±12bc,α
	192	9.60±0.88a,β	2.18±0.18a,β	7.56±0.47ab,α	6.35±0.28a,β	49.51±1.27bcd	19.79±0.39b,αβ	5.00±0.24ab	518±8b,α
	Sign[a]	**	***	**	***	***	***	*	***
ED20	6	13.35±0.45	3.24±0.20b,β	7.67±0.21a	8.05±0.59	48.94±1.15abc	15.05±1.86bc	3.69±0.17a,α	395±11a
	12	12.81±0.91	2.95±0.24ab,β	7.39±0.50a	7.12±0.29	45.97±1.67ab,β	19.08±2.97c,αβ	4.68±0.23b	515±8b
	24	12.86±0.62	2.91±0.17ab,β	7.84±0.24ab	7.33±0.26	48.59±0.14abc,β	15.78±0.85bc,α	4.69±0.14b	541±14b
	48	13.61±0.84	2.95±0.22ab,β	8.79±0.28c	7.35±0.49	52.53±1.37bc	10.26±1.49a	4.53±0.03b	646±31c
	72	11.44±0.33	2.35±0.16ab,β	7.54±0.24a	6.27±0.57α	44.01±0.49a,α	23.47±0.48d,β	4.92±0.13b	634±28c
	120	11.16±1.12β	1.94±0.66ab,β	8.49±0.37bc	5.73±1.48	50.66±2.72abc,α	17.36±1.15bc	4.66±0.27b	603±22c,β
	144	11.08±1.91β	1.85±0.79ab,β	8.85±0.14c	5.57±1.87αβ	54.15±2.89c	14.02±2.27ab	4.48±0.37b	629±8c,β
	168	9.61±2.86,β	1.54±0.94a,αβ	7.91±0.17ab	4.80±2.20αβ	47.66±3.45abc	23.64±2.33d	4.84±0.39b	609±15c,β
	192	9.89±2.34β	1.63±0.81a,β	8.48±0.20bc,β	5.12±2.04αβ	51.97±5.88bc	18.25±1.14bc,α	4.66±0.30b	602±15c,β
	Sign[a]	ns	**	***	ns	**	***	***	*******
ED50	6	11.97±0.53c	2.55±0.12d,α	7.10±0.32ab	7.61±0.33d	49.82±1.13a	16.81±1.34b	4.13±0.15a,β	394±41a
	12	11.87±0.55c	2.39±0.11d,α	7.25±0.24abc	7.18±0.44cd	49.86±1.64a,γ	16.50±1.60b,α	4.95±0.18bc	504±31b
	24	11.25±0.72c	2.27±0.11d,α	7.32±0.34abc	7.00±0.21cd	50.34±0.84a,γ	16.85±0.26b,α	4.96±0.16bc	512±13b

48	12.07±1.06c	2.35±0.18d,α	8.37±0.57bc	7.05±0.30cd	54.55±2.06bc	10.79±2.50a	4.81±0.22b	616±16c
72	11.07±1.66c	1.83±0.32c,α	8.59±0.76c	6.78±0.36c,αβ	58.26±0.53d,γ	9.24±1.86a,α	4.23±0.37a	623±20c
120	8.26±0.96b,α	0.93±0.17b,α	8.09±0.46abc	4.33±0.33b	56.04±1.31cd,β	17.30±1.53b	5.05±0.13bc	594±11c,β
144	7.24±0.94ab,α	0.73±0.09ab,α	7.93±0.61abc	3.69±0.14b,α	58.17±0.99d	17.25±1.17b	4.99±0.24bc	601±12c,β
168	5.50±0.64a,α	0.43±0.05a,α	6.84±0.54a	2.81±0.12a,α	52.41±0.69ab	26.47±0.83d	5.53±0.30c	602±10c,β
192	5.18±0.42a,α	0.43±0.02a,α	7.03±0.42ab,α	2.89±0.24a,α	56.79±2.08cd	22.39±1.63c,β	5.28±0.21bc	596±10c,β
Sign[a]	***	***	**	***	***	***	***	***
Sign[b]	ns,ns,ns,ns, ns,*,*,*,*	**,**,**,**, **,*,*,*,*	ns,ns,ns,ns, ns,ns,ns,ns,*	ns,ns,ns, ns, *,ns,*,*,*	ns,**,**,ns, ***,**,ns,ns,ns,	ns,*,**,ns, ***,ns,ns,ns,*	*,ns,ns,ns, ns,ns,ns,ns,ns	ns,ns,ns,ns, ns,**,**,***,***

All data are expressed as average value ± standard deviation (n=3). [a,b]Sign: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively, for the differences ([a]) among maceration times for each treatment and ([b]) among treatments for each maceration time. Different Latin letters within the same column indicate significant differences ([a]) (Tukey-b test; $p < 0.05$). Different Greek letters within the same column indicate significant differences ([b]) (Tukey-b test; $p < 0.05$). ED20= enzyme dose of 20 mg/kg, ED50= enzyme dose of 50 mg/kg. Dp-3-G= delphinidin-3-glucoside, Cy-3-G= cyanidin-3-glucoside, Pt-3-G= petunidin-3-glucoside, Pn-3-G= peonidin-3-glucoside, Mv-3-G= malvidin-3-glucoside.

Table 2. Anthocyanin profile and content of Nebbiolo winegrapes during the maceration process with and without enzymatic treatment.

Treatment	Maceration time (h)	Dp-3-G (%)	Cy-3-G (%)	Pt-3-G (%)	Pn-3-G (%)	Mv-3-G (%)	∑Acetyl (%)	∑Cinnamoyl (%)	Total (mg/L)
Control	6	7.95±0.41	14.29±1.06f	5.76±0.04a,β	39.03±0.39	28.42±1.21a	2.26±0.61abc	2.29±0.07a	279±16a
	12	7.12±0.57	13.08±0.50e	5.57±0.31a	38.48±1.96	29.55±1.20a	2.87±0.06bc	3.33±0.31b	325±15b
	24	6.93±0.55	12.29±0.31de	5.81±0.27a	38.10±2.08	30.99±0.96ab	2.09±0.28ab	3.79±0.22bc	335±13b
	48	7.89±0.67	12.21±0.35de	6.55±0.28b	36.54±1.99	29.72±1.02a	2.86±0.15bc	4.23±0.27c	401±4c
	72	7.68±0.60	11.63±0.26cd	6.56±0.31b	36.36±2.02	30.75±0.79ab	2.87±0.21bc	4.14±0.27c	392±22c
	96	7.56±0.53	11.09±0.27bc	6.74±0.23bc	35.88±1.85	31.50±1.27ab	2.96±0.05c	4.26±0.15c	382±16c
	144	7.42±0.67	10.15±0.26ab	7.18±0.20bc	35.49±1.62	33.61±1.15bc	2.58±0.23abc	3.56±0.35bc	348±16b
	168	7.41±0.45	10.01±0.25a	7.29±0.22c	35.61±1.87	34.54±1.16c	1.90±0.52a	3.25±0.28b	345±9b
	192	7.32±0.45	9.72±0.31a	7.34±0.17c	35.20±1.87	34.85±1.05c	2.22±0.28abc	3.35±0.28b	332±12b
	Sign[a]	ns	***	***	ns	***	**	***	***
ED20	6	6.72±0.68	14.30±0.98b	5.03±0.15a,α	44.55±2.25c	26.55±2.28a	1.02±0.43a	1.82±0.19a	255±14a
	12	6.09±0.44	12.44±0.79ab	4.98±0.28a	41.84±1.67bc	28.31±2.13ab	3.02±0.15c	3.34±0.52b	314±18b
	24	5.99±0.50	11.67±1.00ab	5.16±0.40a	41.00±2.28abc	29.54±1.87ab	2.87±0.23bc	3.79±0.33b	333±14bc
	48	6.85±0.38	11.64±0.71ab	5.97±0.25b	39.66±1.86ab	28.74±1.89ab	2.85±0.09bc	4.29±0.24b	427±17e
	72	6.80±0.26	11.07±0.44ab	6.06±0.13b	39.35±1.69ab	29.71±1.86abc	2.86±0.20bc	4.16±0.39b	405±13de
	96	6.80±0.46	10.42±0.77ab	6.51±0.20bc	38.85±0.98ab	31.17±1.20abc	2.19±0.81abc	4.06±0.35b	403±16de
	144	6.82±0.56	9.13±2.11a	6.99±0.37c	37.37±0.92ab	33.37±1.38bc	2.32±1.05abc	3.99±1.04b	377±25cde
	168	6.78±0.54	8.85±2.37a	7.12±0.53c	37.12±1.28a	34.49±2.84bc	2.03±0.26abc	3.61±0.63b	366±32bcd
	192	6.76±0.66	8.68±2.59a	7.20±0.59c	37.08±1.41a	35.74±3.81c	1.53±0.27ab	3.01±0.57ab	355±28bcd
	Sign[a]	ns	**	***	***	**	**	***	******
ED50	6	6.88±0.51	13.92±0.97b	5.18±0.16a,α	43.31±2.47b	26.53±2.10	1.93±0.59	2.25±0.40a	279±26a
	12	6.39±0.68	12.30±0.59ab	5.23±0.36a	41.28±2.74ab	28.47±1.69	2.93±0.37	3.40±0.32b	332±25ab
	24	6.39±0.36	11.63±0.98ab	5.44±0.34a	40.44±2.39ab	29.51±1.75	2.72±0.47	3.87±0.50b	354±29ab

48	7.62±0.64	11.77±0.64ab	6.40±0.34ab	38.92±2.53ab	28.31±1.67	2.97±0.21	4.00±0.59b	459±46b
72	7.40±0.50	11.12±0.45ab	6.37±0.26ab	39.22±2.25ab	29.50±1.61	2.63±0.07	3.77±0.46b	439±38b
96	7.76±0.56	10.72±0.56ab	6.87±0.18b	38.14±1.69ab	30.01±0.74	2.79±0.05	3.71±0.37b	437±51b
144	7.50±0.93	8.80±2.85ab	7.51±0.68b	36.14±2.12ab	33.88±4.38	2.95±0.50	3.23±0.08ab	396±78ab
168	7.05±0.90	8.32±3.27a	7.64±0.80b	36.08±3.33ab	36.12±5.82	1.84±0.65	2.96±0.20ab	383±75ab
192	7.07±1.28	8.11±3.40a	7.61±0.70b	35.30±3.66a	36.61±6.76	2.25±0.74	3.04±0.21ab	374±79ab
Sign[a]	ns	*	***	*	ns	ns	***	*
Sign[b]	ns,ns,ns,ns,ns, ns,ns,ns,ns	ns,ns,ns,ns,ns, ns,ns,ns,ns	**,ns,ns,ns,ns, ns,ns,ns,ns	ns,ns,ns,ns,ns, ns,ns,ns,ns	ns,ns,ns,ns,ns, ns,ns,ns,ns	ns,ns,ns,ns,ns, ns,ns,ns,ns	ns,ns,ns,ns,ns, ns,ns,ns,ns	ns,ns,ns,ns,ns, ns,ns,ns,ns

All data are expressed as average value ± standard deviation (n=3). [a,b]Sign: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively, for the differences ([a]) among maceration times for each treatment and ([b]) among treatments for each maceration time. Different Latin letters within the same column indicate significant differences ([a]) (Tukey-b test; $p < 0.05$). Different Greek letters within the same column indicate significant differences ([b]) (Tukey-b test; $p < 0.05$). ED20= enzyme dose of 20 mg/kg, ED50= enzyme dose of 50 mg/kg. Dp-3-G= delphinidin-3-glucoside, Cy-3-G= cyanidin-3-glucoside, Pt-3-G= petunidin-3-glucoside, Pn-3-G= peonidin-3-glucoside, Mv-3-G= malvidin-3-glucoside.

Table 3. Skin mechanical properties of Cabernet Sauvignon and Nebbiolo winegrapes before and after the maceration process with and without enzymatic treatment.

Treatment	Maceration time (h)	Cabernet Sauvignon		Nebbiolo		Sign[c]
		F_{sk} (N)	W_{sk} (mJ)	F_{sk} (N)	W_{sk} (mJ)	
Control	0	0.571±0.105	0.284±0.073	0.571±0.086	0.250±0.078	ns,ns
	192	0.335±0.144b	0.156±0.132b	0.421±0.129b	0.185±0.078b	*,ns
	Sign[a]	***	***	***	**	
ED20	0	0.605±0.097	0.304±0.085	0.605±0.119	0.264±0.093	ns,ns
	192	0.124±0.074a	0.035±0.054a	0.101±0.034a	0.023±0.011a	ns,ns
	Sign[a]	***	***	***	***	
ED50	0	0.548±0.122	0.255±0.084	0.593±0.095	0.272±0.105	ns,ns
	192	0.076±0.021a	0.008±0.008a	0.089±0.021a	0.018±0.016a	ns,ns
	Sign[a]	***	***	***	***	
	Sign[b]	ns,***	ns,***	ns,***	ns,***	

All data are expressed as average value ± standard deviation (n=30). [a,b,c]Sign: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively, for the differences ([a]) among maceration times for each treatment and variety, ([b]) among treatments for each maceration time and variety, and ([c]) among varieties for each treatment and maceration time. Different Latin letters within the same column indicate significant differences ([b]) (Tukey-b test; $p < 0.05$). ED20= enzyme dose of 20 mg/kg, ED50= enzyme dose of 50 mg/kg. F_{sk}= berry skin break force, W_{sk}= berry skin break energy.