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IMPACT OF SEVERAL PRE-TREATMENTS ON THE EXTRACTION OF PHENOLIC COMPOUNDS IN WINEGRAPE VARIETIES WITH DIFFERENT ANTHOCYANIN PROFILE AND SKIN MECHANICAL PROPERTIES

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ABSTRACT

This study was performed to evaluate and compare the effects of different pre-treatments of whole grape berries (freezing with dry ice or in a cold room, steam blanching with different exposure times, and microwave heating with different exposure times and microwave power density) on total content of some phenolic compounds and the composition of individual anthocyanins released into the pulp during the treatment and those extracted during the maceration step. Two red winegrape varieties with different proportions of di- and tri-substituted anthocyanins were used (Nebbiolo and Barbera, respectively). Pulp extracted anthocyanins were more significantly influenced by the pre-treatment. The results highlighted that freezing with dry ice, followed by freezing in a cold room and steam blanching for 5 min, have a great potential from an industrial point of view. They facilitated the extraction of anthocyanins in the must prior to maceration, when compared with the control samples, increasing their total content (+37.8-83.6%), and modifying the anthocyanin profile through the enrichment in the most stable compounds (+2.8-6.6% malvidin derivatives) in detriment of others more prone to oxidation (-0.8-11.0% cyanidin derivatives). In Nebbiolo winegrapes, an improved extraction of low and high molecular weight flavanols into the pulp was also observed (+60.4-73.4%). Significant relationships between the phenolic composition of treated berries and the corresponding skin mechanical properties were also studied, but they were variety dependent. Discriminant analysis permitted a correct classification of the samples according to the variety and pre-treatment.

Keywords: Grape pre-treatments; Anthocyanins; Flavanols; Extraction; Skin mechanical properties; Dry ice

INTRODUCTION

The biosynthesis of grape anthocyanins depends on several factors, including maturity, climate, seasonal variations, soil, variety and viticultural practices.[1-3] Nevertheless, the grapevine genome determines the characteristic anthocyanin profile of each variety. In fact, the anthocyanin fingerprinting enables the chemotaxonomic differentiation of red winegrape varieties.[4-7]

The degradation of the cell-wall polysaccharide structures during grape ripening is a fundamental step to increase the release of anthocyanins from grape skins during winemaking, and the chemical composition of berry skin cell-walls at harvest is important for the anthocyanin extractability.[8-10] On the other hand, the mechanical properties of berry skin also influence the anthocyanin extraction from the grape skin into the must/wine. Therefore, the anthocyanin extraction can be successfully predicted by instrumental texture analysis. In particular, the berry skin break force can be considered the best mechanical parameter to estimate anthocyanin extraction kinetics with adequate reliability.[11]

However, these colored compounds are only partially extracted during fermentation/maceration. In the initial phase of maceration, 3'-hydroxylated anthocyanins are preferentially extracted from winegrape varieties with hard skins and may be easily oxidized by the enzymes present in the juice.[12] Once released, anthocyanin compounds suffer chemical reactions (oxidation, hydrolysis, cycloaddition, condensation and polymerization), may be adsorbed by the yeasts and also fixed again onto the solid parts of the grapes. This affects the color stability and taste of the must/wine. Particularly, the formation of flavanol-anthocyanin complexes promotes the color stability and contributes favorably to the sensory characteristics by decreasing the astringency perception.[13,14] Therefore, the extraction of flavanols is also of great relevance for the color of the final product.

Alternative techniques to traditional maceration have been used to promote the diffusion of phenolic compounds from the berry skin and their solubilization in the must. The rate and extent of

the extraction of anthocyanins and proanthocyanidins are influenced by the enological technique applied.[15] In particular, the use of pectolytic enzymes, maceration temperature and time, and ethanol content affect the cell and membrane permeability in grape berries.[16-21]

Grape freezing and heat treatment prior crushing do not constitute maceration techniques themselves, but are possible pre-treatments aiming at the preparation of the grapes to encourage release of phenolic compounds from the skins. Grape freezing causes the berry cells to burst, breaking the cell membranes and thus releasing preferentially water-soluble compounds into the must.[22] Consequently, the anthocyanin extraction from the skins is enhanced.[18,23-25] The thermal treatment of grape berries also damages the hypodermal cell membranes, favoring preferential anthocyanin release into the must.[22] Nevertheless, some authors reported that the heat can cause partial thermal degradation of anthocyanins[26] and adverse effects on the sensory attributes of the final product.[27]

The use of microwave heating as an alternative to conventional grape blanching and drying has been recently reported in the literature.[27-29] Moreover, the advantages of microwave-assisted extraction of anthocyanins in alcoholic and/or acidic medium from grape skins have been investigated.[30,31] Microwave energy has a high penetration power, which allows rapid heating throughout the sample while reducing both the exposure time and the impact of temperature on thermolabile compounds and sensory characteristics.[32] Lin and Brewer[33] also demonstrated that microwaves enable an efficient heat transfer with little or without water, which reduces nutrient losses compared with the traditional method. In fact, microwave hydrodiffusion permits the rapid juice extraction by internal heating of the water present inside the grape until its boiling point because of the expansion of the cells and consequent cell-wall rupture.[34] The same principle could be applied to the release of the cell constituents into the surrounding juice enhancing the extraction efficiency. In this sense, many attempts were carried out to produce a grape juice enriched in polyphenols.[35]

The new enological trends aim at implementing the production of high quality red wines and colored juices from the exploitation of the intrinsic chromatic characteristics of the grapes and their preservation in the final product. Therefore, the aim of this work was to evaluate and compare the impact of different treatments on winegrapes prior to crushing, such as grape freezing, steam blanching and microwave heating, on the skin mechanical properties and the extraction of anthocyanins from red winegrapes. Many experimental conditions influencing the anthocyanin release from berry skins were investigated. In particular, the study was carried out on *Vitis vinifera* L. cv. Nebbiolo and Barbera to assess the effect in grape berries with a high proportion of di- and-tri-substituted anthocyanins, respectively, because of the different trend of these compounds to be lost during winemaking. Given the relevance for color, the extraction of low and high molecular weight flavanols from the skin was also evaluated. These are important and well-known Italian varieties, giving grapes used for the production of renowned red wines that are commercialized worldwide.

MATERIALS AND METHODS

Grape samples. Twenty whole bunches of red grape cultivars *Vitis vinifera* L. cv. Nebbiolo and Barbera were harvested at technological maturity (24.0 ºBrix, 8.41 g/L tartaric acid and 24.3 ºBrix, 6.66 g/L tartaric acid, respectively) from as many vines in commercial vineyards located in the same growing zone (Piedmont, Cuneo province, north-west Italy) in 2012. Once in the laboratory, for each variety, a subsample of approximately 1.5 kg of grapes (1000-1200 berries) was randomly selected by picking berries with attached pedicels, cut in the proximity of the receptacle, from different positions in the cluster (shoulders, middle and bottom). The presence of the pedicel prevents grape must losses. For each subsample, the berries were sorted according to their density by flotation using different saline solutions (from 100 to 190 g/L sodium chloride corresponding to densities comprised between 1069 and 1125 kg/m^3) and following the protocol described by Rolle et al.[2] This densimetric sorting allows obtaining more homogeneous samples and minimizing the

possible ripening effect among berries. The comparative study was carried out on the berries belonging to the most representative class with a density of 1107 kg/m^3 and a relative weight of 60% w/w. The sorted berries were washed with water and visually inspected; those with damaged skins were discarded. For each variety studied, seven sets of 30 sorted berries (three replicates of ten berries)[36] were randomly selected to apply the different pre-treatments, and another one of 30 sorted berries was used as control sample (untreated grapes, CS).

Grape pre-treatments. Three grape pre-treatments were studied at different experimental conditions: steam blanching, freezing and microwave heating. Each replicate of sorted berries was weighed by means of a technical balance (Gibertini E1700, Modena, Italy). Therefore, three replicates of each experiment were performed for a total of seven experiments. The grape berries were treated using the following methods:

Steam blanching. The berries were treated with the vapor released during water heating at 100 ºC for 1 min (HS1) or 5 min (HS5). Subsequently, the grapes were quickly cooled at 4 °C in a fridge down to ambient temperature (20 ± 1 °C).

Freezing. The berries were frozen using two different cooling methods: direct freezing in a freezer at -18 ºC for 2 days (DF), or by adding 3 mm thick dry ice pellets in the same amount as the berries weight (DI) as proposed by Busse-Valverde et al.[15] for crushed grapes. Then, the grapes were defrosted at ambient temperature.

Microwave heating. Microwave treatments were conducted by using a 2450 MHz microwave oven with maximum delivered power of 1000 W (Panasonic NE 1037). The berries were arranged in a single layer on the rotating glass plate and placed in the center of the oven. The treatments were performed at two different microwave power densities and exposure times using 1 W/g for 30 s (MW1D30), 1 W/g for 60 s (MW1D60) or 2 W/g for 60 s (MW2D60), thereby preventing grape must losses during the treatment.[34] The grapes were quickly cooled at 4 °C in a fridge down to ambient temperature.

Pulp and surface temperatures of grape berries were measured immediately after heating pre-treatments (HS1, HS5, MW1D30, MW1D60, MW2D60) employing two different techniques. Pulp temperature was measured inserting a T-type thermocouple (0.6 mm diameter and 10 mm long junction) in the centre of two berries. A DeltaOHM HD32.8.16 thermocouple datalogger recoded data. Surface temperature was evaluated adopting the infrared (IR) thermography technique. IR images were acquired by an Avio TVS-500 camera and analyzed with the Goramtec Termography Studio (GTS 4.8) software. Emissivity parameter was assessed heating a sample of ten grape berries at 30, 40, 50 and 60 °C in a thermostatic chamber and acquiring, for each condition, an IR image once the thermal equilibrium was achieved. The cooling time of the berries was evaluated considering the pulp temperature for each heating pre-treatment. In particular, the temperature was monitored during the entire cooling process, and the cooling time was assumed as the time required to the three thermocouples to achieve the temperature of 20±0.5 °C. The same technique was followed to assess the thawing time for DF and DI pre-treatments and the freezing time for DI pre-treatment. In this last case, the pulp temperature of grape berries was measured till they achieved the same temperature of dry ice.

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 grape texture analysis. The berry skin hardness was assessed by a puncture test using a SMS P/2N needle probe (Stable Micro Systems), a test speed of 1 mm/s and a penetration depth of 3 mm.[37] Each berry (untreated or treated) was individually punctured in the lateral face, 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}.[37] 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 skin mechanical properties were

calculated from force-distance curves using the Texture Exponent software package (Stable Micro Systems).

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), (+)-catechin and cyanidin chloride were supplied from Extrasynthèse (Genay, France).

Technological ripeness parameters. Soluble solids concentration (ºBrix) was measured using an Atago 0-32 Brix temperature compensating refractometer (Atago Corporation, Tokyo, Japan), and titratable acidity (g/L tartaric acid) was estimated using the OIV method.[38]

Extraction and determination of phenolic compounds. Once the berries have been punctured, the berry skins were manually removed from the pulp using a laboratory spatula. The pulp was introduced into a tube containing 100 mg sodium metabisulphite, weighed and subsequently diluted (9:1, m/m) with 5 mol/L sulphuric acid.[39] 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. In the resulting solution, pulp phenolic compounds were determined in untreated berries and compared to those extracted during the grape pre-treatment. The skins were weighed and quickly immersed into 25 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. After remaining 48 h at 25 ºC, the solution was used for determining extracted skin phenolic compounds during the maceration step.[40] In these conditions, an extraction time of 48 h was sufficient to achieve a plateau for all compounds investigated.[11,40] The residual berry skins were quickly immersed into 25 mL of a new hydroalcoholic buffer containing 600 mg/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 phenolic compounds.[40] Total contents of anthocyanins (expressed as mg malvidin-3-O-glucoside chloride/kg grape, as TA), proanthocyanidins (expressed as mg cyanidin chloride/kg grape, as PRO) and flavanols reactive to vanillin (expressed as mg (+)-catechin/kg grape, as FRV) were determined by spectrophotometric methods[39,41] using an UV-1800 spectrophotometer (Shimazdu Corporation, Kyoto, Japan).

The determination of the anthocyanin profile was performed after the berry skin or pulp extract had been submitted to reversed-phase solid-phase extraction using a 1 g SEP-PAK C_{18} cartridge (Waters Corporation, Milford, MA, USA) with methanol as the eluent.[42] The HPLC-DAD system and chromatographic conditions were those used in a previous work.[42] 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), and a Spectra Focus diode array detector (DAD, Spectra Physics Analytical, Inc.) operating at 520 nm were used. The mobile phases were: A = formic acid/water (10:90, v/v); B = formic acid/methanol/water (10:50:40, v/v), working at a flow-rate of 1 mL/min. 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.[7] Individual anthocyanins were expressed in percentages. All of the analyses were performed in duplicate and then averaged.

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

RESULTS AND DISCUSSION

As a consequence of minimizing the ripening effect by densimetric sorting of berries, all the berries belonging to the same density class of 1107 kg/m^3 have similar soluble solids concentration

of 24.2 ± 0.2 and 24.2 ± 0.1 ºBrix for Barbera and Nebbiolo winegrapes, respectively, and titratable acidity value of 8.74 ± 0.11 and 6.81 ± 0.03 g/L tartaric acid, respectively.

Grape pre-treatments. Figure 1 shows pulp and surface temperatures of grape berries measured immediately after heating pre-treatments by steam and microwave energy (HS1, HS5, MW1D30, MW1D60 and MW2D60). The temperatures achieved increased with longer heating times and/or higher microwave power density. The cooling time of the berries to achieve the temperature of 20±0.5 °C was 8, 16, 21, 26 and 30 min, respectively. The freezing time for the DI pre-treatment (-77 ºC) was 38 min, whereas the thawing time for the DF and DI pre-treatments was 85 and 220 min, respectively.

Total anthocyanins and flavanols. Total content of phenolic compounds released from the skin to the pulp during the grape pre-treatment, extracted skin phenolic compounds during the maceration step and non-extracted skin phenolic compounds for the two red winegrape varieties studied is shown in Table 1. As regards total skin anthocyanins (sum of pulp, extracted skin and non-extracted skin anthocyanins) of Barbera grapes, the concentration obtained (753±52 mg/kg) was lower than that reported for berries with higher contents of reducing sugars.[41] This agreed with the significant increase (16-31%) in the concentration of total anthocyanins (TA) in Barbera grapes with increasing the sugars content between 235 and 269 g/L for most of the production zones evaluated.[41] The concentration of total skin anthocyanins in Nebbiolo grapes (440±29 mg/kg), although low, was usual for this variety as shown in other works performed on berries containing a similar content of reducing sugars or belonging to the same density class.[2,42] In addition to the ripening effect, the influence of the growing location on the content of TA has been observed for both Barbera and Nebbiolo varieties in several studies.[41,42]

For Nebbiolo untreated berries, the average anthocyanin extraction yield was 80.5% (100-non-extracted skin anthocyanins, expressed as percentage), this value being lower than that published (91.1-94.2%) for different harvest dates and berry densities.[2] However, the average percentage obtained of non-extracted skin anthocyanins (19.5%, Table 1) agreed with that reported

(19.9%) for Nebbiolo berries with hard skins.[11] For Barbera grapes, no study has been previously carried out on the extraction yield of anthocyanins from the skin into the pulp or a wine-like solution, and only the cellular maturity index (EA%) was determined in some works.[41]

The sum of pulp, extracted skin and non-extracted skin contents of proanthocyanidins (PRO) and flavanols reactive to vanillin (FRV) (602±116 and 200±39 mg/kg for Barbera grapes, and 2700±288 and 1104±98 mg/kg for Nebbiolo grapes, respectively) was in the range published for total contents in the skin of the same varieties,[2,41] with the exception of FRV in Barbera grapes for which the values obtained in the present work were higher. The influence of the growing location on the content of FRV in the skin was very evident.[41] For Nebbiolo untreated berries, the average extraction yield of PRO and FRV was 75.0 and 78.9% (100-non-extracted skin compounds, expressed as percentage), respectively, these values agreed with those reported for different harvest dates and berry densities.[2]

TA contents in the pulp lower than 18 and 13 mg/kg for Barbera and Nebbiolo untreated berries, respectively, could be due in part to the inevitable manipulation of the sample during the step of removing the skin from the pulp. Only direct freezing of berries in a freezer (DF) and by adding dry ice (DI) facilitated the releasing of skin anthocyanins to the pulp for both Barbera and Nebbiolo winegrapes, as well as the pre-treatment of the berries by steam blanching for 5 min (HS5) for Nebbiolo. For the Barbera variety, the highest content of pulp TA was obtained when the pre-treatment with dry ice was used (67.7 mg/kg). For the Nebbiolo variety, the highest contents were associated with the pre-treatment by steam blanching for 5 min (74.8 mg/kg), but they were not significantly different to the ones corresponding to the pre-treatment with dry ice (60.9 mg/kg). For each variety, there were no significant differences in the content of extracted skin TA among treated and untreated berries. Therefore in Barbera winegrapes, the percentage of non-extracted anthocyanins was significantly lower for the DI pre-treatment than for untreated berries (CS) or the treated with heat (HS or MW). On the other hand, non-extracted TA were significantly lower for the

HS5 pre-treatment in Nebbiolo winegrapes although did not differ from those remaining in the skin after berries freezing (DF and DI).

As occurred for TA in Nebbiolo winegrapes, the DF, DI and HS5 pre-treatments facilitated the releasing of PRO and FRV from the skin to the pulp. The lowest percentages of non-extracted skin PRO and FRV corresponded to the HS5 pre-treatment, although they did not differ significantly from those remaining in the skin after berries freezing (DF and DI) or shorter steam blanching (HS1). In Barbera winegrapes, significant differences were only found in the percentages of non-extracted skin PRO and FRV. In this case, the percentage of non-extracted skin PRO was significantly lower for freezing pre-treatments (DF and DI), as well as that of FRV for the DF pre-treatment, than for CS berries. Therefore, the improved extraction of anthocyanins and flavanols from the skin could favor the long-term color stability through the formation of pigmented polymers.[13,14] Furthermore, an increase in the proportion of PRO (high molecular weight flavanols) released from the skin usually leads to a softening effect on the mouthfeel because of the lower astringency perception.[15]

Other studies revealed that the wines obtained using different low temperature prefermentative treatments (grape freezing in a freezer, must freezing with dry ice and cold maceration) show higher concentrations of TA at the end of fermentative maceration than the control wine.[15,18,19,43] Particularly, freezing may provide better results in winegrape varieties characterized by skins having a more rigid structure.[18] The use of dry ice also facilitated the extraction of high molecular weight PRO from skins by degrading the structures.[15] Freezing with dry ice facilitates the extraction of phenolic compounds probably due to a quick thermal shock that induces the formation of ice crystals within the berry skin. The increased volume of the intracellular liquids causes the breakage of the cell walls and membranes. An additional advantage of dry ice is the protective effect against oxidation of extracted anthocyanins before fermentation, as it sublimates to gaseous carbon dioxide displacing completely the oxygen present in the medium and inhibits the activity of polyphenoloxidases enzymes.[15,22,25] The carbon dioxide saturation maintains

the inhibitory effect even when temperature reaches normal fermentation values.[18] However, other works reported that the effectiveness of these treatments depends on the grape ripeness, the berry size and the composition of the skin cell walls.[20,23,44]

In the scientific literature, dry ice is usually added to the must after grapes crushing, whereas the uncrushed grapes are directly frozen in a freezer. The comparative studies carried out on the use of these two prefermentative cold treatments provide contradictory results. Gil-Muñoz et al.[18] pointed out that must freezing with dry ice leads to higher contents of TA at the end of alcoholic fermentation. However, Heredia et al.[25] justified lower anthocyanin contents on the basis of a local effect on the skin just in the mass being directly in contact with dry ice. When prefermentative cold maceration of the must with the solid parts (crushed grapes) was carried out, higher extraction of TA was achieved with the addition of dry ice instead of keeping the sample in the freezer.[20] In the present study, the two pre-treatments (DF and DI) were applied to uncrushed berries, and the release and solubilization of anthocyanins into the pulp for Barbera and Nebbiolo winegrapes were also more favored using the DI pre-treatment as compared with the DF pre-treatment.

According to the results obtained for steam blanching of whole berries, TA, PRO and FRV released into the pulp during the pre-treatment seem to increase with increasing heating time for the Nebbiolo variety. In some works, higher concentrations of anthocyanins in wines were obtained by heating partially dejuiced grapes at 90-95 °C for 1 min,[45] by mash thermal treatment at 50 ºC for 15 min[46] or by mash prefermentative heating up to 65 ºC and cooling down for 24 h (30 ºC)[47] than using the untreated grapes. On the contrary, other researchers reported lower content of TA but higher one of total flavanols in the wine obtained by mash prefermentative heating at 65 ºC for 8 h than in the control wine.[16] Therefore, temperature and time of heating seem to be important factors. Although the degradation of monomeric anthocyanins occurs more rapidly with increasing temperature, shorter treatment time at higher temperatures might be ideal for enhancing the extraction of thermally labile compounds.[26] Mild blanching of the berries can inactivate polyphenoloxidases enzymes, protecting anthocyanins against enzymatic degradation.[48] On the

other hand, the water content is the most common absorbing phase for microwave energy and, therefore, plays a key role during the microwave treatment of grapes. In the present work, significant differences were not found in the content of pulp TA for microwave treated berries (MW) in relation to untreated berries (CS). According to the results obtained, the anthocyanin degradation was not observed using the thermal pre-treatments of whole berries with steam or microwave energy, probably due to the short treatment times and/or low microwave power densities.[49]

Anthocyanin profile. The qualitative composition of skin anthocyanins released into the pulp before and after the grape pre-treatment, extracted skin anthocyanins during the maceration step and non-extracted skin anthocyanins for Barbera and Nebbiolo winegrapes is shown in Tables 2 and 3, respectively. For Barbera untreated grapes, malvidin derivatives were the predominant anthocyanin compounds (about 62%) and the richness in tri-substituted anthocyanin forms ranged from 77% to 92% (Table 2). However, the anthocyanin profile was slightly different as a function of the extraction easiness of anthocyanins (Table 2). In particular, peonidin derivatives were the second most abundant pulp anthocyanins (11%), whereas petunidin derivatives were for extracted and non-extracted skin anthocyanins (13% and 20%, respectively). Furthermore, simple glucoside (free or unacylated) forms accounted for around 75% of extracted and non-extracted skin anthocyanins, but they achieved 92% of pulp anthocyanins mainly at the expense of acetylated forms. Although the general profile of extracted and non-extracted skin anthocyanins in Barbera untreated winegrapes was similar, non-extracted skin anthocyanins showed slightly lower percentages of malvidin derivatives, which resulted in higher abundance of the other tri-substituted anthocyanins. Despite some small differences attributable to the growing zone and seasonal variability,[6] the anthocyanin profile of Barbera untreated grapes was similar to the extractable anthocyanin profile previously reported in other works,[5,36,41] particularly for extracted and non-extracted skin anthocyanins. The works published to date did not consider pulp anthocyanins during the manipulation step of the berry skin.

Regarding the Nebbiolo variety, Table 3 shows that untreated winegrapes were rich in di-substituted anthocyanin forms with percentages ranging from 63% to 81%. The major anthocyanin compounds were peonidin derivatives (about 45%). Malvidin derivatives were also abundant extracted and non-extracted skin anthocyanins (about 23%), although lower than peonidin forms. However, cyanidin derivatives (35%) predominated over malvidin derivatives (13%) in pulp anthocyanins. This constitutes an important enological aspect because of the high percentage of di-substituted anthocyanin forms easily extracted in the pulp (77-81%). As occurred in Barbera untreated grapes, simple glucoside forms accounted for 97% of pulp anthocyanins, whereas they only reached percentages comprised between 84% and 88% of extracted and non-extracted skin anthocyanins. The qualitative composition of extracted and non-extracted skin anthocyanins for Nebbiolo untreated grapes was quite similar and agreed, with some small differences, with the extractable anthocyanin profile previously reported in the scientific literature.[2,5,42,50]

When the profile of extracted skin anthocyanins was compared with that of pulp anthocyanins in untreated and treated berries, greater percentages of delphinidin, petunidin and malvidin derivatives were usually observed in the former for both Barbera and Nebbiolo varieties, whereas the percentages of cyanidin and peonidin forms were lower. The diffusion of di-substituted anthocyanins is faster than the one corresponding to tri-substituted anthocyanins,[12] which justifies the higher relative abundance of the first compounds in the pulp, but the percentage of tri-substituted anthocyanins increases during the first days of maceration.[12] On the other hand, the proportion of unacylated forms in extracted skin anthocyanins was lower in favor of higher proportions of acetylated and cinnamoylated glucosides. Acylated anthocyanins are very important because they participate in intramolecular copigmentation processes, protecting the flavylium cation.[18] In the present work, some exceptions were found to this general behavior, particularly in malvidin and peonidin derivatives for Barbera and Nebbiolo winegrapes, respectively. Therefore, for a given variety the different pre-treatments studied on whole berries may influence the anthocyanin profile of the final product, favoring preferential releasing of a certain anthocyanin

form from the skin into the must. This fact is of great importance because the reactivity of the different anthocyanins depends on their structure. Particularly, 3´-hydroxylated molecules (delphinidin, cyanidin and petunidin) are more prone to oxidation and thermolabile,[51,52] and therefore the juices/wines with higher proportions of these molecules are more sensitive to color degradation. In fact, anthocyanins without ortho-hydroxylated groups can interact with flavanols and ethanal, leading to a stable red pigmentation. In varieties characterized by an important presence of di-substituted anthocyanins, such as Nebbiolo, a remarkable loss of these anthocyanin compounds has been also noticed during winemaking.[53] The next step was to evaluate the effect of each grape pre-treatment studied on the profile of pulp, extracted skin and non-extracted skin anthocyanin compounds for Barbera and Nebbiolo berries.

The different pre-treatments of the berries caused changes of different magnitude in the anthocyanin profile of Barbera grapes if compared with untreated berries (Table 2). Most of significant differences were found in pulp anthocyanins. Freezing with dry ice (DI) was the grape pre-treatment that affected more significantly the profile of pulp anthocyanins. Although the proportions of tri-substituted anthocyanins like delphinidin and petunidin forms experienced a significant decrease, total tri-substituted anthocyanins were more abundant in the berries treated with dry ice because of a significant decrease in the percentage of cyanidin derivatives and the largest increase in the percentage of malvidin derivatives (+6.4%). This effect of the DI pre-treatment on the profile of pulp anthocyanins agreed with the observed in wines made from winegrapes rich in tri-substituted anthocyanins.[54] The DI pre-treatment also induced the greatest decrease in the relative abundance of simple glucoside anthocyanins in favor of the more significant increase in acetylated glucosides (+2.3%). The influence of freezing of the berries in a freezer (DF) on the profile of pulp anthocyanins was similar to the one corresponding to the DI pre-treatment but to a much lesser extent. Microwave heating at 2 W/g for 60 s (MW2D60) also promoted a marked increase in the percentage of total tri-substituted anthocyanins as a consequence of the lowest percentages of cyanidin and peonidin derivatives released from the skin into the pulp during the

berries pre-treatment (decreasing -3.0% and -1.7%, respectively). The MW2D60 pre-treatment and steam blanching for 5 min (HS5) caused an important increase in the percentage of malvidin derivatives. In the case of the HS5 pre-treatment, the lowest percentages of other tri-substituted anthocyanins like delphinidin and petunidin derivatives were obtained (decreasing -2.8% and -1.9%, respectively). Microwave heating at 1 W/g for 60 s (MW1D60) was the grape pre-treatment giving the highest proportion of di-substituted anthocyanins and the greatest decrease in the percentage of malvidin derivatives in the pulp (-1.9%). Microwave heating using any experimental condition investigated permitted a significant increase in the proportion of acetylated glucoside anthocyanins at the expense of simple glucoside forms.

The effect of the different pre-treatments applied to the berries on the anthocyanin profile of Nebbiolo winegrapes is shown in Table 3. As already mentioned for the Barbera variety, most of significant differences among untreated and treated berries were found in pulp anthocyanins. The most influential grape pre-treatments were DI, DF and HS5. The DI pre-treatment caused the largest increase in the percentage of malvidin compounds (+6.6%) and the greatest decrease in the proportion of cyanidin derivative pulp anthocyanins (-11.0%). Although this last treatment also favored the significant increase in the percentage of peonidin derivatives, total tri-substituted anthocyanins were more abundant in the berries treated with dry ice. Furthermore, the more significant increase in the proportion of acetylated glucoside pulp anthocyanins at the expense of free glucosides corresponded to the DI pre-treatment. On the other hand, the greatest increase in the percentage of peonidin derivatives (+6.3%) was associated with the DF pre-treatment, but it was balanced with a significant reduction in the proportion of cyanidin derivatives and an important increase in the percentage of malvidin forms of pulp anthocyanins. This fact implied that the relative abundance of total tri-substituted anthocyanins was not significantly different among freezing pre-treatments (DF and DI). The percentage of these compounds also agreed with the obtained in the pulp after the HS5 pre-treatment of the berries, as a consequence of the highest increase found in the proportions of delphinidin and petunidin derivatives (+1.2% and +0.8%,

respectively) together with a significant reduction in the percentage of cyanidin derivatives and an important increase in malvidin forms in relation to untreated berries. However, the percentage of peonidin derivatives increased significantly. Furthermore, the lowest proportion of free glucoside pulp anthocyanins (decreasing -0.9%), in favor of acetylated glucosides, corresponded to the HS5 pre-treatment. The anthocyanin profile of the berries steam blanched for 1 min (HS1) or microwave heated (MW) was not significantly different to the one corresponding to untreated berries.

For each variety, only a few significant differences were found in the profile of extracted and non-extracted skin anthocyanins among untreated and treated berries. Because the grape pre-treatment can cause changes in the skin tissues that facilitate the extraction of anthocyanins during the maceration step, the profile of extracted skin anthocyanins for Barbera winegrapes showed some significant changes affecting mainly petunidin derivatives (+2.1%) when the DI pre-treatment was applied (Table 2). For the Nebbiolo variety (Table 3), the only significant difference was the lowest percentage of simple glucosides (-1.7%) when the berries were submitted to the HS5 pre-treatment in favor of cinnamoylated glucosides. Regarding the qualitative composition of anthocyanins that remain in the skin after maceration, the most important changes were observed for Nebbiolo berries where the greatest decrease in the percentages of delphinidin derivatives and simple glucosides (about -1.4% and -2.3%, respectively), in favor of cinnamoylated glucosides, was obtained after the DI and HS5 pre-treatment.

Regarding general trends, some aspects are particularly relevant. Freezing of whole berries (DF and DI) improved the extraction of malvidin derivatives from the skin into the pulp for both Barbera and Nebbiolo winegrapes, which agreed with the increased extraction of malvidin-3-glucoside into the wine using different prefermentative cold maceration treatments of crushed berries.[23,54] Since malvidin derivatives are the most stable forms of anthocyanins (particularly acylated forms), their higher diffusion into the pulp may contribute favorably to the color stability of the resulting juice/wine. When acylation was considered, higher percentages of acylated glucosides were observed in pulp anthocyanins after the DI pre-treatment of Barbera and Nebbiolo

winegrapes than after the DF pre-treatment. Heredia et al.[25] have also found lower abundance of monoglucosides during cryomaceration with dry ice when compared with refrigeration of the berries in a freezer.

According to the results obtained, the effect of heating time and/or microwave power density depended on the variety. Microwave power density affected mainly the anthocyanin profile of Barbera winegrapes, whereas the effect of treatment time was particularly important for the Nebbiolo variety using steam as heating source. Other work showed that, after alcoholic fermentation, the wine obtained by prefermentative mash heating at 65 ºC for 8 h contains less malvidin-3-glucoside and more delphinidin-3-glucoside and petunidin-3-glucoside than the control wine, even though malvidin-3-glucoside is the most abundant monomeric anthocyanin in Okuzgozu grapes.[16] These results disagreed with those obtained in the present work. The variety and the experimental conditions of heating may be very influential factors on the anthocyanin profile of the resulting juices/wines and, therefore, on their color stability.

Instrumental texture analysis. The skin mechanical properties of Barbera and Nebbiolo winegrapes before and after the different pre-treatments studied are shown in Table 4. The values obtained in untreated berries are comprised in the usual range for these varieties, particularly from Piedmont growing zone.[41,42,50] The magnitude of the effect of the different freezing and heating pre-treatments on the instrumental texture parameters defining the skin hardness (F_{sk} and W_{sk}) depended on the variety. For Barbera winegrapes, freezing with dry ice (DI) reduced significantly the skin break energy (W_{sk}) achieving the lowest values in relation to berries untreated and treated with any other methodology studied. No significant difference was found in the skin break force (F_{sk}) among untreated and treated berries for the Barbera variety. For Nebbiolo winegrapes, the hardest skins were found after the thermal pre-treatment of the berries by steam blanching for 5 min (HS5), with values of F_{sk} and W_{sk} being significantly higher than those ones corresponding to untreated and differently treated berries. A possible explanation could be the caramelization of sugars closer to the

skin, which occurs at high temperatures. The DI pre-treatment also promoted a significant increase in the values of W_{sk} in relation to untreated berries.

Instrumental texture parameters of the skin have been proposed as anthocyanin extractability markers.[11] However, the relationships between the skin mechanical properties and the anthocyanin extraction yield are variety dependent. The chemical composition of grape skin cell-walls may determine the mechanical resistance of the berry skin to the anthocyanin release.[8] In Nebbiolo grapes, higher values of F_{sk} facilitated more complete anthocyanin release from the skin.[11] In the present work, significant correlations (correlation factor $R>0.5$, $p<0.05$) were found for each variety between the value of W_{sk} in treated whole berries and the corresponding content of TA (n=21, 7 treatments x 3 replicates of 10 berries). So, the amount of pulp TA and the percentage of non-extracted skin TA were better correlated with the value of W_{sk} for Nebbiolo ($R=0.736$, $p<0.001$ and $R=-0.721$, $p<0.001$, respectively) than for Barbera winegrapes ($R=-0.553$, $p<0.01$ and $R=0.615$, $p<0.01$, respectively), as shown in Table 5. Furthermore, the relationships were inverse for the two varieties. The results obtained agreed with those reported by Rolle et al.[11] for the Nebbiolo variety, the higher the skin hardness the higher the extracted anthocyanins. Because higher skin hardness probably involves greater cell wall fragility, the higher the W_{sk} values the higher the amounts of pulp flavanols (PRO and FRV) ($R=0.640$, $p<0.01$ and $R=0.681$, $p<0.001$, respectively) for Nebbiolo winegrapes, and therefore the lower the percentages of non-extracted skin flavanols ($R=-0.751$, $p<0.001$ and -0.755, $p<0.001$, respectively). As occurred for TA in Barbera winegrapes, the content of PRO (high molecular weight flavanols) released into the pulp was negatively correlated with the value of W_{sk} ($R=-0.576$, $p<0.01$), whereas no significant correlation was found for FRV (low molecular weight flavanols).

The berry skin hardness also affected the individual anthocyanin composition of the hydroalcoholic extracts for Nebbiolo skins.[11] Only in the early phases of the anthocyanin diffusion, harder skins (more fragile) exhibited significantly higher percentages of extracted petunidin-3-glucoside (+0.8%), cyanidin-3-glucoside (+3.6%) and peonidin-3-glucoside (+6.0%) derivatives at

the expense of malvidin-3-glucosides (-9.6%). After macerating for 10 min, no significant differences were found in the extracted anthocyanin profile of skins with different hardness. Hence the values obtained of F_{sk} in the present work for Nebbiolo winegrapes correspond to hard skins, higher proportions of peonidin derivatives in pulp anthocyanins (+1.6-6.3%) are justified for treated berries if compared with untreated berries. On the other hand, a higher number of significant relationships between the berry skin mechanical properties and the anthocyanin profile were associated with pulp anthocyanins (Table 5), probably due to the fact that they were more quickly released from the skin. Considering only the most significant correlations (Table 5, $R>0.675$, $p<0.001$), in Nebbiolo treated winegrapes, the percentages of delphinidin and petunidin glucosides increased with increasing the values of F_{sk} and W_{sk}, whereas those of cyanidin decreased with increasing W_{sk}, for pulp anthocyanins. However, the most important variation in pulp anthocyanins with W_{sk} corresponded to the percentages of cyanidin and malvidin glucosides. Harder skins of Nebbiolo treated berries provided lower relative abundance of cyanidin glucosides (sensitive to oxidation reactions) and higher percentage of malvidin glucosides (highly stable). Furthermore, the proportion of unacylated glucosides decreased significantly, and the relative amount of cinnamoylated forms increased with rising the value of W_{sk} for extracted skin anthocyanins in Nebbiolo treated berries. In Barbera treated winegrapes, the significant correlations were less numerous and weaker than in Nebbiolo ones (Table 5, $R<0.670$, $p>0.001$) with general inverse trends for the two varieties.

Multivariate analysis. Discriminant analysis based on the contents of TA, PRO and FRV, and the composition of individual anthocyanins released into the pulp, extracted during maceration and remaining in the skin (as defined in the materials and methods section), and the skin mechanical properties for treated and untreated Barbera and Nebbiolo winegrapes (Tables 1-4) showed a very clear classification of the berries according to the pre-treatment (Figure 2a). A perfect clustering of scores of the samples for each pre-treatment was observed. The two first canonical functions accounted for 91.0% of total variance in the original data. Function 1 explained 79.5% of the

variability and was mainly related to the percentages of cyanidin and peonidin derivatives of pulp anthocyanins, particularly unacylated forms. The highest positive values of function 1 corresponded to the berries frozen (DF and DI pre-treatments). Function 2 accounted for 11.6% of the cumulative variance and was mainly associated with the relative abundance of pulp petunidin derivatives, extracted skin cyanidin and peonidin derivatives and non-extracted skin cyanidin derivatives, as well as the content of extracted skin TA and PRO, the thermal pre-treatment HS5 corresponding to the highest positive values. The standardized coefficients of canonical functions are shown in Table 6. Although the differentiation of berries untreated and treated by other thermal pre-treatments was not so clear, 95.8% of the samples were correctly classified. When discriminant analysis was carried out separately on Barbera and Nebbiolo winegrapes (Figure 2b), a perfect clustering of scores of the samples for each variety and pre-treatment was observed. The two first canonical functions accounted for 99.2% of total variance in the original data. Function 1 explained 97.7% of the variability and was mainly related to the content of pulp PRO, extracted skin TA and PRO and non-extracted skin TA. The highest positive and negative values of function 1 were associated with Barbera and Nebbiolo winegrapes, respectively. Therefore, function 1 permitted a good differentiation of the samples according to the variety. Function 2 accounted for 1.6% of the cumulative variance and was mainly associated with the percentage of cyanidin derivatives of extracted skin anthocyanins and the relative abundance of petunidin derivatives of non-extracted skin anthocyanins, particularly unacylated and acetylated forms. The highest positive values of function 2 corresponded to freezing pre-treatments (DF and DI) and the thermal pre-treatment HS5, whereas the highest negative values were associated with untreated berries (CS) and the thermal pre-treatment HS1. The standardized coefficients of the seven first canonical functions are shown in Table 7. In this case, 100% of the samples were correctly classified. Therefore, function 2 facilitated the differentiation of untreated berries and those submitted to the different pre-treatments studied.

In conclusion, different pre-treatments of whole berries (freezing with dry ice, freezing in a freezer and steam blanching for 5 min) enhanced mainly the releasing of anthocyanins from the skin into the pulp. Nevertheless, the magnitude of the effect depended on the grape pre-treatment, experimental conditions used, anthocyanin compound and variety. The varietal effect may be related to the changes occurring in the skin tissue during the pre-treatment, which affected the facility to break the cell-walls and to release anthocyanins. In fact, the significance and strength of the correlations between the berry skin mechanical properties and the phenolic composition of treated berries depended strongly on the variety studied. Freezing with dry ice can be considered a promising treatment for industrial processing of uncrushed grape berries, not only because of increased total content of pulp extracted anthocyanins but also the modified anthocyanin profile in favor of more stable compounds. It is particularly important for winegrape varieties with a high proportion of di-substituted anthocyanins, such as Nebbiolo. For this variety, the use of dry ice also facilitated the release of low and high molecular weight flavanols from the skin into the pulp, which may lead to more colored juices/wines with lower astringency perception. In future works, the practical application of the most advantageous pre-treatments will be thoroughly evaluated by means of chemical, chromatic and sensory quality of the juices and/or wines obtained. Particularly, the production of grape juices rich in anthocyanins could be of great interest for offering a product with higher nutritional value, better chromatic characteristics and improved acceptance by consumers.

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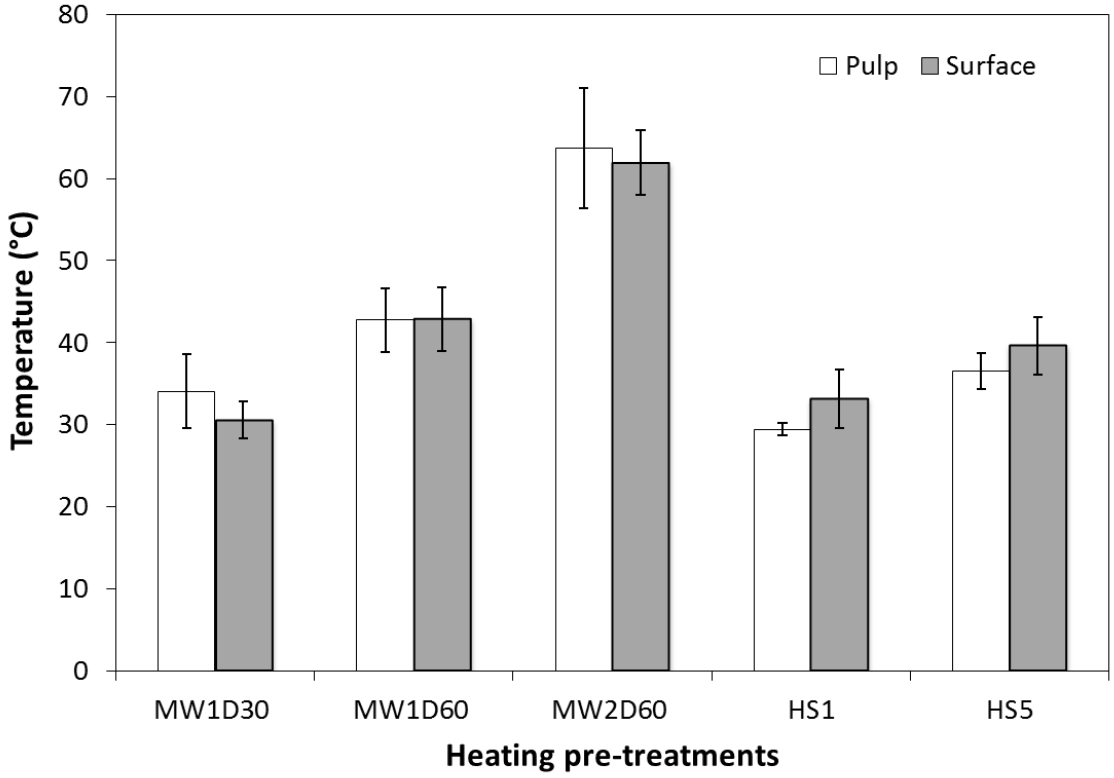
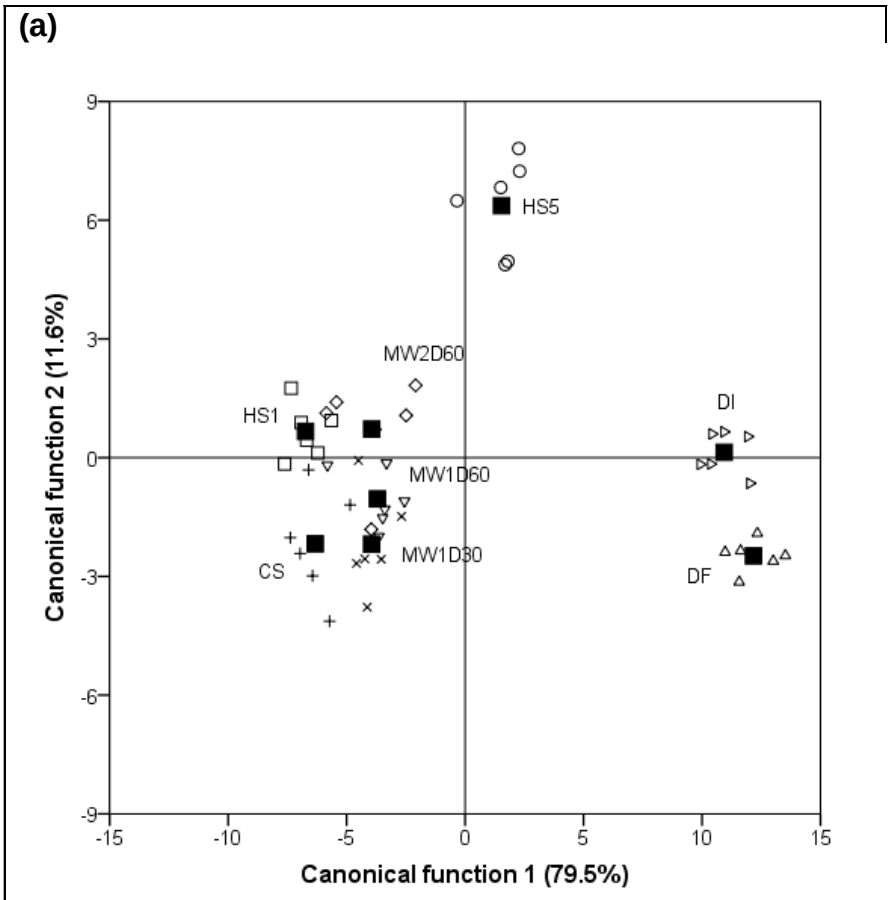


Figure 1. Pulp and surface temperatures of grape berries measured immediately after heating pre-treatments. MW1D30 = microwave heating at 1 W/g for 30 s, MW1D60 = microwave heating at 1 W/g for 60 s, MW2D60 = microwave heating at 2 W/g for 60 s, HS1 = steam blanching at 100 ºC for 1 min, HS5 = steam blanching at 100 ºC for 5 min.


(a)
Canonical function 2 (11.6%)
Canonical function 1 (79.5%)
HS5
MW2D60
HS1
DI
MW1D60
MW1D30
CS
DF
9
6
3
0
-3
-6
-9
-15
-10
-5
0
5
10
15

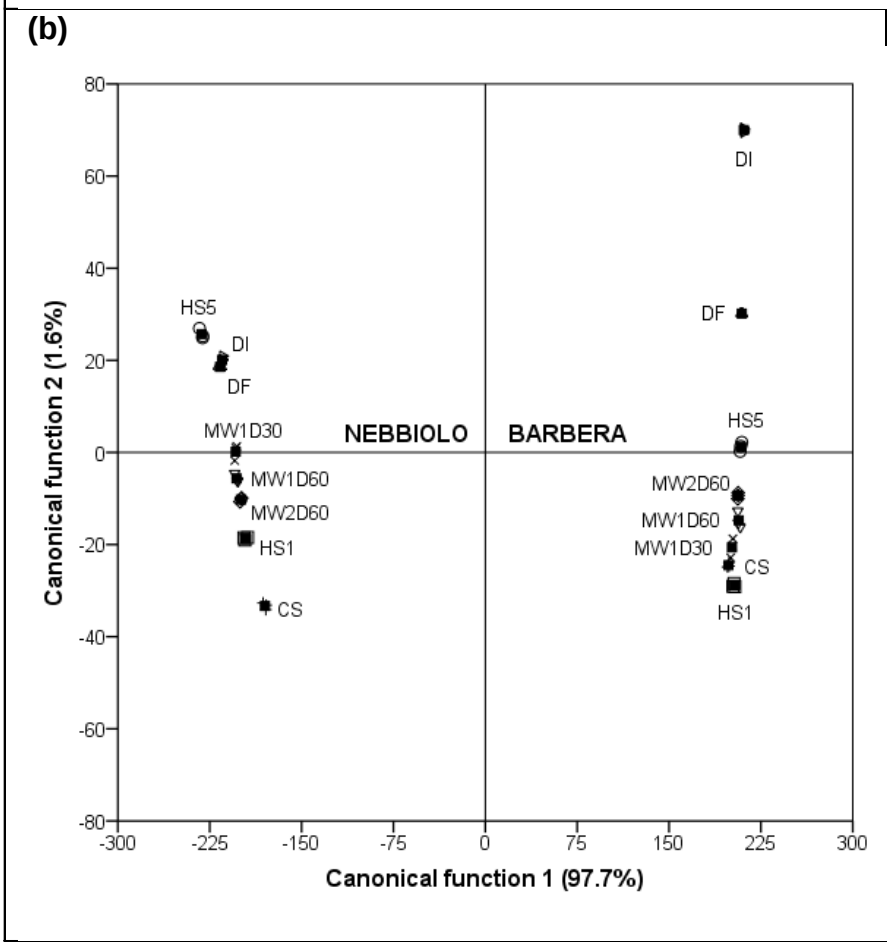

(b)
Canonical function 2 (1.6%)
Canonical function 1 (97.7%)
NEBBIOLO
BARBERA
HS5
DI
DF
MW1D30
MW1D60
MW2D60
HS1
CS
80
60
40
20
0
-20
-40
-60
-80
-300
-225
-150
-75
0
75
150
225
300

Figure 2. Projection of winegrape berries untreated and submitted to the different pre-treatments studied in the plane defined by the two first canonical functions considering Barbera and Nebbiolo varieties simultaneously (a) and separately (b). HS1 = steam blanching at 100 ºC for 1 min, HS5 = steam blanching at 100 ºC for 5 min, DF = direct freezing in a freezer at -18 ºC for 2 days, DI = freezing by adding dry ice, MW1D30 = microwave heating at 1 W/g for 30 s, MW1D60 = microwave heating at 1 W/g for 60 s, MW2D60 = microwave heating at 2 W/g for 60 s, CS = control.

Table 1. Total Content of Phenolic Compounds in Barbera and Nebbiolo Winegrapes Before and After Different Pre-Treatments.

Parameter[a]	Grape pre-treatment[b]								
	HS1	HS5	DF	DI	MW1D30	MW1D60	MW2D60	CS	Sign[c]
					Barbera				
Pulp TA (mg/kg)	10.8±2.0a	28.6±8.2ab	44.0±14.3b	67.7±8.3c	15.8±3.0a	20.5±2.4a	23.5±6.9a	17.8±0.4a	***
Extracted skin TA (mg/kg)	481.6±29.8	451.1±19.6	474.2±12.5	470.1±16.8	451.6±6.2	474.3±18.5	450.6±15.4	449.6±18.6	ns
Non-extracted skin TA (%)	34.6±3.7bc	36.3±1.8bc	31.2±0.3ab	28.6±3.1a	37.9±1.2c	34.3±2.2bc	37.0±1.4bc	37.9±2.5c	***
Pulp PRO (mg/kg)	25.2±3.2	37.9±16.4	56.1±14.2	60.3±16.1	34.0±9.5	30.3±1.6	32.0±9.7	43.6±11.0	ns
Extracted skin PRO (mg/kg)	350.9±3.8	315.6±7.5	378.5±39.8	362.5±26.5	295.8±80.0	350.2±14.5	332.7±7.3	230.8±82.5	ns
Non-extracted skin PRO (%)	37.5±1.2ab	41.3±4.0ab	27.8±4.2a	29.8±1.7a	45.2±11.7ab	36.8±2.2ab	39.4±0.4ab	54.4±11.9b	*
Pulp FRV (mg/kg)	3.4±0.9	7.7±5.9	10.4±1.6	8.0±1.2	4.7±0.4	6.4±0.8	4.8±0.9	3.9±1.1	ns
Extracted skin FRV (mg/kg)	164.5±0.8	160.7±9.6	167.7±8.4	160.0±5.6	163.3±2.2	167.3±4.7	165.6±1.1	154.7±2.1	ns
Non-extracted skin FRV (%)	16.2±0.0ab	15.9±1.9ab	11.1±3.4a	16.1±3.4ab	16.1±0.9ab	13.3±2.0ab	14.9±0.1ab	20.8±0.5b	*
					Nebbiolo				
Pulp TA (mg/kg)	14.8±1.5a	74.8±17.1c	44.2±11.1b	60.9±9.0bc	19.6±4.2a	22.8±3.1a	21.0±2.1a	12.3±0.3a	***
Extracted skin TA (mg/kg)	339.2±14.6	314.2±14.0	333.8±10.5	314.5±16.8	345.9±10.4	331.8±13.6	340.3±1.2	342.3±6.7	ns
Non-extracted skin TA (%)	19.6±3.0c	11.7±1.1a	14.2±0.1ab	14.8±1.8ab	17.0±1.5bc	19.5±2.4c	18.0±0.7bc	19.5±1.5c	***
Pulp PRO (mg/kg)	292.3±95.7a	639.3±69.9b	655.7±50.5b	629.5±1.7b	317.2±179.8a	333.3±53.2a	380.5±15.0ab	249.4±8.6a	**
Extracted skin PRO (mg/kg)	1886.3±70.7	1670.3±110.1	1626.4±0.6	1585.5±2.0	1801.9±158.0	1797.1±0.9	1774.6±63.0	1776.4±31.7	ns
Non-extracted skin PRO (%)	19.3±0.9abc	14.5±1.5a	15.5±1.9ab	18.0±0.1abc	21.5±0.8cd	21.1±2.0cd	20.2±1.8bcd	25.0±1.5d	**
Pulp FRV (mg/kg)	89.4±44.0a	252.9±45.4b	193.1±34.0ab	246.5±11.1b	106.7±64.1a	103.3±24.3a	118.3±12.3a	67.2±2.2a	**
Extracted skin FRV (mg/kg)	854.7±30.2b	716.7±53.6a	761.1±43.9ab	704.7±13.2a	785.7±45.3ab	780.3±9.7ab	794.7±4.6ab	804.3±15.1ab	*
Non-extracted skin FRV (%)	14.5±1.2ab	12.2±0.7a	13.6±0.9ab	13.9±0.2ab	19.2±1.7c	20.0±1.3c	17.3±1.5bc	21.1±1.6c	***

[a]TA = total anthocyanins, PRO = proanthocyanidins, FRV = flavanols reactive to vanillin. [b]HS1 = steam blanching at 100 ºC for 1 min, HS5 = steam blanching at 100 ºC for 5 min, DF = direct freezing in a freezer at -18 ºC for 2 days, DI = freezing by adding dry ice, MW1D30 = microwave heating at 1 W/g for 30 s, MW1D60 = microwave heating at 1 W/g for 60 s, MW2D60 = microwave heating at 2 W/g for 60 s, CS = control. All data are expressed as average value ± standard deviation (n=3). Different Latin letters within the same row indicate significant differences ([c]) among pre-treatments according to Tukey-b test ($p < 0.05$). [c]Sign: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively.

Table 2. Anthocyanin Profile of Barbera Winegrapes Before and After Different Pre-Treatments.

Compound	Grape pre-treatment[a]								
	HS1	HS5	DF	DI	MW1D30	MW1D60	MW2D60	CS	Sign[b]
					Pulp anthocyanins (%)				
Σ Delphinidin derivatives	6.58±0.39d	3.54±0.68a	4.85±0.56abc	4.53±0.33ab	6.18±0.65cd	5.74±0.29bcd	5.79±0.80bcd	6.34±0.72cd	***
Σ Cyanidin derivatives	9.53±0.70cd	8.87±0.83bcd	7.74±0.98abc	7.11±0.72ab	8.70±1.02bcd	10.65±0.26d	6.64±0.46a	9.62±0.88cd	***
Σ Petunidin derivatives	10.50±0.57d	8.04±0.70a	8.89±0.48abc	8.46±0.44ab	10.28±0.52cd	9.62±0.27bcd	10.21±0.69cd	9.93±0.49cd	***
Σ Peonidin derivatives	10.09±1.22ab	12.08±0.58b	12.54±1.37b	10.30±0.72ab	10.44±1.25ab	12.70±0.14b	9.23±0.52a	10.97±1.22ab	**
Σ Malvidin derivatives	63.30±1.21ab	67.47±1.85bc	65.98±2.51abc	69.59±1.64c	64.40±3.00abc	61.29±0.45a	68.13±2.38bc	63.15±2.38ab	**
Σ Simple glucosides	91.53±0.32bc	92.12±0.37c	90.45±0.25ab	89.92±0.71a	90.65±0.28ab	90.23±0.33ab	90.87±0.96abc	92.04±0.41c	***
Σ Acetyl glucosides	1.22±0.08abc	1.13±0.06ab	2.68±0.12d	3.17±0.40e	1.36±0.11bc	1.66±0.12c	1.47±0.25bc	0.88±0.03a	***
Σ Cinnamoyl glucosides	7.24±0.25abc	6.75±0.36a	6.87±0.14ab	6.91±0.35ab	7.99±0.37bc	8.12±0.21c	7.67±0.82abc	7.08±0.41abc	**
					Extracted skin anthocyanins (%)				
Σ Delphinidin derivatives	9.55±0.60	9.48±0.46	9.55±0.55	10.04±0.43	9.30±0.59	9.55±0.27	9.54±0.31	9.54±0.13	ns
Σ Cyanidin derivatives	3.58±0.26ab	4.61±0.47b	3.32±0.54a	4.18±0.15ab	3.93±0.50ab	3.47±0.47a	3.64±0.26ab	3.80±0.34ab	*
Σ Petunidin derivatives	13.21±0.37a	13.75±0.40a	13.97±0.21a	15.42±0.20b	13.33±0.64a	13.42±0.04a	13.45±0.31a	13.37±0.09a	***
Σ Peonidin derivatives	5.08±0.31ab	6.09±0.46b	4.67±0.77a	5.63±0.40ab	5.66±0.50ab	4.97±0.51ab	5.07±0.34ab	5.39±0.57ab	*
Σ Malvidin derivatives	68.57±1.29	66.06±1.56	68.50±1.66	64.73±0.61	67.78±2.16	68.59±1.26	68.30±1.17	67.90±0.82	ns
Σ Simple glucosides	75.22±3.21	75.24±0.99	72.73±0.56	75.74±0.44	74.51±2.09	75.60±3.91	76.48±1.59	74.59±0.50	ns
Σ Acetyl glucosides	15.83±3.30	16.45±0.90	17.80±0.29	15.28±0.58	16.40±1.75	15.28±3.67	14.93±1.69	16.72±0.43	ns
Σ Cinnamoyl glucosides	8.95±0.35ab	8.31±0.20a	9.47±0.48b	8.98±0.15ab	9.09±0.43ab	9.12±0.24ab	8.59±0.10ab	8.69±0.41ab	*
					Non-extracted skin anthocyanins (%)				
Σ Delphinidin derivatives	15.30±1.49	14.21±0.83	13.69±0.97	13.30±0.54	14.82±1.12	14.53±0.86	15.35±0.73	15.53±0.23	ns
Σ Cyanidin derivatives	3.91±0.30ab	5.07±0.55b	3.62±0.68a	4.72±0.08ab	4.24±0.53ab	3.77±0.71ab	3.84±0.26ab	4.10±0.42ab	*
Σ Petunidin derivatives	19.35±0.83	19.54±0.44	19.20±0.24	19.50±0.43	19.36±0.69	19.38±0.27	19.84±0.49	19.56±0.26	ns
Σ Peonidin derivatives	4.70±0.24	5.65±0.32	4.55±0.71	5.45±0.39	5.10±0.44	4.64±0.49	4.52±0.24	5.03±0.59	ns
Σ Malvidin derivatives	56.75±2.81	55.53±2.02	58.94±2.28	57.03±0.59	56.49±2.39	57.67±2.20	56.47±1.50	55.78±0.87	ns
Σ Simple glucosides	74.52±2.63	73.30±1.28	71.68±1.29	74.88±2.69	72.78±1.48	70.81±1.57	74.02±1.10	75.33±3.67	ns

Σ Acetyl glucosides	11.33±1.55	12.79±1.01	12.67±0.26	10.66±1.52	13.05±0.71	14.49±0.60	12.62±1.16	12.25±2.96	ns
Σ Cinnamoyl glucosides	14.15±1.73	13.91±0.62	15.65±1.36	14.47±1.66	14.17±0.82	14.69±0.98	13.36±0.21	12.42±0.72	ns

[a]HS1 = steam blanching at 100 ºC for 1 min, HS5 = steam blanching at 100 ºC for 5 min, DF = direct freezing in a freezer at -18 ºC for 2 days, DI = freezing by adding dry ice, MW1D30 = microwave heating at 1 W/g for 30 s, MW1D60 = microwave heating at 1 W/g for 60 s, MW2D60 = microwave heating at 2 W/g for 60 s, CS = control. All data are expressed as average value ± standard deviation (n=3). Different Latin letters within the same row indicate significant differences ([b]) among pre-treatments according to Tukey-b test ($p < 0.05$). [b]Sign: *,**,*** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively.

Table 3. Anthocyanin Profile of Nebbiolo Winegrapes Before and After Different Pre-Treatments.

Compound	Grape pre-treatment[a]								
	HS1	HS5	DF	DI	MW1D30	MW1D60	MW2D60	CS	Sign[b]
					Pulp anthocyanins (%)				
Σ Delphinidin derivatives	3.89±0.39a	5.84±0.33b	4.38±0.60a	4.83±0.36ab	4.06±0.37a	4.10±0.48a	4.47±0.63a	4.63±0.50a	**
Σ Cyanidin derivatives	36.58±3.07b	24.05±0.84a	24.31±1.30a	23.91±2.11a	32.45±2.73b	32.68±1.83b	32.74±1.19b	34.86±1.72b	***
Σ Petunidin derivatives	3.43±0.15a	4.47±0.15b	3.68±0.42a	4.01±0.30ab	3.67±0.19a	3.54±0.31a	3.86±0.24ab	3.69±0.12a	**
Σ Peonidin derivatives	45.44±1.00ab	48.53±0.78cd	50.10±1.69d	47.70±1.89bcd	46.66±0.59abc	46.80±0.61abc	45.48±1.02ab	43.85±0.49a	***
Σ Malvidin derivatives	10.66±1.64a	17.11±1.02bc	17.53±1.74bc	19.55±3.37c	13.17±2.64ab	12.89±1.50ab	13.45±1.77ab	12.97±1.63ab	***
Σ Simple glucosides	97.13±0.10cd	95.96±0.22a	97.17±0.30d	96.22±0.15ab	96.53±0.36abc	96.56±0.22abcd	96.75±0.28bcd	96.88±0.12cd	***
Σ Acetyl glucosides	0.36±0.06a	0.73±0.02b	0.72±0.11b	0.93±0.06c	0.45±0.06a	0.38±0.05a	0.38±0.06a	0.35±0.03a	***
Σ Cinnamoyl glucosides	2.50±0.04ab	3.31±0.20c	2.11±0.20a	2.86±0.17bc	3.02±0.34bc	3.06±0.17c	2.87±0.26bc	2.77±0.11bc	***
					Extracted skin anthocyanins (%)				
Σ Delphinidin derivatives	6.12±0.54	6.89±0.21	6.73±0.24	6.60±0.72	6.05±0.57	6.20±0.16	6.42±0.57	6.76±0.19	ns
Σ Cyanidin derivatives	20.75±2.69	20.40±1.49	17.19±1.21	18.72±1.26	17.45±2.39	17.14±1.52	18.99±2.82	18.15±0.14	ns
Σ Petunidin derivatives	5.01±0.53	5.12±0.17	5.49±0.08	5.44±0.55	5.03±0.40	5.19±0.18	5.18±0.16	5.42±0.06	ns
Σ Peonidin derivatives	48.11±2.49	47.47±0.52	47.33±1.16	48.08±3.31	47.49±2.93	48.02±1.10	45.99±0.83	46.26±0.63	ns
Σ Malvidin derivatives	20.02±4.19	20.11±1.46	23.26±2.16	21.15±3.28	23.98±4.28	23.44±1.77	23.42±2.89	23.40±0.52	ns
Σ Simple glucosides	89.60±0.85b	86.46±0.24a	88.19±0.64b	88.15±0.55b	88.85±0.72b	88.65±0.21b	88.30±0.78b	88.12±0.08b	***
Σ Acetyl glucosides	4.29±0.43	4.88±0.32	4.62±0.32	4.45±0.34	4.39±0.34	4.79±0.06	4.63±0.14	4.88±0.07	ns
Σ Cinnamoyl glucosides	6.10±0.47a	8.66±0.10c	7.18±0.36b	7.40±0.23b	6.76±0.38ab	6.56±0.27ab	7.07±0.65ab	7.00±0.02ab	***
					Non-extracted skin anthocyanins (%)				
Σ Delphinidin derivatives	6.99±0.74ab	6.51±0.10a	6.83±0.20ab	6.27±0.58a	6.69±0.35ab	7.05±0.29ab	7.02±0.62ab	7.75±0.18b	*
Σ Cyanidin derivatives	20.86±2.10	20.43±1.78	17.45±1.34	18.81±0.98	17.64±2.73	17.67±1.32	19.62±2.92	18.72±0.46	ns
Σ Petunidin derivatives	5.36±0.59	5.05±0.21	5.60±0.04	5.34±0.49	5.35±0.33	5.63±0.28	5.48±0.17	5.96±0.11	ns
Σ Peonidin derivatives	47.53±2.66	48.22±0.53	47.57±1.21	48.75±3.26	47.11±2.84	47.86±1.59	45.58±0.73	45.03±1.21	ns
Σ Malvidin derivatives	19.26±3.38	19.80±1.99	22.55±2.35	20.83±3.15	23.21±5.07	21.79±1.82	22.29±3.00	22.54±0.80	ns

Σ Simple glucosides	86.31±1.09c	82.31±0.42a	82.78±1.58ab	82.10±0.76a	85.07±1.37bc	84.84±0.60bc	85.20±0.80bc	84.54±0.41abc	***
Σ Acetyl glucosides	3.57±0.45	3.77±0.33	3.59±0.53	3.78±0.52	3.88±0.51	4.13±0.12	3.59±0.12	4.12±0.18	ns
Σ Cinnamoyl glucosides	10.13±0.66a	13.92±0.10b	13.63±1.06b	14.12±0.27b	11.06±0.86a	11.03±0.50a	11.20±0.70a	11.34±0.32a	***

[a]HS1 = steam blanching at 100 ºC for 1 min, HS5 = steam blanching at 100 ºC for 5 min, DF = direct freezing in a freezer at -18 ºC for 2 days, DI = freezing by adding dry ice, MW1D30 = microwave heating at 1 W/g for 30 s, MW1D60 = microwave heating at 1 W/g for 60 s, MW2D60 = microwave heating at 2 W/g for 60 s, CS = control. All data are expressed as average value ± standard deviation (n=3). Different Latin letters within the same row indicate significant differences ([b]) among pre-treatments according to Tukey-b test ($p < 0.05$). [b]Sign: *,**,*** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively.

Table 4. Skin Mechanical Properties of Barbera and Nebbiolo Winegrapes Before and After Different Pre-Treatments.

Parameter	Grape pre-treatment[a]								
	HS1	HS5	DF	DI	MW1D30	MW1D60	MW2D60	CS	Sign[b]
					Barbera				
F_{sk} (N)[c]	0.837±0.118	0.874±0.146	0.767±0.134	0.831±0.150	0.777±0.150	0.797±0.142	0.840±0.126	0.852±0.123	ns
W_{sk} (mJ)[d]	0.859±0.189bc	0.921±0.214c	0.781±0.270bc	0.574±0.231a	0.726±0.205b	0.779±0.208bc	0.925±0.188c	0.803±0.229bc	***
					Nebbiolo				
F_{sk} (N)[c]	0.635±0.110b	0.785±0.129c	0.510±0.106a	0.603±0.121b	0.570±0.084ab	0.580±0.144ab	0.628±0.123b	0.575±0.077ab	***
W_{sk} (mJ)[d]	0.468±0.140abc	0.657±0.171d	0.504±0.208bc	0.528±0.176c	0.386±0.100a	0.387±0.135a	0.461±0.128abc	0.410±0.093ab	***

[a]HS1 = steam blanching at 100 ºC for 1 min, HS5 = steam blanching at 100 ºC for 5 min, DF = direct freezing in a freezer at -18 ºC for 2 days, DI = freezing by adding dry ice, MW1D30 = microwave heating at 1 W/g for 30 s, MW1D60 = microwave heating at 1 W/g for 60 s, MW2D60 = microwave heating at 2 W/g for 60 s, CS = control. All data are expressed as average value ± standard deviation (n=30). Different Latin letters within the same row indicate significant differences ([b]) among pre-treatments according to Tukey-b test ($p < 0.05$). [b]Sign: *** and ns indicate significance at $p < 0.001$ and not significant, respectively. [c]F_{sk} = skin break force, [d]W_{sk} = skin break energy.

Table 5. Pearson's Correlation Coefficients Between Skin Mechanical Properties and Phenolic Composition of Treated Barbera and Nebbiolo Winegrapes.

Compound[a]	Barbera		Nebbiolo	
	F_{sk} (N)[b,c]	W_{sk} (mJ)[b,d]	F_{sk} (N)[b,c]	W_{sk} (mJ)[b,d]
	Pulp phenolic compounds			
TA (mg/kg)	ns	-0.553**	0.433*	0.736***
PRO (mg/kg)	ns	-0.576**	ns	0.640**
FRV (mg/kg)	ns	ns	ns	0.681***
Σ Delphinidin derivatives (%)	ns	ns	0.731***	0.775***
Σ Cyanidin derivatives (%)	ns	ns	ns	-0.692***
Σ Petunidin derivatives (%)	ns	ns	0.717***	0.724***
Σ Peonidin derivatives (%)	ns	ns	ns	ns
Σ Malvidin derivatives (%)	ns	ns	ns	0.597**
Σ Simple glucosides (%)	0.468*	0.608**	-0.519*	ns
Σ Acetyl glucosides (%)	ns	-0.665**	ns	0.605**
Σ Cinnamoyl glucosides (%)	ns	ns	0.514*	ns
	Extracted skin phenolic compounds			
TA (mg/kg)	-0.456*	ns	ns	-0.455*
PRO (mg/kg)	ns	ns	ns	-0.456*
FRV (mg/kg)	ns	ns	ns	-0.454*
Σ Delphinidin derivatives (%)	ns	ns	ns	0.595**
Σ Cyanidin derivatives (%)	ns	ns	ns	ns
Σ Petunidin derivatives (%)	ns	-0.648**	ns	ns
Σ Peonidin derivatives (%)	ns	ns	ns	ns
Σ Malvidin derivatives (%)	ns	ns	ns	ns
Σ Simple glucosides (%)	ns	ns	-0.546*	-0.678***
Σ Acetyl glucosides (%)	ns	ns	ns	ns

Σ Cinnamoyl glucosides (%)	-0.614**	-0.453*	0.553**	0.721***
	Non-extracted skin phenolic compounds			
TA (%)	ns	0.615**	ns	-0.721***
PRO (%)	ns	ns	ns	-0.751***
FRV (%)	ns	ns	ns	-0.755***
Σ Delphinidin derivatives (%)	ns	0.451*	ns	ns
Σ Cyanidin derivatives (%)	ns	ns	ns	ns
Σ Petunidin derivatives (%)	0.500*	ns	ns	ns
Σ Peonidin derivatives (%)	ns	ns	ns	ns
Σ Malvidin derivatives (%)	-0.461*	ns	ns	ns
Σ Simple glucosides (%)	ns	ns	ns	-0.549**
Σ Acetyl glucosides (%)	ns	ns	ns	ns
Σ Cinnamoyl glucosides (%)	ns	ns	ns	0.622**

[a]TA = total anthocyanins, PRO = proanthocyanidins, FRV = flavanols reactive to vanillin. [b]Sign: *,**,*** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively (n=21). [c]F_{sk} = skin break force, [d]W_{sk} = skin break energy.

Table 6. Standardized Coefficients of Canonic Discriminant Functions for Barbera and Nebbiolo Winegrapes Considered Simultaneously.

Parameter[a]	Function						
	1	2	3	4	5	6	7
				Pulp phenolic compounds			
TA (mg/kg)	0.144	1.841	0.696	0.106	1.148	2.075	0.248
FRV (mg/kg)	2.052	1.100	2.965	1.768	-0.791	4.980	-1.199
Σ Delphinidin derivatives (%)	-0.639	-3.546	-1.361	-2.309	0.798	2.250	-0.036
Σ Cyanidin derivatives (%)	-8.930	-0.999	1.870	-2.664	3.169	6.336	-4.794
Σ Petunidin derivatives (%)	-4.343	8.550	2.744	9.475	-2.922	-7.608	0.547
Σ Peonidin derivatives (%)	7.191	-2.894	3.927	5.085	11.810	-1.986	5.815
Σ Simple glucosides (%)	10.389	-0.065	-5.460	-0.748	0.259	1.666	0.221
Σ Acetyl glucosides (%)	7.349	-3.378	-2.210	0.404	-1.146	0.200	-0.538
				Extracted skin phenolic compounds			
TA (mg/kg)	1.244	6.135	1.896	4.564	3.356	13.140	5.936
PRO (mg/kg)	-5.531	10.516	6.538	12.672	0.186	9.027	-1.470
Σ Delphinidin derivatives (%)	-0.188	2.548	0.811	-0.883	-0.410	1.666	-1.107
Σ Cyanidin derivatives (%)	1.264	-6.596	-8.635	-2.407	-1.189	1.009	12.490
Σ Peonidin derivatives (%)	-1.543	9.009	2.455	-3.817	-9.353	5.289	6.042
Σ Simple glucosides (%)	-1.931	0.365	1.216	0.218	-1.324	2.868	-2.595
				Non-extracted skin phenolic compounds			
TA (%)	2.655	1.518	2.454	3.909	2.437	12.145	3.501
Σ Cyanidin derivatives (%)	1.483	7.712	1.873	5.326	0.059	-9.420	-7.925
Σ Acetyl glucosides (%)	1.285	1.628	2.631	-0.585	1.132	-1.225	-0.616
				Skin mechanical properties			
F_{sk} (N)	-3.058	2.912	1.433	-0.709	-1.105	-0.002	1.101
W_{sk} (mJ)	0.501	0.548	-2.584	0.658	1.450	0.861	-1.867

[a]TA = total anthocyanins, PRO = proanthocyanidins, FRV = flavanols reactive to vanillin, F_{sk} = skin break force, W_{sk} = skin break energy.

Table 7. Standardized Coefficients of Canonic Discriminant Functions for Barbera and Nebbiolo Winegrapes Considered Separately.

Parameter[a]	Function						
	1	2	3	4	5	6	7
			Pulp phenolic compounds				
TA (mg/kg)	4.339	1.427	1.845	-0.624	-2.529	2.809	0.976
PRO (mg/kg)	-6.219	2.861	2.189	-1.654	1.589	0.624	1.060
Σ Delphinidin derivatives (%)	-0.126	-5.192	0.701	2.026	-1.453	-1.412	1.073
Σ Cyanidin derivatives (%)	0.327	-5.585	-1.274	0.880	-0.450	0.239	0.559
Σ Petunidin derivatives (%)	-0.048	1.579	0.326	-2.553	1.452	0.652	-1.087
Σ Peonidin derivatives (%)	-0.350	2.124	0.285	-0.685	1.087	-0.859	0.551
Σ Simple glucosides (%)	-0.023	2.365	-0.715	-0.212	-1.257	-0.091	0.073
Σ Acetyl glucosides (%)	-0.174	3.846	-1.845	-0.205	-0.524	-0.824	0.410
			Extracted skin phenolic compounds				
TA (mg/kg)	8.200	2.807	3.240	-2.432	-3.096	3.579	3.202
PRO (mg/kg)	-9.838	5.677	1.446	-3.020	6.352	-0.589	0.951
FRV (mg/kg)	2.770	-2.576	-1.479	0.086	-4.358	1.136	-1.035
Σ Delphinidin derivatives (%)	4.905	-5.230	6.835	7.082	-3.178	4.945	2.428
Σ Cyanidin derivatives (%)	3.529	6.584	0.671	-5.988	-0.220	-0.360	-1.900
Σ Petunidin derivatives (%)	-2.162	0.155	-2.718	-1.396	2.317	-0.995	-2.589
Σ Peonidin derivatives (%)	4.024	-1.767	7.144	4.539	-1.269	4.788	0.694
Σ Simple glucosides (%)	2.058	-13.482	5.693	20.092	-3.961	4.514	-0.145
Σ Acetyl glucosides (%)	1.397	-13.246	4.681	19.334	-4.221	3.719	-0.406
			Non-extracted skin phenolic compounds				
TA (%)	7.929	-1.548	4.026	0.620	-3.171	3.369	2.191
PRO (%)	-4.980	0.746	0.599	-1.046	2.605	-0.254	0.654
FRV (%)	0.034	1.890	-0.671	-0.162	0.269	0.743	0.766
Σ Delphinidin derivatives (%)	-5.084	-2.537	-3.024	-3.006	-4.992	-1.518	-1.264
Σ Cyanidin derivatives (%)	-2.781	-0.490	-3.842	1.553	1.513	-1.195	0.432

Σ Petunidin derivatives (%)	2.824	6.289	1.493	-0.717	4.005	-0.572	1.317
Σ Peonidin derivatives (%)	-5.049	1.806	-4.467	-2.711	1.798	-3.436	-0.817
Σ Simple glucosides (%)	0.350	12.254	-5.398	-9.922	8.486	-2.902	-0.654
Σ Acetyl glucosides (%)	0.136	9.404	-4.406	-6.819	7.056	-2.074	-1.314
			Skin mechanical properties				
F_{sk} (N)	0.012	0.530	-0.034	-1.271	-2.063	1.675	1.077
W_{sk} (mJ)	0.075	-2.081	0.989	0.083	1.633	-0.278	-1.104

[a]TA = total anthocyanins, PRO = proanthocyanidins, FRV = flavanols reactive to vanillin, F_{sk} = skin break force, W_{sk} = skin break energy.