Application of enzyme preparations for extraction of berry skin phenolics in withered winegrapes

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Abbreviated title: Enzymes for extraction of withered grape skin phenolics

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ABSTRACT

Postharvest withering of grapes strongly affects the content and extractability of phenolic compounds in the production of sfursat, fortified and passito wines. This work evaluated the effectiveness of enzymes applied individually and/or in multi-enzyme blends, on the extraction of anthocyanins, oligomeric flavanols and polymeric flavanols from withered grape skins during simulated maceration. The study was performed on *Vitis vinifera* L. cv. Nebbiolo and Barbera because of their different skin phenolic profile and cell wall composition. Our findings highlight that the relationship between skin mechanical properties (berry skin break force and energy) and extraction yield of phenolic compounds is variety dependent. Significant correlations were found between the skin softening associated with cell wall degradation and the extraction of anthocyanins and flavanols in Nebbiolo, for which polygalacturonase, individually or in multi-enzyme blends, plays an important role. In Barbera, the extractability of phenolic compounds was not affected by the presence of exogenous enzymes.

Keywords: postharvest withered grapes; macerating enzymes; phenolic compounds; extractability; skin mechanical properties; cell wall composition.

1. Introduction

In the oenological sector, postharvest withering of grape berries is used for the production of reinforced, fortified and *passito* wines. In addition to the changes in cell metabolism due to water loss, postharvest grape dehydration affects the chemical composition of the berries, including the content of phenolic compounds and their extractability (Rolle, Giacosa, Río Segade, Ferrarini, Torchio, & Gerbi, 2013; Torchio et al., 2016). The diffusion of these compounds from grapes into the must-wine begins during grape crushing, and it mainly occurs throughout alcoholic fermentation/maceration due to the contact between solid (skin, seeds and pulp) and liquid (juice) phases. Their content and structure may be modified by both unfavourable (adsorption and degradation) and favourable (condensation) reactions (Morata, Gomez-Cordoves, Suberviola, Bartolome, Colomo, & Suarez, 2003; Romero-Cascales, Fernández-Fernández, López-Roca, & Gómez-Plaza, 2005). As stated by other authors (Bautista-Ortín, Busse-Valverde, Fernández-Fernández, Gómez-Plaza, & Gil-Muñoz, 2016), the extractability and final concentration of phenolic compounds at the end of the maceration process mainly depend on their content and localization in the berry, anatomical structure of skin layers, grape variety and ripeness, as well as on maceration conditions (i.e. temperature, duration, alcohol level, concentration gradient between grape skins and must-wine).

The grape skin is formed by three layers: i) the cuticle is the outermost tissue, which is composed of hydroxylated fatty acids and is covered by hydrophobic waxes; ii) the epidermis is a layer of a regular tilling of cells; and iii) the inner layer is the hypodermis consisting of several cell layers, which contain most of skin phenolic compounds (Pinelo, Arnous, & Meyer, 2006). In grape skins, phenolic compounds may be distinguished depending on their localization in skin cells (Pinelo et al., 2006). Cell wall linked phenolic compounds are mainly polymeric flavanols (mean polymerization degree of ca. 28, Souquet, Cheynier, Brossaud, & Moutounet, 1996), which are linked or entangled, via hydrogen bonds or hydrophobic interactions, to the polysaccharides of the cell wall, giving rise to polysaccharide-phenol complexes. Non cell wall phenolic compounds

include both those occurring in cell vacuoles (in free form inside the vacuoles or linked to proteins forming the vacuolar inclusions) and in cell nucleus (Fontes, Gerós, & Delrot, 2011).

Skin cell wall is a barrier for the diffusion of phenolic compounds from grapes into the must-wine, and it is made up of 30% neutral polysaccharides (cellulose, xyloglucan, arabinan, galactan, xylan and mannan), 20% acidic pectin substances (of which 62% are methyl esterified), about 15% insoluble proanthocyanidins and <5% structural proteins (Lecas & Brillouet, 1994; Pinelo et al., 2006). The release of skin phenolic compounds requires the cleavage of the middle lamella binding the cells together, which is mainly composed of pectin.

The degradation of grape skin cell walls can be facilitated by the use of exogenous enzymes, thus increasing the extraction of phenolic compounds. Although pectinases were the first macerating enzymes applied in oenology, over the last years the use of commercial preparations with mixed enzymatic activities (pectinases, cellulases and proteases) has become a very common practice to achieve a more complete breakdown of the skin cells (Bautista-Ortín, Martínez-Cutillas, Ros-García, López-Roca, & Gómez-Plaza, 2005). Nevertheless, Apolinar-Valiente, Romero-Cascales, Gómez-Plaza and Ros- García (2016) found that the effectiveness of purified polygalacturonase and cellulase in improving the cell wall degradation of Syrah and Cabernet Sauvignon grapes is variety dependent as a consequence of the different composition and morphology of skin cell wall material. Furthermore, compositional differences in the enzyme preparations used can lead to contradictory results (Romero-Cascales, Fernández-Fernández, Ros-García, López-Roca, & Gómez-Plaza, 2008). A recent study performed on fresh grapes has demonstrated that this degradation by enzymes causes the skin softening, so that the decrease of skin mechanical properties is significantly related to the anthocyanin extraction yield (Río Segade, Pace, Torchio, Giacosa, Gerbi, & Rolle, 2015).

Although the use of macerating enzymes has been investigated by numerous authors in fresh grapes, studies concerning the effect on the extraction of phenolic compounds from partially dehydrated grape berries have not yet been carried out. Therefore, the main aim of this study was to

93 evaluate, for the first time, the effectiveness of several macerating enzymes, applied as single or
94 multi-enzyme blends, on the extraction of anthocyanins, oligomeric flavanols and polymeric
95 flavanols from withered grape skins during simulated maceration. This could provide knowledge on
96 the individual effect of each single enzyme activity and on which multi-enzyme blend further
97 enhances the extraction effectiveness. The relationship between the extraction yield and skin
98 mechanical properties was also assessed for the first time in withered grapes. The study was
99 performed on *Vitis vinifera* L. Nebbiolo and Barbera varieties, which were chosen for their
100 distinctive content and profile of phenolic compounds (Río Segade et al., 2014), as well as for their
101 different skin cell wall composition, because variety differences could influence the selection of
102 macerating enzymes.
103
104 **2. Materials and methods**
105 *2.1. Grapes and withering process*
106 In 2015, whole bunches of *Vitis vinifera* L. cv. Barbera and Nebbiolo red winegrapes were
107 harvested at experimental vineyards located in Alba (Piedmont region, north-west Italy) when about
108 24 °Brix were reached. Healthy bunches were placed in perforated boxes (30 cm x 20 cm, about
109 2 kg of grape berries per box) in a single layer for correct aeration. They were then partially
110 dehydrated up to 20% weight loss (percentage usually used to produce fortified wines) in a
111 thermohygrometrically controlled chamber at 20 ºC and 80% average relative humidity (RH). The
112 withering process lasted 26 days. At the end, Barbera grapes had 307 g/L of reducing sugars, 9.97
113 g/L of tartaric acid and 1.79 g/L of malic acid, whereas Nebbiolo grapes contained 287 g/L of
114 reducing sugars, 7.90 g/L of tartaric acid and 1.97 g/L of malic acid. The skins of withered Barbera
115 and Nebbiolo winegrapes were characterized according to their phenolic composition, mechanical
116 properties and cell wall composition.
117
118 *2.2. Chemical and mechanical analysis of withered grape skins*

2.2.1. Phenolic composition

Five replicates of 10 grape berries (17.9±0.4 g for each replicate) were randomly selected for each variety. The skins were manually removed from the pulp using a laboratory spatula, accurately weighed and quickly immersed into 50 mL of a hydroalcoholic buffer at pH 3.2 containing 14% v/v ethanol, 5 g/L of tartaric acid and 2 g/L of sodium metabisulphite (Torchio et al., 2016). After homogenization for 1 min at 8000 rpm using an Ultraturrax T25 high-speed homogenizer (IKA Labortechnik, Staufen, Germany) and centrifugation for 15 min at 3000 × g at 20 °C with a PK 131 centrifuge (ALC International, MI, Italy), the supernatant was used for the determination of phenolic compounds by spectrophotometric methods (Torchio, Cagnasso, Gerbi, & Rolle, 2010). The content of total anthocyanins (TA) was determined after dilution with an ethanol:water:37% hydrochloric acid 70:30:1 (v/v) solution and expressed as mg of malvidin-3-glucoside chloride/g of skin. Flavanols reactive to vanillin (FRV) were quantified after reaction with 4% m/v vanillin in methanol:37% hydrochloric acid medium and expressed as mg of (+)-catechin/g of skin. Proanthocyanidins (PRO) were transformed into cyanidin by acid hydrolysis at 100 °C using a ferrous salt ($FeSO_4$) as catalyst (Bate-Smith reaction) and expressed as mg of cyanidin chloride/g of skin. Malvidin-3-glucoside chloride was purchased from Extrasynthèse (Genay, France), whereas cyanidin chloride and (+)-catechin were supplied by Sigma (Milan, Italy). An UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used.

The anthocyanin profile of berry skins was determined by HPLC-DAD using the chromatographic system and conditions previously reported (Río Segade et al., 2014). The hydroalcoholic extracts were diluted 1:2 with 0.1 M hydrochloric acid, filtered through 0.45 μm PTFE membrane filters (Pall Corporation, Port Washington, NY, USA) and directly injected (50 μL) in the HPLC-DAD system. The separation was performed in a LiChroCART analytical column (25 cm × 0.4 cm i.d.) purchased from Merck (Darmstadt, Germany) and packed with LiChrospher 100 RP-18 (5 μm) particles supplied by Alltech (Deerfield, IL, USA). The mobile phases were formic acid/water (10:90, v/v) and formic acid/methanol/water (10:50:40, v/v). The amounts of individual

anthocyanins were expressed as percentages. Delphinidin-3-glucoside chloride, cyanidin-3-glucoside chloride, petunidin chloride, peonidin-3-glucoside chloride and malvidin-3-glucoside chloride were purchased from Extrasynthèse (Genay, France).

2.2.2. Mechanical properties

A total of 30 whole berries were randomly selected for each winegrape variety. A TA.XTplus texture analyzer (Stable Micro Systems, Godalming, Surrey, UK), equipped with a HDP/90 platform, a 5 kg load cell and a P/2N needle probe, was used to assess experimentally the skin hardness by measuring skin break force (N, as F_{sk}) and skin break energy (mJ, as W_{sk}) (Rolle, Torchio, Zeppa & Gerbi, 2008). For this purpose, a puncture test was individually performed on the lateral face of each berry at a test speed of 1 mm/s and a penetration depth of 3 mm. All data acquisitions were made at 500 points per second.

2.2.3. Isolation and chemical analysis of cell wall material

Cell wall material was isolated according to the method proposed by De Vries, Voragen, Rombouts and Pilnik (1981) and adapted by Apolinar-Valiente, Romero-Cascales, López-Roca, Gómez-Plaza and Ros-García (2010). Briefly, all skins of 300 berries were manually removed from the pulp using a laboratory spatula, freeze-dried and then manually ground with a mortar and pestle. The resulting fine powder (5 g) was suspended in boiling water for 5 min, homogenized for 1 min at 10000 rpm and then centrifuged for 15 min at 3000 × g. The solid residue was repeatedly treated with fresh 70% v/v ethanol for 30 min at 40 °C and centrifuged until no sugars were detected in the supernatant according to the Dubois test (reaction with phenol and concentrated sulphuric acid). After washing the alcohol-insoluble solid twice with 96% v/v ethanol and once with acetone, it was dried overnight at 20 °C under an air stream. The recovered cell wall was accurately weighed and manually ground.

The chemical composition of the cell wall material was determined according to the methodology used by Apolinar-Valiente et al. (2016) and Castro-López, Gómez-Plaza, Ortega-Regules, Lozada and Bautista-Ortín (2016). A set of four replicates (10 mg each) was treated with 72% v/v sulphuric

171 acid for 1 h at 30 ºC, followed by hydrolysis with 1 M sulphuric acid for 3 h at 100 ºC. In the
172 resulting solution, uronic acids were determined by the spectrophotometric 3,5-dimethylphenol
173 assay using galacturonic acid (Sigma, St Louis, MO, USA) as a standard, and total glucose was
174 quantified using an enzymatic kit (R-Biopharm, Darmstadt, Germany). Klason lignin was
175 determined gravimetrically. In other set of four replicates (10 mg each), non-cellulosic glucose was
176 determined using the enzymatic method in the resulting solution from direct hydrolysis with 1 M
177 sulphuric acid for 3 h at 100 ºC. The content of cellulosic glucose was calculated as the difference
178 between total glucose and non-cellulosic glucose contents. In a third set of four replicates (10 mg
179 each), proteins and total phenolic compounds were extracted with 1 M sodium hydroxide for 10 min
180 at 100 ºC. Proteins were spectrophotometrically determined using the Coomassie brilliant blue
181 reagent and bovine serum albumin (J.T. Baker, Deventer, the Netherlands) as a standard, whereas
182 phenolic compounds were determined by the spectrophotometric Folin reagent assay using gallic
183 acid (Sigma, St. Louis, MO, USA) as a standard. All results were expressed as mg/g of cell wall.
184
185 *2.3. Characterization of macerating enzymes*
186 The enzyme treatments applied during the maceration process, as single enzymes or combined in
187 multi-enzyme blends achieving a total dosage of 0.03 g/L, are listed in Table 1 and further
188 described in Table S1. The activity (U/mg $_{BSAeq}$) of each enzyme at the concentration varying from
189 0.006 g/L to 0.03 g/L was determined at the maceration conditions (0.03 M tartaric buffer, pH 3.2,
190 at 25 °C) as described in the section 2.3.2. A blank correction was always carried out using a
191 sample without enzyme. All enzymatic assays were conducted in triplicate. The total amount of
192 proteins in the commercial enzymes was determined following the method proposed in the section
193 2.2.3.
194 *2.3.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*
195 The electrophoretic profile of each commercial enzyme preparation was analyzed by means of SDS-
196 PAGE (Laemmli, 1970) on precast commercial gels of 4–15% (Bio-Rad, Richmond, California,

197 USA) using a vertical electrophoresis apparatus (Mini-Protean Tetra cell, Bio-Rad). Standard
198 molecular weight (Precision Plus Protein Standards, Kaleidoscope, Bio-Rad) ranged from 10 to 250
199 kDa. The gel run at a constant voltage of 200 V. Protein bands in the gels were stained with
200 Coomassie Blue G-250, and the destaining was done in deionised water.

201 *2.3.2. Enzyme activities determination*

202 *Pectinesterase assay.* The enzymatic assay of pectin methylesterase (PME) was performed by
203 stirring 10 mL of 1% w/v pectin in 0.03 M tartaric buffer (pH 3.2) and 20 mM sodium hydroxide
204 titrating solution. Assays were started by adding the PME enzyme solution. One Unit of PME
205 activity (U) was taken as the amount of sodium hydroxide (mEq) consumed per min to keep
206 constant pH value (pH 7.5) at 25 °C (Polydera, Galanou, Stoforos, & Taoukis, 2004).

207 *Pectin lyase assay.* The determination of pectin lyase (PL) activity was carried out
208 spectrophotometrically, monitoring the increase of absorbance at 235 nm (A_{235}) due to the
209 formation of a conjugated double bond of the Δ4:5 unsaturated uronide formed during the reaction
210 (Busto, García-Tramontín, Ortega, & Perez-Mateos, 2006). Enzyme was mixed with 5 mL of 1%
211 w/v pectin in 0.03 M tartaric buffer (pH 3.2) and filled up to 10 mL with the aforementioned buffer.
212 Solutions were preincubated at 25 °C for 10 min. One Unit of PL activity (U) was defined as the
213 amount of enzyme that produced an increase of one unit of A_{235} per minute at 25 °C.

214 *Polygalacturonase assay.* Polygalacturonase (PG) activity was measured by the determination of
215 the galacturonic acid released from polygalacturonic acid (Miller, 1959). Enzyme was mixed with
216 10 mL of 1% w/v polygalacturonic acid in 0.03 M tartaric buffer (pH 3.2). The reaction mixture was
217 incubated at 25 °C. The resulting galacturonic acid was determined by the 3',5'-dinitrosalicylic
218 (DNS) acid method as reported by Kashyap, Chandra, Kaul and Tewari (2000). Calibration
219 standards of galacturonic acid (Sigma) were prepared in 0.03 M tartaric buffer (pH 3.2). One Unit
220 of PG activity (U) was defined as the amount of enzyme required to release 1 μmol of galacturonic
221 acid from polygalacturonic acid per minute under the assay conditions.

Cellulase assay. Cellulase (C) activity was measured following the method reported by Ghose (1987) and expressed as filter paper units (FPU). This method was modified determining the reducing sugars released in 60 min, at 25 °C, from a mixture (10 mL) of cellulase enzyme solution and 0.03 M tartaric buffer (pH 3.2) in the presence of 0.5 g Whatman No. 1 filter paper. The released sugars were determined by the DNS method as glucose equivalent (Miller, 1959). One Unit of C activity (U) was defined as the amount of enzyme releasing 1 μmol of reducing sugars in 1 min.

Protease assay. Protease (PA) activity was tested using a tripeptide chromogenic substrate (Bz-Phe-Val-ArgpNA), purchased from Bachem (Switzerland), at a concentration of 0.22 mM solubilized in 0.03 M tartaric buffer (pH 3.2). Papain cleaves the synthetic substrate via the hydrolysis of the ester bond between amino groups in the N-terminal position and pNA, whose release was detected spectrophotometrically at 410 nm. The enzymatic activity was determined by measuring the change of absorbance vs time. One Unit of PA activity (U) was defined as the amount of enzyme releasing 1 μmol of pNA in 1 min at 25°C.

2.4. Simulated maceration of withered grape skins

The effect of different enzyme preparations consisting of single enzyme activity or combined enzyme activities was evaluated on the phenolic compound extraction and mechanical properties of the berry skins during the maceration process. For each of the ten maceration tests conducted (control and nine enzyme preparations), three replicates of 20 berries were randomly selected for each winegrape variety. The skins were manually removed from the pulp using a laboratory spatula, accurately weighed and punctured. Afterwards, they were quickly immersed into 100 mL of a buffer solution at pH 3.2 containing 5 g/L of tartaric acid (control), which was also added with the nine preparations composed of the following enzyme activities: PME, PL, PG, C, PA, PME+PL+PG, PME+PL+PG+C, PME+PL+PG+PA and PME+PL+PG+C+PA. The total dosage for each enzyme preparation was 0.03 g/L. To simulate the fermentation/maceration process, the skins

were macerated at 25 ºC during 9 days in the buffer solutions with and without enzyme addition, and ethanol was progressively added daily until reaching a final content of 14% v/v ethanol at the sixth day (Río Segade et al., 2016).

2.4.1. Extraction kinetics of phenolic compounds

Solution aliquots were taken at different maceration times (3, 6, 9, 24, 48, 72, 144 and 216 h) and used for monitoring the extraction kinetics of phenolic compounds. The extraction yield (%) of TA, FRV and PRO was calculated as the extracted content at each maceration time divided by the content in berry skins. After 216 h, the residual berry skins were quickly immersed into 100 mL of a hydroalcoholic buffer at pH 3.2 containing 14% v/v ethanol, 5 g/L of tartaric acid and 2 g/L of sodium metabisulphite, homogenized for 1 min at 8000 rpm and centrifuged for 15 min at 3000 × g at 20 °C. Non-extracted skin phenolic compounds were determined in the supernatant. The contents of extracted and non-extracted TA, FRV and PRO, as well as the anthocyanin profile, were determined following the methodology previously described (section 2.2.1).

Kinetic parameters (maximum extraction yield, as E_{max}; extraction rates, as k; half-time extraction, as $t_{1/2}$) for the extraction of TA, FRV and PRO were calculated using the pseudo-first order equation proposed by Sant'Anna, Marczak and Tessaro (2013) to model experimental data.

2.4.2. Skin mechanical properties before and after maceration

To evaluate the effect of macerating enzymes on skin hardness, three replicates of 20 berry skins were individually punctured before maceration, whereas three replicates of 10 berry skins were individually punctured after maceration for each test (Río Segade et al., 2015). The texture analyzer, experimental conditions and measured mechanical parameters were previously described (section 2.2.2).

2.5. Statistical analysis

Statistical analyses were performed using the SPSS statistics software package (IBM Corporation, Armonk, NY, USA). The Tukey-b test for $p < 0.05$ was used to establish significant differences by

one-way analysis of variance (ANOVA). Pearson's correlation coefficients were calculated to determine significant relationships.

3. Results and discussion

Regarding the use of commercial enzyme preparations during the maceration process to promote the release of phenolic compounds from the skins of fresh winegrapes, some contradictory results have been published in the scientific literature. These discrepancies have been mainly attributed to different enzymatic activities and variety effect (Ortega-Regules, Ros-García, Bautista-Ortín, López-Roca, & Gómez-Plaza, 2008; Romero-Cascales et al., 2008). Taking into account that this is the first study on the use of macerating enzymes for withered grape skins, it is very important to characterize the berry skins according to the chemical composition and mechanical properties, as well as to know the activities and purity of the single enzyme preparations used.

3.1. Chemical and mechanical analysis of withered grape skins

The differences in skin composition and mechanical properties between withered Barbera and Nebbiolo winegrapes are shown in Table 2. Regarding the main phenolic compounds, the content of TA was noticeably higher in Barbera, whereas Nebbiolo skins were characterized by a significantly greater content of both FRV and PRO. In relation to the anthocyanin profile, unacylated forms predominated in the two varieties used, although Nebbiolo was less rich in acylated glucosides. Barbera is prevalent in trisubstituted derivatives with a profile characterized by the high presence of malvidin-3-glucoside, whereas Nebbiolo is rich in disubstituted forms with a prevalence of peonidin-3-glucoside. In general, these results were in accordance with those previously reported for fresh and partially dehydrated grapes (Ferrandino, Carra, Rolle, Schneider, & Schubert, 2012; Río Segade et al., 2015; Torchio et al., 2016).

Moreover, skin cell wall composition emphasized the differences between withered Barbera and Nebbiolo winegrapes (Table 2). The former variety presented a significantly higher content of total

300 glucose and cellulosic glucose than Nebbiolo, as well as a lower content of uronic acids. This could
301 indicate that the berry skins of withered Barbera grapes contained smaller amounts of pectic
302 polysaccharides, as suggested for Syrah grape skins by Apolinar-Valiente et al. (2016). No
303 significant differences were observed between the two varieties studied in terms of proteins, total
304 phenols and lignin amounts. It has been stated that the composition of skin cell walls depends
305 greatly on the grape variety (Apolinar-Valiente et al., 2016; Ortega-Regules et al., 2008).
306 Despite the differences found in the cell wall composition, the skin instrumental texture parameters
307 of partially dehydrated Barbera and Nebbiolo winegrapes agreed in terms of F_{sk} and W_{sk} (Table 2).
308 This could be due to the high variability associated with the measurements performed directly on
309 withered whole grapes (Rolle et al., 2013).
310 The selection of two grape varieties with distinctive skin chemical composition will permit a better
311 assessment of the effect of macerating enzymes on the extractability of phenolic compounds. In
312 fact, skin cell wall composition is an important factor affecting the extractability of anthocyanins
313 and flavanols (Quijada-Morín, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón, 2015;
314 Ortega-Regules, Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2006) because the
315 cell walls form a limiting barrier for the diffusion of phenolic compounds.
316
317 *3.2. Characterization of macerating enzymes*
318 The five commercial enzymes used in this study were characterized by a different amount of total
319 proteins (Table S1), which ranged from 0.089 mg $_{BSAeq}$/mL (Pectinesterase) to 44 mg $_{BSAeq}$/mL
320 (Cellulase ACx 3000L). They are monocomponent preparations, as proved by the SDS-PAGE
321 profile (Figure S1) that provides their protein fingerprint. Moreover, a dominant band was observed
322 for each single enzyme preparation, thus indicating the high purity of the commercial enzymes
323 used. Therefore, these biocatalysts were suitable for the present study.
324 Although the use of macerating enzyme preparations composed of combined enzymatic activities
325 (pectinases, cellulase and proteases) has become a very common oenological practice (Bautista-

Ortín et al., 2005), no clear conclusions were reached on the mechanisms and ability of each enzymatic activity to break down the cell wall. For this reason, the commercial biocatalysts selected were used, as monocomponent (single enzyme activity) or known multi-enzyme blends (combined enzyme activities), to investigate their single and multiple effects during the maceration process of withered grape skins. The specific activities measured in the different commercial enzymes are reported in Table 1.

The first three enzymes (PME, PL and PG) act degrading the pectin fraction, which represents one of the main components of the cell wall. Methylesterases, being obtained from orange peel, remove methoxyl groups from pectin. Instead depolymerases by *Aspergillus* strains (lyases and hydrolases) cleave the bonds between galacturonate units (Romero-Cascales et al., 2008). PL depolymerizes highly esterified pectin, while PG hydrolyzes bonds adjacent to free carboxyl groups in low methylated pectin and pectate. Cellulase degrades the skin cell wall polysaccharides that form cellulose, thus increasing colour and promoting the release of tannins bound to cell walls (Amrani Joutei, Ouazzani Chahdi, Bouya, Saucier, & Glories, 2003). In addition, a native plant cysteine protease, papain from *Carica papaya* L. latex, was also tested. Proteases catalyse the degradation of proteins from the cellular membrane and may favour the extraction of phenolic compounds located inside the vacuoles (Barka, Kalantari, Makhlouf, & Arul, 2000). The mixed enzyme activities investigated were only pectinolytic activities or combined with cellulase and protease activities.

3.3. Extraction kinetics of phenolic compounds

The extraction kinetics of phenolic compounds (TA, FRV and PRO) from withered Barbera and Nebbiolo grape skins were evaluated throughout simulated macerations, without the addition of enzymes (control) and with the addition of various single and combined enzyme preparations (Table 3). The maceration process itself exerted a remarkable effect towards partially dehydrated Barbera and Nebbiolo grape skins, inducing a significant extraction yield of phenolic compounds. Independently on the presence of macerating enzymes, TA, FRV and PRO extraction followed

quite similar trends in the two winegrape varieties during the maceration period, which lasted 9 days. The extraction yield of TA and PRO increased from the beginning of maceration until achieving the maximum value at 72 h, and then slowly decreased (Fig. S2 and S3). Several studies proved that the maximum anthocyanin extraction is usually achieved within the first days of maceration (Bautista-Ortín et al., 2005; Rolle, Torchio, Zeppa, & Gerbi, 2008). The subsequent decrease could be due to two different phenomena: chemical reactions involving TA and PRO (polymerization and oxidation), and adsorption onto grape skins (Bautista-Ortín et al., 2016). Contrariwise, the extraction kinetics of FRV followed a hyperbolic behaviour until the end of maceration.

For each variety and treatment, experimental data were modeled (Sant'Anna et al., 2013), and the kinetic parameters estimated ($E_{max,}$ k, $t_{1/2}$) are reported in Table 3. Taking into account the different extraction behaviour above described, experimental data for TA and PRO were fitted from the beginning of maceration until the following 72 h, whereas for FRV all data were modeled. The corresponding values of regression coefficient (R^2), varying between 0.949 and 1.000, revealed that the pseudo-first order equation satisfactorily fitted the experimental data. Therefore, the kinetic parameters estimated can be used to describe the extraction kinetics of phenolic compounds during simulated maceration of withered grape skins.

With the exception of cellulase treated samples, faster extraction kinetics of TA, FRV and PRO was observed in withered Nebbiolo grape skins, according to the higher k values and the corresponding lower $t_{1/2}$ with respect to Barbera. Among the different enzymatic treatments applied in withered Barbera grape skins, neither the single enzymes nor the multi-enzyme blends allowed a significant variation in the E_{final} values of TA, FRV and PRO. Ortega-Regules et al. (2006), Ortega-Regules et al. (2008) and Hernández-Hierro et al. (2014) proved that the difficulty for the anthocyanin extraction from Monastrell and Tempranillo could be ascribable to some grape skin characteristics (high amount of cell wall material, high content of cellulosic glucose and low content of uronic acids), which were also found in withered Barbera grape skins (Table 2).

Moreover, the treatments carried out using C enzyme, as single enzyme or in the multi-enzyme blend composed of PME+PL+PG+C, allowed to obtain a significantly faster extraction of FRV with respect to both the control sample and the other enzymatic treatments, as it appears from the highest k values (0.043 and 0.030 h^{-1}, respectively) and the corresponding lowest $t_{1/2}$ (16.0 and 23.5 h, respectively). In agreement with our findings, Guerrand, Aloisio, Palacios, Santiago, Macías, & Navascues (2003) proved that, when the enzyme preparation has higher cellulase and hemicellulase activities, the rate of extraction is faster. The positive effect exerted by C enzyme on the extraction kinetics of FRV from withered Barbera grape skins could be ascribable to the release of flavanols bound to cell walls (Amrani Joutei et al., 2003). Cellulose is degraded by cellulase, opening up the cell wall structure and, therefore, facilitating the release of pectic polymers (Panouillé, Thibault, & Bonnin, 2006). Taking into account that the galacturonan rich-fraction of skin cell wall material has a high affinity for low molecular mass flavanols (Quijada-Morín et al., 2015), the release of this fraction promoted by the use of cellulase could have accelerated the extraction of FRV.

In Barbera samples, the only remarkable difference in the extraction yield of PRO was observed with the multi-enzyme blend PME+PL+PG+C+PA, whose application during maceration significantly increased the E_{max} value by means of a slower extraction mechanism, as it results from the lower k value (0.005 h^{-1}) and the corresponding higher $t_{1/2}$ (141.2 h). As already reported for FRV, the use of single C enzyme also allowed a faster extraction of PRO according to the highest k value (0.041 h^{-1}) and the corresponding lowest $t_{1/2}$ (17.1 h).

In withered Nebbiolo grape skins, all the enzymatic treatments improved the extraction of phenolic compounds (TA, FRV and PRO), with a remarkable increase of the values of E_{final} and E_{max} with respect to the control sample (Table 3). A significant effect was exerted by PG as single enzymatic activity, as well as by the enzyme preparation composed of only pectinolytic activity (PME+PL+PG) or combined with cellulase (PME+PL+PG+C) or with protease (PME+PL+PG+PA). For TA and FRV, PG and the above mentioned enzymatic blends raised both E_{final} and E_{max} values with respect to the control sample, without affecting the extraction velocity, as

reported by the k values and $t_{1/2}$. However, a slower extraction of PRO was observed achieving the lowest k values (ranging from 0.024 to 0.045 h^{-1}) and the corresponding greatest $t_{1/2}$ (ranging from 15.5 to 26.1 h). The extraction mechanism of TA was accelerated only by the use of PL as single enzyme, with the highest k value (0.117 h^{-1}) and the corresponding lowest $t_{1/2}$ (5.9 h). As reported by Río Segade et al. (2015), a macerating enzyme preparation containing PL could be useful for shortening the time needed to release TA from grape skins.

The combined effect observed on the extraction of TA, FRV and PRO from withered Nebbiolo grape skins, when the multi-enzyme blends containing pectinolytic activities were used, could be related to the high amount of the pectin fraction into the cell wall, which is the specific substrate of these biocatalysts. The fact that most of polymeric flavanols are bounded to the pectic fraction of cell walls (Ruiz-Garcia, Smith, & Bindon, 2014), that withered Nebbiolo grape skins are rich in PRO and that probably the degradation by PG occurs late could be the causes of slowing down even if increasing their release.

Independently on the enzyme preparation used, the extracted content of TA, FRV and PRO into the wine-like solution at the end of simulated maceration (Table 4) was not proportional to their initial contents in withered grape skins. This observation agreed with the findings described by other authors (Romero-Cascales et al., 2005), who demonstrated that the content of TA in the wine is not correlated with that found in the grapes because degradation, polymerization and adsorption processes can occur simultaneously to the extraction.

Data reported in Table 4 confirmed that the effect exerted by the tested macerating enzymes on the extraction of skin phenolic compounds is variety-dependent. In withered Barbera grape skins, although significant differences were not observed in the extracted content of TA, FRV and PRO among enzyme treatments, the lowest amount of TA remaining in the skins at the end of maceration was found for PG and PA single enzyme activities, followed by the combination of enzymes with pectinolytic activities (PME+PL+PG). This could involve higher losses of the anthocyanins released throughout maceration with respect to the control sample. In withered Nebbiolo grape

skins, cell wall material might re-adsorb a significant amount of phenolic compounds during simulated maceration as shown by the significantly higher contents of phenolic compounds found in the skins at the end of maceration (Río Segade et al., 2015).

At the end of maceration, Barbera skin extracts showed some differences in the anthocyanin profile among enzyme treatments (Table 5). The application of PG as single enzyme significantly reduced the percentage of trisubstituted anthocyanins and, concurrently, it increased the relative abundance of acylated compounds with respect to the control sample. Likewise, the pectinolytic-enzyme preparation composed of PME+PL+PG activities also promoted the extraction of acylated anthocyanins. Trisubstituted derivatives are the most stable forms of anthocyanins (Cheynier, Souquet, Kontek, & Moutounet, 1994), and their less presence in the extracts may contribute unfavorably to the color stability. This small negative aspect could be compensated with the higher presence of acylated anthocyanins, which protect the flavylium cation due to their participation in intramolecular copigmentation processes (Gil-Muñoz, Moreno-Pérez, Vila-López, Fernández-Fernández, Martínez-Cutillas, & Gómez-Plaza, 2009). Contrariwise, the anthocyanin profile of withered Nebbiolo grape skins at the end of maceration was not affected by the enzyme treatment (Table 5). Río Segade et al. (2015) also reported that the anthocyanin profile of fresh Nebbiolo skins was independent on the addition of enzymes throughout the maceration process.

3.4. Effect of macerating enzymes on skin mechanical properties

Berry skin hardness is strongly linked to the extractability of phenolic compounds (Rolle et al., 2008). The relationship is variety dependent because the structure and chemical composition of the cell wall influence the mechanical properties of grape skins (Ortega-Regules et al., 2006; Río Segade et al., 2014). Nevertheless, the decrease of skin hardness throughout maceration could be used as a measurement of the skin cell wall disassembly, for which enzymes can play a key role (Río Segade et al., 2015). Therefore, instrumental texture parameters defining skin hardness were determined, for the first time in the present study, before and after enzyme-assisted simulated

maceration of withered Barbera and Nebbiolo grape skins to assess the enzyme effect from the mechanical point of view (Table 6).

In withered grapes, data reported in Table 6 showed that the maceration carried out without the addition of enzymes caused the decrease of the skin mechanical properties for the two varieties studied. This degradative effect was more evident in Barbera, inducing a 2-fold higher diminution of F_{sk} and about a 48-fold higher decrease of W_{sk} with respect to that observed in Nebbiolo. This phenomenon probably masks the effectiveness of exogenous enzymatic activities in withered Barbera grape skins. Similar findings have been found by Río Segade et al. (2015), comparing fresh Nebbiolo and Cabernet Sauvignon skins, who also reported lower skin softening in the former variety during maceration when no enzymatic treatment was applied.

The mechanical properties of withered Barbera grape skins significantly decreased with the addition of PG as single macerating enzyme if compared with the control sample. Also for withered Nebbiolo grape skins, the application of PG, as single enzyme or in the multi-enzyme blends, during maceration caused a significant decrease of F_{sk} (from 73.8% to 79.8%) and of W_{sk} (from 81.0% to 89.4%). A slight but significant skin softening (about 30%) was also observed applying PL only in Nebbiolo. The efficacy of pectinolytic-enzyme preparations in disassembling Nebbiolo skin cell wall is probably related to the abundant amount of the pectin fraction. As ascertained by other authors, macerating enzymes mainly act degrading pectic polysaccharides of the skin cell wall (Romero-Cascales, Ros-García, López-Roca, & Gómez-Plaza, 2012), mimicking the natural phenomena occurring during fruit ripening (Rosli, Civello, & Martínez, 2004).

Finally, the correlation between the variation percentage (%Δ) of skin mechanical properties due to maceration and the extraction yield of phenolic compounds was investigated for each variety considering all untreated and enzyme-treated samples (n=30, 10 treatments × 3 replicates of 20 berry skins each). In withered Barbera grape skins, a mild significant correlation was observed only between $\%\Delta W_{sk}$ and the corresponding extraction yield of FRV ($R=0.377$, $p < 0.05$), whereas significant positive relationships were found between the skin softening and the extraction yield of

TA (R=0.662-0.686, $p < 0.001$), FRV (R=0.791-0.793, $p < 0.001$) and PRO (R=0.856, $p < 0.001$) in Nebbiolo. Therefore, the higher the skin softening associated with the degradation of pectic polysaccharides of the cell walls, the higher the extractability of phenolic compounds. These data were in accordance with the findings reported in other studies performed on the use of macerating enzymes in fresh grape skins (Río Segade et al., 2015), but in the case of withered grape skins the relationships were variety dependent probably due to the different chemical composition of grape skin cell walls (Hernández-Hierro et al., 2014; Ortega-Regules et al., 2006).

4. Conclusions

The advantages of using macerating enzymes for the extraction of phenolic compounds from partially dehydrated grape berries were evaluated for the first time in this study. The effectiveness of individual and combined macerating enzymes in improving the extractability of phenolic compounds from postharvest withered grape skins, as well as the relationships between skin mechanical properties and the extraction yield were variety dependent. In Nebbiolo, polygalacturonase activity, either as single enzyme or in multi-enzyme blends, affected the mechanical properties of berry skin during simulated maceration, increasing the skin softening as a result of the degradation of cell walls rich in pectin substances. This skin softening was strongly linked to a higher extraction of anthocyanins, oligomeric flavanols and polymeric flavanols, without affecting the anthocyanin profile. The ability of polygalacturonase to release polymeric flavanols bounded to the pectic fraction of cell walls caused a lengthening of extraction time, whereas the extraction speed was not influenced for the compounds mostly present in the vacuoles. Although skin softening was also observed with the use of polygalacturonase single enzyme in Barbera, no remarkable advantages were revealed applying macerating enzymes, probably due to the significant degradative and extractant effects associated with the own maceration process in this variety having skin cell walls rich in cellulose and with low content of uronic acids. Therefore, the chemical

507 composition and morphology of skin cell walls may play a significant role in the effectiveness of
508 macerating enzymes to facilitate the extraction of phenolic compounds.
509 From an oenological point of view, our findings show that the addition of macerating enzymes is a
510 useful tool to increase the extractability of phenolic compounds from withered Nebbiolo grape
511 skins. Polygalacturonase as single enzymatic activity and combined in multi-pectinolytic enzyme
512 blends enhanced the degradation of cell walls facilitating the release of phenolic compounds from
513 the skins during maceration. This may further promote the formation of anthocyanin-flavanol
514 complexes and therefore improving wine colour stability.
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Table 1
Enzymatic activities (U/mg $_{BSAeq}$) determined at pH 3.2 and 25 °C in the enzyme preparations used.

Treatment	PME[a] Pectin methylesterase	PL Pectin lyase	PG Polygalacturonase	C Cellulase	PA Protease
PME	30±2	-	-	-	-
PL	-	214±10	-	-	-
PG	-	-	3.75±0.25	-	-
C	-	-	-	0.07±0.02	-
PA	-	-	-	-	0.20±0.04
PME+PL+PG	25±2	211±7	0.54±0.08	-	-
PME+PL+PG+C	22±2	142±5	0.37±0.05	0.05±0.01	-
PME+PL+PG+PA	20±1	179±6	0.33±0.05	-	0.14±0.03
PME+PL+PG+C+PA	15±1	109±5	0.23±0.03	0.03±0.01	0.16±0.04

[a]PME substrate: pectin solution (1% w/v). PL substrate: pectin solution (0.5% w/v). PG substrate: polygalacturonic acid (1% w/v). C substrate: Whatman No. 1 filter paper (0.5g). PA substrate: Bz-Phe-Val-ArgpNA (0.22 mM).

Table 2
Berry skin composition and mechanical properties of withered Barbera and Nebbiolo winegrapes.

Parameter	Barbera	Nebbiolo	Sign
Phenolic compounds[a]			
TA (mg/g skin)	10.69±1.17	3.83±0.17	***
FRV (mg/g skin)	1.37±0.65	7.64±0.44	***
PRO (mg/g skin)	9.90±0.79	21.02±1.70	***
Anthocyanin profile[a]			
Σ Disubstituted G (%)	8.1±0.9	48.3±1.0	***
Σ Trisubstituted G (%)	68.6±0.9	35.2±1.8	***
Σ Acylated G (%)	23.3±0.7	16.4±0.9	***
Cell wall composition[b]			
Skin CW (mg/g fresh skin)	62.4	50.6	-
Proteins (mg/g CW)	83.3±4.2	85.7±2.3	ns
Total phenols (mg/g CW)	63.9±2.6	65.1±4.4	ns
Total glucose (mg/g CW)	249.9±5.8	180.7±4.6	***
Cellulosic glucose (mg/g CW)	199.2±3.9	164.2±4.3	***
Uronic acids (mg/g CW)	127.2±10.7	160.0±14.8	*
Lignin (mg/g CW)	322.7±28.0	359.3±4.9	ns
Mechanical properties[c]			
F_{sk} (N)	0.957±0.500	0.890±0.195	ns
W_{sk} (mJ)	1.400±0.867	1.050±0.406	ns

All data are expressed as average value ± standard deviation. [a](n=5). [b](n=4). [c]Directly measured on whole berries (n=30). Sign: *, *** and ns indicate significance at $p < 0.05$, 0.001 and not significant, respectively. TA = total anthocyanins, FRV = flavanols reactive to vanillin, PRO = proanthocyanidins, G = glucoside, CW = cell wall, F_{sk} = berry skin break force, W_{sk} = berry skin break energy.

Table 3. Final extraction yield and kinetic parameters estimated by pseudo-first order equation of untreated and enzyme-treated withered Barbera and Nebbiolo grape skins during simulated maceration.

Compound	Treatment	Barbera					Nebbiolo				
		E_{final} (%)	E_{max} (%)	k (h^{-1})	$t_{½}$ (h)	R^2	E_{final} (%)	E_{max} (%)	k (h^{-1})	$t_{½}$ (h)	R^2
TA	Control	41.3±1.5	46.7±1.9bc	0.048±0.009	14.5±1.2	0.993	36.2±0.4a	53.3±0.5a	0.086±0.005ab	8.1±0.6bc	0.999
	PME	34.3±1.7	35.0±2.1a	0.050±0.015	13.9±1.0	0.981	44.1±4.8ab	60.7±0.9cd	0.079±0.007ab	8.8±0.8bc	0.998
	PL	44.2±2.2	50.3±3.0c	0.055±0.018	12.5±0.9	0.976	42.0±1.7ab	57.4±1.3b	0.117±0.014c	5.9±0.5a	0.994
	PG	44.6±3.7	43.8±1.4b	0.065±0.011	10.6±1.0	0.993	46.6±1.8ab	60.5±0.3cd	0.096±0.003b	7.2±0.4bc	1.000
	C	42.7±2.3	45.2±1.8bc	0.066±0.015	10.5±1.1	0.986	41.4±0.7ab	58.8±0.1bc	0.091±0.001ab	7.6±0.6bc	1.000
	PA	39.1±11.5	40.8±2.1ab	0.054±0.015	12.8±0.8	0.982	42.2±4.7ab	57.5±0.5b	0.089±0.005ab	7.8±0.7bc	0.999
	PME+PL+PG	42.4±4.1	40.8±2.3ab	0.050±0.013	13.8±1.1	0.985	50.9±4.3b	66.0±0.8f	0.077±0.006a	9.0±0.8c	0.999
	PME+PL+PG+C	36.9±4.4	37.5±1.7a	0.060±0.015	11.5±0.7	0.984	48.7±3.0b	62.8±0.7e	0.080±0.005ab	8.7±0.6bc	0.999
	PME+PL+PG+PA	40.4±4.2	44.3±2.7bc	0.043±0.011	16.2±2.0	0.987	47.7±6.0b	63.2±0.6e	0.074±0.004a	9.4±0.5c	0.999
	PME+PL+PG+C+PA	42.3±5.8	44.6±2.0bc	0.051±0.011	13.6±1.1	0.989	46.9±4.9ab	62.3±0.4de	0.082±0.003ab	8.5±1.0bc	1.000
	Sign	ns	***	ns	ns		***	***	***	***	
FRV	Control	80.9±8.7	88.4±7.6abc	0.010±0.003a	70.7±1.5e	0.971	56.5±4.8a	56.4±1.1a	0.031±0.004	22.6±1.0	0.990
	PME	64.3±6.1	70.4±5.5a	0.011±0.004a	61.1±1.3d	0.964	67.0±3.9ab	64.0±2.8b	0.022±0.005	21.0±0.7	0.972
	PL	87.0±15.6	103.9±6.0c	0.005±0.003a	126.8±2.5f	0.968	67.8±4.9ab	70.7±3.2bc	0.030±0.007	22.2±0.8	0.966
	PG	84.3±11.8	84.0±3.9ab	0.018±0.004ab	37.5±1.8c	0.977	86.8±8.2cd	87.7±2.2de	0.031±0.005	22.0±0.6	0.987
	C	64.0±8.3	65.7±1.6a	0.043±0.010c	16.0±0.6a	0.974	68.2±1.8ab	68.5±2.3bc	0.032±0.007	22.0±1.0	0.971
	PA	75.5±14.1	84.2±3.8ab	0.009±0.001a	74.0±2.0e	0.994	60.8±4.6a	64.5±2.6b	0.041±0.010	19.0±0.8	0.967
	PME+PL+PG	88.8±7.7	98.5±7.3bc	0.012±0.003a	59.7±1.6d	0.978	92.9±3.8d	93.2±3.2e	0.030±0.005	22.8±0.9	0.983
	PME+PL+PG+C	85.6±13.8	78.4±4.0ab	0.030±0.009bc	23.5±1.0b	0.954	82.6±11.7bcd	85.4±2.9d	0.035±0.007	19.6±0.4	0.980
	PME+PL+PG+PA	80.8±27.7	84.5±2.5ab	0.017±0.002ab	41.7±2.0c	0.990	82.6±4.0bcd	83.9±2.6d	0.031±0.005	22.3±1.1	0.985
	PME+PL+PG+C+PA	75.7±12.3	79.0±3.3ab	0.016±0.003ab	44.0±1.5c	0.982	72.7±2.1abc	74.4±1.7c	0.033±0.004	20.8±0.5	0.991
	Sign	ns	***	***	***		***	***	ns	ns	
PRO	Control	45.2±7.3	44.6±5.2a	0.036±0.021ab	24.2±1.0b	0.949	56.4±4.4a	57.9±2.4a	0.075±0.020b	9.2±0.8a	0.979
	PME	37.8±5.6	55.7±10.2a	0.020±0.009ab	35.4±2.1c	0.984	69.5±3.9abc	65.8±2.4ab	0.046±0.009ab	15.2±1.2bc	0.993
	PL	43.6±3.7	60.6±7.5a	0.026±0.011ab	26.8±2.1b	0.980	72.2±5.8bc	72.8±5.1abc	0.061±0.022ab	11.3±0.7b	0.967
	PG	53.9±7.8	61.5±13.3a	0.024±0.015ab	29.3±1.5b	0.962	80.5±5.8cd	87.7±3.3cde	0.045±0.008ab	15.5±0.0bc	0.994
	C	49.7±4.4	51.9±3.6a	0.041±0.013b	17.1±1.0a	0.981	61.9±2.2ab	74.0±5.3bc	0.039±0.012ab	17.7±1.3bc	0.983
	PA	45.8±7.8	53.7±7.2a	0.022±0.008ab	31.6±1.2bc	0.988	61.1±4.6ab	73.1±6.7bc	0.040±0.018ab	17.4±0.8bc	0.965
	PME+PL+PG	50.8±5.6	78.6±10.6a	0.015±0.012ab	47.7±1.3d	0.974	92.0±8.0d	89.4±2.9def	0.029±0.006a	24.9±0.8c	0.996
	PME+PL+PG+C	43.4±6.2	76.3±14.5a	0.014±0.005ab	49.9±1.2d	0.994	89.0±7.4d	103.5±8.2f	0.024±0.006a	26.1±1.2c	0.994
	PME+PL+PG+PA	50.8±12.2	69.5±13.8a	0.020±0.009ab	34.9±1.0c	0.985	92.1±0.9d	98.7±7.7ef	0.028±0.007a	24.7±0.6c	0.992
	PME+PL+PG+C+PA	50.2±10.8	95.9±14.1b	0.005±0.002a	141.2±3.2e	1.000	79.0±0.5cd	79.3±3.4bcd	0.049±0.010ab	14.2±0.6bc	0.990
	Sign	ns	***	*	***		***	***	***	***	

All data are expressed as average value ± standard deviation (n=3). Different Latin letters within the same column indicate significant differences among treatments according to Tukey-b test ($p < 0.05$). Sign: *, *** and ns indicate significance at $p < 0.05$, 0.001 and not significant, respectively. TA = total anthocyanins, FRV = flavanols reactive to vanillin, PRO = proanthocyanidins. E_{final} = final extraction yield; E_{max} = maximum extraction yield; k = extraction rates; $t_{1/2}$ = half-time extraction.

Table 4
Phenolic composition of untreated and enzyme-treated withered Barbera and Nebbiolo grape skins at the end of simulated maceration.

Compound (mg/g skin)	Treatment	Barbera		Nebbiolo	
		Extracted	Non-extracted	Extracted	Non-extracted
TA	Control	4.41±0.16	1.86±0.03bc	1.38±0.02a	0.35±0.01a
	PME	3.67±0.18	1.63±0.13abc	1.69±0.19ab	0.45±0.04c
	PL	4.72±0.24	1.76±0.08abc	1.61±0.07ab	0.36±0.03ab
	PG	4.76±0.40	1.40±0.13a	1.78±0.07b	0.39±0.02abc
	C	4.56±0.24	1.75±0.07abc	1.58±0.03ab	0.43±0.03bc
	PA	4.18±1.23	1.46±0.09a	1.61±0.18ab	0.43±0.01bc
	PME+PL+PG	4.53±0.44	1.53±0.11ab	1.95±0.17b	0.43±0.04bc
	PME+PL+PG+C	3.94±0.47	1.75±0.16abc	1.86±0.12b	0.40±0.01abc
	PME+PL+PG+PA	4.32±0.45	1.63±0.17abc	1.83±0.23b	0.41±0.03abc
	PME+PL+PG+C+PA	4.52±0.62	1.98±0.17c	1.79±0.19b	0.41±0.04abc
	Sign	ns	***	**	**
FRV	Control	1.11±0.12	0.13±0.03abc	4.75±0.79a	0.57±0.07ab
	PME	0.88±0.08	0.09±0.08abc	5.12±0.30ab	0.64±0.07bc
	PL	1.19±0.21	0.05±0.05ab	5.18±0.38ab	0.86±0.07d
	PG	1.15±0.16	0.05±0.05ab	6.64±0.63cd	0.63±0.05bc
	C	0.88±0.11	0.00±0.00a	5.21±0.14ab	0.54±0.07ab
	PA	1.03±0.19	0.17±0.11abc	4.65±0.35a	0.83±0.06d
	PME+PL+PG	1.22±0.11	0.25±0.06c	7.10±0.29d	0.40±0.09a
	PME+PL+PG+C	1.17±0.19	0.17±0.12abc	6.31±0.90bcd	0.79±0.05cd
	PME+PL+PG+PA	1.11±0.38	0.23±0.05bc	6.31±0.31bcd	0.62±0.07b
	PME+PL+PG+C+PA	1.03±0.17	0.05±0.05ab	5.55±0.16abc	0.46±0.02ab
	Sign	ns	**	***	***
PRO	Control	4.47±0.73	2.02±0.20a	11.85±0.92a	2.73±0.05
	PME	3.74±0.56	2.15±0.06a	14.60±0.83abc	3.20±0.15
	PL	4.31±0.36	2.43±0.27ab	15.18±1.23bc	2.63±0.14
	PG	5.34±0.78	2.08±0.20a	16.92±1.22cd	2.78±0.22
	C	4.92±0.44	2.13±0.12a	13.01±0.46ab	3.11±0.30
	PA	4.53±0.77	2.02±0.30a	12.84±0.96ab	2.92±0.18
	PME+PL+PG	5.03±0.56	2.41±0.04ab	19.33±1.69d	3.09±0.26
	PME+PL+PG+C	4.30±0.62	2.29±0.18ab	18.69±1.55d	2.96±0.23
	PME+PL+PG+PA	5.02±1.21	2.26±0.34ab	19.35±0.20d	3.01±0.11
	PME+PL+PG+C+PA	4.97±1.07	2.74±0.24b	16.60±0.11cd	2.74±0.20
	Sign	ns	*	***	*

All data are expressed as average value ± standard deviation (n=3). Different Latin letters within the same column indicate significant differences among treatments according to Tukey-b test ($p < 0.05$). Sign: *, **, *** and ns indicate significance at $p < 0.05$, 0.01, 0.001 and not significant, respectively. TA = total anthocyanins, FRV = flavanols reactive to vanillin, PRO = proanthocyanidins.

Table 5
Anthocyanin profile of untreated and enzyme-treated withered Barbera and Nebbiolo grape skins at the end of simulated maceration.

Compound (%)	Treatment	Barbera		Nebbiolo	
		Extracted	Non-extracted	Extracted	Non-extracted
Σ Disubstituted G	Control	5.7±1.3	4.8±1.3	36.2±3.1	35.4±3.2
	PME	6.0±1.1	6.8±1.7	34.8±5.0	33.4±3.7
	PL	6.0±0.4	6.0±0.7	35.2±4.7	34.2±4.7
	PG	5.1±0.3	5.9±0.6	38.6±4.2	37.5±3.0
	C	5.9±0.6	5.9±0.3	37.8±3.0	37.3±3.8
	PA	4.1±0.1	4.0±0.2	37.4±1.5	37.5±2.1
	PME+PL+PG	5.0±1.4	5.8±1.9	38.0±2.7	36.5±2.1
	PME+PL+PG+C	6.5±0.8	6.3±1.1	39.8±2.5	38.1±2.3
	PME+PL+PG+PA	5.7±0.5	5.9±0.4	40.0±0.9	39.5±1.4
	PME+PL+PG+C+PA	5.5±0.5	5.6±0.4	37.8±2.6	36.7±2.8
	Sign	ns	ns	ns	ns
Σ Trisubstituted G	Control	74.6±1.3b	60.8±1.3b	54.5±3.3	48.3±4.0
	PME	74.1±1.1ab	58.4±1.1ab	55.5±4.5	49.1±2.3
	PL	73.2±1.1ab	58.3±0.4ab	55.1±4.1	48.4±4.3
	PG	72.0±0.2a	57.1±1.7a	51.8±3.7	44.3±2.4
	C	73.5±0.2ab	60.5±0.3b	53.0±3.1	45.5±4.1
	PA	74.4±0.6b	60.4±0.9b	53.2±1.8	44.8±2.4
	PME+PL+PG	72.8±1.0ab	57.1±1.9a	52.1±2.6	45.1±2.1
	PME+PL+PG+C	73.2±0.4ab	58.7±1.0ab	50.4±2.4	43.5±2.4
	PME+PL+PG+PA	73.3±0.3ab	60.3±0.2b	50.2±0.8	42.1±2.1
	PME+PL+PG+C+PA	73.1±0.2ab	60.0±0.4ab	52.6±2.5	45.9±3.2
	Sign	*	**	ns	ns
Σ Acylated G	Control	19.8±0.1a	34.3±1.4	9.3±0.2	16.3±0.8
	PME	19.9±0.6a	34.8±0.7	9.7±0.7	17.6±1.5
	PL	20.9±0.7abc	35.7±1.0	9.7±0.8	17.4±0.9
	PG	22.9±0.1c	37.0±2.0	9.7±0.5	18.2±0.8
	C	20.6±0.7ab	33.7±0.2	9.2±0.3	17.2±0.8
	PA	21.5±0.6abc	35.6±0.9	9.4±0.8	17.7±1.2
	PME+PL+PG	22.2±1.7bc	37.1±2.2	9.9±0.4	18.4±0.2
	PME+PL+PG+C	20.3±0.6ab	35.0±1.1	9.8±0.7	18.5±0.5
	PME+PL+PG+PA	21.0±0.3abc	33.8±0.4	9.8±0.2	18.5±0.7
	PME+PL+PG+C+PA	21.4±0.4abc	34.5±0.7	9.6±0.3	17.4±0.8
	Sign	**	*	ns	ns

All data are expressed as average value ± standard deviation (n=3). Different Latin letters within the same column indicate significant differences among treatments according to Tukey-b test ($p < 0.05$). Sign: *, ** and ns indicate significance at $p < 0.05$, 0.01 and not significant, respectively. G = glucoside.

Table 6
Mechanical properties of untreated and enzyme-treated withered Barbera and Nebbiolo grape skins before and after simulated maceration.

Parameter	Treatment	Barbera			Nebbiolo		
		Before[a]	After[b]	%Δ[b]	Before[a]	After[b]	%Δ[b]
F_{sk} (N)	Control	0.888±0.111	0.726±0.054bc	18.2±6.1ab	0.770±0.018	0.695±0.082c	9.7±10.6a
	PME		0.834±0.177c	6.0±20.0a		0.759±0.076c	1.4±9.9a
	PL		0.490±0.042b	44.8±4.7b		0.529±0.039b	31.3±5.1b
	PG		0.215±0.066a	75.7±7.4c		0.156±0.018a	79.8±2.4c
	C		0.833±0.158c	6.2±17.8a		0.683±0.046c	11.2±6.0a
	PA		0.615±0.017bc	30.7±1.9ab		0.699±0.056c	9.3±7.2a
	PME+PL+PG		0.485±0.111b	45.3±12.5b		0.166±0.021a	78.4±2.8c
	PME+PL+PG+C		0.607±0.104bc	31.6±11.7ab		0.172±0.016a	77.6±2.1c
	PME+PL+PG+PA		0.638±0.093bc	28.1±10.4ab		0.198±0.031a	74.2±4.1c
	PME+PL+PG+C+PA		0.621±0.073bc	30.1±8.2ab		0.202±0.022a	73.8±2.9c
	Sign		***	***		***	***
W_{sk} (mJ)	Control	0.538±0.085	0.460±0.060bc	14.4±11.1ab	0.376±0.006	0.375±0.087c	0.3±23.1a
	PME		0.548±0.164bc	-1.9±30.4ab		0.363±0.045c	3.4±12.1a
	PL		0.292±0.014ab	45.7±2.6bc		0.239±0.038b	36.5±10.2b
	PG		0.113±0.060a	79.0±11.1c		0.040±0.009a	89.4±2.3c
	C		0.632±0.189c	-17.5±35.0a		0.317±0.036bc	15.8±9.7ab
	PA		0.359±0.013ab	33.2±2.4bc		0.318±0.041bc	15.4±11.0ab
	PME+PL+PG		0.278±0.091ab	48.4±16.9bc		0.049±0.014a	87.0±3.7c
	PME+PL+PG+C		0.356±0.080ab	33.7±15.0bc		0.050±0.006a	86.8±1.6c
	PME+PL+PG+PA		0.387±0.086bc	28.0±15.9ab		0.067±0.012a	82.1±3.3c
	PME+PL+PG+C+PA		0.366±0.056ab	31.9±10.3bc		0.071±0.016a	81.0±4.2c
	Sign		***	***		***	***

All data are expressed as average value ± standard deviation. [a]Skin mechanical properties before maceration were the same for all treatments. [a]Three replicates of 20 berry skins (n=3). [b]Three replicates of 10 berry skins (n=3). Different Latin letters within the same column indicate significant differences among treatments according to Tukey-b test ($p < 0.05$). Sign: *** indicates significance at $p < 0.001$. F_{sk} = berry skin break force, W_{sk} = berry skin break energy, Δ% = variation percentage during maceration.

Supplementary Material

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