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Role of cell wall deconstructing enzymes in the proanthocyanidin–cell wall adsorption–desorption phenomena

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ABSTRACT

The transference of proanthocyanidins from grapes to wine is quite low. This could be due, among other causes, to proanthocyanidins being bound to grape cell wall polysaccharides, which are present in high concentrations in the must. Therefore, the effective extraction of proanthocyanidins from grapes will depend on the ability to disrupt these associations, and, in this respect, enzymes that degrade these polysaccharides could play an important role. The main objective of this work was to test the behavior of proanthocyanidin–cell wall interactions when commercial maceration enzymes are present in the solution. The results showed that cell wall polysaccharides adsorbed a high amount of proanthocyanidins and only a limited quantity of proanthocyanidins could be desorbed from the cell walls after washing with a model solution. The presence of enzymes in the solution reduced the proanthocyanidin–cell wall interaction, probably through the elimination of pectins from the cell wall network.

1. Introduction

Proanthocyanidins (commonly called tannins by winemakers) are one of the main determinants of red wine quality. Some sensory attributes such as color, body and astringency are directly associated with the qualitative and quantitative composition of wine proanthocyanidins (PAs). In the grape, they are located in the skin and seeds and are transferred to the must/wine during the maceration step of winemaking. However, previous studies have shown that the quantities found in wines are frequently lower than expected (Adams & Scholz, 2007; Busse-Valverde, Bautista-Ortín, Gómez-Plaza, Fernández-Fernández, & Gil-Muñoz, 2012; Busse-Valverde et al., 2010), which may not only be related to the fact that PAs are not extensively extracted from the skin and seeds but also to the finding that a substantial proportion of them are adsorbed by the skin and pulp cell walls (CWs) in suspension in the must after crushing the grapes and that finally precipitates during settling (Bindon, Smith, Holt, & Kennedy, 2010).

The existence of interactions between proanthocyanidins and cell wall material, more precisely with the polysaccharides that are the main components of the cell walls, has been demonstrated and investigated by several research groups (Cai, Gaffney, Lilley, & Haslam, 1989; McManus et al., 1985; Riou, Vernhet, & Doco, 2002) as has the possible enological implications such interactions may have (Bautista-Ortin, Cano-Lechuga, Ruiz-García, & Gómez-Plaza, 2014; Bindon, Smith, Holt, et al., 2010; Le Bourvellec, Bouchet, & Renard, 2005). Therefore, the effective extraction of proanthocyanidins from grapes will depend on having enough knowledge of the nature of these interactions and on the ability to disrupt or manage these associations. As regards the nature of these associations it has been found that electrostatic or ionic interactions do not appear to play any part in the association between proanthocyanidins and cell-wall material, rather the adsorption mechanism seems to involve the establishment of weak interactions, more precisely hydrogen bonds and hydrophobic interactions (Le Bourvellec, Le Quere, & Renard, 2007). Studies have pointed to an increase in association with increasing ionic strength, suggesting the presence of hydrophobic interactions, and a notable decrease with increasing temperature, indicating hydrogen bonding (Le Bourvellec, Guyot, & Renard, 2004). Renard, Baron, Guyot, and Drilleau (2001) also demonstrated that washing the proanthocyanidins + CW complexes with 8 M urea, a chaotropic reagent, or an acetone:water 60:40 solution, resulted in total re-extraction of the procyanidins, while washing with buffer led only to partial re-extraction and adding NaCl actually decreased this

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re-extraction. All of this indicated that the bonds involved were most probably H-bonds or hydrophobic interactions, while ionic interactions were apparently absent.

During winemaking neither high temperatures nor urea can be used to limit the interactions. However, maceration enzymes, a very common enological product, may have a significant role on the extent of these interactions. Maceration enzymes may be used for improving must volume and clarification, for enhancing filterability and, especially in red winemaking processes, for increasing the degradation of the skin cell walls, the limiting barrier for the extraction of phenolic compounds. They exert their effect by deconstructing the polysaccharide network of the cell wall and allowing the extraction of the phenolic compounds located inside the vacuoles. However, the effect of these enzymes over the complexes that PAs and cell walls (CWs) form, whether they limit or favor the interactions and/or participate in the desorption processes of these compounds from cell walls have not been deeply studied.

2. Material and methods

2.1. Chemicals

Chromatographic solvents were of high-performance liquid chromatography (HPLC) grade, and chemicals were of analytical reagent grade. Acetonitrile, acetone, chloroform, methanol, ethanol, formic acid and trifluoroacetic acid were from Merck (Darmstadt, Germany). The phloroglucinol reagent and tris–HCl equilibrated phenol pH 6.7 were sourced from Sigma Aldrich (MO, USA). Sodium acetate was from J.T. Baker (Deventer, Netherlands). The standards (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate and (−)-epigallocatechin were obtained from Extrasynthese (Genay, France). Galacturonic acid was from Sigma (St. Louis, MO, USA) and Bovine serum albumin (BSA) fraction V from J.T. Baker (Deventer, Holland). For the glucose determination, a enzymatic analysis kit from R-biopharm (Darmstadt, Germany) was used.

2.2. Instrumentation

The HPLC apparatus was a Waters 2695 (Waters, Milford, MA) equipped with a Syrahstem autosampler, and a Waters 2996 photodiode array detector (Waters, Milford, MA).

2.3. Grapes

Vitis vinifera L. cv. Monastrell grapes were sampled from a commercial vineyard located in Jumilla, Murcia (Spain). Grapevines grafted in 2005 onto 110R rootstock trained to a bilateral cordon system and trellised to a three-wire vertical system. The vineyard was drip irrigated. Grapes were sampled at two stages of ripeness, veraison (14 °Brix) and harvest (25 °Brix), for the 2013 growing season. To obtain a representative vineyard sample, a 1000-berry sample was collected from three rows distributed within the vineyard block, and then pooled and transported to the laboratory, where they were stored at −20 °C until analysis.

2.4. Cell wall material

Purified cell walls (CW) were extracted from fresh skins of *V. vinifera* L. cv. Monastrell. Cell walls were isolated following the method of De Vries, Voragen, Rombouts, and Pilnik (1981) and adapted by Ruiz-García, Smith, and Bindon (2014). Briefly, skins were extracted in 70% v/v acetone to remove proanthocyanidins. The acetone-extracted residues were washed in additional 70% v/v acetone, followed by Milli-Q water. Acetone-extracted skin material was then homogenized under liquid nitrogen. Thereafter, acetone-insoluble residues from skins (1 g) were extracted in 50 mL of tris–HCl equilibrated phenol pH 6.7, and then washed in 80% v/v methanol (100 mL), and in acetone (100 mL) to remove phenol. Samples were then extracted with slow shaking for 30 min in 1:1 v:v methanol/chloroform (50 mL) and washed in methanol (100 mL), and in acetone (100 mL). The insoluble residue was then lyophilized. Recovered CW was manually ground to a fine particle size with a mortar and pestle and then frozen at −20 °C until used.

2.5. Analysis of cell wall composition

Uronic acids were determined in the sulfuric acid cell wall hydrosilate by the colorimetric 3,5-dimethylphenol assay after cell walls pretreatment (30 °C, 1 h) with aq. 72% sulfuric acid followed by hydrolysis with 1 M sulfuric acid (100 °C, 3 h). Pure galacturonic acid was used as a standard.

The proteins and total phenolic compound content of the cell wall material were determined after extraction with 1 M NaOH (100 °C, 10 min) by the colorimetric Coomassie Brilliant Blue assay and by the colorimetric Folin–Ciocalteau reagent assay, respectively. Bovine serum albumin (BSA) fraction V and pure gallic acid were used as standards, respectively.

The total glucose was determined using a kit for glucose enzymatic analysis from R-biopharm (Darmstadt, Germany) after pretreatment (30 °C, 1 h) with aqueous 72% sulfuric acid, followed by hydrolysis with 1 M sulfuric acid (100 °C, 3 h). Hydrolysis using only 1 M sulfuric acid (100 °C, 3 h) was used to determine non-cellulosic glucose. Cellulosic glucose was obtained by difference between the total glucose and non-cellulosic glucose content. The acid-insoluble residue obtained after pretreatment and hydrolysis was used to estimate the content of lignin (Klason lignin).

2.6. Proanthocyanidins used in the interaction studies

The proanthocyanidin used for the experience was seed-derived commercial tannin (TanReactive, Agrovin, Alcazar de San Juan, Spain) with a purity of 56%, an degree of polymerization of 2.77 and a galloylation percentage of 7.5%. The purity of the commercial tannin was estimated spectrophotometrically following the method of Ribéreau-Gayon, Glories, Maujean, and Dubourdieu (1998) after acid hydrolysis of the samples and the degree of polymerization and galloylation percentage were calculated with the phloroglucinolysis method described in Section 2.8.

2.7. Binding reactions between tannins and cell wall material with and without enzyme

Test 1: Skin CWs from veraison and ripen grapes were separately weighed into 3 mL tubes. CW samples were then combined with the enological tannin previously dissolved in a model solution (12% ethanol and pH 3.6 adjusted with trifluoroacetic acid) at a concentration of 2 g/L. The reaction volume was 2.5 mL and the final CW concentration was 13 mg/mL. The samples were shaken at 300 rpm in an orbital shaker at room temperature for 90 min. Six replicates were performed for each state of ripeness of the cell walls. A blank without CW was also included. After the binding reaction, samples were centrifuged at 13,000 rpm and the supernatant was transferred to a new tube. Samples were then dried under vacuum at 35 °C. Recovered tannin was reconstituted in 250 μL of methanol and then analyzed by phloroglucinolysis and size exclusion chromatography (SEC). The precipitates comprising the CW–proanthocyanidin complexes were redissolved, in triplicate, with 2.5 mL of the model solution containing a commercial enzyme (Enozym Vintage, supplied by Agrovim S.A., Spain, at a

dose of 15 mg/L) and treated under the same conditions described above to monitor any possible desorption of CW-bound proanthocyanidins. Also, the redisolution of the CW–proanthocyanidin complexes with model solution without any enzyme was tested.

Test 2: Skin CWs were weighed into 3 mL tubes. CW samples were then combined with the enological tannin previously dissolved in a model solution that also contained a commercial maceration enzyme (Enozym Vintage, Agrovin S.A., Alcazar de San Juan, Spain) at a dose of 15 mg/L. Six replicates were performed for each state of ripeness of the cell walls. After the binding reaction, the same procedure described for test 1 was applied.

2.8. Analysis of proanthocyanidins using the phloroglucinolysis reagent

The PA content of the control sample (2 g/L) and those PAs remaining in solution after the interaction with the CWs were analyzed using the phloroglucinol reagent, according to the method described by Kennedy and Jones (Kennedy & Jones, 2001) with the modifications described below. A solution of 0.2 M HCl in methanol, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid, was prepared (phloroglucinolysis reagent). The methanolic extract was left to react with the phloroglucinolysis reagent (1:1) in a water bath for 20 min at 50 °C and then combined with 2 volumes of 200 mM aqueous sodium acetate to stop the reaction.

HPLC analysis followed the conditions described by Busse-Valverde et al. (2010). Samples (10 μL injection volume) were injected on an Atlantis dC18 column (250 × 4.6 mm, 5 μm packing) protected with a guard column of the same material (20 mm × 4.6 mm, 5 μm packing) (Waters, Milford, MA). The elution conditions were as follows: 0.8 mL/min flow rate; oven temperature, 30 °C; solvent A, water/formic acid (98:2, v/v), and solvent B, acetonitrile/solvent A (80:20 v/v). Elution began with 0% B for 5 min, linear gradient from 0 to 10% B in 30 min and gradient from 10 to 20% in 30 min, followed by washing and re-equilibration of the column.

Proanthocyanidin cleavage products were estimated using their response factors at 280 nm relative to (+)-catechin, which was used as the quantitative standard. These analyses allowed determination of the recovery by mass of the total proanthocyanidin content, the apparent mean degree of polymerization (mDP) and the percentage of each constitutive unit. The mDP was calculated as the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in moles) divided by the sum of all flavan-3-ol monomers (in moles).

The percentage of conversion was calculated gravimetrically from the percentage of conversion of tannins to known proanthocyanidin subunits.

To determine the naturally occurring proanthocyanidin monomers (catechin, epicatechin and epicatechin-3-O-gallate), the methanolic extract was analyzed without reacting with the phloroglucinolysis reagent.

2.9. Analysis of proanthocyanidins by size exclusion chromatography

An adaptation of the method described by Kennedy and Taylor (2003) was used for size exclusion chromatography (SEC). The method used two PLgel (300 × 7.5 mm, 5 μm, 500 (effective molecular mass range of up to 4000 using polystyrene standards) by 100 Å (effective molecular mass range of 500–30,000 using polystyrene standards) columns (styrene divinyl benzene copolymers in ethyl benzene) connected in series and protected by a guard column containing the same material (50 × 7.5 mm, 5 μm), all purchased from Polymer Labs (Amherst, MA, USA). The amount of sample injected was 40 μg. The isocratic method used a mobile phase consisting of N,N-dimethylformamide containing 1% glacial acetic acid, 5% water and 0.15 M lithium chloride. The flow-rate was maintained at 1 mL/min with a column temperature of 60 °C and elution was monitored at 280 nm.

2.10. Statistical analysis

All the statistical analyses were made using Statgraphics Centurion XVI (Statpoint Technologies Inc., Warrenton, VA, USA).

3. Results and discussion

The extension of CW–PAs interaction and the effect of maceration enzymes in limiting the adsorption and/or favoring the desorption of these PAs have been studied in an ethanolic model solution. For a better understanding of the action of the enzyme on the CWs interaction capacity, and given that this interaction capacity changes along with the grape ripening (Bindon, Bacic, & Kennedy, 2012), we have worked with two different CWs, extracted from veraison or ripe grapes.

3.1. Cell wall characterization

The results concerning the polysaccharide and non-polysaccharide composition of veraison and ripe skin CWs showed that differences were slight (Table 1). Ripe skin CWs (rCWs) showed the highest values of proteins and non-cellulosic glucose, while veraison skin CWs (vCWs) presented the highest lignin content. Ortega-Regules, Ros-García, Bautista-Ortín, López-Roca, and Gómez-Plaza (2008) also found that only small changes in the content of CWs cellulose, lignin, protein and total polyphenols during grape maturation could be observed. Moreover, they also found that the concentration of pectins (measured as uronic acids) barely changed during maturation. It has been stated that the decrease in uronic acids (if detected) is not usually very pronounced, because, simultaneously with their solubilization, there seems to be an active synthesis of pectins, especially of homogalacturonan (Rosli & Civello, 2004).

3.2. Reactivity assays

The reaction of CWs with the PA in solution led to a 52% and 32% decrease in the PA content of the solution when rCWs or vCWs were used, respectively (Table 2), indicating that rCWs retained more proanthocyanidins than vCWs. Furthermore, those of higher molecular mass (MM) were preferentially retained, as can be concluded from the lower mDP of the tannins that remained in solution. These differences in the adsorption properties of CWs from ripe or unripe grape skins were previously observed by Bindon et al. (2012), whose studies showed that the onset of veraison was characterized by skin cell walls that showed a low level of PA adsorption, especially of those of high molecular mass, and that as ripening progressed, skin cell wall isolates showed an increasing retention of PAs.

However, as stated above, and reflecting the results of previous studies (Ortega-Regules et al., 2008), changes in the polysaccharide composition in grape skin CWs following the preveraison period were minimal and not sufficient to completely explain the observed differences in PA adsorption properties. As reported by Bindon et al. (2012), it seems that the slight chemical changes that occur in the CW composition are accompanied by physical and spatial changes in cell walls during maturation, especially an increase in porosity. This enhanced cell wall porosity may explain not only the highest adsorption of tannins in CWs from ripe grape skins but also the increased affinity for higher molecular weight PAs, since enhanced cell wall porosity would facilitate penetration

Table 1
Composition of major components in cell walls from ripe (rCW) and veraison (vCW) grape skin (proteins are expressed as mg bovine serum albumin/g cell wall, total phenolic compounds (TP) as mg galic acid/g cell wall).

	mg protein/g CWM	mg TP/g CWM	mg non-cellulose glucose/g CWM	mg cellulose/g CWM	mg UA/g CWM	mg lignin/g CWM
rCW	100.7b	44.7a	29.8b	117.8a	138.7a	568.3a
vCW	90.3a	45.6a	9.1a	119.3a	136.6a	598.9b

UA, uronic acids; rCW, skin cell walls from ripe grapes, vCW, skin cell walls from veraison grapes. Different letters within the same column indicate significant differences ($p < 0.05$).

Table 2
Results of the adsorption and desorption of proanthocyanidins (PAs) and cell walls from ripe grape skins (rCW) or veraison grape skin (vCW).

		Adsorbed PAs (mg/L)*	PAs in solution (mg/L)	PAs in solution (mDP)
Adsorption	PA	0	1126.6 ± 152.6	2.8 ± 0.1
	rCW–PA	575.4 ± 56.5	551.2 ± 86.5	2.2 ± 0.1
	vCW–PA	336.0 ± 24.4	760.6 ± 91.4	2.6 ± 0.1
Desorption	(rCW–PA)	471.3 ± 13.3	104.1 ± 23.3	2.8 ± 0.1
	(rCW–PA) + enzyme	505.3 ± 5.9	70.1 ± 15.9	2.4 ± 016
	(vCW–PA)	232.9 ± 8.3	106.1 ± 5.8	3.4 ± 0.1
	(vCW–PA) + enzyme	250.7 ± 9.6	85.3 ± 6.9	3.0 ± 0.1

mDP, mean degree of polymerization.
* Calculated as the difference between PA concentration in the solution before and after the interaction with CWs.

of large PA molecules into a more open cell wall framework (Le Bourvellec, Watrelot, Ginies, Imberty, & Renard, 2012).

However, it should not be ruled out the fact that the different chemical composition of rCWs and vCWs, although small, may also play a role in the observed differences. The higher content of proteins in cell walls obtained from ripe grape skin may contribute to the differences observed in the adsorption of PAs between the two types of cell walls. The possibility that cell wall-bound protein, particularly the hydroxyproline rich glycoproteins which accumulate in cell walls during ripening, may strongly adsorb PAs has been suggested previously (Hanlin, Hrmova, Harbertson, & Downey, 2010). In the same way, Bindon et al. (2012) suggested that, as maturation progressed, there was an increased access of PAs to form hydrogen bonds with structural proteins within the skin cell wall pores. On the other hand, the higher proportion of lignin in cell walls from unripe grape skin may decrease the interaction with large PAs, since this may represent a greater CW structural rigidity, exposing a smaller surface area for interaction with high molecular mass PAs. In this case, saturation of the available binding sites on the CW surface might occur, while smaller PA molecules might still penetrate the interior of the CW structure (Bindon, Smith, & Kennedy, 2010).

The original PA sample and the remaining PAs in solution after the adsorption process were also analyzed using SEC (Fig. 1A). This technique provides complementary information concerning the composition and molecular mass distribution of the tannins. The profiles showed that the differences observed between the different samples largely coincided with the results observed with the phloroglucinolysis analysis. The total area (see Table 4) varied between 0.58 for the original seed-derived PA in solution and 0.22 for those tannins remaining in solution after the reaction with rCWs (an area decrease of 62%), the tannins remaining after the interaction with vCWs showing an intermediate value of 0.36, which represents an area decrease of 38%. The decrease on the area corresponding to those tannins with the higher molecular mass (those eluting earlier, between 10 and 11.8 min) was much more pronounced in rCWs than in vCWs (a decrease of 82% compared with a decrease of 45.5% for vCWs), reflecting the lower mDP observed for those tannins remaining in solution when the phloroglucinolysis analysis was used.

The second step of this experiment was to determine the role of maceration enzymes in the desorption of the PAs linked to CWs. The enzyme used was a commercial preparation whose main activities were polygalacturonase (546.6 U/g), pectin lyase (2.8 U/g) and pectinmethyl esterase (7.3 U/g) and also some secondary activities such as β-glucanase and proteases (www. Agrovin.com). We chose this enzyme since we have experience working with it in real wine samples and information on the effect, on real vinifications, on proanthocyanin extraction (Busse-Valverde et al., 2010), cell wall degradation (Apolinar-Valiente, Romero-Cascales, Gómez-Plaza, López-Roca, & Ros-García, 2015) and polysaccharide liberation (Apolinar-Valiente et al., 2013) is available. In this second step, the CW–PA complexes formed in the first step were recovered, thoroughly washed and once again introduced into a new model solution, in the presence of the commercial maceration enzyme. The results were compared with a control (the same CW–PA complexes in a solution without the enzyme) to determine the effect of the enzyme, if any, on the tannin desorption process (Table 2). In the case of the rCWs, we observed that, after 90 min and when the enzyme was not present, only 104.1 mg/L was measured in solution, a value that indicates that only 18% of the bound PAs could be found again in the solution (meaning they were desorbed), whereas, 106.1 mg/L were measured in the solution containing the vCWs, representing 31% of the estimated vCW-bound PAs. Therefore, although similar quantities of PAs were measured in the solution, proportionally, less PAs desorbed from rCWs than from vCWs. Possibly, the lower content of lignin in rCWs and their greater porosity provided a flexible conformation, thus permitting stronger interactions with the PAs (Hanlin et al., 2010). In a study of the reactivity of PAs with apple CWs, (Renard et al., 2001) also observed that these compounds were not easily desorbed, since even after extensive washing, those bound to apple CWs were retained.

Contrary to expected, only 12% of the PAs bound to rCWs were found in the solution when this solution contained the commercial enzyme, a percentage that reached 25% for vCWs. In both cases, recoveries were lower than when the enzyme was absent. The studies of Le Bourvellec et al. (2004) and Le Bourvellec, Guyot, and Renard (2009) also found that washing cell walls with a buffer in the presence of an enzyme (pectin lyase), the re-extraction level of CW-bound PAs was very low, particularly if the bound PAs presented a high degree of polymerization. A similar effect was also observed by Renard et al. (2001) with other enzymes since the effect of applying a combination of polygalacturonase, pectin

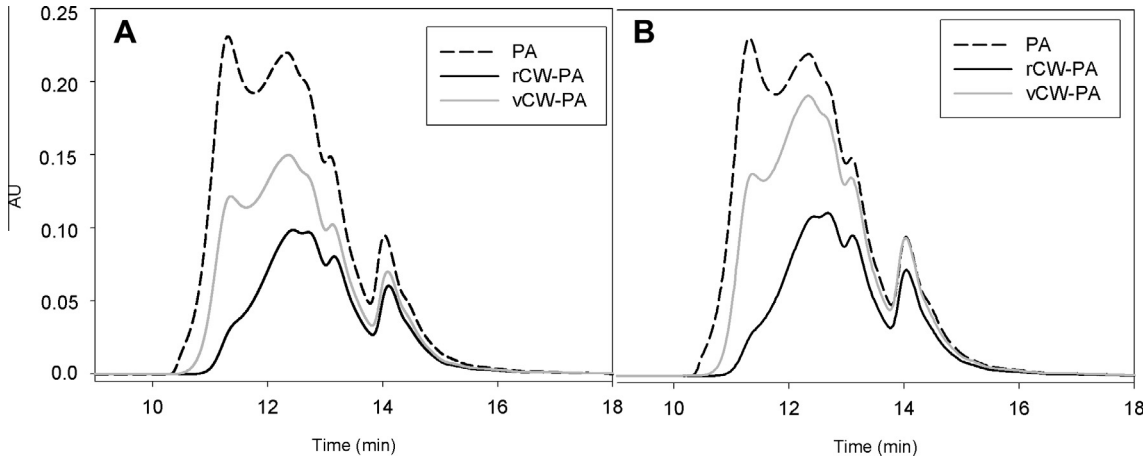

Fig. 1. Comparison of the size exclusion chromatogram of the original proanthocyanidin in solution and that for those remaining after the addition of rCW or vCWs (A) and that of the original proanthocyanidins in solution and those remaining after the addition of CWs and a maceration enzyme (B).

methyl esterase and cellulase (a composition similar to that of our commercial enzyme) to CW–PA complexes was very limited as regard PA desorption. Therefore, the results of these authors agree with ours, both as regards the limited desorption of PAs and the fact that we also found a slightly higher re-extraction from vCWs-PA complexes, since their adsorbed PAs were of lower molecular mass than those retained in rCWs and easily extracted.

There are two possible explanations of these observations. The first is the failure of the enzyme to access the polysaccharide network due to steric impediments caused by the PAs occupying the CW surface and therefore, not modifying the CW structure, although this possibility does not explain why the level of desorbed PAs is lower in the presence of the enzyme. The second possibility was that the lower recovery of PAs in solution when the enzyme was present, compared with the control solution without the enzyme, could be attributed to liberated polysaccharides (appearing from CWs after the reaction with the maceration enzyme) reacting with desorbed PAs and therefore they would not be measured in solution. This would also explain the lower mDP of the PAs in solution when the enzyme was used, since polysaccharides present a much higher affinity of high molecular mass PAs (Hanlin et al., 2010). However, deeper studies need to be done in this area.

The results of the SEC (Fig. 2A and Table 4) complemented this information. Similarly to the results observed with the phloroglucinolysis analysis, the area of the PAs found in solution was similar in both rCWs and vCWs (although the quantities of bound PAs in both CWs differed, so proportionally, as already mentioned, vCWs desorbed more PAs than rCWs). However, the profiles were quite different. The PAs desorbed from vCWs presented a higher area corresponding to high molecular mass PAs, indicating than these compounds were more easily desorbed from vCWs than from rCWs, and this is coincident with the higher mDP of these PA measured by phloroglucinolysis analysis compared with those desorbed from rCW. Probably, the vCW conformation led to weaker bonds with PAs, especially in the case of high molecular mass PAs. When enzyme was present in the solution, the SEC analysis also agreed with the results of the phloroglucinolysis analysis, showing that less PAs were desorbed (lower area).

The estimated percentage that each PA fraction represents in the desorbed PAs, compared with the composition of the adsorbed tannins, is also included in Table 4. These data showed that, in general, the smaller PAs were more easily liberated in the desorption process, probably due to this lower molecular mass fraction having fewer interactions with the CW matrix. By contrast, the higher molecular mass PA molecules occupy a number of sites and cooperativity could result in a stronger net retention (Renard et al., 2001), especially in the more flexible and porous rCWs.

In an attempt to go further in our studies, in a second experiment (Test 2), the CWs were placed in a solution containing both the commercial tannin and the maceration enzyme, as occurs during vinification, when maceration enzymes are used (Table 3). When the enzyme was in the solution, the adsorption of PAs to rCWs decreased (from 52% to 46%) compared with the previous study and it did so to a larger extent when vCW were used (32 to 12%), so the presence of the enzyme limited the adsorption of PAs, especially in vCWs.

The studies of Ruiz-García et al. (2014) indicated that only a minor loss of pectin (which in our study may have resulted from the presence in the solution of the maceration enzyme presenting pectolitic activities) may lead to a significant drop in the polysaccharides binding capacity, although the results also suggest that the cell wall from the skin of ripe grapes, with more pores and cavities, still retains a relatively high proanthocyanidin binding capacity, independent of the contribution of the pectic fraction, which may explain the lower decrease in the adsorption capacity of rCWs. The presence of the enzyme did not change the fact that the high molecular mass PAs were again preferentially bound, especially in rCWs, since the degree of polymerization of the proanthocyanidins in solution decreased.

The results of the size exclusion chromatography (Fig. 1B) again agreed with those of the phloroglucinolysis analysis. After the interaction with rCWs in the medium containing the enzyme, the remaining tannins represented 42% of the original tannins whereas with vCWs, it represented 77% of the original tannins, indicating an adsorption of only 23%. Again, the greatest differences between rCWs and vCWs were in the percentage of adsorption of high molecular mass tannins, which in the case of rCWs was 89%.

These results suggest that some of the positive action of maceration enzymes, such as increasing the tannin content of wine, is not only related with the effect of enzymes in disaggregating skin (Segade et al., 2015) and seed (Bautista-Ortin et al., 2013) structures and thus facilitating a greater extraction of these compounds, but also to lower adsorption of the extracted tannins on must-suspended CWs, especially in immature grape CWs. In this way, our results also throw more light on the previous findings of Gómez-Plaza, Romero-Cascales, and Bautista-Ortín (2010), who observed that the use of macerating enzymes might be of interest in the vinification of less than fully ripe grapes, since they would favor better extraction of the phenolic compounds from skin

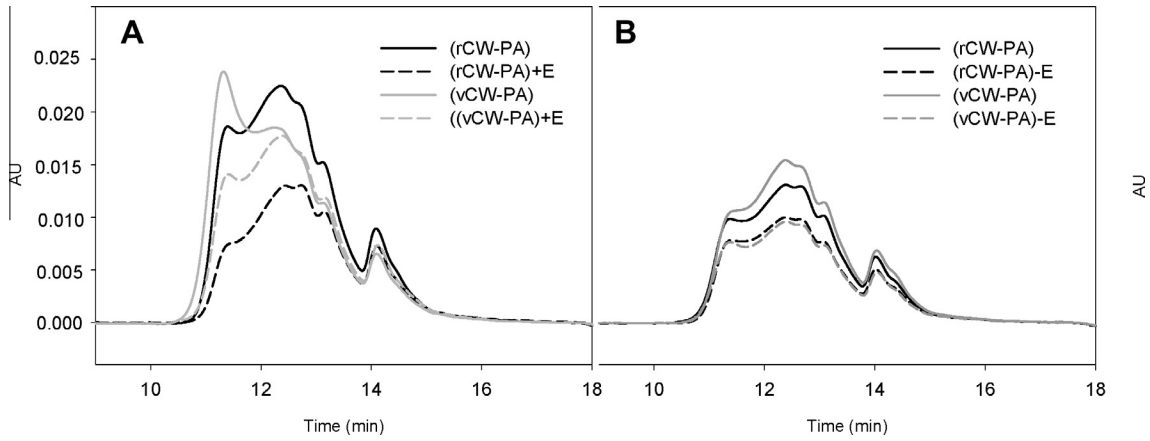

Fig. 2. Comparison of the size exclusion chromatogram of the proanthocyanidin desorbed from CWs in presence or absence of a maceration enzyme (A) and those desorbed from enzymatic pretreated-CWs in presence or absence of a maceration enzyme (B).

Table 3
Results of the interaction of proanthocyanidins (PA) and cell walls from ripe grape skins (rCW) or veraison grape skin (vCW) when a maceration enzyme was included in the solution.

		Adsorbed PAs (mg/L)*	PAs in solution (mg/L)	PAs in solution (mDP)
Adsorption	PA	0	1126.6 ± 152.6	2.8 ± 0.04
	rCW–PA	522.1 ± 26.5	604.5 ± 57.6	2.1 ± 0.04
	vCW–PA	138.5 ± 24.4	988.1 ± 71.4	2.5 ± 0.03
Desorption	(rCW–PA)	456.7 ± 13.3	65.4 ± 668	2.5 ± 0.1
	(rCW–PA) + enzyme	474.5 ± 5.9	47.5 ± 1.9	2.6 ± 0.1
	(vCW–PA)	53.9 ± 8.3	84.5 ± 19.9	2.7 ± 0.1
	(vCW–PA) + enzyme	76.72 ± 9.6	61.8 ± 4.1	2.7 ± 0.1

mDP, mean degree of polymerization.
* Calculated as the difference between PA concentration in the solution before and after the interaction with CWs.

Table 4
Total area measured in the size exclusion chromatography analysis for the original PA and those remaining in solution after the interaction (adsorption and desorption) with CWs and area corresponding to high molecular mass PAs (those eluting from 10 to 11.8 min), medium molecular mass (eluting form 11.8 to 13.6) and those corresponding to low molecular mass PAs (eluting from 13.6 to 16.00 min) when the experiment was conducted in absence or in presence of a maceration enzyme.

			Total area	Area corresponding to high MM PAs	Area corresponding to medium MM PAs	Area corresponding to low MM PAs
Absence of maceration enzyme	Adsorption	PA	0.58	0.22	0.29	0.054
		rCW–PA	0.22	0.04	0.14	0.037
		vCW–PA	0.36	0.12	0.20	0.042
	Desorption	(rCW–PA)	0.052	0.017 (9.4)*	0.030 (20.0)	0.005 (29.4)
		(rCW–PA) + enzyme	0.032	0.007 (3.9)	0.019 (12.7)	0.005 (29.4)
		(vCW–PA)	0.048	0.021 (21.0)	0.023 (25.5)	0.004 (33.5)
		(vCW–PA) + enzyme	0.041	0.013 (13.0)	0.023 (25.5)	0.005 (41.7)
Presence of maceration enzyme	Adsorption	PA	0.58	0.17	0.32	0.069
		rCW–PA	0.24	0.020	0.16	0.050
		vCW–PA	0.42	0.095	0.27	0.059
	Desorption	(rCW–PA)	0.032	0.007 (4.7)*	0.019 (11.9)	0.005 (26.3)
		(rCW–PA) + enzyme	0.026	0.005 (3.3)	0.015 (9.4)	0.004 (21.1)
		(vCW–PA)	0.036	0.009 (12.0)	0.023 (46.0)	0.005 (50.0)
		(vCW–PA) + enzyme	0.024	0.005 (6.7)	0.014 (28.0)	0.004 (40.0)

* The number in parenthesis indicate the estimated proportion of high, medium and low MM PAs liberated from those adsorbed in the CWs.

vacuoles into the must, the enzyme being more effective than in the most mature grapes. Our present results indicate that not only do enzymes lead to the better extraction of grape proanthocyanidins but also to a lower adsorption to cell walls, especially in unripe grapes.

After recovery and washing the CW-adsorbed PAs complexes and placing them in another two different solutions (with or without enzyme, as we did in the first experiment), the quantities of recovered tannins were very low, although in the case of vCW (given that the adsorbed quantities were already low), the unbound tannins represented now a 61% of the estimated bound tannins. When the enzyme was present, the same observation as described for the previous study was applicable and its presence did not help to increase the desorption of bound PAs.

The results of the SEC analysis were agreed closely with the results of the phloroglucinolysis analysis (Fig. 2B and Table 4). Similar areas were observed for those PAs desorbed from rCW and vCWs, and lower areas when the enzyme was present. However, contrary to what was observed in test 1, the profiles of the desorbed PAs were now very similar, as was the calculated mDP.

4. Conclusions

From these experiments, the high affinity of CWs for PAs in solution is evident, especially in the case of rCWs, probably due to their lower lignin content, higher protein content and a more flexible conformation. In the wineries, this high affinity will largely limit proanthocyanidin content in the wines, moreover when the results also showed that this interaction is not easily reverted, especially under the mild conditions found during vinification.

Also, we can conclude that the use of techniques which may degrade the cell wall pectic fractions during vinification, such as the use of maceration enzyme will favor an increase in the proanthocyanidin content of must and wine, not only by favoring the extraction of these compounds from skin cell vacuoles but also by promoting a lower adsorption on CWs.

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