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ARTICLE

Interaction between Grape-Derived Proanthocyanidins and Cell Wall Material. 1. Effect on Proanthocyanidin Composition and Molecular Mass

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Insoluble cell wall material was prepared from the skin and flesh of commercially ripe *Vitis vinifera* L. cv. Shiraz berries and then combined in suspension with preveraison skin and seed proanthocyanidin containing solutions. Analysis of proanthocyanidins before and after fining with cell wall material by phloroglucinolysis provided information on recovery by mass, subunit composition, and mean degree of polymerization, whereas proanthocyanidin molecular mass distribution was determined by gel permeation chromatography. Cell wall material from flesh showed the highest affinity for proanthocyanidin, binding up to 47% and 57% w/w of total seed and skin proanthocyanidin respectively. Comparison of the molecular mass distribution of skin or seed proanthocyanidin before and after fining indicated that affinity of cell walls for proanthocyanidin increased with increasing proanthocyanidin molecular mass. Initial results of subunit composition of skin and seed proanthocyanidin mixtures following fining with cell wall material showed that the % galloylation decreased, suggesting a preference for seed-derived proanthocyanidins. Subsequent experiments suggest that fining with insoluble cell wall material is size-based and does not have a specific affinity for seed-derived proanthocyanidins.

KEYWORDS: ***Vitis vinifera*; grape; proanthocyanidin; tannin; cell wall; polysaccharide; phloroglucinolysis; gel permeation chromatography; molecular mass**

INTRODUCTION

In the making of red wines, the optimal extraction of phenolics from the skin and seed of grape berries is crucial to ensure stable color formation and to impart desirable mouthfeel properties. The extraction of proanthocyanidins (PAs), in particular, is known to vary considerably, and is dependent upon cultivar, ripeness and origin within the berry (*1*−*7*). Another aspect which may affect the extraction of proanthocyanidins is their interaction with insoluble cell wall material (CWM).

Within the developing grape berry, some association of PAs with skin CWM has been reported to occur, although most PA is localized within the cell vacuoles (*8*). For intact cells, cell walls are a barrier to the diffusion of intracellular phenolic material (*9*). Studies on the extraction of skin PAs in model hydroalchoholic solution have shown that extraction is incomplete, with only 23% of available skin PA recovered in 12% v/v ethanol solution (*6*). Comparison of the extractable and inextractable PA composition and mean degree of polymerization (mDP) indicated that extractable PA had a lower mDP, while inextractable PA had both higher mDP and subunit galloylation (*6*). This work suggests that one limitation in PA extractability is its mDP and potentially galloylation.

Apart from the cell wall presenting a diffusional barrier, it has been shown that a significant portion of otherwise extractable PA (22%) can be bound by grape CWM in suspension (*10*). Similarly, apple CWM in apple juice was found to have an affinity for PA polymers (*11*, *12*). This interaction was found to be driven by noncovalent interactions, namely, hydrogen bonding and hydrophobic interactions. It was proposed by the authors that PA interaction with CWM was modulated by flavan-3-ol subunit composition, the stereochemistry of which might lead to changes in PA conformation. An important conclusion from apple cell wall studies was that cultivars rich in high mDP PAs produced juices with lower extractable PA, and with a greater proportion of PA associated with insoluble CWM. This finding has significant implications for wine production, and has the potential to explain the discrepancies often observed between total PA concentration in grape tissues and the quantity of PA in wine (*6*).

As yet, the interaction of grape-derived PA with CWM is not well understood, and the question remains as to the affinity and selectivity of grape-derived CWM for PA. This study was undertaken to begin to address these questions using a model experiment whereby PA composition was altered by varying the proportion of skin- and seed-derived PA, in order to determine the degree of selectivity of CWM for PA .

MATERIALS AND METHODS

Instrumentation. An Agilent model 1100 HPLC (Agilent Technologies Australia Pty Ltd., Melbourne, Australia) was used with Chemstation software for chromatographic analyses.

Preparation of Grape CWM. Grape bunch samples were obtained at commercial ripeness (23 °Brix) from a 9-year-old Shiraz (clone 712)

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Table 1. Proanthocyanidin (PA) Standards for GPC Prepared from Preveraison Grape Skin and Seed PA (nd = Not Determined)

		skin PA			seed PA		
fraction	solvent system[a]	mDP[b]	est MM[c]	MC (%)[d]	mDP[b]	est MM[c]	MC (%)[d]
1	60% v/v methanol	nd[e]	nd[e]	nd[e]	1.94	573	26
2	75% v/v methanol	4.33	1277	56	2.36	739	64
3	90% v/v methanol	6.75	2006	75	2.87	946	72
4	10% v/v acetone; 80% v/v methanol	11.10	3305	74	6.15	1944	75
5	20% v/v acetone; 65% v/v methanol	17.28	5158	72	9.80	3090	73
6	30% v/v acetone; 40% v/v methanol	23.29	6947	76	12.32	3876	67
7	60% v/v acetone	30.15	8996	75	15.15	4750	49

[a] Each solvent contained 0.05% v/v TFA. [b] Mean degree of polymerization (mDP) based on subunit composition from phloroglucinolysis. [c] Estimated molecular mass based on subunit composition from phloroglucinolysis. [d] Mass conversion = % recovery of PA subunits by phloroglucinolysis based on the gravimetric mass. [e] An insignificant amount of material was isolated in the first fraction.

vineyard on own roots in the McLaren Vale region of South Australia (35°15′S, 138°30′E). The pruning applied was 35−40 buds/vine giving an average vineyard yield of 6.5 t/ha. For juice yield determination, a fresh 200-berry sample was collected and the juice was expressed by gentle pressing. Destemmed grape berries were frozen at −20 °C for not longer than 3 months prior to analysis. Immediately prior to processing, 700 berries were frozen for 1 h at −80 °C and then manually separated into skin and flesh components using a scalpel. Seeds were removed from the flesh component. During processing, skin and flesh components were kept frozen with liquid nitrogen to limit oxidation. Processed samples were stored at −80 °C until extraction.

For CWM extraction, frozen flesh was homogenized at 8000 rpm for 20 s in a Retsch Grindomix GM200 (Retsch GmbH & Co, Haan, Germany) to form a slurry, and 400 mL was immediately added to 400 mL of 40 mM HEPES pH 7 at 4 °C and stirred for 15 min to remove water-soluble material. The samples were then centrifuged twice at 8000*g* for 20 min at 4 °C, and the insoluble residue was retained. The HEPES-extracted flesh material and untreated frozen skins were then extracted in 70% v/v acetone to remove PA. Acetone-extracted residues were washed in additional 70% v/v acetone, followed by Milli-Q water (Millipore Corporation, Billerica, MA). Acetone-extracted skin material was then homogenized in a Retsch Grindomix GM200 (Retsch GmbH & Co., Haan, Germany) and then finely milled under liquid nitrogen in a IKA A11 basic grinder (IKA-Werke GmbH & Co. KG, Staufen, Germany). Thereafter, CWM was prepared from acetone-extracted skin and flesh residues according to the method of Vidal et al. (*14*), with the following modifications. Acetone-insoluble residues were extracted in Tris-HCl equilibrated phenol pH 6.7 (Sigma-Aldrich, St. Louis, MO), and then washed twice in 80% v/v ethanol, and three times in acetone to remove phenol. Samples were then extracted with slow shaking for 30 min in 1:1 v:v methanol: chloroform, and the insoluble residue was then lyophilized. Recovered CWM was manually ground to a fine particle size with a mortar and pestle and then frozen at −20 °C until used.

Preparation of PA Molecular Mass Standards for Chromatography. Whole preveraison skin and seed PA (*Vitis vinifera* L. cv. Pinot noir) was isolated according to a previously published method (*15*). For preparation of standards for gel permeation chromatography (GPC), 0.9 g of preveraison grape skin PA or 0.65 g of seed PA was dissolved in 50 mL of 60% (v/v) HPLC grade methanol containing 0.05% v/v trifluoroacetic acid (TFA) and then applied (∼18.3 mL/min) to a glass column (Michel-Miller, 300 × 21 mm, Vineland, NJ) containing Sephadex LH20 chromatography resin (Amersham, Uppsala, Sweden) to an approximate bed volume of 93 mL. The column was equilibrated with 60% v/v methanol containing 0.05% v/v TFA. The applied PA was fractionated using the solvent system described in *16*, but 0.02% v/v formic acid was replaced with 0.05% v/v TFA (**Table 1**). The eluted PA fractions were concentrated under reduced pressure at 35 °C to remove organic solvents and then lyophilized to a dry powder.

Binding Reaction of Skin and Seed PA Isolates with CWM. Lyophilized flesh and skin CWM was transferred into 1.5 mL polypropylene centrifuge tubes in 6 mg and 13 mg quantities. CWM was combined with whole preveraison skin and/or seed PA (2 g/L) in 1 mL of a solution containing 12% v/v ethanol and 0.01% v/v TFA and allowed to interact for 1 h, with shaking, at 32 °C. For PA mixtures, the skin:seed PA ratio was varied as 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 0:100. Additionally, whole preveraison skin or seed PA was combined 1:1 by mass with a lower mDP PA fraction 3 (**Table 1**) of seed or skin PA respectively to a final concentration of 2 g/L and the reactions performed as described above. Each reaction was performed in duplicate. For each reaction, a blank reaction without CWM was run in order to account for possible loss in PA recovery due to self-association, precipitation or oxidation. Additionally, a CWM blank without PA was included. Following the binding reaction, samples were centrifuged at 16000*g* for 20 min and the supernatant transferred to a new 1.5 mL centrifuge tube. Samples were then dried under vacuum at 35 °C in a Heto vacuum centrifuge (Heto-Holten A/S, Allerod, Denmark). Residual PA was then reconstituted in 100 μL of 100% methanol, and then analyzed by phloroglucinolysis and GPC.

Acid Catalysis of PA in the Presence of Excess Phloroglucinol (Phloroglucinolysis). Whole skin and seed PA isolates, skin molecular mass fractions and residual PA from the binding reaction were characterized by phloroglucinolysis (*15*) to determine subunit composition and mDP. To accommodate both high sample throughput and small sample size, the reaction volume was reduced from that in the original method. In a 0.2 mL PCR tube (Eppendorf, Hamburg, Germany), 25 μL of PA in methanol was added to an equal volume of 0.2 N HCl, 100 g/L phloroglucinol (Sigma Aldrich, St. Louis, MO) and 20 g/L ascorbic acid (Sigma Aldrich, St. Louis, MO) in methanol to give a final maximum PA concentration of 5 g/L. The phloroglucinolysis reaction was then carried out at 50 °C for 25 min, cooled and then neutralized and analyzed by RP-HPLC according to the conditions outlined in the original method using (−)-epicatechin (Sigma Aldrich, St. Louis, MO) as the quantitative standard.

Gel Permeation Chromatography (GPC). The GPC method previously described (*16*) allowed for size distribution determination of the PA isolates. Either preveraison skin or seed PA fractions of known mDP (**Table 1**) were used as standards for calibration, and a second-order polynomial fit with the time at 50% elution for each was used to predict mean molecular mass. Prior to analysis, PA samples prepared in methanol were diluted with 4 volumes of HPLC-grade mobile phase (*N*,*N*-dimethylformamide containing 1% v/v glacial acetic acid, 5% v/v water and 0.15 M lithium chloride). The maximum amount of PA injected onto the column was 40 μg.

RESULTS AND DISCUSSION

Subunit Composition and Molecular Mass Distribution of Skin and Seed PA Fractions. Preveraison seed and skin PAs were selected for this study due to their high degree of conversion by phloroglucinolysis (*17*−*19*). The compositional characteristics of the PA samples used for reaction with CWM changed as the proportion of skin and seed PA varied (**Table 2**). As expected for mixtures containing both seed and skin PA, the proportion of epigallocatechin decreased with a decline in the proportion of skin-derived PA, whereas the proportion of epicatechin-3-*O*-gallate increased with seed-derived PA (*7*, *17*, *18*).

The mass conversion for the seed PA fraction was not as high as previously reported for preveraison PA (*17*). As the proportion of seed PA in the samples increased, the mDP decreased, from an

average mDP of 17 in 100% skin PA sample, to mDP 7 for the 100% seed PA sample. These mDP values represent a smaller range of PA sizes compared to previous investigations involving binding to CWM (*11*, *12*). However, the use of GPC enabled visualization of the molecular mass (MM) distribution of PAs (*16*). Due to the use of skin-based PAs for the initial GPC calibration, the average MM of the PA samples determined by phloroglucinolysis compared with that by 50% elution using GPC showed better agreement for the PA samples dominated by skin-derived material. Because of this, the GPC method was useful for comparing MM distributions within each experiment, but its accuracy declined as the proportion of seed PA increased. For example, the MM distribution determined by GPC for the reconstituted PA samples (**Figure 1A**) accurately shows a shift from higher to lower MM as the proportion of seed PA increased, but does not accurately reflect the absolute distribution, which should have an expected average MM (at 50% elution) which is 55% lower according to the results by phloroglucinolysis. Because of this and unless otherwise specified, the MM distribution for each reconstituted PA sample reacted with CWM was interpreted within each experiment.

Table 2. Properties of Reconstituted Seed and Skin Proanthocyanidin (PA) Samples

PA ratio[a]	mDP[b]	est MM[c]	MC (%)[d]	% galloylation	% tri-OH[e]	MM[f]
100:0	17.13	5094	87.6	2	25	6401
90:10	16.19	4842	85.0	3	23	6264
75:25	13.60	4104	80.5	5	20	6047
50:50	11.42	3509	75.0	10	13	5828
25:75	9.57	2970	75.3	12	8	5677
10:90	7.67	2411	79.0	16	3	5378
0:100	7.51	2374	74.5	17	0	5301

[a] All data represent analysis of PA of equivalent final gravimetric mass. Ratio represents the proportion of skin:seed PA. [b] Mean degree of polymerization (mDP) based on subunit composition from phloroglucinolysis. [c] Estimated molecular mass based on subunit composition from phloroglucinolysis. [d] Mass conversion = % recovery of PA subunits by phloroglucinolysis based on the gravimetric mass. [e] % trihydroxylated (prodelphinidin) PA subunits. [f] Molecular mass (MM) based on 50% elution volume of proanthocyanidins using GPC.

The GPC elution profile of the 100% skin and 100% seed PA samples, and a 50:50 combination of seed:skin PA (**Figure 1B**) shows a slight increase in the contribution of some higher molecular mass material (10.5 min) in the 100% skin PA fraction, which decreases from 100% skin to 100% seed. The 100% seed sample shows a greater relative proportion of later eluting, lower molecular mass material (12−14 min) than in the skin sample. However, as discussed, the differences in the GPC profiles of the prepared fractions were minor when skin PA standards were used to estimate MM.

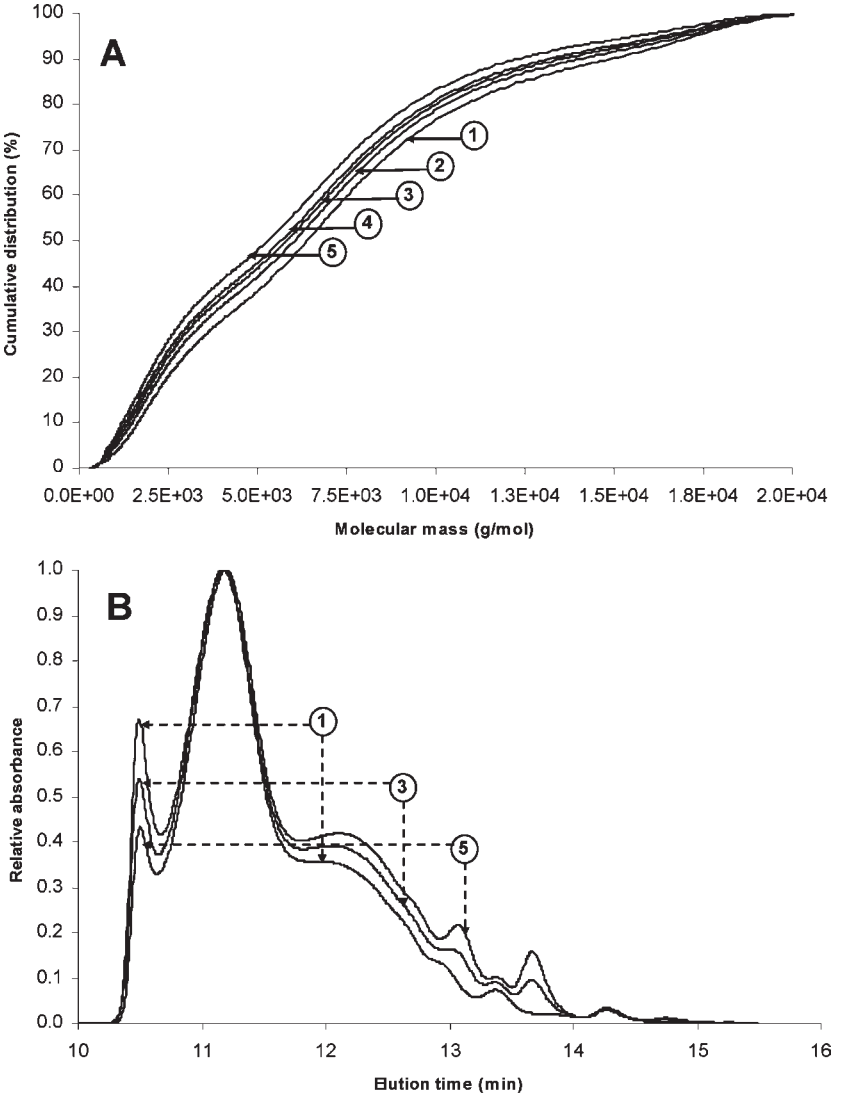

Figure 1. (**A**) Cumulative molecular mass distribution and (**B**) GPC elution profile of untreated skin and seed proanthocyanidin fractions in ratios of skin:seed 100:0 (1) 75:25 (2) 50:50 (3) 25:75 (4) and 0:100 (5).

Yield of Grape Flesh and Skin CWM. The yield of flesh and skin CWM is shown in **Table 3**. The yield of CWM from grape berries has been previously reported in various ways: as proportional quantities of sugar moieties and protein, as the dry weight yield following phenol extraction, or as the acetone-insoluble dry weight yield (*10*, *12*, *14*, *20*−*24*). The gravimetric yield of CWM expressed per berry in the current study is therefore difficult to compare directly with other research. The gravimetric yield of skin CWM of 6 mg/berry in this study is within the range of values reported for Shiraz skin (*21*−*23*). A single study on Shiraz flesh CWM reported a yield of 3 mg/berry, which is significantly lower than that reported here, at 9 mg/berry (*22*). Future investigations will seek to understand factors affecting flesh CWM concentration.

While the CWM was not characterized in this study, it is likely that it contained polysaccharides and unextractable proteins, as well as lignin and other polyphenolics (*14*, *23*, *24*). Since the current study sought to explore the interaction of PA with CWM in a concentration range reflecting conditions found during vinification, the yield of CWM was expressed on an estimated must yield of 0.5 mL/berry (calculated from the juice yield). Preliminary experiments explored the binding of two higher mDP skin PA fractions 5 and 7 (**Table 1**) with flesh CWM, and showed that, within the range of CWM dry weight concentrations expected in grape, the PA would be completely removed from solution (data not shown). Therefore, in order to explore selectivity of CWM binding to PA, flesh CWM concentrations lower than that expected in must were selected.

Table 3. Dry Weight Yield of Grape Flesh and Skin Cell Wall Material (CWM) per Berry (0.99 g/Berry) and per mL Juice Yield (0.5 mL/Berry)

berry compositional parameter	flesh	skin
berry fresh wt (mg fresh wt/berry)	670	160
CWM per berry (mg dry wt/berry)	8.83	6.29
CWM per mL juice (mg dry wt/mL)	17.54	12.58

Effect of CWM on PA Concentration, Molecular Mass Distribution, and Composition. As the proportion of seed PA increased in the unfined control solutions, there was an observed decrease in PA concentration from 2.0 mg/mL per reaction which was due to decreasing mass conversion of the seed-derived PA material by phloroglucinolysis (**Table 2**) as opposed to loss of PA due to self-association and/or precipitation (**Figure 2A**). The addition of 13 mg/mL flesh CWM to the PA solution showed the greatest decrease in PA removed by mass, and the total PA amount bound to CWM declined progressively as the proportion of seed PA in the solution increased, from 57% removed for 100% skin PA to 47% removed for 100% seed PA. For skin CWM addition, no pattern of PA mass decrease was observed as the proportion of skin:seed PA changed, and PA removed was 36% and 17% for 13 mg/mL and 6 mg/mL CWM respectively (**Figure 2A**). When comparing flesh and skin CWM binding of PA, skin CWM removed less PA than flesh CWM at the same CWM concentration.

The observed mDP for the residual PA relative to the untreated control decreased with 13 mg/mL flesh CWM addition (**Figure 2B**). As the proportion of seed PA increased, the decrease in mDP was reduced, from 31% in 100% skin PA to 19% for 100% seed PA. The reduction in mDP was less pronounced as CWM concentration decreased, or when skin CWM was used instead of flesh CWM. At an addition of 13 mg/mL skin CWM,

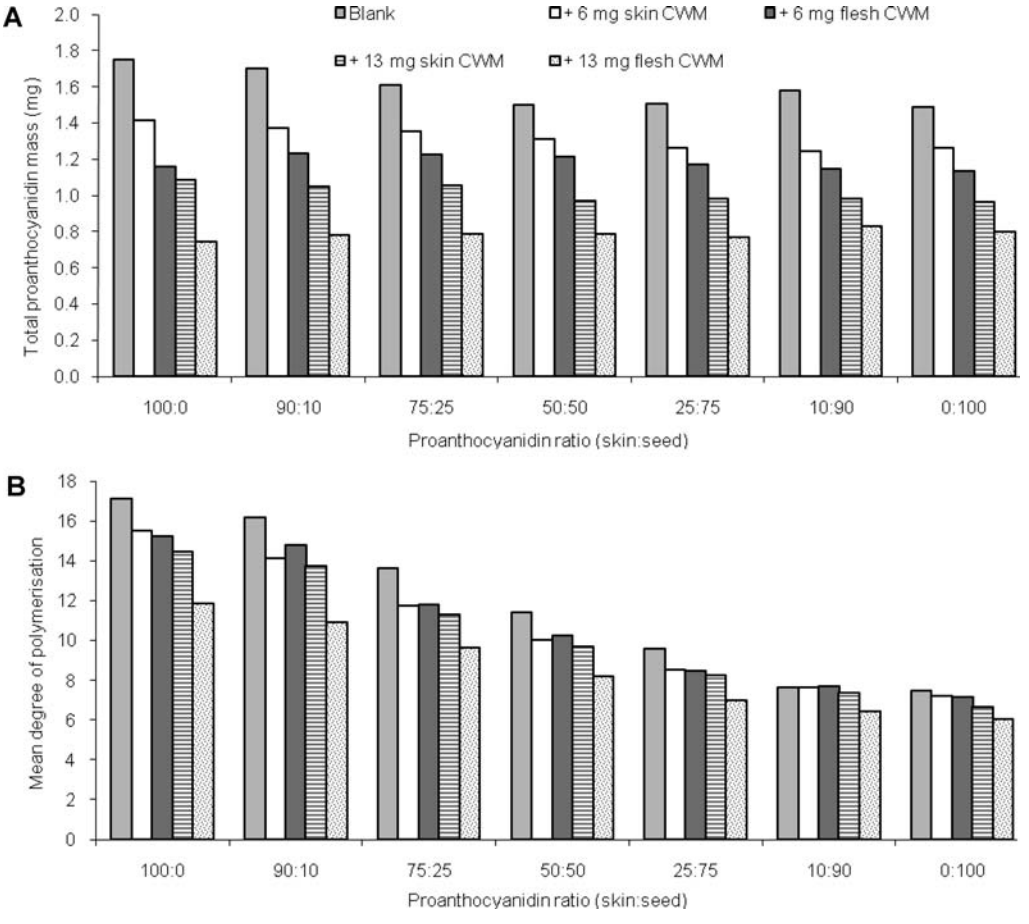

Figure 2. (**A**) Proanthocyanidin mass (mg) and (**B**) mean degree of polymerization (flavan-3-ol units) (2 mg/mL) in different proportions of skin:seed before and after reaction with 6 or 13 mg/mL flesh and skin cell walls ($N = 2$; SD <5% for all samples).

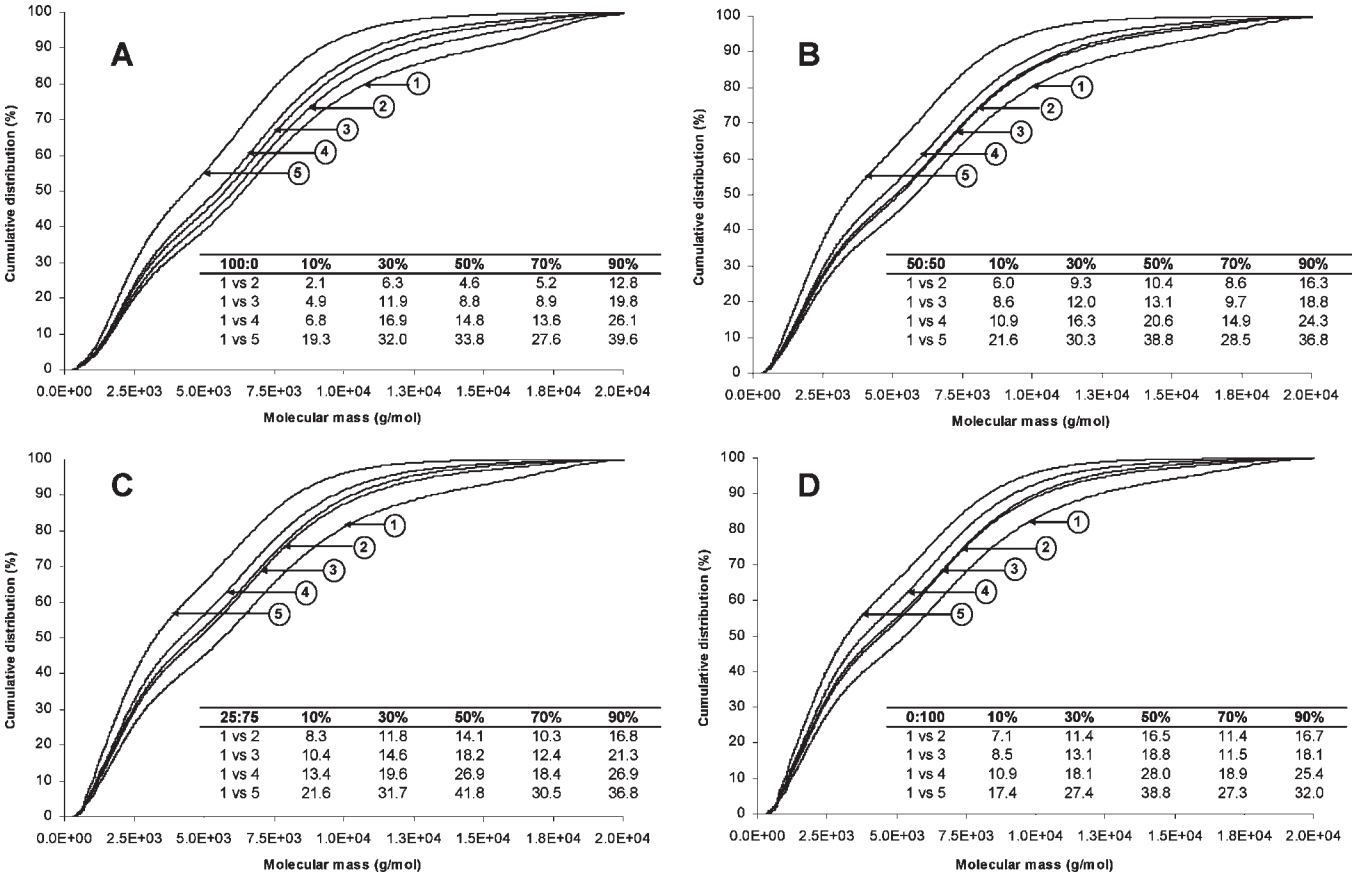

Figure 3. Cumulative molecular mass distribution of (**A**) 100:0; (**B**) 50:50; (**C**) 25:75; and (**D**) 0:100 skin:seed proanthocyanidin. Plots represent untreated control (1) and treatment with 6 mg of skin CW (2), 6 mg of flesh CW (3), 13 mg of skin CW (4) and 13 mg of flesh CW (5). Table inset shows the percentage decrease in the molecular mass average at 10, 30, 50, 70 and 90% elution from the untreated control for plots 2−5.

6 mg/mL flesh or skin CWM, the decrease in mDP was insignificant for PA solutions containing either 90 or 100% seed PA.

Regardless of the composition of seed and skin PA proportions, GPC showed that the average MM decreased with increasing CWM concentration, consistent with the mDP changes observed by phloroglucinolysis (**Figure 3**). For the 100% skin PA (**Figure 3A**), the greatest decrease in PA occurred in the higher MM range (90% elution), with less material removed by CWM in the lower MM range (below 50% elution). For 100% skin PA, the selectivity for higher MM was similar for both flesh and skin CWM, although it decreased from flesh to skin CWM. As the proportion of seed PA increased in the PA samples (**Figure 3B−D**), the observed decrease in average MM was a result of PA removal across the entire MM range. Regardless of the proportion of seed PA in the reaction, 13 mg/mL flesh CWM produced the greatest reduction in average MM and consistently removed a greater proportion of lower MM material (< 2500 g/mol).

An increased affinity of higher MM PA for CWM (*11*, *12*) and proteins (*25*−*33*) is well documented. The mechanism for this interaction has been proposed to be due to both hydrophobic interactions and hydrogen bonding (*12*, *27*). For PAs, an increase in the number of hydrogen bonding sites would increase with molecular size thus increasing affinity. However, it should be noted that the conformational structure and flexibility of the PA molecule could also affect the PA surface area, which is dependent not only on average MM but also on PA composition, the degree of oxidation and the solvent used (*27*, *28*, *31*, *34*, *35*).

To compare the interaction of seed and skin PA with CWM, seed and skin PA GPC standards (**Table 1**) were used to predict the MM of the respective PA system to provide more accurate MM information for the respective PA types. After CWM addition and removal from the individual PA system, it was observed that, at an equivalent MM, seed PA had a stronger affinity for CWM (**Figure 4**). A potential explanation for this observation, as suggested by Kennedy and Taylor (*16*), is that seed PA has a larger hydrodynamic volume than skin PA of equal MM and thus an increase in the number of available hydrogen bonding sites per molecular mass unit. The variation in seed and skin PA subunit composition, or perhaps PA branching may lead to the observed differences in affinity for CWM.

GPC is a size-based separation method, and therefore, the molecular mass of analytes is determined by their elution time in relation to appropriate molecular mass standards. It is critical that analytes do not interact with the column stationary phase, or errors in molecular mass determination will result. Based upon work on PAs and other wine related phenolics (*16*), the GPC method used in the current study should be effective at size-based separation. Thus, to explore the relationship between PA size and affinity for CWM, the proportional loss of PA following fining with CWM was determined by monitoring PA loss as a function of GPC elution time. From **Figure 5** it can be seen that, on a size basis, seed and skin PA have a similar affinity for CWM, with PAs eluting before ~11.5 min having a similar affinity for CWM. This elution time corresponded to a molecular mass of 5123 and 2900 for skin and seed PA, respectively. For material eluting between 11.5 and 12.8 min, skin PAs were removed from solution to a greater extent, although the differences were minor. For PA elution after 12.8 min, the results were difficult to interpret, yet PAs eluting in this region represent a minor proportion of the total (**Figure 1B**). Overall, these results suggest that PA interaction with CWM is a size-based interaction.

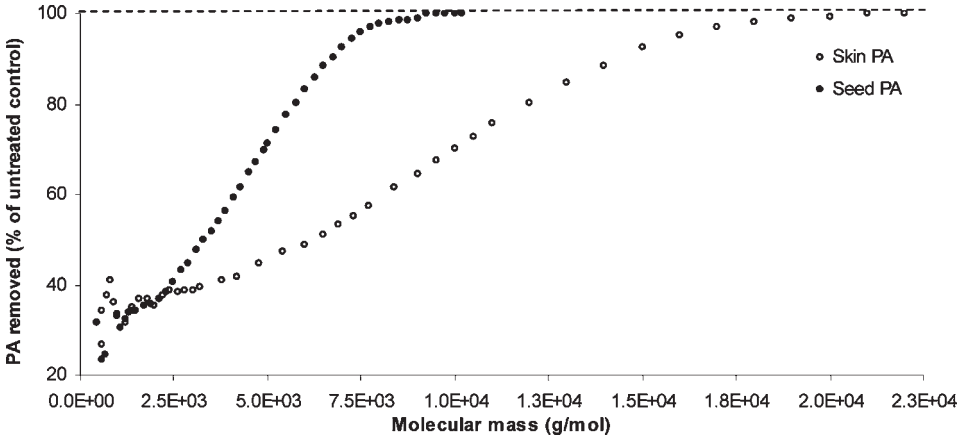

Figure 4. Percentage removal of whole preveraison skin and seed proanthocyanidin after reaction with flesh cell wall material and as a function of molecular mass.

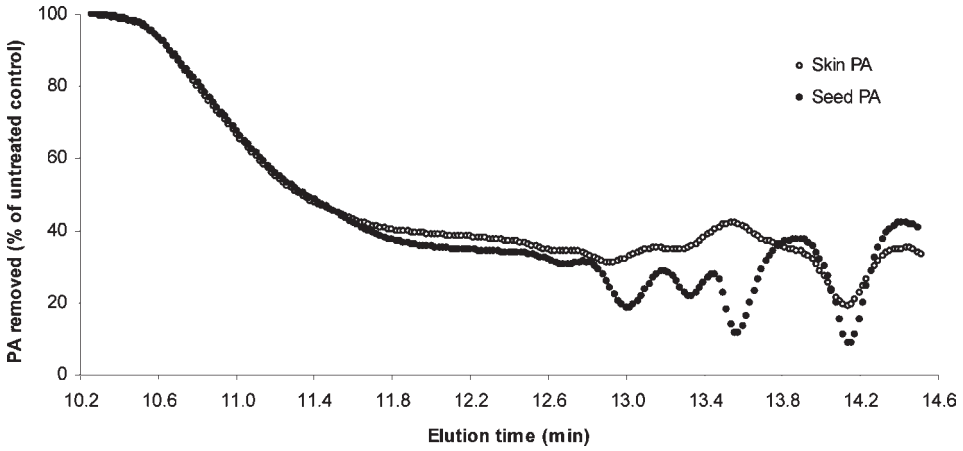

Figure 5. Percentage removal of whole preveraison skin and seed proanthocyanidin after reaction with flesh cell wall material and as a function of elution time.

A recent study has investigated the conformation of PA of different mDP (*35*), and has shown that, in hydroalcoholic solutions, PA polymers that are less than 13 nm in length (24−43 epicatechin units) are generally rigid, linear structures. Given this, it would be expected that the skin and seed PAs used in this study would be rigid and linear based on their chain length. That study found that divergence from the proposed linear structure would occur if there was PA branching in the molecule (*35*). Branching could not be detected in low mDP (< 7) PAs, but the study used PAs which were 95% epicatechin in their subunit composition, and as yet little data exists on the effect of epicatechin-3-*O*-gallate, catechin or epigallocatechin on PA polymer conformation. Given the increased proportion of epicatechin-3-*O*-gallate-containing subunits in seed PA, the C-3 galloylation may result in a more extended conformation for seed PA, or variations in interflavanoid bond position. To date, little is known about structure−conformation relationships for more complex PA polymer systems.

The subunit composition of the skin and seed PAs by phloroglucinolysis and the MM distribution by GPC was compared before and after CWM fining. It was found that for the treatments with higher proportions of either skin or seed PA, and regardless of the added CWM source, changes in the composition of individual PA subunits were minor (**Table 4**). This suggests that the composition of PAs is consistent across the size distribution. Small changes in the proportion of extension to terminal subunits led to the observed decrease in residual PA mDP. For the 50:50 skin:seed PA experiment which had a more varied subunit composition, CWM addition led to decreases in the proportion of epicatechin-3-*O*-gallate extension subunits leading to a net decrease in galloylation (**Table 4**, **Figure 6**). This decrease in extension subunit galloylation decreased as the mixture became dominant in either skin or seed PA.

Manipulation of PA Size Distribution and Subsequent Effect of CWM Fining on PA Composition. Since our results indicated that PA interaction with CWM is a size based interaction (**Figure 5**), further experiments were conducted by combining PAs of varying size distribution and seed or skin origin and then fining the mixtures with CWM. Initially, 100% skin or seed PA was combined with a low mDP fraction of seed or skin PA respectively (1:1 w/w). The skin PA:seed PA mDP 3 showed a 38% or 31% reduction in PA amount (data not shown) when added to 13 mg/mL flesh or skin CW respectively. The expected subunit composition of this sample, however, did not change significantly following CWM fining, and it was determined that the expected subunit compositional variation with PA size was minor (**Figure 6**). Nevertheless, a small decrease in the % galloylation was observed. A 1:1 seed PA:skin PA mDP 7 combination (**Figure 7A** and **Figure 7B**) resulted in a 34% or 30% reduction in PA mass when fined with flesh or skin CWM respectively, and showed a decrease in PA galloylation of 20% after fining (data not shown).

To maximize the size difference between seed and skin PA, and to test the hypothesis that CWM interaction with PAs is based primarily upon size, a skin PA fraction high in mDP (∼30) was combined with a seed PA fraction low in mDP (∼3) (**Figure 6**, **Figure 7C** and **Figure 7D**). The results of fining with CWM were consistent with a size-based interaction in that we were able to effectively increase seed-derived PAs based upon subunit composition (i.e., a decrease in

Table 4. Proanthocyanidin (PA) Composition by Phloroglucinolysis before and after Treatment with Flesh and Skin Cell Wall Material (CWM) ($N = 2$; SD <5% for All Samples)

		extension[a]				terminal[a]		
treatment	ratio[b]	% EGC	% C	% EC	% ECG	% C	% EC	% ECG
control	100:0	25.05	2.55	64.56	2.01	5.66	0.00	0.18
+ 13 mg of flesh CWM	100:0	24.52	2.91	62.31	1.81	8.15	0.00	0.30
+ 6 mg of flesh CWM	100:0	25.06	2.70	63.69	1.99	6.28	0.00	0.28
+ 13 mg of skin CWM	100:0	25.50	2.94	62.62	2.02	6.64	0.00	0.28
+ 6 mg of skin CWM	100:0	25.03	2.80	63.65	2.08	6.12	0.00	0.33
control	50:50	14.20	4.74	64.43	7.09	4.85	1.03	3.66
+ 13 mg of flesh CWM	50:50	13.49	5.60	63.58	5.11	7.36	1.47	3.39
+ 6 mg of flesh CWM	50:50	13.84	5.32	65.17	5.94	5.80	1.32	2.61
+ 13 mg of skin CWM	50:50	15.16	5.49	63.43	5.61	5.93	1.66	2.71
+ 6 mg of skin CWM	50:50	13.94	5.12	64.97	6.02	5.74	1.44	2.78
control	0:100	0.00	7.43	67.66	11.59	5.25	2.49	5.59
+ 13 mg of flesh CWM	0:100	0.00	8.44	65.73	9.30	6.88	2.81	6.84
+ 6 mg of flesh CWM	0:100	0.00	7.87	67.54	10.60	5.47	2.82	5.71
+ 13 mg of skin CWM	0:100	0.00	8.15	66.63	10.13	5.78	3.65	5.66
+ 6 mg of skin CWM	0:100	0.00	7.70	67.68	10.76	5.27	2.90	5.69

[a] Percent composition of PA subunits (in moles), and with the following subunit abbreviations: EGC, epigallocatechin; C, catechin; EC, epicatechin; ECG, epicatechin-3-*O*-gallate. [b] Ratio represents the proportion of skin:seed PA.

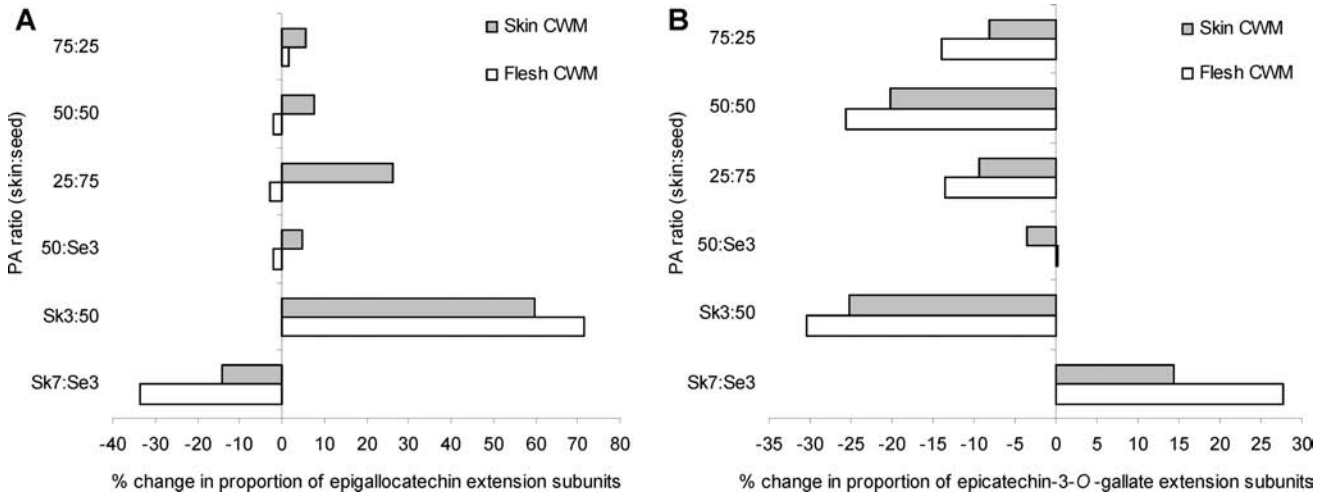

Figure 6. Decrease in the proportion of (**A**) epigallocatechin and (**B**) epicatechin-3-*O*-gallate extension PA subunits following reaction with skin or flesh CWM. Reconstituted PA samples were of unfractionated skin or seed PA unless indicated as follows: Se3 = seed PA fraction 3, mDP 3; Sk3 = skin PA fraction 3, mDP 7; Sk7 = skin PA fraction 7, mDP 30. The concentration of all reconstituted PA samples was 2 mg/mL before reaction with 13 mg/mL skin or flesh CWM ($N = 2$; SD <5% for all samples).

epigallocatechin and an increase in epicatechin-3-*O*-gallate extension subunits). These data are consistent with the hypothesis that there is a binding preference of CWM for larger-sized PA. Comparison of the GPC elution profiles of PAs before and after reaction with CWM confirmed this, showing selective removal of larger (earlier eluting) material (**Figure 7B** and **Figure 7D**). These results suggest that the observation by other authors of the role of PA galloylation in imparting increased binding capacity of PA to CWM (*12*), and protein (*25*, *27*−*29*, *32*, *33*, *36*) can be largely explained by the variation in the size of galloylated material, as opposed to specific selectivity for galloylated material.

Binding Mechanism of PA with Flesh and Skin CWM. Cell wall structure and composition is an important determinant of CWM affinity for PA (*13*, *37*−*39*). In studies using model polysaccharides, the association of PA with CWM has been proposed to be primarily due to the high affinity of PA for pectin (*37*, *38*). More recent work has shown that the affinity of PA for pectins within insoluble apple cell wall increased for more highly methylated pectins (*39*). In the current study, differences between the PA binding affinity of skin and flesh CWM were observed. For *Vitis vinifera* cv. Shiraz grape tissues, most studies on CWM composition have only focused on skin CWM (*21*, *23*, *24*). A comparison of flesh and skin CWM in *Vitis vinifera* cv. Shiraz showed very similar composition in terms of their respective neutral sugar composition, content of uronic acids and protein, and the degree of methylation, although polyphenol content of the skin material was higher (*22*). An increasing association of higher mDP PA with CWM in the inner cell compartment has been reported for *Vitis vinifera* L. cv. Cabernet Sauvignon skins during grape ripening, which was proposed to limit its PA extractability (*8*). It is therefore possible that, for skin CWM, some bound PA remain after CWM preparation, thereby reducing available binding sites. Future studies will investigate this phenomenon, and attempt to elucidate the structural and/or chemical differences between flesh and skin CWM and their influence on the binding capacity for PA.

Conclusion. The findings reported here may have significant implications for vinification. During grape crushing and fermentation, the presence of PA bound to CWM represents a barrier to PA extraction. Second, insoluble polysaccharides in must could

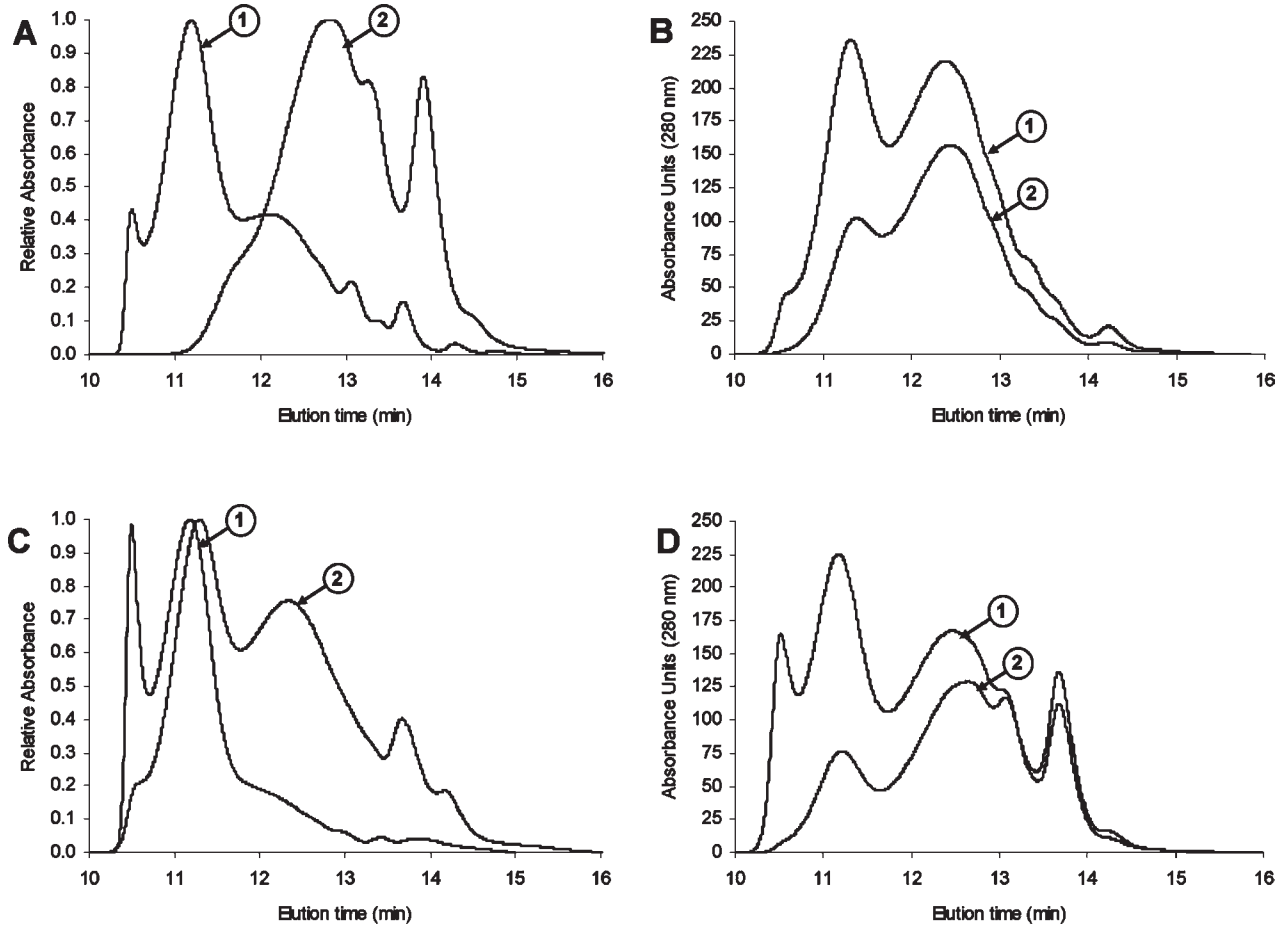

Figure 7. GPC elution profiles showing the following: (**A**) Relative absorbance of (1) seed PA and (2) fraction 3 skin PA mDP 7. (**B**) Comparison of (1) untreated 50:50 (w/w) seed PA and fraction 3 skin PA mDP 7 and (2) following reaction with 13 mg/mL flesh CWM. (**C**) Relative absorbance of (1) skin PA fraction 7, mDP 30, and (2) fraction 3 seed PA mDP 3. (**D**) Comparison of (1) untreated 50:50 (w/w) skin PA fraction 7, mDP 30, and fraction 3 seed PA mDP 3 and (2) following reaction with 13 mg/mL flesh CWM.

potentially bind to and remove a significant amount of otherwise extractable PA. As a consequence, CWM has the potential to significantly alter the composition of PA remaining in solution through its increased binding affinity for larger-sized PA. This might have implications for wine sensory properties such as mouthfeel, astringency and bitterness and affect the concentration of stable pigmented polymers in wine. A further area which needs to be addressed is the effect of viticultural factors, namely, grape variety, maturity and vineyard management practices, on both the composition of insoluble CWM in grapes and also their effect on PA extractability and composition in wine.

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