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ARTICLE

Interaction between Grape-Derived Proanthocyanidins and Cell Wall Material. 2. Implications for Vinification

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Proanthocyanidins (PAs) were isolated from the skins, seeds and flesh of commercially ripe grapes, and from wine and marc produced from the same source. In the grape berry, skin PAs accounted for 54% of the total extractable PA, while seed and flesh-derived PA accounted for 30% and 15% of the total, respectively. Following fermentation, 25% of the fruit PA was found in the wine, while 27% was found in the pericarp isolated from marc, and 48% was unaccounted for (either remaining in the seed or adsorbed to lees). To investigate the role that cell wall material (CWM) has on PA extraction during fermentation, CWM isolated from skin and flesh were combined with PA in model suspensions. In general, the affinity of flesh CWM for PA increased with increasing PA molecular mass (MM); however, this relationship was not observed for the interaction of skin CWM with skin PA. Subsequent experiments suggest that the differences in the interaction of flesh and skin CWM with PA of higher MM (>15000 g/mol) may be limited by the structure of the CWM. Observed variations in the composition between skin and flesh CWM may explain the differences in PA interaction at high MM. Among wine-derived PA, no higher MM material was detected, suggesting that, during vinification, higher MM PA are nonextractable and/or are removed from the wine by interaction with CWM.

KEYWORDS: Proanthocyanidin; tannin; skin; flesh; wine; polysaccharide; grape; phloroglucinolysis; marc; gel permeation chromatography; molecular mass

INTRODUCTION

In grapes, proanthocyanidins (PAs) are present in the skins, seeds and flesh (*1*−*5*). The composition of the PAs is unique to the tissue in which they occur. In grape skins, PAs are more highly polymerized, and contain a higher proportion of epigallocatechin, and a small proportion of epicatechin-3-*O*-gallate (*3*). Seed PAs have a lower mean degree of polymerization (mDP) than those derived from skin, and have a relatively high proportion of epicatechin-3-*O*-gallate, both as a terminal and as an extension subunit (*4*). The localization of skin and seed PA has been shown to be either within the cytoplasmic compartment (vacuole) or associated with cell wall material (*6*−*8*). In the developing grape berry, PAs have been reported to be synthesized to a maximum amount near veraison (ripening), a process which is closely regulated by gene expression (*8*−*10*). After the maximum PA level is reached, the extractable amount of both skin- and seed-derived PA declines (*1*, *3*, *4*, *8*, *10*). For seeds, this may be either due to programmed oxidation (*4*) or due to increased association with cell wall components, rendering the PA nonextractable (*8*). The observed mDP of seed PA has been generally found to decrease during ripening, and in some cases to remain unchanged (*4*, *8*). For skin PA, however, reports of changes in mDP following acid catalysis in the presence of excess phloroglucinol (phloroglucinolysis (*2*)) have been variable, showing increases or decreases or remaining unchanged as ripening progressed (*3*, *8*, *10*). This variability in reported results may be due to changes in the extent to which PA can be cleaved into its component subunits, since their conversion declines during ripening (*2*, *3*). Factors which can modify PA molecules during grape ripening and limit their conversion by phloroglucinolysis include oxidation (*2*) and the incorporation of anthocyanin (*3*), both of which may vary according to the environmental conditions in which the grapes are grown. The information provided by phloroglucinolysis is limited because of incomplete conversion of the PA molecule to its constitutive subunits, and does not include information on the size distribution of the PA. For size distribution determination, gel permeation chromatography has provided an additional tool to characterize PA (*11*).

PAs have several important roles in wine including their ability to complex with other molecules, namely, anthocyanin, proteins and polysaccharides, and also because of their contribution of "astringency" and "mouthfeel" sensory attributes to wines (*12*−*17*). An increase in PA galloylation has been associated with the perception of "coarse" notes in wines, whereas this perception is reduced with an increase in the proportion of epigallocatechin (*17*). The incorporation of anthocyanin into PAs decreases perceived astringency (*18*). From these perspectives, PA extraction from grapes and structure modification during vinification will significantly affect the sensory properties of wine. Extraction of PA from the grape during fermentation and maceration is influenced by, among other factors, grape variety, ripeness, PA composition, the extent of berry crushing, skin contact time, enzyme addition, and by the ethanol content of the wine (*19*−*26*).

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Model extraction studies have shown that grape ripeness has a limited effect on PA concentration and composition, but does affect polymer size (*21*, *26*). The type of PA extracted during fermentation and maceration therefore has significant implications for wine composition in terms of its size distribution and subunit composition. In ethanol solution at or below 12%, PAs form colloid complexes which become less stable, leading to precipitation (*27*, *28*). From this, it could be inferred that as fermentation progresses and ethanol content in the solution increases, the stability of PAs in colloid complexes will increase. At lower ethanol concentration extracted PA can be lost due to self-association and precipitation, but generally this would be assumed to be low at low PA concentrations.

The ability of PAs to associate with grape-derived cell wall material (CWM) in suspension has recently been demonstrated, and may explain the observed change in PA concentration, composition and size distribution observed during fermentation and maceration (*12*). The loss of PA bound to insoluble CWM which is removed from solution during wine settling would first limit the final concentration of free PA in wine, but also potentially modulate the composition of PA remaining in solution due to the higher affinity of CWM for PAs of higher molecular mass (*12*).

The aim of the current study was to investigate the interaction of ripe grape and wine PAs with grape CWM in model solution to address two hypotheses: first, that the association of higher molecular mass (MM) PAs with skin CWM *in situ* limits their extraction during vinification and thus affects the MM distribution of PAs in wine; second, that suspended grape CWM has the capacity to selectively bind solubilized PAs in wine, thereby modulating wine PA composition. This study used a single grape system, tracking the yield and composition of PAs from grape to wine using gravimetric recovery, phloroglucinolysis and gel-permeation chromatography.

MATERIALS AND METHODS

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

Small-Scale Winemaking. *Vitis vinifera* L. cv. Shiraz grape samples were obtained at commercial ripeness (23 °Brix) from a vineyard in the McLaren Vale region of South Australia during the 2009 vintage as previously reported (*12*). Grapes were hand-plucked from bunches and divided into triplicate 1 kg lots, then sealed in individual snap-lock bags and crushed gently by hand. Grapes were then placed into 2 L Schott bottles, which were sealed and remained at room temperature until inoculation. Based on pH and yeast assimilable nitrogen (YAN) measures for the juice, no tartaric acid or nitrogen additions were required. *Saccharomyces cerevisiae* yeast from the AWRI culture collection No. 1537 (VIN 13, Anchor Foods, South Africa) was inoculated at 2×10^6 cfu/mL. Inoculated ferments were sealed with a bottle cap containing ports for sampling and for gas escape and then placed at 21 °C for the duration of fermentation. Gas escape tubes were immersed in a dilute solution of potassium metabisulphite (PMS: wine grade, Winequip, Magill, South Australia) to ensure that oxygen was excluded from the ferment while allowing CO_2 to escape. Ferments were mixed by gentle rolling three times a day to ensure fruit contact with the wine to allow extraction. Ferments were weighed daily to monitor fermentation progress by CO_2 loss and were sampled daily after mixing to determine total soluble solids (as °Brix) during the early stages of fermentation or sugar concentration using Clinitest tablets (Bayer Healthcare, Basel, Switzerland) at later stages of ferment, following manufacturer's instructions. Fermentations were pressed after 7 days using a small stainless steel screw press. The solid residue (marc) was retained and frozen at −20 °C. Following pressing, wines were kept at 21 °C until fermentation was complete: less than 2 g/L sugar by Clinitest tablets. Once fermentation was complete, wines were racked into smaller bottles with no ullage and cold settled at 4 °C for 14 days. Following this the finished wines were racked into bottles with no ullage. Analysis was completed on finished wines after 6 months in storage at 20 °C.

Preparation of Grape and Marc Cell Wall Material. Three fresh 200-berry samples from each winemaking replicate were collected and the juice was expressed by gentle pressing, to determine total juice yield. A further sample of destemmed grape berries was 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, seed and flesh components using a scalpel. During processing, the respective components were retained on ice, and otherwise kept frozen at −80 °C with liquid nitrogen to prevent sample degradation. Frozen flesh material was homogenized at 8000 rpm for 20 s in a Retsch Grindomix GM200 (Retsch GmbH & Co, Haan, Germany), and 400 mL of the slurry was immediately added to 400 mL of 40 mM HEPES pH 7 buffer (4 °C) and stirred for 15 min to remove buffer-soluble material as previously described (*12*). PA in the buffer extract was absent. The samples were then centrifuged twice at 8000*g* for 20 min at 4 °C, and the insoluble residue was retained. The buffer-extracted flesh material and untreated frozen skins and seeds were then extracted in 70% v/v acetone in a ratio of 1:4 (w/v) for 18 h as described previously (*12*). Marc from the three winemaking replicates was pooled, and then manually separated into pericarp and seed components. Although the pericarp consisted primarily of skin material, a small amount of material may have remained, and is hence not referred to as "skin". A subfraction of the marc pericarp material was extracted in a ratio of 1:4 (w/v) 70% v/v acetone for 18 h. Acetone-extracted residues of skin, flesh and marc pericarp were washed in additional 70% v/v acetone, followed by Milli-Q water (Millipore Corporation, Billerica, MA) and then lyophilized. CWM was prepared from the acetone-extracted skin and flesh residues using the extraction protocol described previously (*12*). 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 then lyophilized. Dry CWM was kept frozen at −20 °C until used.

Preparation of PA Fractions from Grape Tissues, Marc, and Wine. 70% v/v acetone extracts from skin, flesh, seed, and marc were concentrated under reduced pressure at 35 °C to remove acetone. A 20 mL wine sample of each replicate was concentrated to approximately 5 mL under a nitrogen stream. Concentrated grape extracts and wines were then made up to 50 mL with a final concentration of 60% v/v methanol containing 0.05% v/v trifluoroacetic acid (TFA) and then fractionated as previously described (*11*). Briefly, samples were applied (~18.3 mL/min) to a glass column (Michel-Miller, 300 mm × 21 mm, Vineland, NJ) containing Sephadex LH20 chromatography resin (Amersham, Uppsala, Sweden) to an approximate bed volume of 93 mL, and low molecular weight phenolics were eluted with 300 mL of 60% (v/v) HPLC grade methanol containing 0.05% v/v TFA. PA was then eluted with 250 mL of 70% v/v acetone containing 0.05% v/v TFA. The column was re-equilibrated with 60% v/v methanol containing 0.05% v/v TFA after each sample. The PA fractions eluted were concentrated under reduced pressure (35 °C) to remove organic solvent, and the aqueous fraction was then extracted with hexane to remove residual lipophilic material. Absence of monomeric phenolics, in particular anthocyanin, from the PA fractions was confirmed by HPLC (*29*). The aqueous fraction was then frozen in liquid nitrogen, lyophilized to a dry powder and weighed. Dried PA isolates were stored in the dark and under nitrogen at −20 °C prior to analysis. Isolated grape skin PA was further fractionated into 7 molecular mass (MM) subclasses (F1−F7) on Sephadex LH20 chromatography resin (Amersham, Uppsala, Sweden) according to the conditions outlined previously (*12*). An additional high MM PA isolate from preveraison skin tissue (cv. Pinot noir) was also prepared as previously described (*12*).

Acid Catalysis in the Presence of Excess Phloroglucinol (Phloroglucinolysis). Skin, flesh, seed, wine and marc PA isolates and skin molecular weight fractions were characterized by phloroglucinolysis (*2*) 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 concentration of PA of 5 g/L.

The phloroglucinolysis reaction was then carried out at 50 °C for 25 min, 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.

Binding Reaction of PA Isolates with CWM. Flesh and skin CWM were weighed into 1.5 mL centrifuge tubes in 6 mg and 13 mg quantities. CWM samples were then combined with PA isolates from either skin, seed, flesh or wine at 2 g/L containing 12% v/v ethanol and 0.01% v/v TFA, in a 1 mL reaction volume, and shaken for 1 h at 32 °C. An additional experiment was performed using 2 g/L skin PA fractions F4 and F7 with flesh and skin CWM in a 13 mg/mL final reaction volume, containing either 12%, 15% or 20% v/v ethanol with 0.01% v/v TFA. Each reaction was performed in duplicate. For each reaction, a PA standard blank of the respective PA combination without CWM was included 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 to monitor desorption of CWM-bound PA, if any. Following the binding reaction, samples were centrifuged at 16000*g* and the supernatant was 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). Recovered PA was then reconstituted in 100 μL of methanol, and then analyzed by phloroglucinolysis and GPC.

Direct Phloroglucinolysis of Insoluble Material from Marc, Skin, and Flesh. Phloroglucinolysis was performed directly on insoluble, lyophilized material from the acetone-extracted skin, flesh and marc (pericarp) material as well as purified CWM from skins and flesh. In a 20 mL volumetric flask vessel, 1.9 mL of a methanol solution containing 0.1 N HCl, 50 g/L phloroglucinol and 10 g/L ascorbic was added to 50 mg of insoluble residue or CWM, sealed and stirred for 25 min at 50 °C in a water bath. Analyses were performed in duplicate. After incubation, the reactions were stopped on ice, neutralized with sodium acetate (*2*) and stirred. An aliquot of the supernatant was removed, centrifuged at 16000*g* for 10 min and then analyzed by HPLC (*2*). The recovery of a spiked 1 mg/mL seed PA/CWM mixture from the direct phloroglucinolysis reaction was 97%, indicating that CWM did not affect the reaction efficiency with regard to PA depolymerization and adduct formation.

Gel Permeation Chromatography (GPC). The GPC method previously described (*11*) allowed for size distribution determination of the PA isolates. Preveraison skin PA fractions of known mDP (by phloroglucinolysis) were used as standards for calibration. For calibration, a second order polynomial was fit with the PA elution time at 50% for each standard (*12*). For analysis, PA samples in methanol were diluted with 4 volumes of the HPLC mobile phase. The maximum amount of PA injected onto the column was 40 μg.

Partitioning of PA Fractions in Octanol and Tris-HCl Buffer. 1 g/L solutions of skin PA fractions F4 and F7 were prepared in 10% v/v ethanol containing 0.1% v/v formic acid. A 50 μL aliquot was added to 450 μL of 20 mM Tris-HCl pH 7.4 buffer which had been presaturated with octanol. This was then covered with 500 μL of octanol which had been presaturated with Tris-HCL buffer. Each fraction was prepared in triplicate. The samples were vortexed, then gently shaken for 30 min at 22 °C and centrifuged for 5 min at 1500*g*. For the octanol and Tris-HCl phases, 300 μL of each was transferred to a Greiner 96 well microtiter plate (Interpath Services Ltd., West Heidelberg, Victoria, Australia). The absorbance at 280 nm of each aliquot was determined using a Spectramax M2 plate reader (Molecular Devices Ltd., Auburn, Victoria, Australia) using 300 μL of presaturated octanol or Tris-HCl buffer as the sample blank for the corresponding phase. The partition coefficient in octanol/water (buffer) was determined as the $\log_{10}$ of the ratio of 280 nm absorbance of octanol/Tris-HCl buffer for each PA fraction.

Crude Fractionation of CWM. A crude gravimetric analysis of the CWM composition was performed using an adaptation of the forage fiber analysis method (*30*). This allowed for the determination of broad categories of CWM components, namely, neutral-detergent-soluble fiber (NDSF), acid-detergent-soluble fiber (ADSF), cellulose and lignin. Pretreatment of CWM with amylase was not performed due to the negligible content of starch (*31*). Duplicate samples of 50 mg of skin or flesh CWM were treated with 1.5 mL of neutral-detergent solution (*30*) at 100 °C, shaking for 1 h for the determination of NDSF. The samples were then centrifuged at 16000*g* for 20 min, and the supernatant was discarded. The insoluble residue was washed 3 times in 1.5 mL of boiling water and 100% v/v acetone respectively. Samples were recentrifuged between washes, and care was taken to prevent loss of insoluble material during the transfer of supernatants. The insoluble neutral-detergent residue was dried at 100 °C for 18 h and then weighed. The residue was then crushed to a fine powder and a subportion treated with 1.5 mL of acid-detergent fiber solution for the determination of ADSF (*30*) and the same procedure followed as for NDSF. For the determination of cellulose and lignin, the procedure was repeated using 300 mg samples of skin and flesh CWM. The sequential NDSF−ADSF procedure was repeated as described without the NDSF drying step. The dried residue of this reaction was crushed to a powder and treated with 5 mL of 72% w/v sulfuric acid, shaking at 32 °C for 3 h. Following this, 45 mL of milli-Q water was added, and the sample was centrifuged at 20000*g* for 20 min and then washed twice in 50 mL of 70% (v/v) acetone followed by 50 mL of acetone. The insoluble residue was then dried at 100 °C for 18 h and weighed.

Table 1. Gravimetric Recovery, Subunit Composition and Molecular Mass of Acetone-Extractable PA from Ripe Grapes and the Corresponding Wines and Marc (Grape Pericarp)

	skin	flesh	seed	wine	marc
extractable PA (g/L)[a]	4.05 (54)	1.11 (15)	2.29 (30)	1.84 (25)	2.00 (27)
extension subunits[b]					
EGC−P	47.4	13.8	0.0	34.8	32.1
C−P + EC−P	44.4	71.6	64.0	49.7	56.1
ECG−P	5.0	7.6	15.4	3.8	6.4
terminal subunits[b]					
C	2.9	3.3	5.4	6.9	4.1
EC	0.0	1.3	6.9	3.7	0.0
ECG	0.2	2.4	8.3	1.0	1.3
% trihydroxylation	47.4	13.8	0.0	34.8	32.1
% galloylation	5.3	10.0	23.7	4.8	7.7
mDP (n)[c]	31.8	14.2	4.8	8.6	18.5
MM (g/mol)[d]	9741	4382	1583	2611	5678
MM by GPC 50% (g/mol)[e]	6491	5852	2902	2802	5897
mass conversion (%)	50	40	80	20	40

[a] PA concentration expressed as g per L wine yield; values in parentheses represent % of total extractable PA from whole grape berries. [b] Percent composition of PA subunits (in moles) with the following subunit abbreviations: (−P), phloroglucinol adduct of extension subunit; EGC, (−)-epigallocatechin; C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-*O*-gallate. [c] Mean degree of polymerization in epicatechin units. [d] Molecular mass as determined by phloroglucinolysis. [e] Molecular mass as determined by GPC at 50% PA elution.

RESULTS AND DISCUSSION

Recovery and Composition of Acetone-Extractable and Non-extractable PA from Grape Tissues, Grape Marc and Wine. Due to the poor mass conversion of PA extracted from ripe grapes and wine when analyzed by phloroglucinolysis (*3*), the recovery of acetone-extractable PA from grape tissues, wine and marc was determined gravimetrically (**Table 1**). Skin PA represented the largest proportion of extractable grape-derived PA at 54% w/w, followed by seed (30% w/w) and flesh (15% w/w). The composition of skin and seed PA was as expected from the literature (*2*−*4*) with skin PA containing epigallocatechin extension subunits, and having a higher mDP and proportionally lower galloylation than seed PA. Flesh PAs have been previously reported (*1*, *5*), but to the best of our knowledge, the characterization of flesh PA subunit composition has not. Flesh PA contained a higher proportion of galloylated subunits and a lower proportion of epigallocatechin extension subunits and had a lower mDP than skin PAs. It should be noted that although partial contamination of flesh PA by either skin or seed PA could have occurred during sample preparation, the buffer wash which preceded acetone extraction of flesh material indicated that negligible PA was present by HPLC, and therefore it is more likely that the current results provide flesh-specific PA information.

Table 2. Recovery and Subunit Composition of Acetone-Inextractable PA Determined by Direct Phloroglucinolysis on Grape and Marc Cell Wall Material

	skin CWM		flesh CWM		marc CWM
	ACE	PHE	ACE	PHE	ACE
inextractable PA (g/kg CWM)[a]	89.8	81.0	24.2	14.6	44.4
extension subunits[b]					
EGC−P	43.15	43.75	15.33	14.68	36.29
C−P + EC−P	48.72	49.14	77.79	79.49	55.83
ECG−P	6.19	5.63	4.03	3.32	5.23
terminal subunits[b]					
C	1.94	1.49	2.12	1.99	2.35
EC	0.00	0.00	0.00	0.00	0.00
ECG	0.00	0.00	0.73	0.52	0.29
% trihydroxylation	43.15	43.75	15.33	14.68	36.29
% galloylation	6.19	5.63	4.76	3.84	5.52
mDP (n)[c]	51.62	67.33	35.06	39.78	37.87
MM (g/mol)[d]	15827	20593	10517	11871	11530
mass conversion (%)[e]	8.98	8.10	2.42	1.46	4.44

[a] PA concentration expressed as g/kg of CWM extracted in acetone (ACE) or acetone + phenol (PHE). [b] Percent composition of inextractable PA subunits (in moles) with the following subunit abbreviations: (−P), phloroglucinol adduct of extension subunit; EGC, (−)-epigallocatechin; C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-*O*-gallate. [c] Mean degree of polymerization in epicatechin units. [d] Molecular mass as determined by phloroglucinolysis. [e] Mass conversion based on the recovery of PA subunits as g per 100 g CWM, also represents % PA of CWM.

The recovery of PA from wines indicated that 25% of the total grape-extractable PA were extracted from handpicked grapes after gentle hand crushing and small-scale fermentation (**Table 1**). Subunit composition of the wine PA showed that it was similar in composition to skin PA, having a higher proportion of epigallocatechin extension units and a lower galloylation than seed PA. However, this does not exclude the possibility that some wine PA may have been derived from flesh and seed, albeit proportionally smaller. By comparison with skin PA, the mDP of wine PA as determined by phloroglucinolysis and GPC was significantly lower. This result indicates that a significant proportion of the higher MM skin PA was either not extracted during fermentation or, if extracted, was removed from solution. Analysis of the PA from marc pericarp showed that 27% of total acetone-extractable grape PA remained in the pericarp marc following fermentation, which excludes that from seed marc, or potentially lost as lees which were not determined. The composition of extractable marc pericarp PA had an mDP, and % epigallocatechin intermediate between that of extractable skin and flesh PA.

The content and composition of nonextractable grape skin, flesh and marc PA was determined by direct phloroglucinolysis on acetone-extracted CWM or purified CWM (**Table 2**). Acetone-extracted skin CWM had a higher proportion of nonextractable PA than that from flesh, at 8.1% and 1.4% by dry mass respectively. Comparison of nonextractable PA before and after extraction of CWM with phenol showed that a small amount of nonextractable PA was lost during the purification of CWM, and was most likely PA that was associated with cytoplasmic protein. In terms of the composition of extension subunits, the nonextractable PA from skin and flesh was similar to the extractable PA from the corresponding source (**Tables 1**, **2**). However, the mDP determined by phloroglucinolysis was significantly higher in the nonextractable PA, by 39% and 60% for acetone-extracted skin and flesh CWM respectively (data not shown). This may indicate that the PA which remains nonextractable is of a higher mDP, but may also be due to a reduced efficiency of the phloroglucinolysis reaction to cleave terminal subunits bound to CWM, or unknown "terminal subunits". The latter possibility may be as a result of covalent bonding between PA and CWM, the detailed structure of which is as yet an unknown. The composition of the nonextractable PA in acetone-extracted marc CWM had a subunit composition and mDP intermediate between that of skin and flesh. Since the marc pericarp CWM would be derived from both skin and flesh components, this would be expected. The similarity of the nonextractable PA composition in this material to that from acetone-extracted skin and flesh CWM indicates that the fermentation process did not significantly alter desorption of PA from CWM.

Interaction between Grape- and Wine-Derived PA with Flesh and Skin CWM. PA from skin, seed, flesh and wine were combined with purified flesh and skin CWM in model suspension to determine the effect of the interaction in terms of subunit composition and MM distribution. A similar study was reported previously using the same flesh and skin CWM, but using preveraison seed and skin PA instead of that isolated from ripe grapes (*12*). That initial study made use of PA which had a high mass conversion, which allowed an accurate measure of PA recovered before and after reaction with CWM by phloroglucinolysis. For the current study, it should be noted that apart from seed PA, the mass conversion for the ripe grape PA was low (**Table 1**). The interpretation of the results in the current study is more limited due to a reduction in known PA-containing material by mass.

CWM Effect on Ripe Seed PA Subunit Composition and Molecular Mass Distribution. The interaction of ripe grape seed PA with CWM produced very similar results to that previously reported for preveraison seed PA (*12*). Compared to skin CWM, flesh CWM had a higher affinity for seed PA, binding 44% and 25% after a 13 mg/mL and 6 mg/mL CWM addition respectively (**Figure 1 A**), whereas skin CWM removed 30% and 17% seed PA after a 13 mg/mL and 6 mg/mL CWM addition respectively. Consistent with the previous study using preveraison seed PA (*12*), the effect of CWM addition on seed PA mDP by phloroglucinolysis was minor, and was reduced a maximum of 10% by a 13 mg/mL addition of flesh CWM. There was a small effect of CWM addition on the % galloylation of seed PA, which decreased slightly following reaction with flesh and skin CWM. The effect of CWM addition on seed PA elution profile and cumulative MM distribution was studied by GPC (**Figure 2 A**,**B**). For the addition of both CWM types there was a preferential removal of seed PA across both the intermediate and high MM range, but below 50% elution the effect was minor for most of the CWM treatments, except for the addition of 13 mg/mL of flesh CWM. That treatment caused a significant shift in the MM distribution toward a lower average MM (at 50% elution). This would account for the small change in mDP observed by phloroglucinolysis. The preferential removal of the higher MM range PA in seed PAs is in agreement with previous work (*12*).

CWM Effect on Ripe Skin and Flesh PA Subunit Composition and Molecular Mass Distribution. The interaction of grape skin PA with flesh CWM showed the highest affinity of the PA samples studied (**Figure 1 B**) with 78% and 56% bound by 13 mg/mL and 6 mg/L flesh CWM solutions respectively. For the addition of skin PA to skin CWM, the effect was smaller, with 52% and 32% bound by 13 mg/mL and 6 mg/mL skin CWM respectively. The magnitude of the effect for both flesh and skin CWM is greater than that reported previously for the reaction with preveraison skin PA (*12*). Examination of the effect of CWM addition on the phloroglucinolysis mDP of the skin PA samples showed that both were significantly reduced by flesh CWM, but this was minimal for skin CWM addition despite a significant loss of PA material in the latter. This result differed from that previously reported for preveraison skin PA with the same source of skin CWM (*12*). For both flesh and skin CWM addition, the effect on subunit composition in terms of the proportion of galloylation or epigallocatechin was minor.

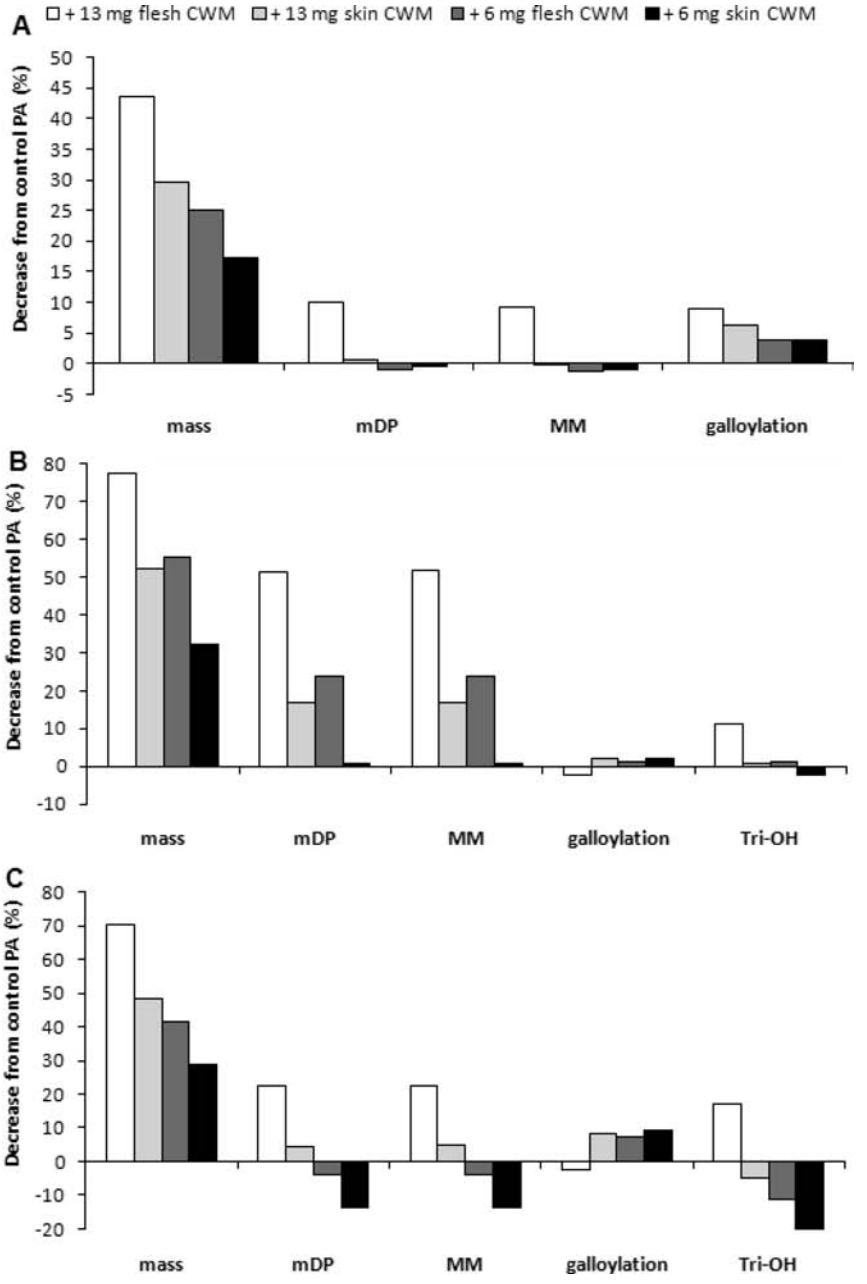

Figure 1. PA recovery following reaction of 2 mg/mL (**A**) seed, (**B**) skin and (**C**) flesh PA with either flesh or skin CWM at 6 mg/mL or 13 mg/mL. Histograms show the % difference in PA mass, mean degree of polymerization (mDP), molecular mass (MM), % epicatechin-3-*O*-gallate (galloylation) or % epigallocatechin (Tri-OH) from the control, determined by phloroglucinolysis ($N = 2$; SD <5% for all samples).

The unexpected response observed for the interaction of skin PA with CWM was further explored using GPC (**Figure 2 C**,**D**) and revealed that for flesh CWM addition that the response was as expected according to previous findings using preveraison skin PA (*12*), with PA of higher MM being preferentially removed from solution by CWM. However, for the addition of skin CWM, it was evident that for the highest MM material, skin PA had a poor affinity for a 13 mg/mL skin CWM addition and no affinity at a 6 mg/mL addition. Conversely, the significant loss of skin PA from solution, albeit greater than that observed for seed PA (**Figure 1 A**,**B**), was preferentially in the lower MM range. As a result of this, rather than showing a shift of the MM distribution toward a lower average MM as observed for flesh CWM addition (**Figure 2 D**), the MM distribution was shifted toward a higher average MM by skin CWM addition. This observation was unexpected based on previous work with preveraison skin PA (*12*), and a detailed discussion will follow in this paper.

The addition of flesh PA to either flesh or skin CWM showed similarity to the effect observed for skin PA, in that there was a poor affinity for higher MM PA with addition of 6 mg/mL or 13 mg/mL skin CWM (**Figure 2 E**,**F**). It is interesting to note that this effect was also observed for a 6 mg/mL addition of flesh CWM. As such, the flesh PA MM distribution was changed little by CWM addition, apart from when 13 mg/mL flesh CWM was added and significantly removed PA from the higher MM range. By observing the changes in subunit composition (**Figure 1 C**), a high affinity for flesh-derived PA by both flesh and skin CWM was observed with 70% and 50% removed respectively for a 13 mg/mL CWM addition. Despite this high affinity, changes in mDP were smaller, decreasing 22% after a 13 mg/mL flesh CWM addition, unchanged by either 6 mg/mL flesh CWM or 13 mg/mL

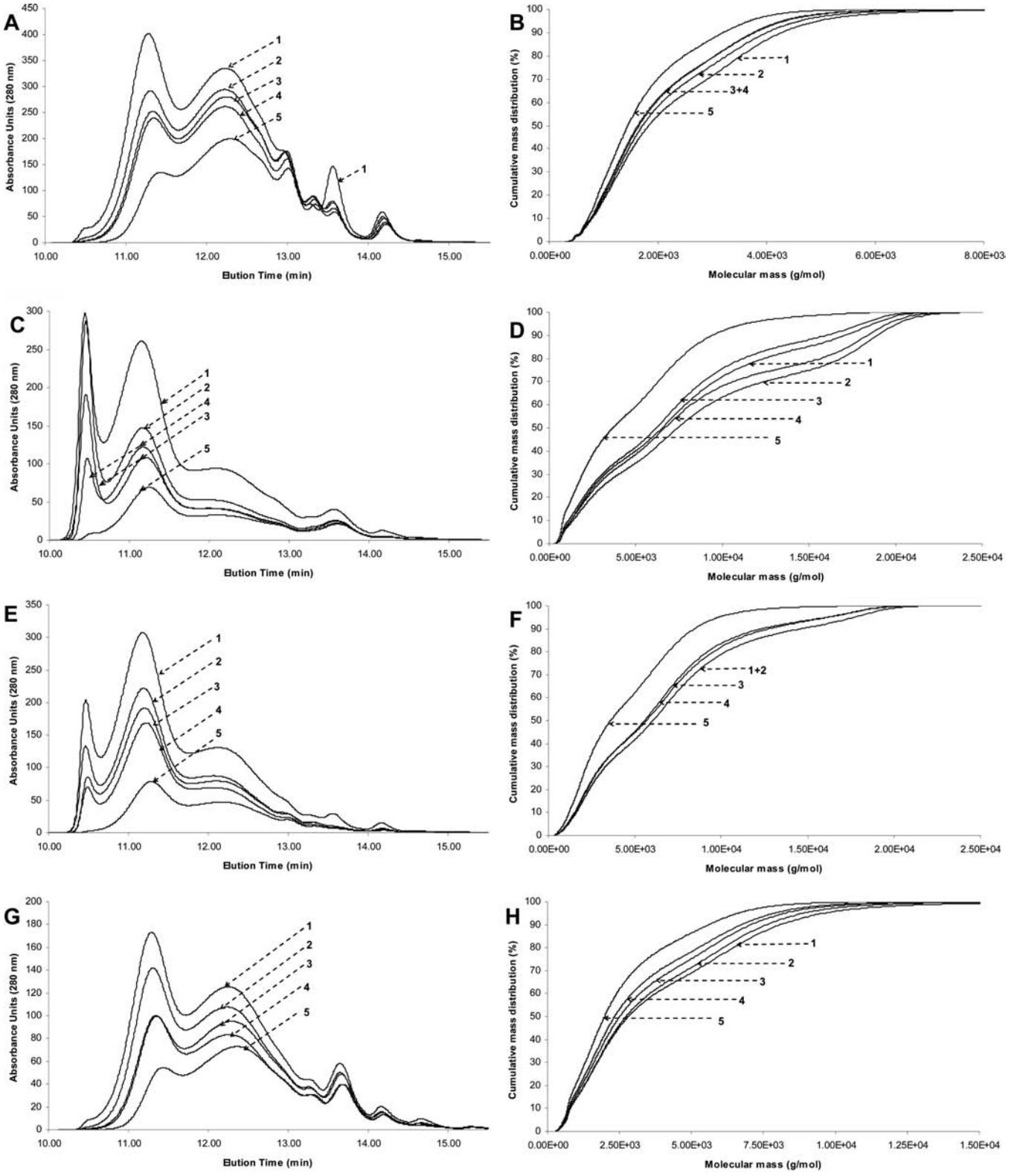

Figure 2. Analysis of PA before and after addition of CWM as elution profile (EP) and cumulative mass distribution (CD) by gel permeation chromatography. (**A**) EP of seed PA; (**B**) CD of seed PA; (**C**) EP of skin PA; (**D**) CD of skin PA; (**E**) EP of flesh PA; (**F**) CD of flesh PA; (**G**) EP of wine PA; (**H**) CD of wine PA. Plots represent untreated control (1) and treatment with 6 mg/mL skin CWM (2), 6 mg/mL flesh CWM (3), 13 mg/mL skin CWM (4) and 13 mg/mL flesh CWM (5).

skin CWM addition; and increased 14% with 6 mg/mL skin CWM addition. The proportion of epigallocatechin changed in a similar manner to the change in mDP, with a reduction in the proportion of epigallocatechin with a 13 mg/mL flesh CWM addition (**Figure 1 C**).

Wine PA Subunit Composition and Molecular Mass Distribution. The MM distribution of the wine PA was similar to that for seed PA, showing an absence of the higher MM PAs present in skin and flesh PA, hence having a narrower MM range (**Figure 2 G**,**H**). The subunit composition of wine PA following reaction with

Table 3. Subunit Composition, Molecular Mass and Partition Coefficients in Octanol/Water of Skin PA Fractions Isolated from Ripe and Preveraison Grape Material

	ripe skin PA		preveraison skin PA
	F4	F7	PV7
extension subunits[a]			
EGC-P	32.91	50.60	29.09
C−P + EC−P	53.86	42.95	65.46
ECG−P	6.44	4.60	2.14
terminal subunits[a]			
C	5.71	1.76	3.18
EC	0.67	0.00	0.00
ECG	0.40	0.09	0.13
% trihydroxylation	32.91	50.60	29.10
% galloylation	6.84	4.69	2.30
mDP (n)[b]	14.74	53.99	30.15
MM by subunit (g/mol)[c]	4511	16493	8996
MM by GPC 50% (g/mol)[d]	6752	15755	8996
mass conversion (%)	44.16	53.14	75.00
log $P_{oct/wat}$[e]	−1.13	−0.89	nd

[a] Percent composition of PA subunits (in moles) with the following subunit abbreviations: (−P), phloroglucinol adduct of extension subunit; EGC, (−)-epigallocatechin; C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-*O*-gallate. [b] Mean degree of polymerization in epicatechin units. [c] Molecular mass as determined by phloroglucinolysis. [d] Molecular mass as determined by GPC at 50% PA elution. [e] Partition coefficient of PA in buffered octanol and water determined by spectrophotometric absorbance at 280 nm; nd = not determined.

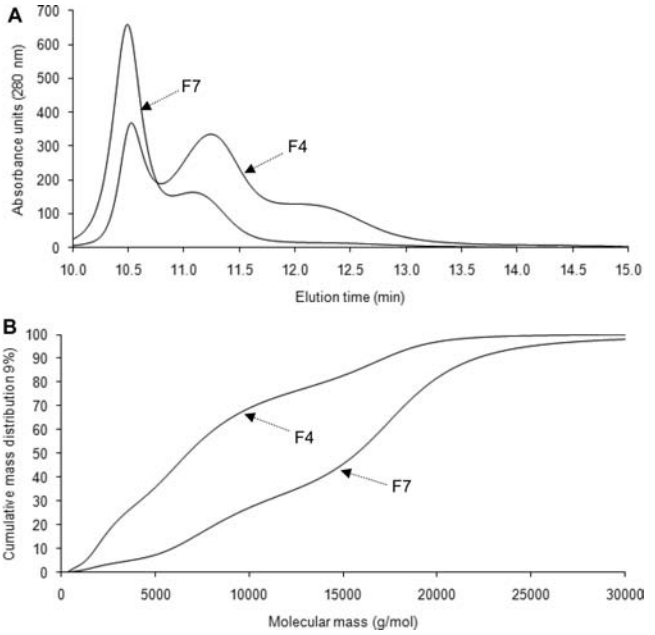

Figure 3. Analysis of skin PA fractions F4 and F7 before and after addition of CWM as (**A**) elution profile and (**B**) cumulative mass distribution by gel permeation chromatography.

CWM is not reported in detail, due to its low mass conversion (**Table 1**). However, an assay of the recovery of wine PA after reaction with CWM showed a high affinity for flesh CWM, with a 13 mg/mL and 6 mg/mL addition removing 58% and 29% of PA respectively (data not shown). As with grape-derived PA samples, wine PA showed a lower affinity for skin CWM with a 13 mg/mL and 6 mg/mL addition removing 32% and 7% of PA from solution respectively (data not shown). A reduction in wine PA MM distribution was observed following the addition of both types of CWM. However, the reduction in MM distribution was minimal for the 6 mg/mL skin CWM treatment. The binding study involving wine PA with CWM is of interest, in that it demonstrates a lack of higher MM PA material transferred from grape to wine. This reiterates the earlier statement that this material either is poorly extracted or, if extracted, is removed from solution by suspended CWM during vinification. Furthermore, the poor affinity of higher MM skin and flesh PAs for skin CWM demonstrated here provides some evidence that binding to the cell wall may not limit the extraction of this material during winemaking, except that the cell wall represents a physical barrier to diffusion. These findings suggest that grape flesh CWM, suspended during fermentation, may significantly alter the MM and compositional properties of extracted PA during winemaking. Second, this study demonstrates that the addition of skin and flesh CWM to wine PA has the ability to selectively remove some PAs, which may be of interest as a fining agent to alter the sensory properties of wine PA as previously suggested (*32*).

Comparison of Skin PA Fractions Differing in Molecular Size on the Interaction with CWM. We reported in a previous study the increasing affinity of higher MM preveraison skin PA for skin and flesh CWM (*12*). From that study, the preveraison skin PA used had an MM of 6401 g/mol at 50% elution and 15474 g/mol at 90% elution. For the skin PA isolated in the present study, the MM was 6491 g/mol at 50% elution and 17119 g/mol at 90% elution, (**Figure 2 D**). The ripe skin PA sample isolated in this study therefore has a similar average MM to the preveraison PA material, but a higher proportion of high MM material. In order to better understand the relatively low affinity of ripe skin PA for skin CWM, the binding experiments were repeated using subfractions of skin PA which were distinct in terms of their composition and MM distribution (**Table 3**, **Figure 3**). Two distinct skin PA fractions F4 and F7 from ripe grapes were selected for the experiment, having an MM of 6752 g/mol and 15755 g/mol at 50% elution respectively. F4 and F7 had low octanol/water partition coefficients, being essentially hydrophilic in nature though the F7 fraction was slightly more hydrophobic. A high MM preveraison skin PA (PV7) (**Table 3**) which was isolated in a previous study (*12*) was included for comparison with the ripe skin PA fractions. When compared to PV7 and from a size distribution standpoint, it is evident that F7 had a higher proportion of high MM material. The elution profiles of F7 and PV7 are shown in **Figure 4**, together with their respective responses to skin CWM addition at 13 mg/mL. It is evident that, for the reaction of F7 from ripe grapes with skin CWM, the earlier-eluting, higher MM material is poorly removed, while for PV7, the expected pattern was followed with the higher MM material being preferentially removed. This result supports the observation of the interaction of whole ripe skin PA with skin CWM, implying that a poor affinity for CWM exists in the higher MM range.

The experiment was repeated, with the addition of ethanol from the original concentration at 12% (v/v) to 15% and 20% (v/v) respectively. The aim of this experiment was 2-fold. First, the addition of ethanol decreases hydrophobic interactions, which have been shown to drive the effectiveness of the interaction of CWM higher in pectin (*34*, *35*). Second, the possibility for self-association of higher MM PA could occur at lower ethanol concentration, although it has not been conclusively demonstrated for grape PA (*27*, *28*). A higher ethanol concentration might limit the self-association of PA molecules, thus increasing their solubility and association with CWM. The average MM of the F4 and F7 fractions before and after fining with skin or flesh CWM at different ethanol concentrations are shown in **Figure 5A**. The significance of the differences in PA MM at 50% elution following CWM addition is shown in **Figure 5B**. It is evident that F7 had a higher affinity for flesh CWM than F4, and that for both, the effectiveness of the interaction was reduced by ethanol.

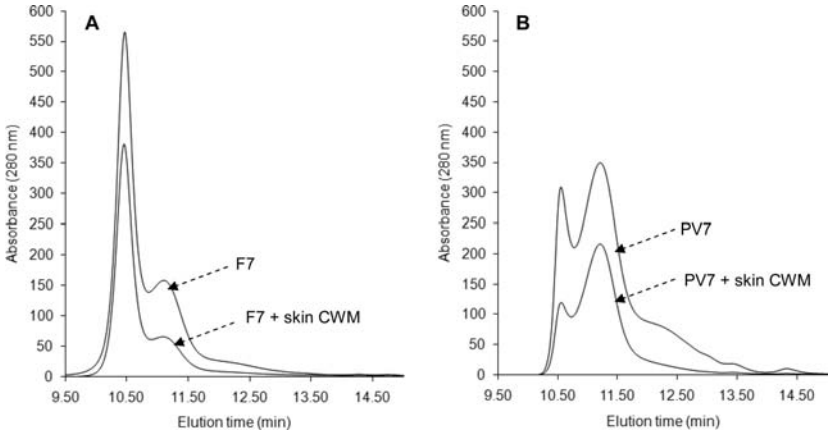

Figure 4. Elution profiles of 2 mg/L (**A**) ripe skin PA fraction F7 and (**B**) preveraison skin PA fraction PV7 before and after addition of 13 mg/mL skin CWM determined by gel permeation chromatography.

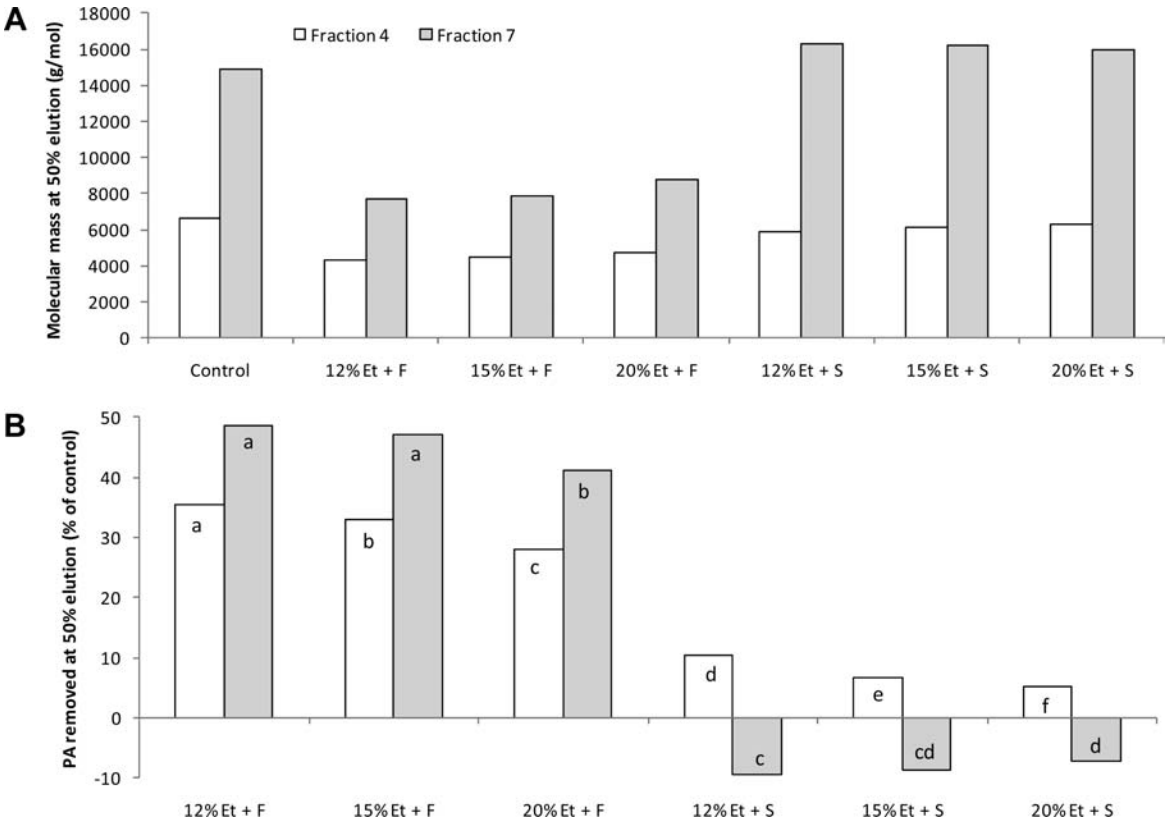

Figure 5. Change in skin PA fractions F4 and F7 following a reaction of 2 mg/mL PA with flesh (F) or skin (S) cell wall material with addition of 12, 15 or 20% ethanol (Et) determined by gel permeation chromatography. (**A**) PA molecular mass at 50% elution. (**B**) % difference in PA molecular mass at 50% elution after reaction with cell wall material (ANOVA: $P < 0.0001$; posthoc Student's t test; $N = 2$; different letters in the histogram bars of F4 or F7 indicate significant differences within each fraction).

For the interaction with skin CWM, the average MM of the PA sample was decreased after reaction with F4 and increased after reaction with F7. In this interaction, ethanol addition decreased the effectiveness of the association of F4 with skin CWM, but did not for F7. This result would suggest that, for the lower MM fraction F4, hydrophobic interaction contributes significantly to the interaction with both flesh and skin CWM, but for F7 evidence for hydrophobic interaction was evident with flesh CWM only. It appears that, for F7, the hydrophobic interaction with skin CWM may be limited.

Effect of Cell Wall Composition on Affinity for PA. To interpret the findings reported in this study, an understanding of the nature of CWM structure in the interaction with PA is necessary. In previous work (*34*, *35*), the importance of CWM structure and composition was demonstrated, with higher affinities for PA demonstrated for covalently-linked pectin over xyloglucan and cellulose. It was proposed that the structure of CWM, either flexible/folded or rigid/impervious, allowed either for bonding of PA within hydrophobic cavities created by folding in pectin or a weaker surface interaction with limited binding sites on cellulose. For pectin and xyloglucan, greater affinity for PA was found as PA MM increased, but this was not the case for cellulose (*34*). The importance of the porous nature of CWM in the interaction with PA was demonstrated in a study on CWM drying, such that harsh drying altered the 3-dimensional structure of the polysaccharide, rendering it impervious to PA and having a reduced affinity (*36*).

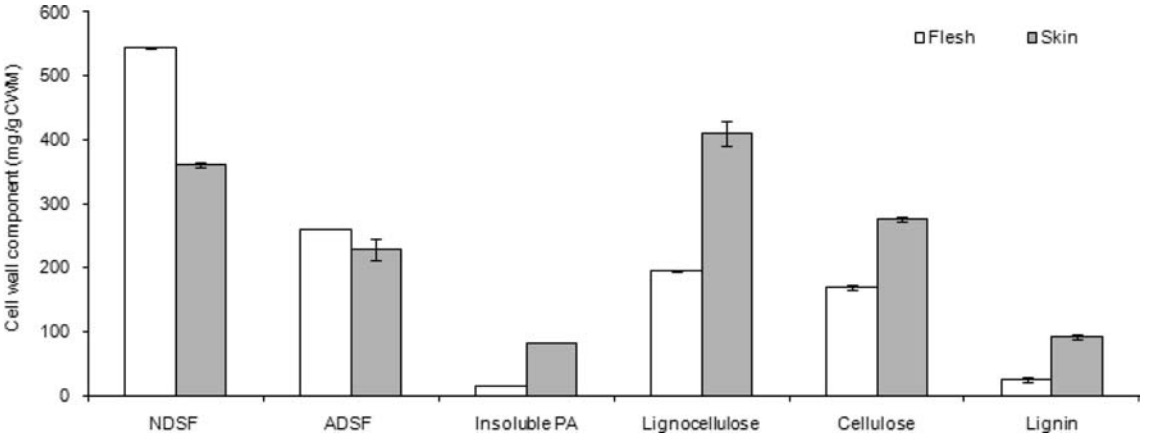

Figure 6. Compositional information of fractionated flesh and skin cell wall material. NDSF = neutral detergent soluble fiber; ADSF = acid detergent soluble fiber; insoluble PA was determined by direct phloroglucinolysis on cell wall material; lignocellulose represents the sum of cellulose + lignin; lignin may contain contamination of cutin or PA ($N = 2$; data points are the mean ± SE).

For *V. vinifera* cv. Shiraz, studies on CWM structure and composition are limited and have generally been on the neutral and acidic sugar composition of skin polysaccharides (*5*, *37*−*39*). A single study which compared flesh and skin CWM for Shiraz grapes revealed that, for neutral sugar composition, content of uronic acids and protein, and the degree of methylation, the two CWM sources were similar (*5*). The current study undertook to more broadly distinguish the CWM composition isolated from flesh and skin, and also to incorporate an estimate of the degree of CWM lignification. Little data exists for the degree of lignification of grape tissues. Some have reported a lignin/cutin content of up to 29% (w/v) in grape pomace, which has been proposed to contain high amounts of hydrolysis-resistant protein and nonextractable PA (*31*, *40*, *41*). A detailed study of Shiraz grape skins (*37*) indicated that the lignin content of CWM was as high as 45% w/w, with 49% w/w being monocarbohydrates. A discrepancy between the hydrolysis agent used, either TFA or HCl, gave the composition of cellulose at either 10% or 50% of monocarbohydrate respectively, and was thus inconclusive. In the current study, a crude fractionation method which has been extensively used for the characterization of forage fiber digestibility (*30*) was used. The NDSF component is represented primarily by insoluble CWM-bound pectins and CWM-bound protein (*42*), since most cytoplasmic protein would have been removed in the phenol extraction step of CWM preparation (*43*). NDSF was higher in the grape flesh CWM than the skin CWM (**Figure 6**). The ADSF component consists primarily of hemicelluloses, although some cellulose may have been hydrolyzed under the experimental conditions, and this was equivalent for flesh and skin CWM. Lignocellulose, the residue of CWM following the removal of ADSF, was significantly higher in skin CWM than flesh, as were cellulose and lignin respectively. The estimates of lignin and cellulose in the skin CWM were lower than previously reported (*37*), although it should be noted that the methods used for the CWM analysis differed. Although preliminary, the data confirm that significant compositional differences between flesh and skin CWM exist which may confer distinct structural characteristics to each. The higher proportion of pectin, and lower cellulose and lignin content in flesh CWM may impart a higher flexibility to the material, allowing for a higher contact surface area for interaction with higher MM PA (*34*, *35*). However, the higher proportion of cellulose and lignin in skin CWM may represent a greater structural rigidity, allowing for only a surface-mediated interaction with high MM PA. In this case, saturation of the available binding sites on the CWM surface might occur, while smaller PA molecules might still penetrate the interior of the CWM structure. This is demonstrated in the current study in that the addition of skin CWM to skin PA allowed some binding at 13 mg/mL in the higher MM range, but at 6 mg/mL this was absent. A further factor which might reduce the availability of binding sites for PA on the CWM surface is the presence of nonextractable PA (**Table 2**, **Figure 6**). Although the data reported in this study does not account for the possibility of covalently bound PA subunits to CWM, it has shown that a significant proportion of high MM PA remains bound to CWM after extraction in 70% (v/v) acetone. The presence of bound PA on the surface of the skin CWM may therefore further reduce its affinity for PA.

Implications of Grape CWM−PA Interaction for Vinification. A recent review of literature on grape PA extractability has drawn attention to the potential implications of the CWM−PA interaction in removing free PA in wine during the fermentation process (*32*, *44*). Model experiments have verified that CWM, especially flesh-derived CWM, can remove a significant portion of PA from the wine during vinification (*12*). In a wine system, the observed increase in wine PA concentration over time is the result of a complex sequence of events (**Figure 7**). The bulk of extractable PA in grape tissues is present in the cytoplasmic compartment, most likely as vacuolar inclusions (*6*). When representing the path of movement of PA out of the vacuole as a rate constant k_E (**Figure 7**), it will be determined by the concentration of vacuolar PA and the concentration of PA and other solutes in the surrounding medium which designates the concentration gradient. Limitations to k_E will be conferred by the permeability of the cell wall, the higher cellular structure of the organ, and the ability of PA to adsorb to cell walls and possibly other cell contents, e.g., cytoplasmic protein (k_A). The point of adsorption could be either intracellular or extracellular. The possibility that adsorbed PA may desorb is accounted for as a desorption constant (k_D). Apart from the obvious factors which would influence the extraction process such as wine ethanol concentration or PA concentration, which have already been mentioned, various factors in vinification may influence the rate constants defined here. For example, the degree of berry crushing has been shown to significantly alter skin PA extraction (*23*) such that k_E and the final PA concentration increases. However, the profile of extraction is not affected by the crushing and, regardless of the total skin PA extracted, has been shown to reach a plateau over which no further extraction occurs. The authors suggest that this may reflect continued adsorption of extracted PA to suspended CWM, and possibly yeast cells (*23*). Berry crushing may therefore

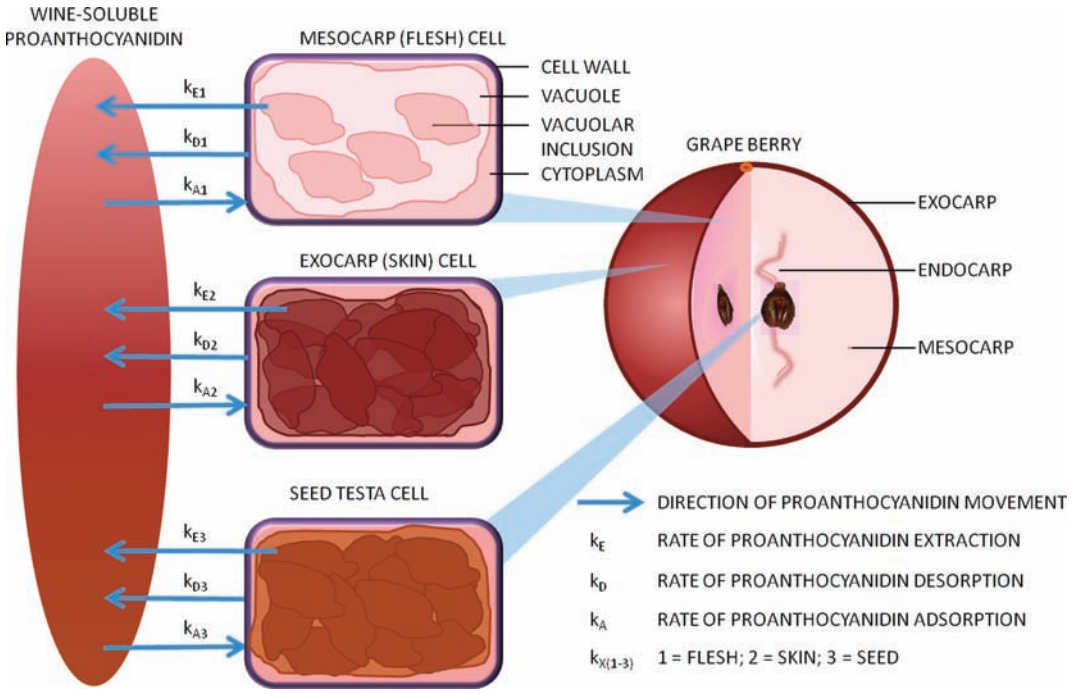

Figure 7. Model of proanthocyanidin extraction, adsorption and desorption during vinification.

affect initial k_E but due to maceration during fermentation may not affect the final release of CWM into suspension, thus k_A remains unchanged. However, a saturation of available binding sites on suspended CWM with the PA in solution may exist, such that k_A becomes static at a certain point during fermentation. There are many additional variables that are relevant from a winemaking standpoint but have not been investigated in the context of this study including, for example, fermentation time and temperature, enzymes, and yeast strain.

We have shown that flesh CWM has high selectivity for higher MM PA, however ripe skin CWM has shown poor affinity for very high MM skin PA. This means that although skin CWM may not adsorb high MM PA, it would be adsorbed by suspended flesh CWM. It is not clear from the literature as to the extent of extraction of higher MM PAs during fermentation. To propose a rate constant k_D to the model in **Figure 7** is therefore more complex. Flesh PA has been shown to be present at a significant concentration in this study, but it is most likely not completely extracted at the lower ethanol concentrations in wine due to its affinity for CWM in 12% ethanol, such that k_D would be relatively small. Studies on grape PA composition have used strong solvents such as 70% acetone, and may therefore not represent what is extractable from the whole tissue during fermentation and maceration (*3*, *10*−*12*). A proportion of higher MM skin PA remains bound to skin CWM (*6*), but this may partially be released with acetone which disrupts hydrogen bonding. The current study has shown that higher MM skin PAs are absent in wine and although they poorly associate with skin CWM, KD for release of skin PA from skin CWM may be low. Further research will reveal whether at wine ethanol concentration higher MM PAs are in fact desorbed from skin CWM. The rate constant k_D can also be applied to PA which adsorbs to CWM during vinification, and may later be partially desorbed as ethanol concentration in the wine increases. A further important point is that little is known about the structural modification of PA, notably skin PA during grape ripening. The current study has compared the binding properties of preveraison and ripe skin PA, and made comparisons based on subunit composition and MM. However, other modifications of PA, whether from oxidation or incorporation of non-PA material, may alter both the reactive sites available for binding and the structural conformation and dimensions of the PA molecule. This may affect k_E, k_A and k_D.

In summary, further research is needed to better understand the underlying relationship between CWM structure and their selection affinity for specific PAs, given that the composition of both grape skin and flesh CWM changes significantly during ripening, namely with a loss of structural rigidity and degradation (solubilization) and deesterification of pectin (*39*). A significant area of future work will be an examination of the effect of grape ripeness on CWM composition and affinity for PA, and to more completely understand k_E in relation to k_A and k_D. In light of the current results, differences in CWM structure may alter the interaction with PA, and modulate the PA composition of the associated wines. The implications of the current study suggest that CWM has the potential to regulate the mouthfeel properties of wine PA.

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