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ARTICLE

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Ripening-Induced Changes in Grape Skin Proanthocyanidins Modify Their Interaction with Cell Walls

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Supporting Information

ABSTRACT: Proanthocyanidins were isolated from the skins of Cabernet Sauvignon grapes at different stages of grape development in order to study the effect of proanthocyanidin modification on the interaction with grape cell wall material. After veraison, the degree of proanthocyanidin polymerization increased, and thereafter was variable between 24 and 33 subunits as ripening progressed. Affinity of skin cell wall material for proanthocyanidin decreased with proanthocyanidin ripeness following veraison. A significant negative relationship ($R^2 = 0.93$) was found for average proanthocyanidin molecular mass and the proportion of high molecular mass proanthocyanidin adsorbed by skin cell wall material. This indicated that as proanthocyanidin polymerization increased, the affinity of a component of high molecular mass proanthocyanidins for skin cell wall material declined. This phenomenon was only associated with skin proanthocyanidins from colored grapes, as high molecular mass proanthocyanidins of equivalent subunit composition from colorless mutant Cabernet Sauvignon grapes had a higher affinity for skin cell wall material.

KEYWORDS: proanthocyanidin, anthocyanin, tannin, color, grape, ripening, skin, flesh, cell wall, polysaccharide, phloroglucinolysis, gel permeation chromatography, molecular mass

INTRODUCTION

Grape berries accumulate significant quantities of proanthocyanidins (condensed tannins), which are phenolic polymers consisting of flavan-3-ol subunits, primarily epicatechin as an extension subunit and catechin as a terminal subunit.[1−3] Grape skin proanthocyanidins differ from those derived either from the seed or from the flesh in the proportional composition of their constitutive subunits, being richer in epigallocatechin (prodelphinidin) as an extension subunit, and containing smaller amounts of (−)-epicatechin-3-*O*-gallate as either a terminal or extension subunit.[2−4] A further distinguishing characteristic of grape skin proanthocyanidins is their average molecular mass or mean degree of polymerization (mDP), which is significantly higher than that found in seeds. Proanthocyanidins are heterogeneous biomolecules, and exist in a diverse range of mDPs from small oligomers to large polymers, which in the case of skin proanthocyanidin can be greater than 70 subunits.[4,5] This heterogeneity imparts diverse, size-dependent chemical properties to proanthocyanidins.[4,6]

In general, research has shown that gene expression for the biosynthesis of flavan-3-ol precursors to proanthocyanidins is active up until the onset of ripening (veraison), and is down-regulated by the hormone abscisic acid (ABA) which signals the onset of veraison.[7−9] In accordance with these observations, some studies have found that skin proanthocyanidin content in grape berries declines during ripening.[9−11] However, based on a range of literature sources and analytical methodologies, there is little consensus on the timing of and progression of skin proanthocyanidin accumulation through grape ripening, which has been reported to increase, decrease or remain constant.[9−14] Furthermore, information on changes in the degree of skin proanthocyanidin polymerization during berry development is inconsistent, reporting increases or decreases with the progression of ripening or in response to environmental phenomena.[2,10−13] The potential association of grape proanthocyanidins with other biomolecules such as polysaccharides,[4,6,14,15] their modification by oxidation[16] or subcellular partitioning[17−19] may partially account for variability in the reported data, since this could decrease the extent to which proanthocyanidin is cleaved to its subunits for compositional analysis. Hence, the information provided by subunit composition following acid catalysis alone[9−11] is limited by incomplete conversion of the proanthocyanidin molecule, and does not provide information on the relative size classes of the heterogeneous proanthocyanidin polymer. For resolution of proanthocyanidin molecular size distribution, gel permeation chromatography has provided an additional tool.[20]

To date, while the biosynthesis of flavan-3-ol precursors to proanthocyanidins has been studied in grapes, little is known of the subcellular site of, and the mechanisms which facilitate, their polymerization in skin cells. The localization of grape proanthocyanidins has been shown to be either within the cytoplasmic compartment (vacuole), or associated with cell wall material.[17−19]

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Skin proanthocyanidins in the vacuole are thought to be less polymerized than those associated with the cell wall, with their subcellular organization shifting from the vacuole to the cell wall during the later stages of berry ripening.[17] In studies on other plant species it has been proposed that this could be mediated by vesicle trafficking, in which proanthocyanidins are transported via vesicles from the vacuole to the cell wall, later fusing with it.[21]

The mechanism of proanthocyanidin polymerization is unknown, and could occur in the vacuole under the acidic conditions induced by tonoplast proton pumps (H^+-ATPases).[21,22] Following transport of glycosylated flavan-3-ol precursors from the cytoplasm through the tonoplast,[23] glucose is cleaved and the subsequent condensation of a flavan-3-ol terminal subunit to a quinone methide or protonated carbocation form would occur under acidic pH, yielding a proanthocyanidin molecule.[24,25]

While pigmentation of proanthocyanidin conjugates may represent an artifact of extraction and processing, research has shown that red pigmentation of skin proanthocyanidin isolates increases with the progression of ripening.[13] Furthermore, it has recently been demonstrated that anthocyanin and proanthocyanidin can occur simultaneously within subvacuolar compartments of cultured grape cells.[19] While not conclusively demonstrating that anthocyanins could be incorporated as proanthocyanidin subunits *in situ*, it does allow for the question of proanthocyanidin polymerization to be revisited, especially in a system such as red grapes where anthocyanin is in excess of other flavonoids. Direct condensation of anthocyanin-proanthocyanidin or proanthocyanidin-anthocyanin could facilitate proanthocyanidin polymerization under acidic conditions through the continuous hydrolysis and condensation of interflavan bonds, potentially allowing incorporation of anthocyanin (or anthocyanidin) into the proanthocyanidin structure.[26] The degree of pigmentation of the anthocyanin-proanthocyanidin or proanthocyanidin-anthocyanin product and its resistance to attack by bisulfite would be variable due to the nature of the interflavan bond, the acidity of the medium, the structure of the polymer with respect to inter- and intramolecular bonding, and the degree of oxidation.[16,24,26] While this type of reaction has been shown to occur with monomers up to the level of oligomers in grapes, it has not been shown to occur in proanthocyanidin polymers *in vivo*.

A recent study[4] has shown that changes in skin proanthocyanidin structure and properties from preveraison (green) to ripe (red) grapes altered its binding properties with grape-derived cell wall material. For preveraison proanthocyanidin from skin or seed, cell wall material had greater affinity for higher molecular mass polymers irrespective of their origin within the grape berry.[6] For proanthocyanidins from ripe grape skins, however, those of higher molecular mass showed reduced affinity for skin cell wall material.[4] While opening up the question as to whether the higher levels of polymerization occurring during grape ripening might alter the conformational structure of high molecular mass skin proanthocyanidins, a further, obvious visual difference was that of the relative degree of pigmentation of the proanthocyanidin isolates from ripe grapes. The present study has been designed to track changes in proanthocyanidin polymerization and pigmentation during grape ripening, and to explore whether associated changes in proanthocyanidin binding properties with cell wall material could be observed. Since the association of proanthocyanidins and anthocyanin could potentially be an artifact of the extraction process, an additional experiment aimed to determine whether colorless proanthocyanidins combined with anthocyanin produced a colored product, and if this altered its binding properties with cell wall material.

MATERIALS AND METHODS

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

Grape Sampling and Preparation for Analysis. *Vitis vinifera* L. cv. Cabernet Sauvignon grape samples were obtained from two commercial vineyards (A and B) in the Langhorne Creek growing region of South Australia. From vineyard A (Orlando-Wyndham), grape samples were obtained at different stages of ripeness from preveraison (green) to commercial harvest for the 2009 and 2010 growing seasons. To obtain a representative vineyard sample, a 200-berry and a 100-berry sample were collected from three rows distributed within the vineyard block, and then pooled. The 100-berry sample was processed fresh, and the skins were separated from flesh and seed, while kept on ice. Skin material was weighed, frozen in liquid nitrogen and stored at −80 °C until used.

For a separate experiment, duplicate samples of wild-type Cabernet Sauvignon and two mutant bud-sports, Malian and Shalistin, were obtained in the 2009 season from a second vineyard B (Cleggett Wines). The Malian and Shalistin mutants have been described in detail in ref 27 and differ from the wild-type phenotype in having reduced or absent anthocyanin content respectively. Twenty-bunch samples of each genotype were sourced from two spatially distinct vineyard blocks. From each bunch sample, a 200-berry sample and a 100-berry sample were obtained. For vineyard B, the 100-berry samples were frozen whole at −80 °C, and skins from these samples were later peeled while the berries were frozen. Skins were kept frozen in dry ice during processing, weighed, and stored at −80 °C until analyzed. For both vineyards, the fresh 200-berry sample was pressed by hand in a small plastic bag to express the juice, and centrifuged at 1730*g* for 5 min to clarify the juice from insoluble material. The juice total soluble solids (Brix) were determined using a digital refractometer.

Extraction and Preparation of Skin Proanthocyanidins. For frozen skin samples from vineyard A and B, proanthocyanidins were isolated and purified from using an adaptation of previously published methods.[2,20] Skins were extracted in 70% v/v acetone and concentrated under reduced pressure at 35 °C to remove acetone and then extracted with hexane to remove residual lipophilic material. The aqueous fraction was then recovered using a separatory funnel. The aqueous skin extracts were then made up to a final concentration of 60% v/v methanol containing 0.05% v/v trifluoroacetic acid (TFA) and then applied (∼18.3 mL/min) to a 300 mm × 21 mm glass column (Michel-Miller, Vineland, NJ, USA) 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. Proanthocyanidin 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 proanthocyanidin fractions eluted were concentrated under reduced pressure at 35 °C to remove organic solvent and the aqueous fraction recovered (≅5 mL). The aqueous fraction was frozen in a pretared 12 mL test tube at −80 °C, lyophilized to a dry powder and weighed. Dried proanthocyanidin isolates were stored in the dark and under nitrogen at −20 °C prior to analysis.

Binding Reaction of Proanthocyanidin Isolates with Cell Wall Material. The grape skin and flesh cell wall material used for the binding reaction with proanthocyanidin isolates was the same as that described previously.[4,6] Flesh and skin cell wall material were weighed

into 1.5 mL centrifuge tubes in 13 mg quantities. Cell wall material samples were then combined with proanthocyanidin isolates from skin at different ripeness stages or genetic origin 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. Each reaction was performed in duplicate. For each reaction, a proanthocyanidin standard blank of the respective proanthocyanidin combination without cell wall material was included in order to account for possible reduction in proanthocyanidin recovery due to self-association, precipitation or oxidation. Following the binding reaction, samples were centrifuged at 16000*g* and a 500 μL aliquot of 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). Recovered proanthocyanidin was then reconstituted in 100 μL of methanol, and analyzed by phloroglucinolysis and gel permeation chromatography (GPC).

Acid Catalysis in the Presence of Excess Phloroglucinol (Phloroglucinolysis). Skin proanthocyanidin isolates and proanthocyanidins recovered from the binding reactions were characterized by phloroglucinolysis[1] to determine subunit composition and mDP. The high-throughput method described in refs 4 and 6 was followed. The phloroglucinolysis reaction was carried out at 50 °C for 25 min, neutralized and analyzed by RP-HPLC according to the conditions outlined in the original method[1] using (−)-epicatechin (Sigma Aldrich, St. Louis, MO, USA) as the quantitative standard.

Gel Permeation Chromatography. The GPC method was adapted from that previously described[20] to allow for increased size distribution resolution of high molecular mass proanthocyanidins. The 100 Å column described in the original method was replaced by a 300 mm × 7.5 mm, 5 μm, 10^4 Å column (Varian Inc., Mulgrave, Victoria, Australia). The column arrangement and chromatographic conditions were the same as the original method. The preveraison skin proanthocyanidin fractions of known mDP (by phloroglucinolysis) used as standards for calibration were the same as previously.[4] For calibration, a second order polynomial was fitted with the proanthocyanidin elution time at 50% for each standard.[20] For GPC analysis, proanthocyanidin samples in methanol were diluted with 4 volumes of the HPLC mobile phase. The maximum amount of proanthocyanidin injected onto the column was 40 μg.

Spectrophotometric Determination of Relative Proanthocyanidin Color. Proanthocyanidin isolates were prepared (0.63 g/L) in various solutions: 0.1 N HCl (A_{520}HCl), model wine buffer pH 3.4 of 0.5% (w/v) tartaric acid, 12% (v/v) ethanol (A_{520}buffer), and model wine buffer with 0.375% (w/v) sodium metabisulfite ($A_{520}SO_2$).[28] A 300 μL aliquot of each were transferred to a Greiner 96 well microtiter plate (Interpath Services Ltd., West Heidelberg, Victoria, Australia). The absorbance at 520 nm of each aliquot was determined using a Spectramax M2 plate reader (Molecular Devices Ltd., Auburn, Victoria, Australia) with correction for a 1 cm path length. Spectrophotometric measurements were taken after 3 h for A_{520}HCl, and after 1 h for A_{520}buffer and $A_{520}SO_2$.

A further experiment was performed using a 50 μL solution of 10 g/L proanthocyanidin in 0.1 N HCl/methanol, which was then heated to 50 °C for 25 min in a heating block as described for the high-throughput phloroglucinolysis.[4,6] Following the heating reaction, the proanthocyanidin samples were cooled on ice and then diluted to 0.63 g/L in 0.1 N HCl. The absorbance of a 300 μL aliquot was then determined at 520 nm as outlined above (A_{520}50 °C). While not estimating a quantitative yield of anthocyanidin by autoxidation,[29] the method allowed for a comparison of the relative degree of red color liberated from proanthocyanidins under the conditions used for phloroglucinolysis.

In order to determine the contamination of proanthocyanidin fraction with monomeric anthocyanin and/or incorporation of anthocyanin to the polymer, the fractions were analyzed by HPLC.[30] The ratio of total proanthocyanidin absorbance at 520 to 280 nm was determined ($A_{520/280}$), excluding the contribution of contaminating monomeric material, if any.

Isolation of Monomeric Anthocyanins and Reaction with Proanthocyanidin. Methanolic eluant obtained from the proanthocyanidin isolation procedure containing lower molecular mass phenolics was retained and used to isolate anthocyanins for reaction with proanthocyanidin. Methanol was removed under reduced pressure at 35 °C, and the aqueous extract acidified to 0.05% TFA. Amberlite FPX66 resin (Dow Chemical Company, Spring House, PA, USA) was prepared by washing in Milli-Q water, filtered, and added directly to the aqueous extract. The sample was sealed and stirred for 1 h and then filtered through glass wool. The resin was then rinsed twice in Milli-Q water containing 0.05% TFA to remove soluble nonphenolic material. Two fractions were obtained from the adsorbed component by sequentially fractionating in 60% v/v methanol 0.05% TFA followed by 95% v/v methanol 0.05% TFA.

The fractions were analyzed by HPLC,[30] and 60% methanol fraction was found to contain a higher proportion of monoglucoside anthocyanins (82%) and was free of proanthocyanidin. The methanol from this fraction was removed under reduced pressure (35 °C) and then made up to 70% (v/v) acetone to a final anthocyanin concentration of 98 mg/L. Powdered colorless Shalistin grape skin proanthocyanidin was then added to this solution in a ratio of 1:1.3 (w/w) proanthocyanidin: anthocyanin (which approximated the ratio in grape skins, data not shown) and stirred overnight under nitrogen, in the dark. Acetone was then removed under reduced pressure at 35 °C, and the aqueous extract made up to 60% (v/v) methanol 0.05% (v/v) TFA. A further sample of colorless Shalistin skin proanthocyanidin was made up in the same solvent. Samples were loaded to a to a 300 mm × 21 mm glass column (Michel-Miller, Vineland, NJ, USA) containing Sephadex LH20 chromatography resin (Amersham, Uppsala, Sweden) to an approximate bed volume of 40 mL. Proanthocyanidin was isolated following sequential elution with 150 mL (v/v) 60% methanol, 0.05% TFA, followed by 150 mL (v/v) 70% acetone. The 70% acetone fraction was concentrated under reduced pressure (35 °C), and the aqueous portion dried under a stream of nitrogen. The proanthocyanidin isolated which had been reacted with anthocyanin was designated Shalistin-A.

The isolates were reacted then with skin cell wall material or analyzed spectrophotometrically at 520 nm determined as described. Additionally, the isolates were refractionated on the same column which had been equilibrated with 60% (v/v) methanol, 0.05% (v/v) TFA and lower molecular weight proanthocyanidins eluted with 150 mL of 90% (v/v) HPLC grade methanol containing 0.05% (v/v) TFA. High molecular mass proanthocyanidins were recovered by sequentially eluting with 150 mL of (v/v) 20% acetone, 65% methanol, 0.05% TFA, followed by 150 mL of (v/v) 70% acetone, 0.05% TFA. The 70% acetone fraction was concentrated under reduced pressure (35 °C), and the aqueous portion dried under a stream of nitrogen. The recovered proanthocyanidin was reacted with skin cell wall material as described, either alone or in 1:1 (w/v) combination with a low molecular mass preveraison proanthocyanidin (2008 g/mol) to give a final reaction concentration of 2 g/L. This low molecular mass proanthocyanidin has been previously described[6] and was designated as F3 for the current study. Proanthocyanidin extracts recovered from the binding experiments were analyzed by GPC.

Statistical Analysis. Significant differences in binding response between proanthocyanidins were determined from duplicate experiments using a one-way analysis of variance (ANOVA), followed by a post hoc Student's *t* test. The JMP 5.0.1 statistical software package (SAS, Cary, NC, USA) was used for all statistical analysis. Linear regression analysis was performed using Microsoft Excel 2007 Software.

RESULTS AND DISCUSSION

Composition of Skin Proanthocyanidins at Different Ripeness Stages. For the 2009 and 2010 seasons, the ripening

Table 1. Gravimetric Recovery, Color, Subunit Composition and Molecular Mass of Proanthocyanidins Isolated from Cabernet Sauvignon Grape Skins at Sequential Ripeness Stages for the 2009 and 2010 Seasons[a]

		proanthocyanidin[c]		proanthocyanidin color[d]							Molecular mass		terminal subunits[i]			extension subunits[i]		
sampling date[b]	soluble solids, °Brix	mg/berry	mg/g fresh wt	A_{520}HCl	A_{520}50 °C	A_{520}buffer	$A_{520}SO_2$	$A_{520/280}$	MC,[e] %	mDP[f]	subunit[g]	GPC 50%[h]	C	EC	ECG	EGC-P	(C+E)-P	ECG-P
2009 Season																		
9/1/2009	nd	0.90	1.67	0.11	0.54	nd	nd	nd	84.9	17.8	5365	4440	91.1	5.4	3.6	60.9	36.4	2.7
10/2/2009	21.0	1.13	1.31	0.93	1.58	0.25	0.13	0.12	70.7	23.7	7153	7401	77.1	18.1	4.8	59.2	38.2	2.6
23/2/2009	22.4	0.97	1.09	1.32	2.27	0.59	0.28	0.13	60.9	24.6	7453	7252	72.4	21.8	5.8	61.5	35.5	3.0
2/3/2009	23.4	1.23	1.34	1.29	2.62	0.59	0.30	0.14	63.1	23.9	7231	7614	70.2	24.5	5.3	60.3	36.6	3.1
12/3/2009	24.8	1.49	1.51	1.07	2.01	0.52	0.28	0.13	66.2	27.2	8249	8720	67.1	26.6	6.3	62.2	34.8	3.0
19/3/2009	25.0	1.32	1.33	1.08	1.78	0.52	0.29	0.12	69.2	33.1	10039	9477	74.0	23.5	2.5	63.0	34.1	2.9
26/3/2009	25.8	1.21	1.18	1.08	1.83	0.50	0.24	0.13	62.9	28.2	8552	8004	67.4	27.3	5.3	61.7	35.2	3.0
2010 Season																		
15/1/2010	nd	2.72	4.59	0.30	0.49	nd	nd	nd	71.1	17.3	5225	4548	91.0	4.4	4.6	51.2	45.6	3.2
26/1/2010	13.2	1.24	1.45	0.17	0.71	0.11	0.09	0.07	77.2	25.2	7574	9792	90.7	6.3	3.0	50.6	46.2	3.1
9/2/2010	18.0	1.18	1.27	0.49	1.01	0.21	0.14	0.06	71.4	27.4	8272	9153	85.9	11.3	2.8	52.2	44.7	3.1
16/2/2010	19.8	1.28	1.31	0.71	1.34	0.35	0.15	0.08	69.7	27.2	8190	7932	84.0	12.3	3.7	52.6	44.5	2.9
23/2/2010	21.7	1.29	1.27	1.00	1.73	0.53	0.20	0.10	68.4	25.6	7710	6936	81.9	14.9	3.2	52.5	44.6	2.9
2/3/2010	23.8	1.41	1.42	0.95	1.64	0.50	0.22	0.11	62.8	25.6	7707	6829	78.0	18.1	3.9	51.9	45.1	3.0
10/3/2010	24.0	1.48	1.49	0.85	1.61	0.48	0.21	0.10	69.3	28.1	8477	7889	79.7	17.6	2.8	52.9	44.1	3.0
17/3/2010	26.1	1.74	1.62	0.95	1.66	0.48	0.25	0.11	69.3	26.9	8099	8085	73.8	23.4	2.9	50.4	46.2	3.5

[a] Contamination of monomeric anthocyanin was ≤1%; nd = not determined. [b] Day/month/year. [c] Gravimetric proanthocyanidin recovery expressed as content per berry or concentration per g berry fresh weight. [d] Spectrophotometric proanthocyanidin color measures: in 0.1 N HCl, in 0.1 N HCl after heating in 0.1 N HCl/MeOH at 50 °C, in model wine buffer and in the presence of bisulfite; or proanthocyanidin 520 nm/280 nm absorbance ratio by HPLC excluding monomeric anthocyanin. [e] Mass conversion based on % recovery of proanthocyanidin by phloroglucinolysis based on the gravimetric mass. [f] Mean degree of polymerization in epicatechin units. [g] Molecular mass as determined by phloroglucinolysis. [h] Molecular mass as determined by GPC at 50% proanthocyanidin elution. [i] Percent composition of proanthocyanidin 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.

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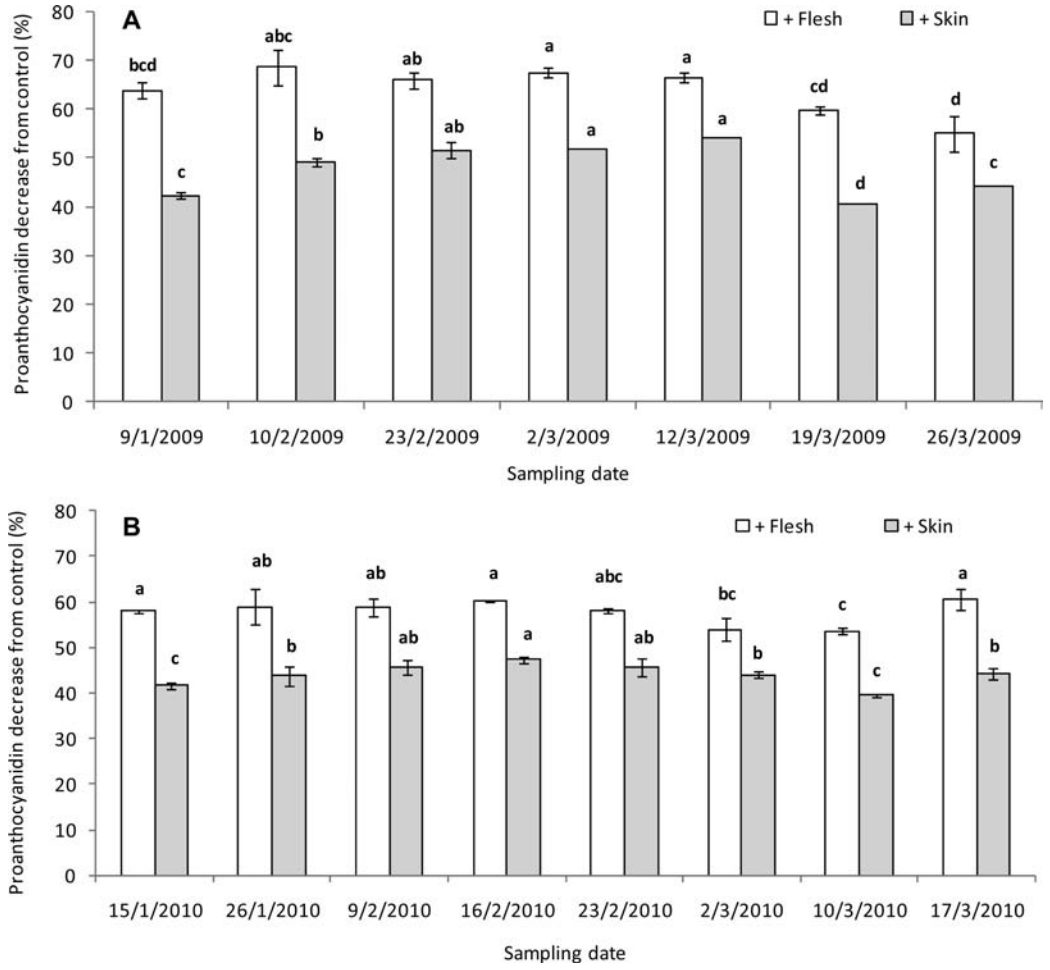

Figure 1. Proanthocyanidin removed from solution following reaction of 13 mg/mL flesh and skin cell wall material with 2 mg/mL Cabernet Sauvignon skin proanthocyanidins at different ripeness stages from (A) 2009 season and (B) 2010 season ($N = 2$; ±SE of the mean; ANOVA: $P < 0.05$ followed by post hoc Student's t test; different letters in the histogram bars indicate significant differences).

pattern over the two seasons was similar, showing an early rapid increase in juice soluble solids as °Brix, which then plateaued and increased slowly thereafter, reaching a commercial ripeness of approximately 26 °Brix (Table 1). For the range of skin proanthocyanidins isolated at different grape ripeness stages, both seasons included a preveraison stage, which was sourced in mid-January. The second season of the study included an additional veraison sample on 26 January, which corresponded with approximately 30% colored berries.

The changes in skin proanthocyanidin content (mg/berry) were not consistent between seasons, increasing from the preveraison measure in 2009 and then declining toward commercial ripeness (Table 1), and in 2010 being highest preveraison, declining to the postveraison measure and then increasing toward commercial harvest. When expressing this data as concentration (mg/g fresh wt) which includes berry weight information, it is evident that the preveraison measure was highest for both seasons, although this was greater in 2010. For postveraison sampling dates, the expression of proanthocyanidin recovery data as either content or concentration showed similar trends. According to other studies, it would be expected that the preveraison or veraison proanthocyanidin measure would be highest, followed by a decline as ripening progressed.[10,11] However, since the current study primarily aimed to recover proanthocyanidin gravimetrically, in order to provide an accurate measure of the % conversion by mass to its subunits (MC), comparison with other studies reporting only subunit composition by phloroglucinolysis[10,11] is limited. The incorporation of nonproanthocyanidin material into proanthocyanidins during ripening has been reported in other studies,[2,13] and this may contribute differentially to the final mass of the recovered proanthocyanidin, and may account for the variability in content observed during ripening and between seasons. For both seasons of the study, the mass conversion of the isolated proanthocyanidins was a minimum of 61% (Table 1). In 2009, the mass conversion decreased following the preveraison measure and was then variable between sampling dates whereas, in 2010, the mass conversion was highest at veraison.

For both seasons, the mean degree of polymerization (mDP) was lowest at the preveraison stage, increasing thereafter. Changes in mDP were variable during the ripening period, in 2009 increasing to a maximum of 33 (Table 1) and then declining at commercial ripeness. In 2010 the highest observed mDP was 28, although mDP did not vary significantly from veraison onward. Changes in mDP were reflected in the average molecular mass determined by subunit composition. Molecular mass by GPC (50% elution) followed a similar trend to the molecular mass determined by phloroglucinolysis. The correlation between

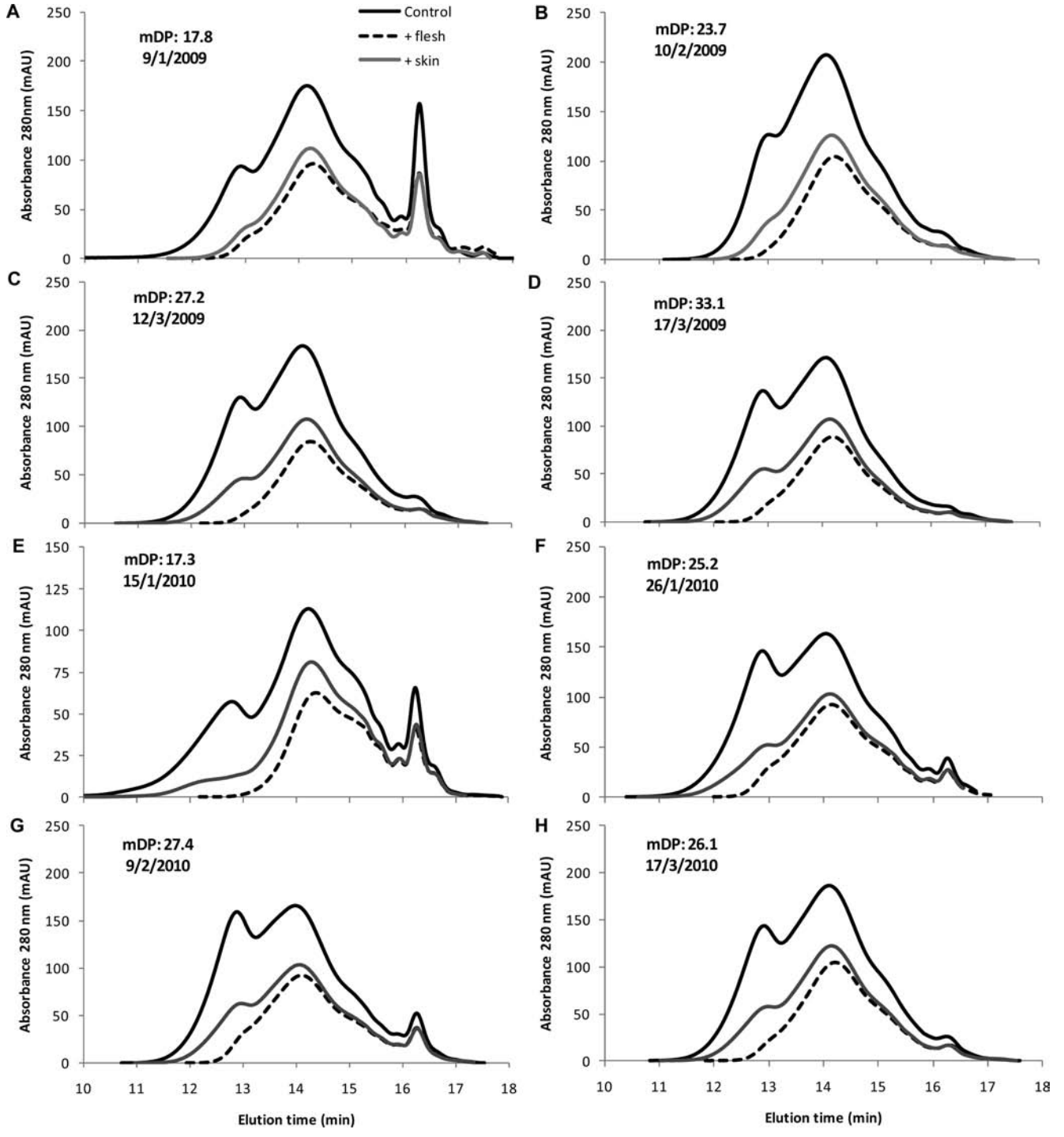

Figure 2. Gel permeation chromatogratograms of skin proanthocyanidins isolated from different ripeness stages before and after addition of flesh or skin cell wall material from the 2009 (A−D) and 2010 (E−H) seasons.

the two measures for proanthocyanidin samples combined from both seasons was $R^2 = 0.71$ (data not shown).

Subunit composition was determined as a proportion of either terminal or extension subunits (Table 1). For both seasons, the proportion of (+)-catechin as a terminal subunit declined as ripening progressed, while the proportion of (−)-epicatechin increased. As a proportion of terminal subunits, (−)-epicatechin-3-*O*-gallate was low, at between 2.5% and 6.5% over both seasons, and did not show a pattern which reflected the progression of ripening. For the extension subunits, the proportion of (−)-epigallocatechin was consistent during ripening, being slightly higher in the 2009 season at 60−63%, and was 50−53% in 2010. Similarly, the (+)-catechin and (−)-epicatechin as extension subunits did not change significantly during ripening for either season, and were proportionally higher in 2010, reflecting the differences between seasons observed for (−)-epigallocatechin. As observed for the terminal subunits,

(−)-epicatechin-3-*O*-gallate as an extension subunit was proportionally low for both seasons, between 2.6% and 3.5%.

Color of Skin Proanthocyanidins at Different Ripeness Stages. It was noted that there were strong visual color differences between preveraison, veraison and ripening skin proanthocyanidins. Therefore, various properties of spectrophotometrically measured color at 520 nm of isolated proanthocyanidins for the 2009 and 2010 seasons were determined (Table 1). For both seasons, an increase in A_{520}HCl was observed from preveraison to the onset of ripening, which was more apparent in the 2010 season due to the inclusion of samples taken at veraison. However, proanthocyanidin color did not increase as ripening progressed, but was highest shortly after veraison. Following this, A_{520}HCl declined slightly. For all isolated proanthocyanidins, contamination with monomeric anthocyanin was negligible, at ≤1% (w/w), and showed no correlation ($R^2 \leq 0.2$) to any measure of proanthocyanidin color at 520 nm (data not shown). The contribution of incorporated anthocyanin to the proanthocyanidin polymer color was observed by determining the $\mathbf{A}_{520/280}$ and $A_{520}SO_2$ values and showed that significant relationships were found with the spectrophotometric measurements A_{520}HCl ($\mathbf{A}_{520/280}$ $R^2 = 0.79$; $A_{520}SO_2$ $R^2 = 0.85$) and A_{520}buffer ($\mathbf{A}_{520/280}$ $R^2 = 0.58$; $A_{520}SO_2$ $R^2 = 0.75$) (data not shown). It was of interest to determine if the color increase following acid-catalyzed depolymerization and autoxidation of proanthocyanidin subunits was related to spectrophotometrically measured color from incorporated anthocyanin, since in its bound form it may have reduced absorbance at 520 nm. For the preveraison proanthocyanidins, it is evident for both the 2009 and 2010 seasons that A_{520}50 °C increased from the A_{520}HCl measure (Table 1), which can be concluded to be due to autoxidation of procyanidin and prodelphinidin subunits in the reaction.[29] However, from veraison onward, the increase in A_{520}50 °C was more significant, and a significant relationship ($R^2 = 0.77$) was found for this value and both the $A_{520/280}$ ratio and $A_{520}SO_2$ (data not shown).

Interaction of Skin Proanthocyanidins of Different Ripeness with Flesh and Skin Cell Wall Material. The skin proanthocyanidin isolates from the 2009 and 2010 seasons were combined with purified flesh and skin cell wall material in model suspensions to determine the effect of the interaction in terms of molecular mass distribution. The effect of cell wall material addition on proanthocyanidin concentration and composition was determined by observing changes in the proanthocyanidin which remained in solution, in comparison with a control solution. Similar studies using this cell wall material have been reported previously.[4,6] Since the current study undertook to explore previous observations in greater detail using a diverse set of skin proanthocyanidin isolates, the cell wall material source was thus kept constant as for previous work.[4,6] As observed in refs 4 and 6, the affinity of flesh cell wall material for proanthocyanidin was higher than for skin cell wall material, removing a range of 55−69% of proanthocyanidin from solution by mass compared to 40−54% for skin cell wall material addition (Figure 1A and 1B).

The reaction of flesh cell wall material with the 2009 skin proanthocyanidin series revealed that the % removal of proanthocyanidin from solution by mass was similar for all apart from the final two ripeness stages, for which cell wall material affinity for proanthocyanidin was lower (Figure 1A). For the 2010 proanthocyanidin series, a similar trend was observed to 2009, but at commercial harvest the % removal by flesh cell wall material was equivalent to that for proanthocyanidins from the earliest ripeness stages (Figure 1B). For the addition of skin cell wall material, similar responses were observed for the 2009 and 2010 seasons, with the % removal of proanthocyanidin from solution increasing for skin proanthocyanins sourced at veraison or immediately postveraison, and then declining as ripening progressed. Interestingly, for both flesh and skin cell wall material addition there were no or minor differences in the % removal of preveraison proanthocyanidins compared to those isolated at the later ripeness stages.

In general, minor changes in the proanthocyanidin removed by cell wall material were observed in terms of total mass, but greater differences in the response of proanthocyanidin molecular mass distribution were found for the range of isolates studied. The use of GPC elution chromatograms allowed for visualization of the molecular mass distribution of the respective proanthocyanidins, before and after addition of cell wall material (Figure 2). The changes in proanthocyanidin average mDP and molecular mass (Table 1) observed with grape ripening in the 2009 season are reflected in the loss of a significant proportion of lower molecular mass material (later eluting) (Figure 2A) and the progressive increase of higher molecular mass material (earlier eluting) (Figure 2B−D). For the interaction with flesh cell wall material, it can be seen that there is consistent removal of the higher molecular mass proanthocyanidin fraction irrespective of proanthocyanidin ripeness or average molecular mass (mDP), an observation consistent with previous findings.[4,6] However, the higher molecular mass proanthocyanidins from postveraison grapes showed a decreased affinity for skin cell wall material. This observation is consistent with the findings of ref 4 for ripe *Vitis vinifera* cv. Shiraz skin proanthocyanidin. It should be noted, however, that the current study made use of only a single concentration (13 mg/mL) at which skin cell wall material was reacted with proanthocyanidin, which is the same as the highest level of addition used previously.[4,6] For a lower skin cell wall material addition of 6 mg/mL,[4] there was no affinity for higher molecular mass ripe skin proanthocyanidin material. For the 2010 skin proanthocyanidin series, the changes in proanthocyanidin elution profile from preveraison to ripe skin proanthocyanidin were similar to those observed for 2009, showing the loss of lower molecular mass material (Figure 2E) and the proportional increase of higher molecular mass material (Figure 2F−H). For preveraison skin proanthocyanidin, its loss from solution through interaction with both skin and flesh cell wall material was greatest from the higher molecular mass range. As for 2009, the skin cell wall material reaction with ripe proanthocyanidins for the 2010 series showed a decreasing affinity for proanthocyanidin in the higher molecular mass range, but this was not enhanced as ripening progressed. Thus, the elution profiles observed following skin cell wall addition at veraison (Figure 2F) and at commercial harvest (Figure 2H) were similar. However, it is evident from proanthocyanidin compositional analysis that proanthocyanidin mDP was similar throughout the 2010 season, unlike 2009 where greater differences were observed (Table 1).

The extent of molecular mass decrease at 50% elution by GPC (corresponding to the average proanthocyanidin molecular mass) and at 90% elution by GPC (corresponding to the highest molecular mass material) was compared between proanthocyanidins for their interaction with either flesh or skin cell wall material. For both seasons studied, the average decrease in molecular mass at 50% elution was 32%, and at 90%, elution was 51% following proanthocyanidin reaction with flesh cell wall

Table 2. Gravimetric Recovery, Color, Subunit Composition and Molecular Mass of Proanthocyanidins Isolated from the Grape Skins of Wild-Type Cabernet Sauvignon and Two of Its Mutant Clones at Commercial Harvest for the 2009 Season[a]

Cabernet Sauvignon genotype	site	sampling date[b]	soluble solids, °Brix	proanthocyanidin[c] mg/berry	proanthocyanidin[c] mg/g freshwt	proanthocyanidin color,[d] A_{520}HCl	MC,[e] %	mDP[f]	Molecular mass subunit[g]	Molecular mass GPC 50%[h]	terminal C	terminal EC	terminal ECG	extension subunits[i] EGC-P	extension subunits[i] (C+E)-P	extension subunits[i] ECG-P
wild-type	block 1	2/3/2009	22.7	1.10	1.86	0.67	73.5	36.4	11038	8251	75.8	21.4	2.8	67.8	30.2	2.1
wild-type	block 2	2/3/2009	22.6	1.23	2.24	0.88	67.1	31.2	9452	nd	72.1	23.0	4.9	65.6	31.9	2.5
Malian	block 2	19/3/2009	22.9	1.79	1.76	0.28	60.4	33.0	10002	7387	75.5	22.1	2.4	65.6	31.9	2.5
Malian	block 3	2/3/2009	24.8	2.66	4.25	0.36	55.6	37.3	11315	nd	81.6	15.4	3.0	65.2	32.2	2.6
Shalistin	block 1	2/3/2009	22.4	1.61	1.26	0.43	65.1	36.0	10884	8035	89.0	7.1	3.9	66.8	31.2	1.9
Shalistin	block 2	2/3/2009	22.8	1.82	1.43	0.16	68.8	28.2	8528	nd	86.4	10.6	3.0	64.3	33.3	2.3

[a] Two sample replicates of each genotype were collected from spatially distinct blocks; contamination of monomeric anthocyanin was ≤1%; nd = not determined. [b] Day/month/year. [c] Gravimetric proanthocyanidin recovery expressed as content per berry or concentration per g berry fresh weight. [d] Spectrophotometric proanthocyanidin color measures in 0.1 N HCl before and after heating in 0.1 N HCl/MeOH at 50 °C. [e] Mass conversion based on % recovery of proanthocyanidin by phloroglucinolysis based on the gravimetric mass. [f] Mean degree of polymerization in epicatechin units. [g] Molecular mass as determined by phloroglucinolysis. [h] Molecular mass as determined by GPC at 50% proanthocyanidin elution. [i] Percent composition of proanthocyanidin 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.

material (data not shown), with insignificant differences found between isolates. Similarly, the addition of skin cell wall material to the various proanthocyanidins reduced their molecular mass at 50% elution by 19% and did not vary with grape ripeness stage. Greater variability was found for the proanthocyanidin molecular mass decrease at 90% elution following reaction with skin cell wall material, ranging from 50% for preveraison proanthocyanidins to 14% for the higher molecular mass postveraison isolates (data not shown). However, there was inconsistency in the pattern observed for the two seasons of isolated skin proanthocyanidins studied, indicating that the progression of ripening alone was not the driver of the observed changes.

To attempt to quantify these observations in terms of proanthocyanidin size, linear regression analysis for average proanthocyanidin molecular mass determined at 50% elution and the % loss of molecular mass at either 50% elution or 90% elution by GPC was determined. For the reaction of proanthocyanidins with skin cell wall material, no relationship was observed for the decrease in molecular mass at 50% elution using linear regression analysis (R^2 = 0.01, data not shown). A significant negative relationship was found at 90% elution when the response of postveraison proanthocyanidins alone was analyzed (R^2 = 0.87) or when preveraison proanthocyanidins were included in the analysis (R^2 = 0.93) (data not shown). This result indicates that the decrease in the affinity of skin cell wall material for high molecular mass proanthocyanidin is related to proanthocyanidin molecular size. Thus, where changes in average proanthocyanidin molecular mass are associated with ripening, this might account for the reduction in the affinity of high molecular mass proanthocyanidins for skin cell wall material. While allowing for the quantitative characterization of this phenomenon, previously reported,[4] this result does not give insight into the mechanism which induces the reduction in high molecular mass proanthocyanidin affinity for skin cell wall material. While not being directly associated with grape ripeness in terms of its progression, it may indirectly result from the response of subcellular organizational and biochemical changes at the onset of ripening which occur from veraison onward.

Comparison of Cell Wall Material Interaction with Proanthocyanidins from Grapes Differing in Their Anthocyanin Content. The coloring of extracted grape proanthocyanidins from preveraison through ripening in red grapes has been reported,[2,13] but anecdotal evidence indicates that the association of colored material with proanthocyanidins is an artifact of the extraction process rather than occurring within the grape berry cell itself.

An outcome of the initial experiments in this study and previous work[4] has revealed that changes to grape skin proanthocyanidin occurring after veraison, associated with higher levels of polymerization, render this material less reactive to skin cell wall material. One possibility of this is that integration of anthocyanins into larger molecular mass proanthocyanidins might reduce the interaction with cell wall material. For the range of Cabernet Sauvignon skin proanthocyanidins discussed in the previous section, their spectrophotometric properties at 520 nm showed no relationship to the interaction with cell wall material (data not shown). Nevertheless, the possibility that the interaction of anthocyanin and proanthocyanidins might alter its binding properties with cell wall material was further tested.

In order to compare skin proanthocyanidins which were similar in molecular mass and composition, but differed in their color properties, proanthocyanidins were sourced from the skins of wild-type

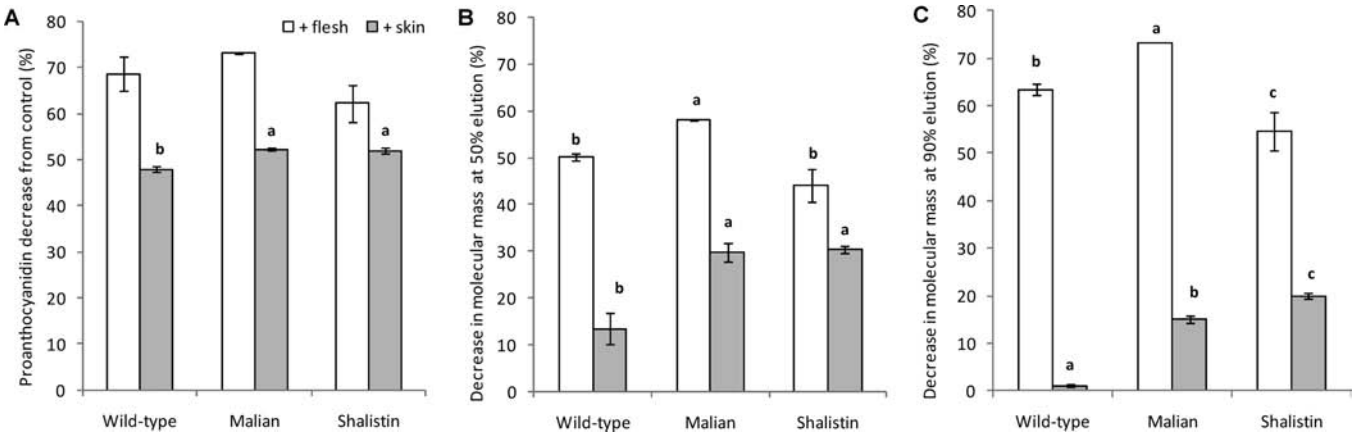

Figure 3. Change in proanthocyanidin properties following reaction of skin proanthocyanidins of wild-type, Malian and Shalistin genotypes of Cabernet Sauvignon with flesh and skin cell wall material: (A) proanthocyanidin mass removed from solution; (B) change in proanthocyanidin molecular mass at 50% elution; (C) change in proanthocyanidin molecular mass at 90% elution ($N = 2$; ± SE of the mean; ANOVA: $P < 0.05$ followed by post hoc Student's t test; different letters in the histogram bars indicate significant differences).

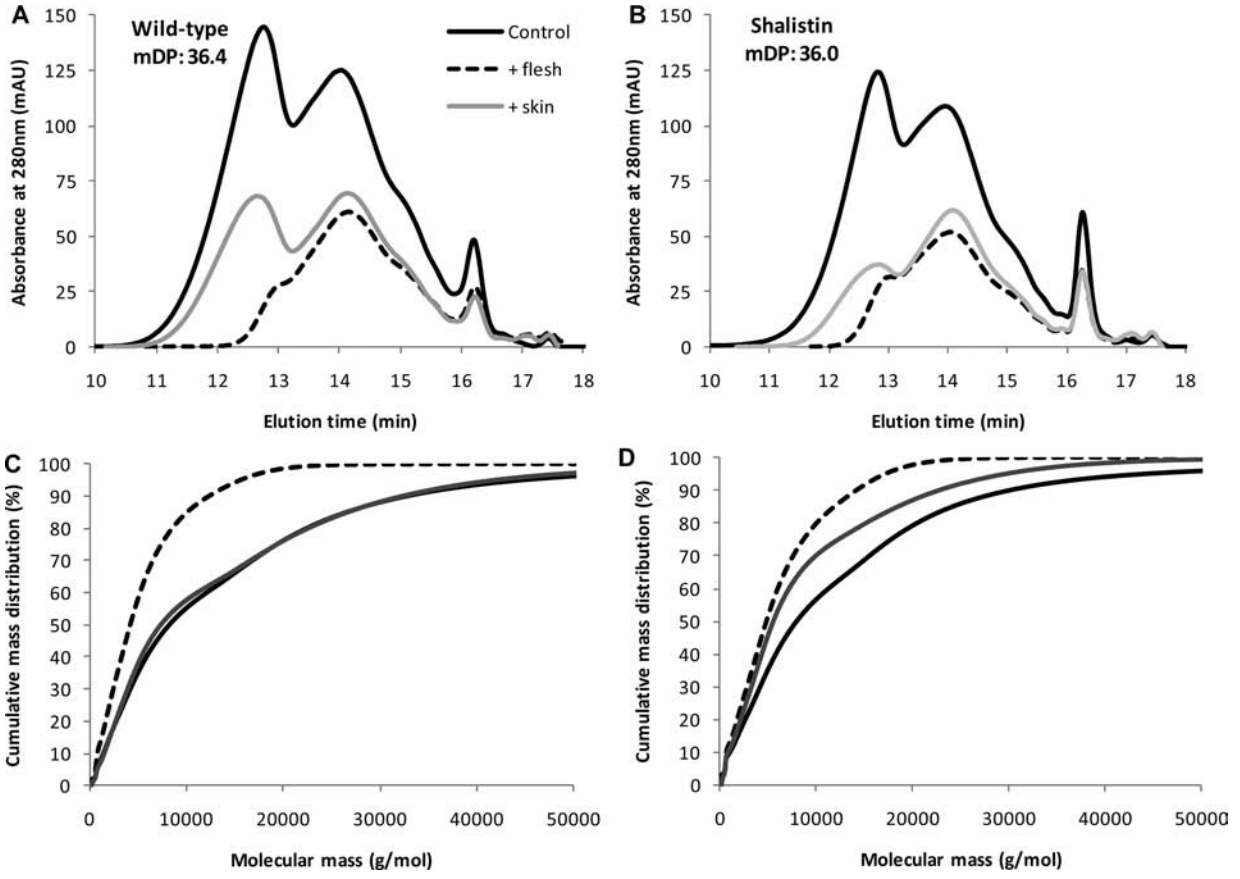

Figure 4. Gel permeation chromatography analysis of skin proanthocyanidins from two Cabernet Sauvignon genotypes before and after addition of flesh and skin cell wall material: (A) elution profile of wild-type; (B) elution profile of Shalistin; (C) cumulative mass distribution of wild-type; (D) cumulative mass distribution of Shalistin.

Cabernet Sauvignon, and two of its mutants Malian and Shalistin which had undergone progressive loss of their ability to synthesize anthocyanin. These mutant phenotypes and genotypes have been characterized in detail in ref 27. Comparison of proanthocyanidin content from the wild-type sample (Table 2) was within the range of proanthocyanidin content reported for Cabernet Sauvignon (Table 1), but when compared in terms of concentration was higher, due to a smaller berry weight. Malian and Shalistin grapes had higher proanthocyanidin levels than the wild-type on a per berry basis (Table 1), but for the Shalistin samples this was due to a slightly larger berry size. Despite small differences in proanthocyanidin content, comparison of the mDP and subunit composition of these grape samples revealed that they were similar (Table 2). Although Malian grapes have small quantities of anthocyanin in the surface

layer of the epidermis,[27] the A_{520}HCl of proanthocyanidins extracted from them did not differ significantly from the colorless Shalistin proanthocyanidins of which one sample had a high baseline A_{520}HCl absorbance, but did not contain anthocyanin by HPLC (data not shown). Both Malian and Shalistin proanthocyanidins had lower 520 nm color than wild-type proanthocyanidins.

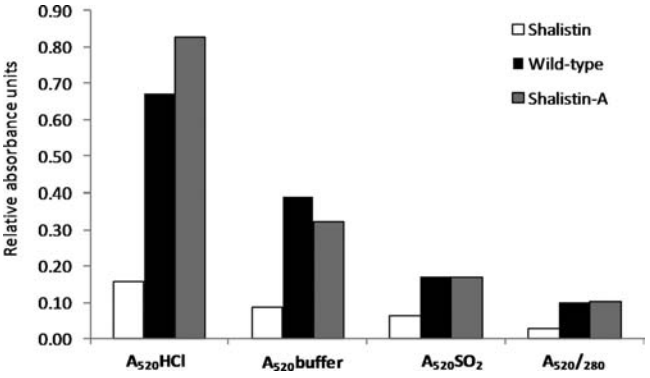

Figure 5. Spectrophotometric properties of Cabernet Sauvignon skin proanthocyanidin isolates from wild-type, Shalistin and Shalistin-A.

For comparison of their binding properties with flesh and skin cell wall material, proanthocyanidins of similar mDP of each variety were selected, for which the corresponding molecular mass by GPC is shown (Table 2). In terms of their % removal by mass following reaction with flesh cell wall material, proanthocyanidins from the three varieties did not differ (Figure 3A). Loss of wild-type proanthocyanidin was lower following reaction with skin cell wall material than those isolated from the other genotypes (Figure 3A). When compared in terms of its selectivity for proanthocyanidins of different molecular mass by GPC, flesh cell wall material reduced Malian proanthocyanidin molecular mass at 50% and 90% elution the most significantly compared with the other isolates (Figure 3B, C). Reaction with flesh cell wall material reduced wild-type and Shalistin proanthocyanidin molecular mass similarly at 50% elution, but at 90% elution, Shalistin was the least significantly reduced. Conversely, reaction with skin cell wall material reduced wild-type proanthocyanidin the least at both 50% and 90% elution, with the responses of Malian and Shalistin proanthocyanidins to skin cell wall material addition being similar. To further demonstrate this interaction, GPC elution profiles and cumulative mass distribution for wild-type and Shalistin skin proanthocyanidins following reaction with flesh and skin cell wall material are shown (Figure 4). The preferential removal of high molecular mass proanthocyanidin from

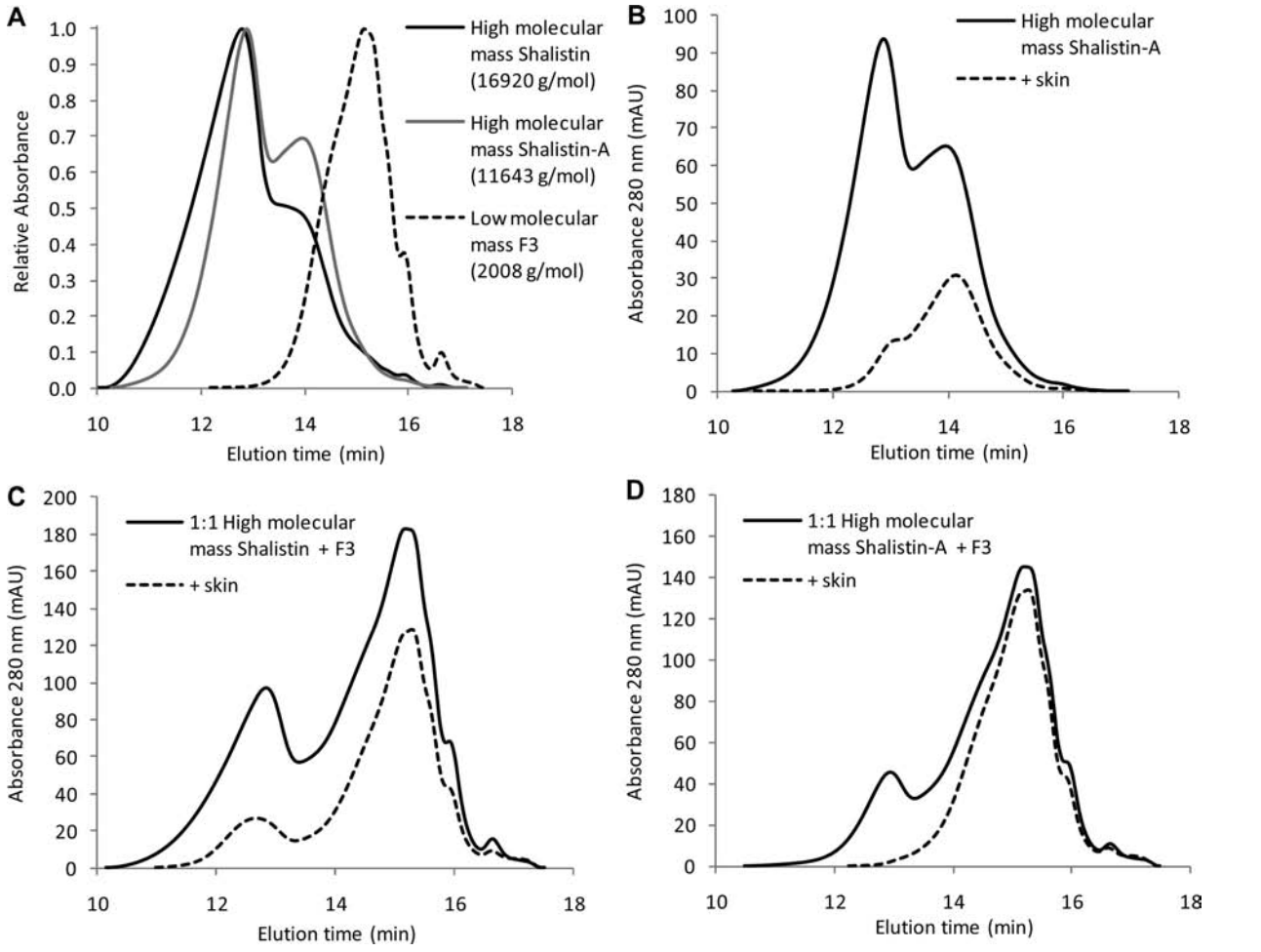

Figure 6. Gel permeation chromatography analysis of high molecular mass proanthocyanidin fractions isolated from Shalistin or Shalistin-A extracts and a low molecular mass proanthocyanidin F3 before and after addition of skin cell wall material: (A) elution profiles of Shalistin, Shalistin-A and F3; (B) Shalistin-A before and after addition of skin cell wall material; (C) 1:1 Shalistin + F3 before and after addition of skin cell wall material; (D) 1:1 Shalistin-A + F3 before and after addition of skin cell wall material.

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solution is evident following reaction with flesh cell wall material for both varieties (Figure 4A,B) and was reflected in a significant shift in the cumulative mass distribution to a lower molecular mass range relative to the control (Figure 4C,D). For the reaction with skin cell wall material on the other hand, the difference between the two proanthocyanidin types is evident, with a greater proportion of high molecular mass proanthocyanidin remaining in solution for the wild-type (Figure 4A,B). In terms of cumulative mass distribution, Shalistin proanthocyanidin was shifted toward a lower average molecular mass relative to the control following reaction with skin cell wall material, albeit smaller than that observed following interaction with flesh cell wall material (Figure 4C). On the other hand, there was no change in the cumulative mass distribution of wild-type proanthocyanidin before and after reaction with skin cell wall material (Figure 4D). An obvious assumption from this study is that the primary difference between these proanthocyanidin samples is the presence of anthocyanin in the proanthocyanidin. The A_{520}HCl and A_{520}buffer absorbances of these ripe wild-type proanthocyanidin samples were similar to the reported values for other wild-type Cabernet Sauvignon proanthocyanidins (Tables 1 and 2).

In order to determine whether the observed differences might be imparted from anthocyanin association with the proanthocyanidin during the extraction process, Shalistin proanthocyanidin was combined with anthocyanin following the standard extraction procedure to produce a colored proanthocyanidin isolate (Shalistin-A). The A_{520}HCl and A_{520}buffer absorbances of the Shalistin-A proanthocyanidin were similar to the reported values for wild-type proanthocyanidin, while Shalistin proanthocyanidin absorbances were low (Figure 5). It is of interest to note that $A_{520}SO_2$ and the ratio $A_{520/280}$ were equivalent for wild-type and Shalistin-A proanthocyanidins (Figure 5) showing that the extraction procedure itself may account for incorporation of anthocyanin to the proanthocyanidin polymer as an artifact.

When the Shalistin-A proanthocyanidin was reacted with skin cell wall material, the higher molecular mass material was preferentially removed, 54% higher than the Shalistin control (result not shown). This finding was contrary to the hypothesis that anthocyanin incorporation as an artifact of the extraction could limit the association of high molecular mass proanthocyanidins with skin cell walls. In order to more accurately verify this finding, the experiment was repeated, and high molecular mass proanthocyanidin isolated from both pure Shalistin proanthocyanidin and Shalistin-A by sequential fractionation. The average molecular mass at 50% elution of the Shalistin-A proanthocyanidin was slightly lower than that isolated from the Shalistin proanthocyanidin (Figure 6A). Therefore, in order to confirm selectivity information in the presence of skin cell wall material, the two high molecular mass fractions were also reacted with skin cell wall material in the presence of a low molecular mass preveraison proanthocyanidin fraction F3 (Figure 6A,C,D).

The results of the Shalistin-A proanthocyanidin following reaction with skin cell wall material confirmed preliminary observations, that higher molecular mass material was preferentially removed (Figure 6B). Further evidence was obtained when the high molecular mass fractions were reacted in the presence of the F3 proanthocyanidin (Figure 6C,D). At 90% elution, the high molecular mass Shalistin-A fraction was more significantly removed (67%) than the high molecular mass Shalistin fraction (40%) (result not shown). While this experiment confirms that colorless proanthocyanidins will associate with anthocyanin as an artifact of the extraction process, it has shown that the converse occurred in terms of the expected result of proanthocyanidin affinity for skin cell wall material. Rather than reducing the affinity of high molecular mass proanthocyanidin for skin cell wall material, it increased binding affinity. This result first shows that the presence of anthocyanin in solution with proanthocyanidin during extraction may significantly alter its color and binding properties for cell wall material, and warrants further investigation. Second, the data indicate that the poor affinity of higher molecular mass proanthocyanidin from ripe, red grapes for skin cell wall material may not simply be due to an artifact of anthocyanin incorporation to proanthocyanidin.

While the skin proanthocyanins isolated from both red Cabernet Sauvignon grapes and poorly colored or colorless mutants have similar subunit composition and size distribution characteristics, they have demonstrated very different binding properties, which could be conferred by their conformation. Future work will explore the subcellular organization of proanthocyanidins in terms of composition, potential incorporation of non-flavan-3-ol subunits, and molecular size. In addition, the mode of proanthocyanidin polymerization, in particular the potential role of oxidation in conferring differences in proanthocyanidin composition and conformation, constitutes a significant frontier of future research.

Significance of Skin Proanthocyanidin Modification for Vinification. The changes occurring in skin proanthocyanidin structure following veraison, which modify both their molecular mass and binding properties with cell wall material, may have implications for their extractability during vinification. The progression of ripening showed for skin cell wall material, in particular, that affinity for proanthocyanidin generally decreased, which indicates that increased extraction of proanthocyanidin might be expected from grape skins during vinification of riper grapes. Furthermore, a reduced affinity of skin cell wall material for higher molecular mass proanthocyanidins was found for isolates from postveraison grape samples. In cases where the progression of ripening is associated with greater levels of proanthocyanidin polymerization, the extraction of higher molecular mass proanthocyanidins may be favored due to a poor affinity for skin cell wall material. It is of significance to note, however, that, despite showing small decreases in the affinity for skin proanthocyanidins from riper grapes, the adsorption of proanthocyanidins by flesh cell wall material remained high. This means that the presence of flesh material in must during vinification may represent a significant sink for extracted proanthocyanidins, preferentially adsorbing those of higher molecular mass. The interaction between skin and flesh cell wall material in the selective adsorption of proanthocyanidins is therefore complex, and wine proanthocyanidin properties will be dependent upon both aspects. While this study has highlighted the importance of proanthocyanidin structure in modifying the interaction with cell walls, further research is required to model the conditions which occur during vinification. Another area of future work will be to look at changes in skin and flesh cell wall material composition during grape development, in order to observe how this might influence their respective binding properties with proanthocyanidins.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Regression analysis of spectroscopic properties of skin proanthocyanidins from different grape ripeness stages and linear regression analysis of skin

proanthocyanidin average molecular mass. This material is available free of charge via the Internet at http://pubs.acs.org.

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