Composition of Grape Skin Proanthocyanidins at Different Stages of Berry Development

James A. Kennedy,*[,†,‡] Yoji Hayasaka,[‡,§] Stéphane Vidal,[‡,§] Elizabeth J. Waters,[‡,§] and Graham P. Jones[†,‡]

Department of Horticulture, Viticulture and Oenology, Adelaide University, Waite Campus, PMB 1, Glen Osmond, South Australia 5064, Australia, and Cooperative Research Centre for Viticulture, PO Box 154, Glen Osmond, South Australia 5064, Australia, and The Australian Wine Research Institute, Waite Campus, PMB 1, Glen Osmond, South Australia 5064, Australia

The composition of grape (*Vitis vinifera* L. cv. Shiraz) skin proanthocyanidins has been determined at different stages of berry development. Beginning approximately 3 weeks after fruit set and concluding at commercial ripeness, the composition of isolated skin proanthocyanidins was determined using the following analytical techniques: elemental analysis, UV−Vis absorption spectroscopy, reversed-phase HPLC after acid-catalysis in the presence of excess phloroglucinol, gel permeation chromatography, electrospray ionization mass spectrometry (ESI-MS), and ^{13}C NMR. On the basis of these analyses, berry development was correlated with an increase in proanthocyanidin mean degree of polymerization, an increase in the proportion of (−)-epigallocatechin extension subunits, and increases in the level of anthocyanins associated with the proanthocyanidin fraction. Additionally, data acquired from ESI-MS of the isolates following acid-catalysis in the presence of excess phloroglucinol is consistent with pectin-bound proanthocyanidins.

Keywords: *Proanthocyanidin; tannin; anthocyanin; pigmented tannin; pectin; polygalacturonate; galacturonic acid; composition; Vitis vinifera; grape; berry development*

INTRODUCTION

Proanthocyanidins (condensed tannins) are polymeric flavonoid compounds widely distributed in the plant kingdom including the grape (*Vitis vinifera* L.). Present in skin (*1*), seed (*2*), and stem (*3*) tissues of the fruit and extracted during red wine making (*4*), proanthocyanidins provide red wine with astringency (*5*, *6*) and are considered to be important to the long-term color stability of red wine (*7*, *8*). Because these attributes contribute so much to overall red wine quality (*9*), an understanding of the relationship between proanthocyanidin composition and the sensory properties of red wine is critically important.

Grape skin tissue contains, in addition to proanthocyanidins, other flavonoid compounds including flavonols and anthocyanins (*10*). As a consequence of these components, crude extracts of skin proanthocyanidins are typically complex. With the uncertain nature of existing purification techniques, completely defining skin proanthocyanidins and the extraneous material associated with them has been historically difficult.

The need to more fully characterize skin proanthocyanidins is understood when the color of the purified material is considered. Proanthocyanidins obtained from commercially ripe red grape varieties and purified by adsorption chromatography are red in appearance (*7*). The red color is considered to be due to associated anthocyanins, which are difficult to completely remove from proanthocyanidins. It is unclear whether these associations are covalent or noncovalent. Furthermore, if there are covalent associations present, it is unclear whether the association is an artifact of isolation or whether pigmented proanthocyanidins (pigmented tannins) are actually formed in the grape during development (*7*, *11*). Making this determination is of fundamental importance in wine science because of the increasing role of pigmented tannin, with age, to overall red wine color. If the formation of pigmented tannin does take place during berry development, and if the polymeric form is extracted during winemaking, vineyard management practices could become an important aspect of red wine color stability.

Another example of the need to more completely characterize skin proanthocyanidins is demonstrated by their retention properties relative to other proanthocyanidins when analyzed by normal-phase high performance liquid chromatography (*12*, *13*). When fractions with the same elution times of grape skin and seed proanthocyanidins are collected and analyzed by thiolysis, their apparent mean degree of polymerization is very dissimilar. This suggests that there are aspects of proanthocyanidin composition that are not understood and which could affect their sensory properties.

The development of the grape berry follows a double sigmoid growth pattern (*14*), with each growth period differing considerably in biochemical activity and subsequent berry composition (*15*). On the basis of grape seed polyphenols (*16*), the proanthocyanidins are present during both growth periods. From this, it was concluded that studying skin proanthocyanidin composition during these growth periods could provide gross information on the compositional changes that proanthocyanidins

* Corresponding author (Tel: +1-541-737-9150; fax: +1-541-737-1877; e-mail: James.Kennedy@orst.edu; current address: Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331).

† Adelaide University.

‡ Cooperative Research Centre for Viticulture.

§ The Australian Wine Research Institute.

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undergo during fruit ripening. Studying proanthocyanidin composition in the grape provides knowledge of their composition prior to extraction into wine, and this study is thus designed to investigate anecdotal observations about the association of grape quality with wine quality (*17*, *18*).

MATERIALS AND METHODS

Chemicals. All chromatographic solvents were HPLC grade and were purchased from BDH (Kilsyth, Vic., Australia). Potassium metabisulfite was also purchased from BDH. (+)-Catechin, (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, and ascorbic acid were purchased from Sigma (Castle Hill, NSW, Australia). Phloroglucinol and sodium acetate were purchased from Aldrich (Castle Hill, NSW, Australia), while the flavan-3-ol-4-phloroglucinol adducts were isolated and characterized as previously described (*19*).

Proanthocyanidin Isolation. *Vitis vinifera* L. cv. Shiraz (syn. Syrah) grape berries grown during the 2001 harvest season on the Waite Campus at the University of Adelaide were used as the source material for proanthocyanidins. Samples were collected at three specific times during berry development: three weeks after fruit set, véraison (the commencement of fruit ripening), and commercial maturity. The sample collected at véraison was divided into unpigmented and pigmented berries. All samples (∼5 kg) consisted of randomly collected fruit clusters within the same vineyard block.

Briefly, proanthocyanidin purification was carried out as follows. Isolated skins were extracted in 2:1 acetone/water. Afterward, acetone was removed under reduced pressure, and the aqueous portion was extracted with chloroform, freeze-dried to a powder, and purified by adsorption chromatography using Toyopearl TSK HW 40-F size exclusion media (Sigma; Castle Hill, NSW, Australia). Adsorbed proanthocyanidins were rinsed (∼3 column volumes) with 1:1 methanol/water containing 0.1% v/v trifluoroacetic acid to remove low molecular weight contaminants. The sample collected at commercial maturity was rinsed with an additional 7 column volumes of this solvent in an attempt to remove apparent anthocyanin impurities. After rinsing the samples, proanthocyanidins were eluted with 2:1 acetone/water with 0.1% v/v trifluoroacetic acid added. The acetone was evaporated, and the aqueous residue was freeze-dried to a powder. With the exception of the chloroform extraction, proanthocyanidin isolation and purification has been previously described (*19*).

Acid-Catalysis in the Presence of Phloroglucinol. Proanthocyanidin subunit composition, apparent mean degree of polymerization (mDP), and conversion yield were determined by acid-catalysis in the presence of excess phloroglucinol. This procedure in addition to analysis and quantitation of products has been described previously (*19*).

^{13}C NMR Spectroscopy. Purified proanthocyanidins (100 mg/mL, 1:1 acetone-d_6/D_2O) were characterized by ^{13}C NMR (150 MHz, Varian Inova), with chemical shifts (δ) in ppm referenced internally with acetone-d_6. The proton-decoupled, inverse-gated sequence, with 80° pulse length (12.5 μs), 36 000 Hz spectral width, 10 K data points, 1.5 s acquisition time, relaxation delay of 4 s, 64 K scans, and 5 Hz line broadening was carried out at a temperature of 298 K.

Mass Spectrometry. Mass spectrometric analyses were carried out with a API-300 mass spectrometer equipped with an electrospray ion source (PE Sciex, Thornhill, Ontario, Canada). Individual proanthocyanidin isolates were dissolved (100 μg/mL) in methanol/acetonitrile (1:1) and then directly infused into the electrosprayer of the electrospray ion source at a flow rate of 5 μL/min using a syringe pump (Cole-Parmer, Vernon, IL). Proanthocyanidins were also directly infused (100 μg/mL, 5 μL/min) after acid-catalysis in the presence of phloroglucinol followed by dilution with methanol/acetonitrile (1:1). Electrospray mass spectra ranging from *m*/*z* 200 to 3000 were taken in positive ion mode and negative ion mode with a step size and dwell time of 0.2 amu and 0.5 ms, respectively. The electrospray needle potentials were set at 5000 V for positive mode and −4500 V for negative mode and the orifice potentials varied between ± 60 V and ± 120 V. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 12 units, respectively. The ion signals were consecutively accumulated (10 scans) using multichannel-acquisition mode within Sample Control software 1.3 version (PE Sciex).

Gel Permeation Chromatography. Proanthocyanidins were analyzed by gel permeation chromatography (GPC) using a method similar to a previously published method (*20*), with some exceptions. Two columns were connected in series (TSKgel G3000 H_{XL} particle size 6 μm followed by G2500 H_{XL} particle size 5 μm, both 300 × 7.8 mm i.d.) and were protected by a guard column (TSKgel H_{XL}-L, particle size 5 μm, 40 × 6 mm i.d.) purchased from Supelco (Castle Hill, NSW, Australia). Separation was carried out under isocratic conditions using dimethylformamide (0.6 mL/min) as the mobile phase. Run time was 40 min, and eluting peaks were monitored at 280 and 520 nm. The chromatographic system was calibrated using polystyrene, catechin, and malvidin-3-glucoside standards.

UV/Vis Spectrophotometry. Solutions of pigmented proanthocyanidins (5 g/L) were prepared in aqueous 0.1 N HCl and in 50% aqueous acetone or methanol (0.1 N HCl). Spectra were recorded as soon as practically possible to eliminate potential interference by formation of anthocyanidin degradation product. Aqueous proanthocyanidin solutions were also exposed to potassium metabisulfite as previously described (*21*). All spectra were obtained using a Cary 100 spectrophotometer (Varian, Palo Alto, CA).

Elemental Analysis. For C, H, and N determination, approximately 2 mg of sample was accurately weighed (0.1 μg) into a tin cup. The cup was sealed and compressed before placing it into a Carlo Erba EZ 1108 elemental analyzer (CE Instruments, Milan, Italy). Elemental analysis was determined in the normal mode of operation.

RESULTS AND DISCUSSION

Four skin proanthocyanidin isolates were obtained from berries sampled at distinct stages of berry development (*16*): green berries sampled 3 weeks after berry set corresponding to Eichorn and Lorenz (E-L) growth stage 29 (*22*) (green berries), green berries at véraison (véraison-green), red berries at véraison (véraison-red), and red, commercially ripe berries (red berries).

The isolates varied in their appearance: two (green and véraison-green berries) were light buff in color, while the véraison-red and red berries were distinctly red, with the red berries being redder in appearance despite additional rinsing during purification. The red appearance suggested that anthocyanins were present either as strongly associated noncovalent contaminants or as covalent adducts.

Proanthocyanidin Composition. The four isolates were subjected to acid catalysis in the presence of excess phloroglucinol to determine proanthocyanidin subunit composition. Under these conditions, the isolates were converted into (−)-epigallocatechin-(4β → 2)-phloroglucinol (**1**), (−)-epicatechin-(4β → 2)-phloroglucinol (**2**), (+)-catechin-(4α → 2)-phloroglucinol (**3**), (−)-epicatechin-3-*O*-gallate-(4β → 2)-phloroglucinol (**4**), and (+)-catechin (**5**) (Figure 1, Table 1), similar to previous results obtained for cv. Shiraz (*19*, *23*). In contrast to previous reports (*1*), the terminal subunits (−)-epicatechin and (−)-epicatechin-3-*O*-gallate were either insignificant or absent in all isolates.

For extension subunits, the proportion of (−)-epigallocatechin was lower (25.1%) for green berries than for the remaining isolates (∼29.3%). This change in skin proanthocyanidin extension subunit composition during berry development is in contrast to that of seed proan-

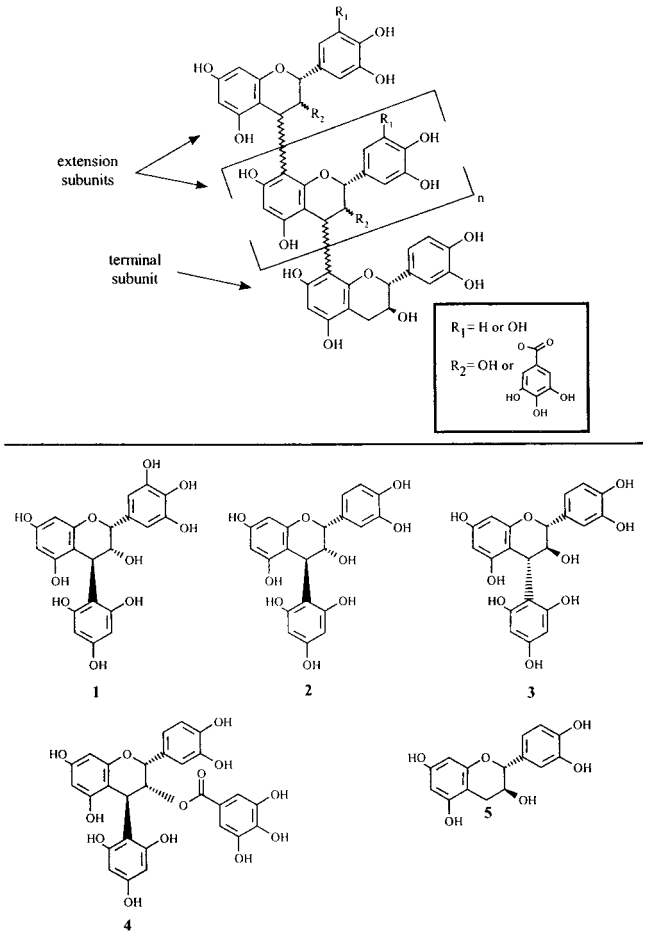

Figure 1. Generalized skin proanthocyanidin structure and subunit products found in *Vitis vinifera* L. berries following acid-catalysis in the presence of phloroglucinol.

Table 1. Summary of Grape Skin Proanthocyanidin Composition Following Acid Catalysis in the Presence of Phloroglucinol ± Standard Deviation (n = 5)

compound	green berries	véraison green	véraison red	red berries
1	21.7[a] ± 0.1	26.7 ± 0.1	26.3 ± 0.2	28.4 ± 0.2
2	56.3 ± 0.1	56.5 ± 0.1	57.3 ± 0.2	60.3 ± 0.2
3	3.1 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.5 ± 0.1
4	5.2 ± 0.1	4.7 ± 0.1	4.7 ± 0.1	5.1 ± 0.2
5	13.8 ± 0.1	9.4 ± 0.1	8.8 ± 0.1	3.7 ± 0.1
mDP[b]	7.3 ± 0.1	10.6 ± 0.1	11.3 ± 0.1	27.0 ± 1.0
% yield[c]	83.3 ± 1.0	79.7 ± 2.2	68.4 ± 1.0	71.3 ± 1.2

[a] Values are given in proportional composition (mole %). [b] Mean degree of polymerization. [c] Conversion yield into known proanthocyanidin subunits (by mass).

thocyanidins isolated from the same variety, which were invariant (*16*).

Proanthocyanidin Proportion. On the basis of conversion yields from the acid-catalysis/phloroglucinol assay, the isolates were confirmed to be predominantly proanthocyanidin in nature. Beginning with green berries and ending with red berries, the conversion yield decreased from 83 to 70% by mass. This reduction in conversion yield with berry development has been observed in grape seed proanthocyanidins (*16*) and suggests that skin proanthocyanidins are affected by berry development.

Proanthocyanidin mDP. Various methods have been used to determine the mDP of proanthocyanidins (*24*), and in this study, several techniques were used. These analyses indicated that the average molecular weight of proanthocyanidins increased with berry development.

From the acid-catalysis/phloroglucinol assay, molecular weight increase was seen as an increase in the proportion of extension subunits relative to terminal subunits (mDP increase of 3.7 times from greed to red berries). The mDP for red berries (27.0) is in general agreement with previously observed values for cv. Shiraz grape skin proanthocyanidins (*23*), which varied from 27 to 33 (determined by thiolysis).

^{13}C NMR studies, in addition to providing structural information, indicated trends in mDP. Here, the proportional increase in mDP during berry development was seen as a change in the proportion in the C-3 signal of extension subunits relative to that of the terminal subunits (δ 67 versus δ 72 ppm, respectively) [Figure 2] (*25*). For green berries, the calculated mDP was determined to be 4.5, varying somewhat from the mDP of 7.3 determined by the acid catalysis/phloroglucinol assay. Possible explanations for this discrepancy are that the integrated values for C-3 of the subunits is incorrect, possibly due to the downfield shifts created by galloylation (*26*) or variations in T_1 effects. Conversely, the presence of (4 → 6) interflavonoid bonds, which would be more resistant to acid catalysis (*27*), could have contributed to the discrepancy if a significant proportion of terminal subunits were linked in this way. Unfortunately, the mDP for proanthocyanidins isolated from more developed berries could not be determined with any accuracy because of the weak terminal subunit C-3 signal. Additional evidence obtained by ^{13}C NMR that indicated that the proanthocyanidins increased in average molecular weight during berry development was the general decline in sharp signals and a concomitant broadening of peaks with increased berry development (*28*).

From the GPC data, there was a proportional increase in early eluting material as the berry developed, also indicating an increase in average molecular weight (Figure 3). This technique has been used to analyze underivatized proanthocyanidins (*20*), although from the results here, it is difficult to determine the exact molecular weight distribution because of differences between the retention properties of polystyrene standards and the polyphenols (~10× based on the limited data set). In addition, a "dip" in the chromatograms occurred, suggesting a bimodal proanthocyanidin distribution, inconsistent with previous results for proanthocyanidins (*29*). Nevertheless, the results indicate that an increase in molecular weight distribution was associated with berry development. First, the convex elution pattern (the region of the chromatogram between points **1** and **2** in Figure 3) of lower molecular weight material observed in green berries became increasingly concave with berry development. Second, the proportion of high molecular weight material increased with berry development. On the basis of an arbitrarily selected integration division at ~18 min, the proportion of high molecular weight material increased in the order: 27.7, 37.4, 42.5, and 59.5% for green, véraison-green, véraison-red, and red berries, respectively.

GPC also provided information not obtainable by the other analytical methods. For véraison-green and véraison-red berries, material eluting at ~29 min was consistent with a flavan-3-ol monomer, indicating that the elimination of low molecular weight material during sample cleanup was incomplete. This suggests that for

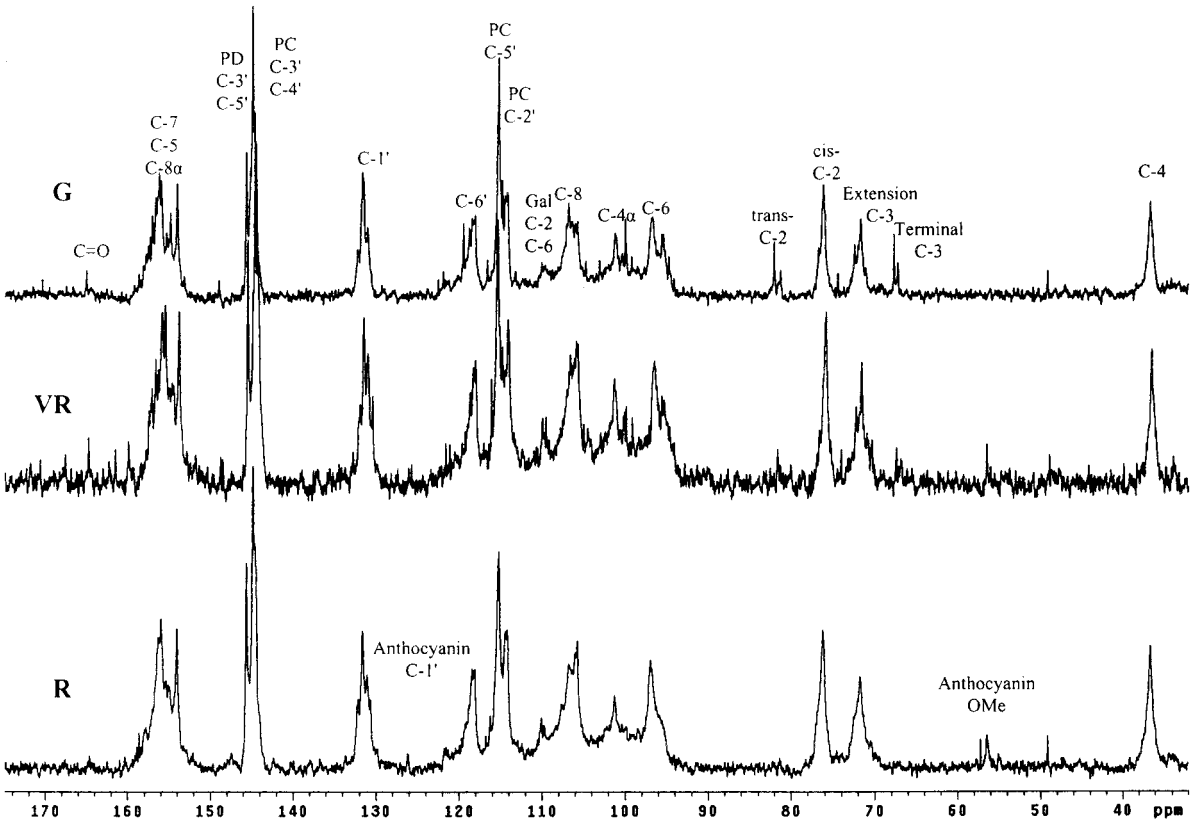

Figure 2. ^{13}C NMR spectra for skin proanthocyanidins isolated from green (G), véraison-red (VR), and red berries (R). Structural features are indicated.

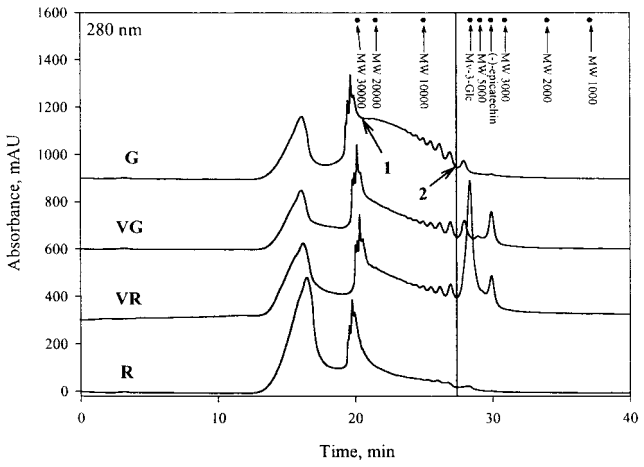

Figure 3. GPC chromatogram of skin proanthocyanidins isolated from green (G), véraison-green (VG), véraison-red (VR), and red berries (R) and monitored at 280 nm. Retention times for polystyrene molecular weight standards are indicated, while the vertical line indicates the integration cutoff for the polymeric material.

these two samples, the mDP was higher than predicted by the acid catalysis/phloroglucinol assay.

As a further indicator of changes in average molecular weight, the ionization intensity by ESI-MS of proanthocyanidin isolates declined with berry development (data not shown). Considering that the isolates had a similar composition, a decline in ESI-MS intensity suggests an increase in mDP. All of these data, when combined, provide strong evidence that the mDP of proanthocyanidins increased during berry development.

Partial Characterization of Material Associated with Proanthocyanidins. An increase in the proportion of uncharacterized material was observed during berry development based on differences in conversion yield in the acid-catalysis/phloroglucinol assay of isolates into known proanthocyanidin subunits. An attempt was made to characterize this portion of the isolates.

Pigmentation. The pigmentation was the most obvious portion of the isolates that was not proanthocyanidin, and differences in conversion yield were associated with differences in physical appearance. That is, red-pigmented proanthocyanidins (véraison-red and red berries) had a lower conversion yield than unpigmented proanthocyanidins (green and véraison-green berries). The véraison samples were of particular interest because the corresponding berries were collected at nearly the same stage of berry development (véraison-green berries not obtained from second crop). Interestingly, while the subunit compositions and mDP were very similar for véraison-green and véraison-red berries, the conversion yields were considerably different. Because of the importance of pigmented polymers to long-term red wine color stability, the pigmentation in véraison-red and red berries was investigated to determine its nature.

On the basis of their spectral properties, the pigmentation in véraison-red and red berries was not the same, and both samples were different from previously published data for pigmented tannins (*7*). For pigmented tannins isolated from cv. Shiraz, Somers (*7*) reported the following: $\lambda_{max} = 535-540$; $\lambda_{min} = 475-480$; $E_{min}/E_{max} = 0.79$; $E^{1\%}_{1cm} = 24$. Although the λ_{max} for isolated samples was similar in both studies, the λ_{min} was considerably different. In the present study, the λ_{min} resembled the absorption spectra of anthocyanins more so than those of the pigmented tannin isolates observed

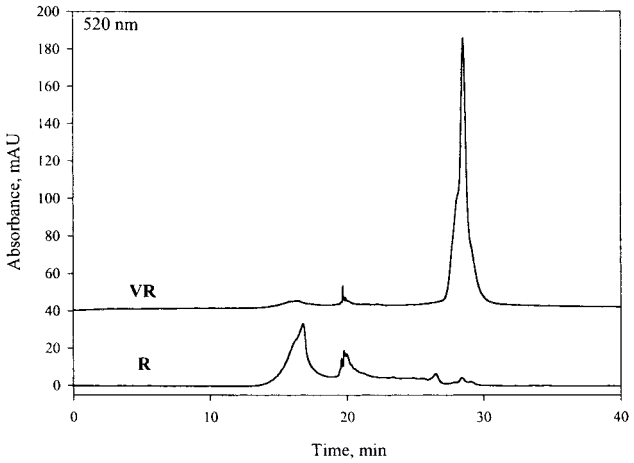

Figure 4. GPC chromatogram of pigmented skin proanthocyanidins isolated from véraison-red (VR) and red berries (R) and monitored at 520 nm.

by Somers (*7*). One explanation for the difference, as noted by Somers (*11*), is that in the process of isolation under acidic conditions, degradation of tannin and/or anthocyanin is occurring. The extraction procedure used in the present study was not acidic, and therefore degradation was probably minimized. The E_{min}/E_{max} of 0.79 reported by Somers (*7*) was considerably higher than the present samples (0.18 and 0.34 for véraison-red and red berries respectively), and based on the changes in E_{min}/E_{max} during the aging of wine, an increase in this value is associated with advanced age and presumably degradation (*7*, *30*). From spectral evidence, the results indicate that the pigmentation, in the current study, is not the same as the pigmentation observed in previously investigated tannins.

The spectral properties of isolates obtained from véraison-red and red berries were compared with anthocyanins (*31*). In the presence of potassium metabisulfite, aqueous proanthocyanidin solutions from véraison-red and red berries were rapidly (<1 s) decolorized, leaving a colorless solution for véraison-red and a pale persistent pink-yellow color for red berries. These results are consistent with the behavior of anthocyanins. Also, the E_{440}/E_{vismax}% value of an acidic methanol solution of the véraison-red berry isolate (26%) was similar to malvidin-3-glucoside (20%), the major anthocyanin in cv. Shiraz (*32*). For red berries, however, the value was considerably different (45%). The λ_{max} for véraison-red (528 nm) and red berries (526 nm) in aqueous solution were similar to anthocyanins, although bathochromically shifted from malvidin-3-glucoside (*33*). This could be explained by the effect of co-pigmentation between anthocyanins and proanthocyanidins (*34*).

Additional analyses confirmed that the proanthocyanidin pigmentation was different for véraison-red and red berries. By GPC (Figure 4), the earlier elution of pigment in red berries provided very good evidence for differences in molecular weights of these pigments. From ESI-MS of véraison-red and red berry isolates following acid-catalysis in the presence of phloroglucinol, and for véraison-red berries (Figure 5A) signals at *m*/*z* 493 and 639 corresponding to the $[M]^+$ ions of malvidin-3-glucoside and its 6″-coumarate ester were present, consistent with the presence of anthocyanin. These signals were absent in red berries (Figure 5B). Significantly, readily identifiable signals that could be associated with anthocyanin−proanthocyanidin adducts were not observed in red berries.

A comparison of véraison-red and red berry with green berry isolates by ^{13}C NMR spectroscopy (Figure

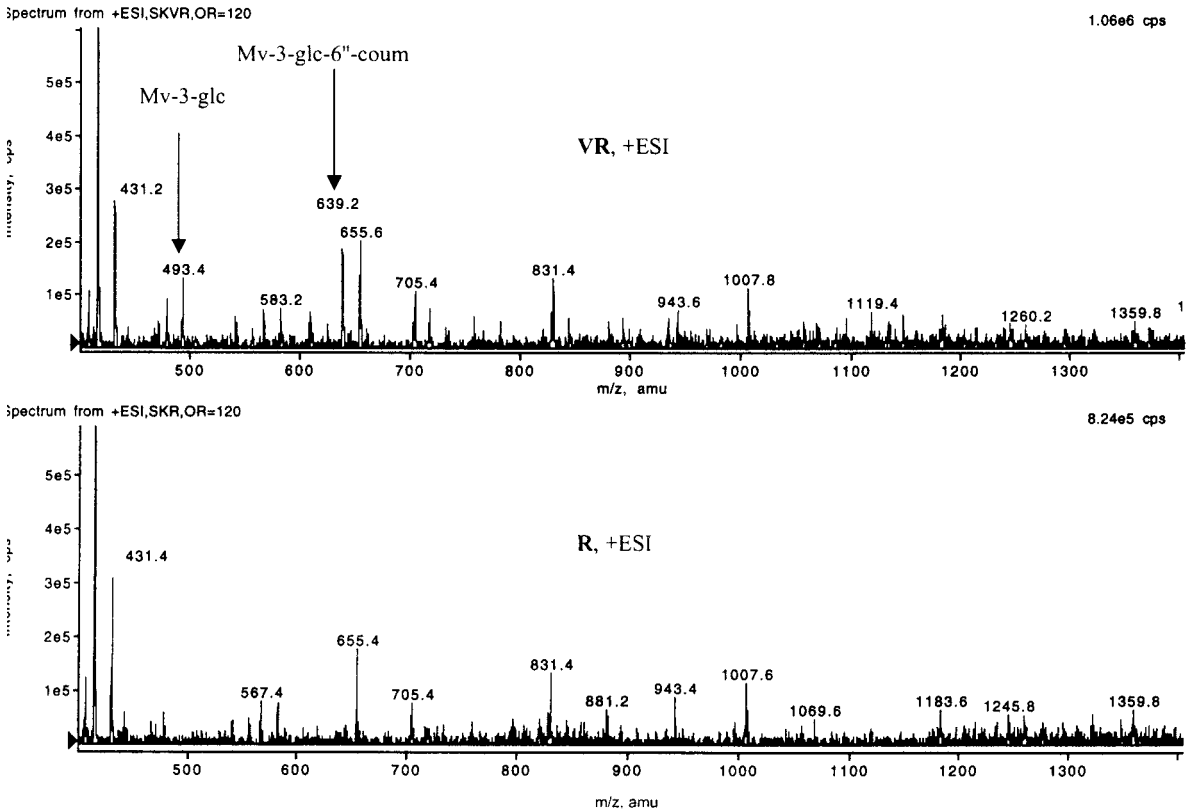

Figure 5. ESI mass spectra of véraison-red (VR) and red berry (R) skin proanthocyanidins after acid catalysis in the presence of phloroglucinol and with ion monitoring in the positive ion mode.

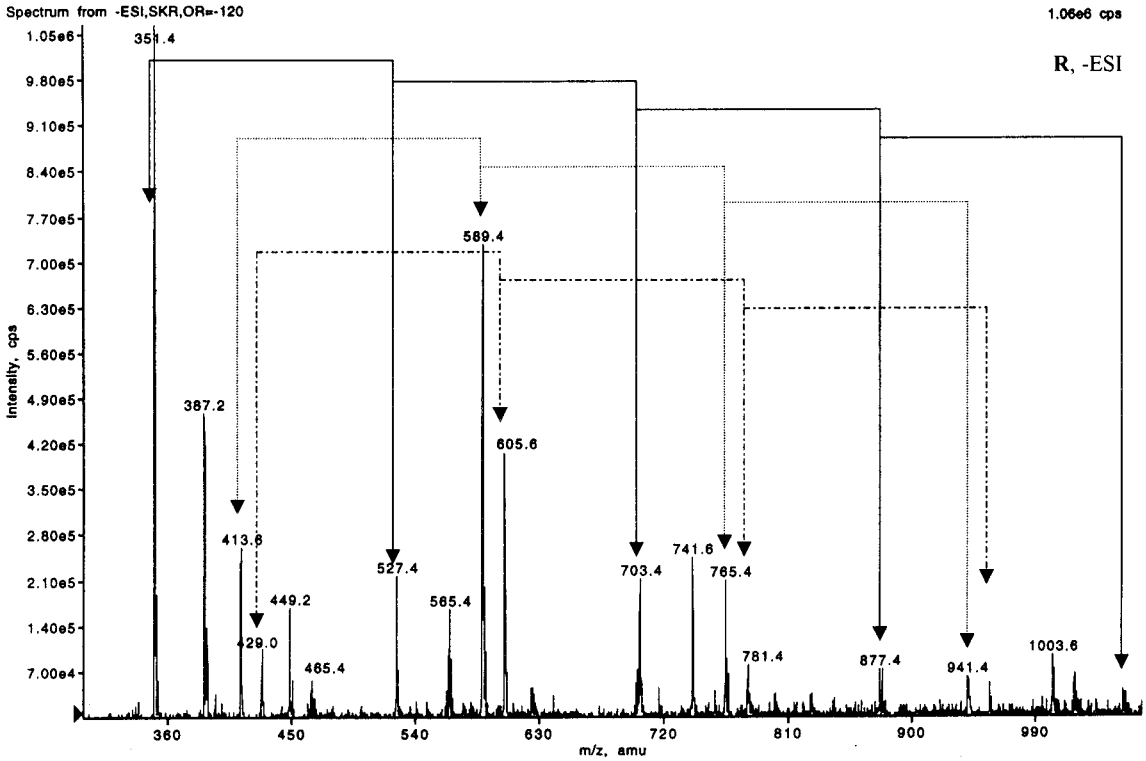

Figure 6. ESI mass spectra of red berry skin (R) proanthocyanidins after acid catalysis in the presence of phloroglucinol with ion monitoring in the negative ion mode, and 176 mass unit separation between vertical lines within each series.

2) indicated that there were additional signals present (indicated on red berry isolate) in the pigmented isolates. Specifically, the signals at δ 56.5 and 57.0 ppm are consistent with the methoxy signal of malvidin-3-glucoside (*35*). On the basis of broadness of the signal envelope, the signal in red berries is associated with a polymer, whereas for véraison-red the assignment is much more speculative, consistent with the results obtained from GPC. Because of its sharpness, it is likely to arise from monomeric anthocyanin. Another signal, which is consistent with C-1′ of the anthocyanin pseudobase, was also observed. Other signals that could be associated with an anthocyanin were obscured by the proanthocyanidin signals.

These data indicate that monomeric anthocyanins were probably responsible for the pigmentation in véraison-red berries. On the basis of GPC and ^{13}C NMR, the data also indicate that the pigmentation in red berries is polymeric. The formation of pigmented proanthocyanidin in the grape is suggested by these data. However, these results do not exclude the possibility that covalently bound pigmentation is an artifact. In the absence of in vivo observations, pigmented proanthocyanidin formation in the grape is likely to remain speculative given the general reactivities of proanthocyanidins and anthocyanins. Nevertheless, regardless of whether pigmented proanthocyanidins are formed in the grape, these data are consistent with the pigmentation in red berries being covalently linked. The rapid bleaching of red berries by bisulfite ion suggests that most of the presumed anthocyanin is unsubstituted at position 4. On the basis of the proposed connectivity between anthocyanins and proanthocyanidins (*36*), a reasonable conclusion from the current results would be that the anthocyanins in red berries are covalently associated with proanthocyanidins through the A-ring (C-8 or C-6) of the anthocyanin. However, there is some evidence that this linkage is unstable under the acidic conditions employed in the phloroglucinol assay (*37*), suggesting that an alternative linkage between the polymeric material and anthocyanins exists.

Assuming that the pigmented proanthocyanidin isolates contain anthocyanins, and given the known molar absorptivity of malvidin-3-glucoside (*38*), an estimate of the amount of pigment present in the proanthocyanidins was made based on pigment absorption in acidified methanol. The calculated amount of pigment associated with the proanthocyanidin was approximately 1−2.5%. These are highly approximate numbers, but based on them, the anthocyanin (flavylium form) contribution to the unknown material was negligible.

Other Associated Material. Elemental analysis indicated that the isolates contained very little nitrogen (0.03−0.14%). Therefore, little if any proteinaceous material was present. Additionally, despite the differences in conversion yield, there was little difference in sample C (54.08−54.85%) and H (4.10−4.18%) content and with no apparent trend, indicating that the associated material had a similar C/H/O composition to the isolates. Additionally, it was noted that the C/H content differed from previous results for proanthocyanidin polymers, which have a C and H content of 52.6% (C) and 5.3% (H) for hydrated polymers and 60.0% (C) and 4.2% (H) for anhydrous polymers (*25*).

GPC analysis of equal concentrations of the individual proanthocyanidin isolates showed that the integrated peak area at 280 nm declined during development. The peak area for red berry at 280 nm was 85.4% that of the green berry, similar to the differences in conversion yield (red berry was 84.0% of green berry). This suggests that the differences in conversion yield were due to non-280 nm absorbing material.

Because a significant portion of the proanthocyanidin isolates was unknown and because of the potential importance of the extraneous material, further ESI-MS experiments on the acid-catalyzed/phloroglucinol iso-

lates were conducted (Figure 6). The data revealed several ionization series separated by a mass difference of 176 units within each series. This mass spectral pattern is consistent with pectin (*39*), and given its abundance in plant cell walls including the grape (*40*), this evidence strongly suggests that pectins composed of repeating D-galacturonic acid subunits are present in the isolates. Specifically, the presence of a series beginning at *m*/*z* 351, which is consistent with the expected mass for the $[M-H]^-$ ion for a galacturonic acid dimer minus H_2O, is present. The fact that the masses of these fragments are 18 mass units less than those expected for galacturonic acid oligomers suggests that these fragments originated from larger molecules.

Two particularly interesting series were also present in the ESI-MS spectra. Beginning at *m*/*z* 413, which is consistent with the expected mass for the $[M-H]^-$ ion for compounds **2** and **3**, and *m*/*z* 429, which is consistent with the expected mass for the $[M-H]^-$ ion for compound **1**, sequential increases of 176 mass units are consistent with the covalent association of pectins with proanthocyanidins. Parent and daughter ion scans were also consistent with pectin being covalently associated with proanthocyanidins (data not shown). Attempts to resolve the discrepancy in the elemental composition of isolates by incorporation of pectin were unsuccessful. Possible explanations for the discrepancy could be varying degrees of hydration and/or the presence of inorganic cations. In addition to providing additional evidence to confirm that covalent association of pectins with proanthocyanidins is grape derived, additional information is needed to resolve the elemental composition of the isolates.

The association of proanthocyanidins with polysaccharides has been proposed by others (*41*, *42*). However, the extractability of the associated material has been questioned. The present results are consistent with covalently associated proanthocyanidins and pectins being extracted. Given the ability of pectins to modify the perception of astringency of phenolics (*43*, *44*), these results have important implication for red wine quality. Furthermore, the existence of pectins in proanthocyanidin isolates in addition to the apparent association of anthocyanins with polymeric material raises fundamental questions about the nature of the bonding between anthocyanins and the polymeric material.

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