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Berry Integrity and Extraction of Skin and Seed Proanthocyanidins during Red Wine Fermentation

FIORELLA K. CERPA-CALDERÓN AND JAMES A. KENNEDY*

Department of Food Science and Technology, Oregon State University, Corvallis, Oregon 97331

In this study, microscale fermentations were conducted on *Vitis vinifera* L. cv. Merlot. Five treatments were established varying from 0−100% crushed fruit (25% increments × 5 replicates). Caps were kept submerged throughout the experiment, and fermentation temperatures were maintained at 25 °C. Samples were collected throughout fermentation and from the free run and press wine at the time of pressing. Proanthocyanidins were determined by acid-catalyzed depolymerization in the presence of phloroglucinol, followed by reversed phase, high performance liquid chromatography (RP-HPLC). Total proanthocyanidin extraction increased with time in all treatments. In addition, crushing increased the rate at which proanthocyanidins were extracted. When the extraction of skin and seed proanthocyanidins were monitored separately, skin proanthocyanidin extraction rate exceeded that for seed proanthocyanidins and followed a Boltzmann sigmoid extraction model. The highest proanthocyanidin concentration for skin (435 mg/L) and seed (344 mg/L) was observed for the 75% crushed fruit treatment at the time of pressing (17 days). The highest skin proanthocyanidin proportion (79%) was observed for the 75% crushed fruit treatment on day 9 with a total proanthocyanidin concentration of 439 mg/L. For all treatments, skin proanthocyanidin extraction reached a plateau concentration prior to pressing, with the plateau concentration increasing with crushing. Seed proanthocyanidin concentration increased throughout maceration.

KEYWORDS: Proanthocyanidin; phloroglucinolysis; maceration; extraction; red wine fermentation

INTRODUCTION

Flavonoids are a group of phenolic compounds found in many plants and plant products including grapes and wine. The most relevant flavonoids in red wine from a wine quality standpoint include the flavan-3-ols, anthocyanins, and their reaction products. Flavan-3-ols exist as monomers, and their polymers (proanthocyanidins), are found in grape skin, seed and stem tissue, and are produced in the berry during the first phase of berry growth (*1*−*3*). Anthocyanins are almost exclusively restricted to the grape skin tissue and accumulate during fruit ripening (*4*).

Seed proanthocyanidins consist of (+)-catechin (C), (−)-epicatechin (EC), and (−)-epicatechin-3-*O*-gallate subunits (ECG) (*1*). Skin proanthocyanidins, in addition to C, EC, and ECG, contain (−)-epigallocatechin (EGC) subunits (*2*). Subunits are linked by C4−C8 and/or C4−C6 interflavonoid bonds, and skin proanthocyanidins have a higher degree of polymerization and a lower proportion of ECG subunits than those found in seed (*1, 2*).

Combining chemical and sensory information, it is predicted that the balance of bitterness in a grape berry is seed-derived because of the presence of flavan-3-ol monomers. The bitterness in seed tannins appears to be related to the relative proportion of low molecular weight tannins (*5*). The relative lack of ECG within the skin proanthocyanidin structure together with the presence of EGC has been speculated to confer a softening effect on the mouthfeel of wine (*6, 7*).

Because of the importance of proanthocyanidins to red wine quality and because of the varied sources of proanthocyanidins and their predicted variation with regard to sensory impact, research in this area has been a perennial interest (*8*−*15*). These studies have varied from the general analysis of total phenolic extraction into wine (*8, 11*) to the specific analysis of proanthocyanidin extraction from individual skin and seed tissues (*9, 10, 12*−*15*). Despite the considerable amount of research, historically it has not been possible to monitor both skin and seed proanthocyanidin extraction simultaneously during fermentation and maceration.

Recently, a procedure was developed that allows for the simultaneous monitoring of skin and seed proanthocyanidin extraction (*16*). This method has been used to investigate the influence of various grape and wine production practices on skin and seed proanthocyanidin composition in wine including prefermentation soak (*16*), vine vigor (*17*), fruit shading (*18*), fruit maturity (*19*), variety (*20*), and small-scale fermentations (*21*). The objective of this study was to understand the influence of berry integrity on the extraction of skin and seed proanthocyanidins using this analytical approach in conjunction with a small-scale fermentation technique.

* Corresponding author. Tel: +1-541-737-9150. Fax: +1-541-737-1877. E-mail: james.kennedy@oregonstate.edu.

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MATERIALS AND METHODS

Grapes. *Vitis vinifera* L. cv. Merlot (~100 kg) grapes harvested during the 2006 growing season and obtained from the Oregon State University experimental vineyard, located in Alpine, Oregon were harvested on October 6th. The selection of cv. Merlot was based upon the desire to be able to remove berries from the rachis without compromising berry integrity. For fruit analysis, 10 clusters were selected randomly and stored at −35 °C until processed.

Chemicals. All solvents were HPLC grade. Acetonitrile, ethanol, methanol, glacial acetic acid, ascorbic acid, potassium metabisulfite, and potassium hydroxide were purchased from J. T. Baker (Phillipsburg, NJ). Phloroglucinol, (+)-catechin hydrate, and (−)-epicatechin were purchased from Sigma (St. Louis, MO). Orthophosphoric acid (85% w/w) and hydrochloric acid (37% w/w) were purchased from E. M. Science (Gibbstown, NJ). Anhydrous sodium acetate and ammonium phosphate monobasic were purchased from Mallinckrodt (Phillipsburg, NJ). Distilled water was purified to HPLC grade using a Millipore Milli-Q water purification system (Bedford, MA).

Instrumentation. An Agilent 1100 HPLC (Palo Alto, CA) consisting of a vacuum degasser, autosampler, quaternary pump, diode array detector, and column heater was used. A computer workstation with Chemstation software was used for chromatographic analysis. Soluble solids were measured using an Atago model WM-7 digital wine refractometer (Bellevue, WA). An Innova model 2300 platform shaker from New Brunswick Scientific (Edison, NJ), Labconco centrivap concentrator (Kansas City, MO), and Büchi model R-205 rotary evaporator (New Castle, DE) were used for the extraction and concentration of phenolics.

Proanthocyanidin Extraction from Fruit. Frozen berries were separated from the cluster stem, and random samples of 150 berries were collected (×5 replicates) and weighed. Seeds and skins were separated from the berry and then separately rinsed with water, weighed, freeze-dried, and then reweighed. The number of seeds was recorded. Each replicate of seeds and skins were then placed separately into 250 mL Erlenmeyer flasks with 2:1 v/v acetone/water (1 mL/g fresh berry weight). Flasks were covered with aluminum foil and placed on a platform shaker for 24 h at room temperature. Extracts were then filtered through Whatman #1 filters and evaporated under reduced pressure at 35 °C using a water aspirator, to remove acetone. The aqueous solution was adjusted to a volume of 100 mL with water and stored at −20 °C until analyzed.

Analysis of Grape Proanthocyanidins. For analysis, 5 mL of skin or seed extract was dried using a centrivap concentrator and then dissolved in methanol (2 mL). Phloroglucinolysis reagent was prepared as described previously (*22*), although the concentration of phloroglucinol and HCl was doubled. Ascorbic acid was excluded from the reagent after analyses indicated that it could be excluded without compromising sample integrity. For reaction, 1 mL of seed or skin methanolic extract was combined with 1 mL of reagent and reacted at 50 °C for 20 min. To stop the reaction, one volume of this solution was then combined with five volumes of 40 mM aqueous sodium acetate. Cleavage products were analyzed by reversed-phase HPLC as described previously (*23*). Proanthocyanidin concentration, composition, and mean degree of polymerization (mDP) were determined as previously described (*22*).

Winemaking. A microscale fermentation method described by Sampaio et al. (*21*) was used for fermenting fruit. Fruit was sorted and destemmed by hand to avoid crushing and then divided into 25 uniform lots. Five treatments were established (×5 replicates, 2.8 kg fruit/replicate), with each treatment containing a different proportion of crushed berries (0−100%, 25% increments). A hand-operated crusher (Mini 40 × 40, Marchisio S.P.A., Italy) was used to crush the proportion of fruit within each treatment requiring crushing. Following crushing, the crushed berries had broken skins, but the seeds remained within the berry. For each replicate, the fraction of crushed fruit was combined and mixed with the corresponding fraction of whole berries. Each replicate of fruit was placed into the fermentor with dry ice (~100 g, to sparge the system of oxygen) and 50 mg/L sulfur dioxide. Fermentors were then equipped with fermentation traps, kept at 10 °C for 36 h, were then allowed to warm to 20 °C, and finally inoculated with yeast (QA23, Lallemand Inc., CA, USA) following the manufacturer's instructions. Musts were fermented at 25 °C, and once caps had formed, they were kept submerged with the help of a plastic screen.

Table 1. Summary of Grape Berry Data (Means ± SEM, $n = 5$)

berry weight (g/berry)	1.30 ± 0.02
soluble solids at harvest (°Brix)	23.3
fresh skin weight (mg/berry)	132.25 ± 5.77
dry skin weight (mg/berry)	27.87 ± 2.02
fresh seed weight (mg/seed)	63.00 ± 0.60
dry seed weight (mg/seed)	44.30 ± 0.58
seed per berry	1.71 ± 0.01

Fermentation progress was monitored daily with a digital hydrometer and thermometer (DMA 35N, Anton Paar, Austria). Samples were collected every two days from the center of the fermentation vessels, through the air-lock opening using a 50 mL plastic syringe fitted with a 40 cm polypropylene tube. Samples were taken throughout fermentation and on the day of pressing (15 days after inoculation), and were stored at −20 °C until analyzed. Pressing was performed using a 2000 mL Erlenmeyer filtration flask equipped with a 6000 mL Büchner funnel. The pomace was transferred to the funnel and covered with a high density plastic sheet. After securing the plastic sheet with a rubber band, a pressure of 1.7 bar was applied in 10 cycles of 2 min each, with stirring of pomace between cycles. The free run and press wine volumes were recorded. Free run fractions were kept separate from press wine.

Analysis of Proanthocyanidins in Wine. For wine proanthocyanidin analysis, 5−10 mL wine was dried in a centrivap concentrator. After drying, extracts were dissolved in water and applied to a C18-SPE column (1 g Alltech) after activation with 12 mL of methanol followed by 12 mL of water. After sample application, the column was washed with 6 mL of water and eluted with 10 mL of methanol.

For phloroglucinolysis, the methanolic isolate was evaporated under reduced pressure (using a water aspirator) at 40 °C, reconstituted with 2 mL of methanol, and then treated as described above for seed and skin extracts. The proportion of seed and skin proanthocyanidin extracted into wine was calculated using a previously described method (*16*). The percent skin proanthocyanidin extracted from the fruit into the wine was calculated on the basis of the relative proportion of EGC and EC extension subunits as compared to the skin proanthocyanidin.

Statistical Analyses. Statistical analysis of data was performed using analysis of variance (ANOVA) and the least significant difference (LSD) test to determine statistically different values at a significance level of $\alpha = 0.05$ or less. All statistical analyses were performed using Statgraphics Plus version 5.1.

RESULTS AND DISCUSSION

Grape Data. The average berry weight for harvested fruit was 1.3 g/berry (**Table 1**). Upon the basis of the average soluble solids (23.3 °Brix), the fruit would likely be considered by most to be under-ripe from a commercial standpoint. However, it was decided to harvest the fruit in order to avoid dilution of soluble solids due to rainfall. Fresh and dry skin weights are also shown in **Table 1**. Seeds were slightly green and averaged 1.71 per berry.

Seed and skin proanthocyanidin concentration and composition were determined in berry extracts to provide information on the potential proanthocyanidin contents available for extraction and also to provide requisite compositional information (**Table 2**). The concentration of skin and seed proanthocyanidin was 846 mg/kg and 644 mg/kg fruit, respectively. The ratio of seed/skin proanthocyanidin was much lower than the reported values for other varieties (*17, 24*). Variable ratios have been reported for Merlot, both higher and lower (*25, 26*). Many factors could have led to the observed differences between the seed and skin proanthocyanidin including, for example, berry

Table 2. Skin and Seed Proanthocyanidin Composition (mol %), Concentration in Fruit, and Mean Degree of Polymerization (mDP)[a]

	extension[b]				terminal[b]				
	EGC	C	EC	ECG	C	EC	ECG	mg/kg fruit	mDP
skin	46.22 ± 0.01	1.45 ± 0.01	49.79 ± 0.01	2.55 ± 0.01	92.66 ± 0.01	4.86 ± 0.01	2.48 ± 0.01	845.78 ± 19.80	23.50 ± 1.61
seed		10.15 ± 0.01	73.80 ± 0.01	16.05 ± 0.01	39.20 ± 0.01	38.85 ± 0.01	21.94 ± 0.01	644.41 ± 83.83	5.23 ± 0.13

[a] Data are expressed as means ± SEM ($n = 5$). [b] Mole percent with the following subunit abbreviations: EGC: (−)-epigallocatechin, C: (+)-catechin; EC: (−)-epicatechin; ECG: (−)-epicatechin-3-O-gallate.

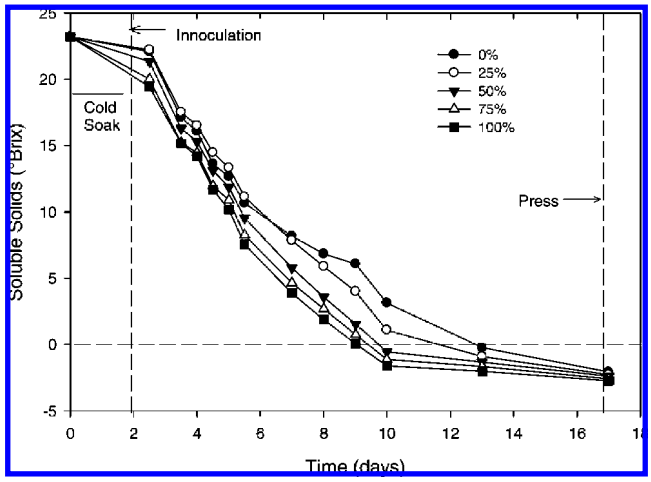


Figure 1. Influence of berry crushing on the progress of fermentation, with individual treatment curves shown: 0% crushed fruit (●), 25% crushed fruit (○), 50% crushed fruit (▼), 75% crushed fruit (Δ), and 100% crushed fruit (■).

size (*27*), postvéraison variation in seed tannins (*28*), and high yield (*29, 30*).

Compositionally and consistent with expectation, skin proanthocyanidins contained EGC, while those from seed did not (**Table 2**). Skin proanthocyanidins had a lower proportion of ECG than seed proanthocyanidins. Skin proanthocyanidin terminal subunits were almost exclusively (+)-catechin (C) as expected, with very small amounts of EC and ECG present as well. The skin extension subunits were composed mainly of EC but also contained EGC as mentioned. The amount of EGC was considerably higher than previously published data for cvs Carmenere (*20*), Pinot noir (*19*), and Shiraz (*24, 32*), but consistent with data published for Merlot (*2*). A high proportion of EGC in skin proanthocyanidins has been observed in other varieties including Cabernet Sauvignon and Cabernet Franc (*32, 33*). Skin proanthocyanidin mean degree of polymerization (mDP) was higher than seed proanthocyanidin mDP (**Table 2**). This observation appears to be a generally observed difference across several varieties (*14, 17, 19, 22, 28, 32*).

For seed proanthocyanidins, terminal subunits were composed of C and EC (~40 mol % each) and ECG (~20 mol%). Extension subunits were composed mostly of EC. These results are consistent with previous work (*28*).

Fermentation. It was important for wines to be made in a consistent manner so that differences in wine composition could be attributed to the percentage of crushed fruit used, and it was found that microscale fermentations could provide that level of control (*21*). Fruit was crushed on day 0 and yeast was pitched on day 2 (**Figure 1**). Fermentation rates for all treatments were similar until day 5 when, at that point, increased crushing led to an increase in fermentation rate. Overall, the analysis of replicates indicated very good reproducibility, consistent with previous work using this method (*21*).

Table 3. Volume of Free Run and Press Wine (mL, ± SEM ($n = 5$))

treatment	free run wine	press wine
0% crush	645 ± 23	1151 ± 14
25% crush	741 ± 6	1141 ± 11
50% crush	818 ± 19	1104 ± 16
75% crush	863 ± 15	1055 ± 11
100% crush	1052 ± 23	945 ± 26

On day 13, when the soluble solids for all treatments fell below 0 °Brix, a decision was made to continue macerating for four more days in order to increase phenolic extraction. Wines were pressed on day 17. The relative volume of press wine

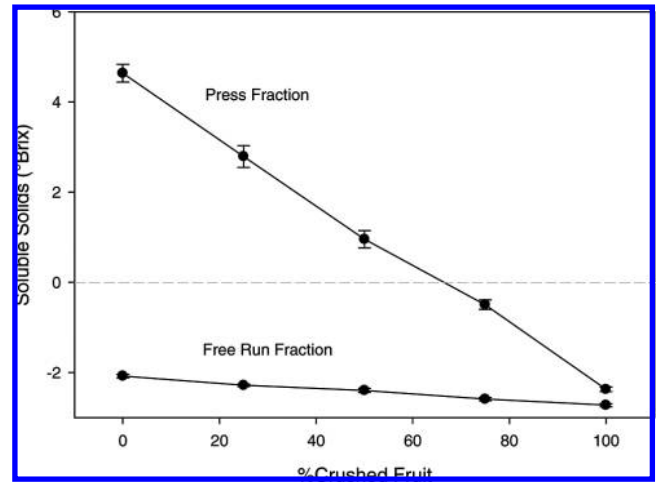


Figure 2. Soluble solids versus % crushed fruit for the different treatments at the time of pressing ($N = 5$, ± SEM).

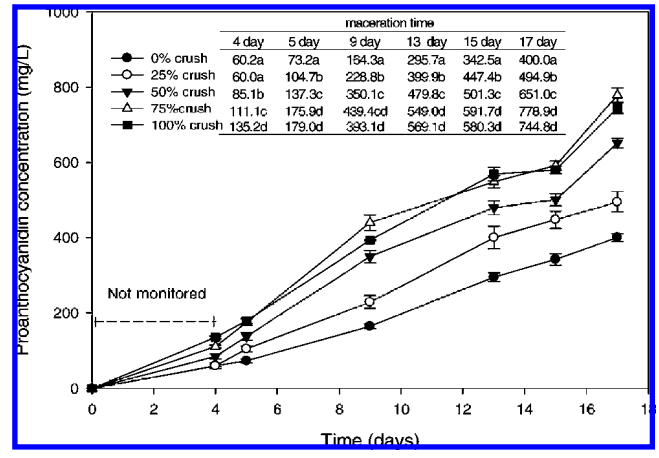


Figure 3. Total proanthocyanidin concentration in samples collected during fermentation. Five treatments were conducted with different proportions of whole and crushed fruit: 0% crushed fruit (100% whole berries) (●), 25% crushed berries (○), 50% crushed berries (▼), 75% crushed berries (Δ), and 100% crushed berries (■). Data are expressed as the means ($N = 5$, ± SEM), and values sharing the same letter within each column are not significantly different at $p = 0.05$ or greater.

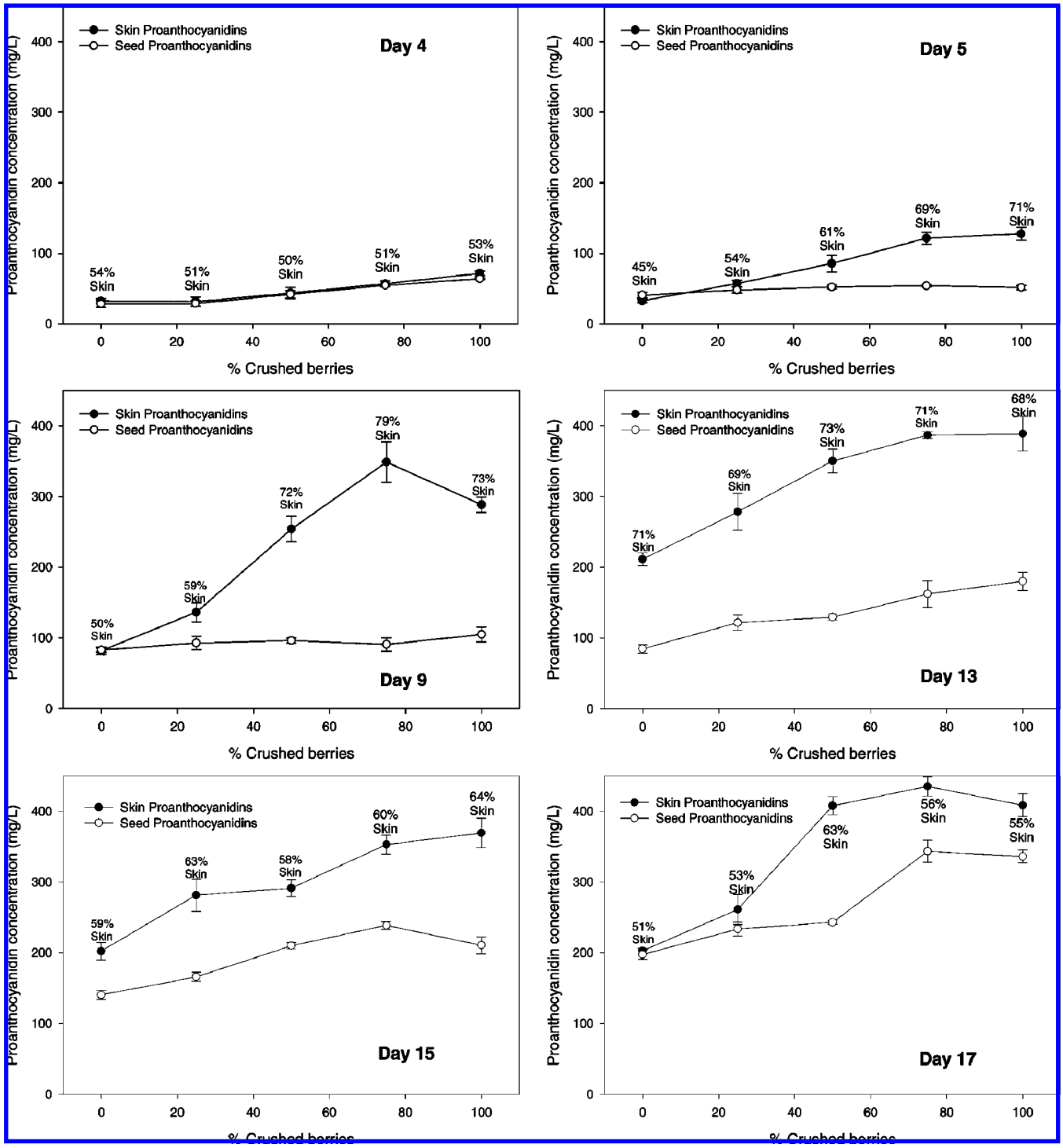
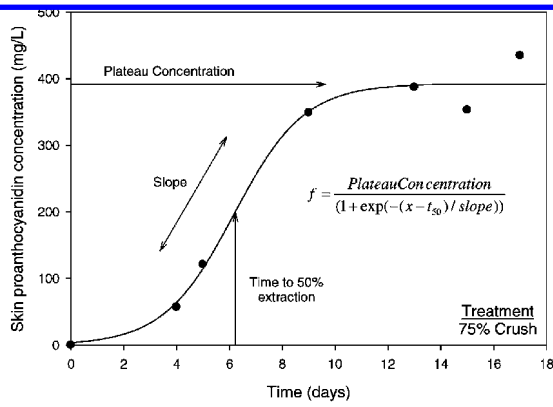


Figure 4. Skin and seed proanthocyanidin concentrations (mg/L) as a function of crushed berry percentage for samples collected during fermentation. Data are expressed as means ($N = 5$, ± SEM). Values indicated over skin proanthocyanidin data represent the percent contribution of skin proanthocyanidins to the total proanthocyanidins in the wine.

fractions were considerably higher than what would normally be expected for a commercial fermentation (**Table 3**). As these were small scale fermentations and the cap was submerged and undisturbed, it was somewhat anticipated that the press volume proportion would be higher. Nevertheless, it was felt that a comparison of the free and press fractions could provide meaningful information about the presumed proanthocyanidin extraction inside and outside of the grape berry.

Consistent with the large proportional volume of press wine, a significant quantity of berries remained whole at the time of pressing. The proportion of whole berries increased with a reduction in the level of crushing. Along with this observation, the soluble solids in press fractions were higher in treatments with more whole berries (**Figure 2**). The soluble solids for the press fractions varied from 4.6 to −2.4 °Brix. Based solely on fermentation progress, the observed increase in soluble solids with a reduction in crushing suggests that lower phenolic extraction would be present in the treatments with less crushed berries.

Proanthocyanidin Extraction during Fermentation. The total proanthocyanidin concentration was analyzed on days 4, 5, 9, 13, 15, and 17 (**Figure 3**). Before day 4, it was not possible to determine proanthocyanidin concentration due to insufficient



treatment	plateau concentration (mg/L)	time to 50% extraction (days)	slope	model fit (R)
0% crush	219.5	9.4	2.08	0.9864
25% crush	287.4	8.7	2.09	0.9911
50% crush	358.5	7.4	1.89	0.9575
75% crush	392.3	6.2	1.33	0.9818
100% crush	398.3	6.9	2.06	0.9931

Figure 5. Boltzmann sigmoid extraction model for skin proanthocyanidin extraction with relevant parameters and their values for the individual treatments indicated.

liquid in the treatment with 0% crushed fruit. On day 4, 48 h after inoculation, differences between treatments existed despite the low overall extraction. By day 17, the highest proanthocyanidin extraction was reached in the treatment with 75% crush (779 mg/L) followed by the 100% crush treatment (745 mg/L). For all treatments, there was a general increase in proanthocyanidin concentration with time. The rate of proanthocyanidin extraction increased with crushing, although for higher crush levels, the rate of extraction appeared to slow.

The general proanthocyanidin extraction pattern observed in all treatments (**Figure 3**) was consistent with the first portion of a two-term tannin extraction pattern summarized by Boulton (*34*). Although, most of the extraction data followed an exponential growth to maximum, the maxima were not observed within the monitoring period.

Various factors have been identified that influence the extraction of phenolic compounds including prefermentation cold soak, thermal vinification, solubility of specific phenolic compounds, diffusion out of the berry, fruit maturity, fermentation temperature, maceration time, alcohol concentration, enzyme usage, concentration of sulfur dioxide, cap management, berry size, seeds per berry, and the extent of berry breakage as seen in the present study (*16, 35, 36*). The extraction of phenolic compounds out of the grape and into the fermentor is consistent with a diffusion-controlled process. By diffusion, the compounds move from a region of high concentration toward a region of lower concentration. In this case, phenolic compounds diffuse from the plant cell into the wine, and the rate of diffusion should depend on factors such as temperature, molecular weight, concentration gradient, cell permeability, surface area, and ethanol concentration. Increasing these variables would be expected to positively influence the rate of diffusion except molecular size (*37, 38*).

In this study, the variable being investigated (degree of crushing) was one that is varied in wine production. We are not, however, inferring that the degree of crushing would be considered causal. There are many fundamental factors that vary with crushing (e.g., cellular integrity and diffusive currents). For example, although all fermentors had the same initial soluble solids concentration and final alcohol concentration (data not shown), the rate of alcohol production increased with crushing. Because of this, an increase in crushing likely resulted in an increase in alcoholic maceration. Rather than determine the causality for the observed differences in proanthocyanidin extraction, the major purpose of this study was to determine the differences in skin and seed proanthocyanidin extraction as a function of berry crushing.

Extraction of Skin and Seed Proanthocyanidins. The composition of extracted proanthocyanidins was determined by phloroglucinolysis, and the subunits present in grape tissue were also present in the samples. To provide information on the relative extraction of proanthocyanidins from the seed and skin tissues, the next portion of this study separated out these extraction curves upon the basis of the proanthocyanidin compositional data (data not shown) and using the method developed by Peyrot des Gachons and Kennedy (*16*).

When skin and seed proanthocyanidin extractions were determined separately (**Figure 4**), their individual extraction curves were different. Skin proanthocyanidin extraction was generally similar to the overall tannin extraction described by Boulton (*34*), although there was an apparent initial lag period. For seed proanthocyanidins, the extraction rate was similar for all treatments until day 9 at which point the rate of extraction increased. This observation is consistent with conventional observations on seed proanthocyanidin extraction where it is generally considered that late in fermentation, seed proanthocyanidin extraction increases. This increase is thought to be due to an increase in alcohol concentration per se, although direct evidence for this has not been presented.

Figure 4 compares skin and seed proanthocyanidin extraction over time with respect to crushing. Early seed and skin proanthocyanidin extraction was similar. Although early extraction was low, an increase in extraction rate was associated with an increase in crushed fruit. This is in agreement with previous studies and general understanding of extraction (*34, 37*). After day 4, the extraction of skin and seed proanthocyanidins deviated, when skin proanthocyanidin extraction rate increased relative to those found in seed.

For individual treatments, the highest proportion of skin proanthocyanidin extraction was reached near day 9 for 75% and 100% crushed fruit and around day 13 for the other treatments (**Figure 4**). The highest proportion of skin proanthocyanidin (79%) was found on day 9 for 75% crushed fruit. After day 13, the rate of extraction for skin proanthocyanidins slowed and eventually reached a plateau near 400 mg/L.

The general extraction of skin proanthocyanidins is consistent with the Boltzmann sigmoid equation as shown in eq 1:

$$f = \frac{Plateau\ Concentration}{(1+\exp(-(x-t_{50})/slope))} \quad (1)$$

where the concentration at time x is a function of the following: *Plateau Concentration* is the difference between the minimum (initially ~0 mg/L) and maximum concentration, t_{50} is the time that it takes to reach 50% of *Plateau Concentration*, and *slope* is the slope of the extraction curve. From **Figure 5**, the skin proanthocyanidin extraction with time is shown. From this, it can be seen that the model is a reasonable fit for extraction.

An interpretation for this model would be that the initially slow extraction represents the period of time required for the proanthocyanidins to diffuse out of the plant cell and into the fermentor. As crushing increases, the time required for the proanthocyanidins to diffuse out of the plant cell and the overall barrier to diffusion are reduced. Finally, a plateau concentration is reached where the proanthocyanidin concentration has reached an apparent maximum. This plateau is of interest because its

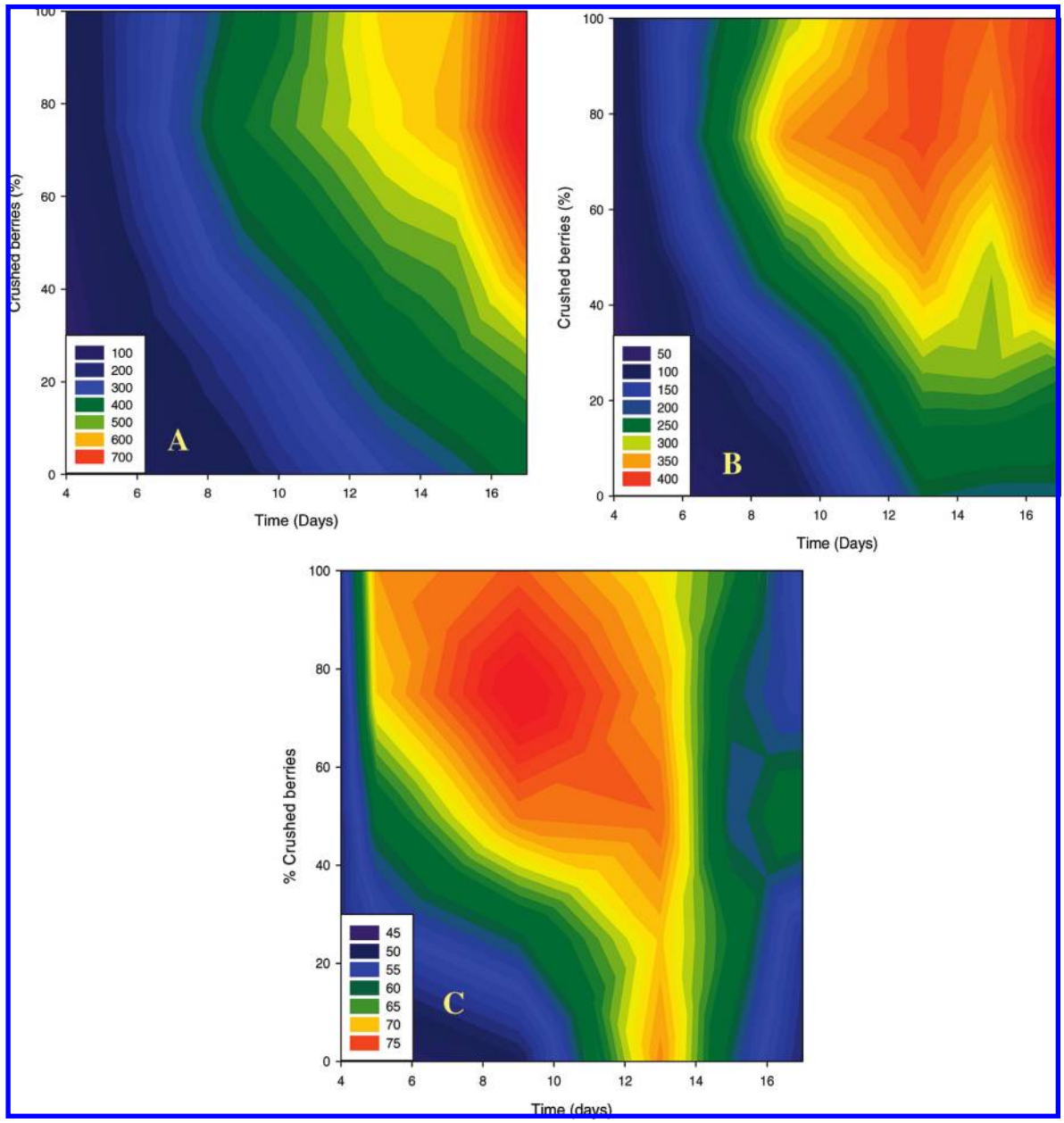


Figure 6. Total proanthocyanidin concentration (mg/L; **A**), skin proanthocyanidin concentration (mg/L; **B**), and percent skin proanthocyanidin (**C**) in samples collected as a function of time and % crushed berries.

value increased with crushing. One potential explanation for the observed plateau concentration increase with crushing could be that physical injury of fruit led to an acceleration in cell wall degrading enzyme activity and ultimately, cell permeability. The influence of mechanical injury on cell wall breakdown and phenolic extraction are not well understood. The increase in cell wall degradation upon mechanical injury has been observed in tomato (*39*) and given that enzyme-mediated cell wall disassembly is generally observed in fruits during ripening (*40*) could provide an explanation for the observed variation in the proanthocyanidin plateau concentration.

Although the initial seed proanthocyanidin extraction was similar to that observed in the skin tissue, it is uncertain if the Boltzmann sigmoid model would fit the observed data. The primary reason for this is that the seed proanthocyanidin extraction did not reach a plateau during the study period (**Figure 4**). There was, however, a pronounced lag period, and if a plateau is assumed, the Boltzmann sigmoid equation fits the data (data not shown).

Overall, **Figure 6** provides a good summary of proanthocyanidin extraction. The variables include total proanthocyanidin extraction (**Figure 6A**), skin proanthocyanidin extraction (**Figure 6B**), and % skin proanthocyanidin extraction (**Figure 6C**). As an example, if it is the desire of the winemaker to maximize the total proanthocyanidin as well as the proportion of skin proanthocyanidin, the results of this experiment indicate that increased fruit crushing and early pressing lead to a more desirable skin proanthocyanidin concentration and proportion. In this example, it is likely that the wine would have to be pressed prior to reaching dryness, and this could present a problem for assessing correct proanthocyanidin extraction. Moreover, an increase in crushing leads to acceleration in fermentation rate and this might not be desirable.

Historically, an understanding of total proanthocyanidin concentration in the fermentor has been the primary means for assessing astringency quality. It seems apparent from this study, that there can be not only a significant variation in quantity but also in composition of proanthocyanidins in red wine made from

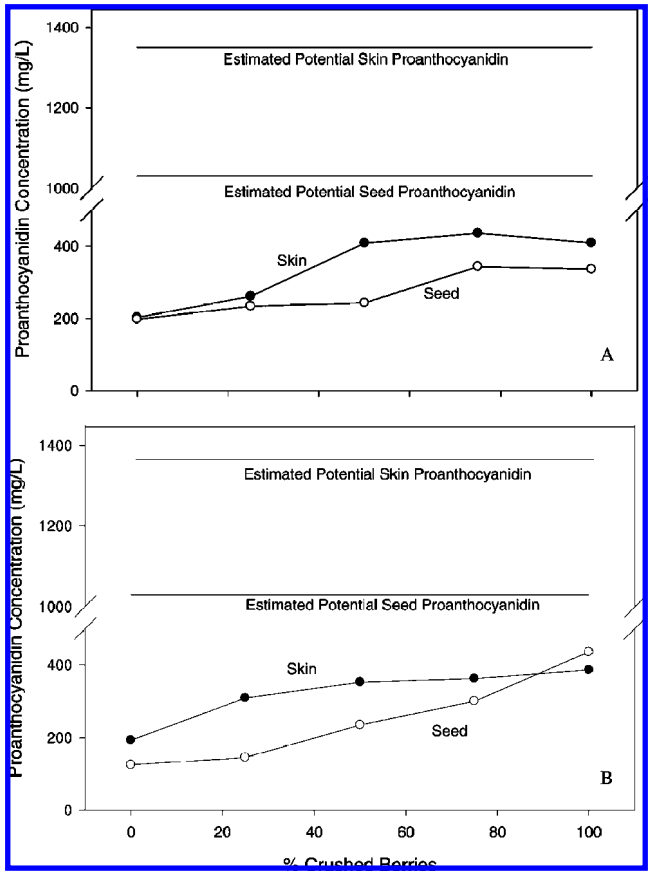


Figure 7. Skin and seed proanthocyanidin concentration (mg/L) of free run wine (**A**) and press wine (**B**) in comparison with the estimated potential proanthocyanidin concentration.

different levels of crushed berries. Upon the basis of the individual sensory properties of skin and seed proanthocyanidins (*5*−*7*), an understanding of total quantity and composition are needed in order to have a better understanding of the significance of grape-based proanthocyanidins to wine astringency quality.

Composition of Press Wine. The analysis of the press fraction was important because of the overall volume of press wine observed in this study (**Table 3**). As mentioned above, although the proportional volume of press wine in this study is higher than would be observed under commercial circumstances, this somewhat theoretical approach to berry crushing (i.e., small volumes, absolute whole berry, no cap management) provided a potential opportunity to compare the extraction dynamic between whole and broken berries assuming that press wine provided information on the proanthocyanidin content of juice contained within a berry.

From **Figure 7**, it was observed that the skin proanthocyanidin concentration in press fraction was similar to the free run fraction. Skin proanthocyanidin extraction for the free run and press wines increased with crushed fruit. In addition, above a 50% crush, the concentration of skin proanthocyanidin seemed to reach an apparent plateau in both free run and press wine. Because of the experimental conditions, it is likely that carbonic maceration occurred in the unbroken berries. It is likely that the carbonic maceration led to the partial breakdown of the berry tissue and that this then led to an increase in skin proanthocyanidin extraction within the grape berry (*34*).

In contrast to skin proanthocyanidins, seed proanthocyanidin extraction behaved differently for free run and press wines (**Figure 7**). For free run wines, there seemed to be only two levels of extraction of about 200 mg/L for 0, 25, and 50% crushed fruit, and around 340 mg/L for 75 and 100% crushed fruit. For the press wines, seed proanthocyanidin extraction increased linearly with the proportion of crushed fruit and at 100% crush was higher than the concentration of skin proanthocyanidin.

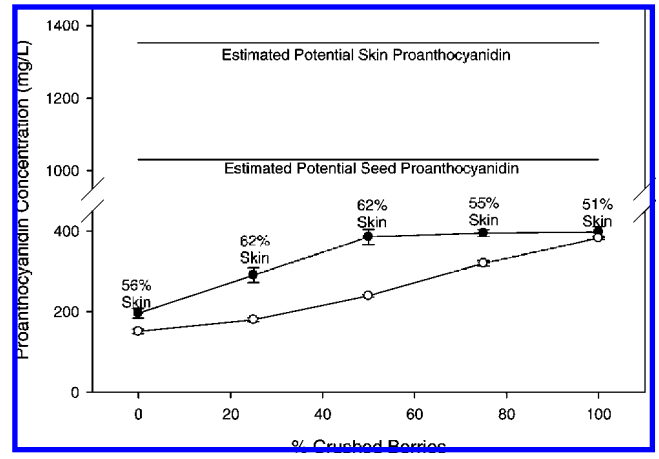


Figure 8. Influence of crushed berry percentage on the theoretical summation of press and free run wine for skin and seed proanthocyanidin extraction with the estimated potential proanthocyanidin extraction shown ($N = 5$, ± SEM). The value indicated over skin proanthocyanidin data represents the percent contribution of skin proanthocyanidins to the total proanthocyanidins in the wine.

The estimated theoretical skin and seed proanthocyanidin extraction was calculated from the grape data (**Table 2**). The yield of wine was assumed to be 0.71 L/Kg fruit on the basis of industry approximations. This conversion was near the observed volume (free run plus press wine) for this experiment (0.68 L/Kg, **Table 3**). Upon the basis of these data, the potential total concentration for skin and seed proanthocyanidin was 1351 mg/L and 1029 mg/L, respectively. At most therefore, only 32% of the available skin proanthocyanidin was extracted from the skin tissue. For seed proanthocyanidins, the maximum extraction reached by the 100% crush treatment was 42%.

Upon the basis of the volumes of the free run and press wine (**Table 3**), the combined concentration of skin and seed proanthocyanidin is shown in **Figure 8A** and **B**. Overall seed extraction increased linearly with crushing. Skin extraction could be described by two trends: extraction increasing linearly up to 50% crushed fruit and reaching an apparent plateau beyond. One potential explanation for the apparent plateau could be that the proanthocyanidins are becoming modified and their modification results in the formation of products that are no longer observable using this analytical approach. Upon the basis of previous work (*41*), the conversion yield of high molecular weight phenolic isolates to known proanthocyanidin subunits varies with maceration time, although this study found that the conversion yield increases with maceration time. Nevertheless, variations in conversion yield may influence the results of this study as suggested by Peyrot des Gachons and Kennedy (*16*).

Another potential explanation is that proanthocyanidins are becoming adsorbed onto the grape and yeast cell walls or are simply not available for extraction under wine conditions. The influence of cell walls or polysaccharides on the adsorption of proanthocyanidins has been investigated (*42*−*44*). In the grape, there is some evidence that proanthocyanidins can associate with cell wall material (*31, 45*), and as observed by others (*46*), it is clear that proanthocyanidin extraction during wine production is complex.

The maximum extraction from the skin did not exceed 400 mg/L, which is ∼30% of the total proanthocyanidin available

in the skins, similar to work conducted by Fournand et al. (*46*) (**Figure 8**). It was not clear from this experiment if a longer maceration would have resulted in the release of additional skin proanthocyanidins. On the contrary, it seems clear that seed proanthocyanidin extraction would have continued with additional time. The results of this study improve our understanding of how crushing influences the extraction of proanthocyanidins during fermentation and also provides some direction for influencing the composition of skin and seed-derived proanthocyanidins in wine. This research has also confirmed that additional research on understanding the barriers to proanthocyanidin extraction and/or retention is needed.

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Supporting Information Available: Seed and skin proanthocyanidin extraction data in tabular form for samples collected during fermentation and for free and press wines. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric proanthocyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781–784.
(2) Souquet, J. M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509–512.
(3) Souquet, J. M.; Labarbe, B.; Le Guernevé, C.; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems. *J. Agric. Food Chem.* **2000**, *48*, 1076–1080.
(4) Cacho, J.; Fernandez, P.; Ferreira, V.; Castells, J. E. Evolution of five anthocyanidin-3-glucosides in the skin of the Tempranillo, Moristel, and Garnacha grape varieties and influence of climatological variables. *Am. J. Enol. Vitic.* **1992**, *43*, 244–248.
(5) Cheynier, V.; Fulcrand, H.; Brossaud, F.; Asselin, C.; Moutounet, M. Phenolic composition as related to red wine flavor. In *Chemistry of Wine Flavor*; Waterhouse, A. L., Ebeler, S. E., Eds.; American Chemical Society: Washington, DC, 1998; 1−22.
(6) Vidal, S.; Francis, L.; Guyot, S.; Marnet, N.; Kwiatkowski, M.; Gawel, R.; Cheynier, V.; Waters, E. J. The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. *J. Sci. Food Agric.* **2003**, *83*, 564–573.
(7) Vidal, S.; Francis, L.; Noble, A.; Kwiatkowski, M.; Cheynier, V.; Waters, E. Taste and mouth-feel properties of different types of tannin-like polyphenolic compounds and anthocyanins in wine. *Anal. Chim. Acta* **2004**, *513*, 57–65.
(8) Berg, H. W.; Akiyoshi, M. The effect of contact time of juice with pomace on the color and tannin content of red wines. *Am. J. Enol. Vitic.* **1956**, *7*, 84–90.
(9) Singleton, V. L.; Draper, D. E. The transfer of polyphenolic compounds from grape seeds into wines. *Am. J. Enol. Vitic.* **1964**, *15*, 34–40.
(10) Meyer, B.; Hernandez, J. R. Seed tannin extraction in Cabernet Sauvignon. *Am. J. Enol. Vitic.* **1970**, *21*, 184–188.
(11) Ribereau-Gayon, P. Chemistry of Red Wine Color. In Advances in Chemistry Series 137; Chemistry of Winemaking; Webb, A. D., Ed.; American Chemical Society: Washington, DC, 1974; 50−87.
(12) Oszmianski, J.; Romeyer, F. M.; Sapis, J. C.; Macheix, J. J. Grape seed phenolics: Extraction as affected by some conditions occurring during wine processing. *Am. J. Enol. Vitic.* **1986**, *37*, 7–12.
(13) Kovac, V.; Alonso, E.; Bourzeix, M.; Revilla, E. Effect of several enological practices on the content of catechins and proanthocyanidins of red wines. *J. Agric. Food Chem.* **1992**, *40*, 1953–1957.
(14) Sun, B. S.; Pinto, T.; Leandro, M. C.; Ricardo-da-Silva, J. M.; Spranger, M. I. Transfer of catechins and proanthocyanidins from solid parts of the grape cluster into wine. *Am. J. Enol. Vitic.* **1999**, *50*, 179–184.
(15) Gonzalez-Manzano, S.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. *Anal. Chim. Acta* **2004**, *513*, 283–289.
(16) Peyrot des Gachons, C.; Kennedy, J. A. Direct method for determining seed and skin proanthocyanidin extraction into red wine. *J. Agric. Food Chem.* **2003**, *51*, 5877–5881.
(17) Cortell, J. M.; Halbleib, M.; Gallagher, A. V.; Righetti, T. L.; Kennedy, J. A. Influence of vine vigor on grape (*Vitis vinifera* L. cv. Pinot noir) and wine proanthocyanidins. *J. Agric. Food Chem.* **2005**, *53*, 5798–5808.
(18) Cortell, J. M.; Kennedy, J. A. Effect of shading on accumulation of flavonoid compounds in (*Vitis vinifera* L.) pinot noir fruit and extraction in a model system. *J. Agric. Food Chem.* **2006**, *54*, 8510–8520.
(19) Pastor del Rio, J. L.; Kennedy, J. A. Development of proanthocyanidins in *Vitis vinifera* L. cv. Pinot noir grapes and extraction into wine. *Am. J. Enol. Vitic.* **2006**, *57*, 125–132.
(20) Fernandez, K.; Kennedy, J. A.; Agosin, E. Characterization of *Vitis vinifera* L. Cv.Carménère grape and wine proanthocyanidins. *J. Agric. Food Chem.* **2007**, *55*, 3675–3680.
(21) Sampaio, T.; Kennedy, J. A.; Vasconcelos, M. C. Use of microscale fermentations in grape and wine research. *Am. J. Enol. Vitic.* **2007**, *58*, 534–539.
(22) Kennedy, J. A.; Jones, G. P. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J. Agric. Food Chem.* **2001**, *49*, 1740–1746.
(23) Kennedy, J. A.; Taylor, A. W. Analysis of proanthocyanidins by high-performance gel permeation chromatography. *J. Chromatogr., A* **2003**, *995*, 99–107.
(24) Downey, M. O.; Harvey, J. S.; Robinson, S. P. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Aust. J. Grape Wine Res.* **2003**, *9*, 15–27.
(25) Bourzeix, M.; Weyland, D.; Héredia, N. Étude des catéchins et des procyanidols de la grappe de raisin, du vin et d'autres dérivés de la vigne. *Bull. O.I.V.* **1986**, *669−670*, 1171–1254.
(26) Ribereau-Gayon, P. Evolution des composes phenoliques au cours de la maturation du raisin. II. Discussion des resultats obtenus en 1969, 1970 et 1971. *Conn. Vigne Vin* **1972**, *6*, 161–175.
(27) Roby, G.; Harbertson, J. F.; Adams, D. O.; Matthews, M. A. Berry size and vine water deficits as factors in winegrape composition: Anthocyanins and tannins. *Aust. J. Grape Wine Res.* **2004**, *10*, 100–107.
(28) Kennedy, J. A.; Matthews, M. A.; Waterhouse, A. L. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry* **2000**, *55*, 77–85.
(29) Bravdo, B.; Hepner, Y.; Loinger, C.; Cohen, S.; Tabacman, H. Effect of irrigation and crop level on growth, yield and wine quality of cv. Cabernet Sauvignon. *Am. J. Enol. Vitic.* **1985**, *36*, 132–139.
(30) Jackson, D. I.; Lombard, P. B. Environmental and management practices affecting grape composition and wine quality: A review. *Am. J. Enol. Vitic.* **1993**, *44*, 409–430.
(31) Kennedy, J. A.; Hayasaka, Y.; Vidal, S.; Waters, E. J.; Jones, G. P. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric. Food Chem.* **2001**, *49*, 5348–5355.
(32) Kennedy, J. A.; Matthews, M. A.; Waterhouse, A. L. Effect of maturity and vine water status on grape skin and wine flavonoids. *Am. J. Enol. Vitic.* **2002**, *53*, 268–274.
(33) Moutounet, M.; Rigaud, J.; Souquet, J. M.; Cheynier, V. Structural characterization of grape berry tannins: some examples of the effect of vine variety, "terroir", and vine training. *Bull. O.I.V.* **1996**, *783−784*, 433–443.
(34) Boulton, R. Red Wines. In *Fermented Beverage Production*; Lea, A. G. H., Piggott, J. R., Eds.; Kluwer Academic/Plenum: New York, NY, 2003; 107−137.

(35) Ribereau-Gayon, P.; Sudraud, P.; Milhe, J. C.; Canbas, A. Recherches technologiques sur les composes phenoliques des vins rouges. *Conn. Vigne Vin* **1970**, *4*, 133–144.

(36) Sacchi, K. L.; Bisson, L. F.; Adams, D. O. A review of the effect of winemaking techniques on phenolic extraction in red wines. *Am. J. Enol. Vitic.* **2005**, *56*, 197–206.

(37) Seader, J. D.; Henley, E. J. *Separation Process Principles*, 2nd ed.; John Wiley and Sons, Inc.: Hoboken, NJ, 2006; 66−116.

(38) Kennedy, J. A.; Peyrot des Gachons, C. Phenolic extraction in red wine production. *Pract. Winery Vineyard* **2003**, July/August, *38−46*.

(39) Van-Linden, V.; Ndaka-Sila, D.; Duvetter, T.; De-Baerdemaeker, J.; Hendrickx, M. Effect of mechanical impact-bruising on polygalacturonase and pectinmethylesterase activity and pectic cell wall components in tomato fruit. *Postharvest Biol. Technol.* **2008**, *47*, 98–106.

(40) Vicente, A. R.; Saladié, M.; Rose, J. K. C.; Labavitch, J. M. The linkage between cell wall metabolism and fruit softening: Looking to the future. *J. Sci. Food Agric.* **2007**, *87*, 1435–1448.

(41) Aron, P. M.; Kennedy, J. A. Compositional investigation of phenolic polymersisolated from *Vitis vinifera* L. cv. Pinot noir during fermentation. *J. Agric. Food Chem.* **2007**, *55*, 5670–5680.

(42) Le Bourvellec, C.; Guyot, S.; Renard, C. Non-covalent interaction between procyanidins and apple cell wall material. Part I. Effect of some environmental parameters. *Biochim. Biophys. Acta* **2004**, *1672*, 192–202.

(43) Le Bourvellec, C.; Renard, C. Non-covalent interaction between procyanidins and apple cell wall material. Part II: Quantification and impact of cell wall drying. *Biochim. Biophys. Acta* **2005**, *1725*, 1–9.

(44) Le Bourvellec, C.; Bouchet, B.; Renard, C. Non-covalent interaction between procyanidins and apple cell wall material. Part III: Study on model polysaccharides. *Biochim. Biophys. Acta* **2005**, *1725*, 10–18.

(45) Amrani-Joutei, K.; Glories, Y.; Mercier, M. Localization of tannins in grape berry skins. *Vitis* **1994**, *33*, 133–138.

(46) Fournand, D.; Vicens, A.; Sidhoum, L.; Souquet, J. M.; Moutounet, M.; Cheynier, V. Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages. *J. Agric. Food Chem.* **2006**, *54*, 7331–7338.

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