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Direct Method for Determining Seed and Skin Proanthocyanidin Extraction into Red Wine

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A method was developed for determining the amount of seed and skin proanthocyanidin extraction into wines by direct measurement. This method was based upon the analysis of proanthocyanidin cleavage products after acid catalysis in the presence of excess phloroglucinol. On the basis of the analysis of proanthocyanidin extracts from grape tissues, two observations were made as follows: (i) the seed and skin proanthocyanidin extension subunit compositions were considerably different from each other, and (ii) their composition did not vary with extraction time. Thus, by comparing the proportional extension subunit composition of proanthocyanidins in wine relative to their proportional composition in corresponding grape seed and skin, it was possible to determine the contribution of each to wine. To provide additional information, the procedure was used to investigate seed and skin proanthocyanidin extraction during commercial-scale fermentations that had undergone 4 or 10 day low temperature prefermentation skin contact prior to the onset of fermentation. The results for both fermentations indicated that the proportion of skin tannin declined during fermentation and also showed that at the end of fermentation the amount and proportion of skin tannin were the same.

KEYWORDS: Proanthocyanidin; tannin; extraction; red wine; *Vitis vinifera*; grape seed; grape skin

INTRODUCTION

Proanthocyanidins (condensed tannins) are polymeric flavonoid compounds and essential red wine constituents. Initially present in the solid part of the grape skin (*1*), seed (*2*), and stem (*3*), they are extracted during red wine production (*4*, *5*). Proanthocyanidins are primarily responsible for the astringent properties of red wine (*6*, *7*). Astringency varies with the degree of proanthocyanidin polymerization (*8*, *9*) and galloylation (*10*). Skin proanthocyanidins differ from seed proanthocyanidins by the presence of prodelphinidins, their higher degree of polymerization, and their lower proportion of galloylated subunits. Although proanthocyanidin amounts are greater in seeds, skin proanthocyanidins are considered to be more readily extracted because of their apparent localization (*11*). Because of the overall importance of proanthocyanidins to wine quality, it is important to understand the influence of winemaking practices on proanthocyanidin extraction from each grape part to fully understand their effect on the organoleptic properties of wine.

Numerous studies have investigated enological practices such as maceration time (*12*, *13*), enzyme treatment (*14*), and fermentation temperature (*15*) on proanthocyanidin extraction, but prior to this investigation, it was extremely difficult to determine the contribution of proanthocyanidins from each grape tissue to wine. Several authors have studied it indirectly by comparing proanthocyanidin amounts in wine made with or without pomace contact (*4*), by adding supplementary quantities of seed (*16*) or by isolating seed and stems from pomace after fermentation (*5*). All of these studies are of interest but are time consuming to investigate, difficult, and approximate.

The purpose of this investigation was to develop an analytical method for measuring proanthocyanidin extraction from seed and skin directly in wines, based upon the analysis of proanthocyanidin cleavage products after acid catalysis in the presence of excess phloroglucinol (*17*).

MATERIALS AND METHODS

Chemicals. Acetonitrile, and methanol were high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Santa Clara, CA). Also purchased from Fisher scientific were glacial acetic acid and sodium acetate. Phloroglucinol was purchased from Sigma (St. Louis, MO). (+)-Catechin (C) was purchased from Aldrich (St. Louis, MO). The distilled or reverse osmosis water used in all solutions was purified to HPLC grade using a Millipore Milli-Q water system (Bedford, MA). Purified proanthocyanidin extracts from grape seed and skin tissues were prepared as described previously (*17*).

Extraction of Proanthocyanidins into Model Wine. Isolated skins (8.8 g) and seeds (5.0 g) from Pinot noir grapes were combined with 35 (skin) or 45 mL (seed) of model wine solution (13% v/v ethanol, 3 g/L tartaric acid, pH 3.5, with NaOH) in 50 mL plastic centrifugation tubes equipped with screw caps. Extraction times were targeted to cover the range of extracted proanthocyanidin concentrations that would be expected to occur in an actual fermentation. For each extraction time, three replicates were analyzed for each tissue. After decanting the extract away from the grape tissues, extracts were prepared for analysis as described below.

Standard Addition of Proanthocyanidins. Purified seed and skin proanthocyanidins isolated from Pinot noir grapes were added to 10

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Table 1. Composition of Proanthocyanidin Extension Subunits Extracted into Model Wine at Specific Times ($n = 3$)

			extension subunit proportion[a]			
time[b]	concentration[c]	mDP[d]	EGC	C	EC	ECG
			seed			
24	478.1 ± 67.9[e]	1.78 ± 0.12		0.133 ± 0.010	0.787 ± 0.006	0.080 ± 0.013
48	978.4 ± 99.9	1.79 ± 0.09		0.146 ± 0.009	0.780 ± 0.002	0.074 ± 0.010
			skin			
24	445.2 ± 70.5	11.63 ± 2.04	0.273 ± 0.008	0.035 ± 0.004	0.677 ± 0.005	0.015 ± 0.004
48	579.7 ± 59.0	14.09 ± 0.55	0.267 ± 0.005	0.033 ± 0.001	0.680 ± 0.001	0.020 ± 0.001

[a] mol equivalent. [b] Hours. [c] mg/L catechin equivalents. [d] Mean degree of polymerization. [e] ± SD ($n = 3$).

mL of both a model wine and a red wine in five different proportions: 0/100, 25/75, 50/50, 75/25, and 100/0% of skin/seed proanthocyanidin. For each, their final concentration was 0.75 g/L.

Commercial Fermentations. Commercial Pinot noir wines were made at Willakenzie Estate winery (Oregon, U.S.A.). Grapes were harvested from one vineyard block at 24 °Brix, divided equally into two 2.7 metric ton open-top fermentors, and underwent a cold prefermentation skin contact time for 4 or 10 days prior to alcoholic fermentation. The pomace was punched down once a day during the prefermentation period and twice during alcoholic fermentation.

Wine samples were collected daily during alcoholic fermentation following punchdown and prepared as previously described, although caffeine was not used (*18*, *19*). Following sample preparation, isolated proanthocyanidins from wine were contained within a methanol solution.

Acid Catalysis in the Presence of Excess Phloroglucinol. Isolated proanthocyanidins underwent acid-catalyzed cleavage in the presence of phloroglucinol as previously described (*17*), with the following change: the cleavage reagent was concentrated to allow for dilution when combined with the proanthocyanidin containing methanol solution (1 volume of proanthocyanidin solution was combined with 1 volume of reagent).

Reversed Phase HPLC Analysis of Extracts. Following acid-catalyzed cleavage of proanthocyanidins, analysis of products was carried out by reversed phase HPLC using a previously described method (*20*).

Calculation of Skin and Seed Proanthocyanidin Proportion. At the time of harvest, a random berry sample was collected from the vineyard to be studied as previously described (*21*). Skins and seeds from berries were isolated, extracted (2:1 acetone:water, 24 h), and analyzed to determine the relative composition of epigallocatechin and epicatechin extension subunits (*22*). The proportion of skin and seed proanthocyanidin extracted during wine production was then determined by first determining the relative molar amounts of epigallocatechin and epicatechin extension subunits in the wine isolates and then comparing these results with those determined for the grape isolates.

Determination of Total Proanthocyanidin Amount in Wines. The monomeric flavan-3-ol concentration in wine (C and (−)-epicatechin (EC)) was determined (*23*) and subtracted from the sum of extension and terminal proanthocyanidin subunits determined by the phloroglucinol method (*17*) to provide an estimate of the total amount of proanthocyanidins extracted. This quantity was expressed in C equivalents.

RESULTS AND DISCUSSION

Proanthocyanidin Extraction from Grape Tissues. Previous studies have shown that the composition of proanthocyanidins can vary with respect to molecular weight (*1*, *2*). Given that the rate of proanthocyanidin diffusion out of grape tissue is expected to vary with molecular weight, this experiment was conducted to determine the effect of time on proanthocyanidin composition. The results indicated that the composition of proanthocyanidin extension subunits did not vary with extraction time at concentrations that would be expected in wine (**Table 1**), consistent with proanthocyanidin composition not varying with diffusion. This experiment was also conducted under shorter extraction times, and the results also indicated that the extension subunit composition is invariant with time (data not shown). Finally, a comparison of proanthocyanidin extension subunit composition obtained from model wine extracts compared favorably with proanthocyanidins obtained from 2:1 acetone:water extract solutions. It was important to make this determination given that the acetone:water solvent system was used for the extraction of grape tissues.

These results suggest that the compositional variability previously observed (*1*, *2*) may be due to adsorptive differences during chromatographic purification. In addition to the lack of variability with respect to diffusion observed in the current study, it was noted that the composition of proanthocyanidins from skin and seed was considerably different, consistent with previous studies (*22*, *24*).

Given that the extension subunit composition was invariant with extraction time and that the seed and skin extension subunit composition was considerably different, it was conceptually possible to determine the proportion of seed and skin proanthocyanidins extracted into red wine by comparing the proportional proanthocyanidin extension subunit composition in wine relative to the proportional extension subunit composition in the corresponding grapes.

Measurement of Proanthocyanidins in Wine after Standard Additions. Purified seed and skin proanthocyanidins isolated from Pinot noir grapes were added to both a model wine and a red wine in five different proportions (0/100, 25/75, 50/50, 75/25, and 100/0% skin/seed proanthocyanidins), with their final concentration corresponding to 0.75 g/L. The proanthocyanidins in these modified wines were isolated and analyzed. The proportional extension subunit composition determined in the modified model wines and red wines were then compared to the expected proportional extension subunit composition calculated from the purified seed and skin analysis (**Table 2**).

The values determined in both the model wines and the red wines were similar to the expected values for (−)-epigallocatechin (EGC) and EC, but they differed for C and (−)-epicatechin-3-*O*-gallate (ECG). A possible explanation for this observation could be that the minor amounts present for these extension subunits result in a higher measured error. Assuming this, only EGC and EC extension subunits were used in subsequent analyses. In this case, the measured values were very similar to the expected values (**Figure 1**). Thus, by measuring the relative molar amount of the extension subunit EGC and EC in wine, it should be possible to determine the relative proportion of seed and skin proanthocyanidins present in wines.

To determine the method reproducibility, an actual commercial-scale red wine fermentation was monitored during fermentation (discussion to follow). On the basis of triplicate analyses (**Table 3**), the analytical method was determined to be reproducible over a broad composition of must/wine.

Table 2. Proportional Extension Subunit Composition of Model Wines and Red Wines in Comparison with Expected Proportions after the Addition of Proanthocyanidins with Known Skin/Seed Proportion

skin/seed (wt %)	EGC			C			EC			ECG		
		result			result			result			result	
	expected	model	wine	expected	model	wine	expected	model	wine	expected	model	wine
0/100				0.093[a]	0.106	0.099	0.772	0.787	0.795	0.135	0.097	0.106
25/75	0.082	0.076	0.075	0.077	0.090	0.075	0.736	0.761	0.760	0.106	0.073	0.091
50/50	0.164	0.151	0.156	0.061	0.071	0.057	0.700	0.723	0.720	0.076	0.056	0.067
75/25	0.245	0.230	0.232	0.045	0.052	0.036	0.663	0.680	0.689	0.047	0.038	0.043
100/0	0.327	0.324	0.334	0.029	0.031	0.017	0.627	0.634	0.635	0.017	0.011	0.015

[a] Values are given in molar fraction.

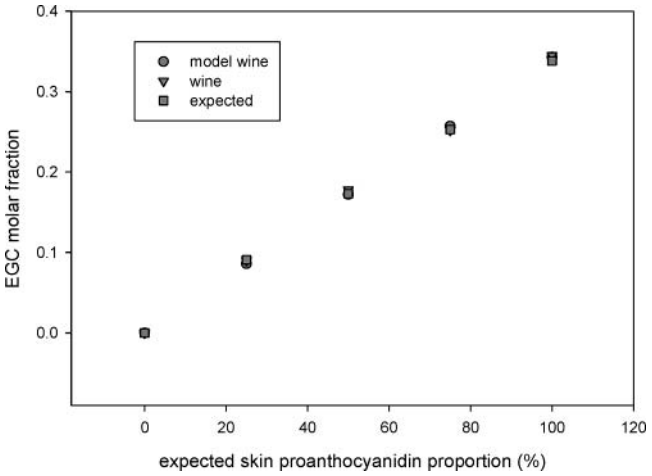

Figure 1. Comparison of the EGC molar fraction (considering only EGC and EC extension subunits) determined in model wine and wine with expected skin proanthocyanidin proportion (%) after the standard addition of proanthocyanidins.

Table 3. Change in Measured Skin Proanthocyanidin Proportion (%) during Alcoholic Fermentation, ± SD ($n = 3$)

day	skin proanthocyanidin (%)	day	skin proanthocyanidin (%)
1	91.1 ± 0.1	5	72.3 ± 0.2
2	82.0 ± 0.2	6	64.2 ± 0.3
3	76.6 ± 0.3	7	56.8 ± 0.2
4	76.7 ± 0.4	8	56.6 ± 0.2

Proanthocyanidins Extraction from Seed and Skin during Red Wine Production. This experiment was conducted in commercial-scale fermentations. Pinot noir grapes were harvested, divided equally into two fermentors, and underwent a prefermentation cold skin maceration for 4 or 10 days prior to the commencement of alcoholic fermentation. After fermentation started (day 1) and until the dry wine was pressed (day 8), wine samples were taken daily and seed and skin proanthocyanidins were analyzed. The results presented in **Figure 2** were consistent with general thoughts on the extraction of proanthocyanidins in red wines. Specifically, skin proanthocyanidins make up the early portion of extraction during fermentation as they are more readily extracted (*11*, *25*); then with the appearance of ethanol, seeds become an increasingly important source of proanthocyanidins (*26*, *27*).

The trends in extraction were similar in both 4 and 10 day low temperature prefermentation skin contact experiments, and the relative proportion of seed and skin proanthocyanidins at the end of fermentation was the same, suggesting that additional low temperature prefermentation skin contact time had little effect on proanthocyanidin composition or concentration (**Figure 3**).

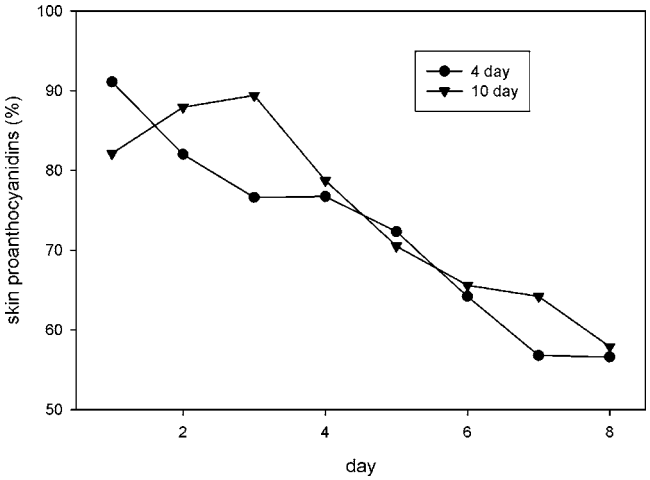

Figure 2. Decrease of skin proanthocyanidin percentage during alcoholic fermentation in a 4 and 10 day low temperature prefermentation skin contact.

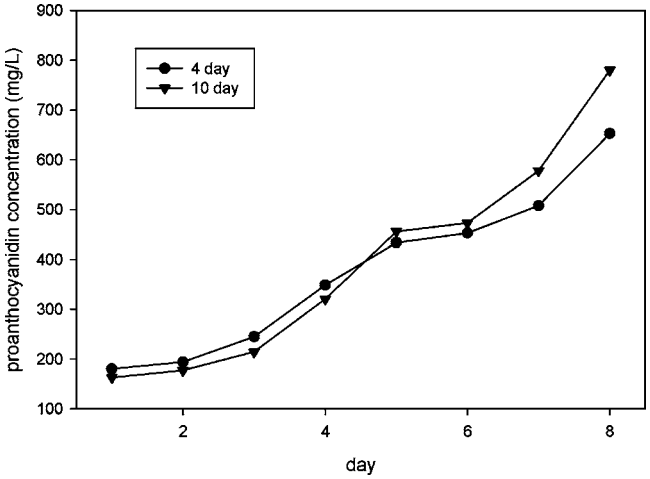

Figure 3. Increase of total proanthocyanidin concentration during alcoholic fermentation in a 4 and 10 day low temperature prefermentation skin contact.

Knowing the total wine proanthocyanidin concentration and the seed and skin proanthocyanidin proportion, it was possible to determine the seed and skin proanthocyanidin concentration individually (**Figure 4**). Through the alcoholic fermentation, the seed and skin proanthocyanidin changes were similar in both 4 and 10 day low temperature prefermentation skin contact experiments.

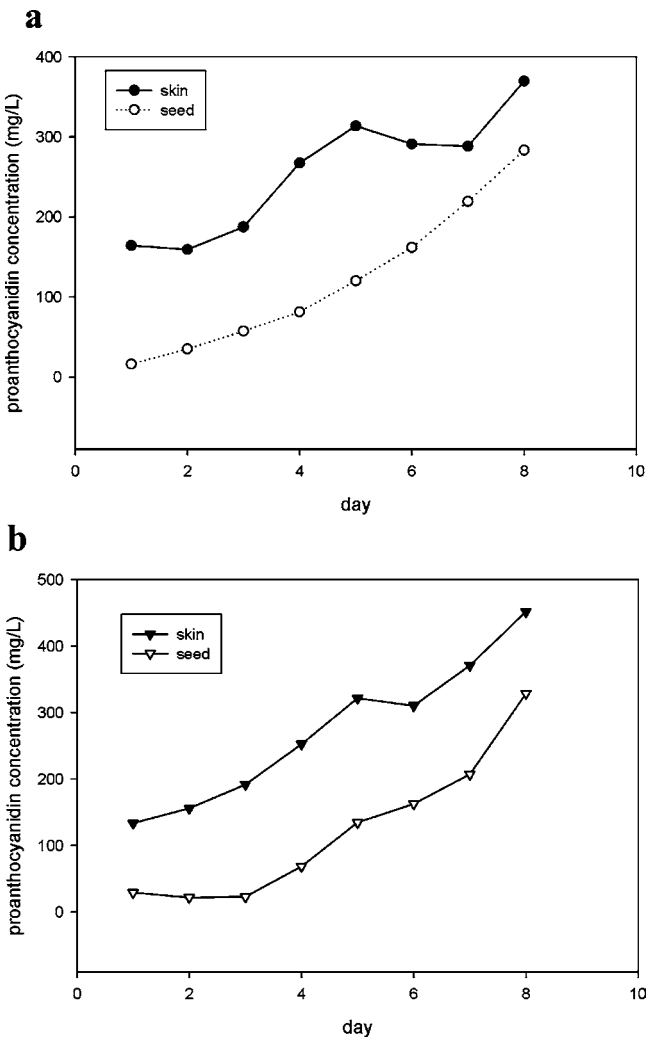

Figure 4. Increase in skin and seed proanthocyanidin concentration during alcoholic fermentation during a fermentation with 4 (a) and 10 day (b) low temperature prefermentation skin contact prior to fermentation.

CONCLUSION

By analyzing proanthocyanidins cleavage products after acid catalysis in the presence of excess phloroglucinol, the results of this study indicate that it should be possible to measure seed and skin proanthocyanidin extraction individually and directly during red wine fermentations. It must be stressed however that while this analytical approach appears to have promise, red wine fermentations are particularly complex; therefore, it is not possible at this time to determine this method's full utility. For example, during the development of this method, it has become clear that proanthocyanidin composition has a fairly large degree of variation within a specific variety, indicating that fruit analysis will be essential for every experiment (Pinot noir, Oregon, vintage 2002, vineyard variability study: the EGC molar fraction based upon EGC and EC extension subunit analysis varies from 0.221 to 0.318 for skin isolates). Additionally, recent experiments in this laboratory indicate that skin and seed proanthocyanidins differ in their potential to become oxidized. This would affect the outcome of this method and therefore restricts its use to conditions where oxidation is controlled. Undoubtedly, given the complex nature of this subject, many other potential variables could affect the analytical results (i.e., berry size, variety, physiological state of berry development, microbial flora). Additional work is now underway to provide additional information on the utility of this method.

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LITERATURE CITED

(1) Souquet, J. M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.

(2) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781−784.

(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) Kantz, K.; Singleton, V. L. Isolation and determination of Polymeric polyphenols in wines using Sephadex LH-20. *Am. J. Enol. Vitic.* **1991**, *42*, 309−315.

(5) Sun, B. S.; Pinto, T.; Leandro, M. C.; Ricardo da Silva, J. M.; Spranger, M. I. Transfer of catechins and proanthocanidins from solid parts of the grape cluster into wine. *Am. J. Enol. Vitic.* **1999**, *50*, 179−184.

(6) Noble, A. C. Bitterness and astringency in wine. In *Developments in Food Science 25. Bitterness in Foods and Beverages*; Rouseff, R. L., Ed.; Elsevier: New York, 1990; pp 145−158.

(7) Gawel, R. Red wine astringency: A review. *Aust. J. Grape Wine Res.* **1998**, *4*, 74−96.

(8) Lea, A. G.; Arnold, G. M. The phenolics of cider: bitterness and astringency. *J. Sci. Food Agric.* **1978**, *29*, 478−483.

(9) Porter, L. J.; Woodruffe, J. Haemanalysis: the relative astringency of proanthocyanidin polymers. *Phytochemistry* **1984**, *23*, 1255−1256.

(10) Cheynier, V.; Rigaud, J.; Ricardo da Silva, J. M. In *Plant Polyphenols: Synthesis, Properties, Significance*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: New York, 1992; p 281.

(11) Amrani Joutei, K.; Glories, Y.; Mercier, M. Localisation des tanins dans la pellicule de baie de raisin. *Vitis* **1994**, *33*, 133−138.

(12) Ribéreau-Gayon, P.; Sudraud, P.; Milhe, J. C.; Canbas, A. Recherches technologiques sur les composés phénoliques des vins rouges. *Conn. Vigne Vin* **1970**, *4*, 133−144.

(13) Scudamoresmith, P. D.; Hooper, R. L.; McLaran, E. D. Color and phenolic changes of Cabernet Sauvignon wine made by simultaneous yeast/bacterial fermentation and extended pomace contact. *Am. J. Enol. Vitic.* **1990**, *1*, 57−67.

(14) Wightman, J. D.; Price, S. F.; Watson, B. T.; Wrolstad, R. E. Some effects of processing enzymes on anthocyanins and phenolics in Pinot noir and Cabernet Sauvignon wines. *Am. J. Enol. Vitic.* **1997**, *48*, 39−48.

(15) 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.

(16) Kovac, V.; Alonso, E.; Revilla, E. The effect of adding supplementary quantities of seeds during fermentation on the phenolic composition of wines. *Am. J. Enol. Vitic.* **1995**, *46*, 363−367.

(17) 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.

(18) Kennedy, J. A.; Waterhouse, A. L. Analysis of pigmented high-molecular mass grape phenolics using ion-pair, normal-phase high-performance liquid chromatography. *J. Chromatogr. A* **2000**, *866*, 25−34.

(19) Vrhovsek, U.; Mattivi, F.; Waterhouse, A. L. Analysis of red wine phenolics: comparison of HPLC and spectrophotometric methods. *Vitis* **2001**, *40*, 87−91.

(20) Kennedy, J. A.; Taylor, A. W. Analysis of proanthocyanidins by high-performance gel permeation chromatography. *J. Chromatogr. A* **2003**, *995*, 99−107.
(21) Kennedy, J. A.; Matthews, M. A.; Waterhouse, A. L. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry* **2000**, *55*, 77−85.
(22) Kennedy, J. A.; Troup, G. P.; Pilbrow, J. R.; Hutton, D. R.; Hewitt, D.; Hunter, C. A.; Ristic, R.; Iland, P. G.; Jones, G. P. Development of seed polyphenols in berries from *Vitis vinifera* L. cv. Shiraz. *Aust. J. Grape Wine Res.* **2000**, *6*, 244−254.
(23) Lamuela-Raventos, R. M.; Waterhouse A. L. A direct HPLC separation of wine phenolics. *Am. J. Enol. Vitic.* **1994**, *45*, 1−5.
(24) 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.
(25) Meyer, J.; Hernandez, R. Seed tannin extraction in Cabernet Sauvignon. *Am. J. Enol. Vitic.* **1970**, *21*, 184−188.
(26) Singleton, V. L.; Draper D. E. The tranfer of polyphenolic compounds from grape seeds into wines. *Am. J. Enol. Vitic.* **1964**, *15*, 34−40.
(27) Thorngate, J. H.; Singleton, V. L. Localization of procyanidins in grape seeds. *Am. J. Enol. Vitic.* **1994**, *45*, 259−262.

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