Effect of Ethanol on Grape Seed Proanthocyanidin Extraction

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Abstract: Proanthocyanidins are found in the seeds and skins of winegrapes and are extracted into the must-wine during maceration. For seed proanthocyanidins, extraction is generally thought to be possible only in the presence of ethanol. This study examined the extraction of seed proanthocyanidins in model solutions with increasing concentrations of ethanol, from 0 to 15% v/v. Spectrophotometric and chromatographic results showed that ethanol was not required for proanthocyanidin extraction, although its presence increased the rate of extraction. Extraction dynamics indicated that alcohol increased the rate of proanthocyanidin extraction for the initial six days of maceration, after which, even in the absence of ethanol, the extraction rate was nearly identical for all treatments. These findings suggest that extraction time is an important consideration when managing techniques, such as cold soak, which are thought not to affect seed proanthocyanidin extraction.

Key words: grape, seed, tannins, wine, proanthocyanidin, maceration, extraction

Tannins play an important role in red wine quality since they are responsible for astringency and bitterness mouthfeel properties. Grape tannins are proanthocyanidins, which are found in skin and seed tissue. In skin, they are mainly located in the skin cell vacuoles, and in seed they are found in the epidermis, the outer integument, and the inner integument (Cadot et al. 2006). Seed proanthocyanidins contain no prodelphinidins (Cortell et al. 2005) and a higher proportion of galloylated procyanidins (Labarbe et al. 1999, Prieur et al. 1994) and the mean degree of polymerization (mDP) is lower than skin proanthocyanidins (Cheynier et al. 1997, Moutounet et al. 1996).

Skin proanthocyanidins have frequently been described as "soft" or "ripe," contrary to seed proanthocyanidins, which have been associated with more aggressive and less desirable sensory descriptors like "green" or "hard." Studies have shown that the mDP and galloylation of wine proanthocyanidins are important structural variables that affect wine astringency perception. The percentage of galloylation has been positively correlated with astringency, and a strong positive correlation has been found between mDP and astringency (Vidal et al. 2003). However, Chira et al. (2009) found that the correlation between mDP and astringency could be modulated by the presence of epigallocatechin, so that skin proanthocyanidins provide a softer sensation than seed proanthocyanidins.

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During maceration, proanthocyanidins are extracted from skin and seeds. In general, skin proanthocyanidins have been reported as being more readily extractible, whereas extraction from seeds requires longer maceration and is favored by the presence of ethanol (Canals et al. 2005, Llaudy et al. 2008, Gonzalez-Manzano et al. 2004). Wine proanthocyanidins can be manipulated by winemaking practices (Lee et al. 2008), with several of these practices based on the assumption that ethanol from fermentation is necessary to disorganize the outer lipidic layer that covers and isolates the seeds, meaning that seed proanthocyanidins are extracted at the end of alcoholic fermentation (Ribéreau-Gayon et al. 1998). In this way, short maceration periods have been used when wines with a high concentration of skin proanthocyanidins are desired, since most of the extraction occurred at low alcohol concentrations. Low temperature prefermentative macerations are also designed to increase the extraction and stabilization of the polyphenolic compounds (anthocyanins and proanthocyanidins) from skins during this prefermentative phase, avoiding the extraction of the more aggressive seed proanthocyanidins. However, in 1964 researchers demonstrated that water alone could extract an important amount of seed extractable polyphenols (Singleton and Draper 1964).

In this study, we used a model solution to determine how ethanol and time affect the amount and characteristics of proanthocyanidins extracted from seeds and to deepen our knowledge concerning the expected behavior of seed proanthocyanidins when using different techniques during winemaking.

Materials and Methods

Seeds from grapes of *Vitis vinifera* cv. Monastrell at commercial maturity (24.5 Brix) were extracted by hand, cleaned, and dried with cellulose paper, placed into hermetic bags filled with nitrogen and stored refrigerated at 4°C for a maximum of 24 hr before maceration. Simulated maceration assays were performed at room temperature by placing 100 g seeds in 830 mL amber glass flasks filled (to the top) with model wine

solutions (adjusted to pH 3.6 with tartaric acid and 50 mg/L SO_2) with increasing concentrations (0, 5, 10, and 15% v/v) ethanol in triplicate. Each flask was vigorously shaken twice a day and sampled every two days (refilling with the original model solution, sparging with nitrogen to avoid oxidation) for up to 10 days. All assays were conducted in triplicate.

The absorbance measurements to determine total phenolic index were directly made in an extract after filtering and diluting 100-fold in a Helios Alpha spectrophotometer (Thermo Spectronic, Waltham, MA) with 1 cm path-length glass cells. Proanthocyanidin determination was carried out according to the method described by Kennedy and Jones (2001) with some modifications. To this end, 10 mL extract was concentrated under reduced pressure at 50°C and then redissolved in methanol in a volumetric flask. Then 100 µL methanolic extract was reacted with 100 µL phloroglucinolysis reagent (a solution of 0.2 N HCl in methanol, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid) in a water bath for 20 min at 50°C and then combined with 2 vol 200 mM aqueous sodium acetate to stop the reaction. The flavan-3-ol monomer content present in the extract was measured, exchanging the phloroglucinolysis reagent for methanol.

HPLC analysis of proanthocyanidins followed the conditions described previously (Kennedy and Taylor 2003). The HPLC apparatus was a Waters 2695 system (Waters, Milford, MA) equipped with an autosampler and a Waters 2996 photodiode array detector. Samples (10 µL injection volume) were injected on two Chromolith RP-18e (100 x 4.6 mm, 5 µm packing) columns connected in series and protected by a guard column (Purospher STAR RP-18e, 4 x 4 mm, 5 µm packing), all of them from Merck (Darmstadt, Germany). The elution conditions were as follows: 3 mL/min flow rate; oven temperature, 30°C; solvent A, water/acetic acid (99:1, v/v); and solvent B, acetonitrile/acetic acid (99:1 v/v). Eluting peaks were monitored at 280 nm and the elution began with 3% B for 4 min, a linear gradient from 3 to 18% B in 10 min, followed by washing and reequilibration of the column. Proanthocyanidin cleavage products were estimated using their response factors relative to (+)-catechin, which was used as the quantitative standard. These analyses allowed determination of the total proanthocyanidin content, the apparent mDP, and the percentage of each constitutive unit. The mDP was calculated as the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in moles) divided by the sum of all flavan-3-ol monomers (in moles).

Statistical analyses. Significant differences in the proanthocyanidin concentration and composition for each variable at the different extraction times and for the different ethanol contents were assessed by multivariate analysis of variance (MANOVA). The LSD test was used to separate the means ($p < 0.05$) using the statistical package StatGraphics 5.0 Plus (StatPoint Technologies, Warrenton, VA).

Results and Discussion

The evolution over 10 days of tannin concentration in the model solutions differing in ethanol concentration is shown (Figure 1). Results show that ethanol is not necessary to release seed tannins since, after 10 days, a substantial proanthocyanidin concentration existed in the solution containing 0% ethanol, representing 72.60% of the maximum obtained in the solution containing 15% alcohol. The 5 and 10% ethanol concentrations showed intermediate values. These results are similar to those found for extractable seed phenols (Singleton and Draper 1964). From a practical point of view, these same authors also demonstrated that the same behavior was observed regardless of the temperature of extraction, the only difference being the amount of seed phenols extracted, which was 30% lower at 11.2°C than at 30°C. These findings indicate that a considerable amount of seed proanthocyanidins would be extracted when performing prefermentative macerations during winemaking.

Two different zones can be observed in the plot representing tannin evolution in the different model solutions. From day 0 to day 6, all evolutions can be fitted to a sigmoidal plot, the regression coefficients (r^2) ranking from 0.99 (for the 15% ethanol plot) to 0.82 (for the 0% ethanol plot). From day 6 to 10, the plots can be perfectly fitted to a linear equation ($y = ax + b$) with a very high r^2 (the lowest at 0.98 for the 0% ethanol plot), showing an almost identical slope.

According to Cerpa-Calderón and Kennedy (2008), the general extraction of skin proanthocyanidins is consistent with the Boltzmann sigmoid equation:

$$f = \frac{\text{plateau concentration}}{1+e^{-((x-t50)/\text{slope})}}$$

However, according the authors, it was uncertain if the Boltzmann sigmoid model would fit seed proanthocyanidin extraction, as seed proanthocyanidin extraction did not reach a plateau during the study period (Cerpa-Calderón and Kennedy 2008). Reflecting those results, we observed that the data from the first six days fit the Boltzmann equation but then, as the rate of extraction increased again, it did so linearly.

Adapting the interpretation of the given model (Cerpa-Calderón and Kennedy 2008) and for the first six days, the

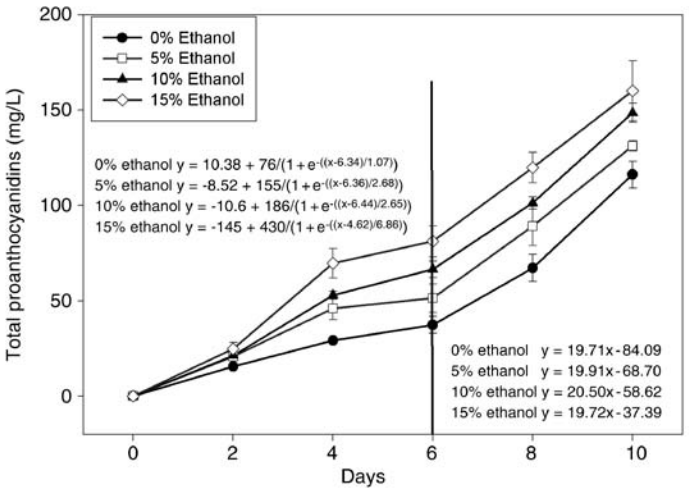

Figure 1 Total proanthocyanidin concentration in samples collected during maceration. Data expressed as means (n = 3 ± SEM).

initially slow extraction represents the period of time required for the proanthocyanidins to diffuse out of the plant cell and into the solution. After this time, the extraction was more pronounced. Singleton and Draper (1964) also described a lag period before the phenolic substances were extracted at a maximum rate. As ethanol content increases, so does the rate of maximum extraction. Finally, a small concentration plateau was reached, the value of which increased with ethanol content. One explanation for the observed concentration increase with ethanol is that ethanol leads to acceleration in the degradation of the outer protective layer of seeds.

From day 6 to day 10, proanthocyanidin extraction followed a linear equation. Interestingly, the slope of the individual equations was nearly the same at all ethanol concentrations, which could indicate that not only an effect of ethanol but also an effect of time was significant. Over time, seed cells may become increasingly hydrated and leaky and, when they reached a certain hydration level, proanthocyanidin extraction rate becomes constant and does not depend on ethanol concentration. Therefore, prior to day 6 there is an ethanol effect; after day 6 the ethanol effect is absent.

A two-way analysis of variance was also used to study the effect of ethanol and time of extraction on the proanthocyanidin composition of the different model solutions (Table 1, Table 2). As previously described, total phenols (OD280) and total proanthocyanidins increased with maceration time. The composition and characteristics of the extracted proanthocyanidins were similar to those reported by Prieur et al. (1994) for the second of five seed proanthocyanidin fractions obtained by preparative chromatography after extraction with acetone (fraction IIa). The findings of these authors suggest that only a fraction of the total bulk of seed proanthocyanidins was extracted under hydroalcoholic conditions, the others remaining unextracted. Singleton and Draper (1964) estimated that less than half of the seed proanthocyanidins would be extracted under typical winemaking conditions, and, in this way,

Table 1 Multivariate analysis of variance of the optical density at 280 nm (OD280), the mean degree of polymerization (mDP), and the concentration of monomeric and polymeric proanthocyanidins (mg/L) in the different model solution as affected by maceration time and alcohol content.

	OD280	mDP	Monomeric catechin	Monomeric epicatechin	tC	tEC	tECG	extC	extEC	extECG	Total
Days											
2	1.39a[a]	5.37b	2.28a	3.17a	1.71a	1.18a	1.00a	2.22a	8.16a	0.93a	20.67a
4	2.28b	4.22a	4.18b	6.22b	5.13b	3.80b	2.17b	5.11b	20.69b	2.17b	49.46b
6	2.99c	4.06a	5.41c	6.83b	6.97c	4.55b	2.71b	5.70b	24.41c	2.54b	59.13c
8	4.06d	4.28a	7.99d	11.22c	9.30d	7.97c	3.65c	8.75c	41.14d	4.37c	94.70d
10	4.85e	4.46a	11.02e	15.89d	13.49e	10.66d	5.08d	12.68d	62.95e	7.21d	138.992e
% Alcohol											
0	2.10a	4.43a	4.75a	7.04a	5.51a	4.55a	1.21a	5.29a	22.98a	1.82a	53.12a
5	2.76b	3.93a	6.18b	8.63b	6.99b	6.03b	2.00b	6.51b	28.85b	2.55b	67.74b
10	3.52c	4.33a	6.71c	9.18bc	8.01c	6.06b	3.31c	7.22c	33.80	3.81c	78.10c
15	4.23d	5.22b	7.06c	9.82c	8.78c	5.87b	5.17d	8.55d	40.29d	5.60d	91.16d

Abbreviations: mDP: mean degree of polymerization; tC: terminal (+)-catechin; tEC: terminal (-)-epicatechin; tECG: terminal (-)-epicatechin gallate; extC: extension (+)-catechin; extEC: extension (-)-epicatechin; extECG: extension (-)-epicatechin gallate.
[a]Different letters within the same column indicate significant differences ($p < 0.05$).

Table 2 Multivariate analysis of variance of the percentage (all values reported in %) of the different monomeric and polymeric proanthocyanidins in the model solution as affected by maceration time and alcohol content.

	Monomeric catechin	Monomeric epicatechin	tC	tEC	tECG	extC	extEC	extECG	Total monomers	Total polymers	Terminal subunits	Extension subunits
Days												
2	10.94c[a]	15.36c	8.39a	5.75a	4.73b	10.85d	39.51a	4.47ab	26.29c	73.71a	18.87a	54.84a
4	8.55a	12.85b	10.40b	7.75b	4.16ab	10.40c	41.60b	4.29a	21.40b	78.60b	22.31bc	56.29ab
6	9.38b	11.65a	11.79c	7.69b	4.26ab	9.78b	41.41b	4.05a	21.03b	78.97b	23.74c	55.23a
8	8.72a	11.88a	9.84b	8.44b	3.72a	9.32a	43.79c	4.60ab	20.30ab	79.70bc	21.99b	57.71bc
10	8.04a	11.67a	9.76b	7.84b	3.47a	9.13a	45.10d	4.96b	19.71a	80.29c	21.09b	59.2c
% Alcohol												
0	9.05b	13.36b	10.33b	7.44b	2.89a	10.54c	42.61bc	3.79a	22.41b	77.59a	20.65a	56.94b
5	9.61b	13.01b	10.26.	9.05c	3.01a	9.89b	41.41a	3.76a	22.62b	77.38a	22.32b	55.06a
10	9.28b	12.63b	10.12ab	7.61bc	4.51b	9.48a	41.77ab	4.59b	21.91b	78.09a	22.25b	55.84ab
15	8.33a	11.73a	9.45a	5.88a	5.85c	9.67ab	43.33c	5.76c	20.06a	79.94b	21.18ab	58.76c

Abbreviations: tC: percentage of terminal (+)-catechin; tEC: percentage of terminal (-)-epicatechin; tECG: percentage of terminal (-)-epicatechin gallate; extC: percentage of extension (+)-catechin; extEC: percentage of extension (-)-epicatechin; extECG: percentage of extension (-)-epicatechin gallate.
[a]Different letters within the same column indicate significant differences ($p < 0.05$).

a theoretical seed proanthocyanidin extraction of 9.2%, 7.2%, and 8.7% was obtained in wines from Monastrell, Cabernet Sauvignon, and Syrah grapes, respectively (Busse-Valverde et al. 2010). Higher theoretical seed proanthocyanidin extraction was found from Merlot grapes (42%) (Cerpa-Calderón and Kennedy 2008).

No increase in mDP was observed as maceration time increased, a result consistent with those reported elsewhere (Llaudy et al. 2008). The percentage of monomeric compounds (catechin and epicatechin) decreased with time (they are readily extracted at the beginning of the maceration process) and the percentage of polymeric proanthocyanidins increased. However, very small differences in terminal and extension subunit composition were observed during maceration.

When the effect of the ethanol content was observed, mDP changed little, only increasing significantly when the solution contained 15% ethanol, whereas an increase in OD280 (50%) and total proanthocyanidins (40%) was evident with increasing ethanol concentrations. Consistent with the increase in total proanthocyanidins, all the different fractions of monomer compounds and polymeric compounds increased. The percentage of monomeric compounds decreased as the alcohol content increased, whereas the percentage of polymeric proanthocyanidins increased; terminal epicatechin-3-*O*-gallate doubled its concentration and extension epicatechin-3-*O*-gallate increased its percentage, although to a lesser extent. Geny et al. (2003) found a higher proportion of epicatechin-3-*O*-gallate in the proanthocyanidins located in the seed cell wall fraction than in the inner part of the cell, so that a greater degradation of the cell walls with increasing alcohol content could promote a higher release of more galloylated proanthocyanidins. Similarly, Koyama et al. (2007) indicated that the galloylated proanthocyanidins, which present higher hydrophobicity, might be selectively trapped by internal components of the seed cells and be released with the aid of increased ethanol concentrations. This effect was also observed in wines, where the percentage of galloylation increased during the second part of fermentation, when alcohol reached high values (Busse-Valverde et al. 2011), which may be of sensory importance since astringency increases with galloylation (Vidal et al. 2003). Overall, the percentage of terminal and extension subunits changed little.

Conclusions

Results indicate that ethanol is not essential for the extraction of seed tannins, although their extraction is more intense and faster when ethanol is present, especially at the beginning of the maceration period, since from day 6 to 10 the rate of extraction was similar for all solutions. The main effect of ethanol is probably to help disorganize the outer lipidic layer that protects seeds. However, time also has an important role, increasing seed cells hydration and leakiness. It appears that, once a certain level of seed cell hydration is reached, tannins are extracted independently of alcohol content. The mDP did not change with longer maceration times and very little with increasing ethanol content, probably because only a small fraction of the total seed tannin content is extracted in hydroalcoholic solutions. The proportion of galloylated proanthocyanidin did not increase with maceration time but almost doubled its value as the alcohol content increased, probably due to the different binding and localization these more complex molecules could have on the seed.

From a practical point of view and considering 10 days as a usual prefermentative maceration time in techniques such as prefermentative cold soak, the diffusion kinetics found in this research will be of interest to winemakers. Prefermentative maceration not only would extract a considerable amount of seed tannins but also, in some way, would prepare seeds for a linear extraction during fermentative maceration. These results confirm those obtained by Alvarez et al. (2005) and Busse-Valverde et al. (2010, 2011), who found increased seed PA content and galloylation in wines after applying low temperature prefermentative macerations.

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