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Composition and Cellular Localization of Tannins in Grape Seeds during Maturation

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Cell walls were isolated from seeds of grape berries (*Vitis vinifera* L.), and proanthocyanidin composition was determined over the course of ripening for different levels of vine water status. During the ripening period the tannins from the cell walls were always more polymerized than those from the inner part of the cell. At maturity this difference becomes more significant compared to véraison, due to a significant increase in the mean degree of polymerization of the cell wall tannins. The tannin composition was typical of grape seed tannins and was quite similar in the two cell fractions studied, but the epicatechin gallate proportion was significantly higher in the cell wall fraction. There were no significant effects of water deficit on composition and polymerization of seed tannins.

KEYWORDS: ***Vitis vinifera* L.; grape seeds; cell wall; tannins; water status**

INTRODUCTION

The development of the grape berry follows a double-sigmoid growth pattern (*1*), with each growth period differing considerably in biochemical activity and subsequent berry composition (*2*). During the first period of berry growth, the pericarp and seed cell number increases, and during this stage, the seed approaches its full size. Véraison is characterized by little change in berry size, whereas the seed embryo develops with a concomitant hardening of the seed coat. During ripening, berry size increases, whereas many seeds are already fully developed (*3*).

Past véraison, seeds change color from an initial green to a light buff and finally to a dark brown at harvest, and seed growth could continue 40 days prior to harvest (*4*). Changes in grape seed tannin composition during maturity were reported by Kennedy et al. (*4*). These compounds, also called proanthocyanidins, are polymers of flavan-3-ol units such as catechin (**Figure 1**). Despite the importance of these molecules in the production of wine, there is little information available on the cellular localization. According to Amrani et al. (*5*), during grape ripening, tannins bind to the proteins of the internal surface of tonoplast and the cell wall polysaccharides. The chemical composition of cell walls isolated from seeds and the polyphenolic changes that occur during ripening have not been defined. Cell walls are mainly responsible for the integrity and texture of tissues and, during winemaking, they constitute a diffusion barrier for phenolic and aromatic compounds. It is, however, important to chemically elucidate the mechanism whereby seed polyphenols change during fruit development.

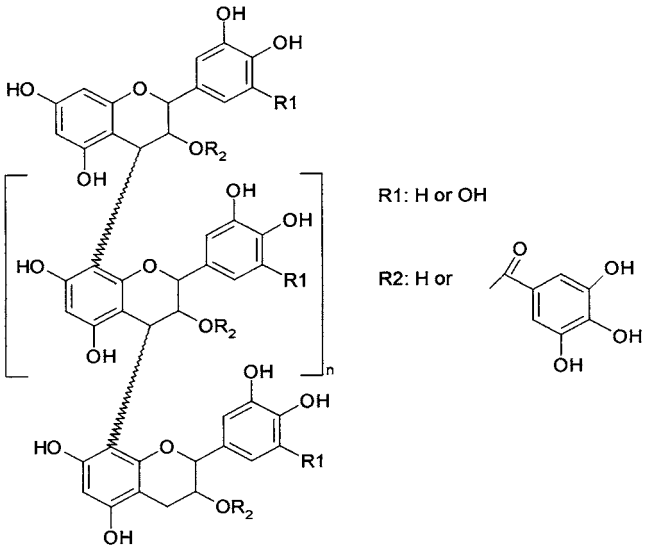

Figure 1. Tannin (proanthocyanidin) structures in *V. vinifera* L. berries.

In the work reported here we have developed a method for the separation of cell walls from the inner content of grape berry seed cells. The distribution and composition of tannins were investigated in the two fractions obtained. The effect of water status on these variables was also examined.

MATERIALS AND METHODS

Plant Material. At véraison (berry softening and color change) and maturity, berries of Cabernet Sauvignon were collected in a vineyard at Tudela (Spain) in 2001. The vineyard was planted in 1985 and grafted onto SO_4 rootstock. Vines were planted 3 m between the rows and 1.7 m in the row and trained to bilateral cordons with vertically shoots.

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Three irrigation treatments were established in a randomized block, replicated three times, with each replicate consisting of five vines, and with four buffer rows separating treatments.

Three irrigation levels were applied during all of the different developmental periods and corresponded to (A) double irrigation, 60 L of water per plant and per week; (B) control, 30 L of water per plant per week; and (C) minimal irrigation, 15 L of water per plant per week. Vine water status was monitored by measuring the stem water potential with a pressure chamber as described by Choné et al. (*6*). Stem water potential was measured with a pressure chamber equipped with a digital manometer (SAM Precis 2000, Gradignan, France) at the middle of the day on bagged mature leaves (in a black plastic bag for 2 h).

Sample Collection and Expression of Results. Three by ten× bunches of grape samples were collected from each treatment. For each sample, after the 100-berry sample had been weighed, seeds were collected, weighed, and stored at −80 °C until extraction.

Isolation of Cell Walls from Grape Seeds. To process samples, seeds were removed from berries and rinsed well with distilled water. The cell wall isolation procedure was done according to the method of Harris (*7*) adapted to our plant material. Four grams of seeds was suspended in 20 mL of 0.2 M Tris-HCl buffer, pH 7.5, containing 2.5% EDTA and homogenized in an ice-cold mortar. The samples were then centrifuged at 9000*g* for 15 min at 4 °C. The supernatant was filtered through a 3 μm PTFE filter, and the pellet was resuspended in 10 mL of 0.2 M Tris-HCl buffer, pH 7.5, containing 2.5% EDTA and recentrifuged at 9000*g* for 15 min at 4 °C. The supernatant was filtered through 0.45 μm PTFE filters and added to the previous supernatant; this fraction was designated the "internal cell fraction". The pellets were washed with 0.1% Triton X solution to remove membranous contaminants, and this fraction was called the "cell wall fraction". During the isolation procedure, cell walls were examined under a light microscope to confirm their purity. All fractions were stored at −80 °C until use.

Extraction of Tannins. One gram of cell wall fractiof or 1 mL of internal cell fraction was adjusted to 100 mL with MeOH/12 N HCl (99.9:0.1 v/v). These solutions were then stirred at 20 °C for 14 h for the cell wall fraction and for 1 h for the internal cell fraction.

Extracts were filtered through 3 μm PTFE filters, concentrated 40 times for the cell wall fraction and 100 times for the internal cell fraction, and stored at −20 °C until HPLC analysis.

Thioacidolysis and HPLC Analysis of Tannins. Mean degree of polymerization (mDP) and subunit composition of procyanidins in the fractions were analyzed by reversed phase HPLC after thiolysis as described previously by Saucier et al. (*8*). The thiolysis reagent was composed of benzyl mercaptan/2 NHCl /MeOH (0.5:2:7.5 v/v/v). One hundred microliters of reagent and 100 μL of tannin fraction were placed in a 2 mL HPLC sealed vial and heated at 90 °C for 3 min (*9*). Before HPLC injection, 150 μL of water was added to the vial with a microsyringe. HPLC analysis conditions were the following: column, 250 × 4.6 mm, 5 μm, ODS (Beckman, Roissy CDG, France); flow rate, 1 mL/min; solvent A, water/acetic acid (95:5 v/v); solvent B, MeOH/acetic acid (95:5 v/v); gradient, 30−100% B from 0 to 35 min, 100% B from 35 to 40 min, 100 to 30% B from 40 to 45 min; injection volume, 20 μL; detection wavelength, 280 nm.

RESULTS AND DISCUSSION

Phenolic Composition and Cellular Localization of Tannins in Seed Cells during Maturation. *Extraction.* The different parts of the seed cells (inner parts and cell walls) were first separated and frozen at −80 °C until use. A first series of experiments was done to set up an easy method of phenolic compound extraction. The goal was to have a reproducible solid−liquid extraction of the tannins from the extracts and to have a solution sufficiently concentrated to allow direct thioacidolysis HPLC analysis. Acidified methanol was chosen because it is a good solvent for tannins. The different extracts were then left under agitation in this solvent, and the kinetics of tannin extraction were followed by UV spectrophotometry. The results obtained (**Figure 2**) show that the extraction is very fast for the extraction of the inner part of the seed cells. The UV absorbance is stable over time, so we chose 1 h of extraction time for these extracts. Concerning the cell walls, the absorbance increases during the first 10 h until a plateau is reached. We then chose 14 h of extraction as the required time for the extraction of cell walls for future analyses.

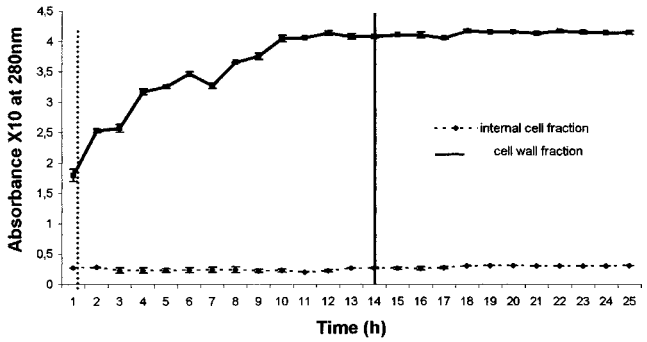

Figure 2. Kinetics of phenolic extraction for the different seed fractions. Each point represents the mean of two 100-berry samples, and error bars represent the standard deviation of the mean.

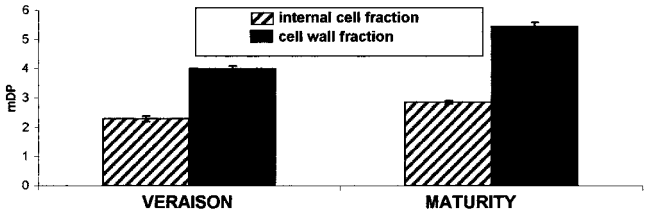

Figure 3. mDP in different parts of cells of seeds at véraison and maturity. Each point represents the mean of three 100-berry samples, and error bars represent the standard deviation of the mean.

Evolution of Mean Degree of Polymerization of Procyanidins. The mDP of the tannins in the different parts of the seed cells was analyzed by reversed phase HPLC after thioacidolysis at two different ripening stages (véraison and maturity). The results shown in **Figure 3** indicate that regardless of ripening period, the tannins from the cell walls are always more polymerized than those from the inner part of the cell. At maturity this difference becomes more significant compared to véraison. This phenomenon is in fact due to an important increase of mDP for the cell wall tannins, whereas the size of the inner cell tannins remains quite constant. These results give for the first time molecular information on tannin structure in relation to their localization in the cell. This information could explain the microscopic observation previously obtained on skin cells (*5*, *10*), which showed a tannin aggregation increase close to the skin cell walls during ripening. A similar phenomenon could exist in seed cell walls, which could be due to the higher mDP of the tannins bound to the cell wall and thus to a higher hydrophobicity.

On average, our results suggest a slight increase of seed tannin mDP during maturity. These results contradict previous results obtained from Cabernet Sauvignon grown in California (*4*). In a similar way, irreversible adsorption could occur between the galloylated tannins and the cell wall. In another fruit (apple), it has been shown in vitro that selective adsorption of higher molecular tannins onto cell walls can occur (*11*). This could be due to genetic (difference in Cabernet Sauvignon clone or rootstock) or environmental ("terroir") factors.

Composition of Procyanidins during Ripening. The flavan-3-ol composition of the tannins in the different part of the seed cells was also analyzed by reversed phase HPLC after thioacidolysis. The corresponding results are presented in **Figure 4**

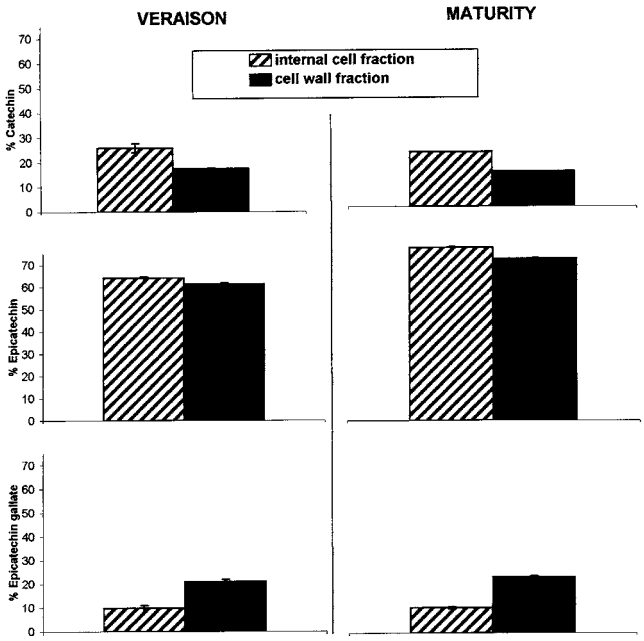

Figure 4. Tannin composition in different parts of cells of seeds at véraison and maturity. Each point represents the mean of three 100-berry samples, and error bars represent the standard deviation of the mean.

for the different ripening stages. The three flavanol units found are typical of grape seed tannins: epicatechin, catechin, and epicatechin gallate were found (in decreasing order of amount) in accordance with previous studies on grape seed tannins (*12*, *13*). The tannin composition is quite similar in the two cell fractions studied, but the epicatechin gallate proportion is significantly higher in the cell wall fraction. This may be due to a different biosynthesis in the different parts of the cell, or it is possible that a diffusion process exists between the two parts (*5*, *14*). It has been shown that galloylation of the tannins increases their affinity for proteins (*15*).

Plant Water Status and Seed Tannin Composition. Three irrigation levels were applied during all of the different developmental periods and corresponded to A, 60 L of water per plant per week; B, 30 L of water per plant per week; and C, 15 L of water per plant per week.

The stem water potentials (Ψ) at véraison and maturity for the different treatments are shown in **Table 1**. Throughout ripening, 30 L/plant/week (control) maintained a midday Ψ between -0.9 MPa at veraison and -1.2 MPa at maturity. Irrigation treatments established significant differences in stem potential at veraison and maturity. With minimal irrigation (15 L/plant/week) Ψ values were -1.1 MPa at véraison and -1.3 MPa at maturity, and with double irrigation (60 L/plant/week) Ψ values were -0.6 MPa at véraison and -0.7 MPa at maturity.

Mean berry weight at harvest was 1.88 g for the treatment with 30 L/plant/week (**Table 1**). No significant difference was observed with 15 L/plant/week, whereas 60 L/plant/week increased the berry weight by 10% (to 2.11 g). For all treatments, fresh seed mass was not significantly different between véraison and maturity (**Table 1**). On the other hand, double irrigation (60 L/plant/week) drastically increased the mean seed weight.

Many authors (*16*−*18*) have found that berry weight was increased by increasing the amount or the frequency of irrigation, which correlates with the results of our study, but to our knowledge, no study has examined the effect of water status on seed weight.

The mDP and the flavan-3-ol composition of the tannins in the different parts of the seed cells were analyzed by reversed phase HPLC after thioacidolysis at two different ripening stages and with three different water statuses.

Table 1. Effect of Water Status on Stem Potential, Berry and Seed Weight, and mDP on Different Parts of Cells of Seeds, at Véraison and Maturity[a]

	water status		
	15 L/plant/ week	30 L/plant/ week	60 L/plant/ week
Ψ: stem potential (MPa)			
véraison	–1.100 ± 0.035	–0.935 ± 0.065	–0.675 ± 0.056
maturity	–1.321 ± 0.102	–1.212 ± 0.163	–0.746 ± 0.108
fresh wt of one berry (g)			
véraison	1.84 ± 0.03	1.86 ± 0.02	1.92 ± 0.05
maturity	1.85 ± 0.15	1.88 ± 0.10	2.11 ± 0.18
fresh wt of 100 seeds (g)			
véraison	5.04 ± 0.16	4.98 ± 0.13	5.76 ± 0,19
maturity	5.10 ± 0.12	5.06 ± 0.11	5.91 ± 0.16
mDP in internal cell fraction			
véraison	2.35 ± 0.05	2.33 ± 0.13	2.51 ± 0,19
maturity	2.71 ± 0.06	2.85 ± 0.05	3.15 ± 0.06
mDP in cell wall fraction			
véraison	3.35 ± 0.25	4.03 ± 0.11	3.25 ± 0,45
maturity	5.55 ± 0.16	5.45 ± 0.15	6.05 ± 0.05

[a] Each value represents the mean of three 5-shoot samples for stem potential and three 100-berry samples for other parameters with standard deviation of the mean.

Concerning the effect on water status, these results also clearly show that water has no significant influence on seed tannin mDP (**Table 1**) and on tannin composition (data not shown), regardless of ripening stage. To our knowledge, no previous work has reported these facts on seeds, but similar trends were observed for skin tannins (*12*).

The composition and mDP have been measured for the first time in two different parts of grape seed cells. The variables studied were two ripening stages (véraison and maturity) and water status. The main results obtained show that mDP and epicatechin-gallate proportion are always higher in cell walls than in the inner part of the cells. The modification of water status (irrigation) had no significant effect on tannin size or composition in seeds. These results would have to be confirmed on other parts of the berry (pulp and skin). Future work is needed to explore the influence of variety and vintage on tannin biosynthesis as well as environmental (terroir) factors.

LITERATURE CITED

(1) Coombe, B. G. The regulation of set and development of the grape berry. *Acta Hortic.* **1973**, *34*, 261−273.
(2) Brady, C. J. Fruit ripening. *Annu. Rev. Plant Physiol.* **1987**, *38*, 155−178.
(3) Coombe, B. G. Relationships of growth and development to changes in sugars, auxins, and gibberellins in fruit of seeded and seedless varieties of *Vitis vinifera*. *Plant Physiol.* **1960**, *35*, 241−250.
(4) Kennedy, J. A.; Matthews, M. A.; Waterhouse, A. L. Changes in grape seed polyphenols during fruit ripening. *Phytochemistry* **2000**, *55*, 77−85.
(5) Amrani Joutei, K.; Glories, Y.; Mercier, M. Localisation des tanins dans la pellicule de baie de raisin. *Vitis* **1994**, *33*, 133−138.
(6) Choné, X.; Trégoat, O.; Van Leeuwen, C.; Dubourdieu, D. Déficit hydrique modéré de la Vigne: parmi les 3 applications de la chambre à pression, le potentiel tige est l'indicateur le plus précis. *J. Int. Sci. Vigne Vin* **2000**, *34*, 169−176.
(7) Harris, P. J. Cell walls. In *Isolation of Membranes and Ganelles from Plant Cells*; Hall, J. L., More, A. L., Eds.; Academic Press: New York, 1983; pp 25−53.

(8) Saucier, C.; Mirabel, M.; Daviaud, F.; Longieras, A.; Glories, Y. Rapid fractionation of grape seed proanthocyanidins. *J. Agric. Food Chem.* **2001**, *49*, 5732−5735.

(9) Matthews, S.; Mila, I.; Scalbert, A.; Pollet, B.; Lapierre, C.; Herve du Penhoat, C. L. M.; Rolando, C.; Donnelly, D. M. X. Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. *J. Agric. Food Chem*. **1997**, *45*, 1195−1201.

(10) Colin, L.; Cholet, C.; Geny, L. Relationships between endogenous polyamine, cellular structure and arrested growth of grape berries. *Aust. J. Grape Wine Res.* **2002**, *8*, 101−108.

(11) Renard, C. M. G. C.; Baron, A.; Guyot, S.; Drilleau, J. Interaction between apple cells walls and native apple polyphenols: Quantification and some consequences. *Int. J. Biol. Macromol.* **2001**, *29*, 115−125.

(12) Kennedy, J. A.; Matthews, M. A.; Waterhouse, A. L. Effect of maturity and wine water status on grape skin and wine flavonoids. *Am. J. Enol. Vitic*. **2002**, *53*, 268−274.

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

(14) Darné, G. Recherches sur la composition en anthocyanes des grappes et des feuilles de Vigne. Thèse d'état, Université de Bordeaux I, 1991.

(15) Cheynier, V.; Prieur, C.; Guyot, S.; Rigaud, J.; Moutounet, M. In *Wine—Nutritional and Therapeutic Benefits*; ACS Symposium Series 661; Watkins, T. R., Ed.; American Chemical Society: Washington, DC, 1997; pp 81−93.

(16) Stevens, R. M.; Harvey, G.; Aspinall, D. Grapevine growth of shoots and fruit linearly correlate with water stress indices based on root-weihted soil matric potential. *Aust. J. Grape Wine Res.* **1995**, *1*, 58−66.

(17) McCarthy, M. G. Developmental variation in sensitivity of *Vitis vinifera* L. (Shiraz) berries to soil water deficit. *Aust. J. Grape Wine Res.* **2000**, *6*, 136−140.

(18) Ojeda, H.; Deloire, A.; Carbonneau, A. Influence of water deficits on grape berry growth. *Vitis* **2001**, *40*, 141−145.

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