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Composition and Cellular Localization of Tannins in Cabernet Sauvignon Skins during Growth

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For two successive years, cell walls were isolated from the internal part of skin cells of *Vitis vinifera* L. cv. Cabernet Sauvignon grape berries grown in a vineyard. Procyanidin localization and composition were determined over the course of development. Tannins were mainly localized in the inner cell fraction, due to their biosynthesis and storage. Cell wall tannins always exhibited a higher mean degree of polymerization as compared to the internal cell fraction, which had a constant mDP. The mDP of cell wall tannins also tended to increase at the end of maturation. Our results suggest tannin polymerization near the cell wall but an aggregation in the vacuole during growth. The tannin composition was typical of skins, and small differences were noted between the two cell parts. Surprisingly, epigallocatechin-3-gallate was also detected, although in a very small amount. Epicatechin was present in significant proportions in both fractions, especially as an extension subunit, while epigallocatechin was likewise abundant, also as a terminal subunit. Last, procyanidin composition and organization seemed to be characteristic of the Cabernet Sauvignon variety.

KEYWORDS: ***Vitis vinifera* L.; skins; cell walls; procyanidins; ripening**

INTRODUCTION

Grape skins are crucial tissues for berry quality. As a physical barrier between the vine and its environment, skins are submitted to both internal and external influences. Changes in berry metabolism and external factors induce significant modifications in their structure and composition. Another major characteristic of grape skin tissue is the accumulation of polyphenols, which confer essential properties to grapes and wine, such as astringency. Anthocyanins accumulate during véraison, or color change. Tannins, or procyanidins, are synthesized precociously, but their accumulation and organization are modified throughout maturation. They can also contribute indirectly to grape and wine color by acting as copigments for anthocyanins (*1*). Anthocyanins and tannins also have a role in response to pathogens (*2*). These molecules are synthesized in the cytoplasm and stored in the vacuole. They can be associated with cell walls, which ensure the integrity and structure of the skin (*3*). Amrani (*4*) observed that the organization of tannins varied according to their localization and changed during ripening; aggregated structures were always present near the cell wall, while tannins in the vacuoles became condensed. A previous study (*5*) on the localization of procyanidins in grape seeds, an important source of tannins, showed differences between the two parts of the cell with regard to composition and polymerization on the one hand and between stages of development on the other hand. Skin tannins are important for winemaking from the time they are first released into the must (*6*). Phenolic extractability depends on their interaction with cell walls (*7*) that limit their diffusion and the release of aromatic compounds. It is well-known that skin tannins accumulate and change their organization during fruit growth, but despite their essential role in wine-making, little information is available as to their cellular distribution and organization. In the present work, we separated the cell walls from the inner part of Cabernet Sauvignon skin cells, and the distribution of polyphenols was investigated. We focused on tannin distribution and composition in these two fractions at different stages of development. Experiments were conducted under the same conditions for two successive vintages.

MATERIALS AND METHODS

Plant Material and Sample Collection. The experiment was conducted on berries (*Vitis vinifera* L. cv. Cabernet Sauvignon) grown in 2004 and 2005 in a vineyard in the Pessac-Léognan appellation near Bordeaux (France) and collected at different stages of development. The vineyard was planted in 1990 and grafted onto 101-14 rootstock. Row direction was north/south, planting density was 6500 vines per ha, and Guyot double pruning was used. During the 2004, five samplings of grape clusters were made: at the end of the first period of active growth (corresponding to berry touch), 48 days after anthesis (DAA) or stage 33 as defined by Eichorn and Lorenz (*8*); at three different points during the color change period:− 50% (phenological

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stage 36), 80%, and 100% (phenological stage 37) red ripe (RR), corresponding, respectively, to 63, 65, and 71 DAA; and at maturity (harvest), or 110 DAA, corresponding to the harvest, or stage 38. During 2005, eight samplings were made: at two green stages:− pea-sized berries and berry touch, 36 and 56 DAA, respectively, corresponding to stages 31 and 33 of the scale defined by Eichorn and Lorenz; at four points during the color change period: 10% (phenological stage 35), 50%, 80%, and 100% RR, corresponding, respectively, to 68, 70, 73, and 78 DAA; during fruit maturation: −2 weeks after the end of the color change (98 DAA); and at maturity (110 DAA, stage 38).

Random samples of five grape clusters on 10 vines were selected at each stage and immediately frozen in liquid nitrogen, and the berries were stored at −80 °C until analysis. The berry skins were carefully removed with razor blades and then analyzed.

Isolation of Cell Walls from Skins. The cell wall isolation procedure followed the method described by Harris et al. (*9*), and already described by Gény et al. (*4*), with slight modifications. Twenty-five skins were ground to a fine powder under liquid nitrogen and homogenized in 20 mL of 0.2 M Tris-HCl buffer, pH 7.5, containing 2.5% of EDTA. 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 then resuspended in 10 mL of homogeneization buffer and recentrifuged under the same conditions. The supernatant was filtered through a 0.45 μm PTFE filter and added to the previous supernatant. We designated this fraction the internal cell fraction. The pellets were washed with 0.1% Triton X-100 solution to remove membranous contaminants, and we referred to this fraction as the cell wall fraction. Fractions were examined under a light microscope after appropriate coloration (neutral red for membrane staining and carmine stain for cell walls) so as to confirm their purity and then stored at −80 °C until extraction. Isolations were done twice, but both fractions were analyzed at the same time.

Phenolic Compound Extraction and Assays. The extraction of phenolic compounds from internal cell fractions was adapted from Gény et al. (*5*). Twenty-five milliliters of fraction was mixed with 25 mL of MeOH/12 N HCl (99.9:0.1; v/v) and stirred for 14 h at room temperature. Extraction of the cell wall fraction was performed according to Gagné et al. (*10*). Two successive macerations were carried out for 3 h each, at room temperature and accompanied by stirring in 40 mL of MeOH/12 N HCl (99.9:0.1; v/v). After incubation, total phenolics were directly measured at 280 nm after diluting to 1:100 (*11*). The data are expressed in units of absorbance at 280 nm per gram of fresh weight ($A_{280\ nm}\ g^{-1}$ FW) in an extraction volume of 40 mL and represent the mean ± standard deviation (SD) of three replicates. Tannin and anthocyanin contents were determined by spectrophotometry according to the Ribéreau−Gayon and Stonestreet methods (refs *12* and *13*, respectively). The data are expressed in milligrams of total tannins per gram of fresh weight (internal cell fresh weight or cell wall fresh weight g^{-1} FW) or in milligrams per berry (/berry) and represent the mean ± standard deviation (SD) of three replicates.

Thioacidolysis and HPLC Analysis of Tannins. Subunit composition and mean degree of polymerization (mDP) of procyanidins in fractions were analyzed by reversed-phase HPLC after thiolysis as previously described by Atanasova et al. (*14*). Phenolic compound extracts (2 mL) were purified by adsorption chromatography using Toyopearl TSK HW-50F (Tosoh Corporation, Tokyo, Japan). Adsorbed proanthocyanidins were rinsed with 50 mL of H_2O/EtOH/TFA (55:45:0.005; v/v/v) to remove anthocyanins and low molecular contaminants and then eluted with 50 mL of acetone/H_2O. Samples were evaporated dry and then redissolved in 2 mL of MeOH. One hundred microliters was added to 100 μL of thiolysis reagent, composed of benzylmercaptan/2 N HCl/MeOH (0.5:2:7.5; v/v/v), and incubated for 5 h at 60 °C. Immediately before HPLC injection, 150 μL of a 4-methylcatechol solution at a concentration of 100 mg L^{-1} (Sigma, Saint Quentin Fallavier, France) was added to stop the reaction. HLPC analysis conditions were the following: column 250 mm × 4.6 mm, 5 μm, ODS (Beckman, Roissy CDG, France); flow rate, 1 mL min^{-1}; solvent A, H_2O/acetic acid (95:5; v/v); solvent B, MeOH/acetic acid (95:5; v/v); gradient, 30−40% B from 0 to 15 min, 40−70% B from 15 to 30 min, 70−100% B from 30 to 40 min, 100% B from 40 to 45 min, 100 to 30% B from 45 to 46 min, and 30% B from 46 to 50 min; injection 50 μL; and detection wavelength 280 nm. Flavanols and thioethers were identified according to their retention time and their absorption spectrum, in comparison with commercial molecules (Sigma, Saint Quentin Fallavier, France) and thiolysis products previously purified and characterized in the laboratory.

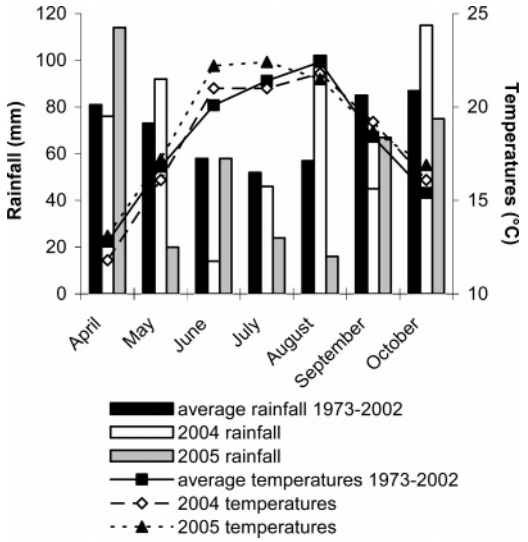

Figure 1. Comparison of rainfall (mm) and temperatures (°C) between 2004, 2005, and the 1973–2002 period from April to October.

RESULTS AND DISCUSSION

Composition and cellular localization of polyphenols, especially procyanidins, were studied in the skins of Cabernet Sauvignon berries harvested in 2004 and 2005 in the Bordeaux region. These two vintages were characterized by very different climatic conditions (**Figure 1**), which are well-known for greatly affecting phenolic compound biosynthesis (*15*, *16*). The ripening period in 2004 was rainy, with moderate temperatures. On the contrary, a precocious and continuous drought occurred throughout berry formation and growth in 2005 (except in June) with higher temperatures than the average temperature for the last three decades, thereby delaying ripening (*17*). The particularity of the 2005 vintage explains the shift in sampling dates: the middle of véraison was reached 63 DAA in 2004 but 70 DAA in 2005. Color change was complete 71 DAA in 2004 and 1 week later in 2005. However, moderate temperatures and rainfall in the fall of 2005 favored the completion of maturation after ripening. This explains why the maturity stage occurred on the same dates for both years. In addition, yields were particularly high in 2004, but with less phenolic maturity than in 2005 (*18*, *19*). The 2004 wines were quite tannic, but these tannins were rough and astringent. Yields were low in 2005, which produced powerful but not astringent tannins that were easy to extract, along with fresh, fruity aromas (*19*). In this study, we looked for a possible relationship between these data and tannin organization in skin cells.

Accumulation and Localization of Polyphenols during Ripening. Polyphenol contents were determined according to their localization. The fractionation method used to separate cell walls from the inner part of the cell did not prevent an unspecific adsorption of tannins on cell walls, which was an inevitable artifact of this approach. But, this method (*5*) and other similar techniques (*7*, *20*) were validated in numerous publications as an efficient way to study the organization of grape skin cells at a chemical level. Moreover, the results presented here support previous microscopic observations, bringing unquestionable evidence on tannin localization.

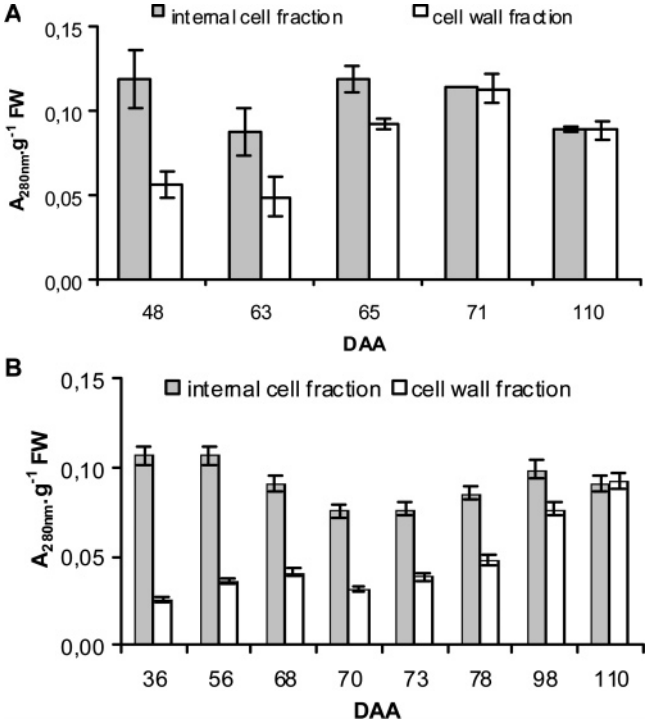

Figure 2. Total phenolics in different cell fractions of grape skins throughout growth of berries harvested in 2004 (**A**) and 2005 (**B**). The data represent the mean of three measures per 25 berry sample, in an extraction volume of 40 mL, ± standard deviation (error bars).

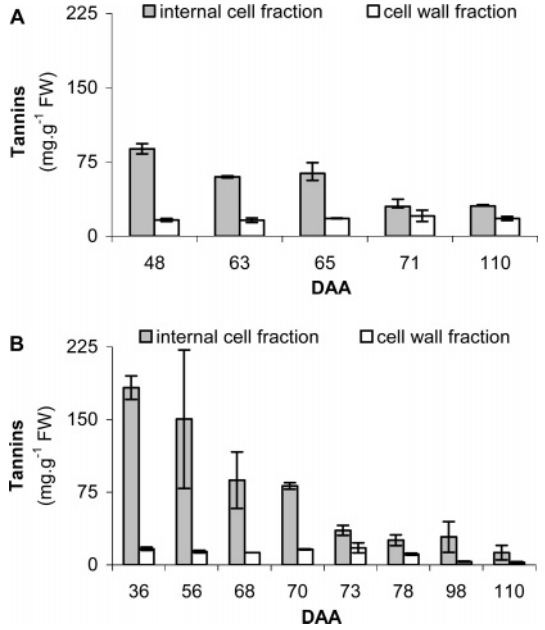

Figure 3. Tannin concentrations, expressed in milligram per gram of fresh weight, in different parts of skin cells throughout the growth of berries harvested in 2004 (**A**) and in 2005 (**B**). Each point represents the mean of three measures per 25 berry sample ± standard deviation (error bars).

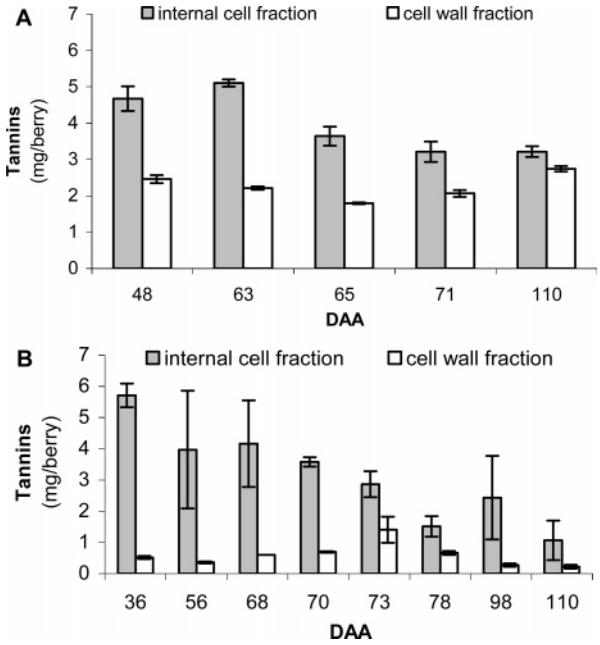

Figure 4. Tannin concentrations, expressed in milligrams per berry, in different parts of skin cells throughout the growth of berries harvested in 2004 (**A**) and in 2005 (**B**). Each point represents the mean of three measures per 25 berry sample ± standard deviation (error bars).

Total polyphenols, directly measured at 280 nm (**Figure 2**), developed differently in both fractions. In internal cell fractions, the accumulation pattern can be divided in three parts: the first growth period was characterized by a decrease in total polyphenols, followed by an increase from the end of véraison, and then, stabilization occurred at maturity. This appeared more clearly in 2005 when more samplings were analyzed. In cell wall fractions, a constant increase was observed. These profiles are similar for both years. Polyphenols were more abundant in internal cell fractions than in cell wall fractions up to the end of véraison, when amounts became equivalent. This is particularly noticeable in 2005, where absorbance of the cell wall fraction went from 5-fold to 2-fold the absorbance of the internal cell fraction.

The accumulation of tannins was similar in 2004 and 2005, as opposed to anthocyanin patterns (data not shown). When data are expressed per gram of fresh weight (**Figure 3**), contents decreased from early stages of development to maturity in internal cell fractions but remained nearly constant in cell wall fractions. When expressed per berry (**Figure 4**), accumulation was quite similar during the two growing seasons; tannin contents decreased continuously in the internal cell part and remained constant in the cell wall. We noticed that tannins accumulated in the cell wall fraction during the first active growth period and reached a maximum at 80% red ripe (73 DAA) in 2005, suggesting biosynthesis (achieved at véraison), when anthocyanin biosynthesis had not yet begun. This is in accordance with the results of Downey et al. (*21*) regarding Shiraz skins and with expression patterns of genes involved in the anthocyanin pathway established by Boss et al. (*22*). Decreasing tannin contents were measured from the end of véraison to maturity. During ripening, cell walls degrade by active hydrolysis of structural polysaccharides and lose pectin-bound calcium (*3*). This leads to a decreasing relative proportion of cell walls in grape skins. Whatever the expression of data, the tannin content decreased in both fractions, probably due to tannin catabolism. These results also suggest a change in cellular tannin organization. Previous microscopic observations have shown an increasing aggregation of tannins in the skin during ripening (*4*): from diffuse structures in the vacuoles of green berry skin, they form condensed masses on vacuole membranes. Either polymerization or the auto-association of tannins without strong binding between molecules may account for the formation of these structures and the limit of their extractability, as well as the fact that measured amounts are lower than expected in the internal cell fraction, when compared to measurements done

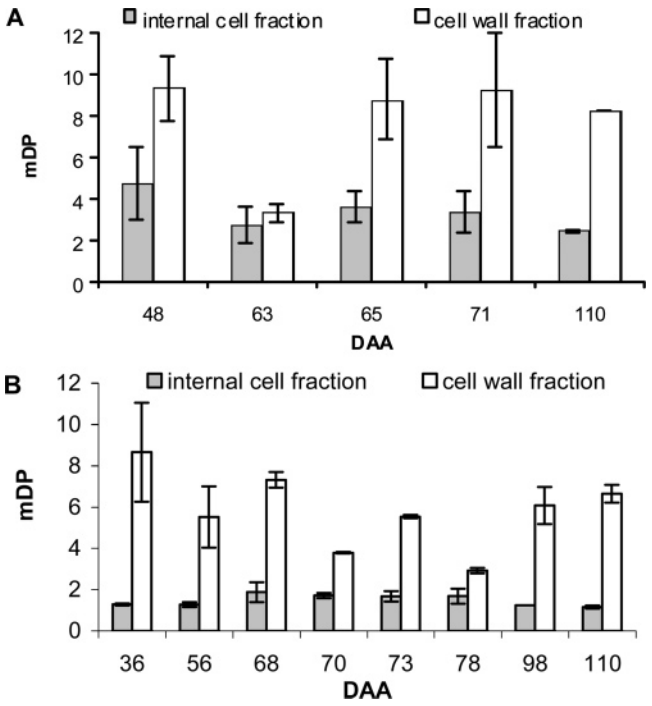

Figure 5. Mean degree of polymerization (mDP) of tannins from different skin cell fractions throughout the growing season for grapes harvested in 2004 (**A**) and 2005 (**B**). The data represent the mean of two determinations per 25 berry sample ± standard deviation.

on whole skin cells (data not shown). Tannins being synthesized in the vacuole, an intracellular transfer to cell walls may occur, explaining the decreasing contents in intracellular fractions, but no increasing contents were detected in the cell walls (*4*). A simultaneous polymerization that limits extractability could occur near cell walls and help the measured contents to remain constant. In addition, the drop in tannin content during the second growth period (beginning with véraison) may imply the hypothesis of tannin catabolism: a part of the accumulated pool could be used for anthocyanin biosynthesis (*23*), in accordance with the different accumulation of anthocyanins and tannins observed (data not shown). Furthermore, the determination of the mean degree of polymerization is the most direct way to investigate the mechanisms underlying these changes.

Proportions of anthocyanins and tannins were then calculated from these results (data not shown). The results were similar between the two fractions. Tannins were always present in a higher concentration, and an average of 80% of tannins was located in the vacuole. Anthocyanins were also mainly located in the inner cell fraction. They represent between 20 and 40% of polyphenols in all fractions. Our results contradict previous ones, showing that anthocyanins represent more than half of the total polyphenol content (*24*). However, the determined values exactly match with amounts measured in whole skins (data not shown).

Mean Degree of Procyanidin Polymerization during Berry Development. The mDP values (**Figure 5**) analyzed in both types of fractions are close to mDP values measured in seeds (*5*) but less than those for other grape varieties, especially Shiraz (*25*, *21*). It is important to keep in mind that results concerning mDP are largely dependent on the type of extraction technique used (*25*). Tannins in the internal cell fraction were less polymerized than those in the cell wall. The cell wall mDP was twice (in 2004) or three times (in 2005) the inner cell mDP. Its value remained constant in both years, whereas the mDP of the internal cell fraction in 2005 was smaller than that of 2004.

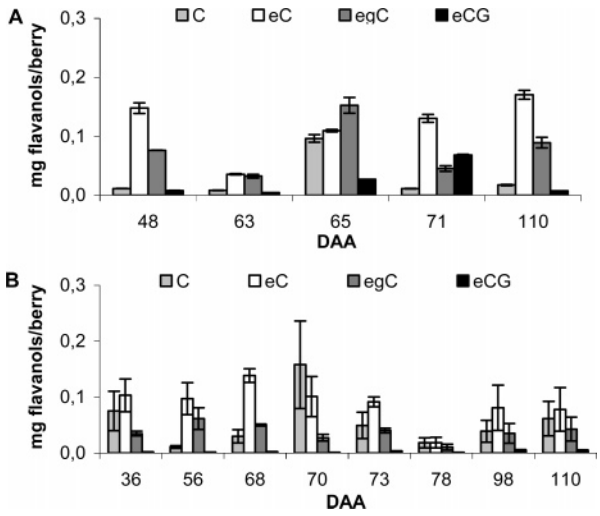

Figure 6. Tannin composition of the cell wall fraction of skins throughout the growing season for berries harvested in 2004 (**A**) and 2005 (**B**). The data represent the mean of two determinations per 25 berry sample ± standard deviation.

This can be related to vintage particularities: 2004 tannins were difficult to extract and conferred astringency and herbaceous characteristics to wine, whereas the tannins were fairly round and smooth in 2005 (*18*). The mDP of the internal cell fraction was nearly constant all through the growing season. These results suggest that the mass of tannins formed in vacuoles during ripening result from auto-association of tannins rather than polymerization. For cell wall fractions, mDP is more variable. For both years, the middle of véraison (63 DAA in 2004 and 70 DAA in 2005) was characterized by a 50% drop in mDP. At peak color change, the biosynthetic pathway leading to anthocyanins has already been activated. Since the biosynthetic pathway largely coincides with the tannin pathway, some precursors may be used to synthesize flavanols, which are then translocated to cell walls. This transient increase in free monomers could explain the decrease of the cell wall mD. But, no increase in tannin contents is observed to support that hypothesis. Other mechanisms are probably involved in the control of mDP during véraison. It is noticeable that a similar drop occurred at 78 DAA in 2005. The delay in the maturation process of this vintage could explain this profile: a delayed activation of the anthocyanin pathway may influence the mDP decrease. In our study, mDP tended to increase and then stabilize at the end of ripening, in accordance with microscopic observations of aggregated tannins close to the cell wall (*26*, *27*) and with data from Shiraz skins (*24*). Moreover, De Freitas et al. (*28*) have demonstrated that the mDP value depends on the grape variety. This is in accordance with our results, showing a similar mDP for both years for Cabernet Sauvignon, despite different growth conditions. The extractability of tannins could also be limited by polymerization, which would explain why the tannin content decreased. A significant increase in cell wall polymerization from véraison to maturity and lower mDP in grape seeds were reported: −two to three for inner cell mDP and −four to six for cell wall mDP (*5*). Skin tannins are thus more polymerized than seed tannins. It is well-known that condensed tannins react most strongly to proteins, so this polymerization could help strengthen cell walls, thereby protecting berry skins from external stress. This must be taken into account on an organoleptic level, as previous studies showed that the increase in astringency could be correlated with an increase in mDP (*29*).

Procyanidin Composition during Maturation. The composition of each fraction was determined at different stages of

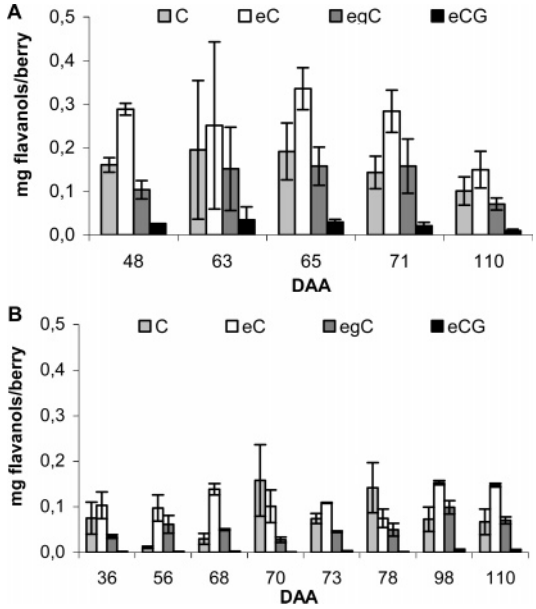

Figure 7. Tannin composition of the internal cell fraction of skins throughout the growing season for berries harvested in 2004 (**A**) and 2005 (**B**). The data represent the mean of two determinations per 25 berry sample ± standard deviation.

development. The results for cell wall fractions and internal cell fractions are presented in **Figures 6** and **7**, respectively. Four flavanol units typical of grape skin tannins were significantly detected: epicatechin, epigallocatechin (prodelphinidin), catechin, and epicatechin-3-gallate. This last unit has already been characterized in grape skins, especially in Shiraz, where they represent less than 6% of total flavanols (*21*, *24*). Except for the 100% red ripe point in cell walls in 2004 (71 DAA), our measures matched with these data since we found less than 5% of epigallocatechin-3-gallate, whatever the fraction or stage of development. As expected, epigallocatechin was significantly detected as a specific skin flavanol but was absent from seed tannins. Fraction composition was similar between years, but differences existed between the two types of cell fractions, in accordance with previous results on Cabernet Sauvignon seed tannins (*5*). Moreover, epicatechin represented nearly 65% of the procyanidins in grape seeds, regardless of the part of the cell or stage of development. Skin and seed fractions showed similarities in composition, which may be related to the Cabernet Sauvignon variety. The main differences between skins and seeds consist of the significant level of epigallocatechin and very low amount of epicatechin-3-gallate in the skins. With regard to cell wall fractions (**Figure 6**), epicatechin is predominant, while catechin and epigallocatechin are present in variable amounts depending on the degree of maturation. In all instances, epicatechin-3-gallate is always present in negligible amounts and dramatically inferior in skins as compared to seeds. This is surprising since galloylation increases the affinity of tannins for proteins and thus for cell walls. It has been shown that epicatechin-3-gallate in seeds was significantly involved in the structure of tannins associated with cell walls (*5*). Inner cell fractions (**Figure 7**) also exhibited very low levels of galloylated tannins, which can be correlated with their organization and with data on other grape varieties. For instance, Cabernet Franc (*30*) or Merlot (*31*) skins are characterized by a very small degree of galloylation. We found that the other three major flavanols were involved in equivalent amounts. However, epicatechin was the main monomer, followed by catechin, as observed in seed tannins. When expressed in milligrams per

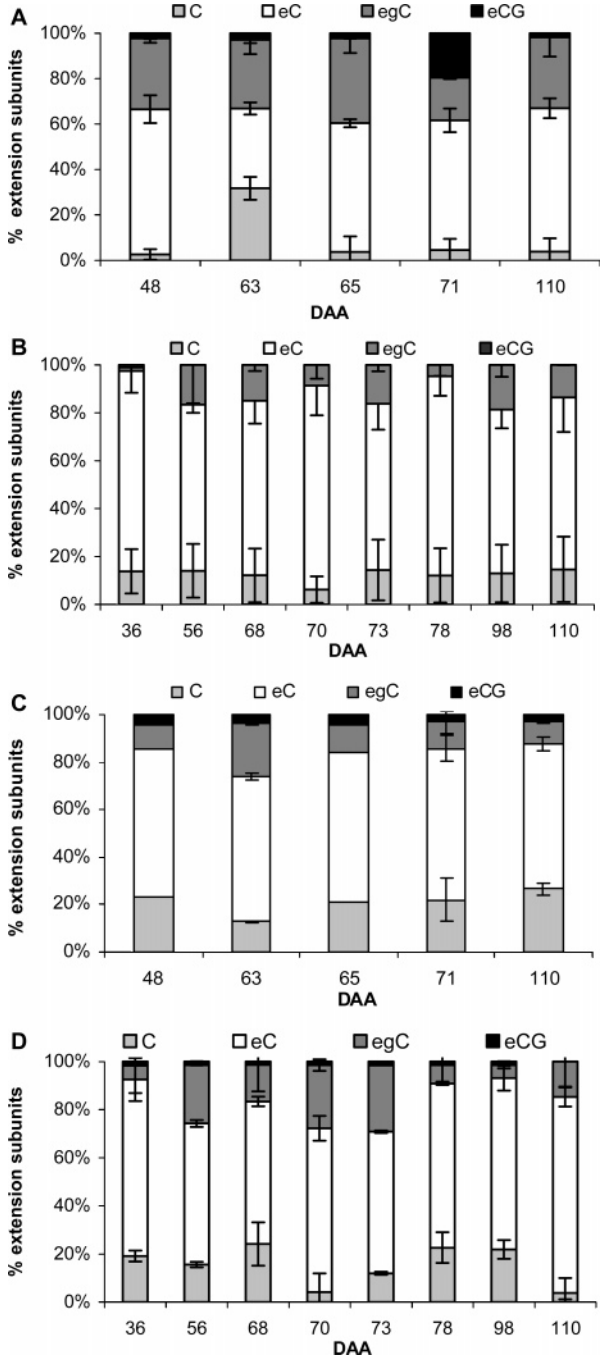

Figure 8. Percentage of different flavanols as extension subunits in cell wall fractions (**A** and **B**) and internal cell fractions (**C** and **D**) of skins throughout the growing season for berries harvested in 2004 (**A** and **C**) and 2005 (**B** and **D**). The data represent the mean of two determinations per 25 berry sample ± standard deviation.

berry, tannin contents in inner cell fractions remained quite constant or slightly increased during maturation. However, contents fluctuated in cell wall fractions, especially in 2004, when they accumulated at 50% red ripe (65 DAA), possibly due to the activation of the biosynthetic pathway. In 2005, the tannic content in both types of fractions paralleled the accumulation of skin tannins: the amount increased up to peak véraison (70 DAA) and then decreased. This argued in favor of the assumption that biosynthesis continued during the first growth period.

The polymer composition was also investigated: the proportions of different flavanols were determined as extension subunits (**Figure 8**) and as terminal subunits (**Figure 9**). Our

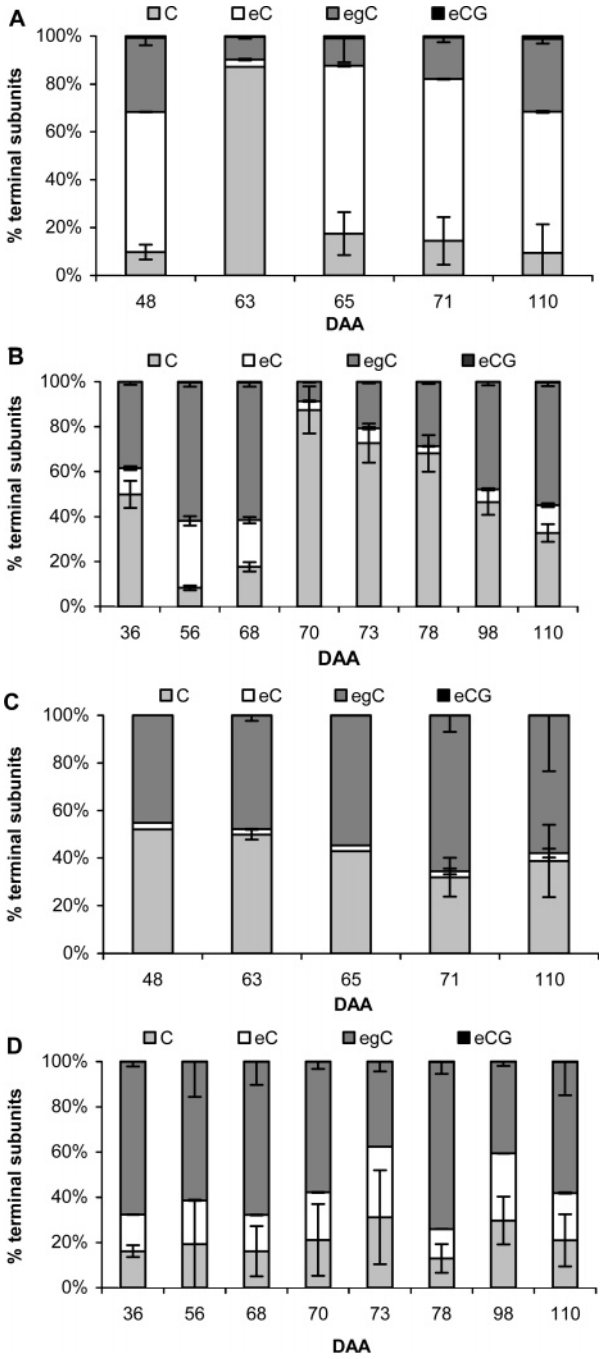

Figure 9. Percentage of different flavanols as terminal subunits in cell wall fractions (**A** and **B**) and internal cell fractions (**C** and **D**) of skins throughout the growing season for berries harvested in 2004 (**A** and **C**) and 2005 (**B** and **D**). The data represent the mean of two determinations per 25 berry sample ± standard deviation.

investigation revealed that the composition of terminal and extension subunits was similar in both cell fractions. For both of these, epicatechin was the main extension subunit, whereas epigallocatechin and catechin were present in nearly equal and constant amounts. Epicatechin-3-gallate was only detected as an extension subunit. The terminal subunit composition fluctuated for cell wall fractions, while it remained constant for internal cell fractions. Epigallocatechin was always the main flavanol in the inner cell, whereas epicatechin or epigallocatechin were the main flavanols of in cell wall tannins. One major change occurred at 50% red ripe (65 DAA in 2004 and 70 DAA in 2005): catechin became largely predominant, replacing epicatechin (2004) or epigallocatechin (2005). In 2005, the presence of catechin slightly decreased until maturity. Further studies, in particular, expression studies of the leucoanthocyanidin reductase that catalyzes catechin formation, are needed to elucidate this change.

This paper presents the first results on the composition and polymerization of tannins in two different parts of the grape skin cells. These parameters were studied over the course of two successive vintages (from green berry to harvest). Our study showed that the identified flavanols were typical of those found in grape skins, with significant amounts of prodelphinidins. Cell wall fractions exhibited a higher mDP value than internal cell fractions. Both had a similar composition, with low epicatechin-3-gallate content. All these data seemed to be typical of the Cabernet Sauvignon variety. In addition, our results suggest a tannin biosynthesis during the first active growth period followed by transport from the inner cell to the cell wall. Considering their organization throughout maturation, an aggregation may occur in the vacuole and a polymerization near the cell wall. Finally, these results emphasize the influence of vintage characteristics on the organoleptic properties of tannins and thus on fruit and wine quality.

ABBREVIATIONS USED

C, catechin; eC, epicatechin; eCG, epicatechin-3-gallate; egC, epigallocatechin; FW, fresh weight; mDP, mean degree of polymerization.

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