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Accumulation and Extractability of Grape Skin Tannins and Anthocyanins at Different Advanced Physiological Stages

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Quantitative and qualitative modifications of tannins and anthocyanins in grape skin were investigated at different dates of harvest, from berries sorted on the basis of their density. Free anthocyanins accumulated until 170 g/L of sugars in pulp before undergoing a slight decrease. Changes in anthocyanin composition were observed with increasing sugar levels in the pulp that reflected structural differences between classes of anthocyanins. The proportion of methoxylated anthocyanins continued to increase in the skin as sugar accumulated while the proportion of coumaroylated anthocyanins initially increased (up to 200 g/L of sugars in the pulp) and then rapidly decreased. In comparison, no major quantitative nor qualitative change was observed for tannins, except for a slight increase of the mean degree of polymerization. Whatever the physiological stage of the pulp, the extraction yield of skin phenolics into hydroalcoholic solution for 5 h was lower than 77% for anthocyanins and 38% for proanthocyanidins. For both classes of compounds, no clear evolution in these extraction yields could be observed as sugars accumulated in pulp (from 162.6 to 275.0 g/L). Nevertheless, some structural features within each family of compounds significantly influenced extractability, for example, a lower extraction yield for coumaroylated anthocyanins and for tannins with a high degree of polymerization. Finally, no direct relationship could be found in extraction media between the amounts of all red pigments (measured in acidic conditions) and the color intensity at 520 nm (measured in wine-like model solutions).

KEYWORDS: Grape skin; ripening; tannins; proanthocyanidins; anthocyanins; biosynthesis; pulp sugar content; extractability; HPLC; extraction yield

INTRODUCTION

Condensed tannins (proanthocyanidins) and anthocyanins constitute the two most abundant classes of phenolics in the berry skins. Condensed tannins are polymeric flavan-3-ols that mainly comprise subunits of (−)-epicatechin, but also significant amounts of epigallocatechin, (+)-catechin, and epicatechin-3-*O*-gallate (*1*−*4*). These different subunits are linked by C4−C8, and to a lesser extent C4−C6, interflavan bonds. Anthocyanins are responsible for the color of red and black varieties. Most *Vitis vinifera* varieties produce non-acylated glucoside, acetylglucoside, coumaroylglucoside (and to a lesser extent caffeoylglucoside) derivatives of delphinidin, cyanidin, petunidin, peonidin, and malvidin (*5*−*11*). Each variety of grape has a particular anthocyanin profile so that anthocyanin analysis has been proposed for the varietal authentification of grapes and wines (*7*, *12*−*14*). Both anthocyanins and tannins are partly extracted from grape skins during wine making and can undergo structural transformations through many reactions with significant influence on wine sensory characteristics as they are involved in astringency, bitterness, color intensity, and color stability (*15*−*20*).

Accumulation of anthocyanins in skins starts at véraison and is at a maximum around harvest (*4*, *11*, *21*, *22*). Some studies describe a decline in total anthocyanins just before harvest (*6*, *23*) and/or during over-ripening (*10*).

On the other hand, proanthocyanidins mainly accumulate before véraison (*24*). Whether total amounts and/or composition of tannins change or not in skins during ripening is not clear. Some authors observed an increase in total tannins after véraison (*21*, *25*) and others a decrease (*2*, *26*, *27*), but the analytical methods used were not always the same. Similarly, both an increase (*3*, *4*) and a decrease (*2*, *25*) in the mean degree of polymerization (mDP) have been reported.

Because of their impact on the sensory characteristics of wine, extraction of anthocyanins and proanthocyanidins during wine making has often been investigated (*28*−*31*). However, few studies have examined the impact of ripening stage of the grapes on extractability of phenolic compounds into the wine (*32*, *33*).

The objective of this work is first to investigate anthocyanin and proanthocyanidin quantitative and compositional modifications in grape skins during sugar accumulation in pulp and then

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to follow over the same period the evolution in their extractability into a model hydroalcoholic solution. This approach aims at determining whether the date of harvest may have an influence on the skin phenolic extraction at a quantitative and/or compositional level, irrespective of the impact of pulp, seeds, or yeasts present in actual maceration processes.

MATERIALS AND METHODS

Grape Samples. *Vitis vinifera* var. Shiraz grape samples were harvested weekly from 1 to 10 weeks after véraison from a vineyard located at Montpellier (France), in 2004. To improve the physiological homogeneity of the different samples, berries were calibrated according to their density (i.e., total soluble solids). Density was estimated by flotation of berries in ten different salt solutions (from 100 to 190 g/L NaCl) so that the difference in total soluble solids of two consecutive berry batches was about 17 g/L (i.e., 1 vol % in potential titratable alcohol). For each date, the class of berries retained after density sorting was not necessarily the major one. For the first weeks after véraison, the less dense classes were selected, and for the last samples, the most dense classes were selected, so that the physiological differences between the first (1 week after véraison) and the last sample (10 weeks after véraison) were emphasized. A representative sample of 100 sorted berries was squeezed for measurements of juice pH (using a calibrated pH-meter), total acidity (by titration with NaOH), and total soluble solids (°Brix, using a hand-held refractometer).

A second sample of 100 sorted berries was manually peeled, and the skins were immediately frozen in liquid nitrogen, freeze-dried, and dry-crushed in liquid nitrogen with a freezer mill (Bioblock, Ilkirch, France) for later determination of anthocyanin and tannin contents. Powders were stored under vacuum in the dark at +4 °C in the presence of P_2O_5.

Finally, a third sample of 180 sorted berries (2 × 90) was also manually peeled for hydroalcoholic extraction on fresh material.

Chemicals. Methanol and acetonitrile were HPLC grade and were purchased from Merck (Darmstadt, Germany). (+)-Catechin, (−)-epicatechin, phloroglucinol, and L-ascorbic acid were purchased from Sigma (St. Louis, MO). All flavan-3-ol-4-phloroglucinol adducts were isolated and characterized as previously described (*34*, *35*).

Extraction in Hydroalcoholic Solutions. Ninety freshly isolated skins were placed in 150 mL of a hydroalcoholic solution containing 12 vol % ethanol, 2 g/L potassium hydrogen tartrate, and 100 mg/L SO_2 (to limit oxidation of phenolic compounds throughout extraction). Flasks were placed under argon and gently stirred at 27 °C for 5 h. In these conditions, a 5-h-long extraction was sufficient to achieve a plateau for all the investigated compounds. The pH value of the extraction medium varies from 3.8 (0 h) to 4.2 (5 h) for all samples. At regular intervals (5, 10, 20, 30, 60, 120, 180, 240, and 300 min), 1 mL of extraction medium was taken and centrifugated (10 min, 10 000*g*) for spectrophotometric measurements (total phenols, total flavanols, total red pigments, and absorbance at 520 nm). At the end of the extraction, residual solid material was first rinsed with a hydroalcoholic solution and then immediately freeze-dried and dry-crushed in liquid nitrogen with a freezer mill. The powders obtained were used for the analysis of non-extracted phenolic compounds. Extraction media were also frozen in liquid nitrogen after centrifugation (without evaporation) for later determination of extracted compounds. All hydroalcoholic extraction experiments were performed in duplicate.

Preparation of Skin Extracts before and after Hydroalcoholic Extraction. Powders obtained from initial skins (50 mg) and from residual solid parts after hydroalcoholic extraction (25 mg) were extracted in the dark with 3 × 0.5 mL acetone/water (60:40, v/v) containing 0.05% trifluoroacetic acid. A fourth extraction with 0.5 mL of the same solvent was performed over a 12 h period at +4 °C. After centrifugation (15 min, 10 000*g*), an aliquot (200 μL) of pooled extraction supernatants was dried under vacuum at 35 °C for 120 min. Dried samples were either redissolved in 20% MeOH containing 1% HCl before spectrophotometric measurements (total phenols, total flavanols, and total red pigments) and HPLC analysis of free anthocyanins or used for acid-catalyzed degradation of proanthocyanidins in the presence of phloroglucinol.

Spectrophotometric Analysis. Total red pigments in skin extracts and hydroalcoholic solutions were measured at 520 nm after dilution in appropriate amounts of 1 N HCl at pH 1.0 (to be between 0.02 and 1 absorbance unit) and are expressed as mg/L malvidin-3-*O*-glucoside equivalents. Color extracted in hydroalcoholic media was assayed at 520 nm without dilution in cuvettes with appropriate optical path length (to be between 0.02 and 1 absorbance unit) and was also expressed as mg/L malvidin-3-*O*-glucoside equivalents. Total phenols were measured by the method of Folin-Ciocalteu, using gallic acid as a standard (*36*). Samples were diluted in appropriate amounts of H_2O (to be within the 10−80 mg/L range in gallic acid equivalents) and 2.5 mL of Folin-Ciocalteu reagent 0.1× (1:9 Folin-Ciocalteu reagent/water mixture) was added. After 3 min at room temperature, 2 mL of 7% (w/v) Na_2CO_3 was added, and the mixture was placed at 50 °C for 5 min. Absorbance at 750 nm was then measured after cooling. Total flavanols were assayed with dimethyl-aminocinnamaldehyde (DMACA), using (+)-catechin as a standard (*37*). Samples were diluted in appropriate amounts of MeOH (to be within the 2−16 mg/L range in (+)-catechin equivalents), and 200 μL of that solution were added in 1 mL of DMACA (0.1% w/v in a cold 9:1 MeOH/HCl mixture). Absorbance at 640 nm was read after 10 min.

HPLC Analysis of Free Anthocyanins. Free anthocyanins were analyzed by HPLC using a Waters Millenium HPLC-DAD system (Milford, MA). The column consisted of a reversed-phase Atlantis dC18 (Waters, Milford, MA; 5 m packing, 250 mm × 2.1 mm i.d.) protected by a SecurityguardTM cartridges C18 (Phenomenex, Torrance, CA; 4.0 mm × 2.0 mm i.d.). Oven temperature was set at 30 °C. The mobile phase was a linear gradient of water/acetonitrile/formic acid (80:15:5; solvent B), in water/formic acid (95:5; solvent A), at a flow rate of 0.25 mL/min. Proportions of solvent B were as follows: 0−15 min, 0−2%; 15−30 min, 2−10%; 30−40 min, 10−20%; 40−45 min, 20−30%; 45−50 min, 30−40%; 50−55 min, 40−50%; 55−60 min, 50−80%; and 60−65 min, 80−0%. The elution was monitored on a Waters 996 photodiode array (240−600 nm) and Millenium 32 software. All analyses were performed in duplicate. Anthocyanins were identified according to their retention time and UV−vis spectra (*38*). Quantification was carried out from peak areas at 520 nm using malvidin-3-*O*-glucoside as an external standard.

HPLC Analysis of Tannins after Acid-Catalyzed Degradation in the Presence of Phloroglucinol. Dried extracts of initial skins and residual solid material after hydroalcoholic extraction as well as the corresponding hydroalcoholic extraction medium (after evaporation) were dissolved in 100 or 200 μL of MeOH containing 0.2 N HCl, 50 g/L phloroglucinol, and 10 g/L ascorbic acid and heated for 20 min at 50 °C. Then, an equivalent volume of aqueous 200 mM sodium acetate was added to stop the reaction (*34*). Released terminal subunits and extension subunit−phloroglucinol adducts were analyzed by HPLC, using a reversed-phase Atlantis dC18 column (Waters, Milford, MA; 5 μm packing, 250 mm × 4.6 mm i.d.) protected by a guard column of the same material (20 × 4.6 mm i.d.; Waters, Milford, MA) and with a SecurityguardTM cartridge C18 (Phenomenex, Torrance, CA; 4.0 mm × 3.0 mm i.d.). The mobile phase was a linear gradient of water/acetonitrile/formic acid (80:18:2; solvent B) in water/formic acid (98:2; solvent A), at a flow rate of 0.25 mL/min at 30 °C. Proportions of solvent B were as follows: isocratic for 5 min with 0%; 5−35 min, 0−10%; 35−65 min, 10−20%; 65−70 min, 20−100%; and 70−75 min, 100−0%. The proanthocyanins were detected by two successive detectors. The first one consisted of a photodiode array 1100 (Agilent Technologies, Waldbronn, Germany) and was used at 280 nm for the quantitative determination of extension subunit−phloroglucinol adducts. The second one, a spectrofluorimeter Shimadzu (Kyoto, Japan), was used to improve accuracy of the quantitative determination of terminal subunits. Excitation and emission wavelengths were 275 and 322 nm, respectively, as previously described (*39*). Chromatograms were monitored on a Chemstation A.10.02 software (Agilent Technologies, Waldbronn, Germany). Each analysis was performed in triplicate. The mDP of proanthocyanidins as well as all qualitative data was calculated on a molar basis.

Table 1. Physiological Characteristics of Densimetrically Sorted Berries of *Vitis vinifera* L. Cv. Shiraz Grown in Montpellier, France, in the 2003–2004 Season, from 1 Week to 10 Weeks after Véraison

date of harvest	physiological data on the homogeneous berry sample selected on a densimetric basis						
weeks after véraison	berry weight (g)	°Brix	sugar content (g/L)	potential alcohol (vol %)	pH	titratable acidity (mequiv/L)	sugar to acidity ratio (g/mequiv)
1	1.89	14.2	127.3	7.56	3.07	191.0	0.67
2	2.20	17.4	162.6	9.66	3.26	130.0	1.25
3	2.30	18.0	169.3	10.06	3.21	116.8	1.45
4	2.29	19.5	186.8	11.10	3.41	99.5	1.88
5	2.26	20.1	193.4	11.49	3.43	97.9	1.98
6	2.20	20.8	201.1	11.97	3.49	85.4	2.36
7	1.96	22.7	222.4	13.21	3.67	76.5	2.92
8	1.84	24.2	240.6	14.30	3.67	73.5	3.28
9	1.70	26.8	270.4	16.07	3.72	79.0	3.42
10	1.62	27.2	275.0	16.34	3.79	73.2	3.76

RESULTS AND DISCUSSION

Physiological Data on Berry Samples. As the physiological characteristics of grape berries in a vineyard are very heterogeneous (*40*), samples were harvested and sorted out, as described above, as a function of the berry density (directly related to their content in total soluble solids). The physiological ripeness of the berry samples selected at each harvest date was assessed by measurement of berry weight (g/berry), °Brix, pH, and titratable acidity (mequiv/L). °Brix is directly related to the sugar content (g/L) and potential titratable alcohol (vol %). The sugar content of the 10 samples ranged between 127.3 and 275.0 g/L, corresponding to a potential titratable alcohol of 7.56 and 16.34 vol %, respectively, confirming that the sampling provided berries with contrasted physiological patterns (**Table 1**). The maximum mean berry weight was achieved for a sugar content of about 170−190 g/L. In agreement with previous studies performed on non-sorted berries (*2*, *10*, *23*), the berry weight then decreased as the sugar content increased, because of water loss.

Anthocyanin Composition in Berry Skins as a Function of the Pulp Sugar Content. Skin extracts were analyzed by spectrophotometric methods to quantify total red pigments and by HPLC methods to quantify free anthocyanins. Both total red pigments and free anthocyanins accumulated rapidly until 170 g/L of sugars in pulp (18.0 °Brix), as described in previous studies performed on non-sorted berries (*10*). Afterward, while the amount of total red pigments remained nearly unchanged, free anthocyanins slightly decreased (**Figure 1**), suggesting a conversion of free anthocyanins to derived pigments. The presence of such compounds in grape skins has been previously reported (*3*, *4*, *32*, *41*).

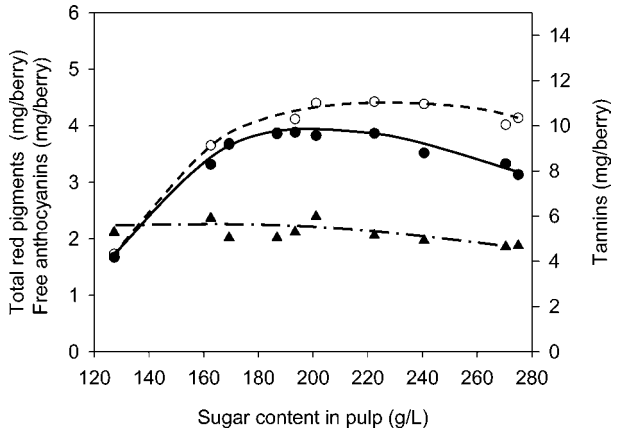

Figure 1. Amounts of total red pigments (○), free anthocyanins (●), and proanthocyanidins (▲) in skins as a function of pulp sugar content.

Skin free anthocyanin composition significantly changed as the pulp sugar content increased. As shown in **Figure 2**, when anthocyanins were grouped as a function of their B-ring substitution pattern (aglycone structure), the amounts of delphinidin, cyanidin, and petunidin derivatives reached a maximum before those of peonidin and malvidin derivatives (for sugar levels in the pulp of 170 and 200 g/L, respectively). As a consequence, the proportion of two of the three methoxylated forms, peonidin and malvidin, continually increased as sugars accumulated in pulp, the third one (petunidin) appearing to be an intermediate form between delphinidin and malvidin. The percentages of peonidin plus malvidin derivatives were 73, 80, and 84% at 127, 201, and 275 g/L of sugars in pulp, respectively. This suggests that methyltransferase activity (converting cyanidin to peonidin and delphinidin to petunidin and malvidin (*5*,

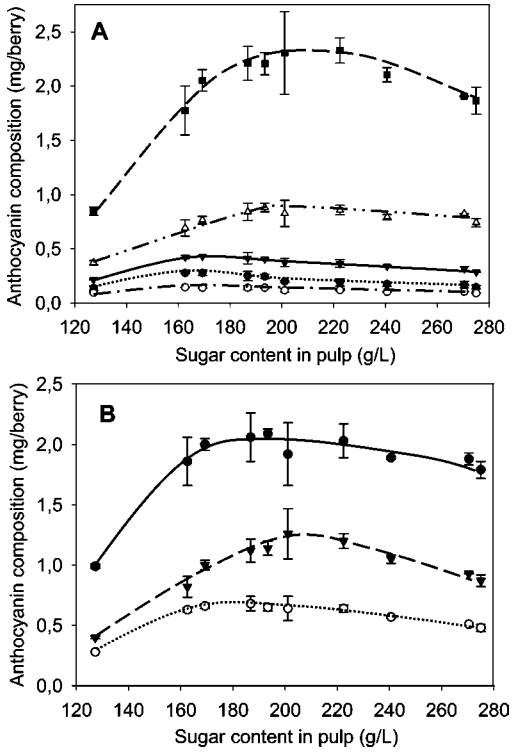

Figure 2. Changes in amounts of the different free anthocyanin species. **A**, Anthocyanins grouped as a function of their B-ring substitution pattern: (■), malvidin derivatives; (△), peonidin derivatives; (▼), petunidin derivatives; (◆), delphinidin derivatives; (○), cyanidin derivatives. **B**, Anthocyanins grouped as a function of their C-ring substitution pattern: (●), non-acylated derivatives; (○), acetylglucoside derivatives; (▼), coumaroylglucoside derivatives. Results are mean values of duplicates.

10, *42*)) increased during grape ripening compared to other activities upstream in the flavonoid pathway.

Surprisingly, the proportions of 3′-substituted and 3′,5′-substituted aglycones remained constant (27 and 73%, respectively) whatever the physiological stage, as if activity of flavonoid 3′-hydroxylase was rigorously constant relative to that of flavonoid 3′,5′-hydroxylase. The ratio between 3′-substituted and 3′,5′-substituted aglycones seems to rather be a function of the cultivar, for example, 23:77 for Graciano, 5:95 for Tempranillo, 8:92 for Cabernet Sauvignon, or 12:88 for Tannat (*8*, *9*), although cultural practices, types of soil, and climatic conditions sometimes appear to significantly modulate these ratios (*22*, *43*).

The amounts of non-acylated glucoside and acetylglucoside derivatives reached a maximum before those of coumaroylglucoside derivatives (no caffeoyl esters were detected in fresh skins; **Figure 2**) so that their proportions were 59:17:24, 50:17:33, and 57:25:28, at 127, 201, and 275 g/L of sugars in pulp, respectively. Non-acylated derivatives were predominant (>50%) but coumaroylglucoside derivatives were also present in high quantities (>24%), representing one-third of free anthocyanins at maturity (potential titratable alcohol between 12 and 13 vol %).

The degradation rate observed at high sugar content in pulp (over 200 g/L) was higher for coumaroylglucoside derivatives (123 μg/berry/week) than for non-acylated glucoside derivatives (77 μg/berry/week) or acetylglucoside derivatives (59 μg/berry/week). Coumaroylglucoside derivatives might be hydrolyzed to restore the non-acylated forms, or the cinnamoyltransferase activity might decrease compared to the other activities involved in anthocyanin biosynthesis. Coumaroylglucoside derivatives may also be more reactive and consequently more involved in the formation of derived pigments than other anthocyanins. Their higher reactivity has been previously observed during fermentation, where the rate of formation of *p*-coumaroylvitisins was estimated to be higher than that of non-acylated vitisins (*44*).

Proanthocyanidin Composition in Berry Skins as a Function of the Pulp Sugar Content. Tannins were analyzed in skin extracts by acid catalysis in the presence of excess phloroglucinol. No increase in tannin content could be observed as sugars accumulated in pulp (**Figure 1**). Considering a recent work from Bogs et al. (*45*), these results indicate that proanthocyanidin and anthocyanin biosynthesis occur sequentially in skins and that both pathways do not work together, maybe because of regulation phenomena between on one hand UDP−glucose/flavonoid 3-*O*-glucosyltransferase, leading to anthocyanins, and on the other hand anthocyanidin reductase and leucoanthocyanidin reductase, both leading to flavan-3-ols. Changes in skin tannin content in our study were different from those reported in a previous work on the same cultivar with the same analytical method (*2*), in which an important decrease was observed (ca. 60%) on the same range of sugar contents. Other works also described a decrease in tannins in Pinot Noir (ca. 25%) and in Shiraz (30 to 50%), but no clear trend in Cabernet Sauvignon (*4*, *26*). In sharp contrast, some other studies, performed on Merlot and Tempranillo cultivars by spectrophotometric methods, described a significant increase in total tannins (*21*, *25*).

(−)-Epicatechin and epigallocatechin accounted for about 60−70% and 20−30% of total subunits, respectively, in agreement with previous studies performed on Merlot, Shiraz, and Pinot Noir (*1*−*3*, *40*) (**Figure 3**). The other subunits ((+)-catechin and epicatechin-3-*O*-gallate) were present at less than 8%. Note that in our case, monomers of (+)-catechin and (−)-epicatechin were included in tannin analyses because they did not significantly impact on the overall tannin composition (data not shown). Whatever the physiological stage studied, (−)-epicatechin constituted more than 65% of the extension subunits whereas (+)-catechin constituted more than 86% of the terminal subunits, in agreement with Downey et al. (*2*).

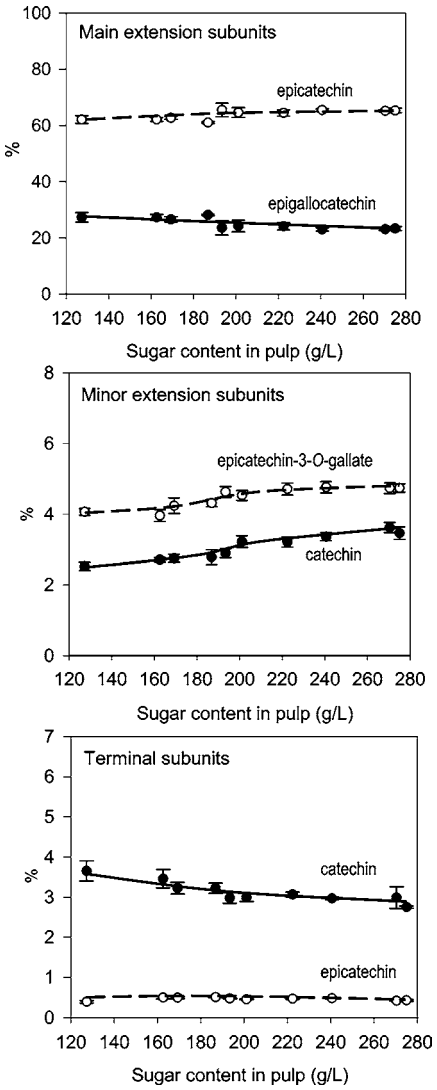

Figure 3. Changes in proanthocyanidin composition in skins as a function of pulp sugar content. Results are mean values of triplicates.

Proanthocyanidin composition slightly changed as sugars accumulated in the pulp (**Figure 3**). Concerning the extension subunits, epigallocatechin decreased from 28 to 23% whereas catechin increased from 2.5 to 3.5% together with epicatechin-3-*O*-gallate (from 4.0 to 4.8%). We also observed a slight increase in average polymer length (from 25 to 31 units). All these minor compositional modifications were in good agreement with a previous work performed on Shiraz (*2*). Regarding the change in mDP, the literature is somewhat contradictory, some authors describing an increase (*3*, *4*) and some others a decrease (*2*). In our opinion, all the disagreements found in literature might result from different methodological approaches (isolation procedures, technique of analysis) rather than from actual differences, although some other parameters can influence tannin structure (vintage, vine water status, and type of soil, for example) (*4*, *22*, *40*, *46*).

Kinetics of Hydroalcoholic Extraction of Skin Phenolic Compounds as a Function of the Pulp Sugar Content. Hydroalcoholic extractions were performed on berries in the

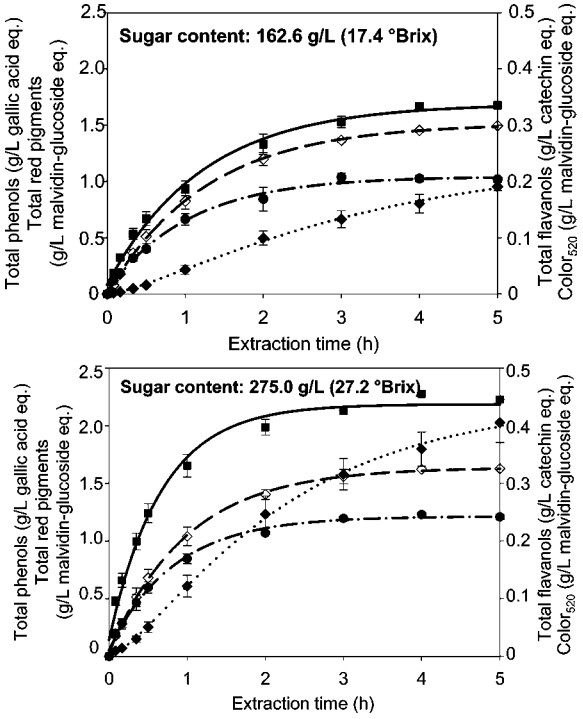

Figure 4. Extraction in a model hydroalcoholic solution of total phenols (■), total flavanols (●), total red pigments (◇), and color at 520 nm (◆) from skins isolated from berries with two contrasted pulp sugar contents. Kinetics were performed in duplicate.

162.6−275.0 g/L sugar content range. For each physiological stage, fresh skins of 90 berries were extracted for 5 h in a hydroalcoholic solution as described above. At regular intervals, 1 mL of extraction medium was taken for spectrophotometric determinations of total phenols, total flavanols, total red pigments (pH 1.0), and absorbance at 520 nm (A520). Spectrophotometric results were fitted (SigmaPlot 9.0 software) with a classical diffusion equation for total phenols, total flavanols, and total red pigments ($y = y_0 + a[1 - e^{-bx}]$) and with a Chapman type equation for the evolution in A520 ($y = y_0 + a[1 - e^{-bx}]^c$). The coefficient y_0 (mg/L) can be considered as the amount of compounds adsorbed onto skin after peeling, whereas coefficient a (mg/L) corresponds to the maximum extractable amount by actual diffusion (y_0 values are low relative to those of a). Coefficient b (h^{-1}) corresponds to the initial relative rate of diffusion, and coefficient ab (mg/(L·h)) corresponds to the slope of the tangent at point (0, 0). Other possible applications of these models could be for instance the study of the impact of pH, temperature, or ethanol concentration on the extraction of phenolic compounds. In our case, the sigmoidal pattern of A520 was due to an important decoloration by SO_2 at the beginning of the extraction and by copigmentation phenomena (increase of Boulton's index (*47*)) after 2 h, resulting in color enhancement (data not shown). As an example, the curves obtained for two berry samples with contrasted sugar contents are given in **Figure 4**. We observed that the ripeness grade (on a sugar content basis) mainly influenced the color extraction (A520) and to a lesser extent total phenols but unexpectedly neither the extraction of total red pigments (pH 1.0) nor that of total flavanols. We can thus conclude that there is no direct relationship between total red pigments and color in extraction media, as earlier observed by Canals et al. (*32*), probably because of copigmentation and/or various amounts of polymeric pigments.

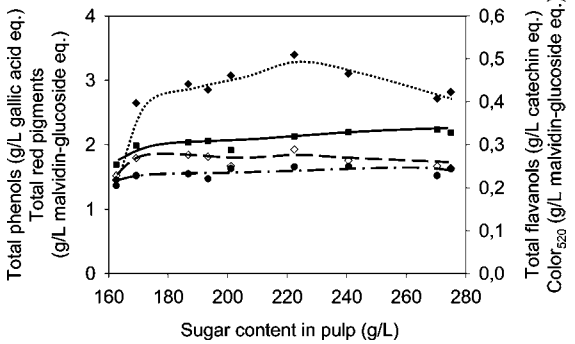

Figure 5. Changes in the maximum extracted amounts of total phenols (■), total flavanols (●), total red pigments (◇), and color at 520 nm (◆) in hydroalcoholic solution as a function of pulp sugar content.

The mathematical model allowed determination of the coefficient $a + y_0$, corresponding to the maximum extracted amounts (mg/L) in our conditions. As shown in **Figure 5**, the maximum extracted amounts ($a + y_0$) of total phenols tended to increase throughout sugar accumulation (+30% between 162.6 and 275.0 g/L) whereas A520 was maximum for a pulp sugar content of 222.4 g/L (+100% between 162.6 and 222.4 g/L). In contrast, no significant increase in extracted flavanols and total red pigments (pH 1.0) could be observed. The slightly lower amounts of extracted red pigments in the first sample (162.6 g/L of sugars) were probably related to a lower content in the skins (**Figure 2**). Canals et al. (*32*) obtained similar results with extraction experiments performed during several days without continuous shaking, describing first an increase in extracted anthocyanins between 160 and 180 g/L of sugars in pulp (and not in extractability as written in the paper, as no anthocyanin measurement was performed on skin before model extraction) and then constant extracted amounts between 180 and 192 g/L of sugars.

By comparing the amounts of extracted red pigments at the end of the hydroalcoholic extractions with the amounts of red pigments initially present in skins (quantified after extraction with acidified 60% acetone as described above), we could see that the extraction yield remained constant whatever the pulp sugar content. About 70% of total red pigments (free and derived anthocyanins) were extracted in the hydroalcoholic solutions (data not shown).

Extractability of Free Anthocyanins from Skin as a Function of the Pulp Sugar Content. Model extraction media and residual solid parts were analyzed at the end of the 5-h-long hydroalcoholic extraction. Extracted and non-extracted free anthocyanins were quantified by direct HPLC analysis and compared to initial content in skins. About 97% of the amounts initially present in skins were recovered in extraction media and residual solid parts. The extraction yield of free anthocyanins remained constant (77%) whatever the sugar content in the pulp (**Figure 6A**).

Some anthocyanin species presented different behaviors during extraction (**Figure 6B**,**C**). While the aglycone structure had very limited impact (only a slightly better extractability for cyanidin derivatives), this was not the case of C-ring substituting groups. Coumaroylglucoside derivatives turned out to be much less extractable than the other C-ring derivatives (67% instead of 81% for non-acylated glucosides or acetylglucosides). As shown in **Figure 7**, the non-extracted anthocyanins (23% of the free anthocyanins initially present in the skin) were considerably enriched in coumaroylglucoside derivatives, which were present at nearly equal amounts with non-acylated derivatives. On the other hand, the extracted anthocyanins (77% of the free anthocyanins initially present in skin) were mainly

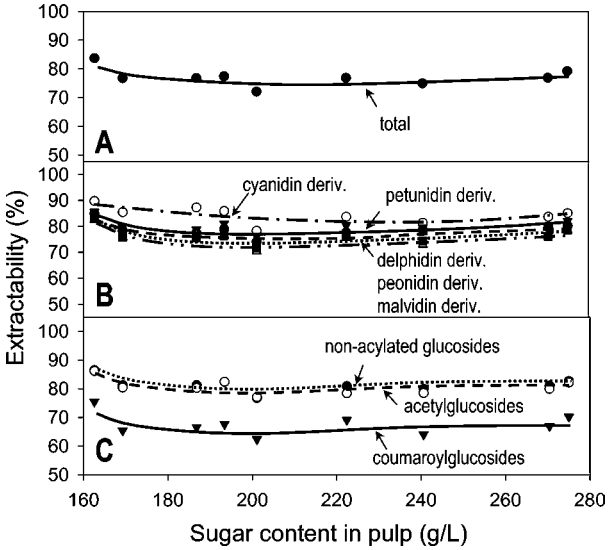

Figure 6. Extractability (%) in a model hydroalcoholic solution of total free anthocyanins (**A**), free anthocyanins grouped as a function of their B-ring substitution pattern (**B**), and free anthocyanins grouped as a function of their C-ring substitution pattern (**C**). Extractability corresponds to the amounts of anthocyanins recovered in hydroalcoholic media at the end of extraction compared with the amounts initially present in skins.

constituted of the non-acylated forms. These results are consistent with previous studies performed on berries from different varieties and on the corresponding wines in which the authors systematically observed a higher ratio of non-acylated glucosides to coumaroylglucosides (*7*, *48*).

Approximately 3.2% of total anthocyanins in the non-extracted fraction were present as caffeoylglucoside derivatives. In spite of their higher polarity compared to coumaroylglucosides (due to the presence of a second hydroxyl group), the fact that they have been detected neither in the skins nor in extracted fractions seems to indicate that their extraction yield is unexpectedly low. Whether acylated anthocyanidins have specific functions in fruit is still unknown. However, coumaroyl- and caffeoylglucoside anthocyanidins may interact more easily with the surrounding matrix components.

Extractability of Proanthocyanidins from Grape Skin as a Function of the Pulp Sugar Content. Model extraction media and residual solid parts were analyzed at the end of the 5-h-long extraction for quantitative determination of proanthocyanidins. Extracted and non-extracted proanthocyanidins were analyzed by HPLC after acid-catalyzed cleavage in the presence of excess phloroglucinol and compared with the tannins initially present in skin. About 85% of the tannins present in skin were recovered in extraction media and residual solid parts. The extraction yield calculated on the basis of the amounts of tannin recovered in hydroalcoholic solution was consequently lower (23 ± 3%) than that calculated on the basis of the amounts recovered in residual solid parts (38 ± 6%). However, this extraction yield remained constant whatever the pulp sugar content (data not shown).

The structural features of extracted and non-extracted proanthocyanidins were quite different (**Table 2**). Tannin extractability appeared to be mainly dependent on the size of the molecules, the mDP being much higher in the non-extracted fractions. To a lesser extent, the galloylation rate was also higher in non-

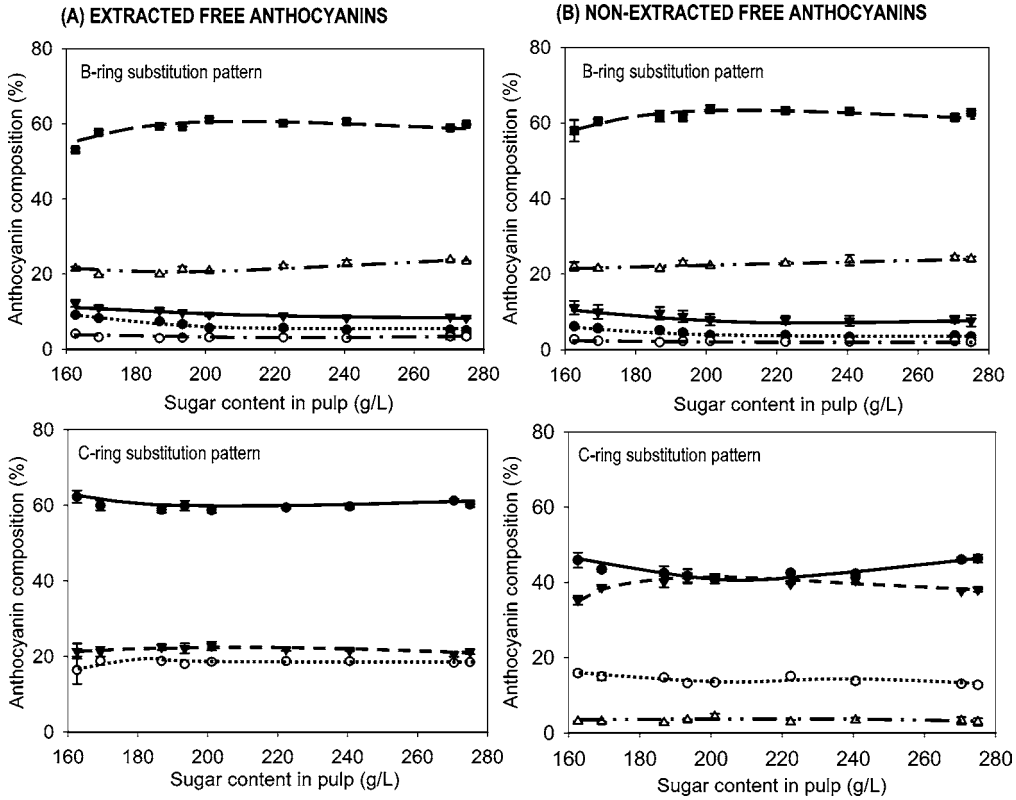

Figure 7. Changes in free anthocyanin composition in hydroalcoholic solutions (**A**) and in residual solid parts (**B**) as a function of pulp sugar content. When anthocyanins are grouped as a function of their B-ring substitution pattern: (■), malvidin derivatives; (△), peonidin derivatives; (▼), petunidin derivatives; (●), delphinidin derivatives; (○), cyanidin derivatives. When anthocyanins are grouped as a function of their C-ring substitution pattern: (●), non-acylated derivatives; (○), acetylglucoside derivatives; (▼), coumaroylglucoside derivatives; (△), caffeoylglucoside derivatives. Results are mean values of model extraction duplicates.

Table 2. Mean Characteristics of Extracted and Non-Extracted Proanthocyanidins in a Model Hydroalcoholic Solution throughout Ripening

	extracted tannins	non-extracted tannins
% yield	23 ± 3	62 ± 6
mDP	19 ± 3	55 ± 3
galloylation (%)	3.7 ± 0.2	4.8 ± 0.2
trihydroxylation (%)	27 ± 1	26 ± 2

extracted compounds. On the other hand, the B-ring trihydroxylation rate had no effect.

The compositional discrepancies between extracted and non-extracted compounds very slightly changed as the pulp accumulated sugars (from 162.6 to 275.0 g/L). We only observed a slight increase in the mDP of extracted proanthocyanidins (from 15 to 22) consistent with a concomitant decrease in non-extracted ones (from 59 to 52).

Vidal et al. previously evaluated the mouth-feel properties of different proanthocyanidin fractions in wine-like solutions and showed that the higher the mDP, the higher the overall astringency (*49*). Consequently, the minor compositional changes in extracted tannins observed in our study cannot account for the loss of astringency usually observed throughout ripening. It is thus reasonable to suggest that more complex phenomena have to be considered to explain this mouth-feel evolution.

Kennedy et al. (*3*) observed an increase in material associated with proanthocyanidins during berry development. An attempt to characterize this portion by electrospray ionization mass spectrometry revealed that, in addition to anthocyanins, proanthocyanidins were also covalently associated to pectins. Amrani-Joutei et al. also showed, by light and transmission electron microscopy and chemical analysis, that a fraction of proanthocyanidins was bound to the cell wall polysaccharides (*50*). Moreover, Le Bourvellec et al. demonstrated the existence of noncovalent interactions between proanthocyanidins and apple cell wall material in aqueous solutions (*51*−*53*), and Riou et al. observed that some polysaccharide fractions modify tannin aggregation in model wine-like solutions (*54*). Given the ability of soluble pectins from *Citrus* and acidic polysaccharides from apple juice to reduce the perception of astringency in persimmon and in wine, respectively (*55*, *56*), all these studies lead to the conclusion that further investigations have to be made on this topic in grapes, especially the ability of cell wall oligo- and polysaccharides to be extracted in wine and interfere with tannin perception when berries ripen.

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