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Validation of an Extraction Method on Whole Pericarp of Grape Berry (*Vitis vinifera* L. cv. Shiraz) to Study Biochemical and Molecular Aspects of Flavan-3-ol Synthesis during Berry Development

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An extraction method on grape berry was optimized for the total flavan-3-ol content measurement with regard to the nature of the sample and the duration of its extraction. This extraction was performed for the first time on the whole pericarp. Flavan-3-ol extractions were achieved on Shiraz ripe samples of pericarp versus skin within different durations: the best results were obtained for the whole pericarp and 1 h duration. Therefore, this more convenient protocol was used to investigate the flavan-3-ol content at different stages through berry development, in parallel with the abundance of transcripts involved in their biosynthesis. Furthermore, flavan-3-ol extractions on pericarp analysis confirmed their presence in both pulp and skin. For the first time, the flavan-3-ol biosynthesis in pulp was demonstrated with both biochemical and transcriptomic analyses since the presence of leucoanthocyanidin reductase (LAR2) and anthocyanin reductase (ANR) transcripts was revealed by real-time PCR. In addition, the percentage of epigallocatechin was different in pulp and skin.

KEYWORDS: Grape berry; pericarp; pulp; skin; flavan-3-ols; proanthocyanidins; extractability; HPLC; real-time PCR; leucoanthocyanidin and anthocyanin reductases; cinnamyl alcohol dehydrogenase; flavonoid 3′,5′-hydroxylase

INTRODUCTION

The flavonoid biosynthetic pathway gives rise to plant secondary metabolites of great interest for grape and wine organoleptic properties (*1, 2*). As recently reviewed, the major flavonoids of grape berry are flavan-3-ols (*3*) and anthocyanins (*4*), involved, respectively, in astringency and bitterness and in color. Flavan-3-ol biosynthesis occurs early during green stage, whereas anthocyanins are synthesized after *véraison* (*5–9*). The former ones occur in grape berry as both free monomers [(+)-catechin (Cat), (−)-epicatechin (Ec), (−)-epicatechin-3-*O*-gallate (EcG), and (−)-epigallocatechin (Egc)] and proanthocyanidin polymers (PA) (*10*). In grape seeds, PA contain predominantly Ec, Cat, and EcG subunits, linked by interflavan bonds (mostly C4−C8 but also C4−C6) (*11*). Flavan-3-ols from grape skins are rather large polymers with a mean degree of polymerization (mDP) around 30; they contain all of the above cited free monomers with lower amounts of EcG than in seeds but also Egc and trace amounts of (+)-gallocatechin subunits (*12*). In grape pulp, trace amounts of monomers and procyanidin dimers (*13, 14*) and trimers (*14*) were also reported in different cultivars. The presence of PA in pulp was recently confirmed in white and red cultivars (*15*).

In the flavonoid biosynthetic pathway, the role of leucoanthocyanidin reductase (LAR) and anthocyanin reductase (ANR) is well known to produce Cat and Ec monomers and terminal subunits (TU), respectively, while mechanisms involved in the synthesis and polymerization of extension subunits (EU) from leucoanthocyanindin are not yet established (*16*). Isolation and functional characterization of genes encoding LAR and ANR have been recently reported in grape skins and leaves (*17*). However, these authors emphasize the impossibility of measuring gene expression and flavan-3-ol content on skin from flowering to ripeness in a continuous way because peeling green berries at very early stages is impossible (*17*).

Our aim was therefore to compare from both biochemical and transcriptomic points of view the flavonoid metabolic pathway throughout ripening in the skin and pulp of grape berry. Since a specific protocol has been already developed to quantify anthocyanins on whole pericarps (major tissues involved in the winemaking) (*18*), a similar approach is there proposed to prepare common samples for further analyses of both flavan-3-ol and anthocyanin. A specific biochemical method was thus developed to quantify flavan-3-ols on whole pericarps because of two restraints. First, rapid dissection of grape berry into seeds, skins, and pulps in the field is quite impossible at green stages; second, quick separation of pericarps from seeds is essential to

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Table 1. Physiological Characteristics of *Vitis vinifera* L. cv. Shiraz Berries Sampled with Compartment Preparations Performed in 2004 in Montpellier, France

date	developmental stage	DAA[a]	average berry weight (g)	weight of each batch of 50 berries (g)	weight of skins (g)	weight of pulpes (g)	weight of pericarps (g)	°Brix
25/06/04	green	25	0.469	24.9	5.0	13.4		
				22.0			19.3	
22/07/04	*véraison*	52	0.976	52.1	7.0	40.3		
				45.5			41.9	
				93.5				
07/09/04	ripe	99	1.737	80.2	16.0	58.3	72.3	21.6
11/10/04	ripe	133	1.783	88.2	15.1	NP[b]		26.4
				90.1			76.2	

[a] DAA: days after anthesis. [b] NP: compartment not prepared.

avoid any transcriptomic modification due to RNA turnover (*19*). This method was adapted from several other protocols applied to skins and seeds either during berry development (*6, 9, 20, 21*) or at harvest (*22, 23*). This new method was applied to pericarp and skin at ripe stage, and data was analyzed by several one- or two-factor ANOVA. Further investigations at different developmental stages on the flavan-3-ol compartment distribution confirmed the presence of PA in pulp as recently demonstrated in other cultivars at ripe stage (*15*). Thus, this method used to investigate the flavan-3-ol content of grape pericarp during berry development suggests a different metabolic pathway for flavan-3-ols in pulp versus skin. Finally, this study demonstrates the presence in pulp of transcripts involved in flavan-3-ol biosynthesis, for the first time.

MATERIALS AND METHODS

Chemicals. Acetonitrile was HPLC grade purchased from Merck (Darmstad, Germany); acetone was normapur TM purchased from Prolabo, (Fontenay-sous-Bois, France). (+)-Catechin, (−)-epicatechin, phloroglucinol, and L-ascorbic acid were purchased from Sigma (St Louis, MO). Each phloroglucinol derivative was obtained and characterized as previously described (*23*).

Grape Samples. Grapevine berries of *Vitis vinifera* L. Shiraz cultivar were collected from the experimental vineyard of Montpellier SupAgro (Centre International d'Etudes Supérieures en Sciences Agronomiques, 43°86 north, 3°53 east, France) during summer 2004 on 30 vinestocks of homogeneous vigor. The grapevines were 8 years old, grafted on Fercal rootstock, trained on a Lyre system, pruned as cordon, and grown in 70 L pots containing perlite and sand (90:10, v/v). Minerals were brought with water at a 4.5 L/day rate by a drip irrigation system, and all plants were at maximal evapotranspiration. Anthesis (50% caps off) and *véraison* (green berries at the beginning of softening) occurred respectively on June 1st and July 22nd, 2004 (52nd day after anthesis [DAA]).

One hundred healthy berries were collected at green stage (25th DAA), *véraison* (52nd DAA), and at two ripe stages (99th and 133rd DAA) (**Table 1**). For the three first samples, after removing berry pedicels and seeds, two groups of half berries were constituted. The whole pericarp (pulp + skin) was processed for the first group, whereas pulp and skin were processed separately for the second group. Thus for each developemental stage, three different batches were obtained, i.e., whole pericarp (skin + pulp), skin, and pulp. The second ripe stage (133rd DAA) was used to optimize the flavan-3-ol extraction method. Removing pedicels and seeds was first achieved on berries selected according to their health, softness, color, and size; two groups of half berries were further used for preparing pericarp and skin batches. All batches were rapidly frozen in liquid nitrogen, then ground under liquid nitrogen to a fine powder in a ball mill (Dangoumill 300, Lonjumeau, France) and stored at −80 °C until used for extraction and/or transcriptomic analysis. All biochemical results are expressed in mg/berry.

Flavan-3-ol Extractions. They were carried out with either one-step extractions (1 h, 3 h, 24 h) or one three-step extraction (1 h + 1 h + 22 h) on powder aliquots of the second ripe sample (133rd DAA), respectively, 0.25 g of pericarp (P) or 0.05 g of skin (S). For each one-step extraction (1 h, 3 h, or 24 h), aliquots were mixed with 750 μL of the extraction solution (acetone/water (70:30, v/v) containing 0.05% trifluoroacetic acid, to allow a constant pH all along the experiment and to prevent oxidation of polyphenols (*23, 24*), and 50 μL of a *p*-hydroxy methyl ester solution (3g/L in methanol) as internal standard for HPLC analyses. Samples were stirred on a Stuart Tube Rotator SB3 (Bibby Sterilin, Stone, UK) in a dark at room temperature during extraction and centrifuged (13,000*g*, 15 min, +4 °C), and the supernatant was recovered.

For the three-step extraction (1 h + 1 h + 22 h), 250 μL of extraction solution and 50 μL of a *p*-hydroxy methyl ester solution were added to powder aliquots of pericarp (P) and skin (S). After 1 h of stirring, samples were centrifuged (13,000*g*, 15 min, +4 °C), and supernatant was recovered and kept at +4 °C. The two additional steps of extraction (each using 250 μL of the same extraction solution) were further performed for 1 and 22 h. Finally, the three supernatants were pooled.

Immediately after the extraction(s), 200 μL of each supernatant was dried under vacuum at +35 °C for 2 h (Genevac, Ipswich, U.K.) and resuspended in 100 μL of reagent solution (0.25 g phloroglucinol, 0.05 g ascorbic acid, and 5 mL acidified methanol [0,2 N HCl]) for acid catalysis in the presence of excess phloroglucinol (*21, 22*). After incubation (+50 °C, 20 min), the reaction was stopped by adding 100 μL of a sodium acetate buffer (200 mM, pH 7.5). Samples were then centrifuged before injection onto the HPLC system.

In parallel, phloroglucinolysis was also performed on each extraction pellet to determine the amount of proanthocyanidins remaining in plant tissue residues (*21, 22, 25*). After extraction and centrifugation, each pellet was washed in 1 mL of extraction solution, centrifuged once more, dried under vacuum at +35 °C for 2 h, and then resuspended in 500 μL of phloroglucinolysis reagent. After incubation (+50 °C, 20 min), the reaction was neutralized by adding additional 500 μL of sodium acetate buffer. Samples were then centrifuged before injecting supernatants onto the HPLC system.

For the three other samples (25th, 52nd, and 99th DAA), only 1 h extractions were performed on powder aliquots of each compartment [i.e., pericarp (P), pulp (pulp), and skin (S)]. According to the developmental stage, the weight of each compartment was adjusted to take into account their relative proportion with regard to whole berry. Ratio of powder (mg) versus total volume of extraction solution (μL) was optimized as well to avoid any solvent saturation. Thus, powder aliquots of P, pulp, and S were, respectively, at green stage (25th DAA) 0.15 g, 0.20 g, and 0.04 g; at *véraison* (52nd DAA) 0.20 g, 0.20 g, and 0.04 g; and at ripe stage (99th DAA) 0.25 g, 0.25 g, and 0.05 g.

HPLC Analyses. HPLC runs were performed on an Agilent 1100LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector and coupled on line to an RF-10A XL model fluorimeter detector (Shimadzu, Kyoto, Japan). The separation was carried out using an Atlantis dc18 (250 × 4.6 mm, 5 μm) analytical column (Waters, Milford, MA) protected by both an Atlantis dc18 (20 × 4.6 mm, 3 μm) precolumn (Waters, Milford, MA) and a Phenomenex (4 × 3 mm) guard column (Phenomenex, Torrance, CA).

Table 2. Primers Used for Real-Time PCR and Expected Size for Amplified Fragment[a]

TC number	putative function	forward primer	reverse primer	expected size[b]
TC55506	LAR1	CACATGCATGCGATTAGTCC	ACGAATTTCGCCCATGTTAC	102
TC61972	LAR2	GCCAGCTCCCCAATCTTATT	TCTGCAGTTTCTTTGATTGAGTTC	146
TC58767	ANR	GCTTGTGAAAGAGGGGTTCA	TGCTCCTTTTAGCCAAGACA	132
TC52678	EF1alpha	GAACTGGGTGCTTGATAGGC	AACCAAAATATCCGGAGTAAAAGA	150
TC58374	CAD	ATAGGTTTGTGATCGATGTGG	ACACCATGCCTCAAAACAAG	103

[a] Tentative Contig (TC) numbers are from DFCI Grape Gene Index: http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape. [b] Base pairs.

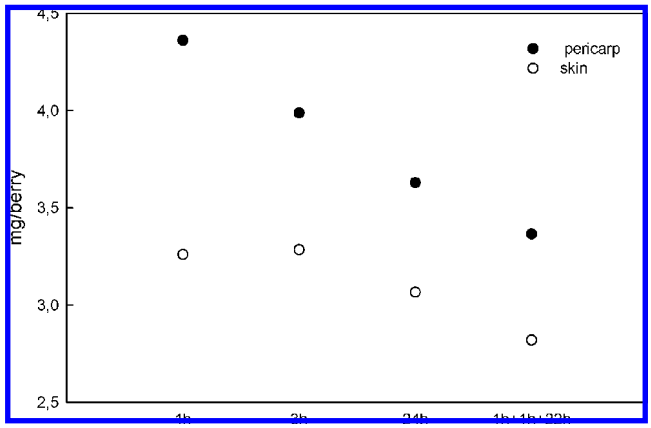

Figure 1. Interaction plot of total flavan-3-ols in pericarp and skin versus extraction durations at ripe stage (133rd DAA). Data are the mean values of triplicate extractions.

For ripe stages, 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 1 mL/mn at 30 °C as previously used by Souquet et al. (*23*) and Fournand et al. (*9*). For green and *véraison* stages, elution conditions were modified only for the gradient of solvent B as follows: isocratic for 5 min with 0% B; from 0 to 7.5% B in 8 min; isocratic for 1.5 min with 7.5% B; from 7.5 to 9% B in 11.5 min; isocratic for 2.5mn with 9% B; from 9 to 10% B in 6.5 min; from 10 to 60% B in 30 min; and finally washing and reequilibrating of the column. Both methods were developed to separate TU, including free monomers, and EU (i.e., phloroglucinol adducts) of proanthocyanidins using UV-DAD (λ = 280 nm) and fluorimeter detector (λ_{ex} = 275 nm and λ_{em} = 322 nm) (*26*). For all of the different units, the quantitative determinations were performed using a fluorimeter detector except for phloroglucinol-EcG adducts whose concentrations were calculated using UV−DAD detection. Concentrations of subunits were determined from standard curves calculated from pure monomers and purified phloroglucinol adducts (*9, 23*). Polymer length was estimated by the mean degree of polymerization (mDP) calculated as the ratio between the sum of EU and TU to the sum of TU. The mDP, the percentage of galloylation [EcG (%)], and the percentage of epigallocatechin subunits [Egc (%)] are calculated on a molar basis.

Real-Time PCR. Total RNA was extracted as previously described (*27*). The RNA was accurately quantified with Ribogreen reagent (Molecular probes, Leiden, Netherlands). A triplicate reverse transcription was performed on 500 ng of total RNA from each development stage using the Superscript II RT kit (Invitrogen, Fischerbioblock, Illkirch, France) according to the manufacturer's instructions. Triplicates were further pooled.

Specific oligonucleotide primer pairs were designed with Primer3 software (**Table 2**). Specific annealing of the oligonucleotides was controlled on dissociation kinetics performed at the end of each PCR run. The efficiency of each primer pair was measured on a PCR product or plasmid serial dilutions. The PCR was performed in triplicate on 1 μL of cDNA from each berry development stage, using a model 7300 Sequence Detection System (Applied Biosystems, Warrington, UK) and the SYBR-Green PCR Master kit (Applied Biosystems Applera France, Courtaboeuf, France). EF1 alpha was chosen as the constant control transcript as in Terrier et al. (*28*) and confirmed by Reid et al. (*29*). The difference between the cycle threshold (*Ct*) of the target gene and EF1 alpha was used to obtain the normalized expression of the target gene, calculated as 2 exp-($Ct_{target} - Ct_{EF1alpha}$).

Statistics. Data is expressed as the arithmetic average ± standard deviation of three replicates. ANOVA (one-factor and two-factor) and tests (Fisher and Tukey) were carried out with R software.

RESULTS AND DISCUSSION

Our extraction method was set up on pericarp versus skin at the second ripe stage (133rd DAA), considering on the one hand Downey et al. (*21*) and on the other hand Souquet et al. (*23*), further adapted by Fournand et al. (*9*). Since earlier work has shown the presence of proanthocyanidins remaining in the pellet after extraction and thus protocols being performed with direct acid-catalyzed cleavage on pellets (*21, 22, 25*), our extraction method was also achieved on both supernatant and pellets.

Comparative Study of Flavan-3-ol Extractions on Shiraz Pericarp versus Skin within Four Different Durations. Table 3 presents total (supernatant and pellet) flavan-3-ol contents extracted from both pericarp and skin of the second ripe sample (133rd DAA) with four duration protocols (three one-step extractions, two short (1 h, 3 h) or one long (24 h) durations; a unique three-step extraction, two short (1 h + 1 h) plus one long (22 h) durations). A two-factor ANOVA set on extraction duration and compartment preparation shows that both factors significantly modify the extracted amounts. For total flavan-3-ols, i.e., proanthocyanidins and monomers, a highly significant effect of the extraction duration factor occurs at the $p < 0.001$ threshold. Pairwise comparisons (Tukey Test) between all coupled extraction durations precisely highlights a significant difference between 1 and 1 h + 1 h + 22 h ($p < 0.1$). Extraction efficiency of all flavan-3-ols was maximal for two one-step extractions (1 h, 3 h) and minimal for the three-step extraction (1 h + 1 h + 22 h), whatever the type of involved tissue. Several explanations could be proposed. For the three-step protocol, each volume of extraction solution was equal to 1/3 of the total volume used for a one-step extraction. Although a possible saturation of this lesser volume due to a higher weight/volume ratio might occur at the first step, the two last extractions plus the final direct acid catalysis on pellet should be efficient enough to overcome this difficulty. At higher weight/volume ratio, the solvent might be less efficient in inhibiting enzymatic oxidation as suggested by Cheynier et al. (*30*). Moreover, during the first step of extraction, a physical transformation of cells might also increase the adsorption of flavan-3-ols on macromolecular compounds (polysaccharides, proteins,...) as suggested by Mané et al. (*15*). Nevertheless, as our protocols have taken supernatant and pellet flavan-3-ol contents into account, direct acid catalysis on pellet should have released these possibly adsorbed polymers regardless of the duration involved. For each flavan-3-ol subunit, a highly significant effect of the extraction duration occurs at the $p < 0.001$ threshold except for EcG EU with a less significant effect at the $p < 0.01$ threshold. Short extraction

Table 3. Total Flavan-3-ol Contents (Supernatant and Pellet) Extracted within Four Durations from Shiraz Berry Compartments (Pericarp and Skin) at 133rd Day after Anthesis[a]

flavan-3-ols	duration	berry compartment: pericarp	berry compartment: skin
	terminals subunits		
catechin	1 h	0.0929 ± 0.0015	0.0749 ± 0.0019
	3 h	0.0846 ± 0.0070	0.0773 ± 0.0033
	24 h	0.0802 ± 0.0016	0.0758 ± 0.0019
	1 h + 1 h + 22 h	0.0761 ± 0.0014	0.0674 ± 0.0025
	[b]two-factor ANOVA: compartment $p < 0.001$; duration $p < 0.001$; compartment-duration $p < 0.05$		
epicatechin	1 h	0.0234 ± 0.0008	0.0146 ± 0.0002
	3 h	0.0206 ± 0.0008	0.0151 ± 0.0005
	24 h	0.0192 ± 0.0005	0.0150 ± 0.0004
	1 h + 1 h + 22 h	0.0170 ± 0.0005	0.0129 ± 0.0005
	two-factor ANOVA: compartment $p < 0.001$; duration $p < 0.001$; compartment-duration $p < 0.001$		
total terminals subunits	1 h	0.1163 ± 0.0020	0.0895 ± 0.0016
	3 h	0.1052 ± 0.0077	0.0924 ± 0.0038
	24 h	0.0994 ± 0.0019	0.0907 ± 0.0023
	1 h + 1 h + 22 h	0.0931 ± 0.0019	0.0803 ± 0.0031
	two-factor ANOVA: compartment $p < 0.001$; duration $p < 0.001$; compartment-duration $p < 0.01$		
	extension subunits		
catechin	1 h	0.1373 ± 0.0042	0.0860 ± 0.0022
	3 h	0.1261 ± 0.0108	0.0870 ± 0.0026
	24 h	0.1215 ± 0.0034	0.0863 ± 0.0032
	1 h + 1 h + 22 h	0.1149 ± 0.0011	0.0784 ± 0.0024
	two-factor ANOVA: compartment $p < 0.001$; duration $p < 0.001$; compartment-duration $p < 0.05$		
epicatechin	1 h	2.5249 ± 0.0903	1.6594 ± 0.0642
	3 h	2.3289 ± 0.1957	1.6867 ± 0.0594
	24 h	2.1718 ± 0.0778	1.6495 ± 0.0652
	1 h + 1 h + 22 h	2.0229 ± 0.0178	1.4167 ± 0.0549
	two-factor ANOVA: compartment $p < 0.001$; duration $p < 0.001$; compartment-duration $p < 0.05$		
epicatechin-3-*O*-gallate	1 h	0.1413 ± 0.0046	0.1516 ± 0.0139
	3 h	0.1511 ± 0.0309	0.1532 ± 0.0038
	24 h	0.1306 ± 0.0060	0.1428 ± 0.0077
	1 h + 1 h + 22 h	0.1168 ± 0.0089	0.1238 ± 0.0042
	two-factor ANOVA: compartment *ns*; duration $p < 0.01$; compartment-duration *ns*		
epigallocatechin	1 h	1.4418 ± 0.0470	1.2743 ± 0.2368
	3 h	1.2769 ± 0.1259	1.2651 ± 0.0396
	24 h	1.1069 ± 0.0743	1.0970 ± 0.0640
	1 h + 1 h + 22 h	1.0185 ± 0.0248	1.1211 ± 0.0298
	two-factor ANOVA: compartment *ns*; duration $p < 0.001$; compartment-duration *ns*		
total extension subunits	1 h	4.2453 ± 0.1397	3.1714 ± 0.3006
	3 h	3.8830 ± 0.3621	3.1920 ± 0.0301
	24 h	3.5307 ± 0.1510	2.9756 ± 0.1391
	1 h + 1 h + 22 h	3.2730 ± 0.0184	2.7401 ± 0.0902
	two-factor ANOVA: compartment $p < 0.001$; duration $p < 0.001$; compartment-duration *ns*		
	total flavan-3-ols		
	1 h	4.3616 ± 0.1409	3.2609 ± 0.3022
	3 h	3.9882 ± 0.3693	3.2844 ± 0.0338
	24 h	3.6302 ± 0.1528	3.0663 ± 0.1414
	1 h + 1 h + 22 h	3.3662 ± 0.0200	2.8203 ± 0.0932
	two-factor ANOVA: compartment $p < 0.001$; duration $p < 0.001$; compartment-duration *ns*		
mDP[c]	1 h	36.4	35.1
	3 h	36.8	34.3
	24 h	35.5	32.6
	1 h + 1 h + 22 h	35.1	33.9
	two-factor ANOVA: compartment $p < 0.001$; duration *ns*; compartment-duration *ns*		
EcG (%)[d]	1 h	2.2	3.2
	3 h	2.5	3.2
	24 h	2.4	3.2
	1 h + 1 h + 22 h	2.3	3.0
	two-factor ANOVA: compartment $p < 0.001$; duration *ns*; compartment-duration *ns*		
Egc (%)[e]	1 h	32.2	38.2
	3 h	31.3	37.9
	24 h	29.7	35.1
	1 h + 1 h + 22 h	29.5	39.1
	two-factor ANOVA: compartment $p < 0.001$; duration $p < 0.05$; compartment-duration *ns*		

[a] Data is expressed as mean values of three replicates ± standard deviation ($n = 3$; mg/berry). [b] Statistical analysis: two-factor ANOVA (compartment, duration) and interaction between factors; effect significance at a probability level of 5%; *ns*, not significant. [c] Calculated mean degree of polymerization. [d] Percentage of galloylation. [e] Percentage of Egc.

durations do not noticeably modify the composition of the measured proanthocyanidins, i.e., all kinds of subunits are affected in the same way by the modification of the extraction protocol. Therefore, 1 h is long enough to optimize the extraction

Table 4. Supernatant and Pellet Contents of Total Terminal and Extension Subunits in Shiraz Berry Compartments (Pericarp and Skin) at 133rd Day after Anthesis within Four Durations[a]

flavan-3-ols	duration	berry compartment: pericarp	berry compartment: skin
	(A) terminals subunits		
supernatant[a]	1 h	0.1113 ± 0.0022	0.0870 ± 0.0016
	3 h	0.1008 ± 0.0073	0.0892 ± 0.0023
	24 h	0.0962 ± 0.0022	0.0888 ± 0.0021
	1 h + 1 h + 22 h	0.0892 ± 0.0018	0.0787 ± 0.0029
	[b]one-factor ANOVA: compartment $p < 0.05$		
pellet[a]	1 h	0.0050 ± 0.0001	0.0025 ± 0.0001
	3 h	0.0044 ± 0.0010	0.0032 ± 0.0016
	24 h	0.0033 ± 0.0003	0.0019 ± 0.0002
	1 h + 1 h + 22 h	0.0039 ± 0.0001	0.0015 ± 0.0001
	one-factor ANOVA: compartment $p < 0.05$		
	extension subunits		
supernatant[a]	1 h	3.9269 ± 0.1386	3.0452 ± 0.3190
	3 h	3.5739 ± 0.3649	3.0368 ± 0.0293
	24 h	3.3086 ± 0.1583	2.8706 ± 0.1222
	1 h + 1 h + 22 h	2.8567 ± 0.0428	2.6232 ± 0.0875
	one-factor ANOVA: compartment *ns*		
pellet[a]	1 h	0.3184 ± 0.0097	0.1262 ± 0.0194
	3 h	0.3092 ± 0.0314	0.1552 ± 0.0594
	24 h	0.2222 ± 0.0080	0.1051 ± 0.0176
	1 h + 1 h + 22 h	0.4163 ± 0.0317	0.1168 ± 0.0072
	one-factor ANOVA: compartment $p < 0.01$		
	(B) pellet/total (%)[c]		
terminal subunits	1 h	4.3	2.8
	3 h	4.2	3.5
	24 h	3.3	2.1
	1 h + 1 h + 22 h	4.2	1.9
	two-factor ANOVA: compartment $p < 0.01$; subunit $p < 0.01$		
extension subunits	1 h	7.5	4.0
	3 h	8.0	4.9
	24 h	6.3	3.5
	1 h + 1 h + 22 h	12.7	4.3
	two-factor ANOVA: compartment $p < 0.01$; subunit $p < 0.01$		
	(C) mDP[d]		
supernatant	1 h	35.3	34.7
	3 h	35.3	33.8
	24 h	34.4	32.2
	1 h + 1 h + 22 h	32.1	33.1
	two-factor ANOVA: compartment *ns*; fraction $p < 0.001$		
pellet	1 h	63.0	49.6
	3 h	70.6	49.4
	24 h	67.5	52.9
	1 h + 1 h + 22 h	97.9	74.7
	two-factor ANOVA: compartment *ns*; fraction $p < 0.001$		
	EcG (%)[e]		
supernatant	1 h	2.1	3.1
	3 h	2.4	3.2
	24 h	2.4	3.1
	1 h + 1 h + 22 h	2.2	3.0
	two-factor ANOVA: compartment $p < 0.01$; fraction $p < 0.01$		
pellet	1 h	2.8	4.0
	3 h	4.0	3.5
	24 h	3.3	5.1
	1 h + 1 h + 22 h	3.2	3.3
	two-factor ANOVA: compartment $p < 0.01$; fraction $p < 0.01$		
	Egc (%)[f]		
supernatant	1 h	33.1	38.8
	3 h	32.3	38.7
	24 h	30.6	35.7
	1 h + 1 h + 22 h	30.7	39.6
	two-factor ANOVA: compartment $p < 0.01$; fraction $p < 0.001$		
pellet	1 h	21.8	22.7
	3 h	19.5	21.6
	24 h	17.1	20.0
	1 h + 1 h + 22 h	21.2	26.7
	two-factor ANOVA: compartment $p < 0.01$; fraction $p < 0.001$		

[a] Data is expressed as mean values of three replicates ± standard deviation ($n = 3$; mg/berry). [b] Statistical analysis: one-ANOVA (compartment) and two-factor ANOVA (compartment, subunit; compartment, phloroglucinolysis fraction), and interactions between factors; effect significance at a probability level of 5%; *ns*, not significant. [c] Relative ratios of ≪pellet TU/ total TU≫ and of ≪pellet EU/ total EU≫. [d] Calculated mean degree of polymerization (mDP). [e] Percentage of galloylation. [f] Percentage of Egc.

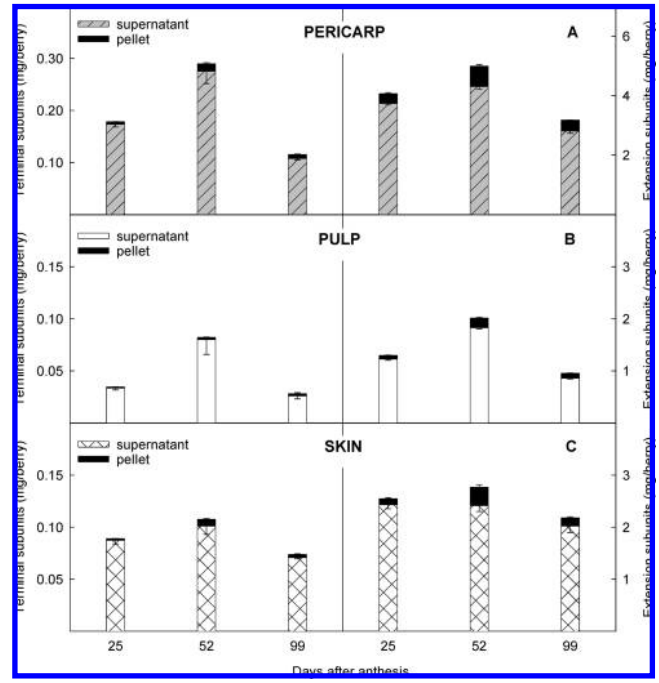

Figure 2. Levels of total terminal and extension subunits present in both acetone extracts (supernatant) and direct acid catalysis of residues (pellet) of Shiraz pericarp (**A**), pulp (**B**), and skin (**C**) at green stage (25th DAA), *véraison* (52nd DAA), and ripe stage (99th DAA). Data are the mean values (±SD) of triplicate extractions.

of flavan-3-ols on whole pericarp, thus confirming previous optimization of flavan-3-ol extractions on seeds, pulp, and skin (*15*).

For total flavan-3-ols, a highly significant effect of the compartment preparation factor also occurs at the $p < 0.001$ threshold (**Table 3**). For each flavan-3-ol subunit and for mDP, EcG (%), and Egc (%), a highly significant effect of this factor still occurs ($p < 0.001$) except for EcG EU and Egc EU for which no effect is obviously underlined. For all parameters, pericarp amounts are always the highest except for EcG EU and Egc EU. Therefore, this data demonstrates the relevance of flavan-3-ol extractions on pericarp for the first time.

A weak interaction between these two factors is only observed for Cat and Ec in both TU and EU at different thresholds (**Table 3**) and is further confirmed by a pairwise comparison (Tukey test) (**Figure 1**). Despite its weakness, this interaction is slightly stronger at the short durations, and higher amounts are still extracted in whole pericarp.

The distribution of PA after each extraction between the acetone extract and the pellet was further analyzed on the pericarp and skin with several one or two-factor ANOVAs (**Table 4**). No significant effect was found for interactions for both two-factor ANOVAs. The effect of the compartment preparation factor is significant at different thresholds for all parameters in both supernatants and pellets except for total EU in supernatants and mDP. Whatever the total subunits (TU or EU) or the fraction involved, pericarp amounts (**Table 4A**) and pericarp relative ratios of pellet subunits/total subunits (**Table 4B**) are respectively higher than skin ones. The effect of the subunit factor on these pellet ratios is also significant and allows to discriminate EU from TU (**Table 4B**). For all durations, all amounts released from the pellets are lower than those readily extracted in supernatants as found by Downey et al. (*21*) on Shiraz skin samples with similar °Brix value (**Table 4A**). Pericarp amounts released from pellets are lower in TU [relative

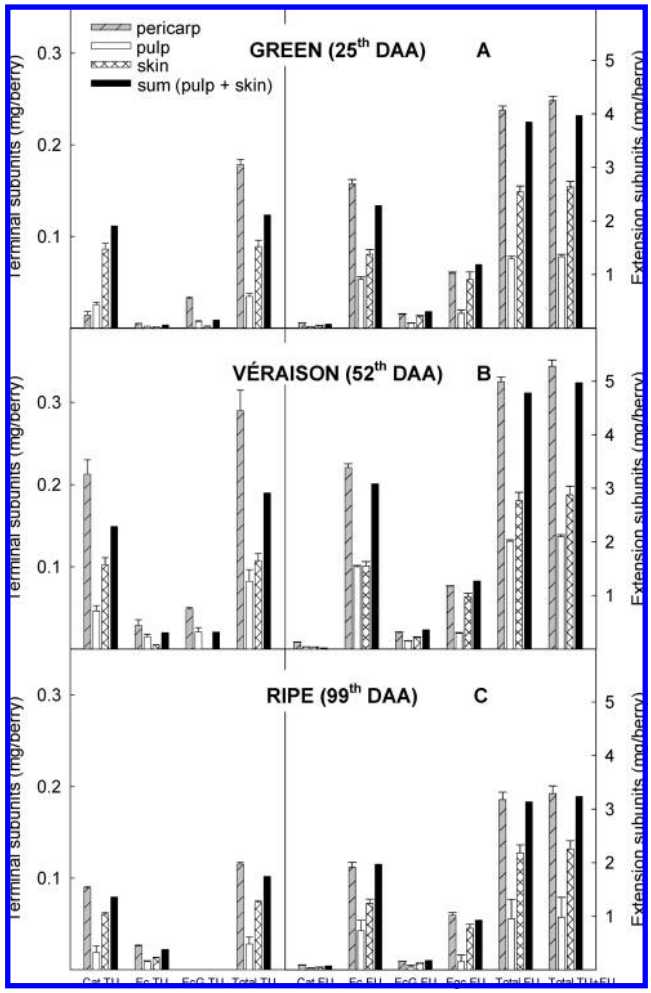

Figure 3. Total (supernatant + pellet) terminal and extension subunits (total TU and total EU) and total flavan-3-ols, [catechin (Cat); epicatechin (Ec); epicatechin-3-*O*-gallate (EcG); epigallocatechin (Egc)] of Shiraz pericarp, pulp, and skin at green stage (25th DAA) (**A**), *véraison* (52nd DAA) (**B**), and ripe stage (99th DAA) (**C**). The 4th histograms are the sums of (pulp + skin) mean values. Data are the mean values (±SD) of triplicate extractions.

ratios of ≪pellet TU/ total TU≫ (3−4%)] than in EU [relative ratios of ≪pellet EU/ total EU≫ (6−13%)] (**Table 4B**). To a lesser extent, this difference is also observed for skin samples [TU (2−4%) − EU (3−5%)], but these percentages do not match the same ratios found by Downey et al. in previous cited work [TU (30%) − EU (13%) (*21*)]. The effect significance of the fraction factor is higher for mDP and Egc (%) than for EcG (%) (**Table 4C**). For both pericarp and skin, much larger mDP values and higher EcG (%) in pellets are in accordance with results from Le Bourvellec et al. studying PA adsorption on the apple cell wall material (*31*). The highest mDP values for both pericarp and skin were encountered in pellets of the three-step extraction, thus confirming previous cited hypotheses about deep changes (lower inhibition of enzymatic oxidation or increase of flavan-3-ol/macromolecular compound adsorptions) occurring along this protocol. These results show the efficiency of the one-step extraction(s) compared to the three-step protocol and also emphasize the importance of pellet analysis.

Analysis of Flavan-3-ol Accumulation at Three Development Stages in Pericarp, Pulp, and Skin. Since extracted amounts of total flavan-3-ols at ripe stage (133rd DAA) were still higher in whole pericarp than in skin, further investigations were undertaken for the first time on flavan-3-ol contents of the three berry compartments at three earlier developmental stages with 1 h extractions on both supernatants and pellets, to be subsequently related to transcriptomic analysis.

Pericarp, pulp, and skin from berries collected at green stage (25th DAA), *véraison* (52nd DAA), and ripe stage (99th DAA) (**Table 1**) were analyzed. The general distribution of flavan-3-ols is presented in pericarp (**Figure 2A**), pulp (**Figure 2B**), and skin (**Figure 2C**) for both amounts of total subunits (TU and EU) and supernatant/pellet. A similar pré-*véraison* rise occurs in both pericarp and pulp, whereas this increase is lower in skin. At *véraison*, amounts released from pellets are still higher for all compartments and both TU and EU. Decrease arises after *véraison* in the three compartments, although this decline is slightly higher in pericarp and pulp than in skin. For this last compartment, similar results were obtained by Downey et al. despite a 1.5-fold higher EU level at *véraison* in their data (*21*). This post-*véraison* decrease has been previously attributed to an increase in stable associations between PA and other cellular components such as polysaccharides, lignins, and proteins by several authors (*10, 20, 21, 32, 33*). For the first time, our results reveal that post*véraison* rearrangements also occur in pulp. Surprisingly, total subunit (TU and EU) amounts for both pericarp and skin are much lower at 99th DAA than at 133rd DAA (**Table 3**). This result could suggest a better extractibility due to a possible modification ot these former associations during latest ripeness stages. **Figure 3** presents the amounts of total flavan-3-ols, TU, and EU at the three developmental stages (**A**−**C**) for each compartment and the sum (pulp + skin) of mean values. Despite lack of feasibiliy of any statistical analysis on the sum of mean values, **Figure 3** analysis shows that for total TU, total EU, and total flavan-3-ol, pericarp levels are higher than (pulp + skin) amounts at 25th DAA (**A**) and 52nd DAA (**B**), and to a lesser extent at 99th DAA (**C**). This difference could mainly be attributed to the contribution of the content of Ec EU, the major EU. The presence of each PA subunit in pulp at the three developmental stages is unquestionably confirmed, thus strenghtening previous work (*15, 34*) and suggesting either a diffusion between the two compartments or a flavan-3-ol synthesis occurring in pulp as well as in skin with rather comparable levels of total flavan-3-ols at 52nd DAA (2.1 and 2.9 mg/berry, respectively). In TU, Cat is present in pulp and skin during berry development, whereas EcG was mostly detected in pulp at 25th DAA and 52nd DAA. In EU, Egc amounts are much higher in skin whatever the developmental stage. This data questions whether flavan-3-ol biosynthesis may occur in a different way in each compartment.

Flavan-3-ol composition was analyzed in each compartment during berry development to further investigate this hypothesis (**Table 5**). In pulp as in skin during berry development, as for Kennedy et al. (*20*) on Shiraz skin, predominancy of Cat among TU and both molar percentage of Cat and EcG EU are confirmed but an obvious difference between these two compartments occurs in proportions of the two major EUs. While the ratio Ec/Egc is almost constant in skin (∼1.7), it increases in pulp until *véraison* during flavan-3-ol biosynthesis (from ∼3.5 to ∼5.5) and is rather constant until ripe stage (∼5). This result strongly suggests a lower activity of flavonoid 3′,5′-hydroxylase (F3′5′H) occurring in pulp at the level of either its transcript expression or its enzymatic activity and resulting thus in a difference in the composition of final PA polymers for this compartment. Comparable results during ripening were obtained for skin [ratio Ec/Egc almost constant (∼2.1)] by Kennedy et al. (*20, 22*). In pericarp, flavan-3-ol proportions are different from skin ones, especially for the ratio Ec/Egc, which is constant

Table 5. Proanthocyanidin Composition (Supernatant and Pellet) of Shiraz Berry Compartments (Pulp, Skin, and Pericarp) at Three Different Develomental Stages[a]

develpomental stage	DAA[b]	berry compartment	terminal subunits			extension subunits				Ec/Egc	mDP[d]	EcG (%)[e]	Egc (%)[f]
			Cat	Ec	EcG	Cat	Ec	EcG	Egc				
		pericarp	3.3 ± 0.2	0.2 ± 0.1	0.5 ± 0.0	2.4 ± 0.1	66.4 ± 1.0	4.0 ± 0.0	23.2 ± 1.0	2.9	25.1	4.5	23.0
green	25	pulp	2.0 ± 0.1	0.1 ± 0.0	0.4 ± 0.0	2.0 ± 0.1	70.7 ± 2.3	4.5 ± 0.2	20.3 ± 2.6	3.5	40.1	4.9	20.3
		skin	3.6 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	2.2 ± 0.9	54.4 ± 1.6	5.6 ± 0.2	34.1 ± 2.8	1.6	27.6	5.7	34.2
		pericarp	4.1 ± 0.2	0.6 ± 0.1	0.6 ± 0.0	2.4 ± 0.1	66.8 ± 0.1	3.9 ± 0.1	21.6 ± 0.5	3.1	18.9	4.5	21.6
véraison	52	pulp	2.3 ± 0.3	0.7 ± 0.1	0.7 ± 0.2	1.9 ± 0.1	76.0 ± 1.3	4.7 ± 0.2	13.7 ± 0.7	5.5	27.4	5.4	13.7
		skin	3.7 ± 0.2	0.2 ± 0.0	0.0 ± 0.0	1.3 ± 0.1	56.2 ± 0.7	5.1 ± 0.1	33.5 ± 0.9	1.7	25.6	5.1	33.5
		pericarp	2.8 ± 0.1	0.8 ± 0.0	NA[c]	2.7 ± 0.0	60.1 ± 0.4	3.2 ± 0.3	30.4 ± 0.8	2.0	27.6	3.2	30.4
ripe	99	pulp	2.0 ± 0.2	1.0 ± 0.2	NA[c]	2.5 ± 0.1	75.9 ± 0.4	3.4 ± 0.2	15.3 ± 0.3	5.0	34.3	3.4	15.3
		skin	2.8 ± 0.2	0.6 ± 0.0	NA[c]	2.2 ± 0.2	57.2 ± 2.5	3.6 ± 0.3	33.6 ± 3.2	1.7	29.5	3.6	33.6

[a] Values are given in proportional composition (mole% ± standard deviation, $n = 3$). [b] DAA: days after anthesis. [c] NA: not analyzed at ripeness. [d] Calculated mean degree of polymerization. [e] Percentage of galloylation. [f] Percentage of Egc.

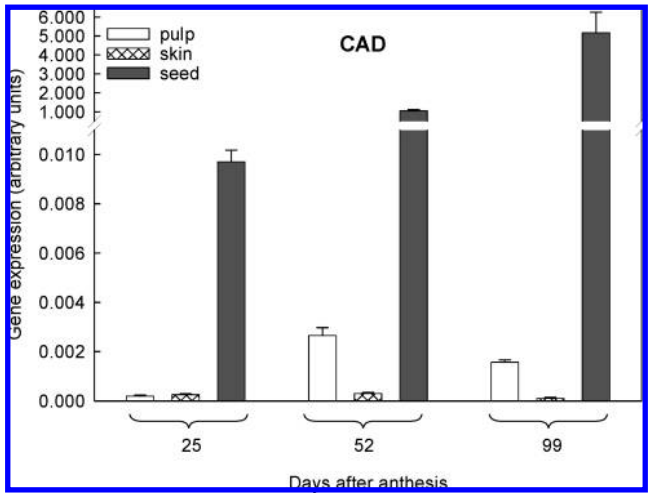

Figure 4. Expression profile of a CAD transcript in Shiraz pulp, skin, and seeds at green stage (25th DAA), *véraison* (52nd DAA), and ripe stage (99th DAA). Expression was determined by real-time PCR using EF1 alpha as reference. Data are the mean values (±SD) of three replicates.

during biosynthesis (~3.0) but decreasing to ~2.0 at a ripe stage (99th DAA). For this ratio, pericarp value is intermediate between skin and pulp ones during flavan-3-ol biosynthesis and gets closer to the skin value during ripening. At these three stages, higher mDP values are obtained in pulp compared to skin, essentially thanks to greater supernatant mDP values in pulp (data not shown). At 52nd DAA, higher mDP values were also found in pulp versus in skin as in three other red cultivars [Mourvèdre, Grenache, and Muscat de Hambourg (*34*)] but in contrast with the half-fold lower mDP values in pulp compared to skin for the two red champenois cultivars [Pinot Noir, and Pinot Meunier (*15*)]. All six red cultivars exhibited lower Egc (%) in pulp than in skin, the largest value being found in Shiraz pulp (15.3) and the lowest ones (1.7−1.9) in the Pinot cultivars. In skin, the highest value for this percentage was also found in Shiraz (33.6) compared to Mourvèdre (15.6), Grenache (10.2), Muscat de Hambourg (12.1), Pinot Noir (14.5), and Pinot Meunier (13.2). Lower Egc (%) values in pulp than in skin were also observed for some white cultivars [(Maccabeo, Grenache blanc, and Muscat Frontignan) (*34*), Chardonnay (*15*)]. This data strongly suggests that the flavonoid 3′,5′-hydroxylase (F3′5′H) activity is also differentially regulated according to the cultivars. For pericarp at the three developmental stages, the Egc (%) is always intermediate between skin and pulp, and surprisingly, mDP and EcG (%) values are the lowest ones. These latest results contrast with the pericarp mDP value at 133rd DAA, higher than the pericarp mDP value at the 99th DAA but also greater than skin mDP at the 133rd DAA (**Table 3**).

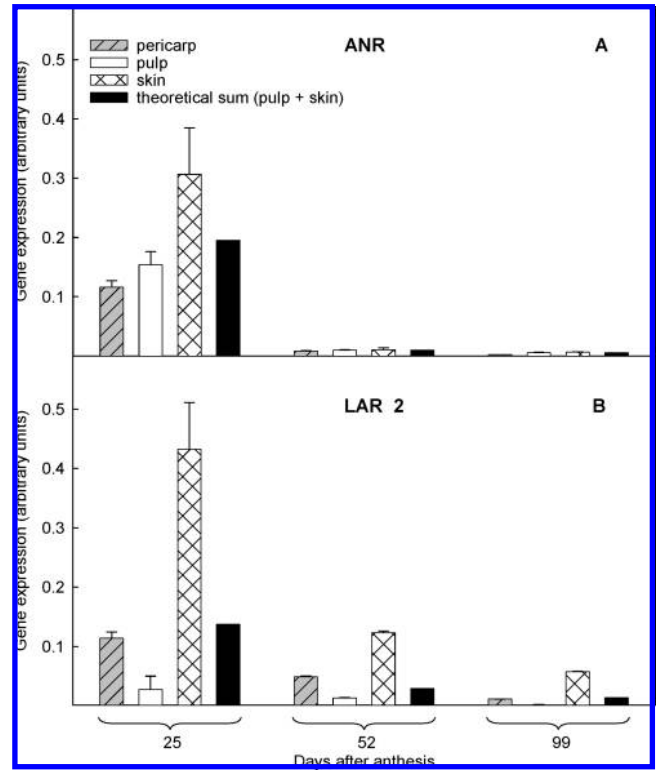

Figure 5. Expression profile of ANR (**A**) and LAR2 (**B**) transcripts in Shiraz pericarp, pulp and skin at green stage (25th DAA), *véraison* (52nd DAA) and ripe stage (99th DAA). The 4th histograms are a theoretical pericarp (pulp + skin) expression mean values. Expression was determined by real-time PCR using EF1 alpha as reference. Data is the mean values (±SD) of three replicates.

Absence of Cross Contamination of Tissue Samples. In order to validate our result and to definitively confirm the presence of PA in the pulp, we had to prove that our pulp sample was not contaminated by any skin or seed. At green stage until *véraison*, the skin is very tiny and can be easily separated from pulp. From *véraison* to ripeness, skin and pulp are a little bit more difficult to separate: skin and pulp next to skin are so stuck together that the pulp sample is generally reduced because some pulp remains sticking to the skin. However, no spreading from skin has occurred since anthocyanins, known to be easily extracted even without solvent, are not detected in pulp. To examine a possible contamination of pericarp and/or pulp samples by residual seeds, in our microarray data (Terrier, unpublished results), we detected genes whose expressions are restricted to seeds, whatever the developmental stage. A gene

of cinnamyl alcohol dehydrogenase (CAD, tentative contig TC58374) appears to meet this criteria. Its expression (**Figure 4**) is 50, 400, and 3000 times more important in the seeds than in the pulp for the three developmental stages investigated. It can be considered that its expression is restricted to seeds and is hardly detectable in any other part of the berry. If our pulp samples had been contaminated by seeds, such a type of discriminant expression profile could not have been obtained.

Occurence of Anthocyanidin Reductase (ANR) and Leucoanthocyanidin Reductase (LAR2) in Pulp. To establish a relationship between the presence of proanthocyanidins in pulp and the expression of transcripts involved in their biosynthesis, real-time PCR of ANR and two isogenes of LAR (LAR1, LAR2), involved, respectively, in Ec TU and Cat TU biosynthesis, were assessed on pericarp, pulp, and skin at the three developmental stages. Unfortunately nowadays, the molecular and biochemical mechanisms involved in PA EU synthesis and in PA polymerization are still unknown. As already shown by Bogs et al. (*17*) expression of LAR1 (data not shown) is very low in pericarp and quite restricted to seeds. **Figure 5** presents expression profiles for ANR (**A**) and for LAR 2 (**B**). A theoretical pericarp expression value was calculated taking expression in skin and pulp and weight of each compartment into account. Measurement of expression in whole pericarp highly reflects this theoretical value. Therefore, it can be concluded that extracting RNA from pericarp is a valuable tool to measure gene expression through berry development, even for genes preferentially expressed in the skin. Our results also confirm those of Bogs et al. (*17*) concerning the constant decrease of expression of both genes all along berry development. However, for the first time, we have demonstrated the presence of these transcripts (especially ANR) in pulp. A relationship between proanthocyanidins and transcripts could be established with the comparison of ratios (skin versus pulp) of transcript levels for one gene at green stage toward its enzyme product. For ratio calculation, results were expressed in arbitrary units/g FW or mg/g FW for transcripts or subunits, respectively. Ratios for ANR (2 times more expressed in skin than in pulp at green stage) and Ec TU (5.7 times more concentrated in skin than in pulp) point out that Ec amount in pulp is lower than expected. Furthermore, the over representation of the ANR transcript in pulp when compared to LAR2 should result in a higher proportion of Ec TU in pulp, which was not observed. Thus, post-transcriptional regulations should be assumed for this gene in pulp. Ratios between skin and pulp for LAR2 expression (19.7) and Cat TU (23.9) show that no post-transcriptional regulation seems to occur for this gene. Further biochemical, enzymatic, and transcriptomic investigations during berry development are needed to understand those differences in the flavonoid metabolic pathway.

Flavan-3-ol biosynthesis in pulp has been demonstrated for the first time with both biochemical and transcriptomic analyses, showing also a difference in PA composition when compared to skin, especially in the ratio Ec/Egc. Thus, this study leads to the conclusion that flavan-3-ols of skin and pulp should be taken into account in the estimation of tannin potential of the cultivars for either red or white winemaking. Further investigations on major cultivars involved in winemaking would be of interest.

ABBREVIATIONS USED

ANR, anthocyanin reductase; CAD, cinnamyl alcohol dehydrogenase; Cat, (+)-catechin; DAA, day after anthesis; Ec, (−)-epicatechin; EcG, (−)-epicatechin-3-*O*-gallate; EcG (%), percentage of galloylation; Egc, (−)-epigallocatechin; Egc (%), percentage of epigallocatechin subunits; EU, extension subunits; F3′5′H, flavonoid 3′,5′-hydroxylase; LAR, leucoanthocyanidin reductase; mDP, mean degree of polymerization; PA, proanthocyanidin polymers; TU, terminal subunits.

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LITERATURE CITED

(1) Glories, Y. Anthocyanins and tannins from wine: organoleptic properties. *Prog. Clin. Biol. Res*. **1988**, *280*, 123−134.

(2) Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grains*; CRC Press Inc.: Boca Raton, FL, 1993; p 362.

(3) Ferreira, D.; Slade, D.; Marais, J. P. J. Flavans and Proanthocyanidins. In *Flavonoids: Chemistry, Biochemistry and Applications*; Andersen, O. M. and Markham, K. R. Eds.; CRC Press LLC: Boca Raton, FL, 2006; pp 553−616.

(4) Andersen, O. M.; Jordheim, M. The anthocyanins. In *Flavonoids: chemistry, biochemistry and applications*; Andersen O. M. and-Markham,K. R. Eds.;CRC Press LLC:Boca Raton, FL, 2006; pp 471−551.

(5) Gonzalez-Neves, G.; Barreiro, L.; Gil, G.; Franco, J.; Ferrer, M.; Moutounet, M.; Carbonneau, A. Anthocyanic composition of Tannat grapes from the south region of Uruguay. *Anal. Chim. Acta* **2004**, *513*, 197–202.

(6) Ojeda, H.; Andary, C.; Kraeva, E.; Carbonneau, A.; Deloire, A. Influence of pre- and postveraison water deficit on synthesis and concentration of skin phenolic compounds during berry growth of *Vitis vinifera* cv. Shiraz. *Am. J. Enol. Vitic*. **2002**, *53*, 261–267.

(7) Downey, M. O.; Harvey, J. S.; Robinson, S. P. The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes. *Aust. J. Grape Wine Res*. **2004**, *10*, 55–73.

(8) Cadot, Y.; Minana Castello, M. T.; Chevalier, M. Flavan-3-ol compositional changes in grape berries (*Vitis vinifera* L. cv Cabernet franc) before veraison, using two complementary analytical approaches, HPLC reversed phase and histochemistry. *Anal. Chim. Acta* **2006**, *563*, 65–75.

(9) Fournand, D.; Vicens, A.; Sidhoum, L.; Souquet, J. M.; Moutounet, M.; Cheynier, V. Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages. *J. Agric. Food Chem*. **2006**, *54*, 7331–7338.

(10) Cheynier, V.; Prieur, C.; Guyot, S.; Rigaud, J.; Moutounet, M. The Structures of Tannins in Grapes and Wines and Their Interactions with Proteins. In *Wine Nutritional and Therapeutic Benefits*; Watkins, T. Ed.; ACS Symposium Series 661; American Chemical Society: Washington, DC, 1997; p 81−93.

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

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

(13) Bourzeix, M.; Weyland, D.; Heredia, N. Etude des catéchines et des procyanidolS de la grappe de raisin, du vin et d'autres dérivés de la vigne. *Bulletin de l'OIV* **1986**, *669−670*, 1171–1253.

(14) Ricardo-da-Silva, J. M.; Rosec, J. P.; Bourzeix, M.; Mourgues, J.; Moutounet, M. Dimer and trimer procyanidins in Carignan and Mourvèdre grapes and red wines. *Vitis* **1992**, *31*, 55–63.

(15) Mané, C.; Souquet, J. M.; Ollé, D.; Verriès, C.; Véran, F.; Mazerolles, G.; Cheynier, V.; Fulcrand, H. Optimization of simultaneous flavanol, phenolic acid and anthocyanin extraction from grapes using an experimental design; application to the characterization of Champagne grape varieties. *J. Agric. Food Chem*. **2007**, *55*, 7224–7233.

(16) Dixon, R. A.; Xie, D. Y.; Sharma, S. B. Proanthocyanidins - a final frontier in flavonoid research. *New Phytol*. **2005**, *165*, 9–28.

(17) Bogs, J.; Downey, M. O.; Harvey, J. S.; Ashton, A. R.; Tanner, G. J.; Robinson, S. P. Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. *Plant Physiol.* **2005**, *139*, 652–663.

(18) Ageorges, A.; Fernandez, L.; Vialet, S.; Merdinoglu, D.; Terrier, N.; Romieu, C. Four specific isogenes of the anthocyanin metabolic pathway are systematically co-expressed with the red colour of grape berries. *Plant Sci.* **2006**, *170*, 372–383.

(19) Terrier, N.; Sauvage, F. X.; Ageorges, A.; Romieu, C. Changes in acidity and in proton transport at the tonoplast of grape berries during development. *Planta* **2001**, *213*, 20–28.

(20) Kennedy, J. A.; Hayasaka, Y.; Vidal, S.; Waters, E. J.; Jones, G. P. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric. Food Chem.* **2001**, *49*, 5348–5355.

(21) Downey, M. O.; Harvey, J. S.; Robinson, S. P. Analysis of tannins in seeds and skins of Shiraz grapes throughout berry development. *Aust. J. Grape Wine Res.* **2003**, *9*, 15–27.

(22) Kennedy, J. A.; Jones, G. P. Analysis of proanthocyanidin cleavage products following acid-catalysis in the presence of excess phloroglucinol. *J. Agric. Food Chem.* **2001**, *49*, 1740–1746.

(23) Souquet, J.-M.; Mazauric, J.-P.; Meudec, E.; Preys, S.; Morel-Salmi, C.; Cheynier, V. Comparison of Different Methods of Depolymerization for the Characterization and Quantification of Proanthocyanidins in Grape Seed, Grape Skin and Wine, *Proceedings of the XXIInd International Conference on Polyphenols. Polyphenol Communications 2004, Helsinki, Finland, 2004*; Hoikkala, A., Soidinsalo, O., Wähälä, K., Eds.; pp 693−694.

(24) Kallithraka, S.; Garciaviguera, C.; Bridle, P.; Bakker, J. Survey of solvents for the sxtraction of grape seed phenolics. *Phytochem. Anal.* **1995**, *6*, 265–267.

(25) Matthews, S.; Mila, I.; Scalbert, A.; Donnelly, D. M. X. Extractable and non-extractable proanthocyanidins in barks. *Phytochemistry* **1997**, *45*, 405–410.

(26) Carando, S.; Teissèdre, P.-L.; Cabanis, J.-C. HPLC coupled with fluorescence detection for the determination of procyanidins in white wines. *Chromatographia* **1999**, *50*, 253–254.

(27) Sarni-Manchado, P.; Verries, C.; Tesniere, C. Molecular characterization and structural analysis of one alcohol dehydrogenase gene (GV-Adh1) expressed during ripening of grapevine (*Vitis vinifera* L.) berry. *Plant Sci.* **1997**, *125*, 177–187.

(28) Terrier, N.; Glissant, D.; Grimplet, J.; Barrieu, F.; Abbal, P.; Couture, C.; Ageorges, A.; Atanassova, R.; Leon, C.; Renaudin, J. P.; Dedaldechamp, F.; Romieu, C.; Delrot, S.; Hamdi, S. Isogene specific oligo arrays reveal multifaceted changes in gene expression during grape berry (*Vitis vinifera* L.) development. *Planta* **2005**, *222*, 832–847.

(29) Reid, K. E.; Olsson, N.; Schlosser, J.; Peng, F.; Lund, S. T. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biol.* **2006**, 6.

(30) Cheynier, V.; Osse, C.; Rigaud, J. Oxidation of grape juice phenolic compounds in model solutions. *J. Food Sci.* **1988**, *53*, 1729–1732.

(31) Le Bourvellec, C.; Guyot, S.; Renard, C. M. G. C. Non-covalent interaction between procyanidins and apple cell wall material. Part I. Effect of some environmental parameters. *Biochim. Biophys. Acta* **2004**, *1672*, 192–202.

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

(33) Escribano-Bailon, M. T.; Guerra, M. T.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. Proanthocyanidins in skins from different grape varieties. *Z. Lebens.-Unters. Forsch.* **1995**, *200*, 221–224.

(34) Souquet, J.-M.; Véran, F.; Mané, C.; Cheynier, V. Optimization of Extraction Conditions on Phenolic Yields from the Different Parts of Grape Clusters. Quantitative Distribution of Their Proanthocyanidins, *Proceedings of XXIII*rd *International Conference on Polyphenols, Polyphenols Communications 2006*, F. Daayf, A. El Hadrami, L., Adam, G. M. Balance, Eds. Winnipeg, Manitoba, Canada, 2006; pp 245−246.

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