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Impact of Extended Maceration and Regulated Deficit Irrigation (RDI) in Cabernet Sauvignon Wines: Characterization of Proanthocyanidin Distribution, Anthocyanin Extraction, and Chromatic Properties

L. Federico Casassa,[†] Richard C. Larsen,[‡] Christopher W. Beaver,[†] Maria S. Mireles,[†] Markus Keller,[§] William R. Riley,[∥] Russell Smithyman,[∥] and James F. Harbertson*[,†]

[†]School of Food Science, Washington State University (WSU), Irrigated Agriculture Research and Extension Center, 24106 North Bunn Road, Prosser, Washington 99350-8694, United States

[‡]Viticulture and Enology Program, Washington State University, Irrigated Agriculture Research and Extension Center, 24106 North Bunn Road, Prosser, Washington 99350-8694, United States

[§]Department of Horticulture, Washington State University, Irrigated Agriculture Research and Extension Center, 24106 North Bunn Road, Prosser, Washington 99350-8694, United States

[∥]Ste. Michelle Wine Estates, Prosser, Washington 99350-8694, United States

Supporting Information

ABSTRACT: The impact of extended maceration (EM) was studied in Cabernet Sauvignon grapes sourced from a vineyard subjected to four regulated deficit irrigation (RDI) treatments: (I) 100% replenishment of crop evapotranspiration (100% ET_c), (II) 70% ET_c, (III) 25% ET_c until véraison, followed by 100% ET_c until harvest, and IV) 25% ET_c. Each vineyard replicate was made into wine with two replicates designated as controls (10-day skin contact) and two as extended maceration (EM, 30-day skin contact). The mean degree of polymerization (mDP), size distribution, concentration, and composition of wine proanthocyanidins (PAs) and monomeric flavan-3-ols of 90 fractions were characterized by preparative and analytical HPLC techniques. The maceration length imparted a larger effect on most chemical parameters. The RDI treatment had no effect on the extraction patterns of anthocyanins, PAs, and/or on the origin of the PAs extracted into the wines. Conversely, EM led to anthocyanin losses and increased PA extraction during maceration, with ∼73% of seed-derived PAs. Accordingly, the concentration of monomeric flavan-3-ols, oligomeric ($2 \leq mDP < 5$) and polymeric PAs ($mDP \geq 5$) was higher in EM wines. The size distribution of the wines' PAs revealed two major peaks as a function of concentration at mDP 2 (22−27% of total PAs mass) and at mDP 6−7 (12−17% of total PAs mass) and was found to follow a non-normal Rayleigh-type distribution.

KEYWORDS: *extended maceration, regulated deficit irrigation, anthocyanin extraction, proanthocyanidin distribution*

INTRODUCTION

Phenolic compounds are ubiquitous in plant-derived food and beverage products. These are functional biomolecules possessing a specific three-aromatic ring system defined by a C6−C3−C6 structure bearing diverse hydroxyl and nonhydroxyl substitutions.[1] In *Vitis vinifera* L., phenolics are synthesized via the phenyl-propanoid biosynthetic pathway, which is modulated by both biotic and abiotic factors, with irrigation practices being among them.[2,3] From a chemical and sensory standpoint, the two most relevant phenolic classes in grapes and wines are anthocyanins and proanthocyanidins. Anthocyanins occur as vacuolar components in the skin tissue (and in the mesocarp of the *teinturier* varieties) and are present as monomers of six glycosylated forms, including malvidin, cyanidin, petunidin, peonidin, delphinidin, and pelargonidin.[4,5] Glycosylation typically occurs at the C3 position and renders the molecule water-soluble, thus facilitating their early extraction during maceration.[4,6]

Proanthocyanidins (PAs) are present in seeds, skins, and stem/rachis as oligomers and polymers of four flavan-3-ol subunits: (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, and (−)-epicatechin-3-*O*-gallate.[7,8] The average number of constitutive flavan-3-ol monomers in the PA structure, which are linked by covalent C4→C8 (or less commonly C4→C6) interflavanic bonds, is referred to as mean degree of polymerization (mDP). In wines of five *V. vinifera* cultivars, the polymeric ($mDP \geq 5$) PA fraction accounted for 77− 95% of the total PA distribution.[9] However, in the previous study fractionation was performed in C18 Sep-Pak cartridges and quantification was achieved by the vanillin essay, which lacks specificity in wine extracts.[10,11] In a separate study, the polymeric fraction represented 77−84% of the total PA distribution and showed a mDP variable from 6.3 to 13, but the presence of oligomers, particularly B-type dimers, was also observed.[12] Wines also contain a non-negligible amount of monomeric flavan-3-ols, with their content varying from 29 to 41 mg/L up to 189 mg/L, of which catechin alone represents 60−73% of the total flavan-3-ol content.[12,13]

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Because of the intrinsic heterogeneity of grape and wine PAs, structural characterization remains a challenge. To date, many different chromatographic techniques have been employed to fractionate polydisperse extracts of PAs. Sephadex LH-20[14,15] and silica stationary phases[16,17] have arguably been the two most used means of separation. However, such methods cannot resolve beyond the pentamer level and coelution occurs as polymers become increasingly substituted.[18] High molecular weight PAs can be enriched by solid phase extraction employing sorbents such as C18, XAD, or PVPP.[9,12] Direct assessment of native PAs by gel permeation chromatography has also been investigated.[19] However, a shortcoming common to these techniques is the lack of high molecular weight PA standards to build a standard curve that would allow quantification.[10]

Currently, analysis of skin, seed, or wine PAs is carried out by acid-catalyzed cleavage of the interflavanic bond in the presence of a nucleophilic reagent such as toluene-α-thiol,[9] benzylmercaptan,[20] or phloroglucinol.[7] This method allows the computation of the apparent mDP albeit with limitations. For example, the relative distribution of oligomers and polymers in grape and wine extracts is polydisperse and not centered in the mDP value.[21,22] As a result, phloroglucinolysis followed by HPLC is insufficient to provide information about the polymer size distribution.

Extended maceration (EM) consists of extending the contact of seeds, skins, and stems (when present) with the wine after alcoholic fermentation is completed. Reported benefits of this technique include enhanced phenolic extraction, particularly from seeds,[23−25] and stabilization of wine color.[23] However, there is to date no published information on the effects of EM on the wine PA distribution and composition.

Arid climates afford grape growers unique control over the vineyard water status through the application of specific irrigation protocols. Among the available irrigation techniques, regulated deficit irrigation (RDI) restricts water application to provide less than the full evapotranspirative demand of a vineyard.[26] RDI is utilized in many arid viticultural regions as a way of not only conserving water but also controlling shoot growth, berry size, and yield, which leads to changes in fruit and wine composition.[26,27] However, compositional changes in grapes do not consistently translate into the corresponding wines.[27,28] Presently, it is not clear how RDI affect the extraction of the major phenolic classes in commercially ripe fruit or if the application of winemaking techniques known to selectively increase the extraction of certain phenolic classes can result in synergistic or antagonistic chemical effects.

The present study is framed on a larger field experiment and was undertaken to address two objectives. First, we aimed to uncover possible interactions between the application of EM and four different RDI treatments on the extraction and evolution of anthocyanins and PAs. Second, the distribution of wine monomeric flavan-3-ols and PA material exhaustively isolated by monitoring the elution at 280 nm on a XAD resin was characterized to understand how the skin contact treatments and the RDI treatments affect these parameters.

MATERIALS AND METHODS

Vineyard Site and Experimental Design. The experiment was conducted during the 2011 growing season in the Cold Creek vineyard of Ste. Michelle Wine Estates, SE of Mattawa, Washington (latitude 46° 57′ N, longitude 119° 89′ W). Own-rooted *V. vinifera* cv. Cabernet Sauvignon (clone 8) were planted in 1981 with a vine by row spacing of 2.13 m by 3 m in north−south oriented rows. Vines were trained to a bilateral cordon, spur-pruned to 70 nodes per vine, and shoots were positioned between two foliage wires 30 cm above the cordon. The vineyard was drip-irrigated using pressure-compensated emitters (flow rate 4 L/h) spaced 1.14 m apart. The root zone was irrigated to field capacity before bud-break, but irrigation was interrupted before bloom to control shoot growth.[26] Four irrigation treatments were imposed at fruit set (growth stage 27).[29] The current industry standard for regulated deficit irrigation (RDI) was used as a control to replenish 70% of full-vine evapotranspiration (ET_c) through harvest and is referred to as 70% ET_c. This standard was derived from a reference crop (grass) evapotranspiration (ET_0) provided by the Washington State University AgWeatherNet weather station in Desert Aire, WA (USA), and a variable crop coefficient, K_c (from ∼0.3 at the start of treatments to ∼0.8 in early August to ∼0.4 by harvest) developed for fully irrigated Cabernet Sauvignon vines in eastern Washington: $ET_c = ET_0 \times K_c$.[30] Three other RDI treatments were also imposed from fruit set to harvest to replenish 100% ET_c, 25% ET_c, and 25% ET_c from fruit set to véraison (beginning of ripening) followed by 100% ET_c from véraison to harvest (25/100% ET_c). The total cumulative irrigation supplied (in mm) relative to the 100% ET_c treatment was 28%, 39%, and 76% lower in the 70% ET_c, 25/100% ET_c, and 25% ET_c treatments, respectively (Supporting Information Table 1). The experiment was designed as a randomized complete block with four replicated blocks ($n = 4$) and the irrigation treatments randomly applied (≥6 rows each) within each block. Midday stem water potentials (Ψ_s), yield components, and pruning weights (a measure of plant vigor) were obtained from two vines per treatment replicate ($n = 8$) as previously reported.[26] Recorded Ψ_s and total irrigation water applied during the growing season, as well as yield components, are reported in Supporting Information Tables 1 and 2, respectively.

Winemaking. On October 12, 300 kg of fruit from each vineyard replicate were manually harvested for a total of 4800 kg (4 RDI treatments × 4 replicates each) and transported to the Washington State University (WSU) research winery facility. Two skin contact treatments were applied in duplicate using two of the four field replicates of each RDI treatment: control wines, with a 10-day skin contact period, and extended maceration wines (EM), with a 30-day skin contact period, affording a total of 16 wines. The fruit was destemmed using a Gamma model 40 RM destemmer crusher (Toscana Enologica Mori, Italy) and pumped to 300 L stainless steel jacketed fermentors (Ghidi, Italy) with a positive-displacement pump (Francesca, Imola, Italy). Sulfur dioxide (SO_2) was added at a rate of 50 mg/L during the fermentor filling process. Diammonium phosphate was added to raise the yeast assimilable nitrogen to 225 mg/L. Musts were inoculated 4 h after crushing with selected dry yeast (Lalvin EC-1118, Lallemand, Montreal, Canada) at a rate of 250 mg/L. Malolactic bacteria (Lalvin VP41, Lallemand, Montreal, Canada) were added 48 h after yeast inoculation at a rate of 10 mg/L. Sugar consumption during fermentation was monitored daily with a hand-held densitometer (DMA 35N, Anton Paar, Graz, Austria), and tank temperatures were maintained at 26 ± 2 °C at the fermentation peak using a web-based fermentation system (TankNet, Acrolon Technologies, Sonoma, CA, USA). Cap management consisted of a whole-volume tank pump-over followed by a 5 min punch down twice a day during active fermentation. Alcoholic fermentation was completed (reducing sugars <2 g/L) after 9−10 days in all wines (Supporting Information Figure 1). During postfermentation, EM wines received one 1 min punch down per day, after which the tanks were sealed and sparged with N_2 (30 L/min × 3 min). Wines were pressed after completion of the skin contact time allotted for each treatment, and free run wines were transferred to 100 L stainless steel tanks (Ghidi, Italy). Malolactic fermentation (MLF) was monitored by enzymatic analysis of L-malic acid (Unitech Scientific, Hawaiian Gardens, CA, USA) and was completed (<0.1 g/L of malic acid) within 20−25 days after completion of alcoholic fermentation. After completion of MLF, the wines were racked, adjusted to 35 mg/L free SO_2, and cold-stabilized for 60 days at 0 ± 2 °C. Prior to bottling, the wines were adjusted to 0.5 ppm molecular SO_2 and membrane-filtered using a 0.45 μm pore size cartridge (Vitipore II Plus, Millipore Corporation, Billerica, MA, USA). The wines were bottled at room temperature (20 ± 1 °C) in 750 mL bottles sparged with N_2 gas before

and after filling (20 psi × 5 s), immediately sealed with screw-cap closures (Stelvin Saranex liner, Amcor corporation, Zurich, Switzerland), and stored at 10 ± 2 °C.

Chemical Analysis: Reagents. Reagents for determination of protein precipitable PAs and total phenolics are reported elsewhere.[31] Phloroglucinol, L-ascorbic acid, L-malic acid (97%), L-lactic acid (85%, 13 M), glucose, and fructose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid (99.7%, 17 M) and HPLC-grade solvents were obtained from Merck (Darmstadt, Germany).

Fruit and Wine Basic Analysis. For fruit analysis, three 15-cluster replicates were randomly selected from each replicate vineyard block. For each replicate, berries were separated from the clusters and placed onto a table where two sets of 30 berries were selected at random. In one of the 30-berry sets, the juice was extracted for 20 s using an IKA A11 analytical mill (Fisher Scientific, Waltham, MA, USA) and the pulp solids and liquid were transferred to 50 mL tubes, centrifuged (5000*g* × 6 min at 5 °C), and the supernatant analyzed for basic chemistry.[24] In the second 30-berry set, phenolics in the fruit were analyzed as described previously,[31] with results expressed on a fresh weight (FW) basis. Ethanol concentrations were measured using a digital infrared spectrophotometer (Anton Paar, Graz, Austria), and free and total SO_2 levels throughout the study were determined with the FIAstar system (Foss Analytical, Hilleroed, Denmark).

Spectrophotometric Analysis. Must and wine samples were treated with 1 mM sodium azide to inhibit microbial activity, centrifuged (5000*g* × 5 min) and filtered through 0.22 μm filters (Fisher Scientific, Westboro, MA, USA) prior to analysis. Spectrophotometric measurements were carried out with an Agilent 8453 UV−visible spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Anthocyanins, small polymeric pigments (SPP), and large polymeric pigments (LPP) were measured as previously detailed.[31] PAs in the fruit, pomace, and wines were analyzed by protein precipitation with bovine serum albumin (BSA). For pomace samples, a published protocol was used to analyze PAs recovered in the skins and seeds collected from the pomace of each replicate.[24] Characterization of wine color was undertaken by means of the Cie-Lab system using MSCV software (Grupo de Color de La Rioja, Logroño, Spain). To explore overall chromatic differences between treatments, the Cie-Lab color difference (ΔE^*) between any given pair of wines was calculated as previously described[32] at day 30 (pressing of EM wines), 250, and 400 postcrushing.

HPLC-DAD-ESI-MS^n Analysis in Wines. An Agilent 1100 series HPLC-DAD system (Agilent Technologies, Palo Alto, CA, USA) was used for all chromatographic separations. Samples were filtered through cellulose acetate 0.22 μm syringe filters (Fisher Scientific, Westboro, MA, USA) prior to analysis.

Organic Acids and Reducing Sugars. Malic, lactic, and acetic acid were measured by reverse phase HPLC using a previously described method.[33] Glucose (Glu) and fructose (Fru) were separated by HPLC and detected using a refractive index (RI) detector (model G1362A).

Proanthocyanidin Fractionation. Fractionation of wine PAs was based on a published procedure,[22] with modifications. Ethanol was removed from wine samples (400 mL) by rotary evaporation under reduced pressure at 30 °C (Buchi R-200, Flawil, Switzerland) to a final volume of 300 mL. Samples (n = 16) were independently loaded onto a flash chromatography system (Buchi C-620, Flawil, Switzerland) which consisted of a glass column (46.5 cm × 4.3 cm) packed with Amberlite XAD7HP resin (Dow Chemical, Midland, MI, USA) to an approximate bed volume of 675 cm^3. The system was operated at a flow rate of 120 mL/min. Eluate was monitored at 280 nm using a UV-photometer (Buchi C-635, Flawil, Switzerland). The column was washed with 3 L of deionized water to remove sugars and organic acids. Monomeric flavan-3-ols and PAs were eluted with 1.225 L of 80% methanol and 700 mL of pure methanol, using a collection threshold of 0.08 absorbance units. The obtained eluate (∼1.9 L) was rotary evaporated to eliminate methanol to a final volume of 300 mL, which was then dried using a nanospray drier (ADL311S, Yamato scientific, Tokyo, Japan) to yield a fine dark red−purple powder (0.8518 to 1.1585 g), heretofore referred to as wine PA crude extract. An aliquot of the PA crude extract of each wine (375 ± 0.5 mg) was resuspended in 3 mL of a 3:1 mixture of mobile phase A and B from the preparative HPLC system, centrifuged (14000*g* × 10 min), and the supernatant submitted to preparative-scale HPLC using a 1260 Infinity preparative HPLC-DAD system (Agilent Technologies, Waldbronn, Germany). Separations were achieved at room temperature using a binary gradient of 0.05% formic acid in acetonitrile (mobile phase A) and 0.05% formic acid in a 85:8:7 mixture of methanol, water, and 2-propanol (mobile phase B). The column selected was a Luna HILIC preparative column (250 mm × 21.2 mm, 200 Å, 5 μm particle size, Phenomenex, Torrance, CA, USA), protected by a guard column of the same packing material. The flow rate was 15 mL/min and the gradient conditions were as follows: 0−45 min 30% B, 45−60 min 30% B, 65−90 min 100% B, 91−100 min 0% B, followed by a postrun equilibration time of 5 min at initial conditions. Injection volume was 1800 μL. The eluate was monitored by DAD at 280 and 520 nm, and fractions were collected using an automatic fraction collector (G1364B) at 1 min intervals from 0 to 90 min, yielding 90 × 15 mL fractions.

Phloroglucinolysis. Phloroglucinolysis was performed as previously described,[7,22] with modifications. Herein, separations were performed using an Atlantis C18 column (250 mm × 4.6 mm, 5 μm particle size, Waters, Mildford, MA, USA) protected by a guard column of the same material. A 1 mL aliquot of each fraction was evaporated to dryness under reduced pressure at room temperature using a SpeedVac system (Savant ISS110, Thermo Fisher Scientific, Asheville, NC, USA), resuspended in 33 μL of phloroglucinol reagent, sonicated for 10 s, and heated in a water bath (50 °C × 20 min). The reaction was quenched by addition of 33 μL of aqueous sodium acetate (200 mM), vortexed for 5 s, and centrifuged (14000*g* × 5 min). The supernatant was transferred to 200 μL glass insets, placed in brown HPLC vials, and injected in the HPLC system within 6 h after the end of the reaction. Identification was performed by ESI-MS^n with a 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), as previously reported.[25] Following HPLC separation, the concentration of the released extension and terminal subunit and free monomers was determined using a 6-point catechin standard curve (r^2 = 0.999) and published conversion factors for each subunit relative to catechin.[22] The mDP was calculated as reported.[7] Fractions of the same mDP were pooled to report PA concentration, percent conversion yield, and the proportion of constitutive subunits at individual mDP values. The average subunit composition and the mDP of each wine crude extract without fractionation was also determined.

Determination of the Percent Conversion Yield. The conversion yield (%) of each fraction was determined as described previously,[22] with minor modifications. Briefly, a 100 μL aliquot of each fraction prior to phloroglucinolysis was resuspended in 900 μL of a 3:1 mixture of the mobile phases used for HPLC separation (mobile phase A, 2% formic acid in water; mobile phase B, 20% A in acetonitrile). The absorbance of each fraction was measured at 280 nm using 1.5 mL UV cuvettes. The total PA concentration of the fraction determined by UV−vis spectrophotometry was expressed as catechin equivalents using a 6-point catechin standard curve (r^2 = 0.998).

Sampling Protocol. Spectrophotometric analysis of anthocyanins and PAs were performed at 2-day intervals during skin contact, at pressing (day 10 and 30), and at days 60, 120, 200, 270, and 400 postcrushing. Proanthocyanidin fractionation and distribution was performed on all the 16 wines (8 treatments × 2 replicates) at day ∼250.

Data Analysis. The harvest fruit comparison of the RDI treatments was analyzed by one-way analysis of variance (ANOVA). A fixed-effect two-way ANOVA with interaction (df = 15) and a 5% level for rejection of the null hypothesis was used to analyze the effects of RDI regime, skin contact time, and their interaction for all the chemical parameters. Fisher's LSD test was used as a posthoc comparison of means. For anthocyanin and PA extraction, least significant difference (LSD) is given when differences between any treatments are significant (p < 0.05). Data analysis was performed with XLSTAT v. 2011 (Addinsoft, Paris, France). Distribution fitting of PAs by size was performed using the software EasyFit Professional version 5.5 (MathWave Technologies, San Diego, CA, USA). The Kolmogorov−Smirnov test was used for the calculation of the goodness of fit (GOF).

Table 1. One-Way ANOVA of Berry Weight, Basic Fruit Chemistry, and Phenolic Composition of cv. Cabernet Sauvignon Grapes of the Different Irrigation Treatments at Harvest (Means ± SEM (n = 4))

treatment	berry weight (g)	Brix	pH	titratable acidity (g/L)	skin anthocyanins[c] (mg/g FW)	skin PAs[d] (mg/g FW)	seed PAs[d] (mg/g FW)
100% ET_c	1.33 ± 0.04 a[a]	25.4 ± 0.2 a	3.44 ± 0.01 a	7.49 ± 0.62 a	0.91 ± 0.11 b	0.61 ± 0.04 b	2.97 ± 0.16 ab
70% ET_c	1.31 ± 0.04 a	25.8 ± 0.2 a	3.48 ± 0.01 a	7.55 ± 0.57 a	0.82 ± 0.02 b	0.66 ± 0.01 b	2.55 ± 0.31 b
25/100% ET_c	1.13 ± 0.05 b	25.3 ± 0.2 a	3.47 ± 0.01 a	6.59 ± 0.61 a	1.12 ± 0.07 a	0.88 ± 0.07 a	2.92 ± 0.36 ab
25% ET_c	0.99 ± 0.02 c	25.5 ± 0.2 a	3.49 ± 0.02 a	6.89 ± 0.22 a	1.28 ± 0.04 a	0.82 ± 0.03 a	3.54 ± 0.13 a
p-value	**<0.0001**[b]	0.899	0.201	0.528	**0.006**	**0.003**	0.108

[a]Within a column, values followed by the same letter are not significantly different according to Fisher's LSD test at $p < 0.05$. [b]Significant p-values ($p < 0.05$) are shown in bold. [c]Determined by HPLC-DAD-MS. [d]Determined by protein precipitation (Harbertson et al. 2003).

Table 2. Two-Way ANOVA Showing Mean (± SEM) Values of Basic Analysis at Bottling of Cabernet Sauvignon Wines Subjected to Four Different RDI Regimes (Vineyard Treatment) and Two Skin Contact Treatments with Their Interaction

ANOVA parameter	titratable acidity (g/L tartaric acid)	pH	ethanol (% v/v)	reducing sugars (g/L Glu + Fru)	malic acid (mg/L)	acetic acid (g/L)	free SO_2 (mg/L)
RDI Treatment (RDI)							
100% ET_c	5.71 ± 0.06 ab[a]	3.79 ± 0.01 b	13.99 ± 0.03 b	0.10 ± 0.03 a	30 ± 0.00 a	0.50 ± 0.02 a	40 ± 3 ab
70% ET_c	5.74 ± 0.03 ab	3.84 ± 0.03 a	14.57 ± 0.05 a	0.16 ± 0.00 a	30 ± 0.00 a	0.50 ± 0.03 a	41 ± 2 a
25/100% ET_c	5.66 ± 0.03 b	3.76 ± 0.01 b	13.97 ± 0.09 b	0.18 ± 0.04 a	30 ± 0.00 a	0.51 ± 0.04 a	38 ± 4 b
25% ET_c	5.85 ± 0.04 a	3.75 ± 0.01 b	14.64 ± 0.08 a	0.16 ± 0.07 a	30 ± 0.00 a	0.50 ± 0.03 a	39 ± 3 ab
p-value	0.129[b]	**0.012**	**<0.0001**	0.614	0.841	0.954	**0.002**
Skin Contact (SC)							
control	5.72 ± 0.03 a	3.76 ± 0.01 b	14.21 ± 0.12 a	0.14 ± 0.02 a	30 ± 0.00 a	0.45 ± 0.01 b	40 ± 5 a
EM[c]	5.75 ± 0.04 a	3.81 ± 0.01 a	14.36 ± 0.12 a	0.16 ± 0.04 a	28 ± 0.00 a	0.55 ± 0.01 a	39 ± 3 a
p-value	0.519	**0.011**	0.316	0.706	0.064	**0.002**	0.821
RDI × SC Interaction							
p-value	0.969	0.413	0.588	0.573	0.274	0.921	0.281

[a]Within a column, values followed by the same letter are not significantly different according to Fisher's LSD test at $p < 0.05$. [b]Significant p-values ($p < 0.05$) are shown in bold. [c]Extended maceration.

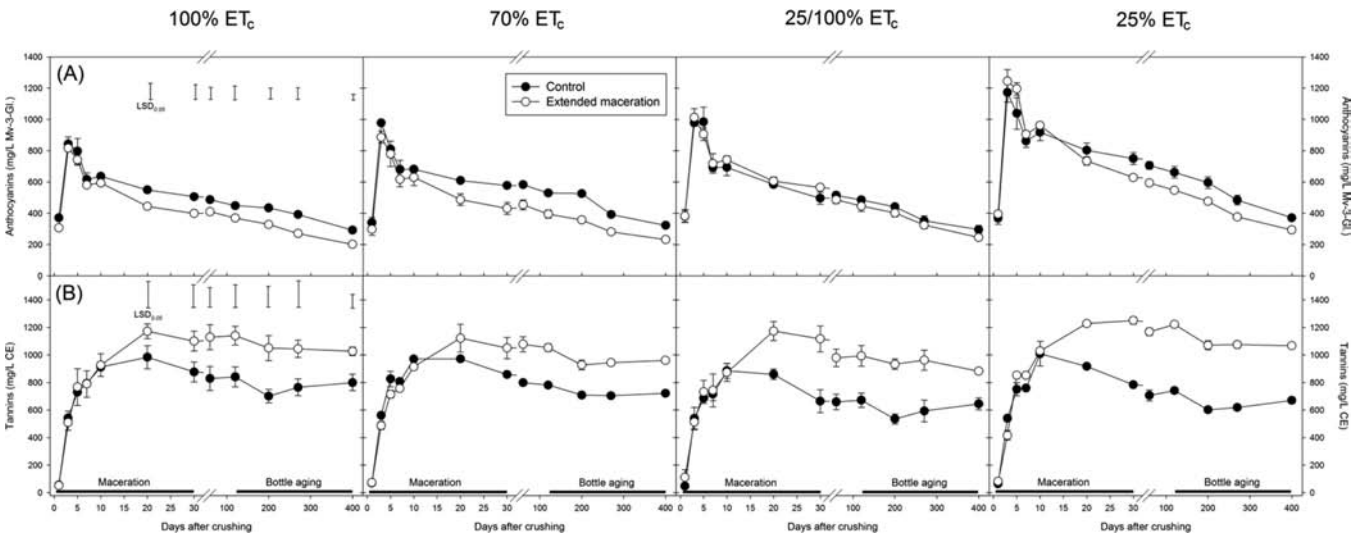

Figure 1. Extraction and evolution of (A) anthocyanins and (B) protein precipitable PAs during maceration and bottle aging of Cabernet Sauvignon wines obtained with a combination of four RDI and two skin contact treatments. CE: catechin equivalents. Mean values ± SEM (n = 2). Solid vertical bars in the top left of (A) and (B) represent the calculated LSD ($p < 0.05$) between any two given wines.

RESULTS AND DISCUSSION

Berry Basic and Phenolic Chemistry and Wines' Basic Chemistry. Measurements of Ψ_s and pruning weights indicated that the RDI treatments had the desired effect, namely to reduce vine water status (Supporting Information Table 1) and, by limiting shoot growth, plant vigor (Supporting Information Table 2). Abnormally low yields were observed in the 70% ET_c treatment due to a comparatively lower shoot number (possibly due to unintentional errors during pruning, data not shown). As a result, the effect of the RDI treatments on yield components was

Table 3. Two-Way ANOVA Showing Mean (± SEM) Values of PA Content Recovered on the Pomace, and Estimated Proportion of Skin and Seed PAs Extracted into Cabernet Sauvignon Wines Subjected to Four Different RDI Regimes (Vineyard Treatment) and Two Skin Contact Treatments with Their Interaction

ANOVA parameter	skin PAs (mg/g FW)	seed PAs (mg/g FW)	proportion of skin-derived PAs (%)	proportion of seed-derived PAs (%)
RDI Treatment (RDI)				
100% ET_c	0.12 ± 0.02 a[a]	1.46 ± 0.43 b	27 ± 6 a	73 ± 6 a
70% ET_c	0.12 ± 0.02 a	1.31 ± 0.18 b	37 ± 4 a	63 ± 4 a
25/100% ET_c	0.16 ± 0.03 a	2.06 ± 0.23 ab	37 ± 5 a	63 ± 5 a
25% ET_c	0.20 ± 0.04 a	2.71 ± 0.11 a	43 ± 5 a	57 ± 5 a
p-value	0.262[b]	**0.035**	0.451	0.451
Skin Contact (SC)				
control	0.11 ± 0.02 b	2.02 ± 0.09 a	45 ± 3 a	55 ± 4 b
EM[c]	0.19 ± 0.02 a	1.75 ± 0.11 b	28 ± 3 b	73 ± 3 a
p-value	**0.026**	**0.031**	**0.008**	**0.008**
RDI × SC Interaction				
p-value	0.778	0.741	0.678	0.678

[a]Within a column, values followed by the same letter are not significantly different according to Fisher's LSD test at $p < 0.05$. [b]Significant *p*-values ($p < 0.05$) are shown in bold. [c]Extended maceration.

Table 4. Two-Way ANOVA Showing Mean (± SEM) Values of Small Polymeric Pigments (SPP) and Large Polymeric Pigments (LPP) at Days 30, 250, and 400 Post-Crushing in Cabernet Sauvignon Wines Subjected to Four Different RDI Regimes (Vineyard Treatment) and Two Skin Contact Treatments with Their Interaction

	day 30		day 250		day 400	
ANOVA parameter	SPP (AU @ 520 nm)	LPP (AU @ 520 nm)	SPP (AU @ 520 nm)	LPP (AU @ 520 nm)	SPP (AU @ 520 nm)	LPP(AU @ 520 nm)
RDI Treatment (RDI)						
100% ET_c	1.53 ± 0.08 c[a]	0.60 ± 0.04 b	1.64 ± 0.10 c	0.93 ± 0.15 b	2.59 ± 0.10 c	0.87 ± 0.11 b
70% ET_c	1.78 ± 0.15 b	0.54 ± 0.04 b	1.95 ± 0.11 b	1.04 ± 0.14 ab	2.93 ± 0.15 b	0.91 ± 0.07 b
25/100% ET_c	1.81 ± 0.08 b	0.42 ± 0.11 b	2.04 ± 0.07 b	0.80 ± 0.09 b	2.99 ± 0.12 b	0.78 ± 0.12 b
25% ET_c	2.34 ± 0.17 a	0.90 ± 0.11 a	2.58 ± 0.15 a	1.41 ± 0.13 a	3.71 ± 0.18 a	1.49 ± 0.13 a
p-value	**<0.0001**[b]	**0.006**	**<0.0001**	**0.041**	**<0.0001**	**0.005**
Skin Contact (SC)						
control	2.05 ± 0.13 a	0.55 ± 0.07 b	2.20 ± 0.14 a	1.09 ± 0.12 a	3.27 ± 0.17 a	1.02 ± 0.07 a
EM[c]	1.67 ± 0.11 b	0.68 ± 0.09 a	1.91 ± 0.13 b	1.02 ± 0.12 a	2.84 ± 0.11 b	1.01 ± 0.16 a
p-value	**0.001**	0.094	**0.009**	0.492	**0.001**	0.867
RDI × SC Interaction						
p-value	0.377	0.181	0.852	0.338	0.766	0.238

[a]Within a column, values followed by the same letter are not significantly different according to Fisher's LSD test at $p < 0.05$. [b]Significant *p*-values ($p < 0.05$) are shown in bold. [c]Extended maceration.

less clear-cut (Supporting Information Table 2) and should be tested over at least three growing seasons. Berry weight was affected by the RDI treatment (Table 1) and, relative to the control treatment (100% ET_c), was reduced by 30% in 25% ET_c and by 16% in 25/100% ET_c.

There was no effect of the RDI treatment on the basic chemistry of the berries (Table 1). Relative to the 100% ET_c treatment, skin anthocyanins and skins PAs increased by 40% and 34%, respectively, in the berries of the 25% ET_ctreatment, whereas skin PAs increased by 44% in 25/100% ET_c (Table 1). However, when expressing the results on a per berry basis to assess the phenolic content beyond the effect of berry size,[34] no differences in the anthocyanin content within treatments were found ($p = 0.095$) and only a higher content of skin PAs in the 25/100% ET_c ($p = 0.011$) was evidenced (data not shown). For seed PAs, differences on a FW basis between 70% ET_c and 25% ET_c were observed, but no clear trend as a function of RDI could be identified. Similarly, no differences were observed when expressing the seed tannin content on a per berry basis ($p = 0.533$, data not shown).

A two-way ANOVA on the basic chemistry of the finished wines (Table 2), pooled as a function of both the skin contact treatments and the RDI treatments, showed no interactive effect between these two factors and uncovered only minor effects of the RDI treatment (pH, ethanol content, and free SO_2). and the skin contact treatment (pH and volatile acidity).

Anthocyanin and PA Extraction and Evolution during Bottle Aging. The extraction and evolution of anthocyanins (Figure 1A) and protein precipitable PAs (Figure 1B) was followed during maceration and bottle aging in the individual wines to assess the kinetics of extraction of these phenolic classes as affected by the skin contact and the RDI treatments. Extraction patterns of both phenolic classes during maceration agreed with the literature. Anthocyanins peaked at day 5 postcrushing and

Table 5. Double Entry Table Showing the Evolution of Cie-Lab Color Difference (ΔE^*) in the Individual Wines at Days 30, 250 (Single Underline), and 400 (Italic and Underlined) in Cabernet Sauvignon Wines Subjected to Four Different RDI Regimes and Two Skin Contact Treatments. Values Are Presented As Mean of Two Replicates ($n = 2$)

RDI treatment		100% ET_c			70% ET_c		25/100% ET_c		25% ET_c	
RDI treatment	skin contact	C		EM	C	EM	C	EM	C	EM
100% ET_c	C			**5.92, 6.31, *6.61***	**5.17**, 4.13, *4.19*	**6.57**, 6.11, *5.46*	4.68, 2.92, *2.89*	1.38, 2.64, *1.73*	**14.41**, 12.49, *11.81*	**12.85**, 10.71, *8.84*
	EM[b]	**5.92**,[a] 6.31, *6.61*			**10.25**, 6.11, *10.72*	1.75, 0.74, *1.77*	**9.45**, 9.19, *9.49*	**6.48**, 3.87, *5.22*	**19.69**, 18.23, *18.19*	**17.87**, 16.14, *15.85*
70% ET_c	C	**5.17**, 4.13, *4.19*		**10.25**, 6.11, *10.72*		**11.25**, 9.91, *9.39*	1.06, 1.43, *1.40*	4.45, **6.44** , *5.52*	**9.91**, 8.43, *7.66*	**8.07**, 6.92, 4.71
	EM	**6.57**, 6.11, *5.46*		1.75, 0.74, *1.77*	**11.25**, 9.91, *9.39*		**10.47**, 8.93, *8.28*	**7.39**, 3.54, *3.89*	**20.78**, 17.75, *16.80*	**19.04**, 15.66, *13.76*
25/100% ET_c	C	4.68, 2.92, *2.89*		**9.45**, 9.19, *9.49*	1.06, 1.43, *1.40*	**10.47**, 8.93, *8.28*		4.03, **5.41**, *4.39*	**10.84**, 9.80, *9.02*	**8.91**, 8.25, *6.11*
	EM	1.38, 2.64, *1.73*		**6.48**, 3.87, *5.22*	4.45, **6.44**, *5.52*	**7.39**, 3.54, *3.89*	4.03, **5.41**, *4.39*		**13.43**, 14.47, *12.99*	**11.83**, 12.43, *9.97*
25% ET_c	C	**14.41**, 12.49, *11.81*		**19.69**, 18.23, *18.19*	**9.91**, 8.43, *7.66*	**20.78**, 17.75, *16.80*	**10.84**, 9.80, *9.02*	**13.43**, 14.47, *12.99*		2.50, 2.69, *3.05*
	EM	**12.85**, 10.71, *8.84*		**17.87**, 16.14, *15.85*	**8.07**, 6.92, 4.71	**19.04**, 15.66, *13.76*	**8.91**, 8.25, *6.11*	**11.83**, 12.43, *9.97*	2.50, 2.69, *3.05*	

[a]Numbers in bold indicate a ΔE^* resulting in a chromatic difference discernible by the human eye between any given pair of wines (Pérez-Magariño and González-Sanjosé 2003). [b]Extended maceration.

decreased afterward following approximately a second-order kinetic.[24,25,35] Protein precipitable PAs peaked or slightly decreased at the time of pressing then remained stable during bottle aging. Interestingly, the pattern of extraction of anthocyanins and PAs was unaffected by the RDI treatment. Nevertheless, quantitative differences were observed within some RDI treatments. For example, at day 400, the 25% ET_c-control wines retained higher concentration of anthocyanins ($p = 0.001$), whereas the concentration of PAs was lower in the controls of both the 25/100% ET_c and 25% ET_c treatments ($p < 0.001$).

The effect of the skin contact treatments on the extraction patterns of anthocyanins and PAs was more evident than that of the RDI treatments. With the sole exception of the 25/100% ET_c treatment, EM led to a decrease in anthocyanins, which was evidenced after pressing and progressed along bottle aging. Losses of anthocyanins in the EM wines occurred mainly between day 10 and 30. The factors that explain such losses include adsorption to fermentation solids, oxidative degradation, and formation of polymeric pigments and pyranoanthocyanins, as further discussed elsewhere.[24,25,36] As expected, the EM treatments promoted PA extraction and at day 400 EM wines had significantly higher PA content than their control counterparts at any given RDI treatment ($p = 0.002$).

Analysis of PAs recovered from seed and skin pomace after maceration provided further insights into the origin of the total PA mass extracted into the wines and the separate effects of the skin contact and RDI treatments (Table 3). Although the overall effect of the RDI treatment on the proportion of skin- and seed-derived tannins was not significant for a two-way ANOVA model, a one-way ANOVA on the individual wines showed a significantly higher proportion of skin-derived tannins in the control wines of the 70% ET_c and 25% ET_c treatments (Supporting Information Table 5). On the other hand, the skin contact treatment had a consistent effect on the proportion of skin- and seed-derived PAs extracted. Thus control wines showed an equivalent proportion of skin- and seed-derived PAs. EM wines, on the other hand, had a PA content mainly derived from seeds (~73%). Similarly, in a report in Merlot wines, EM for 20 days resulted in wines with 79% of seed-derived PAs.[24] In a separate study, also in Merlot, EM wines had between 73 and 80% of seed-derived PAs after 30 days of maceration,[25] consistent with the data in this study. Overall, increased PA extraction from seeds during EM masked any compositional differences in the PA content of the different RDI treatments.

Small (SPP), Large Polymeric Pigments (LPP), and Overall Color Differences. A two-way ANOVA was performed on the values of SPP and LPP at selected time-points, namely at 30 (pressing of EM wines), 250 (~3 months of bottle aging), and 400 (~9 months of bottle aging) days postcrushing with results summarized in Table 4. There was no interactive effect of RDI and the skin contact treatments on the formation of either SPP or LPP. However, there were significant individual effects of both the RDI and the skin contact treatments. When analyzed on the basis of the RDI treatments, the formation of both SPP and LPP was favored in the 25% ET_c wines, consistent with a higher concentration of anthocyanins in the fruit of this treatment (Table 1) and in the corresponding wines (Figure 1A). SPP are formed primarily by reaction of anthocyanins with miscellaneous compounds, including (but not limited to) acetaldehyde, pyruvic acid, and flavan-3-ol monomers or dimers,[37] resulting in low molecular size pigments that do not precipitate the BSA protein used in the method to assess PAs.[31]

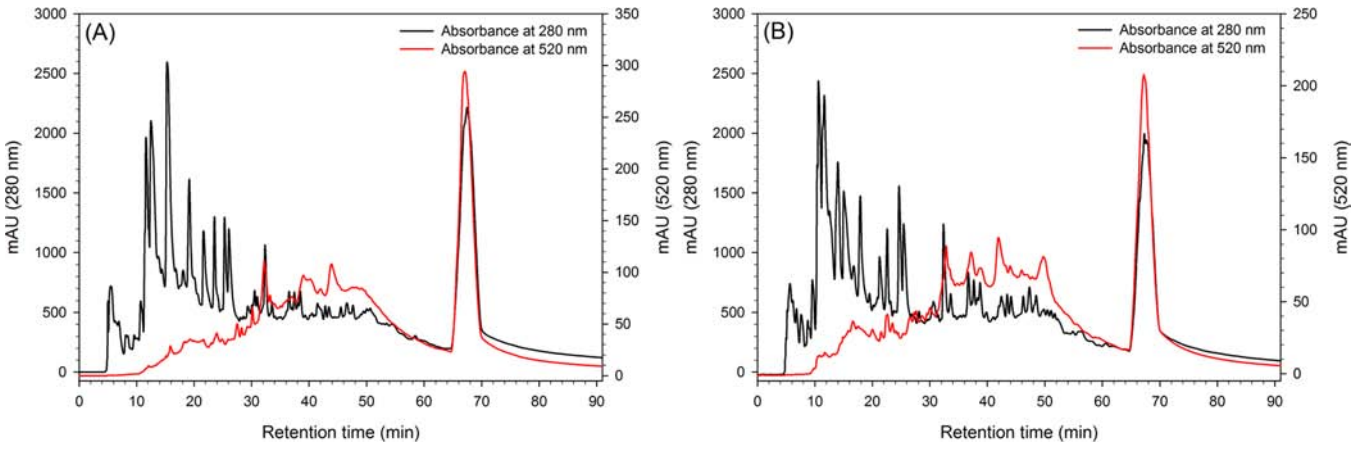

Figure 2. Representative chromatograms recorded during the preparative fractionation of Cabernet Sauvignon wine crude extracts on Luna HILIC stationary phase. (A) 100% ET_c: control wine. (B) 100% ET_c: EM wine.

Conversely, LPP are pigmented PAs that coprecipitate with the BSA protein, and they reportedly contribute to perceived astringency.[25,38] From this perspective, higher astringency ratings were anticipated in the wines of the 25% ET_c treatment.

With regard to the skin contact treatment, EM wines did not favor SPP formation and actually had lower levels of SPP, suggesting that prolonged maceration does not add to SPP synthesis. Alternatively, the comparatively lower anthocyanin concentration in EM wines (Figure 1A) can also account for the lower SPP content in these wines.

Chromatic differences between the wines are shown in a double entry table (Table 5). ΔE^* values between any given pair of wines (r and s) were calculated based on the values of Lightness (L^*), a^* (green/red component), and b^* (blue/yellow component) using the equation

$$\Delta E^*_{r,s} = [(\Delta L^*_{r,s})^2 + (\Delta a^*_{r,s})^2 + (\Delta b^*_{r,s})^2]^{1/2} \quad (1)$$

where: $\Delta L^*_{r,s} = (L^*_r - L^*_s)$; $\Delta a^*_{r,s}$ and $\Delta b^*_{r,s}$ are defined in the same fashion as $\Delta L^*_{r,s}$.[32]

Chromatic differences discernible by the human eye ($\Delta E^* > 5$) and in favor of the control wines were observed in the contrast of these wines against the EM wines for the 100% ET_c and 70% ET_c treatments at any given time, consistent with a comparatively higher concentration of anthocyanins and SPP in the control wines of these two treatments. No chromatic differences were observed between the control and EM wines for the 25/100% ET_c and 25% ET_c treatments. For the 25/100% ET_c wines, this was anticipated, as a comparable anthocyanin concentration in both control and EM wines was observed (Figure 1A). For the 25% ET_c treatment, the levels of anthocyanins were higher in the control wines and a perceptible difference in chromaticity favorable to the control should be expected. Nevertheless, the general feature of comparatively higher formation of both SPP and LPP in the wines of the 25% ET_c treatment (Table 4) may be clouding the relationship between anthocyanin concentration and perceived color. Chromatic differences were also observed within some of the RDI treatments, in particular in the contrast of 25% ET_c against 100% ET_c, but also in the contrast of 25% ET_c against either 25/100% ET_c and/or 70% ET_c.

Flavan-3-ol Concentration and Proanthocyanidin Distribution. Figure 2 presents two representative HPLC chromatograms recorded after the preparative fractionation of the wine extracts on the stationary phase of the Luna HILIC column for control (Figure 2A) and EM wines (Figure 2B). Under hydrophilic interaction chromatography (HILIC) mode, separation of oligomeric and polymeric PAs occurs based on polymer length, with monomeric flavan-3-ols eluting in the early fractions.[39] Here, the preparative isolation of wine PAs was undertaken employing HILIC with a stationary phase specifically selected to increase the retention of high molecular weight PAs. Upon elution, a total of 90 × 15 mL fractions were collected and subsequently submitted to phloroglucinolysis. Monomeric flavan-3-ols were detected in fractions 11, 12, 14, and 15, although they also appeared intermittently in fractions 10, 13, 16, 17, 20, and 21, as previously reported on a similar separation of grape seed PAs using a HILIC stationary phase.[40] In the present work, the presence of monomeric flavan-3-ols was initially detected in the chromatograms as isolated terminal subunits. Further confirmation and quantification was achieved by analyzing the same fractions without acid catalysis.[8] On the basis of relative retention times previously reported under HILIC conditions,[39,40] fractions 11, 12, 14, and 15 were first assigned as mixtures of catechin and epicatechin units. Likewise, fractions 10, 13, 16, 17, 20, and 21 were assigned as mixtures of trace amounts of catechin, epicatechin, and epicatechin-3-*O*-gallate. Additional characterization of these fractions by MS/MS^n confirmed these results. Oligomers with an mDP between 2 and 4 were detected starting in fractions 23 to 26 through fractions 48 to 68. Polymeric PAs (mDP ≥ 5) were detected starting in fractions 48 to 68 up to fraction 90.

The concentration and composition of monomeric flavan-3-ols, and the PA size distribution and composition of the wines grouped as a function of the RDI treatment (Figure 3) and the skin contact treatment (Figure 4), was obtained upon qualitative and quantitative analysis of the fractions after phloroglucinolysis. The results are presented expressing the concentration as catechin equivalents (CE). When the wines were analyzed on the basis of the RDI treatment (Figure 3), the concentration of monomeric flavan-3-ols was on average 288 ± 23 mg/L. Of these monomers, catechin alone represented 57% of the monomer concentration, followed by epicatechin (26%) and epicatechin-3-*O*-gallate (16%). A one-way ANOVA on the monomeric content revealed no differences between treatments ($p = 0.783$). The oligomeric fraction (2 ≤ mDP < 5) represented 53% of the total PA content of the wines, i.e., 739 ± 36 mg/L. No treatment effect was observed ($p = 0.872$). The polymeric fraction (mDP ≥ 5) represented the remaining PAs mass (641 ± 36 mg/L on average), and, again, no treatment effect was observed (p =

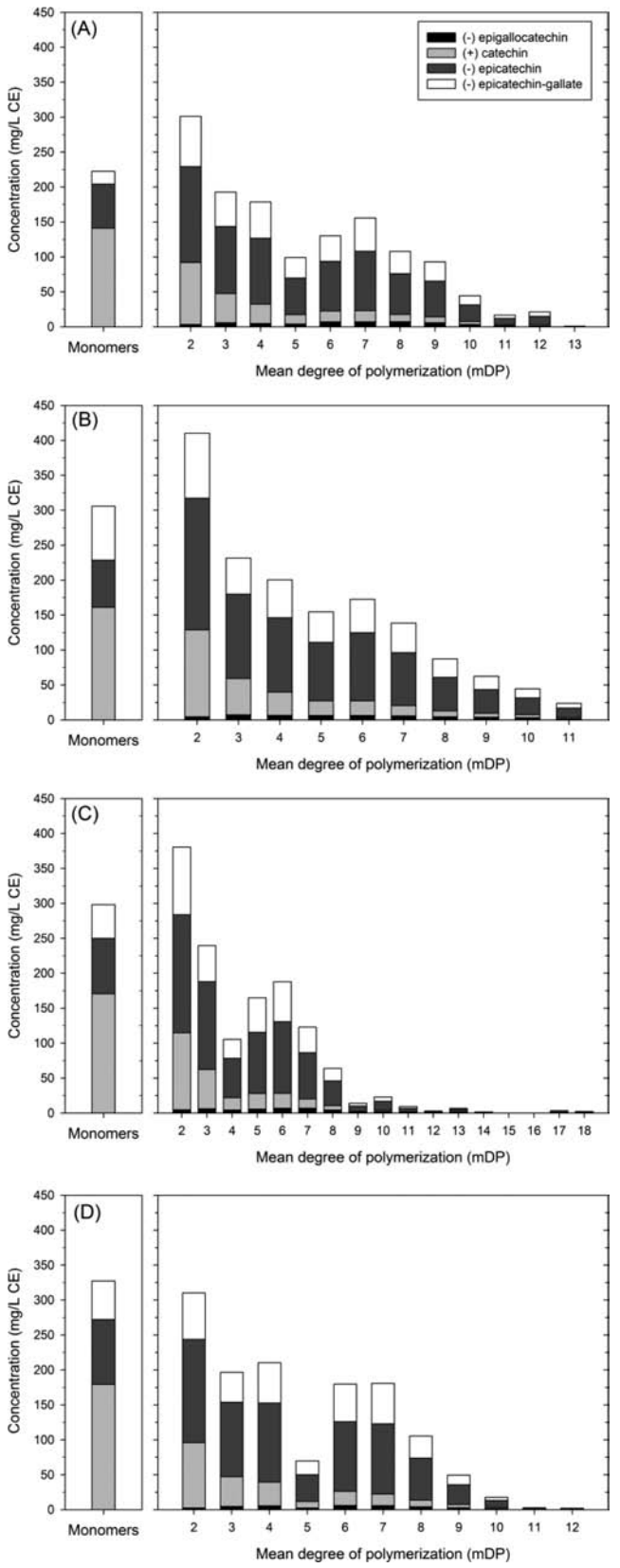

Figure 3. Monomer concentration and composition and proanthocyanidin size distribution by concentration and composition in Cabernet Sauvignon wines grouped as a function of the RDI treatment. (A) 100% ET_{σ}, (B) 70% ET_{σ}, (C) 25/100% ET_{σ}, (D) 25% ET_{c}. CE: catechin equivalents. For the sake of clarity, error bars are not included.

0.971). For both oligomeric and polymeric PAs, epicatechin was the predominant constitutive unit. Epicatechin represented about 45% of the composition of PAs with an mDP of 2 and up to 63% of the composition of PAs with and mDP of 15. The third most abundant constitutive unit, epicatechin-3-*O*-gallate, was evenly distributed within the PA distribution, accounting on average for 29% of the constitutive units. Conversely, the contribution of epigallocatechin to the total PA content increased steadily from 1% on average for mDP 2, up to 22% for mDP 17. As epigallocatechin is proportionally higher in skins but virtually absent in seeds,[8,41] the increased contribution of this subunit alongside with the increase in mDP suggests a concomitant contribution of skin PA for the larger PA material of the distribution. Lastly, when the flavan-3-ol composition at each mDP value was analyzed on a percentage contribution basis as a function of the RDI treatment, no significant effect was uncovered (Supporting Information Table 3). This suggests that the RDI treatment had no quantitative or compositional effect on the PA distribution of the final wines.

The content of flavan-3-ols and the distribution of the wines' PAs as affected by the two skin contact treatments are shown in Figure 4. The concentration of monomeric flavan-3-ols showed a clear treatment effect: for EM wines, monomeric flavan-3-ols were present at a concentration of 347 ± 37 mg/L, whereas in control wines the concentration of monomers was on average 229 ± 22 mg/L. A two-tailed unpaired *t*-test for $p < 0.05$ revealed that these differences were significant ($p = 0.0116$). It has been shown that the release of higher concentration of flavan-3-ol monomers from the seeds occurred only after prolonged skin contact.[42] This supports the results presented here, wherein EM wines had concentrations of catechin and epicatechin of 198 ± 57 and 94 ± 15 mg/L, respectively. The skin contact treatment was also a determining factor on both the concentration of oligomeric and polymeric PA of the wines. EM significantly increased the concentration of both oligomers (799 ± 26 mg/L) and polymers (791 ± 95 mg/L), relative to the control wines, in which these two fractions were present at concentrations of 425 ± 48 mg/L and 488 ± 38 mg/L, respectively ($p < 0.0001$ and $p < 0.0109$, respectively).

Unlike what was observed for the RDI treatments, the skin contact time had certain effect on the subunit composition of the PA distribution. When the flavan-3-ol composition at each mDP value was assessed on a percentage basis as a function of the skin contact treatment, EM wines showed a significantly higher proportion of epicatechin-3-*O*-gallate in fractions with mDP 4 up to mDP 12 (Supporting Information Table 3). The fact that the flavan-3-ols content and the percentage contribution of epicatechin-3-*O*-gallate were significantly higher in EM wines, combined with the observation that the proportion of seed-derived PAs on the wines was on average 73% (Table 3), adds further evidence that the monomeric, oligomeric, and polymeric PA concentration and composition of wines produced under EM conditions is primarily the result of extraction from seeds.

In addition to differences in concentration, the PA distribution was broader in control wines, as it extended up to an mDP of 18 whereas for EM wines it extended up to an mDP of 12 (Figure 4). Previously, the mDP distribution of seeds and skins PAs in Cabernet Sauvignon was found to extend from mDP 2 to mDP 15 and from mDP 4 to 76, respectively.[22] Therefore, in the present study, the shorter PA distribution in EM wines is consistent with a larger contribution of seed PAs. Conversely, the broader mDP distribution in control wines compares favorably

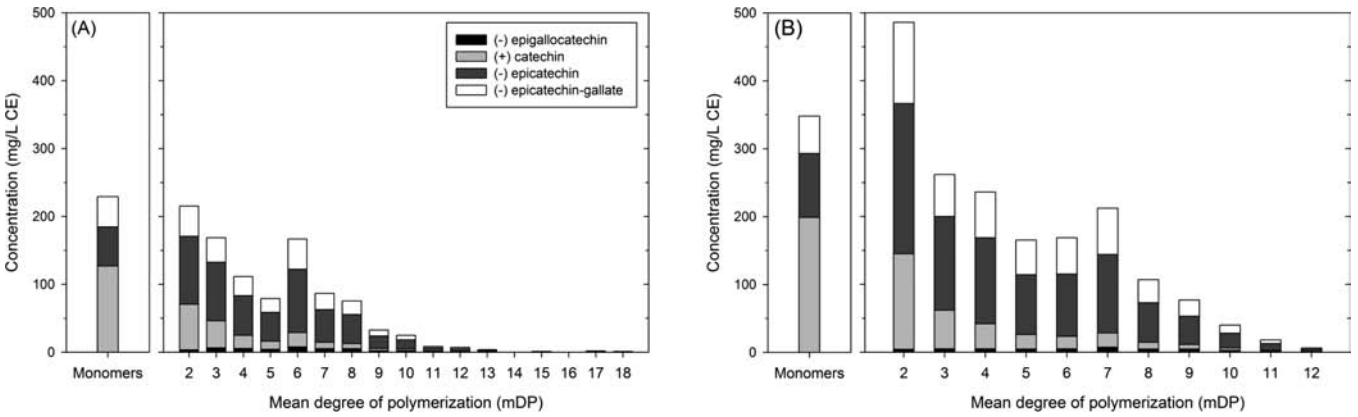

Figure 4. Monomer concentration and composition and proanthocyanidin size distribution by concentration and composition in Cabernet Sauvignon wines grouped as a function of the maceration length treatment. (A) Control wines, (B) extended maceration wines. CE: catechin equivalents. For the sake of clarity, error bars are not included.

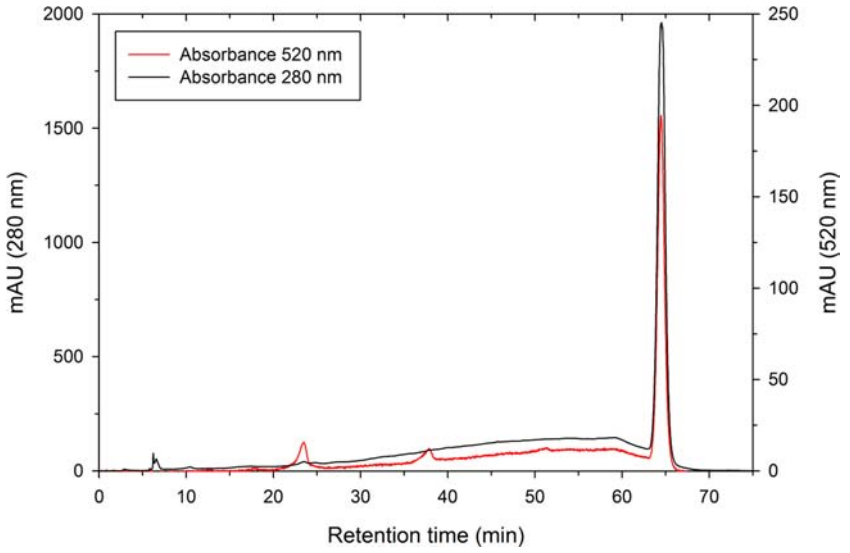

Figure 5. HPLC chromatograms showing absorbance at 280 and 520 nm recorded after injection of previously collected fractions 64−70 (tentatively assigned as polymeric pigments) on a polystyrene divinylbenzene reverse phase column.

with a comparatively higher contribution of skin PAs in these wines, as also revealed by analysis of the pomace (Table 3).

Inspection of the size distribution of the wines' PAs revealed two major peaks as a function of concentration. Proanthocyanidins of mDP 2 and 6 represented 22% (215 ± 28 mg/L) and 17% (166 ± 12 mg/L), respectively, of the total PA distribution in control wines (Figure 4A). For EM wines PAs of mDP 2 and 7 represented 27% (486 ± 42 mg/L) and 12% (212 ± 31 mg/L), respectively, of the total PA distribution (Figure 4B). These results, together with two previous studies, strongly suggest a bimodal distribution of wine PAs.[9,22] Consequently, we attempted to fit a probability distribution to the PA distribution in the individual wines pooled together as a function of the skin contact treatment, which was the determinant factor on defining the concentration of the different PA fractions. The parameters of the distributions with the three best goodness of fit (GOF) as well as the probability density function histograms for the distributions with the highest GOF values are provided as Supporting Information (Supporting Information Table 10 and Figure 2, respectively). Over 55 probability distributions tested, the Rayleigh distribution ranked first as a function of the GOF values for both control and EM wines. For control wines, the normal distribution ranked no. 16 and the GOF was not significant for α values of 0.05, 0.2, and 0.1. For EM wines, the normal distribution ranked no. 14 and once again the GOF parameter was not significant for α values of 0.05, 0.2, and 0.1. The Rayleigh distribution had means of 4.89 and 4.63 for control and EM wines, respectively, and was positively skewed and leptokurtic for both wines. Subsequent analysis of the wines' crude extracts (i.e., without fractionation) revealed an mDP of 4.86 ± 0.07 for control wines and an mDP of 3.78 ± 0.03 for EM wines. By contrasting the means obtained after fitting of the Rayleigh distribution (Supporting Information Table 10) with the mDP values of the crude (i.e., intact) extracts, it is evident that for control wines the deviation from normality was not practically relevant. However, for EM wines, the mDP of the crude extracts was underestimated by the standard phloroglucinolysis procedure.

From the current results and others based on PA depolymerization by acid catalysis,[9,22,25] it seems evident that large PA material (mDP > 20) either derived from seed or skin tissue is poorly or not retained in the wine matrix during maceration. It has been argued that at the solvent conditions encountered during standard winemaking conditions (ethanol

Table 6. Mean Conversion Yield Percentage (± SEM) of Flavan-3-ols Monomers and Proanthocyanidin Fractions in Cabernet Sauvignon Wines after Acid Catalysis in Presence of Phloroglucinol (n = 16)

	percentage conversion yield
flavan-3-ols monomers	14 ± 1
Proanthocyanidins	
mDP 2	20 ± 1
mDP 3	22 ± 1
mDP 4	27 ± 1
mDP 5	29 ± 1
mDP 6	34 ± 2
mDP 7	34 ± 2
mDP 8	37 ± 2
mDP 9	35 ± 3
mDP 10	36 ± 5
mDP 11	34 ± 3
mDP 12	25 ± 6
mDP 13	17 ± 6
mDP 14	11 ± 0
mDP 15	26 ± 0
mDP 17	13 ± 1
mDP 18	15 ± 1

concentrations between 10 and 15% v/v) only small polymers are extracted, while larger polymers remain bound to the cell wall matrix.[43] A series of studies conducted by Bindon and colleagues had shown a significant relationship between the PA molecular mass and the proportion of PA adsorbed by the mesocarp and skin cell wall polysaccharides, the end result being that higher molecular mass PA (>15000 g/mol) are not extractable and/or removed from the wine by interaction with the cell wall components.[44,45]

Nonetheless, the possibility that the analytical procedure in itself is failing to account for the actual polymer(s) size(s) cannot be ruled out. For example, the occurrence of A-type linkages as extension or terminal subunits in wine PAs during the winemaking process may be problematic as this linkage, unlike the more common B-type, remains stable during acid catalysis.[46] Should this kind of linkage occur during wine aging, then it may lead to an artificial variation of the calculated mDP because these units would be either unaccounted or accounted as terminal subunits if coelution with legit terminal subunits occurs.

Analysis of the preparative chromatograms in Figure 2A,B also indicate the presence of anthocyanins as confirmed by the absorbance at 520 nm. The spectral features and retention time of the peak eluting between 64 and 70 min is consistent with pigmented polymers featuring a comparatively higher 280 to 520 nm absorbance ratio compared to that of intact anthocyanins. The polymeric and pigmented nature of this peak was confirmed by two independent methods. After collecting the peak using preparative HPLC chromatography, a known HPLC method for quantification of polymeric pigments was carried out[47] and confirmed that the peak contained polymeric pigments (Figure 5). Furthermore, the fraction was subjected to the same BSA precipitation and bisulfite bleaching method as described in the Materials and Methods section. Upon this analysis, it was found that the fraction precipitated the BSA protein and 71% of its absorbance was due to LPP. The mDP of this polymeric pigmented material varied between 5 and 10 for control wines and between 4 and 10 for EM wines (data not shown).

Analysis of the conversion yield percentages revealed that for monomeric flavan-3-ols, only 14% of the material was recovered after phloroglucinolysis (Table 6). For oligomers, these yields averaged 23%, whereas for polymers the conversion yields averaged 27%. The conversion yields reported in the present study are higher than the ones reported in Cabernet Sauvignon wines following the same analytical methodology, which varied between 11% for PAs with an mDP of 5 to 14% for PAs with an mDP of 15.[22] Notwithstanding, the low conversion yields of fractions with mDP 13, 14, 17, and 18 are consistent with oxidative polymerization reactions during wine aging, leading to new PAs structures that are no longer amenable to acid-catalyzed depolymerization.[11,48] Considering that for these fractions 83−91% of PA material remains uncharacterized, these results should be taken cautiously.

In summary, the present study uncovered a larger effect of the skin contact treatments on most chemical parameters relative to that of the RDI treatments. Although the pattern of extraction of anthocyanins and PAs was unaffected by the RDI treatments, quantitative differences that mirrored those found in the fruit, particularly for anthocyanin chemistry, did occur. Chromatic differences as well as formation of SPP and LPP were favored in the 25% ET_c wines and, to a lesser extent, in the 25/100% ET_c wines. Nevertheless, the application of EM superseded differences in anthocyanins and PA content within the RDI treatments, leading to anthocyanin losses but promoting PA extraction from seeds during extended maceration.

Analysis of PAs distribution by molecular size revealed that PAs in wines follow a bimodal distribution that can be fitted to a Rayleigh distribution. The RDI treatments had no major effect on PA distribution and concentration. Likewise, the qualitative composition, e.g., proportion of catechin, epicatechin, epigallocatechin, and epicatechin-3-*O*-gallate, was relatively unaffected by the RDI treatment. However, the concentration of monomeric, oligomeric, and polymeric flavan-3-ols was higher in EM wines whose PA extraction was mainly derived from seeds. Accordingly, a higher percentage contribution of epigallocatechin-3-*O*-gallate in the PA distribution of EM wines, confirming a predominant contribution of seed PAs in these wines, was observed.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Fermentation and temperatures curves recorded during fermentation/maceration; probability density function histograms for the three distributions with the best goodness of fit; one-way ANOVA analysis of pre- and postvéraison midday stem water potential (Ψ_s) and total irrigation applied, yield components and pruning weights, percentage contribution of flavan-3-ols subunits for monomers and proanthocyanidins of the different mDP values, mean values of basic analysis at bottling, mean values of PA content recovered on the pomace, and estimated proportion of skin and seed PAs extracted, mean values of small polymeric pigments (SPP) and large polymeric pigments (LPP) at days 30, 250, and 400 postcrushing, Cie-Lab color parameters at day 30 postcrushing, Cie-Lab color parameters at day 250 postcrushing, Cie-Lab color parameters at day 400 postcrushing. Summary of the probability density distributions with the best goodness of fit (GOF) and main statistical parameters of each distribution. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +1-509-786-9296. Fax: +1-509-786-9370. E-mail: jfharbertson@wsu.edu.

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Notes

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ABBREVIATIONS USED

C, control; BSA, bovine serum albumin; DAD, diode-array detection; EM, extended maceration; GOF, goodness of fit; MS, mass spectroscopy; PAs, proanthocyanidins; RDI, regulated deficit irrigation

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