



Changes in grape seed polyphenols during fruit ripening

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Abstract

The quantity and characterization of extracted flavan-3-ol monomers and procyanidins was determined in seeds from *Vitis vinifera* cv. Cabernet Sauvignon berries, over the course of ripening and at different levels of vine water status. The per berry extractive yield of all polyphenols decreased with maturity, and followed second-order kinetics. The flavan-3-ol monomers decreased most rapidly, followed by the procyanidin extension units and finally, the terminal units. The relative proportion of procyanidin extension units did not vary with maturity. During fruit ripening, the mean degree of polymerization of extracted procyanidins is unchanged when analyzed intact by HPLC, but decreases by thiolytic degradation. The proportion of extracted procyanidins resistant to acid catalyzed thiolysis increased with maturity. Changes in vine water status affected polyphenol amounts, indicating that cultural practices can be used to influence composition. Oxidation of the seed polyphenols during fruit ripening, could explain these observations. © 2000 Published by Elsevier Science Ltd.

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1. Introduction

The growth of the grape berry is characterized by two successive sigmoid curves, with a plateau in-between corresponding to three stages of development (Coombe, 1973) (Fig. 1). During stage I, characterized by the first period of berry growth, the pericarp and seed cell number increases. During this stage, the seed approaches its full size. During stage II, characterized by little change in berry size, the seed embryo develops with a concomitant hardening of the seed coat (Coombe, 1960). Stage III, characterized by the second period of berry growth, is the ripening stage when sugars rapidly accumulate. The inception of stage III (termed véraison) is characterized by berry softening and anthocyanin accumulation (red varieties). By véraison, much of the seed is fully developed.

The seed plays an important role during the making

of red wine, contributing flavan-3-ol monomers and procyanidins (collectively called seed tannins by wine-makers) to the final product. These compounds are important sensory components, providing red wine with bitterness and astringency (Robichaud and Noble, 1990).

Fruit maturity is important to the overall quality of red wine, yet it is unclear to what extent the seed tannins change during this period. There is evidence that extractable flavan-3-ol monomers (M_r : 290–442) and low molecular weight seed tannins (M_r : <900) decrease during fruit ripening (Czochanska et al., 1979; Romeyer et al., 1986). However, the molecular weight of grape seed tannins can exceed 3000 (Foo and Porter, 1981; Prieur et al., 1994), and no information on molecular weight and compositional changes during this period exists. Some evidences, based on seed weight gain during fruit ripening, indicate that the seed continues to develop during this time (Staudt et al., 1986), and this development could affect seed tannins.

Research on other plant species indicates that significant changes in procyanidin composition occur with

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respect to maturity and cultural practice, and can also influence the ability of procyanidins to act as astringents (Bate-Smith, 1973; Mole et al., 1986; Koupai-Abyazani et al., 1993). To investigate the effect of fruit maturity on grape seed polyphenols, changes in polyphenol amount, composition and molecular weight were determined during fruit ripening.

In addition to fruit maturity, environmental conditions (such as vine water status) have been associated with differences in grape polyphenols. It is clear that vine water status affects fruit growth (Matthews et al., 1987), the concentration of total phenolics (Matthews and Anderson, 1988), and wine sensory attributes (Matthews et al., 1990). Yet, it has not been established whether changes in seed polyphenol composition result from changes in irrigation practice. Therefore, this investigation was also conducted to determine the changes that grape seed polyphenols undergo with respect to grapevine water status.

2. Results and discussion

2.1. Berry development

Three treatments were established to investigate (in addition to maturity) the effect of vine water status on seed polyphenols. For the standard irrigation (SI) treatment, water was applied weekly (8 h \times 4 l/h/vine) beginning 2 weeks before véraison, and continued until 1 October. For the double irrigation (DI) treatment, water was applied at twice the SI rate (8 h \times 8 l/h/vine). For the minimally irrigated (MI) treatment, water was applied (8 h \times 4 l/h/vine) when the midday leaf water potential (Ψ_l), fell below -1.6 MPa, occurring twice during the growing season.

The irrigation treatments established large differ-

ences in vine water status (Fig. 2). Prior to irrigation, Ψ_l for SI vines decreased from ca. -0.85 to -1.2 MPa, where it remained throughout fruit ripening. For DI vines, Ψ_l increased to ca. -0.8 MPa, and remained through most of fruit ripening. For MI vines, Ψ_l declined during fruit ripening, reaching ca. -1.6 MPa at the end of September. Thus, during fruit ripening, Ψ_l for the DI and MI vines were ca. 0.2 – 0.4 MPa greater and lower, respectively, than the SI vines.

Véraison for all treatments occurred on 19 August. Fruit growth in the SI and DI treatments was similar, but was reduced in the MI treatment (Fig. 3A). Final berry mass was 18% less in the MI treatment than the other treatments. In all the treatments, berries attained maximum size approximately 30 days prior to harvest, with no evidence of berry contraction.

Visually, the seeds changed color during fruit ripening from an initial green to a light buff, and finally to a dark brown at harvest. At véraison, seed mass was greatest in the DI and least in the MI treatment (Fig. 3B). Seed growth continued past véraison in all treatments and was essentially complete ca. 40 days prior to harvest. At harvest, there were no treatment differences in seed weight (28.4 mg/berry) or seed number (1.17 seeds/berry). These data indicate that the seed were still developing after véraison. The relationship between seed growth and fruit maturation varies among varieties (Coombe, 1960; Staudt et al., 1986; Peynaud and Ribereau-Gayon, 1971), and could indicate differences (among varieties) in seed polyphenols as well.

Differences in sugar accumulation among treatments were greater early in ripening than later (Fig. 3C). Sugar accumulation started earlier in MI and was greatest in MI on 19 September. However, the rate of increase in $^{\circ}$ Brix (measurement of sugar concentration;

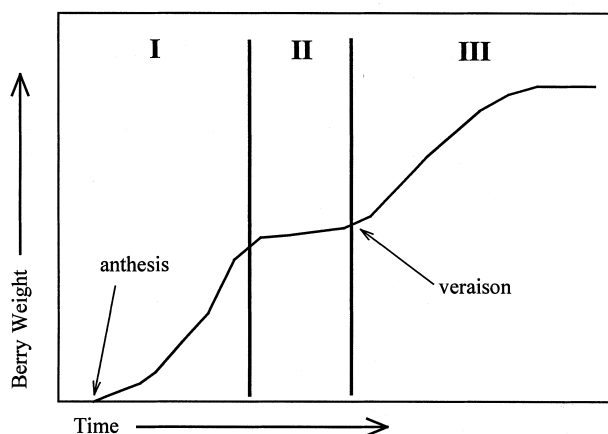


Fig. 1. The development of a grape berry through the growing season follows a double sigmoid ripening pattern, which can be divided into three growth stages.

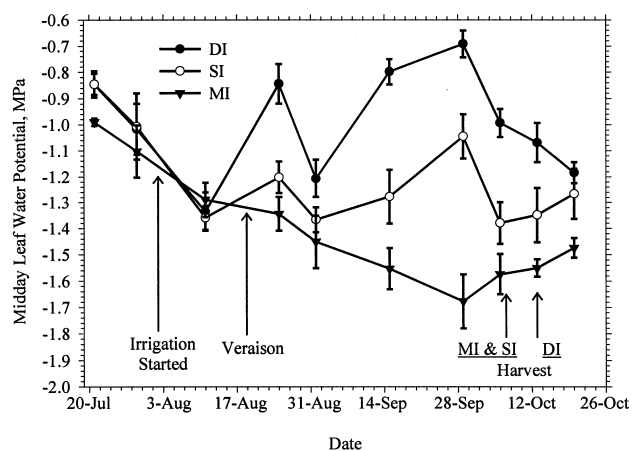


Fig. 2. Midday leaf water potential during fruit ripening, for treatments with double irrigation (\bullet), standard irrigation (\circ), and minimal irrigation (\blacktriangledown), with error bars representing twice the standard error.

equivalent to % w/w sucrose) slowed thereafter, eliminating the difference between MI and SI. °Brix was lower in DI than in other treatments throughout ripening; thus, to the extent that °Brix indicates fruit maturity, maturity was delayed in DI ca. 1 week. The fruit was allowed to ripen for an additional 10–13 days after commercial maturity before collecting the last sample.

2.2. Changes in polyphenols during fruit ripening

2.2.1. Flavan-3-ol monomers

The observed flavan-3-ol monomers included (+)-catechin (C), (–)-epicatechin (eC), and (–)-epicatechin-3-*O*-gallate (eCG), flavan-3-ol monomers being

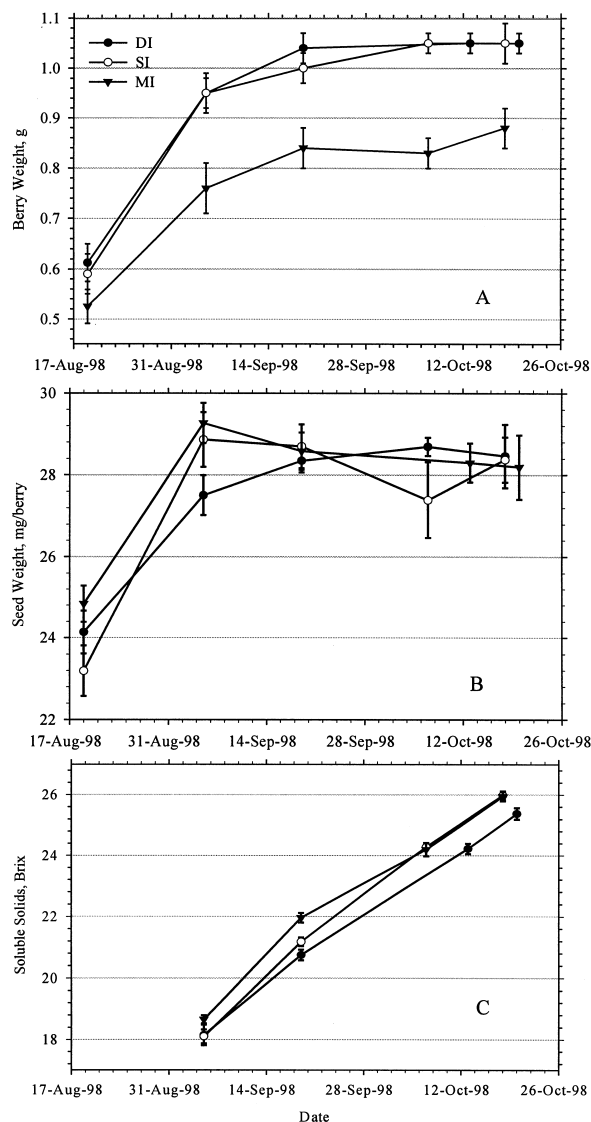


Fig. 3. (A) Berry weight, (B) soluble solids and (C) seed weight during fruit ripening, for treatments with double irrigation (●), standard irrigation (○), and minimal irrigation (▼), with error bars representing twice the standard error.

the major in grape seed (Su and Singleton, 1969). Gallic acid levels remained low throughout the season suggesting that eCG hydrolysis did not contribute to the eC pool (Singleton et al., 1966).

Flavan-3-ol monomers declined dramatically during fruit ripening in all treatments (Fig. 4). For the SI treatment total flavan-3-ol monomers decreased 90% from 1861 nmol/berry at véraison to 180 nmol/berry on the last sample date. The total flavan-3-ol monomers were significantly less in MI than in SI and DI at all sample dates. However, differences were greatest at véraison, suggesting that the lower Ψ_l of MI vines before véraison (Fig. 2) was responsible for the treatment differences in flavan-3-ol monomers. Cultural practices that alter environmental conditions such as light (Dokoozlian and Kliewer, 1996), temperature (Hale and Buttrick, 1975) or water status (Hardie and Considine, 1976; Matthews et al., 1988) prior to véraison can significantly affect grape composition as much or more than post-véraison phenomenon.

Assuming a constant seed compartment volume during ripening, the decline in total flavan-3-ol monomers closely followed second-order kinetics in all treatments despite large changes in metabolism, composition, and environmental conditions such as air temperature (Table 1). The rate constant was increased

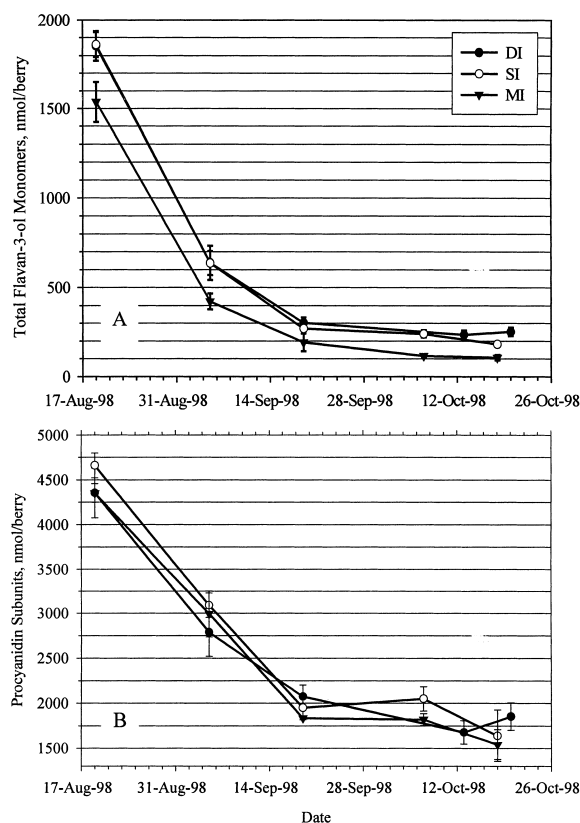


Fig. 4. The total extractable flavan-3-ol monomers (A), and procyanidin subunits (B), for all treatments during fruit ripening.

by water deficits, being 1.3 \times and 2.7 \times greater in the SI and MI treatments, respectively, than in the DI treatment. The rate constants for individual flavan-3-ol monomers were increased by water deficits as well, but less for eCG than that for eC and C. In all the treatments, the rate constant was ca. 25 \times and 1.2 \times greater for eCG and C, respectively, than for eC. Thus, the relative composition of C:eC:eCG changed from 65:27:8 at véraison to 48:51:1 at harvest (Fig. 5A).

2.2.2. Procyanidins

2.2.2.1. Amount and composition. The quantity of extracted procyanidins per berry was determined by acid-catalyzed degradation in the presence of benzyl mercaptan (thiolysis) (Rigaud et al., 1993; Matthews et al., 1997). The quantities of extracted material declined from a high value at véraison (Fig. 4B). For the SI treatment, the amounts declined 65% from 4659 nmol/berry on 19 August (véraison), to 1641 nmol/berry on 18 October. The losses were similar for the DI and MI treatments over the same period of time, declining by 57 and 65%, respectively. Much of the decline in procyanidins (as well as the flavan-3-ol monomers) occurred in the first month after véraison.

As with the flavan-3-ol monomers, the decline in procyanidins followed second-order kinetics, and increased with water deficit, although differences were less apparent (Table 1). There was an increase in rate of 1.3 \times from the DI to the SI and MI treatments.

Procyanidin terminal subunits decreased from 535 nmol/berry at véraison to 273 nmol/berry on the last sample date, for the SI treatment (Fig. 5B). The fractional composition of the individual subunits changed with maturity: eCG decreased (38–18%), C did not change (ca. 43%), and eC increased (17–41%) (Fig. 5B inset), but not in response to water deficits (data not shown). Since procyanidins would be expected to undergo acid-catalyzed degradation in the cell vacuole

Table 1

Second-order rate constants and correlation coefficients (shown in parentheses) for the decrease in flavan-3-ol monomers and procyanidins (per berry) of fruit that was double irrigated (DI), standard irrigated (SI), and minimally irrigated (MI)

	Second-order rate constant, $\text{m}^{-1} \text{s}^{-1}$		
	DI	SI	MI
Flavan-3-ol monomers:			
C	1.49 (0.94)	2.05 (0.96)	4.55 (0.98)
eC	1.24 (0.87)	1.70 (0.93)	3.03 (0.98)
eCG	33.57 (0.96)	50.85 (0.98)	61.58 (0.94)
Total (C + eC + eCG)	0.69 (0.92)	0.96 (0.96)	1.89 (0.98)
Procyanidins:			
	0.06 (0.89)	0.08 (0.90)	0.08 (0.93)

(Hemingway and McGraw, 1983; Davies, 1997), electrophilic extension subunit intermediates and nucleophilic terminal subunits would be formed (Hemingway, 1989). Assuming that flavan-3-ol monomers and procyanidins occupy the same vacuolar space, terminal subunit and flavan-3-ol monomer composition would approach the same value at equilibrium. As predicted the composition of the terminal units approached that of the flavan-3-ol monomers (compare insets of Fig. 5A and B).

As with the flavan-3-ol monomers and the procyanidin terminal subunits, the extractable extension subunits declined with maturity (Fig. 5C). However, while

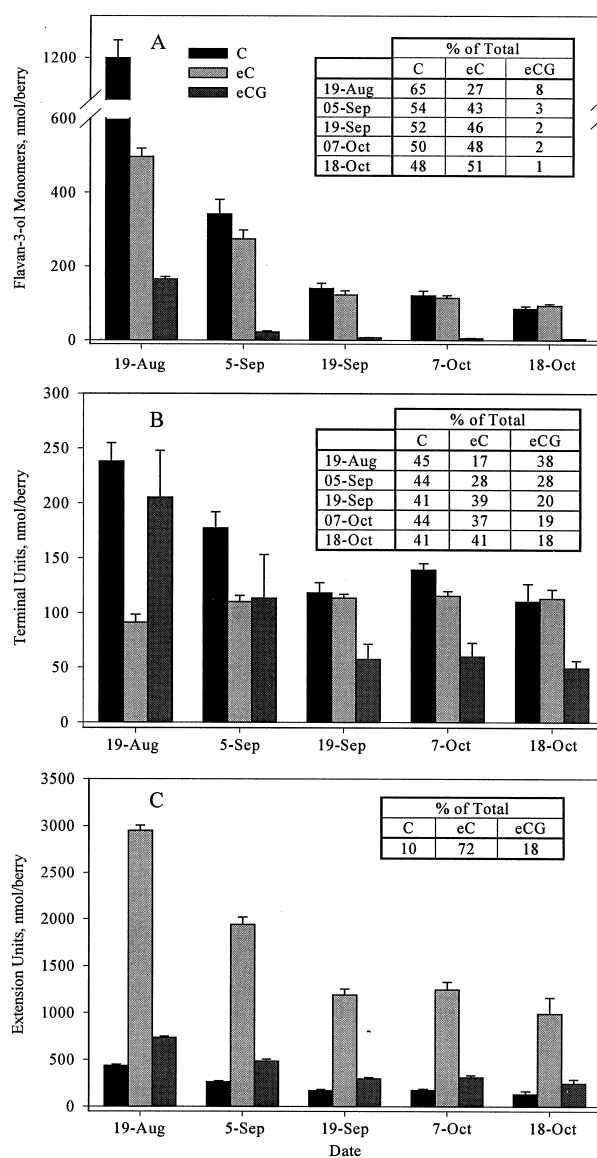


Fig. 5. (A) The flavan-3-ol monomer, (B) procyanidin terminal unit, and (C) procyanidin extension unit composition during fruit ripening, for the SI treatment, with error bars representing the standard error. The percent of total for individual flavan-3-ol monomers and procyanidin subunits is given in tabular form within each graph.

the composition of the flavan-3-ol monomers and of the terminal subunits changed during fruit ripening, the composition of extension subunits did not (Fig. 5C inset). From this, it can be concluded that the terminal subunits can change in the plant cell independent of the extension units (again, consistent with acid-catalyzed degradation); and furthermore, that each of the extension subunits are equally susceptible to events which result in a decrease in their extractable amounts.

The quantity of extracted procyanidins was also determined by normal-phase HPLC, which left the procyanidins intact. For the SI treatment, procyanidins declined during fruit ripening, from an initial total of 2.42 to 1.12 mg/berry on the last day of sampling (eC eq.), a 54% reduction (Fig. 6A). This decline is similar to the decline in thiolysis products (ca. 65%, Fig. 4B). With the exception of procyanidins containing over 30 subunits, the procyanidins declined during fruit ripening. Procyanidins containing over 30 subunits remained essentially constant (ca. 200 $\mu\text{g}/\text{berry}$). For the procyanidins that declined, those containing 6–10 subunits declined the least during fruit ripening

(53.5%), followed by those containing 2–5 and 11–15 subunits (60.4 and 62.9% decline, respectively). Procyanidins containing 16–20 and 21–25 subunits declined the most (71.6 and 73.6% decline, respectively).

Proportionally, the distribution of procyanidins changed during fruit ripening. Procyanidins containing 10 or fewer subunits changed very little (ca. 17% for 2–5 subunits and 24% for 6–10 subunits, Fig. 6B). Procyanidins containing 11–25 subunits declined (ca. 47–35%), those containing 25–30 subunits changed very little (ca. 3.5%), and those containing more than 30 subunits increased significantly from 9 to 21%.

Given the abundance of flavan-3-ol monomers and low molecular weight procyanidins (2–15 subunits) during fruit ripening (ca. 2–3 molar equivalents, Fig. 7), and under conditions of acid-catalyzed degradation (*vide supra*), the procyanidin molecular weight should decline with a concomitant decline in flavan-3-ol monomers. While there was a *proportional* decline in flavan-3-ol monomers and procyanidins containing 16–30 subunits during fruit ripening (Figs. 6B and 7), the proportion of procyanidins containing over 30 subunits increased during the same period. These observations are inconsistent with predictions involving the polyphenols alone.

Acid-catalyzed degradation of procyanidins involving additional, much higher molecular weight nucleophiles, could explain the observations. These nucleophiles could be protein or carbohydrate in nature, the products of which would likely become insoluble.

2.2.2.2. Estimated mean degree of polymerization. The estimated procyanidin mean degree of polymerization (mDP) was determined in two ways. First, polymeriz-

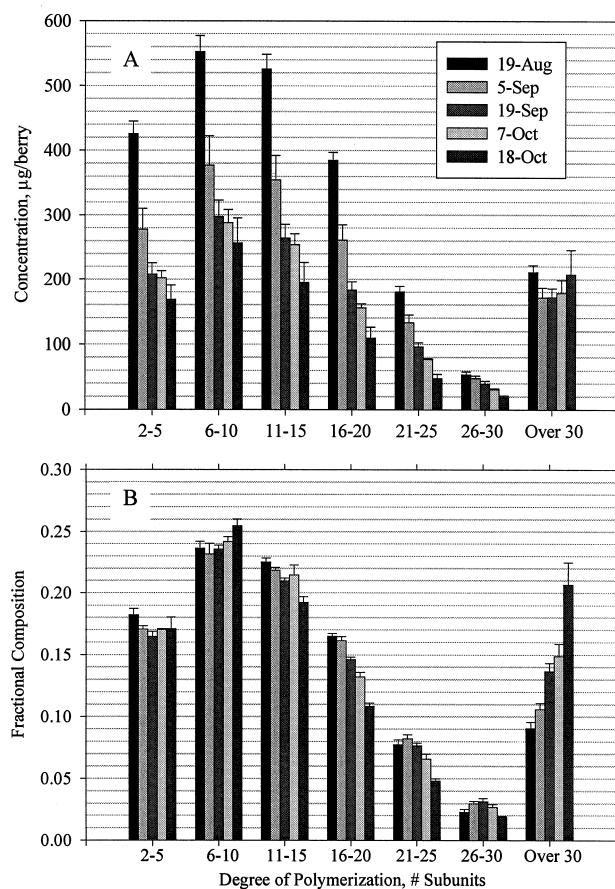


Fig. 6. The quantity of extracted procyanidins (A), determined by normal-phase HPLC and categorized by degree of polymerization; and the relative proportion of each category (B) for the SI treatment during fruit ripening, with error bars representing the standard error.

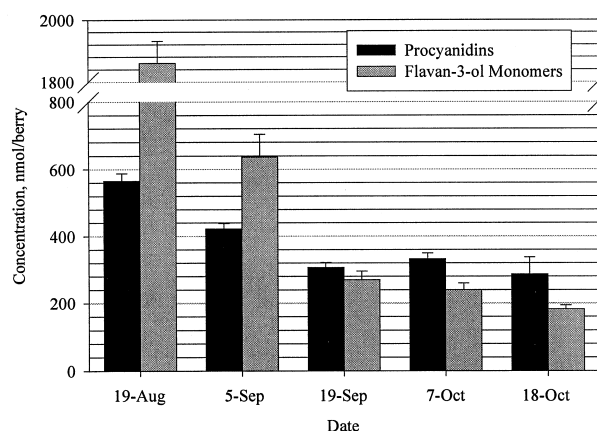


Fig. 7. The moles per berry of extracted flavan-3-ol monomers and procyanidins (estimated) for the SI treatment during fruit ripening, with error bars representing the standard error. Procyanidin concentrations were determined by dividing the total extension unit concentration by the mean degree of polymerization (mDP-thiolysis).

ation was studied by normal-phase HPLC, which separates the procyanidins by size. Gallation increases size and retention time in this method, but the extent of gallation cannot be measured. However, relative measures are valid as long as subunit gallation does not change and the subunit composition of extension units did not change during maturation, while terminal units, a small proportion of total mass, did change as noted above. From the molecular weight distribution of the procyanidins, the predicted mDP is between 11 and 15 subunits throughout fruit ripening, although there was a proportional increase in procyanidins containing more than 30 subunits (Fig. 6B).

Secondly, the estimated mDP for the procyanidins was determined by thiolysis. The results indicate that the mDP decreased with respect to grape maturity (Fig. 8). At véraison, the mDP was 8.29 subunits, declining to 5.63 at the end of the study. This observation is inconsistent with the conclusions by normal-phase HPLC (Fig. 6B).

One explanation for this inconsistency is that covalent adducts not susceptible to thiolysis are being formed during fruit ripening. If the extension and terminal units are equally susceptible to adduct formation, observed mDP would decrease during fruit ripening. At véraison, for example, the mDP was 8.29 indicating that for every terminal unit there are 7.29 extension units, and the probability of an extension unit being involved in a reaction would be greater. Each subunit involved in such a reaction would not appear as an expected thiolysis product and thus would be lost in the tabulation of extension and terminal units. While a reduced mDP would be observed by thiolysis, these reactions would lead to a higher mDP by normal-phase HPLC since the reaction products would be increasing in molecular size.

Also, if this hypothesis is valid, and combined with acid-catalyzed degradation, an asymmetrical molecular

weight distribution would result. This is consistent with the results by normal-phase HPLC (Fig. 6B)

2.2.2.3. Susceptibility to thiolysis. If the above hypothesis is valid, extracted procyanidins should become less susceptible to thiolysis during fruit ripening. To determine the extent of procyanidin thiolysis, the total concentration of the products determined by thiolysis (in eC equivalents) was compared to the total concentration determined by normal-phase HPLC (Fig. 6A) to arrive at a fractional yield. For the SI treatment, and at véraison, 78% of the procyanidins were converted to known thiolysis degradation products, decreasing to 57% in the final sample (Fig. 9). Upon close inspection of chromatograms, many small peaks were observed that increased in size relative to known thiolysis products during fruit ripening. These results were similar for all treatments (data not shown). This revealed a troubling aspect of thiolysis, or more accurately, its inability to fully explain proanthocyanidin structure.

3. Conclusion

Grape seed polyphenols decrease dramatically during ripening with a 90% decrease in flavan-3-ol monomers and a 60% decrease in procyanidins. The results described above, specifically:

1. changing seed coat color
2. decreasing flavan-3-ol monomers and procyanidins
3. decreasing flavan-3-ol monomers in the order $eCG \gg C > eC$
4. inconsistency between the procyanidin mDP when analyzed intact and the procyanidin mDP when determined by thiolysis.
5. decreasing thiolytic yield
6. second-order decline in polyphenols

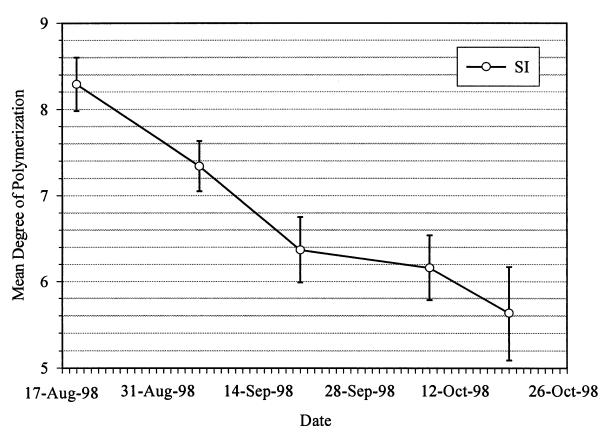


Fig. 8. Mean degree of polymerization during fruit ripening, for the SI treatment, with error bars representing twice the standard error.

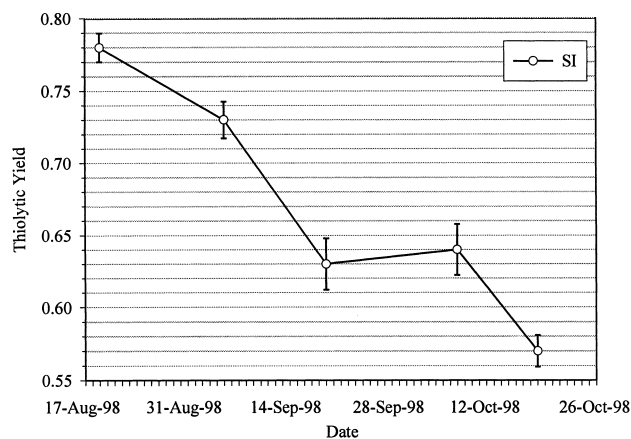


Fig. 9. Thiolytic yield during fruit ripening, for the SI treatment, with error bars representing twice the standard error.

are consistent with an oxidative process. Seed coat development processes in other plant species which are related to seed longevity, are also consistent with an oxidative process (Marbach and Mayer, 1974; Werker et al., 1979; Halloin, 1982). Direct evidence for oxidation, however, needs to be acquired.

The products of oxidation would likely be polyphenols cross-linked with other polyphenols, carbohydrates, or proteins; and it would be expected that the phenolic functional groups would become modified. Given the importance of phenolic functional groups in procyanidin/protein interactions (Hagerman et al., 1998), it would be expected that their sensory properties (i.e.: astringency) would be modified as well. Given the importance of astringency in red wine, it is important to chemically elucidate the mechanism whereby seed polyphenols change during fruit ripening.

Seed growth and polyphenols per berry were less sensitive to water deficits than berry growth and sugar accumulation. There were no significant effects of water deficits on procyanidins or their polymerization. However, water deficits decreased the amounts of flavan-3-ol monomers and greatly increased their rate of loss during fruit ripening.

4. Experimental

4.1. Grape cultivation

Treatments were imposed in a commercial, drip irrigated vineyard [*Vitis vinifera* cv. Cabernet Sauvignon (UC Davis, FPMS clone #8) grafted onto 110 Richter rootstock] planted in 1989 on the valley floor in the Napa Valley, near Oakville, California, USA (latitude: 38°27' N; longitude: 122°25' W; elevation: 65 m). Vines were planted 1 m within the rows and 1.8 m between rows, and trained to bilateral cordons with shoots trained vertically. Fertilization, pest control and other vineyard operations were consistent with accepted commercial vineyard practices.

The three irrigation treatments were established in a randomized block design, replicated five times, with each replicate consisting of 18 vines, and with two buffer rows separating treatments. Vine water status was monitored by measuring the midday leaf water potential of fully mature, sun-exposed, excised leaves, with a pressure chamber as described by Matthews et al. (1987).

4.2. Chemicals

With the exception of HCO₂H (AR grade), extraction and chromatography solvents were HPLC grade. (+)-Catechin and (–)-epicatechin were obtained from

Sigma. (–)-Epicatechin-3-*O*-gallate was isolated from aq. Me₂CO extracts of grape seeds collected at véraison. Benzyl mercaptan was obtained from Aldrich. Thioether standards were prepared as previously described (Matthews et al., 1997), and standard spectra (UV, mass, ¹H NMR) were consistent with previous data (Prieur et al., 1994; Matthews et al., 1997).

4.3. Sample preparation

Beginning at véraison, 300 berry samples were collected randomly, keeping the peduncles attached to minimize oxidation. Five samples were collected at ca. 10-day intervals. The fourth sample was collected when grapes were commercially mature (ca. 24 °Brix). Samples were kept at 4°C until processed.

To process samples, peduncles were removed from berries, and separated into two equal samples. To one sample, berries were counted, weighed, and their volume was determined by measuring H₂O displacement in a graduated cylinder. The seeds were removed from the berries, rinsed well with H₂O, their appearance noted, then placed into a 250 ml Erlenmeyer flask. One milliliter of 66% v/v aq. Me₂CO solution was added for every gram fresh weight. Flasks were covered with septa, sparged with N₂, and placed on an orbital shaker for 24 h at 20°C at 100 RPM. After extraction, solutions were filtered with Whatman #1 filters, then evaporated under reduced pressure to remove Me₂CO. The aq. solutions were brought to 50 ml with distilled–deionized H₂O and then stored at –20°C until analyzed. Extracted seeds were dried in a vacuum oven, and then counted and weighed.

For the second sample, berries were crushed and their sugar concentration was measured using a hand-held refractometer. The sample was then discarded.

To prepare samples for chromatography, aq. extracts were first thawed. For flavan-3-ol monomer analysis, the extract was filtered through 0.45 µm PTFE filters into HPLC vials, and then analyzed.

For the analysis of procyanidins, it was necessary to first change the solvent to methanol. Three milliliter of the aq. extract was placed in a 15 ml centrifuge tube, followed by 3 ml of a 0.1 M caffeine solution to precipitate the majority of the procyanidins. The contents were centrifuged briefly, and the supernatant was applied to an equilibrated solid-phase extraction (SPE) column filled with 1 g of C₁₈ solid support (18% carbon load). The ppt. was resuspended with H₂O, centrifuged, and the supernatant applied to the SPE column. The column was rinsed with 12 ml H₂O and the adsorbed phenolics were eluted with 18 ml MeOH into a 100 ml round bottom flask. The ppt. was dissolved in a minimum amount of MeOH and also transferred to the 100 ml round bottom flask. The MeOH was evaporated under reduced pressure at 37°C, and the

residue was dissolved in 3 ml MeOH, followed by filtration through 0.45 μm PTFE filters. For the analysis of intact procyanidins, MeOH extracts were transferred to HPLC vials and analyzed by normal-phase HPLC.

To determine the composition of procyanidins, it was necessary to thiolize them. Samples were prepared as follows: 900 μl of the MeOH/procyanidin solution was placed into a 1.8 ml HPLC vial. To this was added 900 μl of an 0.08 M HCl solution in MeOH, containing 12% v/v benzyl mercaptan. The HPLC vial was capped and placed in a 55°C H₂O bath for 7 min. The thiolized material was cooled and analyzed by reversed-phase HPLC within 12 h. This method of preparation is similar to a previously developed method (Rigaud et al., 1991).

4.4. HPLC analysis

The reversed-phase HPLC method used to analyze flavan-3-ol monomers has been previously described (Lamuela-Raventos and Waterhouse, 1994). Identification was made with authentic standards.

Except for the absence of ion-pair reagent, the HPLC method used to analyze intact procyanidins was similar to a previously developed method (Kennedy and Waterhouse, 2000). Eluting peaks were monitored at 280 nm. Quantitation was made using an eC standard. Molecular weights were approximated by comparing retention times of grape seed polyphenols with an equation generated from a procyanidin extract isolated from cacao beans. A plot of the retention time for individual procyanidin oligomers isolated from cacao beans, versus their subunit length, followed a natural logarithmic equation. This equation was then used to approximate the subunit length of polyphenols isolated from grape seeds.

Thiolized procyanidins were analyzed by reversed-phase HPLC. The column was a LiChrospher RP-18 (particle size 5 μm , 250 \times 4 mm i.d.) purchased from EM Science (Gibbstown, NJ), protected by a guard column (10 \times 4 mm) containing the same material. Prior to injection, the MeOH in samples was reduced with H₂O to 20% v/v MeOH to minimize peak tailing. The method utilizes a binary gradient with mobile phases containing: (A) 1 ml 85% H₃PO₄ in 1 l H₂O and (B) MeOH. Elution conditions were as follows: flow rate is 1 ml/min; linear gradients are from 20 to 70% B for 35 min, 90% B for 5 min and reequilibration with 20% B for 5 min. Eluting peaks were monitored at 280 nm. The flavan-3-ol monomers consisted of monomer flavan-3-ols and procyanidin terminal subunits. Terminal subunits were determined by subtracting flavan-3-ol monomers determined prior to thiolysis.

Terminal and extension subunit identities were deter-

mined by comparison with authentic thiolysis product standards. An eC standard was used to quantify subunits. For eCG and its corresponding thioether, the calculated amounts were adjusted downward (by a factor of 4.01) to compensate for their higher molar absorptivities.

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