Dynamics of anthocyanin and flavonol profiles in the 'Crimson Seedless' grape berry skin during development and ripening

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Abstract

'Crimson Seedless' grapes (*Vitis vinifera* L.) do not develop adequate berry colour in different parts of the world including Australia and USA leading to serious economic losses to the growers. In the present study, various anthocyanins and flavonols were identified in the skin of the 'Crimson Seedless' grape berries using LC/PDA/ESI-MS and their changes in the berry skin during development and ripening of 'Crimson Seedless' grape berries were investigated during 2005-06 and 2006-07. Eleven anthocyanins and two flavonols were identified in the berry skin using LC/PDA/ESI-MS. Of the anthocyanins identified, four anthocyanins including cyanidin 3-*O*-(6″-*O*-acetyl)-glucoside, peonidin 3-*O*-(6″-*O*-acetyl)-glucoside, malvidin 3-*O*-(6″-*O*-acetyl)-glucoside and malvidin 3-*O*-(6″-*O*-coumaroyl)-glucoside were not reported earlier. During both the years, the concentration of the 3-*O*-glucosides of delphinidin, petunidin, peonidin, and malvidin as well as the acetyl and coumaroyl esters of the 3-*O*-glucosides of cyanidin, peonidin, and malvidin in the berry skin increased during berry development and ripening. During 2006-07, the concentration of cyanidin 3-*O*-glucoside in the berry skin increased during the early stages of berry ripening and subsequently declined till harvest while in 2005-06, the concentration increased during the initial phase of berry ripening and remained relatively stable thereafter till harvest. The concentration of total anthocyanins in the berry skin was higher during 2006-07 as compared to 2005-06. During both years, the concentration of quercetin 3-*O*-glucoside in the berry skin increased during berry development and ripening while the concentration of quercetin 3-*O*-glucuronide in the berry skin decreased during the same period. To the best of our knowledge, this is the first report on the evolution of different anthocyanins and flavonols in the 'Crimson Seedless' berry skin during berry development and ripening.

Key words: Anthocyanins; Flavonol; *Vitis vinifera*; HPLC and Berry growth

1. Introduction

Berry skin colour is an important quality parameter in table grapes (Mizuno et al., 2006). In the USA, poor berry colour development in 'Crimson Seedless' grapes causes serious economic losses and leads to 30 per cent or more of the fruit being unharvested (Dokoozlian et al., 1995). Additionally, in Western Australia, this cultivar does not develop desirable crimson red colour when grown in areas where high or low night temperatures prevail during berry development and ripening (Cameron, 2001).

Flavonoids are a group of polyphenolic compounds including anthocyanins, flavonols and flavan-3-ols (Montealegre et al., 2006), that are largely responsible for the development of colour in grape berries (Kanellis and Roubelakis-Angelakis, 1993). Although anthocyanins are the main compounds responsible for the colour development in the red grapes (Gao and Cahoon, 1995; Nunez et al., 2004) flavonols increase the colour by stabilizing the coloured form of the anthocyanin molecule through co-pigmentation (Boulton, 2001).

The accumulation of anthocyanins in the berry skin occurs in three stages; beginning with an initial slow anthocyanin accumulation which is followed by a rapid increase, ending in a stabilisation stage before a decline at the end of ripening (Gholami, 2004; Mateus et al., 2002). Flavonol concentrations in grapes were found to be highest at flowering followed by a decrease between flowering and berry set, and then they remained constant throughout berry development (Downey et al., 2003).

The red cultivars of grape (*V. vinifera* L.) contain 3-*O*-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin along with the glucoside esters of acetic, coumaric and caffeic acid (Regules et al., 2006). Diglucosides are distinctively

absent in the skin of berries of vinifera grapes (Mazza, 1995). The amount of anthocyanins present in the skin of the grape berries depends on the cultivar, maturity stage and seasonal conditions, production area, and cultural practices (Mazza and Miniati, 1993). The anthocyanin profiles of various *V. vinifera* cultivars have been reported such as 'Tempranillo' (Hebrero et al., 1988), 'Tannat' (Neves et al., 2004), 'Graciano' (Nunez et al., 2004), 'Cabernet Sauvignon' and 'Pinot noir' (Wulf and Nagel, 1978), 'Flame Seedless', 'Emperor' and 'Red Globe' (Carreno et al., 1997), and 'Crimson Seedless' (Cantos et al., 2002). Further, the evolution of different anthocyanins during the ripening of the grape berries has been reported in a number of cultivars including 'Cabernet Sauvignon' (Regules et al., 2006; Ryan and Revilla, 2003), 'Merlot' (Regules et al., 2006), 'Syrah' (Regules et al., 2006; Roggero et al., 1986), 'Monastrell' (Fernandez-Lopez et al., 1992; Regules et al., 2006), 'Tempranillo' (Cacho et al., 1992; Ryan and Revilla, 2003), 'Touriga Nacional' and 'Touriga Francesa' (Mateus et al., 2002), 'Reliance' (*Vitis vinifera* L. x *V. labrusca* L.) (Gao and Cahoon, 1995), 'Moristel and Garnacha' (Cacho et al., 1992) using High Performance Liquid Chromatography (HPLC). However, no information is available in the literature regarding the accumulation of different anthocyanins in the skin of Crimson Seedless grape berries during development and ripening.

The 3-*O*-glucosides of kaempferol, quercetin and myricetin are the major flavonols present in red grapes and of these, quercetin 3-*O*-glucoside and quercetin 3-*O*-glucuronide are dominant (Cheynier and Rigaud, 1986). Flavonol composition in ripe berries has been reported in the cultivars 'Cinsault' (Cheynier and Rigaud, 1986) and 'Crimson Seedless' (Cantos et al., 2002) and their evolution during berry development has been reported only in 'Shiraz' and 'Chardonnay cultivars' (Downey et al., 2003). To the best of our knowledge, no research has been reported on the evolution of flavonols in 'Crimson Seedless' grape berries during development and ripening.

Cantos et al. (2002) reported the presence of 3-*O*-monoglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin as the major anthocyanins and quercetin 3-*O*-glucoside and quercetin 3-*O*-glucuronide as the main flavonols in the skin of the 'Crimson Seedless' grape berries at harvest. However, the presence of acetyl ester of the monoglucosides in the grape berry skin has not been yet reported. As the *V. vinifera* cultivars are known to contain acetyl derivatives along with the 3-*O*-monoglucosides and their coumaroyl esters, a further research is needed to fully characterize the anthocyanin profile in the skin of 'Crimson Seedless' berries. It is envisaged that the comprehensive knowledge of accumulation of individual anthocyanins in the berry skin is essential to the understanding of colour development (Gao and Cahoon, 1995). These observations prompted to investigate the anthocyanin profile and the patterns of accumulation of individual anthocyanins and flavonols in the skin of 'Crimson Seedless' berries during berry development and ripening.

2. Materials and methods

2.1. Plant materials

Seven years old 'Crimson Seedless' (*V. vinifera* L.) vines grafted on Ramsey rootstock growing in the commercial vineyard located in Swan valley (latitude -31°51′; longitude 115°59′), Western Australia were used for the experiment during 2005-06 and 2006-07. The soil was classified as Cruse sand loam. The grapevines were spaced 3.3m between the rows and 2.7m within the vines and directed east- west. The vines were pruned in early September and gibberellic acid (GA_3) at the concentration of 1 mg L^{-1} was applied when bunches were 40-80 % in flower. All the shoots were tipped 20 and 60 days after flowering. Bunch size was adjusted to 80-100 berries per bunch at fruit set and crop load

was adjusted after fruit set to 35-40 bunches per vine. Grapes were harvested when sugar: acid ratio exceeded 30:1 and bunches had acceptable crimson red colour.

2.2. Sampling

One hundred berries were randomly collected from bunches of whole grapevine at 10- day intervals commencing from one week before veraison till ripening. On the same day the berries were peeled manually and the skin was stored at -20 $^{\circ}$C for later analysis of total anthocyanins and individual phenolics. Single grapevine was treated as an experimental unit and replicated four and three times during 2005-06 and 2006-07 respectively.

2.3. Berry weight

The sampled berries from each replication were weighed and average berry weight was calculated on each sampling date commencing from one week before veraison till harvest.

2.4. Total anthocyanins

Berry skin anthocyanins were extracted in 100 mL aqueous methanol (95 %)/ concentrated HCl (97:3 v/v) by sonicating for an hour. The extract was centrifuged for 10 minutes at 11180 g at 4 $^{\circ}$C and anthocyanin absorbance was determined from the supernatant solution at 520 nm using a UV/vis spectrophotometer (model 6405; Jenway spectrophotometer, Dunmow, Essex, UK). Total anthocyanin concentration was determined using the molecular weight of 449.2 g mol^{-1} and molar extinction coefficient of 23900 L cm^{-1} mol^{-1} for cyanidin-3-*O*-glucoside (Serrano et al., 2006).

146 *2.5. Individual Phenolics*

147 *2.5.1 Extraction of phenolic compounds*

148 Phenolics were extracted from the berry skin (1.0 to 2.0 g ground in mortar and
149 pestle in the presence of 300 mg acid washed white quartz sand (-50 + 70 mesh, Sigma-
150 Aldrich, Castle Hill, NSW, Australia)) using 10 mL solution of methanol/formic acid (97:3
151 v/v). The extracts were centrifuged at 5000 g for 5 min, filtered through 0.45 µm membrane
152 filters (Fluoropore™ Membrane filters, Millipore, Ireland) and analyzed by High
153 Performance Liquid Chromatography (HPLC).

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155 *2.5.2. Identification of anthocyanins and flavonols*

156 High Performance Liquid chromatography /photodiode array, electrospray mass
157 spectrometry (LC/PDA/ESI-MS): Analysis was performed on a ThermoFinnigan LCQ
158 Deca XPplus instrument equipped with an ESI probe and surveyor LC pumps, autosampler
159 and photodiode array detector (PDA). Chromatography was performed using a
160 Phenomenex Hydro Synergi column (4 µ, 150 mm x 2.0 mm) with 5% formic acid in
161 MilliQ water (solvent A) and 5% formic acid, 15% water and 80% acetonitrile (solvent B)
162 as eluting solvents at a flow rate of 0.18 mL/min. The gradient program started at 10%B
163 proceeding to 35% (35 min), 60% (60 min), held at 60% (60 min), 10% (61.01 min) and
164 10% (70 min). The injection volume was 20 µL. Photodiode array detection was set at 350
165 and 520 nm. MS and MS/MS analysis was performed in negative ion mode with the
166 instrument parameters set as sheath gas flow 84 (arbitrary units), auxillary gas flow 20
167 (arbitrary units), spray voltage 5 (V), capillary temperature 300 (ºC), capillary voltage -15
168 (V), tube lens offset-30 (V), multipole 1 offset 5 (V), lens voltage 16 (V), multipole 2 offset
169 7 (V), multipole RF amplitude 400 (V), entrance lens 60 (V).

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2.5.3. HPLC quantification

HPLC analysis was performed using a modification involving a Symmetry® C_{18} column (3.9 x 150 mm i.d. 5 µm) and column temperature of 30°C of the method previously described by Cantos et al. (2002). Briefly, 20 µl extract obtained as above was injected into the Waters HPLC system (Waters 1525 Binary HPLC pump fitted to Waters 2487 Dual Wavelength Absorbance Detector and Waters 717 plus Autosampler; Waters Corp., Milford, Mass., USA). Separation was achieved on Symmetry® C_{18} column (3.9 x 150 mm i.d. 5µm) proceeded by Symmetry C_{18} guard column of the same stationary phase. Both the column and the guard column were kept at 30 °C during analysis. The mobile phase consisted of HPLC grade methanol (solvent B) and 5 % (v/v) formic acid (solvent A) at a flow rate of 1 mL/minute. The gradient was according to the following programme: linear gradient from 2% B to 32% in 30 min, from 32 % to 40 % in 10 min, from 40 % to 95 % in 10 min, isocratic at 95 % B for 5 min, from 95 % B to 2 % B in 5 min, and then isocratic for 10 minutes. Anthocyanidin 3-monoglucosides were quantified at 520 nm against there respective external standards, acylated anthocyanidin 3-monoglucosides were quantified at 520 nm as malvidin 3-glucoside equivalents and flavonols at 350 nm as quercetin 3-glucoside equivalents.

2.6. Chemicals

The standards used for HPLC including cyanidin 3-glucoside and peonidin 3-glucoside (Polyphenols Laboratories, Sandnes, Norway), delphinidin 3-glucoside, petunidin 3-glucoside, malvidin 3-glucoside chloride and quercetin 3-glucoside (Apin chemicals Ltd, Oxon, UK), Sand, white quartz (Sigma, Castle Hill, NSW, Australia). All the solvents used for HPLC analysis of phenolic compounds were of HPLC grade including methanol

(Mallinckrodt chemicals, Phillipsburg, USA) and formic acid (Merck, Darmstadt, Germany).

2.7. Statistical analysis

The data were subjected to analysis of variance (ANOVA) using Genstat 9 release (Lawes Agricultural Trust, Rothamsted Experimental Station, UK) from each year. Experimental data were analysed by using one way ANOVA. The least significant differences (Fisher's LSD) were calculated following significant F test ($P \leq 0.05$). All the assumptions of analysis were checked to ensure the validity of statistical analysis. The data of two years were not pooled because error mean squares over years were heterogeneous.

3. Results

3.1. Berry growth

With the onset of berry ripening, which corresponds to day 60 after full bloom in both the years, an increase in berry weight was observed. The berry weight increased from 3.40 g berry^{-1} at veraison to 6.80 g berry^{-1} at approximately 40 days after veraison representing 100 % increase before ending at 6.46 g berry^{-1} at harvest, during the year 2005 -06 (Fig. 2). During 2006 – 07, the berry weight increased gradually from 3.72 g berry^{-1} at veraison to 6.79 g berry^{-1} at approximately 30 days after veraison which represented an increment of 82.52 % before finishing at 7.04 g berry^{-1} at harvest. During 2006-07, the berry weight increased during the early phase of the berry ripening and remained relatively constant near the harvest while in 2005-06, the berry weight decreased slightly at harvest. The trends in changes of berry weight during development and ripening were similar in both years (Fig. 2).

3.2. Changes in total anthocyanins

Total anthocyanins in the berry skin increased at the onset of veraison, reached a peak level and remained comparatively stable till harvest (Fig. 3). The total anthocyanin levels in the berry skin varied during both years of experiment. During 2005-06 and 2006-07, total anthocyanins gradually increased from 339 µg g^{-1} to 1157 and 211 µg g^{-1} to 2359, respectively. At harvest, the concentration of total anthocyanins in the grape berry skin was two- fold higher in 2006-07 than in 2005-06.

3.3. Identification of anthocyanins and flavonols

Typical LC chromatograms obtained with photodiode array detection at 520 and 350 nm are shown in Fig. 1A and 1B. MS and MS-MS analysis confirmed the identity of the anthocyanins, and flavonols associated with the peaks appearing on the LC chromatograms. Table 1 shows the molecular ions and the fragment ions detected and associated with each peak. The anthocyanidin 3-*O*-monoglucosides including delphinidin 3-*O*-glucoside (Dp3glc), cyanidin 3-*O*-glucoside (Cn3glc), petunidin 3-*O*-glucoside (Pt3glc), peonidin 3-*O*-glucoside (Pn3glc) and malvidin 3-*O*-glucoside (Mv3glc) were identified on the basis of retention times by comparison with authentic standards, and confirmed using LC/PDA/ESI-MS. The identity of the acylated forms of these monoglucosides namely cyanidin 3-*O*-(6″-*O*-acetyl)-glucoside (Cn3Acglc), peonidin 3-*O*-(6″-*O*-acetyl)-glucoside (Pn3Acglc), malvidin 3-*O*-(6″-*O*-acetyl)-glucoside (Mv3Acglc), cyanidin 3-*O*-(6″-*O*-coumaroyl)-glucoside (Cn3Cmglc), peonidin 3-*O*-(6″-*O*-coumaroyl)-glucoside (Pn3Cmglc), and malvidin 3-*O*-(6″-*O*-coumaroyl)-glucoside (Mv3Cmglc) was confirmed using LC/PDA/ESI-MS. Delphinidin 3-*O*-(6″-*O*-acetyl)-glucoside, delphinidin 3-*O*-(6″-*O*-coumaroyl)-glucoside and peonidin 3-*O*-(6″-*O*-caffeoyl)-glucoside were

suspected to be present in small quantities but the levels were too low for absolute confirmation. Eleven different anthocyanins including the monoglucosides and their acylated forms were identified in the Crimson Seedless grape skin extract but only nine of these were detected in quantifiable levels.

The two flavonols detected, identified and quantified from the berry skin were the 3-*O*-glucoside and 3-*O*-glucuronide of Quercetin (Table 1).

3.4. Evolution of anthocyanidin 3-O-monoglucosides

The evolution of Dp3glc, Pt3glc, Pn3glc, and Mv3glc in the grape berry skin was similar in both the years increasing throughout grape berry development and ripening whereas the changes in Cn3glc during berry ripening differed in both the years (Fig. 4). During 2005-06, the concentration of Cn3glc in the berry skin increased during the early phase of the berry ripening and subsequently remained relatively stable until harvest. However, during 2006-07, the concentration of Cn3glc in the berry skin increased during the early stages of berry ripening, reached a peak level and decreased thereafter till harvest.

3.5. Evolution of acylated anthocyanins

The concentrations of acyl derivates of the monoglucosides in the grape berry skin increased from veraison till ripening (Fig. 5). At harvest, the concentrations of the acylated anthocyanins in the berry skin were higher in 2006-07 than 2005-06.

3.6. Evolution of flavonols

The trends in accumulation of quercetin 3-*O*-glucoside (Q3glc) in the grape berry skin were similar between the two years (Fig. 6). During both the years, the concentration of Q3glc in the berry skin increased from veraison till approximately 30 days after

veraison, followed by a decrease prior to an increase at harvest. However, at harvest, the level of Q3glc was higher in the year 2005-06 than in 2006-07. In both the years, the concentrations of quercetin 3-*O*-glucuronide (Q3glr) decreased during grape berry ripening prior to stabilization near the harvest (Fig. 6). At harvest, similar to Q3glc, the concentration of Q3glr in the berry skin was higher in 2005-06 than 2006-07.

4. Discussion

4.1. Berry growth

As expected, the berry weight increased during the berry development and ripening phase during both the years but in 2005-06 the berry weight decreased slightly at harvest. Similarly increased berry weight during development and ripening has been reported in the literature (Dreier et al., 1998; Esteban et al., 1999; Rogiers et al., 2006) and some reports describe a decrease in berry weight close to the harvest (McCarthy, 1997; McCarthy, 1999). The rapid increase in berry weight during the post-veraison stage may be ascribed to the pericarp cell enlargement and the influx of solutes, particularly sugars and water, into the grape berry mainly through the phloem (Coombe, 1992; Creasy et al., 1993; Dreier et al., 1998; Harries et al., 1968; Mullins et al., 1992; Rogiers et al., 2000; Winkler et al., 1974). Berry weight stabilization or a slight reduction in the weight at the later stages of berry ripening or at harvest may be attributed to the slow influx of solutes into the berry through the phloem as reported (Rogiers et al., 2006).

4.2. Total anthocyanins

The accumulation of total anthocyanins substantially increased in the skin of the ‘Crimson Seedless’ grape berries during the ripening period. The concentrations of

anthocyanins in the skin of 'Crimson Seedless' berries increased from veraison till approximately 30 days after veraison, and remained relatively stable thereafter till harvest. Similarly, Pirie and Mullins (1980), reported that anthocyanins levels in the skin of Shiraz berries increased throughout berry ripening and became relatively stable near the harvest. However, Boss et al. (1996), Jeong et al. (2004) and Esteban et al. (2001) reported an increase in the anthocyanin accumulation in the skin of grape berries from veraison till harvest while Somers (1976) found a decrease in the anthocyanin concentration near the harvest. The variation in the concentration of total anthocyanins in the skin of the 'Crimson Seedless' berries between both years may be ascribed to the seasonal conditions particularly temperature during berry growth and development. Similarly Cacho et al. (1992) reported that the weather conditions heavily influence the anthocyanin content of the grape cultivars.

4.3. Anthocyanins identified

In the present study, eleven anthocyanins including the 3-*O*-monoglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin as well as the acetyl and coumaroyl esters of Cn3glc, Pn3glc and Mv3glc were identified in the skin of the Crimson Seedless grape berries (Figures 1a, 3, 4 and Table 1). Previously seven anthocyanins have been reported in the skin of Crimson Seedless berries (Cantos et al., 2002). Our experimental data indicate the presence of four more anthocyanins in the skin of Crimson Seedless berries namely Cn3Acglc, Pn3Acglc, Mv3Acglc and Mv3Cmglc (Figures 1a, 4 and Table 1). The presence of anthocyanidin 3-*O*-monoglucosides along with their acyl derivatives in the berry skin have been demonstrated in different grape cultivars including Tempranillo, Garnacha and Cabernet Sauvignon (Arozarena et al., 2002), Shiraz (Boss et al., 1996), Cabernet Sauvignon, Merlot, Syrah and Monastrell (Regules et al., 2006), and Cabernet Sauvignon

(Wulf and Nagel, 1978). On the other hand, Pinot noir completely lacks acylated anthocyanins (Fong et al., 1974; Wulf and Nagel, 1978).

4.4. Anthocyanin profile

In agreement with the findings of Cantos et al., (2002), Pn3glc was the major anthocyanin found in 'Crimson Seedless' berries which is contrary to the general perception that Mv3glc is the main anthocyanin of the grapes (Fernandez-Lopez et al., 1992; Roggero et al., 1986). Cantos et al. (2002) reported that the concentration of Mv3glc and Dp3glc was higher as compared to Cn3glc and Pt3glc in the berry skin, respectively. Our experimental data indicate that, at harvest, the concentrations of Pt3glc in the berry skin were higher as compared to Dp3glc while the concentrations of Cn3glc and Mv3glc in the berry skin varied between both years. During 2006-07, the concentration of Mv3glc in the berry skin was higher than Cn3glc, similar to the results of Cantos et al. (2002) but the concentrations of these two anthocyanins in the berry skin were similar in 2005-06. It has been suggested that the anthocyanin profile of berry skin may be directly related to the intensity of colour as evident from the higher concentrations of the anthocyanins which are produced at the end of the biosynthetic pathway namely Pn3glc, Mv3glc and Pt3glc, these being predominant in the higher pigmented cultivars (Carreno et al., 1997; Fernandez-Lopez et al., 1998). The concentration of Mv3glc was higher in the berry skin than Cn3glc during 2006-07 which was more highly pigmented than 2005-06. It has been reported that the cyanidin and peonidin levels are higher in red grape cultivars whereas delphinidin and malvidin levels are higher in black grapes [cited in Mizuno et al., (2006)]. Our data indicate that the colour of grape berries is related to the relative concentration of Cn3glc and Mv3glc in the grape berry skin. In the biosynthetic pathway it is known that cyanidin is a precursor to the other anthocyanidins and is converted into peonidin by the action of 3′-*O*-

methyltransferase or into delphinidin by the action of 3′-hydroxylase. Delphinidin is further converted via petunidin to malvidin by the action of 3′5′-*O*-methyltransferase (Pomar et al., 2005). In low pigmented cultivars, it has been hypothesized that there is a restricted activity of 5′-hydroxylase which results into lower concentration of trisubstituted anthocyanins (Fernandez-Lopez et al., 1998). As the activity of the enzymes depends on the temperature, possibly the temperature differences between the years may be a cause of variation in the relative concentrations of Cn3glc and Mv3glc in the berry skin of 'Crimson Seedless' grapes (Figure 6). We have found that the concentrations of 3-*O*-monoglucosides in the 'Crimson Seedless' berry skin were much higher as compared to their acyl derivatives. Similarly, a higher abundance of 3-*O*-monoglucosides in the total anthocyanin in the grape berry skin as compared to their esters has also been reported (Boss et al., 1996; Pomar et al., 2005; Regules et al., 2006).

4.5. Evolution of anthocyanidin 3-monoglucosides

The evolution of Pt3glc, Pn3glc, and Mv3glc was similar during both the years, increasing throughout berry ripening. Similar trends in the concentrations of these anthocyanins in berry skin during ripening in various grape cultivars have also been reported (Boss et al., 1996; Cacho et al., 1992; Esteban et al., 2001; Fernandez-Lopez et al., 1992).

During 2006-07, the concentration of Cn3glc in the berry skin increased for a few weeks after veraison and subsequently decreased till harvest. Likewise, Roggero et al. (1986), Cacho et al. (1992) and Esteban et al. (2001) reported similar trends and found an increase in the concentration of Cn3glc during the early stages of berry ripening followed by a gradual decline in the berry skin of different grape cultivars. The decrease in Cn3glc

367 may be due to its continued transformation into more stable pigments including Pn3glc and
368 Mv3glc and marks the end of anthocyanin biosynthesis in the grape berry skin (Roggero et
369 al., 1986). Nevertheless, Fernandez-Lopez et al. (1992) reported a drop in the concentration
370 of Cn3glc during the berry ripening before peaking at harvest. We did not find a similar
371 drop in the concentration of Cn3glc during berry ripening in 2005-06 which may be
372 ascribed to the variation in climatic condition prevailing during the experimental years
373 (Figure 6).

374 During 2006-07, the concentration of Dp3glc in the berry skin increased during the
375 ripening phase. Likewise, continuous increase in the concentration of Dp3glc in the berry
376 skin during berry ripening has been reported (Esteban et al., 2001; Fernandez-Lopez et al.,
377 1992). However, Roggero et al. (1986) reported that the concentration of Dp3glc in the
378 grape berry increased during the early stages of berry ripening reached a maximum value
379 and subsequently decreased till harvest due to its conversion into Pt3glc and Mv3glc by the
380 action of methyl transferases.

381 Figure 3 shows a large increase in most anthocyanins from days 20 to 30 in 2006-
382 07. This phenomenon may possibly be ascribed to the lower temperature that prevailed
383 during that period of berry maturation and ripening (Figure 6). It is generally known that
384 grape berries develop more colour under cooler temperatures (Winkler et al. 1974).

385 *4.6. Individual anthocyanins in the skin from veraison to ripening*

386 At the onset of ripening, the concentrations of Pn3glc and Cn3glc in the berry skin
387 were higher as compared to the other anthocyanins including Mv3glc. As the ripening
388 progressed, the concentration of Mv3glc increased and became similar or even higher than
389 Cn3glc. Possibly, at the initiation of berry colour development, the activity of flavonoid 3′,

5′-hydroxylase is much lower as compared to flavonoid 3′-hydroxylase which resulted in the partial blockage of the anthocyanin biosynthetic pathway leading to the formation of trisubstituted anthocyanins. With the advancement of the ripening, the activity of this enzyme may have increased resulting in higher concentration of trisubstituted anthocyanins particularly Mv3glc as compared to Cn3glc in the berry skin. Nevertheless, all the anthocyanins appeared in the grape berry skin at the commencement of veraison and has also been reported in other *V. vinifera* cultivars (cited in Gonzales-SanJose et al., (1990)).

4.7. Evolution of acylated anthocyanins

The combined concentration of Pn3Cmglc and Mv3Cmglc in the berry skin increased throughout ripening during both the years. During both years, the concentration of Cn3Cmglc and Pn3Acglc in the berry skin increased from veraison till harvest. Earlier, Fernandez-Lopez et al. (1992) and Boss et al. (1996) reported an increase in the concentrations of these acylated anthocyanins in the berry skin throughout berry ripening in different grape cultivars.

In conclusion, eleven different anthocyanins and two flavonols were identified using LC/PDA/ESI-MS in the skin of the ‘Crimson Seedless’ grape berries. Peonidin 3-*O*-glucoside was the major anthocyanin present in the skin of the grape berries. During both the years, the concentration of all anthocyanins increased in the berry skin during berry development and ripening. The concentration of Q3glc in the berry skin increased from veraison till harvest. However, the concentration of Q3glr in the berry skin declined during berry development and ripening.

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References

Arozarena, I., Ayestaran, B., Cantalejo, M.J., Navarro, M., Vera, M., Abril, I.,Casp, A., 2002. Anthocyanin composition of Tempranillo, Garnacha and Cabernet Sauvignon grapes from high- and low-quality vineyards over two years. Europ. Food Res. Tech. 214, 303-309.

Boss, P.K.,Davies, C.,Robinson, S.P., 1996. Analysis of the expression of anthocyanin pathway genes in developing *V. vinifera* L. cv Shiraz grape berries and the implications for pathway regulation. Plant Physiol. 111, 1059-1066.

Boulton, R., 2001. The copigmentation of anthocyanins and its role in the color of red wine: A critical review. Am. J. Enol. Vitic. 52, 67-87.

Cacho, J.,Fernandez, P.,Ferreira, V.,Castells, J.E., 1992. Evolution of five anthocyanidin-3-glucosides in the skin of the Tempranillo, Moristel and Garnacha grape varieties and influence of climatological variables. Am. J. Enol. Vitic. 43, 244-248.

Cameron, I., 2001. Crimson Seedless promise WA table grape boon. Journal of Agriculture. 42, 1-5.

Cantos, E.,Espin, J.C.,Barberan, F.A.T., 2002. Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC - DAD - MS - MS. J. Agric. Food Chem. 50, 5691-5696.

437 Carreno, J.,Almela, L.,Martinez, A.,Lopez, J.A.F., 1997. Chemotaxonomical classification
438 of red table grapes based on anthocyanin profile and external colour. Food Sci.
439 Tech. 30, 259-265.
440 Cheynier, V.,Rigaud, J., 1986. HPLC separation and characterization of flavonols in the
441 skins of *Vitis vinifera* var.Cinsault. Am. J. Enol. Vitic. 37, 248-252.
442 Coombe, B.G., 1992. Research on the development and ripening of the grape berry. Am. J.
443 Enol. Vitic. 43, 101-110.
444 Creasy, G.L.,Price, S.F.,Lombard, P.B., 1993. Evidence for xylem discontinuity in Pinot
445 noir and Merlot grapes: dye uptake and mineral composition during berry
446 maturation. Am. J. Enol. Vitic. 44, 187-192.
447 Dokoozlian , N.,Luvisi, D.,Moriyama , M.,Schrader , P., 1995. Cultural practices improve
448 color, size of 'Crimson Seedless '. Cal. Agric. 49, 36-40.
449 Downey, M.O.,Harvey, J.S.,Robinson, S.P., 2003. Synthesis of flavonols and expression of
450 flavonol synthase genes in the developing grape berries of Shiraz and Chardonnay
451 (*Vitis vinifera* L.). Aust. J. Grape Wine Res. 9, 110-121.
452 Dreier, L.P.,Hunter, J.J.,Ruffner, H.P., 1998. Invertase activity, grape berry development
453 and cell compartmentation. Plant Physiol. Biochem. 36, 865-872.
454 Esteban, M.A.,Villanueva, M.J.,Lissarrague, J.R., 1999. Effect of irrigation on changes in
455 berry composition of Tempranillo during maturation. Sugars, organic acids, and
456 mineral elements. Am. J. Enol. Vitic. 50, 418-434.
457 Esteban, M.A.,Villanueva, M.J.,Lissarrague, J.R., 2001. Effect of irrigation on changes in
458 the anthocyanin composition of the skin of cv Tempranillo (*Vitis vinifera* L.) grape
459 berries during ripening. J. Sci. Food Agric. 81, 409-420.

460 Fernandez-Lopez, J.A.,Almela, L.,Munoz, J.A.,Hidalgo, V.,Carreno, J., 1998. Dependence
461 between colour and individual anthocyanin content in ripening grapes. Food Res.
462 Int. 31, 667-672.
463 Fernandez-Lopez, J.A.,Hidalgo, V.,Almela, L.,Roca, J.M.L., 1992. Quantitative changes in
464 anthocyanin pigments of *Vitis vinifera* cv Monastrell during maturation. J. Sci. Food
465 Agric. 58, 153-155.
466 Fong, R.A.,Webb, A.D.,Kepner, R.E., 1974. Acylated anthocyanins in a hybrid Vitis
467 variety. Phytochem. 13, 1001-1004.
468 Gao, Y.,Cahoon, G.A., 1995. High performance liquid chromatographic analysis of
469 anthocyanins in the Red Seedless table grape Reliance. Am. J. Enol. Vitic. 46, 339-
470 345.
471 Gholami, M., 2004. Biosynthesis of anthocyanins in Shiraz grape berries. Acta Hortic. 640,
472 353-360.
473 Gonzales-SanJose, M.L.,Barron, L.J.R.,Diez, C., 1990. Evolution of anthocyanins during
474 maturation of Tempranillo grape variety (*Vitis vinifera*) using polynomial regression
475 models. J. Sci. Food Agric. 51, 337-343.
476 Harries, J.M.,Kriedemann, P.E.,Possingham, J.V., 1968. Anatomical aspects of grape berry
477 development. Vitis. 7, 106-119.
478 Hebrero, E.,Buelga, C.S.,Gonzalo, J.C.R., 1988. High performance liquid chromatography-
479 Diode array spectroscopy identification of anthocyanins of *Vitis vinifera* variety
480 Tempranillo. Am. J. Enol. Vitic. 39, 227-233.
481 Jeong, S.T.,Goto-Yamamoto, N.,Kobayashi, S.,Esaka, M., 2004. Effects of plant hormones
482 and shading on the accumulation of anthocyanins and the expression of anthocyanin
483 biosynthetic genes in grape berry skins. Plant Sci. 167, 247-252.

484 Kanellis, A.K.,Roubelakis-Angelakis, K.A. 1993. Grape, p. 189-234. In: G. Seymour, J.
485 Taylor, and G. Tucker (eds.). Biochemistry of Fruit Ripening. Chapman & Hall,
486 London.
487 Mateus, N.,Machado, J.M.,De, F.V., 2002. Development changes of anthocyanins in *Vitis*
488 *vinifera* grapes grown in the Douro Valley and concentration in respective wines. J.
489 Sci. Food Agric. 82, 1689-1695.
490 Mazza, G., 1995. Anthocaynins in grapes and grape products. Cri. Rev. Food. Sci. Nut. 35,
491 341-371.
492 Mazza, G.,Miniati, E. 1993. Grapes, p. 149 -199. Anthocyanins in Fruits, Vegetables, and
493 Grains. CRC Press, Inc.
494 McCarthy, M.G., 1997. The effect of transient water deficit on berry development of cv.
495 Shiraz (*Vitis vinifera* L.). Aust. J. Grape Wine Res. 3, 102-108.
496 McCarthy, M.G., 1999. Weight loss from ripening berries of Shiraz grapevines (*Vitis*
497 *vinifera* L. cv. Shiraz). Aust. J. Grape Wine Res. 5, 10-16.
498 Mizuno, H.,Hirano, K.,Okamoto, G., 2006. Effect of anthocyanin composition in grape skin
499 on anthocyanic vacuolar inclusion development and skin coloration. Vitis. 45, 173-
500 177.
501 Montealegre, R.R.,Peces, R.R.,Vozmediano, J.L.C.,Gascuena, J.M.,Romero, E.G., 2006.
502 Phenolic compounds in skins and seeds of ten grape *Vitis vinifera* varieties grown in
503 a warm climate. J. Food Comp. Analy. 19, 687-693.
504 Mullins, M.G.,Bouquet, A.,Williams, L.E. 1992. Biology of the grapevine. Cambridge
505 University Press, Cambridge, Great Britain.
506 Neves, G.G., Barreiro, L., Gil, G., Franco, J., Ferrer, M., Moutounet, M.,Carbonneau, A.,
507 2004. Anthocyanic composition of Tannat grapes from the south region of Uruguay.
508 Analy. Chim. Acta. 513, 197-202.

509 Nunez, V.,Monagas, M.,Cordoves, M.C.G.,Bartolome, B., 2004. *Vitis vinifera* L.cv.
510 Graciano grapes characterized by its anthocyanin profile. Posthar. Biol. Tech. 31,
511 69-79.
512 Pirie, A.J.G.,Mullins, M.G., 1980. Concentration of phenolics in the skin of grape berries
513 during fruit development and ripening. Am. J. Enol. Vitic. 31, 34-36.
514 Pomar, F.,Novo, M.,Masa, A., 2005. Varietal differences among the anthocyanin profiles of
515 50 red table grape cultivars studied by High performance liquid chromatography. J.
516 Chrom. A. 1094, 34- 41.
517 Regules, A.O.,Cascales, I.R.,Roca, J.M.L.,Garcia, J.M.R.,Plaza, E.G., 2006. Anthocyanin
518 fingerprint of grapes: environmental and genetic variations. J. Sci. Food Agric. 86,
519 1460-1467.
520 Roggero, J.P.,Coen, S.,Ragonnet, B., 1986. High performance liquid chromatography
521 survey on changes in pigment content in ripening grapes of Syrah. An approach to
522 anthocyanin metabolism. Am. J. Enol. Vitic. 37, 77-83.
523 Rogiers, S.Y.,Greer, D.H.,Hatfield, J.M.,Orchard, B.A.,Keller, M., 2006. Solute transport
524 into shiraz berries during development and late-ripening shrinkage. Am. J. Enol.
525 Vitic. 57, 73-80.
526 Rogiers, S.Y.,Keller, M.,Holzapfel, B.P.,Virgona, J.M., 2000. Accumulation of potassium
527 and calcium by ripening berries on field vines of *Vitis vinifera* (L) cv. Shiraz. Aust.
528 J. Grape Wine Res. 6, 240-243.
529 Ryan, J.-M.,Revilla, E., 2003. Anthocyanin composition of Cabernet Sauvignon and
530 Tempranillo grapes at different stages of ripening. J. Agric. Food Chem. 51, 3372-
531 3378.

Serrano, M., Valverde, J.M., Guillen, F., Castillo, S., Romero, D.M.,Valero, D., 2006. Use of Aloe vera gel coating preserves the functional properties of Table grapes. J. Agric. Food Chem. 54, 3882-3886.

Somers, T.C., 1976. Pigment development during the ripening of the grape. Vitis. 14, 269-277.

Winkler, A.J.,Cook, J.A.,Kliewer, W.M.,Lider, L.A. 1974. General Viticulture. University of California Press, London, United Kingdom.

Wulf, L.W.,Nagel, C.W., 1978. High pressure liquid chromatographic separation of anthocyanins of *Vitis vinifera*. Am. J. Enol. Vitic. 29, 42-49.

CAPTION TO FIGURES

Figure 1: HPLC- DAD chromatograms of skin extracts of 'Crimson Seedless' grapes; A: Anthocyanins at 520 nm; B: Flavonols at 350 nm. Peak 1: Delphinidin 3-*O*-glucoside; peak 2: Cyanidin 3-*O*-glucoside; peak 3: Petunidin 3-*O*-glucoside; peak 4: Peonidin 3-*O*-glucoside; peak 5: Malvidin 3-*O*-glucoside; peak 6: Peonidin 3-*O*- (6″-*O*- acetyl)-glucoside; peak 7: Cyanidin 3-*O*-(6″-*O*- coumaroyl)-glucoside; peak 8: Peonidin 3-*O*- (6″-*O*- coumaroyl)-glucoside; peak 9: Malvidin 3-*O*-(6″-*O*- coumaroyl)-glucoside; peak 10: Quercetin 3-*O*-glucuronide; peak 11: Quercetin 3-*O*-glucoside

Figure 2: Changes in berry weight during berry development and ripening of 'Crimson Seedless' grapes; n = 3 replicates and 4 replicates during 2006-07 and 2005-06, respectively. Vertical bars represent standard error of means. LSD ($P \leq 0.05$): (2006-07) = 0.49; (2005-06) = 0.81

Figure 3: Changes in the levels of anthocyanins in the skin of 'Crimson Seedless' grape berries during berry development and ripening. n = 3 replicates and 4 replicates during 2006-07 and 2005-06, respectively. Vertical bars represent standard error of means. LSD ($P \leq 0.05$): Total anthocyanins: (2006-07) = 1377.2; (2005-06) = 463.9; Pn3glc: (2006-07) = 861; (2005-06) = 419.8; Cn3glc: (2006-07) = 182.9; (2005-06) = 57.57; Mv3glc: (2006-07) = 139; (2005-06) = 59.70; Pt3glc: (2006-07) = 25.98; (2005-06) = 10.25; Dp3glc: (2006-07) = 21.06; (2005-06) = 7.26.

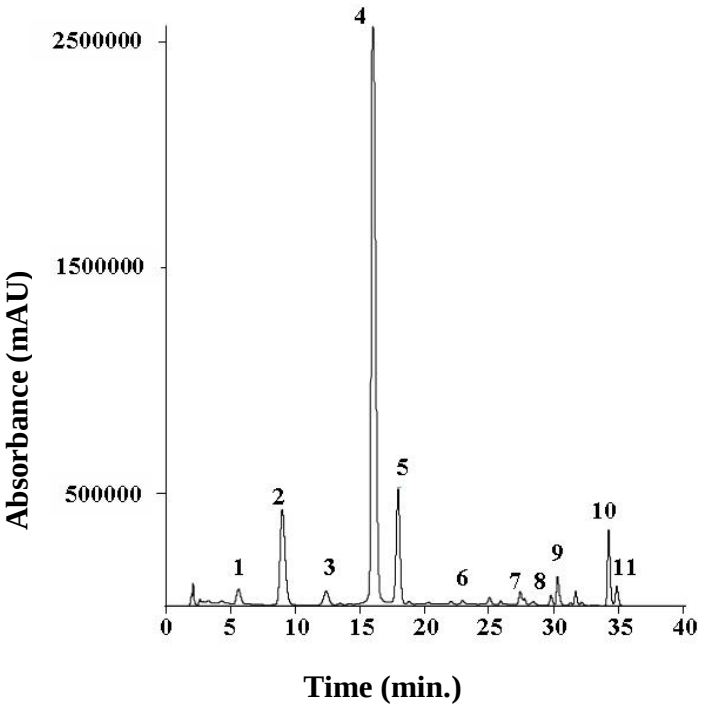
Figure 4: Changes in the levels of acylated anthocyanins in the skin of 'Crimson Seedless' grape berries during berry development and ripening; n = 3 replicates and 4 replicates during 2006-07 and 2005-06, respectively. Vertical bars represent standard error of means. LSD ($P \leq 0.05$): Pn3Cmglc + Mv3Cmglc: (2006-07) = 39.01; (2005-06) = 23.29; Cn3Cmglc: (2006-07) = 5.97; (2005-06) = 5.03; Pn3Acglc: (2006-07) = 6.29; (2005-06) = 3.14

Figure 5: Changes in the levels of flavonols in the skin of 'Crimson Seedless' grape berries during berry development and ripening; n = 3 replicates and 4 replicates during 2006-07 and 2005-06, respectively. Vertical bars represent standard error of means, LSD ($P \leq 0.05$): Q3glc: (2006-07) = 28.48; (2005-06) = 46.09; Q3glr: (2006-07) = 21.63; (2005-06) = 23

Figure 6: Average daily temperature recorded from the Swan Valley experimental site in 2005-06 and 2006-07 using Tinytag*Plus* Gemini Data loggers.

Fig. 1 (Brar et al.)

(A)



(B)

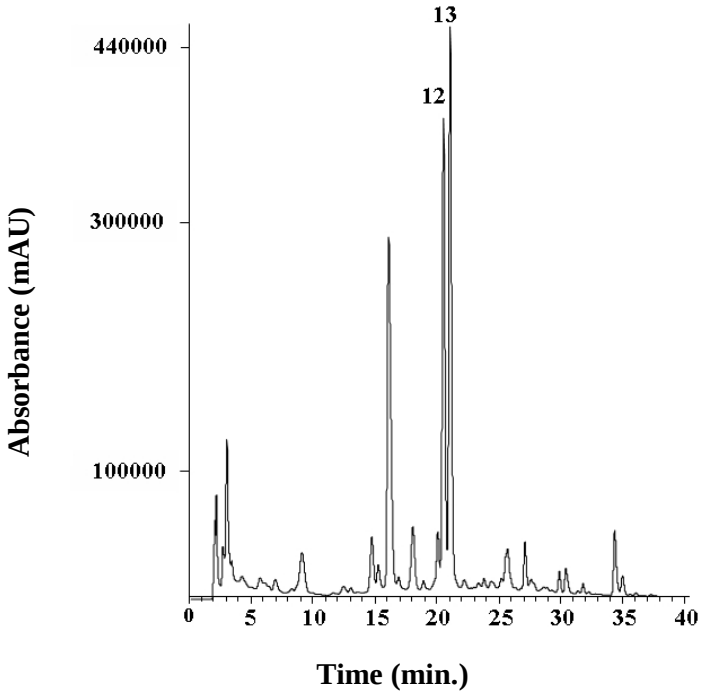


Fig. 2 (Brar et al.)

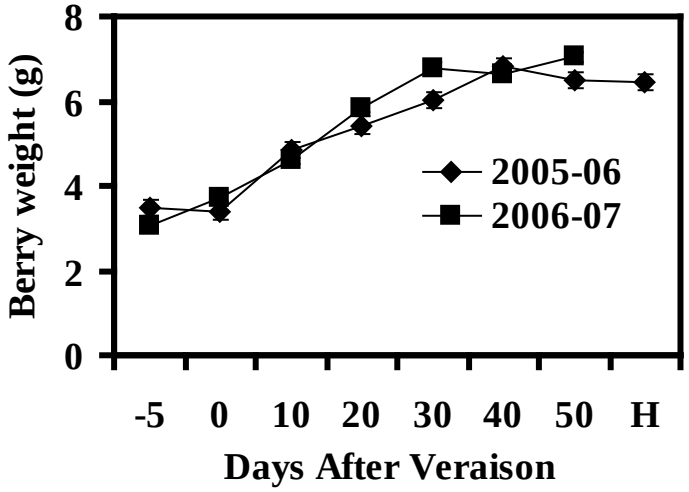


Fig. 3 (Brar et al.)

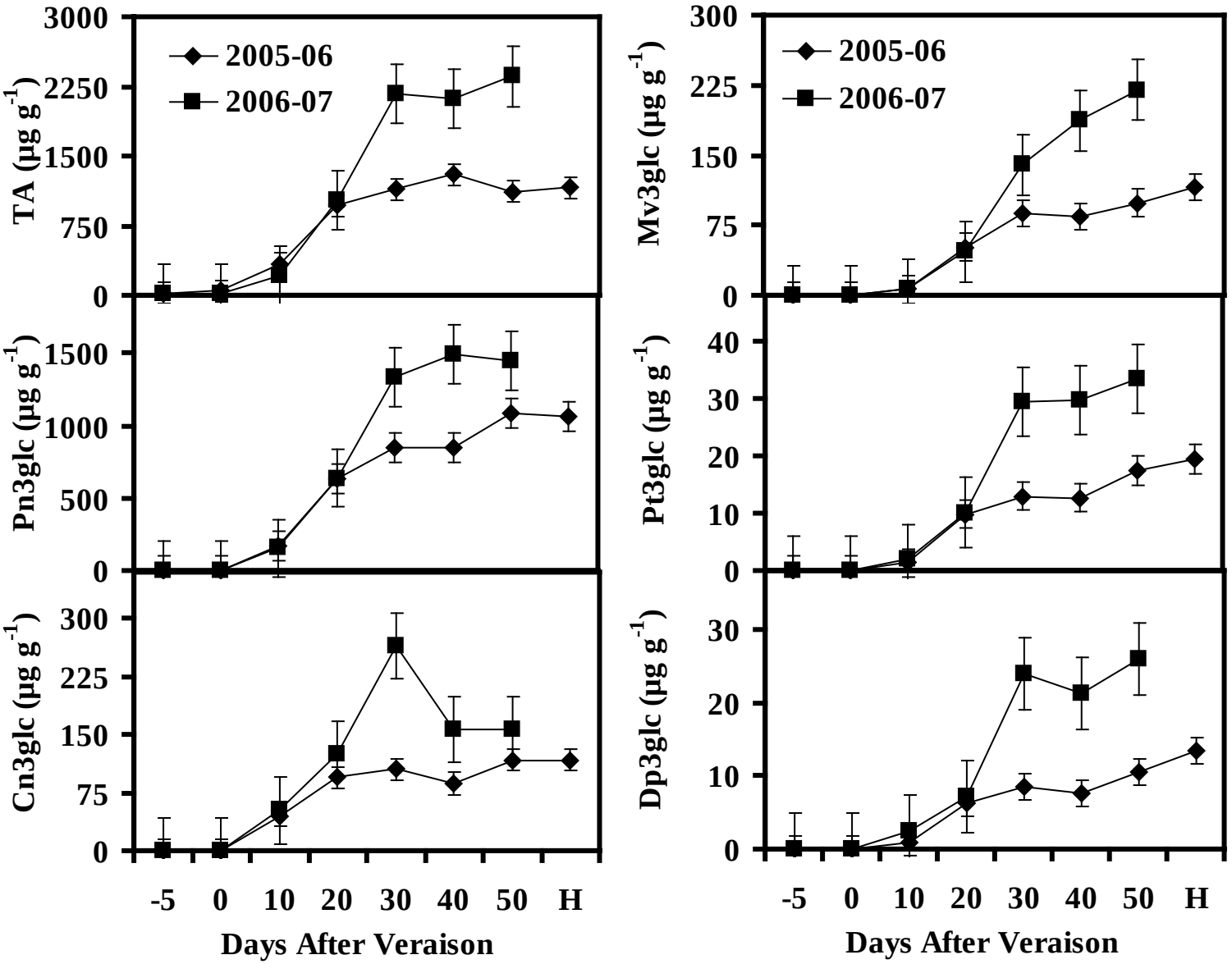


Fig. 4 (Brar et al.)

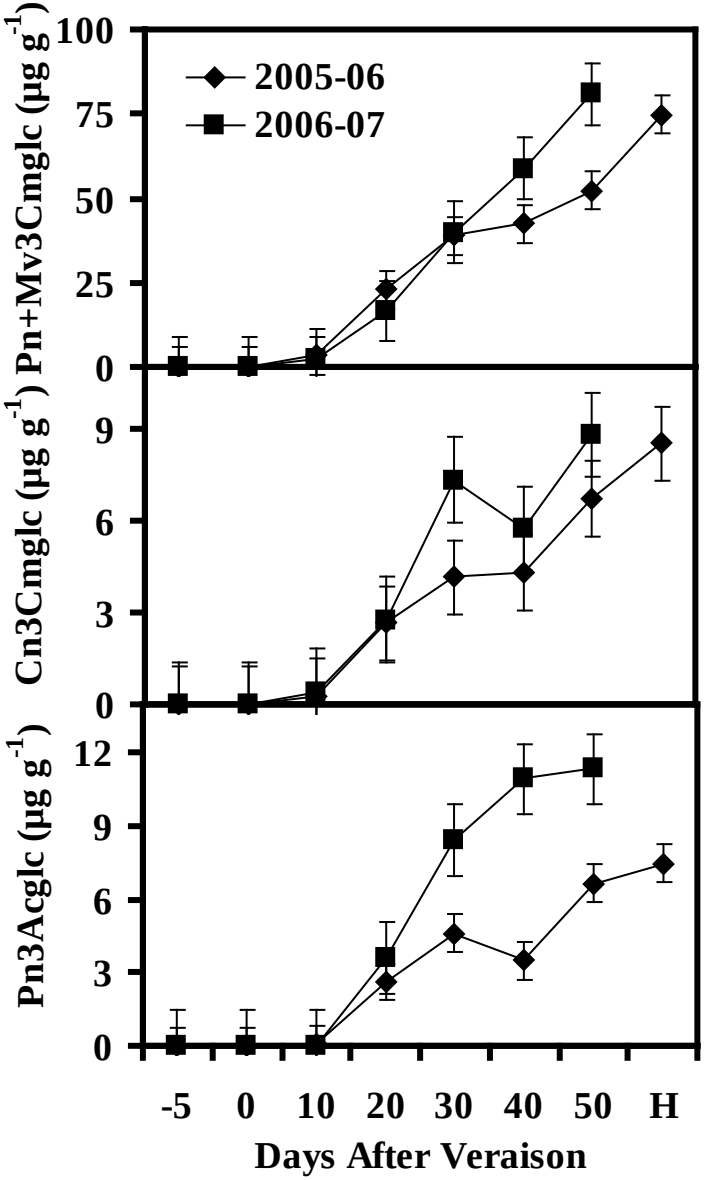


Fig. 5 (Brar et al.)

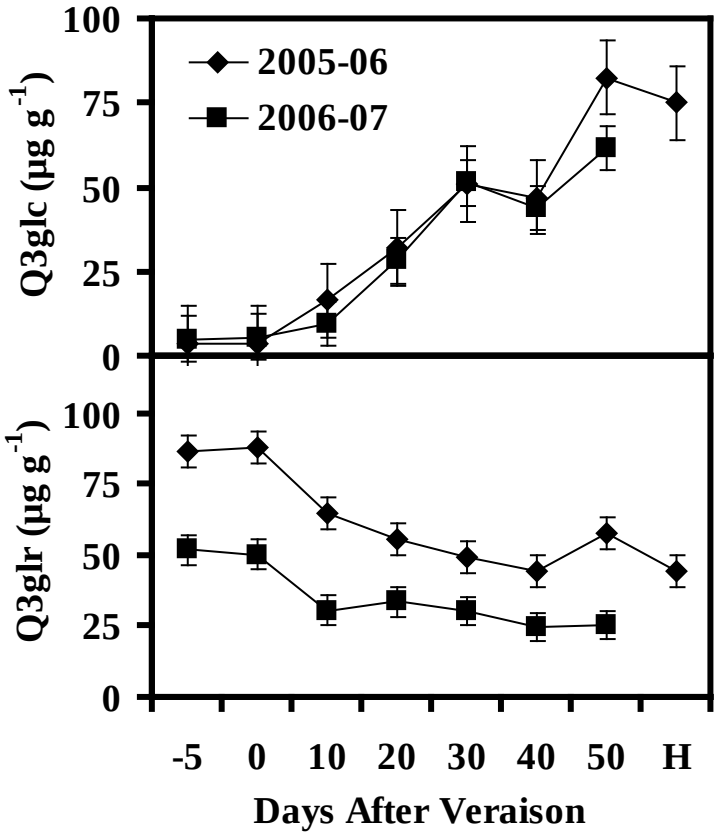


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Fig. 6 (Brar et al.)

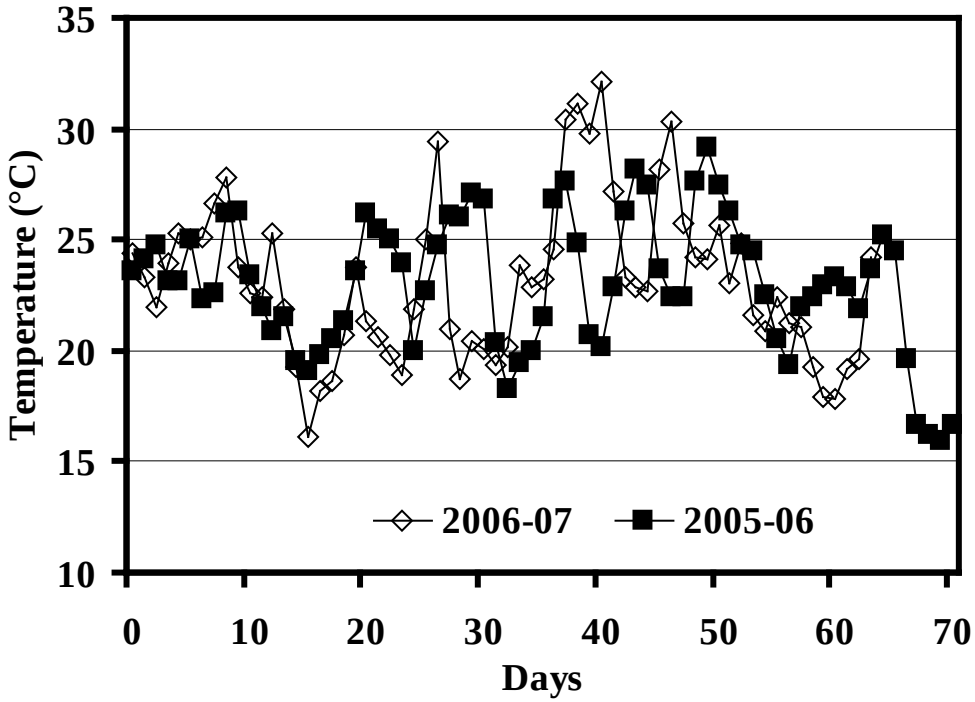

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Table 1

HPLC/PDA/MS anthocyanin and flavonol profile in the skin of ‘Crimson Seedless’ grape

HPLC Peak	***Compound***	***[M-H]⁻, m/z***	***Fragment ions m/z***
1	Delphinidin 3-*O*-glucoside **(Dp3glc)**	463	301
2	Cyanidin 3-*O*-glucoside **(Cn3glc)**	447	285
3	Petunidin 3-*O*-glucoside **(Pt3glc)**	477	315
4	Peonidin 3-*O*-glucoside **(Pn3glc)**	461	299
5	Malvidin 3-*O*-glucoside **(Mv3glc)**	491	329
6	Cyanidin 3-*O*-(6″-*O*-acetyl)-glucoside **(Cn3Acglc)**	489	447, 285
7	Peonidin 3-*O*-(6″-*O*-acetyl)-glucoside **(Pn3Acglc)**	503	461, 299
8	Malvidin 3-*O*-(6″-*O*-acetyl)-glucoside **(Mv3Acglc)**	533	491, 329
9	Cyanidin 3-*O*-(6″-*O*-coumaroyl)-glucoside **(Cn3Cmglc)**	593	447, 285
10	Peonidin 3-*O*-(6″-*O*-coumaroyl)-glucoside **(Pn3Cmglc)**	607	461, 299
11	Malvidin 3-*O*-(6″-*O*-coumaroyl)-glucoside **(Mv3Cmglc)**	637	491, 329
12	Quercetin 3-*O*-glucuronide **(Q3glr)**	477	301
13	Quercetin 3-*O*-glucoside **(Q3glc)**	463	301