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ANTHOCYANIN PROFILE IN BERRY SKINS AND FERMENTING MUST/WINE, AS AFFECTED BY GRAPE RIPENESS LEVEL OF *VITIS VINIFERA* CV. SHIRAZ/R99 [1]

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Abstract

The release of anthocyanin compounds from berry skins into the wine is affected by several viticulture and oenological practices. Ripeness level seems to play a significant role. The study aimed to evaluate the extraction kinetics of individual anthocyanins from grape skins into wine under controlled conditions of small-scale vinification of Shiraz grapes, harvested at three ripeness levels (23, 25 and 28 °Brix). The anthocyanin profile of intact berry skins, fermenting musts/wines and crushed skins during the period between crushing and pressing was analysed by HPLC. Ripeness level greatly impacted on the skin:juice ratio (ranging from 169 ± 4.5 g L^{-1} at 23 °B, 185 ± 7.2 g L^{-1} at 25 °B and 256 ± 10.7 g L^{-1} at 28 °B) and on grape anthocyanin concentration (ranging from 0.80 ± 0.13 g kg^{-1} at 23 °B, 0.78 ± 0.07 g kg^{-1} at 25 °B and 1.21 ± 0.13 g kg^{-1} at 28 °B) and profile (%) at harvest. In addition, it affected the rate and amount of individual anthocyanin extraction from skins into wine and their transformation during fermentation, consistent with the relevant chemical group classification (3-glucoside, 3-acetylglucoside, 3-*p*-coumaroylglucoside). At pressing, the anthocyanin concentration of the three wines was similar (319 mg L^{-1}, on average), but the anthocyanin profile was different, particularly the ratio of 3-glucoside:3-*p*-coumaroylglucoside derivatives, which was on average 3.55 at 23 °B and 25 °B and 2.6 at 28 °B. Viticultural choices, such as to harvest earlier or later, influencing the chemical and physical composition of the berries, may influence the extraction kinetics of individual anthocyanins and their destiny in the fermenting must, offering to winemakers different basic wines suitable for the production of different wine products. These findings are valuable to improve viticultural and oenological practices for Shiraz wine production, allowing the improvement of wine quality and the purposeful creation of different styles of wine.

Keywords: Anthocyanidin acetyl-glucosides, Anthocyanidin p-coumaroyl-glucosides, Fermentation, · Anthocyanin extractability, · ·Over-ripening.

Introduction

Phenolic compounds contained in vacuoles of the grape skin cells are released into the fermenting must during the alcoholic fermentation process of vinification [1]. Research carried out by means of model solutions or small-scale vinification have shown that oenological practices, such as duration and temperature of fermentation, juice pH, amount of SO_2, and enzyme additives, as well as alcohol concentration may influence the phenol and anthocyanin extractability during fermentation [2-16]. Vine vigour, cultural practices, and the ripeness level of the grapes may also impact on concentration and extractability [17-27, 10]. However, since the variety has a considerable effect on the amount and profile of anthocyanins [28-31, 12], results are not easily comparable and explicable. Skin morphological and structural characteristics seem to exert a direct effect on the extractability of phenolic compounds [32-34, 27]. Furthermore, during vinification molecules may undergo different kinds of degradation and/or polymerization with other molecules (copigmentation) [1], which may then lead to differences in the anthocyanin profiles of young wines. It was shown that grapes with different ripeness levels may produce wines with very different colour characteristics, regardless of the anthocyanin concentration in the grape [21, 40, 41]. The significant variation in the ratio between anthocyanins and tannins of skins and seeds with grape ripeness level [24, 26] as well as the anthocyanin composition and the pH of the must, also play a role. More coloured grapes would therefore not always produce more coloured wines.

The manner and rate of diffusion of anthocyanin molecules from the skins to the wine seem to be influenced by their structure and, in particular, by the number of hydroxyl groups in rings B and C of the molecule; the 3'-hydroxylated anthocyanins appear to diffuse faster into the wine than 3',5'-di-hydroxylated compounds, whereas the p-coumaroyl derivatives diffuse more slowly than mono-glucoside or acetyl derivatives [3, 23, 35-36]. Once anthocyanins have been extracted into the wine, they may react with tannins to produce different kinds of polymers or undergo reactions of polymerization, transformation, and degradation as a function of the medium conditions; this leads to a different intensity, hue and stability of the wine colour [1, 37].

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Despite numerous studies concerning the relationship between grape and wine characteristics, more information is required to improve viticultural and oenological practices, which may lead to better wine quality and the purposeful creation of different styles of wine.

The aim of this study was to evaluate the impact of grape ripeness level on the extraction kinetics of individual anthocyanins from grape skins into the wine during small-scale vinification of Shiraz grapes.

Materials and Methods

Vineyard. The study was done in 2009 in a Shiraz (clone SH 9C/101-14 Mgt) vineyard that was planted during 2003 to four row orientations, i.e. North-South (NS), East-West (EW), North-East-South-West, and North-West-South-East, replicated five times on flat terroir with clayey loam soil at the Robertson experiment farm of ARC Infruitec-Nietvoorbij in the Breede River Region, Robertson (33°5'S/19°54'E/159m), South Africa. The experiment was laid out on a total surface of approximately 3 ha. Vines were spaced to a fixed distance of 1.8 x 2.7 m and pruned to two buds per spur. The vertical shoot positioned canopies had approximately four leaf layers (side to side) and were uniformly managed. Vines were supplementary irrigated every 7 days due to the region receiving low winter rainfall (av. 150–300 mm/annum). Forty kg of grapes per replicate were sampled from the NS and EW orientations, respectively, at each of three ripeness levels, corresponding to 23, 25 and 28 Brix (°B). Sampling dates were 2, 12 and 24 March.

Winemaking. Grapes from each replicate were crushed and destemmed and sugar content, titratable acidity and pH of the must assessed immediately after. Volume of the juice was measured and 50 ml L^{-1} SO_2 added. Skin contact was allowed for 1 hour before addition of 30 g hL^{-1} re-hydrated pure yeast (VIN 13). As nutrient source, di-ammonium phosphate was also added at 50 g hL^{-1}. Fermentation was conducted in food grade plastic containers at controlled temperature of 24 °C. The pomace cap was punched down three times per day throughout the 5-day fermentation period. Pomace was then pressed with a balloon press at 2 bar. Pressed and free-run wine were mixed and allowed to further ferment at 25 °C until dry (less than 5 °B). During fermentation, temperature, sugar content and potential alcohol of the wines were measured.

Sampling before crushing. Ten berries of each replicate were sub-sampled from a 200 berry sample of bunches used for winemaking. After weighing, skins were carefully removed from pulps with a scalpel and immediately immersed in 40 mL of an extracting solution containing 12 % ethanol, 5 g L^{-1} D-tartaric acid, and 2 g L^{-1} sodium metabisulfite, with pH adjusted to 3.2 with addition of 1N NaOH [38]. Skin mass was obtained by subtracting container tare weight from gross weight (container plus skins).

Sampling and extraction during vinification. A 200 mL-sample of fermenting wine containing crushed skins was taken during vinification at 6, 24, 48, 72, 96 and 120 hours after crushing from each replicate, the latter samples being taken after pressing (P). Ten skins from each replicate were cleaned from pulp residues, weighed, immersed in 40 mL of the extracting solution described above, and homogenised by means of a macerator (Ultra-Turrax T25 - IKA Labortechnik, Staufen, Germany) for 60 sec at 8000 r.p.m. Samples were then kept at room temperature for 4 hours, centrifuged at 3500 r.p.m. for 15 min and supernatants stored at 4 °C until HPLC analyses. Wine samples (fermenting must) were centrifuged as described for skins and stored at 4 °C until further analyses.

Anthocyanin purification and HPLC analytical conditions. Skin, must and wine samples were purified by pushing samples through a C_{18} Sep-Pack cartridge (Waters, Milford, USA), followed by elution with methanol [38]. Anthocyanin profiles were analysed by HPLC [39] using a Hewlett Packard series 1100 HPLC, equipped with a series 1200 automatic degasser, quaternary pump, auto sampler, UV detector, and a LiChroCart 250-4 Purospher RP-18 column (Merck, Darmstadt, Germany), measuring 25 x 0.4 cm with 5 µm particle size. Formic acid:water (10:90, v/v) was used as solvent A and formic acid:methanol:water (10:50:40, v/v/v) as solvent B. Anthocyanin compounds were identified by comparing the retention time of each chromatographic peak with available data in literature [39]. Anthocyanins were quantified at 520 nm using malvidin 3-*O*-glucoside chloride (Extrasynthèse, Genay, France) as external standard. Total anthocyanin concentration was calculated as the sum of concentrations of mono-glucoside anthocyanins and acetyl and *p*-coumaroyl derivatives.

Statistics. Data were subjected to a one-way analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC).

Results and discussion

Evaluation of berries and must. As ripening progressed from the first to the third harvest, berry mass decreased by 20 % due to significant dehydration; this also affected the recovery of juice at crushing, which, in turn, decreased from 60 % to 46 % during this period (Table 1). Dehydration and concomitant shrinking of the berries may be enhanced by warm ambient temperature and low humidity conditions as well as water deficit management to increase concentration during the later phases of ripening. It is likely that for Shiraz in particular, dehydration may also proceed in absence of elevated temperatures [40]. With the increase in sugar concentration from 23 °B to 25 °B, the berry skin mass declined by about 10 %, but only a slight decrease was observed with further progression of ripening from 25 °B to 28 °B. The reduction in berry size with ripening changed the ratio between the extracting solution (the juice) and the amount of skins, as already observed [41]. The acidity decreased from 6.2 g L^{-1} to 5.0 g L^{-1}, whereas the pH increased from 3.72 to 4.26, indicating an over-ripe condition [40]. This was paralleled by an increase in skin total anthocyanins per berry, indicating further anthocyanin formation, from 25 °B to 28 °B. This is in agreement with that found by Hunter et al. [40]. It has been suggested that high air temperature conditions may induce anthocyanin degradation [42]. The observed increase in anthocyanin concentration may be related to a cooler temperature at the final ripening stage that may have allowed a maintenance or only minor degradation of anthocyanins. It is, however, more likely that extractable anthocyanin increased with an increase in the softening of the berry with ripening [1].

Table 1. Berry and skin mass, juice recovery at crushing (litres of juice per kg of grape) and berry composition at different ripeness levels.

Ripeness level	Skin mass (mg)	Berry mass (g)	Juice recovery (L kg $^{-1}$)	Titratable acidity (mg L^{-1})	pH	Skin total anthocyanins (mg berry $^{-1}$)
23 °B	189 ^{a}	1.85 ^{a}	0.60 ^{a}	6.16 ^{a}	3.72 ^{c}	1.49 ^{ab}
25 °B	173 ^{b}	1.68 ^{b}	0.55 ^{b}	5.68 ^{b}	3.93 ^{b}	1.31 ^{b}
28 °B	167 ^{b}	1.44 ^{c}	0.46 ^{c}	4.99 ^{c}	4.26 ^{a}	1.74 ^{a}

For each parameter different letters indicate significant differences at $p \leq 0.05$.

Skin anthocyanin composition and profile before crushing. Anthocyanin concentration, when expressed on a per kg basis (about 0.80 g kg^{-1} at 23 °B and 25 °B and 1.20 g kg^{-1} at 28 °B) (Figure 1), was affected by the concentration per berry, berry mass and number of berries per kg. Taking into account the decrease of the berry mass due to dehydration (Table 1), the equality of the amount of free anthocyanin as well as acetyl and *p*-coumaroyl derivatives observed for 23 °B and 25 °B (Figure 1), was mainly evidence of a rate of anthocyanin degradation; the latter was less evident from 25 °B to 28 °B.

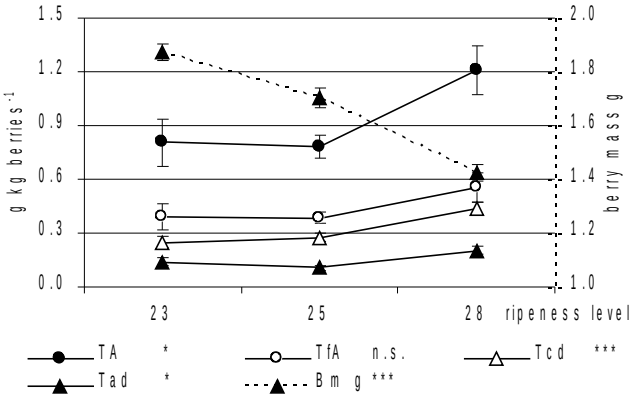

Figure 1. Anthocyanin concentrations in the berry skin at different ripeness levels (g/kg of berries); TA = Total anthocyanins, TA = total anthocyanins, TfA = Total free mono-glucoside anthocyanins, Tad = Total acetyl derivatives, Tcd = Total *p*-coumaroyl derivatives; Bm = Berry mass. (Mean of 6 replicates ± standard error). The ANOVA results for each parameter are reported in the key legend: ns = non-significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

The skin anthocyanin profile at each of the three harvest times showed similar percentages of malvidin 3-glucoside and malvidin coumaroyl-glucoside (about 30 % of total) and a moderate proportion of malvidin acetyl-glucoside and peonidin 3-glucoside (about 10 % of total) (Figure 2). This profile corresponds to those found by Guidoni et al. [43] and Ristic et al. [26], but it is inconsistent with reports of higher [44-45] or

comparable [30, 46-47] proportions of acetyl glucoside derivatives, with respect to *p*-coumaroyl derivatives. This shows that the Shiraz anthocyanin profile may change, depending on different growth or environmental conditions.

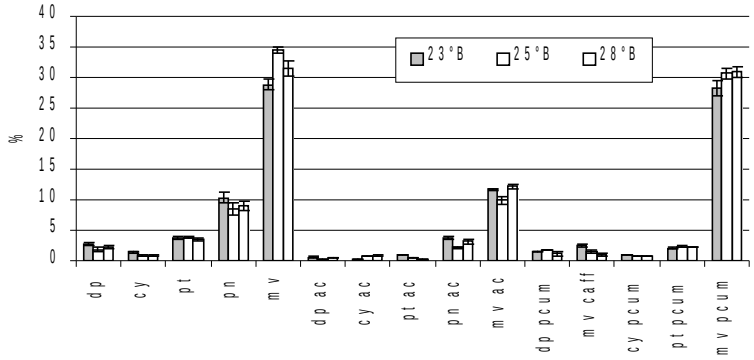

Figure 2. Anthocyanin profile of berry skins at different ripeness levels (means ± standard error, n=6). (dp: delphinidin 3-glucoside; cy: cyanidin 3-glucoside; pt: petunidin 3-glucoside; pn: peonidin 3-glucoside; mv: malvidin 3-glucoside; ac: acetyl derivative; pcum: *p*-coumaroyl derivative; caff: caffeoyl derivative).

Fournand et al. [23] detected a decrease in the amount of free anthocyanin in Shiraz berries with an increase in sugar concentration. The present study seems to confirm this evidence, even with different sampling methods, cultivation practices and environmental conditions. The impact of cultural practices, climate and vineyard microclimate on the cultivar anthocyanin profile is documented [45, 48-49]. It seems that the anthocyanin profile of Shiraz is generally more affected by the environment, season and cultural conditions than that of other cultivars. Knowing the anthocyanin profile of Shiraz in specific conditions would therefore be very useful to both viticulturists and oenologists.

Furthermore, in the present study some differences were observed between the anthocyanin profile of the berries depending on ripeness level. Malvidin 3-glucoside and malvidin *p*-coumaroyl glucoside, were present in minor percentages at 23 °B compared to the higher ripeness levels, whereas the free and acetate forms of other anthocyanins (peonidin 3-glucoside in particular) were higher (Figure 2 & Table 3). The high percentage of malvidin 3-glucoside found at 25 °B appeared not to occur at the expense of *p*-coumaroyl derivatives, but of acetyl derivatives, which seemed to be more affected by degradation reactions. The percentage of malvidin 3-glucoside decreased from 25 °B to 28 °B, whereas *p*-coumaroyl derivatives kept stable, indicating that the higher concentration found at 28 °B (Figure 1) was manly due to the synthesis of derivative forms.. It was suggested that non-acylated glucosides and acetyl-glucosides are sensitive to degradative processes induced by high temperature, whereas malvidin coumaroyl-glucoside would be more stable under such circumstances [42, 45, 5-51]. A likely reason for the stability of *p*-coumaroyl-derivatives may be attributable to their participation in a kind of intramolecular copigmentation [52].

Fermentation trend. The least ripe grapes (23 °B) only started to ferment approximately 48 hours after crushing (Hac) (Figure 3); a pre-fermentation maceration of 48 hours at 21-22 °C therefore took place. Anthocyanin extraction that occurred in aqueous medium depended on cellular maturation [1] that was, apparently, already sufficient at 23 °B.

For the other two ripeness levels, an immediate increase in temperature and alcohol concentration occurred, with 90 % of the must sugar from the second and third harvests fermented after 72 h and 96 h, respectively (Figure 3). At pressing, a low residual sugar content was found for the highest ripeness level. The temperature of the fermenting juice varied from 20 °C to 30 °C, depending on the stage of fermentation and ripeness level, and reached the highest value after 48 hours (Figure 3).

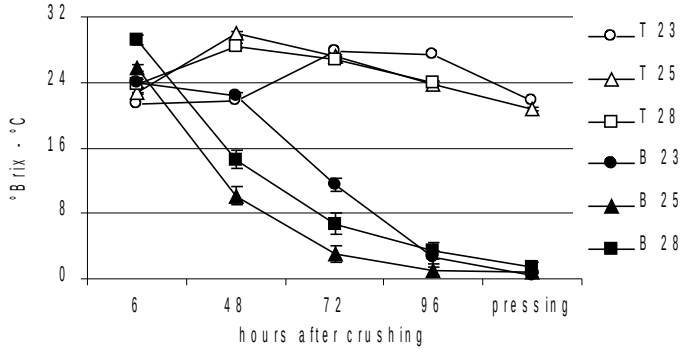

Figure 3. Changes in temperature, (T, °C) and sugar content (B, Brix) (above) and potential alcohol (%) (below) during fermentation of Shiraz grapes at different ripeness levels (mean of 6 replicates ± standard error).

Juice anthocyanin concentration and profile during vinification. Changes in the anthocyanin concentration of skins and wine were analysed quantitatively and qualitatively during the fermentation period and after pressing (Figures 4 and 5). To estimate the level of extraction of anthocyanins from the skins to the juice, an '*extraction efficiency index*' (EEI) was calculated, dividing the skin anthocyanin concentration before crushing (expressed in mg kg^{-1}) by that of the fermenting must (in mg L^{-1}) at the time of peak concentration, i.e. either 72 or 96 Hac (depending on the ripeness level and the type of molecule) (Figure 4).

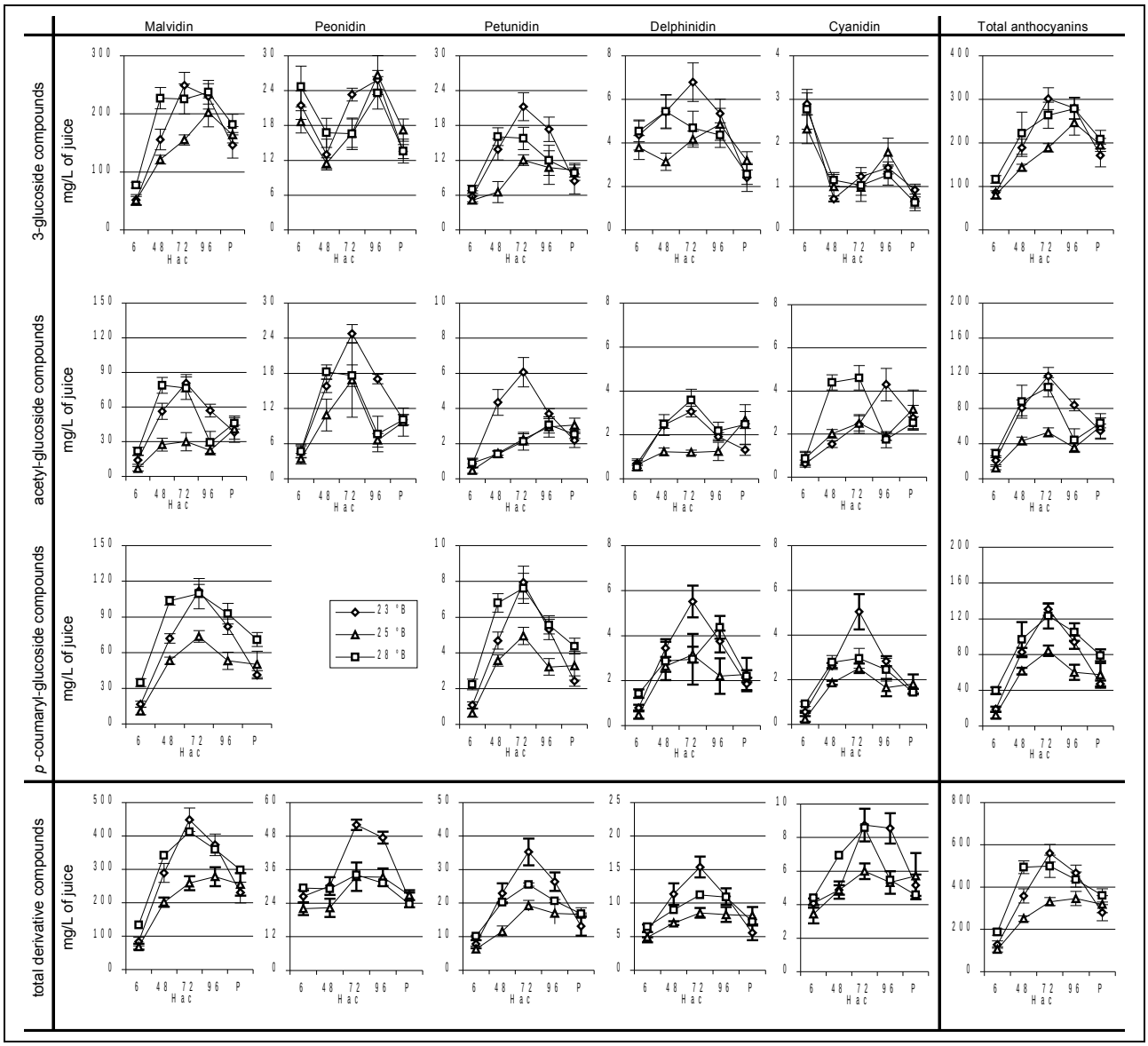

Figure 4. Changes in individual anthocyanins and their derivatives in fermenting must, as affected by ripeness level; Hac = Hours after crushing; B = berry skins before crushing; P = skins after pressing. (Mean of 6 replicates ± standard error; for ANOVA results see Table 4).

The EEI was higher when a pre-fermentation maceration occurred (for grapes at 23 °B) (EEI = 0.65 ± 0.6), consistent with the observation that anthocyanin extraction from the skins increased in an aqueous medium [1]; no difference was observed in the other cases (0.46 ± 0.03 and 0.44 ± 0.05 for 25 °B and 28 °B, respectively). This finding has high practical significance, particularly when conditions in the vineyard are not favourable to anthocyanin formation, and when higher anthocyanin extraction is required when harvesting at a lower ripeness level; the latter is often necessary in order to avoid bunch rot, unfavourable grape composition (low acidity, high pH, loss of aroma, etc.) and high alcohol wines or to produce a different style of wine [53]. At the same time, this result showed that the skin concentration and the ratio of juice:skin (which incidentally was much lower at 28 °B) were not the only factors determining the extraction efficiency of anthocyanin compounds from the skins to the wine.

The quantitative juice anthocyanin level at the extraction peak time (500 mg L^{-1} for 28 °B and 352 mg L^{-1} for 25 °B) was only proportional to the concentration in the grapes when similar winemaking conditions occurred (25 °B *vs* 28 °B) (Table 1). The pre-fermentation maceration stimulated a higher extraction of anthocyanin at a lower ripeness level (23 °B) (peak at 560 mg L^{-1}), even though the concentration in the skin was similar to that of 25 °B and lower than that at 28 °B. The lower level of extraction of the faster fermentations (25 °B and 28 °B) may be attributed to the rapid increase in alcohol concentration that probably slowed down the extraction of compounds from the skins [1]. In addition, the lower ratio of juice:skins at the higher ripeness level may, perhaps against popular believe, have prevented complete anthocyanin extraction as a function of saturation. With the progressive increase of ethanol in the medium, and in parallel with the extraction, the anthocyanins may undergo hydrolytic [55] and oxidative degradation, precipitate as monomers or as polymers embedded in insoluble molecules, or may be absorbed by the yeast (especially the non-methylated anthocyanins) [3, 54]. In our study, this decline seemed to depend on the characteristics of the original grapes and on the ripeness level, in addition to the molecule examined and to the conditions of vinification.

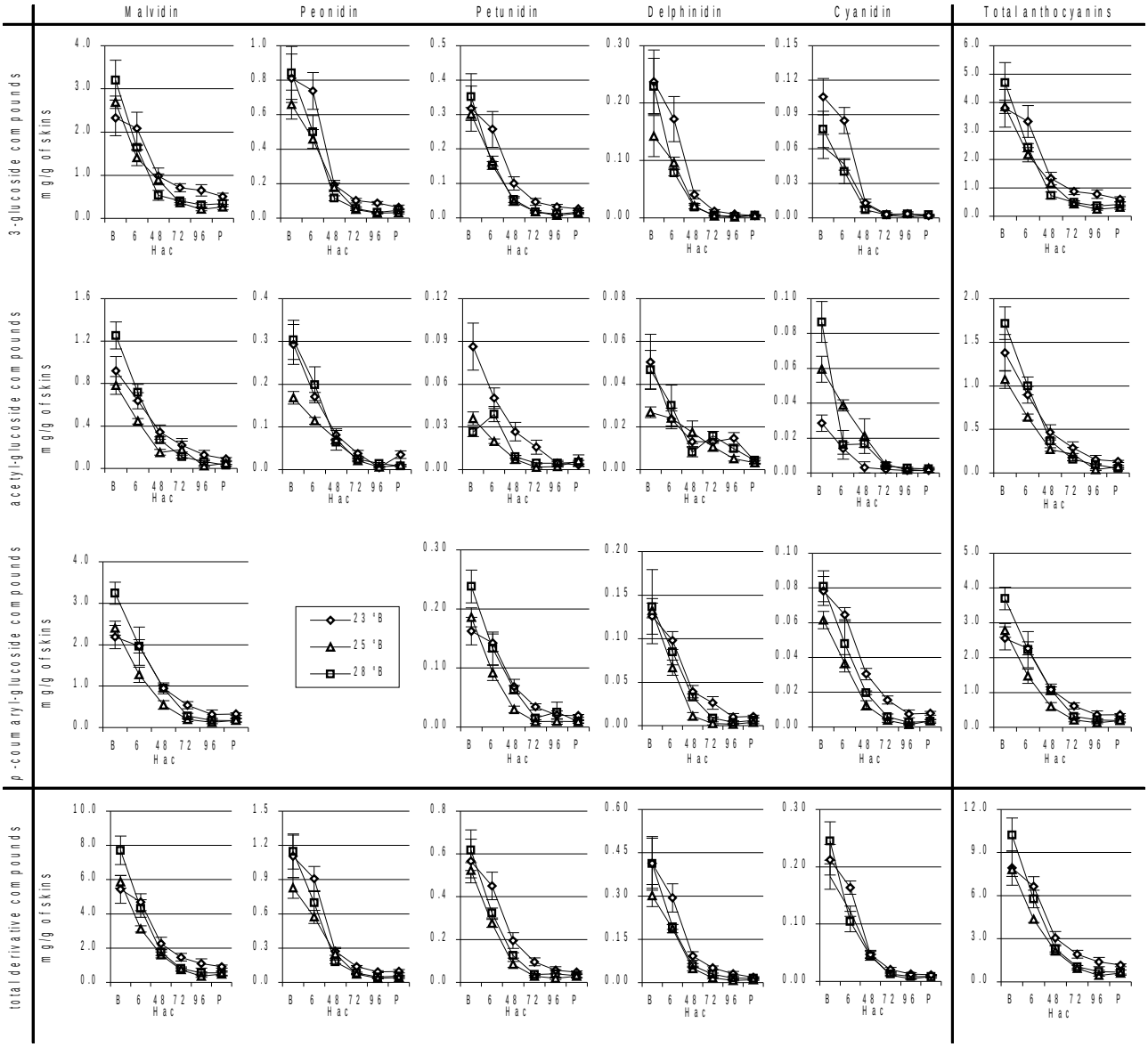

Figure 5. Changes in individual anthocyanins and their derivatives in berry skins during fermentation, as affected by ripeness level; Hac = Hours after crushing; B = berry skins before crushing; P = skins after pressing. (Mean of 6 replicates ± standard error; for ANOVA results see Table 4).

At 23 °B, the anthocyanin concentration reached a peak after 72 Hac for almost all compounds; the subsequent decline affected 43 % of the free compounds, 53 % of the acetyl and 64 % of the *p*-coumaroyl derivatives (Figure 4). At pressing (P), the total anthocyanin concentration in the wine from the 23 °B grapes

was 279 mg L^{-1}, which corresponded to 50 % of that recorded at peak time). The remaining anthocyanin concentration in the extracted skins (Figure 5), on the contrary, was high (1.15 mg g^{-1} of skin) when compared to that from the grapes at 25 °B and 28 °B (0.6 and 0.7 mg g^{-1} of skin, respectively). This finding demonstrates that the ripeness level may affect the chemical-physical condition of the berries, impacting on the fermentation pattern and on the release of the polyphenol molecules; the level of skin softness may therefore have played a role in anthocyanin extractability [56].

At 25 °B, the free forms were extracted into the wine until 96 Hac and the subsequent decrease of the concentration was modest; the concentration of coumaroyl derivatives, after peaking at 72 Hac, decreased rapidly (-32%), but the extraction of acetyl derivatives reached a maximum after pressing (Figure 5). Due to the different behaviour of the individual anthocyanins, the total concentration of anthocyanins remained constant during the later stages of fermentation, thus, after pressing, it reached a level similar to that of the other ripeness levels. It is interesting to note that, before pressing (at 96 hac), the wine profile (Table 3) was very different from that of the original grape skins (Table 2) and from of the other ripeness levels (Table 3). At pressing, however, differences were less evident. The data confirmed that the extraction of acetyl derivatives was probably delayed as a consequence of the ethanol concentration in the extracting medium [3]. Due to their localisation, these molecules may not have undergone the usual degradations known for molecules already diffused into the wine: this resulted in a relatively high anthocyanin concentration in the wine after pressing (Figure 4, Table 4). However, the behaviour of the anthocyanin derivatives may have also been affected by the level of tannins present in the juice during fermentation, but this aspect was not in the scope of this study.

Table 2 Proportional changes (%) in free anthocyanins and their derivatives in the **berry skins** during fermentation as affected by the ripeness level (RL) and ANOVA results relative to the RL and Hac factors and their interaction.

RL	Hac	Tma†	Tad	Tcd	Dpd	Cyd	Ptd	Pnd	Mvd
23°B	Bry	47.3 b	17.4 a	32.8 a	5.0 a	2.8 a	7.0 a	14.0 a	71.2 b
	6	49.2 b	13.6 b	35.0 a	4.3 ab	2.6 a	6.6 a	14.5 a	72.0 b
	48	43.3 b	14.9 ab	36.0 a	3.0 bc	1.6 b	6.2 a	11.9 a	77.2 a
	72	46.1 b	14.4 ab	32.1 a	3.0 bc	1.1 c	4.8 b	12.7 a	78.3 a
	96	63.4 a	12.5 b	19.3 b	3.3 bc	1.0 c	4.0 b	13.0 a	78.7 a
	P	51.0 b	11.7 b	31.9 a	1.6 c	1.0 c	4.1 b	11.9 a	81.4 a
25°B	Bry	48.5 a	14.2 ab	35.6 a	4.2 a	2.6 a	6.7 a	11.6 abc	75.0 b
	6	49.6 a	14.8 ab	33.8 a	4.3 a	2.8 a	6.3 ab	13.6 ab	72.9 b
	48	53.8 a	12.9 ab	28.3 a	2.3 b	2.2 a	3.9 c	14.4 a	77.2 b
	72	48.0 a	22.1 a	22.0 a	1.7 b	1.2 b	2.9 c	9.0 bc	85.2 a
	96	57.0 a	8.1 b	27.8 a	1.7 b	0.9 b	4.0 c	8.9 bc	84.4 a
	P	51.3 a	11.9 ab	32.0 a	1.8 b	1.3 b	4.7 bc	8.0 c	84.2 a
28°B	Bry	47.1 ab	16.9 a	35.1 ab	4.0 a	2.2 a	6.0 a	12.2 a	75.3 b
	6	44.8 ab	17.3 a	35.7 ab	3.5 ab	1.8 bc	5.7 a	12.7 a	76.3 b
	48	31.5 b	16.2 a	47.1 a	2.6 bc	2.0 ab	5.5 a	9.2 a	80.6 ab
	72	48.6 a	15.2 a	28.1 b	2.9 abc	1.2 cd	3.5 a	10.3 a	82.1 a
	96	49.7 a	13.9 a	28.4 b	2.2 c	1.3 cd	4.8 a	7.7 a	84.0 a
	P	61.7 a	7.8 b	26.6 b	2.2 c	1.2 d	4.5 a	8.9 a	83.1 a
RL		***	***	***	***	***	***	**	***
Hac		n.s.	n.s.	n.s.	***	n.s.	***	***	***
RL x Hac		n.s.	*	n.s.	n.s.	*	n.s.	n.s.	n.s.

*† Statistical significance is displayed for each ripeness level separately, superscript different letters indicate significant differences ($p \leq 0.05$) between Hac (Hours after crushing). For ANOVA results: n.s. = non-significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.*

†Hours after crushing. Tma= Total mono glucoside anthocyanins, Tad = Total acetyl derivatives, Tcd = Total p-coumaryl derivatives, Dpd = Delphinidin derivatives, Cyd = Cyanidin derivatives, Ptd = Petunidim derivatives, Pnd = Peonidin derivatives, Mvd = Malvidin derivatives.

At 28 °B, the extraction trend was similar to that of the grapes at 25 °B, but the peak of the anthocyanin concentration of the former was higher (Figure 4). The anthocyanin decrease after peaking comprised 25 % of the free compounds, 39 % of acetyl and 36 % of p-coumaroyl derivatives; overall the anthocyanins declined by 28 %, reaching 360 mg L^{-1} at pressing. The high amount of malvidin 3-glucoside in the wine demonstrated its lower involvement in the oxidative reactions when it diffuses into the wine. The differences highlighted as a function of the level of grape ripeness (Table 4) did not change this trend. As found for the 25 °B, a higher concentration of acetyl anthocyanins was found in the wine after pressing. A similar behaviour was observed at 23 °B, showing that pressing could improve the anthocyanin extraction from the skins, but that the ripeness level may also play a significant role.

Table 3 Proportional changes (%) in free anthocyanins and their derivatives in **the wine** during fermentation as affected by the ripeness level (°RL) and ANOVA results relative to the RL and Hac factors and their interaction. (See table 2 for abbreviation).

RL	Hac	Tma†	Tad	Tcd	Dpd	Cyd	Ptd	Pnd	Mvd
	6	67.3 ^{a}	16.7 ^{b}	14.8 ^{d}	4.5 ^{a}	3.5 ^{a}	5.9 ^{a}	22.0 ^{a}	64.2 ^{b}
	48	52.9 ^{c}	21.8 ^{a}	23.6 ^{a}	3.1 ^{b}	1.4 ^{b}	6.4 ^{a}	8.1 ^{b}	81.0 ^{a}
23°B	72	53.7 ^{c}	20.8 ^{ab}	23.5 ^{a}	2.7 ^{bc}	1.6 ^{b}	6.2 ^{a}	8.8 ^{b}	80.6 ^{a}
	96	59.8 ^{b}	18.3 ^{b}	20.2 ^{b}	2.3 ^{cd}	1.8 ^{b}	5.6 ^{a}	8.7 ^{b}	81.6 ^{a}
	P	60.8 ^{b}	19.4 ^{ab}	17.4 ^{c}	1.9 ^{d}	1.9 ^{b}	4.5 ^{b}	9.3 ^{b}	82.4 ^{a}
	6	74.3 ^{a}	12.0 ^{b}	12.8 ^{b}	4.3 ^{a}	3.3 ^{a}	5.9 ^{a}	20.6 ^{a}	66.0 ^{b}
	48	57.1 ^{c}	17.0 ^{a}	24.4 ^{a}	2.8 ^{b}	1.9 ^{b}	4.7 ^{a}	9.2 ^{b}	81.4 ^{a}
25°B	72	57.2 ^{c}	15.8 ^{ab}	25.3 ^{a}	2.6 ^{bc}	1.8 ^{b}	5.8 ^{a}	11.1 ^{b}	78.7 ^{a}
	96	70.7 ^{b}	10.7 ^{b}	17.4 ^{b}	2.4 ^{cd}	1.5 ^{b}	4.9 ^{a}	9.8 ^{b}	81.4 ^{a}
	P	63.1 ^{b}	17.6 ^{a}	17.4 ^{b}	2.5 ^{bc}	1.7 ^{b}	5.2 ^{a}	9.4 ^{b}	81.1 ^{a}
	6	62.4 ^{a}	14.9 ^{b}	21.0 ^{a}	3.4 ^{a}	2.3 ^{a}	5.4 ^{a}	15.9 ^{a}	72.9 ^{b}
	48	53.6 ^{b}	21.3 ^{a}	23.8 ^{a}	2.2 ^{b}	1.7 ^{b}	4.9 ^{a}	7.2 ^{b}	84.0 ^{a}
28°B	72	52.9 ^{b}	20.9 ^{a}	24.7 ^{a}	2.3 ^{b}	1.7 ^{b}	5.2 ^{a}	7.4 ^{b}	83.4 ^{a}
	96	64.1 ^{a}	9.9 ^{c}	24.2 ^{a}	2.5 ^{b}	1.2 ^{b}	4.7 ^{a}	7.4 ^{b}	84.2 ^{a}
	P	57.6 ^{ab}	17.7 ^{ab}	22.0 ^{a}	2.0 ^{b}	1.3 ^{b}	4.6 ^{a}	7.0 ^{b}	85.1 ^{a}
RL		***	***	***	***	***	***	***	***
Hac		***	***	***	***	***	***	**	***
RL x Hac		n.s.	*	***	***	n.s.	n.s.	n.s.	n.s.

*† Statistical significance is displayed for each ripeness level separately, superscript different letters indicate significant differences ($p \leq 0.05$) between Hac (Hours after crushing). For ANOVA results: n.s. = non-significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.*

The 3'-hydroxilated anthocyanins (peonidin 3-glucoside and cyanidin 3-glucoside) reached maximum concentration very quickly, after 6 Hac, they being known as the more rapidly extractable molecules [3] (Figure 4). In the subsequent 24 hours, however, their concentration decreased sharply by 30 %; this behaviour was expected because, being rapidly extracted, the molecules are present in the juice during the first stage of fermentation, when the oxidative reaction, through polyphenol oxidase (PPO) activity, is more intense [35]. The rapid degradation was not affected by the medium (aqueous or alcoholic) in which the compounds were extracted. Subsequently, however, the concentration of particularly peonidin 3-glucoside increased again, showing that its extraction was not complete during the first stages. Furthermore, as the fermentation continued and the PPO activity decreased, the oxidative degradation of the molecule was diminished, allowing the concentration to increase during the final stage of maceration-fermentation. As mentioned above, during the early stages of extraction and in the presence of low alcohol, yeasts may absorb anthocyanins. It is possible that, as the alcohol increases, and due to the very low affinity with cellular structures of the yeast, some molecules (particularly peonidin 3-glucoside) may again be released. This may be another explanation of the increase in peonidin 3-glucoside concentration observed in the wine during the second part of fermentation (Figure 4) and of the fact that the anthocyanins extracted from the yeast-containing medium after racking showed a profile with high percentages of malvidin-3-glucoside and its derivatives (83 %) and low percentages of peonidin derivatives (7 %). The susceptibility of the molecules to

the oxidative degradation appeared related to the rapidity of their diffusion into the juice/wine as observed for petunidin and delphinidin derivatives in particular. The level of ripeness also affected the molecule degradation by influencing the speed of extraction.

Table 4 ANOVA results for the anthocyanin compounds measured in the berry skins during fermentation (mg/g of skins, see figure 5) and in the fermenting must (mg/L of juice, see the figure 4).

	Factors	TA	Tma	Tad	Tcd	Dpd	Cyd	Ptd	Pnd	Mvd
SKINS	ripeness level	***	**	***	***	**	*	***	***	***
	Hac†	***	***	***	***	***	***	***	***	***
	ripeness level x Hac	**	n.s.	**	**	n.s.	**	n.s.	n.s.	***
MUST	ripeness level	***	***	***	***	***	***	***	***	***
	Hac	***	***	***	***	***	***	***	***	***
	ripeness level x Hac	**	**	***	*	n.s.	***	n.s.	***	***

n.s. = non-significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

At 96 Hac the free-run wines had an anthocyanin content of 436 mg L^{-1} (28 °B), 346 mg L^{-1} (25 °B) and 467 mg L^{-1} (23 °B) and from 60 - 70 % of the molecules were in the free glucoside form (Table 3). At pressing, the wine anthocyanin concentration decreased further, reaching 360 mg L^{-1} for the 28 °B (- 17 %), 319 mg L^{-1} for the 25 °B (- 8 %) and 280 mg L^{-1} for the 23 °B (- 32 %) ripeness level (Figure 5), most probably because oxidative reactions other than the reactions between anthocyanins and phenols extracted from the skin during pressing and involving particularly the free anthocyanins, may have taken place during pressing because of the high presence of oxygen in the wine (Di Stefano, 2011; personal communication). The prominent decrease observed for the 23 °B ripeness level was mainly due to reactions involving the acetyl and p-coumaroyl derivatives that decreased 52 % and 64 %, respectively, between the extraction peak time and pressing. After pressing, approximately 60 % of the molecules were in the free glucoside form of which more than 80 % was represented by malvidin 3-glucoside and its derivatives (Table 3). Coumaroyl derivatives represented, on average, 19 % of total anthocyanins, but they were particularly high at 28 °B (Table 3). In the extracted skins at 23 °B and 25 °B, these compounds represented 32 % of the total and at 28 °B less than 27 % (Table 2); this again demonstrated the reduced extraction of these compounds from the skins and that their extraction may be enhanced when grapes are over ripe. As already observed [23, 29, 30], the anthocyanins present in wines mainly comprised the non-acylated forms (Table 3), even though they always represented more then 50 % of the total anthocyanin amount in the berry skins (Table 2). It is possible that, once extracted, the acetyl derivatives more easily interact with components of the surrounding matrix or, as reported by the previous authors, but not confirmed by our results, are more difficult to extract from the skins.

The highest anthocyanin concentration in the wine after pressing was measured when the highest anthocyanin concentration also was found in the skins, confirming, at least from a quantitative point of view, the positive relationship between the concentration of anthocyanins in the wine and that in the original grapes [26, 40]. The main differences concerned the concentration of malvidin 3-glucoside derivatives.

Skin anthocyanin profile during vinification. Monitoring the remaining skin anthocyanin contents during fermentation provides insight into the rate at which the compounds are extracted during vinification. It is a useful tool for describing the extraction of individual anthocyanins from the skins, regardless of the destiny of the molecules in the wine (Figure 5 and Table 4). At 23 °B, extraction was slow for the first six hours, with the exception of some acetyl derivatives; after that, the decrease was faster, but at the end of maceration 14.5 % of the total anthocyanin was still not extracted. At 25 °B and 28 °B, the concentration decrease in the skins was more rapid and regular; after pressing, 7.8 % and 6.6 % of the anthocyanins present in the skins of the whole berries remained in the skins probably because of a higher degradation of the cellular structures due to over ripening of the grapes. The skin profile at the end of anthocyanin extraction generally included a higher proportion of malvidin derivatives (83 % *vs* 74 %) and a lower percentage of acetyl derivatives (10 % *vs* 16 %) with respect to the intact skins (Table 3); more than 50 % of the malvidin derivatives were represented by malvidin 3-glucoside. The percentage of *p*-coumaroyl derivatives was very similar to that of the intact skins at 23 °B and 25 °B, confirming previous observations [23, 29-30]. Nevertheless, in the extracted skins of the 28 °B berries, the percentage of *p*-coumaroyl derivatives was particularly low compared to that of the original grapes. This value was consistent with the level measured in the must where,

on the contrary, particularly high levels were found. This anomalous behaviour may have been related to the ripeness level and/or to the skin physical characteristics, which may lead to a higher rate of extraction when berries are over-ripe [27, 33, 40, 57].

Conclusion

The study provides information about the extraction of anthocyanins from the skin into the wine during fermentation at different ripeness levels of Shiraz grapes. The level of ripeness affected the skin:juice ratio and recovery during crushing because of the natural dehydration of the grapes as ripening proceeded. The grape anthocyanin profile changed with an increase in the proportion of malvidin 3-glucoside and its p-coumaroyl derivatives at the late ripening stage (25 °B and 28 °B). The ripeness level also affected the rate and pattern of extraction of the individual compounds during winemaking; the physical characteristics of the skins and the chemical changes during fermentation clearly played an important role.

Pre-fermentation maceration, which occurred for the 23 °B grapes, resulted in a rapid but incomplete extraction of compounds that further underwent significant degradation in the wine; the extracted skins still contained a considerable amount of coumaroyl derivative forms. In contrast, over-ripeness (28 °B) led to a high recovery of anthocyanin, particularly p-coumaroyl derivatives, and nearly complete skin depletion; at the same time, a reduced incidence of anthocyanin degradation was observed during the latter stages of fermentation. The intermediate level of maturation (25 °B) led to poor extraction of compounds from the skins, but they were not readily involved in further transformation processes.

Both extracted skin and wine anthocyanin profiles were very different from those of the original grapes, the latter containing a higher proportion of malvidin and its derivatives and of free forms with differences among ripening level. The study confirmed that malvidin and 3'-5' hydroxylated anthocyanins are not easily extracted from the skins, but that once extracted, it seems stable and less susceptible to reactions occurring in the wine. The dissimilar behaviour in extraction shown by different classes of anthocyanins (acetyl forms in particular), also as a consequence of ripeness level, confirmed that the extraction of anthocyanins from the skins and their behaviour in the wine are affected by numerous factors. Systematic approaches would be required to further investigate the impact on the anthocyanin profile of the complex matrix in which extraction takes place during vinification in any situation.

The results showed that viticultural/oenological choices, like earlier or later harvesting, could modify the physical and chemical nature of the berries as well as the extraction kinetics of individual anthocyanins, with potential effects on the wine colour. This aspect should be of interest in any situation where an impact on the colour of the wine (and other quality compounds), is required.

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