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Survey on the content of vitisin A and hydroxyphenyl-pyranoanthocyanins in Tempranillo wines

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ABSTRACT

Little is known about the content and development of pyranoanthocyanins, pigments mainly formed during red wine ageing, in commercial wines. Some of the major pyranoanthocyanins in a wide selection of 1–10 years-old Spanish Tempranillo wines and also in a 29 years wide-vertical series of Tempranillo wines from an individual cellar have been determined. Great variability in pyranoanthocyanin concentrations was found (range, mg/l): vitisin A, 0–10.76; pinotin A, 0–4.26; and malvidin 3-glucoside-4-vinylphenol, 0.03–1.37. Vitisin A and malvidin 3-glucoside-4-vinylphenol were already present in 1–2 years-old wines, whereas pinotin A was only detectable in a few of the 1 and 2 years-old wines. Vitisin A tended to decrease with wine age, while hydroxyphenyl-pyranoanthocyanins showed the reverse trend. However, the aforementioned trends were interrupted by various temporary maxima, most likely due to some "refreshment" of the oldest wines (i.e., addition of young wine), as suggested by unexpected high concentrations of malvidin 3-glucoside, in contrast to the results found in the wine vertical series. The effects of addition of young wine on aged wine pyranoanthocyanin concentrations were confirmed by wine refreshment experiments.

1. Introduction

The actual colour of a red wine is the result of a combination of different physical and chemical processes which sometimes overlap, and begin to develop as soon as anthocyanins are extracted from grape skins to fermenting must. After the maceration period, and also during and after alcoholic fermentation, anthocyanins and other phenolic wine constituents react to give a great variety of new monomeric, oligomeric and polymeric compounds. Next to the physical phenomenon of copigmentation, which is partly responsible for colour enhancement, the contribution of polymeric anthocyanin-derived pigments to the colour of red wine is very important, especially in aged wines. The study of the pigments contributing to red wine colour emerged around 50 years ago and has attracted great attention, especially within the last decade. The initially suggested anthocyanin-derived wine pigments were tannin-anthocyanin polymeric pigments that could explain the precipitation of colouring matter observed during red wine storage and ageing. However, wine polymeric pigments represent a heterogeneous and not well-characterised group of reaction products. Another group of anthocyanin-derived wine pigments correspond to monomeric and oligomeric pigments that are formed in great numbers by the reaction of anthocyanins and molecules of low molecular weight. The latter pigment group is easier to study, and nowadays the following classification can be suggested: non-acylated and acylated native anthocyanins; anthocyanins acylated with lactic acid; dimeric anthocyanins; flavanol–anthocyanin direct and acetaldehyde-mediated reaction pigments; flavanol–dimeric anthocyanin direct reaction pigments; and pyranoanthocyanins (Alcalde-Eon, 2008; Alcalde-Eon, Boido, Carrau, Dellacassa, & Rivas-Gonzalo, 2006; Alcalde-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2004, 2006).

Among the newly formed red wine pigments, the class of pyranoanthocyanins plays an increasing role during wine ageing

Abbreviations: mv-3-glc, malvidin 3-glucoside; mv-3-glc-4-VP, malvidin 3-glucoside-4-vinylphenol (pyranoanthocyanin resulted from the reaction of malvidin 3-glucoside and *p*-coumaric acid); pinotin A or mv-3-glc-4-VC, malvidin 3-glucoside-4-vinylcatechol (pyranoanthocyanin resulted from the reaction of malvidin 3-glucoside and caffeic acid); vitisin A, pyranoanthocyanin resulted from the reaction of malvidin 3-glucoside and pyruvic acid.

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because these relatively small molecules remain in solution, in contrast to the classical flavanol–anthocyanin polymeric pigments that tend to precipitate. In addition, most pyranoanthocyanins differ from their anthocyanin precursors, especially in their colour. With the exception of the so-called portisins, most pyranoanthocyanins show a hypsochromically shifted maximum of absorption, in comparison to native grape anthocyanins (Rentzsch, Schwarz, & Winterhalter, 2007a). This shift causes a red-orange colour of pyranoanthocyanins, in contrast to the red-purple colour of genuine anthocyanins, like malvidin 3-glucoside. Moreover, the newly formed pyran ring stabilises the colour of pyranoanthocyanins at varying pH values, and hardly any loss of red colour intensity is observed within the pH range 1–5. Under the same conditions the genuine grape anthocyanins lose up to 90% of their initial colour (Mazza & Miniati, 1993). A further difference between grape anthocyanins and pyranoanthocyanins is related to their development during storage. Anthocyanins reach a maximum in concentration after 2–3 days of skin maceration and, afterwards, they are constantly degraded until an almost complete disappearance after several years of ageing. In contrast to this, the greatest amount of pyranoanthocyanins is produced during processing and storage of red wines (Rentzsch, Schwarz, Winterhalter, & Hermosín-Gutiérrez, 2007b; Romero & Bakker, 2000; Schwarz, Hofmann, & Winterhalter, 2004; Schwarz, Quast, von Baer, & Winterhalter, 2003b).

Pyranoanthocyanins result from the reaction of a native grape anthocyanin and molecules bearing a polarisable double bond. Pyranoanthocyanin structures are diverse and several pathways have been proposed for their formation (Rentzsch et al., 2007a). Some relevant pyranoanthocyanins emerge from the reaction of anthocyanins and yeast metabolites, such as pyruvic acid (e.g., vitisin-type pyranoanthocyanins; **1** in Fig. 1) or hydroxycinnamic acids (e.g., hydroxyphenyl-type pyranoanthocyanins; **2** in Fig. 1). In the latter case, formation of these hydroxyphenyl-pyranoanthocyanins seems to develop in part during fermentation through yeast-mediated decarboxylation of hydroxycinnamic acids (Fulcrand, Cameira dos Santos, Sarni-Manchado, Cheynier, & Favre-Bonvin, 1996) and, more prominently, by direct reaction of free hydroxycinnamic acids (Schwarz, Picazo-Bacete, Winterhalter, & Hermosín-Gutiérrez, 2005; Schwarz, Wabnitz, & Winterhalter, 2003c) after release from their respective tartaric acid esters (Rentzsch et al., 2007b).

Published data about the content and development of pyranoanthocyanins in commercial wines are scarce (Rentzsch et al., 2007b; Schwarz et al., 2003b, 2004). The initial aim of this work was to make a survey on the concentrations of some of the most commonly occurring pyranoanthocyanins (vitisin A and the hydroxyphenyl-pyranoanthocyanins pinotin A and malvidin 3-glucoside-4-vinylphenol) in a wide set of commercial wines elaborated using grapes of the *Vitis vinifera* Tempranillo variety, the most common Spanish red grape variety. As a consequence of the preliminary results we obtained, a second objective of our work was to evaluate the effect of ageing (a period of 1–10 years was covered) in the content of pyranoanthocyanins in wines, paying special attention to some factors that can influence the levels of hydroxyphenyl-pyranoanthocyanins in aged Tempranillo wines, namely, the occurrence of their respective precursor molecules (free hydroxycinnamic acids) and the addition of young red wines to aged wines (the oenological practice known as "refreshment"). To help in achieving the latter objective, a vertical series of Tempranillo wines elaborated in the same cellar over a period of 29 years was considered as reference for anthocyanin evolution during ageing, and also experiments of refreshment and further accelerated ageing were performed.

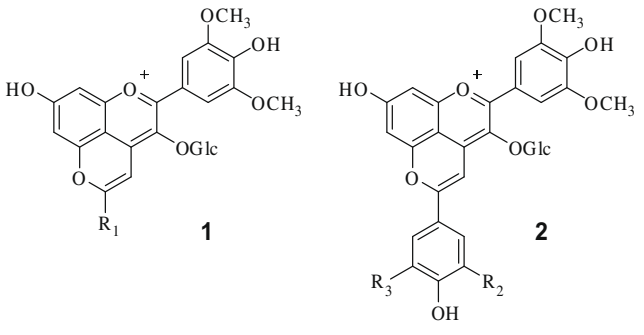

Fig. 1. Chemical structure of wine pyranoanthocyanins derived from malvidin 3-glucoside: **1**, vitisin-type pyranoanthocyanins (R_1 = COOH, vitisin A; R_1 = H, vitisin B); **2**, hydroxyphenyl-type pyranoanthocyanins (R_2 = R_3 = H, malvidin 3-glucoside-4-vinylphenol or mv-3-glc-4-VP; R_2 = H and R_3 = OH, malvidin 3-glucoside-4-vinylcatechol or pinotin A; R_2 = H and R_3 = OCH_3, malvidin 3-glucoside-4-vinylguaiacol or mv-3-glc-4-VG).

2. Materials and methods

2.1. Chemicals and wine samples

All solvents were of HPLC quality and all chemicals of analytical grade (>99%). Water was of MilliQ® quality. The HPLC commercial standards used for quantification were: malvidin 3-glucoside (PhytoLab, Vestenbergsgreuth, Germany); and caffeic and *p*-coumaric acids (Merck, Darmstadt, Germany). Wine samples were divided into two sets: the first was a vertical series of wines supplied by one individual cellar (Vinícola de Castilla, Manzanares, located in the southern middle Spanish region called La Mancha) elaborated with similar quality grapes and winemaking conditions and covering the vintages period 1979–2007 (48 samples with the exception of years 1981, 1982, 1985, 1988 and 1992); the second wine set corresponded to commercial wines purchased at local wine stores, which were also produced in the winemaking region of La Mancha. The sampling of the commercial wines (106 samples) covered a wide range of ageing times, from the youngest wines (1 year old) up to the oldest available wines (10 years old). As the set of commercial wines was collected and analysed at three different times (September 2005, September 2006, and November 2008) the wine samples were grouped by age (Table 1), that is, by calculating the difference in years between analysis time and vintage of elaboration. Only red wines subjected to Origin Denomination control with a declaration as "Tempranillo (or its synonym Cencibel) wines" were selected; a control which implies that at least 85% of the wine has to be made from Tempranillo grapes.

2.2. Wine refreshment experiments

Young wine (2007 vintage; 150 ml) was added to old Tempranillo wine (2002 vintage; 850 ml) and then was gently homogenised. Two different young wines were assayed, one was a Tempranillo wine and the other was a Petit Verdot wine. After that, the resulting refreshed Tempranillo wine was distributed in 25-ml dark glass bottles, flushed with nitrogen to remove air in the headspace, and sealed. The refreshed wines were submitted to an accelerated ageing process following a described procedure (Ugliano, Siebert, Mercurio, Capone, & Henschke, 2008), consisting in maintaining them in darkness at 30 °C in an oven. Samples were taken in triplicate each week for HPLC analysis.

Table 1
Mean values ± standard deviations for the content (mg/l) of pyranoanthocyanins and related compounds (their precursors, malvidin 3-glucoside, free and bound hydroxycinnamic acids) in commercial Tempranillo wines grouped by age.

Compound	1 year (n = 5)	2 years (n = 9)	3 years (n = 4)	4 years (n = 19)	5 years (n = 16)	6 years (n = 20)	7 years (n = 17)	8 years (n = 6)	9 years (n = 6)	10 years (n = 4)
mv-3-glc	62.9^{a} ± 11.0	18.7^{b} ± 15.8	9.6^{b} ± 12.5	33.0^{b} ± 28.7	13.8^{b} ± 27.1	13.7^{b} ± 19.1	24.3^{b} ± 26.6	12.2^{b} ± 14.7	6.1^{b} ± 13.0	11.0^{b} ± 20.1
Vitisin A	3.21 ± 2.97	0.88 ± 0.26	0.67 ± 0.20	3.99 ± 2.60	3.23 ± 4.06	2.10 ± 1.68	1.64 ± 2.08	2.99 ± 1.87	1.64 ± 0.84	1.43 ± 0.44
Pinotin A	0.45 ± 0.62	0.34 ± 0.34	0.42 ± 0.28	1.51 ± 1.16	0.64 ± 0.48	1.04 ± 0.87	1.02 ± 0.92	0.88 ± 0.25	0.81 ± 0.65	0.78 ± 0.98
mv-3-glc-4-VP	0.17 ± 0.09	0.15 ± 0.07	0.10 ± 0.10	0.52 ± 0.34	0.28 ± 0.32	0.36 ± 0.35	0.29 ± 0.32	0.36 ± 0.21	0.23 ± 0.18	0.24 ± 0.31
Caftaric acid	39.6 ± 11.4	37.1 ± 14.8	29.7 ± 2.5	27.8 ± 9.0	28.4 ± 9.3	25.0 ± 8.7	25.6 ± 14.4	26.2 ± 8.8	29.7 ± 12.9	31.2 ± 15.0
Coutaric acid	19.9 ± 8.8	20.5 ± 8.0	19.10 ± 5.3	18.3 ± 7.4	19.6 ± 13.8	16.0 ± 7.8	17.5 ± 13.3	26.9 ± 14.3	16.6 ± 16.9	19.0 ± 18.7
Caffeic acid	13.6^{ab} ± 2.0	11.6^{ab} ± 2.1	8.9^{a} ± 3.2	17.7^{b} ± 8.2	11.5^{ab} ± 3.9	13.8^{ab} ± 5.0	11.8^{ab} ± 4.4	11.4^{ab} ± 1.8	13.4^{ab} ± 3.9	15.1^{ab} ± 3.7
p-Coumaric acid	4.5^{ab} ± 2.1	4.3^{ab} ± 2.2	2.3^{a} ± 1.0	9.5^{b} ± 6.3	4.6^{ab} ± 2.7	5.8^{ab} ± 3.3	5.6^{ab} ± 3.8	6.8^{ab} ± 2.1	5.4^{ab} ± 4.6	5.1^{ab} ± 4.3

Different letters in the same row indicate significant differences according to the test of Student–Newman–Keuls (α = 0.05).

2.3. Identification and quantitative analysis of anthocyanins, pyranoanthocyanins, and hydroxycinnamic acid derivatives by HPLC with electrospray ionisation multiple mass spectrometry (HPLC–ESI-MS^{n})

HPLC separation, identification and quantification of Tempranillo wine phenolics were performed on an Agilent 1100 Series system (Agilent, Waldbronn, Germany), equipped with DAD (G1315B) and LC/MSD Trap VL (G2445C VL) electrospray ionisation mass spectrometry (ESI-MS^{n}) system, and coupled to an Agilent Chemstation (Version B.01.03) data-processing station. The mass spectral data were processed with the Agilent LC/MS Trap software (version 5.3). The wine samples were injected (50 µl) after filtration (0.20 µm, polyester membrane, Chromafil PET 20/25, Macherey–Nagel, Düren, Germany) on a reversed-phase column Zorbax Eclipse XDB-C18 (4.6 × 250 mm; 5 µm particle; Agilent, Germany), thermostatted at 40 °C. The chromatographic conditions were adapted from the OIV method for analysis of anthocyanins in red wines (Office International de la Vigne et du Vin, 2003). The solvents were water:acetonitrile:formic acid (87:3:10, v/v/v, solvent A; 40:50:10, v/v/v, solvent B), and the flow rate was 0.63 ml/min. The linear gradient for solvent B was: 0 min, 6%; 15 min, 30%;

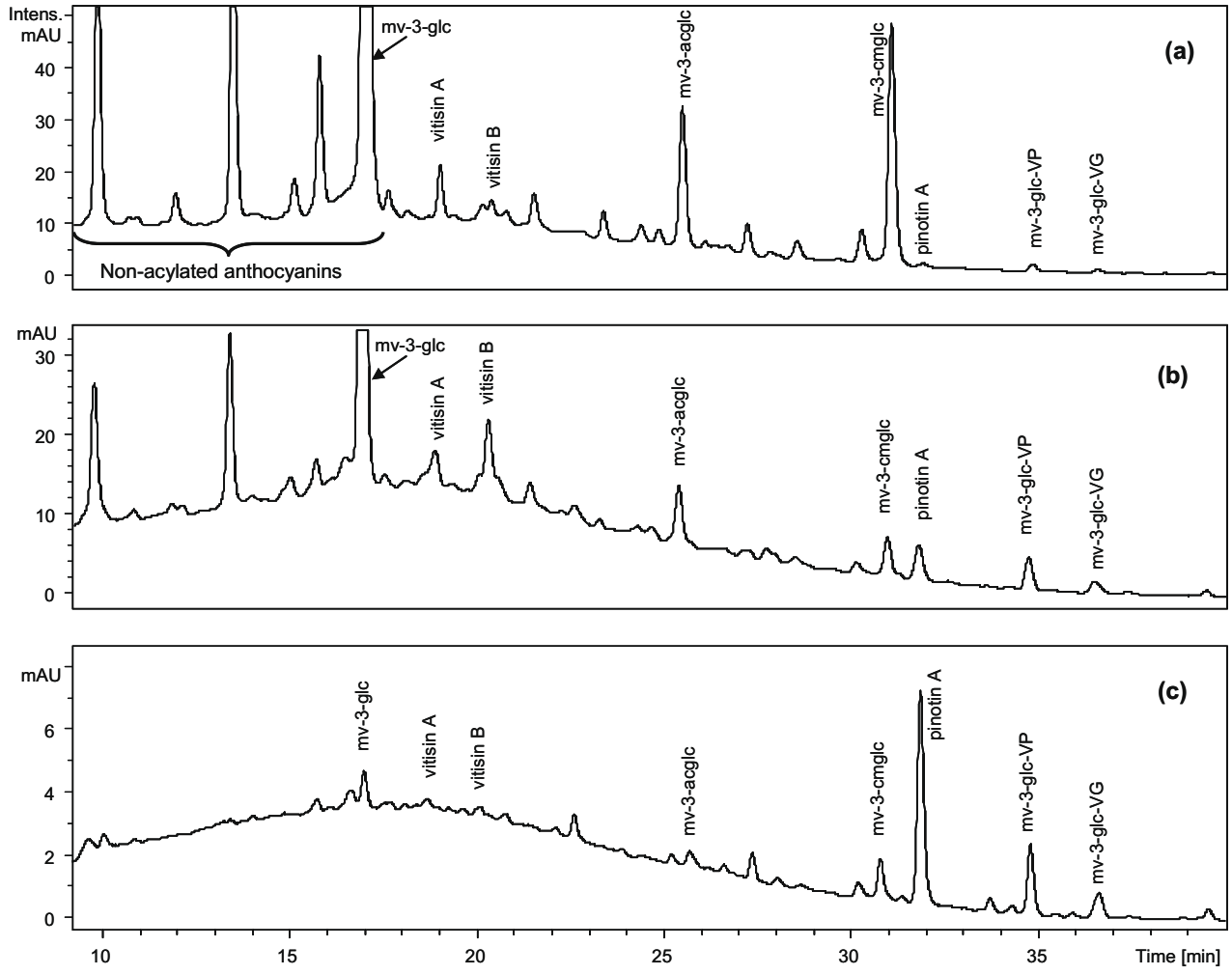

Fig. 2. Changes in anthocyanins and pyranoanthocyanins of Tempranillo wines during ageing (a) 2 year-old wine; (b) 4 year-old wine; (c) 7 year-old wine. Peak assignation: mv, malvidin; glc, glucoside; ac, acetyl; cm, *p*-coumaroyl; VP, vinylphenol; VG, vinylguaiacol.

30 min, 50%; 35 min, 60%; 38 min, 60%; 46 min, 6%. For identification, ESI-MS^n was used employing the following parameters: positive ion mode; dry gas, N_2, 11 ml/min; drying temperature, 350 °C; nebuliser, 65 psi; capillary, −2500 V; capillary exit offset, 70 V; skimmer 1, 20 V; skimmer 2, 6 V; scan range, *m/z* 50–1200. For quantification, different DAD-chromatograms were extracted at 520 nm (malvidin 3-glucoside, mv-3-glc), 510 nm (vitisin A and hydroxyphenyl-pyranoanthocyanins), and 320 nm (hydroxycinnamic acid derivatives). Vitisin A and pinotin A used as reference standards were prepared as previously described, by reaction of mv-3-glc with pyruvic acid (Schwarz et al., 2003b) or isolation from Pinotage wine (Schwarz, Jerz, & Winterhalter, 2003a), respectively. Malvidin 3-glucoside-4-vinylphenol (mv-3-glc-4-VP) used as standard was isolated from the reaction mixture of mv-3-glc with *p*-coumaric acid using similar conditions as described for the synthesis of pinotin A (Schwarz & Winterhalter, 2003).

2.4. Statistical data analysis

ANOVA analysis of the wine data grouped by age was performed (Student–Newman–Keuls test, $\alpha = 0.05$; SPSS version 10.0, SPSS Inc., Chicago, IL), in order to look for significant differences. The wine data were also submitted to principal components analysis (SPSS version 10.0, SPSS Inc.), to test the possibility of grouping, paying attention to its composition in pyranoanthocyanins and also their precursors.

3. Results and discussion

3.1. Tempranillo wine vertical series

Reversed-phase HPLC can successfully separate red wine pigments, which are not of a polymeric nature. Usually, the HPLC-chromatogram at 520 nm of a young Tempranillo red wine is strongly dominated by native grape anthocyanins. Additionally some minor peaks that correspond to pyranoanthocyanins, especially those derived from pyruvic acid (vitisin A) and acetaldehyde (vitisin B), are detectable (Fig. 2a). The ageing induces a decrease in the anthocyanin concentration in Tempranillo wines while, simultaneously, an increasing importance of hydroxyphenyl-pyranoanthocyanins can be observed (Fig. 2b). It is common that, after an ageing time of 5–7 years, the anthocyanins are completely degraded and the chromatogram is usually dominated by pyranoanthocyanins (Fig. 2c). Analysing a vertical series of wines elaborated in the same winery over 29 years, the native malvidin 3-glucoside (mv-3-glc) almost disappeared in wines of 6 or more years old (Fig. 3a), accounting for 55–57 mg/l in the first year of ageing (vintage 2007) and then strongly decreasing its concentration below 15 mg/l for wines 2–5 years old (vintages 2002 to 2006). However, relatively high concentrations of mv-3-glc were found for some wines of the 2003 vintage and all the wine samples of the 2002 vintage, which could be very likely explained by the typical variations found in biological materials and also as a result of the many conditions that can influence the content of anthocyanins in wine (for instance, some years the same vineyards can notably vary in the content of grape anthocyanins, due to unexpected variations in the grape maturation process). Regarding the content of pyranoanthocyanins in the wines of this vertical series, the general trend found for vitisin A was a decrease over ageing of its content that was frequently interrupted by various temporary maxima and minima (Fig. 3b), in close accordance to the behaviour reported for a similar vertical series of Chilean Cabernet Sauvignon wines (Schwarz et al., 2003b). In the case of the hydroxyphenyl-pyranoanthocyanins malvidin 3-glucoside-4-vinylcatechol (mv-3-glc-4-VC, or pinotin A; Fig. 3c) and malvidin 3-glucoside-4-vinylphenol (mv-3-glc-4-VP; Fig. 3d), their concentrations initially tended to

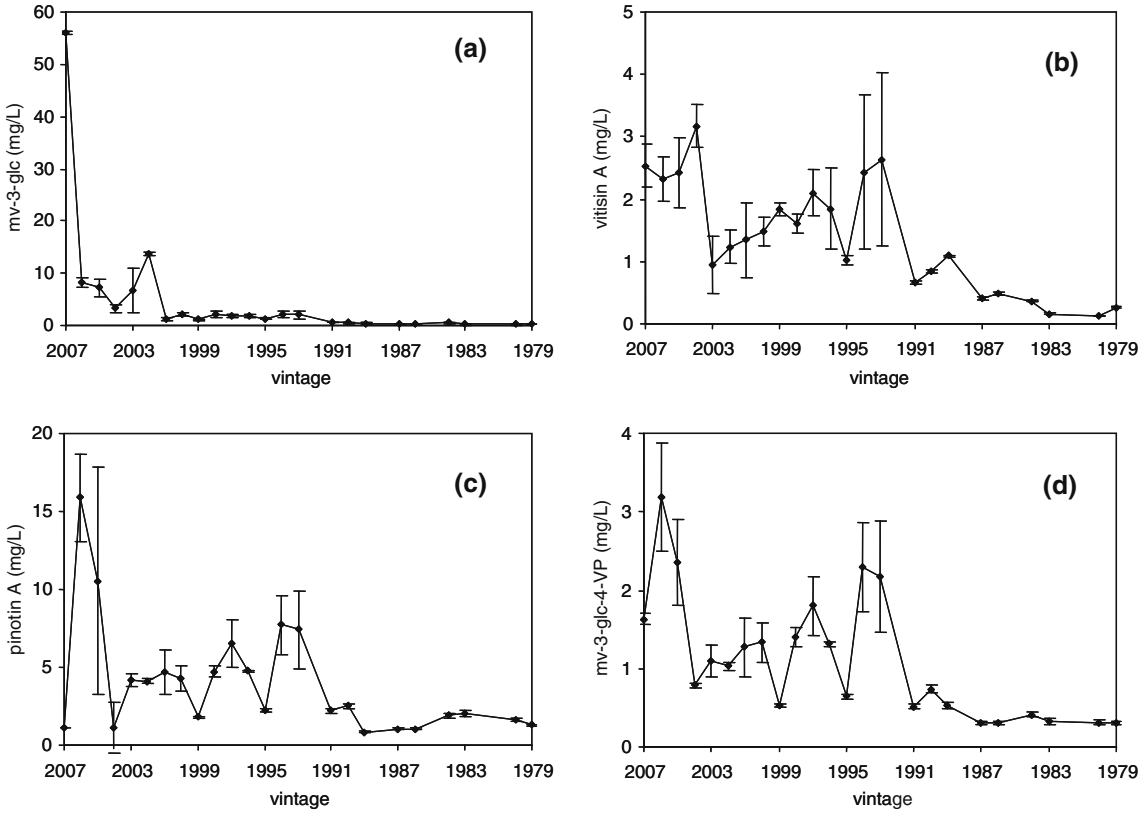

Fig. 3. Tempranillo wine vertical series. Variation with wine age of the concentrations (mg/l) of (a) malvidin 3-glucoside (mv-3-glc); (b) vitisin A; (c) pinotin A (malvidin 3-glucoside-4-vinylcatechol); (d) malvidin 3-glucoside-4-vinylphenol (mv-3-glc-4-VP).

show a strong increase within the first 2 years of ageing (wines from the 2007 and 2006 vintages), reaching the highest values found for pinotin A (17.7 mg/l) and mv-3-glc-4-VP (3.8 mg/l), and continued with an abrupt decrease over the next 2 years of ageing (wines from the 2005 and 2004 vintages) that further tended to a new increase during the next 11 years of ageing (wines from the 2003 to 1993 vintages; concentrations increased up to 9.9 and 2.9 mg/l for pinotin A and mv-3-glc-4-VP, respectively), although this trend suffered two interruptions with minima values (wines from the 1999 and 1995 vintages). Finally, wines older than 15 years showed the lowest concentrations of both hydroxyphenyl-pyranoanthocyanins (around 1–2 and 0.3–0.7 mg/l for pinotin A and mv-3-glc-4-VP, respectively) and they did not change much over the next 14 years of ageing (wines from the 1992 to 1979 vintages). The trend shown by the content of both pinotin A and mv-3-glc-4-VP over ageing was quite similar, but a remarkable difference was the relative initial value of their concentrations: pinotin A was almost a trace compound (less than 1 mg/l) in 1 year-old wines (2007 vintage), whereas mv-3-glc-4-VP already accounted in the first year of ageing for a remarkably high concentration (mean value of 1.6 mg/l) and only a few wines (2006, 2005, 1997, 1994 and 1993 vintages) showed higher values than this initial concentration.

3.2. Vitisin A content in Tempranillo wines

Commercial Tempranillo wines showed a great variability in their content of pyranoanthocyanins (Table 1). Concentrations of the different pyranoanthocyanins detected were (range and mean value, in mg/l): vitisin A, 0–10.76 (2.43); pinotin A, 0–4.26 (0.92); and mv-3-glc-4-VP, 0.03–1.37 (0.32). These wide concentration ranges led to no significant differences when the contents of pyranoanthocyanins were statistically analysed by the ANOVA test of Student–Newman–Keuls with regard to the wine age (Table 1). Also the principal component analysis of the data failed in grouping of wine samples by age (data not shown).

The pyranoanthocyanin vitisin A is formed during alcoholic fermentation by a reaction of pyruvic acid with malvidin 3-glucoside (mv-3-glc). Although the occurrence of vitisin A in wine was first reported in 1997 (Bakker & Timberlake, 1997; Bakker et al., 1997), there are still very little data available on its content in commercial wines and also its evolution during ageing. In the case of Tempranillo wines, only a few papers have reported concentrations of vitisin A around 0.5–6.0 mg/l in 6–30 months-old wines (Revilla & González-Sanjosé, 2001a, 2001b). The reported vitisin A content for other commercial and experimental wines is usually within the aforementioned range (Asenstorfer, Markides, Illand, & Jones, 2003; Schwarz et al., 2003b), with the exception of young port wines that reached up to 9.5–15.4 mg/l of vitisin A, due to a special winemaking technique (Mateus & de Freitas, 2001; Romero & Bakker, 2001). Therefore, the content of vitisin A in most of the wines that were analysed in our investigation (the 48 samples of the vertical series, and 96 commercial samples out of 106) was within a typical range (i.e., between 0 and 5.61 mg/l), while only 10 of the commercial wine samples showed unexpectedly high values (6.38–10.76 mg/l).

With regard to the degradation rate of vitisin A during wine ageing, the results reported for a vertical row of Chilean Cabernet Sauvignon wines (wines from the same cellar covering 16 years of ageing) showed that maximum concentrations of vitisin A were reached within the first year of storage, when pyruvic acid is still available, as it was only produced during alcoholic fermentation by yeast. After this period, vitisin A concentration slowly decreased from 5 to 1 mg/l (Schwarz et al., 2003b). The results found for the vertical series of Tempranillo wines were in this line, although the vitisin A concentration range was lower than that found for Chilean Cabernet Sauvignon wines, starting around 3 mg/l for 1 year-old wines and finishing around 0.2 mg/l after 29 years of ageing. In contrast, the set of commercial Tempranillo wines did not show clearly this trend (Table 1) and relative maximum concentrations of vitisin A were detected not only in 1 year-old wines (mean value of 3.21 mg/l), but also in 4 year (mean value of 3.99 mg/l) and 8 year (mean value of 2.99 mg/l) wines. However, in the aforementioned study the general decline of vitisin A in Chilean Cabernet Sauvignon wines was also interrupted by various temporary fluctuations (Schwarz et al., 2003b), which were observed in our Tempranillo vertical series of wines (Fig. 3b). With the exception of the maxima found in 4 and 8 years-old commercial Tempranillo wines, the concentration of vitisin A showed a slight tendency to decrease over three ageing periods, i.e., 1–3, 4–7 and 8–10 years of ageing (Table 1), although the observed high standard deviations suggest the need for further confirmation. One likely explanation for this observation is that the commercial Tempranillo wines were produced in different cellars using different yeast strains and winemaking techniques, resulting in different amounts of pyruvic acid, which is required for the formation of vitisin A (Monagas, Gómez-Cordovés, & Bartolomé, 2007). Together with variations in the initial contents of native grape anthocyanins, these different conditions result in different initial concentrations of vitisin A that can explain the observed maxima. In relation to the latter, the attempt to correlate the content of vitisin A to the concentration of mv-3-glc in the wines of our study was not successful (r^2 value of 0.266), as the initial concentration of vitisin A and mv-3-glc remained unknown. This indicates that the current content of vitisin A in a wine is the result of several factors. A correlation of the vitisin A content with wine age appears to be inherently hampered by the varying initial composition of wines. In addition, the lack of an extensive database of vitisin A content in wines and the necessary studies dealing with the factors affecting the kinetics of degradation of vitisin A (i.e., temperature, redox potential, interaction with other wine constituents) make the interpretation of the significance of vitisin A content in a single wine not yet possible.

3.3. Hydroxyphenyl-pyranoanthocyanins content in Tempranillo wines

In relation to hydroxyphenyl-pyranoanthocyanins, it is noticeable that pinotin A was not detectable in many of the 1 year-old wines and only traces appeared in several of the 1- and 2-years-old wines (Table 1). Most of the Tempranillo wine samples contained pinotin A at a concentration below 2.5 mg/l (100 samples of commercial wines and 27 samples of the vertical series wines), and the rest of the samples showed higher concentrations, ranging within 2.6–5.7 mg/l (6 samples of the commercial wines and 13 samples of the vertical series wines); moreover, 8 of the wines of the vertical series contained very high amounts of pinotin A, within the range of 6.1–17.8 mg/l. The only data available for comparison are that of commercial Pinotage wines (Schwarz et al., 2004). In these wines high contents of pinotin A within the range 0.15–17.93 mg/l and mean values of 2.6–5.3 mg/l were detected that were explained by the naturally high contents of caffeic acid in this grape variety. The results obtained for the analysed Tempranillo wines supported the hypothesis that pinotin A is almost exclusively produced after the release of caffeic acid from its tartaric ester (Rentzsch et al., 2007b), as an acceptable linear correlation was found for the contents of both pinotin A and its precursor, caffeic acid (Fig. 4a); a similar correlation was found for the vertical series wines (r^2 = 0.680; data not shown). This confirmed the results of previous studies carried out with Pinotage wines, that pointed out that the formation of pinotin A in aged wines ($\leqslant$5 years) largely depends on the caffeic acid concentration (Schwarz et al., 2004). It is also important to note that, despite the similar mean

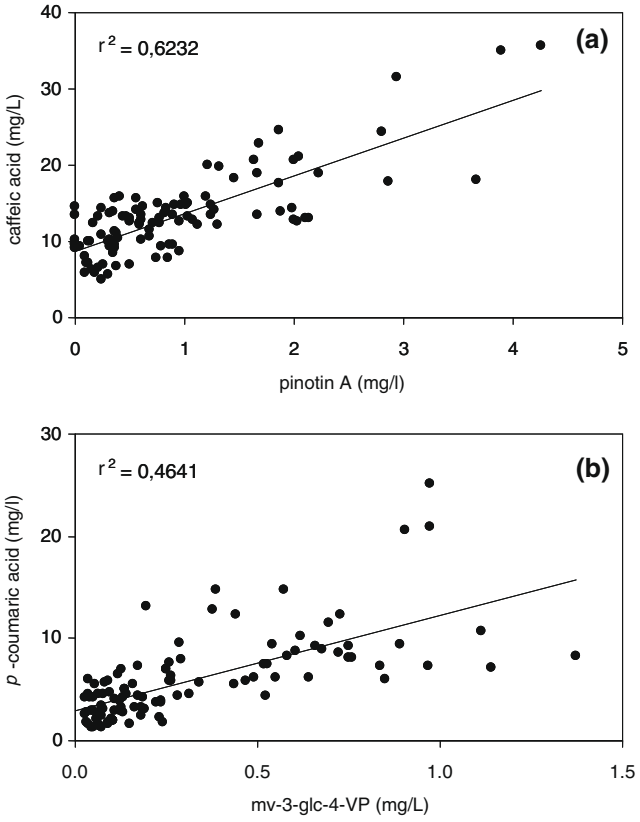

Fig. 4. Correlation of free hydroxycinnamic acid concentration (mg/l) to the corresponding concentration (mg/l) of hydroxyphenyl-pyranoanthocyanins in Tempranillo wines (a) caffeic acid *vs.* malvidin 3-glucoside-4-vinylcatechol (mv-3-glc-4-VC, or pinotin A); (b) *p*-coumaric acid *vs.* malvidin 3-glucoside-4-vinylphenol (mv-3-glc-4-VP).

concentrations found for caffeic acid in commercial Tempranillo wines over the ageing time (9–18 mg/l; Table 1), the formation of pinotin A was not remarkable in the first and second year of ageing. Unlike *p*-coumaric acid, caffeic acid is not decarboxylated by an enzymatic side activity of the yeast (Chatonnet, Dubourdieu, Boidron, & Lavigne, 1993), which is the main reaction pathway for formation of hydroxyphenyl-pyranoanthocyanins in young wines. However, Morata, González, and Suárez-Lepe (2007) recently reported on the formation of pinotin A in red musts supplemented with caffeic acid, which were fermented by selected yeast strains possessing intermediate and high hydroxycinnamate decarboxylase activity; the formation of pinotin A by the enzymatic pathway during alcoholic fermentation must be carefully revised. The observed slow development of pinotin A could be due to the fact that the reaction partner of caffeic acid to form pinotin A, i.e., the anthocyanin mv-3-glc, is likely to be involved in other competing reactions, such as pigment polymerisation. This may be one reason why the correlation of the concentration of pinotin A with the combined concentrations of caffeic acid and mv-3-glc did not improve much (data not shown), as compared to the correlation of pinotin A to caffeic acid concentration alone.

In contrast to pinotin A, mv-3-glc-4-VP was already present in the youngest Tempranillo wines. To our knowledge, this is the first time that data on the concentration of mv-3-glc-4-VP have been reported for commercial Tempranillo wines. Previously reported data mainly corresponded to experimental wines. Concentration of mv-3-glc-4-VP in commercial Tempranillo wines rarely exceeded 1.0 mg/l (only 3 samples out of the 106 commercial samples contained mv-3-glc-4-VP within the range 1.1–1.4 mg/l) and most of the youngest wines (1 and 2 years old) showed concentrations below 0.3 mg/l, a value rather similar to the 0.30–0.36 mg/l reported for Grenache wines after completion of malolactic fermentation (Rentzsch et al., 2007b). However, the Tempranillo wines of the vertical series showed higher contents of mv-3-glc-4-VP (29 samples contained less than 1.0 mg/l, and the content of the other 19 samples ranged within 1.1–3.8 mg/l), as also observed for the content of pinotin A. These results can be explained by an initial formation of this pyranoanthocyanin *via* enzymatic decarboxylation of *p*-coumaric acid during the alcoholic fermentation, followed by a direct reaction of released *p*-coumaric acid over the ageing time (its mean concentration significantly increased from 2.3–4.5 to 4.6–9.5 mg/l; Table 1). Our results suggest that mv-3-glc-4-VP is formed after enzymatic decarboxylation of *p*-coumaric acid, during alcoholic fermentation, reaching concentrations no higher than 0.3 mg/l. This concentration is increased during ageing by the pure chemical formation of mv-3-glc-4-VP when *p*-coumaric acid is released from coutaric acid. However, the maximum concentration of mv-3-glc-4-VP detected is around 3.8 mg/l in Tempranillo wines, an amount significantly lower than the maximum value found for pinotin A (17.7 mg/l) in the same wines. These values correspond to the proportions of the precursor *p*-coumaric and caffeic acids and their bound forms (coutaric and caftaric acids, respectively) that are present in these wines (Table 1). Due to the two different pathways of formation of mv-3-glc-4-VP, the correlation between the concentrations of this hydroxyphenyl-pyranoanthocyanin and its precursor, the *p*-coumaric acid, was poor in both sets of commercial wines (Fig. 4b) and vertical series wines (r^2 = 0.458; data not shown). The formation of mv-3-glc-4-VP in aged wines seems, obviously, not to be solely under the control of *p*-coumaric acid concentrations, as its initial concentration is determined by enzymatic activity. As found for pinotin A, the formation of mv-3-glc-4-VP was poorly influenced by mv-3-glc concentration (data not shown).

3.4. Refreshment experiments of aged Tempranillo wine

On the basis of the hypothesis of formation of hydroxyphenyl-pyranoanthocyanins by direct reaction of hydroxycinnamic acids with anthocyanins, their content in Tempranillo wines is expected to increase with ageing time, as has been demonstrated for Pinotage wines (Schwarz et al., 2004). This was the initial trend shown in commercial Tempranillo wines, but an unexpected maximum content of hydroxyphenyl-pyranoanthocyanins appeared in 4 years-old wines, followed by a new decrease and a further slight increase (Table 1). Associated with this unexpected maximum, there was a high content of mv-3-glc in 4 years-old wines and, in a lesser extent, in 7 years-old wines (Table 1), opposite to the normal trend of evolution for anthocyanins during wine ageing as indicated the study of the vertical series of Tempranillo wines (Fig. 3a). The latter results can partly be explained by higher initial values of the precursors, but more likely is the use of a winemaking technique referred to as the "refreshment" of old wines; that is the addition of young red wines to aged wines with the aim of improving some sensory properties. This assumption is backed up by the observed high concentration of mv-3-glc (more than 50 mg/l) found in several of the wines of 4 years old, or older.

For supporting the refreshment hypothesis, we carried out experiments of addition of young wine (2007 vintage) to aged Tempranillo wines (2002 vintage). The aged Tempranillo wine contained mv-3-glc in trace amounts and only free caffeic and *p*-coumaric acids were observed (Table 2); this wine already contained vitisin A and also important amounts of pinotin A and mv-3-glc-4-VP. No further formation of pinotin A and mv-3-glc-4-VP was expected in this aged wine, due to the lack of one of the reactants, the native grape anthocyanins (e.g., mv-3-glc). Two different young

Table 2
Composition (mg/l) in pigments and hydroxycinnamic acids of the aged and young wines used in the refreshment experiments. nd, not detected.

Wine	Tempranillo 2002	Tempranillo 2007	Petit Verdot 2007
mv-3-glc	0.90	52.9	50.8
Vitisin A	0.79	6.49	13.9
Pinotin A	25.4	0.99	7.64
mv-3-glc-4-VP	1.74	1.36	1.55
Caftaric acid	nd	23.1	5.87
Coutaric acid	nd	8.41	1.60
Caffeic acid	13.9	9.99	41.5
p-Coumaric acid	8.90	5.59	13.2

wines were assayed, one was a Tempranillo young wine and the other was a Petit Verdot young wine, because a single-variety wine subjected to Origin Denomination must contain at least 85% (v/v) of the wine of the respective grape variety, but there is no restriction about the remaining 15% wine volume. Both young wines contained high amounts of mv-3-glc, and their contents in free hydroxycinnamic acids and also in pyranoanthocyanins were as expected for young wines, especially in the case of young Tempranillo wine (Table 2). To accelerate the ageing process, refreshed wines were kept in darkness at 30 °C in an oven for several weeks. In both cases a constant decline in mv-3-glc concentration was observed during ageing (Figs. 5a and 6a). It could be expected that the disappeared mv-3-glc had reacted with the free caffeic and *p*-coumaric acids, but no immediate increase in the contents of pinotin A and mv-3-glc-4-VP were observed. Moreover, a characteristic delay in the increase of the content of these two hydroxyphenyl-pyranoanthocyanins was observed, 2 weeks in the case of aged Tempranillo wine refreshed with young Tempranillo (Fig. 5c and d) and up to 4 weeks in the case of the wine refreshed with young Petit Verdot wine (Fig. 6c and d), thus suggesting a different response of the refreshment treatment based on the compositional differences (regarding competing substances reacting with mv-3-glc) between the added young wines. After this delay, the concentrations of pinotin A and mv-3-glc-4-VP increased during ageing, reaching a maximum after 5–6 weeks of ageing (the concentrations at this moment were twice the initial concentrations for each hydroxyphenyl-pyranoanthocyanin). The subsequent evolution of the concentration of both hydroxyphenyl-pyranoanthocyanins was different according to the added young wine. They were decreasing in the case of the wine refreshed with young Tempranillo wine, whereas they remained almost constant in the case of the wine refreshed with young Petit Verdot. The aforementioned results were in agreement with the results reported for Pinotage wines (Schwarz et al., 2004), which clearly indicated that high amounts of mv-3-glc present in young wines are rapidly consumed by various competing reactions (i.e., formation of polymeric flavanol–anthocyanin pigments) and only a very small percentage is converted into hydroxyphenyl-pyranoanthocyanins (i.e., pinotin A). The over-proportional production of hydroxyphenyl-pyranoanthocyanins commences when interfering reactions become less likely, due to a lower concentration of the reactants, such as flavanols and anthocyanins, while the hydroxycinnamic acids concentrations remains rather stable, or even increase, throughout the ageing process. An increase of mv-3-glc concentration in aged Tempranillo wines, by means of the previously mentioned "refreshment", introduces a very efficient competitor in the formation of polymeric pigments, thus helping to maintain the current concentrations of pinotin A and mv-3-glc-4-VP in the wines. The refreshment treatment and its influence on pyranoanthocyanin

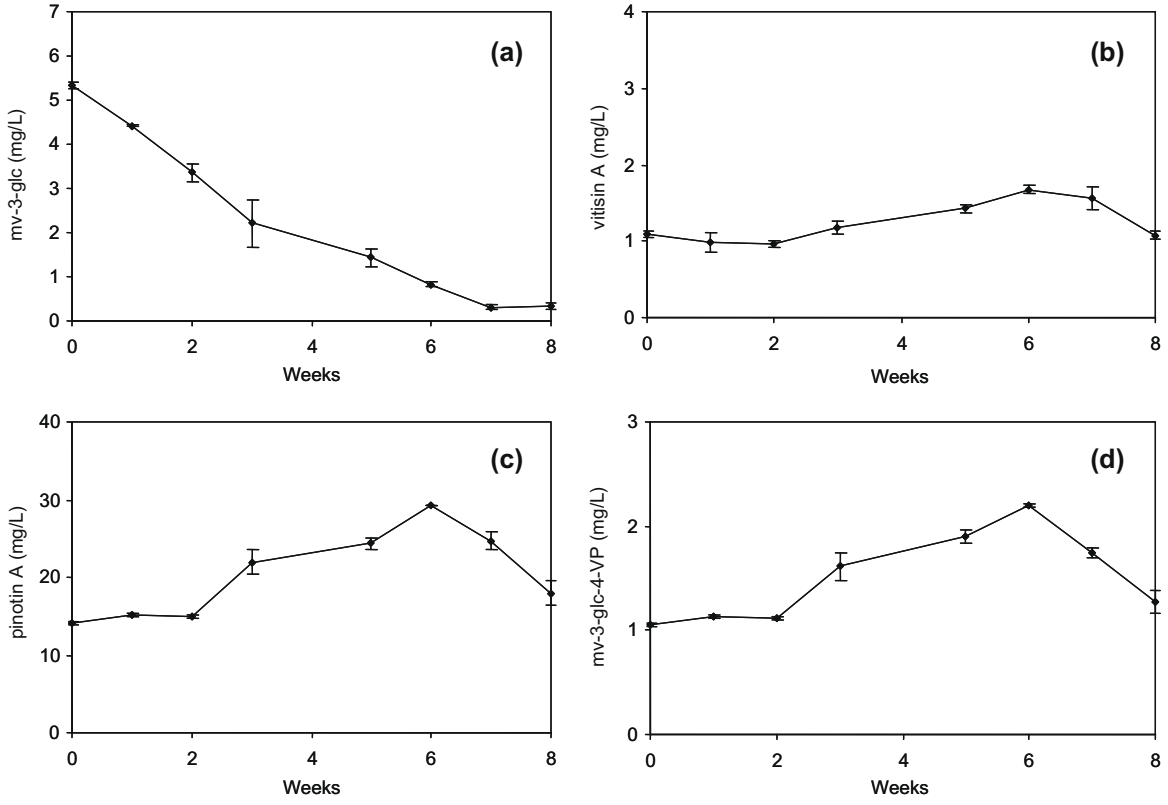

Fig. 5. Refreshment of aged Tempranillo wine (2002 vintage) with 15% of young Tempranillo wine (2007 vintage). Variation during accelerated ageing at 30 °C of the concentrations (mg/l) of (a) malvidin 3-glucoside (mv-3-glc); (b) vitisin A; (c) pinotin A (malvidin 3-glucoside-4-vinylcatechol); (d) malvidin 3-glucoside-4-vinylphenol (mv-3-glc-4-VP).

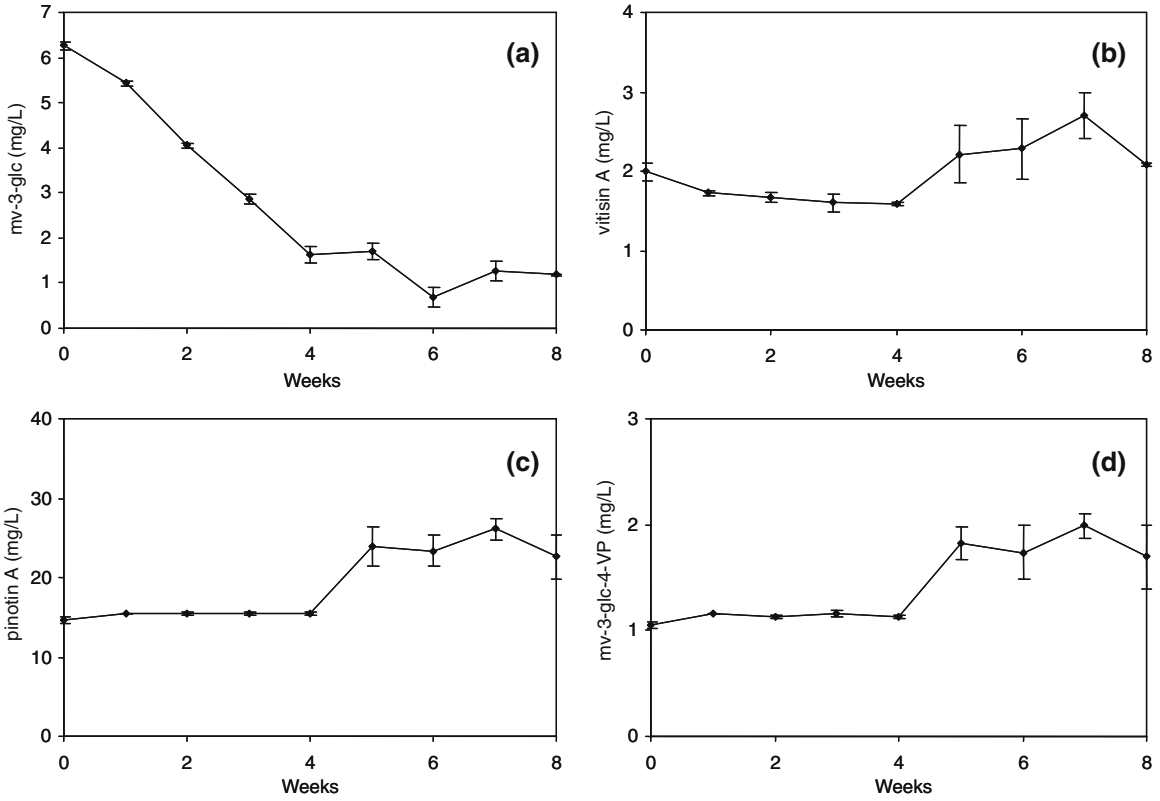

Fig. 6. Refreshment of aged Tempranillo wine (2002 vintage) with 15% of young Petit Verdot wine (2007 vintage). Variation during accelerated ageing at 30 °C of the concentrations (mg/l) of (a) malvidin 3-glucoside (mv-3-glc); (b) vitisin A; (c) pinotin A (malvidin 3-glucoside-4-vinylcatechol); (d) malvidin 3-glucoside-4-vinylphenol (mv-3-glc-4-VP).

development make it impossible to use the hydroxyphenyl-pyranoanthocyanin concentration for the determination of the age of commercial Tempranillo wines, as reported for Pinotage wines (Schwarz et al., 2004). Finally, vitisin A content in refreshed wines was also affected by the addition of the young wine (Figs. 5b and 6b), as occurred to pinotin A and mv-3-glc-4-VP. However, a slight decrease in vitisin A content was observed in the first week of ageing and the maximum contents reached were only one and a half times the initial concentrations. The results suggest that added young wine can also be a source of pyruvic acid that can lead to the formation of vitisin A, as discussed for the formation of pinotin A and mv-3-glc-4-VP. Free pyruvic acid is present in wines and is very likely that this alcoholic fermentation metabolite accounted for higher concentrations in young wines, as compared to aged wines. Moreover, the increase of the concentration of vitisin A in red wines by means of pyruvic acid addition has been reported (Romero & Bakker, 2000; Mateus and de Freitas, 2001). The initial decrease in vitisin A content could be connected to the formation of portisin-type pigments, a class of pyranoanthocyanin pigments formed by reaction of vitisin A with both flavanols, in the presence of acetaldehyde and free hydroxycinnamic acids, as has been recently reported (Mateus, Oliveira, Santos-Buelga, Silva, & de Freitas, 2004; Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & de Freitas, 2003; Oliveira, de Freitas, Silva, & Mateus, 2007).

4. Conclusion

In this survey we have contributed to the, so far, only scarce data available for pyranoanthocyanin contents in 1–10 year-old commercial wines elaborated using grapes of the *V. vinifera* Spanish Tempranillo variety. The results show a great variability in the concentrations found for the most common pyranoanthocyanins (vitisin A, pinotin A and mv-3-glc-4-VP).

Vitisin A is formed from pyruvic acid, a yeast metabolite only produced during alcoholic fermentation, and malvidin 3-glucoside (mv-3-glc). Vitisin A was already present in high amounts in the youngest wines (1–3 years old) and its concentration tended to decrease with wine age, although various temporary maxima at 4 and 8 years interrupted this trend. In addition, no correlation of vitisin A content with the concentration of mv-3-glc was found. These results suggest that the content of vitisin A in a wine is the result of several factors which still remain unclear, making the interpretation of the significance of the vitisin A content in a single wine not yet possible.

No pinotin A was detectable in many of the 1 year-old wines and several of the 2 year-old wines. In contrast, mv-3-glc-4-VP was already present in 1 year-old wines. Both hydroxyphenyl-pyranoanthocyanins increased their concentration during the ageing period, as a consequence of not only the releasing of their respective precursors (i.e., caffeic and *p*-coumaric acids), but also the availability of anthocyanins, which were not involved in competing reactions (i.e., polymeric pigment formation). Supporting the latter was the unexpectedly high concentrations of pinotin A and mv-3-glc-3-VP found in several of the detected "refreshed" wines (more than 4 year-old wines having a mv-3-glc concentration higher than 50 mg/l, due to the addition of younger wine, in order to improve their sensory properties after a long ageing period). Confirmation of the effects of refreshment on wine pyranoanthocyanins contents were obtained by model refreshment experiments, involving the addition of young wines to aged wines (ratio of 15:85, v/v) and submitting the refreshed wines to an accelerated ageing. A characteristic delay was observed previous to the increase in the content of not only pinotin A and mv-3-glc-4-VP, but also vitisin A.

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