Interactions between Grape Anthocyanins and Pyruvic Acid, with Effect of pH and Acid Concentration on Anthocyanin Composition and Color in Model Solutions

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The formation of vitisin A, an anthocyanin formed naturally in small quantities in maturing port wines, was studied in model wine solutions at a range of pH values (2.0−4.5) and pyruvate concentrations [molar ratios of pyruvic acid to total anthocyanins (PA/TA) ranging from 12.20 to 172.40]. Additionally, the effect of vitisin A formation on the color changes of these model wines was evaluated. Vitisin A was formed through the interaction between malvidin 3-glucoside and pyruvic acid, and vitisin A in acylated forms, having the 6-position of the sugar acylated with acetic acid (3-acetylvitisin A) and *p*-coumaric acid (3-*p*-coumarylvitisin A), formed through the interaction between pyruvic acid and malvidin 3-acetylglucoside and malvidin 3-*p*-coumarylglucoside, respectively; their identities were confirmed by spectral analysis and FABMS. The maximum formation of these new anthocyanin derivatives was at pH 2.7−3.0, at the higher pyruvic acid concentration (PA/TA of 172.40 units). The vitisins A caused changes in the color of the solution and expressed about 11 times (pH 3) to 14 times (pH 2) more color than the normal anthocyanins. On aging, the model solutions changed from a bluish red, attributable to the main anthocyanins present, to a slightly more orange red, attributable to the vitisin compounds. The aged models containing vitisins A were all much redder than the more red-brown color of the models aged without pyruvic acid.

Keywords: *Model wine solutions; anthocyanins; pyruvic acid; pH; vitisin A; reaction*

INTRODUCTION

Anthocyanins are a widespread source of naturally occurring colorants of foods and a major determinant of red wine quality. The influence of processing variables on the color quality of red wines and the effect of color on overall quality have been demonstrated (Timberlake and Bridle, 1976). The color changes in red wines due to condensation reactions between anthocyanins and other phenolic compounds naturally occurring in wines are well documented, and until recently these were the reactions thought to exert the greatest influence on the color changes in red wines during maturation. Three mechanisms have been proposed, summarized by Ribéreau-Gayon (1982). The first mechanism involves the reaction of a 4-carbonium ion of procyanidin with anthocyanin (carbinol base form) at position 6 or 8, thus forming a dimer. The second mechanism is the reverse of the first one, with an anthocyanin 4-carbonium ion reacting with procyanidin at the 6- or 8-position. This dimer can undergo further condensation reactions. Evidence for the occurrence of mechanism 2 is the formation of xanthylium salts in stored grape juices and wines (Timberlake and Bridle, 1976). Santos-Buelga et al. (1995) showed in model solutions the formation of brown polymers, in the absence of acetaldehyde, by condensation of anthocyanins with flavan 3-ols, which, by oxidation, formed yellow xanthylium derivatives. The third mechanism involves the condensation of anthocyanin and procyanidin by the intermediary of acetaldehyde, a reaction first proposed by Timberlake and Bridle (1976). Further evidence for this mechanism has since been reported (Bakker et al., 1993; Picinelli et al., 1994; García-Viguera et al., 1994; Rivas-Gonzalo et al., 1995).

Until recently the main changes in anthocyanin composition during red wine maturation and the resulting color changes in red wines were believed to be due to the formation of condensation pigments between anthocyanins and flavan 3-ols, occurring both with and without acetyl bridges, depending on the availability of acetaldehyde. Only free acetaldehyde is available for such reactions; in the presence of sulfur dioxide acetaldehyde will bind reversibly to form aldehydebisulfite (Bakker et al., 1986). Formation of acetaldehyde by coupled oxidation of ethanol is known to occur even in the presence of small amounts of sulfur dioxide (Wildenradt and Singleton, 1974) and has also been shown to occur in maturing port wines (Bakker et al., 1986).

However, recent publications reported the formation of new malvidin-derived pigments, indicating that anthocyanins undergo even more complex changes in wines than hitherto had been studied in polymerization reactions. Earlier, Bakker (1985) suggested the formation of a number of new peaks during changes of red port wine, eluting soon after malvidin 3-glucoside. Properties of one of these anthocyanins reported by Bakker (1985) included resistance to color bleaching by sulfur dioxide. This stability in the presence of sulfur dioxide is consistent, by analogy with flavylium salt studies (Timberlake, 1968; Timberlake and Bridle, 1968), with the presence of a 4-substituent on the anthocyanin molecule. Bakker et al. (1997) reported the

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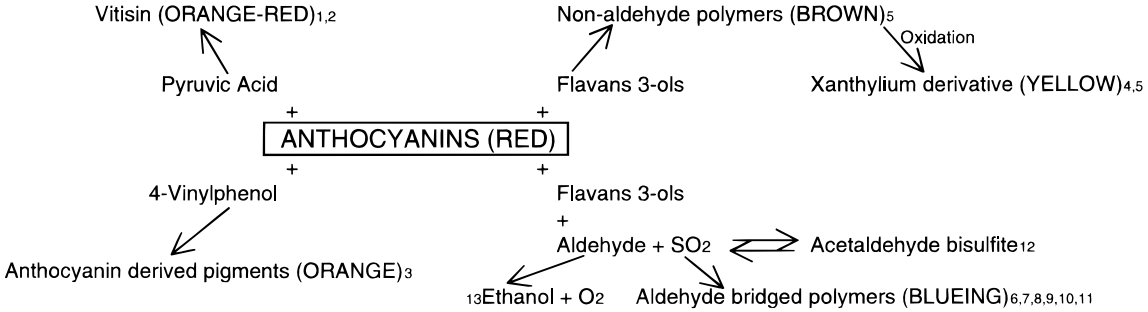
Scheme 1. Anthocyanin Reactions Shown To Occur in Wines, Influencing the Color Changes during Maturation[a]



[a] Red wine references: [1]Bakker et al. (1997); [3]Fulcrand et al. (1996); [4]Liao et al. (1992); [10]Ribéreau-Gayon et al. (1983); [11]Sims and Morris (1986); [12]Bakker et al. (1986); [13]Wildenradt and Singleton (1974). Model solution references: [2]Fulcrand et al. (1998); [3]Fulcrand et al. (1996); [5]Santos-Buelga et al. (1995); [6]Timberlake and Bridle (1976); [7]Bakker et al. (1993); [8]Rivas-Gonzalo et al. (1995); [9]García-Viguera et al. (1994).

isolation of this anthocyanin-type pigment, named vitisin A, from aging red wines and, after purification, have fully identified this compound using a combination of FABMS and ^1H and ^{13}C NMR. They suggested that vitisin A is based on malvidin 3-glucoside with an additional C_3O_2 between position 4 and the 5-hydroxyl of the molecule.

The isolation and structural determination of four new anthocyanin pigments found in red port wines in trace amounts were reported by Bakker and Timberlake (1997). Two pigments were identified as the 3-glucoside (vitisin A) and the 3-acetylglucoside (acetylvitisin A) of malvidin containing a $C_3H_2O_2$ grouping, linking carbon 4 and the 5-hydroxyl group of its molecule (vitisidin A). The other two anthocyanins were identified as the 3-glucoside (vitisin B) and the 3-acetylglucoside (acetylvitisin B) of malvidin containing a CH=CH moiety linking carbon 4 and the 5-hydroxyl group of its molecule (vitisidin B or decarboxy vitisidin A). Most remarkable were their different properties, because unlike other anthocyanins these novel compounds were found to be wholly or partly resistant to bleaching by sulfur dioxide and express more color up to pH 7 than malvidin 3-glucoside. Detailed spectral measurements at both UV and visible wavelengths indicated the formation of stable quinonoidal bases, confirming that there could be little formation of the colorless carbinol base forms.

Further evidence of such reactions was published by Fulcrand et al. (1996), who reported the formation of two new malvidin-derived compounds detected in red wine, which were synthesized in reactions with malvidin 3-glucoside and 4-vinylphenol. Structural elucidation suggested the formation involving cyclization between C-4 and the hydroxy group at C-5 of the flavylium form and the vinylphenol double bond. Fulcrand et al. (1998) also reported the formation of pigments generated by reaction between anthocyanins and pyruvic acid, and one of these compounds generated ^1H and ^{13}C NMR signals similar to those reported by Bakker et al. (1997), although the assignments of some of the signals were different, leading to a slightly different structure being proposed. In red wines all of the above reactions have been shown to occur, as shown in Scheme 1. The extent to which these reactions occur will depend on factors such as wine-making techniques, composition, pH, storage temperature, storage time, acetaldehyde concentration, sulfur dioxide concentration, aeration during storage, and availability of other compounds such as pyruvic acid and 4-vinylphenol. A better understanding of all these factors, and how they may interact, may give us more control over the type of reaction we would like to be prevalent during wine maturation.

The formation of vitisin A as a result of a reaction of malvidin 3-glucoside with pyruvic acid is in agreement with observations made in our laboratory, that these compounds are more abundant in fortified port wine than red table wine (Bakker, 1985). Port wine is made by stopping halfway its fermentation by the addition of fortifying grape spirit, thus maintaining natural sweetness (Reader and Dominguez, 1995). Pyruvic acid, an intermediate of the Embden−Meyerhof−Parnas scheme, is excreted by yeast during fermentations, sometimes in high concentrations. In wine fermentation the maximum concentration occurs when approximately half the sugar has been fermented (Whiting and Coggins, 1960). Subsequently, pyruvate is taken up by the yeast and metabolized further. Because red table wines are generally made by allowing the fermentation to go to dryness, the concentrations of pyruvic acid would be lower in red table wines than in fortified port wines. A range of 11−460 mg/L (mean = 71 mg/L) of pyruvic acid in wines has been recorded (Lafon-Lafourcade and Peynaud, 1966).

The color properties of the vitisins are expected to influence the red wine color and may explain some of the observations made by many enologists regarding the color changes in red wines during maturation. The hypsochromic spectral shifts of both vitisins from malvidin3-glucoside indicate a shift to orange-brown (Bakker and Timberlake, 1997). These authors also reported a browner color of the vitisins than malvidin 3-glucoside at typical wine pH values, and they suggested that the vitisins will make a color contribution at a lower wavelength, that is, toward the orange-brown region, than malvidin 3-glucoside. The greater color expression in aqueous solutions of the vitisins than malvidin 3-glucoside at pH values more typical for red wine (~3.8) may also be significant in wine. In further studies Bakker et al. (1998) reported a significant increase in the percentage of vitisin A from 3.5 to 4.7% in young wines to between 25 and 36% of the anthocyanins after 24 months and concluded that even though the concentrations were quite small, the stronger red color expression of vitisin A becomes an important factor, contributing a redness to the older wines.

The aim of this study was to study in model wines the formation of vitisin A, an anthocyanin formed by reaction between pyruvic acid and malvidin 3-glucoside, and in particular the effect of pyruvate concentra-

Table 1. Concentrations of Main Anthocyanins Determined during Storage at 30 °C of Model Solution (pH 3.5) Containing Grape Skin Extract and Pyruvic Acid (PA/TA = 235 Units)

storage time (days)	anthocyanin concn (mg/L)	malvidin 3-glucoside (mg/L)	malvidin 3-acetylglucoside (mg/L)	malvidin 3-*p*CAglucoside (mg/L)	A[a] (mg/L)	B[a] (mg/L) (vitisin A)	C[a] (mg/L) (3-acetylvitisin A)	D[a,b] (mg/L) (3-*p*-CAvitisin A)
0	65.36	23.70	8.82	18.43	0	0	0	1.08
1	50.24	20.07	6.28	16.84	0	0.40	0.19	1.39
2	57.23	21.37	7.43	13.92	0	1.07	0.46	2.36
3	53.63	20.05	6.75	12.29	0	1.76	0.67	2.63
4	51.40	18.87	6.42	10.95	0.20	2.18	0.85	3.24
7	43.83	14.94	4.90	8.20	0.27	3.28	1.24	4.25
9	39.64	13.03	3.78	7.01	0.38	3.97	1.55	3.41
11	34.93	11.25	3.19	5.60	0.36	4.45	1.64	3.75
14	29.25	8.94	2.45	3.84	0.43	4.55	1.78	3.75

[a] Peaks A−D are the new peaks formed during storage. [b] c(mg/L) = c(mg/L) of unidentified peak + c(mg/L) of 3-*p*-CAvitisin A.

tion and pH. Additionally, the effect of vitisin A formation on the color changes of these model wines was evaluated.

MATERIALS AND METHODS

Chemicals. Pyruvic acid was purchased from Sigma (St. Louis, MO). Anthocyanins were extracted from Touriga National grape skins (Vilariça) with methanol containing 3% (v/v) formic acid. The methanolic solution was concentrated by rotary evaporator (Büchi) at 30 °C and dried under vacuum. The grape skin extract was kept in the desiccator until used.

Model Solutions. The dry grape skin extract was dissolved in 10^{-3} M HCl containing 20% methanol (Fulcrand et al., 1996), to investigate the formation of vitisin A from the reaction between malvidin 3-glucoside and pyruvic acid. The solution was filtered (0.45 μm membrane filter), and the pH was adjusted to 3.5. The solution was divided into two portions; one was kept as the control, and to the second one was added pyruvic acid in a molar ratio of pyruvic acid to total anthocyanins (PA/TA) of 235 units. Both solutions were kept in stoppered vials in the presence of air (3 mL of sample in a 15 mL vial) and allowed to react in the incubator in the dark at 30 °C.

To study the effect of pH and concentration of pyruvic acid on the formation of vitisin A, a potassium hydrogen tartrate (Merck) buffer (0.02 M, pH 3.5) containing 10% ethanol (v/v) was used as a model wine base (Bakker et al., 1993). The pH 3.5 buffer mixtures were adjusted to give four other pH values, by addition of Na_2CO_3 (pH 3.7 and 4.5), tartaric acid (pH 3 and 2.7), and HCl (pH 2). Different concentrations of pyruvic acid were added, obtaining molar ratios PA/TA of 0, 12.20, 59.50, and 172.40. The pH values of all model solutions were determined after the addition of pyruvic acid and adjusted again to the pH value desired with NaOH. All solutions were filtered, and duplicate samples in the presence of air (10 mL of sample in a 25 mL stoppered vial) were incubated in the dark at 25 °C.

Samples from separate vials were analyzed periodically by HPLC and spectrophotometry.

HPLC. Samples were analyzed on a Hewlett-Packard 1090 M Series II chromatograph with an autoinjector (25 μL). A Hypersil ODS column, 2.1 mm × 100 mm (5 μm), was used at 40 °C, with diode array detection at 280 and 520 nm. The elution conditions were as follows: flow rate = 0.3 mL/min; solvent A, water with 1% of perchloric acid; solvent B, methanol. The mobile phase consisted initially of 80% of A and 20% of B; using a linear gradient, the concentration of the latter solvent was increased to 50% during 30 min, to 98% at 30.5 min, held for 2 min at 98% of B to wash the column, and then returned to the initial conditions (20% of B) to re-equilibrate for 10 min. Malvidin 3-glucoside was used as an external standard to quantify the anthocyanin peaks in the model solutions. The absorbance (A) of this standard (16 mg/L in 1 N HCl) was measured at 520 nm in a 10 mm glass cell, and the concentration (c) of anthocyanins expressed as malvidin 3-glucoside chloride (molecular weight = 529) was calculated using a molar absorptivity value of 28000 (Niketic-Aleksic and Hrazdina, 1972); thus, c (mg/L) = 18.9A. The quantification of the vitisins was done also using the external standard of malvidin 3-glucoside, even though the molar absorptivity of the vitisin compounds was not determined. This may well lead to some error. However, the HPLC solvents have a very low pH value (pH 1.0), which means that most of the anthocyanins are in the colored flavylium salt form (Brouillard and Delaporte, 1978). Thus, the increased color expression at higher pH values reported for the vitisin compounds (Bakker and Timberlake, 1997; Bakker et al., 1997) does not interfere with the HPLC quantification. The data of each measurement are the average of duplicate samples.

Colorimetric Measurements. Absorption spectra of solutions in 1 mm path length glass cells were carried out using a Philips PU8740 UV−vis spectrophotometer, equipped with a computer software program to calculate the L^*, a^*, b^*, chroma, and hue angle parameters by scanning the solutions from 380 to 770 nm. L^*, a^*, and b^* values describe a three-dimensional color space. The vertical axis L^* is a measure of lightness, from completely opaque (0) to completely transparent (100), whereas on the hue-circle a^* is a measure of redness (or $-a^*$ of greenness) and b^* of yellowness (or $-b^*$ of blueness). From a^* and b^* values other chromaticity parameters can be calculated. The hue angle (degrees) expresses the color nuance and is calculated from $h = \text{arctg}(b/a)$ (McLaren, 1980); when the angle increases from 0° to 60°, the color changes from violet-red through red to reddish brown. The chroma (c) was obtained as $(a^2 + b^2)^{1/2}$. The data of each measurement are the average of duplicate samples.

FABMS Analysis. A model solution containing the new peaks was retained on an ODS minicolumn (Sep-Pak cartridge C_{18}, Waters, Millipore, Watford, U.K.) washed with aqueous 3% formic acid, eluted with methanolic 3% formic acid, and dried at room temperature under reduced pressure in a desiccator. A small sample of anthocyanin was dissolved in HCl (1 M), placed on a clean copper-tipped probe, dried under vacuum, and mixed with glycerol on the probe before insertion in the FAB source. Spectra were obtained using an MS890 mass spectrometer (Kratos Analytical Ltd., Manchester, U.K.), operated at a resolution of 1000, with the gun producing a 5−7 kV beam of xenon atoms, using a scan speed of 30 s per decade. The spectra were acquired using the DS90 data system (Kratos Analytical Ltd.).

Statistical Analysis. Linear regression, analysis of variance, and standard deviation were done using SPSS.

RESULTS AND DISCUSSION

Formation and Identity of Vitisins Formed in Model Solution. The evolution of the anthocyanins in a model system, consisting of dilute HCl (10^{-3} M) at pH 3.5 in the presence of pyruvic acid (PA/TA = 235 units) and storage for 14 days at 30 °C, is shown in Table 1. Malvidin 3-glucoside was the main anthocyanin present, forming 36.3% of the total, followed by malvidin 3-*p*-coumarylglucoside (28.2%) and malvidin 3-acetylgluco-

Table 2. Retention Times of Compounds by HPLC Using a Linear Gradient with Two Solvents, 1% $HClO_4$ in H_2O and Methanol

anthocyanin	retention time (min)	λ_{max}[a] (nm)	$\Delta\lambda_{vismax}$ (nm)
malvidin 3-glucoside	13.39	526, 278, 350	
malvidin 3-acetylglucoside	23.72	532, 280	
malvidin 3-*p*-CAglucoside	29.97	538, 282	
peak A	14.99		
peak B (vitisin A)	16.39	514, 272, 300, 370	−12[b]
peak C (3-acetylvitisin A)	17.92	520, 270, 300, 370	−12[c]
peak D (3-*p*-CAvitisin A)	22.66	520, 306, 264−398	−18[d]

[a] The absorbance maxima (λ_{max}) were determined using the same solvent composition in which the HPLC analysis was started [1% $HClO_4$ in H_2O and methanol (80:20)]. In the model setup the molar ratio pyruvic acid/total anthocyanins was 235 units, stored at pH 3.5 and 30 °C. [b] From malvidin 3-glucoside. [c] From malvidin 3-acetylglucoside. [d] From malvidin 3-*p*-CAglucoside.

side (13.5%). It was evident that during the period of study, the concentrations of these main anthocyanins decreased (37, 56, and 44%, respectively, after 7 days) and four new compounds appeared, eluting between malvidin 3-glucoside and malvidin 3-acetylglucoside, indicating that they are less polar than malvidin 3-glucoside. These changes of the anthocyanin composition occurred only in the presence of pyruvic acid, whereas in the control solution some losses of anthocyanins (8.4, 58, and 24%, respectively, after 7 days) were observed, but no new compounds were formed; these losses are due to the general instability of anthocyanins and possibly some precipitation as well as their participation in other reactions as discussed above. These observations indicate an interaction between pyruvic acid and anthocyanins.

A new anthocyanin, labeled peak A, appeared after 4 days (Table 1) and had a retention time 1.6 min greater than that of malvidin 3-glucoside (Table 2). Because its concentration remained low, with only 0.43 mg/L being formed after 14 days, its spectrum could not be measured.

On the second day, two new compounds were detected, labeled peaks B and C (Table 1). Peak B eluted 3 min later than malvidin 3-glucoside, and its UV−vis spectrum (Table 2), recorded in 1% $HClO_4$ in H_2O and methanol (80:20), using a diode array detector, had a λ_{max} of 514 nm, exhibited a hypsochromic spectral shift of 12 nm from the λ_{max} of 526 nm of malvidin 3-glucoside, and had three absorbance peaks at 272, 300, and 370 nm. The retention time and the UV−vis spectrum obtained for peak B identified it as vitisin A. Differences in values for λ_{max} for both malvidin 3-glucoside and vitisin A reported here compared with those reported by Bakker and Timberlake (1997) can probably be attributed to differences in solvents in which the spectra were recorded. The spectrum was similar to that of malvidin 3-monoglucoside−pyruvic acid adduct reported by Fulcrand et al. (1998), who recorded the spectra for malvidin 3-glucoside and the new anthocyanin in a mixture of water, formic acid, and acetonitrile. FABMS analysis further confirmed the identity of this compound as vitisin A, giving a molecular ion at *m*/*z* 561 and an ion at 399 after the loss of glucose, results that were in agreement with the values reported by Bakker and Timberlake (1997) for this anthocyanin.

One would assume that if vitisin A was formed as a result of reaction between malvidin 3-glucoside and pyruvic acid, an excess of pyruvic acid would allow the formation of two further detectable vitisins A, derived from the other main anthocyanins present in the model solution, malvidin 3-acetylglucoside and malvidin 3-*p*-coumarylglucoside.

In Table 1 we observe the formation of two new compounds, labeled peaks C and D, eluting 4.53 and 9.27 min later than malvidin 3-glucoside, respectively (Table 2). Peak C appeared the second day, eluted 1.53 min later than vitisin A, and increased until a concentration of 1.78 mg/L after 14 days. Its UV−vis spectrum showed a λ_{max} at 520 nm (Table 2), a value typical of maximum absorption in the visible region for any anthocyanin (Sudraud, 1958), exhibiting a hypsochromic spectral shift of 12 nm from the λ_{max} of 532 nm of malvidin 3-acetylglucoside. Also, it showed another three absorbance peaks at 270, 300, and 370 nm, and its spectrum was very similar to the vitisin A spectrum, in agreement with the spectra of vitisin A and 3-acetylvitisin A reported by Bakker et al. (1997). In addition, the FABMS analyses showed a molecular ion at *m*/*z* 603, just the sum of the mass of vitisin A aglycon (399) and the mass of the acetylglucoside group (204), in agreement with the *m*/*z* reported by Bakker and Timberlake (1997). In accordance with the above results, peak C is likely to be 3-acetylvitisin A.

The second largest new compound, peak D, eluted 1.06 min before malvidin 3-acetylglucoside (Table 2). From the spectral changes observed during the period of study, the new compound coeluted with an existing anthocyanin, but the latter seemed to disappear. From the second day of study its UV−vis spectrum started to change. In the beginning, peak D showed a λ_{max} at 520 nm and another absorption peak at 282 nm together a shoulder in the 308−348 region. Whereas the visible absorption remained at 520 nm, the absorption peak at 282 nm and the shoulder changed and, after 14 days, this compound showed a maximum UV absorption at 306 nm and a shoulder in the 264−398 nm region (Table 2), exhibiting a hypsochromic spectral shift of 18 nm from the λ_{max} of 538 nm of malvidin 3-*p*-coumarylglucoside. The FABMS of this anthocyanin, unknown in the literature, showed a molecular ion (M^+) of 707.0 mass units, which corresponds exactly to the 3-*p*-coumarylglucoside group with the vitisin A aglycon. Therefore, the likely identity of peak D is 3-*p*-coumarylvitisin A.

It is interesting to note that after 14 days, 19.2% of malvidin 3-glucoside was converted into vitisin A and 20.2% of malvidin 3-acetylglucoside was converted into 3-acetylvitisin A (peak C), whereas 20.3% of malvidin 3-*p*-coumarylglucoside was converted into 3-*p*-coumarylvitisin A (peak D). This would seem to indicate comparable reactivities of the three main anthocyanins under the conditions tested.

The above results show that the presence of pyruvic acid led to the formation of vitisin A, derived from all three main anthocyanins, accompanied by the loss of the main pigments. This formation is in agreement with the results reported by Fulcrand et al. (1998), who reported a new class of stable red pigments, identified as pyruvic acid derivatives of grape anthocyanins.

Formation of Vitisins in Model Wines, Effect of pH and Pyruvic Acid Concentration. In a second experiment the effect of pH value (2, 3, and 4.5) and the molar ratio PA/TA (0, 12.20, and 59.50) on the formation of vitisin A and other vitisin-type compounds was investigated in model wine buffer solutions stored

Table 3. Reaction Rates with Respect to the Loss of Total Anthocyanins and Main Anthocyanins in Model Systems[a]

compound	pH	PA/TA ratio 0	12.20	59.50
anthocyanins	4.5	0.039 (0.996)[b]	0.016 (0.974)	0.015 (0.973)
anthocyanins	3	0.016 (0.944)	0.012 (0.978)	0.011 (0.951)
anthocyanins	2	0.007 (0.907)	0.006 (0.913)	0.005 (0.888)
malvidin 3-glucoside	4.5	0.033 (0.994)	0.012 (0.981)	0.012 (0.982)
malvidin 3-glucoside	3	0.014 (0.932)	0.011 (0.983)	0.014 (0.989)
malvidin 3-glucoside	2	0.005 (0.894)	0.003 (0.803)	0.004 (0.892)
malvidin 3-acetylglucoside	4.5	0.033 (0.996)	0.013 (0.963)	0.013 (0.974)
malvidin 3-acetylglucoside	3	0.022 (0.979)	0.019 (0.993)	0.025 (0.989)
malvidin 3-acetylglucoside	2	0.051 (0.931)	0.065 (0.996)	0.063 (0.989)
malvidin 3-pCAglucoside	4.5	0.044 (0.982)	0.019 (0.975)	0.023 (0.988)
malvidin 3-pCAglucoside	3	0.017 (0.917)	0.013 (0.939)	0.023 (0.933)
malvidin 3-pCAglucoside	2	0.008 (0.901)	0.009 (0.937)	0.008 (0.979)

[a] Parameters employed were pH values of 4.5, 3, and 2, molar ratios of pyruvic acid/total anthocyanins of 0, 12.20, and 59.50 units, and storage temperature of 25 °C. [b] Value of r^2.

Table 4. Increase (+) or Decrease (−) in the Anthocyanin Concentrations (Milligrams per Liter) after 154 Days of Study in the Absence and Presence of Pyruvic Acid (PA/TA = 12.20 and 59.50 Units) at 25 °C[a]

pH	PA/TA	TA	MG	MAG	MCG	PA	PB	PC	PD
4.5	0	−51.22 (2.74)[b]	−19.03 (0.89)	−6.68 (0.45)	−13.40 (0.59)	0.00 (0.00)	−0.62 (0.14)	0.00 (0.00)	−0.75 (0.07)
4.5	12.20	−45.68 (3.69)	−45.76 (1.29)	−5.89 (0.78)	−12.48 (0.94)	0.00 (0.00)	−0.62 (0.01)	−0.16 (0.01)	−0.58 (0.13)
4.5	59.50	−42.37 (2.81)	−14.77 (0.69)	−5.56 (0.59)	−11.57 (0.99)	0.00 (0.00)	−0.43 (0.14)	−0.19 (0.00)	−0.41 (0.10)
3	0	−49.22 (4.68)	−18.13 (1.49)	−6.17 (0.48)	−11.80 (0.89)	0.00 (0.00)	−0.84 (0.24)	−0.29 (0.00)	−0.79 (0.00)
3	12.20	−42.46 (3.42)	−16.01 (1.09)	−5.78 (0.37)	−10.37 (0.80)	+0.37 (0.13)	−0.03 (0.30)	+0.01 (0.03)	−0.20 (0.21)
3	59.50	−37.79 (3.89)	−15.88 (0.84)	−5.55 (0.41)	−10.53 (0.87)	+0.52 (0.13)	+1.93 (1.24)	+0.38 (0.39)	+1.23 (0.57)
2	0	−37.76 (0.54)	−12.76 (0.11)	−6.45 (0.13)	−8.80 (0.23)	0.00 (0.00)	−0.44 (0.12)	−0.17 (0.00)	−0.83 (0.04)
2	12.20	−31.18 (1.63)	−10.13 (0.78)	−6.02 (0.07)	−7.85 (0.29)	0.00 (0.00)	−0.44 (0.33)	−0.18 (0.00)	−0.37 (0.08)
2	59.50	−28.34 (0.72)	−10.90 (0.32)	−5.69 (0.07)	−7.85 (0.17)	+0.31 (0.00)	+1.41 (0.18)	−0.18 (0.00)	+0.63 (0.09)

[a] Total anthocyanins (TA), malvidin 3-glucoside (MG), malvidin 3-acetylglucoside (MAG), malvidin 3-*p*-coumarylglucoside (MCG), peak A (PA), vitisin A (PB), 3-acetylvitisin A (PC), and 3-p-coumarylvitisin A (PD). [b] Standard deviation of the differences.

at 25 °C. On this occasion we found that peaks B (vitisin A) and C (3-acetylvitisin A) appeared from the beginning; this may be because the wet methanolic extract of grapes was stored for 1−2 months in a refrigerator before use, a condition that possibly allowed the formation of these natural compounds like in red wine. Small concentrations of pyruvic acid have been reported in the grape (Blouin and Peynaud, 1963). Earlier analyses of red fortified port wine pigments by HPLC revealed a number of small unidentified peaks eluting soon after malvidin 3-glucoside (Bakker, 1985), and this author reported that one of those, vitisin A, was found in small amounts in some red wines and at trace levels in stored grapes (Bakker et al., 1997).

A general decrease in the concentration of anthocyanins occurred at all pyruvic acid concentrations and pH values studied, concurrent with the formation of new colored compounds in most model solutions. The reaction rates k (days^{-1}) are shown in Table 3. The control sample generally showed a faster rate of anthocyanin loss at all pH values than the solutions containing pyruvic acid. The linear loss of anthocyanins in all model systems indicates a first-order reaction with respect to this loss, confirming the finding of Baranowski and Nagel (1983) and Bakker et al. (1993).

The loss of the three main anthocyanins (malvidin 3-glucoside, malvidin 3-acetylglucoside, and maldivin 3-*p*-coumarylglucoside) was dependent on the pH value. Generally, the reaction rate increases with the pH value, with one only exception: the loss of malvidin 3-acetylglucoside was faster at the lowest pH value. At pH 4.5 the control sample without pyruvic acid showed a faster rate of loss for the three anthocyanins than the models containing pyruvic acid, indicating a protective effect of pyruvic acid on anthocyanin loss at this pH. The behavior at pH 3 and 2 was different. At pH 3, the losses were faster when the molar ratio PA/TA had a value of 59.50 units than at the ratio of 12.20 for the three anthocyanins, possibly due to the formation of vitisins at this pH. However, at pH 2 the pyruvic acid concentration did not influence the loss of malvidin 3-glucoside and malvidin 3-*p*-coumarylglucoside, but the loss of malvidin 3-acetylglucoside was slower in the absence of pyruvic acid than in its presence.

In the literature, we find that the k value for the loss of malvidin 3-glucoside had been reported as 0.005 days^{-1} (Bakker et al., 1993), but in their model solutions the initial concentration of this compound was 125.3 mg/L at pH 3.7, whereas losses were monitored during storage at 20 °C. In the experiments reported here the initial concentration of malvidin 3-glucoside was ∼20 mg/L, with pH values of 2, 3, and 4.5, and losses were monitored during storage at 25 °C. At a higher storage temperature one would expect a higher k value, and indeed by linear extrapolation between the pH values a k of 0.023 days^{-1} at pH 3.7 would be expected at 25 °C, compared to the reported value of 0.005 days^{-1} at 20 °C. It seems that the values reported here are in line with the value reported to date.

The changes in the anthocyanin concentrations after 154 days, in this second experiment, are shown in Table 4. At pH 4.5, the results show that all of the initial anthocyanins present decreased in concentration during the study, including vitisin A (Figure 1) and 3-*p*-coumarylvitisin A (Figure 2). It is interesting to note that peak A was never formed at pH 4.5. However, the presence of pyruvic acid had some protective effect on the losses of anthocyanins, as was observed in differences in the k values (Table 3) and in the smaller decrease in concentration of each compound in the presence of pyruvic acid (Table 4), an effect that increased with the pyruvic acid concentration.

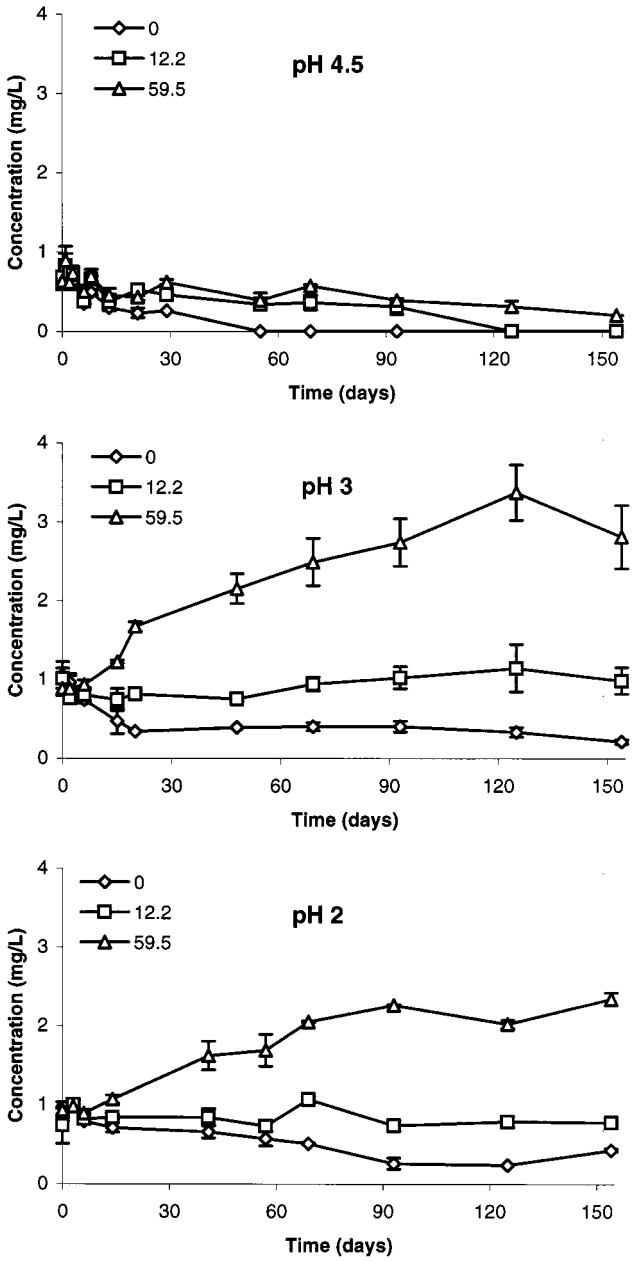


Figure 1. Evolution of the vitisin A concentration in model wines at pH 4.5, 3, and 2 in the absence and presence of pyruvic acid (PA/TA = 12.20, and 59.50 units), during 154 days at 25 °C. Each point is the average of two measurements; where error bars are not visible, determinations are within the size of the symbols on the graph.

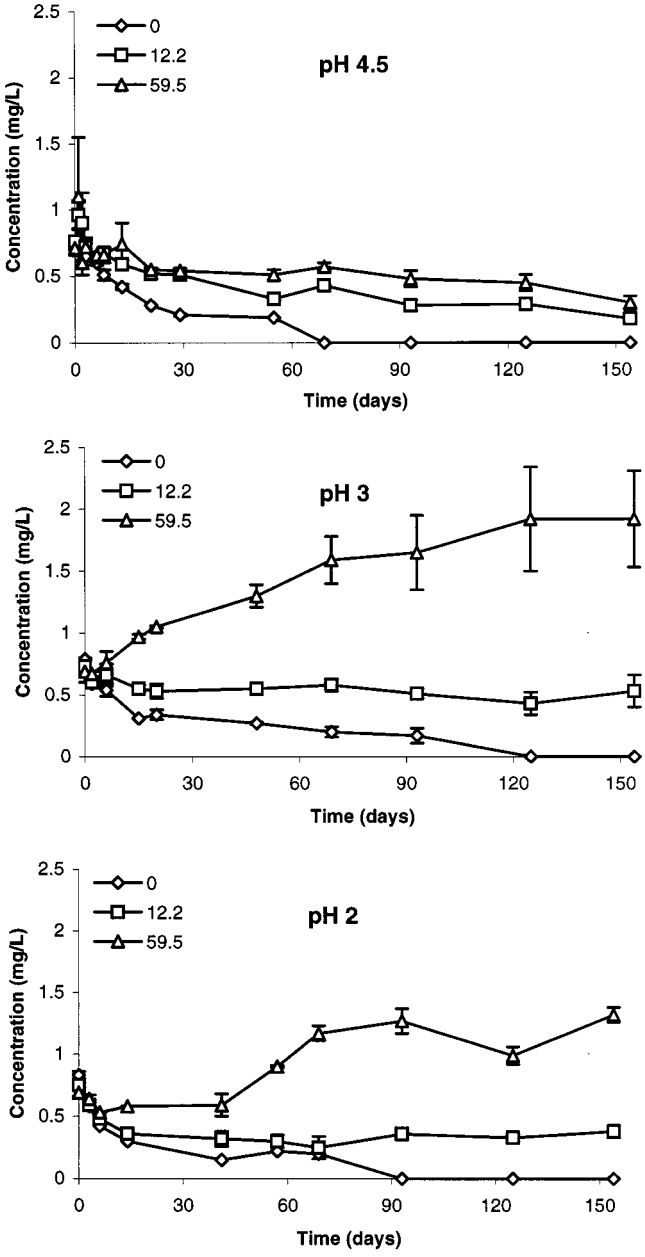


Figure 2. Evolution of the 3-*p*-coumarylvitisin A concentration in model wines at pH 4.5, 3, and 2 in the absence and presence of pyruvic acid (PA/TA = 12.20 and 59.50 units), during 154 days at 25 °C. Each point is the average of two measurements; where error bars are not visible, determinations are within the size of the symbols on the graph.

However, at pH 3 and 2, interesting changes were observed. Peak A started to increase after 48 days, with a maximum concentration of 0.37−0.52 mg/L after 154 days at pH 3 (Table 4). At pH 2, peak A appeared after 69 days but had totally disappeared after 93 days at a PA/TA value of 12.20 units, but when the PA/TA value was 59.50, the peak A concentration was 0.31 mg/L after 154 days (Table 4). At pH 2 and 3 the vitisin A concentration at the molar ratio PA/TA of 12.20 units remained more or less constant (Figure 1), which suggests that the formation of vitisin A was able to replace any loss of the initial concentration present in the solution. At pH 2 and 3 at the higher pyruvic acid concentration, with PA/TA ratio of 59.50, vitisin A was clearly formed (Figure 1). At pH 3 vitisin A attained a maximum concentration of 3.37 mg/L after 125 days; it increased by 27.05% of the total anthocyanins at the initial concentration, but later its concentration started to decrease. At pH 2, the vitisin A concentration increased to 2.34 mg/L in 154 days, an increase of 10.06% of the total anthocyanins at the initial concentration. At pH 2 the concentration changes of the main anthocyanins were smaller than at pH 4.5 and 3 (Table 4); therefore, the experiments at pH 2 were continued after 154 days, obtaining the maximum concentration of vitisin A at the PA/TA of 59.50 units after 184 days (2.49 mg/L), but thereafter the concentration started to decrease.

The 3-acetylvitisin A was also formed at pH 3 in the presence of pyruvic acid; the maximum concentration was 0.76 mg/L after 125 days, at the higher PA/TA ratio. However, at pH 2, this compound was not formed and

the small initial concentration was lost after 3−10 days. Likewise, at pH 2 the loss of malvidin 3-acetylglucoside was the most rapid (Table 3). One possibility is the hydrolysis of the acetyl group from the glucose, converting this anthocyanin to malvidin 3-glucoside. Anderson et al. (1970) identified acetic acid as an acylating agent of anthocyanin pigments in grapes and reported that facile hydrolysis of anthocyanin acetates occurs upon the exposure to trace quantities of mineral acid during the extraction processes, and some authors working on chemotaxonomic studies have reported that the extraction of some acylated anthocyanins under acid conditions may cause their partial or total hydrolysis (Van Wyck and Winter, 1994). Possibly the much lower losses of malvidin 3-glucoside at pH 2 (Table 3) could in part be attributed to the formation of malvidin 3-glucoside by hydrolysis of malvidin 3-acetylglucoside. Therefore, the lack of the formation of the new acetyl-derived anthocyanin at pH 2 could be because the loss of any acetyl-derived anthocyanins may be much faster than at a higher pH, or there is simply insufficient malvidin 3-acetylglucoside left to form detectable concentrations of 3-acetylvitisin A.

The second highest concentration of anthocyanin formed in the model solutions was 3-*p*-coumarylvitisin A. At the molar ratio PA/TA of 12.20 units (Figure 2) the initial concentration of 3-*p*-coumarylvitisin A decreased a little during 9 days at pH 3 and during 14 days at pH 2, maintaining a concentration of ∼0.53 mg/L at pH 3 and ∼0.32 mg/L at pH 2 until the end of the experiments. However, at the highest pyruvic acid concentration (PA/TA = 59.50 units), 3-*p*-coumarylvitisin A started to increase from day 6 at pH 3, reaching a concentration of 1.92 mg/L after 154 days, and from day 57 at pH 2, with a maximum concentration of 1.79 mg/L after 184 days.

Thus, it seems that pyruvic acid does react with all three major anthocyanins in wines, forming vitisin A, 3-acetylvitisin A, and 3-*p*-coumarylvitisin A; the formation occurred at fairly low pH values, pH 2.0−3.0, and tended to be enhanced at high pyruvic acid concentrations.

Effect of Vitisins on Color of Model Wines. The effect of the new vitisin A derived compounds on the color of the model wines still needs to be established, although it has been speculated that they may contribute significantly once wines have lost most other anthocyanins (Bakker and Timberlake, 1997). This is because vitisins tend to be the largest group of anthocyanins left but also because at wine pH vitisins express more color than, for example, malvidin 3-glucoside. In real wines significant contributions to the color are also expected to be made from polymers formed during maturation, but in these models there was little evidence of their presence, and in any estimation of the color of vitisins the contribution of polymers has been ignored. The CIELAB 76 measurements give color information closely associated to human vision (McLaren, 1980), using data collected over the entire visible range of wavelengths. However, using the entire data set at each pH value, there were good linear correlations between tint and hue angle and between color density and *L** (Table 5). A similar result has been reported by Bakker et al. (1986) for color changes in young port. The results here provide support that tint and color density, traditionally used for wine color assessments, compare well with CIELAB 76 measurements in these model systems. Only the CIELAB 76 data will be discussed for this paper.

Table 5. Correlations (r^2) between CIELAB 76 Data (Hue Angle and *L) and Tint and Color Density[a]**

pH	tint−hue angle	color density−*L**
4.5	0.995 (n = 22)	0.961 (n = 29)
3	0.942 (n = 27)	0.978 (n = 27)
2	0.994 (n = 26)	0.984 (n = 26)

[a] The model grape skin extract solutions contained pyruvic acid in three different concentrations (PA/TA = 0, 12.20, and 59.50 units). The experiment was carried out at 25 °C.

Table 6. Increase (+) or Decrease (−) in the Color Parameters after 154 Days of Storage at 25 °C[a]

pH	PA/TA [b]	*L**	chroma	hue angle (deg)
4.5	0	−1.58 (0.10)[c]	−0.08 (0.04)	+65.96 (0.19)
4.5	12.20	−3.24 (0.14)	+0.32 (0.03)	+39.91 (0.13)
4.5	59.50	−3.34 (0.14)	+0.34 (0.09)	+39.71 (1.33)
3	0	−4.36 (6.03)	−9.86 (0.77)	+19.22 (4.24)
3	12.20	−7.21 (9.50)	−7.92 (1.46)	+9.81 (5.65)
3	59.50	0.00 (0.00)	−8.05 (0.45)	+12.19 (0.16)
2	0	+2.72 (0.11)	−12.82 (0.26)	+7.06 (0.08)
2	12.20	+1.10 (0.91)	−9.55 (1.49)	+4.08 (0.09)
2	59.50	+0.00 (0.00)	−8.80 (0.55)	+5.19 (0.14)

[a] The model grape skin extract solutions were studied at three pH values (2, 3, and 4.5) and three molar ratios PA/TA (0, 12.20, and 59.50). [b] PA/TA, molar ratio pyruvic acid/total anthocyanins. [c] Standard deviation of the differences.

As expected, the initial *L** value depended on the pH value, with the solutions prepared at the lower pH value being darker (lower *L** values) than the solutions at higher pH values, due to the amount of anthocyanins expressed in the flavylium salt form at the various pH values (Timberlake, 1980). During storage all samples changed from a purplish red toward brown-red, as indicated by the increases in hue angle (Table 6). Generally, the changes are greater for samples stored at higher pH values; for example, for the pH 4.5 samples the hue angle increased from 350° to 55° for the control and to ∼29° for the samples containing pyruvic acid, whereas at pH 2.0 the increases in hue angle were from 349° to 354° for the control and to 353° for the samples containing pyruvic acid.

Interestingly, all samples containing pyruvic acid changed less toward brown-red than their respective controls, although the presence of a high concentration of pyruvic acid led to the formation of measurable concentrations of vitisin A only in the samples stored at pH 2.0 and 3.0; however, these results are in agreement with the fact that the presence of this acid caused less anthocyanin losses (Table 4). When measurable concentrations of vitisins A were formed (pH 2 and 3), the increase in hue angle was considerably less than when none was formed at pH 4.5 (Table 6).

Bakker and Timberlake (1997) reported that the hue angles of both pure vitisin A and B in buffer solutions were considerably browner than that of malvidin 3-glucoside and the hypsochromic spectral shifts of both pure solutions of vitisin A and B indicate a shift to orange-brown. They also showed that the vitisins had a higher hue angle at wine pH than malvidin 3-glucoside; however, the hue angle of the pure vitisin solutions did not change much when the pH was reduced toward 2. Further examination of our hue angle data show that the percent vitisin A of the total anthocyanins did correlate well with the hue angle (Table 7), in particular, for the samples in which a considerable amount of

Table 7. Correlations (r^2) between Vitisin (Percent) and Hue Angle (Degrees) during 154 Days of Storage ($p < 0.001$)[a]

description	pH 2.0	pH 3.0	pH 4.5
all data	NS	NS	NS
control	NS	0.888	NS
PA/TA = 12.20	0.876	0.866	NS
PA/TA = 59.50	0.982	0.969	0.756

[a] The model grape skin extract solutions contained pyruvic acid in three different concentrations (PA/TA = 0, 12.20, and 59.50 units), and three pH values (2.0, 3.0, and 4.5 units) were assayed. The experiment was carried out at 25 °C.

vitisin A was formed, that is, the high addition of pyruvic acid at pH 2 and 3. When all of the data at each pH value were combined there was no significant correlation, indicating that the changes in hue angle depended on the reaction conditions used. Thus, although the pure vitisins were reported to be browner than malvidin 3-glucoside (Bakker and Timberlake, 1997), in aged model wine solutions the vitisins contributed to maintaining a much redder color than when malvidin 3-glucosides were lost in typical wine-browning reactions.

During 154 days of storage, the samples at pH 4.5 all became a little darker, as shown by decreases in L^* value (Table 6). The control sample and the sample with a molar ratio PA/TA of 12.20 units stored at pH 3 also became darker, but no change in L^* value for the sample with higher pyruvic acid concentration was observed. At pH 2 the samples tended to become slightly lighter, as indicated by small increases in L^* value, although again there was no change in L^* value for the sample containing molar ratio PA/TA of 59.50 units. The observation that some of the samples became darker, even though there was a general loss in the anthocyanin concentration and no formation of vitisins A, seems to indicate that polymerization reactions gave soluble brown compounds which expressed more color than the red anthocyanins.

The highest amount of vitisin A was formed in the two samples that did not change in L^* value during storage (pH 3.0 and 2.0, with a molar ratio PA/TA of 59.50 units, Figure 1). Examining the changes in these samples during 154 days of storage in more detail shows that at pH 3 the total anthocyanin concentration decreased almost 38 mg/L (Table 4), whereas the vitisin A concentration increased by 1.93 mg/L, 3-acetylvitisin A increased by 0.38 mg/L, and 3-*p*-coumarylvitisin A increased by 1.23 mg/L. Because there was little browning in these samples, which could have contributed to the samples becoming darker as seen above, it would seem to indicate that the expected decrease in color intensity due to the loss of total anthocyanins during storage was compensated by the formation of ~3.54 mg/L vitisins. It would seem that at pH 3 the vitisins express ~11 times more color than the normal anthocyanins. At pH 2 the total anthocyanin concentration decreased by 28.3 mg/L (Table 4), whereas the vitisin A increased by 1.41 mg/L and 3-*p*-coumarylvitisin A increased from 0.63 mg/L. With the L^* value remaining constant, the formation of ~2 mg/L vitisins compensated for the loss of total anthocyanins, indicating that the vitisins express at pH 2 ~14 times more color than the normal anthocyanins. Bakker and Timberlake (1997) previously showed that the change in color intensity (L^* value) and hue angle over the pH range 2−5 was very small, which is in agreement with the relatively small change in color contribution of the visitins in the samples at the two different pH values.

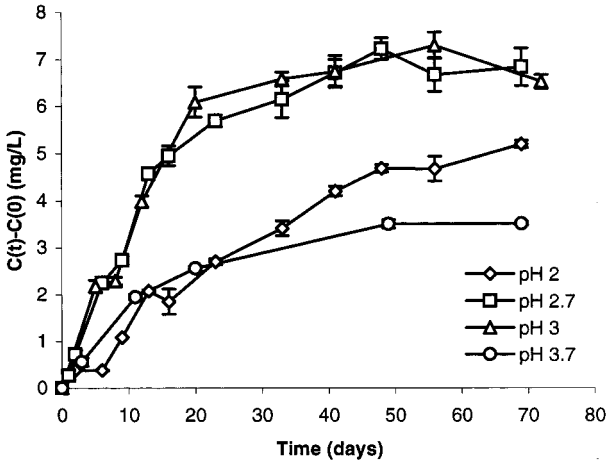


Figure 3. Evolution of the vitisin A concentration in model wines at pH values between 2.0 and 3.7 in the presence of pyruvic acid (PA/TA = 172.40 units), during 70 days at 25 °C. Each point is the average of two measurements; where error bars are not visible, determinations are within the size of the symbols on the graph.

Finally, the chroma parameter was very dependent on the pH value, showing a higher change in its value with the low pH; its decrease was greater in the absence of pyruvic acid in the medium (Table 6). Earlier, Bakker et al. (1993) and Picinelli et al. (1994) had reported similar results for chroma values when they studied model solutions. The presence of pyruvic acid brings about less change on the value of this parameter.

Overall, it seems that the addition of pyruvic acid does influence the color changes, with the amount of vitisins formed influencing the color of the samples. There is a significant correlation percent vitisin and hue angle, and in aged model wine solutions the vitisins contributed to maintaining a much redder color than when malvidin 3-glucosides were lost in typical wine-browning reactions. Samples with added pyruvic acid seem to brown less than their respective controls.

Comparison of Vitisin A Formation at Different pH Values. We have shown the formation of vitisins, derived from malvidin 3-glucoside, malvidin 3-acetylglucoside, and malvidin 3-*p*-coumarylglucoside, through the addition of pyruvic acid in a model solution of grape extract. A higher pyruvic acid concentration enhanced this formation, but the effect of pH value, influencing color expression of anthocyanins (Brouillard, 1982), was not too clear. Trying to clarify this point, another experiment was done with a higher concentration of pyruvic acid (molar ratio PA/TA of 172.40 units) and with the range of pH values including a pH value typical for wine (2, 2.7, 3, and 3.7). The results for the formation of vitisin A are shown in the Figure 3, indicating a maximum formation at pH ~2.7−3, whereas a smaller amount of vitisin A was formed at lower and higher pH values tested. The pK value at 25 °C of pyruvic acid is 2.49, so if the acidic form of pyruvic acid was the limiting factor in the formation of the vitisins, then a greater formation at pH 4.5 than at pH 2.0 would be expected.

The synthetic pathway for the formation of these compounds proposed by Fulcrand et al. (1998) suggested a reaction between the undissociated form of pyruvic acid and the flavylium form of malvidin 3-glucoside. This would lead one to expect that the greater amount of vitisins would be formed at the lowest pH tested, pH

2, because the greatest amount of the flavylium salt form would be present, whereas a great amount of pyruvic acid would be in the undissociated form. However, we observe the greatest formation at pH 2.7−3.0. One possible explanation is the availability of pyruvic acid, because this compound is not very stable (von Korff, 1969) and polymerizes and decomposes over time. Possibly the slower formation of vitisin A at pH 2 than at pH 3 is due to an increased instability of pyruvic acid at low pH, leading to a lower pyruvic acid concentration to be available for these reactions at low pH. At pH 4.5 the theoretical concentration of the flavylium forms of the anthocyanins is expected to be very low (Timberlake, 1980) and may have become the limiting factor for any vitisin formation. Further studies investigating the stability of pyruvic acid in wines and wine-like solutions are needed to gain a better understanding in the reaction mechanism between anthocyanins and pyruvate acids. In wines the pyruvate concentrations are very much lower than these used for our studies (Whiting and Coggins, 1960), but appreciable amounts of vitisin A have been detected in red table wines (Bakker et al., 1998) and fortified port wines (Bakker and Timberlake, 1997).

Conclusions. Vitisin A was formed through the interaction between malvidin 3-glucoside and pyruvic acid. Likewise, vitisin A occurs in acylated forms, having the 6-position of the sugar acylated with acetic acid (3-acetylvitisin A) and *p*-coumaric acid (3-*p*-coumarylvitisin A), formed through the interaction between pyruvic acid and malvidin 3-acetylglucoside and malvidin 3-*p*-coumarylglucoside, respectively. A range of pH values between 4.5 and 2 units was assayed, and the results indicated that the formation of these new colored compounds was favored in acid solutions, with a maximum formation at pH 2.7−3.0 and a higher pyruvic acid concentration (PA/TA of 172.40 units). Obviously, the presence of the vitisins A caused changes in the color of the solution and expressed about 11 times (pH 3) to 14 times (pH 2) more color than the normal anthocyanins. Even relatively small concentrations of vitisin may exert an influence on the wine color, changing the model solutions from a bluish red, attributable to the main anthocyanins present, to a slightly more orange-red, attributable to the vitisin compounds. The aged models containing vitisins A were all much redder than the more red-brown color of the models aged without pyruvic acid.

Further investigations to study the influence of temperature, acetaldehyde, etc., on the formation of these compounds will allow us to shed more light on one of the pathways of port wine maturation, during which the monomeric anthocyanins rapidly disappear, accompanied by a color change from brick red to a more tawny hue.

ABBREVIATIONS USED

FABMS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PA/TA, molar ratio of pyruvic acid to total anthocyanins; UV−vis, ultraviolet−visible.

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