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Vitisin A Content in Chilean Wines from *Vitis vinifera* Cv. Cabernet Sauvignon and Contribution to the Color of Aged Red Wines

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Vitisin A was prepared from malvidin 3-glucoside and pyruvic acid in model wine medium, isolated by countercurrent chromatography, and purified by preparative high-performance liquid chromatography (HPLC). The synthesized compound was used as a reference standard to quantify vitisin A in Chilean wines from *Vitis vinifera* cv. Cabernet Sauvignon, including a vertical row of wines from the same vineyard over 16 years. Maximum vitisin A content was reached within the first year of storage. Importantly, up to half of the initial amount of vitisin A in young wines was still present in 15 year old wines. Although vitisin A was found to be much more stable as compared to other monomeric C-4 underivatized anthocyanins, it also slowly degrades after reaching its peak concentration. The "color activity concept" was applied to vitisin A, malvidin 3-glucoside, malvidin 3-(6″-acetylglucoside), and polymeric pigments isolated by countercurrent chromatography in order to estimate their contribution toward the overall color expression of wines. It was found that vitisin A is only a minor contributor to the visually perceived color of aged red wines (color contribution ∼ 5%). The major contributor is the polymeric fraction (color contribution ∼ 70−90%).

KEYWORDS: Vitisin; anthocyanins; countercurrent chromatography; color activity concept; Cabernet Sauvignon

INTRODUCTION

Vitisin A belongs to a group of minor pigments that have been detected in red wine but not in fresh grapes. This anthocyanin-derived pigment is formed by reaction between malvidin 3-glucoside and pyruvic acid, which is present in relatively high amounts during fermentation (*1*). The occurrence and structure of vitisin A were first reported in 1997 (*2*, *3*). A revised structure (**Figure 1**) bearing a carboxyl group at C-10 was proposed shortly thereafter by Fulcrand and co-workers (*1*) and was confirmed by Mateus et al. (*4*). The latter structure is also supported by liquid chromatography/mass spectrometry (LC/MS) analyses, where apart from the cleavage of the glucose moiety, a loss of 44 mass units is observed in negative ion mode, which can be explained by decarboxylation (*1*).

The color expressed by anthocyanins is strongly dependent on the pH value of the solution. In highly acidic media, the anthocyanins occur mainly as their red to bluish-red flavylium cations. An increase in pH leads to the formation of colorless carbinol bases, which in turn are in an equilibrium with the open chalcone form. In neutral and alkaline medium, the blue quinonoidal base is responsible for the perceived color. The importance of substitution at C-4 on the stability of anthocyanins in solution is well-documented (*5*−*7*). In the case of vitisin A, the newly introduced pyran ring between C-4 and the hydroxyl group attached to C-5 of the malvidin base structure shields vitisin A from hydration, thus delaying the formation of the colorless carbinol base, which at wine pH is already the predominant form of malvidin 3-glucoside. It has been reported that at pH 3, vitisin A exhibits an approximately 11 times higher color strength than normal anthocyanins (*8*). However, the authors ignored in their calculations the possible formation of polymeric pigments, which also contribute to overall color.

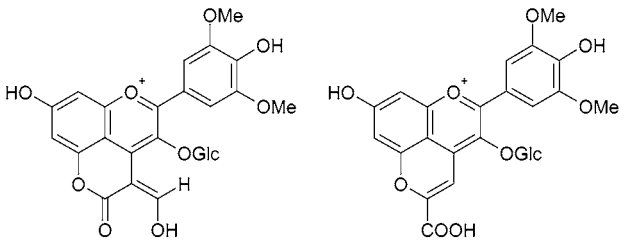

Figure 1. Structures of vitisin A as proposed by Bakker and Timberlake (*3*) (left) and Fulcrand et al. (*1*) (right).

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Because of the additional pyran ring, vitisin A distinguishes itself from malvidin 3-glucoside by a hypsochromic shift of its visible absorbance maximum of ~15 nm to around 510 nm and also by an increased absorbance between 350 and 400 nm (*1*). These changes in spectral properties result in a more orange-red or orange-brown color of vitisin A in contrast to the bluish-red of malvidin 3-glucoside. Vitisin A is also resistant against bleaching by sulfur dioxide (*3*).

The dependence of vitisin A formation on pH, temperature, and concentration of acetaldehyde and pyruvic acid in model wine solutions was subject to ample studies (*8*−*10*). It was found that maximum vitisin A formation occurred at pH values between 2.7 and 3.0, in the presence of a high excess of pyruvic acid and at low temperatures (10−15 °C), whereas at higher temperature (32 °C) the formation of polymeric pigments was favored. The presence of acetaldehyde diminished the amount of vitisin A formed by enhancing the formation of polymeric pigments. The resulting solutions containing vitisin A were reported to be much redder in comparison to the brownish solutions aged without the addition of pyruvic acid, thus suggesting that vitisin A as well as acetyl- and coumaroylvitisin A have a significant impact on the color of the aged model wine solutions.

The objective of the present study was to generate sufficient amounts of vitisin A by reaction of malvidin 3-glucoside with pyruvic acid in model wine medium. This authentic reference will then be used for (i) confirmation of the proposed structure, (ii) quantification of vitisin A in Chilean wines from *Vitis vinifera* cv. Cabernet Sauvignon, and (iii) clarification of its contribution to the color of aged red wines.

MATERIALS AND METHODS

Chemicals. All solvents were of high-performance liquid chromatography (HPLC) quality, and all chemicals were of p.a. grade.

Isolation of Malvidin 3-Glucoside. XAD-7 extracts from various young red wines were prepared by solid phase extraction (*11*). Wines were diluted with an equal volume of water to decrease the ethanol content. The solution was then poured onto a glass column (100 cm × 6 cm) filled with Amberlite XAD-7, and the column was washed with water to remove sugars, organic acids, proteins, and salts. Phenolic compounds (like the anthocyanins) were retained by the resin and eluted using a mixture of methanol−acetic acid (19:1, v/v). Methanol was evaporated in vacuo, and the aqueous phase was lyophilized.

The lyophilizate was separated with a CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research Corp., Baltimore, MD) equipped with three coils connected in series (sample loop, 20 mL; total volume, 850 mL; speed, 800 rpm). The solvent system consisted of *n*-butanol/TBME/acetonitrile/water (2/2/1/5, v/v/v/v, acidified with 0.1% TFA). Between 2 and 3 g of the XAD-7 extract (dissolved in 10 mL each of mobile and stationary phase prior to injection) was separated in a single run yielding around 10% of malvidin 3-glucoside of ~80% purity as determined by HPLC at 530 nm (*11*−*13*).

Synthesis and Isolation of Vitisin A. Malvidin 3-glucoside (900 mg) was dissolved in 500 mL of model wine medium (saturated aqueous solution of potassium hydrogen tartrate, 10% EtOH, adjusted to pH 3.2 with tartaric acid). A 200-fold excess (45 g) of sodium pyruvate (Merck, Germany) was added, and the solution was kept at 10 °C for 6 weeks. Progress of the reaction (increase of vitisin A and decrease of malvidin 3-glucoside) was monitored weekly by HPLC according to the procedure previously published (*14*).

After completion of the reaction (consumption of ~90% of malvidin 3-glucoside), ethanol was removed by rotary evaporation in vacuo. An XAD-7 extract was prepared from the remaining solution as described above to remove excess sodium pyruvate. After freeze-drying, 780 mg of a dry residue was obtained and separated by CCC (solvent system as above; flow rate, 3.5 mL/min) in three consecutive runs. The combined fractions enriched in vitisin A (140 mg) were purified by semipreparative HPLC. The HPLC system consisted of a Knauer 64 pump (Berlin, Germany) and a variable UV/vis detector ($\lambda = 510$ nm). The sample was injected through a Rheodyne valve 7125 (Techlab, Erkerode, Germany) with a 200 μL loop. Purification was achieved by isocratic elution with water/acetonitrile/formic acid (80/10/10, v/v/v, 6.0 mL/min) on a Luna RP-18 (Phenomenex, Aschaffenburg, Germany) column (250 mm × 10 mm) equipped with a guard column (50 mm × 10 mm) of the same material.

Synthesis of Methylvitisin A. Fifty milligrams of malvidin 3-glucoside and 200 mg of pyruvic acid methyl ester (200 equivalents) were dissolved in 30 mL of water. The solution was stored in the dark at 35 °C and analyzed daily by HPLC.

Structure Elucidation. The sample solution was delivered directly by a syringe pump 74900 (Cole-Parmer, Vernon Hills, IL) into the electrospray ionization (ESI) source of an Esquire ion trap ESI-LC-MS system (Bruker Daltonik, Bremen, Germany) at a flow rate of 300 mL/h. ESI spectra were measured both in positive and in negative ion mode. MS parameters: dry gas, N_2, 4.0 L/min; dry temperature, 300 °C; nebulizer, 10 psi; capillary, −3500 V; end plate offset, −500 V; skimmer 1, 30 V; skimmer 2, 10 V; capillary exit offset, 60 V; trap drive, 60; accumulation time, 200 ms; and scan range, 50−1000 *m*/*z*.

^{1}H and ^{13}C NMR and DEPT spectra were measured on a Bruker AMX 300 spectrometer at 300.1 and 75.4 MHz, respectively. HMQC and HMBC experiments were performed on a Bruker AM 360 instrument. Solvent was a mixture of methanol-d_4 and TFA-d_1 (19:1, v/v).

Quantification of Anthocyanins in Chilean Cabernet Sauvignon Wines. Reference standards of the 3-glucosides of delphinidin, petunidin, peonidin, and malvidin as well as malvidin 3-(6″-acetylglucoside) were isolated by CCC (*11*, *13*) and purified by HPLC. Calibration curves were obtained for these standards as well as for vitisin A in different concentration ranges. A Shimadzu (Kyoto, Japan) HPLC system equipped with two LC-10 AD_{VP} pumps, a SIL-10 AD_{VP} autosampler, a SCL-10 A_{VP} controller, a CTO-10 A_{VP} column oven, a DGU-12 A degasser, and a SPD-10 AV detector, controlled by Class-VP version 6.12 SP 1 software, was used. Analyses were carried out on a RP-12 Synergi MaxRP 4 μm 250 mm × 4.6 mm column (Phenomenex). Solvent A was 10 mmol/L potassium dihydrogen phosphate, adjusted to pH 1.60 with phosphoric acid, solvent B was solvent A/acetonitrile (50/50; v/v) 0% B from 0 to 5 min, then linear gradient from 0 to 17% B at 5−10 min and 17 to 55% B at 10−60 min, then back to initial conditions at 60−65 min. The detection wavelength was 520 nm, the flow rate was 0.5 mL/min, and the injection volume was 20 μL (50 μL for older wines). Wines were membrane filtered (0.45 μm) prior to injection.

A total of 25 Cabernet Sauvignon wines from various producers of the vintages 1999 to 2002 were analyzed for their anthocyanin content in September 2002, i.e., the youngest wines (vintage 2002) were approximately 6 months old. Wines were either bought in local supermarkets (13 samples) or directly supplied by the wineries (12 samples). The vertical row of wines (vintages 1987 to 2002) was the Cabernet Sauvignon "Don Melchor" (Concha y Toro Winery, Puente Alto Vineyard, Maipo Valley, Chile).

Isolation of Polymeric Fractions from Red Wine. XAD-7 extracts were prepared from three different aged red wines (Cabernet Sauvignon, 1997, California; Pinotage, 1996, South Africa; Portugieser, 1997, Germany) and polymers isolated by CCC (solvent system as above; flow rate, 3.0 mL/min; ref *13*). The polymeric fraction eluted first, followed by malvidin 3-glucoside (chromatograms not shown). The fraction was freeze-dried, and then, the yield and the concentration of polymers in the red wines were calculated.

Determination of Visual Detection Limits, Color Activity Values (CAVs), and Percentage Color Contribution (PCC). Vitisin A, malvidin 3-glucoside, malvidin 3-(6″-acetylglucoside), and the polymeric fraction isolated from a 1997 Portugieser red wine were dissolved in model wine medium (saturated aqueous solution of potassium hydrogen tartrate, 10% EtOH, adjusted to pH 3.6 with tartaric acid). The solutions were diluted to various concentrations above and below the estimated visual detection limit. Four milliliters of the solution was pipetted into plastic cuvettes (1 cm × 1 cm; liquid layer thickness, 4

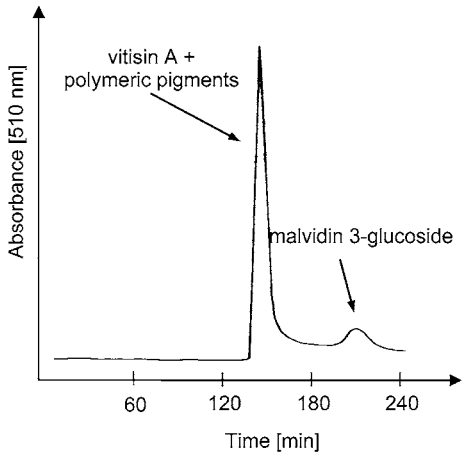

Figure 2. CCC isolation of vitisin A from the reaction mixture.

cm) and placed into a white polystyrene holder. Ten cuvettes containing blank model wine and the sample solutions in decreasing concentration (from left to right) were arranged alternating in the holder in such a way that the sample solutions were always placed between two blanks. Five judges were asked to specify the last solution that displayed any color in comparison to the surrounding blanks under ambient light conditions. The visual detection limits were then calculated as the average concentration. The dilution factors for the aged red wines were determined accordingly.

As all four sides of the cuvettes were covered by the holder, viewing was only possible directly from above and any reflections from adjacent cuvettes were prevented—reflections and the possibility of various viewing angles were the most critical points in a first trial experiment, which was performed in standard glass test tubes.

A CAV was calculated for the pigments by dividing the concentration of the respective pigment by its visual detection limit. PCC of a pigment was expressed as PCC = (CAV/dilution factor) × 100 (*15*).

RESULTS AND DISCUSSION

Preparation of Vitisin A. Vitisin A was prepared by reaction of malvidin 3-glucoside with pyruvic acid in model wine medium. The progress of the synthesis was monitored by HPLC. After 3 weeks, the ratio of remaining malvidin 3-glucoside to the newly formed pigment was approximately 1:1, and after 6 weeks, 90% of the malvidin 3-glucoside had reacted. An XAD-7 extract was prepared and separated by CCC (**Figure 2**).

Two fractions were obtained. The first contained mainly vitisin A and polymeric pigments, the second one unreacted malvidin 3-glucoside. Twenty milligrams of pure vitisin A was obtained after preparative HPLC. Given the large amount of starting material, the yield of vitisin A in our reaction appears very low (<3%). A possible explanation is that a significant portion of polymeric pigments was formed during the reaction as no other distinct peaks were observed in the diode array HPLC chromatograms.

Molecular mass $[M]^+$ of the isolated compound was determined to be at *m*/*z* 561 (in negative mode *m*/*z* 559 $[M - 2H]^-$). Upon fragmentation in the positive ion mode, a loss of 162 mass units indicated the cleavage of a hexose moiety. The remaining aglycon had a mass of *m*/*z* 399 and its fragmentation pattern followed with elimination masses of 16, 32, 44, and 61, largely the typical pathway for malvidin-derived pigments (*16*), although losses of 15 and 33 mass units were also observed. In negative ion mode, a loss of 44 mass units could be attributed to the cleavage of a carboxyl group. Further fragmentation of the resulting ion (*m*/*z* 515) produced again a loss of 162 u. Our results are in line with the data of Fulcrand et al. (*1*). Results of the NMR experiments are given in **Table 1**.

Table 1. NMR Data for Vitisin A of ^{1}H, ^{13}C, and DEPT Experiments[a]

position	^{13}C (ppm)	^{1}H (ppm)	*J* (Hz)	DEPT 90/135
2	165.5			q
3	136.0			q
4	110.8			q
4a	110.8			q
5	154.3			q
6	101.8	7.17	d, 2.0	CH
7	169.6			q
8	101.7	7.32	d, 2.0	CH
8a	154.3			q
9	107.1	7.97	s	CH
10	154.4			q
COOH	159.6			q
1′	120.0			q
2′, 6′	110.2	7.73	s	CH
3′, 5′	149.5			q
4′	145.0			q
OMe	57.4	3.99	s	CH_3
1″	105.4	4.71	d, 7.5	CH
2″	77.9	3.36	NR	CH
3″	75.7	3.62	NR	CH
4″	71.8	3.22	NR	CH
5″	79.3	3.14	NR	CH
6″	63.0	3.37, 3.72	NR	CH_2

[a] Spectra were recorded in methanol-d_4 and TFA-d_1 (19:1, v/v); d, doublet; s, singlet; NR, not resolved; q, quaternary.

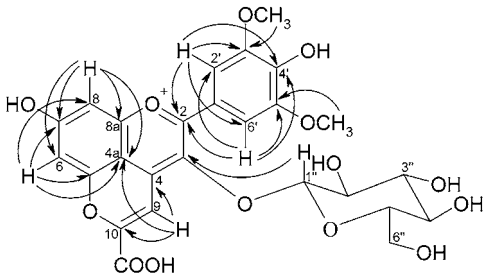

Figure 3. Long-range H–C correlations observed in HMBC experiment.

The ^{1}H NMR spectrum showed signals for H-6, H-8, H-2′, H-6′, and two methoxy groups, thus confirming the structure to be derived from malvidin-3-*O*-β-glucoside. However, the signal for H-4 was missing and the anomeric glucose proton shifted upfield to 4.71 ppm with a coupling constant of 7.5 Hz, proving β-configuration. The remaining glucose resonances appeared between 3.14 and 3.72 ppm. The only other signal observed was a singlet at 7.97 ppm, assigned to H-9. The ^{13}C NMR spectrum displayed a total of 26 resonances, six of them being related to β-D-glucose and another eight signals to the unchanged B-ring of the malvidin-based structure. All aromatic protons and connectivities were assigned in the A-, B-, and C-ring of the molecule by means of two-dimensional NMR techniques (HMQC, HMBC). Only a single proton (H-9) is available for the structural elucidation of the newly formed pyran ring, and we observed a rapid deuterium exchange for this signal, resulting in a lower sensitivity. While our data were otherwise in total agreement with the proposed structure, we were unable to detect all of the theoretical possible long-range correlations between H-9 and C-3, C-4, C-4a, C-10, and the carboxyl group as published by Fulcrand et al. (*1*). However, Mateus et al. (*4*) also did not observe the cross-peak between H-9 and C-3 and only a weak correlation of H-9 with the carboxyl group, which was completely absent for the coumaroylated vitisin A. The long-range H−C correlations are illustrated in **Figure 3**.

Additional evidence for the correctness of the structure bearing a carboxyl group at the newly formed pyran ring was

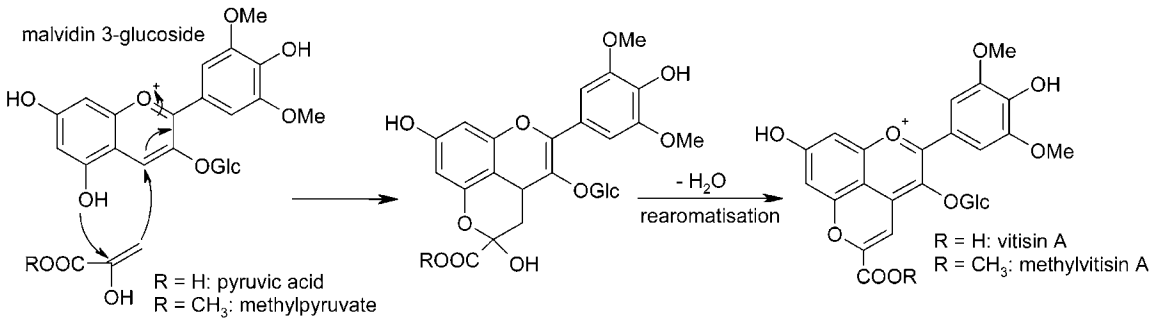

Figure 4. Reaction mechanism as postulated by Fulcrand et al. (*1*) leading to the formation of vitisin A and its methyl ester.

Table 2. Calibration Details for Quantification of Anthocyanins[a]

	anthocyanins					
	mv 3-glc	peo 3-glc	pt 3-glc	del 3-glc	mv 3-acglc	vitisin A
calibration points	7	5	6	6	6	6
calibration range (mg/L)	1.63–318.75	0.23–112.99	1.03–77.24	0.12–29.00	1.07–139.37	0.11–56.28
linear correlation coefficient (R^2)	0.9993	0.9991	0.9989	0.9996	0.9994	0.9993

[a] mv, malvidin; peo, peonidin; pt, petunidin; del, delphinidin; glc, glucoside; ac, 6″-acetyl.

Table 3. Average Values, SDs, and Coefficients of Variation (CV) for the Anthocyanins in the Analyzed Cabernet Sauvignon Wines of Various Vintages

	mv 3-glc			peo 3-glc			pt 3-glc			del 3-glc			mv 3-acglc			vitisin A		
vintage	∅ (mg/L)	SD (mg/L)	CV (%)	∅ (mg/L)	SD (mg/L)	CV (%)	∅ (mg/L)	SD (mg/L)	CV (%)	∅ (mg/L)	SD (mg/L)	CV (%)	∅ (mg/L)	SD (mg/L)	CV (%)	∅ (mg/L)	SD (mg/L)	CV (%)
1999 ($n = 3$)	25.1	8.8	35.3	4.5	1.5	32.4	4.5	0.6	13.3	3.1	1.2	38.2	11.8	10.2	86.5	3.0	0.3	11.0
2000 ($n = 7$)	51.8	20.1	38.8	8.8	3.9	43.7	7.5	3.1	40.5	4.9	2.4	49.3	25.5	10.9	42.7	2.8	1.4	49.6
2001 ($n = 6$)	92.5	26.0	28.1	28.0	21.9	78.1	13.0	5.2	40.2	8.2	3.3	40.5	55.0	17.2	31.2	2.5	1.0	38.9
2002 ($n = 9$)	121.0	30.5	25.2	22.2	7.9	35.4	20.6	4.3	20.9	16.4	4.9	29.6	64.0	20.2	31.5	5.5	2.9	53.3

drawn from the fact that the pyruvic acid methyl ester could be successfully reacted with malvidin 3-glucoside. Although Bakker et al. (*2*) never suggested a reaction mechanism starting from pyruvic acid and leading to their proposed structure of vitisin A, with a keto group at C-10, it is obvious that such a pathway would require dehydration and loss of the hydroxyl part of the acid function. Therefore, formation of a methyl ester of vitisin A from methylpyruvate should not be possible. Our experiment proved the opposite. The reaction between malvidin 3-glucoside and methylpyruvate proceeded rapidly. The reaction product had a molecular mass of *m*/*z* 575, 14 u more than vitisin A. Both in positive and in negative ion mode, the glucose moiety was split off first. Further fragmentation of the aglycon (*m*/*z* 413) in positive mode followed with losses of 16, 32, 44, 61, and 89 u exactly the malvidin pattern (*16*). In negative ion mode, a fragment of 44 u did not occur, proving that a free carboxyl group was not present. The predominant elimination mass from the aglycon (*m*/*z* 411) was 15 u, indicative of the cleavage of a methyl group. Other fragments were observed at *m*/*z* 381 (−30 u), 368 (−43 u), and 353 (−58 u). The formation of methylvitisin A can follow exactly the same reaction mechanism as postulated by Fulcrand et al. (*1*) (**Figure 4**).

While the visible absorbance maximum of vitisin A was hypsochromically shifted toward 512 nm in comparison to 524 nm of malvidin 3-glucoside, the absorbance maximum of methylvitisin A exhibited a slight bathochromic shift to 532 nm.

Vitisin A Content of Chilean Cabernet Sauvignon Wines. Details on the calibration range and linear correlation coefficients for the various anthocyanins are presented in **Table 2**. Mean values and standard deviations (SDs) of the analytical results for the examined Chilean Cabernet Sauvignon wines are summarized in **Table 3**. The analytical data for the 25 single wines are provided as Supporting Information.

The average vitisin A concentration was found to be 5.5 ± 2.9 mg/L in the 2002 wines. A strong decrease was observed during the first year, after which vitisin A stayed relatively constant at about 50% of its initial concentration. All other anthocyanins showed a steady decrease. In wines from the 1999 vintage, only 20% of the initial concentration was still present.

The analysis of the vertical row (= a sequence of wines obtained from the same vineyard over a period of several years) of the "Don Melchor" wines from 1987 to 2002 gave a different picture (**Table 4**). The underivatized anthocyanins degraded with a similar rate as observed in the other wines. In contrast, the vitisin A content decreased much slower. We observed an in the end steady decline, interrupted however by various temporary minima and maxima (**Figure 5**). They can be explained partly by the differing yearly growing conditions, resulting in, e.g., an above or below average initial amount of malvidin 3-glucoside present in the grapes. The pyruvic acid content in the musts and wines will also directly influence vitisin A formation and is in turn a function of yeast strain and vitamin B_1 (thiamine) supply (*17*).

Table 4. Anthocyanin Content in the Vertical Row of Cabernet Sauvignon Wines (Vintages 1987 to 2002)[a]

	anthocyanin content (mg/L)					
vintage	mv 3-glc	peo 3-glc	pt 3-glc	del 3-glc	mv 3-acglc	vitisin A
1987	ND	ND	ND	ND	ND	0.71
1988	ND	ND	ND	ND	ND	2.44
1989	ND	ND	ND	ND	ND	2.94
1990	ND	ND	ND	ND	ND	0.93
1991	ND	ND	ND	ND	ND	1.57
1992	ND	ND	ND	ND	ND	1.32
1993	3.06	ND	ND	ND	ND	3.31
1994	3.71	ND	ND	ND	ND	2.41
1995	5.18	ND	ND	ND	ND	3.33
1996	4.64	ND	ND	ND	0.68	3.51
1997	7.63	1.13	2.26	0.93	2.29	3.83
1998	10.63	1.76	2.81	1.51	3.30	3.23
1999	29.69	4.46	4.57	2.60	13.27	2.83
2000	35.56	4.22	5.23	3.33	15.91	5.29
2001	74.86	15.03	9.14	6.11	43.64	4.27
2002	124.53	21.57	15.07	11.37	87.74	4.15

[a] ND, not detected.

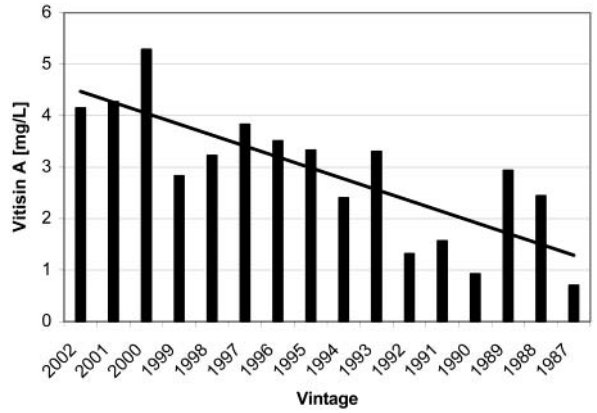

Figure 5. Course of the vitisin A content in the vertical row (vintages 1987–2002) of Chilean Cabernet Sauvignon Wines from a single vineyard.

It was shown that a deficiency in thiamine during fermentation leads to a strong accumulation of pyruvic acid in the wines, while through a sufficient supply of vitamin B_1 the pyruvic acid decarboxylase activity of the yeast is maintained; hence, only small amounts of pyruvic acid remain in the wine (*18*, *19*). Pyruvic acid can also be bound by SO_2, and it was found that the amount of sulfur dioxide also has an impact on vitisin A formation (*20*, *21*).

The stability of vitisin A as compared to the genuine anthocyanins was well-demonstrated. While malvidin 3-glucoside was no longer detectable in wines aged for 10 years and more, only 45% of vitisin A (as calculated from the regression curve in **Figure 5**) degraded during the same time. The vitisin A concentration in the three oldest wines of the vertical row (1987−1989) ranged from 13 to 55% of its maximum concentration in the sample of the vintage 2000 wine.

So far, there have been no papers on vitisin A in commercial red table wines. The only information on the content of vitisin A (expressed as malvidin 3-glucoside) comes from papers on experimental red wines, made from *Vitis vinifera* cv. Tempranillo and Shiraz grapes. In a study on the influence of sulfur dioxide and must extraction, initial concentrations of 5−6 mg/L followed by a decrease of about 50% during 2 years of storage have been reported (*20*). In another investigation on the influence of oak wood on anthocyanin composition, Revilla and Gonzáles-Sanjosé found concentrations of combined vitisin A type pigments in the range of 0.5−1.8 mg/L after 30 months of storage (18 months in bottle) (*22*). In a study on the use of pectolytic enzymes, vitisin A concentrations were between 1 and 2 mg/L after fermentation and increased up to about 6 mg/L during the first 6 months of storage, after which a slow decline was observed (*23*). Asenstorfer et al. (*21*) reported that the vitisin A concentration declined after 6 months in wines that underwent malolactic fermentation (MLF) and after 12 months in wines made without MLF.

Initial concentrations of vitisin A in these previous studies are comparable with our findings. The main difference is that maximum vitisin A content was reached after only 6 months as compared to the maximum observed after 30 months in the vertical Don Melchor row. However, this increase can also be explained by an initially higher vitisin A content in vintage 2000, possibly due to technological reasons or because of a higher anthocyanin content of the grapes and is not necessarily a result of vitisin A formation during 3 years of storage. The degradation of vitisin A in our Cabernet Sauvignons was also much slower as compared to previous studies, as even in wines of about 15 years of age up to 50% of the maximum concentration in younger wines was detected.

In Port wine production, the fermentation is stopped by adding wine spirit after approximately half of the sugar has been fermented. At this stage, the concentration of pyruvic acid in the wine is much higher as compared to dry red table wines (*24*). Therefore, higher concentrations of vitisin A are expected in Port wines, but the papers are inconsistent. In 32 natural Port wines analyzed after various storage times (2−6 years), the maximum vitisin A concentration was 2.22 mg/L, unexpectedly well below our results during the same period of time. In eight Port wines (stored 4 years and longer), vitisin A was not detectable at all, but in most of the wines, it was the major or even only monomeric anthocyanin detected. Because of the lack of a standard, the authors used malvidin 3-glucoside for quantification (*25*).

In another study (*26*), using authentic reference standards, the vitisin A concentration was monitored in four Port wines obtained by microvinification and stored for 38 months in oak barrels. The stability of the vitisin type pigments was found to be much higher as compared to other monomeric anthocyanins, which is in line with our findings. No malvidin monoglucosides were detectable after 30 months of storage, but small amounts of vitisin A (0−3 mg/L) were still found in some wines after 38 months, although a significant decrease of its initial content (10−20 mg/L) was observed.

The same authors found concentrations between 9.5 and 15.4 mg/L of vitisin A in 10 commercial Port wines when they were bottled after 6 months of storage. This amount decreased only slightly by 9−18% within the next 18 months, as opposed to a decrease of 70% in Port wines stored in oak barrels during the same period of time. A possible explanation given was that the reductive environment in the bottle prevented further oxidation reactions. Thus, the initial vitisin A concentration in Port wines is about three times higher, whereas the degradation rate in bottled Ports and table wines appears similar.

In our investigation, we measured the concentration of vitisin A during long-term storage by analysis of a vertical row of wines from a single vineyard. However, climatic conditions, time of harvesting, course of fermentation, and winemaking technology may slightly differ from year to year and thus could influence the wine composition. Ideally, a wine from one vintage would be analyzed during storage over years, which is for the apparent reason difficult to accomplish. Nonetheless, the influence of

different vintages was kept to a minimum by analyzing a wine derived from the same vineyard, thus eliminating effects of different growing regions, soils, etc. and minimizing climatic and technological factors. The remaining fluctuations are the most likely reason for the alternating decline in vitisin A concentration.

Our observation on the evolution of the vitisin A concentration during the first years of storage is in line with earlier kinetic studies on vitisin A formation in Port wines (*26*) and model wine solutions (*8*). In both studies, the maximum vitisin A concentration was reached a couple of months after fermentation before it began to decline. The exact time when the maximum was reached could not be determined in our investigation as the wines were analyzed only at a given time and not monitored in regular intervals. Thus, we observed an overall declining vitisin A content over the 16 investigated vintages. The slow degradation rate of vitisin A as compared to other anthocyanins can be explained on one hand by its higher stability, a result of the newly formed pyran ring protecting C-4 from nucleophilic attack. On the other hand, vitisin A can be constantly regenerated as long as sufficient amounts of malvidin 3-glucoside and pyruvic acid are available, thus compensating for its simultaneous incorporation into polymeric pigments.

Contribution of Vitisin A to the Color of Aged Red Wines. Several authors (*20*, *23*, *27*) have speculated on the importance of vitisin A toward the color of aged red wines. So far, the color activity concept (*15*), which allows identification of key colorants in mixtures and exact calculation of their percentage contribution to total color, has been applied to the major anthocyanins in red wine but not to vitisin A (*13*). A pure standard was not available to determine its concentrations and visual detection limit. Reliable methods for quantification of polymeric pigments are also not at hand. Estimations of their color contribution are based on photometric measurements, e.g., after bisulfite bleaching of the monomeric compounds (*7*), but this method does not give absolute amounts. In addition, because of the resistance of C-4-substituted anthocyanins against sulfur dioxide, these are mistakenly quantified as polymers by this method. It has been shown that polymeric pigments can be isolated from red wine extracts by countercurrent chromatography. This fraction obtained by CCC eluted as an unresolved hump from a reversed-phase HPLC column and was free of monomeric anthocyanins, and NMR and MS analyses revealed the polymeric nature of the pigments (*13*).

It has been reported that polymeric pigments contribute to as much as 50% of color intensity in 1 year old wine, the percentage further increasing with storage time (*7*). In experimental red wines, the percentage contribution of polymeric pigments to color was reported to increase from approximately 60 to over 90% within 2 years (*20*). During the same period, the vitisin A concentration of total monomeric anthocyanins increased from 4 to around 25%. Despite the large excess of polymeric pigments, the authors stressed the importance of vitisin A to the red color of the aged wine.

To estimate the contribution of polymeric pigments, vitisin A, and remaining monomeric anthocyanins to the overall color in aged wines, the visual detection limits for polymeric pigments, vitisin A, malvidin 3-glucoside, and malvidin 3-(6″-acetylglucoside) were determined first. The results are summarized in **Table 5**.

At a typical red wine pH of 3.60, the visual detection limit for vitisin A is well below its mean concentration in red wine, approximately four times lower than for malvidin 3-glucoside and about five times lower in regard to malvidin 3-(6″-acetylglucoside). In comparison, the detection limit for the polymeric fraction (obtained by CCC) from a 1997 Portugieser wine of 0.94 mg/L is more than 27-fold higher. These findings are not directly comparable to the results published by Degenhardt et al. (*13*) because the liquid layer thickness used for determination of the visual detection limits was not specified in this study. However, the ratios of their detection limits for polymers:malvidin 3-(6″-acetylglucoside):malvidin 3-glucoside were 6.8:2.2:1 and thus very similar to ours with 6.8:1.3:1. Comparative values for the visual detection limit of vitisin A were not available.

Table 5. Visual Detection Thresholds for Anthocyanins and Polymeric Fraction

anthocyanin	initial concn (mg/L)	avg dilution of last colored solution	visual detection limit (mg/L)
malvidin 3-glucoside	152	1100	0.138
mv 3-(6″-acetylglucoside)	85	467	0.182
vitisin A	250	7267	0.034
polymers	1345	1433	0.939

Table 6. Dilution Factors, CAVs, and PCC of the Pigments to the Color of Aged Red Wines

	Pinotage, 1996	Cabernet Sauvignon, 1997
dilution factor	1400	1060
c (polymers) (mg/L)	1228	716
c (mv 3-glc) (mg/L)	3.69	9.47
c (mv 3-acglc) (mg/L)	0.89	5.69
c (vitisin A) (mg/L)	2.15	1.1
CAV (PCC) polymers	1307.8 (93.4%)	763 (71.9%)
CAV (PCC) mv 3-glc	26.7 (1.9%)	68.6 (6.5%)
CAV (PCC) mv 3-acglc	4.9 (0.35%)	31.3 (2.9%)
CAV (PCC) vitisin A	63.2 (4.5%)	32.4 (3.1%)

In the next step, the concentration of the four pigments was measured in two aged red wines and subsequently the dilution factors of the two wines (dilution at which the wines are just distinguishable from blank model solution) were determined. Polymeric pigments were isolated by CCC and eluted well-separated from monomeric anthocyanins and derived pigments (e.g., C-4 substituted derivatives) due to their higher polarity. Monomeric anthocyanins were quantified by HPLC. CAVs and PCCs of each pigment were calculated. The results are given in **Table 6**.

Obviously, the color activity concept can only serve as an approximation, as the pigments are being evaluated in an isolated environment and possible copigmentation effects are not taken into account. In addition, the detection limit for the polymeric pigments cannot be treated as an absolute value as the visual properties of this heterogeneous class may differ depending on its composition, age, and degree of polymerization. Nonetheless, our results clearly show that the major impact on the color of aged red wines comes from the polymeric pigments, which, e.g., in the case of a 1996 Pinotage wine, contributed with 93% to overall color. In contrast, the PCC of vitisin A is well below 5% and thus in the same range as the PCC of remaining malvidin 3-glucoside and malvidin 3-(6″-acetylglucoside).

CONCLUSIONS

Synthesis of vitisin A in model wine medium yielded sufficient amounts for structural characterization by multiple analytical methods. Our results, obtained by mass spectrometry, one-/two-dimensional NMR spectroscopy, and model reactions

confirm the structure bearing a carboxyl group as proposed by Fulcrand et al. (*1*) (cf. **Figure 1**).

The maximum vitisin A concentration is already reached shortly after fermentation, followed by a slow decline. The most likely explanation is that the initial concentration of pyruvic acid is too low to ensure a constant formation of vitisin A over years of storage and to compensate for losses due to polymerization.

By using the color activity concept, it could be calculated that polymeric pigments are the most important contributors to the color of aged red wines (70−90%). Although vitisin A may be the largest discrete peak in a chromatogram of an older wine, it contributes to less than 5% of overall color.

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Supporting Information Available: Analytical data on the anthocyanin content of the 25 Chilean Cabernet Sauvignon wines (vintages 1999−2002). This material is available free of charge via the Internet at http://pubs.acs.org.

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