Preparative Isolation of Anthocyanins by High-Speed Countercurrent Chromatography and Application of the Color Activity Concept to Red Wine

Andreas Degenhardt, Sandra Hofmann, Holger Knapp, and Peter Winterhalter*

Institute of Food Chemistry, Technical University of Braunschweig, Schleinitzstrasse 20, DE−38106 Braunschweig, Germany

Red pigments were isolated from wine and grape-skin extracts using preparative high-speed countercurrent chromatography (HSCCC) and identified by NMR and MS techniques. Four solvent systems were developed in order to separate anthocyanins with different polarities. Malvidin-3-glucoside was the major component present in young red wines, and up to 500 mg of pure malvidin-3-glucoside could be obtained from a single bottle of a red wine. Other isolated pigments were the malvidin- and peonidin-3,5-diglucosides, as well as acetyl-, coumaroyl-, and caffeoyl-derivatives of anthocyanins. Furthermore, condensed red wine pigments formed from malvidin-3-glucoside (vitisin A and acetylvitisin A) were isolated on a preparative scale. Isolated compounds were used as standards for quantification of anthocyanins in a range of red wines. The "color activity concept" was applied to red wine, and visual detection thresholds were determined for some of the isolated anthocyanins. Mono-glucosides were found to exhibit lower visual detection thresholds than di-glucosides and acylated anthocyanins.

Keywords: *Red wine; color; anthocyanins; high-speed countercurrent chromatography; color activity concept*

INTRODUCTION

Red wine color is an important quality marker and can be measured by various techniques (Kreuz et al., 1998). Apart from simple visual assessment of wines, photometric measurements are common practice in the color evaluation of red wines. More recently, tristimulus measurements have been used in place of photometric measurements, because the results obtained by tristimulus measurements are considered to be close to human vision (Bakker et al., 1986; Heredia et al., 1997, 1998; Kreuz et al., 1998; Romero and Bakker, 1999). Another technique for color evaluation is the "color activity concept" (Hofmann, 1998). After determination of visual threshold values and quantification of the colorants in the food system, so-called "color activity values" (CAV) are determined. In this way, the color contribution of individual compounds to the overall color of the system is evaluated and key colorants can be identified.

In young red wines, the color is based on the presence of monomeric anthocyanins (Mirabel et al., 1999). With aging of the wine, the anthocyanin profile shifts from monomeric anthocyanins to poorly characterized polymeric pigments formed from condensation of anthocyanins with colorless phenolics such as (−)-epicatechin, (+)-catechin, and phenolic acids (Liao et al., 1992). This causes a change in the perceived color. Aged wines show an increase in yellow color at $\lambda \sim 420$ nm and a shift from the brightness and purple tints of young wines to a more brownish color (Heredia et al., 1997). Recently, red wine pigments formed from anthocyanins and pyruvic acid (so-called vitisins) were identified. They are considered to be partly responsible for the color of aged wines (Bakker and Timberlake, 1997; Bakker et al., 1997; Fulcrand et al., 1998; Romero and Bakker, 1999).

The aim of the current study was to identify key colorants in young and old red wines by using the color activity concept. In addition, an attempt was made to estimate the color contribution of polymeric anthocyanins and condensed pigments (vitisins). For the isolation of large quantities of standard compounds, high-speed countercurrent chromatography (HSCCC) was used. HSCCC is an all-liquid chromatographic technique, in which the separation of compounds occurs under gentle operating conditions through partitioning between two immiscible liquid phases. In recent years, this technique has seen a renaissance in natural product analysis (Conway and Petroski, 1995). It is especially suited to preparative separations of even very polar compounds (e.g., anthocyanins) and allows isolation of up to several hundred milligrams of pure compounds within a couple of hours (Degenhardt et al., 2000a).

MATERIALS AND METHODS

Wines. Materials used for the analyses were Dornfelder, 1999, Rheinhessen, Germany; Ruby Red, 1998, California; Spätburgunder (Pinot Noir), 1997, Baden, Germany; Portugieser, 1996, Rheinhessen, Germany; Merlot, 1990, France; and grape skin extract (Dr. Marcus GmbH, Geesthacht, Germany).

Model Wine Solution. Dilution experiments with standard compounds and wines, respectively, were carried out with a model wine solution consisting of 10% aqueous ethanol (v/v) buffered to pH 3.6.

Extraction, Purification, and Isolation of Anthocyanins. Anthocyanins were isolated from either a commercial

* Corresponding author. E-mail: p.winterhalter@tu-bs.de. Fax: ++49 531 391 7230.

10.1021/jf0007481 CCC: $19.00
Published on Web 11/01/2000

grape skin extract or young red wines. Isolation and clean up of the anthocyanin mixture was performed on an Amberlite XAD-7 resin (Fluka, Buchs, Switzerland) according to the procedure described by Degenhardt et al. (2000b). HSCCC separations were carried out with the XAD-7 isolates. A high-speed model CCC-1000 manufactured by Pharma-Tech Research Corporation (Baltimore, MD) was equipped with 3 preparative coils (total volume, 850 mL). Details about the operation of the HSCCC apparatus were described earlier (Degenhardt et al., 2000a). The amount of sample injected varied from 300 mg to 750 mg. Stationary phase retention was in the range of 45−75%.

Solvent Systems used for the Separation of Red Wine Pigments. Solvent I was medium polar, for mono-glucosides and acylated di-glucosides, and consisted of *tert*-butyl-methyl ether (TBME)−*n*-butanol−acetonitrile−water (2/2/1/5) plus 0.1% trifluoroacetic acid (TFA). Solvent II was polar, for vitisins and di-glucosides, and consisted of ethyl acetate−*n*-butanol−water (2/3/5) plus 0.1% TFA. Solvent III was nonpolar, for coumaroyl- and caffeoyl-mono-glucosides, and was a mixture of ethyl acetate−water (1/1) plus 0.1% TFA. Solvent IV was medium polar, for acetylated anthocyanins, and was ethyl acetate−*n*-butanol−water (4/1/5) plus 0.1% TFA. Assignment of the polarity of the solvent systems is based on data published by Oka et al. (1991).

HPLC Analysis. A Jasco ternary gradient unit LG-980-02, with degasser and UV-975 detector, was used. Malvidin-3-glucoside, malvidin-3-(6″-acetylglucoside), malvidin-3-(6″-coumaroylglucoside), malvidin-3,5-diglucoside, malvidin-3-(6″-coumaroylglucoside)-5-glucoside, peonidin-3-glucoside, and peonidin-3,5-diglucoside were isolated from red wine or grape skin extract by HSCCC as standards for HPLC. Delphinidin-3-glucoside was isolated from blackcurrant juice according to a procedure described by Degenhardt et al. (2000a). Four-point calibration curves were set up, and anthocyanins in red wine were quantified using a detection wavelength of 520 nm. Nonavailable standards were cyanidin-3-glucoside and petunidin-3-glucoside which were expressed as peonidin-3-glucoside. Peonidin-3-(6″-acetylglucoside) was expressed as malvidin-3-(6″-acetylglucoside), peonidin-3-(6″-coumaroylglucoside) was expressed as the respective malvidin derivative, and malvidin-3-(6″-caffeoylglucoside)-5-glucoside was expressed as malvidin-3-(6″-coumaroylglucoside)-5-glucoside. Because of the low amount of the respective compounds present in the examined wines, the error introduced by this simplification is not considered to be significant. Red wines were filtered (0.2 μm) prior to injection. The column was a RP-18 5-μm LUNA 150 × 4.6 mm (Phenomenex, Aschaffenburg, Germany), flow rate was 0.8 mL/min, and solvents were water−formic acid−acetonitrile (87/10/3, v/v/v, solvent A) and water−formic acid−acetonitrile (40/10/50, v/v/v, solvent B). The linear gradient was from 94% A and 6% B to 80% A and 20% B in 20 min; to 60% A, 40% B in 15 min; to 40% A, 60% B in 5 min; to 30% A, 70% B in 6 min; and back to initial conditions (Holbach et al., 1997).

Color Analysis. Visual detection thresholds were determined in a triangle test by duplicate analysis. The test was carried out in daylight against a white background. The standards were dissolved in a model wine solution of 10% ethanol (buffered to pH 3.6) and stepwise diluted. The concentration of the colorant at which a difference between the diluted sample and the two blanks could just be detected visually was defined as the "visual detection threshold". The dilution factor is determined by diluting the red wine sample with model wine solution in a triangle test until almost complete loss of the color was achieved (for details cf. Hofmann, 1998).

Proton Magnetic Resonance Spectroscopy (^{1}H NMR). All experiments were performed on a Bruker AMX 300 spectrometer (300 MHz). Spectra were recorded in CD_3OD−CF_3COOD (19:1, v/v). Assignments were made on the basis of spectral data published by Pedersen et al. (1993), van Calsteren et al. (1991), and Kim et al. (1989).

Electrospray Ionization−Ion Trap Multiple Mass Spectrometry (ESI−MS/MS). Bruker Esquire-LC-MS/MS with electrospray ionization in the positive mode was used. Samples were measured by continuous infusion of an acetonitrile−2% formic acid solution (9/1, v/v) at a flow rate of 4 μL/min. Dry gas was nitrogen with a gas flow of 4 L/min (350 °C); the nebulizer was set at 10 psi. MS and MS^n parameters were optimized on the total ion current in MS mode for each compound. Typical values were capillary, −3000 V; end plate, −2500 V; capillary exit, 110 V; skim 1, 35 V; skim 2, 8 V. MS/MS experiments were performed with different fragmentation amplitudes.

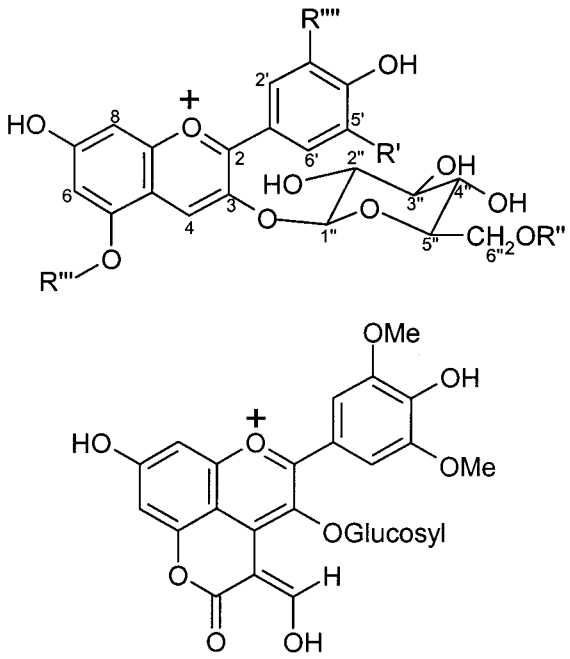

Figure 1. Top, structures of isolated anthocyanins. Malvidin-3-glucoside (**1**): R′, OMe; R″, H; R‴, H; R⁗, OMe. Malvidin-3,5-diglucoside (**2**): R′, OMe; R″, H; R‴; glucose; R⁗, OMe. Malvidin-3-(6″-acetylglucoside) (**3**): R′, OMe; R″, acetate; R‴, H; R⁗, OMe. Malvidin-3-(6″-coumaroylglucoside) (**4**): R′, OMe; R″, coumarate; R‴, H; R⁗, OMe. Malvidin-3-(6″-coumaroylglucoside)-5-glucoside (**5**): R′, OMe; R″, coumarate; R‴, glucose; R⁗, OMe. Peonidin-3-glucoside (**6**): R′, H; R″, H; R‴, H; R⁗, OMe. Peonidin-3,5-diglucoside (**7**): R′, H; R″, H; R‴, glucose; R⁗, OMe. Peonidin-3-(6″-coumaroylglucoside)-5-glucoside (**8**): R′, H; R″, coumarate; R‴, glucose; R⁗, OMe. Malvidin-3-(6″-acetylglucoside)-5-glucoside (**9**): R′, OMe' R″, acetate; R‴, glucose; R⁗, OMe. Malvidin-3-(6″-caffeoylglucoside) (**12**): R′, OMe; R″, caffeoate; R‴, H; R⁗, OMe. Delphinidin-3-(6″-acetylglucoside) (**13**): R′, OH; R″, acetate; R‴, H; R⁗, OH. Petunidin-3-(6″-acetylglucoside) (**14**): R′, OH; R″, acetate; R‴, H; R⁗, OMe. Bottom, structure of vitisin A according to Bakker et al., 1997.

RESULTS AND DISCUSSION

Isolation of Different Groups of Anthocyanins Using HSCCC. Anthocyanin standards (for structures see Figure 1) were isolated by HSCCC on a preparative scale from red wine, grape skin extracts, and other fruit juice extracts. An example demonstrating the separation power of HSCCC using the previously published standard solvent system for anthocyanin separation, i.e., *tert*-butyl-methyl ether (TBME)−*n*-butanol−acetonitrile−water (2/2/1/5) plus 0.1% trifluoroacetic acid (TFA) (solvent I), is shown in Figure 2, left side (Degenhardt et al., 2000b). From this Californian red wine (Ruby Red) a mixture of malvidin-3,5-diglucoside (**2**) and peonidin-3,5-diglucoside (**7**) was isolated as the major pigment. As further anthocyanin standards malvidin-3-(6″-acetylglucoside) (**3**) and compounds **5** and **8** could be obtained. The latter pigments were identified as malvidin-3-(6″-coumaroylglucoside)-5-glucoside (**5**) and

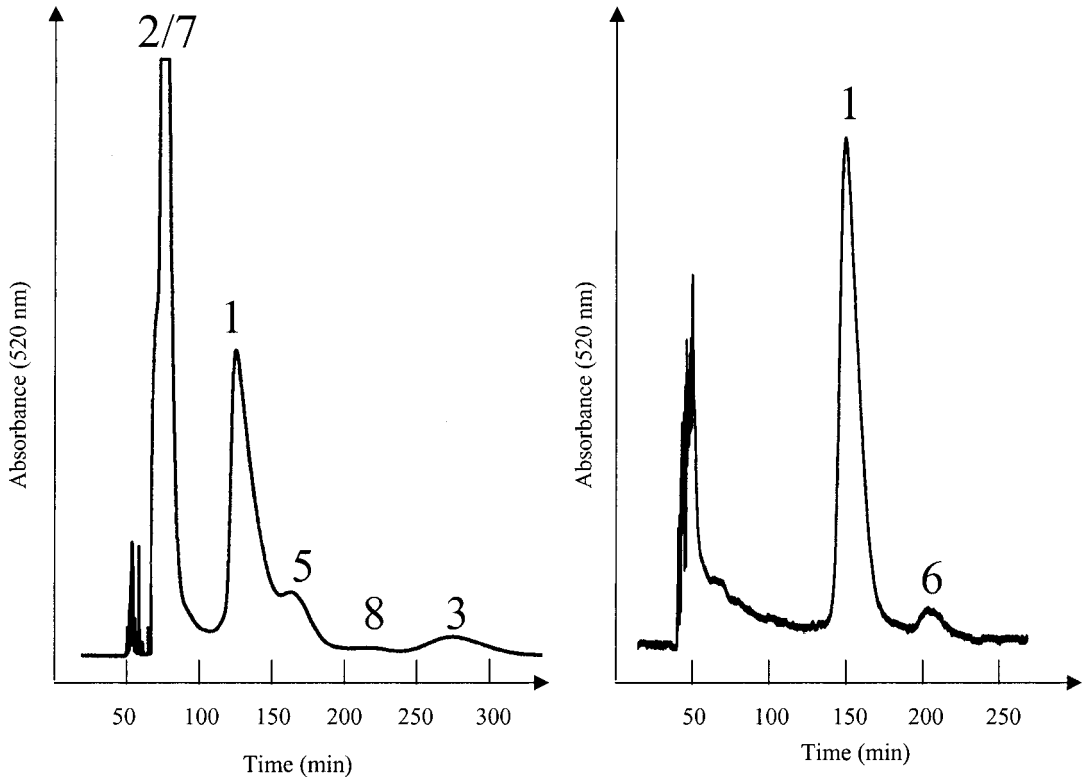

Figure 2. Left side, HSCCC separation of a Californian red wine (Ruby Red) (solvent system I): malvidin-3,5-diglucoside (**2**), peonidin-3,5-diglucoside (**7**), malvidin-3-glucoside (**1**), malvidin-3-(6″-coumaroylglucoside)-5-glucoside (**5**), peonidin-3-(6″-coumaroylglucoside)-5-glucoside (**8**), and malvidin-3-(6″-acetylglucoside) (**3**). Right side, HSCCC separation of a German Spätburgunder red wine (solvent system I): malvidin-3-glucoside (**1**) and peonidin-3-glucoside (**6**). For structures cf. Figure 1.

the respective peonidin derivative (**8**). In contrast to the Ruby Red, the Pinot Noir (Spätburgunder) wine showed a different, much simpler profile by HSCCC (cf. Figure 2, right side). With solvent system I only two compounds could be isolated, i.e., malvidin-3-glucoside (**1**) and peonidin-3-glucoside (**6**). In an effort to obtain pure standards for all of the different groups of anthocyanins, additional solvent systems had to be developed.

Separation of Di-Glucosides. To separate compounds **2** and **7** a slightly more polar system was employed, namely ethyl acetate−*n*-butanol−water (2/3/5) acidified with 0.1% TFA as shown in Figure 3 (left side). The diglucosides were unambiguously identified by ^{1}H NMR spectroscopy. Although no baseline separation was achieved, pure compounds could be obtained by discarding overlapped fractions. The isolated compounds are useful as standards for HPLC analysis, because they allow the detection of hybrid grapes or the adulteration of wines (Holbach et al., 1997). In addition, compound **9** was obtained in pure form and, on the basis of its ESI−MS/MS spectrum, tentatively identified as malvidin-3-(6″-acetylglucoside)-5-glucoside (**9**).

Separation of Vitisins. By using solvent system II the red pigments vitisin A (**10**) and acetylvitisin A (**11**) (cf. Figure 1 for structure) could be obtained on a preparative scale from a German Dornfelder wine (cf. Figure 3, right side). Compounds **10** and **11** were identified by ESI−MS analysis (Bakker and Timberlake, 1997; Fulcrand et al., 1998). Because impurities were detected in the ^{1}H NMR spectra, further purification steps by preparative HPLC are required to obtain pure vitisin standards. Due to the low concentration of vitisins present in all of the wines under investigation, a better alternative for obtaining larger quantities of vitisin A and related compounds is model reactions of malvidin-3-glucoside (**1**) with various reagents.

Separation of Acylated Pigments. In the 1999 Dornfelder wine acylated pigments were present. Because of their greater hydrophobicity, solvent system I could not elute these acylated compounds which were retained in the stationary phase on the CCC coil. Therefore, the more hydrophobic solvent system III (ethyl acetate−water, 1/1, acidified with 0.1% TFA) was used for the elution and allowed separation of malvidin-3-(6″-coumaroylglucoside) (**4**) and malvidin-3-(6″-caffeoylglucoside) (**12**) (chromatogram not shown). Compound **4** was unequivocally identified by NMR, whereas compound **12**, because of the low amount present, could only be tentatively identified on the basis of its ESI−MS/MS spectrum.

Separation of Acetylated Anthocyanins. Application of solvent system IV (ethyl acetate−*n*-butanol−water, 4/1/5, acidified with 0.1% TFA) resulted in the isolation of various acetylated pigments i.e., malvidin-3-(6″-acetylglucoside) (**3**), delphinidin-3-(6″-acetylglucoside) (**13**), and petunidin-3-(6″-acetylglucoside) (**14**) from a grape skin extract (chromatogram not shown).

Isolation of a Polymeric Anthocyanin Fraction. A polymeric anthocyanin fraction (PF2) from the 1990 Merlot was isolated from the XAD-7 isolate by HSCCC with solvent system I. The majority of colored material eluted with the solvent front and was concentrated in two fractions. All other known monomeric anthocyanins (including vitisins) remained in the stationary organic phase on the coil of the HSCCC instrument. Attempts to characterize the polymeric fraction PF2 by ESI−MS failed. The MS spectra varied with time, thereby confirming the assumption that the sample is polymeric and polydisperse in nature. Preliminary NMR studies

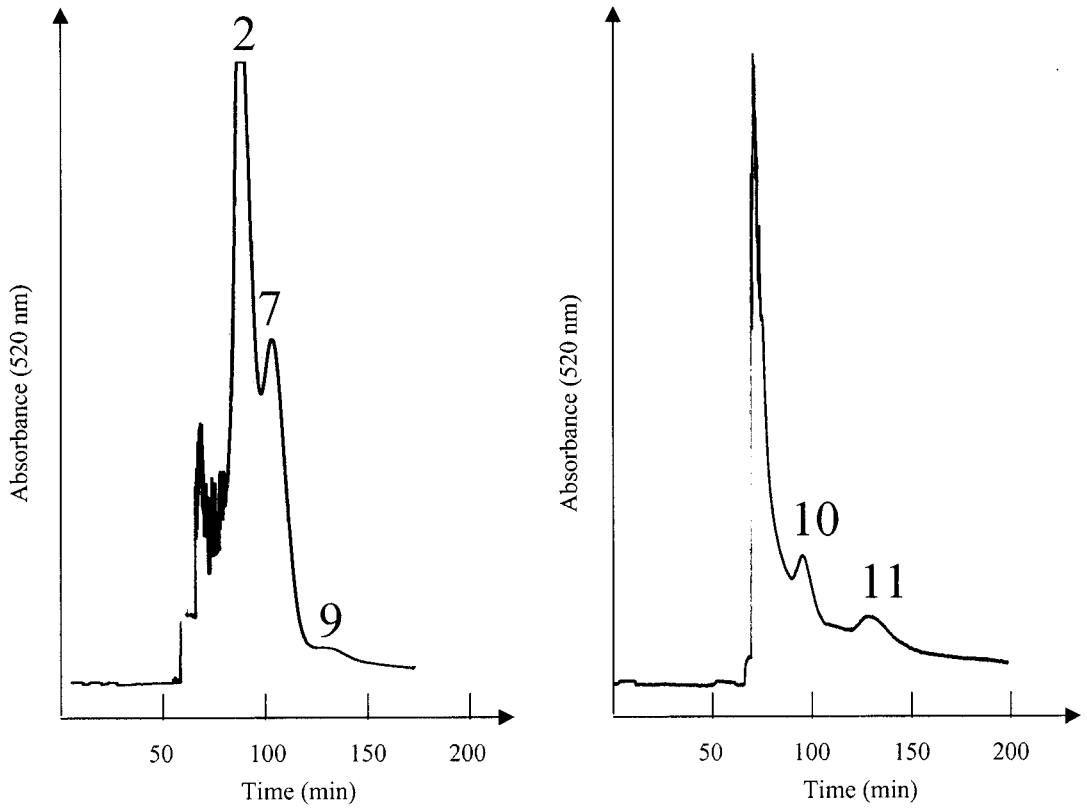

Figure 3. Left side, HSCCC separation (solvent system II) of coeluting compounds malvidin-3,5-diglucoside (**2**) and peonidin-3,5-diglucoside (**7**) from Figure 2, and malvidin-3-(6″-acetylglucoside)-5-glucoside (**9**). Right side, HSCCC separation of a German Dornfelder red wine (solvent system II): vitisin A (**10**), acetylvitisin A (**11**).

of the pigments revealed "polymer-like" ^{1}H NMR spectra (Bailey et al., 1992). HPLC analysis showed that this fraction is free of known monomeric anthocyanins and eluted from a reversed-phase HPLC packing as a convex "hump"; a phenomenon also encountered in the case of black tea polymers, so-called "thearubigins" (Bruschi et al., 1999). The polymeric fraction PF1 was isolated from the 1998 Californian wine with solvent system II and eluted similarly with the solvent front.

With regard to the preparative capability of HSCCC, it has to be stressed that separation of a XAD-7 isolate from a single bottle (0.75 L) of a German Dornfelder red wine yielded up to 500 mg of pure malvidin-3-glucoside (**1**) as shown by ^{1}H NMR, ESI−MS, and HPLC analysis of the isolated pigment.

Quantification of Anthocyanins in Red Wines Using the Isolated Compounds as Standards. Monomeric anthocyanins in red wines were determined using a HPLC method with detection at 520 nm. Wines were injected after filtration and appropriate dilution of the original wine, and the analytical results are shown in Table 1. Total contents of monomeric anthocyanins are in the range of 10 to 1465 mg/L. The 1990 French wine contained as little as 10 mg/L of monomeric anthocyanins. The Pinot Noir exhibited a simple anthocyanin profile containing the four mono-glucosides malvidin-3-glucoside (**1**), peonidin-3-glucoside (**6**), petunidin-3-glucoside, and delphinidin-3-glucoside, whereas the Dornfelder variety contained 9 major monomeric anthocyanins (Table 1).

Vitisin A (**10**) was present in each of the wines and represented the major peak in the 1990 wine. Quantitatively (expressed as malvidin-3-glucoside), the vitisin A content in this wine is ca. 7 mg/L. It is noteworthy that vitisin A is also present in the youngest wine (1999 Dornfelder) in similar amounts.

Color Evaluation of Wines. Hofmann (1998) developed the color activity concept in order to elucidate impact compounds for the color of Maillard reaction mixtures. The author showed that this concept originating from flavor research (Rothe and Thomas, 1963) is applicable to color vision. The color activity value of a compound is defined as

$$\text{color activity value (CAV)} = \frac{\text{concentration [mg/L]}}{\text{visual detection threshold [mg/L]}} \quad (1)$$

The concentration of monomeric anthocyanins was determined by HPLC (Table 1). The visual detection thresholds of isolated compounds are given in Table 1 (last column) and were established using a triangle test. For CAV > 1, a compound contributes to the color of a system. A calculated percentage color contribution (PCC) correlates CAVs to the overall color of the mixture using the following relationship

$$\text{percentage color contribution (PCC)} = \frac{\text{concentration (CAV)}}{\text{dilution factor}} \times 100 \quad (2)$$

Dilution factors for red wines are listed in Table 4 at the end of this paper. A dilution factor of 320 means that a 320-fold dilution of the 1998 Californian wine results in a color that is just above the visual detection threshold. The dilution factor is another measure for the color intensity of wines and ranges from 160 to 1280 for the red wines tested in the study. With color

Table 1. HPLC Analysis of Red Wines (contents in mg/L)

	variety					
compound[a]	Dornfelder 1999	Ruby Red 1998	Spätburgunder (Pinot Noir) 1997	Portugieser 1996	Merlot 1990	visual detection threshold, mg/L
mv-3-glc (**1**)	718	98	128	115	<1	1.3
peo-3-glc (**6**)	100	3	7	6	<1	1.4
pt-3-glc	105	-	4	15	-	n.d.
cy-3-glc	8	-	-	-	-	n.d.
del-3-glc	34	4	1	6	-	0.70
mv-3-glc-ac (**3**)	204	52	-	18	2	2.9
peo-3-glc-ac	39	-	-	-	-	n.d.
mv-3-glc-coum (**4**)	218	-	-	36	8	5.7
peo-3-glc-coum	39	-	-	-	<1	n.d.
mv-3,5-diglc (**2**)	-	257	-	-	-	4.7
peo-3,5-diglc (**7**)	-	128	-	-	-	5.0
mv-3-glc-caff-5-glc	-	2	-	-	-	n.d.
mv-3-glc-coum-5-glc (**5**)	-	2	-	-	-	n.d.
PF1	n.d.[b]	n.d.	n.d.	n.d.	n.d.	8.3
PF2	n.d.	n.d.	n.d.	n.d.	n.d.	9.5
total of monomeric anthocyanins	1465	546	140	196	10	-

[a] Abbreviations used: mv, malvidin; glc, glucoside; peo, peonidin; pt, petunidin; cy, cyanidin; del, delphinidin; ac, acetyl; coum, coumaroyl; diglc, di-glucoside; caff, caffeoyl; PF1, polymer fraction from Ruby Red; PF2, polymer fraction from Merlot. [b] n.d., not determined.

intensity measured by photometry (Mazza et al., 1999; results not shown) the same ranking of wines was obtained. Consequently, determination of dilution factors represents an easy and quick method to assess and compare the color intensity of red wines.

Color Evaluation of the Isolated Anthocyanins. Visual detection thresholds for the isolated compounds were in the range of 0.70 to 9.5 mg/L (Table 1). The monoglucosides proved to be most intense in color, whereas a second sugar residue on the aglycone or a substitution on the sugar moiety increased the visual detection threshold. Giusti et al. (1999) have compared color intensity of anthocyanins with different substitution patterns by L*, hue, and chroma values. They found hyperchromic effects when substitution with cinnamic acid derivatives occurred. In addition, molar absorptivity was reported to increase with double substitution at C-3 and C-5 in comparison to C-3 monosubstituted anthocyanins. This latter finding is apparently in contrast with the results obtained by our visual analysis, in which the mono-glucosylated anthocyanins showed the lowest visual detection thresholds. The most reasonable explanation for this discrepancy is pH: whereas the experiments of Giusti et al. (1999) were carried out at pH 1.0, our experiments were performed at pH 3.6, which is the pH value of wine. Our results are in agreement with the findings of Mazza and Miniati (1993) who have also reported that anthocyanin 3-glycosides are more colored than the 3,5- and 5-glycosides. At the pH value of wine (ca. 3.6), mono-glucosides might be expected to be more intensely colored than di-glucosides because, without copigmentation, di-glucosides (e.g., malvidin-3,5-diglucoside) exist to a larger extent in the colorless hemiacetal and *cis*-chalcone structures (Mazza and Brouillard, 1990; Dangles et al., 1993). An interesting observation was made in the case of the Californian wine. Although higher in concentration, the color contribution of the di-glucosides was found to be less important compared to that of the monoglucosidic anthocyanins. This is caused by the different color intensities found using the color activity concept (Table 3).

In general, among monomeric anthocyanins, malvidin-based pigments are the most important contributors to red wine color. With regard to the color contribution of the polymeric fraction some unexpected results were obtained. For the isolated polymeric fraction PF2 from the 1990 French red wine a visual detection threshold of approximately 9.5 mg/L was determined. The polymeric fraction PF1 from the 1998 Ruby Red had a similar detection limit of 8.3 mg/L (Table 1). It must be stressed that color evaluation of these polymeric fractions can serve only as an approximation, because this is a heterogeneous and not well-characterized fraction. Nevertheless, our findings contradict the results of Ribéreau-Gayon et al. (1983) who found that anthocyanins in the condensed form (with tannins) are more colored than in the free form. Because of the considerably higher visual detection thresholds of the polymeric fraction, our results indicate that condensed forms are less colored than monomeric anthocyanins.

Determination of Color Activity Values. CAVs rank the anthocyanins on the basis of their relative effectiveness in generating the overall color of red wines. To evaluate the percentage contribution of each anthocyanin, the CAV is divided by the dilution factor of the whole red wine, which was defined to be 100% color activity (Hofmann, 1998). Tables 2 and 3 list the calculated values for the 1999 Dornfelder and the 1998 Californian wine, respectively. It was found that the monomeric anthocyanins (without vitisins) make up 69% of the color in the case of the Dornfelder variety, versus 57% for the Californian Ruby Red. The contribution of monomeric anthocyanins to the color of the oldest wine of the study (1990 Merlot) was found to be negligible (Table 4).

The color activity concept is an approximation. It looks at compounds in an isolated environment; other interactions are not considered. The difference to 100% color is therefore most likely due to the content of vitisins and polymeric pigments, as well as copigmentation effects. Intermolecular copigmentation effects are difficult to investigate because wine is a complex system with many copigments present (Liao et al., 1992). This fact is further complicated as copigmentation is dependent on various factors, i.e., molar ratio of copigment to anthocyanin, anthocyanin concentration, pH, temperature, presence of metal ions, and composition of the

Table 2. Color Activity Values (CAVs) and Percentage Color Contribution for 1999 Dornfelder (Abbreviations Same as for Table 1)

compound	content of total monomeric anthocyanins (%)	color activity value (CAV)	percentage color contribution (%)
del-3-glc	2.3	48.6	3.8
cy-3-glc[a]	0.5	5.7	0.4
pt-3-glc[a]	7.2	75.0	5.8
peo-3-glc	6.8	71.4	5.6
mv-3-glc	49.0	552.3	43.1
peo-3-glc-ac[b]	2.7	13.4	1.1
mv-3-glc-ac	13.9	70.3	5.5
peo-3-glc-coum[c]	2.7	6.8	0.5
mv-3-glc-coum	14.9	38.2	3.0
sum	100	881.7	68.8

Compounds not available, [a]expressed as peo-3-glc, [b]expressed as mv-3-glc-ac, [c]expressed as mv-3-glc-coum.

Table 3. CAVs and Percentage Color Contribution for 1998 Californian Ruby Red (Abbreviations Same as for Tables 1 and 2)

compound	content of total monomeric anthocyanins (%)	CAV	percentage color contribution (%)
del-3-glc	0.7	5.7	1.8
peo-3-glc	0.6	2.1	0.7
mv-3-glc	17.9	75.4	23.6
mv-3-glc-ac	9.5	17.9	5.6
peo-3,5-diglc	23.4	25.6	8.0
mv-3,5-diglc	47.1	54.7	17.1
mv-3-glc-caff-5-glc	0.4	n.d.	-
mv-3-glc-coum-5-glc	0.4	n.d.	-
sum	100	181.4	56.8

Table 4. Dilution Factors for Wines and Percentage Color Contribution of Monomeric Anthocyanins (Excluding Vitisins)

wine	dilution factor	percentage color contribution of monomeric anthocyanins (excluding vitisins)
Dornfelder, 1999	1280	68.8
Ruby Red, 1998	320	56.8
Spätburgunder (Pinot Noir), 1997	160	47.1
Portugieser, 1996	640	18.6
Merlot, 1990	640	0.2

medium (Mazza and Miniati, 1993). Timberlake and Bridle (1976) found that color augmentation increased with increasing molar ratios of copigments to anthocyanins and could amount to about 20%. Rutin is a very effective copigment found in red wine and copigmentation starts at ratios of copigment to anthocyanin of about 1 (Brouillard and Dangles, 1994). Chlorogenic acids and catechin are weaker copigments and typical molar ratios used to investigate copigmentation effects range from 10 to 100. Apart from intermolecular copigmentation effects, intramolecular copigmentation might also be possible. Cinnamic acid residues of anthocyanins stack parallel with the anthocyanidin, thus protecting against nucleophilic attack (Brouillard and Dangles, 1994). However, Brouillard and Dangles (1994) rule out intramolecular copigmentation effects for red grape anthocyanins due to their structures. Intermolecular association is mostly taking place. An interesting fact is that color enhancement by copigmentation is most prominent at pH values of about 3.6, i.e., the pH value of many red wines.

Self-association is another factor which may influence color expression of anthocyanin mixtures (Mazza and Miniati, 1993) and is also not taken into account by the model.

Color Contribution of Vitisins and Polymeric Pigments. According to Romero and Bakker (1999), vitisin A and related compounds contribute to a large extent to the color of aged red wines. The authors found for vitisin A in aqueous solutions a greater color expression than for normal anthocyanins, contributing a redness to older wines. In the 1990 wine, vitisin A represents the most abundant of the known, chromatographically resolved pigments. However, its concentration is very low and in a range similar to that in the other wines. According to the color activity model, color contribution of vitisin A requires either a low visual detection threshold or a high concentration of the pigment. Because vitisin A concentrations are roughly in the same order of magnitude as the visual detection thresholds of other monomeric anthocyanins, it is unlikely that vitisin A contributes significantly to the color of wines. The final evaluation of the color contribution of vitisins will require sufficient amounts of pure vitisin reference compounds to enable an accurate determination of visual detection thresholds. As far as the aged French red wine from 1990 is concerned, a far greater contribution to the color has to be assigned to polymeric anthocyanins (Table 4). However, quantification of colored polymers in red wine will be difficult to accomplish unless a countercurrent chromatography (CCC) system is developed that allows separation of the complex polymer fraction.

Summary. The color of young red wines is dominated by malvidin-based monomeric anthocyanins. As the wine ages, this picture changes: monomeric anthocyanins are almost nondetectable and polymeric fractions represent the majority of colored material. It is suggested that the color contribution of vitisins may have been overestimated for older wines. However, wine is a complex system and matrix effects (i.e., copigmentation) remain unaccounted for by the color activity concept. The color activity concept is a useful tool for the study of key colorants in complex mixtures and helps to understand the contribution of individual pigments to the overall color of wine.

Measurement of anthocyanin content is, in general, important for research as well as for industrial applications. HSCCC provides a tool for the chemist to isolate large quantities of pure standards which will lead to a more accurate quantification because many anthocyanin references are not commercially available.

ACKNOWLEDGMENT

Mrs. C. Balcke is thanked for measuring ESI−MS spectra. Mrs. C. Stingl and Mrs. M. Messerer are gratefully acknowledged for running NMR spectra.

LITERATURE CITED

Bailey, R. G.; Nursten, H. E.; McDowell, I. Isolation and analysis of a polymeric thearubigin fraction from tea. *J. Sci. Food Agric.* **1992**, *59*, 365−375.

Bakker, J.; Timberlake, C. F. Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines. *J. Agric. Food Chem.* **1997**, *45*, 35−43.

Bakker, J.; Bridle, P.; Timberlake, C. F. Tristimulus measurements (CIELAB 76) of port wine colour. *Vitis* **1986**, *25*, 67−78.

Bakker, J.; Bridle, P.; Honda, T.; Kuwano, H.; Saito, N.; Terahara, N.; Timberlake, C. F. Identification of an anthocyanin occurring in some red wines. *Phytochemistry* **1997**, *44*, 1375−1382.

Brouillard, R.; Dangles, O. Anthocyanin molecular interactions: the first step in the formation of new pigments during wine aging? *Food Chem*. **1994**, *51*, 365−371.

Bruschi, L.; Copeland, E. L.; Clifford M. N. Unexpected hyperchromic interactions during the chromatography of theafulvins and simple flavonoids. *Food Chem*. **1999**, *67*, 143−146.

Conway, W. D.; Petroski, R. J. (Eds.) *Modern Countercurrent Chromatography*; ACS Symposium Series 593; American Chemical Society: Washington, DC, 1995.

Dangles, O.; Saito, N.; Brouillard, R. Anthocyanin intramolecular copigmentation effect. *Phytochemistry* **1993**, *34*, 119−124.

Degenhardt, A.; Knapp, H.; Winterhalter, P. Separation and purification of anthocyanins by high-speed countercurrent chromatography and screening for antioxidant activity. *J. Agric. Food Chem*. **2000a**, *48*, 338−343.

Degenhardt, A.; Knapp, H.; Winterhalter, P. Rapid isolation of malvidin 3-glucoside from red wine by high-speed countercurrent chromatography. *Vitis* **2000b**, *39*, 43−44.

Fulcrand, H.; Benabdeljalil, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry* **1998**, *47*, 1401−1407.

Giusti, M. M.; Rodríguez-Saona, L. E.; Wrolstad, R. E. Molar absorptivity and color characteristics of acylated and nonacylated pelargonidin-based anthocyanins. *J. Agric. Food Chem*. **1999**, *47*, 4631−4637.

Heredia, F. J.; Troncoso, A. M.; Guzmán-Chozas, M. Multivariate characterization of aging status in red wines based on chromatic parameters. *Food Chem*. **1997**, *60*, 103−108.

Heredia, F. J.; Francia-Aricha, E. M.; Rivas-Gonzalo, J. C.; Vicario, I. M.; Santos-Buelga, C. Chromatic characterization of anthocyanins from red grapes − I. pH effect. *Food Chem*. **1998**, *63*, 491−498.

Hofmann, T. Studies on the influence of the solvent on the contribution of single maillard reaction products to the total color of browned pentose/alanine solutions − a quantitative correlation using the color activity concept. *J. Agric. Food Chem*. **1998**, *46*, 3912−3917.

Holbach, B.; Marx, R.; Ackermann, M. Determination of anthocyanin composition of red wines using high-pressure liquid chromatography (HPLC). *Lebensmittelchemie* **1997**, *51*, 78−80.

Kim, J. H.; Nonaka, G.-I.; Fujieda, K.; Uemoto, S. Anthocyanidin malonylglucosides in flowers of *Hibiscus syriacus*. *Phytochemistry* **1989**, *28*, 1503−1506.

Kreuz, S.; Flak, W.; Kernbauer, E.; Pitschmann, M.; Schaber, R. Qualitative assessment of wines by tristimulus measurements and related techniques. *Mitt. Klosterneuburg* **1998**, *48*, 25−33.

Liao, H.; Cai Y.; Haslam E. Polyphenol interactions. Anthocyanins: co-pigmentation and colour changes in red wines. *J. Sci. Food Agric*. **1992**, *59*, 299−305.

Mazza, G.; Brouillard, R. The mechanism of co-pigmentation of anthocyanins in aqueous solutions. *Phytochemistry* **1990**, *29*, 1097−1102.

Mazza, G.; Miniati, E. *Anthocyanins in Fruits, Vegetables, and Grains*; CRC Press: Boca Raton, FL, 1993.

Mazza, G.; Fukumoto, L.; Delaquis, P.; Girard, B.; Ewert, B. Anthocyanins, phenolics, and color of Cabernet Franc, Merlot, and Pinot Noir wines from British Columbia. *J. Agric. Food Chem*. **1999**, *47*, 4009−4017.

Mirabel, M.; Saucier, C.; Guerra, C.; Glories, Y. Copigmentation in model wine solutions: occurrence and relation to wine aging. *Am. J. Enol. Vitic*. **1999**, *50*, 211−218.

Oka, F.; Oka, H.; Ito, Y. Systematic search for suitable two-phase solvent systems for high-speed countercurrent chromatography. *J. Chromatogr*. **1991**, *538*, 99−108.

Pedersen, A. T.; Andersen, Ø. M.; Aksnes, D. W.; Nerdal, W. NMR of anthocyanins: assignments and effects of exchanging aromatic protons. *Magn. Reson. Chem*. **1993**, *31*, 972−976.

Ribéreau-Gayon, P.; Pontallier, P.; Glories, Y. Some interpretations of colour changes in young red wines during their conservation. *J. Sci. Food Agric*. **1983**, *34*, 505−516.

Romero, C.; Bakker, J. Interactions between grape anthocyanins and pyruvic acid, with effect of pH and acid concentration on anthocyanin composition and color in model solutions. *J. Agric. Food Chem*. **1999**, *47*, 3130−3139.

Rothe, M.; Thomas, B. Flavor compounds from bread. *Z. Lebensm.-Unters.-Forsch*. **1963**, *119*, 302−310.

Timberlake, C. F.; Bridle, P. Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. *Am. J. Enol. Vitic*. **1976**, *27*, 97−105.

Van Calsteren, M.-R.; Cormier, F.; Do, C. B.; Laing, R. R. ^{1}H and ^{13}C NMR assignments of the major anthocyanins from *Vitis vinifera* cell suspension culture. *Spectroscopy* **1991**, *9*, 1−15.

Received for review June 21, 2000. Revised manuscript received September 8, 2000. Accepted September 11, 2000.

JF0007481