The Copigmentation of Anthocyanins and Its Role in the Color of Red Wine: A Critical Review

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Copigmentation is a solution phenomenon in which pigments and other noncolored organic components form molecular associations or complexes. It generally results in an enhancement in the absorbance and in some cases, a shift in the wavelength of the maximum absorbance of the pigment. Copigmentation has not previously been taken into account in traditional wine color measures, in the relationship between color and pigment analysis, or in spectrophotometric assays for anthocyanin content. It is now apparent that copigmentation can account for between 30 and 50% of the color in young wines and that it is primarily influenced by the levels of several specific, noncolored phenolic components or cofactors. Copigmentation is of critical importance in understanding the relationship between grape composition and wine color, the variation in color and pigment concentration between wines, and in all reactions involving the anthocyanins during wine aging. This review focuses on the importance of the individual pigments and cofactors, the strength of their interactions, and their relative abundance in grapes and wines. A simple mathematical analysis of the solution equilibrium is developed to explain the nonlinear deviation from Beer's law. When solved for typical wines, this function provides estimates of the apparent association constant, K, and the apparent molar extinction of the copigmented form, Ec, in natural mixtures. These measures allow the fraction of the anthocyanins which is in the copigmented form to be estimated. The significance of this phenomenon on pigment extraction and color retention during fermentations, on the rate of subsequent pigment polymerization, on the possible protection of anthocyanins from oxidation, and in the possible involvement on perceived mouthfeel and astringency of wines are suggested. Aspects of the copigmentation phenomenon that are poorly understood are identified and some research directions are suggested.

Key words: Copigmentation, anthocyanins, red wine color, pigment complexes, cofactors

The phenomenon of copigmentation is due to molecular associations between pigments and other (usually noncolored) organic molecules in solution. These associations cause the pigments to exhibit far greater color than would be expected from their concentration. The phenomenon has long been recognized in flowers and fruits. Willstatter and Zollinger [122,123] noted the intensification of color on the addition of tannin to acidic oenin (malvidin 3-glucoside) solutions, the major pigment that they had isolated from Alicante grapes. They also observed that the effect was not found in similar cyanin (cyanidin 3,5-diglucoside) solutions. Copigmentation was studied more extensively by Robinson and Robinson [94], who ranked almost 30 different cofactors by their ability to provide a blue shift to acidic oenin (malvidin 3-glucoside) solutions. They noted, "it is evidently the result of the formation of weak additive complexes, which are dissociated at elevated temperature or by the action of solvents." In an early spectrophotometric study of color in red wines, Boutaric et al. [22] noted that the color exhibited deviations from Beer's law when it was diluted. They suggested that this was due to "a state of micelle complexes, between the coloring materials and a number of other mineral and organic constituents in wine."

This association between the pigments and their copigmentation cofactors (also referred to as "copigments" by some authors) involves the anthocyanin glucosides, and certain phenolic acids, flavonoids, and, in particular, derivatives of the flavonol and flavone subgroups. It accounts for almost half of the observed color of young red wines [79]. As well as resulting in higher absorbance values (a hyperchromic shift), certain cofactors lead to a bathochromic shift in the wavelength at which the maximum absorbance is observed, typically 5 to 20 nm higher, providing a blue-purple tone in an otherwise red solution.

The extent of the spectral shift is not directly related to the enhancement in color and vice versa. Some combinations, such as quercetin and oenin [94], show primarily a wavelength shift, while other pairs, such as protocatechuic acid and cyanidin 3,5-

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diglucoside, show primarily a color enhancement [5]. In general, most combinations display both of these features to some extent [5,11-14,72], and there is presently no known relationship for the prediction of these effects. The earlier reviews of copigmentation by Osawa [83] and Mazza and Brouillard [72] provide additional examples.

The potential color enhancement is fixed for a given pigment-cofactor pair and the observed color in solution depends on the concentration of pigment, the molar ratio of cofactor to pigment, pH, the extent of nonaqueous conditions, and the anions in solution. In most natural mixtures such as juices and wines, it is expected that there will be competition between the various cofactors and pigments with some kind of preference or order for these associations.

There appears to be a minimum concentration of anthocyanin (approximately 35 µM) before significant copigmentation is detectable [4, 68]. This corresponds to 18.5 mg/L as malvidin 3-glucoside, so most red wines (300 to 500 mg/L) are expected to be in the concentration range of significant copigmentation while most blush and rose wines (5 to 50 mg/L) are not. That is why the blue and purple tones are absent in these wine types, yet the red wines made from the corresponding grapes can often display this trait.

The color exhibited by the anthocyanins when they are in these copigmentation complexes can be severalfold that of the free form, and the actual enhancement depends primarily on the nature of the pigment, the cofactor, the ratio of cofactor to pigment, and the pH. The equilibrium nature of this complex formation leads to a nonlinear relationship between color and concentration when diluted, leading several authors [22,105,107,110] to note that red wines do not obey Beer's law. The copigmentation complex is easily disrupted by dilution with a model wine buffer, with the components of the complex returning to their free pigment and cofactor forms in order to satisfy a new equilibrium position. This feature can be used to distinguish the color due to copigmentation from that due to the monomeric and polymeric pigments and has now been introduced into a more comprehensive assay for the color of solutions such as wine [19] that exhibit copigmentation. Somers and Verette [109] have referred to this loss in color due to dilution as the "color synergism factor," but there has been no attempt to include it into the earlier assay [107], even though it significantly affects the estimates of the anthocyanin content.

In young red wines made from *Vitis vinifera* grapes, copigmentation seems to result in both a higher pigment concentration and an enhancement in the color of those pigments. The color enhancement has been found to be between two and ten times that expected from the pigment alone, with typical values being four and six times (Boulton, unpublished data). Both effects seem to be pronounced in the juices and wines of the Teinturier cultivars (for example, Gamay Teinturier, Alicante Bouschet, Rubired, Salvador, Centurion, and some of the red Oberlin crosses) and some non-*vinifera* grapes (such as Noble and Concord). The colors of most berry juices and jams (for example, cranberries, raspberries, blackberries, and blueberries) owe much of their intensity, purpleness, and blueness to the combined effects of mildly acidic pH and copigmentation. Unfortunately, there are few published studies of the levels and nature of the cofactors in these juices. The effect of added chlorogenic acid on the color of strawberries and chokeberries has been demonstrated and the effect of pH on the color due to copigmentation investigated [124]. They found similar responses to those observed in Cabernet Sauvignon wines [70], that is, a local maximum in copigmented color at a pH of 3.3.

Although most of the solution studies have been with monomeric phenols and anthocyanins, other components that have been suggested to have copigmentation effects are C-glycosylxanthones, pectin, and tannin preparations [89]. Other studies [97] have shown that Concord grape seed extract, sugars, protein and iron, tin, or zinc ions had no significant effects on the color of malvidin 3,5-diglucoside solutions at pH of 3.20. It is not clear if the effects attributed to some materials are due to the impurities of monomeric components such as catechin or galacturonic acid that might be present in the natural preparations employed in some of these studies. Some winemakers and researchers believe that it is the tannins in red wine that are responsible for the complexing of anthocyanins and deeper color. However, there has yet to be a clear experimental demonstration of this belief.

The Self-Association of Anthocyanins

A subset of the interactions involving anthocyanins is the case of self-association of molecules in relatively concentrated (>1 mM) solutions [5,58,60-62,63,75,83,111,120]. That might be expected due the effectiveness of the most flavonoids as copigmentation cofactors and the features of aromatic hydroxy, carbonyl, and sugar groups at certain key positions that they have in common with many of the anthocyanins.

Asen et al. [5] reported that by self-association, cyanidin 3,5-diglucoside solutions at 5 mM displayed twice the expected color (200 times that of the 50 µM solution) at pH values of 3.16, 4.12, and 5.10. This can be compared to the copigmentation effect in equimolar solutions (5 mM) of cyanidin 3,5-diglucoside and quercitrin, which gave three times the expected color (300 times that of the 50 µM solution) under the same pH conditions. Unlike copigmentation, self-association is characterized by a hypsochromic shift in the wavelength of the absorbance maximum, that is, toward shorter values [49,60,62].

Hoshino et al. [59,60,62] in studies of various glycosides of malvidin concluded that malvidin 3-glucoside showed less effect of concentration on its circular dichroism (CD) pattern and was therefore less capable of self-aggregation than the 3,5-diglucoside at a pH of 7. That is significant when comparing much of the work that has been performed using the 3,5-diglucoside in both self-association and copigmentation studies. The CD patterns of this self-association display a split into two broad peaks, one positive, the other negative, and both in the colored region of the spectrum. This pattern occurs at pH of 7 [60] and pH of 1.0 [57] with several anthocyanins. These CD spectra have been interpreted as evidence of molecular stacking along either a left- (or a right-) handed helical axis [60-62].

The relationship between anthocyanin concentration and solution color in the case of self-association differs from that of

copigmentation. It is expected that the equilibrium and color response in self-association would be second order in nature, while for copigmentation it would be first order with respect to the anthocyanin concentration. Timberlake [111] has shown similar self-association in an aqueous solution of malvidin 3-glucoside at pH=3.5, displayed a second order increase in color over a tenfold concentration range, estimated to be from 85 to 850 mg/L. This data can be fitted by a second order association between pigment molecules with an association constant of approximately 1000 M^{-1}. Similar measurements in 12% ethanol solutions, or with acylated forms of this pigment, do not appear to have been conducted. The effect of ethanol on the self-association equilibrium is unknown, and there is no report of a bathochromic shift in these self-association studies.

The ability of some anthocyanins to act as cofactors for other anthocyanins, better described as copigmentation rather than self-association, has been demonstrated by Nakayama and Powers [78] and Miniati et al. [75]. The later study reported the color enhancement of a mixture of the 3,5-diglucosides of pelargonidin, cyanidin, and malvidin, each at 75 µM, in water and 10% v/v ethanol solutions. At pH=3.5, the enhancement in absorbance was only 5% in water, but in the ethanol solution, it was found to be 18%. This higher response in ethanol solutions could be due to more extensive association or a larger extinction coefficient of the copigmented forms, or both.

Somers and Evans [108] considered self-association to be responsible for much of the non-Beer's law behavior and proposed dimers linked through the 4 and 8 positions of adjacent anthocyanins to explain the purple color found in many red wines. However, neither self-association nor dimer formation can explain the absence of purpleness in some red wines with medium and high anthocyanin concentrations. These hypotheses are now discounted by the strong instrumental evidence for copigmentation stacking and positive bathochromic shifts due to the cofactors in copigmentation equilibria.

Pigment-Metal Complexes

Copigmentation is also different from the formation of colored complexes between some anthocyanins and certain metal cations such as Al, Fe, Sn, and Cu at levels of 10 mg/L [50,94, 100, 118] and borate [100]. The ability to form such complexes is related to an ortho-dihydroxyl arrangement on the B ring, so that while the glycosides of cyanidin, delphinidin, and petunidin can form, those of malvidin, pelargonidin and peonidin cannot [46,100]. The work of Dangles et al. [32] and Elhabiri et al. [37] has shown that pigment complexes involving aluminum have a color maximum at pH of 4.5, and this can distinguish it from the pH response of either anthocyanins alone or those in copigmented associations. Since malvidin 3-glucoside is the major anthocyanin in most *vinifera* grapes, it is unlikely that pigment-metal complexes play any significant role in the color of their wines.

The blue color of blueberries (*vaccinium* sp.) is associated with aluminum complexes of otherwise red anthocyanins [83]. The blueness and color intensity of juice and wine from some hybrid grapes such as Concord have been attributed to such metal complexes [67], as cyanidin 3,5-diglucoside, and the diglucosides in general, are less ionized (and therefore, less colorful) than the monoglucosides at wine pH. However, it is quite possible that the difference in color noted by these authors could be due to copigmentation as well as metal-pigment complexes. There is presently no evidence that the levels of these metal cations in wine are at all correlated with the levels of copigmentation or the dilution-sensitive aspect of red color [74], and this will not be considered further in this review.

The Nature of the Copigment Complex

While there is only limited understanding of the physical nature of the association in solution, the concept of hydrophobic and π-π interactions causing planar stacks [60-62] is presently favored, while earlier researchers had considered hydrogen bonding to be significant [5,113]. The role of functional groups seems to be due to their π-cloud interactions and steric hindrance of stack formation. Much of this picture has been elucidated by the application of circular dichroism (CD) and proton nuclear magnetic resonance (^{1}H-NMR) methodologies that provide instrumental evidence of π-π interactions, similar to those found in nucleoside stacking [96].

Circular dichroism is measured by noting the difference in absorbance when two beams of circularly polarized light are passed through a solution [47,96]. One beam has a right-handed rotation while the other has a left-handed rotation at each wavelength. It has proven to be useful in detecting modifications in chiral properties due to molecular interactions such as clustering and stacking. The application of CD has been able to establish evidence of stacking, and the interpretation of left- or right-handed spiral stacks can be made from the sign of the first and second Cotton effects [60]. At a pH of 7.0, these authors proposed right-handed spirals for the self-association stacks of pelagonin and cyanin and left-handed spirals for malvin, peonin, and delphin [60,62]. This pattern is altered under acidic conditions (pH=1.0), and all of the 3,5-diglucosides show negative first Cotton effects and, hence, left-handed spiral patterns [57] for self-association. These authors also showed that the addition of a flavone (flavocommelin) to commelin solutions altered the CD spectrum and suggested a one-to-one stacking under these copigmentation conditions [45].

We have determined what appear to be the first CD spectra of a red wine at pH values of 3.6 and 1.0 (Molinski and Boulton, unpublished data), shown in Figure 1. The first important fea-

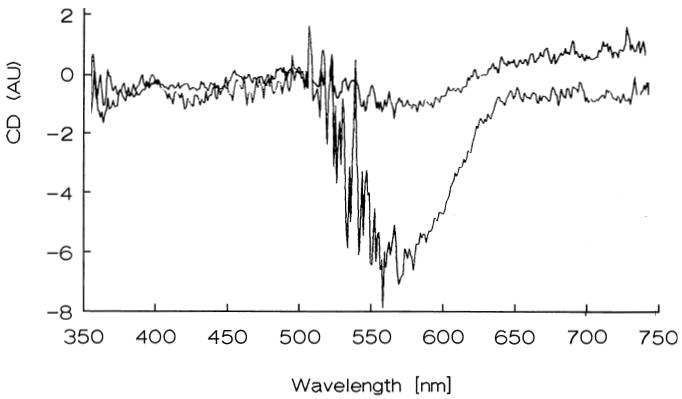

Figure 1 Measured CD visible spectrum of a young Merlot wine at natural and low pH.

ture is the presence of only one negative peak with a minimum at 550 nm in contrast to the two split (opposite) peaks, which characterize self-association. The larger negative difference at pH =1.0 indicates that copigmentation stacking exists and it is far more extensive than under wine conditions. (The presence of copigmentation at this pH would not be expected based on a number of studies, discussed in more detail below). The negative values in the region 500 to 600 nm indicate negative first Cotton effects and, therefore, left-handed spiral copigmentation stacks would be expected. The jagged curve in the 500 to 520 nm regions may be due to the mixture of pigments and cofactors in the stacks (or mixtures of stacks) within this wine. The spectra are different from those observed for the self-association in model solutions [57,60,62], where both positive and negative deviations in the CD spectrum are observed. This is the strongest instrumental evidence yet that copigmentation rather than self-association is responsible for the enhanced, nonlinear color contributions in red wine.

The application of proton NMR has provided further insights into the forms of the anthocyanin equilibria that are involved in such stacks and the shifts that have enabled the association constants of the dimer stack to be estimated. For the case of malvidin 3,5-diglucoside [56,57], the forms involved in the stacking are neutral and ionized quinoidal bases at neutral pH and the association constants were estimated to be 1800 and 400 M^{-1}, respectively. A recent study [63], applying the same techniques with malvidin 3-glucoside at acidic pH values, has further developed the picture by distinguishing two chalcone entities, the E (*cis*) and Z (*trans*) chalcones. The authors concluded that the flavylium cation and the Z-chalcone are the stacking forms with a self-association constant of 3700 M^{-1} for the cations and 3080 M^{-1} for the cation-chalcone copigmentation. This study also estimated the inter-conversion constant for the E and Z chalcone forms and found that the association constants, especially that of the flavylium cation ionization, were concentration dependent. The apparent pKh values ranged from 3.0 (at 0.14 mM) to 3.4 at (0.68 mM) compared to that reported of 2.6 [23,24]. At wine conditions of 200 to 800 mg/L of anthocyanin, these values would be even higher, causing much more of the flavylium cation to be present at wine pH. This finding has an important effect on the use of methods for estimating anthocyanin content, which are based on color measurements, and the need for the pH and ionization functions to be included in them. The corresponding proton NMR studies have yet to be performed on model solutions containing anthocyanins and copigmentation cofactors, such as catechin, myricetin, and quercetin derivatives.

Techniques for the measurements of the size distribution of these copigmentation stacks or aggregates do not appear to exist. The associations will be referred to hereafter as stacks since planar stacking was previously suggested for copigmentation [45] and for the self-association of malvidin 3,5-diglucoside [56,61]. Based on NMR measurements, Hoshino proposed that discrete spiral stacks containing 10 molecules each could be formed, given an angle estimate of 20 degrees. The possibility of perfectly superimposed stacking has been eliminated based on the observed CD patterns which require some angle between adjacent molecules [56].

While the stoichiometry of many copigmentation aggregates seems to be approximately 1:1 between the anthocyanin and the cofactor, a distribution of stacks ranging from 2 to 10 sheets each might be expected. With few exceptions (arginine and histidine, CH-π interactions), all of the significant cofactors have at least one benzene ring in their structure (π-π interactions). The stronger cofactors are typically flavonoid derivatives that contain 3 to 6 hydroxyl groups, with the strongest of them being the 4 position flavones [5,31], based on studies with 3,5-diglucoside anthocyanins. The strongest known cofactors have electron withdrawing groups in the rings, which would favor the face-to-face stacking arrangement.

In an alternative view, Asen et al. [5] considered hydrogen bonding and Williams and Hrazdina [121 have proposed interactions that are end-to-end in nature, typically between the quinoidal oxygen of the anthocyanin and the vicinal dihydroxy of the cofactor. There are, however, no convincing instrumental measurements to support these arrangements, and the changes in the energy states of the molecules would have to be somewhat localized and inductive in nature. The end-to-end configuration is more akin to the carbonyl and oxy anion complexes with metal chelation of Al [32] or with CH-π interactions [82] but not the planar vertical stacking, based on π-π interactions. As the strongest known cofactors are the flavones (myricetin, kaempferol, and quercetin and their derivatives) and the 6 C- and 8 C-glucosyl apigenins (the vitexins and orientins), it is difficult to see how these substitutions into the benzene ring can be compatible with induction effects that would enhance the end-to-end configurations.

At present, there are no quantitative relationships between molecular structure, chiral properties, and either the bathochromic shift in maximum wavelength or the enhanced extinction coefficients that are observed in these solutions. Recent advances in molecular modeling, even for spatial arrangements in vacuum, would appear to be promising in terms of the prediction of likely molecular orientations as the cofactor and pigment approach each other, and the resultant excitation of energy states that might be related to the extinction and wavelength changes. Research into the role of solvent molecules on such structures and interactions has also significantly advanced in the past decade and adds to the interest in considering such approaches in the future. These methodologies would provide a far more convenient way of screening a wide array of components as potential cofactors as well as beginning to build models of mixture interactions and the effects of nonaqueous solvents. The obvious approach, using model solutions, has been limited for many years by the expense or unavailability of many natural cofactors and the difficulty of isolating rare and trace components from natural mixture such as grape juice or wine.

In recent studies of copigmentation [79], it was noted that the UV absorbance (280 nm) of the total phenolics in wine also undergoes a change with dilution and time, suggesting that there might be similar but more general associations between the phenolic components that influence their spectral properties. This effect, while much smaller than that observed with the anthocyanins and of no visual impact, usually leads to about a 20% loss in the absorbance at 280 nm over a three-hour period. This

finding supports the view of a more general phenomenon of aggregation or stacking between cyclic molecules (such as sugars, phenols, terpenes, benzyl derivatives, and pyrazines) in solutions (and in wines). Such a phenomenon might influence other physical and chemical properties such as the headspace concentrations (due to the solution activity and volatility) of certain molecules, the activity of these components in oxidation and polymerization reactions, and the binding of components or complexes to taste receptors and, therefore, the perception of astringency, bitterness, and a range of flavor components.

The Nature of the Cofactor

Early studies of copigmentation with grape pigments noted that "tannin" additions modified the color of oenin (malvidin 3-glucoside) solutions, shifting it toward a blue rather than the more usual red appearance [122,123]. Robinson and Robinson [94] reported ethyl gallate, aesculin, and "tannin" had a strong effect on the bluing of oenin from grapes. Gentisic and protocatechuic acids, vanillin, and quercetin had a moderate effect, and tyrosine, arbutin, salicylic and p-hydroxybenzoic acids, and catechin had only a slight bluing effect. Gallic acid was reported to act similarly [68], but the addition of quercetin, chlorogenic acid, or methyl gallate had no significant effect on the spectra of cyanidin 3-glucoside. These findings, while somewhat confusing, simply illustrate the importance of the anthocyanin concentration and the particular pigment-cofactor pair in observing these effects. Further, chlorogenic acid and quercitrin are known to be good cofactors with cyanidin 3,5-diglucoside at pH of 3.25 [5], and this suggested a poorer response of the cyanidin 3-glucoside to copigmentation under certain conditions. Rutin (quercetin 3-rhamno-glucoside) at 12 mM was shown to be a good cofactor for malvidin 3,5-diglucoside at pH of 3.20, providing about a fivefold increase in color to 1 mM pigment solutions [97]. This study also found that grape seed extract and sugars provided insignificant copigmentation effects under juice and wine conditions. A summary of the more significant studies that will be discussed in further detail below can be found in Table 1.

The most comprehensive investigation of the enhanced color response due to the presence of individual cofactors is that by Asen et al. [5]. They determined the color enhancement (at 520 nm) and shift in wavelength of the absorbance maximum (from 520 nm) in aqueous solutions containing a 3:1 molar ratio of cofactor to cyanidin 3,5-diglucoside, at pH 3.25. They found that the cinnamic acids (caffeic, coumaric, chlorogenic, sinapic, and ferulic) provided enhancements of approximately 60 to 70% with little shift in the wavelength of the maximum absorbance. By comparison, the quercetin glycosides (rhamnose and glucose) caused shifts of 15 to 20 nm and enhancements of 150 to 200% in absorbance. Some of the most potent of the cofactors studied were the 6 or 8 carbon glucosides of tri-hydroxy and tetra-hydroxy apigenin, respectively known as vitexin (and iso-vitexin) and orientin (and iso-orientin). These cofactors gave absorbance increases of 300 and 400% with shifts in wavelength maximum of 15 to 20 nm, like the flavones, when in solution with a 3:1 excess of the cofactor. (It is interesting to note that these components were first isolated from grape leaves several years earlier and were thought to be phytoalexins [119].) A related report of the flavone and flavonol levels in the leaves of several *vinifera* cultivars is that by Hmamouchi et al. [55]. Similar comparisons of many cofactors have been reported by Haslam [48], Chen and Hrazdina [31], and more recently by Cai et al. [30], Mistry et al. [76], and Liao et al. [71]. Dramatic enhancements in absorbance are found as the ratio of cofactor to pigment (cyanidin 3,5-diglucoside) is increased, but these are quite specific to the cofactor.

Some of the literature involving the color in red wines has noted "tannin" binding with pigments, and it has become an essential feature of some descriptions of color equilibria [41-43,86,102]. While the term *tannin* was used by Willstatter [122,123] and Robinson and Robinson [94] to describe tests for pigment responses, it may have been a crude extract that contained significant levels of monomeric cofactors and dimers, which may be responsible for the effects observed. Ribereau-Gayon [89] reported increases in the color intensity when anthocyanin solution was added to one containing "tannins" from pine bark, considered to mirror the situation in wines. The increase in color intensity is due solely to the carryover into absorbance at 520 nm due to the brownness of the tannin preparations used, and there is no evidence of color enhancement in these solutions. Scheffeldt and Hrazdina [97], however, found a very poor color response when a grape seed extract was added to 200 µM malvidin 3,5-diglucoside at a pH of 3.2. This extract would undoubtedly contain significant levels of grape seed tannin and be classified as "tannin" in most tests. It is important to note that virtually all studies of copigmentation phenomena have focused on the monomeric components as cofactors and that there does not appear to be any evidence in model solutions of polymeric phenols (tannins) being copigmentation cofactors.

There has been some suggestion that components from new oak barrels can lead to color enhancement, but it is not clear if this is simply due to adsorption of free sulfur dioxide onto freshly charred surfaces or to some extraction of components that are acting as copigmentation cofactors and complimentary to those already present in the wine. There is evidence that significant amounts of anthocyanins can be adsorbed onto the surface of new oak casks [89] and that any copigmentation effect would have to overcome this effect to show a net increase in color. Part of the confusion may have arisen from the use of the sum of the absorbances at 420 nm and 520 nm [110], as a measure of color intensity. With this measure, any compound that contributes to an increase in "brownness" would be considered in some definitions to increase the "color." Measurements of copigmentation (rather than color density) after the addition of tannin fractions and extracts, from both grape seed and oak wood sources, either in model solution mixtures or directly in young wines should be conducted to clarify this situation.

Perhaps the strongest case against the role of polymeric phenols being involved in the copigmentation of red wines comes from a recent study of almost 100 wines, using partial least squares methodologies, in which more than 95% of the variation in the copigmented color could be accounted for by considering only monomeric phenols as cofactors [36]. This result implies that the variation in copigmentation that can be accounted

Table 1 Summary of pigments, cofactor, and conditions of major copigmentation studies of relevance to wine.

Author(s) and date	Anthocyanin and cofactor	[Anthocyanin] and cofactor/ pigment ratio	Buffer system	Comments Enhancement, $(A-A_0)/A_0$ (%)
Asen et al. 1972 [5]	Cyanidin 3,5-diglucoside and 30 cofactors (amino, benzoic and cinnamic acids, flavan-3-ols, flavonols, flavones)	6 mM Ratio of 3.0	Citrate-phosphate pH = 3.32	Enhancement of 20% arginine, proline, benzoic acids, 60 to 80% caffeic acid, catechin, 220 to 240% quercetin and kaempferol glucosides, 467% swertisin. Wavelength shift (0 to 33 nm).
Asen et al. 1972 [5]	Delphinidin 3-glucoside, 3,5-diglucosides of pelargonidin, cyanidin, peonidin, petunidin, and malvidin with quercitrin	2 mM Ratio of 3.0	Citrate-phosphate pH = 3.32	Wavelength shift (15 to 30 nm). Enhancement of 200% to 560% for the diglucosides, 10% for the monoglucoside.
Asen et al. 1972 [5]	Cyanidin 3,5-diglucoside and quercitrin	50 µM, 500 µM, and 5 mM Ratio of 1.0	Citrate-phosphate pH = 2.12, 3.16, 4.15, and 5.10	Self-association effect 10% at 500 µM and 200% at 5 mM. Wavelength shift independent of pH. No copigmentation effect at pH 2.12 and 5 mM.
Asen et al. 1972 [5]	Cyanidin 3,5-diglucoside and quercitrin	5 mM, 7.5 mM, and 10 mM Ratios of 0.1, 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0	Citrate-phosphate pH = 4.38	Enhancement proportional to cofactor to pigment ratio and increases more than expected with pigment concentration.
Scheffeldt & Hrazdina 1978 [97]	Malvidin 3,5-diglucoside, malvidin 3-glucoside and their acylated forms with rutin	200 µM Ratios of 0 to 12	Tartrate-malate pH = 3.2	Enhancements of 1000% for 3,5-diglucoside,150% for 3-glucoside at ratio of 12. Shifts of 45 nm at ratio of 12.
Williams & Hrazdina 1979 [121]	3,5-diglucosides of cyanidin and malvidin, some acylated forms with rutin	250 µM Ratio of 12	Citrate-HCl for pH 1.0 to 4.0	Enhancements of 500% at pH 1.0 and ratio of 12. pH effects from 1.0 to 7.0. Little acylation effect.
Chen and Hrazdina 1981 [31]	Malvidin 3,5-diglucoside with 23 flavonoid and phloroglucinol derivatives	1 mM Ratio of 4	Phosphate-citrate pH = 3.2	Enhancements of 200% myricetin, 172% fitesin, 167% quercetin, 149% quercitrin, 129% apigenin 7-glucoside, 35% catechin. Shifts from 6 nm catechin to 34 nm myricetin.
Brouillard et al. 1989 [25]	Malvidin 3,5-diglucoside with chlorogenic acid	773 µM Ratios of 0, 1, 5, 10, and 20	Phosphate-acetate pH = 3.65	Effect of temperature 10 to 60°C. Ion strength effect. No shift observed. K_{eq} values. Concentration product analysis given.
Cai et al. 1990 [30]	Malvidin 3,5-diglucoside with chlorogenic acid	100 mM Ratio of 2	Acetate pH = 3.65	Enhancements of 15% B2, 18% methyl gallate and epicatechin, 44% epigallocatechin 3 gallate, 121% pentagalloylglucose, 173% quercetin 3-galactoside. Shifts from 1 nm to 19 nm (quercetin 7-galactoside).
Mazza and Brouillard 1990 [73]	3,5-diglucosides and 3-glucoside of cyanidin and malvidin	85.8, 258 and 773 µM Ratios of 0, 1, 10, 20, 40, 80, 150, and 200	Phosphate-acetate pH = 2.74, 3.64, 4.72, and 5.74	Enhancements of 610% cyanidin 3,5-diglucoside, 1165% malvidin 3,5-glucoside and at pH = 3.6 and R =150, 90% cyanidin 3-glucoside, 144% malvidin 3-glucoside at R = 40. Effect of temperature.
Brouillard et al. 1991 [26]	Malvidin 3,5-diglucoside with chlorogenic acid, caffeine, adenosine, catechin, epicatechin, and gallic acid	30, 60 µM Ratios of 10, 20, 40, 80, 120, and 200 (chlorogenic acid)	Citrate-phosphate HCl for pH <2.5 pH = 0.65 to 8.0	Enhancements of 14 to 68% for pyrimidine derivatives, 50% catechin, 71% epicatechin, 8% gallic acid, 65% pentagalloylglucose at pH 3.6 and ratio of 10. Solvent effects and some K_{eq} also.
Mistry et al. 1991 [76]	Malvidin 3,5-diglucoside with quercetin 3-galactoside	100 µM Ratios of 5, 10, 15, 20, 25, and 30	Acetate pH = 3.65	Enhancement of 550% and shift of 30 nm at R = 30.

Table 1 Continued

Author(s) and date	Anthocyanin and cofactor	[Anthocyanin] and cofactor/ pigment ratio	Buffer system	Comments Enhancement, $(A-A_0)/A_0$ (%)
Liao et al. 1992 [71]	Malvidin 3,5-diglucoside with epicatechin, catechin, catechin-3-O-gallate, catechol, and phloroglucinol	1,2 mM Ratios of 8, 11, 14, 21, 22, 32, and 33	None pH=3.32	Enhancements of 68% Phloroglucinol at R = 32, 151% epicatechin at R = 14, and 221% catechin 3 gallate at R =8. Shifts of 6 nm to 19nm. Unbuffered water and 10% v/v ethanol solutions.
Miniati et al. 1992 [75]	3,5-diglucosides of pelargonidin, cyanidin, and malvidin with gallic acid, catechin, and quercetin	75 µM Ratios of 0, 3, 30, and 0, 1, 10	Phosphate-acetate pH =2.5, 3.5, and 4.5	Aqueous and 10% v/v ethanol. Pool of the 3 pigments. Self-association and copigmentation.
Yoshitama et al. 1992 [129]	Malvidin 3-rhamonoside, 5-glucoside with quercitrin and myricitrin	2.5 mM Ratio of 3.2 of both cofactors	Citrate-phosphate pH = 3.6 and 5.8	Enhancement of 600%. Shift of 50 nm.
Davies and Mazza 1993 [35]	Pelargonidin 3-glucoside, malvidin 3,5-diglucoside and its acylated form monardaein with caffeic, chlorogenic acid, and rutin	258 µM and 5.16 mM Ratio of 5, 10, 20, 40, and 80	Phosphate-acetate pH = 2.7 to 5.7	K_{eq} values estimated. Stack proportions of pigment to caffeic acid 3:2 and rutin, 3:1, at pH 3.7.
Baranac et al. 1996a [11]	Malvidin 3,5-diglucoside with rutin	386 µM Ratio of 0.5, 1, and 2	Phosphate-acetate pH = 3.65	Enhancement of 300% at R = 2. Shift of 24 nm. K_{eq} of 3300. Temperature effect from 10 to 70°C.
Baranac et al. 1996b [12}	Malvidin 3,5-diglucoside with quercetin	300 µM Ratio of 0.5, 0.7, and 1	Phosphate-acetate pH = 2.30, 3.20, and 3.65	Enhancement of 80% at R = 1. Shift of 12 nm. K_{eq} of 650 estimated. Temperature effect from 25–60°C.
Baranac et al. 1996c [13]	Malvidin 3,5-diglucoside with morin (3,5,7,2',4' pentahydroxy flavone)	300 µM Ratio of 1, 2, and 3	Phosphate-acetate pH = 3.65	Enhancement of 100% at R = 2. Shift of 7 nm. K_{eq} of 2300. Temperature effect from 10–70°C.
Baranac et al. 1996d [14]	Malvidin 3,5-diglucoside with apigenin 7-glucoside	386 µM Ratio of 1, 2, and 3	Phosphate-acetate pH = 3.65	Enhancement of 250% at R = 3. Shift of 27 nm. K_{eq} 137. Temperature effect from 20–70°C.
Wilska-Jeszka & Korzuchowska 1996 [124]	Strawberry and chokeberry juice and pigments from strawberry (pelargonidin 3-glucoside) and chokeberry (cyanidin 3-glycosides) with chlorogenic acid	100 µM Ratios of 0, 1, 10, 25, and 50 (w/w)	Britton buffer pH = 2.6, 3.2, 3.4, 3.6, 4.1, 4.5, and 6.0	Enhancements of 68% chokeberry juice, 49% strawberry juice, at pH = 3.4 and R = 50, 38% chokeberry pigments and 23% strawberry pigments at R = 25. Shifts of 6 and 8 nm. 20% loss in 10% v/v ethanol solution.

for by dimmers and tannins (and metals, pectins and all other possible factors) is less than 5% of the variation observed in the copigmented color. It also suggests that wood tannins and seed tannins are not major contributors to the copigmentation phenomenon of young red wines.

It is interesting to note that the levels of flavones, such as quercetin, in young red wines can be in the range 20 to 50 mg/L, while it is difficult to get more than 5 mg/L dissolved into model wine solutions. Due to their strong copigmentation interactions, they appear to be held in wines at multiples of normal solubility, which has implications for their role as superoxide and hydroxy radical scavengers. While (+) catechin and (-) epicatechin are 1.5 times faster than quercetin in the reaction with hydroxy radicals, quercetin is almost three times faster in the reaction with superoxide radicals and more than six times faster with other radicals [49]. This is important to in vitro and in vivo studies using anthocyanins obtained from wines (or other fruit products). They are likely to contain impurities of these very reactive cofactors due to copigmentation, and these may contribute significantly to the results.

The Nature of the Anthocyanin

The anthocyanins of most commercially-important winegrapes have been identified in several studies [1,3, 7,8,10,28,39,51,64,65,67,69,77,84,85,87,95,99,109]. The levels in which they are found in grapes and wines have also been ex-

tensively studied [6,9,10,21,28,41,64,77,81,89,91,92,95,99, 104,106,107,109,128].

The majority of copigmentation studies have used the diglucosides of malvidin and cyanidin as the anthocyanin of choice, due to their commercial availability. In the following comparisons, it is important to understand that the color enhancements noted are the combination of two effects, the strength of the association and the extinction of the copigmented form, and that at present it is not possible to distinguish between them.

Asen et al. [5] compared the shifts and responses for the diglucosides of pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, and delphinidin 3-glucoside, each at 2 mM, in aqueous solutions of 6 mM quercitrin. While the enhancement ranged from 1.1 to 1.69 AU for the diglucosides, with malvidin 3,5-diglucoside displaying the greatest change, it was only 0.47 for delphinidin 3-glucoside. The shifts ranged from 20 nm for the monoglucoside to between 19 and 30 nm for the diglucosides, with malvidin 3,5-diglucoside displaying the greatest shift. Williams and Hrazdina [121] found the pH response of the 3,5-diglucosides of cyanidin and malvidin to be similar with rutin (at 12 times excess) in the pH range 1 to 4. The acylated 3-glucosides of these pigments also displayed similar response curves but, typically, they were displaced about pH unit higher, as might be expected from their pKh values.

Mazza and Brouillard [73] found that the 3,5 diglucosides of cyanidin and malvidin displayed enhancements of 196 and 327%, respectively, at a pH of 3.62 with chlorogenic acid at a ratio of 40:1. The corresponding monoglucosides displayed only 90 and 144% enhancements under identical conditions. However Davies and Mazza [35] found very similar association constants for malvidin 3,5-diglucoside, pelargonidin 3-glucoside, and monardaein (an acylated malvidin 3,5-diglucoside) with chlorogenic acid. The values were 263, 247, and 257 M^{-1}, respectively, indicating that most of the color enhancement was due to differences in the extinction of the copigmented forms. These studies also show that the effects of acylation to be positive but slight, at least for the diglucoside case. The role of acylation may become important in wine copigmentation, as some cultivars, for example Pinot noir and Sangiovese, which are low in the level of copigmentation, are also known to lack acylated pigments.

The Influence of pH

Studies of self-association of pigments have been performed at near-neutral pH [60] as well as at mildly acidic pHs of 3.5 [5,63,111]. The neutral conditions have investigated the interaction of uncharged forms, including the blue quinoidal species. In contrast, under acidic conditions typically seen in wines, the presence of the red flavylium cation would have to be neutralized with either anionic forms of the cofactor or of anthocyanin or of the buffer system.

Asen et al. [5] observed equimolar solutions of cyanidin 3,5-diglucoside and quercitrin at pHs of 2.12, 3.16, and 4.15. The color enhancement was only apparent in the 500 µM and 5 mM solutions and then only at 3.16 and 4.15. In pH 3.16 solutions, the enhancement was about 8% at the 500 µM and 45% at 5 mM concentration, while at pH 4.15, the enhancement was 33% at 500 µM and 270% at 5 mM concentration

It is not clear if all forms of the anthocyanin are involved in the stacks of copigmentation, or only the flavylium and quinoidal base, or the flavylium cations alone. The contribution from both flavylium and quinoidal base would result in a bimodal pH function with one part for the blue quinoidal form and the other for the flavylium form at low pH. This has been observed by Williams and Hrazdina [121], with the flavylium function at pH less than 4 and the quinoidal base from pH 4 to 7.

An alternative view is that the flavylium cations have to be balanced by the anionic anthocyanin forms within a stack in order to maintain a balance in net charge. Under this description, the ionization within the stack would differ from that of the free anthocyanin in solution and show a different pH function altogether.

A third view is that all forms are involved at all pHs, and it is simply the color displayed by the flavylium ions in the stack and the stability of the stack that result in the observed pH variation. The abundance of intact stacks might then be dependent on the pH function of other entities such as the cofactors or even of the major counter ion in the buffer system. The pH function of color in this last case would be the product of the ion fractions of the anthocyanin and the counter ion (such as bitartrate, di-hydrogen phosphate, or chloride), which would display a bell-shaped local maximum, like many enzyme systems.

This function is especially important in understanding the various reports in which the choice of buffer system seems to have influenced the results obtained. Davies and Mazza [35] studied the influence of pH on the color displayed by aqueous solutions of three pigments (pelargonidin 3-glucoside, malvidin 3,5-diglucoside and monardaein, pelargonidin 3,5-diglucoside acylated with malonic acid and coumaric acid) and three cofactors (chlorogenic acid, caffeic acid, and rutin). Solutions of 516 µM to 1.55 mM showed pH maxima generally between 3 and 4 for the phenolic acids, slighter higher for rutin, and with similar results for all pigments. For malvidin-chlorogenic acid points it ranged between 2 to 2.5 at pH 3 and 4 with a maximum of 3.3 at pH 3.6. All solutions showed a similar pH response between 2.0 and 3.0, which may be due to their choice of a phosphate-acetate buffer system. The role of the buffer anion will be considered in more detail in the next section.

The Role of the Buffer Anion

The fact that copigmentation with uncharged cofactors (such as rutin or catechin) shows a similar pH response to the partially ionized phenolic acids (such as chlorogenic and caffeic) suggests one of two possibilities. The first is that the carboxylate of the cofactor plays little if any role, and it is the aromatic ring function that has the most effect. The second is that only the un-ionized forms are involved, and in this case there is a need for an anion to counteract the charge of the flavylium under acidic conditions. The pH function of copigmentation has been found to be bell-shaped in nature [35], with a local maximum near 3.5 for a number of copigment pairs. One explanation of this pH

effect is that it follows the concentration product of the flavylium ion and the singly charged anion of the buffer system. Each of the species is changing in different ways with pH if there is only one component of the buffer. Figure 2 shows the shape of this product for the pH range 2.0 to 4.0 based on malvidin 3,5-diglucoside and the dihydrogen phosphate and acetate anions of the buffer used by Davies and Mazza [35]. Plotted over this on a relative scale is the pH function for copigmentation as measured by these authors. A 1:1 mix of these buffers has been assumed for calculation purposes, but the low pH solutions would have a higher proportion of the phosphate buffer, while those at higher pH would contain more of the acetate buffer. It can be seen that the low pH end of this curve can readily explain the observed pattern, while other effects seem to be playing a role on the higher pH side.

In wines, Levengood [70] measured the color due to copigmentation in four young Cabernet Sauvignon wines in the pH range 3.0 to 3.9. These all showed maxima in copigmented color at around pH of 3.3, with the behavior near the maximum varying slightly between the wines. The corresponding pH function based on malvidin 3-glucoside, using the pKh data of Houbiers et al. [63], and a bitartrate buffer in 12% v/v ethanol is shown in Figure 3 together with the results of the wines, on a relative scale. It is clear that the pH response of the color due to copigmentation has essentially the same characteristic as that of a cross product of the flavylium cation and the bitartrate anion. This is strong evidence of an ion-pairing effect between the cation in the stack and a stabilizing anion nearby. Wines are a mixed buffer, dominated by bitartrate ions, with secondary contributions from bimalate (or lactate), bisuccinate, dihydrogen phosphate, and bisulfate as well as secondary effects due to chloride and nitrate ions. The variation in these components between wines is expected to be significant. The role of inorganic anions in the copigmentation of wine has yet to be established.

The studies by Asen et al. [5], Brouillard et al. [26], and Davies and Mazza [35] are examples in which mixed buffers (dihydrogen phosphate-acetate) have been used at pH values where the scarcity of buffer anions appears to have influenced the results. This has complicated the interpretation of their results due to pH, and explains why these authors find essentially no effect at or below pH of 2 while Williams and Hrazdina [121], Houbiers et al. [63], and our own studies (Figure 1) show that the color due to copigmentation continues to become more pronounced at pH of 1 or less. The explanation lies in the need for a counter anion to compensate for the flavylium cations in the stacks at low pH and the ability of chloride to provide this in the latter system. The scarcity of anions in the phosphate buffer systems at pH less than 3.0 seems to have introduced artifacts into these experiments. This leads to a picture of copigmentation stacks in which the un-ionized and neutral cofactors are involved with flavylium cations that need to form ion pairs with available anions in the solution in order to be stable. It is interesting to look at the literature (Goto et al. [44,45]), in which salt strength, or particularly the presence of Mg cations, was thought to be essential for the stability of commelinin, an anthocyanin-flavone copigment complex. The authors found that sodium chloride in the solution could provide stability to the copigmented complex

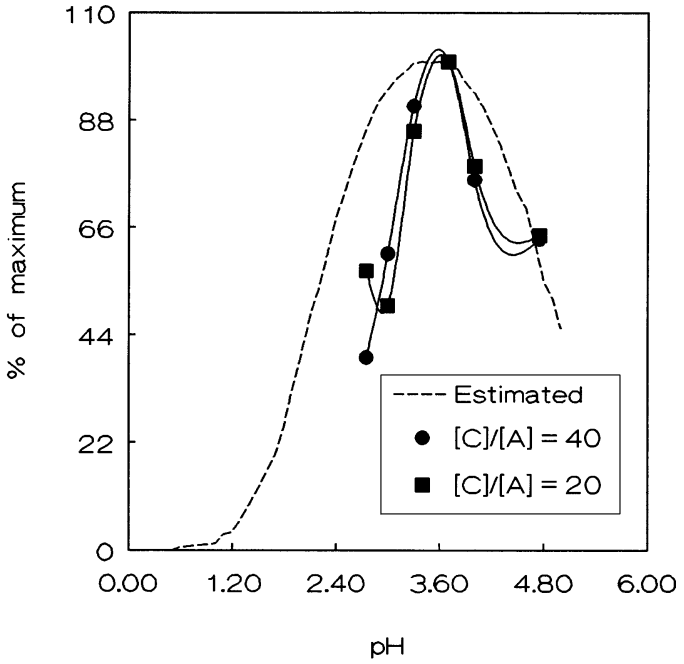

Figure 2 Calculated response of color due to copigmentation at various pH values for a phosphate-acetate buffer and reported values of Davies and Mazza [35] using malvidin 3,5-diglucoside [A] and chlorogenic acid [C] in a phosphate-acetate buffer.

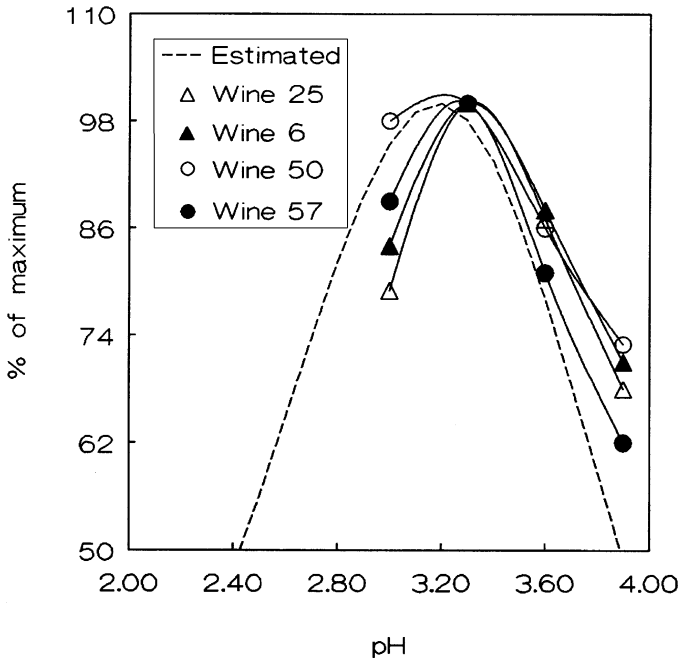

Figure 3 Calculated response of color due to copigmentation at various pH values for a bitartrate buffer and the reported values of Levengood [70] using four Cabernet Sauvignon wines.

just as well as the magnesium salt. This effect on stability may be better explained as the role of the chloride anion, from either NaCl or $MgCl_2$, as being the counter ion that is required for stack stability in this system.

The influence of the buffer anion on the pH response is clearly significant, especially in the region below 3, and this needs to be

taken into account in future studies and in the interpretation of published reports.

The Influence of Ethanol

The role of organic solvents is to disrupt the physical associations such as those found in copigmentation (as suggested by Robinson and Robinson [94], Boutaric et al. [22], and Somers and Evans [108]) and to modify the wavelength of the absorbance maximum. Boutaric et al. [22] showed that the non-Beer's law phenomenon (that is, copigmentation) disappeared when 50% ethanol was used as the diluting solution. Harborne [46] found that most anthocyanins display a bathochromic shift of the maximum wavelength by up to 25 nm in acidified ethanol, when compared to acidified water. The anthocyanin spectra and extinction values obtained in acidified methanol seem to be closest to those under wine conditions. In a study with rose wines, Aubert [6] found significantly lower color (15 to 35% lower) for wines diluted one to ten, with aqueous HCl than those diluted with an acidified ethanol (98%) solution. The dilution would have greatly reduced the color contribution due to copigmentation, but it is not clear if this ethanol effect applies to the free anthocyanins, the polymeric pigment, or both. Based on the absorbance at 520 and 550 nm, the dilution with acidified ethanol provided the most similar color pattern to those of the original wines.

Although copigmentation is present to a significant extent in red wines containing 12 and 14% ethanol, the purple appearance in young fortified red wines such as Port (18 to 21% v/v ethanol) may be due to a combination of copigmentation and ethanol effects. In contrast, the higher initial levels of copigmentation in cold-soaked or heated juices seem to be partly broken down by the ethanol produced during fermentation. The higher solubility of the flavones at higher ethanol levels in fortified wines may have a countering effect that enables them to retain significant copigmentation levels under these conditions.

Somers and Evans [108] showed that a significant loss of color occurred when other nonaqueous solvents were added to wines even though the concentration of anthocyanins was essentially unchanged. This can be explained by the disruption of copigmentation stacks due to the weakening of hydrophobic interactions by the organic solvents used. Brouillard et al. [26] also investigated the effect of several solvents on the color enhancement of model solutions of malvidin 3,5-diglucoside-chlorogenic acid at pH of 5.0 and at a cofactor to pigment ratio of 12. They found that at the level of ethanol in table wines, the loss in copigmented color was about 15%, while for fortified wines the reduction appears to be closer to 20% for these conditions. Miniati et al. [75] studied the influence of ethanol (10% v/v) in solutions of the diglucosides of malvidin, pelargonidin, and cyanidin and mixtures of them. They found between 7 and 20% lower absorbances of several anthocyanin when alone, but not so when they were mixed. The pigment concentrations were 75 µM, with the cofactors gallic acid, catechin, and quercetin with ratios of cofactor to pigment between 1 and 30. They found little effect of ethanol on the color enhancement at a pH of 3.5 for malvidin 3,5-diglucoside with catechin or quercetin.

In the range of concentrations found in wines, the influence of ethanol appears to be minor and of limited importance in the copigmentation phenomenon. It is important, however, when comparing wines to results obtained in aqueous media and especially so for analytical methods for anthocyanins and copigmentation involving buffered dilutions.

The Importance of Copigmentation in Color Measurements

Several methods for the assessment of color in red wines have generally recognized the ionizable and bleachable nature of the anthocyanins and have distinguished these from the polymeric forms that are generally less sensitive to pH and less bleached by bisulfite. Methods that follow this pattern are those presented by Berg and Akiyoshi [17], Berg [15], Ribereau-Gayon and Stonestreet [92], Ribereau-Gayon [88], Somers and Evans [106,107], and Timberlake and Bridle [113].

It is now clear that the contribution of color due to copigmentation is also bleached with SO_2 [70]. It can now be seen why estimation methods for the determination of anthocyanin content using a pH shift (Ribereau-Gayon and Stonestreet [92], Ribereau-Gayon and Nedeltchev [91]) will generally underestimate the true value, even when performed on diluted samples. The common dilution factor of 10 to 1, while adequate for older wines, is usually not sufficient to completely eliminate the effects of copigmentation in most young wines; further, the pH function of the copigmented form does not follow that of the free anthocyanin.

The opposite situation exists for the SO_2 bleaching methods, as they will attribute all of the color loss to free anthocyanins, when as much as half of it will be from copigmented forms in young wines, and these forms have extinction values several times those of the free anthocyanins. The modification of isolating the monomeric pigment pool on a PVPP column (Bourzeix et al. [21]) should provide more accurate estimates of total anthocyanin content, as the copigmentation stacks are expected to be broken up under the solvent conditions of elution and dilution. This should provide the total anthocyanin pool in the absence of copigmentation.

The incorporation of measurements of the SO_2 bleaching of anthocyanins, the acetaldehyde addition to eliminate bleaching due to free SO_2, and a shift to low pH enabled Somers and Evans [107] to propose a set of equations for the estimation total anthocyanin content, the degree of ionization of the anthocyanins at wine pH, and two ratios between monomeric and polymeric fractions, which were referred to as chemical age indices. These equations, like the earlier approaches, are of limited use now, as they try to explain wine color in terms of only monomeric anthocyanins and polymeric pigments. The use of a diluting solution that does not contain ethanol for the pH=1 reading and the assignment of the color changes due to SO_2 bleaching only to monomeric anthocyanins are the main reasons why the anthocyanin contents estimated by this method have been found to be unreliable and generally higher than by other methods. The degree of ionization calculated from these equations, when applied to wines of different pH values, does not follow that known for anthocyanin dissociation, and there are secondary effects of pH on the extinction of the polymers that are not easily accounted

for by the 5/3 factor proposed. Finally, while these authors acknowledge the existence of self-association and copigmentation, the contribution of copigmentation is present in some color readings and absent from others in the difference equations.

Comparisons of the anthocyanin content, determined by HPLC, with those of various spectrophotometric methods (Bakker et al. [9]) have also met with limited success due to failure to account for the contribution of copigmentation in all existing assays. Some of the early HPLC methods for separation of wine anthocyanins (Wulf and Nagel [127], Hrazdina [64], Somers and Verette [109], Singleton [99]) appear to have not completely broken up copigment complexes, which has probably resulted in underreporting of the total anthocyanin levels. (The existence of a broad, poorly resolved "hump" at approximately 45 minutes in many of these separations may be due to residual copigmentation aggregates rather than to the "polymeric" pigment that some authors have considered it to be.)

Bakker et al. [9] found good agreement between the anthocyanin content by the Somers and Evans [107] and HPLC methods when analyzing grape skin extracts but poor agreement when analyzing the corresponding wines. In the wines, the spectrophotometric method generally overestimated the anthocyanin content compared to the HPLC values. The agreement with the skin extracts is probably due to the fact that the copigmentation stacks have been broken apart by dilution and the color behavior follows that expected for free anthocyanins. However, in young red wines, the fraction of the color due to copigmentation can be as high as 50%. If the color (including that due to copigmentation) is used in an anthocyanin assay, it will greatly overestimate their concentration.

The nonlinear behavior of wine color with dilution was noted and studied by Boutaric et al. [22] and more recently by Somers [105]. The link between the changes in color with dilution and the dissociation of copigmented forms has not previously been incorporated into spectral methods and quantitative estimates for the pigment content in wine or, in fact, any fruit juices or other plant extracts. The spectrophotometric method described elsewhere [19] is the first to do so and provides an integration of all of the contributions of the color components in wines.

The use of bleaching in assays for free sulfur dioxide (Burroughs [29], Somers and Evans [107]) should also be avoided because the enhanced color of the copigmented forms will lead to significant overestimation of the anthocyanin content, by a factor of two or more. This in turn underestimates the free sulfur dioxide content by a similar factor. That is why these assays do not agree or correlate with other methods, as can be seen in the data of Burroughs [29].

The Copigmentation Equilibrium

The copigmentation equilibrium can be written

Free anthocyanins	+	Copigmentation cofactors	=	Copigmented anthocyanins
[A-C]		[B-C]		[C]

for a solution containing A moles of anthocyanin and B moles of cofactor. The concentrations of the free forms of these at equilibrium, when [C] moles of copigmented anthocyanin exist, are [A-C] and [B-C], respectively. The equilibrium (or association) constant, K_{eq}, can be written:

$$K_{eq} = \frac{[C]}{[A\text{-}C][B\text{-}C]} \quad (1)$$

The color of a solution containing copigmented anthocyanins, ignoring any self-association effects and assuming the same extent of ionization in the free and copigmented forms, can be written

$$\begin{aligned} AU_{520} &= Ec^*[C]^*\text{fraction} + Ea^*[A\text{-}C]^*\text{fraction} \\ &= (Ec^*[C] + Ea^*[A\text{-}C])^*\text{fraction} \end{aligned} \quad (2)$$

where Ea and Ec are the molar extinction values for the anthocyanin and the copigmented anthocyanin, respectively, and "fraction" is the fraction of the anthocyanin in the flavylium form at the pH of the solution.

The relative enhancement in color due to copigmentation, $(A\text{-}A_0)/A_0$ can then be written

$$\begin{aligned} (A\text{-}A_0)/A_0 &= ((Ec^*[C] + Ea^*[A\text{-}C])^*\text{fraction} - Ea^*[A])/(Ea^*[A]) \\ &= ((r^*[C] + [A\text{-}C])^*\text{fraction} - [A])/[A] \end{aligned} \quad (3)$$

where r is the ratio Ec/Ea, the copigmented anthocyanin extinction, expressed as a multiple of the anthocyanin extinction.

Estimates of Association Constants

One method of estimating the association constants of pigment-cofactor pairs has been proposed and used by Brouillard et al. [25]. It considers a situation in which "n" molecules of cofactor are associated with each molecule of pigment and leads to a relationship between the relative color increase, the concentration product $[A][B]^n$, and the association constant K. A log-log plot of the color increase, $(A\text{-}A_0)/A_0$, and initial concentration product, [A][B], is then used to estimate the constant, K, from the intercept and the cofactor loading, n, from the slope. In some systems, this is essentially unity while in others it suggests some other ratio of cofactor to pigment. These authors used this approach to estimate the association between malvidin 3,5-diglucoside and rutin to be 4000 M^{-1} at pH of 3.10 and at 25°C.

Davies and Mazza [35] studied the influence of pH on the color displayed by aqueous solutions of three pigments, pelargonidin 3-glucoside, malvidin 3,5-diglucoside, and monardaein (pelargonidin 3,5-diglucoside, acylated with malonic and coumaric acids) and three cofactors (chlorogenic acid, caffeic acid, and rutin). The solutions ranged from 516 µM to 1.55 mM in pigment content. The K values were estimated for each pair at pH with maximum color. For malvidin 3,5-diglucoside, the values were 263, 20, and 3913 M^{-1} for the chlorogenic, caffeic acid, and rutin, respectively. For pelargonidin 3-glucoside, they were 247 and 21M^{-1} for chlorogenic and caffeic acid. For monardaein, they were 257 and 53 M^{-1}, respectively. These values suggest the order of association strength is rutin, chlorogenic, and caffeic acid, but the caffeic had greatest color enhancement in the copigmentation. Similar studies with malvidin 3,5-diglucoside have now been conducted by Baranac et al. [11,12,13,14] with rutin, quercetin, morin, and apigenin 7-

glucoside; the K values reported were 3300, 650, 2300, and 137 M^{-1}, respectively.

The reported values of K obtained using this method are in fact a product of the true equilibrium constant, K_{eq}, and the extinction of the copigmented anthocyanin, Ec, as this is imbedded in the $(A-A_0)/A_0$ variable. This issue will be considered in a later section in more detail.

A Simple Analysis of the Nonlinear Behavior of Wine Color

The copigmented anthocyanins can be considered to be in a dissociable equilibrium with free anthocyanins and free cofactor compounds. The dilution of a sample at constant pH and ion strength leads to the progressive dissociation of the copigmented forms. At high dilutions, typically 19:1 or 24:1, virtually all of the copigmented anthocyanins have returned to the ionization equilibrium involving the flavylium cation, the pseudobase, and the chalcone forms. This loss in color with dilution can be used in calculations of the fraction of color that is due to copigmentation.

The equilibrium relationship (eq. 1) can be rearranged to give

$$([C]^2 - ([A]+[B])[C] + [A][B])K_{eq} = [C] \quad (4)$$

which can be solved for [C], the concentration of copigmented anthocyanin, for a given value of K_{eq} and any concentrations of A and B. The amount of copigmented anthocyanins formed, [C], can be found as the positive root of the quadratic equation. For the general case in which [A] does not equal [B]

$$[C] = \frac{([A]+[B]+1/K_{eq}) - \sqrt{[([A]+[B]+1/K_{eq})^2 - 4[A][B]]}}{2} \quad (4a)$$

and for the special case in which equal concentrations of pigment and cofactor exist, [A] = [B]

$$[C] = \frac{(2[A]+1/K_{eq}) - \sqrt{[(2[A]+1/K_{eq})^2 - 4[A]^2]}}{2} \quad (4b)$$

This provides the relationship between the concentration of copigmented anthocyanin [C], the total anthocyanin content [A], and the equilibrium constant K_{eq}. This expression can be applied at other concentrations that have been developed by a series of dilutions to estimate the extent of copigmentation in various wines or other anthocyanin solutions.

For diluted solutions, the concentrations of all components can be written more generally as [ ]/(m+1) for a (m+1)-fold dilution. The expression for the copigmented anthocyanin concentration at any dilution is then

$$[C]_{m+1} = \frac{(2[A]/(m+1)+1/K_{eq}) - \sqrt{[(2[A]/(m+1)+1/K_{eq})^2 - 4[[A]/(m+1)]^2]}}{2} \quad (4c)$$

The response of the color due to copigmentation at various dilutions can be calculated using eqs. 3 and 4c, which can be added to the diluted contributions from the free anthocyanins and the polymeric pigment to provide a general relationship for wine color at any dilution. Such an equation can be used with the absorbance readings at several dilutions to estimate the total anthocyanin content, [A], the apparent association constant, K_{eq}, and the extinction of copigmented anthocyanin. This has been done for many wines from the 1995 harvest in California and compared to estimates from an improved spectrophotometric method for copigmented color and anthocyanin content [19]. Equation 4c also provides the mathematical expression for the non-Beer's law behavior in wines, noted by Boutaric et al. [22] and Somers [105] and illustrated in Figure 4.

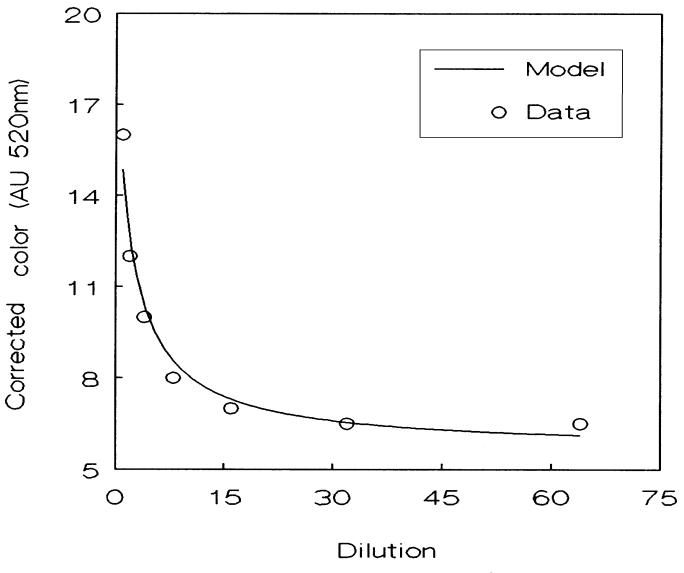

Figure 4 The buffered-dilution curve of color at 520 nm for a Shiraz wine [109] and the best-fit form of eqs. 3 and 4c.

The Influence of the Cofactor/Pigment Ratio

The amount of copigmented anthocyanin [C] in the equilibrium described in eq. 1 can be increased by increasing either the concentration of total anthocyanin or of cofactor or both. It appears that the extent of copigmentation in wine is determined by the quantity of available cofactors, which can be limited by either their concentrations in the grape or, in some cases, their solubility in the juice or wine. In wines, the total anthocyanin content varies from 150 mg/L (350 µM) in some Pinot noir wines to 800 mg/L (1.85 mM) in some Merlot wines. The concentrations of some of the weaker cofactors, such as catechin, can be 150 mg/L (750 µM) and for stronger ones such as myricetin, kaempferol, and quercetin can be 30 mg/L (200 µM). As a result, the cofactor to anthocyanin ratios are likely to be in the range 0.05 to 2, which can have a dramatic influence on the color displayed by a given concentration of anthocyanins.

For any chosen anthocyanin-cofactor pair, the response in color to an increasing ratio of cofactor to anthocyanin follows a sigmoidal rise from the color of the free anthocyanin solution to an upper limit when virtually all of the anthocyanins are in the copigmented form. Typical situations are shown in Figure 5, for different association constants and a given anthocyanin-cofactor pair. It is clear that in most wines the lack of cofactors causes the anthocyanins to display only a small fraction of their potential color (at high cofactor to pigment ratios).

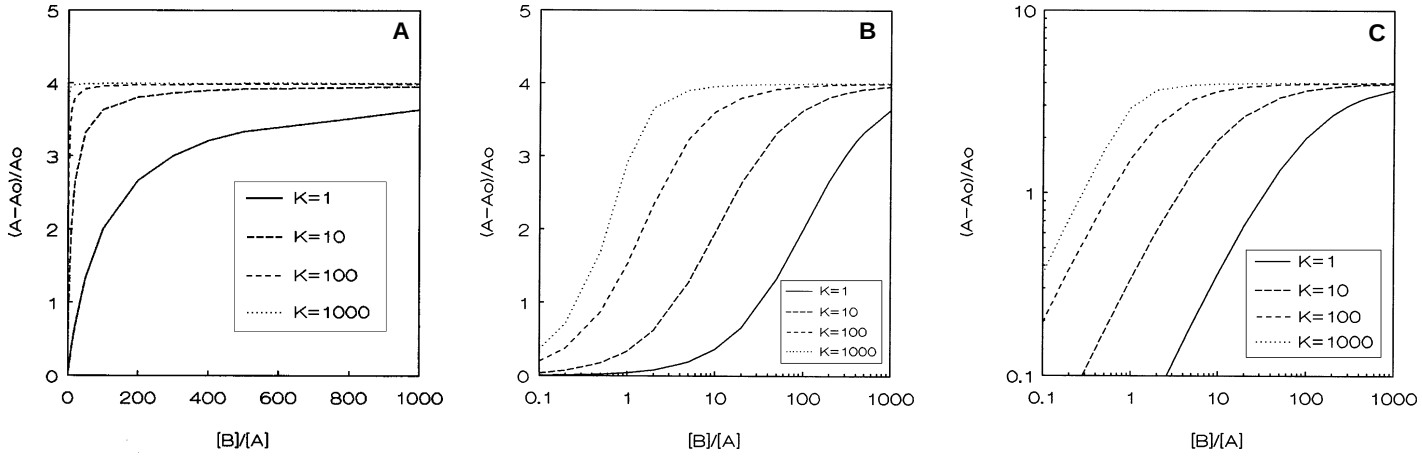

Figure 5 The estimated color response for an aqueous solution of malvidin 3-glucoside (10 mM, pH=3.6) as a function of the molar ratio of cofactor [B], to pigment [A], for 3 values of the association constant, K (linear-linear axes, **A**; log-linear axes, **B**; and log-log axes, **C**).

While the overall relationship is sigmoidal, there are sections of it that appear to be quite linear over small intervals of cofactor-anthocyanin ratio, and this has been observed in a number of studies. Asen et al. [5] studied quercitrin/cyanidin 3,5-diglucoside with ratios of 0.1 to 6 at pH=4.38 and pigment concentrations of 5, 7.5, and 10 mM. At low cofactor to pigment ratios, the extent of color enhancement is small and limited by the availability of cofactor. At intermediate ratios, there is moderate copigment formation and strong response due to the concentration of cofactor. At high ratios, the concentration of copigmentation is large and the system is limited by the availability of free anthocyanin and is weakly responsive to increases in cofactor. All of these solutions exhibited linear responses in color with increasing cofactor-anthocyanin ratio, and the color increase is more than expected from the pigment self-association.

The most comprehensive tabulation of response of various cofactors when present in a 3:1 excess at a pH of 3.32 is that of Asen et al. [5] and another using cofactors of interest to wine conditions is that of Brouillard et al. [26]. Several other cofactor-anthocyanin pairs have been studied and a number of them have used large excesses of cofactor to pigment, a condition that does not appear to occur in wines: Williams and Hrazdina [121], Chen and Hrazdina [31], Cai et al. [30], Davies and Mazza [35], Liao et al. [71], Mazza and Brouillard [73], Miniati et al. [75, Mistry et al. [76], and more recently Baranac et al. [11,12,13,14]. Most of the dramatic effects are observed at very large cofactor to pigment ratios, and the upper levels of these experiments are often limited by solubility of the cofactor. An alternative approach can be found in the studies of Scheffeldt and Hrazdina [97], in which the pigment concentration is increased for a given cofactor level. As would be expected, at high pigment to cofactor ratios, the color due to copigmentation rapidly decreases to that of the pigment alone. The cofactor to pigment ratio and the conditions employed in these studies are summarized in more detail in Table 1.

It is interesting to contrast the curves for the ratio approach, in Figure 5c, with those of the concentration product approach of Brouillard et al. [25], in Figure 6, for the color increases as a function of increasing cofactor levels. For any of the curves, the slope changes dramatically even though it is essentially linear over several small intervals of the ratio. It is also true that each curve has a region in which the slope may be unity, but other regions where it is not. The slope of this curve, unlike Brouillard's concentration product approach, cannot be interpreted to provide information about the ratio of pigment to cofactor in the stacks, as it is generated from a fixed proportion, that of one to one. Inspection of Figure 6 reveals that while at low concentration product values where the cofactor is in excess, the slope is constant, while a region of pigment excess, where the slope decreases to zero, appears at high concentration product values. These conditions do not appear to have been explored in Brouillard's analysis or commented on by previous authors using this approach.

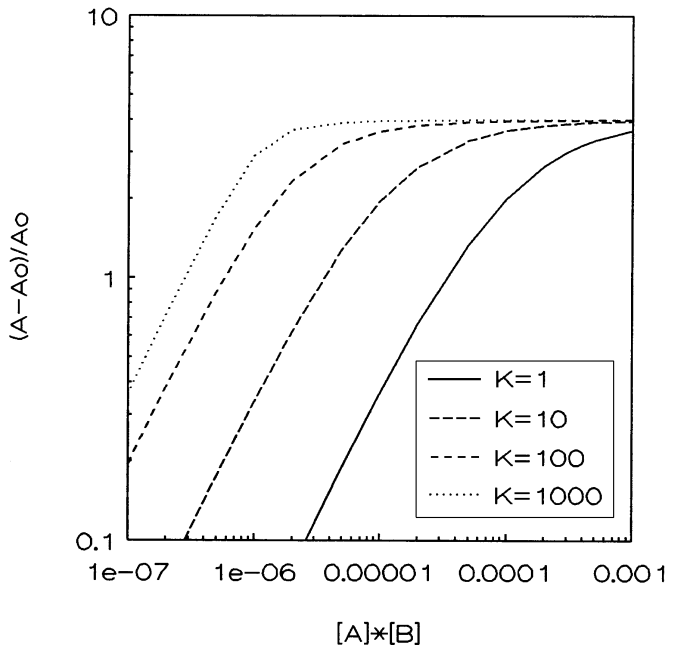

Figure 6 The estimated color response for an aqueous solution of malvidin 3-glucoside (10 mM, pH=3.6) as a function of the molar product of cofactor [B] and pigment [A] for 4 values of the association constant, K.

As a result, there are regions in curves of this type that will be (and have been) interpreted to mean that the ratio of cofactor to copigment is something other than one. Some studies, for example Davies and Mazza [35], in which ratios between 0.38 and 0.86 are reported for malvidin 3,5-diglucoside and caffeic acid, may be examples of this situation.

The ratio approach indicated in Figure 5 also provides (by nonlinear fitting) an estimate of the extinction value of the copigmented anthocyanin, as well as the association constant, based on the one-to-one stacking. (It can be easily modified for other ratios if evidence supports them). In contrast, the concentration product approach, as presented, cannot estimate the extinction value because it is lumped with the association constant in the "apparent K" derived by this method. The K values derived from Figure 6 would be 400 while the true K_{eq} would be 100 and the copigmented enhancement, (Ec-Ea)/Ea, is 4.0 in this example (where Ec = 5*Ea). The concentration product approach uses the intercept to provide K_{eq} when in fact it is providing (Ec-Ea)/Ea*K_{eq} values. As a result, combinations of pigment and cofactor that have higher Ec values will be (and have been) misinterpreted to have high binding constants (Brouillard et al. [25], Mazza and Brouillard [73], Brouillard et al. [26], Davies and Mazza [35], and Baranac et al. [11,12,13,14]). Care should be taken with this approach in future interpretations of copigmentation studies. A wider range of the concentration products should be employed, so as to include the saturation region. The value of $(A-A_0)/A_0$ at saturation then provides the ultimate color of the copigmented form, (Ec-Ea)/Ea, and this, while valuable in itself, would enable the true K_{eq} value to be estimated. This estimation has yet to be done in any of the reports that have been published to date.

Copigmentation Effects in Wines

The copigmentation of anthocyanins in wines will be a competitive equilibrium involving several anthocyanins and many cofactors, with some of the concentrations being determined by initial levels in the grapes and others by limited solubility. It is not clear how dependent the equilibrium is to changes in cofactor concentration, and it will be continually re-adjusting as free pigment is converted into colored polymer, especially during the first year of wine aging and as cofactors are oxidized or hydrolyzed. The role of cofactors and copigmentation during skin contact and fermentation of wines can be seen as twofold. First, the binding of free anthocyanins into copigmented forms enables more pigment to be retained, resulting in higher total anthocyanin content in the wine. Second, the copigmented anthocyanins provide much more color than they would have if they were in the free form. Wines made from grapes low in cofactors will not be able to form much copigmentation and will have low pigment contents. This seems to be the case for Pinot noir and Sangiovese and is why sometimes poorly colored wines result from darkly colored berries. Such wines are generally cherry-red when young and show no sign of purpleness. Other wines, from grapes richer in cofactors, will form more copigmentation, capture more pigment, and have deeper color with the blue and purple tones characteristic of significant copigmentation.

The acylated forms of the non-malvidin pigments seem to be involved in strong copigmentation, which means that wines predominantly malvidin in their anthocyanin makeup may have poorer color. This is partly the reason for poorer copigmentation in Pinot noir (and Sangiovese), which lack the acylated pigments [59,87]. There is a critical need to assess the pigment patterns of all major wine grapes to understand their potential for copigmentation based on anthocyanins, as well as comprehensive surveys of their cofactor pools.

While it would not be expected that the solubility of anthocyanin is limiting the color of red wines, it does appear that the ability to form significant copigmentation stacks parallels the pigment content of wines from Pinot noir, Nebbiolo, Sangiovese, Zinfandel, Cabernet Sauvignon, Cabernet Franc, Merlot, Durif, and Petite Verdot [79,116]. The concentrations of pigment retained in wines after fermentation will be due to a combination of effects, including partitioning of pigments (and cofactors) between the skins and the wine, copigment formation, and adsorption of soluble pigments (and cofactors) onto grape pulp, seeds, and yeast. The formation of copigmentation will enhance the apparent solubility and perhaps reduce the extent of adsorption from solution, with a net result of increased anthocyanin levels in wines with more ability to form copigmented anthocyanins.

Influence of Copigmentation on Color Extraction

The copigmentation phenomenon and its behavior as a dynamic equilibrium is of central importance to the understanding of the limitations to color potential, anthocyanin capturing, and pigment retention in wines. The extraction of pigments from grape skins either prior to, during, or following fermentation is far from complete (typically only 30 to 40%; Singleton and Esau [100] and Van Balen [117]). The evidence suggests that an equilibrium based on adsorption-desorption is established between the concentrations in the wine and the cellular concentration in the skin tissue, with significant reductions as the ethanol content increases. The role of copigmentation is to shift pigments out of the free anthocyanin pool of the adsorption equilibrium, causing more pigment to move from the skins into the wine. The extent to which this can occur now seems to be limited by the concentrations of certain cofactors in the skins at harvest and their solubility under wine conditions.

A widespread misconception is that color is simply extracted with time of contacting or improved mixing and this has led to considerable confusion regarding what determines the pigment, monomeric phenol, and tannin content of a young wine. As a result, a range of alternate contacting and mixing practices exist within almost every wine region, each with its advocates, but without consensus. Many studies have shown that additional contact time between skins and wine cannot provide additional anthocyanin content or color [2,15,16,38,53,54,77,80,89, 90,110,108,117]. It is noteworthy that three of these studies [16,54,89] reported increased tannin levels under extended contact, and that this additional tannin content did not lead to further capturing of the pigments.

The existence of an extraction equilibrium (and therefore limits to pigment extraction) is easily demonstrated by taking skins taken from red wine after the maximum pigment concentration has been attained and putting them into a white juice. While the pigments are not able to move into the wine from which they were taken, where an equilibrium concentration had been reached, they will readily move from the skin tissue into the new juice until a new equilibrium is established.

The extent of this equilibrium, and hence the color of a young wine, would then be controlled by the concentrations of pigments and cofactors in the berries and the juice volume in which they are in contact. Other than the initial rate of movement into the wine, the contacting and mixing practices would have little influence on the final equilibrium concentration of the pigments, which is what is actually observed in virtually all red wines. The role of higher temperature has dual and competing contributions. It enhances the solubility of most species, but especially those cofactors that have limited solubility, enhancing the pool of copigmented pigments. Thermodynamically, however, it favors the dissociation of copigmented forms and may actually cause a loss in the color due to copigmentation. On cooling, and often in the weeks and months following fermentation, precipitation can occur of the excess cofactors, usually resulting in some pigment precipitation and significant color loss.

The influence of the juice volume is to establish the concentration of free anthocyanin that results from the amount of anthocyanins that move from the skins into the juice. This movement will continue until the free anthocyanin equilibrium has been established and the effects on copigmentation are due to the second-order contributions from both the pigment and the cofactor concentrations. That is why berry size seems to be important in the color of some red wines and why there can be effects due to increasing the proportion of skins to juice, either by the addition of other skins or the removal of some juice in the practice of "bleeding." However, these results are not generally true and often no enhancement in color is observed. The effect on final color will be limited if saturation has been reached for the major cofactors [98].

Physical limitations to the establishment of the color equilibrium can result from mass transfer due to the mixing of skins and juice, and while enhanced rates of extraction can be achieved in some methods, the same pigment equilibrium is expected to be established. While some investigators and winemakers do not hold this view, well-controlled studies testing the influence of extraction methods on all of the components involved in the color equilibrium have yet to be reported. There are several studies that have shown the attainment of pigment and color maxima during the first half of traditional fermentations (punched-down or pumped-over) and that further skin contact has no effect in increasing pigment concentration or retention [15,16,38,53,54, 77,80,89,90,108,117]. The use of alternative contacting regimes has yet to demonstrate any ability to modify this equilibrium other than by temperature effects, most of which seem to be short-term. These regimes include the use of complete, or fractional, drawing of the juice from the skins prior to its return (*de l' estage*), soaking of skins prior to the onset of fermentation ("presoak"), keeping of juice and skins together for a period of weeks after the end of fermentation ("extended maceration") or continuous (or periodic) pump-overs, a submerged skin cap, punching-down of the skin cap or rotary fermentors. While all of these treatments have the potential to modify the true extraction of most other phenols and tannin levels, particularly the tannins from seeds, there is no evidence that they can enhance the copigmentation equilibrium or wine color to any significant degree in the long-term. The same seems to be true of various enzyme preparations that have been tested for color enhancement.

The view that this equilibrium is predetermined by the composition of the grapes would suggest that the enhancement of color is not possible, even with enhanced extraction by cell wall breakdown. While they may provide short-term results of higher color, such methods do not seem to provide enhancements that remain after the exposure to barrel surfaces and months of aging at cellar temperatures. It appears to be generally true that the factors controlling the solubility and retention of pigments in young wines are more important than contacting methods in determining wine color. That is one explanation for why a wide range of alternative contacting and extraction practices continue to be used, with no single method being significantly better than others in terms of color retention.

Further evidence that the initial composition is the major influence on final wine color can be found in experiments in which the grape composition has been modified by the addition of colorless cofactors. These additions, of natural grape constituents such as caffeic acid and catechin, to juices prior to fermentation have resulted in significantly enhanced pigment retention in the cultivars Listan negro [33,34] and Pinot noir [27]. These color enhancements are stable (retained for more than a year) and greater than any effects due to temperature, enzyme treatments, or skins-juice contacting methods that have ever been reported.

It is now of great importance to renew the search for relationships between the pigment and phenol composition of grapes and their corresponding wine. Until recently, the color of wine due to the anthocyanins could not be calculated from a chromatographic analysis and the anthocyanin content could not be accurately determined by spectrophotometric methods, both because of the influence of copigmentation. Further, there has been an overdue emphasis on the anthocyanin content of skins. It is now clear that the levels of copigmentation cofactors are at least as important, and in many cases more important. In light of this, it is necessary to reassess many of the conclusions from previous studies and to rethink many of the ideas that have been developed concerning the influence of viticultural conditions and winemaking practices on the color of young and aged red wines.

Cofermentation of Red Grapes from Different Cultivars

The possibility exists in which red grapes that are deficient in certain cofactors might benefit from being fermented in the presence of others that might have an excess of them, especially if they are cofactors, such as the flavones, with limited solubility. The ability to form additional copigmentation during fermentation would give rise to more deeply colored wines than would occur simply by blending the individual wines that had been

fermented separately. The grape mixture may be of different lots of the same cultivar or it may be of different cultivars. Enhanced color will not be seen in all cases since the extent of the effect will depend on the ability of some of the grapes to benefit from additional cofactors and the ability of other grapes in the mixture to provide those cofactors.

Cofermentation of White and Red Grapes

The extension of this reasoning of grape mixtures includes the possibility that certain white cultivars might be rich in cofactors and that when cofermented with red grapes deficient in cofactors, more copigmentation and capturing of the anthocyanins could occur, giving more color in the resultant wine. Because white juices are not usually fermented on their skins, the levels of certain cofactors, especially the slightly soluble flavones present in the skins, will not usually be reflected in the phenolic analysis of these wines. The traditional use of some white grapes, such as Trebbiano or Malvasia, with Sangiovese and other red grapes in certain regions of Italy, are good examples of this. Others examples exist in certain wine regions of France, Spain, and Argentina. The increase in color of a port wine when a white skin extract was introduced was noted by Timberlake [112], and it is an example of color enhancement due to copigmentation, resulting from the cofactors from the white skins.

Gogliotti et al. [40] has shown, based on the wines from six seasons, that the best color enhancement of one-year-old Sangiovese wines occurred when Trebbiano and Malvasia comprised between 5 and 15% of the grape mix and a further 10% was from Canaiolo. The addition of too high a percentage of white grapes will lead to progressive pigment adsorption to the skins and pulp, beyond any color enhancement effects, resulting in a net loss in color. Any concern regarding dilution (or extension of the red wine volume) can be overcome when only the pressed skins are used. However, there will be no advantage in this approach if the red grapes already have sufficient amounts of the cofactors or if the white grapes have insufficient amounts of cofactors to give. It is clear that not all white cultivars will be suitable or acceptable and there are many possible combinations that need to be investigated. Seasonal effects on the outcome of such a practice are to be expected.

Color Loss in the Months after Fermentation

The formation of maximum color by midfermentation and then a loss of some of it in the days and weeks following the end of fermentation is a pattern that has long been known and widely reported [15,38,52-54,77,80,89,90,108,110,117]. This has generally been attributed to a fall in anthocyanin content, although the loss in color is a larger percentage than that observed in the anthocyanin content [90]. This larger loss in color than can be accounted for by the loss in anthocyanins may be due to a breakup of the copigmentation stacks formed earlier in fermentation.

The shape of the anthocyanin concentration curve during fermentation has been described mathematically in terms of an exponential approach to a limiting value, followed by a later, slower depletion to a lower value [18]. This has been postulated to be due to diffusion-controlled extraction phase, which reaches an extraction equilibrium, followed by secondary adsorption phase (to grape pulp, seeds, and yeast) as the ethanol concentration increases toward the end of the fermentation. The additional color loss by adsorption onto stems when they are present has been well documented, and this appears to be an extension of the more general adsorption onto pulp and yeast. Hilgard [53,54] noted the fall in color after fermentation and showed it to be greater in fermentations at 32 and 20°C than at 25°C. It ranged from almost nothing in a Teinturier sample to typically 30% in Cabernet Sauvignon, Zinfandel, and Barbera wines [52].

If the adsorption involves primarily the free anthocyanin forms and not the copigmented forms, wines low in copigmentation would be more easily depleted of their anthocyanins by this action. As the solubility of anthocyanins in winelike solutions is much greater than that found in most wines, it appears that concentrations beyond that of the natural distribution between the skin and the wine levels must be due to the result of copigmentation.

Further loss in color due to adsorption of either free pigments or cofactors onto the surfaces of new barrels is expected (and has been shown in one case [89]). The extent of this would be wine-specific, and it will alter the equilibrium that is in effect during the polymerization reactions that will occur in the subsequent aging of the wine.

The Color Contribution of Wines to Blends

A common challenge for winemakers is to understand the color exhibited by different wines and that displayed when they are blended. This especially true when these are young wines in which most of their color is accounted for by the free and copigmented anthocyanins. The nonlinear loss of copigmented color with dilution applies to each wine in a blend, and the color of the blend will not always be in relation to the volume and color of the wines used. While a small amount of deeply colored young wine might be chosen to add color to older aged red wines, at small additions it will be extensively diluted. The implication based on copigmentation is that it can only give the noncopigmented fraction of its color to the blend, which would typically be 70%, or as little as 50%, of its initial color. Boutaric et al. [22] and Winkler and Amerine [125,126] have both shown the nonlinear nature of the color of red wine blends, when at least one wine is young enough to have significant copigmentation. When there is insignificant copigmentation in either wine, usually in wines that have been aged for a year or more, the color of the blend then follows the expected linear relationship with the volume of the fractions used.

Behavior of Copigmentation toward Fining and Cold-Holding Treatments

The presence of copigmented forms of the anthocyanins calls for a fresh look at the effects of various other winemaking treatments such as fining and cold-stabilization on the color of young red wines. Because the copigmented form is so much more colorful than the free form, any treatments that cause some dissociation of the copigmentation stack can have more effect on color than that due to anthocyanin depletion.

The importance of the cofactors in the extent of copigmentation has already been emphasized and the limited

solubility of some of the more potent ones has been mentioned. The effect of the temperature conditions used during stabilization would be capable of causing a loss in copigmentation and color, with relatively little change in the anthocyanin content. The use of moderate temperatures more like those expected in the marketplace, 10 to 15°C, have been suggested [20] for "tartrate stabilization" of red wines, even though much more severe conditions are generally used in both the domestic and international trade.

A secondary effect appears to be the possible role of the bitartrate anion in the stability of the copigmentation stack at wine pH. The reduction of the bitartrate concentration in wines already low in tartrate levels may cause significant loss in copigmented color due to a shortage of available anions and less stability of the copigmentation stack.

Role of Copigmentation in Oxidation and Aging Reactions

The rates of certain important chemical reactions in wine, namely the oxidation of flavonoids and the polymerization of pigments and flavonoids into larger polymers, are poorly understood and currently cannot be estimated from wine composition. Part of the reason for this is that the understanding of the kinetics of these reactions is limited even for single components, let alone for mixtures. However, a more significant factor may be that the rates are likely to be related to the free concentrations of the phenolic substrates, not their total concentrations. Since many of the flavonoids are moderately strong cofactors, a significant fraction (perhaps 30%) of their total pool is likely to be in the copigmentation stack at any time, thereby establishing a smaller reaction pool than might be expected and, subsequently, a slower reaction rate. The immediate implication is that the rates of polymerization would be slower than an equivalent pool without any copigmentation, and if both an anthocyanin and a flavonoid are involved in the reaction, then it could be second-order in nature, leading to an even more dramatic effect on the rate. Berg [15] suggested that the susceptibility of wine to oxidation was related to the degree of association of its pigments, although there are little data to demonstrate this. A later report of the rates of red polymer formation found that the polymeric pigments in a model solution, prepared by skin extraction, formed at almost twice the rate of those in the corresponding wine [103], a result that would be expected based on the above arguments. This suggests that the level of copigmentation may influence the rates of polymer formation and oxidation, and wines that are higher in copigmentation would react more slowly than those lower in copigmentation, under the same conditions. While there is some anecdotal evidence that this might be true in deeply colored red wines, there are several conflicting factors such as total phenolic content, tannin content, and pH that limit any conclusions at this time. Controlled experiments in which the rates of pigment consumption and polymer formation need to be performed in many different wines to see if the rates are at all influenced by the extent of copigmentation.

The above discussion is based on the premise that the free form of pigments and flavonoids are involved in these reactions. An alternative view is that the stacked form may be a more reactive or preferred arrangement for the limiting step in the anthocyanin polymerization reactions. In this case, the rate of anthocyanin loss during aging would be related to the fraction of pigment in the copigment stack rather than that in the free form, and wines higher in copigmentation would display faster rates of polymer formation.

The rates of oxidation reactions can be expected to be related to the free monomeric concentrations of the corresponding phenols. The effect of copigmentation would be to provide lower free concentrations and slower rates of oxidation, especially for the anthocyanins and the major cofactors. This would partly protect them from oxidation and favor their incorporation into red polymers rather than into brown polymers, a matter of considerable importance in the aging of red wines.

The distinction needs to be made between wines with high levels of copigmentation (due to higher levels of the critical cofactors) and wines high in total phenol or tannin content. Wines that have been overextracted, in terms of tannin or total phenolics, are not usually higher in copigmentation. This is because the movement of the cofactors from the skins into the wine seems to be controlled by an equilibrium, while that of most phenols (and tannins) is more completely extracted. There is essentially no relationship between these measures in the hundreds of wines that we have studied to date, and it is clear that further extraction does not lead to higher levels of cofactors.

Role of Copigmentation in Sensory Analysis

Like the rates of polymerization and oxygen uptake reactions, the astringency and bitterness of red wines cannot be predicted from their component composition. There is considerable confusion as to the relative roles of monomeric and polymeric phenols in both astringency and bitterness, and there is growing evidence that the role of monomeric forms is much greater than has previously been considered. This is especially true of the influence of pH on these attributes and the behavior of the phenolic acids as a group compared to the flavonoids and polymers. Many of the flavonoids appear to be present at levels close to their flavor threshold concentration (Singleton and Noble [101]), and the rates of binding to receptor and saliva proteins would be expected to be related to their free concentrations rather than to their total concentrations. The influence of copigmentation will again be to lower the free pool of such components and thereby lower the rates and extent of these sensory interactions. Again, there is some anecdotal evidence that wines richer in color, especially purpleness, receive higher sensory rating. Such a relationship was proposed many years ago by Somers and Evans [106], based on the panel scores awarded to young Shiraz and Cabernet Sauvignon wines in one region of Australia. Timberlake et al. [114] studied various color measures and "flavor" and "overall quality" in two vintages of Beaujolais wines. They also re-analyzed the data of Somers and Evans [106] and the only measure that was significant in all cases was the "anthocyanin color," that is the color loss due to bisulfite bleaching. As the majority of this measure is due to copigmentation, it adds support to the view that copigmentation is positively correlated with some "quality," but more importantly, "flavor," measures in red wines. Tromp and van Wyk [115] have reinforced this view by

showing that panelists could guess the quality rating of wines based on their color, but they were less successful estimating the color, having tasted the (masked) wine first. It is clear that the anthocyanins themselves provide no significant flavor contribution, and the connection was generally dismissed as being a correlated marker of other, more significant, impact flavor components. However, in terms of the possible softness that might be imparted by the inclusion of some astringent monomeric components into copigmentation stacks, the relationship between the extent of copigmentation (and therefore darker red wine color) and taster preference deserves renewed attention.

As mentioned previously, the 20% loss in the absorbance of wines at 280 nm, observed when they undergo a 20-fold dilution, may be due to the breakup of the copigmented forms, as measured at that wavelength. Alternatively, it may be evidence of a more general but limited association between essentially all of the phenolics components in red wines. This latter scenario would have several ramifications on a range of sensory attributes by acting as a delayed release mechanism for possibly all heterocyclic aroma constituents and of their mouth-feel and flavor. These ideas need to be further investigated.

Some Possible Research Directions

The fundamental studies of copigmentation with malvidin 3-glucoside-cofactor pairs in model solutions using both CD and NMR techniques have yet to be performed. These two methods each have their advantages, and there is considerable information about the association constants and molecular forms involved that needs to elucidated, both in aqueous and winelike (pH 3.3 to 3.6 and 12% v/v ethanol) solutions.

The role of ionic strength, more specifically anion type and concentration, on the stability of copigmentation stacks needs to be further investigated, particularly with respect to the differences between wines and the effects of cold stabilization treatments. The influence of wine pH on the color due to copigmentation also deserves further study so that its contribution in the various assays for anthocyanin content can be further understood.

The present picture of copigmented and free anthocyanins should now be applied to study the rates of polymeric pigment formation and the factors influencing it. These would include pH and oxidation effects, which in the past have been confused with the formation of brown polymers and their contribution to color density.

Our knowledge of this phenomenon in wines is still undeveloped and yet its role in the natural color of red wines can only be described as dramatic. It is now clear why the color of young red wine is not related to the anthocyanin content of the grapes from which it was made. The critical importance of the nature and concentrations of the copigmentation cofactors is now apparent, but there is little understanding of the factors influencing their formation and retention during the maturation of any red wine grapes. Almost all of the studies that have been conducted with an aim of understanding wine color need to be repeated, but with the focus on cofactors and their concentrations, rather than on the anthocyanins. This is as true for grapes as it is for wines. Hopefully, the limited value of experiments that measure the pigments content of berries, without following their movement into the corresponding wine, is now apparent.

There are quite specific patterns of the cofactors and their concentrations in wines that have been made from different cultivars [116]. This will lead to improved grouping of young wines based on their copigmentation components rather than by their pigments alone. There is also considerable variation in the concentration of copigmentation observed within wines of the same cultivar, growing district, and season. The causes of this variation are not yet understood. One study of more than 69 Cabernet Sauvignon wines found that the variation in copigmentation levels of wines made at any of 12 wineries was similar to the variation in the wines from the other wineries, and little different from the overall variation in all of the wines [70]. This suggests that most of the variance in copigmentation is due to the vineyard site and cultural practices rather than to the winemaking practices within a winery. It also supports the notion that the color obtained in a wine is preset at harvest and that there is little effect due to the contacting practices of the winery.

Apart from this study of Cabernet Sauvignon wines from one harvest in one district, there are apparently no similar comprehensive studies with any other cultivars. The effects due to rootstock and clone on copigmentation have not been studied in detail for any cultivar to date and, unfortunately, the same is true for the entire spectrum of cultural practices. The crop level (reported not measured) in the Cabernet Sauvignon study was not significantly correlated with copigmentation in the wines, although this deserves further study in other sites and conditions. Controlled investigations of the effects of light, both intensity and wavelength, on the formation and retention of cofactors in berries have started only recently. The role of root conditions, rootstock, soil moisture, and particularly root temperature would seem to be important in the initiation and control of berry size and the flavonoid synthesis, and may be preset long before veraison, although little attention has historically been given to this aspect in viticulture.

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