Anthocyanin molecular interactions: the first step in the formation of new pigments during wine aging?

Raymond Brouillard & Olivier Dangles

Laboratoire de Chimie des Polyphénols, associé au CNRS, Université Louis Pasteur, Institut de Chimie, 1, rue Blaise Pascal, 67008 Strasbourg, France

The changes in red wine colour on aging are still a challenge. From recent work involving model experiments, it has been concluded that high quality red wine storage conditions (long period of time, permanent contact with wood, low pH values, low temperature and aqueous environment), are all in favour of good molecular associations between the wine anthocyanins and the aromatic planar colourless molecules present in such a medium. In this article, factors which affect anthocyanin non-covalent interactions will be reviewed. They include pigment and copigment concentrations and structures, pH, solvent and temperature. The complex of a pigment with one of its copigments may be the starting point of a covalent linkage between the molecules. This could explain changes in colour and in anthocyanin reactivity observed during the aging of red wines, for instance.

INTRODUCTION

Red wine colour belongs to red wine sensory properties (Ribéreau-Gayon, 1982). Indeed, the visual perception of a red wine is strongly related to its taste and therefore to its quality. Young red wine pigments are essentially those of the red grapes from which the wine has been made. Since among the edible fruits grapes have been the most investigated, such pigments are well known. Red grape pigments are anthocyanins and, as far as *Vitis vinifera* is concerned, these are 3-monoglucosides of various aglycones with malvidin 3-glucoside (oenin) and its derivatives being, in almost all cultivars, the more abundant (Macheix *et al.*, 1990). There are also many colourless phenolics accompanying the anthocyanins within grapes; examples are phenolic acids, tannins, flavanols, flavonols and flavanonols. It is thought that red wine colour changes during maturation and aging are due to the simultaneous existence in young red wines of anthocyanins *and* of many colourless phenolic chemical species present in grapes like the monomeric (+)-catechin and (−)-epicatechin (Timberlake & Bridle, 1976; Somers & Vérette, 1988; Haslam, 1989; Liao *et al.*, 1992; Mazza & Miniati, 1993). In addition, permanent contact with the oak wood of the barrel during maturation results in more phenolic compounds being extracted from the wood into the wine.

It has been reported that a typical effect of aging on red wine colour consists of a steady increase in the A_{420nm}/A_{520nm} absorbance ratio (Ribéreau-Gayon, 1982; Bakker *et al.*, 1986; Somers & Vérette, 1988). These variations in the light-absorption properties of a wine are usually accounted for by formation of specific wine pigments at the expense of the free monomeric anthocyanins initially provided by the berries to the must (Baranowski & Nagel, 1983). Although it is now believed that the anthocyanin flavylium chromophore could remain within the new structure of the wine pigment or could change into a xanthylium ion (Liao *et al.*, 1992), no structural elucidation of a red wine pigment has been published so far. A unique role has been allocated to acetaldehyde which, under certain conditions, may bridge the flavylium nucleus with an already existing polyphenol like (+)-catechin (Singleton *et al.*, 1964; Timberlake & Bridle, 1976). Identifying new pigment structures directly from aged red wines is a formidable challenge to the food chemist because it is expected that combinations between the many anthocyanins of grape with the even larger number of polyphenolics found in those grapes may amount to several tens, if not several hundreds! Therefore, model experiments are necessary to further improve our knowledge in this field, and a good very recent example of such a work has been given by Haslam and his group (Liao *et al.*, 1992).

Pathways by which grape anthocyanins transform into red wine pigments remain to be established. Very recently, Haslam pointed out that copigmentation, the hydrophobic stacking interaction between anthocyanin chromophores and the so-called copigments, could be the first step in the formation of a covalent bond between the pigment (anthocyanin) and its copigment (Liao *et al.*, 1992). Several years ago similar views were put forward by Somers (1971), who also noticed that

the polymeric wine pigments are less sensitive to pH changes and SO_2 bleaching compared to the grape anthocyanins themselves. On their side, Timberlake and Bridle (1976, 1977) seemed to favour a pathway where one more species, acetaldehyde, was necessary for a covalent bond to form between the initial anthocyanin and one of the catechin copigments, for instance. It has not yet been decided whether acetaldehyde-mediated association or only copigmentation is the pathway taken by the anthocyanins during wine aging or if both pathways occur at the same time.

From now on we focus attention on the copigmentation molecular interaction which, probably, has much more to do with the formation of oligomeric red wine pigments than it has been thought over the past two decades. Six years ago, the copigment effect has been reviewed by Mazza and Brouillard (1987). In that paper, the authors rather reflected the classical views on copigmentation. In particular, no theoretical support could be provided to the numerous experimental results which accumulated over the 1970s to 80s. Soon after, a theoretical framework was set up by Brouillard and his group (Brouillard et al., 1989) and new experiments could be devised, thus highlighting many of the most important features of copigmentation. As stated above, copigmentation is the hydrophobic association of an anthocyanin chromophore with the planar electronically unsaturated part of a copigment. This association is easily seen by visible absorption spectroscopy. Indeed, the anthocyanin absorption maximum is shifted both in position (bathochromism) and in intensity (hyperchromism) upon copigment addition to the anthocyanin-containing solution. Factors which affect the copigment effect are now under control (Brouillard & Dangles, 1993). These are pigment and copigment structures and concentrations, acidity, solvent and temperature. Substances like metallic ions or macrocycles, capable of reacting with either pigment or copigment, also influence the intensity of the copigment effect (Dangles & Brouillard, 1992a; Dangles et al., 1992). However, such purely chemical factors are not considered here although they may play a role during wine conservation.

It is the purpose of the present review to outline some of the more important parameters of copigmentation and to stress that the physicochemical conditions of red wine storage should promote good interactions of grape anthocyanins with the remainder of the phenolic pool originating from grapes or from the wood barrel. It is hoped that this review article will be helpful to the food chemist in the planning of new types of experiment, aimed at discovering the fate of red grape anthocyanins in red wine aging processes.

INFLUENCE OF STRUCTURE AND CONCENTRATION

For the copigment effect to become measurable, two concentration-related parameters have to be taken into

consideration. One is merely the concentration of the anthocyanin which has to be usually higher than 10^{-5} M for a sensitive investigation using visible absorption spectroscopy. The second parameter is the copigment-to-pigment molar ratio. Most often large values of this ratio are required, especially when dealing with poor copigments. For common copigments (see Fig. 1) like chlorogenic acid (2), caffeine (3) and (+)-catechin (4) in association with malvin (1), typical molar ratio values range from 10 to 100. In the case of strong copigments like rutin (5), copigmentation starts to operate at lower (close to 1) copigment-to-pigment molar ratios. It has been demonstrated that one molecule of copigment forms a non-covalent stack with one molecule of anthocyanin either in the flavylium form or in the quinonoidal base form (Brouillard et al., 1989; Mazza & Brouillard, 1990). In so doing, the copigment expels a few water molecules from the pigment solvation shells into the bulk water and thus protects the flavylium chromophore from the nucleophilic attack of water (covalent hydration) which leads to the colourless anthocyanin forms (hemiacetal and chalcones) (Brouillard et al., 1990). Moreover, a striking but firmly established feature is that usually a copigment does not associate with the colourless forms of anthocyanins. As a consequence, copigmentation tends to invert the reversible hydration process and to increase the concentration of 'active' coloured forms at the expense of the 'inactive' colourless forms. Thus, on progressive addition of copigment to an almost colourless anthocyanin solution, a large recovery of the colour occurs as well as a change in this colour since the flavylium ion and
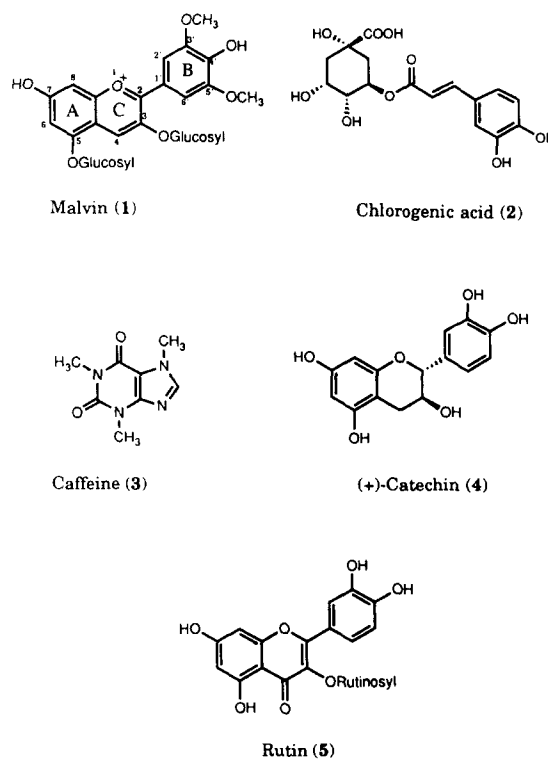

Malvin (1)

Chlorogenic acid (2)

Caffeine (3)
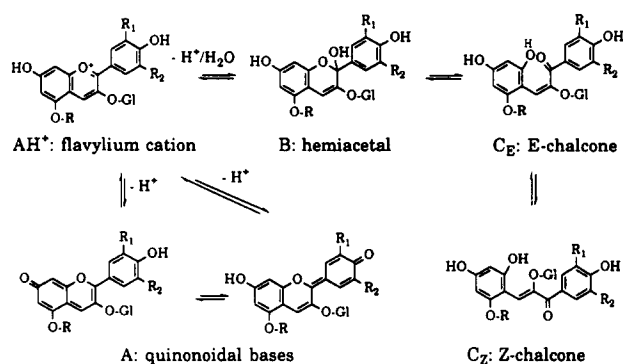
(+)-Catechin (4)

Rutin (5)

Fig. 1.

Fig. 2. The structural transformations of red grape anthocyanins. (R = H, Gl; R_1, R_2 = H, OH, OMe; Gl = glucosyl).

quinonoidal bases are no longer in a pure aqueous environment but associated with the organic copigment. In spite of copigmentation, the hydration reaction usually remains the dominant phenomenon so that the coloured flavylium cation and quinonoidal bases (see Fig. 2) are much less abundant than the colourless species. In fact, the driving force allowing the copigment-to-pigment stacking (van der Waals interactions and solvation effects) is much smaller than that for covalent bonding and this is why the copigment has to produce some kind of mass effect to achieve molecular complexation with the pigment. Although copigmentation is best observed by means of visible absorption and emission spectroscopies (Wigand *et al.*, 1992), it can also be investigated by NMR on more concentrated (say 10^{-3} M) pigment solutions (Cai *et al.*, 1990).

All anthocyanins are affected by the copigment effect and, for instance, the common anthocyanidin 3-glucosides and 3,5-diglucosides are good candidates for copigmentation studies. *Vitis* anthocyanins are of these types. In the case of a few sophisticated anthocyanins intramolecular copigmentation can also arise (Brouillard, 1981). Intramolecular copigmentation only occurs when the pigment and the copigment are parts of a unique molecule, for instance, when the anthocyanin chromophore and a cinnamic acid moiety (copigment) are covalently bound to the same sugar residue. In fact, the sugar residues of anthocyanins are now believed to act as important building blocks in the formation of the pigment–copigment complexes whether these occur inter- or intramolecularly (Dangles *et al.*, 1993). When two (or more) cinnamic esters are present in an anthocyanin, intramolecular copigmentation may be strong enough to prevent intermolecular copigmentation to take place (Dangles *et al.*, 1993). Due to their very structures, red grape anthocyanins are unlikely to show important intramolecular copigment effects and, in this case, intermolecular association is the rule.

Until now copigments tested belong to different chemical families like flavonoids and purines (Asen *et al.*, 1972; Brouillard *et al.*, 1991). As to the required structural features of a good copigment, things are less clear. Nevertheless, it seems that the only necessary, but not sufficient, structural element which has to be

found in a copigment is a planar, π electron-rich ring allowing a large π–π overlap with the flavylium ion and the quinonoidal bases. Such a vertical stacking can be easily demonstrated by the copigment-induced diamagnetic displacements of the anthocyanidin proton peaks in the NMR spectrum of the pigment. As examples of molecules acting as copigments of the common malvin (1), let us mention phloroglucinol and tryptophan which form weak copigmentation complexes whereas chlorogenic acid (2), caffeine (3) and (+)-catechin (4) interact more strongly with this anthocyanin. Finally, one of the most efficient copigments known today is rutin (5) (Dangles & Brouillard, 1992*b*). Hydrogen bonding may reinforce the association whenever possible but, with only one apparent exception (caffeine associated with a synthetic, non-natural anthocyanin, 7-hydroxy-3,4'-dimethoxyflavylium chloride), it is not expected to bring an important contribution to the copigmentation complex-forming process (Wigand *et al.*, 1992).

As pointed out in the introduction, many of the molecules present in mature red grapes are potential copigments and, although the flavanols (+)-catechin and (–)-epicatechin have drawn more attention within the wine context, other grape components, like caftaric acid (caffeoyltartaric acid) as well as the numerous grape flavonols, may well appear in the future as good copigments.

INFLUENCE OF pH

It has been quantitatively demonstrated in our laboratory that the copigment effect reaches its highest point within the pH range 3 to 5, depending upon the investigated pigment-copigment pair (Brouillard *et al.*, 1991). For instance, in the case of malvin (1) copigmented by chlorogenic acid (2), maximal colour augmentation was found close to pH 3·6 (Brouillard *et al.*, 1989). It is strange that the wine acidity range falls within the limits where the copigment effect is at its highest! It is therefore expected that, in red wines, copigmentation is a general phenomenon. One should also note that the influence of pH is essentially related to the structural transformations followed by the anthocyanin itself (Fig. 1). By contrast, changes in the protonation state of a copigment like adenosine do not exert measurable effects on the copigmentation strength, strongly suggesting that neither hydrogen bonding nor electrostatic attraction or repulsion, plays a significant role in the association between anthocyanins and purines (Brouillard *et al.*, 1991).

A rough mechanism can now be suggested for what happens in more acidic media (0·1M HCl, for instance). Water and copigment molecules compete for the immediate neighbourhood of the flavylium chromophore. In the absence of any copigment, the hydration reaction largely prevails only if the solution is slightly acidic or neutral (Fig. 2). In the case of strongly acidic solutions (pH $\leq$ 1), covalent bonding between water and the
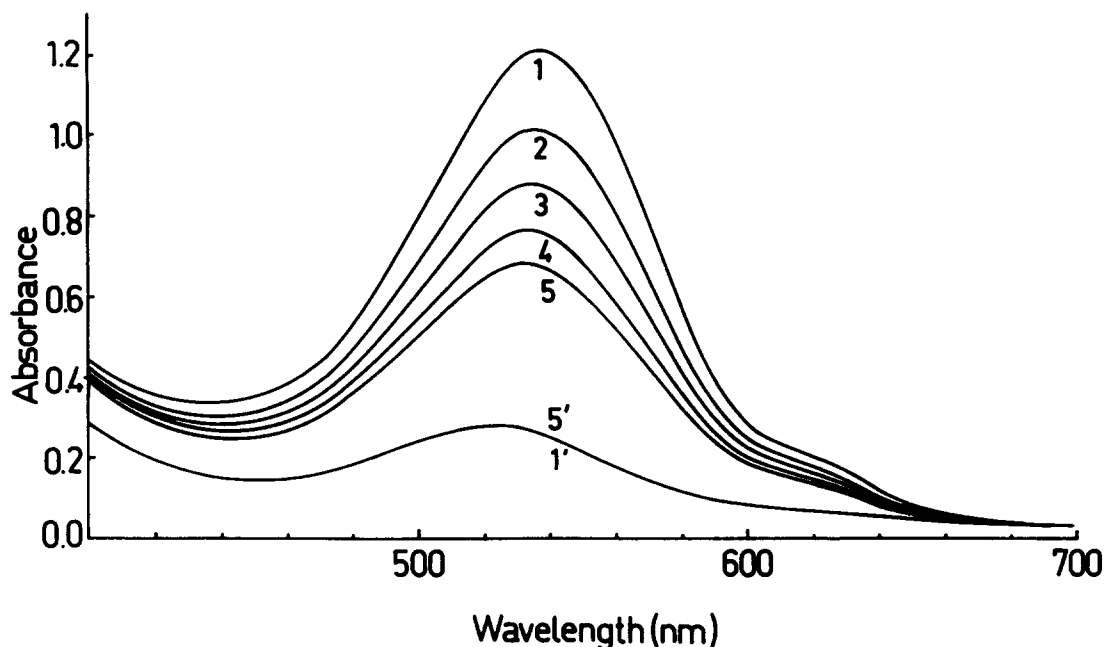
Fig. 3. Temperature-dependence of the visible absorption band of malvin for the malvin-(+)-catechin system at pH 3·5. Malvin (1): 6×10^{-4} M. (+)-catechin (4): 10^{-2} M. The lower spectra concern malvin without copigment at 10°C (1') and 30°C (5'). For the remaining spectra and from top to bottom, temperature values are 10 (1), 15 (2), 20 (3), 25 (4) and 30°C. (Adapted from Dangles & Brouillard, 1992b.)

flavylium ion, which under such conditions is a stable species, has ceased. In the absence of a fading process, the copigment which still associates with the antho-cyanin chromophore is no longer able to generate colour since the initial anthocyanin solution is already highly coloured by itself. What essentially remains from the intense copigment effect seen in moderately acidic media is a bathochromic shift with, this time, a small hypo-chromic shift. Such spectral characteristics no longer feature the copigment effect. In conclusion, let us keep in mind that copigmentation takes place in slightly acidic, neutral and slightly alkaline aqueous solutions.

INFLUENCE OF SOLVENT

This is a factor which has generally been given less at-tention over the past fifty years. Let us mention, how-ever, that it was as early as 1931 that Robinson and Robinson pointed out the determining influence of temperature and composition of the medium on the copigmentation intensity. Recently, using several pig-ment–copigment pairs, we recorded large effects on the copigmentation complexation brought about by chang-ing either the temperature of the solution or its compo-sition (Brouillard et al., 1991; Dangles & Brouillard, 1992b). Concerning the influence of the composition of the medium in which copigmentation occurs, a clear conclusion came out over the past four years: copig-mentation is specific to aqueous media and a decrease in water content always leads to a decrease in the

copigment effect, no matter the nature of the organic cosolvent added to water. For instance, alcoholic cosol-vents like methanol, ethanol or ethylene glycol all re-duce the amount of complexation, the largest reduction being obtained with ethanol. In a 4:1 mixture of water with ethanol, the strength of the association between malvin (1) and chlorogenic acid (2) drops to a quarter of its value in pure water at the same temperature. From our most detailed thermodynamic investigation on cosolvent effects in copigmentation (Dangles & Brouillard, 1992b), the following scheme has emerged: highly polar organic cosolvents like alcohols interact with polyphenolic solutes more strongly than water; in the case of copigmentation and as far as low cosolvent-to-water molar ratios (say, lower than 0·1) are con-cerned, such interactions are stronger with the copigmentation complex than with the independent species. Everything happens as if the hydrophobic stacking with simultaneous release of solvation water molecules were also accompanied by some kind of lipophilic attraction between the cosolvent molecules and the stacks. Therefore, in our model, the copigmen-tation complex has a first solvation shell essentially made of organic cosolvent, the whole being surrounded by a more aqueous environment. However, because of their low entropy, such highly organized pigment–copigment–solvent–cosolvent assemblies would form at high free energy cost so that the overall effect of cosol-vent addition is a weakening of the copigmentation in-teraction. Note that no simple property of the medium was found to correlate with the extent of complexation, although some relationship exists with the solvent po-

larity parameter E_T (30) which itself reaches its highest value in the case of pure water (Reichardt, 1988).

In red wines, copigmentation probably takes place as long as free anthocyanic species remain at a sufficient concentration level. In these media, it is not easy to distinguish a free anthocyanin form from the corresponding copigmented structure. To know if complex formation is the initial step in the aging of red wine, the following experimental procedure may be used. Perform copigmentation with binary ethanol–water solvent systems until the amount of ethanol is sufficient for a complete destruction of the copigmentation complexes. Keep these solutions at low temperatures for several weeks or months. Compare the anthocyanin content in the aged solutions with regard to their initial anthocyanin content. If the anthocyanin composition within the series of aged solutions does not vary from one sample to the other, this certainly means that copigmentation has little to do with the aging mechanisms of red wines. On the contrary, if the anthocyanin in the less alcoholic sample has the more evolved toward some kind of new pigment, copigmentation could really be the first molecular step in the formation of new pigments during aging.

It remains to be said that copigmentation, as a particular case of molecular interactions, is an extremely fast dynamic process and the lifetime of a given complex is probably less than one microsecond. Encounters between partners are numerous but brief. So, for a covalent bond to establish between anthocyanin and copigment a minimum period of time, much longer than a complex mean lifetime, is necessary. This may explain why red wine aging is such a slow phenomenon occurring over months, if not years; it is only from time to time that a complex could be able to escape from the copigmentation equilibrium to give a new pigment molecule (Scheme 1). Increasing or lowering the temperature will have very little effect on the kinetics of complex formation or complex dissociation, but the usual rate-accelerating effect of increasing temperature should be observed in the step where pigment and copigment become covalently linked. However, little is known for the slow reaction drawn on Scheme 1 and for which spectroscopic, kinetic and equilibrium data are scarce (Liao *et al.*, 1992).

anthocyanin + copigment ⇌ complex (fast)
complex ⟶ new pigment (slow)
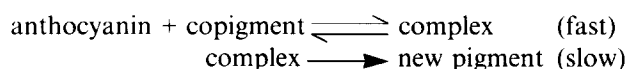
Scheme 1

Very interestingly, it has been suggested that the new pigment could be of the xanthylium type (Timberlake & Bridle, 1976); again that proposal has been convincingly put forward very recently (Liao *et al.*, 1992). In our most recent work (Dangles & Brouillard, 1994) on the 1,3,6,8-tetrahydroxyxanthylium ion, no xanthenol arising from covalent hydration was found and this

could point to highly hydroxylated xanthylium ions being more resistant toward nucleophiles than flavylium ions. This is in agreement with the low sensitivity of wine pigments against colour loss due to added sulfite and pH changes (Somers, 1971). Nevertheless, structural elucidation of the pigment(s) produced in the model systems seems necessary before going further in that field. In the absence of covalent hydration, copigmentation of xanthylium pigments cannot operate and, for instance, the vertical stacking of caffeine onto the 1,3,6,8-tetrahydroxyxanthylium ion or the corresponding quinonoidal forms leads to only minor colour changes. In fact, the most striking characteristics of our xanthylium pigment is its very strong non-covalent dimerization in water. This also is in contrast with anthocyanins for which self-association does not significantly contribute to colour expression.

INFLUENCE OF TEMPERATURE

This may be seen as a less important factor although our opinion is somewhat different. As long as maturation and aging of red wines are concerned, this statement is more or less true. Nevertheless, note that a temperature change produces an astonishingly strong effect on the visible spectrum of a copigmented solution of anthocyanin (Brouillard *et al.*, 1989; Dangles & Brouillard, 1992*b*). The temperature dependence of the visible absorption band of malvin (1) in the presence of (+)-catechin at pH 3·5 is represented on Fig. 3. Since a temperature change has barely any effect on the absorption of free malvin, the observed spectral variations can only arise from a shift in the copigmentation equilibrium. Clearly, an increase in temperature brings about a partial dissociation of the copigmentation complexes and the flavylium ions released in that way become liable to covalent hydration with simultaneous fading. The conversion of highly coloured species (the flavylium ion and its complex with (+)-catechin) into colourless species (hemiacetal) satisfactorily accounts for the large spectral changes which, in turn, allow an accurate determination of the enthalpy change of copigmentation (Dangles & Brouillard, 1992*b*). This thermodynamic parameter is the true measure of the thermal effect on copigmentation. Its negative sign (exothermic process) points to copigmentation complexes becoming less stable when temperature rises. Here again, solvation effects are expected to play a dominant role. Thus, when water molecules from the anthocyanin solvation shells are expelled by the copigment to the bulk where they can form more hydrogen bonds, stabilization ensues. An increase in temperature is expected to cause partial disruptions in the liquid water structure so that the number of hydrogen bonds per water molecule in the bulk is reduced and the above-described phenomenon is less efficient. In other words, it is the very chemical structure of liquid water which acts in favour of complex formation (hydropho-

bic interaction) and the stronger the cohesion of this unique medium, the stronger the copigmentation interaction. In fact, water acts on a flavylium ion in two very different and fundamental ways: firstly, water solvates the hydrophilic sites (phenolic and glycosylic OH groups) of the flavylium ion (non-covalent hydration) and even reacts at its electrophilic C-2 site (covalent hydration) and, secondly, by the specific nature of its liquid state, water promotes the molecular association of the flavylium ion with a copigment. As the temperature lowers, competition between hydration and copigmentation turns in favour of copigmentation as the 'pressure' exerted by the bulk water becomes stronger and stronger.

One more point is how copigmentation can be detected in red wines. The techniques based on pH changes or dilution effects, that are used for measuring the total concentration of anthocyanins in the wine, are not at all suitable. In fact, one should take advantage of the thermal sensitivity of the copigment effect (Brouillard *et al.*, 1989; Dangles & Brouillard, 1992*b*). A typical experiment may be as follows: divide a red wine sample into two subsamples; bring the first subsample close to the wine boiling point, and the second close to its freezing point; then measure the absorbance of each at 520 nm. Compare the absorbances and the larger the difference, the stronger the copigmentation interaction in that particular wine and vice-versa. If needed, small corrections for temperature-induced pH and volume changes could be made. If taking place, pH variations are not so crucial since, in the acidity domain of wines, copigmentation does not much depart from its maximum. Voluntary dilution should be carefully avoided while light scattering, if it occurs, should be kept at a minimum, thus restricting the measurements to the light absorption phenomenon only. The same type of experiment may be performed with anthocyanin-containing beverages from fruit which all possess some kind of natural or added copigments. In the absence of ethanol (fruit juices, for instance), the difference in the 'cold' and 'hot' absorbances should even be larger (Mazza & Brouillard, 1990). In the last case however, it is not known what the influence is on the copigment effect of the large amount of sugar usually found in soft drinks.

CONCLUSION

Maturation and aging of red wines generally happen at relatively low temperatures, in approximately 1:9 ethanol-to-water molar ratio media and at pH values nearing 3·5. Under such conditions good molecular copigmentation interactions between grape anthocyanins and copigments occur. During the development of wine (fermentations, maturation and aging), typical wine pigments are formed, some of them being of the xanthylium type. Formation of xanthylium pigments is very appealing and more consideration should be given to their chemistry without delay. Our own experimental data on 1,3,6,8-tetrahydroxyxanthylium chloride (colour,

precipitation, self-association, low influence of change in pH...) are all in line with the properties described in the literature for wine pigments formed during red wine aging (Dangles & Brouillard, 1994).

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