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Influence of procyanidin structures on their ability to complex with oenin

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Abstract

Intermolecular copigmentation between malvidin-3-*O*-glucoside (oenin) and several procyanidins of a homogeneous series in a wine model solution (pH 3.6, in hydroalcoholic (12%) citrate–phosphate buffer at a ionic strength of 0.2 M) was studied by UV–Vis spectroscopy. The effects of structural factors and stereochemistry of flavan-3-ol on their ability to interact with oenin were analysed. The presence of a galloyl group in the flavan-3-ol structures increased the strength of copigmentation. The strength of the interaction was not related to the degree of polymerisation for (−)-epicatechin, dimer B2 on trimer C1. Dimers with a C4–C6 interflavonoid linkage associated more strongly with oenin than did their C4–C8 analogues. Above all, the extent of copigmentation was influenced by the conformation of each procyanidin and its ability to establish a hydrogen-bonded network with the water medium.

Keywords: Intermolecular copigmentation; Flavanol; Procyanidins; Malvidin 3-*O*-glucoside; UV–Vis spectroscopy

1. Introduction

Anthocyanins and tannins are responsible for several organoleptic characteristics of food and beverages, such as colour and astringency. Anthocyanins are important natural pigments responsible for a wide range of colours from orange to purple. Depending on the pH of the solution, the coloured flavylium cation co-exists with other forms of anthocyanins, causing the colour to fade quickly. Along with proton transfer reactions, leading to the quinonoidal bases, hydration of flavylium ions gives hemiacetals in equilibrium with small amounts of chalcones (Brouillard & Delaporte, 1977). Important stabilisation processes result in the interaction of anthocyanins between themselves (self-association) or with other chemicals of the medium, such as metal cations (metal complexation) and copigments (copigmentation) (Asen, Stewart, & Norris, 1972; Dangles, 1997; Haslam, 1998; Robinson & Robinson, 1931). Hydration is countered by these phenomena; water molecules are removed from the surface of the chromophore, thus stabilising the colour form. Copigmentation of anthocyanins is extremely important, as it is responsible for the increase in absorbance intensity (hyperchromism) and for a positive shift in the visible wavelength (bathochromism). Besides the anthocyanin and copigment types and their relative concentration, copigmentation is shown to be dependent upon ionic strength, pH, solvent, the presence of metal salts or macrocycles and temperature (Mazza & Brouillard, 1990). Those factors have been studied in the case of fruit-derived products (Mazza & Brouillard, 1987) and wine (Brouillard & Dangles, 1994; Dariaz-Martín, Martín Luis, Carillo-López, Lamuela-Raventós, Díaz-Romero, & Boulton, 2002).

Besides UV–Vis spectroscopy, NMR (Dangles & El hajji, 1994; Goto & Kondo, 1991; Houbiers, Lima,

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Maçanita, & Santos, 1998; Wigand, Dangles, & Brouillard, 1992; Yoshida, Toyama, Kameda, & Kondo, 2000) and fluorescence (Alluis, Pérol, El hajji, & Dangles, 2000; Wigand, Dangles, & Brouillard, 1992) are used to study copigmentation. Most of the copigments studied are polyphenols: phenolic acids (caffeic acid, ferulic acid) (Dimitric Markovic, Petranovic, & Baranac, 2000), chlorogenic acid, flavonols (rutin, quercetin 3-β-D-galactoside) (Davies & Mazza, 1993), hydrolysable tannins (gallic acid, pentagalloylglucose) and purines and pyrimidine (Brouillard, Wigand, Dangles, & Cheminat, 1991). Even though grape constituents have drawn attention within the wine context, few procyanidins (condensed tannins) have been studied in detail (Cai, Lilley, & Haslam, 1990; Escribano-Bail, Santos-Buelga, Francia-Aricha, Rivas-Gonzalo, & Heredia, 1999) and above all monomers (Brouillard et al., 1991; Dariaz-Martín, Carillo, Díaz, & Boulton, 2001; Liao, Cai, & Haslam, 1992).

The aim of this work is to evaluate the influence of procyanidin structural factors on the strength of copigmentation with malvidin 3-*O*-glucoside (oenin), the most abundant pigment of *Vitis vinifera* (together with its acylated derivatives), in order to understand the mechanisms of colour expression in young red wines.

2. Materials and methods

2.1. Sample preparation

Anthocyanins were extracted from grape skins with EtOH/H_2O (1:1 v/v) and malvidin 3-*O*-glucoside was purified according to the experimental conditions described elsewhere (Mateus, Silva, Vercauteren, & De Freitas, 2001; Roggero, Coen, Archier, & Rocheville-Divorne, 1987). Malvidin 3-*O*-glucoside separation from the other components was carried out by Toyopearl HW-40 (Tosohaas®, Germany) column chromatography and purified by HPLC semipreparative (Merck-Hitachi® L-7100) chromatography using a C18 ODS column (250 × 4.6 mm i.d.). The solvents were: (A) H_2O/HCOOH (9:1); (B) CH_3CN/H_2O/HCOOH (3:6:1) gradient elution was from 20% to 85% B for 70 min, 85–100% B for 5 min and then isocratic for 10 min at a flow rate of 1 ml/min. Detection was carried out at 520 nm with a DAD (Merck Hitachi L-7450A).

(−)-Epicatechin and (+)-catechin were purchased from Aldrich®. Procyanidin dimers, B3, B6, B4 and B8, were synthesised (Geissman & Yoshimura, 1966) and the remaining procyanidins B1, B2, B5, B7, trimer C1, and esters epicatechin-gallate and B2-3″-*O*-gallate were extracted from grape seeds from *V. vinifera* species and purified according to the experimental conditions described elsewhere (De Freitas, Glories, Bourgeois, & Vitry, 1998). Its separation from the other components was carried out by Toyopearl HW-40 (Tosohaas®, Germany) column chromatography and purified by HPLC semipreparative (Merck-Hitachi® L-7100) using two connected columns Ultrasphere C18 ODS (250 × 4.6 mm i.d.) protected with a guard column packed with the same packing material. The elution system consisted of two solvents, A: 2.5% HOAc in H_2O, B: 80% CH_3CN in A and the following gradients; elution starting with 7% B in A, isocratic for 5 min; 7–20% B in A, 5–90 min; 20–100% B in A, 90–95 min; 100% B, 95–100 min (isocratic), followed by washing (100% B over 10 min) and reconditioning of the column (100–7% B in A over 5 min). Detection was conducted with a DAD (Merck Hitachi L-7450A) at 280 nm. The retention times (R_t, min) of the studied flavanols were the following: (+)-catechin, 29.5; (−)-epicatechin, 48.6; (−)-epicatechin gallate, 74.7; dimers B1, 21.8; B2, 37.7; B3, 23.4; B4, 32.2; B5, 83.7; B6, 39.9; B7, 59.3; B8, 47.0; B2-gallate, 52.5; trimer C1, 50.8. All pigments and copigments were identified by comparison with authentic standards by HPLC. The purity of the obtained compounds was assessed by HPLC-DAD (Merck-Hitachi® L-7450A). These compounds were protected from light during purification and, after freeze-drying, were stored at −18 °C, under argon, until used.

2.2. Copigmentation

All solutions used were prepared in an hydroalcoholic citrate–phosphate buffer solution (12%) at pH 3.6, and the ionic strength was adjusted to 0.2 M by addition of sodium chloride.

Each pigment/copigment solution was prepared by mixing equal volumes of oenin (10^{-4} M) solution and procyanidin solution and citrate–phosphate buffer solution at pH 3.6, corresponding to the adequate pigment to copigment ratio. Typical pigment to copigment ratios were 1:0–1:40. The spectrum of the pigment/copigment mixture was recorded after 30 min and 3 h. Practically no difference was found in the UV–Vis spectrum between 30 min and 3 h. Data reported herein are those recorded after 30 min.

2.3. UV–Vis absorption spectra

Spectrophotometric measurements of oenin solution, with and without copigment, were recorded with a UV–Vis spectrophotometer fitted with a plastic cell (optical path $d = 1$ cm, volume 1 ml). A constant temperature of 22 °C was obtained by use of a water-thermostated bath. The reference solutions in spectrophotometric measurements were pure buffer solution. The methodology of recording the absorbance in the visible range from 360 to 830 nm with a 2 nm slit width, a 1 nm sampling interval and at a medium scan speed was the same in all measurements.

3. Results and discussion

3.1. General

UV–Vis spectroscopic investigations of the homogeneous series of procyanidins, differing in their monomeric constituents ((+)-catechin and (−)-epicatechin), interflavanoid bond linkage (C4–C8 and C4–C6), degree of polymerisation and gallic acid esterification were performed in order to clarify the role of these structural features in the copigmentation effect with oenin (Fig. 1).

The interaction study between oenin (10^{-4} M) and procyanidin (molar ratio 1:5–1:40) was conducted in hydroalcoholic (12%) citrate–phosphate buffer at a ionic strength of 0.2 M at pH 3.6. Even though ethanol has been shown to induce a large reduction in the copigment effect (Dangles & Brouillard, 1992), its inclusion in the experimental conditions described herein attempts to mimic red wine composition. In weakly acidic conditions the hemiacetal and flavylium forms of oenin predominate and quinonoidal bases are only present to a very small extent (Brouillard & Delaporte, 1977).

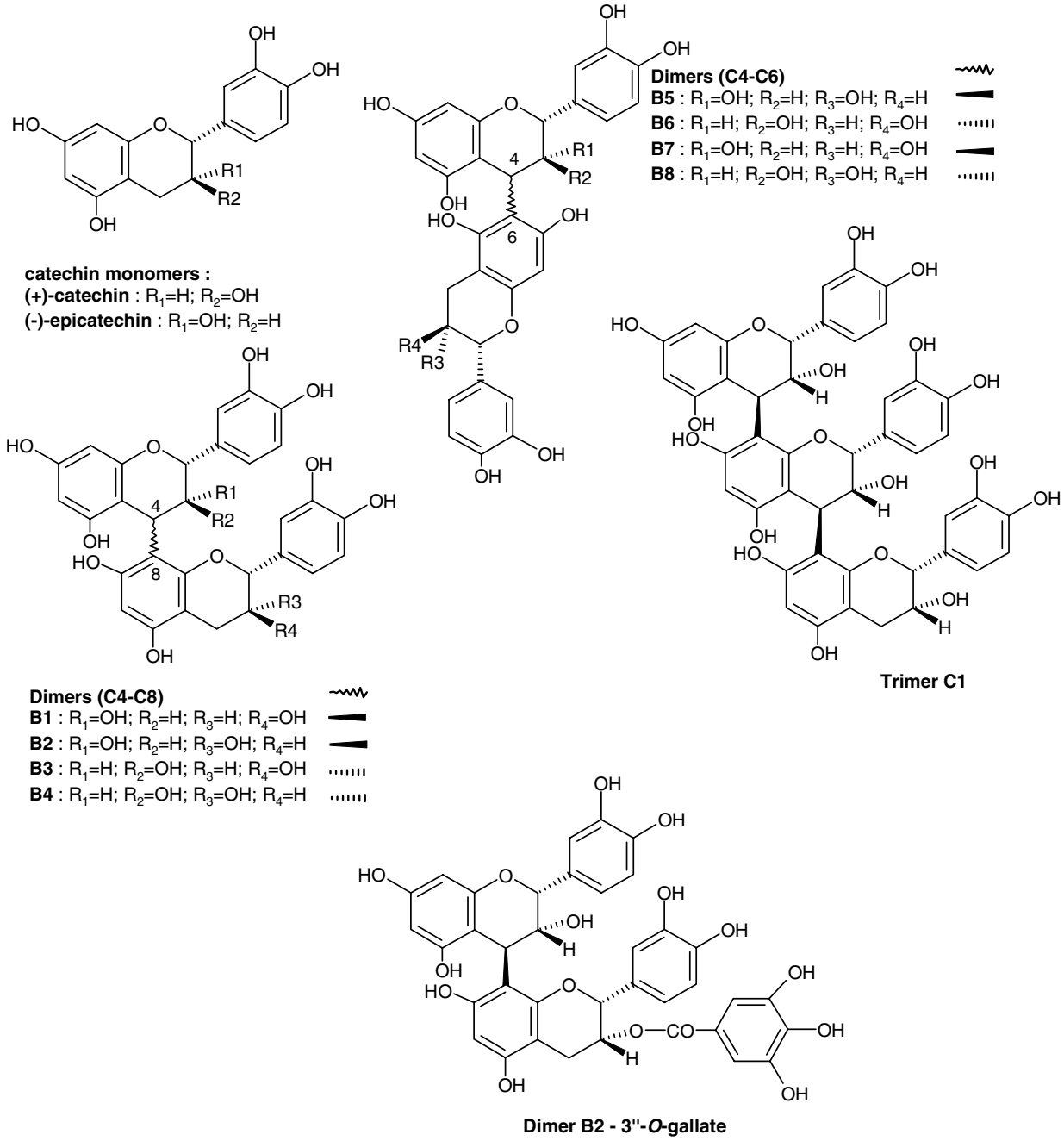

Fig. 1. Chemical structures of catechin monomers and procyanidins.

3.2. Copigmentation of the anthocyanins species

For all copigments tested, the spectra of copigmented malvidin 3-*O*-glucoside solution display an increased absorptivity (hyperchromic effect), the maximum effect observed occurring at the highest molecular ratio. Absorption spectra of oenin and the copigments (−)-epicatechin-3-*O*-gallate and dimer B6, for different pigment to co-pigment ratios (from 1:0 to 1:40), are shown in Fig. 2.

No new spectral bands appear in the spectrum recorded at 30 min and 3 h. Thus only non-covalent interaction of oenin and procyanidin seems to take place. The absence of formation of new adduct was checked by HPLC. Despite secondary structures of anthocyanin being shown to play a role (Dangles & El hajji, 1994; Mistry, Cai, Lilley, & Haslam, 1991) in the mechanism of copigmentation, there is no evidence of modification of the absorption near 350 nm characteristic of chalcone, nor the shoulder around 610 nm, characteristic of the quinonoidal bases. Therefore the complexation with chalcones and quinonoidal bases does not occur in this case.

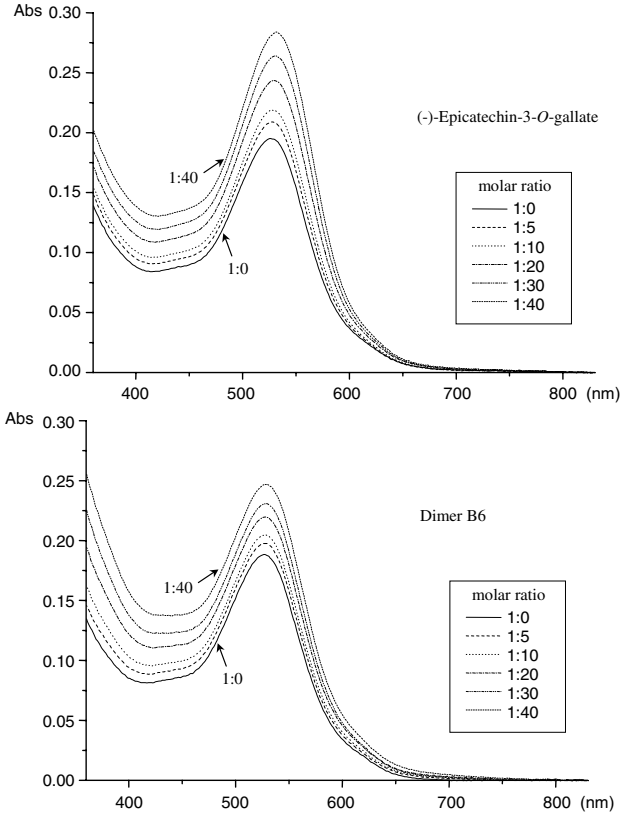

Fig. 2. Visible spectra of the solution after addition of epicatechin-3-*O*-gallate (above) and dimer B6 (below) for different pigment/copigment molar ratios (1:0, 1:5, 1:10, 1:20, 1:30, 1:40). Concentration of oenin, 10^{-4} M; pH 3.6, in hydroalcoholic (12%) citrate–phosphate buffer; ionic strength, 0.2 M.

The bathochromic effect is seen only for monomers ((−)-epicatechin, (−)-epicatechin gallate and (+)-catechin), the maximum shift observed is from 527 to 532 nm for (−)-epicatechin (data not shown).

The graphics of the percentage of increase in absorptivity $(A - A_0)/A_0$ as a function of pigment/copigment molar ratio (5–40) are linear and well correlated (Fig. 3). The slopes of these regressions (Cp) provide

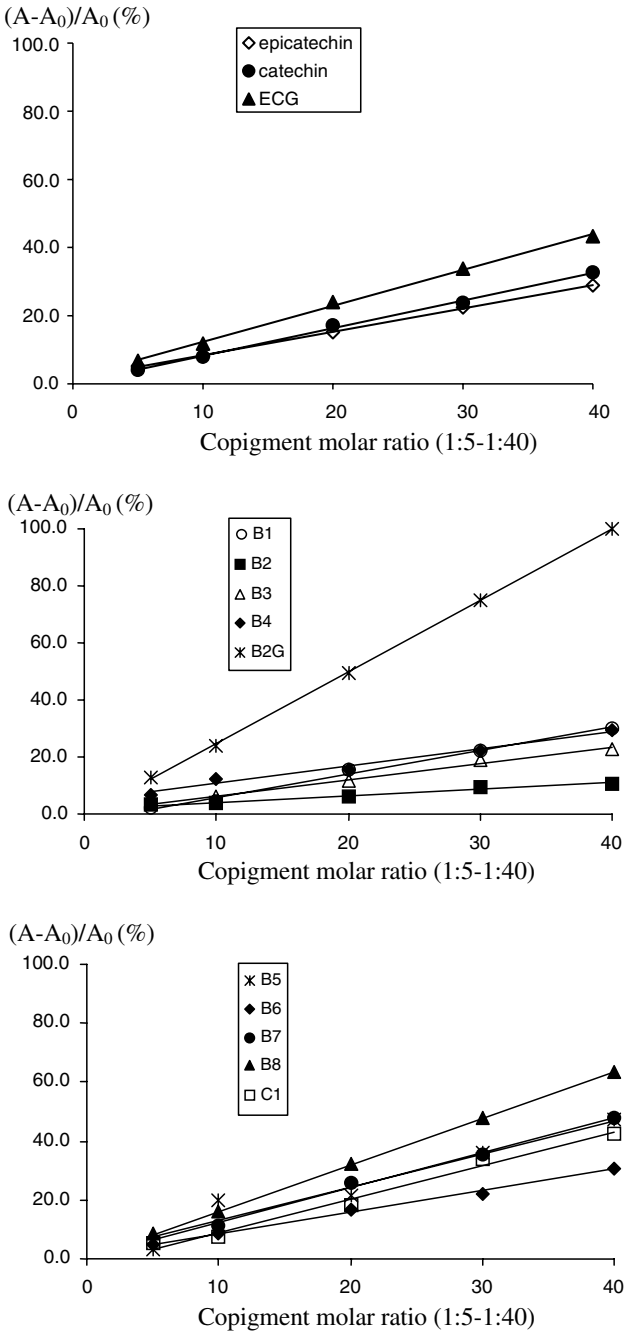

Fig. 3. Plot of the increase in absorptivity $(A - A_0)/A_0$ vs pigment/copigment molar ratio of 1:5–1:40 at λ 525 nm. A_0, absorbance of the oenin solution at λ 525; A, absorbance after addition of procyanidin at λ 525.

Table 1
Copigmentation strength values (Cp) of flavan-3-ol with oenin

Flavan-3-ol	Cp
Monomers	
(+)-catechin	0.81
(−)-epicatechin	0.68
epicatechin gallate	1.05
Dimers C4–C8	
B1	0.82
B2	0.23
B3	0.56
B4	0.60
B2-gallate	2.51
Dimers C4–C6	
B5	1.13
B6	0.72
B7	1.19
B8	1.58
Trimer C1	1.13

important information on the strength of the copigmentation and depend on the copigment (Table 1). These constants were determined for λ 525 nm; at this wavelength no interference in the pigment absorption by a copigment or a cosolvent occurs.

3.3. Influence of molecular structure of procyanidins on the complex strength

The values of Cp found for (+)-catechin are higher than the values for (−)-epicatechin, 0.81 and 0.68, respectively. This result shows the effects of small changes in the conformational structures of these copigments (stereochemistry of C-3 carbon) in their ability to complex with oenin. Copigmentation studies of malvin, which has two glycosyl residues, have shown the predominant pseudo-equatorial position of the catechol ring B for (−)-epicatechin, allowing a more efficient π–π overlap of aromatic groups, comparatively to (+)-catechin in which the axial conformer is a significant one (Brouillard, Wigand, Dangles, & Cheminat, 1991). This conformational feature of catechin monomers does not affect their interaction with oenin in the same way as it affects malvin.

The Cp values found for monomer, dimer and trimer were not proportional to the degree of polymerisation. Although the number of aromatic rings increases proportionally with the number of units, which could result in more groups available to interact, the increase in molecular size seems to impose some conformational restraints in the copigmentation phenomenon. For C4–C8 homologue polymers of (−)-epicatechin studied, represented by dimer B2 and trimer C1, although the Cp value found for the monomer (0.68) is lower than the value measured for trimer C1 (1.13), these constants are above that found for dimer B2 (0.23).

The strength of the association is smaller for procyanidin dimers with a C4–C8 interflavonoid linkage (except for procyanidin dimer B1) than those with a C4–C6 bond. This is presumably due to greater proximity of the upper and lower units for dimers with a C4–C8 interflavonoid bond, resulting in restricted accessibility for the pigment. This is visible for dimer B8 (1.58) and its C4–C8 analogue, dimer B4 (0.60). In fact, previous molecular studies concerning the estimation of the flavan-3-ol conformation in solution, using molecular mechanics and NMR techniques, showed that C4–C6 dimers possess a more flexible and open conformational structure than the respective C4–C8 dimers (Fig. 4) (De Freitas, Glories, & Laguerre, 1998). The conformation of dimer B6 showed an extended conformer where as the conformation of dimer B2 was more compact. This shows a particular conformational feature of C4–C6 dimers,

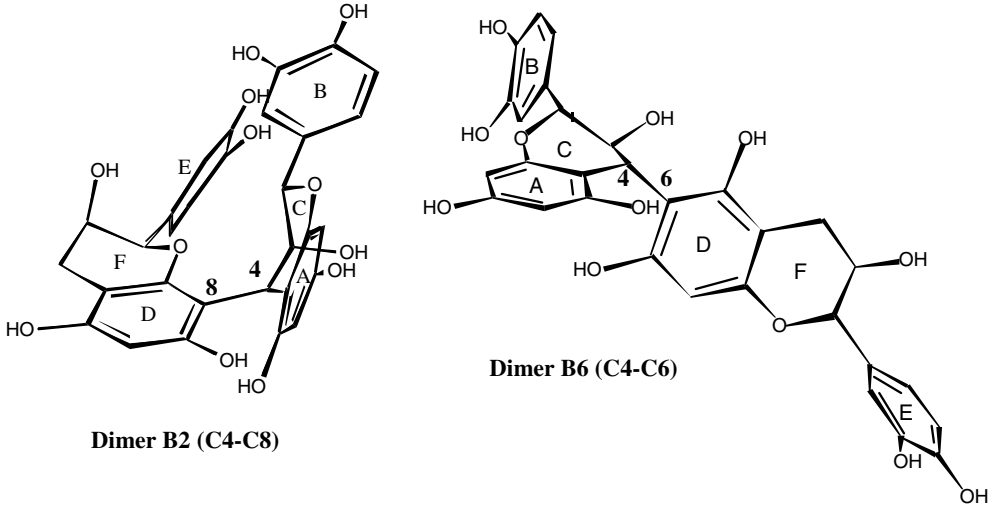

Fig. 4. Preferred conformations of dimers B2 and B6, determined using molecular mechanics using Allinger's MM2* force field parameters (De Freitas et al., 1998).

which could be related to their higher ability to complex with oenin. Dimer B8 was by far the most efficient non-galloylated copigment found in these experimental conditions. For the C4–C8 dimers, the values of Cp found for dimers B3 (0.56) and B4 (0.60) are similar and above that corresponding to dimer B2 (0.23). These results are in agreement with the increase in absorbtivity $(A - A_0)$ promoted by these compounds in their copimentation effect with malvin (Mistry, Cai, Lilley, & Haslam, 1991).

The formation of the weak non-covalent anthocyanin-copigment complex is generally assumed to involve hydrophobic π–π stacking interactions of aromatic groups. The shape adopted by copigments could importantly affect their interaction with anthocyanin.

3.4. Influence of esterification with gallic acid

The values of Cp show that the association is more important for (−)-epicatechin-*O*-gallate than for (−)-epicatechin. Moreover, the strength of the interaction is strongly enhanced for dimer B2-3″-*O*-gallate (B2G) compared to its analogue dimer B2, 2.51 and 0.23 respectively. In Fig. 3, the increase of absorptivity $(A - A_0)$ of oenin/B2-3″-*O*-gallate solution (molar ratio of 1:40) is near of 100%, i.e., the absorbance at 520 nm was practically twice that of the solution without copigment. The copigmentation effect is increased by esterification of the C-3 hydroxyl function by gallic acid, as was previously proposed (Liao, Cai, & Haslam, 1992; Mistry, Cai, Lilley, & Haslam, 1991).

Molecular studies have shown a possible π–π stacking arrangement between the aromatic gallate and catechol rings of dimer B2-3″-*O*-gallate, (Fig. 5) (De Freitas, Glories, & Laguerre, 1998). The substantial enhancement of copigmentation in the case the dimer B2-3″-*O*-gallate compared to its analogue dimer B2 could be explained by the presence of the well exposed planar π–π systems forming a pocket into which the anthocyanin may intercalate (sandwich-type complex), thereby offering an important interaction, (Fig. 5). These results indicate the importance of gallic acid esterification of procyanidins in their ability to interact with anthocyanins. Some studies have reported that condensed tannins, procyanidin dimer B2 and (−)-epicatechin have a low copigmentation effect with malvin and cyanin at a pH value of 3.65 (acetate buffer solution) and room temperature in comparison with hydrolysable tannins, due to the lack of esterification by gallic acid (Mistry, Cai, Lilley, & Haslam, 1991). The presence of galloyl groups influences the copigmentation phenomenon as has been described for interactions with proteins. This is a general feature of tannin interaction (De Freitas & Mateus, 2001).

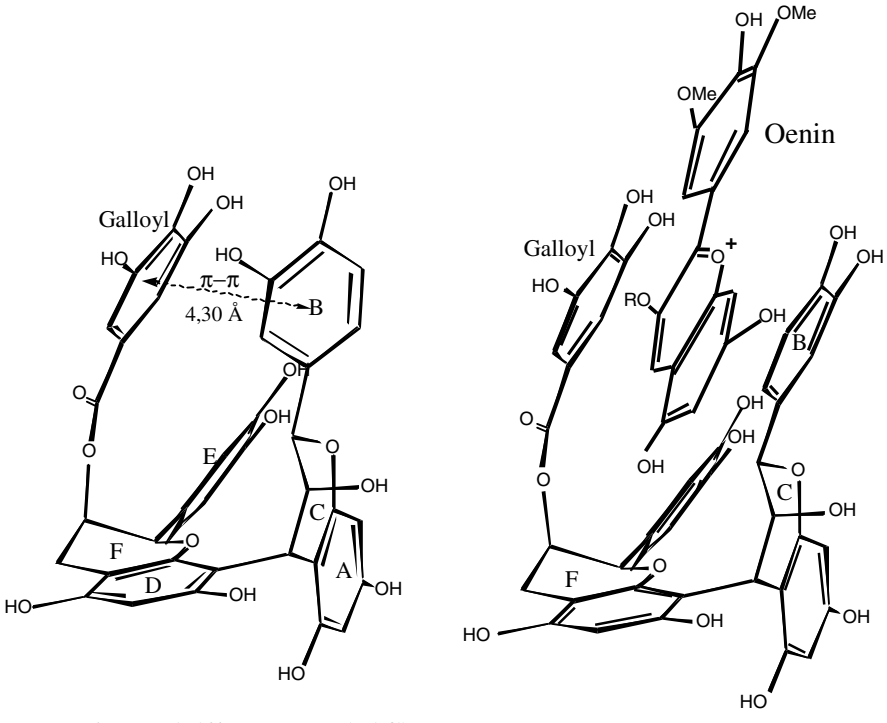

Fig. 5. Preferred conformation of dimer B2-3″-*O*-gallate determined by molecular mechanics using Allinger's MM2* force field parameters (De Freitas et al., 1998). Suggested conformational arrangements of the oenin intercalated between the galloyl ester group and catechol ring B of the dimer B2-3″-*O*-gallate (B2G).

4. Conclusion

Differences of dimer association strength with oenin appear to be related to combinations of their stereoisomeric differences, such as asymmetries of the side group of pyranic rings (2,3-*cis* or -*trans*), type of interflavonoid bond, stereochemistry of the interflavonoid bond, rather than to a particular structural factor. Those different factors may result in the establishment of a special hydrogen-bonded network between water and procyanidins, due to the peculiar projections of the pyran hydroxyl and the phenyl hydroxyl at the periphery of the molecule. Furthermore, existence of different conformers has been described for procyanidin dimers (De Freitas, Glories, & Laguerre, 1998; Hatano & Hemingway, 1997). Thus each procyanidin, with it own conformation, may modify the water network in favour of a more or less important interaction with oenin. Even though procyanidins show a reduced planar structure, they interact with malvidin 3-*O*-glucoside. NMR studies and more especially NOE measurements, should be done to precisely assess their interaction sites.

In the food industry, not only wine, but also several vegetable-and-fruit based beverages are affected by copigmentation that controls colour stabilisation. Therefore it would be interesting to analyse the effect of procyanidin copigmentation on colour variation in terms of hue, lightness and saturation.

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