



# Spectrophotometric study of the copigmentation of malvidin 3-O-glucoside with *p*-coumaric, vanillic and syringic acids



Naim Malaj\*, Bruna Clara De Simone, Angelo Domenico Quartarolo, Nino Russo

Dipartimento di Chimica e Tecnologie Chimiche, Università della Calabria, Ponte Pietro Bucci Cubo 14/C, I-87036 Arcavacata di Rende (CS), Italy

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## ABSTRACT

Anthocyanins are a natural source of pigments in plants and their processed food products have become attractive and excellent candidates to replace the synthetic colourants due to their characteristic intense colours and associated health benefits. The intermolecular copigmentation between anthocyanins and other colourless compounds has been reported to be an important way to enhance and stabilise the colour intensity of aqueous solutions. In the present work we report the equilibrium constant, stoichiometric ratio and the thermodynamic parameters ( $\Delta G^\circ$ ,  $\Delta H^\circ$  and  $\Delta S^\circ$ ) related to the intermolecular copigmentation reactions of the anthocyanin malvidin 3-O-glucoside with one hydroxycinnamic acid (*p*-coumaric acid) and two O-methylated hydroxybenzoic acids (vanillic and syringic acid). Different factors which affect their interactions such as copigment concentration, pH and temperature of the medium are examined at two pH levels (pH = 2.50 and 3.65) corresponding to those of the major food mediums where these reactions take place (fruit juices, wine, jams etc.).

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## 1. Introduction

Anthocyanins are a class of plant secondary metabolites known as a source of colours in plant tissues and their derivatives. Due to their characteristic intense colours and related health promoting benefits (Duthie et al., 2006; He & Giusti, 2010; Pascual-Teresa & Sanchez-Ballesta, 2007; Qin et al., 2009; Sancho & Pastore, 2012; Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010) anthocyanins have become attractive as natural food colourants (Boulton, 2001; Bridle & Timberlake, 1997; Brouillard & Dangles, 1994; Francis, 1989; Pazmiño-Durán, Giusti, Wrolstad, & Glória, 2001; Wallace & Giusti, 2008) to substitute the synthetic ones in processed food products (juice, wine, jam etc.). They are usually water-soluble, which facilitates their extraction (mainly from grapes, berries, apples, radishes, red cabbage, tulips, roses, orchids etc.) and incorporation into water mediums of food interest. However, the wide use of anthocyanins as food dyes has encountered difficulties related to the stability of the colour in different food matrices. In fact, anthocyanins are compounds with relatively low stability and their susceptibility to degradation is influenced by factors such as temperature, light, pH of the medium where they are found, solvents, oxygen, self-association interactions and their reactions of coordination with metallic ions or with other colourless compounds (copigmentation) (Buckow, Kastell,

Terefe, & Versteeg, 2010; Howard, Castrodale, Brownmiller, & Maurouostakos, 2010; Jiménez et al., 2010; Malien-Aubert, Dangles, & Amiot, 2001; Turker, Aksay, & Ekiz, 2004). These last reactions, namely known as copigmentation reactions, can occur through two mechanisms: (a) intramolecular interactions in which the pigment (anthocyanin) is bound covalently to the copigment, usually via acylation reactions and (b) intermolecular interactions in which the pigment and the copigment interact via weak  $\pi$ - $\pi$  interactions. This last mechanism has been indicated as one of the major factors that affects the colour stability in wines and fruit juices and is further examined in the present work.

The copigmentation effect is manifested by an enhancement of colour intensity of the solution and a change of colour tonality when an aqueous solution of pigment (anthocyanin), in slightly acidic mediums, is added to a colourless copigment. The chemical structure of anthocyanins changes depending on the pH of the water solutions. In acidic mediums anthocyanin pigments exist principally in equilibrium between the following species (Scheme 1, Supplementary material): as coloured flavylum cation  $[AH^+]$  at low pHs (pH < 3), as colourless pseudobase or carbinol pseudobase  $[B]$  in tautomerism equilibrium with pale yellow *cis*-chalcone  $[C_E]$  and *trans*-chalcone  $[C_Z]$  forms, and as quinoidal bases  $[A]$  at higher pHs (pH > 3) (Brouillard, Mazza, Saad, Albrecht-Gary, & Cheminatt, 1989). There are still controversies about which of the above species is responsible for the copigmentation reactions, the role of each of them in reactions of copigmentation and the mechanism of the interactions pigment-copigment. However, most of the authors

\* Corresponding author. Tel.: +39 0984 493341; fax: +39 0984492044.

E-mail address: [nmalaj@unical.it](mailto:nmalaj@unical.it) (N. Malaj).

attributed the enhancement of the colour of the solutions to the interactions between the flavylum cation and copigments (Baranac, Petranović, & Dimitrić-Marković, 1996; Brouillard et al., 1989; Dimitrić-Marković, Petranović, & Baranac, 2000; Mazza & Brouillard, 1990). These interactions have been indicated to be the origin of the stabilisation of the flavylum ion and the origin of the changes of colour tendency and intensity, which are accompanied with increases of absorbance in the visible range (hyperchromic shift) and a shift of the wavelength of the maximum absorbance toward higher values (bathochromic shift), therefore both detectable spectrophotometrically (Dangles & Brouillard, 1992).

Since this phenomenon has been observed by Willstätter & Zolinger (1917), extensive investigations have been performed using basically qualitative approaches (Robinson & Robinson, 1931). Brouillard and coworkers (1989) introduced a spectrophotometric method to quantify the extent of the copigmentation effect, proposed the mechanism related to the reactions of copigmentation between malvin and chlorogenic acid and evidenced the factors that affect it (Brouillard et al., 1989; Mazza & Brouillard, 1990). Brouillard's approach has been used by several research groups to study both qualitatively and quantitatively the copigmentation processes between anthocyanins and different classes of natural compounds (Baranac, Petranović, & Dimitrić-Marković, 1997a, 1997b, 1997c; Baranac et al., 1996; Dimitrić-Marković, Petranović, & Baranac, 2005; Dimitrić-Marković et al., 2000; Lambert, Asenstorfer, Williamson, Iland, & Jones, 2011) evidencing the role of different factors (concentration, pH, temperature, solvent) that affect the copigmentation process.

The stabilisation of anthocyanins in different formulations during processing procedures and storage conditions is still a challenge for their wide potential applications as alternative food colourants with beneficial health effects. This is an important topic since the colouration of food products represents one of the main features for product acceptance.

In the present work we report the results of the intermolecular copigmentation between the pigment oenin (malvidin 3-O-glucoside) and the copigments *p*-coumaric, vanillic and syringic acids (Chart 1, Supplementary material) in order to highlight the role of such phenomenon on the colouration of food mediums and to evidence the factors which affect it. The pigment and the copigments involved in this study represent the main constituents of their classes in several fruits, vegetables and their processed derivatives (Castillo-Muñoz, Fernández-González, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez, 2009; He et al., 2012; Lorenzo, Pardo, Zalacain, Alonso, & Salinas, 2005). The principal factors which affect their interactions such as copigment concentration, pH and temperature were examined and the equilibrium constant, stoichiometric ratio together with the thermodynamic parameters related to each copigmentation reaction were determined spectrophotometrically. All the copigmentation reactions were examined at pH values similar to those of fruit juices (apple, cranberry, blueberry, pear, citrus juices etc.), jams and wine, therefore the data reported may be useful to industries which investigate new strategies to ameliorate their food products.

## 2. Materials and methods

Oenin chloride was purchased from Extrasynthese (Extrasynthese, Genay Cedex, France), while *p*-coumaric, vanillic and syringic acids were purchased from Sigma–Aldrich (Milan, Italy). Phosphoric acid 85%, hydrochloric acid 37%, sodium hydroxide, potassium chloride, methanol and acetic acid were purchased from Carlo Erba (Milan, Italy). The experiments were carried out following the method of Brouillard et al. (1989) with a few modifications.

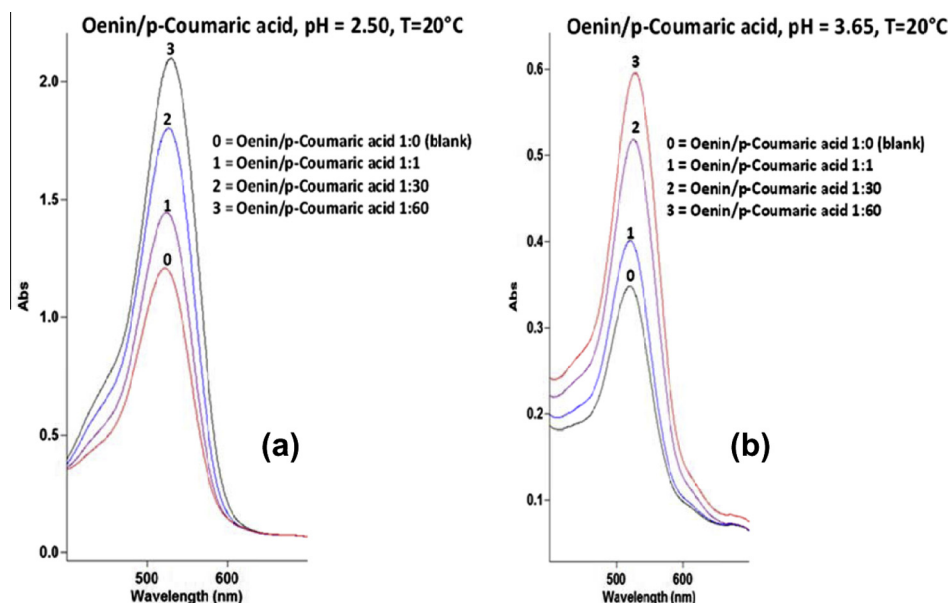
The solution of pigment was prepared at a double concentration of that of analysis ( $2 \times 10^{-4}$  M) by dissolving oenin in aqueous solution of phosphoric acid 0.06 M. The copigments (*p*-coumaric, vanillic and syringic acids), due to their low solubility in water, were first dissolved in methanol (8% v/v<sub>total</sub>) and then diluted with a buffer solution of CH<sub>3</sub>COONa 0.02 M to the desired working concentrations. The ionic strength was adjusted to 0.02 M by adding KCl. The copigmentation process was investigated at pH = 2.50 and 3.65. The pH was measured by a laboratory micro-processor pH meter (HI8521, Hanna Instruments Inc., Woonsocket, RI 02895 USA) and was adjusted to the selected values by adding a few microliters of 10 M HCl or 10 M NaOH. The UV/Vis spectra were acquired with a Varian Cary® 50 UV/Vis Spectrophotometer (Agilent Technologies, Milan, Italy) equipped with a temperature probe accessory and using quartz cuvette with 1 cm length-path. Blank solution, composed of oenin solution (0.5 ml) and the buffer solution (0.5 ml) at the working pigment concentration of  $1 \times 10^{-4}$  M, was registered prior to sample analysis. Then, the solution of pigment (0.5 ml) was mixed with those of copigments (0.5 ml) at increasing concentrations (pigment/copigment molar ratio 1:1, 1:10, 1:30 and 1:60) and stirred in dark for 20 min prior to analysis. Two main experiments were performed:

- (i) Determination of the magnitude of the hyperchromic shift in function of copigment concentration: in this group of experiments the hyperchromic shift was monitored by increasing the molar ratio of copigments while keeping constant that of the pigment and following the variation of absorbance at  $\lambda = 520$  nm, where pure oenin in buffered solution shows its maximum absorbance ( $\lambda_{\text{max}}$ ); this group of experiments allowed the determination of the equilibrium constant (*K*), Gibbs energy variation ( $\Delta G^\circ$ ) and the stoichiometry ratio of the copigmentation reactions involved. Moreover, to register the magnitude of the bathochromic shift in function of copigment concentration, the spectra were registered in the range 400–700 nm and the repositioning of the maximum wavelength absorbance ( $\lambda_{\text{max}}$ ) with respect to blank solution was followed.
- (ii) The behaviour of each couple of pigment/copigment complex was examined, at  $\lambda = 520$  nm, as a function of temperature increase allowing us to determine the variation of enthalpy and entropy associated to the copigmentation reactions involved; in addition, for a visual evaluation of the effect of temperature increase on the copigmentation reactions, their absorption spectra were acquired in the range of 400–700 nm.

## 3. Results and discussion

The copigments involved in the present study show a maximum absorbance in the UV range 200–320 nm ( $\lambda_{\text{max}} \sim 280$  nm), where absorbs also the pigment oenin. Contrary, for copigments no absorption is observed in the visible range 400–700 nm where the absorption of the cationic form of pigment oenin is maximum ( $\lambda_{\text{max}} = 520$  nm) (Dimitrić-Marković et al., 2000). Therefore, it is appropriate to monitor the intermolecular copigmentation between oenin and copigments involved in this latter range (400–700 nm).

Thus, in the first group of experiments (i) the concentration of oenin was kept constant ( $1 \times 10^{-4}$  M) and its copigmentation with increasing amounts of copigments involved (oenin/copigments molar ratio 1:1, 1:10, 1:30 and 1:60) was examined at both pH values. To evaluate the magnitude of the hyperchromic shift, the absorbance of the mixtures of oenin with increasing molar ratio of copigments was registered consecutively at  $\lambda = 520$  nm, while the bathochromic shift ( $\Delta\lambda_{\text{max}}$ ) was monitored by registering the



**Fig. 1.** The intermolecular copigmentation effect is manifested by the hyperchromic and bathochromic shifts observed at both pH values examined. The spectra shown display the change of absorption (400–700 nm) of the flavylium form of oenin as a function of increasing amounts of copigment *p*-coumaric acid at (a) pH = 2.5 and (b) at pH = 3.65. The spectra regarding vanillic and syringic acids are reported in Fig. S1 of the Supplementary material.

spectra in the range of 400–700 nm. In Fig. 1 the absorption spectra (400–700 nm) of oenin and *p*-coumaric acid are shown at pH = 2.50 and 3.65 while the hyperchromic values for all the reactions are summarised in Table 1 (the absorption spectra for oenin/vanillic acid and oenin/syringic acid are reported as Supplementary material, Fig. S1).

The entity  $(A - A_0)/A_0$  was used in Table 1, as proposed by Brouillard and coworkers (1989), as the main experimental parameter to measure the magnitude of the interactions in copigmentation reactions. The addition of increasing concentrations of copigments to a constant concentration of oenin resulted, as can be observed in Table 1 and visualised in Fig. 1 (see also Fig. S1, Supplementary material), in a continuous increase of absorbance (hyperchromic effect) at  $\lambda = 520$  nm, at both pH values. This clearly indicates the pigment–copigment intermolecular interaction, that is the copigmentation between oenin and copigments, and its important role in the colouration of solutions and matrices when they are found together. Observing the values of  $(A - A_0)/A_0$  in Table 1 it is evident that the hyperchromic shift is larger for higher molar ratios of copigments, indicating the dependence of the reactions of copigmentation on their concentration. Concerning the pH dependence, the hyperchromic effect is stronger at pH 3.65 than at 2.50, at all molar ratios examined, in agreement with previous observations (Mazza & Brouillard 1990; Davies & Mazza, 1993; Dimitrić-Marković et al., 2000). The spectra for all the copigmentation reactions at pH 3.65 (Fig. 1(b) and in Supplementary material

Fig. S1(b, d)) show, besides the main hyperchromic shift around  $\lambda = 520$  nm, a tiny shoulder at  $\sim 610$  nm, which is absent at pH 2.50. This shoulder has been observed also by others (Brouillard et al., 1989; Dimitrić-Marković et al., 2000) and used as indicator of the presence of the quinoidal bases with which our copigments interact as well (note the hyperchromic effect with increasing concentration of copigments).

In addition to the hyperchromic effect, increasing the concentrations of copigments led also to a shift of the maximum wavelength absorbance ( $\lambda_{\max}$ ) toward higher values of the spectrum (bathochromic shift,  $\Delta\lambda_{\max} = \lambda_{\max} - \lambda_{520}$ ). As can be observed in Fig. 1 (and Fig. S1, Supplementary material), the extent of the bathochromic shift increases with the increment of the molar ratio of organic acids included in this study (see Table S1 in Supplementary material for the shift values). In fact, the highest values of bathochromic shifts are registered for complexes 1:60, at both pH values investigated. The values of the bathochromic shift resulted higher almost for all complexes at pH 3.65 than at pH 2.50, indicating that the magnitude of the bathochromic shift is influenced also by the pH of the medium.

Evaluating the extent of the hyper- and bathochromic shift in Table 1 and Table S1 (Supplementary material), respectively, it is evident that at all molar ratios and at both pH values, syringic acid showed the highest values followed by vanillic acid and *p*-coumaric acid. If we compare the hyper- and bathochromic shifts observed for the hydroxybenzoic acids, the di-O-methylated one (syringic acid) resulted in a slightly more efficient copigment than the mono-O-methylated one (vanillic acid), indicating a direct correlation of the copigmentation effect with the degree of O-methylation of the hydroxybenzoic acids. Moreover, both methoxylated hydroxybenzoic acids showed a higher contribution to colour enhancement with respect to the hydroxycinnamic acid (*p*-coumaric acid). These findings are in accordance with similar studies which evidenced a stronger copigmentation effect for methoxylated acids than those with hydroxyl substituents (Dimitrić-Marković et al., 2000; Sun, Cao, Bai, Liao, & Hu, 2010).

As mentioned in the introduction section, there are still disagreements about which of the pigment species is responsible for the above hyperchromic and bathochromic effects and what is

**Table 1**

Values of hyperchromic shift, reported as  $(A - A_0)/A_0$ , due to the copigmentation of oenin with increasing molar ratios of *p*-coumaric, vanillic and syringic acids in buffered solutions (at pH = 2.50 and 3.65) acquired at 520 nm.

Copigmentation	pH	$(A - A_0)/A_0 (\lambda_{520})$			
		1:1	1:10	1:30	1:60
Oenin/ <i>p</i> -coumaric acid	2.50	0.0106	0.0619	0.1406	0.2039
	3.65	0.0403	0.1685	0.3564	0.5862
Oenin/vanillic acid	2.50	0.0147	0.0870	0.1748	0.2942
	3.65	0.0674	0.3124	0.6542	1.2242
Oenin/syringic acid	2.50	0.0148	0.0907	0.1766	0.2986
	3.65	0.0860	0.4238	0.9389	1.3801

the role of the other species in the copigmentation reactions. It is generally accepted that at low pH ( $\text{pH} < 3$ ), where flavylum cation is predominant, the hyperchromic and bathochromic effects observed are due to the interactions of the flavylum cation with copigments. The results presented here are a further confirmation of the above attribution (see Tables 1 and S1 and Fig. 1 and S1 for reactions at  $\text{pH} = 2.50$ ). As the pH raises to 3.65, the concentration of the flavylum cation decreases due to its hydration, which produces the colourless carbinol pseudobase (supported by the reduced absorbance of “curves 0” in Fig. 1 and Fig. S1 in Supplementary material), but the hyperchromic and bathochromic effects found are even stronger, in agreement with previously reported data (Brouillard et al., 1989; Dimitrić-Marković et al., 2000; Mazza & Brouillard., 1990).

Furthermore, the evaluation of the magnitude of the hyperchromic shift induced by the addition of increasing molar ratios of copigments to oenin solution and registering consecutively the variation of absorbance at  $\lambda = 520$  nm, allowed to calculate the equilibrium constant for the process of copigmentation using the method described by Brouillard et al. (1989), according to the following equation:

$$\ln[(A - A_0)/A_0] = \ln(K) + n \ln[C_p]_0$$

where  $A_0$  is the absorbance of oenin solution ( $\lambda_{520}$ ) in absence of copigment (absorbance of blank solution),  $A$  is the absorbance of copigmentation mixture (oenin/copigment) at  $\lambda_{520}$  (absorbance of sample),  $K$  is the equilibrium constant of the reaction,  $n$  is the stoichiometric ratio between oenin and copigment involved and  $[C_p]_0$  is the copigment concentration.

According to this method, a straight curve is obtained with slope  $n$  and intercept  $\ln K$  when plotting  $\ln[(A - A_0)/A_0]$  vs  $\ln[C_p]_0$ . In Fig. 2 are reported such plots for each pigment/copigment reaction (built using the data  $(A - A_0)/A_0$  reported in Table 1). From Fig. 2 we can read the values of  $\ln K$  (intercepts) and the stoichiometric ratios  $n$  (slopes) related to each reaction. Thus, the determination of the value of equilibrium constant ( $\ln K$ ) allowed to calculate the variation of Gibbs free energy for the copigmentation process:  $\Delta G^\circ = -RT \ln K$ . In Table 2 the values of  $K$  and  $\Delta G^\circ$  accompanying each copigmentation reaction are shown. In order to standardise the results the thermodynamic parameters were calculated at  $T = 298$  K.

The values of the equilibrium constant obtained, which are an important indicator of the strength of the association pigment–copigment, display a strong dependence on the pH of the medium. As shown in Table 2, the values of  $K$  obtained for the reactions investigated indicate that the interactions pigment–copigment are more favorable at pH 3.65. In fact, the calculated  $\Delta G^\circ$  values are lower at pH 3.65 than at pH 2.50 for the same copigmentation couple. However, all  $\Delta G^\circ$  values are negative suggesting the spontaneous nature of the process of copigmentation between oenin and the copigments involved and indicating syringic acid as the most efficient compigment followed by vanillic acid and *p*-coumaric acid.

The method used in the present work implies that the plot of  $\ln[(A - A_0)/A_0]$  vs  $\ln[C_p]_0$  provides a straight line with a slope ( $n$ ) close to unity denoting the association of one molecule of pigment with one of copigment. In our experiments the stoichiometric ratios ( $n$ ) found (Table 2) at both pH values were lower than the unity but, in accordance with the previous literature data, can be considered approximately 1:1 (Lambert et al., 2011; Mazza & Brouillard., 1990; Sun et al., 2010).

In the second group of experiments (ii) the behaviour of the copigmentation mixtures was examined as a function of temperature. Gradually increasing the temperature of the copigmentation solutions from 20 to 80 °C (in steps of 20 °C) caused a progressive

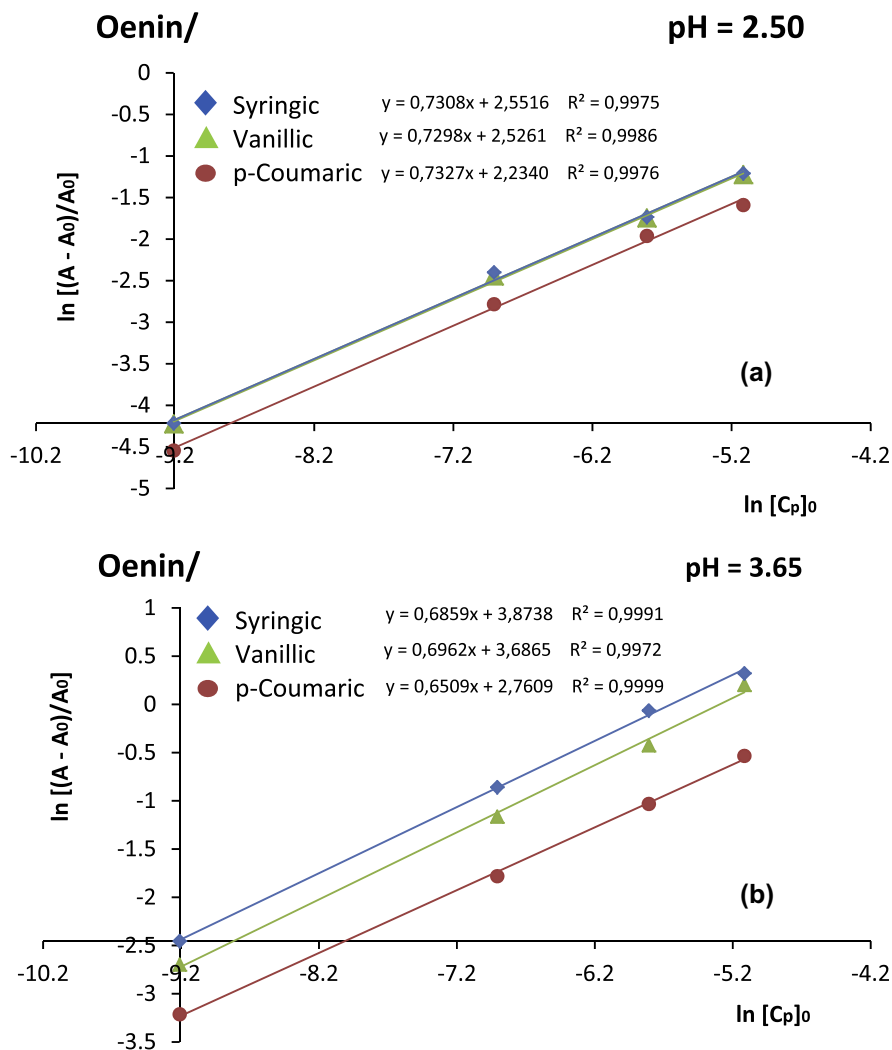
diminution of absorption (hypochromic shift) and a shift of the wavelength of the maximum absorption toward lower values (hypsochromic shift), at both pHs and all molar ratios examined. As an illustrative example, in Fig. 3, the variation of absorbance with increasing temperature of the complex oenin/*p*-coumaric acid 1:60 is reported (see also Fig. S2 for complexes oenin/vanillic and syringic acid, in Supplementary material). The hypochromic shift observed is ascribable to the spontaneous nature of the copigmentation reactions toward product formation (see  $\Delta G^\circ$  values in Table 2), thus the increase of temperature shifts the reaction toward the reactants destroying the established pigment–copigment weak interactions. This is further confirmed by observing “curves 4” in Fig. 3, which represent the absorbance of the copigmentation reactions after cooling the solutions from 80 to 20 °C. Therefore, cooling the reaction mixtures gives rise to the re-establishment of the interactions shifting the reaction equilibrium toward the products. This behaviour of our copigmentation couples is in good agreement with previous literature results (Dimitrić-Marković et al., 2000, 2005; Mazza & Brouillard., 1990; Sun et al., 2010).

Furthermore, the behaviour of each couple pigment/copigment 1:60 was examined at  $\lambda = 520$  nm as a function of the temperature in order to obtain information about the variation of enthalpy and entropy associated to the copigmentation reactions involved. According to the method of Brouillard et al. (1989), the enthalpy of the process of copigmentation for each reaction can be derived by plotting the  $\ln[(A - A_0)/A_0]$  as a function of the reciprocal of the temperature ( $1/T$ ). This plot gives a curve with slope  $-\Delta H^\circ/R$  from which enthalpy values were derived. Fig. 4 shows the plots of  $\ln[(A - A_0)/A_0]$  vs  $1/T$  for the reactions of copigmentation of oenin with pigments at pH 3.65 (the equivalent plots for the reactions at pH = 2.50 are reported in Fig. S3, Supplementary material), while the values of  $\Delta H^\circ$  for all the reactions at both pH levels are summarised in Table 2. Once the  $\Delta H^\circ$  values were determined, the entropy variations ( $\Delta S^\circ$ ) were then calculated from the Gibbs–Helmholtz equation ( $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ ).

The enthalpy variations at pH 2.50 were found to be  $-15.05$ ,  $-12.09$  and  $-13.38$  kJ mol $^{-1}$ , respectively, for the copigmentation of oenin with *p*-coumaric acid, vanillic acid and syringic acid, whereas at pH 3.65 the  $\Delta H^\circ$  values found were  $-18.87$ ,  $-23.75$  and  $-24.15$  kJ mol $^{-1}$ , respectively. At both pH levels the negative values obtained point out the exothermic nature, which is stronger at pH 3.65 for all the reactions investigated. The available data in literature regarding the enthalpy of similar pigment/copigment systems often show discrepancies based on the experimental conditions and methods used to perform the measurements. However, the values of the enthalpy change for our reactions at pH 3.65 are in good agreement with those reported previously for similar pigment/copigment couples (Baranac, Petranović, & Dimitrić-Marković, 1997b; Baranac et al., 1996; Brouillard et al., 1989; Mazza & Brouillard 1990; Sun et al., 2010). On the other hand, our  $\Delta H^\circ$  values are higher than those reported for complexes of oenin with caffeic acid, ellagic acid, catechin, procyanidin B<sub>2</sub> and rutin, which were obtained by a different experimental approach and operating at pH 3.2 (Kunsági-Máté, Szabó, Nikfardjam, & Kollár, 2006).

Entropy changes found at pH 2.50 were  $-31.92$ ,  $-19.57$  and  $-23.68$  J K $^{-1}$  mol $^{-1}$  for the copigmentation of oenin with *p*-coumaric acid, vanillic acid and syringic acid, respectively, while lower values were found at pH 3.65,  $-40.37$ ,  $-49.04$  and  $-48.84$  J K $^{-1}$  mol $^{-1}$ , respectively. The entropy decrease found for our reactions at pH 3.65 is comparable to the values reported by other authors (Baranac, Petranović, & Dimitrić-Marković, 1997a; Sun et al., 2010), and is even more favorable (lower) than those reported for similar systems (Baranac et al., 1997b; Brouillard et al., 1989; Lambert et al., 2011; Mazza & Brouillard 1990). On the other hand, Kunsági-Máté et al. (2006), using a different experimental





**Fig. 2.** Multiple plots of  $\ln [(A - A_0)/A_0]$  vs  $\ln [C_p]_0$  for the copigmentation reactions of oenin with *p*-coumaric, vanillic and syringic acids at pH = 2.50 (a) and 3.65 (b). Intercepts represent the values of  $\ln K$  while slopes the stoichiometric ratios ( $n$ ) of the respective copigmentation reactions.

**Table 2**

Thermodynamic parameters related to the process of copigmentation between oenin and the copigments involved, at pH 2.50 and 3.65.

Copigmentation	pH	$n$	$K$	$\Delta G^\circ$ (kJ mol <sup>-1</sup> ) <sup>a</sup>	$\Delta H^\circ$ (kJ mol <sup>-1</sup> ) <sup>b</sup>	$\Delta S^\circ$ (J K <sup>-1</sup> mol) <sup>c</sup>
Oenin/ <i>p</i> -coumaric	2.50	0.73	9.34	-5.54	-15.05	-31.92
	3.65	0.65	15.81	-6.84	-18.87	-40.37
Oenin/vanillic	2.50	0.73	12.50	-6.26	-12.09	-19.57
	3.65	0.70	39.90	-9.13	-23.75	-49.04
Oenin/syringic	2.50	0.73	12.83	-6.32	-13.38	-23.68
	3.65	0.69	48.12	-9.60	-24.15	-48.84

<sup>a</sup> These values were obtained from the equation:  $\Delta G^\circ = -RT \ln K$ .

<sup>b</sup> Values were obtained from oenin/copigment molar ratio 1:60.

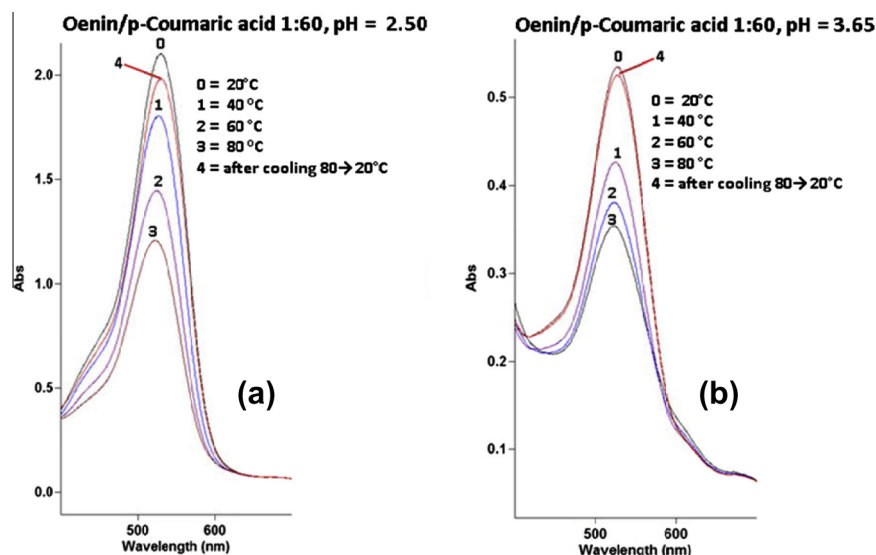
<sup>c</sup> Calculated from the equation:  $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ .

approach and operating at pH 3.2, reported lower values of  $\Delta S^\circ$  for complexes of oenin with caffeic acid, ellagic acid, catechin, procyanidin B<sub>2</sub> and rutin.

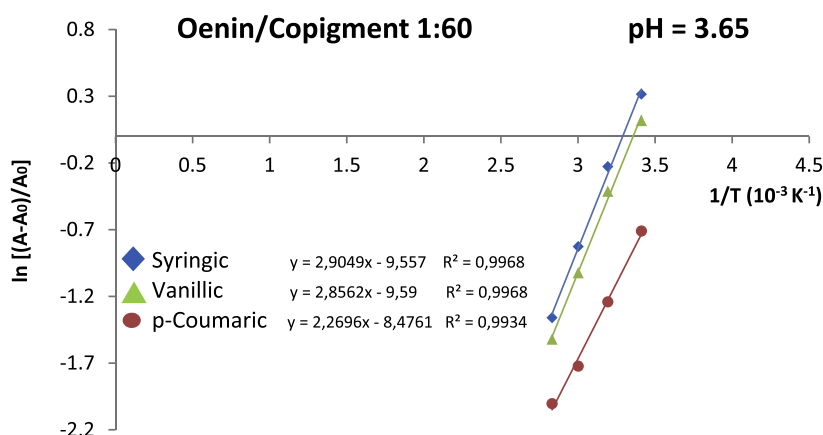
#### 4. Conclusions

The results reported here indicate that oenin (malvidin 3-O-glucoside) gives rise to intermolecular copigmentation interactions with *p*-coumaric, vanillic and syringic acids. The existence of the copigmentation phenomenon was demonstrated by hyperchromic and bathochromic shifts observed when increasing amounts of one

hydroxycinnamic acid and two hydroxybenzoic acids were added to a constant concentration of oenin solution at two pH levels. The copigmentation process was found to be dependent on the chemical structure of the copigment, its concentration, and on the pH and temperature of the medium. Syringic acid showed the highest contribution to the hyperchromic and bathochromic shifts followed by the vanillic acid with a slightly lower contribution and by the *p*-coumaric acid exhibiting the lowest one. Therefore, the results obtained indicate that di-O-methylated hydroxybenzoic acid (syringic acid) is a slightly more efficient copigment than mono-O-methylated (vanillic acid), suggesting a



**Fig. 3.** The response of the copigmentation mixture to the temperature increase. The copigmentation complex oenin/*p*-coumaric 1:60 shows a hypochromic and hypsochromic shift with the temperature increase indicating also the thermal instability of the above reactions (for complexes oenin/vanillic and syringic acid see Fig. S2 in Supplementary material).



**Fig. 4.** The plot of  $\ln [(A - A_0)/A_0]$  as a function of the reciprocal of the temperature ( $1/T$ ) gives a straight line with slope  $-\Delta H/R$ . The values of  $\Delta H$  for complexes of oenin with *p*-coumaric acid, vanillic acid and syringic acid at pH = 3.65 were derived from the slopes of the above curves (the equivalent graph for the reactions at pH = 2.50 is reported in Fig. S3, Supplementary material).

correlation of copigmentation effect with the degree of O-methylation of the hydroxybenzoic acids. Moreover, both methoxylated hydroxybenzoic acids exhibited a higher contribution to colour enhancement with respect to the hydroxycinnamic acid (*p*-coumaric acid). The magnitude of copigmentation, measured as hyperchromic and bathochromic shift, increased with increasing concentration of all copigments, at fixed pH and temperature, establishing a direct correlation with the concentration of copigments. As was previously reported, the influence of pH was significant for the copigmentation process as well. The values of the equilibrium constant for the copigmentation reactions were all found to be smaller at pH = 2.50 than at pH = 3.65 and, as a consequence, the  $\Delta G^\circ$  values indicate that the copigmentation process is more favorable at pH 3.65. However, all the copigmentation reactions studied were spontaneous ( $\Delta G^\circ < 0$ ). Our experiments also showed the thermal instability of the complexes examined. In fact, when the temperature of the copigmentation mixtures was increased progressively by a 20 °C step (20–40–60–80 °C), a decrease of absorbance (hypochromic shift) and a slight shift to shorter wavelengths (hypsochromic shift) were observed, indicating that

the equilibrium of the system is shifting from products toward reactants. Successive cooling shifted the reaction toward the products, re-establishing the copigmentation effect and the absorption returned almost to the initial point, a confirmation of the regeneration of the complex and the almost total absence of thermo-degradation of the reactants. This behaviour of our system with the temperature is another indication of the exothermic nature of the copigmentation process. All the copigmentation reactions were also accompanied by negative values of entropy ( $\Delta S^\circ < 0$ ) signifying a higher ordering of the system when the complex pigment–copigment is formed.

The above data allow us to conclude that the process of copigmentation between oenin and the copigments included in the present study is critically dependent on the chemical structure of copigment, its concentration, the pH of the medium and the temperature. Hence, the industrial processes which plan to use this phenomenon for the improvement of the colour of their products should take into account the strong dependence of the copigmentation reactions on the above factors in all stages of the product cycle (during processing, transportation, commercialisation and storage).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2013.06.017>.

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