

## A spectrophotometric study of the reaction of copigmentation of malvin and tannic acid

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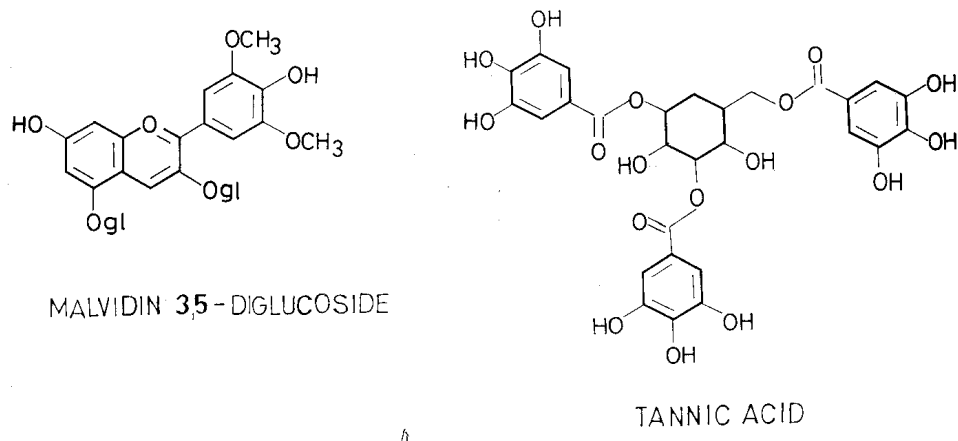
The reaction of copigmentation of malvidin 3,5-diglucoside and tannic acid of the ester type was studied. The interactions of these molecules were observed *via* UV-VIS absorption spectroscopy. It was established that the pH of the medium, the concentration of the copigmentating molecules, and the temperature affect the copigmentation process. The calculated equilibrium constant of the reaction of pH 3.00 is  $K = 226.9$ , and at pH 3.65 it is  $K = 277.0$ . The change of the Gibbs free energy in pH 3.00 buffer is  $\Delta G = -13.4$  kJ/mol, and in pH 3.65 buffer it is  $\Delta G = -13.9$  kJ/mol. The stoichiometric ratio of the components in the copigment is 1:1, at both pH values. It is evident from the calculated values of the thermodynamic functions that the process is thermodynamically favorable in the lower temperature range. Temperature appears as the basic parameter of the thermodynamic feasibility of the process, since the copigmentation process is exothermic ( $\Delta H_{\text{pH}=3.00} = -41.6$  kJ/mol and  $\Delta H_{\text{pH}=3.65} = -41.6$  kJ/mol) and proceeds with a decrease in entropy ( $\Delta S_{\text{pH}=3.00} = -94.4$  J/mol K and  $\Delta S_{\text{pH}=3.65} = -92.7$  J/mol K).

*Key words:* malvin, tannic acid, copigmentation, UV-VIS spectra.

Copigmentation is considered to be one of the more important factors of stabilization of the flavylum chromophore under *in vivo* conditions. It is possible to define it as a phenomenon that makes the color of anthocyanes deeper, more intensive and more stable, being a consequence of the interaction of anthocyanes with a range of organic compounds.

Our previous investigations dealt with the process of copigmentation of the anthocyan molecule, malvidin 3,5-diglucoside (malvin in the subsequent text), with variously substituted flavone compounds.<sup>1-4</sup> In the present work, tannic acid of the ester type (see structure Scheme 1) was chosen as the copigmentating molecule.

The literature describes the role of tannins in the composition of plant tissue<sup>8,9</sup> where they are present to varying degrees, depending on the plant species and the stage of its maturity. The fact that anthocyanes are present in the same natural



Scheme 1. Structural formulae of the compounds.

environments, *i.e.*, plants and fruits, indicates the possibility of their interaction with copigment formation. The objective of the present work was to investigate the equilibrium of the copigmentation reaction of the molecules investigated *in vitro*, and determine the factors that affect the reaction, while defining its thermodynamic parameters.

#### EXPERIMENTAL

The substances used in the present work were malvidin 3,5 diglucoside (97%, Fluka Biochemica) and tannic acid (95%, Aldrich Co.). The spectrophotometric measurements were carried out on a Pye Unicam SP8-100 UV-VIS spectrophotometer. The temperature was measured by a Pye Unicam Temperature Cell Controller, which is an integral part of the spectrophotometer. The measurements were performed in the temperature range from 20 °C to 50 °C ( $\pm 2$  °C). Quartz cuvettes of 1 cm optical path were used for recording the spectra. The methodology of recording the spectra was the same for all measurements. Each spectrum was recorded 30 s after the preparation of the solution. The copigmentation reaction of the molecules under study was followed in buffer solutions at pH 3.00 and pH 3.65.

The pH values were chosen on the basis of our earlier studies,<sup>2</sup> as well as according to available literature data.<sup>5</sup> Namely, it is known that the process of copigmentation occurs primarily in moderately acidic media under *in vivo* conditions. For this reason we studied the reaction in solutions whose pH values were close to those of natural fruit juices.

Buffer solutions of constant ionic strength (0.02 M) were obtained by mixing 0.02 M sodium acetate (p.a., Merck) and 0.06 M phosphoric acid (85%, BDH Poole England). The ionic strength of the solution was adjusted by adding 0.02 M sodium hydroxide (p.a., Merck). The reference solutions in the spectrophotometric measurements were pure buffer solutions of the corresponding pH values.

Measurements of the pH were carried out on an Iskra MA 5730 pH meter with a combined electrode, at 298 K. The standard buffer solution for calibration was a solution of potassium biphthalate (p.a., Merck).

## RESULTS

The process of copigmentation of the above mentioned molecules was followed at two pH values, in buffer solutions of pH 3.00 and pH 3.65. Figure 1 shows the absorption spectra of malvin and tannic acid at pH 3.00 (the absorption spectrum of malvin at pH 3.65 is given in a previous papers.<sup>1</sup> As pointed out earlier, malvin is present in the used buffer solutions in its cationic form.<sup>1-4</sup> It is evident from Fig. 1 that tannic acid does not absorb in the visible range of the spectrum, but only in the UV, in which an intense band at 280 nm appears.

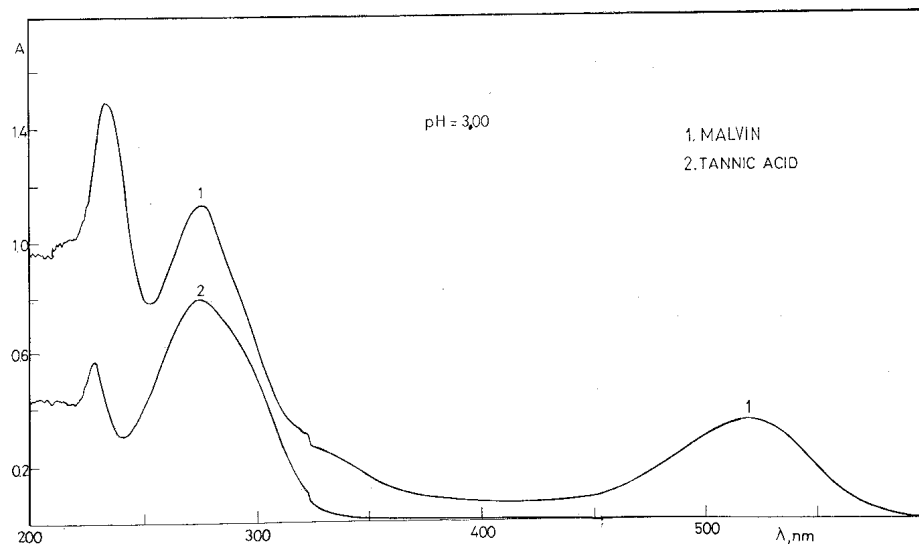


Fig. 1. Absorption spectra of malvin and tannic acid in buffer solution. pH 3.00.

The copigmentation process at the used pH values was followed at a constant malvin concentration of  $1 \times 10^{-4}$  M. The absorption spectra of malvin and the copigments formed at varying mole ratios and pH are presented in Fig. 2. The bathochromic shift of the fundamental absorption maximum of the malvin cationic form, with a slight hyperchromic effect, is the principal proof of copigment formation at the given pH values. From the bathochromic shift, it can be concluded that the optimal mole ratio of the components in the copigment is 1:15 at both pH values and the given malvin concentration ( $\Delta\lambda = 14.0$  nm at pH 3.00,  $\Delta\lambda = 13.0$  nm at the pH 3.65).

The copigmentation reaction can be schematically presented in the following way:<sup>5</sup>



where  $\text{AH}^+$  – flavylium cation and Cp – copigmentating molecule (tannic acid, in the present case).

The equilibrium constant of this reaction is given by Eq. (2):

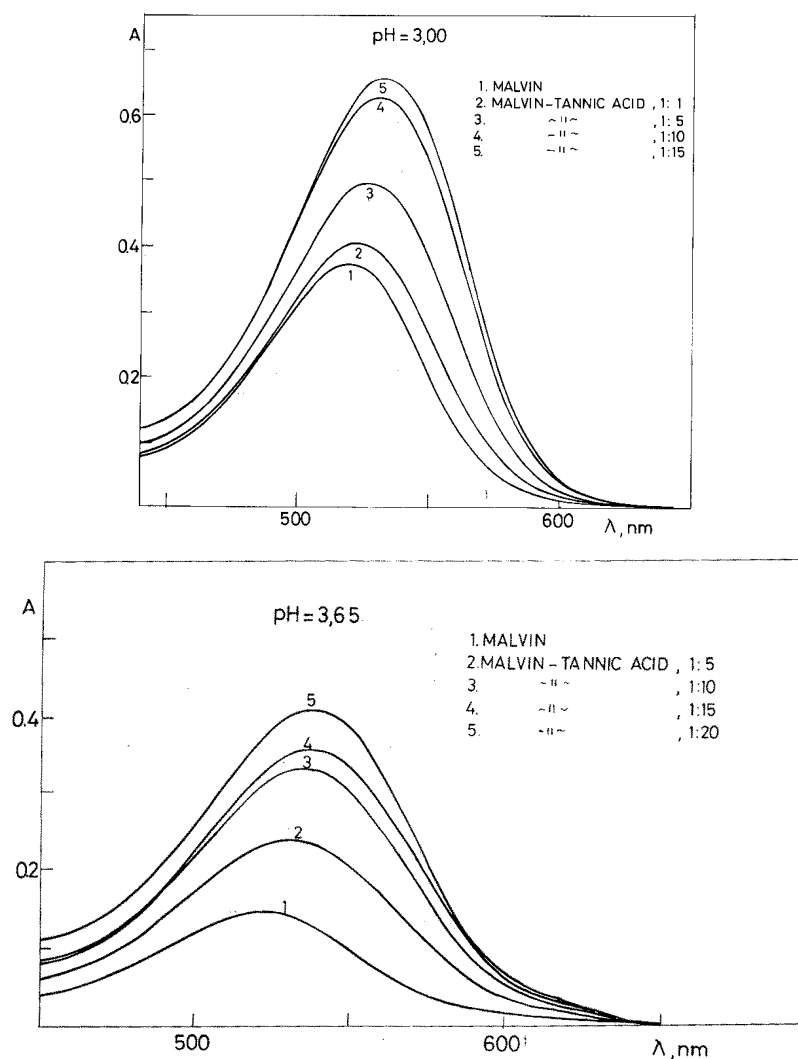


Fig. 2. Absorption spectra of malvin and copigment malvin-tannic acid, different mole ratios in buffer solutions a) pH 3.00 and b) pH 3.65.

$$K = \frac{[\text{AH}(\text{Cp})_n^+]}{[(\text{AH})^+][\text{Cp}^n]} \quad (2)$$

The relationship between the copigmentation reaction equilibrium and the spectroscopic properties that are directly determined is given by Eq. (3):<sup>5</sup>

$$(A-A_0)/A_0 = Kr[\text{Cp}^n] \quad (3)$$

where  $(A-A_0)/A_0$  – the relative change of the intensity of the copigment absorption maximum vs. the malvin absorption maximum,  $K$  – the equilibrium constant of the process,  $n$  – number of free copigmenting molecules that are bound to the flavylum

cation, *i.e.*, the stoichiometric ratio of the components in the copigment,  $[Cp_0]$  – the initial concentration of the copigmenting molecules (tannic acid) and  $r$  – a parameter that represents the ratio of the completely copigmented flavylum cation absorption maximum intensity vs. the absorption maximum intensity of the cation alone,  $(A/A_0)$ .<sup>5</sup>

The logarithm form of Eq. (3), given by Eq. (4) is used to determine the equilibrium constant,  $K$ , and the value of  $n$ :

$$\ln [(A-A_0)/A_0] = \ln (Kr) + n \ln [Cp_0] \quad (4)$$

The dependence of  $\ln [(A-A_0)/A_0]$  on the concentration of tannin,  $(\ln [(A-A_0)/A_0] = f(\ln [Cp_0]))$  (Fig. 3), at the absorption maximum wavelength, is a straight line with a slope equal to  $n$ , and an intercept of  $\ln (Kr)$ . The stoichiometric ratio of the components in the copigment is determined from the slope, while the equilibrium constant is determined from the intercept.<sup>5</sup> The stoichiometric ratio of the components in the copigment was 1:1 at the pH values investigated. The calculated values of the constant were  $K = 226.9$  at pH 3.00, and  $K = 277.0$  at pH 3.65. The values of the standard Gibbs energy, which were calculated from the known relation  $\Delta G = -RT \ln K$ , were found to be  $\Delta G = -13.4$  kJ/mol at pH 3.00, and  $\Delta G = -13.9$  kJ/mol at pH 3.65.

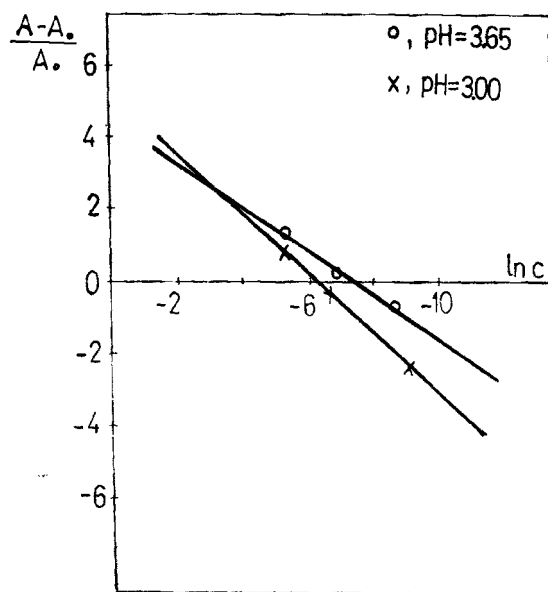


Fig. 3. Plot of  $\ln [(A-A_0)/A_0]$  as a function of tannic acid concentration for different buffer solutions.

The stability of the copigment solution with time was also monitored, at a temperature of 298 K using the mole ratios 1:5 and 1:15, at the studied pH values, with respect to malvin solutions at the same pH values and the same temperature. Although all the solutions were kept in the dark, measured after the same time interval, and in the same manner, the intensity of the color decayed differently as a

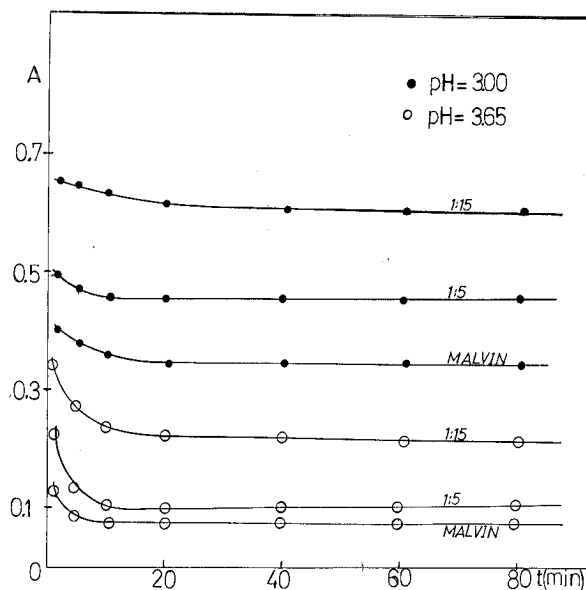


Fig. 4. The change in intensities of the absorption maxima of malvin and the copigments (mole ratios 1:5 and 1:15) as a function of time, in buffer solutions pH 3.00 and pH 3.65.

function of time. This was manifested by a hypochromic effect on the basic absorption maxima of malvin and the copigments. The change of absorbance with time for the solutions investigated is given in Fig. 4. It was established that malvin is more stable at pH 3.00 than at pH 3.65. Malvin is also more stable than the copigments formed, at both pH values. The stability of the copigment is greater at pH 3.00 than at pH 3.65. The solutions with a mole ratio of 1:15 are more stable than the ones with a mole ratio of 1:5.

The thermal stability of the system was also monitored. It was reported in a previous paper<sup>2</sup> that malvin solutions insignificantly change with temperature, hence only the thermal stability of the copigment solution was monitored in the present work. Temperature effects were observed at all mole ratios of copigment formation, and at both pH values. The changes in the absorption spectra of the copigment (1:15) as a function of temperature, at both pH values, are presented in Figs. 5a and 5b. Increasing the temperature to 50 °C evidently caused copigment degradation, manifested by a hypsochromic shift of the band maxima ( $\lambda_{\max, 25\text{ }^{\circ}\text{C}} = 530\text{ nm}$ ,  $\lambda_{\max, 50\text{ }^{\circ}\text{C}} = 524\text{ nm}$ ) with a hypsochromic effect (Fig. 5a, curves 2–6, and Fig. 5b, curves 2–8). After an abrupt decrease of temperature to 20 °C, the equilibrium shifts towards the product of the process, *i.e.*, the copigment (Fig. 5a, curve 7, and Fig. 5b, curve 9).

The values of the enthalpy change of the process ( $\tan \alpha = -\Delta H/R$ ) were determined from the slope of a plot of  $\ln [(A-A_0)/A_0]$  as a function of reciprocal temperature (Fig. 6). The enthalpy change is  $\Delta H = -41.6\text{ kJ/mol}$ , at both pH values. Entropy changes,  $\Delta S$ , were calculated from the Gibbs-Helmholtz equation, and found to be  $\Delta S_{\text{pH}=3.00} = -94.4\text{ J/mol K}$  and  $\Delta S_{\text{pH}=3.65} = -92.7\text{ J/mol K}$ .

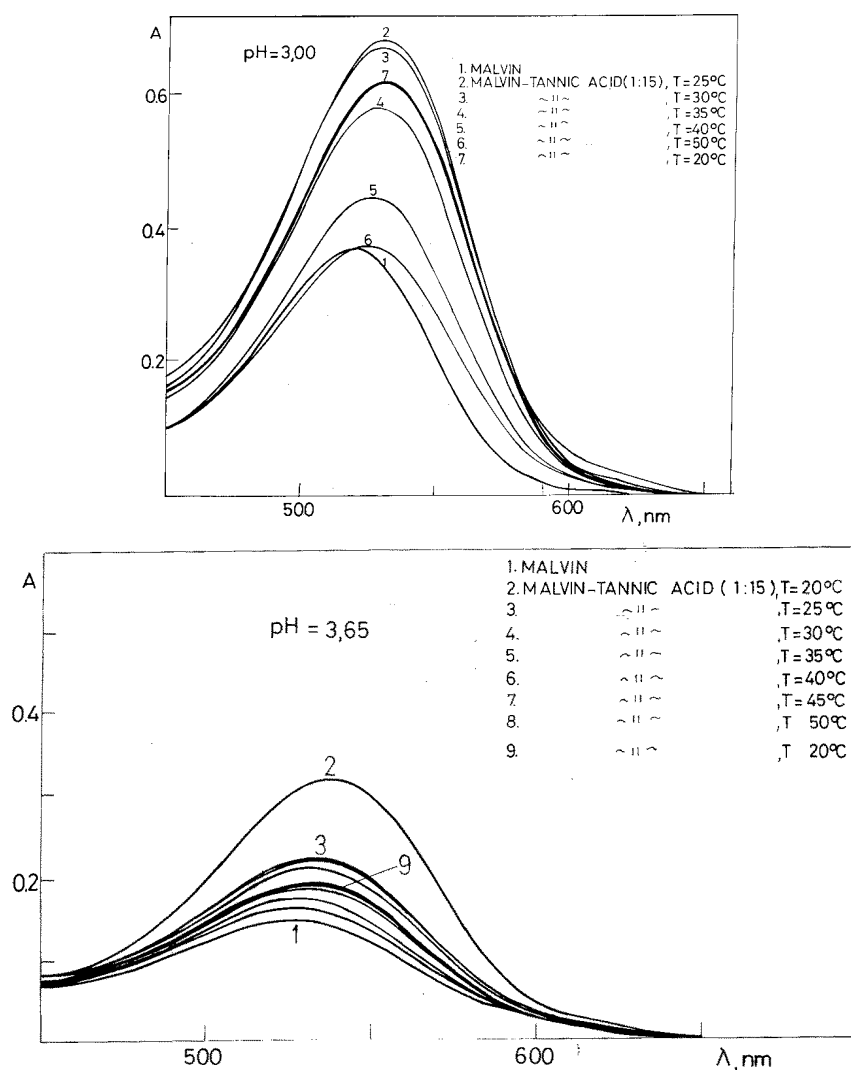


Fig. 5. Change in the absorption spectra of the copigment malvin-tannic acid (mole ratio 1:15) with temperature in different buffer solutions a) pH 3.00 and b) pH 3.65.

The thermodynamic properties of the copigmentation process (mole ratio 1:15) are given in Table I.

TABLE I. Thermodynamic data for the process of copigmentation of malvin and tannic acid in buffer solutions at pH 3.00 and pH 3.65 ( $T = 298$  K).

pH	$n$	$K$	$\Delta G/(\text{kJ/mol K})$	$\Delta H/(\text{kJ/mol K})$	$\Delta S/(\text{J/mol K})$
3.00	1	226.9	-13.4	-41.6	-94.4
3.65	1	277.0	-13.9	-41.6	-92.7

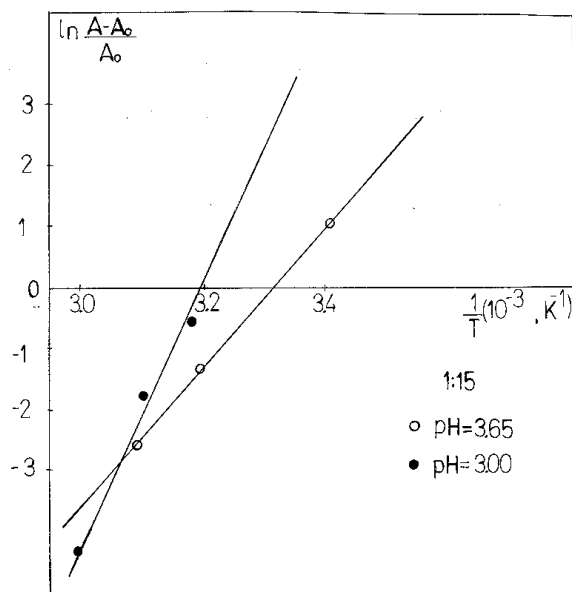


Fig. 6. Plot of  $\ln [(A-A_0)/A_0]$  for a malvin-tannic acid solution (mole ratio 1:15) as a function of reciprocal temperature for different buffer solutions.

#### DISCUSSION

The present work established that a copigmentation process exists between malvin 3,5-diglucoside and tannic acid in buffer solutions at pH 3.00 and pH 3.65. From the results presented it is evident that the process under study, in the buffer media given, is characterized by approximately the same values of the characteristic parameters.

By following the stability of malvin solutions and copigment solutions with time, at both pH values, the malvin solutions, and the copigment solutions were found to have greater stability at the lower pH value, pH 3.00 (Fig. 4).

The proof of copigment formation is the bathochromic shift of the fundamental malvin absorption band, as a result of the increased delocalization of the  $\pi$ -electron system, *i.e.*, the formation of copigments. At a constant malvin concentration ( $1 \times 10^{-4}$  M), the copigment was formed in solutions of various mole ratios (Fig. 2). The mole ratio of components 1:15 (malvin: tannic acid) was found to be optimal, both at pH 3.00 and pH 3.65, since the greatest bathochromic shifts were observed with this ratio ( $\Delta\lambda_{\text{pH}=3.00} = 14$  nm,  $\Delta\lambda_{\text{pH}=3.65} = 13$  nm).

As in systems previously studied,<sup>1-4</sup> the copigment was formed in the stoichiometric ratio 1:1.

We believe that the differences in the values of the reaction equilibrium constant, which is  $K = 226.9$  ( $T = 298$  K) at pH 3.00 and  $K = 277.0$  ( $T = 298$  K) at pH 3.65, are a consequence of a change in the flavylum cation concentration with pH<sup>5</sup> (see Eq. (2)). At pH 3.00 (Fig. 2a, curve 1) the flavylum cation concentration is higher than at pH 3.65 (Fig. 2b, curve 1). The calculated values of the thermody-



namic function  $\Delta G (\Delta G < 0)$ , which are approximately equal at both pH values (see Table I), indicate the spontaneity of the copigmentation process.

It was established, as with previously studied systems,<sup>1-4</sup> that increasing the temperature, under the given conditions and mole ratio, causes the equilibrium of the process to shift towards the initial reactants (Fig. 5a, curves 2–6, and Fig. 5b, curve 2–8). On lowering the temperature the equilibrium of the process shifts in the opposite direction, towards the copigment (Fig. 5a, curve 7 and Fig. 5b, curve 9). Such a temperature effect is a consequence of copigment formation being an exothermic process. The negative change of entropy,  $\Delta S$ , as a measure of system ordering is also proof of copigment formation, under the given conditions. The fact that the thermodynamic functions  $\Delta H$  and  $\Delta S$  are both negative,  $\Delta H < 0$  and  $\Delta S < 0$ , Table I, indicates that copigment formation is only possible at lower temperatures.

As for the structure of the copigments formed, we accept the assumptions of other authors<sup>6,7,11</sup> that bonds are formed *via* hydrogen bonding, but we do not have our own experimental proof of that till now.

The results of the investigation of the copigmentation process *in vitro* help to define better malvin copigmentation with tannic acid *in vivo*. Since it is believed that tannic acid plays an important role in the stabilization of the red wine color in the maturing process,<sup>10</sup> we assume that the results of our work will contribute to a better understanding of this process under natural conditions.

### ИЗВОД

#### СПЕКТРОФОТОМЕТРИЈСКО ИСПИТИВАЊЕ РЕАКЦИЈЕ КОПИГМЕНТАЦИЈЕ МАЛВИНА И ТАНИНСКЕ КИСЕЛИНЕ

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У овом раду испитивана је реакција копигментације малвидин 3,5-диглукозида и танинске киселине естарског типа. Интеракције наведених молекула праћене су преко UV-VIS апсорпционих спектра. Констатовано је да на процес копигментације утиче рН средине, концентрација молекула и температура. На основу израчунатих вредности термодинамичких функција евидентно је да је процес термодинамички повољан у области нижих температура. Температура се јавља као основни параметар термодинамичке условљености процеса пошто је процес копигментације егзотерман ( $\Delta H < 0$ ) и одвија се са опадањем ентропије ( $\Delta S < 0$ ).

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