A Spectrophotometric Study of the Copigmentation of Malvin with Caffeic and Ferulic Acids

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The process of copigmentation of the anthocyanin molecule malvidin 3,5-diglucoside with two organic monocarboxylic phenolic acids, caffeic and ferulic acids, was studied via their absorption electronic spectra. The dependence of the copigmentation process on the pH of the medium, molecular concentration, and temperature was established. The process of copigmentation was observed at two pH values: 2.50 and 3.65. The stoichiometric ratio was 1:1 at both pH values. The copigmentation was characterized by approximately equal values of the equilibrium constant, *K*, within each of the pH values. The temperature was found to be a significant factor that determines the thermodynamic conditions of the copigmentation process, because the process is spontaneous ($\Delta G° < 0$), and results in entropy loss ($\Delta S° < 0$) at both pH values.

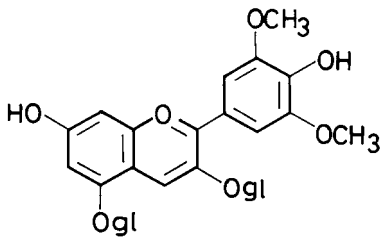
Keywords: *Malvin; caffeic and ferulic acids; copigmentation; UV−Vis spectra*

INTRODUCTION

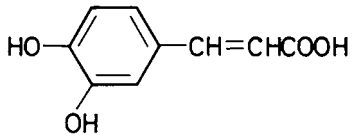
Anthocyanidins and their glycosylated forms, anthocyanins, represent one of the most important and most widely spread groups of plant pigments of the class of flavonoids. This group of pigments is responsible for the existence of most of the red, blue, and purple colors in flowers and fruits (Harborne, 1967). Studies published so far have shown that a change in the pH of the medium, as well as the processes of hydroxylation, methylation, glycosylation, and acylation, can affect the stability and the number of anthocyanins (Swain, 1976; Harborne, 1967; Hoshino et al., 1980). All these effects, however, cannot account for the ample variety of colors and hues in the plant world. This is why it had first been assumed, and later confirmed, that different colors are the consequences of complexation (Bayer et al., 1966; Veselinović et al., 1992; Elhabiri et al., 1997), i.e., copigmentation (Asen et al., 1972 and 1975; Brouillard, 1983), of the flavylium chromophore. Copigmentation is regarded today as one of the significant factors of structure stabilization, i.e., coloration, of anthocyanins under in vivo conditions.

The present work is a follow-up of the research into the copigmentation of malvidin 3,5-diglucoside, as a significant anthocyanin molecule widely spread in nature. As copigmentation molecules we chose two organic phenolic molecules, caffeic and ferulic acid (see the structures in Scheme 1). This choice was made on the basis of the hitherto published data (Hahlbrock, 1975; Barz, 1975), according to which these acids are part of plant tissue, in which they play a significant role. In addition to having a significant metabolic function, cinnamic acids constitute the main acyl groups in the structure of acylated anthocyanins (Harborne, 1967), which are also part of plant tissue. The indications in the literature that acylated anthocyanins are subject to intramolecular copigmentation (Figueiredo et al., 1996; Dangles et al., 1993) led us to investigate the possibilities of intermolecular copigmentation of these molecules.

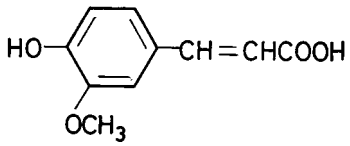
Scheme 1. Structural Formulas of the Compounds



MALVIDIN 3,5-DIGLUCOSIDE



CAFFEIC ACID



FERULIC ACID

The objective of the present work was to study the equilibrium of the reaction of copigmentation of the molecules mentioned, under in vitro conditions, and determine the factors that affect the process, together with determining the thermodynamic parameters.

EXPERIMENTAL PROCEDURES

The substances used in the present work were malvidin 3,5 diglucoside (malvin, 97%, Fluka Biochemica), caffeic acid

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10.1021/jf000038v CCC: $19.00
Published on Web 10/21/2000

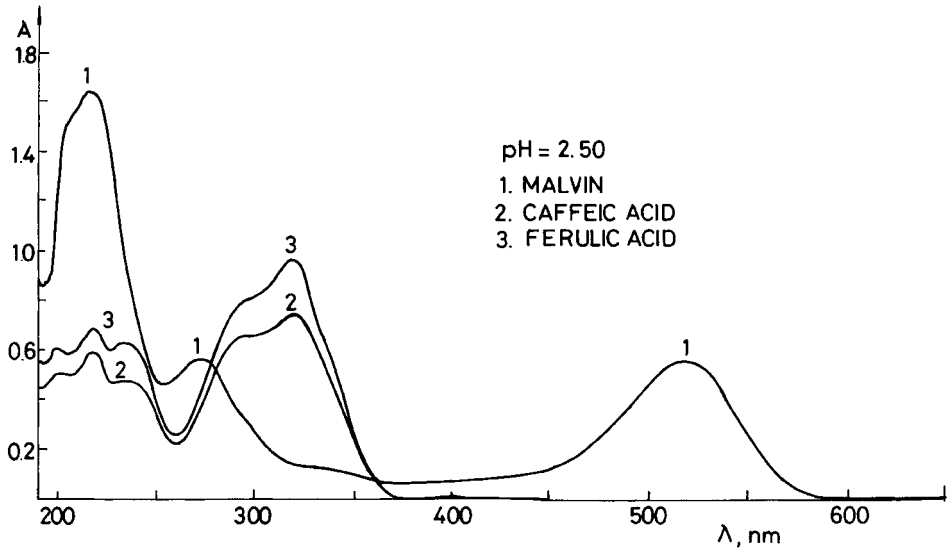

Figure 1. Absorption spectra of malvin and caffeic and ferulic acids in buffered solution at pH 2.50.

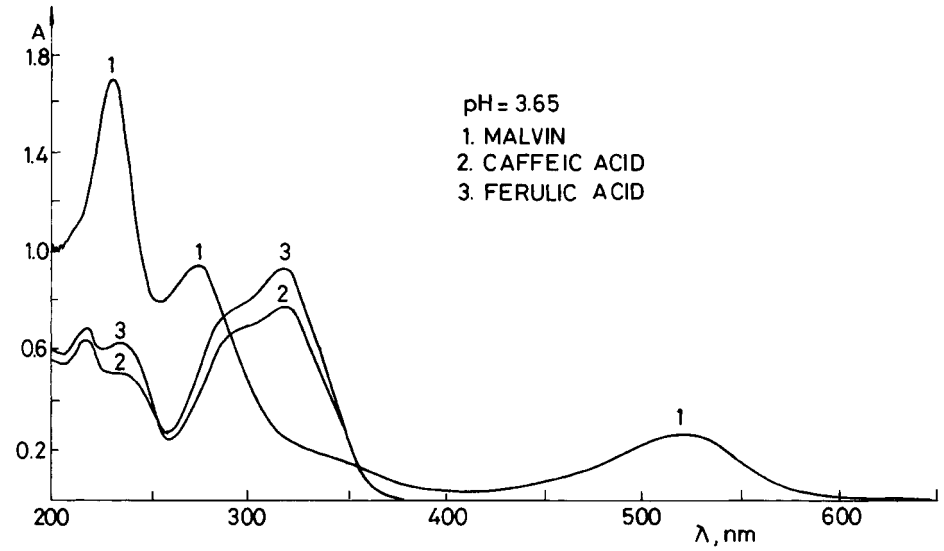

Figure 2. Absorption spectra of malvin and caffeic and ferulic acids in buffered solution at pH 3.65.

(ICN Biochemicals), and ferulic acid (ICN Biochemicals). The spectrophotometric measurements were carried out on a Pye Unicam SP8-100 UV−Vis spectrophotometer. Temperature was measured by a Pye Unicam Temperature Cell Controller, which is an integral part of the spectrophotometer. Quartz cuvettes of 0.1, 0.5, and 1 cm optical path were used for recording the spectra. The methodology of recording the spectra was the same in all the measurements.

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%, Poole England). The ionic strength of the solution was adjusted by adding 0.02 M sodium chloride (p.a., Merck). The reference solutions in 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 diphthalate (p.a., Merck).

RESULTS

The process of copigmentation of these molecules was observed in buffer solutions at pH 2.50 and pH 3.65. Absorption spectra of malvin and of caffeic and ferulic acid in the pH 2.50 buffer solution are presented in Figure 1, and the same spectra obtained in the pH 3.65 buffer are presented in Figure 2. It is observable in both figures that the form of malvin is cationic in these buffer solutions, as emphasized earlier (Baranac et al., 1996). Also, the absorption band of the cationic malvin form appears in the visible part of the spectrum, whereas the absorption bands of the acids appear in the UV range.

Malvin concentration was constant, 1×10^{-4} M, at both pH values. Copigmentation of these molecules, in the studied buffer solutions, manifests in the batochromic and hyperchromic effects on the main malvin absorption peak. Because malvin concentration was constant in all solutions, it is clear that the magnitude of the batochromic shift depended on the concentration of acids. Absorption spectra of malvin and spectra of the copigments formed with caffeic and ferulic acid in the pH 2.50 buffer solution are shown in Figures 3 and 4. Mole ratios of the components in the copigment were 1:20, 1:40, 1:60, and 1:100 (malvin/acid). The respective magnitudes of the batochromic and hyperchromic shifts are given in Table 1. A comparison of the values shows that they are somewhat larger when the copigment is formed in the system malvin−ferulic acid. Absorption

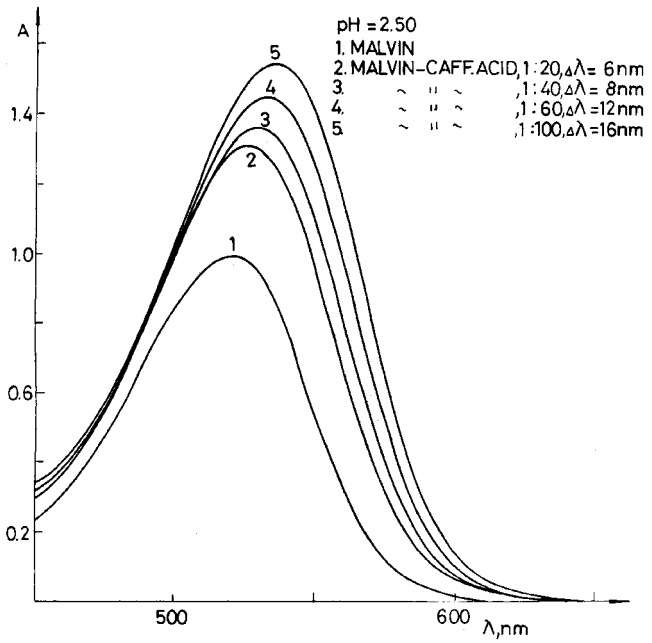

Figure 3. Absorption spectra of malvin and copigmentation complex malvin−caffeic acid, different molar ratios, in buffered solution at pH 2.50.

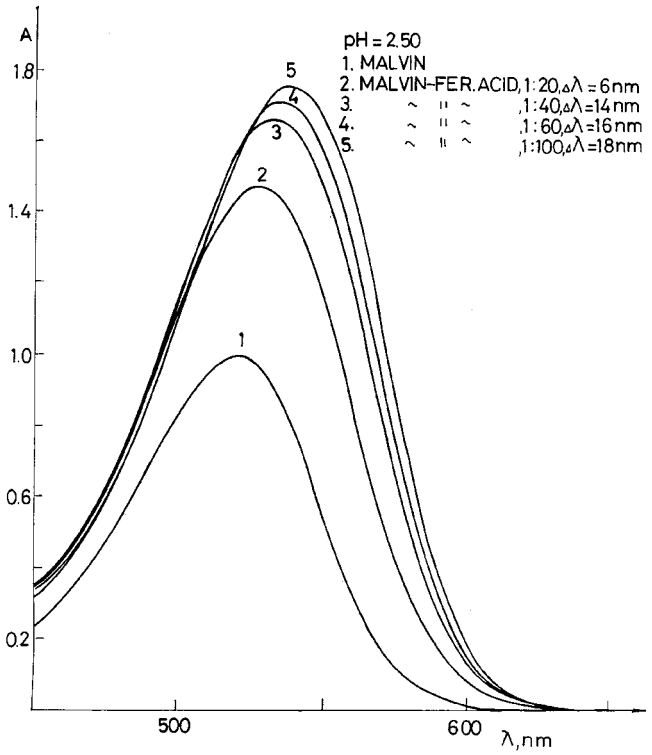

Figure 4. Absorption spectra of malvin and copigmentation complex malvin−ferulic acid, different molar ratios, in buffered solution at pH 2.50.

spectra of malvin and those of copigments formed in the pH 3.65 buffer solution are shown in Figures 5 and 6. Mole ratios of the components were the same as in the pH 2.50 buffer solution. It is observable from Table 1 that at pH 3.65 the values for $\Delta\lambda$ and ΔA are higher in the case of malvin−ferulic acid copigment, at all mole ratios investigated. The absorption spectra of the copigments (mole ratio 1:20) at both pH values, and in the range from 200 to 650 nm, are presented in Figure 7. As observable, the changes occur only on the main

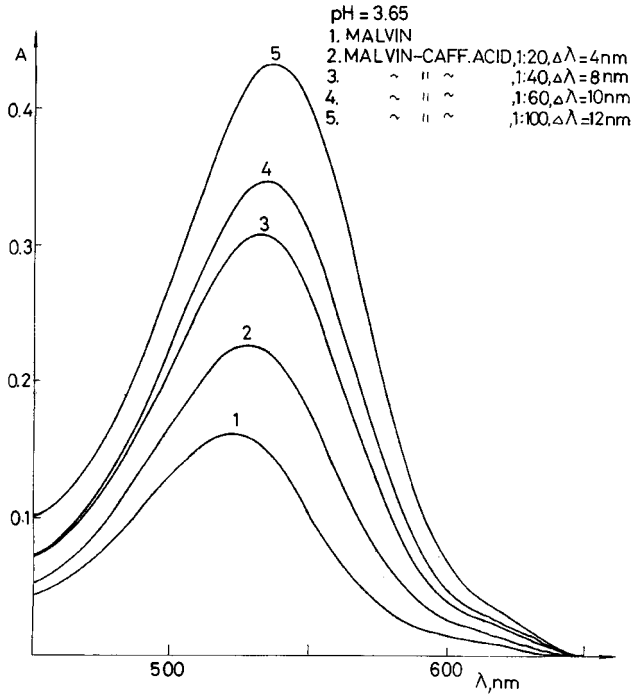

Figure 5. Absorption spectra of malvin and copigmentation complex malvin−caffeic acid, different molar ratios, in buffered solution at pH 3.65.

Table 1. Values of Batochromic and Hyperchromic Shifts of the Main Absorption Peak of Malvin on Complex Formation with Caffeic and Ferulic Acid in Buffer Solutions at pH 2.50 and pH 3.65

copigment	molar ratios	$\Delta\lambda$ (nm)	ΔA
	pH 2.50		
malvin−caffeic acid	1:20	6	0.32
malvin−ferulic acid		6	0.48
malvin−caffeic acid	1:40	8	0.38
malvin−ferulic acid		14	0.68
malvin−caffeic acid	1:60	12	0.47
malvin−ferulic acid		16	0.72
malvin−caffeic acid	1:100	16	0.56
malvin−ferulic acid		18	0.77
	pH 3.65		
malvin−caffeic acid	1:20	4	0.06
malvin−ferulic acid		6	0.19
malvin−caffeic acid	1:40	8	0.15
malvin−ferulic acid		12	0.36
malvin−caffeic acid	1:60	10	0.19
malvin−ferulic acid		14	0.43
malvin−caffeic acid	1:100	12	0.27
malvin−ferulic acid		18	0.63

absorption peak of malvin in the visible range of the spectrum. No new spectral bands appear in the UV range.

The equilibrium constant and the stoichiometric ratio of copigment components were determined for the process of malvin copigmentation. The calculation follows from eq 1 (Brouillard et al., 1989):

$$\ln [(A - A_0)/A_0] = \ln(Kr) + n \ln [Cp_0] \qquad (1)$$

The dependence of $\ln[(A - A_0)/A_0]$ on the concentrations of caffeic and ferulic acid (Figures 8 and 9) ($\ln [(A - A_0)/A_0] = f(\ln [Cp_0])$ yields a line of slope n and intercept $\ln(Kr)$. The slope determines the stoichiometric ratio of the components, and the intercept determines the equilibrium constant. The stoichiometric ratio of copig-

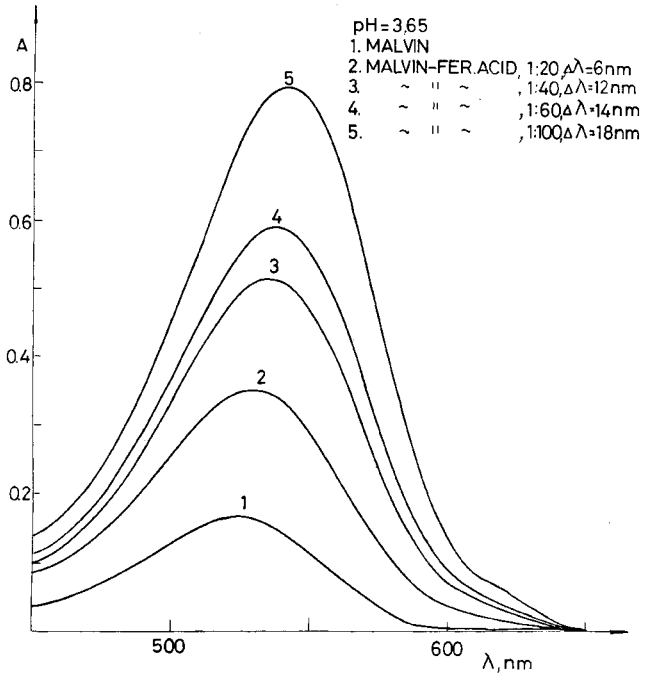

Figure 6. Absorption spectra of malvin and copigmentation complex malvin−ferulic acid, different molar ratios, in buffered solution at pH 3.65.

ment components was 1:1 for both acids and at both pH values. The values calculated for the equilibrium constant at pH 2.50 are $K = 33.3$ (malvin−caffeic acid) and $K = 35.8$ (malvin−ferulic acid). At pH 3.65 the equilibrium constant values are $K = 74.9$ (malvin−caffeic acid) and $K = 88.8$ (malvin−ferulic acid). The magnitude of Gibbs free energy, calculated from the known relationship $\Delta G° = -RT \times \ln K$, at pH 2.50 was $\Delta G° = -8.7$ kJ/mol (malvin−caffeic acid) and $\Delta G° = -8.9$ kJ/mol (malvin−ferulic acid). At pH 3.65 it was found to be $\Delta G° = -10.7$ kJ/mol (malvin−caffeic acid) and $\Delta G° = -11.1$ kJ/mol (malvin−ferulic acid).

Thermal stability of the copigments was also investigated. Increasing temperature produced changes on the copigments formed, which resulted in hypsochromic and hypochromic shifts of the main absorption peaks. This temperature effect was observed at both pH values and at all molar ratios. Changes in the absorption spectra of malvin−caffeic acid and malvin−ferulic acid (molar ratio 1:60) as a function of temperature at pH 2.50 are shown in Figures 10 and 11, respectively. The increase in temperature to 50 °C causes complex decomposition, manifested by a hypsochromic shift of the absorption peak, accompanied by a hypochromic effect (Figures 10 and 11, curves 1−5 and 1−6, respectively). Abrupt cooling to room-temperature shifts the equilibrium toward the product, i.e., the copigment (Figures 10 and 11, curves 6 and 7, respectively). Changes in the absorption spectra of malvin−caffeic acid and malvin−ferulic acid (molar ratio 1:60) as a function of temperature at pH 3.65 are shown in Figures 12 and 13, respectively. The increase in temperature to 50 °C, i.e., to 53 °C, at this pH too, shifts the equilibrium toward the reactants (Figure 12, curves 1−6, and Figure 13, curves 1−5). Abrupt cooling shifts the equilibrium toward product formation (Figure 12, curve 7, and Figure 13, curve 6).

A plot of ln $[(A - A_0)/A_0]$ against reciprocal temperature (Figures 14 and 15) yields a slope from which enthalpy changes of the process ($tg\ \alpha = -\Delta H/R$) were derived. The enthalpy changes at pH 2.50 were found to be $\Delta H° = -36.9$ kJ/mol (malvin−caffeic acid) and $\Delta H° = -43.1$ kJ/mol (malvin−ferulic acid). At pH 3.65 enthalpy changes are $\Delta H° = -49.9$ kJ/mol (malvin−caffeic acid) and $\Delta H° = -55.4$ kJ/mol (malvin−ferulic acid). The Gibbs−Helmholtz equation was used to calculate the values of entropy changes, $\Delta S°$. In the pH 2.50 buffer the entropy changes were found to be $\Delta S° = -94.6$ J/molK (malvin−caffeic acid) and $\Delta S° = -114.8$ J/molK (malvin−ferulic acid). In the pH 3.65 buffer they were $\Delta S° = -131.5$ J/molK (malvin−caffeic acid) and $\Delta S° = -148.6$ J/molK (malvin−ferulic acid). Thermodynamic properties of the processes of copigmentation of malvin with caffeic and ferulic acid (component molar ratio 1:60) are given in Table 2.

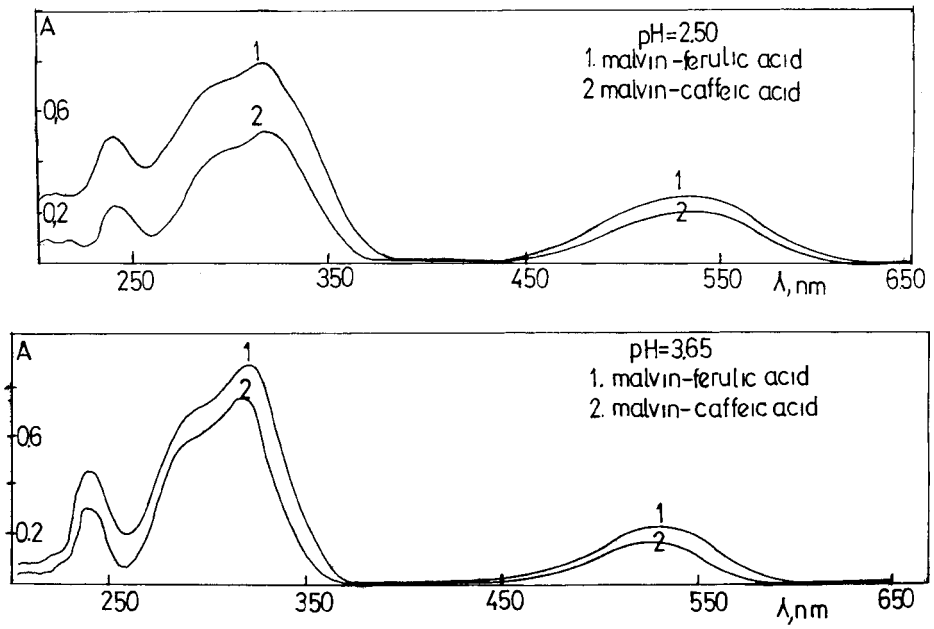

Figure 7. Absorption spectra of the copigmentation complexes malvin−caffeic acid and malvin−ferulic acid, molar ratio 1:20, in buffered solutions at pH 2.50 and pH 3.65.

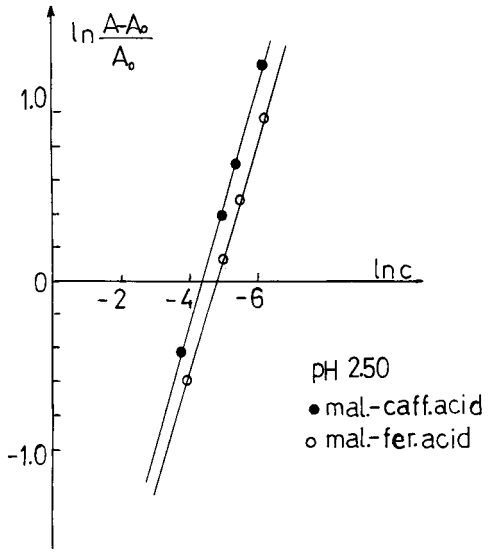

Figure 8. Plot of ln $\{(A - A_0)/A_0\}$ as a function of caffeic and ferulic acid concentration in buffered solution at pH 2.50.

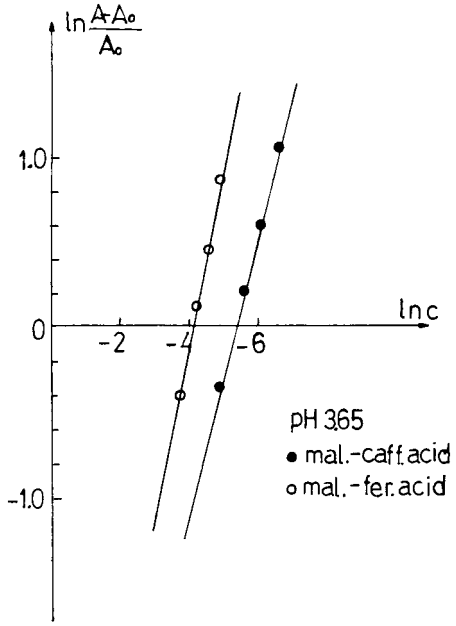

Figure 9. Plot of ln $\{(A - A_0)/A_0\}$ as a function of caffeic and ferulic acid concentration in buffered solution at pH 2.50.

DISCUSSION AND CONCLUSION

From the results presented here it can be concluded that at the two pH values investigated (2.50 and 3.65), malvidin 3,5-diglucoside enters an interaction of copigmentation with the acids studied.

The main proof of complex formation is the batochromic shift of the main absorption band of malvin cationic form, accompanied by a hyperchromic effect. At a constant malvin concentration of 1×10^{-4} M complex is formed at different molar ratios of the reactants (Figures 3−6). It was established that the magnitude of the batochromic shift and the hyperchromic effect depend on the concentration of the acids, at both pH values, and that they are larger for the system malvin−ferulic acid, at all mole ratios investigated (Table 1). Because the copigments formed were observed at high mole ratios of the components it can be assumed that the copigmentation of the molecules investigated is

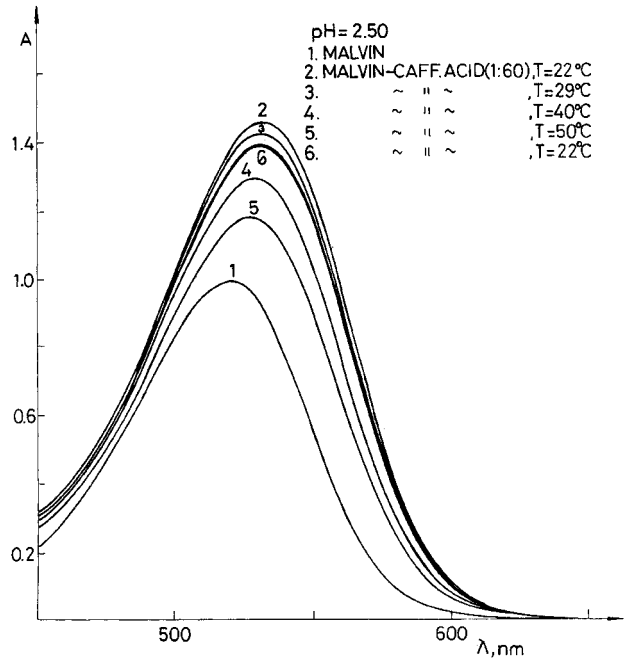

Figure 10. Change in absorption spectra of the copigmentation complex malvin−caffeic acid (molar ratio 1:60) with temperature in buffered solution at pH 2.50.

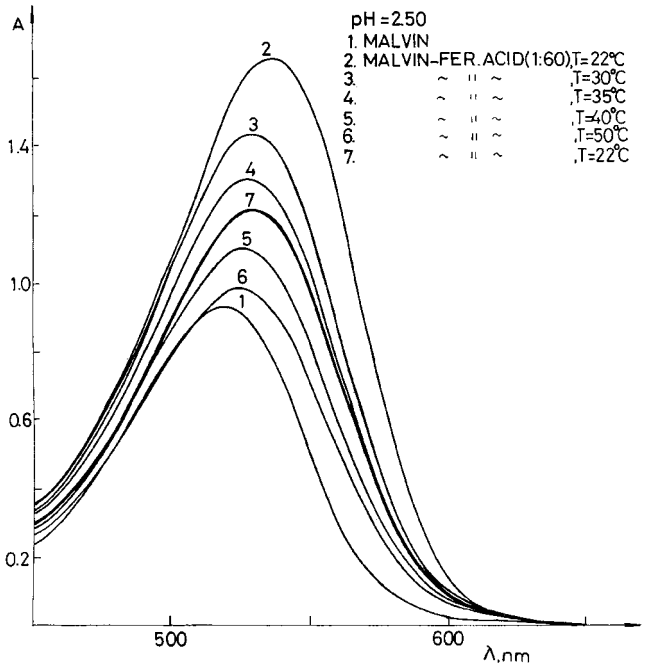

Figure 11. Change in absorption spectra of the copigmentation complex malvin−ferulic acid (molar ratio 1:60) with temperature in buffered solution at pH 2.50.

intermolecular. This is also supported by Brouillard's statement that intermolecular copigments are formed at high mole ratios, whereas intramolecular copigments are formed at significantly lower mole ratios (Dangles et al., 1993).

The proof that the process is really copigmentation, and not acylation, of the malvin molecule, is the fact that the main changes in the absorption spectra, in terms of $\Delta\lambda$ and ΔA increase, at both pH values, occur only on the main absorption of malvin cationic form. It has, namely, been established (Harborne, 1967) that acylation of anthocyanins produces new absorption bands in the UV range of the spectrum. No new

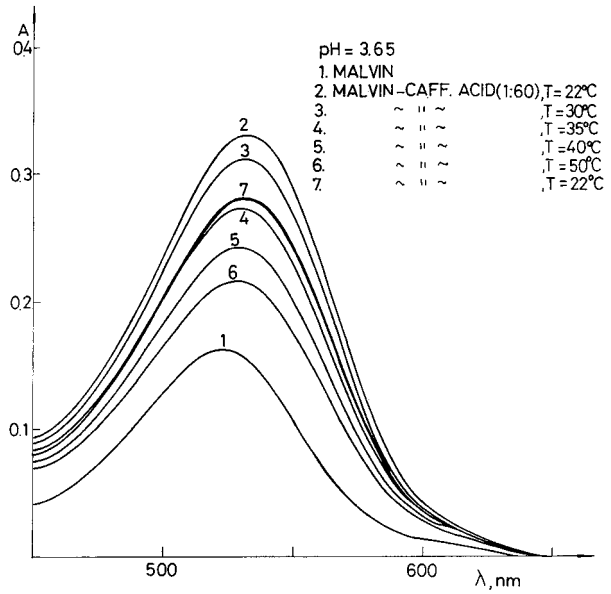

Figure 12. Change in absorption spectra of the copigmentation complex malvin−caffeic acid (molar ratio 1:60) with temperature in buffered solution at pH 3.65.

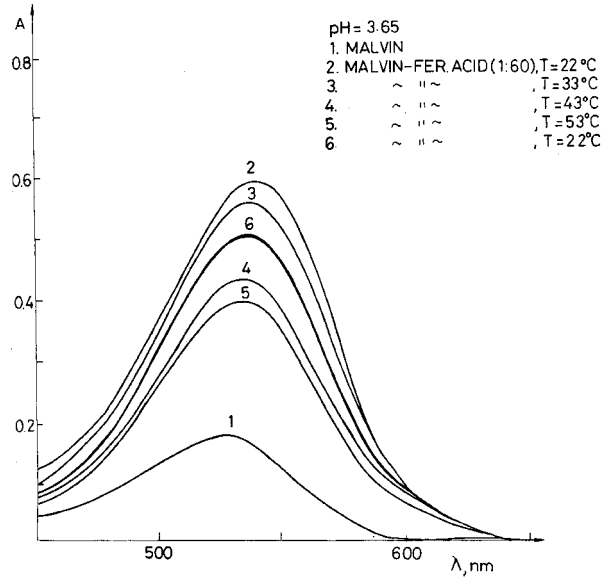

Figure 13. Change in absorption spectra of the copigmentation complex malvin−ferulic acid (molar ratio 1:60) with temperature in buffered solution at pH 3.65.

absorption bands appear in our spectra, as shown in Figure 7, which means that in this case only copigmentation of these molecules takes place.

Observing the behavior and characteristics of the copigments formed reveals a significant effect of the pH value of the environment.

As in the previously studied systems (Baranac et al., 1996, 1997, and 1999), complexes were formed in the stoichiometric ratio 1:1. The calculated equilibrium constants (Table 2) show that the effect of the pH value is significant. At pH 3.65 the value of the equilibrium constant is greater than at pH 2.50. It was also established that the equilibrium constant for the malvin−ferulic acid system is greater than for the malvin−caffeic acid system, at both pH values. The values obtained for thermodynamic function $\Delta G°$ ($\Delta G° < 0$) indicate that the process of copigmentation is spontaneous at the pH values investigated.

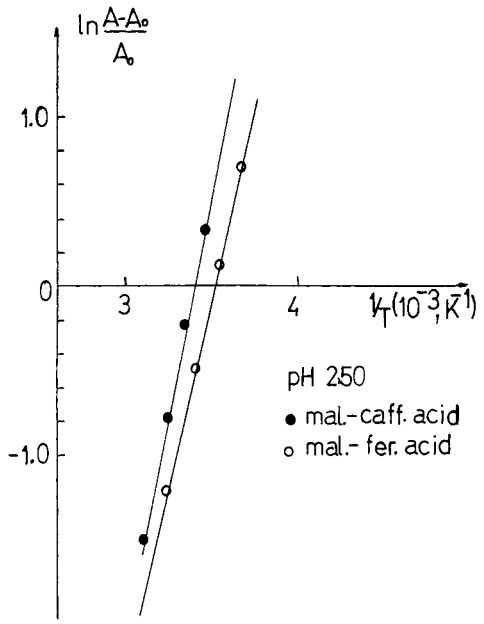

Figure 14. Plot of ln $\{(A - A_0)/A_0\}$ for malvin−caffeic and malvin−ferulic solutions (molar ratio 1:60) as a function of reciprocal temperature for buffered solution at pH 2.50.

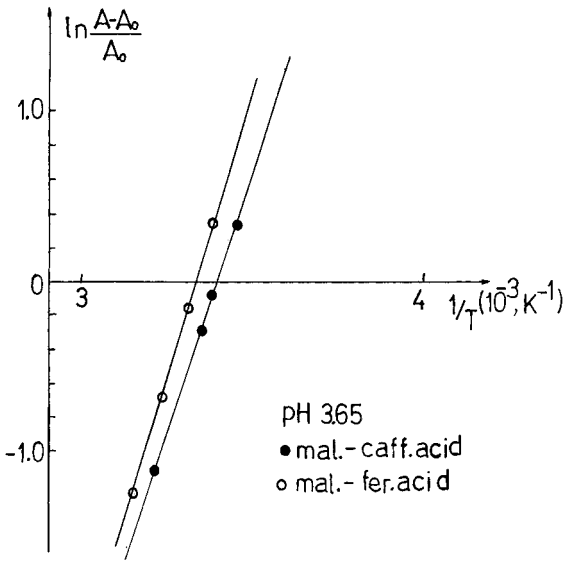

Figure 15. Plot of ln $\{(A - A_0)/A_0\}$ for malvin−caffeic and malvin−ferulic solutions (molar ratio 1:60) as a function of reciprocal temperature for buffered solution at pH 3.65.

Table 2. Thermodynamic Properties of the Process of Copigmentation of Malvin with Caffeic and Ferulic Acid[a]

copigmentation	pH	K	$\Delta G°$ (kJ/mol)	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/molK)	N
malvin−caffeic acid	2.50	33.3	−8.7	−36.9	−94.6	1
malvin−ferulic acid		35.8	−8.9	−43.1	−114.8	1
malvin−caffeic acid	3.65	74.9	−10.7	−49.9	−131.5	1
malvin−ferulic acid		88.8	−11.1	−55.4	−148.6	1

[a] Molar ratio 1:60, T = 298 K.

The effect of temperature, under these conditions, causes a shift of the equilibrium toward the reactants (Figures 10−13). Because abrupt cooling shifts the equilibrium toward product formation it can be concluded that such a dependence on temperature is a consequence of the exothermic copigmentation process, $\Delta H° < 0$. Relatively small changes of $\Delta H°$ in both buffer solutions also indicate low affinity of the reactants,

which was expected, because of large mole ratios of the components applied in the experiments.

The copigmentation was also accompanied by entropy loss, $\Delta S°$, i.e., by gain in the system ordering, which is another proof of complex formation. On the basis of enthalpy changes (Table 2) in the two buffers it can be concluded that the process of copigmentation is possible only at lower temperatures.

The results obtained on the process of copigmentation in vitro show that the affinity of the reactants is low (the bond formed is weak), the reactivity is low, and the complexes formed are stable only at low temperatures. We believe this is of importance in understanding these processes in vivo.

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Received for review January 4, 2000. Revised manuscript received August 2, 2000. Accepted August 3, 2000.

JF000038V