Spectrophotometric Study of Anthocyan Copigmentation Reactions. 2. Malvin and the Nonglycosidized Flavone Quercetin

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Using UV−vis spectrophotometry we have established that a process of copigmentation takes place between an anthocyan molecule, malvin chloride (malvidin 3,5-diglucoside), and a nonglycosidized pentahydroxyflavone, quercetin (3,5,7,3′,4′-pentahydroxyflavone). The kinetic and thermodynamic parameters, by which the process is characterized, were correlated to the structure, i.e., the nature and position of the substituents in the interacting molecules.

INTRODUCTION

As recognized, anthocyanidins and anthocyanosides belong to an important group of plant pigments. One of the factors that stabilizes the color of these compounds in natural media is the process of copigmentation (Nakayama and Powers, 1972; Timberlake and Bridle, 1975). Since the role of molecular structure has been pointed out in the literature (Chen and Hrazdina, 1981), our choice of quercetin was in accordance with that (see structures below). Its structure differs from the previously studied rutin (Baranac et al., 1996) in that it does not contain sugar molecules while having the same number of hydroxy groups. For that reason, the role and effect of the absence of sugar molecules on the copigmentation process characteristics could be studied.
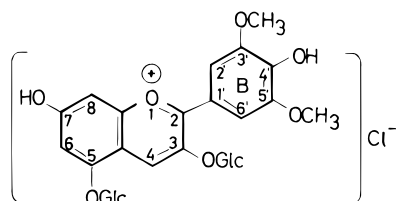
MALVIN CHLORIDE

(MALVIDIN~3,5− DIGLUCOSIDE)
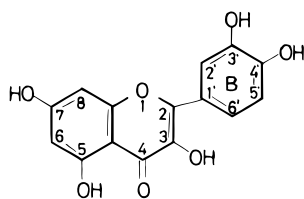
QUERCETIN

(3,5,7,3′,4′-PENTAHYDROXY FLAVONE)

EXPERIMENTAL PROCEDURES

Malvin chloride (malvidin 3,5-diglucoside) (∼97%) ($c = 3.0 \times 10^{-4}$ M) from Aldrich Chemical Co. and quercetin (3,5,7,3′,4′-pentahydroxyflavone) ($c = 3.0 \times 10^{-4}$ M) from Fluka Biochemika were the substances used in this work. UV−vis absorption spectra were recorded by a Pye Unicam SP8-100 spectrophotometer. The temperature was monitored by a Pye Unicam cell temperature controller (range 20−70 °C). Quartz cuvettes of 10 mm path were used.

The buffer solutions, of a constant ionic strength ($I = 0.2$ M), were prepared as mixtures of 0.02 M sodium acetate (Merck) and 0.06 M phosphoric acid (Poole, England). The ionic strength was adjusted by addition of sodium chloride (Merck). The pH of the solutions was measured by an Iskra MA 5730 pH meter, with a combined electrode. Buffer solutions of pH 4.00 and 7.00 were used for the pH meter calibration (NBS standards, Merck).

RESULTS

In the previous paper we have shown that the cation form of malvin, which exists at pH 3.65, takes part in the copigmentation reaction with rutin (Baranac et al., 1996). In order to define the optimal acidic solution for the observation of the copigmentation process, it was necessary to investigate the behavior of malvin in a number of solutions of lower pH values, as well as its stability in time and with temperature.

In this context, we observed malvin UV−vis absorption spectra in different acidic buffer solutions. In Figure 1 it is observable that at pH 2.30 (curve 1) the cation form of its flavylium structure dominates, with an absorption maximum at $\lambda = 520$ nm. After increasing the pH value to pH 3.20 (curve 2), the cation form absorption band is still existent but with reduced intensity. At pH 3.65 (curve 3), the intensity of the cation form absorption band still decreases and bathochromically shifts to $\lambda = 525$ nm. Obviously, the cation form of malvin exists in all acidic buffers used, only its concentration decreases proportionally with the increasing pH of the medium. The absorption spectrum of quercetin, in buffer solution of pH 3.65, is also presented in Figure 1 (curve 4).

Our further investigations dealt with following the stability of acidic malvin solutions in time. The solutions, kept in airtight quartz cuvettes, were measured in time intervals of 2−16 min. The absorbance changes of the longest wavelength maxima are presented in Figure 2 as a function of time, at different pH values. As observable, the most stable malvin solution in the buffer solutions used is the one at pH 2.30 ($k = 1.8 \times 10^{-2}$ min^{-1}) followed by the one at pH 3.65 ($k = 5.0 \times 10^{-2}$ min^{-1}) since their absorbance change is the lowest between two consecutive measurements ($\Delta A = 0.025/$min, $\Delta A = 0.05/$min). Besides the stability in time, we also investigated malvin stability in the same solutions with temperature. The malvin buffer solution at pH 2.30, which showed the best stability in time, was very
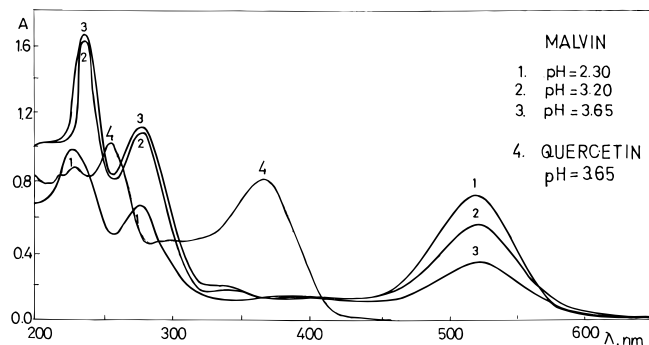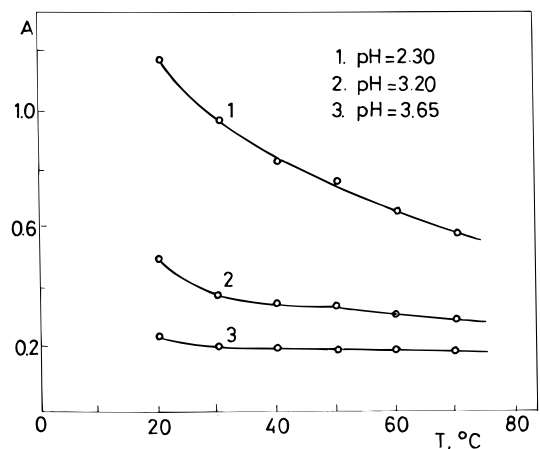
Figure 1. Absorption spectrum of malvin ($c = 3.0 \times 10^{-4}$ M) in different buffer solutions and absorption spectrum of quercetin in a pH 3.65 buffer solution.
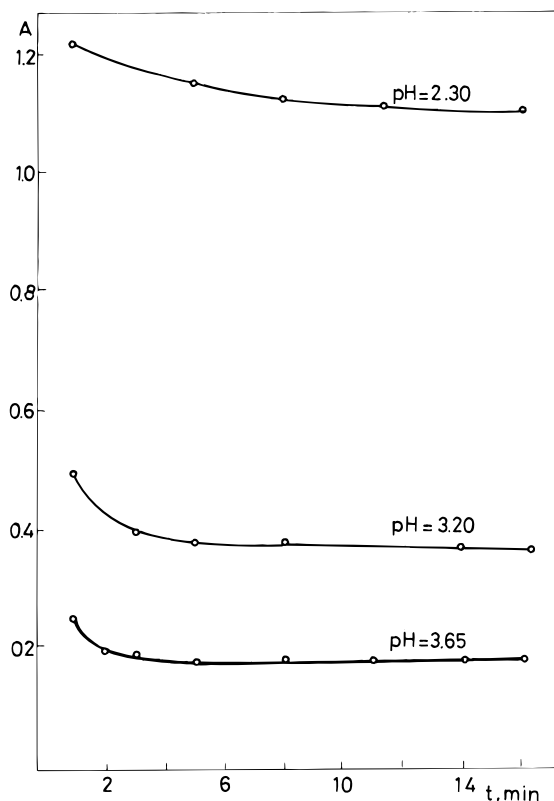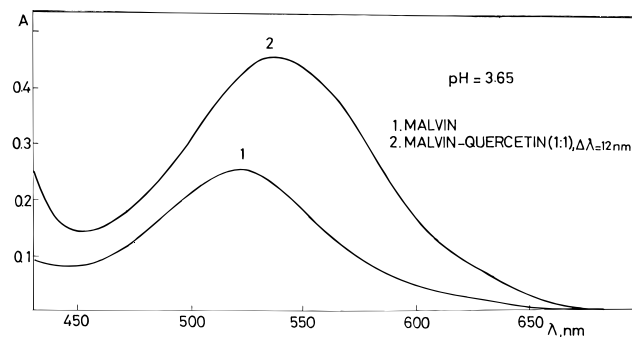

Figure 2. The change in the absorbance of a malvin solution ($c = 3.0 \times 10^{-4}$ M) in time, in different buffers.

Figure 3. The change in the absorbance of a malvin solution ($c = 3.0 \times 10^{-4}$ M) with temperature, in different buffers.

Figure 4. Absorption spectra of malvin ($c = 3.0 \times 10^{-4}$ M) and copigment malvin–quercetin, mole ratio 1:1.
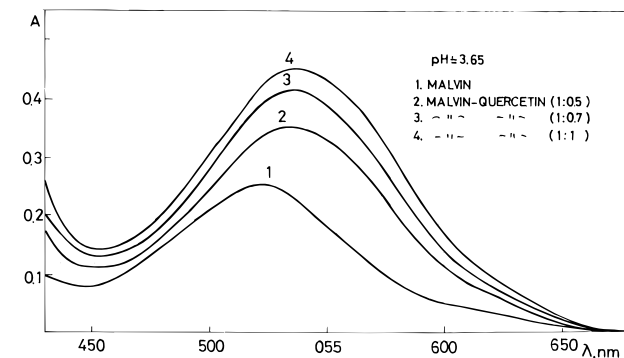
Figure 5. Absorption spectrum of malvin ($c = 3.0 \times 10^{-4}$ M) and copigment malvin–quercetin, mole ratios 1:0.5, 1:0.7, and 1:1.

unstable under a temperature change, as observed in Figure 3. The highest stability with temperature was observed with the pH 3.65 solution and somewhat lower stability with the pH 3.20 solution. In the previous paper (Baranac et al., 1996) we have shown that a temperature increase up to 70 °C does not produce significant changes in the intensity and position of the absorption band of malvin cation in the buffer solution of pH 3.65. Due to the greatest stability shown by time and temperature at pH 3.65, we chose that combination as the optimal one for investigating the copigmentation process. At the same time, the solution of this pH value is the closest in acidity to the solutions of plant juices *in vivo*, which is probably the main reason for its use in research, according to published work (Brouillard et al., 1989).

Our further investigations dealt with copigmentation reactions of quercetin and defining the conditions for them. In Figure 4 the process of copigmentation of the molecules investigated in the pH 3.65 buffer is presented, manifested by a bathochromic shift and a

hyperchromic shift of the malvin cation form absorption band ($\Delta\lambda = 12$ nm, $\Delta A = 0.200$). A change in quercetin concentration affects the magnitude of the shifts of the copigmentation reaction. In order to determine the optimum mole ratio in the copigment formed, for a given malvin concentration, absorption spectra of solutions obtained by mixing the components in varying mole ratios were recorded (Figure 5). The largest bathochromic shift of $\Delta\lambda = 12$ nm, at a malvin concentration of 3.0×10^{-4} M, was observed at a mole ratio of 1:1, which is, at the same time, the optimal mole ratio. An increase of the mole ratio above 1:1 results in precipitate formation. In Figure 6 the dependence of the bathochromic shift of the copigment absorption maximum on quercetin concentration is presented.

Besides the concentration of the molecules in interaction and the pH value, temperature has a significant effect on the reaction. Since the thermal stability of
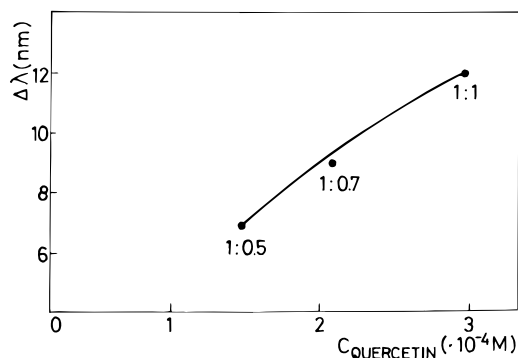
Figure 6. Bathochromic shift of the absorption maximum position of malvin ($c = 3.0 \times 10^{-4}$ M)−quercetin solution as a function of quercetin concentration
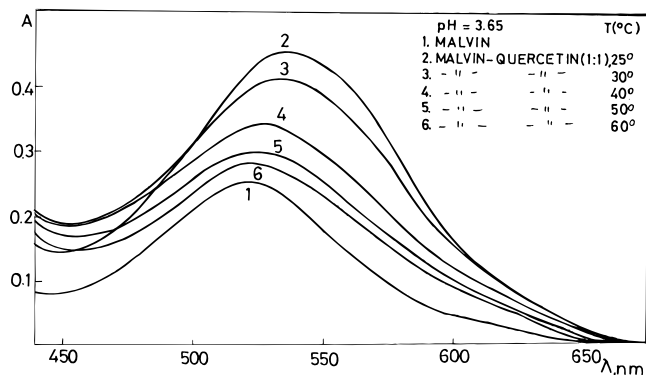
**Figure 7.** Change in absorption spectra of the copigment malvin ($c = 3.0 \times 10^{-4}$ M)−quercetin with temperature (mole ratio 1:1).
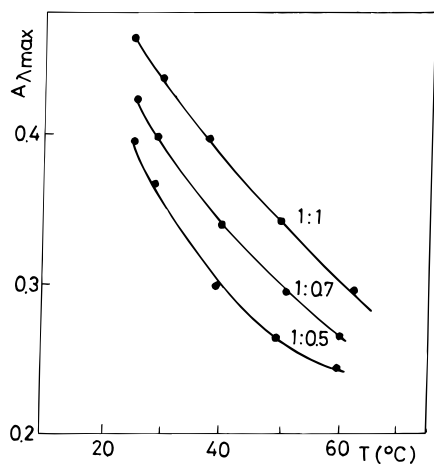

Figure 8. Change in absorption of the copigment as a function of temperature for different malvin ($c = 3.0 \times 10^{-4}$ M)−quercetin mole ratios.

malvin itself has been confirmed, we took to investigating the temperature effect on the copigment formed, at the optimal mole ratio of the components, 1:1 (Figure 7). The system was heated gradually from room temperature, 25 °C, to 60 °C. A temperature increase produces a decrease of the copigment band intensity and its hypsochromic shift. Heating beyond 60 °C leads to occurrence of a voluminous precipitate, which remains after cooling. The effect of temperature is the same at other mole ratios. The change of absorption at absorption maxima as a function of temperature is given in Figure 8, for mole ratios 1:0.5, 1:0.7, and 1:1.

In Figure 9 the dependence of the experimental parameter $\ln[(A - A_0)/A_0]$ (A is absorbance of the copigment solution and A_0 is absorbance of the pure
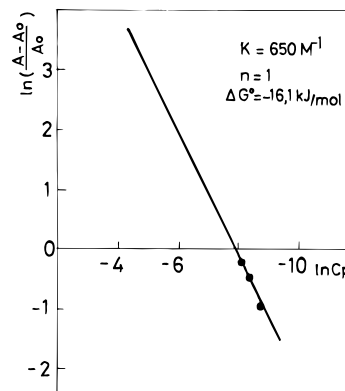
Figure 9. Plot of $\ln[(A - A_0)/A_0]$ as a function of logarithm of quercetin concentration.
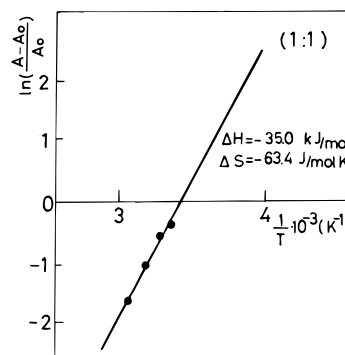
Figure 10. Plot of $\ln[(A - A_0)/A_0]$ as a function of reciprocal temperature (mole ratio 1:1).

malvin solution) as a function of the logarithm of the quercetin concentration determined is presented. From this plot we determined the parameters of the stoichiometric ratio of the components, the equilibrium constant, and the standard Gibbs free energy change. The stoichiometric ratio of the components is 1:1. The constant was determined to be $K = 650$ M^{-1} and the standard Gibbs free energy change $\Delta G° = -16.1$ kJ/mol. From the dependence of the same experimental parameter (Figure 10) on reciprocal temperature, we determined the enthalpy and entropy changes of the process: the enthalpy change is $\Delta H = -35.0$ kJ/mol, and the entropy change is $\Delta S = -63.4$ J/K mol.

DISCUSSION AND CONCLUSIONS

In the initial part of the present work it was concluded that a solution at pH 3.65 is the most stable in time and with temperature, thus being the optimal one for following other copigmentation processes. The bathochromic and hyperchromic effects, which are also characteristic to other investigated systems (Scheffeldt and Hrazdina, 1978; Asen et al., 1972), were the proof of copigment formation between malvin and quercetin. The optimum mole ratio of the components in the copigment is 1:1 (malvin:quercetin), at a malvin concentration of 3.0×10^{-4} M. The stoichiometric ratio of the components in the copigment is, as in the previously investigated system (Baranac et al., 1996), 1:1.

The equilibrium constant for the malvin−quercetin system, determined to be $K = 650$ M^{-1}, is significantly lower than the corresponding value for the malvin−rutin system (Baranac et al., 1996, $K = 3300$ M^{-1}) which, as mentioned, in contrast to quercetin, has two sugar molecules at position 3. We believe that the interaction in the copigmentation process takes place

via hydrogen bonds, which can explain the difference in the constants.

The change of the standard Gibbs free energy, which is $\Delta G° = -16.1$ kJ/mol, indicates that the copigmentation process is spontaneous in the direction of formation of the products. A temperature increase results in a permanent degradation of the copigment, which does not regenerate upon cooling. The reversibility of the copigmentation in the malvin−quercetin system does not exist, in contrast to the malvin−rutin system (Baranac et al., 1996). The enthalpy change of $\Delta H = -35.0$ kJ/mol indicates a greater affinity of the components in the reaction, as compared with the malvin−rutin system ($\Delta H = -26.6$ kJ/mol) (Baranac et al., 1996). By comparing the values obtained for the equilibrium constants, i.e., the $\Delta G°$ values, it was concluded that the copigment formed with quercetin is less stable than the one with rutin, but the affinity of the malvin interaction with quercetin is higher. The negative value of the entropy, $\Delta S = -63.4$ J/K mol, indicates that copigment formation establishes greater order in the system.

The difference in the substituents in rutin (sugar molecules) and quercetin is responsible for the existing differences in the thermodynamic parameters and points to the effect of structure of the reacting molecules on the copigmentation process.

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