Spectrophotometric Study of Anthocyan Copigmentation Reactions

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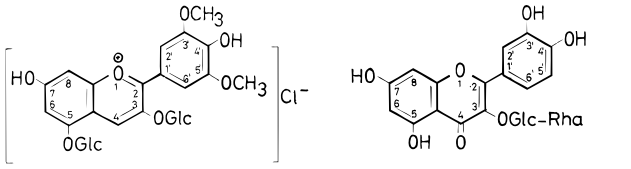
The reaction of malvin chloride (malvidin 3,5-diglucoside) with a flavonoid compound rutin (quercetin 3-rutinoside) is investigated. Reactions of these molecules are observed through UV−vis absorption spectra, to identify the factors that influence the copigmentation as well as the characteristics of the copigment formed. It is established that the copigmentation process takes place in buffer solutions at a specific pH value and that it is conditioned by the mole ratio and temperature. Copigment formation is defined by kinetic and thermodynamic parameters.

Keywords: *Copigmentation; anthocyans; rutin; UV−vis spectra*

INTRODUCTION

Anthocyanidins and anthocyans, as hydroxylated and glycosidized flavylium compounds, are plant pigments responsible for the red, blue, and purple hues of flowers and fruits in nature. Investigating these compounds *in vitro* revealed a change in their structure as a function of the solution pH value, that causes different coloring. Such a structural change is not possible in complex natural media. It is therefore assumed that *in vivo* color changes are caused by other reactions, e.g. complexing reactions of anthocyanins with metal ions (Bayer *et al.*, 1966; Veselinović *et al.*, 1992) or copigmentation reactions (Asen *et al.*, 1972; Scheffeldt and Hrazdina, 1978; Williams and Hrazdina, 1979) with various organic compounds present in higher plants.

The object of the present paper is to investigate the copigmentation reaction between malvin, malvidin 3,5-diglucoside, and rutin, quercetin 3-rutinoside (see structures I and II) and, at the same time, define all factors that influence the reactions and the characteristics of copigments formed.



STRUCTURES OF MALVIN AND RUTIN

EXPERIMENTAL PROCEDURES

The main substances used in the present work are malvin chloride [~97% (Cl)] (malvidin 3,5-diglucoside, $c = 3.86 \times 10^{-4}$ M) from Aldrich Chemical Co. and rutin (quercetin 3-rutinoside, $c = 3.86 \times 10^{-4}$ M) from Fluka Biochemika. Prior to use, rutin is repurified by recrystallization from methanol and characterized by a DSC curve obtained on a DuPont 1090 thermal analyzer.

UV−vis absorption spectra were recorded by a Pye Unicam SP8-100 spectrophotometer in a series of solutions of a constant ionic strength ($I = 0.2$ mol dm^{-3}). The solutions are prepared as mixtures of 0.02 M sodium acetate and 0.06 M phosphoric acid. All solutions are kept in the dark before and after their spectra are recorded.

RESULTS

To follow the reaction of copigmentation through UV−vis absorption, the spectra of malvin are defined in solutions of various pH values. In an acidic medium, at pH 3.65 (Figure 1, curve 1), the spectrum of pure malvin shows a characteristic absorption of the cation form of the flavylium structure, with peak absorption at $\lambda_{max} = 525$ nm, but also at $\lambda = 276$ nm, with an inflection at $\lambda_i = 336$ nm. These would correspond to a pseudobase (B) and chalcone (C), respectively. At decreased acidity, in weakly acidic and neutral buffer solutions, pH 5.00 and 7.90 (Figure 1, curves 2 and 3), the malvin spectrum ceases to show a flavylium structure absorption band. The last two maxima indicate the anhydrobase (curve 2) and ionized anhydrobase (curve 3) form formation. The same figure also contains a spectrum of rutin in a pH 3.65 buffer (dashed curve) with an absorption band at $\lambda = 348$ nm.

An investigation of the stability of a pure malvin solution with temperature, in a pH 3.65 buffer (Figure 2), shows that the main malvin absorption band in the visible range undergoes a slight intensity change ($\Delta A = 0.06$), while the band position remains unchanged.

After the malvin structure and its stability were investigated with respect to temperature, the reaction of copigmentation was followed in a pH 3.65 buffer solution. UV−vis absorption spectra of malvin−rutin solutions in various mole ratios (1:0.5; 1:1; 1:2) are recorded. It is observed that ratio 1:2 is an optimum in this interval (Figure 3). The absorption spectra in Figure 3 show that copigment formation is manifested by a shift of the absorption maximum ($\Delta\lambda = 24$ nm) and by an increase in the intensity of this maximum with respect to the pure malvin spectrum ($\Delta A = 0.93$) (Figure 3, curve 1). The magnitude of the bathochromic shift of the absorption maximum of the copigment formed clearly depends on the concentration of the introduced rutin (Figure 4). Besides the rutin concentration, the temperature effect on the copigment was also investigated. Each malvin−rutin solution is heated from 25 to 70 °C. In Figure 5 only the behavior of the solution

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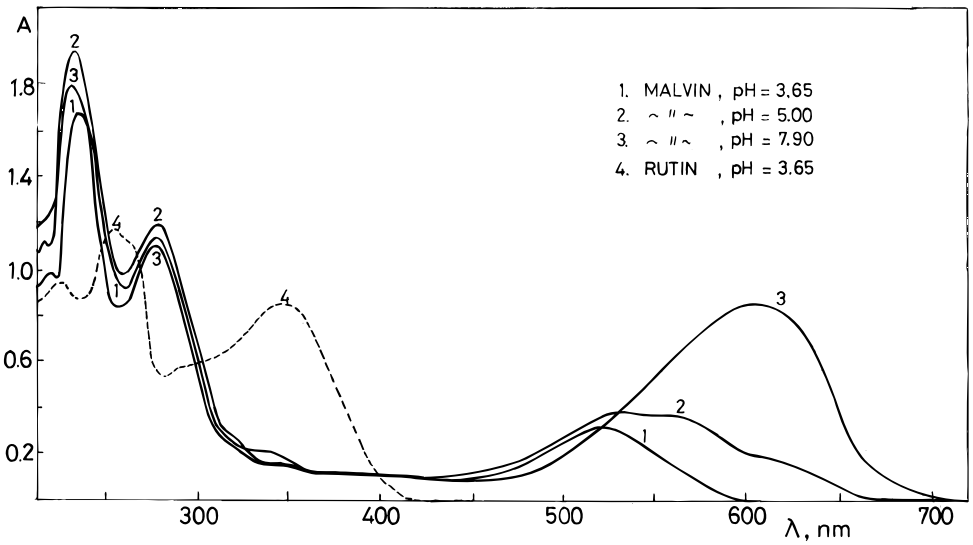

Figure 1. Absorption spectrum of malvin ($c = 3.86 \times 10^{-4}$ M) in different buffer solutions.

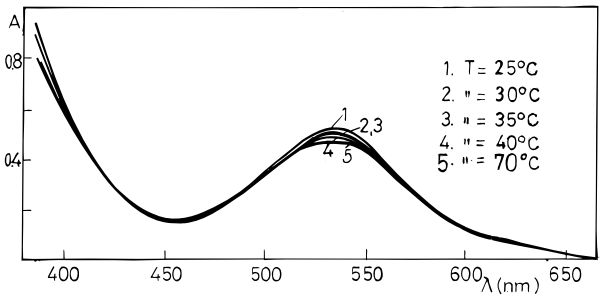

Figure 2. Change of malvin absorption ($c = 3.86 \times 10^{-4}$ M) with temperature in a pH 3.65 buffer solution.

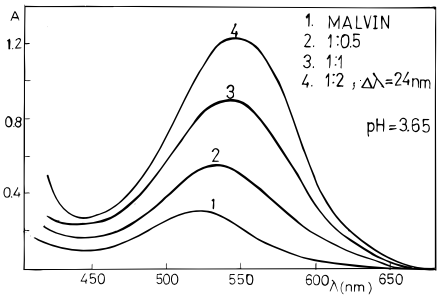

Figure 3. Absorption spectrum of malvin (1) ($c = 3.86 \times 10^{-4}$ M) and copigment malvin−rutin, mole ratios 1:0.5 (2), 1:1 (3), and 1:2 (4).

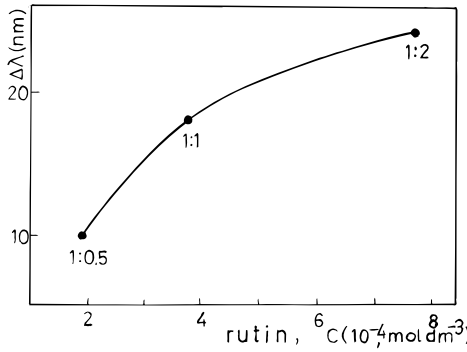

Figure 4. Shift of the absorption maximum position of a malvin ($c = 3.86 \times 10^{-4}$ M)−rutin solution as a function of rutin concentration.

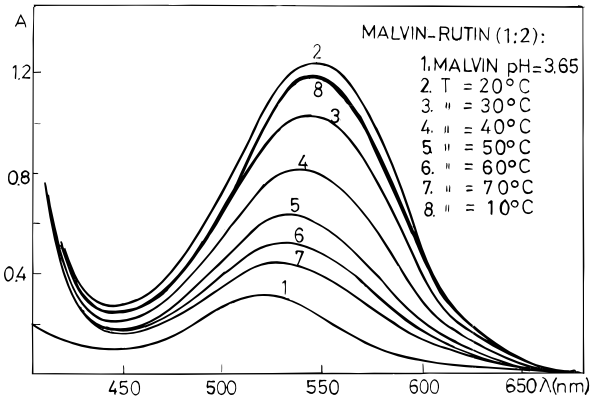

Figure 5. Change in absorption spectra of the copigment malvin ($c = 3.86 \times 10^{-4}$ M)−rutin (mole ratio 1:2) with temperature.

of optimum ratio, 1:2, is shown (curves 2−7). Heating causes a hypsochromic and hypochromic effect on the copigment absorption band. At 70 °C (Figure 5, curve 7) the absorption maximum position is shifted closer to the pure malvin maximum (Figure 5, curve 1). Cooling the solution to 10 °C regenerates the copigment, as is observable in Figure 5, curve 8. The effect of temperature is the same at all mole ratios of malvin and rutin mixtures. The change of absorbance as a function of temperature at wavelengths of absorption maxima (Figure 6) shows that the copigmentation effect decreases with increasing temperature, regardless of the rutin concentration (see the extrapolated parts in Figure 6), which indicates a thermodynamic character of the system, i.e. a negative enthalpy.

From the plot of $\ln(A - A_0/A_0)$ (A = absorbance of the malvin−rutin solution; A_0 = absorbance of free malvin), the parameter that represents a key experimental property and a measure of the copigmentation effect under given conditions, as a function of the analytical rutin concentration ($[C_p]_0$) (Figure 7) values of the copigment equilibrium constant, are derived, together with the stoichiometric ratio of its components. The plot $\ln(A - A_0/A_0)$ versus reciprocal temperature, presented in Figure 8, enables a determination of enthalpy and an assessment of entropy of the copigmentation process.

It is apparent from the plots that the copigment component ratio is 1:1. The value of copigmentation constant, which represents a quantitative measure of the strength of the copigment formed, is 3300 M^{-1}, while the enthalpy change is $\Delta H = -26.6$ kJ mol^{-1}. The

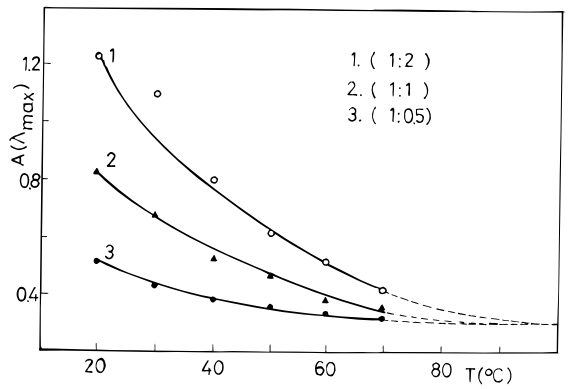

Figure 6. Change in absorption of the copigment as a function of temperature for different malvin ($c = 3.86 \times 10^{-4}$ M)−rutin mole ratios.

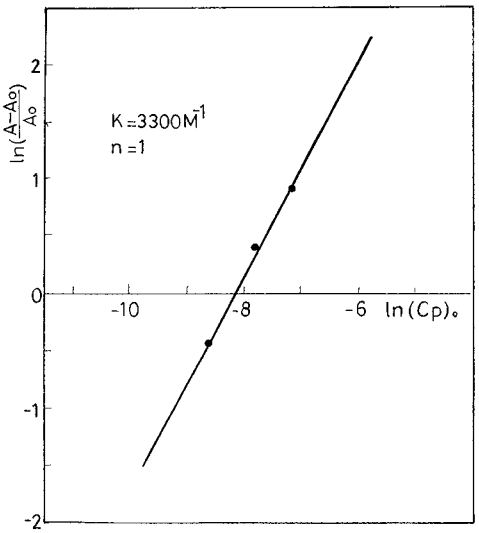

Figure 7. Plot of $\ln(A - A_0/A_0)$ as a function of rutin concentration.

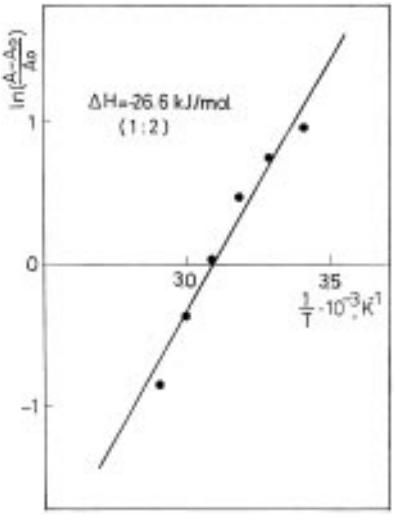

Figure 8. Plot of $\ln(A - A_0/A_0)$ for a malvin ($c = 3.86 \times 10^{-4}$ M)−rutin solution (mole ratio 1:2) as a function of reciprocal temperature.

copigmentation process is accompanied by a decrease in entropy (see Figure 8).

DISCUSSION AND CONCLUSIONS

The possibility of copigmentation as a very important process, especially *in vivo*, that is manifested in deepening and intensifying of anthocyan coloring is established in the present work.

In the context of the interaction between malvin and rutin, the largest bathochromic and hyperchromic effect on the absorption maximum of the malvin cation form is taken as a criterion for defining the optimum conditions for its formation. According to the presented results, the formed copigment malvin−rutin depends on the pH value of the solution and on the rutin concentration. The optimum acidity for its formation is pH 3.65, and the optimum mole ratio of malvin to rutin is 1:2 for the pH and copigment range we have studied. Various mole ratios between malvin and rutin are investigated, and they show a greater affinity between rutin and malvin than between them and other molecules investigated (Brouillard *et al.*, 1989), because the reaction takes place with larger absorption maximum wavelength change even with 10 times lower concentrations. This is supported by the derived equilibrium constant of the copigment (Figure 7) for the stoichiometric ratio 1:2.

The effect of temperature is the same at all observed mole ratios (Figure 6). Lowering the temperature shows a regeneration of the copigment, which indicates a thermodynamic process.

The obtained values of enthalpy change (Figure 8, $\Delta H = -26.6$ kJ mol^{-1}) define the process of copigmentation as a spontaneous and exothermic one. Since the assessed values of entropy change are negative, they indicate an increase in the ordering of the investigated system by the copigmentation process.

Everything presented above indicates a formation of a certain copigment structure that we cannot say more about, at this point. It can only be said that the copigment is formed in the interaction of the malvin cation form (Figure 1, curve 1) and rutin. This is also confirmed by the effect that copigmentation has on the absorption maximum at $\lambda = 525$ nm, which corresponds to the cation form. At pH 3.65, in the aqueous medium that is used, the pseudobase form exists too (Figure 1, curve 1), while an anhydrobase form is absent. Assumptions by other authors, that at pH 3.65 an anhydrobase exists and possibly takes part in the copigmentation process, do not have ground (Sheffeldt and Hrazdina, 1978; Williams and Hrazdina, 1979). Our results show that the anhydrobase is absent from the investigated solution at pH 3.65, so we consider that the copigmentation process takes place primarily with the cation form of malvin. According to literature data, it appears that the role of water is very significant, because copigmentation does not take place after methanol and other molecules are added into the copigment buffer solution (Chen and Hrazdina, 1981). The fact that copigments are situated in plant cell vacuoles, which contain anthocyans and water, supports the assumption about the role of water in the formation and structure of the copigment. We can, for now, on the basis of Brouillard's claim (Brouillard *et al.*, 1989) assume that the essence of copigmentation is a certain process of association of the malvin cation and rutin. It seems that the role of the formed copigment is to protect the cation structure of malvin, which is colored, and important due to that. It is known that the change in malvin structure *in vitro* occurs during the increase of the solution pH value by the process of deprotonation or by its disturbance and transition into a pseudobase by hydration. The latter process is evident in our buffer solution (Figure 1, curve 1) through the existence of

pseudobase band at $\lambda = 276$ nm. Since a change in the pH value is not possible in natural media, it means that only hydration takes place, and it consists of water addition to position 2 of the flavylium nucleus, which causes a disruption in the flavylium structure. We therefore assume that the role of the copigment is exactly in suppressing the hydration and intensifying the coloring. The significance of this paper, with respect to already published data, is in certain kinetic and thermodynamic parameters which undoubtedly better emphasize the essence of the copigmentation process.

These processes are important because, *in vitro*, they can protect these anthocyan molecules that are present in various colored food products. Their investigation has thus a high applicative value.

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