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Spectrophotometric and thermodynamic study on the co-pigmentation interaction between strawberry anthocyanins and quercetin in model systems

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Changes in the visible spectra of strawberry anthocyanins were studied depending on the addition of quercetin as a co-pigment. The investigations were carried out at different temperatures between 20ºC and50 ºC. The solution with a molar pigment:co-pigment ratio of 1:6 exhibits absorption maxima(*Amax*) at 20 and 30 ºC; in solutions with molar ratios of 1:8 and 1:10 *Amax* decreases. At temperatures of 40 and 50 ºC *Amax* gradually increases with the increase in the concentrations of strawberry anthocyanin and quercetin, accompanied by bathochromic and hyperchromic effects. Experiments were performed at pH=3.4 (0.1 M McIlvaine buffer). The thermodynamic parameters of the system, ΔG, ΔH and ΔS were calculated as a function of temperature.

Keywords: Co-pigmentation, anthocyanins, quercetin, thermodynamics, equilibrium constant

INTRODUCTION

Anthocyanins represent one of the most important and most widespread 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 [1]. Unfortunately, accelerated pigment degradation occurs during conventional processing and storage due to the low total content of strawberry anthocyanins [2] and their inherent heat and light sensitivity [3]. Therefore, retention of strawberry color has always been a technological challenge. Co-pigmentation is regarded today as one of the significant factors of structure stabilization and coloration of anthocyanins under *in vivo* conditions. In the last years, pigment:co-pigment couples were studied by different authors and thermodynamic parameters of the systems were determined. Marcovic *et al.* [4] investigated the process of co-pigmentation of the anthocyanin molecule malvidin with two organic acids - caffeic and ferulic acid by electronic absorption spectroscopy. Equilibrium constant, Gibbs energy and entropy of the co-pigmentation process were calculated. Color intensification of anthocyanin solutions in the presence of natural polyphenols (co-pigmentation) was re-interpreted in terms of charge transfer from the co-pigment to the anthocyanin by Da Silva *et al.* [5]. It was also demonstrated, for a large series of anthocyanin-co-pigment pairs, that the standard Gibbs free energy of complex formation linearly decreases on increasing the difference between the electron affinity of the anthocyanin, EA(Anthoc), and the ionization potential of the co-pigment, IP(Cop).Co-pigmentation of malvin was investigated by Olivera *et al.*[6] and Baranc *et al.* [7]. The former authorsstudieda series of naturally occurring colorless organic molecules (co-pigments) by UV-visible absorption and determined the co-pigmentation stability constant. Co-pigment formation was defined by kinetic and thermodynamic parameters obtained by the authors of [7]. Brouillard *et al.* [8] investigated the system caffeoylquinic acid:flavylium cation of malvin chloride(1:1)using visible absorption spectrophotometry. Using UV-vis spectrophotometry, Baranac *et al.* [9] established that a process of co-pigmentation takes place between an anthocyan molecule, malvin chloride and a non-glycosidized pentahydroxyflavone. Oszianski *et al.* [10] investigated the process of co-pigmentation between isolated acylated anthocyanin and rutin. The thermodynamic parameters were related to the structure and position of the substituents in the interacting molecules. Rose petal polyphenols were foundas stabilizing agents for strawberry anthocyanins in real beverage systems by Mollov *et al.* [11] and in heated model systems by Shikov *et al.* [12]. Recently, Shikov *et al.* [13-15] investigated canned and frozen fruits and strawberry anthocyanins in model solutions depending on the addition of rose petal polyphenols acting as co-pigments.

No investigation of the system strawberry anthocyanin and quercetin was found in the literature so far. The present study aimed at performing a spectrophotometric investigation on the system

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pigment:co-pigment to determine its stability at different temperatures using the equilibrium constant of the transfer from the co-pigment to the anthocyanin and other thermodynamic parameters.

EXPERIMENTAL

Chemicals

The co-pigment quercetin dihydrate (97%)was from GmbH&CoAG Company, Germany. The reagents used for preparing the McIlvaine buffer of pH 3.4 - citric acid monohydrate and disodium hydrogen phosphate dodecahydrate - were from Merck (Darmstadt, Germany). The adsorbent resin Amberlite XAD 16N was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). All other reagents and solvents used were of analytical grade.

Extraction, purification and determination of anthocyanins

Strawberry anthocyanins were extracted and purified as described by Shikov *et al*. [12]. Frozen strawberries (*Fragaria×ananassa* Duch. cv. Siabelle, harvest 2014) were thawed and manually squeezed in a beaker. The homogenized purée was extracted overnight at 4 °C using methanol acidified with hydrochloric acid (1%, v/v) at a solvent/solid ratio of 2.5:1 (v/w). The extraction mixture was filtered and the organic solvent was evaporated under vacuum at30 °C. To remove sugars, salts, and amino acids from the crude extracts, the samples were purified using a column (465 × 30 mm i.d.) filled with the adsorption resin Amberlite XAD 16N. Prior to sample application, the resin was conditioned and equilibrated by rinsing with 500 ml of methanol and 1000 ml of water, and acidified with trifluoroacetic acid (TFA, pH 2). Subsequently, 250 ml of the aqueous strawberry extract were applied and the column was rinsed with 1000 ml of acidified water (pH 2). For elution of the pigments, at least 500 ml of a mixture of methanol and acidified water (TFA, pH 2) (95:5, v/v) was applied until the column was colorless. The organic solvent was evaporated from the eluate under vacuum (30 °C). To separate anthocyanins from colorless phenolics, further purification was performed by extracting the aqueous phase three times with the same volume of ethyl acetate. After evaporation and concentration under vacuum (30 ^{0}C), the residue was lyophilized for 72 h.

The total monomeric anthocyanins were assessed by the pH-differential method, as described by Shikov *et al*. [14].The results were expressed as pelargonidin 3-glucoside equivalents.

Preparation of model solutions

Stock solutions of strawberry extract, on the basis of the total anthocyanins, and quercetin dehydrate, were prepared in McIlvaine buffer (0.1 M, pH 3.4).Model solutions of strawberry anthocyanins (1×10^{-4} M)were obtained by mixing equal volumes (5 ml) of the corresponding stock solutions and were left for equilibration (30 min at 25 ^{o}C).

Spectrophotometric measurements

Absorption spectra from 380 to 780 nm were recorded with a Helios Omega UV-Vis spectrophotometer equipped with VISION*lite* software (all from Thermo Fisher Scientific, Madison, WI, USA) using 1 cm path length cuvettes.

Before measurements, the samples were thermostated (VEB MLW PRÜFGERÄTE-WERK Medingen,Sitz Freital, Germany) at 20, 30, 40 or 50 °C, respectively.

Statistical analysis

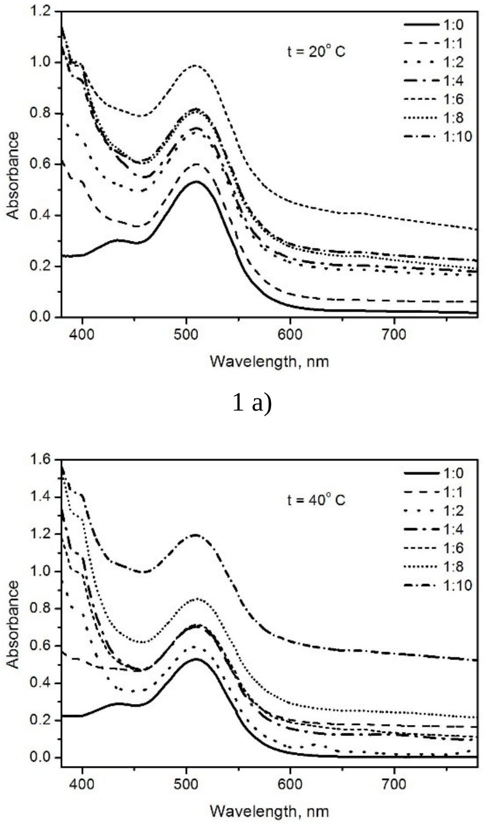
The results reported in the present study are the mean values of at least two determinations and the coefficients of variation were found to be below 2% in all cases. Linear regression analysis was performed using the statistical package of Microsoft Excel.

RESULTS AND DISCUSSION

Since the anthocyanin concentration in the model solutions was constant, the spectral variations (Figure 1) caused by the co-pigmentation reaction depended on the concentration of quercetin. Figure 1a presents the system at 20 ^{0}C. At this temperature some deviation from the results of co-pigmentation effect at different concentrations appeared. With an increase inco-pigment concentrations no bathochromic effect but only hyperchromic effect appeared. The results of this investigation are presented in Table 1. Da Silva *et al*. [5] studied the system malvin:ferulic acid (pigment:co-pigment) and observed similar results. At a ratio between strawberry anthocyanins and quercetin = 1:6,a large absorption maximum appeared, while with other concentrations of co-pigment and at pigment:co-pigment ratios of1:8 and 1:10, the absorption maximum decreased. The same effect was observed in the system at a temperature of 30^{o}C. These results are probably connected with interactions during the co-pigmentation process which stabilized the system at this ratio(1:6). Probably a more stable complex was obtained that decomposed at a higher temperature. In Figure 1b the results of the system strawberry anthocyanins:quercetin at a temperature of 40 ^{0}C are shown. Co-pigmentation of these molecules at this temperature shows expected results - the absorption maximum increases with the increase inco-pigment concentration. In the studied solutions, bathochromic and hyperchromic effects on the main quercetin absorption peak at 40and 50 ^{0}Caremanifested. The respective magnitudes of the bathochromic and hyperchromic shifts at a ratio of 1:10 are observed at a higher temperature.

Table 1. Bathochromic ($\Delta\lambda_{max}$) and hyperchromic (ΔA%) effects in model solutions of strawberry anthocyanins and quercetin at different molar ratio pigment/co-pigment

Molar ratio pigment/co-pigment	$\Delta\lambda_{max}$	ΔA%
	t = 20 ^{0}C	
1:0	-	-
1:1	0	10.7
1:2	0	37.5
1:4	0	58.8
1:6	1	72.3
1:8	0	49.1
1:10	1	58.8
	t = 30 ^{0}C	
1:0	-	-
1:1	1	12.5
1:2	0	52.3
1:4	0	54.3
1:6	1	87.6
1:8	1	74.2
1:10	1	54.3
	t = 40 ^{0}C	
1:0	-	-
1:1	2	43.4
1:2	2	12.9
1:4	1	34.3
1:6	2	35.2
1:8	2	61.4
1:10	1	107.4
	t = 50 ^{0}C	
1:0	-	-
1:1	1	37.0
1:2	1	16.9
1:4	3	31.1
1:6	3	22.6
1:8	3	38.4
1:10	1	140



1 a)

1 b)

Fig.1. Absorption spectra of purified strawberry anthocyanins in model solutions with added quercetin at different pigment:co-pigment molar ratios (1:0, 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10) at 20 ^{0}C (a) and 40 ^{0}C (b).

The equilibrium constant K and the thermodynamic parameters: Gibbs free energy, enthalpy and entropy for the co-pigmentation reaction, are presented in Table 2. The constant was calculated using the following equation: $\ln[(A - Ao)/Ao] = \ln[K] + n \times \ln[C]$, where A and Ao are the absorption maximum values of the anthocyanin solution with and without added co-pigment, respectively; C is the molar co-pigment concentration; K is the equilibrium constant and n is the stoichiometric ratio of the reaction[16]. The dependence of $\ln[(A - Ao)/Ao]$ on the co-pigment concentration, $\ln[(A - Ao)/Ao] = f(\ln[C])$, is a straight line with a slope and intercept equal to n and $\ln[K]$, respectively.

The equilibrium constant exhibited different values at different temperatures.

The thermodynamic parameters: Gibbs free energy, enthalpy and entropy were calculated using the following equations [17]:

$$\Delta G = -RT \ln Kp \qquad (1)$$

where R is the universal gas constant (R = 8.314 J K^{-1} mol^{-1}), T is the absolute temperature (K), ΔG is

Gibbs free energy (kJ mol^{-1}), K – equilibrium constant.

The enthalpy was calculated by the Van't-Hoff equation:

$$\frac{d\ln K}{d(1/T)} = \frac{-\Delta H}{R} \quad (2)$$

ΔH is the enthalpy for the co-pigmentation reaction (kJ mol^{-1}).

Once the Gibbs free energy and the enthalpy were obtained, the entropy was determined by the classical thermodynamic equation (3):

$$\Delta S = \frac{(\Delta H - \Delta G)}{T} \quad (3)$$

ΔS is the entropy for the co-pigmentation reaction (kJ K^{-1} mol^{-1}).

At 30 °C the constant showed the highest value. At the same temperature the Gibbs energy exhibited the highest value – 22.105 kJ mol^{-1}. This result shows that the system is most stable at 30 ^{0}C. At temperaturesabove30 ^{0}C, the stability decreased and interactions between pigment and co-pigment slowed down. At all temperatures negative Gibbs energies were calculated. These results are connected with the spontaneous process of co-pigmentation leading to formation of the products. On the basis of Gibbs energy changes (Table 2) at the four temperatures it can be concluded that the process of co-pigmentation is possible only at temperatures up to30 ^{0}C.

With temperature increase the permanent degradation of the co-pigment does not regenerate upon cooling. There is no reversibility of the co-pigmentation in the malvin-quercetin system, contrary to the malvin-rutin system [9]. In this work the same results were observed as in themalvin-quercetin system [9]. The enthalpy and entropy changes of the process were also negative at all temperatures. It can be concluded that such a dependence on temperature is a consequence of the exothermic co-pigmentation process, ΔH° < 0. The negative value of the entropy, ΔS, indicates that co-pigment formation establishes greater order in the system.

Figures 2-4 present the thermodynamic parameters as depending of different parameters.

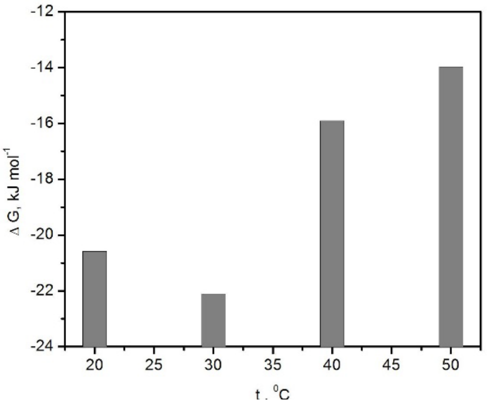


Fig. 2. Temperature dependence of Gibbs free energy for the co-pigmentation interaction between strawberry anthocyanins and quercetin.

The graphical view of Gibbs free energy dependence on the temperature is presented in Figure 2. The stability of the system is determined by the decrease in Gibbs energy to a minimal negative value. On the figure the minimal value is observed at 30 ^{0}C. The second temperature close to the first one was 20 ^{0}C. At the other two temperatures, 40 and 50 ^{0}C, the Gibbs energy increased to positive values, which is connected to the break-down of the pigment: co-pigment bond and destroying of the system.

In Figure 3 a good correlation between Ln of the equilibrium constant and different temperatures is observed. The spots exhibit linear dependence with a deviation at 30 ^{0}C due to the increasein the equilibrium constant at this temperature. According to Brouillard *et al.* [18] the temperature has a major influence on the degradation kinetic plots of Ln(K) against 1/T (K^{-1}) for each of the Hibiscus anthocyanins. These experimental results confirm that with increase of temperature, Gibbs free energy increases to positive values too. Figure 4 presents the linear dependence between enthalpy and entropy change in the strawberry anthocyanins:quercetin system.

Table 2. Equilibrium constants and thermodynamic parameters for the co-pigmentation interaction between strawberry anthocyanins and quercetin at different temperatures and stoichiometric ratio n=1.1:1.

T, ^{0}C	K [M^{-1}]	ΔG [kJ mol^{-1}]	ΔH [kJ mol^{-1}]	ΔS [kJ K^{-1} mol^{-1}]
20	4677.351	-20.585	-25.992	-0.0184
30	6456.542	-22.105	-27.232	-0.0169
40	449.883	-15.897	-25.121	-0.0294
50	181.970	-13.974	-24.867	-0.0337

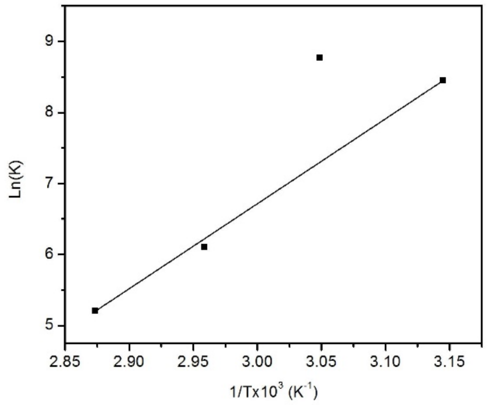


Fig.3. Plot of Ln(*K*) for the co-pigmentation interaction between strawberry anthocyanins and quercetin as a function of reciprocal temperature.

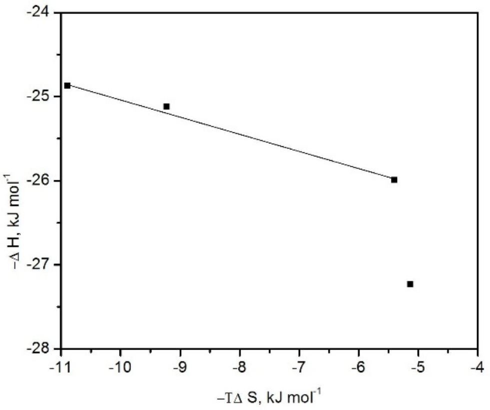


Fig.4. Plot of enthalpy-entropy compensation for the co-pigmentation interaction between strawberry anthocyanins and quercetin.

Similarly, the looser contact between pigment molecules and co-pigments, suggests enthalpy-entropy compensation. The compensation temperature was 30 ^{0}C and at this temperature the maximum value of enthalpy was observed, connected with deviation of the straight line.

CONCLUSIONS

In this study, the thermal stability of isolated strawberry anthocyanins was examined in model solutions in the presence of quercetin as a co-pigment at four temperatures. The study in the temperature range 20–50 ^{0}C revealed that the system was most stable at30 ^{0}C. This result is connected with the Gibbs free energy determined which exhibits the lowest negative value at the same temperature. Based on the experimental and calculated results of this work, further studies would be necessary for finding out appropriate processing conditions in small temperature intervals. More efficient utilization of this pigment is topicalfor food products.

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СПЕКТРОФОТОМЕТРИЧНО И ТЕРМОДИНАМИЧНО ИЗСЛЕДВАНЕ НА КО-ПИГМЕНТАЦИОННИ ВЗАИМОДЕЙСТВИЯ МЕЖДУ АНТОЦИАНИ ОТ ЯГОДИ И КВЕРЦЕТИН В МОДЕЛНИ СИСТЕМИ

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(Резюме)

Изследвана е промяната на видимите спектри на антоциани от ягоди с добавка на кверцетин, като ко-пигмент. Изследванията са проведени при различни температури от 20 до 50 ^{0}C. В разтвора приготвен с моларно съотношение 1:6 пигмент:ко-пигмент са измерени максимални абсорбции при температури 20 и 30 ^{0}C, а в другите два разтвора 1:8 и 1:10 стойностите на измерената абсорбция намаляват. При другите две температури 40 и 50 ^{0}C, измерената абсорбция се повишава с повишаване на концентрацията на разтворите, като са наблюдавани хиперхромен и батохромен ефекти.Експериментите са проведени при pH=3.4 (0.1 M Буфер на Макливаин). Пресметнати са термодинамичните параметри на системата ΔG, ΔH и ΔS като функция на температурата.