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The Copigmentation Interactions between Strawberry Anthocyanins and High Concentration Caffeic Acid with Different Methods

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Running title: **The Copigmentation Reaction between Anthocyanins and Polyphenols**

Abstract
Interactions between strawberry anthocyanins as pigment and caffeic acid as copigment were studied. Investigations were done in different temperatures from 20°C to 50°C at heating system and cooling the same system at 50 to 20°C. The system was investigated with high concentration of caffeic acid from 1:20 to 1:100 molar ratio. The thermodynamic parameters ΔG, ΔH and $\Delta S$of the system were calculated as function of temperature at heating and at cooling. Obtained results confirmed that the interaction between pigment:copigment complex was destroyed at heating to 50 °C"and with following cooling to 20°C was not seen reversibility of the copigmentation process.

Practical applications
The copigmentation process connected with the color stability. Investigation of the system applies to known and protected color in different temperatures.

Key words: anthocyanins, copigmentation effect, caffeic acid, Gibbs free energy, equilibrium constant

Introduction

Copigmentation is a solution phenomenon in which pigments and other noncolored organic components form molecular associations or complexes. It generally results in an enhancement in the absorbance and in some cases, a shift in the wavelength of the maximum absorbance of the pigment (Bulton, 2001).

In model wine solutions (Kunsági-Máté et al.,2008) studied the complexation behavior of malvidin with ellagic and caffeic acid and ferrous and ferric ions. The results show the significant role of caffeic acid in the anthocyanin–polyphenol interaction (also known as copigmentation).

Investigated formation kinetics of malvidin-polyphenol copigmentation complexes was studied (Kunsági-Máté et al., 2009) too.Two reaction channels were examined according to the order of complex formation. Evaluation of the Arrhenius parameters of the reaction shows that the activation energy of the first reaction channel is lower and the frequency factor is higher supporting a higher reaction rate.

The red raspberry extracts were selected as pigments and five phenolic acids include caffeic acid as copigments(Sun et al., 2010). The influences of pH, temperature, structure of anthocyanins and copigments, and molar ratio of anthocyanins to copigments on the copigmentation effect were analyzed with a UV–Visible spectrophotometer. The reaction was thermodynamically defined in terms of ΔH^0, ΔG^0 and ΔS^0 values. Moreover, the calculated thermodynamic data indicated that the copigmentation reaction was more favorable at pH 4.0 than at pH 3.2 for the same anthocyanin and more favorable for cyaniding 3-glucoside than for cyaniding 3-sophorosideat the same ph.

Copigmentation reactions between five anthocyanins and five phenolic acids acting as copigments were investigated (Heinonen et al., 2002). The strongest copigments for all anthocyanins were ferulic and rosmarinic acids.

The color intensity of pelargonidin 3-glucoside increased greatly throughout the storage period with the addition of ferulic and caffeic acids.

Rose petal polyphenols were demonstrated as stabilizing agents for strawberry anthocyanins in real beverage system by (Mollov et al., 2007) and in heated model system by (Shikov et al., 2008). Recently were (Shikov et al., 2012, 2013) investigated canned and frozen fruits and strawberry as anthocyanins in model solutions depending on the addition of rose petal polyphenols acting as copigments.

In the present work the thermodynamics of the molecular association process between strawberry anthocyanins as pigment and caffeic acid as copigment was studied. Our investigations were focused on the determination of the thermodynamic properties of the copigmentation process, i.e. on Gibbs free energy, enthalpy and entropy values. The equilibrium constant was determined by spectrophotometer measurements. Other parameters were calculated using classical thermodynamics equations.

Materials and methods

Chemicals

The copigmentcaffeic acid was from Sigma – Aldrich, 98 % (Germany).The reagents used for the McIlvaine buffer pH 3.4 citric acid monohydrate and disodium hydrogen phosphate dodecahydrate, were from Merck (Darmstadt, Germany). The adsorbent resin AmberliteXAD 16N resin 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 (Shikov et al., 2008). 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 2.5:1 (v/w). The extraction mixture was filtered and the organic solvent was evaporated under vacuum (30 °C). To remove sugars, salts, and amino acids from the crude extracts, samples were purified using a column (465 × 30 mm i.d.) filled with adsorption resin AmberliteXAD 16N. Prior to sample application, the resin was conditioned and equilibrated by rinsing with 500 ml of methanol and 1000 ml of water, acidified with trifluoroacetic acid (TFA, pH 2). Subsequently, 250 ml of the aqueous strawberry extract were applied and the column 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 of the eluate was evaporated

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 °C), the residue was lyophilized for 72 h.
The total monomeric anthocyanins were assessed by the pH-differential method(Shikov et al.,2012).The results were expressed as pelargonidin 3-glucoside equivalents.

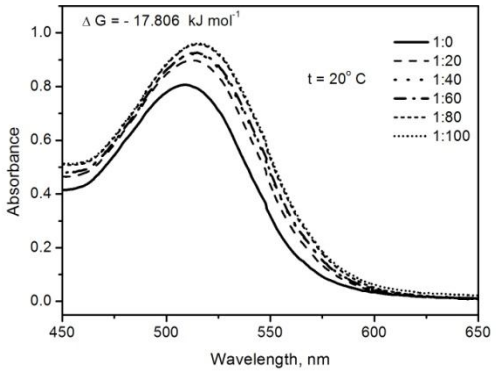
Preparation of model solutions
Stock solutions of strawberry extract, on the basis of the total anthocyanins, and caffeic acid were prepared in McIlvaine buffer (0.1 M, pH 3.4). Model solutions of strawberry anthocyanins (1 x 10^{-4} M)were obtained by mixing equal volumes (5 ml) of the corresponding stock solutions and were left for equilibration (30 min at 25 °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 (VEBMLW Prufgepate-Werk Medingensitz Freital, Germany) at 20, 30, 40 and 50 °C, respectively.

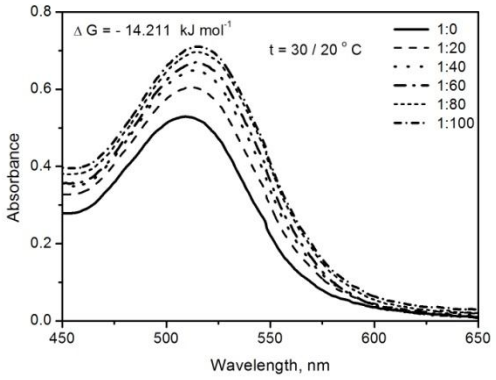
Statistical analysis
The results reported in the present study are the mean values of at least two determinations and the coefficients of variation were found to be below 2.5 % in all cases. Linear regression analysis was performed using the statistical package of Microsoft Excel®.

Results
In this study the model solutions prepared with stoichiometry quantity anthocyanin and variations of copigment change between 1:20 to 1:100 high concentration. Table 1 presented results of bathochromic and hyper chromic effects in the system when heating from 20, 30, and 40to 50^{o}Cand following cooling to 40, 30 and 20^{o}C model solutions. In this model system observed and predictedtwo effects.

Discussion
Figure 1 presented absorbance curves at 20^{o}C at heating and 20^{o}C after cooling the system.



1 a)



1 b)

Figure 1.Absorption spectra of strawberry anthocyanins and caffeic acid high concentration at different molar pigment: copigment ratios (1:0, 1:20, 1:40, 1:60, 1:80 and 1:100) at 20^{o}C (a – at heating) and 30/20ºC (b – at cooling).

Investigation of the system showed that system exhibited high stability (the maximum absorbance exceeded 0.9.) at 20^{o}C, Figure 1 a. In the figure observed increase of absorbance with increase of copigment concentration. At 30^{o}C appeared the similar results.
With increase of temperature to 40 and 50^{o}C maximum absorbance decreases. At 50^{o}C the absorbance maximum is 0.6. After cooling the system from 50 to 20 ^{o}C, restore of absorbance values was not observed. Figure 1 b presented system at 20^{o}C after cooling. The maximum absorbance is around 0.7.
In this investigation was found that with increase of temperature of the pigment: copigment system and following decrease of temperature to 20^{o}C the copigmentation complex was destroyed and not form again when the system cooling.

Table 1. Bathochromic ($\Delta\lambda_{max}$) and hyper chromic ($\Delta A\%$) effects in model solutions of strawberry anthocyanins and caffeic acid at different molar pigment/copigment ratio

Molar ratio pigment/copigment	$\Delta\lambda_{max}$	$\Delta A\%$
t = 20 °C		
1:0	1	0
1:20	4	12.52
1:40	4	21.21
1:60	6	31.48
1:80	9	34.43
1:100	10	35.48
t = 30 °C		
1:0	1	0
1:20	3	12.80
1:40	7	22.04
1:60	8	31.83
1:80	7	32.76
1:100	12	35.96
t = 40°C		
1:0	1	0
1:20	3	6.27
1:40	6	14.95
1:60	6	26.20
1:80	2	25.46
1:100	4	15.50
t = 50°C		
1:0	0	0
1:20	2	7.74
1:40	4	18.11
1:60	6	81.32
1:80	7	20.18
1:100	7	32.64
t = 50/40° C		
1:0	0	0
1:20	2	9.90
1:40	3	20.00
1:60	5	23.05
1:80	4	29.33
1:100	6	20.57
t = 40/30°C		
1:0	0	0
1:20	2	12.33
1:40	4	20.87
1:60	6	25.05
1:80	9	30.75
1:100	6	30.75
t = 30/20°C		
1:0	0	0
1:20	1	14.40
1:40	4	22.72
1:60	4	26.90
1:80	6	31.81
1:100	7	34.28

This phenomena probably exhibited concentration dependence and connected with high concentration caffeic acid in model solutions.

The equilibrium constant K and thermodynamic parameters Gibbs free energy, enthalpy and entropy, for the copigmentation 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 copigment, respectively; C is the molar copigment concentration; K is the equilibrium constant and n is the stoichiometric ratio of the reaction(Brouillard, 1983). The dependence of $\ln [(A - Ao)/Ao]$ on the copigmentconcentration $\ln [(A - Ao)/Ao] = f (\ln[C])$ 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.

Thermodynamic parameters Gibbs free energy, enthalpy and entropy were calculated using the following equations (Brouillard et al., 1994).

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

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 applying the Vant-Hoff equation.

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

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

When the Gibbs free energy and the enthalpy were obtained, the entropy can be determined by thermodynamic equation (3):

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

ΔSis entropy for the copigmentation reaction (kJ K^{-1} mol^{-1}).

The equilibrium constant and calculated thermodynamic parameters are presented in Table 2.

At 20 °C the constant showed the highest value 1202.264 M^{-1}. At the same temperature the Gibbs energy exhibit the most value -17.806 kJ mol^{-1}. At 30°C the Gibbs energy exhibited the similar results. With increase of temperature more den 30°C stability decrease and pigment: copigment couple decompose. At 50°C Gibbs energy is equal to -16.942 kJ mol^{-1}. At 40, 30 and 20°C in following cooling system the Gibbs energy had values generally around -13.000 to -14.000 kJ mol^{-1}.

These results connected with destroy complex system at heating to 50°C and not restore at cooling to 20°C. Gibbs energies are negative at all temperatures. These results connected with spontaneous process of copigmentation. On the basis of Gibbs energy changes in all temperatures it can be concluded that the process of copigmentation is possible only at lower temperatures maximum 30°C (Table 2).

The reversibility of the copigmentation in the malvin-quercetin system does not exist, in contrast to the malvin-rutin system (Baranacet al., 1997). In this work observed the same results as a system strawberry anthocyanin: caffeic acid. The enthalpy and entropy changes of the process were negative at all temperatures at heating and at cooling. It can be concluded that such a dependence on temperature is a consequence of the exothermic copigmentation process, $\Delta H < 0$. The negative value of the entropy, ΔS indicates that the couple formation depending of order/disorder of the system.

In Figures 2 observed calculated thermodynamic parameter Gibbs energies at different temperatures at heating and at cooling. At heating system seen decrease of Gibbs energies in positive values and at cooling the Gibbs energies not restore values and increase more to positive values. Increase Gibbs energies to positive values are proof to decrease stability of investigated pigment: copigmentsystem and in this case connected with destroy of the system. Figure 3 presented dependence between time cooling system and absorbance with different caffeic acid concentration. At concentration 1:0 caffeicacid observed linear dependence. This line exhibited lower absorbance. In that case, no change in the sign in the investigated concentration range of the copigment.

When the total concentration of copigment is raised, the differences in the Ao absorbance values just after mixing of the pigment and copigment solutions essentially reflect the differences in the molar absorption coefficients of the free and complex forms at the selected wavelength. In solution without copigment investigated absorbance is Ao = 0.575 and λ = 501 nm. With increase of copigment concentration to 1:100 A = 0.779 and λ = 510 nm. These experimental results can be seen in Figure 3. In the conclusion from the investigated kinetics is that absorbance exhibited

Table 2. Equilibrium constants and thermodynamic parameters for the copigmentation interaction between strawberry anthocyanins and caffeic acid high concentration at different temperatures and stoichiometric ratio n=1, 1:1.

t, ^{o}C	K, M^{-1}	ΔG, kJ mol^{-1}	ΔH, kJ mol^{-1}	ΔS, kJ K^{-1} mol^{-1}
20	1202.264	-17.806	-26.156	-0.0249
30	1172.195	-17.272	-25. 866	-0.0248
40	870.964	-17.116	-25.365	-0.0263
50	549.541	-16.942	-24.555	-0.0285
50/40	204.174	-13.841	-23.806	-0.0331
40/30	281.838	-13.967	-23.118	-0.0316
30/20	309.029	-14.211	-24.227	-0.0312

linear depending with time and these results connected with first order reaction.

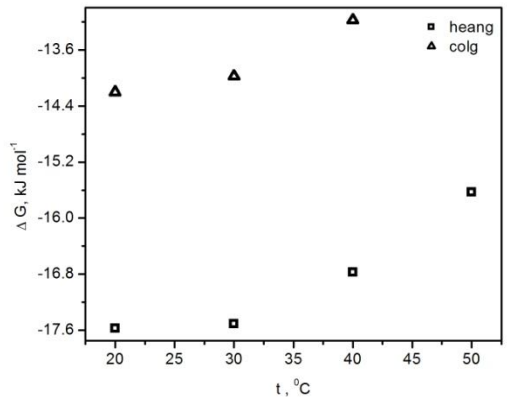


Figure 2. Temperature dependence of Gibbs free energy for the copigmentation interaction between strawberry anthocyanins and caffeic acid with concentration between 1:10 to 1:100 at heating and at cooling.

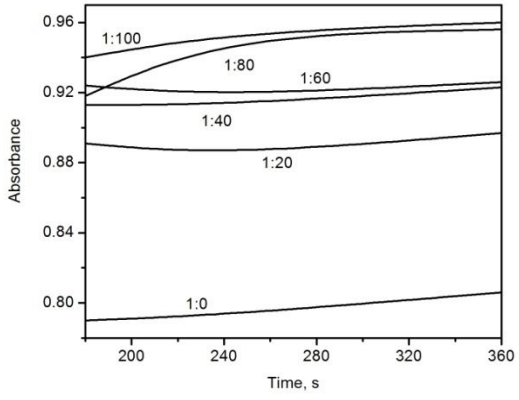


Figure 3. Time-dependence of the strawberry anthocyanin absorbance with different caffeic acid concentration from1:20 to 1:100.

Conclusion

The thermal stability of isolated strawberry anthocyanins was examined in model solutions, with high concentrations caffeic acid as a copigment at different temperatures at heating and at cooling. In the temperature range 20-50^{o}C at heating was confirm that the system was the most stable at 20^{o}C. With increasing temperature to 50 ^{o}C and following decreasing to 20 ^{o}C, pigment: copigment complex was destroyed and not restored again. Calculations of Gibbs free energies at all temperatures confirm the experimental results. Based on the experimental and calculated results of this work, further studies would be necessary for the determination of appropriate concentrations of copigment. Pigment: copigment interactions can be used in food products.

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