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THERMODYNAMIC STUDY ON THE CO–PIGMENTATIOON INTERACTION BETWEEN CYANIDIN 3–GALACTOSIDE AND ROSMARINIC ACID IN MODEL SYSTEMS

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Abstract: Interactions between aronia anthocyanins as pigment and rosmarinic acid as copigment were studied. Investigations were done in different temperatures from 20^0C to 80^0C at heating system and cooling the same system at 80 to 20^0C. The system was investigated with high concentration of rosmarinic acid from 1:1 to 1:50 molar ratio. The thermodynamic parameters ΔG, ΔH and ΔS 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 $80^0 C$ and with following cooling to 20^0C seen reversibility of the copigmentation process.
Keywords: cyanidin; rosmarinic acid; copigmentation; thermodynamic parameters

INTRODUCTION

The production of natural food pigments continues to grow worldwide. Pigments such as anthocyanins, carotenoids, betalains, and chlorophylls have been used to color foods. However, there are challenges related to color losses during food processing, storage, and commercialization due to a low stability of natural pigments compared to synthetic colorants. This review summarizes the most recent studies and patents aimed at enhancing anthocyanin stability in food systems. The stabilizing methods include additions of copigment compounds, such as polymers, phenolic compounds, and metals. In addition, the exclusion of O_2 during processing and storage, hard–panned candy coating methods for blue, green, and brown colors, and various encapsulation techniques were considered. Combining strategies and evaluating new materials capable of stabilizing anthocyanins will enhance their potential for use as value–added natural food pigments [1]. 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 [2]. Although phenolic copigments are rarely colored, they can still influence color expression in red wine copigmentation. Here, prefermentative agents, such as caffeic and rosmarinic acids, were added as copigments during Cabernet Sauvignon red winemaking. The evolution of colors and polyphenols was analyzed by tristimulus colorimetry and high–performance liquid chromatography–tandem mass spectrometry during brewing and aging periods. The results showed that the addition of caffeic and rosmarinic copigments was beneficial to obtain more saturated and vivid hue of wines. The changes in color were correlated with a significant increase in anthocyanins concentration. Moreover, the results demonstrated that the addition of the two phenolic copigments increased the concentration of total phenols and enhanced the copigmentation effect, indicating improved chromatic intensity and color stability of wines. Therefore, the addition of caffeic and rosmarinic acids is a simple and useful enological technique to obtain red wines with high color quality and aging potential [3].

Zhao et al. [4] study investigated the protective effect and mechanism of action of combined use of rosmarinic acid (RA) and xanthan gum (XG) on the stability of anthocyanins (ACNs) in the presence of l–ascorbic acid (pH 3.0). The addition of RA and XG, alone and in combination, significantly enhanced the color stability of ACNs, and the combined use of RA and XG showed the best effect. According to Eiro et al. [5] intermolecular copigmentation reactions are significantly responsible for the manifold color expression of fruits, berries, and their products. These reactions were investigated with five anthocyanins and five phenolic acids acting as copigments. The stability of the pigment–copigment complexes formed was studied during a storage period of 6 months. The study was conducted using a UV–visible spectrophotometer to monitor the hyperchromic effect and the bathochromic shift of the complexes. The greatest copigmentation reactions took place in malvidin 3–glucoside solutions. The strongest copigments for all anthocyanins were ferulic and rosmarinic acids. The immediate reaction of rosmarinic acid with malvidin 3–glucoside resulted in the biggest bathochromic shift (19 nm) and the strongest hyperchromic effect, increasing the color intensity by 260%. The color induced by rosmarinic acid was not very stable. Copigmentation of anthocyanins accounts for over 30% of fresh red wine color, while during storage, the color of polymeric pigments formed between anthocyanins and proanthocyanidins predominates. Rosmarinic acid and natural extracts rich in hydroxycinnamic acids, obtained from aromatic plants (Origanum vulgare and Satureja thymbra), were examined as cofactors to fresh Merlot wine and the effect on anthocyanin copigmentation and wine color was studied during storage for 6months [6].

Copigmentation has been suggested as a main colour stabilising mechanism in plants protecting the coloured flavylium cation from the nucleophilic attack by the water molecule. In this study influence of phenolic compounds

addition (catechol, 4–methyl catechol, (+)–catechin and gallic acid) on stability of red currant juice anthocyanins (copigment:pigment molar ratio 50:1 and 100:1) during 30 days of storage at 4°C was investigated. Stability of anthocyanins was evaluated through determination of anthocyanins, total colour difference (ΔE*), kinetic parameters and anthocyanin retention [7]. Anthocyanins show low–stability when exposed to different food processing conditions. Copigmentation is one of the main reactions contributing to the in vivo color responsible to the stability of anthocyanins. In the aim of holding the red color, copigmentation effect of organic acids (caffeic, ferulic, gallic and tannic acids) combined with anthocyanins in crude Cabernet Sauvignon (Vitis vinifera L.) grape skin extract at pH values (1.0, 2.0, 3.0, 3.3, 3.5, 3.7, 4.0, 4.5) was evaluated in this research [8]. 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 ΔS 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 [9].

Amzad et al. [10] effect of solvents on recovery of polyphenols from the pink fuji apple skin. Dehydrated apple skin powder was used to evaluate the recovery of selected flavones and rosmarinic acid using water, methanol, acetone, chloroform, aqueous 50% methanol, and aqueous 70% acetone at 40°C. The retrieved extracts were subjected to qualitative and quantitative GC–MS analysis. Similar yield of rosmarinic acid (RA) was obtained in aqueous 70% acetone extracts when the periods of extraction were 2, 4, 6 and 8 hours, respectively.

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 [11]. The effect of temperature on the stability of the copigmentation complex of strawberry anthocyanin extract as pigment and caffeic acid as copigment was investigated. The system was studied with a high concentration of caffeic acid 1:20 to 1:100 molar ratio. Different temperatures and copigment concentrations were used for the investigated pigment: copigment interaction and kinetic parameters such as activation energy (Ea), z – factor and degradation rates (k) were calculated.

According to the calculated results, at high temperatures (50°C) destruction of the complex was observed. Decreasing the temperature in the range of 20–30°C did not lead to restoration of the complex, indicating irreversibility of the copigmentation process [12].Thermal degradation and color changes of purified strawberry anthocyanins in model solutions were studied upon heating at 85 degrees C by HPLC–DAD analyses and CIELCh measurements, respectively. The anthocyanin half–life values increased significantly due to the addition of rose (Rosa damascena Mill.) petal extracts enriched in natural copigments. Correspondingly, the color stability increased as the total color difference values were smaller for anthocyanins upon copigment addition, especially after extended heating [13].The stability of anthocyanins from Viburnum opulus fruits, in aqueous and ethanolic extracts, stored under darkness for 7 days at different temperatures (2°C, 37°C and 75 °C) and pH values (pH = 3 and 7), was studied here. Results indicate that the storage degradation of anthocyanins followed first–order reaction kinetics under all investigated conditions [14].

MATERIALS AND METHOD
— Chemicals
Chemicals the copigment rosmarinic 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.

— Plant materials
Aronia fruits were supplied from local growers of aronia fruits, in the stage of full maturity, in august 2020. Fresh fruits were put in polyethylene bags, frozen immediately and stored at – 18°C until extracted.

— Extraction, purification and determination of anthocyanins
Aronia anthocyanins were extracted and purified as described Shikov at al., [13]. Frozen aronia 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 colourless. The organic solvent of the eluate was evaporated under vacuum (30 °C). To separate anthocyanins from colourless 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. The results were expressed as pelargonidin 3–glucoside equivalents.

— **Preparation of model solutions of pigment and co–pigments**

Stock solutions of strawberry extract, on the basis of the total anthocyanins, and rosmarinic acid were prepared in McIlvaine buffer (0.1 M, pH 3.4). Model solutions of strawberry anthocyanins (1 $\times 10^{-4}$ 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 400 to 700 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, 60 and 80 °C, respectively during heating and then for 10 min at 60°C, 40°C, and 20°C during cooling.

— **Modelling of thermodynamic parameters**

Thermodynamic parameters were calculated according to Petrova et al. [9]. After spectrophotometer measurements the equilibrium constant was calculated by the equation:

$$\ln[(A - A_0|A_0)] = \ln[K] + n\ln C \qquad (1)$$

where: A and Ao are the absorption maximum values of the anthocyanin solution with and without co–pigment; C is the molar co–pigment concentration; K is the equilibrium constant and n is the stoichiometric ratio of the reaction. 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].

Gibbs free energy was calculated using the equation

$$\Delta G = -RT\ln Kp \qquad (2)$$

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:

$$\frac{d\ln K}{d(1/T)} = \frac{-\Delta H}{R} \qquad (3)$$

ΔH is enthalpy for the co–pigmentation reaction (kJ mol^{-1}). Once the Gibbs free energy and the enthalpy were obtained, the entropy can be determined by using the following classic thermodynamic equation:

$$\Delta S = \frac{(\Delta H - \Delta G)}{T} \qquad (4)$$

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

RESULTS AND DISCUSSION

In this study the model solutions prepared with stoichiometry quantity anthocyanin and variations of copigment change between 1:1 to 1:50 high concentration. The equilibrium constant and calculated thermodynamic parameters are presented in Table 1.

Table 1: Reaction equilibrium constant and thermodynamic parameters of the copigmentation interactions between black chokeberry anthocyanins and rosmarinic acid

Temperature, °C		K	ΔG [kJ.mol^{-1}]	ΔH [kJ.mol^{-1}]	ΔS [kJ.mol^{-1}]
Heating	20	9705,1	−22,74	−27,22	−0,015
	40	4570,88	−21,93	−27,74	−0,018
	60	390,46	−16,02	−28,28	−0,0379
	80	107,15	−12,94	−25,00	−0,036
Cooling	60	400,86	−16,09	−25,78	−0,030
	40	3419,00	−21,17	−27,41	−0,019
	20	6295,06	−21,67	−26,75	−0,017

At 20°C the constant showed the highest value. At the same temperature the Gibbs energy exhibit the most value −22.74 kJ mol^{-1}. At 40°C the Gibbs energy exhibited the similar results. With increase of temperature more den 40°C stability decrease and pigment: copigment couple decompose. At 80°C Gibbs energy is equal to −12.94 kJ mol^{-1}. These results connected with destroy complex system at heating to 80°C and restore at cooling to 20°C.

These results connected with spontaneous process of copigmentation. 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, ΔH< 0. The negative value of the entropy, ΔS indicates that the couple formation depending of order/disorder of the system.
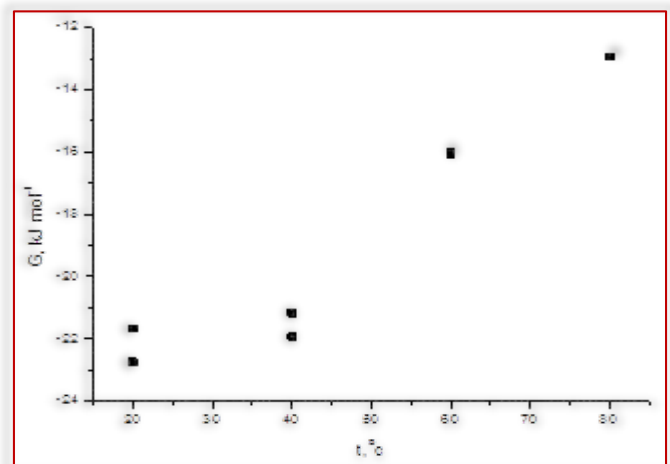

Figure.1. Dependence of thermodynamic parameter Gibbs energies at different temperatures at heating and at cooling

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: copigment system and in this case connected with destroy of the system.

CONCLUSION

The thermal stability of isolated aronia anthocyanins: rosmarinic acid was provide first by heating system to 80°C and then cooling to 20°C. At the same temperatures destruction was observed and after that the observed complex was restored. The thermodynamic parameters exhibited negative values at all temperatures and this is proof for a stable complex. On the basis of thermodynamic parameters obtained in all temperature range it can be concluded that the process of copigmentation is possible only at temperature 40°C or lower.

References

[1]   R. Cortez, D. A. Luna–Vital, D. Margulis, E. Gonzalez de Mejia. Natural Pigments: Stabilization Methods of Anthocyanins for Food Applications.. Comprehensive reviews in food science and food safety. 2021

[2]   J.B. Harborne, Comperative Biochemistry of the. Flavonoids, Academic Press: London, 1967, Chapter 1.

[3]   B. Zhang, X. Wang, Bo Yang, N. Li, J. Niu, X. Shi, S. Han. Copigmentation evidence of phenolic compound: The effect of caffeic and rosmarinic acids addition on the chromatic quality and phenolic composition of Cabernet Sauvignon red wine from the Hexi Corridor region (China). Journal of Food Composition and Analysis, Volume 102, 2021, 104037

[4]   L. Zhao, F. Pan, A. Mehmood, H. Zhang, A. Rehman, J. Li, S. Hao, C. Wang. Improved color stability of anthocyanins in the presence of ascorbic acid with the combination of rosmarinic acid and xanthan gum. Food Chemistry, Volume 351, 2021, 129317

[5]   M. Eiro, M. Heinonen. Anthocyanin color behavior and stability during storage: effect of intermolecular copigmentation. Agric Food Chem. 2002 Dec 4;50(25):7461–6.

[6]   A. Bimpilas, M. Panagopoulou, D. Tsimogiannis, V. Oreopoulou. Anthocyanin copigmentation and color of wine: The effect of naturally obtained hydroxycinnamic acids as cofactors. Food Chem. 2016 Apr 15;197(Pt A):39–46.

[7]   M.Kopjar, V. Piližota. Copigmentation effect of phenolic compounds on red currant juice anthocyanins during storage. Croat. J. Food Sci. Technol. (2009) 1 (2) 16–20.

[8]   C. Gauche, E. da Silva, M.Luiz. Effect of pH on the copigmentation of anthocyanins from Cabernet Sauvignon grape extracts with organic acids. Food Science and Technology Sci. agric. (Piracicaba, Braz.) 67 (1), 2010,41–46

[9]   I. Petrova, V. Shikov, V. Gandova, K. Mihalev, D. Dimitrov. The Copigmentation Interactions between Strawberry Anthocyanins and High Concentration Caffeic Acid with Different Methods. Scientific works of University of food technologies, 2016 vol.63 issue1.

[10]   H. Amzad, S. Salehuddin, R. Mizanur, M. Kabir. The effect of solvents on recovery of polyphenols from the pink fuji apple skin. African Journal of Food Agriculture, Nutrition and Development, Vol. 10, No. 5, May, 2010, pp. 2556–2569.

[11]   J.Baranac, N. Petranovic, J.Dimitric–Markovic. Spectrophotometric study of anthocyan copigmentation reactions. 2. Malvin and the nonglycosidized flavone quercetin. Journal of agricultural and food chemistry (USA) 1997, 1997, 45, 5, 1694–1697.

[12]   I. Petrova, V. Gandova, P. Denev, K. Mihalev,V. Shikov. Kinetic parameters of the copigmentation effect of caffeic acid and strawberry anthocyanins. Bulgarian Chemical Communications, Volume 50, Special Issue C (pp. 184 – 189) 2018.

[13]   V. Shikov, D. Kammerer, K. Mihalev, R. Carle. Heat Stability of Strawberry Anthocyanins in Model Solutions Containing Natural Copigments Extracted from Rose (Rosa damascena Mill.) Petals. Journal of Agricultural and Food Chemistry 56(18): 8521–6, 2008.

[14]   B. Moldovan, L. David,C. Chişbora, C. Cimpoiu. Degradation Kinetics of Anthocyanins from European Cranberrybush (Viburnum opulus L.) Fruit Extracts. Effects of Temperature, pH and Storage SolventMolecules 2012, 17(10), 11655–11666