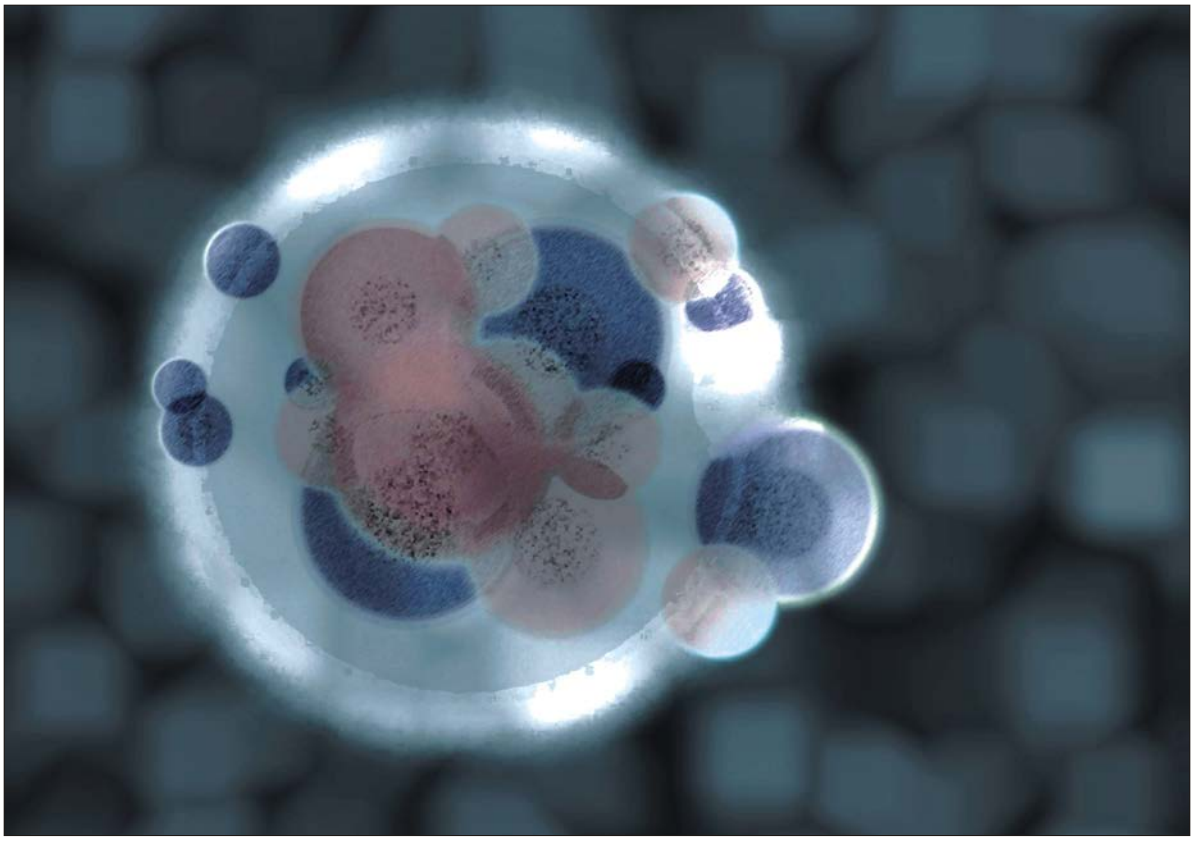

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The Effects of Heating, UV Irradiation and pH on Stability of Siahe Sardasht Grape Anthocyanin-copigment Complex

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Abstract: Neutral solution of isolated anthocyanins are unstable and quickly decolourized by reactions. Thus anthocyanins in living cells may have mechanisms for resistance to maintain colour stability. Copigmentation is the main colour-stabilizing mechanism. In this study copigmentation of Siahe Sardasht grape anthocyanin were investigated with five copigment, tannic acid, coumaric acid, ferulic acid, benzoic acid and caffeic acid. In this research, the dependence of the copigmenation process on the pH, copigment concentration and temperature were established. Also the effects of uv irradiation on colour stability and inhibiting effects of copigmentation against degradation influence of UV irradiation on anthocyanin were evaluated. Copigmentation of anthocyanin resulting in increase in both hyperchromic effects and bathochromic shifts. In this study tannic acid has the biggest hyperchromic effect and benzoic acid has the lowest hyperchromic effect. Four levels of copigment concentration as: 1:0, 1:20, 1:40, 1:60 were examined. The copigmentation effect increased with copigment containe. UV irradiation has the biggest effect on the copigmentation complex in comparison with heating. The suitable pH for copigmentation complex was in pH 3.5. In this study, tannic acid predominate copigment among copigments.

key words: Anthocyanin, colour stability, copigmentation, UV irradiation, Siahe Sardasht grape

INTRODUCTION

Anthocyanins are part of a larger class of molecules of known as flavonoids. They are responsible for the attractive red colours of many fruit products (Mazza and Brouillard, 1987).

Interest in the anthocyanins has increased because of their potential as natural colorant and beneficial health effects. Anthocyanins are used as natural food colorant and beneficial heath effects. Anthocyanins are used as natural colorants for a long time and are therefor as safe. The antioxidative activity of anthocyanins (*in vitro*) has been recently considered to be responsible for the protection against choronic disease *in vivo* (Wang *et al*., 1997). Attractive color is one of the main sensory characteristics of fruit products and this important quality parameter strongly effects consumer behavior. Unfortunately, the color of red juice is unstable and easily susceptible to degradation leading to a dull and brownish juice color (Maarit and Heinnoen, 2004). Anthocyanins have different colors, depending on the pH of the solution (Dao, *et al*., 1998; Bakowska *et al*., 2003) at pH below 2 anthocyanins exist primarily in the form of red flavylium cations. When the pH increases to 6, the flavylium cation converts into purple quinonoidal bases. This compounds are labile and upon nucleophilic attack by water. Transfer to the colourless carbinol pseudobase and chalcone pseudobase. Therefore limiting factor for the incorporation of anthocyanins in foods is the low stability of these pigments which is influenced by several factors:temperature, pH, oxygen, enzymes, ascorbic acid, sulfur dioxide, sugars, their degradation products, the presence of copigments and metallic ions (Mazza and Miniati, 1993).

Isolated anthocyanins neutral aqueous solutions are unstable and quickly decolorized by reactions. Thus anthocyanins in living cells may have mechanisms for resistance to maintain color stability (Yoshida *et al*., 2003).

Copigmentation between anthocyanins and copigments is the main coluor-stabilizing mechanism (Oszmianski *et al*., 2004) and is regarded to day as one of the significant factors of structure stabilization, colorant of anthocyanins under *in vivo* conditions (Marcovic *et al*., 2000). The copigmentation reaction in anthocyanin was first reported by Robinson.

Copigmentation occurs when a colorless molecule with a planar, pi-electron rich moiety (the copigment) interaction with the planar chromophore of the anthocyanin (the pigment) forming a colored complex. The copigments interaction competes with water attack on the flavilium system by covering the sit of attack, blocking waters approach. The complexation of a copigment with

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an anthocyamin causes a hyperchromic effects (ΔA) and a bathochromic shift (Δλ). (Chen and Hrazdina, 1981; Mazza and Miniati, 1993). The hyperchromic effect means an increase in colour intensity while the bathochromic shift consists of a shift of the wavelength of maximum absorbance (Bakowska *et al*., 2003). A wide rang of different molecules has been found to act as copigments. The most common structurally unrelated copigment compounds are flavonoids and other polyphenols, alkaloids, amino acids and organic acids (Brouillar *et al*., 1989).

The most studied group of copigments is: chlorogenic acid (Brouillard *et al*., 1989; Mazza and Brouillard, 1990; Brouillard *et al*., 1991; Bakowska *et al*., 2003).

Flavonoids such as rutin and quercetin (Scheffeldt and Hrazdina, 1978; Chen and Hrazdina, 1981; Baranac *et al*., 1997; Gonnet, 1999; Bakowska *et al*., 2003; Oszmianski *et al*., 2004).

Hydroxy cinamic acids and hydroxyl benzoic acid (Marcovic *et al*., 2000; Brouillard *et al*., 1989) tannic acid (Bakowska *et al*., 2003; Cai *et al*., 1990; Robinson and Robinson, 1931), ferulic acid (Rein and Heinonen, 2004, Eiro and Heinonen, 2002; Marcovic *et al*., 2000; Asen *et al*., 1972) caffeic acid (Wesche-Ebeling *et al*., 2003) caffeine (Wesche-Ebeling, 2003; Darias Martin *et al*., 2001, 2002; Brouillard *et al*., 1991), sinapic acid (Marcovic *et al*., 2005; Rein and Heinonen, 2004, Marcovic *et al*., 2000), amino acids (Asen *et al*., 1972), rosmarinic acid (Rein and Heinonen, 2004).

The free phenolics existing in plant materials can be regarded as potential copigmens (Gao and Mazza 1994) such as chlorogenic acid in blue berries (Skrede *et al*., 2000; Gao and Mazza, 1994) and in raspberries and strawberries (Mattila and Kumpulainen, 2002) ferulic acid and coumaric acid in blue berries (Mattila *et al*., 2004) flavonons in scutellaria baicalensis (Bakowska *et al*., 2003), rosmarinus officinal is extracts (Talcott *et al*., 2003; Delpozo-Insfran *et al*., 2002).

The overall aim of this research to exam enhance and stabilize of anthocyamin with copigments and factors (such as pH, heating, UV irradiation) that effects on copigmentation phenomena. Since there have been only limited reports on the effect of UV irradiation on anthocyanin-copigment complex, our effort was to exam this factor.

In this study five substances were examined as copigments: Tannic acid, caffeic acid, coumaric acid, ferulic acid and benzoic acid. The stability of anthocyanin was investigated at pH 2.5, 3.5, 4.5 under UV irradiation and heating in presence and absence of copigment.

MATERIALS AND METHODS

Sample preparation: The grape cultivar used for this study was Siahe Sardasht variety a major grape of *Vitis vinifera* grows as wild plant on mountains of Sardasht in the south of West Azarbayjan (WA) and is planted at the south of WA province in Iran.

Siahe sardasht grapes were harvested from Sardasht mountains in the south of WA province and carried in ice to our laboratory.

The samples were washed with tap water and sealed in polyethylene bags and finally they were frozen and stored at -18°C.

Extraction procedures: Grape (500 g) were thawed at room temperature and aerated in a warning blender with methanole (250 mL) containing/01% HCl as solvent under a nitrogen atmosphere for 5 min.

The macerated samples were filtered through a filtter paper (Whatman No. 1) under vacuum and residue was extracted with the same solvent until most of the pigments were extracted. The solvent (acidic methanol) was removed and the juice samples were concentrated under reduced pressure with a rotary evaporator (Buchi Rotavapor-r) keeping the temperature of the water bath below 35°C.

Materials: Substances used in the experiments were tannic acid, caffeic acid, ferulic acid, coumaric acid, benzoic acid. All substances were purchased from Merck Chemical Company.

Solutions: Buffered solutions were obtained by mixing sodium acetate (/02 mol dm^{-3}) and phosphoric acid (/06 mol dm^{-3}). The pH value of the most acidic buffer solution was adjusted with HCl (/1 mol dm^{-3}) by using pH meter (METTLER DELTA 340).The ionic strength of the solutions was adjusted by sodium choloride (/02 mol dm^{-3}) (Marcovic *et al*., 2005).

UV-VIS spectra: The juice samples were diluted with distilled water to give an absorbance reading. The absorbance spectra were scanned from 450 to 610 nm. All absorbance reading were made against distilled water as a blank. Spectrophotometric measurements were carried out by using a UV/VIS spectrophotometer (Biochrom S200 UK).

The spectra were recorded in quartz curvets of 1 cm optical path length. Each spectroscopic measurement was repeated three times. For thermal stability colorant solution with and without copigment (10 mL) in glass tubes with screw caps were placed in a water bath at

80°C. For uv stability colorant solutions with and without copigment (10 mL) in glass open cuvetts, were placed under UV light.

RESULTS AND DISCUSSION

General: The addition of phenolic acids to the grape juice improved the juice color by enhancing the anthocyanin color intensity. This addition induced change in bathochromic shifts and hyperchromic effects that manifest coipgmentation of anthocyanin in the buffer solutions. According to Mazza and Brouillard (1990) studies, pH, pigment and copigment concentration, type of anthocyanin, temperature, ionic strength and solvent influence the copigmentation of anthocyanin. The result of addition of copigments at four concentration levels showed that the outcome of copigmentation is dependent on mola ratio, which is in agreement with previous works (Davis and Mazza, 1993; Asen *et al*., 1972). However, the concentration of both anthocyanin and copigment molecules influence the extent of the copigmentation effect (Mazza and Brouillard, 1990). Increasing anthocyanin concentration, increases absorbance and the bathochromic shift.

The magnitude of the increase in absorbance is depend on the copigment to anthocyanin ratio for a given anthocyanin. Increasing the copigment-anthocyanin ratio also increases the magnitude of the hyperchromic and bathochromic shift. Because the anthocyanin concentration was constant in each solution, it seems obvious that the magnitude of the bathochromic shifts and hyperchromic effects depended on the concentration of copigment.

Absorption spectra of anthocyanin and spectra of the copigment formed with four copigments at the pH 3.5 buffer solution are shown in Fig. 1-5. Molar ratio of the copigments were 1:0, 1:20, 1:40 and 1:60.

As shown in this figures, in all of anthocyanin/copigment complex the highest anthocyanin/copigment molar ratio of 1:60 resulted in the strongest copigmentation effect. This is in agreement with other studies (Marcovic *et al*., 2005; Dimitric-Marcovic petranovic and Baranac, 2000). Type of copigment is an important factor that effects on copigmentation. Figure 6 shows absorption spectra of grape anthocyanin with and without copigments. This figure indicates the biggest bathochromic shift was induced by tannic acid with grape anthocyanin (ΔA = ./425, $\Delta\lambda$ =16) .With other acids, this effects are lower and amount to: ΔA = ./242, $\Delta\lambda$ = 12 for ferulic acid ΔA = ./167, $\Delta\lambda$ = 10 for caffeic acid, ΔA = ./093, $\Delta\lambda$ = 9.5 for coumaric acid, ΔA = ./061, $\Delta\lambda$ = 4 for benzoic acid.

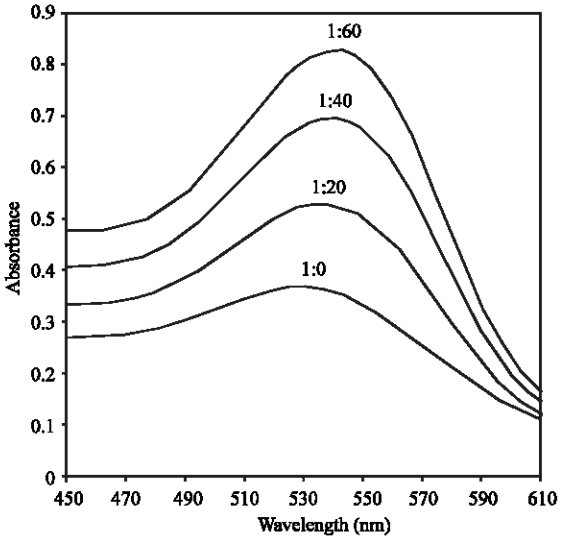

Fig. 1: Absorption spectra of anthocyanin and tannic acid, different molar ratio pH 3.5

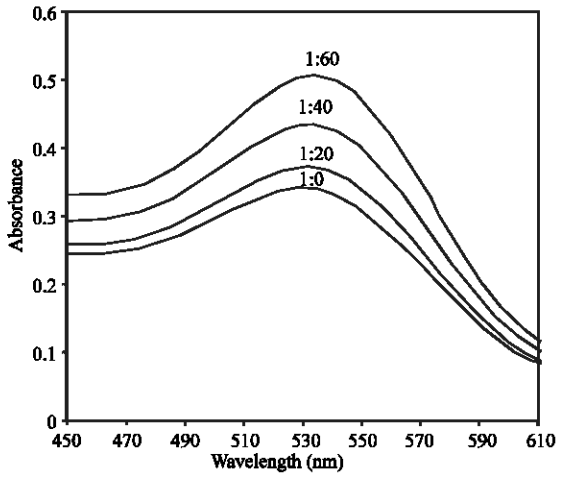

Fig. 2: Absorption spectra of anthocyanin and coumaric different molar ratio pH 3.5

Of the copigments, tannic acid and ferulic acid were the best copigment for grape anthocyanin. Benzoic acid seem to be rather poor copigment in contrast with other acids that is good agreement with results reported by Asen *et al*. (1972).

The influence of heating on the copigmentation effect: In this research it has demonstrated that increasing time and temperature of heating resulted in changes in anthocyanin and the copigmentation complex which resulted an increase in the visible spectrum (hyperchromic effect) and an increase in max (bathochromic shift) in the main absorption peaks.

Table 1: Influence of heating at 80°C on bathochromic shift and hyperchromic effect in solutions of copigments and anthocyanin at pH 3.5[1]

	Δλ (nm)		ΔA	
Copigment	Before	After 60 min 80°C	Before	After 60 min 80°C
Tannic acid	14.5	12.5	./418	./257
Ferulic acid	10.5	7.5	./158	./074
Caffeic acid	7	4	./136	./086
Coumaric acid	6	3	./107	./069
Benzoic acid	3	1	./058	./052

[1]ΔA and Δλ are changes of wavelength and absorbance of visible maximum at 525 nm upon addition of copigments

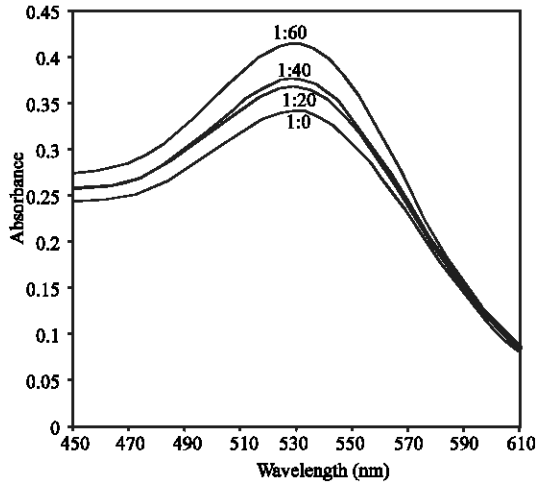

Fig. 3: Absorption spectra of anthocyanin and benozic acid different molar ratio pH 3.5

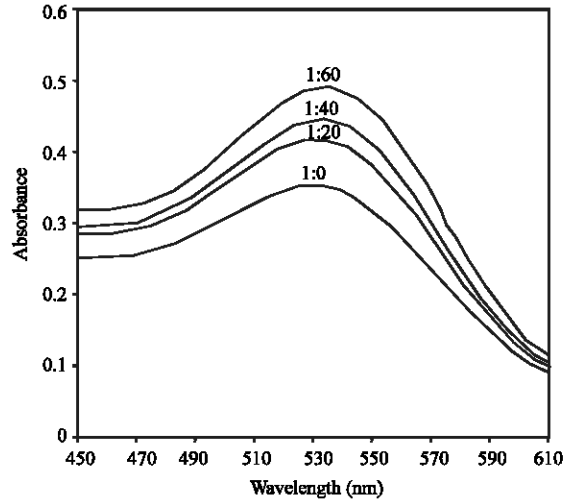

Fig. 4: Absorption spectra of anthocyanin and caffeic acid different molar ratio pH 3.5

This changes are shown in Table 1 as Influence of heating at 80°C on bathochromic shift and hyperchromic effect in solutions of copigments and anthocyanin at pH

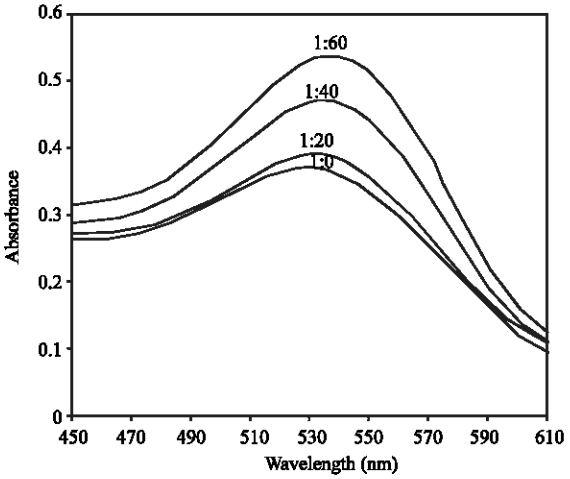

Fig. 5: Absorption spectra of anthocyanin and ferulic acid different molar ratio pH 3.5

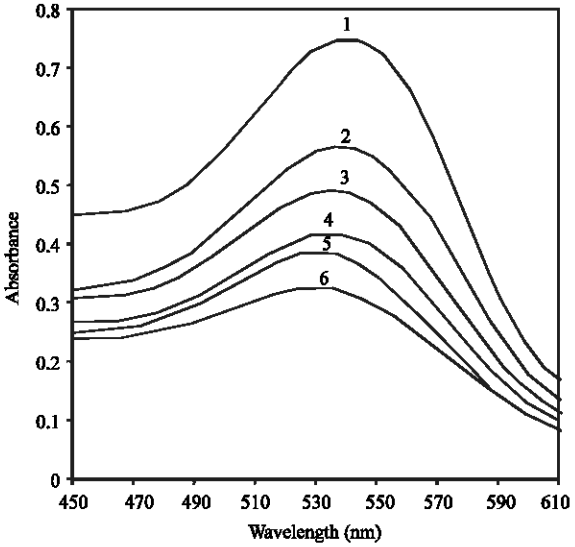

Fig. 6: Absorption spectra of anthocyanin with copigment molar ratio (1:60) pH 3.5

3.5 is shown in Table 1 the highest bathochromic shift was observed for anthocyanin with tannic acid complex. And the lowest bathochromic shift was observed with the complex of anthocyanin and benzoic acid.

In the previous research, Bakowska *et al.* (2003) were reported similar results about effects of heating on copigmentation. Also these results are in good agreement with that found by Kucharska *et al.* (1998), Kucharska *et al.* (1998), Baranac *et al.* (1997), Brouillard and Dangles (1994), Davies and Mazza (1993) and Mazza and Brouillard (1990). Marcovic *et al.* (2000) reported the

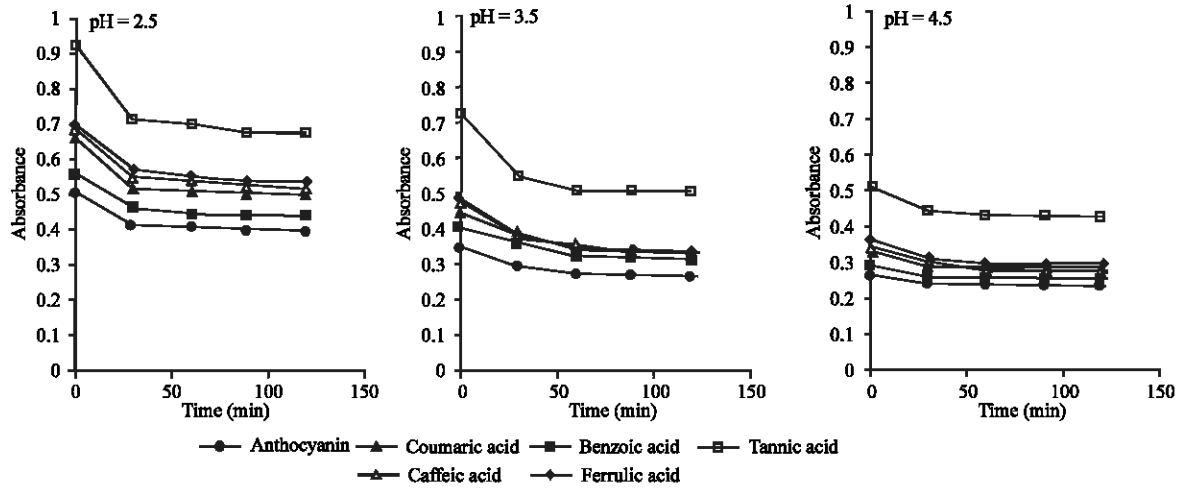

Fig. 7: Influence of time heating at 80°C and pH value (2.5-4.5) on absorbance at 525 nm of Anthocyanin with and without of copigment mol ratio 1:60

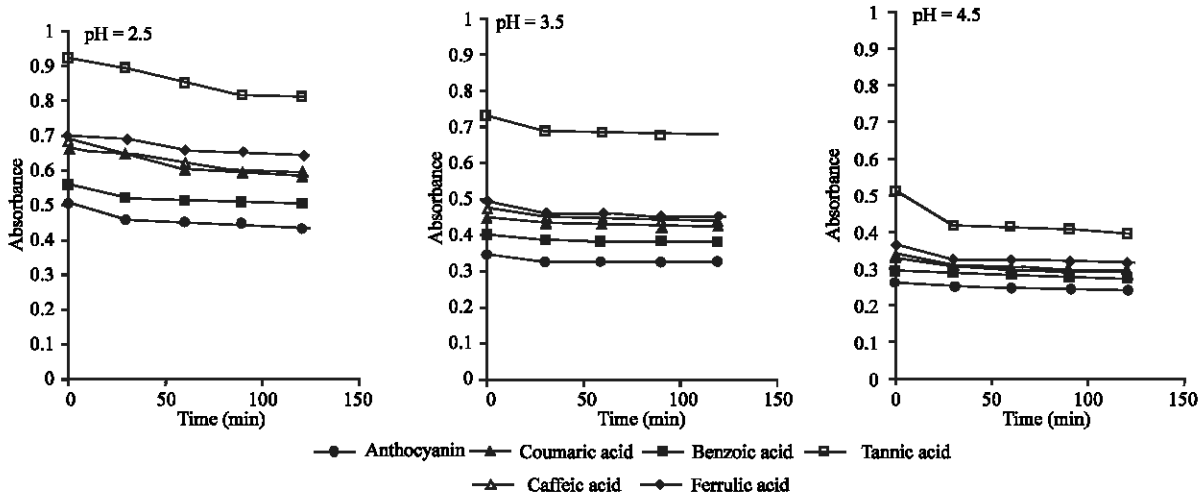

Fig. 8: Influence of UV irradiation and pH value (2.5-4.5) on absorbance at 525 nm of Anthocyanin with and without of copigment mol ratio 1:60

temperature was found to be a significant factor that determine the thermodynamic conditions of the copigmentation process.

In all reported research a temperature increase produces a decrease of the copigment bond intensity and its hyperchromic shift.

Copigment complexes are exothermic and are particulery sensitive to temperature (Dangles and Brouillard, 1992). Although a temperature raise has been shown shift anthocyanin equilibrium to wards the chalcone form, this has negligible effect in the visible range and the large decrease in absorbance with increasing temperature and quantitative recovery of colour on cooling of copigment solution is due to changes the copigment complex (Brouillard *et al*., 1989).

A plot of absorbance at max against temperature, demonstrates that as the boiling point of the solution is approached, the absorbance approaches the value of the anthocyanin alone without copigment. Raising the temperature of the anthocyanin solution without copigment, does not shift the absorbance spectrum in the visible range at all (Dangles and Brouillard, 1992).

The reason for this behaviour is once more related to the unique properties of water and its role in driving the copigmentation reaction. As the temperature is increased the organized lattice structure of liquid water is disruption in the complex, with the result that flavylium ions are released and hydrated to the colourless hemiacetal form.

As the temperature is increased, disruptions in the water structure accrue, leading to a reduction in the extent

Table 2: Influence of 60 min UV irradiation on bathochromic shift and hyperchromic effect in solutions of copigments and anthocyanin at pH3.5[1]

	ΔA (nm)		Δλ	
Copigment	Before	After 60 min UV	Before	After 60 min uv
Tannic acid	14.5	13.5	./418	./272
Ferulic acid	10.5	8	./158	./098
Caffeic acid	7	4.5	./36	./099
Coumaric acid	6	4	./107	./083
Benzoic acid	3	1.5	./058	./055

[1]ΔA and Δλ are changes of wavelength and absorbance of visible maximnm at 525 nm upon addition of copigments

of water hydrogen bonding and a decrease in the effectiveness of the copigmentation complex (Brouillard and Dangles, 1994).

Another factor which effects coipgmentation, is pH. At pH values below a bout 1 for anthocyanin in aqueous solution, addition of copigment will produce a bathochrimic shift, but no increase in absorbance is observed, in fact, a small hypochromic shift occurs. The formation of the copigment complex still occurs, but because hydration by covalent bonding of water to C_2 does not happen at low pH and nor does proton loss, all of the anthocyanin exists in the flavylium form. The optimum pH range for the copigmentation of anthocyanins is between approximately 3 and 5. At pH 1 -2, anthocyanin exists essentially in the flavilium form and the hyperchromic shift in the spectral maximum, observed in the presence of the copigment is due to the interaction of the anthocyanin flavylium cation with the copigment.

At pH 3, there is an important colour loss for anthocyanin alone and significant colour retention for the solution containing the anthocyanin and the copigment. Thus, the copigment effect is to reduce the production of the colourless carbinol psedobase. At pH 4-6, the solution containing only anthocyanin and copigment are still coloured. In this pH range quinoidal bases are formed and, again, colour retention is due to decrease in the amount of the carbinol pseudobase in the solution (Brouillard *et al*., 1991).

As shown in Fig. 7 at pH 2.5 colour of all copigmented anthocyanin solution indicates sharply decrease during first 30 min. Then it decreases mor slowly up to 150 min. This Fig. 7 indicated that colour of anthocyanin with tannic acid has the biggest decrease in contrast of other anthocyanon-copigment solutions. Plot of absorbance of anthocyanin-copigment solution at pH 3.5 shows slow decrease of anthocyanin colour during first 30 min while at pH 4.5 absorbance decrease is slowly all the time. In this exanıs, illustrated that after 120 min all investigated copigments stabilized anthocyanin colour at all values. In anıong investigated copigments, for grape anthocyanin, tannic acid were predominated over other copigments.

Influence of UV irradiation on the copigmentation effect: UV irradiation is one of the several factors that induces anthocyanin degradation.

As can be seen in this Table 2, anthocyanin-tannic acid complex has the biggest hyperchromic effects and anthocyanin with benzoic acid has the lowest hyperchromic effects. As shown in Table 2, UV irradiation induced anthocyanin degradation after 60 min irradiation. This degradation of anthocyanin and decrease of anthocyanin colour identificated by decrease of hyperchromic effects after 60 min.

Anna Bakowska *et al*. (2003) and Kucharska *et al*. (1998) previously investigated the influence of uv irradiation on the stability of the anthocyanin-copigment complex. They found similar results that we obtained in our research. Figure 8 shows influence of UV irradiation time on anthocyanin stability. As can be seen, the absorbance at 525 nm decreases with increasing irradiation time at all investigated pH values. This decrease is famous at pH 2.5. Tannic acid as the copigment, prevented the UV degradation and inhibited the degradation influence of UV and stabilized the anthocyanin better than other copigments. At pH 4.5, absorbance decrease is very slowly during UV irradiation and after 30 min anthocyanin was stabilized at all the time. The results of this investigate were good agreement with the results reported by Bakowska *et al*. (2003) and Kucharska *et al*. (1998) who, studied influence of uv irradiation time on anthocyanin-copigment stability.

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