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Anthocyanin Color Behavior and Stability during Storage: Effect of Intermolecular Copigmentation

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

KEYWORDS: Anthocyanins; phenolic acids; intermolecular copigmentation; storage stability

INTRODUCTION

Anthocyanins are phenolic molecules that are interesting for their diverse color properties. They are responsible for the red, blue, and purple colors in many fruits and berries and food products derived from them (*1*). However, anthocyanin molecules are unstable and highly susceptible to degradation. The color stability of anthocyanins is influenced by pH, temperature, presence of enzymes, light, structure and concentration of the anthocyanins, and the presence of complexing compounds such as other flavonoids, phenolic acids, and metals (*2*).

Anthocyanin copigmentation gives brighter, stronger, and more stable colors than those expressed by anthocyanin alone. Copigmentation is known to be responsible for the profuse color variability of bluish flowers and for stable wine colors (*3*−*9*), through which the phenomenon was first investigated. In food science, copigmentation is considered an important interaction, as color is one of the quality factors strongly affecting consumer acceptance of food. Copigmentation reactions of the more complicated anthocyanin complexes have long been studied with flower and wine colors, but intermolecular copigmentation reactions of the simpler anthocyanins occurring in fruits and berries are yet to be elucidated.

Copigmentation reactions can occur through intramolecular interactions, in which an organic acid, an aromatic acyl group, or a flavonoid (or some combination thereof) is covalently linked to an anthocyanin chromophore (*3*, *10*−*11*), or through loose intermolecular interactions, in which colorless flavonoids or other phenolic compounds (**Figure 1**) react with weak hydrophobic forces with anthocyanins (*12*). The former reactions are predominant in flower vacuoles, and the latter occur mostly in fruits and berries (*13*). Copigmentation is detected both as a hyperchromic effect, where the λ_{max} of the absorption spectrum increases, and as a bathochromic shift, where a shift toward higher wavelength (nm) at λ_{max} of the absorption spectrum occurs (*8*, *12*, *14*, *15*).

The present work consisted of two main objectives: to study the effects of five phenolic acids acting as copigments on five anthocyanins, and to investigate the stability of the formed copigmentation complexes during a storage period of 6 months. It was also of interest to examine the significance of the anthocyanin structure on the copigmentation phenomenon as well as the effect of the molar ratio of the anthocyanin−copigment.

MATERIALS AND METHODS

The anthocyanins used in this study were pelargonidin 3-glucoside (callistephin), cyanidin 3-glucoside (kuromanine), and malvidin 3-glucoside (oenin), obtained from Extrasynthese (Geney, France), and cyanidin 3-*O*-(2″-*O*-*β*-xylopyranosyl-6″-*O*-*β*-glucopyranosyl)-*β*-galactoside and cyanidin 3-*O*-(2″-*O*-*β*-xylopyranosyl-6″-*O*-(6-*O*-((*E*)-coumaroyl)-*β*-glucopyranosyl))-*β*-galactoside, obtained from Polyphenols (Bergen, Norway). The copigments ferulic acid, caffeic acid, rosmarinic acid, chlorogenic acid, and sinapic acid were purchased from Extrasynthese, and gallic acid, rutin, and quercetin were obtained from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Acros

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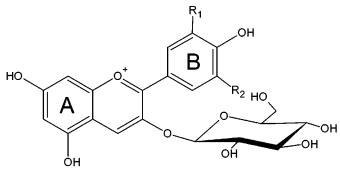
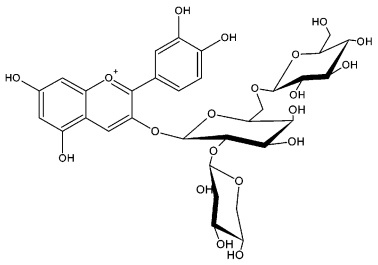
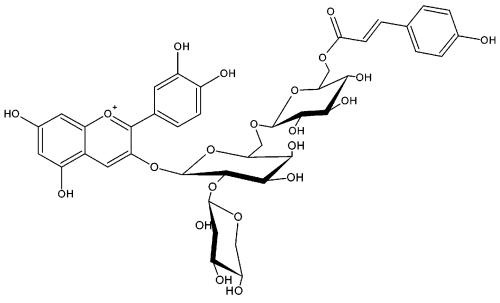


Anthocyanin monoglucosides

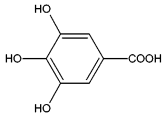
	R1	R2
Pelargonidin 3-glucoside	H	H
Cyanidin 3-glucoside	OH	H
Malvidin 3-glucoside	OCH_3	OCH_3



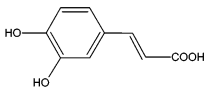
Cyanidin 3-(2″-xylosyl-6″-glucosyl)-galactoside



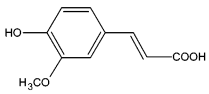
Cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside



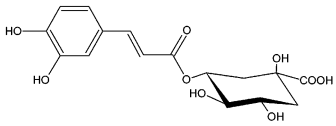
Gallic acid



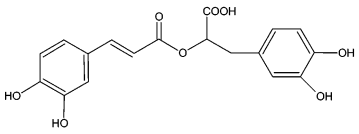
Caffeic acid



Ferulic acid



Chlorogenic acid



Rosmarinic acid

Figure 1. Structures of anthocyanins and phenolic acids used for copigmentation studies.

(Geel, Belgium), and trifluoroacetic acid (TFA) and the other chemicals were purchased from Sigma (Steinheim, Germany).

The anthocyanins were dissolved in 0.5% TFA at the concentration of 0.2 mM and the copigments in 10% DMSO in 0.02 M ammonium acetate at the concentration of 0.02 M. Here, 10% DMSO was used to increase the solubility of the copigments, which are rather insoluble in water. The anthocyanins were diluted 1:1 with the copigment, resulting in the final concentrations of 0.1 mM for anthocyanin and 0.01 M for copigment and a molar ratio of 1:100, which was the molar ratio chosen for the storage studies. Molar ratios of 1:50 and 1:10 were also established, keeping the anthocyanin concentration constant as above. Reference solutions of the anthocyanins were prepared by dissolving them 1:1 with 10% DMSO in 0.02 M NH_4OAc. Immediately after an anthocyanin was dissolved with a copigment, the pH was set to 3.37 $\pm$ 0.02, which is the natural pH of strawberry juice, with 25% ammonia or 10 M HCl, as verified using a Metler Delta 350 pH meter with pHC3359-9 combined electrode (Radiometer). This was also done for the reference solutions. The solutions were equilibrated for 30 min, after which their absorption spectra were recorded by using a UV−visible spectrophotometer (Perkin-Elmer), scanning the visible range from 450 to 600 nm. The solutions were kept at room temperature in daylight in sealed, but not vacuum-sealed, tubes to avoid evaporation for 6 months, during which time their spectra were recorded, first every second week and later once a month. A hyperchromic effect was detected as an increase in the absorbance value at λ_{max} and a bathochromic shift as a shift of the wavelength (nm) of λ_{max}. The pH of the solutions rose only 0.05 during the experiment.

The anthocyanidin, pelargonidin, was also included in the study, but under the conditions used it deteriorated at once and was not usable for the storage nor the copigmentation investigations. From the selected copigments, rutin and quercetin were also not usable since they precipitated as soon as they were introduced into the anthocyanin-containing solutions. Sinapic acid was not usable due to its low solubility.

Statistical analysis of variance (ANOVA) was conducted using Statgraphics plus, version 3.2. Significant ($P < 0.05$) differences between means of three replicates were identified using Tukey's procedure.

RESULTS AND DISCUSSION

Immediate Intermolecular Copigmentation Effect. The strongest immediate copigmentation effect at the 1:100 molar ratio occurred with malvidin 3-glucoside with all five copigments (gallic, ferulic, caffeic, rosmarinic, and chlorogenic acids), detected both as a hyperchromic effect (**Figure 2**) and as a bathochromic shift (**Table 1**). Intermolecular copigmentation in terms of hyperchromic effect took place relatively strongly also in cyanidin 3-glucoside and pelargonidin 3-glucoside solutions (**Figure 2**). The increase in color intensity was more profound with cyanidin 3-glucoside than with pelargonidin 3-glucoside. The acylated anthocyanin, cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside, possessed the strongest color intensity compared to the other anthocyanins (**Figure 2**). On the day of preparation, however, it did not show any statistically significant intermolecular copigmentation, and neither did the trisaccharidic anthocyanin, cyanidin 3-(2″-xylosyl-6″-glucosyl)-galactoside. As Dangles et al. (*16*) have pointed out, two or more cinnamic acid esters present in an anthocyanin induce strong enough intramolecular copigmentation to prevent intermolecular copigmentation from taking place. Our results support the assumption that one acyl group is enough for adequate intramolecular copigmentation and three sugar moieties are enough to prevent intermolecular copigmentation. Hoshino et al. (*17*) showed that the acylated anthocyanin,

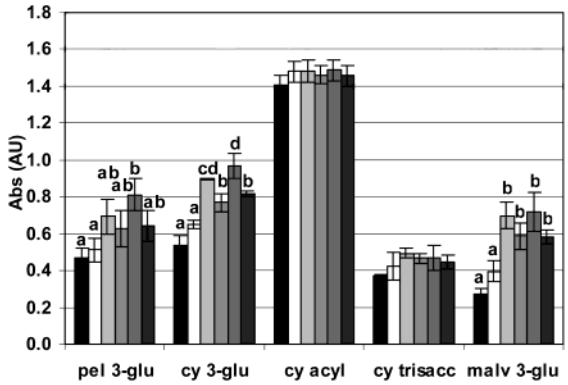


Figure 2. Copigmentation effect of anthocyanins induced by different phenolic acids in a 1:100 molar ratio on the day of preparation. Bars, from left to right, for each anthocyanin, represent anthocyanin alone, anthocyanin + gallic acid, anthocyanin + ferulic acid, anthocyanin + caffeic acid, anthocyanin + rosmarinic acid, and anthocyanin + chlorogenic acid. Columns within each group marked by the same letter or no letter are not significantly different.

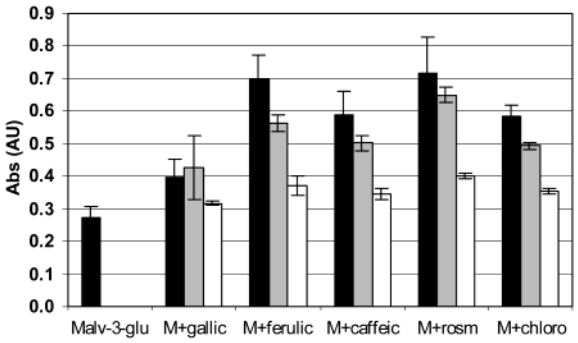


Figure 3. Intermolecular copigmentation effect of malvidin 3-glucoside on the day of preparation induced by different phenolic acids in a molar ratio 1:100 (black bars), 1:50 (gray bars), and 1:10 (white bars), detected as an increase of the absorbance of λ_{max}. The three molar ratio groups were statistically significantly different from each other when all the observations from every acid addition within a molar ratio were pooled together.

delphinidin 3-coumaroyl-glucoside-5-glucoside (awobanin), exhibited stronger intermolecular copigmentation than its unacylated form, delphinidin 3,5-diglucoside (delphin), but this copigmentation was observed with a flavone and not with phenolic acids.

Of the copigments, rosmarinic acid and ferulic acid were the best color enhancers on the day of preparation. They both increased the color intensity of malvidin 3-glucoside by 160%. Rosmarinic acid also enhanced cyanidin 3-glucoside color by 80%, and ferulic acid enhanced it by 70%. Likewise, caffeic acid and chlorogenic acid were relatively good copigments on the day of preparation. In a study conducted by Davies and Mazza (*18*), caffeic acid exhibited 40−50% greater copigmentation effect than chlorogenic acid with a diacylated anthocyanin, pelargonidin 3-coumaroyl-glucoside-5-malonoyl-glucoside (monardein). On the other hand, caffeic acid and chlorogenic acid had similar effects on pelargonidin 3-glucoside, as reported also in our results. The intermolecular copigmentation effect of caffeic acid and chlorogenic acid on the day of preparation was of the same magnitude with all the anthocyanins, including the trisaccharidic forms. Caffeic acid increased the color of the monoglucosidic anthocyanins by 30−110% and chlorogenic acid by 40−110%. Gallic acid produced a moderate color enhancement, increasing the absorbance at λ_{max} by 9−45%.

The most statistically significant bathochromic shift with the monoglucosides was induced by rosmarinic acid, the biggest shift of 18.6 nm occurring with malvidin 3-glucoside. The second highest shift emerged with ferulic acid, ranging from 17.6 nm with malvidin 3-glucoside to 5.5 nm with cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside (**Table 1**). The bathochromic shifts declined only very slightly during the 6-month storage period in most of the copigment−pigment solutions; only solutions containing ferulic acid and caffeic acid reverted close to the starting value of the visible absorbance at λ_{max} (data not shown).

Effect of Anthocyanin and Copigment Concentration. The results of the addition of copigments at three concentration levels showed that the outcome of copigmentation is dependent on molar ratio, which is in agreement with previous works (*8*, *18*). The anthocyanin/copigment molar ratio of 1:100 resulted in the strongest copigmentation effect, compared to molar ratios 1:50 and 1:10 (**Figure 3**). An increase in the concentration of the copigment resulted in both an increment of absorbance at λ_{max} and a bathochromic shift of λ_{max}. The difference in the effect of molar ratio on copigmentation was more pronounced with the copigments that induced the strongest copigmentation. For example, rosmarinic acid enhanced malvidin 3-glucoside color by 260% at a molar ratio of 1:100 and by 150% at 1:10, whereas with a weak copigment, gallic acid, the effect of molar ratio was not noteworthy (**Figure 3**). The effect of molar ratio also varied within the different anthocyanins (data not shown); the effect was stronger with the monoglucosidic anthocyanins. It can be concluded that the higher the copigment excess, the more pronounced the copigmentation effect. However, when the copigmentation concentration exceeds a certain level, no further changes in absorbance can be observed; therefore, the molar ratio cannot be raised to an unlimited extent (*17*).

Copigmentation and Color Stability during Storage. During the 6-month storage period, cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside had the best color stability of the studied anthocyanins. Its color diminished only by 30% from the original (**Figure 4C**). Malvidin 3-glucoside lost its color quickly, being imperceptible after 55 days (**Figure 4D**). Pelargonidin 3-glucoside and cyanidin 3-glucoside retained 20% and 25% of their color (**Figure 4A**,**B**), respectively, and cyanidin 3-(2″-xylosyl-6″-glucosyl)-galactoside had 40% of its color left after 6 months of storage (data not shown). It can be concluded that although the two trisaccharidic anthocyanins expressed weak copigmentation reactions, their structure was evidently protected from degradation by their substituents, and thus they retained their color better than the monoglucosides. This leads to the assumption that it is not only acylation (*19*) but also the sterical conditions given by the glycosylation pattern, in this case all three sugar moieties at position 3, that stabilizes the anthocyanin molecule. Garzón and Wrolstad (*20*) reported that the stability of acylated pelargonidin 3-sophoroside-5-glucoside was weaker than the stability of pelargonidin 3-glucoside. The authors concluded, however, that the solvent used, glycerol, may have an influence on the results. It has also been indicated that glucosylation at position 5 can induce rapid hydration of an anthocyanin (*10*), which in the case of acylated pelargonidin 3-sophoroside-5-glucoside could weaker its stability over pelargonidin 3-glucoside.

Copigment addition increased anthocyanin color stability in general during storage, which is in agreement with the results of other studies (*21*−*23*). Surprisingly, ferulic acid and caffeic

Table 1. Bathochromic Shift of Anthocyanins Reacting with Phenolic Acids, Detected as a Shift of the Absorbance Maximum $\Delta\lambda_{max}$ (nm) on the Day of Preparation ($X \pm$ SD, $n = 3$)[a]

	phenolic acid				
anthocyanin	gallic	ferulic	caffeic	rosmarinic	chlorogenic
pelargonidin 3-glucoside	4.6 ± 0.4a	10.0 ± 0.3c	7.8 ± 0.2b	12.9 ± 0.3d	8.7 ± 0.5b
cyanidin 3-glucoside	5.3 ± 0.5a	12.3 ± 0.1c	10.1 ± 0.2b	15.2 ± 0.2d	10.4 ± 0.2b
malvidin 3-glucoside	7.4 ± 0.4a	17.6 ± 0.2c	14.4 ± 0.2b	18.6 ± 0.3d	14.1 ± 0.3b
acylated cyanidin trisaccharide	2.5 ± 0.1a	5.5 ± 0.4c	4.2 ± 0.3b	6.1 ± 0.2c	4.3 ± 0.3b
cyanidin trisaccharide	2.5 ± 0.5a	7.3 ± 0.6c	4.9 ± 0.3b	8.3 ± 0.1c	5.7 ± 0.2b

[a] Values marked by the same letter within the same row are not significantly different. The anthocyanin concentration was 0.1 mM, and the copigment concentration was 0.01 M, pH 3.37, solvent TFA–NH_4OAc buffer with 5% DMSO.

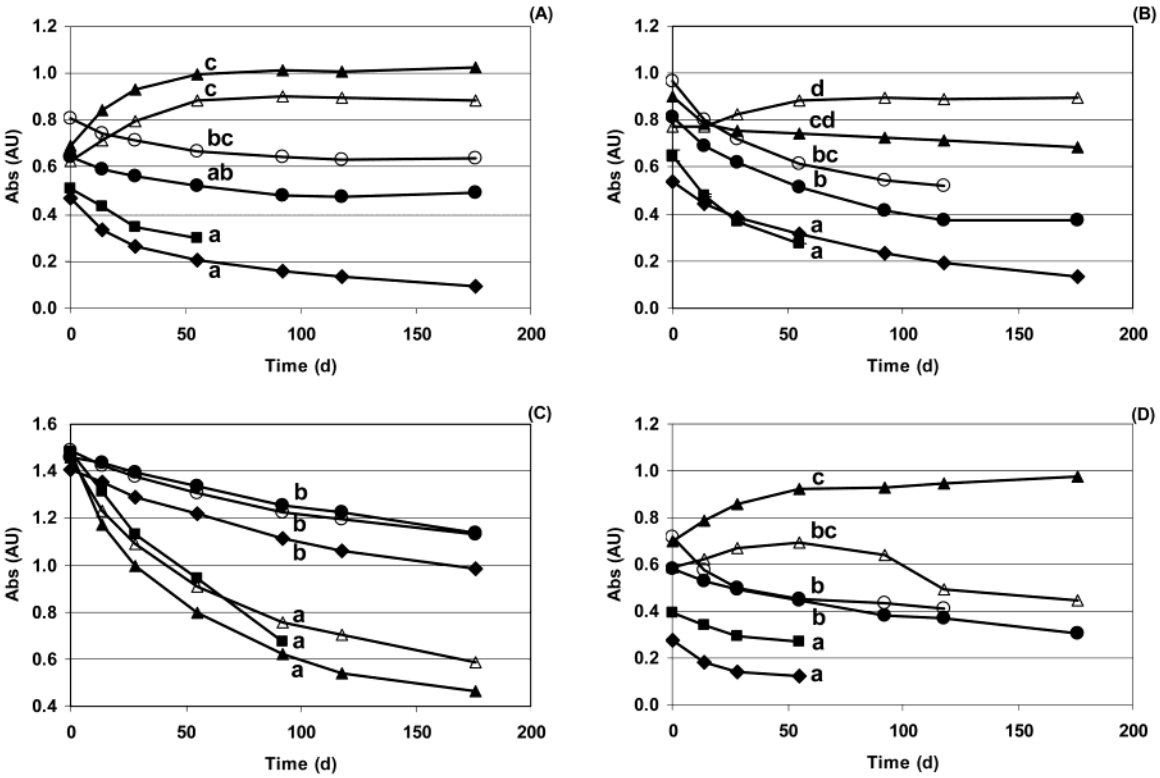


Figure 4. Intermolecular copigmentation effect of pelargonidin 3-glucoside (A), cyanidin 3-glucoside (B), cyanidin 3-(2″-xylosyl-6″-(coumaroyl-glucosyl))-galactoside (C), and malvidin 3-glucoside (D) with different phenolic acids during storage, detected as a change in the absorbance of λ_{max}. Anthocyanin alone, ◆; anthocyanin + gallic acid, ■; anthocyanin + chlorogenic acid, ●; anthocyanin + rosmarinic acid, ○; anthocyanin + caffeic acid, △; anthocyanin + ferulic acid, ▲. Within the specific time point, values marked by the same letter are not significantly different.

acid addition greatly increased the color of pelargonidin 3-glucoside throughout the entire storage period, the anthocyanin color intensity at the end of storage being 220% and 190% of the original intensity, respectively (**Figure 4A**). Caffeic acid also enhanced cyanidin 3-glucoside color throughout the storage period (**Figure 4B**), and ferulic acid enhanced the color of malvidin 3-glucoside (**Figure 4D**). Although rosmarinic acid enhanced anthocyanin colors strongly on the day of preparation, in storage it maintained the color poorly. Chlorogenic acid stabilized the anthocyanin color somewhat. Gallic acid was the weakest copigment with all the anthocyanins, resulting quickly in yellowish solutions. Gallic, ferulic, and caffeic acids decreased the color stability of the acylated anthocyanin during storage, which suggests that these phenolic acids interfere with and diminish the protective intramolecular mechanism of the acylated anthocyanin (**Figure 4C**).

Copigmentation reactions of the acylated anthocyanin and the trisaccharidic anthocyanin during storage were not significant, resulting most probably from their sterically compact structure, which prevents the copigments from intervening with the anthocyanin chromophore to form any kind of intermolecular complexes. Although the monoglucosidic anthocyanin, malvidin 3-glucoside, itself had weak color stability compared to the trisaccharidic anthocyanins for example, it enabled strong copigmentation to take place. This is in accordance with the results of earlier studies, in which malvidin-based anthocyanins exhibited greater copigmentation effects than other anthocyanins (*12*, *18*). According to Mazza and Brouillard (*12*), the copigmentation effect increases with the degree of methoxylation and glycosylation of the anthocyanin chromophore. This was the case with methoxylation in the present study but not with glycosylation; malvidin 3-glucoside exhibited a greater copigmentation effect than cyanidin 3-glucoside, but the trisaccharidic form of cyanidin exhibited weaker copigmentation than its monoglucosidic analogue. Therefore, it seems likely that it is the number of positions of sugar attachments in the anthocyanin chromophore, rather than the actual number of the sugar moieties, that increases the copigmentation effect. Obviously,

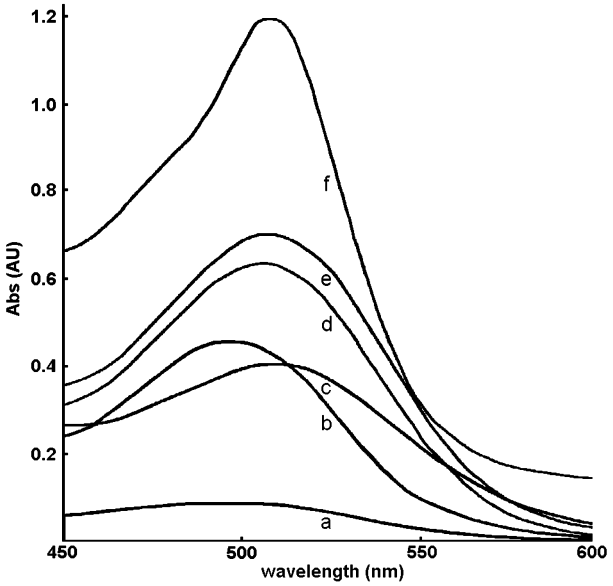


Figure 5. Absorption spectra of pelargonidin 3-glucoside with and without ferulic or chlorogenic acid addition on the day of preparation (T_0) and after 6 months of storage (T_6): (a) pelargonidin 3-glucoside at T_6; (b) pelargonidin 3-glucoside at T_0; (c) pelargonidin 3-glucoside + chlorogenic acid at T_6; (d) pelargonidin 3-glucoside + chlorogenic acid at T_0; (e) pelargonidin 3-glucoside + ferulic acid at T_0; (f) pelargonidin 3-glucoside + ferulic acid at T_6.

the degree of hydroxylation also affects the copigmentation reaction; hence, pelargonidin 3-glucoside produced stable copigmentation color with one free hydroxyl group in the B-ring, but the copigmentation effect was not as strong with cyanidin 3-glucoside, possessing two free hydroxyl groups in the B-ring. These findings of the importance of structure in the outcome of copigmentation reactions, color, and stability of anthocyanins are in agreement with the results of previous studies (*18*, *24*, *25*).

To our knowledge, there are no previous reports on the stability of intermolecular copigmentation complexes of anthocyanins with phenolic acids during storage. The most interesting findings in the present study were the reactions of ferulic and caffeic acids with the monoglucosidic anthocyanins. These copigments increased the color intensity of the monoglucosidic anthocyanins throughout the storage period. However, the standard deviation of the caffeic acid replicates was wide, which reduced their significance somewhat. On the basis of our results, the copigments in this study can be classified into three categories. The behavior and association of caffeic and ferulic acid with the anthocyanins differed from those of chlorogenic and rosmarinic acid, and the behavior and association of gallic acid differed further from those of all the other copigments. Their chemical structures also support this classification. Ferulic and caffeic acids, which are simple cinnamic acids with and without a methoxyl group, respectively, were the best copigments. Chlorogenic and rosmarinic acids, which are conjugated cinnamic acid derivatives, can be considered as moderate copigments, and gallic acid, which is a simple benzoic acid, was the poorest copigment. The calculation of different stoichiometric and equilibrium constants for chlorogenic and caffeic acids by Davies and Mazza (*18*) indicates their difference in the nature of associations with anthocyanins and supports their different classification as copigments. This difference is detectable also in the spectra of these copigments reacting with pelargonidin 3-glucoside (**Figure 5**). Ferulic acid gives a much narrower spectrum with pelargonidin 3-glucoside during storage than chlorogenic acid.

The current results may be of use in development of foods with anthocyanin-rich ingredients. To fully benefit from the phenomenon of intermolecular copigmentation, the promising effects of ferulic acid and caffeic acid on anthocyanin color enhancement and stability should be further investigated in food matrices containing both anthocyanins and these copigments.

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