Copigmentation of Simple and Acylated Anthocyanins with Colorless Phenolic Compounds

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The influence of pH and structure and concentration of copigment and pigment on the copigmentation reaction of anthocyanin-colorless phenolic compounds has been investigated. It has been found that the copigmentation effect displayed by chlorogenic acid, caffeic acid, and rutin was greater for the acylated pelargonidin, monardaein, than for the corresponding pelargonidin 3-glucoside, and malvin solutions exhibited the greatest copigmentation effects. The pH of maximum copigmentation effect was 3.2-3.7 for chlorogenic acid and 3.5-4.7 for caffeic acid and rutin. The equilibrium and stoichiometric constants for eight pigment-copigment combinations were determined and used to quantitate the association between pigments and copigments. The magnitude of the copigmentation effect is interpreted in terms of the acidity constant of the copigment and the hydration constant of the pigment.

INTRODUCTION

A phenomenon which plays a major role in the expression of such a wide range of brilliant colors by anthocyanins in plants is copigmentation, a molecular interaction that occurs between anthocyanins and copigments. Its effects are an increase in the color intensity (hyperchromic effect) and a shift in the wavelength of maximum absorbance toward higher wavelengths (bathochromic shift) (Asen et al., 1971; Osawa, 1982; Brouillard et al., 1989; Mazza and Brouillard, 1990; Goto and Kondo, 1991). Molecules acting as copigments include a large variety of compounds, such as flavonoids, polyphenols, alkaloids, amino acids, organic acids, and the anthocyanins themselves. Of these substances, however, only a few have been investigated in some detail. Colorless flavonoids and polyphenols are frequently found in association with anthocyanins in the vacuoles of the colored cells of higher plant organs (McClure, 1979). Therefore, the copigmentation phenomenon is widespread in nature. It also occurs in fruit and vegetable products such as juices and wines (Mazza and Brouillard, 1987).

In previous studies by Brouillard et al. (1989) and Mazza and Brouillard (1990), it was demonstrated that chlorogenic acid associates with the flavylium cation, AH^+, of malvin and cyanin giving 1:1 molecular complexes. In past studies, however, copigmentation was studied only with simple anthocyanins and at pH 3.6, which corresponds to the domain where the copigment effect exerted by chlorogenic acid on the anthocyanin colored molecules is at its maximum. In the present work, we have addressed the following objectives: (1) to evaluate the copigmentation behavior of a diacylated form of pelargonidin (monardaein) complexed with chlorogenic acid and caffeic acid; (2) to evaluate the effectiveness of three colorless phenolic compounds, chlorogenic acid, caffeic acid, and rutin, as copigments of malvidin 3,5-diglucoside and pelargonidin 3-glucoside; and (3) to elucidate the mechanism by which the copigments chlorogenic acid, caffeic acid, and rutin interact with the pigments pelargonidin monoglucoside, monardaein, and malvidin diglucoside at pH 2.7-5.7.

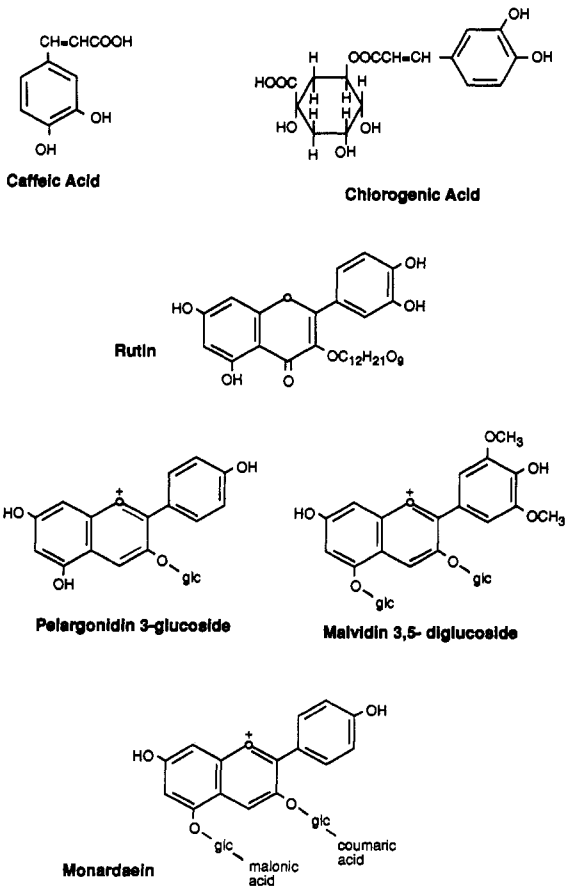

Figure 1. Structures of pigments and copigments used for complexation studies.

MATERIALS AND METHODS

Chlorogenic and caffeic acid were purchased from Sigma (St. Louis, MO). Malvin, pelargonidin 3-glucoside, and rutin were purchased from Carl Roth (Karlsruhe, Germany). Monardaein (Figure 1) was purified by a combination of Sephadex LH20 column chromatography and HPLC from petals of *Monarda fistulosa* grown at the Agriculture Canada Research Station, Morden, MB. All pigments and copigments were analyzed by HPLC for assessment of purity.

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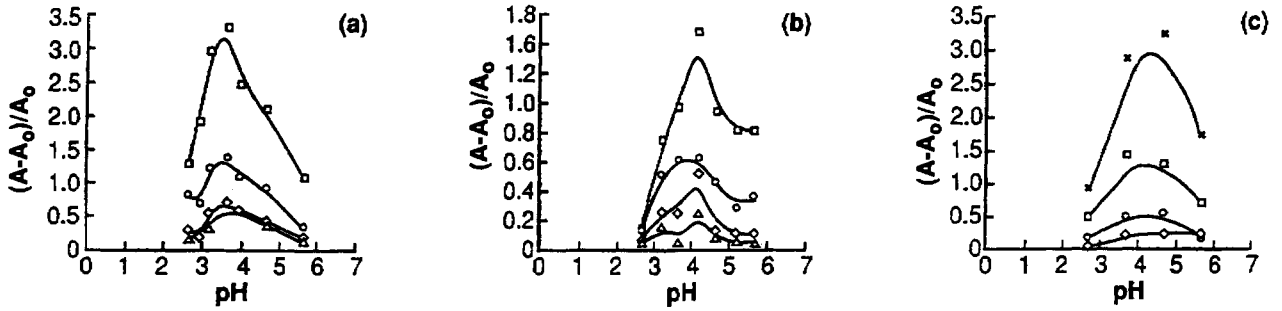

Figure 2. Plots of $(A - A_0)/A_0$ vs pH for malvin (a), pelargonidin 3-glucoside (b), and monardaein (c) copigmented with chlorgenic acid. (Pigment concentration = 2.58×10^{-4} M. Copigment:pigment molar ratios: △, 5; ◇, 10; ○, 20; □, 40; and ×, 80. Solvent: aqueous H_3PO_4–NaOAc buffer. l = 1 cm. Ionic strength = 0.20 M. T = 20 ● 0.5 °C.)

Table I. Influence of Pigment and Copigment Structures on pH of Maximum Copigmentation Effect

pigment	copigment	pH of max copigmentation effect
malvin	chlorogenic acid	3.2–3.7
	caffeic acid	3.7–4.7
	rutin	3.7–4.7
pelargonidin 3-glucoside	chlorogenic acid	3.2–3.5
	caffeic acid	3.5–4.5
	rutin	3.5–4.0
monardaein	chlorogenic acid	3.7–4.7
	caffeic acid	3.2–4.7

The reagents used for preparation of the buffer system were of reagent grade and supplied by Fisher Scientific (Winnipeg, MB), J. T. Baker (Phillipsburg, NJ), and Merck and Co. (Montreal, PQ).

Chlorogenic Acid Copigmentation. Pure anthocyanins (pelargonidin 3-glucoside, malvidin 3,5-diglucoside, and monardaein) were dissolved in 0.06 M aqueous phosphoric acid at concentrations of 5.16×10^{-4} to 1.55×10^{-3} M. Immediately after preparation, each solution was thoroughly mixed in the dark at 20 °C for 30–45 min and diluted to half the original concentration by addition of 0.2 M aqueous NaOAc. Absorption spectra of buffered solutions with and without copigment were monitored in the visible range from 400 to 700 nm with a Beckman DU-50 spectrophotometer connected to an Epson RX-80 printer and an IBM personal computer equipped with a "peak pick" program (Beckman Quant 1 Soft-Pak, Beckman Instruments Inc., Scientific Instruments Division, Irvine, CA). A and A_0 absorbance values, representing the absorbance with and without added copigment, were recorded at 525 nm. At this wavelength, no interference by the copigment in the absorbance occurs. The spectrophotometer was fitted with a magnetic stirring device (GFS Chemicals, Columbus, OH) and a temperature-controlled 1-cm quartz cuvette connected to a 20 ● 0.5 °C water bath. A 2-mL aliquot of anthocyanin solution was placed in a cuvette and, after its spectrum was recorded, a known weight of chlorogenic acid was added.

Following 15–20 min of mixing, the pH of each copigment–pigment solution was adjusted to the desired level and the spectrum of the pigment–copigment was recorded. The pH of each solution of anthocyanin was adjusted to the desired level by injection into the sample cells of a few microliters of 10 N HCl or 10 M NaOH. The pH of the solution was measured directly in the cuvette with a Fisher Accument pH meter Model 825 MP and Fisher Accu-pHast combination glass electrode. The pH meter was calibrated using Fisher pH 4.00 and 7.00 standard buffers at room temperature.

Caffeic Acid Copigmentation. Due to the low solubility of caffeic acid in the mildly acidic H_3PO_4–NaOAc solution, it was necessary to dissolve the caffeic acid in the sodium acetate buffer prior to addition to the phosphoric acid anthocyanin solution. The anthocyanin solution was prepared as described previously; however, the solution was not diluted immediately with sodium acetate. A known weight of copigment in twice the required molality was dissolved in 0.2 M NaOAc. Once thoroughly dissolved, 1 mL of pigment solution in 0.06 M H_3PO_4 and 1 mL of copigment solution in 0.2 M NaOAc were added to the cuvette and mixed thoroughly for 15–20 min. The pH was adjusted as previously described.

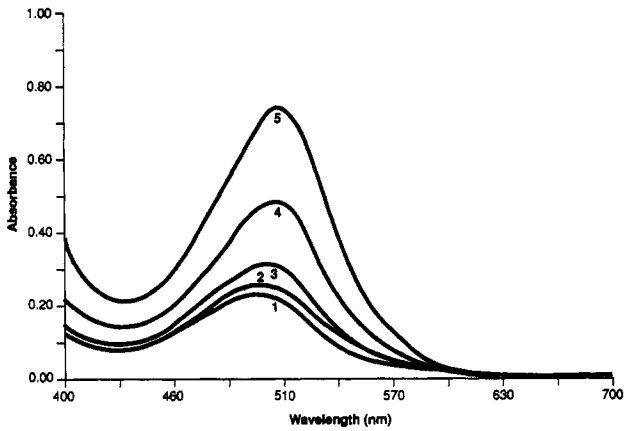

Figure 3. Visible spectra of monardaein (2.58×10^{-4} M) solutions copigmented with chlorogenic acid at (1) 0, (2) 5, (3) 20, (4) 40, and 5 (80) copigment:pigment molar ratios in aqueous H_3PO_4–NaOAc buffer, pH 3.70. (Ionic strength = 0.20 M; l = 1 cm; T = 20 ± 0.5 °C.)

Rutin Copigmentation. Rutin was dissolved in 0.08 M NaOH followed by partial neutralization with 0.01 M HCl prior to addition to the 0.06 M H_3PO_4 anthocyanin solution. The anthocyanin and copigment solutions were prepared in twice the required molarity, and the pH was adjusted as described previously.

Data Analysis. Stoichiometric constants were determined using Statistical Analysis System (SAS) version 6.06 (SAS Institute Inc., Cary, NC), and the data were plotted using Harvard Graphics version 2.30 (SPC Software Publishing Corp., Mountain View, CA).

RESULTS AND DISCUSSION

The magnitude of the copigmentation effect has been found to be dependent upon a variety of factors, including the nature of both the pigment and copigment, and can be measured by the parameter $(A - A_0)/A_0$. As illustrated in Figure 2 and Table I, the chlorogenic acid complexes formed with the three pigments, monardaein, pelargonidin 3-glucoside, and malvin, generally exhibited a maximum copigmentation effect in the pH range 3.2–3.7. Caffeic acid, however, exhibited a maximum copigmentation effect at a slightly higher pH range, approximately pH 3.5–4.7. Rutin exhibited a maximum copigmentation effect at approximately pH 3.5–4.0 for pelargonidin and pH 3.7–4.7 for malvin.

Generally, all of the complexes studied (eight copigment–pigment combinations) exhibited bathochromic and hyperchromic shifts in the maximum absorbance as a result of the copigmentation reaction. Figure 3 illustrates the bathochromic and hyperchromic effects observed for the

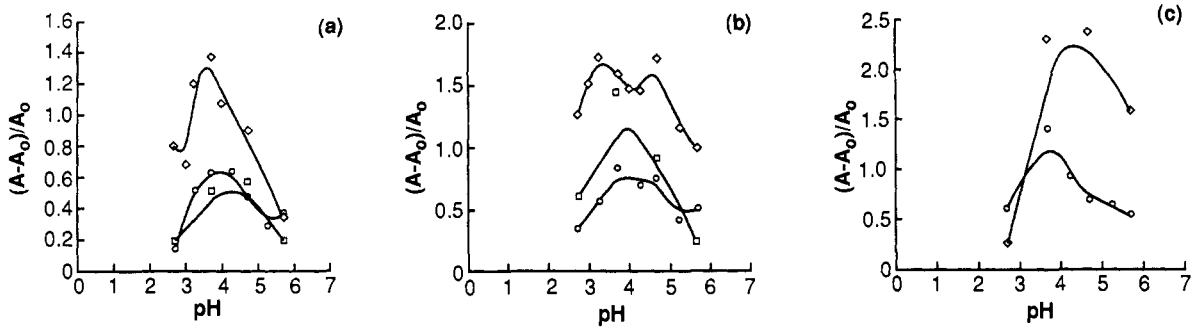

Figure 4. (a) Plots of $(A - A_0)/A_0$ vs pH for malvin (◇), monardaein (□), and pelargonidin 3-glucoside (○) solutions (2.58×10^{-4} M) copigmented with chlorogenic acid (5.16×10^{-3} M). (b) Plots of $(A - A_0)/A_0$ vs pH for malvin, monardaein, and pelargonidin 3-glucoside solutions (2.58×10^{-4} M) copigmented with caffeic acid (5.16×10^{-3} M). (c) Plots of $(A - A_0)/A_0$ vs pH for malvin and pelargonidin 3-glucoside solutions (2.58×10^{-4} M) copigmented with rutin (6.45×10^{-4} M). Solvent, aqueous H_3PO_4–NaOAc buffer, ionic strength = 0.20 M; l = 1 cm; T = 20 ± 0.5 °C.)

Table II. Stoichiometric Constants for Malvin, Pelargonidin 3-Glucoside, and Monardaein Solutions (2.58×10^{-4} M) Complexed with Chlorogenic Acid, Caffeic Acid, and Rutin in Aqueous H_3PO_4–NaOAc Buffer (pH Range 2.7–5.7; Ionic Strength 0.20 M; l = 1 cm; T = 20 ± 0.5 °C)

		anthocyanin		
copigment	pH	malvin	pelargonidin 3-glucoside	monardaein
chlorogenic acid	2.7	0.94	0.67	1.46
	3.7	0.97	1.14	0.99
	4.7	1.05	1.25	1.38
	5.7	1.17	1.22	0.90
caffeic acid	2.7	0.63	0.46	0.79
	3.7	0.86	0.66	0.79
	4.7	0.49	0.67	0.71
	5.7	0.38	0.53	1.23
rutin	2.7	1.10	0.14	nd[a]
	3.7	1.14	0.31	nd
	4.7	0.76	nd	nd

[a] nd, not determined.

complexes formed between monardaein and chlorogenic acid as the copigment concentration increased at pH 3.7.

The malvin–caffeic acid and pelargonidin 3-glucoside–caffeic acid complexes exhibited greater copigmentation magnitudes than the chlorogenic acid based complexes (Figure 4a,b). Likewise, the copigmentation effectiveness of the two copigments was notably different when complexed with monardaein (Figure 4a,b). The caffeic acid–monardaein complexes exhibited 40–50% greater copigmentation magnitude at pH 2.7–4.7 than the corresponding chlorogenic acid complexes. The copigmentation trends exhibited by pelargonidin 3-glucoside as a function of pH were similar to those of monardaein; however, the magnitude of copigmentation exhibited by the pelargonidin complexes was considerably lower.

The copigmentation magnitudes exhibited by the caffeic acid–monardaein complexes were consistently 2-fold higher than the corresponding pelargonidin complexes from pH 2.7 to 4.7, as illustrated in Figure 4b. However, the copigmentation magnitudes exhibited by the malvin–caffeic acid complexes were greater than those of both the monardaein and pelargonidin complexes. The copigmentation magnitude exhibited by the chlorogenic acid–malvin complexes was considerably higher (1.5–3-fold) than that of the corresponding monardaein complexes.

Generally, pigment structure and pH had little influence on the stoichiometric constants (n), determined from plots of $\ln[Cp]_0$ vs $\ln((A - A_0)/A_0)$ and describing the association between chlorogenic acid and the anthocyanin pigments studied (Table II). From pH 3.7 to 5.7, the chlorogenic acid and caffeic acid complexes formed with the three pigments exhibit n values approximating 1 (Figure 5). It has been reported previously that a stoichiometric constant close to unity is characteristic of a 1:1 association between pigment and copigment. The results presented in Table II are in agreement with findings reported in the literature (Brouillard et al., 1989; Mazza and Brouillard, 1990). The caffeic acid complexes, particularly those with malvin and pelargonidin 3-glucoside, exhibited stoichiometric constants closer to 0.5 than 1. This suggests that these molecular species may not associate in a 1:1 ratio. The monardaein solutions exhibited similar stoichiometric constants for chlorogenic acid and caffeic acid complexes, but once again, only the chlorogenic acid based complexes can be described as a 1:1 complex. The caffeic acid based complexes generally do not fit the 1:1 model. It is thus suggested that the lack of the quinic acid moiety in caffeic acid may be conducive to the association of two molecules of this copigment for each molecule of anthocyanin.

Figure 5c illustrates that pigment concentration had little effect on the stoichiometry of the malvin complexes formed with caffeic acid at pH 3.7. Similarly, the effect of pigment concentration on the stoichiometry of the complexes formed with chlorogenic acid was found to be negligible.

The equilibrium constant for the reaction of complexation, K, which is expressed as $[AH(Cp)_n^+]/([AH^+][Cp]^n)$, gives the strength of the association between the copigment and the anthocyanin (Brouillard et al., 1989). In this study, K was determined from the intercept of the plot $\ln((A - A_0)/A_0)$ as a function of $\ln[Cp]_0$ (Figure 5) and measurement of the ratio of absorbance of anthocyanin in 0.2 M aqueous HCl solution containing an excessive amount of copigment (A) and no copigment (A_0), respectively. The values of K determined by this method varied with the pigment–copigment system. At the pH of maximum copigmentation, K was 263 ± 44 M^{-1} for the malvin–chlorogenic acid complex, 20 ± 11 M^{-1} for the malvin–caffeic acid complex, and 3913 ± 170 M^{-1} for the malvin–rutin complex. The K values for pelargonidin 3-glucoside–chlorogenic acid and pelargonidin–caffeic acid complexes were 247 ± 81 and 21 ± 3 M^{-1}, respectively, and the K values for monardaein–chlorogenic acid and monardaein–caffeic acid were 257 ± 25 and 52 ± 33 M^{-1}, respectively. These values of K appear to suggest that the tendency of the three copigments investigated to associate with anthocyanins is high for rutin, intermediate for chlorogenic acid, and low for caffeic acid. Caffeic acid, however, gave copigmentation effects often greater than those of chlorogenic acid. Thus, the tendency of this acid to complex with anthocyanins should also be higher than that of chlorogenic acid. This seeming discrepancy, however, reflects differences in the nature of the association between

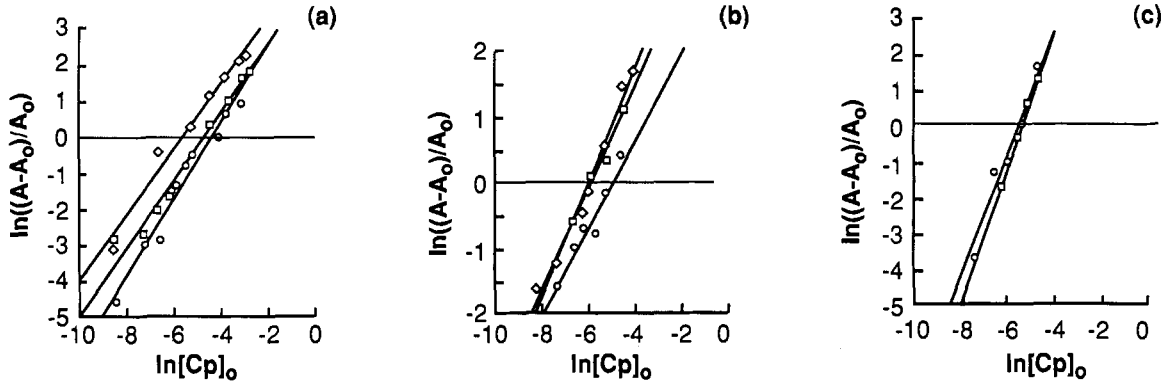

Figure 5. (a) Plots of $\ln((A - A_0)/A_0)$ vs $\ln[Cp]_0$ for 2.58×10^{-4} M malvin (◇), pelargonidin 3-glucoside (○), and monardaein (□) solutions complexed with chlorogenic acid in aqueous H_3PO_4–NaOAc buffer. (b) Plots of $\ln((A - A_0)/A_0)$ vs $\ln[Cp]_0$ for 2.58×10^{-4} M malvin, pelargonidin 3-glucoside, and monardaein solutions complexed with caffeic acid in aqueous H_3PO_4–NaOAc buffer. (c) Plots of $\ln((A - A_0)/A_0)$ vs $\ln[Cp]_0$ for (1) 2.58×10^{-4} M and (2) 7.73×10^{-4} M malvin solutions complexed with caffeic acid in aqueous H_3PO_4–NaOAc buffer; pH 3.7. (Ionic strength = 0.20 M; l = 1 cm; $T = 20 \pm 0.5$ °C; $[Cp]_0$ = analytical concentration of copigment.)

these copigments and the anthocyanins. The higher values of K for chlorogenic acid indicate that this copigment more strongly associates with the flavylium cation AH^+ than with the neutral base A and the anionic quinonoidal base A^-, which in turn interact more strongly with caffeic acid. The K values determined in this study are in general agreement with the values reported by Brouillard et al. (1989), who estimated the K constants for the malvin–chlorogenic acid and malvin–rutin complexes at 390 ± 50 and 4000 M^{-1}, respectively. The slight variation between the values from the two studies probably originates from the difficulty in obtaining reliable A/A_0 ratios due to some diffusion of light for the more concentrated copigment solutions.

It has been previously established that the copigmentation reaction reduces the extent of the hydration of the pyrilium nucleus of the flavylium cation (Brouillard et al., 1989; Mazza and Brouillard, 1990). In the present study, it has been found that the extent to which the copigmentation reaction reduces the hydration reaction is dependent upon pH, copigment and pigment structure, and concentrations of both copigment and pigment. Solutions of nonacylated and acylated pelargonidins exhibited the maximum degree of copigmentation at approximately pH 3.2–3.5 and 3.7–4.7, respectively, when complexed with chlorogenic acid. From this, it can be concluded that chlorogenic acid effectively stabilizes the color of both pelargonidin 3-glucoside and monardaein. Since it has been established that the substitution pattern of the B ring of the pigment affects the interaction between copigment and pigment, the difference in chlorogenic acid copigmentation observed among the three pigment solutions can be attributed to the structural differences among the three pigments. It can thus be concluded that the number of oxygen substituents has a greater influence on the copigmentation magnitude than the presence of diacylation, as found on the monardaein molecule. Generally, the copigmentation observed with pelargonidin 3-glucoside solutions was lower than that with the other pigments studied and can be attributed to the value of the hydration constant, K_h, which is approximately 10-fold smaller than that of the corresponding diglucosides (Sondheimer, 1953; Mazza and Brouillard, 1987). Thus, because of the low hydration constant of the monoglucoside, the recovery of color by the copigmentation reaction is lowered since the hydration reaction occurs at a higher pH than in the diglucoside.

The different effectiveness of the copigments can be ascribed to several factors including differences in stereochemistry or configuration required to bring the copigment into proximity with the pyrylium nucleus and differences in pK values between the copigments. For instance, the higher pK_a value of caffeic acid, 4.5, compared to that of chlorogenic acid, 3.5 (Timberlake, 1959), may partially account for the higher pH at which the maximum copigmentation effect was observed in the caffeic acid–pigment complexes.

The complex formed between malvin and rutin exhibited a dramatic change in the magnitude of copigmentation as a function of pH, indicative of a displacement of the hydration equilibrium in favor of the flavylium cation (Figure 4c). The results reported are in agreement with those of Sadlowski (1985) and indicate that the enhanced copigmentation effect produced can be attributed to a displacement of the equilibrium of the hydration reaction produced by the hydrophobic stacking interaction of the flavylium cation and the copigment.

CONCLUSIONS

Results of this study have demonstrated that pH, copigment and pigment structure, and concentration of both copigment and pigment have a dramatic influence on the copigmentation phenomenon. The three phenolic compounds were shown to interact differently with each of the pigments studied, indicating an influence of both copigment and pigment structure on the magnitude of copigmentation. Complexes formed with monardaein resulted in greater copigmentation magnitudes than the corresponding monoglucoside complexes over all pH levels studied. These differences can be attributed to the presence of acyl substituents attached to the monardaein molecule which result in a higher value of the hydration constant as compared to that of the monoglucoside anthocyanin. Comparison to complexes formed with malvin, however, reveals that the presence of methyl substituents has a greater effect than diacyl substitution on the copigmentation phenomenon.

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