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Iron–Polyphenol Complex Formation and Skin Discoloration in Peaches and Nectarines

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ABSTRACT. **The formation of metallo-pigmentation and copigmentation as potential mechanisms of inking formation was investigated in peach and nectarine skin tissues. Cyanidin-3-glucoside, the most abundant anthocyanin in peaches and nectarines, formed very purple ferric complexes with an anthocyanin/iron molar ratio of two. Greenish metallo complexes between ferric ion and chlorogenic acid, caffeic acid, catechin, or epicatechin formed with an phenolic/iron molar ratio of one. The lack of copigmentation pointed out the importance to focus research on the metallo-phenolics reaction. High intensity of dark color formation was developed with cyanidin-3-glucoside, followed by caffeic acid, chlorogenic acid, catechin, and epicatechin on an equal molar basis. Citric acid acted as a strong iron chelator to prevent and reverse the formation of ferric cyanidin-3-glucoside complexes. The variety of dark and light colored spots observed on the surface of peaches and nectarines is explained by the formation of metallo-pigment complexes.**

Consumers often purchase fresh fruit based on external appearance and nutritional value. Therefore, the presence of abnormal cosmetic skin color or blemishes can reduce sales. Black or brown spots commonly result from either nonenzymic or enzymic reactions involving plant phenolics, oxygen, and environmental contaminants such as metal ions (Cheng and Crisosto, 1994; Crisosto et al., 1993; Denny et al., 1986; Hopfinger, 1989; Phillips, 1988). While enzymatic oxidations of phenolics generally promote brown discoloration in fruit skin (Cheng and Crisosto, 1995; Mellenthin and Wang, 1974), nonenzymic reactions such as formation of metal ion complexes may be the only contributors to peach and nectarine black staining or inking (Cheng and Crisosto, 1994; Denny et al., 1986; Phillips, 1988; Ridley, 1976). Based on previous work, we demonstrated (Cheng and Crisosto, 1994; Crisosto et al., 1993) that fruit skin abrasion damage combined with heavy metals fruit contamination are requirements for peach and nectarine skin discoloration or inking to occur.

Formation of metallo complexes between metal ions and anthocyanins in abraded epidermal cells has yielded darkened discoloration on peaches and nectarines (Cheng and Crisosto, 1994; Denny et al., 1986, Phillips, 1988). Among the metal ions studied, iron showed the greatest induction of dark discoloration (Cheng and Crisosto, 1994). Heavy metals, at very low levels, such as iron, aluminum, and copper may become fruit skin contaminants as a result of preharvest sprays applied to control decay and insect damage on fruit. Heavy metals are contaminants on pesticide formulations at physiological active levels, which can trigger reactions with skin pigments. Preharvest spray intervals for different fungicides have been recommended to reduce fruit contamination and, thus, inking incidence. As we had also observed light brown spots on the green-yellow part of peach and nectarine skins, we believed that nonanthocyanin skin phenolics may also be involved in the inking mechanism. The epidermal tissues of peaches and nectarines are rich in trihydroxylbenzene and *o*-dihydroxylbenzene phenolics (Cheng and Crisosto, 1995; Macheix et al., 1990), which have strong iron chelating abilities (Bayer, 1959; Bayer et al., 1966). In this dark color formation mechanism, the formation of metallo complexes may be hindered by cellular iron chelators (Hughes and Swain, 1962; Jurd and Asen, 1966). Cellular iron chelator, such as citric and malic acids, are commonly present in fresh fruit (van Gorsel et al., 1992; Wills et al., 1984), and its levels in the skin of peaches and nectarines may affect the intensity of iron-copigment reaction.

Another potential mechanism for dark spot formation is the reaction between anthocyanins and other organic metabolites (copigmentation) without metal participation (Brouillard, 1983; Osawa, 1982). At the skin's physiological pH, copigmentation between anthocyanins and copigments could contribute to the discoloration in abraded peach and nectarine skin. It has been reported that in flowers, fruit, and aqueous solutions, copigmentation can produce colors ranging from pink to blue (Brouillard, 1983). The determination of the mechanism involved in inking is essential to develop practical ways to control its incidence.

The purpose of this study was to investigate the formation of metallic complexes in acetate buffer solutions at physiological pH, and then to assess the role of copigmentation, iron-phenolic complex formation and cellular iron chelators in peach and nectarine skin discoloration.

Materials and Methods

PLANT MATERIALS AND CHEMICALS. Freeze-dried peach and nectarine skin powder was prepared as previously described (Cheng and Crisosto, 1994). Cyanidin-3-glucoside (HPLC pure) was purchased from Extrasynthese (Genay, France) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis).

SPECTRAL ANALYSIS. All analyses were conducted in 0.5 M sodium acetate buffer, which does not chelate iron, with pH (3.5 and 4.0) in the range reported for skin homogenates of peaches and nectarines (Cheng and Crisosto, 1994). All solutions were prepared in distilled water except anthocyanin solutions, which were in 0.01% HCl in distilled water. When necessary, dissolving phenolics was aided by warming. All spectral analysis solutions contained 0.9 mL buffer and an aliquot of iron, phenolics and/or organic acid solutions plus distilled water to bring the final volume

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to 1 mL. All solutions were added directly to quartz cuvettes, mixed quickly by inverting, and their absorbance was immediately measured. When cyanidin-3-glucoside was present in the analysis solution, measurements were made 1 min after mixing to allow absorbance stabilization. All spectroscopic measurements were done with a spectrophotometer (UV160; Shimadzu, Columbia, Md.) using a cell held at 25 ± 1 °C by water-isothermal bath and quartz cuvettes with a 10-mm path length and 1.5-mL volume capacity.

CHELATION BETWEEN IRON AND PHENOLIC COMPOUNDS OR ORGANIC ACIDS. Interactions between iron and phenolics were measured using solutions of 0.1 to 5 mM iron ($FeCl_3$) and 0.4 mM chlorogenic acid or 1.0 mM caffeic acid, (+)–catechin, or (–)–epicatechin. Iron and cyanidin-3-glucoside interactions were measured using 0.010 to 0.25 mM iron and 0.05 mM anthocyanin. Interactions between iron and organic acids were measured using 4 mM citric or malic acid and 0.25 to 10 mM iron. Buffer pH was readjusted with NaOH to help maintain the mixed solutions at the desired pH under high iron concentrations.

CHELATION INVOLVING IRON, CITRIC ACID, AND CYANIDIN-3-GLUCOSIDE. Chelation was measured by solution spectral parameters that reflected changes in the visible wavelength. An aliquot of citric acid or iron solution in equal molar amounts was added at different time intervals to acetate buffer (0.5 M, pH 4.0, 0.9 mL) containing 0.05 mM cyanidin-3-glucoside. The spectral parameters of the mixed solutions were determined immediately before, right after, and 1 and 5 min after each solution addition. The data were adjusted for volume changes.

COPIGMENTATION INTERACTIONS INVOLVING CYANIDIN-3-GLUCOSIDE AND PHENOLIC COMPOUNDS OR ORGANIC ACIDS. Acetate buffer solutions (pH 4.0) containing cyanidin-3-glucoside (0.05 mM) and either citric acid (0.25 to 8 mM), malic acid (0.25 to 8 mM), chlorogenic acid (0.025 to 0.4 mM), catechin (0.025 to 0.4 mM), caffeic acid (0.005 to 0.2 mM), or epicatechin (0.005 to 0.2 mM) were prepared and absorption spectra were scanned before and after adding copigments. The concentrations of the copigments were selected based on their ratio to the anthocyanin concentrations previously reported in peach and nectarine skin (Cheng and Crisosto, 1995).

SKIN IRON-BINDING CAPACITY. The iron-binding capacity of skin was estimated by measuring absorbance at 578 nm (for blue iron-galloyl complexes) and 680 nm (for green iron-catechol complexes) according to the method of Brune et al. (1991). Phenolics were extracted by homogenizing skin powder (400 mg) in 10 mL extraction reagent (dimethylformamide and 0.1 M acetate buffer of pH 4.4, 1:1, v/v) for 1 min and centrifuging at 20,000× *g* for 10 min at 4 °C. The pellets were reextracted twice with 5 mL extraction reagent following centrifugation. The supernatants were combined and used as phenolic extracts. One milliliter of the phenolic extract was mixed with 4 mL ferric reagent [5% ferric ammonium sulfate in 1 M HCl (w/v), 50% urea in 0.1 M acetate buffer (pH 4.4) and 1% gum arabic in distilled water (w/v), 1:89:10, by volume]. After 20 min, absorbances of the mixture were measured at 578 and 680 nm against a blank of 1 mL extraction reagent and 4 mL ferric reagent. The background absorbance contributed by compounds in phenolic extracts was determined by reading a mixture of 1 mL phenolic extract and 4 mL ferric reagent against a blank of 1 mL extraction reagent and 4 mL ferric reagent lacking ferric ammonium sulfate. The concentrations of phenolics were determined based on standards of authentic catechin and tannic acid prepared in extraction reagent.

SKIN ORGANIC ACID ANALYSIS. Peach and nectarine skin powder (1 g) was homogenized in 12 mL distilled water and centrifuged at 15,000× *g* for 20 min at 4 °C. The pH of the supernatant was adjusted to ≈8.5 with 60% NH_4OH and brought to a final volume of 10 mL with distilled water. The citric and malic acids in the alkalized supernatants were purified with an anion-exchange column and analyzed by HPLC according to van Gorsel et al. (1992).

STATISTICAL ANALYSIS. Mean differences were analyzed using analysis of variance procedures (ANOVA) of the Statistical Analysis System (SAS) program for the personal computer (SAS Institute, Cary, N.C.).

Results

FORMATION OF IRON–PHENOLIC COMPLEXES. Immediately after mixing, the wavelength of maximum absorbance (λ_{max}) of cyanidin-3-glucoside (0.05 mM) was 515 nm in 0.5 M acetate buffer at pH 4.0, 528 nm in 0.01% HCl methanol, and 510 nm in 50 mM KCl solution at pH 1.0. Solutions of cyanidin-3-glucoside showed an increase in absorbance as ferric ion concentrations increased at pH 3.5 and 4.0. The maximum absorbances (A_{max}) of the ferric cyanidin-3-glucoside solutions were greater at pH 3.5 than at pH 4.0 (Fig. 1A). The absorbance at 690 nm ($A_{690\,nm}$), which was approximately the λ_{max} of iron-phenolate, had a pattern similar to A_{max} at pH 3.5 and pH 4.0, but was greater in less acidic solutions. A shift of λ_{max} was detected as the concentration of iron in the solutions was raised (Fig. 1B). With increasing iron concentration, λ_{max} of the cyanidin-3-glucoside solution shifted from ≈513 nm to ≈530 nm at pH 3.5 and to ≈542 nm at pH 4.0. The shift in λ_{max} following the addition of iron slowed greatly after the iron concentrations exceeded 0.1 mM, a concentration twice that of anthocyanin. Buffer solutions that contained the same $FeCl_3$ concentrations had no absorbance at

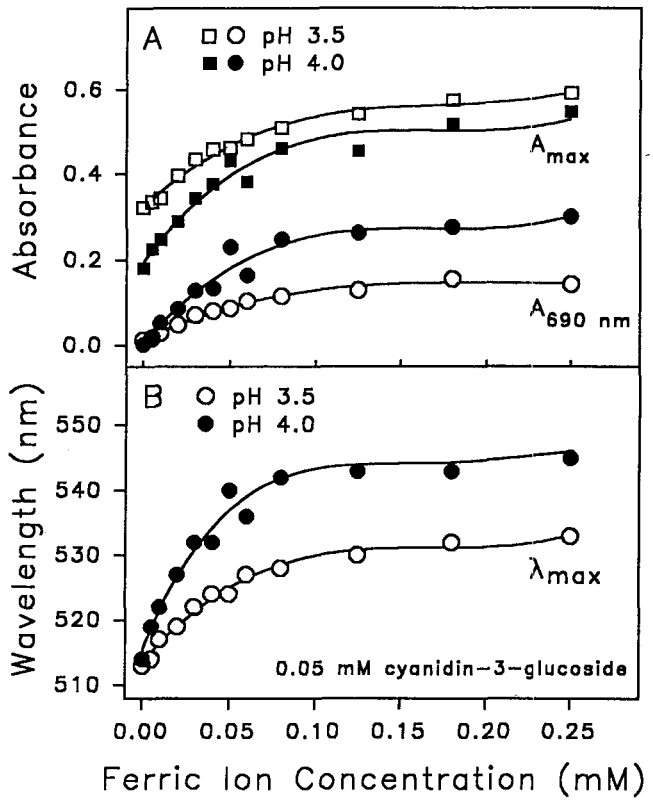

Fig. 1. Photospectrometrical changes of cyanidin-3-glucoside buffer solutions in the presence of increasing concentrations of ferric ion. (**A**) absorbance at 690 nm (A_{690nm}) and maximum absorbance (A_{max}). (**B**) maximum absorption wavelength (λ_{max}). Measurements were made one minute after solution mixing.

visible wavelengths higher than 590 nm (data not shown). The ferric cyanidin-3-glucoside solutions were purple with dark bluish pigments precipitating out of solution when left overnight at room temperature.

Absorbances of chlorogenic acid and caffeic acid solutions began at wavelengths ≥410 nm, and catechin at ≥310 nm, but epicatechin had no detectable absorbance within the 360- to 850-nm wavelength range (data not shown). The addition of iron led to marked changes in the absorbance spectra in the visible wavelength range (i.e., 390 to 790 nm), with peaks around 690 nm. Thus, the absorbance at 690 nm wavelength was selected to study the role of iron in spectral changes (data not shown). The absorbance at 690 nm for solutions of chlorogenic acid, caffeic acid, catechin, and epicatechin increased rapidly as the iron concentration increased until the iron/phenolic molar ratio reached ≈1 (Fig. 2 A–D). The solution absorbance continued to increase as more ferric ions (up to an iron/phenolic ratio of 5) were added to the solutions, but at much lower rates. The increases at $A_{690\,nm}$ were accompanied by an intensified greenish color. All iron–phenolate solutions had greater $A_{690\,nm}$ at pH 4.0 than at 3.5. When the solutions were left overnight at room temperature, they became colorless with dark precipitates. On an equal molar basis and a ferric/phenolic ratio of 5 in a pH 4.0 buffer, cyanidin-3-glucoside gave the highest absorbance at 690 nm (6.0), followed by caffeic acid (0.88), chlorogenic acid (0.63), catechin (0.40), and epicatechin (0.33).

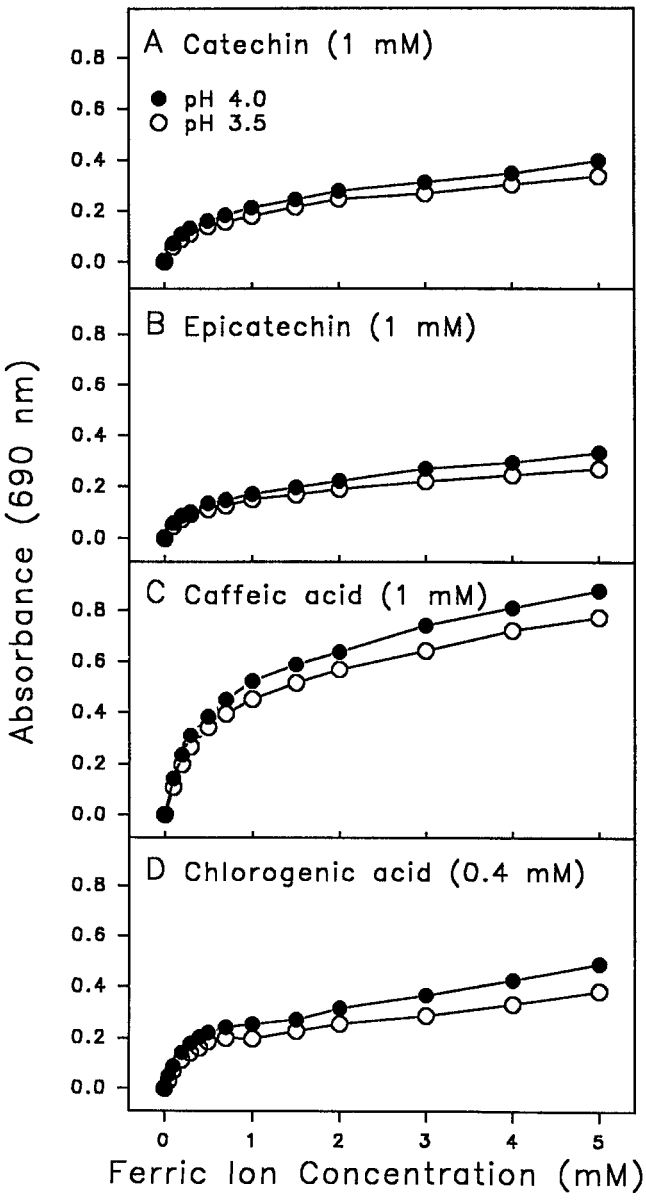

Fig. 2. Changes in absorbance of several phenolic solutions in the presence of increasing concentrations of ferric ion. Measurements were made immediately after solution mixing.

Formation of iron–organic acid complexes. Iron-buffer solutions displayed increased absorptions in the visible range when iron concentration was increased. In contrast, buffer solutions containing only organic acids had no absorption within the range of 360 to 850 nm under our experimental conditions. When organic acid was added to the iron-buffer solution, the iron-related absorption increase was reduced especially at wavelength near 520 nm. Citric acid was more effective than malic acid in postponing and limiting the absorption increase on a equal molar basis (Fig. 3). The absorbance at 520 nm of the organic acid solutions changed little until the iron content was greater than or equal to the citric acid content or greater than or equal to three-fourths of the malic acid content. The stronger iron binding capacity of citric acid compared to malic acid is presumably related to its greater number of carboxyl groups. Once the iron contents were higher than those critical levels, the absorbances of acid-iron solutions increased parallel to but at a lower level than the solutions containing only iron.

Iron-chelation competition between citric acid and cyanidin-3-glucoside. The spectral absorbance of iron-anthocyanin and iron-citrate solutions changed markedly after adding citric acid and anthocyanin, respectively. The shift in λ_{max} from shorter (≈515 nm) to longer (≈535 nm) wavelengths in cyanidin-3-glucoside solutions after adding iron was reversed almost completely when citric acid in an equal amount to the iron was added (Fig. 4). The iron-induced purple color also reverted to the orange red color of the cyanidin-3-glucoside alone. The opposite changes in λ_{max} were observed when citric acid was first added to the anthocyanin solution followed by iron (data not shown).

Copigmentation of cyanidin-3-glucoside. The spectra of cyanidin-3-glucoside solutions (pH 3.5 and 4.0) remained essen-

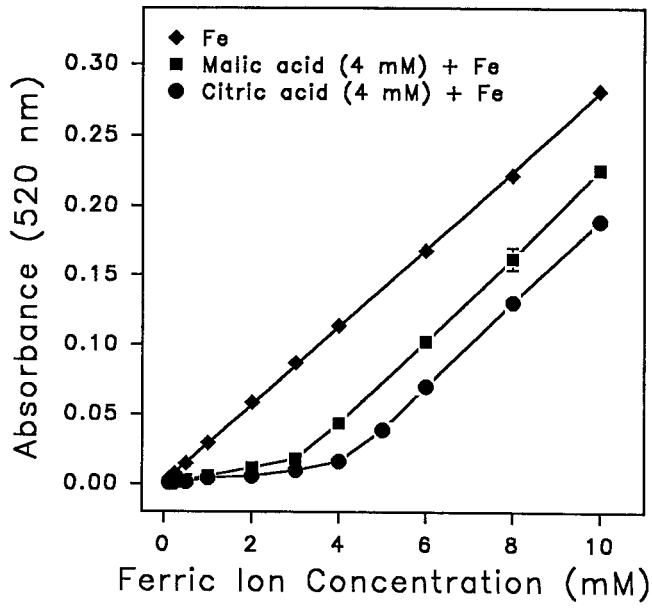

Fig. 3. Changes in absorbance of ferric ion solution in the absence and presence of citric acid and malic acid. Vertical bars represent ±SE. Measurements were made immediately after solution mixing.

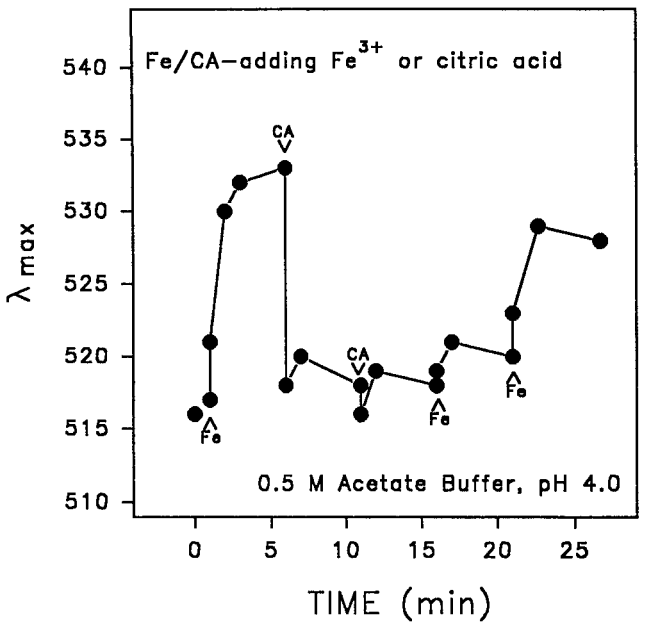

Fig 4 Relationship of ferric ion and citric acid on maximum absorption wavelength (λ_{max}) of cyanidin-3-glucoside buffer solution at pH 4. The addition of each compound equaled 0.05 mM increase in molarity.

tially unchanged over the range of 360 to 850 nm with the addition of citric acid or malic acid (5 to 80, copigment/anthocyanin molar ratio), chlorogenic acid or catechin (0.5 to 8), and caffeic acid or epicatechin (0.1 to 8) (data not shown). The absorbance spectra also indicated no changes when all four phenolic compounds were present together in a solution at their highest concentrations.

Skin tissue iron-binding capacity. Iron-binding capacity levels varied among the peach and nectarine skin. Fruit skin tended to have a higher iron-binding level in catechin equivalent (CE) than in tannic acid equivalent (TE) (Fig. 5). The values for CE and TE iron binding among cultivars ranged from 20 to 46 and from 17 to 39 mg·g^{-1} dry mass, respectively. 'Maycrest' and 'Elegant Lady' had the highest CE and TE. 'Flavorcrest' and 'May Glo' had the lowest CE and TE values, which were about one-half the values for 'Maycrest'.

Skin citric and malic acid content. Among the cultivars examined, citric and malic acid content differed from ≈5 to >12 and from 7 to ≈23 mg·g^{-1} dry mass, respectively (Fig. 6). The cumulative levels of both acids ranged from 15 to 36 mg·g^{-1} dry mass among all of the cultivars. 'May Glo' had the highest citric acid content while 'MayGlo' and 'O'Henry' had the highest malic acid content. 'Maycrest' and 'O'Henry' had citric/malic acid ratios of 0.26 while the other cultivars had ratios in the range of 0.54 to 0.70.

Discussion

At physiological skin cell pH values (i.e., 3.5 and 4.0), ferric ion formed complexes with cyanidin-3-glucoside, chlorogenic acid, catechin, epicatechin, and caffeic acid, compounds that are commonly present in peach and nectarine skin (Cheng and Crisosto, 1995; Macheix et al., 1990). For cyanidin-3-glucoside, the shift of λ_{max} continued until the concentration of ferric ion was about twice that of anthocyanin. This reaction is similar to the 2:1 ligand molar ratio of cyanidin-3,5-diglucoside to aluminum at pH 4.5 (Gabor, 1977). The rapid change in spectral properties of iron–phenolic solutions within an apparent equal ligand molar ratio range indicates the ferric-phenolates formed were initially monocomplexes. This agrees with an early report that showed that ferric caffeic acids are 1:1 complexes at pH between 3.5 to 5.0 (Juul, 1949). The consistent and gradual increase in absorption at 690 nm as the iron concentration in solution exceeded that of anthocyanin and phenolics suggests continuous chelation, which may result from intermolecular association (Bayer, 1958; Bayer et al., 1966). The different rates of increase in A_{690nm} for different phenolics as the iron/phenolic ratio increased implies that the association was affected by the phenolic molecular structure. The higher visible light absorption of ferric phenolic complex solutions at pH 4.0 compared to pH 3.5 was similar to that observed by Jurd and Asen (1966).

The discoloration intensity resulting from metallo phenolic complex formation varied greatly among phenolic compounds. The increase in $A_{690\,nm}$ was greatest with cyanidin-3-glucoside and <15% on a molar basis for the other four phenolic compounds tested. The increase in absorption was higher for the cinnamic acids than the flavan-3-ols. This demonstrated that at on an equal molar basis, contamination by iron will lead to more intense fruit skin discoloration from interactions with cyanidin-3-glucoside, the major fruit tissue anthocyanin (Macheix et al., 1990), than with the other phenolic compounds. The abundance of anthocyanin in skin and its high absorbance response when combined with ferric ions points out the important role of anthocyanin in iron-related color development in damaged fruit skin. This explain why cultivars with high skin red color expressed inking easily.

The greenish and/or bluish pigmentation of iron–phenolates supports our hypothesis that similar discoloration observed on the orange yellow area of peach and nectarine skin surfaces are related to phenolics. All ferric complexes of the four phenolics examined had greenish color in the buffers of pH 3.5 and 4.0. The discoloration may vary if the pH in wounded tissues is higher due to environmental influences such as postharvest handling. Iron and other heavy metal complexes of chlorogenic acid and caffeic acid change from green to brown-green, grey-blue, brown, and blue-purple as the pH of the solution increases from 5.5 to 7.5 (Hughes and Swain, 1962) on areas of the fruit surface with little or no presence of anthocyanin. On poorly red-colored fruit or on fruit

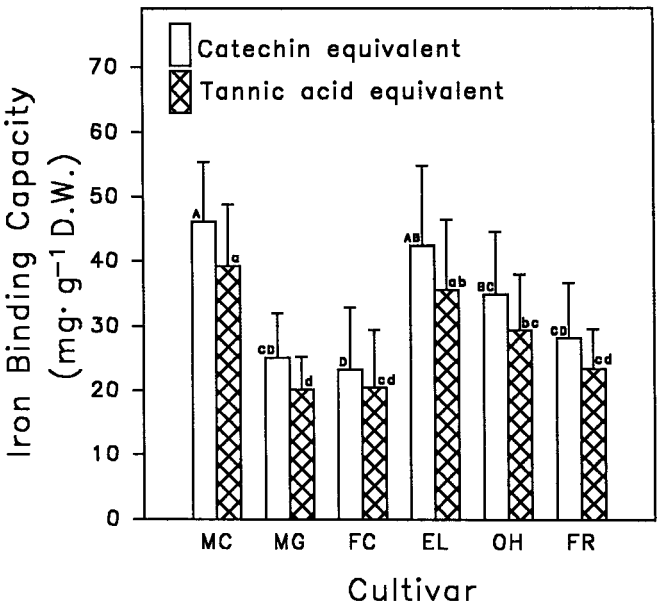

Fig. 5. Phenolic iron-binding capacity of peach and nectarine skin on a dry mass basis. Peaches: MC = 'Maycrest', FC = 'Flavorcrest', EL = 'Elegant Lady', and OH = 'O'Henry'. Nectarines: MG = 'May Glo' and FR = 'Flaming Red'. Vertical bars represent ±SD. Different letters reflect significant differences among means at $P = 0.05$.

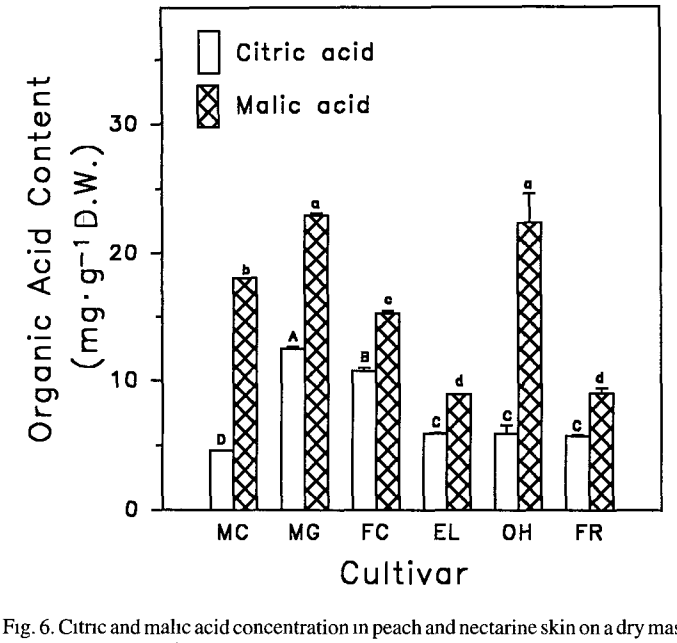

Fig. 6. Citric and malic acid concentration in peach and nectarine skin on a dry mass basis. Peaches. MC = 'Maycrest', FC = 'Flavorcrest', EL = 'Elegant Lady', and OH = 'O'Henry'. Nectarines. MG = 'May Glo' and FR = 'Flaming Red'. Different letters reflect significant differences among means at $P = 0.05$.

surfaces with low red color, the involvement of other phenolics in iron-related discoloration becomes important. Since anthocyanins and phenolic compounds are present in skin tissues, iron-induced discoloration depends mainly on the availability of heavy metal around the abraded skin tissue.

Copigmentation between cyanidin-3-glucoside and phenolic compounds including chlorogenic acid was not observed at the physiological pH values that should favor copigmentation (Asen et al., 1969; Williams and Hrazdina, 1979). At a concentration similar to this previous research (0.035 mM), cyanidin-3-glucoside in this study showed no copigmentation with chlorogenic acid or quercitin with a copigment/pigment molar ratio of 1 to 10 in buffers of pH from 3 to 6 (Jurd and Asen, 1966). The low pigment concentration used on our work and that of Jurd and Asen (1966) may have been the major factor for the lack of copigmentation, which is strongly affected by pigment level (Brouillard, 1983; Osawa, 1982). In these two studies, the pigment/copigment molar ratios used were selected according to phenolic levels determined previously in these tissues (Cheng and Crisosto, 1995). Copigmentation between anthocyanins and chlorogenic acid has been reported at a higher cyanidin-3-glucoside concentration (0.258 mM) (Mazza and Brouillard, 1990). The negative influence of the high ionic strength as a result of high buffer concentration on copigmentation may also have contributed to the lack of copigmentation (Brouillard, 1983; Mazza and Brouillard, 1990).

Peach and nectarine skins are rich in iron-binding phenolic compounds and iron-chelating organic acids. The iron-binding capacity of peach and nectarine skin in equivalents of catechin and tannic acid were high with a catechol equivalent alone of up to 8 $mg \cdot g^{-1}$ dry mass based on an equal molar binding ratio. This agrees with the high skin discoloration sensitivity of abraded fruit exposed to exogenous ferric or ferrous ion contaminants (Cheng and Crisosto, 1994). The skin tissue levels of malic and citric acid together were ≈13 to 35 $mg \cdot g^{-1}$ dry mass, representing iron chelation potentials of 4.5 to >10 mg Fe/g dry mass based on acid levels and the ferric iron binding ratio obtained in this study. Our results demonstrate that citric acid can delay and reverse the formation of ferric-anthocyanin complexes. This agrees with similar reports on the formation of aluminum-cyanidin-3-glucoside and other heavy metal-cyanidin complexes (Bayer et al., 1966; Jurd and Asen, 1966). The potential amount of active organic iron chelators may play a critical role since only a few layers of epidermal cells are injured in abraded skin (Cheng and Crisosto, 1994; Crisosto et al., 1993).

Our results indicate that formation of ferric phenolic complexes induced spectra changes in cyanidin-3-glucoside, chlorogenic acid, caffeic acid, catechin, and epicatechin solutions. The development of purple, blue, green, and other colored metallo complexes by anthocyanin and other phenolics and the high level of phenolic iron-binding potential of skin cells support the importance of exogenous metal ion contamination in the development of skin discoloration of abraded peach and nectarine fruit skin. The lack of copigmentation on peach and nectarine skin pointed out that more work should focus on the interaction between heavy metalic ions and pigments. The iron affinity and the strong iron chelating ability of organic acids such as citric acid merit future study on ways to revert skin discoloration and identify cultivars less susceptible to this problem.

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