Investigating the interaction of ascorbic acid with anthocyanins and pyranoanthocyanins

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Abstract

Juices colored by anthocyanins are subject to color loss related to fortification with ascorbic acid (AA), likely through condensation at carbon-4 (C4) of anthocyanins (ACN). To investigate this mechanism, pyrananthocyanins (PACN), having a fourth-ring covalently occupying C4, were synthesized to compare against ACN (chokeberry extract and cyanidin-3-galactoside). Pyrananthocyanins were synthesized by combining chokeberry anthocyanins with pyruvic acid. AA was added to either chokeberry ACN, cyanidin-3-galactoside or 5-carboxypyranoanthocyanidin-3-galactoside. Samples were stored in the dark for five days at 25°C. Spectral (380-700 nm), color (CIE-L*a*b*) and HPLC data were collected. Extensive bleaching was observed for ACN+AA, with half-life (t1/2) reduced from 35.7 to 0.3 days. An 8-13 fold increase in t1/2 was observed for PACN-AA compared to ACN-AA. A 10 nm hypsochromic shift was observed for PACN-AA, and three new chromophores were formed. The PACN having enhanced resistance to bleaching suggested AA accessibility to C4 played a critical role in ACN bleaching.

Keywords: anthocyanins, pyrananthocyanins, ascorbic acid, bleaching, condensation

Chemical compounds

Chemical compounds studied in this article: Cyanidin-3-galactoside (PubChem CID: 44256700), 5-carboxypyranoanthocyanidin-3-galactoside (PubChem CID: N.A.), pyruvic acid (PubChem CID: 1060).
**Highlights**

- Cyanidin-3-gal was highly susceptible to AA bleaching, followed by chokeberry ACN
- PACNs had significantly greater resistance to AA bleaching than ACN
- These results support C4 as the preferential site of ACN-AA condensation
- PACN-AA interaction resulted in three colored degradation products
- Alternative modes of interaction by AA were possibly observed with PACNs
- Other phenols in chokeberry likely enhanced stability over Cyanidin-3-galactoside
1. Introduction

Consumers commonly use color to make assessments on acceptance and liking, implied flavor, safety and overall quality of food products. Synthetic colorants have been used to correct for natural variation of food items, mask imperfections as well as offer alternative product identities (Sharma, McKone, & Markow, 2010). The innate stability of synthetic colorants over natural pigments has been a driver for their selection in coloring food products. Recently this trend has begun to reverse as consumers have expressed concerns over the safety of synthetic colorants and preference for colorants from natural sources (Kobylewski & Jacobson, 2010).

Anthocyanins are widely viewed as a natural alternative due to their wide spectrum of hue expression, however their application has been limited due to stability (Sigurdson, Tang, & Giusti, 2017).

Anthocyanins (ACN) are a class of water-soluble polyphenols found in many fruits and vegetables. Their color properties are greatly influenced by the substitution patterns on the aglycone structure as well as pH environment. Warm hues including reds are observed at low pH but shift expression to vibrant purple-blues in more alkaline conditions. Their stability is influenced by many factors including pH, heat, enzymes, light, as well as food ingredients including vitamin C (or ascorbic acid, AA). The latter is of significance for beverages and juices which are often fortified with high levels of this micronutrient.

It has long been known that the presence of AA in anthocyanin-colored solutions results in loss of color (Kertesz, 1953). This results in mutual destruction of both the pigment and micronutrient, and it is evident for both major vitamers, ascorbic and dehydroascorbic acid (Markakis & Jurd, 1974). The two components being mutually destructive has led researchers in the past to propose that direct condensation of the compounds might be occurring. This presents
a major hurdle for the food industry to use ACN-based colorants in juices and beverages fortified with the micronutrient. Previous research has proposed that anthocyanin bleaching is the result of condensation of ascorbic acid at the carbon 4 (C4) of the anthocyanin (Poei-Langston & Wrolstad, 1981). It is thought that this site is subject to nucleophilic attack by ascorbic acid. Furthermore, it is thought that ascorbic acid, hydrogen peroxide, and bisulfite follow similar bleaching mechanisms. The proposed condensation is thought to result in an opened C-ring structure, leading to the loss of aromaticity in the chromophore, therefore lacking the original color expression. Direct condensation has been proposed as the major cause for loss of color, but other routes of degradation might also contribute.

Auto-oxidation of AA in solution can give rise to hydrogen peroxide (H₂O₂) formation (Calcutt, 1951). H₂O₂ is a powerful bleaching agent that can further lead to hydroxyl radicals which are highly reactive and cause rapid ACN degradation (Ruenroengklin, Yang, Lin, Chen, & Jiang, 2009). Previous work has found ACN 3,5-substitution to increase pigment stability against ascorbic acid compared to just 3-substitution, likely a result of further restricting access to C4 in between. Viguera and Bridle (1992) reported that Malvidin-3,5-diglucoside experienced slower color loss as compared to Malvidin-3-glucoside. The same authors reported direct substitution of the C4 with phenyl and methyl groups enhanced their stability against ascorbic acid color loss versus typical –H substitution (Viguera and Bridle 1992). Another way to investigate the interaction between ACN and AA is by similar investigation with pyranoanthocyanins.

Pyranoanthocyanins (Figure 1) are formed by anthocyanins undergoing heterocyclic addition of a polar carboxyl-containing compound such as pyruvic acid, acetaldehyde, or catechins which are often byproducts from yeast fermentation (de Freitas & Mateus, 2011). This results in the formation of a fourth ring (D) that covalently occupies C4 and C5 of the pigment.
Pyranoanthocyanins are often found in aged wines\(^1\) and have been reported in red onions\(^2\) and strawberries\(^3\) (\(^1\)De Freitas and Mateus 2011, \(^2\)Fossen and Andersen 2003, \(^3\)Andersen et al. 2004). Previous research on pyranoanthocyanin stability has shown enhanced resistance of these compounds to bisulfite bleaching. Carboxy-pyranoanthocyanins, resulting from synthesis with pyruvic acid, have shown greatly enhanced stability against bisulfite bleaching (up to 250ppm) compared to anthocyanins (He et al 2010). Oligomeric pyranoanthocyanins were shown to exhibit complete resistance to bisulfite bleaching (up to 250 ppm) for 2 days (He et al 2010). Acetyl-pyranoanthocyanins, synthesized with acetaldehyde, have been shown to completely overcome bisulfite bleaching and to experience a hyperchromic shift in response to bisulfite at up to 200 ppm, an unexpected response to a common bleaching agent (Hermos, 2012).

The objective of this study was to increase our understanding of the interactions between anthocyanins and AA while assessing the potential site of action responsible for anthocyanin-ascorbic acid bleaching by comparison to pyranoanthocyanins. It was hypothesized that pyranoanthocyanins, with a covalently occupied C4, would result in less bleaching and better preserved color expression in the presence of AA as compared to ACN. These findings could further support an understanding of the mechanism of interaction between anthocyanin-ascorbic acid and the resulting color loss that hinders anthocyanin’s potential as a colorant.

2. Materials and Methods

2.1. Materials

Powdered chokeberry fruit was provided by Artemis Inc. (Fort Wayne, Indiana, U.S.). Lab grade pyruvic acid used for the synthesis of pyranoanthocyanins was purchased from Sigma Aldrich (St. Louis, Missouri, U.S.). USP grade 3% hydrogen peroxide was manufactured by
Kroger (Cincinnati, Ohio, U.S.). Analytical grade ascorbic acid (99% L-ascorbic acid) was purchased from Sigma Aldrich (St. Louis, Missouri, U.S.). HPLC grade acetonitrile and water were obtained from Fisher Scientific (Hampton, New Hampshire, U.S.) and HPLC grade formic acid from Sigma Aldrich (St. Louis, Missouri, U.S.).

2.2. Methods

2.2.1. Anthocyanin semi-purification (SPE)

Chokeberry powder was mixed with water acidified with 0.01% HCl prior to purification. The solution was loaded onto a Waters Sep-pak C18 cartridge for solid phase extraction (SPE). The column was then washed with acidified water (0.01% HCl) to remove of sugars and acids then followed with ethyl acetate for removal of the more non-polar phenolics. Pigments were recovered from the cartridge with methanol acidified with 0.01% HCl, and the solvent was removed by rotary evaporation (40°C, under vacuum). Pigments were then solubilized and stored in acidified water for future use. This was the only preparatory step for chokeberry treatments.

2.2.2. Pyrananthocyanin synthesis

Pyrananthocyanins were synthesized from the semi-purified chokeberry by addition of pyruvic acid. The extract (1000 μM cyanidin-3-glucoside equivalent) was added to a pH 2.6 citrate buffer that had 0.1% potassium sorbate and 0.1% sodium benzoate to prevent molding. A molar ratio of 1:50 (ACN: pyruvic acid) was followed as previously described (He et al., 2010). The prepared anthocyanin pyruvic acid solution was stored in an incubator in the dark at 35 °C for 10 days (Isotemp, Fisher Scientific, Waltham, MA, US). After the incubation period ended, cyanidin-3-galactoside and 5-carboxypyranocyanidin-3-galactoside, the resulting
pyranoanthocyanin from cyanidin-3-galactoside and pyruvic acid, were isolated from the solution using semipreparatory HPLC.

2.2.3. Anthocyanin and pyranoanthocyanin purification

A reverse phase HPLC system composed of the following modules was used: LC-6AD pumps, CBM-20A communication module, SIL-20A HT autosampler, CTO-20A column oven, and SPD-M20A Photodiode Array detector (Shimadzu, Maryland, U.S.). The reverse-phase column selected was a 250 x 21.2 mm Luna pentafluorophenyl column with 5 µm particle size and 100Å pore size (Phenomenex, California, U.S.). Samples were filtered prior to injection with a Phenex RC 0.45 µm, 15 mm membrane syringe filter (Phenomenex, California, U.S.).

With a flow rate of 10 mL/min and a run time of 30 minutes, peaks were separated and collected. An isocratic system with the following solvents were used: 11:89 (Solvent A: Solvent B v/v) with solvent A being 4.5% formic acid in HPLC grade water and solvent B was HPLC grade acetonitrile. Elution of peaks was monitored at 500 nm. Peaks were manually collected. The two collected peaks were diluted with distilled water and again subjected to SPE semi-purification to remove formic acid and acetonitrile from the HPLC. Rotary evaporation was used to remove methanol, and the pigments were stored in 0.01% HCl in acidified water.

2.2.4. Anthocyanin and pyranoanthocyanin purity

Prior to experimentation, pigments were evaluated for purity by using an analytical HPLC only different from the previously listed one by the use of different pumps (LC-20AD, Shimadzu, MD, US). Purified pigments were filtered using the Phenex RC 0.45 µm membranes. A binary system with 1 mL/min flow rate was used: solvent A: 4.5% formic acid in HPLC grade water and solvent B: HPLC grade acetonitrile. The gradient began with an isocratic flow of 6% solvent B for 17 minutes (elution of primary anthocyanins), increasing to 15% solvent B by 45
minutes (elution of primary pyranoanthocyanins), to 40% solvent B by 50 minutes. A 10 uL injection volume was loaded onto a Phenomenex Kinetix 5µm EVO C18 100 A. 150 × 4.6 mm column and Phenomenex Ultra UHPLC EVO C18 guard cartridge attached.

Purity was expressed in terms of % peak area of targeted pigment as compared to the total area of all peaks present in the max plot (260-700 nm). The isolate of 5-carboxypyranocyanidin-3-galactoside accounted for 94% of the overall area under the curve (AUC), cyanidin-3-galactoside isolate was 92% AUC while chokeberry ACN purity was 35% AUC.

2.2.5. Sample preparation

The semi-purified chokeberry extract, the isolated cyanidin-3-galactoside, and the purified 5-carboxypyranocyanidin-3-galactoside were diluted in pH 3.0 citrate buffer (0.1M adjusted with HCl) until an absorbance of 1.0 at their respective λmax was reached. Levels of AA of 250, 500, and 1000 mg/L were added using a concentrated ascorbic acid stock solution, and a control consisting of each pigment with the absence of AA was maintained. The control consisted of the pigment in the buffer in the absence of AA. An additional test was performed with the same model juice using hydrogen peroxide, a known degradation product of AA. To determine if AA bleaching was due to formation of H2O2, direct addition of it was investigated. In place of added AA, a 62.2 uL of a 0.3% peroxide solution was added to match the molar equivalency of the 500 mg/L AA level which was 2.84 mM.

All samples were brought to the same final volume with additional citrate buffer. The pH of all samples were evaluated using a S220 SevenCompact pH meter (Mettler Toledo, Columbus, OH, U.S.) and were found to be 3.0 ± 0.05. Samples were stored in the dark at 25 °C in an incubator (listed in 2.2.2). UV-Vis spectrophotometry, colorimetry, and HPLC analyses were
conducted over a 5 day period following the addition of ascorbic acid or hydrogen peroxide. UV-Vis spectral data was collected every hour for the first 8 hours, and then at 12hr, and daily from that point on for 5 days. Spectra and color were evaluated with this data. HPLC analyses were conducted on days 0, 1, 3 and 5.

2.2.6. UV-vis spectrophotometry of samples

A SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, California, U.S.) with a 96-well plate (poly-D-lysine coated polystyrene) were used for the evaluation of absorbance from 380-700 nm, 1 nm intervals. Aliquots (200 uL) of samples were loaded into individual wells, and a blank consisted of the same citrate buffer used.

2.2.7. Color analyses of samples

Using UV-Vis spectral data (5 nm steps, 380-700 nm) in combination with software written for color conversion, absorbance data was translated to CIE-L*\(c\)*h* (Farr and Giusti, 2017). The calculations for CIE-L*\(c\)*h* implemented by the software used CIE relative spectral power distribution for a D65 standard illuminants and 10° observer angle function (Commission Internationale de l’Eclairage, 2016).

2.2.8. HPLC monitoring of samples

Prepared solutions were monitored to determine the formation of potential degradation products or profile changes. Using the analytical HPLC system and conditions previously described (2.2.4), chromatograms were monitored with the max plot (260-700 nm), 490 nm (near \(\lambda_{\text{max}}\) of 5-carboxypyranocyanidin-3-galactoside), and 520 nm (near \(\lambda_{\text{max}}\) of cyanidin-3-galactoside).

2.2.9. Statistical analysis of data
Data was organized for means and standard deviations using Microsoft Excel (Redmond, Washington, U.S.). 1-Way ANOVA was performed for each treatment at all time points to determine if a significant change in CIEL\(^*c*h\) and maximum absorbance occurred as well as Tukey’s post-hoc test to determine when the change became significantly different from time 0. 1-Way ANOVA was also performed for each pigment (control, 250, 500, 1000 mg/L AA) at each time point to determine if and when which CIEL\(^*c*h\) and maximum absorbance became significantly different from the control. Software used for ANOVA tests was SPSS (IBM, North Castle, New York, U.S.).

3. Results and Discussion

3.1. UV-Vis Spectrophotometry

Anthocyanins degraded quickly in the presence of ascorbic acid, as seen in Figure 2. Chokeberry extract, containing an ACN profile which is ~79% Cyanidin-3-galactoside, and with anthocyanins representing ~35% of the total AUC in the max plot showed greater resistance to bleaching compared to the purified Cyanidin-3-galactoside (~92% AUC in the max plot). This is likely the result of other chokeberry phenols playing a protective role against AA-induced degradation. Similar observations have been reported by Brenes and et al (2005) with rosemary phenolics added in a model grape juice resulting in less ascorbic acid-induced bleaching. PACN (5-carboxypyranocyanidin-3-galactoside) derived from Cyanidin-3-galactoside showed the least change in absorbance over time. Covalently occupying C4 in 5-carboxypyranocyanidin-3-galactoside resulted in less change in absorbance as compared to Cyanidin-3-galactoside and chokeberry, consistent with other reports of bleaching observed with bisulfites (Oliveira et al., 2006).
All pigments experienced significant changes in maximum absorbance over time but post hoc analysis indicated these changes occurred at different rates. The following times were necessary before a significant difference (p < 0.05) in maximum absorbance from time 0 was found for each pigment at all levels of AA added: Cyanidin-3-galactoside, 2 hours; chokeberry extract, 12 hours, and 5-carboxypyranoxyanidin-3-galactoside, 24 hours. As AA levels increased, the loss in absorbance for each pigment over time also increased, revealing a dose-dependent effect of AA. For 500 mg/L AA treatments over a 5 day period, 5-carboxypyranoxyanidin-3-galactoside saw a reduction of 38% reduction in maximum absorbance, chokeberry a 79% reduction, and Cyandin-3-galactoside an 88% reduction.

Changes in $\lambda_{\text{max}}$ were also observed. For 500 mg/L AA levels, the following hypsochromic changes in $\lambda_{\text{max}}$ occurred: chokeberry, 512 to 511 nm; cyanidin-3-galactoside, 511 to 509 nm, 5-carboxypyranoxyanidin-3-galactoside, 491 to 484 nm. These shifts in $\lambda_{\text{max}}$ are reflected in Figure 3. The change for 5-carboxypyranoxyanidin-3-galactoside correlated with the newly developed peaks discovered during HPLC analysis and resulted in the solution being more orange-red. Hypsochromic changes on ACN (chokeberry and cyanidin-3-galactoside) $\lambda_{\text{max}}$ observed over the 5 days of the AA treatment, were less than 5 nm, regardless of the levels of AA. However, 5-carboxypyranoxyanidin-3-galactoside experienced hypsochromic shifts as large as 10 nm, with shifts becoming more pronounced as AA levels increased.

3.2. Kinetics of degradation

Because of the rapid nature of AA-induced color loss, degradation kinetics were evaluated in terms of change in maximum absorbance at the original $\lambda_{\text{max}}$ over time. Bleaching has been previously reported as a first-order reaction, typical of ACN degradation, and was modeled as such in determining the reaction rate and half-life (Sondheimer & Kertesz, 1952,
The decrease in maximum absorbance correlated with an increase in lightness \( (L^*) \) as well as the decrease in chroma \( (c^*) \). The reduction in maximum absorbance did closely follow first-order kinetics for chokeberry extract and Cyanidin-3-galactoside but slightly deviated for 5-carboxypyranocyanidin-3-galactoside. This suggests that there might be different reactions taking place contributing to the change in maximum absorbance for pyranoanthocyanin-ascorbic acid interaction, but not in the case of anthocyanin-ascorbic acid interaction. The kinetics results for each pigment and AA level can be found in Table 1. With 1000 mg/L AA added, Cyanidin-3-galactoside had a half life of 8 hours, chokeberry extract, 24 hours, and 5-carboxypyranocyanidin-3-galactoside was 64 hours, seen in Table 1.

Without ascorbic acid, 5-carboxypyranocyanidin-3-galactoside had the greatest half life (978 hours), followed by chokeberry extract (858 hours) and then Cyanidin-3-galactoside (546 hours). This order of stability was also exhibited across all AA levels. The enhanced stability and extension of half life for 5-carboxypyranocyanidin-3-galactoside was more evident upon addition of ascorbic acid. Pyranoanthocyanins exhibited a half-life 8-13x higher than Cyanidin-3-galactoside in the presence of AA. Kinetics data supports the hypothesis that C4 is an important site for anthocyanin-ascorbic acid interaction, but it also reinforces the notion that alternative mechanisms play a role in AA-mediated degradation for the pyranoanthocyanin.

### 3.3. Colorimetry

#### 3.3.1 Lightness

Rapid color loss and extensive bleaching of pigments can be seen with CIE lightness \( (L^*) \) in Figure 3. Within 48 hours exposed to 1000 mg/L AA \( L^* \) increased from 77.2 to 96.4 (\( \Delta 19.2 \)) for Cyanidin-3-galactoside; chokeberry, 74.4 to 89.4 (\( \Delta 15.0 \)); and 5-carboxypyranocyanidin-3-
galactoside, 81.7 to 86.6 (Δ4.9). The presence of AA resulted in higher lightness over time, and this was dose dependant. Pyranoanthocyanins showed the least change in L* in response to AA, and Cyanidin-3-galactoside the greatest. An increase in L* represents a lighter color expression and was most evident for chokeberry and cyanidin-3-galactoside.

### 3.3.2 Chroma

Pigment levels were standardized by absorbance at their respective λ_{max}; therefore, chroma values were in close agreement at day 0. Chroma, being a measure of color intensity, is useful for determining the extent of bleaching and is reported in Figure 3. The PACN had less change in chroma compared to Cyanidin-3-galactoside and chokeberry. Chroma decreased with increasing AA levels with the exception of Cyanidin-3-galactoside after 48 hours, likely the result of ascorbic acid and pigment browning playing a larger role at those times. The changes in chroma in response to AA addition followed: Cyanidin-3-galactoside > chokeberry > 5-carboxypyranocyanidin-3-galactoside. All pigments (including controls) experienced a significant change in chroma over 5 days. Post hoc analysis was used for the determination of the time necessary for a change from time 0 and showed differences. For all levels of AA, 5-carboxypyranocyanidin-3-galactoside did not experience a significant change in chroma as compared to the control until 48 hours, for Cyanidin-3-galactoside 2 hours, and chokeberry, 12 hours.

### 3.3.3 Hue angle

Synthesis of pyranoanthocyanins results in a pigment with a lower λ_{max} and higher hue angle compared to the respective anthocyanin, having a more orange-red color expression, as compared to the red color of ACN. This was clearly observed on the initial hue angle values of 5-carboxypyranocyanidin-3-galactoside (50°), a more orange-red hue than Cyanidin-3-
galactoside (16.6°), with a more pure red color (Figure 3). While the initial hue angle for chokeberry and Cyandin-3-galactoside started much lower and more red (<20°) than the PACN, the reaction between ACN-AA resulted in a dramatic color shift towards a yellow coloration. For the 1000 mg/L AA level, large increases in hue angle were observed from day 0 to 5 for chokeberry (17.6° to 53.8°) and cyanidin-3-galactoside (18.7° to 77.5°) while the hue angle change was much smaller for 5-carboxypyranoxyanidin-3-galactoside, changing from 51.0° to 57.1°. Changes in hue angle in the presence of AA were dose dependant. The rapid increase in hue angle for cyanidin-3-galactoside and chokeberry was likely the result of pigment degradation and fading; whereas, for 5-carboxypyranoxyanidin-3-galactoside which better retained original chroma and lightness parameters, new pigment formation may explain hue angle changes.

### 3.3.4 Total color change (ΔE)

Total color changes (ΔE) were calculated as the color change from day 0 to 5 for each respective treatment, and presented in Table 2. Without AA, chokeberry had the smallest ΔE, followed by 5-carboxypyranoxyanidin-3-galactoside and Cyanidin-3-galactoside. Other phenolics present in chokeberry could have enhanced color retention through mechanisms such as copigmentation or radical oxidation by additional phenolics which would have not been possible with isolated 5-carboxypyranoxyanidin-3-galactoside and cyanidin-3-galactoside. This possible explanation could be supported by previous work where quercetin quercitin-3-rhamnoside were found to lead to higher anthocyanin retention in the presence of AA (Shrikhande, Francis, 1974). Chokeberry fruit has previously been reported to have 89 mg/kg of quercetin and further supports why greater stability was observed for the chokeberry treatment as compared to purified Cyanidin-3-galactoside (Häkkinen, Kärenlampi, Heinonen, Mykkänen, & Törrönen, 1999). For all levels of AA addition, chokeberry and Cyanidin-3-galactoside had a ΔE
greater than 29. However, 5-carboxypyranoxyanindin-3-galactoside with AA exhibited a significantly smaller color shift with ΔE’s ranging from 8.7 to 10.9. This three-fold reduction in ΔE resulted in overall better retention of color in response to AA addition.

3.4. HPLC evaluation

To determine the relationship between spectral and color changes with changes in pigment composition, HPLC analysis was utilized. Cyanidin-3-galactoside and the PACN 5-carboxypyranoxyanindin-3-galactoside showed a dramatic reduction (2% and 10% of the target peaks left after 1 day, respectively, while less than 1% were left after 5 days for both with 1000 mg/L AA addition) over time in the presence of AA, followed by and chokeberry extract (32% left after 1 days and only 5% after 5 days for 1000 mg/L AA addition), as seen in Figure 4. This was in contrast to UV-Vis and colorimetric data, which could have suggested greater retention of 5-carboxypyranoxyanindin-3-galactoside over chokeberry anthocyanins.

Analysis of pigments profiles revealed three newly-formed peaks containing a chromophore resulting between 5-carboxypyranoxyanidin-3-galactoside and ascorbic acid. These new peaks appeared within the first 24 hours and can be seen in Figure 4. These new peaks were not formed during the storage of the 5-carboxypyranoxyanidin-3-galactoside control, showing their formation is the result of interaction of PACN with AA. The new peaks had λ<sub>max</sub> of 486 nm and 478, which differed from that of 5-carboxypoxyanidin-3-galactoside (λ<sub>max</sub> of 502 nm) and can explain the change in hue angle towards more orange-red as the new peaks formed. These results suggest alternative sites of interaction between 5-carboxypyranoxyanidin-3-galactoside and AA, given that access to C4 has been blocked.
Auto-oxidation of AA in solution can give rise to H$_2$O$_2$ formation, a powerful bleaching agent that can further lead to rapid ACN degradation (Ruenroengklin et al., 2009). To determine if the changes observed might be the result of H$_2$O$_2$ formed from AA, direct addition of H$_2$O$_2$ was investigated for effect on pigments. Hydrogen peroxide was added to 5-carboxypranocyanidin-3-galactoside and chokeberry extract. HPLC profiles as well as maximum absorbance and $\lambda_{max}$ of the solutions were evaluated. Similar results to those previously described after addition of AA were observed for the chokeberry extract in the presence of H$_2$O$_2$, a rapid reduction in the original peaks (11% remaining after 1 day with H$_2$O$_2$ addition). For 5-carboxypranocyanidin-3-galactoside, the pigment was more resistant to bleaching compared to anthocyanins as seen in with AA addition; however, no newly-formed chromophores developed. There was also no hypsochromic shift in $\lambda_{max}$ with H$_2$O$_2$ as seen with 5-carboxypranocyanidin-3-galactoside and AA. This would imply that these newly formed peaks between 5-carboxypranocyanidin-3-galactoside and ascorbic acid are not the result of PACN interactions with hydrogen peroxide and peroxy radicals, but likely another form of AA-driven interaction.

The formation of three new chromophores is likely the result of AA interaction with PACN at the A, B, or D ring and maintenance of a closed C-ring (Figure 1). It has previously been reported that acetyl pyranoanthocyanins experience both a hyperchromic and hypsochromic shift in response to up to 200 ppm sulfites, and it was proposed this was the result of sulfite covalent linkage at the acetyl group in D-ring, enhancing the molar absorptivity (Hermos, 2012). It is possible that a similar interaction could be taking place with the D-ring carboxylic acid of the 5-carboxypranocyanidin-3-galactoside and ascorbic acid, resulting in this case in a hypochromic and hypsochromic shift. These possible alternative reactions might explain why the resulting degradation products contained a chromophore, versus ACN-AA condensation products.
which preferentially reacted at the nucleophilic C4 and had irreversibly lost aromaticity at the C-ring.

4. Conclusion

Cyanidin-3-galactoside degraded rapidly in the presence of ascorbic acid, followed by chokeberry extract. Other phenols in chokeberry extract likely played a protective role against AA mediated pigment bleaching. The PACN 5-carboxypyranoxyanidin-3-galactoside showed greater resistance to bleaching compared to Cyanidin-3-galactoside and chokeberry extract with a smaller color change (ΔE) and loss in absorbance in response to ascorbic acid. The interaction between PACN-AA resulted in three new chromophores, not observed when PACN interacted with H₂O₂. This suggests that the mechanism for H₂O₂ and AA bleaching are distinct from one another. NMR work is underway to determine the site of reaction for PACN-AA as well the as ACN-AA reactivity. These findings further support the hypothesis that C4 plays an important role as a major – but not the only - site of interaction for AA mediated bleaching of anthocyanins.

5. Acknowledgements

6. Conflicts of Interest

No conflicts of interest known.
7. References

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Table 1. Reaction rates and half life (t½) of pigments modeled with first-order kinetics

| Ascorbic Acid Level | Pigment                                      | k        | t½ (hours) | R²  |
|---------------------|----------------------------------------------|----------|------------|-----|
| Control             | Chokeberry Extract                           | 8.08E-04 | 858        | 0.947 |
|                     | Cyanidin-3-galactoside                       | 1.27E-03 | 546        | 0.957 |
|                     | 5-carboxypyranocyanidin-3-galactoside        | 7.08E-04 | 978        | 0.977 |
| 250 mg/L AA         | Chokeberry Extract                           | 1.02E-02 | 68         | 0.991 |
|                     | Cyanidin-3-galactoside                       | 3.18E-02 | 22         | 0.996 |
|                     | 5-carboxypyranocyanidin-3-galactoside        | 2.69E-03 | 258        | 0.992 |
| 500 mg/L AA         | Chokeberry Extract                           | 1.60E-02 | 43         | 0.991 |
|                     | Cyanidin-3-galactoside                       | 5.90E-02 | 12         | 0.998 |
|                     | 5-carboxypyranocyanidin-3-galactoside        | 4.61E-03 | 150        | 0.965 |
| 1000 mg/L AA        | Chokeberry Extract                           | 2.85E-02 | 24         | 0.999 |
|                     | Cyanidin-3-galactoside                       | 8.64E-02 | 8          | 0.996 |
|                     | 5-carboxypyranocyanidin-3-galactoside        | 1.08E-02 | 64         | 0.998 |

Table 2. Day 0 and 5 colorimetric values (CIEL*e*h*) and total color change (ΔE) of chokeberry, cyanidin-3-galactoside and 5-carboxypyranocyanidin-3-galactoside for all AA levels over time. Numbers are means of 3 replications, followed by (standard deviations). ΔE: total color change from day 0 (control) to day 5.

| Treatment                        | Lightness | Chroma  | Hue Angle | ΔE    |
|----------------------------------|-----------|---------|-----------|-------|
|                                  | Day 0     | Day 5   | Day 0     | Day 5 | Over 5 days |
| Chokeberry Extract               | 75.1 (0.5)| 76.3 (0.5)| 51.6 (0.8)| 48.4 (1.2)| 16.6 (0.5)| 14.9 (0.8)| 3.3 (0.4) |
| 250 mg/L AA                      | 74.7 (0.2)| 88.0 (0.2)| 52.3 (0.4)| 22.8 (0.3)| 17.4 (0.2)| 17.5 (0.5)| 29.9 (0.4) |
| 500 mg/L AA                      | 75.0 (0.3)| 91.1 (0.6)| 51.8 (0.6)| 15.0 (0.4)| 16.6 (0.3)| 34.0 (3.1)| 36.9 (0.2) |
| 1000 mg/L AA                     | 74.4 (0.2)| 92.7 (1.1)| 52.9 (0.2)| 13.3 (0.6)| 17.6 (0.2)| 53.8 (3.1)| 42.0 (2.2) |
| Cyanidin-3-galactoside           | 77.3 (0.1)| 79.4 (0.8)| 51.2 (0.2)| 46.5 (2.1)| 18.6 (0.1)| 15.9 (1.0)| 2.6 (1.1)  |
| 250 mg/L AA                      | 77.5 (0.2)| 93.2 (0.3)| 50.8 (0.2)| 13.9 (0.7)| 18.7 (0.1)| 21.9 (1.2)| 19.3 (0.3) |
| 500 mg/L AA                      | 77.0 (0.1)| 94.4 (0.6)| 51.8 (0.0)| 10.5 (0.4)| 18.8 (0.0)| 51.1 (2.7)| 23.6 (0.2) |
| 1000 mg/L AA                     | 77.2 (0.3)| 94.5 (0.7)| 50.9 (0.1)| 13.7 (1.8)| 18.7 (0.1)| 77.5 (4.5)| 27.6 (0.7) |
| 5-carboxypyranocyanidin-3-galactoside | 82.2 (0.2)| 82.8 (0.6)| 54.3 (0.7)| 49.9 (1.3)| 50.0 (0.2)| 47.5 (0.6)| 2.1 (0.4)  |
| 250 mg/L AA                      | 81.9 (0.1)| 82.8 (0.3)| 55.2 (0.4)| 43.2 (0.2)| 50.7 (0.2)| 46.9 (0.3)| 4.5 (0.2)  |
| 500 mg/L AA                      | 82.1 (0.3)| 83.6 (0.4)| 54.7 (0.3)| 39.2 (0.3)| 50.4 (0.1)| 48.3 (0.4)| 5.0 (0.2)  |
| 1000 mg/L AA                     | 81.7 (0.5)| 83.7 (0.2)| 55.6 (0.7)| 36.2 (0.3)| 51.0 (0.1)| 57.1 (0.7)| 5.2 (0.5)  |