Color and Chemical Stability of a Variety of Anthocyanins and Ascorbic Acid in Solution and Powder Forms

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Ⓢ Supporting Information

ABSTRACT: The color and chemical stabilities of six anthocyanins, including cyanidin 3-glucoside, highly purified and present in semipurified extracts (also containing other anthocyanins) from grape pomace, purple corn, and black rice, were determined in combination with ascorbic acid in solutions at differing pH values (3.0 and 4.0) and temperatures (6−40 °C) and lyophilized powders at different relative humidities (43−98% RH). Color and chemical changes were analyzed using CIELAB measurements and HPLC, respectively. In liquids, stability was inversely related to increasing pH and temperature; for powders, stability was inversely related to RH. The mutual destruction of anthocyanins and ascorbic acid in solution was confirmed, with unexpected new findings showing no significant stabilizing/destabilizing effect based upon anthocyanin structure, including differing flavylium core (three types) and type of acylation (two aliphatic, one cinnamic acid), or final extract purity.

KEYWORDS: *cyanidin 3-glucoside, vitamin C, stability, powders, beverages, color*

INTRODUCTION

Anthocyanins (ACNs) are a class of naturally sourced colorants that are increasingly used in foods as alternatives to artificial dyes, especially for providing red and purple hues.[1] Additionally, several ACNs have been shown to possess health-promoting properties, suggesting an additional role as functional food ingredients.[2] Challenges arise for product developers when selecting ACN-containing extracts as food ingredients. For example, ACNs possess increased sensitivity to a host of chemical and environmental factors, but especially pH, elevated temperature, oxygen, light, and a number of intra- and intermolecular interactions as compared to synthetic dyes.[1,3] An interaction with ascorbic acid (AA) is particularly noteworthy due to the fact that the effect of this interaction is largely negative.[3−8]

Beverages are an example of a food product category wherein ACNs and AA are coformulated, in both ready-to-drink and powdered formulations. A small survey conducted by us of powdered beverages available in the United States (17 samples from 7 different manufacturers/distributors) containing added AA revealed levels from 0.4 to 114 mg of AA per gram of beverage mix, delivering 10% to almost 2000% of the recommended daily nutritional values of vitamin C per serving when reconstituted. The majority of powdered beverages are colored with artificial dyes; however, an increasing number are available containing naturally sourced colors, especially ACNs. With the mainly negative popular press concerning the ingestion of artificial dyes and recent legislative and regulatory activity,[1] the replacement of dyes with naturally sourced alternatives, of which ACNs will be a large component, will surely continue or even accelerate. A significant reduction or removal of AA as a means to prevent ACN color loss is likely to be met with marketing resistance as fortification with vitamin C is a simple and low-cost approach for improving the perceived nutritional quality of these beverages and providing a positive point-of-comparison with fruit juice.

The mechanism behind the destruction of ACNs by AA has been the subject of some debate, but the prevailing hypothesis is that the autoxidation of AA yields free radicals, which cleave the flavylium core structure of ACNs (refer to Figure 1).[3] An alternative proposal that AA condenses with ACNs to generate a colorless product has not been supported with direct chemical evidence.[4,5] Regardless of the precise mechanism, experiments demonstrate that this reactivity is subject to influence by a number of factors, including the exact chemical nature of the ACN,[6] the presence of other molecules, such as simple phenolic acids,[7] and conditions that stabilize AA.[8]

Owing to numerous experimental differences, including purity and concentration of samples, storage conditions, and perhaps even methods of analysis, the direct comparison of ACN stability results between laboratories is often a difficult task. Further complicating the situation is the variety of how the results are presented, which may include one or more of the following: HPLC data for individual components usually expressed as either percentage remaining or lost;[9] UV−vis absorbance values, including measures of total ACN and plots of absorbance maxima;[10,11] CIELAB values (L^*, a^*, b^*, chromaticity, hue angle, and others);[12,13] various kinetic degradation parameters, very often half-life data;[10,14] and even photographic comparisons of actual samples.[11] The aim of this study was to examine the color and chemical stabilities of a variety of ACNs from different sources in the presence of AA in solutions and powders in a single study to enable direct comparisons of treatment effects. The ACNs examined included those with three different flavylium cores as well as several differing acylated and nonacylated ACNs (Figure 1), and sources included highly purified ACNs and semipurified extracts of black rice, purple corn, and grape pomace.
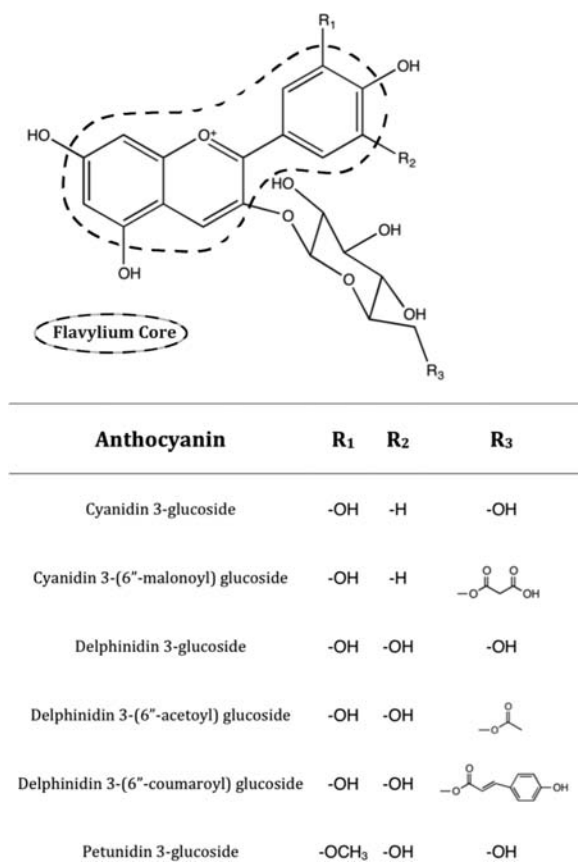
Figure 1. Test anthocyanins.

MATERIALS AND METHODS

Materials. Highly purified cyanidin 3-glucoside (≥95%; C3G), AA, sucrose, glucose, and fructose were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). Cyanidin 3-(6″-malonoyl)glucoside was purified from purple corn extract by Chromadex, Inc. (Irvine, CA, USA). Grape pomace, purple corn, and black rice were obtained from Milne Fruit Products, Inc. (Prosser, WA, USA), Belmont International Trading Corp. (Miami, FL, USA), and The Barry Farm (Wapakoneta, OH, USA), respectively. Amberlite FPX66 resin was purchased from Rohm and Haas (Philadelphia, PA, USA). All chemicals for extraction, purification, and dissolution were of ACS grade, and solvents for chromatography were of HPLC grade or better.

Semipurified Extract Preparation. Grape pomace was freeze-dried at room temperature before extraction, and purple corn and black rice were extracted as received. For the preparation of extracts, plant materials were stirred with 2% v/v aqueous formic acid (1:2 ratio of plant material to acid), the head space was flushed with argon, and the mixtures were allowed to stand for 20−24 h at 5 °C in the dark. The extracts were filtered through grade 2 filter paper followed by a 0.45 μm nylon membrane filter. The clarified extracts were passed down a column containing FPX66 resin (1:1 ratio of extract and resin volumes) previously washed with water and 2% v/v aqueous formic acid. The resin column was then washed with ∼20 L of water, discarding the aqueous washes. Absolute ethanol was introduced onto the column to remove the ACNs, as well as other adsorbed phenolic compounds. Collection was started when color elution was observed and terminated when the eluate was colorless. Ethanol and water were removed under water aspirator vacuum using a rotary evaporator at bath temperatures of <40 °C and in the dark. The remaining water extract was freeze-dried at room temperature, and dried extracts were stored at −20 °C under argon. ACNs, simple sugar content, and AA content of the initial extracts and final freeze-dried products were compared using HPLC, and the identity of the ACNs was confirmed by HPLC-MS using methodologies described below. The resin process routinely reduced the levels of simple sugars and AA to <0.1% without altering the ACNs.

Sample Preparation and Storage. Potassium acid phthalate/hydrochloric acid buffer solutions (pH 3.0 and 4.0) were used to prepare ACN and AA solutions.[15] These pH values were selected on the basis of the small survey of powdered beverage mixes, which revealed a pH range of 2.6−3.9 when reconstituted. The pH of the solutions was verified using a Mettler Toledo SevenMulti pH-meter equipped with an InLab 413 combination electrode (Columbus, OH, USA). The concentrations of test materials were as follows: standard C3G 0.1 mg/mL; grape pomace extract, 1 mg/mL; purple corn extract, 0.6 mg/mL; and black rice extract, 0.3 mg/mL. AA was added at a final concentration of 0.5 mg/mL. Purified cyanidin 3-(6″-malonoyl)glucoside was placed in pH 3.0 buffer at a concentration of ∼0.2 mg/mL in a separate experiment to evaluate its stability at 40 °C. In other separate experiments, AA was added at 0.01−0.05 mg/mL in increments of 0.01 mg/mL and at 0.1−0.5 mg/mL in increments of 0.1 mg/mL to purple corn extract (0.6 mg/mL) at pH 3.0 and stored at 40 °C for 21 and 12 days, respectively. Liquid samples were stored in incubators at 6, 25, and 40 °C in the dark for up to 22 days; samples were removed for analysis usually every second or third day. Aqueous solutions of the respective test materials and AA were prepared in a similar manner, and 10 mL aliquots were freeze-dried in scintillation vials to provide homogeneous powdered storage samples. These powders were placed in glass desiccators containing the following saturated salt solutions to achieve the desired environmental relative humidity (RH) (potassium carbonate, 43% RH; sodium chloride, 75% RH; and potassium sulfate, 98% RH) and stored at 25 °C in the dark for up to 9 weeks; samples were removed for analysis weekly. RH was monitored in each chamber by inclusion of a traceable hygrometer from Control Co. (Friendswood, TX, USA). Additional details of the powder storage test procedure are described elsewhere.[16]

Colorimeter Analysis. CIELAB values, L^*, a^*, b^*, were determined using a Lovibond Tintometer PFX990 (HF Scientific, Inc., Ft. Myers, FL, USA) in a 1 cm glass cuvette, room temperature, 10° standard observer, and D65 standard illuminant. Liquid samples were analyzed directly without further dilution, whereas powder samples were dissolved in 10 mL of 2% v/v aqueous formic acid. Hue angle (h) was calculated using $h = \arctan(b^*/a^*)$. The L^*, a^*, and b^* values were imported into Adobe Photoshop CS5 software (Adobe Systems, Inc., San Jose, CA, USA) and color printed to create color swatches. These color swatches enable visualization of the CIELAB data. The sensitivity of hue angle, lightness values, and the generation of color swatches for detecting color changes were verified in separate experiments, where the concentration of AA was varied from 2 to 83% of the weight of the ACN-containing extract (representing a beverage delivering 4−200% of the daily value of vitamin C per serving; results presented in Supplemental Figures S1−S4 in the Supporting Information).

HPLC Analysis. For the analysis of ACNs, the HPLC was a Waters (Milford, MA, USA) 2695 HPLC equipped with a 2998 PDA detector, Empower 2 software, and a Grace (Deerfield, IL, USA) Prevail C18 5 μ analytical column, 250 mm × 4.6 mm, with a Prevail C18 5 μ guard column, 7.5 mm × 4.6 mm, at 30 °C. The mobile phase was a linear gradient of 2% v/v formic acid in water and methanol starting at 0% methanol and increasing to 25% methanol in 10 min and then to 50% methanol in 30 min, flow rate was 1.0 mL/min, and the injection size was 20 μL. All samples for HPLC were filtered through Millipore (Billerica, MA, USA) Millex LCR 0.45 μm filters. Liquid samples were analyzed directly without further dilution, whereas the entire contents of the vials containing powder samples were dissolved in 10 mL of 2% v/v aqueous formic acid. Detection was at 520 nm. Additional chromatograms were recorded at 360, 320, and 280 nm for the detection of flavonols, conjugated forms of hydroxycinnamic acids, and flavan-3-ols, respectively, as well as other phenolic compounds.[17] ACNs exceeding an arbitrarily set percentage of the total ACN profile of ≥5% were quantified using standard C3G dissolved at 0.025 mg/mL in 2% v/v aqueous formic acid. All samples were analyzed in duplicate.

AA was determined in a fashion similar to ACNs except that the mobile phase was a linear gradient of 2% v/v formic acid in water and methanol starting at 0% methanol and increasing to 25% methanol in 10 min and then to 35% methanol in an additional 10 min. Detection was at 250 nm. The AA standard was 0.5 mg/mL prepared in buffer. All samples were analyzed in duplicate.

For determining simple sugars and confirming the residual AA content in semipurified extracts, the HPLC was a Waters 625 LC system equipped with a 410 differential refractometer and an Agilent 3396 Series III Integrator (Santa Clara, CA, USA). The columns were a Bio-Rad (Hercules, CA, USA) Aminex HPX-87H, 300 mm × 7.8 mm, analytical column and Cation H, 30 mm × 4.6 mm, guard column at 35 °C. The isocratic mobile phase was 0.1% phosphoric acid with a flow rate of 0.8 mL/min. Samples were dissolved in mobile phase, and the injection size for filtered samples was 100 μL. Solutions of sucrose, glucose, fructose, and AA at a concentration of ∼0.5 mg/mL and ∼0.1 mg/mL of each component dissolved in mobile phase were used as standards.

Mass Spectrometry. The identity of the individual ACNs was confirmed using a Waters Acquity UPLC system fitted with a Waters quadrupole time-of-flight (Q-TOF) Premier mass spectrometer (MS) equipped with electrospray ionization (ESI) and controlled by MassLynx V4.1 software (Waters Corp., Milford, MA, USA). A Prevail C18 5 μ, 2.1 mm × 150 mm, column was used. The mobile phase was a linear gradient of 2% v/v formic acid in water (A) and 2% v/v formic acid in acetonitrile (B) starting at 0% (B) and increasing to 50% (B) over 40 min, flow rate was 0.275 mL/min, and the column was at ambient temperature. The Q-TOF was operated under the following instrument parameters in TOF-MS mode to acquire full-scan spectra: positive ESI mode, mass range of 100−1500 Da, desolvation temperature of 300 °C; desolvation nitrogen gas flow of 600 L/h; capillary voltage of 2.8 kV; cone voltage of 30 eV; and collision energy of 5 eV. ACNs were identified on the basis of accurate mass measurements, tandem MS fragmentation consistent with the ACN aglycone and sugar moieties, and comparison with published MS data.[18,19]

Vapor Sorption Analysis. Moisture sorption profiles were generated for freeze-dried purple corn extract powders with and without AA using a TA Q5000 moisture sorption instrument (TA Instruments, New Castle, DE, USA). The samples were equilibrated at 25 °C and 0% RH for 240 min. RH was increased in 5% increments from 43 to 93% RH. The equilibrium criterion was set at 0.01% w/w in 60 min with a maximum of 360 min of equilibrium time.

Powder X-ray Diffraction. Powder X-ray diffraction patterns were collected for freeze-dried purple corn extract powders with and without co-freeze-dried or crystalline AA using a Shimadzu X-ray diffractometer XRD-6000 (Columbia, MD, USA) operating at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation.

Statistical Analysis. Data were analyzed using R version 2.8.0 software (R Foundation, Vienna, Austria). Multiple-comparison methods (Tukey) along with ANOVA and two-way ANOVA were used to determine the statistical significance of the data at α = 0.05.

■ RESULTS AND DISCUSSION

Changes in Color of ACN−Ascorbic Acid Solutions. Plots of hue angle and lightness values for all solutions are presented in Figure 2, and a representative set of color swatches is presented in Figure 3, with the remainder shown in the Supporting Information (Figures S5−S9). Without added AA, highly purified C3G solutions demonstrated excellent color stability across all experimental pH and temperature conditions, except when stored at pH 4.0 and 40 °C, the highest pH and temperature in the study (Figure 2A1). Visual examination of the color swatches revealed this color change to be clearly noticeable after day 9. In the presence of AA, however, there were substantial changes in the hue angle and lightness values of all C3G solutions except those stored at 6 °C (Figure 2B). These changes were observed to occur more rapidly with increased temperature or pH: after 8 days at pH 3.0 and 25 °C, 4 days at pH 3.0 and 40 °C, 6 days at pH 4.0 and 25 °C, and 4 days at pH 4.0 and 40 °C. Both the hue angle results and color swatches revealed that the typical red-blue color of the C3G solutions changed over time to light yellow.

In solutions containing only AA, hue angle and lightness values (Figure 2F) for 25 and 40 °C and especially the color swatches for these temperatures show noticeable changes in color beginning after days 7 and 5, respectively. At a given temperature, color changes were most noticeable at pH 4.0. These initially colorless solutions changed to light yellow, not unlike what was seen in the C3G/AA solutions. This suggests that the color generated by the degradation of AA plays a prominent role in the final color of the binary solutions observed at later stages of storage. Samples held at 6 °C remained colorless throughout storage.

The patterns of the color changes of the various ACN-containing extracts appeared very similar to each other (Figure 2B,C,D). Excellent color stability was again observed at the lowest temperature, 6 °C, at either pH. All solutions stored at the two higher temperatures regardless of pH appeared to be degrading to produce a similar and stable final hue. This was especially noticeable at 40 °C, but even the solutions at 25 °C appeared to be changing in the same manner, albeit more slowly. An example of this progression of color change can be viewed in Figure 3.

The final hue of the ACN-containing extracts/AA binary solutions was different from that observed in the highly purified C3G/AA solutions (Figure 3; Supporting Information Figures S6−S8). This can be best explained by the presence in the extracts of many other substances that also have the potential to degrade and produce color. Furthermore, it is important to note that although the extracts were from very different sources and possess differing ACN profiles, they all showed remarkable similarity as it pertained to loss of ACN color and browning.

Changes in ACN−Ascorbic Acid Concentrations in Solutions. The percentages of ACNs and AA remaining in solutions at the end of each experimental period taken from the HPLC data are presented in Table 1. An example of the treatment effects over time on the stability of ACNs from grape pomace in solution is shown in Figure 4, and additional HPLC plots are shown in the Supporting Information (Figures S10−S14). All peak identifications were confirmed by HPLC-MS. The degradation results for highly purified C3G and AA when stored separately are presented for comparison with results obtained when both were in solution together. Results were generally consistent with those reported by others in that both substances were more stable at lower pH and temperature.[12,15,20,21] One difference in our results was that C3G was equally stable at the lowest storage temperature whether at pH 3.0 or 4.0. Torskangerpoll and Andersen[12] and Cabrita et al.[15] reported C3G to have better stability at the lower of all pH values regardless of storage temperature. The cause of these differences is unknown, but our experiments were conducted at 6 °C compared to 10 °C, the lowest temperature reported in both previous studies.

Highly purified C3G and AA stored together in solution resulted in their mutual destruction, replicating observations reported by others.[22,23] The loss of highly purified C3G was especially pronounced at temperatures above 6 °C; however, stability at pH 3.0 appeared to be slightly better than at pH 4.0 at higher temperatures, although not significantly so. For example, when end-of-storage results were compared for highly purified C3G, the percentage remaining averaged ∼88% for both pH levels at 6 °C without AA, but this average dropped to ∼69% with AA present. At 25 °C and pH 3.0, ∼83% of the highly purified C3G remained when stored without AA, but fell to 3% when AA was present. At this temperature and pH 4.0, the results were ∼67% remaining in the absence of AA and <0.5% when it was present. The differences with and without AA were even more pronounced at 40 °C, at which only a trace amount (≤0.1%) of

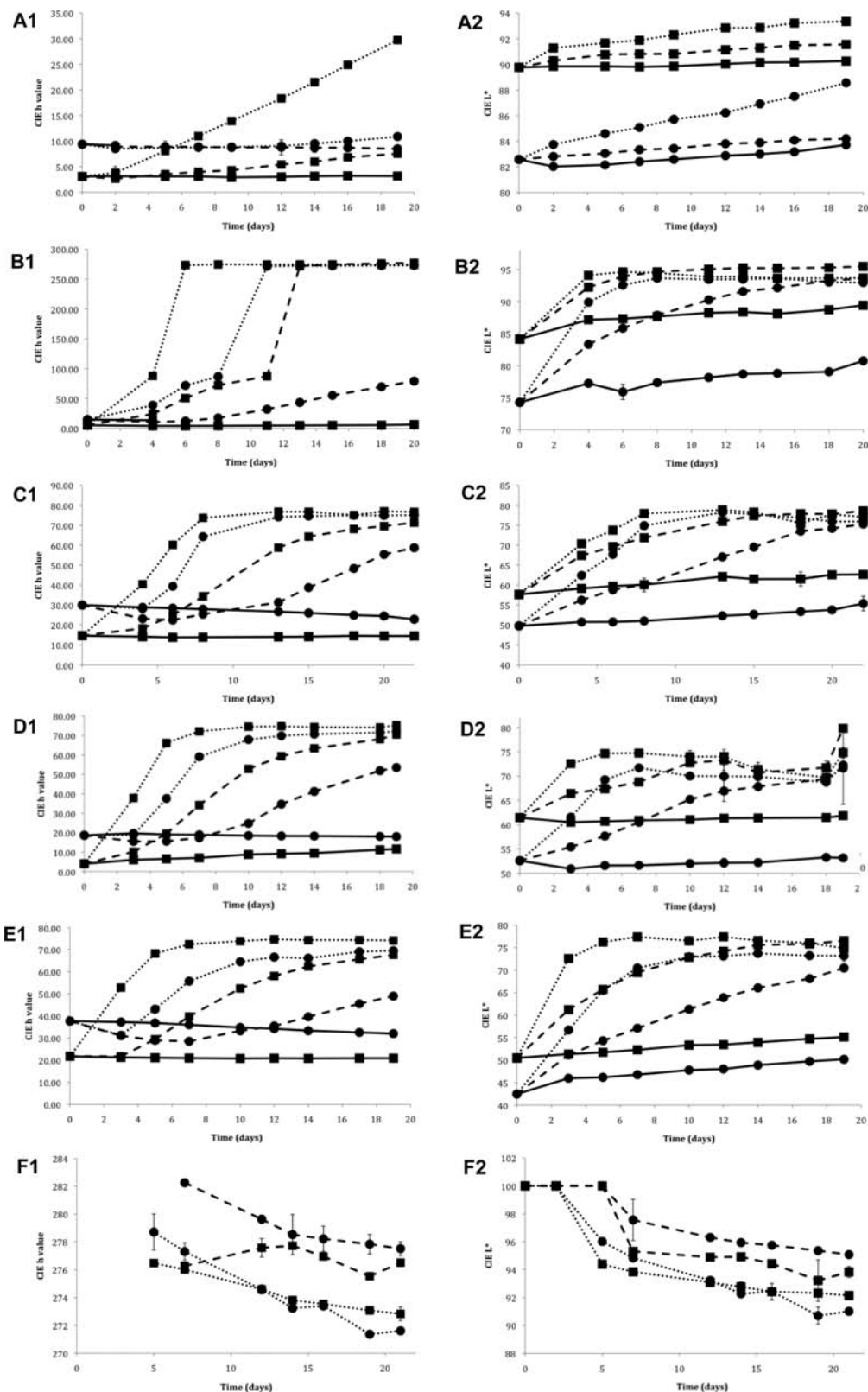**Figure 2.** Changes in hue angle (CIE *h*, series 1) and lightness values (CIE *L**, series 2) of (A1,2) C3G, (B1,2) C3G/AA, (C1,2) black rice extract/AA, (D1,2) purple corn extract/AA, (E1,2) grape pomace extract/AA, and (F1,2) AA in solution at pH values 3.0 and 4.0 and at 6, 25, and 40 °C for up to 22 days: (solid circle, solid line) pH 3, 6 °C; (solid box, solid line) pH 4, 6 °C; (solid circle, dashed line) pH 3, 25 °C; (solid box, dashed line) pH 4, 25 °C; (solid circle, dotted line) pH 3, 40 °C; (solid box, dotted line) pH 4, 40 °C.

highly purified C3G remained at either pH with added AA. The degradation of AA in the presence of highly purified C3G followed a similar trend, but the losses of AA were even greater and more rapid when coformulated with ACN.

The stability of C3G in the presence of AA in solution was similar regardless of its source, percentage of the total ACN profile, or purity (Table 1). In the ACN profiles, C3G comprised 79, 37, and 14% of black rice, purple corn, and grape pomace extracts, respectively. At all three temperatures, the differences between the stability of the highly purified single compound and C3G present in any of the extracts were not significant; however,
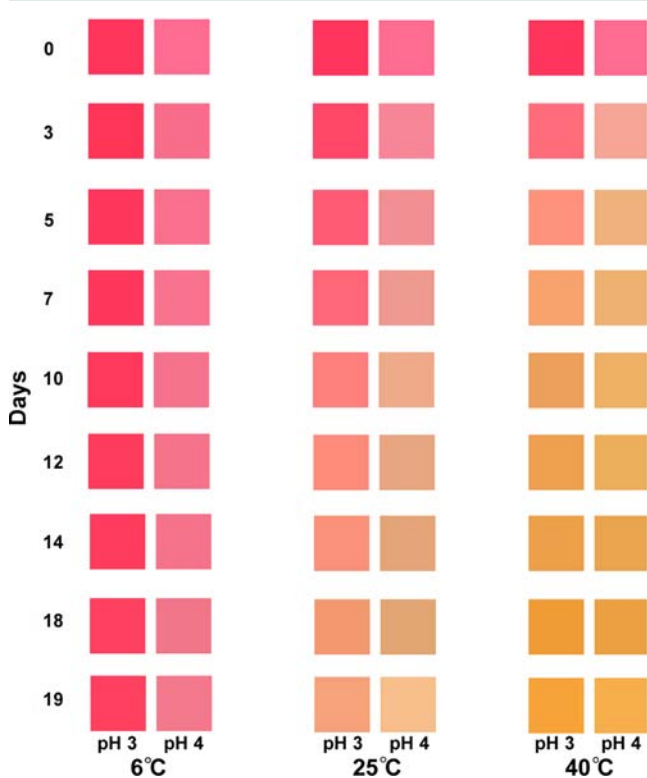
this ACN trended toward being more stable in the grape pomace extract at all pH values and temperatures and less stable in the black rice extract. Specifically, at 6 °C the C3G remaining at the end of the experiments for the highly purified compound and in the three extracts averaged ∼66% at pH 3.0 and ∼71% at pH 4.0. These values had dropped at 25 °C to ∼4% at pH 3.0 and to ∼2% at pH 4.0.

In the grape pomace semipurified extract, there was no significant difference between the stability of ACN monoglucosides containing a different flavylium core structure, whether cyanidin, delphinidin, or petunidin (Table 1). The values were not different at a given pH and temperature. These results are contrary to reports of others suggesting differing stabilities based upon ACN type,[15] but we suspect that the presence of AA was the overriding factor. This is an important finding as it suggests improved stability will likely not be achieved by including ACNs containing differing flavylium cores to color powdered beverages formulated with AA. Naturally, the various flavylium types do provide a different hue, which is an important consideration for obtaining the desirable final product color.

This similarity in loss of ACN stability when in the presence of AA also pertained to the acylated ACNs present in our samples. Many studies have reported the superior stability of acylated ACNs in a variety of systems,[12,20,24,25] but neither the malonoyl derivative in purple corn nor the coumaroyl derivative in grape pomace showed enhanced stability in the presence of AA (Table 1). On the basis of our data, delphinidin 3-(6″-acetoyl)glucoside may be slightly more stable than other acyl derivatives and its nonacylated parent, but the at best 10% greater concentration recorded at the end of some studies may not be a meaningful opportunity for improving beverage color shelf life. As was the case for differing flavylium cores, these findings further suggest that significant improvement to color stability may not be achieved by including certain acylated ACNs to color powdered beverages containing AA. However, this finding needs to be confirmed with extracts containing ACNs with more extensively

Figure 3. Color swatches for grape pomace extract/AA solutions stored over 19 days.

Table 1. End-of-Study (19−22 Days) Anthocyanin and AA Percentages for Solutions[a]

		% remaining − anthocyanin (AA)					
		pH 3.0			pH 4.0		
compound	source	6 °C	25 °C	40 °C	6 °C	25 °C	40 °C
cyanidin 3-glucoside (no AA)	Aldrich, >95%	87.6 ± 0.1 a	82.8 ± 0.1 a,b	48.0 ± 0.2 d	88.1 ± 0.1 a	67.2 ± 0.4 c	32.1 ± 0.1 e
AA (no anthocyanin)	Aldrich	(84.5 ± 0.9 g)	(<0.1 j)	(<0.1 j)	(65.5 ± 1.6 h)	(<0.1 j)	(<0.1 j)
cyanidin 3-glucoside	Aldrich, >95%	67.1 ± 0.2 c	3.1 ± 0.02 f	0.1 ± 0.02 f	70.1 ± 0.5 c	0.4 ± 0.03 f	<0.1 f
		(56.8 ± 0.5 h)	(<0.1 j)	(<0.1 j)	(39.6 ± 1.8 i)	(<0.1 j)	(<0.1 j)
cyanidin 3-glucoside	black rice	58.9 ± 9.3 c	2.1 ± 0.08 f	<0.1 f	70.5 ± 0.6 c	1.7 ± 0.3 f	<0.1 f
		(74.3 ± 10.3 g)	(<0.1 j)	(<0.1 j)	(77.7 ± 1.0 g)	(<0.1 j)	(<0.1 j)
cyanidin 3-glucoside	purple corn	65.2 ± 1.7 c	3.9 ± 0.2 f	0.3 ± 0.02 f	73.8 ± 2.3 c	2.3 ± 0.5 f	0.2 ± 0.03 f
		(60.2 ± 0.7 h)	(<0.1 j)	(<0.1 j)	(56.8 ± 0.7 h)	(<0.1 j)	(<0.1 j)
cyanidin 3-glucoside	grape pomace	73.0 ± 0.3 c	6.9 ± 0.5 f	0.7 ± 0.0 f	71.2 ± 0.7 c	5.5 ± 0.2 f	<0.1 f
		(65.2 ± 3.1 h)	(<0.1 j)	(<0.1 j)	(50.6 ± 3.1 h)	(<0.1 j)	(<0.1 j)
petunidin 3-glucoside	grape pomace	74.3 ± 0.7 c	6.2 ± 1.8 f	<0.1 f	73.7 ± 0.01 c	7.3 ± 1.2 f	<0.1 f
delphinidin 3-glucoside	grape pomace	73.1 ± 0.7 c	6.2 ± 0.8 f	0.3 ± 0.0 f	71.4 ± 0.9 c	4.7 ± 0.2 f	<0.1 f
delphinidin 3-(6″-acetoyl) glucoside	grape pomace	74.6 ± 0.7 b,c	5.7 ± 1.2 f	<0.1 f	75.6 ± 6.7 b	8.4 ± 2.0 f	<0.1 f
delphinidin 3-(6″-coumaroyl) glucoside	grape pomace	64.5 ± 1.7 c	8.2 ± 7.3 f	<0.1 f	64.7 ± 3.3 c	1.5 ± 0.5 f	<0.1 f
cyanidin 3-(6″-malonoyl) glucoside	purple corn	63.9 ± 1.7 c	2.9 ± 0.1 f	<0.1 f	74.0 ± 2.6 c	1.7 ± 0.4 f	<0.1 f

[a]AA values are presented once with the first mention of the source. Lower case letters a−f are comparisons across all storage treatments and sample types for anthocyanins. Lower case letters g−j are comparisons across all storage treatments and sample types for AA. Treatments with the same letters are not significantly different at *p* = 0.05. The identities of all extract anthocyanins were confirmed by mass spectrometry.
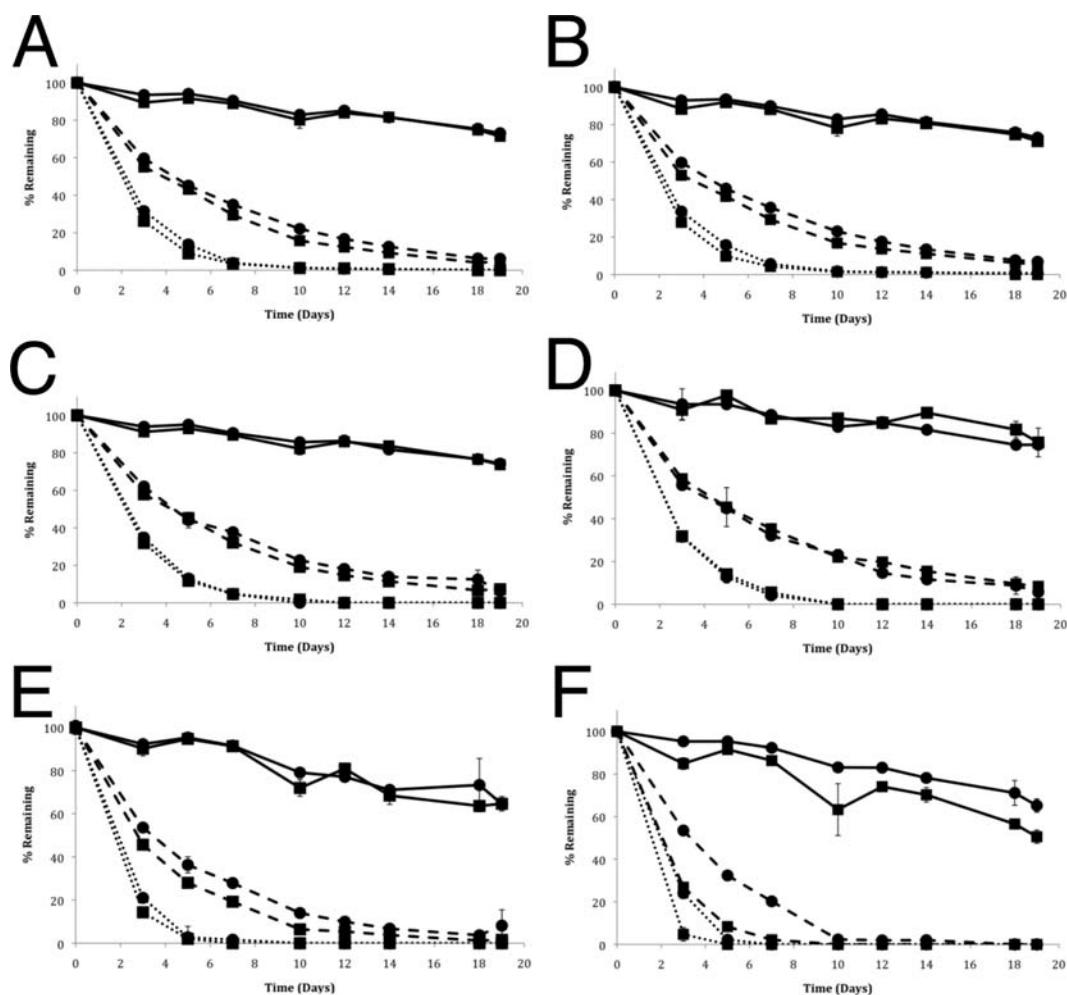
Figure 4. Effects of pH and storage temperature on the stability of grape pomace extract/AA solutions stored over 19 days: (A) delphinidin 3-glucoside; (B) C3G; (C) petunidin 3-glucoside; (D) delphinidin 3-(6″-acetoyl)glucoside; (E) delphinidin 3-(6″-coumaroyl)glucoside; (F) AA; (solid circle, solid line) pH 3, 6 °C; (solid box, solid line) pH 4, 6 °C; (solid circle, dashed line) pH 3, 25 °C; (solid box, dashed line) pH 4, 25 °C; (solid circle, dotted line) pH 3, 40 °C; (solid box, dotted line) pH 4, 40 °C.

substituted mixtures of acylated anthocyanins, such as those present in radish or cabbage.[19,26] Furthermore, because our ACNs were monoglucosides, it would be useful to evaluate representatives with differing sugars as well as those more highly glycosylated such as the types found in black carrot.[27] If verified using a larger variety of acylated and glycosylated ACNs, this observation does mean that developers of powdered beverages may enjoy a greater selection of ACNs when compared with formulating other food products that do require more chemically complex ACNs to maintain color stability. This expanded number of options increases the likelihood of finding ACNs that will provide desirable hues, adequate supply, and favorable cost.

In a separate experiment conducted without the presence of AA, ~68% of the purified cyanidin 3-(6″-malonoyl)glucoside was lost over 22 days of storage at pH 3.0 and 40 °C, being converted primarily to C3G (chromatogram presented in Supporting Information Figure S21). This means that the results for C3G when in the presence of an acylated derivative, as occurs in certain purple corn extracts, could be erroneously high at any time beyond the initial analysis point. This finding may have important implications for other acylated ACNs when both acylated and nonacyl parent compound are quantified at the same time. For example, in food environments that favor the loss

of acyl groups, the product developer may be wise to consider sources which contain mainly the nonacylated parent ACN, especially for products exposed to longer shelf life storage at higher temperatures.

The semipurification procedure employed a resin designed for the concentration of not just ACNs but all other phenolic compounds as well.[28] While not rigorously identifying these other phenolic compounds, chromatographic fingerprints indicated that the type, number, and concentration of phenolic compounds in our extracts from different plant sources varied widely. An inspection of the extract chromatograms at the three wavelengths other than 520 nm revealed the order of levels and numbers of suspected phenolic components to be purple corn > grape pomace > black rice (chromatograms presented in Supporting Information Figure S22). Studies by others have demonstrated that certain of these phenolic compounds may slow the degradation of ACNs and AA.[22,29−32] However, no advantage of any particular extract over another was found in our study for stabilizing ACNs in the presence of AA. It was hoped that selecting a source and/or purification scheme that provided a differing background of phenolic compounds might confer a stabilizing effect; however, it appears that the overriding presence of AA makes any contribution from the natural background of
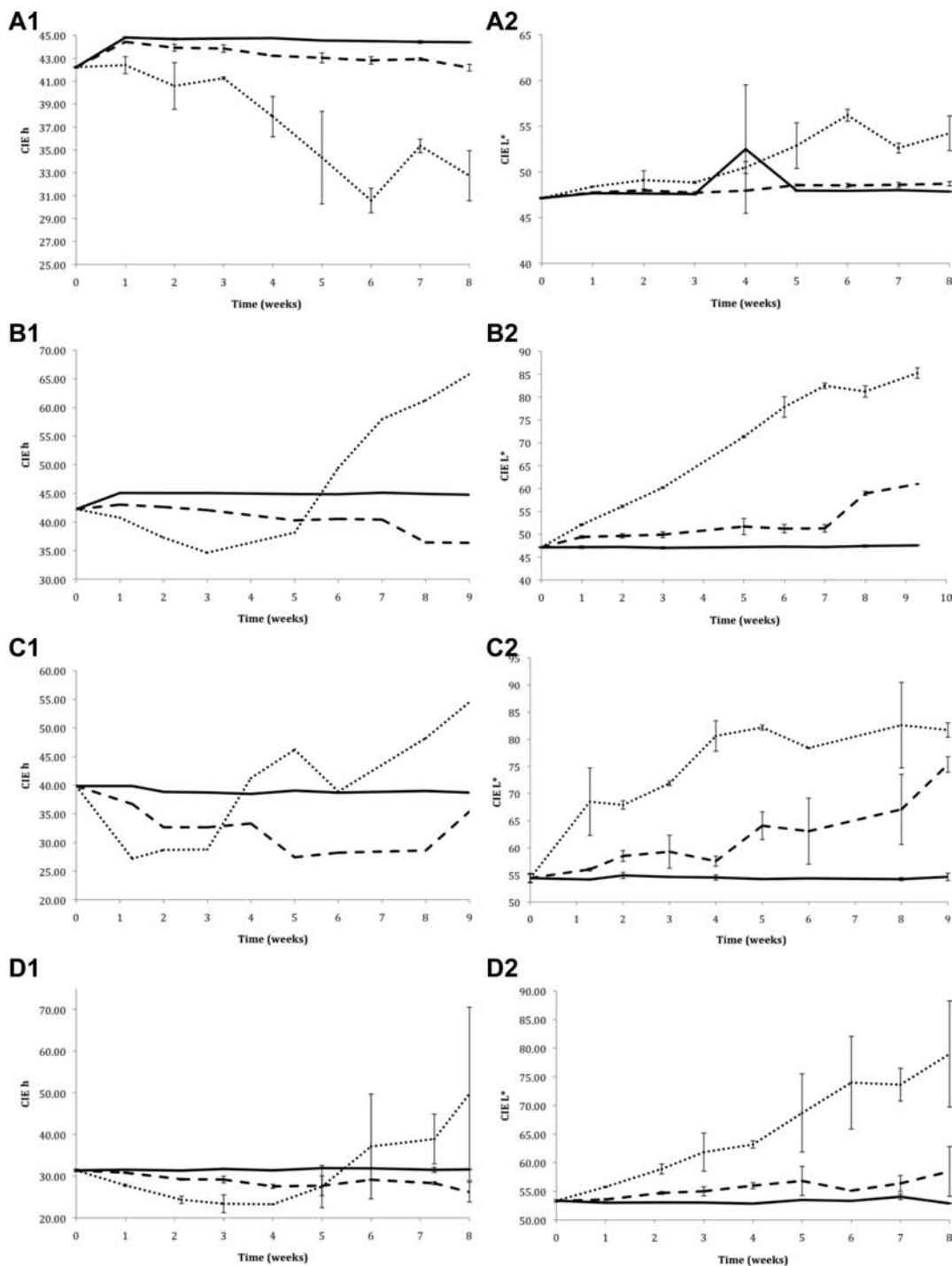
Figure 5. Changes in hue angle (CIE *h*, series 1) and lightness values (CIE *L**, series 2) of (A1,2) purple corn extract, (B1,2) purple corn extract/AA, (C1,2) black rice extract/AA, and (D1,2) grape pomace/AA stored dry at 43, 75, and 98% RH and 25 °C for up to 9 weeks: (heavy solid line) 43% RH; (dashed line) 75% RH; (dotted line) 98% RH.

phenolic compounds mute. It remains to be seen if the addition of exogenous phenolic compounds at even higher concentrations or ones possibly with enhanced protection properties could improve the stability of ACNs coformulated with AA.

In general, AA degradation increased in the presence of the ACN-containing extracts and highly purified C3G compared to when it was stored alone (Table 1; Figure 4F; Supporting Information Figures S11−S14). An exception was the black rice extract, in which AA was stabilized compared to highly purified C3G for both pH values when stored at 6 °C. This effect may be

due to the presence of unidentified agent(s) in this extract that protects AA against destruction.

Changes in Color of ACN−Ascorbic Acid Powders. Whereas many powdered beverage products are designed for single use and packaged in materials, such as foil, which should limit the exposure to changes in environmental humidity, some products are sold in multiuse canisters which with repeated opening and closing by the consumer will subject the powdered beverage product to various degrees of moisture. It is for this reason that these powder studies were undertaken.

Plots of hue angle and lightness values of all samples reconstituted from powders are presented in Figure 5. An example set of color swatches is presented in Figure 6, with the remainder shown in
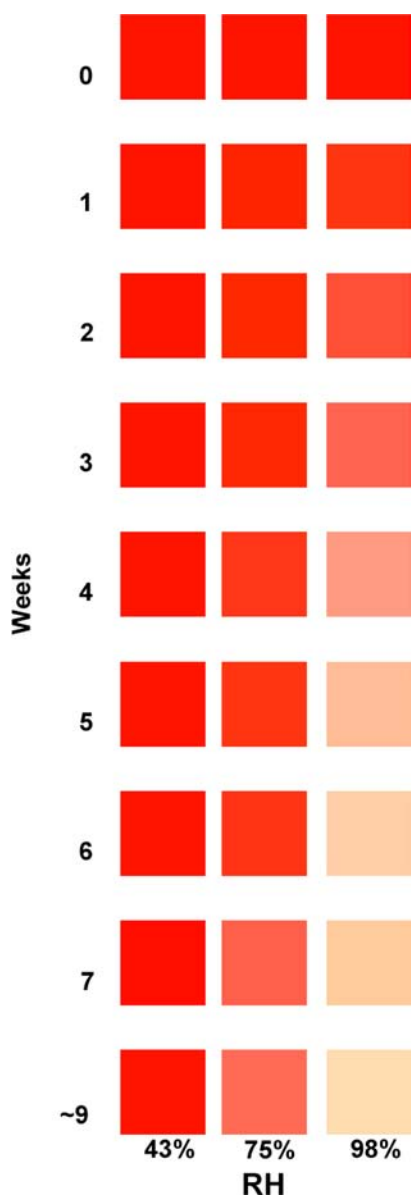

Figure 6. Color swatches for purple corn extract/AA powders stored at 43, 75, and 98% RH over ~9 weeks.

the Supporting Information (Figures S15−S17). When reconstituted, all samples, with and without AA, stored at 43% RH for up to 9 weeks showed no changes in color values judged important to the appearance of the final solution. At 75% RH, the only color change determined to be important to the final appearance of the reconstituted solution occurred with the lightness of the black rice extract containing AA at 9 weeks of storage (Figure 5C2). Considerable changes in hue angles, lightness values, and color swatches occurred with the samples containing AA held at 98% RH. Purple corn extract/AA samples became visibly lighter and browner (Figures 5B and 6) at weeks 4 and 6, respectively. Black rice extract/AA became lighter by week 4 and browner by week 9 (Figure 5C and Supporting Information Figure S17). Grape pomace appeared to perform slightly better (side-by-side comparison of the color swatches)

than the other AA-containing extracts, becoming visibly lighter at week 5 and browner by week 8 (Figure 5D and Supporting Information Figure S16).

Changes in ACN−Ascorbic Acid Concentrations in Powders. The percentages of ACNs and AA remaining in the powders following storage treatment at select relative humidities are presented in Table 2. An example of the treatment effects over time on the stability of purple corn extract/AA powders is shown in Figure 7, and additional HPLC plots are included in the Supporting Information (Figures S18−S20). All peak identifications were confirmed by HPLC-MS.

The stability of ACNs stored in powdered form as compared to when dissolved in solution revealed considerable differences. All ACNs remained stable at 43% RH. Only black rice extract appeared to perform slightly less well at this RH, but not significantly so. Many of the losses at 75 and 98% RH for both ACNs and AA were much higher than expected. However, the samples held at 98% RH and, to a lesser degree, 75% RH often absorbed water to such an extent that some were no longer in powder form, but actually existed as concentrated solutions or pastes. These larger than expected losses may perhaps be explained on the basis of this observation. In addition, the large variability in many of the replicate samples held at these two relative humidities, especially apparent in grape pomace extract combined with AA, was responsible for the loss of significance between certain treatments. However, the lack of statistical significance between certain samples does not change the overall observation that loss of ACNs and AA is positively correlated with increasing humidity.

At 75% RH, C3G in the purple corn extract without AA present appeared to be the most stable, losing about 10% of the ACN in 8 weeks. In the presence of AA about 73% was lost over a similar time period. In grape pomace and black rice, about 37 and 93% of the C3G were lost, respectively. This is very different from solutions for which losses of C3G, although being considerably greater in a shorter period of time, were very similar between extracts.

As observed in solutions, the loss of the different acylated and nonacylated ACNs was very similar in the powders, and usually not significantly different. The notable exception was in purple corn extract without AA, for which at 75% RH, about 84% of the acylated ACN was lost compared to only 10% of the nonacylated parent. The conversion of the acylated derivative to the nonacylated parent is likely part of the reason for this observation; however, we can offer no explanation for the much greater loss of cyanidin 3-(6″-malonoyl)glucoside compared to the other acylated ACNs in this study. Also, as seen with solutions, no differences were observed on the basis of flavylium core type.

Not surprisingly, AA in the presence of ACNs generally showed better stability stored in powder form than when stored in solution. In solutions with ACNs at 25 °C, the AA concentration was reduced to below 10% often in <1 week. In powders at 43% RH, <4% of the added AA was lost in any one extract over the storage period, whereas at 75% RH between 7 and 30% was lost depending upon the extract. The results at 98% RH showed substantial losses of AA of ~70−100%.

The moisture sorption profiles (Figure 8A) of lyophilized purple corn extract with and without AA were characteristic of type II moisture sorption, indicative of amorphous powders.[33] However, the profile also suggested a possible recrystallization event of AA between 70 and 80% RH. Additional experimentation will be required to establish whether stability differences of ACNs in the presence of crystalline or amorphous AA exist, as

Table 2. End-of-Study (8−9 Weeks) Anthocyanin and AA Percentages for Powders[a]

compound	source	% remaining − anthocyanin (AA)		
		43% RH	75% RH	98% RH
cyanidin 3-glucoside (no AA)	purple corn	101.9 ± 0.1 a	89.7 ± 1.7 a	46.9 ± 8.5 b
cyanidin 3-glucoside	purple corn	96.9 ± 0.3 a	26.8 ± 0.1 b,c	0.2 ± 0.08 c
		(95.9 ± 0.01 d)	(76.5 ± 2.2 e)	(<0.1 g)
cyanidin 3-glucoside	black rice	88.2 ± 3.1 a,b	6.96 ± 1.7 b,c	0.7 ± 0.2 c
		(102.5 ± 2.2 d)	(68.4 ± 4.8 e)	(12.2 ± 2.4 f)
cyanidin 3-glucoside	grape pomace	100.1 ± 0.2 a	62.9 ± 24.8 a,b	8.3 ± 10.0 b,c
		(98.1 ± 0.2 d)	(92.5 ± 4.7 d)	(30.7 f)[b]
petunidin 3-glucoside	grape pomace	95.3 ± 3.5 a	60.7 ± 23.6 a,b	9.3 ± 10.7 b,c
delphinidin 3-glucoside	grape pomace	100.0 ± 0.4 a	64.0 ± 30.0 a,b	9.1 ± 11.1 b,c
delphinidin 3-(6″-acetoyl)glucoside	grape pomace	100.4 ± 4.1 a	64.5 ± 21.6 a,b	1.1 ± 1.1 c
delphinidin 3-(6″-coumaroyl)glucoside	grape pomace	96.9 ± 0.8 a	65.5 ± 18.7 a,b	6.7 ± 9.5 b,c
cyanidin 3-(6″-malonoyl)glucoside (no AA)	purple corn	99.6 ± 0.04 a	70.0 ± 2.8 a,b	24.7 ± 3.6 b,c
cyanidin 3-(6″-malonoyl)glucoside	purple corn	93.4 ± 0.4 a	16.1 ± 0.1 b,c	0.2 ± 0.0 c

[a]AA values are presented once with the first mention of the source. Lower case letters a−c are comparisons across all storage treatments and sample types for anthocyanins. Lower case letters d−f are comparisons across all storage treatments and sample types for AA. Treatments with the same letters are not significantly different at $p = 0.05$. The identities of all extract anthocyanins were confirmed by mass spectrometry. [b]Single determination.
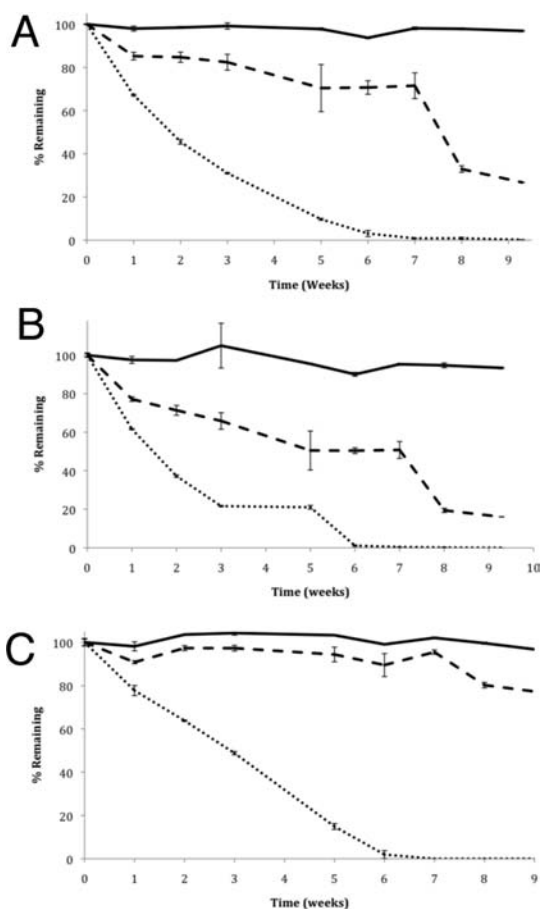

Figure 7. Effects of RH on the stability of purple corn extract/AA powders stored over ~9 weeks: (A) C3G; (B) cyanidin 3-(6″-malonoyl)glucoside; (C) AA; (heavy solid line) 43% RH; (dashed line) 75% RH; (dotted line) 98% RH.

well as the effect of the recrystallization event on the ACN and AA stability. PXRD confirmed that the starting freeze-dried powders were X-ray amorphous, with no evidence of crystalline
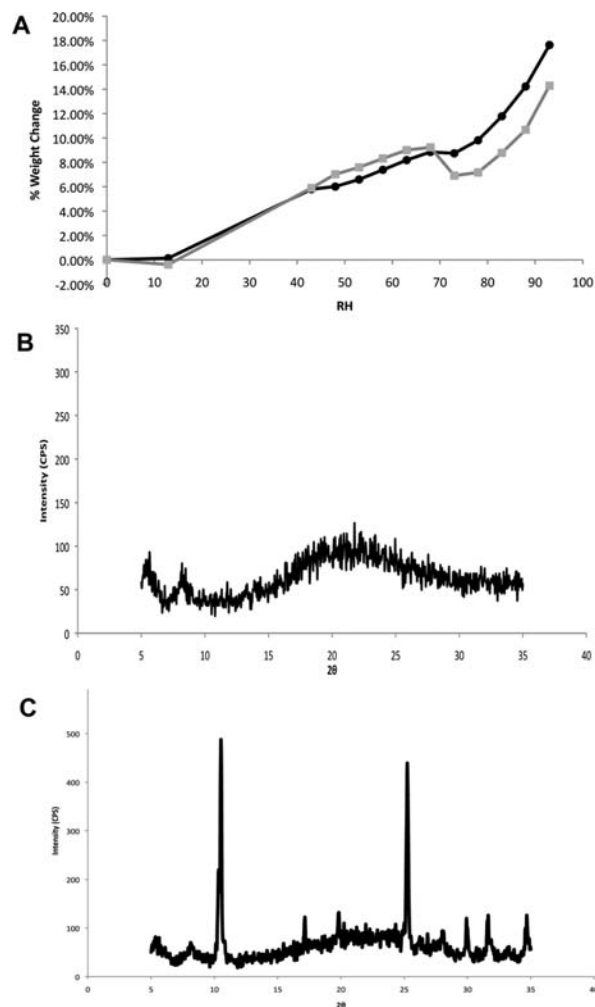

Figure 8. (A) Moisture sorption profile of purple corn extract powders with (gray box) and without (black circle) AA at 25 °C; (B) powder X-ray diffraction pattern of purple corn extract powder freeze-dried with AA; (C) powder X-ray diffraction pattern of freeze-dried purple corn extract powder blended with crystalline AA.

AA in the binary mixtures (Figure 8B). The direct admixture of freeze-dried purple corn extract with crystalline AA (as verified by PXRD; Figure 8C) would likely result in a more stable blend, at least at lower relative humidities. However, the amount of water present at high RH would dissolve the AA regardless of its physical state, explaining the observed loss of both ACNs and AA.[34]

To the best of our knowledge, this is the first study in which the stability of ACNs and AA in solution and powder forms was evaluated together. Our results further demonstrate the deleterious effect of this combination on the color and chemical stabilities of both ACNs and AA when stored in acidic solutions over time and at various temperatures. Increasing environmental RH induced color and chemical changes in powders containing AA colyophilized with ACNs. In liquids, stability was inversely related to increasing pH and temperature; for powders, stability was inversely related to increasing moisture. Also, the research presented here demonstrates that studies conducted on the color properties of ACNs require both qualitative and quantitative color and chemical measurements. Chemical changes in ACNs readily detected by HPLC, such as a loss of acylation, may or may not have important consequences on the product color, a condition readily quantified with various CIELAB measurements. However, precise knowledge of the chemical changes provides the basis for a more rational selection of ACNs. With the acylation example, a food system that promotes the loss of acyl groups may suggest the selection of lower cost, more readily available ACN mixtures containing only nonacylated ACNs. Finally, the results presented here suggest that for powdered beverage products, the solution to the problem of the mutual destruction of ACNs and AA may involve approaches other than the application of specific ACNs with putative superior stability in food systems. In our experiments, ACNs with three different core structures or two differing acylation types (two aliphatic; one cinnamic acid) failed to offer any advantages to color or chemical stability in the presence of AA. However, expanded experimentation including ACNs with increasingly complicated acylation patterns as well as more extensive glycosylation will be required before more elegant solutions to this problem can be suggested, for example, keeping both ingredients separate using encapsulated AA.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

Plots of hue angles, lightness values, and chromatographic results as well as color swatches. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ACN(s), anthocyanin(s); AA, ascorbic acid; CIELAB or CIE, Commission Internationale de l'Eclairage $L^*a^*b^*$ scale; C3G, cyanidin 3-glucoside; hue angle, h or CIE h value; HPLC, high-performance liquid chromatography; L^* or CIE L^*, lightness value; MS, mass spectrometry; PXRD, powder X-ray diffraction; RH, relative humidity

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