Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences

Volume 12 Article 11

Fall 2011

Storage effects of gel encapsulation on stability of chokeberry monomeric anthocyanins, procyanidins, color density, and percent polymeric color

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ABSTRACT

Chokeberries (*Aronia melanocarpa*) are an antioxidant-rich plant product due to their high content of polyphenols, especially anthocyanins and procyanidins. These polyphenols have been shown to provide protection against coronary heart disease, stroke, and lung cancer, as well as against oxidative stress, the main cause behind chronic diseases promoted by free radicals. The objective of this study was to determine the storage effects of gelatin encapsulation on monomeric anthocyanins, procyanidins, color density, and percent polymeric color of three gummy candies of different strengths formulated with a base of 25.4% chokeberry concentrate, 47.6% sucrose, 1.3% Splenda, and 0.025% potassium sorbate. The gum strengths varied by percentages of gelatin and water in the formulations, with 19.1:6.6, 17.8:7.9, and 16.5:9.2 ratios used to produce soft, medium, and hard strength gummies, respectively. Total monomeric anthocyanins, total procyanidins, color density, and percent polymeric color of the gummies were determined 1 day post-processing and after 2, 4, and 6 months of storage at refrigerated and room temperatures. Storage for 6 months at room temperature resulted in dramatic losses of monomeric anthocyanins (80-82%), total procyanidins (48-54%), and color density (76-80%). Anthocyanin losses during storage coincided with marked increases in percent polymeric color values indicating that anthocyanins and procyanidins underwent condensation reactions to form polymers. Refrigerated storage ameliorated losses of monomeric anthocyanins (61-65%), total procyanidins (17-22%), and color density (60-67%) over 6 months of storage compared to samples stored at ambient temperature. Refrigerated storage also ameliorated the increase in polymeric color values observed in samples stored at room temperature indicating condensation reactions responsible for polymer formation were retarded. Gum strength did not have a significant effect on retention of anthocyanins and procyanidins.

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† Luke Howard is the faculty mentor and a professor in the Department of Food Science.

MEET THE STUDENT-AUTHOR

Mary Kordsmeier

I graduated from Conway High School in May 2007. In the fall of 2007, I started as a freshman at the University of Arkansas after receiving the Chancellor's Scholarship and the Arkansas Governor's Distinguished Scholarship. Since my first food science class, I have really enjoyed my time learning and working in the department. I have been very active in the food science field, serving as president of the Food Science Club, student representative for the Ozark Institute of Food Technologists Board, and as a member of the Institute of Food Technologists Student Association. I have gotten to work in both the functional foods lab and the rice processing program, where I have greatly strengthened my research experience. I have also had the honor of receiving the Outstanding Senior Student Award in the food science department.

With the tremendous support and guidance of my honors mentor, Dr. Luke Howard, and his laboratory team, I have been able to complete my thesis project, which was funded by a Student Undergraduate Research Fellowship and the Dale Bumpers College of Agricultural, Food, and Life Sciences Undergraduate Research Fellowship. I graduated with my B.S. degree in food science and a minor in animal science in May 2011. In the summer after graduation, I plan to start my M.S. degree in food science at the U of A, continuing to work under Dr. Howard. I have gained a lot of experience and knowledge during my undergraduate career that I will carry with me through the rest of my life.

INTRODUCTION

The interest in plant products rich in health-protecting bioactive substances such as polyphenols is increasing (Borowska et al., 2009). Polyphenols are secondary metabolites of plants, which contain more than one aromatic ring carrying one or more hydroxyl groups. Fruits, especially berries, contain high levels of polyphenols, particularly one class of polyphenols called flavonoids (anthocyanins, flavonols, flavan-3-ols, and proanthocyanidins) (Howard and Hager, 2002). The chokeberry (*Aronia melanocarpa*) is a rich source of these polyphenols, especially anthocyanins, which account for >50% of the total polyphenols in chokeberry. Chokeberry anthocyanins are a mixture of cyanidin glycosides: 3-galactoside, 3-glucoside, 3-arabinoside, 3-xyloside, of which cyanidin-3-galactoside is predominant (Oszmiański and Wojdylo, 2005). These compounds are not only responsible for the pigments found in berries (Cabrita et al., 2000), but they have also been shown to exhibit antioxidant, antimicrobial, anti-inflammatory, and vasodilatory functions (Kähkönen et al., 2001). Due to limited shelf life and season availability, fresh berries are commonly distributed and consumed in processed forms including jams, jellies, juices, canned, and dehydrated products. Unfortunately, anthocyanins in processed berry products are unstable during storage, due in large part to condensation reactions involving anthocyanins and procyanidins, resulting in the formation of large molecular weight polymeric pigments (Brownmiller et al., 2008; 2009).

Storage temperature plays a key role in anthocyanin stability and antioxidant capacity. Anthocyanin degradation in model solutions doubled with a temperature increase from 10 to 23 °C over 60 days (Cabrita et al., 2000), and the antioxidant content of strawberry jams was reported to be more stable at 4 °C than 20 °C (Wicklund et al., 2005). Visible light is also harmful to anthocyanins and increases their rate of degradation (Jackman and Smith, 1996). Anthocyanin losses in stored blueberry products were accompanied by increased polymeric color values, suggesting that anthocyanins underwent polymerization reactions with procyanidins to form anthocyanin-procyanidin polymers (Brownmiller et al., 2008).

Gel encapsulation is a promising technology that may be useful in stabilizing berry polyphenols during storage. Improved anthocyanin stability has been reported in several studies through the use of natural polymers including pullulan (Gradinaru et al., 2003), cyclodextrin (Mourtzinos et al., 2008), and (1→3,1→4)-β-$_D$-glucan (Xiong et al., 2006). Maier et al. (2009) studied the effects of gelatin and pectin encapsulation on polyphenols in grape pomace extracts. They reported considerable losses in total phenolics

in the gelatin gels, which they attributed to thermal treatment of the gels, while pectin gels had greater anthocyanin retention possibly due to a stabilizing effect of pectin during processing. In addition to improved polyphenol stability, gel encapsulation may provide controlled release of bioactive polyphenols in the human body, and be a useful platform for clinical feeding trials. The objective of this study was to determine the effects of gelatin encapsulation on the stability of chokeberry anthocyanins during six months dark storage at 4 °C and 23 °C.

MATERIALS AND METHODS

Preparation of Gummy Candies. Chokeberry concentrate (70° brix, or 70 g sugars per 100 g solution) was obtained from Mae's Health & Wellness (Omaha, Neb.). Three formulations of chokeberry gummy candies were developed to obtain textures of soft, medium, and hard by varying the gelatin-to-water ratio in the formulation (Table 1).

For the base, sugar was added to chokeberry concentrate and the mixture was boiled on a hot plate until the sugar was completely dissolved. Splenda® and potassium sorbate were then added to the mixture. The mixture was constantly stirred while boiling on the hot plate until the ingredients were completely dissolved. For each gummy, the required amount of Knox gelatine® (Kraft Foods, Inc., Northfield, Ill.) was dissolved in the appropriate amount of water at 40 °C. The melted gelatine mixture was added to the boiling chokeberry mixture. After stirring the mixture was poured into gummy molds (Cakes 'N Things, Inc., Gridley, Ill.) greased with olive oil cooking spray. The gummies were allowed to equilibrate to room temperature, and then were placed in a -20 °C freezer for 10 min. The gummies were placed into Ziploc® bags with half stored in the dark at 4 °C and half stored in the dark at 23 °C. The gummies were sampled in triplicate after 1 day and at 2, 4, and 6 months of storage.

Extraction of Anthocyanins. Anthocyanins in the chokeberry concentrate were extracted by the method of Cho et al. (2004) with modifications. The gummies were soaked in 20 mL of methanol/water/formic acid (60:37:3 v/v/v) for 15 min. The gummy-solvent mixture was homogenized using a Euro Turax T19 Tissuemizer (Tekmar-Dohrman Corp., Mason, Ohio). The samples were filtered through Miracloth (Calbiochem, La Jolla, Calif.), and the filtrates adjusted to a final volume of 100 mL with the extraction solvent.

Monomeric Anthocyanins Analysis by UV-Visible Spectroscopy. Monomeric anthocyanins were analyzed using the pH-differential method of Giusti and Wrolstad (2001). Absorbance values of diluted extracts were measured by a diode array spectrophotometer (Hewlett Packard, Palo Alto, Calif.). The absorbance of the diluted sample (A) was calculated as follows:

$$A = (A_{510\,nm} - A_{700\,nm})_{pH\,1.0} - (A_{510\,nm} - A_{700\,nm})_{pH\,4.5}$$

The total monomeric anthocyanin pigment concentration (mg/kg gummy) in the original sample was calculated using the following formula:

$$\text{Monomeric anthocyanin pigment} = \frac{\left(\begin{array}{l}A \times \text{molecular weight} \times \text{dilution factor} \times \\ \text{extraction volume} \times 1000\end{array}\right)}{(\text{molar absorptivity} \times 1000 \times \text{gummy weight})}$$

Cyanidin-3-glucoside, with a molar absorptivity of 26900 (Jurd and Asen, 1966), and molecular weight of 467.2 (Giusti and Wrolstad, 2001) was used as standard, with results expressed as mg of cyanidin 3-glucoside equivalents/kg gummy. The dilution factor used was 10.

Procyanidin Analysis. Procyanidins (PACs) were analyzed using the 4-dimethylaminocinnamaldehyde (DMAC) colorimetric method of Prior et al. (2010) with one modification. The method uses acetone/water/acetic acid (75:24.5:0.5 v/v/v) to extract PAC, but in order to liquefy gelatin in the gummies, extractions were done using methanol/water/formic acid (60:37:3 v/v/v). The concentrations of PACs in the sample extract were measured using a BioTek Synergy HT microplate reader (Winooski, Vt.), using catechin as standard. The total PAC concentration (mg PAC/kg gummy) in the original sample was calculated using the following formula:

$$\text{Total PACs} = \frac{\left(\begin{array}{l}\text{concentration of PACs in sample extract} \times \\ \text{dilution factor} \times \text{extraction volume} \times 1000\end{array}\right)}{(1000 \times \text{gummy weight})}$$

Color Density and Polymeric Color Analysis. Color density and percent polymeric color were analyzed using the method of Giusti and Wrolstad (2001). Color density was calculated using the control sample according to the following formula:

$$\text{Color density} = [(A_{420\,nm} - A_{700\,nm}) + (A_{510\,nm} - A_{700\,nm})] \times \text{dilution factor}$$

Polymeric color was calculated using the bisulfite-bleached sample as follows:

$$\text{Polymeric color} = [(A_{420\,nm} - A_{700\,nm}) + (A_{510\,nm} - A_{700\,nm})] \times \text{dilution factor}$$

Percent polymeric color was calculated using the following formula:

$$\%\ \text{Polymeric color} = (\text{polymeric color}/\text{color density}) \times 100$$

Color density, polymeric color, and % polymeric color were expressed as unitless numerical values.

Statistical Analysis. Effects of gel strength, storage temperature, and storage time on total anthocyanins, total PAC,

color density, and polymeric color were analyzed by analysis of variance (ANOVA) using JMP® software (SAS Inst. Inc., Cary, N.C.). Significant differences ($P < 0.05$) between means were determined by Student's *t*-test. Pairwise correlations between total anthocyanins, total PAC, color density and polymeric color were also determined using JMP® software.

RESULTS AND DISCUSSION

Monomeric Anthocyanins. Monomeric anthocyanin content was affected by the interaction between storage temperature and storage time ($P < 0.0001$) (Fig. 2) and the interaction between gum strength and storage time ($P = 0.05$) (Fig. 1). Hard gummies had higher retention of total monomeric anthocyanins than medium or soft gummies 1 day after processing. Hard gummies also had higher total monomeric anthocyanins than soft gummies at 4 months storage (Fig. 1).

For both storage temperatures, total monomeric anthocyanins decreased over storage time (Fig. 2). This finding was consistent with a previous study on stability of black currant anthocyanins encapsulated in glucan gel, where refrigerated storage resulted in greater retention of anthocyanins (Xiong et al., 2006). There was a clear temperature effect after 2 months of storage, with greater total monomeric anthocyanin retention in refrigerated gummies at months 2, 4, and 6 compared to those stored at room temperature at each respective sampling point. Total monomeric anthocyanin retentions were not affected by gum strength, with retention values of 46%, 47%, and 46% in soft, medium, and hard gummies, respectively, stored for 4 months at room temperature (data not shown). Anthocyanin retention continued to decrease over storage in gummies stored at room temperature, with retention values of 18%, 20%, and 19% in soft, medium, and hard gummies, respectively, after 6 months of storage (data not shown). With gummies stored in refrigeration, anthocyanin retention was much higher during the first 4 months of storage. Monomeric color loss at 4 months was minimal for refrigerated gummies of all three strengths, with retentions of 87%, 87%, and 84% for soft, medium, and hard gummies, respectively (data not shown). After 6 months of storage at refrigerated temperature, monomeric color retention values decreased to 38%, 39%, and 35% for soft, medium, and hard gummies, respectively. These values are lower than previously reported values for a study on storage effects on anthocyanin retention of grape pomace extracts encapsulated by gelatin, which found retentions of 72% and 80% for gels stored for 24 weeks in the dark at 20 °C and 6 °C, respectively (Maier et al., 2009). This discrepancy may be attributed to differences in fruit structure, type and localization of anthocyanins, and gel type.

Monomeric anthocyanins were found to be more stable over time when stored at refrigerated temperatures. This is consistent with previous studies with aqueous solutions of anthocyanin 3-glucosides as well as strawberry jam, where anthocyanin stability was found to increase with decreased storage temperature (Cabrita et al., 2000; García-Viguera et al., 1999). Anthocyanin degradation in the gummies during storage may have been due to two different reactions. During heating at pH 3.5, a hydrolysis reaction occurs in anthocyanins, whereby the compounds are converted to chalcones, which are aromatic ketones, and then further cleaved to form phenolic acids and aldehydes (Sadilova et al., 2007). This type of degradation may have occurred during thermal processing of the gummies. Another more plausible mechanism involves condensation reactions of anthocyanins with other phenolic compounds, including flavan-3-ols or polyflavan-3-ols, which may occur through direct anthocyanin-tannin reactions (Reed et al., 2005). It was hypothesized that hard gummies would better retain anthocyanins due to limited amount of water in the gummies needed for direct anthocyanin-tannin condensation reactions to occur. However, our results indicated that increased gum strength did not affect anthocyanin retention, suggesting anthocyanins and procyanidins were in close proximity to react regardless of varying moisture content in the gummies.

Total Procyanidins. Total procyanidin (PAC) content was affected by the interaction between storage temperature and time ($P < 0.0001$). Month 4 values were considered outliers due to temperature abuse that caused degradation of procyanidins in the month 4 extracts.

For gummies stored at room temperature, there was a significant loss in procyanidins from 2 to 6 months, with retention dropping to 48% after 6 months storage (Fig. 3). Procyanidin content was significantly greater in gummies stored in refrigeration, with 82% retention at 6 months. These findings indicate room temperature storage was detrimental to procyanidins, which are consistent with a study by Brownmiller et al. (2009) who reported total procyanidin retentions of 11%, 7%, and 22% in blueberry juices, purees, and canned samples after 6 months storage at room temperature. Procyanidins are known to be sensitive to polymerization reactions at higher temperatures (Spanos and Wrolstad, 1990). It is likely that procyanidins reacted with anthocyanins to form polymers, thereby decreasing both monomeric anthocyanin and procyanidin content in the gummies over time. This reaction appeared to be retarded when samples were stored refrigerated.

Color Density and Percent Polymeric Color. Color density was affected an interaction between storage treatment, gum strength, and storage time ($P = 0.02$). Hard gummies had higher color density than soft and medium gummies, with average values of 3.9, 3.5, and 3.5 in hard, medium, and soft gummies, respectively (data not shown). This was expected as monomeric color and color density are highly correlated ($r = 0.94$). Hard gummies stored under refrigeration had significantly higher color density than medium and soft gum-

mies the day after processing. Refrigerated hard gummies also had higher color density than medium gummies stored under refrigeration after 2 months of storage (Fig. 4). This suggests that at lower temperatures, the increased gelatin concentration in the hard gummy may have helped retain some color density. This may be due to the water content of the gummies, as hard gummies contained less water than medium and soft gummies. The water may have caused a dilution effect in the spectrophotometric method, resulting in higher anthocyanin content and color density values in hard gummies at month 0.

As with anthocyanins, there was a clear storage time effect on color density, which decreased over the 6 months of storage for all gum strengths (Fig. 4). After 6 months of storage, color density was lowest in gummies stored at room temperature compared to those stored under refrigeration, indicating color density was more stable at lower temperatures.

Percent polymeric color was measured to determine the amount of polymerized anthocyanins resistant to bleaching with sodium metabisulfite (Fig. 5). A higher percent polymeric color indicates the formation of anthocyanin-procyanidin polymers. Polymeric color was affected by the interaction of storage temperature and time ($P < 0.0001$). The increase in polymeric color was accompanied by a decrease in total procyanidin content ($r = -0.62$), monomeric anthocyanins ($r = -0.78$), and color density ($r = -0.68$), which agrees with findings of previous studies on black raspberry products, processed blackberry products, and processed blueberry products stored for 6 months at room temperature (Hager et al., 2008a; Hager et al., 2008b; Brownmiller et al., 2009). While correlations in this study were only moderately strong, the results of this study agreed with expected trends. Hard, medium, and soft gummies had approximately 20% polymeric color values one day after processing, but differences due to storage temperature were apparent over storage time. The polymeric color of gummies stored at room temperature increased to 35%, 43%, and 47% after 2, 4, and 6 months of storage, respectively. Gummies stored refrigerated had much lower polymeric color values, with values only increasing to 25%, 26%, and 26% after 2, 4, and 6 months of storage, respectively. These results indicate that gummies stored at room temperature had lower anthocyanin retentions as a result of the formation of anthocyanin-procyanidin polymers as is suggested by higher percent polymeric color values.

These results are not surprising, as it has been shown that a sandwich structure between anthocyanins and polyphenols can be easily formed and have high stability at lower temperatures. At higher temperatures, though, the irreversible polymer-like structure of these two compounds forms, which is brown in color, increasing percent polymeric color (Kunsági-Máté et al., 2011). It is not known whether these polymers are readily absorbed *in vivo* to provide antioxidant benefits, as they are most likely large molecular weight compounds. However, it has been suggested that enzymes in the gastrointestinal tract can degrade phenolic compounds into more readily absorbed metabolites, which could provide health benefits upon absorption (Selma et al., 2009). Because the gummies contained sugar, it is possible that the Maillard Browning reaction could have taken place, with the products formed also contributing to increased polymeric color values.

SUMMARY

Anthocyanins and procyanidins were more susceptible to polymerization in gummies stored at room temperature than under refrigeration. Encapsulation by gelatin in hard gummies did not affect anthocyanin and procyanidin degradation over storage time. More research is needed to determine the bioavailability of the anthocyanin-procyanidin polymers formed and to identify treatments to prevent their formation. Chokeberry-containing products should be stored at refrigerated temperatures to better retain beneficial polyphenols.

ACKNOWLEDGEMENTS

Financial support for this project was provided by an Arkansas Student Undergraduate Research Fellowship and a University of Arkansas Dale Bumpers College of Agricultural, Food and Life Sciences Undergraduate Research Grant. I would personally like to thank Dr. Luke Howard, Cindi Brownmiller, Brittany White, and Jeana Boucher for all of their help and guidance over the last couple of years. I could not have completed my thesis without you!

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Table 1. Formulations for soft, medium, and hard gummies.

	Gum Strength					
	Soft		Medium		Hard	
Ingredient	Amount	%	Amount	%	Amount	%
Aronia	100 mL	25.397	100 mL	25.397	100 mL	25.397
Sugar	187.5 g	47.619	187.5 g	47.619	187.5 g	47.619
Splenda	5 g	1.270	5 g	1.270	5 g	1.270
Potassium sorbate	0.0985 g	0.025	0.0985 g	0.025	0.0985 g	0.025
Water	75.175 mL	19.092	69.9 mL	17.752	64.9 mL	16.483
Gelatin	25.975 g	6.597	31.25 g	7.937	36.25 g	9.206
Total		100		100		100

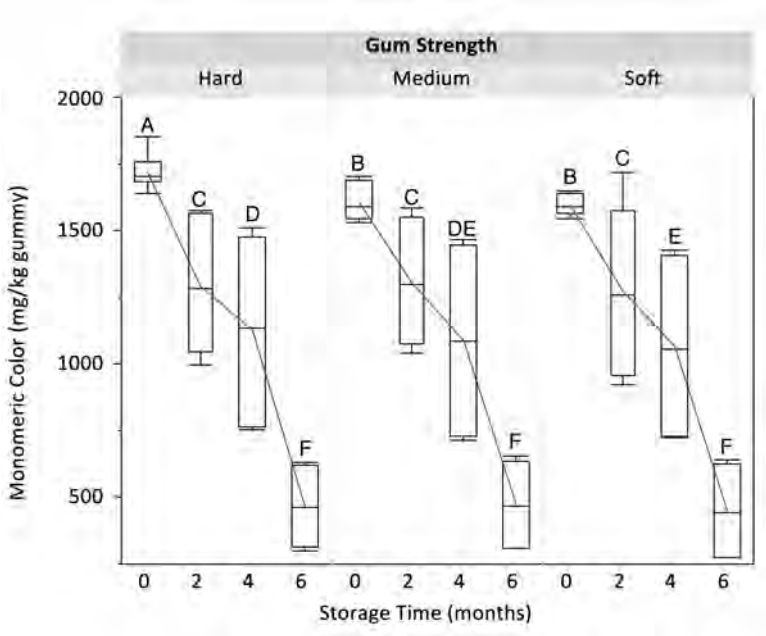

Fig. 1. Total monomeric anthocyanin content of chokeberry gummies of various gum strengths over storage time of 6 months. Letters indicate significant differences in total monomeric anthocyanin content among treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

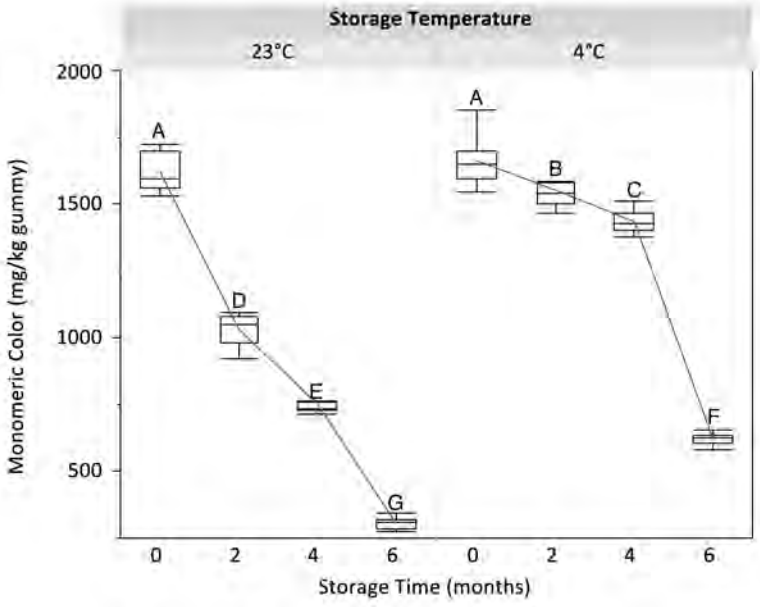

Fig. 2. Total monomeric anthocyanin content of chokeberry gummies stored at refrigerated and room temperatures for 6 months. Letters indicate significant differences in total monomeric anthocyanin content among treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

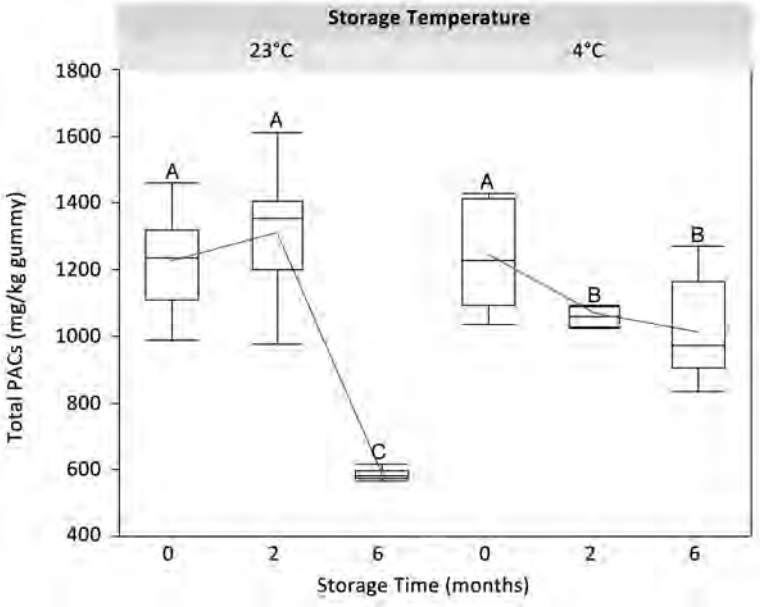

Fig. 3. Total procyanidins in chokeberry gummies stored at refrigerated and room temperatures at 0, 2, and 6 months. Letters indicate significant differences in total procyanidin content among treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

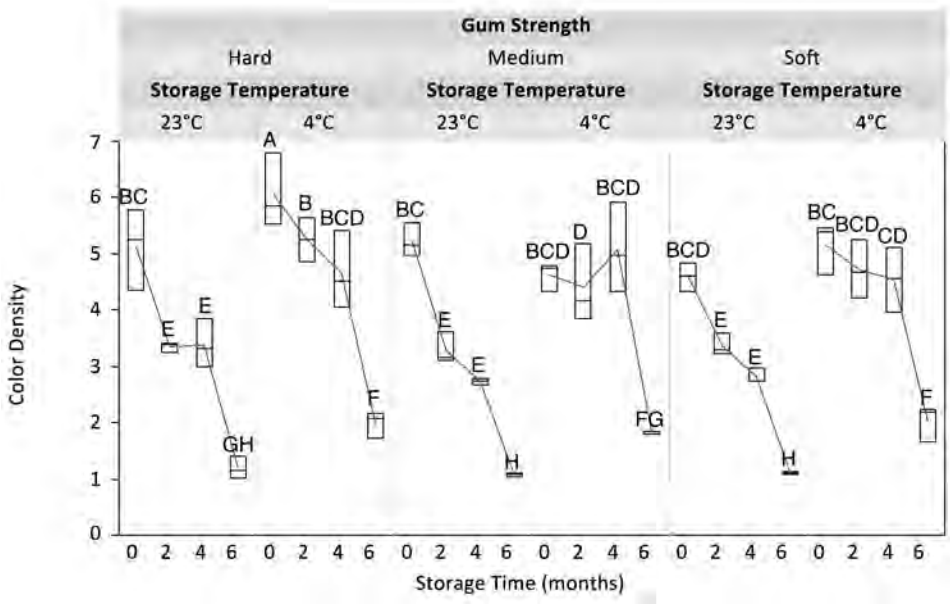

Fig. 4. Color density values for hard, medium, and soft gummies at various storage times and temperatures. Letters indicate significant differences in color density among treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.

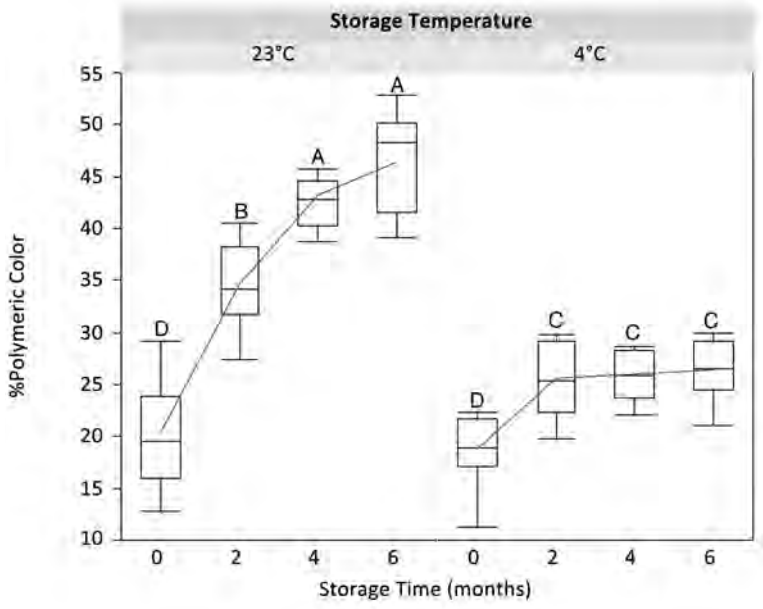

Fig. 5. Percent polymeric color values of chokeberry gummies at various storage times and temperatures. Letters indicate significant differences in percent polymeric color among storage treatments (Student's *t*-test, $P < 0.05$). The bottom and top ends of each box represent the 25th and 75th quantiles, respectively.