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Abstract

This study evaluated the stability of anthocyanins in six blueberry products (gummy, graham bar, oatmeal bar, rice krispy bar, ice pop and juice) prepared with freeze-dried wild blueberry powder during processing and over eight weeks storage. Total anthocyanins were determined by HPLC before processing and at day 0 and 2, 4, 6, and 8 weeks of storage. Thermal processing of gummy and graham bar products resulted in significant losses of anthocyanins (50% and 31%, respectively). An eight-week storage time also resulted in a significant decrease in anthocyanins (7% to 51%) in products stored at ambient temperature. The ice pop, which was stored at -20^{o}C, was the best product for shelf-stability as it experienced no significant decline in total anthocyanins during processing or over the entire shelf-life study. Future research should be conducted to determine the differences in total anthocyanins in the products over time when they are stored under refrigeration. Additionally, polymeric color should be analyzed as this indicator has the potential to further explain the nature of the decreases in anthocyanins observed during storage.

Keywords: anthocyanins; HPLC; blueberry; storage; thermal processing

Introduction and Literature Review

Diet during childhood can influence bone mineral density and susceptibility to chronic disease later in life (Chen et. al., 2010; Gilsanz & Wren, 2007). Population-based studies have shown fruit and vegetable intake is an independent predictor of bone size in children and may contribute to the building of bone mass (Novotny et. al., 2004; Tylavsky et. al., 2004; Lanham, 2006). Unfortunately, many children fail to consume the recommended amount of fruits and vegetables (Kuntz et. al., 2015). The recent popularity of consuming fruits and vegetables in prepared forms is worth exploring as a means to increase the consumption of health-promoting compounds, like the group of polyphenols called anthocyanins in blueberries (Kuntz et. al., 2015). Additionally, anthocyanin-rich products are needed for human clinical trials that require subjects to consume large doses of anthocyanins per day.

Anthocyanins, a class of polyphenols are responsible for the brilliant red, blue, purple and black colors of fruits and vegetables. The six major anthocyanins found in fruits and vegetables, delphinidin, cyanidin, petunidin, peonidin, pelargonidin, and malvidin vary according the substituents (OH or OCH_3) attached at positions R_1 and R_3 on the B ring (Figure 1). Anthocyanins in nature almost always have a sugar (glycoside) or multiple sugars attached at carbon three on the middle heterocyclic ring. Additionally, phenolic acids such as coumaric or caffeic acid, or organic acids such as acetic or malonic acid can be attached to the sugars resulting in the formation of acylated anthocyanins.

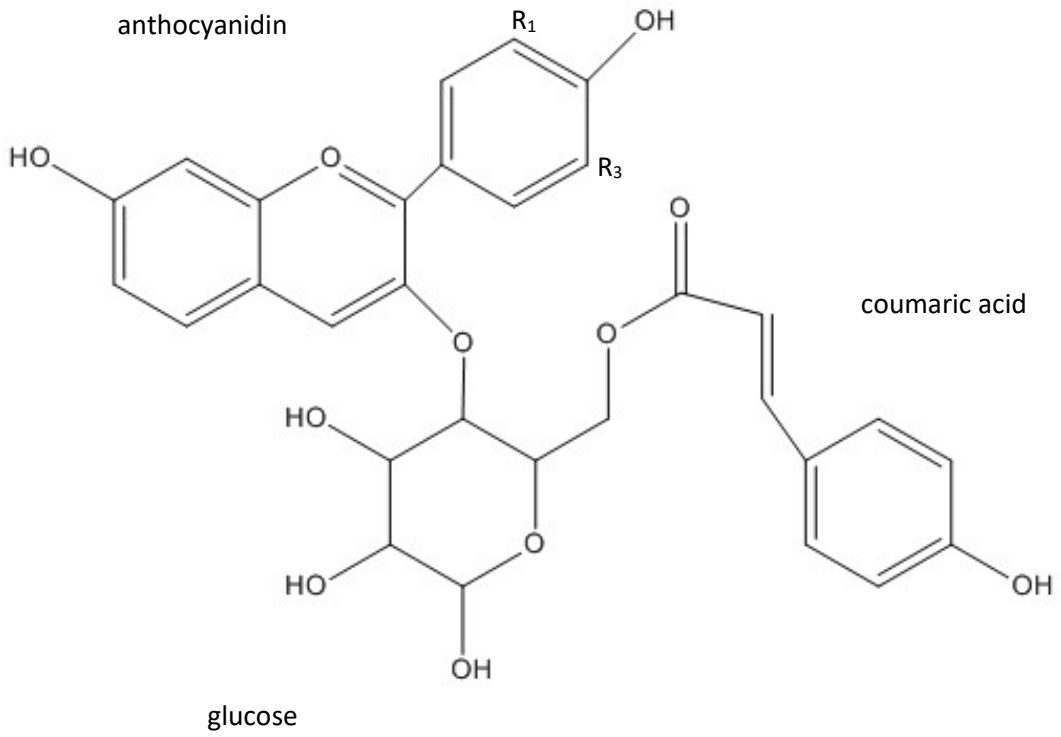

Figure 1 Skeletal structure of an acylated anthocyanin.

The anthocyanin content of berries varies distinctly from one type of berry to another (Lee, Kim, Soung, Vance & Lee, 2015). It is thought that, since the composition of anthocyanins in berries affects their bioavailability and anti-oxidant affects, different anthocyanin-containing berries may affect the same cells differently (Lee, 2015). Blueberries from various sources have been found to contain 20-27 different anthocyanins (Wu & Prior, 2005). Wild blueberries are an especially good source of the anthocyanins petunidin and malvidin, delivering 87.6 mg/100g and 154.6 mg/100g, respectively (Wu et. al., 2006).

The average daily intake of raw blueberries has been estimated to be 0.93 g, yielding 3.39 mg of anthocyanins (Wu et. al., 2006). Intakes of greater than 100 mg of anthocyanins per day could easily be achieved with the regular consumption of blueberries (Wu et. al., 2006). In an *in*

vitro study, malvidin-3-glucoside, one of the major monoglucosides in wild blueberries, was found to be significantly more effective at inhibiting pro-inflammatory genes than epicatechin or chlorogenic acid (Esposito, Chen, Grace, Komarnytsky & Lila, 2014).

Degradation and loss of anthocyanins during processing is of great concern when developing processed blueberry products. Conditions during thermal processing must be considered to ensure the biological activity of anthocyanins is retained in a thermally processed product (Rodriguez-Mateos et. al., 2014). Thermal processing has been found to result in total monomeric anthocyanin losses of 28% to 59% in canned, pureed, and juiced blueberry products (Brownmiller, Prior, & Howard, 2008). Some thermal processing methods are significantly better at preserving anthocyanin content than others. For example, Rodriguez-Mateos and coworkers employed a short baking time of twelve minutes, and a relatively low temperature of 180˚C to develop a baked bun containing freeze-dried blueberry powder (Rodriguez-Mateos et. al., 2014). The anthocyanins in the buns following baking were well-preserved compared to the freeze-dried blueberry powder before thermal processing (Rodriguez-Mateos et. al., 2014).

The objective of this study was to evaluate the stability of anthocyanins in six blueberry products (gummy, graham bar, oatmeal bar, rice krispy bar, ice pop and juice) prepared with freeze-dried wild blueberry powder during processing and over eight weeks storage. A storage period of 8 weeks was selected for this study as it is a valid storage time for products of this type sold commercially.

Materials and Methods

The freeze-dried powder used in the products and as a comparison was HiActives® North American wild blueberry powder 1.5% (FutureCeuticals, Momence, IL, USA). Prepared blueberry

products were previously formulated, produced, and packaged by others in the lab before the start of the shelf-life study. One sample was taken from each of three packages of each product (gummy, graham bar, oatmeal bar, rice krispy bar, ice pop and juice) at each timepoint during the shelf-life study for a total of three samples per product per timepoint. These include: day 0 (immediately after processing) and after 2, 4, 6, and 8 weeks of storage at 21^{o}C (except for the ice pop which was frozen and stored at -20^{o}C) for anthocyanin analysis.

Anthocyanin Extraction and Sample Preparation

Samples of each food product, gummy, graham bar, oatmeal bar and rice krispy bar, excluding the ice pop and juice, were first homogenized with methanol/water/formic (60:37:3, v/v/v) acid solvent using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp., Mason, Ohio, U.S.A.) (Brownmiller et. al., 2008). Homogenized samples were then filtered through Miracloth (Calbiochem, LaJolla, CA, USA) and the filtrates were adjusted to a volume of 150 mL in a volumetric flask (Brownmiller et. al., 2008). The 150 mL filtrate solutions were then poured into beakers to ensure adequate mixing of the filtrates and then transferred from the beakers into labeled 50-mL test tubes. From the 50-mL test tubes, 5 mL was pipetted into separate labeled 50-mL test tubes. The test tubes containing 5 mL of filtrate solution were dried overnight in a Speed Vac® concentrator (ThermoSavant, Holbrook, NY, USA) and then reconstituted with 1 mL of aqueous formic acid solution (Cho, Howard, Prior & Clark, 2004).

Ice pop and juice samples did not require the same extraction method as the other product samples. Both pasteurized and unpasteurized juice samples were evaluated to measure the anthocyanin losses during thermal processing of the juice. The ice pop and juice samples were diluted (200 μL ice pop or juice + 800 μL 5% formic acid) in glass test tubes prior to HPLC

analysis. The reconstituted extracted solutions from the products and the diluted juice and ice pop samples were each passed through 25 mm syringe filters with 0.45 μm nylon membranes (VWR International™, Radnor, PA, USA) before analysis by HPLC.

High-Performance Liquid Chromatography

Chromatographic analysis was performed using an established HPLC method to measure the content of individual anthocyanin monoglycosides (Brownmiller et. al., 2008). Anthocyanin analysis by HPLC was performed according to the method of Cho and coworkers with a 50-μL injection volume of samples using a Waters HPLC system (Waters Corp, Milford, MA, USA) fitted with a model 600 pump, 717 Plus autosampler, and a model 996 photodiode array detector (Cho et al., 2004). For separation, a 4.6 mm x 250 mm Symmetry® C18 column (Waters Corp, Milford, MA, USA) and a 3.9 mm x 20 mm Symmetry® C18 column (Waters Corp, Milford, MA, USA) were utilized (Cho et al., 2004). A linear gradient of 5% formic acid and methanol was used for the mobile phase (Cho et al., 2004). Detection wavelength used for the anthocyanins was 510 nm (Cho et al., 2004). Identification of the anthocyanins in the freeze-dried blueberry powder was previously performed by HPLC-MS. Individual anthocyanin glycosides were quantified as delphinidin, cyanidin, petunidin, peonidin, and malvidin glucoside equivalents using external calibration curves of a mixture of the five anthocyanin glucosides. The concentrations of individual anthocyanins were summed and reported as total anthocyanins. Results are expressed as total mg anthocyanins per gram of blueberry powder in each product.

Statistical Analysis

JMP® statistical software by SAS Institute Inc. was utilized for statistical analysis. One-way ANOVA with mean comparison by student's *t*-test was used to determine significant

differences ($p < 0.05$) in average total mg anthocyanins per gram of blueberry powder in each product during the eight-week shelf-life study to measure anthocyanin stability over time. Significant differences ($p < 0.05$) in average total mg anthocyanins per gram of blueberry powder in each product at day 0 were also compared to average total mg anthocyanins per gram of blueberry powder to evaluate the stability of anthocyanins during processing of the products using a student's t-test.

Results

Effects of Processing

Table 1 Total anthocyanin content of prepared blueberry products

Product	Average mg anthocyanins /g blueberry powder in product	Decrease in total anthocyanins measured (%)
Powder[a]	19.55 ± 1.6[b]	Reference
Gummy	9.7 ± 0.7	50%
Graham bar	13.5 ± 0.7	31%
Rice krispy bar	16.9 ± 1.7	NS[c]
Oatmeal bar	19.2 ± 0.6	NS
Juice, NP[d]	21.81 ± 0.4	NS
Juice, P[e]	21.44 ± 1.3	NS
Ice pop	22.6 ± 0.3	NS

[a]Blueberry powder used to prepare blueberry products
[b]Values represent means (n=3) ± SEMs
[c]NS = no significant difference ($p > 0.05$)
[d]NP = not pasteurized
[e]P = pasteurized

Compared to the unprocessed blueberry powder, there was no significant difference ($p > 0.05$) in the average mg anthocyanins per gram of blueberry powder in any of the prepared products at day 0 (after processing), except for the graham bar and the gummy. The juice also experienced no significant decrease ($p > 0.05$) in total anthocyanins during pasteurization. The

graham bar contained 31% less anthocyanins after processing and the gummy contained 50% less anthocyanins after processing.

Gummy

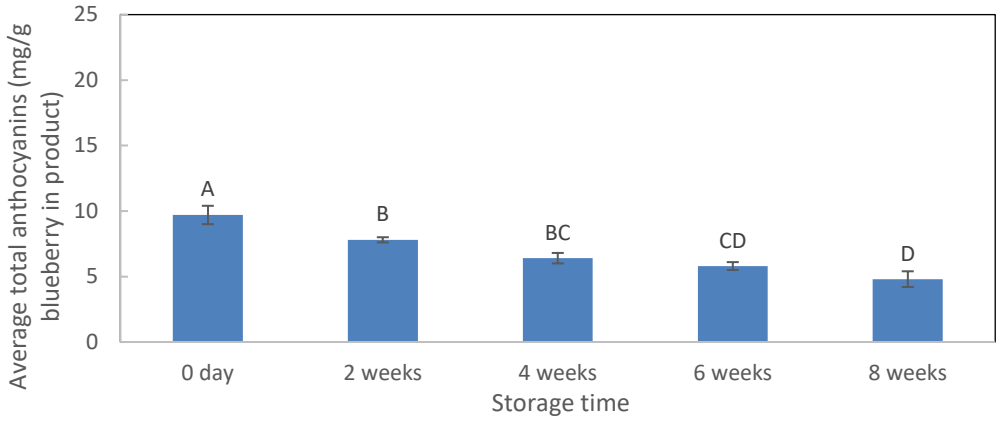

Figure 2 Average total anthocyanins in gummy during an eight-week shelf-life study. Values are mean total anthocyanins in mg/g blueberry in product ± SEMs. Bars with different letters are significantly different ($p < 0.05$).

The gummy experienced a relatively linear decrease in total anthocyanins during the shelf-life study. Between day 0 and week 8, a 51% decrease in anthocyanins was measured.

Graham Bar

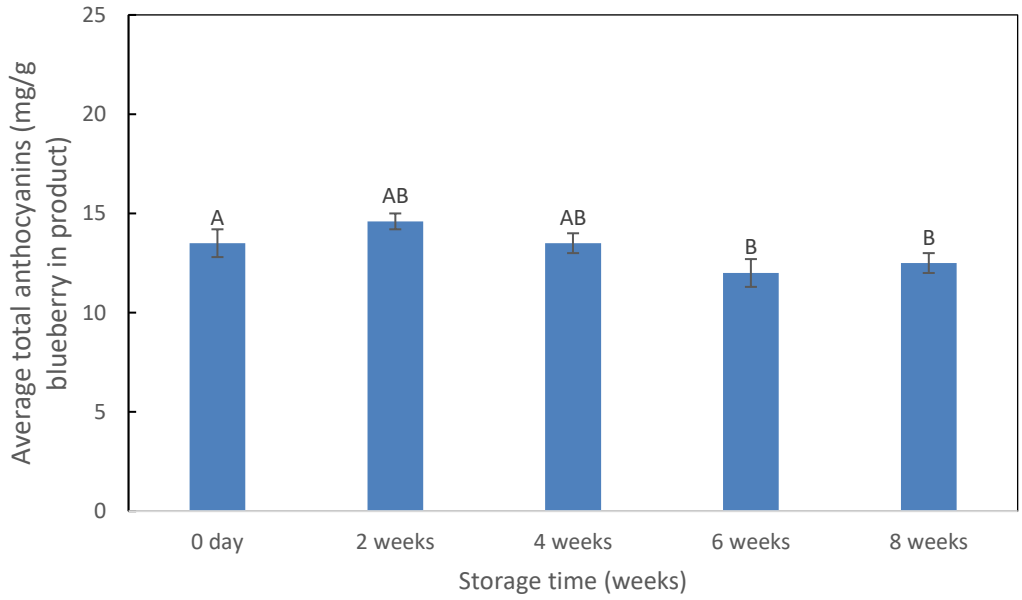

Figure 3 Average total anthocyanins in graham bar during an eight-week shelf-life study. Values are mean total anthocyanins in mg/g blueberry in product ± SEMs. Bars with different letters are significantly different ($p < 0.05$).

The total anthocyanins in the graham bar remained relatively stable from day 0 to week 8. There was no significant decline in anthocyanins until week 6 and a 7% decrease in anthocyanins was measured between day 0 and week 8.

Rice Krispy Bar

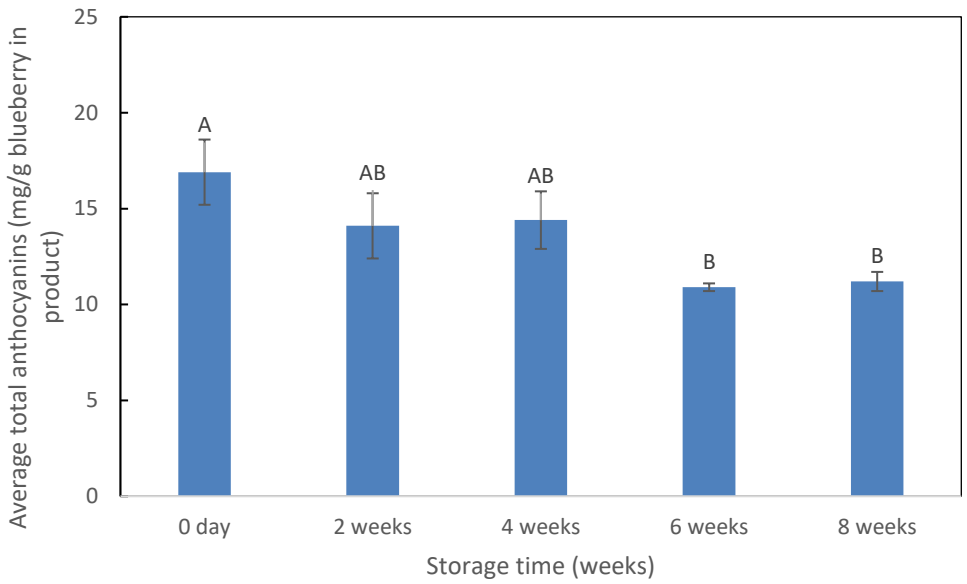

Figure 4 Average total anthocyanins in rice krispy bar during an eight-week shelf-life study. Values are mean total anthocyanins in mg/g blueberry in product ± SEMs. Bars with different letters are significantly different ($p < 0.05$).

Total anthocyanins in the rice krispy bar at day 0 and week 6 and 8 were significantly different ($p < 0.05$). From day 0 to week 8 there was a 34% decrease in total anthocyanins.

Oatmeal Bar

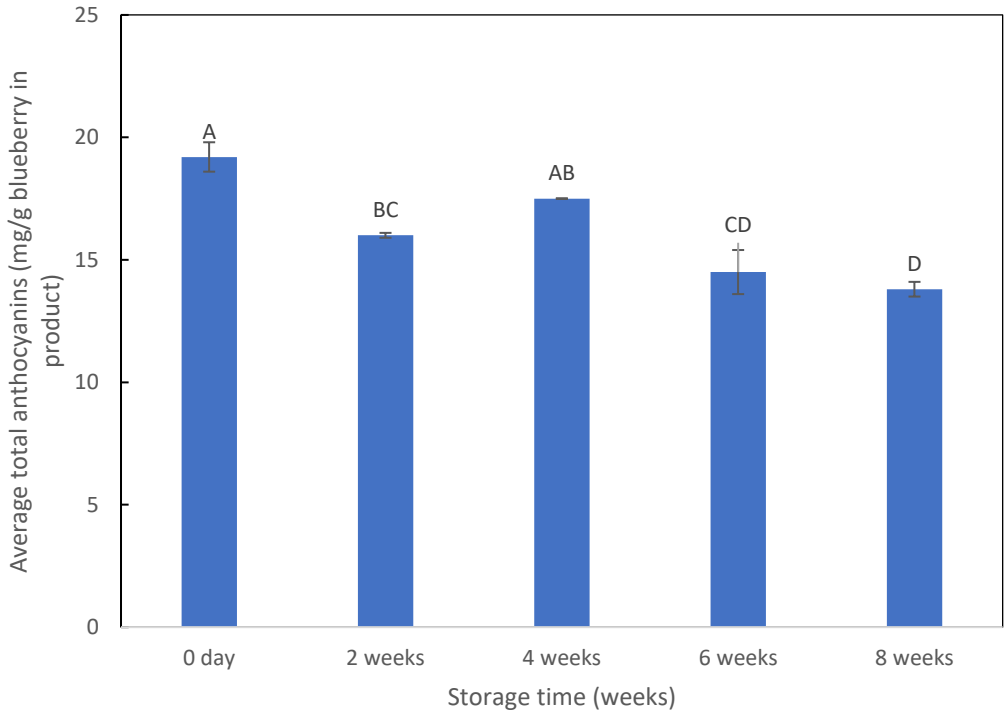

Figure 5 Average total anthocyanins in oatmeal bar during an eight-week shelf-life study. Values are mean total anthocyanins in mg/g blueberry in product ± SEMs. Bars with different letters are significantly different ($p < 0.05$).

Total anthocyanins decreased gradually over the course of the shelf-life study. From day 0 to week 8, a 28% decrease in total anthocyanins was observed.

Juice

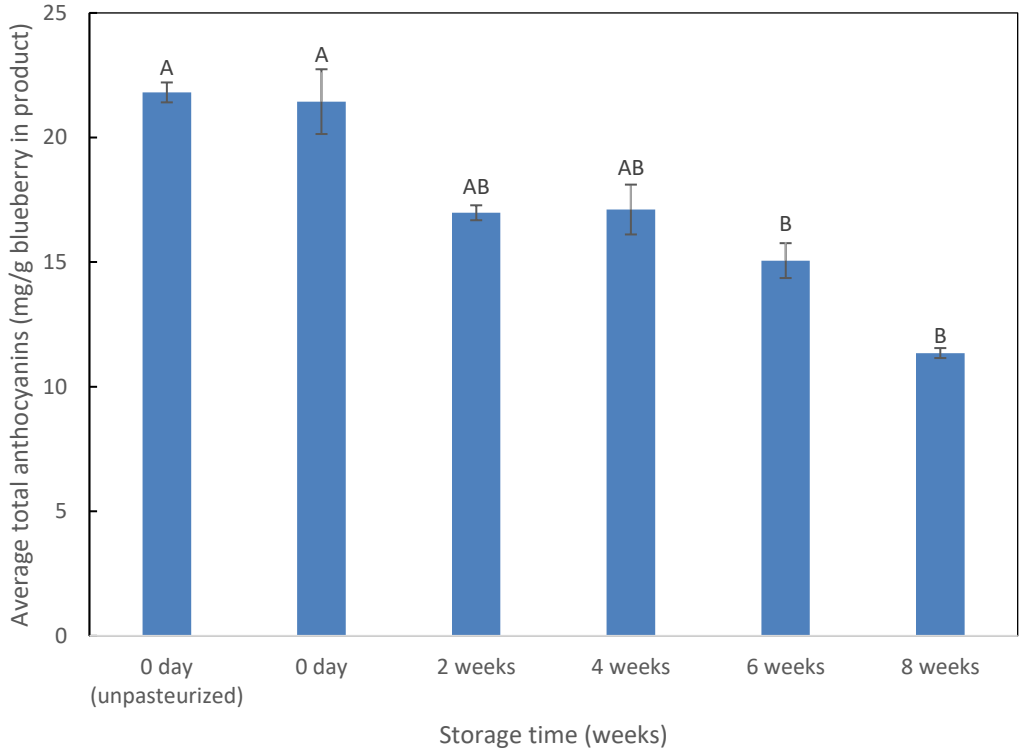

Figure 6 Average total anthocyanins in juice during an eight-week shelf-life study. Juice from day 0 to week 8 was pasteurized. Values are mean total anthocyanins in mg/g blueberry in product ± SEMs. Bars with different letters are significantly different ($p < 0.05$).

No significant decrease ($p > 0.05$) in anthocyanins in the juice was measured until week 6 of the shelf-life study. Between day 0 and week 8, the pasteurized juice experienced a 47% decrease in total anthocyanins.

Ice Pop

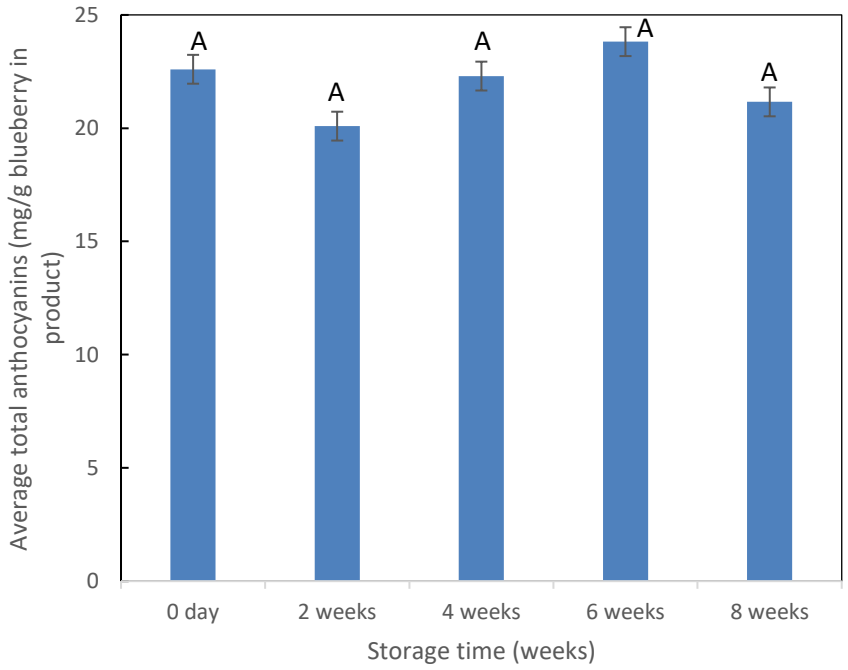

Figure 7 Average total anthocyanins in ice pop during an eight-week shelf-life study. Values are mean total anthocyanins in mg/g blueberry in product ± SEMs. No means were significantly different ($p > 0.05$).

The anthocyanins in the ice pop were stable with no significant change ($p > 0.05$) observed between day 0 and week 8.

Table 2 Total anthocyanin content of blueberry products over eight weeks of storage at 21°C

Product	Storage time (weeks)	Total anthocyanins, mg/g blueberry powder in product
Gummy	0 day	9.7 ± 0.7[d]
	2 weeks	7.8 ± 0.2
	4 weeks	6.4 ± 0.4
	6 weeks	5.8 ±0.3
	8 weeks	4.8 ± 0.6
Graham bar	0 day	13.5 ± 0.7
	2 weeks	14.6 ± 0.4
	4 weeks	13.5 ± 0.5
	6 weeks	12.0 ± 0.7
	8 weeks	12.5 ± 0.5
Rice krispy bar	0 day	16.9 ± 1.7
	2 weeks	14.1 ± 1.7
	4 weeks	14.4 ± 1.5
	6 weeks	10.9 ± 0.2
	8 weeks	11.2 ± 0.5
Oatmeal bar	0 day	19.2 ± 0.6
	2 weeks	16.0 ± 0.1
	4 weeks	17.5 ± 0.0
	6 weeks	14.5 ± 0.9
	8 weeks	13.8 ± 0.3
Juice	0 day, NP[b]	21.8 ± 0.4
	0 day[c]	21.4 ± 1.3
	2 weeks[c]	17.0 ± 0.3
	4 weeks[c]	17.1 ± 1.0
	6 weeks[c]	15.1 ± 0.7
	8 weeks[c]	11.4 ± 0.2
Ice pop[a]	0 day	22.6 ± 0.3
	2 weeks	20.1 ± 0.9
	4 weeks	22.3 ± 1.0
	6 weeks	23.8 ± 2.0
	8 weeks	21.2 ± 1.1

[a]Ice pop was stored at -20°C
[b]NP=Not pasteurized
[c]Pasteurized
[d]Values represent means (n=3) ± SEMs

Discussion

The anthocyanin contents of the prepared food products were compared to that of the freeze-dried blueberry powder used to prepare the products. Because there was no significant difference ($p > 0.05$) in the average mg anthocyanins per gram of blueberry powder in any of the products except for the graham bar and the gummy when compared to the unprocessed blueberry powder, it appeared the methods used to produce the oatmeal bar, rice krispy bar, ice pop, and juice (both pasteurized and unpasteurized) do not cause significant losses of anthocyanins.

The gummy was notably the least stable of all the products analyzed, experiencing a 50% decrease in total anthocyanins during processing and then another 51% decrease by week 8 of the shelf-life study. With the gummy only appearing to contain approximately 25% of the anthocyanins present in the blueberry powder used to formulate it, it does not seem to be as viable of an option for consistent delivery of anthocyanins compared to the other products. Additional research is needed to determine why the anthocyanins were unstable during gummy processing and storage.

The graham bar also experienced a significant 31% decrease in total anthocyanins during processing. Unlike the gummy, the graham bar remained relatively stable over time with no significant decrease until week 6 and only a 7% decrease in total anthocyanins during the entire shelf-life study. While the rice krispy bar did not experience a significant decrease in anthocyanins during processing, it experienced a 34% decrease in anthocyanins during the shelf-life study. The oatmeal bar also experienced a moderate 28% decrease in anthocyanins from day 0 to week 8.

While the thermal processing (pasteurization) of the juice did not significantly decrease the total anthocyanins measured in the product, the anthocyanins declined by 47% over eight weeks of storage. The ice pop was by far the most stable of the products evaluated. There was no significant decline in total anthocyanins during processing or the entire shelf-life study. The stability of the anthocyanins is likely due to the fact the ice pop was stored frozen rather than at ambient temperature like the other products. Freezing presumably suppressed the mechanism(s) responsible for loss of anthocyanins observed in products stored at -20^{o}C.

While a decrease in total anthocyanins was measured in several of the products during processing and storage, the question remains about whether the decrease was due to actual losses as a result of thermal degradation or conversion of anthocyanins into polymerized forms. Previous research found that, while some of the decrease in anthocyanins in blueberry products is due to thermal degradation during processing and storage, polymerization of anthocyanins with other anthocyanins and phenolic compounds also occurs (Brownmiller et. al., 2008). Furthermore, the ability of the blueberry products to scavenge peroxyl radicals (hydrophilic antioxidant capacity) was not found to decline as significantly as total anthocyanin content over storage (Brownmiller et. al., 2008). This suggests these blueberry products likely do not experience as significant of a decline in bioactivity as the decrease in measured total anthocyanins (Brownmiller et. al., 2008). The authors postulated that anthocyanins formed polymeric structures with procyanidins during storage, and these polymeric compounds retained potent hydrophilic antioxidant capacity (Brownmiller et al., 2008).

Conclusions

Thermal processing of gummy and graham bar products resulted in significant losses of anthocyanins (50% and 31%, respectively). An eight-week storage time also resulted in a significant decrease in anthocyanins (7% to 51%) in products stored at ambient temperature, except for the ice pop, which was stored at -20°C. The ice pop was the best product for shelf-stability as it experienced no significant decline in total anthocyanins during thermal processing or the entire shelf-life study. Future research should be conducted to determine the differences in total anthocyanins in the products over time when they are stored under refrigeration. Additionally, polymeric color should be analyzed as this indicator has the potential to further explain the nature of the decreases in anthocyanins that were observed.

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