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The Effect of Freezing as a Storage Method on Anthocyanin Concentration in Blueberries

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Abstract

Blueberries are rich in a water-soluble class of pigments known as anthocyanins which are known antioxidants. Antioxidants help prevent many diseases by stabilizing free radicals, but are prone to losses in food during storage. The goal of this study was to test the effect of freezing as a storage method on anthocyanin concentration and antioxidant activity over time. Blueberries from Canada and Argentina were frozen for up to 5 months and periodically tested for anthocyanin concentration and antioxidant activity. Anthocyanins were extracted using a mixture of methanol, acetic acid, and water, and evaporation under vacuum. They were separated using column chromatography. The concentration was determined by absorbance at 538 nm and the Beer-Lambert law, and antioxidant activity was measured using absorbance at 515 nm and DPPH free radical. Anthocyanin concentration ranged from 3.32 ± 0.40 mg/g in fresh berries to 8.89 ± 3.56 mg/g in berries frozen for 133 days. Concentration directly correlated with the antioxidant activity of anthocyanin on DPPH free radical in that 55.37 and 39.07% DPPH remained after 2 hours of antioxidant/DPPH reaction for the fresh and 133 day samples, respectively. Country of origin did not appear to impact anthocyanin concentration but did play a role in how the anthocyanin reacted with the free radical. Freezing appears to be an acceptable form of storing blueberries for up to 66 days (about 2 months) depending on ice crystal formation.

INTRODUCTION

Anthocyanins are a group of water-soluble pigments responsible for the red, blue, and purple color of many fruits and vegetables including grapes, currants, red cabbage, eggplant, and berries, among others. In fact, anthocyanins make up the largest group of water-soluble pigments in the entire plant kingdom (Navas, Jimenez-Moreno, Bueno, Saez-Plaza, & Asuero, 2012). In addition, these anthocyanins are known antioxidants in that they act as scavengers to stabilize damage-causing free radicals. As a result, they are important in the prevention of many diseases such as cardiovascular disease, diabetes, and cancer (Lohachoompol, Srzednicki, & Craske, 2004). Anthocyanins are also known to help lower cholesterol and may even play a role in improving bone density (Wood, 2011). Given that these pigments are produced in plants and not synthesized in the human body, it is important that they are consumed in the diet (Leong & Oey, 2012).

Blueberries, a fruit commonly consumed in the United States, happen to be a rich source of anthocyanins. In fact, blueberries have one of the most complex anthocyanin profiles with over 25 unique fractions of which cyanidin 3-rutinoside is the most dominant (Navas et al., 2012). Given the incredible nutrition potential of anthocyanins, blueberries have become known as a powerful health food, and Americans are eating three times more of them today than they did 10 years ago (Wood, 2011). In order to optimize the health benefits consumers receive from eating blueberries, it is important to find the best processing and storage conditions that maintain anthocyanin quality.

Processing is known to have detrimental effects on the nutritional quality of sensitive nutrients, especially antioxidants like anthocyanins. For example, pasteurizing or concentrating blueberry juice significantly decreases anthocyanin content and may also change its antioxidant activity. Needless to say, most of the anthocyanins stay in the fruit pulp and are not transferred to the juice (Smith & Charter, 2010). In contrast, processing can also be used to increase the nutritional quality in some foods (Allaith, Ahmed, & Jafer, 2011).

With this in mind, the goal of this study was to test the effect of freezing as a storage technique on the anthocyanin concentration and antioxidant activity in blueberries over a period of months to see if it differed from fresh blueberries. Freezing is already a common storage technique in the United States where blueberries are usually imported from other countries when not in season (during the winter months). This is a reflection of the knowledge that huge losses in water-soluble vitamin and antioxidant content occur over time, so importing fresh berries is not always beneficial (Ketata, Desjardins, & Ratti, 2012). As a result, the hope for this study is that freezing will lock the water-soluble vitamins and antioxidants into the berry as the water in the plant tissue is rendered immobile. This will hopefully decrease losses in anthocyanin concentration and maintain antioxidant activity.

MATERIALS AND METHODS

Fresh blueberries from Canada and Argentina were frozen at -20°C for up to 5 months. Samples were taken about every month for anthocyanin extraction to measure anthocyanin concentration and kinetic activity as per Lohachoompol et al. (2004).

Anthocyanin Extraction

20 g of thawed blueberry sample was blended with 75 mL methanol/acetic acid/distilled water (25:1:24) for 1 min. The mixture was then centrifuged at 3,750 rpm and 20°C for 40 min, and the supernatant was dried under vacuum at 35°C. The dried residue was re-dissolved with 5 mL 3% formic acid in water and adsorbed onto a C18 Sep-Pak cartridge. The column was washed with 5 mL 3% formic acid in water and eluted with 3.5 mL 3% formic acid in methanol. The eluted anthocyanin was then dried under vacuum at 35°C. This adsorption of the anthocyanin residue was critical in purifying the desired fraction of anthocyanin: cyanidin 3-rutinoside, which is the dominant anthocyanin in blueberries (Navas et al., 2012).

Determination of Total Anthocyanins

The dried anthocyanin was re-dissolved with 25 mL methanol/0.1 M hydrochloric acid (85:15) and then diluted as needed with methanol/0.1 M hydrochloric acid to read the absorbance at 538 nm (usually 10^{-1}). The absorbance at 538 nm was used to calculate the anthocyanin concentration by the Beer-Lambert law:

$$C = \frac{A}{\varepsilon l} \times MW \times Dilution\ factor$$

Anthocyanin concentration was expressed as cyanidin 3-rutinoside (mg/g) which has a molar absorptivity of 31,085 at 530 nm, and its molecular weight is 631 g/mol. The cuvette length was 1 cm and the dilution factor ranged from 250-350 depending on the sample.

Antioxidant Activity

0.1 mL of diluted anthocyanin was put into a cuvette with 3.9 mL of DPPH free radical (6×10^{-5} mol/L DPPH in methanol). The absorbance was read at 515 nm at 0 min, 1 min, 5 min, and every 5 min for 2 hours, or until the absorbance leveled off. The amount of DPPH remaining after 2 hours was calculated as a percent of the initial amount present based on the absorbance at 515 nm.

Statistical Analysis

Two to four replicates were performed for each sample, and concentration results were expressed as mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

As mentioned, the absorbance at 538 nm and the Beer-Lambert law were used to determine the concentration of anthocyanin in the blueberry extract. The respective concentrations for each sample of berries are shown here in **Table 1**.

Table 1. Anthocyanin concentration as mg/g cyanidin 3-rutinoside.

Sample	Avg. Concentration (mg/g)
Fresh (Argentina)	3.32 ± 0.40
Frozen for 27 Days	4.74 ± 1.75
Frozen for 34 Days	2.83 ± 1.78
Frozen for 66 Days (Argentina)	2.15 ± 0.03
Frozen for 105 Days	6.72 ± 0.26
Frozen for 133 Days	8.89 ± 3.56

Overall, the anthocyanin concentration increased over time. Every concentration from the frozen berries was higher than that for the fresh except for the samples taken at 34 and 66 days. This general increase over time was most likely the result of ice crystal formation (freezer burn) which began around 66 days, or about 2 months. Because anthocyanins are water-soluble, they were most likely removed from the flesh of the blueberry and locked in the ice crystals. As a result, when the blueberries were thawed prior to extraction, the anthocyanins were probably leeched from the berry, thus increasing the amount found in the extract. Larger ice crystals are more destructive and usually form when freezing is slow and not at a low enough temperature. This would increase leeching upon thawing. As an example, **Figure 1** below shows the difference in appearance between berries frozen for 140 days (product of Argentina) and berries frozen for 179 days (product of Canada). Note the more shriveled appearance of the berries frozen for 179 days.

Figure 1. Thawed blueberries after freezing for 140 days (top) versus 179 days (bottom).

Although freezer burn appeared to play a major role in the anthocyanin concentration change, the structural differences between different anthocyanin fractions may have also been involved. In addition, how these structurally different anthocyanins interact with each other and other compounds in the blueberry tissue may also have affected the anthocyanin concentration (Allaith et al., 2011).

Furthermore, the data were all from the Canadian blueberries except for the fresh and 66 day data which were from those from Argentina. This difference in origin did not prove to be significant in terms of concentration and did not explain the drop in concentration at both 34 and 66 days. The drop at 34 days may have been caused by improperly dividing the duplicates (leading to the larger standard deviation), while the drop at 66 days may have simply been due to a poor extraction. For example, the blueberry tissue may not have been blended enough, or more methanol/acetic acid/water may have been needed. Again, structural differences in anthocyanin fractions may have been responsible for any differences between the blueberries from Canada and Argentina, but country of origin did not appear to affect the change in anthocyanin concentration.

On the other hand, in terms of antioxidant activity, the absorbance values from the anthocyanin/DPPH reaction were used to estimate the antioxidant kinetics of the reaction. **Figures 2** and **3** are the graphical representation of each sample's reaction shown as absorbance over time. The absorbance values used in the graphs were averages of the duplicates for each (2-4 duplicates were done). Also, the absorbance scales differ between the two graphs because a higher concentration of DPPH was unintentionally used for those in the second graph.

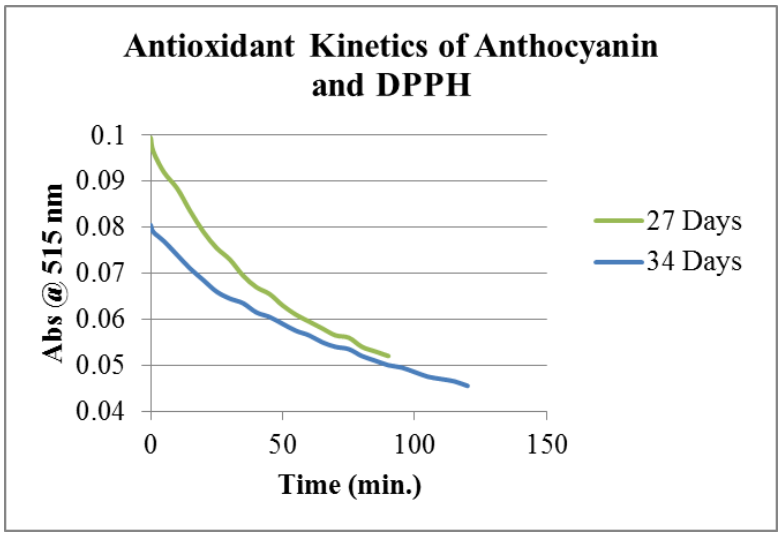

Figure 2. Antioxidant kinetics of anthocyanin in terms of absorbance of the anthocyanin/DPPH reaction over 2 hours for blueberries frozen for 27 and 34 days.

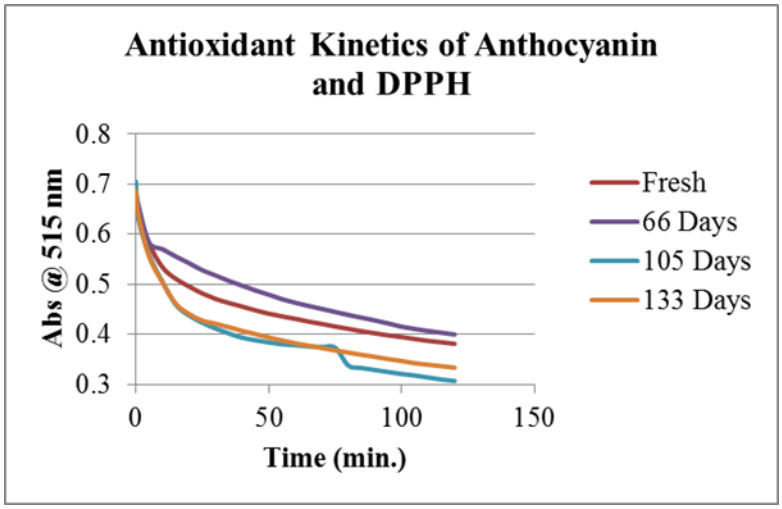

Figure 3. Antioxidant kinetics of anthocyanin in terms of absorbance of the anthocyanin/DPPH reaction over 2 hours for fresh blueberries and berries frozen for 66, 105, and 133 days.

Using the above graphs, the slope of each trend line was found and used as an indicator of the rate of the reaction. A steeper slope indicated a faster reaction which was expected with higher anthocyanin concentration. In addition, the amount of DPPH free radical remaining after the 2 hour reaction was determined as a percent of the initial amount present (as determined by the initial absorbance value at 515 nm). The data for each respective blueberry sample are given below.

Table 2. Average slope and percent DPPH remaining after 2 hours in the antioxidant/free radical reaction of anthocyanin and DPPH as measured by the absorbance at 515 nm.

Sample	Average Slope	% DPPH remaining after 2 hours
Fresh (Argentina)	-0.0018	55.37
Frozen for 27 Days	-0.0005	54.30
Frozen for 34 Days	-0.0003	57.00
Frozen for 66 Days (Argentina)	-0.0019	58.32
Frozen for 105 Days	-0.0023	50.39
Frozen for 133 Days	-0.0020	39.07

The percent DPPH remaining after 2 hours directly reflected the concentration of anthocyanin present (the two different concentrations of DPPH used was not significant because the remaining DPPH was taken as a percent of the initial amount present). In comparing **Tables 1** and **2**, an increase in anthocyanin concentration directly corresponded with a decrease in

remaining DPPH. This was interesting considering the slopes, or rate of each reaction, did not show the same correlation. The blueberries from Argentina (fresh and frozen for 66 days) both had about the same slope, but differed in anthocyanin concentration and percent DPPH remaining. Furthermore, the slopes for the Canadian berries frozen for 27 and 34 days were very close to zero, but actually showed less remaining DPPH when respectively compared to those from Argentina (fresh and frozen for 66 days). This was starkly contrasted by the Canadian berries frozen for 105 and 133 days which had the sharpest slopes at -0.0023 and -0.0020, respectively, as well as the largest decrease in remaining DPPH at 50.39 and 39.07%, respectively. This was, again, most likely due to differences in anthocyanin structure.

Overall, every blueberry sample showed direct correlation between anthocyanin concentration and remaining DPPH, regardless of country of origin. This trend was reflected in the general decrease in remaining DPPH over time. However, country of origin became significant in terms of the rate of the reaction. This may have been a result of different structural compositions of the anthocyanin fractions in the blueberries from each respective country. In addition, freezing may have had different stabilizing effects on the blueberries from different countries resulting in differences in the reaction of the antioxidant with DPPH (Allaith et al., 2011).

The general increase in anthocyanin concentration over time agreed with all of the cited literature with the exception of Lohachoompol et al. (2004), which found no significant difference in anthocyanin concentration or antioxidant activity between frozen and fresh blueberries. In terms of antioxidant activity, the increase in activity with concentration was similar to that found in Michalczyk and Macura (2010).

CONCLUSIONS

Anthocyanin concentration generally increased with freezing time due to leeching from the blueberry tissue as a result of freezer burn. This directly affected the DPPH free radical remaining after the antioxidant/free radical reaction of anthocyanin with DPPH. Furthermore, the country of origin of the blueberries did not appear to significantly affect the change in anthocyanin concentration, but it was significant in how the anthocyanin interacted with the DPPH free radical.

On the whole, freezing appears to be an acceptable form of storage for blueberries for up to 66 days (about 2 months) depending on ice crystal formation, or freezer burn. Freezing faster at lower temperatures could decrease initial ice crystal size while packaging could be adjusted to prevent freezer burn.

This study could have been improved by eliminating a few of the variables such as the country of origin of the blueberries and the differing DPPH concentrations. These factors were not originally intended to be variables in this study but were simply a result of timing and

availability of materials. In addition, extending the time frame of freezing to up to 6 months or even 1 year may improve results. Also, other methods could be used to increase accuracy such as the ferric reducing ability of plasma (FRAP) assay to measure antioxidant activity, or high performance liquid chromatography (HPLC) or gas chromatography (GC) for anthocyanin separation (Allaith et al., 2011), and Folin-Ciocalteau reagent for anthocyanin quantification (Michalczyk & Macura, 2010).

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