Drew University College of Liberal Arts

Effects of freezer storage on soluble solids, anthocyanins, and titratable acidity in *Rubus phoenicolasius* fruits

A Thesis in Environmental Science

By:

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Abstract

Fruit ripening results in fruit softening, which translates to a loss of quality in commercial settings and increased difficulty handling samples in research settings. Ripening also involves chemical changes within a fruit, which can impact flavor and nutritional content and thus also impact consumers' perceptions of quality. While freezing helps preserve the initial postharvest properties of a fruit, there may be limits to how long a fruit can be stored before such properties change. The amount of available literature on the storage potential of *Rubus* fruits is growing, but there is still very little known about the qualities and frozen storage potential of Japanese wineberries (*Rubus phoenicolasius*) in particular. The goal of this study was to observe the effects of different durations of freezer storage on wineberry fruit chemistry, compared to qualities of fresh wineberries, to learn more about the storage potential of both whole wineberry fruits and juice. I sought to observe changes in soluble solids content, total anthocyanin content, and titratable acidity, as concentrations of these substances change as a fruit ripens. I found that during short-term cold storage, there were no changes in whole fruits. However, long-term storage of whole fruits and both short-term and long-term storage of juice resulted in changes to soluble solids and anthocyanin concentrations. These results contribute to determining the storage potential of wineberry samples for commercial and research purposes, but also reveal opportunities for further research on the impacts of cold storage on juice samples. Further research should consider the impact of environmental factors on initial fruit quality and storage potential.

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1. Introduction

1.1. Overview

I conducted an observational study on the fruit chemistry of Japanese Wineberry (*Rubus phoenicolasius*) in the summer, fall, and winter of 2021. Fruits were collected and analyzed in the summer, followed by further sample and data analysis in the subsequent fall semester. The main goal of my research was to observe and compare the effects of freezer storage on wineberry fruit samples under different storage durations. To do so, I collected data on the soluble solids concentration (SSC), total anthocyanin content (TAC), and titratable acidity (TA) of my samples before and after periods of frozen storage. More specifically, I sought to observe the effects of freezer storage on fruit chemistry by comparing the results of tests before and after short-term (14 weeks) freezer storage.

This study is a continuation of research on the *Rubus* genus originally led by Dr. Alex Bajcz at Drew University and thus also included data from samples collected for previous research in the summer of 2019. I utilized these samples to also observe the effects of longer-term freezer storage. Finally, using already-prepared juice samples from analyses conducted in 2019 and 2021, I compared the effects of freezer storage on whole fruits to those on fruit juice.

Below, I will give an overview on my study species, the compounds I chose to study, the process of fruit ripening, and the commercial and scientific relevance of frozen storage. I will then elaborate on the questions and predictions that guided my research.

1.2. Rubus phoenicolasius

R. phoenicolasius is an invasive species originally native to China, Korea, and Japan, now commonly also found in forest, field, and wetland habitats in the eastern United States (Swearingen

et al. 2010). It is a woody shrub characterized by greenish-brown stems covered in small red hairs and prickles and palmately compound leaves with three wide leaflets each, the center leaflet being larger and more distant from the others (Figure 1). Hairs, prickles, and trichomes are also present on the pedicels and the undersides of the leaves. The sepals (Figure 2), which open to reveal the flower then close again around the developing fruit, are also covered in hairs and trichomes; the sepals, hairs, and trichomes start out green and become redder as the fruit inside grows and ripens.

Figure 1. A wineberry cane in autumn, showing the characteristic stem and leaves. The stem is greenish-brown and densely covered with red hairs and prickles. The leaves are palmately compound, with three wide, rounded leaflets, the center one larger than and further away from the others.

Figure 2. Pictures showing the color change in the sepals (also covered with red hairs and trichomes) surrounding the wineberry fruits as they developed.

Like other species in the *Rubus* genus, wineberry is a perennial plant with biennial canes – flexible stems that grow for their first year and produce fruits in their second year. Wineberry fruits

are aggregates of drupelets, formed from multiple ovaries (Graham and Brennan 2018). The terms "fruit" and "whole fruit," as used throughout the paper, refer to the aggregate of drupelets. *R. phoenicolasius* is part of the *Idaeobatus* subgenus, characterized by fruits that fully separate from their receptacles upon harvesting (Graham and Brennan 2018).

Figure 3. Close-up of a cluster of wineberries in summer, featuring an almost ripe wineberry to the top left, an unripe wineberry to the bottom left, and several closed sepals throughout.

Most, if not all, members of the *Rubus* genus produce edible fruits, some of which are well known and sold commercially, like raspberries (*R. idaeus*) and blackberries (*R. allegheniensis*) (Graham and Brennan 2018). Although wineberry fruits are not found in stores, they could serve as a model for other related fruits with regards to fruit chemistry and reactions to freezer storage. Information about their fruit chemistry can also be valuable in further research of wild wineberries in regards to invasiveness, as fruits attract herbivores and frugivores that can then disperse the seeds contained within (Swearingen et al. 2010).

1.3. Fruit Chemistry During Ripening

While there is a plentiful amount of research done on fruit ripening in general, a good portion focuses on individual constituent processes within the ripening process. Most research also

involves tomatoes and strawberries, which are popular agricultural crops and models for two different fruit ripening systems – climacteric (requiring the presence of ethylene) and nonclimacteric (not requiring ethylene), respectively (Giovannoni 2001; Goulao and Oliveira 2008). Perkins-Veazie and Nonnecke (1992) report results that indicate that *R. idaeus* fruits should be classified as non-climacteric; because of their other similar characteristics, it is possible that wineberries could be classified as non-climacteric as well.

There is a smaller amount of research available on the ripening process as a whole, and how individual constituent processes interact with each other. In general, though, it is known that fruits undergo physical and chemical changes during ripening, and often continue to change physically and chemically after detachment from the plant (de Ancos et al. 2006). In recent years, more research has been conducted involving other kinds of fruits, including species in the *Rubus* genus. Because there is very little research involving the specific fruit chemistry and ripening processes of wineberries, I will be referencing what is known about red raspberries (*Rubus idaeus*). These two plants are in the same subgenus (*Idaeobatus*) and have similar fruits, so much of what is known about red raspberries might translate to wineberries as well.

There are many compounds and properties that change during fruit ripening, but for this study I chose to focus on the effects of cold storage on soluble solids (made up of mostly sugars), anthocyanins, and organic acids because of their contributions to the commercial quality of fruits and the ways they change and interact with each other during ripening. They are also relatively easy to measure and quantify. Refractometry was used to measure soluble solids content, quantified in %Brix, which represents the concentration of sugar in solution. Anthocyanin content

was measured via the pH differential method using a spectrophotometer. Organic acid content was quantified as titratable acidity and measured through titration.

The main sugars found in *Rubus* fruits are fructose and glucose, followed by sucrose (Wang et al. 2009). Perkins-Veazie and Nonnecke (1992), Famiani et al. (2005), and Contreras et al. (2020), among others, picked raspberry fruits at different stages of development and maturity (based on color), and assessed the sugar, anthocyanin, and acidity content of each stage to uncover trends in fruit chemistry during ripening. These studies have found that, as a raspberry matures, soluble solids content (representing predominantly sugars) of the fruit tends to increase (Perkins-Veazie and Nonnecke 1992; Famiani et al. 2005; Contreras et al. 2020). Wang et al. (2009) found that raspberry fruits continue to accumulate sugars post-harvest as well, likely due to solubilization of cell wall components.

Anthocyanins are pigments that contribute red and/or purple color to plant tissues (Mazza and Miniati 1993). There are several anthocyanins present in *Rubus* fruits like raspberries and wineberries, but the molecular weight and extinction coefficient of cyanidin-3-glucoside are often used in quantification calculations (Lee et al. 2005), as it is one of the dominant pigments in red fruits (de Ancos et al. 2000). Studies have found that anthocyanin content increases or that color intensifies/darkens as raspberries mature (Perkins-Veazie and Nonnecke 1992; Mazza and Miniati 1993; Famiani et al. 2005; Contreras et al. 2020). Wang et al. (2009) also found that synthesis of anthocyanins in raspberries could continue post-harvest under certain storage conditions. For instance, fruits that were exposed to high intensity light during storage continued to develop their red color, with a stronger effect seen in especially immature fruit (Wang et al. 2009).

The main organic acid in *Rubus* fruits like raspberries and wineberries is citric acid, followed by malic acid (Famiani et al. 2005; Wang et al. 2009). Research has found that titratable acidity (representing acid content) tends to decrease in raspberries as they mature (Perkins-Veazie and Nonnecke 1992; Famiani et al. 2005; Wang et al. 2009; Hancock et al. 2018; Contreras et al. 2020). The acids initially found in raspberry fruits are derived from sugars that are produced in the plant's leaves and transported to the developing fruits (Hancock et al. 2018). The decrease in acidity during ripening can be attributed to several processes, including catabolism and gluconeogenesis, which result in the formation of other compounds (Hancock et al. 2018).

These three classes of compounds also interact in several ways during the ripening process. de Ancos et al. (2000) found that, during freezer storage, the preservation of anthocyanin content depended on several factors, including organic acid and soluble solids contents – fruits from two late-fruiting cultivars of raspberry had lower pH and higher %Brix values than two early-fruiting cultivars, and experienced a decline in anthocyanin content during storage unlike the early-fruiting cultivars. Also, while anthocyanins contribute color to the fruit, other compounds such as organic acids can act as copigments, intensifying color (Mazza and Miniati 1993). The pH of a fruit can also impact the shade color of color observed; in aqueous solutions, anthocyanins appear red at lower pH values and transition to blue and then colorless as pH increases (Mazza and Miniati 1993). As stated above, the dissimilation/catabolism of acids that occurs during ripening via enzymatic activity provides material for processes such as gluconeogenesis and anthocyanin synthesis, potentially also contributing to an increase in sugars and anthocyanins as ripening progresses (Hancock et al. 2018).

1.4. Role of Enzymes

Several enzymes have been found to have a role in fruit ripening. Famiani et al. (2005) found that an increase in the enzyme phosphoenolpyruvate carboxykinase (PEPCK) occurred during raspberry maturation, and corresponded with a decline in citric acid content via dissimilation. Enzymatic activity also has a role in anthocyanin biosynthesis and degradation and resulting color changes within fruits (de Ancos et al. 2000; Giovannoni 2001; de Ancos et al. 2006), as well as the conversion of starch to sugars (Giovannoni 2001).

Polygalacturonase (PG), pectin methylesterase (PME), and cellulase have been found to have a role in fruit ripening and softening (Iannetta et al. 1999; Bapat et al. 2010). Enzymes such as these act on cell wall components, leading to cell wall degradation and a loss of fruit firmness - PG and PME degrade pectin, while cellulase breaks cellulose down into glucose (Bapat et al. 2010). As enzymes modify and degrade cell walls, the sugars they are made of are broken down and dissolved, contributing to increases in dissolved sugar content as a fruit ripens (Goulao and Oliveira 2008).

1.5. Fruit Quality, Health Benefits, and Commercial Value

The compounds I chose to study are not only involved in ripening but are also indicators of fruit quality (taste, color, and firmness) and contribute to nutritional value. The ratio of sugars to acids is considered an important factor in fruit flavor (Klee 2010). Anthocyanin content determines color, which is often used by consumers to assess fruit quality (Mazza and Miniati 1993; de Ancos et al. 2000; Lee et al. 2005). Additionally, *Rubus* fruits have been found to have especially high levels of phytonutrients, including anthocyanins (Moyer et al. 2002); the potential

health benefits of these phytonutrients make *Rubus* fruits and fruit products a valued commercial product (Mazza and Miniati 1993; Graham and Brennan 2018).

Another aspect of fruit quality is firmness – changes in chemistry that occur during ripening also result in softening, especially in already soft fruits like raspberries, leading to a shorter shelf life (Contreras et al. 2020) and a perceived loss of quality. This is commonly countered by keeping fruits in cold temperatures during transportation and storage to slow the advancement of ripening (Giovannoni 2001; Gonzalez et al. 2002; George 2008). Some studies have also looked at the efficacy of various post-harvest treatments, such as nanofilms/edible coatings (Sridhar et al. 2020), cryogenic freezing (Zlabur et al. 2021), and osmotic dehydration (Bonat Celli et al. 2016) in preserving fruit quality. However, cold storage is a more common method for increasing the shelf life of soft fruits like raspberries (Gonzalez et al. 2002; Bonat Celli et al. 2016; Graham and Brennan 2018) and will be the focus of this study.

Cold storage is used both before and after fruits and fruit products get to a consumer: during transportation from farms to stores, within stores, and within consumers' homes. Freezers are also used to hold samples in research labs, or in processing facilities before fruits are turned into juices and other products (Gonzalez et al. 2002; George 2008). Because cold storage is so commonly used to preserve fruits that provide important nutritional value, it is necessary to understand chemical changes that could occur during storage and to determine if there is a limit to how long fruits can be stored before their chemistry, appearance, and/or nutritional value decline.

1.6. Questions and Predictions

Using raspberries as a reference for what wineberry fruit chemistry may be like, I sought to learn more about the impacts of short- and long-term cold storage on the chemistry of wineberry

fruits. I also sought to compare the impacts of cold storage on whole fruits to the impacts on juice/pulp. I predicted that there would be no changes during short-term storage because cold temperatures can slow chemical and enzymatic reactions. However, I predicted that during long-term storage slowed reactions would eventually produce observable changes, and that if changes were observed during long-term storage they would follow the expected patterns that occur during fruit ripening - an increase in sugars and anthocyanins and a decrease in acidity. I also predicted that cold storage of juice samples would result in chemical changes similar to those seen in long-term storage of whole fruits.

2. Methods

2.1. Overview

I predicted that freezer storage would not have a significant effect on the fruit chemistry of wineberries over a period of fourteen weeks. To assess this prediction, I tested samples for soluble solids (primarily sugars) concentration (SSC), total anthocyanin concentration (TAC), and titratable acidity (TA) at Weeks 0, 7, and 14 of freezer storage. Sugars, anthocyanins, and acids were chosen to be measured because their concentrations typically change during fruit ripening. I used digital refractometry to measure soluble solids concentration, spectrophotometry to measure anthocyanin concentration, and titration to measure titratable acidity.

I was also curious about the longer-term effects of cold storage on wineberries. Whole fruit samples collected for previous research in 2019 (Thexton and Bajcz 2021) had been stored in the freezer and I had quantified the soluble solids concentration of these samples in 2019, providing an opportunity to analyze chemistry in older fruit and juice samples. I first compared the SSC values of these samples from 2019 to the Week 0 SSC values from 2021 to determine if the initial

qualities of fruits from these years may have been different. TAC and TA were not measured in 2019, so I was unable to make comparisons between the 2019 and 2021 samples for those properties. I then measured the SSC values of the 2019 samples that had been stored as both whole fruits and as juice, and compared them to the original values measured in 2019, to get a sense of the effects of longer-term cold storage of wineberries.

Because the samples I prepared in the summer of 2021 were also stored as juice in the freezer, I assessed the effects of short-term cold storage on the soluble solids and anthocyanins content of wineberry juice as well.

2.2. Sample Collection

We collected wineberry fruits from sixteen plots found in four parks and hiking trails in northern New Jersey: one plot at Drew University's Zuck Arboretum (DU), six plots at Jockey Hollow at Morristown National Historical Park (JH), four plots at Lewis Morris County Park (LM), and five plots at Old Troy County Park (OT) (Figures 3 and 4). These plots were selected from a list previously studied and described by Thexton and Bajcz (2021). The plots I used were chosen based on relative abundance of *R. phoenicolasius* canes and ease of access from trails.

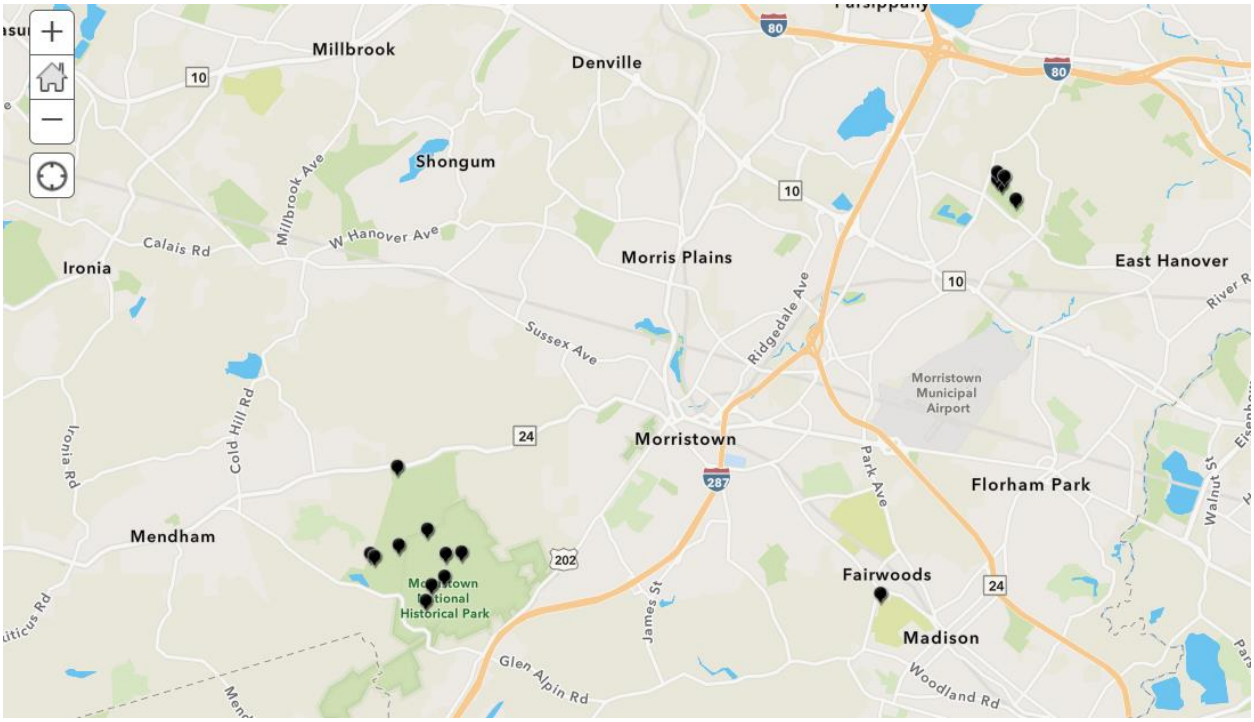

Figure 4. ArcGIS map with black pins marking the locations of our sixteen study plots in northern New Jersey. The plots were spread across four properties: Drew University's Zuck Arboretum, Jockey Hollow at Morristown National Historical Park, Lewis Morris County Park, and Old Troy Park. ArcGIS.com basemap: Community Map. Map contributors: New Jersey Office of GIS, Esri, HERE, Garmin, SafeGraph, GeoTechnologies, Inc, METI/NASA, USGS, EPA, NPS, USDA.

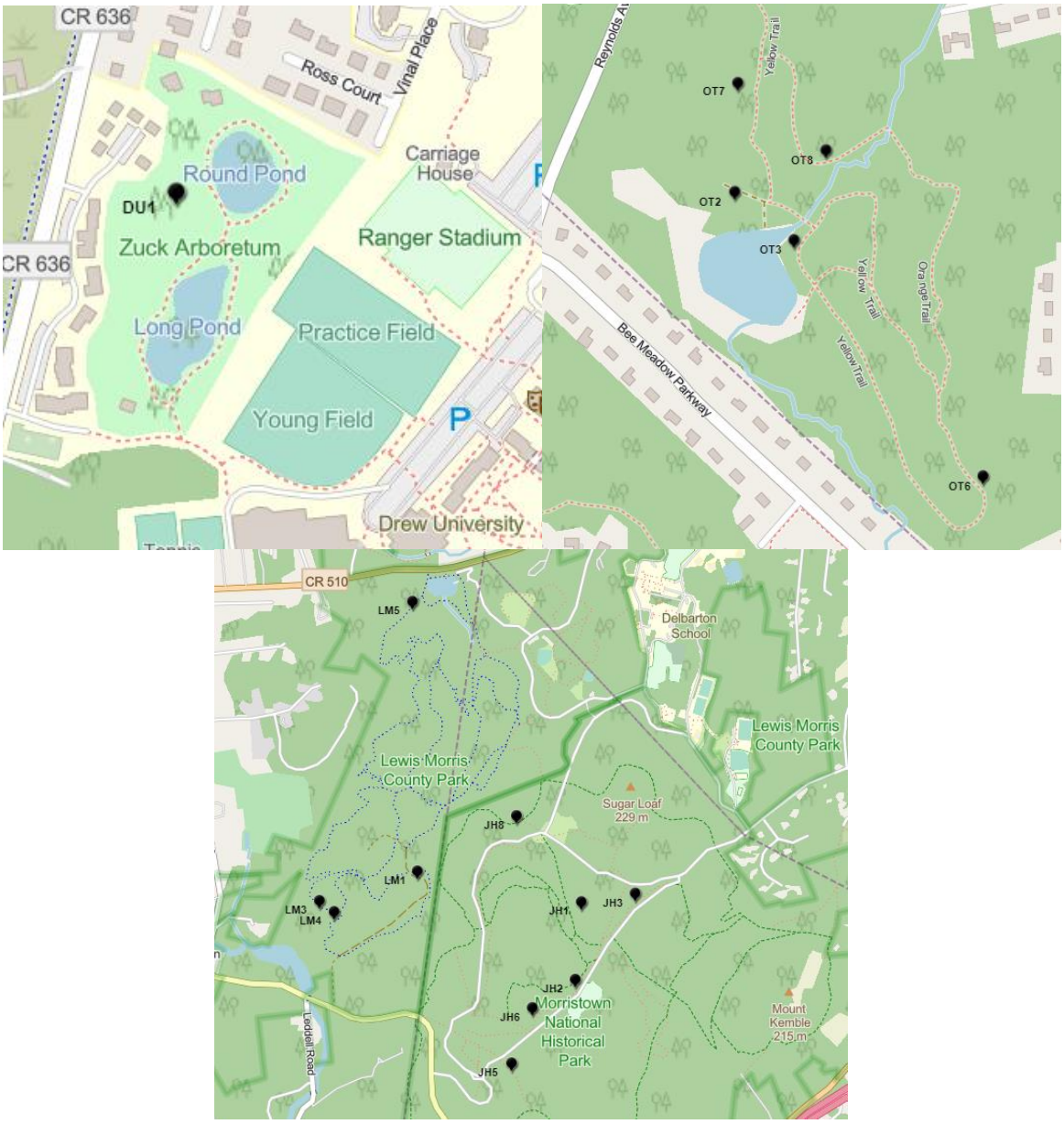

Figure 5. ArcGIS maps showing the plots and properties in more detail. Each black pin marks a plot, and is labeled with a unique plot code already established by Thexton and Bajcz (2021). The Drew University Zuck Arboretum plot is labeled DU, the Jockey Hollow plots are labeled JH, the Lewis Morris plots are labeled LM, and the Old Troy plots are labeled OT.
ArcGIS.com basemap: OpenStreetMap. Map contributors: OpenStreetMap contributors, Microsoft, Esri Community Maps contributors, Esri.

2021 sample collection occurred on four different dates by three teams. The five Old Troy plots were sampled on July 11, 2021. The Zuck Arboretum, four Jockey Hollow Plots, and one Lewis Morris plot were sampled on July 19, 2021. On July 21, 2021, the remaining Jockey Hollow

and Lewis Morris plots were sampled, and additional samples were collected from the same five Old Troy plots. An additional sample was also collected from the Zuck Arboretum on July 23, 2021. Additional samples were only collected from plots if it was determined that the initial sample would not be enough to produce three subsamples for testing.

At each plot, approximately 100-150 ripe fruits were picked. Ripeness was indicated by a crimson red color; paler orange fruits were deemed underripe, and fruits with a deeper, almost burgundy color were deemed overripe. Fruits that fell apart upon picking were discarded, as this was used as another indicator of over-ripeness. Fruits were picked from canes throughout the plot and from various parts of each cane without favoritism based on plant/fruit size/position; no more than three fruits were taken from any one cluster found on a cane to ensure a more representative sample from across the plot. We stored the fruits in labeled and sealed plastic bags in a portable cooler with ice packs for the remainder of each sample collection day. All sample bags were stored overnight in a refrigerator to be tested the morning following their collection. The first testing date was considered Week 0, after which the fruits were stored in a freezer at -20℃.

2.3. 2019 Samples

Samples collected in the summer of 2019 were collected according to the same general procedure described above except that three samples from each plot were collected over the course of three weeks and all ripe fruits present at the time were collected. These wineberry samples from 2019 have also been stored in labeled and sealed plastic bags in the same freezer at -20℃ since their collection.

In November of 2019, as part of previous research, I used digital refractometry as described below to quantify the soluble solids concentrations of the samples from the summer. The fruit juice

samples that I prepared at that time have also been stored in the freezer since they were originally tested. I later thawed and re-centrifuged these again before analyzing them using digital refractometry. The soluble solids measurements I did in 2019 included samples from 36 plots; of these, there were 27 centrifuge tubes in the freezer with enough juice to perform another SSC measurement.

Because I have soluble solids data from 2019, I also used whole fruit samples collected in 2019 to assess the long-term storage potential of wineberries. One plot (OT8) was excluded from this analysis because there were no whole fruits remaining in the freezer from the 2019 collection from that plot. Samples were prepared according to the same procedure detailed below.

2.4. Sample Preparation

For the Week 0 tests, I removed the fruit samples from the refrigerator, selected fruits from their bags without favoritism, and placed them into 50mL centrifuge tubes. After centrifuge tubes were prepared for initial tests, the rest of the fruit samples were stored in their bags in a -20°C freezer for seven weeks, after which they were sampled and tested again at Weeks 7 and 14. The sample bags were spread out in empty shelf spaces within the freezer to prevent damage to the structure of the fruits from overcrowding. When preparing frozen samples, I selected fruits from their bags without favoritism, placed them into 15mL centrifuge tubes, and allowed the fruits to thaw for 30 minutes before crushing. I crushed the fruit samples with a thin tissue muddler until no whole drupelets remained. The samples were centrifuged until there was a clear separation between solids and liquids.

All centrifuge tubes of juice were returned to the freezer after analysis. The Week 0 juice samples were later used to assess the chemistry of juice after short-term freezer storage. After

approximately five months, I took the samples back out to measure soluble solids content and total anthocyanin content.

See Appendix A for the full Sample Preparation SOP.

2.5. Soluble Solids Content (SSC)

Soluble solids content (SSC) was measured using a Laxco RHD-B-102 benchtop digital refractometer. I first calibrated the refractometer using deionized water. To read each sample, I pipetted 0.3mL of fruit juice onto the refractometer lens from one centrifuge tube at a time, and the results were recorded in %Brix. I cleaned the refractometer lens with deionized water and a Kim wipe between each sample. I also created a standard curve using table sugar solutions with concentrations of 0.03, 0.06, 0.09, 0.12, and 0.15 (wt/vol). After reading the standard solutions in the same manner as the samples, I plotted the concentrations and %Brix readings to obtain a standard curve and equation; this equation was used to determine the concentration (wt/vol) of soluble solids in each sample using their %Brix readings.

See Appendix B for the full Refractometry SOP.

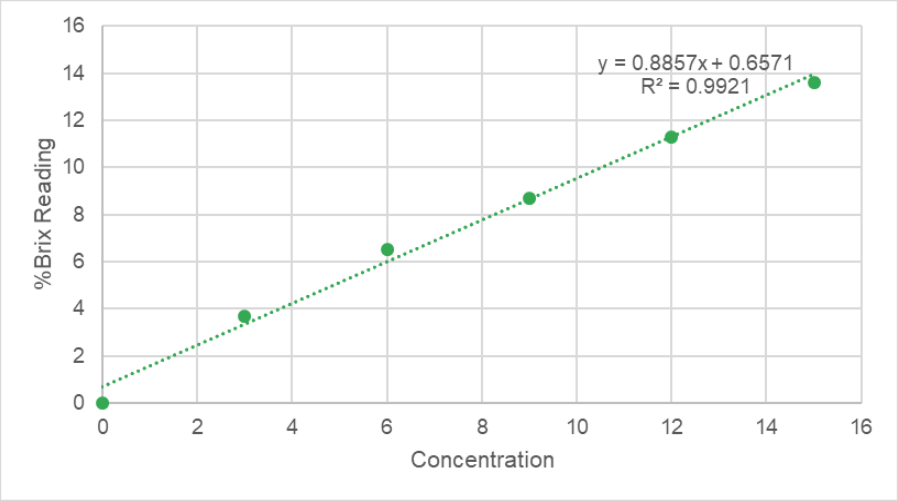

Figure 6. Calibration curve showing the relationship between sugar concentration and %Brix as suggested by the refractometer. The %Brix readings of six standard sugar solutions were plotted against their concentrations. The equation of the resulting trendline was used to calculate the soluble solids concentration of each fruit juice sample from their %Brix readings.

2.6. Total Anthocyanin Content (TAC)

Total anthocyanin content (TAC) was measured via a pH differential colorimetric test using a ThermoScientific Genesys 180 UV-Visible Spectrophotometer. I pipetted each sample into two cuvettes; I added a potassium chloride + hydrochloric acid buffer solution with a pH of 1 to one cuvette, and a sodium acetate + hydrochloric acid buffer solution with a pH of 4.5 to the other. The pH 1 samples had a dilution factor of 10 (0.1mL of sample to 0.9mL of buffer) and the pH 4.5 samples had a dilution factor of 3.33 (0.3mL of sample to 0.7mL of buffer). These dilution factors were chosen for this test because, when reading previously collected samples (not included in this dataset), the absorbance values produced were within the spectrophotometer's linear range. The samples were read at wavelengths 520nm and 700nm, and the absorbances were recorded. 520nm is the wavelength that is absorbed by the anthocyanins; the samples are read at 700nm to measure the "background absorbance," which will be subtracted from the absorbance at 520nm (Lee et al. 2005).

Anthocyanin content was calculated using the following equation:

(1) Anthocyanin equivalents = $\{(A_{TAD} * MW * 1000mg\ anth./g\ anth.)/(EC * PL)\}$

where MW equals the molecular weight of cyanidin-3-glucoside (449.2 g anthocyanin equivalents per mole); EC equals the extinction coefficient of cyanidin-3-glucoside (26,900,000 mL solution per mole per centimeter); and PL equals the path length of the spectrophotometer tube (usually 1 centimeter) and where A_{TAD} is equal to the total absorbance difference, calculated via the equation:

$$A_{TAD} = DF_1(A_{520nm} - A_{700nm})_{pH1} - DF_{4.5}(A_{520nm} - A_{700nm})_{pH4.5}$$

where DF represents the dilution factor of the sample [(mL of buffer + mL of sample)/mL of sample] and A represents the absorbance of the sample at the indicated wavelength. The subscripts 1 and 4.5 refer to the pH of the buffers added to the cuvettes, and the subscripts 520nm and 700nm refer to the wavelengths at which absorbance was measured. The resulting units were milligrams of anthocyanin equivalents per milliliter of fruit juice.

See Appendix C for the full Spectrophotometry SOP.

2.7. Titratable Acidity (TA)

Titratable acidity (TA) was measured via hand titration of each sample. 1mL of juice from each sample was pipetted into a 100mL beaker with 49mL of deionized water. This solution was titrated to a pH endpoint of approximately 8.2 with 0.1N NaOH solution. A pH of 8.2 is referred to as the "phenolphthalein endpoint," as it is the pH endpoint commonly reached when titrating using a phenolphthalein indicator and thus is also used when titrating with a pH probe in place of an indicator (Sadler and Murphy 2010).

The pH was measured using a ThermoScientific ROSS Ultra combination glass pH electrode calibrated using a series of buffer solutions (pH 4, 7, and 10) and connected to a

ThermoScientific Orion Star T900 series titrator to read the pH values. When the sample reached a pH of approximately 7.6-7.9, the pH and burette volume were recorded. An additional drop of NaOH was then added, usually increasing the pH to well above 8.2. If not, another drop was added. The final pH and final burette volume were also recorded. The volume that resulted in the greatest pH below 8.2 was used in the following calculation:

$$(2)\ \%\ \text{citric acid} = [(N \times V_1 \times \text{Eq wt})/(V_2 \times 1000)] \times 100$$

where N is the normality of the titrant, V_1 is the volume of titrant used, Eq wt is the equivalent weight of the predominant acid (in this case, the anhydrous weight of citric acid), and V_2 is the volume of the sample. The resulting units were %citric acid equivalents (wt/vol).

See Appendix D for the full Titration SOP.

2.8. Statistical Analyses

I stored my data in Microsoft Excel and analyzed them using a combination of Microsoft Excel (2016) and IBM SPSS Statistics (Version 28.0.0.0). I used a Repeated Measures ANOVA for each dependent variable (SSC, anthocyanins, and acidity) to check for statistically significant changes over 14 weeks, using time as the factor and plot as the identifier of subjects. I used a paired t-test to compare SSC values from 2019 to SSC values from Week 0 in 2021 to determine whether the initial qualities of fruits from these years were different. I also used paired t-tests for assessing the short-term storage of juice from 2021 and the long-term storage of whole fruits and juice from 2019. Results were considered statistically significant if the p-value was less than 0.05.

3. Results

3.1. Short-term Storage of Whole Fruits -- SSC, TAC, & TA

There was no statistically significant difference in soluble solids content in whole fruits stored for fourteen weeks (Figure 7; Repeated Measures ANOVA: $F_{2, 45} = 0.236$, $p = 0.791$).

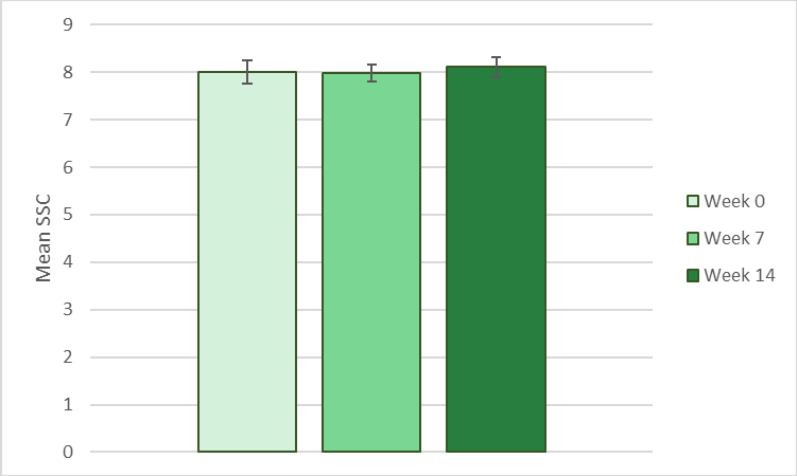

Figure 7. Mean soluble solids content (% sugar in wt/vol) at week 0, week 7, and week 14, with error bars equal to two standard errors. There was no significant change in SSC in whole fruits stored at -20°C for fourteen weeks.

There was no statistically significant difference in total anthocyanin concentration in whole fruits stored for fourteen weeks (Figure 8; Repeated Measures ANOVA: $F_{2, 45} = 0.755$, $p = 0.479$).

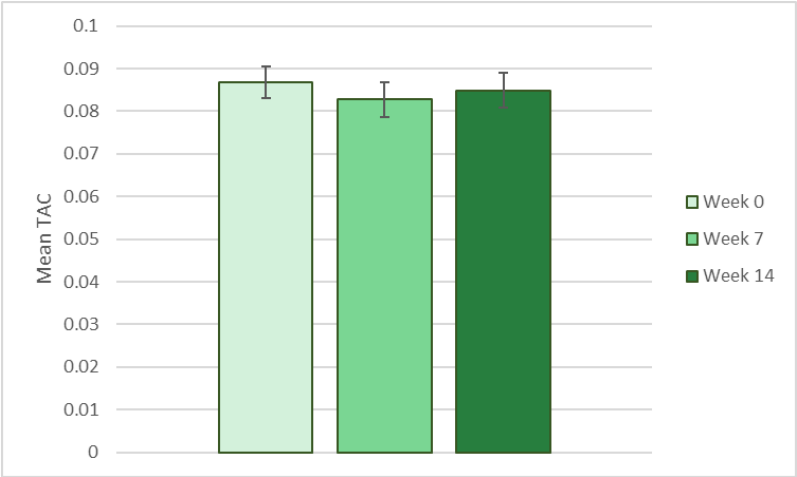

Figure 8. Mean total anthocyanins content (mg cyanidin-3-glucoside equivalents/mL of sample) at week 0, week 7, and week 14, with error bars equal to two standard errors. There was no significant change in TAC in whole fruits stored for fourteen weeks.

There was no statistically significant difference in titratable acidity in whole fruits stored for fourteen weeks, but there was evidence of a trend of slightly increasing titratable acidity (Figure 9; Repeated Measures ANOVA: $F_{2, 45} = 2.929$, $p = 0.069$). *Post-hoc* tests revealed a significant increase in titratable acidity from week 0 to week 7 ($p = 0.024$), but this result was not maintained into week 14.

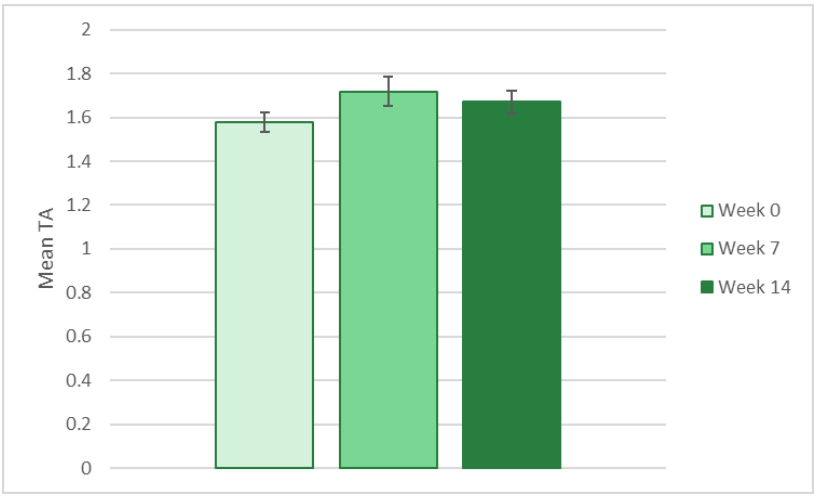

Figure 9. Mean titratable acidity (% citric acid in wt/vol) at week 0, week 7, and week 14, with error bars equal to two standard errors. There was no significant change in TA in whole fruits stored for fourteen weeks, but there was evidence of a trend towards slightly increasing TA.

3.2. Short-term Storage of Juice -- SSC & TAC

There was a significant difference in soluble solids content in samples stored as juice for approximately five months (Figure 10; paired t-test: t = 15.137, df = 15, $p << 0.001$). On average, SSC tended to be lower after storage than before.

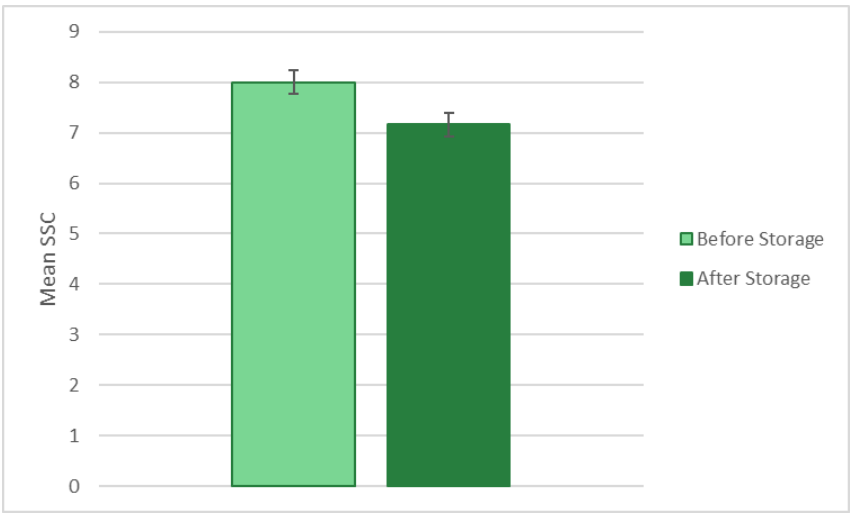

Figure 10. Mean soluble solids content (% sugar wt/vol) before and after approximately five months of cold storage of juice samples collected and prepared in 2021, with error bars equal to two standard errors. There was a significant decrease in SSC after storage.

There was also a significant difference in total anthocyanin content in samples stored as juice for approximately five months (Figure 11; paired t-test: t = -2.682, df = 15, p = 0.017). On average, TAC tended to be higher after storage than before.

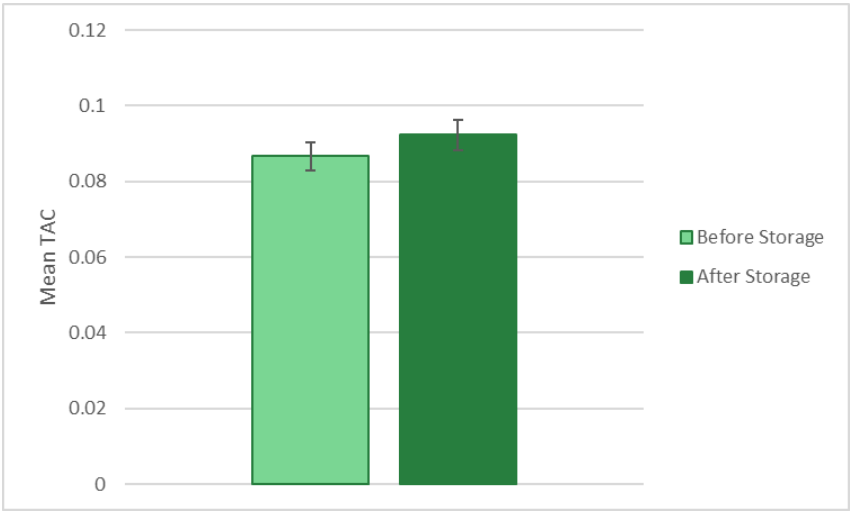

Figure 11. Mean total anthocyanins content (mg cyanidin-3-glucoside equivalents/mL of sample) before and after approximately five months of cold storage of juice samples collected and prepared in 2021, with error bars equal to two standard errors. There was a significant increase in TAC after storage.

3.3. Long-term Storage

Because I did not find a significant difference in soluble solids concentration over a fourteen week period in the 2021 samples, I will treat the November 2019 SSC values as "Week 0" for those samples, as they should not be significantly different than if the samples had been tested in the summer of 2019.

To decide if the 2019 samples could be used as an estimate of long-term freezer storage and be included in any trends witnessed in the 2021 samples, I compared the 2019 "Week 0" SSC values to the 2021 Week 0 SSC values. There was a significant difference in these values (Figure 12; paired t-test: t = -6.905, df = 15, $p << 0.001$), indicating that the overall fruit chemistry in 2019 may be different enough from the fruit chemistry in 2021 that I cannot confidently use the 2019 samples stored in the freezer as an estimate of the changes that would be seen in the 2021 samples after an approximately two and a half year storage period.

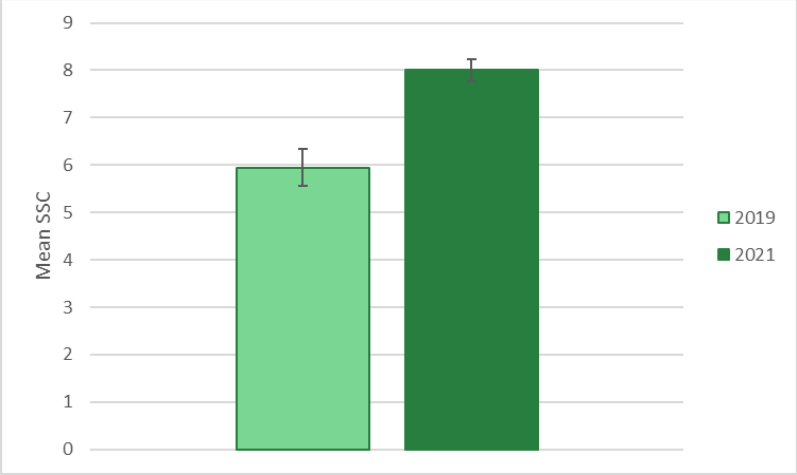

Figure 12. Comparison of average SSC (% sugar in wt/vol) of wineberry samples collected from the same sixteen plots in 2019 and 2021. There was a significant difference in the SSC values of these samples, with the 2021 samples having higher SSC values on average.

3.3.1. Long-term Storage of Whole Fruits -- SSC

When comparing the readings of whole fruit samples (from the same 27 plots) frozen for approximately 28 months to the original SSC values of those samples from analyses done in 2019, there was a significant effect of time on soluble solids content (Figure 13; paired t-test: t = -6.461, df = 25, $p << 0.001$). On average, SSC tended to be higher after storage than before.

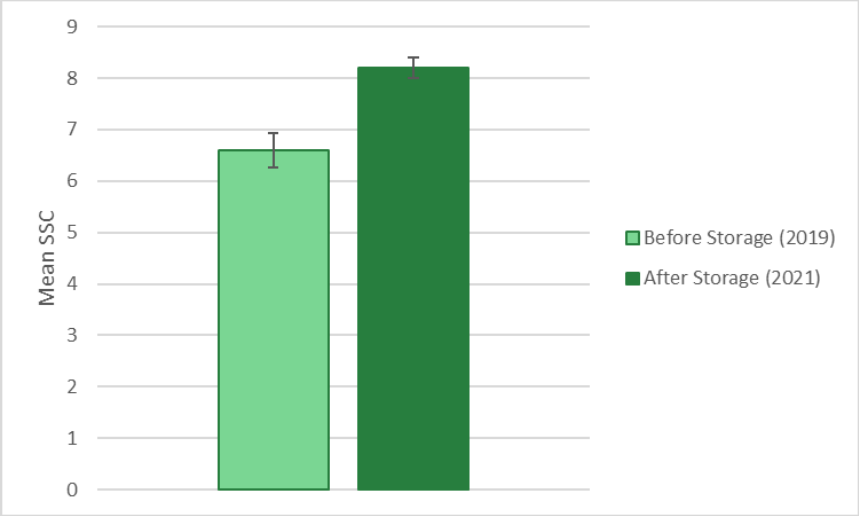

Figure 13. Mean soluble solids content (% sugar in wt/vol) before and after approximately 28 months of cold storage of whole fruit samples collected in 2019, with error bars equal to two standard errors. There was a significant increase in SSC after storage.

3.3.2. Long-term Storage of Juice -- SSC

When comparing the SSC values of the 2019 frozen juice samples to the original SSC values of those same samples (calculated in 2019), there was a significant difference in soluble solids content (Figure 14; paired t-test: t = 3.233, df = 26, $p = 0.003$). On average, SSC tended to be lower after storage than before.

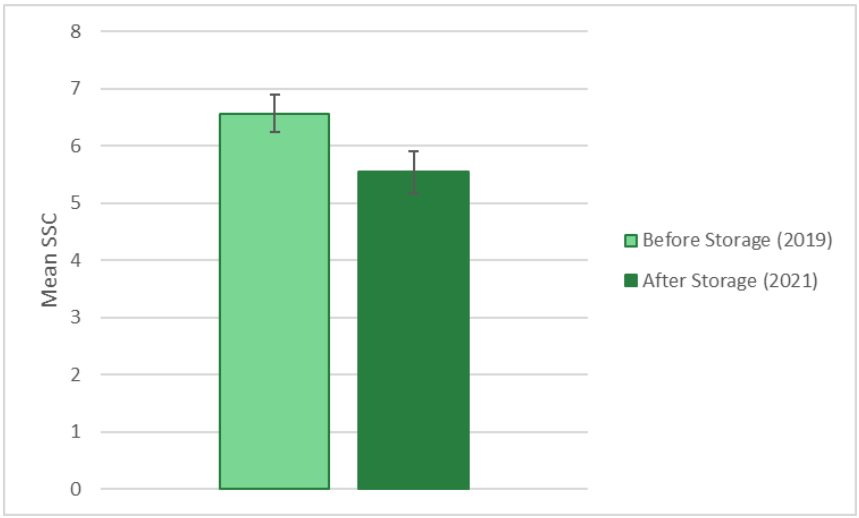

Figure 14. Mean soluble solids content (% sugar in wt/vol) before and after approximately two years of cold storage of juice samples collected and prepared in 2019, with error bars equal to two standard errors. There was a significant decrease in SSC after storage.

4. Discussion

4.1. Fruit Ripening

Under normal fruit ripening conditions, soluble solids content (SSC) and total anthocyanin content (TAC) both increase and titratable acidity (TA) decreases over time for fruits like raspberries and wineberries (Perkins-Veazie and Nonnecke 1992; Mazza and Miniati 1993; Famiani et al. 2005; Wang et al. 2009; Hancock et al. 2018; Contreras et al. 2020). SSC is thought to increase because cell walls break down, putting more sugars into solution within the fruit juice (Wang et al. 2009). The decrease in acidity is thought to be caused by several chemical changes, including the formation of sugars and anthocyanins from dissimilated acids (Mazza and Miniati 1993; Hancock et al. 2018). As such, the increase in anthocyanins could be due to the concomitant breakdown of organic acids. Cold storage, however, is expected to slow these reactions, preserving the initial chemical qualities of the fruit (Gonzalez et al. 2002).

4.2. Short- and Long-term Storage as Whole Fruits

After short-term freezer storage (fourteen weeks at -20°C), there were no statistically significant changes to the SSC, TAC, or TA of whole wineberries. This suggests that the cold temperature slowed the chemical and enzymatic reactions that would normally be taking place within the fruit as part of fruit ripening. Several studies have found that the crystallization of water that occurs during freezing reduces the rate of chemical and biochemical reactions that would normally lead to the ripening/deterioration of fruits (de Ancos et al. 2006; George 2008; Chassagne-Berces et al. 2010). However, these reactions are not completely stopped and continue to occur but at a much slower rate (Gonzalez et al. 2002; de Ancos et al. 2006). As such, it could be possible for changes in fruit chemistry to occur or become more obvious as frozen storage time increases beyond fourteen weeks.

There did appear to be a slight increasing trend in acidity during this period, which does not match the expected changes that would occur during normal ripening. The acidity increased from Week 0 to Week 7, then decreased from Week 7 to Week 14, with an overall slight (but not statistically significant) increase between Weeks 0 and 14. The organic acids in a fruit are accumulated during growth and development on the plant. As ripening continues before and/or after detachment, additional acids do not accumulate; the existing acids break down, contributing to the formation of other compounds (Hancock et al. 2018). As such, this apparent trend is likely best explained as a consequence of random variability between samples. However, further investigation into the fruit chemistry and storage potential of wineberries could help clarify the reality of this trend, or potential sources of variability.

For longer-term storage of whole fruits, only SSC could be assessed. After storage for approximately 28 months at -20°C, there was a significant increase in the SSC of whole wineberries. Normally, as fruits ripen, cell wall-modifying enzymes break down and dissolve cell wall components, leading to the further accumulation of sugars within a fruit post-harvest (Goulao and Oliveira 2008). While the cold temperature of frozen storage probably prevented changes to fruit chemistry in the samples stored for fourteen weeks, it is possible that these slowed changes eventually became evident in samples stored for about 28 months. Gonzalez et al. (2002) also found that changes occurred to the properties of red raspberries during frozen storage, and that some of these changes were more evident as storage time increased. This could be indicative of a limit to how long fruits can be frozen before there is an impact on chemical and physical traits. de Ancos et al. (2006) and George (2008) both reported, based on information from the International Institute of Refrigeration, that the practical storage life of raspberries was 24 months when kept at a consistent -18°C. My wineberry samples were kept at -20°C and likely have a similar storage life. Wineberry samples that were stored for longer than 24 months showed evidence of changes in SSC; because of the general patterns of fruit ripening and how sugars, anthocyanins, and acids interact with each other during the process, it is possible that other changes to fruit chemistry would have been observed during this period of cold storage as well.

Changes observed in samples stored for longer periods of time could also be attributed to the formation of ice crystals. During my time working with the samples, I noticed more intense ice crystals in the sample bags from 2019 than in the sample bags from 2021. It was often difficult to handle the older samples and separate individual fruits because of the extent of the ice crystal formation holding the fruits together, while the 2021 samples tended to stay separated and without

visible ice crystals. There is evidence in other studies of ice crystals disrupting cell membranes and walls of fruit tissue, leading to changes that would similarly occur after enzymatic modification of cell walls (Gonzalez et al. 2002; Chassagne-Berces et al. 2010). Temperature fluctuations during frozen storage can also lead to ice recrystallization, which can create larger and more irregularly-shaped ice crystals (de Ancos et al. 2006). The samples from 2019 were sorted over the course of a few days in the fall of 2019, which required removing them from the freezer for unrecorded periods of time. While care was taken to ensure the samples were not out of the freezer for longer than what was necessary, it is possible that the temperature changed enough to generate larger and/or sharper ice crystals within and around the fruits, causing additional damage to cell membranes and walls. In this case, larger and sharper ice crystals in the older samples may have ruptured cell membranes, further encouraging the solubilization of cell wall components via enzymatic activity.

Additionally, the crystallization of water outside of a fruit can result from a loss of water from within the fruit, perhaps following damage to cell membranes, leading to a concentration of compounds within plant cells (George 2008). This concentration of compounds could appear as an increase in soluble solids/sugar content, for example. All of these processes could have contributed to the increase in soluble solids that was observed in the wineberry samples that were stored in the freezer for about 28 months.

4.3. Short- and Long-Term Storage as Juice

Anthocyanin content was only able to be assessed during short-term frozen storage of juice samples (approximately five months); TAC increased during this time. While anthocyanin content increasing is expected during fruit ripening and storage (Famiani et al. 2005; Wang et al. 2009), it

is difficult to identify a cause for it without knowing if other properties, such as titratable acidity, changed in the samples as well. Acidity was not able to be assessed for these juice samples due to a lack of sample volume needed for titration. However, an increase in anthocyanin content could be the result of the breakdown of organic acids into components needed to synthesize phenolic compounds like anthocyanins (Mazza and Miniati 1993).

During short-term and long-term storage, SSC decreased in frozen wineberry juice samples. This was unexpected based on what is known about sugars during ripening and storage, which is that sugar content tends to increase (Contreras et al. 2020). One hypothesis to explain this is that crushing the fruits into juice disrupts cells so much that compounds that would normally be separated from each other were instead mixed together, and the compounds and enzymes may have (slowly) interacted in unexpected ways. Gonzalez et al. (2002) also suggested that cellular disruption mixed compounds in their samples, resulting in a variety of changes to fruit chemistry. One such potential reaction is fermentation, which would convert sugar into alcohol with the help of naturally-occurring yeast (Saranraj et al. 2017).

4.4. Further Research/Changes

This study's results provide some insights into the fruit chemistry and storage potential of wineberries, but this area of research could benefit from several expansions and changes moving forward. Firstly, more data could be obtained by:

1. Increasing the sample size of plots – there are many accessible wineberry plots in northern New Jersey that were not visited in this study but are included in Thexton and Bajcz's study (2021). Including more plots can increase the sample size, potentially strengthening

evidence of trends in fruit chemistry. Sampling more plots can provide a wider variety of environmental conditions to study as well (the value of which is discussed below);

2. Assessing samples after more storage durations – there was a relatively large time gap between the short-term and long-term storage of the samples in this study. Assessing the fruits at more time points in between fourteen weeks and 28 months could provide more insight into the storage potential of wineberries, by finding the approximate time when chemical changes first begin to become evident; and
3. Expanding on the compounds studied – anthocyanin content and acidity were not assessed in all samples because of the unique circumstances of this study. More consistency in observations across samples will provide a better idea of the changing fruit chemistry, especially when certain compounds can interact with and influence each other. Future research could also look at additional compounds that change during ripening, like flavonoids and antioxidants, or at the enzymes that impact physical and chemical changes, such as PEPCK or cell-wall modifying enzymes. Measuring the alcohol content of fruits and fruit juice could clarify the role of fermentation in changing fruit chemistry as well.

Additionally, collecting corresponding environmental data could provide valuable insight into the initial characteristics of a fruit sample and, potentially, a sample's resiliency to change during frozen storage. For example, Liu et al. (2021) found that peppers grown in soils with different physical and chemical properties had varying nutrient contents. For example, soil pH can impact the availability of certain elements (magnesium, zinc, copper, etc.) to pepper plants and thus the amount of them the plant absorbs (Liu et al. 2021). A similar effect could potentially be seen in *Rubus* fruits. Light levels may have an influence on the anthocyanins in a plant or fruit as

well: Wang et al. (2009) found that red raspberries of different maturity stages exposed to UV light after harvest continued to accumulate anthocyanins. It's possible that light levels pre-harvest could have a similar effect.

Bonat Celli et al. (2016) also suggested that pre-harvest environmental factors like temperature can influence fruit quality, including its response to frozen storage. While temperature data could be difficult to utilize in combination with chemistry data as it would have to be collected continuously before and throughout the fruiting season, it could be valuable for understanding the reactions of fruits to cold temperatures. Yamada et al. (2002) found that plants that were exposed to non-freezing cold temperatures would become acclimated to the cold and thus be more tolerant of freezing and the dehydration that accompanies it. This cold-acclimation also resulted in a reduction of cell wall pore size, which would restrict the movement of and reactions between compounds involved in the transformation of a fruit's chemistry during ripening more than a chilling-sensitive plant with larger cell wall pores. These types of factors can influence fruit chemistry both before harvesting and during storage, and consequently the results of analyses involving changes to fruit chemistry during storage.

Another factor that could influence the impacts of freezer storage on fruit chemistry is harvest time. Gonzalez et al. (2002) found that early-fruiting cultivars of raspberries experienced an increase in SSC after frozen storage, while late-fruiting cultivars experienced a decrease. Similarly, de Ancos et al. (2000) found that early-fruiting cultivars of raspberry experienced an increase in anthocyanin content after frozen storage, while late-fruiting cultivars experienced a decrease. Goulao and Oliveira (2008) also recommend studying cultivars individually, as they may not experience the same changes to fruit chemistry. While there are not necessarily different

cultivars of wineberries because they are not commercially grown, this indicates that further study on wineberries is important for understanding the species' fruit chemistry because it may be different from what is found in the available literature about raspberries, as they may have different harvesting times within the year, as well as different genetics or ecological relationships.

Beyond this, different storage conditions could improve the quality of a future study's results. In this study, the samples from 2021 were able to be spread out in the freezer, while the samples from 2019 were packed very tightly into boxes because there were more of them. As a result, I noticed that the 2021 samples were easier to work with, as the fruits stayed separated; however, in many of the 2019 sample bags, the fruits were stuck together by ice crystals or frozen juice. Also, many of the plot labels fell off of the 2019 sample bags, making them unidentifiable and unusable. Sturdier sample containers, a more reliable labeling method, and assurance that there's enough freezer space relative to the number of samples could all help make the process of analyzing samples easier. This could also improve the results, as there would be less of a potential impact from excessive ice crystallization on the concentration of soluble compounds. It will also be important to ensure that the fruits remain at a consistent temperature once they are stored, as fluctuating temperatures or unintended thawing can impact ice crystal formation (de Ancos et al. 2006) and, by extension, fruit chemistry.

4.5. Applications

This research fills a gap in the knowledge of *Rubus* fruit chemistry and in the storage potential of wineberries in particular. Although wineberries are not commonly found in stores, they are sometimes harvested by individuals who could benefit from knowing the limits of the species' storage potential. Also, wineberries may continue to be studied in the future because of

their invasive nature, and this could include observing their fruits and their interactions with herbivores. It will be crucial for researchers to know how long samples can be stored before their initial properties are no longer preserved. If a study takes several years to complete, the fruits may no longer be the same at the end as they were when first harvested, despite being stored in a freezer. Finally, it is important to note that freezing fruits and fruit products requires energy and money - learning more about how long different items can be preserved can help ensure that resources are not wasted on storing something after its quality has declined.

5. Conclusion

I started this study predicting that short-term freezer storage would not lead to changes in fruit chemistry, and that long-term freezer storage might lead to changes in fruit chemistry that follow the pattern of normal fruit ripening. My results supported these predictions with regards to the storage of whole fruits, showing no significant changes during short-term storage and a significant increase in soluble solids content during long-term storage. However, unexpected changes occurred in the juice samples that were stored, mainly a significant decrease in soluble solids content, revealing opportunities for further research.

Research Contributions

Morgan Zielinski (MZ) designed the experiment, performed laboratory analyses on fruit samples, and wrote the manuscript and SOPs with guidance from Tammy Windfelder and Alex Bajcz (AB). MZ, AB, Erica Cowper, and Robert Zielinski carried out the fieldwork to collect samples. MZ was also assisted by members of Dr. Windfelder's 2021 DSSI group during sample collection.

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Appendix A: Preparation of *Rubus* Samples for Fruit Chemistry Tests

Morgan Zielinski & Dr. Alex Bajcz

Last modified September 2021

General Purpose: To make general-use juice samples from *Rubus* fruits to use in fruit chemistry tests such as refractometry, titration, spectrophotometry, etc.

Equipment and PPE:

15 mL centrifuge tubes + caps
50 mL centrifuge tubes + caps
Deionized water (obtained from HS112)
Permanent marker
Tissue muddler that will fit inside both 15mL and 50mL centrifuge tubes
Disposable nitrile gloves
Lab coat
Kim wipes
Centrifuge that can hold 15mL centrifuge tubes (found in HS129)
Centrifuge that can hold 50mL centrifuge tubes (found in HS112)
Centrifuge tube racks for holding centrifuge tubes upright
Portable cooler and ice packs
Garbage can or plastic bag for waste disposal

Hazards: Stains to skin or clothing

Gloves should be worn during this procedure to protect against staining your hands and to protect the samples from contamination. A lab coat should also be worn to protect one's clothes.

Procedure for preparing 15mL centrifuge tubes:

Steps 1-4 of this procedure should be followed in HS102. Step 5 should be followed in HS129.

1. Before handling fruits, put on nitrile/latex gloves. Transfer the sample bags from up to six plots at a time to the portable cooler with ice packs in it. Label a corresponding number of 15mL

centrifuge tubes with the respective plot codes from the sample bags using a permanent marker. Each tube should be labeled in three places in case part of the label is rubbed off during this procedure. One of the labels should be on a small piece of lab tape stuck to the tube.

2. Remove one bag from the portable cooler. If the berries are not frozen together, shuffle them inside the bag with your fingers before selecting berries, to avoid making a biased sample. If the berries are frozen together, try to separate the clump and take from all parts of the bag when selecting your sample, again to avoid a biased sample. A small empty beaker or centrifuge tube rack can be used to keep the tube upright while selecting berries from the bags. The tissue muddler can also be used at this step to gently push berries down if they do not fall to the bottom of the tube on their own. For fresh samples, fill the tube to about the 13 mL mark to prevent spills when using the tissue muddler in Step 4; for frozen samples, fill the tube to the top (the berries will fall down further, taking up less total space, as they thaw in Step 3). Close the bag of fruit, pressing extra air out, and return it to the portable cooler. Repeat this step for all of the sample bags.

Note: Don't pack the berries too tight in the centrifuge tube. It is okay to push them down slightly with the tissue muddler to fill in empty space, but the berries should not be completely broken while filling the tube.

3. If preparing samples from fresh fruit, proceed to Step 4. If preparing samples from frozen fruit, thaw the fruits in the centrifuge tubes before using a tissue muddler. Pour warm water from the tap into a large beaker and place the full centrifuge tubes in the beaker for 30 minutes before moving on to Step 4. This makes the process of breaking up the fruits easier than if they were still frozen.

4. Use the tissue muddler to break up the berries in the centrifuge tube for at least one minute, until there are no individual intact drupelets remaining. Replace the centrifuge tube's cap and place the tube in a rack. Clean the tissue muddler with a Kim wipe and rinse with DI water over the sink. Repeat Steps 3 and 4 for the remaining plot bags in the cooler. When all sample tubes are prepared, return the sample bags to the freezer.

5. When six centrifuge tubes have been filled and prepared, bring them to the centrifuge in HS129. Plug the centrifuge in and press Stop to unlock the lid. Make sure the centrifuge is balanced by

placing tubes with similar volumes across from each other, then close the lid. If you have an odd number of samples, use a tube of water with a similar volume to the odd sample tube to balance the centrifuge. Centrifuge the samples at approximately 2700-3000rpm for about 3 minutes. When the samples are in the centrifuge and the lid is closed, turn the timer dial to 3 minutes and turn the speed dial to a high speed. Press the button in the middle of the timer dial to start the centrifuge, then adjust the speed dial to the desired speed. When the timer is up, the start button will pop out and the centrifuge will slow down until it reaches 0rpm; press the stop button to unlock the lid. Check the samples to ensure that there is a clear separation between liquids and solids in each tube. If not, place the tubes back in the centrifuge at a slightly higher speed. It may also help to stir the samples again with a tissue muddler before re-centrifuging. After centrifuging, remove the tubes, turn off and unplug the centrifuge, and bring the samples back to HS102.

Note: It isn't possible to over-centrifuge these samples, but it is possible to under-centrifuge them. If unsure, centrifuge at a higher speed/for a longer time.

6. Repeat steps 1-5 for any remaining plot bags.

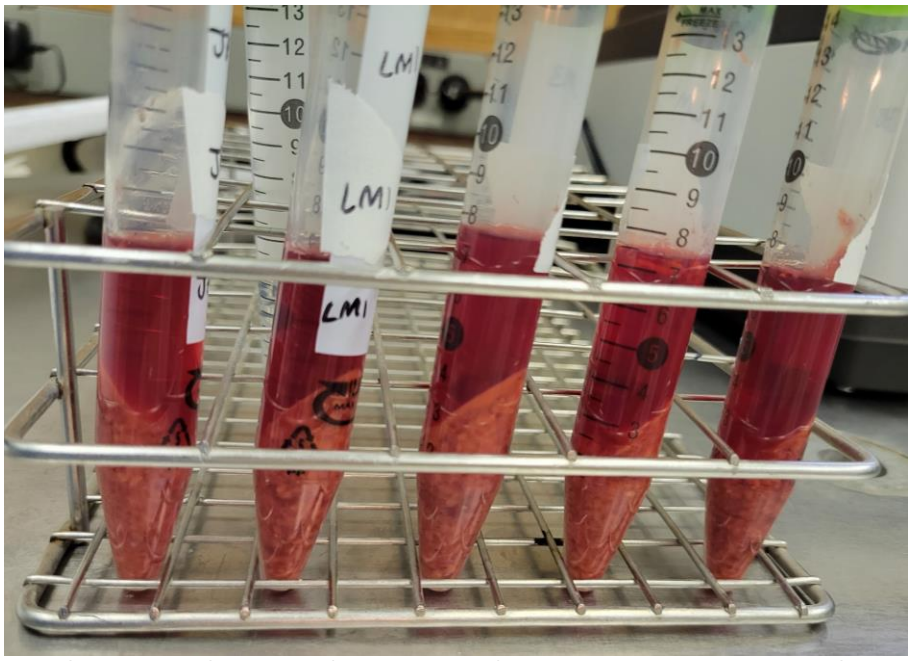

Figure 1. Example of 15mL centrifuge tubes after being centrifuged. Note the clear separation of solids and liquids in each of the tubes.

Procedure for preparing 50mL centrifuge tubes:

Steps 1-4 of this procedure should be followed in HS102. Step 5 should be followed in HS112.

1. Before handling fruits, put on nitrile/latex gloves. Transfer the sample bags from up to eight plots at a time from the freezer in HS102 to the portable cooler with ice packs in it. Label eight 50mL centrifuge tubes with their respective plot codes, using the permanent marker. Each tube should be labeled in three places in case part of the label is rubbed off during this procedure. One of the labels should be on a small piece of lab tape stuck to the tube.

2. Remove one bag from the portable cooler. If the berries are not frozen together, shuffle them in the bag with your fingers before selecting berries to add to your sample. If the berries are frozen together, try to take from all parts of the bag when selecting your sample. For fresh samples, the total volume of berries in the 50mL centrifuge tube should not go past the 40-45mL mark on the tube to prevent spills when using the tissue muddler. For frozen samples, the tube can be filled to the top, as the berries will fall and take up less space as they thaw in Step 3. A small empty beaker or centrifuge tube rack can be used to keep the tube upright while selecting berries from the bags. The tissue muddler can also be used at this step to gently push berries down if they do not fall to the bottom of the tube on their own.

Note: Don't pack the berries too tight in the centrifuge tube. It is okay to push them down slightly with the tissue muddler to fill in empty space, but the berries should not be completely broken while filling the tube.

3. If preparing samples from fresh fruit, proceed to Step 4. If preparing samples from frozen fruit, thaw the fruits in the centrifuge tubes before using a tissue muddler. Pour warm water from the tap into a large beaker and place the full centrifuge tubes in the beaker for 30 minutes before moving on to Step 4. This makes the process of breaking up the fruits easier than if they were still frozen.

4. Use the tissue muddler to break up the berries in the centrifuge tube for at least one minute, until there are no individual solid drupelets remaining. For fresh samples, place more randomly-selected berries in the tube up to the 40-45mL mark and use the tissue muddler again until there are no individual solid drupelets remaining. Replace the centrifuge tube's cap and place the tube in a rack.

Clean the tissue muddler with a Kim wipe and rinse with DI water over the sink. Close the bag of fruit, pressing extra air out, and return it to the portable cooler. Repeat steps 3 and 4 for the remaining plot bags in the cooler. When all of the sample tubes have been prepared, return the sample bags to the freezer in HS102.

5. When eight centrifuge tubes have been filled and prepared, bring them to the centrifuge in HS112. Press and hold the Open button on the centrifuge while opening the lid. Remove the cap from the sample holder. Make sure the centrifuge is balanced by placing tubes with similar volumes across from each other. If you have an odd number of samples, use a tube of water with a similar volume to the odd sample tube to balance the centrifuge. Replace the cap and close the lid. Centrifuge the samples at 5000rpm for 4 minutes. Press the Speed button on the centrifuge, then type 5000 and press enter on the number pad. Press the Time button, then type 4 and press enter on the number pad. Then press Start. When the centrifuge has stopped, press and hold the Open button while opening the lid, then remove the cap. Check the samples to ensure that there is a clear separation between liquids and solids in each tube. If not, place the tubes back in the centrifuge with the same settings. It may also help to stir the samples again with a tissue muddler before re-centrifuging. After centrifuging, remove the tubes, replace the cap on the sample holder, close the lid, and bring the samples back to HS102.

6. Repeat steps 1-5 for any remaining plot bags.

Appendix B: *Rubus* Fruit Juice Total Soluble Solids Quantification via Digital Refractometry

Morgan Zielinski & Dr. Alex Bajcz

Last modified December 2021

General Purpose: To determine the quantity of total soluble solids dissolved in the juice of *Rubus* fruits by measuring the juice's percent Brix.

Theory: Brix (or %Brix) is a measure of the concentration (grams solute/liter water) of dissolved solids in a solution. It technically measures the fraction of all dissolved solids, but for our purposes (and for applications in the culinary world), it is assumed that sugar makes up the majority, if not all of, the dissolved solids. By finding the %Brix reading for a sample of juice from a plot, and comparing these readings to a standard curve, we can estimate the sugar concentration of that plot's fruit. Because sugary foods are often sought out by animals, this type of information is useful for looking at trade-offs between plant defense and fruit quality, as we can compare trends in sugar content to the prevalence of defense characteristics.

Chemical Hazards: Sucrose is a common sugar also known as table sugar. In crystalline form, sucrose dust can be mildly irritating to sensitive tissues. Skin exposure poses no risk, nor does ingestion except in extremely large doses, which can result in digestive irritation. Sucrose is mildly flammable and will burn if exposed to flame.

Equipment and PPE

100mL bottles with caps
Crystalline sucrose or table sugar
Weigh boat
100mL graduated cylinder
Deionized water (obtained from HS112)
Digital scale sensitive to at least 0.01g
Scoopula
Orbital shaker

Permanent marker
Disposable nitrile gloves
Lab coat
Kim wipes
Digital refractometer that auto-corrects for ambient temperature
Digital pipette that can deliver 300μL + pipette tips
Garbage can or plastic bag for waste disposal
Refrigerator (Central storage area)

Chemicals: Sucrose

Hazards: Stains to skin or clothing

Gloves and a lab coat should be worn at all times during this procedure, according to Drew University's lab safety policies.

Procedure:

Steps 1-8 of this protocol should be followed in HS102. Parts of Step 2 should be followed in the central stockroom.

1. Prepare a data sheet with two columns: Plot and %Brix. One sample (corresponding to a row) will be taken from each plot. Fill in the first column of the table with the plot codes (e.g. DU1, DU2, DU3, etc.) found on the sample bags you are using. The %Brix column will be filled with the readings received from the digital refractometer.

2. Prepare a set of standard solutions. Label five 100mL bottles: 3%, 6%, 9%, 12%, and 15% sucrose. Using the balance and a weigh boat in the central stockroom, weigh out X grams of sucrose for each tube, X being equal to the percentage on the corresponding bottle. Return the sucrose/table sugar to its place in the central stockroom. Bring the bottles with the sugar back to HS102. Then, using a graduated cylinder, measure out and add 100mL of deionized water to each bottle. Turn on the orbital shaker in HS102 using the switch on the back, cap the bottles, place them in the holders on the orbital shaker, and press the "start/stop" button. Press the "start/stop" button again to stop the machine when there are no visible sucrose crystals remaining (after about

a minute, depending on the speed). Remember to turn off and unplug the orbital shaker when finished. When not in use, the sugar solutions can be stored in a fridge in the central storage room. However, if the sugar solutions were stored in the fridge before use, make sure they have returned to room temperature before reading them in the refractometer.

Note: You will also need a small beaker of deionized water to act as a 0% sucrose solution, and to calibrate and clean the refractometer.

3. Follow the steps in the Sample Preparation SOP to obtain samples for testing.

4. Remove the refractometer from its carrying case and place it on a clean workstation in HS102. Your workstation should also include: the beaker of deionized water, the set of standard solutions, a box of Kim wipes, a digital pipette set to 300µL, a bag of disposable pipette tips (more of these can be acquired from Central Supply if the cabinet in HS102 doesn't have more), a small surface or container to rest a designated DI-water-only pipette tip, a rack for centrifuge tubes, the prepared samples (kept upright in the rack), and a garbage can or plastic bag for waste disposal.

5. The digital refractometer must be calibrated before use. Turn the refractometer on using the power button on the front, open the cover, and cover the lens with 300µL of deionized water; then wipe it away with a Kim wipe. Add a second dose of 300µL of DI water (using the same DI-water-only pipet tip), close the refractometer's cover, and press and hold the Zero button for 2 seconds. Hit the Zero button again to calibrate the machine and wait; the machine will use DI water as a blank. The screen should read 0.0. If not, clean off the lens and try again, until the machine reads 0.0.

6. Proceed to read each of the standard solutions you have prepared, one at a time. The basic protocol for reading a sample is as follows:

1. Dispense 300µL of sample solution onto the lens, taking care to not let the tip of the pipette touch the lens, as doing so will create bubbles that impact the accuracy of the reading. *A new pipette tip should be used for every single sample,* the previous one being disposed of after one use. This is to prevent cross-contamination of samples. Pipette tips should be

ejected into the waste receptacle using the ejector button on the pipette, rather than removing them by hand.

2. Close the cover and press the Read button. Record the reading in the appropriate location on your data sheet. Fruit sample readings will go in the table; standard solutions can be written to the side or they can be recorded like standard samples but with their concentration in place of the Plot. For fruit juice samples, the Read button should be hit as soon as possible after each sample has been placed on the lens (no later than 10 seconds afterward). Some samples contain solids or colloids that may settle on the lens's surface and increase the reading if given time to do so. A sample should not be placed on the lens if you are unable to take the reading quickly enough.
3. After recording the reading, open the cover and remove the solution from the lens with a Kim wipe as completely as possible. Use the pipette to clean the lens at least once with 300µL of deionized water, wiping the deionized water off with a Kim wipe as well.
4. After a few samples, dispense 300µL of deionized water onto the lens, close the cover, and press Read to ensure that the machine is still calibrated. If the machine does not read 0.0 for the water, it should be re-calibrated; previous samples can then be re-tested to make sure they are accurate.
5. Only pipette tips used for deionized water can be reused - a single tip can be used for this entire procedure unless somehow contaminated, in which case it should be replaced.

7. Follow the sample processing protocol in step 6 above for each fruit sample, keeping in mind the following differences or issues:

1. Only fruit juice should be dispensed onto the lens - solids should be avoided. It can be difficult to tell where the juice is in a vial for darker samples. A tube can be held up to the light in this case - the juice will typically be along one side of the vial due to the orientation of the tubes in the centrifuge. Some samples may have solids floating on top of the juice, which can make it difficult to avoid getting solids in the pipette. Maneuver the pipette tip to be below these solids before taking up liquid, or set the sample aside to be centrifuged again, according to the instructions in the Sample Preparation SOP.

2. Try to take juice samples from about the midway point between the top of the juice and the solids at the bottom whenever possible. The goal is to ensure that samples from the same vial are repeatable, but Dr. Bajcz has anecdotally observed that juice towards the bottom of the vial, near the solids layer, will be thicker and possibly more rich in dissolved solids, resulting in a higher reading, while the reverse may be true for juice at the top of the sample.
3. If there are any bubbles in the juice on the lens, gently remove them with the pipette tip by popping them or "scraping" them out of the depression where the lens is located, but care must be taken to not scratch the lens. Only use a pipette tip for this, and it should be done very gently.
4. Aside from the usual zeroing of the machine in between samples, the refractometer should also be checked with a standard solution a few times throughout a larger batch of samples. If testing smaller batches of samples, read the standard solutions at the beginning of each day of testing. If a different reading is given for the standard solution than what was previously recorded for that solution, clean the lens and make sure that the machine is calibrated correctly by following step 5.

8. To clean up, remember to: 1) Make sure the lens of the refractometer is clean before closing the cover; 2) Turn off and unplug the refractometer, then return it to its case; 3) Turn off the pipette; 4) Put the refractometer, pipette, and bag of pipette tips back in the cabinet in HS102; 5) Make sure all centrifuge tubes have been placed in the freezer in HS102; 6) Wipe down any surfaces that may be sticky from juice; 7) Rinse out any beakers that were used for storage while working with DI water.

9. To prepare a standard curve for calculating soluble solids concentration, plot the %Brix readings of the standard solutions against their concentrations. Insert the best fit line of this graph, the equation of which will be used to calculate the soluble solids concentration of each sample, with %Brix being y, and the soluble solids concentration being x.

Appendix C: pH Differential Colorimetric Anthocyanin Test for *Rubus* Fruits

Morgan Zielinski & Dr. Alex Bajcz

Last modified September 2021

Reference: Lee J, Durst RW, Wrolstad RE. 2005. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *Journal of AOAC International.* 88 (5):1269-1278.

General Purpose: To determine the concentration of anthocyanin molecules in a *Rubus* fruit juice sample.

Theory: Anthocyanins are water-soluble pigments found in plant tissues, including their fruits. Depending on pH, anthocyanins can provide a blue, purple, black, or red color to plant tissues. They have protective qualities within a plant, protecting cells against excess light, extreme temperatures, and oxidative stress. They can also be an indicator of level of ripeness, as fruits often change color as they ripen.

Anthocyanins, being colored pigments, will absorb a certain wavelength of light, in this case 520nm. The samples' absorbances are also measured at 700nm to get the "background absorbance," which can be subtracted from the absorbance at 520nm to get the absorbance value for the sample. Two different pH buffer solutions are added to the samples, as degraded anthocyanins absorb light better at a pH of 4.5 and intact anthocyanins absorb light better at a pH of 1. As such, these two readings give us the relative ratio of intact to degraded anthocyanins.

Chemical Hazards:

Sodium acetate is a common salt that can be stored in a general storage cabinet. Inhaling sodium acetate can irritate the respiratory tract; ingesting large quantities can lead to nausea and vomiting. Contact with skin or eyes can cause irritation.

If significant exposure occurs, flush eyes or skin with water for at least 15 minutes. If ingested, do not induce vomiting; drink 2-4 cups of milk or water. If inhaled, remove yourself from exposure and to fresh air immediately. Seek medical care if symptoms worsen. To protect yourself,

handle sodium acetate carefully so as to avoid creating and/or inhaling dust. Wear goggles, gloves, and a lab coat while using sodium acetate.

Potassium chloride is a common salt that can be stored in a general storage cabinet. Inhaling potassium chloride can irritate the respiratory tract; ingesting large quantities can lead to nausea, vomiting, weakness, and circulatory problems. Contact with skin or eyes can cause irritation.

If significant exposure occurs, flush eyes or skin with water for at least 15 minutes and remove contaminated clothing. If ingested, do not induce vomiting; drink 2-4 cups of milk or water. If inhaled, remove yourself from exposure and to fresh air immediately. Seek medical care if symptoms worsen. To protect yourself, handle solid potassium chloride carefully so as to avoid creating and/or inhaling dust. Wear goggles, gloves, and a lab coat while using potassium chloride.

Reagent-grade hydrochloric acid is a very corrosive clear liquid and should only be stored in its designated corrosives cabinet in the stockroom. Vapors can cause damage to the respiratory tract, nose, teeth, and throat, and chronic exposure to vapors can be fatal. Ingestion will result in chemical burns in the mouth and digestive tract, abdominal pain, vomiting, and potentially death. Contact with skin causes chemical burns and ulceration. Exposure to vapors can cause irritation and burns to the eyes; contact with the liquid can cause severe burns to the eyes. Additionally, hydrochloric acid vapors can cause significant corrosion to metal surfaces and devices; care must be taken around such surfaces and devices to protect them from exposure to vapors. The vapor has a distinctive, acrid smell.

If significant exposure occurs, get medical care immediately. Flush eyes or skin with water for at least 15 minutes and remove contaminated clothing. If ingested, do not induce vomiting; drink water and seek medical care immediately. If inhaled, remove yourself from exposure and seek medical care immediately. To protect yourself, wear goggles, gloves, and a lab coat at all times while handling hydrochloric acid. Avoid breathing in vapors while using hydrochloric acid.

Equipment and PPE:

Lab coat

Disposable nitrile gloves

ThermoScientific Genesys 180 UV-Visible Spectrophotometer
Plastic spectrophotometer tubes/cuvettes (1cm path length)
Digital pipette that can deliver up to 1000 uL (1 mL)
Glass graduated pipettes + pipette aid
Kim wipes
Deionized water
500mL Erlenmeyer flasks
500mL glass bottles + caps
Large glass bottle with cap for chemical/sample waste
Magnetic stir bars
Magnetic stir plate
Digital pH meter with glass electrode + calibration buffer solutions
Disposable weigh boats
Scoopulas
Digital scale sensitive to at least 0.01g

Chemicals: Crystalline sodium acetate (reagent-grade, solid)
Crystalline potassium chloride (reagent-grade, solid)
Hydrochloric acid (reagent-grade)

Hazards: Chemical exposure; stains to hands and/or clothing

Gloves and a lab coat should be worn at all times during this procedure, according to Drew University's lab safety policies. Goggles should also be worn during Steps 1-3.

Procedure:

Steps 1-3 should be followed in the central stockroom. Steps 4-14 should be followed in HS102.

1. Make sure the pH meter in the stockroom is calibrated according to factory instructions. If unsure, ask the Biology Lab Manager for help. Place an empty weight boat on the scale in the central stockroom and weigh out 0.93g of KCl using a scoopula. Carefully place this amount into a 500mL Erlenmeyer flask; the plastic weigh boats can be bent to form a sort of funnel as you

pour. Add deionized water until the total volume is approximately 475mL. Place a magnetic stir bar into the flask and place the flask on a stir plate under the pH meter. Slowly increase the stir plate to about half speed. When the KCl is dissolved (no visible crystals remain), submerge the pH probe into the flask and wait for it to read the initial pH (read the same number for about 2 minutes) before proceeding. Using a graduated pipette and pipette aid, adjust the pH of the solution to 1 +/- 0.01 with hydrochloric acid by pipetting 1mL of HCl into the flask at a time, pausing between each dose to allow the pH reading to equilibrate. Depending on ambient conditions, this could take between 3mL and 20mL of HCl. (Note: contact with water will cause the hydrochloric acid to vaporize, and the fumes may be irritating. Pipet the HCl into the flask very slowly, angling the tip away from your face, and make an effort not to inhale close to the flask as you are adding the HCl. Goggles, gloves, and a lab coat should be worn at all times when working with HCl). Add additional deionized water to the flask to bring the volume to 500mL. Turn off the stir plate and remove the pH probe from the flask; rinse the pH probe over a waste jar using deionized water. Using a funnel, transfer the solution to a 500mL glass bottle, cap, and label with the chemical names and solution pH, plus warning labels for corrosivity from the central supply area.

2. Weigh 27.215g sodium acetate into a 500mL Erlenmeyer flask in a similar manner to Step 1. Add deionized water until the total volume is approximately 475mL. Place a magnetic stir bar into the flask and place the flask on the stir plate under the pH meter. Slowly increase the stir plate to about half speed. When the sodium acetate is dissolved (no visible crystals remain), submerge the pH probe into the flask and wait for it to read the initial pH (read the same number for about 2 minutes) before proceeding. Adjust the pH to 4.5 +/- 0.01 with hydrochloric acid by pipetting 1mL of HCl in the same manner as described in Step 1. Depending on ambient conditions, this could take between 10mL and 15mL of HCl. Add deionized water to the flask to bring the total volume to 500mL. Turn off the stir plate and remove the pH probe from the flask; rinse the pH probe over the waste jar using deionized water. Using a funnel, fully transfer to a 500mL glass bottle, cap, and label with the chemical names and solution pH.

3. Return all chemicals and equipment to their designated spots in the central stockroom. Transfer both buffer solutions to the fume hood in HS102.

4. Prepare a data sheet consisting of X rows, where X is the number of samples to be processed, and five columns. These columns should be titled, left to right: Plot, A520 pH 1, A700 pH1, A520 pH4.5, A700 pH4.5.

5. Follow the steps outlined in the Sample Preparation SOP to obtain juice samples.

6. Empty one of the styrofoam boxes that holds the spectrophotometer cuvettes. Count out the number of cuvettes you will need so that there are two for each sample you have prepared and place them in the box in two or three rows, depending on the number of samples. Extra cuvettes can be placed on a workstation in HS 102 until needed. There will be two spots for each sample because you will add two different pH buffers - one with a pH of 1 and another with a pH of 4.5. You can draw and label (with the sample code and pH) a grid on a piece of paper to help you keep track of which samples are in which cuvettes.

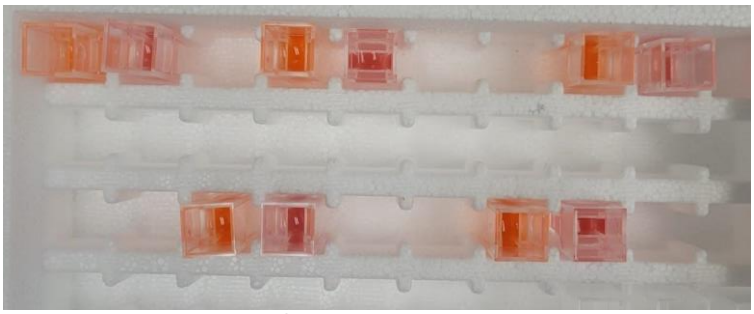

Figure 1. Example of cuvette set up. Each group of two cuvettes corresponds to one plot. The cuvette on the left of each group has had pH 1 buffer added to it; the cuvette on the right of each group has had pH 4.5 added to it.

7. Using the digital pipette, dispense 0.1mL (100uL) of each sample into its respective pH 1 cuvette. Using the same pipette, dispense 0.3mL (300uL) of each sample into its respective pH 4.5 cuvette. You can either reset the pipette to measure 0.3mL or dispense 0.1mL three times into each cuvette.

Note: The pipette tip must be changed for each sample to prevent cross contamination. You can, however, use the same pipette tip for both cuvettes of the same sample.

8. Move the two buffer solutions from the fume hood in HS102 to your workstation. Reset the pipette to measure 0.7mL (700uL), dispense 0.7mL of the pH 4.5 buffer into every pH 4.5 cuvette,

then dispose of the pipette tip. Reset the pipette to measure 0.9mL (900uL), dispense 0.9mL of the pH 1 buffer into every pH 1 cuvette, then dispose of the pipette tip. Close the buffer solutions and return them to the fume hood.

Note: Each spectrophotometer tube will need to be plugged with a gloved finger and inverted after the addition of buffer solution in order to properly mix them. Be very careful so as to not spill any of the very acidic solution. Also, spectrophotometer readings must be taken within 30 minutes of adding the buffer solutions because the stability of the color change reaction is unknown beyond this time period. Do not prepare cuvettes if you will not be able to put them in the spectrophotometer within 30 minutes.

9. Plug in and turn on the spectrophotometer. On the main menu, select Multi-Wavelength, then press the plus sign at the bottom-right corner to set up a new method. Press Setup towards the top-right to add an accessory. Choose the 8 Cell-changer; press the circles so that slot 1 is a blank and slots 2-7 are samples. In the wavelength table, enter 520 into the first column and 700 into the second column. All other columns should be blank. You can also name your method at this point and press the save button at the top right so it can be reused later.

10. Open the spectrophotometer. Fill a cuvette with deionized water and place it in the first slot to act as a blank. Cuvettes must be placed in the spectrophotometer so that the side with a triangle at the top will face the triangle on the machine when the cell-changer turns (See Figures 1 and 2).

11. Choose six cuvettes (three sets of two) to fill slots 2-7. Make sure to only pick cuvettes up with gloved hands, and to wipe the reading frame sides with a Kim wipe to remove any smudges.

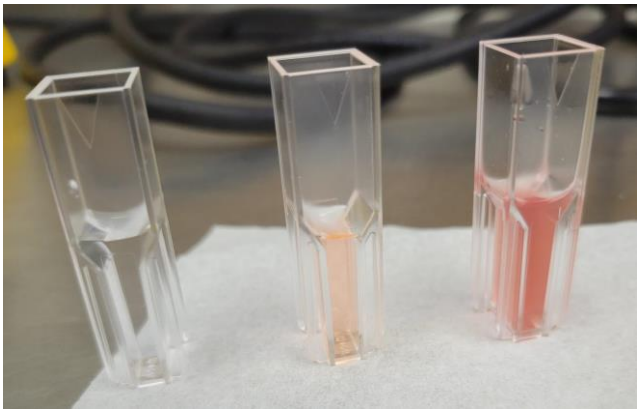

Figure 2. The reading faces, indicated by a triangle, are shown on these cuvettes. The triangle at the top should line up with the triangle on the machine (see Figure 3) when the cuvette is being read.

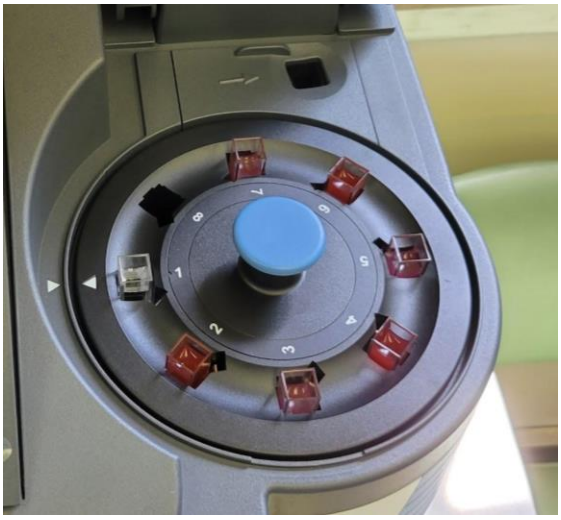

Figure 3. The cell changer on the spectrophotometer. Note the triangles on the cell changer and the machine. These triangles must line up before starting the run so the cell changer starts at slot 1.

12. Close the lid and press Start on the spectrophotometer screen. When the machine is done reading, record the results in their respective spaces on your data sheet.

13. Repeat Steps 11-12 for the remaining samples/cuvettes. If, at the end, you do not have six cuvettes to put in the spectrophotometer, you can change the accessory setup to read the correct number of samples.

14. To clean up, dump the contents of the cuvettes into the waste jar - hydrochloric acid-containing solutions should not be poured down the sink. Use deionized water in a squirt bottle to rinse each cuvette three times, dumping the contents into the waste jar the first time and into the sink the

remaining times. Place the cuvettes face down on a paper towel to dry. Close, turn off, and unplug the spectrophotometer. Wipe down your workstation thoroughly if there were any spills during this procedure. Cap the waste jar, label it with the names of all chemicals it contains, and transfer it to the fume hood in HS102.

15. Use the following equation to calculate the anthocyanin content of the fruits in units of cyanidin-3-glucoside equivalents:

Anthocyanins = $\{(A_{TAD} * MW * 1000mg\ anth./g\ anth.)/(EC * PL)\}$

where A_{TAD} is equal to the total absorbance difference, calculated via the equation:

$A_{TAD} = DF_1(A_{520nm} - A_{700nm})_{pH1} - DF_{4.5}(A_{520nm} - A_{700nm})_{pH4.5}$

where A represents absorbance as measured by the spectrophotometer at the given pH and wavelength. DF_1 and $DF_{4.5}$ are both dilution factors calculated by the following equation:

DF = (XmL buffer + YmL sample)/(YmL sample)

where X and Y are equal to the volumes of buffer solution and sample fluid, respectively, pipetted into the corresponding spectrophotometer tube. The units for the dilution factors are mL solution per mL sample.

Measures of absorbance are unitless.

and where MW equals the molecular weight of cyanidin-3-glucoside (449.2 g anthocyanin equivalents per mole); EC equals the extinction coefficient of cyanidin-3-glucoside (26,900,000 mL solution per mole per centimeter); and PL equals the path length of the spectrophotometer tube (usually 1 centimeter).

The resultant units of this equation are mg of cyanidin-3-glucoside equivalents per mL of sample.

Appendix D: Measuring Titratable Acidity of *Rubus* Fruit Juice

Morgan Zielinski

Last modified September 2021

Reference: Sadler GD, Murphy PA. 2010. pH and titratable acidity. In: Nielsen SS, editors. Food analysis. Fourth Edition. New York: Springer. p. 219-238.

General Purpose: To determine the concentration of acid in a *Rubus* fruit juice sample.

Theory: Titratable acidity is a measure of the total acid concentration of a fruit. The acids in a fruit are an important contributor to its taste, along with the sugar concentration; it is common to use a sugar:acid ratio to describe the flavor of a fruit, especially as it changes as a fruit ripens. Acidity can also affect the color of a fruit, as anthocyanins are pH-dependent pigments, and will change color as acidity changes.

Titratable acidity can be measured by neutralizing the acid in a fruit sample to a pH endpoint of 8.2, using a standard base like sodium hydroxide. Using the volume of titrant needed to neutralize the sample, the normality of the base, the equivalent weight of the predominant acid, and the volume of the sample, you can calculate the percentage of acid in the sample in weight/volume. A pH probe is used to find the endpoint, rather than an indicator solution, because the sample itself is already pigmented, making color changes harder to observe.

Chemical Hazards:

Solid sodium hydroxide is corrosive and should be stored in a closed container in a well-ventilated area away from acids and water/moisture, such as a storage cabinet or fume hood. Inhalation will severely irritate the upper respiratory tract and can cause chemical burns, coughing, and difficulty breathing. Ingestion can damage and chemically burn the digestive tract. It can also cause pain, nausea, and vomiting and induce shock. Contact with eyes can burn and cause blindness; contact with skin can create burns and skin ulcers.

If significant exposure occurs, flush skin or eyes with water for at least 15 minutes; remove contaminated clothing and seek medical care immediately. If ingested, do not induce vomiting; drink water and seek medical care immediately. If inhaled, move yourself to fresh air immediately

and seek medical care. To protect yourself, handle sodium hydroxide carefully so as to avoid creating and/or inhaling dust. Wear goggles, gloves, and a lab coat at all times while handling sodium hydroxide.

Equipment and PPE:

Lab coat
Disposable nitrile gloves
Lab goggles
1L graduated cylinder
Parafilm
50mL burette
Ring stand with clamp attachments to hold burette and pH probe
100mL beakers
Digital pH meter with glass electrode + calibration buffer solutions
Stir plate
Magnetic stir bars
Deionized water (from HS112)
100mL graduated cylinder
Digital pipette that can deliver up to 1000uL (1mL)
Large glass bottle with lid for waste

Chemicals: 0.1N sodium hydroxide

Hazards: Chemical exposure; stains to clothing or skin

Gloves, goggles, and a lab coat should be worn at all times during this procedure, according to Drew University's lab safety policies.

Procedure:

Step 1 should be followed in the central stockroom. Steps 2-11 should be followed in HS102.

1. Prepare a 0.1N sodium hydroxide solution. Measure out 4g of solid sodium hydroxide using a weigh boat on the scale in the central stockroom, and add it to a 1L graduated cylinder. Add water

up to the 1L line on the graduated cylinder. Cover the cylinder opening with parafilm and shake until all NaOH pellets have dissolved. Using a funnel, pour the solution into a bottle, then cap and label the bottle. Carefully transfer the sodium hydroxide solution to the fume hood in HS102.

2. Follow the steps in the Sample Preparation SOP to obtain fruit juice samples.

3. Create a data sheet with X number of rows, with X being the number of samples you will titrate, and 6 columns: Plot Code, Volume of Sample, Initial pH, Starting Volume of Burette, Final Volume of Burette, and Final pH.

4. Plug the pH probe in HS102 into the autotitrator, then plug in and turn on the autotitrator. Using the buffer solutions (pH 4, pH 7, and pH 10) for the pH probe in the central stockroom, calibrate the pH probe by using the autotitrator's calibration feature. Press the Measure button (shaped like a triangular ruler) at the bottom left of the titrator's screen, then press the CAL button. After placing the probe into one of the solutions, Start. Wait for the reading to settle on a number and for a check mark to appear on the right side of the screen, then press Accept. Follow the prompts on the screen to move on to the next calibration. Remember to rinse the probe off after putting it in each solution by using a squirt bottle of deionized water while holding the probe over a waste beaker. Return the buffer solutions to the stockroom.

Note: The pH probe should be calibrated on each new day of titrating.

Figure 1. Example of the calibration screen. Note the checkmark next to the pH reading, indicating that the reading has settled on a number.

5. Set up a ring stand with clamps to hold a 50mL burette and the pH probe. Place a stir plate under the burette and probe and plug it in. Make sure there is enough room for a 100mL beaker to fit on the stir plate under the burette and probe.

Note: The grey stir plates found in the chemistry labs fit best in this setup. If you are having trouble using the stir plate from HS102 for this procedure, ask a chemistry professor if you could borrow one of the grey stir plates.

6. Make sure the burette valve is shut. Using a funnel, carefully pour a small amount of 0.1N NaOH into the burette. With the burette over a waste beaker, turn the knob to allow the NaOH to flow out, rinsing the burette. When the burette is empty, turn the knob to close it; using the funnel, pour more NaOH into the burette. If you are going to titrate many samples, fill the burette to about the 0mL line; if you are only going to titrate a few samples, you can pour closer to the 35mL line on the burette. Record this initial volume as the Starting Volume in the first row of your data table.

7. Place a small stir bar into a 100mL beaker. Using a pipette, dispense 1000uL (1mL) of a juice sample into the beaker. Record this volume and the plot code of this sample in your data table. Using a graduated cylinder, measure 49mL of deionized water and add it to the beaker. Place the beaker on the stir plate; turn the stir plate up to 100 rpm for about 30 seconds to ensure the sample is thoroughly mixed before continuing.

8. Adjust the clamps so that the burette is positioned over the beaker and the pH probe is submerged in the fruit juice solution without touching the bottom/sides of the beaker or the stir bar. Press the measure button (shaped like a triangular ruler) on the autotitrator to see the current reading on the pH probe. Wait for the pH probe to settle on a number (a checkmark will appear next to the number on the screen); record this as the initial pH in your data table.

9. The target endpoint for the titration is a pH of 8.2. Carefully turn the knob on the burette until the flow just barely starts; the ideal flow rate is between one drop per second and one drop every other second. Allow the burette to flow like this until the pH begins to read around 6.0. Close the burette and wait for the pH reading to stabilize. Reopen the burette valve so the flow is about one drop every other second, until the pH reading is around 7.0; then close the burette and allow the

pH reading to stabilize again. Add NaOH one drop at a time, with a few seconds between drops. When the reading reaches a pH of about 7.5-7.9, write down the pH and the volume in the burette as the first "final volume." Add another drop of titrant, after which the pH reading will likely increase above 8.2; close the valve completely and record the pH and the volume in the burette as the second "final volume."

Note: During titration, do not bump the table or move the beaker/pH probe, as this will cause the pH reading to jump, making it difficult to get an accurate reading.

10. To clean up after each sample, spray the pH probe with a squirt bottle of deionized water while holding it over the waste beaker. Place the probe in a beaker of deionized water in between samples, as the probe can be easily damaged if not kept moist. Use a magnetic stick to remove the stir bar from the beaker and rinse it with deionized water over the waste beaker. Pour the fruit sample/NaOH solution into the waste beaker. Do not pour fluids containing NaOH in the sink. Rinse the beaker with deionized water three times, once over the waste beaker and twice over the sink before reusing or storing.

11. Repeat steps 7-10 for the remaining samples. The final volume from the previous titration will be the initial volume for the next titration unless the volume of the burette is below the 45mL mark, in which case the burette should be refilled.

12. To calculate titratable acidity as a percentage of fruit juice volume (wt/vol), use the following equation:

$$\%\text{acid (wt/vol)} = [(N \times V_1 \times \text{Eq wt})/(V_2 \times 1000)] \times 100$$ where:

N = the normality of the titrant (mEq/mL)

V_1 = the volume of titrant (final volume - initial volume) (mL)

The smaller of the two final volumes (that produced a pH slightly below 8.2) should be used in the equation.

Eq wt = the equivalent weight of the predominant acid in the sample (mg/mEq)

V_2 = the volume of the sample (mL)

1000 = converts mg to g