Managing Apple Maturity and Storage to Improve the Quality of Virginia Hard Ciders

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This thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Food Science and Technology

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November 30, 2016

Blacksburg, Virginia

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Abstract

Though the cidermaking process is very similar to that of winemaking, there is a lack of scientific knowledge as to how orchard management practices and fruit storage affect the quality of the resulting cider. This research examined how both varying harvest maturities and post-harvest storage temperatures and durations in apple cultivars Dabinett, Brown Snout, and York impacted fruit quality as well as the chemistry of the juice and cider. Harvest intervals of two weeks before maturity, at maturity, and 2 weeks after maturity resulted in significant differences in fruit quality and juice chemistry, but few of these differences persisted in cider chemistry. Nonetheless, differences in concentration of some individual polyphenols determined by UPLC-MS were observed in ciders made from fruit harvested at different stages. For example, cider made from optimally mature Dabinett had over 250% the concentration of procyanidin B5 that was found in cider made from fruit harvested earlier or later. The storage treatments also resulted in substantial differences in fruit and juice chemistry, but fewer differences in cider chemistry. As with the harvest maturity experiment, differences in individual polyphenols were detected, with ciders made from cv. York having 20% higher epicatechin concentration when stored for 6 weeks at 1°C rather than 10°C. Finally, the accuracy of the Folin-Ciocalteu (FC) assay, commonly used for quantification of total polyphenols in fruit juices and fermented fruit beverages was critically evaluated. Reducing sugars in the sample matrix did not affect the results of the FC assay, whereas the presence of the amino acid tyrosine resulted in significant overestimation of total polyphenols in fruit juice by the FC assay.

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Public abstract

Cider production and consumption in North America has increased rapidly in recent years. Though cidermaking is in many ways similar to winemaking, there is a lack of scientific knowledge as to how orchard management practices and fruit storage affect the quality of cider. This study examined how varying the harvest date and the ways in which apples are stored after harvest but before cidermaking can affect cider quality. This work was conducted using the apple cultivars Dabinett, Brown Snout, and York. We found that differences in fruit maturity at harvest as well as post-harvest storage parameters impacted fruit, juice and cider quality. However, the differences observed in cider quality as a result of these treatments were much more subtle than the observed differences in fruit and juice quality. This work indicates that in order to accurately assess the impact of orchard management and post-harvest treatments on cider quality, cider should be made and evaluated, rather than assuming that the differences observed in fruit and/or juice will directly translate into cider quality differences.

Acknowledgements

First, I would like to thank the Virginia Wine Board for funding this project as well as an additional year of the study.

I would like to sincerely thank Dr. Amanda Stewart for her guidance throughout my graduate experience. Thanks to her, I was constantly challenged but supported throughout my research, and I have grown as a scientist and as a person because of it.

Thank you to Drs. Greg Peck and Andrew Neilson for your advice and assistance throughout this research.

Thank you to the faculty, students, and staff in the Food Science and Technology department. A big thank you to Sihui Ma for her research guidance and, more importantly, her friendship. I would like to especially thank Kayla Moberg and Natalie Pulido for being incredible friends throughout this whole experience.

Of course, all is much more rewarding with the love and support from my family and friends. Thank you, Mom and Dad, for always knowing my strengths and capabilities and for brainwashing me into the person I am today.

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CHAPTER 1: INTRODUCTION

The increased popularity of hard apple cider in recent years has created the opportunity for cider producers to compete for a growing share of the alcoholic beverage market. As with beer and wine, there are ciders of varying quality, style and price to appeal to a variety of consumers. Relatively little research has been dedicated to the science of cider production, especially when compared to the body of research-based knowledge available to commercial producers of beer and wine. The present research project aims to better understand the relationship between cider production practices from orchard to bottle and resulting product quality in order to provide practical resources and recommendations for producers.

Decades of research have been dedicated to wine grape maturity and its impact on the chemical and sensory properties of resulting wines, whereas there is a lack of sufficient information relating apple maturity to cider quality. Apples at varying stages of maturity have different physical and chemical compositions. Riper apples generally are larger and heavier and have higher sugar contents, lower starch content, higher levels of volatile aromas, lower levels of acidity, and increased skin pigmentation [1-3]. The magnitude of these differences is variable across cultivars. However, unripe apples may have a greater long-term storage potential due to lower ethylene concentrations at the onset of storage. It is possible to store apples for 6-13 months before processing, but the chemistry of apples may change during storage and continued maturation, thus necessitating the evaluation of the effects that storage will have on apple, juice, and cider quality[4, 5].

Lastly, one common method of determining total polyphenols in fruit juice, wine, and cider is the use of the Folin-Ciocalteu (FC) assay. There is growing evidence that this method is non-specific to polyphenols and that it may be more accurately represented as a means to measure total antioxidant capacity or reducing compounds, rather than total polyphenols, per se. Therefore, the evaluation of substances found in fruit juice, cider, and wine that may potentially interfere with the FC assay can provide better understanding of the results and provide improved context for the appropriate application of the method and interpretation of results in horticulture and food science research.

1.1 Long-term Objective

The long-term objective of this research area is to quantify the degree to which harvest maturity stage and post-harvest storage conditions of apples affects the quality of cider.

1.2 Overall Objectives

The overall objective of this study is to understand how fruit maturity at harvest and post-harvest storage duration and temperature affect maturity and chemical quality parameters of the fruit, juice, and resulting cider. As cider production volume in Virginia increases, a better understanding of the extent to which harvest maturity and post-harvest storage of apples impact cider quality will be essential to managing logistics of increased production while maintaining cider quality.

1.3 Specific Objectives and Hypotheses

Specific Objective 1

Determine if and to what extent harvest maturity of apples affects physical and chemical qualities of fruit, juice, and cider.

Working Hypothesis: The maturity of the apples will affect fruit, juice, and cider quality.

Specific Objective 2

Determine if and to what extent post-harvest storage of apples affects physical and chemical qualities of fruit, juice, and cider.

Working Hypothesis: The post-harvest storage temperature and duration will affect fruit, juice, and cider quality.

Specific Objective 3

Determine the extent to which the presence of reducing sugars and tyrosine in juice affects the accuracy of the Folin-Ciocalteu (FC) assay in determining total polyphenols.

Working Hypothesis: The presence of reducing sugars and tyrosine in juice does affect the results of the FC assay in an additive manner.

CHAPTER 2: LITERATURE REVIEW

2.1 Definition of Cider

In the United States, the term "cider" often refers to cloudy, unfiltered apple juice. However in England, the term "cider" refers to what Americans know as "hard cider," the fermented, alcoholic beverage. Alcoholic cider can also be described as being "apple wine" due to its production similarities to white wine production [1, 6]. The term "cider" used in this document refers to the alcoholic beverage made from apple juice [7].

There are various styles of ciders made from fermented apple juice. Cider can be still or sparkling, and it can by dry or sweet [1]. Typically, cider has an alcohol content of 5-10%, but sparkling sweet cider can have an alcohol content as low as 1% [6, 8]. In production of both sparkling and sparkling sweet cider, carbon dioxide gas is produced during fermentation resulting in effervescence in the final product. Effervescence can also be obtained by charging still fermented cider with carbon dioxide or by following sparkling wine production methods including methode champenoise or the Charmat process. A sweeter-tasting cider can be produced in two ways. The first is with cider that has not been fermented to dryness, leaving some residual sugar. The second is to add sugar or apple juice back to cider that has been fermented to dryness, a process commonly referred to as back-sweetening [6]. In the United States, ciders flavored with adjuncts such as fruit flavors or hops represent a new category of cider styles that is gaining popularity. Ciders can be further classified by their processes or cultural origins [1].

2.2 History of Cider

Cider in North America had been an important beverage for consumption and trade in the 18th century [9-11]. Because access to unpolluted, palatable drinking water was often not available and importation of other beverages from Europe was expensive, homemade cider became a staple in households for people all ages [12].

Virginia has been regarded as a favorable climate for cider production for hundreds of years. Thomas Jefferson made his own cider at Monticello, which he had brought to the White House during his presidency [1, 13]. Later in life, he wrote that cider was his "main table drink" [13]. A book written by William Coxe in 1817 mentions the James River in Virginia as an optimal location for cider production [14]. It is apparent from this text that Virginian cider has a history that extends for centuries.

Unfortunately, the association between farmers and ciders contributed to a decline of cider consumption in England and North America in the 18th and 19th centuries, respectively. Cider had been perceived as an inexpensive alcohol source for the working class instead of a beverage capable of refinement and complexity [1, 12]. In North America, cider was a targeted beverage in the temperance movement and its acceptance therefore suffered immensely [12, 15].

Despite the decline of cider production and consumption in the past, cider has been rapidly growing in popularity in several global locations, including North America [12]. The increase in popularity since the mid-2000s has resulted in the growth of existing and emergence of new cider producers [16].

2.3 Cider Market and Research Relevance

Although the cider market in the United States is less than 1% the size of the beer market, it is growing rapidly. Cider consumption has increased alongside that of craft beers, and it is capturing the attention of various demographics. Part of its popularity can be attributed to the variety of cider flavors available as well as it being a gluten-free product. Furthermore, the lighter, fruitier taste of cider compared to beer is attractive to both female and newer consumers of alcohol, as well as health-conscious individuals [7, 17].

Sales and production of hard cider have grown tremendously over recent years. Cider has become the fastest growing sector of the alcoholic beverage market. Supermarket sales grew 65 percent from 2011 to 2012 compared to a 5.6 percent growth in wine and a 13 percent growth in craft beer sales [12]. The number of new cidermakers entering the market has dramatically increased since 2008, as represented in Figure 2.1. In 2015, there were 126 new cidermakers in the United States, which is the largest number of new cideries reported in one year in the US to date [16].

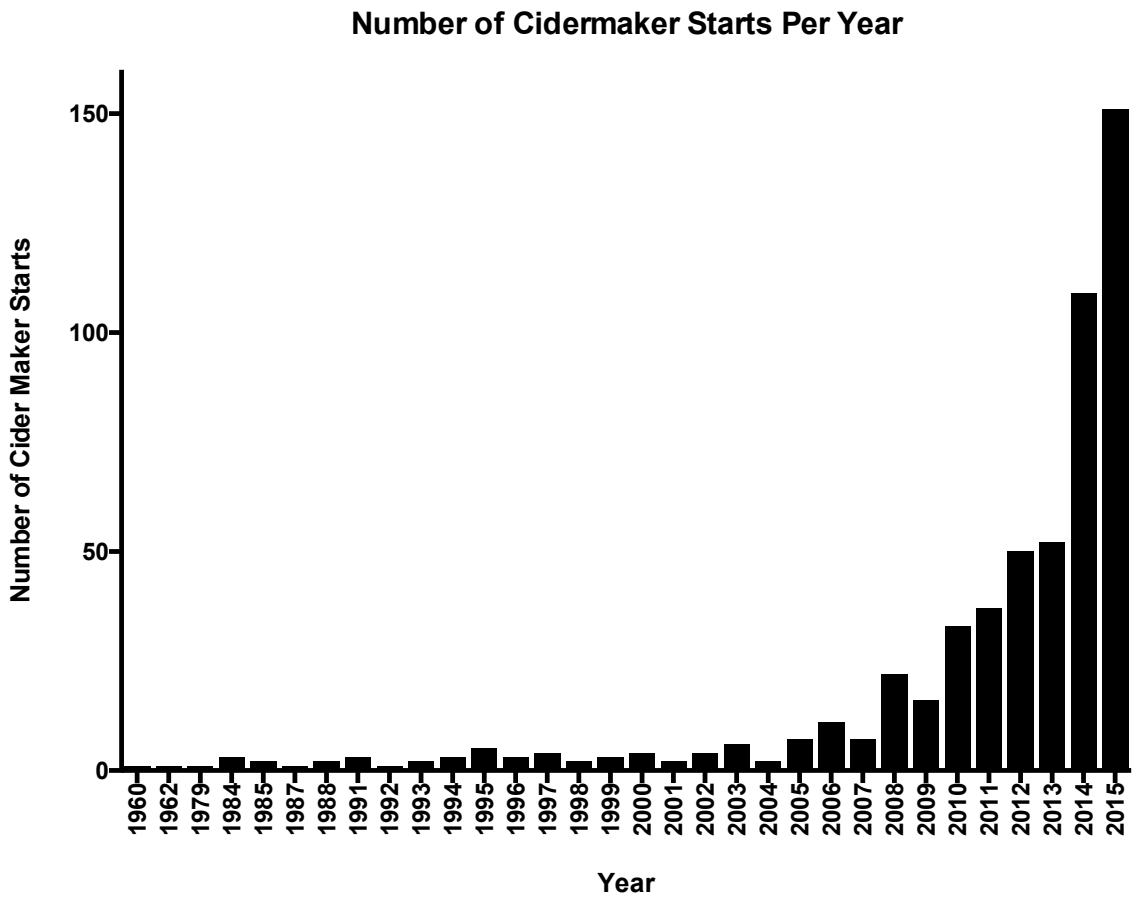

Figure 2.1: Starts of USA Cidermakers by year as of December 2015 [16] (adapted from Cyder Market LLC, 2015).

The top four cider brands in the United States make up 69.2% of the cider market[17] Woodchuck, a brand of Vermont Hard Cider Co, was the largest brand in the United States and increased 40% in sales from 2011 to 2012. Boston Beer's Angry Orchard brand was the fastest growing brand in the market in 2013, and is now the largest brand in the market with 34.6% of the industry[17 7].

According to The Cyder Market, LLC. as of December 2015, cider is being commercially produced in 43 states and Washington D.C. Although the majority of cider producers are in the northeastern, northwestern, western, and mid-northern regions of the

United States, there have been new cider producers emerging in the southwestern and southern regions, including Virginia [16].

2.4 Climacteric Ripening

Apples are climacteric fruits, meaning that the ripening process is promoted by ethylene gas [18]. Ethylene is produced in small amounts in unripe fruit and larger amounts in riper fruit [19]. Its production is autocatalytic, meaning that the presence of some ethylene will promote the biochemical pathway leading to further production of ethylene [2]. Apples that are harvested before they are fully ripe have very small amounts of ethylene and will have slower rates of respiration, fruit softening, and acid degradation. This allows fruit harvested earlier in the maturation process to be stored for longer periods of time compared to apples harvested when they are riper. However, apples harvested earlier may not develop the same concentration of volatile components that provide desirable flavor and aroma characteristics [2, 3, 20]. Cider producers mostly consider the effect of apple maturity and ripening to obtain the desired levels of sugar and malic acid for cider making. As fruit ripens, starch is converted to sugar and malic acid is lost through respiration [1].

A recent study by Alberti et al. examined the effect of harvest maturity on the juice and cider of three dessert cultivars: Fuji, Gala, and Lis Gala. Juice and cider made from unripe, ripe, and senescent apples showed varying levels of sugars, acids, amino acids, phenolic compounds, volatile compounds, and minerals. Sugar content in the juice increased with apple maturity for all three cultivars, but the sugar composition was cultivar-dependent. Fructose was consistently the lowest in the unripe apples compared to

the ripe and senescent apples, and glucose concentration had a greater increase throughout ripening compared to fructose. The malic acid levels in the juice decreased during ripening, but showed no significant difference in the resulting ciders due to the production of additional organic acids during fermentation through yeast metabolism. Total juice nitrogen content determined by the Kjeldhal method decreased as apple maturity increased, but aroma compounds in juice and cider increased with maturity. It was proposed that the nitrogen content decreased as it was used for volatile compound synthesis. Polyphenol compound composition varied across cultivars, ripening stage, and class of polyphenols [3].That work demonstrated that harvest maturity can impact apple composition, but further studies are necessary to draw conclusions as to the extent to which harvest maturity decisions can impact cider composition and quality.

It can be economically and logistically favorable for cider producers to be able to store apples for extended periods of time. This practice reduces the pressure to process immediately at harvest, allowing cider producers more control over processing dates and throughput through the cidery. Furthermore, cider producers could manipulate harvest and storage factors to obtain fruit ideal for a particular style of cider, or to improve characteristics of dual-purpose or dessert-style apples. It is therefore useful to improve our understanding of how apple maturity and post-harvest storage affect quality parameters such as sugar, acid, nitrogen, and phenolic content in fruit, juice, and cider.

2.5 Classification of Apples and Resulting Cider Characteristics

Cider can be made from a wide variety of apple cultivars, but individual cultivars have chemical and sensory differences. Blending ciders made from bitter and non-bitter

apples produces cider with qualities that would not be possible with cider from just one cultivar [12, 21]. It is very difficult to achieve the desired balance between sugars, acids, and polyphenols using a single cultivar [1]. Furthermore, the availability of high-tannin apples is oftentimes limited due to relatively few dedicated plantings of high-tannin cultivars, so cider producers may use a combination of low-tanning fruit and apple juice concentrate to meet the growing demand for cider in the marketplace[22].

According to the USDA National Nutrient Database, apples on average contain approximately 10g sugar/100g of whole, raw apples. On average, 23% of the sugar is glucose, 57% is fructose, and 20% is sucrose and the average moisture content for apples is 86% [23]. According to Lea (2003), to obtain a balanced cider flavor when blending, the juice should contain 70-110g fructose/1L juice, 15-30g glucose/1L juice, and 20-45g sucrose/1L juice, and 2-10g sorbitol/1L juice [1]. The majority of apple acid is malic acid [1, 24]. The pH for cidermaking is recommended to be in the range of 3.3-3.8 and the target range for titratable acidity as malic acid is generally 0.2-0.5g malic acid/100mL[1].

Apples can be further classified into sweet, bittersweet, sharp, and bittersharp categories based on their concentrations of polyphenols and malic acid, measured by weight per volume. Table 2.1 shows the classification of apples based on the amount of acid and tannin content as determined by the Lowenthal Permanganate titration [25]. These classifications were developed originally for English cider apples and provide a general guideline for selection of cultivars that provide the desired levels of acid, sugar, and tannin character [1, 21, 26]. As the term "tannin" is often used in wine and cider literature, we have chosen to use it here in our adaptation of this table from the cider literature. However, moving forward in our research, we prefer to use more specific classes of

chemical compounds, such as procyanidins, to quantify classes of polyphenols contributing bitterness and astringency in cider. Selection of the best analytical method for the next revision of this classification system is a topic of ongoing research.

Table 2.1: Classification of Cider Apples [1] (Adapted from Lea, 2003).

	Acid (g/L)	Tannin (g/L)
Sharp	>4.5	<2.0
Bittersharp	>4.5	>2.0
Bittersweet	<4.5	>2.0
Sweet	<4.5	<2.0

As of 2015, the amount of apples harvested in the United States has increased when compared to a five-year average. The state of Virginia is the sixth largest apple producing state by production volume in the United States with a total of 2,955,326 bushels harvested in 2015 [27]. Nonetheless, due to the limited amount of high-tannin apple cultivar plantings in Virginia, cider makers who wish to incorporate high-tannin cultivars (usually bittersharps or bittersweets) into a cider blend must purchase high-tannin apples, juice, or concentrate from out of state producers. However, there may be economic incentive for some Virginian apple growers to begin planting high-tannin apple orchards. A study by Farris et al. in 2013 examined the potential economic outcome of high-tannin apple growers in Virginia. For a particular set of assumptions relevant to Virginia's industry and published in the report, it was estimated that a grower could net $138,404 per acre over a period of 25 years. Cider producers surveyed expressed that

they would be willing to pay between $12 and $18 per bushel for high-tannin apples compared to between $8 and $13 per bushel for cultivars that can be utilized for more general purposes. Despite the potential for economic gains, growers would still be taking a greater risk by planting high-tannin cultivars than if they were to grow multipurpose cultivars. Multipurpose cultivars could be marketed to not only cider producers, but also for fresh market purposes. High-tannin cultivars also may be subject to biennial bearing and challenges not associated with other lower-tannin cultivars [8].

2.6 Juice Clarification

After pressing apples into juice, the juice is turbid, or cloudy, due to the presence of suspended solids. The suspensions are caused by methoxylated pectin substances that when demethoxylated will sediment at the bottom of the container [28]. There are several ways of clarifying juice, including centrifuging, filtering, and settling as well achieving clarification with pectic enzymes, also known as "pectinases" [29]. Apple juice contains three types of pectins that can be broken up by a mixture of the enzymes pectinesterase, pectingalacturonase, and pectinlyase [29] Some cider makers will not clarify the juice before beginning fermentation due to the belief that removing solid particles may also remove important compounds for yeast nutrition, slowing fermentation rates [30]. In addition, the juice and yeast naturally contain some pectinase activity, so clarification by other means may not always be necessary. However, if there are remaining suspended particles after fermentation, they will be much more difficult to remove than had they been removed before fermentation [1]. This is because methoxylated pectins become more soluble as ethanol concentrations increase.

Heating apple juice before inoculation causes additional problems during clarification. Although properly applied heat treatments kill undesired microorganisms, heat also inactivates the enzyme pectin esterase. This prevents demethylation of the pectins by this enzyme thus inhibiting clarification of the juice [31]. Low-temperature microwave pasteurization has also been shown to destroy pectinases in orange juice [32]. Therefore, if pasteurization is a part of cider processing, it should be done after fermentation.

Long-term storage of fruit prior to juice processing may also create additional problems in juice clarification. A study by Hsu et al. examined Granny Smith apple juice clarity using juices produced from apples that were stored at 1°C for either three months or nine months. The juice produced from apples stored at nine months had a higher level of haze formation than the juice produced from apples shored at the shorter three-month interval. Though the short-term storage treatment had a greater concentration of proteins, Hsu et al. found that the juice from the long-term storage treatment contained phenols and polysaccharides that, with proteins, may contribute to haze formation through interactions [33]. Therefore, the chemical changes of apples during storage may greatly contribute to the resulting clarity in the juice.

2.7 Yeast Selection

Spontaneous fermentation by naturally occurring yeast and bacteria is a common practice in traditional cider making. The yeast and bacteria originate from either the fruit itself or the surfaces of equipment in the cidery [1, 31, 34-36]. In these "natural" fermentations, there are generally several yeast species present that facilitate the alcoholic fermentation.

Metabolism by yeast and bacterial species during the alcoholic fermentation result in a variety of aromas, both desirable and undesirable. Species of *Candidia, Kloeckera, Hansenula, Metschnikowia, Pichia, Torulopsis, Hansenosporia, Rhodotorula,* and *Saccharomyces* are found on the apple surface, though *Saccharomyces* is often present in higher levels on the pressing equipment [1, 4, 31, 35]. Grape wine research has shown that *Candida* and *Kloeckera* species are significant in spontaneous fermentations, but they are unable to tolerate ethanol concentrations higher than 5-6% (v/v) [37-39]. *Saccharomyces* yeast species will dominate when ethanol concentrations are above 5-6%[1, 36-39]. In winemaking, the ethanol concentration is generally above 5-7% after the first half of the fermentation process. However in cider fermentation, the final alcohol may not exceed 5-7%, such that the native yeast and bacteria may remain active and have greater potential to contribute spoilage or off-aromas than in a grape-based wine fermentation [1].

Currently in commercial operations, it is common for cider fermentations to be inoculated with active dried *Saccharomyces* yeast. *Saccharomyces cerevisiae* and *Saccharomyces bayanus* species are the most and second most commonly used wine yeast species, respectively, and interspecific hybrids between the two are common in wine and cider production [40]. Sulfur dioxide is added prior to inoculation in part as an antioxidant and to inhibit growth of any competing microflora. This provides a competitive advantage to the inoculated yeasts, which are selected in part based on resistance to low concentrations of added sulfur dioxide [1, 31]. There are several strains of commercially available active dried yeast that can be selected based on fermentation rates, desired volatile characteristics, low acetic acid and hydrogen sulfide production and overall wine quality. There have been hundreds of volatile compounds identified that

result from yeast metabolism. Specific yeast strains have the potential to alter the aroma and overall quality of the final product [37, 41]. As observed in wines, the utilization patterns of different yeast species may contribute to the types and amounts of aroma compounds present in cider [42].

Lalvin EC1118 is a strain of *Saccharomyces cerevisiae bayanus* that has been isolated from the Champagne region of France. This yeast relatively tolerant to high alcohol concentrations and wide temperature ranges, and it is often employed in enology research [43, 44].

2.8 Yeast Assimilable Nitrogen

Definition and Implications of Yeast Assimilable Nitrogen

Nitrogen is one of the most important yeast nutrients for fermentation progress and completion [15, 42, 44-47]. Yeast assimilable nitrogen (YAN) in apples can vary between cultivars, orchards, harvest years, and orchard management practices including crop load [45, 48].

YAN is composed of nitrogen sources that are readily utilized by yeast [44]. For *Saccharomyces cerevisiae*, the most important nitrogen sources include primary amino nitrogen (PAN), also sometimes referred to as free amino nitrogen (FAN), and ammonia, but low molecular weight peptides may also be utilized as nitrogen sources [42, 44, 46]. For this reason, YAN is usually measured by summings the ammonia and primary amino acid concentrations. In apple juice 86-95% of the total amino acids are composed of asparagine, glutamine, aspartic acid, glutamic acid, and serine [31, 45], although variation in composition has been observed across cultivars. Other prevalent amino acids include α-

alanine, γ-aminobutyric acid, valine, isoleucine, and methylhydroxyproline with others in trace amounts [47].

An insufficient amount of nitrogen in the juice can result in slow or stuck fermentations meaning fermentation may cease to continue even though there is still available sugar for the yeast to ferment [15, 42, 44, 46]. A minimum concentration of 140 mg/L of nitrogen is suggested to avoid incomplete or sluggish fermentations [15, 44], however this value is not universally accepted in the field and remains an active research topic.

Pre-fermentation YAN concentration and composition also have the potential to affect the sensory characteristics of a cider. Several studies have been conducted on YAN concentration and composition and their implications on wine fermentation, flavor and aroma, and many of the same principles can be applied to cider making. YAN concentration and composition have been found to influence final concentrations of glycerol, malic acid, α-ketoglutaric acid, succinic acid higher alcohols, fatty acids, ethyl esters, and acetate esters. These non-volatile and volatile compounds can have significant effects on the aroma and flavor of a cider [42].

The residual nitrogen remaining after juice has been fermented to cider is also important. Availability of nitrogen remaining post-fermentation increases the risk of unwanted bacterial and yeast growth (spoilage) during storage. Residual nitrogen encourages the growth of *Brettanomyces,* which is responsible for several undesirable aromas in wine and cider. Furthermore, *Brettanomyces* can metabolize the amino acids lysine and ornithine into volatile aromas that are described as "barnyard" or "mousy"[44]. The nitrogen content of the cider is dependent on the original nitrogen content of the apples, the amount of exogenous yeast nutrient products added to the juice (if any) as

well as the fermentation conditions. There may be an increase in residual nitrogen content if the finished cider is left on the lees, which may also lead to *Brettanomyces* growth [44, 47]. The lees are mainly considered waste products of cider fermentation and are composed of mostly dead yeast cells, but may also contain plant material, bacterial cells, organic acid salts, and polyphenols [49, 50]. Amino acids are released during yeast autolysis which increases the amount of available nitrogen in the cider [47]

A study by Burroughs (1957) examined the nitrogen contents of apples, juices, and ciders from six different cultivars. Although results varied between cultivars, it was generally found that the amino acids asparagine, γ-aminobutyric acid, valine, and isoleucine that were found in the juice were not found in the resulting cider after fermentation. Other amino acids found in the juice were sometimes present or present in small amounts [47]. This implies that some amino acids are preferentially used by yeast while others may be more likely to remain after fermentation as residual nitrogen. As aforementioned, residual nitrogen in cider post-fermentation increases the likelihood of spoilage by *Brettanomyces*. In addition, the total YAN concentration may impact the preferred order of uptake of amino acids, thus influencing volatile aromas produced during yeast metabolism. Amino acid composition and concentration have both been shown to impact cider aroma and quality, and remain a topic of current research.

Exogenous Nitrogen Additions

Juice YAN can be supplemented through the addition of various commercially available yeast nutrient products. Diammonium phosphate (DAP) is one of the most common additions and can help ensure a complete, timely fermentation and minimal hydrogen sulfide formation [42, 44]. DAP only contains ammonium ions and not amino

acids, whereas other products, such as Fermaid® K (Scott Laboratories, Petaluma, CA, USA), contain amino acids and other yeast nutrients [44, 51].The addition of DAP or amino acid containing products can result in differences in production of volatile aroma compounds by a given yeast strain during fermentation [52]. Addition of excessive DAP can result in altered or negative flavor profiles due to excessive nitrogen available for spoilage organisms [42, 44]. This suggests that further research is needed to determine an optimal YAN concentration and composition for cidermaking, rather than simply working from a minimum value for total YAN concentration.

2.9 Polyphenols

Chemistry of Polyphenols

Spanos and Wrolstad (1992) describes polyphenols as "secondary plant metabolites that have important roles in providing flavor and color characteristics of fruit juices and wines" [53]. Phenols consist of a benzene ring and a hydroxyl group, as shown in Figure 2.2, and polyphenols consist of two or more phenols[54].

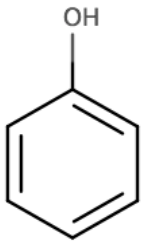

Figure 2.2: A phenol composed of a benzene ring and a hydroxyl group

Apple polyphenols can be classified into four main groups: phenolic acids, flavanols, flavonols, and dihydrochalcones, as represented in Figure 2.3 [53, 55]. An additional group of some importance may be anthocyanins, although anthocyanins are

found in much higher concentrations in highly pigmented foods such as berries or grapes than in apples. Although not found in green or yellow apple varieties, anthocyanins can be present in significant amounts in the skins of red varieties and contribute to the red color[56]. Total and relative concentrations of polyphenols depend largely on the cultivar as well as orchard management conditions [48, 53].

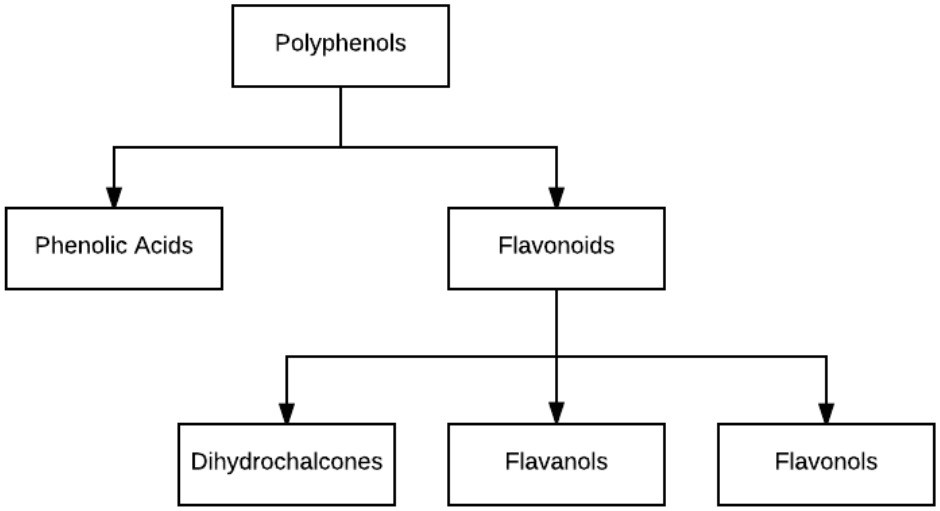

Figure 2.3: Main classes of polyphenols found in apples and their relationship

The most common phenolic acid found in apples is chlorogenic acid, as represented in Figure 2.4 [55]. Chlorogenic acid has an important role in oxidative reactions. Along with catechins, chlorogenic acid can be oxidized by the enzyme polyphenol oxidase (PPO) into *o*-quinones that can further react with other polyphenols resulting in the browning of pigments [57]. This process primarily occurs during pressing and crushing [53, 58]. Phenolic acids and catechins are formed in high concentrations in early development of the apple but decrease in concentration as the apple matures [53].

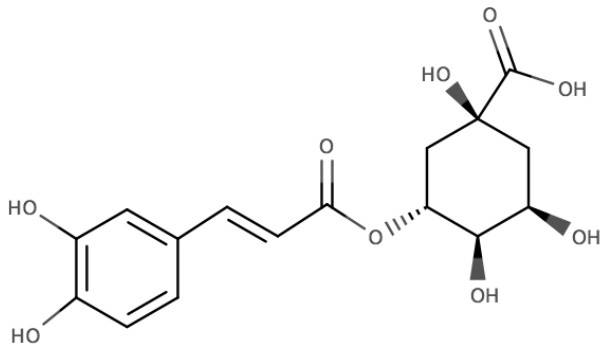

Figure 2.4: The structure of chlorogenic acid, the most prevalent phenolic acid found in apples.

The flavanols group contains mainly (+)-catechin and (-)-epicatechin, with epicatechin having a much higher concentration in apples [58-60]. Procyanidins are oligomeric flavanols and are the most represented polyphenols in the apple and particularly abundant in the flesh [59]. Structures of catechin, epicatechin, PC B1, PC B2, PC B5, PC C1, and Cinn A2 compounds can be found in Figures 2.5-2.11. Other phenolic compounds present in the flesh are phenolic acids, monomeric flavanols, and dihydrochalcones [1, 59, 61, 62]. The structure of phloretin, a dihydrochalcone, can be found in Figure 2.12. The skin contains nearly all of the flavonols and anthocyanins [62, 63]. The structure of quercetin, a flavonol, can be found in Figure 2.13. Relative to anthocyanins, dihydrochalcones and flavonols are minor contributors of pigmentation and are in much lower concentrations compared to flavanols and phenolic acids [55, 64].

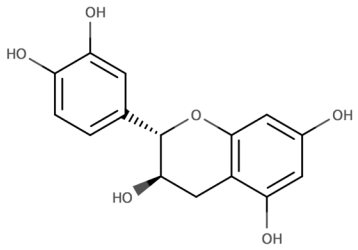

Figure 2.5: Structure of catechin

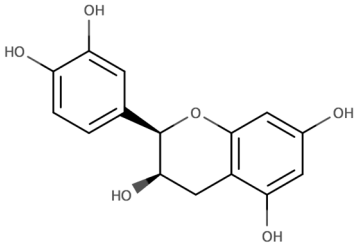

Figure 2.6: Structure of epicatechin

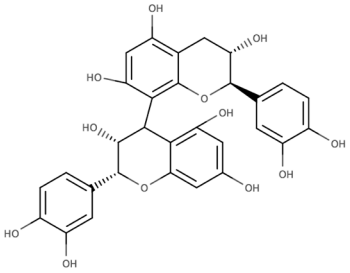

Figure 2.7: Structure of PC B1

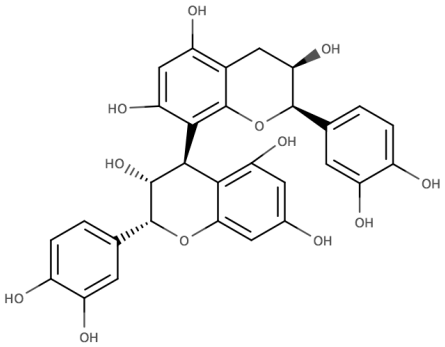

Figure 2.8: Structure of PC B2

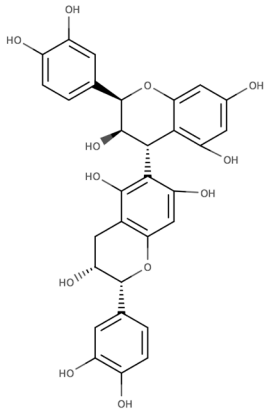

Figure 2.9: Structure of PC B5

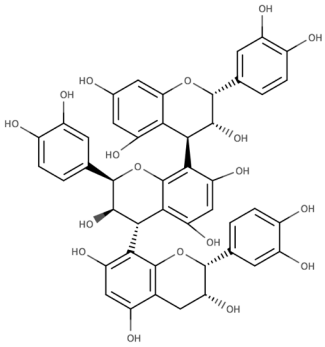

Figure 2.10 Structure of PC C1

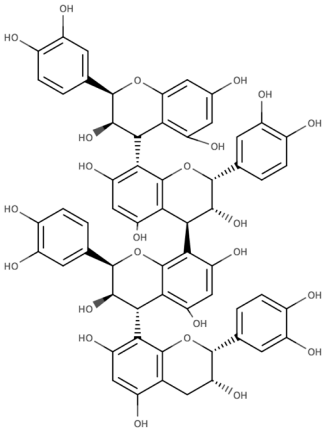

Figure 2.11: Structure of Cinn A2

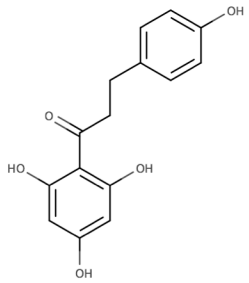

Figure 2.12: Structure of phloretin

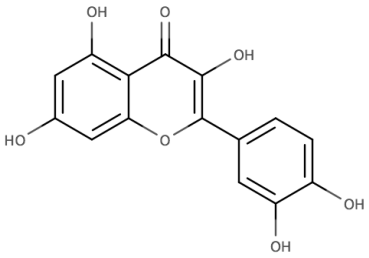

Figure 2.13: Structure of quercetin

Sensory Significance

Polyphenols are important compounds that impact organoleptic qualities of cider, particularly bitterness and astringency. Cider apples contain higher levels of polyphenols compared to dessert apples where the bitter, tannic components were bred out over generations [1, 59]. The exact composition and concentrations of polyphenols vary among cultivars with bitter apples containing up to ten times higher concentrations of total polyphenols compared to non-bitter apples [1, 61].

Astringency is a tactile characteristic defined as a dry, rough, and puckering sensation in the mouth [65]. The sensation of astringency develops over time as polyphenolic compounds precipitate salivary proteins [26, 66, 67]. The perception of bitterness, in contrast, is mediated by taste receptors on the tongue [26, 66]. In the wine literature, astringency will sometimes be described as the sensation imparted by "soft

tannins" while bitterness will be described as the taste associated with "hard tannins" [26]. These terms are used to describe sensory perception and are not structure definitions of the compounds themselves. However, the degree of polymerization of flavanols is an important determinant of the organoleptic effects of compounds. Monomers produce a stronger bitter taste than oligomers or polymers. In contrast, astringency increases as degree of polymerization of procyanidins increases until the molecules are large enough to be insoluble in saliva [26, 66].

The structure of polyphenol compounds is also a determinant of sensory impact. For example, (+)-catechin and (–)-epicatechin are structurally very similar except the stereochemistry of a single hydroxyl group [68]. A study by Thorngate and Noble (1995) found that both bitterness and astringency were perceived as greater and more persistent in (-)-epicatechin compared to (+)-catechin when both compounds were dissolved in water [68]. Kallithraka et al. (1997) further tested these findings and found these properties to be true in grape wine as well [69]. Gardner suggested that the lipophilicity of (-)-epicatechin increases its solubility at the taste receptor compared to (+)-catechin, and its perception is therefore stronger [70]. This observation leads us to believe that subtle changes in flavanol chemistry resulting from storage, processing and fermentation (without resulting in significant differences in total polyphenol concentration) could still result in significant sensory differences in cider.

According to Lea, cider apple juice will ideally contain 300-700mg/L chlorogenic acid, 100-200mg/L phloridzin, and 1000-1500mg/L epicatechin and procyanidins for ideal sensory expression of polyphenols in ciders[1]. These numbers, however, are only

generalizations and will of course vary tremendously based on cider style and desired characteristics.

Health Effects

The impact of polyphenols on human health is a topic of much current research. Apples and apple products are important sources of polyphenols in the human diet [71]. Studies have shown that polyphenols may contribute to the preventing or moderating the effects of several diseases including cancer and cardiovascular diseases [71-80]. Antioxidant effects of polyphenols largely attributed to free radical scavenging[73, 80] had historically been credited for the association between a diet rich in polyphenols and positive health outcomes. However, recent evidence suggests that other mechanisms may be involved in the antioxidant effects, such as the direct interaction with enzymes, influencing a series of redox reactions in cells[73], and furthermore that physiological mechanisms far beyond antioxidant effects play a significant role in the health benefits associated with dietary consumption of polyphenols. Increased polyphenols in the diet have shown a positive impact in decreasing risk of diabetes and obesity [77-79]. Polyphenols also can influence the microbiota of the gastrointestinal tract due to their antimicrobial properties, and microorganisms can potentially increase the bioavailability of polyphenols, though more research is necessary in this subject [81, 82]. The structure of polyphenols impacts their bioactivity, as intestinal absorption of polyphenols decreases as molecular weight increases, among other effects [80]. The concentration of polyphenols as well as their chemical structures and bioavailability all affect the type and degree of human health benefits conferred[80, 83].

Maturity and Storage Effects on Polyphenols

Polyphenols are present at different concentrations throughout maturation, storage, and processing of apples. A study by Mosel and Herrmann (1974) found that catechins and hydroxycinnamic acid derivatives in Golden Delicious and Schöner von Boskoop varieties were most abundant during the early development in the fruit, and their concentrations decreased through the ripening process until full maturity. In addition, they found that as the fruit ripens, the epicatechin to catechin ratio increases. [63]. However, a study by Burda et al. (1990) using Granny Smith apples found that epicatechin and procyanidin B2 were the two most abundant polyphenols found in the apples they evaluated [84]. Mosel and Herrmann reported difficulty in accurately measuring procyanidins in their study due to not having an accurate form of measurement, so this may explain the discrepancy since the most prevalent compounds reported by Burda et. al are indeed procyanidins [63, 84]. Therefore, due to the ability to measure procyanidins, the study by Burda et. al likely better represents the polyphenol composition of apples during ripening.

During apple storage, there may be some decreases in polyphenol concentration. A study by van der Sluis et al. examined four apple cultivars harvested before full maturity and the effects of storage conditions (controlled atmosphere vs. cold storage) on polyphenol concentrations. Controlled atmosphere storage conditions were 1.5℃ , 1.2% oxygen, and 2.5% carbon dioxide for Jonagold, Golden Delicious, and Elstar apples. For Cox's Orange apples, controlled atmosphere storage was 4℃ , 1.2% oxygen, and 0.7% carbon dioxide. Cold storage treatments were held at 4℃. Apples stored in controlled atmosphere storage saw very few and very small changes in polyphenol concentrations

over 52 weeks throughout the cultivars. Cold storage for 25 weeks yielded some apples with slight decreases in catechins, but overall few changes in polyphenol concentrations [85]. These results are fairly consistent with other studies [74, 86-89]. For example, Burda et al. observed significant decreases in total polyphenol concentration during maturation, but few decreases after maturation and during long-term cold storage [84]. However, further research is necessary to better understand changes of both total polyphenol and individual polyphenol concentrations during storage, and whether these trends are consistent across cultivars.

Polyphenol Quantification

Total polyphenol concentration in plant tissues is often measured with the Folin-Ciocalteu (F-C) assay [90, 91], with results being reported as Gallic Acid Equivalents (GAE), where gallic acid is used as the standard. This method uses a colorimetric reagent to react with or compounds to form a blue color [90-93]. The reagent is a mixture of sodium molbdate and sodium tungstate [92]. Reducing compounds undergo oxidation by transferring electrons to the reagent, which reduces the reagent and results in phopsphomolybdic and phosphotungstic acids. Color change due to this reaction is measured using a spectrophotometer as absorbance at 760nm [90, 91, 93].

Despite its common application in horticulture and food science research, there are several important sources of error that can impact results of the F-C assay. Although polyphenols are the most common antioxidants in plants, other compounds in plant tissue (or the cider matrix) with reducing potential may also react with the F-C reagent resulting in overestimation of total polyphenol concentration. Reports have indicated that compounds contributing to this effect may be reducing sugars, aromatic amines, sulfur

dioxide, ascorbic acid, proteins, and several others [90-92]. However, a more recent study by Everette et al. has indicated that reducing sugars may not be sources of interference in the FC assay [92]. Sulfur dioxide and sulfites are common additives to wine products, making it a particular obstacle when determining total polyphenol content in wines [90]. Because of the non-specificity of the FC assay, it is perhaps better considered to be a measure of total reducing power than it is of total polyphenols [92].

2.10 Sulfites

Sulfur dioxide is a commonly used antimicrobial and antioxidant additive used in the production of both wines and ciders [94-96]. It exists in free and bound forms, but only the free form is available to act as an antimicrobial or antioxidant agent [95, 96]. Furthermore, as pH decreases below 5.0, sulfites are converted into the molecular form that can enter and inhibit microorganisms [94, 95]. The amount of bound versus free SO_2 depends largely on the content of acetaldehyde, 2-ketoglutaric acid, pyruvic acid, galacturonic acid, and L-xylozone present in the matrix. Other compounds may also contribute to bound sulfites, but to a lesser extent [95, 96]. Furthermore, cider made from apples free of rot versus apples with high rot incidence tend to have far less sulfite binding capacity [94, 95]. Due to the sulfite-binding compounds produced by molds and bacteria present in rot complex, rotten apples will have a greater number of compounds able to bind sulfites [94].

2.11 Summary

In conclusion, cider production and consumption has dramatically increased over the past decade. Cider can be produced from various apple cultivars to obtain desired characteristics. There are several studies that examine the physical and chemical effects of harvest maturity and post-harvest storage on fruit and juice. However, there is still relatively little research on how apple orchard management practices impact the chemistry of the resulting cider compared to grapes and wine.

CHAPTER 3: MATERIALS AND METHODS

The materials and methods in this chapter describe the experimental process of Chapters 4 and 5 due to the similar experimental methodology between the two chapters.

3.1 Sampling Plan

Three apple cultivars were analyzed for fruit and juice parameters. The cultivars Dabinett and Brown Snout were grown at Cornell's Lansing Orchard in Lansing, NY. The trees were planted in 2003 and grafted on M.9 or G.30 rootstocks. The cultivar York Imperial (Ramey), further referred to as "York" in this study, was grown at Virginia Tech's Alson H. Smith Jr. Agricultural Research and Extension Center in Winchester, VA. These trees were planted in 2000 on M.9 rootstocks. Dabinett and Brown Snout are high-tannin cultivars, and York is a low-tannin culinary apple [97, 98]. These cultivars were chosen to examine the effects of harvest maturity and storage treatments on both high-tannin and low-tannin cultivars, both of which may be used for cider production[1].

There were four biological replicates per treatment per cultivar, composed of fruit harvested from four separate trees. Each replicate per treatment in the experiment was harvested from the same tree for the same replicate number in the other treatments. Fruit and juice analysis was performed on all three cultivars. Only Dabinett and York fruit were fermented into cider due to limited number of fermentation flasks available for use. These cultivars were chosen to process into cider in order to represent a high-tannin and low-tannin cultivar.

3.2 Analysis and Fermentation Plan

The procedures followed to address Objectives 1 and 2 are detailed in the following sections and outlined in Figure 3.1.

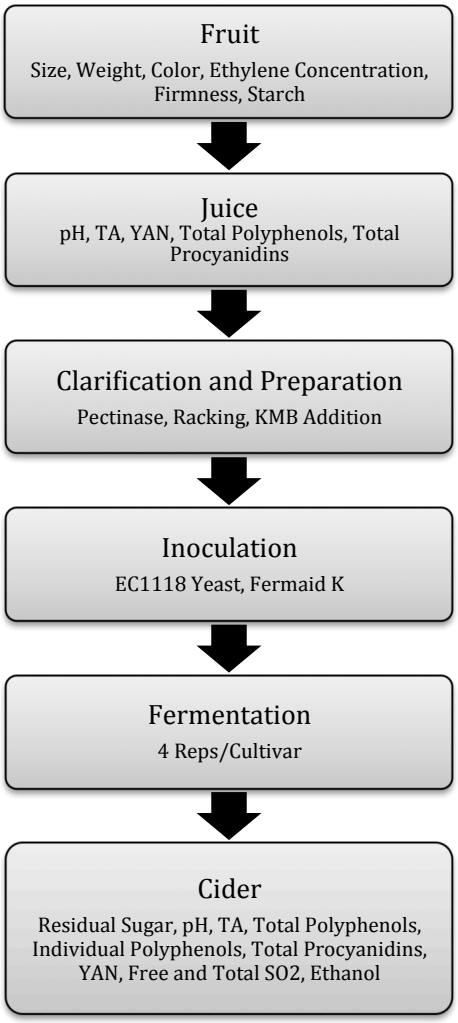

Figure 3.1: Flowchart of the experimental process for Objectives 1 and 2.

3.3 Fruit Analysis

The fruit maturity parameters ethylene concentration, starch-iodine index, fruit firmness, sugar content, pH, and titratable acidity (TA) were determined at the Alson H. Smith Jr. AREC (Winchester, VA). Total phenolic content, total procyanidins, and yeast

assimilable nitrogen (YAN) were determined at the Virginia Tech main campus in the Enology and Fermentation lab (Blacksburg, VA).

The methods of fruit analysis followed those described in Thomposon-Witrick et al., 2014 [64]. Ethylene gas was measured using an Agilent 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA). A gas sample was taken from the core cavity of ten apples and injected into the GC column using nitrogen gas as a carrier gas. Starch-iodine index was determined by staining the stem-side cross-section of an apple sliced equatorially with a 0.22% iodine and 0.88% potassium iodine solution measured in weight per volume. The amount of staining was determined on a the Cornell Starch-Iodine Index1-8 scale[99]. Fruit firmness was measured using a Fruit Texture Analyzer penetrometer (Güss Manufacturing (Pty) Ltd, Strand, S.A.) and a cylindrical 11.1mm diameter tip. Firmness was measured at two points at the equator of the apple after the peel was removed.

3.4 Juice Analysis

Soluble solids content (SSC) was measured in °Brix with a digital PAL-1 refractometer (Atago U.S.A., Inc., Bellevue, WA). The pH was measured using a pH probe (Thermo Scientific Versa Star Benchtop Meter, Model VSTAR30, Thermo Scientific ROSS Ultra Triode Electrode Model 8107BNUMD). TA was measured using an 848 Titrino Plus autotitrator (Metrohm AG, Herisau, CH). The juice was titrated against 0.1N NaOH solution to and endpoint of pH 8.1. Total polyphenol content was measured using the Folin-Ciocalteu method [90]. YAN was measured with a combination of the Primary Amino Nitrogen (NOPA) and Ammonia kits from Megazyme (Megazyme

International, Ireland) [100]. Total procyanidins were measured using the dimethylaminocinnimaldehyde (DMAC) method described below.

3.5 Juicing and Juice Preparation

The apples were juiced using a juicer (The Champion Juicer, Model G5-PG-710, Plastaket Mfg. Inc., Lodi, CA, USA). Pectinase (Pec5L, Scottzyme, Scott Laboratories, Petaluma, CA, USA) was added at a rate of 1ųL/100mL and left to settle overnight in a 18℃ temperature-controlled room. After settling, 750mL of the clarified juice was decanted into autoclaved 1000mL Erlenmeyer flasks for fermentation. Potassium metabisulfite (KMB) (Potassium Metabisulfite, Presque Isle Wine Cellars, North East, PA, USA) was added at 33mg/Lof juice and left for 3 hours before inoculation with yeast.

3.6 Inoculation

Lalvin EC1118 yeast (Scott Labs, Petaluma, CA, USA) was rehydrated in 40℃ deionized water and added to juice according to the manufacturer's recommended procedure at a rate of 250mg/L. After inoculation, Fermaid® K post-hydration nutrient (Scott Labs, Petaluma, CA, USA) was added to each flask at a rate of 24mg nutrient/100mL juice. Rubber bungs and airlocks, sanitized with a KMB and citric acid solution, were used to seal the flasks so that CO_2 gas could escape during the fermentation process, but oxygen and other contaminants could not enter the fermenting sample. The flasks were placed in a temperature-controlled incubation chamber at 18℃ for the duration of the fermentation process. 1L flasks were not stirred as they had

sufficient convection currents to keep the yeast in suspension, but flasks smaller than 1L were stirred twice a day to bring the yeast back into suspension. Smaller flasks were used when juice yield was low due to clarification difficulties.

3.7 Fermentation Monitoring

The progress of the fermentation was monitored by weighing the flasks daily. A decrease in mass is correlated with the production and expulsion of carbon dioxide from the system. Carbon dioxide production is a result of fermentation by yeast. Once the mass showed no day-to-day change, a sample was taken for determination of residual sugar concentration by enzymatic analysis. Fermentation was considered complete when the residual sugar concentration was between 0.2-0.5g/L at which time subsamples were collected and immediately stored at -80°C.

3.8 Sample Collection and Storage

Upon completion of the fermentation process, cider was collected in 50mL and 15mL centrifuge tubes for pH, TA, total polyphenols, total procyanidins, ethanol, total SO_2, free SO_2, and YAN analyses. Additional samples for individual phenolic compounds were collected in 2mL sample tubes with 4 parts sample and 1 part preservative. The preservative consisted of 0.85M glacial acetic acid with and 0.02% weight per volume ascorbic acid. All samples were stored at -80°C until analysis, at which point they were thawed in the refrigerator and then brought to room temperature prior to analysis.

3.9 Cider Analyses

Residual sugar was measured using the Megazyme D-Fructose and D-Glucose kit (Megazyme International, Ireland). The pH was measured using a pH probe (Thermo Scientific Versa Star Benchtop Meter, Model VSTAR30, Thermo Scientific ROSS Ultra Triode Electrode Model 8107BNUMD). TA was measured using titration to an endpoint of pH 8.2 by 0.1M NaOH standardized against HCl [101]. Total phenolic compounds were measured using the Folin-Ciocalteu assay [102], and individual phenolic compounds were measured with Ultrahigh Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS), using methods described in more detail in following sections. Total procyanidins were measured using the DMAC procedure [103]. YAN was measured with a combination of the Primary Amino Nitrogen (NOPA) and Ammonium kits from Megazyme (Megazyme International, Ireland). Free and Total SO_2 were measured at the Enology Analytical Services Laboratory at the Virginia Tech main campus in Blacksburg, VA by the aeration/oxidation method [104]. Ethanol concentration was also measured at the Enology Analytical Services Laboratory using the WineScan™ Fourier Transfer Infrared System (Foss, Eden Prarie, Minnesota, USA).

3.10 DMAC Procedure

Standards were prepared from a 100 mg/L stock solution of PCB2 (Planta Analytica, Danbury, CT) in methanol (Fisher Scientific, Fair Lawn, NJ, USA). Standards of 50ppm, 10ppm, and 1ppm were prepared with dilutions of the stock solution with methanol.

The 1, 10, 50, and 100ppm standards were pipetted in 50uL volumes in duplicate to a Corning UV-Transparent 96-well plate (Fisher Scientific, Fair Lawn, NJ, USA) . Juice samples were also pipetted in 50uL volumes in duplicate to the 96-well microplate. Then, 250uL of DMAC solution was added to each well and the absorbance was read at 640nm using a BioTek Synergy H1 Microplate Reader (BioTek US, Winooski, VT, USA).

3.11 Individual Polyphenols Analysis

UPLC/MS System

Individual polyphenols in cider were analyzed using the UPLC-QDA system (Waters Corporation, Milford, MA, USA). The AQUITY UPLC HSS T3 column (2.1mmx100mm, 1.8µm) (Waters Corporation, Milford, MA, USA) was used in combination with a AQUITY UPLC HSS T3 (2.1x5 mm, 1.7 µm) VanGuard pre-column (Waters Corporation, Milford, MA, USA). Peaks were analyzed using Empower™ Software (Waters Corporation, Milford, MA, USA).

Mobile Phase Preparation

The initial mobile phase was prepared using 95% of Mobile Phase A and 5% Mobile Phase B. Mobile Phase A consisted of 0.1% formic acid (Macron Fine Chemicals, Avantor Performance Materials Inc., Center Valley, PA, USA) in HPLC-grade water (Synergy HPLC Water Purification System, EMD Millipore, Billerica, MA, USA) and Mobile Phase B consisted of 0.1% formic acid in acetonitrile (J.T. Baker, Avantor Performance Materials Inc., Center Valley, PA, USA).

Hydrolysis

Samples were hydrolyzed in order to convert polyphenol glycosides into their aglycones. This was done to limit the number of standards needed for the individual polyphenol analysis using the UPLC/MS.

Cider samples were prepared after fermentation with a 4:1 cider to preservative (0.02% ascorbic acid, 4.77% glacial acetic acid, and HPLC-grade water) ratio and stored at -80°C until analysis. Once thawed, 1mL of the cider-preservative solution was pipetted into 50mL centrifuge tubes and immediately flushed with nitrogen. Then, 1μL of a single sample was pipetted onto pH paper to approximate the pH of the original sample. The pH of all samples were then adjusted to approximately 2.0 using 1μL of 1M HCl and then subsequently flushed with nitrogen. Samples were then hydrolyzed in an oven at 100°C for 10 minutes in order to render polyphenols from their glycosidic to aglycone. Samples were kept on ice until extraction.

Extraction

After cooling samples on ice for several minutes, 2mL of 100% ethyl acetate was pipetted into the 50mL centrifuge tube containing the 1mL sample. Samples were vortex mixed and the layers were then allowed to separate for 30 seconds. The top ethyl acetate layer was extracted using a disposable transfer pipette and dispensed into a second 50mL centrifuge tube. This extraction of the ethyl acetate layer was repeated three times for each sample. The extracted ethyl acetate layer was then dried under nitrogen gas for several hours using the N-EVAP™111 Nitrogen Evaporator (Organomation, Berlin, MA, USA) until all solvent was visibly removed. Samples were stored at -80°C until analysis. For the analysis, samples were reconstituted in 1mL of 95% Mobile Phase A and 5%

Mobile Phase B and then filtered using 1mL Luer-Lok Syringe Tips (BD, Franklin Lakes, NJ, USA) and 0.22um PTFE filters (MicroSolv, Eatontown, NJ, USA) into 12x33 mm UPLC/MS vials (Waters Corporation, Milford, MA, USA)

Standard Preparation

The authentic standards used were catechin, epicatechin, chlorogenic acid, quercetin, phloretin (Sigma-Aldrich, St. Louis, MO), procyandin B1 (PCB1), procyanidin B2 (PCB2), procyanidin B5 (PCB5), procyanidin C1 (PCC1), and cinnamtannin A2 (Planta Analytica, Danbury, CT). These standards were chosen due to their known presence in apples as well as their importance in sensory and health studies [105, 106]. Standards were prepared from a 0.1ug/mL standard in methanol solution stored at -80°C until analysis. Standards were prepared from a 0.1ug/mL standard in methanol solution. A serial dilution using 95% Mobile Phase A and 5% Mobile Phase B was then completed to obtain a total of 8 calibration standards. A diagram of the standard preparation is presented in Figure 3.2.

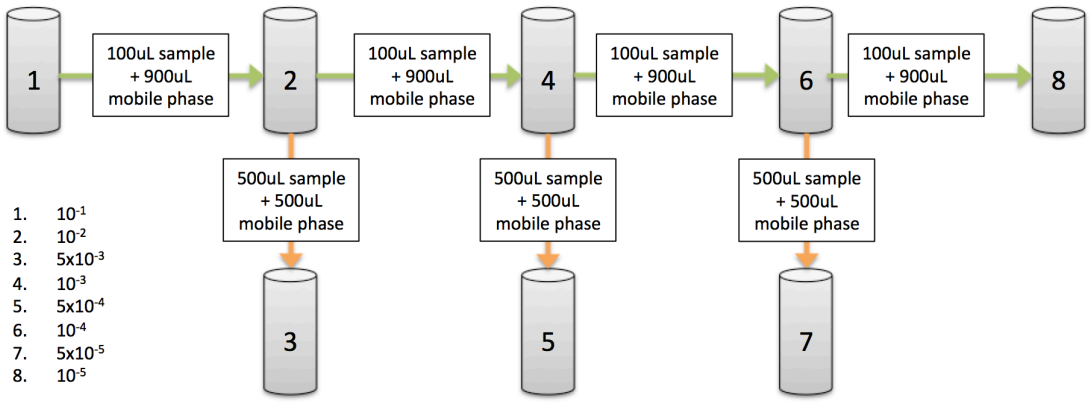

Figure 3.2. Standard Serial Dilution Schematic for UPLC/MS Analysis of Polyphenols

Injection

Standards and samples were incubated in the autosampler at 4°C and injected into the column in 10µL volumes and eluted at 43°C. The flow rate was 0.6mL/min following the gradient in Table 3.1. The probe temperature was 600°C, the capillary voltage was 0.8kV, and the mass range was from m/z 200 to 1250 (centroid). Retention times, molecular weights, and single ion recording (SIR) channels for each compound are presented in Table 3.2.

Table 3.1: UPLC mobile phase gradients for the separation of polyphenols.

Minute	% Mobile Phase A	% Mobile Phase B
0 (Initial)	95	5
0.5	95	5
6.5	65	35
7.5	65	35
8.6	20	80
8.7	20	80
14.6	95	5

Table 3.2: Retention times, molecular weights, and SIR channels for polyphenols.

Compound	Retention Time (min)	Molecular Weight (g/mol)	SIR (m/z)
Catechin	2.892	290.092	289.09
Epicatechin	3.614	290.092	289.09
PC B1	2.476	578.07	577.13
PC B2	3.363	378.04	577.13
PC B5	4.762	578.136	577.13
PC C1	3.889	866.218	865.21
Cinn A2	4.047	1154.808	576.4
Phloretin	7.440	274.26	273.05
Quercetin	6.673	302.23	301.01
Chlorogenic Acid	2.992	354.31	353.06

Peaks were analyzed using the Apex Track function of the Empower Software and were smoothed using a mean smoothing level of 9.

3.12 Statistical Analysis

One-way ANOVA was used to determine if treatments significantly differed from one another. GraphPad Prism v.6 (La Jolla, CA) was used to perform unpaired one-way analysis of variance (ANOVA) tests with significance defined as $p<0.05$. The null hypothesis was that no means differ: $H_0= \mu_1 = \mu_2 \ldots = \mu_n$. The alternative hypothesis, H_a, was that at least one mean differs. Tukey's Honestly Significant Difference (HSD) test at $p<0.05$ was used for post-hoc mean separation.

CHAPTER 4: THE EFFECT OF HARVEST MATURITY OF APPLES ON FRUIT, JUICE, AND CIDER QUALITY

4.0 Abstract

Relative to grapes and winemaking, little is known about how apple maturity at harvest impacts the quality of hard cider. This investigation examined how various harvest maturities impacted the fruit quality of York, Dabinett, and Brown Snout apples as well as the juice and cider chemistry. Harvest intervals of two weeks before maturity, at maturity, and 2 weeks after maturity resulted in significant differences in fruit quality and juice chemistry, but few of these differences persisted in the cider chemistry. Nonetheless, differences in concentration of some individual polyphenols determined by UPLC-MS were observed in ciders made from fruit harvested at different stages. For example, cider made from optimally mature Dabinett had over 250% the concentration of procyanidin B5 that was found in cider made from fruit harvested earlier or later, indicating the potential for harvest maturity to impart sensory differences in astringency and bitterness of cider.

4.1 Introduction

The impact of harvest maturity of wine grapes on wine quality has been the subject of extensive research in recent decades. Considerably less research has investigated the impact of apple harvest maturity on the resulting cider quality. As previously mentioned in Chapter 1, apples undergo a series of physiological changes as a result of climacteric ripening. These changes include an increase in sugar concentration, decrease in acidity, and an increase in volatile aroma compounds [1, 2]. However, it is not

yet clear if these and other maturation effects persist into the cider made from apples harvested at different maturity.

This study aims to determine whether harvesting apples at varying maturity affects the chemistry of fruit, juice, and cider.

4.2 Materials and Methods

Materials and methods for apple, juice, and cider analysis will follow the procedures outlined in Chapter 3.

Experimental Treatments

As represented in Figure 4.1, the first treatment consisted of apples harvested 14 days before typical fruit maturity (H1), as defined by commercial dessert fruit production practices, and was examined based on the resulting fruit, juice, and cider quality. The second treatment consisted of apples harvested at maturity (H2) and was examined based on the resulting fruit, juice, and cider quality. The third treatment included apples harvested 14 days after maturity for York and 13 days after maturity for both Brown Snout and Dabinett (H3) and was examined based on the resulting fruit, juice, and cider quality. There were four biological replications for each treatment per cultivar. Biological replicates were obtained by harvesting fruit from the same tree for each individual replicate. Fruit was harvested from the top, bottom, inside, and outside of the tree to obtain a representative sample.

Fruit quality parameters were measured at the Alson H. Smith, Jr. Agricultural Research and Extension Center in Winchester, VA in addition to several juice quality

parameters including SSC, pH, TA, and SSC:TA. Frozen juice samples were sent to the Blacksburg campus and further analyzed for total polyphenols, PAN, and ammonia.

Due to complications that come from working between two campuses, fruit was not able to processed immediately into cider after harvest. There was therefore a 14-day interval for the first York treatment, a 13-day interval for the second York treatment, and a 13-day interval for the last York treatment. In addition, there was 16-day interval between harvest and processing for all of the Dabinett treatments. Because this delay between harvesting and processing was consistent for all three harvest intervals, it is unlikely to negatively impact this study.

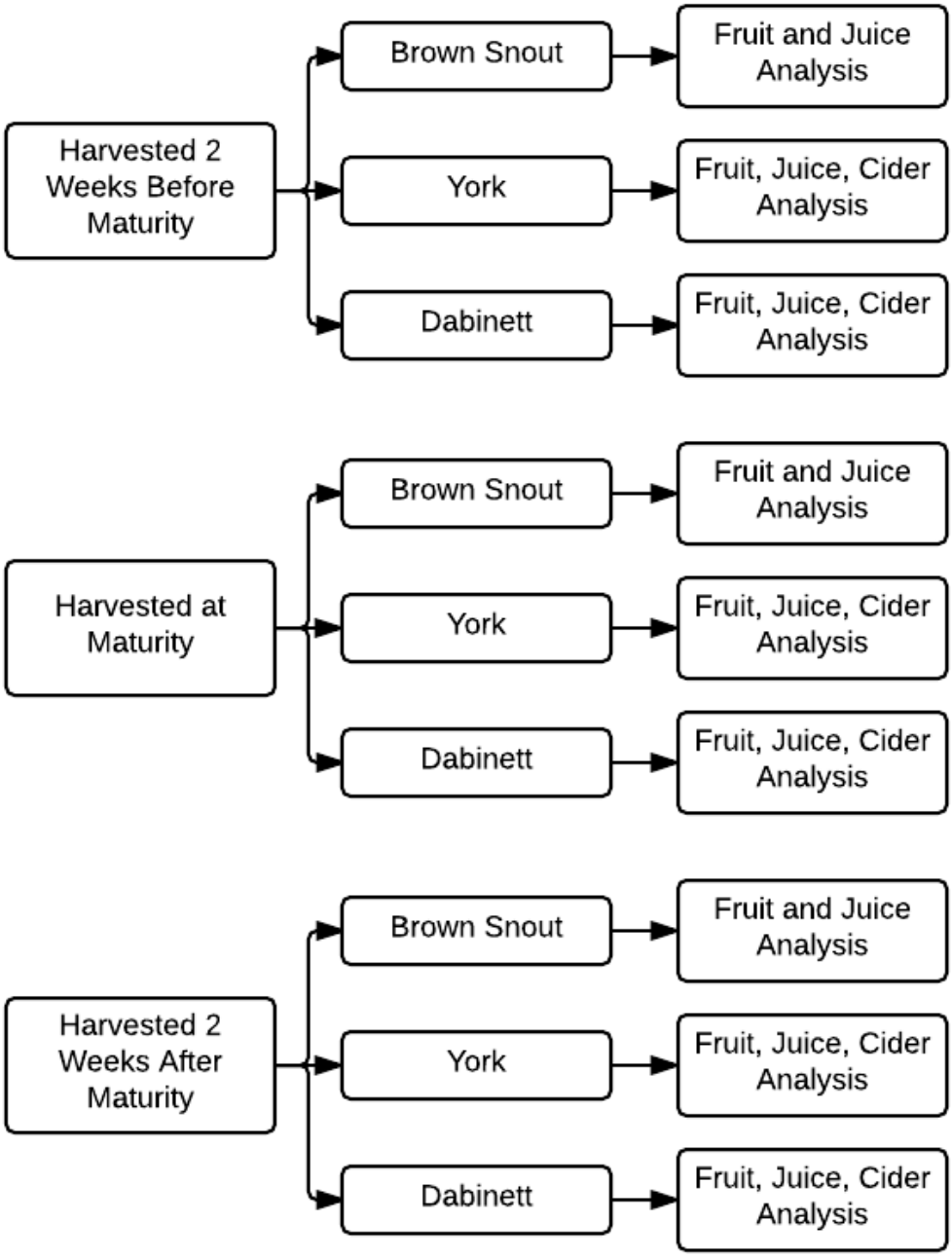

Figure 4.1: Outline of harvest maturity experiment, showing experimental treatments, cultivars, and analyses performed on fruit from each cultivar and treatment.

4.3 Results and Discussion

Results are presented in Tables 4.1 – 4.4 as mean ± standard error for *n*=4 treatment replicates. Letters were assigned to represent significant differences ($p<0.05$) between treatments using one-way ANOVA followed by Tukey's HSD test. Common letters between treatments indicate no significant difference.

Treatments are designated as H1 (2 weeks before maturity), H2 (at maturity), and H3 (2 weeks after maturity).

4.31 Fruit Maturity and Chemistry

Fruit maturity and chemistry parameters are reported in Table 4.1, and discussed in the following paragraphs.

Table 4.1: Effects of Harvest Date on Fruit Maturity and Quality. Mean separation was analyzed separately for each cultivar. Values are represented as mean ± standard error for n=4 replicates.

	Harvest	Fruit Firmness (N)	Fruit Diameter (mm)	Fruit Weight (g)	Starch-Iodine Index (1-8)	Color*	Ethylene (ppm)
York	H1	99.2±1.3^{a}	72.6±0.9^{b}	148.7±5.2^{b}	1.2±0.1^{b}	45.9±3.5^{b}	1.71±0.88^{b}
	H2	86.7±1.3^{b}	77.9±0.9^{a}	179.9±6.6^{a}	1.9±0.2^{a}	75.3±2.6^{a}	8.08±8.07^{ab}
	H3	87.2±1.3^{b}	78.2±1.1^{a}	185.3±6.8^{a}	2.3±0.2^{a}	81.3±2.1^{a}	11.73±3.66^{a}
Dabinett	H1	129.0±1.3^{a}	50.6±0.7^{a}	49.2±2.0^{b}	1.3±0.1^{b}	30.5±2.9^{b}	0.07±0.07^{b}
	H2	115.2±1.8^{b}	52.7±0.8^{a}	59.93±2.6^{a}	2.2±0.1^{a}	57.6±3.2^{a}	5.97±5.59^{ab}
	H3	118.3±1.8^{b}	52.3±0.7^{a}	57.2±2.3^{a}	2.6±0.2^{a}	57.3±3.8^{a}	22.96±4.03^{a}
Brown Snout	H1	100.1±1.3^{a}	42.8±0.7^{b}	28.5±1.3^{b}	1.8±0.1^{b}	2.2±0.1^{c}	0.02±0.01^{c}
	H2	91.6±1.3^{b}	45.5±0.9^{ab}	39.4±2.3^{a}	3.3±0.1^{a}	2.7±0.1^{b}	6.32±0.65^{b}
	H3	89.4±1.3^{b}	48.2±0.9^{a}	44.4±2.3^{a}	3.5±0.1^{a}	2.9±0.1^{a}	17.50±2.60^{a}

*For York and Dabinett, Red Color is measured on a 0-100% scale, and for Brown Snout, Green Background Color is measured on a 1-4 scale.

Fruit Firmness

For all three cultivars, fruit firmness was greatest in the apples harvested 2 weeks before maturity. York fruit firmness decreased 12% from the H1 treatment to the H3

treatment. Dabinett fruit firmness decreased by 8% from the H1 treatment to the H3 treatment. Brown Snout fruit firmness decreased by 11% from the H1 treatment to the H3 treatment. Fruit firmness decreasing with maturity is consistent with previous research [99, 107-109].

Fruit Diameter

In York apples, fruit diameter increased by 7.7% from the H1 treatment to the H3 treatment. In Dabinett apples, there was no significant difference among harvest date treatments. For Brown Snout fruit, there was a 12.6% increase in fruit diameter from the H1 to the H3 fruit. Fruit diameter increasing as fruit reaches maturity is consistent with other previously conducted research. Furthermore, once maturity is reached, the rate of growth decreases, so smaller differences between mature and over-mature fruit is also expected [110, 111].

Fruit Weight

Fruit weight increased by 21% for York fruit, 22% for Dabinett fruit, and 38.2% for Brown Snout fruit between the H1 and H3 treatments. There was no difference between the fruit harvested at maturity and the fruit harvested 2 weeks after maturity.

Fruit weight increasing as fruit reaches maturity is to be expected, and once fruit reaches maturity, the rate of weight increase is expected to decrease [111]. Therefore, these results are consistent with previous studies.

Starch-Iodine Index

The starch-iodine index (1-8) increased from the H1 treatment to the H2 treatment by 58% for York fruit, 69% for Dabinett fruit, and 83.3% for Brown Snout fruit. There

were no differences in the fruit harvested at maturity and the fruit harvested 2 weeks after maturity. These results indicate the breakdown of starch into sugar as apples mature and are consistent with previous research [99, 107].

Color

In both York and Dabinett apples, red color values increased from the H1 treatment to the H2 treatment by 64% for York fruit and 89% for Dabinett fruit. There was no significant difference between the fruit harvested at maturity and those harvested 2 weeks after maturity.

An increase in red pigment between the first and second treatments is expected, as anthocyanin concentrations in the skin are known to increase during ripening due to the stimulation of enzymes involved in phenolic synthesis by ethylene gas [112, 113]. The absence of a significant difference between the second and third treatments could be due to the rate of anthocyanin accumulation reaching a plateau along with the rate of ripening. Another explanation could be that the harvest interval was not large enough to recognize more significant changes for these two cultivars.

In Brown Snout, green background color (1-4) was significantly different between each treatment. There was a 23% increase in green color value from the H1 to the H2 fruit. There was a 7% increase in green color value from the H2 to the H3 fruit.

Color change from green to yellow significantly increased among treatments as ripening progressed. This change in pigment is to be expected as chlorophyll is degraded during ripening [114].

Ethylene Concentration

Ethylene concentration increased between the H1 to H3 treatments by 586% for York fruit and 32,700% for Dabinett fruit, and 87,400% for Brown Snout fruit.

Ethylene concentration generally increased with increasing maturity, which is expected for climacteric fruits such as apples. Ethylene concentrations increasing with maturity have been previously observed, as well as patterns of ethylene production varying between cultivars [2, 18, 19, 99, 115].

4.32 Juice Chemistry

Results from the juice analysis are presented in Table 4.2 and discussed in the following sections.

Table 4.2: Effects of Harvest Date on Juice Quality. Mean separation was analyzed separately for each cultivar. Values are represented as mean ± standard error for n=4 replicates.

	Harvest	SSC (°Brix)	pH	TA (g/L Malic Acid)	SSC:TA	Total Polyphenols (mg/L GAE)	Total Procyanidins (mg/L PC B2 equivalents)	PAN (mg N/mL)	Ammonia (g/L)
York	H1	9.4±0.5^{ab}	3.5±0.1^{a}	5.0±0.1^{a}	1.9±0.1^{a}	363±9^{b}	4±2^{a}	44±2^{a}	ND
	H2	7.9±0.3^{b}	3.5±0.1^{a}	5.2±0.2^{a}	1.5±0.1^{a}	424±4^{a}	7±2^{a}	50±3^{a}	ND
	H3	9.5±0.2^{a}	3.4±0.1^{a}	4.9±0.4^{a}	2.0±0.2^{a}	292±19^{c}	4±1^{a}	38±9^{a}	ND
Dabinett	H1	11.1±0.4^{a}	4.5±0.1^{a}	1.3±0.1^{a}	8.7±0.8^{a}	1718±141^{a}	26±6^{a}	47±4^{a}	ND
	H2	11.3±0.0^{a}	4.4±0.1^{a}	1.3±0.1^{a}	8.9±0.4^{a}	1420±125^{a}	22±3^{a}	31±5^{a}	ND
	H3	10.6±0.4^{a}	4.3±0.2^{a}	1.3±0.1^{a}	8.6±0.8^{a}	1190±161^{a}	12±5^{a}	36±9^{a}	ND
Brown Snout	H1	11.5±0.1^{a}	4.2±0.1^{a}	2.0±0.2^{a}	5.9±0.5^{a}	1259±90^{a}	13±3^{a}	65±4^{a}	ND
	H2	10.3±0.3^{b}	4.1±0.2^{ab}	2.4±0.1^{a}	4.3±0.2^{a}	1122±113^{a}	24±9^{a}	41±11^{ab}	ND
	H3	11.7±0.2^{ab}	3.8±0.1^{ba}	2.8±0.2^{a}	4.3±0.4^{a}	899±101^{a}	13±4^{a}	31±5^{b}	ND

Soluble Solids Content

The SSC of the York juice decreased by 16% from H1 to H2 then increased by 17% from H2 to H3. The SSC of the Dabinett juice was not statistically significant between treatments. The SSC of the Brown Snout juice decreased by 10% from H1 to H2 then increased by 12% from H2 to H3.

Typically, SSC increases with fruit maturity, as seen in the York juice treatments, and has been observed in other studies [99, 116]. Dabinett juice showed no significant differences between treatments, which may be attributed to harvest intervals being too short in duration to result in differences in fruit chemistry. Brown Snout juice had the highest SSC in the juice made from the most immature apples and the lowest SSC in the juice made from the apples harvested at commercial maturity. Like with the Dabinett juice, this could possibly be attributed to the harvest intervals being too short to result in clear significant differences, but could also be due to cultivar varitions or environmental conditions. More studies on Brown Snout fruit are needed to understand how SSC behaves throughout fruit maturation in this cultivar.

pH

The pH in York and Dabinett juices showed no statistical differences between treatments. The Brown Snout juice pH decreased by 10% from H1 to H3.

It is expected that pH would be lowest in the least ripe fruit and highest in the ripest fruit due to the breakdown of malic acid during maturation [24]. The absence of significant differences between York and Dabinett juices could once again be attributed to harvest intervals that are not long enough to allow for significant differences to develop between treatments. Brown Snout juice followed a trend opposite of what was

expected, with pH decreasing at later harvest date. These results are difficult to explain but could possibly be due to cultivar-dependent factors. A study by Bragas et al. saw a similar result when measuring total acidity in Fuji Suprema, Lisgala, and Gala apple juice. Fuji Suprema and Lisgala juice increased in total acidity as fruit maturity increased, but this was not the case for Gala juice. As expected, juice made from immature Gala fruit had a greater amount of acidity in unripe fruit than in ripe and senescent fruit. However, the senescent fruit had a significantly greater amount of total acidity than the ripe fruit [116]. This indicates that acidity may be generally correlated with maturity, but several variables including apple cultivar may contribute to a deviation from this general correlation.

Titratable Acidity

There were no titratable acidity differences between treatments in all three cultivars. Titratable acidity would be expected to decrease during maturation for the reasons mentioned in the pH section. However, no significant differences were found between treatments, which could potentially be attributed to harvest intervals too short to allow for such differences to occur.

SSC:TA

There were no significant SSC:TA differences between treatments in any of the three cultivars.

Total Polyphenols by FC Assay

Total polyphenols in York juice increased from H1 to H2 by 17% then decreased from H2 to H3 by 31%.

The maturation process could in part explain these differences. Some polyphenols begin to degrade throughout maturation, while others, such as anthocyanins, increase in concentration [63, 112, 113]. Individual polyphenols must be examined to determine if this was the case.

Dabinett and Brown Snout juice did not show statistical differences in polyphenol concentrations.

Total Procyanidins

There were no significant differences in total procyanidins between any of the treatments in all three cultivars.

Primary Amino Nitrogen (PAN)

There were no significant differences in PAN concentration between treatments in both York and Dabinett juices.

In Brown Snout juice, PAN concentration from the H1 and H3 treatments decreased by 52.3%. This is consistent with other studies regarding primary amino nitrogen content of juice made from apples of varying maturities and is likely due to amino acids being used to synthesize volatile compounds during maturation [3, 116].

Ammonia

Ammonia was not detectable in any of the cultivars for any of the treatments.

4.13 Cider Chemistry

Results from the cider analysis are presented in Table 4.3 and are discussed in the following sections. Ciders were made from two cultivars: York and Dabinett. Brown

Snout was not further processed from juice into cider in 2015 due to equipment constraints, thus no data for Brown Snout cider are available.

Table 4.3: Effects of Harvest Date on Cider Quality. Mean separation was analyzed separately for each cultivar. Values are represented as mean ± standard error for n=4 replicates.

	Harvest	pH	TA (g/L Malic Acid)	Residual Sugar (g/L)	Total Polyphenols by FC (mg/L GAE Equivalents)	Total Procyanidins (mg/L)	PAN (mg N/mL)	Ammonia (mg/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Ethanol (% v/v)
York	H1	3.6±0.0[a]	4.7±0.8[a]	0.21±0.10[a]	226±13[a]	ND	3±2[a]	0±0[a]	1±0[a]	4±3[a]	5.9±0.3[a]
	H2	3.6±0.0[a]	6.7±0.5[a]	0.98±0.39[a]	192±10[a]	ND	1±1[a]	0±0[a]	1±0[a]	2±0[a]	6.6±0.1[a]
	H3	3.6±0.1[a]	5.6±0.4[a]	0.48±0.17[a]	244±16[a]	ND	7±1[a]	0±0[a]	1±0[a]	1±0[a]	6.7±0.3[a]
Dabinett	H1	4.2±0.0[a]	3.7±0.4[a]	0.51±0.22[b]	1594±126[a]	10±0[a]	7±3[a]	0±0[a]	4±3[a]	12±5[a]	6.7±0.3[a]
	H2	4.2±0.0[a]	6.4±1.4[a]	1.64±0.01[a]	1654±69[a]	11±2[a]	26±7[a]	0±0[a]	2±0[a]	13±3[a]	7.2±0.1[a]
	H3	4.2±0.0[a]	5.3±0.4[a]	1.48±0.19[a]	1294±111[a]	5±1[b]	14±4[a]	0±0[a]	1±0[a]	5±0[a]	7.3±0.4[a]

pH

There were no significant differences in pH between treatments in both York and Dabinett ciders.

Titratable Acidity (TA)

There were no significant differences in TA between treatments in both York and Dabinett ciders. Similarly, Alberti et al. observed no significant differences in TA between ciders made from apples of varying maturities and explained that this equalization of acidity is caused by the synthesis of organic acids during fermentation [3]. Though it is possible that this also occurred in this study, it is unlikely because no TA differences were observed between the treatments in the juice, either.

Residual Sugar

There were no significant differences in residual sugar concentration in York ciders.

In Dabinett cider, the residual sugar concentration increased from the H1 cider to the H2 cider by 222%. Despite these differences, all three Dabinett ciders resulted in below 2.0 g/L residual sugar and would be considered to have completed fermentation to dryness (an acceptable RS value for a commercially acceptable dry cider).

Total Polyphenols by the FC Assay

There were no significant differences in total polyphenol concentration between treatments in both York and Dabinett ciders. Dabinett juice before fermentation did not show any significant differences between treatments, but all three treatments for York juices did significantly differ. This indicates that York juice underwent significant chemical changes throughout its processing into cider to eliminate the polyphenolic

concentration differences between cultivars. One such change that may have contributed to these results is the precipitation of polyphenols involved in haze formation in the juice. Polyphenols in juice may bind to pectin or proteins and create haze, and clarification measures such as pectinase additions and overnight settling aim to eliminate that haze [117]. Therefore, it is possible that juice clarification before fermentation and further precipitation during fermentation was responsible for the lack of persistence of these significant differences in total polyphenol concentration between juice and cider for the York cultivar.

Total Procyanidins

Procyanidins were not detectable in any of the York cider treatments.

Dabinett total procyanidin concentrations decreased by 50% from H1 to H3.

Concentrations of catechins are known to increase during fruit development but tend to decrease during further stages of maturation [53]. If this pattern extends to procyanidin dimers, this could explain why the fruit harvested before and at maturity had higher concentrations of procyanidins than the fruit harvested 2 weeks after maturity.

Residual Primary Amino Nitrogen (PAN)

There were no significant differences in residual (post-fermentation) PAN concentration between treatments in both York and Dabinett ciders.

Residual Ammonia

There were no significant differences in residual (post-fermentation) ammonia concentration between treatments in both York and Dabinett ciders.

Free SO_2

There were no significant differences in free SO_2 concentration between treatments in both York and Dabinett ciders.

Total SO_2

There were no significant differences in total SO_2 concentration between treatments in both York and Dabinett ciders.

Ethanol

There were no significant differences in ethanol concentration between treatments in both York and Dabinett ciders.

4.14 Individual Polyphenols in Cider

The results of the individual polyphenols analysis are presented in Table 4.4. Differences in individual polyphenols found between treatments were polyphenol and cultivar dependent.

Table 4.4: Effects of Harvest Maturity on Individual Polyphenols in Cider (µg/mL). Mean separation was analyzed separately for each cultivar. Values are represented as mean ± standard error for n=4 replicates.

	Harvest	Catechin	Epicatechin	PC B1	PC B2	PC B5	PC C1	Cinn A2	Quercetin	Phloretin	Chlorogenic Acid
York	H1	13.7±2.0^{ab}	44.7±2.1^{a}	24.5±3.5^{a}	39.5±3.7^{a}	2.82±0.26^{a}	7.83±1.33^{a}	3.05±0.44^{a}	2.84±0.30^{a}	0.62±0.11^{a}	136±3^{a}
	H2	8.4±1.2^{b}	34.0±4.6^{b}	13.3±2.5^{a}	21.5±3.4^{b}	1.82±0.20^{b}	3.39±0.87^{a}	2.04±0.23^{a}	1.40±0.19^{b}	0.09±0.09^{b}	128±7^{a}
	H3	14.9±1.4^{a}	45.2±4.6^{a}	22.0±3.5^{a}	36.1±3.9^{ab}	2.87±0.29^{a}	7.23±1.47^{a}	7.38±3.92^{a}	2.38±0.19^{a}	0.32±0.12^{ab}	138±5^{a}
Dabinett	H1	35.9±2.4^{a}	204±9^{ab}	119±10^{a}	167±11^{ab}	19.0±2.7^{ab}	138±12^{ab}	82±10^{ab}	1.79±0.17^{a}	2.04±1.20^{a}	174±3^{a}
	H2	36.7±5.4^{a}	215±15^{a}	132±21^{a}	189±18^{a}	24.0±4.3^{a}	162±25^{a}	102±21^{a}	2.32±0.72^{a}	2.60±0.94^{a}	178±1^{a}
	H3	19.8±5.6^{a}	142±23^{b}	59±25^{a}	94±27^{b}	6.7±4.2^{b}	55±32^{b}	29±21^{b}	0.88±0.06^{a}	0.27±0.11^{a}	153±6^{b}

Catechin

In York ciders, the catechin concentration decreased by 39% from H1 to H2 and increased by 77% from H2 to H3. Previous research has mostly reported a decrease in catechin in fresh apples and juice during maturation in other apple cultivars, though a wide variety of catechin concentration trends resulting from various maturation stages occur in the fruit, juice, and cider depending on the cultivar [58, 63, 118]. Therefore, though these results are somewhat unusual, they are not completely contradictory to previous research as catechin concentrations are somewhat variable throughout maturity in some cultivars [58].

There were no significant differences in catechin concentration between treatments in Dabinett ciders. Longer harvest intervals may be necessary to observe significant differences between treatments.

Epicatechin

Epicatechin concentrations in York ciders decreased from H1 to H2 by 24% and increased from H2 to H3 by 33%. Previous research regarding apples and juice has observed varying trends in polyphenol concentration, including increases in epicatechin production during the early stages of ripening, sometimes followed by decreases after maturation, as well as decreases during maturation [58, 63, 118]. Therefore, though these results are unusual in their pattern, it is not unusual that different cultivars respond differently to varying harvest maturities.

In Dabinett ciders, the concentration of epicatechin increased from H2 to H3 by 34%. This pattern correlates with previous research on epicatechin in fresh apples during maturation [63, 118]. It also corresponds with the decrease in procyanidin dimers PC B2, PC

B5, PC C1, and total procyanidins. This could indicate epicatechin and epicatechin product degradation during the maturation process between maturity and senescence.

PC B1

There were no significant differences between cider treatments in PC B1 concentrations in either the York or Dabinett ciders.

PC B2

In York cider, the concentration of PC B2 decreased by 46% from H1 to H2. In the Dabinett cider, the concentration of PC B2 decreased 50% from H2 to H3. These results correspond to the total procyanidin results from the Dabinett cider where total procyanidins were significantly lower in the H3 treatment compared to the H2 treatment. Therefore, the decrease in total procyanidins from the H2 treatment to the H3treatment may be due to the different concentrations of PC B2.

PC B5

In York cider, PC B5 concentrations decreased by 36% from H1 to H3 ciders.

In Dabinett cider, there was a 72% decrease in PC B5 concentration from H2 to H3 ciders. Again, this trend correlates with that of the total procyanidins, indicating that the concentration of PC B5 may have had a significant effect on the concentrations and differences in concentrations of total procyanidins in the final cider.

PC C1

There were no significant differences in York ciders in regards to PC C1 concentrations. In Dabinett ciders, PC C1 concentrations decreased from the H2 to H3 ciders by 66%. Again, this trend correlates with that of the total procyanidins, indicating

that PC C1 concentrations also may have a significant impact on the concentration of total procyanidins.

Cinnamtannin A2

There was no significant difference in Cinn A2 concentrations between York cider treatments.

In Dabinett ciders, Cinn A2 concentrations decreased by 72% from H2 to H3 ciders.

Quercetin

Quercetin concentrations in York ciders decreased from H1 to H2 ciders by 51% and then increased from H2 to H3 ciders by 70%.

There were no significant differences in quercetin concentrations between Dabinett cider treatments.

Phloretin

Phloretin concentrations in York ciders decreased from H1 to H2 ciders by 86%. A decrease in dihydrochalcones during ripening has been observed in previous research [62].

There were no significant differences in phloretin concentrations between Dabinett cider treatments.

Chlorogenic Acid

Chlorogenic acid concentrations did not significantly differ between treatments in York ciders.

In Dabinett ciders, chlorogenic acid decreased by 12% from H1 to H3 ciders. Chlorogenic acid is most abundant during fruit development [97, 118]. Therefore, it is reasonable to expect that less mature fruit may contain higher levels of chlorogenic acid compared to more mature fruit, and these differences may persist in the final cider.

Summary of Individual Polyphenols Results

Individual polyphenol composition in York ciders varied between harvest maturity treatments. It is unknown how polyphenol glycosides were individually affected by theses treatments, as only the aglycones were measured. Individual polyphenols PC B1, PC C1, chlorogenic acid, and Cinn A2 showed no significant differences between treatments. The other six polyphenols quantified in this study, catechin, epicatechin, PC B2, PC B2, phloretin, and quercetin, however, did show differences, with the H2 treatment concentrations being significantly lower than one of the other treatments. In Dabinett ciders, PC B1, catechin, quercetin, and phloretin concentrations did not differ between treatments. However, the other six polyphenol concentrations in the H2 treatment were significantly higher than in the cider made from fruit harvested 2 weeks after maturity. These contrasting findings between cultivars could result from different mechanisms or timing of polyphenol development during apple maturation, as well as polyphenol oxidase (PPO) activity and oxidation. PPO reacts differently to different classes of polyphenols, as well as differently in each cultivar [119].

Some polyphenols are especially relevant as sensory components. Flavonoids, commonly known as “condensed tannins”, are present in higher quantities in bittersweet apples compared to sweet apples. Dabinett and Brown Snout are considered bittersweet cultivars and should therefore have higher concentrations of flavonoids compared to

York, a sweet cultivar. Bittersweet varieties are considered as such due to their tannin concentrations being greater than 0.2% as measured by the Lowenthal Permanganate titration. In this study, Dabinett and Brown Snout juice procyanidins measured by the DMAC method show lower values than would be expected for bittersweet varieties [1]. This could be due to several factors, including a loose definition of what is considered "tannins" in bittersweet classification and the limitation of the DMAC method for quantifying only procyanidins and not other flavonoids. However, in general and in this study, both Dabinett and Brown Snout juices contain higher levels of total polyphenols and total procyanidins compared to York juice.

As addressed in Chapter 2, flavanols in cider contribute to different sensory perceptions based largely on the degree of polymerization. Monomers, such as catechin and epicatechin, impart bitterness and astringency. As the degree of polymerization increases, bitterness decreases and the tactile sensation of astringency increases. It would be expected that cider treatments with higher concentrations of monomers would be both bitterer and slightly more astringent. This would be especially true concerning epicatechin concentrations, for epicatechin has a stronger sensory impact than catechin in both bitterness and astringency [69]. Therefore, the H2 York treatment and the H3 Dabinett treatment would be expected to be less bitter and less astringent than the other treatments due to their lower concentrations in monomers as well as PC B2 and PC B5 dimers. York cider treatments did not differ in concentrations of the trimer PC C1, but the Dabinett H3 treatment had a lower concentration of PC C1. It would therefore be expected that the H3 treatment might be less astringent compared to other treatments. Lastly, the concentration

of tetramer Cinn A2 did not vary between York cider treatments, but was lower in the H3 treatment in Dabinett ciders, once again indicating an expected decrease in astringency.

It is also important to consider the sensory thresholds of phenolic compounds. In a study by Delcour et al., several phenolic compounds were tasted in various water solutions. Catechin had a sensory threshold of 46.1 mg/L. Procyanidin B3 had a lower sensory threshold compared to catechin at 17.3 mg/L, and the trimer and tetramer mixture had an even lower sensory threshold at 4.1mg/L. However, a mixture of catechin with trimers and tetramers had an even lower sensory threshold of 3.6 mg/L. The presence of catechin may therefore increase the perception of overall tannins in the cider matrix [120]. Though the study conducted by Delcour et al. does not address all of the polyphenols measured in this study, it provides insight as to how varying degrees of polymerization may affect sensory thresholds. Based on these values, the sensory threshold of catechin in ciders from this study, which ranged from 8.4±1.2 to 14.9±1.4 mg/L in York ciders and 19.8±5.6 to 36.7±5.4 mg/L in Dabinett ciders, may not have been reached, but perhaps the presence of catechin would enhance the sensory effects of other more highly polymerized polyphenols.

Lastly, the interaction of polyphenols with other components in the cider matrix may affect the sensory perception. In wines, astringency perception has been found to decrease with increased ethanol concentrations while bitterness perception increases with increased ethanol concentrations [121-123]. The cider samples did not significantly differ in ethanol concentration in this study, so it is unlikely that it would greatly contribute to the sensory perception of tannins. In addition, pH and acid levels can affect the sensory perception of polyphenols. In wine and model solutions, an increase in acidity correlates

with an increase of astringent characteristics [121-124]. There were no differences in pH and TA between the treatments in York and Dabinett ciders, so this would likely not be a contributing factor in perceived bitterness and astringency. There are several factors that impact the perception of bitterness and astringency in wine, and it is likely that these factors, and perhaps others that have not yet been identified, also impact the sensory perception of polyphenols in cider [122].

4.4 Conclusion

Fruit, juice, and cider quality parameters were all affected to varying degrees by the harvest date of the fruit.

With the fruit, one or more treatments significantly differed from the others in nearly all fruit quality parameters measured in all three cultivars. Though the trends were not identical between cultivars, there were several consistencies regarding the fruit's response to increasing maturity including a decrease in fruit firmness, an increase in fruit weight, an increase in fruit diameter, a decrease of starch, an increase in red color for York and Dabinett, an increase in yellow color for Brown Snout, and an increase in ethylene concentration. If these trends carry over to other apple cultivars, these results indicate that fruit maturity at the time of harvest is especially important for fresh market apple producers, and this finding is in agreement with previous work in fresh market fruit production.

In the juice, there were relatively fewer general trends within cultivars. The effects of harvest maturities on juice quality parameters seem to largely be cultivar-dependent. In cider, there were even fewer differences between treatments in both York

and Dabinett ciders. These findings indicate that although fruit maturity at harvest significantly affects many quality parameters of the fruit, it has a more limited effect on quality parameters of juice and cider made from that fruit. Unlike grape harvesting for wine, or even apple harvesting for fresh market fruit, cider producers may have a greater flexibility in harvesting schedules without compromising the quality of the final product.

The results from this study show that varying harvest maturities may have different effects on individual cultivars at each stage of the cider-making process, and emphasize the importance of evaluating cider, rather than fruit for research aimed at improving cider quality through targeted orchard management. Further research on additional cultivars is necessary to better understand the effect of apple maturity on cider chemistry. In addition, it would be beneficial to conduct sensory studies on the appearance, aroma, and taste of cider to evaluate whether the chemical changes alter the sensory experience. Furthermore, the impact of harvest maturities on volatile aroma compounds and amino acid compositions should be examined in future work.

CHAPTER 5: THE EFFECTS OF POST-HARVEST STORAGE OF APPLES ON FRUIT, JUICE, AND CIDER QUALITY

5.0 Abstract

Apples are often stored for several months before processing, and a sizeable body of research has addressed the impacts of post-harvest storage on the quality of fresh apples. However, there is not sufficient scientific information about how post-harvest storage of apples impacts cider quality. This investigation examined how various fruit storage treatments impacted the fruit quality of York, Dabinett, and Brown Snout apples as well as the juice and cider chemistry. Storage treatments of 2 weeks at 4°C, 6 weeks at 1°C, 6 weeks at 10°C, and 4 months at 1°C resulted in several differences in fruit quality and juice chemistry, but few of these differences persisted in the cider chemistry. Differences in individual polyphenols were detected, with ciders made from cv. York having 20% higher epicatechin concentration when stored for 6 weeks at 1°C rather than 10°C. Dabinett ciders had no significant differences in individual polyphenols between treatments.

5.1 Introduction

Unlike wine grapes, apples have the potential to be stored for extended periods of time after harvest and prior to processing. Long-term post-harvest storage (6-13 months) of apples is common in fresh market and processing apples, and has been the topic of extensive research[4, 5, 125]. However, there is little research regarding the effects of long-term storage of apples on the quality of cider made from stored apples. Post-harvest storage of fruit allows cider producers greater flexibility in processing schedules after

harvest, and is at times employed with the intention of improving fruit or cider quality. Sweating, a process of which apples are exposed to a warm, dry environment after storage is a common practice aimed to increase sugar concentration by dehydration, yet there is a lack of research concerning the process. This research aims to determine if, or to what extent, fruit storage duration and conditions affect the chemistry of the resulting cider.

5.2 Materials and Methods

Materials and methods used in the experiments described in this section were previously presented in detail in Chapter 3 of this thesis. A diagram of the experimental treatments for this Objective and analyses for each cultivar can be found in Figure 3 of this chapter.

Experimental Treatments

Three cultivars, York, Dabinett, and Brown Snout, were studied using four different experimental treatments. Biological replicates were obtained by harvesting fruit from the same tree for each individual replicate. Fruit was harvested from the top, bottom, inside, and outside of the tree to obtain a representative sample. Fruit from one tree was then randomly mixed and divided into four lots to make four different storage treatments. The first of these treatments (2W-4C) examined apples processed two weeks after harvesting after storage in a 4℃ cooler. This treatment was originally intended to by processed immediately after harvest but was instead stored due to complications in transferring materials between campuses. The second treatment (6W-1C) examined

apples stored at 1℃ and processed 6 weeks after harvesting. The relative humidity, expressed as mean ± standard deviation in this cooler was 86±3%. The third treatment (6W-10C) examined apples stored at 10℃ and processed 6 weeks after harvesting. Relative humidity of this cooler was 92±2%. The fourth and final treatment (4M-1C) examined apples stored at 1℃ and processed 4 months after harvesting. The relative humidity of this cooler was 83±4%. The apples from the second, third, and fourth treatments were stored in walk-in coolers at the Agricultural Research and Extension Center (AREC) in Winchester, VA. They were brought to the Virginia Tech main campus in Blacksburg, VA and maintained at room temperature for 24 hours before processing to mimic the sweating process. There were four biological replications for each treatment per cultivar with each replication consisting of apples harvested from a single tree, and the plantings are described in detail in Chapter 3, Materials and Methods. There were therefore four fermentations for each treatment, making a total of 32 fermentations.

An unexpected challenge in this study was clarifying the juice from the apples from the 6W-10C and 4M-1C treatments for both Dabinett and York cultivars. Only small amounts of juice were able to be clarified using pectinase and overnight settling. Because the juice yield was relatively small, the York juice for these treatments was fermented in 250mL flasks instead of 1L flasks, and the Dabinett juice was fermented in 500mL flasks.

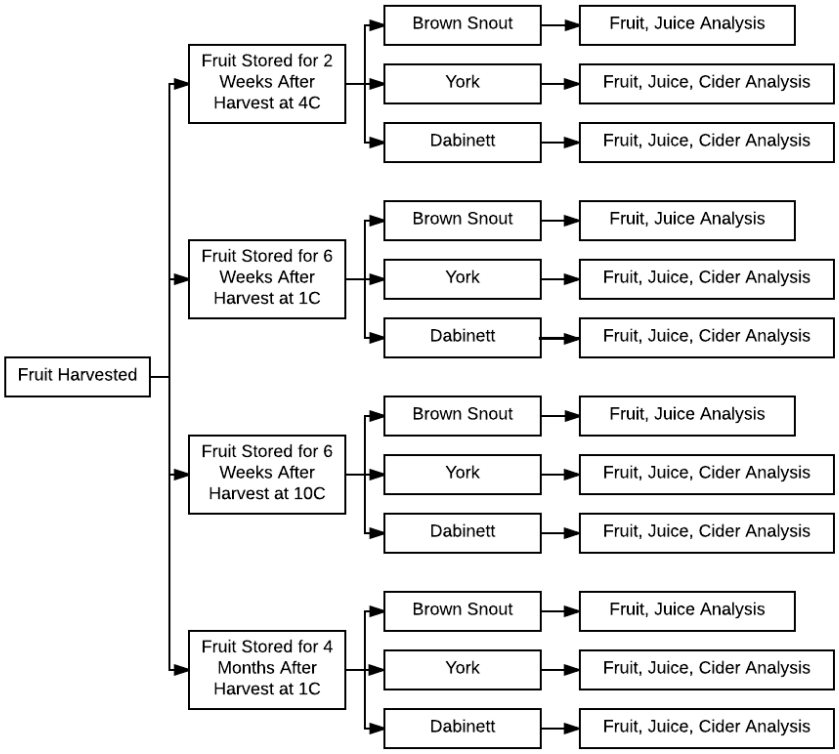

Figure 5.1: Objective 2 Flow Chart showing experimental treatments, cultivars, and analyses performed on fruit from each cultivar and treatment.

5.3 Results and Discussion

Statistical analysis was conducted to compare each treatment within each single cultivar. Result values are represented as mean ± standard error for n=4 replicates. Letters were assigned to represent significant differences ($p<0.05$) between treatments using one-way ANOVA followed by Tukey's HSD test.

5.31 Fruit Maturity and Chemistry

Results from fruit analysis are represented in Table 5.1 and are discussed in the following sections.

Table 5.1: Effects of Post-Harvest Storage Duration and Temperature on Fruit Quality. Mean separation was analyzed separately for each cultivar. Values are represented as mean±standard error for n=4 replicates.

	Storage Condition	Fruit Firmness (lbs)	Fruit Diameter (mm)	Fruit Weight (g)	Starch (1-8)	Color*	Ethylene (ppm)
York	2W-4C	89.4±0.9^{a}	74.7±1.0^{a}	160±6^{a}	2.1±0.2^{c}	65.4±3.2^{a}	1.47±0.16^{c}
	6W-1C	81.8±1.3^{b}	73.2±0.9^{a}	150±6^{ab}	4.3±0.2^{b}	89.1±16.0^{a}	98.48±20.33^{b}
	6W-10C	70.3±1.3^{c}	73.5±0.8^{a}	156±6^{ab}	6.9±0.2^{a}	73.3±3.0^{a}	236.56±35.97^{a}
	4M-1C	64.9±1.3^{d}	73.1±0.9^{a}	144±5^{b}	6.0±0.6^{a}	77.4±3.1^{a}	71.56±8.57^{b}
Dabinett	2W-4C	126.8±1.3^{a}	47.1±0.7^{b}	43±2^{b}	3.3±0.2^{d}	47.3±3.0^{b}	19.62±5.71^{c}
	6W-1C	100.1±2.2^{b}	48.0±0.9^{b}	46±2^{ab}	5.2±0.2^{c}	56.7±3.9^{ab}	51.52±15.96^{b}
	6W-10C	71.2±1.8^{c}	50.3±1.0^{a}	51±3^{a}	7.7±0.1^{b}	59.9±4.4^{ab}	304.00±41.78^{a}
	4M-1C	74.3±0.9^{c}	51.2±0.7^{a}	51±2^{a}	8.0±0.0^{a}	61.3±3.7^{a}	239.15±28.13^{a}
Brown Snout	2W-4C	102.8±2.2^{a}	43.0±0.9^{a}	35±2^{ab}	3.4±0.2^{c}	3.0±0.1^{b}	14.87±1.63^{c}
	6W-1C	81.4±1.3^{b}	45.4±0.6^{a}	39±1^{a}	6.1±0.2^{b}	2.9±0.1^{b}	133.64±29.53^{b}
	6W-10C	63.6±1.8^{c}	44.9±0.8^{a}	38±2^{a}	8.0±0.0^{a}	3.4±0.1^{a}	324.06±59.11^{a}
	4M-1C	ND	44.1±0.7^{a}	31±2^{b}	8.0±0.0^{a}	3.5±0.1^{a}	151.94±20.76^{b}

*For York and Dabinett, Red Color is measured on a 0-100% scale, and for Brown Snout, Green Background Color is measured on a 1-4 scale.

Fruit Firmness

York fruit firmness decreased by 27% from the 2W-4C treatment to the 4M-1C treatment. Dabinett fruit firmness decreased by 41% from the 2W-4C treatment to the 4M-1C treatment. Brown Snout fruit firmness decreased throughout storage to the point where the fruit penetrometer was unable to penetrate the flesh of the fruit.

Fruit firmness decreasing during storage and increased temperatures is consistent with previously conducted research [126-129]. Relative humidity is also an important factor, as low relative humidity results in faster desiccation and a breakdown of texture.

Fruit Diameter

Fruit diameter did not significantly differ between treatments in York and Brown Snout fruit.

Dabinett fruit diameter increased during storage, however these increases were likely not biologically significant.

Fruit Weight

The fruit weight of York apples decreased by 10% between the 2W-4C and 4M-1C treatments. This is likely due to dehydration during storage which indicates that weight loss was mostly due to water loss.

The fruit weight of Dabinett apples increased by 19% between the 2W-4C and 4M-1C treatments. However, the increase of weight is unlikely to be biologically significant.

The fruit weight of Brown Snout apples decreased from the 6W-1C and 6W-10C treatments to the 4M-1C treatment by 18-20%. This is consistent with fruit experiencing dehydration during longer storage times.

Starch-Iodine Index

The Starch-Iodine Index rating increased by 229% between the 2W-4C and 6W-10C treatments and by 186% between the 2W-4C and 6W-10C treatments for York apples. For Dabinett apples, the rating increased from the the 2W-4C and 4M-1C treatments by 142%. For Brown Snout apples, the rating increased among the 2W-4C treatment and both the 6W-10C and 4M-1C treatments

The breakdown of starch into sugars during storage is due to apples continuing to mature due to climacteric ripening. The York and Brown Snout 6W-10C fruit had a more similar starch-iodine index value to the 4M-1C fruit compared to the 6W-1C fruit, indicating that the increased temperature accelerated the maturation process in storage.

Color

There was no significant difference in color in York apple treatments.

For Dabinett fruit, red color measurement increased by 30% between the 2W-4C and 4M-1C treatments.

Red color development is caused by increased concentrations of anthocyanins during maturation. Ethylene gas stimulates enzymes involved in the synthesis of phenolic compounds, including anthocyanins [112, 113]. Therefore, the significant increase in red color between the 2W-1C fruit and the 4M-1C fruit can be explained by fruit continuing to develop red pigment as a result of continual ethylene production during ripening in storage.

For Brown Snout fruit, green color increased by 17% from the 2W-4C and 6W-1C treatments compared to the 6W-10C and 4M-1C treatments.

Color change from green to yellow is caused by the loss of chlorophyll during ripening [114]. The 6W-10C fruit and 4M-1C fruit both had a decreased level of green color compared to the other two treatments, indicating an advanced level of ripening.

Ethylene Concentration

For York fruit, ethylene concentration in the 6W-10C treatment was 15,993% greater than in the 2W-4C fruit. Ethylene concentration in the 4M-1C fruit was 4,768% greater than in the 2W-4C fruit. These results are expected due to the climacteric nature of apples [19].

Dabinett ethylene concentrations increased from the 2W-4C treatment to the 6W-10C and 4M-1C treatments by 1119-1449%. Ethylene concentration was significantly lower in the 2W-4C treatment compared to the other three treatments which is again expected for climacteric fruits [19].

For Brown Snout fruit, ethylene concentration was highest in the 6W-10C treatment compared to all three other treatments.

Ethylene concentration typically increases with maturity as the presence of ethylene initiates further ethylene synthesis. It is therefore expected that the fruit analyzed at harvest will have the lowest ethylene concentration among treatments as seen with the York and Dabinett fruit. However, fruit from the 6W-10C treatment had significantly higher ethylene concentrations compared to all other treatments in York and Brown Snout fruit, and Dabinett fruit from the 6W-10C treatment was not significantly different from the 4M-1C fruit. Again, these results show that temperature can have just as much, and sometimes more, of an effect than time on ripening during storage. Ethylene production increases with temperature up to a maximum of rate at 28°C [19].

5.32 Juice Chemistry

Results from juice analysis are presented in Table 5.2 and discussed in the following sections.

Table 5.2: Effects of Post-Harvest Storage Duration and Temperature on Juice Quality. Mean separation was analyzed separately for each cultivar. Values are represented as mean±standard error for n=4 replicates.

	Storage Condition	SSC (°Brix)	pH	Titratable Acidity (g malic acid/L)	SSC:TA	Total Polyphenols (mg/L GAE equivalents)	Total Procyanidins (mg/L PC B2 equivalents)	PAN (mg N/L)	Ammonia (mg N/L)
York	2W-4C	9.7±0.2^{ab}	3.7±0.0^{a}	4.5±0.2^{ab}	2.2±0.1^{b}	455±24^{b}	6±2^{a}	60±10^{a}	ND
	6W-1C	9.3±0.5^{b}	3.4±0.1^{a}	4.8±0.2^{a}	2.0±0.2^{b}	547±18^{ab}	17±7^{a}	73±21^{a}	ND
	6W-10C	10.2±0.2^{ab}	3.4±0.2^{a}	2.7±0.2^{b}	3.9±0.4^{a}	447±26^{b}	10±5^{a}	51±7^{a}	ND
	4M-1C	10.8±0.3^{a}	3.4±0.1^{a}	3.3±0.2^{b}	3.3±0.2^{a}	588±32^{a}	4±2^{a}	40±7^{a}	ND
Dabinett	2W-4C	11.8±0.4^{b}	4.7±0.0^{a}	1.1±0.1^{a}	10.7±0.6^{a}	1760±112^{b}	19±4^{a}	38±8^{a}	ND
	6W-1C	11.5±0.3^{b}	4.7±0.0^{a}	1.3±0.1^{a}	9.1±1.2^{a}	1698±90^{b}	28±13^{a}	34±10^{a}	ND
	6W-10C	11.6±0.7^{ab}	4.5±0.1^{ab}	1.1±0.1^{a}	11.2±1.0^{a}	1800±163^{b}	46±13^{a}	30±9^{a}	ND
	4M-1C	14.3±0.7^{a}	4.4±0.0^{b}	1.4±0.1^{a}	10.5±0.8^{a}	2493±170^{a}	46±17^{a}	29±8^{a}	ND
Brown Snout	2W-4C	12.9±0.1^{c}	4.3±0.1^{a}	2.3±0.0^{b}	5.6±0.1^{a}	1322±29^{a}	13±5^{b}	31±6^{a}	ND
	6W-1C	12.4±0.6^{c}	4.0±0.1^{ab}	2.6±0.1^{b}	4.8±0.4^{a}	1344±150^{a}	56±12^{a}	20±4^{a}	ND
	6W-10C	15.9±0.3^{b}	4.0±0.1^{b}	2.8±0.1^{ab}	5.8±0.3^{a}	1714±204^{a}	65±18^{a}	28±13^{a}	ND
	4M-1C	17.0±0.2^{a}	4.2±0.1^{ab}	3.2±0.1^{a}	5.4±0.1^{a}	1509±53^{a}	57±7^{a}	24±6^{a}	ND

Soluble Solids Content

For York juice, SSC increased from the 6W-1C treatment juice to the 4M-1C juice by 16%.

For Dabinett juice, SSC increased from the 2W-4C and 6W-1C treatments to the 4M-1C treatment by 21-24%.

Brown Snout juice increased in SSC from the 2W-4C and 6W-1C treatments to the 4M-1C treatment by 32-37%.

These data shows that juice made from the 2W-4C fruit had a significantly lower SSC compared to the juice made from the 4M-1C fruit in all three cultivars. This increase in SSC could potentially be attributed to concentration by dehydration during storage. Furthermore, the juice made from the 6W-10C fruit was not more similar to either the 2W-4C or 4M-1C treatments indicating that storage time had more of an impact on SSC than temperature for this parameter.

pH

York juice pH did not significantly differ among treatments.

Dabinett juice pH decreased from the 2W-4C and 6W-1C juice to the 4M-1C juice by 6%. This is a relatively small change in pH and may not be biologically significant. It is expected that juice pH would increase with increased storage as seen from these results as apples continue to mature during storage.

Brown Snout juice had a decrease in pH from the 2W-4C treatment to the 6W-10C treatment of 7%. However, it is possible that this pH decrease is not biologically significant. These data indicate that the fruit stored for 6 weeks in 10°C conditions may have progressed more through maturation than the other stored apples, and as the fruit was respiring, it was consuming malic acid and therefore the pH increased.

Titratable Acidity

In the York juice, the TA from the 6W-1C treatment was 178% greater than the TA from the 6W-10C treatment. Again, changes in acidity can be attributed to malic acid consumption for fruit metabolism during respiration.

Contrasting with the pH results, Dabinett juice showed no significant differences among treatments.

TA increased in the Brown Snout juice by 39% from the 2W-4C to the 4M-1C treatments. Though it would be expected that malic acid concentrations would decrease throughout the process of fruit respiration, it is possible that the acid was concentrated through dehydration during the 4 month storage period. The decrease in fruit firmness observed in this treatment supports this hypothesis.

SSC:TA

York juice increased in its SSC:TA ratio from the 2W-4C and 6W-1C treatments to the 6W-10C and 4M-1C treatments by 50-95%.

Dabinett and Brown Snout juices showed no significant differences in SSC:TA between treatments.

It would be expected the SSC:TA ratio would increase throughout storage and at higher temperatures as sugar concentrations are expected to rise and malic acid concentrations are expected to fall, thereby increasing the SSC:TA ratio[130]. This trend was observed in the York cultivars, but not in the Dabinett or Brown Snout cultivars. The balance of sugars and acids is an important characteristic of ciders, and indicates that storage will not greatly influence the cider apples balance of sugar and acid.

Total Polyphenols by FC Assay

Total Polyphenols by FC Assay in York juice increased by 29-31% from the 2W-4C and 6W-10C treatments to the 4M-1C treatment.

Total polyphenols in Dabinett juice were significantly higher in the 4M-1C treatment compared to all other treatments.

Brown Snout juice did not show any significant differences in total polyphenol concentration between treatments.

It would be expected that total polyphenols would either not significantly change or slightly decrease with storage time based on previous research, as seen with the Brown Snout fruit [63, 84]. However, these results indicate that in York and Dabinett fruit, the 4M-1C treatments had higher concentrations of polyphenols than some of or all of the other treatments. This could be in part a result of concentration of polyphenols caused by dehydration of fruit during storage or an increase in compounds that cause interferences in the FC assay, which are examined more thoroughly in Chapter 6 of this thesis.

Total Procyanidins

Total procyanidins did not significantly differ among treatments for neither York nor Dabinett juice.

In Brown Snout juice, total procyanidin concentrations were significantly lower in the 2W-4C treatment compared to all other treatments. This could potentially be attributed to dehydration during storage, resulting in concentration of procyanidins.

Primary Amino Nitrogen

There were no significant differences in PAN between treatments in all three cultivars.

Ammonia

Ammonia was not detectable in any of the juice treatments in any of the cultivars.

4.33 Cider Chemistry

Results from the cider analysis are presented in Table 5.3 and discussed in the following sections. Brown Snout was not further processed from juice into cider in 2015 due to equipment constraints, thus no data for Brown Snout cider are available.

Table 5.3: Effects of Post-Harvest Storage Duration and Temperature on Cider Quality (ug/mL). Mean separation was analyzed separately for each cultivar. Values are represented as mean±standard error for n=4 replicates.

	Storage Condition	pH	TA (g/L)	Residual Sugar (g/L)	Total Polyphenols (mg/L GAE equivalents)	Total Procyanidins (mg/L PCB2 equvialents)	PAN (mg N/L)	Ammonia (mg/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Ethanol (%v/v)
York	2W-4C	3.6±0.0^{a}	5.9±0.3^{a}	0.42±0.03^{a}	260±15^{a}	ND	12±3^{a}	0±0^{a}	0±0^{a}	13±1^{a}	5.7±0.4^{a}
	6W-1C	3.6±0.0^{a}	5.1±0.4^{a}	0.38±0.11^{a}	363±33^{a}	ND	16±2^{a}	0±0^{a}	0±0^{a}	12±1^{b}	5.9±0.1^{a}
	6W-10C	3.6±0.0^{a}	5.6±0.4^{a}	0.01±0.01^{b}	286±22^{a}	ND	15±4^{a}	0±0^{a}	0±0^{a}	6±1^{c}	6.5±0.4^{a}
	4M-1C	3.6±0.0^{a}	6.4±1.4^{a}	0.21±0.10^{ab}	315±27^{a}	ND	17±1^{a}	0±0^{a}	0±0^{a}	7±1^{c}	6.8±0.1^{a}
Dabinett	2W-4C	4.1±0.0^{a}	3.7±0.1^{a}	1.29±0.14^{a}	2049± 125^{a}	8±1^{c}	40±13^{a}	0±0^{a}	1±0^{a}	20±1^{a}	7.1±0.4^{a}
	6W-1C	4.0±0.0^{a}	4.4±0.8^{a}	1.32±0.07^{a}	2104±96^{a}	10±1^{bc}	57±2^{a}	0±0^{a}	0±0^{ab}	8±2^{b}	6.9±0.2^{a}
	6W-10C	4.0±0.1^{a}	3.9±0.2^{a}	0.92±0.29^{a}	1997±91^{a}	13±0^{ab}	47±5^{a}	0±0^{a}	0±0^{ab}	12±2^{b}	7.5±0.2^{a}
	4M-1C	4.0±0.0^{a}	4.5±0.1^{a}	0.51±0.22^{a}	2464±207^{a}	14±1^{a}	69±4^{a}	0±0^{a}	0±0^{b}	11±1^{b}	7.8±0.3^{a}

pH

The pH did not significantly differ between treatments in both York and Dabinett ciders. The pH in both York and Dabinett juices significantly differed between some treatments, but these differences did not persist into the final cider.

Titratable Acidity

TA did not significantly differ between treatments in both York and Dabinett ciders. The TA in York juices significantly differed between treatments, but these differences did not persist into the final cider.

Residual Sugar

Though the residual sugar levels statistically differed between treatments, it is unlikely that these differences are biologically different. Residual sugar was below 2 g/L for all treatments, so all treatments may be considered to have been fermented to completion.

Residual sugar did not significantly differ in any of the Dabinett cider treatments.

Total Polyphenols

There were no significant differences in total polyphenol content between treatments in York or Dabinett ciders.

Total Procyanidins

Procyanidins were not detectable in York ciders.

In Dabinett ciders, total procyanidins were 75% higher in the 4M-1C treatment compared to the 2W-4C treatment.

Primary Amino Nitrogen

There were no significant differences in PAN concentration between treatments in either the York or Dabinett ciders.

Ammonia

There were no significant differences in ammonia concentration between treatments in either York or Dabinett ciders.

Ethanol

There was no significant difference in ethanol concentration between treatments in either the York or Dabinett ciders

5.34 Individual Polyphenols

Results from the individual polyphenols analysis are presented in Table 5.4 and discussed in the following sections.

Table 5.4: Effects of Post-Harvest Storage Duration and Temperature on Individual Polyphenols in Cider. Mean separation was analyzed separately for each cultivar. Values are represented as mean±standard error for n=4 replicates. Values are represented as ug/mL.

	Storage Condition	Catechin	Epicatechin	PC B1	PC B2	PC B5	PC C1	Cinn A2	Quercetin	Phloretin	Chlorogenic Acid
York	2W-4C	14.6±1.6^{ab}	86±5^{ab}	39.8±5.1^{b}	62.0±5.6^{ab}	3.79±0.76^{a}	18.1±5.8^{ab}	2.4±0.8^{b}	2.99±0.39^{a}	0.34±0.17^{a}	150±7^{a}
	6W-1C	19.9±1.7^{a}	104±4^{a}	68.1±5.9^{a}	89.0±5.5^{a}	6.23±0.77^{a}	29.9±4.7^{a}	11.2±1.3^{a}	2.11±0.11^{ab}	0.44±0.04^{a}	150±3^{a}
	6W-10C	13.5±1.3^{ab}	76±4^{b}	34.4±3.8^{b}	49.9±4.1^{b}	2.76±0.34^{a}	3.8±2.1^{b}	2.4±1.1^{b}	1.58±0.42^{bc}	0.31±0.20^{a}	142±2^{a}
	4M-1C	12.5±1.6^{b}	75±7^{b}	37.7±6.7^{b}	59.2±9.1^{b}	3.58±1.07^{a}	13.0±4.9^{ab}	6.5±1.3^{ab}	0.76±0.30^{c}	0.64±0.08^{a}	137±4^{a}
Dabinett	2W-4C	52.7±2.2^{a}	249±13^{a}	202±9^{a}	216±9^{a}	38.0±2.1^{a}	217±14^{a}	149±15^{a}	1.08±0.44^{a}	2.86±0.19^{a}	200±5^{a}
	6W-1C	55.1±3.4^{a}	248±13^{a}	209±15^{a}	224±13^{a}	40.5±3.4^{a}	237±21^{a}	159±22^{a}	0.92±0.35^{a}	1.38±0.19^{a}	205±5^{a}
	6W-10C	55.3±2.4^{a}	247±12^{a}	216±10^{a}	223±11^{a}	43.1±2.2^{a}	232±13^{a}	163±16^{a}	1.26±0.32^{a}	2.83±0.75^{a}	204±7^{a}
	4M-1C	56.2±3.4^{a}	250±14^{a}	224±9^{a}	233±6^{a}	48.1±3.0^{a}	251±9^{a}	172±8^{a}	1.21±0.11^{a}	2.54±0.80^{a}	222±5^{a}

Catechin

Catechin concentrations in York ciders were greater in the 6W-1C treatment compared to the 6W-10C treatment by 47% of the 6W-10C value. The sensory threshold of catechin in water has been reported to be approximately 46mg/L [120]. Though there were significant differences in catechin concentrations, these concentrations remain to be below reported sensory thresholds in water and are unlikely to cause a difference in sensory perception.

There were no significant differences in catechin concentrations between Dabinett cider treatments. Catechin concentrations remaining stable throughout varying storage durations and conditions is consistent with previous research [85, 87].

Epicatechin

Epicatechin concentrations were 36-39% greater in the 6W-1C cider treatments compared to the 6W-10C and 4M-1C cider treatments. It is unclear what the sensory threshold of epicatechin is. However, if it is similar to the sensory threshold of catechin, these results may be of sensory significance. This could indicate that cold fruit storage is beneficial in retaining bitterness and astringency compounds in ciders.

There were no significant differences in epicatechin concentrations between Dabinett cider treatments.

PC B1

In York cider, PC B1 concentrations were significantly higher in the 6W-1C cider compared to all other treatments.

There were no significant differences between Dabinett treatments in PC B1 concentrations.

PC B2

PC B2 concentrations in York ciders were 51-78% higher in the 6W-1C cider compared to the 6W-10C and 4M-1C ciders.

There were no significant differences in PC B2 concentrations in Dabinett ciders.

PC B5

PC B5 concentrations did not significantly differ between treatments in either York or Dabinett ciders.

PC C1

PC C1 concentrations in York ciders exhibited a 450% increase in the 6W-10C ciders compared in the 6W-1C.

There were no significant differences in PC C1 concentrations in Dabinett ciders.

Cinn A2

Cinn A2 concentrations in York ciders were significantly greater in the 6W-1C ciders by 367% of the Cinn A2 concentrations in the 6W-10C and 4M-1C ciders.

There were no significant differences in Cinn A2 concentrations between any of the Dabinett cider treatments.

Quercetin

The concentration of quercetin in decreased by 47-75% from the 2W-4C cider treatments to the 6W-10C and 4M-1C treatments.

There were no significant differences between treatments in quercetin concentrations in Dabinett ciders. The concentration of quercetin and quercetin glycosides in fruit has been shown to be constant throughout storage in other apple cultivars [85, 87]. However, there is not sufficient cider research with which to compare.

Phloretin

There were no significant differences in phloretin concentrations between cider treatments in both York and Dabinett ciders. This is consistent with previous research on different apple cultivars that has shown that concentrations of phloretin glycosides do not significantly change during fruit storage for this time period [87]. However, there is not sufficient research concerning apple storage and resulting ciders with which to compare.

Chlorogenic Acid

There were no significant differences in York or Dabinett cider treatments in regards to chlorogenic acid concentrations. Previous research with other cultivars in storage has shown that chlorogenic acid concentrations in fruit do not significantly change during either maturation or storage, but this has yet to be studied in ciders[87, 131].

Summary of Individual Polyphenols

Though not statistically significant, York total polyphenols had among the highest concentrations measured in both the juice and cider in the 6W-1C treatment. It is therefore not surprising that some classes of individual polyphenols may be more abundant in this treatment compared to the other three treatments. The individual polyphenols that are significantly higher in the 6W-1C treatment compared to one or more of the other treatments all belong to the flavonoids group which, as mentioned in

previous chapters, is an important class of polyphenols due to their sensory impact. It would be expected that the elevated levels of flavonoid monomers, dimers, trimers, and tetramers would make this cider treatment both more bitter and more astringent compared to the other treatments, particularly the 6W-10C and the 4M-1C treatments.

York cider individual polyphenols showed some variation between fruit storage treatments while the Dabinett cider individual polyphenols showed no significant difference in concentrations between treatments. Previous work on other apple cultivars has shown that some polyphenol concentrations may remain relatively constant throughout fruit storage, while others may be affected based on the polyphenol class and apple cultivar [84-87]. It is evident from this research that the concentrations of individual polyphenols in ciders made from different cultivars respond very differently during fruit storage. However, it is not clear as to why some York polyphenols showed an increase in concentration in the 6W-1C treatment, whilst the other storage treatments were more often more similar to the 2W-4C treatment. Additional studies on the York cultivar at varying storage temperatures and durations are necessary to better understand these results. Future studies including longer storage durations at two different temperatures could determine whether the results from this study are more duration or temperature dependent. Furthermore, it would be interesting to examine whether other dessert cultivars respond similarly as did the York cultivar while in storage. Lastly, sensory evaluation methods should be considered for future studies to determine whether different fruit storage treatments lead to sensory differences in ciders.

5.4 Conclusion

The extent of chemical and physical variation we observed between storage treatments generally decreased from apples to juice to cider. All apple quality parameters in all three cultivars showed significant differences between treatments. As discussed, these differences generally correlate with previous research on the effect of post-harvest storage on apple maturity and quality.

Differences were also found between treatments in the juice. These differences are largely cultivar-dependent, and there were fewer differences in the juice compared to the fruit. Furthermore, there were even fewer differences in cider chemistry between treatments. Again, these differences are very cultivar-dependent.

The results of this study are useful for cider producers, as they provide insight to the chemical changes of the fruit, juice, and cider resulting from fruit storage. Unlike wine grapes, which must be processed immediately after harvest, apples have the potential to be stored for a period of several months before being processed into juice and cider. Since post-harvest storage of apples has been demonstrated to impact fruit quality and juice chemistry, a similar impact was expected in cider chemistry. Our results showed that, compared to fruit and juice quality parameters, fewer differences persisted into cider chemistry when cider was made from fruit subjected to different post-harvest storage regimes. These results suggest that cider producers may have more flexibility with regards to post-harvest storage, while maintaining similar cider quality. Further research on this topic should include more cultivars, various harvest years, and extended storage times to more accurately evaluate storage effects on the fruit, juice, and cider.

CHAPTER 6: EVALUATION OF THE FOLIN CIOCALTEU ASSAY FOR MEASUREMENT OF TOTAL POLYPHENOLS IN JUICE

6.0 Abstract

The Folin-Ciocalteu (FC) assay is commonly used for quantification of total polyphenols in food and beverage research. There are several compounds that potentially interfere with the accuracy of the assay, and many of these compounds are present in juice. Reducing sugars and tyrosine were both evaluated for additive effects when quantifying total polyphenols. Reducing sugars in the sample matrix did not affect the results of the FC assay. The presence of the amino acid tyrosine, however, resulted in significant overestimation of total polyphenols in fruit juice by the FC assay where 1 mg/L tyrosine resulted in 2.4 mg gallic acid equivalents (GAE)/L measured by the FC assay.

6.1 Introduction

The Folin-Ciocalteu (FC) Assay is a common method for quantifying polyphenols in juice and wine. This method uses a colorimetric reagent made of sodium molbdate and sodium tungstate that oxidizes phenolic compounds. The reagent is reduced by compounds in the sample to phopsphomolybdic and phosphotungstic acids to form a blue color measured as absorbance at 760nm [90-93].

Despite its widespread acceptance and use in horticulture and food science research, there are many concerns over the specificity of the FC Assay for determination of total polyphenols in samples with complex matrices, such as fruit juice, cider or wine. The FC reagent has been reported to react with other compounds including amino acids,

ascorbic acid, sulfites, and potentially many others [90, 92, 93, 132]. Reducing sugars have also been thought to contribute in an additive manner to the results of the FC Assay, but a recent study by Everette et al. has indicated that this may not be the case [90, 92, 93]. Furthermore, galacturonic acid, a significant component of pectins, has been shown to create additive interferences [133].

The long-term objective of this study is to better understand how the reactivity of compounds in the juice matrix with the FC reagent affects juice polyphenol quantification. Knowledge of interfering compounds in the juice matrix may better future polyphenol quantification methods. Two hypotheses were tested: (1) reducing sugars interfere in an additive manner when using the FC Assay to quantify total polyphenols (2) tyrosine interferes in an additive manner when using the FC Assay to quantify total polyphenols. These hypotheses were tested using a combination of Solid Phase Extraction (SPE), the FC Assay, reducing sugar analysis, and the dimethylaminocinnimaldehyde (DMAC) method.

6.2 Part 1: Evaluation of the Effect of Reducing Sugars on the FC Assay

The impact of sugars in the sample matrix on results of the FC Assay was assessed by removing sugars from the sample matrix via solid phase extraction (SPE) and chemically characterizing the resulting eluents and wash fractions. Parameters of interest were residual sugar, total procyanidins by DMAC, pectin and total polyphenols by FC. Figure 6.1 presents a diagram of the experimental approach.

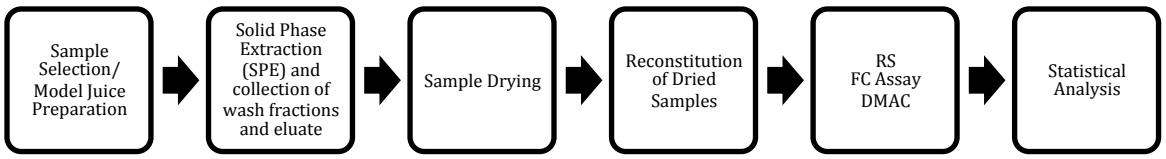

Figure 6.1: Outline of the experimental procedures followed in Part 1.

6.21 Materials and Methods

One commercial apple (CA) juice (Kroger 100% Juice, Natural Apple from Concentrate), one commercial grape (CG) juice (Santa Cruz Organic, Concord Grape juice from Concentrate), one lab-pressed Dabinett cider apple juice from Cornell's Lansing Orchard in Lansing, NY (LA), one lab-pressed Cabernet Sauvignon grape juice from the Virginia Tech Alson H. Smith Jr. Agricultural Research and Extension Center in Winchester, VA (LG), and one model juice (MJ) were used for the analysis. The commercial juices did not contain added ascorbic acid, which is known to be an interfering substance in the FC Assay [90, 92, 132]. The model juice was formulated as follows: 18.7 g glucose (Sigma-Aldrich, Col., St. Louis, MO, USA), 60 g fructose (Sigma-Aldrich, Col., St. Louis, MO, USA), 7.5 g malic acid (Sigma-Aldrich, Col., St. Louis, MO, USA), 11.2 mg catechin (Sigma-Aldrich, Col., St. Louis, MO, USA), 18.7 mg epicatechin (Sigma-Aldrich, Col., St. Louis, MO, USA), and 167 mg quercetin (Sigma-Aldrich, Col., St. Louis, MO, USA) to 1L deionized water. Initial Solid Soluble Content (SSC) was measured by a PAL-1 refractometer (Atago U.S.A., Inc., Bellevue, WA) for each sample for comparison with the results of the residual sugar assay. Sugar, acid, catechin, and epicatechin values were based off of values found in the literature [1, 3,

[134]. The concentration of quercetin is much higher than normally found in apple juice, but was chosen for a more measureable concentration [134]

The initial reducing sugar concentration of the juice samples was first measured using the Megazyme D-Fructose and D-Glucose kit (Megazyme International, Ireland). Dilutions using dilution factors of 10, 100, and 1000 were made as needed so that sugar concentrations of the juice fell within the linear range of the kit. Total polyphenols were measured using the FC Assay, and total procyanidins were measured using the DMAC method.

SPE was used to isolate the polyphenols from the sugars in the juice matrix. Typically, SPE protocols for sample prep follow the general procedure represented in Figure 6.2. First, the column is conditioned with a solvent that wets the adsorption media, and then the column is equilibrated with water. It is important that the samples are loaded soon after this step, as it will decrease the effectiveness of the column if it dries. As the sample runs through the column, the analyte (compound of interest that is to be isolated for further analysis in the typical SPE sample prep procedure) and potentially other compounds are retained in the adsorption media. Next, a specific solvent that will not extract the analyte is passed through the column in a "wash" step. This step removes compounds that may have physically remained on the column (but were not adsorbed) from the "load" step to further isolate the analyte. Lastly, the analyte is collected in an "elution" step, and the resulting liquid is known as the "eluate". The number of wash and elution steps is dependent on each experiment and the analytes being recovered.

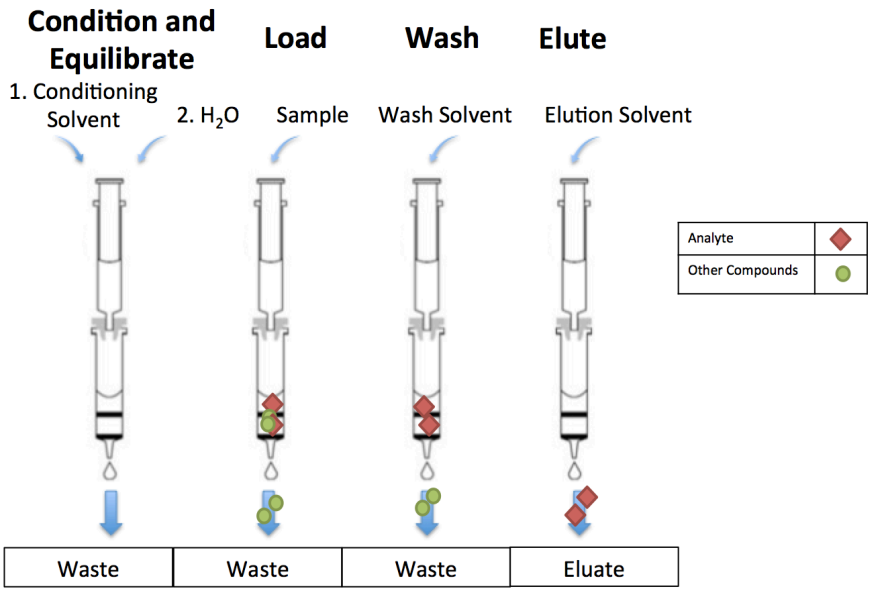

Figure 6.2: General procedure for SPE with a conditioning and equilibration step followed by loading, washing, and eluting.

This experiment employed SPE for a different purpose. Since both the sugars and the polyphenols are compounds of interest with regards to our critical evaluation of the FC assay, they can both be considered "analytes" in this case. Therefore, there were four elution steps and four eluates, rather than three washes and one eluate. The purpose of collecting fractions of these compounds is to examine how they interact individually with the FC reagent and how that compares to the total polyphenols measured by the FC assay in the original complex juice matrix. Separation of compounds present in the matrix allows the measurement of total polyphenols by FC from one specific compound or class of compounds, rather than the additive value obtained through measuring a combination of antioxidant substances.

A diagram of the experiment is shown in Figure 6.3. For SPE, the Oasis HLB 1cc (30mg) Extraction Cartridges were first conditioned with methanol and then equilibrated with water. A vacuum was applied to the SPE manifold thus causing the liquid to flow

through the cartridge. This process was applied each time a column was conditioned, equilibrated, washed, or eluted. Then, 0.5mL of the juice sample was loaded into the cartridge. The resulting eluate (Eluate 1) was collected in a tube and then placed on dry ice. A new collection tube was placed under the cartridge and the cartridge was washed with 1mL 0.5% MeOH. The eluate (Eluate 2) was collected in the tube and was then placed on dry ice. A new collection tube was placed under the cartridge and the cartridge was washed with 2mL 100% MeOH. The resulting eluate (Eluate 3) was placed in the Speed Vap to remove solvent. Eluate 3 did not need to be freeze-dried as the previous step was sufficient for removing all of the solvent. A new collection tube was placed under the cartridge and the cartridge was washed with a solution containing 70% acetone, 28% water, and 2% glacial acetic acid. The eluate (Eluate 4) was collected and placed into the Speed Vap. Eluates 1, 2, and 4 were freeze dried, and all tubes were stored at -80°C until analysis.

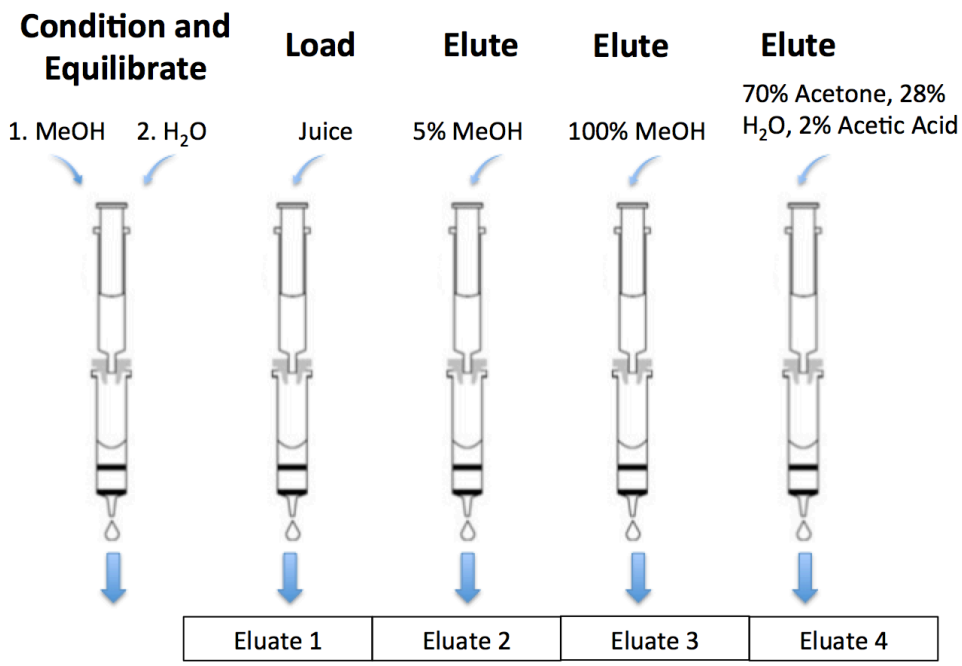

Figure 6.3: Diagram of the SPE method used to isolate polyphenol compounds from sugars in the juice matrix where Eluates 1 and 2 are expected to have the higher sugar concentrations and Eluates 3 and 4 are expected to have the higher polyphenol concentrations, based on the SPE cartridge chemistry as designed for sample prep methods.

Eluate 1 and 2 were reconstituted using 0.1mL of the methanol:water solution and Eluate 3 and 4 were reconstituted using 0.2mL of the methanol:water solution. The collected eluate and original samples were analyzed using residual sugar analysis, FC assay, and DMAC procedures. Results were back-calculated to represent the concentration in the original juice. A diagram of the sample preparation and analysis steps is presented in Figure 6.4.

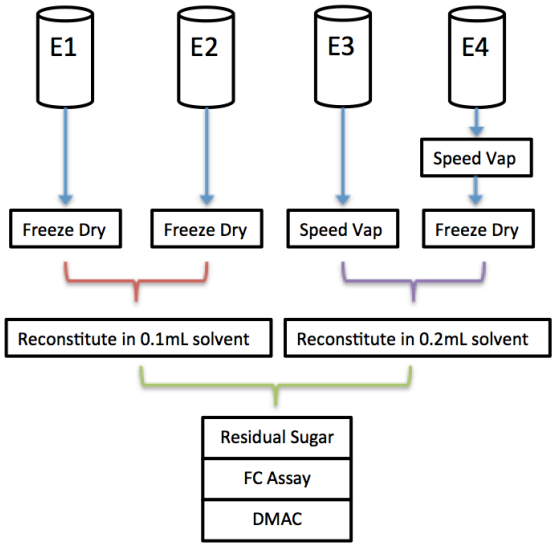

Figure 6.4: Diagram representing the sample preparation and analysis steps after SPE.

Lastly, the presence of pectin in the original samples (juices or model solutions before SPE) was determined using a method described by Zoecklein et al. [135]. Juice was diluted 2:1 in a solution of two parts 95% ethanol and 1% HCl and one part juice..After several minutes, the mixture was visually inspected for gel formation, which indicates the presence of pectin. Pectin cannot be quantified or characterized by this method, but it shows the general presence of pectin nonetheless.

6.22 Results and Discussion

Results from the FC assay are presented in Table 6.1.

Table 6.1: Concentration of total polyphenols by FC ųg/mL GAE equivalents)

Juice Sample	Original Sample	E1	E2	E3	E4	Total (E1-E4)
Commercial Apple	162.1±5.7	91.6±2.2^{a}	27.9±1.8^{b}	25.9±1.3^{b}	1.0±0.5^{c}	146.2±2.0
Commercial Grape	1147.1±6.4	86.3±14.5^{b}	48.5±7.3^{b}	644.0±42.3^{a}	79.3±20.2^{b}	858.0±34.7
Lab Apple	860.6±23.0	81.9±8.8^{b}	8.1±3.6^{b}	570.0±143.1^{a}	68.0±6.9^{b}	728.0±156.1
Lab Grape	300.1±5.7	47.1±1.9^{b}	19.9±4.0^{c}	112.7±5.8^{a}	4.6±0.5^{c}	184.2±8.3
Model Juice	16.7±3.2	1.8±0.3^{a}	0±0^{a}	34.5±18.5^{a}	4.1±2.6^{a}	40.4±16.3

Values are reported as mean ± standard error for n=4 replicates. Common letters assigned to values indicates no significant difference between values.

The CA juice had a significantly higher concentration of polyphenols in E1, and this eluate represented 63% of the polyphenols in the juice. The polyphenol concentration decreased by 70% from E1 to E2. In contrast, the CG, LA, and LG juices had significantly higher concentrations of polyphenols from the third elution (E3) and represented 75%, 78%, and 61% of the total juice polyphenols, respectively. These data are represented in Figures 6.6-6.8. Lastly, the model juice did not have significantly different concentrations between treatments due to large variances and this data is represented in Figure 6.9. Large variances are likely a result of the insolubility of quercetin in the water-based solution. Despite the variances, 85% of the polyphenols measured by FC were presented in E3.

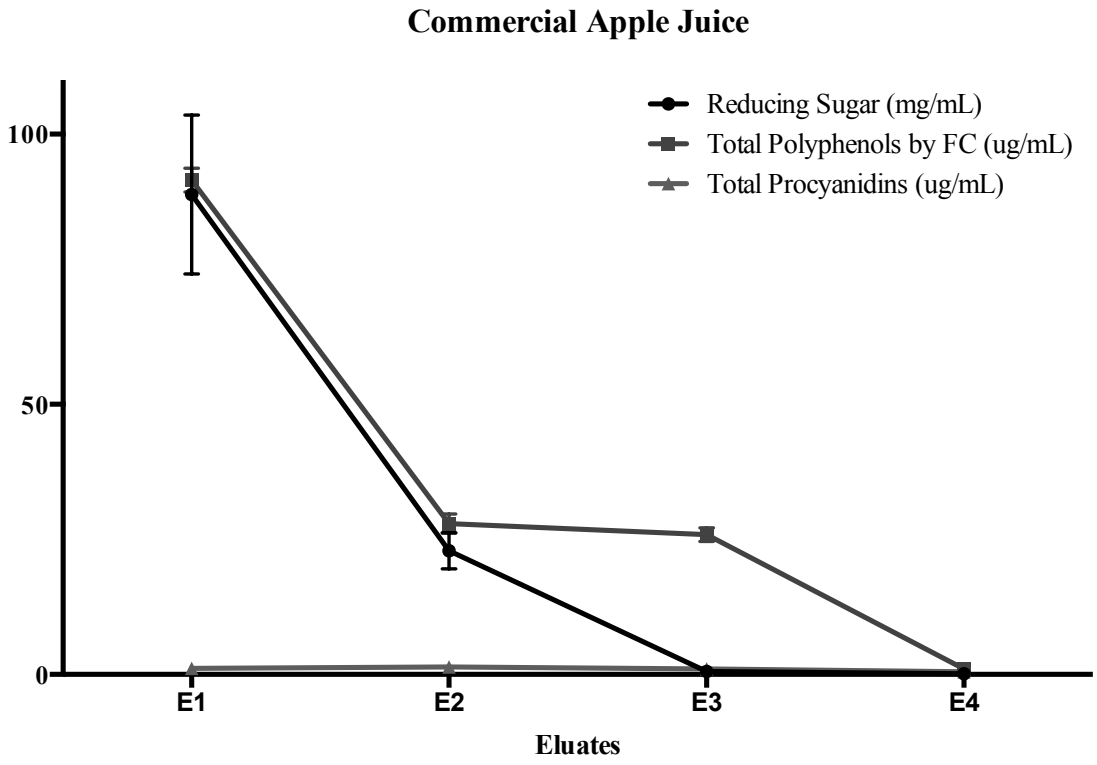

Figure 6.5: Representation of commercial apple juice total polyphenols by FC (as measured by the FC Assay), sugar concentration, and total procyanidins of Eluates 1-4 (E1-4) obtained by Solid Phase Extraction (SPE). Total polyphenols by FC concentration was highest in E1.

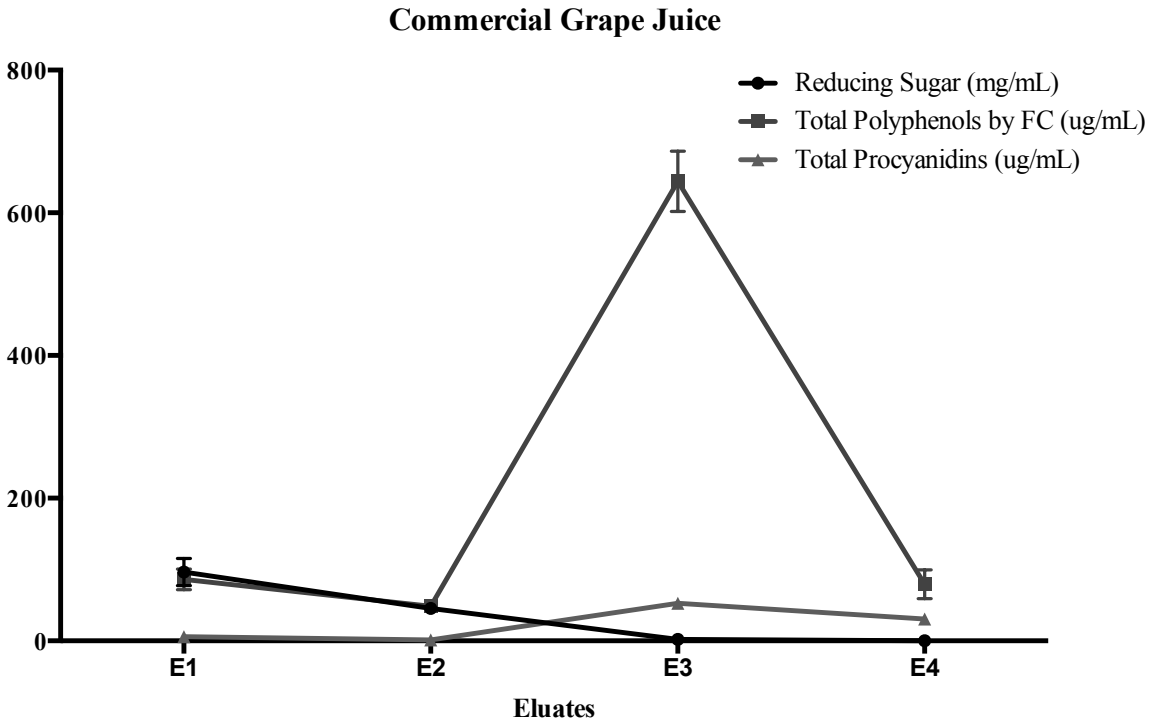

Figure 6.6: Representation of commercial grape juice total polyphenols by FC (as measured by the FC Assay), sugar concentration, and total procyanidins of Eluates 1-4 (E1-4) obtained by Solid Phase Extraction (SPE). Total polyphenols by FC concentration was highest in E3.

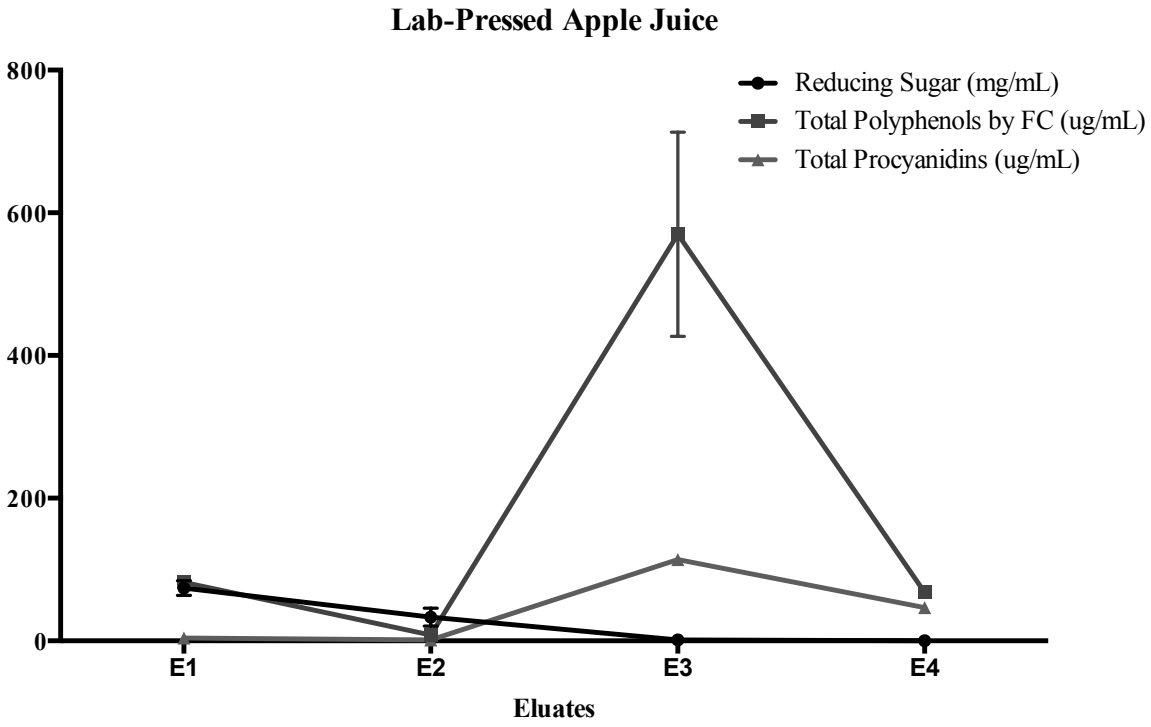

Figure 6.7: Representation of lab-pressed apple juice total polyphenols by FC (as measured by the FC Assay), sugar concentration, and total procyanidins of Eluates 1-4 (E1-4) obtained by Solid Phase Extraction (SPE). Total polyphenols by FC concentration was highest in E3.

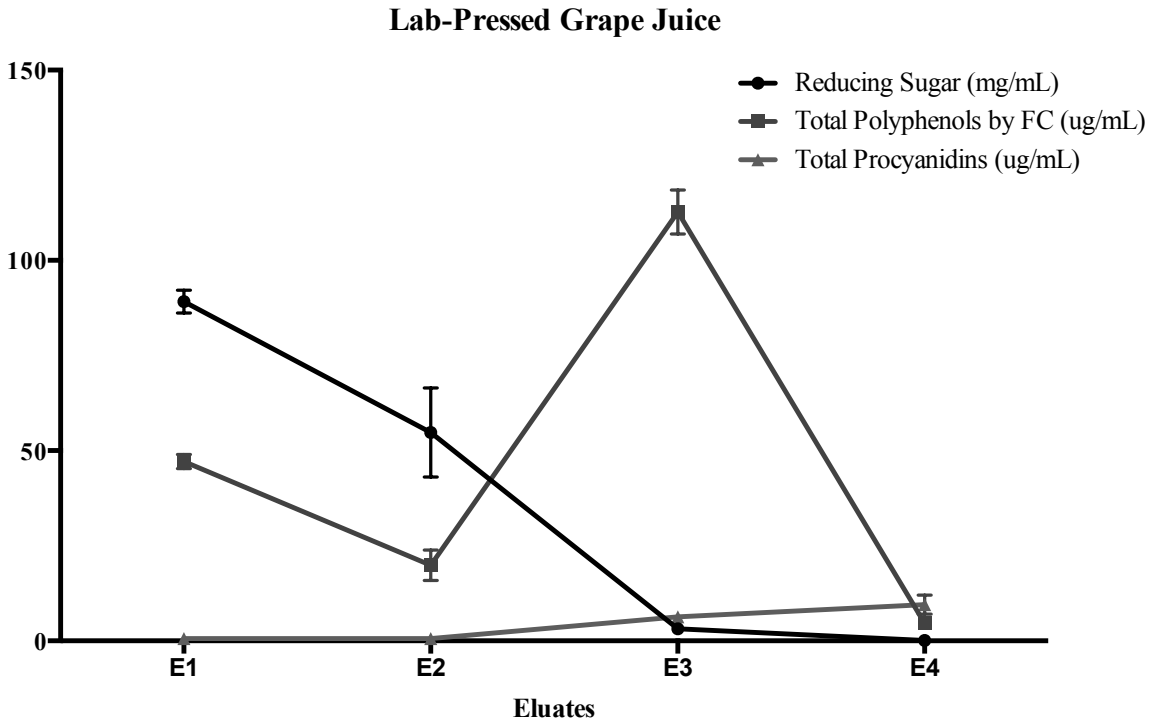

Figure 6.8: Representation of lab-pressed grape juice total polyphenols by FC (as measured by the FC Assay), sugar concentration, and total procyanidins of Eluates 1-4 (E1-4) obtained by Solid Phase Extraction (SPE). Total polyphenols by FC concentration was highest in E3.

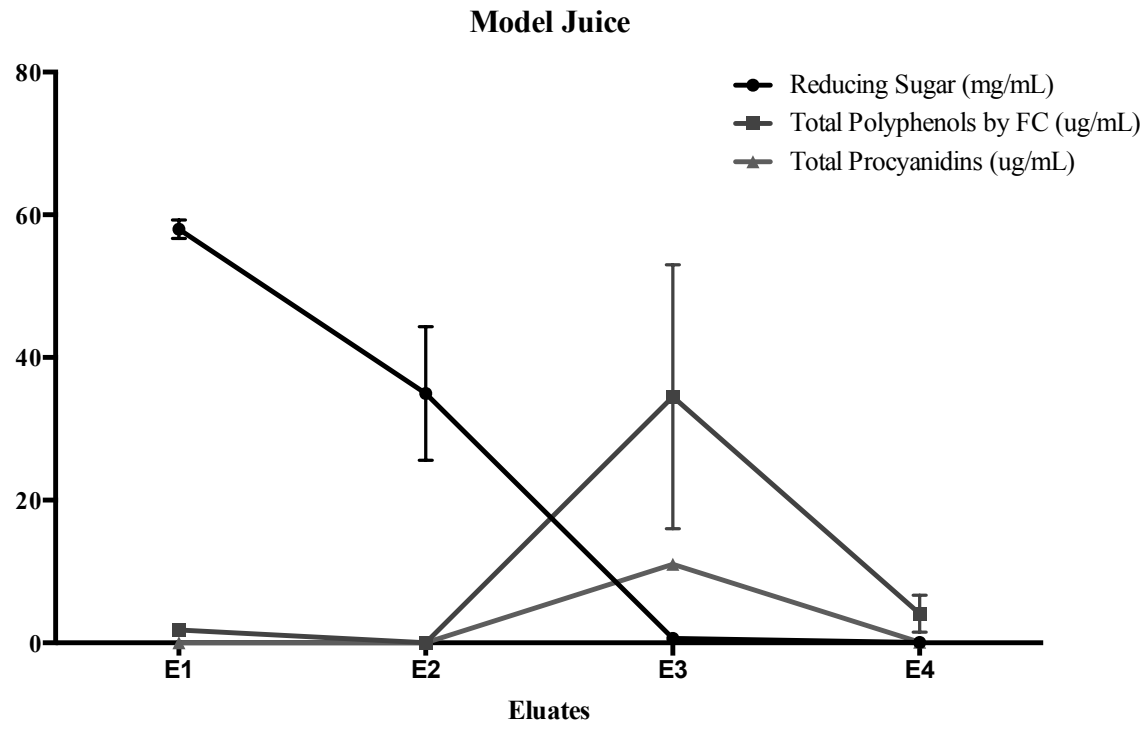

Figure 6.9: Representation of model juice total antioxidant compounds (as measured by the FC Assay), sugar concentration, and total procyanidins of Eluates 1-4 (E1-4) obtained by Solid Phase Extraction (SPE). No eluate was definitively higher in antioxidant compounds due to large variances likely attributed to insolubility of quercetin in solution.

Based on these results, it is likely that this method was successful in isolating polyphenols from the reducing sugars during the third elution for the CG, LA, and LG juices as was intended with the experimental design. However, this was not the case for the CA and MJ juices. For the MJ juice, it is likely that the high variation in polyphenol concentration across replicates due to insolubility of quercetin led to high variance and was the reason the eluates were not significantly different from one another. For the CA juice, the concentration and composition of polyphenols may affect the reliability of this method to isolate and quantify polyphenols. For example, the elution solutions used in this experiment may be more suitable for extraction of some polyphenols compared to others, making the composition of polyphenols an important factor. It is also possible that

this method is only suitable for juices with relatively higher concentrations of polyphenols since isolation during the third elution occurred most effectively in the juices with higher polyphenol concentrations. Lastly, there could be unexpected interfering compounds in the juice matrix that caused higher concentrations of total polyphenols by FC in the first eluate.

Results of the residual sugar analysis are presented in Table 6.2.

Table 6.2: Concentration of Reducing Sugars as the sum of Glucose and Fructose (mg/mL)

Juice	Original Sample	E1	E2	E3	E4	Total (E1-E4)
Commercial Apple	111.12±0.03	88.83±14.73^{a}	22.90±3.38^{b}	0.55±0.02^{b}	0.12±0.02^{b}	112.39±16.59
Commercial Grape	135.14±2.25	96.53±19.00^{a}	45.67±7.19^{b}	2.20±0.86^{c}	0.09±0.02^{c}	144.48±14.37
Lab Apple	106.70±0.64	74.06±10.30^{a}	33.13±12.89^{b}	1.42±0.20^{b}	0.15±0.08^{b}	108.76±7.62
Lab Grape	195.47±0.24	89.18±3.03^{a}	54.78±11.73^{b}	3.15±0.66^{c}	0.08±0.03^{c}	147.19±14.02
Model Juice	81.41±0.26	58.00±1.30^{a}	34.95±9.38^{b}	0.63±0.11^{c}	0.07±0.04^{d}	93.64±10.44

Values are reported as mean ± standard error for n=4 replicates. Common letters assigned to values indicates no significant difference between values.

For all five juices, the residual sugar concentration was significantly greater in the first eluate compared to the other three eluates. This indicates that this method was successful at isolating (i.e. removing from the sample) the majority of the reducing sugars during the first elution.

Total procyanidins results from the DMAC analysis are presented in Table 6.3.

Table 6.3: Concentration of Procyanidins (µg/mL PC B2 equivalents)

Juice	Original Sample	E1	E2	E3	E4	Total (E1-E4)
Commercial Apple	2.9±0.1	1.1±0.1^{a}	1.4±0.6^{a}	1.0±0.2^{a}	0.5±0.0^{a}	4.0±0.7
Commercial Grape	72.4±1.4	6.0±0.3^{c}	1.5±0.2^{c}	52.7±1.6^{a}	30.5±2.1^{b}	83.0±7.2
Lab Apple	63.0±0.9	4.0±0.2^{c}	1.2±0.3^{c}	113.8±4.1^{a}	46.8±1.3^{b}	165.8±3.4
Lab Grape	7.9±0.2	0.6±0.2^{b}	0.6±0.6^{b}	6.3±0.3^{a}	9.5±2.5^{a}	12.7±1.1
Model Juice	2.1±0.1	0±0^{b}	0±0^{b}	11.0±0.2^{a}	0.1±0.0^{b}	11.1±0.2

Values are reported as mean ± standard error for n=4 replicates. Common letters assigned to values indicates no significant difference between values.

There were noticeable discrepancies between the total procyanidin concentration values calculated from the original juice sample compared to the sum of procyanidins by DMAC calculated by adding the content present in all four eluates. For this reason, it is difficult to accurately assess the data. There were no significant differences in procyanidin concentrations between eluates in the CA juice, indicating that this method was either unsuccessful at isolating this class of polyphenols in the CA juice or there was another compound in the first eluate that caused a higher reading of total polyphenols by FC. In the CG and LA juices, procyanidin concentrations were significantly higher in the third eluate followed by the fourth eluate. In LG juice, procyanidin concentrations were significantly higher in the first and second eluates compared to the other two eluates. In the MJ juice, concentrations were significantly higher in the third eluate compared to the other three treatments. Though there are inconsistencies between juice types and in quantification, the general trend of these results still can provide some insight. For CG, LA, and MJ juices, the majority of procyanidins were isolated in either the third or the third and fourth eluates. Therefore, adjustment of this method through alternative solvent or SPE cartridge selections may be a strategy to isolate and quantify total procyanidins more accurately using the FC assay.

The pectin test revealed the presence of pectin in the CA, CG, LA, and LG juices, but not the MJ juice. This test does not classify or quantify the pectin, and further identification tests and turbidity tests would be necessary to better understand the composition of pectin in the juices. Use of the Megazyme Pectin Identification kit (Megazyme International, Ireland) could provide information about the types of pectins found in a variety of juices. However, this kit requires a powdered form of the juice, so a

successful dehydration procedure is necessary for this method. Freeze-drying is a difficult means of achieving a powder due to the high amounts of sugar in the juice creating a sticky mass. Turbidity could be measured using a turbidimeter, and this would provide a means of quantification of solid suspended particles, but not specifically pectin.

6.23 Evaluation of the Effect Reducing Sugars on the FC Assay

Seven different sugar solutions were made to assess the reactivity of the Folin-Ciocalteau's phenol reagent (Sigma-Aldrich, Co., St. Louis, MO, USA) with reducing sugars. The solutions were made with DI water and contained the following concentrations of sugars: 100g/L glucose (Sigma-Aldrich, Co., St. Louis, MO, USA), 100g/L fructose (Sigma-Aldrich, Col., St. Louis, MO, USA), 50g/L glucose plus 50g/L fructose, 50g/L glucose, 50g/L fructose, 25g/L glucose plus 25g/L fructose, and 100 g/L sucrose (Fisher Scientific, Fair Lawn, NJ, USA) as a control. The sugar solutions were then analyzed using the FC assay method. All of the sugar solutions were undetectable using this method, as shown in Table 6.4.

Table 6.4: Effect of Reducing Sugars in water on FC Assay

Solution	GAE (mg/L)
100g Glucose	ND
100g Fructose	ND
50g Glucose + 50g Fructose	ND
50g Glucose	ND
50g Fructose	ND
25g Glucose + 25g Fructose	ND
100g Sucrose	ND

Results are represented with n=4 replicates

This indicates that despite previous reports that reducing sugars, such as glucose and fructose, may interfere with the FC assay, they may not interfere, at least in an

additive manner, to the results of polyphenol quantification. These results are consistent with recent research by Everette et al. where several compounds were tested for reactivity with the FC assay, and glucose and fructose were not reactive [92]. Therefore, the SPE method of isolating polyphenols from reducing sugars may not be the most effective approach for reducing interferences when quantifying polyphenols using the FC assay. Other substances in the juice matrix may still cause interferences [92].

6.24 Potential Sources of Error

Data collected during this experiment could have been subject to one or more sources of error. It is possible that some components of interest, such as polyphenols remaining on the column after the final wash, decreasing the final yield of those components. In addition, each column was washed or eluted at a slightly different rate, meaning that when the vacuum was applied, some columns had been dry for seconds before others had finished. This may be caused by the number of columns on the manifold, the relative location of the columns on the manifold, the tightness of seal of each column to the manifold, the order in which juice and solvents were loaded, the viscosity of the sample, or variations in sorbent packing. This could possibly cause the amount of analytes in each eluate to differ between replicates, and this could also affect the moisture and therefore efficacy of the sorbent.

The FC Assay results may have been affected by this specific SPE method. Fractionation of the juice may have in some instances resulted in a higher total polyphenols value read if additive compounds which lead to false high readings (i.e. reducing compounds) were eluted at the same time, whereas there may have been a

decrease in observed concentration values if oxidizing compounds were eluted in the same step. Further research would be required to determine the retention properties of other potentially interfering substances and their effects on the FC assay.

Furthermore, the presence of pectin in the juice samples could have been responsible for some analytical errors in the FC and DMAC analyses. Polyphenols may associate with pectins in the juice matrix [136]. In the original juice sample, the presence of pectins may have caused a decrease in total polyphenols to be measured due to pectin-polyphenol association making polyphenols unavailable to reduce the reagent in the assay. Also, if procyandins were to associate with pectins, they would precipitate into the methanol solution used in the DMAC analysis. This would make a more heterogeneous solution and make sampling inconsistent between replications.

The model juice was not a successful model for evaluating this SPE method for isolating polyphenols. Quercetin was selected for this experiment due to its presence in apple juice, commercial availability and relatively low cost. However, low solubility of quercetin in the primarily water-based model solution was a challenge. Quercetin visibly precipitated out of solution after vortex mixing, and controlled, homogenous sample extraction was therefore not possible. In addition, this separation could potentially explain inconsistencies between each SPE replication. Because the residual sugar results of the model juice correlate with those of the other juices examined, there is reason to believe that it is likely the inconsistent dissolution of quercetin that resulted in a poor model juice. In the future, solvents should be selected on their ability to solubilize quercetin in solution, or other polyphenol compounds with improved solubility should be

considered. Lastly, eluent and solvent (for the model solution) optimization could also decrease experimental error,

6.3 Part 2: Evaluation of the Effect of Tyrosine on the FC Assay

Following up on the results from the experiment described in Part 1 of this chapter, an experiment was conducted on the effect of tyrosine on the FC assay in a model juice solution. Previous literature has shown that tyrosine has an additive effect to the results on the FC Assay [92]. Tyrosine is an amino acid found in grape and apple juice, albeit usually in relatively low quantities compared to other amino acids [137, 138]. However, some grape varieties have more substantial tyrosine concentrations. Values have been reported as high as 35mg/L in Thompson Seedless grape juices and 30mg/L in Chardonnay grape juices[134, 139]. Carlos Muscadine grapes grown in Florida were reported to have tyrosine concentrations mostly between 50mg/L and 70mg/L, but fruit from some vineyard blocks contained tyrosine concentrations as high as 140mg/L (Stewart, unpublished). Tyrosine has been previously reported as one of the most prevalent amino acids in Muscadine grapes[140]. The range of tyrosine concentration observed in some fruit juices is certainly high enough to potentially cause significant overestimation of total polyphenol concentration by FC assay, thus we systematically investigated the effect of tyrosine on the FC assay.

6.31 Materials and Methods

The experimental process for this experiment is presented in Figure 6.9.

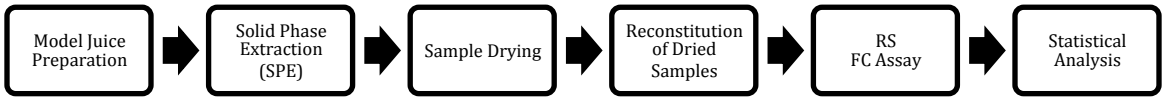

Figure 6.9: Diagram outlining the experimental procedure for Part 2.

Three model solutions were made using glucose, tyrosine, and epicatechin in deionized water. Each solution contained 50mg/mL glucose (Sigma-Aldrich, Col., St. Louis, MO, USA), In addition, "Solution A" contained 25µg/mL tyrosine (Sigma-Aldrich, Col., St. Louis, MO, USA), "Solution B" contained 25µg/mL epicatechin (Sigma-Aldrich, Col., St. Louis, MO, USA), "Solution C" contained both 25µg/mL tyrosine and 25µg/mL epicatechin. The solution compositions are also represented in Table 6.5.

Table 6.5: Starting concentrations of glucose, tyrosine, and epicatechin in model solutions A-C.

	Glucose (mg/mL)	Tyrosine (µg/mL)	Epicatechin (µg/mL)
Solution A	50	25	0
Solution B	50	0	25
Solution C	50	25	25

These tyrosine concentrations are similar to what has been reported in previous work with grape juices, but are much higher than the concentration of tyrosine reported in apple juices.

Each of the three model solutions was processed by SPE prior to FC analysis following the procedure diagramed in Figure 6.2. First the columns were conditioned using 1mL methanol then equilibrated used 1mL water. Next, 0.5mL of the sample was

loaded into the column and the eluate (E1) was collected then freeze-dried. Then, the column was eluted using 1mL 0.5% methanol and the eluate (E2) was collected then freeze-dried. The column was then eluted using 2mL of 100% methanol and the eluate (E3) was collected then speed evaporated. Lastly, the column was eluted using 2mL of a 70:28:2 acetone:water:glacial acetic acid solution and the eluate (E4) was collected, speed evaporated, then freeze-dried. Samples were stored in the -80°C freezer until analysis.

The samples were reconstituted using 200mL of 0.1N HCl. Samples were sonicated for 10 minutes then analyzed using the Residual Sugar Kit and the FC Assay.

6.32 Results and Discussion

The results from Part 2 are presented in Tables 6.6 and 6.7.

Table 6.6: Concentration of Reducing Sugars as Glucose (mg/mL) in model solutions A-C including starting solutions, eluates 1-4 from SPE, and the sum of eluates 1-4 .

Model Solution	Original Sample	E1	E2	E3	E4	Total (E1-E4)
Solution A	50.28±0.64	27.59±1.34^{a}	22.70±0.65^{b}	0.80±0.19^{c}	0.14±0.03^{c}	51.23±1.89
Solution B	53.16±0.68	27.43±1.31^{a}	22.48±1.51^{b}	1.44±0.45^{c}	0.09±0.01^{c}	51.08±1.78
Solution C	50.57±0.56	24.94±1.31^{a}	18.59±4.57^{a}	0.95±0.22^{b}	0.13±0.01^{b}	50.85±1.28

Values are reported as mean ± standard error for n=4 replicates. Common letters assigned to values indicates no significant difference between values.

Table 6.7: Concentration of antioxidant compounds reduced by the Folin reagent using the FC Assay (µg/mL GAE equivalents) in model solutions A-C including starting solutions, eluates 1-4 from SPE, and the sum of eluates 1-4 **.**

Model Solution	Original Sample	E1	E2	E3	E4	Total (E1-E4)
Solution A	60.0±1.0	40.3±1.9^{a}	18.3±0.6^{b}	0.8±0.8^{c}	0±0^{c}	59.5±0.7
Solution B	22.5±0.8	4.7±0.9^{b}	0.5±0.2^{c}	7.0±0.3^{a}	0±0^{c}	12.2±1.3
Solution C	59.2±1.6	38.7±1.4^{a}	14.0±1.6^{b}	12.3±1.0^{b}	0±0^{c}	65.0±1.4

Values are reported as mean ± standard error for n=4 replicates. Common letters assigned to values indicates no significant difference between values.

As expected the majority of the glucose was found in Eluates 1 and 2 for all three solutions. This corresponds with the pattern found in Part 1 of this study. Residual sugar concentrations per eluate were fairly consistent across all three solutions, and would therefore not likely interfere with the differences found in the experiment.

More variation in antioxidant compound concentration as measured by FC between solutions and eluates were observed compared to the sugar concentration measurements. The solutions containing tyrosine had a much higher measured total polyphenols by FC than the solution without, and the concentration of epicatechin measured by FC was much lower than the added concentration. Solution C, which contained 25ug/mL of both tyrosine and epicatechin, did not have a significantly higher measured value compared to Solution, which only contained 25ug/mL tyrosine. Solution B, which contained 25µg/L epicatechin, had a much lower reading than Solutions A and C. This is represented in Figure 6.10.

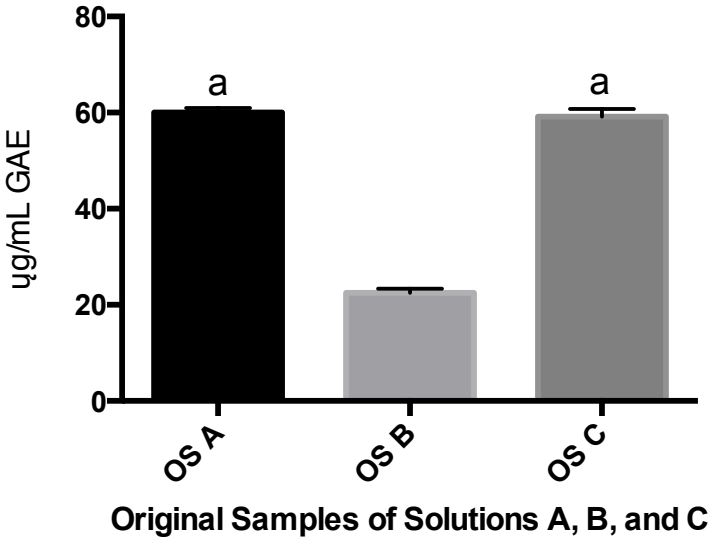

Figure 6.10: Concentration of total polyphenols by FC of the Original Samples of Solutions A, B, and C. Results were analyzed using one-way ANOVA then Tukey's HSD test. Common letters represent no significant differences between samples.

Though the concentrations of total polyphenols by FC in the original samples were higher then expected, these values closely correlated with the totals calculated following SPE for Solution A and Solution C. The total from Solution B was lower than the concentration measured from the original sample. It is unlikely that this decrease after SPE was due to retention of epicatechin on the column since the final elution resulted in a measurement of 0 µg/mL GAE. Perhaps a better explanation for this result involves poor solubility of epicatechin in the reconstitution solution, or that the FC assay underestimates the measurement of epicatechin. Epicatechin is soluble in water, but research has shown that it is more soluble in methanol, ethanol, and 70% ethanol solutions[141]. Using an organic solvent in the sample solutions or during reconstitution may have improved the measurement of epicatechin using the FC Assay.

Despite the challenges in accurately measuring epicatechin using the FC Assay during this experiment, there are useful results obtained through the SPE process. The total polyphenols by FC measured for Solution A was E1, then E2, and lastly E3 and E4 as presented in Figure 6.11. This shows that the compound oxidized by the FC reagent was not well retained by the column and was mostly collected in the first and second elutions. In contrast, the total polyphenols by FC measured in Solution B was highest in E3, then E1, and then E2 and E4, presented in Figure 6.12. This shows that the majority of the analyte, epicatechin, was retained in the column until the third elution, though some of it was not retained during the first elution. Lastly, the total polyphenols by FC measured in Solution C was significantly highest in E1 followed by E2 and E3. E4 was the lowest, as presented in Figure 6.13. The SPE of Solution C shows a higher concentration in E1 due to higher concentrations of tyrosine. E2 likely contained mostly

tyrosine, and E3 likely contained mostly epicatechin. By E4, both analytes had been completely eluted. The total polyphenols by FC of Solution C closely resembles the totals of Solution A and B summed together. Graphs comparing E1, E2, E3, E4, and totals are presented in Figures 6.14-6.18.

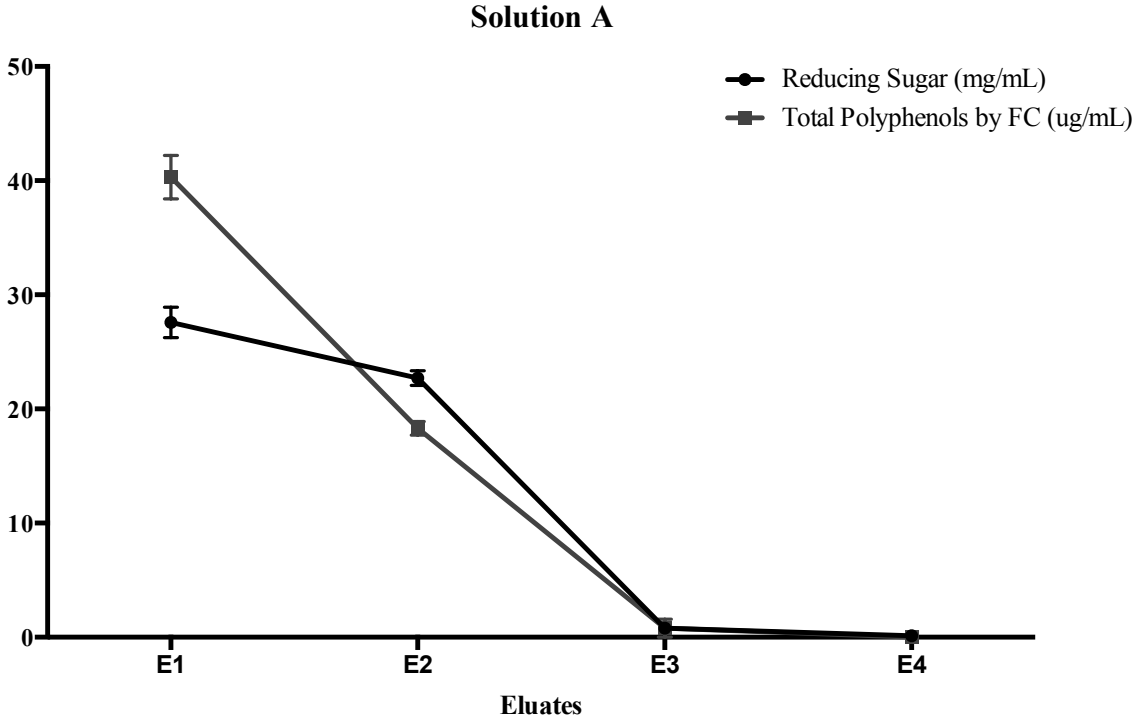

Figure 6.11: Concentrations of total polyphenols by FC and sugar in eluates 1-4 made through SPE. Solution A contained 50mg/L glucose and 25mg/L tyrosine.

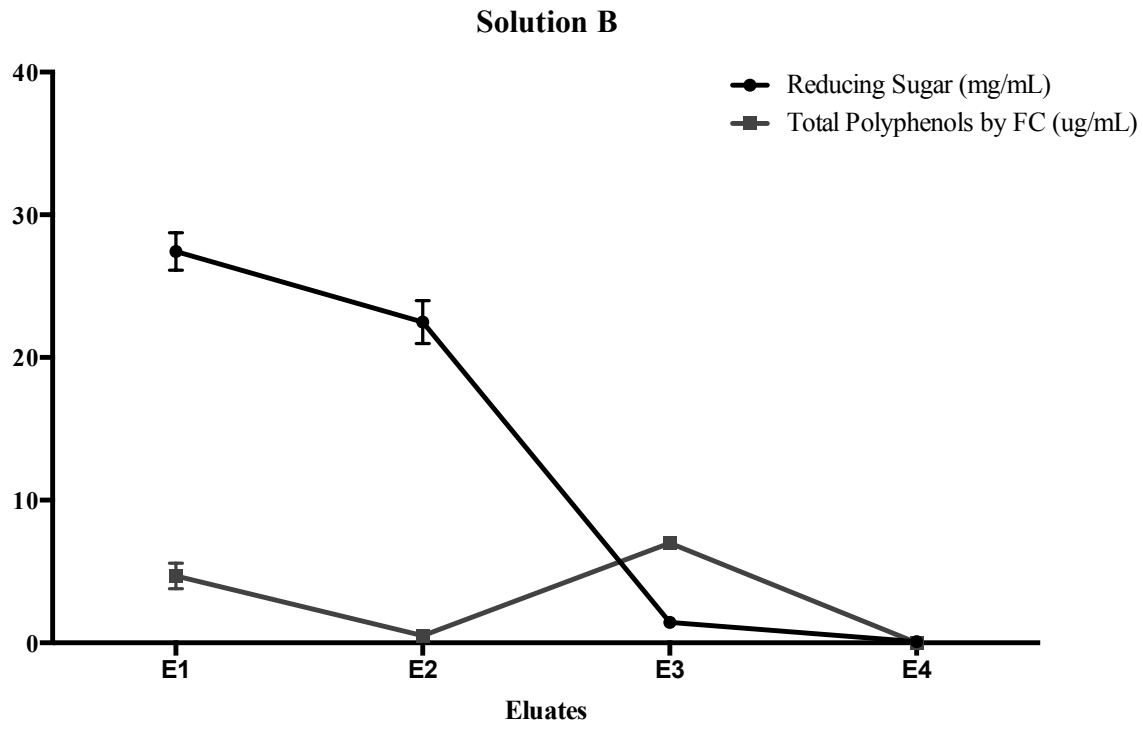

Figure 6.12: Concentrations of total polyphenols by FC and sugar in eluates 1-4 made through SPE. Solution B contained 50mg/L glucose and 25mg/L epicatechin

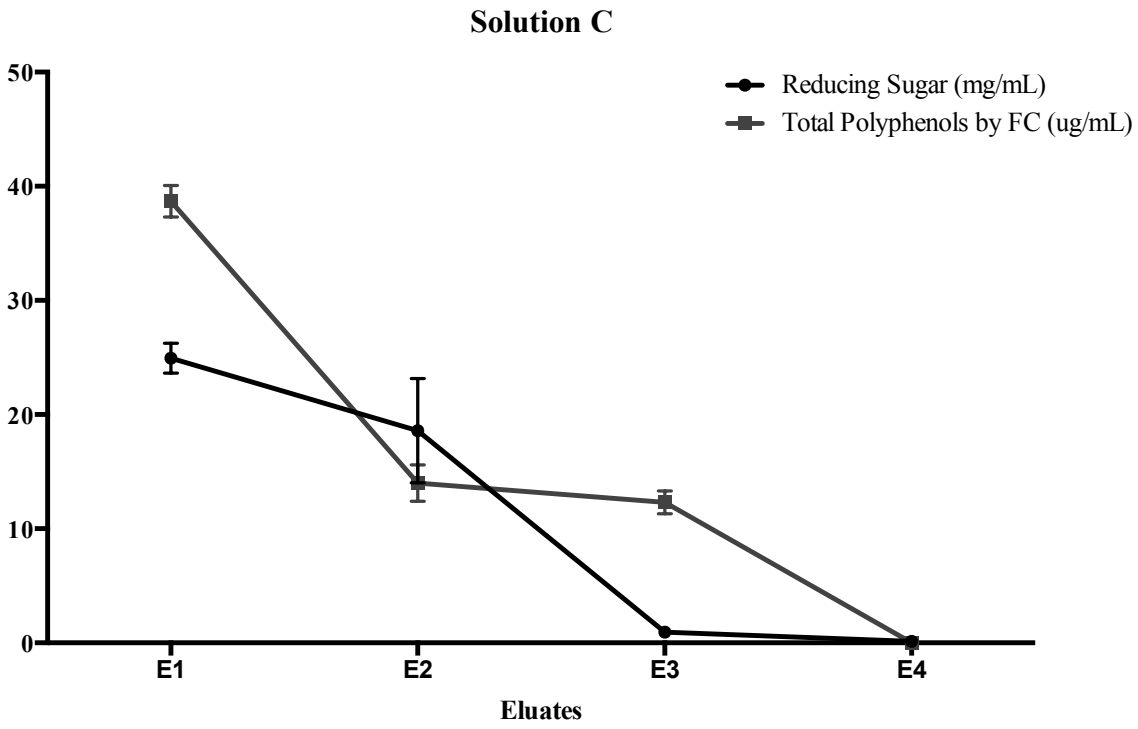

Figure 6.13: Concentrations of total polyphenols by FC and sugar in eluates 1-4 madse through SPE. Solution C contained 50mg/L glucose, 25mg/L tyrosine, and 25mg/L epicatechin.

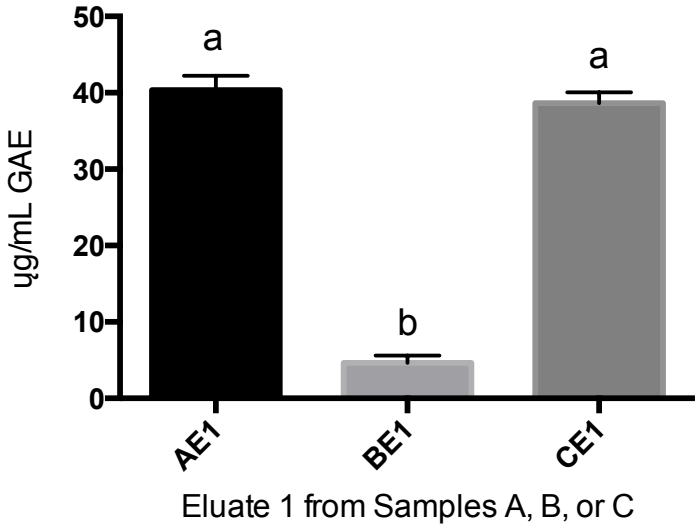

Figure 6.14: Concentration of total polyphenols by FC of Eluate 1 in Solutions A, B, and C. Results were analyzed using one-way ANOVA then Tukey's HSD test. Common letters represent no significant differences between samples.

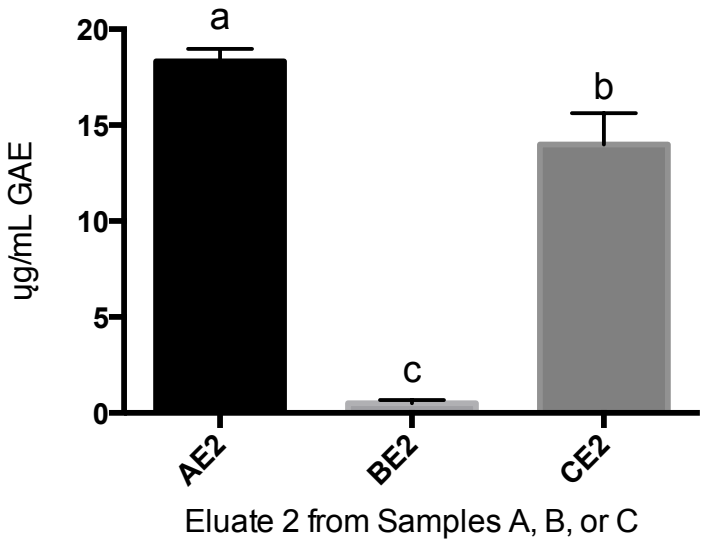

Figure 6.15: Concentration of total polyphenols by FC of Eluate 2 in Solutions A, B, and C. Results were analyzed using one-way ANOVA then Tukey's HSD test. Common letters represent no significant differences between samples.

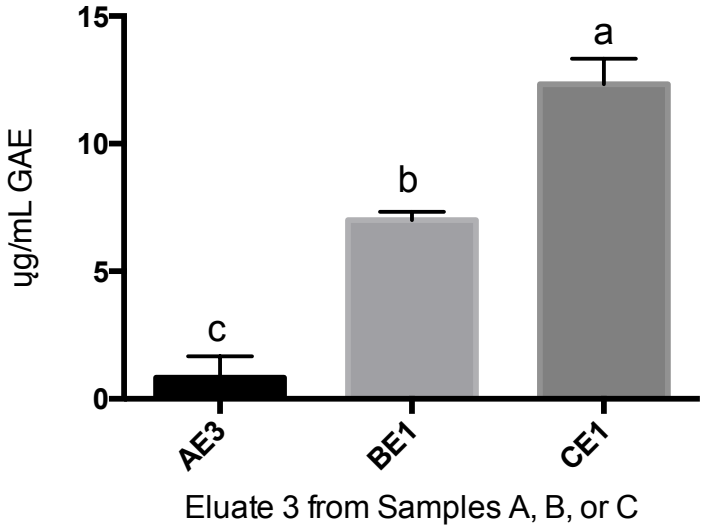

Figure 6.15: Concentration of total polyphenols by FC of Eluate 3 in Solutions A, B, and C. Results were analyzed using one-way ANOVA then Tukey's HSD test. Common letters represent no significant differences between samples.

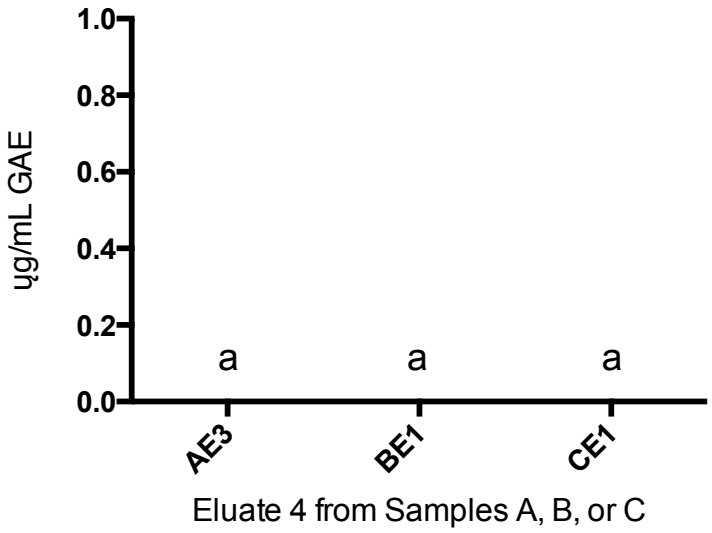

Figure 6.17: Concentration of total polyphenols by FC of Eluate 4 in Solutions A, B, and C. Results were analyzed using one-way ANOVA then Tukey's HSD test. Common letters represent no significant differences between samples.

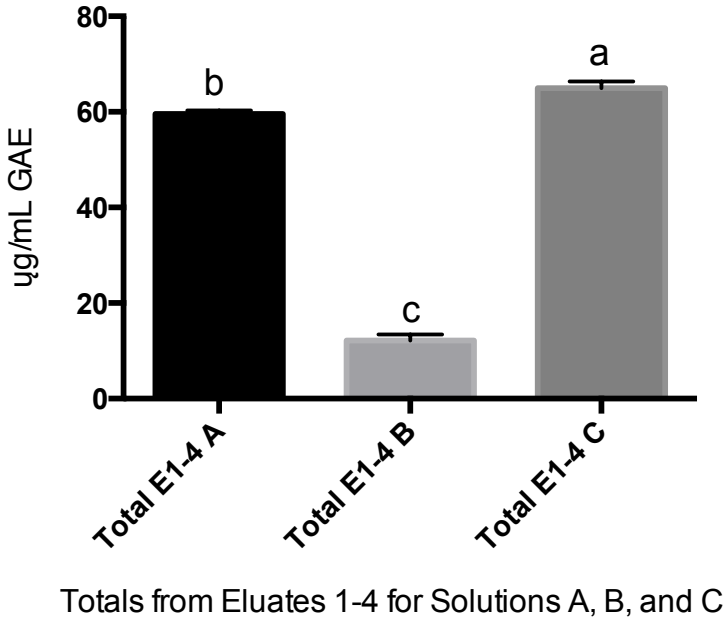

Figure 6.18: Concentration of total polyphenols by FC of the sum of Eluates 1-4 in Solutions A, B, and C. Results were analyzed using one-way ANOVA then Tukey's HSD test. Common letters represent no significant differences between samples.

In general, these data indicate that the FC reagent reacts with tyrosine to a significant degree. Though tyrosine is present in only small quantities in apple juices,

larger concentrations of tyrosine found in some grape juices could significantly affect the accuracy of the FC assay for determination of total polyphenols in fruit juice. The results from Solution A of this study indicate that each unit of tyrosine resulted in an approximate reading of 2.4 units GAE. Assuming a consistent, linear trend, a grape juice sample containing 30 mg/L tyrosine would have a FC result of 72 mg/L from the tyrosine concentration alone. If the FC assay were used to estimate total polyphenols of this juice, the result would be an overestimation of total polyphenols due to this presence of tyrosine. Further research is necessary to determine if there is linearity between tyrosine concentrations and their interferences with the FC assay. Also, further characterization of tyrosine concentrations in a variety of juices would be helpful to be able to estimate tyrosine interferences when quantifying total polyphenols using the FC assay.

6.4 Conclusion

In Part 1, solid phase extraction was effective for separating phenolic compounds from sugar in the commercial grape juice, the lab apple juice, and the lab grape juice, but it was not successful for the commercial apple juice due to an unknown interfering compound. However, it is unlikely that reducing sugars actually affected the FC assay results in an additive manner due to the inability to detect pure sugar solutions by the FC assay. A better understanding of components in the juice matrix that affect the FC assay is necessary to further optimize isolation and quantification of polyphenols.

Part 2 investigated one such interfering compound, tyrosine, and found that it is highly reactive with the FC reagent. Though present in small quantities in apple juices, tyrosine is more prevalent in some grape juices and could potentially have an additive

effect on the FC assay when measuring total polyphenols. The use of SPE in this experiment partly reduced interferences, providing a more accurate measurement of polyphenols.

There is a need for a better understanding of the reactivity of the FC reagent with compounds present in fruit juices if it is to be used to quantify total polyphenols. There are several compounds with antioxidant capacities present in fruit juice, besides polyphenols. These compounds include but are not limited to vitamin derivatives, amino acids, sulfites, metals, salts, nucleotide bases, and potentially many more [90, 92, 93, 132]. Further evaluation of the reactivity of these compounds and their presence in various juices can lead to more accurate results for total polyphenols in fruit juice and fermented fruit beverages such as cider and wine using the FC assay.

CHAPTER 7: SUMMARY AND CONCLUSION

The results of this research provide valuable insights regarding apple maturity and storage and the resulting effects on fruit, juice, and cider chemistry. In addition, the Folin-Ciocalteu (FC) Assay has been critically evaluated with regards to compounds in fruit juice, wine, and cider (specifically sugars and tyrosine) that may interfere with its accuracy in quantification of total polyphenols.

Fruit maturity at harvest affected fruit, juice, and cider qualities to variable extents. Fruit quality parameters were the most variable between treatments in all three cultivars studied, and the different maturation treatments also affected the chemical composition of the juice, though to different degrees among cultivars. Cider chemistry was more consistent, regardless of fruit maturity at harvest, being largely unchanged except for individual polyphenol composition. Procyanidins in cider, which are important compounds affecting sensory characteristics, seemed to be particularly affected by fruit harvest maturity. However, sensory evaluation was beyond the scope of this project, and further research is required to determine whether sensory impact would be imparted by the differences in procyanidin concentration that we observed. In addition, increasing the interval between harvests to greater than two weeks could provide an opportunity to examine whether larger differences in harvest maturities result in greater differences in the resulting cider. However, it would be difficult to increase the harvest intervals due to the risk of fruit drop and fruit rot later in the maturation period.

Post-harvest storage of apples also affected fruit, juice, and cider qualities. Fruit and juice quality parameters were greatly affected by the storage treatments, as expected. Cider chemical analysis revealed that few of those differences persisted into the cider,

regardless of storage treatment. Dabinett ciders showed no differences in individual polyphenol composition between fruit storage treatments, while York ciders made from fruit from different storage treatments reflected more differences in individual polyphenol concentration. However, it is unlikely that many of the individual polyphenol changes observed in cider resulting from our storage treatments would significantly affect the sensory characteristics because the differences observed between compounds are smaller than the sensory thresholds. The results from this study indicated that post-harvest storage of apples will not greatly impact the quality of the final cider, especially as compared to the significant impact of storage on fruit and juice quality. Future studies should include a sensory evaluation of the effects of post-harvest fruit storage on cider quality as well as evaluation of more cultivars, since the treatment effects were not consistent across cultivars in our study (York, Dabinett).

Lastly, the evaluation of the FC Assay using Solid Phase Extraction (SPE) to separate components of the juice matrix has resulted in a better understanding of potentially interfering compounds found in juice, wines, or ciders. Reducing sugars do not seem to directly interfere when using the FC Assay for quantifying total polyphenols in juice. However, there are potentially several other compounds that are present in juice that do interfere. One such compound is the amino acid tyrosine. Tyrosine had a large additive effect to the FC Assay results, indicating that its presence in the juice matrix can potentially greatly inflate the estimation of total polyphenols by the FC Assay. Therefore, high tyrosine concentration in fruit juice may lead to overestimation of polyphenol concentration by FC. Future research should continue to identify compounds in the juice, cider or wine matrix that may potentially interfere with the FC Assay, and strive to

develop quick and practical alternatives to FC for total polyphenol quantification in fruit juice, wine and cider. Also, quantifying tyrosine concentration in grape and apple cultivars could help to identify circumstances in which the FC Assay would be especially inaccurate for quantifying polyphenols, for example, in Muscadine grape juice.

Overall, this research provides cider producers with insights as to how orchard management, harvest maturity and post-harvest storage decisions may or may not impact cider quality. Finding that the relationship between harvest maturity or post-harvest storage of apples has minimal effects on the final cider chemistry, as compared to the effects on fruit quality, potentially allows cider producers an added measure of flexibility in their harvest and storage practices without compromising quality.

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