Development of Flavonoid Compounds in Norton and Cabernet Sauvignon Grape Skins
during Maturation

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The undersigned, appointed by the dean of the Graduate School,
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SAUVIGNON GRAPE SKINS DURING MATURATION

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DEVELOPMENT OF FLAVONOID COMPOUNDS IN NORTON AND CABERNET SAUVIGNON

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ABSTRACT

Norton is an important grape cultivar that is native to Missouri and grown widely because of disease resistance and wine quality. Wine quality is related to levels of flavonoids, and vineyard practices influence flavonoid accumulations. However, little research has examined accumulation of flavonoids in Norton fruit over the growing season. A two-year (2012-2013) study was initiated using Norton and Cabernet Sauvignon vines at Mountain Grove, MO, and a one year study in (2013) using Norton vines at Rocheport, MO. Cabernet Sauvignon, a well-studied grape cultivar was monitored for flavonoid accumulation as a comparison to Norton. Berry samples were collected at six stages of maturation from green berries at 43 days after flowering (DAF) to harvest (125 DAF). Levels of sugars, acids, and flavonoids such as anthocyanins, tannins, and total phenolics were estimated from berry skins at each harvest date. Compared to Cabernet Sauvignon, Norton had 15% higher sugars, 9% higher acids, 72% higher anthocyanins, 40% lower tannins, and 9% lower total phenolics averaged over six stages. High levels of anthocyanins in Norton would contribute to higher quality wine based on color. However, lower tannin levels will result in a less astringent wine; tannins are added during fermentation to adjust astringency. Lower total phenolic content can decrease the stability of wine limiting storage. Documentation of Norton berry flavonoid

content will allow future research to determine how vineyard practices can alter the concentration of flavonoids during berry development.

CHAPTER I

LITERATURE REVIEW

Introduction

Grape is an economically, agronomically, and culturally important fruit crop cultivated for making wine, juice, jam, and raisins. Despite the importance of grapes, less is known about the ripening process in this fruit than in many other fruit crops. The difficulty in working with grapes is in fact that grapes are non-climacteric fruit (Coombe 1992). However, climacteric fruits such as tomato, apple, melon, and banana, ripening is mediated by ethylene (Alexander and Grierson 2002). In contrast, grapes ripen while the berries are still expanding, and as with many non-climacteric fruits, ethylene has minimal, if not, on ripening of berries (Robinson and Davies 2000).

The Missouri wine industry continues to increase in importance. Wine production has increased from 2.65 million liters (702,000 gallons) in 2005, to 3.38 million liters (894,391 gallons) with an estimated value at \$31 million in 2007 (Hodgen 2008). With an increase to 3.50 million liters (925,000 gallons) in 2009, Missouri wineries totaled \$49.8 million, having a retail value of \$77.8 million (Stonebridge Research Group 2010). In 2013, Missouri ranked 12th in grape production and 8th in wine production in the USA (Hodgen 2008). There are 121 operating wineries in Missouri, with approximately two wineries opened each year (Beedle 2012). Ninety percent of Missouri grape production is for wine (USDA 2000). Cultivars grown in Missouri include:

Catawba, Cayuga, Chambourcin, Chardonnay, Concord, Norton, St. Vincent, Seyval Blanc, Traminette, Vidal Blanc, and Vignoles, (Stonebridge Research Group 2010).

Norton is the oldest American wine grape and the state grape of Missouri. Although its true parentage is unknown, Norton is believed to be derived from *V. aestivalis*, *V. labrusca* L., and *V. vinifera* cv. Chasselas (Parker et. al 2009). Aestival refers to of or relating to summer, this is why Norton' nickname is "Summer Grape" (Roberts 2006). Norton vine was discovered near Richmond, Virginia in 1835 by Dr. Daniel Norborne Norton (Ambers and Ambers 2004). The cultivar, has gained considerable attention in the Midwest and Eastern United States for its potential as a quality wine grape. Currently, Norton is the most widely planted cultivar in Missouri comprising 19.3% of the total bearing acreage (USDA-NASS 2012). Some of the primary growing regions in Missouri include Ozark Highlands, Ozark Mountains, Hermann, and Augusta. Norton has resistance to phylloxera (*Daktulosphaira vitifoliae*), powdery mildew (*Erysiphe necator*), and tolerance to Pierce's Disease (*Xylella fastidiosa*), cold tolerance, and less subject to late spring freezing (Main 2005). Norton has the potential to produce high quality wines compared to other American cultivars. However, Norton juice has undesirable characteristics, such as high pH, potassium, malic acid, and titratable acidity (Main 2005).

Fruit development and ripening are critical factors that dictate the selection of optimal harvest times. The grape berry is composed of skin, pulp, and seeds, which range from 5 to 10, 74 to 90, and 0 to 6% by weight, respectively (Rankine 2007). Berries contain sugars, organic acids, tannins, anthocyanins, minerals, and aromatic

compounds, which consist of essential chemical components of wine (Kennedy 2002).

The major flavonoid compounds in grapes are anthocyanins and tannins which influence wine color, astringency, bitterness, stability, and structure (Kennedy 2002, Margalit 1997). Nearly all flavonoid compounds in grapes are extracted from the skin and seeds, with anthocyanins mostly occurring in skins, and tannins present in both seeds and skin (Kennedy 2002, Margalit 1997). Sugars and acids are primarily found in the pulp, but they are also present in the grape skins (Coombe and McCarthy 2000, Possner and Kliewer 1985). In finished wine, sugars add to the complexity of wine, producing alcohol content, and are often perceived to soften acidity. Acids are present in developing grapes and generated during the process of winemaking, are critical for wine balance, microbial stability, color, and aging rate (Margalit 1997). The accumulation of the above compounds changes with development stage of berries, climatic conditions, water availability, and light.

Norton is a relatively young variety (179 years old), and limited information is available on the chemical profile of Norton grape (Ambers and Ambers 2004, Ali et al. 2011). In comparison, Cabernet Sauvignon, an old world grape, has been extensively studied. Studies are necessary to further evaluate and determine the development of grape fruit composition. Understanding development of various components in the berry is important to implementing the best viticultural practices for improving wine quality and diversifying wine styles. Future research will enable winemakers, and viticulturists to design effective strategies for canopy management and harvest

decisions. Further research is necessary to determine if grape fruit composition is effected by site location.

This thesis research is divided into three parts:

A) Extraction Assay

Objective:

- 1) To compare 50% ethanol and 70% acetone as an extraction solvent for flavonoids from Norton grape skins.
- 2) To compare the extraction for flavonoids from Norton grape skins for extraction time points.

B) Comparison of cultivars

Objective:

- 1) To compare the development of total phenols, tannins, and anthocyanins during hard, green berries through harvest for Norton and Cabernet Sauvignon grape berry skins.
- 2) To compare the development of soluble solids for Norton and Cabernet Sauvignon, and organic acids for Norton vines only during hard, green berries through harvest.

C) Comparison of site locations

Objective:

- 1) Flavonoids such as anthocyanins, tannins, and total phenolics were compared during grape berry development for Norton hard, green berries through harvest at two locations.
- 2) Soluble solids and organic acids were compared during hard, green berries through harvest at two locations.

Grapevine Phylogenetics

Grapes belong to the family *Vitaceae* which includes approximately 1,000 species within 17 genera (Guner et al. 2008, Keller 2010). *Vitis* and *Muscadinia* genera

are of greatest economic importance. In terms of acreage and production, *Vitis* is the most important genus. The *Vitis* genus is comprised of 60 species, which can be separated into Eurasian in Europe, Mideast and eastern Asia, and American species in the Northern Hemisphere in North America (Guner et al. 2008, Keller 2010, Mullins 1992). *Vitis* is distinguishable from other species within the genus using floral characteristics: petals joined at the tip and detached from the base to combine as a calyptra or "cap" (Gleason et al. 1963). There are as many as 40 Eurasian species, but *Vitis vinifera* L., is the most economically important. *Vitis* plant species have $2n = 38$ chromosomes and species of *Muscadinia*, have $2n = 40$ chromosomes (Jackson 2008, Jansen et al. 2006). *V. vinifera* encompasses the cultivars Cabernet Sauvignon, Cabernet Franc, Malbec, Merlot, Pinot Noir, Shiraz, and Tempranillo. Cultivars of *V. vinifera* and *V. labrusca* hybrids are commonly grown in the eastern U.S. for wine production. Hybrid grapes are primarily crosses between *V. vinifera*, *V. labrusca*, *V. riparia*, and *V. aestivalis* (Jackson 2008). Although Norton's true parentage is unknown, it's believed to be derived from *V. aestivalis*, *V. labrusca* L., and *V. vinifera* (Parker et. al 2009).

Growth and Development

Grape maturity is defined by the physiological development of the berry on the vine (Bisson 2001). Viticulturists have determined that grape berries advance through three stages during their development after pollination: Stage I (green berry), Stage II (arrest of green berry development and pause before onset of ripening), and Stage III (veraison or ripening) (Bisson 2001). Berry growth follows a double sigmoid curve pattern (Figure 1.2.) (Coombe and McCarthy 2000, Kennedy 2002, Robinson and Davies

2000). Stage I represents the first phase of rapid berry growth. This period lasts approximately 3 to 4 weeks after bloom (Kennedy 2002). The texture of the berry is hard, while the color remains green due to the presence of chlorophyll (Dookozlian 2000). During Stage I, berry size increases rapidly and acids, tannins, and minerals accumulate; sugar levels remain low. Stage II represents the second growth period known as the lag phase of berry growth. While berries remain hard, chlorophyll content decreases and acids accumulate. This stage often lasts 2 to 3 weeks (Dookozlian 2000). Stage III, the second phase of rapid berry growth and fruit ripening, is characterized by the softening and color change of the berry (Kennedy 2002). In the absence of chlorophyll, anthocyanins begin to accumulate in the berry skin (Dookozlian 2000). During Stage III, acids decrease in concentration, while sugar, flavor and aromatic compounds accumulate. (Coombe and McCarthy 2000). This stage continues for 6 to 8 weeks and berry growth is limited to cell enlargement (Dookozlian 2000).

Water relations

Water is essential for plant growth and impacts vegetative growth, yield, and fruit composition, but also correlates carbohydrate supply to the berry (Iland et al. 2011, Roubelakis-Angelakis 2009). Water uptake varies significantly throughout grape berry development. The amount of water that accumulates each day is the sum of water moved into the berry from the xylem, phloem sap, and adsorption through epidermal tissues. After veraison, the contribution of xylem water is reduced by embolism blockage. The blockage is established at the time of berry expansion and indicates that the majority of the water that accumulates in a berry is derived from phloem sap

(Coombe 1992). Iland et al. (2011) reported that fruit set is the most sensitive stage to water availability with insufficient amounts affecting fruit set and berry size. Throughout the growing season each vine generally requires between 406 and 914 mm of water, depending upon climate, cultivar, and soil conditions (Winkler et al. 1974). During the harvest stage, water contributes 70 to 80 % of the berry fresh weight (Dokoozlian 2000). Excessive water during the growing season results in excessive vegetative growth, and insufficient water reduces berry size and yield (Iland et al. 2011).

Berry Minerals

Mineral composition of grape berries is an important determinant in fruit development and chemical composition of wine. Grape berries have 17 nutrients considered essential for growth and development; these nutrients are important in the structure of compounds and activating enzymes (Marschner 1995). Mineral nutrients accumulate in the berry through: root cation uptake; translocation from roots to shoots; re-translocation of cations from shoot downward to roots; the mineral nutrient reserve; and the number of berries and berry growth rates in relation to vine vigor (Etchebarne et. al 2009). Factors that impart soil nutrient accumulation include soil levels, plant function, vine microclimate, and cultural practices. However, there is a direct impact of berry nutrition effecting must (seeds, stems, skins, pulp) and wine composition.

Nitrogen is critical for fruit yield, ripening, and adequate berry and wine quality (Perez-Alvaraz et al. 2013, Schreiner 2005). Grapevines have high N demand because the nutrient is essential for vegetative growth and development, specifically the building of amino acids, nucleic acids, and pigments (Bates and Wolf 2008). The required N level

for grapevines ranges from 30 to 80 kg/ha (Conradie 1980, Hanson and Howell 1995).

These nitrogen compounds are found in the pulp, skin, and seeds of berries. During the initial growth stage of the berry, ammonium ions account for more than half of the total nitrogen in the fruit, while amino acids remain relatively low (Dokoozlian 2000). After veraison, amino acids increase significantly in the berry because ammonium concentrations decline. The amino acid concentration of the berry can increase from two-to five-fold during the ripening process, with extensive variations depending upon variety and fruit maturity (Dokoozlian 2000).

Grape berries are very rich in potassium, which is essential for grape berry growth and development (Mpelasoka et al. 2003). Deficiencies in potassium result in reduction of fruit set and unevenly ripened berries (Rogiers et al. 2006). Inorganic minerals in the soil are taken up by the roots and are transported directly to the berry or re-mobilized from the trunk or roots via the xylem (before veraison) or phloem (Dokoozlian 2000). The mineral cation levels increase two- to three-fold during ripening with potassium as the dominant mineral (Dokoozlian 2000). Potassium is present in relatively high concentrations in all parts of the berry. The potassium concentration ranges between 1,200 and 2,000 mg/L of berry juice (Dokoozlian 2000). According to Possner and Kliewer (1985), reported levels of potassium content increased at a rate of 0.04 mg/berry over the course of fruit development. Potassium levels reached 5 mg/berry at the end of the ripening process. However, potassium showed a higher concentration in green berries (Possner and Kliewer 1985). Vine nutrition is not well understood for Norton because the roots are very efficient in removing potassium from

the soil. Therefore, potassium fertilizer should not be used unless potassium deficiency is measured in the vine. The addition of potassium fertilizer can increase juice pH and potassium in Norton grapes (Morris et. al 1987).

Calcium is present in the cell walls and is important in providing cell structure (Keller 2010, Possner and Kliewer 1985). Disease resistance on the vine can be modulated by high levels of Calcium (Rogiers et al. 2006). Calcium is considered to be known as the xylem-mobile mineral and accumulates prior to veraison, indicating that the xylem contribution to berry growth is diminished after veraison (Rogiers et al. 2006). In contrast with potassium, the calcium content increases for only 30 to 40 days following anthesis, reaching a level of about 0.1 mg/berry and remains constant through berry ripening (Possner and Kliewer 1985). The highest concentrations of Calcium were found in the skin and center portion of the pulp. However, there was no significant difference in concentration of calcium observed between the skin and pulp of the berry (Possner and Kliewer 1985).

Magnesium is a structural component of chlorophyll and is involved in protein synthesis. Magnesium is considered to be known as a phloem-mobile mineral and is accumulated throughout the growth and ripening of the berry (Rogiers et. al 2006). However, rates of accumulation were the highest after veraison. Magnesium increased steadily until 70 days after flowering until a level of 2.6 µg/berry, then declined to final levels of 1.0 micrograms per berry (Rogiers et. al 2006).

Sugars

Sugars provide the foundation to build compounds in the berry, such as organic and amino acids. The primary sugars within grape berries are glucose, fructose, and sucrose. Sucrose is the major translocated sugar in the grapevine, but glucose and fructose make up the bulk of the sugar in the grape berry at all stages of development (Hardy 1968). These sugars primarily accumulate in the pulp, but they are also present in grape skins (Coombe and McCarthy 2000, Possner and Kliewer 1985). During the early stages of berry development, the total sugar concentration is very low and glucose levels exceed that of fructose by 5-fold (Hardy, 1968). During berry ripening, sucrose is transported into the berries via the phloem from leaf photosynthesis (Davies et al. 1996). Sucrose is converted primarily to glucose and fructose that continue to develop throughout Stage III (Ribereau-Gayon et al. 2000). At harvest, glucose and fructose concentrations are between 150 and 250 g/L (Ribereau-Gayon et al. 2000). Concentrations of sucrose range from 0.2 to 5.0 % of fresh berry weight (Kliewer 1967). Glucose and fructose are present in grape berries at harvest, each ranging from 8 to 12 % of fresh berry weight (Dokoozlian 2000).

Sugars are typically measured as percent total soluble solids (degrees Brix) using a refractometer (Jogaiah et al. 2012). The refractometer is a primary tool used to determine grape maturity and optimal harvest date. For the species *Vitis aestivalis*, total soluble solids were 25.5 and 20.9 for 1965 and 1966 in Kansas respectively (Kliewer 1967). Reported ranges of Norton for Brix were 20 to 25 (Main 2005, Main and Morris 2008, Morris and Main 2010). Reported ranges of Norton for glucose and fructose are 77.6 to 93.6, and 79.8 to 137.8 g/L, respectively (Main and Morris 2004, 2008).

Acids

Organic acids are present in all plants, supporting many aspects of cellular metabolism (Sweetman et al. 2009). Tartrate, malate, and citrate are the major organic acids present in grape pulp and skin (Coombe and McCarthy 2000, Possner and Kliewer 1985). Tartaric and malic acid comprise 90% of total acidity inside the berry (Dokoozlian 2000, Walker et al. 2003). The accumulation of these acids occurs typically at the beginning of grape berry development, reaching their highest concentrations near the onset of ripening inside the berry (Dokoozlian 2000). Tartaric acid content peaks during Stage I and remains fairly stable until Stage III when concentrations decline. Malic acid accumulates at the end of Stage I and then begins to decline with berry ripening (Coombe and McCarthy 2000, Possner and Kliewer 1985). Although both acids decline during Stage III, the loss of tartaric acid is not as rapid and has been associated with an increase in berry size. In contrast, reductions in malic acid concentration after veraison result from respiration, enzyme degradation, and dilution (Dokoozlian 2000). Green berries showed a definitive gradient in malic acid, malate gradient, with concentrations lower in the skin and increasing toward the seeds (Possner and Kliewer 1985). Titratable acidity (TA) of grape juice measures: organic acids, inorganic acids, and amino acids (Ribereau-Gayon et al. 2000). TA is measured in g/L with an autotitrator. This instrument is a primary tool used to assess grape maturity and determine harvest date. Reported ranges of titratable acidity for Norton at harvest were 7.9 to 15.8 g/L: for tartaric, malic, and citric acid the levels were 6 to 10, 3.2 to 7.4, and 0.5 to 1.0 g/L, respectively (Jogaiah et al. 2012, Main and Morris 2004, 2008).

pH

The pH of berry Juice is the formulation for stabilizing wine. Changes in berry pH follow a similar pattern as sugars accumulate in berries; stable levels early in the season until veraison, when pH begins to increase due to cation and acid concentrations (Hrazdina et al. 1984). This increase in berry pH coincides with the decrease in acids (Amerine and Ough 1980). The measurement of pH is an important maturity index at grape harvest due to the influence on color, taste, microbial and chemical stability, sulfur, and clarity of wine (Amerine and Ough 1980). Reported pH ranges of Norton for pH are 3.3 to 3.9 (Main 2005, Main and Morris 2008, Morris and Main 2010).

Flavonoids

Flavonoids are important constituents in the berry that contribute to wine color, astringency, bitterness, stability, and structure (Dookoozlian 2000, Kennedy 2002, Margalit 1997). There are several other contributions of flavonoids such as a nutritive value of grape products, and their potential health benefits as antioxidants (Dokoozlian 2000). The main flavonoid compounds in grapes are anthocyanins and tannins. Grape phenolic compounds are mainly found in the skins, pulps, and seeds and compounds are partially extracted during winemaking (Revilla and Ryan 2000). The above compounds are synthesized in the skin, and in the seeds of the berry when present, however, the pulp does not contain color pigments (Adams 2006). Total phenolic content for Norton was 1.8 mg/g of fresh grape, respectively (Hogan et al. 2009).

Anthocyanins

Increasing the amount of anthocyanins in the berry increases grape quality, which is advantageous to both the grape grower and the winemaker. Anthocyanins function as the red-colored phenols that pigment the wine, and are required to give the long-term color of red wine (Kennedy 2007). Anthocyanin is the term describing the simple flavonoid ring structure. The color is based on the fully conjugated 10 electron A-C ring π -system, with some contribution of the B ring (Waterhouse 2002). During berry set, anthocyanins begin to form in the skin but the greatest accumulation is during Stage III (Fournand et al. 2006, Kennedy 2002, Hrazdina et al. 1984). Research has shown a decline in total anthocyanins just before harvest and during over-ripening (Roggero et al. 1986, Ryan et al. 2003).

Anthocyanins are found in trace quantities in grapes and wine because they can be unstable (Waterhouse 2002). The colored pigment such as anthocyanin reacts with tannins to produce a stabilized anthocyanin or pigmented tannin which stabilizes longer in wine than the original form (Waterhouse 2002). Therefore the stabilized color produced perseveres in most red wines more than a few years old (Waterhouse 2002). However, in some wines the monomeric forms found in the grape are retained. The anthocyanin that is converted to other colored forms as the wine ages is called malvidin-3-glucoside (Waterhouse 2002). *V. vinifera* cultivars contain: 3-monoglucoside, 3-acetylglucoside, and 3-p-coumaroglucoside derivatives from the five anthocyanins: cyanidin, peonidin, delphinidin, petunidin, and malvidin-monoglucoside/diglucoside (Ali et al. 2011, Boss et al. 2009). Malvidin-diglucoside is the major anthocyanin in Norton while malvidin-monoglucoside contributes to the anthocyanin content in Cabernet

Sauvignon (Ali et al. 2011). Malvidin is considered the most abundant anthocyanin in red wine. Reported levels of Norton for anthocyanins are 2.7 to 3.4 mg/g of berry wt. (Jogaiah et al. 2012). Total anthocyanin content for Norton was 0.93 mg/g of fresh grape, respectively (Hogan et al. 2009).

Tannins

Developing methods that are useful in predicting the quality of red wine is of utmost importance to winemakers; tannin content is for the quality of red wine. Grape tannins are polymers made of similarly structured phenolic sub-units that are fused together like a chain and contribute to the astringency and mouthfeel of wine (Kennedy 2007). Tannins are a key constituent to form pigmented polymers in association with the anthocyanins to stabilize pigments, resulting in long-term stability (Kennedy 2007). The pro-anthocyanins that are sub-units for structure of the tannin polymers are synthesized in plants through the flavonoid pathway (Kennedy 2007). The specific classes of tannins found in grapes are generally referred to as condensed tannins (Kennedy 2007). In most red wines, nearly all tannins present are grape-derived, and belong to this class of tannins (Kennedy 2007). In grapes, the particular sub-unit structure in tannins can have differences as well as a variation in polymer length. Grape skins contain longer polymers, averaging around 20 to 30 sub-units in length in a ripened berry, while the tannins in grape seeds are 4 to 6 sub-units long (Downey 2003).

Tannins can be found inside the skins and seeds of the berry and also in the stem and tissues of the berry (Kennedy et al. 2001). Tannins present in the skins accumulate during Stage I, reaching the highest accumulation at one to two weeks before veraison,

and declining during Stage III. However, seed tannins accumulate their highest levels during State II (one to two weeks after veraison) (Downey 2003, Kennedy 2002). A decline in tannins is primarily due to oxidation of more bitter seed tannins (Downey 2003, Kennedy 2002). Tannin synthesis in the seeds continues after veraison, and contributes to maturity of the seed coat as well as promotes change in berry color from green to brown (Kennedy 2007). Research has shown that there are 11% in the skin and 89% in the seed by berry weight for grape tannins (Kennedy 2007). Reported levels of tannins in Norton were 2.7 to 3.4 mg/g of berry weight (Jogaiah et al. 2012).

Analytical Methodology

Soluble solids (Brix), titratable acidity (TA), pH, and total tannins/anthocyanins/phenolics will all undergo analytical analyses. Brix are measured by using a refractometer, and pH and TA are measured by a titroprocessor and pH meter (Mazza et. al 1999). Liquid chromatography represents the most important technique used for the estimation of contents of organic acids, and sugars in grapes and wine (Jogaiah et al. 2012). The separation and quantification of organic acids and sugars can be performed either by high performance liquid chromatography (HPLC) or also by ion exchange chromatography (IC) (Mateo et al. 2005). The Thermo Scientific Spectrophotometer analyzes the absorbance for the color measurement of total anthocyanins and also the absorbance for total tannins based on methods described by Iland et al. (1996, 2000). Total anthocyanins are capable of being measured at an absorbance of 520 nm. The methyl cellulose precipitable (MCP) assay measures total grape tannins at an absorbance of 280 nm (Sarneckis et. al 2005, Smith 2005, Mercurio

et. al 2006). The high performance liquid chromatography-mass spectrometry (HPLC-MS) allows the phenolic compounds such as anthocyanins and tannins to be separated and analyzed on specific color pigments, and astringent compounds. Previous literature Kennedy et al. (2001), used ^{13}C NMR spectroscopy, mass spectrometry, gel permeation chromatography, electrospray ionization mass spectrometry (ESI-MS), and UV-Vis absorption spectroscopy for the analytical analysis phenolic compounds.

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Figure 1.1. Molecular phylogeny of the genus *V. vinifera* compared to *V. aestivalis*, and other *Vitis* species (Trondle et al. 2010).

Figure 1.2. Grape berry development. Depiction of grape berry development after bloom at 10 day intervals. Modified from (Coombe and McCarthy 2000, Dokoozlian 2000, Kennedy 2002, Robinson and Davies 2000).

Grapevine growth stages – The modified E-L system

Figure 1.3. Grapevine growth stages (Coombe 1995).

CHAPTER II

METHODOLOGY FOR OPTIMUM EXTRACTION OF FLAVONOIDS IN NORTON GRAPE

BERRY SKINS

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Assessment of the phenolic content of grape skins is an important element in understanding how flavonoids impact wine quality. A study was initiated using seven-year-old Norton vines established at Mountain, Grove, MO, in 2012. Mature berries were collected at harvest (120 days after flowering; DAF) to quantify levels of anthocyanins, tannins, and total phenolics. An experimental method using ethanol as an extraction solvent for flavonoids was compared to acetone using an accepted assay method (Harberston-Adam's Assay). Flavonoid extraction was conducted from 1 to 48 hr to optimize extraction efficiency and compound levels were estimated spectrophotometrically. Extraction with acetone compared to ethanol recovered up to 105% anthocyanins, 164% total phenolics, and significantly greater levels of tannins (325%). Acetone was approximately 30% more effective than ethanol for extraction of flavonoids. Flavonoid extraction with ethanol is not an effective solvent for

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for Norton grape skins and may lead to under reporting and incorrect results. The optimum time for flavonoid extraction was 3 hr using both methods.

Nomenclature: Norton (*Vitis aestivalis*).

Key Words: anthocyanins, tannins, phenolics.

Introduction

Flavonoids are a class of compounds that contribute to different aspects of wine quality. The major flavonoids found in grapes are anthocyanins and tannins.

Anthocyanins are important to wine quality because of their contribution to color and appearance (Lee et al. 2005). Tannins are an important quality parameter because of their contribution to astringency and color stability during ageing (Hanlin et al. 2010).

Quantification of flavonoid content in grape berries is important for identifying optimum harvest time and decision-making on processing grapes for wine. Analysis of flavonoids in developing grape berries can suggest to viticulturists and winemakers vineyard management practices that can alter flavonoid content to optimize wine quality and style as well as the proper time of harvest. For example, low levels of anthocyanins can be impacted by low light involvement inside the canopy (Downey et al. 2006a). However, there are management practices such as shoot and leaf removal to open the canopy for sun exposure in order for anthocyanin biosynthesis (Downey et al. 2006a).

Flavonoids are extracted primarily from skins of grape berries, with several techniques commonly used. The most common technique is solvent extraction (solid-liquid and liquid-liquid extraction techniques), and this has been proven to be reliable and efficient (Dai and Mumper 2010). However, the efficiency of solvent extraction can be affected by many factors such as type of solvent, solvent concentration, time, etc. (Cacace and Mazza 2003). A variety of solvents and methods have been used previously to quantify flavonoids from grapes and wine of *Vitis vinifera* species. Solvents used on *V.*

vinifera to extract flavonoids included 50% ethanol and 70% acetone (Harbertson et al. 2002, Kallithraka et al. 1995, Sarneckis et al. 2006).

Several methods have been developed to quantify flavonoid content. There are spectrophotometrically based methods such as those described by method pH differential (AOAC method 2005.02) (Lee et al. 2005), Hagerman-Butler Assay (Hagerman and Butler 1978), Harbertson-Adams Assay (Harbertson et al. 2003), Folin-Ciocalteu Assay (Sanchez-Rangel et al. 2013). There are also chromatography based methods using high-performance liquid chromatography (HPLC) to measure individual and total flavonoids (Downey et al. 2003, Kennedy et al. 2001, Kennedy and Waterhouse 2000, Peng et al. 2002, Price et al. 1995). However, HPLC can be relatively expensive and not cost effective. Furthermore, extraction and quantification methods can differ, impacting reported data.

A commonly used assay, Harbertson-Adams, is a direct adaption of the Hagerman-Butler method that has been widely used for over 30 years and the accepted standard in studies of flavonoid testing (Harbertson et al. 2002, Hagerman and Butler 1978). Harbertson-Adams assay was developed to provide wineries with an inexpensive, reliable, and readily accessible measurement of tannin, anthocyanin, and total flavonoid concentration in wine (Harbertson et al. 2002, 2003). The assay has been widely adopted at many wineries and winery lab services with precision and reproducibility. However, there are also claims that the Harbertson-Adams assay cannot provide winemakers with useful data due to lack of precision and recovery (Brooks et al. 2008, 2013). As with any analytical procedure, the Harbertson-Adams assay requires time and

effort to ensure that a specific lab or analyst can produce reproducible results.

Furthermore, Brooks et al. (2008, 2013) claim of failure, due to large variation between non-accredited winery labs, has demonstrated lab performance fallibility, not method invalidity.

The measurement of flavonoids in the grape berry at harvest is not indicative of the level of flavonoids extracted into the wine (Harbertson et al. 2002, Hanlin et al. 2010). High colored grapes do not necessarily produce highly colored wines, which may be related to berry skin extraction into wine (Romero-Cascales et al. 2005). Furthermore, tannin extraction is difficult during vinification and analysis (Hanlin et al. 2010, Main and Morris 2007). Low tannin level in wine was speculated to result from reduced extraction of tannin from grape berries, which was a result of tannins binding with other components in the grape berry cell wall, such as proteins and polysaccharides (Amrani-Joutei et al. 1994, Cheynier et al. 1997, Kennedy et al. 2000, Downey et al. 2003, Hazak et al. 2005, Fournand et al. 2006, Cerpa-Calderon and Kennedy 2008). The importance of flavonoid quantification suggests a closer examination of the efficiency of extraction.

Norton is considered a *V. aestivalis*-derived variety and is an important cultivar in the Midwest. Grapes result in quality red wine that is comparable to wines made from *V. vinifera* grapes (Ali et al. 2011). Anthocyanin and tannin levels are significantly higher in Norton berries than in those of *V. vinifera* (Hogan et al. 2009, Munoz-Espada et al. 2004, Ali et al. 2011). These higher levels of anthocyanins and tannins are important because they contribute to quality red wine (Ali et al. 2011).

Traditional extraction techniques listed above have not been validated on Norton. Limited information on Norton led us to speculate that there would be differences between extraction and quantification methods; we hypothesized that the difference in extraction solvent may account for much of the discrepancy. Previous methods only used 1 and 24 hr extraction time points, and we speculated that a 24 hr extraction time would allow some degradation of the flavonoid compounds. The objectives of this study were to compare the effectiveness of two solvents at multiple extraction times for optimum quantification of flavonoids from Norton grape skins.

Materials and Methods

Chemicals. 95% (+)-Catechin, 98% bovine serum albumin (BSA), 99% sodium dodecyl sulfate (SDS), 99% triethanolamine (TEA), 97% ferric chloride hexahydrate (FeCl_3), 99.7% glacial acetic acid, 99% malic acid, hydrochloric acid (37% HCl), sodium hydroxide solution (5.0M NaOH), 100% ethanol, and 99.5% acetone were purchased from Sigma (Saint Louis, MO).

Equipment. Samples were analyzed on a Spectronic Genesys UV-Vis spectrophotometer (Thermo Scientific, Madison, WI) using 2-mm path length UVettes (Brandtech Scientific, Essex, CT).

Flavonoid Extraction. In 2012, grapes were collected at harvest (120 days after flowering) from seven-year-old Norton vines at the Missouri State Fruit Experiment Station in Mountain Grove, MO. Sixty berries from twelve vines in three replicated areas were removed from the rachis and the skins were removed from the pulp and

seeds by razor blade and immediately frozen in liquid nitrogen. Frozen skins from each replication were ground with an Oscillating Mill (Model MM400, Retsch, Haan, Germany) under liquid nitrogen to a uniform particle size, and 2 g aliquots of skins weighed and stored in conical tubes. Ground samples were stored at -80°C for analysis.

Optimum extraction was developed by using different solvents and time intervals for determination of flavonoids from harvested Norton grape samples. Extraction time intervals were based upon previous studies that determined optimum efficiency of extraction (Lapornik et al. 2005, Spigno et al. 2007). The 2 g grape skin samples were thawed for 5 min and incubated in 50% ethanol or 70% acetone. Intervals for incubation included 1, 3, 6, 12, 24, and 48 hr. Three replicates of each solvent/incubation interval for flavonoid extraction were carried out with 2 g grape skin samples in conical tubes. Tubes with ethanol contained 10.0 mL of 50% ethanol in water (v/v) and the tubes with acetone contained 18.0 mL of 70% acetone in water (v/v). Upon incubation, tubes were vortexed for 30 sec. Samples were placed on a rotary shaker (150 rpm) (Henry Throemner LLC., Thorosare, NJ), for the necessary extraction interval. After incubation tubes were centrifuged at 10,000 rpm (model 5804 R, Eppendorf AG, Hamburg) for 10 min. The supernatant from tubes containing ethanol was placed in a cuvette and flavonoid content was measured directly using a spectrophotometer. Acetone was removed from samples using a rotary evaporator (Model Rotavapor-R, Huchi, Switzerland) at 38°C for 20 min. Remaining residue was washed with 10 mL of 50% ethanol, an aliquot added to a cuvette, and sample used immediately for analysis.

Flavonoid Quantification. Flavonoids were measured in each sample using the

Harberston-Adams Assay (Harbertson et al. 2003, Downey et al. 2006b). The complete protocol for analysis of flavonoids is available on the University of California, Davis website (<http://boulton.ucdavis.edu/uv-vis/adamsassay.htm>). Essentially, 2 g grape skins were centrifuged at 10,000 rpm for 10 min to pelletize solids. Liquid supernatant was placed in a cuvette for analysis. UV-visible spectra were collected with a spectrophotometer at 510 and 520 nm using polystyrene cuvettes. Flavonoid content was quantified against a standard curve using catechin and expressed as milligrams of catechin equivalents per gram fresh weight of skin.

Statistics. Flavonoid content from each treatment were subjected to an (ANOVA) using the PROC MIXED procedure of SAS (SAS; version 9.2; Cary, NC, USA). Orthogonal contrasts were used to determine whether differences in flavonoids were related to extraction method. Means were separated by Fisher's Protected least significant differences (LSD) test, $P \leq 0.05$.

Results and Discussion

Flavonoid extraction from Norton berry skins with acetone was more efficient than ethanol at each time interval (Figure 2.1). Acetone recovered 105% more anthocyanins than ethanol extraction. Furthermore, total phenolics recovered 164% more by acetone, and tannins were recovered at a significantly high amount at 318% more by acetone than by the ethanol extraction. Overall acetone was 30% more efficient than ethanol from extraction of flavonoids.

Anthocyanin values in Norton grape skins across all time points varied from 10.4 to 7.2 mg/g skins for acetone and 9.9 to 6.0 mg/g skins for ethanol (Figure 2.1). Maximum recovery was achieved by using the acetone system at 3 hr (Table 2.1). Anthocyanin levels were higher for acetone vs ethanol at 2 out of 6 time points, with higher recovery for the 1 and 3 hr extractions. Anthocyanin levels were highest in grape skins after 3 hr extraction, but declined from 6 through 48 hr. For acetone, the drop in efficiency from 3 to 48 hr was 31%, whereas for ethanol the anthocyanin levels dropped 39% over the same period. The reduction in anthocyanin extraction over time is likely the result of instability, which indicates thermal degradation or polymerization (Gao et al. 1997, Pinelo et al. 2005) that may influence the quantity detected.

Total phenolic levels in Norton grape skins across all time points varied from 9.6 to 6.6 mg/g skins for acetone, and 6.8 to 5.3 mg/g skins for ethanol (Figure 2.1). Acetone recovered an optimal quantity of 9.6 mg/g skin for total phenolics at 3 hr (Table 2.1). Total phenolic levels were higher for acetone vs ethanol at 4 out of the 6 time points. Concentrations of total phenolics were highest after 3, 24 and 48 hr for acetone and 6 hr for ethanol, and declined thereafter. For acetone, the drop in efficiency from 3 to 24 hr was 3% and for 3 to 48 hr was 4%, whereas for ethanol the total phenolic levels dropped 22% over 6 to 48 hr extraction times. The reduction in total phenolic extraction over time is likely a result of degradation or oxidation (Lapornik et al. 2005, Spigno et al. 2007, Zhang et al. 2001).

Tannin levels in Norton grape skins across all time points varied from 3.3 to 1.9 mg/g skins for acetone, and 1.0 to 0.6 mg/g skins for ethanol (Figure 2.1). Maximum

tannin recovery was achieved with a 3 or 24 hr acetone extraction (Table 2.1). However, the 12 hr acetone extraction time had a lower recovery than these two time periods. Tannin levels were higher for acetone vs ethanol at 4 out of the 6 time points. Concentrations of tannin were highest after 3 and 24 hr for acetone and 6 and 24 hr for ethanol. For acetone, the drop in efficiency from 3 to 24 hr was 7%, whereas for ethanol the anthocyanin levels increased 2% from the 6 to 24 hr. The increase in tannin extraction length over time likely resulted because tannins need an extended maceration period for optimum extraction (Alonso et al. 1991, Hanlin et al. 2010, Jayaprakasha et al. 2001, Main and Morris 2007).

Previously 24 hr incubation in acetone has been the accepted extraction time for Cabernet Sauvignon (*Vitis vinifera*) grape skins using Harbertson-Adams assay (Seddon and Downey 2008, Harbertson et al. 2002, 2003). Research from Kallithraka et al. (1995) and Seddon and Downey (2008) found that total phenolic levels were 7.0 to 10.4 mg/g skin for acetone in comparison to Norton levels 9.33 mg/g skin. Reported levels of tannins extracted in acetone for 24 hr using Cabernet Sauvignon skins were 3.0 to 4.0 mg/g skin in comparison with 3.36 mg/g skin for Norton (Seddon and Downey 2008). However, tannin levels in Norton (.89 mg/g skin) were lower than previously reported levels for Cabernet Sauvignon extracted with ethanol for 1 hr (1.5 to 2.0 mg/g skin) (Seddon and Downey 2008). Acetone is more effective in flavonoid extraction than ethanol, consistent with previously published reports (Kallithraka et al. 1995, Seddon and Downey 2008).

Conclusions

The study demonstrates that both solvent and timing to obtain optimal extraction affects the results of flavonoid measurement in grape skins, likely because of the complex nature of anthocyanins, total phenolics, and tannins and the differences in the analytical methodologies. Acetone was clearly superior to ethanol, with optimal extraction at 3 hr time point for Harbertson-Adams Assay using Norton grape skins. The optimum extraction of flavonoids follows use of acetone extraction for 3 hr. Acetone recovered 105% more anthocyanins than ethanol. Total flavonoids were recovered 164% more by acetone, and tannin recovery was significantly higher at 318% more by acetone than by the ethanol extraction. This study demonstrates that ethanol is not an appropriate solvent for flavonoid extraction from Norton grape skins.

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Table 2.1. Comparison of ethanol and acetone extraction methods for anthocyanins, total phenolics, and tannins from mature Norton skins.^x

	Time (Hr)					
	1	3	6	12	24	48
-----50% Ethanol-----						
Anthocyanins ^y	9.92 ± 0.35 ab	9.91 ± 0.22 ab	8.73 ± 0.10 a	7.21 ± 0.60 ab	7.84 ± 0.21 ab	6.03 ± 0.11 b
Total Phenolics ^y	6.38 ± 0.68 a	5.88 ± 0.18 a	6.81 ± 0.52 b	6.51 ± 1.15 d	6.14 ± 0.62 c	5.30 ± 0.87 e
Tannins ^y	0.89 ± 0.32 a	0.98 ± 0.28 a	1.01 ± 0.45 a	0.71 ± 0.10 a	1.03 ± 0.15 a	0.66 ± 0.10 a
-----70% Acetone-----						
Anthocyanins ^y	10.13 ± 0.45 d	10.45 ± 0.79 a	9.85 ± 0.29 c	8.54 ± 0.34 bc	8.31 ± 0.46 ab	7.24 ± 0.23 ab
Total Phenolics ^y	6.66 ± 0.89 a	9.63 ± 0.17 a	8.26 ± 1.16 a	8.71 ± 0.88 b	9.33 ± 0.38 b	9.21 ± 0.65 c
Tannins ^y	2.46 ± 0.35 cd	3.12 ± 0.18 ab	2.36 ± 0.20 cd	1.98 ± 0.10 d	3.36 ± 0.22 a	2.71 ± 0.66 bc

^x Flavonoid values are the mean from 2g of berry skins averaged over three replications and one experiment, values within columns represent means ± standard deviation. Flavonoid values within columns with different letters were significantly different by Fisher's protected LSD at P=0.05.

^y Anthocyanins, total phenolics, and tannins are measured in mg/g skin.

Figure 2.1. Comparison of ethanol and acetone extraction methods for anthocyanins (A), total phenols (B), and tannins (C) from mature Norton grape skins. Asterisk (*) indicates a significant difference in the amount of compounds in berry skins between extraction methods at a specific time point. Means followed by the same letter within a method across time points or lacking letters are not significantly different using Fisher's Protected LSD at $P=0.05$. Vertical bars indicate standard error of the means.

CHAPTER III

DEVELOPMENT OF FLAVONOIDS, SUGARS AND ACIDS IN NORTON AND CABERNET SAUVIGNON GRAPE BERRY SKINS.

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Norton is an important grape cultivar grown commercially in Missouri and adjacent states because of its heat tolerance while ripening, low risk of winter injury, and disease resistance while also producing quality wine. Flavonoid accumulation in berries is critical for making assessments of vineyard practice, vintage, and ultimately winemaking. Traditional metrics such as sugars, pH, and titratable acidity do not provide the winemaker a full understanding of ripeness and the berry's potential to make quality wine. However, little research has examined accumulation of flavonoids in Norton fruit maturation. A two-year (2012-2013) study was initiated using Norton and Cabernet Sauvignon vines at Mountain Grove, MO, with a second site added in 2013, using 10-year-old Norton vines at Rocheport, MO. Cabernet Sauvignon, a well-studied grape cultivar was monitored for comparison to Norton. Berry samples were collected at six stages of maturation from green berries 43 days after flowering (DAF), to harvest

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(125 DAF); levels of sugars, and flavonoids such as anthocyanins, tannins, and total phenolics were quantified from berry skins using Harbertson-Adams Assay. The accumulation of soluble solids ($^{\circ}$ Brix) was linear through berry maturation and peaked at 21.6 (degree Brix) by 114 DAF for both cultivars. Anthocyanins reached a maximum of 12.2 mg/g skin in Norton which was more than 2-fold the 5.2 mg/g skin concentrations measured in Cabernet Sauvignon at 125 DAF. Tannins reached a peak level of 10.7 mg/g skin for Cabernet Sauvignon by 40 and 67 DAF during 2012 and 2013, respectively. In 2012, tannin levels were 3.9 mg/g skin for Norton, and decreased until harvest. However, in 2013 tannin levels increased throughout the season, reaching a maximum of 2.8 mg/g skin at harvest. Total phenolics in Norton berries during 2012 reached 7.9 mg/g skin by 74 DAF (veraison) and remained relatively stable through harvest. In 2013, total phenolics accumulated in Norton berries and reached levels of 11.2 mg/g skin by 114 DAF. Cabernet Sauvignon berries accumulated 21.3 mg/g skin total phenolics at 40 DAF and levels decreased until 120 DAF (harvest).

Nomenclature: Norton, Cabernet Sauvignon

Key Words: flavonoids, sugars, acids, anthocyanins, tannins, total phenolics.

Introduction

In assessing fruit quality, ripeness, and developing appropriate management strategies, it is essential to understand the development of fruit chemistry for compounds important to perceived quality. Although berry chemistry development in *Vitis vinifera* species has been well studied, interspecific hybrids including those with *Vitis aestivalis* heritage have not. Hybrids are important especially in the Midwest for cold hardiness and resistance to most pathogens (Ali et al. 2011). Among compounds important to wine quality are flavonoids (contributing to color and astringency), as well their development in relationship to other basic fruit chemistry parameters such as sugars and acids (Margalit 1997, Lee et al. 2005). Due to lack of research into hybrid flavonoid development, many hybrid growers use assumptions created from *V. vinifera* research to manage their vineyards. Understanding flavonoid development in different varieties and species is important to viticulturists and winemakers for their ability to optimize viticulture practices and continue growth for improving wine quality.

In regions where *V. vinifera* cultivars are unsuitable due to climate or disease considerations (Ali et al. 2011), interspecific hybrids are often utilized for wine production (Reisch et al. 1993). In Missouri hybrid varieties produced more than \$1.6 million in total economic value to the state in 2009 (Stonebridge Research Group 2010). This includes value of grape crops, winery revenue, jobs, and the boost to tourism Missouri wine gives the state. The economic value has tripled since 2005 because of the number of wineries doubling from 50 to 97 by 2009 (Stonebridge Research Group 2010). One of the most widely planted cultivars in Missouri is Norton, which comprises 19.3%

of the total bearing acreage (USDA-NASS 2012). Norton continues to gain considerable attention in the Midwest and Eastern United States for its potential as a quality wine grape, and its potential to produce wine of comparable quality to *V. vinifera* cultivars. Despite the economic importance of Norton, even its true parentage is unknown. It is believed to be derived from *Vitis aestivalis*, *Vitis labrusca* L., and *Vitis vinifera* cv. Chasselas (Parker et al. 2009).

The genus *Vitis* and its various species that are of interest to the winemaker are particularly *V. vinifera*. Although *V. vinifera* produces quality wines, there other species found throughout the world. For example, in the Midwest, hybrids such as Norton are aimed at making a better vine for resistance to disease, yield, and quality. Norton has developed a remarkably high level of resistance to most fungal pathogens such as: phylloxera, powdery mildew, and tolerance to Pierce's Disease, while Cabernet Sauvignon remains susceptible to those pathogens (Ali et al. 2011, Main 2005). This enables viticulturists to grow Norton with minimal application of pesticides in regions with high disease pressure. Furthermore, Norton offers consistency in resistance to winter damage and late spring freezing in regions that *V. vinifera* cannot survive in (Main 2005). This suggests that even though *V. vinifera* cultivars can result in quality wines in specific regions, this doesn't mean cultivars will make a quality wine everywhere. The extreme climate conditions in growing regions of *V. vinifera* leads to limited development of flavonoids during the growing season which results in lower levels of flavonoids, and also susceptibility to disease that cannot compete with hybrids such as Norton (Ali et al. 2011). This is important because the total amounts of

anthocyanin and phenolic content are significantly higher in Norton than in those of *Vitis vinifera* (Hogan et al. 2009, Munoz-Espada et al 2004). A recent study by Ali et al. (2011) found that anthocyanin levels in Norton berry skins were considerably higher (11.6 mg/g fresh weight; FW) than Cabernet Sauvignon berry skin (2.7 mg/g FW) in the Midwest.

Anthocyanins are the glycosylated flavonoid compounds found in grapes that are responsible for color and are often related to wine quality (Lee et al. 2005).

Anthocyanins and their α -glycones, anthocyanidins, account for the red, purple, and blue hues present in berry skins and wine. The main anthocyanins in wine are cyanidin, peonidin, delphinidin, petunidin, and malvidin (Waterhouse 2002). The major anthocyanin found in Norton is malvidin-diglucoside, whereas malvidin-monoglucoside contributes to anthocyanin levels in Cabernet Sauvignon (Ali et al. 2011). Anthocyanins are extracted from the berry skins during crushing, pressing, and fermentation of grapes, resulting in the finished product red wine, where they play a fundamental role for quality control purposes. However, dark blue/black grapes do not necessarily produce dark colored wines, which may be related from extraction and stability (Romero-Cascales et al. 2005).

Another sub-class of flavonoids important to wine quality and storage potential are tannins. These contribute astringency and mouthfeel to wines as well as stabilize color and redox status during extended aging (Hanlin et al. 2010, Margalit 1997, Waterhouse 2002). Astringency contributes to the oral sensation described as drying, roughing, and puckering, whereas, mouthfeel gives texture to the wine (Gawel 1998).

However, with time the tannins tend to soften while enhancing the wine's body and flavor making a quality wine. Tannins can be found in the skins and seeds of the berry and also in the stem and tissues of the berry (Kennedy et al. 2001). Tannins found in wine can be separated into hydrolysable tannins and condensed tannins. Hydrolysable tannins in the wine are derived from the use of additives such as oak barrels, oak chips, and oak powder (Boulton et al. 1998). Condensed tannins are extracted from grape seeds, skins, and stems during winemaking (Boulton et al. 1998, Monagas et al. 2003). Tannins react with anthocyanin's to form a stabilized anthocyanin or pigmented tannin which persists much longer in the wine than the initial form (Kennedy 2007, Waterhouse 2002). For wines that are aged to improve quality, stabilizing color is important (Kennedy 2007, Waterhouse 2002).

While berry flavonoid concentrations are primarily a factor of cultivar, other parameters such as sunlight, air temperature, and the length of growing season are also important (Spayd et al. 2002). Solar radiation is one of the most essential factors for grape berry ripening as it drives photosynthesis and accumulation of anthocyanin as well as tannin development (Mazza et al. 1999). Significant differences in anthocyanin concentration between sun exposed and shaded clusters of Cabernet Sauvignon were found by Crippen and Morrison (1986). More recently, Price et al. (1995) reported that wines made from Pinot Noir clusters highly exposed to sun had 60% higher anthocyanin concentration than wines from shaded clusters, and 14% more than wines made from moderately exposed clusters. Additionally, the intensity of solar radiation also determines the berry temperature (Spayd et al. 2002).

Air temperature during grape berry development also influences flavonoid levels in grapes. With increased temperature, metabolic processes in the plant increase, resulting in greater accumulation of flavonoids (Ebadi et al. 1995, Dokoozlian and Kliewer 1996). However, at high temperatures many metabolic processes stop or are significantly reduced (Jones 1992). In grapevines it is thought that this temperature is ~30°C (Coombe 1987). Previously published research has suggested that temperatures too cold or too warm can be associated with poor color and low tannins (Bergqvist et al. 2001, Spayd et al. 2002,). A cool night temperature (10 or 15°C) did not reverse the effect of hot day temperature on berry color (Spayd et al. 2002). Grapes from vines held at warmer day (25°C) and cool night (15°C) temperatures developed less color than those from vines held at cool day and night temperatures (both 15°C) (Spayd et al. 2002). Separating the effects of temperature and sunlight on grape berry composition is difficult because many of the biochemical pathways are both light and temperature sensitive (Spayd et al. 2002).

Norton is a relatively young (179-years-old) cultivar with limited information available on the chemical profile of the grape (Ambers and Ambers 2004). However, studies are necessary to understand the development of flavonoids to optimize viticulture practices and continue growth for improving wine quality and diversifying wine styles for regions such as the Midwest that are relying on these cultivars for production. Future research will enable winemakers, and viticulturists to design effective strategies for canopy management and harvest decisions, as well as determining how site selection affects grape fruit composition. The objective of this

study was to characterize the accumulation of flavonoids such as anthocyanins, tannins, and total phenolics in grapes from hard green berries to maturity over a two year period. As a comparison, flavonoid content to a well-studied cultivar Cabernet Sauvignon was used as a model for comparison.

Material and Methods

Location

Two sites were included in this study, Mountain Grove, MO (MG) and Rocheport (RO), MO. Samples were taken from MG site in 2012 and 2013, where the RO site was only sampled in 2013. Samples at MG were collected from seven-year-old Norton, own rooted or Cabernet Sauvignon on 3309 rootstock. All vines were grown in a research vineyard at Missouri State Fruit Experiment Station in MG (37° 9' 18" N, 92° 16' 4" W). The soil at this site was 74.1% Wilderness gravelly silt loam with a fragipan at 0.38 to 0.74 m depth, and 25.9% Viraton silt loam (fine-loamy, siliceous, mesic Typic Fragiudalfs) with a fragipan at 0.46 to 0.84 m depth (USDA 2012) (Table A.1). Samples were collected during 2013 growing season from ten-year-old Norton, own rooted in a commercial vineyard in RO (38° 58' 15" N, 92° 33' 3" W). The soil at this site was Menfro silt loam (Fine-silty, mixed, superactive, mesic Typic Hapludalfs) with 2.0 m depth (USDA 2012) (Table A.1).

Three replicates each comprising twelve adjacent vines were arranged in a completely randomized design and trained to a High Bilateral Cordon System at 1.8 m with 2.4 m between vines and 3.0 m between rows; row direction was north and south.

Twelve vines per replicate were distributed throughout the four rows in a 122 m plot.

Vines were spur pruned and balanced using a 20 + 20 formula (20 nodes retained for the first 0.45 kg of pruning weight and 20 nodes left with additional 0.45 kg dormant pruning's removed) during February to March of each respective year. Shoot thinning, downward shoot positioning, and leaf removal was performed annually on Norton and Cabernet Sauvignon, and cluster thinning was done 1 time per year in June on Cabernet Sauvignon. Drip irrigation programming, pest, and weed management was carried out at MG by the Missouri State Fruit Experiment Station staff. Vines were fertilized with N as ammonium nitrate (75.8 kg actual N/ha) and K₂O potash (136.7 kg/ha) annually during the spring.

Three replicates of twelve vine plots were arranged in a completely randomized design and trained to a High Bilateral Cordon System at 1.8 m with 4.9 m between vines and 3.0 m between rows; row direction was east and west. Vines were long spur pruned and balanced leaving approximately 7 nodes for every 0.25 to 0.30 m annually during February to March, 2014. Shoot thinning was performed 1 to 2 times per year, downward shoot positioning was done 2 to 3 times per year, and leaf removal was done 1 to 2 times per year from June through August. Vines were fertilized with N as mono ammonium nitrate (16.5 kg actual N/ha), P (168.0 kg/ha), K (280 kg/ha), S (95.2 kg/ha), B (16.8 kg/ha), Mn (28.0 kg/ha), and Cu (5.6 kg/ha) during spring 2013. Vines were not irrigated; weed and pest management followed commercially accepted practices.

Weather Data

Data were obtained from the University of Missouri Agriculture Electronic Extension Board (AgEBB) weather station located in Mountain Grove, and Columbia, MO. This information was used to determine growing degree days (GDD) with a basic temperature of 10°C starting April 1st (Winkler et al. 1974). Historical weather data were obtained from the University of Missouri Science and Technology and National Weather Service COOP weather station (235834) in Mountain Grove, and (231790) in Columbia, MO.

Vine and Harvest Data

Vine growth, number of dormant pruning weights, and nodes were collected annually (2013 and 2014) during February in MG. Exposed canopy surface area (ECSA) were measured by hand annually (2012 and 2013) during harvest for Norton vines only (Table 3.3). The canopy was divided into height (m), width (m), and length in row direction (m), respectively. ECSA data were computed as described by Schultz (1995). Fruit, yield, and cluster number per vine were collected and cluster weight, berry weight, and berries per cluster were determined at harvest (Table 3.4). Grapes were harvested on 11 Sept 2012 and, 8 October 2013 for Norton, and 11 Sept 2012 and, 3 October 2013 for Cabernet Sauvignon based on measurements of soluble solids, titratable acidity, and pH before harvest. Fruit, yield, and cluster number per vine were collected and cluster weight, berry weight, and berries per cluster were determined at harvest in RO (Table 3.5). Grapes were harvested on 9 Oct 2013 based on harvesting parameters listed above.

To assess canopy architecture and quantify cluster exposure, canopy data were collected during veraison and harvest for Norton in MG/RO and Cabernet Sauvignon vines in MG during 2013 using enhanced point quadrat analysis (EPQA) (Table 3.6, Table 3.7). EPQA measurements were performed simultaneously to photosynthetic photon flux (PPF) measurements with data compiled into EQPA-CEM Toolkit version 1.6 (Smart and Robinson 1991, Meyers and Vanden Huevel 2008). Insertions were made at 0.20 m intervals per vine. EPQA data were analyzed using EQPA-CEM Toolkit version 1.6 (Meyers and Vanden Huevel 2008). A 2 m fiberglass rod (20 mm diameter) was positioned horizontally into the center of the vine canopy to serve as a guide for the measurements. PPF was recorded at the surface height of the fruit zone inside the canopy interior (1.5 m above ground), using a 0.8 m AccuPAR ceptometer (Model LP-80, Decagon Devices, Inc, Pullham, WA). The ceptometer was placed below the cordon parallel to the vine row at each measurement point, and two measurements were taken on both sides of the cordon. Readings were collected from 11:00 am to 1:00 pm Central Daylight Time (CDT) on clear days at the stage of veraison and again prior to harvest.

Vine nutritional status was evaluated from 100 petioles from each replication at veraison in 2013 (Table 3.1, 3.2). Macro (N, Ca, Mg, P, K) and micronutrients (Fe, Zn, Mn, Cu) analyses were conducted by the University of Missouri Soil and Plant testing Laboratory using standard protocols (Nathan and Sun 2006).

Grape Analysis

One hundred and fifty individual berry samples of Norton at MG/RO and one hundred individual berry samples for Cabernet Sauvignon for each replication were

collected for six stages to evaluate juice characteristics. Juice was pressed by hand and then homogenized at ambient temperature using a Stomacher Model 400 circulator (Seward, Worthington, West Sussex, UK). The homogenate was pressed through two layers of grade 40 cheesecloth and centrifuged at 10,000 rpm ambient temperature for 5 min (model 5804 R, Eppendorf AG, Hamburg). Juice pH was measured with a temperature compensating pH probe and Orion 3 star meter (Thermo Scientific, Fort Collins, CO) (Appendix A.4). Titratable acidity were measured with a Compact Titrator (Model G20, Mettler-Toledo, Schwerzenbach, Switzerland) using 2 mL juice sample diluted to 50 mL, degassed and deionized water titrated to an endpoint of 8.2 pH with 0.1 N NaOH (Iland et al. 1996).

For determination of soluble solids, individual berry samples were collected annually (2012 and 2013) at each stage and an average of twelve berries for each replication. Soluble solids were measured with a temperature compensating portable refractometer REF-113 (Quangdong, P. R. China). Three hundred and fifty individual berry samples at each replication were collected from both sides of the vine, and all parts (top, bottom, middle, sides) of the cluster during the 2012 and 2013 seasons for determination of flavonoids and organic acids (tartaric, malic, and citric acid). Samples were frozen immediately in the field with liquid nitrogen and transported to the laboratory on dry ice and stored at -80°C. Samples were removed from the -80°C freezer for partial thawing berries and then skin was removed from the berries using a razor blade and re-frozen in liquid nitrogen prior to crushing. One hundred and ten individual berry samples for each replication were removed from the -80°C freezer and weighed.

Five individual berry samples from each replication were weighed for whole berry, pulp, and skin and diameter measurements were taken (Table A.2, A.3). For analysis, sixty skin frozen samples per replication were crushed with an Oscillating Mill (Model MM400, Retsch, Haan, Germany) and 2 g of frozen skins were weighed and removed for extraction of flavonoids.

Anthocyanins, total phenols, and tannins of each sample were analyzed using the Harbertson-Adams Assay (Harbertson et al. 2003). The complete protocol for analysis of these parameters is available on the University of California, Davis website (Downey et al. 2006). Berry skin samples were centrifuged at 10,000 rpm for 10 min to remove precipitates before analysis. The assay was performed directly on extracted grape samples (as described in previous section). All spectra were collected within 12 hr of performing the analytical analysis. UV-visible spectra were collected with a Spectronic Genesys 2 UV-Vis spectrophotometer (Model 336002, Thermo Scientific, Madison, WI) using polystyrene cuvettes (Fisher Scientific, Waltham, MA).

Samples for organic acid determination of Norton and Cabernet Sauvignon berries were thawed overnight at 4°C. Juice was extracted from berries as previously described and 2 mL were mixed with 0.1 g polyclar #10 (PVPP) before placement on the rotary shaker for 5 min. Samples were centrifuged at 13,000 rpm for 3 min and the supernatant clarified using a 0.45 µm filter prior to analysis. Organic acids were determined by high performance liquid chromatography (HPLC) as described by Jogaiah et al. (2012). Analysis was conducted on a Varian HPLC system equipped with a Varian 355 diode array (Varian, USA). The 100% aqueous mobile phase was pumped at 1

ml/min through an Agilent Hi-Pex H column (6.5x300mm, 8 μ m) (Agilent, Santa Clara, CA) held at 65°C. Calibration was conducted using authentic standards (Sigma Aldrich, St. Louis, MO).

Statistics

Soluble solids, citric, tartaric and malic acid, anthocyanin, total phenolic, and tannin data were subjected to an analysis of variance (ANOVA) using the PROC MIXED procedure of SAS (version 9.2; Cary, NC, USA). Year was considered a random factor for citric and malic acid; therefore data were pooled for site-years. Due to different tartaric, soluble solids, anthocyanin, total phenolic, and tannin levels at MG in 2012-2013 and RO in 2013, data were analyzed separately. Means were separated using Fisher's Protected (LSD) at $P \leq 0.05$. Means with a year by cultivar stage interaction were analyzed by year using PROC MIXED procedure.

Results

Soluble Solids

Total soluble solids (°Brix) in berries during development were similar during the 2012 and 2013 growing season for Norton and Cabernet Sauvignon (Figure 3.1). Over a two-year period, total soluble solids ranged from 3 to 23 degrees brix for Norton at MG (Figure 3.1A). Levels of soluble solids increased over the growing season for both years except for stage two, brix levels were also similar for Cabernet Sauvignon during 2012 and 2013 (Figure 3.1B).

Levels of total soluble solids were slightly higher for Norton compared to Cabernet Sauvignon over both growing seasons (Figure 3.1). Relative soluble solid values for Norton were 2-fold greater than Cabernet Sauvignon at the beginning of the season. As harvest, relative soluble solid quantities for Norton were only 1.1-fold greater than Cabernet Sauvignon. Levels of soluble solids for both cultivars increased at stage four, but remained significantly different for the rest of the growing season.

During 2013, the range of total soluble solids ranged from 3 to 24 degrees brix at RO (Figure 3.1A). Soluble solid levels were initially lower for stages one through three, increased between stages three and four, and then remained constant thereafter. Levels of soluble solids for RO were 10% less than levels for MG at stage 5.

Organic Acids

Citric and malic acid levels for Norton followed a similar trend for all developmental stages during the 2012 and 2013 growing seasons; however, tartaric acid followed a different trend during those years (Figures 3.2A, 3.3A, 3.4A). Over a two-year period, the range of citric, tartaric, and malic acid ranged from 0.2 to 0.7, 2 to 5, and 6 to 14 g/L for Norton at MG. Tartaric acid levels were highest for hard, green berries and decreased over the growing season for both years, whereas, citric and malic acid levels began and ended at approximately the same level for both years. Relative differences of tartaric acid content between years were detected at stage 3. Citric and malic acid content was combined between years because of no relative differences. Levels of citric, tartaric, and malic acid for Cabernet Sauvignon were all significantly different throughout the growing season during 2012 and 2013, but levels all followed a

similar trend (Figures 3.2B, 3.3B, 3.4B). The ranges of citric, tartaric, and malic acid were 0.1 to 0.3, 2 to 9, and 5 to 20 g/L in 2012 and 2013, respectively. Levels of each acid were higher in newly developing berries and decreased over the growing season. Citric acid levels reached a peak at stage two and three for both site years, with levels between 0.32 and 0.37 g/L (Figure 3.2B). Levels declined by harvest, with final concentrations of 0.2 g/L. Tartaric acid levels were significantly different between stages one through five, and unchanged at stage six (harvest). For malic acid, levels significantly changed between stages three through five, and unchanged at harvest.

Norton exhibited higher levels of citric acid compared to Cabernet Sauvignon, but lower levels of tartaric and malic acid over both the 2012 and 2013 growing seasons (Figures 3.2, 3.3, 3.4). Relative citric acid quantities for Norton were 1.9-fold greater than Cabernet Sauvignon at the beginning of the growing season. However, tartaric and malic acid quantities for Norton were 1.7- and 1.4-fold lower, respectively than Cabernet Sauvignon at the beginning of the season. At harvest, relative citric acid quantities for Norton were only 1.7-fold greater than Cabernet Sauvignon. However, tartaric and malic acid quantities were only 1.2-fold, 1.1-fold lower, respectively than Cabernet Sauvignon at harvest.

Anthocyanins

Anthocyanins levels in 2012 were 19.7% lower than accumulated levels in 2013 (Figure 3.5A). Over a two year period anthocyanin levels reached a peak of 10 to 12 mg/g skin for Norton at MG. Anthocyanin levels for both years remained low until stage three (veraison). A significant change in anthocyanin content wasn't observed until

stage three through six for both years. All stages were similar in MG except for stage four, with 77% less anthocyanins in 2012. Whereas, RO had 31% less anthocyanin content than MG.

Anthocyanins initially were detected in Cabernet Sauvignon at veraison and reached levels up to 4.5 mg/g skin in 2012 and 2013 (Figure 3.5B). The range of anthocyanins over a two-year growing season ranged from 0 to 4.6 mg/g skin for Cabernet Sauvignon at MG. Levels of anthocyanins remained low for stages one to three and increased by stage four; levels remained similar levels for stages four through six in both years.

Total Phenolics

Levels of total phenolics for Norton in 2012 remained similar over the growing season, whereas in 2013, total phenolics were 33% higher after veraison (stage 3) (Figure 3.6A). Over a two-year period, the range of total phenolics were 6 to 11 mg/g skin for Norton at MG. Phenolic levels remained similar with no significant changes for 2012 until harvest. However in 2013, phenolic levels initially were lower for stages one through three, increased for stages four and five, and remained unchanged through harvest. Changes in phenolic content were not significantly different after stage one (42 DAF) in 2012. For 2013, there was no significant increase after stage 4 (92 DAF). For RO, phenolic levels remained low for stages one through three, then increased through harvest.

Levels of total phenolics were similar for the 2012 and 2013 growing seasons for Cabernet Sauvignon (Figure 3.6B). The range of total phenolics over the two-year

growing season were 8 to 23 mg/g skin for Cabernet Sauvignon at MG. Phenolic levels were higher at the beginning of the season, and decreased over the growing season for both years. Changes in phenolic content were not significantly different after stage two (64 DAF) in 2012.

Tannins

Tannin levels in Norton grape skins varied widely throughout 2012 and 2013, but final levels were within 17% of each other (Figure 3.7A). Over a two-year period, the range of tannins were 1 to 4 mg/g skin for Norton at MG. Levels of tannins for 2012 were highest at the beginning of the season, and remained similar through harvest. Changes in tannin content were not significantly different after stage one for 2012 and 2013. Tannin levels for 2013 were 73% less than 2012 levels for stage one. For RO, tannin levels were lowest for stages one through four, increased for stage five and remained unchanged through harvest.

Cabernet Sauvignon tannin levels were similar throughout the 2012 and 2013 growing season (Figure 3.7B). Tannins ranged from 2 to 12 mg/g skin over the two-year growing season for Cabernet Sauvignon at MG. Tannin levels were initially higher and then decreased over the growing season for both years. For 2012, changes in tannin levels were not significantly different after stage two (64 DAF). Tannin levels for 2013 were 30% less than 2012 levels at stage one.

Petiole nutrient content

Macro and micronutrient content in petioles was monitored to determine if levels were sufficient to promote berry development. Nutrients in petioles from Norton

and Cabernet Sauvignon vines at MG were in sufficient ranges during 2013, with the exception of Mg, K, and Zn (Tables 3.1) (Bates and Wolf 2008). Magnesium and Zinc levels were above recommended values for both, Norton and Cabernet Sauvignon. Values were similar between Norton and Cabernet Sauvignon, except potassium levels in Norton were 47% less compared to Cabernet Sauvignon.

Nutrient in petioles from Norton vines at RO were in sufficient ranges during 2013, with the exception of Ca, Mg, K, Fe, and Zn (Table 3.2) (Bates and Wolf 2008). Levels were significantly different for all nutrient elements in petioles for MG and RO. Mg content was low (<0.35%) in Norton vines. Potassium levels in RO were not only high, but excessive (>2%); in contrast iron levels were below recommended values, while zinc levels were above recommended values.

Vegetative Growth

The pruning weights and nodes per vine over a 2-year period were significantly different between Norton and Cabernet Sauvignon (Table 3.3). Norton pruning weights and nodes per vine retained were 30% greater in 2012 than 2013. The pruning weights and nodes per vine retained of Cabernet Sauvignon were 47% greater in 2013 than 2012. Exposed canopy surface area (ECSA) was similar between the 2-year periods for Norton. However, no ECSA data were collected on Cabernet Sauvignon vines.

Enhanced point quadrat analysis (EPQA) (Table 3.6) data during 2013 showed Norton and Cabernet Sauvignon vines had very low cluster exposure to sunlight in MG. However, symmetric calibrated flux metrics (CEFS and LEFS) were significantly different at veraison. Traditional PQA metrics (LLN, PIC, and PIL) for both cultivars were different.

EPQA data during 2013, showed Norton vines had lower cluster sun exposure for MG than RO (Table 3.7). However, symmetric calibrated flux metrics (CEFS and LEFS) were significantly different for veraison.

Fruiting Characteristics

Fruit yield for 2012 and 2013 Norton vines in MG were relatively similar (Table 3.4). Vines in 2013 produced 2.6% more fruits than 2012. Average clusters per vine, berry weight, and berries per cluster were similar over the 2-year period, however, in 2013, these parameters for Norton were greater than 2012. Fruit yield for 2013 Norton vines in MG, and RO were different (Table 3.5). Due to the size in cordon length, MG vines were 45% smaller than RO vines. Due to the significant difference in cordon lengths between locations, data were different for yield, and clusters/vine. Fruit yield had the greatest loss at 62% with Norton vines from MG compared to RO Norton vines. In contrast, berry weight, berries/cluster, and cluster weight were similar between the two locations.

Discussion

During berry development, sugars accumulate and are needed for alcoholic fermentation during wine processing. Sugar accumulation commences at veraison, continues linearly until harvest, and is represented as a double sigmoidal curve (Figure 3.1) (Downey et al. 2003). Downey et al. (2004) reported that soluble solid levels for Shiraz grapes ranged from 5 to 25 Degrees Brix, coinciding with levels for Norton and Cabernet Sauvignon (3.6 to 23.6, and 3.9 to 20.5 degrees brix) (Figure 3.1). Sugar levels

for Norton and Cabernet Sauvignon at veraison ranged (9.2 to 11.8 and 10.5 to 11.3 degrees brix) (Figure 3.1), coinciding with previously reported levels of Cabernet Sauvignon and Shiraz berries at (12.5 and 10.0 degrees brix) (Pirie and Mullins 1977). Norton vines exhibited a range at harvest from 20 to 25 Brix (Main 2005, Main and Morris 2008, Morris and Main 2010), coinciding with Norton levels at (22.3 to 23.6 degrees brix) (Figure 3.1A). However, Cabernet Sauvignon levels at harvest were (19.3 to 20.5 degrees Brix) (Figure 3.1B), which were lower than previously reported levels at (22.2 degrees Brix) (Pirie and Mullins 1977). This could be due to geographical location and variation in environmental conditions (Figures 3.9, 3.11). Cabernet Sauvignon does not adapt well to extreme high temperatures that the Missouri climate has typically, ultimately shutting down early berry development when half the growing season is left for Cabernet Sauvignon growth cycle.

Organic acids such as citric, tartaric, and malic accumulate in the skins and reached a maximum at the onset of veraison (Figure 3.2, 3.3, 3.4). However, once the acids reach their peak level; they will start to decline post-veraison throughout the rest of the growing season (Dokoozlian 2000). Norton and Cabernet Sauvignon followed a similar trend from previous studies (Dokoozlian 2000), showing all organic acids reaching their highest levels during stage one and two and decreased over the growing season for both years and both sites. Once synthesized, tartaric acid is believed to be stable, since no enzyme capable of degrading it has been found in the berry (Dokoozlian 2000). The decrease in tartaric acid concentration is attributed to a dilution effect, since berry volume increases while the amount of tartrate per berry remains constant

(Dokoozlian 2000). In contrast, several enzymes capable of metabolizing malate are present in the berry, and the acid is readily respired to CO₂ and H₂O (Dokoozlian 2000, Sweetman et al. 2009). Reductions in malic acid concentration after veraison result from respiration and enzyme degradation (Dokoozlian 2000, Sweetman et al. 2009).

The ranges of organic acids: citric, tartaric, and malic for Norton over a two year study were 0.2 to 0.7, 2 to 5, and 6 to 14 g/L, and 0.1 to 0.3, 2 to 9, and 5 to 20 g/L for Cabernet Sauvignon. Citric and tartaric acid levels at harvest for Norton (0.29 to 0.41 and 1.6 to 2.2 g/L) (Figures 3.2A, 3.3A) and Cabernet Sauvignon (0.18 to 0.21 and 2.1 to 2.5 g/L) (Figures 3.2B, 3.3B) were significantly lower than previously reported levels of Norton (0.5 to 1 and 6 to 10 g/L) (Harris 2013, Jogaiah et al. 2012, Main and Morris 2004, 2008). However, malic acid levels for Norton (6.8 to 8.4 g/L) (Figure 3.4A) were significantly higher than previously reported levels for Norton (3.2 to 7.4 g/L), whereas, levels of Cabernet Sauvignon coincided (6.2 to 7.0 g/L) (Figure 3.4B) with several published reports (Harris 2013, Jogaiah et. al 2012, Main and Morris 2004, 2008). Furthermore, Kliewer (1967) reported that *Vitis aestivalis* species contained higher amounts of malic acid than tartaric acid. Main (2005) reported that Norton contained malic acid levels up to three times higher in Norton than in other *Vitis vinifera* species, posing a problem for winemaking.

Temperature is a key factor in controlling berry acid content (Dokoozlian 2000, Sweetman et al. 2009). During the initial stages of fruit development, the optimum temperature for acid synthesis ranges between 68° and 77°F (20° and 25°C) (Dokoozlian 2000). Fruit acidity at harvest is negatively correlated with temperature during the

ripening period. For example, grapes ripened at low temperatures have greater total acidity, particularly malic acid, compared to grapes ripened at higher temperatures (Dokoozlian 2000). Malic acid levels are reduced upon exposure to direct sunlight and exposure with berry temperature as reported by Pereira et al. (2006) and Spayd et al. (2002), which coincided with 2012 results for Norton and Cabernet Sauvignon with levels at 6.8 and 6.2 g/L. The 2012 growing season experienced extreme drought conditions with higher temperatures. However in 2013, malic acid levels significantly increased for Norton at both locations and Cabernet Sauvignon to 7.9-8.4 and 7.0 g/L, respectively. This is due to malic acid benefiting from a reduced temperature compared to 2012, and the 2013 growing season had higher precipitation (Figure 3.9, 3.11). In contrast, the lower levels of tartaric acid in Norton and Cabernet Sauvignon could be due to a variation among region and years, with lower levels of acidity found in warmer climates which Missouri has (Dokoozlian 2000).

Anthocyanins accumulate in berry skins at veraison, and levels increased to full maturity (Figure 3.5). However, after reaching a maximum value, anthocyanins decline (Draghici et al. 2011). Development of anthocyanins followed a similar trend from previous studies (Ali et al. 2011, Deluc et al. 2009) showing anthocyanins reaching their highest levels in Cabernet Sauvignon after veraison, and remained steady until harvest. In Norton, however, anthocyanin levels continued to increase steadily until harvest. The ranges of anthocyanins for Norton and Cabernet Sauvignon over a two-year study were 0 to 1.8 and 0 to 0.6 mg/g berry wt (Figures 3.5A, 3.5B). These levels of anthocyanins corresponded with previously reported levels (0 to 2.5 mg/g berry wt) for Shiraz skins

(Downey et al. 2004). Veraison levels of anthocyanins from previously reported levels (0.03 mg/g berry wt.) for Cabernet Sauvignon and coincided with Cabernet Sauvignon and Norton levels (0.01 to 0.03 and 0.02 mg/g berry wt.) (Figures 3.5A, 3.5B) during 2012-2013 (Pirie and Mullins 1977). Previously reported levels of Norton anthocyanins at harvest were 1.7 to 1.9 mg/g berry wt. (Figure 3.5A), coinciding with Norton at both locations (1.2 to 1.8 mg/g berry wt.) (Harris 2013). However, Cabernet Sauvignon harvest levels (0.3 to 0.5 mg/g berry wt.) (Figure 3.5B) were lower than previously reported levels (1.11 to 1.24 mg/g berry wt.) by Draghici et al. (2011).

Assumptions for the differences in anthocyanins have been suggested including differences in cultivar, site, season, as well as differences in geographical location and climate (Draghici et al. 2011). Many researchers suggest non-genetic factors such as several environmental conditions or viticultural practices have a greater effect on the concentration of anthocyanins rather than on their relative composition (Arozarena et al. 2002, Pomer et al. 2005). Furthermore, the pattern of anthocyanin levels through berry development can vary year after year depending upon climatic conditions such as rain, cool temperatures, and moisture (Draghici et al. 2011). This coincided with higher levels of anthocyanins for Cabernet Sauvignon during 2012 drought conditions, whereas, lower levels in 2013 growing season with cooler temperatures, and high rainfall (Figure 3.9). However, enhancing anthocyanin accumulation in grapes can be achieved by decreasing the crop load, leaf area ratio, reduced air temperatures, and sprinkler cooling (Pirie and Mullins 1977).

During berry development, the amount of total phenolics increases from veraison to full maturity, and post-harvest a slight decrease was registered (Draghici et al. 2011). The pattern of total phenolics accumulation coincided with the trend of Norton levels for 2012-2013 at both locations (Figure 3.6). Levels of total phenolics at veraison for Norton and Cabernet Sauvignon were 0.7 to 1.2 and 1.2 to 1.8 mg/g berry wt. (Figures 3.6A, 3.6B) coinciding with levels of Shiraz and Cabernet Sauvignon skins (1.1 and 0.7 mg/g berry wt.) (Pirie and Mullins 1977). Previously reported levels of total phenolics for Norton at harvest were 1.2 to 1.5 mg/g berry wt. (Figure 3.6A), coinciding with Norton levels (1.1 to 1.7 mg/g berry wt.) during 2012-2013 growing season at both locations (Harris 2013, Hogan et al. 2009). Whereas, Cabernet Sauvignon levels were lower (1.0 to 1.6 mg/g berry wt.) (Figures 3.6B) during harvest, coinciding with previously reported levels for Shiraz and Cabernet Sauvignon (2.3 and 2.8 mg/g berry wt.) (Pirie and Mullins 1977).

An assumption for the lower levels could be due to influence of geographical location in addition to varietal differences. Missouri climate cannot produce quality Cabernet Sauvignon due to extreme climate differences compared to California. In particular, grape phenolics may be significantly affected by growing conditions related to the geographical region of the cultivar such as soil composition, irrigation, light intensity, temperature, etc. (Cantos et al. 2002, Kanner et al. 1994). Analyzed data from EPQA results could have also played a role on delay of anthocyanin development for Cabernet Sauvignon due to excessive number of leaf layers in the canopy. The variation in the phenolic compounds of grape skins, the rate of phenolics decreasing during the

ripening period, the extractability during wine fermentation, and the wine color characteristic have to be considered when grapes in different maturity stages are used in winemaking (Draghici et al. 2011).

Tannin levels for Norton in 2012 were higher at the beginning of the growing season and decreased as berries fully ripened. However, levels for Cabernet Sauvignon during both years of this study were higher at the start of the growing season, decreased at veraison and then remained relatively similar through harvest (Figure 3.7). This coincides with results from Adams (2006), because ripening was in proportion to berry growth during phase III). However, in 2013, Norton levels at both locations were lowest at the beginning of the season, but increased towards harvest (Figure 3.7). This could be due environmental parameters such as cooler air temperature and higher rainfall during the 2013 growing season, which may have caused tannins to accumulate later in berry development stages. Other studies have also reported that higher concentrations of tannins can be found at harvest in the skin of fruit from low vigor vines (Cortell et al. 2005, Ristic et al. 2007). From EPQA data, a direct impact from light exposure on tannin accumulation may have resulted in lower levels in shaded than exposed fruit (Downey et al. 2004).

The results from this study for Norton 2012, and Cabernet 2012-2013 demonstrate a pattern of accumulation in grape skin that is similar in previous research (Kennedy et al. 2000a, Kennedy et al. 2000b, Downey et al. 2003). Tannin concentration was the highest at four to five weeks pre-veraison for Norton during 2012, and Cabernet Sauvignon 2012-2013, coinciding with previously reported research (Hanlin and Downey

2009). However, previously reported research also coincided with a decrease observed in tannin levels toward harvest (Kennedy et al. 2000, Downey et al. 2003). A decrease in tannin has been previously attributed to tannins being more difficult to extract, possibly due to interactions with cell wall material (Cheynier et al. 1997, Kennedy et al. 2001, Downey et al. 2003). Results from this study suggest that the majority of tannin synthesized occurs at or before fruit set and that the decrease in tannin levels might be a result of tannins becoming increasingly intertwined within the cell wall as cell expansion occurs. Lower tannin content could also be explained by oxidation (Kennedy et al. 2000a). At harvest, skin tannin concentration reported here for Cabernet Sauvignon was similar to previous studies (Downey et al. 2003, Hanlin and Downey 2009, Gagne et al. 2006).

Norton harvest levels for tannins were lower (2.5 to 3.4 mg/g skin) (Figure 3.7A), and Cabernet Sauvignon levels were higher (4.3 to 4.5 mg/g skin) (Figure 3.7B) than previously reported levels reported tannin levels (3.5 mg/g skin) for Cabernet Sauvignon, and (4.6 mg/g skin) for Shiraz berries at harvest stage (Harbertson and Downey 2009). However, on a per berry basis, harvest levels of Cabernet Sauvignon (0.4 to 0.6 mg/g berry wt.) (Figure 3.7B) coincided with previous research (0.7 mg/g berry wt. (Hanlin and Downey 2009). For two years, reported levels of tannins ranged from (0.3 to 0.7 mg/g berry wt.) in Cabernet Sauvignon and (0.4 to 1.1 mg/g berry wt.) in Syrah berries over development stages (Harbertson et al. 2002), coinciding with Norton levels (0.01 to 0.56 mg/g berry wt.) (Figure 3.7A), and Cabernet Sauvignon levels (0.33 to 1.40 mg/g berry wt.) (Figure 3.7B) throughout the growing season. Further research

will help determine how vineyard management practices can be used to influence the concentration of tannin during berry development across sites, seasons, and cultivars.

This practice may preclude any need addition of tannins to grapes at harvest.

Pruning weights for Norton at MG during the 2012 season (0.53 kg/vine) were 32% higher than those the following season (Table 3.3.). The number of buds retained after pruning that year resulted in a large crop load in 2013, which limited vegetative growth. This type of growth/yield relationship was described by Partridge (1925). During the 2013 season at MG, pruning weights for Cabernet Sauvignon were 48% lower than those the previous season (Table 3.3.). It is likely that the higher rainfall during the 2013 growing season resulted in vigorous vegetative growth. In addition to these differences, pruning weights in both years of this study were lower than those reported by Morris and Main (2010) for Norton on a non-divided canopy system (1.4 kg/vine), which may be attributed to high cropping. The number of buds retained for Norton vines over a 2 year period were significantly lower (16 to 23 no. of nodes retained/vine) than previously reported (55 nodes retained/vine) (Main and Morris 2008, Morris and Main 2010). Exposed surface canopy area (ESCA) data ($1.2 \times 10^6 \text{ m}^2/\text{ha}$) coincided with previously reported levels (0.8 to $1.2 \text{ m}^2/\text{ha}$) (Schultz 1995).

Micronutrients (N, Ca, Mg, P, K) in Norton and Cabernet Sauvignon petioles (Table 3.1.) were 0.97, 2.09, 1.34, 0.15, and 0.55%, 1.16, 1.85, 1.25, 0.17, and 1.03% with adequate ranges of 0.8 to 1.2, 1.3 to 2.5, 0.35 to 0.75, 0.14 to 0.3, and 1.2 to 2.0% (Bates and Wolf 2008). Whereas, macronutrients (Fe, Zn, Mn, Cu) in Norton and Cabernet Sauvignon petioles were 49.30, 57.00, 120.30, and 8.70ppm, 41.50, 59.34,

90.67, and 8.43 ppm with adequate ranges of 30 to 100, 25 to 1000, 25, and 5 to 15 ppm (Bates and Wolf 2008). Lower levels of potassium in Norton compared to Cabernet Sauvignon were likely deficient in direct response to excessive Mg content. However, higher magnesium levels are not detrimental to grape vines; it is in direct competition with other nutrients and is known to inhibit the uptake of K, resulting in K deficiency (Bates and Wolf 2008).

Macro (N, Ca, Mg, P, K) and micronutrients (Fe, Zn, Mn, and Cu) in Norton petioles (Table 3.2.) in RO were (0.68, 2.60, 0.24, 0.71, 2.64%), (26.53, 67.00, 213.17, 5.87 ppm), respectively. with adequate ranges reported by Bates and Wolf (2008). The main difference in nutrient content between site locations could be due to management practices, where RO is a commercial vineyard and MG is a research vineyard. Low levels of magnesium resulted in deficiency symptoms as observed on the leaves of the vine with a bright red color. Interestingly, only P and K were negatively affected by higher crop (21.22 kg/vine) in RO. This suggests that supplemental fertilizer may be required in addition to N supplied by ammonium nitrate to produce adequate yield and fruit composition in subsequent growing seasons.

The significant differences in traditional PQA metrics could be due to many factors: canopy size, canopy management, and yield. Norton vines were relatively smaller based on pruning weight than Cabernet Sauvignon vines, and produced less yield. Due to relative differences in the size of the vines, LLN, PIC, PIL, should be different, as Cabernet Sauvignon vines are significantly larger (Table 3.6). Enhanced point quadrat analysis (EPQA) (Table 3.6) data during 2013 showed Norton and

Cabernet Sauvignon vines both having lower cluster exposure to sunlight, but no significant differences between leaf sun exposure (CEFA, and LEFA respectively).

The significant differences in traditional PQA metric could be due to many factors: canopy size, canopy management, and yield. MG vines were relatively smaller than RO vines producing fewer yields. Due to the relative difference in size of the vines, LLN, PIC, and PIL should be different, as RO vines are significantly larger due to cordon length (Table 3.7). EPQA (Table 3.7) data during 2013 showed Norton having lower cluster sun exposure for MG than RO, but no significant differences between leaf sun exposure (CEFA, and LEFA respectively).

Norton grapevines typically produce vigorous vegetative growth and low yields with small fruit clusters (Hendrick 1908, Wagner 1945, Walker et al. 2003). Fruit yield of Norton grapevines at MG during the two years of this study (Table 3.4) coincided with previously reported levels (8 kg/vine) for non-divided canopy systems (Morris and Main 2010). However, clusters per vine were lower (124.5 to 125.7 clusters/vine) than previously reported (139 to 201 clusters/vine) on Norton vines for both non-divided and divided canopy systems (Main and Morris 2008, Morris and Main 2010). Fruit yield of Norton vines at RO during 2013 growing season (Figure 3.5), was higher (21.2 kg/vine) than previously reported levels (8 kg/vine) on Norton vines for non-divided canopy systems (Main and Morris 2008, Morris and Main 2010). Clusters per vine, and cluster weight (281.3 clusters/vine, 76.9 g) were higher than previously reported levels (139 clusters/vine, 58.5 g) on Norton vines for non-divided canopy systems (Main and Morris, Morris and Main 2010).

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Table 3.1. Nutrient content in petioles of Norton and Cabernet Sauvignon grapevines collected at veraison in Mountain Grove, MO in 2013.^x

Nutrient	Cabernet Sauvignon 2013	Norton 2013	Sufficiency Ranges ^y
N (%)	1.16 ± 0.18	0.97 ± 0.04	0.8 to 1.2
Ca (%)	1.85 ± 0.07	2.09 ± 0.08	1.3 to 2.5
Mg (%)	1.25 ± 0.13	1.34 ± 0.12	0.35 to 0.75
P (%)	0.17 ± 0.22	0.15 ± 0.01	0.14 to 0.3
K (%)	1.03 ± 0.07	0.55 ± 0.11	1.2 to 2.0
Fe (ppm)	49.30 ± 1.15	41.50 ± 2.36	30 to 100
Mn (ppm)	120.30 ± 25.12	90.67 ± 6.02	25 to 1,000
Zn (ppm)	57.00 ± 1.13	59.34 ± 4.71	25
Cu (ppm)	8.70 ± 0.52	8.43 ± 0.52	5 to 15

^x Means represent 12 vine plots and 3 replications for each cultivar, values within columns represent means ± std dev.

^y Sufficiency ranges reported from (Bates and Wolf 2008).

Table 3.2. Nutrient content in petioles of Norton grapevines collected at veraison in Mountain Grove, MO and Rocheport, MO in 2013.^x

Nutrient	MG Norton 2013	RO Norton 2013	Sufficiency Ranges ^y
N (%)	0.97 ± 0.04	0.68 ± 0.03	0.8 to 1.2
Ca (%)	2.09 ± 0.08	2.60 ± 0.15	1.3 to 2.5
Mg (%)	1.34 ± 0.12	0.24 ± 0.02	0.35 to 0.75
P (%)	0.15 ± 0.01	0.71 ± 0.05	0.14 to 0.3
K (%)	0.55 ± 0.11	2.64 ± 0.04	1.2 to 2.0
Fe (ppm)	41.50 ± 2.36	26.53 ± 1.05	30 to 100
Mn (ppm)	90.67 ± 6.02	213.17 ± 50.35	25 to 1,000
Zn (ppm)	59.34 ± 4.71	67.00 ± 5.52	25
Cu (ppm)	8.43 ± 0.52	5.87 ± 0.31	5 to 15

^x Means represent 12 vine plots and 3 replications, values within columns represent means ± std dev.

^y Sufficiency ranges reported from (Bates and Wolf 2008).

Table 3.3. Vegetative characteristics of Norton and Cabernet Sauvignon grapevines in Mountain Grove, MO in 2012 and 2013.^w

Variable	----Norton----		----Cabernet Sauvignon----	
	2012	2013	2012	2013
Pruning wt. (kg) ^x	0.53 ± 0.22	0.36 ± 0.24	1.10 ± 0.43	2.10 ± 0.72
No. of nodes retained/vine	23.0 ± 9.55	16.0 ± 10.66	49.0 ± 19.16	92.6 ± 31.73
ECSA (m ² /ha) ^y	1.2x10 ⁶ ± 9.2x10 ⁴	1.2x10 ⁶ ± 1.3x10 ⁵	ND ^z	ND

^w Means represent 12 vine plots and 3 replications.

^x Pruning weight of one year-old canes recorded in March following growing season.

^y ECSA - Exposed Canopy Surface Area.

^z ND- no data collected.

Table 3.4. Fruiting characteristics of Norton grapevines at harvest in Mountain Grove, MO in 2012 and 2013.

	2012	2013
Yield (kg/vine) ^y	7.91 ± 2.33	8.12 ± 2.26
Clusters/vine ^y	124.51 ± 32.06	125.67 ± 31.53
Cluster wt. (g) ^y	60.74 ± 11.28	66.41 ± 16.15
Berry wt. (g) ^y	0.97 ± 0.07	1.05 ± 0.00
Berries/cluster ^z	62.76 ± 6.39	63.31 ± 3.15

^x Means represent 12 vine plots and 3 replications, values within columns represent means ± standard deviation.

^y Cluster wt. = average weight per replication. Reported fruiting characteristics of Norton on a non-divided canopy system for yield, clusters/vine, and cluster weight are 8.0 kg/vine, 139 clusters/vine, and 58.5 g, respectively (Morris and Main 2010).

^z Berry wt. = individual berry fruit weight per replication.

Table 3.5. Fruiting characteristics of Norton grapevines at harvest in Mountain Grove, MO, and Rocheport, MO in 2013.^x

	Mountain Grove	Rocheport
Yield (kg/vine) ^y	8.12 ± 2.26	21.22 ± 3.94
Clusters/vine ^y	125.67 ± 31.53	281.31 ± 62.98
Cluster wt. (g) ^y	66.41 ± 16.15	76.99 ± 13.20
Berry wt. (g) ^z	1.05 ± 0.00	1.16 ± 0.01
Berries/cluster	63.31 ± 3.15	66.25 ± 2.28
Cordon length (cm)	243.06 ± 35.62	441.53 ± 60.98

^x Means represent 12 vine plots and 3 replications, values within columns represent means ± std.

^y Cluster wt. = average weight per replication. Reported fruiting characteristics of Norton on a non-divided canopy system for yield, clusters/vine, and cluster weight are 8.0 kg/vine, 153 clusters/vine, and 58.5 g, respectively (Morris and Main 2010).

^z Berry wt. = individual berry fruit weight per replication.

Table 3.6. Enhanced Point Quadrat Analysis (EQPA) and Calibrated Exposure Mapping (CEM) tools on the canopy of Norton and Cabernet Sauvignon grapevines at veraison and harvest expressed by traditional metrics and calibrated flux metrics in Mountain Grove, MO in 2013.^a

	-----Norton-----		-----Cabernet Sauvignon-----	
	2012	2013	2012	2013
	Veraison	Harvest	Veraison	Harvest
Traditional metrics				
PG ^b	2.37 ± 1.90	5.48 ± 1.41	4.87 ± 1.57	6.03 ± 1.74
LLN ^b	1.98 ± 0.08*	1.77 ± 0.20*	2.78 ± 0.18*	2.23 ± 0.06*
PIL ^b	26.83 ± 2.03	23.00 ± 5.04*	39.33 ± 2.53	26.80 ± 1.61*
PIC ^b	45.18 ± 3.31*	45.03 ± 5.31*	68.93 ± 4.02*	69.52 ± 7.32*
Calibrated flux metrics				
EP ₁ ^b	0.24 ± 0.14	0.20 ± 0.06	0.29 ± 0.02	0.26 ± 0.03
CEFA ^b	0.27 ± 0.16	0.40 ± 0.06	0.20 ± 0.15	0.31 ± 0.05
CEFS ^b	0.05 ± 0.07*	0.10 ± 0.09	-0.14 ± 0.14*	-0.03 ± 0.25
LEFA ^b	0.45 ± 0.01	0.49 ± 0.05	0.41 ± 0.01	0.48 ± 0.01
LEFS ^b	-0.02 ± 0.02*	-0.02 ± 0.05	0.03 ± 0.04*	0.03 ± 0.06

^a Means represent 12 vine plots and 3 replications, values within columns represent means ± std. Reported from (Smart & Robinson 1991).

^b PG: percent gaps; LLN: leaf layer number; PIL: percent interior leaves; PIC: percent interior clusters; EP₁: canopy calibration coefficient; CEFA: cluster exposure flux availability; CEFS: cluster exposure flux symmetry; LEFA: leaf exposure flux availability; LEFS: leaf exposure flux symmetry.

* Asterisk (*) indicates significant difference in metrics between Norton and Cabernet Sauvignon.

Table 3.7. Enhanced Point Quadrat Analysis (EQPA) and Calibrated Exposure Mapping (CEM) tools on the canopy of Norton grapevines at veraison and harvest expressed by traditional metrics and calibrated flux metrics. Grapes were located in Mountain Grove, MO, and Rocheport, MO in 2013.^a

	MG Norton	MG Norton	RO Norton	RO Norton
	Veraison	Harvest	Veraison	Harvest
Traditional Metrics				
PG ^b	2.37 ± 1.90	5.48 ± 1.41	3.73 ± 1.02	3.61 ± 0.92
LLN ^b	1.98 ± 0.08*	1.77 ± 0.20*	2.15 ± 0.05*	2.72 ± 0.18*
PIL ^b	26.83 ± 2.03	23.00 ± 5.04*	26.74 ± 1.01	38.39 ± 3.66*
PIC ^b	45.18 ± 3.31*	45.03 ± 5.31*	66.11 ± 2.84*	74.02 ± 0.33*
Calibrated flux metrics				
EP ₁ ^b	0.24 ± 0.14	0.20 ± 0.06	0.13 ± 0.04	0.29 ± 0.02
CEFA ^b	0.27 ± 0.16	0.40 ± 0.06	0.22 ± 0.01	0.26 ± 0.01
CEFS ^b	0.05 ± 0.07*	0.10 ± 0.09	0.32 ± 0.10*	0.20 ± 0.02
LEFA ^b	0.45 ± 0.01	0.49 ± 0.05	0.42 ± 0.01	0.41 ± 0.03
LEFS ^b	-0.02 ± 0.02*	-0.02 ± 0.05	-0.08 ± 0.01*	-0.05 ± 0.00

^a Means represent 12 vine plots and 3 replications, values within columns represent means ± std dev. Reported from Smart & Robinson 1991.

^b PG: percent gaps; LLN: leaf layer number; PIL: percent interior leaves; PIC: percent interior clusters; EP₁: canopy calibration coefficient; CEFA: cluster exposure flux availability; CEFS: cluster exposure flux symmetry; LEFA: leaf exposure flux availability; LEFS: leaf exposure flux symmetry.

* indicates a significant difference in the metrics between cultivar locations using PROC GLM.

Table 3.8. Grape berry physical components of Norton and Cabernet Sauvignon grapevines on a mg/g skin wt. to mg/g berry wt. in Mountain Grove, MO in 2012 to 2013, and Rocheport, MO in 2013.^x

Stage	Anthocyanins		Total Phenolics		Tannins	
-----Mg/g berry weight-----						
Norton 2012						
1	0.00	0.00	8.53	0.93	4.04	0.44
2	0.00	0.00	6.24	0.94	3.73	0.56
3	0.13	0.02	7.93	1.18	3.10	0.46
4	1.32	0.22	5.41	0.89	3.20	0.53
5	7.07	1.18	8.04	1.35	3.01	0.50
6	10.10	1.20	10.04	1.19	3.36	0.40
Norton 2013						
1	0.00	0.00	6.20	0.62	1.07	0.11
2	0.00	0.00	6.79	0.74	1.29	0.14
3	0.11	0.02	6.09	1.05	1.22	0.21
4	5.65	0.75	9.37	1.25	2.24	0.30
5	10.43	1.18	11.79	1.33	2.79	0.32
6	12.58	1.83	10.36	1.50	2.79	0.40
Norton 2013 RO						
1	0.00	0.00	2.04	0.15	0.16	0.01
2	0.00	0.00	4.05	0.49	0.74	0.09
3	0.12	0.02	4.26	0.69	1.02	0.17
4	3.89	0.51	6.08	0.79	1.17	0.15
5	10.38	1.39	9.84	1.31	2.42	0.32
6	12.80	1.47	12.19	1.40	2.50	0.29
Cabernet Sauvignon 2012						
1	0.00	0.00	22.24	2.34	12.83	1.35
2	0.00	0.00	23.37	2.80	11.69	1.40
3	0.13	0.01	11.75	1.18	5.28	0.53
4	3.36	0.29	10.06	0.88	4.66	0.41
5	3.96	0.37	12.91	1.19	5.76	0.53
6	3.72	0.35	11.06	1.04	4.33	0.41
Cabernet Sauvignon 2013						
1	0.00	0.00	20.35	2.06	9.03	0.91
2	0.00	0.00	18.18	2.22	9.33	1.14
3	0.25	0.03	13.60	1.83	7.62	1.03
4	3.93	0.46	8.04	0.93	2.81	0.33
5	4.59	0.59	11.14	1.44	4.42	0.57
6	3.55	0.49	11.34	1.56	4.52	0.62

^x Means represent 12 vine plots and 3 replications, values within columns.

Figure 3.1. Changes in levels of soluble solids (°Brix) in Norton (A) and Cabernet Sauvignon (B) grape berries throughout 2012 and 2013 at Mountain Grove, MO. Soluble solid levels for Norton were determined across 6 developmental stages from stage 1(42 and 37 days after flowering (DAF) for 2012 and 2013, respectively) to stage 6 (106 and 120 DAF for 2012 and 2013, respectively). Soluble solid levels for Cabernet were quantified for 2012-2013 from stage 1 (41 and 38 DAF) to stage 6 (101 and 107 DAF). Means followed by the same letter throughout stages by year are not significantly different by Fisher's Protected LSD at $p=0.05$.

Figure 3.2. Changes in levels of citric acid in Norton (A) and Cabernet Sauvignon (B) grape berries throughout 2012 and 2013 at Mountain Grove, MO. Acid levels for Norton were determined across 6 developmental stages from stage 1(42 and 37 days after flowering (DAF) for 2012 and 2013, respectively) to stage 6 (106 and 120 DAF for 2012 and 2013, respectively). Acid levels for Cabernet were quantified for 2012-2013 from stage 1 (41 and 38 DAF) to stage 6 (101 and 107 DAF). Means followed by the same letter throughout stages by year are not significantly different by Fisher's Protected LSD at $p=0.05$.

Figure 3.3. Changes in levels of tartaric acid in Norton (A) and Cabernet Sauvignon (B) grape berries throughout 2012 and 2013 at Mountain Grove, MO. Acid levels for Norton were determined across 6 developmental stages from stage 1(42 and 37 days after flowering (DAF) for 2012 and 2013, respectively) to stage 6 (106 and 120 DAF for 2012 and 2013, respectively). Acid levels for Cabernet were quantified for 2012-2013 from stage 1 (41 and 38 DAF) to stage 6 (101 and 107 DAF). Means followed by the same letter throughout stages by year are not significantly different by Fisher's Protected LSD at $p=0.05$.

Figure 3.4. Changes in levels of malic acid in Norton (A) and Cabernet Sauvignon (B) grape berries throughout 2012 and 2013 at Mountain Grove, MO. Acid levels for Norton were determined across 6 developmental stages from stage 1(42 and 37 days after flowering (DAF) for 2012 and 2013, respectively) to stage 6 (106 and 120 DAF for 2012 and 2013, respectively). Acid levels for Cabernet were quantified for 2012-2013 from stage 1 (41 and 38 DAF) to stage 6 (101 and 107 DAF). Means followed by the same letter throughout stages by year are not significantly different by Fisher's Protected LSD at $p=0.05$.

Figure 3.5. Changes in levels of anthocyanins in Norton (A) and Cabernet Sauvignon (B) grape berries throughout 2012 and 2013 at Mountain Grove, MO. Anthocyanin levels for Norton were determined across 6 developmental stages from stage 1(42 and 37 days after flowering (DAF) for 2012 and 2013, respectively) to stage 6 (106 and 120 DAF for 2012 and 2013, respectively). Anthocyanin levels for Cabernet were quantified for 2012-2013 from stage 1 (41 and 38 DAF) to stage 6 (101 and 107 DAF). Means followed by the same letter throughout stages by year are not significantly different by Fisher's Protected LSD at $p=0.05$.

Figure 3.6. Changes in levels of total phenolics in Norton (A) and Cabernet Sauvignon (B) grape berries throughout 2012 and 2013 at Mountain Grove, MO. Total phenolic levels for Norton were determined across 6 developmental stages from stage 1(42 and 37 days after flowering (DAF) for 2012 and 2013, respectively) to stage 6 (106 and 120 DAF for 2012 and 2013, respectively). Total phenolic levels for Cabernet were quantified for 2012-2013 from stage 1 (41 and 38 DAF) to stage 6 (101 and 107 DAF). Means followed by the same letter throughout stages by year are not significantly different by Fisher's Protected LSD at $p=0.05$. Means without letters are not significantly different within a stage.

Figure 3.7. Changes in levels of tannins in Norton (A) and Cabernet Sauvignon (B) grape berries throughout 2012 and 2013 at Mountain Grove, MO. Tannins levels for Norton were determined across 6 developmental stages from stage 1(42 and 37 days after flowering (DAF) for 2012 and 2013, respectively) to stage 6 (106 and 120 DAF for 2012 and 2013, respectively). Tannin levels for Cabernet were quantified for 2012-2013 from stage 1 (41 and 38 DAF) to stage 6 (101 and 107 DAF). Means followed by the same letter throughout stages by year are not significantly different by Fisher's Protected LSD at $p=0.05$. Means without letters are not significantly different within a stage.

Figure 3.8. Monthly rainfall for April to September 2012 and 2013 with historical average precipitation (1971-2000) in Mountain Grove, Missouri.

Figure 3.9. Average daily temperature, historic (1971-2000) average daily temperature, and precipitation from April to September 2012-2013 for Mountain Grove, Missouri.

Figure 3.10. Monthly rainfall for April to September 2013 with historical average precipitation (1971-2000) in Rocheport, Missouri.

Figure 3.11. Average daily temperature, historic (1971-2000) average daily temperature, and precipitation from April to September 2013 for Boone County, Rocheport, Missouri.

APPENDIX

Table A1. Soil characteristics of Norton grapevines in Mountain Grove, MO and Rocheport, MO in 2013.

	MG	RO	Sufficiency Ranges ^x
pHs	6.7	6.9	6.0
O.M. (%)	3.2	5.3	3 to 5
P (ppm)	28	123	20 to 50
Ca (ppm)	940	1898	500 to 2000
Mg (ppm)	210	116	150 to 250
K (ppm)	75	275	75 to 100
CEC (meg/100g ^y	6.6	11.2	5 to 20 ^y

^x Sufficiency ranges reported from (Bates and Wolf 2008).

^y Sufficiency range on silt loam reported from Dami et. al. (2005).

Table A2. Grape berry physical components of Norton and Cabernet Sauvignon grapevines in Mountain Grove, MO in 2012 and 2013.^x

Norton 2012					
Stages	Diameter (cm) ^y	Pulp (g) ^y	Skin (g) ^y	Whole Berry (g) ^y	110 Berries (g) ^z
1	0.90 ± 0.02	0.65 ± 0.42	0.05 ± 0.05	0.50 ± 0.44	70.41 ± 3.60
2	0.57 ± 0.50	0.47 ± 0.41	0.09 ± 0.08	0.58 ± 0.50	58.29 ± 1.80
3	1.04 ± 0.07	0.78 ± 0.07	0.15 ± 0.02	0.98 ± 0.06	88.05 ± 1.75
4	1.11 ± 0.11	0.72 ± 0.03	0.15 ± 0.02	0.90 ± 0.08	95.17 ± 3.05
5	1.07 ± 0.11	0.64 ± 0.20	0.15 ± 0.04	0.89 ± 0.19	95.84 ± 3.05
6	1.12 ± 0.04	0.91 ± 0.17	0.12 ± 0.01	1.00 ± 0.05	100.46 ± 2.79
Norton 2013					
Stages	Diameter (cm) ^y	Pulp (g) ^y	Skin (g) ^y	Whole Berry (g) ^y	110 Berries (g) ^z
1	0.86 ± 0.21	0.57 ± 0.03	0.07 ± 0.02	0.69 ± 0.03	72.54 ± 1.92
2	0.86 ± 0.05	0.71 ± 0.02	0.09 ± 0.01	0.83 ± 0.02	90.58 ± 2.04
3	0.86 ± 0.05	0.79 ± 0.10	0.14 ± 0.01	0.82 ± 0.13	91.23 ± 3.63
4	1.20 ± 0.26	0.83 ± 0.14	0.15 ± 0.01	1.12 ± 0.11	121.00 ± 12.65
5	1.12 ± 0.03	0.87 ± 0.17	0.13 ± 0.03	1.15 ± 0.11	115.01 ± 0.55
6	1.00 ± 0.02	0.75 ± 0.10	0.15 ± 0.06	1.06 ± 0.01	113.79 ± 4.86
Cabernet Sauvignon 2012					
Stages	Diameter (cm) ^y	Pulp (g) ^y	Skin (g) ^y	Whole Berry (g) ^y	110 Berries (g) ^z
1	0.95 ± 0.07	0.77 ± 0.09	0.09 ± 0.03	0.89 ± 0.07	90.98 ± 2.67
2	1.01 ± 0.06	0.84 ± 0.05	0.11 ± 0.01	0.96 ± 0.01	99.70 ± 0.96
3	1.18 ± 0.02	0.76 ± 0.13	0.10 ± 0.02	0.95 ± 0.05	97.16 ± 2.83
4	1.32 ± 0.01	1.48 ± 0.10	0.15 ± 0.02	1.68 ± 0.05	166.54 ± 3.01
5	1.33 ± 0.05	1.51 ± 0.04	0.16 ± 0.05	1.69 ± 0.08	168.28 ± 6.86
6	1.33 ± 0.06	1.45 ± 0.11	0.16 ± 0.07	1.72 ± 0.04	181.66 ± 1.06
Cabernet Sauvignon 2013					
Stages	Diameter (cm) ^y	Pulp (g) ^y	Skin (g) ^y	Whole Berry (g) ^y	110 Berries (g) ^z
1	1.01 ± 0.05	0.81 ± 0.10	0.10 ± 0.02	0.99 ± 0.17	88.28 ± 2.98
2	1.26 ± 0.17	0.91 ± 0.08	0.14 ± 0.05	1.12 ± 0.07	118.27 ± 2.51
3	1.00 ± 0.04	0.74 ± 0.24	0.15 ± 0.06	1.14 ± 0.32	114.08 ± 4.05
4	1.03 ± 0.17	1.03 ± 0.41	0.17 ± 0.04	1.51 ± 0.10	162.31 ± 1.63
5	1.29 ± 0.12	0.99 ± 0.13	0.19 ± 0.02	1.49 ± 0.06	164.84 ± 5.02
6	1.13 ± 0.18	0.99 ± 0.30	0.22 ± 0.01	1.61 ± 0.04	171.01 ± 2.53

^x Means represent 12 vine plots and 3 replications, values within columns represent means ± std.

^y Means represent 5 berries per vine, 60 total berries per replication.

^z Means represent 110 berries per vine, 1,320 berries per replication.

Table A3. Grape berry physical components of Norton grapevines from Mountain Grove, MO and Rocheport, MO in 2013.^x

	Norton 2013 MG	Norton 2013 RO
Stage	Diameter (cm) ^y	Diameter (cm) ^y
1	0.86 ± 0.21	1.01 ± 0.06
2	0.86 ± 0.05	0.98 ± 0.07
3	0.86 ± 0.05	1.17 ± 0.14
4	1.20 ± 0.26	1.18 ± 0.19
5	1.12 ± 0.03	1.03 ± 0.29
6	1.00 ± 0.02	1.33 ± 0.02
Stage	Pulp (g) ^y	Pulp (g) ^y
1	0.57 ± 0.03	0.79 ± 0.07
2	0.71 ± 0.02	0.78 ± 0.07
3	0.79 ± 0.10	1.10 ± 0.19
4	0.83 ± 0.14	1.01 ± 0.21
5	0.87 ± 0.17	1.00 ± 0.13
6	0.75 ± 0.10	0.95 ± 0.06
Stage	Skin (g) ^y	Skin (g) ^y
1	0.07 ± 0.02	0.07 ± 0.01
2	0.09 ± 0.01	0.11 ± 0.04
3	0.14 ± 0.01	0.19 ± 0.03
4	0.15 ± 0.01	0.16 ± 0.02
5	0.13 ± 0.03	0.17 ± 0.05
6	0.15 ± 0.06	0.14 ± 0.02
Stage	Whole Berry (g) ^y	Whole Berry (g) ^y
1	0.69 ± 0.03	0.93 ± 0.09
2	0.83 ± 0.02	0.95 ± 0.08
3	0.82 ± 0.13	1.19 ± 0.04
4	1.12 ± 0.11	1.22 ± 0.09
5	1.15 ± 0.11	1.29 ± 0.13
6	1.06 ± 0.01	1.22 ± 0.07
Stage	110 Berries (g) ^z	110 Berries (g) ^z
1	72.54 ± 1.92	96.15 ± 2.05
2	90.58 ± 2.04	103.24 ± 6.00
3	91.23 ± 3.63	114.28 ± 11.84
4	121.00 ± 12.65	124.82 ± 4.64
5	115.01 ± 0.55	126.70 ± 3.73
6	113.79 ± 4.86	130.19 ± 1.12

^x Means represent 12 vine plots and 3 replications, values within columns represent means ± std.

^y Means represent 5 berries per vine, 60 total berries per replication.

^z Means represent 110 berries per vine, 1,320 berries per replication.

Table A4. Changes in levels of pH in Norton (A) and Cabernet Sauvignon (B) grape berries throughout 2012 and 2013 at Mountain Grove, MO. pH levels for Norton were determined across 6 developmental stages from stage 1(42 and 37 days after flowering (DAF) for 2012 and 2013, respectively) to stage 6 (106 and 120 DAF for 2012 and 2013, respectively). pH levels for Cabernet were quantified for 2012-2013 from stage 1 (41 and 38 DAF) to stage 6 (101 and 107 DAF). Means followed by the same letter throughout stages by year are not significantly different by Fisher's Protected LSD at $p=0.05$.