Abstract

BRADISH, CHRISTINE MERRILL. Measured Variation in Flavonoid Composition of North Carolina Red Raspberry through Targeted Metabolite Profiling. (Under the direction of Dr. Gina Fernandez and Dr. Penelope Perkins-Veazie.)

Polyphenols in raspberry and other berry crops contain powerful antioxidants, and consumption of these compounds may help prevent and/or moderate chronic diseases. Targeted metabolite profiling methods are useful in identification of key compounds that contribute to antioxidant properties and human health benefits, and for breeding tailored functional foods. In these studies, metabolomic variation was determined among three fall-fruiting red raspberry cultivars ('Autumn Britten', 'Caroline', and 'Nantahala') grown at three North Carolina locations differing in temperature (harvest season max/min averages of 32°/21°, 28°/16°, 24°/14° C) and elevation (214, 630, 917 m above sea level). 'Nantahala' is a recent release from the Rubus breeding program at North Carolina State University, and was specifically bred for the mountainous regions of the southeastern United States.

Ten flavonoid compounds were detected in samples from all cultivars and locations grown under polytunnel cultivation by targeted metabolite analysis using liquid chromatography-time of flight-mass spectrometry (LC-TOF-MS). LC-MS is accurate to 50-100ppm and a mass range of less than 1500 Da, and is useful for identifying and quantifying a wide range of polar compounds; however analysis can be costly. Of those compounds, cyanidin-3-glucoside, cyanidin-3-sophoroside, cyanidin-3-rutinoside, cyanidin-3-sambubioside, and quercetin-3-glucoside were quantified with external standards. Variation in flavonoid composition was primarily due to genotype, and associated with locations with varying night temperatures and hours of exposure to temperatures over 29°C. Multivariate analysis using principal components analysis showed clear separation among cultivars, and a

characteristic flavonoid compound was associated with each cultivar in the corresponding loading plot. 'Caroline' was highest in cyanidin-3-sophoroside, 'Autumn Britten' was highest in cyanidin-3-rutinoside, and 'Nantahala' was highest in cyanidin-3-sambubioside, indicative of its purple raspberry lineage. Quercetin-3-glucoside levels increased the most with elevated temperatures.

Assays analyzing total anthocyanins, total phenolics, and Ferric Reducing Antioxidant Power (FRAP) of samples were used to determine cultivar, location, cultivation, harvest date, and temperature effects in all collected samples. Total anthocyanins, total phenolics, and antioxidant capacity of fruit samples were positively correlated, with differences among samples attributed to cultivar and location effects. 'Caroline' had the highest antioxidant capacity and phenolic content among the three cultivars. Within each cultivar, elevated levels of total phenolics and antioxidant capacity were found at the two warmest harvest locations.

In a second study, high performance liquid chromatography (HPLC) was used to compare the same samples from all grown in both field and polytunnel cultivation. HPLC is a less expensive approach in comparison to LC-MS, and works well to characterize anthocyanins; however it is limited in its ability to distinguish among compounds with similar retention times or absorbance peaks. As was seen previously, variation in flavonoid composition was primarily attributed to genotype. Eight anthocyanins were measured in 'Autumn Britten' and 'Caroline', and four were measured in 'Nantahala' at 520nm. Two ellagitannins were measured in all samples at 280nm. Peaks were quantified as cyanidin-3-glucoside equivalents and gallic acid equivalents, respectively, and identified by comparison to previous studies.

Specific flavonoid quantification versus total anthocyanin and total phenolic trends indicate that genetic and environmental effects vary starting with individual metabolite composition and working up to the whole plant level. The broad fluctuations in the berry metabolome due to genetic and environmental factors must be realized by health researchers, as the health benefits could be altered due to these fluctuations. Changes in flavonoid content must be accounted for or noted by health researchers, and metabolomic techniques allow for the tracking of these changes. Additionally, these methods and results can be utilized in breeding programs to identify key metabolites contributing to antioxidant properties and health benefits for humans, and further understanding and enhancement of nutritional compounds in berry fruits.

Measured Variation in Flavonoid Composition of North Carolina Red Raspberry through Targeted Metabolite Profiling

by
Christine Merrill Bradish

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APPROVED BY:

_____	_____
Allan Brown, PhD	Wei Jia, PhD
_____	_____
Gina Fernandez, PhD Co-chair of Advisory Committee	Penelope Perkins-Veazie, PhD Co-chair of Advisory Committee

Dedication

To my Aunt Carol, who has persevered in her educational journey despite any obstacles that come her way (even Algebra!). Keep up the good work, and you are an inspiration to many each and every day.

To my little sister Molly, keep fighting, and thanks for reminding me to have fun. I love the artwork ☺

Biography

Christine Merrill Bradish was raised in Smithfield, Virginia. Growing up surrounded by agriculture and wetlands, she gained an interest and respect for nature, and spent a great amount of time outdoors playing in the woods and fishing and boating with her father and her sister. Always interested in learning, she worked diligently in grade school, graduating Salutatorian of her high school class. She received a full merit scholarship to Old Dominion University in Norfolk, VA for her undergraduate studies, and chose to study plant sciences after taking several influential biology courses. She earned a Bachelor's of Science with a major in Biological Sciences and a minor in Applied Mathematics, graduating Summa cum Laude. Christine worked as a produce clerk at a local grocery store, gaining a firsthand interest in horticultural crops and postharvest biology. She was accepted into the graduate program in horticultural science at North Carolina State University, as the first Master of Science student associated with the Kannapolis North Carolina Research Campus. She then received a Kannapolis Scholars Transdisciplinary Training Program in Functional Foods Fellowship, sponsored by the USDA. This fellowship requires a research project be conducted that actively engages multiple departments, universities, and/or specialties. Christine developed a transdisplinary project that combined metabolomics and horticultural science, researching raspberry nutritional compounds at the North Carolina Research Campus in Kannapolis engaging the NC State Plants for Human Health Institute and UNC Greensboro Center for Research Excellence in Bioactive Food Components. Her research poster received first place at the 2011 Berry Health Benefits Symposium in Westlake Village,

CA. Also during her graduate education at NC State, she found a true passion for the art and science behind plant breeding and agricultural research, and a deep appreciation for the connection between fruit and vegetable consumption and health. Her ultimate career goal is to work as a fruit breeder, and to collaborate with health researchers to develop functional foods through an understanding of breeding and genetics that may one day be the answer to the treatment and prevention of chronic diseases.

Acknowledgements

To my advisors Penny Perkins-Veazie and Gina Fernandez, I cannot adequately express in writing the gratitude that I have for everything you two have done for me over the past two years. Working with you has opened doors and led me down paths I could only dream of going down before now. You have given me opportunities to learn, travel, and experience; and have been amazing teachers and mentors.

Penny, before working in postharvest I never thought that my job for so many years as a produce clerk would be an important contributing experience to my studies. It has been amazing to see and contribute to the research and development that goes into marketing fresh fruits and vegetables. Additionally, working with nutritional compounds in berries has really opened my eyes to the important connections between diet and health, and I definitely want to continue my research in this.

Gina, I am so glad that you have exposed me to the world of small fruits breeding. Plant breeding is the perfect combination of my love for fieldwork, math, and molecular laboratory techniques, and I feel that I have truly found something I can happily make a career of. I am so glad to be a part of a breeding program that is so important with the increasing market for berries in the east, and with the possibility of climate change that will require more and more plants to be acclimated to warmer temperatures.

Thank you both so much for always being available to answer questions and provide moral support. Additionally, thank you for supporting me and providing me the opportunity

to travel to conferences, research stations, and educational events. Getting exposure to the professional world and being able to present my research publicly has been amazing for my confidence, not to mention that I have now been to wonderful places I would have never made it to on my own.

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office and to keeping each other motivated and healthy. Hopefully we can be grumpy old professors together one day.

Thank you to my parents, Bryan and Karen Bradish. Your continued love and guidance have made this all possible for me; and I respect and care about you more and more each day. To Molly, I could not ask for a better sister, and I am so glad to have you and the love and support we have given each other throughout our lives. To my boyfriend Joshua, without your continued encouragement and unconditional love for me I would not be half as successful as I am today. To Miss Susan, I am so happy to have your love and support, and I am thankful to have you as part of my extended family. Even though I am a state away, I will be there for you now and in the future, and I am thinking of you and your health daily.

My faith has kept me strong, and God has led me wisely to my chosen vocation. I am ever grateful to Father David and Sherran Lasalle; you are like family to me, and your endless stewardship and support has greatly influenced the person I am today. I am truly blessed.

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Literature Review

Introduction

Red raspberries (*Rubus idaeus* L.) are an important perennial fruit crop, grown for both fresh and processing markets. Recently, raspberries and other berry fruits have gained popularity with health researchers and health-savvy consumers because of their flavonoid content and high antioxidant capacity, as regular consumption of fruits and vegetables high in these compounds is associated with the prevention and treatment of chronic disease (Kassim et.al., 2009). Flavonoid levels and antioxidant capacity can be influenced by many genotypical and environmental factors, including cultivar, growing region, soil, irrigation, sunlight, and cultivation system (Anttonen et.al., 2005; Freeman et.al., 2011; Kafkas et.al., 2008; Kahkonen et.al., 2003; Ozgen et.al., 2008). Metabolomics is a relatively new field of study that has been introduced to the plant sciences as a way to measure chemical composition, and to distinguish the sources of variation in composition (Fukusaki & Kobayashi, 2005). It has great potential for measuring chemical composition of berry fruits, and determining the differences causing fluctuations in flavonoid levels and antioxidant capacity.

Crop Background

Raspberries (*Rubus idaeus* L.) belong to *Rosaceae* (Pritts & Handley, 1989) and *Rubus*, which includes blackberries, arctic berries, and several ornamental species (Jennings, 1988). *Rubus* is very diverse, and is divided into 12 subgenera; four of these which contain valuable fruit crops (Deighton et.al., 2000). The raspberry subgenus, *Idaeobatus*, is classified by fruit

that separate from the receptacle when mature. There are over 200 species of raspberries found and cultivated in North America, Europe, and Asia (Jennings, 1988). Raspberries can be red (*R. idaeus*), yellow (*R. idaeus)*, purple (*R. neglectus)*, or black (*R. occidentalis)*. *Rubus idaeus* subsp. *vulgatus* is the European subspecies of red raspberry, and *Rubus idaeus* subsp. *strigosus* is the American subspecies (Tokuşoğlu & Stoner, 2011).

Plant Description

Raspberry plants have a perennial root system and grow biennial canes that can be erect, semi-erect, or trailing (Pritts & Handley, 1989). Raspberry cultivars are classified as floricane (summer) or primocane (fall) fruiting (Weber, 2006). Floricane varieties flower and fruit on lateral shoots of second year canes, and primocane varieties fruit on the terminal ends of first year canes that switch from vegetative to reproductive growth after reaching a certain number of nodes. These primocane varieties are also known as 'everbearing' (Fernandez et.al., 1998). Primocane fruiters are typically grown only for the fall crop, but if left to grow, primocane fruiting varieties will produce a low-yielding early summer crop on the second-year canes, in addition to the fall crop on the first-year canes (Fernandez & Krewer, 2008).

Fruit Description

Raspberries are an aggregate fruit composed of many drupelets adhered together in a round to conical shape (Hui, 2006; Tokuşoğlu & Stoner, 2011). An aggregate fruit has many ovaries contained in a single flower, (Kays & Paull, 2004; Pritts & Handley, 1989) and a

drupe is a fruit that develops from a single ovary, and has one seed inside. Basically, every drupelet is its own fruit, composed of several hundred cells, with its own vascular supply for nutrients and water. Hairs arise from trichomes in the epidermal cells, and these hairs help the drupelets stick together. The fleshy drupelet has parenchyma cells towards the middle, oval shaped cells towards the outside, and one to three layers of supportive collenhyma cells under the epidermis (Jennings, 1988; Iannetta et al., 1999; Stewart et al., 2001). During ripening, abscission layers form between each drupelet and the receptacle (Jennings, 1988). The fruit is hollow because of separation from the receptacle at harvest (Hui, 2006).

Figure 1.1 The aggregate fruit of red raspberry (*Rubus idaeus* L.)

Development and Ripening

Development in raspberry has three distinct stages, typical of drupe fruits, and each stage is 10-12 days long. The first and last stages show accelerated growth due to mitosis and cell enlargement, respectively; and the second stage shows little growth as the embryo and seed develop (Jennings, 1988). There is a steady increase in fresh weight and a decrease in percent dry weight throughout development, except in the second stage when seed

maturation occurs (Iannetta et. al., 1999). After the seed matures, the fruit undergoes the irreversible processes of ripening and senescence, accompanied by changes in texture, flavor, pigments, and cellular composition (Graham et al., 2009; Kays & Paull, 2004). There is a fine line between ripening and senescence. Both are genetically regulated, ripening being more associated with the characteristic eating quality of a fruit and senescence more associated with cell death and quality degradation (Brady, 1987). Raspberry ripening occurs between 30 and 36 days after pollination, and is dependent on genetic and environmental factors. For example, in wild raspberry populations ripening occurs later than in some domestic varieties, and is further influenced by altitude (Graham et. al., 2009; Jennings, 1988). Gene up-regulation, enzyme formation, molecules available for respiration, and energy are all required for ripening to take place (Kays & Paull, 2004).

Color and Flavor Development

Color is one of the best external indicators of ripeness and freshness in raspberry (deAncos et.al., 1999). During ripening, changes in color occur due to chlorophyll degradation and pigment assembly. Chlorophyll loss is controlled by up-regulation of genes coding for the degradation enzymes chlorophyllase, chlorophyll oxidase, and chlorophyll peroxidase. Ethylene plays a part by inducing gene expression for chlorophyllase synthesis and abscission cell formation (Kays & Paull, 2004). The main color pigments in raspberry are anthocyanidins, a class of water-soluble flavonoid phenolics (Hui, 2006). Phenolics are important to the ripening process, not only for color development, but also for

polymerization of lignin and volatile production for flavor (Graham et al., 2009). The anthocyanins cyanidin (with glycosides of sophorose, glucose, glucuorutinose, and rutinose), and pelargonidin (with glycosides of sophorose and glucose) are the most common types of pigments found in red raspberry (Hui, 2006). Anthocyanin collection in the cell vacuole throughout ripening is controlled and affected by light intensity, quantity, and up-regulation of genes dictating structure synthesis of pigments. Recently, QTL for transcription factors regulating the phenylpropanoid pathway and anthocyanin production have been located on chromosomes 1 and 3 (Graham et. al., 2009). In raspberry, anthocyanins are present at high concentrations throughout the fruit (Kays & Paull, 2004). Red raspberries intended for the fresh market have the best fruit quality when harvested at the bright red stage (Tokuşoğlu & Stoner, 2011). Red fruit color is not only an indication of maturity and fruit quality to the consumer, but also functions as an attractant to bird and mammal seed dispersers (Steyn, 2009).

Raspberry fruits are soft and juicy, and have a distinct flavor and aroma (Wang et.al., 2009). Flavor is a combination of taste and aroma, and changes in both are necessary for full characteristic raspberry flavor. Changes in taste occur with increasing sugars and decreasing acidity in the fruit, and both of these components are indispensable to raspberry flavor (Kader, 2002; Kays & Paull, 2004). Fructose and glucose sugars are found in the highest quantities in raspberry, and sucrose in lesser quantity. Titratable, organic acids are a small part of raspberry composition, but they play a large role in non-volatile flavor (deAncos

et.al., 1999; Wang et.al., 2009). The organic acid in highest concentration is citric acid (90% of total acid composition), followed by small amounts of malic, isocitric, succinic, and fumaric acid (Wang et.al., 2009). Starch accumulation occurs in small amounts during ripening. Taste tests indicate that most consumer prefer a raspberry with high levels of both soluble solids and titratable acidity. High acidity and low sugar content result in tart flavor, and when the opposite occurs the berries have a bland flavor (deAncos et.al., 1999; Wang et.al., 2009). Throughout ripening, sugars move from the leaves to the fruit, and organic acids provide carbon skeletons for respiration and the assembly of new molecules. After harvest, loss of acids from respiration can give a flavor perception of increased sweetness; however, sugar accumulation is not taking place. Volatile synthesis during ripening causes changes in aroma (Kader, 2002; Kays & Paull, 2004). Key volatiles contributing to characteristic raspberry odor and flavor are α-pinene, citral, β-pinene, phellanderene, linalool, α-ionone, carryophyllene, and β-ionene (Hui, 2006).

Cultivar Background

In the proposed study, flavonoid composition of the primocane-fruiting cultivars ‘Nantahala’, ‘Caroline, and ‘Autumn Britten’ will be examined. 'Nantahala' was released from North Carolina State University in 2010. It fruits later than most primocane-fruiting cultivars, and is recommended for the mountains of North Carolina and neighboring states. The fruit are described as 'firm, uniform, and conical to ovate', with an average berry weight of 3.5 grams. It is the cross of NC 245, which is derived from 'Algonquin' x 'Royalty', and 'Rossana'

(Ballington et.al., 2010; Fernandez et.al., 2009). 'Royalty' is a backcross between a hybrid purple and red raspberry, and was released in 1982 from Cornell University (Sanford & Ourecky, 1982).

'Caroline' was released from the University of Maryland in 1998. It fruits 1-3 weeks earlier than standard primocane-fruiting cultivars, and the fruit are described as 'firm, red, symmetrical, and truncated conic', with an average berry weight of 1.9 grams. This cultivar is the cross of GEO-1, which is derived from 'Autumn Bliss' x 'Glen Moy', and 'Heritage' (Swartz et.al., 1998). In a press release from a 2002 study in Ohio, 'Caroline' raspberries in particular were reported to be richer in nutritional compounds than comparable red raspberry cultivars 'Lauren', 'Killarney', and 'Heritage'. On average, 'Caroline' had 32% more beta carotene, 35% more vitamin A, 47% more vitamin E, 37% more vitamin C, and 56% higher antioxidant levels than the other three cultivars (Funt, 2002).

'Autumn Britten' is a licensed variety from the East Malling Research Station in Kent, England. It is the from the same cross as 'Autumn Bliss', which was released in 1989, but has better fruit quality and slightly lower yields (Weber, 2006). The fruit are described as 'large, oval-conical in configuration, medium to dark red, and commonly present as fairly large individual drupelets', with an average berry weight of 3.5 - 4 grams. The female parent is derived from several species and cultivars, including *Rubus arcticus, R. occidentalis,* and *R. idaeus* 'Lloyd George', 'Norfolk Giant', 'Pynes Royal', 'Malling Landmark', 'Malling

Promise', and 'Burnetholm'. The male parent is a *R. strigosus* selection (Keep, 1989; Weber, 2006). 'Autumn Bliss' has high noted levels of cyanidin-3-rutinoside (deAncos et.al., 1999).

Raspberry Production

Domestic

The United States is the world's third largest raspberry producer, with Washington, Oregon, and California leading production (USDA, 2007). These states have mild summers where raspberries are not exposed to heat stress, yet have cold enough winters to satisfy chilling requirements. The demand for raspberries exceeds the supply, with 10,000 – 12, 000 tons/year being imported from Canada, Chile, and Mexico (Darnell et.al., 2006).

North Carolina

Raspberries have great potential as a high value crop; however high chilling requirements and summer temperatures are obstacles to commercial production in North Carolina and the southern United States. Recommended raspberry varieties are best suited to high elevation sites where temperatures are consistently cooler. There are three distinct climate regions in North Carolina: the Western mountains, central Piedmont foothills, and Eastern coastal plain. Most raspberry production occurs in the mountains, where cool summer temperatures and consistently cold winter temperatures allow for success with the most varieties. While many cultivars can be grown successfully for commercial production, two primocane varieties recommended for this region are 'Caroline' and 'Nantahala'.

The ideal leaf temperature for raspberries is 16-20°C and the ideal soil temperature is 22-27°C; above these temperatures photosynthesis shuts down. Heat stress can result in stunted growth, smaller fruit size, and increased risk of winter injury due to lessened carbohydrate storage (Fernandez et. al., 1998). In the Piedmont region of North Carolina, summer temperatures are often above 32°C (Fernandez, personal communication), curtailing successful production of many varieties; however the heat-tolerant cultivars 'Mandarin' and 'Dormanred' are recommended for commercial production there (Fernandez et. al., 1998; Fernandez & Krewer, 2008). Raspberries are typically not grown in the coastal plain because of low yields from the high summer temperatures and lack of winter chilling temperature requirements (Fernandez et. al., 1998).

High Tunnel Production

High tunnels are used in the United States to provide fruits, vegetables, and flowers a moderate level of protection from the elements (Casey et.al., 2009). High tunnels are large, unheated, hoop-shaped structures covered in a single layer of 6-mil polyethylene greenhouse grade clear plastic, and offer a level of environmental protection somewhere between field and greenhouse production systems (Kadir et.al., 2006; Heidenreich et.al., 2008). There are numerous benefits of high tunnels, which is why they have been adopted for wide usage in raspberry production in the United States and Canada; the majority with fall-fruiting primocane cultivars. In California alone over 4000 acres of raspberries were grown under high tunnels in 2007, which is more than 90% of the total acreage (Demchak, 2009). High

tunnel production for small fruits is different to field production, with changes in irrigation, fertilization, pest control, season of ripening, spacing and pruning needed (Demchak, 2009).

High tunnels have proven to be a large advantage to the raspberry industry by extending the harvest season, increasing yields, and improving overall fruit quality with minimal economic inputs (Demchak, 2009; Heidenreich et.al., 2008). Marketable yields are also significantly increased because fruit is protected from moisture, frost, and wind. However, it is not known whether high tunnel production has any effect on the production of plant primary or secondary metabolites, important in plant defense systems and containing antioxidants that are beneficial to human health (Thompson et.al., 2009). It is possible that a high tunnel system may affect the metabolite composition of fruit crops due to changes in light wavelength penetration (Kassim et.al., 2009).

Temperatures in high tunnels remain higher and more stable than outside conditions, extending the harvest season and protecting high-value crops from winter frost damage (Carey et.al., 2009). Additionally, soil temperatures stay higher in the tunnel, allowing for earlier plantings and harvests. Both floricane and primocane raspberries have a season extended by three to four weeks because of frost-free conditions in the tunnels (Heidenreich et.al., 2008). In a study of primocane blackberries in high tunnel and field production, tunnel fruit was harvested for three weeks longer, yield was 47% higher in double-tipped primocanes, and fruit was 32% heavier (Thompson et.al., 2009).

High tunnel systems also have effects on light exposure and photosynthetic rates of plants. Light penetration is reduced by about 10% within the tunnel compared with field conditions, which may or may not have an effect on phytochemical production (Kadir et.al., 2006). Even though sunlight penetration is reduced and exposure is indirect, light is more evenly diffused to the leaves of the plants, and photosynthetic rates are higher in tunnel produced fruit (Demchak, 2009; Thompson et.al., 2009). Additionally, the plastic-covering provides a wind barrier. A field study of raspberries showed that plants stopped photosynthesis in windy conditions (Demchak, 2009), indicating that tunnels could increase photosynthetic hours per day (Heidenreich et.al., 2008).

Improved fruit quality is usually obtained in high tunnels with fewer pesticide inputs (Demchak, 2009). High tunnel structures shelter fruit from rainfall, keeping the plants dry and preventing the growth of fungal pathogens. For primocane caneberries, a yield increase of at least 50% can be expected with high tunnel production due to season extension and reduction in gray mold occurrence (Heidenreich et.al., 2008). A trial of tunnel blackberries and raspberries produced 82-98% more market-acceptable fruit compared with field fruit (Kadir et.al., 2006). Additionally, primocane red raspberries grown in tunnels in Pennsylvania had marketable yields two to three times of those grown in the field in a five year trial of primocane-fruiting raspberries; and superior fruit quality and shelf life was obtained without the application of fungicides (Demchak, 2009). The need for fungicides will depend on production area, however, because high humidity conditions in some areas

can promote powdery mildew and rusts even in tunnels. Pest pressure in high tunnels is usually less severe compared with fields, however pests that do occur in high tunnels are those usually associated with greenhouses (Heidenreich et.al., 2008).

Postharvest Considerations

Production of fresh market raspberries is limited, as they are one of the most soft and perishable fruits, having a storage life of only 3-6 days at 0°C and 90-95% relative humidity conditions (Dris & Sharma, 2003; Darnell et.al., 2006; Kader, 2002; Wang et.al., 2009). Raspberries are non-climacteric; therefore they do not continue ripening after harvest and must be picked at maturity to ensure maximum flavor, quality, nutritional value, and optimum levels of sugars and volatile compounds (Wang et.al., 2009). Most of the physical and chemical changes that occur postharvest are deteriorative. Softening is one of the key texture changes that occurs during ripening and contributes to the short postharvest life of raspberry (Dris & Sharma, 2003; Kays & Paull, 2004; Wang et.al., 2009).

Raspberries and Human Health

One cup of fresh raspberries contains 60 calories, 8 grams of dietary fiber, and significant portions of recommended daily allowances for potassium, vitamin C, and folate (Perkins-Veazie & Collins, 2001). These compounds have shown effects on weight management, controlling blood pressure and cholesterol, improving cognitive brain function, slowing age-related eyesight degeneration, and reducing the risk of cardiovascular disease and stroke

(Kassim et.al., 2009; Perkins-Veazie & Collins, 2001). In addition to having health beneficial properties, these compounds also play a role in raspberry color and flavor. The strong flavor and aroma of raspberries are indicative of phenolic compounds, including anthocyanins, proanthocyanins, flavanols, flavan-3-ols, and phenolic acids (Kassim et.al., 2009; Perkins-Veazie & Collins, 2001).

These antioxidant compounds stop or limit damage to cellular DNA, proteins, and lipids caused by reactive oxygen species (ROS). ROS occur naturally in the body or are created during inflammation and exposure to toxic chemicals and ultraviolet radiation. They aggravate and cause various cancers and chronic diseases such as diabetes, arthritis, and cardiovascular disease. Epidemiological studies with large population groups suggest that there is a strong connection between consumption of antioxidant-rich foods and decreased risk for chronic diseases, such as cardiovascular disease and some cancers (Wang et.al., 2009). There have been many in-vitro trials that show berry antioxidants' usefulness as a disease preventative, anti-inflammatory, antibiotic, and anti-cancerous compound (Rao & Snyder, 2010; Heinonen, 2007).

Antioxidants

Antioxidants are defined as any compound present at low concentrations that delays or prevents oxidation of a more prevalent, oxidizable substrate (Hatier & Gould, 2009). Antioxidants can donate an electron or a hydrogen atom, deactivate and limit the oxidative damage caused by free radicals or reactive oxygen species (ROS), and act as transition metal

chelating agents. ROS are produced during normal metabolic processes and with exposure to environmental stressors. In the plant, ROS are produced during photosynthesis and respiration in the chloroplasts, mitochondria, and peroxisomes (Hatier & Gould, 2009).

Over time, plants have protected themselves from oxidative damage by developing complex intracellular antioxidant systems (Hatier & Gould, 2009). Within the plant cell, antioxidants are important to maintaining an oxidation and reduction balance of chemical reactions; extracellularly they serve many additional, human-health related functions (Stoner et.al., 2008). In berry fruits, phenolic acids, anthocyanins, vitamin C, and other flavonoids are the most significant antioxidants (Anttonen et.al., 2005; Freeman et.al., 2011; Sablani et.al., 2010). Because antioxidant capacity is indicative of health-beneficial phenolic compounds, it is often used to gauge the 'healthfulness' of different fruits and vegetables. The major antioxidants found in red raspberry are anthocyanins and ellagitannins, composing up to 85% of total phenolics and 50% of total antioxidant power (Deighton et.al., 2000; Heinonen, 2007; Rao & Snyder, 2010). Antioxidant capacity of fresh raspberries is positively correlated with total phenolics and flavonoids, and increased consumption of antioxidant-rich foods such as raspberries is associated with reduced disease risk (Michalczyk et.al., 2009; Rao & Snyder, 2010).

Measuring Antioxidant Capacity

Several methods have been developed and multiple studies done to measure antioxidant capacity and characterize the behavior of antioxidant compounds (Heinonen, 2007). The oxygen radical absorbace capacity (ORAC) and total radical-trapping antioxidant parameter (TRAP) methods are hydrogen atom transfer assays, measuring the competitive ability of an antioxidant compound to compete with a substrate for peroxyl radicals. The Trolox equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), and cupric ion reducing antioxidant capacity (CUPRAC) methods are electron transfer assays, which measure the ability of an antioxidant compound to reduce an oxidant probe, that changes color when reduced. The color change is measured as light absorbance with a spectrophotometer, and the extent of the color change is dependent on the antioxidant concentration (Dai & Mumper, 2010). Antioxidant capacity assays are reported in Trolox equivalents. Trolox is a well characterized vitamin E analogue that is appropriate for use as a standard (Diamanti et.al., 2010). In berries, antioxidant activity of the phenolic molecules increases with the number of hydroxyl substitutions available to donate or accept protons, and these molecules can have up to four times the antioxidant power of Trolox (Cao & Prior, 1997). Using assays such as these that measure the combined antioxidant activity of a complex biological sample, such as a whole berry extract, are beneficial because they compensate for interactions among the different antioxidant compounds and within the whole food (Dai & Mumper, 2010). A drawback of tests such as these is that they only take place in only one sample mixture. The antioxidant capacity of the analyzed compound is still

unknown in other mediums, such as a complex lipid membrane or protein environment (Heinonen, 2007; Kahkonen et.al., 2003). In assays of raspberry species, TEAC values were 0 – 25.3 µmol Trolox equivalents (TE)/g compared to 190 – 66,000 µmol TE/L in the FRAP assay, indicating that more than one assay of antioxidant capacity is needed in order to avoid misleading conclusions (Deighton et.al., 2007).

Phenolic Compounds

Phenolic compounds are the most abundant secondary metabolites in plants (Dai & Mumper, 2010). Biosynthesis is often in response to environmental stimuli, such as light, temperature, humidity, or salinity; or homeostatic cues like gene signaling, hormonal activity, or nutrition (Thompson et.al., 2009; Von Roepenack-Lahaye et.al., 2004). Over 8000 phenolic compounds have been identified, including phenolic acids, tannins, stilbenes, lignans, and flavonoids (Dai & Mumper, 2010; Thompson et.al., 2009). Structurally, phenolic compounds are classified aromatic rings with attached hydroxyl groups (Stoner et.al., 2008). Like the name suggests, polyphenols consist of phenolic aglycons and attached substituents (Maatta-Riihinen et.al., 2004). Differences in molecular structure arise from variation in "types and oxidation levels of heterocylic rings, substitution patterns of hydroxylation, glycosylation by sugars, acylation by organic and phenolic acids, and conjugation to form polymers" (Rao & Snyder, 2010).

Phenolic Content of Raspberry

Total phenolic content is measurable by the Folin-Ciocalteu method, in which phenolic compounds are oxidized by a yellow molybdotungstophosphoric heteropolyanion reagent, and the subsequent blue-colored product can be measured with a UV-spectrophotometer. Total phenolics measurements are expressed in Gallic acid equivalents (GAE) or chlorogenic equivalents. Gallic acid makes a good reference standard because it is inexpensive, easy to obtain, and has well-characterized stability and solubility. This assay is relatively robust, however some other substances than phenols can be oxidized, such as aromatic amines, sulfur dioxide, and ascorbic acid, that may affect the measured phenolic content (Singleton & Rossi, 1965; Slinkard & Singleton, 1977). A range of values (112 – 359 GAE/ 100g FW) have been reported in raspberry (Anttonen et.al., 2005; Kafkas et.al., 2008; Michalczyk et.al., 2009; Wang & Lin, 2000).

Flavonoids

Flavonoids are low molecular weight phenolics commonly found in plants (Deighton et.al., 2000). Over 4000 flavonoids have been identified in nature, varying widely in polarity and solubility depending on attached sugars and functional groups; the six major subgroups of flavonoids are flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (Lin & Harnly, 2008). Flavonoids are secondary plant metabolites associated with plant defense systems and found in many berry species. The human health benefits of flavonoids is of interest because of their chemical actions after ingestion and their antioxidant, anti-

inflammatory, antiallergic, antiulcer, antibiotic, and anticarcinogenic properties *in-vitro* (Cao & Prior, 1997; Thompson et.al., 2009; Wang et.al., 2009; Winkel-Shirley, 2001).

Flavonoid Biosynthesis

Flavonoids are derived from phenyalanine, an amino acid produced in from the shikimate pathway, and malonyl-CoA, from fatty acid synthesis (Thompson et.al., 2009).

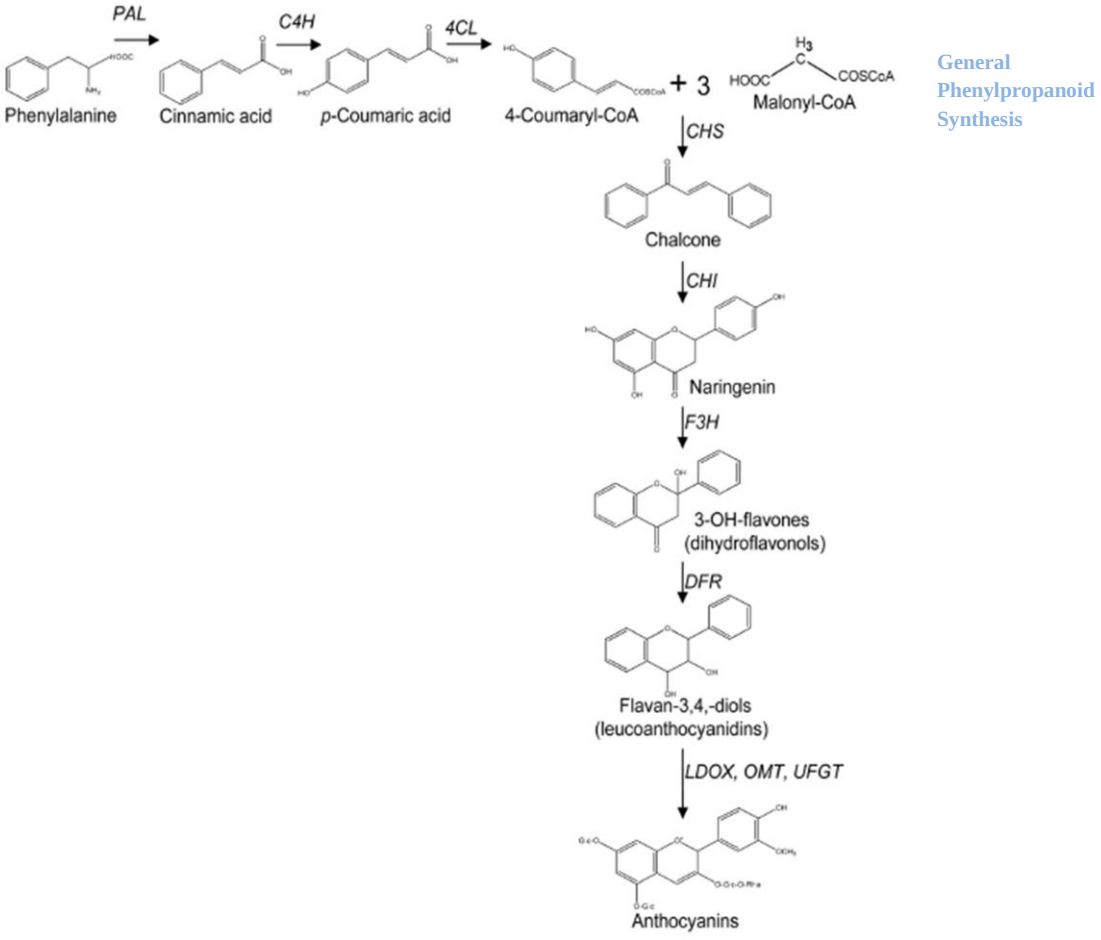


Figure 1.2 The flavonoid biosynthetic pathway for anthocyanins (Kassim et.al. 2009).

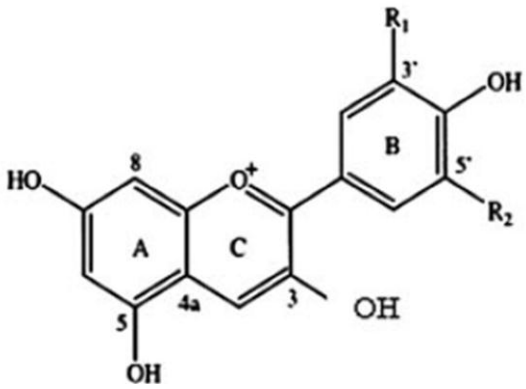
The flavonoid biosynthetic pathway has evolved with higher plants. Parts of the pathway in different plant families and orders have been modified, added or removed throughout time; however the initial steps of the pathway are found in rudimentary plants like bryophytes, which suggest that the primary reason for flavonoid development is chemical signaling, rather than the more special or complex mechanisms associated with polyphenols. The flavonoid pathway is thought to be built into a linearly functioning group of enzymes and controlled by interactions with membrane proteins (Winkel-Shirley, 2001).

Anthocyanins

The word 'anthocyanin' is derived from two Greek words: *anthos* meaning 'flower', and *kyanos* for 'blue' (Von Elbe & Schwartz, 1996). This is fitting, considering the main color pigments in red, blue, and purple fruits, vegetables, and flowers are the anthocyanins, a class of water-soluble flavonoid phenolics (Heinonen, 2007; Hui, 2006; Kahkonen et.al., 2003). There are over 600 naturally-occurring anthocyanins, with molecular weights ranging from 400 – 1200 Da. Berry fruits have relatively high concentrations of these in comparison to other fruits and vegetables (Prior & Wu, 2006; Tian et.al., 2005). Anthocyanins pigments and flavonol copigments serve many functions other than coloration within the plant, including UV protection, feeding inhibition, antimicrobial activities, male fertility influences, pollinator attractants, seed dispersers, and signaling agent of plant-microbe interactions (Steyn, 2009; Winkel-Shirley, 2001). Anthocyanins have attracted interest to human health researchers because of their anti-inflammatory, anti-radiation, and anti-tumor effects, along

with their antioxidant activities in blood vessels and lipids within the human body (Kahkonen et.al., 2003; Tian et.al., 2005).

Anthocyanins are derived from flavyium, and the basic carbon skeleton contains two benzoyl rings ‘A’ and ‘B’ separated by a heterocyclic ring ‘C’. The most common anthocyanidins are cyanidin, with approximately 50% prevalence in nature, delphinidin, malvidin, and pelargonidin with ~12% prevalence each, and petunidin and peonidin with ~7% prevalence each(Rao & Snyder, 2010). ‘Anthocyanidins’ are the aglycone form of ‘anthocyanins.’ Anthocyanins are distinguished from one another by variation in sugars attached to the third or fifth carbon, including glucose, galactose, xylose, glucuronic acid, and arabinose. Of the hundreds of anthocyanins existing in nature, cyanidin-3-glucoside is the most common (Rao & Snyder, 2010).



	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3
Malvidin	OCH_3	OCH_3

Figure 1.3 Molecular structure of common anthocyanins.

Anthocyanins and their glycosides can be further glycosylated or acylated by caffeic, *p*-coumaric, sinapic, *p*-hydroxybenzoic, ferulic, malonic, malic, succinic, and acetic acids; however anthocyanin molecules normally exist as nonacylated monoglycosides within the plant (Kahkonen et.al., 2003; Rao & Snyder, 2010; Springob et.al., 2003; Von Elbe & Schwartz, 1996). Glycosidic variation can be indicative of parentage and phylogeny within and among plant families (Jennings & Carmichael, 1980).

Visible anthocyanin color is caused by excitation of the molecule by light, and the different colors result from how much the electrons within the molecule are able to move when excited. Double bonds and increasing substitutions on the anthocyanin molecule cause visible wavelength elongation and hue deepening from red to violet to blue. Anthocyanin color can also be affected by pH, interactions with metals, and flavonol copigmentation (Von Elbe & Schwartz, 1996). Variation in pH changes flayvium ion concentration, thereby changing anthocyanin color. Lower pH correlates with more intense red coloration in red raspberry (deAncos et.al., 1999; Perkins-Veazie & Collins, 2001). Anthocyanins have strong antioxidant properties due to the phenolic hydroxyl groups attached to their ring structures, and the ability of anthocyanins to neutralize free radicals is positively associated with the number of hydroxyl groups attached to the B-ring, particularly at the third carbon. (Michalczyk et.al., 2009; Wang et.al., 2009).

Anthocyanin Synthesis and Accumulation

Anthocyanin collection in the cell vacuole occurs throughout ripening, and can be influenced by genetic and environmental factors. Anthocyanin biosynthesis can also be initiated by a number of factors, including high light intensity, UV radiation, nutrient deficiencies, high or low temperatures, environmental pollutants, high concentrations of certain ions, insects and diseases, drought, herbicide damage, hormonal activity, and up-regulation of genes dictating structural synthesis of pigments (Hatier & Gould, 2009; Kassim et.al., 2009; Wang et.al., 2009). Cell-signaling pathways controlling anthocyanin synthesis are closely related to stress-signalling pathways and other phenolic synthetic pathways (Steyn, 2009). Proteomics and genetics studies have coded the genes and enzymes involved in anthocyanin biosynthesis in several crop species. A recent study on red raspberry found quantitative trait loci (QTL) at two linkage groups associated with anthocyanin biosynthesis (Kassim et.al., 2009).

Anthocyanins are colorless when synthesized in the cytosol or endoplasmic reticulum, but turn red once they are transported into the low pH vacuole. (Hatier & Gould, 2009). Anthocyanidins are fairly unstable, and must be modified almost immediately through glycosylation, methylation, or acylation in the cytosol. In addition to stability, anthocyanidin modification is thought to signal carrier molecules responsible for anthocyanin transport to the vacuole. Cytosolic glycosylation is controlled by the enzyme family of glycosyltransferases (UGTs). Specific UGTs are sometimes associated with specific glycosides and flavonoids (Springob et.al., 2003; Yonekura-Sakakibara et.al., 2009).

Anthocyanin composition appears to be a unique, distinguishable characteristic among cultivars (Mazza & Miniati, 1993, as reviewed by Ozgen et.al., 2008). In raspberry, anthocyanins are present at high concentrations throughout the entire fruit (Kays & Paull, 2004). Anthocyanins accumulate during fruit ripening and in postharvest storage, with both concentration and/or composition changing throughout fruit development. In raspberry, fruit color changes from green to pink to red with corresponding decreased chlorophyll and increased anthocyanins. Light and cool temperatures contribute to anthocyanin synthesis and accumulation through effects on biosynthetic pathway enzymes and activity in the cell nucleus (Ozgen et.al., 2008; Steyn, 2009). Light can stimulate anthocyanin synthesis, and exposure to light in postharvest storage can improve color accumulation. This practice has been adopted by the strawberry industry, where fruit is picked immature to allow transit, better fruit firmness, decreased decay, and increased shelf-life (Wang et.al., 2009).

Anthocyanin Stability and Degradation

Anthocyanin degradation can occur during extraction, processing, and storage. Anthocyanin stability and decomposition is affected by several factors, including glycosylation, acylation, pH, light exposure, surrounding sugars, metals, and organic acids, oxygen concentration, the presence of other reactive anthocyanins, and degradative enzyme activity. Anthocyanins are the most stable at low pH (<4.0). Chemical structure is also important, as methyl groups and sugar attachments add stability, and hydroxyl groups lessen stability. Petunidin and malvidin are more stable than pelargonidin, cyanidin, and delphinidin because they have fewer

reactive hydroxyl groups (Michalczyk et.al., 2009; Von Elbe & Schwartz, 1996). Temperature also plays a role in anthocyanin degradation, with increased temperatures correlating with loss of flesh color in red pears (Steyn, 2009).

Anthocyanin Composition in Red Raspberry

Most fruits containing anthocyanins have two prevalent aglygones. Red raspberries have predominantly cyanidin glycosides, which are polar compounds with maximum absorption around 516 nm. Orange and yellow raspberries have predominantly pelargonidin glycosides, which are less polar compounds whose maximum absorption is around 504 nm. Different concentrations of these pigments contribute to fruit color, and visual color differences result from the changes in maximum absorption wavelength. Peonidin, delphinidin, and petunidin have been found in lesser amounts in certain wild and domesticated raspberry species (Deighton et.al., 2000; Maatta-Riihinen et.al., 2004). Fall-fruiting cultivars studied by deAncos et.al. in 1999 also contained small traces of malvidin-3-glucoside and delphinidin-3-glucoside in addition to the expected concentrations of cyanidin and pelargonidin. The phenolic profile of selected raspberry cultivars can range in complexity from as few as three to more than nine different anthocyanins within the fruit (deAncos et.al., 1999).

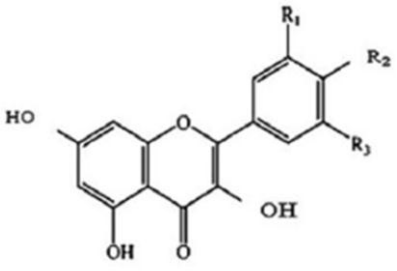
Cyanidin-3-sophoroside is the most commonly found anthocyanin in cultivated red raspberry, especially in European cultivars (Maatta-Riihinen et.al., 2004). Other anthocyanins found in red raspberry include cyanidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-glucosylrutinoside (Michalczyk, 2009; Ochiman & Skupien, 2008; Wang et.al.,

2009). Cyanidin-3-glucoside and cyanidin-3-rutinoside also show promising potential for preventing macular degeneration, as they were found to stimulate the regrowth of important membrane proteins in the rods of frog eyes (Rao & Snyder, 2010). Because antioxidant power appears to be affected by the relative anthocyanin (Gonzalez et.al., 2003), identification and quantification of anthocyanin profiles is important.

Individual anthocyanins can be measured using high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) technologies (Tian et.al. 2005). Advantages of using HPLC to measure anthocyanins is that they are the only group of flavonoids with discrete absorbance around 280 and 520nm, and their elution order is fairly predictable based on aglycone and glycoside structure (Durst & Wrolstad, 2001; Lin & Harnly, 2008). Total anthocyanins can be measured quickly and simply using the pH differential method developed by Giusti and Wrolstad (2001). The total anthocyanins test is informative and inexpensive, but it is less accurate and provides little structural information (Rao & Snyder, 2010). Meauring total anthocyanin content is often done in conjunction with quantifying individual anthocyanins. Total anthocyanin values for red raspberry are reported as 19 – 96 mg anthocyanins / 100g fresh weight (Anttonen et.al., 2005; Michalczyk, et.al, 2009; Ochiman & Skupien, 2008).

Quercetin and Kaempferol

The flavonols comprise the largest subgroup of flavonoids, and quercetin and kaempferol are the most prevalent of these, with over 200 glycosides reported (Lin & Harnly, 2008). Both have antioxidant properties and are found in relatively small amounts in red raspberry: quercetin as glycosides with rhamnose, glucose, and glucuronose on the third carbon, and kaempferol as a third carbon glucuronose glycoside (Rao & Snyder, 2010; Seeram, 2008-2). Quercetin content in red raspberry has been measured between 0.32-1.80 mg/100g fresh weight (Anttonen et.al., 2005). In laboratory and clinical studies, quercetin has been shown to effectively reduce blood pressure and slow the growth of human breast cancer cells (Anttonen et.al., 2005). Another study by Duthie et.al. (1997) found that 10 and 50µM concentrations of quercetin prevented DNA damage caused by ROS, and a similar study by Wilms et.al. (2005) found quercetin to prevent the formation of cancer-associated DNA adducts in human lymph cells. Additional studies showed that quercetin hindered growth and increased apoptotic rate in lung cancer cells at feasible *in vivo* doses (Stoner et.al., 2008).



Quercetin: R1=OH, R2=OH, R3=H
Kaempferol: R1=H, R2=OH, R3=H

Figure 1.4 Molecular structure of the raspberry flavonols quercetin and kaempferol.

Factors Affecting Phenolic Content and Antioxidant Capacity

Measures of total phenolics, anthocyanins, quercetin and kaempferol, and antioxidant capacity in red raspberry are affected by many genetic and environmental factors, including cultivar, climate, field site, harvest season, temperature, relative humidity, light intensity, fertilizer applications, harvest maturity, postharvest handling, processing, storage, wounding, diseases, and stress (Anttonen et.al., 2005; Freeman et.al., 2011; Kafkas et.al., 2008; Kahkonen et.al., 2003; Ozgen et.al., 2008). These factors, in addition to differences in extraction methods and quantification equipment, pose a challenge for the accurate and reproducible measurement of phenolic compounds in berries (Heinonen, 2007; Rao & Snyder, 2010; Stoner et.al., 2008).

Genotype

Phytochemical composition varies widely among raspberry cultivars, and research suggests that genotype contributes significantly to these differences (Ochiman & Skupien, 2008). Raspberry cultivars with similar genetic backgrounds often have similar phenolic profiles, querectein levels, and antioxidant capacity (Freeman et.al., 2011; Ozgen et.al., 2008). Stewart et.al. (2007) determined that phenolic composition was genetically controlled in a 'Glen Moy'*x* 'Latham' cross, because for the vast majority of the progeny, concentrations of quercetin, pelargonidin, and cyanidin fell between those of the parents, independent of location (Stewart et.al., 2007).

Ripening Season

In many studies it has been found that primocane-fruiting raspberry cultivars have higher anthocyanin levels and antioxidant capacity than floricane-fruiting cultivars (Anttonen et.al., 2005; deAncos et.al., 1999; Funt, 2002; Gonzalez et.al., 2003; Kafkas et.al., 2008; Wang & Lin, 2000).

Environmental and Cultural Effects

Location or harvest season effects on phenolic content could be caused by a number of environmental and cultural effects. In several fruit species, high temperatures decrease anthocyanin content, while cool night temperatures contribute to anthocyanin accumulation and pigmentation (Perkins-Veazie & Collins, 2001; Ozgen et.al., 2008). Additionally, Nitrogen deficiencies in the soil are associated with increased anthocyanin levels, either through an internal stress-response or because of reduced chlorophyll production (Steyn, 2009).

A study by Freeman et.al. (2011) found total phenolics found total phenolics in red raspberry to be highest at the beginning and end of the season, and antioxidant capacity to be highest at the end of the season. These variations could be due to internal compositional changes such as interactions among phenolic compounds, or to external factors such as weather and pests. This result may also be significant to the findings above that primocane cultivars have higher levels of bioactives and antioxidant capacity compared with floricane cultivars, as later-

season crops are given nutrients and irrigation several months before harvest, and this extended nourishment period could be a contributing factor to better fruit quality.

Processing and Storage

The effects of processing and storage on red raspberry phenolic content are important to consider with health considerations and with experimental sample storage in mind. Freezing can increase or decrease total anthocyanins, and storage temperatures above 0°C tend to increase anthocyanin content. Quercetin appears to be stable in cold storage for up to nine months (Anttonen et.al., 2005; Gonzalez et.al., 2003).

Freezing is well-known as a good way to preserve the quality of fruit over an extended period of time. With freezing raspberries, total anthocyanins and phenolics start to degrade over time; however the correlations between anthocyanins, phenolics, and antioxidant capacity remain strong, with $R^2 > 0.80$ (Gonzalez et.al., 2003). Freeze-drying is considered superior to other drying methods for preserving food quality. In a study of freeze-dried raspberries over 10 months, antioxidant capacity and anthocyanin content were not significantly reduced (Michalczyk et.al., 2009).

The bioactive content and antioxidant capacity of six primocane-fruiting red raspberries analyzed a 0, 7 days at 5°C or 90 days at -20°C (Freeman et.al., 2011). Total phenolics and antioxidant capacity were higher in refrigerated berries in comparison with fresh or frozen samples; however there were no significant differences among any of the storage effects. A

strong correlation between antioxidant capacity and total phenolics (R^2= 0.81) was maintained throughout storage. The increases in total phenolics and antioxidant capacity during refrigerated storage were correlated with decreased in titratable acidity, and this may be because the carbon skeletons of the acids were disassembled and used for the synthesis of phenolic compounds, or used for respiration.

Stage of Maturity and Plant Part

Harvest maturity and stage of fruit development greatly influences the phenolic composition of red raspberry. Anthocyanins and sugars accumulate while chlorophyll and non-volatile organic acids are lost during fruit ripening (Perkins-Veazie & Collins, 2001). Different growers may harvest at various stages of ripeness, and inexperienced pickers may harvest fruit that is immature or overripe, all contributing to variation in phenolic content and antioxidant capacity of the fruit (Ozgen et.al., 2008).

Many studies have been done the maturity effects of red raspberry anthocyanin and phenolic contents. During ripening, raspberry accumulates cyanidin-3-glucoside, cyanidin-3-sophoroside, cyanidin-3-glucosylrutinoside, and cyanidin-3-rutinoside (detectable in pink fruit), and loses ellagic acid, quercetin-3-glucoronide, quercetin derivative, and kaempferol-3-glucoronide. Pelargonidins accumulate last in red fruit,and total anthocyanin content also significantly increases during this time, while a decrease in total phenolics is seen as fruits ripen and gain red color (Rao & Snyder, 2010; Wang & Lin, 2000; Wang et.al., 2009).

Studies on the Health Benefits of Raspberry

Berries are the chosen food of study for many health researchers because they are richer in bioactive compounds and have higher antioxidant capacity than many other fruits and vegetables (Stoner et.al., 2008). As knowledge about the health benefits of berries grows, so does the need to qualify and quantify these health-beneficial compounds in blood and tissues after consumption (Tian et.al., 2005). In order to completely comprehend how raspberry compounds affect human health, we must understand how they are absorbed, metabolized, and used within the body (Prior & Wu, 2006). Additionally, there must be an understanding of all the factors that can contribute to variation in phenolic content and antioxidant capacity, as variation could affect results in clinical dosing trials, supplements, or *in-vitro* studies (Ozgen et.al., 2008).

Metabolomics

Metabolomics has emerged as an important tool for comparative, detailed phenotyping through measures of chemical composition in many different organisms (Fukusaki & Kobayashi, 2005). In the past, phenotype was only observable in visible traits; but with the advancements of technology, including metabolomics, our comprehension of the phenotype has been expanded to include quantitative measurements at the molecular level (Fiehn, 2002). Metabolomics measures a broad range of metabolites at a given time, and determines the causes behind changes in metabolite composition against a "biological frame of reference" (Stewart et.al., 2007). Metabolites are the low molecular weight final products of

metabolic reactions that are necessary for normal cell growth and function. Metabolites are widely assorted in terms of molecular structure, weight, polarity, solubility, volatility, and other physical and chemical properties. The 'metabolome' is the entire collection of metabolites produced by in a living system; and can be defined at the organelle, cell, tissue, or whole organism level (Dobson et.al., 2008; Dunn & Ellis, 2005; Fiehn, 2002). The metabolome of an organism is designated by genotypic information, arising from the genome, transcriptome, and proteome. Metabolomics data can be used in conjunction with transcriptomics and proteomics to have a more complete understanding of the mechanisms behind chemical composition. Because of its direct connection and usefulness to these other fields, metabolomics is often described as bridging the 'genotype-phenotype' gap (Fukusaki & Kobayashi, 2005; Harrigan et.al., 2007). 'Targeted' metabolomic analysis is the measurement of only specified metabolites, and 'untargeted' analysis measures all metabolites within an organism, cell, or tissue at a specific time point or over time (Fukusaki & Kobayashi, 2005; Harrigan et.al., 2007).

Metabolomic Equipment

With the advancement of metabolomics technologies, metabolites can now be detected accurately and continuously by retention time and exact mass. The most frequently used metabolomics tools are high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) (Fernie & Schauer, 2008). These

techniques are advantageous because of their ability to provide structural information in addition to quantitative information (Smith et.al., 2005).

Mass spectrometry (MS) in particular is commonly used because it qualifies and quantifies metabolites quickly and accurately. To ensure the detection of the maximum number of metabolites, compound separation must be done through either chromatography or electrophoresis before MS is performed. After separation, MS works by ionizing the sample molecules, grouping them according to their mass-to-charge (*m/z*) ratio, and detecting them with a mass analyzing system (Dunn & Ellis, 2005). GC-MS is useful for measuring less polar primary metabolites, including amino acids, fatty acids, organic acids, and carbohydrates. LC-MS can be used to measure a wide range of more polar secondary metabolites, such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates, and polyamines (t'Kindt et.al., 2009). HPLC measures metabolite concentrations based on UV-light absorbance and retention time. It is used as the separation step for LC-MS analysis, or can be used alone to measure metabolite metabolites with aromatic rings or double bonds (Fiehn 2002). HPLC is limited in its ability to distinguish among compounds with similar retention times or absorbance peaks (Mullen et.al., 2002). LC-MS is appropriate for measuring a wide array of metabolites, and when used for metabolite profiling can be key to determining variation due to genotype and environment (Dan et.al., 2008). When compared to NMR, LC-MS has better resolution, easier operation and is less costly (Lin & Harnly, 2008). LC-MS is accurate to 50-100ppm concentration and a mass range of less than 1500 Da. There are several types of LC-MS used in metabolomic

studies, each combing a different type of ionization equipment and mass analyzer. LC-TOF-MS is a combination of an HPLC separation step, followed by ionization by electrospray ionization (ESI) and detection by a time-of-flight mass analyzer (Dan et.al., 2008; Von Roepenack-Lahaye, 2004). LC-MS has been used in many studies to identify and quantify plant phenolics. One of the earliest studies on red raspberry using LC-MS was where Mullen et.al. characterized eight anthocyanin compounds in 'Glen Ample', exhibiting the ability to measure anthocyanins that had been misidentified using HPLC alone (Mullen et.al., 2002).

Limitations

As with any technology, metabolomics equipment has several limitations that must be considered. Chemical standards are often expensive or difficult to isolate, and without them metabolite identification cannot be done with complete certainty (Vorst et.al., 2005). If a single standard is available, it is reasonable to express other values in terms of that standard (Durst & Wrolstad, 2001). Because anthocyanin absorbance varies with pH and solvents used, results are often variable with HPLC analysis. However, when coupled with MS, HPLC shows improved sensitivity to detect intact anthocyanins, and can measure these compounds in plant samples, bodily fluids and tissues (Tian et.al., 2005). In MS, metabolites in low concentrations may be hard to detect due to low signal-to-noise ratios (Fiehn, 2002; Tian et.al., 2005). Ionization suppression is a major limitation of all MS analyses, affecting ionization of metabolite compounds and causing issues with accurate quantification and reproducibility (Dunn & Ellis, 2005). Ionization suppression is caused mainly by the co-

elution or contamination of metabolites during chromatography, and it can be prevented by allowing adequate separation time during this step (Fukusaki & Kobayashi, 2005).

Plant Metabolomics

It is projected that over 200,000 metabolites exist within the plant kingdom. Needless to say, the metabolome of a plant is very complex, and great qualitative and quantitative diversity exists in the metabolome between and among species, with some metabolites exclusive to certain plant families or species. Understanding these differences can reveal much about cellular regulations and responses to external stimuli, and how metabolic composition has contributed to the adaptation of certain plants to harsh environments or stringently-defined ecological niches (Fiehn, 2002; Keurentjes et.al., 2006). Metabolomics techniques are becoming more common in the plant sciences, as they have usefulness for evaluating genotypic and phenotypic variation within a population, monitoring changes in complex chemical regulation throughout plant development, and comparing differences in metabolite composition among varying species or growth conditions (Fiehn et.al., 2000; Harrigan et.al., 2007). When combined with multivariate analysis tools, metabolomics datasets have been useful for identifying different plant genotypes and making comparisons between them (Xie et.al., 2008). There are several steps involved in a metabolomic analysis, including 'plant cultivation, sampling, extraction, derivatization, separation and quantification, data matrix conversion, data mining, and bioscience feedback' (Fukusaki & Kobayashi, 2005). Each of

these steps must be well-thought-out to ensure experiment accuracy, reproducibility, and the clearest possible outputs (Fiehn, 2002; Liu et.al., 2011).

All of the phenolic compounds detailed in previous sections are considered secondary metabolites, and can be quantified using metabolomics techniques (Morreel et.al., 2006; Von Roepenack-Lahaye et.al., 2004). The metabolome changes throughout plant and fruit development; and is altered by various biotic and abiotic stressors, along with mutations and transgenic occurrences (Fukusaki & Kobayashi, 2005; Harrigan et.al., 2007). Metabolomics can be useful for uncovering connections among plant biosynthetic pathways that are only visible at the metabolome level. For example, groups of co-regulated metabolites have been discovered using metabolite profiling of plants grown in different environments (Fiehn et.al., 2000; Morreel et.al., 2006). Additionally, metabolomics may pick up on genetic mutations that are only visible at the chemical level (Fiehn, 2002) or distinguish among plants harvested at different stages of maturity (Vorst et.al., 2005). Changes in metabolomic composition and biosynthetic pathways are controlled by enzymes, and using proteomics in conjunction with metabolomics can help identify these enzymes and how they directly alter the metabolome (Von Roepenack-Lahaye, 2004).

Importance to Plant Breeding

Metabolomics, in combination with proteomics and transcriptomics, has the potential to advance plant breeding now and in the future (Stewart et.al., 2007). Many quality-defining

characteristics of both crops and ornamentals are directly associated with secondary metabolomic composition. Plants have evolved wide genotypic variations, and these broad variations are displayed in the plant phenotype and metabolome. The ability to measure broad metabolomic variation and heritability in a population enhances our ability to determine associations among metabolites and their biosynthetic pathways, and is valuable for the selection of traits of economic importance, such as yield and disease resistance. Additionally, these analyses are employed in metabolite fingerprinting techniques to eliminate undesirable traits (Dobson et.al., 2008; Fernie & Schauer, 2008).

The health of the plant and the human health benefits the plant can confer are directly related to metabolome content (Fernie & Schauer, 2008). Because variation in antioxidant compounds and human essential nutrients is evident in the metabolome, metabolite profiling techniques could be especially useful to measure variation among a population or species in order to develop nutritionally-enhanced cultivars (Harrigan et.al., 2007). For raspberries, with metabolomics and marker-assisted breeding strategies employed, the nutritional value and antioxidant capacity could potentially be improved (Kassim et.al., 2009). Additionally, using data on the metabolic response of a plant to biotic and abiotic stressors, can be important to breeding resistant crops (Fernie & Schauer, 2008).

Many of the previously described studies on red raspberry anthocyanins incorporated metabolomics to some degree in order to analyze the effects of various genetic and

environmental factors on phenolic composition. One study in particular by Stewart et.al. (2007) showed the usefulness of metabolomics techniques in raspberry breeding for improved nutritional value. In this study, LC-MS metabolomic technology was used to measure health-beneficial polyphenolic compounds in the segregating population of a 'Glen Moy' *x* 'Latham' cross. By cultivating the population in two different locations, genetic and environment effects on the metabolome were determined by comparison to the parental values. Understanding how genetic and environmental factors affect levels of health-beneficial compounds is critically important for breeding more nutritious crops. Using these methods, progeny with particularly high levels of health-beneficial compounds can be selected for and incorporated into the germplasm base when breeding for nutritional enhancement (Stewart et.al., 2007).

Limitations

As with any type of analysis, metabolomics studies are constrained by the procedure, the equipment, and size and class of metabolites that can be extracted, separated, and detected (Von Roepenack-Lahaye et.al., 2004). One of the biggest factors that restricts metabolomics studies is high costs. Regardless of this, metabolomic analyses are cheaper than transcription analysis in crops where expression chips are unavailable (such as raspberry), and known genome sequences are not needed beforehand (Fernie & Schauer, 2008). Both repeatability of experiments and reproducibility of results are challenging with metabolomics experiments. Even if the methods are clearly defined, experiment repetition is difficult because of possible

causes for variation at every step of the analysis. Reproducible results are often hard to obtain because of slight variations in mass spectrometry equipment used in different laboratories, along with uncontrollable variations in plant growth or differences in sampling techniques or timing (Fukusaki & Kobayashi, 2005). In the majority of cases, variation in biological samples far exceeds variation in technical equipment (Von Roepenack-Lahaye et.al., 2004). Other limitations include difficulty obtaining chemical standards (Vorst et.al., 2005) and inability to identify metabolite peaks (Fernie & Schauer, 2008).

Data Mining

Independent of the type of analytical platform used, in order to take advantage of the information that metabolomics studies can provide, powerful statistical tools are needed for data analysis and interpretation. These datasets are usually large, complex, and multifaceted, and therefore special data mining tools are required to analyze the results. Because there is so much variation among individual plant samples, multivariate statistical tools are especially useful for eliciting correlations among samples and metabolite concentrations, gene function, and genotype (Fave et.al., 2009; Fiehn et.al., 2000; Vorst et.al., 2005).

Principal Components Analysis

Principal components analysis (PCA) is a multivariate statistical tool that condenses the dimensionality of and analyzes variation within large, complex datasets, such as those produced from metabolomic analytical platforms (Dan et.al., 2008; Stewart et.al., 2007). It is

very useful for the identification of clusters, similarities, and differences within these datasets, and displays interpretable results in both tables and graphs (Gonzalez et.al., 2003; Stewart et.al., 2007). PCA can be done with very little previously known information about the dataset, and it simplifies multivariate datasets without the loss of valuable information (Dan et.al., 2008; Fiehn et.al., 2000; Gonzalez et.al., 2003). PCA produces two visual plots: scores and loading. The scores plot is a visualization of the differences among samples, where each sample is plotted on a graph in which the first two or three principal components make up the axes. The loading plot explains the contribution of each variable to the total variance, and shows key variables causing variation in the dataset (Fukusaki & Kobayashi, 2005).

PCA can provide useful information about the metabolome of plants and animals by combining data from each sample into a single metabolic profile. From there, the individual metabolite profiles from each sample are compared, and the metabolites causing the greatest variance among profiles are identified. A 'metabolic phenotype' can be assigned within a dataset based on how the samples cluster together, and those samples which cluster by genotype can be used to make connections between metabolite concentrations and gene function (Fiehn et.al., 2000).

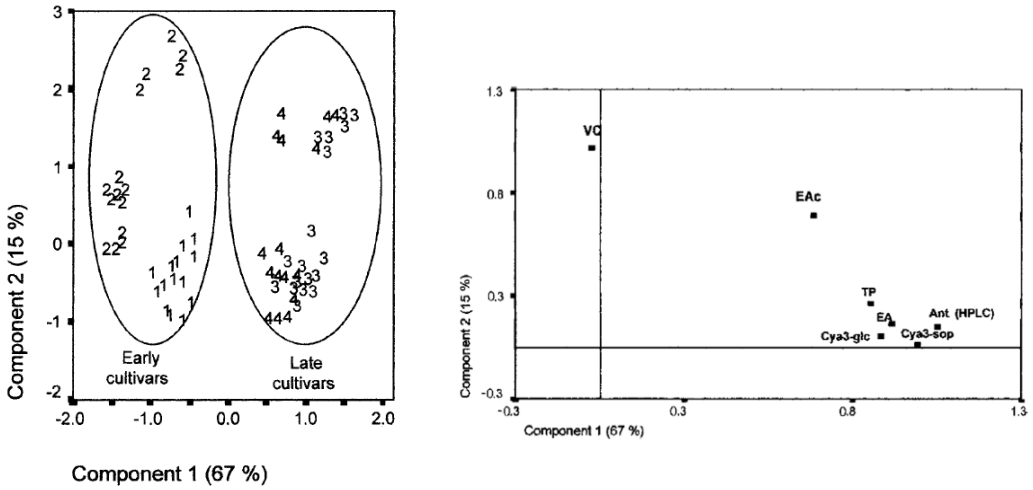


Figure 1.5 PCA scores plot (left) and loading plot (right) from the study on storage effects on phenolic content and antioxidant capacity of Spanish red raspberry cultivars. The scores plot shows clear separation on component 1 between floricane and primocane fruiting cultivars, while the loading plot shows that separation is due primarily to anthocyanin content for component 1 and vitamin C content for component 2 (Gonzalez et.al. 2003).

Several of the studies discussed above made use of PCA for sample separation and to determine key metabolites controlling variation. In the study by Gonzalez et.al.(2003), a PCA dataset was assembled based on antioxidant capacity and measures of individual and total phenolic composition in four red raspberry cultivars either fresh or frozen for 0 - 12 months. In the corresponding scores plot, the first principal component accounted for 67% of total variance, due mainly to values of total phenolics, total anthocyanins, cyanidin-3-glucoside, and cyanidin-3-sophoroside; and separation was visible between summer and fall-fruiting cultivars. In the study by Stewart et.al.(2007), PCA analysis of the polyphenolic profiles of the 'Glen Moy' *x* 'Latham' cross showed clear separation based on the concentration of cyanidin-3-sophoroside and cyanidin-3-rutinoside. This could be useful

when breeding for higher levels of a certain phytochemical, allowing for the screening of progeny with particularly high levels of the desired compound (Stewart et.al., 2007).

Gaps in Knowledge

It is not known whether high tunnel production has any effect on the production of plant metabolites, like flavonoids, that are important in plant defense systems and have antioxidant activities beneficial to human health (Thompson et.al., 2009). It is possible that a high tunnel system may affect metabolite composition due to changes in light wavelength penetration (Kasssim et.al., 2009). In one study by Kassim et.al. (2009), only small significant variations in pelargonidin composition and concentration were apparent between raspberries grown under field and polytunnel cultivation; however further research is needed to compare the metabolite profiles of plants grown under field and tunnel conditions, and to determine if flavonoid composition or antioxidant capacity are affected by production system.

There have been many studies done examining the effects of processing and storage on raspberry phenolic content and degradation. There has been a lesser amount of research done examining the differences in phenolic composition and antioxidant capacity among varying genotypes and environmental conditions for red raspberry as there has been on other crops (Wang & Lin, 2000), showing a need for a better understanding of sources of variation in phenolic composition.

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Chapter 1: **Comparison of flavonoid composition of red raspberries (*Rubus idaeus* L.) grown in the Southern U.S. using targeted metabolite analysis.**

Christine M. Bradish,*[†‡] Penelope Perkins-Veazie,[‡] Gina E. Fernandez, [†] Guoxiang Xie[§], and Wei Jia,[§]

*Corresponding author: Telephone: (919) 515-1220; Fax: (919) 515-7747; e-mail: cmbradis@ncsu.edu

[†]Department of Horticultural Science, North Carolina State University, Campus Box 7609, Raleigh, NC 27695

[‡]NC State University Plants for Human Health Institute, North Carolina Research Campus, 600 Laureate Way, Kannapolis, NC 28081

[§]Department of Nutrition, University of North Carolina at Greensboro, North Carolina Research Campus, 500 Laureate Way, Kannapolis, NC 28081

Abstract

Raspberry flavonoid compounds have significant antioxidant activities, and regular consumption of fresh and processed raspberry products may help prevent and/or moderate chronic diseases. Targeted metabolite profiling methods are useful to identify key compounds contributing to these antioxidant properties and human health benefits, and for tailored breeding for functional foods. In this study, metabolomic variation was determined among three fall-fruiting red raspberry cultivars (‘Autumn Britten’, ‘Caroline’, and ‘Nantahala’) grown at three North Carolina locations differing in elevation and average day/night temperatures. ‘Nantahala’ was specifically bred for the mountainous regions of the southern United States. Ten flavonoid compounds were detected by targeted metabolite analysis using liquid chromatography-time of flight-mass spectrometry (LC-TOF-MS). Of those, cyanidin-3-glucoside, cyanidin-3-sophoroside, cyanidin-3-rutinoside, cyanidin-3-sambubioside, and quercetin-3-glucoside were quantified against authentic external standards. Variation in flavonoid composition was primarily attributed to genotype, and also associated with night temperature and hours of exposure to temperatures over 29°C. ‘Nantahala’ had particularly high levels of cyanidin-3-sambubioside, indicative of its purple raspberry lineage. Quercetin-3-glucoside levels increased the most with elevated temperatures. Total anthocyanins, total phenolics, and antioxidant capacity of fruit samples were positively correlated, with differences among samples attributed to cultivar and location effects.

Keywords: Raspberries, *Rubus idaeus*, anthocyanins, antioxidant capacity, FRAP, LC-MS, LC-TOF-MS, phenolics, flavonoids, PCA, targeted metabolite profiling

Introduction

The international and domestic market for raspberries has grown due in part to consumer interest in more dietary intake of fruits and vegetables for nutrition and health (Kassim et.al., 2009; Ozgen et.al., 2008). Red raspberries in particular have a high fresh and processed market value (Rao & Snyder, 2010; Tokuşoğlu & Stoner, 2011). Red raspberries contain a range of vitamins, minerals, and phytochemicals that are essential for health and related to reduced disease risk (Kassim et.al., 2009). These phytochemicals can function as antioxidants that slow or stop damage to cellular DNA, proteins, and lipids caused by reactive oxygen species (ROS) (Wang et.al., 2009). The major antioxidants found in red raspberry are anthocyanins and ellagitannins, which compose up to 85% of total phenolics and 50% of total antioxidant power (Deighton et.al., 2000; Heinonen, 2007; Rao & Snyder, 2010). Numerous *in-vitro* trials with raspberry have demonstrated effectiveness for disease prevention, anti-inflammation, antibiotic, and anti-cancer. (Rao & Snyder, 2010; Seeram et.al., 2006).

Metabolomics is a field of study that has emerged as an important tool for comparative, detailed phenotyping in many organisms (Fukusaki & Kobayashi, 2005). In the past, phenotype, which includes measurable physical, chemical, and molecular characteristics, was only observable in outwardly-visible traits. However with the

advancements of analytical technology, including metabolomics, the observable phenotype has been expanded to include quantitative measurements at the molecular level (Fiehn, 2002). Metabolomics technologies have progressed such that metabolites can now be detected accurately and continuously by retention time and exact mass (Fernie & Schauer, 2008). Target compound analysis is a metabolomics technique which measures only specific metabolites or groups of metabolites, and is often employed in the plant sciences (Fukusaki & Kobayashi, 2005). One of the earliest metabolomics-type studies on red raspberry was done in 2002, where Mullen et.al. used LC-MS targeted analysis to characterize eight anthocyanin compounds in 'Glen Ample' that had been previously misidentified using HPLC alone (Mullen et.al., 2002). Because variation in antioxidant compounds and human essential nutrients is evident at the metabolite level, these related profiling techniques could be especially useful to measure variation among a population or species in order to develop nutritionally-enhanced cultivars (Harrigan et.al., 2007). Employing metabolomics and marker-assisted breeding strategies in raspberry provides an opportunity to improve the nutritional value and content of bioactive compounds (Kassim et.al., 2009).

Most compositional studies on raspberry have been done with fruit grown in temperate climates with cool production seasons (Anttonen & Karjalainen, 2005; Deighton et.al., 2000; Freeman et.al., 2011; Gonzalez et.al., 2003; Kafkas et.al, 2008; Kassim et.al., 2009; Maatta-Riihinen et.al., 2004; Mullen et.al., 2002; Remberg et.al., 2010; Stewart et.al., 2007). Most red raspberry accessions are not acclimated to grow in warm climates, where high summer temperatures can lead to heat stress and mild winters (>10°C) can interfere with

chilling hour accumulation. The optimum temperatures for red raspberry growth are 18-21°C (air) / 24-27°C (soil). Above these temperatures, photosynthesis shuts down and plant and fruit size are reduced (Fernandez et.al.; Jennings, 1988). Heat stress is evidenced by smaller berry size, lower yields and poor fruit quality of red raspberries where temperatures may exceed 32°C during the summer fruiting season . Higher than ideal temperatures have been shown to decrease anthocyanin content in several fruit species (Ozgen et.al., 2008). In contrast, a study on 'Glen Ample' raspberries found that as postflowering temperature increased from 12 - 24°C, total phenolics, total anthocyanins, and antioxidant capacity significantly increased; however these findings were partially attributed to decreasing fresh weight with increasing temperature (Remberg et.al., 2010). Effects of daily temperature ranging from 20 to 25 °C in the field on raspberry fruit anthocyanins and phenolics is not known.

In this study, we used LC-TOF-MS based targeted analysis to detect and measure flavonoid compounds in three primocane fruiting cultivars of red raspberry grown under polytunnel cultivation in central and western North Carolina. Based on previous research (Anttonen & Karjalainen, 2005; Freeman et.al., 2011; Wang et.al., 2009), we expected to see differences among cultivars and locations. By assembling a metabolite profile of flavonoid compounds in raspberry, we expect to track the warm climate effects on raspberry antioxidants and flavonoids, and simultaneously decipher genotype *x* environment variation.

Materials and Methods

Plant Materials. Primocane-fruiting red raspberry cultivars 'Autumn Britten', 'Caroline', and 'Nantahala' were harvested from July 22 to September 20, 2010 from three research stations located in North Carolina with varying elevations and temperature fluctuations (see **Table 2.1**). Standard practices for raspberry cultivation in North Carolina were followed (Fernandez et.al.). Fruit was grown in replicated trials under quonset-style rounded top high tunnels covered in polyethylene greenhouse-grade plastic at each location. Fruit samples were frozen immediately after harvest for 24 hours at -20°C, and then stored at -80°C until lyophilization. Freeze-dried samples were stored at -20°C until used.

Table 2.1. Locations of replicated trials where fruit samples of each cultivar were harvested. Each location varies in elevation and growing season daily maximum and minimum temperatures from July 15 to September 20, 2010.

Location	Latitude, Longitude	Elevation	Avg. Daily High (°C)	Avg. Night Temp (°C)
1. Piedmont Research Station Salisbury, NC	35.697 N, -80.622 W	214 m	32°C	21°C
2. Mountain Horticultural Crops Research Station Fletcher, NC	35.427 N, -82.559 W	630 m	28°C	16°C
3. Upper Mountain Research Station Laurel Springs, NC	36.402 N, -81.297 W	917 m	24°C	14°C

Standards. Cyanidin-3-sambubioside, cyanidin-3-rutinoside, and cyanidin-3-sophoroside were obtained from Polyphenols Laboratories AS (Sandnes, Norway). Cyanidin-3-glucoside was purchased from Extrasynthese (Genay, France). Quercetin-3-glucoside was obtained from Sigma-Aldrich (St. Louis, MO).

Sample Preparation. For each sample, twenty grams of freeze-dried raspberries were homogenized with a mortar and pestle, and seeds were separated from raspberry powder through a 2mm mesh sieve. Two extractions were performed, where solvent containing five milliliters of LC-MS grade methanol in water (60:39) with 1% formic acid was added to 100 mg of powder, and vortexed for one minute to mix. Samples were centrifuged at 4°C for 20 minutes at 2790 x *g*. Supernatant from each extraction was filtered through Whatman no.1 paper, pooled, and stored at -80°C in 15ml brown glass tubes until analysis.

HPLC Conditions. Prepared extracts were filtered through 0.2 µm PTFE membranes, and 50µL of each sample was diluted in 450 µL of LC-MS grade methanol. Stock solutions for standard curve calculation were prepared from 0.005 - 10 µg/mL for each standard. Five µL aliquot samples were injected at ambient temperature into an Agilent 1200 Series HPLC system equipped with a binary solvent delivery manager and a sample manager (Agilent Corporation, Santa Clara, CA), and fitted with an Agilent ZoRbax Eclipse XDB-C_{18} (4.6 x 150 mm, 5 µm particle size) chromatography column. The column temperature was maintained at 30°C with a flow rate of 0.4 mL/min. The mobile phases consisted of 2% formic acid in water for A and 2% formic acid in acetonitrile for B, with elution gradient: 0-2 min, 2% B; 2-15 min, 2-20% B; 15-30 min, 20-45% B; 30-50 min, 45-98% B; 50-60 min, 98% B; 60-70 min, 2% B. Each sample was run in duplicate, with means averaged.

TOF-MS Conditions. Mass spectra analysis was performed on an Agilent 6220 MSD/TOF mass spectrometer equipped with a dual-spray electrospray ionization (ESI) source (Agilent Corporation, Santa Clara, CA). Data was collected from both positive and negative ESI

modes, scanning a 50-1000 *m/z* range. In positive ion mode capillary voltage was set at 3500 V, nebulizer pressure at 45 psi, drying gas temperature at 325°C, and drying gas flow at 11 L/min. The same conditions were used in negative ion mode, with capillary voltage decreased to 3000 V. Raw data was processed using the Agilent MassHunter Qualitative Analysis software (Agilent Corportation, Santa Clara, CA), and compound identification was done based on mass spectra, retention time compared to authentic external standards, accurate mass data, and previously reported findings (Mullen et.al., 2002; Kassim et.al., 2009; Remberg et.al., 2010; Wang et.al., 2009). For those compounds which standards were available, concentration was reported as mg/g dry weight calculated from the prepared standard curve (**Table 2.2**). Mass spectra and retention time are reported for all identified compounds (**Table 2.3**).

Measurement of Total Anthocyanins. Total anthocyanins of the prepared extracts were determined by the pH-differential method described by Giusti and Wrolstead (2001) using a Shimadzu UV-2450 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) set to read absorbance at 510 and 700nm, through 1-cm path length disposable cuvettes. Samples were replicated and analyzed in triplicate, with means calculated. Total anthocyanins were reported as mg cyanidin-3-glucoside equivalents /L.

Total Phenolics Measurement. Total phenolics of the prepared extracts were determined by the Folin-Ciocalteu method described by Singleton and Rossi (1965). Absorbance of samples and gallic acid standards was read at 765nm by spectrophotometer (Shimadzu UV-2450, Shimadzu Scientific Instruments, Columbia, MD), and samples were analyzed in

triplicate, with means calculated. Total phenolics were reported as mg Gallic acid equivalents (GAE)/100g fresh weight.

Antioxidant Capacity Measurement. Antioxidant capacity of the prepared extracts was determined by the ferric reducing antioxidant power (FRAP) assay initially described by Benzie and Strain (1996). Absorbance of samples and Trolox standards was read at 593nm by spectrophotometer (Shimadzu UV-2450, Shimadzu Scientific Instruments, Columbia, MD), and samples were replicated and analyzed in triplicate, with means calculated. Antioxidant capacity was reported as µmoles Trolox equivalents / g fresh weight.

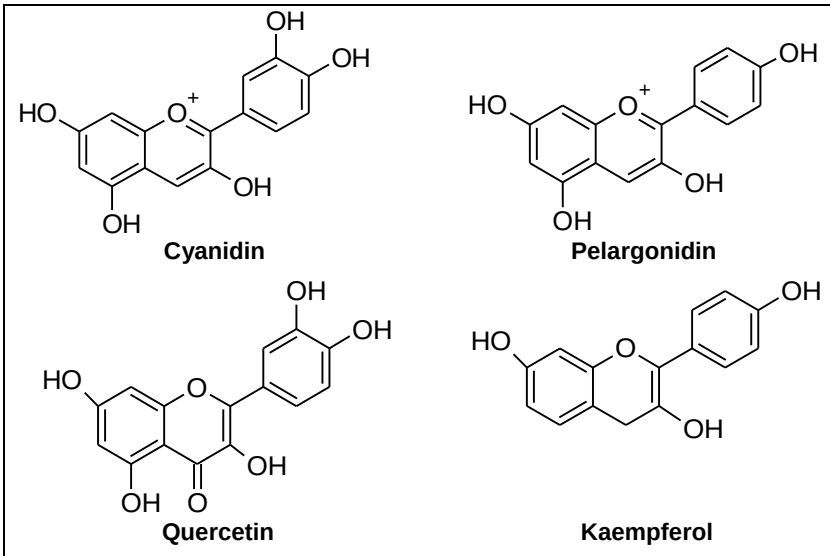

Figure 2.1a. Aglycones of detected flavonoid compounds in red raspberry samples. Cyanidin and pelargonidin are the primary anthocyanin compounds, and quercetin and kaempferol are flavonol compounds found in smaller quantities.

Statistical Analysis. SAS (SAS Institute, Cary, NC), JMP Genomics 8.0.2 (SAS Institute, Cary, NC), and SIMCA P+ 12.0 (Umetrics, Umeå, Sweden) statistical software programs

were used to perform statistical analyses. The experimental design was 3x3 factorial (3 cultivars, 3 locations). One-way analysis of variance (ANOVA) and analysis of covariance (ANCOVA) were used to evaluate differences in mass abundance of detected compounds, and to examine cultivar, location, time and temperature effects on the concentration of each quantified and detected metabolite and total phenolics, anthocyanins, and antioxidant capacity. LSMEANS with a Bonferroni correction was used to make pair-wise comparisons among sample groups (cultivars, locations, harvest dates, average day/night temperatures). Multivariate datasets collected from LC-TOF-MS were analyzed using Principal Component Analysis (PCA).

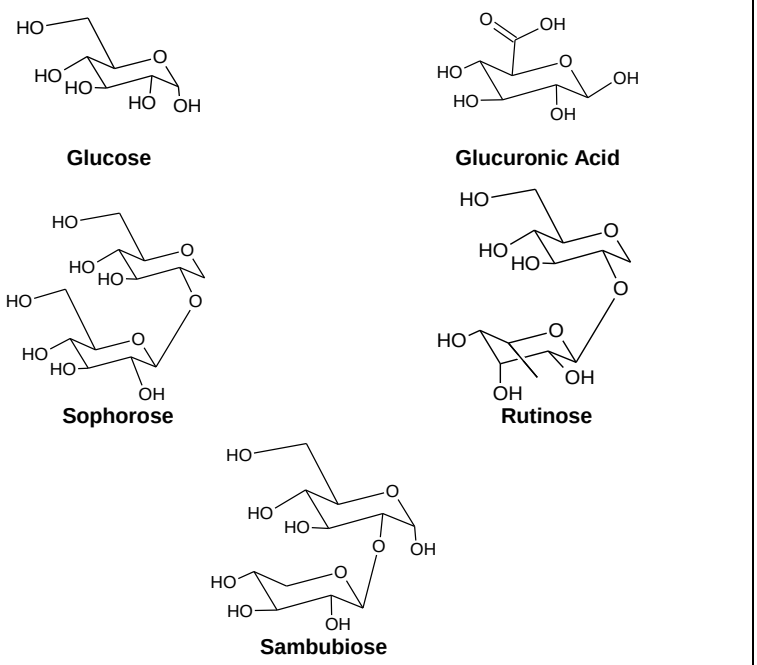

Figure 2.1b. Glycosides detected of the identified compounds (**Table 3**), normally found attached to the hydroxyl group on the third or fifth carbon of the flavonoid aglycone.

Using a multi-dimensional vector approach and eigen analysis linear algebra, PCA determines those basic eigenvectors that most contribute to total variance. The eigenvectors are calculated through linear combinations of the standardized variables, and the eigenvector that correlates with the largest eigenvalue has the same direction as the first principal component, and so on with the remaining principal components. When the samples are plotted in a two or three dimensional space over the main principal components, those contributing the most to total variance provide the best sample separation (Fiehn 2002; Fukusaki & Kobayashi, 2005; Gonzalez et.al., 2003).

Results and Discussion

Using LC-TOF-MS, ten flavonoid compounds were detected in the fruit samples (**Table 2.3**), and five of those were quantified using external standards (**Table 2.2**). The majority of compounds were detected in > 90% of the samples, while cyanidin-3-(2^{G}-glucosylrutinoside) was detected in 72% of the samples and pelargonidin-3-rutinoside was detected in 52% of the samples. Anthocyanin accumulation in red raspberry has been shown in previous research to be mainly under genetic control, with some environmental modifiers present (Anttonen & Karjalainen, 2005; Freeman et.al., 2011; Kassim et.al., 2009; Ozgen et.al., 2008). Our study indicates the same effect, as the flavonoid profiles were not dramatically different among the three harvest locations, and significant temperature and location effects were found in only a few compounds.

Table 2.2. Concentration (mg/g DW) of flavonoid compounds in three raspberry cultivars quantified by LC-QTOF-MS and comparison to external standards.*

Compound	Autumn Britten	Caroline	Nantahala
Cyanidin-3-glucoside	2.510_{b}	5.039_{a}	2.552_{b}
Cyanidin-3-rutinoside	0.132_{a}	0.044_{b}	0.036_{b}
Cyanidin-3-sambubioside	2.763_{b}	0.832_{b}	8.909_{a}
Cyanidin-3-sophoroside	1.716_{c}	5.878_{a}	2.622_{b}
Quercetin-3-glucoside	0.031_{a}	0.028_{ab}	0.014_{b}

*Data values for each cultivar are the mean of 18 samples, averaged across harvest locations. Concentrations within the same row labeled with the same letter are not significantly different ($p<0.05$).

Pelargonidin-3-glucoside, pelargonidin-3-sophoroside, and pelargonidin-3-rutinoside were detected using accurate mass and retention time data (**Table 2.3**), and by comparison to previous studies (Kassim et.al., 2009; Mullen et.al., 2002). Examination of ion abundance versus *m/z* data indicated that no significant variation among cultivars for pelargonidin-3-glucoside. 'Autumn Britten' is particularly abundant in pelargonidin-3-sophoroside, and 'Autumn Britten' and 'Nantahala' had similarly higher amounts of pelargonidin-3-rutinoside in comparison to 'Caroline' (**Figure 2.2**). Pelargonidin is the last anthocyanin to accumulate during ripening (Rao & Snyder, 2010), and may be affected by general fruit load and partitioning of resources. Freeman et al. (2011) found that phenolic levels in red raspberry were highest at the beginning and end of the harvest season. For each of the cyanidin glycosides measured, significant differences among flavonoid composition were attributed to genotype, with some covariate effects due to temperature changes and location. Cyanidin-3-glucoside and cyanidin-3-sophoroside, generally characterized as important red raspberry anthocyanins (Anttonen & Karjalainen, 2005), were the most abundant flavonoids quantified in the samples. Levels of these two compounds were consistently highest ($p<0.0001$) in

'Caroline' (**Table 2.2, Figure 2.2**) suggesting that 'Caroline' contained higher concentrations of glucose and/or glucose-associated enzymes.

Table 2.3. Mass spectral data of detected compounds using LC-QTOF-MS.

Compound	Formula	R_t (min)	$M^{+/-}$ (*m/z*)
Cyanidin-3-glucoside	$C_{21}H_{20}O_{11}$	24.5	447.09^-
Cyanidin-3-(2^G-glucosylrutinoside)	$C_{33}H_{40}O_{20}$	24.0	755.18^-
Cyanidin-3-rutinoside	$C_{27}H_{30}O_{15}$	24.6	593.15^-
Cyanidin-3-sambubioside	$C_{26}H_{28}O_{15}$	24.1	579.14^-
Cyanidin-3-sophoroside	$C_{27}H_{30}O_{16}$	23.6	609.15^-
Kaempferol-3-glucuronide	$C_{21}H_{18}O_{12}$	26.4	461.16^-
Pelargonidin-3-glucoside	$C_{21}H_{20}O_{10}$	26.0	433.03^+
Pelargonidin-3-rutinoside	$C_{27}H_{30}O_{14}$	25.3	577.11^-
Pelargonidin-3-sophoroside	$C_{27}H_{30}O_{15}$	25.2	596.06^+
Quercetin-3-glucoside	$C_{21}H_{20}O_{12}$	29.0	463.09^-

A single glucose molecule attached to a flavonoid aglycone creates a glucoside, and two bonded glucose molecules form the sophorose glycoside (Jennings, 1988; Jennings & Carmichael 1980). For 'Caroline', a significant positive linear correlation (p=0.048) was established between cyanidin-3-glucoside concentration and average hours of exposure to temperatures over 29°C during the harvest period (~7 days prior to harvest date). Additionally, a significant inverse linear correlation (p=0.023) was established between cyanidin-3-sophoroside concentration and average night temperature, and cyanidin-3-sophoroside concentration and average hours of exposure to temperatures over 29°C (p=0.043), which is consistent with other studies showing significant decreases in cyanidin-3-sophoroside with increasing temperatures (Kassim et.al., 2009; Remberg et.al., 2010). It

also may be that cooler night temperatures are important for the accumulation of some anthocyanins, but not for others.

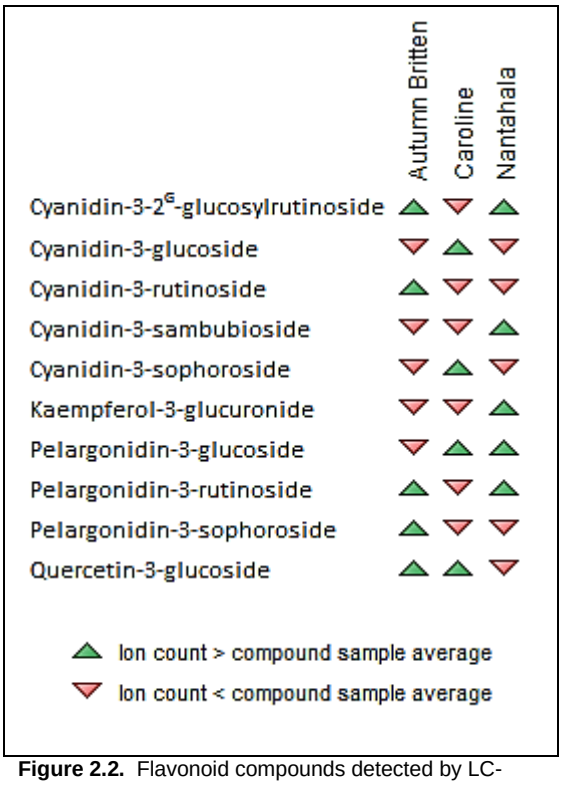

Figure 2.2. Flavonoid compounds detected by LC-QTOF-MS analysis. Ion counts for each compound were averaged across all samples, and averages for each cultivar are reported as above average or below average, indicating approximate genotypical variation.

The contrasting temperature relationships seen between cyanidin-3-glucoside and cyanidin-3-sophoroside in 'Caroline' suggest that high temperatures may have some negative impact on the enzymes responsible for the formation of sophorose or its attachment to the cyanidin aglycone.

Cyanidin-3-rutinoside was significantly higher (p<0.0001) in 'Autumn Britten' than 'Caroline' or 'Nantahala' (**Table 2.2, Figure 2.2**). deAncos et.al. (1999) found that 'Autumn

Bliss', which has the same parentage as 'Autumn Britten', had high levels of cyanidin-3-rutinoside (deAncos et.al., 1999). Higher temperatures throughout the harvest season also seem to enhance the production of cyanidin-3-rutinoside, as a significant interaction between cultivar and location was present (p=0.0004), with the highest levels found in 'Autumn Britten' samples from locations 1 and 2 (**Table 2.1**). Minor variations among concentrations of cyanidin-3-glucoside, cyanidin-3-sophoroside, and cyandin-3-rutinoside may also be due to slight differences in harvest maturity, as these three compounds are found to increase throughout ripening (Wang et.al., 2009).

Cyanidin-3-sambubioside was found in significantly higher concentrations (p=0.0005) in 'Nantahala' versus the other two genotypes. Cyanidin-3-sambubioside is typically found in higher levels in black and purple raspberries,(Jennings, 1988), and this correlates with the breeding background of 'Nantahala'. The cultivar is one-quarter 'Royalty' (Ballington et.al., 2010; Fernandez et.al., 2009), a backcross between a hybrid purple and red raspberry (Sanford & Ourecky, 1982). Both 'Autumn Britten' and 'Nantahala' clearly show that "knowledge of a genotype's anthocyanin sugars frequently provides evidence of its probable ancestry" (Jennings & Carmichael, 1980). Knowing the distinct anthocyanin profiles associated with different genotypes of red raspberry can be important for authentication of botanical products or to help in screening wild and domesticated germplasm bases for related genotypes and species with similar or enhanced health benefits and antioxidant power (Ozgen et.al., 2008).

The flavonols quercetin-3-glucoside and kaempferol-3-glucuronide were detected in each analyzed sample (**Table 2.3, Figure 2.2**). By comparing ion counts between the two compounds, it was determined that quercetin-3-glucoside is more abundant than kaempferol-3-glucuronide, consistent with the findings of a previous study of 'Glen Ample' red raspberries (Mullen et.al., 2002). Both quercetin and kaempferol decrease during ripening (Wang et.al., 2009), consistent with our fully ripened fruit samples. A significant genotypic effect (p=0.013) was found for quercetin-3-glucoside, quantified using an external standard (**Table 2.2**). 'Autumn Britten' had higher levels of quercetin-3-glucoside than 'Nantahala'. In contrast, no significant differences were found among cultivars for relative abundance of kaempferol-3-glucuronide. In addition to genotypic variation, quercetin levels are particularly sensitive to environmental variations such as light intensity, temperature, and soil conditions (Anttonen & Karjalainen, 2005). In our study, the significant variation in quercetin-3-glucoside levels is attributed to location and temperature effects among the measured samples.

A significant interaction between the effects of cultivar and location (p=0.015) is present for quercetin-3-glucoside, with the highest levels seen in 'Autumn Britten' and 'Caroline' grown at locations 1 and 2 (**Table 2.1**). Higher levels of quercetin-3-glucoside at these two warmer locations are further explained by significant covariate relationships between cultivar and hours of exposure over 29°C during the harvest period (p=0.014), and cultivar with average night temperatures (p=0.03). For each genotype evaluated, as night temperatures increased from 14.2 - 25°C, and time above optimal growth conditions during

the harvest period increased from 0 – 7.6 hours, levels of quercetin-3-glucoside also increased.

In addition to employing univariate statistical tools to measure cultivar and location effects on individual flavonoid compounds, PCA was utilized to analyze variation among the samples based on the constructed flavonoid profiles as a whole. In this instance, the samples clearly separated into three groups corresponding to the three genotypes involved (**Figure 2.3a**), verifying that flavonoid composition is a distinguishing characteristic among cultivars (Ozgen et.al., 2008). The first principal component, plotted on the x-axis, explains 79% of total sample variance, and is associated with concentrations of cyanidin-3-sambubioside. The second principal component, plotted on the y-axis, explains 18% of variance, and is designated by the difference in concentrations of cyanidin-3-sophoroside/glucoside and cyanidin-3-rutinoside. The corresponding loading plot (**Figure 2.3b**) shows the primary compounds responsible for the separation, which also correspond with the characteristic compounds defined for each cultivar above using ANOVA analysis. 'Autumn Britten' falls in the third quadrant of the scores plot in **Fig. 2.3a** and corresponds to cyanidin-3-rutinoside in **Fig. 2.3b**. Cyanidin-3-rutinoside was found to be significantly higher in 'Autumn Britten' compared to the other two cultivars. The same is true of 'Caroline' and cyanidin-3-sophoroside and cyanidin-3-glucoside in second quadrant and 'Nantahala' and cyanidin-3-sambubioside in the first quadrant. PCA gave similar results in a previous study (Stewart et.al., 2007), where clear separation occurred among progeny of a 'Glen Moy' *x* 'Latham' based on concentrations of cyanidin-3-sophoroside and cyanidin-3-rutinoside. These types

of results show that PCA can be useful in breeding for functional foods, by allowing for quick selection and screening for individuals with high concentrations of phytochemicals of interest (Stewart et.al., 2007).

Assays measuring total anthocyanins (**Figure 2.4a**), total phenolics (**Figure 2.4b**), and antioxidant capacity (**Figure 2.4c**) showed significant cultivar effects ($p < 0.0001$), with some interacting location effects ($p = 0.0001$). 'Autumn Britten' and 'Caroline' had significantly higher total anthocyanin values than 'Nantahala', and the highest measurements were seen in samples from location 2. Total phenolics and antioxidant capacity assays showed similar significance patterns, with the highest values for 'Caroline', followed by 'Autumn Britten' and 'Nantahala'. Total phenolics and antioxidant capacity went up with increasing harvest season temperatures, as the highest measurements were seen in 'Caroline' and 'Autumn Britten' samples from the two warmest locations at location 1 and 2. These measurements positively correlate with the increases in cyanidin-3-glucoside, cyanidin-3-rutinoside, and quercetin-3-glucoside seen with increased day and night temperatures at different locations. In a similar study, total anthocyanins, total phenolics, and FRAP, measured on a fresh weight basis, increased significantly as postflowering temperature increased from 12-24°C in 'Glen Ample' red raspberries grown in a climate-controlled phytotron (Remberg et.al., 2010). Our study shows that temperature may affect phytochemical accumulation throughout the entire harvest season, in addition to during fruit development.

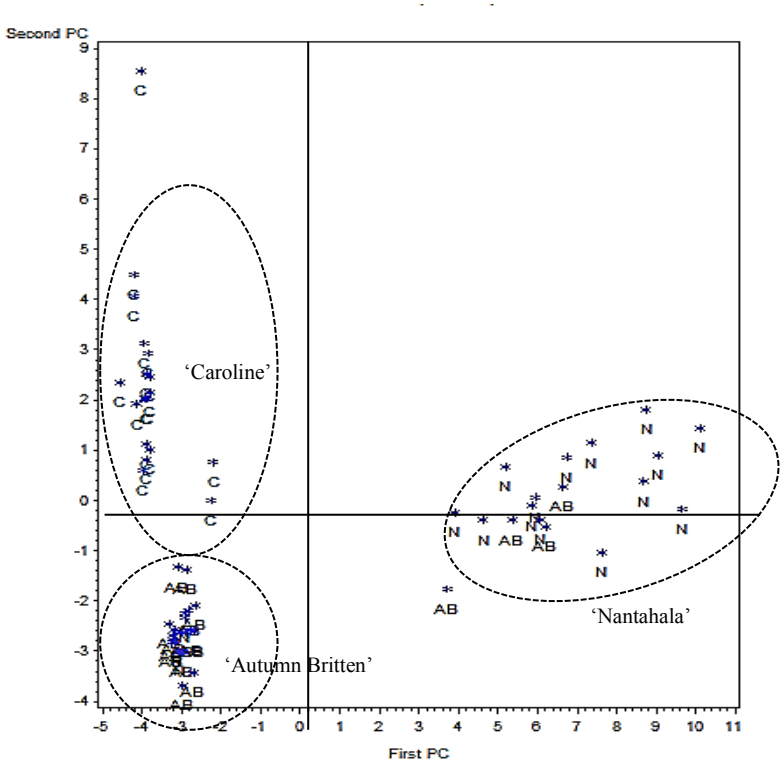

Figure 2.3a. PCA scores plot of samples characterized by LC-QTOF-MS. Each point represents a sample, with 18 samples per cultivar measured. Clustering of the samples by genotype shows significant genotypical control of flavonoid composition

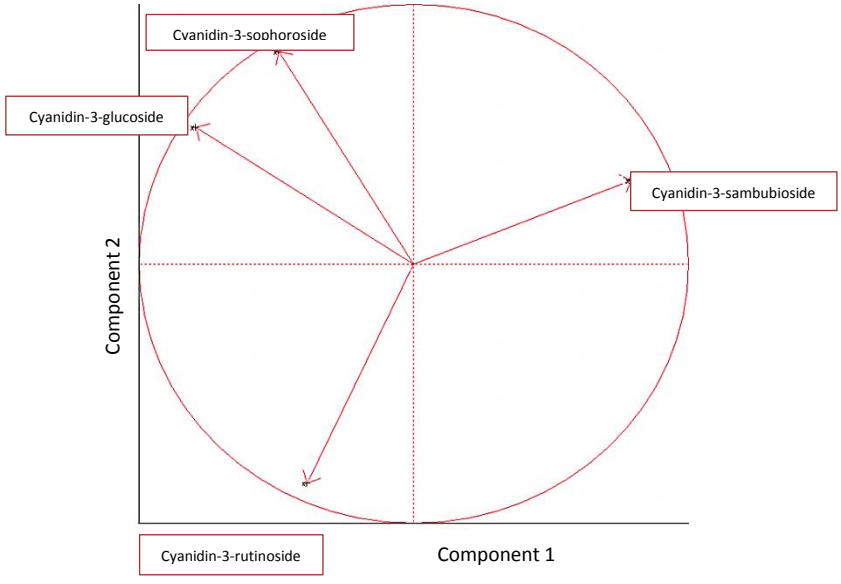

Figure 2.3b. PCA loading plot corresponding wth the scores plot in **Figure 3a**. Compounds detected by external standards are mapped and used to create a multivariate dataset and metabolite profile of the raspberry samples.

Further, these effects may become more pronounced at above-optimal production temperatures (> 27°C). Some concentrating effects may be seen as berry weight decreases and temperature increases (Remberg et.al., 2010); however in our study measurements were taken from lyophilized samples. With interest in breeding for increased health-beneficial properties and the possible need for adaptation to temperature extremes associated with climate change, it is important to understand how temperature affects phytochemical content and antioxidant power, so functionally enhanced fruits can be developed without compromising fruit quality or yield.

Significant correlations ($p < 0.0001$) were found between total phenolics and total anthocyanins ($R^2=0.544$) and total anthocyanins and antioxidant capacity ($R^2=0.596$), indicative of the contribution of anthocyanin compounds to total phenolic composition and overall antioxidant reducing power of the samples (**Figure 2.5a,b**). Additionally, a strong correlation ($p < 0.0001$) was found between total phenolics and antioxidant capacity ($R^2=0.912$), showing the combined contribution of anthocyanins and other phenolics to antioxidant reducing ability of the samples (**Figure 2.5c**). Similar correlations have been found in other studies with raspberries (Deighton et.al., 2000; Freeman et.al., 2011; Gonzalez et.al., 2003; Stewart et.al., 2007), showing the consistent ability of raspberry fruits of different genetic profiles and production environments to deliver health-beneficial antioxidant activity *in-vitro* and *in-vivo*.

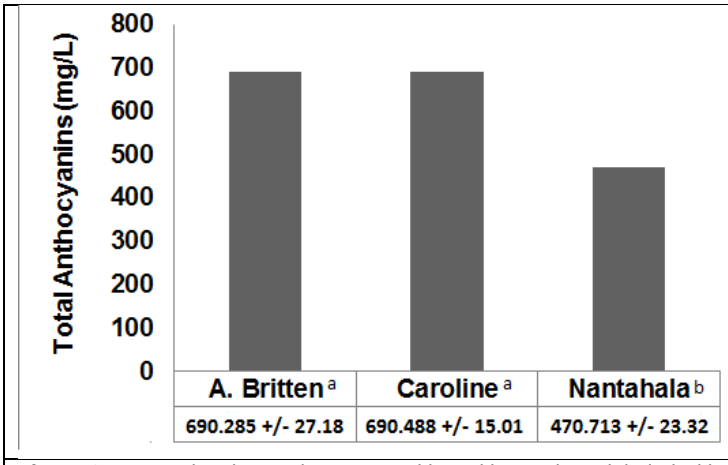

Figure 2.4a. Total anthocyanins averaged by cultivar. Those labeled with the same lowercase letter are not significantly different.

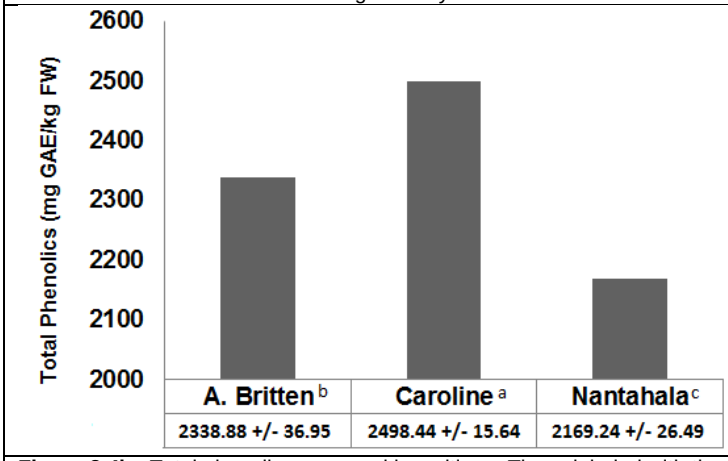

Figure 2.4b. Total phenolics averaged by cultivar. Those labeled with the same lowercase letter are not significantly different.

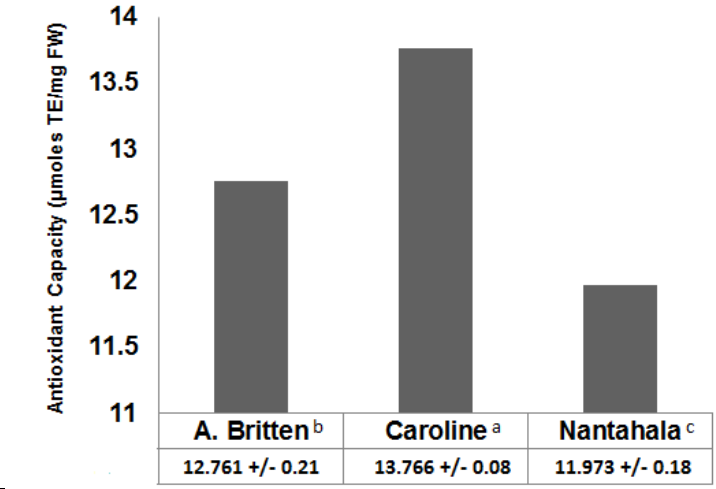

Figure 2.4c. Antioxidant capacity averaged by cultivar. Those labeled with the same lowercase letter are not significantly different.

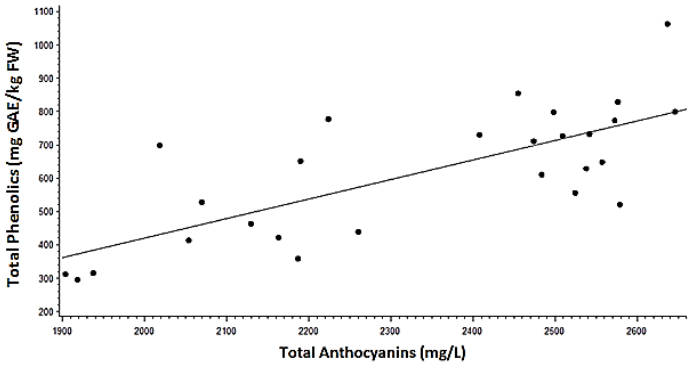

Figure 2.5a. Correlation plot between total anthocyanins and phenolics (R^2 = 0.544). Each point represents the average of three replicates.

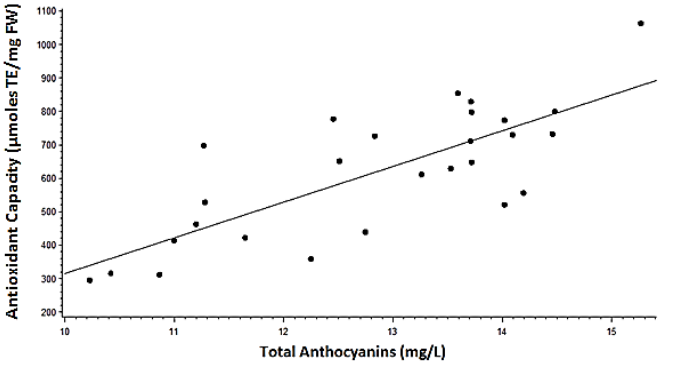

Figure 2.5b. Correlation plot between total anthocyanins and antioxidant capacity (R^2 = 0.596). Each point represents the avg. of three replicates.

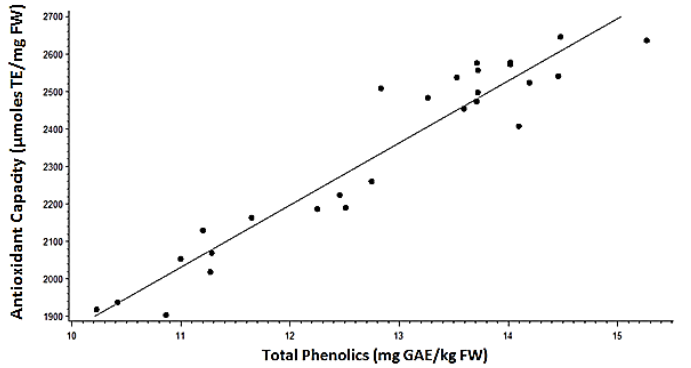

Figure 2.5c. Correlation plot between total phenolics and antioxidant capacity (R^2 = 0.912). Each point represents the avg. of three replicates.

An additional factor that must be considered in this study is the use of polytunnel cultivation. High tunnels have proven to be a large advantage to the raspberry industry

through harvest season extension, with increased yields and improved fruit quality (Demchak, 2009; Heidenreich, 2008). Red raspberries grown in North Carolina under high tunnels show better fruit quality and higher yields because the fruit are kept dry, causing fewer incidences of fungal pathogens (Fernandez, unpublished data). Conditions within the high tunnel are similar to the field, but with reduced light exposure and slightly warmer temperatures. Kassim et al. (2009) found that levels of cyanidin-3-sophoroside, cyanidin-3-glucoside, and pelargonidin-3-rutinoside were significantly lower in fruit of red raspberry grown under high tunnels compared with those grown in an open field. Tunnel production may explain the low detection of pelargonidin-3-rutinoside in our study. The better fruit quality of tunnel-grown fruit, combined with targeted breeding for enhancement of health-beneficial compounds could mitigate tunnel effects on reduced flavonoid content. More research is needed to determine how high tunnel cultivation affects flavonoid content among red raspberry cultivars.

When evaluating the health-benefits of raspberry phytochemicals in laboratory or clinical trials, or when breeding for enhanced antioxidant or phenolic levels, there must be an understanding of variation caused by genotype and environment. Certain cultivars and locations may be more amicable to the accumulation or enhancement of phytochemicals than others (Tokuşoğlu & Stoner, 2011), and these differences must be accounted for in both health research and food processing. Temperature effects on flavonoid composition could ultimately alter the results of laboratory and clinical trials examining the health benefits of whole berries and extracts, along with affecting the potency of botanicals, dietary

supplements, and processed food products (Ozgen et.al., 2008). By using targeted metabolite analysis and practical, well-characterized laboratory assays, both genetic and environmental effects on flavonoid composition in red raspberry have been identified. The results of this study suggest that growing raspberries in a warmer climate may promote the accumulation of health-beneficial flavonoid compounds, and that temperature effects must be accounted for when breeding for enhanced phytochemical content.

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Appendix

A. Methods Appendix

This appendix functions as a reference and record of the laboratory techniques and method development involved in the experiments performed for the thesis research.

1. Fruit Production and Harvest

Three primocane-fruiting red raspberry cultivars were selected from variety trials in North Carolina for this study. 'Autumn Britten', 'Caroline', and 'Nantahala' were harvested red-ripe, into clamshells, from July 22 to September 20, 2010 at three research stations located in North Carolina with varying elevations and temperature fluctuations (**Figure A.1**), measured in average night temperature and hours of exposure to temperatures over 29°C (**Table A.1**). At each location, fruit was grown in replicated trials in conventional field production and under quonset-style rounded top high tunnels covered in polyethylene greenhouse-grade plastic. Standard cultivation practices and pesticide regimes were followed at each growing site (Fernandez et.al.).

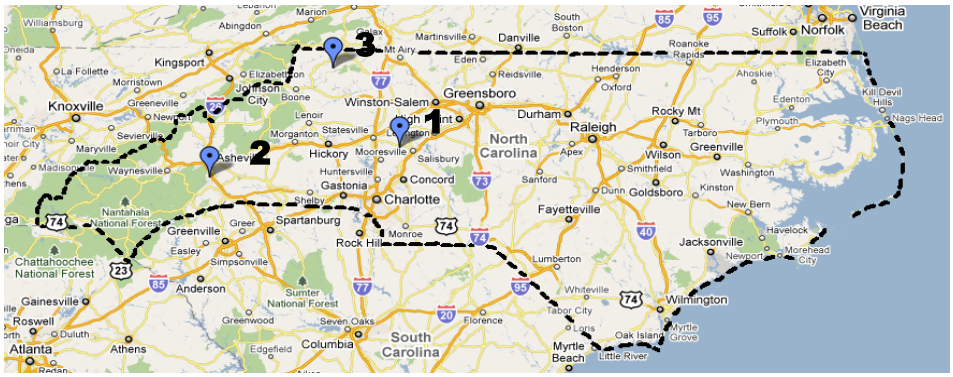

Figure A.1. Map of harvest locations in central and western North Carolina. For more detailed information on each location, see **Table 2.1** on page 97.

Table A.1. Harvest dates and temperature data for each location during the 2010 season. Cultivars harvested from field and tunnel varied for each date.

Station	Harvest Date	Average Hours Over 29°C	Average Night Temperature (°C)
PRS	22-Jul	5.7	23.30
PRS	26-Jul	3.3	22.09
MHCRS	3-Aug	3	21.87
PRS	5-Aug	4.2	23.43
UMRS	19-Aug	0	20.42
PRS	19-Aug	7.6	24.94
MHCRS	24-Aug	0.3	21.09
UMRS	26-Aug	0	18.13
PRS	26-Aug	4.5	22.81
UMRS	30-Aug	0	16.94
MHCRS	31-Aug	0	18.76
UMRS	7-Sep	0	14.14
MHCRS	14-Sep	0.1	15.03
UMRS	20-Sep	0	14.86

2. Postharvest Handling and Storage

It is essential that sample materials for any metabolomic or compositional analyses be stored properly to ensure the most precise, accurate, and reproducible results (Dunn & Ellis, 2005). Storage conditions must be such that enzyme activity is stopped and metabolite composition is unaffected. Some sample materials are sensitive to heat and light, or can be easily oxidized when left out in the open (Durst & Wrolstad, 2001). Freezing is one of the most common ways to store samples, and at -80°C changes in metabolite composition are prevented from occurring. Extraction consistency and metabolite degradation rates can be affected by the natural variation in water content among plant samples, or by thawing and

refreezing of stored materials. Freeze-drying is highly recommended for improved consistency in extractions and analyses because all moisture is removed from sample materials, and enzymes and mobile molecules within the cell are unable to function (Fiehn, 2002; t'Kindt et.al., 2009). Additionally, freeze-drying is a fast freezing process, and the small ice crystals that form are less harmful to the plant tissue, and therefore enzyme activity and metabolite composition are not as affected (Freeman et.al., 2011). Phenolic composition at the time of harvest is best preserved if samples are immediately frozen and subsequently freeze-dried (Lin & Harnly, 2008).

Freezing poses some potential issues, such as metabolite degradation, production of additional metabolites due to handling or bruising, or changes in metabolite composition due to repetitive freezing and thawing (Dunn & Ellis, 2005). Extracted material can be stored at 4°C if used within 48 hours, or in excess of a year if stored below -18°C (Rodriguez-Saona & Wrolstad, 2001). Anthocyanins in particular are most stable stored at pH < 2, in their flavylium-cation form (Lin & Harnly, 2008). Freeze-drying can cause the permanent adsorption of metabolites to cell walls and cell membranes, making extraction difficult. Additionally, freeze-dried samples must be stored in a dry environment to prevent absorption of water from the environment and rehydration of certain enzymes that may affect analysis (Fiehn, 2002; t'Kindt et.al., 2009).

In our study, fruit was transferred after harvest from clamshells to resealable plastic bags and transported in coolers back to the laboratory in Kannapolis, NC. Each sample was weighed, and frozen immediately at -20°C. After 24 hours, samples were moved to a -80°C freezer and stored until lyophilization. After freeze-drying, samples were re-weighed and stored at -20°C until extraction and analysis. From these measurements, dry weight percentages (**Figure A.2**) were determined for each cultivar. The average dry weight among all of the samples was used to estimate fresh weight equivalents (g) for the calculation of total anthocyanins, total phenolics, and antioxidant capacity.

Table A.2. Dry weight percentage by cultivar.

Cultivar	Dry Weight (%)
Autumn Britten	13.94
Caroline	13.74
Nantahala	16.52
Average	**14.74**

3. Homogenization

Sample materials should be homogenized into small, uniformly sized particles, using various tools and techniques depending on the number of samples needed and the types of tissues being broken down. Homogenization into a fine powder increases sample surface area, and therefore reduces extraction time (Rodriguez-Saona & Wrolstad, 2001).

A number of different sample homogenization methods were experimented with to determine the optimum method for sample preparation in this study. A mortar and pestle alone was appropriate to crush lyophilized flesh material; however the addition of liquid nitrogen was

needed to homogenize the seed material. If left intact, seeds could be separated from flesh material through a 2mm mesh kitchen sieve (**Figure A.3**).

To determine the best method for sample homogenization, a small experiment was performed on a representative subset of fruit samples to analyze the contribution of seed and flesh material, along with preparation method, to anthocyanin concentration. The samples were subjected to five various homogenization treatments. For each treatment, ten milliliters of the laboratory's standard methanol in water (60:37) containing 3% formic acid were added to 100mg fruit samples, vortexed for one minute to mix, and filtered through Whatman no.1 papers. Absorbance was measured as the difference between 510 and 700nm. Anthocyanins are absorbed at 510nm, and at 700nm, no anthocyanins are absorbed. We take a reading at this wavelength in order to cancel out any haze or sediments that may be reflecting light in the extracts. All measurements are done using a Shimadzu UV-2450 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). From this experiment, it was determined that seed material made negligible contribution to anthocyanin content (**Table A.3**); and therefore seeds were excluded from the final prepared samples.

Table A.3. Measured absorbance at 510nm by spectrophotometer of 100mg raspberry fruit extracts to determine contribution of seed and flesh to anthocyanin composition and to optimize sample homogenization.

Treatment	Absorbance 510 – 700nm
Powdered flesh material	0.340
Powdered flesh material and intact seeds together	0.254
Intact seeds alone	0.168
Whole berry (seed and flesh) ground in liquid N	0.148
Seeds ground in liquid N	0.114

In the final samples, twenty grams of freeze-dried raspberries were homogenized with a mortar and pestle, and seeds were separated from powdered flesh material through a 2mm mesh sieve (**Figure A.3.**).

Figure A.2. Freeze-dried whole raspberry fruit.

Figure A.3. Separation of seeds from freeze-dried flesh material and its homogenization into powder.

Figure A.4. Homogenized flesh material from raspberry fruit samples, stored at -20°C until use.

4. Sample Concentration

Prior to this study, our laboratory had not performed extractions or assays on freeze-dried berry material; therefore a concentration linearity test was performed to determine dry weight equivalents for experiments done traditionally on fresh-frozen material. A representative subset of fruit samples was chosen (one of each cultivar 'Autumn Britten', 'Caroline', and 'Nantahala'), and weighed out in samples of raspberry powder ranging from 100 – 300mg. Ten milliliters of 60:37:3 extraction solvent was added to each sample, vortexed for one minute, and filtered through Whatman no.1 papers. Absorbance readings were taken at 510 and 700nm (**Figure A.5**).

There is a strong linear relationship between extract concentration and absorbance at 510-700 nm (R^2=0.9955). Because of this linearity, the median value at 200mg/10ml was chosen to be the final extraction concentration throughout the entire study. Additionally, the absorbance values at this concentration were aligned with those for dark-red raspberry cultivars extracted from fresh-frozen material.

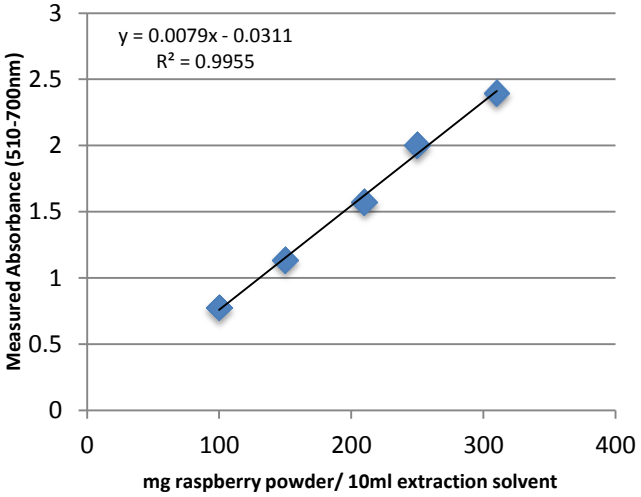

Figure A.5. Linear relationship between mg of lyophilized raspberry powder extracted in solvent and absorbance at 510nm measured by spectrophotometer.

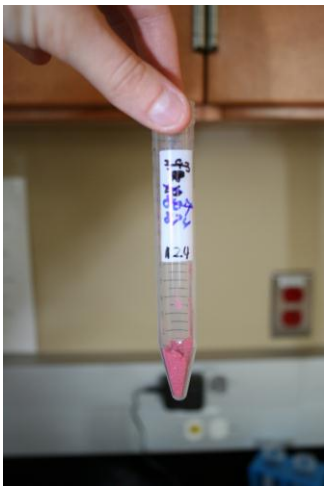

Figure A.6. Freeze-dried raspberry powder weighed out in 200mg increments for extraction.

This concentration is easy to prepare, and is easily diluted for more sensitive equipment such as high performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS).

5. Extraction

After an optimized homogenization and concentration method was developed, the samples consisting of 200mg of raspberry flesh powder in 10ml of solvent were put through several

rounds of small experiments to determine an extraction method that provided the best peak resolution and sample absorbance for the different analytical techniques being used in our studies. These included tests for the efficacy of sonication, centrifugation and filtration, the best solvent system, and the number of extractions needed.

Sonication

A representative subset of fruit was chosen and weighed out into 200mg samples. Ten milliliters of 60:37:3 extraction solvent or a water control was added to each sample and vortexed for one minute, and sonicated for 15 minutes in a Branson 3510 Ultrasonic Cleanser (Emerson Industrial Automation, Danbury, CT). Absorbance was measured at 510 and 700nm using a spectrophotometer. When compared with untreated samples (**Table A.4**), sonication was shown to have a slightly negative effect on absorbance, independent of extraction solvent used.

Table A.4. Measured effects of sonication on absorbance at 510nm. Percent difference is measured in regards to the first reading.

Test	% Difference
Sonication – none (water solvent)	-3.5
Sonication – none (acidified methanol solvent)	-6.4

Based on these results, sonication was not deemed beneficial to the extraction process, and was therefore left out of the final sample preparations.

Centrifugation & Filtration

Samples were extracted as described in the section above, vortexed for one minute, then either centrifuged for 20 minutes at 2790 x *g* and 4°C, filtered through Whatman no.1 papers, or both.

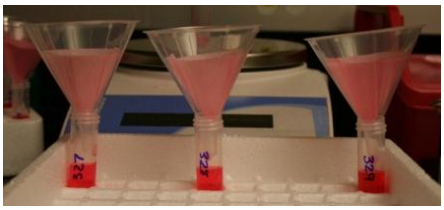

Figure A.7. Filtration of raspberry extracts through Whatman no. 1 papers.

Absorbance was measured at 510 and 700nm using a spectrophotometer. When compared with untreated samples (**Table A.5**), centrifugation and filtration alone were shown to have slightly negative effects on absorbance, independent of extraction solvents used; however for the acidified methanol preparation, the use of both centrifugation and filtration had a positive effect on absorbance.

Table A.5. Measured effects of centrifugation and filtration on absorbance at 510nm. Percent difference is measured in regards to the first reading.

Test	Solvent	% Difference
Centrifuge	Water	-18.5
Filter	Water	-41.5
Centrifuge & Filter	Water	-9.3
Centrifuge	Acidified methanol	-5.8
Filter	Acidified methanol	-0.73
Centrifuge & Filter	Acidified methanol	5.7

In order to have the best peak resolution and cleanest samples possible, both centrifugation and filtration were used in the final optimized preparation.

Solvent System

In this experiment, the efficacy of five different solvent systems containing different ratios of methanol: water: formic acid plus a water control (**Figure A.8**) were tested. A representative subset of fruit was chosen and weighed out into 200mg samples. Ten milliliters of solvent were added to each sample, vortexed for one minute, centrifuged for 20 minutes at 2790 x *g* and 4°C, and filtered through Whatman no.1 papers. Absorbance was measured at 510 and 700 nm using a spectrophotometer.

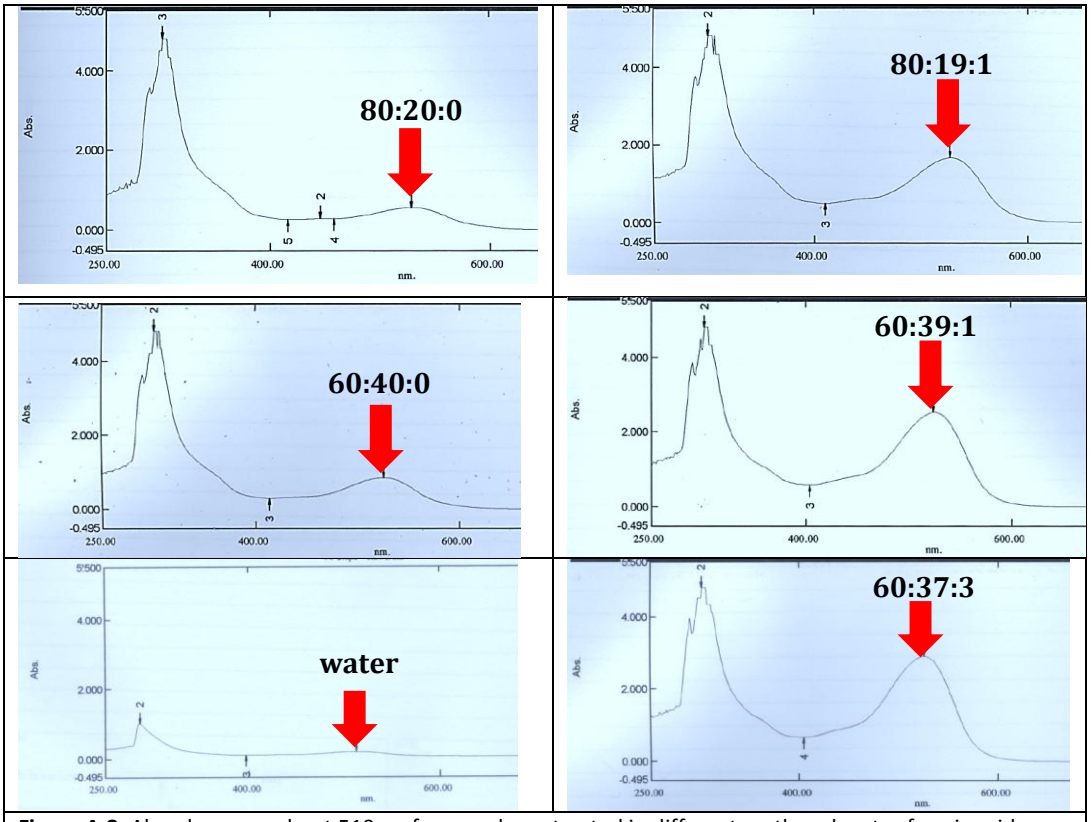

Figure A.8. Absorbance peaks at 510nm for samples extracted in different methanol:water:formic acid mixtures. Acidifying the methanol clearly stabilizes anthocyanins in the fruit, without causing compound breakdown from acid hydrolysis.

Samples should be extracted in a solvent appropriate for the type of compounds being targeted for analysis. Anthocyanins are polar molecules, and therefore they should be extracted in a polar organic solvent (Rodriguez-Saona & Wrolstad, 2001). Methanol is the polar solvent most appropriate for the extraction of lower molecular weight compounds, such as flavonoids and anthocyanins (Dai & Mumper, 2010). A small amount of acid can be added to the extraction solvent to stabilize anthocyanin molecules; however too high a concentration of acid can disassemble cell membranes and dissolve pigments. The best way to avoids issues with this is to use strong acids in concentrations of < 1%, or to use weaker acids like formic, citric, tartaric, phosphoric or acetic, in concentrations of 0.5 – 3.0% (Dai & Mumper, 2010; Kahkonen et.al., 2003; Rodriguez-Saona & Wrolstad, 2001).

As can be clearly seen from the chromatograms, the addition of formic acid to the extraction solvent system greatly improves the peak resolution and absorbance at 510nm, by stabilizing the anthocyanins within solution. Additionally, the 60% methanol solutions show better peak resolution than the 80% methanol solutions. When the two best solvents, 60:37:3 and 60:39:1, are compared side-by-side, the 60:37:3 has a 14.1% higher absorbance (not visible outright when comparing the chromatograms). Even though the 60:37:3 has better initial peak resolution, the higher formic acid content puts it at higher risk for breakdown through acid hydrolysis in storage, and the more sensitive systems such as HPLC or LC-MS require lower concentrations. Therefore our final samples were extracted in a 60:39:1 solvent system, which gave adequate peak resolution and absorbance without risking acid hydrolysis or damage to sensitive equipment.

Number of Extracts

In this experiment, the number of extractions needed to obtain the best absorbance and peak resolution were tested using HPLC. A representative subset of fruit was chosen and weighed out into 100mg samples. Five milliliters of 60:39:1 solvent were added to each sample, vortexed for one minute, centrifuged for 20 minutes at 2790 x *g* and 4°C, and filtered through Whatman no.1 papers. This process was repeated for two more extractions, and prepared extracts were filtered for HPLC through 0.2 µm PTFE membranes (**Figure A.9**).

Figure A.9. Extracts being filtered for HPLC

Ten µL aliquot samples were injected at ambient temperature into an Agilent 1200 Series HPLC system equipped with a binary solvent delivery manager and a sample manager (Agilent Corporation, Santa Clara, CA), fitted with a Suplecoil LC-18 reversed-phase (4.6 x 250 mm, 5 µm particle size) analytical chromatography column. The mobile phases consisted of 5% formic acid in water for A and methanol for B, with flow rate of 1ml/min and elution gradient: 0-5 min, 10-15% B; 5-15 min, 15-20% B; 15-20 min, 20-25% B; 20-25 min, 25-30% B; 25-45 min, 30-60% B; 45-47 min, 60-10% B, 47-60 min, 10% B.

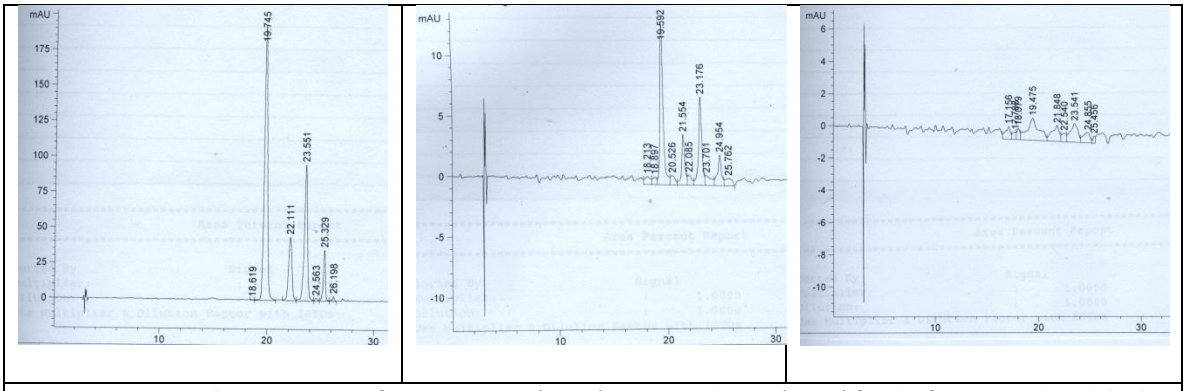

Figure A.10. HPLC chromatograms of retention time (x-axis) versus peak area (y-axis) for the first, second, and third extraction (shown left to right, respectively) for 0.02g/ml raspberry extracts, showing relative contribution of each extraction to absorbance spectra.

Concentrations of the commercial standard for cyanidin-3-glucoside were prepared at 0.0625, 0.125, 0.25, and 0.5 mg/ml, and 5 µl were injected as external standards to assemble a standard curve. Peak area was recorded at 520nm, and the anthocyanins in the samples were quantified in cyanindin-3-glucoside equivalents against the calibrated standard curve. The contribution of each extraction to total peak area was calculated (**Table A.6**).

Table A.6. Cumulative contribution of number of extractions to peak area and percentage peak area for HPLC absorbance spectra at 520 nm. Peak area measurements and percentages are averaged over samples from each of the three cultivars.

Extract	Σ peak area	Σ % peak area
1	1.798	85.99
2	1.979	94.65
3	2.091	100.00

From these results it was determined that two extracts were adequate for approximately 95% peak area recovery. Therefore, in the finalized methods, two extractions were used.

6. pH Differential Assay

Total anthocyanins of the prepared extracts were measured by the pH-differential method originally described by Giusti and Wrolstad (2001). In this method, the difference in absorbance of anthocyanin-containing samples is measured at pH 1.0 and pH 4.5, between which anthocyanin molecules change structural form (**Figure A.11**). At pH 1.0, raspberry anthocyanins are in their red flayvium cation form, and light absorption, and hence coloration, is at a maximum. At pH 4.5, anthocyanins are in their colorless hemiketal form. Measuring the absorbance at the known maximum absorbance wavelength for the anthocyanin of interest, and taking the difference between the two readings allows for quantification of only anthocyanins contributing to fruit coloration, while leaving out degraded pigments or other impurities in the samples (Giusti & Wrolstad 2001; Prior & Wu 2006; Von Elbe & Schwartz 1996).

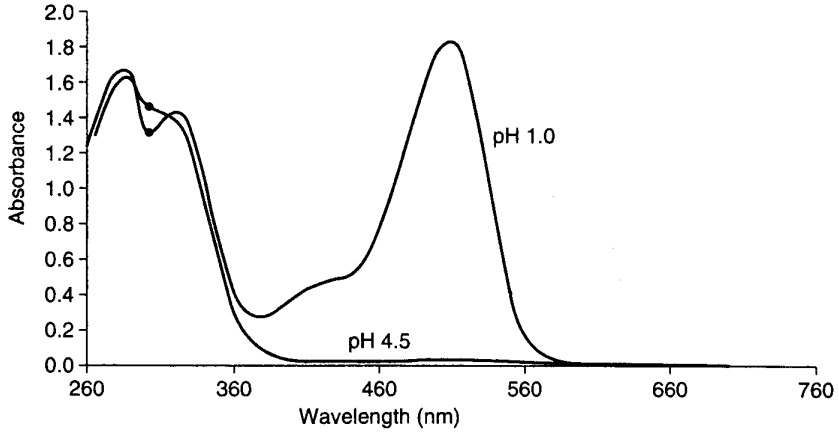

Figure A.11. The spectral characteristics of anthocyanins in their colored oxonium and colorless hemiketal forms. Taken from (Giusti and Wrolstad, 2001).

In order to further understand the originally described methods, and to better adapt our laboratory methods to this project, small experiments were done to determine the best wavelength at which to take total anthocyanin readings, proper blanks to load in the spectrophotometer, the necessity of pH 4.5 buffer solution, the linearity of assay concentration, and the linearity of the assay over time.

Anthocyanin Maximum Absorbance Peaks

To get an accurate estimate of total anthocyanins in a sample, you must measure the absorbance at the known maximum absorbance wavelength for the predominant anthocyanin of interest, and know the molar absorptivity of that anthocyanin. Therefore, you must be somewhat familiar with the composition of your sample, or have spectrophotometric equipment that is equipped for spectral analysis so the maximum absorbance wavelength, and therefore predominant anthocyanin, can be determined. Currently, in our laboratory, total anthocyanins are calculated in cyanidin-3-glucoside equivalents, where the maximum absorbance wavelength is 510nm and the molar absorptivity is 26900; however cyanidin-3-sophoroside is the predominant anthocyanin in raspberry. Unfortunately, cyanidin-3-sophoroside standards are difficult to acquire, and the molar absorptivity of cyanidin-3-sophoroside in pH 1.0 buffer is unknown. To better understand and align the methods developed by Giusti and Wrolstad with our own, and to confirm that 510nm and cyanidin-3-glucoside equivalents would be appropriate substitutions for maximum absorbance wavelength and molar absorptivity in total anthocyanin calculations, the maximum absorbance of three specific anthocyanins found in raspberry (cyanidin-3-glucoside,

cyanidin-3-rutinoside, pelargonidin) and a representative subset of fruit samples were measured using a spectrophotometer.

The anthocyanin samples were measured out from authentic chemical standards (Sigma-Aldrich, St.Louis, MO), and diluted in 60:39:1 extraction solvent. The fruit samples were prepared according to our finalized preparation and extraction methods. To 0.6ml of each prepared sample, 2.4ml of pH 1.0 buffer was added, vortexed for one minute, and allowed to sit at room temperature for one hour. The absorbance spectrum of each sample was measured from 250 – 800nm, and the maximum absorbance wavelength was recorded.

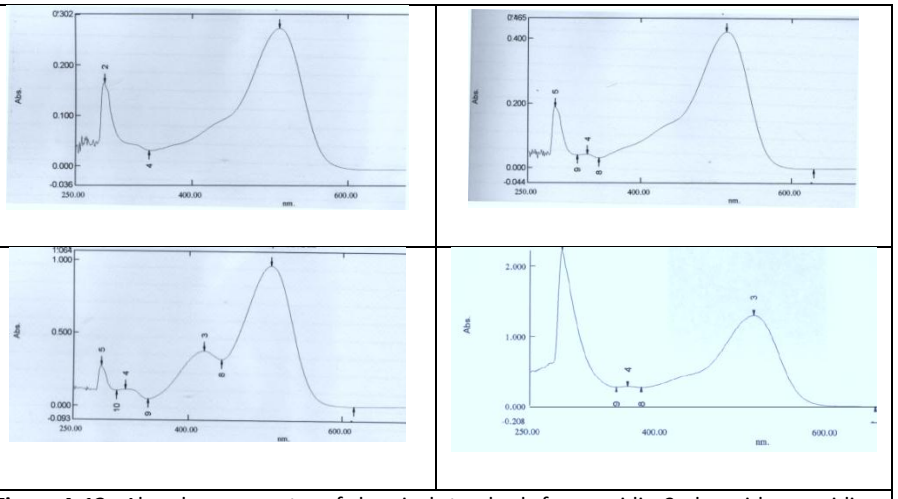

Figure A.12. Absorbance spectra of chemical standards for cyanidin-3-glucoside, cyanidin-3-rutinoside, pelargonidin, and a raspberry sample (shown left to right, respectively).

Maximal absorbance wavelengths for cyanidin-3-glucoside, cyanidin-3-rutinoside, and pelargonidin were 513, 514-515, and 507, respectively. In the literature, they are reported to be 510, 510, and 505, respectively (Giusti & Wrolstad, 2001). On average, the Autumn Britten, Caroline, and Nantahala samples peaked at 514, 513 and 513 nm, respectively. The

differences we found in comparison with reported values were minimal, and could have been due to sample impurities, small pH differences in our buffer solution, or minor calibration and alignment errors in the spectrophotometric equipment. According to these results, and in general, cyanidin-3-glucoside is still appropriate to be used in the raspberry samples, as it is the most predominantly occurring anthocyanin in nature and it is considered the standard for the pH differential assay when molar absorptivity values are unknown.

Spectrophotometer Blanks

When using a spectrophometer, samples and blanks are placed into disposable 1-cm path length cuvettes. The solvent that is used in the blank cuvette depends on the absorbance spectrum of the solvent for the assay being run. For some assays, a deionized water blank is appropriate, while for other assays, the blank solvent should exactly match that being used in the samples. To determine the appropriate blank for the pH differential assay, the absorbance spectrums of deionized water, pH 1.0, and pH 4.5 buffers were compared against a deionized water blank from 250 – 800nm (**Figure A.13**) using a spectrophotometer.

When examining the results, it is seen that the absorbance spectra among the three solvents are not different; and therefore the deionized water blank is sufficient for this assay. Giusti and Wrolstad confirmed these results in their methods, and suggest that using a deionized water blank is less time-consuming and extends the procedure unnecessarily (2001).

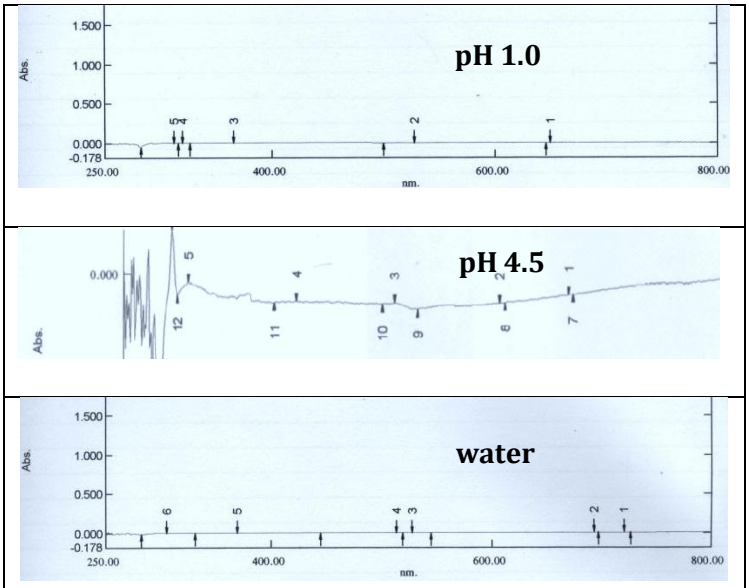

Figure A.13. Absorbance spectra of pH 1.0, 4.5, and water to determine the best blank against which to run samples for the pH differential assay.

Interfering Compound Test

Anthocyanins are pH sensitive, and at pH 4.5 in the pH differential assay, anthocyanin-containing samples are in their colorless hemiketal forms. Any absorbance that is picked up by the spectrophotometer is a measure of impurities or degraded pigments, and is accounted for in the calculations of total anthocyanins. However in our laboratory, the pH differential method had been modified to exclude the use of pH 4.5 buffer solutions, only measuring the absorbance of anthocyanin-containing compounds in pH 1.0 solution at 510 and 700nm, and taking the difference between the two readings. Because of the use of freeze-dried materials versus fresh material, and the use of differently prepared materials in different concentrations, it was worth testing the necessity of pH 4.5 buffer solution in this study.

A representative subset of samples was prepared in duplicate according to the finalized preparation and extraction methods. To 0.6ml of each prepared sample, 2.4ml of pH 1.0 or pH 4.5 buffer was added, vortexed for one minute, and allowed to sit at room temperature for one hour. The absorbance spectrum of each sample was measured at 510 and 700nm, and the total anthocyanins were calculated with and without the inclusion of pH 4.5 buffer (**Figure A.14**).

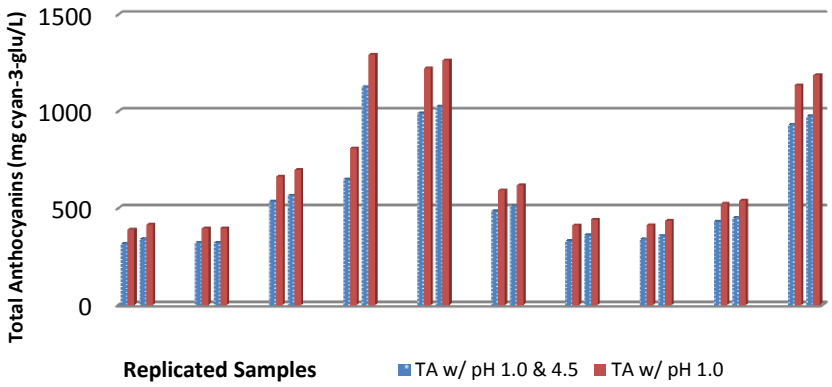

Figure A.14. Total Anthocyanins (mg/L) with and without the use of pH 4.5 buffer. Without accounting for impurities by subtracting the absorbance value of samples in pH 4.5 buffer, total anthocyanins measurements are approximately 22% lower.

For our samples, when both pH 1.0 and pH 4.5 buffers were used to perform the pH differential assay, total anthocyanins values were approximately 22% higher. This could have been due to fine particulates that could not have been filtered from the raspberry powder, or from degraded compounds present in the samples. These results were consistent among for all the samples tested, and it was deemed worthwhile to use both buffers when

performing the assay for this study. In the finalized methods, both pH 1.0 and pH 4.5 buffers were used to perform the pH differential assay for the calculation of total anthocyanins.

Concentration Linearity

Prior to this study, our laboratory had not performed the pH differential assay on freeze-dried berry material; therefore a concentration linearity test was performed to determine the best extract concentration to use for this experiment that is done traditionally on fresh-frozen material in our laboratory. A representative subset of fruit samples was chosen and weighed out in samples of raspberry powder ranging from 100 – 300mg, and were prepared and extracted according to our finalized procedures. To 0.6ml of each prepared sample, 2.4ml of pH 1.0 or pH 4.5 was added, vortexed for one minute, and allowed to sit at room temperature for one hour. Absorbance readings were taken at 510 and 700nm, and total anthocyanins were calculated. Averages of total anthocyanins by concentration were taken, and the linear relationship between the two were determined (**Figure A.15**).

There is a strong linear relationship between extract concentration and total anthocyanins (R^2=0.9975). Because of this linearity, the median value at 0.02g/ml was chosen to be the final extraction concentration throughout the entire study. This concentration does not have to be diluted from the originally prepared extract, further simplifying the assay.

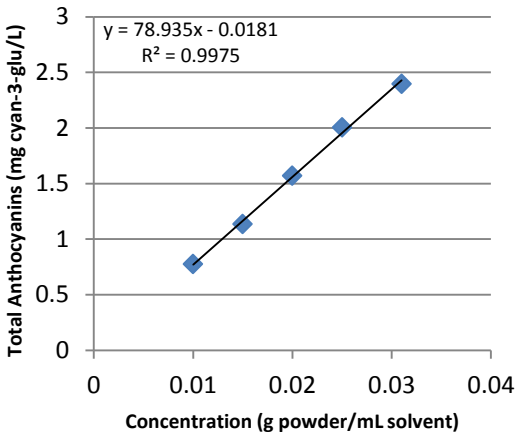

Figure A.15. Linearity of the pH differential assay when run at varying extract concentrations. Based on these results, the original extract does not need further diluting.

Increasing Absorbance over Time

In Giusti and Wrolstad, it is suggested that all absorbance measurements "should be made between 15 min and 1hr after sample preparation, since longer standing times tend to increase observed readings" (2001). Currently in our laboratory, total anthocyanins samples are left to sit in the refrigerator at 4°C for two hours before being absorbance is measured, in order to make sample analysis convenient when being run simultaneously with FRAP and Folin-Ciocalteu assays. The cooler temperatures slow the rate of increase in readings; however for this study we do not need to let the samples sit for that long. To determine the increase in absorbance over time, measurements were taken at 15 minute intervals over two hours on samples sitting at room temperature.

A representative subset of samples was prepared in duplicate according to the finalized preparation and extraction methods. To 0.6ml of each prepared sample, 2.4ml of pH 1.0 or pH 4.5 buffer was added, vortexed for one minute, and allowed to sit at room temperature for two hours. Every 15 minutes, the absorbance spectrum of each sample was measured at 510 and 700nm, with the difference between the two readings taken (**Figure A.16**).

There is a strong linear relationship between time elapsed after sample preparation and measured absorbance (R^2=0.9957). Because of this linearity, the median value at one hour was chosen to be the final resting time for samples throughout the entire study. This timing was convenient with the other assays, and allowed for minimal increase in absorbance values.

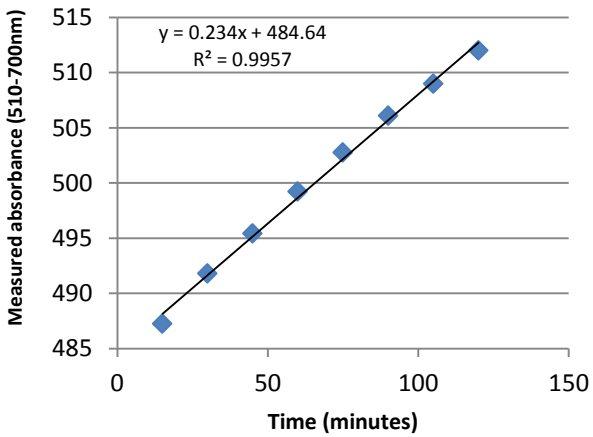

Figure A.16. Linearity of the pH differential assay in samples tested in 15 minute increments from over a 2 hour period of time.

Over the entire course of two hours, the absorbance values only increased by 5.08%, so waiting less or more time to measure absorbance would not drastically affect total anthocyanin values.

Finalized Methods

Raspberry fruit samples were weighed out into 200mg samples. Ten milliliters of solvent were added to each sample, vortexed for one minute, centrifuged for 20 minutes at 2790 x *g* and 4°C, and filtered through Whatman no.1 papers. To 0.6ml of each prepared sample, 2.4ml of pH 1.0 or pH 4.5 buffer was added, vortexed for one minute, and allowed to sit at room temperature for one hour. The absorbance of each sample was measured at 510 and 700nm, and total anthocyanins were calculated as:

Total Anthocyanins (mg cyanidin-3-glucoside equivalents/L) = (A * MW * 1000) / (Ɛ * 1) * DF

Where:

A = Absorbance pH 1.0 (510-700nm) - Absorbance pH 4.5 (510-700nm)

MW = Molecular weight = 449.2

Ɛ = Molar absorptivity = 26900

DF = Dilution factor = 41.85

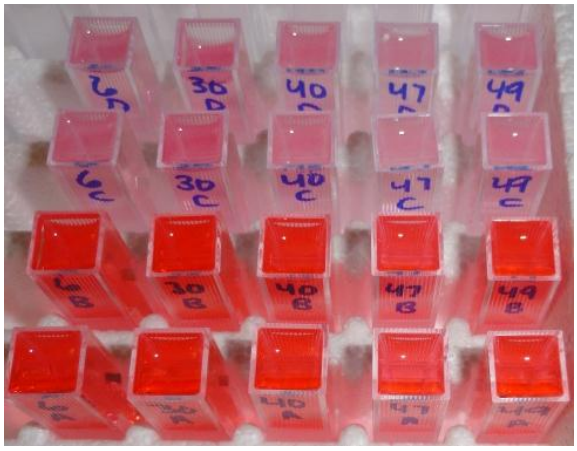

Figure A.17. Prepared samples for the pH differential assay in pH 1.0 (bottom) and pH 4.5 (top) buffer solutions, in 3ml disposable cuvettes.

7. FRAP Assay

Antioxidant capacity of the prepared extracts was measured by the ferric reducing antioxidant power (FRAP) method originally described by Benzie and Strain (1996). The FRAP assay is an electron transfer assay, which measures the ability of an antioxidant compound to reduce an oxidant probe, which changes color when reduced. In this assay, a ferric to ferrous ion reduction occurs at low pH, and causes a colored ferrous-tripyridyltriazine (TPTZ) complex to form. The color change is measured as light absorbance with a spectrophotometer at 593nm, and the extent of the color change is dependent on the antioxidant concentration (Benzie & Strain, 1996; Dai & Mumper, 2010). FRAP assays are reported in Trolox equivalents. Trolox is a well characterized vitamin E analogue that is appropriate for use as a standard (Diamanti et.al., 2010).

In order to further understand the original methods, and to better adapt our laboratory methods to this project, small experiments were done to determine proper blanks to load in the spectrophotometer and the linearity of assay concentration.

Spectrophotometer Blanks

As mentioned above, when using a spectrophometer, samples and blanks are placed into disposable 1-cm path length cuvettes. The solvent that is used in the blank cuvette depends on the absorbance spectrum of the solvent for the assay being run. For some assays, a deionized water blank is appropriate, while for other assays, the blank solvent should exactly match that being used in the samples. To determine the appropriate blank for the FRAP assay, the spectrums of water and FRAP solvent (consisting of 10 parts sodium acetate, 1 part TPTZ, and 1 part $FeCl_3$) were compared from 250 – 800nm (**Figure A.18**).

The differences in absorbance are clear when examining the spectra of water and FRAP solutions. At 593nm, the absorbance of the water blank was 0, while the absorbance of the FRAP solution blank was 0.034. This difference was enough to cause differences in the calculations of antioxidant capacity, and therefore FRAP solution was used as a blank for the finalized samples in the assay.

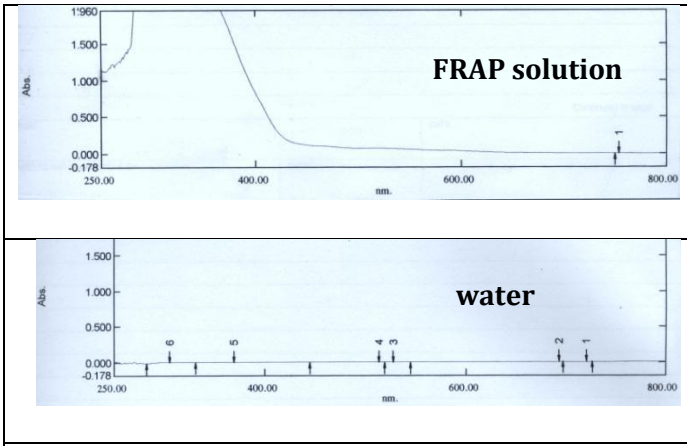

Figure A.18. Absorbance spectra of FRAP solution and water to determine the best blank against which to run samples for the assay.

Concentration Linearity

Prior to this study, our laboratory had not performed the FRAP assay on freeze-dried berry material; therefore a concentration linearity test was performed to determine the best extract concentration to use for this experiment that is done traditionally on fresh-frozen material in our laboratory. A representative subset of fruit samples was chosen and weighed out in samples of raspberry powder ranging from 100 – 300mg, and were prepared and extracted according to our finalized procedures. To 100µl of each prepared sample, 3.0ml of FRAP solution was added, vortexed for one minute, and allowed to sit at room temperature for one hour. Absorbance readings were taken at 593nm. Averages of absorbance readings by concentration were taken and the linear relationship between the two were determined (**Figure A.19**).

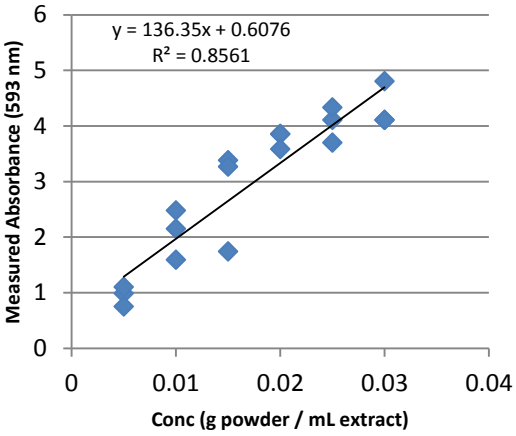

Figure A.19. Linearity of the FRAP assay when run at varying extract concentrations. A stronger linear relationship is seen when cultivars are evaluated individually.

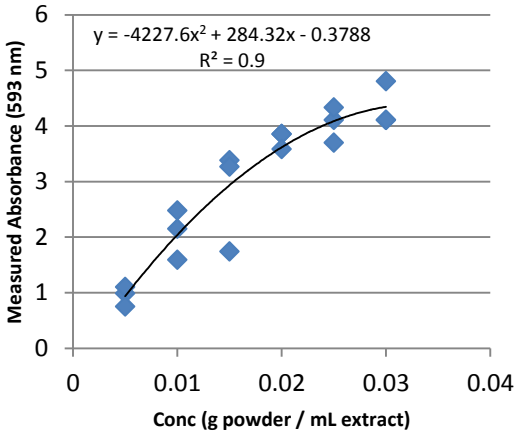

Figure A.20. Quadratic correction for the FRAP analysis has a slightly higher significance; however at the concentration we are using (0.02 g/ml), the line is still basically linear.

When examining **Figure A.19,** there is an evident linear relationship, however the significance of the relationship is relatively low (R^2=0.8561). When a quadratic correction is performed (**Figure A.20**), the significance increases only slightly (R^2=0.90). When the linearity relationship graphs for samples averaged across cultivars are looked at individually the significance values increase substantially for Autumn Britten (R^2= 0.925) and Caroline (R^2=0.9235), and slightly for Nantahala (R^2=0.8753). Additionally, the original methods written by Benzie and Strain describe the absorbance changes associated with the FRAP assay as "linear over a wide concentration range with antioxidant mixtures" (1996). With this in mind, it is best to consider the FRAP assay as linear, and take the median extract concentration of 0.02g/ml as the final extraction concentration throughout the entire study. This concentration does not have to be diluted from the originally prepared extract, further simplifying the assay.

Finalized Methods

Raspberry fruit samples were weighed out into 200mg samples. Ten milliliters of solvent were added to each sample, vortexed for one minute, centrifuged for 20 minutes at 2790 x *g* and 4°C, and filtered through Whatman no.1 papers. FRAP solution, consisting of 10 parts sodium acetate, 1 part TPTZ, and 1 part $FeCl_3$, was prepared, and to 100µl of each prepared sample, 3ml of FRAP solution was added, vortexed for one minute, and allowed to sit at room temperature for one hour. The absorbance of each sample was measured at 593nm, and antioxidant capacity was calculated as:

Antioxidant Capacity (µmoles Trolox Equivalents /mg FW) = M * A * DF * V / W /1000

Where:

M = slope of Trolox standard curve = 478.47

A = Absorbance at 593nm

DF = Dilution factor = 1

V = Volume of solvent extract = 1ml

W = Sample fruit weight = 0.135684 g

8. Total Phenolics Assay

Total phenolics of the prepared extracts were measured by the Folin-Ciocalteu method, originally described by Singleton and Rossi (1965). In this assay, phenolic compounds are oxidized by a yellow molybdotungstophosphoric heteropolyanion reagent, and the subsequent blue-colored product can be measured with a UV-spectrophotometer at 726nm. Total phenolics measurements are expressed in Gallic acid equivalents. Gallic acid makes a good reference standard because it is inexpensive, easy to obtain, and has well-characterized stability and solubility. This assay is relatively robust, however some other substances than phenols can be oxidized, such as aromatic amines, sulfur dioxide, and ascorbic acid, that may affect the measured phenolic content (Singleton & Rossi, 1965; Slinkard & Singleton, 1977)

In order to further understand the methods originally described by Singleton and Rossi, and to better adapt our laboratory methods to this project, small experiments were done to determine proper blanks to load in the spectrophotometer and the linearity of assay concentration.

Spectrophotometer Blanks

As described above, when using a spectrophometer, samples and blanks are placed into disposable 1-cm path length cuvettes. The solvent that is used in the blank cuvette depends on the absorbance spectrum of the solvent for the assay being run. For some assays, a deionized water blank is appropriate, while for other assays, the blank solvent should exactly match that being used in the samples. To determine the appropriate blank for the Folin-

Ciocalteu assay, the spectrums of water and phenolics solvent (consisting of 1 part Folin-Ciocalteu reagent, 1 part sodium carbonate, and 8 parts water) were compared from 250 – 800nm (**Figure A.21**).

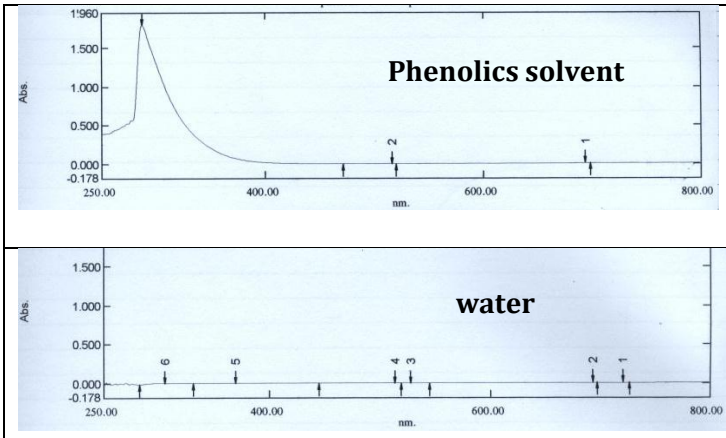

Figure A.21. Absorbance spectra of Folin-Ciocalteu reagent and water to determine the best blank against which to run samples for the assay.

The differences in absorbance are clear when examining the spectra of water and phenolics solutions. At 726 nm, the absorbance of the water blank was 0.003, while the absorbance of the phenolics solution blank was 0.0005. This difference was not enough to cause differences in the calculations of total phenolics, and therefore a water blank was determined to be adequate for use as a blank for the finalized samples in the assay.

Concentration Linearity

Prior to this study, our laboratory had not performed the Folin-Ciocalteu assay on freeze-dried berry material; therefore a concentration linearity test was performed to determine the best extract concentration to use for this experiment that is done traditionally on fresh-frozen

material in our laboratory. A representative subset of fruit samples was chosen and weighed out in samples of raspberry powder ranging from 100 – 300mg, and were prepared and extracted according to our finalized procedures. To 0.3µl of each prepared sample, 2.7ml of phenolics reagents were added in several steps (see finalized methods below), vortexed for one minute, and allowed to sit at room temperature for two hours. Absorbance readings were taken at 726nm. Averages of absorbance values by concentration were taken, and the linear relationship between the two were determined (**Figure A.22**).

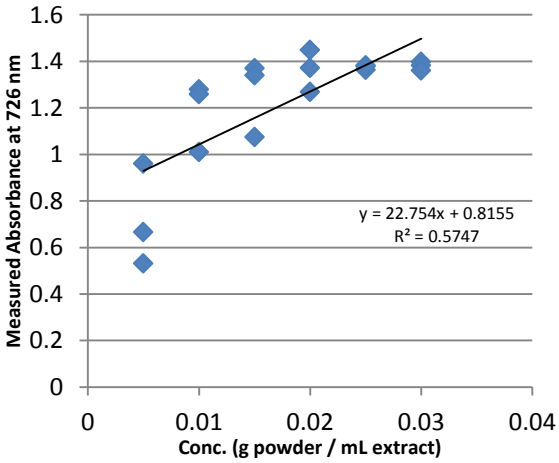

Figure A.22. Linearity of the total phenolics assay when run at varying extract concentrations.

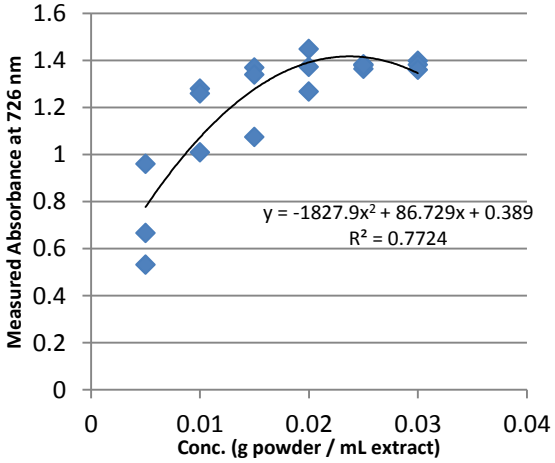

Figure A.23. Quadratic correction for the FRAP analysis has a slightly higher significance; however the R^2 value is still low.

When examining **Figure A.22,** the linear relationship is not significant (R^2=0.5747). When a quadratic correction is performed (**Figure A.23**), the significance increases only slightly (R^2=0.7724). We hypothesized that these poor results were due to assay saturation, so the samples were diluted and a new linearity test was performed (**Figure A.24**).

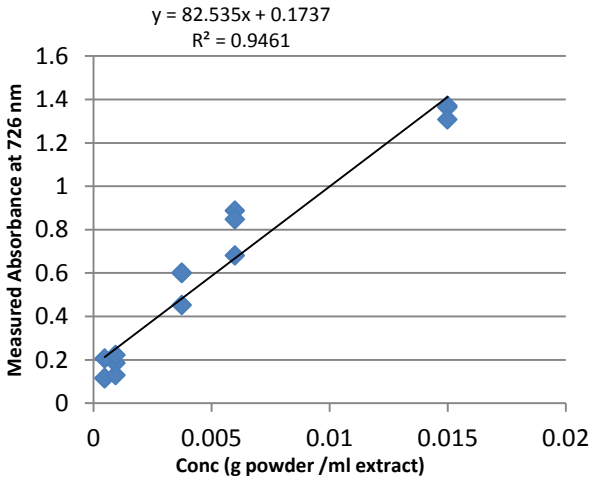

Figure A.24. Linearity of the total phenolics assay when run at various diluted extract concentrations. These concentrations significantly impact the R^2 value, and improve the linearity of the assay.

From this graph, it is clear that the assay is linear, but becomes saturated at higher concentrations and no longer fits a linearity scheme. With better linearity and absorbance resolution seen in the diluted phenolics samples, the finalized samples were diluted two times to 0.01 grams raspberry powder per milliliter extraction solvent.

Finalized Methods

Raspberry fruit samples were weighed out into 200mg samples. Ten milliliters of solvent were added to each sample, vortexed for one minute, centrifuged for 20 minutes at 2790 x *g* and 4°C, and filtered through Whatman no.1 papers. To 150µl of each prepared sample, 150µl of 60:39:1 extraction solvent was added and vortexed for one minute to dilute. To 0.3ml of each diluted sample, 0.3ml of Folin-Ciocalteu reagent was added, vortexed for one minute, and allowed to stand for three minutes. Next, 0.3ml of sodium carbonate was added,

vortexed for one minute and allowed to sit for seven minutes. Finally, 2.1ml of deionized water was added, vortexed for one minute, and allowed to sit at room temperature for two hours. The absorbance of each sample was measured at 726nm, and antioxidant capacity was calculated as:

Total Phenolics (mg Gallic Acid Equivalents/ kg FW) = M * A * DF

Where:

M = slope of Gallic acid standard curve = 117.62

A = Absorbance at 726nm

DF = Dilution factor = 15.74

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B. Comparison of anthocyanin composition in tunnel and field grown red raspberry through HPLC-DAD analysis

Plant Materials. Primocane-fruiting red raspberry cultivars 'Autumn Britten', 'Caroline', and 'Nantahala' were harvested from July 22 to September 20, 2010 from replicated trials at three research stations located in North Carolina with varying elevations and temperature fluctuations (see **Table 2.1**). Standard practices for raspberry cultivation in North Carolina were followed (Fernandez et.al.). Fruit was grown in replicated trials under quonset-style rounded top high tunnels covered in polyethylene greenhouse-grade plastic, or in standard field cultivation at each location. Fruit samples were frozen immediately after harvest for 24 hours at -20°C, and then stored at -80°C until lyophilization. Freeze-dried samples were stored at -20°C until used.

Standards. Cyanidin-3-glucoside and Gallic acid were obtained from Extrasynthese (Genay, France).

Sample Preparation. For each sample, twenty grams of freeze-dried raspberries were homogenized with a mortar and pestle, and seeds were separated from raspberry powder through a 2mm mesh sieve. Two extractions were performed, where solvent containing five milliliters of LC-MS grade methanol in water (60:39) with 1% formic acid was added to 100 mg of powder, and vortexed for one minute to mix. Samples were centrifuged at 4°C for 20

minutes at 2790 x *g*. Supernatant from each extraction was filtered through Whatman no.1 paper, pooled, and stored at -80°C in 15ml brown glass tubes until analysis.

HPLC Conditions. Prepared extracts were filtered through 0.2 µm PTFE membranes. Stock solutions for standard curve calculation were prepared from 0.0625 – 0.5 mg/mL for each standard. Ten µL aliquot samples were injected at ambient temperature into an Agilent 1200 Series HPLC system equipped with a binary solvent delivery manager and a sample manager (Agilent Corporation, Santa Clara, CA), and fitted with a Suplecoil LC-18 reversed-phase (4.6 x 250 mm, 5 µm particle size) analytical chromatography column. The mobile phases consisted of 5% formic acid in water for A and methanol for B, with flow rate of 1ml/min and elution gradient: 0-5 min, 10-15% B; 5-15 min, 15-20% B; 15-20 min, 20-25% B; 20-25 min, 25-30% B; 25-45 min, 30-60% B; 45-47 min, 60-10% B, 47-60 min, 10% B. The column temperature was held at 30ºC, and the solvent flow rate was 1 mL/min. Anthocyanins were detected at 520 nm, and their presence was verified at 280 nm. From the prepared standard curves and recorded peak area of samples, concentration of the anthocyanins and phenolic acids was calculated and reported as mg/g dry weight cyanidin-3-glucoside (if detected at 520 nm) or gallic acid (if detected at 280 nm) equivalents, respectively. Compound identification was performed based on chromatogram structure, retention time, comparison to cyanidin-3-glucoside or caffeic acid external standards, and previously reported findings (Maatta-Riihinen et.al., 2004; Mullen et.al., 2002). Chromatogram structure and retention time are reported for all identified compounds (**Table B.1, Figure B.1-6**).

Measurement of Total Anthocyanins. Total anthocyanins of the prepared extracts were determined by the pH-differential method described by Giusti and Wrolstead (2001) using a Shimadzu UV-2450 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) set to read absorbance at 510 and 700nm, through 1-cm path length disposable cuvettes. Samples were replicated and analyzed in triplicate, with means calculated. Total anthocyanins were reported as mg cyanidin-3-glucoside equivalents /L.

Total Phenolics Measurement. Total phenolics of the prepared extracts were determined by the Folin-Ciocalteu method described by Singleton and Rossi (1965). Absorbance of samples and gallic acid standards was read at 765nm by spectrophotometer (Shimadzu UV-2450, Shimadzu Scientific Instruments, Columbia, MD), and samples were analyzed in triplicate, with means calculated. Total phenolics were reported as mg Gallic acid equivalents (GAE)/100g fresh weight.

Antioxidant Capacity Measurement. Antioxidant capacity of the prepared extracts was determined by the ferric reducing antioxidant power (FRAP) assay initially described by Benzie and Strain (1996). Absorbance of samples and Trolox standards was read at 593nm by spectrophotometer (Shimadzu UV-2450, Shimadzu Scientific Instruments, Columbia, MD), and samples were replicated and analyzed in triplicate, with means calculated. Antioxidant capacity was reported as μmoles Trolox equivalents / g fresh weight.

Statistical Analysis. SAS (SAS Institute, Cary, NC) and JMP Genomics 8.0.2 (SAS Institute, Cary, NC) statistical software programs were used to perform statistical analyses.

One-way analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) were used to evaluate differences in overall anthocyanin composition, and to examine cultivar, location, and tunnel vs. field effects on the concentration of each quantified and detected anthocyanin or phenolic acid and total phenolics, anthocyanins, and antioxidant capacity. LSMEANS with a Bonferroni correction was used to make pair-wise comparisons among sample groups (cultivars, locations, cultivation types).

Results. At 520 nm, the HPLC-DAD detected either four or eight anthocyanins, depending on cultivar. For both 'Autumn Britten' and 'Caroline', eight anthocyanins each were detected, and for 'Nantahala', four anthocyanins were detected (**Figure B.2**). At 280 nm, the HPLC-DAD detected two phenolic acids each for all three cultivars (**Figure B.1**). From preliminary statistical analysis, it appears that the primary contributing factor to variation in anthocyanin concentration is cultivar. For the majority of detected anthocyanin and phenolic acid compounds, there appears to be no significant difference in concentration between field and tunnel grown fruit samples; however further analysis is needed.

Table B.1. Spectral data from HPLC-DAD analysis performed on raspberry samples grown in field and tunnels. Compounds marked with an * were found in all samples.

Compound	R_t (min)	DAD (nm)
Ellagitannin 1*	11.9	280
Ellagitannin 2*	12.8	280
Cyanidin-3-sophoroside*	26.2	520
Cyanidin-3-(2^G-glucosylrutinoside)	27.9	520
Anthocyanin 3*	28.7	520
Cyanidin-3-glucoside*	29.6	520
Anthocyanin 5	30.8	520
Cyanidin-3-rutinoside	31.6	520
Anthocyanin 7*	32.4	520
Anthocyanin 8	34.3	520

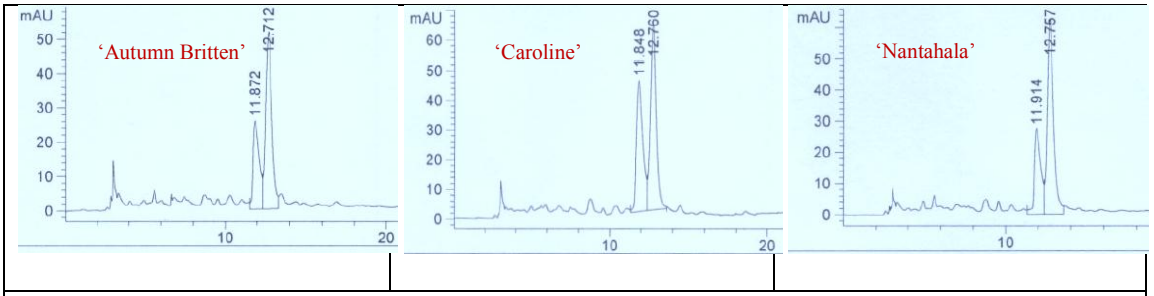

Figure B.1. Chromatographic pattern at 280 nm for 'Autumn Britten', 'Caroline', and 'Nantahala', showing ellagitannins present in all raspberry samples.

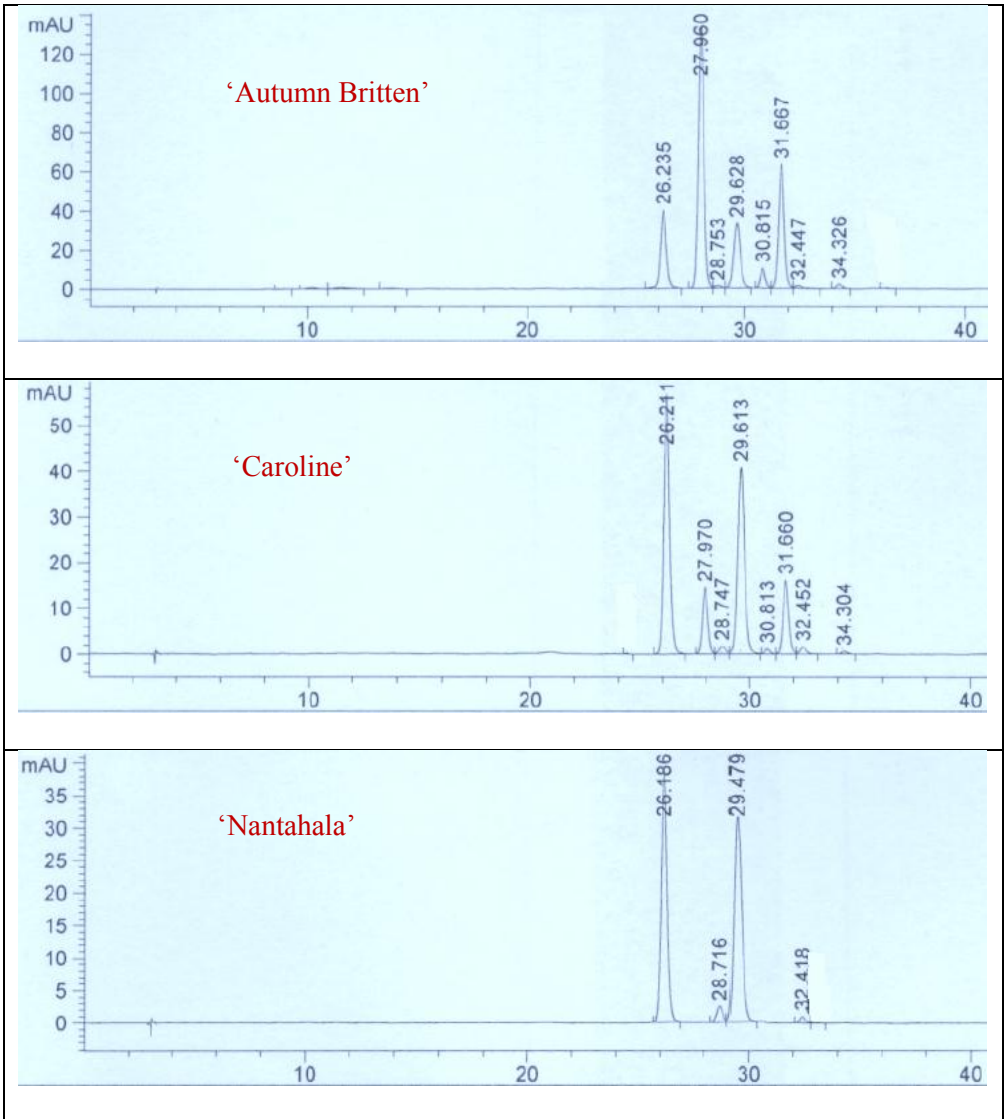

Figure B.2. Chromatographic pattern at 520 nm for 'Autumn Britten', 'Caroline', and 'Nantahala', showing anthocyanins present in raspberry samples.

References Cited

Maatta-Riihinen, K., A. Kamal-Eldin, and A. Torronen. 2004. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (family Rosaceae). Journal of Agricultural and Food Chemistry 52: 6178-6187.

Mullen, W., J. McGinn, M. Lean, M. MacLean, P. Gardner, G. Duthie, T. Yokota, A. Crozier. 2002. Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. Journal of Agricultural and Food Chemistry 50: 5191-5196.

C. SAS Outputs for Reference

Principal Components Analysis

The PRINCOMP Procedure

Observations 54
Variables 5

Simple Statistics

	x2	x3	x4	x9	x10
Mean	3.367373562	0.0242624369	0.0706823441	4.168146579	3.40528090
StD	1.455903672	0.0196968679	0.0686800990	4.906281197	2.191419786

Covariance Matrix

	x2	x3	x4	x9	x10
x2	2.11965550	0.01167444	-0.01143737	-2.58354626	2.41095527
x3	0.01167444	0.00038797	0.00055191	-0.04114023	-0.00143549
x4	-0.01143737	0.00055191	0.00471696	-0.19346142	-0.06560099
x9	-2.58354626	-0.04114023	-0.19346142	24.07159518	-2.02863285
x10	2.41095527	-0.00143549	-0.06560099	-2.02863285	4.80232068

Total Variance 30.998676285

Eigenvalues of the Covariance Matrix

	Eigenvalue	Difference	Proportion	Cumulative
1	24.6408153	18.9210925	0.7949	0.7949
2	5.7197227	5.0834413	0.1845	0.9794
3	0.6362814	0.6346206	0.0205	0.9999
4	0.0016608	0.0014647	0.0001	1.0000
5	0.0001961		0.0000	1.0000

Eigenvectors

	Prin1	Prin2	Prin3	Prin4	Prin5
x2	-.125439	0.466400	0.875515	-.008676	-.011600
x3	-.001698	-.000430	0.013602	0.038002	0.999184

x4	-.007370	-.016385	0.017066	0.998961	-.038246
x9	0.985271	0.161604	0.055173	0.009002	0.000650
x10	-.115971	0.869532	-.479535	0.021824	0.005875

Factor Analysis with Principal Components Analysis

The FACTOR Procedure
Initial Factor Method: Principal Components

Prior Communality Estimates: ONE

Eigenvalues of the Covariance Matrix: Total = 30.9986763 Average = 6.19973526

	Eigenvalue	Difference	Proportion	Cumulative
1	24.6408153	18.9210925	0.7949	0.7949
2	5.7197227	5.0834413	0.1845	0.9794
3	0.6362814	0.6346206	0.0205	0.9999
4	0.0016608	0.0014647	0.0001	1.0000
5	0.0001961		0.0000	1.0000

2 factors will be retained by the NFACTOR criterion.

Factor Pattern

	Factor1	Factor2
x2	-0.42769	0.76615
x3	-0.42789	-0.05224
x4	-0.53268	-0.57057
x9	0.99685	0.07877
x10	-0.26270	0.94896

Variance Explained by Each Factor

Factor	Weighted	Unweighted
Factor1	24.6408153	1.71248452
Factor2	5.7197227	1.82198944

Final Communality Estimates and Variable Weights
Total Communality: Weighted = 30.360538 Unweighted = 3.534474

Variable	Communality	Weight

x2	0.76990304	2.1196555
x3	0.18582158	0.0003880
x4	0.60929761	0.0047170
x9	0.99991953	24.0715952
x10	0.96953219	4.8023207

The FACTOR Procedure
Initial Factor Method: Principal Components

Residual Correlations With Uniqueness on the Diagonal

	x2	x3	x4	x9	x10
x2	0.23010	0.26412	0.09493	0.00430	-0.08373
x3	0.26412	0.81418	0.15024	0.00495	-0.09609
x4	0.09493	0.15024	0.39070	0.00182	-0.03436
x9	0.00430	0.00495	0.00182	0.00008	-0.00157
x10	-0.08373	-0.09609	-0.03436	-0.00157	0.03047

Root Mean Square Off-Diagonal Residuals: Overall = 0.10900445

x2	x3	x4	x9	x10
0.14646070	0.15936764	0.09051141	0.00349179	0.06600428

Partial Correlations Controlling Factors

	x2	x3	x4	x9	x10
x2	1.00000	0.61023	0.31662	0.99996	-1.00000
x3	0.61023	1.00000	0.26639	0.61132	-0.61008
x4	0.31662	0.26639	1.00000	0.32499	-0.31490
x9	0.99996	0.61132	0.32499	1.00000	-0.99994
x10	-1.00000	-0.61008	-0.31490	-0.99994	1.00000

Root Mean Square Off-Diagonal Partials: Overall = 0.67037986

x2	x3	x4	x9	x10
0.78621672	0.54526608	0.30658972	0.78726483	0.78600940

Factor Analysis with a Varimax Rotation

The FACTOR Procedure
Rotation Method: Varimax

Orthogonal Transformation Matrix

	1	2
1	-0.30617	0.95198
2	0.95198	0.30617

Rotated Factor Pattern

	Factor1	Factor2
x2	0.86030	-0.17258
x3	0.08128	-0.42334
x4	-0.38007	-0.68179
x9	-0.23021	0.97310
x10	0.98382	0.04046

Variance Explained by Each Factor

Factor	Weighted	Unweighted
Factor1	7.4933813	1.91207558
Factor2	22.8671567	1.62239837

Final Communality Estimates and Variable Weights
Total Communality: Weighted = 30.360538 Unweighted = 3.534474

Variable	Communality	Weight
x2	0.76990304	2.1196555
x3	0.18582158	0.0003880
x4	0.60929761	0.0047170
x9	0.99991953	24.0715952
x10	0.96953219	4.8023207

ANOVA Mixed Model with Fixed GEI

The Mixed Procedure

Model Information

Data Set	WORK.GEI
Dependent Variable	x2
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Gen	3	1 2 3
Rep	3	1 2 3
Env	3	1 2 3

Dimensions

Covariance Parameters	2
Columns in X	16
Columns in Z	9
Subjects	1
Max Obs Per Subject	27

Number of Observations

Number of Observations Read	27
Number of Observations Used	27
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	-6.79657077	
1	1	-7.07093206	0.00000000

Convergence criteria met.

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate	Standard Error	Z Value	Pr > Z
Rep(Env)	0.002957	0.006237	0.47	0.3177
Residual	0.02022	0.008253	2.45	0.0072

Fit Statistics

-2 Res Log Likelihood	-7.1
AIC (smaller is better)	-3.1
AICC (smaller is better)	-2.3
BIC (smaller is better)	-2.7

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Env	2	6	2.60	0.1539
Gen	2	12	46.74	<.0001
Gen*Env	4	12	1.73	0.2086

---------- Effect=Gen Method=LSD(P<0.05) Set=1 ----------

Obs	Gen	Estimate	Standard Error	Letter Group
1	2	1.1813	0.05074	A
2	1	0.6315	0.05074	B
3	3	0.6094	0.05074	B

ANOVA Models

The GLM Procedure

Class Level Information

Class	Levels	Values
factor1	3	ABritt Caroline Nantahal

Number of Observations Read	54
Number of Observations Used	54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	75.5083830	37.7541915	52.27	<.0001
Error	51	36.8333586	0.7222227		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.672131	25.23738	0.849837	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	52.27	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	52.27	<.0001

The GLM Procedure

Class Level Information

Class	Levels	Values
factor2	3	MHCRS PRS UMRS

Number of Observations Read 54
Number of Observations Used 54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	7.2233119	3.6116560	1.75	0.1837
Error	51	105.1184296	2.0611457		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.064298	42.63468	1.435669	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor2	2	7.22331192	3.61165596	1.75	0.1837

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor2	2	7.22331192	3.61165596	1.75	0.1837

The GLM Procedure

Class Level Information

Class	Levels	Values
factor1	3	ABritt Caroline Nantahal
factor2	3	MHCRS PRS UMRS

Number of Observations Read	54
Number of Observations Used	54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	82.7316949	20.6829237	34.23	<.0001
Error	49	29.6100467	0.6042867		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.736429	23.08502	0.777359	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	62.48	<.0001
factor2	2	7.22331192	3.61165596	5.98	0.0048

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	62.48	<.0001
factor2	2	7.22331192	3.61165596	5.98	0.0048

The GLM Procedure

Class Level Information

Class	Levels	Values
factor1	3	ABritt Caroline Nantahal
factor2	3	MHCRS PRS UMRS

Number of Observations Read	54
Number of Observations Used	54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	8	86.6304825	10.8288103	18.95	<.0001
Error	45	25.7112591	0.5713613		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.771134	22.44730	0.755884	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	66.08	<.0001
factor2	2	7.22331192	3.61165596	6.32	0.0038
factor1*factor2	4	3.89878759	0.97469690	1.71	0.1653

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	66.08	<.0001
factor2	2	7.22331192	3.61165596	6.32	0.0038
factor1*factor2	4	3.89878759	0.97469690	1.71	0.1653

The GLM Procedure

Class Level Information

Class	Levels	Values
factor4	3	Aug July Sep

Number of Observations Read	54
Number of Observations Used	54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	7.1619689	3.5809844	1.74	0.1864
Error	51	105.1797727	2.0623485		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.063752	42.64712	1.436088	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor4	2	7.16196890	3.58098445	1.74	0.1864

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor4	2	7.16196890	3.58098445	1.74	0.1864

The GLM Procedure

Class Level Information

Class	Levels	Values
factor1	3	ABritt Caroline Nantahal
factor4	3	Aug July Sep

Number of Observations Read	54
Number of Observations Used	54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	81.2511578	11.6073083	17.17	<.0001
Error	46	31.0905837	0.6758823		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.723250	24.41430	0.822121	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	55.86	<.0001
factor4	2	3.20224974	1.60112487	2.37	0.1049
factor1*factor4	3	2.54052514	0.84684171	1.25	0.3016

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor1	2	57.35337938	28.67668969	42.43	<.0001
factor4	2	3.10084855	1.55042427	2.29	0.1123
factor1*factor4	3	2.54052514	0.84684171	1.25	0.3016

ANCOVA Models

The GLM Procedure

Class Level Information

Class	Levels	Values
factor1	3	ABritt Caroline Nantahal

Number of Observations Read	54
Number of Observations Used	54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	80.3458072	16.0691614	24.11	<.0001
Error	48	31.9959343	0.6665820		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.715191	24.24574	0.816445	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	56.64	<.0001
factor33	1	3.05524579	3.05524579	4.58	0.0374
factor33*factor1	2	1.78217849	0.89108925	1.34	0.2723

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor1	2	3.44448369	1.72224184	2.58	0.0860
factor33	1	3.56832927	3.56832927	5.35	0.0250
factor33*factor1	2	1.78217849	0.89108925	1.34	0.2723

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	82.4548152	16.4909630	26.49	<.0001
Error	48	29.8869263	0.6226443		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.733964	23.43304	0.789078	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	60.64	<.0001
factor5	1	2.91913908	2.91913908	4.69	0.0354
factor5*factor1	2	4.02729320	2.01364660	3.23	0.0481

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor1	2	28.63548525	14.31774262	23.00	<.0001
factor5	1	2.05924888	2.05924888	3.31	0.0752
factor5*factor1	2	4.02729320	2.01364660	3.23	0.0481

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	79.7280674	15.9456135	23.47	<.0001
Error	48	32.6136741	0.6794515		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.709692	24.47868	0.824289	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor1	2	75.50838296	37.75419148	55.57	<.0001
factor7	1	2.23379994	2.23379994	3.29	0.0761
factor7*factor1	2	1.98588454	0.99294227	1.46	0.2420

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor1	2	0.08793868	0.04396934	0.06	0.9374
factor7	1	2.13353566	2.13353566	3.14	0.0827
factor7*factor1	2	1.98588454	0.99294227	1.46	0.2420

Correlation Models

The GLM Procedure

Number of Observations Read 54
Number of Observations Used 54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	6.6971948	6.6971948	3.30	0.0752
Error	52	105.6445468	2.0316259		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.059614	42.32828	1.425351	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor33	1	6.69719478	6.69719478	3.30	0.0752

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor33	1	6.69719478	6.69719478	3.30	0.0752

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	8.257232451	2.70019506	3.06	0.0035
factor33	-0.020788252	0.01144968	-1.82	0.0752

The GLM Procedure

Number of Observations Read 54
Number of Observations Used 54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	4.2634661	4.2634661	2.05	0.1581
Error	52	108.0782754	2.0784284		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.037951	42.81306	1.441676	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor5	1	4.26346613	4.26346613	2.05	0.1581

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor5	1	4.26346613	4.26346613	2.05	0.1581

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	3.133324617	0.25533113	12.27	<.0001
factor5	0.109520303	0.07646818	1.43	0.1581

The GLM Procedure

Number of Observations Read 54
Number of Observations Used 54

The GLM Procedure

Dependent Variable: x2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	2.3124530	2.3124530	1.09	0.3007
Error	52	110.0292886	2.1159479		
Corrected Total	53	112.3417416			

R-Square	Coeff Var	Root MSE	x2 Mean
0.020584	43.19776	1.454630	3.367374

Source	DF	Type I SS	Mean Square	F Value	Pr > F
factor7	1	2.31245299	2.31245299	1.09	0.3007

Source	DF	Type III SS	Mean Square	F Value	Pr > F
factor7	1	2.31245299	2.31245299	1.09	0.3007

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Intercept	2.254753785	1.08254890	2.08	0.0422
factor7	0.056793741	0.05432710	1.05	0.3007

LSMEANS Procedures

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Bonferroni

factor1	x2 LSMEAN	LSMEAN Number
ABritt	2.13952140	1
Caroline	4.76791359	2
Nantahal	2.52011143	3

Least Squares Means for effect factor1
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: x2

i/j	1	2	3
1		<.0001	0.8962
2	<.0001		<.0001
3	0.8962	<.0001	

factor2	x2 LSMEAN	LSMEAN Number
MHCRS	3.70143499	1
PRS	3.19027128	2
UMRS	2.53584014	3

Least Squares Means for effect factor2
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: x2

i/j	1	2	3
1		0.4018	0.0107
2	0.4018		0.4067
3	0.0107	0.4067	

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Bonferroni

factor1	factor2	x2 LSMEAN	LSMEAN Number
ABritt	MHCRS	2.81776043	1
ABritt	PRS	1.93360346	2
ABritt	UMRS	1.66720031	3
Caroline	MHCRS	5.52465074	4
Caroline	PRS	5.20779018	5
Caroline	UMRS	3.57129985	6
Nantahal	MHCRS	2.76189382	7
Nantahal	PRS	2.42942019	8
Nantahal	UMRS	2.36902027	9

Least Squares Means for effect factor1*factor2
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: x2

i/j	1	2	3	4	5	6	7	8	9
1		1.0000	1.0000	<.0001	0.0351	1.0000	1.0000	1.0000	1.0000
2	1.0000		1.0000	<.0001	0.0014	0.9303	1.0000	1.0000	1.0000
3	1.0000	1.0000		<.0001	0.0031	0.8587	1.0000	1.0000	1.0000
4	<.0001	<.0001	<.0001		1.0000	0.2127	<.0001	<.0001	0.0006
5	0.0351	0.0014	0.0031	1.0000		1.0000	0.0254	0.0224	0.0401
6	1.0000	0.9303	0.8587	0.2127	1.0000		1.0000	1.0000	1.0000
7	1.0000	1.0000	1.0000	<.0001	0.0254	1.0000		1.0000	1.0000
8	1.0000	1.0000	1.0000	<.0001	0.0224	1.0000	1.0000		1.0000
9	1.0000	1.0000	1.0000	0.0006	0.0401	1.0000	1.0000	1.0000	

Mixed Models with Location as a Random Effect

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
factor2	0.08614
Residual	2.0611

Fit Statistics

-2 Res Log Likelihood	193.9
AIC (smaller is better)	197.9
AICC (smaller is better)	198.1
BIC (smaller is better)	196.0

Model Information

Data Set	WORK.LCMSNEW
Dependent Variable	x2
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
factor1	3	ABritt Caroline Nantahal
factor2	3	MHCRS PRS UMRS

Dimensions

Covariance Parameters	2
Columns in X	4
Columns in Z	3
Subjects	1
Max Obs Per Subject	54

Number of Observations

Number of Observations Read	54
Number of Observations Used	54
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	136.80633822	
1	1	131.28955602	0.00000000

Convergence criteria met.

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
factor2	0.1671
Residual	0.6043

Fit Statistics

-2 Res Log Likelihood	131.3
AIC (smaller is better)	135.3
AICC (smaller is better)	135.5
BIC (smaller is better)	133.5

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
factor1	2	49	62.48	<.0001

The Mixed Procedure

Model Information

Data Set	WORK.LCMSNEW
Dependent Variable	x2
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
factor1	3	ABritt Caroline Nantahal
factor2	3	MHCRS PRS UMRS

Dimensions

Covariance Parameters	2
Columns in X	8
Columns in Z	3
Subjects	1
Max Obs Per Subject	54

Number of Observations

Number of Observations Read	54
Number of Observations Used	54
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	136.41483327	
1	3	129.82551809	0.00193377
2	1	129.77825430	0.00021507
3	1	129.77343815	0.00000356
4	1	129.77336328	0.00000000

Convergence criteria met.

The Mixed Procedure

Covariance Parameter
Estimates

Cov Parm	Estimate
factor2	0.1998
Residual	0.5105

Fit Statistics

-2 Res Log Likelihood	129.8
AIC (smaller is better)	133.8
AICC (smaller is better)	134.0
BIC (smaller is better)	132.0

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
factor1	2	46	27.89	<.0001
factor5	1	46	2.47	0.1231
factor5*factor1	2	46	3.87	0.0279

The Mixed Procedure

Model Information

Data Set	WORK.LCMSNEW
Dependent Variable	x2
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
factor1	3	ABritt Caroline Nantahal
factor2	3	MHCRS PRS UMRS

Dimensions

Covariance Parameters	2
Columns in X	8
Columns in Z	3
Subjects	1

Max Obs Per Subject 54

Number of Observations

Number of Observations Read	54
Number of Observations Used	54
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	142.73876961	
1	3	138.21099063	0.00009251
2	1	138.20854193	0.00000084
3	1	138.20852086	0.00000000

Convergence criteria met.

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
factor2	0.1915
Residual	0.5829

Fit Statistics

-2 Res Log Likelihood	138.2
AIC (smaller is better)	142.2
AICC (smaller is better)	142.5
BIC (smaller is better)	140.4

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
factor1	2	46	0.00	0.9993
factor7	1	46	0.10	0.7580
factor7*factor1	2	46	2.21	0.1214