Cultivated and Wild Highbush Blueberry Composition and Influence of Brown Marmorated Stink Bug Infestation on Its Anthocyanin and Phenolics Accumulation

Thesis

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Abstract

Blueberry was firstly domesticated almost 100 years ago. In the past century, the cultivated blueberries have gradually owned some desirable characteristics by selection. Field observations on blueberry revealed differences in insect selection preference exhibited between cultivated and wild highbush blueberries. However, little is known about the link between insect selection and domestication.

There are a number of pests that can infect blueberries, and the brown marmorated stink bug (BMSB) is a potential serious pest for them. Some studies have focused on the blueberries feeding injury caused by BMSB, few works have studied the response of the blueberry plant to this pest, so little is known about influence of BMSB infestation on blueberry secondary metabolites, especially anthocyanin and phenolics accumulation.

In this research, cultivated and wild highbush blueberries in New Jersey were analyzed comparatively to determine their quality attributes including pH, total soluble solids and individual weight, as well as their content of anthocyanin and phenolics. Samples were collected from 8 different locations to determine the interaction between domestication and location. Result showed that cultivated highbush blueberry had bigger fruit weight and higher pH value than wild blueberries, while wild blueberries were with higher...
anthocyanin and phenolic content. The growing location also affected pH and phenolic content of blueberry.

The influence of BMSB infestation on highbush blueberry sugar, anthocyanin and phenolic accumulation was also determined in this experiment. Blueberries provided by the Marucci Blueberry and Cranberry Research and Extension Center in New Jersey were either mechanically damaged or infected by 0, 2, 5 or 10 adult or nymph BMSB and were collected at select time points from 3 different plots. UV-Vis spectrophotometry was applied for anthocyanins and phenolics quantitative analysis. The HPLC chromatographs were recorded to determine compositional differences among samples. The results showed that when samples were compared at similar maturity degree, the 2 or 5 nymph BMSB infestation stimulated blueberries to produce more phenolic compounds, and modified the proportion of certain individual anthocyanins, with little effect on anthocyanin content and sugar profile.
Dedication

This document is dedicated to my family.
Acknowledgments

I would like to express my gratitude to my advisor, Monica Giusti, for her mental support, encouragement, knowledge and patience with my mistakes and progress throughout the duration of my degree. I appreciate her vast knowledge and skill in many areas (e.g., anthocyanin and phenolics extraction, purification and HPLC analysis), and her assistance in writing thesis.

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Highbush blueberries are widely grown across the northern states of the United States and welcomed in the grocery store. It is also the first domesticated blueberry crop, marketed in 1916, represented a significant advance in blueberry quality and launched the modern blueberry industry. The highbush blueberries could be separated as cultivated and wild based on their domestication. Their composition and content of phytochemical compounds and fruit quality vary widely. Published data mostly concern the cultivated varieties (Giovanelli and Buratti, 2009), a few data concern the composition of wild highbush blueberries.

Blueberries have drawn special attention because of their high antioxidant capacity and high concentration of anthocyanin and other phenolic compounds as well as their sweet flavor and vibrant color, which labels the fruit as one of the most desirable and nutritious among fresh fruits and vegetables (You et al, 2010). Significant positive relationships have been reported between antioxidant capacity and the content of anthocyanin and phenolics in ripen blueberries (Kalt et al, 2001). Anthocyanins were hypothesised to play important roles of increasing cells’ capacity of absorbing oxygen radicals, preventing the generation of free radicals, stimulating the expression of Phase II detoxification enzymes,
decreasing lipid peroxidation, inhibiting mutagenesis by environmental toxins and carcinogens, reducing cellular proliferation by modulating signal transduction pathways and effecting cell cycle regulator proteins (Wang & Stoner, 2008). So blueberries are an important source of dietary antioxidants.

Natural phenolic compounds as secondary metabolites also play a major role in plant growth and reproduction, contributing to the color and sensory characteristics of fruits, as well as providing protection against pathogens and predators (Balasundram et al, 2006).

Insect herbivory is a particularly common source of injury to plants. Pest infestation is a continual threat to the survival and reproduction of host plants, possibly causing water and nutrient deficiency and metabolic disturbs in plants (Silva et al, 2005). Moreover, a mechanical damage caused by pest is a potential infection site for pathogens. Rather than acting as passive victims in these interactions, plants can use several strategies to defense against damage cause by insect herbivory (Karban, 1997). Their defense ability can be obtained by direct defense such as secreting chemical compounds including toxin, antifeedant, volatile or nectar that repel insect, reduce plant digestibility or indirect defense such as attracting natural enemies of the plants, as well as increase plant tolerance (Howe and Jander 2008). The combined effects of defense provide resistance to insect feeding. Plants direct defense relies heavily on secondary metabolites like nitrogen compounds, terpenoids and phenolics to thwart insect attack. They are the by-products during the synthesis of primary metabolic products. In regard to pests, these secondary
metabolites are involved in by inhibiting enzymes activity and protein synthesis and affect DNA repair mechanisms (Silva et al, 2005).

Every plant has the future potential for disease and insect attack, the same with blueberry. Although nearly 200 diseases and more than 300 insect species have been reported on blueberries, only 20 to 25 ever become abundant enough to cause economic losses, and only 5 or 6 of these are chronic problems that require control every year. Usually, aphids, bugs and flies are the most common blueberry pests that can attack the buds, destroy the fruit, or threaten survival of the plant.

Brown Marmorated Stink Bug (BMSB), *Halyomorpha halys*, introduced from Asia, has been expanding its range since its first discovery in the mid-1990s in Pennsylvania (Hoebeke and Carter 2003). Since then, the distribution of BMSB has been rapidly spread and, as of 2015 it has been officially detected in 42 states in United States and 2 provinces in Canada. Differ from native stink bug, the BMSB lacks the natural enemies and egg parasitoids in North America, which increase the difficulty of BMSB management.

BMSB obtain nutrients from the host by inserting into food with its “piercing-sucking” mouthpart, injecting saliva enzymes and sucking out fluids. This invasive species feed on tree fruits, small fruits, vegetables, ornamentals, and seeded crops such as corn and soybeans. They have also seen in blueberries from the end of the 2010 and added recently to the pest management guide for blueberries in New Jersey (Wiman et al, 2015).
The overall goal of this study was to evaluate the blueberries quality attributes and composition including individual weight, pH, total soluble solids, anthocyanin and phenolic, as affected by domestication and BMSB infestation. First we compared the above quality parameters between cultivated and wild highbush blueberries. We also evaluated the anthocyanin, phenolic content and sugar profile in highbush blueberry treated by mechanical damage or BMSB infestation. We hope our results could guide farmers to plant blueberries more appreciated by the market.
2.1 Blueberry

2.1.1 Botanical Character and Variety of Blueberry

Blueberries are indigo-colored berries that native to North America and have been around for more than 13000 years. They belong to the section *Cyanococcus* within the genus *Vaccinium* (including cranberries and bilberries) (Hill, 1952). The leaves can be either deciduous or evergreen, 1-8cm long and 0.5-3.5cm broad. The flowers are bell-shaped, white, pale pink or red. The fruits grow in clusters and range in size from 5cm to 16mm with a flared crown in the end. The mature blueberries should have uniform blue, black, bluish-black or purple color. Most blueberries (except wild blueberries) usually have a grayish waxy cover the surface of skin, which is called bloom. The bloom on the skin is a protective coating. The cultivated blueberries are sweet when mature, with variable acidity, while wild blueberries are tart and tangy. Fruiting times are affected by local condition such as altitude and latitude, so the peak of crop can vary from May to the late summer in the northern hemisphere. Blueberries are climacteric fruits that can be harvested from semiwild or wild bushes.
There are five main types of blueberries grown in the United States: northern highbush (Vaccinium corymbosum), southern highbush (a complex hybrid of two species), rabbiteye (Vaccinium virgatum), lowbush (Vaccinium angustifolium) and half-high (crosses between northern highbush and lowbush blueberries) (Moore et al, 2014). The northern highbush (Vaccinium corymbosum) is most common cultivated blueberry grown in the Pacific Northwest and United States, while the lowbush (Vaccinium angustifolium) is commonly referred to as “wild blueberry”.

**Northern highbush blueberries** (Vaccinium corymbosum) are the most commonly cultivated forms of blueberries and the type sold most often in the grocery store. They are produced in temperate regions throughout North America, particularly the Pacific northwest, the Great Lakes region, and the Atlantic states of the United States.

Commercial highbush blueberries were first domesticated and cultivated in New Jersey, USA in the early 20th century (Rivera et al, 2015). The plants grow within a range of 4-9 feet. Cultivated highbush blueberries have often been hybridized to produce larger size berries, which U.S. consumers seem to prefer.

Experienced a long-term artificial cultivation, highbush blueberries have formed a lot of different cultivars like Duke, Patriot, Bluecrop, Elliott, Jersey and so on, and show differences on harvest season, yield and commercial value. For example, Bluecrop is a kind of mid-season harvest variety, with vigorous and upright bush, medium to large size berries that can resist cracking, good flavor and are suit for small farm or home garden growing. While Elliott is late harvest, with medium size berries and susceptible to
cracking and softening, make it not appropriate for small farm or home garden growing (Moore et al, 2014).

Lowbush blueberries (Vaccinium angustifolium) are native to North America and localized in eastern Canada and the northeastern United State. Since they grow wild, the commercial lowbush blueberries are composed of many genetically and phenotypically different clones (Wilhelmina, 2001). They are low-growing shrubs typically grow less than 2 feet in height and often stay even lower, at 8-12 inches from the ground. Other than cultivated berries, wild berries tend to be darker and shinier, with almost no bloom outside and purplish interior. Lowbush species produce berries of a smaller size than highbush. Their great subtlety and depth of flavor make them generally first frozen and then used mainly as an ingredient in processed food. There is evidence supporting that lowbush blueberries contain higher level of phenolics and antioxidant capacity than cultivated blueberries.

2.1.2 Blueberry Nutritional Value and Health Benefits

Blueberries are rich source of the essential dietary mineral manganese, vitamin C, vitamin K and dietary fiber while low in fat and sodium, making them an excellent choice to help meet nutrient needs and keep fit. One serving of blueberries (148g) delivers 14mg of Vitamin C and 29mg of Vitamin K- almost 25% and 32% respectively of one’s daily requirement, can help aid collagen formation and promote wound healing and a health immune system (FDA Reference Values for Nutrition Labeling). Manganese is necessary in bone development and in converting proteins, carbohydrates and fats into energy
Blueberries are also characterized by a high content and wide diversity of phenolic compounds including flavonoids (anthocyanins, flavonols, flavones, flavanols, flavanones, and isoflavonoids), stilbenes, tannins, and phenolic acids, display potential health promoting effects. Depending on the varieties and growing condition, every 100g blueberries contain 181.1-473mg phenolics (Prior, 1998). The antioxidant capacity of phenolic compounds is three to fivefold higher than ascorbic acid, results in the high antioxidant capacity of blueberries (Rice, 1997). In a survey of 22 different fruits and vegetables, blueberries had the highest antioxidant capacity when measured with the oxygen radical absorbing capacity (ORAC) assay (Wilhelmina, 2001). Those compounds are essential to owing the antioxidant capacity by helping to scavenge the free radicals and chelate the trace metals and they can reduce lipid peroxidation and DNA oxidation (Farah, 2007). They can reduce the risk of cardiovascular diseases and cancer, as well as other disorders. Anthocyanins, which show a significant positive relationship with ORAC antioxidant capacity, are also highly concentrated in blueberries: every 100g blueberries contain 62.6-235.4mg anthocyanins (Prior, 1998). The high anthocyanin content gives the blueberries not only their wonderful color but also the amazing antioxidant and anti-inflammatory capacities.
Lots of investigations for the diverse phytonutrients of blueberries in *vitro* and *vivo* have been done. The results demonstrate that these compounds could significantly protect against free radicals and oxidative stress, lead blueberries possess potential disease-preventive actions including novel antioxidant, antibacterial, antiviral and antiangiogenic properties, enhancing nervous system, brain health, immune health, regulating blood sugar and lipid profile, reducing hypertension and impacting hormone metabolism (Zafra-Stone et al, 2007). It has also been well demonstrated that the combination of phytonutrients may exhibit additive or synergistic effects on health benefits in human subjects.

2.2 Phenolics in Plant

2.2.1 Definition, Classification, Function and Resources

In organic chemistry, phenols, sometimes called phenolics, are a class of chemical compounds consisting of one or more hydroxyl groups (—OH) bonded directly to an aromatic hydrocarbon group. Depending on the amount of hydroxyl group, they could range from simple phenolic compounds to highly polymerized compounds (Bravo, 1998). Despite this structural diversity, phenolic compounds are often referred to as ‘polyphenols’ (Balasundram, 2005). Most natural occurring phenolic compounds are present as conjugates with polysaccharides and proteins (Klepacka et al, 2011). Several thousands of phenolic compounds have been identified in plants, thus there are various classification schemes. A commonly used scheme is according to the number of carbons
shown in table 1 (Harborne et al, 1999). Of these, phenolic acids, flavonoids and tannins are regarded as the main dietary phenolic compounds (King & Young, 1999).

<table>
<thead>
<tr>
<th>Class</th>
<th>Structure</th>
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<tbody>
<tr>
<td>Simple Phenolics, benzoquinones</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>Hydroxybenzoic acids</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>Acetophenones, Tyrosine derivatives, Phenylacetic Acids</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Hydroxycinnamic acids, Phenylpropenes, Coumarins, Isocoumarins, Chromoes</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>Naphthoquinones</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>Xanthonoids</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;1&lt;/sub&gt;-C&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>Stilbenoids, Anthraquinones</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;2&lt;/sub&gt;-C&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>Chalconoids, Flavonoids, Isoflavonoids, Neoflavonoids</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;3&lt;/sub&gt;-C&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>Halogenated algal phenolic compounds</td>
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<tr>
<td>Lignans, Neolignans</td>
<td>(C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>Biflavonoids</td>
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<td>Lignins, Catechol melanins, Flavolans, Polyphenolic proteins, Polyphenols</td>
<td>(C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;n&lt;/sub&gt;, (C&lt;sub&gt;6&lt;/sub&gt;)&lt;sub&gt;n&lt;/sub&gt;, (C&lt;sub&gt;6&lt;/sub&gt;-C&lt;sub&gt;3&lt;/sub&gt;-C&lt;sub&gt;6&lt;/sub&gt;)&lt;sub&gt;n&lt;/sub&gt;</td>
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Table 2.1: Main classes of phenolic compounds regarding to their carbon chain.

Natural phenolic compounds have been considered the most important and numerous secondary metabolites and a major class of antioxidants found almost in all plants, and often highly concentrated in vegetables and fruits (You et al, 2011). These substances are synthesized during the normal growth and reproduction of the plant, as well as in response to diverse environment, providing protection against oxidative stress by donating an electron to the free radical and convert it into an innocuous molecule (Haminiuk et al, 2012). Pathogen and insect attack can stimulate the compounds run up at the infection site and conversely limit the development of pathogen and insect. Besides,
they also contribute towards the color and sensory characteristics of fruits and vegetables (Chao et al, 2004).

Phenolic compounds have been associated with the protection against reactive oxygen species by scavenging the free radicals from cell metabolism (Kurosumi et al., 2007). Studies have suggested that a diet rich in phenolic compounds could avoid the oxidative damage that leads to age-related diseases, such as Parkinson’s disease (Haminiuk et al, 2012). Phenolic compounds can also be consumed as anti-allergen, anti-inflammatory, antibiotic, antioxidant or dietary supplements (Balasundram et al, 2005), lowering the incidence of non-communicable diseases (NCDs) like cancer, cardiovascular disease, chronic respiratory diseases and diabetes (Haminiuk et al, 2012). Berries, tea, olive oil, vegetables, fruits and fruit-based drinks contain considerable levels of phenolic compounds. There are wide variations between the total phenolics contents of the different fruits and vegetables. Even within the same fruits or vegetables, differences on phenolic content are still remarkable, this can be explained by the degree of ripeness, species, climate, processing and storage variation and other factors (Belitz et al, 2009).

2.2.2 Phenolic Acids

Phenolic acids, one of the most important dietary polyphenols, are abundant in foods. These substances consist of two subgroups, the hydroxybenzoic and hydroxycinnamic acids. In contrast to other phenolic compounds, the hydroxybenzoic and hydroxycinnamic acids present an acidic character owing to the presence of one carboxylic group in the molecule (Annie & Jean-Jacques, 2003).
Hydroxybenzoic acids with a C$_6$-C$_1$ structure, are found in various fruits and occur most frequently as esters. The most common phenolic acids found in this category are gallic, \( p \)-hydroxybenzoic, protocatechuic, vanillic and syringic acids (Haminiuk et al, 2012).

Gallic acid is the most frequently encountered phenolic acid, mainly found in the form of complex sugar esters in gallotannins such as 2-O-digalloyl- \( \text{tetra-O-galloyl-glucose} \) (Crozier et al, 2009). The major sources of gallic acid in the human diet are grapes, wine, mangoes and tea (Crozier et al, 2009).

Hydroxycinnamic acids including caffeic, ferulic, \( p \)-coumaric and sinapic acids commonly have a C$_6$-C$_3$ skeleton (Balasundram et al, 2006). Hydroxycinnamic acids are rarely present in plants in a free state but mostly occur as simple esters with organic acid or glycosides (Rice-Evans et al, 1996). The two widespread dietary hydroxycinnamic acids are caffeic acid and ferulic acid. Caffeic acid is found at high levels in many fruits and vegetables and in coffee. Ferulic acid is associated with dietary fiber and is linked through ester bonds to hemicelluloses. Ferulic acid is the major phenolic acids in wheat bran (5mg/g) and vegetables (Scalbert and Williamson, 2000).

The antioxidant capacity of phenolic acids depends on the numbers and positions of the hydroxyl groups along with the carboxyl functional groups (Balasundram et al, 2006). Hydroxycinnamic acids exhibit higher antioxidant activity compared to the corresponding hydroxybenzoic acids. The higher activity of the hydroxycinnamic acid could be due to the CH=CH-COOH group, which ensures greater H-donating ability and
radical stabilization than the –COOH group in the hydroxybenzoic acids (Rice-Evans et al, 1996)

2.2.3 Flavonoids

Over five thousands of flavonoids constitute the largest group of plant polyphenols, accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne et al, 1999). They are present particularly in the epidermis of leaves and the skin of fruits (Crozier et al, 2009). Flavonoids are low molecular weight compounds, composed of fifteen carbon atoms, arranged in a C₆-C₃-C₆ configuration. Essentially the structure consists of two aromatic rings A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring C (Figure 2.1) (Balasundram et al, 2006). Variations in substitution patterns to ring C result in several flavonoid subclasses: flavonols, flavones, flavanones, flavanols (or catechins), isoflavones and anthocyanidins (Hollman & Katan, 1999), of which flavones and flavonols are the most widely occurring and structurally diverse (Harborne et al., 1999). The oxygenation, alkylation, glycosylation, acylation and sulfation of rings A and B lead to the difference within each class of flavonoids (Pietta, 2000). Flavonoids that exist in foods are usually glycosylated or esterified rather than as free compounds. When flavonoids are linked to one or more sugar molecules, they are known as flavonoid glycosides (Haminiuk et al, 2012). The linked sugar is often glucose or rhamnose, but can also be galactose, arabinose, xylose, glucuronic acid, or other sugars (Karakaya 2004). Both sugars and hydroxyl groups increase the water solubility of
flavonoids, other substituents, such as methyl groups and isopentyl units, make flavonoids lipophilic (Crozier et al, 2009).

![Flavonoid](image)

**Figure 2.1:** Chemical structure of flavonoid.

Flavonoids are recognized as natural antioxidants because of their high redox potential and their ability to chelate metals, therefore contributing significantly to the health benefit of fruits (Tsao & Yang, 2003). The degree of glycosylation directly affects the antioxidant activity of flavonoids. Usually, the aglycone forms of myricetin and quercetin can increase their activity when compared to the corresponding glycosylation (Rice-Evans et al, 1996; Haminiuk et al, 2012). The antioxidant capacity of flavonoids also depends on the degree of hydroxylation and the positions of the double bond and –OH groups. The maximum effectiveness for radical scavenging requires the 3-OH group attached to the 2,3-double bond and adjacent to the 4-CO in the C ring (Rice-Evans et al, 1996).

It is already established that flavonoids are responsible for plant stress defense, protecting leaf cells from photo-oxidative, pathogens and wounding damage (Winkel-Shirley, 2002).
They also make contributions to the yellow, red and blue colors in fruits and flowers (Lampila et al, 2009).

The dietary flavonoids consumption of U.S adults is about 189.7mg/d, mainly from flavanols (83.55), followed by flavanones (7.6%), flavonols (6.8%), anthocyanidins (1.6%), flavones (0.8%) and isoflavones (0.6%) (Chun et al, 2007).

Flavonols are the most ancient and widespread flavonoids. Flavonols are scavengers of superoxide anions, singlet oxygen and lipid peroxyradicals. Hertog et al (1993) reported that high intake of flavonols had inversely relationship to coronary heart disease mortality. Quercetin, the most frequently occurring flavonol in foods, inhibits oxidation and cytotoxicity of low-density lipoproteins (LDL) in vitro (Hertog et al, 1997). Onions are the main dietary sources of flavonols (Ross and Kasum 2002).

The main flavanols are catechins and proanthocyanidins. Catechins are very abundant in tea. An infusion of green tea contains 1g/L catechins (Lee et al. 1995). Other sources are red wine (270mg/L) and chocolate (Scalbert and Williamson 2000). Proanthocyanidins are polymeric flavanols. They are present in plants as complex mixtures of polymers with an average degree of polymerization between 4 and 11. They are responsible for the astringency of food and are usually present in association with flavanol catechins. It has been confirmed that flavanol rich food can induce vasodilation via activation of the nitric oxide system, protect humans against coronary disease (Fisher et al, 2003).
Natural flavones such as apigenin, luteolin are mainly found in cereals and herbs. Unlike flavonols, flavones have little or no direct antioxidant capacity based on research. Even so, Wenzel et al (2000) proved that flavo

Isoflavones differ from flavone in location of the phenyl group. Soybeans are the richest source of isoflavones in human food, 1g of defatted soybeans contains approximately 2mg of isoflavones (Messina et al, 1994). The major isoflavones in soybeans are genistein and daidzein (Wang and Murphy 1994). In plant tissue, they most often occur as glycosides or their respective malonates or acetyl conjugates. They have received considerable attention due to their role in influencing estrogen and preventing breast cancer as well as antioxidant and antifungal capacity. Barnes and Messina (1991) pointed out that soybean-rich food could bind towards estrogen receptors to inhibit mammary tumorigenesis and reduce breast cancer. In vitro and vivo studies, isoflavones can inhibit lipoxygenase action and UV light-induced oxidation and prevent peroxidative hemolysis of erythrocytes (Naim et al, 1976; Fleury et al, 1992).

Flavanones are generally glycosylated by a disaccharide at position seven to give flavanone glycosides. Flavanones are the most abundant flavonoids in citrus fruit. Naringin is the predominant flavanone in grapefruit and hesperidin is the primary flavanone in orange. These flavanones occur only rarely in other plants and therefore might be considered to be unique to citrus (Ameer et al, 1996).

Anthocyanins are the most important water-soluble pigment. They produce red, blue or purple color for fruits like cherries, strawberries, raspberries and blackberries and
flowers. Their hue is dependent on the pH value and presence of copigments. Their contents in fresh fruits vary from 0.15 to 4.5mg/g (Clifford 1996). They are also highly concentrated in red wines for 26mg/L (Frankel et al, 1995). Anthocyanins in petals are intended to attract pollinators, whereas anthocyanins in seeds and fruits may aid in seed dispersal. Anthocyanins and other flavonoids can also be important as antioxidant and as protection against damage from UV irradiation (Holton and Cornish 1995).

2.2.4 Tannins

Tannins are the third class of polyphenols that are found in fruits and are mostly present as phenolic polymers (Haminiuk et al, 2012). They are a group of polyhydroxy-flavan-3-ol oligomers and polymers with carbon–carbon linkages between flavanol subunits (Schofield et al., 2001). These relatively high molecular weight compounds can be subdivided into hydrolysable and condensed tannins (Balasundram et al, 2006). The former are esters of gallic acid, while the latter (also known as proanthocyanidins) are polymers of polyhydroxyflavan-3-oligomers linked by carbon–carbon bonds. Gallotannin or tannic acid is a type of hydrolysable tannin found in fruits. Condensed tannins (Proanthocyanidins) are the major phenolic compounds found in grape, especially in skins and seeds. Proanthocyanidins, when in contact with salivary proteins, are responsible for the astringency of fruits and bitterness of chocolate (El Gharras, 2009).
2.3 Anthocyanin

2.3.1 Definitions and Chemical Structure

Anthocyanins are water-soluble pigments derived from flavonoids via the phenylpropanoid pathway. They can be found in all higher plants, mostly in flowers and fruits, but also in leaves, stems and roots, giving the red, blue and purple colors of fruits and vegetables. In food, the main sources of anthocyanins are berries, such as blueberries, blackberries and chokeberries, and some vegetables, such as purple corn.

![Anthocyanidin](image)

**Figure 2.2**: Chemical structure of anthocyanidin.

<table>
<thead>
<tr>
<th>Name</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delphidin</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Petunidin</td>
<td>OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Peonidin</td>
<td>OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OCH₃</td>
<td>OCH</td>
</tr>
</tbody>
</table>

**Table 2.2**: The six most common anthocyanidin skeletons in higher plants.
Anthocyanins are derived from anthocyanidins by adding sugars including glucose, galactose, rhamnose, xylose or arabinose. The basic core structure of anthocyanidins is flavylum ions, a structure with totally 7 side groups that could be either hydrogen atom, hydroxide or a methoxy group (Figure 2.2). The sugar components of anthocyanins are usually conjugated to the anthocyanidin skeleton. So anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium of flavylum salts. With different combination of side groups, there are 27 naturally occurring anthocyanidins. Among them, only 6 anthocyanidins are common in higher plants—pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp) (Table 2.2). However, the differences between the nature and number of sugars attached, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars leads to a large number of anthocyanins formed. Based on the several reviews to date, it is estimated that more than 600 anthocyanins have been isolated from plants. The glycosides of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. The following four classes of anthocyanidin glycosides are common: 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides. So the most widespread anthocyanin is cyanidin 3-glucoside. Anthocyanins are not always permanent; they could disappear with changes in temperature, photoperiod or other signals. In contrast to other flavonoids, the anthocyanins carry a positive charge in acidic solution (Wang and Stoner 2008). The color of anthocyanins depends on acidity of the fruits and its structure. At pH 1–3, the flavylum cation is red colored, at pH 5, the
colorless carbinol pseudobase is generated, and at pH 7–8 the blue purple quinoidal base is formed. The acylated anthocyanins display better stability due to intramolecular copigmentation (Malien-Aubert et al, 2001).

2.3.2 Function of Anthocyanin

2.3.2.1 The Role of the Plant Itself

The most significant function of anthocyanins is the ability to impart color to the plant. In flowers, bright-reds and purples play a definite role in the attraction of animals for pollination. In fruits, the color skins also attract the animals to eat the fruits and disperse the seeds. And hence, they are of considerable value in the co-evolution of these plant-animal interactions. A study by Scogin and Freeman (1987) shown that the anthocyanins expressed in the flowers influenced the types of pollinators that visit the flowers.

Since the late 19th century, botanists had noticed that intense sunlight and UV radiation could induce anthocyanins production and they proposed explanations that anthocyanins can protect the photosynthetic structures against high light and UV damage (David, 2002). The finding that developing leaves and senescing leaves may be susceptible because of their immature photosynthetic machinery and chlorophyll breakdown led to the hypothesis that anthocyanins might afford protection against excess light (Miranda et al, 1981; Matile 2000). During the past decade fluorescence measurements have produced more evidences that the anthocyanins protect plant tissues by reducing photoinhibition, a phenomenon known as decline the photosynthetic efficiency of plants under bright and cold environment. Anthocyanins act as “sunscreen” by soaking up
radiant energy at waveband around blue-green and UV light. Such kind of protection mechanism could be found in young, developing leaves or old, senescing leaves of various plant species, behaving as transiently anthocyanin accumulation as a result of photoinduction. So compared with green leaves, red leaves would be more tolerant to photoinhibition. In addition, it can also be induced in mature leaves of some plants when undergoing nutrient deficiency, UVB radiation and high light, especially when temperature is low (Chalker-Scott, 1999).

Anthocyanins present in some plants may also be defensive molecules along with other flavonoids in the resistance of plants to herbivores and pathogens (Harborne, 1988). There are plenty of examples of anthocyanins accumulation attribute to insect or fungal attack: Sorghum halepense feeding by Sipha flava could produce a reddish spot on the leaves, which was identified as anthocyanin (Monique, 2003); insect feeding on floral tissues could make Petunia hybrida synthesize large amount of different anthocyanins (Johnson et al, 2003). Most scientists believed that these transiently produced anthocyanins access the resistance by decreasing insect fitness. For example, Hedin et al. (1983) found that Cyanidin 3-glucoside, the most widespread anthocyanin, was shown to protect cotton leaves against the tabacco budworm. Cesar et al. (2001) suggested that anthocyanins produced by Sorghum halepense after aphid Sipha flava infestation could reduce the aphid fecundity; Johnson et al. (2003) proposed that delphinidin 3-glucoside, another kind of anthocyanin, could reduce the growth of fall armyworms and cabbage loopers while adding cyanidin 3-glucoside to fall armyworms diet could significantly
inhibited its growth. Other anthocyanins can also defend the plant from a wide range of potential herbivores. A second hypothetically potential protection of anthocyanin is that they may decrease the attraction of host plant for insects. Prokopy et al. pointed out that insects prefer healthy green or yellow leaves as food or spawning sites. Based on the published studies, most insects may not easily distinguish what humans perceive as red because within their visual range, they can hardly capture such kind of chromatic optical cue. Based on the foregoing behavioral test, there was a concept that the presence of anthocyanins in leaves other than to be optical warning signals, their accumulation may mask the leaves to avoid giving a perceptible green signal (Panagiota, 2006). Corresponding observations in the field showed that juvenile, old and senescing leaves can accumulate anthocyanins transiently, performing a reddish color. So Stone (1979) concluded that red leaves could mimic unhealthy or immature leaves of low nutritive value to be less preferred by leaf-eating insects.

2.3.2.2 Function on Human Health

Anthocyanins also possess known pharmacological properties due to their high antioxidant activity. The antioxidant activity of anthocyanins is mainly due to the presence of hydroxyl groups in position 3 of ring C and also in the 3', 4' and 5' positions in ring B of the molecule (Wang 2008). The phenolic structure empowers the anthocyanins to directly scavenge reactive oxygen species (ROS) such as superoxide, singlet oxygen and peroxide, increase the oxygen-radical absorbing capacity of cells, stimulate the expression of Phase II detoxification enzymes, reduce the formation of
oxidative adducts in DNA, decrease lipid peroxidation, inhibit mutagenesis by environmental toxins and carcinogens, and reduce cellular proliferation by modulating signal transduction pathways (Wang and Stoner, 2008).

Series studies on *vitro* cell culture and *vivo* animal model as well as data from human therapeutics have proved that the consumption of anthocyanins can lower the risk of cardiovascular disease, obesity, arthritis and cancers.

The *vitro* studies of anthocyanin from Italian red wine done by Ghiselli showed that the anthocyanin was the most effective polyphenolic compound both in scavenging reactive oxygen species and in inhibiting lipoprotein oxidation and platelet aggregation as well as the key component to protect against cardiovascular disease. Supplementary of rats with cyaniding-3-glucoside-containing purple carrot juice could improve dyslipidaemia and glucose tolerance, decrease cardiac stiffness, altered plasma lipid profile and finally decrease the risk of cardiovascular disease (Poudyal et al, 2010).

The definition of obesity is the excess body fat accumulation resulting from various metabolic disorders. It can increase the risk of hypertension, hyperlipidemia, heart disease and type 2 diabetes (Tsuda et al, 2003). The ability of anthocyanins to prevent obesity and diabetes has been studied using various models. When rats were fed by cyaniding 3-glucoside-rich purple corn extract, they gained less body weight and tissue weights when compared with high fat diet. This result proved that anthocyanins as diet supplement could improve high fat diet induced insulin resistance and may suppress obesity and diabetes (Tsuda et al, 2003).
Anthocyanins have been reported to modulate inflammation-induced pain. Rats fed 400mg/kg anthocyanins extracted from tart cherries had less thermal hyperalgesia and paw edema compared to control animals (Tall et al, 2003). Further research suggested that anthocyanins can specifically inhibit histone acetyltransferase (HAT) activities. Accordingly, anthocyanins decrease the expression and function of NF-kappa B, resulting in a reduction in inflammation-induced arthritis. (Seong et al, 2011).

In a study on testing the effect of anthocyanins on tumors, the anthocyanins from red wine could suppress the growth of HCT-15 cells and AGS cells, which are derived from human colon cancer and gastric cancer. The suppression rate by the anthocyanins was significantly higher than that of other flavonoids and their derivatives (Kamei et al, 1998). In most *vivo* studies on animals, rats fed on anthocyanin-rich extract (e.g., black raspberry, cherry, petal) or purified delphinidin or cyanidin-3-glucoside had significantly fewer cecal, esophageal, skin, lung or liver tumors than untreated rats (Stoner et al, 2007, Kang et al, 2003, Afaq et al, 2007, Ding et al, 2006, Obi et al, 1998).

Following the recognition that anthocyanins are more effective than rutin, a primary dietary supplement in humans, in decreasing capillary permeability and fragility and in their anti-inflammatory and antioxidant activities (Wagner, 1985), it is possible that anthocyanins may replace rutin and its derivatives in the treatment of illnesses. At present, the daily intake of anthocyanins in the U.S. diet is estimated to be between 180 and 215 mg whereas the intake of other dietary flavonoids such as genistein, quercetin and apigenin is only 20–25 mg/day (Wang, 2008).
2.3.2.3 Food Colorants

Food Colorants have been used on foods and drinks industry since historical times. Synthetic dyes, commonly concerned with adverse behavioral and neurological effects, are becoming less popular among consumers (He et al, 2010). Recent trial concluded that the artificial colors like sunset yellow, carmoisine in the diet could lead to increased hyperactivity in 3-year-old and 8/9-year-old children (McCann, 2007). Therefore anthocyanin-based natural colorants attract interest because of their vivid colors in orange, red, purple and blue, safety characteristic and potential nutritional effect. They are primarily used in beverage industry, as substitutes for FD&C red #40 (allura red), the most widely used synthetic colorant. They can also be applied on fruit preparations, confectionary and ice cream.

However, there are still some limitations in the use of anthocyanins in food. Anthocyanins are water soluble, which restricts the use. Their structures also undergo reversible pH-dependent transformation in the solution. During processing, formulation and storage conditions, when the acidity changes, the color changes. Anthocyanins are also susceptible to pH, temperature and light. However, Giusti et al (2003) proved that the acylated anthocyanins, rich in radishes, red potatoes and red cabbages, could remarkably enhance the stability to pH changes, heat treatment and light exposure than the nonacylated anthocyanins. Hence, the increased stability of these pigments together with their nutritional value could expand their applications in food industry.
2.4 Blueberry Pest

There are a number of pests that can affect blueberries, including aphids, brown marmorated stink bug, spotted wing drosophila and so on. The green peach aphids can be found on new growth blueberry plants. Feeding by large numbers of aphids causes yellow to yellow and become stunted and distorted. Chronic infestation can lead to reduce yield ad fruit quality. Aphids have also been identified as the vector of blueberry scorch virus, which can cause plant death.

The brown marmorated stink bug is not currently serious pest in United States, but a potential serious pest due to its growing presence and its wide host range. It is a sucking insect that uses its proboscis to pierce the host plant to feed. It secretes saliva then sucks up the plant juices, leaving a necrotic area at the feeding site.

The spotted wing drosophila is a new and exotic pest. Egg laying by the female damages and scars the fruit skin. Fruits also soften, collapsing and bruising at the damage site.

2.5 Brown Marmorated Stink Bug

2.5.1 Background Introduction

The brown marmorated stink bug, Halyomorpha halys (Stål) (Hemiptera: Pentatomidae), is an invasive species native to China, Japan, Korea, and Taiwan (Hoebek and Carter 2003). It was discovered in the United States in the mid-1990s in or near Allentown, PA for the first time (Hoebek and Carter 2003). After that, the distribution of H. halys has grown steadily and, as of 2015 it had been officially detected in 42 states and 2 provinces
in Canada (www.stopbmsb.org). This invasive species has emerged as a significant horticultural pest in tree fruit in the mid-Atlantic region, with a broad host range reported on vegetables, row crops, ornamentals, and potential risks to small fruit like blueberries and grapes (Leskey et al. 2012).

2.5.2 Life Cycle

Brown marmorated stink bug (BMSB) overwinters as adults in a protective sleeplike state, aggregates in and around houses. The majority of BMSB become inactive below 9°C (Lee et al, 2013). It emerges in the spring, and begins mating in about two weeks. BMSB are able to produce one to six generations in a single year depending on the temperature and diet. Photoperiod exposure in both the adult and nymph stages affects pigmentation, body size, feeding frequency, developmental rate and lipid accumulation of adults in lab study (Lee et al, 2013).

The BMSB has five nymphal stages, or instars, ranging from 2.4 mm to 12 mm in length. Unlike the adults who blend in very well with bark, the nymphs are more brightly colored with red and black. The first instars, which have a "tick-like" appearance, are not very active and remain around the hatched egg mass. Nymph is characterized by dark reddish eyes and yellowish-red abdomen which is also striped with black. The legs and antennae of the nymphs are black with white banding. The eggs of the BMSB are often laid on the underside of leaves and a light green in color. They are elliptical in shape and are often
deposited in a mass of approximately 28 eggs.

(http://njaes.rutgers.edu/stinkbug/identify.asp)

2.5.3 Host Range

BMSB is a polyphagous pest. A total of 106 host plants in 45 families have been reported with many from *Fabaceae* and *Rosaceae* (Lee et al, 2013). In the spring, BMSB adults emerge from overwintering sites and become active during warm sunny days. During this time, adult bugs can be found on virtually any plant that exposes them to the sun. Trees, shrubs, and ornamental plants that are near BMSB overwintering shelters often serve as the best places to observe early bug activity. Tall plants and trees tend to have more bugs on them than plants lower to the ground. As adult bug activity increases throughout the month of May and as mating, egg laying, and nymphal development occurs throughout the summer, BMSB can be found on a wide range of plant species. Host plants for BMSB including, but not limited to grapes, orchard crops (including apple, hazelnut, peach and pear), ornamentals (including catalpa, paulownia and tree of heaven), small fruits (including blueberry, cane berry and raspberry), vegetables (including eggplant, lima beans, okra, pepper, snap beans, sweet corn and tomatoes) and field crops (www.stopbmsb.org). Generally, fruits of cultivated species are unsuitable food for eggs or their nymphs, whereas they can be host plant for adults (Funayama 2004). Oda et al. (1980) separated hosts of BMSB into two categories: breeding and food plants. Breeding plants were defined as those on which adults, nymphs and eggs were observed, whereas food plants were those on which only adults were observed feeding. In addition,
Funayama (2002, 2004) suggested that multiple host plants were necessary for normal BMSB development.

2.5.4 Damage on Host

BMSB causes significant damage to many economically important crops worldwide including apple, pear, peach, persimmon and soybean. The following is a summary of the damage and economic loss taken by BMSB feeding injury.

**Blueberry.** Feeding by BMSB damage the fruits by causing increased levels of external discoloration, and internal damage in the form of tissue necrosis. Exposure of berries to BMSB is also associated with decreasing berry weights and lower soluble solids in fruits (Wiman et al, 2015).

**Pear.** Pears are susceptible to BMSB feeding injury throughout the growing season (Lee et al, 2013). Wang and Wang (1988) suggested that BMSB feeding on fruit with 3-cm diameter severely affected fruit development and caused deformity.

**Peach.** Damage is first observed in early May on young peach fruit (Lee et al, 2013). Fruit exposed to BMSB were deformed and showed gummosis, brown spots or abscission. All injury symptoms severely affected marketability of peaches. BMSB injury to peach has been reported to range from 50 to 70% when orchards were not properly managed (Lee et al, 2013).

**Other Crops.** Fukuoka et al. (2002) reported damage to and injury levels of 90% on cucumber, saya pea, and eggplant, 70% on sweet corn, 60% on asparagus and edamame
bean, 8% on pepper, and 80% on strawberry. BMSB damage was also reported on jujube, cherry, and tea (Lee et al. 2013).

2.6. Plant Resistance to Pest Infestation

Insects that feed on plant leaves, sap, pollen, nectar or resins are often called phytophagous insects. Phytophagous insects use chewing or sucking mouthpart to obtain nutrients from their host plants. Approximately two-thirds of all known herbivorous insects, such as Thysanura, Odonata and all Polyneoptera eat leaves and cause damage by mouthparts evolved for chewing, snipping, or tearing (Schoonhoven, 1998). Other piercing-sucking herbivores such as Paraneoptera, bees and Lepidoptera present sucking tubes or sucking pumps that suck the liquid content from lacerated cells. Aphids, whiteflies, and other Hemiptera insert specialized stylets between cells to establish a feeding site in the phloem (Gregg, 2008). Insect feeding causes wounding of the plant, which could be a potential infection site for pathogens and lead to a continual threat to the survival of the plants (Philippe, 2000). Rather than acting as passive victims in these interactions, plants respond to pest infestation with three defense strategies: direct and indirect defenses and tolerance (Karban, 1997). These three mechanisms work together to influence the population dynamics of pests.

**Direct defenses** include any plant traits (e.g., primary and secondary metabolites) that can affect the susceptibility and increase the fitness of host plants by themselves. The foundations of direct defenses against phytophagous insects are secondary metabolites, reducing nutrient value and disrupting physical structures. As the by-products of primary
metabolic products, secondary metabolites are not directly involved in normal plant
growth or reproduction but have influence on plants’ long-term survivability especially
on defenses against phytophagous insects. They are generally classified as nitrogen
compounds, terpenoids and phenolics. The insecticidal activity of nitrogen compounds
(including cyanogenic glucosides, glucosinolates, alkaloids and benzoazinoids) is either
due to their abilities as block DNA repair mechanisms or inhibit enzyme activities.
Terpenoids often interfere with phytophagous insects’ metabolism by acting as
antifeedant, toxins or as modifiers of insect development (Richard, 1994). Phenolics,
including aromatic amino acids, flavonoids, isoflavonoids and tannins, not only act as
feeding deterrents and inhibitor of enzymes for insects, but also create a barrier to
defense against fungal pathogens after insect infestation (Richard, 1994). Reducing
nutrient value ability is achieved by either removing essential nutrient or inhibiting
digestion of herbivorous insects. Like human, insects cannot synthesize all the amino
acids that they need and must obtain these amino acids (including arginine, lysine,
methionine and threonine) from the diet. The amino acid degradation enzymes can
induced by insect feeding, keep active in the insect gut, destroy the essential amino acids
and finally impair the development of the insect (Ming Shun, 2008). The other way to
reduce nutrient value is to inhibit the digestion of insects. When attack by phytophagous
insects, plants can produce various protein inhibitor of insect digestive enzymes and can
stably exist in the insect gut. The development of insect will be impaired (Ming Shun,
2008). Changing physical characteristic is another important method of performing
resistance. Most vascular plants can secrete smooth and slippery wax film and crystals,
alter the texture of the plant tissue and impede insect to populate leaf surfaces. Sometimes, plants protect themselves by thorn and spines. These sharp structures can inhibit insects from contacting the leaf surface, limit their movement, damage their mouthpart and cause more efficient delivery of the plant’s toxins (Gregg, 2008).

**Indirect defenses** are plant defensive traits that protect against phytophagous insects by enhancing the attraction of natural enemies of the insects (Becerra, 2007). Such attraction is due to the release of volatile organic compounds (VOC) by plants. When a plant is attacked, it can release such kind of compound as food cue, and attract the predators to the damaged plant and to feeding the insect (Philipp, 2000)

**Tolerance** of plant can be described as the decrease degree of plant fitness after insect infestation. A plant genotype is regarded as tolerant if it can support an insect infestation with little reduction of crop yield. Although the genotype should be responsible for their tolerance, the mechanism for such variation is not clearly enough (Andre, 2002).
Chapter 3. Comparative Quality Attributes, Anthocyanin and Phenolics Content of Wild and Cultivated Highbush Blueberries From Different Locations

3.1 Abstract

Recent interest in the possible protective effects of dietary antioxidant compounds against human degenerative disease has prompted investigation of foods such as blueberries (Vaccinium sp.), which have a high antioxidant capacity. Cultivated and wild highbush blueberry (‘Bluecrop’) from different plantations were analyzed for their quality attributes such as pH, total soluble solids and individual weight, their content of anthocyanins, and total phenolic compounds, to evaluate the variation in these parameters. The samples were extracted by acetone then analyzed by UV-Vis spectroscopy and HPLC. Result shown that cultivated and wild highbush blueberries were with consistent total soluble solids content. The cultivated blueberry had higher fruit weight and pH value than wild blueberries. The total anthocyanin and phenolics for cultivated ranged from 74.7 to 116.5mg/100g FW and 188 to 362 mg/100g FW. For wild blueberry, their anthocyanin and phenolic content were 0.75 times and 2 times higher
than cultivated. The growing location also affected pH and phenolic content of blueberry. In general, domestication and growing location would work together to influence blueberry quality.

3.2 Introduction

Blueberries have been of specific interest among scientists because of their high antioxidant capacity. In a survey of 22 different fruits and vegetables, blueberries had the highest antioxidant capacity. Phenolic compounds, which include anthocyanins, possess antioxidant properties and are highly concentrated in blueberries. Significant positive relationships were reported between ORAC antioxidant capacity and the content of anthocyanins and total phenolics in ripe blueberries. Because blueberries are important sources of antioxidants, studies indicated that blueberries in human diet can protect against cardiovascular diseases, cancer and aging-related disorders (Bunea et al, 2011).

There are several species of blueberries in the United States, such as highbush, lowbush and rabbiteye. Commonly, the term ‘wild blueberries’ refers to the lowbush blueberries (Vaccinium angustifolium), typically smaller in size; while ‘cultivated blueberries’ are typically the highbush blueberries (Vaccinium corymbosum L.). They are both native to North America and have been commercially produced for many years. Lowbush blueberry production is localized in eastern Canada and the northeastern United States, whereas highbush blueberries are produced in temperate regions throughout North America, particularly the Pacific northwest, the Great Lakes region, and the Atlantic states of the United States (Kalt et al, 2001).
The cultivated and wild highbush blueberries are considered as the same species and share the common ‘highbush blueberry’ name, however, the production systems for cultivated and wild blueberries are distinctly different. The wild blueberries are the ancestors of the cultivated blueberries, and they are used for the ‘artificial’ selection of blueberries. The wild blueberry reproduces by cross pollination. Each seed gives rise to a plant with a different genetic make-up. So there is no variety in the wild plants because a variety is used to designate a plant that has been domesticated. This is why within each species, there can be significant differences in growth, color, the size and shape of the leaves, resistance to diseases, maturity, flavor, productivity, size, firmness and shape of the fruit. In contrast, cultivated highbush blueberries are grown on plantations, using varieties that have been bred for their production and food characteristics. The market for fresh blueberry fruit is met almost entirely by cultivated highbush blueberries, whereas wild blueberries are often incorporated in traditional medicine and cuisine (Mikulic-Petkovsek et al, 2012). Thus, it is important to characterize the fruit quality and nutrient substance in these blueberries and to identify the influence taken by domestication.

The purpose of the study was to compare the fruit quality and composition including individual weight, pH, total soluble solids, anthocyanin and phenolic between cultivated and wild blueberries. We also identified the growing location influence on blueberry quality and its interaction with domestication on blueberry quality, anthocyanin and phenolic content.
3.3 Materials and Methods

3.3.1 Sample Collection

The cultivated highbush blueberry plants ‘Bluercrop’ and wild blueberry were grown in 8 locations called ‘Berenato’, ‘Bill’, ‘Weels’, ‘Thompson’, ‘Doyle’, ‘Merlino’, ‘Miller 1’ and ‘Moore’ in New Jersey, during 2014. For sample collection of each kind, just ripe fruits were collected.

Fruit samples were harvested from each type in each location three times from late June till mid July 2014 (n = 48). After picking the appropriate maturity, samples were placed in polyethylene bags and transported to the lab in a frozen state. All the samples were stored at -20°C until extraction and analysis of compounds.

3.3.2 Determination of Fruit Quality Attributes

‘Quality Attribute’ measurements including individual weight, total soluble solids and pH were determined during sample extraction procedure. The average fresh weight of berries was determined by the quotient between the weight of whole berry samples and number of berries per sample. Soluble solids were determined using a hand refractometer from the juice of berries, standardized with distilled water and expressed as °brix. Three replications were conducted. pH measurement was conducted by pH meter.

3.3.3 Sample Extraction

Blueberry samples from different locations were weighed and blended with appropriate amount of liquid nitrogen and extracted by acetone for the analysis of total anthocyanin.
and total phenolics. Blueberries along with 0.2 volume distill water and certain amount of liquid nitrogen was homogenized in a blender for 2 min. After that, 1.8 volumes of pure acetone was added to blueberry powder and homogenized for 2 min. The blend was then vacuum filtered using a Buchner funnel with 70mm Whatman #4 filter paper (Rodriguez-Saona and Wrolstad, 2001). The anthocyanin solution was poured into a separatory funnel with 1.5 volumes of chloroform. The chloroform/anthocyanin solution was gently mixed and then placed at room temperature (20°C) for 24 hours to allow for maximum separation. The bottom layer of chloroform and polar solvents was discarded and the top layer (anthocyanin/phenolic concentrate) was collected. Residual acetone was evaporated using a Buchi rotary evaporator. The final volume of sample was documented for future calculation.

3.3.4 Determination of Total Anthocyanin Content

Anthocyanin contents were estimated by the pH differential method as mentioned by Giusti and Wrolstad (2001). The absorbance of anthocyanin extracts was measured in a spectrophotometer at $\lambda_{\text{max}}$ and at 700 nm using the molar extinction coefficient for cyanidin-3-glucoside of 29,600. The contents of total anthocyanin were expressed as milligrams of cyanidin-3-glucoside equivalent (c3g) per 100 g of fresh weight.

3.3.5 Determination of Total Phenolic Content

Total soluble phenolics were spectrophotometrically determined with Folin-Ciocalteu reagent according to Slinkard and Singleton’s method (1977) using gallic acid as the
standard. Absorbance was read at 765nm. Contents of total phenolic content were calculated using a regression equation of gallic acid and expressed as mg gallic acid equivalents (CAE) per 100 g of fresh weight.

3.3.6 HPLC-MS Analyses of Anthocyanins

Qualitative analysis of blueberry anthocyanins was conducted in a high performance liquid chromatography (HPLC) system equipped with LC-20AD pumps and a SIL-20AC autosampler coupled to an LCMS Shimadzu 2010 EV mass spectrometer using a SPDM20A Photodiode Array Detector. A reverse phase Symmetry C18 (150 * 4.6mm) column (Kinetex) was used. Samples were first purified by C18 column to remove the sugars and acids, then filtered through a 0.45μm RC membrane filter (Phenomenex). Samples were analyzed with a flow rate of 0.8ml/min and a 75μl injection volume. Samples were semipurified using C18 column. The measurement of anthocyanins was performed using the Shimadzu HPLC equipped with LC-20AD pumps. Separation of anthocyanins for blueberries was achieved using a linear gradient from 0-1 min, 6% to 6% B; 1-20 min, 6% to 11.5% B; 20-28min, 11.5% to 16.5% B; 28-33 min, 16.5% to 30% B; 33-37 min, 30% to 10% B; 37-42min, 10% to 10% B. The mobile phase consisted of solvent (A) 4.5% (v/v) formic acid in HPLC water and solvent (B) 100% acetonitrile. Anthocyanins were measured at 520nm. Spectral data of anthocyanins was collected at 250-700nm.
3.3.6 Statistical Analysis

The statistic calculations were performed with SPSS 11.0 by SPSS Inc. (2001). All data passed the normality and equal variance tests. Significances of differences were conducted with a Tukey-HSD multiple-comparison test ($p \leq 0.05$).

3.4 Results and Discussion

3.4.1 Determination of Fruit Quality Attributes

3.4.1.1 Fruit Weight

Means for individual weight for cultivated and wild blueberries that were grown in 8 different locations in 2014 were presented in Figure 3.1. The individual weight of cultivated blueberry varied from 1.05g to 1.89g when harvested in different locations. The highest individual weight of fruits was found in Merlino while the lowest was found in Bill. The individual weight of wild blueberries was in the interval of 0.18g to 0.39g depending on the location that they harvested.

The ANOVA result for individual fruit weight showed that the domestication effect was significant (Table 3.2). The average individual weight for cultivated blueberry was 1.55 ± 0.37, while for wild was 0.27 ± 0.06. It was obvious that wild blueberries were smaller and lighter, meanwhile, had a much narrower range in fruit weight. However, there was insufficient evidence to suggest that the location could cause variation for fruit individual weight ($P = 0.17$).
3.4.1.2 Total Soluble Solid

The average brix for cultivated and wild blueberries were 9.8 ± 1.5 and 10.1 ± 2.2, respectively (Table 3.1). The cultivated and wild blueberries were with almost the same brix value, which meant that the cultivation methods showed no effect on total sugar content. The significance probability values (0.635, 0.104) suggested that total soluble solids exhibited no variability among cultivated and wild blueberries harvested in different locations. Generally brix value was a constant parameter, only maturity degree would affect it a lot. So this result also explained why brix value was always chosen as a standard to judge the fruit ripeness.
3.4.1.3 PH

Other than brix value, difference on pH was found between cultivated and wild blueberries. The average pH for cultivated and wild blueberries were 3.97 ± 0.62 and 3.48 ± 0.54, respectively (Table 3.1). When fruits were with homogeneous maturity degree, the cultivated had higher pH value than wild, which led to a sour and tart flavor for wild blueberries. Difference was also found in the location of cultivated and wild blueberries. For cultivated blueberry, the highest pH value was gotten in ‘Merlino’ reaching 4.83 and the lowest was found in ‘Moore’ with 3.32. Wild blueberry collected in ‘Doyle’ had a very high pH value, 4.31, which was higher than most of highbush samples. However, one thing to note was its brix value. It was as high as 12.2, the highest among all the samples that were collected. In other word, they had the highest maturity degree. It might be the high maturity degree that led to a high pH value. The interaction between cultivar and location was not significant enough (P = 0.053).
<table>
<thead>
<tr>
<th>Location</th>
<th>Individual Weight (g)</th>
<th>PH</th>
<th>TTS (Brix°)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultivated</strong></td>
<td><strong>Highbush</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berenato</td>
<td>1.52 (0.39)</td>
<td>4.62 (0.33)</td>
<td>9.0 (1.8)</td>
</tr>
<tr>
<td>Bill</td>
<td>1.05 (0.23)</td>
<td>4.30 (0.42)</td>
<td>9.6 (2.4)</td>
</tr>
<tr>
<td>Weels</td>
<td>1.40 (0.51)</td>
<td>3.65 (0.38)</td>
<td>9.1 (1.3)</td>
</tr>
<tr>
<td>Thompson</td>
<td>1.47 (0.40)</td>
<td>3.54 (0.23)</td>
<td>9.6 (2.0)</td>
</tr>
<tr>
<td>Doyle</td>
<td>1.81 (0.19)</td>
<td>4.07 (0.25)</td>
<td>10.7 (1.0)</td>
</tr>
<tr>
<td>Merlino</td>
<td>1.80 (0.11)</td>
<td>4.83 (0.56)</td>
<td>10.7 (1.2)</td>
</tr>
<tr>
<td>Miller 1</td>
<td>1.45 (0.18)</td>
<td>3.42 (0.26)</td>
<td>9.0 (2.0)</td>
</tr>
<tr>
<td>Moore</td>
<td>1.89 (0.22)</td>
<td>3.32 (0.21)</td>
<td>10.6 (1.3)</td>
</tr>
<tr>
<td><strong>Overall mean</strong></td>
<td><strong>1.55 (0.37)</strong></td>
<td>3.97 (0.62)</td>
<td>9.8 (1.5)</td>
</tr>
<tr>
<td><strong>Wild Highbush</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berenato</td>
<td>0.27 (0.046)</td>
<td>2.98 (0.13)</td>
<td>9 (0.7)</td>
</tr>
<tr>
<td>Bill</td>
<td>0.29 (0.035)</td>
<td>2.95 (0.42)</td>
<td>8.5 (2.3)</td>
</tr>
<tr>
<td>Weels</td>
<td>0.26 (0.042)</td>
<td>3.51 (0.29)</td>
<td>9.0 (2.6)</td>
</tr>
<tr>
<td>Thompson</td>
<td>0.38 (0.035)</td>
<td>3.62 (0.22)</td>
<td>10.8 (1.1)</td>
</tr>
<tr>
<td>Doyle</td>
<td>0.18 (0.067)</td>
<td>4.31 (0.33)</td>
<td>12.2 (1.8)</td>
</tr>
<tr>
<td>Merlino</td>
<td>0.23 (0.037)</td>
<td>3.67 (0.20)</td>
<td>7.9 (1.1)</td>
</tr>
<tr>
<td>Miller 1</td>
<td>0.29 (0.030)</td>
<td>3.41 (0.46)</td>
<td>11.1 (1.7)</td>
</tr>
<tr>
<td>Moore</td>
<td>0.29 (0.057)</td>
<td>3.44 (0.37)</td>
<td>11.8 (2.6)</td>
</tr>
<tr>
<td><strong>Overall mean</strong></td>
<td><strong>0.27 (0.065)</strong></td>
<td>3.48 (0.45)</td>
<td>10.1 (2.5)</td>
</tr>
</tbody>
</table>

Table 3.1: Mean (standard deviation) individual fruit weight (g), pH and total soluble solid (°brix) of cultivated and wild blueberries, harvested in 8 locations in 2014.

<table>
<thead>
<tr>
<th>Significance</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Location</td>
</tr>
<tr>
<td>Individual Weight (g)</td>
<td>0</td>
</tr>
<tr>
<td>PH</td>
<td>0</td>
</tr>
<tr>
<td>Total Soluble Solid (Brix°)</td>
<td>0.635</td>
</tr>
</tbody>
</table>

Table 3.2: Significance probability of type-dependent and location-dependent differences in individual weight, pH and total soluble solids of cultivated and wild blueberries, harvested in 8 locations in 2014.
3.4.2 Determination of anthocyanin content and profile

Table 3.4 reported analytical data of anthocyanin and phenolic content for cultivated blueberries (V. corymbosum, var. Bluecrop) and wild blueberries. One-way ANOVA showed that the anthocyanin content of blueberry depended on the domestications. The anthocyanin content indicated that cultivated fruits, despite differences between the 8 locations, had much less anthocyanin content than the wild ones. The mean anthocyanin content of cultivated and wild blueberries were 91.5 ± 30.6mg / 100g FW and 160.5 ± 49.7mg / 100g FW, respectively (Table 3.3). Wild blueberry had significant higher levels of anthocyanin (P = 0) when compared with cultivated type, irrespective of the location (Table 3.4). The highest anthocyanin contents for cultivated and wild blueberries were obtained in ‘Weels’ and ‘Merlino’, respectively. The overall anthocyanin content of wild fruit exceeded that of cultivated fruit by 75.4%. Early studies reported 69 – 117mg/100g FW for highbush cultivar ‘Bluecrop’ and 100 – 114mg/100g FW for wild, which was similar with our results (Kalt et al, 2001). In the study by Prior et al (1998), the total anthocyanin content of six cultivated highbush blueberry varieties from 93 to 235mg/100g FW, which would be explained by different extraction method that they used.

There was no interaction between location and anthocyanin content among either cultivated highbush ‘Bluecrop’ or wild blueberries (P = 0.409).

HPLC analysis of anthocyanins allowed to identify and quantify the compounds listed in Figure 3.2, which showed the HPLC anthocyanin profile of cultivated ‘Bluerop’ and wild
blueberries. Here, samples harvested from ‘Thompson’ were used as an example and samples harvested from other locations followed the similar result.

Figure 3.2: Chromatograms of cultivated ‘Bluecrop’ and wild highbush blueberries harvested at Thompson, detected at 520nm. Peak identification: 1. Dp-3-gal; 2. Dp-3-glu; 3. Cy-3-gal; 4. Dp-3-arab; 5. Cy-3-glu; 6. Pt-3-glu + Cy-3-arab; 7. Pn-3-gal; 8. Pn-3-glu; 9. Mv-3-gla; 10. Mv-3-glu; 11. Mv-3-arab. Acylated anthocyanins didn’t identify.

The cultivated and wild blueberries showed different anthocyanin compositions, as showed in Figure 3.2. Dp-3-gal, Dp-3-glu, Cy-3-gal, Dp-3-arab and Cy-3-glu were all found in lower concentrations in ‘Bluecrop’ cultivated blueberry, and Mv-3-gla, Mv-3-glu and Mv-3-arab were found in much higher concentrations in ‘Bluecrop’ cultivated blueberry. In general terms, delphinidin and malvidin derivatives were the most representative forms in blueberries (Giovanelli and Buratti, 2009). Wild blueberry showed higher concentrations in almost all the identified compounds, as expected from
the higher total anthocyanin content; this conclusion does not apply to malvidin glycosides, especially Mv-3-gla and Mv-3-ara, which are present in significantly lower concentrations in wild fruits than in the cultivated ones. Delphinidin glycosides are the most abundant anthocyanins in wild berries.

<table>
<thead>
<tr>
<th>Location</th>
<th>Anthocyanin Cultivated Highbush</th>
<th>Phenolics</th>
<th>Phenolics Cultivated Highbush</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berenato</td>
<td>78.1 (42.9)</td>
<td>188 (10.6)</td>
<td></td>
</tr>
<tr>
<td>Bill</td>
<td>115.0 (34.0)</td>
<td>227 (35.3)</td>
<td></td>
</tr>
<tr>
<td>Weels</td>
<td>116.5 (28.1)</td>
<td>206 (63.9)</td>
<td></td>
</tr>
<tr>
<td>Thompson</td>
<td>82.1 (27.1)</td>
<td>236 (24.4)</td>
<td></td>
</tr>
<tr>
<td>Doyle</td>
<td>78.3 (23.0)</td>
<td>243 (40.8)</td>
<td></td>
</tr>
<tr>
<td>Merlino</td>
<td>113.5 (24.4)</td>
<td>352 (10.1)</td>
<td></td>
</tr>
<tr>
<td>Miller 1</td>
<td>74.7 (19.1)</td>
<td>190 (18.3)</td>
<td></td>
</tr>
<tr>
<td>Moore</td>
<td>81.6 (44.9)</td>
<td>195 (45.2)</td>
<td></td>
</tr>
<tr>
<td>Overall mean</td>
<td>91.5 (30.6)</td>
<td>218 (49.6)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Location</th>
<th>Anthocyanin Wild Highbush</th>
<th>Phenolics</th>
<th>Phenolics Wild Highbush</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berenato</td>
<td>140.5 (43.6)</td>
<td>323 (69.9)</td>
<td></td>
</tr>
<tr>
<td>Bill</td>
<td>184.9 (47.5)</td>
<td>373 (56.8)</td>
<td></td>
</tr>
<tr>
<td>Weels</td>
<td>135.6 (63.0)</td>
<td>347 (119.9)</td>
<td></td>
</tr>
<tr>
<td>Thompson</td>
<td>185.3 (65.5)</td>
<td>414 (96.8)</td>
<td></td>
</tr>
<tr>
<td>Doyle</td>
<td>148.1 (15.2)</td>
<td>623 (70.9)</td>
<td></td>
</tr>
<tr>
<td>Merlino</td>
<td>206.0 (40.7)</td>
<td>564 (25.6)</td>
<td></td>
</tr>
<tr>
<td>Miller 1</td>
<td>160.3 (60.8)</td>
<td>349 (67.4)</td>
<td></td>
</tr>
<tr>
<td>Moore</td>
<td>133.9 (29.6)</td>
<td>281 (68.4)</td>
<td></td>
</tr>
<tr>
<td>Overall mean</td>
<td>160.5 (49.6)</td>
<td>403 (130.4)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Anthocyanin (mg c3g / 100g FW) and phenolic (mg GAE / 100g FW) contents of cultivated and wild blueberries, harvested in 8 locations in 2014.
<table>
<thead>
<tr>
<th>Type</th>
<th>Significance Probability</th>
<th>Location</th>
<th>Type * Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanin</td>
<td>0</td>
<td>0.409</td>
<td>0.781</td>
</tr>
<tr>
<td>Phenolic</td>
<td>0</td>
<td>0</td>
<td>0.22</td>
</tr>
</tbody>
</table>

Table 3.4: Significance probability of type-dependent and location-dependent differences in total anthocyanin and total phenolics of cultivated and wild blueberries, harvested in 8 locations.

3.4.3 Determination of Phenolic Content

The total phenolic content for cultivated and wild blueberries were presented in Table 3.3. With regard to the phenolic content of cultivated and wild blueberries, data showed that cultivated blueberries were more homogenous, ranging from 188 to 352 mg/100 g FW, while wild blueberries ranged from 281 to 623 mg/100 g FW. The average total phenolic content in cultivated blueberry was 218 mg GAE/100 g FW while in wild blueberry was 403 mg GAE/100 g FW. So the wild blueberry had a much higher concentration of total phenolic content than cultivated blueberry. In wild fruits, anthocyanins accounted for more than 50% of total phenolics, and were present in the pulp as well as in the skin, the pulp of wild berries was red-colored. On the contrary, the pulp of the cultivated varieties didn’t contain red polyphenols (Giovanelli and Buratti, 2009). So that was why phenolic content was higher in wild berries than cultivated berries.

Similar results on anthocyanin and phenolic content in cultivated highbush blueberries had been reported by Prior et al. (1998), they reported concentrations of total anthocyanins ranging from 62 to 157 mg/100 g FW and total phenolics ranging from 233 to 273 mg/100 g FW. The small difference of the present results in comparison to the one
reported in the literature may arise due to different cultivation and climate conditions or using different extraction solvent in sample preparation. The anthocyanin and phenolic content in wild blueberries were much closer to lowbush blueberry (*Vaccinium angustifolium*) species than highbush species. Lee et al. (2004) evaluated the polyphenolic content in various blueberry species. In lowbush blueberries total phenolics varied from 489 to 702 mg/100g FW and total anthocyanins from 176 to 311 mg/100g FW, which was very similar to the results we got for wild highbush blueberries.

Phenolic contents also showed a significant variability among different locations (Table 3.4). Location, as a synthesis environmental factor, can also influence the synthesis of the compounds, especially shown in pH and total phenolic content. The temperature, irradiation, herbivory, and pathogenic infection can all work together as a parameter called location to influence blueberry fruit quality (Kalt et al, 2001). The larger difference in total phenolic content was found in wild blueberry with values from 281 (‘Moore’) to 623 (‘Doyle’) mg GAE/100g FW.

Results obtained in this study showed that wild blueberries grown in New Jersey had much higher antioxidant content and might have higher potential antioxidant capacity. However, their output can’t fully account for the market. Their smaller size would be difficult to harvest, transport, store and process mechanically. And they couldn’t consistently yield larger fruits with better mouth feel.

Still now, we have known that domesticated blueberry emphasize things like fruit size and flavor at the cost of less antioxidant compounds, we still want to explore the other consequences of domestication. For example, has domestication changed blueberries’
defenses against herbivory? If so, it could affect the composition of insect communities that form around these plants.

3.5 Conclusion

Results obtained in this study showed that in a discussion of factors that affected blueberry quality attributes, the cultivar was the first thing to be considered. In the present study, cultivated blueberries showed significant differences on fruit quality with wild blueberries. Cultivated blueberries had an average bigger size and higher pH value, associated with less sour and tart mouth feel. However, wild highbush blueberry grown in New Jersey represented a very interesting source of phenolics and anthocyanins. With respect to some cultivated varieties (Blucrop), commonly available on the North American market, wild blueberries have much higher antioxidant content. Total phenolic and total anthocyanin concentrations are, respectively 0.75 fold and 2 fold higher in the wild fruits. The anthocyanin profiles of the varieties investigated show some differences that allow to differentiate between cultivated and wild blueberries, in particular delphinidin and malvidin content.

Besides, location, as a synthesis environmental factor, can also influence the synthesis of the compounds and eventually influence blueberry fruit quality. It seems that domestication and environmental factors can work together to influence blueberry mouthfeel and antioxidant compounds content. Such kind of differences may not only affect commercial value, but may also influence insect pollination and pest infestation preference.
Chapter 4: Influence of Brown Marmorated Stink Bug Infestation on Blueberry Anthocyanin and Phenolic Accumulation

4.1 Abstract

Highbush blueberries are an economically important crop worth over $60 million in New Jersey (NJ). However, the brown marmorated stink bug (BMSB), *Halyomorpha halys* (Hemiptera: Pentatomidae) can damage the blueberries, leading to leaf stippling, seed loss and also transmission of plant pathogens. Some studies have focused on the blueberries feeding injury caused by BMSB, but not much information is available on the response of blueberry to this pest, so little is known about influence of BMSB infestation on blueberry anthocyanin and phenolics accumulation. This research aims to determine if the attack of BMSB adults or nymphs could impact blueberry anthocyanin and phenolics content and profile. Blueberries were provided by Marucci Blueberry and Cranberry Research and Extension Center in New Jersey. Samples were either mechanically damaged or infested by 0, 2, 5 or 10 adult or nymph BMSB and berries were collected at select time points from 3 different plots. Composition of samples with similar maturity was evaluated. Phenolics (including anthocyanins) were extracted with 70% acetone and partitioned with chloroform. C18 cartridge was used to separate sugars from phenolics.
UV-Vis spectrophotometer was applied for anthocyanins and phenolics quantitative analysis. The HPLC chromatographs were recorded to determine compositional differences among samples. The results showed that when samples had relatively consistent maturity degree, the certain amount of nymph BMSB infestation (2 or 5) stimulated mature blueberries to produce more phenolic compounds while had little effect on anthocyanin level, which could be explained by biochemical stress response. Such kind of injury also influenced the anthocyanin profile.

4.2 Introduction

Blueberry is one of the most welcomed fruits in the United States. They are not only popular because of their delicious mouth taste, but also as having one of the highest antioxidant capacities among all fruits, vegetables, spices and seasonings. Antioxidants are essential to optimizing health by helping to combat the free radicals that can damage cellular structures as well as DNA. The high antioxidant capacity is due to high content of both flavonoid and nonflavonoid phenolics (Cao et al, 1996).

The brown marmorated stink bug (BMSB), Halyomorpha halys (Hemiptera: Pentatomidae) has become a potential serious pest of blueberry in the United States, due to its growing presence and its wide host range (DeFrancesco, 2011). It was initially discovered in eastern Pennsylvania in the mid-1990s. Similar to other stink bugs, the nymphs and adults have a piercing-sucking type of mouthpart. In order to obtain the nutrients of the liquid part of the fruit, stink bugs use these mouthparts in a straw-like
fashion by piercing the fruit. Small necrotic spots on fruit and leaf surfaces often result from feeding damage, and it may be compounded by secondary infections and scarring as the fruit matures.

The resistance of plants to insect pests involves many chemical factors and may occur or induced under any situation (Nutt et al., 2004). Phenolics, a group of secondary metabolites, have been reported to play very important roles on resisting environmental stress like water shortage, pollution and herbivory (Hahlbrock & Scheel, 1989; Simmonds, 2003). In regard to insect-pests, phenolics act as digestion inhibitors, and producing free radicals (Appel, 1993).

To date, very little is known about the physiological response of blueberry to BMSB. In this research, we evaluated blueberry responses to the infestation of BMSB adults and nymphs, in terms of fruit sugar composition, total anthocyanin and phenolics content.

4.3 Materials and Methods

4.3.1 Plant Material and Fruit Sample Collection

Highbush blueberry plants were grown at the P.E. Mariucci Blueberry and Cranberry Experiment Station in Chatsworth, NJ. In this study, the highbush blueberry cultivars used for adult BMSB test and nymph BMSB test were ‘Bluecrop’ (mid-season ripening cultivar) and ‘Elliott’ (late-season ripening cultivar), respectively. All of the blueberry samples were collected during the 2013 season. ‘Bluecrop’ had 4 different harvest dates:
May 30th, June 6th, June 16th and June 26th (n = 60); while ‘Elliott’ had 5 different harvest
dates: July 9th, July 16th, July 24th, July 31st and August 1st (n = 75). Fruits with different
treatments were collected from three different plots per cultivar. In each plot, samples
were randomly harvested from bushes, given a total of three samples per treatment for
each cultivar. Immediately following harvest, samples were placed in polyethylene bags
and transported to the lab in a frozen state. All the samples were stored at -20°C until
extraction and analysis of compounds.
Gallic acid, anhydrous sodium carbonate, Folin-Ciocalteau reagent and ethanol were
purchased from Fisher.

4.3.2 Sample Extraction and Quality Attributes Determination

‘Quality Attribute’ measurements including total soluble solids and pH were determined
during sample extraction procedure. Blueberry samples with different treatments were
weighed using a Denver Instrument SI-2002 gram scale, blended with appropriate
amount of liquid nitrogen and extracted by acetone for the analysis of total anthocyanin
and total phenolics. Blueberries along with 0.1 volume distill water and certain amount of
liquid nitrogen was homogenized in a blender for 2 min. Then a manual refractometer,
standardized with distilled water and expressed as °brix was used to analyze the total
soluble solids of samples. Another 0.1 volume distill water was added to samples to
measure the pH value by pH meter. After that, 1.8 volumes of pure acetone was added to
blueberry powder and homogenized for 2 min. The blend was then vacuum filtered using
a Buchner funnel with 70mm Whatman #4 filter paper (Rodriguez-Saona and Wrolstad,
2001). The anthocyanin solution was poured into a separatory funnel with 1.5 volumes of chloroform. The chloroform/anthocyanin solution was gently mixed and then placed at room temperature (20°C) for 24 hours to allow for maximum separation. The bottom layer of chloroform and polar solvents was discarded and the top layer (anthocyanin/phenolic concentrate) was collected. Residual acetone was evaporated using a Buchi rotary evaporator. The final volume of sample was documented for future calculation.

4.3.3 Determination of Total Anthocyanin Content

The total anthocyanin content was estimated by using a pH differential method (Giusti and Wrolstad, 2001). Buffer solutions were prepared using 0.025M potassium chloride at pH 1.0 and 0.4M sodium acetate buffer at pH 4.5. Absorbance was measured using UV-2450 Shimadzu Spectrophotometer at $\lambda_{\text{max}}$ and 700nm in buffers at pH 1.0 and 4.5, using $A = [(A_{\text{max}} - A_{700})pH1.0 - (A_{\text{max}} - A_{700})pH4.5]$, with a molar absorptivity ($\varepsilon$) of cyaniding-3-glucoside (C-3-G) of 29600. Results were expressed as milligrams of equivalent C-3-G per gram of fresh weight, using Monomeric anthocyanin pigment (mg/liter) = $(A \times MW \times DF \times 1000) / (\varepsilon \times 1)$.

4.3.4 Determination of Total Phenolic Content

According to Slinkard and Singleton (1997), total soluble phenolics in blueberry extractions were spectrophotometrically determined with Folin-ciocalteu reagent using gallic acid as a standard. First, the gallic acid standard was made by diluting 0.02ml,
0.04ml, 0.08ml, 0.16ml, 0.24ml, 0.32ml and 0.40ml of gallic acid solution (5.0g/L) into 2ml of total volume of distilled water in cuvettes. Absorbance was measured at 765nm using the UV-2450 Shimadzu Spectrophotometer. Total phenolics were expressed as gallic acid equivalents (GAE). Then 20μl of sample and 20μl of each gallic acid dilution were added to separate 3ml plastic cuvettes, one set for the standard curve and one set for sample testing. All of the cuvettes were then filled with 1.58ml of distilled water. Next 100μl of FC reagent was added and allowed to sit for 1-8 min. Lastly, 300μl of a 20% sodium carbonate solution was pipetted into each cuvette and was let to sit for two hours. After each addition of solution, the cuvettes were covered in parafilm and gently rotated upside down to mix thoroughly for a better chemical reaction. After two hours, the cuvettes were tapped to get rid of bubbles. A blank was used to calibrate/autozero the spectrophotometer. The gallic acid standards were measured at 765nm to create a standard curve and then samples were measured.

4.3.5 HPLC-MS Analyses of Anthocyanins

Qualitative analysis of blueberry anthocyanins was conducted in a high performance liquid chromatography (HPLC) system equipped with LC-20AD pumps and a SIL-20AC autosampler coupled to an LCMS Shimadzu 2010 EV mass spectrometer using a SPDM20A Photodiode Array Detector. A reverse phase Symmetry C18 (150 * 4.6mm) column (Kinetex) was used. Samples were filtered through a 0.45μm RC membrane filter (Phenomenex). Samples were analyzed with a flow rate of 0.8ml/min and a 75μl injection volume.
Samples were semipurified using C18 column. The measurement of anthocyanins was performed using the Shimadzu HPLC equipped with LC-20AD pumps. Separation of anthocyanins for blueberries was achieved using a linear gradient from 0-1 min, 6% to 6% B; 1-20 min, 6% to 11.5% B; 20-28 min, 11.5% to 16.5% B; 28-33 min, 16.5% to 30% B; 33-37 min, 30% to 10% B; 37-42 min, 10% to 10% B. The mobile phase consisted of solvent (A) 4.5% (v/v) formic acid in HPLC water and solvent (B) 100% acetonitrile. Anthocyanins were measured at 520nm. Spectral data of anthocyanins was collected at 250-700nm.

Mass spectrometry analysis was performed under positive ion mode with the following settings: Nebulizing gas flow, 1.5L/min; interface bias, +4.5 kV. A full scan was performed with a mass range from 50-500m/z and Selective Ion Monitoring (SIM) was used to search for molecular ions of the common anthocyanidins under positive ion mode.

4.3.6 HPLC-RID Analysis of Sugars

The content and amount of sugars were detected using HPLC. Samples were first purified by C18 column to remove anthocyanins and phenolics, then the obtained extract was filtered through a membrane filter with pore size of 0.2μm. Second, extract was placed in a vial and tested by Semi-prep HPLC (Shimadzu, Japan) equipped with monosaccharide calcium column (250 * 4.6 mm, particle size - 5μm) and autosampler SIL-20A. Sugars were detected with a refractive index detector RID-10A (Shimadzu). HPLC water was used as eluent while column temperature was held at 80°C. The flow rate was 0.6ml/min.
Injection volume of samples was 50μl. Calibration curve was acquired after two repeated HPLC runs of six standard solutions of reference compounds.

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<td>( R^2 )</td>
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Table 4.1: Calibration equation of reference compounds used for HPLC analysis.

4.3.7 Statistical Analysis

Statistical analysis was performed using One-way Analysis of Variance (ANOVA). Significantly different means were compared using LSD multiple comparisons test with an alpha level \( P \leq 0.10 \) acceptance of the null hypothesis and a 90% confidence interval to determine significant variables on total phenolics and total monomeric anthocyanin. All statistical data were analyzed using SPSS software.

4.4 Results and Discussion

4.4.1 Blueberry samples infected by adult BMSB

4.4.1.1 Sample selection

Most harvested blueberries contained a considerable amount of immature fruits that were not sorted out prior to analyze. As the plant maturity status reflects physiological and biochemical process of plant tissue, the ripening degree of blueberry was related to the anthocyanin and phenolic content. So difference in maturity degree prevented direct
statistical comparison and comparison could only be drawn within mature samples or immature samples.

The maturity of blueberries is directly related to their total soluble solids and color. Therefore these inhomogenous samples were grouped by total soluble solid contents (brix value) and surface color (anthocyanin content). The average brix for ‘Bluecrop’ highbush blueberry was 9.8 ± 1.5 (shown in Chapter 3), thus 5th and 95th percentiles of brix value were between 6.9 and 12.7. Here, the standard for mature samples were the samples’ brix value higher than 6.9, meanwhile, the anthocyanin content was not zero. The left samples were grouped as immature samples.

Here, most samples were grouped into immature samples. The numbers of replication for mature samples treated by 2, 5 or 10 adult BMSB were 1, 2 and 1, respectively. The sample size was not big enough to meet the minimal requirement for quantitative statistical analysis, so these mature samples were just applied for anthocyanin profile analysis. According to Prior et al. (1998), Wang and Lin (2000), Moyer et al. (2002), Kalt et al. (2003) and Castrejón et al. (2008), immature highbush blueberry green fruits contain very low or undetectable amounts of anthocyanins, then linearly increased through fruit maturation. Our results gotten by UV-Vis spectroscopy and HPLC analysis also showed that at greener maturity stages, blueberry fruits contained undetectable anthocyanins. So the immature samples were only applied for total phenolic content comparison.
4.4.1.2 Determination of anthocyanin profile

Because the number of replication for mature samples was not enough for statistical analysis, meanwhile the immature samples had no or little anthocyanin content, so quantitative comparison for anthocyanin content among different treatments couldn’t be done under such condition. Only anthocyanin profile differential could be determined by HPLC-PDA-MS.

Through comparing the m/z of each anthocyanin molecule and its fragmentation to the values in available published works, anthocyanins can be identified without the aid of standards. The LC-MS chromatograms (Figure 4.1) shown that no matter whether the fruit were infected by pest or not, the cultivar ‘Bluecrop’ extraction always had 14 main peaks, which meant that there were 14 different kinds of anthocyanins in ‘Bluecrop’ and its composition couldn’t be changed by mechanical damage or BMSB infestation. Each of the anthocyanin proportion in the total anthocyanins was shown in Table 4.2. It was obviously that the individual anthocyanin proportion in total peak area varied with each treatment. The most obvious differences were shown on peak 10 and peak 11. Based on HPLC-MS identification, peak 10 and peak 11 was Mv-3-glu and Mv-3-arab, respectively. The ratio between Mv-3-glu and Mv-3-arab in blue ripe fruits significantly varied among the different treatments in the order 5 adults (1.9) > 2 or 10 adults (1.3) > mechanical damage (1.1) > control (1.0)
Table 4.2: Individual anthocyanin proportion in Bluecrop and Elliott blueberry treated by adult BMSB or nymph BMSB, respectively. Determinations were performed by HPLC analysis. Peak area proportion higher than 1 percentage was shown in Table. ND: not detected.
Figure 4.2: Chromatograms of ‘Elliott’ with / without BMSB infestation, detected at 520nm. Peak identification: 1. Dp-3-gal; 3. Cy-3-gal; 4. Dp-3-arab; 6. Pt-3-glu + Cy-3-arab; 8. Pn-3-glu; 9. Mv-3-gla; 10. Mv-3-glu; 11. Mv-3-arab.

4.4.1.3 Determination of phenolic content

The comparison of total phenolic content among different treatments was shown in Table 4.3. In immature blueberries, 5 adult BMSB infestation or mechanical damage could result in lower total phenolic content in blueberry (P ≤ 0.10). In the meantime, samples infected by 10 BMSB shown a significant higher phenolic content than infected by 5 BMSB.

It is important to mention that whole fruits harvested at intermediate ripe stages (from 75% red to blue stages) had lower TPH contents (average of 50%) than those detected in fruits at ripe or green stages, blueberry fruits harvested at greener stages had higher TPH than those harvested as ripe blue fruits (Ribera et al, 2010). Similar results have been
previously reported by Connor et al. (2002a), Kalt et al. (2003) and Castrejón et al. (2008), who observed that TPH contents in highbush blueberry and cranberry ripe fruits decreased up to 30 and 50%, respectively, compared with the levels found in immature fruits. So the phenolic content in blueberry during its maturation process was actually a dynamic process. The more exact classification of blueberry maturity, the more precise results we could get. When Ribera et al (2010) talked about the phenolic compound content among maturity stages in highbush blueberries, they separated fresh fruit at six different maturity stages based on their surface color at harvest: green, 25% red, 50% red, 75% red, 100% red and blue. So when samples were just separated as mature and immature, the standard deviation was so big that influenced the statistic data analysis. So more uniform samples was necessary to do further analysis and to get a more accurate result.
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*, ** The significantly different at $P \leq 0.10$, or 0.05, respectively.

Table 4.3: Total phenolic content (mg GAE / 100g FW) of immature blueberry treatment by mechanical damage or adult BMSB infestation.
4.4.2 Blueberry samples treated by nymph BMSB

4.4.2.1 Sample selection

Some harvested blueberries contained a considerable amount of immature fruits that were not sorted out prior to analyze. As the plant maturity status reflects physiological and biochemical process of plant tissue, the ripening degree of blueberry was related to the anthocyanin and phenolic content. Here, comparison could only be done within mature samples or immature samples.

Therefore these inhomogenous samples were grouped by total soluble solid contents (brix value) and surface color (anthocyanin content). The average brix for ‘Bluecrop’ highbush blueberry was 9.8 ± 1.5 (shown in Chapter 3), thus 5th and 95th percentiles of brix value were between 6.9 and 12.7. Here, the standard for mature samples were the samples’ brix value higher than 6.9, meanwhile, the anthocyanin content was not zero. The left samples were grouped as immature samples.

Here, most samples were grouped into mature samples and the sample size of immature sample was not big enough to do statistic data analysis, so just mature samples were retained for anthocyanin and phenolic content comparison, as well as anthocyanin profile analysis.
4.4.2.2 Determination of anthocyanin content and profile

Because blueberries contain large amounts of sugars, phenolic acids, flavonols and anthocyanins, a solid-phase extraction procedure using C18 column was developed to crudely remove sugars and acids and obtain a anthocyanin-enriched extract.

Date on the average anthocyanin content was presented in Figure 4.3. Although the controlled samples had the lowest average total anthocyanin content, there was no major difference exist among different treatments.

Simmonds (2003) concluded in his review that there was a debate in the relationship between the expression of anthocyanin and pest feeding. In the experiment done on sorghum leaves, the level of anthocyanin would increase after aphids feeding, however, some other experiment questioned their role in making a plant more resistance to attack by insects. Based on our data, the anthocyanin increased little after BMSB infestation.

And an experiment with bigger sample size consistent maturity degree is necessary to get a more precise result.

The profile of anthocyanin in control and treated blueberry samples was presented in Figure 4.2, and the percentage contribution to the total anthocyanins was presented in Table 4.2. Identification of individual anthocyanins becomes quite easy with retention times and UV-Vis and mass spectral data. The total anthocyanin fraction for treatment group was mainly composed of 8 individual anthocyanins (Table 4.2), the same with control group. However, the proportion of individual anthocyanin was varied with
treatments. For comparison, the big differences were shown in peak 1 (Dp-3-gal), peak 4 (Dp-3-arab) and peak 9 (Mv-3-gal). Dp-3-gal proportion in mechanical or BMSB damaged samples ranged from 17.6% to 21.7%, which was much higher than 13.9% in control group. Similar relationship was shown in Dp-3-arab. Its content was high in treatment group and low in control group. In contrast, the proportion of Mv-3-gal was high in control group (33.3%), while lower in BMSB infected samples (25.5% to 29%).

![Graph showing anthocyanin content in blueberries]

Figure 4.3: Mean total monomeric anthocyanin contents (mg c3g / 100g FW) of mature ‘Elliott’ blueberry fruit, treated by 0, 2, 5 or 10 BMSB nymphs or mechanical damage.

4.4.2.3 Determination of phenolic content

In mature blueberries, the phenolic content of blueberry was associated with the blueberry treatments. The infestation with BMSB nymphs on blueberry could significantly stimulate its production of phenolic compounds (P = 0.097) (Figure 4.4). The level of phenolics was higher in most attacked samples. This result was consistent
with previous experiments, which found increased phenolic production response in other plant species driven by pest infestation (Silva et al., 2005; Ghumare and Mukherjee, 2003).

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* The mean significantly different at 0.05 level.

Table 4.4: Total phenolic contents (mg GAE / 100g FW) of mature ‘Elliott’ blueberry fruit, treated by 0, 2, 5 or 10 BMSB nymphs or mechanical damage.
Figure 4.4: Total phenolic contents (mg GAE / 100g FW) of mature ‘Elliott’ blueberry fruit, treated by 0, 2, 5 or 10 BMSB nymphs or mechanical damage. * The significantly different at P ≤ 0.05.

Significant changes in the amounts of phenolics occur in both 2 and 5 nymphs infected samples when compared with control group (Table 4.4). In this study, the blueberry samples showed the same response degree to 2 nymphs infestation and 5 nymphs infestation. Plants accumulating higher rates of phenolics tend to inhibit herbivore development (Ghumare & Mukherjee, 2003; Beninger et al., 2004). However, in this study, when more stress was given (infected by 10 nymph BMSB), there was no significant difference in mean phenolic content when compared with control group, and their phenolic content level was not as high as other samples fed by less BMSB (2 to 5 nymphs). It seems to be a result of insufficient stress-bearing capacity. When plants were infected by small amount of nymph BMSB (2 to 5) that they could sustain, their immunity system began to kick in, producing more phenolic compounds to protect
against feeding damage, act as digestion inhibitors and produce free radicals. However, too many pests would reduce efficiency, and fruits couldn’t produce phenolic compounds as much as before.

There was an example to support this hypothesis. Boone et al (2011) found out that the role of tree defense in constraining mountain pine beetle reproductive and attack could range from crucial to inconsequential depending on beetle population density. When beetle populations were at lower level, trees with higher concentration of induced monoterpenes were less likely to be attacked. However, these relationships were lost as beetle populations got larger. Such density-dependent influences on the efficacy of host defenses may partially explain the result in our experiment.

When fruits hurt by nipper, their average phenolic content was higher than controlled samples, however, such stimulation was not significant enough.

4.4.2.4 Determination of sugar composition

Blueberries could be used for juice and jam processing, for bakery, dairy and convenience foods, but it is in the best for fresh consumption as a dessert fruit. Factor that determines exactly the flavor of the berries is sugar accumulation, especially fructose (Kafkas et al., 2006). It has higher sweetness level compared to glucose and sucrose, therefore more acceptable to consumers who mostly prefer sweet taste (Šně et al., 2011).

Main sugars in blueberries are fructose and glucose (Šně et al., 2011), and small amount of sucrose. During the ripening process the level of sucrose is also decreasing which is
replaced by growing amount of simple sugars like fructose and glucose (Wang et al., 2009). This also confirmed by our HPLC results. Since the samples analyzed here varied from ripe to overripe, the soluble solid contents changed from 7.0 to 13.0, the total fructose and glucose contents also varied along with fruits maturity degree. So simply comparing the fructose and glucose contents among different treatments was unwise under certain condition. Generally mature blueberry fruits had a relative consistent fructose/glucose ratio, so fructose/glucose ratio could be compared under this situation and one-way ANOVA was applied for data comparison. Results showed that the fructose/glucose ratios there were no significant differences among samples (p = 0.383) (Figure 4.5). The control group had a relative higher fructose/glucose ratio (0.88) while mean ratio among nymph BMSB infected samples were varied from 0.73 to 0.80. This result was not accurately enough because of less replication for each treatment and should be further verified by more sample analysis.
Maturity had a marked effect on the total anthocyanins, and the total phenolics of the blueberries. The anthocyanin and phenolics content varied during maturation process. In general, total anthocyanin content was higher in fully ripe berries and lower or not available in unripe green samples; meanwhile, total phenolic content was higher in fully ripe or unripe green samples while relative lower in half-ripe samples. So the evaluation of the total anthocyanin and phenolics should be done under the same maturity degree.

When blueberry fruits under same maturity degree, generally, small amount of nymph BMSB infestation would stimulate the fruit to produce more phenolics, such kind of stimulation was not obvious when blueberry under higher feeding pressure (10 BMSB infestation). This suggested that the increase in phenolics could make the plants more
resistant to attack by insects. However, such kind of stimulation was not obvious on anthocyanin content. In addition, this study demonstrated that both adult and nymph BMSB infestation could change the individual anthocyanin proportion.
Chapter 5. Overall Conclusion and Future Work

The cultivated and wild highbush blueberries showed big differences on fruit quality, anthocyanin and phenolic content. Generally, cultivated blueberry is heavier and not too sour, with less anthocyanin and phenolic content. The wild blueberry has the opposing attributes: lower fruit weight, lower pH and higher anthocyanin and phenolic content. Moreover, the blueberry quality can also be influenced by location. The temperature, irradiation, herbivory, and pathogenic infection can all work together to affect quality. Although different in their relative amounts, cultivated and wild blueberries both have a high antioxidant content compared to other fruits.

We also demonstrated whether certain amount of BMSB infestation could stimulate blueberry to produce more phenolics and anthocyanins than health berries, and whether such kind of infestation could change the profile of anthocyanin and sugar. For mature Elliott blueberry, the 2-5 nymphs BMSB infestation could increase its phenolic content while could hardly influence its anthocyanin content. However, when more BMSB nymphs infected blueberry plants, such stimulation was not significant. In other words, a small quantity of BMSB could stimulate phenolic compounds accumulation, while large quantity of BMSB could inhibit such promotion. Moreover, the anthocyanin content
didn’t change a lot among different treatments, so it seems that the phenolics accumulation is due to other phenolic compounds increase other than anthocyanins.

Studies can continue on sugar profile in cultivated and wild highbush blueberries, and build up relationship between blueberry fruits quality and pest preference. Furthermore, we should find out which phenolic compounds should be responsible for BMSB – blueberry reaction. Previous studies on crops like wheat and barley had verified that the level of phenolic acids including ferulic acid and salicylic acid would increase as a response to pest infestation (Abdel-Aal et al, 2001; Chaman et al, 2003). Future study could apply FT-IR on blueberry phenolic compounds quantitative and qualitative analysis, and establish relationship between other phenolic compounds like phenolic acids and BMSB infestation. Recent research was limited to BMSB, we still need to undertake more experiment to be clear on whether the activity of phenolics is insect specific.
References


