

Concentration and Content of Secondary Metabolites  
in Fruit and Leaves of Haskap (*Lonicera caerulea* L.)

by

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## ABSTRACT

The University of Saskatchewan (UofS) has been conducting crosses of *Lonicera caerulea* and releasing genotypes for fruit production under the name “Haskap”. The primary objectives of the UofS Haskap Breeding Program are to improve fruit flavor, increase fruit size and facilitate mechanical harvest. A more recent additional objective is to increase the concentration and content of compounds with the potential to enhance human health in the fruit and leaves of new haskap genotypes. As a first step to meet this additional objective, this project surveyed the secondary metabolites present within fruit and leaves of haskap. Genotypes tested included genotypes released by the UofS Haskap Breeding Program, unnamed genotypes with potential for use in breeding programs, genotypes acquired from germplasm repositories as well as genotypes of *Lonicera caerulea* subsp. *villosa*. Secondary metabolites were selected for further study if they were both linked to human health and were found at sufficient concentrations in haskap to allow for quantification. Chlorogenic acid, quercetin (three glycosides), loganin and secologanin matched these selection criteria. HPLC and mass spectrometry methodologies were developed to allow for quantification and identification of the target secondary metabolites in methanolic extracts of haskap fruit and leaves. Concentrations of the selected secondary metabolites decreased with fruit development, but the overall content (concentration x fruit weight) increased. In fruit tested at harvest maturity, the highest concentrations of many compounds of interest occurred in the widely grown cultivar Tundra. The concentrations of secondary metabolites in haskap leaves also decreased over the growing season, however at the end of the season, substantial amounts of secondary metabolites were still present in the leaves. The fruit and leaves of *Lonicera caerulea* subsp. *villosa* germplasm had a different quercetin

profile than the other *Lonicera caerulea* genotypes surveyed. The concentrations of secondary metabolites in the fruit of the various genotypes were negatively correlated with the individual fruit weight produced by each genotype. The concentrations of some of the metabolites in fruit and leaves of *Lonicera caerulea* subsp. *villosa* also varied with the geographic site of origin of the genotypes. Post-harvest treatment of haskap fruit with UVC did not enhance the secondary metabolite profile.

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## LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CA	Chlorogenic acid
DPF	Days post fertilization
DNA	Deoxyribonucleic acid
DW	Dry Weight
FRAP	Ferric reducing antioxidant potential
FW	Fresh weight
GDD <sub>5</sub>	Cumulative growing degree days using base 5°C
HBA	Hydroxybenzoic acids
HCA	Hydroxycinnamic acids
HPLC	High Pressure Liquid Chromatography
kJ/m <sup>2</sup>	Kilojoules per meter squared
mg/g	Milligrams per gram
MS	Mass Spectrometry
<i>m/z</i>	Mass to charge ratio
OMAFRA	Ontario Ministry of Agriculture, Food and Rural Affairs
ORAC	Oxygen radical absorbance capacity
Q3G	Quercetin-3-glucoside
Q3R	Quercetin-3-rutinoside
Q3S	Quercetin-3-sambubioside
SS	Soluble solids
TA	Titrateable acidity
<i>t<sub>R</sub></i>	HPLC retention times
UV/vis	Ultra violet and visible light
UVA	Ultra violet A
UVC	Ultra violet C
UofS	University of Saskatchewan
$\lambda_{\text{Max}}$	Absorbance maximum

## **THESIS STYLE**

This thesis is presented in a paper style format. The thesis starts with a chapter outlining the background and reasoning for the project. This chapter establishes the overall goals of the project. The second chapter reviews current information pertaining to haskap and the secondary metabolites in haskap fruit and leaves. Chapters 3 through 7 are written as individual papers. These chapters relate to one another but can stand as individual studies. Between these chapters, transition sections are used to help the reader transition from one chapter to the next. Chapter 8 is an overall discussion which integrates the results presented in chapters 3 through 7. Chapter 9 presents overall conclusions from the entire project.

## 1.0 GENERAL INTRODUCTION

### 1.1 Haskap

*Lonicera caerulea* is a fruit-bearing member of the honeysuckle family. The species is widely distributed over the Northern hemisphere (Bors 2009c), which has led to the adoption of a number of common names for the species including; “Haskap”, “Blue Honeysuckle”, “Blue Berried Honeysuckle”, “Honeyberry”, “Sweet Berry Honeysuckle”, “Swamp Fly Honeysuckle”, “Mountain Honeysuckle” and “Mountain Fly Honeysuckle” (Thompson 2006, Bors 2009c).

Haskap is an anglicization of the first recorded common name “hah-shika-pu” given to *Lonicera caerulea* by the Ainu people, an indigenous group inhabiting Northern Japan and South Eastern Russia (Hokkaido and Kuril islands) (Thompson 2006). Currently “haskap” is the primary name used to identify the *Lonicera caerulea* which are cultivated in North America. North American grower associations use this name when identifying their producer groups such as “Haskap Canada”, “Haskap Growers Association of Nova Scotia” and “Haskap Association of Ontario”. The *Lonicera caerulea* genotypes produced by the University of Saskatchewan (the subject matter for this thesis) are also marketed under the name “haskap”. For those reasons, for the purposes of this thesis, *Lonicera caerulea* and all of its subordinate subspecies and genotypes will be referred to as “haskap”.

The first cultivation of *Lonicera caerulea* was most likely by the Ainu people who made selections from wild genotypes (Miyashita and Hoshino 2010, Hummer et al. 2012), mostly from the subsp. *emphylocalyx* (Thompson 2006). Local folklore in the Hokkaido region has long attributed high nutritional and medicinal value to haskap fruit (Hummer et al. 2012). In the 1980’s, the Japanese government attempted to improve haskap by collecting and crossing superior wild lines of *Lonicera caerulea* subsp. *emphylocalyx* (Miyashita and Hoshino 2010). In

general the goals of these early breeding efforts were to improve fruit flavor and to increase the size of the haskap fruit. This effort eventually led to the development of Japan's first haskap cultivar "Yufutsu" (Tanaka et al. 1994).

In Russia, domestication of haskap began as early as 1900, with domestication efforts intensifying around 1950 (Skvortsov 1986). At that point, Russian botanists led by A.K.Skvortsov began to collect diverse germplasm, mostly from *Lonicera caerulea* subspecies *edulis* and *kamtschatica* (Thompson 2006), and conducted crosses between them. The major objectives of the Russia breeders were increased productivity and larger fruit size (Plekhanova 2000). These early collection and breeding programs eventually led to the release of a large number of haskap genotypes (Hummer 2012).

At present, the major haskap breeding program in the United States is run by a retired professor from Oregon State University, Dr. M. Thompson. Her program began in the mid 1990's and focused on evaluating Russia *Lonicera caerulea* selections, mostly from the subspecies *kamtschatica* and *edulis*. However this material was not adapted to the temperate climate in Oregon. In 2000 the program collected seeds from selections of cultivated haskap growing in Hokkaido Japan. This collection consisted of material from *Lonicera caerulea* subspecies *emphylocalyx* (Thompson 2006) which were more adapted to the climatic conditions of Oregon. Due to the origin of the material, the breeder retained the native Japanese name "haskap" for this fruit crop. The Oregon breeding program subsequently focused on creating cultivars for commercial production, small U-pick operations and back-yard gardeners in the North West USA. (Thompson and Chaovanalikit 2002, Thompson 2006). Selection criteria for genotypes destined for hobby gardeners and U-pick operations were large fruit size, mild taste and enhanced storability (Thompson 2006). The Oregon State Haskap Breeding Program has

cooperated extensively with the UofS Haskap Breeding Program since its inception, and bilateral exchanges of germplasm have occurred (Bors 2009a).

The UofS Fruit Program acquired four haskap genotypes for a nursery in the United States (One Green World Nursery, Portland, Oregon) in 1997 and began its first crosses in 2001 after the acquisition and assessment of additional haskap genotypes from Russia (Bors 2009a). By 2009, the program had amassed a large collection of germplasm which included wild collected material from the Kuril Islands of Japan and improved germplasm from the Oregon State program. The program has also acquired material from the Vavilov Institute, a major germplasm repository in Russia. This acquisition included representatives of several different haskap subspecies, including *L. pallasii*, *altaica* and *venulosa*. The initial breeding strategy for the UofS program was to hybridize the highly productive Russia types with the large fruited Japanese types in the hope of producing hybrids combining the best traits of both parental types (Bors 2012). The UofS Program has also collected > 1400 specimens of wild *Lonicera caerulea* subsp. *villosa* from across Canada, with the hope that this material could be intercrossed with the current genotypes to produce cultivars better adapted to the Canadian climate while also meeting market expectations for fruit quality (Bors 2012).

The present breeding strategy of the UofS Haskap Breeding Program is to make crosses within the diverse germplasm in their collection in an attempt to bring together superior traits (Bors 2012). The main objectives of the program are to improve haskap fruit quality characteristics such as flavor and size, to increase total fruit yield and to improve ease and efficiency of mechanical harvesting (Bors 2009a). Breeding for ease of mechanical harvest involves selecting for traits such as compact bush shape, uniform fruit ripening and durable fruit (Bors 2009a). The UofS Haskap Breeding Program was the first program to include ease of



mechanical harvest as one of the breeding objectives, and purchased a mechanical harvester to test damage from mechanical harvest in future haskap cultivar releases (Bors 2009a, Bors 2009b). The UofS has been active in releasing new haskap genotypes and is presently believed to have the largest and most genetically diverse holding of haskap material in the world. Since its inception the program has released nine genotypes of haskap (Tundra, Borealis, Indigo Treat, Indigo Jem, Indigo Yum, Honey Bee, Aurora, Boreal Blizzard, Boreal Beauty and Boreal Beast) and most genotypes of haskap presently grown in North America commercially or by hobby gardeners arose from the UofS Haskap Breeding Program. Recently the program has also expanded its breeding objectives to include enhancing the quantities of secondary metabolites present in the haskap fruit which have the potential to improve human health (Bors 2012, Rupasinghe et al. 2012). However, it is difficult to increase the concentrations of these compounds without an adequate understanding of the secondary metabolite profiles of haskap fruit and leaves. In addition, there is little information currently available on how these profiles may change based on genotype, developmental stage of the fruit and leaves or the geographic origin of the plant.

## **1.2 Secondary metabolites in haskap**

Haskap fruit contains a range of vitamins and minerals important in human health, but these nutrients are not unique to haskap and other fruit represent equivalent or better sources of these compounds (Plekhanova 1989). Consumption of haskap fruit has been shown to alleviate certain chronic diseases, which suggests the fruit might contain compounds with additional health benefits. For example, animal models have been used to assess the effect of *Lonicera caerulea* subsp. *kamtschatica* fruit consumption on tumour growth (Gruia et al. 2008) and UVA

damage to skin cells (Svobodova et al. 2008). In these studies, consumption of haskap fruit or extracts made from the fruit reduced oxidative damage associated with induced chronic disease. The beneficial health effects observed in these studies were believed to be linked to the presence of certain secondary metabolites within the haskap fruit. The fruit of haskap were found to contain compounds such as hydroxycinnamic acids and flavonols which are produced by the phenylpropanoid pathway (Chaovanalikit et al. 2004, Ochmian et al. 2010, Zadernowski et al. 2005). Some of these compounds have been shown to have beneficial effects when consumed by humans (Nieman et al. 2010).

As the benefits of secondary metabolites, such as hydroxycinnamic acids and flavonols, in the human diet become better appreciated this generates a demand for fruit containing significant quantities of these compounds. Marketing agencies have developed the term “superfruit” for fruit that contain high concentrations of compounds believed to be beneficial. The fruit of haskap has been shown to be comparable to fruit crops sometimes referred to as “superfruit” such as blueberries and bilberries, black currents, and black berries based on the total antioxidant capacity per unit of fruit weight (Chaovanalikit et al. 2004). Rupasinghe et al. (2012) found that the haskap genotypes Borealis, Tundra and Indigo Gem had greater concentrations of both total flavonoids and total phenolics compared to store-bought samples of lowbush blueberry, lingonberry and blackberry. Bakowska-Barczak et al. (2007) found that fruit of an unnamed haskap cultivar had the greatest concentration of total phenolic content of 13 fruit crops grown in Western Canadian, including bilberry, lingonberry, wild raspberry and wild strawberry.

To further enhance the market value of it releases, the UofS Haskap Breeding Program has included the goal of increasing the concentration and content of these secondary metabolites

to its overall breeding objectives for haskap. The secondary metabolites targeted for enhancement should: a) have established potential to improve human health, b) will need to be found at appreciable concentrations in the fruit and/or leaves of the new haskap releases, and c) must have value in the marketplace. Potential market value would be indicated if nutritional supplements consisting of the specific secondary metabolite are already available in the marketplace or if the specific compounds are being marketed as the principal health beneficial compound in comparable fruit products.

The secondary metabolite profile of haskap fruit of a few Russian and Polish genotypes have been studied. The profile included anthocyanins, polyphenols and phenolic acids (Chaovanalikit et al. 2004, Zadernowski et al. 2005, Ochmian et al. 2010). However the secondary metabolite profiles of the haskap genotypes developed at the UofS Haskap Breeding Program, which uses a wider germplasm base, are as yet untested. The metabolite profiles of *Lonicera caerulea* subsp. *villosa* also have not been studied. It is also not clear if there are any major differences amongst the secondary metabolite profiles of different subspecies of *Lonicera caerulea*. The changes in the metabolite profiles that occur during fruit development of haskap are not known, nor have the differences in metabolite profile between fruit and leaf tissues been established. A study investigating the secondary metabolite profile present in the fruit and leaves of diverse haskap genotypes throughout the development period may yield answers to questions such as: a) are the secondary metabolites found in all germplasm or are they genotype specific? b) is the secondary metabolite profile of haskap stable across genotypes, environments and developmental stages? c) what quantities of the individual phenylpropanoid and terpenoid compounds that have shown to have health enhancing effects when consumed by humans are found in haskap fruit and leaves? d) what is the optimum time to harvest haskap fruit (and

possibly leaves) to maximize the total concentration and/or content of the target secondary metabolites? e) are the secondary metabolite profiles in the fruit of haskap related to geographic site of origin of the genotypes? f) can these profiles be enhanced by post-harvest treatments such as UVC irradiation? Answers to these questions could be valuable within a breeding program with the intent of enhancing the secondary metabolite profile of haskap.

### **1.3 Increasing the concentration and content of valuable, health beneficial secondary metabolites in haskap**

The major factors governing the concentration and content of secondary metabolites in all plants, including haskap, are genetics, developmental stage and environment (Jaakola and Hohtola 2010). The effects of certain environmental factors on the concentration and content of secondary metabolites in other fruit bearing crops have been studied. Increased light exposure (UV and white light) (Lois 1994, Lavola 1998, Suzuki et al. 2005, Cohen and Kennedy 2010, Takahashi and Badger 2011), reduced temperature (Suzuki et al. 2005, Cohen and Kennedy 2010) and reduced water availability (Suzuki et al. 2005, Cohen and Kennedy 2010) increased the concentrations of phenylpropanoid compounds in many fruit. Production of terpenoid compounds such as the monoterpenoids can also be induced by insect herbivory (Quintero and Bowers 2013). While the effects of some isolated environmental factors on the concentration of secondary metabolites have been researched, the effects of their interactions (i.e. real time climatic conditions which involve interactions of many individual climatic factors) are hard to predict, measure and control. Thus it is often more consistently effective to try to increase secondary metabolite concentrations and content through improved genetics. Breeding programs

must identify and cross individuals which exhibit high concentrations of the target secondary metabolites in the fruit in the hope of creating offspring that produce even higher amounts.

While consuming fruit rich in health-enhancing secondary metabolites is a popular objective, domestication of fruit species tends to produce genotypes with lower concentrations of secondary metabolite in the fruit than in wild genotypes (Frary et al. 2000). This stems from the drive during domestication and improvement to select for increased fruit yields and increased fruit size. Most secondary metabolites within fruit are either confined to skin tissues or are found at higher concentrations in the skin versus the flesh of the fruit (Petkovsek et al. 2010). As larger fruit generally have a smaller surface area (skin) to volume ratio, selecting for large fruit generally leads to a decrease in the concentration of secondary metabolites. Many secondary metabolites protect the plants from stress. As fruit crops are brought under production conditions the need for these protective compounds may be reduced (Petkovsek et al. 2010) and the energy that went into their formation can instead be directed to other aspects of plant growth, development and yield. For this reason it may be beneficial to survey pure subspecies or wild collected representatives of *Lonicera caerulea*, as this un-improved or wild germplasm may still possess genetics conducive to the production of fruit with high concentrations of secondary metabolites. For example the wild collected *Lonicera caerulea* subsp. *villosa* germplasm held by UofS Haskap Breeding Program may contain genetics conducive to producing fruit with high concentrations of secondary metabolites. It may be beneficial to survey the secondary metabolite profiles in both the fruit and leaves of these genotypes to identify genotypes with superior genetics for the production of secondary metabolites. Differences in secondary metabolite profiles of plants collected over a wide geographic area could also be used to identify locations for future plant collection missions.

Improvement of new lines through standard breeding and selection techniques takes many years. In the meantime there may be potential to enhance the secondary metabolite profile of haskap via post-harvest technologies. For example, UVC irradiation treatments may increase secondary metabolite concentrations in haskap fruit, in effect mimicking the response that would have occurred in nature under stressful conditions.

Another option to increase the amount of secondary metabolites available from a haskap crop would be to utilize haskap leaves as well as the fruit as a harvestable source of these metabolites. Leaves of some plant species contain far greater concentrations of secondary metabolites than the fruit (Harris et al. 2007). Haskap leaves are known to contain quercetin and chlorogenic acid derivatives as well as monoterpenoid iridoids (Machida et al. 1995) and thus the leaves of haskap may represent a potential source of valuable secondary metabolites. It may also be possible to extract secondary metabolites from leaves and add them to processed haskap products to increase the concentrations of secondary metabolites within these products. From a breeding perspective it may also so be possible to use the concentrations of secondary metabolites present in the leaves as a screening tool for early selection of genotypes that will produce fruit with high concentrations of secondary metabolites.

#### **1.4 Goals of the project**

The major goal of the project was to increase our understanding of the secondary metabolite profiles within the fruit and leaves of haskap. This information could be used to aid in breeding efforts aimed at maintaining or ideally enhancing the secondary metabolite profiles in any newly released haskap genotypes. Key gaps in the knowledge base that will be addressed by this project include: a) what compounds are present in haskap fruit and leaves and at what concentrations, b)

determination of how the metabolite profile changes over the course of the growth and development of haskap fruit and leaves, c) comparison of the secondary metabolite profiles within diverse haskap genotypes, including a large collection of *Lonicera caerulea* subsp. *villosa*, a subspecies which has never been investigated, d) comparisons of the profiles of haskap genotypes collected over diverse geographic site of origin and e) to determine if the profiles could be enhanced by post-harvest irradiation with UVC.

## 2.0 LITERATURE REVIEW

### 2.1 Botany of haskap

*Lonicera caerulea* (haskap) is a fruit-bearing hardy shrub in the *Caprifoliaceae* (Honeysuckle) family. Unlike the *Rosaceae* (rose) and *Rutaceae* (citrus) families, the *Caprifoliaceae* family has only a few species which have been domesticated for fruit production. Until recently the *Caprifoliaceae* family also contained the genus *Sambucus* (elderberry) and *Viburnum* (highbush cranberry), but both of these genera have now been moved to the *Adoxaceae* family (Bell et al. 2001). This leaves *Lonicera caerulea* standing somewhat apart from other fruit bearing species.

The wide geographic range of *Lonicera caerulea* has led to a substantial number of naturally occurring polymorphic variants. The correct scientific designation for these polymorphic variants has been disputed (Skvortsov 1986, Lamoureux et al. 2011). Early literature referred to these polymorphic variants as distinct species (i.e. *Lonicera villosa*) or varieties (*Lonicera caerulea* var. *villosa*) (Skvortsov 1986). More recent literature refers to these polymorphic variants as subspecies (i.e. *Lonicera caerulea* subsp. *villosa*) (Lamoureux et al. 2011). However as these variants are easily hybridized and create fertile progeny, the natural variants of *Lonicera caerulea* may be better classified as one species.

#### 2.1.1 Morphology

Cultivated genotypes of *Lonicera caerulea* produce a dense, upright bushy plant, 2m or more in height with a diameter of 1.5-2m when fully mature (Hummer et al. 2012). *L. caerulea* produces between 12-15 upright branches which arise from the crown giving the plant a rounded shape (Plekhanova 1992). A well-established *L. caerulea* plant can have a root system extending



1.5m in diameter (Renata 2001). The root system is mainly located in the upper 0.5m of the soil profile (Renata 2001). Wild genotypes of *L. caerulea* show a great range of variability in size and growth habit (Renata 2001).

Floral tissues of *L. caerulea* appear with the development of the first vegetative material. Flowers develop in the axils of the lowest three nodes of the emerging second year shoots (Hummer et al. 2012). Axillary buds of *L. caerulea* are arranged in a vertical series of three, with the lower two buds containing the flower initials for the following year (Hummer et al. 2012). The uppermost bud usually remains dormant for 3-5 years (Renata 2001). When buds emerge, they give rise to a new vegetative shoot which can grow 50-90cm in a single season (Plekhanova 1989, Hummer et al. 2012).

The flowers of *L. caerulea* have two fused ovaries; giving the appearance of twinned flowers. The fruit weights range from 0.3 to 3.8g depending on the genotype and climatic conditions (Hummer et al. 2012, Bors 2015) and also bears two scars from the abscission zone of the flowers, which is helpful for identification. Flowers are usually self-incompatible and require a compatible pollinator to set for fruit (Plekhanova 2000), however a self-compatible variety “Yufutsu” has been released in Japan (Tanaka et al. 1994).

The bark of *L. caerulea* is brown but becomes tan coloured as the wood matures. Once the wood reaches 2-3 years of age the bark begins to erode in long narrow strips; this is a characteristic of the honeysuckle family (Plekhanova 1989).

### 2.1.2 Life cycle

In Saskatoon, SK (52°N, 106°W) *Lonicera caerulea* plants begin to leaf out and bloom in early May and continue to leaf out for the next 28-30 days (Bors 2012). Trials of advanced haskap selections conducted in Saskatoon showed considerable variation in bloom time (Bors 2012). As *L. caerulea* blooms early in the season, climatic conditions (specifically temperature) can have a large influence on flowering times, and consequently there is considerable variation in bloom time among genotypes. Differences of up to two weeks have been observed for bloom times for the same genotype in differing years at the UofS Field Research Lab (B. Bors personal communication, July 16, 2013.). In haskap grown near St. Petersburg Russia leaf bud break occurred when temperatures were in the range of 2.5–3.8°C and flowering occurred once the temperature were between 9.5-11.7°C (Plekhanova 1989). Flowers remain viable for approximately 48 hours after opening and the flowering period for an individual genotype can last from 7-15 days (Bors 2012). Fruit harvest dates vary with bloom time but usually occurs during late June and early July in Saskatoon (Bors 2012).

Rapid shoot growth of haskap starts in early May and lasts for 15-20 days. Mature plants grow an average rate of 5-6mm per day, while young plants achieved growth rates of 9-15mm (Plekhanova 1989). Growth rates slowed to a rate of 1mm per day for the next 10-15 days (Plekhanova 1989). This gives approximately 39-50 days of shoot growth. The length of fruit bearing shoots can be 8-15cm in a mature plant and 15-35cm in young plants. Flower initials are formed as the shoot growth slows. Setting of flower buds occurs about 35-45 days after harvest (Plekhanova 1989).

*L. caerulea* can begin to fruit within two years of planting a one year old greenhouse-grown plug, with the time required depending somewhat on genotype, climate and management

practices. Fruit yields increase as the plants increase in size (Plekhanova 1992). Cultivated haskap plants typically begin to bear substantial amounts of fruit after 4 years in the field, with full bearing occurring after 7-8 years (Plekhanova 1992). Maximum yields of between 2 and 5 kg of fruit per plant can be harvested after 8-15 years and yields can remain optimum for the next 10-12 years (Plekhanova 1992). Yields plateau as the plants reach an equilibrium between new growth and branch die back. At 20-25 years of age, dying off becomes predominant but productivity can be restored by aggressive pruning or removal of older stems and branches to encourage new growth (Plekhanova 1989).

### 2.1.3 Natural habitat

*Lonicera caerulea* is a cold hardy perennial distributed through most of boreal regions of the northern hemisphere (Plekhanova 2000). As outlined by Plekhanova (1994) and Renata (2001), the species has many subspecies occurring at different geographical locations. Ploidy levels of *L. caerulea* can differ with location (see section 2.2.2). Subspecies of *L. caerulea* can be found in Europe, Asia, and North America (Plekhanova 1989).

*L. caerulea* is native to a variety of habitats such as wet areas near rivers and fens, in boreal forest and on heath barrens (Bors 2012). The bush is considered shade tolerant and is generally slow growing. The limit to its Southern distribution appears to be limited soil moisture availability and inadequate winter chilling (Plekhanova 1989, Hummer et al. 2012). Within Canada, *L. caerulea* subsp. *villosa* is found in all provinces except British Columbia. It is more common in more Northern areas, usually within the Boreal forest (Bors 2012). Plants found in their natural habitat can have longer life spans than cultivated individuals (Plekhanova 1989), which appears to reflect the slower growth and development typical of native environments.

## 2.2 Genetics of haskap

### 2.2.1 Subspecies

*Lonicera caerulea* is distributed across the Northern hemisphere. Due to its wide geographical distribution, *L. caerulea* has many different polymorphic variants as outlined by Skvortsov 1986. However there has been and continues to be debate as to the taxonomic status of these polymorphic variants. Plekhanova (1989) regarded these polymorphic variants as individual species (i.e. *Lonicera villosa*). Tanaka et al. (1994) regarded the polymorphic variants as natural varieties (i.e. *Lonicera caerulea* var. *villosa*). More recently the polymorphic variants have been referred to as subspecies (i.e. *Lonicera caerulea* subsp. *villosa*) (Lamoureux et al. 2011). For the purposes of this thesis, the subspecies designation proposed by Skvortsov (1986) will be used.

The center of diversity of *L. caerulea* is believed to be in central China (Plekhanova et al. 1992). In the haskap germplasm collection held at the Valilov Institute in Russia the accessions are mainly grouped by subspecies (Lamoureux et al. 2011). Lamoureux et al. (2011) found that representatives of the subspecies *altaica*, *pallasii* and *stenantha* were clustered together on the genetic map, while representatives of *kamtschatica* were divided into two distinct clades. Representatives of the subspecies *venulosa* appear to be intermixed throughout the genetic tree, raising questions about the homology of this subspecies. Despite the debate over the level of these polymorphic variants, members of the different subspecies of *Lonicera caerulea* appear to cross readily and produce fertile offspring (Bors 2009c) and thus meet the literal definition of a single species.

### 2.2.2 Ploidy

*Lonicera caerulea* has a base chromosome set of  $n=9$ . Subspecies *edulis* and *boczkarikovae* are diploid, while subsp. *pallasii*, *altacia*, *stenantha*, *kamtschatica*, *venulosa* and *emphyllocalyx* are tetraploid (Plekhanova 1989, Lamoureux et al. 2011). Lamoureux et al. (2011) gave evidence that the diploid subspecies *edulis* and *boczkarikovae* cluster together within the phylogenetic tree of *L. caerulea*, while individual tetraploid subspecies tend to form their own groups. *L. caerulea* subsp. *villosa* was stated to be diploid by Plekhanova (1989), however this data may have been from an incorrectly labeled specimen (Bors 2012). Research into the ploidy level of subsp. *villosa*, as well as the other diverse germplasm held at the UofS, is currently underway. Plekhanov (2000) indicated tetraploid haskap are significantly more productive than diploids, mostly due to greater flower numbers and larger fruit size. Plekhanov (2000) also reported that tetraploid individuals have longer life spans than diploids. Hexaploid and octaploid variants of *L. caerulea* have been artificially created using colchicine (Miyashita and Hoshino 2015), however no details are reported as to whether these plants were fertile or had commercial potential.

Natural populations of *L. caerulea* in Asia contain both diploid and tetraploid individuals (Plekhanova 1992). *L. caerulea* populations with different ploidy levels tend to inhabit different areas over the Asian subcontinent, with tetraploids dominating northern and alpine habitats and diploids being confined to more southern areas (Plekhanova 1992). A study of the distribution of diploid and tetraploid types of *L. caerulea* in the Hokkaido region of Japan found diploid populations were confined mainly to the lowland areas, while tetraploid populations had a wider distribution from lowland to alpine regions (Miyashita et al 2011). As tetraploid plants have

twice the chromosome levels of diploid it would be likely that the tetraploid haskap may have greater genetic diversity.

## **2.3 Commercial cultivation of haskap**

### *2.3.1 Site selection*

Site selection for cultivation of haskap follows most of the general recommendations guiding selection of land for horticultural production. While haskap is considered a shade tolerant species it should be grown in full sun for maximum fruit production (Bors 2009c). *L. caerulea* is extremely winter hardy; Imanishi et al. (1998) estimated the LT50 of *L. caerulea* of overwintering buds of *L. caerulea* at -40°C. The LT50 was negatively correlated with concentrations of the trisaccharide raffinose and the tetrasaccharide stachyose in the buds. *L. caerulea* plants have repeatedly been exposed to temperatures of -40°C in the Saskatoon field plots with minimal damage (Bors 2012). Open flowers of *L. caerulea* can avoid frost and are viable after be exposed to temperatures as low as -8°C (Plekhanova 2000). This allows *L. caerulea* to be grown on a south facing slope with little fear of frost damage to flowers opening in early spring. Planting wind breaks is still suggested as a means to mitigate desiccation damage during the winter and to protect open flowers from wind damage (Bors 2009c).

### *2.3.2 Soil type/pH*

In the wild, *L. caerulea* can be found growing in a range of soil classes (Bors. 2012). Under cultivation, *L. caerulea* is tolerant of a range of soil types and pH levels. Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) suggests a soil pH range of 5.5 - 8.0 for cultivation of haskap in Ontario (OMAFRA 2012). Although *L. caerulea* can be found in the

wild growing in poorly drained bogs and fens, plants respond positively to well-drained soils with some organic matter (Plekhanova 1992).

### 2.3.3 Fertility

Little information is currently available regarding the soil fertility requirements of cultivated *L. caerulea*. Skupien et al. (2007) characterized the crop as having “low” fertility requirements. Ochmian et al. (2010) indicated that yearly application of 40 kg N per ha was a normal practice for haskap production in Poland. Reports from Russia indicate that the plant does not have a heavy requirement for nitrogen, and excess nitrogen may actually hinder root growth (Belosohova and Belosohov 2010). Currently, OMAFRA recommends yearly applications of 30-50 kg N/ha for haskap production in Ontario (OMAFRA 2012), however no information is supplied as to the basis for this recommendation.

### 2.3.4 Planting practices

In Canada most growers plant haskap in spring. At the UofS, planting is usually done in June, August or September, while the month of July is generally avoided due to excessive heat. If plants must be planted in hot weather shade cloth can be employed to protect the plants. Dormant plants are recommended for spring and fall plantings. However as most plant are supplied by greenhouses the plants are usually actively growing when planted. Haskap is generally planted as one year old greenhouse grown rooted plugs. Haskap are planted at a spacing of between 0.75-1m between plants within the row if the objective is to form a solid hedge row suited to mechanical harvest. A spacing of 1.3m between plants produces individual bushes which are better suited to hand harvest (Bors 2009c, OMAFRA 2012). In Slovakia a 1.5

m within row spacing was used (Matuskovic et al. 2009). Spacing between rows is also governed by the chosen method of harvesting. If machines are to be utilized to harvest the crop, the between row spacing must be large enough to accommodate the harvester (Bors 2012, OMAFRA 2012). If plants are to be hand harvested, the between row spacing could be as small as 1.3 m. Haskap is not sensitive to planting depth, although it is believed that deeper planting may lead to a bigger, deeper root system (Bors 2009c). As an obligate out-crosser, haskap requires two compatible genotypes within the same orchard to successfully produce fruit. The Ontario Ministry of Agriculture and Rural Affairs suggest a ratio of one pollinator plant to eight production plants (OMAFRA. 2012). It is recommended that new haskap orchards should be irrigated, as small plants are susceptible to drought damage during the first few years after planting (Bors 2009c).

#### *2.3.5 Pollination*

Haskap requires cross pollination from compatible genotypes to successfully set fruit. Honey bees have been employed to help insure adequate pollination of the high plant densities generally seen in commercial haskap plantings. Bumble bees can also be effective in pollination and commercial hives are available (Bors 2012). One of the common names of haskap was Swamp Fly Honeysuckle, which suggests that flies have been observed pollinating haskap (Bors 2012).

#### *2.3.6 Pruning/vegetation management*



At present there are no recommended pruning regimes for haskap that are based on scientific research. However renewal pruning is recommended to maintain the productivity of older plants (>20 years) (Plekhanova 1989). Pruning systems employed in other fruit production systems may be applicable for haskap, such as those recommended for cherries and Saskatoon berries (B. Bors personal communication, June 11, 2012.).

### *2.3.7 Pests and diseases*

There are few pests or diseases known to afflict cultivated haskap under field conditions. Plants can suffer from powdery mildew (*Erysiphales* sp.) when grown in the greenhouse or shade house (Bors 2012) or in climates with high relative humidity (OMAFRA 2012). Unidentified stem borers and leaf miners have been observed on haskap grown in Ontario (OMAFRA 2012). The leaves can suffer from sun scald, which usually occurs at the midpoint of the season after vegetative growth has slowed (Bors 2012). The major threat to fruit production of haskap is small birds who eat the fruit or knock the fruit to the ground (Bors 2012). Small birds can be excluded from the orchard with bird netting but construction and maintenance of netting systems is costly.

### *2.3.8 Harvest*

Haskap fruit are ready to harvest approximately 6 weeks after fertilization of the flower (Jurikova et al. 2009), at which time the entire berry has developed a red/blue coloration. While machine harvest is a standard practice in large scale agricultural settings, operations such as u-pick and/or small scale operations often rely on hand harvesting. Hand harvest usually causes less damage to harvested fruit and a greater proportion of the total fruit yield can be harvested, as

pickers can harvest the same plant multiple times over the course of several days. Hand harvesting of haskap can be complicated by the fact that fruit often show a red/blue coloration before they are fully ripe (Bors 2012), so pickers must be trained to pick based on both color and ease of removal from the bush. In countries with high labour costs (such as Canada) hand picking may not be a financially viable option and haskap fruit are therefore often harvested by machine. Fu et al. (2011) investigated alternatives to hand picking of haskap, and found total harvesting rates per hour could be increased by vibrating the bush over a portable trap catch. Several different types of machine harvesters are presently employed by haskap producers. Black currant (sideways) harvesters and modified high-bush blueberry (over the row) harvesters are currently the most popular options for harvesting haskap (Bors 2009d). The UofS employs a Joanna-3 currant harvester which uses a combination of shaking action plus a blower fan and conveyer system to remove, clean and direct fruit from the ripe haskap fruit from the bushes into stackable plastic trays (Bors 2009d). Generally haskap fruit are taken in a one-time harvest.

Once-over mechanical harvesters remove fruit based on the ease of separation from the plant. Thus if plants are to be machine harvested the genotypes should have a removal force for a mature fruit that is approximately in line with the force exerted by the desired harvester. Large differences between the fruit separation force created by the harvester and the separation force required for that genotypes could result in harvest of immature fruit or the plants may retain mature fruit even after harvest. Thus improvements in the ease of mechanical harvest of haskap can be achieved through the conventional breeding of new haskap genotypes with fruit pull force requirements in line with that of the selected harvester. Pruning the lower branches to produce a more upright bush stature may improve the efficiency of mechanical harvest. The desired upright bush stature may also be achieved through breeding and selection.

### *2.3.9 Post-harvest*

There are several avenues for post-harvest processing of haskap fruit, depending on the desired end-product. Haskap destined for sale as fresh fruit must be cooled immediately and then quickly transported to market. Even under ideal conditions fresh haskap fruit has a shelf life of only a few days (B. Bors personal communication, June 11, 2012). However, haskap fruit can maintain its quality even after several weeks in a cooler, and fresh haskap fruit can be found for sale in Japan during haskap harvest season (B. Bors personal communication, May 17, 2012.). For haskap destined for sale in more distant markets or for use in processing, the fruit is immediately frozen to at least -20°C. Some producers have also started to juice the fruit immediately after harvest. Pasteurized haskap juice can be stored in a refrigerated state for periods beyond that of fresh fruit. Haskap are used in diverse products ranging from wines and liqueurs to cookies, jams, jellies and even salad dressing.

## **2.4 Fruit chemistry of haskap**

### *2.4.1 Sugar and titratable acidity*

The soluble solid content and titratable acidity of haskap fruit varies widely depending on fruit maturity, genotype and growing conditions. Table 2.1 summarizes the soluble solids and titratable acidity levels reported for mature, harvest-ready haskap fruit. The greatest range of values reported in literature come from reports where data were collected over many growing seasons (Pokorna Jurikova and Matuskovic 2007) or from diverse germplasm (Bors 2012). Based on the ranges presented in Table 2.1 there is considerable variability amongst genotypes with regard to soluble solids and titratable acidity. While the total content of soluble solids and

titratable acidity are important aspects of fruit quality, the ratio between these two parameters is also an important determinant of fruit flavor (Ortiz et al. 2009).

**Table 2.1.** Range of soluble solids (SS) and titratable acidity (TA) content reported for fruit of haskap.

<b>Genotypes tested</b>	<b>% SS</b>	<b>TA (g citric acid/ 100 g FW)</b>	<b>Reference</b>
Wojtek and Czarna	9.8-12.1	2.48-3.61	Ochmian et al. 2008
Czarna and progeny	9.8-13.5	2.28-3.55	Skupien et al. 2009
Wojtek and Brazowa	9.3-13.2	2.81-3.74	Ochmain et al. 2010
Approximately 90 genotypes of diverse origin	11.4-25.6	0.99-3.44	Bors 2012
Czelabinka, Duet, Jolanta and Wojtek	13.4-16.9	1.43-2.14	Wojdyo et al. 2014

#### 2.4.2 Minerals

Table 2.2 displays the concentrations of mineral elements reported in haskap fruit. The predominant mineral element in haskap fruit is potassium, followed by roughly similar amounts of phosphorus and calcium and lesser amounts of magnesium and iron. Haskap fruit also contain trace amounts of manganese, copper and zinc.

**Table 2.2.** Range or average concentration of mineral elements reported for fruit of haskap.

<b>Genotypes tested</b>	<b>Parameter</b>	<b>Range or average (mg/100g FW)</b>	<b>Reference</b>
Five Japanese haskap genotypes	Calcium	38.4	Tanaka and Tanaka 1998
	Iron	0.61	
<i>L.c.</i> subsp. <i>edulis</i> and <i>kamtschatica</i>	Calcium	43-168	Pokorna Jurikova and Matuskovic 2007
	Magnesium	47-95	
	Phosphorous	170-280	
	Potassium	1020-1480	
25 commercial genotypes grown in Russia	Calcium	266	Lefevre et al. 2011
	Iron	2.9	
	Magnesium	116	
	Potassium	1780	
Borealis, Tundra and Indigo Gem	Calcium	140-520	Rupasinghe et al. 2012
	Copper	0.34-0.64	
	Magnesium	80-150	
	Manganese	10.45-12.30	

### 2.4.3 Vitamins

Table 2.3 summarizes the vitamin C content reported for haskap fruit. The vitamin C content of haskap fruit was comparable to other fruit considered to be rich sources of this nutrient, such as oranges, strawberries and kiwi (Terahara et al. 1993, Wojdylo et al. 2014).

**Table 2.3.** Range of vitamin C concentration reported for fruit of haskap.

<b>Genotypes tested</b>	<b>Range (mg/100g FW)</b>	<b>Reference</b>
Czelabinka, Duet, Jolanta and Wojtek	4.9-32.1	Wojdylo et al. 2014
5 genotypes of Japanese haskap	44.3	Tanaka and Tanaka 1998
Czarna and progeny	50-105	Skupien et al. 2009
Wojtek and Czarna	40.5-98.0	Ochmian et al. 2008
<i>L.c.</i> subsp. <i>edulis</i> and <i>kamtschatica</i>	28.6-86.8	Pokorna Jurikova and Matuskovic 2007
22 genotypes of <i>Lonicera caerulea</i> subsp. <i>kamtschatica</i>	9.7-92.3	Matuskovic et al. 2009

#### 2.4.4 Antioxidant activity of haskap

Rupashinghe et al. (2012) determined ORAC (oxygen radical absorbance capacity) values of fruit from UofS haskap genotypes to range between 237 – 262  $\mu\text{mol/g}$  FW Trolox equivalents. This study also tested the FRAP (ferric reducing antioxidant potential) values from these same fruit and found a range of 27- 46  $\mu\text{mol/g}$  FW Trolox equivalents. These values were greater than those found in lowbush blueberry, lingonberry and blackberry (Rupashinghe et al. 2012). Bakowska-Barczak et al. (2007) found fruit of an unnamed haskap cultivar to have the greatest ORAC (95  $\mu\text{mol/g}$  FW Trolox equivalents) of any of the 13 fruit crops tested from fields in Western Canada including bilberry, lingonberry, wild raspberry and wild strawberry.

While tests for ‘in vitro’ antioxidant capacity are good indicators of the total amount of phenylpropanoid compounds in fruit, they do not provide any information on the actual health benefits of these compounds. At one point it was widely believed that phenylpropanoid compounds worked to quench reactive oxygen within the human body, which is similar to its role in plants (Prior et al. 1998). It is now understood that these compounds do not quench reactive oxygen in the human body but rather interact with stress resistance pathways to induce beneficial effects observed after consumption (Howitz et al. 2003). For this reason greater emphasis should be placed on quantification of the individual phenylpropanoid compounds proposed to interact with the human body, as opposed to measurement of the total antioxidant capacity.

Animal models have been used to assess the effect of consumption of *L. caerulea* fruit on tumour growth (Gruia et al. 2008), UVA damage to skin cells (Svobodova et al. 2008) and oxidative damage to human endothelial cells and rat hepatocytes (Palikova et al. 2009). A diet

augmented with cultivated *Lonicera caerulea* fruit reduced oxidative damage associated with these induced chronic diseases.

#### 2.4.5 Phenylpropanoids and polyphenol derivatives

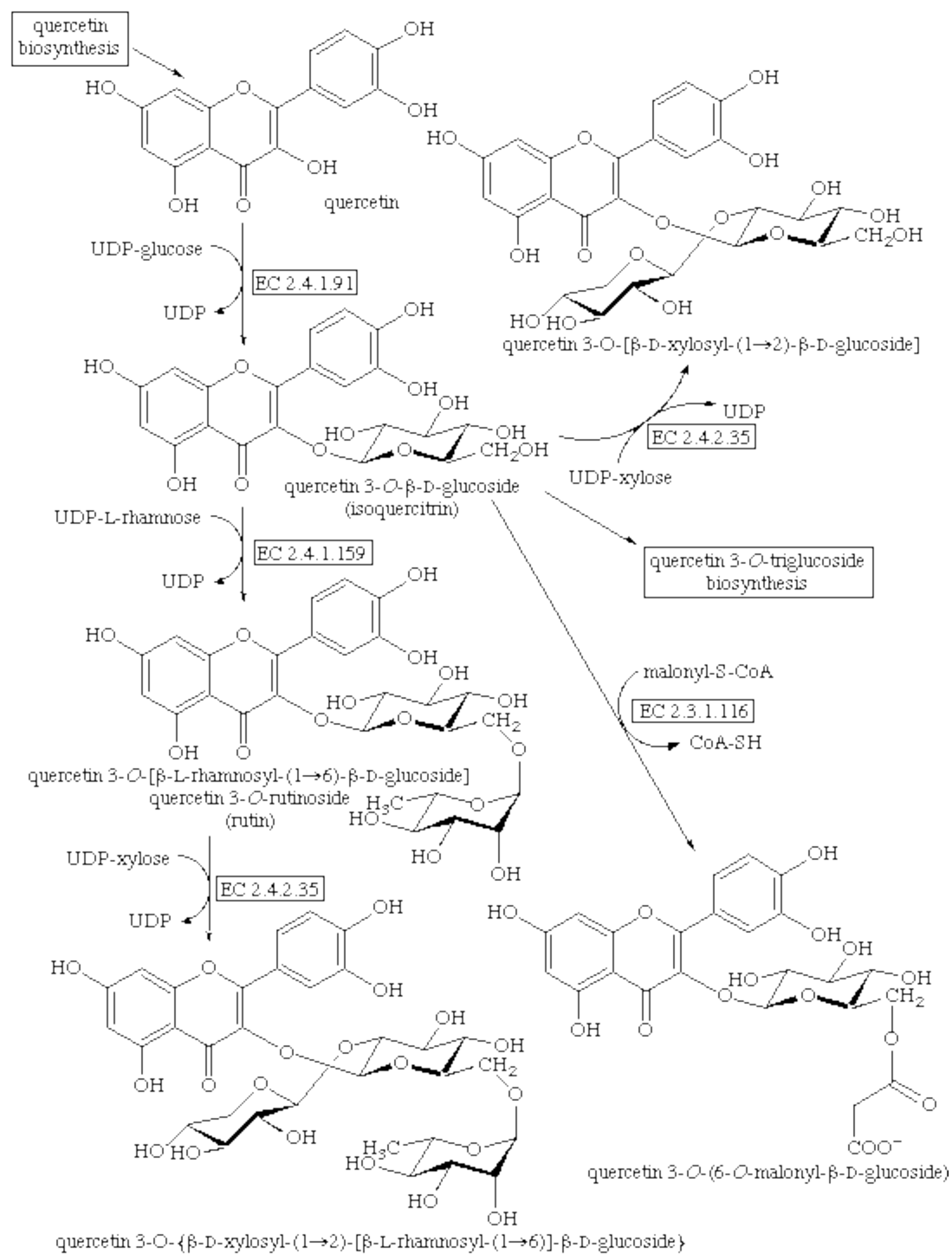
Fruit of *Lonicera caerulea* contain high concentrations of several phenolic compounds, including hydroxybenzoic acids (HBA), hydroxycinnamic acids (HCA) and polyphenolic compounds. Haskap fruit (*L. caerulea* subsp. *kamstchatica*) had greater concentrations of both HBAs and HCAs (1.63 and 3.73 mg/g DW respectively) than blueberries (*Vaccinium myrtillus*), black mulberry (*Morus nigra*), European June berry (*Amelanchier ovalis*), blackberry (*Rubus plicatus*) and black currant (*Ribes nigrum*) (Zadernowski et al. 2005). The major HBA in *L. caerulea* fruit was salicylic acid (75% of the profile), while meta-coumeric acid (53% of the profile) and para-coumeric acid (16% of the profile) were the major HCA (Zadernowski et al. 2005).

Polyphenols, like HBAs and HCAs, are produced from the phenylpropanoid pathway, but are chemically more complex than phenolic acids, as they contain multiple phenol groups. These characteristics may make the polyphenols more potent elicitors of the observed beneficial health effects (Howitz et al. 2003). The fruit of *L. caerulea* contain several polyphenols, including flavonols, anthocyanins and a single flavone. The anthocyanin compounds are responsible for the deep blue colour of haskap fruit. Cyanidin-3-glucoside is the major anthocyanin in *L. caerulea* fruit, accounting for up to 90% of the total anthocyanin pool in certain genotypes (Ochmian et al. 2010). Smaller amounts of cyanidin-3,5-diglucoside, cyanidin-3-rutinoside, peonidin-3-glucoside, peonidin-3-rutinoside and pelargonidin-3-glucoside have also been reported in haskap fruit (Chaovanalikit et al. 2004). The only flavonol known in *L. caerulea* fruit is quercetin

(Skupien et al. 2009), which takes the form of quercetin-3-rutinoside and quercetin-3-glucoside. In addition, the flavone luteolin-7-glucoside is also present in *L. caerulea* fruit (Ochmian et al. 2010).

The biosynthetic pathway for the quercetin glycosides is shown in Fig 2.1.

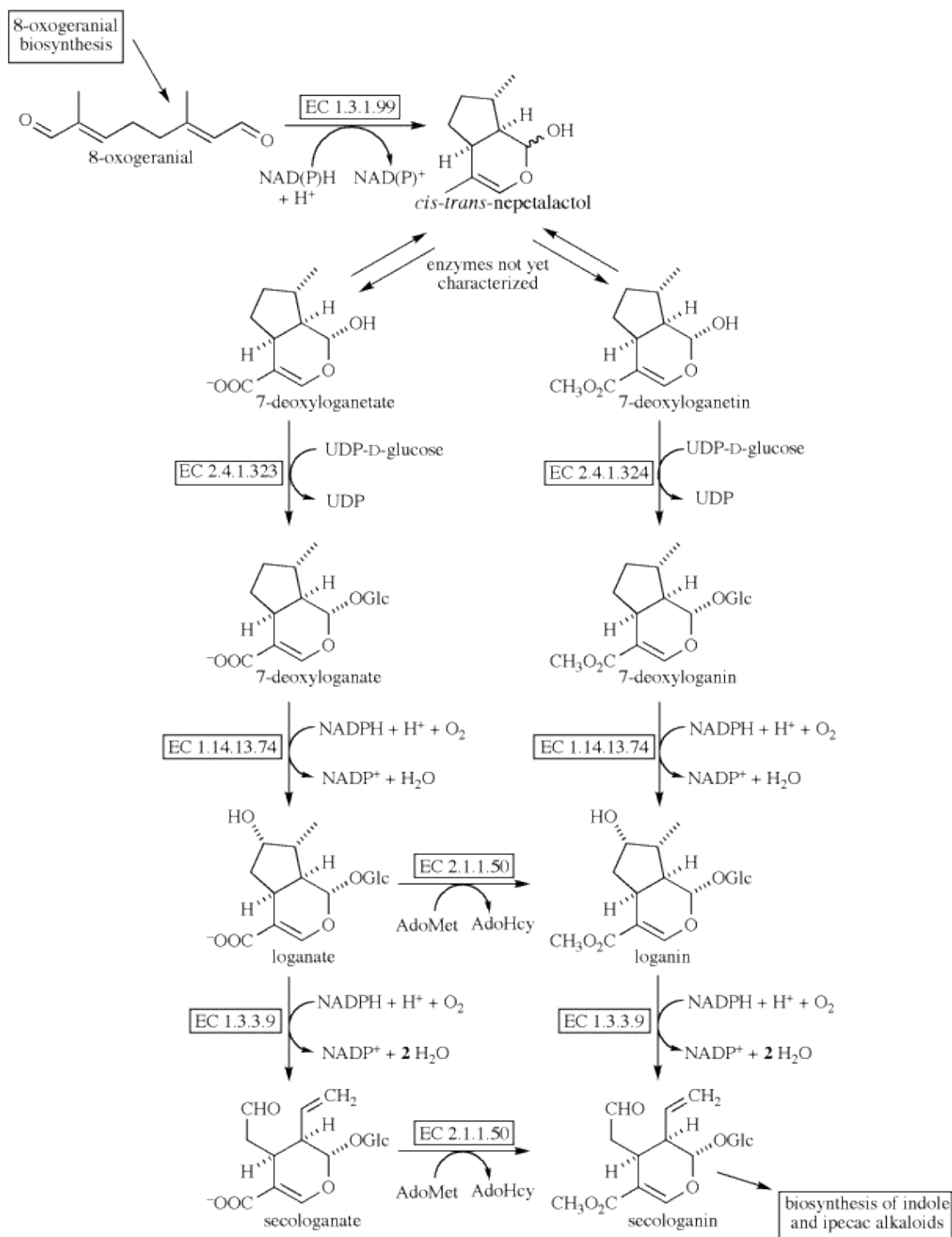




**Figure 2.1.** Biosynthetic pathway for quercetin glycosides. Taken from <http://www.enzyme-database.org/reaction/phenol/querc3gly.html>

#### 2.4.6 Terpenoids

In addition to phenylpropanoid compounds, the leaf tissues of haskap have been shown to contain iridoid glucosides (Machida et al. 1995). The presence of these bitter compounds in the leaves is thought to act as a feeding deterrent to herbivores (Bowers et al. 1992, Whitehead and Bowers, 2012). Iridoids exhibit diverse biological activity and are commonly found in plant species used as folk medicines (Tundis et al., 2008, Park et al. 2011a, 2011b, Yu et al. 2012). The biosynthetic pathway for the iridoid compounds loganin and secologanin is presented in Fig 2.2. Machida et al. (1995) identified loganin, secologanin and sweroside in the leaf tissues of haskap. The same study also identified two bis-iridoids (two iridoid compounds linked together) in haskap leaves. A later study by the same author identified a third bis-iridoid compound in leaf tissues of haskap (Machida and Kikuchi 1995). Only one iridoid compound (7-oxologanin) has been identified in fruit of haskap (Anikina et al. 1988). There is no information available as to the concentration of any iridoid glucoside in either the fruit or leaf tissues of haskap.



**Figure 2.2.** Biosynthetic pathway for secologanin. Taken from <http://www.enzyme-database.org/reaction/terp/loganin.html>

#### 2.4.7 Phenylpropanoids in human health and nutrition

Haskap fruits are known to contain at least two quercetin derivatives; quercetin-3-rutinoside and quercetin-3-glucoside (Ochmian et al. 2009). The pharmacological activity of quercetin and its derivatives have been extensively studied. Quercetin has been shown to be an activator of the sirtuin enzyme in humans (Howitz et al. 2003). This enzyme has been shown to be involved in the aging process of mice through processes such as histone deacetylation, DNA repair and the methylation of inactive genes (Oberdoerffer et al. 2008). Kampkotter et al. (2008) showed that absorption of quercetin can extend the lifespan of the nematode *C. elegans* by approximately 15%. Quercetin has also been shown to stimulate mitochondrial biogenesis within mammalian tissues. In mice fed 25mg/kg quercetin for seven days, the expression of two key mitochondrial biogenesis genes doubled relative to mice fed a placebo. This increased enzyme activity resulted in double the concentration of mitochondrial DNA within muscle and brain tissues. Mice fed quercetin also showed a 50% increase in maximal endurance and increased levels of voluntary activity; both of these responses could be attributed to increased mitochondrial activity (Davis et al. 2009). A study using human subjects also gave evidence that feeding 1g/day quercetin for a 2-week period increased specific markers of mitochondrial biogenesis and resulted in an improvement in 12-min treadmill time trial performance over a placebo (Nieman et al. 2010). Due to their effect on mitochondria, flavonols (specifically quercetin) have been suggested as supplements for high performance athletes and for fitness enthusiasts (Nieman et al. 2010).

Haskap fruit also contain chlorogenic acid, which is a hydroxycinnamic acid derivative consisting of caffeic acid linked by an ester to quinic acid. The consumption of chlorogenic acid has shown the ability to improve insulin resistance in mice (Ma et al. 2014). Chlorogenic acid

consumption in humans has also been suggested as a treatment to reduce high blood pressure (Zhao et al. 2012, Onakpoya et al. 2015).

#### 2.4.8 Terpenoids in human health and nutrition

The pharmacological activity of the iridoid glucoside loganin has been well studied. Loganin is present in several plant species, including *Lonicera japonica* (Flos Lonicerae), *Cornus officinalis* (Corni fructus) (Park et al. 2011a, 2011b, Jiang et al. 2012, Youn et al. 2012) and *Strychnos nux vomica* (Kwon et al. 2009), all of which are common ingredients in natural medicines. Loganin has been shown to reduce scopolamine-induced memory loss in rats and it may be effective in alleviating other types of memory impairment (Kwon et al. 2009). Loganin was also effective at inhibiting the activity of  $\beta$ -secretase, a key protease involved in the production of the  $\beta$ -amyloid aggregates that cause Alzheimer's disease (Youn et al. 2012). In addition to its activity as a neuroprotective agent, loganin has also shown inhibitory effects against diabetes-associated liver disorders (Tundis et al. 2008). Recent studies have suggested the use of loganin to reduce oxidative stress, to improve liver function (Park et al. 2011a, 2011b) and to reduce nephropathy (Tundis et al. 2008, Jiang et al. 2012) in diabetics.

The pharmacology of secologanin is not as well understood as that of its loganin precursor. However, secologanin plays an important role in the biosynthesis of biologically important indole alkaloids such as reserpine, ajmalicine, vincristine and vinblastine (Yamamoto et al. 2000). Derivatives of secologanin have been shown to be anti-inflammatory, antiallergenic, antiarthritic and analgesic (English and Williams 2010).

## 2.5 Influence of genotype on concentrations and content of secondary metabolites

The major factors governing the total concentration of secondary metabolite compounds in fruit crops are developmental stage, genotype and climate (Bowers et al. 1992, Anttonen and Karjalainen 2005, Howard et al. 2003) and in most cases there are significant interactions amongst these factors. As a general rule, there is usually a significant effect of genotype in any study quantifying the concentration of secondary metabolites in either fruit (Awad et al. 2001, Giovanelli and Brenna 2007, Castrejon et al. 2008, Ochmian et al. 2008, Ranalli et al. 2009, Skupien et al. 2009, Ochmain et al. 2010, Raudsepp et al. 2010) or leaves (Marak et al. 2002, Qian et al. 2007, Liu et al. 2014). Secondary metabolite concentrations in various haskap genotypes are presented in Table 2.4. From this table it is apparent that there are differences in the secondary metabolite profile amongst the haskap genotypes. For example there is an order of magnitude difference between the concentration of total flavonol derivatives reported in haskap fruit by Rupasinghe et al. (2012) and those reported by Ochmian et al. (2010) and Skupien et al. (2009). While the concentrations of secondary metabolite differ based on genotype, these differences are very difficult to predict. In addition most reports originate from different locations and growing conditions; this further complicates the understanding of genetic influences. In most cases genotypes need to be grown at the same location and analyzed using the same methodology to ascertain which genotypes produce superior quantities of secondary metabolites. At present there are few studies which have tested diverse haskap germplasm from the same location in the same study. However Chaovanalikit et al. (2004) investigated the concentrations of total flavonols and total hydroxycinnamic acid in fruit of 10 genotypes of *L. caerulea*, including representatives of the *L. caerulea* subspecies *boczkamikovae*, *edulis*, *stenantha*, *pallasii* and *kamtschatica* all grown at the same site. This study showed significant

differences in the secondary metabolite profile amongst the various genotypes. The largest differences in secondary metabolite concentrations occurred when comparing improved versus unimproved genotypes.

**Table 2.4.** Range of phenylpropanoid concentrations reported in fruit of haskap.

Genotypes tested	Compound concentration (mg/g dry weight)			Reference
	Anthocyanin	Chlorogenic acid	Flavonols	
11 genotypes of <i>Lonicera caerulea</i>	7.73-22.60	*	*	Thompson and Chaovanalikit 2002
22 genotypes of <i>Lonicera caerulea</i> subsp. <i>kamtschatica</i>	39.73-127.87	*	*	Matuskovic et al. 2009
Czarna and progeny	8.17-15.11	1.32-1.89	1.09-1.60	Skupien et al. 2009
Wojtek and Brazowa	7.09-12.34	1.04-1.63	0.62-1.41	Ochmian et al. 2010
12 genotypes of <i>Lonicera caerulea</i> subsp. <i>kamtschatica</i>	*	*	20.40-26.73	Rop et al. 2011
8 <i>Lonicera caerulea</i> subsp. <i>edulis</i> genotypes	14.3-45.9	1.02-3.82	1.53-2.73	Kusznierewicz et al. 2012
Borealis, Indigo Gem and Tundra	*	*	39.62-46.61	Rupasinghe et al. 2012
Czelabinka, Duet, Jolanta, Wojtek and four seedlings of <i>L.c.</i> subsp. <i>kamtschatica</i>	*	0.76-2.94	0.45-1.58	Wojdjo et al. 2014

\* Indicates that the parameter was not measured in that study

## **2.6 Developmental stage, environmental and management factors influencing the concentration and content of secondary metabolites in plants**

Accumulation of secondary metabolites in plants is widely thought to be an adaptive response to biotic and abiotic stresses. This is true of both the phenylpropanoid pathway (Dixon and Paiva 1995, Winkel-Shirley 2002, Suzuki et al. 2005) and the terpenoid pathway (Bartram et al. 2006).

Flavonoid compounds such as quercetin are found throughout the plant kingdom and have been shown to be induced by several environmental factors. Accumulation of flavonols can be induced by environmental stresses such as increased light exposure (UV and white light) (Lois 1994, Lavola 1998, Suzuki et al. 2005, Cohen and Kennedy 2010, Takahashi and Badger 2011), reduced temperature (Suzuki et al. 2005, Cohen and Kennedy 2010), reduced water availability (Suzuki et al. 2005, Cohen and Kennedy 2010) and biotic stress such as pathogen infection (Shimezu et al. 2012) and through competition as allelopathic chemicals (Weston and Mathesium 2013). The interactions amongst these factors is complex and environmental effects on the accumulation of secondary metabolites are sometimes further modified by the developmental stage of the plant and/or of the fruit. A short review of the literature on the environmental and developmental effects on concentration and or content of secondary metabolites is presented in the following sections.

### *2.6.1 Developmental stage*

The chemistry of fruit changes during fruit development. These changes involve both primary metabolites such as sugar, starch and acidic compounds, as well as secondary metabolites such as the compounds produced from the phenylpropanoid and terpenoid pathways.



Regulation of the genes associated with the phenylpropanoid pathway has been studied during fruit development in several major fruit crops (Halbwirth et al. 2006, Castellarin et al. 2007, Zifkin et al. 2012). During development of highbush blueberry fruit, chalcone synthase appeared to be most active during the early and late stages of fruit development. The activity of flavanone hydroxylase increased steadily as the fruit began to soften and develop color (Zifkin et al. 2012). Within the fruit of strawberry there appeared to be two phases of metabolism of flavonol compounds. Chalcone synthase was active early in fruit development, while flavonol synthase did not reach peak activity until the final stages of fruit maturity (Halbwirth et al. 2006, Almeida et al. 2007). Within the fruit of the grape variety Cabernet Sauvignon, there were two major peaks in the activity of flavonol synthase during fruit development. The first peak occurred immediately prior to coloration of the fruit, while the second peak occurred immediately prior to maturation of the fruit (Castellarin et al. 2007). Within the fruit of Satsuma mandarin (*Citrus unshiu*), the flavonol synthase gene was most active during the early stages of fruit development, with gene activity peaking 103 day after flowering. Thereafter expression of the gene was markedly reduced (Moriguchi et al. 2002). There appears to be no clear pattern for the activity of the genes responsible for the creation of the polyphenol backbone (chalcone synthase) and subsequent metabolism into flavonols (flavonol synthase) among these different fruit species.

The relationship between the concentrations of secondary metabolites and fruit development for a variety of species is presented in Table 2.5. The literature suggests that there are two distinct patterns for this relationship. In members of the *Vaccinium* genus, the concentration of secondary metabolites appears to decline as fruit development progresses (Vvedenskaya and Vorsa 2004, Castrejon et al. 2008, Zifkin et al. 2012, Gibson et al. 2013). This

also appears to be true of apples (Awad et al. 2001, Reay and Lancaster 2001). Strawberries and grapes exhibit the opposite relationship; with secondary metabolite concentrations increasing as the fruit develop (Kennedy et al. 2002, Martinez-Luscher et al. 2004, Halbwirth et al. 2006, Mahmood et al. 2012). This difference may be due to differences in the tissues present within the fruit i.e. the fruit of strawberry are largely receptacle tissues whereas blueberry fruit are largely comprised of pericarp tissues. In most fruit, secondary metabolites are found in the highest concentrations in the skin tissues (Awad et al. 2001). As the fruit expands, the ratio of skin tissues to flesh tissues decreases; this would decrease the concentration of secondary metabolites on a per unit weight basis. Zifkin et al. (2012) observed a decrease in quercetin concentration from 50-10 mg/100g FW during development of highbush blueberry. Fully developed fruit of lowbush blueberry have approximately 1/3 the concentration of flavonols observed in immature fruit (Gibson et al. 2013). The concentration of total flavonols decreases by approximately 50% over the course of development of apple fruit (Awad et al. 2001). The concentration of the iridoid compound oleuropein in very immature olive fruit is approximately twice that in mature fruit (Ranalli et al. 2009). A similar decrease in concentrations of several different iridoid compounds was found by Gutierrez-Rosales et al. (2012) during their study of olive development.

**Table 2.5.** Relationship between secondary metabolite concentration and fruit development of haskap.

Genotypes tested in study	Compound concentration (mg/g dry weight)			Reference
	Anthocyanin	Chlorogenic acid	Flavonols	
11 genotypes of <i>Lonicera caerulea</i>	7.73-22.60	*	*	Thompson and Chaovanalikit 2002
22 genotypes of <i>Lonicera caerulea</i> subsp. <i>kamtschatica</i>	39.73-127.87	*	*	Matuskovic et al. 2009
Czarna and progeny	8.17-15.11	1.32-1.89	1.09-1.60	Skupien et al. 2009
Wojtek and Brazowa	7.09-12.34	1.04-1.63	0.62-1.41	Ochmian et al. 2010
12 genotypes of <i>Lonicera caerulea</i> subsp. <i>kamtschatica</i>	*	*	20.40-26.73	Rop et al. 2011
8 <i>Lonicera caerulea</i> subsp. <i>edulis</i> genotypes	14.3-45.9	1.02-3.82	1.53-2.73	Kusznierewicz et al. 2012
Borealis, Indigo Gem and Tundra	*	*	39.62-46.61	Rupasinghe et al. 2012
Czelabinka, Duet, Jolanta, Wojtek and four seedlings of <i>L.c.</i> subsp. <i>kamtschatica</i>	*	0.76-2.94	0.45-1.58	Wojdyo et al. 2014

\* Indicates that the parameter was not measured in that study

### 2.6.2 Light quantity and quality

One of the first established physiological roles of phenylpropanoids and more specifically flavonols in plants is to absorb UV light, thus protecting the photosynthetic apparatus of the leaf tissues (Takahashi and Badger 2011). It is therefore not surprising that exposure to light, especially UV wavelengths, has been shown to elicit phenylpropanoid production. The concentrations of quercetin-3-glucoside in leaves of *Ligustrum vulgare* were increased by 100%

by exposing the plants to 30% versus 100% full sunlight intensity and increased UV (240-380nm) irradiance (Agati et al. 2011). Exposure to increased intensity of light did not have a significant effect on the concentration of iridoid within leaves of *Ligustrum vulgare*, however exposure to increased UV light did increase the concentration of iridoids by approximately 10% (Agati et al. 2011). The concentration of rutin (quercetin-3-rutinoside) in leaves of tartary buckwheat was significantly increased by exposure of the leaves to UVB (302nm 3.4 kJ/m<sup>2</sup>) radiation (Suzuki et al. 2005). Exposure to UV light (UVB filter) increased the content of flavonol derivatives in skins of the Malbec grape cultivar by 38% (Berli et al. 2011). The concentration of total flavonols increased by 400%, and the number of gene transcripts for flavonol synthase were increased by 300% in skins of the grape cultivar Tempranillo following exposure to increasing intensity (0-9.66 kJ/m<sup>2</sup> day) of UV light (311-313nm) (Martinez-Luscher et al. 2004). However the concentrations of flavonol derivatives containing three hydroxyl substitutions on the B ring of the flavonol structure were decreased by this treatment. Spayd et al. (2002) found that the concentration of quercetin-3-glucosides in sun-exposed clusters of the grape cultivar Merlot to be 3-4.5 times greater than the concentrations in clusters shaded by UV screens. However reduction in exposure to UV appeared to have no significant effect on the concentrations of total anthocyanin in the fruit (Spayd et al. 2002). This effect could not be attributed to any differences in temperature of the cluster caused by the UV screens. Tattini et al. (2004) showed that concentrations of iridoids and quercetin-3-rutinoside in leaves of *Ligustrum vulgare* increased with increasing exposure to solar radiation (Tattini et al. 2004).

### 2.6.3 Temperature

Lower temperatures increased the concentration of anthocyanin in skins of the grape cultivar Merlot by approximately 20%, however there was no consistent effect of temperature on the concentration of quercetin-3-glucoside (Spayd et al. 2002). Azuma et al. (2012) demonstrated that the concentrations of anthocyanins and flavonols in the skin of the grape cultivar Pione were increased by 700% and 250% respectively by low temperatures (15 vs 35°C), however the activity of chalcone synthases was increased by low temperatures under both high or low light conditions. The concentration of rutin (quercetin-3-rutinoside) in leaves of tartary buckwheat was increased by 190% after exposure of the leaves to -5°C for 5 mins (Suzuki et al. 2005). Wang and Zheng (2001), investigating the effects of day and night temperatures on the concentration of anthocyanin and flavonol compounds in strawberry fruit, found that concentrations of these compounds were increased under elevated day and night (30/22°C) growing temperatures. The concentration of anthocyanin and flavonol compounds in tomato leaves decreased to 1/10 and 1/3 of their initial levels with increased growing temperatures (12°C versus 30°C), however the activity of chalcone synthase and dihydroflavonol reductase (anthocyanin metabolism) showed variable activity with increasing temperature (Lovdal et al. 2010). Neugart et al. (2012) showed a negative relationship between growing temperature and the concentration of flavonol compounds in the leaves of kale. Leyva et al. (1995) showed increases in the relative abundance of the mRNA responsible for expression of both phenylalanine ammonia-lyase and chalcone synthase after transferring *Arabidopsis* plants from 20°C to 4°C; however this increase in mRNA expression occurred only in the presence of light.

#### 2.6.4 Water status and irrigation

Martinez-Luscher et al. (2004) gave evidence that in wine grape water deficiency (27% water holding capacity of the soil) vs control (80% water holding capacity in the potting soil) increased concentration of flavonols with two or three hydroxyl substitutions on the B ring of the flavonol structure. The concentration of quercetin-3-glucoside in leaves of *Ligustrum vulgare* were increased by 20% after application of  $\text{Na}^{2+}$  to the root zone to simulate salt stress (Agati et al. 2011). However the  $\text{Na}^{2+}$  application did not affect the dry weight concentration of iridoid within the leaves. In another study on *Ligustrum vulgare*, leaf concentrations of iridoid compounds were increased by 100% in well-watered plant versus those experiencing drought (Tattini et al. 2004). This same study also indicated that drought did not affect the leaf concentrations of quercetin-3-rutinoside. The concentration of rutin (quercetin-3-rutinoside) in leaves of tartary buckwheat increased by 20% upon exposure of the plants to drought stress (Suzuki et al. 2005)

The total fresh weight concentration of anthocyanin compounds in the skins of the grape cultivar Aglianico were 30% higher in non-irrigated versus irrigated plants but the total concentration of flavonols was reduced by 10% under non-irrigation conditions (Sofo et al. 2012). The concentration of phenolic compounds in skins of the grape variety Tempranillo increased by 15% in non-irrigated versus irrigated vines; the content of phenolic compound per berry however was increased in fruit from irrigated vines (Esteban et al. 2001). The concentration of flavonols and anthocyanins were increased by 50% and 20% respectively in fruit of the wine grape cultivar Cabernet Sauvignon by withholding irrigation (Kennedy et al. 2002). The same study indicated that the content of both flavonols and anthocyanins were

highest in treatments with standard irrigation versus double or minimal irrigation (Kennedy et al. 2002).

### 2.6.5 Fertility

Fertilizer application can have variable effects on the metabolism of compounds from the phenylpropanoid pathway within fruit tissues. Fertilizer studies with multiple mineral elements are often hard to interpret because they tend to fail to give linear responses to increasing fertilization rate and/or show multiple interactions between the different mineral elements. The concentration of phenolic compounds within the skins of the wine grape cultivar Tempranillo were significantly affected by the application of nitrogen and potassium fertilization, however the responses were not linearly related to the amount of fertilizer applied and showed strong interactive effects (Delgado et al. 2004). The concentration of polyphenols in potato tubers was highest with moderate amounts of nitrogen and high levels of potassium and magnesium (Hamouz et al. 2006). Increased nitrogen fertilization reduced total phenolic content in the genotypes Dark Opal, Genovese and Sweet Thai of basil (*Ocimum basilicum*) by approximately 2.5 times (Nguyen and Niemeyer 2008). Fertility regimes leading to nitrogen deficiency increased the concentration of flavonols and anthocyanin compounds in tomato leaves. The activity of flavonol synthase in the tomato leaves was also up-regulated by nitrogen deprivation (Lovdal et al. 2010). Plants of a wild genotype of *Plantago lanceolata* provided with more fertility had lower concentrations of iridoid compounds in both leaf and root tissues (Darrow and Bowers 1999).

### 2.6.6 Pruning/vegetation management

When the vines were more intensely pruned the concentration of quercetin was increased in the skins of grapes and a negative relationship between grape skin quercetin concentrations and leaf area of the vine was determined (Belsic et al. 2010). This response may have been driven by increased light infiltration into the canopy following pruning. Pruning increased the content of total phenolic compounds in a study of fruit of the Shiraz wine grape (Bindon et al. 2008).

### 2.6.7 Biotic stress

Phenylpropanoid compounds such as flavonols have diverse roles in plants and are often implicated as chemical defence mechanisms to protect plants during interactions with pathogens or insects or during competition from other plants (Treutter 2005).

A commercial source of the flavonol kaempferol was an effective inhibitor of spore germination and mycelium growth of the rice pathogen *Pyricularia oryzae* at concentrations as low as 7 µg/mL (Padmavati et al. 1997). This same study indicated that kaempferol was ineffective at inhibiting the growth of the rice pathogen *Xanthomonas oryzae* but naringenin, a related flavonone, was effective (Padmavati et al. 1997). Leyva et al. (1995) showed inoculation of Arabidopsis plants with the pathogen *Pseudomonas syringae* increased mRNA coding for chalcone synthase; this response occurred only in the presence of light. After infection of *Plantago lanceolata* by the fungal pathogen *Diaporthe adunca* the dry weight concentrations of iridoid glucosides in leaves, spikes, stalks and roots were increased by 97, 37, 24 and 17% respectively (Marak et al. 2002)



In addition to protection from pathogens, there is evidence that flavonoids can have negative effect on insect feeding. Several flavonoids isolated from Jersey cudweed (*Gnaphalium affine*) showed anti-feedant activity against the common cutworm *Spodoptera litura* (Morimoto et al. 2000). Flavonol glycosides isolated from several different pine species were effective at reducing the weight and increasing mortality when fed to second instars of the gypsy moth (*Lymantria dispar*) (Beninger and Abou-Zaid 1997). Lattanzio et al. (2000) showed a positive correlation between the concentration of flavonoid glycosides and resistance of cowpea seedling (*Vigna unguiculata*) genotypes to aphids. The concentration of iridoid compounds in leaves of *Plantago lanceolata* was increased by herbivore damage (Darrow and Bowers 1999). Herbivory by generalist insects also significantly increased the total concentration of iridoid glucosides in *Penstemon vigatus* (Quintero and Bowers 2013). Wang et al. (2014) showed that foliar herbivory of *Plantago lanceolata* by cutworms (*Spodoptera lineatus*) induced metabolism of iridoid glucosides in the leaf tissues. Several compounds isolated from leaves of sweet chestnut (*Castanea sativa*), including the flavonols quercetin, rutin and kaempferol, were effective at reducing seed germination of *Raphanus sativus*. This result indicates that these compounds may act allelopathically to protect the plant against competition from other plants.

**3.0 DEVELOPMENT OF A METHODOLOGY FOR INVESTIGATION OF  
SECONDARY METABOLITE PROFILE OF FRUIT AND LEAVES OF HASKAP  
(*Lonicera caerulea* L.)**

**3.1 Abstract**

The goal of this study was to develop a method for isolation, identification and quantification of secondary metabolites in haskap. The secondary metabolite profiles of haskap leaves and fruit were analyzed via HPLC/MS. The following compounds were identified in fruit and leaves: Chlorogenic acid, quercetin-3-glucoside, quercetin-3-rutinoside, quercetin-3-sambubioside, loganin and secologanin. This is the first record of the two iridoid glucosides, loganin and secologanin in fruit tissues as well as the first report of the flavonol glycoside, quercetin-3-sambubioside in any tissue of haskap. The presence of these compounds within haskap fruit is potentially of commercial importance, as they all have the potential to enhance human health. This isolation and identification methodology could also be utilized in studies investigating the impact of growing conditions and production practices on the secondary metabolite concentration and/or content of haskap.

## 3.2 Introduction

### 3.2.1 Commercial interest in haskap

Haskap fruit have been used for centuries in Asia (Rupasinghe et al. 2012) to treat fevers, headaches and respiratory infections (Qian et al. 2007). Cultivation of haskap for use as a food crop was introduced to North America in 2000 (Thompson. 2006). Commercial interest in haskap as a fruit crop stems from it being a fast growing, extremely winter hardy (Imanishi et al. 1998), early ripening crop (Skupien et al. 2009), that is tolerant of a range of soil conditions (Bors 2009d). Haskap produces attractive, tasty fruit that can hharvest by hand or machines (Bors 2009d). In addition to these valuable agronomic traits, haskap fruit contain high concentrations of phenolic and polyphenolic compounds which give the fruit a high antioxidant capacity (Rupasinghe et al. 2012); a valuable trait when marketing to health conscious consumers.

### 3.2.2 Secondary metabolites of haskap fruit and leaf tissues

The profile of secondary metabolites present in the fruit and leaves of *Lonicera caerulea* has been explored by Machida et al. (1995), Zadernowski et al. (2005) and Wojdylo et al. (2013). Haskap fruit contained higher concentrations of phenylpropanoid compounds such as phenolic acids (Zadernowski et al. 2005, Rupasinghe et al. 2012) and flavonoids (Bakowska-Barczak et al. 2007, Rupasinghe et al. 2012) than fruit such as blueberry, blackberry, raspberry and saskatoon berry. The phenolic acid profile of haskap can be classified as either hydroxybenzoic (C6-C1) derivatives or hydroxycinnamic (C6-C3) derivatives. These compounds are differentiated by the number of carbon molecules attached to the benzene rings. The major hydroxybenzoic derivatives of haskap fruit are salicylic, gentisic and protocatechuic acids

(Zadernowski et al. 2005). The hydroxycinnamic derivatives in haskap fruit consist of chlorogenic acid, caffeic acid and both meta- and para- formations of coumaric acid (Zadernowski et al. 2005, Svarcova et al. 2007). In addition, haskap fruit contained minor amounts of several dicaffylquinic acid derivatives (Ochmain et al. 2010).

The flavonoid compounds in haskap fruit have also been identified (Ochmian et al. 2009). Flavonoid compounds take the general formula C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> and include compounds such as flavonols, flavones, flavan-3-ols and anthocyanins. The flavonol profile of haskap is limited to quercetin, which generally is attached to either a glucose or rutinose sugar moiety (Ochmain et al. 2010). Rhamnoside (Chaovanaliket et al. 2004) and galactoside-bound (Kusznierewicz et al. 2010) quercetin compounds were also reported in haskap fruit. Ochmain et al. (2012) identified trace amounts of two unknown flavonol compounds in fruit of haskap. The lone flavone compound previously discovered in fruit of haskap is luteolin, which can be found bound to a rutinoside and a glucoside (Wojdylo et al. 2013). The major flavan-3-ols found in haskap fruit are catechin and procyanidin (Wojdylo et al. 2013). The majority of the anthocyanin in haskap is in the form of cyanidin, which can account for 80% of the total anthocyanin profile (Chaovanalikit et al. 2004). Other minor anthocyanins found in haskap fruit include pelargonidin, delphinidin and peonidin (Svarcova et al. 2007). The major glycoside bound to the anthocyanin compounds in haskap fruit is glucose, with rutinoside and diglucosides making up the remainder of the pool (Wojdylo et al. 2013).

Leaves of haskap were found to contain many of the same compounds as the fruit tissues (Machida et al. 1995). In addition to phenylpropanoids, the leaves of haskap (*Lonicera caerulea* subsp. *emphylocalyx*) have been shown to contain iridoid glucosides including loganin, secologanin, secologanin dimethyl acetyl and sweroside (Machida et al. 1995). While studying

the leaves of haskap, Machida et al. (1995) found two bis-iridoid compounds, named caeruleosides A and B, which are comprised of two iridoid compounds coupled by an acetyl linkage. A subsequent study by the same authors identified a third iridoid, caeruleoside (Machida and Kikuchi 1995) in haskap leaves. The iridoid glucosides are thought to act as feeding deterrents to herbivores (Bowers et al. 1992, Whitehead and Bowers 2012) and are also formed in response to attack by pathogens (Marak et al. 2002). Iridoids exhibit diverse biological activity when consumed by humans, including neuroprotective (Tundis et al. 2008) and anti-inflammatory effects (Tundis et al. 2008, Park et al. 2011). Iridoid compounds are also commonly found in plant species used as folk medicines (Park et al. 2011, Yu et al. 2012). There is a single report of an iridoid compound, 7-oxologanin, in haskap fruit (Anikina et al. 1988) but other iridoid compounds maybe present.

This study isolated and identified compounds present in methanolic extracts of fruit and leaves of haskap, and determined compounds that warranted further study.

### 3.2.3 Goals of the UofS Haskap Breeding Program

The overall goal of the UofS Haskap Breeding Program is to produce new genotypes with improved productivity and fruit quality traits. One of the fruit quality traits of interest is an enhanced concentration and content of secondary metabolites with established potential to enhance human health.

The goal of the current study was to develop methodology for identification and quantification of secondary metabolites in haskap. This methodology would then be used to examine the secondary metabolite profile of the fruit and leaves of *Lonicera caerulea*. These profiles would be used to select specific compounds for further study. The criteria for selection

of secondary metabolites for more intensive study were; a) the compounds could be identified based on comparisons against pure authentic standards or reference spectra, b) the compounds are found in concentrations high enough to be monitored consistently, c) these compounds are already available in the marketplace as a nutrition supplements, or the compounds are being marketed as the primary health beneficial compound in currently available foods, d) compounds have already been shown to have beneficial effects on human health when consumed.

### **3.3 Methodology**

#### *3.3.1 Test material*

As profiles of secondary metabolites are affected by genotype (Wojdylo et al. 2014), environment (Qian et al. 2007) and developmental stage (Awad et al. 2001), a screening study should evaluate haskap fruit from different germplasm and at different development stages. To this end, immature fruit of the commercial standard haskap cultivar Tundra and mature fruit of North American wild type representatives of *Lonicera caerulea* subsp. *villosa* were tested. Tundra is a cross between Kiev #8 and Tomichka which likely involved several *Lonicera caerulea* subspecies including *Lonicera caerulea* subsp. *kamtschatica* and *Lonicera caerulea* subsp. *turczaninowii*. *Lonicera caerulea* subsp. *villosa* has not previously been studied for the secondary metabolite profile of its fruit. These samples represented extremes of fruit development stages were genetically diverse as their parents were from different continents. A methodology capable of identifying and quantifying secondary metabolites in these diverse samples should be usefull for assessing the metabolite profiles of *Lonicera caerulea* germplasm.

Plants used as sources of test material were grown at the UofS Horticultural Field Research Station in Saskatoon SK (52°N, 106°W). The site is on a dark brown chernozem soil with high inherent fertility. Annual management of the haskap plots consisted of hand weeding, with a late fall application of the herbicide Casoron (dichlobenil) and sporadic use of the herbicide Lontrel (chlorpyralid) for control of perennial weeds. The plot was not irrigated.

Five year old plants of *Lonicera caerulea* cv. 'Tundra' and three year old plants of several wild-collected lines of *Lonicera caerulea* subsp. *villosa* were the source of the fruit and leaves evaluated in this study. The wild lines were originally collected from NS (43.82°N, 65.22°W), ON (50.77°N, 90.51°W) and MB (55.67°N, 97.84°W), along with two specimens collected from SK (53.68°N, 105.26°W) and (54.51°N, 103.76°W). These lines were producing enough fruit to provide a sample size sufficient for testing. For the cultivar Tundra immature fruit (approximately 30 berries per line) were collected one week after fertilization of the flowers (late May). Fully mature fruit (approximately 20 berries per line) were collected (42 days after fertilization) in early June. The Tundra fruit were harvested from 10 plants and pooled for analysis. As only a few small plants were available for each of the wild genotypes, fruit from the five different sources of *Lonicera caerulea* subsp. *villosa* were pooled prior to analysis.

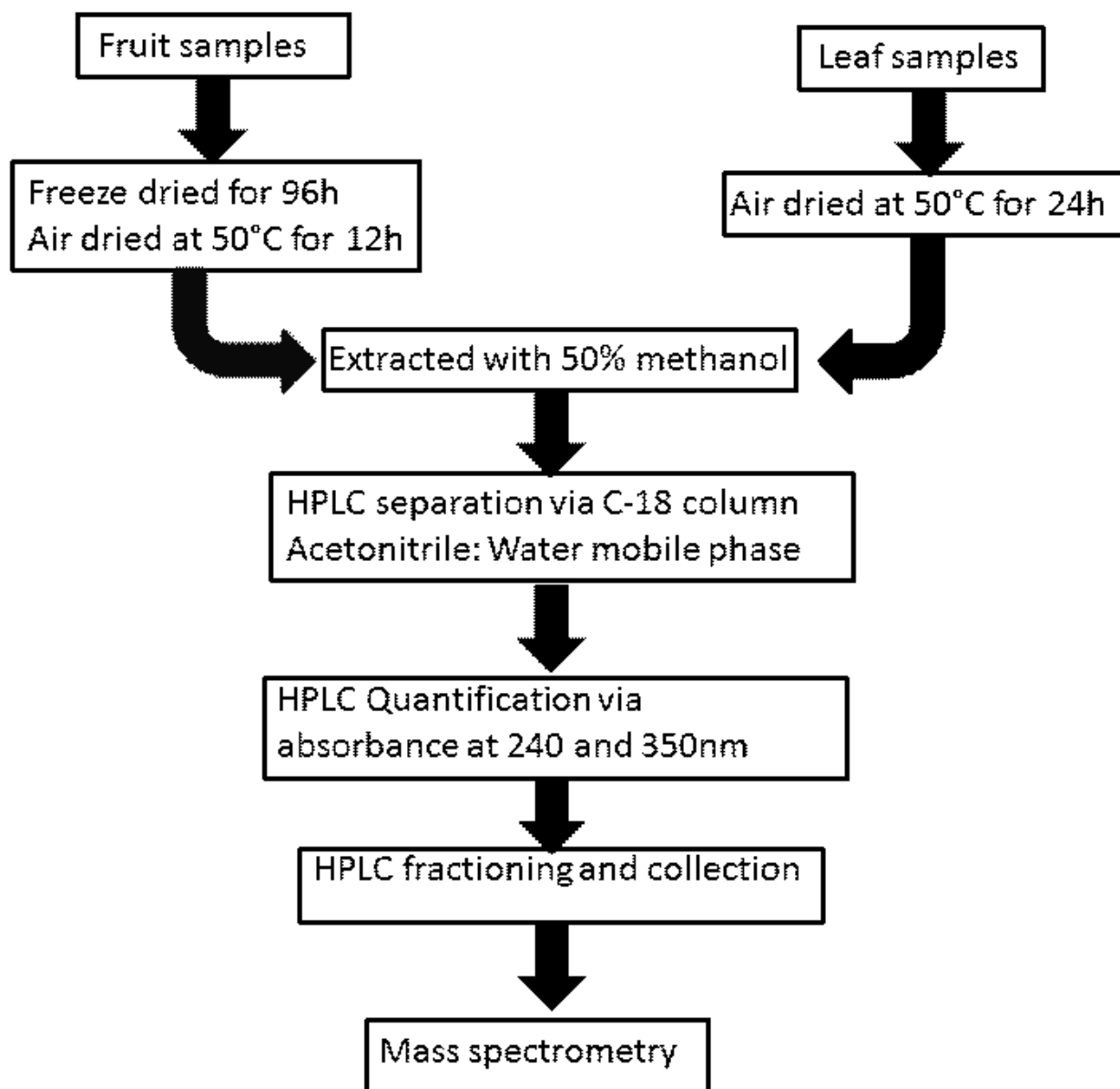
A random sample (throughout the canopy) of mature leaves was collected from the haskap plants at the same time as the mature fruit samples were harvested (early June). The leaf samples were pooled similar to the fruit. Fresh leaves of loquat were acquired from a private *Citrus* breeder (M.P.M. Niar). These leaves were used as reference source of quercetin-3-sambubioside (Ferrerres et al. 2009).

### 3.3.2 *Plant tissue processing*

A flow diagram of the sample preparation and analysis methodologies used in this study is presented in Figure 3.1. Immediately after harvest, the haskap fruit were placed in a -40°C freezer (Convion, Winnipeg, MB, Canada) and held at that temperature until analyzed (ca. two months). Frozen fruit were placed in a freeze dryer (Labconco, Kansas City, MO, USA) for approximately 96 h, then further dehydrated in a drying oven (Precision Scientific, ThermoFisher Scientific, Ottawa, ON, Canada) at 50°C for 12 h. It was necessary to fully dehydrate plant tissues before extraction to achieve the concentrations of compounds necessary for both HPLC fractioning and mass spectrometric analysis. The dehydrated fruit were then ground in a mortar and pestle under liquid nitrogen.

Leaf tissues collected from the haskap and loquat plants were placed in a drying oven (Precision Scientific) at 50°C until a constant dry weight was achieved (24 h). The dried leaf tissue was then ground using a micro hammer mill (Culatti AG, Zürich, Switzerland) and held at room temperature until analyzed.





**Figure 3.1** Flow diagram of sample preparation and analysis methodologies used for this study. Extraction and HPLC methodologies were modified from Qian et al. 2007.

### 3.3.3 Extraction procedure

Qian et al. (2007) showed that a 1:1 methanol:water extract was effective for simultaneous extraction of 13 secondary metabolite compounds from floral bud and stem tissues of *Lonicera japonica*. These compounds included hydroxycinnamic acids, flavonoids and

monoterpene iridoids. As this methodology appeared simple and effective for the separation and quantification of secondary metabolites in *Lonicera* plant tissues it was adopted in this study.

Dried ground plant material, (0.25 g for leaves and 0.5 g for fruit), was agitated in 5 mL of 50% (v/v) methanol (VWR, Mississauga, ON, Canada) in a shaking water bath for one hour at 60°C.

The resulting extract was centrifuged at 5,000 g for 10 mins at room temperature. The supernatant was then decanted. The remaining insoluble fraction was then washed with 5 mL 50% methanol and was again decanted and added to the previous decantation. The 10 mL extract was then passed through a 0.45 µm PTFE membrane syringe filter (VWR, Mississauga, ON, Canada) prior to HPLC analysis.

#### 3.3.4 High pressure liquid chromatography (HPLC) procedure

Compounds in the crude methanolic extracts of haskap leaves and fruit as well as the loquat leaves were separated using the HPLC method developed by Qian et al. (2007). This method had achieved effective separation of phenylpropanoid and terpenoid compounds extracted from floral bud and stem tissues of *Lonicera japonica*. The HPLC set-up used a Waters system equipped with a 2695 separations module and 1525 binary pump, 2998 photodiode array detector and fraction collector III (Waters Corp., Milford, MA, USA). The HPLC system utilized two coupled 100 mm x 4.6 mm i.d. (5 µm pore size) reverse phase Gemini C18 columns (Phenomenex, Torrance, CA, USA). These octadecyl silica columns (C18) are used for separation of non-polar compounds and are have been shown to provide effective separation of plant secondary metabolites such as phenylpropanoids and terpenoids from extracts of *Lonicera* species (Qian et al. 2007, Wojdylo et al. 2014). The column temperature was maintained at 30°C for the duration of the 40 min run. The mobile phases consisted of two solvents. Solvent A was

99.9% acetonitrile, with 0.1% (v/v) trifluoroacetic acid (TFA). Solvent B consisted of deionized water: acetonitrile (90:10, v:v with 0.1% TFA). The extracts were analyzed using the following gradient of solvents at a flow rate of 1 mL min<sup>-1</sup>: 0 to 22% A for 30 min, followed by 22 to 80% A for 10 min, followed by a 10 min reconditioning of the column using 100% solvent B. The injection volume was 10 µL and compound detection occurred based on absorbance at 240 and 350 nm as suggested by Qian et al. (2007). Eluted fractions (0.5 ml) were collected from six runs of each type of haskap tissue and were pooled to provide enough material to allow MS analysis.

HPLC data were interpreted and analyzed using Empower software (version 2.0, Waters). Chromatograms from crude extracts of haskap fruit and leaves were compared to authentic standards for chlorogenic acid, quercetin-3-rutinoside, quercetin-3-glucoside, loganin and secologanin. Authentic standards of chlorogenic acid, quercetin-3-rutinoside, quercetin-3-glucoside, loganin and secologanin were purchased from Sigma-Aldrich, St. Louis, MO, USA. HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from VWR, Mississauga, ON, Canada. A crude methanolic extract from leaves of loquat was used as a source for quercetin-3-sambubioside, as no pure standard of this compound was available. Compounds eluted during HPLC were identified based on comparison of their retention times and spectral signatures relative to the standards. Since trifluoroacetic acid (TFA) is known to interfere with ionization of compounds when using electrospray ionization mass spectrometry (ESI-MS), samples to be used for mass spectrometry analyses were separated as described above, but TFA was not included in the mobile phase. Adequate peak separation was achieved without the TFA. Absorption spectra for the compounds were detected using a diode array (Waters Corp) operating over a wave length spectrum of 190 - 500nm.

### 3.3.5 Mass spectrometry (MS) procedure

The mass spectrometry methodology described below was developed by the author and a senior MS technician (Ms. Deborah Michel) for the Drug Discovery and Development Research Group at the UofS. Sample fractions corresponding to the key peaks in the HPLC profile were analyzed by ESI-MS and were compared to authentic standards.

Fragmentation was performed by collision-activated dissociation, using nitrogen as the collision gas. The declustering potential was 15 V and collision energy was optimized (40 to 45 V) to ensure the formation of fragment ions while maintaining the presence of the precursor ion. All other parameters utilized during MS analysis were maintained for MS/MS analysis. Fragment ions were detected using a linear ion trap set to dynamic fill.

Data were acquired using a Hybrid Triple Quadrupole/Linear Ion trap mass spectrometer (4000 QTRAP® MS/MS system; AB SCIEX, Concord, ON, Canada) equipped with an electrospray ionization (ESI) source. The instrument was operated in the positive ion mode for the iridoids and the negative ion mode for quercetin-3-sambubioside under optimized ESI-MS conditions (declustering potential 30 V, entrance potential 10 V, ion spray voltage 5500 V, curtain gas 10, ion source gas 1 (GS1) 12 and 2 (GS2) 40 in full scan mode (scan range  $m/z$  200-1000). All sample fractions were introduced by direct infusion. Compound identification was based on the  $m/z$  value of the molecular ion and the fragmentation pattern

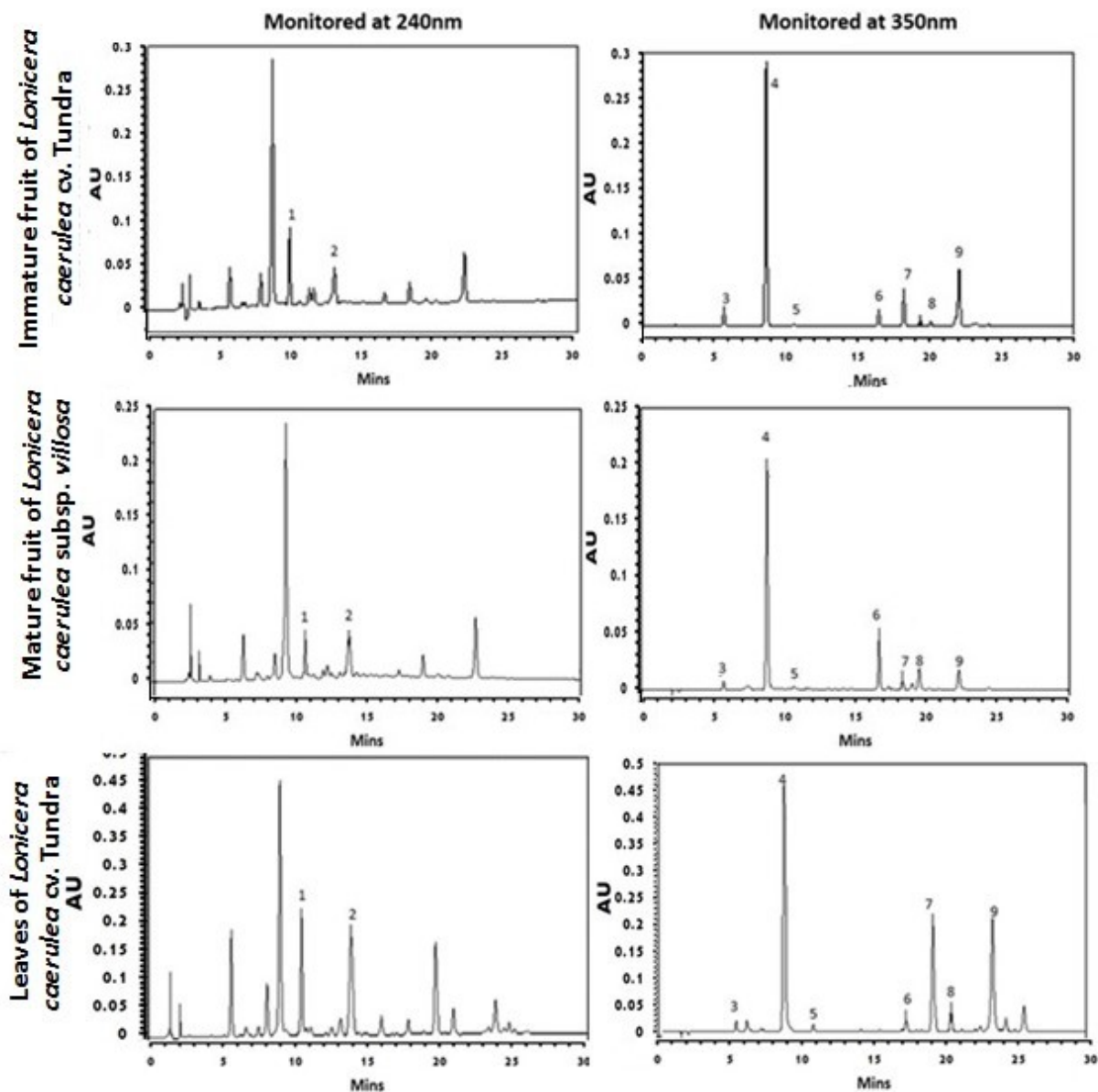
## 3.4 Results

### 3.4.1 Chromatographic separation

Representative HPLC chromatographs of methanolic extracts taken from the immature fruit and leaves of haskap cultivar Tundra, as well as mature fruit of *Lonicera caerulea* subsp. *villosa* are shown in Figure 3.2. The chromatographs show that immature fruit, mature fruit and the leaves of haskap contain a similar array of secondary metabolites. HPLC chromatographs from the leaves also featured several peaks not present in the fruit tissues, however these peaks did not represent a large portion of the total profile or could not be properly identified. Compounds previously reported in haskap fruit (peaks 3, 4, 5, 7, 8 and 9) were identified by comparison of their retention times and the  $\lambda$  absorbance spectra of the isolated peaks relative to authentic standards and also by comparison with published values for retention times and the  $\lambda$  absorbance spectra (Ochmain et al. 2010, Wojdylo et al. 2014). Previously unreported compounds labeled 1, 2 and 6 in Figure 3.2 were also compared to a range of standards based on their retention times and UV/vis absorbance spectra. These unknown compounds were also collected and analyzed via mass spectrometry. Other unidentified peaks were present in chromatographs from extracts of haskap fruit, however their retention times did not match any authentic standard or previously published values for retention times.

Authentic standards for loganin and secologanin had HPLC retention times of 10.7 and 13.8 mins respectively. Peaks with similar retention times (peaks 1 and 2 from Figures 3.2) were obtained by HPLC separation of crude extracts of both the immature fruit of cultivar Tundra, mature fruit of *Lonicera caerulea* subsp. *villosa* and leaves of haskap cultivar Tundra (Figure 3.2 and Table 3.1). Characteristics of the HPLC peak which was tentatively identified as quercetin-3-sambubioside obtained from crude extracts of fruit and leaves of haskap cultivar Tundra

(Figure 3.2) and subsp. *villosa* (Figure 3.2) and from leaves of Loquat (*Eriobotrya japonica*) are shown in Table 3.1 along with literature values for the daughter ion fragmentation patterns of quercetin-3-sambubioside (Zhang et al. 2012). The HPLC analysis suggests that peaks 1, 2 and 6 from Figure 3.2 isolated from haskap fruit are loganin, secologanin and quercetin-3-sambubioside respectively. The two iridoid glucosides loganin and secologanin have previously been reported in haskap leaves (Machida et al. 1995).



**Figure 3.2.** HPLC chromatograph at 240nm and 350nm of methanolic extracts of immature fruit of *Lonicera caerulea* cv. ‘Tundra’, mature fruit of *Lonicera caerulea* subsp. *villosa* and leaves of *Lonicera caerulea* cv. ‘Tundra’ displaying peaks for compounds tentatively identified as 1) loganin and 2) secologanin 3) neochlorogenic acid, 4) chlorogenic acid, 5) luteolin-7-glucoside, 6) quercetin-3-sambubioside, 7) quercetin-3-rutinoside, 8) quercetin-3-glucoside and 9) 3,5-dicaffyl quinic acid.

**Table 3.1.** Comparison of unidentified HPLC peaks from fruit and leaves of haskap (*Lonicera caerulea* cv. ‘Tundra’), wild-type haskap (*Lonicera caerulea* subsp. *villosa*), loquat (*Eriobotrya japonica*) and *Eucommia ulmoides* with authentic standards. HPLC retention times ( $t_R$ ), UV/vis absorbance maxima ( $\lambda_{Max}$ ), MS mass to ion charge ratio ( $m/z$ ) and MS-MS fragment ion charges of isolated compounds are presented. Numbering scheme continued from Figure 3.2.

Peak #	Compound	Source	$t_R$ (min)	UV/visible absorbance spectra	Mass spectra ( $m/z$ value)	Product ion fragmentation
1	Loganin	Standard	10.7	238.0	[M+Na] <sup>+</sup> = 413	219, 285
		Haskap ( <i>Lonicera caerulea</i> cv. ‘Tundra’) leaves	10.7	238.0	N/A	N/A
		Haskap ( <i>Lonicera caerulea</i> cv. ‘Tundra’) fruit	10.6	239.2	[M+Na] <sup>+</sup> = 413	219, 179, 285
2	Secologanin	Standard	13.8	236.9	[M+Na] <sup>+</sup> = 411	249, 255
		Haskap ( <i>Lonicera caerulea</i> cv. ‘Tundra’) leaves	13.8	238.0	N/A	N/A
		Haskap ( <i>Lonicera caerulea</i> cv. ‘Tundra’) fruit	13.7	238.0	[M+Na] <sup>+</sup> = 411	249, 255
6	Quercetin-3-sambubioside	Loquat ( <i>Eriobotrya japonica</i> ) leaves	17.2	255.8, sh* (354.7)	N/A	N/A
		Haskap ( <i>Lonicera caerulea</i> subsp. <i>villosa</i> ) leaves	17.1	255.8, sh* (354.7)	N/A	N/A
		Haskap ( <i>Lonicera caerulea</i> subsp. <i>villosa</i> ) fruit	17.1	255.8, sh* (354.7)	[M-H] <sup>-</sup> =595	301
		<i>Eucommia ulmoides</i> leaves (Zhang et al. 2012)	UN	254, 350	[M-H] <sup>-</sup> =595	301

\*sh indicates a shoulder peak on the UV/vis absorbance spectra, UN indicates information not analyzed, [M+Na]<sup>+</sup> indicates the mass of the compound plus the mass of a sodium ion, [M+H]<sup>-</sup> indicates the mass of the compound minus on hydrogen ion.



### 3.4.2 UV/visible absorbance spectra

UV/visible absorbance of sample fractions corresponding to the HPLC peaks that had been tentatively identified as (1) loganin, (2) secologanin and (6) quercetin-3-sambubioside (Figure 3.2) were determined over the 190 to 700 nm range and compared with those for authentic standards of these compounds. The  $\lambda_{\max}$  absorbance values of the compounds isolated from haskap fruit and leaves tentatively identified as loganin, secologanin and quercetin-3-sambubioside were close but not exact matches for the  $\lambda_{\max}$  absorbance of the standards. The differences in  $\lambda_{\max}$  absorbance values between the standards and compounds extracted from haskap tissues could be caused by contamination of the chromatographic peaks by small amounts of unknown co-eluting compounds. The similarity of the spectral signatures of loganin and secologanin is due to the similarity of the chemical structures of these two iridoids.

UV/visible absorbance spectra of compounds 3) neochlorogenic acid, 4) chlorogenic acid, 5) luteolin-7-glucoside, 7) quercetin-3-rutinoside, 8) quercetin-3-glucoside and 9) 3,5-dicaffeoyl quinic acid from Figure 3.2 matched previously published values (Wojdylo et al. 2014) and the UV/visible absorbance spectra generated when authentic standards of these compounds were run through the system

The  $\lambda_{\max}$  absorbance of other unidentified peaks in the chromatographs for haskap fruit extracts were also determined over the range of 190 to 700 nm. The  $\lambda_{\max}$  absorbance spectra of these compounds were not characteristic of phenylpropanoid compounds. Some compounds present in the chromatographs monitored at 240 nm (Figure 3.2) showed  $\lambda_{\max}$  absorbance spectra characteristic of iridoid glucosides, however without authentic standards these compounds could not be conclusively identified.

### 3.4.3 Mass spectra and product ion fragmentation

The full mass spectrum for the authentic loganin standard demonstrated peaks with  $m/z$  values of 413 ( $[M+Na]^+$ ), 429 ( $[M+K]^+$ ), 803 ( $[M+M+Na]^+$ ) and 819 ( $[M+M+K]^+$ ) (Table 3.1). Daughter ions from the  $m/z = 413$  ( $[M+Na]^+$ ) fragment showed  $m/z$  values of 185 ( $[Glc+Na-H_2O]^+$ ), 201 ( $[Agly+Na-MeOH-H_2O]^+$ ), 203 ( $[Glc+Na]^+$ ), 219 ( $[Agly+Na-MeOH]^+$ ), 233 ( $[Agly+Na-H_2O]^+$ ), 251 ( $[Agly+Na]^+$ ) and 285 ( $[retro-Diels-Alderfragment]^+$ ) (Madhusudanan et al. 2000) (Table 3.1). The mass spectrum of the compound that formed peak number 1 of the HPLC profile of haskap fruit extract which had tentatively been identified as loganin showed a similar, though more complex, mass spectrum, with a base peak of  $m/z$  413 and others at 389 ( $[M+H]^+$ ), 429 ( $[M+K]^+$ ), 803 ( $[M+M+Na]^+$ ) and 819 ( $[M+M+K]^+$ ). Daughter ions from the  $m/z$  413 ( $[M+Na]^+$ ) fragment of the compound demonstrated a fragmentation pattern similar to that produced by the loganin standard (Table 3.1). Although other peaks were also evident in the daughter ion fragmentation pattern of the extract from haskap fruit, the data were consistent with loganin being the component in this major eluted peak.

When the authentic secologanin standard was analyzed by mass spectrometry, it generated major peaks at  $m/z$  389 ( $[M+H]^+$ ), 411 ( $[M+Na]^+$ ), 427 ( $[M+K]^+$ ) and 799 ( $[M+M+Na]^+$ ) (Table 3.1). Peak number 2 isolated from the haskap fruit extract that eluted from the HPLC column at 13.7 mins and had tentatively been identified as secologanin also had major MS peaks at  $m/z$  389 ( $[M+H]^+$ ), 411 ( $[M+Na]^+$ ), 427 ( $[M+K]^+$ ), 799 ( $[M+M+Na]^+$ ) and 815 ( $[M+M+K]^+$ ). Daughter ions from the  $m/z$  411 ( $[M+Na]^+$ ) fragment from both the secologanin standard and the compound isolated by HPLC from the haskap fruit extracts showed similar fragmentation patterns (Table 3.1). These spectral characteristics are consistent with secologanin being peak number 2 eluting from the HPLC at 13.7 mins.

Analysis of the HPLC fraction from crude extracts of haskap fruit which produced peak number 6 that had eluted with a retention time of 17.1 mins generated a full mass spectra with a major peak at  $m/z$  595 ( $[M-H]^-$ ) (Table 3.1). Daughter ions from  $m/z$  595 ( $[M-H]^-$ ) showed only one major peak at  $m/z$  301 ( $[aglycone-H]^-$ ). These peak characteristics are consistent with this compound being the quercetin aglycone (Table 3.1) (Zhang et al. 2012).

### **3.5 Discussion**

#### *3.5.1 Development of a methodology for quantification of secondary metabolites in haskap*

The identification of loganin, secologanin and quercetin-3-sambubioside in extracts obtained from haskap fruit and leaves was accomplished by comparison of HPLC retention times, UV/vis spectra and MS daughter ion fragmentation patterns relative to authentic standards and published values (Ochmain et al. 2010, Wojdylo et al. 2014).

This is the first report of loganin, secologanin and quercetin-3-sambubioside being present in fruit tissues of haskap. Only one other report of an iridoid compounds in haskap fruit tissues exists; 7-oxologanin was isolated from a chloroform-methanol extract of fruit of *Lonicera caerulea* (Anikina et al. 1988). Additional iridoid compounds may be present in the fruit of haskap, especially since a range of different iridoid compounds have been identified from haskap leaves using nuclear magnetic resonance (Machida et al. 1995). However further characterization of the iridoid profile of haskap fruit is hampered by the unavailability of authentic standards for monoterpenoid iridoids. The possibility also exists that bis-iridoids such as caeruleoside A are present in haskap fruit tissues, as the two monoterpenoid precursors of this compound (loganin and secologanin) are present (Machida et al. 1995).

The methodology for extraction, purification, quantification and identification of secondary metabolites outlined in this study was shown to be simple and effective and thus can be used for investigation of genetic, maturity and environmental effects on the secondary metabolites in haskap tissues.

### 3.5.2 Implications of results for use and marketing of haskap

The selection criteria for secondary metabolites meriting further study were; a) the compounds could be identified with the use of pure authentic standards or reference spectra b) the compounds are found in concentrations high enough to be monitored consistently, c) these compounds are already available in the marketplace as a nutrition supplements, or the compounds are being marketed as the primary health beneficial compound in currently available foods, d) compounds have been shown to have beneficial effects on human health when consumed. Using these criteria, six compounds present in the haskap fruit have been identified for further study; chlorogenic acid, three quercetin glycosides as well as loganin and secologanin. These compounds met all the selection criteria. It is potentially important to note that all of these compounds could be identified in both immature and mature fruit as well as the leaves of commercial genotypes of haskap (cv. Tundra) as well as wild type *Lonicera caerulea* subsp. *villosa*. Quercetin compounds have shown a variety of potential beneficial biological activities.

In addition to the compounds produced from the phenylpropanoid pathway, haskap fruit also contain monoterpenoid iridoids (Chapter 3). The pharmacology activity of these iridoids is not as well understood for the flavonols or chlorogenic acid, however the iridoid loganin has shown the ability to inhibit  $\beta$ -secretase, a key protease in the production of the  $\beta$ -amyloid

aggregates that cause Alzheimer's disease (Youn et al. 2012). Loganin has also been shown to reduce scopolamine-induced memory loss in rats and it may be effective in alleviating other types of memory impairment (Kwon et al. 2009). This compound has also shown a protective effect on the liver of individuals with diabetes (Jiang et al. 2012).

Identification of the iridoid glucosides loganin and secologanin within fruit tissues of haskap is potentially important from a marketing perspective, as these compounds are believed to have health beneficial effects when consumed by humans (Tundis et al. 2008). The identification of iridoids in the fruit tissues gives producers and processors of haskap a new set of potential health beneficial compounds to add to the already well known health enhancing potential of the flavonol and chlorogenic acid compounds found in this fruit (Rupasinghe et al. 2012). Iridoid compounds are only found in a few cultivated edible fruit crops such as *Vaccinium* (cranberry, lingonberry and bilberry) (Jensen et al. 2002), noni (Kamiya et al. 2005), gardenia (*Gardenia jasminoides*) (Yu et al. 2009), *Kingelia Africana* (Gouda et al. 2003), *Genipa Americana* (Ono et al. 2005) and Calabash tree (*Crescentia cujete*) (Kaneko et al. 1997).

Table 3.2 indicates the compounds identified in haskap fruit and which of the selection criteria they met or failed. Several secondary metabolite compounds identified in the HPLC profile of haskap do not meet one or more of the previously discussed selection criteria and will not be addressed in subsequent studies included in this thesis. These compounds include neochlorogenic acid, 3,5-dicaffylquinic acid and luteolin. In all three of these cases their exclusion from further study is due to the prohibitively low concentrations of these compounds found in the haskap fruit and/or leaves. In the case of the neochlorogenic acid and 3,5-dicaffylquinic acid there also appears to be little evidence of potential health benefits of these compounds in the literature or in the market place. In addition to the six secondary metabolites

identified in this study, subsequent studies will also investigate the concentration and content of the anthocyanin compounds as they are valuable due to their contribution to the colour of haskap which is important in marketing of many fruit crops.

**Table 3.2.** Compounds identified in fruit and leaves of haskap cultivar (*Lonicera caerulea* cv. ‘Tundra’) and wild-type haskap (*Lonicera caerulea* subsp. *villosa*) and their status with regard to selection criteria. Peak numbering scheme continued from Figure 3.2.

Peak #		Implicated in improving human health*	Appreciable concentration*	Value in the market place	Selected for further study
1	Loganin	+	+	+	+
2	Secologanin	+	+	+	+
3	Neochlorogenic acid	-	-	-	-
4	Chlorogenic acid	+	+	+	+
5	Luteolin-7-glucoside	+	-	+	-
6	Quercetin-3-sambubioside	+	+	+	+
7	Quercetin-3-rutinoside	+	+	+	+
8	Quercetin-3-glucoside	+	+	+	+
9	3,5-dicafflyquinic acid	-	-	-	-

\*See text for definition of these criteria.

### 3.5.3 Importance of the results to the UofS Haskap Breeding Program

One of the goals of the a Haskap Breeding Program may be to increase the concentration of valuable, health enhancing secondary metabolites in fruit of new haskap genotypes.

Identification of the presence of iridoid compounds in the haskap fruit in this study creates another avenue by which the health benefits of haskap fruit could be enhanced. Previously there had been no awareness of these compounds being available in haskap and thus increasing their concentration in haskap fruit has not been a part of previous breeding efforts. The identification of quercetin-3-sambubioside in haskap fruit tissues adds another quercetin conformer to the

already rich pool of phenylpropanoid compounds known to occur within haskap (Chaovanalikit et al. 2004). The fruit of haskap is already known to contain quercetin-3-glucoside and quercetin-3-rutinoside (Ochmain et al. 2010, Wodjdyo et al. 2014) and quercetin-3-rhamnoside has also been reported (Wodjdyo et al. 2014). Quercetin-3-sambubioside differs from quercetin-3-rutinoside by only the substitution of a xylosyl residue to the sugar moiety. Due to the fact that quercetin-3-sambubioside was isolated from *Lonicera caerulea* subsp. *villosa*, representatives of this subspecies may be useful in breeding applications to increase the concentration of this valuable compound in new haskap genotypes.

From a human health perspective, the significance of the difference in glycoside residues between quercetin-3-sambubioside and the other quercetin glycosides is unknown, as our current understanding of the mechanism of polyphenol absorption into human tissues is limited. However there is evidence that the glycoside moiety may not be a factor in determining the rate or degree of absorption after consumption (Fernandes et al. 2013)

### **3.6 Conclusions**

The methodology presented in this study has proven effective for the separation, identification and quantification of secondary metabolite compounds in fruit and leaves of haskap. This method will be useful for further investigation of the influence of factors such as maturity and environmental effects on the secondary metabolites in haskap tissues. In addition we have identified for the first time the presence of the iridoid compounds loganin and secologanin and the flavonoid compound quercetin-3-sambubioside in the fruit of haskap (*Lonicera caerulea*). These results may help haskap producers to market their fruit and will guide haskap breeder's seeking to increase the concentration or content of valuable secondary

metabolites in new haskap genotypes. We have also identified potentially valuable secondary metabolite compounds for further study. Some important questions stemming from these results are; a) do the concentration or content of these secondary metabolite compounds differ in different genotypes? b) Are there changes in the concentration or content of these compounds during fruit development? c) What are the levels at standard harvest maturity? d) What are the impacts of environmental or production practices on the concentration or content of these secondary metabolites?

### **3.7 Transition**

Chapter 3 was intended to establish a methodology to prepare, extract, separate, identify and quantify the concentration and content of secondary metabolite compounds from methanolic extracts of the fruit and leaves of haskap (*Lonicera caerulea*). The chapter also selected compounds found in the secondary metabolite profile of haskap for further study in the following chapters. The selection criteria used was based on; a) the compounds could be identified with the use of pure authentic standards or reference spectra, b) the compounds were found in concentrations high enough to be monitored consistently, c) the compounds are already available in the marketplace as a nutrition supplements, or the compounds are being marketed as the primary health beneficial compound in currently available foods, d) the compounds have been shown to have beneficial effects on human health when consumed.

This chapter furthered the overall objective of this project, which was to increase our understanding of the secondary metabolite profiles within haskap. It identified which compounds are present in the fruit and leaves of haskap and at what concentrations. This chapter also identified individual secondary metabolites which have market value that were present at



significant concentrations within the fruit and leaves of haskap. Establishing a methodology capable of identification and quantification of the secondary metabolites and selecting which compounds to monitor was necessary to close other gaps in the knowledge base as outlined in section 1.1. The next chapter addresses the second key knowledge gap outlined in section 1.1; understanding changes in the metabolite profile of the fruit of haskap which occur over the course of fruit growth and development.

## **4.0 EFFECT OF DEVELOPMENTAL STAGES ON CONCENTRATION AND CONTENT OF SECONDARY METABOLITES IN FRUIT OF SELECTED HASKAP (*Lonicera caerulea* L.) GENOTYPES**

### **4.1 Abstract**

Haskap fruit (*Lonicera caerulea* L.) have been shown to contain a range of secondary metabolites valued for their potential to improve human health. These compounds include chlorogenic acid, several quercetin derivatives and at least two iridoid glucosides. While previous studies have reported on the total concentration of some of these compounds in mature haskap fruit, there is currently no information on the changes in concentration and content of these secondary metabolites that occurs during development of the haskap fruit. Such information would be helpful to haskap breeding programs which seek to increase the concentration and content of these compounds in fruit of new genotypes. This information would also be important in identifying the optimal stage to harvest haskap fruit to obtain maximum concentration and/or content of valuable secondary metabolites. The objective of this study was to evaluate the concentration and content of selected secondary metabolites during fruit development in haskap. Two named releases of haskap developed by the UofS (Tundra and Borealis) were selected for study, along with four genotypes chosen because they are among the most often used parents in the UofS Haskap Breeding Program. Fruit were sampled over the course of development in two concurrent years (2011 and 2012) at the UofS Horticulture Research Plots in Saskatoon, SK. Fruit weight increased linearly with time measured in accumulated heat units (GDD<sub>5</sub>) since anthesis. Fruit samples were extracted with 1:1 mix of methanol and water and analyzed by HPLC. The concentration (mg/g DW) of most selected

secondary metabolites decreased logarithmically over the course of fruit development. However, the total content (mg/fruit) of these same compounds generally increased linearly over the course of fruit development. The data indicates that the optimum time to harvest haskap fruit is when fruit weight reaches its peak; this stage of development also coincides with the fruit having the best flavor profile and the maximum content of most of the secondary metabolites monitored. The haskap cultivar Tundra contained the highest concentrations of chlorogenic acid, total quercetin and total anthocyanin of the genotypes tested. The genotype 77-87 contained the highest concentrations of total iridoids of the genotypes tested. This suggests that there may be potential to improve the overall concentration of secondary metabolites by crossing cv. Tundra and genotype 77-87. Tundra also contained the highest content of chlorogenic acid and total anthocyanin of the genotypes tested. Of the genotypes tested 22-14 contained the highest quercetin content, while 77-87 contained the greatest content of total iridoids. Again it appears that gains in the overall content of secondary metabolites in haskap fruit can be achieved by crossing cv. Tundra with either 22-14 or 77-87.

## **4.2 Introduction**

### *4.2.1 Selection criteria for secondary metabolites and their potential health beneficial effects*

The results presented in Chapter 3 indicate the presence of a range of secondary metabolites in the fruit of haskap. Some of these compounds have market value as they have shown the ability to improve human health. Some of these beneficial compounds have been selected for further study. The selection criteria for secondary metabolite compounds to be investigated in this study (outlined in Chapter 3) were; a) the compounds could be identified based on comparisons with pure authentic standards or reference spectra, b) the compounds are

found in concentrations high enough to be monitored consistently, c) these compounds are already available in the marketplace as a nutrition supplements, or the compounds are being marketed as the primary health beneficial compound in currently available foods, d) compounds have been shown to have beneficial effects on human health when consumed. The selected compounds were chlorogenic acid, quercetin glucosides (quercetin-3-sambubioside, quercetin-3-rutinoside and quercetin-3glucoside) and iridoid glucosides (loganin and secologanin).

#### *4.2.2 Effect of stage of maturity on secondary metabolites in fruit*

The chemistry of fruit changes during development, both in terms of primary metabolites such as sugars (Usenik et al. 2008), starch (Awad et al. 2001) and malic acid (Castrejon et al. 2008), as well as the secondary metabolites compounds produced from the phenylpropanoid (Gibson et al. 2013) and terpenoid pathways (Ranalli et al. 2009). The relationship between secondary metabolite quantities and fruit development can be markedly different when measured on a concentration (mg per unit weight) versus a content (mg per fruit) basis. In general, the concentration of most secondary metabolites decreases as the fruit develop (Awad et al. 2001, Reay and Lancaster 2001, Jaakola et al. 2002, Vvedenskaya and Vorsa 2004, Castrejon et al. 2008, Xu et al 2008, Jemai et al. 2009, Ranalli et al. 2009, Zifkin et al. 2012, Gibson et al. 2013). This likely reflects a dilution effect as fruit size increases. Examples of the types of changes in the concentration of secondary metabolites seen in a range of important fruit crops are presented in Tables 4.1-4.3.

The concentrations of chlorogenic acid in fruit generally decrease during fruit maturity (Table 4.1), with the magnitude of the decrease dependent on the species.

**Table 4.1.** Changes in the concentrations (mg/g DW) of chlorogenic acid in different fruit crops during development.

<b>Species or genotype</b>	<b>Earliest sampling point (mg/g DW)*</b>	<b>Harvest maturity (mg/g DW)</b>	<b>Percent change</b>	<b>Reference</b>
Lowbush blueberry several wild clones	5	2	-60%	Gibson et al. 2013
Apple cv. Elstar (skins)	2	1	-50%	Awad et al. 2001
Apple cv. Jonagold (skins)	5	1	-80%	Dragovic-
Apricot cv. Madjarska	0.025	0.023	-8%	Uzelac et al. 2007
Apricot cv. Velika rana	0.021	0.019	-10%	

\*The first sampling point after fertilization of the flower

As seen from Table 4.2, the concentrations of total quercetin also tend to decrease as fruit develop. The reduction in concentration over the course of development appears to be in the range of between 50-80%, depending on the species and cultivar. By contrast the concentration of flavonoid compounds is more variable during fruit development. The concentration of flavonol compounds increased during the development of strawberry fruit (Halbwirth et al. 2006, Mahmood et al. 2012) and in the fruit of four species of mulberry (Mahmood et al. 2012). The concentration of total flavonols in skins of the grape cultivar Tempranillo also increased with maturation of the fruit (Martinez-Luscher et al. 2004).

**Table 4.2.** Changes in the dry weight concentrations (mg/g DW) of total quercetin in in different fruit crops during development.

<b>Species or genotype</b>	<b>Earliest sampling point (mg/g DW)</b>	<b>Harvest maturity (mg/g DW)</b>	<b>Percent change</b>	<b>Reference</b>
Highbush blueberry cv. Reka	1.5	0.5	-66%	Castrejon et al. 2008
Highbush cv. Puru	1.8	0.2	-90%	
Highbush cv. Bluecrop	0.8	0.1	-78%	Zifkin et al. 2012
Highbush cv. Berkeley	1	0.5	-50%	
Highbush blueberry cv. Rubel	3.6	0.7	-80%	
Lowbush blueberry several wild clones	1.68	0.66	-60%	Gibson et al. 2013
Bilberry wild clone	0.87	0.13	-85%	Jaakola et al. 2002
American cranberry cv. Ben Lear	3.0	2.3	-20%	Vvedenskaya and Vorsa 2004.
Apple cv. Elstar (skins)	10	5	-50%	Awad et al. 2001
Apple cv. Jonagold (skins)	14	8	-40%	
Apricot cv. Madjarska	0.05	0.04	-20%	Dragovic-Uzelac et al. 2007
Apricot cv. Velika rana	0.03	0.02	-20%	

**Table 4.3.** Changes in the concentrations (mg/g DW) of total iridoid in different fruit crops during development.

<b>Species or genotype</b>	<b>Earliest sampling point (µg/g DW)</b>	<b>Harvest maturity (µg/g DW)</b>	<b>Percent change</b>	<b>Reference</b>
Olive cv. Leccino	17.5	8.9	-50%	Ranalli et al. 2009
Olive cv. Cassanese	57.4	43.9	-25%	
Olive cv. Dhokar	23.3	1.3	-95%	Jemai et al. 2009
Olive cv. Chemlali	36.7	10.0	-75%	

The concentration of the iridoid compound oleuropein decreased during the maturation of fruit of seven genotypes of olive (Ranalli et al. 2009). The two olive genotypes presented in Table 4.3 represent the two genotypes with the greatest and least overall change iridoid concentrations during fruit development.

Anthocyanin concentrations in some fruit generally increase after the mid-point of fruit development (Wang and Lin 2000, Awad et al. 2001, Reay and Lancaster 2001, Kennedy et al. 2002, Castrejon et al. 2008). This change is visually obvious, as the anthocyanin compounds have a red/blue coloration.

#### 4.2.3 Effect of stage of maturity on content of secondary metabolites in fruit

Concentrations of secondary metabolites generally decrease during development, while the mass of the fruit increases. In most cases the degree of increase in fruit mass is greater than the reduction in concentration, resulting in a net increase in content (mg/fruit) of secondary metabolites as the fruit develop. An example of this change is presented in Table 4.4 which reports content of quercetin during development of several fruit crops.

**Table 4.4.** Changes in the content (mg/fruit) of total quercetin in different fruit crops during development.

Species or genotype	Earliest sampling point (mg/fruit)	Harvest maturity (mg/fruit)	Reference
Apple cv. Elstar	0.2	2.2	Awad et al. 2001
Apple cv. Jonagold	0.2	3	
Grape cv. Cabernet Sauvignon	0.04	0.09	Kennedy et al. 2002
Strawberry cv. Elsanta	0	1	Halbwirth et al. 2006

As the concentration of anthocyanin generally increases during fruit development, it is not surprising that the anthocyanin content of fruit also increases as the fruit increases in size (Wang and Lin 2000, Awad et al. 2001, Reay and Lancaster 2001, Kennedy et al. 2002, Castrejon et al. 2008).

#### *4.2.4 Breeding Haskap for enhanced secondary metabolite profile*

Although the presence of flavonols and chlorogenic acid within haskap fruit has been well documented (Wojdylo et al. 2014), there have been no studies of the changes to the concentration and content of these compounds that occurs during fruit development. The iridoid compounds that were newly identified in haskap fruit (see Chapter 3) will also be investigated in haskap through the fruit development period from anthesis to harvest maturity and beyond. This knowledge would be useful in breeding programs seeking to increase the concentration and/or content of these secondary metabolites in haskap. If the germplasm shows variation for the secondary metabolite profile then breeding efforts could be profitable. However if the diverse germplasm have similar metabolite profiles, breeding efforts should be directed elsewhere. The germplasm selected for study represents some of the most valuable breeding parents in the U of S collection and information gained from this study may be relevant for future genotypes produced from this material. The resulting data will also be useful to growers as it will help determine the optimum time of harvest for haskap fruit given the objective of simultaneously maximizing yields, fruit quality and flavor parameters as well as potentially optimizing the levels of valuable, health enhancing secondary metabolites in the fruit.

#### *4.2.5 Hypotheses*

a) As the fruit develops, the concentration of the selected secondary metabolites will decline while the overall content (mg/fruit) will increase. b) The concentration and content of selected secondary metabolites is expected to vary amongst genotypes of haskap



## 4.3 Methodology

### 4.3.1 Description of test material

The haskap genotypes outlined in Table 4.5 were selected for analysis of the changes in concentration and content of selected secondary metabolites that occur during fruit development. The cultivar Tundra was sampled most intensively as it is presently the standard haskap cultivar in commercial production in Canada. The other haskap breeding lines from the UofS were selected for their superior agronomic and fruit quality. These genotypes were among the most often used parents in the U of S Haskap Breeding program at the time of this study. These genotypes were sampled less intensely than Tundra.

Tundra and Borealis are full siblings derived from a cross between Kiev #8 (Blue Velvet) and Tomichka (Blue Belle). Kiev #8 is an open pollinated seedling of a plant gathered from the Kurile Islands, while Tomichka is one of the first haskap cultivar released in Russia. While Tundra and Borealis are full siblings, they display different canopy architecture characteristics. The fruit of Tundra is more exposed to the sun while the fruit of Borealis are held deeper within the canopy. 3-03 is an open pollinated seedling of a plant gathered from the Kurile Islands which shares  $\frac{1}{4}$  of its genetic background with Tundra and Borealis. It is a half sibling of Kiev #8. 22-14 was derived from open pollinated seeds from the Bibai region of Hokkaido, Japan (original mother plant named Bibai #8). 44-83 was derived from open pollinated seeds from the Chitose region of Hokkaido (original mother plant named Chitose #8). Genotypes 41-83 and 22-14 originated from different farms in Hokkaido and are not know to share common genetics. 77-87 was a hybrid between Japanese and Russian parents (22-40 and Czech #17). Czech #17 is also known as Berry Blue in North America but was bred from Russian stock in the Czech Republic.

Genotypes 41-83, 22-14 and 77-87 were collected and selected by Dr. Maxine Thompson at Oregon State University.

All plants were grown at the UofS Horticultural Field Research Station as previously described (Chapter 3.3.1). The experiment was conducted during the 2011 and 2012 growing seasons.

**Table 4.5.** Origin of haskap (*Lonicera caerulea*) genotypes evaluated for changes in concentration and content of valuable secondary metabolites during fruit development.

<i>Lonicera caerulea</i> genotype	Origin	Selection criteria
Tundra	U of S	Fruit quality, yield, ease of mechanical harvest
Borealis	U of S	Fruit quality, yield and fruit within canopy
3-03	Jim Gilbert	Superior plant stature and yield
41-83	Maxine Thompson	Fruit quality, yield, ease of mechanical harvest
77-87	Maxine Thompson	Fruit quality, yield, ease of mechanical harvest
22-14	Maxine Thompson	Fruit quality and fruit size

#### 4.3.2 Maturity assessment

Individual flowers of the selected haskap genotypes were marked the day they first opened (day 0). These flowers acted as a visual guide for selection of fruit when sampling throughout development, as the visual characteristics were used to estimate the age of fruit included in each sample.

#### 4.3.3 Sampling procedure

Thirty berries of Tundra (3 per plant, 10 plants per rep), were sampled every 4-5 days from initial fertilization until the fruit reached full size. This development stage was labelled as “harvest maturity” as it corresponds to the stage at which growers most commonly harvest haskap fruit. Sampling of the Tundra fruit continued for 5 days after harvest maturity to study the

changes in fruit size and quality that occurred if harvest is delayed. Twenty berries of the remaining five genotypes (10 berries per plant, 2 plants per rep) were sampled every 7 days from initial fertilization through to harvest maturity. The experiment used a randomized block design with three repetitions. Immediately after sampling, the berries were placed in a -30°C freezer and held at that temperature until further processed.

#### *4.3.4 Fruit processing*

Fruit were processed in the manner described in section 3.3.2.

#### *4.3.5 Fruit extraction procedure*

Methanolic extracts from the fruit samples were prepared in the manner described in section 3.3.3., with the following exception; 0.25g of fruit tissues was used for extraction of each sample.

#### *4.3.6 High pressure liquid chromatography (HPLC) procedure*

The secondary metabolites present in the methanolic extracts obtained from the fruit samples were assessed via HPLC, using the method described in section 3.3.4.

#### *4.3.7 Anthocyanin quantification*

Anthocyanin quantification in the haskap fruit samples was achieved using a modified version of the methodology described by Abdel-Aal and Hucl (1999). Ground berry samples (0.1 g) were weighed in a polypropylene tube and extracted with 10mL acidified methanol (methanol:HCl 1.0M (85:15,v/v) for 60 mins at 50°C. After the 30 min point of the extraction the

tube was vortex mixed for 10 seconds. The tubes were then centrifuged at 5000 rpm for 10 mins and the supernatant decanted. The supernatant was diluted to 1/30 with methanol:HCl 1.0M (85:15,v/v) and the absorbance of the resulting solution was measured at 535 nm with a spectrophotometer (Dynamica, Newport Pagnell UK). Absorbance of the samples was compared with an anthocyanin standard curve created using cyanidine-3-glucoside (Extrasynthase, Lyon, France). As haskap fruit only begin to develop colored anthocyanin compounds after the mid-point of their development, only berries which had developed a red or blue colour were tested for their anthocyanin concentration (4-5 weeks after fertilization of the flower until harvest maturity).

#### *4.3.8 Determination of titratable acidity and soluble solids*

Analysis of titratable acidity in the haskap fruit was accomplished using a HI 84432 Fruit Juice Titratable Acidity Meter (Hanna Instruments, Laval Quebec Canada). Measurements of total soluble solids in the fruit were conducted using a digital brix meter (Atago Co. LTD, Tokyo Japan). Testing for titratable acidity and soluble solids only began once fruit had begun to develop the obvious red/blue color associated with the onset of ripening.

#### *4.3.9 Calculation of cumulative growing degree days from days post-fertilization*

Although days post-fertilization was used to assign the initial sampling dates, a time-based tracking system usually does not provide the most accurate predictor of growth of fruit. A more accurate predictor of maturity of fruit can be achieved based on the accumulation of heat units (Stanley et al. 2000). Heat unit accumulation was assigned to each sampling date by summing the total number of degrees above a base temperature of 5°C over the duration of the

fruit development period. A base temperature of 5°C was chosen as haskap is adapted to cool growing conditions (Imanchi et al. 1998). Details of flowering for the six haskap genotypes tested are presented in Table 4.6.

#### *4.3.10 Statistics*

Regression analysis was used to explore the relationship between the concentration and content of secondary metabolites in the haskap fruit and the stage of fruit development based on cumulative growing degree days since flowering. Data sets from both 2011 and 2012 were combined to create the regression models. Regression model assignment was based on the structure of the residuals versus fits generated from a linear model (model  $Y=X$ ) or a linear regression based on natural log transformed data sets (model  $Y=\ln(X)$ ) in SAS (Proc Reg) (Clewer and Scarisbrick 2001). Models were selected based on the goodness of fit ( $R^2$ ) and the structure of the residuals. For all responses to increasing GDD, a linear model was considered first. If this model did not show a reasonably good fit statistic ( $R^2 < 0.25$ ) and/or there was an obvious structure to the residuals, then an appropriate logarithmic or second order polynomial model was considered. If either a logarithmic or second order polynomial model improved the overall goodness of fit statistic and/or provided a better fit to the residuals over the linear model then that model was accepted. In general the same model was used for each response variable amongst the genotypes, even if one or two of the genotypes showed a relatively low  $R^2$  statistic for that model. Only linear, logarithmic and second order polynomial models were tested, as these models can be explained from a biological context (Clewer and Scarisbrick 2001). ANOVA was used to test the effects of genotype on the fruit quality parameters evaluated at harvest maturity. Tukey's test was used for means separation at  $\alpha=0.05$ . ANOVA was also used to test the effects of year on fruit weight.

## **4.4 Results**

### *4.4.1 Fruit development period*

In 2011 it took 42 days from fertilization until the fruit reached full size (harvest maturity) for the six haskap genotypes, while in 2012 49 days were required. Unfavorable weather conditions during the spring of 2012 increased the time required for the haskap fruit to develop in that growing season. Even though the ripening period in 2011 was on average 7 days shorter than in 2012, the berry weights for all genotypes in 2011 were greater or equal to 2012 (data not shown). While the length of time required for full fruit development was not consistent across the two years of testing, the actual amount of heat ( $GDD_5$ ) required to achieve full fruit development was similar in the two years for the genotypes Tundra, Borealis, 41-83, 77-87 and 22-14 (Table 4.6). By contrast, the genotype (3-03) showed considerable variability in the  $GDD_5$  required for fruit development over the two years of testing. This is mostly likely due to a cool period during flowering in 2012 which pushed the maturation of fruit back farther into July, when Saskatoon usually experiences warm temperatures.

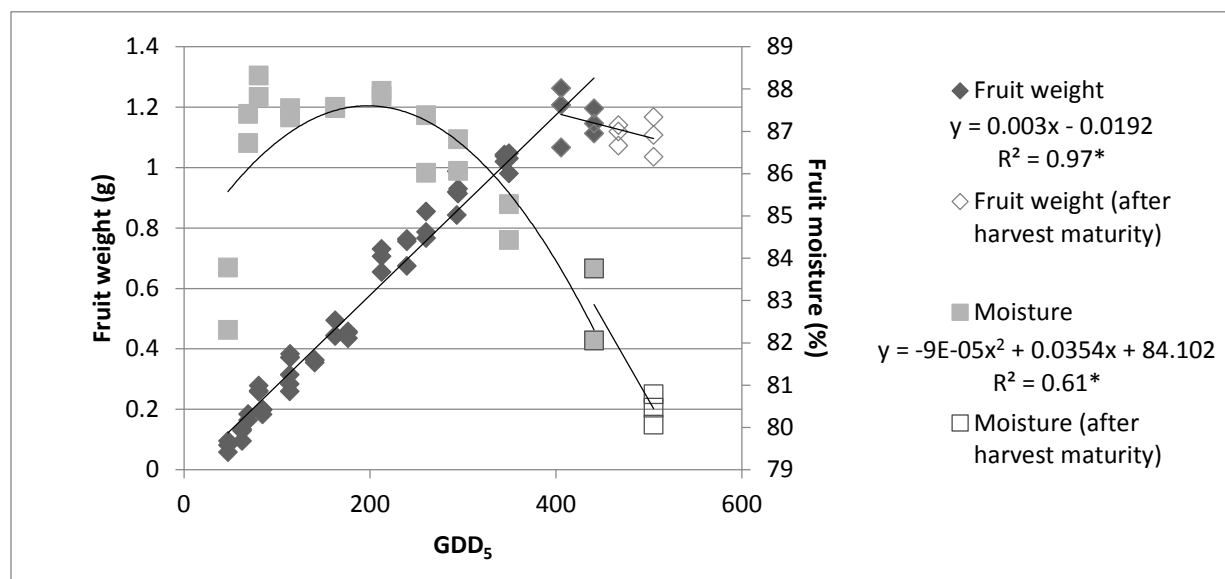
For the purposes of this study harvest maturity was defined as the point when the fruit reach maximum mass and full blue colour. The average  $GDD_5$  required for full fruit development of the haskap genotypes tested was 473.6 with a standard deviation of 67.5 (n=12).

**Table 4.6.** Flowering date, date when the fruit reached harvest maturity and cumulative growing degree days of base 5°C (GDD<sub>5</sub>) from flower to full fruit maturity for six genotypes of haskap (*Lonicera caerulea*) in 2011 and 2012.

Genotype	Year	Peak flowering	Fruit maturity		Difference between years (%)	
			Days from flowering	GDD <sub>5</sub> from flowering	Days	GDD <sub>5</sub>
Tundra	2011	May 18	42	405.4	15	8.1
	2012	May 13	49	440.9		
Borealis	2011	May 20	42	408.8	15	10
	2012	May 17	49	454.1		
3-03	2011	May 31	42	475.5	15	18.3
	2012	May 28	49	582.1		
41-83	2011	May 25	42	430.2	15	6.6
	2012	May 18	49	460.7		
77-87	2011	May 27	42	459.9	15	7.3
	2012	May 21	49	496.2		
22-14	2011	May 31	42	568.8	15	5.9
	2012	May 30	49	604.3		
Mean	2011	May 25	42	440.9	15	13
	2012	May 21	49	506.4		
	Average	May 23		473.6		

#### 4.4.2 Fruit weight during development

Fruit weight in cultivar Tundra increased linearly with increasing GDD<sub>5</sub> from flowering through until harvest maturity, after which fruit weight began to decrease due to dehydration (Figure 4.1). The fruit weight of the other five genotypes also increased linearly with heat accumulation (Appendix Table A.1) through to the point where the fruit reached maximum size. Berry weights at harvest maturity of the different genotypes averaged from 1.14 - 2.12g in 2011 and 0.87 - 1.53g in 2012. The genotype 3-03 had the smallest fruit in both years, while 22-14 produced the largest fruit. Growing season had a significant effect on fruit weight of the haskap fruit at harvest maturity; on average fruit weights were higher at harvest maturity in 2011 (1.45g/fruit) then in 2012 (1.24g/fruit)



**Figure 4.1.** Berry weight and percent moisture content for fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons for fruit weight and during the 2012 growing season for fruit moisture. Fruit weight and moisture have also been modeled beyond the stage of harvest maturity. \* indicates significance at  $\alpha=0.05$

#### 4.4.3 Fruit moisture content

The moisture content of the haskap fruit was measured over the course of fruit development in 2012 (Figure 4.1 and Appendix Table A.2). Percent moisture in the haskap fruit showed a parabolic response to increasing GDD<sub>5</sub> post-fertilization (Appendix Table A.2), with the highest moisture content occurring at the mid-point of fruit development. The percentage moisture ranged from a low of 80.9% to a peak 89.8%. Fruit moisture content declined rapidly after the fruit reached harvest maturity (Figure 4.1)

#### 4.4.4. Changes in concentration and content of phenylpropanoids and monoterpenoids during fruit development

The concentrations and contents of the seven selected valuable secondary metabolites were measured over the course of the fruit development period in six genotypes of haskap over



the course of two growing seasons. The concentrations of the secondary metabolites found in the haskap fruit generally decreased logarithmically as the fruit developed (Appendix Table A.3 – A.9). Averaged across the six haskap genotypes tested, the concentration of chlorogenic acid, total quercetin and total iridoid compounds in fully developed fruit was 10, 39.8 and 9.1% respectively of that contained in the immature fruit. Exceptions to this pattern were; a) the concentration of quercetin-3-glucoside in Tundra and Borealis fruit responded in a parabolic fashion to increasing GDD<sub>5</sub>, with the lowest concentration occurring at the mid-point of fruit development (Appendix Table A.3 and Figure 4.2). b) the concentration of total anthocyanin increased linearly with fruit development, starting at the onset of red/blue coloration of the fruit. Of the secondary metabolites studied, chlorogenic acid occurred at the highest concentration in samples taken early in fruit development. As expected, in fully developed haskap fruit anthocyanin was present at higher concentrations than the other compounds studied.

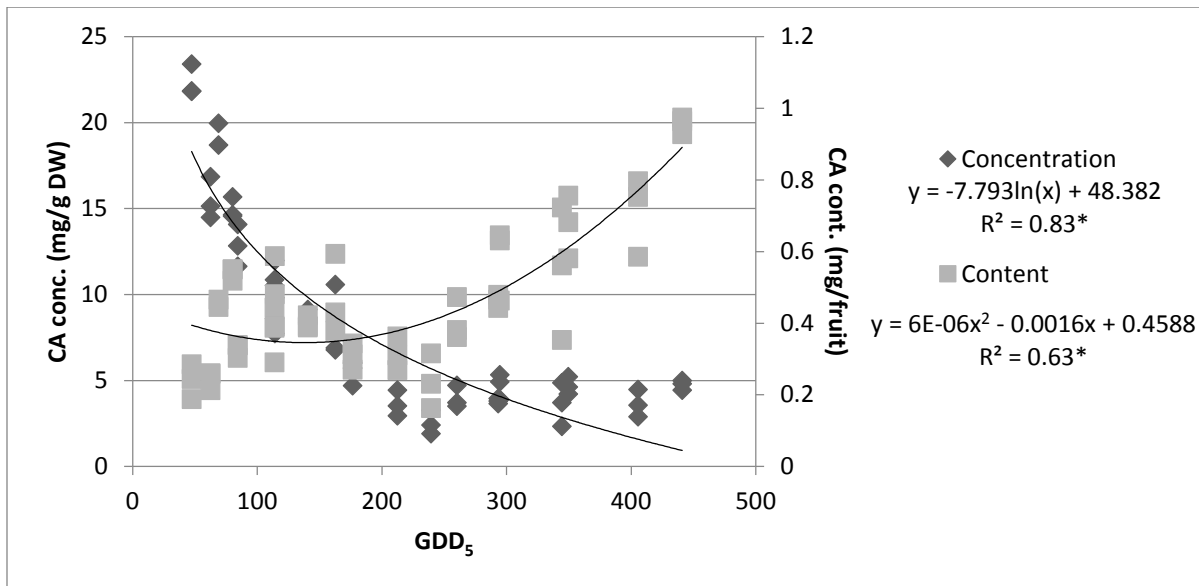
The content (concentration x fruit mass) of all the secondary metabolites studied in the haskap fruit increased with increasing GDD<sub>5</sub>; in most cases this increase was linear. The increase in the content of chlorogenic acid however followed the pattern of a second order polynomial in genotypes Tundra, Borealis and 41-83. There was no significant relationship between the stage of fruit development and the content of chlorogenic acid in fruit of the genotypes 3-03, 77-87 and 22-14. The content of secologanin showed a negative linear relationship with increasing fruit maturity, however this relation was only statistically significant for the genotypes Borealis, 3-03 and 41-83. The content of loganin in fruit of the genotypes Borealis and 41-83 did not show a significant relationship with the stage of fruit development.

Averaged across the six haskap genotypes tested, the content of chlorogenic acid, total quercetin and total iridoid in fruit at harvest maturity were respectively 166, 693 and 145% of the

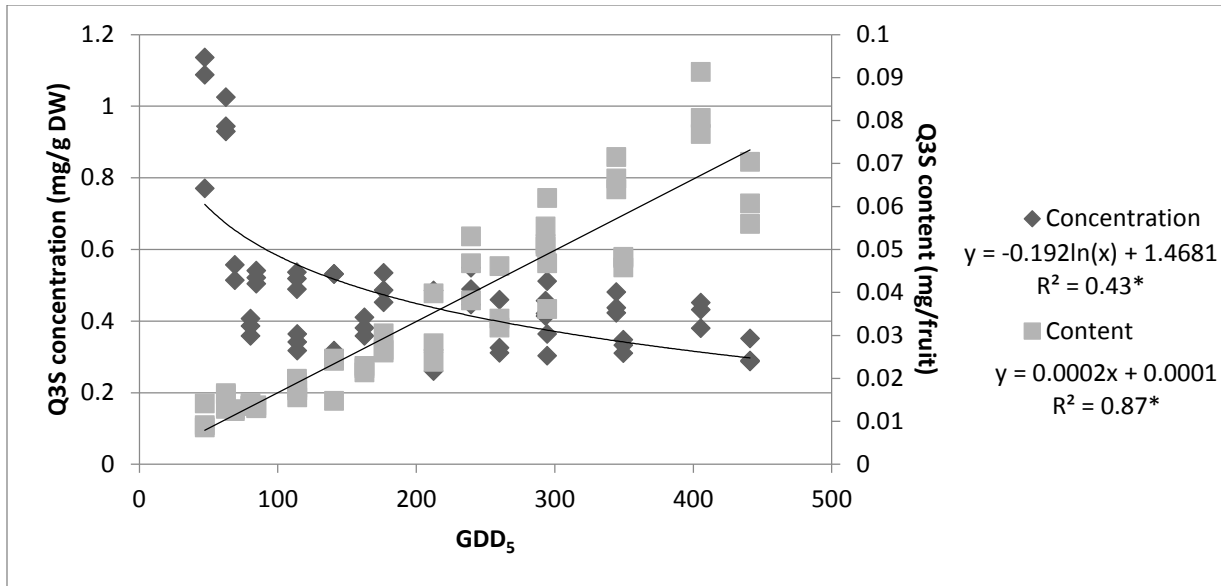
content of the fruit at the first sampling point (between 30 to 60 GDD<sub>5</sub>). The chemical class with the highest content in mature haskap fruit was anthocyanin, followed by chlorogenic acid, total quercetin and total iridoid compounds.

There appeared to be greater variability in the concentrations of secondary metabolites in haskap fruit early in fruit development versus at the mid-point or later stages of growth. This variability was apparent for all the secondary metabolites monitored (Figs 4.2-4.7), and in all the genotypes tested (data not shown).

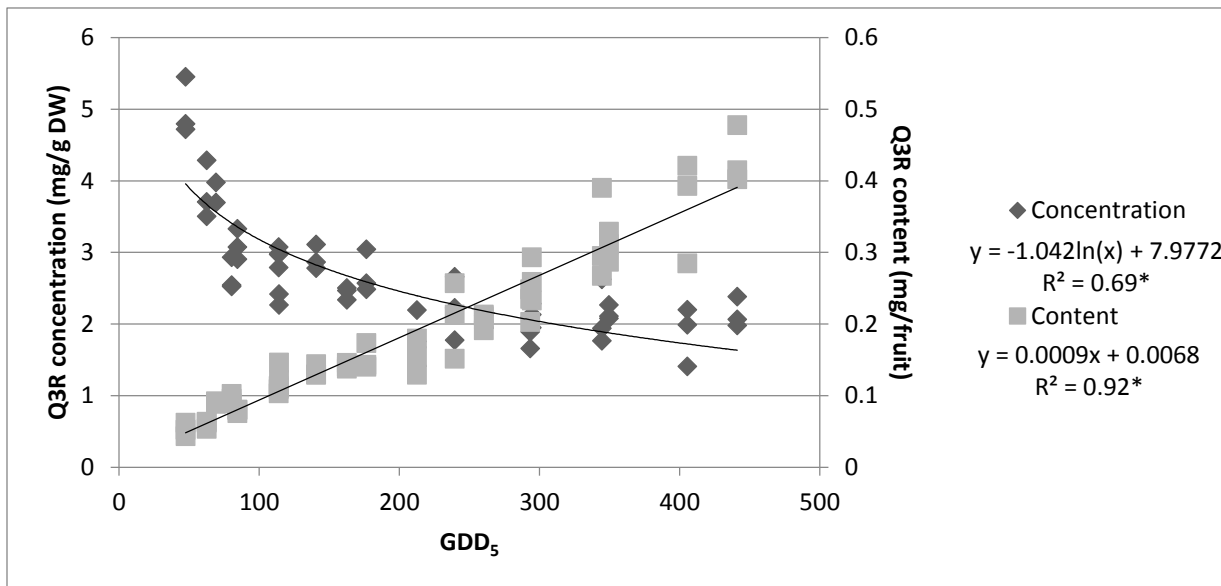
Fruit of the haskap cv. Tundra were monitored after harvest maturity to determine if there were any further changes in the concentration or content of the secondary metabolites. The data indicated that there were no further changes aside from those attributable to dehydration (data not shown).



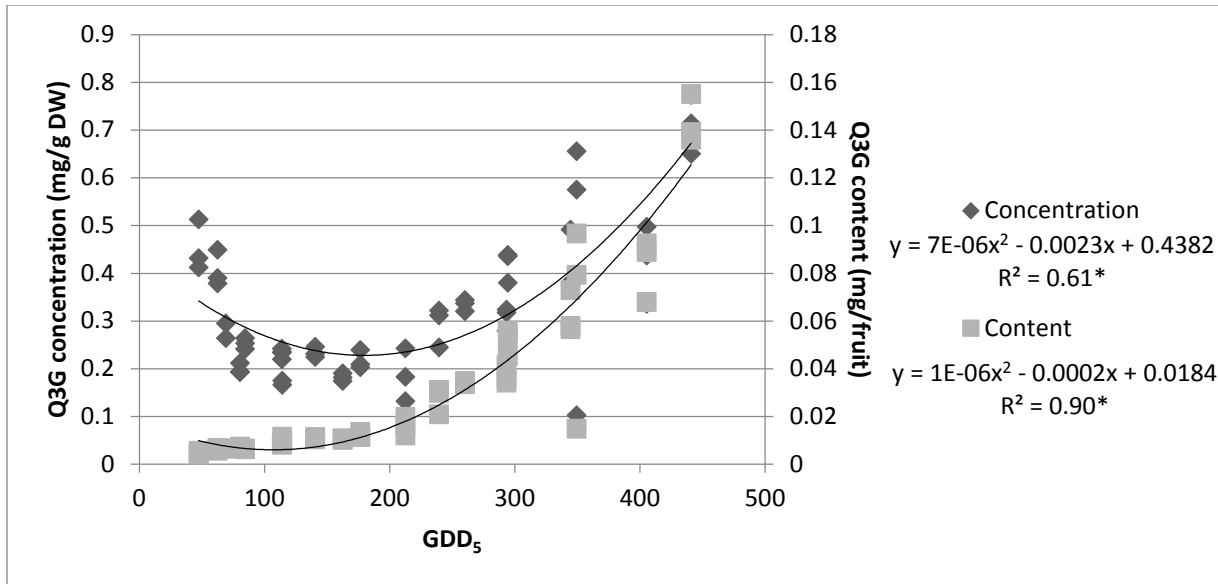
**Figure 4.2.** Chlorogenic acid (CA) concentration and content in fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$



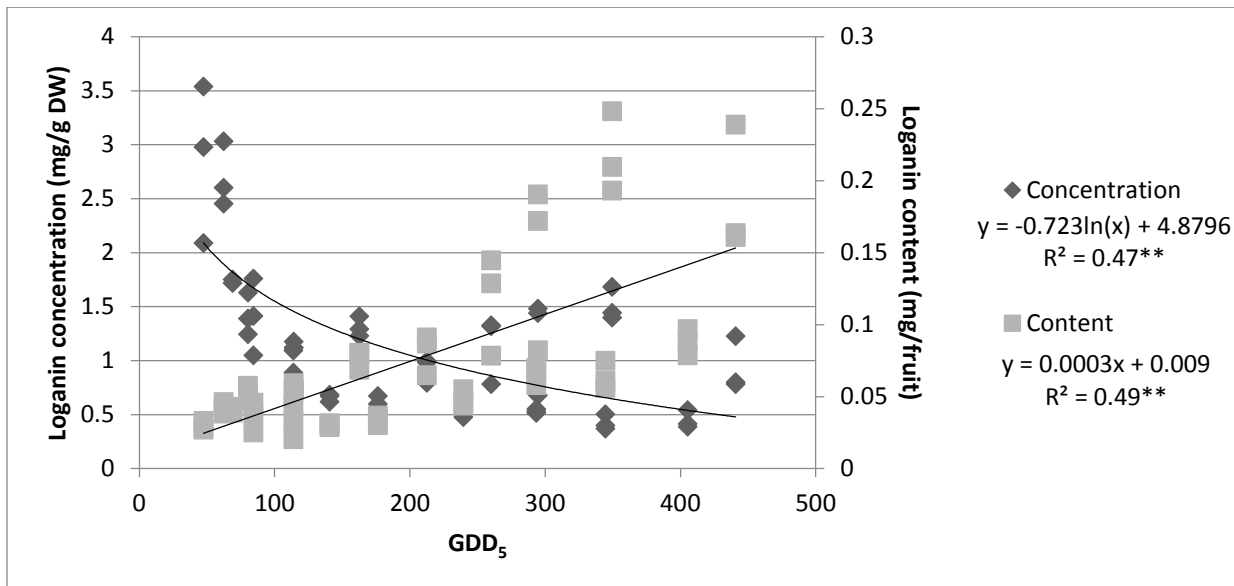
**Figure 4.3.** Quercetin-3-sambubioside (Q3S) concentration and content in fruit of haskap (*Lonicera caerulea* cv. 'Tundra') based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$



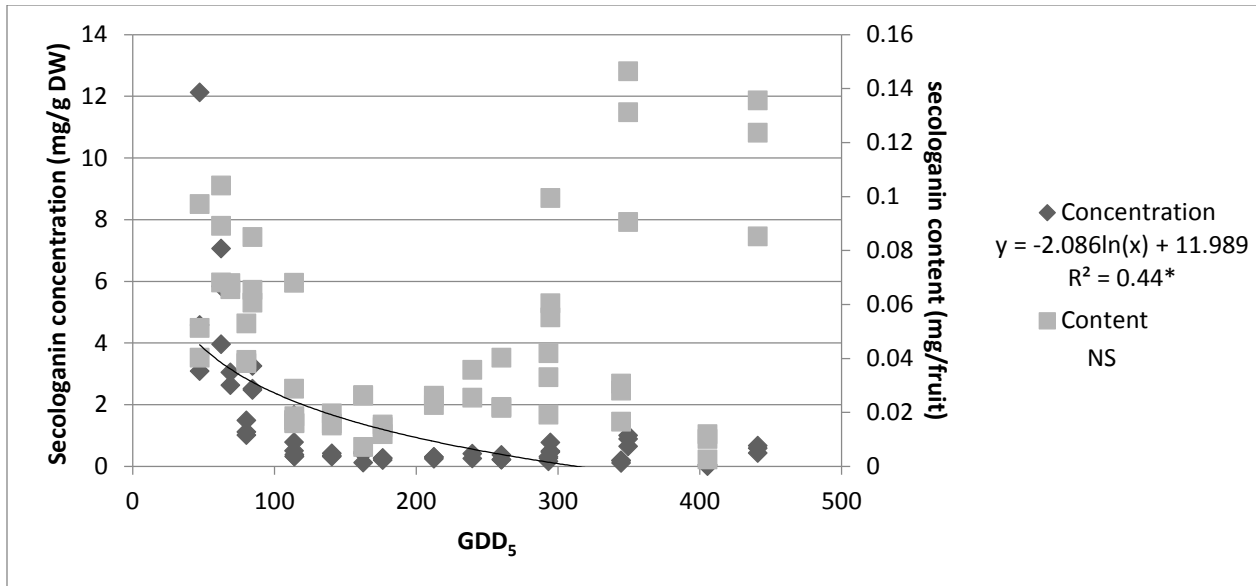
**Figure 4.4.** Quercetin-3-rutinoside (Q3R) concentration and content in fruit of haskap (*Lonicera caerulea* cv. 'Tundra') based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$



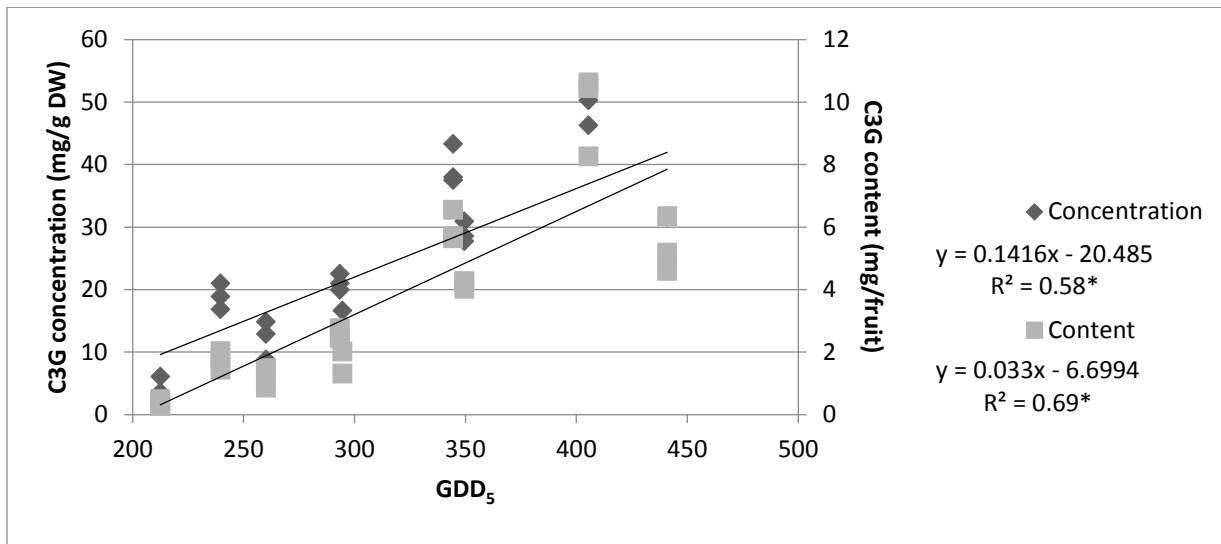
**Figure 4.5.** Quercetin-3-glucoside concentration and content in fruit in haskap (*Lonicera caerulea* cv. ‘Tundra’) based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$



**Figure 4.6.** Loganin concentration and content in fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$



**Figure 4.7.** Secologanin concentration and content in fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$

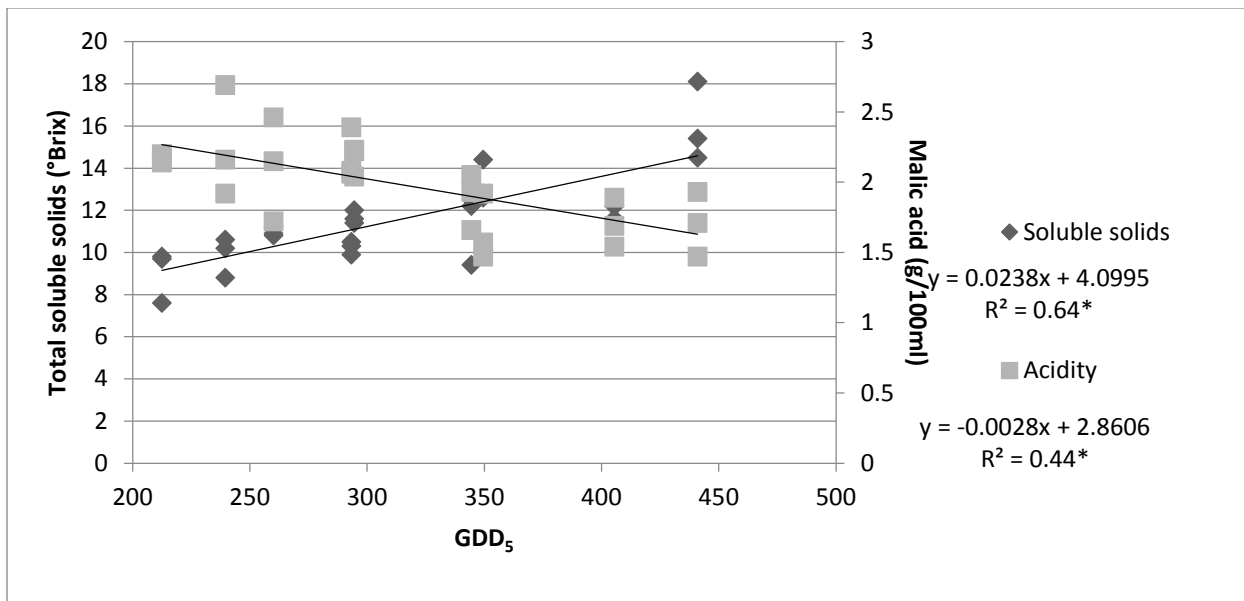


**Figure 4.8.** Anthocyanin concentration and content in fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$

#### 4.4.5 Soluble solids and titratable acidity

Soluble solid content and titratable acidity are important aspects of fruit quality as they are major factors determining fruit flavor. The concentration of titratable acids and soluble solids were measured in haskap fruit commencing with the first appearance of blue colour and ending

at harvest maturity. The soluble solids content in the fruit of genotype Tundra increased in a linear manner as the fruit developed (Appendix Table A.10 and Figure 4.9). This was also true of the other five haskap genotypes, except in the genotype 3-03 which did not show a significant relationship between stage of fruit development and soluble solids content. Titratable acidity (measured as malic acid equivalents) was negatively correlated with increasing fruit development in the genotype Tundra (Appendix Table A.11 and Figure 4.9). A significant negative linear relationship between the stage of fruit development and titratable acidity was only observed for the genotypes Borealis, 41-83 and 77-87. Total soluble solids and total acidity of haskap fruit were variable across genotypes during the later stages of fruit development.



**Figure 4.9.** Total soluble solid (°Bx) and titratable acidity (in malic acid equivalents) in fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on cumulative growing degree days post-fertilization (base 5°C). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$

#### *4.4.6 Seasonal variance in the concentration of secondary metabolites in haskap fruit tested at harvest maturity.*

Growing season had a significant impact on the concentrations quercetin-3-sambubioside, loganin and secologanin in the fruit of haskap at harvest maturity over the two growing seasons tested. On average, fruit grown in 2011 had higher concentrations of quercetin-3-sambubioside and secologanin, but fruit produced in 2012 had higher average concentrations of loganin. The concentrations of chlorogenic acid, quercetin-3-rutinoside and quercetin-3-glucoside in the fruit of haskap were not significantly affected by the growing season.

#### *4.4.7 Genotypic variance in quality parameters of haskap fruit tested at harvest maturity*

Differences amongst haskap genotypes for fruit quality parameters were assessed at harvest maturity. Harvest maturity was defined as the point at which the fruit reached maximum weight and had developed full blue colour. This was the most logical point to designate as harvest maturity as haskap fruit are sold by weight and harvesting at this point would maximize returns for the crop, assuming no significant decreases in fruit quality past this point. There were significant differences amongst the genotypes for mass, percent moisture, soluble solids and titratable acidity of the fruit at harvest maturity (Table 4.7). The genotype 22-14 produced the largest fruit at harvest maturity. Fruit of 22-14 also had the highest soluble solid content and the highest titratable acidity at harvest maturity. The highest moisture content at harvest maturity was found in fruit of genotype 3-03. There were also significant differences amongst the six haskap genotypes tested in terms of the concentration and content of the secondary metabolites measured at harvest maturity (Table 4.8 and 4.9). The highest concentrations of chlorogenic acid, total quercetin and total anthocyanin at harvest maturity were found in fruit of the commercial

standard cultivar Tundra. Fruit of genotype 77-87 had the highest concentration of the iridoid compounds at harvest maturity. The highest content of chlorogenic acid and total anthocyanin at harvest maturity occurred in fruit of the cultivar Tundra, while the highest content of quercetin derivatives were found in fruit of genotype 22-14. The fruit of genotype 77-87 had the highest content of iridoid compounds.

**Table 4.7.** Fruit mass, % moisture, soluble solids and titratable acidity at harvest maturity in fruit of six selected haskap (*Lonicera caerulea*) genotypes. Data were pooled from the 2011 and 2012 growing seasons. Mean values in each column followed by the same letters are not significantly different at  $\alpha=0.05$  using Tukey's test.

Genotype	Fruit mass (g)	% moisture	Soluble solids (°Bx)	Titratable acidity (malic acid g/100mL)
Tundra	1.17 ± 0.03 d	82.9 ± 0.21 d	14.0 ± 1.20 b	1.70 ± 0.07 c
Borealis	1.36 ± 0.03 b	85.2 ± 0.19 b	13.1 ± 0.58 b	2.12 ± 0.12 b
3-03	1.01 ± 0.09 e	86.7 ± 0.74 a	11.4 ± 0.18 c	2.15 ± 0.22 b
41-83	1.26 ± 0.03 c	83.7 ± 0.23 c	13.7 ± 0.73 b	2.14 ± 0.09 b
77-87	1.45 ± 0.09 b	84.5 ± 0.50 b	12.8 ± 0.57 b	1.64 ± 0.07 c
22-14	1.83 ± 0.13 a	84.8 ± 0.62 b	16.2 ± 0.40 a	2.54 ± 0.16 a

**Table 4.8.** Concentrations of secondary metabolites at harvest maturity in fruit of six selected haskap (*Lonicera caerulea*) genotypes. Data were pooled from the 2011 and 2012 growing seasons. Mean values in each column followed by the same letters are not significantly different at  $\alpha=0.05$  using Tukey's test.

Genotype	Chlorogenic acid (mg/g DW)	Total quercetin (mg/g DW)	Total iridoid (mg/g DW)	Total anthocyanin (mg/g DW)
Tundra	4.19 ± 0.33 a	2.94 ± 0.19 a	1.00 ± 0.23 ab	38.07 ± 5.25 a
Borealis	2.62 ± 0.24 b	1.88 ± 0.18 cd	1.23 ± 0.39 ab	27.71 ± 6.55 ab
3-03	1.38 ± 0.12 d	1.83 ± 0.09 d	0.84 ± 0.07 b	19.79 ± 3.50 bc
41-83	1.11 ± 0.07 e	1.73 ± 0.08 d	0.28 ± 0.05 c	13.95 ± 2.54 cd
77-87	2.08 ± 0.13 c	2.28 ± 0.09 b	1.35 ± 0.17 a	22.81 ± 2.39 b
22-14	1.24 ± 0.11 de	2.07 ± 0.10 c	0.31 ± 0.04 c	12.38 ± 1.36 d

\* Total quercetin includes quercetin-3-glucoside, quercetin-3-rutinoside and quercetin-3-sambubioside. Total iridoid includes loganin and secologanin



**Table 4.9.** Total contents of secondary metabolites at harvest maturity in fruit of six selected haskap (*Lonicera caerulea*) genotypes. Data were pooled from the 2011 and 2012 growing seasons. Mean values in each column followed by the same letters are not significantly different at  $\alpha=0.05$  using Tukey's test.

Genotype	Chlorogenic acid (mg/fruit)	Total quercetin (mg/fruit)	Total iridoid (mg/fruit)	Total anthocyanin (mg/fruit)
Tundra	0.83 ± 0.06 a	0.59 ± 0.04 b	0.20 ± 0.05 b	7.59 ± 1.07 a
Borealis	0.42 ± 0.04 b	0.30 ± 0.03 d	0.20 ± 0.06 b	4.38 ± 1.00 bc
3-03	0.19 ± 0.02 c	0.25 ± 0.03 d	0.11 ± 0.01 c	2.79 ± 0.60 d
41-83	0.25 ± 0.02 c	0.40 ± 0.03 c	0.06 ± 0.01 d	3.21 ± 0.63 cd
77-87	0.48 ± 0.05 b	0.53 ± 0.04 b	0.30 ± 0.03 a	4.31 ± 0.69 bc
22-14	0.45 ± 0.05 b	0.74 ± 0.04 a	0.11 ± 0.01 c	4.50 ± 0.64 b

\* Total quercetin includes quercetin-3-glucoside, quercetin-3-rutinoside and quercetin-3-sambubioside. Total iridoid includes loganin and secologanin

## 4.5 Discussion

### 4.5.1 Fruit development

The difference in the rate of accumulation of growing degree days between 2011 and 2012 caused differences in the length of the development period for the haskap fruit. However the GDD<sub>5</sub> from anthesis to maturity was consistent across the two test years. Carlson and Handcock (1991) indicated that a heat unit accumulation model of fruit growth was superior to that of a calendar-day based model for prediction of harvest dates in highbush blueberry. GDD<sub>5</sub> was also shown to be a better predictor of harvest date and fruit size at harvest compared to daily mean temperature for three apple genotypes in New Zealand (Stanley et al. 2000). This study showed that GDD was a superior predictor of harvest date of a range of haskap genotypes. Five of the six genotypes showed smaller percent difference between years when using the heat accumulation model as opposed to simply using the calendar date.

The weight of each developing haskap fruit increased linearly with heat accumulation during fruit development until it reached at peak, after which fruit weight decreased due to dehydration. In other crops the increase in fruit size during development generally follows a sigmoidal pattern, be it single, double or triple sigmoidal when plotted against time (Coombe et al. 1976) or accumulated growing degrees (Godoy et al. 2008). The growth of fruit involves two phases. The first phase occurs from fertilization of the flower to the mid-point of fruit development. This phase is characterized by rapid cell division. The second phase is characterized by the enlargement of the cells created during the first phase. During the mid-point of development (referred to as veraison in grapes), there is a lull in weight accumulation as the metabolism of the developing fruit switches from cell division to cell enlargement (Coombe et al. 1976). However, this experiment showed no indication of a sigmoid pattern when growth of haskap fruit was plotted against cumulative growing degree days. It is possible that there was an insufficient number of sampling dates in this study to observe a sigmoidal pattern in growth of haskap fruit. The mid-point in development is also the point at which the haskap fruit begin to produce the anthocyanin compounds which can be visually observed by the development of red/blue color. In most of the haskap genotypes tested in this project, the first signs of color change occurred once 200 GDD<sub>5</sub> had accumulated.

Soluble solids content of haskap fruit increased as the fruit developed. In general the increase in soluble solids that occurs as haskap fruit develop reflects the accumulation of monosaccharides such as fructose (Kader 2008). By contrast the content of titratable acidity decreased in haskap fruit as they developed. The decrease in acidity that occurs as the fruit develops reflects conversion of organic acids into sugars or aromatics compounds (Kader 2008).

The change in colour of the berry from green to red/blue occurred earlier and with less accumulated GDD<sub>5</sub> in the genotypes Tundra and Borealis than in the other four genotypes tested. This may reflect the parental germplasm used to create these lines. Both Tundra and Borealis come from the same cross between *Lonicera caerulea* subsp. *kamtschatica* and the cultivar Tomichka, an open pollinated Russian variety. *Lonicera caerulea* subsp. *kamtschatica* is known as a mountain type which is adapted to a short, cool growing season (Bors 2009c). This adaptation to a cool, short growing season may have contributed to the early colouration trait observed in these two genotypes. This result may be of interest to haskap breeders as the two genotypes which displayed early red/blue coloration also had the greatest concentration of anthocyanin in the fruit at harvest maturity. The two genotypes which displayed early red/blue coloration also had higher concentrations of both chlorogenic acid and total iridoid compounds when compared to the genotypes that did not display early red/blue coloration. The genotype Tundra showed early red/blue coloration and also contained relatively high concentrations of quercetin derivatives. This result suggests that anthocyanin quantification might be an inexpensive method to screen for high concentrations or content of secondary metabolites in haskap fruit. The genotype 3-03 also contains subsp. *kamtschatica* in its lineage but it did not show early development of red/blue color, nor did it contain high concentrations of any of the secondary metabolites monitored in this study. Thus the linkage between subsp. *kamtschatica* was not an absolute indicator of early ripening or high concentrations of secondary metabolites in fruit.

#### 4.5.2 Effects of stage of fruit development on concentration and content of secondary metabolites

The concentration of chlorogenic acid in the fruit of the six haskap genotypes tested showed a logarithmic pattern of decline with increasing GDD<sub>5</sub> during fruit development. A similar pattern of decreasing chlorogenic acid concentration during fruit maturation has been shown in apple (Awad et al. 2001), apricot (Dragovic-Uzelac et al. 2007), *Citrus poonensis* and *Citrus paradise* (Xu et al. 2008).

The concentration of the three quercetin derivatives identified in haskap fruit tissues also decreased logarithmically with increasing GDD<sub>5</sub> from initial fertilization. This pattern was consistent for the individual quercetin glycosides as well as for the entire quercetin pool. All genotypes showed the same pattern of decreasing quercetin concentrations as the fruit developed. A similar pattern of decreasing concentration of flavonols during fruit development has been seen in apple (Awad et al. 2001, Reay and Lancaster 2001), highbush blueberry (Castrejon et al. 2008, Zifkin et al. 2012), lowbush blueberry (Gibson et al. 2013), bilberry (Jaakola et al. 2002) and American cranberry (Vvedenskaya and Vorsa 2004). Zifkin observed a decrease of approximately 80% in the concentration of quercetin from early to late fruit development in highbush blueberry. Gibson et al. (2013) found that the concentration of quercetin in lowbush blueberry decreased by approximately 66% from initial fertilization to fully mature fruit. The reduction in quercetin concentration of approximately 60% observed within this study is comparable with the responses observed in other crops.

While the concentration of quercetin decreased during development of the haskap fruit, the content of all three quercetin derivatives in the fruit of all six haskap genotypes tested showed a linear pattern of increase with fruit development. This pattern of increasing flavonol

content during fruit maturity has also been shown in other crops such as apple (Awad et al. 2001), grape (Kennedy et al. 2002) and strawberry (Halbwirth et al. 2006).

The concentration of the two iridoid glucosides, loganin and secologanin, also appeared to follow a logarithmic pattern of decline during fruit development in haskap. A similar decrease in the concentration iridoid compounds during fruit maturity has been shown in olive (Jemai et al. 2009, Ranalli et al. 2009, Gutierrez-Rosales et al. 2012), gardenia fruit (*Gardenia jasminoides* Ellis) (Chen et al. 2010) and genipap (*Genipa Americana* L.) (de Sousa Bentes and Mercadante 2014). In their study of Genipap, de Sousa Bentes and Mercadante (2014) observed a 90% decrease in the concentration of total iridoids over the course of fruit development. In this study, the concentrations of total iridoids in haskap fruit also dropped by approximately 90% from fertilization through to harvest maturity. The relationship between the content of iridoid compounds and fruit maturity observed in this study varied amongst the haskap genotypes tested and also between compounds. At harvest maturity the fruit of genotype 77-87 contained a 50% higher content of total iridoids than the next highest genotype. The content of loganin increased with increasing fruit development, while the content of secologanin decreased. The reasons for this difference in the response of these two compounds to increasing fruit development are not clear, considering that loganin is the metabolic precursor to secologanin.

There appears to be a greater variance in the concentration of secondary metabolites in the fruit of different haskap genotypes during early stages of fruit development as compared to the concentrations at the mid-point or at harvest maturity. This is most likely explained by the fact that the fertilization of the ovary of a flower leads to a suite of developmental changes within the flower/developing fruit (de Dios et al. 2006), including the metabolism of secondary metabolites (Wang et al. 2009).

The concentration and content of anthocyanin compounds in the fruit of haskap both increased as the fruit developed. An increase in anthocyanins is a common occurrence in ripening fruit (Wang and Lin 2000, Awad et al. 2001, Reay and Lancaster 2001, Kennedy et al. 2002, Castrejon et al. 2008) as a red/blue coloration helps to attract animals which consume the fruit and disperse the seeds. Within the fruit of haskap the maximum concentration and content of anthocyanin coincided with the stage of fruit maturity which also had the highest sugar and lowest acidity and thus the greatest fruit flavor (Tables 4.11-4.13). This suggests that anthocyanin content may be a useful visual indicator of maturity in haskap. However, the fruit of haskap appears to develop red/blue coloration in epidermal skin tissues ahead of development of this coloration within the pulp tissues of the fruit (Bors 2009c). While results from this study indicate that anthocyanin concentration increases during development of haskap fruit, reaching a maximum at harvest maturity, which tissues within the fruit are developing colored anthocyanin compounds at which point during development is still unknown. Red/blue coloration of the outside of the fruit may still provide an accurate predictor of harvest maturity in haskap, however the relationship between anthocyanin and fruit quality would have to be investigated based on visual appearance of the fruit, and not necessarily a measure of total concentration or content of anthocyanin. Future work to develop a coloration index for haskap fruit in relation to the optimum stage of fruit maturity for harvest could be valuable.

#### *4.5.3 Genotypic variance in fruit parameters of haskap fruit at harvest maturity*

There were obvious differences amongst the six genotypes of haskap tested for fruit mass, moisture content, soluble solids, titratable acidity and the concentration and content of secondary metabolites at harvest maturity. Genotypic effects are common in studies of secondary

metabolites in other fruit species, and have been previously shown in studies of secondary metabolite concentrations in mature haskap fruit (Thompson and Chaovanalikit 2002, Ochmian et al. 2010, Rop et al. 2011, Rupashinge et al 2012, Wojdylo et al. 2014). In this study a 4 fold range in chlorogenic acid concentration (1.1-4.1 mg/g DW), a 1.5 fold range of total quercetin (1.7-2.9 mg/g DW), and a 3 fold range in total anthocyanin (12-38 mg/g DW) were observed in mature fruit of the six haskap genotypes evaluated. The values reported in this study for chlorogenic acid and quercetin concentrations in fruit of the cultivar Tundra appear to be greater than the upper ranges reported by Wojdylo et al. (2014). The concentration of anthocyanin observed in the fruit of Tundra tested in this study also falls within in the range reported by Kusznierevicz et al. (2012). These results indicate that the cultivar Tundra, which is presently the industry standard due to its yield and flavor, also has a substantial concentration of secondary metabolites in its fruit tissues. The concentrations of secondary metabolites in the fruit of the other five haskap genotypes tested in this study generally fell into the range reported by Wojdylo et al. (2014) and Kusznierevicz et al. (2012).

The flavonol concentrations in haskap fruit reported by Rop et al. (2011) and Rupashinge et al. (2012) are considerably lower than the concentrations observed in this study, however Rop et al. (2011) and Rupashinge et al. (2012) used fresh haskap fruit in their analysis which can make it difficult to achieve full extraction of flavonol compounds.

There were also genotypic differences in the patterns of change in the concentration and content of secondary metabolites during development of the haskap fruit. The quercetin-3-glucoside concentration in genotypes Tundra and Borealis showed a parabolic response curve during fruit development, with the minimum concentration occurring during mid-development, while concentrations in the other four haskap genotypes tested gave a negative linear response

with increasing fruit maturity. Fruit of Tundra and Borealis also began accumulating anthocyanin after fewer accumulated GDD<sub>5</sub> than the other four genotypes. Quercetin-3-glucoside is a precursor to cyanidin-3-glucoside, the major anthocyanin in haskap fruit (Chaovanalikit et al. 2004). It is possible that the different pattern of change in quercetin-3-glucoside may be related to early onset of the development of blue coloration of fruit in these genotypes. The genotypes Tundra and Borealis also contained the highest concentrations of anthocyanin compounds at harvest maturity of the genotypes studied. This result again indicates that the cultivar Tundra is well suited as the industry standard, as haskap is sometimes marketed as “the blue-rrr berry” (Phytocultures Ltd.). In this case the ‘bluer’ is used to imply that the fruit crop has more anthocyanins than other blue colored fruit.

#### *4.5.4 Potential importance of concentrations of secondary metabolites to haskap breeding*

Ideally the objective of increasing secondary metabolite concentrations could be achieved without compromising fruit size, yield or quality. This study provided basic information on the metabolism of secondary metabolites within developing fruit of haskap which until this point has been lacking. This study has confirmed that the concentration of valuable secondary metabolites generally decreases over the course of development of haskap fruit. In some cases (chlorogenic acid and secologanin) this reduction in concentration is large enough to also reduce the total content of secondary metabolites in the developing fruit. For these compounds, maximizing the total content harvested will mean a compromise in fruit size and quality, as fruit would have to be harvested before they reach harvest maturity. However for other compounds (quercetin derivatives and loganin) the increase in fruit size during development is more than sufficient to offset any reduction in concentration of the target compounds. This differential response in the



concentration and content of the various secondary metabolites during fruit development has been shown in other crops (Awad et al. 2001). From a breeding perspective, this result indicates that the common breeding objective of selecting for increased fruit size will likely increase the total content of some compounds (quercetin and the iridoid compound loganin) in fully developed fruit. For other compounds (chlorogenic acid and secologanin), increasing the concentration of these compounds in the fruit, especially late in development will represent the most effective means of increasing their total content at harvest maturity.

In addition to enhancing our understanding the metabolism of secondary metabolites during development, this study also showed that cumulative growing degree days (base 5°C) provides an accurate tool for predicting fruit development in various genotypes of haskap.

#### **4.6 Conclusions**

During development, haskap fruit increase in mass at a linear rate determined by the accumulation of heat units. The average number of GDD<sub>5</sub> required to go from anthesis to harvest maturity was 482 but ranged from 405.4 to 604.3 amongst the six haskap genotypes tested over the two growing seasons. The secondary metabolite profile of fruit of haskap changes considerably during development. The concentrations of most of the valuable secondary metabolites selected for testing decreased in a logarithmic pattern during fruit development, but the total content of these compounds generally increased in a linear manner as the fruit developed. There were some exceptions to these trends, both across the range of secondary metabolites tested and across the range of genotypes evaluated. Harvesting the crop once maximum fruit weight was reached would produce highest yields, with the best flavor profile

(maximum soluble solids and minimum titratable acidity) and with the highest yields of most secondary metabolites.

This study has shown that the cultivar Tundra which is presently the industry standard produced good fruit size, has a good sugar to acid ratios and is early ripening. This cultivar also produces fruit with high concentrations of secondary metabolites. There were significant differences in concentration and content of valuable secondary metabolites amongst the haskap genotypes evaluated in this study. This suggests that gains can be made in secondary metabolite quantities by conventional breeding and that these gains can be achieved using some of the parental material evaluated in this study. Efforts to breed haskap with an enhanced profile of valuable secondary metabolites will be based on identification and crossing of plants with inherent high concentrations of these compounds at harvest maturity. Increasing the content of secondary metabolites however can be accomplished by selecting for increasing concentration of the target compounds and/or larger fruit size.

There also appears to be some differences in the metabolism of both cyanidin-3-glucoside and its precursor quercetin-3-glucoside in Tundra and Borealis versus the other genotypes, however the reason for these differences is not entirely clear. The early development of red/blue coloration by Tundra and Borealis should be of interest to haskap breeders, as these genotypes also produced the greatest concentrations of the highly pigmented anthocyanins.

When breeding for altered concentration and/or content of secondary metabolites, it is important to remember that these compounds all have a role within the plant. An understanding of these roles will help direct breeding strategies that include breeding to alter concentrations and/or content of these secondary metabolites. For example, selection for increased quantities of

secondary metabolites in the fruit may interfere with or alter the physiological processes that normally occur within the developing fruit and could result in altered appearance or flavor.

In the next Chapter the concentration of selected secondary metabolites will be monitored in leaf tissues over the course of the growing season. Combined with results from this Chapter, this should provide better understanding of the overall secondary metabolite profile of the haskap plant. The observation that the concentrations of some secondary metabolites in the fruit of the genotypes Tundra and Borealis appear to respond differently during fruit development when compared to the other haskap genotypes would indicate that there are genotypic differences in the metabolism of these compounds. It may be beneficial to test the responses of representatives of pure subspecies of *Lonicera caerulea* to understand if these differences are contributed by a specific subspecies within the lineage of Tundra and Borealis.

#### **4.7 Transition**

This chapter used the methodology developed in Chapter 3 to monitor changes in the secondary metabolite profile of the fruit of haskap. This information helped to further the overall objective of the project which was to increase our understanding of the secondary metabolite profiles within haskap fruit and leaves. This chapter addressed a key knowledge gap (section 4.1.), specifically to determine changes in the secondary metabolite profile over the course of development of the haskap fruit. In addition, this chapter increased our understanding of the secondary metabolite profiles within haskap fruit by comparison of the concentration and content of these compounds amongst haskap genotypes. This chapter identified harvest maturity to be a representative time point at which to quantify the concentration and content of the secondary metabolites. This information may be helpful in closing other key knowledge gaps in this project. Fruit tissues are the highest value tissues of most fruit crops, therefore quantification of

secondary metabolites within the fruit of haskap seems like a logical starting point to further the goals of the study and provide information that had value to producers.

As outlined in Chapter 3, the leaves of haskap also contain many of the same compounds as the fruit. Monitoring changes in the secondary metabolite profile in these tissues would help to further the overall goal of the project. The next chapter investigated the changes in the concentrations of secondary metabolites in haskap leaves over the course of the season, with the aim of further increasing our knowledge of the secondary metabolite profile of haskap.

**5.0 EFFECT OF STAGE OF DEVELOPMENT ON CONCENTRATIONS OF  
VALUABLE SECONDARY METABOLITES IN LEAVES OF SELECTED HASKAP  
(*Lonicera caerulea* L.) GENOTYPES**

**5.1 Abstract**

Haskap leaves (*Lonicera caerulea* L.) have previously been shown to contain a range of secondary metabolites including chlorogenic acid, several quercetin derivatives and at least two iridoid glucosides. These compounds are valued for their potential to enhance human health. In other plant species the concentrations of these and other valuable secondary metabolites have been shown to be greater in leaf tissues than in the fruit. Thus leaf tissues may represent an additional or alternative source of valuable secondary metabolites in haskap. Currently there is no information on the concentrations of secondary metabolites in haskap leaves, nor is there any information regarding the changes in the concentrations of these compounds that occurs over the course of the growing season. This information would be helpful in determining the optimal time to harvest leaf material to maximize yields of secondary metabolites, while also minimizing damage to the plant. In addition, comparisons of secondary metabolite concentrations between fruit and leaf tissues may provide a possible early screening method to identify haskap seedling with high concentrations of secondary metabolites in fruit tissues, based on analysis of the leaves during the 2 year juvenile period during which time most seedlings do not produce fruit. The current study evaluated the concentrations of six valuable secondary metabolites in leaves of six selected haskap genotypes. Leaf samples taken at intervals over the course of the 2011 and 2012 growing seasons were extracted with 50% methanol and analyzed by HPLC. The concentrations

of most of the selected secondary metabolites in the haskap leaves decreased over the course of the growing season, except that the concentrations of chlorogenic acid in leaves was similar at the beginning and end of the season. By the end of the growing season when the fruit has been harvested and most of the growth and development of the leaves had finished, there were still substantial concentrations of secondary metabolites remaining in the haskap leaf tissues. Thus it may be possible to harvest these leaf tissues with minimal impact on fruit yield or the growth and development of the plant in the subsequent crop year. There were significant differences amongst the selected haskap genotypes for the concentrations of the various secondary metabolites found in the leaves sampled. This means that there is potential for breeding within this germplasm to create new haskap genotypes with increased concentrations of secondary metabolites in the leaves. A weak positive relationship was observed between the concentrations of secondary metabolites in the fruit and leaf tissues of the various genotypes tested. This suggests that by screening the secondary metabolite profiles of seedlings breeders may not need to wait until new lines start to fruit in order to select lines with desirable metabolite profiles.

## **5.2 Introduction**

The leaves of some crops contain high concentrations of valuable secondary metabolites, and in many cases the concentrations of these compounds in the leaves are greater than in the fruit tissues (Harris et al. 2007). As the mass of leaf tissue is substantial, leaves may therefore represent an excellent additional raw material source for extraction of valuable secondary metabolites. There are also times when leaf material can be regarded as waste material, such as pruning waste or late in the season when deciduous plants begin to lose their leaves in preparation for winter. Leaves could be harvested at these times without damaging or stressing

the plant, as at this point in the season the leaves are in the process of natural senescence. It is also possible that the leaves of haskap may be more efficient to harvest or to extract than fruit tissues. For this reason there is increasing interest in quantifying secondary metabolites in leaves, especially in species such as haskap where the fruit are known to contain high amounts of valuable secondary metabolites (Rupasinghe et al. 2012, Wojdylo et al. 2014).

### *5.2.1 Effect of stage of the growth season on secondary metabolites in haskap leaves*

The concentrations of secondary metabolites in leaf tissues vary with the species, stage in the growing season and the compounds monitored. A review of the literature regarding the leaf concentrations of the six secondary metabolites selected for evaluation in this project illustrates some of these differences. In leaves of bilberry the concentrations of chlorogenic acid derivatives in the leaves stayed fairly constant over the course of the growing season, while the concentrations of flavonol glycosides increased over the season (Martz et al. 2010). Witzell et al. (2003) observed a parabolic change in the concentrations of both quercetin and chlorogenic acid over the course of the growing season in bilberry leaves, with the maximum concentrations occurring in early August. In leaves of alder (*Alnus incana* (Moench) and birch (*Betula pubescens* (Ehrh) the concentrations of chlorogenic acid decreased by 66% and 50% respectively over the course of the growing season (Kotilainen et al. 2012). In the same study, concentrations of total flavonoids decreased by 50% and 66%, respectively over the course of the growing season. The total quercetin concentration in apple leaves increased by 50% over the course of the growing season (Petkovsek et al. 2010). The concentrations of nine different quercetin glucosides, as well as total flavonols, decreased in a logarithmic pattern in the leaves of oak over the course of the growing season (Salminen et al. 2004). The concentrations of total

flavonoids in leaves of *Lantana camara* showed a parabolic response to increasing leaf maturity, with the maximum concentrations occurring at 80% expansion of the leaf (Bhakta and Ganjewala 2009). The concentration of quercetin in leaves of *Cyclocarya paliurus* stayed fairly consistent over the course of the growing season (Fang et al. 2011). Flavonoid concentrations increased during development of oat primary leaves (Knogge and Weissenbock 1986).

There is relatively little information on the changes in iridoid concentrations that occur in leaves during the growing season. The concentration of the iridoid compound oleuropein decreased by 60% during maturation of olive leaves (Ranalli et al. 2006).

#### *5.2.2 Breeding haskap for enhanced secondary metabolite profile*

There is currently no information on the concentrations of valuable secondary metabolites in leaves of haskap. Changes in secondary metabolite concentration in leaves over the course of the growing season in leaves of haskap are also unknown. This knowledge would be useful to a haskap breeding program seeking to increase the concentration and content of these secondary metabolites in haskap. The aim of this study was to investigate the concentrations of selected secondary metabolite compounds in the leaves of haskap over the course of a 125 day growing season. An additional objective was to investigate the leaf tissues of haskap as a possible source of valuable secondary metabolites and to determine the optimum the time of leaf harvest to maximize yields while minimizing damage to the plants. In addition this study investigated the relationship between concentrations of secondary metabolites in the leaves relative to the concentrations found in the fruit of haskap at harvest maturity.



## **5.3 Methodology**

### *5.3.1 Description of test material*

Descriptions of the test site and the annual field management practices and germplasm used in this experiment are presented in section 3.3.1.

### *5.3.2 Sampling procedure*

The cultivar Tundra was sampled most intensively as it is presently the standard cultivar in commercial haskap production in Canada. Five additional haskap breeding lines in use by the UofS were selected for testing (Borealis, 3-03, 41-83, 77-87 and 22-14). These lines possess superior agronomic characteristics and/or fruit yield, traits which make these lines potentially valuable parents in a breeding program. Thirty randomly selected stems of Tundra were sampled approximately every 14 days, from initial flowering (day post-flowering, DPF) until mid-fall. Initial flowering of the plant was used as the start of the sampling period as this synchronized sampling of the leaves with sampling of the fruit (previous chapter) thereby facilitating comparisons between the two. Leaves of haskap were fully expanded at the first sampling date (14 DPF). Twenty stems of the remaining five genotypes were sampled monthly from initial flowering to mid-fall. Leaves tested during the final sampling date were beginning to dehydrate in preparation for leaf abscission and the onset of winter dormancy.

### *5.3.3 Leaf processing*

Leaf and stem tissues collected from the haskap plants were held in a drying oven (Precision Scientific) at 50°C until a constant dry weight was achieved (24 h). After drying, the

leaves were removed from the stems and the stem portion was discarded. The dried leaf tissue was then ground using a micro hammer mill (Culatti AG, Zürich, Switzerland), with the resulting powder stored at room temperature until analyzed.

#### *5.3.4 Leaf extraction procedure*

Methanolic extracts from the leaf samples were prepared in the same manner used to extract the haskap fruit (see section 3.3.3.), with the following exception; 0.1g of leaf tissues was used for extraction of each sample.

#### *5.3.5 High pressure liquid chromatography (HPLC) procedure*

The secondary metabolites present in the methanolic extracts obtained from the leaf samples were assessed via HPLC, using the same method used to analyze extracts obtained from haskap fruit (see section 3.3.4.)

#### *5.3.6 Statistics*

Regression analysis was used to explore the relationship between the concentrations of secondary metabolites in the leaves and the stage of the growing season (measured as days post-flowering) using the methodology outlined in section 4.3.10.

## 5.4 Results

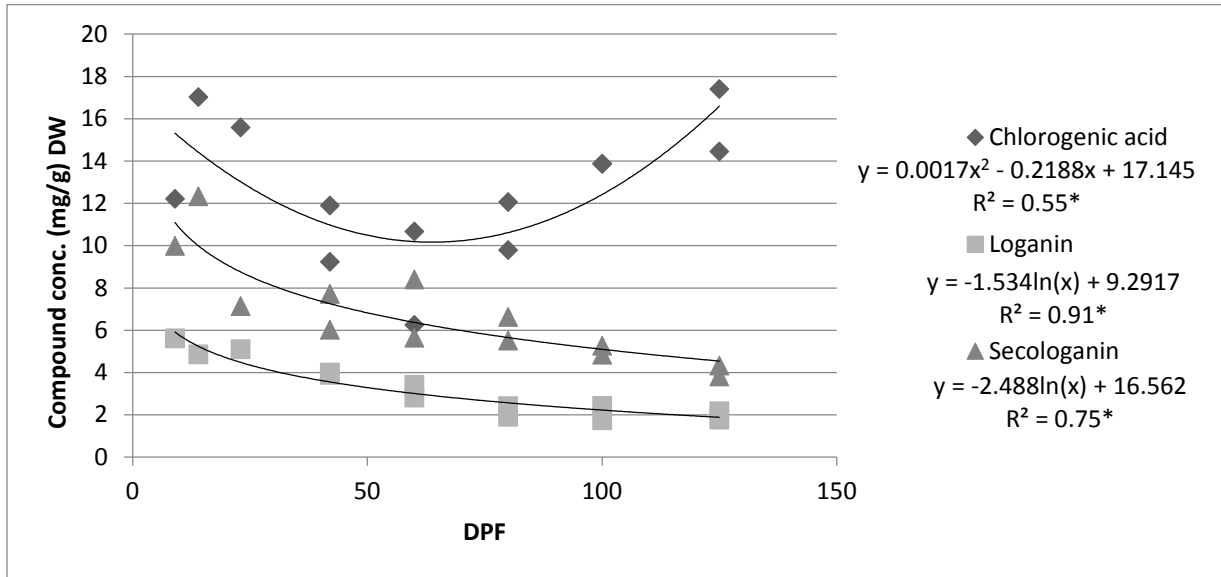
### *5.4.1 Changes in concentrations of phenylpropanoids and monoterpenoids in haskap leaves over the course of the growing season*

In the leaves of the genotype Tundra the concentration of chlorogenic acid showed a parabolic relationship with increasing days post-fertilization (DPF), with the lowest concentration occurring around mid-season (Fig 5.3). This pattern held for the chlorogenic acid concentration in leaves of all the other haskap genotypes tested, except for 22-14 and 77-87, where there was no significant relationship between DPF and the concentration of chlorogenic acid (Appendix Table A.12.). Averaged across the genotypes tested, the concentration of chlorogenic acid at the end of the growing season was 92.7% of that found in the leaves early in the season.

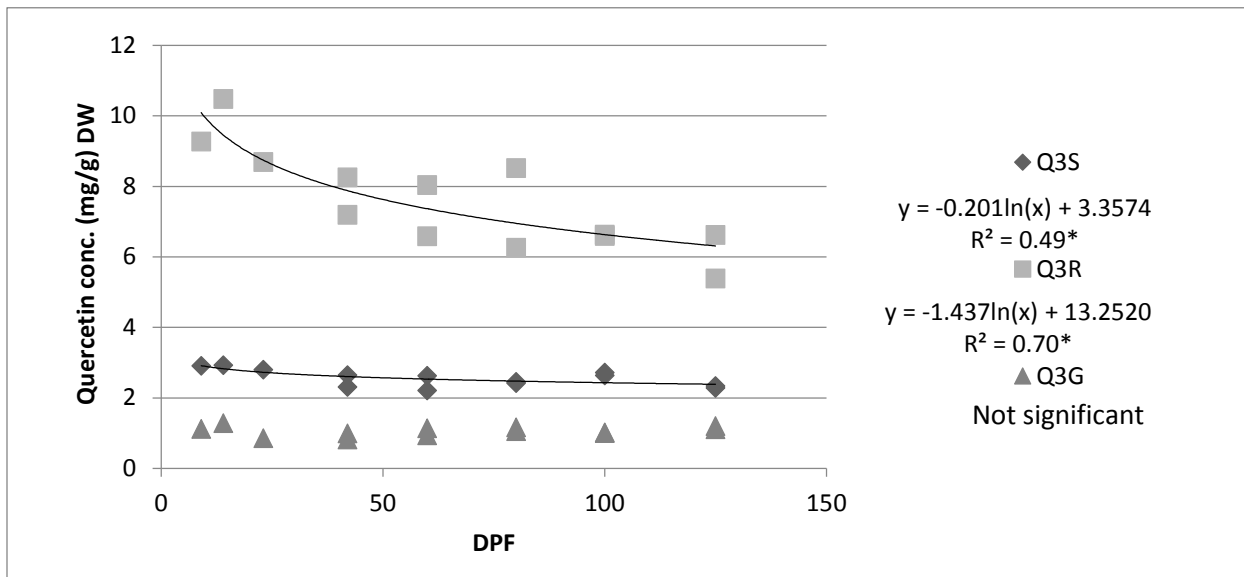
The concentrations of both iridoid derivatives (loganin and secologanin) in the leaves of haskap genotype Tundra decreased in a logarithmic pattern over the course of the growing season (Fig 5.3). This pattern was also observed for the other haskap genotypes tested (Appendix Table A.16.-A.17.). Averaged across the haskap genotypes tested, the concentration of total iridoid compounds in the leaves at the end of the growing season was 32.3% of that found in the leaves early in the season.

The total concentration of quercetin decreased in the leaves of the haskap genotype Tundra over the course of the season; this was also true for the individual quercetin compounds (Fig 5.4). This pattern held true for the other haskap genotypes tested (Appendix Table A.13.-A.15.) with a few exceptions; there was no significant relationship between the concentration of quercetin-3-sambubioside in the leaf tissues and DPF for genotype 77-87. There was also no significant relationship between the concentration of quercetin-3-glucoside in the leaves and

DPF for genotypes Tundra and 22-14. Averaged across the haskap genotypes tested the concentration of total quercetin in the leaves at the end of the growing season was 77.7% of that found in the leaves early in the season.



**Figure 5.1.** Chlorogenic acid, loganin and secologanin concentrations in the leaves of haskap (*Lonicera caerulea* cv. 'Tundra') based on days post-fertilization of the flower (DPF). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$ .



**Figure 5.2.** Quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G) concentrations in leaves of haskap (*Lonicera caerulea* cv. 'Tundra') based on days post-fertilization of the flower (DPF). Data were pooled from the 2011 and 2012 growing seasons. \* indicates significance at  $\alpha=0.05$ .

*5.4.2 Seasonal variance in the concentration of secondary metabolites in haskap leaves tested at the end of the growing season.*

Growing season was a significant factor in the concentrations of chlorogenic acid, quercetin-3-sambubioside, quercetin-3-glucoside, loganin and secologanin during the two seasons tested. On average, the leaves contained higher concentrations of all secondary metabolites in 2012 except chlorogenic acid where higher average concentrations were found in leaves produced in 2011 (Data not shown).

*5.4.3 Genotypic variance in the concentration of secondary metabolites in haskap leaves tested at the end of the growing season.*

Of the valuable secondary metabolites quantified in haskap leaf tissues, the most abundant at the end of the season (125 DPF) were chlorogenic acid and secologanin (Table 5.7). There were significant differences amongst the haskap genotypes tested for concentrations of secondary metabolites in the leaves at the end of the season (Table 5.7). The greatest concentration of chlorogenic acid in leaf tissues at the end of the season occurred in genotype Tundra, the greatest concentration of quercetin derivatives occurred in leaves of genotype 77-87 and the greatest concentration of iridoid compounds occurred in leaves of genotype 3-03.

There were differences amongst the haskap genotypes tested as to which quercetin glucoside was most prevalent within the leaf tissues at the end of the growing season. In the genotype Tundra, quercetin-3-rutinoside was the most prevalent quercetin derivative; its concentration was about 4 fold greater than the quercetin-3-sambubioside. However, in the genotypes Borealis, 41-83 and 22-14, quercetin-3-sambubioside was the most prevalent

quercetin derivative. Genotypes 77-87 and 3-03 had approximately equal concentrations of both quercetin-3-rutinoside and quercetin-3-sambubioside in their leaf tissues at the end of the growing season.

**Table 5.1.** Means, standard errors and mean comparisons for the concentration (mg/g DW) of secondary metabolites at the end of the season (125 DPF) in leaves of six selected haskap genotypes tested averaged over the 2011 and 2012 growing seasons. Mean values followed by the same letters in each column are not significantly different at  $\alpha=0.05$  using Tukey test.

Genotype	CA	Q3S	Q3R	Q3G	Loganin	Secologanin
Tundra	29.27±5.74 a	2.98±0.58 d	12.18±2.13 a	4.39±0.89 c	5.90±1.03 bc	9.02±1.64 ab
Borealis	19.42±3.04 b	5.22±2.09 c	2.93±0.18 b	7.31±0.95 ab	7.72±2.27 ab	6.45±1.12 bc
3-03	19.33±3.20 b	4.89±1.18 c	9.62±1.29 a	7.39±0.79 a	11.73±1.63 a	11.53±1.62 a
41-83	19.03±3.07 b	6.68±1.62 b	2.54±0.35 b	6.02±0.39 b	3.69±0.31 c	6.29±1.24 bc
77-87	20.26±3.41 ab	7.99±2.68 a	10.68±1.17 a	7.13±0.60 a	3.67±0.33 c	4.26±3.97 c
22-14	15.91±1.74 b	8.28±2.73 a	2.61±0.58 b	4.80±0.28 c	2.97±0.59 c	5.12±1.18 c

Abbreviations: chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

#### 5.4.4 Comparisons of concentrations of secondary metabolites between haskap leaf and fruit tissues

The relationship between secondary metabolite concentrations in leaf tissues at different points throughout the season and the concentrations of these same compounds in haskap fruit tested at harvest maturity is presented in Table 5.2. This data were pooled from the six genotypes tested. The analysis suggests that there is a significant positive linear relationship between the concentration of chlorogenic acid in haskap fruit at harvest maturity and the concentrations of chlorogenic acid present in the leaf tissues at 42 and 80 DPF. There was also a positive linear relationship between the total quercetin concentrations in the fruit at harvest maturity and the concentration of total quercetin in the leaves at 42 DPF. Iridoid concentrations

in haskap fruit at harvest maturity and the total iridoid concentrations in the leaves were also positively correlated when the leaves were sampled at 42 and 125 DPF.

**Table 5.2.** Pearson’s correlation coefficients for the relationship between secondary metabolite concentrations in haskap leaf tissues at different days post-fertilization (DPF) in the growing season and the concentrations in the fruit at harvest maturity. Data were averaged over the 2011 and 2012 growing seasons and pooled from the six haskap genotypes tested.

<b>Leaf sampling date</b>	<b>Description of stage</b>	<b>Chlorogenic acid</b>	<b>Total quercetin</b>	<b>Total iridoid</b>
14 DPF	Rapid shoot growth	0.26	0.28	-0.10
42 DPF	Shoot growth slows, fruit at harvest maturity	0.54*	0.38*	0.58*
80 DPF	Shoot growth stopped, terminal buds forming, fruit harvested	0.37*	0.09	0.18
125 DPF	Leaves dehydrating in preparation for abscission	0.32	0.22	0.36*

\* indicates significance at  $\alpha=0.05$ . Total quercetin includes quercetin-3-glucoside, quercetin-3-rutinoside and quercetin-3-sambubioside. Total iridoid includes loganin and secologanin

## 5.5 Discussion

### 5.5.1 Secondary metabolite profile of haskap leaves

HPLC chromatographs of methanolic extracts of haskap leaves revealed the secondary metabolite profile of the leaves to be similar but more complex than the profile observed in haskap fruit (Fig 3.2). Leaf tissues contained significant quantities of all six of the valuable secondary metabolites selected for study as outlined in Chapter 3. Machida et al. (1995) had already reported the presence of many of these secondary metabolite compounds in leaves of *Lonicera caerulea* subsp. *emphyllocalyx*, including chlorogenic acid, several quercetin glucosides and several iridoid glucosides.

Leaves of haskap contained approximately 10x greater concentrations of most of the selected secondary metabolites when compared to the fruit tissues. Harris et al. (2007) observed

a similar relationship between the relative concentrations of chlorogenic acid and several quercetin derivatives in fruit and leaf tissues of lowbush blueberry. This result indicates that leaves of haskap could represent an additional rich source of valuable secondary metabolites. Machida et al. (2005) used nuclear magnetic resonance to detect the presence of two bis-iridoid compounds in the leaves of haskap, it is possible these compounds were also present in the leaf tissues analyzed in this study however no standards are currently available for these compounds and thus they could not be properly identified.

#### *5.5.2 Changes in concentration of secondary metabolites in haskap leaves over the growing season*

The occurrence and concentration of secondary metabolites within plants are mediated by environment and developmental changes and can also be influenced by interactions with other organisms (Witzell et al. 2003). The concentrations of the six selected secondary metabolites measured over the course of the growing season in leaves of six genotypes of haskap generally decreased over the course of the season, but there were some notable exceptions. The concentrations of chlorogenic acid in the leaf tissues showed a parabolic relationship with increasing DPF in this study, which is similar to the pattern change of chlorogenic acid concentration observed over the course of the growing season in leaves of *Coffea pseudozanguebariae* (Bertrand et al. 2003). By contrast, the concentration of chlorogenic acid in apple leaves increased steadily over the course of the growing season (Petkovsek et al. 2010), while the concentration of chlorogenic acid in the leaves of bilberry showed an inverse parabolic response with the progression of the season (Witzell et al. 2003). Clearly the pattern of change in chlorogenic acid concentration over the course of the growing season varies among different



fruit crops. These differences in developmental pattern may reflect differences in environmental conditions, as well as differences in genetics. Chlorogenic acid is a hydroxycinnamic acid that occurs across the plant kingdom. The compound is known to function in pathogen resistance (del Moral 1972) and is a substrate for polyphenol oxidase (PPO). This enzyme is often localized in the cell walls and can form a polymer which serves as a barrier to the invasion of the plant tissues by pathogens (Kader et al. 1997). Bertrand et al. (2003) suggested that chlorogenic acid may act as a precursor for monolignols which go on to form lignin polymers. This idea was also proposed by Escamilla-Trevino et al. (2013). Witzell et al. (2003) suggested that the changes in chlorogenic acid concentration observed in bilberry leaves over the course of the growing season were due to interactions between the plant and its environment, specifically due to interactions with herbivores. The importance of chlorogenic acid in the resistance of chrysanthemum to attack by thrips was shown by Leiss et al. (2013). Chlorogenic acid also sits on the metabolic pathway to isochlorogenic acid and other dicaffeoylquinic acids, some of which are known to have antifungal properties (Bazzalo et al. 1985, Stange et al. 2001).

It is possible that the decrease in the concentration of chlorogenic acid observed in haskap leaves at mid-season may be due to polymerization of this compound by PPO or by further metabolism into dicaffeoylquinic acids. However, it is more likely that the reduction in chlorogenic acid concentration observed at mid-season was due to its use in lignin production. The vegetative growth of haskap plants is generally finished by mid-season (Bors 2009c). At this point there would be no further need for further deposition of lignin in the leaf tissues and any further consumption of chlorogenic acid for this purpose would cease.

The concentrations of all three quercetin derivatives decreased logarithmically in the leaves of haskap over the course of the growing season. The concentration of both rutin and total

flavonoids also decreased over the course of the season in rabbiteye blueberry (*Vaccinium ashi*) leaves (Zhu et al. 2013) and the concentration of both total and individual quercetin compounds decreased over the course of the growing season in leaves of oak (*Quercus rubra*) (Salminen et al. 2004). In contrast, the concentration of quercetin derivatives in leaves of apple increased over the course of the season (Petkovsek et al. 2010). In leaves of bilberry the concentration of quercetin showed an inverse parabolic response as growing season progressed (Witzell et al. 2003). This again shows there is variability between plants species in the response of secondary metabolite concentrations in the leaves over the growing season.

The decline in quercetin concentration observed in haskap leaves over the course of the season is most likely attributable to the age of the tissues combined with the physiological role of these compounds in plants. The best known function of the flavonoids is the absorption of damaging UV light, thereby protecting the sensitive photosynthetic machinery of the leaf (Takahashi and Badger 2011). As the leaf ages, the photosynthetic apparatus becomes less efficient and thus there are fewer resources allocated to protect these aging tissues from potential UV damage. Also as leaves become shaded by new growth, less UV light reaches them and therefore less UV protection is required.

The concentration of iridoid compounds in the haskap leaves also decreased logarithmically over the course of the season. A similar decrease in iridoid concentration through leaf maturity was observed in *Plantago lanceolata* (Bowers et al. 1992) and in leaf tissues of olive (*Olea europaea*) (Ranalli et al. 2006). The main physiological function of iridoid glucosides in plants is believed to be as a means of protection against feeding by herbivores (Darrow and Bowers 1999). The compounds appear to act as deterrents to generalist herbivorous insects, as the iridoids polymerize proteins when in the presence of glucosidase enzymes (Kim et

al. 2000). As leaf tissues become older, less efficient, and less valuable to the plant, there is a corresponding reduction in production of these metabolically costly compounds (Bowers et al. 1992).

### *5.5.3 Genotypic variance in concentrations of secondary metabolites in leaves of haskap*

While the results of this study show that the concentrations of most secondary metabolites in haskap leaves are lowest at the end of the season, this point in the season may still represent the most appropriate time to harvest leaf material. The end point of the growing season is when plants naturally begin to prepare for winter dormancy; in haskap the plant defoliates as part of this process. By this point in the growing season the leaves of haskap have fulfilled their photosynthetic role and leaves would now be considered a waste product. If this point in the season represents the most appropriate time to harvest leaf tissues, this stage is therefore the most appropriate point in time to evaluate differences in secondary metabolite concentrations amongst the genotypes tested. There were significant differences amongst the haskap genotypes tested in the concentrations of secondary metabolites found in the leaves at the end of the growing season. The industry standard genotype Tundra contained the greatest concentration of chlorogenic acid in its leaves, while the greatest concentration of quercetin and iridoid compounds occurred in leaves of genotypes 77-87 and 3-03 respectively. This result indicates that while Tundra was well suited as an industrial standard in terms of the concentration and content of secondary metabolites in its fruit (see Chapter 4), other genotypes may be better suited for extraction of certain secondary metabolites from the leaf tissues. Genotypic differences in the concentrations of secondary metabolites in leaves are not uncommon. Islam et al. (2003) found a 12 fold range in the concentrations of chlorogenic acid in leaves of 1389 accessions of sweet potato. A 2 fold

range (15.9-29.2 mg/g DW) was found over the six haskap genotypes tested in this study. Liu et al. (2014) also observed variations in the leaf concentrations of several flavonol compounds amongst three genotypes of black current.

There were differences in the major quercetin derivatives found in the leaves of the haskap genotypes tested. The genotypes Tundra, 3-03 and 77-87 carried quercetin-3-rutinoside as the major quercetin derivative in its leaf tissues, while the genotypes 41-83 and 22-14 carried quercetin-sambubioside as the major derivative. The genotype Borealis carried the majority of its quercetin pool in the leaves as quercetin-3-glucoside. These differences in the major quercetin derivative might be explained by differences in the ancestry of the genotypes tested. However, the genotypes Tundra and Borealis are full siblings and thus developed from the same parental material, yet they differ as to which major quercetin derivative dominates the quercetin pool in the leaf tissues. In order to better understand these results, it may be beneficial to explore the quercetin profile of the parental material used for this cross.

#### *5.5.4 Importance of the results to the UofS Haskap Breeding Program*

An understanding of the production of secondary metabolism compounds in leaf tissues of haskap could aid in understanding the potential role of these compounds on a whole plant basis and could also possibly aid in developing strategies to breed for higher concentrations or content of these valuable secondary metabolites. In addition, information on the profiles of secondary metabolites in the leaves may also aid in early selection of haskap genotypes rich in secondary metabolites. Haskap plants require 2-3 years before they produce sufficient fruit to allow for quality analysis. The ability to screen juvenile plants for secondary metabolite

concentration based on the secondary metabolite profiles of their leaf tissues could save time and expense for the breeding program.

From an industry standpoint the information presented in this Chapter indicates that leaf material from haskap is a more concentrated source of valuable secondary metabolites than the fruit tissues. In the industry standard cultivar Tundra, the average concentrations of chlorogenic acid, total quercetin and total iridoid in leaves harvested at the end of the season were respectively 7, 6.5, 15 times greater than in fruit taken at harvest maturity. This indicates that; a) haskap leaves could serve as an excellent alternative source for extraction of valuable secondary metabolites and b) it may also be possible for products developed from haskap fruit to be fortified with valuable secondary metabolites extracted from leaf tissues.

#### *5.5.5 Comparisons of concentrations of secondary metabolites between leaves and fruit*

Comparisons between the secondary metabolite concentrations in leaf and fruit tissues indicated that there are several points through the growing season when concentrations in the leaf tissues may provide an indicator of the concentration of secondary metabolites which will occur in the fruit at harvest maturity. At 42 DPF there was a significant linear relationship between concentrations in leaf tissues and fruit at harvest maturity for all the secondary metabolites quantified. This suggests that 42 DPF may be the most appropriate time to screen the leaves of haskap as a predictor of the secondary metabolite profile that could be expected in the fruit. This point in the growing season also coincides with fruit harvest in haskap which may make sampling more convenient. However, none of the correlations observed in this study provided a goodness of fit statistic ( $R^2$ ) greater than 50% and thus the concentrations of secondary metabolites in leaf tissues were at best a weak predictor of secondary metabolite concentrations

in the fruit. As this experiment served as a preliminary run, and used only six genotypes a follow up experiment with more genotypes would be useful to further assess the potential to predict the concentrations of secondary metabolites in the fruit of haskap based on the concentrations in leaves.

## 5.6 Conclusions

The secondary metabolite profile of the leaves of haskap are similar to the profile observed in the fruit, however the leaves contain greater concentrations of most secondary metabolites monitored in this study when compared to the fruit. The concentrations of most of the valuable secondary metabolites selected for testing in haskap leaves decreased in a logarithmic pattern over the course of the season. However there were still relatively high concentrations of secondary metabolites present in the leaf tissues at the end of the season. At the end of season these leaves could be harvested for extraction of the secondary metabolites without damaging the plant. We observed sufficient differences amongst the genotypes evaluated in this study to suggest that gains in secondary metabolite quantities in the leaves could be achieved by conventional breeding. There also appears to be some differences in the major form of quercetin metabolized in leaves amongst the haskap genotypes tested. It may be beneficial to test the leaves of representatives of pure subspecies of *Lonicera caerulea* to understand if these differences in quercetin derivative are contributed by specific subspecies. The results of this study also indicated that there may be some limited potential to use leaf concentrations of secondary metabolites as a predictor of fruit secondary metabolite concentrations.

## **5.7 Transition**

This Chapter monitored changes in the secondary metabolite profile of leaves of haskap using the methodology developed in Chapter 3. The chapter addressed a key knowledge gap (section 4.1.); specifically to determine the changes in the secondary metabolite profile that occur over the course of the season in haskap leaves. In addition, the chapter increased our understanding of the secondary metabolite profiles within haskap leaves by comparison of the concentration of these compounds amongst haskap genotypes. This chapter also investigated the relationship between the concentrations of secondary metabolites found in haskap fruit at harvest maturity (Chapter 4) versus the concentrations found in leaf tissues at different days post-fertilization in the growing season. This information was used in the subsequent chapter to assign sampling periods for one time analysis of the profiles of secondary metabolites in fruit and leaves of different haskap subspecies and genotypes collected over diverse geographic sites of origin.

## **6.0 INFLUENCE OF GEOGRAPHIC SITE OF ORIGIN ON CONCENTRATIONS OF VALUABLE SECONDARY METABOLITES IN FRUIT AND LEAVES OF WILD HASKAP (*Lonicera caerulea* subsp. *villosa* L.)**

### **6.1 Abstract**

The fruit and leaves of haskap (*Lonicera caerulea* L.) have previously been shown to contain a range of secondary metabolites including chlorogenic acid, several quercetin derivatives and at least two iridoid glucosides, all of which are valuable due to their potential ability to enhance human health. The UofS Haskap Breeding Program has one of the world's largest collections of haskap genotypes, including representatives from the *Lonicera caerulea* subspecies *pallasii*, *altacia* and *venulosa*, as well as *Lonicera stenantha*. In addition, the collection contains about 1400 specimens of the North American *Lonicera caerulea* subsp. *villosa*, collected from different locations across Canada. Specimens of all of these haskap genotypes have been replanted in a common orchard at the UofS collection. The current study evaluated the concentrations of six selected secondary metabolites in fruit and leaves of 71 haskap genotypes from the UofS collection. The objective was to provide information on the variability in the concentrations of secondary metabolites that exists within the different *Lonicera caerulea* subspecies in the collection and to determine whether there are relationships between sites of origin and the presence and/or concentrations of secondary metabolites in the fruit and leaves of haskap. The relationship between the concentrations of secondary metabolites in the fruit and leaves of haskap was again evaluated to assess if it is possible to predict the concentrations of secondary metabolites in the fruit based on concentrations in leaf tissues. Fruit and leaf samples were taken from the 71 genotypes at fruit harvest maturity during the 2012 and 2013 growing



seasons. Dried fruit and leaves were extracted with 50% methanol and analyzed by HPLC. The fruit and leaves of representatives of *Lonicera caerulea* subsp. *villosa* showed a different quercetin profile than the other *Lonicera caerulea* genotypes surveyed, however the iridoid profile appeared similar across the genotypes tested. The concentrations of all secondary metabolites in the fruit were negatively correlated with average fruit weight. The concentrations of several of the selected secondary metabolites in fruit and leaves varied with the latitude and longitude of the site of origin. This information may be useful in planning future plant collection expeditions. There was a positive correlation between the concentrations of the selected secondary metabolites in fruit tissues when compared with concentrations in leaf tissues of representatives of *Lonicera caerulea* subsp. *villosa* but the strength of the correlation was likely too weak to be of use as an early selection tool.

## 6.2 Introduction

Haskap (*Lonicera caerulea*) is a fruit-bearing member of the honeysuckle family. The plant inhabits a wide geographic distribution and has several naturally occurring variants (Lamoureux et al. 2011). Opinions differ as to whether these variants represent varieties, subspecies or true species (Plekhanova 1989, Plekhanova 1992, Renata 2001, Lamoureux et al. 2011). This thesis has used the naming system proposed by Lamoureux et al. (2011) which refers to the variants as subspecies. Currently, the UofS has the largest germplasm collection of *Lonicera caerulea* in the world. The collection contains specimens of the *Lonicera caerulea* subspecies *edulis*, *kamtschatica*, *emphylocalyx*, *pallasii*, *altacia* and *venulosa*. The collection also contains Russian genotypes and seedlings which were bred from multiple subspecies. The UofS also holds the world's most comprehensive collection of the North American subspecies

*Lonicera caerulea* subsp. *villosa*. The overall objective of this project was to assess the secondary metabolite profile of *Lonicera caerulea* to determine the presence and concentration of secondary metabolite with the potential to enhance human health. There are currently no reports on the secondary metabolite profile of *Lonicera caerulea* subsp. *villosa* (save for Chapter 3 of this thesis) or reports on the concentrations of secondary metabolites to be found in fruit or leaves of diverse *Lonicera* germplasm.

### 6.2.1 *The effect of geographic site of origin on concentrations of secondary metabolites*

The concentrations of secondary metabolites in fruit are known to differ when plants adapted to different locations are grown in a common garden. Uleberg et al. (2012) found that when bilberry plants were grown at a single test site, plants collected from southern parts of Norway contained greater concentrations of chlorogenic acid and two other hydroxycinnamic acid derivatives when compared to fruit from plants collected at northern sites. Oleszek et al. (2002) indicated that white pine collected from across Europe and planted in a common garden differed in their concentrations of flavonoids in the needles, with needles from plants collected in northern latitudes containing less flavonoids than the needles coming from trees that originated from more southern locations in Finland. A variety of factors are believed to influence the metabolism of flavonoids in plants, including photoperiod, light quality, UV-light exposure and temperature (Jaakola and Hohtola 2010). All of these factors should however be consistent when plants are grown in a common garden.

Zheng et al. (2012) stated that the effects of latitude on the profile of secondary metabolites in plants can be explained by the short-term effect of environmental factors as well as long term, heritable adaptations of plants to the environment. While site of origin refers only to

a geographic location and thus can only give a general sense of the possible environmental conditions or the plant's long term adaptations to those environmental conditions, it can give an indication as to potential reason for the observed differences amongst plants collected from different locations. At the very least, understanding the relationship between geographic location of origin and the concentrations of secondary metabolites can help guide future plant collection expeditions looking for genotypes which possess traits of potential value in a breeding program.

### 6.2.2 Introducing *Lonicera caerulea* subsp. *villosa* into haskap breeding programs

The UofS Haskap Breeding Program has one of the largest collections of haskap germplasm in the world, including more than 1400 wild specimens of *Lonicera caerulea* subsp. *villosa* which is the North American subspecies. One possible goal of the breeding program may be to incorporate this germplasm into new haskap genotypes, with the idea that *Lonicera caerulea* subsp. *villosa* may possess traits that could enhance adaptation to the Canadian climate. Incorporating this germplasm into the breeding program may produce new genotypes with more rapid growth and higher or more consistent fruit yields due to superior adaptation to the Canadian environment. An additional goal of the breeding program is to increase the concentration and content of valuable secondary metabolites in new haskap genotypes. There is currently no information on the secondary metabolites profile of *Lonicera caerulea* subsp. *villosa*, save the data presented in Chapter 3 of this thesis. A survey of the secondary metabolite quantities within the *Lonicera caerulea* subsp. *villosa* germplasm could facilitate more efficient and effective incorporation of this germplasm into the breeding program. Understanding the effects of the site of origin on secondary metabolite concentrations may also aid in developing collection strategies when searching for wild genotypes with secondary metabolite profiles of

potential value to the breeding program. To build on the data presented in Chapters 4 and 5, comparisons of secondary metabolite concentrations between fruit and leaves will also be addressed. This relationship may be useful as an evaluation tool for early screening of *Lonicera caerulea* genotypes with high concentrations of secondary metabolites.

### 6.2.3 Hypotheses

a) The concentration of secondary metabolites in fruit and leaves of wild haskap, grown in a common orchard, will be effected by the latitude and longitude of the site of origin of the test material. b) The concentration of all secondary metabolites measured in fruit tissues will be positively correlated with the concentrations of the same secondary metabolites in leaf tissues.

## 6.3 Methodology

### 6.3.1 Description of test material

Approximately 1400 *Lonicera caerulea* subsp. *villosa* plants were collected between 2008 and 2013 across the Canadian distribution of *Lonicera caerulea* subsp. *villosa*, which ranges from Alberta to Newfoundland (Bors 2009c). The northern and southern limits of *Lonicera caerulea* subsp. *villosa* are not currently known. The collected plants (collected as whole plants or cuttings) were relocated to the UofS Horticulture Field Research Station in Saskatoon, Saskatchewan Canada (52.13 °N, 106.67°W). Conditions at the site and crop management practices within the orchard housing the collection were presented in section 4.3.1. Seventy-one unique genotypes from this collection were selected for study in 2012 and again 2013. Each plant selection was represented by a single plant. The key selection criterion for inclusion in this study was that the plants had to be producing fruit. This criterion eliminated

genotypes that were collected in 2012 and 2013 as they were too young/small to produce fruit. This criterion also eliminated plants with very limited fruit production potential. The sites of origin of the selected lines are presented in Fig 6.1. Harvest maturity of the fruit was selected as the stage to sample the plant material for several reasons; a) This is the most important stage of development to evaluate secondary metabolite concentrations, as this is when the fruit are typically harvested (Chapter 5), b) Only plants which have high concentrations of secondary metabolites at harvest maturity are likely to be selected as parents in a breeding program, c) Most plants only produced enough fruit to allow for a single sampling. Leaves were sampled at the same time as the fruit. This allowed assessment of the relationship between concentrations of secondary metabolites in fruit and concentrations of secondary metabolites in the leaves. A preliminary experiment indicated that the highest correlation between concentrations of secondary metabolites in the fruit and the leaves occurred when samples were taken once the fruit had reached harvest maturity (Chapter 5).

In 2013 a survey of fruit and leaf tissues of other subspecies of *Lonicera caerulea* was also conducted. Samples of *Lonicera caerulea* subsp. *pallasii*, *altacia* and *venulosa* as well as *Lonicera stenantha* obtained from the collection maintained at UofS Horticulture Field Research Station were processed and analyzed as previously described. Information on the sources of these materials is presented in Table 6.1.



**Figure 6.1.** Site of origin of haskap (*Lonicera caerulea* subsp. *villosa*) plants tested.

**Table 6.1.** Region of collection and original collector of the haskap subspecies tested in this study.

<i>Lonicera caerulea</i> subspecies	Region of seed or plant collection	Original collection
<i>pallasii</i>	White Sea	Vavilov Institute of Plant Industry
<i>altacia</i>	Altay	Vavilov Institute of Plant Industry
<i>venulosa</i>	Primorye	Vavilov Institute of Plant Industry
<i>villosa</i>	Canada	University of Saskatchewan
<i>Lonicera stenantha</i>	Middle Asia	Vavilov Institute of Plant Industry

### 6.3.2 Sampling procedure

Fruit were sampled at harvest maturity, as outlined in section 4.3.2. Leaves were sampled at the same time, as outlined in section 5.3.2.

### 6.3.3 Plant tissue processing

Fruit and leaves were processed in the manner described in section 3.3.2.

#### 6.3.4 Extraction procedure

Methanolic extracts from the fruit and leaf samples were prepared in the manner described in section 3.3.3., with the following exception; 0.25g of fruit and 0.1g of leaf tissue were used in extraction of each sample.

#### 6.3.5 High pressure liquid chromatography (HPLC) procedure

The secondary metabolites present in the methanolic extracts obtained from the fruit and leaf samples were assessed via HPLC, using the method described in section 3.3.4.

#### 6.3.6 Statistics

ANOVA was used to test the effects of genotype and growing season on the quality parameters of fruit and leaves. Regression analysis was used to explore the relationships between the longitude and latitude of the site of origin and fruit weight, and the concentrations of secondary metabolites in fruit and leaf tissues of *Lonicera caerulea* subsp. *villosa*. Data sets from 2012 and 2013 were combined to create the regression models. Only linear models were considered (model  $Y=X$ ) SAS (Proc Reg), as application of logarithmic or second order polynomial models did not improve the overall goodness of fit statistic and/or did not provide a better fit for the residuals.

## 6.4 Results

### 6.4.1 The effects of genotype and growing season on the concentrations of secondary metabolites in fruit and leaves of haskap.

In general both the genotype and the growing season were significant factors in determining the concentrations of secondary metabolites in fruit and leaves of the various *Lonicera caerulea* subspecies tested. There were a few exceptions; the concentrations of chlorogenic acid and quercetin-3-glucoside were not significantly affected by growing season in the fruit of the haskap genotypes tested. The concentrations of quercetin-3-rutinoside in leaf tissues were also not significantly affected by growing season in the genotypes tested (data not shown).

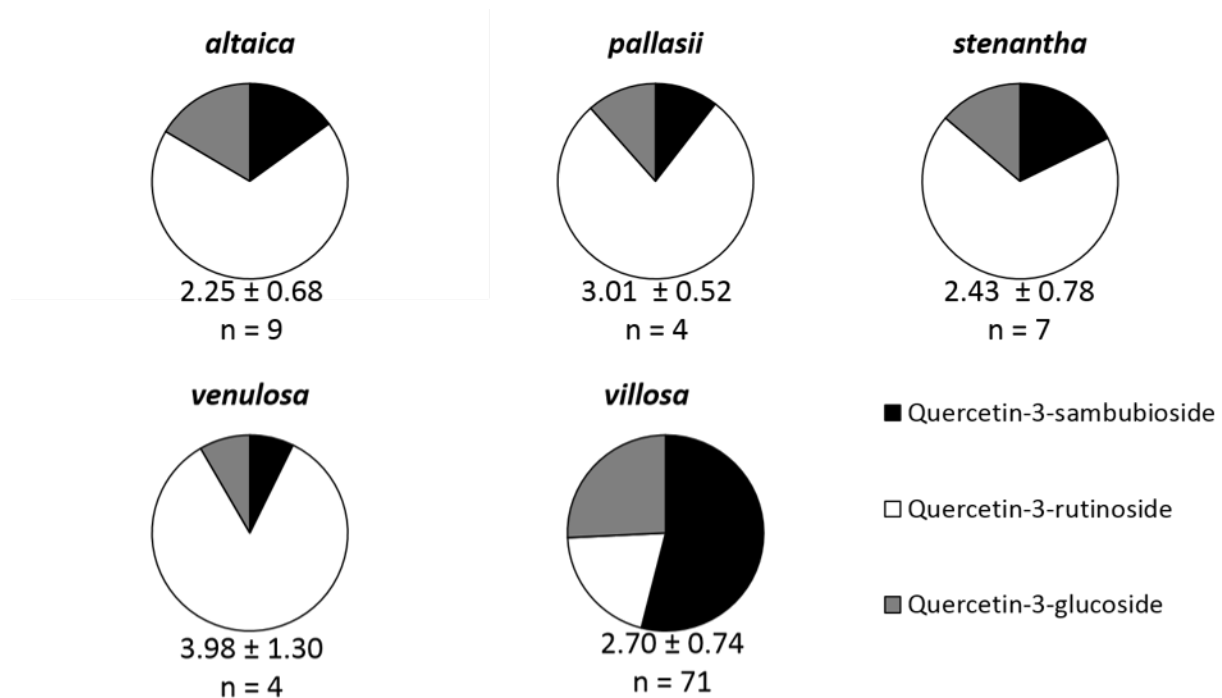
### 6.4.2 Quercetin profiles of subspecies of *Lonicera caerulea*

Total quercetin concentrations in fruit from various *Lonicera caerulea* subspecies ranged from a low of 1.94 mg/g DW for *Lonicera caerulea* subsp. *altacia* to as high as 3.93 mg/g DW in *Lonicera caerulea* subsp. *venulosa* (Fig 6.2). In leaf tissues, total quercetin concentrations ranged from 17.2 mg/g DW in *Lonicera caerulea* subsp. *altacia* to 27.6 mg/g in *Lonicera caerulea* subsp. *villosa*. Based on the samples taken in 2013, the quercetin profile of *Lonicera caerulea* subsp. *villosa* differed from that of the other subspecies. *Lonicera caerulea* subsp. *villosa* had quercetin-3-sambubioside as the major quercetin derivative, while the other subspecies had quercetin-3-rutinoside as the major quercetin derivative. This was true in both the fruit and leaf tissues (Figs 6.2 and 6.3).

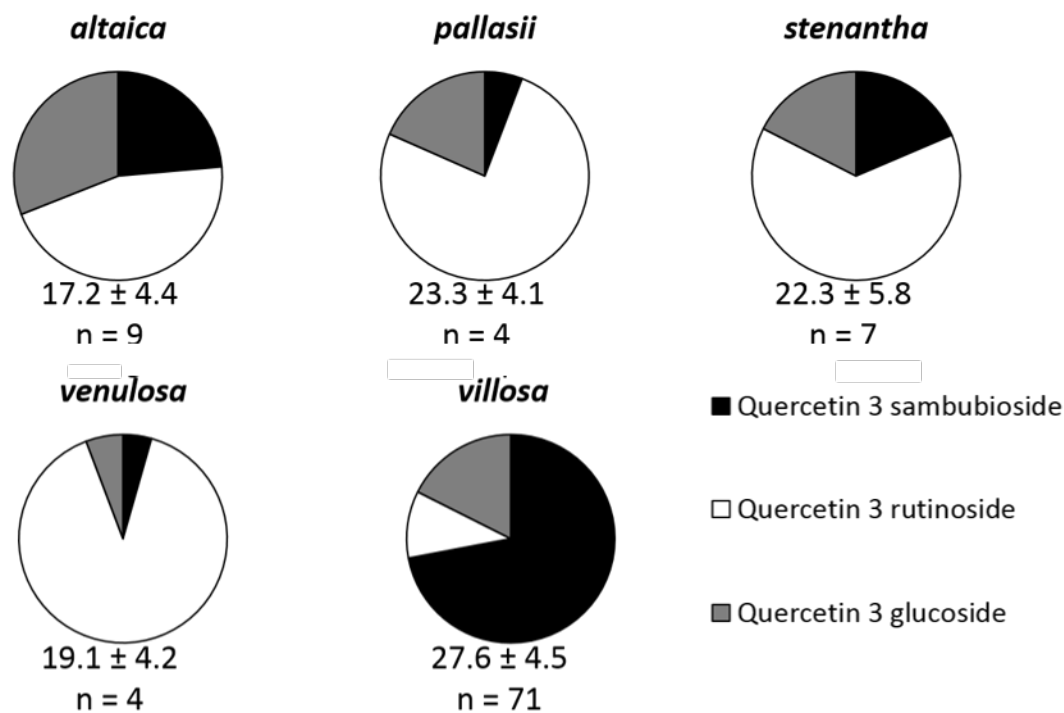
The *venulosa* subspecies showed the greatest range in total quercetin concentrations in the fruit tissues (2.89 to 5.89 mg/g DW), which was almost three times the range observed for



subsp. *pallasii*. By contrast, the range in total quercetin concentration within the leaves was similar for all the genotypes tested. The total concentration of iridoid compounds in the fruit ranged from 2.9 mg/g DW in *Lonicera caerulea* subsp. *venulosa* to 10.5 mg/g DW in *Lonicera caerulea* subsp. *pallasii*. Leaf concentrations of iridoids ranged from 11.1 mg/g DW in *Lonicera caerulea* subsp. *pallasii* to 27.5 mg/g DW in *Lonicera stenantha*. In leaf tissues the major iridoid in all *Lonicera caerulea* subspecies appeared to be secologanin, while in the fruit tissues the major iridoid compound was loganin (data not shown). Due to the differences in secondary metabolite profile between representatives of *Lonicera caerulea* subsp. *villosa* and the other *Lonicera* subspecies, only *Lonicera caerulea* subsp. *villosa* was considered in the following sections.



**Figure 6.2.** Fruit quercetin glycoside profiles and total quercetin concentrations in mg/g dry weight  $\pm$  standard deviation in five subspecies of haskap (*Lonicera caerulea*) sampled at harvest maturity in 2013. \*n indicates sample size



**Figure 6.3.** Leaf quercetin glycoside profiles and total quercetin concentrations in mg/g dry weight  $\pm$  standard deviation in five subspecies of haskap (*Lonicera caerulea*) sampled at harvest maturity in 2013. \*n indicates sample size

#### 6.4.3 Correlations between concentrations of plant secondary metabolites within fruit of wild haskap (*Lonicera caerulea* subsp. *villosa*)

Correlation values for the relationship between the concentrations of individual secondary metabolites found in the fruit of wild haskap are presented in Table 6.2. The concentrations of specific phenylpropanoid derivatives within the fruit appeared to be positively correlated with one another. Chlorogenic acid concentrations were positively correlated with the concentrations of the individual quercetin compounds and with the concentrations of the two monoterpenoid iridoids. The concentration of the individual quercetin compounds were positively correlated with one another. Similarly, the concentrations of secologanin and its loganin precursor were positively correlated.

**Table 6.2.** Correlation values for the concentrations of secondary metabolites in the fruit of 71 haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142

	<b>CA</b>	<b>Q3S</b>	<b>Q3R</b>	<b>Q3G</b>	<b>Loganin</b>	<b>Secologanin</b>
CA	1.00					
Q3S	0.43*	1.00				
Q3R	0.30*	0.28*	1.00			
Q3G	0.51*	0.43*	0.52*	1.00		
Loganin	0.17*	0.28*	0.05	0.16*	1.00	
Secologanin	0.22*	0.40*	-0.22*	0.22*	0.33*	1.00

\*represents a correlation value significant at  $P < 0.05$ .

Abbreviations: chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

#### 6.4.4 Correlations between concentrations of plant secondary metabolites within leaves of wild haskap (*Lonicera caerulea* subsp. *villosa*)

Correlation values for the relationship between the concentrations of individual secondary metabolites within the leaves of the 71 wild haskap (*Lonicera caerulea* subsp. *villosa*) genotypes tested are presented in Table 6.2. The results of the comparisons were similar to the trends seen when the fruit were analyzed. Concentrations of chlorogenic acid were positively correlated with the concentrations of all other compounds monitored within the leaf tissues. The concentrations of individual quercetin compounds were positively correlated with one another; a similar trend was observed in the fruit tissues. While concentrations of both iridoid compounds in the leaves were positively correlated with one another, the concentrations of these compounds were negatively correlated with the concentration of all three quercetin derivatives.

**Table 6.3.** Correlation values for the concentrations of secondary metabolites in the leaves of 71 haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142

	CA	Q3S	Q3R	Q3G	Loganin	Secologanin
CA	1.00					
Q3S	0.21*	1.00				
Q3R	0.14	0.18*	1.00			
Q3G	0.10	0.50*	0.50*	1.00		
Loganin	0.19*	-0.01	-0.10	-0.21*	1.00	
Secologanin	0.16*	-0.01	-0.44*	-0.32*	0.60*	1.00

\*represents a correlation value significant at  $P < 0.05$ .

Abbreviations: chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

#### 6.4.5 Correlations between concentrations of plant secondary metabolites in fruit versus leaf tissues of wild haskap (*Lonicera caerulea* subsp. *villosa*)

The concentrations of all secondary metabolites monitored were positively correlated in the leaf and fruit tissues of the 71 genotypes of *Lonicera caerulea* subsp. *villosa* sampled (Table 6.3). However the  $R^2$  value of the correlations were consistently less than 0.5, thus these relationships have only limited predictive value. Thus the concentrations of secondary metabolites in leaves of *Lonicera caerulea* subsp. *villosa* are not an overly accurate predictor of the concentrations of these some secondary metabolites in fruit tissues within the same plant.

**Table 6.4.** Correlation coefficients for relationship between secondary metabolite concentrations in the fruit and leaf tissues of haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142

Compound	Correlation value	Significance
CA	0.22	<0.01
Q3S	0.20	<0.01
Q3R	0.56	<0.01
Q3G	0.31	<0.01
Loganin	0.25	<0.01
Secologanin	0.42	<0.01

Abbreviations: Chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

6.4.6 Correlations between fruit weight of wild haskap (*Lonicera caerulea* subsp. *villosa*) and site of geographic origin of the test genotypes

Fruit fresh weight of 71 genotypes of *Lonicera caerulea* subsp. *villosa* collected from across Canada and then grown at a common site ranged from 0.12-0.48g, with a mean of 0.28g and a standard deviation of 0.07. Fruit fresh weight of the 71 genotypes of *Lonicera caerulea* subsp. was negatively correlated with increasing North latitude of the site of origin (Table 6.4), but did not show a significant relationship to the longitude of the site of origin (Table 6.4). The concentrations of all secondary metabolites quantified in the fruit were negatively correlated with fruit fresh weight (Table 6.5).

**Table 6.5.** Correlation coefficients for the relationships between geographic site of origin and fruit weight in wild haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142.

Coordinate	Correlation value	Significance
Latitude	-0.21	0.01
Longitude	0.04	0.67

**Table 6.6.** Correlation coefficients for the relationship between fruit weight and the concentration of secondary metabolites in fruit of haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142.

Compound	Correlation value	Significance
CA	-0.22	0.01
Q3S	-0.38	<0.01
Q3R	-0.19	0.03
Q3G	-0.12	0.16
Loganin	-0.08	0.38
Secologanin	-0.11	0.22

Abbreviations; Chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

6.4.7 Correlations between concentrations of secondary metabolites in the fruit of wild haskap (*Lonicera caerulea* subsp. *villosa*) and geographic site of origin of the test genotypes

Correlation values for the relationship between the concentrations of secondary metabolite in the fruit of wild *Lonicera caerulea* subsp. *villosa* versus the latitude and longitude of the site of origin are presented in Tables 6.6 and 6.7. Concentrations of chlorogenic acid in the fruit were positively correlated with increasing North latitude of the site of origin.

Concentrations of loganin were negatively correlated with both increasing North latitude and increasing West longitude. Concentrations of secologanin were positively correlated with increasing North latitude of the site of origin.

**Table 6.7.** Correlation coefficients for the relationship between North latitude and concentration of secondary metabolites in fruit of haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142

Compound	Correlation value	Significance
CA	0.24	<0.01
Q3S	0.14	0.11
Q3R	-0.15	0.09
Q3G	0.21	0.01
Loganin	-0.17	0.04
Secologanin	0.23	<0.01

Abbreviations; Chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

**Table 6.8.** Correlation coefficients for the relationship between West longitude and concentration of secondary metabolites in fruit of haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142

<b>Compound</b>	<b>Correlation value</b>	<b>Significance</b>
CA	0.02	0.79
Q3S	0.14	0.11
Q3R	-0.39	<0.01
Q3G	-0.03	0.73
Loganin	-0.39	<0.01
Secologanin	0.07	0.39

Abbreviations; Chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

*6.4.8 Correlations between concentrations of secondary metabolites in the leaves of wild haskap (Lonicera caerulea subsp. villosa) and geographic site of origin of the test genotypes*

Correlation values for the relationship between geographic site of origin and the concentrations of secondary metabolites in leaves of wild *Lonicera caerulea* subsp. *villosa* are presented in Tables 6.8 and 6.9. Concentrations of chlorogenic acid in the leaves of *Lonicera caerulea* subsp. *villosa* genotypes tested were positively correlated with increasing North latitude of the site of collection. The concentrations of quercetin-3-sambubioside were also positively correlated with increasing North latitude. Concentrations of the major quercetin derivative quercetin-3-sambubioside were positively correlated with increasing West longitude; while concentrations of the two minor derivatives quercetin-3-rutinoside and quercetin-3-glucoside were negatively correlated with increasing West longitude. Concentrations of both iridoid compounds in the leaves of *Lonicera caerulea* subsp. *villosa* were positively correlated with both increasing North latitude and increasing West longitude.

**Table 6.9.** Correlation coefficients for the relationship between North latitude and concentration of secondary metabolites in leaves of haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142

Compounds	Correlation value	Significance
CA	0.20	0.01
Q3S	0.32	<0.01
Q3R	-0.12	0.14
Q3G	0.01	0.89
Loganin	0.28	<0.01
Secologanin	0.41	<0.01

Abbreviations; Chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

**Table 6.10.** Correlation coefficients for the relationship between West longitude and concentration of secondary metabolites in leaves of haskap (*Lonicera caerulea* subsp. *villosa*) genotypes collected from across Canada, grown under common field conditions and sampled in 2012 and 2013. n=142

Compounds	Correlation value	Significance
CA	0.08	0.36
Q3S	0.20	0.01
Q3R	-0.59	<0.01
Q3G	-0.18	0.03
Loganin	0.21	0.01
Secologanin	0.61	<0.01

Abbreviations; Chlorogenic acid (CA), quercetin-3-sambubioside (Q3S), quercetin-3-rutinoside (Q3R) and quercetin-3-glucoside (Q3G).

## 6.5 Discussion

### 6.5.1 Secondary metabolite profiles of *Lonicera caerulea* subspecies

This study surveyed the secondary metabolite profiles of the fruit and leaves of representatives of several subspecies of haskap (*Lonicera caerulea*) held at the UofS. Special attention was paid to *Lonicera caerulea* subsp. *villosa*, the North American subspecies. The UofS has the world's largest holding of this subspecies. To date this subspecies has not appeared



in the lineage of any new genotypes of haskap. Specimens from across the Canadian distribution of *Lonicera caerulea* have been collected from the wild and placed in a common orchard at the UofS. This situation grants an opportunity to understand the genetic (and heritable) components of secondary metabolite profile of *Lonicera caerulea* subsp. *villosa*, with the environmental effects being held consistent across the genotypes.

All of the *Lonicera caerulea* subspecies tested contained the six valuable secondary metabolites previously selected for monitoring. One clear difference observed between the various subspecies tested was in the major quercetin derivative contained within the fruit and leaves. Representative plants of *Lonicera caerulea* subsp. *altacia*, *pallasii*, *venulosa* and *Lonicera stenantha* had the majority of their quercetin pool as quercetin-3-rutinoside with the other two derivatives (quercetin-3-rutinoside and quercetin-3-glucoside) accounting for a much smaller share of the quercetin pool. *Lonicera caerulea* subsp. *villosa* however appeared to be a markedly different; in this subspecies quercetin-3-sambubioside was the major quercetin constituent, while the concentrations of quercetin-3-rutinoside and quercetin-3-glucoside were relatively minor. In higher plants, sugar moieties are generally added to flavonols by the action of UDP sugar:glucosyltransferases (UGTs). Four classes of flavonoid-specific UGTs are known in higher plants; each class is responsible for glycosylation of a different site on the flavonoid backbone (Noguchi et al. 2009). The glycosylation of a sugar moiety on a flavonoid (e.g. quercetin-3-O-rhamnosyl-(1→6)-glucoside) is controlled by group IV flavonoid UGTs (Noguchi et al. 2009). The sugar donor specificity of group IV flavonoid UGTs could be altered by substitution of a single amino acid residue in the relevant gene (Noguchi et al. 2009). The fact that all subspecies of *Lonicera caerulea* were capable of metabolizing both quercetin-3-sambubioside and quercetin-3-rutinoside indicates that there are likely two competitive UGTs

able to add sugar moieties to the quercetin-3-glucoside. Why *Lonicera caerulea* subsp. *villosa* has developed a more active metabolism of quercetin-3-sambubioside is unclear, however *Lonicera caerulea* subsp. *villosa* is the only subspecies tested naturally found in North America.

#### 6.5.2 Relationship between concentrations of secondary metabolites in fruit and leaf of haskap (*Lonicera caerulea*)

The concentrations of all quercetin derivatives were positively correlated with each other within both the fruit and leaf tissues of the various haskap subspecies examined in this study. This may indicate that the metabolism of quercetin in haskap is under feed-back inhibition. This result is especially interesting for quercetin-3-sambubioside and quercetin-3-rutinoside, as these compounds both have quercetin-3-glucosides as their immediate precursor. Loganin and secologanin concentrations within berry and leaf tissues of haskap were also positively correlated. Again this may indicate that the pathway which metabolises these iridoid compounds may be under feed-back regulation.

One noticeable difference between the secondary metabolite profiles of the fruit and leaf tissues of haskap was the relationship between phenylpropanoid and terpenoid metabolism. Within fruit tissues there was a positive relationship between the total concentration of quercetin and the total concentration of iridoids; but within the leaf tissues this relationship was negative. Within leaf tissues it is possible that this negative relationship is due to competition for the carbohydrate pool between the phenylpropanoid and terpenoid metabolic pathways. Both of these pathways create compounds that protect the plant; flavonoids protect against UV damage (Takahashi and Badger 2011) and the iridoids deter insect herbivory (Quintero and Bowers 2013). Thus there may be a trade-off for allocation of resources depending on whether the plant

is adapted to an area with high light intensities or to an area with heavy insect pest pressure. This trade-off between phenylpropanoid and terpenoid metabolism has also been shown in the trichomes of sweet basil (Xie et al. 2008). Control of the relative rate of carbohydrate flux into these pathways was proposed as a mechanism to control the overall secondary metabolite profile within the trichomes (Xie et al. 2008). As the concentrations of total quercetin and total iridoids are positively correlated within the fruit of haskap this may indicate that fruit tissues are not as limited in their carbohydrate metabolism as the leaf tissues.

The concentrations of all secondary metabolites monitored in haskap fruit were positively correlated with their concentrations in the leaves. This may indicate that the compounds share similar physiological roles within the leaf and fruit tissues. It was hoped that leaf concentrations of secondary metabolites in the leaves might be a good predictor of concentrations in the fruit, as this would allow for selection of genotypes with high concentrations of secondary metabolites in the fruit during the 2-5 year juvenile phase before the haskap plant are capable of producing fruit. While the correlations between the concentration in leaves and fruit were statistically significant, the actual predictive value was relatively low (typically explaining less than 50% of the observed variability). This low predictive power indicates that selection for high fruit concentrations of secondary metabolites based on concentrations in the leaves may not be possible.

### *6.5.3 Influence of geographic site of origin on secondary metabolites*

The objective of this experiment was to compare the secondary metabolite profiles of a range *Lonicera caerulea* subsp. *villosa* germplasm, with the intent of finding germplasm which had higher concentrations of secondary metabolites with the potential to improve human health.

However concentrations of secondary metabolites in plant tissues are highly influenced by environment (Jaakola and Hohtola 2012). In this study, the specimens were removed from their natural habitat and grown at a single location. This eliminated variance in secondary metabolites profiles due to site specific environmental factors such as temperature, photoperiod, rainfall pest pressure and fertility and instead isolated the differences due to genetic effects. The observed phenotype would therefore reflect the heritable adaptations of the plant to a specific environment which could be passed on and thus useful in a breeding program.

The geographic site of origin had a significant effect on berry fresh weight, with plants collected from more northern locations genetically programmed to produce smaller fruit. Seed size within fruit species is known to increase with proximity to tropical areas (Moles and Westoby 2004). Seed weight and fruit weight have been shown to be positively correlated in grape (Roby and Matthews 2004) and potato (Almekinder et al. 1995). Thus the negative relationship observed between fruit weight and Northern latitude may be due to differences in seed size within the fruit. In the North there may be less pressure to rapidly establish seedlings, and thus the advantage of large seeds with large endosperms may be lost.

Many secondary metabolites are metabolized within the skin tissues of fruit. As such, small berries with a higher surface area to volume ratios (greater skin to flesh ratio) would be expected to have a higher content of secondary metabolite per berry. The skin mass of grapes is known to be positively correlated with anthocyanin content and skin mass is believed to be the major factor determining the amount of anthocyanin in grape (Melo et al. 2014). It is therefore not unexpected that the concentration of all secondary metabolites in the fruit of haskap was negatively correlated with the weight of the individual fruit.

The concentration of several secondary metabolites in the fruit of wild *Lonicera caerulea* subsp. *villosa* was correlated with the geographic site of origin of the genotypes tested. Chlorogenic acid concentrations in the fruit were positively correlated with increasing Northern latitude at the site of collection. It is possible that this observed effect may be due to the previously discussed relationship between site of origin and fruit size. Smaller fruit from Northern locations would have a greater skin to flesh ratio. The results of this study are in contrast to the results of Uleberg et al. (2012) who indicated that fruit of bilberry plants collected in southern Norway contained greater chlorogenic acid concentrations than those collected from a northern site when plants were grown in a common plot. However the observations of Uleberg et al. (2012) were restricted to only a few clones.

The geographic origin of the *Lonicera caerulea* subsp. *villosa* tested was also correlated with the concentrations of several secondary metabolites within the leaf tissues. Similar to the fruit tissues, chlorogenic acid concentrations in the leaves were positively correlated with increasing North latitude of the site of collection. The total concentration of quercetin in the leaves was also positively correlated with increasing North latitude of origin; this was due mostly to increasing concentrations of quercetin-3-sambubioside. The concentrations of the other two quercetin derivatives did not correlate with the latitude at the site of collection. This result is in contrast to the relationship observed by Oleszek et al. (2002) who showed a negative association between flavonol concentrations and increasing latitude (between 40 and 60°N) of the site of collection in white pine. That study suggested that the observed differences in flavonol concentrations may be due to differences in the necessity of protection from UV light.

There was a significant relationship between West longitude and the concentrations all three quercetin derivatives within the leaves of haskap; however the type of relationship varied

amongst the derivatives. The concentration of the major quercetin derivative (quercetin-3-sambubioside) was positively correlated with increasing West longitude of the site of origin, while concentrations of the two minor conformers were both negatively correlated with increasing longitude. The total concentration of the quercetin derivatives however was not significantly correlated with the longitude of the collection site. This result was unexpected, as the concentrations of the individual quercetin derivatives were positively correlated with one another within leaf tissues.

The concentrations of iridoid compounds in haskap leaves were influenced by geographic site of origin of the genotypes tested. Loganin and secologanin concentrations in haskap leaves were positively correlated with both increasing North latitude and increasing West longitude at the site of origin. The increased concentration of iridoids with increasing North latitude in most likely connected to increased competition for both water and nutrients. Darrow and Bowers (1999) gave evidence that iridoid compounds are induced in *Plantago lanceolata* by insect feeding damage. The concentrations of iridoids in plant tissues including leaves are also increased in nutrient poor soil. Northern soils are often nutrient poor as they are young soils. Reduced water availability increased the concentrations of root produced iridoids in *Scrophularia ningpoensis* seedlings (Wang et al. 2010), while high temperature stress reduced root iridoid concentrations in the same plant (Liang et al. 2014).

#### 6.5.4 Importance of the results to haskap breeding

The results indicate that *Lonicera caerulea subsp. villosa* has a different quercetin profile in both its fruit and leaf tissues than other *Lonicera caerulea* subspecies. Incorporation of *Lonicera caerulea subsp. villosa* genetics into new haskap genotypes should therefore increase

the concentration of the major quercetin derivative (quercetin-3-sambubioside) in both fruit and leaves. There also appears to be a positive correlation between the concentrations of individual quercetin derivatives in haskap tissues, indicating that increasing the concentration of quercetin-3-sambubioside would have no negative effect on the concentrations of the other quercetin derivatives. Thus, incorporating the *Lonicera caerulea* subsp. *villosa* germplasm into a breeding program may aid in increasing the concentration of both quercetin-3-sambubioside and total quercetin in new haskap genotypes.

Currently, increasing fruit size in new haskap genotypes is one of the primary objectives of many fruit breeding programs. The concentrations of secondary metabolites in wild collected haskap fruit were shown to be negatively correlated with fruit weight. This would indicate that there is possibly a trade-off between the breeding objectives of maximizing concentrations of secondary metabolites in the fruit while also selecting for large fruit size.

It was hoped that the positive correlation between concentrations of secondary metabolites in the leaves and fruit of haskap observed in Chapter 5, would also be observed in the *Lonicera caerulea* subsp. *villosa* germplasm, as this relationship may have provided a tool for earlier selection of haskap germplasm with high concentrations of secondary metabolites. While this study again showed a positive relationship between fruit and leaf concentrations of the secondary metabolite compounds monitored in *Lonicera caerulea* subsp. *villosa*, the relationship was weak and thus had limited predictive power. Had the relationship been stronger it may have been possible to estimate secondary metabolite concentrations that could be expected in fruit tissues by assessing the concentrations found in leaf tissues of juvenile plants. This would have allowed early screening of juvenile plants for evaluated levels of secondary metabolite. Currently the breeding program must wait 3-5 years until the haskap produce enough fruit to allow for

quality assessments. Development of an early screening tool for enhanced metabolite profiles would save time and expense in selecting superior haskap germplasm.

#### 6.5.5 Using the results to direct further plant collection

The results of this study suggest potential target areas for future missions to look for additional, potentially valuable *Lonicera caerulea* subsp. *villosa* germplasm. Given the goals of the UofS Fruit Breeding Program to increase fruit size and to increase the concentrations/content of secondary metabolite in new genotypes of haskap, future collecting efforts to locate germplasm with high fruit size should concentrate in more southern locations of the *Lonicera caerulea* subsp. *villosa* distribution. Conversely, germplasm with genetics leading to accumulation of high concentrations of secondary metabolites in fruit and leaves could more likely be found in more northern locations. Collection missions looking for germplasm with high concentrations of terpenoids should focus on more westerly populations of *Lonicera caerulea* subsp. *villosa*.

## 6.6 Conclusions

The fruit and leaves of *Lonicera caerulea* subsp. *villosa* appeared to contain a different quercetin profile than other subspecies tested in this study. It is possible that utilizing representatives of this subspecies in a breeding program could increase the concentration of quercetin-3-sambubioside in both fruit and leaves of new genotypes. It also appears that increasing the concentration of quercetin-3-sambubioside in haskap fruit may be achieved without decreasing the concentrations of the other two quercetin derivatives in the fruit. However



increases in the quercetin-3-sambubioside concentrations may alter other aspects of fruit quality not monitored in this study. The concentrations of secondary metabolite in haskap leaves tissues were not a strong predictor of the concentrations found in the fruit. Selecting for small fruit size may be a good strategy to increase the concentrations of secondary metabolites in haskap fruit, however if this increase occurs at the expense of fruit yield, ease of harvest or quality aspects such as flavor, this selection strategy will likely need to be reassessed. There were differences in the concentrations of secondary metabolites in fruit of *Lonicera caerulea* subsp. *villosa* plants collected from different sites of origin. As these differences occurred even when all genotypes were grown under the same set of conditions this indicates that the differences were genetic and therefore heritable. Differences in secondary metabolite concentrations in fruit and leaves of *Lonicera caerulea* subsp. *villosa* collected from different sites of origin are mostly likely a response to long term selective pressure, related to the proposed roles of the secondary metabolites in protecting the plant against biotic and abiotic stress. The data presented may be useful to a haskap breeding program when planning future plant gathering expeditions, especially if the goal of the expedition is to find wild plants with genetics conducive to the production of high concentrations of secondary metabolites in the fruit or leaves.

## **6.7 Transition**

The UofS Haskap Breeding Program has a large and diverse assemblage of haskap genotypes all being grown in the same orchard under the same environmental conditions. This collection offered an opportunity to test and compare the secondary metabolite profiles of the fruit and leaves of these genotypes under a uniform set of environmental conditions. This allows for isolation of the genetic, heritable contribution to the concentration of secondary metabolite in

the fruit and leaves of haskap. Assessment of this collection also allowed other key knowledge gaps for the project outlined in section 1.4 to be addressed, such as comparison of the secondary metabolite profiles within diverse haskap genotypes and comparisons of the profiles of genotypes collected over diverse geographic sites of origin. The chapter also investigated a number of other relationships which furthered the main goal of the project which was to increase knowledge of the secondary metabolite profile of haskap. The relationship between fruit size and the concentrations of secondary metabolites was found to be negative, while the correlation amongst the concentrations of individual secondary metabolite compounds appeared to be positive. This chapter also built upon information from Chapters 4 and 5 by reassessing the relationship between secondary metabolites in the fruit of haskap and the concentration of these same compounds in the leaves. This chapter employed the chemical methodology established in Chapter 3, and used the sampling times established in Chapters 4 and 5.

While chapter 6 indicated there was sufficient genetic variability to drive a breeding program for enhanced metabolic profile of haskap, breeding programs take considerable time and resources to achieve results. Chapter 7 will investigate a post-harvest technology that has shown potential to increase the concentrations of secondary metabolites in other fruit. This technology may represent a method to further alter and enhance the secondary metabolite profile of haskap, and thus may potentially aid in increasing our understanding of the secondary metabolite profiles of haskap.

## **7.0 EFFECT OF POST-HARVEST UVC IRRADIATION ON THE CONCENTRATION OF VALUABLE SECONDARY METABOLITES IN FRUIT OF HASKAP (*Lonicera caerulea* L.)**

### **7.1 Abstract**

One of the goals of the UofS Haskap Breeding Program is to increase the concentrations of valuable secondary metabolites in the haskap fruit. However, achieving significant improvement through breeding can take years and therefore the Program is looking at other means to enhance the quality of haskap. Post-harvest UVC irradiation has been used to increase the concentrations of a range of secondary metabolites in various fruit species. To evaluate the potential to use post-harvest UVC irradiation to enhance the secondary metabolite profile in haskap fruit, freshly harvested samples of immature, ripe and over-ripe fruit of the haskap cultivar Tundra were exposed to 0, 5, 10 or 20 mins of UVC irradiation (760mW/m<sup>2</sup> intensity at 280 nm). The treated fruit were then stored for 5 days at room temperature before assessment of the effects of the UVC treatment on the concentrations of selected secondary metabolites in the fruit. Responses to UVC treatment varied depending on the developmental stage of the fruit at the time of treatment. In immature fruit, chlorogenic acid and 3,5-dicaffeoylquinic acid concentrations increased after UVC irradiation, while in ripe and over-ripe fruit only secologanin concentrations increased after UVC irradiation. The concentration of quercetin-3-rutinoside in the haskap fruit decreased as the duration of exposure to UVC irradiation increased, regardless of the developmental stage of the fruit at the time of treatment. Overall the use of post-harvest UVC irradiation appeared to have only limited potential to increase the total concentrations of secondary metabolites in mature haskap fruit.

## 7.2 Introduction

### 7.2.1 *Enhancing the secondary metabolite profile of haskap*

One of the goals of the UofS Haskap Breeding Program is to develop new haskap genotypes with higher concentrations of secondary metabolites which have been shown to enhance human health. However breeding haskap to enhance the concentrations/content of secondary metabolites in the fruit will take years of effort with no guarantee of success. In the short term it may be possible to use post-harvest technologies to increase the concentration of secondary metabolites in haskap fruit. One technology which has shown some promise to increase secondary metabolite concentration in other fruit crops is to treat the fruit with UVC (180-280 nm) irradiation after harvest. Irradiation of fruit tissues with UVC light is known to trigger the salicylic acid-mediated defence pathway (Shama 2007). In grape, post-harvest UVC irradiation has been used to induce the phenylpropanoid resveratrol (Cantos et al. 2003, Venditti and D'hallewin 2014), consumption of which is known to reduce coronary heart disease (Cantos et al. 2003). Wen et al. (2015) observed an increase in the concentrations of flavonols after 30 mins of post-harvest irradiation of grapes with UVC at an intensity of  $100 \mu\text{W}/\text{cm}^2$  for up to 1 hour. Increases in the concentrations of phenolics in the fruit of elderberry have also been observed after 30 sec pulses of post-harvest UVC irradiation at an intensity of  $1.2 \text{ W}/\text{cm}^2$  (Murugesan et al. 2010). Post-harvest UVC irradiation at intensities of  $370 \text{ kW}/\text{cm}^2$  for 30 sec also increased the concentration of phenolic compounds in tomatoes (Jagadeesh et al. 2011). However, Cantos (et al. 2000) observed little effect of UVC irradiation at  $2.3 \text{ kW}/\text{cm}^2$  for 30 minutes on the concentrations of flavonols and chlorogenic acid in grape skins; this was true of both mature and immature grape skins. Venditti and D'hallewin (2014) indicated that duration and intensity of UVC irradiation, as well as the developmental stage of the fruit at the time of

treatment, were key factors governing the responses of plant tissues to post-harvest UVC irradiation.

UVC irradiation of the fruit may induce the *de novo* production of compounds. The concentrations of resveratrol increased by 40-70 fold in fruit of *Vitis riparia* after UVC irradiation at  $76\mu\text{W}/\text{cm}^2$  for 5 mins (Kaban, 2012). The methodology developed by Kaban (2012) was intended to screen grape genotypes for their ability to produce high concentrations of resveratrol. It may be possible that there are similar compounds in the fruit of haskap which are only metabolized after UVC irradiation.

The goal of this Chapter was to evaluate the effect of post-harvest UVC irradiation of immature, mature and over-ripe haskap fruit on the concentrations of secondary metabolites found in the fruit.

### *7.2.2 Hypotheses*

a) Increasing duration of UVC exposure will increase the concentration of secondary metabolites in haskap fruit. b) Developmental stage of the fruit at the time of UVC irradiation treatment will determine which secondary metabolites are elicited by the treatment.

## **7.3 Methodology**

### *7.3.1 Description of test material*

The haskap genotype Tundra was used in this study as it is presently the standard cultivar in commercial production in Canada. Haskap fruit used in this study were produced during 2013. Characteristics of the production site and crop management practices used to grow the fruit were presented in section 3.3.1. Characteristics of genotype Tundra are presented in Table 4.1.

### *7.3.2 Sampling procedure*

Fruit were harvested at 16 day post fertilization (DPF) (green berry), 42 DPF (harvest maturity) and 70 DPF (over-ripe berry). The fruit were collected in the manner described in section 4.3.2. UVC treatments were performed immediately after harvest of the fruit.

### *7.3.3 UVC irradiation treatments*

Freshly harvested fruit (10 per treatment) were placed between two 30 W UVC lamps (Cole-Parmer Inc, Montreal, QC) spaced 20 cm apart which generated  $76 \mu\text{W}/\text{cm}^2$  intensity at 254 nm wavelength. The fruit were exposed to the UVC treatment for 0, 5, 10 or 20 mins. These UVC treatments were modified from the methodology developed by Takayanagi et al (2004) who used two 15 W UVC lamps placed at a distance of 10 cm apart to enhance the resveratrol concentrations of wine grapes. Fruit were then left for 5 days at room temperature on a lab benchtop to allow the elicitation of secondary metabolites. This procedure was based on the findings of Kaban (2012) who found that the greatest concentrations of secondary metabolites in UVC treated grapes occurred when fruit were left for 5 days at room temperature following irradiation.

### *7.3.4 Extraction procedure*

Methanolic extracts from the haskap fruit samples were prepared in the manner described in section 3.3.3.

### *7.3.5 High pressure liquid chromatography (HPLC) procedure*

The secondary metabolites present in the methanolic extracts obtained from UVC irradiated haskap fruit were assessed via HPLC, using the method described in section 3.3.4. 3,5-dicaffeoylquinic acid was also included in the analysis as in Chapter 3 (Fig 3.2) this compound was identified in fruit of the haskap cultivar Tundra.

### *7.3.6 Statistics*

Type 3 tests of fixed effects were used to assess the effects of the fruit developmental stage and duration of UVC irradiation on the concentrations of secondary metabolites in the fruit of haskap. Tukey's test was used for means separation at  $\alpha=0.05$ .

## **7.4 Results**

### *7.4.1 Colour assessment*

Green fruit harvested at 16 DPF that were treated with post-harvest UVC irradiation developed a slight purple colouration during the 5 day elicitation period that followed the UVC treatment. A similar degree of colour change was observed for all durations of UVC treatment. No change in colour was observed when more mature berries were treated with UVC, as blue colouration had already developed in these fruit prior to harvest.

### *7.4.2 UVC effect on the concentrations of phenylpropanoids*

Two-way ANOVA revealed that there was a significant effect of fruit developmental stage on the concentrations of all the secondary metabolites found in the haskap fruit. This result was expected given the findings reported in Chapter 4.3. The ANOVA analysis also revealed a

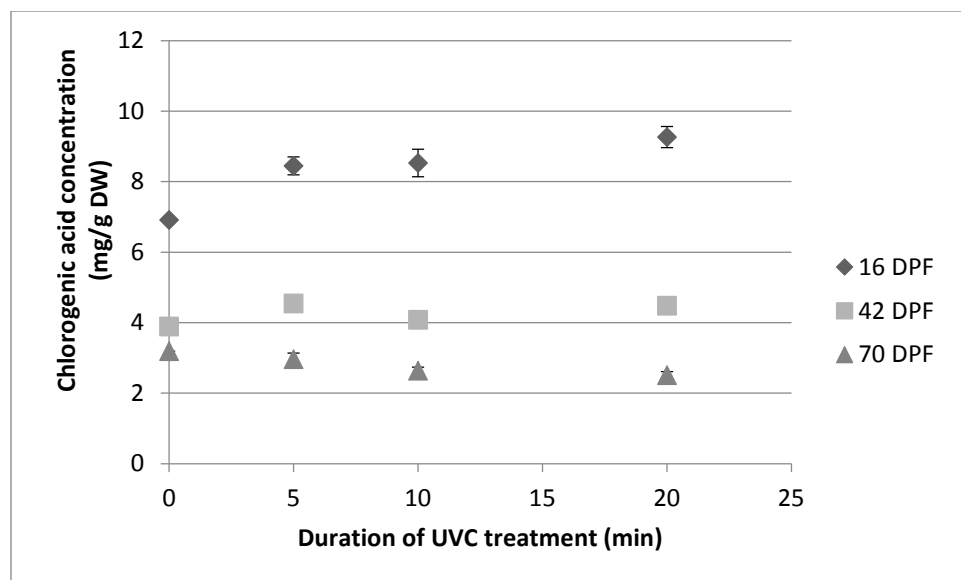
significant interaction between DPF and duration of UVC treatment for the concentrations of chlorogenic acid, 3,5-dicaffeoylquinic acid, secologanin and quercetin-3-rutinoside.

Concentrations of both chlorogenic acid (Table 7.1 and Fig 7.1) and 3,5-dicaffeoylquinic acid (Table 7.2 and Fig 7.2) gave similar responses to UVC elicitation, depending on the developmental stage of the fruit at the time of treatment. When immature fruit (16 DPF) were treated with UVC, the concentrations of both chlorogenic acid and 3,5-dicaffeoylquinic acid increased with an increasing duration of irradiation. In mature fruit (42 DPF), increasing durations of UVC treatment did not affect concentrations of either of these compounds. In overripe fruit (71 DPF), the concentration of chlorogenic acid decreased with increasing durations of UVC irradiation, while the concentration of 3,5-dicaffeoylquinic acid did not give a clear response pattern to increasing exposure to UVC.

**Table 7.1** Type 3 tests of fixed effects of chlorogenic acid concentration in fruit of haskap (*Lonicera caerulea* cv. 'Tundra') based on effects of stage of fruit development at exposure and length of exposure to UVC irradiation.

<b>Effect</b>	Numerator DF	Denominator DF	F value	Pr > F
Stage of fruit development	2	16	289.43	<0.01
Duration of UVC treatment	3	16	3.01	0.06
Stage x Duration	6	16	2.97	0.03

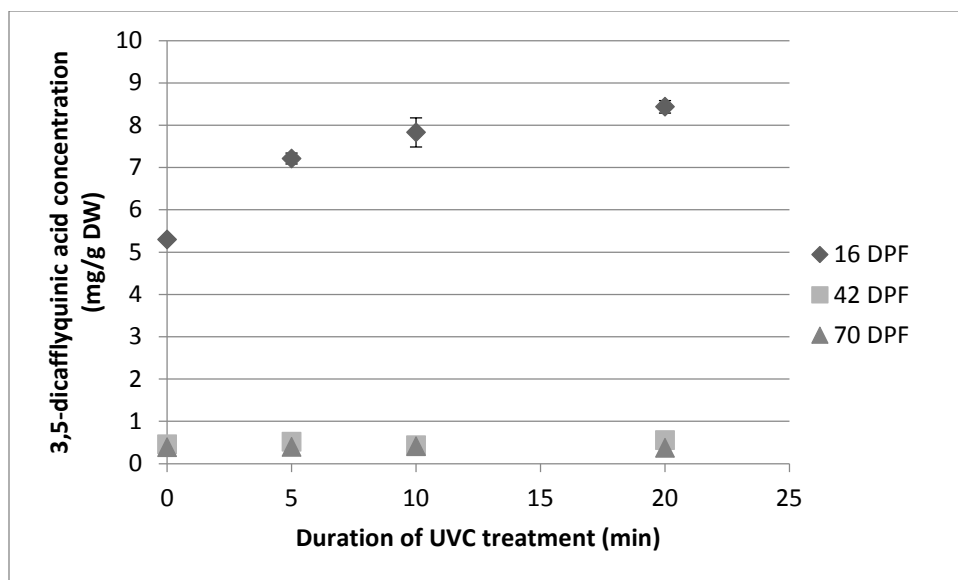




**Figure 7.1** Chlorogenic acid concentrations in haskap (*Lonicera caerulea* cv. ‘Tundra’) fruit exposed to UVC irradiation for 0, 5, 10 or 20 mins at different days post fertilization of the flower (DPF). Bars represent standard error at  $\alpha = 0.05$ .

**Table 7.2.** Type 3 tests of fixed effects of 3,5-dicaffylquinic acid concentration in fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on effects of stage of fruit development at exposure and length of length of exposure to UVC irradiation.

Effect	Numerator DF	Denominator DF	F value	Pr > F
Stage of fruit development	2	16	6850.98	<0.01
Duration of UVC treatment	3	16	54.42	<0.01
Stage x Duration	6	16	53.68	<0.01

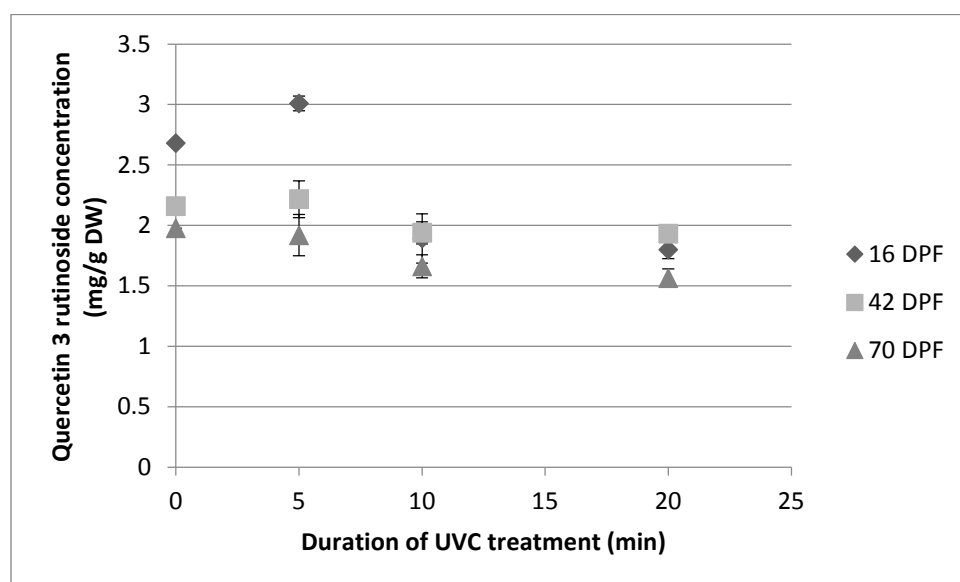


**Figure 7.2.** 3,5-dicafflylquinic acid concentrations in haskap (*Lonicera caerulea* cv. ‘Tundra’) fruit exposed to UVC irradiation for 0, 5, 10 or 20 mins at different days post fertilization of the flower (DPF). Bars represent standard error at  $\alpha = 0.05$ .

While only the concentration of quercetin-3-rutinoside showed a statistically significant interaction between DPF at time of treatment and the length of UVC irradiation (Fig 7.3), the overall response pattern was similar for all three quercetin compounds evaluated. In immature fruit, 5 mins of UVC irradiation gave the maximum concentration of quercetin-3-rutinoside; while longer exposure resulted in concentrations significantly lower than the untreated control. Within ripe fruit, there was no significant effect of UVC irradiation on the concentration of any of the quercetin derivatives or the concentration of the total quercetin pool. UVC irradiation times had no significant effect on quercetin concentration in overripe haskap fruit, however the data shows a trend of decreasing quercetin concentrations with increasing durations of UVC irradiation.

**Table 7.3.** Type 3 tests of fixed effects of quercetin-3-rutinoside concentration in fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on effects of stage of fruit development at exposure and length of exposure to UVC irradiation.

Effect	Numerator DF	Denominator DF	F value	Pr > F
Stage of fruit development	2	16	15.47	<0.01
Duration of UVC treatment	3	16	17.95	<0.01
Stage x Duration	6	16	4.28	<0.01



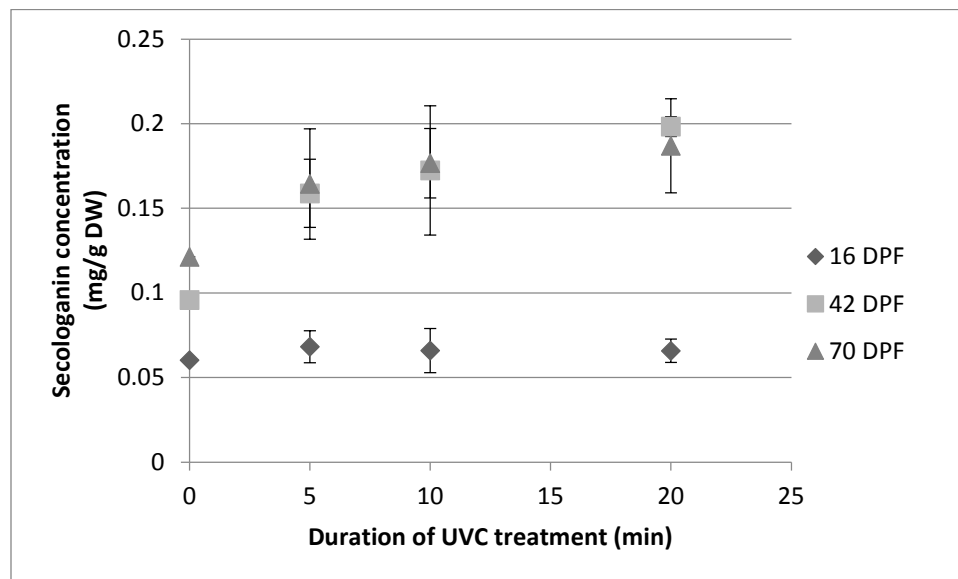
**Figure 7.3.** Quercetin-3-rutinoside concentrations in haskap (*Lonicera caerulea* cv. ‘Tundra’) fruit exposed to UVC irradiation for 0, 5, 10 or 20 mins at different days post fertilization of the flower (DPF). Bars represent standard error at  $\alpha = 0.05$ .

#### 7.4.3 UVC effect on the concentrations of monoterpenoids

The concentration of the iridoid compound loganin in the haskap fruit was not affected by the duration of post-harvest UVC irradiation. The concentration of secologanin however increased with increasing durations of UVC irradiation in both ripe and over ripe haskap fruit (Table 7.4 and Fig 7.4). Immature fruit showed no appreciable change in secologanin concentration in response to UVC treatment.

**Table 7.4.** Type 3 tests of fixed effects of secologanin concentration in fruit of haskap (*Lonicera caerulea* cv. ‘Tundra’) based on effects of stage of fruit development at exposure and length of exposure to UVC irradiation.

Effect	Numerator DF	Denominator DF	F value	Pr > F
Stage of fruit development	2	16	31.20	<0.0001
Duration of UVC treatment	3	16	6.59	0.0041
Stage x Duration	6	16	0.86	0.5432



**Figure 7.4.** Secologanin concentrations in haskap (*Lonicera caerulea* cv. ‘Tundra’) fruit exposed to UVC irradiation for 0, 5, 10 or 20 mins at different days post fertilization of the flower (DPF). Bars represent standard error at  $\alpha = 0.05$ .

## 7.5 Discussion

In this study the concentrations of six secondary metabolite compounds were quantified in the fruit of the haskap cultivar Tundra after post-harvest irradiation with UVC light. The compounds tested included metabolites from the phenylpropanoid pathway (chlorogenic acid, 3,5-dicaffeoylquinic acid, quercetin-3-sambubioside, quercetin-3-rutinoside and quercetin-3-glucoside) and the terpenoid pathway (loganin and secologanin). The concentrations of some of

these compounds were also increased in leaves of the closely related *Lonicera japonica* after exposure to UVC light (Ning et al. 2012). Concentrations of phenylpropanoid compound have been increased after UVC exposure in other fruit crops (Murugesan et al. 2010, Jagadeesh et al. 2011).

While the concentration of anthocyanin compounds were not measured in the haskap fruit, there was a visual color change from green to red when freshly harvested immature haskap fruit were exposed to UVC irradiation. UVC light is known to activate the metabolism of UV absorbing compounds (Austin and Noel 2002). This color change did not occur in mature haskap fruit, or else the change could not be detected by the human eye, as mature fruit already had developed substantial red/blue color.

The effects of post-harvest UVC irradiation on secondary metabolites in the fruit of haskap largely depended on the compound being tested and the developmental stage of the fruit at the time of UVC treatment. Compounds from the phenylpropanoid pathway did not share similar patterns in the changes in the concentrations of secondary metabolites that occurred after post-harvest UVC irradiation. The concentration of chlorogenic acid and 3,5-dicaffeoylquinic acid increased or was unchanged after post-harvest UVC irradiation of haskap fruit, while the concentration of another phenylpropanoid compound, quercetin-3-rutinoside, decreased.

UVC irradiation is known as an activator of pathogen resistance in plants (Cantos et al. 2003, Venditti and D'hallewin 2014). In this study the concentrations of both chlorogenic acid and 3,5-dicaffeoylquinic acid were increased in immature fruit of haskap following increasing durations of exposure to UVC. The accumulation of chlorogenic acid derivatives has been associated with pathogen resistance in other plant species. Ghanekar et al. (1984) showed the inhibitory effect of chlorogenic acid derivatives against the pathogenic bacterium *Erwinia*

*carotovora*. Bazzalo et al. (1985) showed isochlorogenic acid to be a potent inhibitor of mycelial growth of the pathogenic fungus *Sclerotinia sclerotiorum* growing on sunflower. This study also found that isochlorogenic acid was present in healthy tissues, but its concentration increased upon infection by the pathogen. Strange et al. (2001) showed that extracts from the peel of sweet potato (*Ipomoea batatas*), inhibited the growth of the pathogenic fungus *Rhizopus stolonifer*. The principle fungistatic compound in the extract was shown to be isochlorogenic acid.

Short term exposure of haskap fruit to UVC irradiation increased the concentrations of quercetin derivatives in the fruit, but this effect was observed only in immature fruit. UVC light is known to activate chalcone synthase (Schmelzer et al. 1988) and the synthesis of UV absorbing compounds (Austin and Noel 2002). Longer durations of UVC irradiation however decreased the concentration of quercetin in haskap fruit at all developmental stages. This is in contrast to the results of Wen et al. (2015), who observed increases in the concentrations of flavonols in grapes after 30 mins of post-harvest UVC irradiation; that duration of treatment corresponds to the longest treatment used in this study. The reduction of the concentration of quercetin-3-rutinoside in fruit of haskap when the duration of UVC exposure was increased may be due to degradation of these compounds by excessive UV light (Fahlman and Krol 2009).

The results from this experiment indicate that not only is there a difference between the concentrations of secondary metabolites at different stages of development of the fruit of haskap, but there are also potentially differences in the way these tissues respond to pathogen attack or other biotic or abiotic stresses at different stages in development of the fruit. In ripe haskap fruit the iridoid secologanin may serve the same role as the two chlorogenic acid derivatives did in immature fruit. The adaptive advantage of this change in the nature of the chemical defense response is not immediately clear.

Many studies attempting to increase the concentration of secondary metabolites in fruit using post-harvest UVC irradiation focus on a single compound, such as those attempting to increase the concentration of resveratrol in wine grapes (Cantos et al. 2003, Kaban, 2012, Venditti and D'hallewin 2014). It is possible that the concentrations of compounds such as quercetin also decreased after post-harvest UVC irradiation in those studies but the concentrations were not monitored. While UVC treatment of ripe haskap fruit increased secologanin concentration, this increase was not nearly as dramatic as the increase in resveratrol concentration of grapes observed by Kaban (2012) using a similar methodology.

The results from this study suggest that there is little potential to use UVC irradiation as a means to increase the overall secondary metabolite profile of haskap fruit. The only significant enhancement of the profile occurred when immature fruit were treated. However, haskap fruit are typically harvested when fully mature, by which time the fruit appeared to be relatively unresponsive to UVC treatment. There also appears to be limited potential to enhance the efficacy of the UVC treatment by increasing the duration of exposure, as extended UVC irradiation actually decreased the concentrations of some of the valuable compounds. As this experiment was only conducted on one genotype of haskap (Tundra) it is possible that post-harvest UVC irradiation of fruit of other haskap genotypes may produce a different result. It is possible that short durations of higher intensity UVC such as those used by Murugesan et al. 2010 and Jagadeesh et al. 2011, might be more effective at increasing the secondary metabolite concentrations of haskap fruit post-harvest.

Both hydroxycinnamic acid and iridoid appear to be produced following UVC treatment, with the compound induced depending on the stage of fruit development at the time of UVC exposure. It also reemphasizes the importance of stage of development on the production and

profile of secondary metabolites in fruit. This was also observed in Chapter 4, where the concentrations of secondary metabolites in the haskap fruit decreased during fruit development. It is unclear why both compounds (assuming their role is similar) are not produced simultaneously when fruit are irradiated with UVC, as an additive approach to defensive compounds would seem to be more effective at reducing potential damage. It is also possible that UVC irradiation is triggering a number of defensive responses within the plant, in addition to defence from UVC irradiation. UVC irradiation is known to trigger chemical responses resembling those that occur during pathogen attack (Cantos et al. 2003). Concentrations of 3,5-dicaffeoylquinic acid within the fruit tissues of haskap were consistently found at low levels across the germplasm tested. However following UVC irradiation of immature haskap fruit, the concentration of this compound increased to a consistently detectable level. 3,5-dicaffeoylquinic acid is a chlorogenic acid derivative which is essentially two caffeic acid attached to one quinic acid. Thus 3,5-dicaffeoylquinic may act similarly to chlorogenic acid when consumed by humans. Even when the concentration of 3,5-dicaffeoylquinic acid in haskap fruit was enhanced by post-harvest exposure to UVC irradiation, the concentrations do not indicate that haskap fruit would be a rich source of this compound. This compound was one of the few secondary metabolites whose concentration was increased by post-harvest UVC irradiation of haskap fruit.

## **7.6 Conclusion**

The use of post-harvest UVC irradiation appeared to have limited potential to increase the total concentrations of secondary metabolites in mature haskap fruit.



## 8.0 GENERAL DISCUSSION

### *8.1 Improvement of the methodology to quantify secondary metabolites in haskap*

The development of an analytic method to quantify secondary metabolite concentrations in the fruit and leaves of haskap was a necessary first step to complete the objectives outlined for this thesis. The HPLC method developed by Qian et al. (2007) was selected as it was known to be an effective means for the separation and quantification of phenylpropanoid and iridoid compounds in tissues of *Lonicera japonica*. While this method was able to differentiate the key target compounds and worked well with all the haskap germplasm and tissues tested in this study, the sample preparation and analysis process were both time consuming (1 hour per sample) and expensive (approximately \$5 a sample). While simpler, faster and less expensive methods, such as spectrophotometry, are available for quantification of phenolic acid and flavonoids (Rupasinghe et al. 2012), these tests do not allow for the separation and quantification of individual compounds. The greater degree of specificity provided by the more time consuming and expensive HPLC analysis was necessary in this thesis in order to quantify individual compounds. The procedure used also provided useful information such as the observation that *Lonicera caerulea* subsp. *villosa* contains a different quercetin pool than the other subspecies tested. As the results of this thesis have already established these differences in the profile of secondary metabolites in haskap, the use of faster, simpler, less expensive tests may be sufficient for future analysis of the secondary metabolite profile in haskap fruit and/or leaves. For example, a Folin-Cioaltea's test for total polyphenols would be less costly and time consuming than an HPLC analysis, as the Folin-Cioaltea test can be run on a table top spectrophotometer (Rupasinghe et al. 2012). These tests are fast (8 samples a min) and inexpensive (approximately

10¢ a sample). Unfortunately no such simple test is currently available for the quantification of iridoid compounds.

Haskap fruit are typically harvested once they reach full size, have turned a dark blue and have developed an optimum flavor profile. This thesis has also established that fruit harvested at this stage also have the greatest content of the targeted secondary metabolites.

### *8.2 Importance of stage of development on production of secondary metabolites in haskap*

Through the growing season, the concentrations of secondary metabolites in the leaves of haskap stayed fairly consistent, whereas the concentrations of the same compounds decreased as the fruit developed. This decline reflected a dilution effect as the fruit rapidly increased in size. The fruit of haskap on average increased approximately 10 fold in weight from the initial sampling date (approximately 7 days after fertilization of the flower) to the end of fruit development, while the concentration of secondary metabolites decreased by 66% in the case of quercetin and 80% in the case of chlorogenic acid over this period. Total content (concentration x fruit weight) of secondary metabolites in the fruit increased by 200% in the case of chlorogenic acid and by 400% increase for quercetin. This indicates that further production of secondary metabolites occurred during development of the haskap fruit. This increase is most likely the result of the production of skin tissues as the fruit grow (Awad et al. 2000). If increasing skin mass is indeed linked to increased concentrations and content of secondary metabolites in haskap fruit it might be beneficial to find fruit sizes/shapes which maximize the amount of skin on the fruit or to develop genotypes with thicker skin.

The compounds monitored in this thesis, and in a more general sense, most secondary metabolites, including alkaloids, often serve protective roles within plants, protecting plants from

herbivory, pathogens and/or high amounts of UV light. Differences in the secondary metabolite profiles of the fruit and leaves of haskap over the course of the growing season can be explained by considering the roles that these compounds serve within the plant. In leaf tissues a significant amount of energy is expended in the accumulation of chemical defence compounds that protect the leaves from being consumed by pests. Unlike fruit, the leaves of plant tissues do not require dissemination by animals and thus concentrations of these protective compounds persists throughout the year. In haskap fruit tissues an array of defence compounds seemed to be present at high concentrations early in the development of the fruit, before the onset of red/blue color development. After that, the concentrations of these protective compounds decreased rapidly. It is likely that several factors are involved in the observed decrease in concentration of secondary metabolites, including dilution and catabolism. As fruit are produced to eventually be consumed it would be logical that production of compounds that protect against herbivory should decrease as the fruit approach maturity. However, the observed reduction in the concentration of secondary metabolites that occurred during development of haskap fruit may represent a problem for haskap producers, as the greatest fruit size and best flavor occur if the fruit are allowed to reach an advanced stage of maturity before harvest. By that time the concentrations of all secondary metabolites (except anthocyanin) in the fruit are considerably lower than those observed in immature, green fruit. However, as noted previously, total content (concentration x weight) of most secondary metabolites increased in haskap fruit throughout their development. Thus the greatest total yield of secondary metabolites from a haskap orchard would occur at harvest maturity. This information may be helpful for those interested in growing and then processing haskap fruit as a source of secondary metabolites.

### *8.3 Implications of increasing fruit size on concentrations of secondary metabolites in haskap*

The observed negative relationship between fruit size and the concentration of secondary metabolites in haskap fruit leads to a potential conflict between breeding objectives. Producers are more inclined to grow genotypes that produce large fruit, as large fruit are easier to hand harvest and have greater consumer appeal in fresh markets. However, if consumers or processors want higher concentrations of secondary metabolites in the fruit, this study showed that smaller fruit contain higher concentrations of secondary metabolites than larger fruit (Table 6.6). It may therefore be profitable for producers to sort haskap fruit by size. Smaller fruit with higher skin to volume ratios can be used for processing or to enrich foods with secondary metabolites, while larger fruit with smaller skin to volume ratios could be sold as fresh fruit. In addition, haskap skin material that is a by-product of pressing haskap for juice or for fermented products could represent a concentrated source of secondary metabolites that could be extracted or used to enrich other products.

While breeding for higher concentrations of the selected secondary metabolites would improve the health benefits associated with consuming haskap, it is possible that very high concentrations of some of the secondary metabolites may impart an off flavor to the fruit. For example, chlorogenic acid is an acidic compound and elevated concentrations of this compound may make the fruit sourer. There are also some indications that iridoid compounds can impart bitter flavors to fruit (Anikina et al. 1988), however it is unknown at what concentrations these flavors would be perceivable or unacceptable to the consumer. The flesh of the berry is where the majority of the sugars are produced. Breeding to increase the ratio of skin to flesh may cause the haskap fruit to be less sweet and bitterer.

#### *8.4 Comparison of secondary metabolite profile of haskap to that of “superfruit”*

Marketing agencies have developed the term “superfruit” for fruit that contain high concentrations of compounds believed to be beneficial. The total antioxidant capacity of haskap has already been shown to be similar in magnitude to “superfruit” such as blueberries and bilberries, black currants, and black berries (Chaovanalikit et al. 2004). However to date few studies have compared the relative concentrations of other beneficial secondary metabolites in the fruit of haskap to the concentrations found in popular “superfruit”.

In this study, chlorogenic acid was found at higher concentrations than any of the other compounds monitored (except anthocyanin in mature fruit). These high concentrations were present at all developmental stages in both the fruit and leaves of all the haskap genotypes evaluated. There is currently no daily recommended intake levels provided for chlorogenic acid in Canada’s Food Guide (Health Canada 2011). Coffee beans are presently the most common dietary sources of chlorogenic acid. Farah et al. (2005) found concentrations between 8.2-10.9 mg/g DW chlorogenic acid in medium roasted coffee beans. However the content of the chlorogenic acid which is extracted in coffee through percolation of the beans depends on water temperature, volume and extraction method. Chlorogenic acid supplements derived from coffee bean are commercially available. Concentrations of chlorogenic acid in fruit of the six improved genotypes of haskap ranged from 1.1-4.2 mg/g DW while chlorogenic acid concentrations in the fruit of wild collected haskap ranged from 2.7-11.3 mg/g DW. Thus the fruit of haskap could represent a source of chlorogenic acid that is as concentrated as coffee beans. Gibson et al. (2013) reported a concentration of chlorogenic acid of 2 mg/g DW in lowbush blueberry fruit. Awad et al. 2001 reported concentrations of chlorogenic acid in apple skins of approximately 1

mg/g DW. As the concentrations of chlorogenic acid are greater in haskap than in these superfruit, haskap should be able to claim the same title.

This study showed that three quercetin glycosides were present at all developmental stages of the fruit and leaf tissues of all the haskap germplasm tested. Similar to chlorogenic acid, there are currently no recommendations for daily intake of quercetin or flavonols in Canada's Food Guide (Health Canada 2011). However quercetin supplements derived from vegetative tissues of plants such as *Euonymus alatus* and *Nelumbo nucifera* are commercially available at a suggested intake level of 0.8-1.3 g/day. The total quercetin concentrations of fruit of the six improved genotypes of haskap ranged from 1.7- 2.9 mg/g DW, while the concentrations of total quercetin in fruit of the wild collected haskap ranged from 0.9-6.97 mg/g DW. A person would have to consume approximately 220 grams of dried "Tundra" fruit (approximately 1.5 kg of fresh fruit) to reach the minimum recommended daily intake of quercetin provided by these supplements. While the concentration of quercetin in the fruit of haskap is not as high as those in the purified supplements, the concentrations in haskap do appear to be higher than those found in other fruit. Hakkinen et al. (1999) reported the concentrations of quercetin in 18 different fruit species, with the maximum concentrations of approximately 1 mg/g DW occurring in fruit of highbush blueberry, followed by cranberry, lingonberry, chokeberry, crowberry, sea buckthorn and rowanberry. All other fruit tested showed concentrations lower than 0.3 mg/g DW. Castrejon et al. (2008) reported a range of total quercetin between 0.1-0.5 mg/g DW in fruit of highbush blueberry, while Zifkin et al. (2012) reported a total quercetin concentration of 1.5 mg/g DW (assuming 15% moisture) for highbush blueberry fruit. Gibson et al. (2003) reported a total quercetin concentration of 0.7 mg/g DW for lowbush blueberry. As the total quercetin concentrations observed in haskap in this study were

greater than in these reputed “superfruit”, it is again seems reasonable to suggest that haskap qualifies as a “superfruit” based on its quercetin concentrations.

The monoterpene iridoids loganin and secologanin were found in all the haskap genotypes tested. While these iridoids were found at high concentrations early in the development of the fruit, as the fruit grew the concentrations declined to 25% of the initial levels due to dilution and changes in surface area to volume ratio of the fruit. Total concentrations of iridoids in the haskap leaves also decreased by approximately 50% over the course of the growing season. There are no recommendations for daily intake of iridoid compounds presented in the Canada’s Food Guide (Health Canada 2011) and no iridoid supplements are commonly available. However, noni (*Morinda citrifolia*) fruit juice is being promoted for its high content of iridoid compounds (Tahitian Noni 2013). The total iridoid concentration of fruit of the six improved genotypes of haskap ranged from 0.3-1.4 mg/g DW, while total iridoid concentration ranged from 1.3-14.5 mg/g DW in fruit of wild collected haskap. Deng et al. (2010) observed a concentration of approximately 5 mg/g DW iridoid compound in fruit of noni. While cultivated haskap is not presently as rich a source of iridoids as noni, this study has identified wild lines with much higher concentrations of iridoids and these could easily be included in a breeding program.

While most fruit gain the title of “superfruit” based on the presence of high concentrations of a single beneficial nutrient, this study has shown that the fruit of haskap contain high concentrations of more than one group of beneficial compounds. This may set haskap above other “superfruit” currently on the market.

The known health benefits of consumption of anthocyanins are less well studied due to fewer studies with pure compounds and questions about the bioavailability of anthocyanins when

ingested as fruit extracts. However, high concentrations of anthocyanins like cyanidin-3-glucoside are important quality aspect for haskap fruit, as the associated blue color enhances the appearance of the commodity, especially in processed products. Some of the strategies currently employed in marketing of haskap use the deep blue color as a selling feature. For example, the plant propagating business Phytocultures (Clyde River, PEI) is marketing haskap as the “Blue-rrr Berry”. The concentration of total anthocyanin in fruit of the six improved genotypes of haskap ranged from 12.4-38.1 mg/g DW at harvest maturity. Prior et al. (1998) investigated different *Vaccinium* species (often considered “superfruit”) and found concentrations of anthocyanin in the fruit of *V. corymbosum* ranged from 9.4-35.3 mg/g DW, fruit of *V. ashei* ranged between 13.6-28.1 mg/g DW, fruit of *V. angustifolium* ranged from 13.7-28.7 mg/g DW. This project has demonstrated that the concentrations of anthocyanin found in haskap (Table 4.8) fruit are greater than in the fruit of most commonly available members of the blueberry family. There also appears sufficient variability within the available germplasm that further increases in the concentrations of anthocyanin in haskap fruit should be possible through breeding.

#### *8.5 Implications of the results on germplasm diversity for haskap breeding*

The average concentrations of chlorogenic acid found in the fruit of the industry standard cultivar “Tundra” in this study are greater than those reported in any previous study of haskap (see Table 2.4.), except the upper range reported by Skupien et al. (2009), where the values reported were approximately equal to those found in this study (assuming a moisture content of 85%). The concentrations of total quercetin in the fruit of “Tundra” at harvest maturity in this study were also comparable to the upper ranges reported in previous research (assuming a fruit moisture content of 85%) (Ochmian et al. 2010, Kusznierevicz et al. 2012, Wojdyo et al. 2014).



These results indicate that the present commercial standard cultivar Tundra combines high yield potential and good flavor with an excellent profile of secondary metabolites. As this cultivar combines many excellent traits it should be a key component of future breeding programs.

While fruit of haskap cv. Tundra contained high concentrations of all secondary metabolites measured in this study, this study has also shown that there are opportunities to further improve on Tundra in terms of increased concentrations of the selected secondary metabolites in the fruit. Fruit of some *Lonicera caerulea* subsp. *villosa* germplasm contained twice as much of one or more of the selected metabolites than cv. Tundra. Crossing Tundra with these selections could produce offspring with enhanced metabolite profiles, however as the *Lonicera caerulea* subsp. *villosa* genotypes had small fruit size and a lower yield potential than Tundra there may be a corresponding reduction in the yield potential and fruit quality in the offspring arising from this cross. Thus it may be beneficial for a haskap breeding program attempting to pursue the goal of enhancing the secondary metabolite profile in a separate breeding effort. While one program would continue to breed for large fruit size for markets where this is important, the other program would focus on developing new genotypes of haskap for use in markets focused on nutritional value. These genotypes would be selected for high concentrations and yields of secondary metabolites in the fruit with less emphasis on fruit size, shape or flavor. This second program would likely be based on *Lonicera caerulea* subsp. *villosa* genotypes and/or other accessions that have been identified in this study as having exceptionally high levels of valuable secondary metabolites.

The profile of quercetin derivatives was consistent within the fruit and leaves of each haskap genotypes tested but differed amongst genotypes. For example, within *Lonicera caerulea* subsp. *villosa*, the fruit and leaves contained quercetin-3-sambubioside as the major quercetin

derivative, while representatives of other *Lonicera caerulea* subspecies contained quercetin-3-rutinoside as the major quercetin derivative. It is unknown if different quercetin glycosides differ in their absorption or utilization in the human body after consumption. Leaf tissues contained far greater concentrations of both iridoids than the fruit, even at the earliest stage of fruit development. Within the fruit the concentrations of the iridoid loganin were consistently higher than the concentrations of secologanin. However within the leaf tissues secologanin was consistently found at greater concentrations than loganin. This observation was consistent across the *Lonicera caerulea* germplasm tested, regardless of subspecies and breeding history (improved vs unimproved genotypes). It therefore appears that there are some subtle differences in the secondary metabolite profile of different tissues of haskap.

#### *8.6 Implications of the results on future plant collecting projects for haskap in Canada*

As the distribution of *Lonicera caerulea* within Canada encompasses a very large land area, it may help the efficiency of future collection projects if there was some indication as to where one should look for genotypes that have high concentrations of secondary metabolites. As this study was able to keep differences in the environmental effects on growing conditions to a minimum during sampling, locations identified would contain plants with genetics conducive to the production of secondary metabolites. This project showed that Northern areas of Canada may be a good location to scout for haskap germplasm capable of producing high concentrations of secondary metabolites in the fruit and leaves. Fruit produced by haskap originating in Northern areas are generally smaller than fruit produced by haskap originating in Southern areas (Table 6.5). The higher concentrations of secondary metabolites observed in haskap genotypes from the North may simply be a reflection of the fact that the smaller fruit have more skin/unit volume

than larger fruit. The skins of fruit are rich in secondary metabolites. The increased concentrations of secondary metabolites in fruit of haskap originating in Northern areas may also be an adaptive response to stresses associated decreased availability of water and nutrients that are characteristic of young soils (Jaakola et al. 2010) such as peat soils where haskap are found in nature. Plants in Northern areas may also be exposed to longer summer day lengths and greater UV light intensities which could drive the accumulation of metabolites with a protective function (Jaakola et al. 2010). It also appears that Western regions are likely to contain plants which produce high concentrations of secondary metabolites in their leaves.

#### *8.7 Methodologies to speed haskap breeding for enhanced secondary metabolite profiles*

Haskap has a 2-3 year juvenile period during which no or few fruit are produced. This juvenile period slows the progress of breeding, as fruit are needed to evaluate the overall quality of any new cross. An indicator of the concentration of secondary metabolites expected within fruit that could be assessed during this juvenile stage would be of value to breeders as an early selection tool. Since the secondary metabolites found in haskap fruit are also found in the leaves, the secondary metabolite profile of the leaf tissues could potentially be used as an early predictor of the secondary metabolite profile expected in the fruit. Within genotypes of *Lonicera caerulea* subsp. *villosa*, concentrations of secondary metabolites in the fruit were positively correlated with the concentrations found in the leaves. However the strength of the correlation was not strong ( $r = 0.20-0.56$ ) and thus the predictive value of the relationship was limited. As there are presently few tools available for early screening in haskap breeding programs, these relationships could nonetheless be of some value.

The concentrations of most secondary metabolites monitored within fruit of *Lonicera caerulea* subsp. *villosa* were significantly influenced by both the genotype and environmental factors, such as growing season conditions. There were some exceptions to this trend; the concentration of chlorogenic acid and quercetin-3-glucoside in fruit of *Lonicera caerulea* subsp. *villosa* tested did not differ significantly over two years of testing. In a practical sense this result generally means that secondary metabolite concentration within fruit or leaves of new haskap genotypes should be monitored for at least two growing seasons to get a valid representation of the secondary metabolite profile. The year to year variability in secondary metabolite concentration within haskap fruit observed in this project indicates that growers and processors should also expect some year to year variability in the secondary metabolite concentrations found in the crop. Differences in the site of production and/or management practices used in cultivation of the crop will also likely cause additional changes to the concentrations and content of secondary metabolites within haskap. The differences observed in the secondary metabolite profiles amongst the genotypes tested in this study appeared nonetheless to be relatively stable. This stability of genotypic differences is another indication of the potential to breed haskap for improved metabolite profiles.

#### *8.8 Management practices to alter the secondary metabolite profile in haskap fruit*

The improvement of haskap through breeding takes time. In the meantime it would be possible to enhance the secondary metabolite profiles of haskap via the use of post-harvest technologies. In other fruit crops like wine grapes, post-harvest irradiation of the fruit with UVC increased the concentrations of valuable secondary metabolites (Cantos et al. 2003). However in this study, post-harvest irradiation of mature haskap fruit for varying time intervals with UVC

failed to increase the overall concentrations of the selected secondary metabolites, except that irradiating the mature fruit did increase the concentration of the iridoid secologanin. By contrast, treatment of immature fruit with UVC increased concentrations of two chlorogenic acid derivatives. These differences in response to UVC treatment indicate an apparent redundancy within the role of secondary metabolites from diverse pathways.

It is unknown why post-harvest irradiation of haskap fruit was relatively ineffective at increasing the concentrations of secondary metabolites. It is unlikely that the intensities or duration of the UVC treatments were insufficient, as extended durations of exposure actually resulted in the lowest concentrations of secondary metabolites. Other post-harvest treatment methodologies with the potential to enhance secondary metabolite profile of haskap could include application of ozone or chitosan treatments of the fruit are known to elicit defence responses in plants and may be worthwhile for future studies.

#### *8.9 Modeling the secondary metabolite profile of haskap*

It might be possible to develop models which correlated aspects of the secondary metabolite profile (concentration and content) relative to the site of origin of the haskap germplasm. Studies in which fruit material is simply collected at different geographic locations and then assayed for its secondary metabolite profile can only provide information on the resultant phenotype. These studies cannot determine whether the observed differences amongst the tested materials are due to genetic differences or are simply a reflection of differing growing conditions at the collection sites. As breeding programs rely on heritable effects, it is crucial to eliminate or control for environmental effects on the traits of interest. In an effort to address this problem this project examined the secondary metabolite profiles of the collection of wild *Lonicera caerulea* subsp.

*villosa* held at the UofS. This collection contains hundreds of plants that have been collected from the wild, but as these genotypes are now growing in a common location, the environmental effects are held constant. This approach made it possible to identify plants with genetics conducive to enhancing specific aspects of the secondary metabolite profile of new haskap genotypes. . These models could be useful a) to direct germplasm collecting missions as outlined in 8.7, and b) as a starting point for studies of the function these metabolites serve to adapt the germplasm to its native habitat.

This project also developed models to illustrate the changes that occur in the concentrations and content of various secondary metabolites in haskap leaves and fruit over the course of the growing season. In general the concentrations of secondary metabolites decreased by approximately 80% over the course of fruit development; this decreased followed a logarithmic pattern. The concentrations of these metabolites in leaves linearly decreased to approximately 50% of initial. These models would be helpful to anyone attempting to harvest haskap fruit to maximize the concentrations and/or content of secondary metabolites. These models would also benefit anyone attempting to process leaf tissues for the content of secondary metabolites. While the models developed generally showed a good fit for the data they were based on only two years of data from a single production site, using one set of management practices. These models could be made more robust if trials were conducted over more growing seasons and/or across multiple test sites and/or management regimes. The models were developed using only six selected genotypes of haskap. These models could be strengthened by repeating the experiment using additional genotypes, including representatives of pure subspecies of *Lonicera caerulea*.

## 9.0 CONCLUSIONS

Up to this point, investigations into the secondary metabolite profile of haskap have been mostly limited to testing the ripe fruit of a small number of genotypes. This thesis has created a substantial knowledge base of the secondary metabolite profile of haskap by studying all the major compounds contained within methanolic extracts of fruit and leaves of over 100 haskap genotypes originating from collections of both improved hybrids and wild collected genotypes encompassing several different subspecies. This study discovered that there are high concentrations of iridoid and phenylpropanoid compounds in the leaves of haskap and revealed that even at the end of the growing season leaves may represent an excellent source of secondary metabolites. In addition, this thesis is the first to investigate the changes in the secondary metabolite profile that occurs over the development of haskap leaves and fruit. The relationship between stage of fruit development, fruit size and the concentrations of secondary metabolites in haskap fruit was established

This thesis presents the first ever investigation of the secondary metabolite profile of *Lonicera caerulea* subsp. *villosa*. This subspecies displayed high concentrations of secondary metabolites and had a different quercetin profile when compared with other *Lonicera caerulea* subspecies.

Many studies on the secondary metabolite profile of haskap fruit use chemical analysis to measure the total antioxidant activity within the fruit. However these measurements can only give an idea of the concentrations of the individual compounds produced from the phenylpropanoid pathway which have antioxidant potential. In this study concentrations of the individual compounds were monitored for the major compounds within the secondary metabolite

profile of haskap. During the course of this study, two new iridoid glucosides were identified in haskap fruit and a quercetin glucoside was identified for the first time in both the fruit and leaves of haskap.

Finally, this project is the first to investigate the metabolite profile of the fruit and leaves of haskap plants collected from the wild and then grown in a common orchard. This approach effectively isolates the effects exerted by genotype from effects related to environmental conditions at the site of collection or production.

This thesis has identified germplasm with valuable secondary metabolites, improved methodology, and also shown that haskap is highly diversified. All of these should help towards the continuing success of the Haskap Breeding Program at the UofS in its efforts to breed haskap with higher nutritional value.



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## 11.0 APPENDIX

**Appendix Table A.1.** Equation of the line, R square values and significance levels for the relationships between cumulative growing degree days (base 5°C) from time of flowering to harvest maturity and fruit weight (g) for six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Fruit weight (y) = GDD<sub>5</sub> (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = 0.0030x - 0.0192$	0.97*
Borealis	$y = 0.0035x - 0.0695$	0.96*
3-03	$y = 0.0020x - 0.0314$	0.83*
41-83	$y = 0.0031x - 0.0486$	0.94*
77-87	$y = 0.0032x - 0.0506$	0.93*
22-14	$y = 0.0034x - 0.1527$	0.85*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.2.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and % moisture content in fruit of six selected haskap genotypes during the 2012 growing seasons.

<b>Genotype</b>	<b>Moisture (y) = GDD<sub>5</sub> (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = -9E-07x^2 + 0.0004x + 0.8410$	0.61*
Borealis	$y = -5E-07x^2 + 0.0002x + 0.8560$	0.63*
3-03	$y = -2E-07x^2 + 9E-05x + 0.8745$	0.35*
41-83	$y = -8E-07x^2 + 0.0004x + 0.8260$	0.74*
77-87	$y = -1E-06x^2 + 0.0006x + 0.8177$	0.66*
22-14	$y = -8E-07x^2 + 0.0004x + 0.8305$	0.32*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.3.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and chlorogenic acid concentration (mg/g DW) and content (mg/fruit) in fruit of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

Genotype	Response	Chlorogenic acid (y) = GDD <sub>5</sub> (x)	R <sup>2</sup> value
Tundra	Concentration	$y = -7.793\ln(x) + 48.3820$	0.83*
	Content	$y = 6E-06x^2 - 0.0016x + 0.4588$	0.63*
Borealis	Concentration	$y = -11.22\ln(x) + 67.0830$	0.89*
	Content	NS	NS
3-03	Concentration	$y = -6.851\ln(x) + 42.0840$	0.82*
	Content	NS	NS
41-83	Concentration	$y = -8.279\ln(x) + 49.2280$	0.90*
	Content	$y = 2E-06x^2 - 0.0013x + 0.4217$	0.21*
77-87	Concentration	$y = -10.580\ln(x) + 64.0860$	0.92*
	Content	NS	NS
22-14	Concentration	$y = -10.040\ln(x) + 62.0670$	0.92*
	Content	NS	NS

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.4.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and quercetin-3-sambubioside concentration (mg/g DW) and content (mg/fruit) in fruit of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

Genotype	Response	Quercetin-3-sambubioside (y) = GDD <sub>5</sub> (x)	R <sup>2</sup> value
Tundra	Concentration	$y = -0.192\ln(x) + 1.4681$	0.43*
	Content	$y = 0.0002x + 0.0001$	0.87*
Borealis	Concentration	$y = -0.464\ln(x) + 3.0924$	0.87*
	Content	$y = 0.0001x + 0.0159$	0.68*
3-03	Concentration	$y = -0.597\ln(x) + 4.0307$	0.84*
	Content	$y = 8E-05x + 0.0194$	0.56*
41-83	Concentration	$y = -0.842\ln(x) + 5.7912$	0.79*
	Content	$y = 0.0003x + 0.0324$	0.73*
77-87	Concentration	$y = -0.586\ln(x) + 4.1455$	0.89*
	Content	$y = 0.0003x + 0.0153$	0.85*
22-14	Concentration	$y = -1.083\ln(x) + 7.6073$	0.88*
	Content	$y = 0.0006x - 0.0134$	0.81*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.5.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and quercetin-3-rutinoside concentration (mg/g DW) and content (mg/fruit) in fruit of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

Genotype	Response	Quercetin-3-rutinoside (y) = GDD <sub>5</sub> (x)	R <sup>2</sup> value
Tundra	Concentration	$y = -1.042\ln(x) + 7.9772$	0.69*
	Content	$y = 0.0009x + 0.0068$	0.92*
Borealis	Concentration	$y = -0.385\ln(x) + 3.1338$	0.53*
	Content	$y = 0.0004x - 0.002$	0.87*
3-03	Concentration	$y = -1.553\ln(x) + 10.4860$	0.85*
	Content	$y = 0.0002x + 0.0582$	0.52*
41-83	Concentration	$y = -0.279\ln(x) + 2.3288$	0.56*
	Content	$y = 0.0003x - 0.0034$	0.86*
77-87	Concentration	$y = -0.944\ln(x) + 7.0174$	0.77*
	Content	$y = 0.0006x + 0.0215$	0.84*
22-14	Concentration	$y = -0.153\ln(x) + 1.7535$	0.32*
	Content	$y = 0.0006x - 0.0562$	0.85*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.6.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and quercetin-3-glucoside concentration (mg/g DW) and content (mg/fruit) in fruit of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

Genotype	Response	Quercetin-3-glucoside (y) = GDD <sub>5</sub> (x)	R <sup>2</sup> value
Tundra	Concentration	$y = 7E-06x^2 - 0.0023x + 0.4382$	0.61*
	Content	$y = 1E-06x^2 - 0.0002x + 0.0184$	0.90*
Borealis	Concentration	$y = 5E-06x^2 - 0.0027x + 0.5937$	0.50*
	Content	$y = 0.0002x - 0.0062$	0.86*
3-03	Concentration	$y = -0.174\ln(x) + 1.3068$	0.53*
	Content	$y = 8E-05x + 0.0011$	0.66*
41-83	Concentration	$y = -0.203\ln(x) + 1.4385$	0.73*
	Content	$y = 0.0001x + 0.0010$	0.81*
77-87	Concentration	$y = -0.142\ln(x) + 1.1151$	0.51*
	Content	$y = 0.0001x - 0.0036$	0.82*
22-14	Concentration	$y = -0.245\ln(x) + 1.7355$	0.65*
	Content	$y = 0.0002x - 0.0158$	0.77*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.7.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and loganin concentration (mg/g DW) and content (mg/fruit) in fruit of six selected haskap genotypes pooled from the 2011 and 2012 growing seasons.

Genotype	Response	Loganin (y) = GDD <sub>5</sub> (x)	R <sup>2</sup> value
Tundra	Concentration	$y = -0.723\ln(x) + 4.8796$	0.47*
	Content	$y = 0.0003x + 0.0090$	0.49*
Borealis	Concentration	$y = -0.793\ln(x) + 4.9313$	0.68*
	Content	NS	NS
3-03	Concentration	$y = -0.383\ln(x) + 3.0457$	0.42*
	Content	$y = 0.0002x + 0.0123$	0.64*
41-83	Concentration	$y = -0.910\ln(x) + 5.5338$	0.80*
	Content	NS	NS
77-87	Concentration	$y = -2.446\ln(x) + 15.5430$	0.92*
	Content	$y = 0.0002x + 0.1076$	0.25*
22-14	Concentration	$y = -0.682\ln(x) + 4.3534$	0.71*
	Content	$y = 9E-05x + 0.0134$	0.47*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.8.** Equation of the line, R square values and significance levels for relationship between cumulative growing degree days post-fertilization (base 5°C) and secologanin concentration (mg/g DW) and content (mg/fruit) in fruit of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

Genotype	Response	Secologanin (y) = GDD <sub>5</sub> (x)	R <sup>2</sup> value
Tundra	Concentration	$y = -2.086\ln(x) + 11.9890$	0.44*
	Content	NS	NS
Borealis	Concentration	$y = -4.050\ln(x) + 23.0160$	0.74*
	Content	$y = -0.0003x + 0.0999$	0.52*
3-03	Concentration	$y = -1.618\ln(x) + 9.5584$	0.50*
	Content	$y = -4E-05x + 0.0286$	0.21*
41-83	Concentration	$y = -2.512\ln(x) + 14.3300$	0.72*
	Content	$y = -0.0002x + 0.0821$	0.43*
77-87	Concentration	$y = -2.645\ln(x) + 15.5300$	0.64*
	Content	NS	NS
22-14	Concentration	$y = -3.116\ln(x) + 18.6140$	0.47*
	Content	NS	NS

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.9.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and total anthocyanin concentration (mg/g DW) content (mg/fruit) and in fruit of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Response</b>	<b>Anthocyanin (y) = GDD<sub>5</sub> (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	Concentration	$y = 0.1416x - 20.4850$	0.58*
	Content	$y = 0.033x - 6.6994$	0.69*
Borealis	Concentration	$y = 0.0365x + 1.0631$	0.47*
	Content	$y = 0.0149x - 2.1975$	0.37*
3-03	Concentration	NS	NS
	Content	$y = 0.0064x - 0.9508$	0.20*
41-83	Concentration	$y = 0.0531x - 10.3980$	0.34*
	Content	$y = 0.0146x - 3.5372$	0.41*
77-87	Concentration	$y = 0.0426x + 1.4191$	0.19*
	Content	$y = 0.0193x - 4.3198$	0.45*
22-14	Concentration	$y = -0.0206x + 21.8310$	0.21*
	Content	NS	NS

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.10.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and total soluble solids (°Bx) in fruit of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Soluble solids (y) = GDD<sub>5</sub> (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = 0.0238x + 4.0995$	0.64*
Borealis	$y = 0.0163x + 6.5989$	0.41*
3-03	NS	NS
41-83	$y = 0.019x + 5.5662$	0.33*
77-87	$y = 0.0138x + 6.5616$	0.31*
22-14	$y = 0.0127x + 9.5494$	0.43*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.11.** Equation of the line, R square values and significance levels for the relationship between cumulative growing degree days post-fertilization (base 5°C) and titratable acidity (malic acid g/100ml) in fruit of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Titratable acidity (y) = GDD<sub>5</sub> (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = -0.0028x + 2.8606$	0.44*
Borealis	$y = -0.0017x + 2.9267$	0.20*
3-03	NS	NS
41-83	$y = -0.003x + 3.4897$	0.37*
77-87	$y = -0.0035x + 3.3626$	0.57*
22-14	NS	NS

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.12.** Equation of the line, R square values and significance levels for the relationship between days post-fertilization (DPF) and chlorogenic acid concentration (mg/g DW) in leaves of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Chlorogenic acid (y) = DPF (X)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = 0.0017x^2 - 0.2188x + 17.1452$	0.55*
Borealis	$y = 0.0019x^2 - 0.3158x + 19.7659$	0.75*
3-03	$y = 0.0013x^2 - 0.1678x + 12.0684$	0.74*
41-83	$y = 0.0022x^2 - 0.3402x + 20.2151$	0.74*
77-87	NS	NS
22-14	NS	NS

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.13.** Equation of the line, R square values and significance levels for the relationship between days post-fertilization (DPF) and quercetin-3-sambubioside concentration (mg/g DW) in leaves of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Quercetin-3-sambubioside (y) = DPF (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = -0.201\ln(x) + 3.3574$	0.49*
Borealis	$y = -0.557\ln(x) + 8.0791$	0.40*
3-03	$y = -0.499\ln(x) + 7.5195$	0.51*
41-83	$y = -0.793\ln(x) + 11.2910$	0.43*
77-87	$y = -0.146\ln(x) + 9.7843$	NS
22-14	$y = -0.793\ln(x) + 12.8590$	0.45*

\* indicates significance at  $\alpha=0.05$ .



**Appendix Table A.14.** Equation of the line, R square values and significance levels for the relationship between days post-fertilization (DPF) and quercetin-3-rutinoside concentration (mg/g DW) in leaves of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Quercetin-3-rutinoside (y) = DPF (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = -1.437\ln(x) + 13.2520$	0.70*
Borealis	$y = -0.411\ln(x) + 3.2538$	0.87*
3-03	$y = -0.983\ln(x) + 9.4276$	0.87*
41-83	$y = -0.302\ln(x) - 0.3004$	0.85*
77-87	$y = -0.768\ln(x) + 8.5048$	0.59*
22-14	$y = -0.301\ln(x) + 2.2462$	0.71*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.15.** Equation of the line, R square values and significance levels for the relationship between days post-fertilization (DPF) and quercetin-3-glucoside concentration (mg/g DW) in leaves of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Quercetin-3-glucoside (y) = DPF(x)</b>	<b>R<sup>2</sup> value</b>
Tundra	NS	NS
Borealis	$y = -0.502\ln(x) + 4.5190$	0.78*
3-03	$y = -0.318\ln(x) + 3.7967$	0.66*
41-83	$y = -0.403\ln(x) + 3.7259$	0.86*
77-87	$y = -0.316\ln(x) + 3.4911$	0.58*
22-14	NS	NS

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.16.** Equation of the line, R square values and significance levels for the relationship between days post-fertilization (DPF) and loganin concentration (mg/g DW) in leaves of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Loganin (y) = DPF (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = -1.534\ln(x) + 9.2917$	0.91*
Borealis	$y = -1.740\ln(x) + 9.8285$	0.90*
3-03	$y = -3.034\ln(x) + 19.9810$	0.90*
41-83	$y = -2.087\ln(x) + 11.0700$	0.93*
77-87	$y = -2.241\ln(x) + 11.4590$	0.81*
22-14	$y = -1.264\ln(x) + 6.8452$	0.89*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Table A.17.** Equation of the line, R square values and significance levels for the relationship between days post-fertilization (DPF) and secologanin concentration (mg/g DW) in leaves of six selected haskap genotypes. Data are pooled from the 2011 and 2012 growing seasons.

<b>Genotype</b>	<b>Secologanin (y) = DPF (x)</b>	<b>R<sup>2</sup> value</b>
Tundra	$y = -2.488\ln(x) + 16.5620$	0.75*
Borealis	$y = -3.486\ln(x) + 19.4990$	0.80*
3-03	$y = -3.200\ln(x) + 20.9590$	0.94*
41-83	$y = -3.631\ln(x) + 19.3270$	0.85*
77-87	$y = -1.567\ln(x) + 8.8810$	0.60*
22-14	$y = -1.721\ln(x) + 10.3820$	0.61*

\* indicates significance at  $\alpha=0.05$ .

**Appendix Figure A.1.** Full mass spectrum of the HPLC fraction obtained from the mature fruit of haskap (*L. caerulea* var. *villosa*) eluting at 17.1 min. Enhanced product ion spectrum from the precursor ion at  $m/z$  595 ( $[M-H]^-$ )

