Characterization of Fruit Development and Ripening of *Vaccinium angustifolium* Ait. in Relation to Microclimate Conditions

by

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In loving memory of Isabelle Gibson

who offered unconditional love and support throughout my life.

She believed in all my projects and

is greatly missed.

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Abstract

Berry ripening in lowbush blueberry (*Vaccinium angustifolium* Ait.) is influenced by developmental, physiological and climatic factors resulting in a heterogenous mix of maturities at harvest. This study characterizes the physico-chemical changes which occur during fruit ontogeny and links ripening patterns to micoclimate. Individual clones in five commercial fiels were followed in the 2006 and 2007 growing seasons. Phenolic acids, flavonols, and flavan-3-ols decreased and anthocyanins increased with maturity. Peak maturity consistently occurred at 1200 accumulated growing degree days (GDD). There was a sharp decline in fruit retention at the end of the growing season suggesting a date after which harvested yield declines but no consistent pattern was detected between years or fields.The consistency of GDD accumulation in relation to ripening pattern suggests GDDs can be used as a predictive ripening index. The physico-chemical nature of ripe berries indicates ripe berries could be harvested earlier than is currently the practice.

List of Abbreviations Used

AAPH	2.2'-Azobis (2-amidinopropane) dihydrochloride
ABA	Abscisic acid
AIP	2-aminoindan-2-phosphonic acid
ANS	Anthocyanidin synthase
C3-Gal	Cyanidin-3-galactoside
C3-Glu	Cyanidin-3-glucoside
Caf	Caffeic acid
Cat	Catechin
CHI	Chalcone Isomerase
Chl	Chlorogenic acid
CHS	Chalcone Synthase
D3-Gal	Delphinidin-3-galactoside
D3-Glu	Delphinidin-3-glucoside
DFR	Dihydroflavonol 4-reductase
DW	Dry weight
EGC	Epigallocatechin
Epicat	Epicatchin
F3'5'H	Flavonoid 3', 5'-hydroxylase
F3'H	Flavonoid 3'- hydroxylase
F3H	Flavanone-3-hydroxylase

F-C	Folin-Ciocalteu
Fer	Ferulic acid
FRAP	Ferric Reducing Antioxidant Power
HAT	Hydrogen atom transfer
HPLC	High performance liquid chromatography
IAA	Indole 3-Acetic Acid
LDOX	Leucoanthocyanidin dioxgenase
M3-gal	Malvidin-3-galactoside
M3-Glu	Malvidin-3-glucose
NSAC	Nova Scotia Agricultural College
ORAC	Oxygen Radical Absorption Capacity
P3-Gal	Petunidin-3-galactoside
P3-Glu	Petunidin-3-glucoside
PAL	Phenylalanine ammonia lyase
PAR	Photosynthetically active radiation
Pfr	Phytochrome far red
PIF3	Phytochrome interacting factor 3
Ppm	Parts per million
Pr	Phytochrome red
PTFE	Polytetrafluoroethylene
Q3-gal	Quercetin-3-galactoside

Q3-Glu	Quercetin-3-glucoside
Q3-Rha	Quercetin-3-rhamnoside
Q3-Rut	Quercetin-3-rutinoside
SET	Single electron transfer
SSC	Soluble solid content
TA	Titratable acidity
TPTZ	Sodium acetate trihydrate, 2,4,6-tris(2-pyridyl)-S-triazine
UFGT	UDP glucose-flavonoid 3-*O*-glucosyl transferase

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Chaper 1: Introduction

Introduction

Lowbush blueberries (*Vaccinium angustifolium* Ait.) are low growing shrubs indigenous to eastern North America (Vander Kloet 1978). Both First Nations and early settlers used blueberries as a food crop, and the commercial sale of blueberries was established in the early 1800's (Wood 2004). Canning of the fruit was introduced in 1869 and the first frozen blueberries were processed in the 1930's (Wood 2004). Lowbush blueberries are one of Nova Scotia's important commercial agricultural crops. In the year 2000, blueberries represented thirty-two percent of the provinces agri-food exports, with the crop grown on 38,000 acres (Nova Scotia Agriculture Fisheries and Aquaculture, 2005). Currently Nova Scotia produces about eighteen million kilograms of berries annually, which are sold to both the domestic and export markets (Wild Blueberry Producers Association of Nova Scotia 2007). The cultivation of the lowbush blueberry crop is unique in that fields are not developed through planting, but rather cultivation occurs in areas where established clones are found (Eaton and Hall 1961). A clone is an individual blueberry plant consisting of multiple aerial shoots connected through an underground rhizome. Blueberry clones express a high degree of variability in stem height, stem colour, flower colour, fruit set, and fruit size (Burgher- MacLellan and MacKenzie 2004). Commercial fields contain a noticeable patchwork of clones in an irregular pattern (Burgher- MacLellan and MacKenzie 2004). Clones expand through the growth of the rhizome into bare patches and the subsequent production of aerial shoots (Eaton and Hall 1961). Clone expansion is a slow process, as rhizomes grow between five and forty centimetres a year (Kender and Eggert 1966).

The slow growth pattern is continued above ground, as the aerial stems undergo a season of vegetative growth before producing flower buds in the fall. These buds over-winter and fruit development occurs in the second year (Barker and Collins 1963). In established uncultivated plants, new growth is vegetative and berry development occurs on older stems. This annual growth pattern results in a bushy appearance and decreased fruit set (Barker and Collins 1963). In contrast, cultivated fields are pruned after fruit harvest to maintain a two year production cycle, with vegetative growth in the first year and fruit production in the second year (Eaton and McIsacc 1997).

Flower development and fruit set are dependent on the interplay of biotic and abiotic factors. Within the over-wintering bud, the flower primordia are developmentally staggered (Bell and Burchill 1955). At bud break, the flower stem elongates, forming a raceme type inflorescence (Hall *et al.* 1979). Along this stem, the most mature flowers will be found at the tip of the stem and the younger flowers towards the base of the stem (Bell and Burchill 1955). A raceme is one of three broad architectural inflorescence types found in nature, along with panicles and cymes (Prusinkiewicz *et al.* 2007). All other infloresences types such as spikes, corymbs, and catkins are variations on three broad types, resulting from differences in the arrangement of lateral meristems and internode length (Prusinkiewicz *et al.* 2007). Inflorescence structure within a plant family tends to vary within an architectural type, suggesting a developmental mechanism based on genetic control (Prusinkiewicz *et al.* 2007). The limited architectural types and repetition of architectural types between unrelated plant families suggests an adaptive selection influence to inflorescence type (Prusinkiewicz *et al.* 2007). The raceme type

inflorescences are selected for in latitudes with variable length growing seasons between years and in pollinator limited environments (Prusinkiewicz et al 2007).

On a raceme, alternate flowers or floral branches develop off a main axis (Prusinkiewicz *et al.* 2007). In lowbush blueberry, the raceme develops from floral buds at the end of a vegetative stem (Bell 1950). Each floral bud contains between seven to ten flower primordia, which continue to develop after bud break in the spring (Bell 1950). This arrangement results in a cluster of flowers at the tip of a stem (Bell 1950), which increases the attractiveness of the display to pollinators (Harder *et al.* 2004). The timing of bud break and rate of further flower development is dependent on early spring weather conditions (Cappiello and Dunham 1994). Early spring weather also influences when pollinators hatch and how active they are, which determines the rate at which individual flowers are pollinated (Hall *et al.*1963). The movement of insect pollinators within and among inflorescences is dependent on insect species (Javovek *et al*. 2002) and inflorescence type (Jordan and Harder 2006). On a raceme, a bee will approach the inflorescence at the lowest open flower and move vertically through the structure (Jordan and Harder 2006 Jovovek et al. 2002). The staggered development of flowers along the blueberry inflorescence (Harder *et al*. 2004) and a decrease in pollinator attractiveness with increasing floral age (Wood 1962) help to promote outcrossing, which is needed for viable seed production (Harder *et al.* 2004). An increase in the number of times a flower is pollinated leads to a larger number of seeds in the berry, increasing the overall size of the berry (Barker *et al.* 1963). Berry size determines the rate of berry ripening, where larger berries ripen at a faster rate than smaller berries (Barker *et al.* 1963).

Along the inflorencence the blueberry flowers hang in a pendant fashion, with downward facing bells (Desjardins and De Oliveria 2006). The flower corolla is formed from fused petals and contains ten stamens and a single pistle (Hall *et al*. 1979). The pistle is composed of fused carples, which situates the fruit as a simple fleshy fruit (Nabors, 2004). The ovary is an inferior ovary, sitting below the petals and sepals (Hall *et al.* 1979). Flowers open and are receptive for seven days, with declining fertility over time (Hall *et al.* 1979). After pollination the corolla undergo abscission leaving an exposed stamen (Hall *et al.* 1979). A slight swelling of the ovary at this stage indicates a fertilized ovule and a set fruit. Within the ovary, there are ten pseudolocules, containing multiple ovules, which when fertilized develop into seeds (Hall *et al.* 1979). The blueberry fruit develops from the ovary, which consists of three tissue types, the exocarp, mesocarp and endocarp (Nabors, 2004). All three tissue layers soften during ripening, which is characteristic of true berries (Nabors, 2004). Fruit ripening is characterized by physico-chemical changes that make the fruit attractive and palatable (Gortner *et al.* 1967). Ripening involves a decrease in fruit acidity, decreases in most phenolic compounds, an increase in soluble solids and an accumulation of anthocyanins (Eck 1986). Blueberry fruit progress through three growth stages, two stages of fast growth, separated by a period of slow growth, which results in a double sigmoid growth curve. The first stage is initiated with fertilization and in this stage, the berry diameter increases rapidly as a result of cell division, this is followed by a decrease in growth rate during embryo and endosperm development. The second fast growth period results from a rapid increase in pericarp size, at the end of which the berry has attained its full size (Ismail and Kender 1974; Eck 1986). The length of time berries spend in each growth stage is strongly tied to weather (Eck 1986; Barker *et al.* 1963).

The main climatic factors which influence ripening are temperature, light and soil moisture. Ripening rate is linked to the seasonal temperature, with warmer conditions promoting faster ripening (Hall and Aalders 1968). Anthocyanin accumulation is dependent on the growth stage at which berries are exposed to light and the amount of direct sunlight a berry receives throughout the season (Zhou and Singh 2002; Dokoozlian and Kliewer 1996). For proper anthocyanin production blueberries must receive sunlight in the first growth stage. In subsequent growth stages increases in sunlight result in increases anthocyanin accumulation and changes the composition of accumulated anthocyanins (Vvedenskaya and Vorsa 2004; Kalt *et al.* 1995). Soil moisture influences berry acidity and firmness, where berries harvested in wet conditions lack firmness (Seymour *et al.* 2004).

As the biotic and abiotic factors act on individual berries, the rate of ripening is inconsistent among berries, stems and clones. This leads to a field of mixed berry maturities, including green, red, blue, and over-mature berries, on any particular date. Selective hand picking is a time and labour intensive process, and hand rakes were introduced to Nova Scotia in the 1950's (Kinsman 1986). Both hand rakes and the more recent mechanical harvesters remove all berries from the plant, and as a result the crop is harvested from a field once. Berries are then sorted into maturity stages during processing. If the berries are to be frozen, the most desirable berries are separated out through hydro-density separation, before being individually quick frozen (Kinsman 1993).

As there are limited uses for berries from the non-ripe berry stages, predicting the optimal ripening date could allow producers to maximise the harvest of fruit in prime

condition. This thesis aims to characterize the physiochemical changes during ripening; to relate the changes in berry ripening to local weather conditions; and to determine factors influencing the optimal harvest time.

Literature Review

Plant growth happens when adequate light, temperature and soil moisture are present, and changing conditions can enhance or impede development (Nabors 2004). The biotic community around the plant can also influence growth, through competition for soil nutrients or, sunlight and provide vectors for pollination and seed dispersal (Nabors 2004). To place fruit ripening, a terminal stage of development, in context, this chapter describes the ecological community in which lowbush blueberries are found, followed by plant growth and development. The general physiochemical changes which occur during fruit ripening are outlined and the processes by which climate interact with fruit development are presented.

Ecology of Lowbush Blueberry

North American native lowbush blueberry plants grow in association with seventy one different plant species, in two types of ecological communities (Hall *et al.* 1979). In wooded areas, blueberries are part of the herb-dwarf shrub understory and on exposed barrens they form part of the low growing ground cover (Hall *et al.* 1979). Throughout the Maritimes, wild blueberries are most abundant in disturbed habitats, such as clearcut woods, areas affected by fire, or abandoned farmland (Hall *et al.* 1979).

Blueberries maintain their place in the communities through their extensive rhizome system (Hall *et al.* 1979). The rhizome is a modified stem which grows horizontally through the soil and acts as both a food storage and growth expansion system

(Stern 2006). The rhizome is the source for both adventitious roots, which help with nutrient acquisition, and new aerial shoots, which allow for lateral expansion (Stern 2006; Barker and Collins 1962). The rhizome also allows the blueberry to remain established in a community when faced with interspecies competition. Blueberries can be outcompeted by other species that grow vigorously or by taller species that shade out blueberry sprouts (Vander Kloet 1978), but when outcompeted for light, new shoots can emerge from the rhizome in regions where the plant is facing less competition (Hall *et al.* 1979). For example, in the barrens habitat blueberries are found growing in association with juniper (*Juniperus communis* L.) and chokeberry (*Pyrus melanocarpa* (Michx. Willd.). As juniper spreads outward it will shade existing blueberries, which respond with decreased vegetative growth where the plants meet, while new aerial shoots sprout in the senescent core of the juniper (Hall *et al.* 1979).

Lowbush blueberries tolerate marginal habitats, including acidic soils with a pH of four to five, or well drained sandy soils. Both conditions can restrict photosynthetic ability through the mineral composition, low phosphorus and high manganese, or water availability (Taiz and Zeiger 1998). Typically, blueberries grow well on podsols, acidic, or sandy soils low in iron and found below a decomposing organic layer (Hall *et al.* 1964; Jackson *et al.* 1972). Small gravel and stones in the soil promote increased surface area of the roots, as branching only occurs when the root growing tip is impeded or injured (Jackson *et al.* 1972).

Blueberry Growth and Development

In wild lowbush blueberry fruit initiation and subsequent ripening occurs only after a plant is established and matured. Lowbush blueberry require rhizome establishment, and vegetative expansion occur prior to fruiting.

Individual plants within native lowbush blueberry stands are established from animal dispersed seeds (Eaton 1957). Seeds are transported when ripe berries are eaten and the fleshy part of the berry digested, leaving intact seeds to be deposited a considerable distance away from the point of origin (Eaton 1957). The American robin (*Turdus migratorius* L.) is the primary blueberry seed disperser, but berries are also eaten by black bears (*Ursus americanus* Pallis.) and other small mammals (Hall *et al.* 1979). The large home ranges of the berry consumers results in a patchy distribution of native stands (Hall *et al.* 1979).

When a seed is deposited into favourable soil, it will germinate into a 'mother plant' which consists of an underground tap root and a single above ground stem (Eaton and Hall 1961). In the first growing season, the root elongates and develops a crown from which rhizomes emerge (Eaton and Hall 1961). At the same time a leafy aerial shoot emerges and nodes develop along its length (Eaton and Hall 1961). Elongation ends when the distal, or last formed leaf, withers around mid-summer (Bell 1950). When the shoot tip drops off, approximately two weeks after the withering appeared, the axillary buds swell and begin to develop (Bell 1950). Buds located at the top of the shoot develop into flower buds with a characteristic spherical shape. Any buds below the flower buds will have a sharp pointed elongate shape, characteristic of vegetative buds (Bell 1950). In the second year, the flower buds open, and the vegetative buds elongate, undergoing the same

development as the original shoot; vegetative elongation, withering and bud development (Barker and Collins 1963). Over time, an untended plant produces more branches and fewer flower buds and becomes bushy in nature (Barker and Collins 1963).

After a period of three or four years the 'mother plant' develops an underground rhizome (Hall *et al.* 1979). The rhizome functions in nutrient accumulation, nutrient storage and the colonization of new areas by growing laterally through the soil (Kender and Eggert 1966; Eaton and Hall 1961). How far the rhizome expands in a year depends on the type of soil it is growing in and its depth (Hall *et al.* 1972; Hall *et al.* 1979). Organic soils promote greater expansion than do mineral soils (Hall *et al.* 1979). Rhizome expansion is promoted when the rhizome is deeper in the soil (Hall *et al.* 1972). Expansion of a rhizome can range from five to 40 cm in a growing season (Kender and Eggert 1966).

As the rhizome expands it will develop new roots, and aerial shoots emerge from dormant buds found along its length (Eaton and Hall 1961). It is unclear what triggers new shoot growth from rhizomes, although seasonality, temperature and an interplay between the hormones auxin and indole 3-acetic acid (IAA) is suspected in initiation (Barker and Collins 1962; Barker and Collins 1963). Light may also play a role as *in vitro* cultures were found to be seasonally sensitive to light (Barker and Collins 1962). During longer day length cycles, rhizomes exposed to brief light would switch from horizontal, growth to vertical, growth (Barker and Collins 1962).

All the shoots associated with the underground rhizome are genotypically identical and phenotypically similar, and are termed a clone (Kender and Eggert 1966).

As the rhizome expands, more aerial shoots are initiated and an approximate age can be estimated from the clone diameter. A diameter of ten centimetres suggest a clone has had under ten years of growth, twenty years of growth will result in a sixty centimetre clone and forty years of growth result in a clone diameter of one hundred centimetres (Vander Kloet 1976). This slow growth pattern has led to a unique production method for a commercial crop whereby fields are developed through land clearing and the promotion of existing plants, in contrast to transplanting seedlings to a specific site (Eaton and Hall 1961). The promotion of existing plants also results in a heterogeneous mix of clones growing in close proximity (Burgher-MacLellan and MacKenzie 2004). In an established field the clones can be distinguished through physical characteristics such as stem colour, stem height, leaf color, flower colour, berry colour and berry size; as well as less visible traits such as berry yield (Burgher- MacLellan and MacKenzie 2004; Hepler and Yarborough 1991).

The patchy nature of a field is maintained as lowbush blueberry is largely self-sterile and requires animal vectors for pollination and seed distribution (Aalders and Hall 1961; Hall *et al.* 1979). This leads to high genetic variability in fields with many small clones in close physical proximity and increased viability as pollinator are more likely to move gametes to a different clone (Burger- MacLellan and MacKenzie 2004).

Commercial fields of lowbush blueberries were originally pollinated by native insects, but increased stem and flower densities have led producers to supplement pollination with honey (*Apis mellifera* L.) and leaf cutter bees (*Megachile spp*.) (Eaton and Murray 1997). Pollinators are sensitive to climate; for example, under cold wet

conditions pollinators do not forage and therefore fruit set could decrease (Hall *et al.* 1982).

Flower Development

Lowbush blueberry is a perennial plant with a biennial flowering process. Individual stems elongate through vegetative growth in the first year. Flower buds develop in the fall of the first year, overwinter, resume growth and development after dormancy and open in the spring of the second year (Bell and Burchill 1955). Flower buds originate on the stem as undifferentiated meristematic tissue, in the leaf axils attachment sites (Bell and Burchill 1955). Through early summer the bud enlarges and the meristematic tissue develops multiple flower primordia. A developing bud contains seven to ten flower primordia arranged with the younger flowers below the older flowers. When the buds break in the spring, the older flowers all appear to be at the same maturity stage, while the youngest flowers are developmentally delayed (Bell 1950).

By early summer of the first year, floral parts are distinguishable. In each flower primordia the meristematic tissues are differentiated in sequence from the outside in. The developmental order is sepals, petals, stamens, carpels, and pistil. This corresponds to the same relative position in mature flowers (Bell 1950). In late summer the sepal, petal and stamen primordia are elevated to their positions above the developing ovary. This is followed by development of the stylar wall and upper portions of the ovary above the apical meristem. The stylar canal is the last structure to develop; the canal opens at the stigma, travels vertically to the top of the placental primordia, and radially into each loculus. Reproductive tissues in the ovules and anthers are found in the bud by early fall (Bell and Burchill 1955).

By autumn, the oldest flower primordia have developed into florets, in which all the flower parts are recognizable, while the youngest flower primordia remain as undifferentiated meristematic tissue, or as meristematic tissues that is just beginning to differentiate (Bell and Burchill 1955). In the winter differentiation of the youngest flowers continued into mid- December, after which there is a dormant period until late February (Bell 1950).

Floral growth begins again in early spring of the second year, with meiosis in the microspores of the anther and in the megaspore of the ovules. Pollen grains mature about a month after the resumption of growth, and the ovary is ready for fertilization in late spring (Bell and Burchill 1955). Once open, the pistil remains receptive for about a week; however fertility decreases with increasing age (Wood 1962).

Flowers begin to open in early spring and full bloom develops two to four weeks later (Bell and Burchill 1955). Flowers are small, bell shaped and face downward with the stigma extending below the stamens (Desjardins and De Oliveria 2006). Flower parts are typically grouped in sets of five, with five sepals, five petals and ten stamens arranged as two whorls of five attached to the corolla, or fused petals (Hall *et al.* 1979). The orientation of the flower and position of the reproductive structures decrease the possibility of self-fertilization and require insect mediation for optimal pollination (Desjardins and De Oliveria 2006). If fertilization occurs through pollination, berry development is initiated. Failure of the zygote or proembryo to develop normally results in the presence of an imperfect seed (Bell 1957). The ratio of perfect to imperfect seeds influences berry size and ripening rate (Ismail and Kender 1974)

Berry Development and Ripening

Fruit ripening occurs at the end of the maturation stage of plant development and is initiated by pollination and fruit set. Individual berry growth is characterized as having a double sigmoid growth curve, where each of the three portions of the curve corresponds to different stages of development and can be related to fruit ripening (Eck 1986). Ripening is characterized by physical and chemical changes that make the fruit attractive and palatable, typically affecting flavours, texture and taste (Gortner *et al.* 1967). There are three ripening stages, pre-maturation, maturation and senescence (Ismail and Kender 1974). A mature berry has reached its full size, with internal chemistry attractive to animal dispersers (Ismail and Kender 1974; Nabors 2004).

The first growth stage occurs in the pre- mature berry, is initiated at fertilization and can last between thirty and sixty days (Eck 1986; Ismail and Kender 1974; Bell 1957). After fertilization, berry tissues undergo rapid cell division leading to endosperm and zygote formation. These changes are externally evidenced by increased berry diameter (Eck 1986; Bell 1957). The second berry growth stage also occurs in the pre-mature berry and is characterized by the embryo growing to penetrate the endosperm plug and the initiation of seed development (Bell 1957). In the second stage there are few outward signs of development, colour remains unchanged and berry diameter increases at a slower rate (Ismail and Kender 1974). The duration of this stage is size dependent; large berries complete stage II growth after eight days, while small berries remain in stage II for 25 days (Ismail and Kender 1974). The third growth stage occurs in the mature berry. In this stage there is a rapid increase in pericarp tissue and water uptake as the berry grows to its full size (Ismail and Kender 1974). Changes in size are accompanied by

changes in colour, from green to red to blue, and biochemical changes such as increases in pH, sugar composition and soluble solid content (Eck 1986). The duration of stage III is similar to stage II and size dependent (Ismail and Kender 1974). Ripening and senescence is the last ripening stage and occurs after the third growth stage (Ismail and Kender 1974; Bell 1957). Senescence is characterized by a decrease in berry firmness (Ismail and Kender 1974).

While all berries pass through each of the three growth stages, they do not all mature at the same rate (Eck 1986). The range of developmental stages within an inflorescence is translated to wide variability in berry maturities on stems, within clones and throughout fields. At any one time a field will contain a majority of berries at similar maturity stages, but a harvest of these berries will result in all maturity stages being collected.

Physical Changes During Ripening

Berry firmness decreases as berries progress from pre-maturation to mature. This change corresponds with a decrease in turgor, a weakening of the cell wall, a decrease in the starch reserves and an increase in soluble solids (Tucker 1993). As berries reach senescence, firmness decreases (Ballinger *et al.* 1973). At all maturity stages larger berries are softer than smaller berries (Ballinger *et al.* 1973).

The colour change that occurs as blueberry fruit mature is caused by anthocyanin synthesis and accumulation (Manning 1993). Unripe fruit are generally green in colour, as a result of high chlorophyll concentrations. As the berries undergo their second increase in size, they also change from green, through red to a blue colour (Ismail and Kender 1974). During this change, chlorophyll is broken down and anthocyanin production

increases (Tucker 1993). The fruit colour at maturity is determined by the type and concentration of the anthocyanins present and can range from orange to deep purple (Tucker 1993).

Biochemical Changes During Ripening

During maturation berries loose acidity and gain sugar resulting in a sweeter flavour when ripe. The composition of fruit phenolics also changes with maturity. In immature berries the composition of phenolic discourages predators from harvesting the berries, while at maturity the phenolic compounds encourages animals to eat and distribute the berries away from the parental plant.

Soluble solid concentrations are low during pre-maturation, increase during maturation and ripening and peak at the end of ripening. Fruit pH remains level until mid-way through ripening, at which point it increases. Total acidity decreases steadily as the berries pass through each stage of development (Ismail and Kender 1974). This decrease is the result of organic acids being utilized during respiration (Tucker 1993).

The sugar content of lowbush blueberries increases with physiological maturity (Barker *et al.* 1963). A fruit acquires sugar from mobilization of starches from within the fruit or through assimilate import. Glucose and fructose are found in every maturity stage, but sucrose is found only in ripe fruit (Barker *et al.* 1963). In lowbush blueberry, sugar concentration is correlated with berry size and the darkness of the surface colour; larger berries have lower sugar levels, and berries with lower sugar levels have a paler surface colour (Kalt *et al.* 1995). However, there is no direct correlation between berry size and surface colour (Kalt *et al.* 2001a).

At the initiation of the maturation stage of ripening, a peak in the respiration rate; and an increased rate of ripening with exposure to ethylene are characteristic of climacteric fruit (Adams-Phillips *et al*. 2004). Blueberries are considered weak climacteric fruits as they do show a peak in respiration rate that corresponds with the onset of maturity, but only a few clones respond to the application of ethephon, a commercial ethylene generating agrochemical, with an increased rate of maturity (Ismail and Kender 1974; Hall and Forsyth 1967; Eck 1986). Application of an ethylene inhibitor to lowbush blueberries did not result in increased berry yield or weight, (Percival and MacKenzie 2007), further suggesting lowbush blueberries are not a climatric fruit. In non-climactric fruits, ethylene inhibition would not extend the ripening period as non-climacteric fruit are not dependent on ethylene generation to complete maturation (Adams-Phillips *et al*. 2004).

Fruit Phenolic Compounds

The colour change in lowbush blueberries results from a change in the type and amount of anthocyanins in the berry (Tucker 1993). Anthocyanins are a group of secondary metabolites found in plant stems, leaves, flowers and fruit (Koes *et al.* 1994). Anthocyanins are widespread within the plant as they have a variety of functions, dependent on the tissue type and maturity (Koes *et al.* 1994). Anthocyanins function to attract pollinators, block UV light, protect against fungal invasion, reduce stress damage and provide astringency, or a drying feel and unpalatable taste (Harborne 2003; Koes *et al.* 1994).

Anthocyanins are classed as polyphenolic compounds, and more specifically, a type of flavonoid (Hrazdina 1982). Phenolics are grouped together because of their

common structure of an aromatic ring with at least one attached hydroxyl group (Taiz and Zeiger 2006). Monophenols, such as phenolic acids, contain a single phenyl ring, and polyphenols, such as stilbenes and flavonoids, contain two rings linked by a C-2 or C-3 carbon bridge, respectively (Macheix *et al.* 1990). The great diversity of phenolic compounds results from the structure of the functional groups attached to the aromatic ring, which can include additional ring structures (Taiz and Zeiger 2006). This group is further classified by the functional group found at the C-3 carbon position and the degree of saturation of the carbon ring (C^1 ring). In the case of anthocyanins, a hydroxyl group is found on the C-3 carbon (Tucker 1993).

All flavonoid compounds are derived from products of primary plant metabolism, phenylalanine and acetyl CoA. Both compounds act as substrates for other biosynthetic and metabolic processes in primary and secondary metabolism (Manning 1993). The first step is the conversion of phenylalanine to 4-coumaroyl CoA acid through the shikimic acid pathway. At the same time, the malonic acid pathway converts acetyl CoA to malonyl CoA. Chalcone synthase joins 4-coumaroyl CoA and malonyl CoA to form naringenin chalcone. From this point onward the pathway is committed to flavonoid biosynthesis; however, this can still result in diverse products such as tannins, stilbenes or anthocyanins (Mol *et al.* 1996). The multiple potential pathways result in competition for substrates and intermediates and are regulated through both substrate availability and from external signals.

The six major anthocyanidins are delphinidin, pelargonidin, cyanidin, petunidin, malvidin, and peonidin (Tucker 1993). Figure 1-1 illustrates anthocyanin biosynthesis. The production of all anthocyanins starts with the interaction of naringenin chalcone and

chalcone synthase (CHS) to produce naringenin. From here there are three potential pathways. In the first pathway, naringenin flavanone interacts with flavanone-3-hydroxylase (F3H) to produce dihydrokaempferol, which is the precursor for pelargonidin. In the second potential pathway, naringenin interacts with flavonoid 3'-hydroxylase (F3'H) to produces eriodictyol which can interact with F3H to produce dihydroquercetin, the precursor to cyanidin and quercetin. In the third pathway naringenin interacts with flavonoid 3', 5'-hydroxylase (F3'5'H) to produce pentahydroxy-flavanone, which can react with F3H to produce dihydromyricetin, the precursor to delphinidin and myricetin. Delphinidin is the precursor to petunidin and malvidin and cyanidin is the precursor to peonidin (Manning 1993; Cortell and Kennedy 2006).

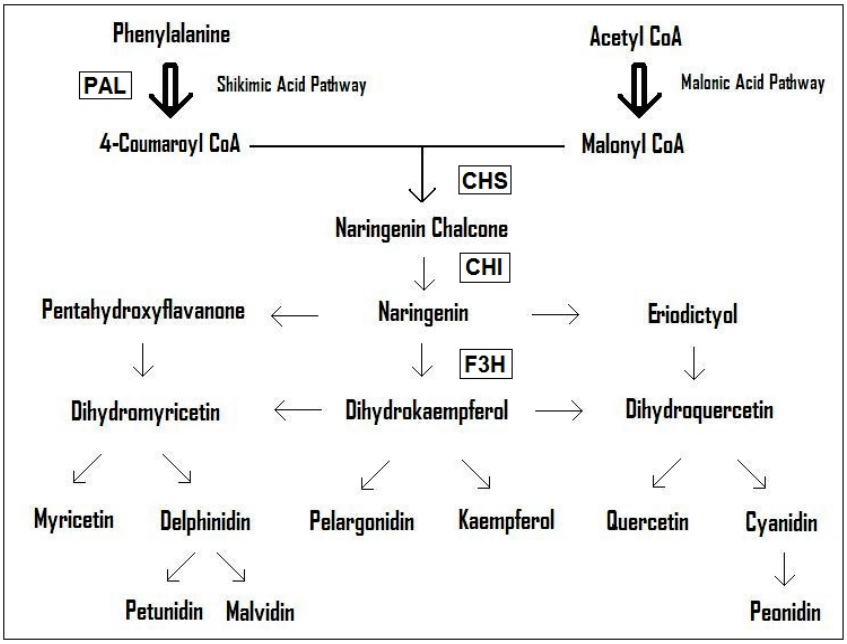

Figure 1-1: Simplified anthocyanin biosynthesis pathway. The anthocyanidins are delphinidin, petunidin, malvidin, pelargonidin, cyanidin and peonidin. The production of naringenin chalcone is the first step that commits the pathway to flavonoid biosynthesis (Manning 1993; Mol *et al.* 1996). Key enzymes are indicated in boxes beside pathway steps. Abbreviations are as follows, PAL (phenylalanine ammonia lyase), CHS (chalcone synthase), CHI (chalcone isomerase), F3H (flavanone 3-hydroxylase).

Each anthocyanidin can undergo glycosylation, to form, 3-galactosides, 3-glucosides and 3- arabinosides (Holton and Cornish 1995). Further diversity results as the glycoside forms undergo methylation or acylation (Tarusico *et al.* 2004). Production of anthocyanins is thought to be under genetic and hormonal control (Tucker 1993). Genetic control is exerted through expression of genes that code for specific enzymes, or through the expression of regulatory genes that control the transcription rate of the enzymatic genes (Jaakola *et al.* 2002). In billberry (*Vaccinium myrtillus*), six key enzymes have been identified, phenylalanine ammonia-lyase (PAL), chalcone synthase, flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP glucose-flavonoid 3-*O*-glucosyl transferase (UFGT), although other enzymes are also involved (Jaakola *et al.* 2002; Cortell and Kennedy 2006). Accumulation of anthocyanins in the billberry fruit requires the expression of all genes expressing the six enzymes (Jaakola *et al.* 2002).

Regulation of colour accumulation through the synthesis of PAL and ANS has been described in strawberries, and appears to be common to soft fruit (Manning 1993). In both grape and billberry, PAL and ANS are activated in early fruit development, the level of enzyme production then decreases during stage II berry growth (Boss *et al.* 1996; Jaakola *et al.* 2002). The early synthesis of PAL and ANS may result in precursor compounds for other flavonol classes, such as isoflavonoid and proanthocyanidins (Boss *et al.* 1996). Colour accumulation occurs in stage III berry growth, and corresponds with an increase in UFGT production (Boss *et al.* 1996; Jaakola *et al.* 2002).

While the regulation of the biosynthesis of anthocyanins appears similar in the soft fruit, the specific anthocyanins found in the fruit are diverse and lead to a range of

colours at maturity. The red colouration of strawberries results from the ratio of pelargonidin- 3-glucoside to cyanidin-3-glucosides (Kalt *et al.* 1993), while the blue of lowbush blueberries result from the mix of delphinidin, cyanidin, petunidin, peonidin and malvidin (Gao and Mazza 1994). Lowbush blueberries also show significant interspecies variation that may relate either to gene expression, environmental effects or interactions between the gene and environmental factors (Kalt *et al.* 1995).

Environmental Influences on Blueberry Fruit Development

On a large scale, latitude and prevailing climate conditions affect the timing of plant development (Hall *et al.* 1979). In general, growth in southern regions is slightly advanced of growth in northern areas (Hall *et al.* 1979). However, regional microclimates can mask this trend between locations of less than one hundred kilometres apart (Bell 1953).

Light, temperature and moisture influence blueberry growth and development in both positive and negative ways. Light is needed for photosynthesis and the initiation of anthocyanin synthesis but is also a source of UV radiation, which can damage deoxyribonucleic acid (DNA) (Taiz and Zeiger 1998). Moisture is needed for respiration and nutrient transport but flooding can result in root rot (Taiz and Zeiger 1998). Temperature plays a role in developmental timing but frost can damage developing floral buds (Hicklenton *et al.* 2002; Olson and Eaton 2001). Each climate factor plays a role in plant vegetative and root growth, berry development and berry ripening.

<u>Light</u>

Both light intensity and light duration affect vegetative growth and floral bud development in the lowbush blueberry (Hall *et al.* 1979). Photoperiods of sixteen hours or

greater promotes vegetative growth and decreases floral bud development (Hall 1958; Aalders *et al.* 1969). Between eight and sixteen hours of light results in variable floral bud development and some vegetative growth and below eight hours of light exposure, vegetative growth decreases and floral buds develop (Hall 1958; Aalders *et al.* 1969).

Under both field and laboratory conditions, increased light intensity results in increased photosynthesis and development. In forested conditions, blueberry plants required a canopy of less than fifty percent shade before flowering; where plants were ninety percent shaded vegetative growth but no flowering occurred, and at ninety-five percent shade, plants exhibited no new growth (Hall 1955). Under controlled conditions, increased light intensity resulted in a higher apparent photosynthetic rate, indicating an increased rate of carbon fixation (Bonn *et al.* 1969). Decreasing the light intensity resulted in plants with smaller stem diameters, increased branching and fewer floral buds (Hall and Ludwig 1961; Hall 1958).

Light drives photosynthesis and assimilate accumulation in plants (Krause 2006). The relationship between light and photosynthesis is not linear, instead there is a point at which the plant is light saturated and light is no longer the limiting factor in photosynthesis (Krause 2006). Lowbush blueberry clones differ in how much light is required for light saturation, ranging between 500-800 $\mu mol \bullet s^{-1} \bullet m^{-2}$ (Hickleton *et al.* 2000b). If light intensity continues to increase beyond the light saturation levels, photosynthesis can be impaired due to photoinhibition (Taiz and Zeiger 1998). Highbush blueberries grown in Chile were found to have increased yields after being shaded by coloured nets (Retamales *et al.* 2008). Coloured nets decreased the photosynthetically active radiation (PAR) received by the plants by 29%, which resulted in increased yields

through greater fruit set. Black nets decreased PAR by 53%, which promoted vegetative growth (Retamales *et al.* 2008). Shading prevented the plants from becoming photoinhibited, while the quality of light coming through the netting triggered different plant responses (Retamales *et al.* 2008). In northern latitudes, overlapping leaves and branches prevent plants from being fully light saturated (Hickleton *et al.* 2000b).

The photosynthetic rate of plants varies with time of day, age of the plant, lifecycle stage and season (Hall *et al.* 1972; Forsyth *et al.* 1965; Hickleton *et al.* 2000b). Photosynthesis is greatest in the morning, as low water stress results in greater stomatal opening and increased gas exchange (Taiz and Zeiger 1998). Increased photosynthesis in the morning may facilitate the replenishment of carbohydrate reserves used in respiration during the night (Hall *et al.* 1972). Once leaves have fully developed, newer leaves were found to have a higher rate of apparent photosynthesis than older leaves (Forsyth and Hall 1965). New shoots have a greater photosynthetic ability than overwintered shoots, possibly because apical meristems act as a stronger assimilate sink than non growing tissues (Hickleton *et al.* 2000b).

A plant's photosynthetic potential is low in the spring as a result of young leaves not having complete photosynthetic capabilities (Hicklenton *et al.* 2000b). By early summer the leaves are fully functional and increases in day length result in peak photosynthetic capability. Photosynthetic capability gradually decreases as the day lengths shorten into fall (Hicklenton *et al.* 2000b). In field grown blueberries, the timing of the peak and duration of assimilate accumulation differs when the plants are in a vegetative year versus a fruiting year (Hickleton *et al.* 2000b). In a vegetative year, the assimilate accumulation is maintained into the fall and is transferred to vegetative and

floral buds, roots and rhizomes. In a fruiting year, assimilate accumulation declines after fruit harvest, with the loss of berries as a sink (Hickleton et al. 2000b).

Light is required for the full expression of the genes involved in anthocyanin biosynthesis (Dokoozlian and Kliewer 1996; Zhou and Singh 2002; Mancinelli 1985). In both grapes (*Vitis vinifera* L.) and cranberries (*Vaccinium oxycoccos* L.), fruit exposed to only dark conditions have reduced anthocyanin pigmentation (Dokoozlian and Kliewer 1996; Zhou and Singh 2002). Exposure to light increases the biosynthesis of anthocyanin regulating genes resulting in anthocyanin accumulation and a darker pigmentation (Zhou and Singh 2002). While short bursts of irradiation result in small quantities of anthocyanins, prolonged exposure to light is needed for the large amounts of anthocyanins found in ripe fruit. However, the longer exposure does not have to be continuous to result in the accumulation of anthocyanins (Mancinelli 1985).

There are two models proposed for the initiation of plant anthocyanin biosynthesis. The first suggests that initiation results from exposure to red light, and the second has biosynthesis being initiated from exposure to UV and blue light (Mancinelli 1985). In both models incoming light interacts with phytochromes localized in specific plant tissues, such as the leaves, flowers or fruit and induces a conformational shift. The conformational shift is translated through a signal transduction pathway and initiates anthocyanin biosynthesis (Manicelli 1985).

The biochemical steps between phytochrome activation and the initiation of anthocyanin production are not clear. It is suspected that signal transduction involves the phosphorylation of proteins, activation of heterotrimeric GTP-binding proteins and

changes in phosphatidylinositol and calcium metabolism (Huub *et al.* 1997). It is known that plants have a range of different phytochromes that respond to different wavelengths of light (Kim *et al.* 2003). Incoming light induces a conformational change from phytochrome red (Pr) to phytochrome far red (Pfr). The Pfr form can interact reversibly with a signalling factor such as phytochrome interacting factor 3 (PIF3). PIF3 is bound to the G box element of the CHS gene promoter and acts as a transcription factor that regulates the expression of the chalcone synthase (CHS) gene, an early step in anthocyanin synthesis (Kim *et al.* 2003). PIF3 can act as both a positive or negative regulator of light responses, although the mechanism for determining the direction of response is not clear. However, the response may result from light quality and duration (Kim *et al.* 2003). Pr-Pfr phytochrome is implicated as the initiation trigger for anthocyanin biosynthesis in cranberries. When kept at a constant temperature, anthocyanin production was initiated and maintained by exposure to red light; while exposure to UV light did not result in anthocyanin accumulation (Zhou and Singh 2002).

Grape growth follows a double sigmoid growth pattern, similar to blueberries (Ismail and Kender 1974; Dokoozlian and Kliewer 1996). In grapes, the initiation of anthocyanin production occurs in stage III (Dokoozlian and Kliewer 1996). Grapes grown with no light in any of the three stages produced berries with the lowest anthocyanin content and grapes given light in all three stages produced berries with the highest anthocyanin content. However, when grapes were grown with no light in stages I and II and given light at stage III they had a lower anthocyanin concentration then when grown with light in stages I and II but no light at stage III (Dokoozlian and Kliewer 1996). This implies the critical stages for receiving light which results in anthocyanin production is

while the berries are still green and before they begin to mature (Dokoozlian and Kliewer 1996). Similarly experiments with tomatoes showed that light is not essential for the induction or accumulation of lycopene, the carotenoid colour pigment, found in the pericarp after the immature green stage (Alba *et al.* 2000).

Anthocyanin biosynthesis in grapes follows a similar pathway to that of bilberry (Boss *et al.* 1996; Jaakola *et al.* 2002). Similar to bilberry, regulation of the pathway occurs through enzymatic gene expression and many of the enzymatic steps are the same, except leucoanthocyanidin dioxgenase (LDOX) is found in grapes instead of anthocyanidin synthase (ANS) as the second to last step in the pathway (Boss *et al.* 1996; Jaakola *et al.* 2002). During stage I growth in grapes all enzymatic genes except UFGT, the last step in the pathway, are expressed. In stage II growth no enzymatic genes are expressed. Stage III growth corresponds with ripening and colour accumulation, and in this stage PAL gene expression is absent but all subsequent enzymatic gene including UFGT are expressed (Boss *et al.* 1996). This pattern suggest exposure to light in stage I results in PAL expression and the accumulation of proanthocyanidins as a percursor pool that is utilized at the start of stage III for the production of anthocyanins (Bogs *et al.* 2005).

Temperature

Plant development is intricately linked to temperature through both direct and indirect effects. Directly, the rate of biochemical reactions such as photosynthesis and anthocyanin accumulation, are regulated by the temperature at which they are occurring (Mori *et al.* 2005; Taiz and Zeiger 1998). Further, key developmental events, such as winter dormancy and bud break occur around specific temperature thresholds (Rowland

et al. 2008; Cappiello and Dunham 1994). Temperature can also have indirect effects on development, for example by influencing pollinator activity (Hall *et al.*1963).

Biochemically, changes in temperature influence the rate at which CO_2 is fixed (Taiz and Zeiger 1998). In controlled laboratory studies, photosynthesis can occur between 13-29^{o}C (Forsyth and Hall 1965). In the field the optimal temperature for net photosynthesis, or the rate of photosynthesis minus the respiration rate, is around 25^{o}C (Glass *et al.* 2005a)

In relation to berry ripening, temperature seems to be a more important factor than ambient light levels (Shutak *et al.* 1956). In growth chamber experiments, increased temperatures affected the developmental rate, but not berry size at maturity (Hall and Aalders 1968). Cooler ambient temperatures on Cape Breton Islands, as compared to mainland Nova Scotia, result in decreased fruit set and slower berry development (Hall *et al.* 1963)

Fruit flavonoid and anthocyanin synthesis are directly affected by temperature (Kalt *et al.* 2001b; Kalt *et al.* 2003). Blueberries from cooler geographic regions are found to have increased flavonoid synthesis and after harvest, blueberries will continue to accumulate anthocyanins at room temperature (20-22^{o}C) but pigmentation is inhibited by cooler or warmer temperatures (Kalt *et al.* 2001b; Kalt *et al.* 2003).

In grapes, increased night temperatures have been found to affect only the flavonoid biosynthetic pathway and not the time to maturity (Mori *et al.* 2005). Temperature increases in growth stages I and II do not impede colouration, whereas an increase in temperature at the start of growth stage III results in a decrease in colouration

(Mori *et al.* 2005). This is because increased temperatures inhibit UFGT activity, the last step in anthocyanin biosynthetic pathway and the step that is responsible for colouration (Mori *et al.* 2005). It is unclear how UFGT activity is inhibited, but it is possible that increased temperatures destabilize the enzyme, substrate or structural gene, inducing a conformational change (Mori *et al.* 2005). Changes in temperature can also shift the composition of the anthocyanins found in the fruit (Cohen *et al.* 2008). Decreased daytime temperatures resulted in an increase of cyanidin and peonidin and a decrease in acylated forms (Cohen *et al.* 2008).

Cold acclimation, dormancy and deacclimation are all directly related to seasonal temperature (Rowland *et al.* 2008). Cold acclimation occurs in response to two environmental cues, short photoperiods and low temperatures and appears to coincide with the accumulation of a dehydrin-like protein (Arora *et al.* 1997). In the greenhouse, researchers could trigger cold adaptation by subjecting plants to short day lengths and a constant 4^{o}C temperature for half of the required chilling hours that would normally trigger dormancy (Arora *et al.* 1997). In the field, cold adaptation occurred when fluctuating cold temperatures resulted in half the chilling hours being met (Arora *et al.* 1997). The degree of cold hardiness has a genetic component. Cultivars with germplasm adapted to southern latitudes were less tolerant of freezing conditions than cultivars that contained lowbush blueberry germplasm (Rowland *et al.* 2005). Further, cold adaptation varied with plant age, in that older or more developed flowers were better able to tolerate and recover from freezing (Hicklenton *et al.* 2002).

Another direct effect is between temperature and growth. In areas with shorter day lengths and cooler average temperatures, plants exhibit less vegetative growth and fewer

flower buds develop (Hall *et al.* 1963). Cold winter conditions can lead to shoot tip damage and less vegetative growth in the following year (Hall *et al.* 1979). Under laboratory conditions an increase of 11^{o}C resulted in stems growing three times longer than control plants (Hall and Ludwig 1961).

Warmer temperatures promote the formation of larger floral buds with an increased number of floral primordia and increases the developmental rate within the bud (Hall *et al.* 1970). Conversely, cooler temperatures resulted in the formation of fewer flower buds formed (Hall *et al.* 1970). Floral buds are most sensitive to temperature in early spring (Hall *et al.* 1979). During the winter, plants and buds are protected from frost damage by the snow cover (Hall *et al.* 1979). However, as snow cover melts in the spring and temperatures become more variable, there is an increased risk of winter injury and frost damage. Plants appear to be most sensitive just before flowers open. The effects of freezing at this stage are temperature and duration dependent (Hicklenton *et al.* 2002). Temperatures below 0 ^{o}C adversely affect fruit set (Hicklenton *et al.* 2002). In controlled experiments the greatest damage occurred two to four hours after freezing and buds were always damaged when the temperature dropped below -3^{o}C (Hicklenton *et al.* 2002). Tissue necrosis was evident in flowers that were open when frozen, and closed flowers often failed to open normally after being frozen despite having no visible damage (Hicklenton *et al.* 2002). Examination of floral buds taken from the field after a spring frost in Nova Scotia showed that ninety percent of sampled fruit buds sustained tissue damage that would interfere with fruit set and half of unopened floral buds had damage to placental tissue that would likely affect fruit set (Olson and Eaton 2001).

Indirectly temperature can influence the activity of pollinators, which will affect the percent fruit set, as well as berry size. Cool, wet weather in the spring when the flowers bloom dampens the activity of pollinators and results in lower fruit set (Eaton and Murray 1997). Cool weather can also increase the mortality of the hatch, resulting in fewer active pollinators (Stubbs *et al.* 1994). Temperature can also have an indirect effect by controlling the severity of disease outbreaks. Cool weather in the spring limits the spread of *Botrytis cinerea* blight from the flower corollas to the ovaries, while spring temperatures above 20^{o}C, combined with dampness, can result in rapid spread of the blight (Hilderbrand *et al.* 2001).

Moisture

All plants require water as a principal component of photosynthesis (Taiz and Zeiger 1998). In the light reaction of photosynthesis, water is oxidised and the electrons used to produce NADPH and ATP (Taiz and Zeiger 1998). Insufficient access to water results in decreased photosynthetic efficiency and is termed water stress (Taiz and Zeiger 1998). In lowbush blueberries water stress results in stomata closing, decreasing CO_2 availability and lowered photosynthetic ability (Taiz and Zeiger 1998; Hicklenton *et al.* 2000a).

Lowbush blueberries are generally drought tolerant, because of a deep root system, which is further extended through mycorrhizal associations, and lateral transport though the rhizome system (Glass *et al.* 2005b). A mature lowbush blueberry plant can have a root system which extends below forty centimetres in depth (Hall 1957; Hicklenton *et al.* 2000b). In coastal areas the water uptake through the roots is further

supplemented by atmospheric water gain, and plants can acquire as much as twenty two percent of their water uptake from fog and mist (Starr and Yarborough 2006).

Different clones and different plant life stages exhibit varying abilities to tolerate water stress (Hicklenton *et al.* 2002b; Glass *et al.* 2005b). Water stress in the vegetative year results in increased vegetative and root growth and decreases the number of floral primordial established within a floral bud and subsequent flowers and fruit in the following year (Benoit *et al.* 1984; Glass *et al.* 2005a; Glass *et al.* 2005b). In the crop year, water stress results in smaller fruit, with increased soluble solid content (Hickenton *et al.* 2002; Glass *et al.* 2005b). In grapes, water stress has been shown to alter the polyphenols in wines (Peterlunger *et al.* 2005; Chalmers *et al.* 2008). However, it is unclear if this is due to a shift in the biosynthetic pathway or changes to the anthocyanin content during aging (Chalmers *et al.* 2008).

Particular berry stages are also sensitive to too much moisture. There is an indirect effect when rain occurs when flowers are receptive to pollination, as pollinators are less active in cold and wet conditions (Hall *et al.*1963). Fewer pollination events results in fewer seeds and smaller berries at maturity (Barker *et al.* 1963). Excess moisture when berries are mature can result in softer berries, which could lead to handling damage at harvest (Seymour *et al.* 2004).

Influence of Climate on Berry Yield

Nova Scotia has a modified continental climate where the broad temperature range experienced over a year is moderated by ocean temperature. The extent of moderation decreases with distance from the coast, such that interior regions have warmer summers and colder winters than coastal regions (Kinsman 1993).

The eight climate regions of Nova Scotia are delineated by prevailing winds, temperature regimes and altitude (Kinsman 1993). Coastal areas include, the Atlantic coastal region, the Bay of Fundy region and the Northumberland Shore. The Atlantic coastal region and the Bay of Fundy region both have cool summers and warm winters. The Northumberland strait is a shallow body of water that does not provide the same moderating effect as the Bay of Fundy or Atlantic ocean, resulting in warmer summers, colder winters and a delayed spring in the Northumberland shore region (Kinsman 1993). Interior climatic zones include the Annapolis valley region, Western Nova Scotia, Eastern Nova Scotia, Northern Nova Scotia and the Cape Breton highlands. Climate in these regions is strongly influenced by altitude, the low point of Nova Scotia occurs in the Annapolis valley, and the greatest altitude occurs in the Cape Breton highlands. The Annapolis valley is generally warmer than the Cape Breton highlands (Kinsman 1993). The majority of Nova Scotia blueberry production occurs in the Northern and Eastern Nova Scotia regions (Kinsman 1993).

Fluctuations in crop yield have been linked to periodic weather events such as early warming followed by a cold period (Hall *et al.* 1979), frost damage in the spring (Olson and Eaton 2001), and to prevailing weather conditions (Hall *et al.* 1963). While broad climate conditions were not a good predictor of provincial yields, localized weather conditions were weakly correlated with regional yields (Hall *et al.* 1982). For example, heavy rainfall during bloom correlated with a decrease in yield in Newfoundland, while sunshine during pollination and warm temperatures in the month after pollination correlated with increased yield in Nova Scotia (Hall *et al.* 1982). This suggests a links between microclimate and yields in local fields.

Growing Degree Days (GDD) is the most common measure to link a climate variable, temperature, to crop development (Wang 1960). Growing degree days are a measure of heat accumulation over time, above a base temperature (Carlson and Hancock 1991). The base temperature is the lowest temperature at which the plant is physiologically active (Carlson and Hancock 1991). Rough models use generalized base temperature, such as 5^{o}C for legumes, but more accurate models choose crop and cultivar specific base temperatures (Gordon and Bootsma 1993; Carlson and Hancock 1991).

Growing degree days can be used as a general measure of a growing season, by comparing the number of heat units accumulated to a specific date and determining if they are more or less than the heat units accumulated to that same date in previous years (Gordon and Bootsma 1993). Choosing one date can be misleading as a cold spring followed by a hot summer can accumulate the same number of GDD as a warm spring followed by a cold summer (Wang 1960). Since plants can respond differently to the same temperature at different developmental stages, the two scenarios can have different results (Wang 1960).

Growing degree days can also be linked to specific developmental processes such as flowering time, crop maturity, or optimal ripeness (Bonhomme 2000). Further, growing degree days have been used to anticipate insect development or fungal sporulation, allowing producers to predict the best times to spray crop protectants (Bonhomme 2000). Once the number of growing degrees required for a specific developmental process has been determined, the accumulation of heat units can be monitored and used to predict optimal harvest dates (Wang 1960; Gordon and Bootsma 1993).

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Chapter 2: Characterization of Fruit Ripening of Lowbush Blueberry: Changes of Selected Phenolic Compounds and Physico-Chemical Parameters

Abstract

Major polyphenolics and selected physico-chemical parameters in relation to fruit maturity of lowbush blueberry were examined. Eighteen major phenolic compounds classified as phenolic acids, flavonols, flavan-3-ols or anthocyanins were determined in berries of four maturity stages: (i) green, (ii) pink/red, (iii) blue and (iv) over-mature. Overall, dry weight-based concentrations of flavonols, flavan-3-ols and phenolic acids decreased during ripening while anthocyanins increased. Chlorogenic acid was the most abundant phenolic acid, and quercetin-3-galactoside the most abundant flavonol in all maturity stages. Epicatechin was the most abundant flavan-3-ol in the green stage, but catechin was the most abundant in red, blue and over mature berries. Rapid increase of concentrations of glucose and fructose and decrease of organic acids were observed during fruit development and ripening. Among the six organic acids that were found, quinic acid was the most abundant throughout the fruit ontogeny. Soluble solids, pH, and density increased with maturity, while titratable acidity decreased. The total antioxidant capacity measured using FRAP and ORAC assays remained similar in all four maturity stages of lowbush blueberry.

Introduction

Fruit phenolics are synthesised through two different pathways using precursors from primary metabolism (Manning 1993). The shikimic acid pathway produces the major groups of simple phenolics and lignins. The malonic acid pathway produces the precursor, malonyl CoA, which combines with products of the shikimic acid pathway results in the major classes of flavonoids (Figure 2-1) (Manning 1993). During synthesis there is a diversity of routes a substrate can follow. Early on, the substrate can be used as a precursor for reactions in either primary or secondary metabolism. Once committed to phenolic biosynthesis, a secondary metabolic pathway, the substrate can be transformed into any of the major classes of phenolic compounds and there is further diversity within a class (Macheix *et al.* 1990). For example, there are at least seventeen naturally

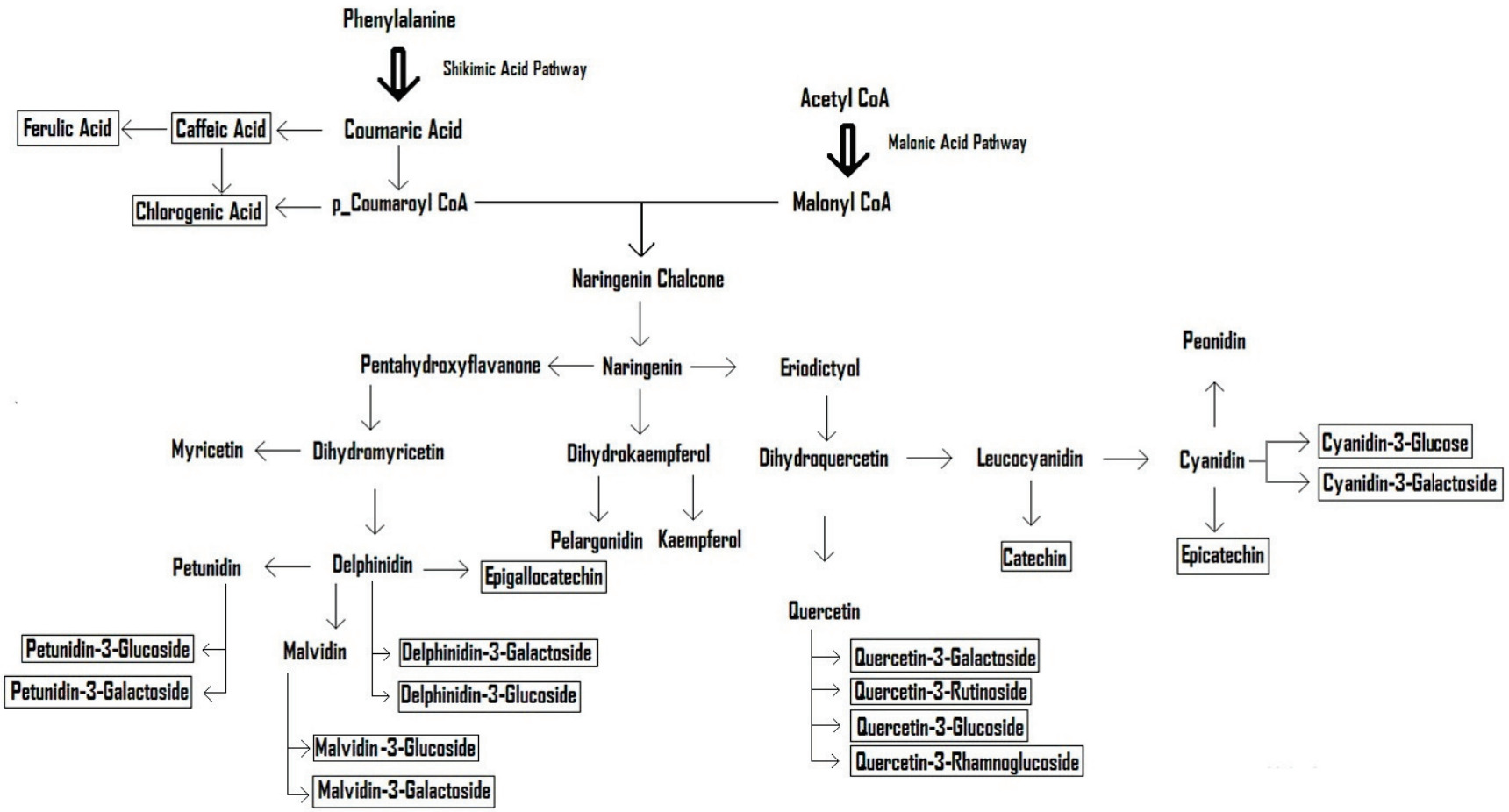

Figure 2-1: General polyphenolic biosynthetic pathway. Compounds in boxes indicate compounds of interest in this study. Not all intermediates are listed.

occurring anthocyanidins, which can then be further modified through the attachment of different sugars (Mazza and Miniati 1993). The multiple potential pathways result in substrate competition and are regulated through the production and activation of enzymes (Machiex *et al.* 1990). The biosynthesis of phenolic compounds in fruit is strongly influenced by genetics, maturity, and environmental conditions (Machiex *et al.* 1990).

A genetic basis to phenolic biosynthesis is evident, as at maturity cultivars express the same phenolic profiles, while the composition and concentration of phenolic classes varies among species (Machiex *et al.* 1990; Kahkonen *et al.* 2001; Tarusico *et al.* 2004). Anthocyanins predominated in billberry, cranberry and blueberry, while flavonols and procyanidins were the primary constituent in cowberry (Kahkonen *et al.* 2001; Percival 2006). Total phenolics and antioxidant capacity of berries are also variable among species, as these measures reflect the phenolic composition present during sampling (Prior *et al.* 1998). Fruit of the *Vaccinium* genus contain high levels of phenolic compounds compared to other fruit sources (Kalt *et al.* 2008; Machiex et al. 1990), and lowbush blueberries had consistently higher antioxidant capacity, than highbush blueberries (Kalt *et al.* 2001a).

Maturation effects on the phenolic biosynthesis are evident as antioxidant capacity and total phenolic content increase with berry maturity (Prior *et al.* 1998). During ripening, total phenolic content tends to increase at the initiation of fruit set, and then depending on the fruit, the total phenolic content will remain at peak levels through maturation, decrease slightly through maturation or will undergo a second increase when anthocyanins are accumulated (Machiex *et al.* 1990). Even when total phenolics stay the same through maturation, the composition of sub-classes that contribute to total phenolics

can change. In cranberry, flavonols and proanthocyanidins decreased during fruit maturation and anthocyanins increased once ripening was initiated (Vvedenskaya and Vorsa 2004).

During ripening, blueberries undergo a distinct colour change from green in the immature stage to blue at full maturity (Ismail and Kender 1974). The colour change occurs as a result of simultaneously occurring chlorophyll degradation and anthocyanin accumulation in the skin of the berry (Brouillard *et al.* 1997). Immature red highbush blueberries contain all fifteen anthocyanins; the glucosides, galactosides and arabinosides of cyanidin, delphinidin, malvidin, peonidin and petunidin. However, the galactoside of peonidin is not present in ripe berries (Makus and Ballinger 1973). Mature lowbush blueberries contain all fifteen anthocyanins (Gao and Mazza 1994). The amount and mixture of anthocyanins present determines the specific berry colour (Gao and Mazza 1994). Large quantities of peonidin will result in red colouration, while the presence of delphinidin results in purple-blue pigmentation (Gao and Mazza 1994). Ripe lowbush blueberries exhibit a range of colours at maturity, corresponding to variations in the anthocyanin content of different clones (Kalt *et al.* 1995).

Environmental factors affect the polyphenolic biosynthesis pathway through the initiation, inhibition and turnover rate of enzymes (Machiex *et al.* 1990). Light exposure has the strongest influence acting both directly and indirectly (Machiex *et al.* 1990). Indirectly, increased light increases photosynthesis, which results in the synthesis of carbohydrate and other carbon structures that can be used as the precursors of phenolic synthesis and their glycosylation (Machiex *et al.* 1990). Directly, exposure to light activates phenylalanine ammonia lyase (PAL), the first and key regulatory enzymatic step

in phenolic biosynthesis, while a PAL inhibitor, possibly 2-aminoindan-2-phosphonic acid (AIP), is activated by exposure to dark conditions (Machiex *et al*. 1990; Appert *et al.* 2003). Bilberry, which also belongs to the *Vaccinium* genus, showed greater expression of five flavonoid pathway genes, flavanone 3-hydroxylase (F3H), PAL,chalcone synthase (CHS), dihydroflavonol 4-reductase (DFR) and anthocyanidin 3-hydroxylase (ANS) when grown under direct light as compared to shaded conditions (Jaakola *et al.* 2004).

Temperature influences are evident as blueberries from cool geographic regions were found to have high antioxidant capacity, the result of increased flavonoid synthesis (Kalt *et al.* 2001b). Post harvest, anthocyanin accumulation continues at room temperature but is inhibited by cooler or warmer temperatures (Kalt *et al.* 2003). It is unclear how temperature affects the accumulation of phenolics. Both the first enzymatic step, PAL and the last enzymatic step (UDP Glc-flavonoid 3-O-glcosyl transferase (UFGT)) in anthocyanin biosynthesis have been proposed to be inhibited by increased temperature (Machiex *et al.* 1990; Mori *et al.* 2005). Possible mechanisms for inhibition include conformational changes in the enzyme, or inhibitory feedback loops, in which the product of the reaction compatitvely inhibit the active site for the enzyme (Machiex *et al.* 1990; Mori *et al.* 2005; Sato *et al.* 1982). The competitive inhibitors for PAL are cinnamic acid derivatives (Sato *et al.* 1982).

Processing and storage conditions impact the recovery of total phenolics, total anthocyanins and the antioxidant capacity of lowbush blueberry fruit (Kalt *et al.* 2000; Lee and Wrolstad 2004). Increasing the temperature to 60 ^{o}C and storage in oxygen rich environments results in increases in total anthocyanin (Kalt *et al.* 2000; Zheng *et al.* 2003). High oxygen rich environments increase total phenolics (Zheng *et al.* 2003).

Antioxidant capacity decreases as pH increases and increase with increasing oxygen levels (Zheng *et al.* 2003).

Three standard methods of measuring antioxidant capacity are, the Ferric Reducing Antioxidant Power (FRAP), the Folin-Ciocalteu (F-C) and, the Oxygen Radical Absorption Capacity (ORAC) assays. F-C can also be used to estimate the total phenolic content (Prior et al. 2005). ORAC and FRAP assays use different methods to measure antioxidant capacity, the FRAP assay measures the antioxidant capacity based on a single electron transfer (SET), while ORAC measures antioxidant capacity based on hydrogen atom transfer (HAT) (Ou *et al.* 2002). In the FRAP assay, the electron moves from the antioxidant to the iron oxide, which changes the UV absorption spectrum of the solution. The change in the absorption spectrum can be related to antioxidant capacity (Ou *et al.* 2002; Singleton *et al.* 1999). With this methodology, the measured values indicate the amount of transmittance through the solution. As antioxidants are added to the solution, the colour changes from clear to blue and less of the source light is passed through the solution, and the measured transmittance decreases (Prior *et al.* 2005; Ndhlala *et al.* 2010). In the ORAC assay, a radical generator combines with oxygen to form a peroxyl radical, which combines with a substrate (fluorescence probe) to initiate a radical chain sequence. When antioxidants are present in the solution, the peroxyl radial removes hydrogen from the antioxidant, resulting in a stable fluoresce probe, which stops or reduces radical chain sequences (Ou *et al.* 2002). In the ORAC assay, higher antioxidant potentials are indicated by increasing values, as additional antioxidants in the solution react with the fluorescence probe to form increasing amounts of a measured non-fluorescent product (Prior *et al.* 2005; Ndhlala *et al.* 2010). The F-C assay works through

a mix of electron and hydrogen atom transfers. The reaction starts with the phenolic compound being oxidized and releasing a hydrogen atom. The hydrogen atom then interacts with molybdate, changing the colour of the solution, which can be related to antioxidant capacity (Ndhlala *et al.* 2010).

While polyphenolic content has been characterized among and within species, and some subclasses have been examined in relation to maturity, there has been no description of the accumulation of different polyphenolic classes during fruit ontogeny. Therefore, the objective of this study was to understand the accumulation of major polyphenolic classes and other fruit quality attributes during fruit ontogeny of lowbush blueberry.

Materials and Methods

Plant Materials and Chemicals

Three distinct clones were marked at one location, Debert, Nova Scotia and fruits were harvested from July to October 2007. The clones consisted of one unnamed wild clone, and two planted clones, Chignecto and a hybrid of 79-12 and Brunswick. These clones were chosen for their large size to increase the likelyhood of sufficient quantaties of berries would be collected from each of the maturity classes. All three clones were wild pollinated and managed with regular industry practices by the Wild Blueberry Institute of Nova Scotia.

Individual berries (approximately 500 g) in four maturity classes, green, pink/ red, blue and over-mature (berries which remained blue but were in poor condition due to splitting, a decrease in firmness or a shrivelled nature) were hand harvested. As the maturity classes follow in succession, only a few berries between 15 g and 100 g of each maturity class were collected at a time, this ensured subsequent maturity classes would

develop on the clone. Collection dates were sporadic, at least weekly at the start in July and increasing to daily in September and October. Berries were transported in coolers to the Nova Scotia Agricultural College. Chemical measures were determined from a composit of fruit collected over time. The first 100 g, in five 20 g replicates, of fresh fruit from each maturity stage was processed for density using water displacement. The remainder of the samples were immediately frozen at -20 ^{o}C. Frozen berries were used to determine polyphenolic profiles, sugar and organic acid content, antioxidant capacity, titratable acidity, soluble solid content and pH.

The liquid chromatography standards used for the polyphenolic analysis were purchased as follows: caffeic acid (Caf), ferulic acid (Fer), quercetin-3-rutinoside (Q3-Rut), epigallocatechin (EGC), catechin (Cat), epicatchin (Epicat), cyanidin-3-glucoside (C3-Glu), petunidin-3-glucoside (P3-Glu), petunidin-3-galactoside (P3-Gal), delphinidin-3-glucoside (D3-Glu), delphinidin-3-galactoside (D3-Gal), malvidin-3-glucose (M3-Glu) and malvidin-3-galactoside (M3-gal) from ChromaDex (Santa Ana, CA. U.S.A). Quercetin-3-galactoside (Q3-gal), and quercetin-3-rhamnoside (Q3-Rha) were purchased from Indofine Chemical Co. (Hillsborough, N. J., U.S.A). Quercetin-3-glucoside (Q3-Glu) and chlorogenic acid (Chl) were purchased from Sigma-Aldrich (St. Louis, MO, U. S. A.) and cyanidin-3-galactoside (C3-Gal) from Extra-synthese (Paris, France).

Gallic acid, sodium carbonate, sodium acetate trihydrate, 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), Trolox, fluorescin, Folin-Ciocalteu reagent, fluorescein sodium salt, ferric chloride and phosphate buffer were obtained from Sigma-Aldrich (St. Louis, MO). 2.2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Walco

Chemical Products Co. Inc., (Buffalo, NY, U. S. A.). Hydrochloric acid, and 96-well microplates were purchased from Fisher Scientific (Ottawa, ON).

Sample Preparation for Phenolics and Antioxidant Capacity Analysis

Three replicates of 50 g frozen berries were used to determine the polyphenolic profiles and antioxidant capacity. The extraction procedure and solution were optimized in a previous trial (Appendix B). The berry samples were freeze dried in a freeze dryer (model 2085C0000, Kinetics Thermal systems, Stone Ridge, NY) for 60 hours. For the first 24 hours the temperature was kept at – 25^{o}C. The berries were then removed, partly crushed and returned to the freeze dryer. The temperature was set to -25^{o}C for five hours, then raised to 5^{o}C for 16 hours, and then raised to room temperature for the remaining time.

Upon removal the berries and containers were weighed to determine the percent moisture loss, then ground to a powder using a coffee grinder (model DCG 12BCC, Cuisinart, Woodbridge, ON). Approximately 0.3 g of powdered berries was mixed with 15 ml of extraction solution (MeOH:Acetone:Water:Formic acid-40:40:20:0.1). Samples were sonicated (VWR 750D Sonicator) in fifteen minute increments, with a ten minute break between rounds, for a total of 45 minutes. After sonication, the samples were centrifuged (Precision Durafuge model 3001, Colonical scientific, Richmond, VA, USA) at 5000 rpm for 15 minutes. Two millimetres of the supernatant was filtered through 0.22 µm nylon filters before use for high performance liquid chromatography (HPLC) analysis.

HPLC Mass Spectrometery Analysis

Concentrations of phenolic acids, flavan-3-ols, flavonols, and anthocyanins were determined using HPLC coupled to electrospray ionization and triple quadrupol mass spectrometry as described by Rupasinghe *et al.* (2008).

Electrospray ionization in negative ion mode was used for flavonols, flavan-3-ols and phenolic acids as follows: Q3-Rut m/z 448 → 301, Q3-Gal and Q3-Glu m/z 463 → 301, Q3-Rha m/z 447 → 301, Chl m/z 353→ 191, Caf m/z 179 →35, Fer m/z 193 → 134, EGC m/z 457 → 169, Cat m/z 289 →109, and Epicat m/z 290 → 109. Positive ion mode electrospray ionization was used for the anthocyanins as follows C3-Glu m/z 448.8 → 286.8, C3-Gal m/z 449 → 287, D3-Glu m/z 465 → 302.6, D3-Gal m/z 465 → 302.6, P3-Glu and P2-Gal m/z 479 → 316.8, M3-Glu and M3-Gal m/z 493 → 330.8.

Total Phenolic and Total Antioxidant Capacity Assays

The sample extracts were also used in three assays: FC that measures total phenolics, FRAP, and ORAC that measure the total antioxidant capacity. The procedures described by Huber and Rupasinghe (2009) were used for the above three assays.

Determination of Sugar and Acid Profiles

Freeze dried berry powder (1.5 g) was mixed with 15 ml of extraction solvent (MeOH:Acetone:Water:Formic acid - 40:40:20:0.1). Samples were sonicated twice for 15 minutes with a 10 minute interval between them, for a total of thirty minutes. Eight millilitres was removed and the solvent was removed to complete dryness (UV vacuum system, Thermo Electra Corporation, Waltham, MA). The samples were re-dissolved in 5 ml of water and vacuum filtered through a C-18 Bond Elut 500 mg SEP cartridge (Varian Canada Inc., Mississauga, ON). Approximately 1.5 ml of eluate was collected and filtered

through a 0.22 µm polytetrafluoroethylene (PTFE), nylon syringe filter attached to a 3 ml luer lock syringe. The sample was split between two HPLC vials for sugar and acid analysis. The filtered extract was analysed for sugar using the procedure outlined in Forney *et al.* (2007). The organic acid content was determined using a Beckman System Gold HPLC with a Synergi Hydro-RP (250 x 4.6mm, 4 micron) column (Phenomenex, Torrance, CA) and a photodiodearray detector using 0.01M H_2SO_4 (pH 2.5) as the mobile phase at a flow rate of 0.5 ml/min.

Determination of Physico-Chemical Properties

Other measures, such as pH, soluble solid content (SSC) and titratable acidity (TA) were determined from juiced berries. The pool of berries collected over time was separated into three replicates, thawed and juiced. To obtain the juice, frozen berries were thawed for four hours, then squeezed through a cheesecloth lined garlic press. The pH was determined by using a pH meter (Acuumet, model 10, Denver Instruments Company, CO). Soluble solid content was measured with a digital refractometer (Model 300016, Sper Scientific, Scottsdale, AZ) and titratable acidity with an automated titrator (785 DMP Titrino, Metrohm, Riverview, FL).

Data Transformations and Statistical Analysis

Berries from each clone were harvested in small quantities between July and October, ensuring some berries were left on the plant to develop into the subsequent maturity stages. Once collected, berries were sorted into maturity stages. This resulted in a pool for each maturity stage which was mixed by date. For each clone, three replicates from each maturity stage pool were taken, totalling thirty-six samples.

To allow for comparisons across groups and among clones, polyphenolic results were standardized to a dry weight basis (mg/100 g) using the following conversion from ppm.

DW (mg/100 g) = (ppm*ml of extraction solution*100 g)/ g of powdered berries

Sugar and acid data were converted to percent DW (mg/100 mg DW) by dividing the ppm by the concentration factor (amount of DW analyzed/ ml reconstituted) and multiplying by 100.

For statistical analysis, the data was normalized using log transformations. The statistics program SYSTAT 10 (SYSTAT, Chicago) was used to calculate MANOVA within the polyphenolic classes and ANOVAs for individual compounds and sub-class totals. Changes in the polyphenolic class totals across maturity were examined by fixing the clone variable and pooling the compounds in each of the four phenolic compound classes, flavan-3-ols, flavonols, phenolic acids, and anthocyanins. SYSTAT 13 (SYSTAT, Chicago) was used to calculate ANOVAs for sugar, acid, and physico-chemical measures. Changes across maturity stages within each compound were examined using ANOVAs to determine if there were significant differences overall and a Tukeys post-hoc test was used to determine the pattern of differences among the maturity stages. This procedure was repeated for total phenolics, total anthocyanins and the physico-chemical measures.

The relationship among total phenolics, total anthocyanins, and the phenolic class totals was examined using a Pearson correlation analysis.

Results

Distribution of Phenolic Compounds Among Maturity Stages

In the green stage, phenolic acids made up 65% of the measured phenolic compounds, flavonols 20%, flavan-3-ols 14%, and anthocyanins 1%. In red berries the majority, 56%, of phenolic compounds were anthocyanins, 31% were phenolic acids, 11% flavonols and 2% flavan-3-ols. Blue and over mature berries were similar to each other with concentrations of 79-80 % anthocyanins, 15% phenolic acids, 4-5 % flavonols and 1% flavan-3-ols.

As the berries ripened, the total quantified flavonols, phenolic acids, and flavan-3-ols decreased and total quantified anthocyanins increased (Table 2-1). A MANOVA indicated that changes in concentration within all compounds resulted from both clone and maturity differences ($p < 0.0001$). There was no significant interaction between clones and maturity classes.

Overall there was no significant difference among any of the maturity stages for total quantified phenolics (Table 2-1). However, there were significant differences among maturity stages within specific phenolic classes. Total flavonols were not significantly different between green and red berries, nor between blue and over mature berries, but were significantly lower ($p < 0.028$) between the immature and mature stages. Total phenolic acids and flavan-3-ols decreased with maturity, with significant differences between green, red and blue classes ($p < 0.0001$) but no difference between blue and over-mature stages. Anthocyanins increased with maturity, with significant differences among green, red and blue maturity stages ($p < 0.0001$) and no significant differences between blue and over mature stages.

Within each phenolic class, individual compounds exhibited wide variability in the amount they increased or decreased within maturity stages (Table 2-1). Chlorogenic acid (Chl) was the most abundant phenolic acid in all maturity stages, approximately 100 times higher in concentration than either Caf or Fer (Table 2-1). Q3-Gal was the most abundant flavonol in all maturity stages, with amounts more than double the other flavonols in all maturity stages (Table 2-1). Epicat was the most abundant flavan-3-ol in green berries, but Cat was most abundant in red, blue and over- mature berries. However, both compounds showed decreases of over ten times between green and red maturity stages. In green berries, the galactoside and glucoside of cyanidin was the most abundant. In red, blue and over-ripe berries the glucosides and galactosides of petunidin and delphinidin were the most abundant (Table 2-1).

Among the flavonols, there was no significant difference between Q3-Rutino and Q3-Rham in any maturity stage (Table 2-1). Q3-Gal was significantly different from all other flavonols in all maturity stages (Table 2-1). In the green and over-mature stage, Q3-Glu was not significantly different from Q3-Rutino and Q3-Rham, while in the red and blue stage, Q3-Glu was significantly different from all other maturity stages (Table 2-1). Within phenolic acids, Chl was significantly different from caf and fer in all maturity stages (Table 2-1). Within the flavan-3-ols ECG was significantly different from all cat and epicat in all maturity stages (Table 2-1). In the red and over-mature maturity stage there was no significant difference between cat and epicat, while in green and blue maturity stage epicat was significantly different from cat (Table 2-1). Within the

Table 2-1: Mean concentration (mg/100g DW) and standard deviation (± values) of the major phenolic compounds found in four maturity stages of lowbush blueberries.

		Maturity Stages				Comparison among clones
		Green	**Red**	**Blue**	**Over mature**	At the P > 0.05 level
Flavonol	Q3-Rutinoside	30 ± 41 $^{a\,A}$	10 ± 13 $^{a\,A}$	6 ± 7 $^{a\,A}$	5.4 ± 4.1 $^{a\,A}$	(1,2) 3
	Q3-Galactoside	107 ± 50 $^{a\,B}$	81 ± 32 $^{a\,B}$	50 ± 17 $^{b\,B}$	37 ± 10 $^{b\,B}$	1 (2,3)
	Q3-Glucoside	25 ± 17 $^{a\,A}$	15± 7 $^{a\,C}$	10 ± 4 $^{b\,C}$	7 ± 2 $^{b\,A}$	(1,2) 3
	Q3-Rhamnoside	6 ± 5 $^{a\,A}$	2 ± 2 $^{a\,A}$	1 ± 1 $^{a\,A}$	3 ± 3 $^{a\,A}$	(1,3) 2
	Total quantified Flavonols	168 ±107 ^{a}	108 ± 45 ^{a}	66 ± 22 ^{b}	52 ± 7 ^{b}	1 (2,3)
Phenolic Acids	Chlorogenic	533 ± 91 $^{a\,A}$	307 ± 32 $^{b\,A}$	200 ± 25 $^{c\,A}$	182 ± 6 $^{c\,A}$	(1,2,3)
	Caffeic	4± 3 $^{a\,B}$	2 ± 1 $^{b\,B}$	1 ± 0.3 $^{b\,B}$	0.8 ± 0.1 $^{b\,B}$	1 (2,3)
	Ferulic	7 ± 4 $^{a\,B}$	2 ± 1 $^{b\,B}$	1 ± 0.3 $^{b\,B}$	1± 0.2 $^{b\,C}$	1 (2,3)
	Totals quantified Phenolic acids	543 ± 91 ^{a}	311 ± 33 ^{b}	202 ± 25 ^{c}	184 ± 6 ^{c}	(1,2,3)
Flavan-3-ols	ECG	0.8 ± 0.1 $^{a\,A}$	0.5 ± 0.1 $^{b\,A}$	0.4 ± 0.3 $^{a\,A}$	1 ± 0.5 $^{c\,A}$	(1,2,3)
	Catechin	39 ± 13 $^{a\,B}$	9 ± 3 $^{b\,B}$	5 ± 2 $^{b\,B}$	6 ± 4 $^{b\,B}$	1 (2,3)
	Epicatechin	80 ± 20 $^{a\,C}$	7 ± 2 $^{b\,B}$	2 ±1 $^{c\,C}$	5 ± 2 $^{d\,B}$	(1,2,3)
	Total quantified Flavan-3-ol	119 ± 29 ^{a}	16 ± 5 ^{b}	8 ± 3 ^{c}	12 ± 6 ^{c}	(1) (2) (3)
Anthocyanins	Cy-3-Glucoside	2 ± 0.3 $^{a\,A}$	59 ± 20 $^{b\,A}$	76 ± 52 $^{b\,A}$	55 ± 45 $^{b\,A}$	(1,2,3)
	Cy-3-Galactoside	2 ± 1.3 $^{a\,A}$	68 ± 9 $^{b\,A}$	69 ± 29 $^{b\,A}$	53 ± 30 $^{b\,A}$	(1,2,3)
	Del-3-Glucoside	0.9 ± 0.1 $^{a\,B}$	81 ± 23 $^{b\,A}$	151 ± 90 $^{b\,B}$	122 ± 87 $^{b\,B}$	(1,2,3)
	Del-3-Galactoside	0.9 ± 0.1 $^{a\,B}$	96 ± 12 $^{b\,A}$	163 ± 56 $^{c\,B}$	135 ± 62 $^{c\,B}$	(1,2,3)
	Pet-3-Glucoside	0.4 ± 0.1 $^{a\,C}$	116 ± 24 $^{b\,A}$	261 ±151 $^{c\,B}$	244 ± 159 $^{b\,B}$	(1,2,3)
	Pet-3-Galactoside	0.3 ± 0.1 $^{a\,C}$	87 ± 22 $^{b\,A}$	179 ± 57 $^{c\,B}$	188 ± 72 $^{d\,B}$	(1,2,3)
	Mal-3-Glucoside	0.7 ± 0.1 $^{a\,D}$	34 ± 11 $^{b\,B}$	95 ± 48 $^{c\,A}$	110 ± 58 $^{c\,B}$	(1,2,3)
	Mal-3-Galactoside	0.7 ± 0.1 $^{a\,D}$	24 ± 10 $^{b\,B}$	66 ± 19 $^{c\,A}$	103 ± 30 $^{d\,B}$	(1,2,3)
	Total quantified Anthocyanin	8 ± 2 ^{a}	565 ± 70 ^{b}	1060 ± 490 ^{c}	1011 ± 534 ^{c}	(1) (2) (3)
Total	**Total phenolics by HPLC/ MS**	839 ± 127 ^{a}	999 ± 77 ^{a}	1335 ± 501 ^{a}	1259 ±542 ^{a}	1 (2,3)

Lower case superscript letters indicate Tukeys mean comparison results within rows. Uppercase superscript letters indicate Tukeys mean comparison results within columns. Different letters indicate significant difference at the p< 0.05 level. Numbers contained within brackets indicate clones that were not significantly different at the p< 0.05 level. N= 9 (mean of three clones of triplicates). *

*Table abbreviations as follows: Q3-Rut (quercetin-3-rutinoside), Q3-Gal (Quercetin-3-galactoside), Q3-Glu (Quercetin-3-glucoside), Q3-Rha (quercetin-3-rhamnoside), EGC (epigallocatechin), Cy-3-Glu (cyaniding-3-glucoside), Cy-3-Gal (cyanidin-3-galactoside), Pet-3-Glu (petunidin-3-glucoside), Pet-3-Gal (petunidin-3-galactoside), Del-3-Glu (delphinidin-3-glucoside), Del-3-Gal (delphinidin-3-galactoside), Mal-3-Glu (malvidin-3-glucose) and Mal-3-gal (malvidin-3-galactoside)

anthocyanins, there was no significant difference between the glucoside and galactoside of any anthocyanin, in any maturity class (Table 2-1). In the green maturity stage the four anthocyanins were each significantly different from each other. In the red maturity stage malvidin was significantly different from cyanidin, petunidin and delphinidin, which were not significantly different from each other (Table 2-1). In the blue stage, cyanidin and malvidin are grouped together and significantly different from delphinidin and petunidin, between which there were no significant difference (Table 2-1). In the over-mature stage, cyanidin was significantly different from all other anthocyanins, and there was no significant difference between delphinidin, malvidin and petunidin (Table 2-1).

Changes in Antioxidant Capacity Through Fruit Ontogeny

The antioxidant capacity of each maturity stage was related to its total phenolic content. Both the F-C and ORAC assays indicated significant maturity stage differences among green berries and all other maturity stages, and no significant differences among red, blue and over-mature stages (Table 2-2). Antioxidant capacity measured by FRAP assays was not significantly different among any maturity stages ($p < 0.05$) (Table 2-2). Each assay suggested differing relationships among the clones. Each clone was significantly different in the F-C assay, while the ORAC assay suggested there was no significant difference among the clones. The FRAP assay found no difference between clones one and three, and clone two to be significantly different from each of the other clones (Table 2-2).

Table 2-2: Total phenolic content and antioxidant capacity determined from three assays, of the four maturity stages of lowbush blueberries. Standard deviations are presented as ±values

	Maturity Stage				Comparison among clones
	Green	**Red**	**Blue**	**Over mature**	
FC (mg GAE/g)	261± 91 [a]	132 ± 17 [b]	120 ± 37 [b]	165 ± 56 [b]	(1) (2) (3)
FRAP (mg TE/g)	302 ± 97 [a]	151 ± 23 [a]	125 ± 62 [a]	160 ± 60 [a]	(1,3) 2
ORAC (µm TE/g)	57 ± 0.4 [a]	195 ± 28 [b]	165 ± 51 [b]	206 ± 45 [b]	(1,2,3)

Superscript letters within rows, indicate the results of the Tukeys post-hoc test. Different letters indicate significant difference at the $p< 0.05$ level. Numbers contained within brackets indicate clones that were not significantly different at the $p< 0.05$ level. Abbreviations, FC (Folin- Ciocalteu), FRAP (Ferric Reducing Antioxidant Power), ORAC (Oxygen Radical Absorbance Capacity), GAE (Gallic Acid Equivallents), TE (Trolox Equivallents).

Other Physico-Chemical Parameters

The two most abundant sugars in lowbush blueberry were glucose and fructose; only trace amounts of galactose and sucrose (<1% DW) were found in all stages (Table 2-3). There were equal percentages of glucose and fructose in the green and red maturity stages, and slightly more fructose in the blue and over-mature berries. The percent of total sugars increased from around eight percent in the green stage to 36 percent in both blue and over-mature stages (Table 2-3). There were no differences in percent sugar or percent total sugar among clones (Table 2-3).

Both soluble solid content (SSC) and fruit density increased as berries matured from green to red to blue. There was no significant difference in either parameter between blue and over mature fruit (Table 2-3). The SSC of green stage berries was 7 ^{o}Brix, and increased to 10 ^{o}Brix when the berries were fully mature (Table 2-3). The fruit density of green berries was 0.98 mg/ml and 1 mg/ml in red berries. Both immature stages floated in pure water. Blue berries sank in pure water, with a density of 1.03 mg/ml. Over mature fruit had a density of 1.02 mg/ ml (Table 2-3).

Table 2- 3: Sugar and organic acid content (mg/ ml DW), soluble solid content (^{o} Brix), density (mg/ml), pH and titratable acidity (citric acid equivalents, g/l) of four maturity stages in lowbush blueberries. Standard deviations are presented as ± values.

		Maturity Stage				Comparison among clones
		Green	**Red**	**Blue**	**Over mature**	
Sugars	Glucose	4.2 ± 6.8 [a]	13.2 ± 2.0 [b]	17.8 ±3.4 [b]	16.9 ±1 [b]	(1,2,3)
	Fructose	4.2 ± 6.7 [a]	13.3 ±1.7 [b]	18.1 ±3.3 [b]	19.4 ±1.5 [b]	(1,2,3)
	Galactose	0.02±0.02 [a]	0.02±0.01 [a]	0.05±0.02 [a]	0.06±0.04 [a]	(1,2,3)
	Sucrose	0.01±0.03 [a]	0.1±0.03 [a]	0.07±0.02 [a]	0.14±0.03 [b]	(1,2,3)
Total Sugars (% DW)		8.5±13.4 [a]	26.6±3.6 [b]	36.0±6.77 [b]	36.5±2.5 [b]	(1,2,3)
Physico-chemical measures	SSC	7 ± 0.4 [a]	8 ± 0.3 [b]	10 ± 0.8 [c]	11 ± 2 [c]	(1,2,3)
	Density	0.98 ± 0.03 [a]	1.00 ± 0.03 [b]	1.03 ± 0.02 [c]	1.02 ± 0.02 [c]	(1,2,3)
Acids	Quinic acid	9.5 ± 2.9 [a]	4.9 ± 0.3 [a]	2.9 ±0.5 [b]	1.7 ± 0.2 [c]	(1,2,3)
	Citric acid	0.8 ± 0.7 [a]	1.8 ± 0.7 [b]	1.2 ± 0.1 [b]	0.6 ± 0.1 [a]	(1,2) 3
	Malic acid	0.5 ± 0.1 [a]	0.3 ± 0.1 [b]	0.1 ± 0.01 [c]	0.4 ± 0.1 [d]	(1,2,3)
	Tartaric acid	Not detected	Trace	Trace	Trace	NA
	Shikimic acid	0.1 ± 0.06 [a]	0.04 ± 0.01 [b]	0.02 ± 0.01 [b]	0.01 ± 0.003 [b]	(1,3) 2
	Succinic acid	0.2 ± 0.1 [a]	0.2 ± 0.06 [a]	0.1 ±0.05 [a]	0.1 ± 0.06 [a]	(1,3) 2
Total Acids (%DW)		11.1 ± 3.1 [a]	7.2 ± 0.7 [b]	4.4 ± 0.5 [c]	2.8 ± 0.32 [d]	
Physico-chemical measures	pH	3.2 ± 0.03 [a]	3.2 ± 0.1 [a]	3.3 ± 0.1 [b]	3.63 ± 0.1 [c]	(1,2,3)
	TA	0.2 ± 0.02 [a]	0.1 ± 0.02 [b]	0.06 ± 0.01 [c]	0.04 ± 0.01[d]	(1,2,3)

Superscript letters within rows, indicate the results of the Tukeys post-hoc test. Different letters indicate significant difference at the $p< 0.05$ level. Numbers contained within brackets indicate clones that were not significantly different at the $p< 0.05$ level. Abbreviations as follows, SSC (soluble solid content), and TA (Titratable acidity).

Five organic acids were found in lowbush blueberry, (-) - quinic, citric, L (-) - malic, (-) - shikimic, and succinic (Table 2-3). L (+) - tartaric acid was not detected in green berries and was found at trace levels in red, blue and over-mature berries. (Table 2-3). Quinic was the most abundant acid in all ripeness stages, 9.5 percent DW in the green stage, 4.9 percent in red berries, 2.9 percent in blue berries and 1.7 percent in over-mature berries. Citric acid was the next most abundant acid, 0.8 percent DW in the green stage, 1.8 percent in the red stage, 1.2 percent in blue blueberries and 0.6 percent in over-mature berries (Table 2-3). The percent DW of malic, shikimic and succinic were less than one percent in all maturity stages. The total percent acids decreased as berries ripened, the green stage contained 11.1 ± 3.1 percent DW, red berries contained 7.2 ± 0.7 percent, blue berries had 4.4 ± 0.5 percent and over-mature berries contained 1.7 ± 0.2 percent DW (Table 2-3). There were no differences among clones for quinic, malic or total acids. For citric acid clone 3 was different from clones 1 and 2. Clone 2 was different from clones 1 and 3 for both shikimic and succinic acid (Table 2-3).

The pH of green and red berries was the same at 3.2, the pH became more basic in the ripe (3.3) and over mature berries (3.6). Titratable acidity decreased and was significantly different among maturity stages (Table 2-3). Green berries had an average of 0.2 g/l citric acid equivalents, red 0.1, blue 0.06 and over mature 0.04 g/l citric acid equivalents (Table 2-3).

Correlation Analysis Among Phenolics and Antioxidant Capacity

When all maturity stages were considered together, significant positive correlations occurred between flavan-3-ols and phenolic acids (R= 0.89), and negative

correlations between flavan-3-ols and anthocyanins (R= -0.88) and phenolic acids and anthocyanins (R= -0.88). None of the phenolic classes were significantly correlated with any of the antioxidant capacity measures (Table 2-4). In the green stage flavonols were negatively correlated with flavan-3-ols (R= -0.91). Total phenolics were positively correlated with flavonols (R= 0.83) and anthocyanins (R= 0.90). The FRAP assay was negatively correlated with flavonols (R= -0.79) and positively correlated with flavan-3-ols (R=0.74) (Table 2-4). In the red stage flavan-3-ols were significantly correlated with anthocyanins (R=0.84) and the FRAP assay was correlated with phenolic acids (R=0.80). In the blue stage total phenolics were positively correlated with flavan-3-ols (R=0.76) and anthocyanins (R=0.96). The FRAP assay was positively correlated with flavan-3-ols (R=0.96) (Table 2-4). In the over mature berries total phenolics were significantly correlated with flavan-3-ols (R= 0.98) and anthocyanins (R=0.99), and the FC assay was positively correlated with flavan-3-ols (R=0.97) and anthocyanins (R=0.95). The FRAP assay was also positively correlated wtih flavan-3-ols (R=0.92) and anthocyanins (R=0.91). ORAC did not significantly correlate with any of the phenolic groups (Table 2-4).

Discussion

Lowbush blueberry clones are phenotypically and genotypically variable (Burgher-MacLellan and MacKenzie 2004). The three clones that were examined in this study had significant differences in their fruit constituents such as flavonols, phenolic acids, flavan-3-ols, total phenolics and organic acids ($p \leq 0.05$) (Table 2-3). These results agree with the results found by other researchers (Kalt and MacDonald 1996; Kalt *et al.*

Table 2-4: Pearson correlations among phenolic sub-classes and antioxidant capacity.

Maturity		Flavonols	Flavan-3-ols	Phenolic Acids	Anthocyanins	Total Phenolics	FC	FRAP	ORAC
Overall (all maturity stages combined)	Flavonols	1							
	Flavan-3-ols	0.47	1						
	Phenolic Acids	0.58	0.89*	1					
	Anthocyanins	-0.55	-0.88*	-0.88*	1				
	Total Phenolics	0.28	-0.01	-0.03	0.37	1			
	FC	0.42	0.72	0.62	-0.61	0.10	1		
	FRAP	0.14	0.86*	0.71	-0.69	-0.06	0.75*	1	
	ORAC	-0.55	-0.58	-0.62	0.78	0.37	-0.36	-0.34	1
Green	Flavonols	1							
	Flavan-3-ols	-0.91*	1						
	Phenolic Acids	-0.23	0.49	1					
	Anthocyanins	0.69	-0.53	0.32	1				
	Total Phenolics	0.83*	-0.57	-0.33	0.90*	1			
	FC	0.39	-0.29	0.61	0.71	0.68	1		
	FRAP	-0.79*	0.74*	0.55	-0.32	-0.47	0.21	1	
	ORAC	-0.06	-0.01	0.03	0.24	0.01	0.24	0.35	1
Red	Flavonols	1							
	Flavan-3-ols	-0.33	1						
	Phenolic Acids	-0.42	-0.01	1					
	Anthocyanins	-0.49	0.84*	0.36	1				
	Total Phenolics	0.64	0.47	-0.13	0.33	1			
	FC	-0.45	-0.24	0.19	-0.18	-0.62	1		
	FRAP	-0.65	0.40	0.80*	0.66	-0.10	0.20	1	
	ORAC	-0.43	0.13	0.32	0.31	-0.22	0.42	0.65	1

Superscript * indicate significant values based on a Bonefitti post-hoc test.

Table 2-4 (Con't): Pearson correlations among phenolic sub-classes and antioxidant capacity.

Maturity		Flavonols	Flavan-3-ols	Phenolic Acids	Anthocyanins	Total Phenolics	FC	FRAP	ORAC
Blue	Flavonols	1							
	Flavan-3-ols	-0.35	1						
	Phenolic Acids	-0.47	0.61	1					
	Anthocyanins	0.23	0.72	0.16	1				
	Total Phenolics	0.31	0.76*	0.29	0.96*	1			
	FC	0.15	0.74	0.13	0.73	0.79*	1		
	FRAP	-0.27	0.96*	0.45	0.70	0.74	0.85*	1	
	ORAC	-0.49	0.60	0.42	0.36	0.31	0.31	0.55	1
Over Mature	Flavonols	1							
	Flavan-3-ols	0.26	1						
	Phenolic Acids	0.43	-0.18	1					
	Anthocyanins	0.31	0.98*	-0.24	1				
	Total Phenolics	0.42	0.98*	-0.12	0.99*	1			
	FC	0.34	0.98*	-0.13	0.95*	0.92*	1		
	FRAP	0.29	0.92*	-0.28	0.91*	0.91*	0.96*	1	
	ORAC	-0.19	0.46	-0.64	0.49	0.41	0.49	0.44	1

Superscript * indicate significant values based on a Bonefitti post-hoc test.

1995). There were no differences among clones for anthocyanins, sugars, SSC, density, pH or TA (Table 2-1). These are in contrast with results reported by Kalt and MacDonald (1996) who found significant differences between clones. However, sugar, density, pH and TA are all inter-dependent and closely associated measures, therefore it is expected that clonal similarities found in the sugar measurements, in this study, or differences found in the Kalt and MacDonald (1996) study should be reflected in the associated physico-chemical measures.

All three clones in this study exhibited a pattern of decreasing total phenolic acids, flavonols and flavan-3-ols with fruit maturity, and increased total anthocyanins with maturity (Table 2-1). This pattern of decreasing non-anthocyanin phenolics and increasing anthocyanins has been noted in other *Vaccinium* species, such as highbush blueberry, cranberries (*Vaccinium macrocarpon* Ait.), and bilberry (*Vaccinium myrtillus* L.), as well as in other fruit such as the camu camu fruit (*Myrciaria dubia* (H.B.K) McVaugh) (Castrejon *et al.* 2008; Vvedenskaya and Vorsa 2004; Jaakola *et al.* 2002; Chirinos *et al.* 2010). The changing pattern of phenolic consitutents with maturity paired with no significant difference in total phenolic among maturity stages (Table 2-1) suggest a shift in the biosynthetic pathway as berries mature.

In ripe berries, anthocyanins are the major phenolic constituent, comprising 80% of the total phenolics (1,060 mg/ 100 g DW). Ripe berries are dark blue-purple in colour and this is due to the composition and concentration of anthocyanins present (Mazza and Miniati 1993). According to an evolutionary perspective, the pigmentation of fruit may act as a signal to animals indicating the berries are ready to be eaten, and enabling seeds to be widely dispersed (Hall *et al.* 1979). The high concentration of anthocyanins in ripe

berries is consistent with other *Vaccinium* species, and lowbush blueberry studies (Kahkonen *et al.* 2001; Tarusico *et al.* 2004; Kalt *et al.* 2008)

While some of the phenolic classes have been reported from ripe (blue) or partly ripe (red) lowbush blueberries, the quantification of individual compounds at each stage of maturity has not been previously reported. While additional phenolics, such as proanthocyanidins have been reported in the blueberry literature (Forney and Kalt 2011), this scope of this study was limited to monomeric flavonoids.

Quercetin-3-galactoside was the most abundant flavonol in all maturity stages. This is similar to what is reported in other *Vaccinium* species (Tarusico *et al.* 2004). Quercetin compounds have two functions in *Vaccinium* species; they can act as anthocyanin co-pigments or as free radical scavengers (Gao and Mazza 1994; Maatta-Riihinem *et al.* 2005).

Chlorogenic acid was the most abundant phenolic acid in each maturity stage. This is consistent with other literature (Gao and Mazza 1994). The high concentrations of chlorogenic acid in blueberries may be because it acts antioxidant free radical scavenger to reduce oxidative stress (Grace and Logan 2011). Chlorogenic acid is widespread in plant tissues and levels have been found to fluctuate with environmental stresses such as high light, low temperatures and water stress (Grace *et al.* 1998). In peach, chlorogenic acid has been found to inhibit the growth of the pathogen *Monilinia fructicola* (Winter) Honey (Hamauzu 2006). Blueberry stems, flowers and berries are affected by different *Monilinia* species (Eaton and Hall 1961; Hall *et al*. 1979) and it is possible high levels of chlorogenic acid in the berries act as a plant defence compound or mechanism Further,

chlorogenic acid is known to act as an anthocyanin co-pigment increasing the colour intensity of anthocyanins between pH 2 -7 (Mazza and Brouillar 1990).

Flavan-3-ols made up 14% of the total phenolics in the green berries, and only 1-2% in subsequent maturity stage. Flavan-3-ols are known to have astringent properties, and their high concentration in immature fruit would help deter animals from taking berries with immature seeds (Maatta-Riihinen *et al.* 2005). Flavan-3-ols also have a protective role against fungal attacks. Cranberries grown in areas prone to fungal attack have elevated levels of flavan-3-ols early in ovary development and fruit set (Vvedenskaya and Vorsa 2004).

Ripe blueberries have been shown to contain fifteen anthocyanins, but the most abundant are the glucoside and galactoside of delphinidin, malvidin, petunidin, cyanidin and peonidin (Francis *et al.* 1966; Gao and Mazza 1994). The glucoside and galactosides of four anthocyanidins were examined in this study.

While there were significant differences among anthocyanins in all maturity stages, no one anthocyanin was more abundant than the others in every maturity stage (Table 2-1). Cyanidin was most abundant in the green stage; cyanidins, delphidin and petunidin in the red stage; delphindin, and petunidin in the blue and over-mature stage (Table 2-1). A similar finding was reported by Kalt *et al.* (2001a). In terms of maturity, about 70-fold increase in anthocyanin concentration occurred between green and red berries. This is to be expected as the colour change in berries results from the accumulation of anthocyanins (Mazza and Miniati 1993).

In the green berries cyanidins were most abundant. In red, blue and over-mature maturity stages, delphinidin and petunidin were the most abundant. While cyanidins, delphinidins, and petudins all decreased slightly between blue and over-mature berries, malvidins continue to increase (Table 2-1). While there was no significant difference among clones for any individual anthocyanin, the anthocyanin total were significantly different among all three clones ($p < 0.05$). Clonal differences in anthocyanin content are expected, as ripe lowbush blueberries are highly variable in their fruit colour (Gao and Mazza 1994; Kalt *et al.* 1995).

Despite the changing nature of the phenolic profile among maturity stages (Table 2-3), the total phenolic contents of the berries were not significantly different among maturity stages ($p = 0.06$). Further investigation would be needed to determine the biochemical mechanism(s) underlying the changes of polyphenolic profile over fruit maturity and ripening. It would be interesting to understand the transcriptional regulation of biosynthesis of subclasses of flavonoids during blueberry fruit ontogeny.

There is a great deal of interest in the antioxidant power of blueberry fruit (Kalt *et al*. 2001b), as a high antioxidant capacity has been shown to have health benefits (Prior *et al.* 1998). In this study three antioxidant assays were used to measure the antioxidant capacity of the four maturity stages (Table 2-2). The three assays measure antioxidant capacity through different pathways, ORAC is a HAT based assay, FRAP is a SET based assay, and F-C is a mix of HAT and SET reactions (Prior *et al*. 2005). In the ORAC assay, increasing values indicate increasing antioxidant capacity, while in both FRAP and F-C assays, increasing antioxidant capacity is noted with decreasing values.

In this study, the FRAP assay indicated no significant differences between maturity stages (Table 2-2). While both the F-C and ORAC assays resulted in the same pattern of antioxidant capacity among maturity classes. These assays indicated that there were no significant differences in antioxidant potential between red, blue and over-mature stages, while the green stage was significantly different from all other maturity stages (Table 2-2). While both assays suggested the same antioxidant capacity pattern between maturity stages, they do not correlate well overall (R= -0.36) or in any particular maturity stage (Table 2-4), suggesting the antioxidant capacity of each maturity stage results from the presence of different compounds. F-C did not significantly correlate with any phenolic class overall, or in the green, red or blue stages (Table 2-4). In the over mature berries F-C was significantly correlated with flavan-3-ols (R= 0.98) and anthocyanins (R= 0.95) (Table 2-4). The ORAC assay was not significantly correlated with any phenolic class either overall or in any maturity stage (Table 2-4). The FRAP assay correlated with the flavan-3-ols (R= 0.86) overall, and with flavonols (R= -0.79) and flavan-3-ols (R= 0.74) in the green stage, phenolic acids (R= 0.80) in the red stage, and flavan-3-ols (R= 0.96; R=0.92) and anthocyanins (R=0.70; R=0.91) in both the blue and over-mature stages. These results suggest the antioxidant capacity of flavan-3-ols and anthocyanins can be characterized by either SET or HAT based assays, while the antioxidant capacity of flavonols and phenolic acids should be characterized by SET based assays. FRAP and F-C assay results were also found to correlate in a study of ripe apple skins (Huber and Rupasingh 2009).

In a study of over nine hundred vegetable samples representing thirteen types, little correlation was found between FRAP and ORAC measures, possibly because of

how phenolic compounds act as antioxidants within the FRAP reaction (Ou *et al.* 2002). The reaction time of phenolics with the FRAP oxidizing reagent, the colour of the phenolic compounds, and the potential of other compounds contributing to the reduction capacity could all negatively impact the accuracy of the FRAP values (Ou *et al*. 2002). Similarly the accuracy of the F-C assay can be affected by the presence of non-phenolic organic and some inorganic compounds (Ndhlala et al. 2010).

ORAC, FRAP and total phenolic values were not significantly different among red, blue and over-mature stages of blueberry fruits. In highbush blueberry, total phenolics decreased with maturity (Kalt *et al.* 2003), but no difference in ORAC related to maturity was found (Kalt *et al.* 2001a). Rabbiteye blueberries (*Vaccinium ashei* Rehd.) had decreasing ORAC values between early ripe berries and ripe berries left on the bush longer (Prior *et al.* 1998). Both blackberry and strawberry had decreased ORAC values with maturity (Wang and Lin 2000). In Saskatoon berries (*Amelanchier alnifolia* Nutt.), total phenolic content decreased as anthocyanin concentrations increased with maturity (Rogers and Knowles 1997). Camu camu fruit have a pattern of increasing ORAC from unripe fruit (green) to immature fruit (green/ red) and then decreasing ORAC from immature fruit (green/ red) to mature fruit (red) (Chirinos *et al.* 2010). In raspberries (*Rubus idaeus* L.), ORAC increased with maturity, similar to what was found in this study (Wang and Lin 2000). Specific anthocyanins are known to have different antioxidant capacity (Wang *et al.* 1997), and it is likely that other phenolic compounds which contribute to the overall antioxidant capacity also vary in their individual antioxidant capacity. The variability in ORAC capacity is likely a reflection of the composition of total phenolics. In this study ORAC and anthocyanin content were

positively correlated (R= 0.78), when all maturity stages were considered together (Table 2-5). Several studies have found ORAC values to be highly correlated with total phenolics and total anthocyanins in blueberries (Prior *et al.* 1998; Tarusic *et al.* 2004; Kalt *et al.* 2001b). In contrast, a study of antioxidant capacity across several blueberry cultivars found an inconsistent relationship among ORAC, total phenolics and total anthocyanins (Clark *et al.* 2002). There was no significant difference in ORAC values among clones (Table 2-2). The ORAC assay was positively correlated with anthocyanin content (all groups combined R=0.78) the ORAC values are likely a reflection of the anthocyanin pattern, where there were also no differences among clones.

Four sugars were found in all lowbush blueberry maturity stages, glucose, fructose, galactose and sucrose (Table 2-3). Glucose and fructose were the most dominant sugars, with galactose and sucrose contributing less than 0.2 mg/ ml DW (Table 2-3). Glucose and fructose increased as berries turned from green to red stage and thereafter were similar in all maturity stages, which is in agreement with the literature (Barker *et al.* 1963; Kushman and Ballinger 1968; Ismail and Kender 1974). We found glucose and fructose to remain at the same concentration in over mature berries as in ripe fruit (Table 2-3). Kalt and MacDonald (1996) looked at a combined glucose and fructose measure in over-ripe fruit and found percent sugar continued to increase in this stage.

The majority of sugar accumulation in blueberry fruit results from the translocation of sugars produced during photosynthesis, from the leaves to the berries (Tucker 1993; Cano-Medrano and Darnell 1997). Photosynthesis in blueberries results in sucrose production (Cano-Medrano and Darnell 1997), while maturing fruit aquire equal quantities of glucose and fructose (Table 2-3). Sucose is actively transported into phloem

and transported to the stem-berry interface (Daie 2011). The conversion of sucrose to glucose and fructose results in the establishment of the concentration gradient and allows berries to continue to act as a sink as sugars are aquired (Cano-Medrano and Darnell 1997). Two enzymes, soluble and insoluble acid invertase, hydrolyze sucrose to fructose and glucose in a 1:1 ratio (Cano-Medrano and Darnell 1997). Insoluble acid invertase hydrolyzes sucrose within the cell wall, and soluble acid invertase hydrolysis sucrose within the cell vaculole (Cano-Medrano and Darnell 1997). Soluble and insoluble acid invertase activity is promoted by the hormone abscisic acid (ABA), which is released by developing seeds (Kobashi *et al.* 2002). In rabbiteye blueberries there appears to be a link between seed number, berry size, ABA level, and soluble acid invertase activity, which results in larger berries gaining sugars at a faster rate than smaller berries (Kobashi *et al.* 2002).

The increase in berry sugars with maturity enhances sweetness (Passam et al. 2011), and along with colour, may signal seed dispersers that berries are optimally ripe (Wilson and Thompson 1981). Lowbush blueberries seeds are primarily dispersed by American robins, which eat the berries and pass them through their guts before being deposited away from the parental plant (Eaton 1957). The predominance of glucose and fructose as berry sugars may have evolved to facilitate seed distribution by the American robin (Darnell *et al.* 1994). Experimental studies have found that American robins avoid foods containing sucrose and preferentially eat foods containing glucose and fructose (Martinez Del Rio *et al.* 1988). Sucrose based foods cause indigestion in robins as they lack the enzyme sucrase, and are unable to digest this sugar (Darnell *et al.* 1994).

Organic acids influence fruit flavours (Passam *et al.* 2011), and the presence of organic acids can diminish the perceived sweetness of the fruit (Wu *et al.* 2005). Five organic acids, quinic, citric, malic, shikimic and succinic, were found in all maturity stages; tartaric acid was not detected in green berries and was found in trace amounts in red, blue and over-mature berries (Table 2-3). Quinic acid was the most abundant in each stage. Quinic acid concentrations were not significantly different between the green and red stage, and significantly different between green/ red, blue and over-mature stages (Table 2-3). Quinic acid is a precursor to chlorogenic acid (Albertini *et al.* 2006). The high levels of quinic acid found in this study may be related to the high levels of chlorogenic acid found, as both show the same pattern of distribution between maturity stage and clones (Table 2-3, Table 2-4). Citric and malic acids were the next most abundant, followed by succinic and shikimic (Table 2-3). This is different from what has been previously reported, where citric acid has been the primary acid, followed by malic and quinic acids and where succinic acid has not been reported at all (Kalt and MacDonald, 1996; Kushman and Ballinger 1968). Citric acid increased between green and red stages, was not significantly different between red and blue stages and decreased between blue and over-mature stages (Table 2-3). This is different than the pattern found by Kalt and MacDonald (1996), who found citric acid to decrease between red and blue berries and then increase in over-ripe berries. However, an increase in citric acid until the second growth stage has also been reported in peaches (Etienne *et al.* 2001). In this study, malic acid decreased from green to blue berries and then increase to near original levels in over-ripe berries, while Kalt and MacDonald (1996) found a continuous decrease across maturity stages.

The paired decrease in berry acidity and increase in sugar concentrations with maturity (Table 2-3), would result in a sweeter flavour (Wu *et al.* 2005). In blueberries the decrease in organic acids is thought to result from organic acid catabolism, paired with dilution due to increased berry size (Famian *et al.* 2005).

Soluble solids and pH, increased with maturity, while titratable acidity decreased with maturity (Table 2-3). This pattern has been previously reported in ripening blueberry fruit (Ismail and Kender 1974).

Conclusion

Eighteen phenolic compounds that belong to the flavonols, flavan-3-ols, phenolic acids and anthocyanins were examined during fruit ontogeny using four distinguishable maturity stages of lowbush blueberry. Concentrations of flavonols, flavan-3-ols and phenolic acids decreased with maturity, while that of anthocyanins increased.

Total phenolics measured by HPLC/ MS, and antioxidant measure (FRAP) were not significantly different among the four maturity stages, while F-C and ORAC were not significantly different among red, blue and over mature berries, but the F-C and ORAC of these stages were significantly different from that of the green stage.

Glucose and fructose concentrations increased, while organic acid concentrations decreased with fruit maturity. The physico-chemical measures of soluble solid content, pH and density increased with maturity, while titratable acidity decreased.

The isolation of specific compounds, or nutracuticles, from food is being promoted by the Government of Canada as a way to promote health benefits and reduce health care costs. Since lowbush blueberries are harvested as a mixed maturity crop,

isolation of nutracuticles could be obtained from the non-ripe maturity stages. Currently, processing often includes hydro-density sorting to divert green, red and over-mature maturity stages, from being included in the packaging of fresh or frozen blueberries. Green berries would be a good source of phenolic acids, flavonols and flavan-3-ols; Red berries could supply phenolic acids, flavonols and some anthocyanins and over-mature berries could be a good source of anthocyanins depending on condition.

Development of targeted compounds from lowbush blueberry might also be accomplished through the promotion of biosynthetic pathways through climate manipulation. For this to be a productive line of research, the genetic regulation of the phenolic biosynthetic pathway would have to be elucidated. This study illustrates how selected compounds in four phenolic classes change with maturity and could act as a framework for further research.

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Chapter 3: Influence of Microclimate on Fruit Ripening

Abstract

The biology of lowbush blueberries, as well as their method of harvest, results in the collection of berries in a range of maturity stages. The distribution of berries in each maturity stage is influenced by the local climate throughout the growing season. This study aims to develop a simple physical, chemical or climatic tool which would allow producers to predict when a field would yield the greatest abundance of ripe berries. Physico-chemical properties of berries of fifteen clones distributed equally among five fields, were studied for ten dates in 2006 and eleven dates in 2007 between Julian day 184 and 261. Berries collected from each clone were used to determine the percent maturity stage by weight and the mean berry size. Three transects were established in each field to collect samples of mixed clone origin. These samples were left unsorted and used to determine the chemical nature of a mixed sample, and correlated against percent maturity. On three dates ripe berries were sorted out of the transect samples and their physico-chemical nature determined, in order to assess the stability of this class across the growing season. To relate the percent blue to climate, ten soil moisture readings were taken in each field on each sample date, and growing degree days were calculated from the temperature recorded at three Environment Canada weather stations. Growing degree days were calculated from June 1st, using a base temperature of 5^{o}C. The pattern of change in percent maturity was consistent among fields and between years. The physico-chemical nature of ripe berries was found to be stable across sampling dates. These two results suggest harvest can occur three weeks earlier than is currently the practice. The physico-chemical nature of the mixed berry maturity sample was significantly different across the dates ($p < 0.001$), however, no measure correlated well against percent blue stage fruit. The physico-chemical nature of the mixed samples did not provide a basis for a simple test to gauge the optimal ripeness in a field. Plotting percent blue stage fruit against accumulated growing degree days indicates percent blue consistently peaks around 1200 growing degree days, which could provide the basis for remotely tracking field ripeness.

Introduction

Lowbush blueberry are native to North-Eastern North America. In the Atlantic region, Quebec, and Maine, the harvesting of mature fruit is an important horticultural practice for both the fresh and frozen-fruit markets. Nova Scotia produced 16, 277, 600 kilograms in 2005; 12, 608, 000 kilograms in 2006; and 12, 179, 000 kilograms in 2007 (Province of Nova Scotia 2007). During these years the crop was valued at approximately $25, 000, 000 per annum (Province of Nova Scotia 2007).

Commercial fields in Nova Scotia are managed on a two year cycle. After the berry harvest, fields are pruned back, most often by mowing, forcing the plant to develop new shoots (Eaton and McIsacc 1997). The new shoots have a season of vegetative growth after which flower buds develop and over-winter (Bell and Burchill 1955). Pollination occurs early in the second year followed by fruit development and ripening (Bell 1957).

Lowbush blueberry fruit have three developmental stages; pre-maturation, maturation, and senescence (Ismail and Kender 1974). Fruit ripening occurs at the end of the maturation stage of plant development. Ripening is characterized by chemical changes that make the fruit attractive and palatable, typically affecting flavours, texture and taste (Gortner *et al.* 1967). During ripening, fruit acidity decreases, pH and soluble solids increase and anthocyanins are accumulated in the berry skin (Eck 1986).

Variations in the physiological development of, and environmental influences on, individual berries results in a crop of mixed maturities (Eck 1986). A floral bud contains seven to ten flower primordia, which develop in a sequential manner (Bell and Burchill 1955). At bud break in the spring, the most mature flowers are found towards the base of the bud and younger flowers towards the tip (Bell and Burchill 1955). This acropedial flower development is the start of the variability in the harvested crop. The degree of variability in berry maturity is increased by environmental influences during the growing season. The frequency of pollination influences the berry size, with increased fertilization resulting in larger berries (Eck 1986). This is relevant as large berries ripen faster than smaller berries (Eck 1986). Sun exposure is a second environmental factor influencing development. Berries accumulate anthocyanins and colour faster when exposed to

sunlight (Zhou and Singh 2002). Since exposure to environmental influences is unevenly distributed, within a clone and across a field, maturity variability widens as the growing season progresses.

The wide variability in berry maturity found on the stem is also reflected within a clone, among clones, and throughout a field. In August, when most of the harvest occurs, a field will consist of mostly mature berries, but will also contain small percentages of two classes of immature berries, green and red, and one class of senescence or over-ripe berries. The use of mechanical harvesters results in a field being picked only once and all maturity stages collected.

Variability in berry maturity throughout the growing season means that calendar date is not effective at predicting the optimal time to harvest (Aalders *et al.* 1972), and thus development of a ripening index would be useful to producers. In highbush blueberries (*Vaccinium corymbosum*), one approach to developing a ripening index was to examine the sugar: acid ratio of a mixed maturity sample. This system worked well to predict general ripeness in the early stages but did not perform well when a large percentage of the fruit were overripe (Woodruff *et al.* 1959). A second approach was to use light transmittance as a non-destructive technique (Dekazos and Birth 1970). However, in lowbush blueberries the high variability in pigment content of ripe fruit makes this technique impractical (Kalt *et al.* 1995).

Another study found that no single macro-climatic variable or combination of variables proved predictive of yield in the Atlantic provinces (Hall *et al.* 1982). Heavy rainfall during bloom correlated with a decrease in yield in Newfoundland, while

sunshine during pollination and warm temperatures in the month after pollination correlated with increased yield in Nova Scotia (Hall *et al.* 1982). However, the correlations were weak, and the authors suggested that variability in physiological development at fixed calendar dates masked the effects of climate on ripening (Hall *et al.* 1982).

In other crops, including fruit crops, growing degree days (GDD) have been used to predict ripening date (Carlson and Hancock 1991). GDD are a measure of heat units accumulated during a day in which a plant has the potential to be physiologically active (Baskerville and Ermin 1969). GDD are calculated from either the date of planting, in annual crops, or from a set date in perennial crops (Carlson and Hancock 1991). All models consider the heat accumulated above a base temperature and some models also consider an upper temperature threshold, at which plant development may be inhibited by temperature (Carlson and Hancock 1991). The base temperature is crop and cultivar specific (Carlson and Hancock 1991).

Growing degree day models have not previously been applied to lowbush blueberry. However, optimization of GDD models in cane berries, such as raspberries, cranberries and highbush blueberries have all placed the base temperature around 4.5-5 ^{o}C (Black *et al.* 2008; Vanden Heuvel and DeMoranville 2009; Carlson and Hancock 1991). The optimization of 5^{o}C as a base temperature in other berry crops suggested it could be used as a starting point in lowbush blueberry. Further work should be done to confirm this is the best base temperature.

The ability to predict ripening date would allow producers to maximise the harvest, by obtaining most fruit in prime condition. The aim of this study was to find a predictor of maturity in terms of physical, chemical or microclimate factors. Three aspects of blueberry ripening were examined; 1) How does the ripening process change across date; 2) How stable is the ripe/ blue maturity stage across the growing season; and 3) Are there linkages between ripening and microclimate.

Methods

Field Location and Plant Selection

Five fields were used in each of two seasons. Since blueberries are produced on a two year production cycle, fields in the same vicinity or split fields were used in 2006 and 2007. The five fields used in 2006 were Farmington (Cumberland county; 45^{o} 34.6' N; 63^{o} 53.8' W), Athol (Cumberland county; 45^{o} 40.8' N; 64^{o} 13.3' W), Diligent River (Cumberland county; 45^{o} 24.6' N; 64^{o} 26.9' W); Debert (Colchester county; 63^{o} 26.6'N; 45^{o} 26.3'W), and Mount Thom (Pictou county; 62^{o} 59.4' N, 45^{o} 29.2'W). In 2007 split fields were used in Farmington, Debert and Mount Thom, along with fields at Southampton (Cumberland county; 45^{o} 35.9' N; 64^{o} 14.8' W), and Diligent River (Cumberland county; 45^{o} 24.3'N, 64^{o} 27.3'W). The five fields were chosen because they differ in microclimate and historically ripen on different dates; the earliest fields (Athol and Debert) ripen in early August, and the late fields (Mt. Thom and Diligent River) ripen a month later in early September (L. Eaton, Personal Communication).

In each field five clones were chosen based on various phenotypic traits such as; stem colour, stem height, flower colour and leaf colour. Sixty tags, marked with a date and a stem identifier were haphazardly distributed throughout the clone. Six stems were

then collected by clipping at ground level on each of the ten (2006) or eleven (2007) dates throughout the season. Stems were placed in individual collection bags and kept cool during transport to the Nova Scotia Agricultural College (NSAC) where they were transferred to a fridge at 4^{o}C. The physical measures were taken within one week of collection.

Three transects that spanned a number of clones were also set up in each field. Approximately 500 g of berries were collected along each transect, on each date. Berries were frozen for later chemical analysis.

Changes by Date

The Julian date calendar system was used to compare dates between years, produce graphs and complete statistical analysis. The Julian date calendar system labels January 1 as day 1 and so on until day 365 on December 31st.

The number of berries on each stem were counted and sorted into maturity stages at each of ten sampling dates in 2006 and eleven dates in 2007 (Appendix A). The four maturity stages were based on colour and skin condition state and the classification is similar to that of Ismail and Kender (1974), but with fewer gradations. In this classification scheme, there were two under-ripe groupings, immature green, immature red, one ripe group, mature blue and one over-ripe group. Green berries were small, and firm with smooth skin. Berries were classified as red, if more than 70% of their surface was red. Red berries were less firm than green berries, had smooth skin. To be considered in the blue class, berries had to be completely blue. Berries were less firm than in the previous two classes and retained a smooth skin texture. The last maturity stage was

over-ripe berries. There was no colour difference between blue berries and over-mature berries, but, a berry was considered over mature if the skin was broken, if the berry was soft to the touch or if the berry had a wrinkled appearance.

The size of each green, red and blue berry was measured in mm to two decimal places using digital callipers (STM G911073, CT, USA). Mean berry size, regardless of maturity stage, at each date, in each field, in both years was determined from the clone samples. An ANOVA was used to determine if berry size changed significantly during the season or among fields. The weight of each maturity stage was taken with a digital balance (Denver Instruments, Bohemia, NY). The weights were used to calculate the percent of each maturity stage, on each date, for each field, in each year.

In 2006, the physical and chemical nature of mixed clone samples was determined. Samples from the transect lines, of mixed clone origin, were used to measure density, pH, soluble solid content (SSC), and titratable acidity (TA). Fresh berries, in 20 g replicates to a maximum of 100 g, were used to measure density, using water displacement. pH, SSC and TA were determined from thawed, juiced berries. To obtain the juice, frozen berries were thawed for four hours, and squeezed through a cheesecloth lined garlic press. pH was determined by using a pH meter (Acumet, model 10, Denver Instruments, Bohemia, NY). Soluble solid content was measured with a digital refractometer (Model 300016, Sper Scientific, Scottsdale, Arizona) and titratable acidity was measured with an automated titrator (785 DMP Titrino, Metrohm, Riverview, Florida).

For data collected in 2006, ANOVAs were used to determine if there were significant changes in density, SSC, pH, and TA among collection dates. A Pearson correlation test was then used to determine the association between the physical and chemical parameters to the percent green, red, blue and over mature fruit in a field. The ANOVA and Pearsons correlation were used to determine if any one parameter from a mixed maturity sample could be used as a ripening measure. All statistical tests were performed using SYSTAT 10 and SYSTAT 13 (SYSTAT, Chicago, IL).

The Stability of the Physico-Chemical Nature of Ripe Blue Stage Berries Over Time

To determine if the blue maturity stage was stable across ripening date, blue berries were compared at three points in the growing season. The first time point was early in the season when the percent blue in a field was increasing, the second time point was mid-season, when the percent blue was unchanged on either side of the time point, and the third time point occurred on the last sampling date, when the percent blue was decreasing. In 2006 the time points occurred on July 18th (Julian day 199), August 8th (day 241) and September 12th (day 251) and in 2007 the time points occurred on July 31st (day 214), August 21st (day 233) and September 11th (day 254).

Size and density were determined from fresh berries, while pH, SSC and TA were determined from thawed frozen berries. ANOVAs were used to compare size, pH, SSC, and TA across dates, among fields, between years and an interacting factor between the variable fields and years.

Ripening and Climate

Soil moisture and air temperature were the two climatic variables measured in this study. Ten soil moisture readings were collected on each of the sampling dates in each field, using a hydrosence moisture probe (Hydrosense I portable soil moisture system Campbell Scientific, Edmonton, AB).

Daily maximum and minimum temperatures were recorded from permanent Environment Canada weather stations located at Nappan (45^{o} 45.57' N and 64^{o} 14.49' W) for the Farmington and Athol fields; Parrsboro (45^{o} 24.8' N and 64^{o} 20.82' W) for the Diligent River field; and Debert (45^{o} 25.0 N and 63^{o} 28.0 W) for the Debert and Mount Thom fields. Growing degree days were calculated using the Baskerville-Emin method, in which the base temperature is subtracted from the average of daily maximum and minimum temperature (Baskerville and Emin 1969). Growing degree days were calculated from June 1^{st} (Julian Day 152) with a base temperature of 5^{o}C. June 1^{st} was chosen, as this date is well past when the chilling requirement for lowbush blueberry has been met in all of the fields in this study. The percent blue present in each field was regressed using a second order polynomial line of best fit against GDD, using excel 2007 (Microsoft, Redmond WA).

Results

Changes by Date, Maturity Patterns

The change in the percent maturity in all fields followed a predictable pattern across a growing season (Figure 3-1). On the first sampling dates, green berries were the most abundant maturity stage by weight. Green berries declined over the first month, but

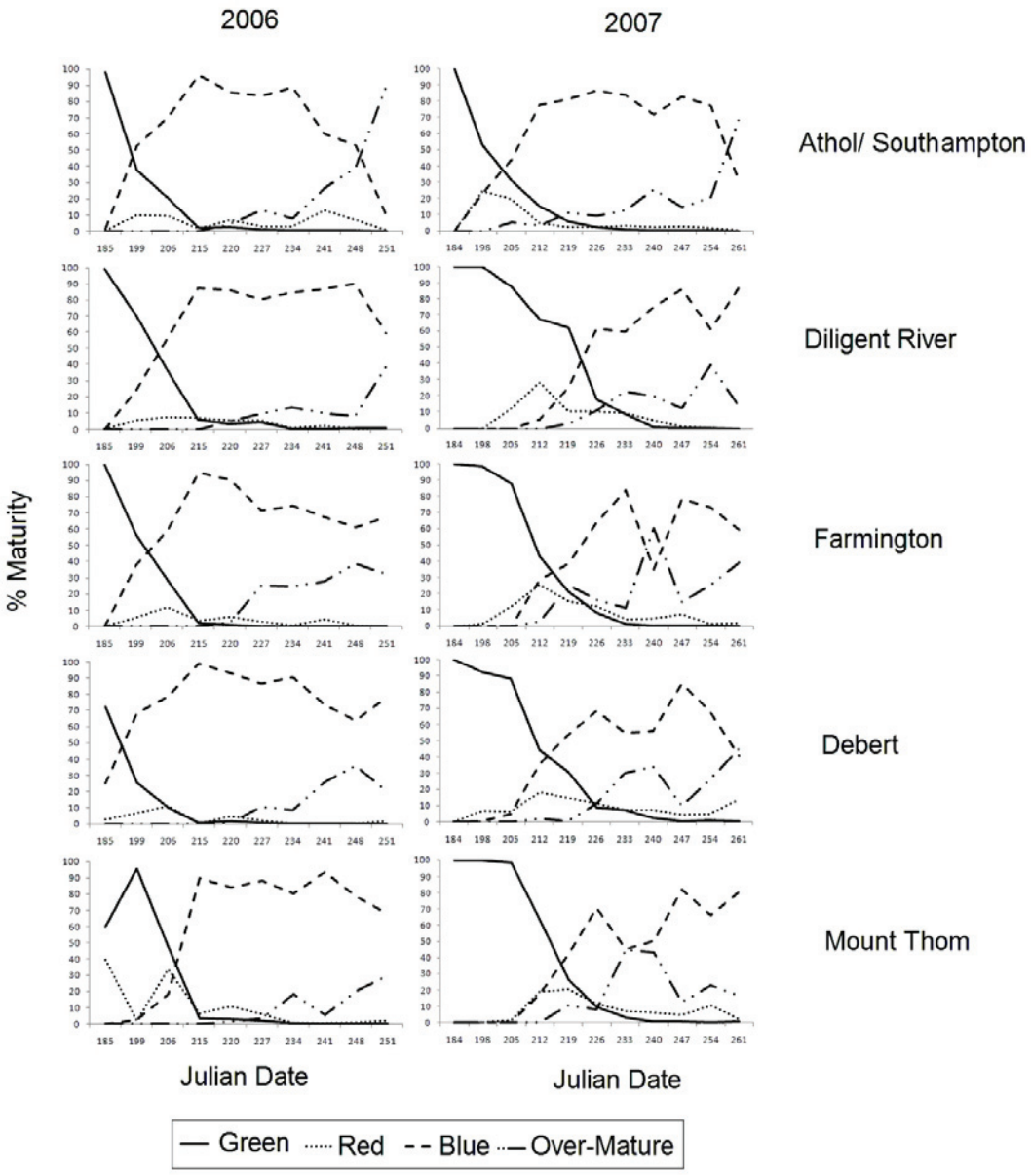

Figure 3-1: Changes in percent maturity across growing season. Graphs on the left are from 2006 and graphs on the right are from 2007.

remained at minimal levels (< 10% by weight) throughout the remainder of the season (Figure 3-1). Immature red berries were found at a low level across the sampling dates, usually comprising less than 20% by weight, but increased to 40% by weight of a sample on one occasion (Mount Thom, 2006) (Figure 3-1). Mature blue blueberries increased in the first month, levelled off at 80-90% by weight for a month and then decreased. Over mature berries appeared in a field after the first month, and then increased through the remaining sampling dates (Figure 3-1). These percent maturity patterns were similar in all fields and between years. However, in 2007 green berries were retained longer and the development of blue berries was delayed (Figure 3-1). This delay at the beginning of the season resulted in blue berries being retained and fewer over mature berries being present in the last sampling dates.

Changes by Date, Size

On the first sample dates (July 3rd and 4th (day 184/ 185)), fields contained only green berries. Green berries tend to be the smallest of the maturity stages, with a mean size of 3.93 ± 1.28 mm. Red berries had a mean size of 5.51 ± 1.51 mm. The mean size of blue blueberries was 7.73 ± 1.51 mm. Because of the wrinkled and squished nature of over-mature berries, no size measurements were taken.

The mean berry size of all berries collected on a particular date, regardless of maturity stage was significantly different among dates in all fields and in both years ($p < 0.001$) (Table 3-1). The mean size of a mixed berry sample increased with date (Table 3-1). The mean berry size on July 4th (Julian date 185) in 2006 was around 4 mm, and

increased to 7mm by September 12th (Julian day's 255). On July 3rd (day 184) in 2007 the mean berry size was 2.5 mm, which increased to 8 mm by September 11th (day 254).

Table 3-1: Mean berry size (mm) of a mixed maturity stage sample. Dates are presented in Julian Day, which increase across the growing season. Standard deviations are presented as ± values. *

Field	Year	185/ 184	199/ 198	206/ 205	215/ 214	220/ 219	227/ 226	234/ 233	241/ 240	248/ 247	255/ 254	261
F	06	4.0± 1	5.6± 0.6	6.5± 1.8	6.7± 2	7.1± 1.7	7.4± 1.6	8.1± 1.5	7.7± 1.8	7.3± 1.6	7.8± 1.3	
	07	2.6± 1.1	3.8± 1.2	4.1± 1.4	5.0± 1.7	5.8± 2.1	6.5± 2.1	7.4± 2.2	7.3± 1.7	8.1± 1.7	8.1± 1.6	8.3± 1.5
A	06	4.3± 0.9	4.9± 1.7	5.8± 2	5.8± 2	6.1± 2.2	6.7± 2	6.8± 2	6.4± 2.1	6.0± 1.5	7.1± 1.3	
	07	3.4± 1.0	4.1± 1.4	5.3± 1.8	6.6± 2.2	7.2± 2.1	7.6± 2.3	7.3± 2.4	7.4± 2.4	8.4± 1.9	8.0± 1.7	8.3± 1.2
DR	06	3.8± 1.1	4.5± 1.5	5.3± 1.8	5.6± 2.5	6.5± 2.1	6.1± 2	6.2± 2. 5	7.1± 2.1	6.5± 1.9	6.8± 1.6	
	07	2.3± 0.7	3.7± 1.1	3.9 ± 1.2	4.5± 1.5	5.7± 1.6	6.3± 2.3	7.1± 2.1	7.3± 1.6	7.9± 1.6	8.3± 1.7	8.6± 1.4
D	06	4.5± 1	5.5± 1.9	6.0± 2.1	6.4± 2.1	6.5± 2	7.1± 2	7.2± 1.6	6.9± 1.9	5.4± 1.4	6.2± 1. 6	
	07	2.9± 1.1	3.7± 1	4.2± 1.3	5.0± 1.7	5.5± 1.7	6.2± 1.8	5.7± 2	6.2± 2	7.2± 1.5	6.9± 1.5	5.8± 1.5
MT	06	4.0± 1	4.4± 1.2	5.4± 1. 4	5.7± 2.0	6.3± 2.2	7.0± 2.3	7.2± 2.4	7.9± 1.6	7.2± 2.1	7.4± 1.8	
	07	2.6± 1	4.0± 1.0	4.3± 1.2	4.9± 1.6	5.7± 1.9	6.4± 2.1	6.6± 2	7.1± 2.1	7.4± 2	7.9± 1.7	7.9± 1.9

The five fields are abbreviated as follows: Farmington (F), Athol (A), Diligent River (DR), Debert (D) and Mount Thom (MT).
* There was a significant difference inberry size between dates in every field, $p < 0.001$.

Changes by Date, Mixed Maturity Samples

Since a harvest of berries at any particular date consisted of berries of mixed maturity, it was useful to examine how the physical and chemical nature of a mixed sample changed over a growing season. The intent was to determine if there was a simple physical or chemical measure growers could use to determine if their field was at an optimal point for

harvest. When all maturity stages were considered together, the mean berry size increased with date.

Berry density increased steadily over all dates in all fields. In early July berries were lighter than water, with a density around 0.85 g/ml. By August, berries sank in water with a density just over 1 g/ml (Table 3-2). The soluble solid content of the mixed berries collected in July was around 6 ^{o} Brix, and the mixed berry samples collected in August had a soluble solid content that was almost doubled, around 12 ^{o} Brix (Table 3-2). However, soluble solids did not increase steadily across the dates, instead, an increase between two dates could be followed by a decrease between the next two dates (Athol, August 1st (day 215), August 8th (day 220) and August 15th (day 227)). Titratable acidity decreased incrementally across each season. Early in the season the titratable acidity was around 0.1 citric acid equivalents and towards the end of the season, titratable acidity values were around 0.04 citric acid equivalents. Mixed berries also became more basic as the season progresses. In July, a mixed sample had a pH of around 2.9, and at the end of the season the pH of a mixed sample was around 3.25.

There was a significant difference in each of the chemical traits among dates, in all fields ($p < 0.001$). A correlation analysis was used to determine if the changes among dates was indicative of ripeness (Table 3-3). Percent blue in a field did not correlate strongly with any of the factors directly, but percent blue did have a strong negative correlation with the percent green in a field (R= -0.84) and percent green had a strong positive correlation with titratable acidity (R= 0.88).

Table 3-2: Chemical traits of mixed berry samples across 10 sampling dates, in 2006, given in Julian days. Standard deviations are presented as ± values.

		Sample date (Julian day)									
Trait	Field	185	199	206	215	220	227	234	241	248	255
Fruit Density (g/ml)	F *		0.83 ±0.01	0.81 ±0.14	0.90 ±0.05	0.96 ±0.04	0.97 ±0.03	1.02 ±0.01	1.0 ±0.01	0.98 ±0.02	0.99 ±0.01
	A *	0.86 ±0.05	0.89 ±0.14	0.96 ±0.02	0.96 ±0.02	0.99 ±0.03	0.95 ±0.03	1.01 ±0.04	1.01 ±0.03	0.99 ±0.01	1.01 ±0.00
	DR *	0.82 ±0.03	0.85 ±0.02	0.93 ±0.02	0.96 ±0.01	0.93 ±0.01	0.93 ±0.03	0.90 ±0.15	1.0 ±0.02	1.01 ±0.01	1.0 ±0.02
	D *	0.87 ±0.02	0.94 ±0.00	0.95 ±0.02	0.97 ±0.02	1.0 ±0.03	0.96 ±0.03	1.0 ±0.01	1.02 ±0.03	0.98 ±0.02	1.07 ±0.17
	MT *	0.86 ±0.00	0.94 ±0.04	0.87 ±0.02	0.97 ±0.01	0.97 ±0.01	1.0 ±0.05	0.98 ±0.03	1.01 ±0.03	1.01 ±0.03	1.0 ±0.01
SSC (^{o}Brix)	F *		8.9 ±0.3	9.0 ±0.8	10.3 ±0.2	10.5 ±0.3	12.1 ±1	10.3 ±0.5	9. 7 ±0.3	10.17 ±1.3	10.5 ±0.6
	A *	6.7 ±0.4	9.8 ±0.3	10.1 ±0.7	9.7 ±0.2	11.4 ±0.6	10 ±0.3	9.9 ±0.4	10.5 ±0.5	9. 7 ±0.5	11.3 ±0.4
	DR *	6.6 ±0.1	9.2 ±0.1	8.2 ±0.3	12.2 ±1.3	11.9 ±0.5	10.8 ±0.5	11.5 ±0.5	11.4 ±0.6	11.2 ±0.2	11.0 ±0.2
	D *	6.7 ±0.2	10.1 ±0.2	8.2 ±0.2	10.6 ±0.3	11.4 ±0.5	10.8 ±0.7	11.3 ±0.1	10.9 ±1	10.1 ±0.4	12.1 ±0.3
	MT *	6.9 ±0.0	10.1 ±0.4	8.1 ±0. 7	13.3 ±1.3	11.3 ±0.6	10.8 ±0.2	11.7 ±0.3	11.6 ±1.1	12.4 ±1.1	12. 7 ±0.7
TA (Citric acid equiv., g/l)	F *		0.13 ±0.1	0.13 ±0.2	0. 07 ±0.1	0.06 ±0.01	0.05 ±0.01	0.05 ±0.01	0.04 ±0.0	0.04 ±0.01	0.04 ±0.0
	A *	0.17 ±0.0	0.1 ±0.01	0.07 ±0.01	0.08 ±0.01	0.06 ±0.01	0.07 ±0.01	0. 06 ±0.01	0.04 ±0.0	0. 06 ±0.01	0.07 ±0.01
	DR *	0.15 ±0.03	0.13 ±0.02	0.13 ±0.02	0.01 ±0.0	0.07 ±0.0	0.06 ±0.01	0.08 ±0.02	0.05 ±0.0	0.04 ±0.01	0.05 ±0.01
	D *	0.2 ±0.01	0.1 ±0.0	0.08 ±0.0	0.06 ±0.0	0. 06 ±0.0	0.06 ±0.02	0.05 ±0.0	0.06 ±0.0	0.05 ±0.0	0.08 ±0.02
	MT *	0.2 ±0.0	0.17 ±0.1	0.13 ±0.02	0.11 ±0.01	0. 09 ±0.01	0.06 ±0.0	0.07 ±0.0	0.06 ±0.0	0.05 ±0.01	0.06 ±0.0
pH	F *		2.7 ±0.0	2.8 ±0.1	2.9 ±0.0	3 ±0.0	3.3 ±0.1	3.2 ±0.0	3.4 ±0.1	3.3 ±0.0	3.4 ±0.0
	A *	2.9 ±0.1	2.8 ±0.0	2.9 ±0.0	2.9 ±0.0	3.2 ±0.0	3.1 ±0.0	3.2 ±0.1	3.3 ±0.1	3.2 ±0.1	3.3 ±0.0
	DR *	2.8 ±0.1	2.8 ±0.1	2.9 ±0.1	2.8 ±0.1	3.0 ±0.1	3.1 ±0.0	3 ±0.1	3.1 ±0.1	3.2 ±0.1	3.2 ±0.1
	D *	2.8 ±0.0	2.8 ±0.0	2.9 ±0.0	3.1 ±0.0	3.2 ±0.1	3.2 ±0.1	3.3 ±0.1	3.2 ±0.1	3.3 ±0.1	3.2 ±0.1
	MT *	2.7 ±0.0	2.8 ±0.0	2.7 ±0.1	3 ±0.0	2.9 ±0.1	3 ±0.0	3.1 ±0.1	3.1 ±0.0	3.2 ±0.1	3.1 ±0.0

Fields are abbreviated as follows: Farmington (F), Athol (A), Diligent River (DR), Debert (D) and Mount Thom (MT).

* indicate fields with significant differences between dates (Density $p < 0.03$; SSC, TA and pH $p < 0.001$)

Table 3-3: Pearson correlation coefficients among physical and chemical features and the percent maturity over all dates. O M are over mature berries.

	SSC	Density	TA	pH	% Green	% Red	% Blue	% O M
SSC	1							
Density	0.69	1						
TA	-0.67	-0.77	1					
pH	0.49	0.76	-0.79	1				
% Green	-0.74	-0.76	0.88*	-0.64	1			
% Red	-0.38	-0.39	0.44	-0.50	0.25	1		
% Blue	0.67	0.57	-0.72	0.37	-0.83*	-0.34	1	
% O M	0.29	0.49	-0.47	0.68	-0.40	-0.29	-0.12	1

Superscript * indicate significant correlation values as determined with a Bonefitti post-hoc test.

The Stability of the Physico-Chemical Nature of Ripe Blue Stage Berries Over Time

Ripe blue stage berries were taken at three times during the growing season in each of 2006 and 2007, with the objective to sample as the percent blue was increasing, at the plateau and decreasing. In 2006 the mean percent blue among fields were 37% on the first date, 88% on the second date and 56% on the third date. In 2007, not all fields were in decline by the last sampling date, and the percent blue increased between the three dates. In 2007, the first sample had 33% blue, the second contained 65% and the third sample 69%. Sampling from where the percent blue was increasing would promote the collection of berries that had recently entered into the blue maturity class, while the blue stage berries collected at the second date would likely have been in the blue stage for a longer period of time. Berry collection at the third point, where percent blue was decreasing would promote the collection of berries just prior to their recruitment into the over-mature stage. It would be expected that changes in the physico-chemical nature of the berry would be greatest as the berry changed into subsequent maturity classes. Further, if leaving the berries on the stem resulted in improved yield through an increase in size or weight, or improved taste attributes through an increase in sugars or a decrease

in acids, producers would have reason to wait until the end of the growing season to harvest.

In most fields blue berry size remained stable across the growing season (Table 3-4). In 2006, there was no significant difference among dates in three fields (Farmington $p= 0.252$, Athol $p= 0.376$, and Mount Thom $p= 0.763$). However, there was a significant difference among dates ($p< 0.0001$) at both Debert and Diligent River. In both cases berry size decreased among the dates (Table 3-4). A decrease in mean size would result if recruitment into the blue stage was primarily from smaller berries which ripened at a slower rate, (Ismail and Kender 1974) while larger blue stage berries transitioned into the over-mature stage (Figure 3-1). In 2007, there was no significant difference in size among dates in any of the fields (Farmington $p= 0.22$, Southampton $p= 0.50$, Diligent River $p= 0.67$, Debert $p= 0.11$, and Mount Thom $p= 0.06$).

With respect to the other chemical measures, there was no significant difference in density or SSC within fields in either year, between years, or among fields (Table 3-4). There was also no significant difference in pH, or TA within fields in either year, between years or among fields in 2006 or 2007. This suggests the chemical nature does not change as berries are recruited into and out of the blue stage.

Ripening and Climate

Temperature and precipitation trends from three Environment Canada weather stations, Debert, Parrsboro and Nappan Nova Scotia were examined over two years. There are only slight differences between the temperature trendlines between years and among fields. As of 2007 June 29th (Julian day 180) ambient temperature was approximately 5^{o}C cooler than 2006. In both years the peak temperature occurred around

Table 3-4: Physical and chemical traits of ripe blueberries at three time points in the growing season in five fields. Measurement units are, size (mm), Density (g/ ml), SSC (^{o}Brix), and TA (citric acid equivalents, g/l). Standard deviations are presented as ± values.

2006	**Farmington**			**Athol/ Southampton**			**Diligent River**			**Debert**			**Mount Thom**		
Julian Day	199	220	255	199	220	255	199	220	255	199	220	255	199	220	255
% Blue	38	90	68	53	86	9	24	86	59	68	93	78	2	85	68
Size *	7.9 ± 1.0	7.6 ± 1.3	7.8 ± 1.3	7.6 ± 1.4	7.4 ± 1.5	7.3 ± 1.1	8.3 ± 0.8	7.63 ± 1.3	7.1 ± 1.4	7.6 ± 1.3	7.3 ± 1.4	6.5 ± 1.3	8.7 ± 0	7.9 ± 1.2	7.9 ± 1.3
Density	0.81	0.98	0.97	1.02	0.97	1.01	0.88	0.99	1	0.94	0.98	0.96	0.98	0.98	0.98
pH	2.8	3.09	3.23	2.93	2.99	3.33	2.89	2.98	3.13	2.91	3.07	3.21	2.8	2.96	3.11
SSC	9.6	11.2	7.7	11.1	10.7	11	11.7	11.5	11.7	11	11.4	11.7	10.6	12.5	11.9
TA	0.09	0.06	0.04	0.1	0.09	0.06	0.1	0.06	0.05	0.05	0.09	0.06	0.15	0.1	0.06
2007	**Farmington**			**Athol/ Southampton**			**Diligent River**			**Debert**			**Mount Thom**		
Julian Day	214	233	254	214	233	254	214	233	254	214	233	254	214	233	254
% Blue	28	83	73	78	84	77	5	59	61	36	55	68	17	45	66
Size	7.9 ± 1.0	8.4 ± 1.4	8.3 ± 1.4	8.32 ± 1.16	8.24 ± 1.3	8.4 ± 1.3	9.0 ± 0.3	8.5 ± 1.5	8.5 ± 1.5	7.6 ± 1.0	7.4 ± 1.3	7.2 ± 1.2	8.4 ± 1.0	8.0 ± 1.3	8.35 ± 1.4
Density	1.03	1	1.02	1.04	1	1.03	1.05	1.02	1.03	1.01	1	1.06	1.01	1.01	1.02
pH	3.21	3.36	3.41	3.38	3.53	3.55	3.21	3.24	3.40	3.29	3.36	3.43	3.12	3.31	3.47
SSC	11.2	11.4	11.1	11.2	11.6	10.6	10.8	11.1	10.9	10	11.4	11.4	10.6	11.8	11.2
TA	0.07	0.06	0.05	0.06	0.04	0.04	0.08	0.06	0.06	0.06	0.07	0.05	0.08	0.07	0.05

* Indicates a significant difference between dates for some fields, $p<0.0001$ for Debert and Diligent river. There was no significant difference between dates for Farmington, Athol/ Southampton or Mount Thom.

July 19th (day 200), with 2007 remaining about 2°C cooler than 2006. After day 200, 2006 cooled down more rapidly until August 28th (day 240), after which the temperature patterns were similar in both years. The Parrsboro field temperatures peaked at a lower temperature than the other two fields in both years.

The precipitation patterns from 2006 and 2007 were reflected in the field soil moisture readings (Figure 3-2). In 2007 the field soil moisture increased across the season, while in 2006, the soil moisture was similar across the season or decreased. Farmington gained and retained the most soil moisture across the season, while Mount Thom was the driest field at all dates, in both years (Figure 3-2).

Growing Degree Days (GDDs) were calculated from the mean daily temperature and reflect the mean temperature curves for a localized area. GDDs are accumulated slowly in the spring and late summer and, more rapidly in the summer. This pattern resulted from how the difference between the daily maximum and minimum temperature, along with the difference between the mean temperature and the base temperature, influenced the accumulation of heat units in any one GDD. Fewer heat units are accumulated when the average temperature is closer to the base temperature. Re-setting the accumulated GDD to zero at the start of each seven day period, allows the seasonal pattern of GDD accumulation to be illustrated. As GDD affect the developing berry these patterns are presented for the course of the growing season.The pattern of weekly GDD accumulation differed in 2006 and 2007 (Figure 3-3)In 2006 weekly accumulated GDD peaked on day 200 and decreased through the rest of the season. In 2007 weekly accumulation of GDD peaked around day 210.

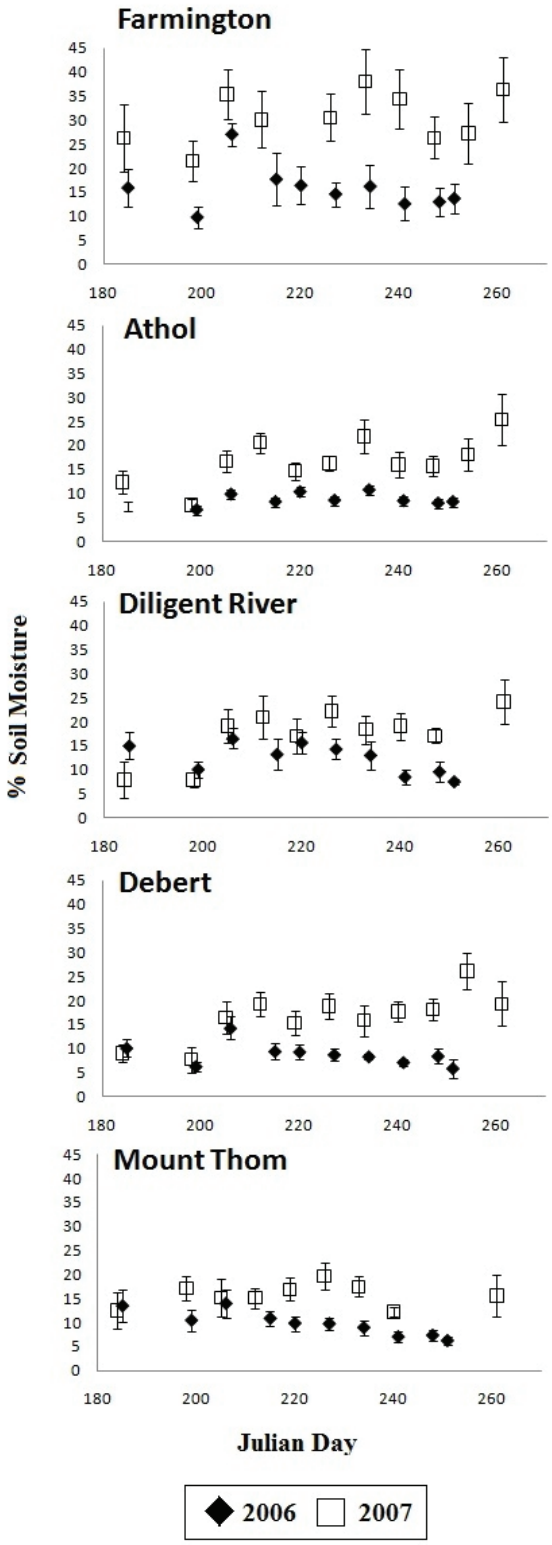

Figure 3-2: Mean % soil moisture and standard deviations across growing season in five fields in two years, 2006 and 2007. Error bars are standard deviations.

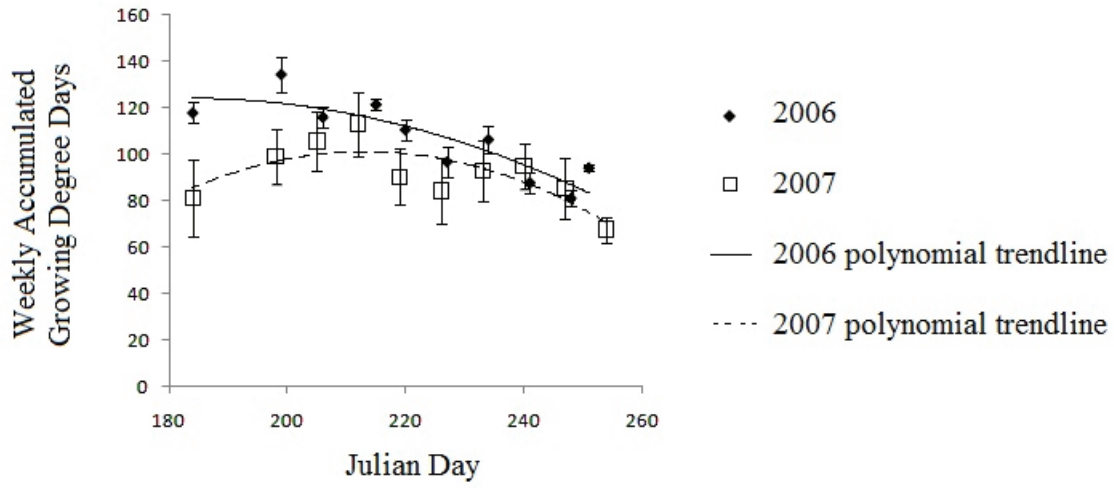

Figure 3-3: Weekly accumulation of growing degree days over the course of berry development. GDDs were calculated from 0 at the start of each week.

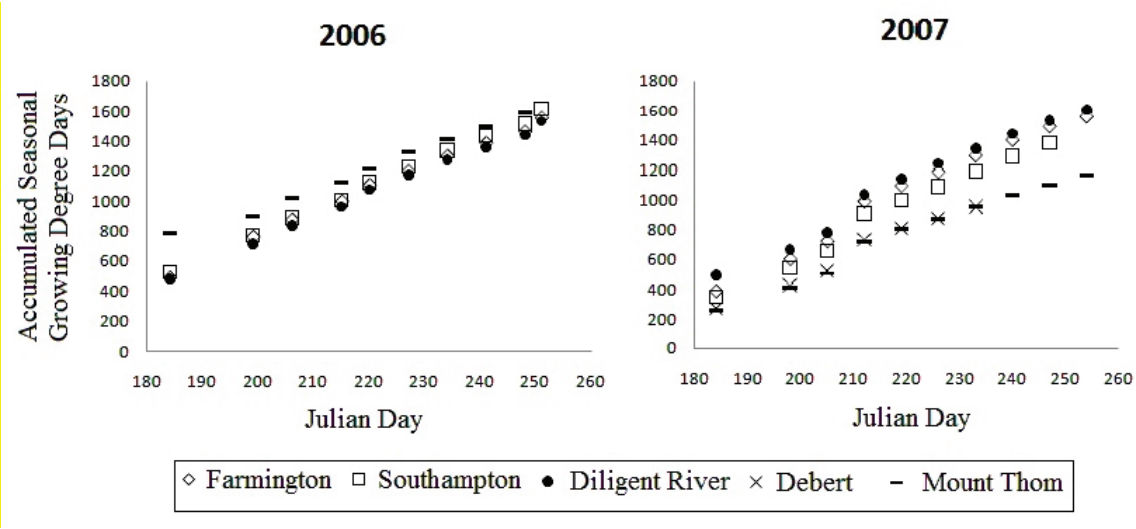

Figure 3-4: Trends of accumulated seasonal growing degree days over two years. Data was collected from three Environment Canada weather stations located at Nappan, Debert and Parrsboro.

Seasonal accumulation of GDD from June 1st (day 152) was variable between years and weather stations (Figure 3-4). At June 29th (day 180) in 2007 each field had accumulated about 200 fewer growing degree days than at the same time in 2006. In both

years the Parrsboro area accumulated fewer growing degree days than the Nappan and Debert areas.

When 2006 and 2007 percent blue and GDD data in each field were plotted, the line of best fit was parabolic. In 2006 the percent blue peaked between 80-90%, while in 2007 the percent blue peaked at between 70-90%. In each field, and each year the line of best fit peaked between 1200 to 1400 growing degree days (Figure 3-5).

Discussion

Fruit ripening is an irreversible event, meaning once started it continues until the fruit reaches senescence and begins to decay (Ismail and Kender 1974). In lowbush blueberry individual fruit on the same stem, on the same plant and within a field can ripen at different rates (Eck 1986). The rate of ripening in individual berries is linked to plant physiology and weather conditions (Hall *et al.* 1979). The onset and rate of ripening of individual fruit is closely linked to ambient temperature. In cold conditions fruit will begin to ripen at a later date and will progress to maturity over a longer period of time; and as the ambient temperature increases ripening rate also increases (Eck 1986). In turn, the ambient temperature is affected by the amount of wind, rain and humidity at any particular time, in any particular location.

Physiological events such as bud break, flower pollination, fruit set and fruit ripening are not synchronized in lowbush blueberry and each stage is influenced by the microclimate in which it occurs, resulting in fields of mixed maturities. However, while individual berries ripen at different rates, broad scale trends are still evident.

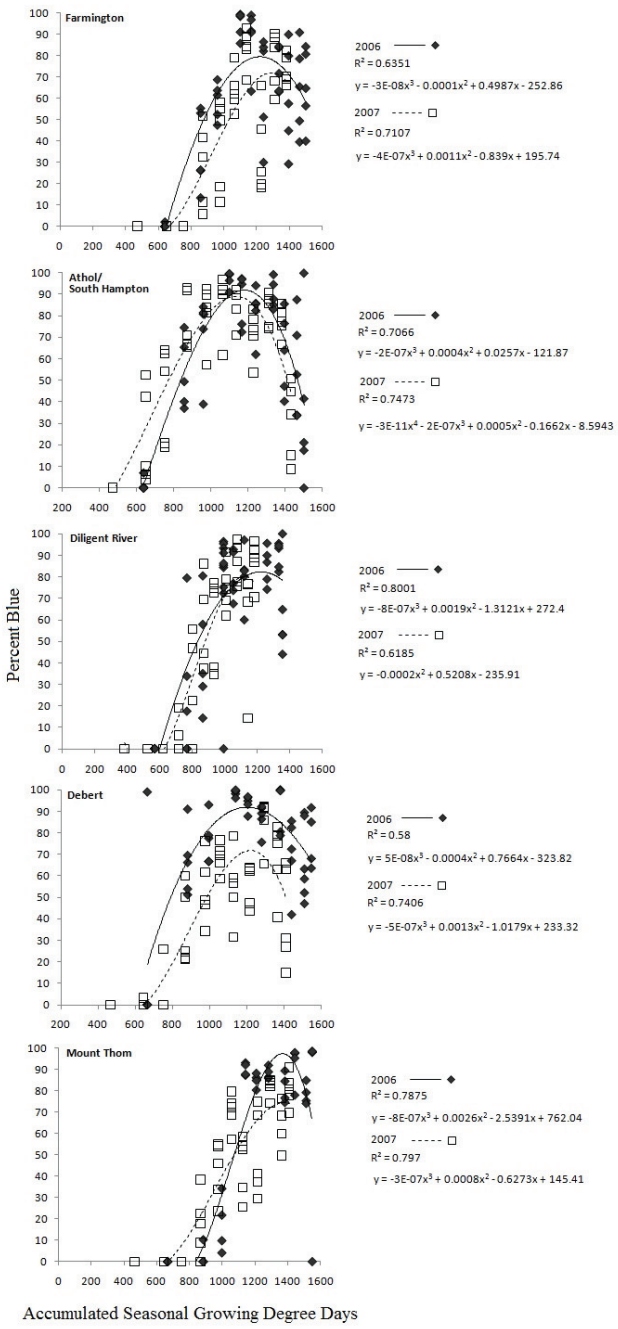

Figure 3-5: Accumulation of percent blue berries in relation to GDDs for five fields and two years. Trend lines were fitted through regression of percent blue against GDD using a second order polynomial.

Changes by Date

The first maturity stage berries pass through during ripening is the immature green stage. During this stage, berry development includes the external change of increased size through an increase in cell division in the pericarp (Eck 1986). Berry development begins immediately after fertilization and lasts between thirty and sixty days (Eck 1986). In 2006, the percent green decreased from 100 to less than 10 percent between July 1st (day 182) and August 1st (day 213). In 2007, the decline occurred at different rates among fields. There was less than ten percent green in Farmington by Aug 7th (day 219). Athol retained more than ten percent green until August 21st (day 233). Diligent River, Debert and Mount Thom, all decreased to less than ten percent green on August 14th (day 226). The delay in ripening from green to blue between 2006 and 2007 is between one and two weeks in all fields (Figure 3-1). Since ripening was delayed in all fields, it is likely due to a large scale climate factor. The mean temperature in 2006 for May, June and July was 12, 16 and 20^{o} C respectively, and in 2007 the mean temperature the same three months was 10, 14 and 18^{o}C. Colder temperatures are known to delay ripening under both greenhouse conditions (Hall and Aalders 1968) and field conditions (Hall *et al.* 1964).

In 2006 green berries were found in the fields until at least August 5th (day 217) (Farmington), and more often persisted across all sampling dates, while in 2007 green berries were found until August 21st (day 233) in all fields (Figure 3-1). There are several factors which contribute to the persistence of berries in the first developmental stage, green, for more than half the growing season. Acropedial development of flowers results in the flowers being receptive to fertilization in a scattered manner, with young flowers being fertilized and starting berry development days or weeks after the earliest flowers

(Bell and Burchill 1955; Bailey 1947). Berry development could equally be delayed by low pollination rates as fewer fertilization events results in smaller berries, which ripen at a slower rate than larger berries (Aalders and Hall 1961). The green berry class might also persist as cooler temperatures delay ripening (Hall and Aalders, 1968). Lastly, a late season green berry may receive fewer nutritive resources from the parental plant, in favour of berries which began to ripen earlier and ones in later maturity stages, which would also delay ripening (Lee 1988).

The second maturity stage is marked by berries turning red, as they accumulate anthocyanins in the skin (Ismail and Kender 1974). Red berries were found in the fields after the second sampling date July 18th (day 199), and persisted until the last sampling date. Berries in the red stage ranged between zero and thirty percent of the sample across sampling dates (Figure 3-1). The red stage is a fast transitional stage, lasting about five days (Young 1952), a shorter period of time than that between sampling dates in this study. Peaks in the percent red in a field all occured in July, early in the growing season; this corresponds with the time period when the majority of green berries transition to mature fruit (Figure 3-1). Mature, blue berries began to accumulate in the fields on the second sampling date July 17th (day 198). In all cases, the percent blue by weight increased over a two or three week period, remained level or decreased slightly during the following month and then declined sharply (Figure 3-1). In 2006 the pattern was consistent in all fields, the percent blue peaked around August 1st (day 215). In Athol and Debert fields, the plateau lasted until Aug 22 (day 234), in Diligent River, Mount Thom the plateau lasted until September 5th (day 248), and Farmington started to decline as soon as it peaked on August 8th (day 220), but had no sharp drop off at the end of the season

(Figure 3-1). As the percent maturity does not peak around a single date, the harvest could occur anywhere within a three to four week time frame.

In 2007 there was more variability among the fields, and the pattern of an increase, plateau and decrease was not observed in all fields. In Southampton the plateau extended between July 24th (day 205) and September 11th (day 254), Diligent River and Farmington, August 21st (day 233) to September 11th (day 254), while both Debert and Mount Thom had a plateau for the same period of time, between August 14th (day 226) to September 11th (day 254).

The percent blue curves were more consistent among fields in 2006, while in 2007 there was more variability in the timing of the increase, leveling off and decrease in percent blue. The pattern in Farmington looked the same in both years, while in 2007 the increase in percent blue was delayed by a week in all other fields. Temperatures were depressed in June and July of 2007, which resulted in delayed development of green berries and subsequent stages, including mature blue. The percent blue began to increase on August 3rd (day 215), and in 2007, the mean August temperature was slightly higher than the mean 2006 temperature (17^{o}C vs. 16.5^{o}C). The increase in temperature would help drive the physiological changes associated with development between red and blue maturity stages. These changes include an increase in size, resulting from pericarp cell proliferation, and water uptake (Ismail and Kender 1974; Bell 1957); a decrease in firmness resulting from weakening cell walls (Tucker 1993); and a continued change in colour, as the anthocyanin composition and concentration changes in the berry skin (Kalt *et al.* 1995).

In 2007 the plateau period was not level in four of the five fields, instead there were steep decreases between dates followed by an increase to previous levels at subsequent dates. Farmington and Diligent River decreased for one sampling day on August 28th (day 240) while Debert and Mount Thom decreased for two consecutive sampling dates, August 21st (day 233) and August 28th (day 240) (Figure 3-1).

The decrease in the percent blue could relate to two weather systems, which passed over the province on August 18th (day 230) and August 24th (day 236) 2007. On August 18th (day 230) the Farmington field received 21.1 mm of rain and the Nappan weather station reported maximum wind gusts of 37 km/ hr, considered a 'fresh breeze' on the beaufort scale. A fresh breeze will make small trees sway (Van Dorn 1974). On the 19th (day 231) the maximum wind gusts reached 56 km/ hours, considered a moderate gale (Van Dorn 1974). On the 24th (day 236), Farmington received 28 mm of rain and the Nappan weather station recorded maximum wind gusts of 39 km/ hour. In Diligent River there was 33.1 mm of rain on the 19th of August (day 231), and the Parrsboro weather station recorded maximum wind speeds of 39 km/ hr and 48 km/ hr on the 19th. On the 24th, 23.1 mm of rain fell and the maximum wind speed was 32 km/hour. The in-field weather station did not record rainfall amount for Athol, Debert or Mount Thom. The weather conditions in Athol, would have been similar to that of Farmington and the Debert and Mount Thom conditions would be similar to the conditions found at Environment Canada's Debert station. On the 18th of August (day 230), 21 mm of rain was recorded, and wind gusts of 33 km/hr and 52 km/ hr on the 19th (day 231) On the 24th (day 236), 49.4 mm of rain fell and the maximum wind gusts were 44 km/ hr.

As berries ripen, they develop an abscission layer between the fruit and the stem, allowing the berry to be picked or shed for dispersal (Gonzales- Carranza *et al.* 1998). The combined wind and rain events of August 18th, 19th, and 24th (day 230, 231, 236) may have knocked berries with well developed abscission layers off the stem partly explaining the dip in the percent blue berries on the sampling dates immediately after these events. However, there was also an increase in over-mature berries during the same time frame, suggesting some of the berries which remained on the stem were classed as over-mature fruit, possibly incorrectly. Berry softening results from a decrease in turgor pressure resulting from leaky cell walls (Tucker 1993) or from a rapid increase in water providing the substrate needed to dissolve the soluble pectins located in the middle lamella (Proctor and Peng 1989). Under conditions where there is a large amount of rainfall, followed by a drying period, berries can also shed water through transpiration or by a return of excess water to the parental plant (Matthews and Shackel 2005). Since the collection dates around the dip in percent blue occurred in wet conditions, it's possible the berries had a rapid uptake in water, making them soft without the corresponding break down in cell wall components. Under dry conditions on the September 4th (day 247) collection date, the excess water would have been reduced, leading to an increase in the blue maturity stage. As well there would be some development of the green and red berries to the blue maturity stages. The Athol field only shows a slight dip in the percent blue on August 21st (day 233) and no dip on August 28th (day 240). This field is at a low elevation, and is a steeply sloping field, sheltered on three sides by woods and a home, this location may have minimized the impact of the weather events.

The traditional classification of Athol as an early ripening field was not supported by the date at which the percent blue reached the plateau, in either year (Figure 3-1). Consideration of Mount Thom as a late ripening field, could be because it does not have a steep drop off point, as late as September 18th (day 261) (Figure 3-1).

In both 2006 and 2007, the increase in percent over-mature berries paralleled the decrease in percent blue berries. Berries were considered over-mature if they had a soft texture, split skins, or a shrivelled appearance. Soft texture, and split skins occur in senescent berries under wet conditions, while shrivelling occurs under dry conditions. Over-mature berries with split skins can result from a rapid uptake in water. In Rabbiteye berries, splitting results from water uptake through the peel or pedicle (Marshall and Spiers 2009). Specific cultivars are more or less susceptible to splitting, which appears to be influenced by the firmness and elasticity of the fruit. Increased firmness resulted in increased splitting (Marshall and Spiers 2009). Over-mature berries with a shrivelled appearance results from moisture loss. In highbush berries, shrivelling is apparent after the berry has lost between two and three percent of its fresh weight value (Nunes *et al.* 2004). The mechanisms that result in berry water loss are still under investigation (Krasnow *et al.* 2008). In grapes, shrivelling is associated with a backflow of water from the berry into the xylem and the extrusion of solutes into the apoplastic space (Krasnow et al. 2008). The loss of water from the berry results in a volume change, but berry surface area remains the same causing the berry to take on a shrivelled appearance.

Trends in Mixed Berry Samples

When all berries were considered together, the mean size of the berries increased between July 3rd (day 184) and August 8th (day 220) in both years (Table 3-1). In 2006, this was the period during which berry composition in each field changed from primarily green to mostly blue. During this transition, berries grow to their full size through the development of pericarp tissue (Eck 1986). After August 8th (day 220), the berry composition remained as primarily blue berries and the decrease in berry size reflected the smaller diameter of late ripening berries, as over-mature berries were not measured for size.

In 2006, the chemical traits of mixed berry samples were examined, in hopes of determining a quick measure that would identify when a field was at optimal ripeness. The chemical measures examined were density, soluble solid content (SSC), titratable acidity (TA) and pH.

Berry density was significantly different among dates in all fields except Debert (all p values < 0.03; in Debert $p= 0.097$). Berry density changed with maturity due to changes in internal berry chemistry. Commercial production of both rabbit-eye (*Vaccinium ashei* Reade) and lowbush blueberries have a mixed maturity harvest and are sorted through hydro-density (Patten *et al.* 1989). During density sorting, berries were immersed in salt brines at various concentrations that float or sink particular maturity stages (Patten *et al.* 1989). In this study green berries had a lower density than water and floated in pure water, the density of red berries wasclose to that of water, and blue blueberries had a higher density than water and sunk (Table 2-3). The maturity of kiwi

fruit (*Actinidia deliciosa*) can also be determined using density; in this species, changes in density are due to the balance of starch and sugars, along with the presence and size of the intercellular spaces (Jordan *et al.* 2000). In lowbush blueberries, starch content decreases with maturity as the cell wall is broken down, and the sugar content increases with maturity (Tucker 1993). Composition of ripe highbush blueberries is roughly 83% water, 15% sugar, 1.2% of protein and fat and 0.3% ash (Skupien 2006). The composition of lowbush blueberries is expected to be very similar, and the sugar compliment consists of glucose and fructose in equal amounts (Barker *et al.* 1963; Table 2-3). The density of glucose and fructose is very similar, and at a concentration of 15% would be 1.06 g/cm^3 (Lide 1998). As the concentration of sugars decreases, the density of the berry also decreases and approaches 1.00 g/ cm^3, the equivalent to water (Lind 1998). Therefore as berries mature from green to blue and acquire sugars, the density of the berry increases, causing the berry to sink. Over-mature berries contain a similar amount of sugar to blue berries and would also sink however, the condition of the skin in over-mature berries, caused them to leak in the water solution, creating inaccuracies.

The density of the mixed sample was a reflection of the maturity stages included in the mix at each date. On the early dates, the samples contained mostly green berries, which float. As the mix changed to be composed of mostly red berries, the density increased to be near that of water, and would float or sink depending on if the mix contained red and green berries or red and blue berries. A composition of mostly blue berries, as was found from August to September, would sink. A mix of blue and over-mature berries would float or sink depending on the condition of the over-mature berries. Over-mature berries have a similar sugar content to blue berries (Table 2-3) and both

would sink in water. However, when over-mature berries were in poor condition, they leaked into the water, changing the density of the solution, which changed how the maturity stages reacted to the solution. If the solution gains sugars, the blue maturity stage would have an equal density, and begin to float. Since the density of a mixed sample is a reflection of the composition of the sample, and does not strongly correlate with any one maturity stage (Table 3-3), this method would be more complicated than a visual inspection of the field, as is currently the practice.

SSC, TA and pH were all significantly different among dates in all fields ($p<0.001$). In each field SSC increased between July 4th (day 185) and August 15th (day 227), this corresponded with the change in the mixed composition from mostly green to mostly blue. SSC is a measure of the amount of dissolved solids in a juiced sample, and in fruit it is roughly equivalent to the amount of sugar and dissolved anthocyanins in a sample (Saftner 2008). The amount of sugar increased as berries changed from green to blue (Table 2-3). Between August 15th (day 227) and Aug 22nd (day 234) there was a slight decline in SSC, which subsequently fluctuated around 10 ^{o} Brix on the latter sampling dates. The decline around August 15th (day 227) corresponded with an increase in over-mature berries in the mixed sample. The condition of over-mature fruit was not consistent; some berries had broken skins, while others were wrinkled and dry. The sugar content of any particular sample would have been influenced by the condition of the over-mature fruit and the amount of retained sugars in the over-mature fruit. While SSC had the strongest positive correlation with percent blue ($R^2=0.64$), and the values of SSC across the season increased, the values on consecutive sampling days fluctuated widely

(Table 3-2). As such, no specific value of SSC reflected an optimal harvest composition with greater accuracy than the current practice.

Titratable acidity and pH are both measures of berry acidity; TA is an indication of the total acids, while pH is a measure of hydrogen and hydronium ions (Sadler and Murphy 2003). TA and pH reflect the presence of acids in opposite numerical order, an increase in TA indicates an increase in the amount of acid present, while an increase in pH indicates that the berries are becoming more basic and there are fewer hydrogen and hydronium ions present (Sadler and Murphy 2003). While the two measures are strongly correlated (R= -0.78, Table 3-3), the measuring system results in a negative correlation.

Fruit acids influence taste through both composition and concentration; different acids are perceived as bitter, astringent or sour (Rubico and McDaniel 1992), while high concentrations increase the perception of sour notes (Saftner *et al.* 2008). An unpleasant taste in un-ripe fruit helps prevent the early harvest of berries by dispersers, and allows seeds to fully mature (Gould & Lister 2006). As berries mature, the acids can be converted to sugars or channelled into the respiration pathway (Dai *et al.* 2009). In the mixed berry samples, pH increased in all fields from about 2.71 in July to about 3.10 in September (Table 3-2). TA decreased with sampling date, generally going from 0.1 citric acid equivalents in July to 0.04 in August (Table 3-2). There was a sharp decline in TA July 18th (day 199) and July 25th (day 206) (Athol, Debert), July 25th (day 206) and Aug 1st (day 215) (Farmington), and between Aug 1st (day 215) and Aug 8th (day 220) (Diligent River; Mount Thom) (Table 3-2), which corresponded with a switch between green berries being the largest fraction of the mixed sample, to blue berries being the largest component of the mixed berry samples (Figure 3-1). In three of the fields, these

dates corresponded with the period prior to when the percent blue had peaked and levelled off in the fields, and in the last two fields, Diligent River and Mount Thom, it corresponded with the date at which the percent blue levelled off. Titratable acidity was strongly correlated with the percent green in the field (R= 0.88), which suggests TA is a promising measure to indicate optimal berry composition. However, the inconsistent results among fields may limit the usefulness of measuring TA as a ripening measure. As well, TA largely reflected the decrease in percent green in the field, this again may be as accurately judged by visual inspection.

Correlation analysis of the mixed berry chemical factors and the percent maturity found the percent green strongly correlated with TA (R= 0.88; Table 3-3) and inversely correlated with SSC (R= -0.67). Percent blue was strongly inversely correlated with percent green (R= -0.83), and weakly correlated with SSC (R= 0.64) and inversely with TA (R= -0.72). None of these values suggest that measuring the chemical nature of a mixed sample would lead to increased accuracy in predicting the optimal time to harvest, over judging a field by eye.

The Stability of the Physico-Chemical Nature of Ripe Blue Stage Berries Over Time

A large proportion of berries were ripe in the field around Aug 1st (day 213) in 2006, and between July 31st (day 212) and Aug 14th (day 226) in 2007. If characteristics of blue berries harvested early in the season are the same as blue berries harvested late in the season, producers would have a longer time frame in which to harvest their crop.

There was no difference in size among the three dates, in three of the five fields in 2006 (Table 3-4). Diligent River ($r= 0.001$) and Debert ($r< 0.001$) both show a decrease

in size between July 18th (day 199) and September 12th (day 255) (Table 3-4). In 2007, there was no difference in size among the three dates, in any of the fields (Table 3-4). A trend of decreasing size as the season progresses was the result of smaller berries taking longer to ripen and appearing in the mature blue class around the same time larger mature berries moved into the over-mature class.

There was no significant difference in any of the chemical measures, density, pH, SSC, and TA, within fields in either year, between years or among fields.

The stability of the chemical nature and berry size across dates indicates harvest could occur as soon as the percent maturity peaks. In 2006 the percent blue first peaked on July 25th (day 215), and fields were harvested around August 21st (day 233). In 2007 the first peak in percent blue occurred on August 14th (day 226) and berry harvest occurred around September 5th (day 248) in 2007. This was an extension of between two and three weeks in each year. Waiting additional weeks before harvesting, does not increase the percent blue in the field (Figure 3-1), the berry size (Table 3-4) or improve the chemical nature of the berries (Table 3-4).

Ripening and Climate

Berry ripening is initiated at fruit set and continues irreversibly until berry senescent (Prasanna *et al.* 2010). Physiological and climate factors influence the ripening rate of individual berries and result in a harvestable crop of mixed maturity berries. The variability in ripening rate has hindered the development of a ripening index for lowbush blueberries. Early attempts involved counting the number of days between bloom and full maturity; however, differences among individual berries, clones, fields and regions meant

it was not possible to determine a set number of days at which berries reach maturity (Bailey 1947). When ripening was compared to broad scale, provincial weather patterns, no one climate factor was found to strongly correlate with ripening in all areas (Hall *et al.* 1982). The lack of a statistical correlation was attributed to local ripening patterns being masked by the extent of the study area and climate patterns (Hall *et al.* 1982). Since berry ripening rate is influenced by localized weather factors, comparing localized climate and ripening rates should reduce the amount of variability between these two measures, allowing ripening patterns to be evident.

Temperature and soil moisture are both climate variables which directly affect ripening (Taiz and Zeiger 2006) and around which ripening indices could be built. Researchers studying other crops including grains, root vegetables and fruit crops have been successful in determining optimal ripeness through the use of growing degree days (GDDs) (Carlson and Hancock 1991). GDDs are calculated based on the difference between the minimum temperature at which a plant is physiologically active, and the maximum ambient temperature measured over a time period (Carlson and Hancock 1991). For annual crops GDD accumulation usually starts at a specific physiological event, such as planting date. In perennial crops, the timming of physiological events is more variable and GDD are calculated from set dates (Carlson and Hancock 1991). June 1st was chosen as a date by which all chilling requirements would have been met, and the date around which the average low temperature is at or above the 5^{o}C base temperature.

The same type of work to link soil moisture to plant physiology has not been done. An index based on soil moisture could be developed if both the amount of soil moisture available to a plant, and how much water a plant uses for photosynthesis and

other metabolic process were known variables. Growth would occur when the available soil moisture was greater than the plants water use. Since metabolic activity is also linked to temperature, soil moisture units could be calculated from the first date above 5^{o}C, with sufficient soil moisture. This would be a relatively simplistic model which would not account for lowbush blueberry adaptations to drought stress or moisture storage in the extensive rhizome system (Glass *et al.* 2005), and as such might not relate well to percent maturity. In this study the soil moisture and percent blue were not correlated overall (R= -0.08) or when grouped by years (2006 R= -0.09; 2007 R= 0.26). The lack of correlation with percent blue or a repeated pattern in soil moisture between field or years did not suggest soil moisture to be a viable basis for a maturity index at this time.

Linking percent maturity with GDD for the five fields and two years resulted in a pattern of the peak percent blue occurring at approximately 1200 GDD, with the exception of Mount Thom where the peak occurred around 1400 GDD (Figure 3-5). The peak percent blue differed between the two years, most distinctly in Farmington, Debert and Mount Thom, with lower levels in 2007. This is consistent with the percent maturity by date (Figure 3-1), and is likely the result of colder temperature across the 2007 growing season. Colder temperatures could result in lower percent maturity curves in a number of ways. Colder temperatures in the spring could decrease pollinator activity (Eaton and Murray 1997), resulting in fewer pollination events and smaller berries (Ismail and Kender 1974). Since the percent maturity curves were calculated on a by weight basis, smaller berries would weigh less and the percent maturity curve would be reduced. Alternatively the percent maturity could be decreased if the number of floral primorida were reduced on the raceme. Colder temperatures during floral development has been

shown to result in a fewer number of floral primordial (Hall *et al.* 1970). Receptive flowers could also be reduced if there was a spring frost (Hicklenton *et al.* 2002). Finally, under controlled conditions, berries ripened faster in warmer temperatures (Hall and Aalders, 1968), so the overall number of ripe berries would be lower in cooler temperatures.

Counting from June 1st (day 152) Nappan reached 1200 GDD around September 12th (day 225) in 2006 and August 23rd (day 235) in 2007. Debert reached 1200 GDD around August 8th (day 220) in 2006 and August 28th (day 240) in 2007, Debert reached 1400 GDD around August 28th (day 240) in 2006 and September 17th (day 260) in 2007. Parrsboro reached Julian day 1200 around August 28th (day 240) in 2006 and September 17th (day 260) in 2007 (Data not shown). The Nappan weather station covers the Farmington and Athol fields, Parrsboro covers the Diligent River field, and the Debert weather station covers the Debert and Mount Thom fields. With the exception of Diligent River and Mount Thom, these dates in 2006 occur towards the start of the percent blue plateau (Figure 3-1). In Diligent River and Mount Thom in 2006 there was a slightly higher peak in percent maturity at the end of the plateau period then at the start of the period (Figure 3-1).

In 2007, the plateau region of percent blue was more variable, in the Farmington, Athol/ Southampton and Debert fields the GDD peak occurred on August 28th (day 240) (Figure 3-1). Both the Athol/ Southampton and Debert fields had a higher peak in percent blue on September 11th (day 254), while the Farmington field had a higher percent blue on August 21st (day 233) (Figure 3-1), by which time the field had accumulated 1100 GDDs (Figure 3-5). Diligent River reached 1200 GDD around September 17th (day 260)

in 2007, and Mount Thom did not reach 1200 GDDs (Figure 3-5). The percent blue curves for these fields were also still increasing on these dates (Figure3-1), suggesting these fields didn't fully ripen in 2007.

While 1200 GDDs did not consistently match up with the first peak in percent blue, it did always occur somewhere along the plateau in percent blue (Figure 3-1; 3-5). Tracking growing degree days could be done remotely, with on site weather stations, which should also increase the accuracy of the measure. Further, GDDs could be forecasted based on predicted weather, allowing for the development and implementation of harvesting plans.

There are several avenues of research that could improve the GDD model. The base temperature should be optimized for lowbush blueberry. 5^{o}C was a useful place to start because of its use in raspberries and highbush blueberry but it should be confirmed that lowbush blueberries are physiologically active at this temperature. It should also be determined if there is an upper temperature threshold at which photosynthesis is limited in lowbush blueberry. As blueberries are a perennial plant, it is reasonable to use a fixed date from which to start accumulating GDDs, however futher improvements in the model could be achieved if this date was optimized. Lastly, the percent maturity was calculated from the highest value of percent blue regardless of where it occurred within the plateau of percent blue over time. Since the physico-chemical nature of berries does not change along the plateau, the GDD model should

Conclusion

Consistent patterns in the percent maturity were seen in both years and all fields. Percent green was highest on the earliest sampling dates (July 4th (day 185) 2006 and July 3rd (day 184) 2007) and decreased to low levels mid season (Aug 1st (day 215) 2006; between Aug 14th- 21st in 2007 (day 226-231). In some fields, the green maturity stage persisted across all sampling dates. Red berries remain at low percentages across the entire season. Over-mature berries began to increase mid-season (Aug 15th (day 227) in 2006 and Aug 21st (day 233) in 2007) and peak towards the last sampling dates. The maturity pattern in ripe blue berries was to increase in early July, level off between the end of July and the end of August and to decrease into September. While the overall pattern was consistent, the timing of the increase can be influenced by the seasonal temperature and large weather events can disrupt the plateau by dislodging berries or by pre-maturely softening berries.

The chemical nature of blue stage blueberries remained constant across ripening date. Berry size remained the same or decreased as the growing season progresses. The number of berries in a field also remained stable. These factors suggest lowbush blueberries could be harvested over a wider period of time than is currently the practice.

There is a positive relationship between the percent blue and heat unit accumulation. The percent blue peak between 1200- 1400 GDD accumulated. All fields are within the percent blue stage plateau at 1200 GDD, therefore fields can be considered optimally ripe at 1200 GDD. The consistency of this measure among fields and between years suggests GDDs would be an effective ripening index for lowbush blueberry.

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Chapter 4: Pre-Harvest Fruit Loss in Lowbush blueberries

Abstract

Lowbush blueberry exhibit scattered ripening resulting from differences in location, nutrition, the degree of pollination and the amount of sunlight received by individual fruit. Harvest occurs only once and fruit shatter, the loss of ripe fruit prior to harvest, represents a potential yield reduction to the producer. This study assessed fruit shatter in five Nova Scotia commercial lowbush blueberry fields during 2006 and 2007. Transects spanning several clones were established in five commercial blueberry fields in Colchester, Cumberland and Pictou Counties, Nova Scotia. Stems were sampled biweekly between the first two sample dates and weekly for the remaining dates. Differences in berry numbers were determined by comparing the number of berries collected at the sampling date to the initial number observed and were converted to percent fruit retention. In both years and in every field there was a trend of increasing fruit loss as the season progressed. The mean percent loss, averaged across fields but analysed separately by year was significantly different among dates (ANOVA $p < 0.001$). Piecewise regression was used to determine the date at which there was a significant change in fruit retention. The break occurred on Aug 28th in 2006 and on September 4th in 2007. With the exception of Diligent River in 2007, the sharp decline in berry retention occurred a week or more after harvest for all fields in both years. The harvest in Diligent River in 2007 occurred in the same week as the decline in berry retention. To determine the relationship between the percent loss and mixed maturities in the field a general linear model was fit. Thirty six percent of the variation in the percent retained is related to field, the amount of over mature berries and to a lesser extent the amount of blue berries. Based on these results, fruit shatter is not a serious concern for the wild blueberry industry, although the decrease in fruit retention at the end of the growing season might limit the date that fields can be harvested.

Introduction

The lowbush blueberry is a unique crop in that it is not composed of a single variety and the harvest typically contains fruits of mixed maturities (Burgher- MacLellan and MacKenzie 2004). The mixed maturities are the result of both physiological and climacteric influences on berry development (Bell 1950; Bell and Burchill 1955; Hall *et al.* 1982). Physiological factors include floral development, pollination rate and seed development. Climate factors include the temperature and precipitation.

The lowbush blueberry produces fruit in a biennial fashion, with fruit development initiated in the fall of the vegetative year, when floral buds are formed at the tip of a vegetative stem (Bell 1950). Each bud contains between seven to ten flower primordia (Bell 1950), which continue to develop as they overwinter. In spring the buds open and the oldest flowers are all at roughly the same maturity stage, while the youngest flowers are incompletely developed (Bell 1950; Bell and Burchill 1955). The developmental difference at the floral stage is the first factor that contributes to subsequent uneven berry development.

Pollination is the second factor to lead to mixed berry maturities at harvest. Pollination occurs in the spring and is insect mediated (Eck 1986). In commercial fields natural pollinators are supplemented with introduced bees (Eaton and Murray 1997). A cold or wet climate can depress the pollination rate by being unfavourable for flight by the pollinator (Eaton and Murray 1997). A decrease in pollination leads to fewer viable seeds and smaller berries that do not ripen as quickly (Eck 1986). If a flower is not pollinated the petals and anthers are dropped leaving an exposed pistil, known as a 'pinhead'. No further berry development occurs in a pinhead. Successful fertilization initiates events in both the ovary and the ovule that lead to mature fruit; specifically, endosperm and zygote development within the ovule which forms the set berry (Eck 1986; Bell 1957).

The last physiological influence on ripening rate is the number of viable seeds that develop in the berry. Fertilization triggers both endosperm and zygote formation (Bell 1957). However, in blueberry endosperm and zygote formation are not matched developmental processes; instead the endosperm may develop faster than the zygote or

the zygote may be mostly formed prior to the formation of endosperm (Bell 1957). When the endosperm and zygote do not develop at the same rate, one of the tissue layers can begin to degenerate prior to the zygote penetrating the endosperm plug, resulting in an nonviable seed (Bell 1957). Berries are capable of ripening with only two perfect seeds but gain weight, berry diameter and ripen faster with increasing numbers of perfect seeds (Eck 1987; Aalders and Hall 1961).

In some fruit, mostly those borne on trees, fruitlet abscission occurs early in the growing season (Bangerth 2000). It is thought that this self regulated thinning process occurs to ensure that the plant can support growing fruit to maturity under current environmental conditions (Bangerth 2000). While pre-harvest fruit loss has not been noted in *Vaccinium* species, the number of retained berries at harvest can be 50-90 percent lower than the number of berries set (Trevett 1959). Some losses can be attributed to disease and predation (Martin *et al.* 2006; Annis and Stubbs 2004; Eaton 1957). Losses due to disease are localized to clustered patches (Martin *et al.* 2006; Annis and Stubbs 2004). Fruit is also taken by both birds and mammals. While birds are known seed dispersers, the percent loss to animal predation is not known for lowbush blueberry (Eaton 1957). In other plant species this loss has been underestimated and mistakenly attributed to climate factors (Conover 1982; Bartuszevige *et al.* 2006).

Fruit drop is only a concern if the majority of the berry loss occurs prior to harvest. The range of berry maturities found in a field does provide flexibility in potential harvest dates, but producers risk losing harvestable yield due to fruit drop if harvest is delayed too long. This study examines whether fruit shatter occurs in non diseased

lowbush blueberry plants, if fruit abscission occurs at any maturity stage prior to fully ripe and whether there is an impact of fruit shatter on harvestable yield.

Methods

Five commercial blueberry fields in Colchester, Cumberland and Pictou counties, Nova Scotia were sampled in both 2006 and 2007. In each field, two transects spanning different clones were established by tagging stems at 20 cm intervals. In 2006, 80 stems were tagged in each field and 100 stems were tagged in each field in 2007. On July 4th (Julian day 185) 2006 and July 3rd (day 184) 2007 the number of set fruit on each stem were counted, assuming enlarged fruit were set and would undergo fruit ripening. The first collection date occurred a week later, and eight (in 2006) to ten (in 2007) stems were cut and bagged. After transport to the lab, the number of berries remaining on each stem was recorded. Collecting continued two weeks later and then weekly thereafter. On the final sampling date (September 12th (day 255) in 2006; and September 25th (day 268) in 2007), all remaining stems were harvested.

Three values were calculated for each stem, the difference in number, the percent loss and the percent retained. At each sample date, the difference in berry number was determined by subtracting the number of berries remaining on the stem from the original set fruit count. Percent loss was calculated by multiplying the difference divided by the original berry number by 100. Percent berry retention was calculated by subtracting the percent lost value from 100. To determine the percent fruit retained by date the percent retained values were averaged across the number of stems sampled in each field on each date.

To determine if there was a difference in the average percent retained among the dates, an ANOVA was performed for the 2006 and separately for 2007. A piecewise regression using the formula (slope1*date + intercept1) * (date<threshold) + (slope2*date+threshold*(slope1-slope2) +intecept1) * (date>=threshold) was completed to determine the date at which retention dropped off. To complete this analysis date was converted to Julian date a calendar system which labels January 1 as day 1 and so on until day 365 on December 31st.

To determine how the percent loss was related to maturity stage a backward stepwise general linear model was fit using year, field, year*field, the amount of blue, and the amount of over mature berries as independent factors . All statistical analysis was done using SYSTAT 10 (SYSTAT Software INC, Chicago).

Results and Discussion

There was considerable variability in the average number of berries retained from week to week during the season (Table 4-1). The greatest fluctuation occurred early in the season with increased counts from one week to the next. Part of the fluctuation can be explained by sampling error related to the estimation of set fruit in the field and where some set fruit were recorded as pinheads. This was less of an issue in 2006 than 2007. The count of set fruit occurred around the same date in both years July 3rd (day 184) in 2007 and July 4th (Julian day 185) in 2006. In 2007 the berries were not as developed. The developmental delay is most likely related to a colder than usual spring. 2007 was approximately 5°C colder than 2006 in early July. Further, only 400 GDD had been accumulated in 2007 compared to 600 GDD at the same time in 2006 (Figure 3- 4). Cold

and wet conditions can delay the appearance of flowers, pollination and berry set (Hall and Aalders 1968; Eaton and Murray 1997).

Table 4-1: Percent retained berries by week for five commercial fields in 2006 and 2007. Sampling date given in Julian Days.

2006										
Sample Date (Julian day)										
	199	**213**	**220**	**227**	**234**	**241**	**248**	**255**		
Farmington	80.1	88.3	74.1	57.7	64.1*	71.4*	53.3	54.0		
Athol	100.6	95.8	78.9	108.1*	88.4	79.9	52.2	35.8		
Diligent River	91.5	77.8	87.3	82.1	76.6*	75.3*	54.0	31.9		
Debert	75.1	70.2	81.6	65.1	69.6*	50.2*	14.2	21.4		
Mt. Thom	93.9	95.3	76.0	57.6	85.7	88.0*	70.6	31.1		
2007										
Sample Date (Julian Day)										
2007	**198**	**212**	**221**	**226**	**233**	**240**	**247**	**254**	**261**	**268**
Farmington	99.3	86.3	58.0	64.4	56.9	53.6	50.0*	44.6	27.4	17.7
Athol	123.4	88.5	93.3	129.5*	79.9	121.2	96.1	68.0	71.5	55.2
Diligent River	106.4	116.0	71.4	135.3	98.8	73.4	84.9*	40.1*	43.8	34.9
Debert	118.2	81.9	95.3	67.9	89.9	80.6*	80.3	63.4	22.4	0
Mt. Thom	112.6	148.2	112.6	71.9	80.6	68.9	76.3	61.9*	58.0	21.7

* indicate approximate harvest date.

Overall, every field showed a decline in the percentage of berries retained on the stem as the growing season progressed (Table 4-1). In 2006, Farmington, Diligent River, Debert and Mt. Thom all showed steady declines, starting with between 75- 94 percent retained and declining to between 21- 54 percent retained. Athol gained berries between tagging and the first sampling day, then declined to 74% on August 8th (day 220). On August 15th (day 227), berry retention jumped to greater than 100%, after which there was a decline to 36%. In 2007, Diligent River and Farmington showed a declining pattern across the season. These fields started with 106 and 99 percent retained and dropped to 34

and 17 percent retained respectively. The other three fields all appeared to gain berries between August 7th (day 219) and 28th (day 240) before declining to 0-56 percent retained on the last sampling date. To determine if there was a difference among the mean percent retained on each date, an ANOVA analysis was completed. In both 2006 and 2007, there was a significant difference among dates ($p > 0.001$).

A decline in berry retention over the course of a growing season is expected; berries are known to detach due to disease and predation (Martin *et al.* 2006; Eaton 1957). It is also possible that berries would detach in climate events such as a hard rain or high windfall, although this has not been investigated specifically in blueberries. Lastly, it is likely that blueberries more readily detached upon maturity, although the literature on lowbush blueberry maturity focuses on the internal chemistry and post harvest effects away from the field.

The ANOVA results indicate that there is a change in retention but does not suggest the date at which retention changes. If lowbush blueberries were behaving like tree fruit and shedding immature fruit to conserve resources, a drop in retention would be expected soon after fruit set (Bangerth 2000). This is unlikely in lowbush blueberries as Table 4-1 indicates that the percent retained remains high, between 80-100 %, for the month after fruit set in all fields and both years.

The trend in average berry retention in both years showed a slight decrease for about three quarters of the season followed by a sharp decline on August 28th (day 240) in 2006 and September 4th (day 247) in 2007 (Figure 4-1). To determine where the slope of the line changed piecewise regression was used. In 2006 the threshold was determined to

be on August 28th (day 240) ± 7 days (R^2 =0.96). In 2007 the threshold value was determined to be on September 4th (day 247) ± 16 days (R^2 = 0.94)

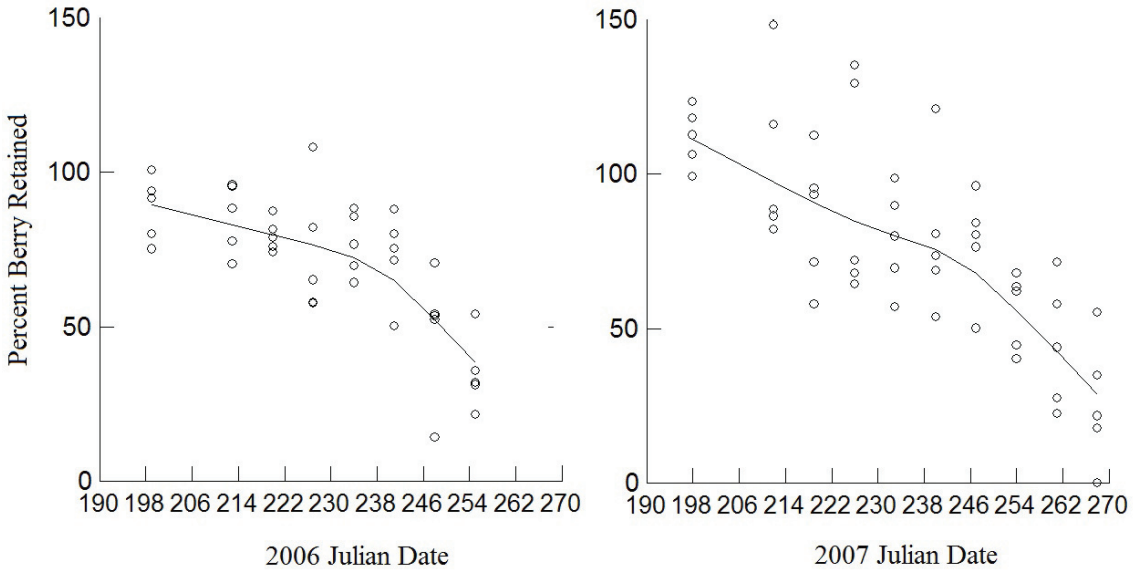

Figure 4-1: Scatter plot of percent berry retention across all fields in 2006 and 2007.

While it appears as if lowbush blueberries do not drop fruit prematurely early in the season, they do experience fruit loss as the season progresses (Table 4-1; Trevett 1959). Physiological and climatic events lead to a crop of mixed maturities as blueberries ripen (Bell and Burchill 1955; Eck 1986; Aalders *et al.* 1972), therefore it is possible that fruit loss is related to the composition of the maturities in the field as over-mature berries are easier to detach from the stem than immature berries (Gonzalez-Carranza *et al.* 1998). Percent blue stage and over-mature stage berries by weight and averaged across all fields were plotted to compare the pattern in percent loss against the trends of both maturity stages (Figure 4-2). In 2006, percent blue stage peaked on July 29th (day 210), decreased slowly to August 23rd (day 235), after which there was a more rapid decrease (Figure 4-

2). In 2007 percent blue stage peaked twice, on August 13th (day 225) and September 7th (day 250) before declining sharply (Figure 4-2). In both years over-mature berries were present on August 3rd (day 215) and increased over the growing season (Figure 4-2).

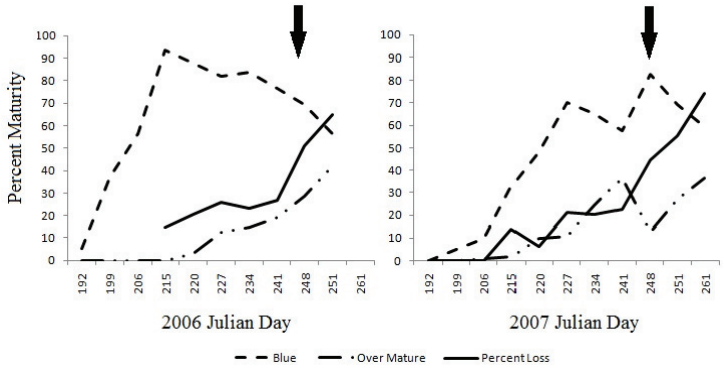

Figure 4-2: Maturity stage in relation to percent loss across dates in 2006 and 2007. Arrows indicate the date by which harvest should occur to minimize berry loss due to fruit shatter.

In 2006 the average percent loss increased as the percent of blue stage blueberries decreased and the percent of overripe stage berries increased. In 2007, the percent loss increased with the amount of overripe berries, but the relationship is not as clear, as there was an increase in overripe berries between August 21st (day 233) and September 4th (day 246) with no corresponding increase in the percent loss (Figure 4-2). This pattern suggested that berry retention may decline in relation to the amount of over mature and ripe berries in a field. To test this relationship a general linear model was fit using backwards stepwise regression. The percent loss is weakly related to three independent factors, percent over-mature stage, percent blue stage and field (R^2= 0.361). The percent over mature stage has the most influence ($p < 0.001$), followed by the differences among the fields ($p=0.004$) and lastly the percent blue stage ($p=0.080$). This suggests that the mix of maturities in the field does influence the amount of fruit shatter and that some fields may be more prone to shatter than other.

To determine if fields behave consistently between years, the largest percent loss among successive dates was examined (Table 4-2). The ranking of fields in 2006 was Farmington (18% decline in percent retained), Diligent River (22% decline in percent retained), Athol (27% decline in percent retained), Debert (36% decline in percent retained) and Mount Thom (40% decline in percent retained). In 2007, the ranking was Farmington (28% decline in percent retained), Mount Thom (40% decline in percent retained), Debert (41% decline in percent retained), Diligent River (44% decline in percent retained) and Southampton (49% decline in percent retained). There is no evident pattern in either the amount of percent loss between dates, or in the order in which the fields experience their largest decline in percent loss.

Table 4-2: Decline in percent retained and the dates between which the decline occurs.

	Largest decline in % retained 2006	**Date Range**
Farmington	18 %	Aug 29- Sep 5 (241- 248)
Athol	27 %	Aug 29- Sep 5 (241- 248)
Diligent River	22 %	Sep 5- Sep 12 (248- 255)
Debert	36 %	Aug 29- Sep 5 (241- 248)
Mt. Thom	40%	Sep 5- Sep 12 (248- 255)
	Largest decline in % retained 2007	**Date Range**
Farmington	28 %	Aug 1- Aug 8 (213- 220)
Southampton	49 %	Aug 15- Aug 22 (227- 234)
Diligent River	44 %	Sep 5- Sep 12 (248-255)
Debert	41%	Sep 12- Sep 18 (254- 261)
Mt. Thom	40 %	Aug 8- Aug 15 (220-227)

In 2006, three fields experienced the largest percent decline between August 29th (day 241) and September 5th (day 248) (Farmington, Athol and Debert), and the largest decline in the other two fields (Diligent river and Mount Thom) occurred between day August 29th (day 241) and September 5th (day 248). This pattern was not repeated in 2007, where each field lost the largest percent retained through different date ranges, in the order of Farmington, Mount Thom, Southampton, Diligent River and Debert (Table 4-2). Based on these results, fields cannot be grouped into fields that shatter earlier or later, or by greater and lesser amounts.

If fields shattered in response to climate, large scale weather events such as occurred on Aug 18th (day 230) and Aug 24th (day 236) of 2007 should be paired with the sharp decline in retention of percent blue. However, only one of the four affected fields, Southampton, (Fig 3-1) had a sharp decline in berry retention around these dates. Both Farmington and Mt. Thom shattered prior to this date and in Debert the decline in berry retention occurred two weeks after the weather system passed over (Table 3-1). An initial comparison of the break point in each year with GDD also does not suggest a strong relationship. By August 28th (day 240) in 2006, between 1300- 1400 GDD had been accumulated and in 2007 1100-1300 GDD had been accumulated by September 5th (day 247). It is possible that a relationship between GDD and fruit shatter is being masked by the measurement scale, and that in field monitoring of GDD would reveal a stronger relationship.

While berry retention does decrease across the season, it does not affect the harvestable yield unless the largest drop in retention occurs before harvest (Table 4-1). Piecewise regression indicated there was a break point between the mean percent

retention on Aug 28 (day 240) in 2006 and September 4th (day 247) in 2007. In 2006 all fields had been harvested just prior to this date (Table 4-1). In 2007 there was greater variability in when these fields were harvested, but at least three fields were harvested around or after September 4th.

In 2007 only two fields, Athol and Debert, were harvested prior to the date at which retention decreased, September 4th (day 247). This suggests that yields could have been increased with an earlier harvest date. Harvest occurred in Farmington on September 4th (day 247) and on this date the percent retention was at 50%. In the prior week retention was at 53%. So an earlier harvest date in this field would have only resulted in a marginal increase. Mt. Thom was harvested on September 11th (day 254) on this date the percent retained was at 62%, while in the week prior the retention was at 73%. Diligent River was harvested sometime between September 4th (day 247) and September 11th (day 254). In this period the retention dropped from 85% retained to 40% retained. In these fields an earlier harvest could have resulted in an increased yield by as much as 45%.

Based on the results of this study it appears that blueberries are not prone to early fruit abscission, and that fruit retention through the growing season is weakly related to the maturity stages present in the fields. In Nova Scotia, significant losses of fruit do not occur until late in the season in lowbush blueberry fields. However, producers should be aware that losses from fruit shatter might be significant in fields that are left for a late harvest.

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Chapter 5: Conclusions

While the general changes that occur in lowbush blueberry during ripening are well documented, how the phenolic classes and specific compounds change during fruit ontogeny have not been previously reported. Three of the four phenolic classes, flavonols, phenolic acids and flavan-3-ols declined on a percent dry weight basis as berries matured, while the fourth class anthocyanins increased with maturity. Overall, the abundance of total phenolics did not change with maturity.

Throughout all maturity stages, quercetin-3-*O*-galactoside was the most abundant flavonol, chlorogenic acid was the most abundant phenolic acid, and epicatechin was the most abundant flavan-3-ol. No one anthocyanin was more abundant than any other anthocyanins across all maturity stages, which reflects the variability in colour among ripe blueberries.

A comparison of three antioxidant measures found F-C and ORAC to suggest a similar pattern across maturity, while the FRAP analysis suggested there was no change in antioxidant capacity with maturity. The three measures were each positively correlated with different phenolic classes, F-C correlated with flavan-3-ols, FRAP with flavan-3-ols and phenolic acids, and ORAC with anthocyanins. Additional work could be completed to determine how specific phenolic classes contribute to the overall antioxidant capacity of blueberries.

The profile of how total sugar and acid changed with maturity was consistent with what was found in the literature. Sugar content increased with maturity and acid content decreased with maturity. Glucose and fructose were the most abundant sugars in all maturity stages, with small amounts of galactose and sucrose contributing to the total

sugar content. Except for galactose, there was a significant difference in sugar content among green berries and all other maturity stages, and no significant difference among red, blue and over-mature berries. Five organic acids were found in all maturity stages, while tartaric acid was found in trace amounts in the red through over-mature stages. Unlike the reported literature, quinic acid was found to be the most abundant acid, followed by citric acid in all maturity stages; in the literature this pattern is reversed. It is unclear why this result was found, but replicates from the samples collected in one year were analyzed separately in two different years and the same pattern occurred. It would be interesting to collect further berries from these clones, to see if the pattern persisted between years. Since all the samples for this analysis came from one field, it is also possible this is a field effect.

Phenolic biosynthesis occurs as a part of plant secondary metabolism, initially using substrates from primary metabolism. Within the pathway phenolic compounds can be use as substrates for downstream reactions resulting in a diversity of end products. The biosynthetic pathway is regulated by a limited number of genes at key steps along the pathway, that are known to be influenced by developmental and environmental factors. Since the amount of total phenolics measured in this study did not change with maturity, it suggests that the biosynthetic pathways is substrate limited and the various phenolic subclasses are preferentially synthesised at the different maturity stages. Further investigation should be carried out to determine how the genetic regulation of the phenolic biosynthetic pathway changes with fruit maturity.

Each phenolic class contributes different traits to the berry, such as astringency or colour, and there is likely an ecological influence on the expression of these compounds.

It would be interesting to look at the genetic expression of this pathway and relate the findings to the ecological role of the phenolic compound. This could lead to additional research into horticultural practices to promote or inhibit the expression of specific phenolic compounds in lowbush blueberry. The ability to manipulate the blueberry plant to produce specific phenolics could result in the development of nutraceuticals, and result in additional maturity classes being desirable at harvest.

The change in percent maturity for all stages was consistent across fields and between years. Fields start off with nearly all green berries, which persist in the field for about a month. Red stage berries appear in as the percent green stage begins to decline, and remain at low levels across the growing season. Blue stage berries begin to increase as the percent green declines, peaks two to three weeks after starting to increase, remain at high level of percent blue for about a month before declining. Lastly, there is a sharp increase in over-mature berries as blue stage berries decline. While the pattern is consistent, the timing and duration of the pattern is strongly influenced by weather. 2007 was a coolder year than 2006 and this is reflected in a delay in the rise in percent blue by a week. Further, and not all fields experienced the decline in percent blue at the end of the season. The physico-chemical nature of blue stage berries was examined to determine if the nature of a blue stage berry changed as the growing season progressed. Size, density, SSC, pH and TA, were not significantly different among the sampling dates in any of the fields, in either year. Since the percent blue peaks in early August, and the physico-chemical nature of the berry is not improved with time, producers could harvest three to four weeks earlier than is currently the practice.

Both physiology and climatic variables lead to fields containing a mix of fruit maturities throughout the growing season. As blueberries are slow growing plants, which require cross pollination for seed development, maintaining fields of mixed varieties is desirable. However, the development of a maturity index would allow producers to judge when the optimal time to harvest would be. Since the fields contain a mix of maturities on all dates, the chemical nature of a mixed sample was examined to determine if there was a simple test which would indicate if there was an optimal mix of maturities in the field. None of the parameters correlated well with percent blue, and would not be more accurate than visually inspecting a field.

Comparing climatic variables to the amount of percent blue in the fields, found that temperature could form the basis of a maturity index, as is used in other crops. Converting temperature into GDD allows the measure to be linked to plant physiology. Tracking the GDD from June 1st found that the percent blue peaked at 1200 GDD and that this was consistent among years and fields. Further, a comparison between the earliest dates at which percent blue peaked to GDDs, indicates that all fields had accumulated between 1000 and 1100 GDD in both years.

The range of GDD through which blue stage berries are at peak levels and the stable physico-chemical nature of the blue stage across the season suggest producers could harvest fields anytime between 1000 and 1200 GDD after June 1st.

A GDD based maturity index could be improved with an accurate determination of the temperature at which blueberries become physiologically active, and whether blueberries have an upper temperature ceiling at which photosynthesis is inhibited.

Accuracy would also be improved with in field weather monitoring, as the berries are responding to micro-climates. A further advantage is that in field monitoring could also be done remotely.

Literature reports suggested lowbush blueberry could lose between 50 and 90 percent of set fruit throughout a growing season. This study found that fields lost between 18 and 40 percent of set fruit, with a greater amount of fruit drop in 2007, than in 2006. In both years a date could be determined after which the greatest amount of fruit drop occurred. In 2006, a sharp decline in berry retention occurred on and after August 28th (day 240), and in 2007 the rate of fruit shatter increased after September 5th (day 247). No consistent pattern in the order in which fields lost fruit or the date on which fields shatter was evident. Further, the date after which fields shatter occurred after the harvest dates for the fields, suggesting fruit drop is not a concern for Nova Scotia producers.

That there is a date after which retention declines sharply suggests there is an end of season boundary on when fields can be harvested with optimal berry yield. An initial comparison of this date with GDD does not suggest a relationship, as the accumulated GDD by these dates overlaps with optimal accumulation of GDD for maximum yield of percent blue. This relationship might be worth revisiting as the GDD model for lowbush blueberries becomes refined and more accurate.

Lowbush blueberry are harvested as a crop of mixed maturities and crop yield gains could be achieved by harvesting when the fields contain a majority of ripe blue class berries. The development of a predictive harvesting index could be useful to producers, allowing them to judge when fields are optimally ripe. The physico-chemical

nature of the ripe berries further suggests harvesting can be occured as soon as the percent blue peaks in a field, typically around the beginning of August. However, calendar date has been demonstrated to be an ineffecting predictive tool to judge the optimal harvest time, and a ripening index should link plant physiology to climate influences. GDD as a predictive model meet these criteria. The usefulness of GDDs is further evidenced by the consistency with which the peak in percent blue occurred at 1200 GDDs among fields and between years. A sharp decline in berry retention was evident in both years, indicating a date afterwhich harvestable yields decline. No detectable pattern was evident in the order or dates through which fields experienced fruit loss and futher work should be done to determine the factors leading to fruit abscission. That there are sharp declines in berry retention at the end of the growing season futher suggests an earlier harvest date would be bennificial to producers.

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Appendix A

Table A-1: Comparison of sampling day, calendar day and Julian day for 2006 and 2007

	Sampling Date	Julian Date	Actual Date
2006	1	185	July 4
	2	199	July 18
	3	206	July 25
	4	215	Aug 1
	5	220	Aug 8
	6	227	Aug 15
	7	234	Aug 22
	8	241	Aug 29
	9	248	Sept 5
	10	255	Sept 12
2007	1	184	July 3
	2	198	July 17
	3	205	July 24
	4	214	July 31
	5	219	Aug 7
	6	226	Aug 14
	7	233	Aug 21
	8	240	Aug 28
	9	247	Sept 4
	10	254	Sept 11
	11	261	Sept 18

Appendix B

Anthocyanins can be extracted from fresh fruit using different extraction media (Kalt et al. 2008; Gao and Mazza 1994; Huber and Rupasinghe 2009). In order to optimize anthocyanin extraction for this project, a comparison was made among three extraction solutions and two fresh fruit processing methods. The methodology used and the results from this optimization are presented below.

Extraction solution 1(E1) was taken from Kalt et al. (2000). The formulation for E1 was MeOH: Acetone: Water: Formic Acid (40:40:20:0.1). Extraction solution 2 (E2) was based on Huber and Rupasinghe (2009). The formulation for E2 was MeOH: Formic Acid (100: 0.1). Extraction solution 3 (E3) was taken from Gao and Mazza (1994) , with a formulation of MeOH: Water: Formic Acid (70:28: 2).

Along with the extraction media, there are various way to process berries for anthocyanin extraction, two common methods are to grind fresh berries in a blender (Kalt et al. 2000) and to freeze dry the fruit (Huber and Rupasinghe 2009). In order to optimize anthocyanin extraction, the two processes were compared, followed by a comparison of the three extraction solutions. E1 was chosen to compare the processing procedures.

Four anthocyanins were compared, the glucoside of cyanidin, delphinidin, malvidin and petunidin.

Plant Collection

One distinct clone was marked at one location, Mount Thom, Nova Scotia and fruits were harvested from July to October 2007. All three clones were wild pollinated and managed with regular industry practices by Bragg group.

Individual berries (approximately 500 g) of the red maturity stage, were hand harvested as they appeared on the clone. Berries were transported in coolers to the Nova Scotia Agricultural College. Red stage berries were used as insufficient numbers of the other maturity stages were collected for a complete analysis.

Chemical standards were purchased as outlined in Chapter 2.

Procedure for Processing Fresh Fruit Through Grinding

Fifty grams of fresh fruit were shredded using a food processor (Magic Bullet, Homland Housewares, California). Approximately 15 g of blended berries was mixed in a Virtis blender with 30-40 ml of extractions solutions, for two minutes. The sample was then vacuum filtered through Wartman glass fibre filter paper in 125 ml vacuum flask. The filtrate was added to a 50 ml volumetric flask and made up to volume with the extraction media. The rest of the extraction followed the procedure outlined in chapter 2.

Procedure for Processing Fresh Fruit Through Freeze Drying

Fifty grams of fresh berry samples were freeze dried in a Freeze Dryer (model 2085C0000, Kinetics Thermal systems, NY) for 60 hours. For the first 24 hours the temperature was kept at – 25^{o}C. The berries were then removed, partly crushed and

returned to the freeze dryer. The temperature was set to -25°C for five hours, then raised to 5°C for 16 hours, and then raised to room temperature for the remaining time.

Upon removal the berries and containers were weighed to determine the percent moisture loss, then ground to a powder using a coffee grinder (model DCG 12BCC, Cuisinart, Ontario). Following freeze drying the berries were powdered, and extraction was completed by mixing approximately 0.3 g of powdered berries with 15 ml of each of the three extraction solutions. The rest of the extraction followed the procedure outlined in chapter 2.

There was a significant difference in the extraction methodology for all four anthocyanins ($p<0.0001$). The freeze dried extraction methodology resulted in better extraction using the freeze dried berries, for all four anthocyanins considered (Figure A-1).

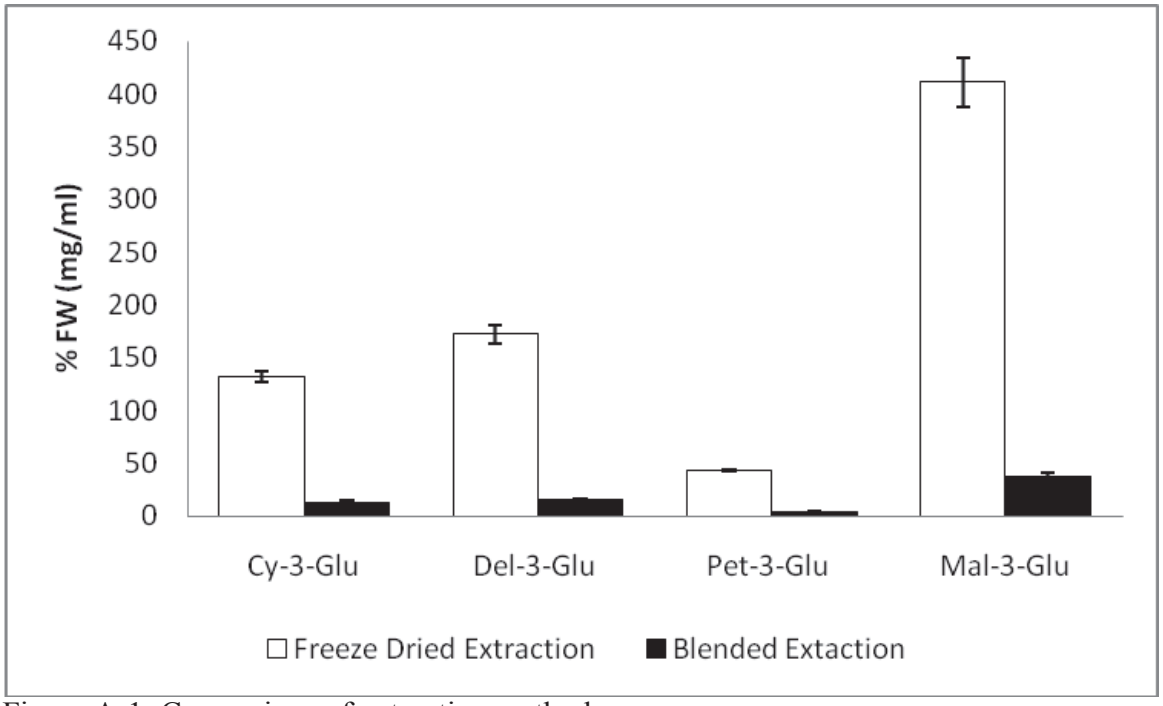

Figure A-1: Comparison of extraction methods

Comparison of Extraction Solutions

For the comparison of extraction solutions, fresh fruit was processed through freeze drying and using the sample procedure as above.

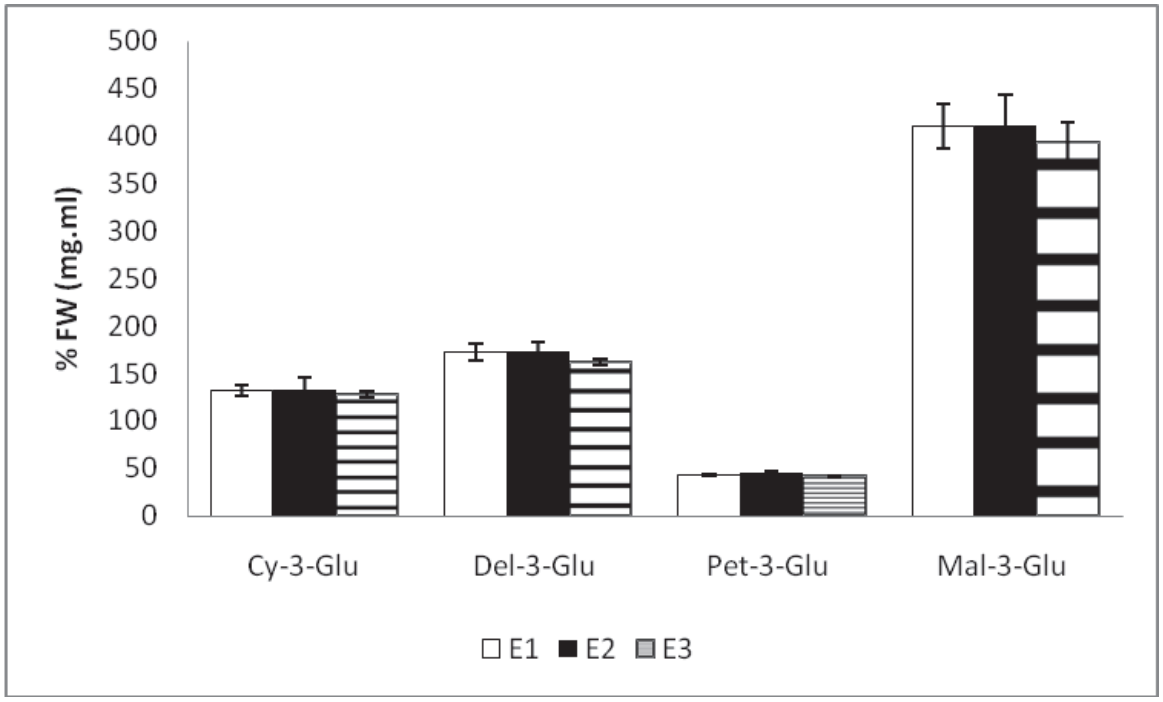

Figure A-2: Comparison of extraction solutions

There was no significant difference between the extractions solutions for any of the four anthocyanins (cyanidin p=0.73; delphinidin p=0.21; petunidin p=0.15; malvidin p=0.63) (Figure A-2). As E1 had been used in the literature for lowbush blueberries, this solution was chosen for use in the rest of the project.

Literature Cited

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