A survey of the quantitative intraspecific variation of anthocyanins, phenolics and
antioxidant capacity in leaves and fruit of
Vaccinium angustifolium Aiton clones in Nova Scotia

by

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Abstract

A total of 135 clones of *V. angustifolium* were sampled from three regions in Nova Scotia; Cumberland County, Hants County and Queens County. The leaves and fruit of each clone were tested for total anthocyanins, phenolics, antioxidant capacity and, in leaves, surface colour, in an effort to determine the quantitative intraspecific variation present among clones, morphological types, regions and (for leaves) growing seasons. Fruit were highly variable in anthocyanin content and antioxidant capacity, suggesting genetic and environmental influences on these components. Leaves demonstrated a broad range of anthocyanin and surface colour values, but no correlation was found between fruit and leaf anthocyanin levels. The three morphological types of *V. angustifolium* did not differ in any of the variables measured. The three regions of Nova Scotia differed in fruit and leaf anthocyanin content and antioxidant capacity (and surface colour in leaves). Leaves harvested during the early, middle and late part of the 1997 growing season differed in % dry weight, anthocyanins, antioxidant capacity and leaf surface colour (hue angle). Correlations were found between anthocyanins, phenolics and antioxidant capacity in fruit, but not in leaves. Early and mid season leaf phenolic levels correlated weakly with total phenolics in fruit. Measurements of leaf surface colour (hue angle) taken from dried, powdered leaves were correlated with leaf anthocyanin content, indicating that leaves which appeared red contained more anthocyanin pigment. There has been little if any work done to date on quantifying the intraspecific variation in the polymorphic *V. angustifolium*, and no previous studies which involved quantitative analysis of anthocyanins, phenolics, antioxidant capacity and surface colour in leaves of this species.

Abbreviations

Abbreviation	Meaning
gDW	gram dry weight
M-3-GE	malvidin-3-glucoside equivalents
M-3-GE/gDW	malvidin-3-glucoside equivalents per gram dry weight
GAE	gallic acid equivalents
GAE/gDW	gallic acid equivalents per gram dry weight
TE	Trolox equivalents
TE/gDW	Trolox equivalents per gram dry weight
MeOH	Methanol
ORAC	Oxygen Radical Absorbance Capacity

Introduction

The wild lowbush blueberry (*Vaccinium angustifolium* Aiton) is a native

North American plant which has been commercially managed for many years in

Eastern Canada and the United States. A member of the Ericaceae, this species

is a low-growing, woody shrub which, when established, can form large colonies

of genetically identical plants which are connected via underground rhizomes

(Vander Kloet, 1988). Primary commercial wild lowbush blueberry growing

regions include Maine, Nova Scotia, New Brunswick, Newfoundland and the Lac-

St.-Jean region of Quebec (Hall and Aalders, 1979).

The province of Nova Scotia produced 22,028,000 lbs of wild lowbush

blueberries in 1997, which is slightly less than the previous five year average of

approximately 29,000,000 lbs (McIsaac, 1997). Wild blueberries are grown

throughout the province, however different regions of Nova Scotia vary in their

potential for growing wild blueberries, depending on the length of their growing

season, temperatures, and the type of terrain (McIsaac, 1997). Approximately

75% of the province's blueberries are currently grown in Cumberland County,

however, there are also many growers in Hants, Queens and Pictou Counties,

and, as the natural conditions of these regions are conducive to growing

blueberries, production there is expected to grow (McIsaac, 1997).

Commercial blueberry fields are exposed to heat and cold, mild to severe

drought conditions, strong winds and depleted soil nutrients. Minimal agricultural

input is made, including herbicide treatments to control weeds and insecticides to control insect pests. In addition, bee colonies are placed in fields during flowering to ensure good pollination rates (McIsaac, 1997). Most growers employ an alternate year production method, so that plants produce fruit only every second year. After the berries have been harvested, fields are mowed or burned to encourage vigorous vegetative growth and branching during the next growing season (McIsaac, 1997).

Commercial blueberry fields are a composite of many genetically and phenotypically different clones (Eaton, 1949). The genetic diversity which exists among wild blueberry clones also distinguishes this crop from the cultivated highbush blueberry (*V. corymbosum* L.), where artificial selection has produced several preferred genetic strains which are usually planted *en masse*, resulting in a genetically uniform crop. Although wild lowbush blueberry selections, such as 'Cumberland' and 'Fundy' (Hall *et al.*, 1988) are available, few have been planted and essentially all wild blueberry growers continue to manage genetically diverse stands of wild blueberry clones.

Clonal, Seasonal and Morphological Variation

Clonal variation is visibly striking in a blueberry field, in as much as clones often differ widely in leaf and fruit colour as well as other characteristics such as plant height, berry size and leaf density. Seasonal changes in pigment levels are also visually manifested in the leaves of *V. angustifolium* in a manner typical of

many herbs, shrubs and trees. In early summer, when the leaves first flush out, they are green with generous tinges of red. In mid summer, the mature leaves are often totally green; with the onset of fall, the leaves take on a range of shades of red. This latter phenomenon is common among plants and is believed to occur as a result of the decomposition of chlorophyll and the increased levels of anthocyanin pigment (Moore, 1965; Raven *et al.*, 1992), the production of which is encouraged by cool nights and sunny days (Deal *et al.*, 1990; Mancinelli, 1985). The hue that leaves acquire in the fall - which ranges from deep purple to bright orange red - varies among individual clones. Indeed, the pronounced colouration in some clones has recently been recognized by ornamental horticulturalists, who have selected certain clones of *V. angustifolium* for their distinct foliage colours (Huttleston, 1990).

In addition to the abundant clonal variation, three morphological types of lowbush blueberries are usually present in a field, and each of these types may be visually distinguished by their leaves and, in some cases, fruit. The "blue" morph has bluish-grey leaves and black fruit; the "green" morph has very green leaves and the fruit are often a medium blue colour and glaucous; the "hairy" morph exhibits hair on the underside of its leaves. Camp (1945) considered these morphological types to be different species, namely *V. brittonii* Porter ex Bicknell, *V. angustifolium* Aiton, and *V. lamarckii* Camp, however Vander Kloet (1978) has shown that they are not distinct biological entities, but merely inconsistent variations of a single polymorphic species, *Vaccinium angustifolium*.

The majority of chemical research conducted on lowbush blueberries, whether it be in the area of food chemistry (Gao and Mazza, 1994;1995; Kalt *et al.*, 1995; Kalt and McDonald, 1996), health research (Kalt and Dufour, 1997; Prior *et al.*, 1998), or taxonomy (Ballinger *et al.*, 1979), focuses on the fruit. A few authors have demonstrated an interest in *Vaccinium* leaves, whether it be for general chemical characterization (Friedrich and Schonert, 1971; Harborne, 1973) or to determine the effects of changing environmental stimuli - such as temperature, nutrients and light - on leaf pigment levels (Lockhart, 1959; Trevett, 1962; Hall *et al.*, 1970; Hall and Stark, 1972). In addition, there has been some research carried out on pigmentation in *V. angustifolium* stems (Wood and Barker, 1963). However, there has been little research into the non-anthocyanin phenolic components of the vegetative tissues of this species, and no examination of the quantitative chemical relationships between leaves and fruit.

The wild lowbush blueberry displays many interesting leaf and fruit variations in the field. Despite some recognition of the immense clonal variation present in the Nova Scotia wild lowbush blueberry (Barker and Wood, 1963; Wood and Barker, 1963; Kalt et al., 1995), little effort has been made to quantify the intraspecific variation present within either the leaves or fruit of *V. angustifolium*. Similarly, the visible changes in *V. angustifolium*'s leaf colouration over the growing season have not been examined. In addition, there has been no research into the phenolic content of the three morphological types of this species.

The Health Benefits of *Vaccinium* Species

Antioxidant compounds absorb potentially damaging oxygen free radicals in the body, and it is purported that increasing one's dietary antioxidant intake will promote good health and decrease the risk of degenerative diseases such as cardiovascular disease and various cancers (Ames et al., 1993; Diplock et al., 1998). Certain fruits and vegetables are believed to be high in dietary antioxidants (Cao et al., 1997). Their benefits are often attributed to their content of antioxidant compounds, including Vitamin C and various tocopherols and carotenoids (Diplock et al., 1998), as well as phenolics and, in particular, a subgroup of phenolics, the flavonoids (Cao et al., 1997).

While almost all fruits and vegetables contain at least some dietary antioxidants, there appear to be some that have particularly high levels of these beneficial compounds. Recent studies (Cao et al., 1996; Wang et al., 1996, Prior et al., 1998), showed that, in a survey of twenty-two different fruits and vegetables, the lowbush blueberry had the highest antioxidant capacity. Researchers have also found that blueberries are distinctive in that they are rich in phenolics, a ubiquitous, chemically diverse group of secondary plant compounds. Among their complement of phenolics, blueberries contain high amounts of anthocyanins, the pigments which impart the red, pink, purple and blue hues of the vegetative and reproductive tissues of most plants, (Francis, 1989) including blueberries. Anthocyanins have been found to possess potent antioxidant properties (Wang *et al.*, 1997). In addition, significant correlations

have been found between the amount of total phenolics and anthocyanins present in blueberries and their antioxidant capacities, as measured by the oxygen radical absorbance capacity assay (Prior *et al.*, 1998).

Despite research which has recently been conducted on the antioxidant capacity of lowbush blueberries, however, the issue of clonal, regional and seasonal effects on anthocyanins, phenolics and antioxidant capacity in berries and leaves has received little attention. In addition, the amount of total anthocyanins and phenolics, and antioxidant capacity, has not yet been reported for a large number of individual clones of *V. angustifolium*. Such measurements would help assess an important aspect of the variation among clones of wild lowbush blueberries.

The fruit and leaves of *Vaccinium* species have a long history of medicinal use. Fruit of a European species, *V. myrtillus* (bilberry) were considered astringent, tonic and antiseptic and were used for centuries in Europe to treat urinary complaints, inflammations, infection and to combat scurvy (Morazzoni and Bombardelli, 1996). The leaves of this species were used as a remedy for diabetes (Grieve, 1992; Morazzoni and Bombardelli, 1996). The bilberry's medicinal qualities continue to be investigated, and this species is used in many health and pharmaceutical products throughout the world (Morazzoni and Bombardelli, 1996; Kalt and Dufour, 1997).

V. angustifolium has been used medicinally for centuries in North America. The fruit have been used to treat stomach complaints and lung

ailments (Turner and Szczawinski, 1978). The leaves were used by Native

Americans as a diuretic and blood tonic (Turner and Szczawinski, 1978) and for

colic, labour, and following miscarriage (Duke, 1986). Although both leaves and

fruit have been used medicinally for centuries, only the latter has received recent

attention for its health effects (Kalt and Dufour, 1997). Given the increased

interest in phytomedicines and natural health supplements, and given that

lowbush blueberry fruit have a high antioxidant capacity, further research into the

potential health benefits of blueberry leaves seems necessary and timely. A

measurement of total phenolic and anthocyanin content, as well as antioxidant

capacity, has not been reported for lowbush blueberry leaves, and would

contribute to the health research which is currently being conducted on members

of the *Vaccinium* genus worldwide.

Chemical and Biological Attributes of Anthocyanins and Phenolics

Phenolics are a large and chemically diverse group of secondary plant

compounds, all of which contain one or more phenol groups, ie. an aromatic ring

containing one or more hydroxyl groups. The flavonoids are a large subgroup of

the phenolics and have in common a diphenyl propane carbon skeleton,

consisting of 15 carbons with 2 aromatic rings connected by a 3 carbon bridge.

Examples of flavonoids include anthocyanins, flavonols, isoflavones and

flavones (Figure 1). These flavonoids are widely distributed in food and non-food

plant species.

Several types of phenolics - including flavonoids - occur in many of the fruits and vegetables of the human diet (Herrmann, 1976), and some have been important to the food industry. For example, the chemistry of anthocyanins, which are responsible for the colour of red wine and many other fruit-derived food products (Francis, 1989), has been intensively studied in food science with regard to colour quality and stability.

Phenolic compounds are believed to play various important ecological roles in plants. The biosynthetic metabolism of certain phenolic compounds appears to be responsive to environmental stimuli (Blank, 1947; McClure, 1975; Bohm, 1987) and certain phenolics are suspected of being involved in some types of stress responses (Chalker-Scott *et al.*, 1989). In some plant species, phenolic and anthocyanin production in leaves and fruit can be triggered by increased light levels (Woodhead, 1981; Mancinelli, 1985; Waterman *et al.*, 1984) and temperatures (Hall and Ludwig, 1961; Kliewer, 1970; Hall and Stark, 1972; Deal *et al.*, 1990); they can also be produced in response to, and help protect plant leaves from, ultraviolet light (McClure, 1975; Dong *et al.*, 1995). Nutrient deficiencies can stimulate the production of certain phenolics in some plants (Dustin and Cooper-Driver, 1992), as can attacks by herbivores and pathogens (Swain, 1979; Friend, 1985).

Environmental effects on phenolic production can differ depending on the tissue being examined; Parks *et al.* (1972) found that *Gossypium* leaf flavonoid chemistry was much more affected by environmental stimuli than that of the

petals. Similarly, Steward *et al.* (1979) found that the amount and proportion of anthocyanins in pointsettias was influenced by a number of factors, including the area of the bract sampled. Production of phenolics may also be linked to a plant's genetic makeup. Although little research has been done at the intraspecific level, it is known that certain phenolics can vary qualitatively and quantitatively within a species, and that genetics may be at least partly responsible for this variation (Bohm, 1987).

The impact of environmental and genetic influences on the phenolic components of the lowbush blueberry could be significant with respect to their potential health properties. A series of studies is required to test whether these factors significantly impact the total phenolic and anthocyanin content as well as the antioxidant capacity, and could be useful to the North American wild blueberry industry. Indeed, given that phenolic and anthocyanin levels can be affected by a variety of environmental factors, it seems plausible that blueberry plants from different regions of Nova Scotia may vary in levels of the aforementioned compounds, and possibly even antioxidant capacity. In addition, the feasibility of artificially selecting for new strains of potentially "healthier" blueberry clones which contain high levels of phenolics and anthocyanins and have a high antioxidant capacity should be assessed. Such an assessment should include studies of the relationship between leaf surface colour and the anthocyanin content and antioxidant capacity in fruit. Finally, studies are needed to discern how the contents of anthocyanins, phenolics and antioxidant capacity

are related in leaves and fruit of *V. angustifolium*.

Research Objectives:

A. To characterize the intraspecific variation in total anthocyanins, total phenolics and antioxidant capacity, as well as surface colour, in leaves and total anthocyanins, total phenolics and antioxidant capacity in fruit of wild lowbush blueberries in Nova Scotia. This objective will be addressed by examining:

      i) clonal variation in fruit and leaves,

      ii) leaf variation over the growing season,

      iii) morphological types and

      iv) clones from different regions.

B. To assess the relationships between total anthocyanin and phenolic content, as well as antioxidant capacity within fruit and leaf tissues of wild lowbush blueberry.

C. To determine the relationship between wild lowbush blueberry leaf surface colour and leaf anthocyanin content.

Materials and Methods

Plant Collection

A total of 135 genotypes of *V. angustifolium* were surveyed. Plant material was collected from three Counties (Queens, Hants and Cumberland; Figure 2) in Nova Scotia, Canada, between May and September, 1997. Three commercial blueberry fields in their fruiting year were selected within each county (Table 1); fields within the same region were not more than 10 km apart. Within each field, fifteen blueberry clones were tagged for harvesting three times during the season. Five clones of each of the three morphological types "green", "blue" and "hairy" were selected to comprise the fifteen tagged clones.

Clones were identified with a weatherproof plastic tag on the first of the three harvest dates (Table 2). The tags were labelled with the clone number, field number and morphological type. A stake marked with flagging tape was also placed in the middle of the clone for easy identification upon return to the field.

Both leaf and fruit material were collected for this study. The leaf material was collected three times during the season (Table 2); the leaf flush in late spring, mature leaves in mid summer (at the time of commercial fruit harvest) and early fall leaves (a few days before fields were mowed or burned). Harvests were conducted at different times for each of the three counties, according to the rate of development of leaves and fruit, which was determined through contact with the commercial growers. For example, Queens County fields were

harvested before those in Hants County, because the former's plants mature earlier due to slightly warmer temperatures (similarly, Hants County was harvested before Cumberland County).

Within each field, leaves were collected from each tagged clone by snipping off leafy stems at their base and placing them in a labelled 9"x12" Ziploc plastic bag; enough stems were collected from each clone to completely fill the bag. Only a small percentage of the clone's area (5-10%) was harvested, so as to prevent any damage to the plant that might affect fruit or leaf harvests later in the season. All three fields within each County were harvested in one day. During the day, samples were stored at ambient temperature (out of direct sunlight), and the bags were left unsealed. The bags and their contents were stored unsealed in a fridge overnight until they could be processed the next day.

Fruit from each tagged clone were hand picked at commercial maturity and placed in labelled plastic containers. All fields within the county were harvested within one day. The samples were kept shaded and at ambient temperature after harvest and were placed in a refrigerator overnight.

Sample Preparation

Leaves:

The day after each leaf harvest, leaves were removed from stems and dead or diseased leaves were discarded. Approximately 15-20 g of leaf material was placed in labelled plastic containers with covers, and stored in a -70°C

freezer. The material was freeze dried as soon as possible in the uncovered containers, using an Edwards (Sussex, UK) freeze drier at approximately -55°C for four days. Freeze dried material was ground for approximately 2 min in an Aromatic Coffee Grinder (Braun Inc., Lynnfield, MA), filtered through a 500 mm sieve, and then stored in labelled plastic air-tight pill bottles. Bottles were packed into plastic bags and vacuum sealed; bags were then stored in the dark at room temperature.

Fruit:

The day after each berry harvest, the fruit material was cleaned of any vegetative material and overripe, broken or green berries. A small subset of the berry sample (approximately 5 g) was transferred into a small Ziploc bag for determination of dry matter content. Approximately 15 g of cleaned berries were placed into labelled plastic bags and stored in a -70°C freezer until use.

Physical Measurements

Leaves:

Leaf surface colour (hue angle), was measured on powdered leaf tissue. Hue angle (0°= red/purple; 90° = yellow; 180° = bluish/green; 270°= blue) is an effective means of providing an intuitive prediction of visual colour appearance (McGuire, 1992). The reflectance colorimeter probe (Minolta Chroma Meter CR200, Minolta, Japan) was placed flatly on the surface of the leaf powder, and

three readings were taken; the mean of the three was recorded.

The dry matter content of the fresh leaf samples was determined by weighing a subsample of the fresh leaves (approximately 1 g) in a pre-weighed, labelled aluminum dish. The dish and its contents were dried to constant weight at 100°C (approximately three days) in a drying oven; samples were then removed and placed in a glass desiccator to cool before their weights were recorded.

Fruit:

The dry matter content of fresh berry samples was determined by crushing and mixing the subset of berries within the Ziploc bag, so as to obtain a uniform sample. A tablespoon of this berry mixture was spooned into a weighed labelled aluminum dish, and the weight was recorded. The dishes were dried to constant weight in a vacuum drying oven, set at 100°C, for four days. The dishes and samples were placed in a glass desiccator to cool, before their weights were recorded. Two dry weight measurements were performed on each berry sample, and the mean was calculated.

Extraction Procedures

Leaves:

To make a leaf extract, approximately 0.4 g of leaf powder was weighed into a labelled low density polyethylene 30 ml centrifuge tube (Nalgene,

Rochester, NY), to which 15 ml of 60% acidified methanol (0.1% formic acid) was added. The contents of the tube were ground in a Virtishear homogenizer (The Virtis Co., Gardiner, New York) with a homogenizing probe (1cm diameter) for two min at 40 rpm. The grinding probe was washed with distilled water and dried between grinding. The extract was vacuum-filtered using Whatman no. 4 filter paper, and brought to 25 ml (using extraction solvent) in a volumetric flask.

The leaf extract was passed through a Waters Sep Pak Vak 3cc C18 cartridge (Waters Scientific, Mississauga, ON) so that chlorophylls, which interfere with the measurement of total phenolics (Appendix 1), were removed. The method for chlorophyll removal is similar to that used in Eskins and Dutton (1979), and was carried out using a vacuum manifold. After the cartridges were pre-conditioned with 4 ml of methanol and 8 ml of water, 3 ml of leaf extract were immediately loaded onto the conditioned cartridge and allowed to drain as waste. Then 11 ml of extract was loaded onto the cartridge, and the filtrate was collected in a clean, labelled test tube. The chlorophylls were retained by the C18 resin while the phenolics and anthocyanins were not. A 1 ml aliquot of this extract was transferred into a labelled Eppendorf tube and stored at -70°C for phenolic and antioxidant capacity analysis. The remaining extract was concentrated 30X at 30°C under vacuum using a Speed Vac Concentrator (Savant Instruments, Hicksville, NY). The concentrated samples, which were dissolved in MeOH containing 0.1% HCl, were stored at -70°C prior to anthocyanin analysis.

Fruit:

Berry extracts were prepared by weighing out approximately 3.0 g of fruit (or a minimum of ten berries) into a Virtis glass container. The weight and number of fruit was recorded, so as to determine the average fruit weight for each sample. Thirty ml of acidified 88% MeOH (0.1% formic acid) was then added, and the mixture was ground in a Virtishear homogenizer (with macro blade attachment) for 2.5 min at 40 rpm. The mixture was transferred to a glass screw-top tube and stored in darkness overnight at room temperature. The next day, the extract was transferred into a centrifuge tube and spun at 15,000 rpm for 5 min in a Dupont Sorvall RC 5C Plus preparative centrifuge (Newtown, CN). Approximately 3 ml of the supernatant was decanted into a labelled amber vial and stored at -70°C until use.

Chemical Measurements

Monomeric anthocyanins in leaf and fruit extracts were measured using an adaptation of the pH differential method of Wrolstad (1976) and a 96-well microplate. Two buffers were used - a 0.2 M HCl and 0.2 M KCl buffer adjusted to pH 1.0 and a 1 M $C_2H_3O_2Na$ buffer adjusted to pH 4.5. Thirty μl of the appropriately diluted sample was added to 270 μl of each buffer in separate microwells and absorbances at 520 and 700 nm were read using a microplate reader (Molecular Devices, Menlo Park, CA). The concentration of monomeric anthocyanins was calculated using the extinction coefficient for malvidin-3-

glucoside (28 000) and the following equations:

$$\text{absorbance} = (A_{520nm}\ pH1.0 - A_{700nm}pH\ 1.0) - (A_{520nm}pH\ 4.5 - A_{700nm}\ pH\ 4.5)$$

$$\text{concentration (mg/L)} = \text{Absorbance/(extinction coefficient x path length)} \times 10^3 \times$$

$$\text{molecular weight x dilution factor}$$

Anthocyanin content was expressed as mg malvidin-3-glucoside equivalents

(M-3-GE) per g of dry weight. Three replicates of the anthocyanin analysis were

performed, and the mean value was taken. Most berry extracts were diluted 4X

with acidified MeOH (.1% HCl) before use in the Wrolstad test; dilution factors for

the concentrated leaf extracts were 1, 2, 4 or 6X.

Total dissolved phenolics were measured on the leaf and berry extracts

using an adaptation of the Folin-Ciocalteu assay (Singleton and Rossi, 1965).

The analysis involved combining 20 µl of appropriately diluted sample, 200 µl of

distilled water and 40 µl Folin-Ciocalteu reagent (diluted 1:3 with water; BDH

Scientific, Toronto, ON). This reagent is an acidic phosphomolybdo-tungstate

solution which oxidizes phenolates, resulting in the production of a blue pigment.

A solution of saturated anhydrous $NaCO_3$ was added (10 µl) to ensure that the

mixture remained alkaline during the reaction. The procedure was adapted for a

96-well microplate and samples were read at 700 nm. Most berry extracts

required a dilution factor of 2X, while the unconcentrated leaf extracts were

diluted 10X. The test was performed three times on each sample, and the mean

was calculated. Gallic acid was used as a phenolic standard, and the total phenolic content is recorded as mg of gallic acid equivalents (GAE) per g of dry weight.

The antioxidant capacity of the leaf and berry extracts was measured using an automated Oxygen Radical Absorbance Capacity (ORAC) assay (Cao et al., 1993, 1995) and carried out on a COBAS FARA II spectrofluorometric centrifugal analyzer (Roche Diagnostic System Inc., Nutley, NJ). The COBAS FARA was equipped with fluorescence filters for an excitation wavelength of 540 nm and an emission wavelength of 565 nm. A 75 mM phosphate buffer stock solution (pH 7.0) was prepared by dissolving 10.2 g of KH_2PO_4 and 2.5 g KOH in ultrapure water in a volume of 1 L. R-Phycoerythrin (R-PE) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma (St. Louis, MO). 2,2' azobis (2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals (Richmond, VI).

In the final assay mixture (0.4 ml total volume), R-PE (16.7 nM) was used as the target of free radical attack. AAPH (4 mM) was used as the peroxyl radical generator and Trolox (1.0 µM), a water soluble analogue of vitamin E, was used as a standard for antioxidant activity.

The ORAC method is based on the inhibition of free radical action by an antioxidant; sample is added to a mixture containing the free radical generator, and the inhibition in the loss of fluorescence of the indicator protein, RPE, is recorded. The total running time for one test is 70 min, and the analyzer was

programmed to record the fluorescence of R-PE every 2 min after addition of

AAPH. Two blank samples (phosphate buffer) and two standard samples were

run along with the $V.$ $angustifolium$ extracts. Final results were calculated using

the ratio between areas under the decay curves of R-PE for the standard and a

sample, and were expressed as μm Trolox equivalents (TE) per gram of dry

weight.

Since aqueous samples were required for the assay, leaf and berry

extracts were dried under vacuum and dissolved in water prior to undergoing

ORAC analysis. Most berry extracts were diluted 400X so as to obtain an ORAC

reading within an acceptable range, and the unconcentrated leaf extracts were

diluted 1500X. Two replicates were performed for each sample, and the mean

value was calculated.

Statistical Analysis

Data were analyzed using Genstat 5 (Payne et $al.$, 1993), unless

otherwise noted. Analysis of variance (ANOVA) was conducted to compare

means of fruit % dry weight, total anthocyanins, total phenolics and antioxidant

capacity among the three regions and morphological types. Means of leaf per

cent dry weight, total anthocyanins, total phenolics, antioxidant capacity and hue

angle were compared among the three regions, morphological types, and the

three harvest dates. Due to the very broad range of outliers, leaf anthocyanin

measurements were converted to a logarithmic scale for the ANOVA, and were

reported as back transformed values. Genstat's LSD (least significant difference) test was used to determine significant differences between individual regions and leaf harvests. For analysis of variance and LSD procedures, only differences of $P<0.05$ are presented, unless otherwise indicated.

Spearman's Rank Correlation Coefficient was used to determine the relationship between total anthocyanins, phenolics and antioxidant capacity in fruit, and total anthocyanins, phenolics, antioxidant capacity and leaf hue angle in leaves. Spearman's Rank Correlation Coefficient is a measure of association between the rankings of two variables, and was used to minimize the effect of extreme outliers on a given relationship. In the case of fruit, 9 means were ranked (n=15; means taken for each morph, for each region); for leaves, 27 means were ranked (n=15; means taken for each morph and region, and for each leaf harvest). The correlation coefficient is calculated from the ranking of each mean, and the t-distribution is used to test the null hypothesis of independance between the samples.

To compare variables between fruit and leaves, the simple product-moment correlation coefficient was used. The coefficient of determination (r^2) was calculated in Sigma Plot version 4.01 (1997, SPSS Inc.), and is a measure of the proportion of the variation of one variable determined by the variation of the other. The following variables were plotted and analysed using the product-moment correlation coefficient: fruit and leaf anthocyanins, phenolics and antioxidant capacities. Leaf hue angle and fruit anthocyanins were also analysed

in this manner.

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Results

A. Intraspecific Variation

Clonal Variation in Fruit and Leaves

In the fruit, total anthocyanins, phenolics and antioxidant capacity showed large variation in the 135 clones of *V. angustifolium* (Figure 3). There was an 8-fold difference in total anthocyanins, with values ranging from 4.61 to 36.4 mg M-3-GE/gDW. Total fruit phenolic values demonstrated a 3-fold difference, and ranged from 11.9 to 40.6 mg GAE/gDW. Fruit antioxidant capacities varied 7-fold, with values from 274 to 1837 µm TE/gDW. In addition, there was a greater spread of anthocyanin and antioxidant values above the 90th percentile as compared to below the 10th percentile.

In the leaves, hue angle, total anthocyanins, phenolics, and antioxidant capacity showed a wide spread of values in the 135 clones of *V. angustifolium* (Figure 4). There was a 4-fold difference in hue angle values, from 27.6 to 114 degrees, with most of the extreme values falling below the 10th percentile (a lower hue angle means a more red leaf, and a higher value means a more green leaf).

Leaf total anthocyanin values ranged from zero to 1.82 mg M-3-G/gDW, with a broad spread in the outliers with values higher than the 90th percentile. As in fruit, leaf phenolic values did not show as broad a spread as anthocyanins, antioxidant capacities and hue angle - they varied over a 2.5-fold range, with values from 63.0 to 159 mg GAE/gDW. Values for leaf antioxidant capacity

ranged 10-fold - from 474 to 5020 μm TE/gDW. There was a fairly equal spread

of outliers which fell above and below the 10th and 90th percentiles, with the

exception of the highest value, which was 2.25 times greater than the mean.

Significant product-moment correlation coefficients were not found

between the anthocyanin contents of leaves and fruit (Table 3) or the leaf and

fruit antioxidant capacity. The total phenolic content of early and mid season

leaves correlated significantly with fruit phenolic content, however the correlation

value was very low.

Seasonal Differences in Leaves

Lowbush blueberry leaves demonstrated significant seasonal differences

in several of the variables measured (Table 5). Mean percent dry weight was

different between harvests (P=<0.001), with the late season leaves having the

highest percent dry weight and early season leaves the lowest. Hue angle was

significantly different (P=<0.001) among the different leaf harvests, with late

season leaves having the lowest mean hue angle (more red) and the mid-

season leaves having the highest mean hue angle value (more green). Mean

anthocyanin content was different in early, mid and late season leaf harvests

(P=<0.001), with the latter exhibiting the highest amount of anthocyanin and the

mid-summer leaves the lowest. The mean antioxidant capacity was also

significantly different (P=<0.001) among leaf harvests; early leaves had the

highest ORAC value. There was no difference among the three harvest dates in

terms of phenolic levels.

Hue angle shows a greater spread of values late in the growing season,
indicating that leaf colour differences are most apparent in the fall (Figure 5).
This trend is also seen in the late leaf anthocyanins (Figure 5).

Morphological Types

No significant differences were found between the three morphological
types (ie. blue, green and hairy) of lowbush blueberry for any of the fruit or leaf
variates measured (Appendix 2a and b).

Regional Differences

Certain fruit and leaf variables demonstrated differences among the three
sampling regions within Nova Scotia. Among the fruit sampled, % dry weight
was significantly different (P=0.017), with Queens County berries having the
highest percent dry matter and Cumberland County the lowest (Table 6). Fruit
anthocyanin levels were highest in Cumberland County and lowest in Queens,
as were the antioxidant capacities as measured by ORAC (P=0.045; P=<0.001,
respectively).

Blueberry leaves tended to demonstrate different regional patterns
compared to fruit (Table 7). The mean leaf anthocyanin and antioxidant capacity
values were significantly different (P=0.019; P=0.002, respectively) among the
three different regions of the province, with leaves in Cumberland County having

the highest values for both variables and those in Hants County the lowest. Hue

angle was also significantly different among regions (P=0.034); mean hue angle

was highest (most green) in Hants and lowest (most red) in Cumberland County.

B. Relationship Between Variables in Fruit and Leaves

Significant correlations were found between fruit anthocyanins and

antioxidant capacity, anthocyanins and phenolics antioxidant capacity and

phenolics. These correlations were not significant in leaves (Table 4).

C. Leaf Surface Colour and Leaf Anthocyanin Content

Using Spearman's Rank Correlation Coefficients, leaf anthocyanin content

and hue angle were found to be negatively correlated (-.856, Table 4) suggesting

that as leaf anthocyanin content increases, leaf hue angle decreases (indicating

a more red hue). This relationship was plotted for all three seasons (Figure 6)

and the individual leaf harvests (Figure 7).

Discussion

A. Intraspecific Variation

Clonal Variation in Fruit and Leaves

Nova Scotia's wild lowbush blueberry clones varied in anthocyanin and phenolic levels, as well as antioxidant capacity, fruit size, % dry weight and leaf surface colour. These findings corroborate the reported polymorphic nature of this species (Vander Kloet, 1978) and this is the first survey of this nature for a large number of individual clones in Nova Scotia.

Mean values obtained in this study were comparable to other reports for most of the fruit variables. Total mean fruit anthocyanin content was 11.83 mg M-3-GE/gDW in this study compared to 11.04 mg M-3-GE/gDW found in a study by Kalt and McDonald (1996) of three named *V. angustifolium* clones. Prior *et al.* (1998) found a mean anthocyanin content of 148.2 mg M-3-GE/100 g fresh weight for wild clones; the fresh weight value found in this study was 199.4 mg M-3-GE/100 g fresh weight. The mean total phenolic level in fruit was reported by Prior *et al.* (1998) to be 398 mg GAE/100g of fresh weight. In this study, mean phenolic content was 412.0 mg GAE/100 g of fresh tissue.

The mean fruit antioxidant capacity was found to be 620 μm TE/gDW in this study. In the study by Prior et al (1998), the mean antioxidant capacity for a clonal mixture of lowbush blueberries was 229.8 μm TE/gDW. The discrepancy between these numbers is large given that the same method for measuring

antioxidant capacity was used for both studies. The extraction procedures did vary between the two studies, although given that the anthocyanin and phenolic values were similar - although somewhat higher - to those of Prior et al (1998), differences in extraction methods does not seem a plausible explanation of the discrepancy in ORAC values. The high ORAC values in this study may be partially attributable to the high % dry weight and small fruit size, the means of which were higher and lower, respectively, than any published values for this species (Kalt et al., 1995; Kalt and McDonald, 1996). However, given the dry weather conditions in Nova Scotia in the summer of 1997, the high % dry weight value and small fruit size in this study is not surprising. A smaller fruit size would mean more anthocyanin-rich peel and less flesh per berry. Given that fruit anthocyanin level and antioxidant capacity are correlated, it is plausible that a smaller berry would have a higher antioxidant capacity.

Fruit anthocyanin values covered a broad range. Sapers et al., (1986a) found that individual berries from many plants of the same cranberry (V. macrocarpon) cultivars demonstrated a 2-3 fold difference in total anthocyanin values. In this study, a 8-fold difference in anthocyanin values was found by comparing a much larger number of individual wild clones. Kalt et al. (1995) found an 11-fold difference in anthocyanin levels in a comparison of 504 V. angustifolium berries from 72 clones (7 berries per clone). In that study, an effort was made to encompass berries with a broad range of surface colours (some slightly underripe/ overripe) so it is not surprising that they found a greater

spread of anthocyanin values.

There was a broader spread of clones with high amounts (greater than the 90th percentile) of fruit anthocyanins than with low values. The fruit antioxidant capacity showed a similar trend, which may be promising in terms of potential breeding strategies. Growers of wild blueberries currently manage wild stands of genetically diverse plants. However, given the recognition recently awarded to the wild blueberry for its high antioxidant capacity, interest in growing berries with high anthocyanin content and/or antioxidant capacity will likely grow. This study suggests that there are wild clones with naturally high fruit anthocyanin and ORAC values. However, clones should be monitered over a number of years to ensure that their fruit is consistently high in phenolic antioxidants. In addition, investigations into the factors that control these components should be conducted. Breeding strategies for increasing anthocyanin content have been previously investigated in cranberries (Sapers *et al.*, 1986b). The authors acknowledged the large variation in values within cultivars, but also emphasized the need for further study of the genetic and environmental factors which increase anthocyanin production in fruit.

There have been few quantitative studies of leaf anthocyanins, although there has been a study of this nature performed on *V. angustifolium* leaves (Hall *et al.*, 1970). The method of measurement and the units used to express anthocyanin content vary widely, thus making a comparison with the values determined in this study difficult. In the study by Hall *et al.* (1970) on

V. angustifolium leaf anthocyanin levels, only the optical density at 535 nm is reported, although the authors did find a 10-fold difference in these values for the three clones studied. The total anthocyanin content in jack pine seedlings was found to range from 14.7 mg/gGW for very purple needles to 0.4 mg/gDW for very green needles (Nozzolillo *et al.*, 1990).

As in fruit, leaf anthocyanin levels in individual clones of any species have yet to be quantified over more than one growing season. At least one study of this nature - conducted on quaking aspen - indicates that high fall leaf anthocyanin content varies considerably from year to year (Chang, 1989). However, given that *V. angustifolium* clones have been named and registered horticulturally according to leaf colour (Huttleston, 1990), it seems probable that leaf pigmentation may be consistent from year to year in some clones.

Leaf phenolic levels of *V. angustifolium* were comparable to those found by other authors studying different species. Several species of woody plants in Singapore were found to have leaf total phenolic levels in the range of 12.4 - 211 mg tannic acid/gDW. (Turner, 1995). Mountain birch leaves were found to have 80-105 mg gallic acid/gDW (Nurmi *et al.*, 1996). Willow leaves were found to have 45-135 mg equivalents of phenolics/gDW (Julkunen-Tiitto, 1985), and eucalyptus leaf phenolics were found to be in the range of 90-202 mg quebracho equivalents/gDW (Cork and Krockenberger, 1991). All of the aforementioned studies used the Folin-Denis or Folin-Ciocalteu method (or a slight modification thereof) for measuring total phenolics. The range of leaf phenolics for this study

was from 64.0 mg GAE/gDW to 159 mg GAE/gDW, which is in the same range as these species.

There is no existing information on the antioxidant capacity of *V. angustifolium* leaves. Similarly, there is no published information regarding the antioxidant capacity of different types of tissues (vegetative/reproductive) of the same species, let alone the same plant. Leaf antioxidant capacity values were much higher (approximately double) than those found in fruit, but were not correlated with either anthocyanin or phenolic values, as they were in fruit. It should be noted that the high antioxidant capacity values found in leaves may not necessarily translate into high antioxidant activity in humans. Indeed, more tests must be performed to determine the link between high ORAC values *in vitro* and effective antioxidant capacity *in vivo*. In addition, further information on which specific compounds (certain phenolics and/or anthocyanins, for instance) affect the ORAC readings most significantly would be of use in assessing the meaning of antioxidant capacity values.

No correlation was found to exist between fruit and leaf anthocyanins and antioxidant capacity in clones of *V. angustifolium*. Thus it cannot be said that a plant which has extremely high leaf anthocyanin content - either in the early, mid or late season - will also have high fruit anthocyanin content in that growing season. Hence there is no easy way to predict - by looking at the leaves of a clone - the fruit's anthocyanin or antioxidant capacity for that season. Such a simple and quick test would have made selecting clones for the purposes of

achieving high beneficial health components in the fruit easier. Indeed, other scientists have investigated a similar test using the anthocyanin content of young vegetative tissues to predict fruit anthocyanin contents in muscadine grapes (Goldy et al., 1987a; 1987b); however, they had only limited success with their method of using young vegetative tissues to predict grape anthocyanin content.

Early and middle leaf phenolics were significantly - although weakly - correlated with total phenolics in fruit. There have been very few studies in which the relationships between phenolic levels of vegetative and reproductive tissues of the same species have been assessed, and this is the first report of such a correlation in V. angustifolium. Hermann (1976) noted that flavonoid concentrations are often much higher in leaves than in other parts of the plant, but did not address the issue of correlation between leaves and other tissues. Leaf flavonoids are believed to be more responsive to environmental stimuli than reproductive tissues (Harborne, 1967; Parks et al., 1972), but again, no studies were performed on relating levels in leaves and other tissues. Further studies are necessary to determine the strength of this trend, whether it is consistent over a number of growing seasons, and whether it holds true for non-fruiting year clones.

Clones of lowbush blueberries demonstrated variability in their anthocyanin levels and antioxidant capacities, indicating that these factors are influenced by genetic and environmental facotrs. Due to the lack of correlation between leaf anthocyanin content and surface colour, however, leaves cannot

be used as an immediate visual indicator of fruit anthocyanin content or antioxidant capacity. A longer term survey is needed to determine whether the high levels of these beneficial health components are consistent from year to year in individual clones.

Seasonal Differences in Leaves

Anthocyanin content and hue angles demonstrated similar patterns. Total anthocyanin levels were highest in the late leaves and lowest in the mid-season leaves. A host of hypotheses have been put forth to explain the phenomenon of increased anthocyanin production in fall leaves. It has been postulated that fall anthocyanin formation may be due to the accumulation of sugars during senescence (Kramer and Kozlowski, 1960) but this has been disputed (Moore, 1965). Another suggestion - more related to the effect of fall temperatures - is that anthocyanins accumulate when the amount of leaf sugar exceeds that required for immediate growth, and that cool night temperatures during the fall reduce dark respiration rates, favouring high sugar content and increasing development and retention of anthocyanins (Levitt, 1972). In addition, Kozukue and Ogata (1972) as well as Engelsma (1970) argued that low temperatures may increase the activity of phenylalanine ammonia lyase (PAL), an important enzyme in anthocyanin biosynthesis.

There have been somewhat fewer hypotheses regarding the anthocyanin "spring flush" colour in young leaves in temperate species. However, it has

been suggested that high levels of free sugars may lead to anthocyanin formation (Harborne, 1967). It has also been postulated that anthocyanins in young leaves provide protection against ultraviolet rays, especially in tropical species (Lee, 1980).

Leaf antioxidant capacity showed significant differences between leaf harvests. Early leaves had the highest antioxidant capacity measurements, and the numbers decreased as the season progressed. It is difficult to determine what caused this pattern, as neither the total anthocyanin content or total phenolics followed a similar trend. These two groups of compounds - especially the latter, in leaves - are generally believed to affect the antioxidant capacity (Prior *et al.*, 1998). It should be noted that both the total anthocyanin and phenolic tests are not qualitative in nature, and that although the level of total phenolics may not vary significantly over the seasons, there may be specific phenolic compounds which fluctuated over the course of the growing season, and these may affect the antioxidant capacity. More qualitative analysis, like High Performance Liquid Chromatography, would be required to determine the link between phenolic and anthocyanin profiles and antioxidant capacity in leaves during the growing season.

No significant seasonal difference was found in phenolic levels of *V. angustifolium* leaves. There appears to be little agreement in the literature on the subject of seasonal changes in leaf total phenolics (Shure and Wilson 1993; Nurmi *et al.*, 1996; Mauffette and Oechel, 1989; Nozzolillo *et al.*, 1990;

Woodhead, 1981). Leaf phenolic levels were not close to being significantly different in this study, indicating that there is no clear pattern in terms of quantitative change in total phenolics over the growing season. Again, however, there may have been substantial quantitative changes in individual leaf phenolic levels which are not detectable using the Folin-Ciocalteu method of measurement.

Morphological Types

The absence of significant differences among the three morphological types of *V. angustifolium* supports Vander Kloet's (1978) conclusion that the three morphs form a single gene pool. Taxonomic differences at the species level can be reflected in the chemical characteristics of certain groups of plants, including the genus *Vaccinium*, as evidenced in the examination of anthocyanin profiles by Ballinger *et al.*, (1979); Ballington *et al.*, (1988); Ballington *et al.*, (1987) for taxonomic purposes. However, studies of a chemotaxonomic nature often employ qualitative rather than quantitative methods, and thus the finding of this study should not be construed as a definitive chemotaxonomic result. A study which examines the anthocyanin profiles of the three morphs would indeed be useful in settling the chemical relationship between them, and it would be interesting to see whether the results mirrored those found in this quantitative survey. Indeed, a survey of the factors examined in this study (region, morphological types, leaf harvest, leaf/fruit comparison) but using qualitative

techniques to analyse anthocyanins and phenolics would be of interest, especially if accompanied by antioxidant capacity measurements. There is a need for in-depth investigation of intraspecific chemical variation in many species (Bohm, 1987), especially known polymorphic ones like *V. angustifolium*.

Although the fruit and leaf colours can be strikingly different between the morphs, especially the "green" and "blue" types, it has been shown that, in highbush blueberries, fruit can appear to be very black (as the fruit of the "blue" morph often does), but that it does not necessarily possess more total anthocyanins (Sapers et al, 1984). The findings of this study thus lend evidence (for lowbush blueberry) to the hypothesis that dark berry colouration is due to a less apparent waxy bloom on the outer surface of the fruit rather than a high anthocyanin content. Similarly, although the leaves of the different morphs (especially green and blue) can appear strikingly different; the hue angle - and thus the leaf colour perceived by an observer - was not significantly different between morphological types.

It can thus be concluded that although the three morphological types of the wild lowbush blueberry may be different in appearance, there is no evidence of quantitative differences in the variables measured in this study.

Regional Differences

Significant differences were noted among regions in certain fruit and leaf components, which could be attributable to differences in the genetic makeup of

plants from the different areas, or to environmental factors. Attributing the patterns seen here to genetic or environmental effects would require the surveyance of plants over a number of years, which was beyond the scope of this study.

The vegetative and reproductive structures of Nova Scotia's wild lowbush blueberry have been shown to be affected by weather. Temperature has been the main subject of study - it has been shown to affect vegetative growth in shoots and rhizomes (Hall and Aalders, 1968; Hall and Ludwig, 1961), fruit production (Hall et al., 1982), leaf anthocyanin content and flower bud formation (Hall et al., 1970). Photoperiod and light intensity have been shown to affect flower bud formation and vegetative growth (Hall and Ludwig, 1961). Also, in a study in which the authors attempted to ascertain why Cape Breton blueberry fields are consistently low producers when compared to Cumberland County fields, the authors tested genetic differences and soil nutrition differences between fields in the two regions and found that these two factors did not significantly affect fruit production. They did find, however, that low temperatures significantly decreased growth and production of blueberry plants in Cape Breton (Hall et al., 1964), indicating that the cooler temperatures in northerly regions of the province affect the growth and fruit production of V. angustifolium in Nova Scotia.

The effect of provenance on the chemistry of Vaccinium berries and leaves has rarely been studied. The anthocyanin levels of cranberry fruit have

been shown to be affected by their growing environment (Zuckerman et al., 1966; Sapers et al., 1986a). In one study, 12 cranberry cultivars growing in different sites in Massachusetts were tested for anthocyanin content, and were found to be significantly different according to location (Zuckerman et al., 1966). In contrast, Ballinger et al. (1981), found that geographic origin did not quantitatively affect anthocyanin content in the fruit of V. stamineum. The findings of this study show that total anthocyanin and antioxidant capacity were highest in fruit from Cumberland County and lowest in fruit from Queens County. Prior et al.(1998) found similar trends: lowbush blueberry fruit harvested from Nova Scotia and Prince Edward Island (PEI) had a higher total anthocyanin content and antioxidant capacity than lowbush blueberries grown in Maine.

The similarity between results of this study and that of Prior et al. (1998), along with the aforementioned studies on effect of temperature on blueberry growth, suggests that temperature may affect total anthocyanin and antioxidant levels in blueberry fruit, although multiple year studies are needed to determine the consistency of this trend. Cumberland County is the most northerly (and coolest) of the three counties studied, and Prior et al. (1998) found that northern berries (from Nova Scotia and PEI) had higher anthocyanin and antioxidant capacities than those from the south (Maine). This would appear to lend support to the hypothesis that increased anthocyanin production in fruit is linked with decreasing temperatures (Hall and Stark, 1972; Kliewer, 1970; Creasy, 1968; Saure, 1990).

To this author's knowledge, no work has been done to determine the effect of temperature on antioxidant capacity in fruit. However, given that anthocyanins in fruit are generally believed to increase with decreased temperature, and given the proposed relationships of anthocyanins and antioxidant capacity values (Prior et al., 1998), and the high anthocyanin content of blueberries, such a study would be of interest.

Leaf anthocyanin and antioxidant capacity also demonstrated significant regional differences. Cumberland County leaves (and fruit) contained the most anthocyanins and had the highest antioxidant capacities, while Hants County leaves had the lowest levels. It would thus appear that the hypothesized link between anthocyanins, antioxidant capacity and temperature is less clear in the leaves, although the literature suggests a connection between red leaf colour and low temperatures. Hall et al. (1970) found an indirect relationship between temperature and anthocyanin content in V. angustifolium leaves, and noted that leaves in Cape Breton and Newfoundland demonstrated an earlier and more vibrant red colouration. Hall and Stark (1972) found a similar relationship in cranberry leaves, and other authors have noted the pattern in non-Vaccinium species (Deal et al., 1990; Nozollilo et al., 1990). Chang (1989) noted that leaves of quaking aspen trees growing at higher elevations coloured more quickly than those at lower elevations. The effect of cool temperatures on antioxidant capacity has not yet been established, although the leaves of certain species of plants from northern climates have been shown to produce more

essential oils and other medicinally useful chemicals - a phenomenon known as "northern vigour" (Branka Barl, personal communication).

Hue angle, a measurement of leaf surface colour, was also found to be significantly different among regions, and the pattern follows that of anthocyanin content, thus suggesting that hue angle measurement reflects the amount of anthocyanin present in a blueberry leaf. The results found in this study thus indicate that not only is anthocyanin content highest in Cumberland County but that the increased pigment amount is visually detectable as well.

It is likely that the regional effects seen in this study may be due to a host of factors - other than temperature - which differ according to region. However, Hall *et al.* (1964) did find that temperature, and not genetic differences or soil quality, affected the production of *V. angustifolium* clones in Cape Breton. In addition, given the pattern of regional effects and the known effects of temperature on anthocyanin content in leaves and fruit, it is plausible that temperature differences may explain some of the regional effects seen in this study. It must be stressed, however, that long-term studies are required to assess the consistency of the regional patterns seen here, and other experiments would be needed to determine the effect of various environmental factors on the anthocyanin, phenolic and antioxidant capacity.

B. Relationships Between Variables in Leaves and Fruit

Significant positive correlations were found in fruit between anthocyanins

and phenolics, anthocyanins and ORAC, and phenolics and ORAC. The high correlation between anthocyanins and phenolics and anthocyanins and antioxidant capacity suggests that anthocyanins are a primary contributor to phenolics in the fruit, and that anthocyanins are a main contributer to antioxidant capacity in the fruit. Such correlations have been found in other studies on a number of *Vaccinium* species, as well as other fruit such as raspberries and strawberries (Prior *et al.*, 1998; Kalt *et al.*, 1998). Conversely, in leaves, the low correlation between anthocyanins and phenolics indicates that anthocyanins are not a main leaf phenolic (which is not surprising), and the non-significant relationship between total anthocyanins, phenolics and antioxidant capacity indicates that antioxidant capacity is not related to either of these two measures in leaves.

C. Relationship Between Leaf Surface Colour and Leaf Anthocyanin Content

Leaf anthocyanin content and hue angle were found to be negatively correlated, indicating that leaves which appear more red do have more anthocyanins and those which appear green have less anthocyanins. In addition, leaf anthocyanins and hue angles demonstrated the same regional and seasonal patterns in the ANOVA. For example, hue angle measurements indicate significant seasonal differences similar to the anthocyanin patterns. However, relationship between hue angle and anthocyanin content was not as

strong in the mid season leaves as it was in the early and late leaves (although it was still significant). This indicates that a broad range of leaf colours is needed to best illustrate the correlation.

The ANOVA patterns, combined with the strong negative correlation coefficient, indicate that measurement of hue angle - even on dried, powdered leaf material - is a useful indicator of anthocyanin content in *V. angustifolium* leaves.

This study indicates that leaves and fruit of *V. angustifolium* clones are highly variable in terms of total anthocyanin content and antioxidant capacity. Long term field and laboratory experiments should be conducted to determine the effects of genetic and environmental influences (such as temperature, water levels and amount of light) on anthocyanin content and antioxidant capacity in this species. Investigations should be conducted on the effect of growing region on anthocyanin and antioxidant levels; the entire geographical range of this species should be sampled for such a study. Studies should also be conducted to determine the anthocyanin and phenolic content as well as the antioxidant capacity in clones during fruiting and vegetative years, and to determine the between-year variation in total anthocyanins, phenolics and antioxidant capacities in individual clones. Tests of this nature would help to determine the feasibility of producing fruit with consistently high anthocyanin and antioxidant levels.

More studies are needed to ascertain which components are responsible for the high ORAC values in leaves. Indeed, investigations should be conducted to determine which anthocyanins, phenolics and other antioxidants strongly affect the ORAC test. *In vivo* studies could then be conducted to determine these components' antioxidant effects in the body.

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Tables

Table 1: Approximate locations of the three sets of fields. Fields within the same region were not more than 10 km apart.

Region	Nearest Town	Latitude/Longitude			
Queens County, NS	Caledonia, NS	44°	22N	65°	02W
Hants County, NS	Rawdon Gold Mines, NS	45°	03N	63°	46W
Cumberland County, NS	Parrsboro, NS	45°	24N	64°	20W

Table 2: Leaf and berry harvest dates for the 1997 growing season.

Harvest	Sample	Queens	Hants	Cumberland
1	Early season leaf	May 27	June 3	June 10
2	Mid season leaf, fruit	July 28	Aug. 11	Aug. 18
3	Late season leaf	Sept. 24	Sept. 26	Sept. 29

Table 3:   Simple product-moment correlation coefficients and significance for

leaf and fruit anthocyanins, phenolics and antioxidant capacity.

Variable	Leaf Harvest	Fruit	r^2 Value	Significant
Total anthocyanins	Early Season	Maturity	0.0065	
	Mid Season	"	0.0071	
	Late Season	"	0.0091	
Total phenolics	Early Season	"	0.041	yes
	Mid Season	"	0.104	yes
	Late Season	"	0.030	
Antioxidant capacity	Early Season	"	0.000	
	Mid Season	"	0.005	
	Late Season	"	0.014	

Table 4:   Spearman's Rank Correlation Coefficient for fruit anthocyanins,

phenolics and antioxidant capacity (ORAC) and leaf anthocyanins,

phenolics, antioxidant capacity and surface colour (hue angle)

Tissue	Variate 1	Variate 2	Spearman's Correlation Coefficient	F probability
Fruit	Anthocyanin	Phenolics	0.883	<0.01
Fruit	Anthocyanin	ORAC	0.833	0.01
Fruit	Phenolics	ORAC	0.750	0.02
Leaf	Anthocyanin	Phenolics	0.172	0.39
Leaf	Anthocyanin	ORAC	0.287	0.15
Leaf	Phenolics	ORAC	0.073	0.72
Leaf	Anthocyanin	Hue angle	-0.856	<0.01

Table 5:   Leaf variable means and significance for the three leaf harvests

Letters in brackets indicate which leaf harvests were significantly

different from each other, p< 0.05; (e = early leaf; m = mid leaf; l =

late leaf; * = all harvests were significantly different from each

other).

Season	%DW	Hue angle (degrees)	Anthocyanins (mgM-3-GE/gDW)	Phenolics (mgGAE/gDW)	ORAC (μmTE/gDW)
Early leaf	30.9 (*)	101 (*)	0.099 (*)	106	2505 (m,l)
Mid leaf	42.2 (*)	107 (*)	0.0593 (*)	110	2009 (e)
Late leaf	49.3 (*)	87.3 (*)	0.236 (*)	111	2161 (e)
Grand Mean	40.8	98.4	0.131	110	2225
Std. Error	0.39	1.24	0.0163	1.68	53.3
F Prob.	<.001	<.001	<.001	0.149	<.001

Table 6: Fruit variable means and significance for the three regions in Nova

Scotia. Letters in brackets indicate which leaf harvests were

significantly different from each other, p< 0.05; (e = early leaf; m =

mid leaf; l = late leaf; *= all harvests were significantly different

from each other).

	g/berry	%DW	Anthocyanins (mgM-3-GE/gDW)	Phenolics (mgGAE/gDW)	ORAC (μmTE/gDW)
Cumberland	0.281	16.0 (Q)	14.1 (Q)	28.6	758 (*)
Hants	0.27	16.5 (Q)	12.0	24.9	638 (*)
Queens	0.277	18.0 (H,C)	9.39 (C)	20.9	465 (*)
Std. Error	0.017	0.352	1.01	1.86	13.7
F Probability	0.196	0.017	0.045	0.07	<0.001

Table 7: Leaf variable means and significance for the three regions in Nova

Scotia. Letters in brackets indicate which leaf harvests were

significantly different from each other, p< 0.05; (e = early leaf; m =

mid leaf; l = late leaf; * = all harvests were significantly different

from each other).

	%DW	Hue angle (degrees)	Anthocyanins (mgM-3-GE/gDW)	Phenolics (mgGAE/gDW)	ORAC (µmTE/gDW)
Cumberland	40	93.5 (H)	0.182 (H)	104	2454 (H)
Hants	41	103 (C)	0.079 (C)	103	1872 (Q, H)
Queens	41.3	99	0.133	121	2350 (H)
Std. Error	1.94	1.87	0.020	6.5	69.2
F Probability	0.88	0.034	0.019	0.154	0.002

Figures

Figure 1:   Structures of some major flavonoid classes

ANTHOCYANIN

R, R' = CH₃ or OH
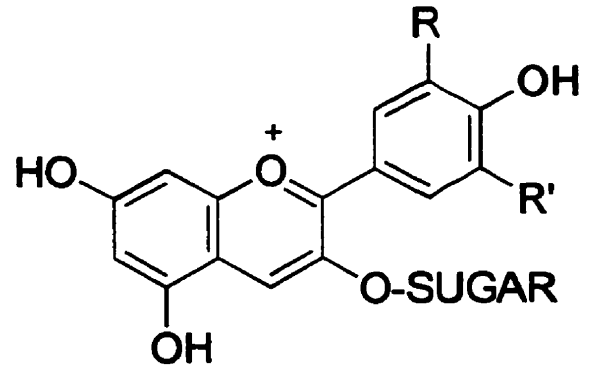
FLAVONOL
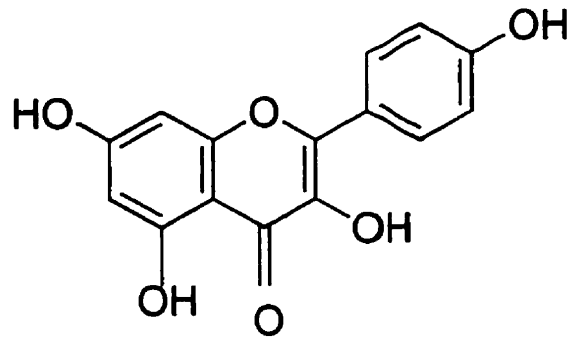
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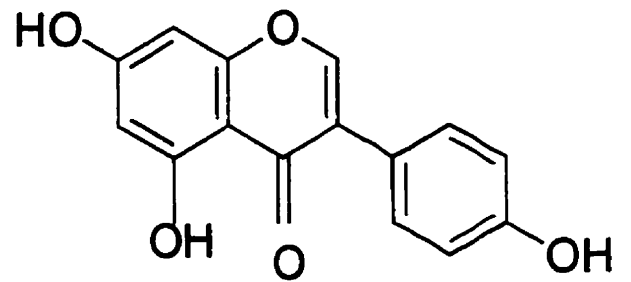
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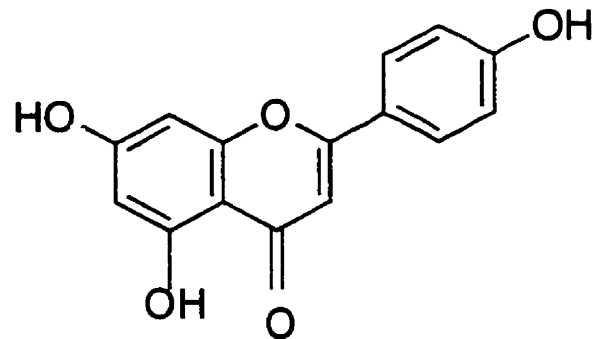
Figure 2:   Map of Nova Scotia, showing the three sampling regions
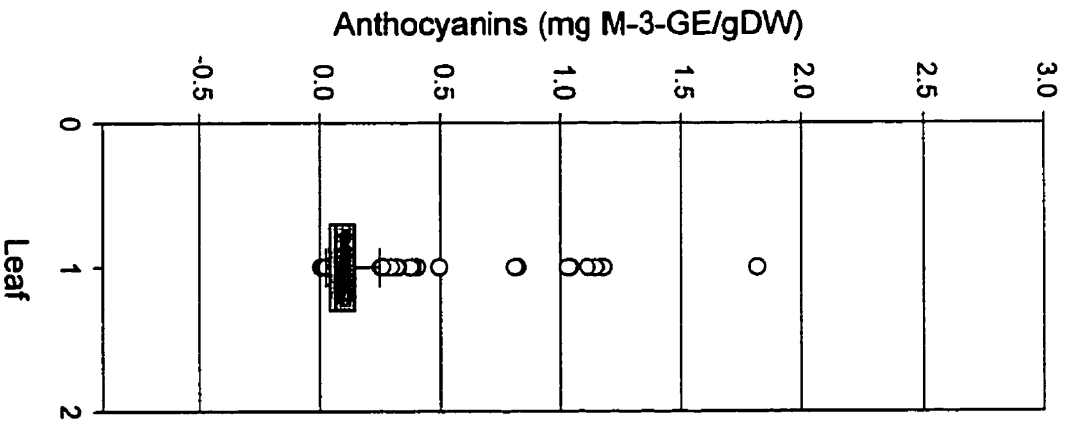
(1=Cumberland County, 2=Hants County, 3=Queens County)
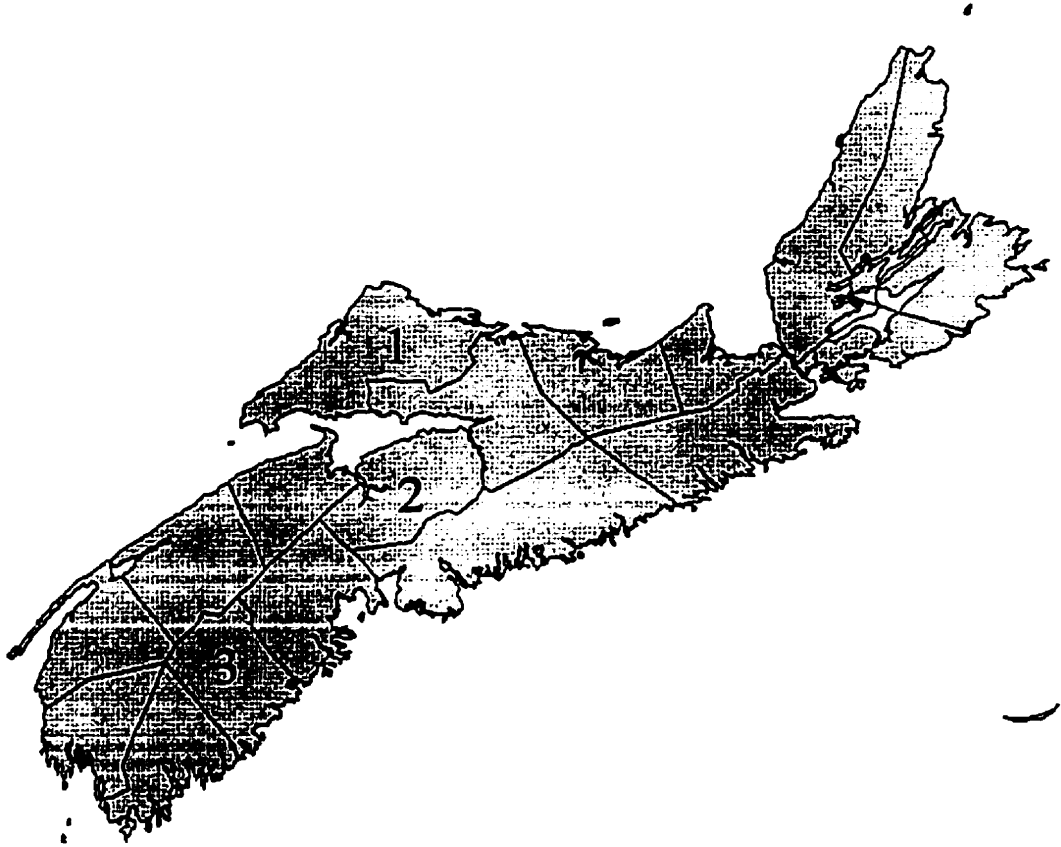
Figure 3: Box plot showing range of values for anthocyanins, phenolics and antioxidant capacity in fruit of 135 *V. angustifolium* clones

The median is indicated by the mid line of the shaded box, while the 75th and 25th percentiles are the upper and lower boundaries, respectively, of the box. Error bars indicate the 90th and 10th percentiles, and all points which lie outside these percentiles are indicated by dots.

Anthocyanins (mg M-3-GE/gDW)

anthocyanin

Total phenolics (mg GAE/gDW)

phenolics

Antioxidant capacity (µm TE/gDW)

antioxidant capacity
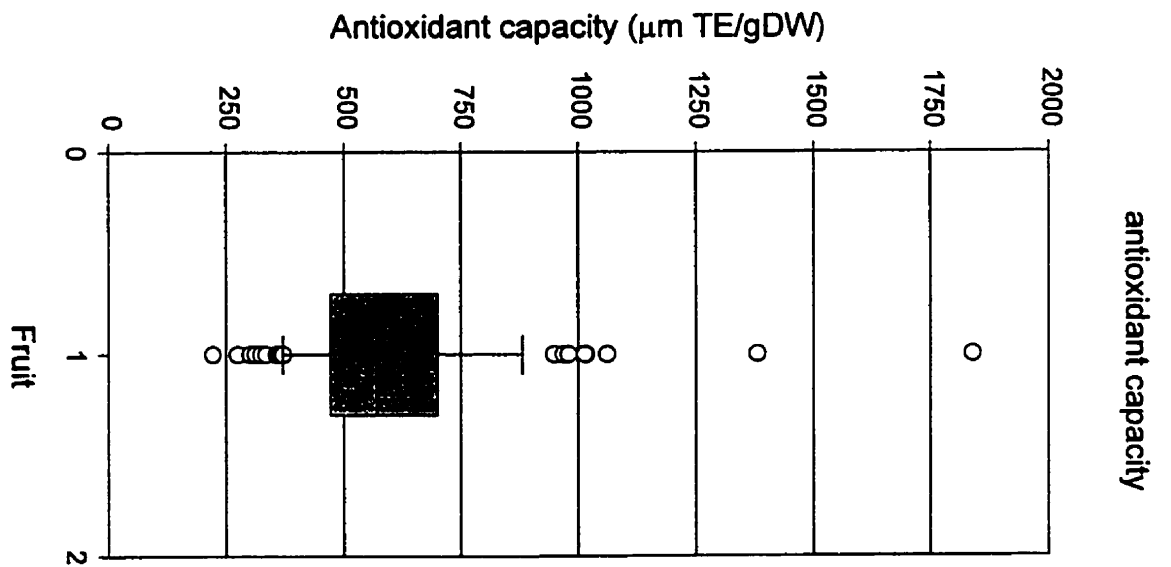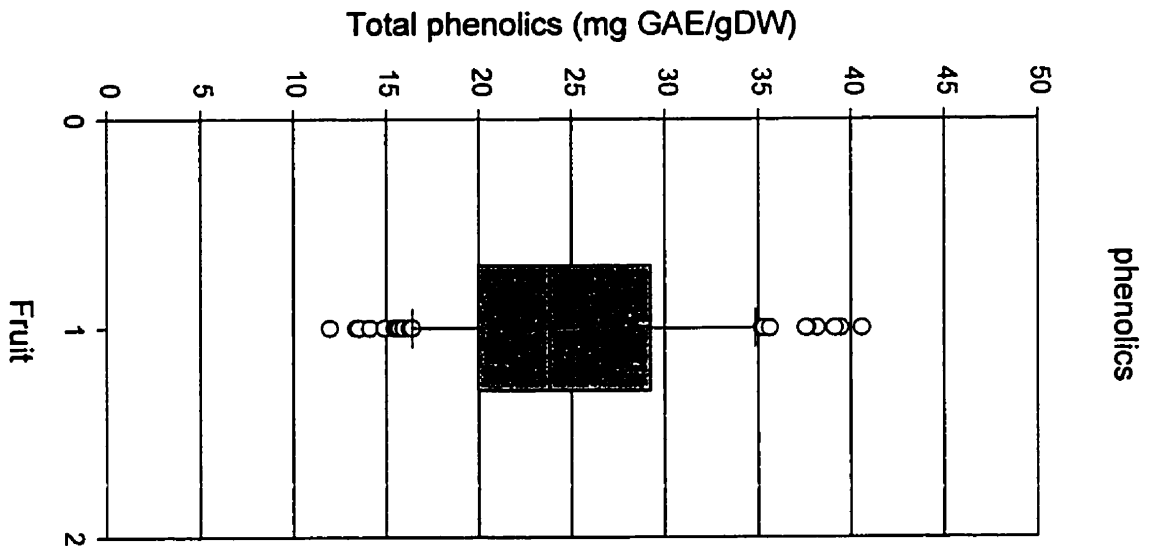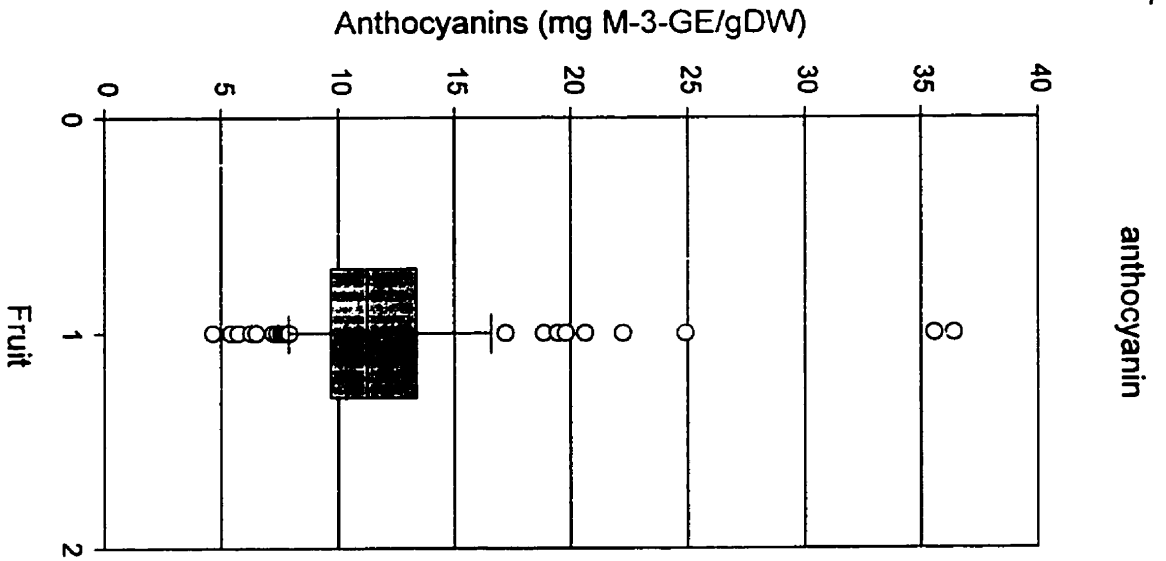
Figure 4:   Box plot showing range of values for surface colour, anthocyanins,

phenolics and antioxidant capacity in leaves of 135 *V. angustifolium*

clones

The median is indicated by the mid line of the shaded box, while the

75th and 25th percentiles are the upper and lower boundaries,

respectively, of the box. Error bars indicate the 90th and 10th

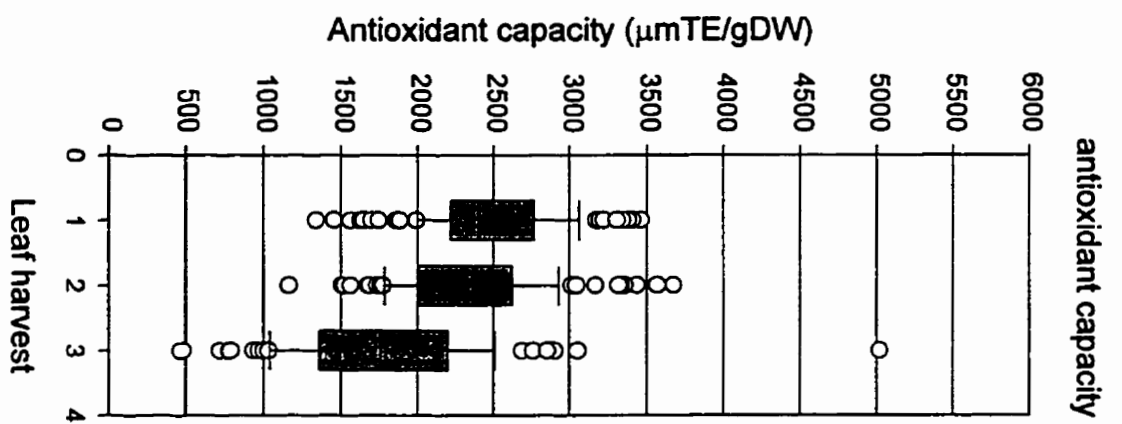percentiles, and all points which lie outside these percentiles are
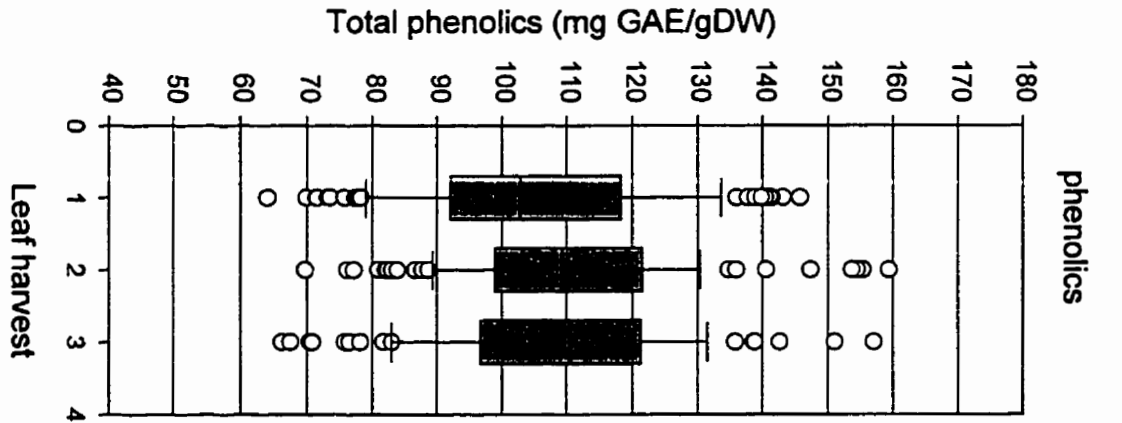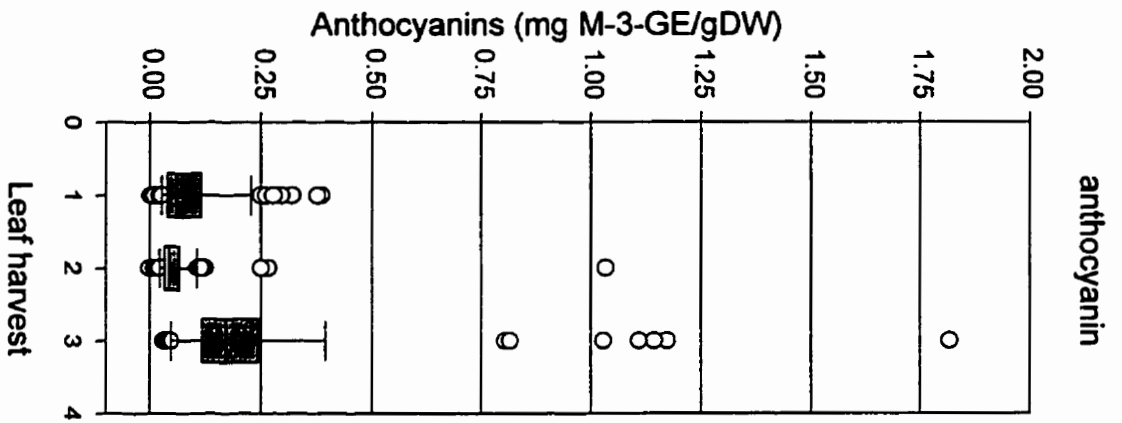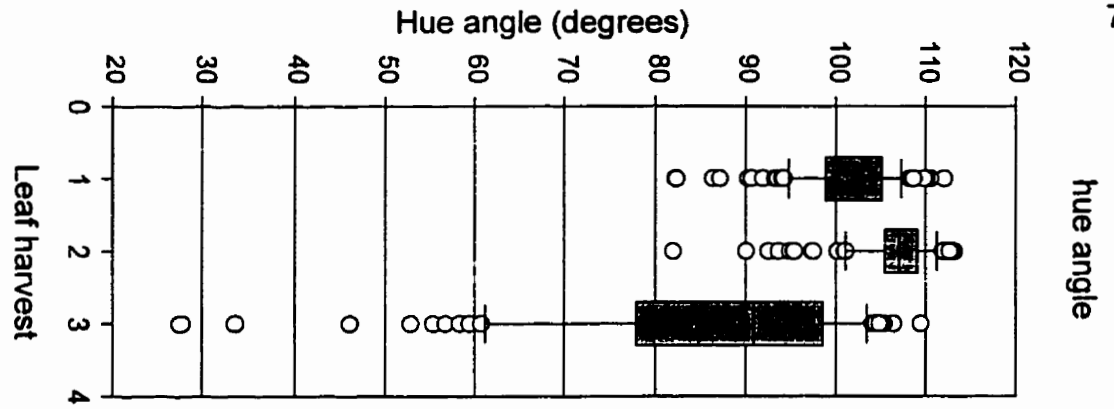
indicated by dots.

Figure 5:   Box plot showing range of values for leaf surface colour,

anthocyanins, phenolics and antioxidant capacity for three leaf

harvests in 135 clones of *V. angustifolium*

The median is indicated by the mid line of the shaded box, while the

75th and 25th percentiles are the upper and lower boundaries,

respectively, of the box. Error bars indicate the 90th and 10th

percentiles, and all points which lie outside these percentiles are

indicated by dots.

Hue angle (degrees) — hue angle, Leaf harvest

Anthocyanins (mg M-3-GE/gDW) — anthocyanin, Leaf harvest

Total phenolics (mg GAE/gDW) — phenolics, Leaf harvest

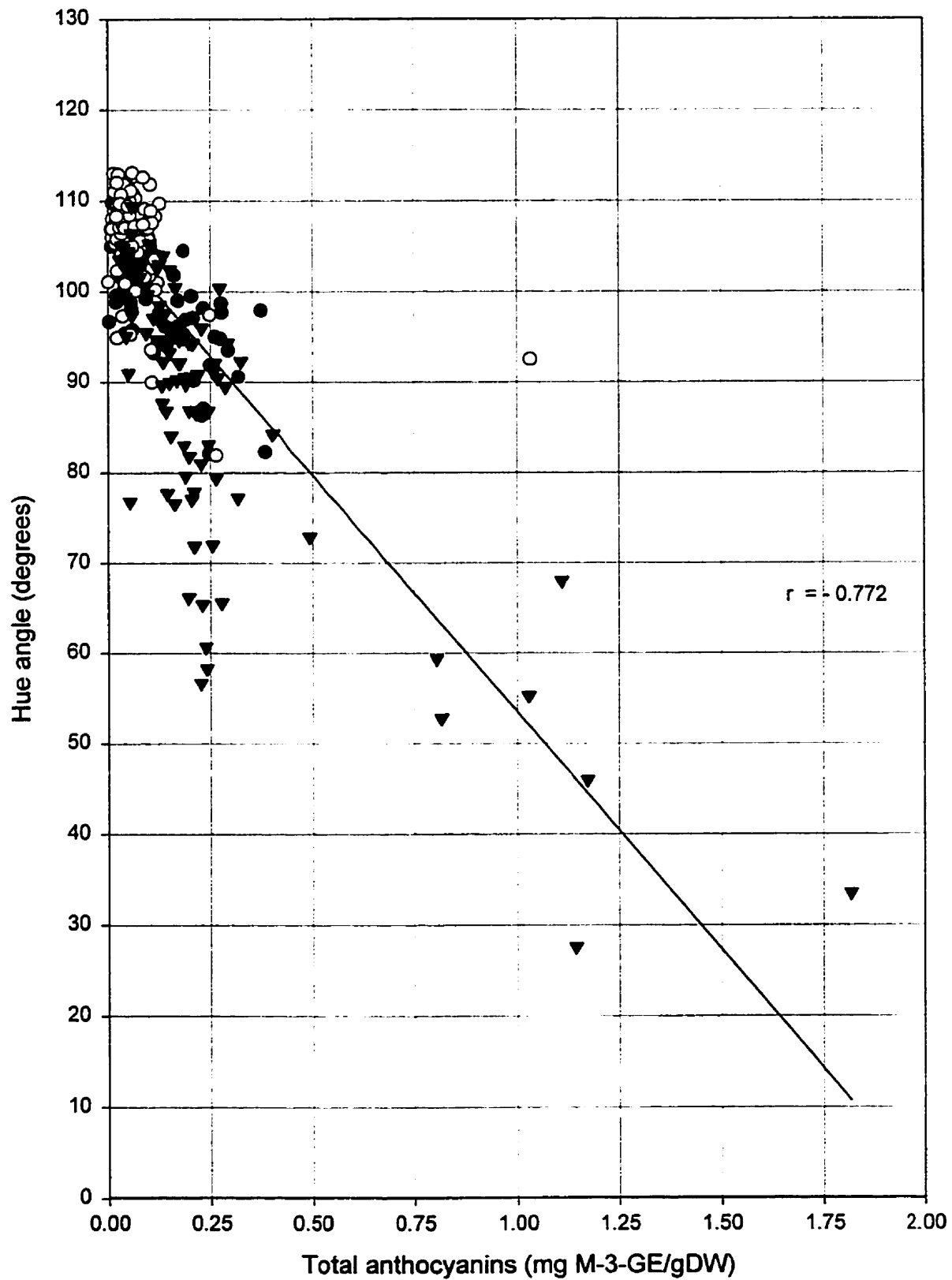Antioxidant capacity (μmTE/gDW) — antioxidant capacity, Leaf harvest

Figure 6:   Anthocyanin content and hue angle in leaves of 135

V.angustifolium clones for three leaf harvests.

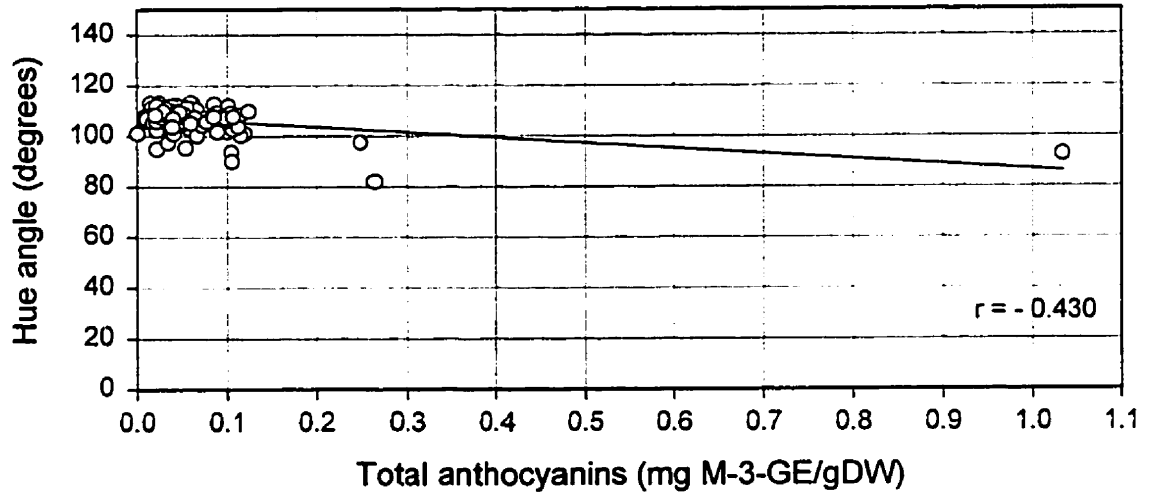Figure 7:   Anthocyanin content and hue angle in leaves of 135

V. angustifolium clones for individual leaf harvests

Early season leaf

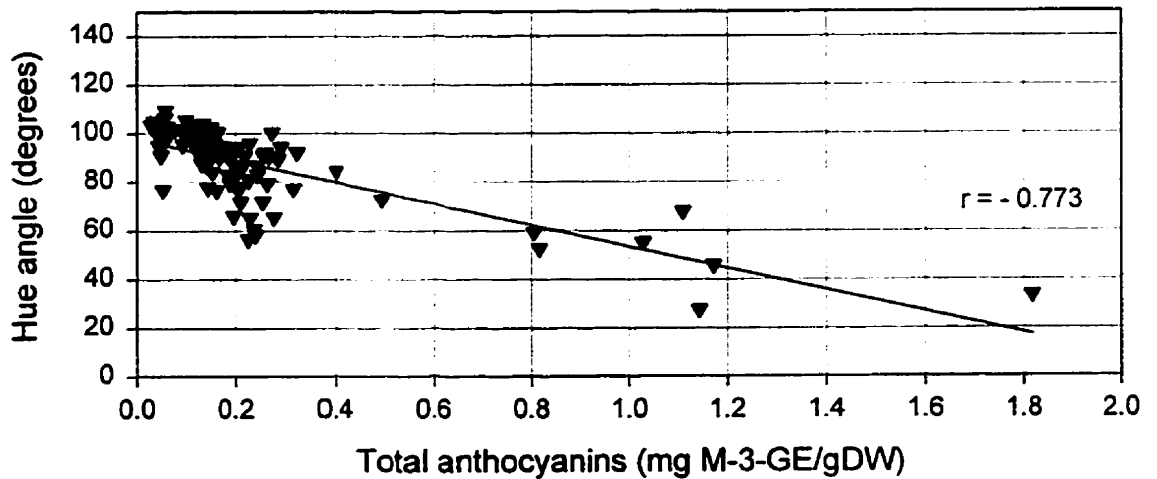r = - 0.730

Total anthocyanins (mg M-3-GE/gDW)

Mid season leaf

r = - 0.430

Total anthocyanins (mg M-3-GE/gDW)

Late season leaf

r = - 0.773

Total anthocyanins (mg M-3-GE/gDW)
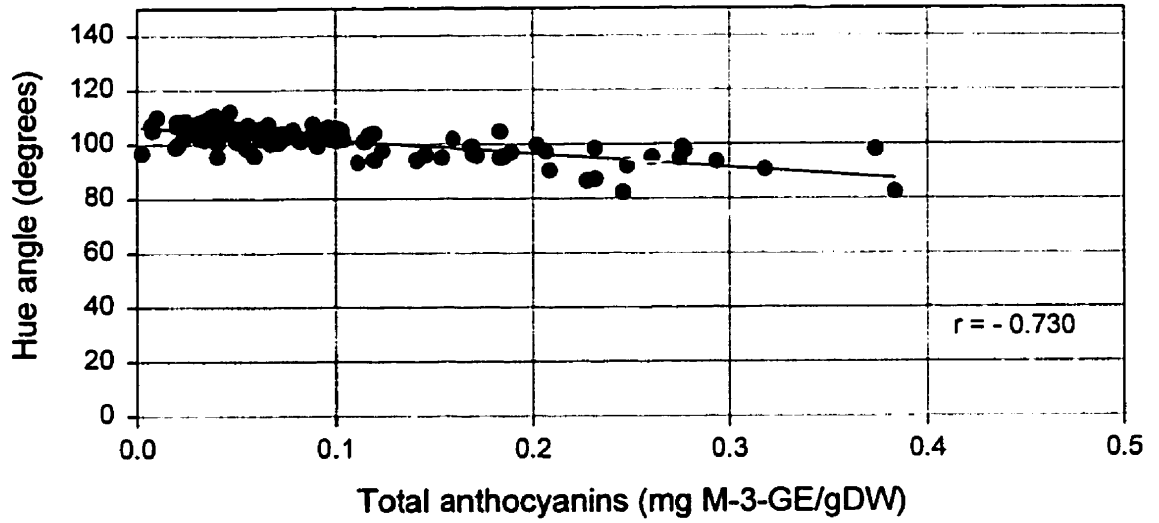
Appendices

Appendix 1: Procedure and results of tests conducted to determine interference

of chlorophyll with Folin-Ciocalteu assay

Procedure

- 3 leaf powders were chosen based on colour - red (#1), brown (#2) and green (#3)

- 8 extracts were made of each of the three powders (as per leaf extraction procedure

in Materials and Methods)

- 5 ml of the 24 leaf extracts were spiked as follows:

    4.75 ml extract

    250 μl of 10 mg/ml gallic acid (GA)

- an aliquot of the spiked extract was removed, and the remaining volume was run

through a C18 Sep Pak and collected into Eppendorf tubes

- an aliquot of the unspiked extract was removed and the remaining volume was run

through a Sep Pak and collected into Eppendorf tubes

- unspiked samples were diluted 10X before phenolic analysis; spiked extracts were

diluted 15X

Appendix 1(continued)

Data showing slight chlorophyll interference in measurement of total phenolics

Spike	Sep Pak		Extract 1 (Red) gGAE/L	Extract 2 (Brown) gGAE/L	Extract 3 (Green) gGAE/L
No	No		2.35 (0.0452)	2.08 (0.0296)	2.21 (0.0266)
No	Yes		2.34 (0.0375)	1.96 (0.0161)	1.98 (0.0265)
		% recovery	99.8%	94.4%	89.7%
Yes	No		3.585 (0.0215)	3.19 (0.0102)	3.34 (0.0139)
Yes	Yes		3.56 (0.0178)	3.07 (0.0087)	3.07 (0.0078)
		% recovery	99.2%	96.37%	92.0%

Gallic acid spike only:

Spike - no spike / no Sep Pak	1.24	1.11	1.13
Spike - no spike / Sep Pak	1.21	1.11	1.085
% recovery	97.9%	100%	96.4%

Appendix 2a :   Fruit variable means and significance for the three

morphological types of *V. angustifolium*

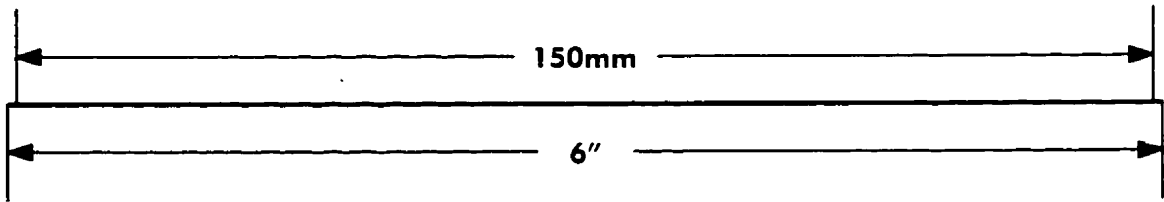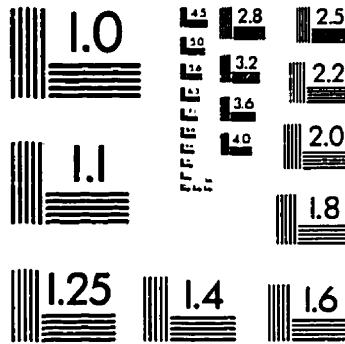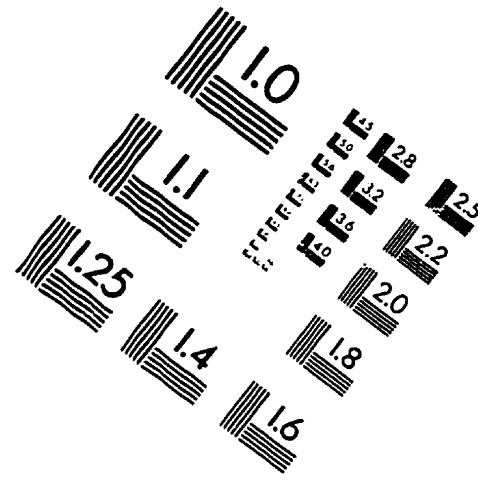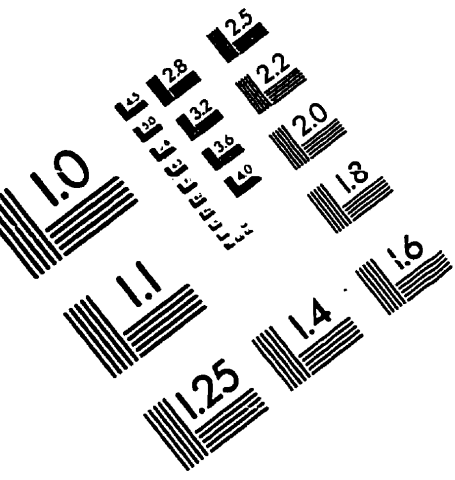|	g/berry	%DW	Anthocyanins (mgM-3-GE/gDW)	Phenolics (mgGAE/gDW)	ORAC (μmolTE/gDW)
Hairy	0.274	17	11.3	25.8	624
Green	0.263	17	12.4	24.7	624
Blue	0.287	16.4	11.8	23.9	612
Grand Mean	0.275	16.8	11.8	24.8	620
Std. Error	0.0089	0.272	0.438	0.885	38.8
F Probability	0.857	0.217	0.223	0.345	0.97

Appendix 2b :   Leaf variable means and significance for the three

morphological types of *V. angustifolium*

	%DW	Hue angle (degrees)	Anthocyanins (mgM-3-GE/gDW)	Phenolics (mgGAE/gDW)	ORAC (μmTE/gDW)
Hairy	43.3	97.1	0.152	108	2150
Green	40.7	99.7	0.125	110	2234
Blue	38.4	98.6	0.117	109	2292
Std. Error	2.12	0.929	0.0116	2	61.8
F Probability	0.296	0.173	0.225	0.766	0.3

Appendix 3: Mean monthly temperatures recorded at Kejimkujik Park, Mount
Uniacke and Parrsboro for 1997.

Site	Region	Mean Monthly Temp. (°C)				
		May	June	July	Aug.	Sept.
Kejimkujik Park	Queens	9.5	15.5	19.4	18.0	14.4
Mount Uniacke	Hants	8.5	13.8	18.6	17.5	13.8
Parrsboro	Cumberland	8.3	13.8	17.5	17.5	14.2

IMAGE EVALUATION
TEST TARGET (QA-3)

1.0

1.1

1.25   1.4   1.6

1.0

1.1

1.25

1.4

1.6

2.8   2.5

3.2   2.2

3.6

4.0   2.0

1.8

|← 150mm →|

|← 6" →|