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**INVESTIGATION OF CYANIDIN AND PELARGONIDIN CONTENTS
IN THE GENUS *FRAGARIA* L.**

BY

LISE BOUCHARD MAHONEY

Bachelor of Science, University of New Hampshire, 1976

THESIS

**Submitted to the University of New Hampshire
in Partial Fulfillment of the
Requirements for the Degree of**

Master of Science

in

Plant Biology

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September 11, 2007
Date

DEDICATION

I dedicate my thesis to my father Gilles E. Bouchard and to my mother Irène M. Bouchard whose qualities of bravery and eagerness to tackle the unknown, and tenacity to see projects to completion were instilled in me. These qualities have been my friends in completing this project.

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I look forward to continuing and growing relationships at the University of New Hampshire as I embark on a Doctor of Philosophy degree program.

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LIST OF ABBREVIATIONS AND UNITS

UNH	University of New Hampshire, Durham NH
USDA	United States Department of Agriculture
NCGR	National Clonal Germplasm Repository, Corvallis Oregon
NPGS PI	National Plant Germplasm System Plant Introduction
FRA=CFRA	NCGR Inventory ID number prefix
HF	UNH Woodman Horticultural Farm
GH	UNH Greenhouses
spp.	Species
ssp.	Subspecies
f.	forma
B	Cultivar 'Bountiful'
PP	Cultivar 'Pink Panda'
B-PP	Accessions of an F ₁ population of 'Bountiful' (B) x 'Pink Panda' (PP)
HPLC	High Performance Liquid Chromatography
FDW	Freeze Dry Weight
aq.	aqueous
ml	milli (10 ⁻³) liter
μl	micro (10 ⁻⁶) liters
μg	micro (10 ⁻⁶) gram
μM	micro (10 ⁻⁶) molar
g	gram
N	Normality, equivalents of solute per liter of solution, ie with HCl, 1N =1M.
COA	Certificate of analysis
ORAC	Oxygen radical absorbance capacity, μM Trolox equivalent

Chemical

[]	concentration
Cyn	Cyanidin
Plr	Pelargonidin
Anth	Anthocyanidin =Cyn and Plr
CPRatio	Ratio of [Cyn] to [Plr]
CCPRatio	Ratio of [Cyn] to [Anth]
HCl	Hydrochloric Acid
FS	Flavone synthase
FLS	Flavonol synthase
F3H	Flavonone 3-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
F3'5'H	Flavonoid 3', 5'-hydroxylase
DFR	Dihydroflavonol 4-reductase
ANS	Leucoanthocyanidin oxygenase
3GT	anthocyanidin 3-O-glucosyltransferase

Statistical

ANOVA	Analysis of Variance
df	Degrees of Freedom
N	Number of samples
SS	Sum of the Squares
MS	Mean square
ME	Mean square for error
F-ratio	Fisher test ratio
P	Probability
SE	Standard Error of Mean
R^2	Multiple Regression Coefficient Squared

ABSTRACT
INVESTIGATION OF CYANIDIN AND PELARGONIDIN CONTENTS IN THE
GENUS *FRAGARIA* L.

by

Lise Bouchard Mahoney

University of New Hampshire, December 2007

Cultivated strawberry (*Fragaria* × *ananassa*) fruit contain primarily pelargonidin and little cyanidin. The antioxidant potential of cyanidin is reportedly twice that of pelargonidin. A High Performance Liquid Chromatography method was developed to assay fruit anthocyanidin content in 87 *Fragaria* accessions, including wild diploids and octoploids, cultivars, and two segregating populations. A cyanidin to pelargonidin ratio (CPRatio) greater than one was found only in diploids *Fragaria vesca* ssp. *americana* (confirming a previous report), *F. viridis*, and *F. iinumae*, and in three wild octoploid accessions: all *F. chiloensis*. The highest cyanidin contents were found in diploid *F. viridis* and in *F. chiloensis* ssp. *lucida*. During fruit ripening, pelargonidin content increased, while cyanidin content held steady. The results hold promise for breeding of cultivars with higher cyanidin content and with potentially higher antioxidant potential.

CHAPTER 1

INTRODUCTION

Strawberries are an economically important and nutritious fruit, with a worldwide annual production of 8 billion pounds (Perez, 2005). The United States accounts for about 29% of the world's production, or approximately 2.3 billion pounds (Perez and Toland, 2006), while New Hampshire grows about 1 million pounds of strawberries per year (Keough, 2007). In the United States, the per capita consumption of strawberries has increased by over 50% in the last ten years to 6.8 pounds, with 5.3 lbs being consumed fresh (Perez, 2005).

The fruit of wild and cultivated strawberry (*Fragaria* L.) contain anthocyanins, which are important secondary metabolites, are antioxidants, and are regarded as health promoting compounds (Moyer et al., 2002). The level of anthocyanins has been correlated to the total antioxidant activity of strawberry fruit (Wang and Lin, 2000), and the antioxidant activity has also been correlated to the type of anthocyanin (Wang et al., 1997). Strawberry fruit contain two predominant types of anthocyanins: cyanidin 3-glucoside and pelargonidin 3-glucoside (Bakker et al., 1994). The antioxidant capacity as measured by oxygen radical absorbance capacity (ORAC, μM Trolox equivalent) of cyanidin 3-glucoside is two to three times that of pelargonidin 3-glucoside (Pietta, 2000; Wang et al., 1997).

Interestingly, cyanidin constitutes only 5% to 10% of the total anthocyanins in the octoploid, cultivated strawberry, *Fragaria × ananassa* (Weston) *Fragaria* Duchesne ex Rozier (Kalt et al., 2001; Kikoku et al., 1995; Wang and Zheng, 2001; Wang et al., 2002) and a few cultivars contain no evident cyanidin (Bakker et al., 1994). In contrast, an early study found a 1:1 cyanidin to pelargonidin ratio in the fruit of *Fragaria vesca* L., a wild diploid species (Sondheimer and Karash, 1956). This early study reported only the ratio of cyanidin to pelargonidin, but did not provide absolute amounts; so it is not known whether the comparatively high cyanidin to pelargonidin ratio reported for *Fragaria vesca* was due to high cyanidin content, low pelargonidin content, or both.

Growth and cultural practices may also impact the levels of strawberry fruit anthocyanins. Studies have shown that the overall levels of anthocyanins in strawberry fruit are affected by temperature (Wang and Zheng, 2001), compost and fertilizer (Wang and Lin, 2003), matted-row culture versus plasticulture (Wang et al., 2002), and carbon dioxide (Wang et al., 2003), but the anthocyanidin content remained within the ranges observed in cultivated strawberries and the respective ratio of cyanidin to pelargonidin content were unaffected. The results suggest that the reported differences between the cyanidin and pelargonidin contents in *F. × ananassa* and in *F. vesca* are likely not the result of environmental effects but rather are due to genetic factors.

Fragaria vesca is considered a likely diploid ($2n=2x=14$) ancestor of the octoploid ($2n=8x=56$) group of strawberry species (Hancock, 1999) the cultivated

strawberry, *F. × ananassa* and its immediate wild progenitors, *F. chiloensis* (L.) Duchesne and *F. virginiana* Mill. The reported presence of a cyanidin to pelargonidin ratio of 1:1 in *F. vesca* prompted the hypothesis that sources of high cyanidin content may also exist in other wild strawberry species, including the octoploid germplasm pool of the cultivated strawberry. If so, gene introgression from wild octoploid into cultivated varieties could afford an opportunity to increase the cyanidin content in the cultivated strawberry through breeding.

Strawberry Germplasm

Fragaria is part of the economically important Rosaceae family. Other economically important members of the Rosaceae family include fruit or nut bearing *Malus* Mill. (apple), *Prunus* L. (almond, apricot), and *Rubus* L. (raspberry) and ornamentals such as *Potentilla* L. (cinquefoil) (Judd et al., 2002).

Fragaria vesca grows across North America from the northern part of British Columbia and south to Central America in San Salvador (Staudt, 1999), on the island of Hawaii (Hancock and Luby, 1993), and in Europe (Hancock and Luby, 1993). *Fragaria vesca* is represented in the Americas by subspecies *americana* (Porter) Staudt, *bracteata* (A. Heller) Staudt, and *californica* (Chamisso and Schlechtendal) Staudt (Darrow, 1966). From Europe to Siberia, *F. vesca* is represented by subspecies *vesca* (Folta and Davis, 2006). The early report of the 1:1 cyanidin to pelargonidin ratio in *F. vesca* concerned plants grown in Geneva, NY, but which originated from one clone from East Malling, England (Sondheimer and Karash, 1956) and therefore were presumably subspecies *vesca*. In addition to *F. vesca*, many other diploid strawberry species

have been identified, including *F. viridis* Duchesne, *F. iinumae* Makino, *F. nilgerrensis* Schlecht. ex J. Gay, *F. daltoniana* J. Gay, *F. nubicola* (Hook. f.) Lindl. ex Lacaita, *F. yezoensis* Hara, *F. mandshurica* Staudt, and *F. nipponica* Makino (Hancock and Luby, 1993). Along with *F. vesca*, several of these diploids are considered possible ancestors to the octoploid strawberry species (Davis et al., 2007; Hancock, 1999).

Fragaria chiloensis grows wild along the Pacific coast from the southern coastline of Alaska to the coasts of Oregon and California, in Hawaii, and in South America. In South America, *F. chiloensis* grows in Ecuador, Peru, and in Chile, but it also occurs at high altitudes where it has escaped from cultivation in areas where it has been under cultivation for many years, as early as 1500 (Darrow, 1966). *Fragaria chiloensis* is represented by four subspecies and two *formae* within the subspecies *chiloensis* (Staudt, 1999), with much fruit color variation including white, yellowish-white, whitish-red and red (Darrow, 1966). The infraspecific taxa in *F. chiloensis* subspecies include ssp. *lucida*, ssp. *pacifica* Staudt, ssp. *sandwicensis* (O. Deg and I. Deg) Staudt, and subspecies *chiloensis* with f. *chiloensis* and f. *patagonica* Staudt (Staudt, 1999).

Fragaria virginiana grows wild throughout North America including Canada and the United States from Alaska to Mississippi (Staudt, 1999). *Fragaria virginiana* is represented by four subspecies, with red fruit with white to red internal color (Staudt, 1999): ssp. *virginiana*, ssp. *glauca* (S. Watson) Staudt, ssp. *grayana* (E. Vilm. ex J. Gay) Staudt, and ssp. *platypetala* (Rydb.) Staudt (Staudt, 1999).

Fragaria × *ananassa*, the primary contemporary cultivated strawberry, originated in France from an accidental pollination of *F. chiloensis* by *F. virginiana*, in the mid-1700's. Initially, five female *F. chiloensis* plants were brought from Concepción, Chile to France in 1712 by a French spy with a background in the sciences, who coincidentally but most appropriately was named Frézier (meaning strawberry in French). The flowers of these plants were described as having many petals and being as large as 1 3/5" in diameter, and the fruit was described as 'whitish-red' and as big as a 'walnut' and sometimes a 'hen's egg' (Darrow, 1966). The source of *F. virginiana* and its voyage to France is less clear, but documents indicate that *F. virginiana* came from eastern North America and most likely from the state of Virginia (Darrow, 1966), thereby named *virginiana*. *Fragaria* × *ananassa* originated, when by chance, by planting alternate rows of *F. virginiana* and *F. chiloensis*, *F. virginiana* donated pollen to *F. chiloensis* resulting in fruit reported as large as 7 1/2" in circumference (Darrow, 1966); approximately 2 3/8" in diameter.

Fragaria × *ananassa* makes up the largest share of strawberry cultivars on the market. The few exceptions are diploid *F. vesca* 'Alpine' and other varieties such as 'Yellow Wonder' and 'Baron Solemacher' from Europe. Most of the *F.* × *ananassa* cultivars were derived from continual breeding and hybridizations of the original cross of *F. chiloensis* and *F. virginiana*. *Fragaria* × *ananassa* cultivars are described as either short-day or day-neutral cultivars. Short-day cultivars form flower buds during photoperiods of short days (long nights), while day-neutral cultivars flower irrespective of the photoperiod.

More recently, octoploid *F. × ananassa* cultivars have been derived through introgression of wild stock originating from different parts of the Americas to introduce new characteristics. For example the day-neutrality of the cultivars 'Seascape' and 'Selva' are the result of introducing *F. virginiana* ssp. *glauca* into their pedigree (Bringhurst, 1991). In addition, there is growing interest in developing cultivars with increased nutritional value, containing higher amounts of vitamins and minerals, and increased antioxidant potential with identification and focus on secondary metabolites and anthocyanins (Crosby and Jifon, 2007; Just, 2004; Khanizadeh et al., 2007; Scalzo et al., 2007; Yoo et al., 2007). The interest in developing cultivars with increased nutritional content is broad but is not limited to fruits. For example, the carrot traditionally known for its high beta-carotene content, has been bred for color and to contain a predominance of secondary metabolites, for example yellow carrots with xanthophylls, red carrots with lycopenes, and purple carrots with anthocyanin compounds (Pike, 2005; Simon, 1997) and all cyanidin derivatives (Kurilich et al., 2005). The purple carrot was derived by interbreeding the cultivated carrot with wild 'Black' carrot germplasm native to Afghanistan, Turkey, and the Middle East (Simon, 2005).

Development of new cultivars is not limited to interspecific breeding. For example, 'Pink Panda' is the result of intergeneric hybridization of *Fragaria × ananassa* with *Potentilla palustris* (L.) Scop. (Ellis, 1962; Ellis, 1991). A new combination name *Fragaria × rosea* (Mabb.) K. Hammer et Pistrick has been proposed (Hammer and Pistrick, 2003) for this intergeneric hybridization. 'Pink Panda' is considered an ornamental strawberry, because of its pink flowers and

poor fruit set. *Potentilla palustris* differs from all the other *Potentilla* species in that it has “a large fruit with a large but non-succulent spongy receptacle” (Ellis, 1962) and is sometimes segregated as *Comarum palustre* L. (Crow and Hellquist, 2000). Interestingly, an F₁ population of a cross between *F. × ananassa* ‘Bountiful’ and ‘Pink Panda’ initially made by Chad Finn, Oregon State University and repeated by Thomas M. Davis, University of New Hampshire (UNH), segregated for flower color, which elicited the question of whether the population was also segregating for cyanidin content.

Anthocyanins

Anthocyanins are the most conspicuous of the flavonoids; comprising a large class of plant secondary metabolites (Holton and Cornish, 1995). Cyanidin, the secondary anthocyanin found in the cultivated strawberry, imparts slight reddish-blue color as is more typical of raspberry, while pelargonidin imparts the typical orange-red color to the strawberry fruit. Anthocyanins contain a sugar moiety usually at the 3-carbon position. Anthocyanidins do not contain any sugar moiety. The basic chemical structure of anthocyanidins consists of two phenolic rings linked by a three carbon chain (Figure 1.1). Both cyanidin 3-glucoside and

Figure 1.1 Chemical structures of anthocyanins in strawberry. Cyanidin 3-O-β-D-glucoside and Pelargonidin 3-O-β-D-glucoside structure and nomenclature differ by only one hydroxyl (OH*) group.

pelargonidin 3-glucoside are very similar in structure, both have an O- β -D-glucoside at the 3 carbon position, and hydroxyl (-OH) groups at the 5, 7 and the 4' carbon positions. Cyanidin 3-glucoside and pelargonidin 3-glucoside differ by only one hydroxyl group, with cyanidin having an additional hydroxyl at the 3' carbon position.

The basic anthocyanin biosynthetic pathway for the synthesis of cyanidin and pelargonidin in plants, as illustrated in Figure 1.2, is well understood (Holton and Cornish, 1995; Moss, 2001). Cyanidin and pelargonidin are both synthesized from dihydrokaempferol. Flavonone 3-hydroxylase (F3H) reduces naringenin and produces dihydrokaempferol. Flavonoid 3'-hydroxylase (F3'H) catalyzes the hydroxylation of the 3' carbon of dihydrokaempferol to yield dihydroquercetin.

Dihydroflavonol 4-reductase (DFR) reduces dihydrokaempferol to produce leucopelargonidin and reduces dihydroquercetin to produce leucocyanidin. Flavonol synthase (FLS) oxidizes the dihydroflavonols to produce kaempferol and quercetin. The F3'H enzyme competes with DFR and FLS (Holton and Cornish, 1995) for substrates, and kaempferol (a flavonol), naringenin (a flavanone), and epigenin (a flavone) all serve as substrates for F3'H (Moss, 2001), competing with dihydroquercetin. These competing pathways are not shown in Figure 1.2. The lack of specificity for substrates and the competition between the enzymes for common substrates results in the availability of multiple paths for the same intermediate (Holton and Cornish, 1995).

Figure 1.2: Anthocyanin biosynthetic pathway. Enzyme abbreviations: FS (flavone synthase), F3H (flavonone 3-hydroxylase), FLS (flavonol synthase), F3'H (flavonoid 3'-hydroxylase), DFR (dihydroflavonol 4-reductase), ANS (leucoanthocyanidin oxygenase), 3GT (anthocyanidin 3-O-glucosyltransferase).

Low levels of flavonols, quercetin and kaempferol; have been reported in strawberry in a number of studies (Heinonen et al., 1998; Wang and Lin, 2003). The presence of these flavonols at low levels in the cultivated strawberry suggests low activity of the FLS enzyme and that dihydrokaempferol is not being

consumed for production of these flavonols. In petunia, high levels of cyanidin derivatives accumulate only in accessions that possess F3'H activity but lack flavonoid 3'5'-hydroxylase (F3'5'H) (not shown in Figure 1,2) and FLS activity (Holton and Cornish, 1995). Similarly, FLS activity may also be low in the cultivated strawberry as indicated by the low contents of quercetin and kaempferol. And that, coupled with the lack of F3'5'H, suggest that a possible cause of the low cyanidin content in the cultivated strawberry is a loss of function in the F3'H gene.

Anthocyanin pathway genes for enzymes chalcone synthase (CHS), chalcone hydroxyl isomerase (CHI), flavonone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) and one *Del*-like regulatory gene have been partially cloned and sequenced in strawberry (Deng and Davis, 2001). The complete CHS, CHI, DFR, ANS, and anthocyanidin 3-O-glucosyltransferase (3GT) gene sequences have been determined for maize, snapdragon and petunia (Holton and Cornish, 1995). The complete sequences of F3H have been determined for snapdragon and petunia (Holton and Cornish, 1995) and more recently in *F. × ananassa* (Almeida et al., 2004). An F3'H encoding gene has been cloned and sequenced for petunia (Holton and Cornish, 1995). On the basis of research on anthocyanin pathway genes in other species, it is expected that the entire complement of anthocyanin pathway genes will soon be isolated from *Fragaria* by virtue of homology to the respective genes in other species. Identifying germplasm differing in cyanidin content at the diploid and/or octoploid levels should make it possible to identify

molecular genetic determinants of cyanidin levels, and to develop molecular tools that breeders could use to select for enhanced cyanidin content.

Anthocyanidin Assays

For the quantitative determination of anthocyanins and anthocyanidins in fruit, High Performance Liquid Chromatography (HPLC) is the commonly accepted technology (Durst and Wrolstad, 2001; Hong and Wrolstad, 1990; Kikoku et al., 1995; Mazza et al., 2004; Merken and Beecher, 2000; Nyman and Kumpulainen, 2001). Methods of HPLC analysis of strawberry anthocyanidins have been described (Bridle and Garcia-Viguera, 1997; Merken and Beecher, 2000; Nyman and Kumpulainen, 2001). Others have described HPLC procedures for the detection of strawberry anthocyanins (Merken and Beecher, 2000; Wang and Lin, 2003; Wu and Prior, 2005). HPLC methods outlined in the literature differ by column use, temperature control, by wavelength of detection, by use of isocratic or gradient elution, and by type and concentration of chemicals in the mobile phases.

The preparation of the fruit sample for assay of the anthocyanidin content of fruit requires hydrolysis of the anthocyanins (which have an attached sugar) to cleave the sugars, thereby forming the respective anthocyanidins (which do not have an attached sugar). By definition, an anthocyanin has attached sugar(s); once the sugar is removed from an anthocyanin, the pigment is known as an anthocyanidin (Harborne, 1958). Acid treatment in combination with heating of the fruit tissue completes the hydrolysis of the anthocyanins. Acid hydrolysis removes glucosides (sugars) (Durst and Wrolstad, 2001; Merken and Beecher,

2000; Nyman and Kumpulainen, 2001), thereby reducing the number of possible forms of anthocyanidins in strawberry to two: cyanidin and pelargonidin. The anthocyanidins are then separated from the fruit tissue by centrifugation and purified using selective molecular absorption, in preparation for separation and assay determination of cyanidin and pelargonidin contents by HPLC.

Project Overview

The focus of this study was to assess cyanidin and pelargonidin contents in a diverse sampling of strawberry diploid and octoploid germplasm (Table 2.1-2.4). Of particular interest were *F. vesca*, *F. chiloensis* and *F. virginiana*. Included in the study was a sampling of diverse diploid species and hybrids to discover if high cyanidin content was really present in *F. vesca* and/or in other diploid accessions. A United States Department of Agriculture (USDA) Supercore Collection held by National Clonal Germplasm Repository (NCGR) to represent the diversity of wild octoploid species of *F. chiloensis* and *F. virginiana* (Hancock et al., 2001) was surveyed to discover if high cyanidin is present in the wild octoploid germplasm pool of the cultivated strawberry. Among the *F. × ananassa* cultivars included in the study were day-neutral varieties, to discover if high cyanidin might exist in some of these varieties. Finally, an interspecific hybrid F_1 population segregating for flower color (pink versus white) was surveyed to determine if the population might also be segregating for cyanidin content. Segregation in the population for cyanidin content would suggest that cyanidin content could be increased in the cultivated strawberry through breeding.

Goals and Objectives

The overall goal of the research was to discover sources of high cyanidin content in wild and or cultivated strawberry species and to evaluate the potential for increases in strawberry fruit cyanidin through breeding. The objectives of this project were to:

- 1) Establish methods and conditions for the assessment of anthocyanidin content in the fruit of strawberry;
- 2) Assess variation in cyanidin and pelargonidin contents in the fruit of diploid and octoploid germplasm including diploid *F. vesca*, *F. viridis* and some diploid hybrids, and octoploid Supercore Collection, and assorted cultivars; and
- 3) Assess variation in cyanidin and pelargonidin in an octoploid population segregating for flower color.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials

A germplasm collection comprised of wild diploid and octoploid species, and an assortment of cultivated diploid and octoploid varieties, was assembled. The word accession (which is widely applied in reference to the members of a germplasm collection) is used generically throughout this thesis to refer to individual wild species, hybrids, cultivated varieties, and to individual members of an F_1 population. Information about the identification and sites of origin of the species, subspecies, and cultivars used in this study is provided in Tables 2.1 through 2.4. The surveyed diploids included *F. vesca*, *F. viridis*, *F. iinumae*, and an assortment of diploid hybrids (Table 2.1). The wild octoploid species consisted of a Supercore Collection of 43 accessions originally compiled by USDA-sponsored investigators to represent the diversity of wild *F. chiloensis* and *F. virginiana* in the Americas (Hancock et al., 2000) (Table 2.2). *Fragaria* × *ananassa* cultivars surveyed for the present study comprised an assortment of short-day and day-neutral cultivars (Table 2.3). The further classification within the short-day cultivars indicates when flowers and fruit will develop during the short-day (long night) photoperiod. In addition, an F_1 population of 43 members resulting from a hybridization of 'Bountiful' (B) and 'Pink Panda' (PP), as well as the two parental cultivars, were surveyed in this investigation (Table 2.4).

Table 2.1 Diploid accessions surveyed.

The diploid species, subspecies (ssp.), and hybrids, the name and/or accession number of each plant, the UNH ID, the origin (original collection site of wild species or origin of hybrids), and the sources of seed or plant material with reference information are provided. Hybrids surveyed were originally generated by T.M. Davis. The CFRA ID accessions numbers are the inventory identifications of the National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon. The PI (Plant Introduction) numbers are the National Plant Germplasm System (NPGS) accession designations.

Plant Identity	UNH ID	Origin	Plant or seed source
<u>F. vesca</u>			
ssp. <i>americana</i>	WC-8	Coos County, NH	UNH (Williamson, 1993)
ssp. <i>vesca</i> 'Baron Solemacher'	BS	European origin, 'Alpine' variety	Seeds obtained from Johnny's Selected Seeds (Yu and Davis, 1995)
ssp. <i>vesca</i> 'Yellow Wonder'	YW	European origin, 'Alpine' variety	Seeds obtained from W. Atlee Burpee and Co (Yu and Davis, 1995)
<u>F. iinumae</u> CFRA1855 (PI 637969)	J-17	Hokkaido, Japan	Collected in Japan by T. M. Davis
<u>F₁ Hybrids</u>			
<i>F. vesca</i> YW × <i>F. nubicola</i> CFRA520 (PI 551851)	YWx520F ₁ C	Europe × Pakistan	(Deng and Davis, 2001)
<i>F. viridis</i> CFRA341(PI 551742) × <i>F. viridis</i> SIB1	<i>F.vir</i> F ₁ D	Germany × Siberia	SIB1 seeds collected by G. E. Crow (UNH) in Siberia.
<i>F. vesca</i> YW × (<i>F. viridis</i> × <i>F. vesca</i> ssp. <i>bracteata</i> (CFRA364)	BQS prefixes	F ₁ population	Progeny derived from a complex and partially undefined pedigree. CFRA364 initially listed by USDA as <i>F. vesca</i> , but later determined to be an interspecific hybrid of <i>F. viridis</i> × <i>F. vesca</i> ssp. <i>bracteata</i> (Yu and Davis, 1995)
<u>Potentilla nepalensis</u> <u>Hook.</u> (PI 436993)		Himalayas	Clone obtained from North Central Regional Station: Ames, Iowa

Table 2.2 Octoploid Supercore Collection surveyed.

A diverse collection of *F. chiloensis* and *F. virginiana* from the Americas (Hancock et al., 2000). Accessions were collected in the wild, unless otherwise indicated. The National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon provided these accessions as runners. The suffix in the Taxon nomenclature for *F. virginiana* ssp. *virginiana* refers to the latitudinal origin of the plant material in the United States; (N) for northern and (S) for southern. NPGS PI # refers to the National Plant Germplasm System Plant Introduction number. The NCGR Inventory CFRA ID numbers of the accessions that produced enough fruit for sampling are underlined and in **bold**.

Taxon	NPGS PI #	NCGR CFRA ID	Plant Origin
<i>F. chiloensis</i> ssp. <i>chiloensis</i> f. <i>chiloensis</i>	551736	<u>372.002</u>	Previously cultivated in Peru
	552038	743.001	Chile
	616544	1104.002	Chile
	602570	1108.002	Chile
	612318	1480.001	Ecuador
<i>F. chiloensis</i> ssp. <i>chiloensis</i> f. <i>patagonica</i>	552091	796.001	Chile
	612316	1088.002	Chile
	612317	1092.002	Chile
	602568	<u>1100.002</u>	Chile
	236579	24.001	Chile, Garden near Lake
<i>F. chiloensis</i> ssp. <i>lucida</i>	551445	34.002	California
	551453	<u>42.001</u>	Washington
	612489	<u>1691.001</u>	Oregon
<i>F. chiloensis</i> ssp. <i>pacifica</i>	551459	48.001	Oregon
	551728	357.002	California
	551735	<u>368.002</u>	Alaska
	551735	<u>368.005</u>	Alaska
	612487	1689.001	B.C., Canada
	612488	1690.001	B.C., Canada
	612490	1692.001	California

Table 2.2 continued.

Taxon	NPGS PI #	NCGR CFRA ID	Plant Origin
<i>F. virginiana</i> ssp. <i>glauca</i>	612496	1698.001	Alaska
	612501	1703.001	Montana
<i>F. virginiana</i> ssp. <i>grayana</i>	612486	<u>1408.001</u>	Mississippi
	612486	<u>1408.002</u>	Mississippi
	612569	<u>1414.001</u>	Mississippi
<i>F. virginiana</i> ssp. <i>glauca</i> × ssp. <i>virginiana</i>	612494	1696.001	S. Dakota
	612495	<u>1697.001</u>	Montana
	612495	<u>1697.002</u>	Montana
<i>F. virginiana</i> ssp. <i>platypetala</i>	551471	58.002	California
	551527	110.001	Oregon
<i>F. virginiana</i> ssp. <i>virginiana</i> (N)	612492	<u>1694.001</u>	Ont., Canada
	612493	<u>1695.001</u>	Ont., Canada
	612493	<u>1695.002</u>	Ont., Canada
	612497	<u>1699.001</u>	Ont., Canada
	612498	<u>1700.001</u>	Minnesota
	612499	<u>1701.001</u>	Minnesota
	612499	<u>1701.002</u>	Minnesota
<i>F. virginiana</i> ssp. <i>virginiana</i> (S)	612570	<u>1435.001</u>	Florida
	612570	<u>1435.002</u>	Florida
	612320	<u>1455.001</u>	Georgia
	612320	<u>1455.002</u>	Georgia
	612323	1557.001	Alabama
	612324	<u>1580.001</u>	S. Carolina
	612325	1620.001	S. Carolina

Table 2.3: Octoploid *Fragaria* × *ananassa* cultivars surveyed.

Cultivars were donated as crowns by Nourse Farms, Deerfield, Massachusetts, with the exception of cultivars 'Delmarvel' and 'Selva' which were obtained from *NCGR (National Clonal Germplasm Repository: Corvallis, Oregon). **Information on these cultivars can be found on NPGS PI# entries or at the alternate sources listed.

Day-length Preference		Variety	Information**
Short-day	Early	Earliglow	551394
	Early	Northeaster	616918
	Mid-Season	Delmarvel*	616589
		Honeoye	551588
		Raritan	551595
	Mid-Late	Jewel	551927
		Seneca	616559
		Sparkle	551559
	Late	Winona	U of M Extension
		Seascape	Patent #PP7,614
Day-neutral		Tribute	551953
		Tristar	551954
		Selva*	551814

Table 2.4: Octoploid B-PP interspecific hybrids surveyed.

Chad Finn Oregon State University donated parents and first 40 of F₁ numbered progeny as runners.

Variety	Accession PI#	Pedigree Description
Bountiful	551855	<i>F. × ananassa</i>
Pink Panda	616586	<i>F. × ananassa × Potentilla palustris</i> . <i>Fragaria</i> 'Frel' US Patent Plant #7598(Ellis, 1991)
B-PP#	Numbered F ₁ population	'Bountiful' × 'Pink Panda' hybrid crosses made by Chad Finn (progeny #1-#40) and by T. M. Davis (progeny #41-201)

Maintenance of Germplasm

All of the germplasm was maintained in the UNH greenhouses. In addition, the octoploids were planted at the UNH Woodman Horticultural Farm.

Experimental Design and Statistical Analysis of Data

An observational unit (replicate) consisted of a sample of fruit from a single pot (in the greenhouse), or a single plot (in the field), containing one or more plants that had been clonally propagated from an original source plant. For each replicate sample, one to five berries were harvested and pooled. The replicates were collected over one to three seasons. In addition to the individual accessions maintained in pots in the greenhouse, field plots (Appendix A) were established, one plot for each of the Supercore Collection accessions (Figure A.1), four plots for each cultivar (Figure A.2), and one plot for each of the B-PP accessions as well as for Bountiful (B) and Pink Panda (PP) (Figure A.3).

A field plot consisted of a section of row where a clone was allowed to propagate itself by runners. Plots were spaced 4' on center within the row. Replication consisted of repeat sampling (on the same or different dates) of the same plot, or pot, and/or sampling of different plots of the same variety (on the same or different dates). Each plot of the Supercore Collection initially consisted of five crowns. Each plot of the cultivars initially consisted of one crown. Each plot of B x PP F₁ progeny and associated parents initially consisted of a division of crowns from one 12" pot. In the context of data analysis by ANOVA (analysis of variance), individual accessions were considered treatments. The plant germplasm collections; *Fragaria* diploids, octoploids (*F. × ananassa*) cultivars,

octoploid 'Supercorers', and the B-PP accessions including 'Bountiful' and 'Pink Panda', were considered as separate treatment groups in the statistical analysis of the data.

Anthocyanidin content in the fruit of each accession was calculated as the average of assay results of two or more replicate samples, unless otherwise noted. Systat®10 (SPSS Inc) was used to analyze the data. For each plant accession, the Standard Error of the Mean (SE) was taken as the square root of the Mean Square for Error (ME) of the respective treatment group divided by the number of replicate samples of the particular accession. Analysis of variance with Fisher test (F-ratio) and probability (P) were used to test hypotheses, and Tukey® tests were used to check within-group pairwise differences. In addition, ANOVA using SYSTAT® 10 was employed to test results for similarity between harvest locations (greenhouse versus field), and to test for similarity between analytical methods used in investigation Phases 1, 2, and 3 (see below).

Phases of Investigation

The methodology for extracting and analyzing the anthocyanidins was developed incrementally as real samples were assayed. The data obtained during the initial methods development Phase of the study are referred to as "Phase 1 and Phase 2" data. Data obtained after the final methodology had been established are referred to as "Phase 3" data. These investigation phases differed with respect to HPLC sample injection volume and/or to centrifuge speed, as detailed subsequently. The ANOVA results on comparison of methods were used to determine whether normalization factors would be required to

enable the combining of Phase 1, Phase 2, and Phase 3 data in the same statistical analysis.

Fruit Sample Collection and Preservation

The fruit was harvested at 'peak' ripeness firm-ripe stage (Wang and Camp, 2000) or 'commercially ripe stage' (Wang et al., 2003), with the exception of fruit harvested earlier for the degree of ripeness study. Harvested fruit were carefully handled in preparation for the HPLC analysis. Fruit samples of strawberry fruit were harvested into labeled zip-lock bags and placed on ice in a cooler. Subsequently, within 1 to 2 hours, each zip-lock bag was individually removed from the cooler, the sample was removed from the bag and weighed in a labeled weigh boat, and the fruit-containing weigh boat was re-inserted into the original zip-lock bag. The fruit was then squashed by pressing the fruit into the weigh boat from outside of the zip-lock bag, in order to expose more tissue surface area so as to facilitate the subsequent freeze drying process. Each zip-lock bag containing squashed fruit in a weigh boat was then placed on ice in a cooler. Once all of the collected fruit in the zip-lock bags had been weighed and squashed, the samples were transferred from the cooler to a -80°C freezer.

The frozen fruit was lyophilized (Nyman and Kumpulainen, 2001; Wang et al., 2003) to minimize the water content, and then stored at -80°C (Wang et al., 2003), as opposed to the -18°C temperature used by Nyman and Kumpulainen (2001). Just prior to freeze drying, fruit samples were taken out of the -80°C freezer and pre-conditioned at -20°C for 1 to 2 hours. The samples were then freeze dried under vacuum at 0.20 to 0.25 mbars with a condenser temperature

of -60°C to -80°C for 48 hours and without the use of a heat plate, so as to minimize any loss of quality as studied by Shishehgarha et al.(2002). The lyophilized samples were weighed to provide dry weight values and returned to storage at -80°C. The lyophilized samples were stored at -80°C for an indefinite amount of time ranging from a day to months.

Fruit Sample Preparation for Anthocyanidin Assay

Hydrolysis of Anthocyanins

The lyophilized fruit was pulverized (Wang et al., 2003) to achieve a uniform composite sample (Phases 2 and 3). The acid hydrolysis process was performed using aqueous 2N HCl (Durst and Wrolstad, 2001), as opposed to the use of 2N HCl in methanol (Nyman and Kumpulainen, 2001), to avoid use of highly hazardous methanol at elevated temperatures and in proximity to non-explosion proof electrical devices. Each 0.5 g sample of lyophilized strawberry fruit was mixed with 7 ml of 2N HCl in a 12 ml glass tube (Phases 1 and 2), or in a 15 ml polypropylene centrifuge tube (Phase 3) (VWR SuperClear™ Catalog #21008), hydrolyzed at 90°C for 50 minutes (Nyman and Kumpulainen, 2001) using a Fisher Scientific Dry Bath Incubator (Phases 1 and 2), or in a Nitrogen Evaporator™ 111 (Organomation Associates, Inc) used as a hot water bath (Phase 3), and then cooled in a separate cold tap-water bath for 5 minutes. The hydrolyzed samples were spun for 20 minutes in a Damon IEC HN-3 centrifuge at 1200 xg and room temperature (Phases 1 and 2), or in an Eppendorf 5804R centrifuge at 15,500 xg and 4°C (Phase 3). The centrifuged samples were

filtered, using syringe-adapted filter housings with F6801 Sigma-Aldrich 100 µm mesh nylon fabric, to recover the liquid extract containing the anthocyanidins.

Extraction of Anthocyanidins

The procedure used to extract the anthocyanidins was patterned after those previously described (Rodriguez-Saona and Wrolstad, 2001; Wang and Lin, 2003; Wang et al., 2003), with modifications as indicated below. Sep-Pak® C18 cartridges (Waters Corp, Ireland) were activated with 2 ml of 0.01% HCl in methanol, but the additional rinses of deionized water (Rodriguez-Saona and Wrolstad, 2001; Wang et al., 2003) or formic acid (aqueous) (Wang et al., 2003) were not employed. Each supernatant sample was poured into a Sep-Pak® C18 cartridge to bind the anthocyanidins, then the sugars and starches were eluted with 10 ml of 3% formic acid (aqueous). The anthocyanidins and other phenolic compounds were recovered from the C18 Sep-Pak® cartridges with 2.5 ml acidified methanol containing 0.01% HCl (Rodriguez-Saona and Wrolstad, 2001), rather than 3% formic acid in methanol (Wang et al., 2003), to minimize the handling of formic acid and the required number of stock solutions (Table B.1).

The recovered anthocyanidins were then filtered with a 0.2 µm syringe filter, in lieu of the 0.45 µm filter (Wang et al., 2003), to prolong the life of the HPLC separation column. Sample aliquot volumes of 750 µl were transferred to 1.5 ml HPLC vials for analysis. In order to minimize decomposition of the anthocyanidins, the processed samples were immediately analyzed by HPLC, or held in a -20°C freezer for up to 2 hours before warming to room temperature for 15 to 20 minutes before analysis by HPLC. The purified samples were stored at

-80°C according to Rodriguez-Saona and Wrolstad (2001), who recommended storage of purified samples at less than -15°C, preferably less at -70°C.

High Performance Liquid Chromatography Method

The separation and quantification of anthocyanidins were conducted following a modified protocol (Miyazawa et al., 1999) with a Hewlett Packard Series 1100 HPLC equipped with a Diode Array Detector. A reverse-phase Nova-Pak®C18 column 150 x 3.9 mm with 4µm particle size equipped with a Nova-Pak®C18 20 x 3.9 mm guard column (Waters Corp, Ireland) (Wang et al., 2003) was used for the separation of cyanidin and pelargonidin. Due to its smaller size (lower cost) and corresponding lower chemical and sample volume requirements, this configuration was preferred to a commonly used reverse-phase C18 250 x 4.6 mm ODS (Durst and Wrolstad, 2001; Miyazawa et al., 1999).

The photodiode array was set to scan the range of 240 to 570 nm with detection at 320, 350 and 528 nm, similar to Wang et al. (2003), whose scan was set at 240 to 550 nm with detection at 320, 350 and 510 nm, as compared with single point detection at 520 nm (Durst and Wrolstad, 2001), or 532 nm (Miyazawa et al., 1999)

The temperature of the columns was maintained at 30°C to operate above fluctuations in the room temperature which might impact results, in contrast to the use of ambient temperature (Durst and Wrolstad, 2001), 20°C (Wang et al., 2003), or 40°C (Miyazawa et al., 1999). Cooling the column was not an option with the utilized HPLC system.

The solvents chosen for mobile phases A and B were formic acid (aqueous) and 100% methanol, respectively, (Miyazawa et al., 1999), in contrast to Wang et al.(2003) mobile phases A 2.5% formic acid (aq) and B (acetonitrile) or mobile phases A (acetonitrile) and B (10% acetic acid, 5% acetonitrile, and 1% phosphoric acid) (Durst and Wrolstad, 2001) to coincide with the chemicals used in the sample preparation and thus minimize the variety of chemicals required.

The mobile phase A concentration of formic acid was reduced to 0.3% v/v from 6.9% v/v (1.5 molar)(Miyazawa et al., 1999) or from 2.5% v/v (Wang et al., 2003) in order to minimize corrosion of the HPLC plumbing system and to extend the life of the column, as well as to minimize use of chemicals, while maintaining an acidic pH for pigment stability and maximum absorption (Cabrita et al., 2000). The mobile phase B methanol was diluted to 95% v/v to reduce gas bubbles in the column at the point of mixing of the two mobile phases.

Isocratic elution as opposed to gradient elution was chosen to maintain steady state and thus minimize the run cycle time to 15 minutes. The mobile phases were optimized to 52% as mobile phase A (0.3% formic acid) and 48% as mobile phase B (95% methanol) in contrast to (Miyazawa et al., 1999) compositions of 14% A containing 1.5 mol/l (6.9%) formic acid and 86% B (100% methanol) and in contrast to gradient elution and alternative mobile phase solvents as described above (Wang et al., 2003). The mobile phase total flow was reduced to 0.7 from 1.5 ml/min (Miyazawa et al., 1999) based on the smaller column size and from 1.0 ml/min (Wang et al., 2003) based on sample peak shape. The initial volume of the sample injection was 20 μ l (Phase 1) in

accordance with Wang et al.(2003) much less than the 50 µl used by Durst and Wrolstad (2001) or 100 µl by Miyazawa et al.(1999). The volume of the sample injections was later optimized to 5 µl (Phases 2 and 3) to reduce the load on the columns, with the intent of reducing the potential for overload and improve peak shape.

The prepared anthocyanidin samples, 20 µl (Phase 1) or 5 µl (Phases 2 and 3), were auto-injected into an isocratic 0.7 ml/min elution stream containing 52% mobile phase A (0.3% formic acid) and 48% mobile phase B (95% methanol).

Preparation of Anthocyanidin Standard Solutions

Standard solutions needed for calibration of the HPLC for assays were prepared from concentrated master stock solutions. The master solutions were prepared by dissolving certified HPLC grade powder standards (Indofine Chemicals, Hillsborough NJ) of pelargonidin chloride and cyanidin chloride in acidified methanol (0.01% HCl) to make master solutions. The master solutions were made at a concentration of 1000 µg/g in acidified methanol as follows: the powder standards were weighed (scale accuracy of four decimal places) and then the required amount of acidified methanol was used to police the powder standard into a dark colored vial. The required amount of acidified methanol was determined as follows:

$$Y \text{ mls of methanol} = \frac{X \text{ gms of powder standard} \times \text{purity of standard}}{(\text{density of methanol} \times 1000 \text{ µg/g})}$$

The purity of the powder standards was obtained from the certificates of analysis (COA).

- Pelargonidin COA = 0.998

- Cyanidin COA = 0.972

The density of methanol was obtained from the manufacturer's technical data sheet.

- Density of the methanol = 0.7875 g/ml

Standard solutions with concentrations of 10, 50, 90, 100, and 150 µg/g of cyanidin and pelargonidin chloride were each made directly from the master solutions. For instance, to make a standard solution concentration of 10 µg/g of acidified methanol, 10 µl of the master batch was added to 990 µl of acidified methanol. Aliquots of the standard solutions were stored at -20°C.

Using freshly made standards, the HPLC was calibrated once during the course of Phase 1 data collection with 20 µl sample injections. At the onset of Phase 2, the standard solutions previously used in the Phase 1 calibration and stored at -20°C were assayed with 20 µl and 5 µl sample injections to develop a calibration curve for 5 µl sample injections (Phases 2 and 3). The integrated HPLC absorbance areas at 5 µl were correlated to concentrations determined from the 20 µl injections. Aliquots of anthocyanidin extract of cultivar 'Jewel' fruit, prepared as described earlier and stored at -80°C, were used as an external standard for most of the assays in Phases 1 and 2, and for all assays in Phase 3, to assure run-to-run repeatability. Cyanidin and pelargonidin were identified by comparing the retention times of the standards with those of the strawberry fruit extract.

Stability of Anthocyanidin Standards and Mixtures

The studies of the stability of anthocyanidin standard solutions at room temperature were conducted on freshly prepared and on cold stored standards and mixtures of standards to 1) determine if freshly prepared standard solutions could be preserved in cold storage and reused at a later time, and 2) to guide the establishment of time constraints for the HPLC analyses. Initially, freshly made standard solutions were used to prepare a set of mixtures containing varying concentrations and proportions of cyanidin chloride to pelargonidin chloride. Aliquots of each mixture were put into cold storage at -80°C , while samples of the freshly made mixtures were immediately assayed over a twenty-two hour period. In parallel, aliquots of the original cyanidin or pelargonidin standards were stored at -20°C . After one week in cold storage, these standard solutions were warmed to room temperature for one hour in preparation for analyses and then were repeatedly analyzed by HPLC over a twenty-four hour period.

CHAPTER 3

RESULTS

Note: Anthocyanidin (anthocyanidin=anth, cyanidin=cyn, and pelargonidin=plr) content of fruits is reported as micrograms of pigment/gram freeze dry weight of fruit tissue ($\mu\text{g/g}$ FDW) $\pm$ SE (where SE = Standard Error of the Mean).

Overview – Investigation Phases

The methodology was developed in phases as real samples were assayed. In Phase 1, 20 μl prepared samples were assayed by the HPLC. Beginning with Phase 2 and continuing thereafter, the volume of prepared samples assayed in the HPLC was reduced from 20 to 5 μl . In Phases 1 and 2, a centrifuge with a maximum speed of 1200 xg was used to extract the pigments from the hydrolyzed samples. The solids were amorphous in the liquid extract and only about half of the supernatant was recovered. In Phases 2 and 3, the lyophilized fruit tissue was pulverized with a mortar and pestle before hydrolysis to expose more anthocyanidin for hydrolysis and extraction. In Phase 3, a new centrifuge permitted spinning samples at 15500 xg , thus improving the recovery of the anthocyanidins. The solids were more clearly separated from the supernatant and were filtered out improving the recovery of the anthocyanidins. Accessions that were sampled during at least two of the three phases were compared in order to develop normalization factors to pool and compare the data

from different phases for ANOVA of the treatment groups, as subsequently described.

Methods Development

Fruit Samples

Initially, none of the germplasm was planted at the horticultural farm, and so only fruit from accessions maintained in the greenhouse could be sampled. The *Fragaria* diploids were primarily maintained in the greenhouse, with only a few planted in the field during the first season. The Supercore Collection was obtained in mid-June of the second season and the runners were immediately rooted in pots in the greenhouse and then planted in the field in late July, and therefore fruit was not available until the third season. The cultivars were obtained and planted early in the second season and so fruit was available from these in both the second and third season. The greenhouse maintained B-PP accessions were divided and planted in the field during the first season and so fruit obtained from these in the first and second season was available for sampling. In the third season, the B-PP field plants were less productive and fruit was more limited.

Fruit Sample Preparation for Anthocyanidin Assay

During the procedures of fruit harvesting, storage, lyophilizing, and freezing, all procedures went smoothly, and no noteworthy problems were encountered. There was no visible evidence of sample degradation during cold storage.

When the anthocyanins in the lyophilized fruit were hydrolyzed and the anthocyanidins extracted, the only difficulty encountered was in the use of the Sep-Pak® C18 cartridges. A thorough activation of the cartridges, as evidenced by an almost translucent appearance of the C18 cartridge, was required, without which the anthocyanidins plugged the cartridge. An incompletely activated cartridge appeared opaque. In addition, the solids had to be filtered out of the hydrolyzed extract following the centrifugation step, to prevent plugging the Sep-Pak® C18 cartridges with solids. Filtering out the solids using a syringe-adapted filter housings with F6801 Sigma-Aldrich 100 µm mesh nylon fabric provided the added benefit of a more complete recovery of the anthocyanidins; only solids with no visible liquid remained in the centrifuge tubes.

The extraction of the filtered anthocyanidin sample was successfully accomplished using Sep-Pak® C18 cartridges that were activated with 2 ml of 0.01% HCl in methanol. There was no evidence of anthocyanidin loss in the liquid effluent, which was colorless.

High Performance Liquid Chromatography for Anthocyanidin Assay

The HPLC calibration was based on detection at the 528 nm wavelength, which was found to maximize the absorption of both cyanidin and pelargonidin (data not shown).

The laboratory room temperature varied considerably from about 18°C to 27°C over the 2 year period of the investigation. Therefore, with the temperature of the columns set at 30°C, conservatively above the upper limit of room temperature range, possible variations in the results due to temperature

fluctuation were avoided. Although a cooler temperature may have been better in terms of the impact on the stability of the anthocyanidins, heating was the only option with the available HPLC equipment

Using the chosen HPLC conditions, cycle time was 15 minutes (Figure 3.1), as compared to prior studies: 45 minutes (Miyazawa et al., 1999), or 32 minutes (Wang et al., 2003), or 25 minutes (Durst and Wrolstad, 2001). The chosen conditions also favored preservation of the initially purchased reverse column, which remained functional throughout the investigation and was used for all the assays.

Figure 3.1 HPLC chromatograms.

Examples of typical chromatograms using 15 minute cycle time. Chromatograms of anthocyanidin extracts of fruit samples with absorption peak heights (mAU) and retention time (minutes) with high cyanidin and low pelargonidin (lower figure) and with low cyanidin and high pelargonidin contents (top figure). Cyanidin and pelargonidin contents are determined by comparing the integration of the area under the respective peaks to the calibration curve.

Calibration of HPLC for Anthocyanidin Assay. An appropriate concentration range for the standards was selected by measuring absorption with a spectrophotometer and diluting the standards until the highest concentration had an Absorbance of less than 1 using a stand-alone spectrophotometer (Spectronic Genesys 5, Milton Roy).

The HPLC integrated absorption peak areas of cyanidin and pelargonidin standard solutions correlated linearly with concentration, with R^2 (multiple regression coefficient squared) of greater than 0.994 with both 20 μl sample injections (Phase 1), and 5 μl sample injections (Phases 2 and 3) (Figure 3.2).

Figure 3.2: HPLC calibration with anthocyanidin standard solutions.

HPLC integrated peak absorption areas, at 528 nm, with 20 μl (Phase 1) and 5 μl injections (Phases 2 and 3) of cyanidin chloride and pelargonidin chloride standard solutions ($\mu\text{g/ml}$ powder standard dissolved in acidified methanol, 0.01% HCl). Linear calibration equations have correlation coefficients of $R^2 > 0.99$.

Stability of Anthocyanidin Standards and Mixtures. The stability of freshly made mixtures of pelargonidin and cyanidin standard solutions was tested at room temperature over time. Slight increases in concentration were observed for each component, with the exception of the cyanidin content in the $[Cyn]_o=51.9$: $[Pel]_o=9.0$ mixture, which decreased slightly in the first eleven hours of the twenty-two hour test (Figure 3.3a and 3.3b). An ANOVA (Table 3.1) of the data from the first eleven hours indicated that at the higher and lower ranges of concentration, $[Cyn]$ and $[Plr]$ responded similarly to time and that neither $[Cyn]$ nor $[Plr]$ changed significantly over this time period. However, a linear regression of concentration with time indicated that concentrations were increasing slightly over time at the lower concentration range, but with low $R^2=0.286$ and $R^2=0.434$ for cyanidin and pelargonidin, respectively. In contrast, at the higher range, cyanidin and pelargonidin concentrations were not changing significantly over time, with even lower respective $R^2=0.004$ and $R^2=0.022$. Following one week of cold storage, the concentrations of the standard solutions and mixtures were initially equivalent to their respective original, pre-storage concentrations, as shown in a t-test pairwise comparison of the data (Table 3.2). However, over the subsequent twenty-four hour period at room temperature, the concentrations of both $[Cyn]$ and $[Plr]$ of the previously cold stored standard solutions and mixtures declined, and the rate of decline increased with the concentration of the starting solution and with time (Figure 3.4a-c). In another comparison of freshly made standards containing comparable concentrations (Figure 3.5), but made at

different times, with one set stored at -20°C and the other set stored at -80°C, the rate of loss at room temperature over twenty-four hours was higher with the standards stored for one week at -80°C as compared to six weeks at -20°C.

Figure 3.3 Stability of freshly made mixed (Cyn and Plr) anthocyanidin mixtures.

Mixtures containing standard solutions were prepared at two levels of concentration ($\mu\text{g/ml}$) of cyanidin (Cyn) and pelargonidin (Plr) in four combinations. Initial concentrations are indicated as cyanidin [Cyn]₀, pelargonidin [Plr]₀, figure legend. Concentrations were measured at the indicated time points in a period of 11 hours.

Table 3.1 Anthocyanidin content of mixtures of freshly made standard solutions at room temperature.

Mixtures containing standard solutions were prepared at two levels of concentration of pelargonidin [Plr] and cyanidin [Cyn] in four combinations of lower [Cyn]_L and [Plr]_L range and higher [Cyn]_H and [Plr]_H ranges. Provided are initial values for concentrations of Cyn, Plr, and Anth (Cyn + Plr), along with means over a period of 11 hours and SE, followed by ANOVAs and linear regressions on the data set.

Mixtures Ranges	N	Anthocyanins (µg/ml)								
		Initial			Mean			SE		
		[Cyn]	[Plr]	[Anth]	[Cyn]	[Plr]	[Anth]	[Cyn]	[Plr]	[Anth]
[Cyn] _L /[Plr] _L	6	5.7	7.7	13.4	6.10	8.17	14.27	0.157	0.189	0.349
[Cyn] _H /[Plr] _L	8	51.6	9.0	60.6	52.19	9.10	61.31	0.405	0.033	0.396
[Cyn] _L /[Plr] _H	8	7.2	54.3	61.5	7.42	54.40	61.84	0.045	0.076	0.112
[Cyn] _H /[Plr] _H	8	51.9	56.2	107.8	52.24	56.04	108.28	0.108	0.105	0.177
Combined										
[Cyn] _L &[Plr] _L	14				6.86	8.70	15.56	0.194	0.150	0.280
[Cyn] _H &[Plr] _H	16				52.21	55.22	107.43	0.202	0.220	0.262
ANOVA (N = 30) Low and High Range										
Dependent Variable	Source	df	SS	MS	F-ratio	P				
[Cyn]	Hours	1	0.85	0.85	1.5	0.231				
R ² =0.999	Cyn level	1	2008	2008	3549	0.000				
	Cyn level × Hours	1	1.41	1.41	2.5	0.127				
	Error	26	14.7	0.6						
[Plr]	Hours	1	1.76	1.76	3.3	0.080				
R ² =0.999	Plr level	1	2115	2115	4001	0.000				
	Plr level × Hours	1	0.4	0.4	0.8	0.381				
	Error	26	13.7	0.5						
Linear Regression test of probability that slope of line b= 0										
Mixtures (µg/ml)	N	Cyn		Plr		Cyn + Plr				
		R ²	P	R ²	P	R ²	P			
[Cyn] _L / [Plr] _L	6	0.946	0.001	0.938	0.001	0.950	0.001			
[Cyn] _H / [Plr] _L	8	0.059	0.562	0.783	0.004	0.021	0.734			
[Cyn] _L / [Plr] _H	8	0.913	0.000	0.466	0.062	0.647	0.016			
[Cyn] _H / [Plr] _H	8	0.319	0.145	0.008	0.835	0.140	0.360			
Combined Ranges										
[Cyn] _L and [Plr] _L	14	0.286	0.049	.434	0.010					
[Cyn] _H and [Plr] _H	16	0.004	0.813	0.022	0.586					

Table 3.2 Anthocyanidin content of standard solutions and mixtures before and after cold storage.

Components consisted of standard solutions of cyanidin (Cyn) and pelargonidin (Plr) and mixtures of cyanidin and pelargonidin. HPLC results of concentration of fresh solutions (Conc_o) and of solutions following one week of cold storage (Conc_a). HPLC results represented as concentration of µg anthocyanidin per ml acidified methanol (0.01% HCl).

Data used in t test		
Standard Solutions or Mixtures	Conc _o (µg/ml)	Conc _a (µg/ml)
<u>Cold Storage at -20°C</u>		
[Cyn]	5.5	6.7
[Cyn]	23.9	27.6
[Cyn]	48.8	57.3
[Cyn]	76.7	85.7
[Plr]	8.7	9.6
[Plr]	26.5	30.2
[Plr]	63.3	72.8
[Plr]	97.7	103.6
<u>Cold Storage at -80°C</u>		
[Cyn] in [Cyn] _o =5.7:[Plr] _o =7.7	5.7	5.8
[Cyn] in [Cyn] _o =51.6:[Plr] _o =9.0	51.6	49.5
[Cyn] in [Cyn] _o =7.2:[Plr] _o =54.3	7.2	7.6
[Cyn] in [Cyn] _o =51.9:[Plr] _o =56.2	51.9	51.3
[Plr] in [Cyn] _o =5.7:[Plr] _o =7.7	7.7	7.4
[Plr] in [Cyn] _o =51.6:[Plr] _o =9.0	9.0	8.0
[Plr] in [Cyn] _o =7.2:[Plr] _o =54.3	54.3	51.8
[Plr] in [Cyn] _o =51.9:[Plr] _o =56.2	56.3	50.4
t test : Conc _o =Conc _a , P = 0.107

Figure 3.4a-c Stability of anthocyanidin standard solutions and mixtures following cold storage.

Concentrations (µg/ml) were measured at the indicated time points over a 26 hour period. Initial concentrations are indicated as cyanidin [Cyn]₀, pelargonidin [Plr]₀, and anthocyanins [Anth]₀. The freshly made standard solutions and mixtures were cold stored and then assayed at room temperature. Both solutions and mixtures were stored for one week prior to analysis; standard solutions of cyanidin or pelargonidin were stored at -80°C, while mixtures containing both cyanidin and pelargonidin were stored at -20°C.

Figure 3.5 Stability standard solutions (Cyn and Plr) at room temperature following cold storage.

Two freshly made standard solutions were stored 1) at -20°C for six weeks or 2) at -80°C for one week, and then compared at room temperature over twenty-four hours.

Cultivar 'Jewel' fruit anthocyanidin extract was stable in storage at -80°C over a three month test period, with respective average cyanidin and pelargonidin contents of 33.6 and 683.0 $\mu\text{g/g}$ including two outliers (Table 3.3 and Figure 3.6), and was used for all Phase 3 assays as the external standard.

Table 3.3 Anthocyanidin content of cultivar 'Jewel' extract.
Extract used as an external standard for three months.

'Jewel' Extract	Number of Analyses	Anthocyanidin (µg/g FDW)				
		Means		SE		
		Cyn	Plr	Cyn	Plr	
Overall	15	33.6	683	0.5	12.5	
ANOVA						
Dependent Variable	Source	df	SS	MS	F-ratio	P
<u>Cyn</u>	Date	12	39.1	3.26	1.45	0.480
	Error	2	4.5	2.25		
<u>Plr</u>	Date	12	31460	2622	4.2	0.208
	Error	2	1250	625

Figure 3.6: HPLC 'Jewel' cultivar external standard.

HPLC results (µg/g FDW) of anthocyanidins extract of fruit from 'Jewel' cultivar used as an external standard. The external standard consisted of 100 µl samples taken from extract aliquots stored at -80°C for three months.

Phases of Investigation

Anthocyanidin Levels in Greenhouse and Field Fruit. In order to validate pooling of greenhouse (GH) and field (HF) fruit in the comparison of data obtained using the different Phase protocols, fruit from nine accessions (Table C.1a and C.1b) obtained from both the GH and HF and analyzed using Phase 3 protocol were compared retrospectively. The average cyanidin content in the (GH) fruit was higher than in the (HF) fruit, 115 µg/g compared to 107 µg/g respectively, but the difference was not significant, $P=0.545$ (Table 3.4 and Figure 3.7). The average pelargonidin content in fruit collected from the GH (559 µg/g) were also higher when compared to fruit collected from the HF (371 µg/g), and the difference was significant, $P=0.004^{**}$. The data consisted of thirty-four data points, twelve from the GH and twenty-two from the HF. From each environment, four B-PP accessions, four Supercore Collection accessions, and

Table 3.4 Anthocyanidin content in greenhouse and horticultural field fruit.
The same protocol was used on ripe fruit accessions from both the greenhouse (GH) and the horticultural field (HF). The mean represents the mean of the means of each accession and the SE = $\sqrt{ME/Number\ of\ Samples}$. The accession sample data used in the statistical analysis is provided in Table C.1.

Location	Number of		Anthocyanin ($\mu\text{g/g FDW}$)			
	Samples	Accessions	Mean		SE	
			[Cyn]	[Plr]	[Cyn]	[Plr]
GH	12	9	115	559		
HF	22	9	107	371		
Overall	34	18	111	465		

Greenhouse versus Field Fruit ANOVA						
Dependent Variable	Source	df	SS	MS	F-ratio	P
[Cyn]	Location	1	363	363	0.382	0.545
	Accession	8	108305	13538	14.234	0.000
	Loc $\times$ Accession	8	3816	477	0.501	0.838
	Error	16	15218	951		
[Plr]	Location	1	221816	221816	11.141	0.004**
	Accession	9	789171	98646	4.954	0.003
	Loc $\times$ Accession	9	258363	32295	1.622	0.195
	Error	16	318570	19911		
[Cyn]/([Cyn]+[Plr])	Location	1	0.004	0.004	1.799	0.199
	Accession	9	0.696	0.087	41.771	0.000
	Loc $\times$ Accession	9	0.064	0.008	3.843	0.011
	Error	16	0.033	0.002

Figure 3.7 Comparison of anthocyanidin content in greenhouse and field fruit.

Mean values $\mu\text{g/g FDW} \pm \text{SE}$ (N=10) of fruit harvested from the two cultural methods. The differences between fruit harvested from the GH and HF were not significant for (P=0.509) cyanidin, and significant P=0.004** for pelargonidin).

one cultivar accession, were tested under the Phase 3 conditions. The individual accessions responded similarly to the cultural method, GH or HF, in both cyanidin ($P=0.838$) and pelargonidin content ($P=0.195$). Retrospectively, the data from the GH and HF data were pooled in the ANOVAs of fruit anthocyanidin content, notwithstanding the significant difference in pelargonidin content between the two cultural methods. The rationale for pooling the data was that 1) the difference in cyanidin content between GH and HF collected fruit samples were not significant, 2) the variability in the cyanidin content were much less than that of the pelargonidin content, and 3) the large variability in pelargonidin may be typical as found in the degree of ripeness study to be described following this Phases of Investigation section.

HPLC Results with Phase 1 and Phase 2 Methods. Anthocyanidin results using sample volumes of 20 μl (Phase 1) compared to 5 μl (Phase 2) injections differed significantly ($P=0.000^{**}$ and $P=0.002^{**}$) for both cyanidin and pelargonidin contents respectively (Table 3.5 and Figure 3.8). In Phase 2, with the 5 μl injections, cyanidin and pelargonidin contents ($\mu\text{g/g}$ FDW) averaged, respectively 44 and 250 $\mu\text{g/g}$ FDW compared to 16 and 100 $\mu\text{g/g}$ FDW (Table 3.5), with the 20 μl injections (Phase 1). The data consisted of sixty data points, sixteen from the Phase 1 and forty-four from Phase 2 (Table C.2). From each phase, thirteen B-PP accessions were tested. All Phase 1 replicates were greenhouse fruit and all Phase 2 replicates were field fruit. In addition to the specific injection volume change in going from Phase 1 to Phase 2 protocol, during Phase 2 the fruit was ground using a mortar and pestle to expose more

tissue to the anthocyanidin extraction and the time required to complete the samples was reduced by about one-half thus reducing the time and temperature exposure of the samples and resultant decomposition. These factors are more difficult to quantify, but combine to improve the effectiveness of the Phase 2 protocol.

HPLC Results with Phase 2 and Phase 3 Methods. A new centrifuge obtained during this study permitted the extraction of pigments from hydrolyzed fruit at 15,500 xg (Phase 3) versus the original 1,200 xg force (Phase 1 and Phase 2), which resulted in significantly different cyanidin and pelargonidin ($P=000^{**}$) contents (Table 3.6 and Figure 3.9). The data consisted of thirty-five data points, twenty-three from the Phase 2 and twelve from Phase 3 (Table C.3). From each phase, five B-PP and two cultivar accessions were tested. All of the samples were horticultural farm fruit. Of the replicated samples, five were B-PP, and two were cultivars. In addition to a new centrifuge obtained in Phase 3, the hydrolyzed samples were filtered to recover about twice the amount of pigment and the duration of sample preparation protocol was reduced by about an additional 25%, thus reducing again the time temperature exposure of the samples.

Application of normalization factors. In the assay of cyanidin and pelargonidin contents respectively, the Phase 2 protocol was 2.75 and 2.50 times more effective than the Phase 1 protocol (Table 3.7). Phase 3 was 1.95 and 2.08 times (Table 3.7) more effective in the extraction of cyanidin and

Table 3.5 Anthocyanidin results with Phase 1 and Phase 2 methods.

HPLC sample volume injections were 20µl (Phase 1) and 5 µl (Phase 2). Samples were centrifuged at 1,200 xg in both Phase 1 and Phase 2. The mean is the mean of the means of each accession. $SE = \sqrt{ME/Number\ of\ Samples}$. The accession sample data used in the statistical analysis is provided in Appendix C, Table C.2.

Phase (Injection Volume) (µl)	Number of		Anthocyanin (µg/g FDW)			
			Mean		SE	
	Samples	Accessions	Cyn	Plr	Cyn	Plr
Phase 1 (20)	16	16	16	100	4.1	37
Phase 2 (5)	44	16	44	250	2.5	22
Overall	60	32	30	175	2.1	19

Phase 1 and Phase 2 ANOVA						
Dependent Variable	Source	df	SS	MS	F-ratio	P
[Cyn]	Phase	1	8983	8983	32.989	0.000**
	Accession	15	5233	349	1.281	0.277
	Phase × Accession	15	1812	121	1.281	0.950
	Error	28	7625	272	0.443	
[Plr]	Phase	1	248716		11.303	0.002**
	Accession	15	744277	49618	2.255	0.031*
	Phase × Accession	15	120573	8038	0.365	0.978
	Error	28	616178	22006		
[Cyn]/([Cyn]+[Plr])	Phase	1	0.001	0.001	0.523	0.476
	Accession	15	0.181	0.012	4.243	0.001
	Phase × Accession	15	0.122	0.008	2.853	0.009
	Error	28	0.077	0.003

Figure 3.8 Anthocyanidin results with Phase 1 and Phase 2 methods. Cyanidin and pelargonidin (µg/g FDW ± SE) results were significantly different ($P=0.000^{**}$ and $P=0.002^{**}$ respectively) with 20 µl (Phase 1) and 5 µl (Phase 2) injections.

Table 3.6 Anthocyanidin results with Phase 2 and Phase 3 methods.

HPLC results of ripe fruit accessions using Phase 2 protocol (1,200 xg) were compared to ripe fruit of the same accessions using Phase 3 (15,500 xg). The represents the mean of the means of each accession and the $SE = \sqrt{ME/Number\ of\ Samples}$. The accession sample data used in the statistical analysis is provided in Appendix C, Table C.3.

Phase (Centrifuge) (xg)	Number of		Anthocyanin ($\mu\text{g/g FDW}$)			
			Mean		SE	
	Samples	Accessions	Cyn	Plr	Cyn	Plr
Phase 2	23	7	56	289	4	37
Phase 3	12	7	109	600	6	51
Overall	35	7	76	426	3	30

Phase 2 and Phase 3 ANOVA

Dependent Variable	Source	df	SS	MS	F-ratio	P
[Cyn]	Phase	1	18312	18312	46.316	0.000**
	Accession	6	17265	2878	7.278	0.000**
	Phase $\times$ Accession	6	4249	708	1.791	0.150
	Error	21	8303	395		
[Plr]	Phase	1	628055	628055	20.459	0.000**
	Accession	6	88293	147132	4.793	0.003**
	Phase $\times$ Accession	6	320920	53487	1.742	0.160
	Error	21	644652	30698		
[Cyn]/([Cyn]+[Plr])	Phase	1	0.002	0.002	0.552	0.466
	Accession	6	0.068	0.011	3.658	0.012*
	Phase $\times$ Accession	6	0.021	0.003	1.110	0.390
	Error	21	0.065	0.003

Figure 3.9 Anthocyanidin results with Phase 2 and Phase 3 methods.

Cyanidin and pelargonidin ($\mu\text{g/g FDW} \pm SE$) results were significantly different for sample preparations made with centrifugal forces, at 1,200 xg (Phase 2) and 15,500 xg (Phase 3), ($P=0.000^{**}$).

pelargonidin respectively from the fruit compared to Phase 2. A conservative normalization factor of 1.90 was applied to both the cyanidin and pelargonidin results from Phase 2 to allow pooling of the Phase 2 and Phase 3 data. In order to pool the Phase 1 and Phase 3 data in the ANOVAs, an overall factor of 5.1 [(the application of the 2.7 factor (Phase 1 to Phase 2) multiplied by the 1.9 normalization factor (Phase 2 to Phase 3)] was applied. Data affected by the normalization factors (Table 3.7) included sixteen out of the eighteen diploid samples (12 from Phase 1 and 4 from Phase 2), none of the Supercore Collection samples, ten out of the seventy-nine octoploid cultivars samples (none from Phase 1 and 10 from Phase 2), and ninety-one of the one hundred and fifty B-PP samples (34 from Phase 1 and 71 from Phase 2).

Anthocyanidin Content in Fruit at Different Degrees of Ripeness. In a pattern that applied across the germplasm (Table 3.8 and Figure 3.10), the cyanidin content was relatively constant ($P=0.971$) and independent of degree of ripeness, while pelargonidin content continued to increase with increasing degree of ripeness treatment ($P=0.023^*$). Each treatment included replicates of accessions including three accessions from the Supercore Collection (one *F. chiloensis*, and two *F. virginiana*), four cultivars, and one B-PP accession. Because the cyanidin and pelargonidin did not respond in the same manner to increasing ripeness, and because the pelargonidin varied in the GH and HF comparison, the absolute contents of cyanidin and pelargonidin were used in the ANOVA instead of a calculated ratio of cyanidin to pelargonidin as used in the early literature (Sondheimer and Karash, 1956).

Table 3.7 Sample data affected by the three different protocols (Phases 1, 2 and 3) and summary of factors applied to the data.

Cyanidin and pelargonidin contents of Phase 1 and Phase 2 data normalized to Phase 3 data, in order to pool data in the ANOVAs. The number of accessions represented in each Phase is indicated in (parenthesis) following the number, N, of fruit samples. The mean represents the mean of the means of each accession and the SE = $\sqrt{ME/Number\ of\ Samples}$.

Sample data distribution:	Phase			Total
	1	2	3	
-Centrifuge force, xg	1,200	1,200	15,500	
-Sample injection volume, µl	20	5	5	
Number of Samples: N				
-Diploids	12 (10)	4 (4)	2 (2)	18 (14)
-Supercore Collection			53 (16)	53 (16)
- Octoploid Cultivars		10 (4)	69 (12)	79 (12)
- B-PP including B and PP	34 (32)	57 (22)	59 (27)	150 (45)
<u>Total number of samples</u>	46 (42)	71 (30)	183 (57)	300 (87)
-Ripening Study			67 (19)	67 (19)

Comparison of data with method	Number of		Anthocyanin (µg/g FDW)			
			Mean		SE	
	Samples	Accessions	Cyn	Plr	Cyn	Plr
Phase 1	16	16	16	100	4.1	37
Phase 2	46	16	44	250	2.5	22
Phase 2/Phase 1 ratio			2.75	2.50		
Phase 1 and 2, P=			0.000**	0.002**	significant	
Phase 2	23	7	56	289	4	37
Phase 3	12	7	109	600	6	51
Phase 3/Phase 2 ratio			1.95	2.08		
Phase 2 and 3, P=			0.000**	0.000**	significant	

Summary of ratios= normalization factors	Cyn	Plr	Applied Cyn & Plr factor	Normalization Factor to Phase 3
Phase 2/Phase 1 ratio	2.75	2.50	2.70	5.1
Phase 3/Phase 2 ratio	1.95	2.08	1.90	1.9

Normalization factor: Normalization factor x data at Phase 1 or Phase 2 conditions =
Results normalized to Phase 3 conditions

Table 3.8 Anthocyanidin content in fruit with increasing degrees of ripeness.

Germplasm included three groups: Supercores (FRA372.002, 1408.001, and 1695.002), cultivars (Delmarvel, Jewel, Seneca, and Sparkle), and only one B-PP (B-PP-59). The three degrees of ripeness were under-ripe (UR), ripe (R), and over-ripe (OR). Anthocyanidin reported as mean values $\pm$ SE ($\mu\text{g/g}$ FDW), where $\text{SE} = \sqrt{\text{ME} / \text{N}}$, ME=Mean square for error of data set, and N=number of samples at each level of ripeness of each group.

Group	Degree of Ripeness	N	Anthocyanin ($\mu\text{g/g}$ FDW)			
			Means		SE	
			Cyn	Plr	Cyn	Plr
Supercores	UR	4	330	224	62	137
	R	4	296	445	62	137
	OR	3	199	444	72	158
Cultivars	UR	6	46	164	51	112
	R	7	54	538	47	103
	OR	8	67	843	44	97
B-PP	UR	1	101	212	124	273
	R	1	111	373	124	273
	OR	1	161	680	124	273
Overall	UR	11	155	190	37	82
	R	12	139	494	36	79
	OR	12	108	730	36	79

Degree of Ripeness ANOVA (N = 35)

Dependent Variable	Source	df	SS	MS	F-ratio	p
<u>Cyn</u>	Ripeness	2	916	458	0.03	0.971
	Group	2	341610	170805	11.06	0.000
	Ripeness $\times$ Group	4	31564	7891	0.51	0.728
	Error	26	401523	15443		
<u>Plr</u>	Ripeness	2	653202	326601	4.38	0.023
	Group	2	152693	76347	1.02	0.374
	Ripeness $\times$ Group	4	258045	64511	0.86	0.498
	Error	26	1941127	74659

Figure 3.10 Anthocyanidin content in under-ripe, ripe, and over-ripe fruit.

Cyanidin and pelargonidin expressed as mean values $\pm$ SE ($\mu\text{g/g}$ FDW), and the ratio of cyanidin to total anthocyanidins in under-ripe (UR), ripe (R), and over-ripe (OR) fruit. Accessions include: Supercore Collection FRA372.002, 1408.001, and 1695.002, Cultivars (Delmarvel, Jewel, Seneca, and Sparkle), and one B-PP (B-PP-59). The $SE = \sqrt{ME/N}$ and N =number of samples at each level of ripeness of each group. The three germplasm groups responded similarly to increasing ripeness ($P=0.728$ for cyanidin, $P=0.498$ for pelargonidin). Cyanidin content in under-ripe, ripe and over-ripe fruit do not differ ($P=0.971$). Overall pelargonidin content in ripe and over-ripe fruit differ from under-ripe fruit (means without a common letter (a and b) differ significantly at $P=0.05$). The ratio of [Cyn] to [Cyn] and [Plr] show a decrease in the ratio with increasing degree of ripeness.

Anthocyanidin Content in Fruit

Fragaria Diploids

Most of the diploid fruit samples (12 of the 18 samples) were assayed using the Phase 1 protocol (Table 3.9). The cyanidin and pelargonidin values in the accessions are provided with the normalization factors (Figure 3.11a) and without the normalization factors (Figure 3.11b). The ANOVA was completed with the data normalized to Phase 3 according to Table 3.7. The ANOVA of the diploid accession data showed significant variability in cyanidin values ($P=0.014^*$), but the variability in pelargonidin values ($P=0.258$) was not statistically significant (Table 3.9 and Figure 3.11). Replicate sampling was lacking with eight of the thirteen accessions and limited to two samples for the five remaining accessions. A wide range of ratios of cyanidin to total anthocyanidin and of anthocyanidin values ($\mu\text{g/g FDW} \pm \text{SE}$) was observed among the *Fragaria* diploids. The [Cyn] to [Plr] ratio varied from 0.56 to 19.28 and the [Cyn] to [Anth] ratio varied from 0.359 to 0.951. A reported [Cyn]:([Cyn]+[Plr]) ratio ≥ 0.5 is equivalent to a ratio of [Cyn]:[Plr] ≥ 1.0 . The ratio of [Cyn] to [Anth] exceeded 0.5 for most accessions (10 out of 12 red fruited accessions). No anthocyanidins were detected in the yellow-fruited 'YW' accession samples. In the red-fruited accessions, the cyanidin content values ranged from 122 $\mu\text{g/g}$ in *F. vesca* 'BS' to 2805 $\mu\text{g/g}$ in *F. viridis* F₁D, with an overall average of 792 ± 86 $\mu\text{g/g}$. The pelargonidin content values ranged from 109 $\mu\text{g/g}$ in *F. vesca* $\times$ *F. viridis* 'BQS37' to 912 $\mu\text{g/g}$ in *F. vesca* spp. *americana* WC8 with an average of 425 ± 72 $\mu\text{g/g}$.

Table 3.9 Anthocyanidin content in *Fragaria* diploids.

The [Cyn] and [Plr] and ratio of [Cyn] to [Cyn] and [Plr] along with the number of samples (N) for each accession is provided. The accession mean represents the mean of the accession means including BQS Yellow. The data is shown with Phase normalization factors applied and Phase 1 data is highlighted **in bold and underlined**.

Accessions		N	Anthocyanidin (µg/g FDW)			
			[Cyn]	[Plr]	[Cyn]/ [Plr]	[Cyn]/ [Anth]
<i>F. vesca</i>						
BS		2	<u>328</u>	<u>447</u>	0.73	0.423
			122	195	0.62	0.384
WC8		1	<u>2111</u>	<u>912</u>	2.31	0.698
YW × 520F1C		1	<u>222</u>	<u>158</u>	1.40	0.583
<i>F. vesca</i> × <i>F. viridis</i> (YW × CFRA364)						
BQS37		1	<u>147</u>	<u>109</u>	1.35	0.574
BQS4		1	211	378	0.56	0.359
BQS9		1	325	242	1.34	0.573
BQS22		2	<u>423</u>	<u>274</u>	1.18	0.607
			1132	960	1.54	0.541
BQS3		2	<u>769</u>	<u>447</u>	1.72	0.632
			<u>1398</u>	<u>785</u>	1.78	0.640
BQS32		2	1093	961	1.53	0.532
			<u>1231</u>	<u>806</u>	1.14	0.605
BQS31		1	<u>1335</u>	<u>509</u>	2.62	0.724
BQS Yellow		2	<u>0</u>	<u>0</u>	NA	
			<u>0</u>	<u>0</u>	NA	
<i>F. viridis</i>						
F ₁ D		1	2805	146	19.28	0.951
<i>F. iinumae</i>						
J-17		1	<u>599</u>	<u>320</u>	1.87	0.652
Overall Accession Mean			<u>792</u>	<u>425</u>		0.591
Overall Standard Error			<u>73</u>	<u>61</u>		0.007
ANOVA (N = 18)						
Dependent Variable	Source	df	SS	MS	F-ratio	P
[Cyn]	Accessions	12	9762273	813523	8.48	0.014
	Error	5	479901	95980		
[Plr]	Accessions	12	1489950	124162	1.85	0.258
	Error	5	336184	67237		
[Cyn]/[Anth]	Accessions	11	0.294	0.027	18.59	0.006
	Error	4	0.006	0.001

Figure 3.11 Anthocyanidin content in ripe fruit of *Fragaria* diploids. Cyanidin and pelargonidin values ($\mu\text{g/g}$ FDW) show variability in cyanidin and insignificant variability in pelargonidin. Diploid *Potentilla nepalensis* flower petals contained only cyanidin, and were included for comparison. The accession data which include Phase 1 results are prefaced with an asterisk (*). Normalization factors that were applied to Phase 1 and Phase 2 data in Figure 3.11a were not applied to Figure 3.11b.

In the accessions of the BQS progeny population with red fruit, the cyanidin values ranged from 147 to 1290 $\mu\text{g/g}$, and pelargonidin values ranged from 109 to 884 $\mu\text{g/g}$. When the statistical analysis was limited to the BQS

progeny population, the differences between the accessions in both cyanidin and pelargonidin values were not significant ($P > 0.05$), while in a Tukey® multiple pairwise comparison (not shown), the difference in ratio of cyanidin to total anthocyanins was only significant ($P = 0.044^*$) between accessions BQS4 and BQS31.

Octoploid Supercore Collection of *Fragaria* Wild Species

Fruit was produced and harvested from at least one accession each of the four *F. chiloensis* subspecies, and three of the five *F. virginiana* subspecies groups. Neither of the two *F. virginiana* ssp. *platypetala* accessions or the two ssp. *glauca* accessions produced any fruit. *F. virginiana* are subdivided as groups rather than subspecies because one of the groups consists of three accessions which are putative hybrids of *F. virginiana* of ssp. *glauca* x ssp. *virginiana*. The inventory ID numbers of the accessions that produced fruit suitable for analyses are underlined and highlighted in bold (Table 2.2). Although inconsistent fruit production resulted in uneven replication, there were at least two replicates for twelve of the sixteen fruiting accessions.

The analysis of the Supercore Collection anthocyanidin content ($\mu\text{g/g}$ FDW $\pm$ SE) indicated significant variability in both cyanidin and pelargonidin contents (Table 3.10 and Figure 3.12). At the level of species, cyanidin content was significantly higher in *F. chiloensis* (270 $\mu\text{g/g}$) than in *F. virginiana* (146 $\mu\text{g/g}$), whereas pelargonidin content was significantly lower in *F. chiloensis* (189 $\mu\text{g/g}$) than in *F. virginiana* (485 $\mu\text{g/g}$) (Table 3.10). However, total anthocyanidins was not significantly different between the two species. In a

Tukey® pairwise comparison (Table 3.11) *F. chiloensis* ssp. *lucida*, had a significantly higher average cyanidin content (592 µg/g), when compared with any of the other subspecies groups. The *F. chiloensis* ssp. *lucida*, FRA1691 accession from Oregon, contained the most cyanidin, with an average content of 707 µg/g.

In comparisons among groups, *F. virginiana* ssp. *virginiana* (North) had the highest pelargonidin value (671 µg/g), but in pairwise comparisons was only significantly higher than *F. virginiana* ssp. *virginiana* grayana (339 µg/g), and *F. chiloensis* ssp. *chiloensis* f. *chiloensis* (133 µg/g) and *F. chiloensis* ssp. *lucida* (151 µg/g) (Table 3.11). *F. chiloensis* ssp. *lucida* from Oregon and *F. virginiana* ssp. *virginiana* (North) contained equivalent amounts of total anthocyanins due to inversely high and low cyanidin and pelargonidin contents.

The variability in cyanidin and pelargonidin contents in the Supercore Collection was to an extent observable in the fruit color. The red color of the fruit of *Fragaria chiloensis* ssp. *lucida* from Oregon (Figure 3.13) was particularly distinct with noticeable bluish/purplish hue and contained the most cyanidin (Figure 3.12).

Table 3.10 Octoploid species Supercore Collection ripe fruit anthocyanidin content.

Germplasm collection includes USDA selected subspecies of *F. chiloensis* and *F. virginiana*. "Origin" refers to original accession collection site. The shaded or boxed data are N totals and means for grouped species or subspecies. Separate ANOVAs were conducted on the individual accessions and on the species and subspecies groups. All Supercore samples were assayed in Phase 3. Individual sample data is provided in Table C.4.

			Anthocyanidin (µg/g FDW)				
Species and subspecies groups	ORIGIN	FRA#	Mean			SE	
			N	Cyn	Plr	Cyn	Plr
<u><i>F. chiloensis</i> ssp.</u>			11	270	189	27	70
<i>pacifica</i>	Arkansas	368	4	91	249	45	115
<i>lucida</i>			3	592	151	52	133
	Washington	42	1	362	52	91	231
	Oregon	1691	2	707	201	64	163
<i>chiloensis</i> f. <i>chiloensis</i>	Peru	372	3	220	133	52	133
<i>chiloensis</i> f. <i>patagonica</i>	Chile	1100	1	167	233	91	231
<u><i>F. virginiana</i> ssp.</u>			42	146	485	14	36
<i>virginiana</i> (North)			17	189	671	22	56
	Ontario	1694	1	165	749	91	231
	Ontario	1699	4	189	835	45	115
	Ontario	1695	5	293	661	41	103
	Minnesota	1701	5	68	454	41	103
	Minnesota	1700	2	245	871	64	163
<i>virginiana</i> (South)			8	122	365	32	82
	South Carolina	1580	5	106	252	41	103
	Georgia	1455	2	112	550	64	163
	Florida	1435	1	222	561	91	231
<i>glauca</i> x <i>virginiana</i>	Montana	1697	7	115	379	34	87
<i>grayana</i>			10	112	339	29	73
	Mississippi	1414	3	77	227	52	133
	Mississippi	1408	7	128	387	34	87
Overall			53	172	424	12	32

Table 3.10 continued.

All Supercore Accessions ANOVA (N = 53)						
Dependent Variable	Source	df	SS	MS	F-ratio	P
<u>Cyn</u>	Accessions	19	909362	47861	5.82	0.000**
	Error	33	271344	8223		
<u>Pir</u>	Accessions	19	2905912	152943	2.87	0.004**
	Error	33	1757137	53247		
<u>Anth</u>	Accessions	19	4158830	218886	2.56	0.009**
	Error	33	2826726	85658		
Species and Subspecies (SSP) Groups ANOVA (N = 53)						
Dependent Variable	Source	df	SS	MS	F-ratio	P
<u>Cyn</u>	Species	1	120193	120193	10.09	0.003**
	SSP (Species)	6	511025	85171	7.15	0.000**
	Error	45	536073	11913		
<u>Pir</u>	Species	1	416921	416921	6.525	0.014*
	SSP (Species)	6	1023900	170650	2.671	0.027*
	Error	45	2875525	63901		
<u>Anth</u>	Species	1	89388	89388	0.824	0.369
	SSP (Species)	6	1846570	307762	2.838	0.020*
	Error	45	4880714	108460

Figure 3.12 Anthocyanidin content in ripe fruit of Supercore Collection *Fragaria chiloensis* and *F. virginiana* subspecies.

Cyanidin and pelargonidin contents reported ($\mu\text{g/g}$ FDW $\pm$ SE) in fruit collected from plants at UNH horticultural farm and greenhouses. At the subspecies level in terms of cyanidin content, *F. chiloensis* ssp. *lucida* significantly different ($P=0.000^{**}$ to 0.031^{*}) from any of the other subspecies with exception of *F. chiloensis* ssp. *pacifica*. In terms of pelargonidin content, *F. virginiana* ssp. *virginiana* (N) differed significantly when compared to *F. chiloensis* ssp. *lucida* ($P=0.038^{*}$) and ssp. *chiloensis* f. *chiloensis* ($P=0.028^{*}$) and also with *F. virginiana* ssp. *grayana* ($P=0.037^{*}$).

Table 3.11 Supercore Collection Tukey® multiple subspecies pairwise comparison of cyanidin and pelargonidin contents in ripe fruit.

The number in the column headings refer to the same species and subspecies as numbered in the rows. Significant differences are underlined and bold. The mean cyanidin and pelargonidin contents for each subspecies are given for ease of comparison. P values are provided in the main body of the table.

Cyanidin	Mean	1	2	3	4	5	6	7	8
<u><i>F. chiloensis</i> subspecies</u>									
1. <i>chiloensis</i> f. <i>chiloensis</i>	220	1.000							
2. <i>chiloensis</i> f. <i>patagonica</i>	167	1.000	1.000						
3. <i>lucida</i>	592	<u>0.003</u>	<u>0.031</u>	1.000					
4. <i>pacifica</i>	91	0.778	0.998	<u>0.000</u>	1.000				
<u><i>F. virginiana</i> subspecies</u>									
5. <i>glauca</i> x <i>virginiana</i>	115	0.857	1.000	<u>0.000</u>	1.000	1.000			
6. <i>virginiana</i> (S)	122	0.883	1.000	<u>0.000</u>	1.000	1.000	1.000		
7. <i>virginiana</i> (N)	189	1.000	1.000	<u>0.000</u>	0.736	0.798	0.833	1.000	
8. <i>grayana</i>	112	0.805	1.000	<u>0.000</u>	1.000	1.000	1.000	0.646	1.000
Pelargonidin	Mean	1	2	3	4	5	6	7	8
<u><i>F. chiloensis</i> subspecies</u>									
1. <i>chiloensis</i> f. <i>chiloensis</i>	133	1.000							
2. <i>chiloensis</i> f. <i>patagonica</i>	233	1.000	1.000						
3. <i>lucida</i>	151	1.000	1.000	1.000					
4. <i>pacifica</i>	249	0.999	1.000	1.000	1.000				
<u><i>F. virginiana</i> subspecies</u>									
5. <i>glauca</i> x <i>virginiana</i>	379	0.848	0.999	0.893	0.991	1.000			
6. <i>virginiana</i> (S)	365	0.870	1.000	0.912	0.995	1.000	1.000		
7. <i>virginiana</i> (N)	671	<u>0.028</u>	0.697	<u>0.038</u>	0.076	0.192	0.116	1.000	
8. <i>grayana</i>	339	0.915	1.000	0.947	0.999	1.000	1.000	<u>0.037</u>	1.000

Figure 3.13 Supercore Collection flower and fruit variability.

Octoploid *Fragaria* × *ananassa* Cultivars

Fragaria × *ananassa* cultivars included nine short-day and four day-neutral varieties. Fruit set in these cultivars was sufficient to allow at least three replications per accession. The anthocyanidin (µg/g FDW) content found in the fruit from the cultivars harvested at peak ripeness varied significantly for cyanidin, but not for pelargonidin (Table 3.12a and Figure 3.14). Overall, the content of cyanidin in the cultivars averaged 60 µg/g, with 'Raritan' having the lowest (27 µg/g) and 'Seascape' having the highest (120 µg/g) cyanidin content. The cultivar 'Seascape' also had the highest cyanidin to total anthocyanidin ratio of 0.19, compared to an overall average ratio of 0.10 in the cultivars. Overall, the content of pelargonidin averaged 597 µg/g, and ranged from a low of 402 µg/g in 'Tribute' to a high of 761 µg/g in 'Tristar'. Day-neutral cultivars, as a group, contained significantly more cyanidin than the short-day cultivars (only because of 'Seascape'), while the pelargonidin contents were not significantly different. In a Tukey® pairwise comparison 'Seascape' was significantly different from the other cultivars in both cyanidin and ratio of cyanidin to total anthocyanidins (Table 3.12b).

Table 3.12a Octoploid *Fragaria* × *ananassa* ripe fruit anthocyanidin content. Varieties are organized by production day-length. Ripe fruit were harvested from plants maintained at UNH. Individual sample data along with Phase protocol in use is provided in Table C.5.

Day-length Preference	Variety	Anthocyanidin (µg/g FDW)					
		N	Cyn/ (Cyn+Plr)	Mean		SE	
				Cyn	Plr	Cyn	Plr
Short-day		55	0.08	52	589	4	33
Early	Northeaster	4	0.09	47	499	14	125
	Earliglow	5	0.09	62	588	13	112
Mid-Season	Raritan	5	0.05	27	532	13	112
	Delmarvel	3	0.06	43	727	16	144
	Honeoye	8	0.07	50	697	10	88
Mid-Late	Jewel	9	0.07	48	671	9	83
	Seneca	7	0.10	53	484	11	94
	Sparkle	9	0.13	74	512	9	83
Late	Winona	5	0.08	50	598	13	112
Day Neutral		24	0.11	77	617	7	50
	Tribute	7	0.10	45	402	11	94
	Tristar	13	0.10	81	761	8	69
	Seascape	4	0.19	120	524	14	125
	Overall	79	0.10	60	597	3	28

***F. ×ananassa* ANOVA (N = 79)**

Dependent Variable	Source	df	SS	MS	F-ratio	P
<u>Cyn</u>	Variety	11	33228	3021	3.86	0.00
	Error	67	52396	782		
<u>Plr</u>	Variety	11	1034694	94063	1.51	0.15
	Error	67	4186658	62487		
<u>Cyn:(Cyn+Plr)</u>	Variety	11	0.066	0.006	5.92	0.00
	Error	67	0.068	0.001		
<u>Cyn</u>	Day	1	10098	10098	10.296	0.002
	Error	77	75526	981		
<u>Plr</u>	Day	1	132263	132263	0.196	0.65
	Error	77	5208089	67638		

Table 3.12b Octoploid *Fragaria* × *ananassa* ripe fruit Tukey® pairwise comparison.

A comparison between cultivars of the cyanidin content and of the CCPRatio (ratio of cyanidin to total anthocyanidins) highlighting significant differences in **bold** and underlined. The column headings refer to the same cultivars as numbered in the rows.

Cyanidin	1	2	3	4	5	6	7	8	9	10	11	12
1 Delmarvel	1.000											
2 Earliglow	0.999	1.000										
3 Honeoye	1.000	1.000	1.000									
4 Jewel	1.000	0.999	1.000	1.000								
5 Northeast	1.000	1.000	1.000	1.000	1.000							
6 Raritan	1.000	0.713	0.951	0.965	0.996	1.000						
7 Seascape	<u>0.027</u>	0.101	0.006	0.003	<u>0.020</u>	0.000	1.000					
8 Seneca	1.000	1.000	1.000	1.000	1.000	0.903	<u>0.014</u>	1.000				
9 Sparkle	0.883	1.000	0.829	0.731	0.894	0.128	0.225	0.942	1.000			
10 Tribute	1.000	0.997	1.000	1.000	1.000	0.993	<u>0.003</u>	1.000	0.671	1.000		
11 Tristar	0.622	0.977	0.381	0.260	0.598	<u>0.023</u>	0.383	0.616	1.000	0.244	1.000	
12 Winona	1.000	1.000	1.000	1.000	1.000	0.971	<u>0.020</u>	1.000	0.936	1.000	0.653	1.000
CCPRatio	1	2	3	4	5	6	7	8	9	10	11	12
1 Delmarvel	1.000											
2 Earliglow	0.982	1.000										
3 Honeoye	1.000	0.976	1.000									
4 Jewel	1.000	0.929	1.000	1.000								
5 Northeast	0.999	1.000	0.999	0.997	1.000							
6 Raritan	1.000	0.671	0.998	1.000	0.915	1.000						
7 Seascape	<u>0.000</u>	<u>0.003</u>	<u>0.000</u>	<u>0.000</u>	<u>0.002</u>	<u>0.000</u>	1.000					
8 Seneca	0.883	1.000	0.771	0.602	1.000	0.313	<u>0.003</u>	1.000				
9 Sparkle	0.195	0.846	0.034	0.013	0.694	0.007	0.076	0.943	1.000			
10 Tribute	0.969	1.000	0.943	0.853	1.000	0.540	<u>0.001</u>	1.000	0.764	1.000		
11 Tristar	0.590	0.999	0.256	0.124	0.986	0.060	<u>0.003</u>	1.000	0.990	0.998	1.000	
12 Winona	1.000	0.999	1.000	1.000	1.000	0.987	<u>0.000</u>	0.979	0.262	0.999	0.768	1.000

Figure 3.14 Anthocyanidin content in ripe fruit of *Fragaria* × *ananassa* cultivars.

Cyanidin and pelargonidin contents (µg/g FDW ± SE) in fruit collected from plants at the UNH horticultural farm. In a Tukey® multiple pairwise comparison, cyanidin content was significantly variable at both the cultivar level ($P=0.000^{**}$) and at the day-length preference level ($P=0.002^{**}$). Pelargonidin content was not significantly variable at either the cultivar level ($P=0.150$) or at the level of day-length preference ($P=0.659$).

Octoploid 'Bountiful', 'Pink Panda' and B-PP Accessions

The fruit cyanidin content in 'Bountiful' (B) was 54 µg/g and in 'Pink Panda' (PP) was 305 µg/g, with respective pelargonidin content of 355 µg/g and 507 µg/g. Fruit set varied considerably but was sufficient to allow at least two replications for all but six of the F₁ plants. In the F₁ population of a cross between B and PP consisting of forty-three individuals (B-PP) accessions, both the cyanidin and pelargonidin contents varied significantly (Table 3.13 and Figure 3.15). The cyanidin content averaged 92 µg/g and varied from 32 to 259 µg/g, and the pelargonidin content averaged 493 µg/g and varied from 139 to 1688 µg/g.

Table 3.13 Octoploid B-PP accessions, ripe fruit anthocyanidin content.

B-PP accessions consist of an F₁ population, which resulted from a hybridization of 'Bountiful' (B), an *F. × ananassa* variety, and 'Pink Panda' (PP), an ornamental cross of *F. × ananassa* x *Potentilla palustris*. Ripe fruit harvested from plants at the UNH greenhouse and field. Individual sample data along with Phase protocol in use is provided in Table C.6.

Plant ID	N	Anthocyanidin (µg/g FDW)				
		Means			SE	
		Cyn	Plr	Cyn + Plr	Cyn	Plr
Bountiful	4	54	355	409	17	123
B-PP-072	2	32	269	301	24	175
B-PP-037	3	44	502	546	19	143
B-PP-112	4	45	162	207	17	123
B-PP-017	5	48	460	508	15	110
B-PP-057	1	49	146	195	33	247
B-PP-109	4	53	168	221	17	123
B-PP-200	3	53	239	291	19	143
B-PP-016	1	55	298	353	33	247
B-PP-201?	2	57	216	272	24	175
B-PP-110	3	58	139	196	19	143
B-PP-005	2	69	398	467	24	175
B-PP-064?	4	71	712	783	17	123
B-PP-103	3	71	438	509	19	143
B-PP-077	7	75	352	427	13	93
B-PP-105	6	77	504	581	14	101
B-PP-101	2	78	414	491	24	175
B-PP-102?	4	80	310	390	17	123
B-PP-059	2	83	382	464	24	175
B-PP-062	5	83	780	863	15	110
B-PP-044	2	83	357	440	24	175
B-PP-038	6	85	372	457	14	101
B-PP-024	8	85	622	706	12	87
B-PP-078	2	87	489	576	24	175
B-PP-104	3	91	295	386	19	143
B-PP-030	5	91	549	641	15	110
B-PP-106	3	92	727	820	19	143

Table 3.13 continued.

Plant ID	N	Anthocyanidin ($\mu\text{g/g}$ FDW)				
		Means			SE	
		Cyn	Plr	Cyn + Plr	Cyn	Plr
B-PP-069	4	93	248	340	17	123
B-PP-023	4	94	513	607	17	123
B-PP-108	1	98	174	272	33	247
B-PP-031	4	100	947	1047	17	123
B-PP-081?	1	100	186	286	33	247
B-PP-007	6	101	517	618	14	101
B-PP-107	2	104	360	464	24	175
B-PP-039	4	104	392	496	17	123
B-PP-036	1	107	772	879	33	247
B-PP-055	5	109	553	662	15	110
B-PP-009	4	125	647	772	17	123
B-PP-068	3	125	590	715	19	143
B-PP-111?	4	127	1688	1815	17	123
B-PP-070	2	128	423	551	24	175
B-PP-067	2	148	421	569	24	175
B-PP-047?	2	162	706	868	24	175
B-PP-003	1	259	659	918	33	247
Pink Panda	4	305	507	812	17	123
Overall	150	92	493		3	20
B-PP ANOVA (N = 150)						
Dependent Variable	Source	SS	df	MS	F-ratio	P
<u>Cyn</u>	BPP	312991	44	7113	6.36	0.000**
	Error	117440	105	1118		
<u>Plr</u>	BPP	1.08E+07	44	246300	4.042	0.000**
	Error	6.E+06	105	60934		
<u>Cyn:(Cyn+Plr)</u>	BPP	0.709	44	0.016	4.915	0.000**
	Error	0.344	105	0.003

Figure 3.15 Anthocyanidin content in the ripe fruit of octoploid B-PP accessions.

Cyanidin and pelargonidin contents ($\mu\text{g/g FDW} \pm \text{SE}$) in fruit of 'Bountiful' (B) and 'Pink Panda' (PP) and of their F₁ population collected from plants at the UNH greenhouse and horticultural farm. The individual pairs of bars in Figure 3.15 represent cyanidin and pelargonidin levels in unique F₁ plants. As a group, there were significant differences in both cyanidin and pelargonidin contents ($P=0.000^{**}$)

CHAPTER 4

DISCUSSION

Overview

The analytical method for determining the contents of cyanidin and pelargonidin in strawberry fruit was developed incrementally after a literature search of currently practiced methods. As detailed in the methods section, alternate procedural options were considered at various stages of the process, and various criteria were employed as a basis for choosing the option to be employed. Among these criteria were consideration for the number of types and quantity of chemicals, the number of single use items, as well as the processing time and consideration of health, safety and the environment. In most instances, limitations on time and resources precluded systematic experimental comparisons among options, and the effort was focused simply on establishing an overall procedure that gave satisfactory and reproducible results. Overall, the final set of procedures outlined in the Materials and Methods chapter appeared to be effective, and these procedures are recommended for use in future investigations.

The methodology was developed in phases distinguished by modifications that were adopted due to changing circumstances. At the outset (Phase 1), a centrifuge with a maximum speed of 1200 xg was used to extract the pigments from the hydrolyzed samples, and these samples were assayed in 20 µl volumes.

A shift to 5 µl assay volumes during the optimization of the HPLC operation marked the transition to Phase 2, and a shift to use of a new centrifuge that permitted an RCF of 15500 xg marked the onset of Phase 3. Accessions that were sampled during at least two of the three phases were compared in order to develop factors that could be used to normalize and pool the data for ANOVA of the treatment groups. A comparison of accession data showed an increase in anthocyanidin recovery with each new phase. Both cyanidin and pelargonidin recovery results were significantly increased, by a factor of 2.7 in going from Phase 1 to Phase 2, and by a factor of 1.9 in going from Phase 2 to Phase 3, such that overall anthocyanidin content measurements were increased by a factor of 5.1 in going from Phase 1 to Phase 3 (Table 3.7). Fortunately, a large percentage of the samples (68%) was analyzed in Phase 3, with less (20%) analyzed in Phase 2 and even less (12%) in Phase 1.

The impact of the phased protocol development on the results for the germplasm is as follows. The results for the diploids were most affected: 65% were analyzed in Phase 1, 25% in Phase 2 and only 10% in Phase 3. All of the fruit from the Supercore Collection was analyzed in Phase 3, and so these results were unaffected by the phased development of the method. The cultivars were analyzed mostly in Phase 3 (87%), with only 13% analyzed in Phase 2. B-PP fruit was collected and analyzed during all three Phases (21% in Phase 1, 38% in Phase 2, and 41% in Phase 3). Therefore, it was the results from the B-PP accessions that provided the most opportunity to evaluate the impact of a shift in methods with the different Phases (Table 3.7).

Methods Development

Sample Preparation for Anthocyanidin Assay

The strawberry fruit was lyophilized for this study, and although no loss or change in color was visible, a prior study found that the total phenolic compounds in strawberry fruit were significantly reduced during freeze-drying compared to freezing at -20°C (Asami et al., 2003). It is possible that the anthocyanidin content of fruit were also reduced during the freeze-drying process used in the present investigation. However, it is likely that both cyanidin and pelargonidin would have been affected similarly, as they were in the stability studies performed on cyanidin and pelargonidin mixtures of standards solutions and on the 'Jewel' extract. Therefore it is assumed any losses incurred in freeze-drying should have affected both pigments similarly and should not have affected the comparisons among accessions that constitute the key conclusions of this study.

Activating the Sep-Pak® C18 cartridge with just the acidified methanol worked very well, as no color was observed in the rinse during the anthocyanidin binding or purification steps, in contrast to observations by (Rodriguez-Saona and Wrolstad, 2001) who included additional rinses and observed color in the rinses, suggesting loss of anthocyanidins. It is important for the cartridges to be thoroughly activated, as was discovered in the present study, and this cannot be overemphasized.

Although prior studies did not include a filtration step, filtering the anthocyanidin supernatant was very effective in preventing solids from plugging the Sep-Pak® C18 cartridge. The filtering also facilitated near perfect recovery

of the extract; only solids with no visible liquid remained in the centrifuge tube and filtering the supernatant is recommended in future investigations.

Controlling the HPLC at 30°C, just above normal range of room temperature, is recommended in future investigations, as the room temperature did tend to vary considerably. There was no evident need to control the column at a higher temperature such as the 40°C set by (Miyazawa et al., 1999) as evidenced by reproducible results with the 'Jewel' cultivar external standard (Table 3.3 and Figure 3.6).

Stability of Anthocyanidin Standard Solutions and Mixtures

The assay results of freshly made mixtures of cyanidin and pelargonidin at room temperature over an eleven hour period showed an increase [Cyn] and [Plr], with the exception of the mixture with $[Cyn]_0=51.9:[Plr]_0=9.0$ containing the highest concentration of cyanidin. These results suggest that two factors acted in opposing ways to affect the concentration results. The generally observed increase in concentration may have been the result of a loss of the sample solvent, methanol, due to evaporation. In the exceptional case, the observed decline in concentration may be explained by a higher degradation rate due to the higher concentration of cyanidin. Thus solvent evaporation may be a constant while solute degradation is a function of concentration.

The degradation of standards and mixtures at room temperature following cold storage was evident, thus the degradation rate was higher following cold storage compared to that of freshly made standards and mixtures and was not offset by any evaporation of the methanol. There was not any evidence of

precipitation of the pigments from the solution. The hypothesis that the evaporation of methanol is masking the degradation rate is supported by evident loss of concentration of the mixture with the highest contents of pelargonidin and cyanidin, as higher degradation rates with increasing concentrations are a typical chemical phenomenon.

The higher degradation rate at room temperature of the standards and mixtures following cold storage, which was more evident following storage at -80°C compared to storage at -20°C , could have been the result of a supercritical sublimation of the solvent at -80°C causing a more rapid degradation. A second comparison of two sets of standard solutions (Figure 3.5) of comparable concentration supports the hypothesis that the cold storage hastened the degradation of the standard solutions and mixtures and that the colder the storage, the higher was the degradation rate.

As a result of the stability studies, fruit samples continued to be analyzed immediately upon completion of sample preparation to assure maximum retention of cyanidin and pelargonidin contents of fruit. In addition, aliquots of cultivar 'Jewel' fruit anthocyanidin extract used as an external standard during Phase 3 were warmed from -80°C to room temperature for only 15 to 30 minutes prior to assay during each HPLC run. The 'Jewel' fruit anthocyanidin extracts were stable in cold storage over a three month test period with respect to their cyanidin and pelargonidin contents (Table 3.3 and Figure 3.6). The stability results of the 'Jewel' extract, as anthocyanidins (without sugars), agreed well with results with results with the individual anthocyanins, cyanidin-3-glucoside and

pelargonidin-3-glucoside reported in the literature, which were stable at a pH of 1-3, even after 60 days of storage, measured by UV max absorbance at 10°C (Cabrita et al., 2000).

Comparison of Greenhouse and Field Fruit

Fruit was collected from both greenhouse and field accessions in order to have fruit for sampling 1) early in the investigation, before any accessions were planted in the field 2) from accessions which rarely produce fruit, and 3) during the off-season. Collecting fruit from both the greenhouse and the field maximized the amount of fruit available for this investigation. The drawback to the combined sampling of field and greenhouse is that another variable was introduced, another potential source of error. Although the ANOVA comparison of greenhouse and field fruit does not show a significant difference between the two cultural systems, it would have been more desirable to have all fruit samples collected from the field in the same two seasons to minimize the sources of error and variability.

Effect of Degree of Ripeness

Although a sharp increase in total anthocyanidin content from underripe to overripe strawberry fruit was documented by others (Cheng and Breen, 1991), the absolute contents of the individual cyanidin and pelargonidin pigments were not measured in their study. In the present study, we observed that the pelargonidin content continued to increase with increasing degree of ripeness, but that cyanidin remained relatively constant. Therefore the individual cyanidin and pelargonidin contents were documented throughout the investigation, in

addition to calculating the cyanidin-to-pelargonidin ratio as initially intended based on the earliest reference of a 1:1 cyanidin to pelargonidin ratio in *F. vesca* (Sondheimer and Karash, 1956). Cyanidin and pelargonidin contents and ratios (Cyn to Plr and Cyn to total Anth) were determined for all sampled germplasm accessions.

Anthocyanidin Content in Fruit

Fragaria Diploids

This set of assays was done early in the investigation and the results were hampered by two factors 1) methodology was being developed and changed and 2) the opportunity for replication was limited by lack of fruit. As a consequence, even though the observed values varied over a wide range, few of the differences were statistically significant. Now that satisfactory methodology has been established it would be desirable to expand the replicate testing of diploids accessions in a future investigation. However, some important conclusions can be drawn from the existing data in relation to the goals of the investigation:

1) High cyanidin to pelargonidin ratios were found in the diploid *Fragaria* germplasm. The tested ratio expressed as $[Cyn]:[Cyn]+[Plr] \geq 0.5$ equivalent to $[Cyn]:[Plr] \geq 1.0$ was exceeded in all but two of the twelve surveyed red-fruited accessions. Of the diploids surveyed, only *Fragaria vesca* ssp. *vesca* BS and one of the *F. vesca* x *F. viridis* BQS accessions had $[Cyn]:[Plr] < 1.0$.

2) High cyanidin content were found in the diploid *Fragaria* germplasm, and a 1:1 $[Cyn]:[Plr]$ ratio was exceeded due to high cyanidin as well as

due to low pelargonidin. In *F. viridis*, the high cyanidin (2805 µg/g) and low pelargonidin (146 µg/g) sample were determined using the Phase 3 protocol and although there were no replicates, the high cyanidin content was consistent with the observation that the red fruit had a bluish/purplish hue. Although the absolute magnitude of the content is uncertain, the data indicate that *F. vesca* ssp. *americana* WC8 contained both high cyanidin and pelargonidin contents of 2111 µg/g and 912 µg/g respectively. The exact magnitude of the high contents of both pelargonidin and cyanidin is uncertain because the WC8 accession fruit sample was analyzed very early in the investigation with only one sample using Phase 1 protocol, and visual observations of fruit color were not photographically documented.

3) In the BQS population, the presence of yellow fruited accessions indicates segregation for anthocyanidin biosynthesis. A prior study suggested that yellow fruit color in *F. vesca* was due to a mutation in the F3H gene (Deng and Davis, 2001). Further, significant difference in cyanidin content between two accessions, BQS4 and BQS31, in a Tukey® multiple pairwise comparison, indicates possible segregation for cyanidin content, which could be ascertained with additional sampling.

Octoploid Supercore Collection of *Fragaria* Wild Species

This set of assays was completed later in the investigation (Phase 3) when the methodology had been developed and had become routine. Additionally, because Supercore accessions generally produced more fruit than

the diploids and the fact that the Supercore Collection was planted in the field, as well as being maintained in the greenhouse, two replicates samples were obtained for twelve of sixteen accessions, translating to at least three replicates for seven out of the eight subspecies that produced fruit.

The low cyanidin content found in *Fragaria virginiana* and in both of the South American *F. chiloensis* accessions (167 and 220 µg/g) surveyed combined with Chile being the well-documented provenance of *F. chiloensis* in *F. × ananassa* and aptly named 'White Chilean' (Dale et al., 2001) support the speculation that high cyanidin content was not lost in *F. × ananassa* but that high cyanidin capability was never introduced.

The results show that higher cyanidin content is present in some wild octoploid germplasm, specifically in *F. chiloensis* ssp. *lucida* (592 µg/g). The data suggest a possible opportunity for breeding higher cyanidin content by introgression of *F. chiloensis* ssp. *lucida* into the cultivated strawberry.

The results also suggest that the genetic determinants of high pelargonidin content in *F. × ananassa* came from *F. virginiana*, wherein pelargonidin content was significantly higher than in *F. chiloensis*. *Fragaria virginiana* generally contained low cyanidin and high pelargonidin contents, as is found in *F. × ananassa*. Therefore, the results also suggest that pelargonidin content could be further increased in *F. × ananassa* through introgression of *F. virginiana* ssp. *virginiana* from the North, which although not statistically significant, generally registered a higher pelargonidin content (671 µg/g) when

compared to the average (365 µg/g) in *F. virginiana* from the South, the reputed provenance of *F. virginiana* in *F. × ananassa*.

Octoploid *Fragaria × ananassa* Cultivars

This set of assays was completed late in the investigation when the methodology development had been completed and become routine; only ten out of seventy-nine total assays were completed during Phase 2, the rest were completed in Phase 3 (Table 3.7). Additionally, this data set contains the most replication, with at least three replicate samples for each accession. Replication was possible because the cultivars produced considerably more fruit than the wild octoploid germplasm and were planted in the field in four replicate plots. As a group, the day-neutrals contained significantly more cyanidin than the short-day cultivars, but this was primarily because of 'Seascape'. In the fruit of cultivars, the anthocyanidin content was comprised of 5% to 10% (by weight) cyanidin, in agreement with other studies (Kalt et al., 2001; Wang et al., 2003), with the exception of 'Seascape', which contained 19% cyanidin. The relatively high cyanidin content in 'Seascape' could be the result of the introgression of *F. virginiana* ssp. *glauca* in its development (Bringhurst, 1991). Although both accessions of *F. virginiana* ssp. *glauca* failed to produce any fruit and the putative hybrid *F. virginiana* ssp. *glauca* x ssp. *virginiana* accessions contained relatively low levels of cyanidin (Table 3.10), *F. virginiana* ssp. *glauca* might have contributed the relatively high cyanidin in 'Seascape'. Survey of fruit collected from the pedigree of 'Seascape' would help to definitively answer this question.

Octoploid 'Bountiful', 'Pink Panda', and B-PP Accessions

This set of assays was conducted throughout the course of this investigation (Table 3.7). Although, the B-PP accessions produced a variable amount of fruit, at a least two replicate samples were obtained from thirty-eight out of forty-three accessions. 'Bountiful', 'Pink Panda' and the B-PP accessions, the F_1 population, were planted in the field in addition to being maintained in the greenhouse. Significant variability in cyanidin and pelargonidin contents is present to suggest that the B-PP F_1 population is segregating for both cyanidin and pelargonidin contents. Although the population was segregating for flower color with varying shades of pink and white, no correlation was observed between fruit cyanidin content and flower color (data not shown). The source of high cyanidin in the B-PP accessions is particularly interesting because 'Pink Panda' is an intergeneric hybrid with *Potentilla palustris*, which has pink flowers (Ellis, 1991). The variability in cyanidin content suggests that high cyanidin can be bred into cultivars by introgression of wild germplasm and even by interspecific hybridization.

CHAPTER 5

CONCLUSIONS

An effective methodology was developed to assess the cyanidin and pelargonidin contents in strawberry fruit. We discovered significant variation in cyanidin content both within and between wild strawberry species, and germplasm accessions were identified that contained high levels and proportions of cyanidin. In the diploids surveyed, two accessions, one each of *F. vesca* and *F. viridis* were identified that contain markedly high cyanidin and with a cyanidin to pelargonidin ratio of greater than one. In the wild octoploid germplasm, an *F. chiloensis* ssp. *lucida* accession (CFRA 1691) was identified with exceptionally high cyanidin content (707 µg/g FDW) and a cyanidin to pelargonidin ratio of greater than one. In contrast, the highest cyanidin content among the cultivated germplasm, in cultivar 'Seascape', was only 120 µg/g FDW, and the cyanidin to pelargonidin ratio was less than one.

It is reasonable to speculate that the generally low cyanidin content in the cultivated strawberry, as found in the present and prior studies, likely results from the absence of high cyanidin content in the particular wild germplasm that comprised its original pedigree. Considering the known ancestors of *F. × ananassa*, wild *F. virginiana* ssp. *virginiana* accessions contained little cyanidin, and *F. chiloensis* accessions surveyed from South America (provenance of *F. chiloensis* in the cultivated strawberry) also contained low

cyanidin. In contrast, an *F. chiloensis* ssp. *lucida* from Oregon was identified that contained exceptionally high cyanidin, as did one accession (WC8) of *F. vesca*, a putative diploid ancestor of the octoploids. Thus, the genetic determinants of high cyanidin content exist in at least some wild germplasm that are closely related to the cultivated species. This reasoning prompts optimism that high cyanidin cultivars could be developed via introgression of genes from specific, high cyanidin wild octoploid accessions.

We also discovered segregation for cyanidin content in an octoploid F_1 population, suggesting that cyanidin content could be increased via selection in breeding populations. The F_1 population which resulted from a cross *F. × ananassa* 'Bountiful' and 'Pink Panda' involved an intergeneric hybridization in the pedigree of 'Pink Panda'. The ornamental strawberry 'Pink Panda' included an intergeneric hybridization of *F. × ananassa* with *Potentilla palustris* (Ellis, 1962). Thus, interspecific and even intergeneric hybridization could be used with advantage as a starting point for introgression of high cyanidin determinants. Overall, the results presented here show that specific wild strawberry germplasm has the potential to be useful in future strawberry breeding programs aimed at enhancing the cyanidin content, and therefore the antioxidant potential, of strawberry cultivars, perhaps contributing to their healthful properties.

The data reported here will be contributed to the GRIN system to add to the descriptions of *Fragaria* accessions held in the USDA germplasm system, and will be submitted for publication in a journal of the American Horticultural

Society. In addition, interesting germplasm in the UNH collection that is not currently held by the USDA-NCGR system will be offered for donation to that system.

Potential Future Implications of this Work

The study focused on two anthocyanins. Because the associated biosynthetic enzymes are known for both cyanidin and pelargonidin, the respective genes can be targeted for molecular marker development. The anthocyanin biosynthetic pathway step at which pelargonidin and cyanidin biosynthesis diverge has not been studied in strawberry. Identification of wild strawberry species with high levels of the cyanidin pigment constitutes a first step toward the dissection of this biosynthetic pathway in strawberry, and the molecular identification and cloning of genes for high cyanidin content. Advances made by this study could lead to the development of molecular tools allowing marker assisted selection for high cyanidin content in strawberry breeding programs. The immediate goal of further work will be to assess the correlation between cyanidin content and the alleles of the F3'H gene, which marks the first committed step toward cyanidin biosynthesis. Projected genetic studies will include the sequencing and comparison the F3'H genes from sources with high and low levels of cyanidin to detect differences in nucleotide sequences which may account for a functional loss in F3'H function in species/varieties with low levels of cyanidin.

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APPENDICES

APPENDIX A

GREENHOUSE POTS AND FIELD PLOT LAYOUTS

Figure A.1 Supercore Collection greenhouse pot and field plot layouts.

A plot of each Supercore Collection accession (shaded-boxed numbers) was planted at the UNH horticultural farm, and a pot of each Supercore Collection accession (circled numbers) was maintained at the UNH greenhouses.

The numbers and the suffix letters within the boxes and circles are the accession NCGR CFRA inventory ID numbers and species designated as 'C' for *F. chiloensis* or 'V' for *F. virginiana*.

A number was pre-assigned for each plot (number 1 through 33) to be planted, at the horticultural farm. Three replicate plots of each of the 11 cultivar (listed as Clone 1, 2, and 3) were planted, at the horticultural farm. Numbers representing a cultivar and clone combination were randomly drawn, and planted in the plots (shaded-boxed numbers) as shown. An additional plot of each cultivar, Clone 4 and Clone 5, was planted at the Rockingham Botanical Gardens and at the Organic Garden respectively. Clone 4 plots are not shown as fruit was not collected from these clones.

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Figure A.3 Bountiful, Pink Panda, and B-PP accessions greenhouse pot and field plot layouts.

A pot (circled letters with numbers) and a plot (shaded-boxed letters with numbers) of 'Bountiful' (B), Pink Panda (PP) and of each BxPP F₁ progeny (B-PP) was respectively maintained at the UNH greenhouses and planted at the UNH horticultural farm. The suffix number following the letters in the green circles or boxes represent an individual numbered F₁ plant.

APPENDIX B

CHEMICAL AND STOCK SOLUTIONS

Table B.1 Chemicals and stock solutions.

Chemicals		Manufacturer / Supplier
Methanol, HPLC grade, 0.2 μ m filtered, specific gravity = 0.7875 g/ml		Fisher Scientific Inc., Fair Lawn NJ
Formic Acid, 88% min by acidimetry ACSgrade		J.T. Baker Analyzed Reagent / VWR International West Chester, PA
Hydrochloric Acid, ACS grade, 12.1N		EMD™ Chemicals / VWR International West Chester, PA
Pelargonidin Chloride Powder Standard, Certificate of Analysis (COA) = 99.8%		Indofine Chemicals Company, Inc., Hillsborough NJ
Cyanidin Chloride Standard, Certificate of Analysis (COA) = 97.2%		Indofine Chemicals Company, Inc., Hillsborough NJ

Stock Solutions	Concentration	Recipe
Acidified methanol pH=1.0	0.01% HCl in Methanol	10 μ l of 12.1N HCl per 100 ml of HPLC grade methanol. 12.1N HCl assumed as containing 100% HCl.
Hydrolysis Acid	HCl 2N	16.5 ml of 12.1N HCl per 100 ml of solution.
Extraction Acid	3% Formic Acid (aq)	34 ml of 88% formic acid in DI water per 100 ml of solution.
Mobile Phase A:	Formic Acid (0.3%), pH 2.8	3400 μ l of 88% formic acid in DI water per liter of solution filtered through 0.2 μ m filter.
Mobile Phase B:	Methanol (95%)	950 ml of methanol and 50 ml of DI water filtered through 0.2 μ m filter.
Pelargonidin Standard Solution	1000 μ g/g in acidified methanol	1000 μ g/g $\times$ 0.7875 g/ml of acidified methanol / 0.998 = 789.1 μ g standard/ml of methanol. Weighed out standard and added amount of methanol required to achieve 1000 μ g/g concentration (1000 μ g/g = 787.5 g/ml).
Cyanidin Standard Solution	1000 μ g/g in acidified methanol	1000 μ g/g $\times$ 0.7875 g/ml of acidified methanol / 0.972 = 810.2 μ g standard/ml of methanol. Weighed

APPENDIX C

DATA

Table C.1a Comparison of GH and HF HPLC results.

[Cyn] and [Plr] concentrations ($\mu\text{g/g}$ FDW) and ratio of [Cyn] to [Cyn] and [Plr] determined using the Phase 3 protocol in ripe fruit collected from accessions in greenhouse (GH) and field (HF). The Run # refers to the HPLC identification in the data records.

Samples	Collection Year	Run #	Accession	No of Berries	Collection Location	[Cyn]	[Plr]	[Cyn]/([Cyn]+[Plr])
1	2006	6153	Bountiful	1	GH	39	430	0.08
2	2005	5091	Bountiful	2	HF	55	426	0.11
3	2005	5092	Bountiful	3	HF	31	291	0.10
4	2005	5093	Bountiful	4	HF	92	273	0.25
5	2006	6134	B-PP-024	?	GH	49	546	0.08
6	2005	5110	B-PP-024	6	HF	65	555	0.10
7	2005	5111	B-PP-024	6	HF	69	474	0.13
8	2005	5117	B-PP-044	3	GH	76	416	0.15
9	2005	5140	B-PP-044	4	HF	90	298	0.23
10	2005	5118	B-PP-077	3	GH	132	802	0.14
11	2005	5108	B-PP-077	2	HF	77	320	0.19
12	2006	6148C	FRA0372	1	GH	215	67	0.76
13	2006	6085	FRA0372	2	HF	181	149	0.55
14	2006	6036	FRA0372	1	HF	264	182	0.59
15	2006	6150A	FRA1697	1	GH	137	475	0.22
16	2006	6131	FRA1697	2	GH	84	315	0.21
17	2006	6133	FRA1697	3	GH	152	847	0.15
18	2006	6093	FRA1697	3	HF	144	405	0.26
19	2006	6007	FRA1697	5	HF	78	215	0.27
20	2006	6008	FRA1697	7	HF	62	163	0.27
21	2006	6048	FRA1697	10	HF	150	230	0.39
22	2006	6132	FRA1700	2	GH	256	1090	0.19
23	2006	6050	FRA1700	2	HF	234	651	0.26
24	2006	6152	FRA1701	1	GH	68	741	0.08
25	2006	6010	FRA1701	5	HF	57	303	0.16
26	2006	6011	FRA1701	5	HF	49	189	0.20
27	2006	6051	FRA1701	4	HF	82	466	0.15
28	2006	6123	FRA1701	14	HF	85	571	0.13
29	2006	6146	Tribute	1	GH	66	298	0.18
30	2006	6137	Tribute	1	GH	80	490	0.14
31	2006	6054	Tribute	2	HF	37	454	0.07
32	2006	6017	Tribute	3	HF	39	385	0.09
33	2006	6084	Tribute	4	HF	46	505	0.08
34	2006	6080	Tribute	5	HF	27	354	0.07

Table C.1b Comparison of GH and HF accession means.

The ripe fruit in this comparison were all assayed using the Phase 3 protocol.

Samples	Accession	Collection Location	No. of Samples	[Cyn]	[Plr]	[Cyn]/ ([Cyn]+[Plr])
1	Bountiful	GH	1	39	430	0.080
2	Bountiful	HF	3	59	330	0.153
3	B-PP-024	GH	1	49	546	0.080
4	B-PP-024	HF	2	67	515	0.115
5	B-PP-044	GH	1	76	416	0.150
6	B-PP-044	HF	1	90	298	0.230
7	B-PP-077	GH	1	132	802	0.140
8	B-PP-077	HF	1	77	320	0.190
9	FRA0372	GH	1	215	67	0.760
10	FRA0372	HF	2	223	166	0.570
11	FRA1697	GH	3	124	546	0.193
12	FRA1697	HF	4	109	253	0.298
13	FRA1700	GH	1	256	1090	0.190
14	FRA1700	HF	1	234	651	0.260
15	FRA1701	GH	1	68	741	0.080
16	FRA1701	HF	4	68	382	0.160
17	Tribute	GH	2	73	394	0.160
18	Tribute	HF	4	37	425	0.078

Table C.2a Comparison of Phase 1 and Phase 2 HPLC results.

[Cyn] and [Pir] concentrations ($\mu\text{g/g}$ FDW) and ratio of [Cyn] to [Cyn] and [Pir] collected from accessions with HPLC sample volume decreased from 20 μl in Phase 1 to 5 μl in Phase 2. Extraction of anthocyanidin conducted with centrifuge RCF of 1,200 $\times\text{g}$ E during both phases. The Run # refers to the HPLC identification in the data records.

Samples	Collection Year	Run #	Phase	Accession	Collection Location	[Cyn]	[Pir]	[Cyn]/ ([Cyn]+[Pir])
1	2004	56	1	B-PP-007	GH	19	131	0.128
2	2005	5044	2	B-PP-007	HF	45	143	0.239
3	2005	5053	2	B-PP-007	HF	79	225	0.260
4	2005	5056	2	B-PP-007	HF	54	268	0.167
5	2004	48	1	B-PP-009	GH	31	172	0.154
6	2005	5033	2	B-PP-009	HF	51	319	0.137
7	2005	5035	2	B-PP-009	HF	63	303	0.173
8	2005	5049	2	B-PP-009	HF	68	292	0.189
9	2004	58	1	B-PP-017	GH	0	91	0.003
10	2005	5040	2	B-PP-017	HF	30	198	0.133
11	2005	5041	2	B-PP-017	HF	24	196	0.109
12	2005	5057	2	B-PP-017	HF	38	332	0.104
13	2005	5059	2	B-PP-017	HF	33	248	0.118
14	2004	26	1	B-PP-023	GH	23	78	0.229
15	2005	5037	2	B-PP-023	HF	72	413	0.149
16	2005	5042	2	B-PP-023	HF	33	134	0.195
17	2005	5048	2	B-PP-023	HF	33	328	0.092
18	2004	50	1	B-PP-024	GH	20	88	0.187
19	2005	5036	2	B-PP-024	HF	24	173	0.122
20	2005	5039	2	B-PP-024	HF	58	353	0.140
21	2005	5054	2	B-PP-024	HF	66	452	0.128
22	2005	5055	2	B-PP-024	HF	60	583	0.094
23	2004	43	1	B-PP-030	GH	16	148	0.098
24	2005	5034	2	B-PP-030	HF	48	196	0.198
25	2005	5038	2	B-PP-030	HF	39	226	0.146
26	2005	5043	2	B-PP-030	HF	42	229	0.156
27	2004	60	1	B-PP-038	GH	27	163	0.142
28	2005	5003	2	B-PP-038	HF	30	72	0.293
29	2005	5006	2	B-PP-038	HF	35	54	0.395
30	2005	5050	2	B-PP-038	HF	41	277	0.130
31	2004	33	1	B-PP-069	GH	22	46	0.322
32	2005	5061	2	B-PP-069	HF	41	118	0.259
33	2005	5062	2	B-PP-069	HF	38	106	0.265
34	2004	34	1	B-PP-070	GH	20	70	0.225
35	2005	5024	2	B-PP-070	HF	82	263	0.237
36	2004	35	1	B-PP-072	GH	0	51	0.006
37	2005	5070	2	B-PP-072	HF	33	151	0.178
38	2004	21	1	B-PP-077	GH	0	10	0.010
39	2005	5004	2	B-PP-077	HF	74	167	0.307
40	2005	5025	2	B-PP-077	HF	45	233	0.163

Table C.2a continued: Comparison of Phase 1 and Phase 2 HPLC results.

Samples	Collection Year	Run #	Phase	Accession	Collection Location	[Cyn]	[PIr]	[Cyn]/ ([Cyn]+[PIr])
41	2005	5026	2	B-PP-077	HF	36	228	0.136
42	2005	5027	2	B-PP-077	HF	20	124	0.138
43	2005	5028	2	B-PP-077	HF	32	142	0.184
44	2004	36	1	B-PP-078	GH	16	34	0.327
45	2005	5063	2	B-PP-078	HF	0	48	0.000
46	2005	5065	2	B-PP-078	HF	49	428	0.103
47	2004	22	1	B-PP-102?	GH	10	35	0.223
48	2005	5073	2	B-PP-102?	HF	50	160	0.239
49	2005	5074	2	B-PP-102?	HF	40	204	0.163
50	2005	5075	2	B-PP-102?	HF	52	200	0.206
51	2004	66	1	B-PP-111?	GH	29	414	0.066
52	2005	5010	2	B-PP-111?	HF	96	1300	0.068
53	2005	5011	2	B-PP-111?	HF	50	655	0.071
54	2005	5016	2	B-PP-111?	HF	45	522	0.080
55	2004	24	1	B-PP-112	GH	8	17	0.319
56	2005	5012	2	B-PP-112	HF	24	132	0.154
57	2005	5013	2	B-PP-112	HF	28	121	0.188
58	2004	20	1	B-PP-200	GH	12	54	0.186
59	2005	5014	2	B-PP-200	HF	29	164	0.151
60	2005	5015	2	B-PP-200	HF	22	74	0.229

Table C.2b Comparison of Phase 1 and Phase 2 accession means.

Samples	Collection Year	Phase	Accession	No. of Samples	Collection Location	[Cyn]	[Pir]	[Cyn]/ ([Cyn]+[Pir])
1	2004	1	B-PP-007	1	GH	19	131	0.128
2	2005	2	B-PP-007	3	HF	59	212	0.222
3	2004	1	B-PP-009	1	GH	31	172	0.154
4	2005	2	B-PP-009	3	HF	61	305	0.166
5	2004	1	B-PP-017	1	GH	0.3	91	0.003
6	2005	2	B-PP-017	4	HF	31	244	0.116
7	2004	1	B-PP-023	1	GH	23	78	0.229
8	2005	2	B-PP-023	3	HF	46	292	0.145
9	2004	1	B-PP-024	1	GH	20	88	0.187
10	2005	2	B-PP-024	4	HF	52	390	0.121
11	2004	1	B-PP-030	1	GH	16	148	0.098
12	2005	2	B-PP-030	3	HF	43	217	0.167
13	2004	1	B-PP-038	1	GH	27	163	0.142
14	2005	2	B-PP-038	3	HF	35	134	0.273
15	2004	1	B-PP-069	1	GH	22	46	0.322
16	2005	2	B-PP-069	2	HF	40	112	0.262
17	2004	1	B-PP-070	1	GH	20	70	0.225
18	2005	2	B-PP-070	1	HF	82	263	0.237
19	2004	1	B-PP-072	1	GH	0.1	51	0.006
20	2005	2	B-PP-072	1	HF	33	151	0.178
21	2004	1	B-PP-077	1	GH	0	10	0.010
22	2005	2	B-PP-077	5	HF	41	179	0.186
23	2004	1	B-PP-078	1	GH	16	34	0.327
24	2005	2	B-PP-078	2	HF	24	238	0.103
25	2004	1	B-PP-102?	1	GH	10	35	0.223
26	2005	2	B-PP-102?	3	HF	47	188	0.203
27	2004	1	B-PP-111?	1	GH	29	414	0.066
28	2005	2	B-PP-111?	3	HF	64	826	0.073
29	2004	1	B-PP-112	1	GH	8	17	0.319
30	2005	2	B-PP-112	2	HF	26	126	0.171
31	2004	1	B-PP-200	1	GH	12	54	0.186
32	2005	2	B-PP-200	2	HF	26	119	0.190

Table C.3a Comparison of Phase 2 and Phase 3 HPLC results.

[Cyn] and [Pir] concentrations ($\mu\text{g/g}$ FDW) and ratio of [Cyn] to [Cyn] and [Pir] with the anthocyanidin extraction conducted with a centrifuge RCF of 1,200 in Phase 2 and increased to 15,500 xg with a new centrifuge in Phase 3. The HPLC sample volume was 5 μl in both phases. The Run # refers to the HPLC identification in the data records.

Samples	Collection Year	Run #	Phase	Accession	No of Berries	Collection Location	[Cyn]	[Pir]	[Cyn]/ ([Cyn]+[Pir])
1	2005	5044	2	B-PP-007	3	HF	45	143	0.24
2	2005	5053	2	B-PP-007	4	HF	79	225	0.26
3	2005	5056	2	B-PP-007	4	HF	54	268	0.17
4	2005	5106	3	B-PP-007	6	HF	87	528	0.14
5	2005	5107	3	B-PP-007	6	HF	87	719	0.11
6	2005	5036	2	B-PP-024	2	HF	24	173	0.12
7	2005	5039	2	B-PP-024	4	HF	58	353	0.14
8	2005	5054	2	B-PP-024	4	HF	66	452	0.13
9	2005	5055	2	B-PP-024	4	HF	60	583	0.09
10	2005	5110	3	B-PP-024	6	HF	65	555	0.10
11	2005	5111	3	B-PP-024	6	HF	69	474	0.13
12	2005	5034	2	B-PP-030	3	HF	48	196	0.20
13	2005	5038	2	B-PP-030	3	HF	39	226	0.15
14	2005	5043	2	B-PP-030	4	HF	42	229	0.16
15	2005	5112	3	B-PP-030	3	HF	131	777	0.14
16	2005	5003	2	B-PP-038	2	HF	30	72	0.29
17	2005	5006	2	B-PP-038	2	HF	35	54	0.39
18	2005	5050	2	B-PP-038	2	HF	41	277	0.13
19	2005	5130	3	B-PP-038	8	HF	104	472	0.18
20	2005	5025	2	B-PP-077	3	HF	45	233	0.16
21	2005	5026	2	B-PP-077	4	HF	36	228	0.14
22	2005	5027	2	B-PP-077	4	HF	20	124	0.14
23	2005	5028	2	B-PP-077	4	HF	32	142	0.18
24	2005	5108	3	B-PP-077	2	HF	77	320	0.19
25	2005	5084	2	Seascape	2	HF	116	422	0.22
26	2005	5132	3	Seascape	2	HF	132	535	0.20
27	2005	5120	3	Seascape	1	HF	222	342	0.39
28	2005	5081	2	Tristar	2	HF	56	377	0.13
29	2005	5007	2	Tristar	2	HF	36	253	0.12
30	2005	5020	2	Tristar	1	HF	35	293	0.11
31	2005	5058	2	Tristar	1	HF	60	713	0.08
32	2005	5083	2	Tristar	3	HF	88	704	0.11
33	2005	5129	3	Tristar	2	HF	133	1053	0.11
34	2005	5131	3	Tristar	2	HF	123	1420	0.08
35	2005	5119	3	Tristar	1	HF	111	692	0.14

Table C.3b Comparison of Phase 2 and Phase 3 accession means.

Samples	Phase	Accession	No. of Samples	[CYN]	[PLR]	[Cyn]/ ([Cyn]+[Plr])
1	2	B-PP-007	3	59	212	0.223
2	3	B-PP-007	2	87	624	0.125
3	2	B-PP-024	4	52	390	0.120
4	3	B-PP-024	2	67	515	0.115
5	2	B-PP-030	3	43	217	0.170
6	3	B-PP-030	1	131	777	0.140
7	2	B-PP-038	3	35	134	0.270
8	3	B-PP-038	1	104	472	0.180
9	2	B-PP-077	4	33	182	0.155
10	3	B-PP-077	1	77	320	0.190
11	2	Seascape	1	116	422	0.220
12	3	Seascape	2	177	439	0.295
13	2	Tristar	5	55	468	0.110
14	3	Tristar	3	122	1055	0.110

Table C.4 Octoploid species Supercore Collection ripe fruit anthocyanidin content.

The Supercore Collection ripe fruit were assayed using the Phase 3 protocol. The Run # refers to the HPLC identification in the data records.

Case	Run	Species subspecies	Origin	Accession CFRA	Number of Berries	Loc	Anthocyanidin (µg/g dry FDW)			
							[Cyn]	[Plr]	[Anth]	[Cyn]/ ([Anth])
<i>F. chiloensis</i>										
1	6001	<i>ssp. pacifica</i>	Alaska	368.002	2	HF	56	205	261	0.213
2	6002	<i>ssp. pacifica</i>	Alaska	368.005	5	HF	88	237	325	0.270
3	6118	<i>ssp. pacifica</i>	Alaska	368.005	3	HF	98	152	250	0.393
4	6124	<i>ssp. pacifica</i>	Alaska	368.005	4	HF	122	400	522	0.235
5	6094	<i>ssp lucida</i>	Washington	42.001	3	HF	362	52	414	0.875
6	6091	<i>ssp lucida</i>	Oregon	1691.001	1	HF	524	159	683	0.768
7	6130	<i>ssp lucida</i>	Oregon	1691.001	3	HF	889	243	1132	0.785
8	6148C	<i>f.chiloensis</i>	Peru	372.002	1	GH	215	67	282	0.762
9	6085	<i>f.chiloensis</i>	Peru	372.002	2	HF	181	149	331	0.548
10	6036	<i>f.chiloensis</i>	Peru	372.002	1	HF	264	182	446	0.592
11	6095	<i>f.patagonica</i>	Chilé	1100.002	2	HF	167	233	400	0.417
<i>F. virginiana</i>										
12	6154	<i>ssp virginiana(N)</i>	Ontario	1694.001	1	HF	165	749	915	0.181
13	6009	<i>ssp virginiana(N)</i>	Ontario	1699.001	4	HF	67	434	501	0.134
14	6069	<i>ssp virginiana(N)</i>	Ontario	1699.001	1	HF	379	1497	1876	0.202
15	6113	<i>ssp virginiana(N)</i>	Ontario	1699.001	5	HF	100	447	547	0.182
16	6122	<i>ssp virginiana(N)</i>	Ontario	1699.001	6	HF	209	961	1171	0.179
17	6097	<i>ssp virginiana(N)</i>	Ontario	1695.001	2	HF	198	321	519	0.381
18	6098	<i>ssp virginiana(N)</i>	Ontario	1695.001	1	HF	314	869	1183	0.266
19	6155	<i>ssp virginiana(N)</i>	Ontario	1695.001	1	HF	176	213	389	0.453
20	6037	<i>ssp virginiana(N)</i>	Ontario	1695.002	2	HF	610	1004	1613	0.378
21	6129	<i>ssp virginiana(N)</i>	Ontario	1695.002	2	HF	169	899	1067	0.158
22	6010	<i>ssp virginiana(N)</i>	Minnesota	1701.001	5	HF	57	303	360	0.160
23	6011	<i>ssp virginiana(N)</i>	Minnesota	1701.001	5	HF	49	189	238	0.204

Table C.4 Octoploid species Supercore Collection ripe fruit anthocyanidin content continued.

Case	Run	Species subspecies	Origin	Accession CFRA	Number of Berries	Loc	Anthocyanidin (µg/g dry FDW)			
							[Cyn]	[Plr]	[Anth]	[Cyn]/ ([Anth])
<i>F. virginiana</i> continued										
24	6051	ssp <i>virginiana</i> (N)	Minnesota	1701.001	4	HF	82	466	548	0.149
25	6123	ssp <i>virginiana</i> (N)	Minnesota	1701.001	14	HF	85	571	655	0.129
26	6152	ssp <i>virginiana</i> (N)	Minnesota	1701.001	1	GH	68	741	809	0.084
27	6050	ssp <i>virginiana</i> (N)	Minnesota	1700.001	2	HF	234	651	885	0.265
28	6132	ssp <i>virginiana</i> (N)	Minnesota	1700.001	2	GH	256	1090	1346	0.190
29	6006	ssp <i>virginiana</i> (S)	S. Carolina	1580.001	5	HF	93	232	325	0.286
30	6049	ssp <i>virginiana</i> (S)	S. Carolina	1580.001	2	HF	109	225	334	0.327
31	6068	ssp <i>virginiana</i> (S)	S. Carolina	1580.001	7	HF	107	244	351	0.305
32	6112	ssp <i>virginiana</i> (S)	S. Carolina	1580.001	3	HF	117	343	460	0.255
33	6121	ssp <i>virginiana</i> (S)	S. Carolina	1580.001	9	HF	103	218	322	0.322
34	6005	ssp <i>virginiana</i> (S)	Georgia	1455.001	2	HF	87	512	599	0.145
35	6067	ssp <i>virginiana</i> (S)	Georgia	1455.001	2	HF	137	588	725	0.188
36	6128	ssp <i>virginiana</i> (S)	Florida	1435.002	1	HF	222	561	784	0.284
37	6150A	ssp <i>glauca</i> * <i>virginiana</i>	Montana	1697.001	1	GH	137	475	612	0.223
38	6093	ssp <i>glauca</i> * <i>virginiana</i>	Montana	1697.001	3	HF	144	405	549	0.262
39	6131	ssp <i>glauca</i> * <i>virginiana</i>	Montana	1697.001	2	GH	84	315	399	0.210
40	6133	ssp <i>glauca</i> * <i>virginiana</i>	Montana	1697.001	3	GH	152	847	999	0.152
41	6007	ssp <i>glauca</i> * <i>virginiana</i>	Montana	1697.002	5	HF	78	215	293	0.265
42	6008	ssp <i>glauca</i> * <i>virginiana</i>	Montana	1697.002	7	HF	62	163	225	0.275
43	6048	ssp <i>glauca</i> * <i>virginiana</i>	Montana	1697.002	10	HF	150	230	380	0.395
44	6066	ssp <i>grayana</i>	Mississippi	1414.001	3	HF	57	175	232	0.247
45	6120	ssp <i>grayana</i>	Mississippi	1414.001	1	HF	70	176	246	0.283
46	6127	ssp <i>grayana</i>	Mississippi	1414.001	1	HF	104	329	433	0.240
47	6003	ssp <i>grayana</i>	Mississippi	1408.001	2	HF	93	185	279	0.335
48	6125	ssp <i>grayana</i>	Mississippi	1408.001	5	HF	98	329	427	0.229

Table C.4 Octoploid species Supercore Collection ripe fruit anthocyanidin content continued.

Case	Run	Species subspecies	Origin	Accession CFRA	Number of Berries	Loc	Anthocyanidin (µg/g dry FDW)			
							[Cyn]	[Plr]	[Anth]	[Cyn]/ ([Anth])
F. virginiana continued										
49	6086	ssp grayana	Mississippi	1408.001	3	HF	176	561	737	0.238
50	6004	ssp grayana	Mississippi	1408.002	1	HF	242	577	819	0.296
51	6065	ssp grayana	Mississippi	1408.002	4	HF	71	170	241	0.296
52	6119	ssp grayana	Mississippi	1408.002	4	HF	91	278	369	0.248
53	6126	ssp grayana	Mississippi	1408.002	5	HF	123	611	734	0.167

Table C.5 Octoploid *Fragaria* × *ananassa* ripe fruit anthocyanidin content.

Most of the assays of the cultivars were conducted using Phase 3 protocol 69 out of 79 assays, with the remaining 10 assays conducted using the Phase 2 protocol. The Run # refers to the HPLC identification in the data records.

Case	Collection Year	Phase	Run #	Accession	No of Berries	Collection Location	Anthocyanidins (µg/g FDW)			
							[Cyn]	[Plr]	[Anth]	[Cyn]/[Anth]
1	2006	3	6138	Delmarvel	1	GH	51	616	667	0.076
2	2006	3	6139	Delmarvel	1	GH	48	634	682	0.070
3	2006	3	6144	Delmarvel	1	GH	47	1210	1257	0.037
4	2006	3	6012	Earliglow	5	HF	65	834	900	0.073
5	2006	3	6027	Earliglow	5	HF	69	664	733	0.094
6	2006	3	6047	Earliglow	3	HF	77	413	491	0.157
7	2006	3	6078	Earliglow	5	HF	56	554	611	0.092
8	2006	3	6116	Earliglow	5	HF	41	476	516	0.079
9	2006	3	6013	Honeoye	5	HF	41	692	733	0.056
10	2006	3	6028	Honeoye	5	HF	57	865	922	0.062
11	2006	3	6045	Honeoye	5	HF	61	820	882	0.070
12	2006	3	6071	Honeoye	5	HF	49	625	674	0.073
13	2006	3	6072	Honeoye	5	HF	44	366	410	0.106
14	2006	3	6106	Honeoye	5	HF	51	707	758	0.067
15	2006	3	6114	Honeoye	5	HF	44	448	492	0.090
16	2006	3	6115	Honeoye	5	HF	51	1056	1108	0.046
17	2006	3	6014	Jewel	5	HF	34	692	727	0.047
18	2006	3	6021	Jewel	5	HF	46	772	818	0.056
19	2006	3	6022	Jewel	1	HF	56	848	904	0.062
20	2006	3	6038	Jewel	3	HF	45	462	508	0.089
21	2006	3	6041	Jewel	2	HF	47	518	565	0.083
22	2006	3	6062	Jewel	5	HF	39	720	759	0.051
23	2006	3	6063	Jewel	4	HF	91	875	967	0.094
24	2006	3	6073	Jewel	5	HF	30	295	325	0.093
25	2006	3	6107	Jewel	3	HF	46	854	901	0.052

Table C.5 Octoploid *Fragaria* × *ananassa* ripe fruit anthocyanidin content continued.

Case	Collection Year	Phase	Run #	Accession	No of Berries	Collection Location	Anthocyanidins (µg/g FDW)			
							[Cyn]	[Pir]	[Anth]	[Cyn]/[Anth]
26	2006	3	6015	Northeast	5	HF	45	422	467	0.096
27	2006	3	6023	Northeast	2	HF	48	539	587	0.082
28	2006	3	6024	Northeast	1	HF	48	662	709	0.067
29	2006	3	6081	Northeast	4	HF	45	371	416	0.108
30	2006	3	6020	Raritan	5	HF	23	328	351	0.065
31	2006	3	6074	Raritan	5	HF	28	734	761	0.036
32	2006	3	6082	Raritan	4	HF	28	464	493	0.058
33	2006	3	6100	Raritan	4	HF	27	467	494	0.055
34	2006	3	6101	Raritan	5	HF	28	667	695	0.040
35	2005	2	5084	Seascape	2	HF	116	422	538	0.216
36	2005	3	5105	Seascape	1	HF	86	294	380	0.227
37	2005	3	5120	Seascape	1	HF	91	308	399	0.227
38	2005	3	5132	Seascape	2	HF	132	535	666	0.198
39	2006	3	6025	Seneca	5	HF	54	495	549	0.098
40	2006	3	6026	Seneca	5	HF	40	373	413	0.097
41	2006	3	6030	Seneca	3	HF	59	524	583	0.101
42	2006	3	6033	Seneca	2	HF	69	444	514	0.135
43	2006	3	6035	Seneca	5	HF	54	558	611	0.088
44	2006	3	6056	Seneca	2	HF	51	748	799	0.064
45	2006	3	6083	Seneca	3	HF	44	246	291	0.152
46	2006	3	6016	Sparkle	5	HF	69	601	670	0.103
47	2006	3	6057	Sparkle	5	HF	154	902	1056	0.146
48	2006	3	6059	Sparkle	5	HF	89	519	608	0.146
49	2006	3	6076	Sparkle	5	HF	61	315	376	0.163
50	2006	3	6077	Sparkle	5	HF	65	558	624	0.105
51	2006	3	6079	Sparkle	5	HF	45	265	310	0.146
52	2006	3	6102	Sparkle	3	HF	70	306	377	0.187

Table C.5 Octoploid *Fragaria* × *ananassa* ripe fruit anthocyanidin content continued.

Case	Collection Year	Phase	Run #	Accession	No of Berries	Collection Location	Anthocyanidins (µg/g FDW)			
							[Cyn]	[Plr]	[Anth]	[Cyn]/[Anth]
53	2006	3	6103	Sparkle	5	HF	70	698	768	0.091
54	2006	3	6117	Sparkle	2	HF	41	441	482	0.086
55	2005	2	5046	Tribute		HF	21	326	347	0.061
56	2006	3	6017	Tribute	3	HF	39	385	424	0.091
57	2006	3	6054	Tribute	2	HF	37	454	491	0.075
58	2006	3	6064	Tribute	4	HF	46	505	551	0.083
59	2006	3	6080	Tribute	5	HF	27	354	381	0.072
60	2006	3	6137	Tribute	1	GH	80	490	570	0.141
61	2006	3	6146	Tribute	1	GH	66	298	364	0.180
62	2005	2	5007	Tristar	2	HF	36	253	289	0.123
63	2005	2	5008	Tristar	1	HF	23	135	158	0.143
64	2005	2	5009	Tristar	1	HF	17	109	126	0.134
65	2005	2	5020	Tristar	1	HF	35	293	328	0.107
66	2005	2	5058	Tristar	1	HF	60	713	773	0.078
67	2005	2	5081	Tristar	2	HF	56	377	433	0.129
68	2005	2	5082	Tristar	3	HF	21	308	329	0.064
69	2005	2	5083	Tristar	3	HF	88	704	791	0.111
70	2005	3	5119	Tristar	1	HF	89	716	806	0.111
71	2005	3	5129	Tristar	2	HF	133	1053	1186	0.112
72	2005	3	5131	Tristar	2	HF	123	1420	1543	0.080
73	2006	3	6018	Tristar	5	HF	75	733	808	0.093
74	2006	3	6046	Tristar	1	HF	138	864	1002	0.138
75	2006	3	6060	Winona	5	HF	42	521	563	0.075
76	2006	3	6061	Winona	5	HF	55	789	844	0.065
77	2006	3	6099	Winona	2	HF	44	403	447	0.099
78	2006	3	6104	Winona	1	HF	49	670	719	0.068
79	2006	3	6111	Winona	3	HF	62	606	669	0.093

Table C.6 Octoploid 'Bountiful', 'Pink Panda' and B-PP Accessions ripe fruit anthocyanidin content.

These accessions were analyzed during the phases of development. The Run # refers to the HPLC identification in the data records.

Case	Collection		Phase	Accession	No of Berries	Collection Location	Anthocyanidins (µg/g FDW)			
	Year	Run #					[Cyn]	[Plr]	[Anth]	[Cyn]/[Anth]
1	2005	5091	3	Bountiful	2	HF	55	426	481	0.114
2	2005	5092	3	Bountiful	3	HF	31	291	322	0.096
3	2005	5093	3	Bountiful	4	HF	92	273	365	0.252
4	2006	6153	3	Bountiful	1	GH	39	430	468	0.083
5	2004	25	1	B-PP-003		GH	52	133	185	0.282
6	2005	5045	2	B-PP-005	4	GH	42	265	307	0.137
7	2005	5047	2	B-PP-005	2	HF	30	154	184	0.163
8	2004	56	1	B-PP-007		GH	19	131	150	0.128
9	2005	5044	2	B-PP-007	3	HF	45	143	188	0.239
10	2005	5053	2	B-PP-007	4	HF	79	225	304	0.260
11	2005	5056	2	B-PP-007	4	HF	54	268	322	0.167
12	2005	5106	3	B-PP-007	6	HF	87	528	615	0.142
13	2005	5107	3	B-PP-007	6	HF	87	719	806	0.108
14	2004	48	1	B-PP-009		GH	31	172	204	0.154
15	2005	5033	2	B-PP-009		HF	51	319	370	0.137
16	2005	5035	2	B-PP-009	4	HF	63	303	367	0.173
17	2005	5049	2	B-PP-009	5	HF	68	292	360	0.189
18	2004	57	1	B-PP-016		GH	0		0	NA
19	2005	5109	3	B-PP-016	4	HF	55	298	353	0.155
20	2004	58	1	B-PP-017		GH	0	91	91	0.003
21	2005	5040	2	B-PP-017	3	HF	30	198	229	0.133
22	2005	5041	2	B-PP-017	3	HF	24	196	219	0.109
23	2005	5057	2	B-PP-017	3	HF	38	332	370	0.104
24	2005	5059	2	B-PP-017	3	HF	33	248	281	0.118
25	2004	26	1	B-PP-023		GH	23	78	102	0.229
26	2005	5037	2	B-PP-023	4	HF	72	413	485	0.149

Table C.6 Octoploid 'Bountiful', 'Pink Panda' and B-PP Accessions ripe fruit anthocyanidin content continued.

Case	Collection Year	Run #	Phase	Accession	No of Berries	Collectio Location	Anthocyanidins (µg/g FDW)			
							[Cyn]	[Plr]	[Anth]	[Cyn]/[Anth]
27	2005	5042	2	B-PP-023	4	HF	33	134	167	0.195
28	2005	5048	2	B-PP-023	4	HF	33	328	361	0.092
29	2004	50	1	B-PP-024		GH	20	88	108	0.187
30	2005	5036	2	B-PP-024	2	HF	24	173	197	0.122
31	2005	5039	2	B-PP-024	4	HF	58	353	410	0.140
32	2005	5039	2	B-PP-024		HF	56	336	392	0.142
33	2005	5054	2	B-PP-024	4	HF	66	452	518	0.128
34	2005	5055	2	B-PP-024	4	HF	60	583	644	0.094
35	2006	6134	3	B-PP-024	?	GH	49	546	594	0.082
36	2005	5110	3	B-PP-024	6	HF	65	555	620	0.105
37	2005	5111	3	B-PP-024	6	HF	69	474	544	0.128
38	2004	43	1	B-PP-030		GH	16	148	164	0.098
39	2005	5034	2	B-PP-030	3	HF	48	196	245	0.198
40	2005	5038	2	B-PP-030	3	HF	39	226	265	0.146
41	2005	5043	2	B-PP-030	4	HF	42	229	272	0.156
42	2005	5112	3	B-PP-030	3	HF	131	777	908	0.144
43	2004	68	1	B-PP-031		GH	33	288	320	0.102
44	2005	5113	3	B-PP-031	3	HF	66	416	481	0.136
45	2005	5145	3	B-PP-031	2	HF	78	608	686	0.114
46	2005	5146	3	B-PP-031	3	HF	92	1341	1432	0.064
47	2004	49	1	B-PP-036		GH	22	156	178	0.122
48	2004	27	1	B-PP-037		GH	0	126	127	0.002
49	2005	5147	3	B-PP-037	4	HF	51	367	419	0.122
50	2005	5148	3	B-PP-037	4	HF	78	513	591	0.132
51	2004	60	1	B-PP-038		GH	27	163	190	0.142
52	2005	5003	2	B-PP-038	2	HF	30	72	102	0.293
53	2005	5006	2	B-PP-038	2	HF	35	54	88	0.395

Table C.6 Octoploid 'Bountiful', 'Pink Panda' and B-PP Accessions ripe fruit anthocyanidin content.

Case	Collection Year	Run #	Phase	Accession	No of Berries	Collection Location	Anthocyanidins (µg/g FDW)			
							[Cyn]	[Pir]	[Anth]	[Cyn]/[Anth]
54	2005	5050	2	B-PP-038	2	HF	41	277	318	0.130
55	2005	5130	3	B-PP-038	8	HF	104	472	576	0.180
56	2005	5133	3	B-PP-038	5	HF	69	187	255	0.269
57	2004	29	1	B-PP-039		GH	2	5	7	0.310
58	2004	45	1	B-PP-039		GH	31	115	146	0.211
59	2005	5114	3	B-PP-039	6	HF	68	398	466	0.147
60	2005	5139	3	B-PP-039	5	HF	81	344	425	0.190
61	2005	5117	3	B-PP-044	3	GH	76	416	492	0.154
62	2005	5140	3	B-PP-044	4	HF	90	298	388	0.232
63	2005	5068	2	B-PP-047?	5	HF	86	302	388	0.222
64	2005	5069	2	B-PP-047?	5	HF	85	442	527	0.161
65	2004	67	1	B-PP-055		GH	28	102	130	0.215
66	2005	5128	3	B-PP-055	5	HF	132	800	932	0.141
67	2005	5134	3	B-PP-055	6	HF	78	322	401	0.196
68	2005	5135	3	B-PP-055	6	HF	141	833	974	0.145
69	2005	5136	3	B-PP-055	8	HF	56	303	359	0.155
70	2005	5141	3	B-PP-057	5	HF	49	146	195	0.251
71	2005	5142	3	B-PP-059	5	HF	54	390	444	0.122
72	2006	6109	3	B-PP-059	9	HF	111	373	483	0.229
73	2004	69	1	B-PP-062		GH	24	77	101	0.237
74	2005	5051	2	B-PP-062	5	GH	39	693	732	0.054
75	2005	5052	2	B-PP-062	4	GH	53	714	766	0.069
76	2005	5149	3	B-PP-062	6	HF	64	409	473	0.136
77	2005	5150	3	B-PP-062	5	HF	55	439	494	0.111
78	2004	32	1	B-PP-064?		GH	18	148	166	0.110
79	2005	5137	3	B-PP-064?	4	HF	80	830	910	0.088
80	2005	5138	3	B-PP-064?	4	HF	69	762	831	0.083

Table C.6 Octoploid 'Bountiful', 'Pink Panda' and B-PP Accessions ripe fruit anthocyanidin content.

Case	Collection Year	Run #	Phase	Accession	No of Berries	Collection Location	Anthocyanidins (µg/g FDW)			
							[Cyn]	[Plr]	[Anth]	[Cyn]/[Anth]
81	2005	5143	3	B-PP-064?	4	HF	46	523	569	0.082
82	2004	70	1	B-PP-067		GH	39	138	177	0.222
83	2005	5144	3	B-PP-067	3	HF	102	160	262	0.390
84	2005	5021	2	B-PP-068	4	HF	82	321	402	0.203
85	2005	5022	2	B-PP-068	3	HF	68	331	399	0.171
86	2005	5023	2	B-PP-068	3	HF	48	281	329	0.145
87	2004	33	1	B-PP-069		GH	22	46	67	0.322
88	2004	62	1	B-PP-069		GH	23	69	91	0.248
89	2005	5061	2	B-PP-069	4	HF	41	118	159	0.259
90	2005	5062	2	B-PP-069	5	HF	38	106	145	0.265
91	2004	34	1	B-PP-070		GH	20	70	90	0.225
92	2005	5024	2	B-PP-070	2	HF	82	263	345	0.237
93	2004	35	1	B-PP-072		GH	0	51	51	0.006
94	2005	5070	2	B-PP-072	3	HF	33	151	183	0.178
95	2004	21	1	B-PP-077		GH	0	10	10	0.010
96	2005	5004	2	B-PP-077	1	HF	74	167	241	0.307
97	2005	5025	2	B-PP-077	3	HF	45	233	279	0.163
98	2005	5026	2	B-PP-077	4	HF	36	228	264	0.136
99	2005	5027	2	B-PP-077	4	HF	20	124	144	0.138
100	2005	5028	2	B-PP-077	4	HF	32	142	175	0.184
101	2005	5118	3	B-PP-077	3	GH	26	53	78	0.327
102	2005	5108	3	B-PP-077	2	HF	77	320	398	0.194
103	2004	36	1	B-PP-078		GH	16	34	50	0.327
104	2005	5065	2	B-PP-078	5	HF	49	428	477	0.103
105	2005	5155	3	B-PP-081?	3	HF	100	186	286	0.350
106	2005	5071	2	B-PP-101	4	HF	41	305	346	0.118
107	2005	5072	2	B-PP-101	4	HF	41	131	171	0.238

Table C.6 Octoploid 'Bountiful', 'Pink Panda' and B-PP Accessions ripe fruit anthocyanidin content.

Case	Collection Year	Run #	Phase	Accession	No of Berries	Collection Location	Anthocyanidins (µg/g FDW)			
							[Cyn]	[Plr]	[Anth]	[Cyn]/[Anth]
108	2004	22	1	B-PP-102?		GH	10	35	45	0.223
109	2005	5073	2	B-PP-102?	5	HF	50	160	210	0.239
110	2005	5074	2	B-PP-102?	5	HF	40	204	243	0.163
111	2005	5075	2	B-PP-102?	4	HF	52	200	252	0.206
112	2004	37	1	B-PP-103		GH	19	68	87	0.219
113	2005	5151	3	B-PP-103	5	HF	67	613	680	0.098
114	2005	5156	3	B-PP-103	5	HF	53	364	417	0.128
115	2004	38	1	B-PP-104		GH	24	67	91	0.265
116	2005	5152	3	B-PP-104	4	HF	63	226	289	0.218
117	2005	5157	3	B-PP-104	4	HF	90	328	418	0.215
118	2004	65	1	B-PP-105		GH	24	180	204	0.120
119	2005	5100	3	B-PP-105	9	HF	36	323	359	0.101
120	2005	5101	3	B-PP-105	9	HF	60	376	436	0.137
121	2005	5102	3	B-PP-105	9	HF	85	470	555	0.152
122	2005	5153	3	B-PP-105	3	HF	83	607	689	0.120
123	2005	5158	3	B-PP-105	5	HF	78	359	437	0.179
124	2004	39	1	B-PP-106		GH	27	212	239	0.112
125	2005	5154	3	B-PP-106	3	HF	99	789	888	0.112
126	2005	5159	3	B-PP-106	4	HF	45	344	389	0.116
127	2004	47	1	B-PP-107		GH	32	117	149	0.213
128	2005	5160	3	B-PP-107	5	HF	51	140	191	0.266
129	2004	64	1	B-PP-108		GH	20	35	55	0.360
130	2004	40	1	B-PP-109		GH	19	45	64	0.300
131	2005	5097	3	B-PP-109	4	HF	48	187	235	0.203
132	2005	5098	3	B-PP-109	4	HF	41	155	196	0.209
133	2005	5099	3	B-PP-109	3	HF	26	109	135	0.191
134	2005	5094	3	B-PP-110	4	HF	57	165	222	0.257

Table C.6 Octoploid 'Bountiful', 'Pink Panda' and B-PP Accessions ripe fruit anthocyanidin content continued.

Case	Collection Year	Run #	Phase	Accession	No of Berries	Collection Location	Anthocyanidins (µg/g FDW)			
							[Cyn]	[Pir]	[Anth]	[Cyn]/[Anth]
135	2005	5095	3	B-PP-110	4	HF	50	93	143	0.348
136	2005	5096	3	B-PP-110	4	HF	66	159	224	0.293
137	2004	66	1	B-PP-111?		GH	29	414	443	0.066
138	2005	5010	2	B-PP-111?	4	HF	96	1300	1396	0.068
139	2004	24	1	B-PP-112		GH	8	17	25	0.319
140	2005	5012	2	B-PP-112	4	HF	24	132	156	0.154
141	2005	5013	2	B-PP-112	4	HF	28	121	149	0.188
142	2004	20	1	B-PP-200		GH	12	54	66	0.186
143	2005	5014	2	B-PP-200	4	HF	29	164	193	0.151
144	2005	5015	2	B-PP-200	4	HF	22	74	96	0.229
145	2005	5017	2	B-PP-201?	4	HF	27	104	130	0.205
146	2005	5018	2	B-PP-201?	5	HF	33	123	156	0.209
146	2005	5005	2	Pink Panda	4	HF	197	304	502	0.393
148	2005	5103	3	Pink Panda	3	HF	189	247	437	0.434
149	2005	5104	3	Pink Panda	5	HF	303	478	781	0.388
150	2005	5127	3	Pink Panda	3	HF	354	723	1077	0.329