J. Amer. Soc. Hort. Sci. 127(1):89–97. 2002.

Genotypic and Environmental Variation in Antioxidant Activity, Total Phenolic Content, and Anthocyanin Content among Blueberry Cultivars

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Additional index words. *Vaccinium* sp., blueberry breeding, genotype × environment interaction

Abstract. Dietary antioxidants may have a role in preventing some of the chronic diseases in humans resulting from free radical oxidation of lipids and other cellular components. Blueberries (*Vaccinium* L. sp.) are considered one of the best fresh fruit sources of antioxidants, and there is the potential to increase the antioxidant activity further through breeding. Thus, the variability of fruit antioxidant activity (AA) was examined among a set of 16 highbush and interspecific hybrid cultivars grown at locations in Minnesota (MN), Michigan (MI), and Oregon (OR) over 2 years (1998 and 1999) to determine effects of genotype, year, and location. Nine cultivars were common to all three locations in both years. Antioxidant activity, total phenolic content (TPH), and total anthocyanin content (ACY), were determined in triplicate samples from each genotype. Cultivars differed significantly ($P \leq 0.05$) in AA, TPH, and ACY both within and over locations. The single location mean AA for all cultivars changed significantly between the 2 years in OR and in MI, while the single location mean for TPH differed between the 2 years in MN and MI. Changes in cultivar rank were significant for AA, TPH, and ACY between years within each location. Significant changes in rank for TPH and ACY were also noted between pairs of locations as well. Pearson's correlation for AA (based on cultivar means) appeared highest between MN and OR ($r = 0.90$) and MN and MI ($r = 0.69$) in 1998; correlations between locations for the combined years were 0.74 for MN and OR, 0.55 for MN and MI and 0.45 for MI and OR. For the group of nine cultivars, AA correlated well with TPH within each location, with r ranging from 0.67 to 0.95 for data from individual and combined years. Correlation of AA with ACY at each location was lower than that for AA with TPH, in both individual and combined years. This study demonstrates significant genotype × environment interaction for AA in blueberry.

The effect of diet on human health is of increasing interest to the scientific community and the general public, as more individuals are taking an active role in maintaining their health to avoid or delay the onset of chronic and degenerative diseases. Recent scientific evidence points to oxidation of lipids and other cellular components as playing a central role in chronic disease processes such as atherosclerotic cardiovascular disease (Esterbauer et al., 1992), neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, cataract formation, immune dysfunction, and carcinogenesis (reviewed by Diplock et al., 1998).

Some epidemiological studies also suggest that antioxidant consumption can positively influence health. Diets high in flavonoid antioxidants have been associated with lower rates of mortality from coronary heart disease (Hertog et al., 1993, 1997; Knekt et al., 1996), lung cancer (Knekt et al., 1997), stroke (Keli et al., 1996), and nonfatal heart attack in smokers (Hirvonen et al., 2001). Increased fresh fruit and vegetable consumption has been associated with reduced risk of stroke (Joshipura et al., 1999), early mortality from stroke (Acheson and Williams, 1983), risk of cardiovascular disease in women (Liu et al., 2000) and reduced risk of some types of cancer (Steinmetz and Potter, 1996; Zhang et al., 2000). A specific role for flavonoid antioxidants in the latter associations has not been defined, and intake of other antioxidants, such as vitamin C and carotenoids, may be a significant factor (Yochum et al., 2000). Persons with high plasma vitamin C levels and those with moderate or high plasma carotenoid levels may have decreased risk of certain types of cataracts (Jacques et al., 1988). Dietary and supplemental vitamin C, vitamin E, and certain carotenoids have been variously reported to decrease the risk of cataracts in some populations (Cumming et al., 2000; Hankinson et al., 1992; Lyle et al., 1999; Mares-Perlman et al.; 1995). Several animal models have demonstrated beneficial health effects of dietary supplementation with components high in antioxidants. For example, in rats, dietary supplementation with fruit [strawberry (*Fragaria ×ananassa* Duchesne) and blueberry (*Vaccinium* sp.)] or vegetable [spinach (*Spinacia oleracea* L.)] extracts with high in vitro antioxidant activity (AA) measured as oxygen radical absorbance capacity (ORAC) slowed and even reversed some of the neurological deficits associated with aging (Joseph et al., 1998, 1999). Supplementation of rat diets with procyanidins from grape (*Vitis vinifera* L.) seeds increased plasma AA and decreased heart muscle damage following reduced coronary artery blood flow and subsequent reperfusion (Facino et al., 1999).

Received for publication 20 Mar. 2001. Accepted for publication 11 Sept. 2001. We thank Frank Martin and Faye Propsom for statistical advice. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

Diets high in fruit and vegetables and those including red wine are typically high in antioxidants, including vitamin C, carotenoids, and phenolic compounds such as flavonoids, anthocyanins, phenolic acids, and catechins and their oligomers (procyanidins). Some studies report that ingestion of diets high in fruit and vegetables (Cao et al., 1998) or consumption of red wine (Maxwell et al., 1994; Nigdikar et al., 1998; Whitehead et al., 1995) can increase plasma or serum AA in humans. Among fresh fruit, blueberries have exceptionally high AA. Prior et al. (1998) found that AA, as determined by ORAC, ranged from 13.9 to 45.9 μmol Trolox equivalents (TE)/g fresh fruit among representative genotypes of lowbush (*Vaccinium angustifolium* Ait.), northern and southern highbush (*Vaccinium corymbosum* L.), and rabbiteye (*Vaccinium ashei* Reade) blueberries. A survey of 12 other fruit (Wang et al., 1996) demonstrated AA ranging from less than 1 μmol TE/g fresh fruit for melon (*Cucumis melo* L.) up to ≈15 μmol TE/g fresh fruit for strawberry. Antioxidant activity correlates well with total phenolic content and anthocyanin content in blueberry (Ehlenfeldt and Prior, 2001; Prior et al., 1998).

Antioxidant intake may be increased by breeding fruit, such as blueberry, with higher AA. The success of breeding depends on identifying genetic variation for the trait, and knowledge of the impacts of environmental parameters on its expression. However, information on the variation among genotypes and the effects of environmental parameters is scant. In their survey of *Vaccinium* genotypes, Prior et al. (1998) showed variation in AA among 19 named cultivars representing several species, but only one cultivar ('Jersey') was grown at multiple locations. Similarly, a survey of 87 highbush blueberry cultivars reported by Ehlenfeldt and Prior (2001) included data collected in only a single year and location.

As part of a study examining variability and heritability of AA in the blueberry breeding population at the University of Minnesota, we examined a set of cultivars that were grown at sites in Minnesota, Michigan, and Oregon. Fruit were collected from all three locations in two consecutive growing seasons to determine the effect of genotype and environment on AA, and the correlation of this trait with TPH and ACY.

Table 1. Mean antioxidant activity of fresh blueberry fruit (μmol Trolox equivalents/g berries) for cultivars harvested in Minnesota (MN), Oregon (OR), and Michigan (MI) in 1998 and 1999, with nine cultivars harvested at all three locations in both years listed first, and cultivars harvested at two of the three locations in both years listed next; yearly means and location means for the subset of nine cultivars and for all cultivars are provided. Analyses of variance (bottom left) and covariance with adjustment for berry weight (bottom right) for the nine cultivars common to all locations.

	Location						
	MN		OR		MI		Cultivar
Cultivar	1998	1999	1998	1999	1998	1999	mean
Bluecrop	27.3	35.3	32.1	27.8	13.6	19.7	26.0
Bluegold	53.1	34.9	61.1	34.2	36.0	36.0	42.6
Bluetta	24.3	27.8	31.8	39.1	17.9	24.0	27.8
Duke	39.8	30.9	37.3	39.2	21.6	20.3	31.5
Jersey	25.9	29.3	22.5	16.7	22.3	20.0	22.8
Northblue	28.9	29.7	23.3	28.1	23.8	28.5	27.1
Northland	39.2	37.6	38.0	32.4	33.5	33.6	35.7
Northsky	21.4	23.0	27.8	28.7	26.6	26.0	25.6
Patriot	26.2	29.0	24.0	27.0	20.0	28.2	25.7
Overall mean (9 cultivars)							29.4
Yearly mean (9 cultivars)	31.8	30.8	33.1	30.4	23.9	26.3	
Location mean (9 cultivars)	31.3		31.7		25.1		
Bounty	23.7	29.9	25.1	22.7	20.5	---	
Chippewa	11.4	25.9	---	25.9	14.9	26.3	
Legacy	---	---	39.2	28.6	22.6	25.7	
Little Giant	---	---	---	47.3	24.0	37.4	
Nelson	42.8	34.8	29.8	34.5	---	18.6	
Northcountry	29.5	29.1	---	35.9	28.6	33.5	
Polaris	21.4	22.9	---	22.3	18.8	19.9	
Yearly mean (all cultivars)	29.6	30.0	32.7	30.7	23.0	26.5	
Location mean (all cultivars)	29.8		31.7		24.8		

Source	df	Mean square	F	*P*	df	Mean square	F	*P*
Berry weight	---	---	---	---	1	392.7	3.1	0.093
Cultivar (C)	8	1395.5	7.7	<0.0001	8	1477.8	11.5	<0.0001
Location (L)	2	1492.7	11.9	0.037	2	1642.1	6.0	0.089
C × L	16	235.4	1.3	0.277	16	204.0	1.6	0.149
Year/loc	3	125.2	6.4	<0.0005	3	272.3	13.8	<0.0001
C × year/L	24	182.9	9.3	<0.0001	24	128.6	6.5	<0.0001
Extract (C × year/L)	108	19.5	4.2	<0.0001	107	19.7	4.3	<0.0001
Error	162	4.6			162	4.6		

Materials and Methods

Fruit. Fruit were harvested during June and July in 1998 and 1999 from nine highbush and interspecific hybrid cultivars (listed in Table 1) common to three locales in the United States: Becker, Minn. (MN), Grand Junction, Mich. (MI), and Aurora or Corvallis, Ore. (OR). Berries were also harvested from six additional cultivars common to two of the three locations in both years (Table 1). Generally, harvest for the same genotype was made from the same plants in both years. About 100 g of sound, ripe berries were harvested when 40% to 70% of the fruit on the bushes were ripe; overripe fruit were discarded. In MN and OR, harvested berries were held in polyethylene bags on ice until frozen at –80 °C the same day. In MI, harvested berries were held 1 to 2 h in polyethylene bags at ambient field temperature prior to freezing at –70 °C, and some leakage of juice occurred. Berries from OR and MI were shipped to St. Paul, Minn., on dry ice without thawing. All fruit was maintained in St. Paul at –80 °C until extraction.

Extractions. Extractions were performed under reduced-light conditions. About 10 g of frozen, fully colored berries were weighed and counted, and allowed to thaw slightly. Ice-cold acidified (0.1% HCl) methanol was added to the berries (1:1, w/v), which were homogenized by grinding for 2 min with a Polytron homogenizer (Kinematica, Luzern, Switzerland). The container and homogenizer probe were rinsed with a second identical volume of acidified methanol. After standing on ice for a minimum of 20 min, the homogenate was filtered by gravity though 11-μm filter paper (P5 qualitative; Fisher Scientific, Pittsburgh, Pa.) and the filtrate collected. The residue was mixed with a third identical volume of acidified methanol and filtered again. Filtrates were combined and the volumes standardized to 30 mL. An 8 mL aliquot was stored at –80 °C until assayed, generally within 2 to 6 months of preparation. Three extracts were prepared per genotype with the exception of 'Little Giant' from OR (1999), for which there were sufficient berries for only a single extract. Duplicate determinations of AA, TPH, and ACY were made on each extract.

Table 2. Mean total phenolic content of fresh blueberry fruit (mg chlorogenic acid equivalents/100 g berries) for cultivars harvested in Minnesota (MN), Oregon (OR), and Michigan (MI) in 1998 and 1999, with nine cultivars harvested at all three locations in both years listed first, and cultivars harvested at two of the three locations in both years listed next; yearly means and location means for the subset of nine cultivars and for all cultivars are provided. Analyses of variance (bottom left) and covariance with adjustment for berry weight (bottom right) for the nine cultivars common to all locations.

	Location						
	MN		OR		MI		Cultivar
Cultivar	1998	1999	1998	1999	1998	1999	mean
Bluecrop	433	461	455	417	295	344	401
Bluegold	760	576	670	476	580	562	604
Bluetta	426	482	593	704	355	477	506
Duke	667	587	622	725	432	395	571
Jersey	516	464	453	350	429	454	444
Northblue	472	495	454	481	368	438	451
Northland	631	574	626	561	610	573	596
Northsky	471	391	425	382	449	391	418
Patriot	406	528	404	569	360	416	447
Overall mean (9 cultivars)							493
Yearly mean (9 cultivars)	531	506	522	518	431	450	
Location mean (9 cultivars)	519		520		441		
Bounty	397	452	405	430	386	---	
Chippewa	373	373	---	360	297	345	
Legacy	---	---	414	518	438	430	
Little Giant	---	---	---	725	499	670	
Nelson	599	528	487	519	---	352	
Northcountry	510	556	---	592	473	520	
Polaris	476	428	---	492	378	382	
Yearly mean (all cultivars)	510	493	501	519	423	450	
Location mean (all cultivars)	502		510		437		

Source	df	Mean square	F	*P*	df	Mean square	F	*P*
Berry weight	---	---	---	---	1	266644.1	20.3	<0.0005
Cultivar (C)	8	222555.4	8.8	<0.0001	8	234765.1	18.2	<0.0001
Location (L)	2	226304.4	25.0	0.013	2	260449.8	8.3	0.060
C × L	16	46143.9	1.8	0.089	16	33815.0	2.6	0.018
Year/L	3	9063.5	4.0	0.009	3	31234.7	14.3	<0.0001
C × year/L	24	25322.9	11.2	<0.0001	24	13118.5	6.0	<0.0001
Extract (C × year/L)	108	2251.8	118.2	<0.0001	107	2189.1	114.9	<0.0001
Error	162	19.0			162	19.0		

Antioxidant activity. The AA assay was based on the methods used by Barclay et al. (1984) to study oxidation of linoleic acid in heterogeneous (lipid-aqueous) systems using lipid- and water-soluble initiators and inhibitors of oxidation; and by Fuhrman et al. (1995) to study susceptibility of human plasma and low-density lipoprotein to lipid peroxidation following consumption of red wine. The aqueous peroxyl radical generator 2,2'-azobis amidinopropane dihydrochloride (AAPH; Wako Chem., Richmond, Va.) (10 μM final concentration) was used to induce oxidation of a linoleic acid methyl ester (Sigma Chem., St. Louis) (3.1 mg·mL^{-1} final concentration) emulsion prepared in 0.1 M sodium phosphate buffer (pH 7.0) containing Tween 20 (1.0% w/v). Antioxidant activity was determined by measuring inhibition of lipid oxidation achieved by addition of diluted blueberry extract to the emulsion (final extract dilution 1:800; total reaction volume of 1 mL.). The oxidation reaction was carried out at 37 °C for 130 min, and terminated by placing the reaction tubes on ice. Oxidation products were detected as malondialdehyde equivalents in a thiobarbituric acid reaction based on the method by Lee et al. (1992) and measured at 535 nm (Beckman DU-50 spectrophotometer, Beckman Instruments, Fullerton, Calif.). An antioxidant standard curve was prepared with each assay, substituting the water-soluble vitamin E analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Aldrich Chem., Milwaukee, Wis.) for the blueberry extract at appropriate dilutions. The standard curve was linear between 0 and 30 μM TE (final concentration). Results are expressed as μmol TE/g fresh fruit.

Total phenolic content. The method of Coseteng and Lee (1987) was used, with an incubation time of 90 min for color development. Results are expressed as chlorogenic acid equivalents (CAE; mg/100 g fresh fruit) which, under the conditions of this assay, were determined to be ≈1.8× those of gallic acid (GAE).

Anthocyanin content. Extracts were diluted in acidified methanol to obtain an absorbance between 0.200 and 1.000 at 530 nm. Because the extracts were freshly prepared from frozen fruit, and did not undergo extensive processing or significant brown-

Table 3. Mean total anthocyanin content of fresh blueberry fruit (mg cyanidin 3-glucoside equivalents/100 g berries) for cultivars harvested in Minnesota (MN), Oregon (OR), and Michigan (MI) in 1998 and 1999, with nine cultivars harvested at all three locations in both years listed first, and cultivars harvested at two of the three locations in both years listed next; yearly means and location means for the subset of nine cultivars and for all cultivars are provided. Analyses of variance (bottom left) and covariance with adjustment for berry weight (bottom right) for the nine cultivars common to all locations.

	Location						
	MN		OR		MI		Cultivar
Cultivar	1998	1999	1998	1999	1998	1999	mean
Bluecrop	118	123	102	108	83	99	105
Bluegold	269	229	216	140	219	182	209
Bluetta	199	223	263	323	164	222	232
Duke	273	274	241	278	201	149	236
Jersey	198	196	157	147	174	185	176
Northblue	160	169	159	165	136	168	160
Northland	250	231	260	245	209	201	233
Northsky	164	152	116	117	162	121	139
Patriot	140	173	143	191	140	144	155
Overall mean (9 cultivars)							183
Yearly mean (9 cultivars)	197	197	184	190	166	163	
Location mean (9 cultivars)	197		187		164		
Bounty	141	197	148	173	159	---	
Chippewa	110	118	---	103	96	110	
Legacy	---	---	110	180	183	181	
Little Giant	---	---	---	205	225	307	
Nelson	179	180	125	126	---	110	
Northcountry	214	220	---	201	185	175	
Polaris	214	189	---	188	163	165	
Yearly mean (all cultivars)	188	191	170	181	167	168	
Location mean (all cultivars)	190		176		168		

Source	df	Mean square	F	*P*	df	Mean square	F	*P*
Berry weight	---	---	---	---	1	89957.8	37.6	<0.0001
Cultivar (C)	8	79831.6	23.3	<0.0001	8	73050.3	30.5	<0.0001
Location (L)	2	29698.0	72.3	0.003	2	33561.3	26.9	0.012
C × L	16	9826.5	2.9	0.010	16	8523.4	3.7	0.003
Year/L	3	410.6	1.1	0.333	3	1248.7	3.5	0.018
C × year/L	24	3431.3	9.6	<0.0001	24	2392.9	6.7	<0.001
Extract (C × year/L)	108	357.7	163.4	<0.0001	107	359.4	164.2	<0.001
Error	162	2.2			162	2.2		

Table 4. Analyses of variance for antioxidant activity of nine blueberry cultivars (listed at the top of Table 1), for each pair of locations at which fruit were harvested in 1998 and 1999.[z]

		MN and MI		MN and OR		MI and OR	
Source	df	Mean square	F	Mean square	F	Mean square	F
Cultivar (C)	8	829.0	7.1^{***}	1215.1	4.9^{**}	982.3	5.5^{**}
Location (L)	1	2095.3	23.9^{*}	8.7	0.8^{NS}	2374.1	13.5^{NS}
C × L	8	200.6	1.7^{NS}	178.9	0.7^{NS}	326.8	1.8^{NS}
Year/L	2	87.6	4.7^{*}	112.3	6.5^{**}	175.8	7.7^{***}
C × year/L	16	117.1	6.3^{***}	250.1	14.4^{***}	250.1	7.8^{***}
Extract (C × year/L)	72	18.6	4.5^{***}	17.3	3.4^{***}	22.8	4.9^{***}
Error	108	4.1		5.0			

[z]MN = Minnesota, MI = Michigan, and OR = Oregon.
^{NS,*,**,***}Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

ing, a pH differential method for determining anthocyanin content (Wrolstad, 1976) was considered unnecessary. Results are expressed as mg cyanidin 3-glucoside equivalents /100 g fresh fruit, using a molar extinction coefficient of 29,600.

Statistical analyses. Analyses of variance (ANOVA) were performed with genotypes and locations as fixed effects, and years and extracts as random effects. Years were nested within locations, and extracts within genotypes. For all analyses, $P = 0.05$. Correlations were estimated on a cultivar mean basis. All analyses were performed using SPSS for Windows, version 8.0 (SPSS, Inc., Chicago).

Results

Means for AA, TPH, and ACY for cultivars that were grown in at least one location in both years are presented by location and year in Tables 1, 2, and 3, respectively. The means for the nine cultivars that were common to all three locations in both years are listed first, and the yearly and location means for this group are also shown separately. The additional seven cultivars listed are those that were grown in two of the three locations in both years. The ANOVA for AA, TPH, and ACY based on the nine common cultivars (Tables 1, 2, and 3, respectively) at all three locations show that all main effects were significant, with the exception of years within locations for ACY (Table 3). Cultivar × year interactions within location were significant for all three variables, but cultivar × location interactions were significant only for ACY.

Results in Table 1 and the ANOVA performed for each pair of locations (Table 4) demonstrate that mean AA of the nine cultivars did not differ significantly between OR and MN or OR and MI, but that MI was significantly lower than MN. The differences between MI and MN were apparent for both combined years (Table 1) and individual years (analyses not presented). Additionally, the individual year paired-location analyses did show significant differences in mean AA for the nine cultivars between OR and MI in both 1998 and 1999, even though the combined year analysis did not. Differences in AA among cultivars in the three-location analysis (Table 1) and each paired-location analysis (Table 4) were evident in individual year analyses within each location (analyses not presented), but remained significant in combined-year analyses only within MI. The ANOVA in Table 4 also show that no significant changes in cultivar rank occurred between any pair of locations. The nine-cultivar mean changed significantly between 1998 and 1999 at OR and MI, but not at MN (Table 1; single-location ANOVA not presented). Within MN and within OR, there was a significant change in cultivar rank between years. The three cultivars with the highest AA in 1998 ('Bluegold', 'Northland', and 'Duke') were the same at MN and OR. At both of these locations, 'Bluegold' showed much lower AA in 1999. This marked change contributed substantially to the observed decreased range in AA among all cultivars from 1998 to 1999 at MN and OR. At MI, 'Bluegold' was the highest-ranking cultivar both years, and the overall range of activity was similar in 1998 and 1999.

As shown in Tables 1, 2, and 3, there were more than nine cultivars common to both years within each location: three in OR, five in MI, and five in MN. With the inclusion of the five additional cultivars in MN, significant differences ($P \leq 0.05$) among the cultivars could be distinguished in combined year analysis (analysis not presented). However, addition of the three cultivars in OR did not alter substantially the analysis for either the main effects or their interactions (analyses not presented). The cultivar × year interaction was significant in MI with the inclusion of five additional cultivars ($P \leq 0.001$); in particular, 'Little Giant', 'Patriot', and 'Chippewa' demonstrated significant rank changes between 1998 and 1999.

Mean TPH by location and year is summarized in Table 2 and shows that the ANOVA for TPH for the nine common cultivars were similar to that for AA. As with AA, the ANOVA for TPH by each pair of locations showed that the mean TPH of the cultivars did not differ significantly between MN and OR for combined years (Table 5) or individual years (analyses not presented), but it did differ significantly between MN and MI. In contrast to AA, however, mean TPH of the nine cultivars also differed significantly between MI and OR. Mean TPH differed between years within MN and within MI (data not presented). Whereas AA showed no cultivar × location interaction in any paired-location analysis, significant changes in cultivar rank or scale were evident for TPH between MI and OR (Table 5). For the other paired analyses (MN and MI, MN and OR), differences among genotypes across years and locations could be distinguished. In both MN and MI, genotypic differences in TPH could be distinguished, but significant change in cultivar rank occurred between years within each of the three locations (analyses not presented). In contrast to the rank changes noted for AA in MN, the three cultivars with the highest TPH in MN remained the same in both years, although the TPH levels decreased. The marked decrease in AA noted in 'Bluegold' between 1998 and 1999 was reflected in the decreased TPH in this cultivar. In MN, rank changes occurred principally among the lower-ranking cultivars. Inclusion of additional cultivars at each location did not change the results of the within-location ANOVA for TPH, except in OR,

Table 5. Analyses of variance for total phenolic content of nine blueberry cultivars (listed at the top of Table 2), for each pair of locations at which fruit were harvested in 1998 and 1999.[z]

		MN and MI		MN and OR		MI and OR	
Source	df	Mean square	F	Mean square	F	Mean square	F
Cultivar (C)	8	160234.4	8.9***	180540.3	5.6**	150479.5	5.9**
Location (L)	1	333007.3	24.9*	120.3	0.01^{NS}	345785.6	66.5*
C × L	8	24525.5	1.4^{NS}	43779.5	1.3^{NS}	70126.9	2.8*
Year/L	2	13387.1	5.4**	8600.4	3.7*	5202.9	2.6^{NS}
C × year/L	16	18040.5	7.2***	18040.5	14.4***	254322.0	12.9***
Extract (C × year/L)	72	2489.9	146.3***	2297.4	100.8***	1968.1	113.6***
Error	108	17.0		22.8		17.3	

[z]MN = Minnesota, MI = Michigan, and OR = Oregon.
^{NS,*,**,***}Nonsignificant or significant at $P \leq 0.05$, 0.01 or 0.001, respectively.

where cultivar differences were distinguishable upon the addition of three cultivars to the analysis (data not presented); however, the cultivar × year interaction remained significant.

Mean ACY for the nine cultivars is summarized in Table 3 by location and year. The ANOVA for ACY (Table 3) shows that location and cultivar were significant main effects, but that mean ACY did not differ significantly between years within each location. Paired-location analyses showed significant changes in rank between MN and OR and between MI and OR (Table 6). Rank changes between years within each location were also noted. Inclusion of additional cultivars at each location altered results of the ANOVA for ACY only in OR; mean ACY differed significantly between years with inclusion of 'Bounty', 'Legacy', and 'Nelson' (analysis not presented).

Anthocyanins are among the phenolic compounds that are significant contributors to the high AA in blueberry (Kalt, 1999; Prior, 1998); they are confined principally to the fruit skin and, thus, variation in AA among cultivars may simply reflect smaller berry size in those cultivars with higher activity. Ehlenfeldt and Prior (2001) demonstrated a correlation of 0.90 between AA (as ORAC) expressed on per weight basis and AA on an estimated surface area basis in highbush blueberry. Thus, we used berry weight rather than an estimate of surface area in our analyses. The relationships between berry weight, a surrogate measure of berry size, and AA, TPH, and ACY are shown in Fig. 1A, B, and C, respectively. Correlations of berry size and AA ($r = -0.28$, $P \leq 0.05$), TPH ($r = -0.44$, $P \leq 0.01$), and ACY ($r = -0.41$, $P \leq 0.01$) were significant. ANOVA performed with berry weight as a covariate demonstrated that weight was a significant factor for TPH and ACY (Tables 2 and 3, respectively) but not for AA (Table 1). Variation among cultivars for TPH and ACY was still highly significant when adjusted for berry weight.

Correlations among AA, TPH, and ACY at each location are given on a cultivar mean basis for the nine common cultivars in Table 7, combining data from both years. In general, correlations among these variables within each location were moderate to high. Correlations of individual variables across locations tended to be higher between OR and MN than MI and MN or MI and OR. Correlation of AA with TPH was high in MN and MI ($r = 0.88$ and 0.89, respectively; $P \leq 0.01$), and moderate in OR ($r = 0.75$, $P \leq 0.01$). AA correlated to a lesser degree with ACY than with TPH in all locations (for AA and ACY, $r = 0.61$ in MN and $r = 0.59$ in MI, $P \leq 0.01$ for both; $r = 0.50$, $P \leq 0.05$ in OR). Inclusion of additional cultivars within each location did not change the correlations substantially (data not presented).

Discussion

Results herein have important implications for blueberry breeding programs that have increased AA as a breeding objective. Variation for AA was significant among the cultivars across locations and years, as well as within individual years in each location. However, ability to distinguish within-location cultivar differences in combined-year analysis was hampered in some locations when only the nine common cultivars were assessed, and improved when a larger set of cultivars was evaluated. While differences in overall mean AA among locations occurred, there was no significant change in rank among locations. However, AA showed considerable genotype × year interaction. If the relative performance in 1 year is only moderately reliable in predicting

Table 6. Analyses of variance for anthocyanin content of nine blueberry cultivars (listed at the top of Table 3), for each pair of locations at which fruit was harvested in 1998 and 1999.[z]

		MN and MI		MN and OR		MI and OR	
Source	df	Mean square	F	Mean square	F	Mean square	F
Cultivar (C)	8	42566.6	15.8***	71800.3	22.6***	55122.8	12.5***
Location (L)	1	56133.1	852.4**	4760.1	8.6^{NS}	28200.8	45.8*
C × L	8	4596.2	1.7^{NS}	11671.4	3.7*	13212.0	3.0*
Year/loc	2	65.9	0.2^{NS}	550.5	1.6^{NS}	615.6	1.8^{NS}
C × year/L	16	2693.4	7.0***	3181.2	9.1***	4419.4	13.1***
Extract (C × year/L)	72	385.3	182.5***	350.8	142.3***	337.0	169.3***
Error	108	2.1		2.5		2.0	

[z]MN = Minnesota, MI = Michigan, and OR = Oregon.
^{NS,*,**,***}Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

relative performance in other years, then selection of genotypes based on AA may require assessments made over several years. Substantial year-to-year variability occurred in some cultivars, notably 'Bluegold' harvested in MN and in OR. Part of the decrease in AA may be attributable to the increased size of the berries in 1999 as compared with 1998, (≈12% in MN and 23% in OR), but the factors contributing to difference in berry size, or contributing more directly to the change in AA, between years are not clear. Even with such year-to-year variability, it should be noted that differences between years at each location were considerably smaller than variation among cultivars. Prior et al. (1998) found no substantial difference in AA in 'Jersey' harvested at sites in Oregon, Michigan, and New Jersey in a single year. Antioxidant activity in 'Jersey' in our study was similar in OR and MI in 1998, but differed between these locations and MN, and showed variability between years. This emphasizes that stability for AA in a single cultivar may be difficult to demonstrate with limited testing across years or locations.

Antioxidant activity, TPH, and ACY were substantially lower in MI than in MN. This may reflect differences in climate and cultural practices among locations, including differences in ultraviolet radiation, high temperature or water stress, or mineral nutrient availability. As these and other environmental variables were not controlled in this study, their effects would be confounded and cannot be addressed. The lower AA, TPH, and ACY in MI might also reflect differences in postharvest handling (e.g., fruit were held for 1 to 2 h at ambient field temperature in MI, but only 15 min at ambient field temperature in MN). This emphasizes the necessity of maintaining consistent conditions during harvest, handling, and analysis of fruit when comparing results of antioxidant studies, and when evaluating genotypes over several years or in different locations in a breeding project. This point is also illustrated by comparing the ranking for AA of 13 of the cultivars tested in this study to ranks reported by Ehlenfeldt and Prior (2001), using ORAC to test blueberry fruit harvested in a single year in New Jersey. These cultivars show substantial differences in rank between these two studies. The studies differ in extraction and assay methods, timing of fruit collection (percentage of ripe berries on the bush), and location of harvest, all of which may have contributed to differences in rank. Studies in our laboratory (Connor, 2001) suggest that timing of fruit collection may not significantly impact AA, as fully mature (100% blue) berries harvested at two different stages of bush matu-

Fig. 1. Correlation of mean berry weight in grams with (**A**) mean antioxidant activity in µmol Trolox equivalents (TE)/g fresh weight (FW), (**B**) total phenolic content in mg chlorogenic acid equivalents (CAE)/ 100 g FW, and (**C**) anthocyanin content in mg cyanidin 3-glucoside equivalents (c3g eq)/100 g FW, based on n = 86 means from 14 blueberry cultivars in 1998 and 1999 in Minnesota (MN), 12 cultivars in 1998 and 16 cultivars in 1999 in Oregon (OR) and 15 cultivars in 1998 and 1999 in Michigan (MI).

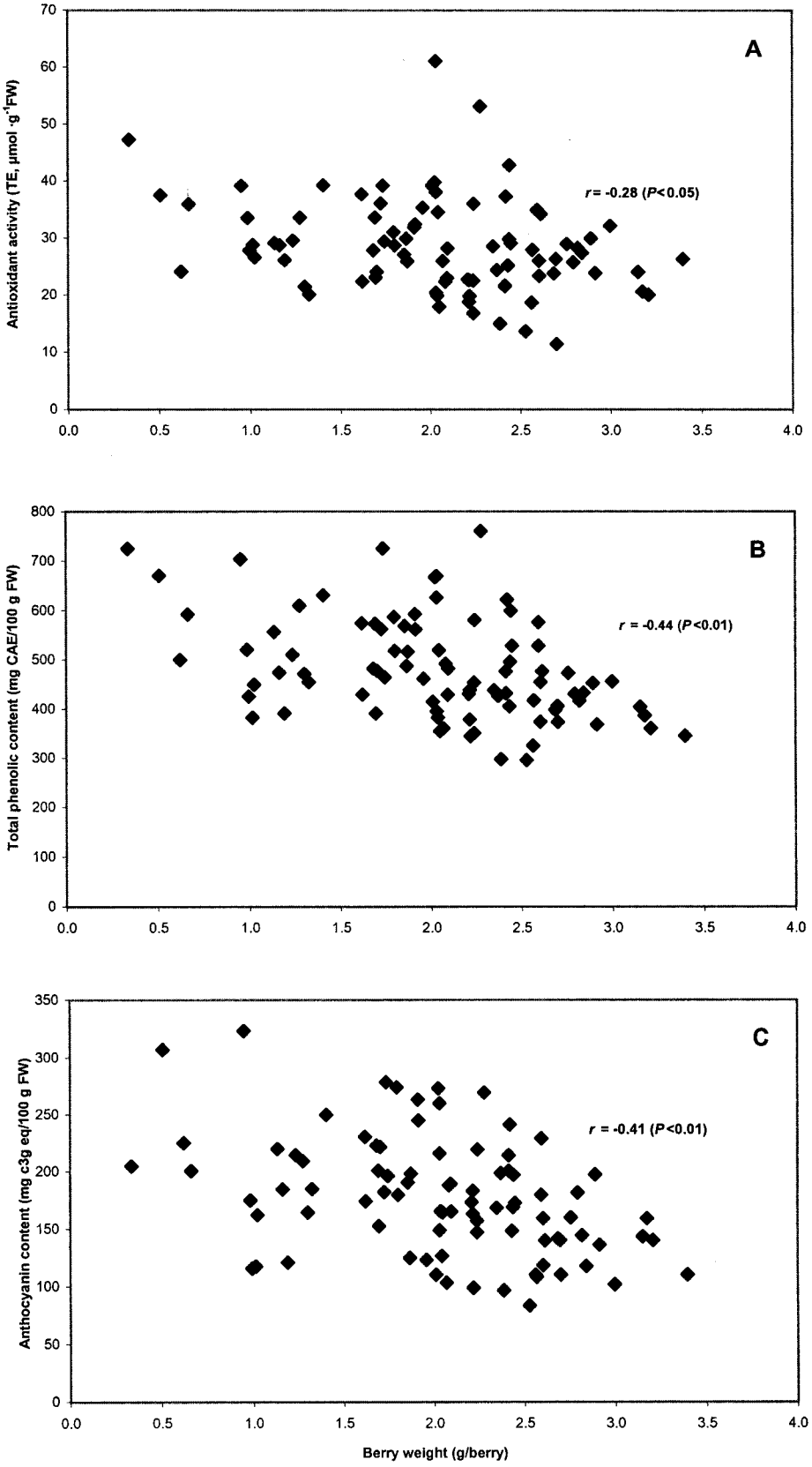

Table 7. Correlations within and among locations for blueberry fruit antioxidant activity (AA), total phenolic content (TPH), and anthocyanin content (ACY), based on nine cultivars common to all three locations, harvested in 2 years at Minnesota (MN), Oregon (OR), and Michigan (MI).

		MN			OR			MI		
Location	Variable	AA	TPH	ACY	AA	TPH	ACY	AA	TPH	ACY
MN	AA	1.0	0.88**	0.61**	0.74**	0.48*	0.31	0.55*	0.62**	0.49*
	TPH		1.0	0.83**	0.75**	0.65**	0.46	0.61**	0.70**	0.65**
	ACY			1.0	0.64**	0.77**	0.75**	0.46	0.68**	0.80**
OR	AA				1.0	0.75*	0.50*	0.45	0.49*	0.46
	TPH					1.0	0.90**	0.24	0.38	0.55*
	ACY						1.0	0.17	0.39	0.67**
MI	AA							1.0	0.89**	0.59**
	TPH								1.0	0.82**
	ACY									1.0

*,**Significant at $P \leq 0.05$ or 0.01, respectively.

rity (30% to 50% vs. 60% to 80% ripe berries on the bush) did not differ significantly for AA.

Another source of variation may be in judging the ripeness of the berries based on color alone, as berry size may increase slightly after full coloration, and could alter AA when expressed on a per-weight basis. However, such variation would be expected to affect all locations and years equally.

One of the cultivars with the highest level of AA in 1998 ('Bluegold') showed substantially lower activity in 1999 in MN and OR. Such marked year-to-year variability is apparently not unusual for some genotypes, as it was also demonstrated by 'Chippewa' grown in MN and MI and by 'Little Giant' in MI.

Analyses of TPH and ACY also showed significant genotype by environment interactions involving both year and location, but in most respects were similar to analysis of AA. Blueberries are high in anthocyanins and other phenolic compounds, including chlorogenic acid, which are the major contributors to their high AA (Kalt et al., 1999; Prior et al., 1998). Thus, a change in AA could be expected to reflect a change in TPH, similar in direction and magnitude. However, plant phenolic compounds do not all have the same antioxidant capacity in vitro (Cao et al. 1997; Rice-Evans et al. 1996; Wang et al. 1997). Thus, if specific blueberry phenolic compounds such as anthocyanins are produced in more or less consistent concentration in the fruit from year to year regardless of the sufficiency of the total phenolic pool, or at the expense of compounds derived from the pool, the AA could conceivably vary while the ACY remains the same. This may explain why significant differences in AA between years at each location were observed for some cultivars despite lack of significant change in ACY. For example, 'Chippewa' grown in MI showed a substantial decrease in AA between 1998 and 1999, accompanied by virtually no change in ACY but a significant change in TPH between the 2 years. However, this reasoning does not completely explain the significant increase in AA in 'Chippewa' between 1998 and 1999 in MN, as ACY and TPH were virtually unchanged between years.

Because anthocyanins are important contributors to AA in blueberry and are confined primarily to the skin, berry size is expected to be a significant factor in AA activity, as the surface area : volume ratio decreases with increasing size. However, in our analyses, berry size accounted for a significant proportion of variation in TPH and ACY, but not in AA. Differences in TPH and ACY among cultivars were still significant when adjustment was made for berry size, using weight as a surrogate measure of size. These findings suggest that selection for high AA in blueberry will not necessarily result in concomitant selection for smaller berry size; in fact, higher levels of AA are apparent over a range of berry sizes (Fig. 1A), and gain in AA could be made while maintaining or possibly increasing size. Skin thickness might also influence AA, but our study did not examine this variable. Ehlenfeldt and Prior (2001) suggest that AA standardized to surface area is a more meaningful measure for breeders to compare blueberry genotypes, but this calculation requires the assumptions that berries are perfectly spherical and that skin thickness does not vary.

The correlation between TPH and AA was high in MN and MI and moderately high in OR. A larger survey of genotypes within MN (n = 52) also showed a high degree of correlation ($r = 0.92$ over 2 years; Connor, 2001) between AA and TPH. This is in agreement with a correlation of 0.92 reported by Prior et al. (1998), and slightly higher than the correlation of 0.76 (with AA expressed on a per weight basis) reported by Ehlenfeldt and Prior (2001). Both of the latter studies used ORAC as a measure of AA. The high correlation between AA and TPH suggests it is feasible to use TPH to screen for AA. Using ACY as a screen for AA is also a possibility, although in our study the correlation between AA and ACY is not as high as that between AA and TPH. Prior et al. (1998) observed a correlation of 0.77 and Ehlenfeldt and Prior (2001) reported a correlation of 0.57 (with AA expressed on a per weight basis) between AA and ACY, whereas we observed a correlation of $r = 0.73$ in a 2-year survey of 52 genotypes (Connor, 2001). In contrast to Prior et al. (1998) and Ehlenfeldt and Prior (2001), we did not use a pH differential method for determining ACY, and our extraction solvents and methods differed from those used in their study. Additionally, our AA assay is based on a mixed (lipid-aqueous) system, whereas the ORAC is an aqueous-based method. Despite these differences in methodology, correlations between AA and ACY in our study and that by Prior et al. (1998) were quite similar. Additionally, AA determined by our assay method correlates well with that obtained by ORAC ($r = 0.92$ for n = 19 extracts; Connor, 2001).

In all analyses, the variation among extracts was significant compared to variation between duplicate analyses of that extract. The source of this variation is probably an effect due to sampling few berries in the 10 g subsample used for each extraction. Using larger subsamples, or changing the procedure so that all subsamples are taken from a single homogenate processed from a larger quantity of fruit, may reduce variability. By decreasing variation among extracts, differences may be detected with fewer samples, or smaller differences among genotypes might be detected. That

genotype differences could still be detected with as little as 30 g of sound fruit is important, because the amount of fruit available for testing may be the limiting factor in some breeding programs.

In summary, our study shows that screening blueberry for genotypic differences in AA is possible using a relatively small quantity of fruit. AA may be assayed directly, or indirectly by using TPH as a surrogate measure of AA. Because of the significant genotype × environment interaction, evaluation of the fruit over several years and locations appears warranted.

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