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Variability in Antioxidant Activity in Blueberry and Correlations among Different Antioxidant Activity Assays

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Abstract. **Variation in antioxidant activity (AA), total phenolic content (TPH), and total anthocyanin content (ACY) was examined in 1998 and 1999 in fruit of 52 (49 blue-fruited and 3 pink-fruited) genotypes from a blueberry breeding population. The species ancestry included *Vaccinium corymbosum* L. (northern highbush blueberry), *V. angustifolium* Ait. (lowbush blueberry), *V. constablaei* Gray (mountain highbush blueberry), *V. ashei* Reade (rabbiteye blueberry), and *V. myrtilloides* Michx. (lowbush blueberry). Using a methyl linoleate oxidation assay (MeLO) on acidified methanolic extracts of the berries, a 5-fold variation was found in AA in 1998 and a 3-fold variation in 1999 among the blue-fruited genotypes. Analyses of variance (ANOVA) revealed variation among genotypes ($P < 0.0001$) in single and combined years, regardless of inclusion of pink-fruited selections and adjustment for berry size. While mean AA of all genotypes did not change between the 2 years, ranking of some genotypes for AA changed significantly between 1998 and 1999. Of the 10 genotypes that demonstrated the highest AA in 1998, four were among the 10 genotypes that demonstrated highest AA in 1999. Similarly, of the 15 genotypes with the highest AA, 10 were the same both years. As with AA, mean TPH of all genotypes did not change between years and ANOVA demonstrated genotypic variation regardless of adjustment for berry size/weight or exclusion of pink-fruited selections. Changes in genotype rank occurred between years. The difference in TPH between lowest- and highest-ranking blue-fruited genotypes was ≈2.6-fold in both 1998 and 1999. Seven of the 10 highest-ranking genotypes were the same both years and TPH correlated with AA ($r = 0.92$, $P < 0.01$) on a genotype mean basis for combined years. ACY correlated less well with AA ($r = 0.73$, $P < 0.01$ for combined years). When genotypes were categorized into six groups according to species ancestry, *V. myrtilloides* and *V. constablaei* x *V. ashei* crosses ranked highest and second highest, respectively, for AA in both years. The groups comprised of *V. corymbosum* genotypes, *V. angustifolium* genotypes, and those with both *V. corymbosum* and *V. angustifolium* in their lineage were indistinguishable from each other. Samples from some of the genotypes were analyzed for oxygen radical absorbance capacity and ferric-reducing antioxidant power, and these aqueous-based antioxidant assays correlated well with the lipid emulsion-based MeLO (all $r \geq 0.90$, $P < 0.01$). The three antioxidant assays may be equally useful for screening in a blueberry breeding program and the choice of assay may depend on the goal of the program and the resources available.**

Oxidation of biomolecules is increasingly emphasized as a common factor in the evolution and progression of chronic diseases in humans, such as atherosclerosis, cancer, and neurodegenerative diseases including dementia. Some epidemiological studies suggest that the risk of these diseases may be reduced in those consuming diets high in fruit and vegetables (Acheson and Williams, 1983; Block et al., 1992; Joshipura et al., 1999; Liu et al., 2000; Steinmetz and Potter, 1991, 1996; Zhang et al., 2000), or diets high in polyphenolic compounds such as flavonoids and phenolic acids (Hertog et al., 1993, 1997; Hirvonen et al., 2001; Keli et al., 1996; Knekt et al., 1997). These compounds have high antioxidant activity (AA). Antioxidants can reduce oxidative molecular and cellular damage by preventing the initial attack of biomolecules by free radicals or by interrupting the perpetuation of free radical species (Terao and Piskula, 1998).

Several studies have examined the in vitro AA of fruit extracts. Berries, which tend to be high in anthocyanins, phenolic acids, flavonols, and flavan-3-ols, are frequently cited as excellent sources of antioxidants (Costantino et al., 1992; Heinonen et al., 1998; Wang et al., 1996). Blueberries rank especially high in AA determined by the oxygen radical absorbance capacity (ORAC) assay (Kalt et al., 1999; Prior et al., 1998). However, the relationship of blueberry AA to that of other fruit may be affected by the method of fruit preparation, including the type of extraction solvents and use of pectinase, and by the assay used to measure AA (Costantino et al., 1992; Heinonen et al., 1998; Prior et al., 1998). Aqueous AA assays can be designed with excellent precision and some, including the ORAC and ferric-reducing antioxidant power (FRAP) assays, have been automated (Benzie and Strain, 1996; Cao et al., 1995) to increase their efficiency. Lipid emulsion-based assays are more difficult to automate for simultaneous sample determination due to the requirement for constant mixing, but these assays may more accurately reflect the complexity of in vivo oxidation (Barclay et al., 1984), in which both initiation and propagation of free radicals may contribute to oxidative damage (Halliwell, 1996).

Breeding fruit and vegetables with higher levels of compounds with AA is one way of increasing dietary antioxidant intake. However, this goal is realistic only if sufficient variability in AA exists in the breeding population. Ehlenfeldt and Prior (2001) demonstrated AA ranging from 4.6 to 31.1 μmol Trolox equivalents (TE)/g fresh weight among 87 northern and southern highbush blueberry cultivars harvested in a single season using ORAC. In a fruit such as blueberry, in which interspecific hybridization is used frequently, both the level of antioxidant activity and the degree of variation may differ among breeding populations. Thus, these parameters must be established in a sample that encompasses all species used in the breeding program.

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In this study, we examined variation in AA, anthocyanin content (ACY), and total phenolic content (TPH) in fruit of 52 genotypes sampled from the blueberry breeding program at the University of Minnesota in 1998 and 1999. Correlations are also reported among three antioxidant assays including both aqueous and lipid-emulsion systems.

Materials and Methods

Fruit. Fruit were harvested in 1998 and 1999 from 52 genotypes grown at the Sand Plain Research Farm, Becker, Minn. (lat. 45º20'N), on a Hubbard loamy sand (sandy, mixed, Udorthentic Haploboroll) with 2% to 3% organic matter and soil pH of 5.5. The species ancestry of the genotypes used included *V. corymbosum* (northern highbush blueberry), *V. angustifolium* (lowbush blueberry), *V. constablaei* (mountain highbush blueberry), *V. myrtilloides* (lowbush blueberry), and *V. ashei* (rabbiteye blueberry) (Table 1). Three of the 52 genotypes were pink-fruited selections (MN494, MN515, and MN676). Fruit was harvested from one additional genotype (MN819) in 1999 and used in the comparison of AA assays. About 100 g of sound, ripe fruit were harvested when 40% to 70% of the fruit on the bushes were ripe. Harvested fruit were held in polyethylene bags on ice in coolers until frozen later in the day. Prior to freezing, all samples were weighed and counted, and the weight/100 berries was calculated. Fruit were held at –80 ºC until extraction. Harvest for the same genotype was made from the same plants in both years.

Extraction. Extracts for all assays were prepared using acidified methanol (0.1% HCl). This solvent maximizes extraction of anthocyanins (Harborne, 1967), and studies in our lab (data not presented) demonstrated that acidified methanol was equivalent to methanol/formic acid/water (Gao and Mazza, 1994) for recovery of total anthocyanins. Preliminary studies (data not presented) also demonstrated no consistent difference in total phenolic content between extracts prepared in acidified methanol and those prepared in 80% ethanol (Coseteng and Lee, 1987). About 10 g of frozen berries was weighed and then thawed slightly at –20 ºC. Ice-cold acidified methanol was added to the berries in a 1:1 ratio (w/v) and ground for 2 min with a homogenizer (Polytron; 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 and the filtrate collected. The residue was mixed with a third 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 ºC until assayed. Extractions were performed under reduced-light conditions. Three extracts were prepared per genotype. Duplicate determinations of AA, TPH, and ACY were made from each extract, except for AA by ORAC, for which duplicate determinations were made on only one of the three extracts prepared for 19 genotypes.

AA assays. In both years, an AA assay was used that measured the ability of blueberry extracts to inhibit peroxyl radical-induced oxidation of linoleic acid methyl ester in an emulsion (MeLO). Two additional AA assays were also used on subsets of the blueberry extracts. One of these (FRAP) measures the ferric-reducing antioxidant power of the extracts in an aqueous system (Benzie and Strain, 1996; Deighton et al., 2000), while the ORAC assay (Cao et al., 1995; Kalt et al., 1999; Prior et al., 1998) measures inhibition of peroxyl radical-induced oxidation of phycoerythrin using an aqueous system.

MeLO is 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. In the current study, 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 ºC for 130 min in a shaking water bath (model 25; Precision Scientific, Chicago) at 100 rpm, 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 on a spectrophotometer (DU-50; Beckman Instruments, Fullerton, Calif.). Color interference by anthocyanins (maximal absorbance 530 nm) in the diluted extracts was assessed and adjusted for, if necessary, by using a color blank, substituting sodium phosphate buffer for the methyl linoleate suspension. Appropriate controls to assess baseline linoleate oxidation and the effect of methanol in the diluted extracts were included. An antioxidant standard curve was prepared with each assay, substituting the water-soluble vitamin E analogue Trolox (Aldrich Chemical, Milwaukee, Wis.) for the blueberry extract at appropriate dilutions. The standard curve was linear between 0 and 30 µM Trolox equivalents (TE) (final concentration). Results are expressed as µmol TE/g fresh fruit.

The ORAC (Cao et al., 1995) of 19 extracts was measured using a COBAS-FARA II analyzer (Roche Diagnostics, Nutley, N.J.) following the procedure as outlined by Kalt et al. (1999) substituting β-phycoerythrin for R-phycoerythrin. Because the ORAC is highly sensitive to methanol, the extracts were evaporated under nitrogen at room temperature and resuspended in deionized water prior to the appropriate dilution (1:400 for blue-fruited cultivars and 1:200 for pink-fruited selections) in 75 mM phosphate buffer (pH 7.0). The ORAC results are expressed as µmol TE/g fresh fruit.

The ferric-reducing antioxidant power (FRAP; originally referred to as the ferric-reducing ability of plasma) of the extracts from fruit collected in 1999 was determined using the modification by Deighton et al. (2000) of the method by Benzie and Strain (1996). Readings of the colored product [ferrous tripyridyltriazine (TPTZ) complex] were taken at 593 nm 4 min after addition of the diluted extracts to the FRAP reagent. Two standard curves, using Trolox (25 to 500 µM) and ferrous ammonium sulfate (50 to 1000 µM), were run with each assay. The standard curves were linear in the ranges tested, with Trolox demonstrating an activity about twice that of ferrous ammonium sulfate, and consistent with Benzie and Strain (1996). The 1% (v/v) dilution of the blueberry extracts in water, when used in the final reaction at a 1:9 dilution with the FRAP reagent, did not produce significant color interference at 593 nm. Results are expressed as µmol TE/g fresh fruit.

Total phenolic content. The method of Coseteng and Lee (1987) was used for total phenolic content, using an incubation time of 90 min for color development. Results are expressed as

Table 1. Combined-year means for antioxidant activity (AA) by methyl linoleate oxidation assay (MeLO),[z] total phenolic content (TPH),[y] and total anthocyanin content (ACY)[x] for 52 blueberry (*Vaccinium* sp.) genotypes harvested in Minnesota in 1998 and 1999, grouped according to species ancestry.

Designation	Genotype	MeLO	TPH	ACY
Group 1 *Vaccinium angustifolium*	Cumberland	29.8	446	164
	Fundy	36.7	599	234
	GR V.a.	41.4	579	186
	Michigan lowbush 1	28.2	400	128
	N70127	35.2	526	174
	N70145	39.4	550	209
	N70249	46.5	665	205
	N7068	42.3	612	202
	Group 1 means	37.4	547	188
Group 2 *V. corymbosum*/*V. angustifolium*	Bluegold	44.0	668	249
	Bluetta	26.0	454	211
	Chippewa	18.6	373	114
	GR 2	52.6	906	428
	MN449	31.2	571	214
	MN452	40.4	742	341
	MN455	27.5	482	165
	MN494	10.4	178	1
	MN496	20.0	407	162
	MN497	30.2	502	194
	MN515	5.2	150	1
	MN61	24.1	453	176
	MN676	11.7	209	1
	MN84	37.7	602	210
	N86158	38.6	645	280
	Northblue	29.3	484	164
	Northcountry	29.3	533	217
	Northland	38.4	602	240
	Northsky	22.2	431	158
	Patriot	27.6	467	156
	Polaris	22.2	452	202
	R2P4	25.4	474	199
	St. Cloud	33.6	552	202
	Group 2 means	30.9[w]	540[w]	214[w]
Group 3 *V. corymbosum*	B1-1	34.9	551	223
	B10	31.4	574	240
	B11	31.3	498	190
	B6	27.4	466	181
	Bluecrop	31.3	447	120
	Bounty	26.8	424	169
	Duke	35.4	627	274
	Friendship	42.3	790	382
	Jersey	27.6	490	197
	N70218	27.1	412	139
	N86161	42.0	808	375
	Nelson	38.8	564	188
	Group 3 means	33.0	549	219
Group 4 *V. myrtilloides*	N69180	62.6	945	259
	N70239	43.8	681	218
	Group 4 means	53.2	813	238
Group 5 *V. constablaei*/*V. ashei*	BY8815	56.1	816	281
	NC1831	45.8	709	219
	NC1832	52.3	831	313
	NC2342	35.7	574	201
	Group 5 means	47.5	732	228
Group 6 *V. constablaei*	N8426	35.6	528	168
	N8428	35.3	573	211
	N87014	43.7	729	290
	Group 6 means	38.2	610	223
Tukey's HSD for genotype[v]		19.2	213	67
Tukey's HSD for group[u]		19.7	268	139

[z]AA expressed in Trolox equivalents, $\mu mol \cdot g^{-1}$ fresh fruit.
[y]TPH expressed in mg chlorogenic acid equivalents/100 g fresh fruit.
[x]ACY expressed in mg cyanidin 3-glucoside equivalents/100 g fresh fruit.
[w]Group 2 mean values excluding pink-fruited selections (MN494, MN515, and MN676). Group 2 mean values with inclusion of these selections are 28.1, 493, and 186 for AA, TPH, and ACY, respectively.
[v]HSD for 2-year genotype means based on 52 genotypes, $P = 0.05$, and mean sample size of 12.
[u]HSD for 2-year species means based on six groups (excluding pink-fruited selections from Group 2), $P = 0.05$ and harmonic mean sample size of 53.665.

mg chlorogenic acid equivalents /100 g fresh fruit which, under the conditions of this assay, were determined to be ≈1.8× gallic acid equivalents.

Anthocyanin content. Berry extracts were diluted (1:99, v/v) in acidified methanol to obtain an absorbance between 0.200 and 1.000 at 530 nm. Because the extracts were freshly prepared from fruit maintained at –80 ºC and did not undergo extensive processing or significant browning, a pH differential method for anthocyanin content 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 genotype, extract, and year as random effects, and species ancestry group as a fixed effect. Extract was nested within genotype and genotype was nested within species ancestry group. Correlations were calculated on a genotype mean basis, except where indicated otherwise. All analyses were performed using SPSS for Windows, version 8.0 (SPSS, Inc., Chicago).

Results

Variation among genotypes. The 2-year means for AA by MeLO, TPH, and ACY for each of the 52 genotypes harvested in both years are listed in Table 1 with genotypes grouped according to their ancestry. The ANOVA for MeLO, TPH, and ACY combined over years are shown in Table 2.

Variation for AA among genotypes was detected both across years ($P < 0.0001$, Table 2) and within each year ($P < 0.0001$, analyses not presented). The mean of the 52 genotypes did not differ between years, although the range in AA decreased from ≈20-fold in 1998 to ≈12-fold in 1999 (data not presented). Genotypes changed rank between years ($P < 0.001$). Seven of the 10 genotypes with the lowest AA in 1998 ('Chippewa', 'Northsky', 'Polaris', and MN496, and the pink-fruited selections MN494, MN515, and MN676) were among the 10 genotypes with the lowest AA in 1999; but only four of the 10 genotypes with the highest AA in 1998 (GR 2, N69180, BY8815, and NC1832) were among the 10 with the highest AA in 1999. Among the 15 highest-ranking genotypes, 10 were the same in both years. Variation among genotypes ($P \leq 0.001$) was still evident when the three pink-fruited selections were excluded from the analysis (analysis not presented). Exclusion of these selections decreased the range of AA to only 5-fold in 1998 and to ≈3-fold in 1999. An ANOVA using the weight of 100 berries, an estimate for berry size, as a covariate demonstrated that berry size accounted for a large proportion of the variation in AA (Table 2). However, even with the adjustment for berry weight, significant variation in AA was still apparent among genotypes ($P < 0.0001$, Table 2). Variation among extracts within genotype was detected in all analyses for AA, but was substantially less than the variation among genotypes.

Variation for TPH among genotypes differed significantly between years ($P < 0.0001$, Table 2) and within each year ($P < 0.0001$, analyses not presented) while the mean of all genotypes did not change between 1998 and 1999. The range in TPH was ≈6-fold in 1998 and 7-fold in 1999; with exclusion of the pink-fruited genotypes, this range was reduced to 2.6 fold each year. Genotype rank changed between years (Table 2), with only five of the 10 genotypes with the lowest TPH in 1998 (Michigan lowbush 1, 'Chippewa', MN494, MN515, and MN676) being among the 10 genotypes with the lowest TPH in 1999. Among the 10 genotypes with the highest TPH in 1998, eight (GR 2, MN452, 'Friendship', N86161, N69180, BY8815, NC1831, and NC1832) were among the 10 with the highest TPH in 1999. Variation among genotypes was apparent even after adjustment for berry weight ($P < 0.0001$) or when the pink-fruited selections were excluded from analysis ($P < 0.0001$, analysis not presented). In all analyses for TPH, variation among genotypes was much greater than variation among extracts within genotype.

Table 2. ANOVA (left) for antioxidant activity by methyl linoleate oxidation assay, total phenolic content, and total anthocyanin content, and analyses of covariance (right) with adjustment for berry weight for 52 blueberry (*Vaccinium* sp.) genotypes harvested in Minnesota in 1998 and 1999.

Source	df	Mean square	F	*P*	df	Mean square	F	*P*
				Methyl linoleate oxidation assay for antioxidant activity				
Berry weight	---	---	---	---	1	13,470.0	11.4	0.001
Genotype	51	1,464.1	9.2	<0.0001	51	1,213.3	8.2	<0.0001
Year	1	13.0	0.1	0.776	1	55.8	0.4	0.541
Genotype × year	51	158.8	9.7	<0.0001	50	147.5	9.1	<0.0001
Extract/genotype × year	208	16.3	3.4	<0.0001	208	16.3	3.4	<0.0001
Error	312	4.8			312	4.8		
				Total phenolic content				
Berry weight	---	---	---	---	1	2,356,838.0	8.9	0.004
Genotype	51	317,692.6	16.3	<0.0001	51	272,422.0	14.3	<0.0001
Year	1	6,042.8	0.3	0.580	1	2,158.9	0.1	0.738
Genotype × year	51	19,506.3	11.8	<0.0001	50	19,013.3	11.5	<0.0001
Extract/genotype × year	208	1,652.7	86.5	<0.0001	208	1,652.7	86.5	<0.0001
Error	312	19.1			312	19.1		
				Total anthocyanin content				
Berry weight	---	---	---	---	1	291,787.7	4.0	0.049
Genotype	51	79,964.5	42.1	<0.0001	51	74,308.6	39.2	<0.0001
Year	1	7,528.4	4.0	0.052	1	6,277.1	3.3	0.075
Genotype × year	51	1,899.0	7.9	<0.0001	50	1,895.2	7.9	<0.000
Extract/genotype × year	208	240.2	91.2	<0.0001	208	240.2	91.2	<0.0001
Error	312	2.6			312	2.6		

Table 3. ANOVA for antioxidant activity by the methyl linoleate oxidation assay (AA), total phenolic content (TPH), and anthocyanin content (ACY) for blueberry (*Vaccinium* sp.) genotypes (excluding pink-fruited selections) harvested in 1998 and 1999 and grouped by species ancestry as outlined in Table 1.

		AA		TPH		ACY	
Source	df	Mean square	F	Mean square	F	Mean square	F
Group	5	4,159.9	4.0^{*}	603,738.8	2.8^{*}	33,036.3	0.6
Gtype[z]/group	43	708.7	5.8^{***}	181,932.6	10.9^{***}	54,385.7	34.7^{***}
Year	1	5.7	0.05^{NS}	7,653.5	0.5^{NS}	1,418.4	0.9^{NS}
Group × year	5	461.4	3.8*	47,321.7	2.8^{*}	5,762.6	3.7^{**}
Gtype/group × year	43	122.7	7.4^{***}	16,649.4	9.6^{***}	1,569.1	6.2^{***}
Extract (gtype/group × year)	196	16.6	3.4^{***}	1,738.3	88.1^{***}	254.9	91.1^{***}
Error	294	4.9		19.7		2.8	

[z]Gtype = genotype.
^{NS,*,**,***}Nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table 4. Pearson's correlation coefficients for antioxidant activity determined by methyl linoleate oxidation assay (MeLO), total phenolic content (TPH), and anthocyanin content (ACY) in 1998, 1999, and combined years; and the correlation of these traits with antioxidant activity determined by ferric-reducing antioxidant power assay (FRAP) in 1999, based on genotype means from duplicate determinations of three extracts from 52 blueberry (*Vaccinium* sp.) genotypes harvested in Minnesota.

	1998			1999			Combined years		
Variable	MeLO	TPH	ACY	MeLO	TPH	ACY	MeLO	TPH	ACY
MeLO	---	0.94^{**}	0.78^{**}	---	0.91^{**}	0.67^{**}	---	0.92^{**}	0.73^{**}
TPH	---	---	0.87^{**}	---	---	0.81^{**}	---	---	0.84^{**}
FRAP	---	---	---	0.90^{**}	0.99^{**}	0.93^{**}	---	---	---

**Significant at $P < 0.01$.

The blue-fruited genotypes showed an ≈4-fold range in ACY in both 1998 and 1999. The ACY of pink-fruited selections MN494, MN515, and MN676 was minimal, and their inclusion expanded the range in ACY values considerably. Variation for ACY among genotypes was detected ($P < 0.001$), regardless of inclusion of pink-fruited genotypes. The P value for the effect of year was 0.052 with all 52 genotypes (Table 2) included in the analysis, and was 0.050 when the pink-fruited selections were excluded (analyses not presented). This contrasts with the clear lack of effect of year on AA and TPH (Table 2). When adjusted for berry weight, the effect of year was nonsignificant ($P = 0.075$). Changes in genotype rank for ACY between years were found with or without adjustment for berry weight ($P < 0.0001$ for both analyses), or when pink-fruited selections were excluded ($P < 0.0001$, analysis not presented). However, nine of the 10 genotypes that ranked highest for ACY were the same in both years and seven of the 10 lowest-ranking genotypes for ACY were the same both years, regardless of the inclusion of the pink-fruited selections. Variation in ACY among extracts within genotype were found, but were substantially smaller than variation among genotypes in all analyses.

Variation among species ancestry groups. Variation for AA among species ancestry groups was detected ($P < 0.05$, Table 3). In both 1998 and 1999, Group 4 (*V. myrtilloides*) and Group 5 (*V. constablaei* x *V. ashei* crosses) demonstrated the highest AA, while Group 2 (genotypes with both *V. angustifolium* and *V. corymbosum* in their lineage) demonstrated the lowest AA (Table 1). Changes in group rank or scale were found between 1998 and 1999 ($P < 0.01$ for group × year interaction). However, in the combined year analysis, the five lowest-ranking groups for AA were not distinguishable from each other by Tukey's studentized range test (HSD). Variation among species ancestry groups for TPH was noted, based on the combined-year analysis ($P < 0.05$, Table 3) and group × year interaction was also detected ($P < 0.05$, Table 3). The rank order of groups was the same for AA and TPH, except for Groups 2 and 1, respectively, occupying the lowest rank in 1998 and in combined years. Species ancestry groups did not differ for ACY, in either combined years (Table 3) or individual years (analyses not presented), regardless of inclusion of pink-fruited selections; however, group × year interaction was detected. Analyses of covariance using berry weight as a covariate demonstrated the importance of this factor, but also showed that variation for AA and TPH among species ancestry groups continued to be significant, even when adjusted for berry size (analyses not presented). Analysis of covariance for ACY also demonstrated the significance of berry weight as a cofactor for this variable, and variation among groups for ACY became significant ($P < 0.01$, analyses not presented) with adjustment for this factor.

Correlations. Correlations between MeLO, TPH, and ACY for both combined and individual years are shown in Table 4. MeLO was highly correlated with TPH in 1998, 1999, and in combined years, while MeLO and ACY showed slightly lower correlations in individual and combined years. Based on assays of three extracts from each fruit sample harvested in 1999, FRAP correlated well with MeLO (Table 4). The absolute values for AA determined by FRAP are ≈40% of those determined by MeLO, while the absolute values for AA determined by ORAC are very similar to those obtained by MeLO (Table 5). Correlations between all pairs of AA assays were ≥0.92 ($P < 0.01$) based on single extracts for the 19 samples listed. Correlations between each AA assay and TPH or ACY were ≥0.91 ($P < 0.01$), except between MeLO and ACY ($r = 0.76$, $P < 0.01$).

Discussion

To develop blueberry cultivars with higher levels of AA, variation for this trait is necessary. This study demonstrates the ample variation in AA that exists among genotypes and among species in a blueberry breeding population. The pink-fruited genotypes included in our study demonstrated detectable AA and low TPH, despite extremely low ACY. Thus, phenolic compounds other than anthocyanins that occur in significant amounts in blueberry, such as chlorogenic acid (Gao and Mazza, 1994; Kalt and McDonald, 1996) and flavonols (Häkkinen et al., 1999) appear to contribute to AA in blueberry. The study also demonstrated year-to-year variability in AA among genotypes, to the extent that changes in rank order occurred. Thus, accurate assessment and selection of genotypes for higher AA will necessitate testing over multiple years. Berry size clearly accounted for a large proportion of the variability in AA. Since anthocyanins are an important contributor to antioxidant activity and are confined principally to the skin in blueberry, the significant effect of berry size on AA variation is expected, by virtue of its effect on the surface area to volume ratio. However, differences in AA among genotypes remained when adjusted for berry size. This suggests that progress in breeding for higher AA can be made while maintaining a desired berry size.

Table 5. Antioxidant activity of blueberry fruit extracts as determined by methyl linoleate oxidation (MeLO),[z] ferric-reducing antioxidant power (FRAP),[z] and oxygen radical absorbance capacity (ORAC)[z] assays; and total phenolic content (TPH)[y] and anthocyanin content (ACY)[x] based on duplicate determinations of single extracts from 18 blueberry (*Vaccinium* sp.) genotypes harvested in 1999, and 'Bluegold' harvested in 1998 and 1999 (top); and Pearson's correlation coefficients for these variables (bottom).

Designation	MeLO	FRAP	ORAC	TPH	ACY
MN819	59.1	19.3	41.0	739	252
BY8815	57.2	24.6	51.9	739	274
Bluegold(1998)	55.4	22.5	46.1	809	292
N70249	52.7	19.9	41.3	734	224
NC1832	51.3	23.8	51.4	845	326
Fundy	40.4	16.7	38.1	663	264
Bluegold (1999)	35.8	15.8	35.8	538	220
Duke	34.9	15.6	37.6	592	268
Nelson	34.9	12.8	28.4	513	162
Bluecrop	34.5	10.6	26.9	460	115
Northland	34.4	16.5	34.9	568	226
Cumberland	32.8	11.8	29.4	452	172
Northblue	30.4	11.8	29.9	452	144
Chippewa	30.4	9.2	29.9	375	124
Jersey	29.6	13.0	32.7	484	202
Bluetta	26.0	12.1	25.3	459	208
MN61	23.8	10.9	24.4	451	169
Polaris	20.1	12.0	25.2	413	191
MN494	11.2	1.8	10.3	150	1
Correlations					
MeLO	---	0.92**	0.92**	0.94**	0.76**
FRAP	---	---	0.99**	0.97**	0.92**
ORAC	---	---	---	0.96**	0.91**
TPH	---	---	---	---	0.92**

[z]Expressed in Trolox equivalents, $\mu mol \cdot g^{-1}$ of fresh fruit.
[y]Expressed in mg chlorogenic acid equivalents/100 g fresh fruit.
[x]Expressed in mg cyanidin 3-glucoside equivalents/100 g fresh fruit.
**Significant at $P < 0.01$.

The species ancestry groups demonstrated variation in AA, TPH, and ACY when adjusted for berry size. Although the number of genotypes in certain groups was small, these results suggest that a higher surface area:volume ratio in the small-fruited genotypes that predominate in groups such as *V. myrtilloides*, did not fully account for the relatively higher AA levels in these groups. The rank changes between years for some of the species ancestry groups, as well as the differences in range of AA within groups between years, makes it difficult to state conclusively that specific species ancestry groups consistently differ for AA. On the other hand, the *V. constablaei,* and *V. constablaei* x *V. ashei* groups had more consistent AA from year to year, and a larger survey of these species or species crosses could confirm whether they exhibit greater year-to-year consistency in AA. Prior et al. (1998) reported an overall range in AA from 13.9 to 45.9 µmol TE/g fresh fruit in their study of northern and southern highbush, rabbiteye, and lowbush blueberry genotypes harvested in a single year, with considerable overlap in AA values among the genotypes of different species. Our study demonstrated a wider range and greater overlap in AA values among species.

The MeLO used in this study measures lipid oxidation occurring in a lipid emulsion. This type of system was developed to investigate free radical and antioxidant interactions at an aqueous-lipid interface, presumably an important site of radical generation in biological systems (Barclay et al., 1984). In contrast, the ORAC assay used by Prior et al. (1998) is an aqueous assay, and measures the alteration of a fluorescent protein by free radicals. Aqueous assays might be expected to detect slightly different antioxidant capabilities than lipid emulsion-based assays, meaning specific compounds or classes of compounds that are highly effective antioxidants in one assay system may be less effective in the other system. For example, in the present study, ACY shows higher correlation with ORAC than with MeLO, which may reflect a more important role for these compounds in aqueous systems than in emulsions. Nevertheless, the correlation between these two AA assays ($r = 0.92$ for $n = 19$) was high. AA as determined by either MeLO or ORAC showed high correlation with AA determined by the aqueous-based FRAP assay, although the assays detect slightly different capabilities (peroxyl radical-scavenging activity versus ferric-reducing ability) that contribute to AA. All three assays also demonstrated high correlation of AA with TPH and ACY in this study. Prior et al. (1998) showed a high correlation of AA by ORAC with TPH and ACY in their study of *Vaccinium* L. sp. The results of our study are in agreement with those findings, with correlations over combined years of $r = 0.92$ ($P \leq 0.01$) between AA and TPH and $r = 0.73$ ($P \leq 0.01$) between AA and ACY. Correlation of ACY with ORAC for single extracts was somewhat higher than that obtained by Prior et al. (1998), but may reflect differences in extraction solvents and ACY assay procedures between the two studies. Use of methanol for extraction and deionized water for reconstitution of evaporated extracts for performance of ORAC in our study could also bias the ORAC assay results. Under these conditions, the activity of antioxidant compounds that are soluble in methanol but insoluble in water, such as vitamin E or vitamin A, would be underestimated in the ORAC assay. These nutrients are present in small quantities in blueberries (U.S. Department of Agriculture, 1998). Insoluble substances that quench fluorescence also may bias results. However, our study did not address these issues.

The lower AA values determined by FRAP compared to MeLO or ORAC may reflect a relative difference in the ability of

the antioxidant compounds in the extracts to quench aqueous peroxyl radicals vs. reduce ferric iron in in vitro systems. The correlation between FRAP and assays based on scavenging activity toward active oxygen species other than peroxyl radicals may be lower than between FRAP and MeLO or ORAC. Heinonen et al. (1998) observed that extracts of blueberry phenolics are less effective than other fruit in preventing copper-catalyzed oxidation of human low-density lipoprotein in vitro. This contrasts to the apparent top ranking of blueberry extracts (prepared in acidified acetonitrile) compared to other fruit for peroxyl radical scavenging activity observed by Prior et al. (1998). Wang et al. (2000) noted that blueberry extracts were the least effective of five small fruit in scavenging hydroxyl radicals and singlet oxygen. Thus, results from antioxidant assays using different free radical generators may not be highly correlated.

The choice of screening assay for a breeding program may depend on which method most reliably distinguishes among genotypes within the crop being screened. The high correlation between TPH and the AA assays used in this study suggests that TPH may be used as a surrogate measure of AA in blueberry. For the purpose of screening blueberry germplasm for AA directly, any of the three AA assays used in this study appear equally effective when using acidified methanol extracts of the berries. Because the relative contribution of different classes of bioactive compounds may differ among fruit crops, however, the correlation between ORAC, FRAP, and MeLO may need to be confirmed for screening other fruit crops. Ideally, the assay of choice for screening germplasm should be simple, inexpensive, rapidly performed, and provide the degree of precision desired. If one of the goals is to screen for fruit antioxidant activity early in the program, when the amount of fruit available is limited, the assay should not require a large sample size. Assays adapted to automated instruments can save technical time and reagent costs, and may permit multiple simultaneous sample determinations, but are cost efficient only if the instrumentation is easily accessible and inexpensive to operate.

Literature Cited

Acheson, R.M. and D.R.R. Williams. 1983. Does consumption of fruit and vegetables protect against stroke? Lancet 321:1191–1193.

Barclay, L.R.C., S.J. Locke, J.M. MacNeil, J. VanKessel, G.W. Burton, and K.U. Ingold. 1984. Autoxidation of micelles and model membranes. Quantitative kinetic measurements can be made by using either water-soluble or lipid-soluble initiators with water-soluble or lipid-soluble chain-breaking antioxidants. J. Amer. Chem. Soc. 106:2479–2481.

Benzie, I.F.F. and J.J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal. Biochem. 239:70–76.

Block, G., B. Patterson, and A. Subar. 1992. Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. Nutr. Cancer 18:1–29.

Cao, G., C.P. Verdon, A.H.B. Wu, H. Wang, and R.L. Prior. 1995. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. Clinical Chem. 41:1738–1744.

Coseteng, M.Y. and C.Y. Lee. 1987. Changes in apple polyphenoloxidase and polyphenol concentration in relation to degree of browning. J. Food Sci. 52:985–989.

Costantino, L., A. Albasino, G. Rastelli, and S. Benvenuti. 1992. Activity of polyphenolic crude extracts as scavengers of superoxide radicals and inhibitors of xanthine oxidase. Planta Medica 58:342–344.

Deighton, N., R. Brennan, C. Finn, and H.V. Davies. 2000. Antioxidant properties of domesticated and wild *Rubus* species. J. Sci. Food Agr. 80:1307–1313.

Ehlenfeldt, M.K. and R.L. Prior. 2001. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. J. Agr. Food Chem. 49:2222–2227.

Fuhrman, B., A. Levy, and M. Aviram. 1995. Consumption of red wine with meals reduces susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. Amer. J. Clinical Nutr. 61:549–554.

Gao, L. and G. Mazza. 1994. Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. J. Food Sci. 59:1057–1059.

Häkkinen, S.H., S.O. Kärenlampi, I.M. Heinonen, H.M. Mykkänen, and A.R. Törrönen. 1999. Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. J. Agr. Food Chem. 47:2274–2279.

Halliwell, B. 1996. Mechanisms involved in the generation of free radicals. Pathologie Biologie 44:6–13.

Harborne, J.B. 1967. Comparative biochemistry of the flavonoids. Academic Press, New York, p. 1–36.

Heinonen, I.M., A.S. Meyer, and E.N. Frankel. 1998. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. J. Agr. Food Chem. 46:4107–4112.

Hertog, M.G.L., E.J.M. Feskens, P.C.H. Hollman, M.B. Katan, and D. Kroumhout. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. Lancet 342:1007–1011.

Hertog, M.G.L, E.J.M. Feskens, and D. Kromhout. 1997. Antioxidant flavonols and coronary heart disease risk. Lancet 349:699.

Hirvonen, T., P. Pietinen, M. Virtanen, M.-L. Ovaskainen, S. Häkkinen, D. Albanes, and J. Virtamo. 2001. Intake of flavonols and flavones and risk of coronary heart disease in male smokers. Epidemiology 12:62–67.

Joshipura, K.J., A. Ascherio, J.E Manson, M.J. Stampfer, E.B. Rimm, F.E. Speizer, C.H. Hennekens, D. Spiegelman, and W.C. Willett. 1999. Fruit and vegetable intake in relation to risk of ischemic stroke. J. Amer. Med. Assn. 282:1233–1239.

Kalt, W., C.F. Forney, A. Martin, and R. L. Prior. 1999. Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruit. J. Agr. Food Chem. 47:4638–4644.

Kalt, W. and J.E. McDonald. 1996. Chemical composition of lowbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 121:142–146.

Keli, S.O., M.G.L. Hertog, E.J.M. Feskens, and D. Kromhout. 1996. Dietary flavonoids, antioxidant vitamins, and incidence of stroke. The Zutphen Study. Arch. Intern. Med. 154:637–642.

Knekt, P., R. Järvinen, A. Reunanen, and J. Maatela. 1997. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Amer. J. Epidemiol. 146:223–230.

Lee, H.-S., D.W. Shoeman, and A.S. Csallany. 1992. Urinary response to *in vivo* lipid peroxidation induced by vitamin E deficiency. Lipids 27:125–128.

Liu, S., J.E. Manson, I-M. Lee, S.R. Cole, C.H. Hennekens, W.C. Willett, and J.E. Buring. 2000. Fruit and vegetable intake and risk of cardiovascular disease in the Women's Health Study. Amer. J. Clinical Nutr. 72:922–928.

Prior, R.L., G. Cao, A. Martin, E. Sofic, J. McEwen, C. O'Brien, N. Lischner, M. Ehlenfeldt, W. Kalt, G. Krewer, and C. M. Mainland. 1998. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. J. Agr. Food Chem. 46:2686–2693.

Steinmetz, K.A. and J.D. Potter. 1991. Vegetable, fruit, and cancer. I. Epidemiology. Cancer Causes Control 2:325–357.

Steinmetz, K.A. and J.D. Potter. 1996. Vegetables, fruit, and cancer prevention: A review. J. Amer. Dietetic Assoc. 96:1027–1039.

Terao, J. and M.K. Piskula. 1998. Flavonoids as inhibitors of lipid peroxidation in membranes, p. 277–293. In: C. Rice-Evans and L. Packer (eds.). Flavonoids in health and disease. Marcel Dekker, Inc., New York.

U.S. Department of Agriculture. 1998. Nutrient database for standard reference. Nutrient Data Lab., Agr. Res. Serv. http://www.nal.usda.gov/fnic/foodcomp.

Wang, H., G. Cao, and R.L. Prior. 1996. Total antioxidant capacity of fruits. J. Agr. Food Chem. 44:701–705.

Wang, S.Y. and H.S. Lin. 2000. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. J. Agr. Food Chem. 48:140–146.

Zhang S.M.M., D.J. Hunter, B.A. Rosner, E.L. Giovannucci, G.A. Colditz, F.E. Speizer, and W.C. Willett. 2000. Intakes of fruits, vegetables, and related nutrients and the risk of non-Hodgkin's lymphoma among women. Cancer Epidemiol. Biomarkers and Prevention 9:477–485.