JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Cultivar Evaluation and Effect of Fermentation on Antioxidant Capacity and *in Vitro* Inhibition of α-Amylase and α-Glucosidase by Highbush Blueberry (*Vaccinium corombosum*)

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ABSTRACT: The berry fruits of highbush blueberry (*Vaccinium corymbosum*) contain bioactive compounds with potential health benefits. The objective was to evaluate blueberries grown in southern Illinois as well as the effect of fermentation, at two different temperatures, on chemical and physical parameters. Fruits from fifteen blueberry cultivars were analyzed. Fruit diameter ranged from 12.8 mm to 18.7 mm, pH from 2.6 to 3.7, reducing sugars from 6.4% to 15.2%, total sugars from 13.9% to 21.6%, total polyphenols from 0.39 to 1.00 mg gallic acid equivalents (GAE)/g blueberry and antioxidant capacity from 5.8 to 10.9 μM Trolox equivalents (TE)/g. *In vitro* α-amylase and α-glucosidase inhibitory capacity relative to the positive control acarbose, a known anti-diabetic drug, showed a range from 91.8 to 103.3% for α-amylase and from 103.2% to 190.8% for α-glucosidase. Wines prepared from several of these blueberry cultivars were analyzed throughout fermentation and compared at room temperature and cold temperature fermentation for pH (3.5 to 6.3), °Brix (13.6 to 29.7), total polyphenols (375.4 to 657.1 μg GAE/mL wine), and antioxidant capacity (4.5 to 25.1 mM TE). The wines were also tested for their *in vitro* capacity to inhibit α-amylase and α-glucosidase and maintained similar inhibitory action as the berries. Highbush blueberry cultivars and their fermented beverages are good natural sources of antioxidants and starch-degrading enzyme inhibitors important for type 2 diabetes management.

KEYWORDS: highbush blueberry, *V. corymbosum*, fermentation, antioxidant, α-amylase, α-glucosidase

INTRODUCTION

In the United States there are increasing numbers of teens and adults that have prediabetes, and currently, 25.6 million people have diabetes.[1] This condition causes severe complications if blood glucose levels are not monitored properly, making diabetes prevention and management a very important health issue. There is a need to develop strategies for diabetes management in order to decrease its incidence and deaths from diabetes-related complications. One therapeutic approach for management of type 2 diabetes is to reduce glucose absorption by inhibiting α-amylase and α-glucosidase involved in starch degradation. Various berries have been investigated for their potential use in the management of diabetes by inhibiting starch-degrading enzymes after the consumption of soft fruits,[2] Brazilian native fruits,[3] strawberries[4] and raspberries.[5] In clinical trials, consumption of whole blueberries and freeze-dried blueberry beverages have been found to exhibit antidiabetic properties by reducing blood glucose concentration[6,7] in both healthy subjects and those with metabolic syndrome. For those with diabetes, natural sources of inhibitors of α-amylase and α-glucosidase would be beneficial in order to avoid potential gastrointestinal side effects[8] caused by commercial α-glucosidase inhibitors.

Berries in general have been noted for having positive health effects due to their high amount of phenolic compounds and antioxidants.[9,10] The phenolic profiles and composition of many fruits and vegetables have been investigated, and blueberries have been found to be rich sources of antioxidants. The high antioxidant activity of blueberries is well documented, and it has been correlated to their anthocyanin[11] and total phenolic content.[12,13] High concentrations of phenolic compounds, such as resveratrol and anthocyanins found in grapes, have shown potential for reducing hyperglycemia.[14]

Specific cultivars of blueberries and their composition have been studied,[15] but not much research explains the compositional changes from processing, including fermentation. Studies have shown that during ripening of berries, especially blueberries, anthocyanins accumulate after harvest and even during storage.[16] Since the bioavailability of anthocyanins is relatively low compared to other flavonoids,[17] and because fermentation is known to increase antioxidant capacity of blueberry juice,[18] wine processing techniques that maximize the amount of skin contact are viable ways to increase the anthocyanin content and antioxidant capacity of berry juice products.[18,19] In addition, ellagic acid, a major sedimentation product formed during fermentation, has been previously shown to have greater losses due to precipitation at higher temperatures of storage for wines produced from muscadine grapes.[20] One specific hypothesis of this study is that fermenting blueberries at colder temperatures will cause less ellagic acid precipitation, having an important implication to wine quality.

To our knowledge, no research has been conducted on the effect of berry fermentation temperature on the inhibition of starch-degrading enzymes. Therefore, there is a need to further investigate different genotypes of berries and the effects of fermentation for

Received: May 1, 2011
Accepted: July 15, 2011
Revised: July 10, 2011
Published: July 15, 2011

their potential use to decrease starch degradation. This research aimed to perform an evaluation of highbush blueberries, *V. corymbosum*, grown at the Dixon Springs Agricultural Center (DSAC) in southern Illinois, including their physical descriptions, total polyphenols, antioxidant capacity, and *in vitro* enzymatic inhibition of α-amylase and α-glucosidase. In addition, the effect of fermentation of blueberries at two different temperatures on these parameters and the *in vitro* potential of the resulting wine for inhibiting starch breakdown were also assessed.

MATERIALS AND METHODS

Chemicals. Compounds used as standards and their purity were as follows: Trolox (≥97%), glucose (≥99.5%), gallic acid (≥98%), and ellagic acid (≥95%). All chemicals and reagents were purchased from Sigma (St. Louis, MO), unless otherwise specified.

Blueberry Cultivars. All berries were grown at the same location under the same environmental conditions, and all of them were harvested when fully ripe, independently of their diameter, during June 2010 from the Dixon Springs Agricultural Center (DSAC) in Simpson, IL. They were immediately washed, dried, and stored in plastic bags at −4 °C. All blueberries grown at DSAC were of the species *Vaccinium corymbosum*. Cultivars studied were Berkley, Blue Chip, Blue Haven, Blue Jay, Bluecrop, Blueray, Bluetta, Collins, Coville, Darrow, Earliblue, North Country, Patriot, Spartan, and Stanley. The results obtained from the blueberries grown at DSAC were compared to a fresh fully ripe commercial blueberry sample (Naturipe Farms, Naturipe Farms LLC, Martinez, GA).

Physical Characterization. One hundred blueberries were randomly picked from each cultivar. The cross-diameter of each berry was measured in duplicate immediately after harvesting using a pair of Vernier calipers (Bel-Art Products Scienceware, Pequannock, NJ 07440−1992). Twenty grams of blueberries from each cultivar listed above were blended in 50 mL of double distilled (dd) water and filtered using Whatman #4 filter paper. The filtrates were stored at 4 °C in test tubes covered with Parafilm before analysis. The filtrates were taken out of refrigeration 10 min before the experiment to allow the samples to reach room temperature, 26.3 °C. A pH meter (Mettler Toledo SevenEasy, Columbus, OH) and pH probe (Mettler Toledo InLab Expert 2m, Columbus, OH) were used to measure the pH of each blueberry extract. Reducing sugars were determined using a modified method originally described by Lindsay.[21] Briefly, 0.5 mL of each extract was transferred into a clean, dry test tube, 0.5 mL of 1.5 N H_2SO_4 was added, and the mixture was boiled in a water bath (100 °C) for 20 min with occasional mixing. Tubes were cooled down, 0.75 mL of 2 N sodium hydroxide was added, and then 1 mL of dinitrosalicylic acid reagent and 2 mL of dd water were added. The test tubes were heated again (100 °C) in a water bath for 5 min and then cooled down, 16 mL of dd water was added, and the absorbance was read at 570 nm in a spectrophotometer (Beckman DU-64 Spectrophotometer, Fullerton, CA) using glucose as a standard.

Extraction and Removal of Reducing Sugars. Berry extraction and fractionation were performed based on procedures developed by Grace et al.[22] Twenty grams of frozen berries were blended with 40 mL repeat washings of 100% methanol acidified with 0.3% trifluoroacetic acid (TFA) and filtered through Whatman #4 filter paper until no further color was detected. The collected hydroalcoholic extract was concentrated using a rotary evaporator (40 °C). Frozen berry extract or wine sample (5 mL) was loaded onto an Amberlite XAD-7 column (30 × 3 cm) preconditioned with acidified water (0.3% TFA). The aqueous layer and resin were washed thoroughly with acidified water (0.3% TFA, ~600 mL) to remove free sugars, pectins, and phenolic acids. The polyphenolic mixture was then eluted with acidified methanol (0.3% TFA) and concentrated on a rotary evaporator (40 °C) to yield a crude-Amberlite extract (CAE). Samples were then freeze-dried and stored at −80 °C.

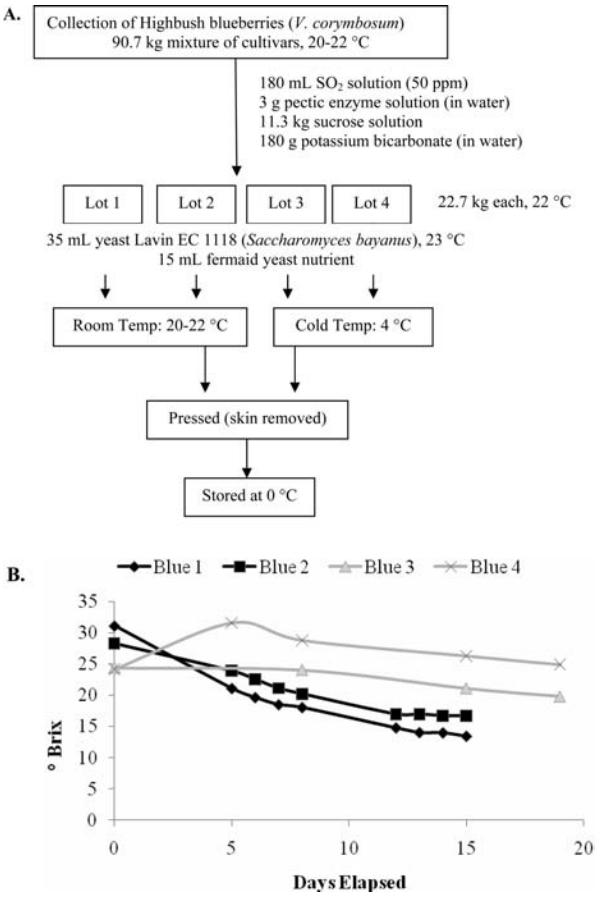

Figure 1. A. Flow diagram of the fermentation of berry juice. B. Fermentation curves for each batch of wine throughout time. Batches 1 and 2 were fermented at room temperature (RT) while 3 and 4 were fermented at cold temperature (CT).

Fermentation of Berry Juice. Upon collection of the fruits from the various cultivars, the berries were stored frozen at −18 °C until use. Berry wines were prepared, as indicated in Figure 1A, using about 90.7 kg of roughly even mixtures of cultivars Blue Chip, Bluecrop, Blue Haven, Blue Jay, Blueray, Bluetta, Collins, Coville, Darrow, Earliblue, Elliot, Jersey, Late Blue, and Spartan. After thawing frozen berries overnight at room temperature (20 − 22 °C), 180 mL of SO_2 solution (50 ppm), 3 g of pectic enzyme solution (dissolved in water), 11.3 kg of sucrose solution, and 180 g of potassium bicarbonate in water were added. This mixture was then divided into four evenly divided lots, about 22.7 kg each. Dry wine yeast Lavin EC 1118 (*Saccharomyces bayanus*) was obtained from Presque Isle Wine Cellars (North East, PA). Yeast EC 1118 was rehydrated in potable water at 40 °C for 20 min with occasional gentle stirring to remove clumps prior to inoculation of the juice. Per manufacturer's recommendations, yeast EC 1118 was inoculated at 40 mg of dry yeast/100 mL and 35 mL of yeast (23 °C) was added to each lot of fruit (22 °C). Fifty grams of Fermaid was dissolved in water, and 15 mL of this nutrient was added to each lot. Lots 1 and 2 were held at room temperature (RT, 20−22 °C); lots 3 and 4 were held at cold temperature (CT, 4 °C) for two weeks until pressed (skin removed); RT ready to press 14 days after yeast was added, CT ready 5 days later. Juice from all treatments was tested each week for pH, °Brix (Figure 1B) and phenolics. Fermented wine has been stored at 0 °C. Results presented are averages of the independent duplicates lots 1 and 2 as room temperature (RT) or 3 and 4 as cold temperature (CT), unless otherwise specified.

Table 1. Physical and Chemical Characteristics of Blueberry Cultivars Grown in Southern Illinois, Dixon Springs Agricultural Center[a]

V. corymbosum cultivar	pH of juice	av diam (mm)	% total sugar	% reducing sugar
Berkley	2.8	16.57 ± 1.76 de	16.9 ± 2.9 bc	11.6 ± 0.2 abc
Blue Chip	2.7	17.58 ± 2.74 b	17.7 ± 0.3 b	12.9 ± 0.5 abc
Blue Haven	2.9	17.17 ± 1.36 bc	17.4 ± 0.1 b	13.6 ± 1.0 ab
Blue Jay	2.9	16.63 ± 1.96 de	15.3 ± 0.7 bc	12.5 ± 1.5 abc
Bluecrop	2.8	16.50 ± 1.14 de	16.1 ± 0.3 bc	13.7 ± 1.7 ab
Blueray	3.0	16.84 ± 2.16 cd	15.3 ± 0.1 bc	10.8 ± 0.0 bcd
Bluetta	3.0	15.20 ± 2.58 f	16.7 ± 0.5 bc	9.3 ± 0.1 cde
Collins	2.8	16.44 ± 1.61 de	16.3 ± 0.4 bc	6.4 ± 0.1 de
Coville	3.0	16.74 ± 2.13 cde	15.6 ± 0.2 bc	7.1 ± 0.0 de
Darrow	3.0	18.49 ± 3.43 a	16.7 ± 0.6 bc	15.2 ± 2.2 a
Earliblue	3.4	15.49 ± 2.46 f	17.8 ± 1.0 b	8.9 ± 0.1 cde
North Country	3.0	12.80 ± 0.99 g	21.6 ± 0.4 a	14.6 ± 1.4 ab
Patriot	2.6	17.46 ± 2.72 b	15.6 ± 0.5 bc	12.7 ± 1.4 abc
Spartan	2.8	18.69 ± 1.84 a	13.9 ± 0.1 c	12.7 ± 0.8 abc
Stanley	3.3	15.00 ± 1.38 f	14.5 ± 0.2 bc	12.0 ± 0.4 abc
commercial sample	3.7	16.28 ± 3.79 e	14.4 ± 0.3 bc	7.3 ± 0.3 de

[a] The data represents the mean ± SD from at least two independent studies and at least a triplicate analysis. Values within a column followed by different letters are significant at $p < 0.05$. Diameter minimum significant difference = 0.5003; % total sugar minimum significant difference = 3.4178; % reducing sugar minimum significant difference = 4.0038.

Total Phenolic Content. Total phenolic content (TP) was quantified using the Folin−Ciocalteu method described originally by Amerine and Ough[23] adapted to a microassay. Briefly, to a 96-well flat bottom plate were added 50 μL of 1 N Folin−Ciocalteu's phenol reagent and 50 μL of either sample, standard or blank; this mixture was allowed to stand for 5 min before the addition of 100 μL of 20% Na_2CO_3. The solution was then allowed to stand for 10 min before reading at 690 nm using a Synergy 2 multiwell plate reader (Biotek, Winooski, VT). Results were expressed as μg gallic acid equivalents (GAE)/g blueberry or μg ellagic acid equivalents (EAE)/mL wine.

Total Anthocyanins. Total anthocyanins were determined using the AOAC Official Method as described previously by Lee.[24] Samples were diluted 1:10 using two different buffers (pH 1.0 KCl buffer and pH 4.5 sodium acetate buffer), and the absorbance was read at 520 and 700 nm on a spectrophotometer (Beckman DU-64 spectophotometer, Fullerton, CA). The results were expressed as total monomeric anthocyanins as cyanidin-3-glucoside equivalents in mg/L using eq 1:

$$\text{total monomeric anthocyanins } (mg/L) = (A \times MW \times D \times 1000)/(\varepsilon \times PL) \quad (1)$$

where $A = (A_{520} - A_{700})\,pH_{1.0} - (A_{520} - A_{700})\,pH_{4.5}$; MW is 449.2 g/moL for cyanidin 3-glucoside; D = dilution factor; PL is the constant at path length 1 cm; ε is 26900 L/mol cm, the molar extinction coefficient for cyanidin 3-glucoside; 1000 is the factor for conversion from g to mg.

Antioxidant Capacity. Antioxidant capacity (AC) was measured by the oxygen radical absorbance capacity (ORAC) assay as described by Prior et al.[25] using 20 μL of Trolox standard, samples, or blank (75 mM phosphate buffer pH 7.4), 120 μL of 116.9 nM fluorescein (final concentration 70 nm/well), and 60 μL of 40 mM AAPH per well. A black walled 96-well plate was read at 485 and 582 nm every 2 min at sensitivity 60 at 37 °C using a Synergy 2 multiwell plate reader (Biotek, Winooski, VT). Results were expressed as mmol of Trolox equivalents (TE) or μmol TE/g blueberry.

α-Glucosidase and α-Amylase Inhibition. The methods for enzymatic inhibition assays were adapted from Apostolidie et al.[26] Briefly for the α-glucosidase assay, in a 96-well plate 50 μL of sample or positive control (1 mM acarbose) was added to 100 μL of a 1 U/mL α-glucosidase solution (in 0.1 M sodium phosphate buffer pH 6.9) and incubated for 10 min. A 50 μL aliquot of a 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution (in 0.1 M sodium phosphate buffer pH 6.9) was added briefly to each well and incubated at 25 °C for 5 min before the absorbance was read at 405 nm. For the α-amylase assay, 500 μL of sample or positive control (1 mM acarbose) was added to 500 μL of 13 U/mL α-amylase solution (type VI-B from porcine pancreas in 0.02 M sodium phosphate buffer pH 6.9) and incubated in test tubes at 25 °C for 10 min before 500 μL of 1% soluble starch solution (dissolved in sodium phosphate buffer and boiled for 10−15 min) was added to each tube and incubated for another 10 min. Finally, 1 mL of dinitrosalicylic acid color reagent was added and the tubes were placed in 100 °C water for 5 min. The mixture was diluted with 10 mL of distilled water and absorbance was read at 520 nm. Results are presented as percent inhibition relative to the positive control acarbose having 100% inhibition.

Wine Quality. Finished wine was measured for quality parameters using 600 μL of wine loaded onto the OenoFoss (FOSS, Denmark) machine and read using the Foss Integrator software. Glucose/fructose, pH, total acid, malic acid, volatile acid, and percent ethanol were determined by Fourier transform infrared (FTIR) spectroscopy. Global calibrations provide accurate readings for ranges between 0 and 15 g/L for glucose/fructose, 2.6 and 4.5 for pH, 1.5 and 8.0 g/L for total acid, 0 and 7 g/L for malic acid, 0 and 1.5 g/L volatile acid (as acetic acid), and 8 and 18% ethanol.

Statistical Analysis. Data were expressed as means of at least three replications. Statistical analysis was conducted using Tukey's Studentized range test groupings using the proc GLM function of SAS version 9.2 (SAS Inst. Inc., Cary, NC). Group means were considered to be significant at $p < 0.0001$ based on minimum significant differences from one-way analysis of variance (ANOVA) with alpha = 0.05.

RESULTS AND DISCUSSION

Physical Characterization of the Blueberry Cultivars. *Diameter.* The physical and chemical characteristics of the different cultivars are presented in Table 1. Darrow and Spartan cultivars were found to have the largest average diameters (18.49 ± 3.43

Table 2. Total Polyphenols, Antioxidant Capacity, Alpha-Amylase Inhibition and Alpha-Glucosidase Inhibition of Blueberry Cultivars Grown in Southern Illinois, Dixon Springs Agricultural Center[a]

V. corymbosum cultivar	total polyphenols (mg GAE/g berry)	antioxidant capacity (μmol TE/g blueberry)	CAE[b] % α-amylase inhibn (%[c])	CAE % α-glucosidase inhibn (%[c])
Berkley	0.75 ± 0.03 b	8.98 ± 0.31 b	97.67 ± 0.28 cd	147.07 ± 0.04 bcd
Blue Chip	0.48 ± 0.01 cd	10.86 ± 0.22 a	103.32 ± 0.34 a	181.66 ± 0.05 ab
Blue Haven	0.55 ± 0.01 bcd	5.81 ± 0.04 g	96.59 ± 0.21 b	157.62 ± 0.02 abc
Blue Jay	0.47 ± 0.06 d	7.50 ± 0.26 cd	101.70 ± 0.62 g	157.76 ± 0.02 cde
Bluecrop	0.47 ± 0.04 d	7.64 ± 0.33 c	91.79 ± 0.82 de	129.07 ± 0.39 abc
Blueray	0.48 ± 0.03 cd	6.14 ± 0.03 fg	92.06 ± 0.34 g	184.70 ± 0.01 ab
Bluetta	0.54 ± 0.01 cd	6.74 ± 0.12 ef	95.78 ± 0.21 ef	133.12 ± 0.07 cde
Collins	0.39 ± 0.03 d	7.02 ± 1.55 cde	96.95 ± 0.00 cde	193.61 ± 0.02 a
Coville	0.54 ± 0.31 bcd	10.76 ± 0.13 a	97.08 ± 0.49 cde	190.78 ± 0.01 a
Darrow	0.69 ± 0.14 bc	10.77 ± 0.45 a	97.49 ± 0.49 cd	155.24 ± 0.14 abc
Earliblue	0.75 ± 0.04 b	9.06 ± 0.32 b	98.29 ± 0.84 c	103.22 ± 0.15 f
North Country	1.00 ± 0.02 a	9.07 ± 0.04 b	94.84 ± 0.43 f	113.91 ± 0.09 def
Patriot	0.65 ± 0.03 bcd	6.77 ± 1.90 ef	101.12 ± 0.40 b	161.04 ± 0.07 abc
Spartan	0.69 ± 0.01 bc	6.86 ± 0.27 de	97.67 ± 0.68 cd	184.03 ± 0.01 ab
Stanley	0.66 ± 0.00 bc	7.07 ± 0.71 cde	101.39 ± 0.93 b	161.99 ± 0.11 abc
commercial sample	0.47 ± 0.03 d	5.88 ± 0.26 g	86.80 ± 0.39 h	75.54 ± 0.19 f

[a] The data represents the mean ± SD from at least two independent studies and at least a triplicate analysis. Values within a column followed by different letters are significant at $p < 0.05$. Total polyphenols minimum significant difference = 0.2347; antioxidant capacity minimum significant difference = 0.6731; α-amylase minimum significant difference = 1.5997; α-glucosidase minimum significant difference = 40.424. [b] CAE = crude Amberlite extract; contains no reducing sugars that would interfere with assay. [c] Positive control acarbose was considered as 100% inhibition.

and 18.69 ± 1.84 mm, respectively). North Country, on the other hand, had the smallest diameter (12.80 ± 0.99 mm). Eleven of the cultivars grown at DSAC were found to have higher average diameters than the commercial sample (16.28 ± 3.79 mm). The commercial sample showed the greatest variability in size. Despite variation in average diameters, all blueberries sampled were categorized as "large"[27] since all of the average diameters fell above 12 mm. It was found that diameter and total sugar had a strong negative correlation ($r = -0.568$); as the diameter increased in these highbush blueberry cultivars, the total sugar content decreased.

pH. The commercial sample was found to have the highest pH (3.7), while Patriot showed the lowest (2.6). The pH among all cultivars ranged from 2.6 to 3.4 as seen in Table 1. This range is close to that found in the literature, from 2.85 to 3.49.[28]

Reducing Sugars. The range of reducing sugars across the blueberry cultivars was found to be as low as 6.4 ± 0.1% in Collins to 15.2 ± 2.2% in Darrow (Table 1). All other cultivars grown at DSAC were found to have higher reducing sugars than the commercial sample. The reducing sugar content of commercial blueberries was not significantly different from the value of 7.1% reported in the literature.[28]

Total Sugars. Similarly, total sugar content varied across the blueberry cultivars from 13.9 ± 0.1% in Spartan to 21.6 ± 0.4% in North Country (Table 1). With the exception of Spartan, fourteen out of the fifteen cultivars grown at DSAC had comparable or higher content of total sugars than the commercial sample. The total sugar content in the commercial sample was approximately twice the value reported in the literature of 7.3%.[29]

Total Polyphenol Content. Total polyphenols ranged from 0.39 ± 0.03 mg GAE/g berry in Collins to 1.00 ± 0.02 mg GAE/g blueberry in North Country (Table 2). The phenolic content could be affected by the diameter of the blueberry, as most polyphenolic compounds are located in the skins of the fruits; however no significant correlation was found (results not shown). Overall, most of the TP values for the blueberry cultivars were slightly below the reported average of 0.95 mg GAE/g blueberry[13] with exception of Collins.

Antioxidant Capacity. AC varied from 10.86 ± 0.22 μmol TE/g blueberry in Blue Chip to 5.81 ± 0.04 μmol TE/g blueberry in Blue Haven (Table 2). Most blueberry cultivars grown at DSAC showed higher AC values than the commercial sample (5.88 ± 0.26 μmol TE/g blueberry), except Blue Haven. Factors that have been known to impact antioxidant capacity include the total anthocyanin content, total phenolic content, and fruit maturity, as well as growing and postharvest storage conditions.[30] There was a strong positive correlation ($r = 0.70$) between total polyphenolics and antioxidant capacity, suggesting the antioxidant activity is due to phenolic compounds found in the berries.

Blueberry Cultivar and Enzyme Inhibitory Capacity. *α-Amylase.* The effectiveness of α-amylase inhibition of the blueberry fruits from the different cultivars was measured using acarbose, an anti-hyperglycemic drug, as a positive control having 100% inhibition. In comparison to acarbose, all blueberry extracts showed similar α-amylase inhibition capabilities, ranging from 91.79 ± 0.82% for Bluecrop to 103.32 ± 0.34% for Blue Chip (Table 2). All blueberries grown at DSAC were found to have higher percent enzyme inhibition than the commercial sample (86.80 ± 0.39% inhibition). It is expected that the α-amylase inhibitory activity increases as the total polyphenolic content rise in a fruit as previously seen for small fruits;[2] there was a strong positive correlation between total polyphenolic content and α-amylase inhibitory activity ($r = 0.85$). Our preliminary studies with *Vaccinium* showed that a proanthocyanidin-enriched fraction had the lowest IC_{50} value ($IC_{50} = 31.4$ μg/mL) for α-amylase, suggesting a higher enzyme inhibitory potential, in comparison to the anthocyanin-enriched fraction ($IC_{50} > 100$ μg/mL).

Table 3. Chemical Characteristics of Blueberries Throughout Fermentation[a]

					α-amylase inhibition		α-glucosidase inhibition	
	time points	pH	°Brix	total polyphenols (μg GAE/mL wine)	% inhibn[b]	%/g GAE	% inhibn[b]	%/g GAE
RT	week 0	6.3 a	29.7 a	362.3 ± 6.9 c	103.11	4.35 a	314.3	13.3 a
	week 1	3.5 b	19.1 cde	563.3 ± 7.4 ab				
	pressed	3.6 c	15.5 e	657.1 ± 6.1 a				
	fermented	3.8 c	13.6 de	590.0 ± 26.7 ab	92.21	3.81 b	308.4	12.7 a
CT	week 0	4.5 c	26.3 abc	357.4 ± 27.7 c	102.89	4.19 a	319.4	13.0 a
	week 1	3.5 c	22.5 ab	524.2 ± 13.4 c				
	pressed	3.6 c	20.6 cd	378.5 ± 7.9 c				
	fermented	3.8 c	20.0 bc	423.3 ± 35.0 c	91.71	3.74 b	311.6	12.7 a

[a] The data represents the mean ± SD from at least two independent studies and at least a triplicate analysis. Values within a column followed by different letters are significant at $p < 0.05$. Time points: Week 0 = day yeast was added; no fermentation occurred. Week 1 = 7 days after yeast added. Pressed (skin removed): RT ready to press (low Brix) 14 days after yeast, CT ready 5 days later. Fermented = stored in cold room at 0 °C. Values are averages of independent duplicates for each temperature treatment. RT = room temperature (20−22 °C); CT = cold temperature (4 °C). pH minimum significant difference = 0.2876; °Brix minimum significant difference = 6.0291; total polyphenols minimum significant difference = 100.52; α-amylase minimum significant difference = 1.8067; α-glucosidase minimum significant difference = 19.755. [b] Positive control acarbose is 100% inhibition.

α-Glucosidase. As seen in Table 2, all blueberry cultivar extracts were found to have higher α-glucosidase inhibitory capabilities than acarbose. Additionally, most blueberry cultivars grown at DSAC had significantly higher percent inhibition than the commercial sample (75.54 ± 0.19%) with the highest inhibition seen by Collins (193.60 ± 0.02% inhibition) and Coville (190.78 ± 0.01% inhibition). It is desirable for starch degrading inhibitory agents to have a high α-glucosidase inhibition and a moderate α-amylase inhibition to avoid gastrointestinal side effects.[7] This makes highbush blueberry cultivars a good natural alternative for inhibiting starch degradation. There was a positive correlation ($r = 0.47$) between total polyphenolic content and α-glucosidase inhibition. Regarding α-glucosidase, our preliminary studies with *Vaccinium* showed that a proanthocyanidin-enriched fraction had the lowest IC_{50} value (IC_{50} = 25.0 μg/mL), suggesting again a higher enzyme inhibitory potential, in comparison to the anthocyanin-enriched fraction (IC_{50} >100 μg/mL).

Combining all results from cultivar evaluation, after sugar elimination, blueberries grown at DSAC have hypoglycemic effects comparable to acarbose, a known anti-hyperglycemic drug. Tannins normally present in fruits of red color such as red grapes, red wines, raspberries, and strawberries are not present in as high amounts in blueberries.[2] Because tannins contribute to the α-amylase inhibition, the effectiveness of the blueberry extracts to inhibit α-amylase is lower than the effectiveness to inhibit α-glucosidase. It was found that smaller *V. corymbosum* berries contained higher levels of total phenolics, anthocyanins, and flavonoids than larger genotypes,[31] although we found nonsignificant correlations.

To summarize our findings on the blueberry cultivars, out of the fifteen studied, Blue Chip and Coville exhibited the most hypoglycemic potential having high antioxidant capacity, high total polyphenol content, high α-glucosidase inhibition, and moderate α-amylase inhibition. Overall, all cultivars had high antioxidant capacity measured as μmol Trolox equivalents and high total polyphenol content measured as mg gallic acid equivalents. The commercial sample showed considerably lower antioxidant capacity, α-amylase inhibition and α-glucosidase inhibition. A lower antioxidant capacity for the commercial sample could be due to postharvest handling, storage, and transportation conditions which may have resulted in an oxidation of compounds having antioxidant activity. The phenolic profile of the blueberry cultivars needs to be further studied in order to attribute the enzyme inhibitory activity to a certain polyphenolic compound.

Berry Wine Analysis: Changes of Berry Juice Due to Fermentation. *Quality.* Finished blueberry wine samples fermented at room temperature (RT) had a pH of 3.8 ± 0.13, total acid content of 6.2 ± 0.25 g/L, malic acid content of 4.5 ± 0.07 g/L, volatile acid content measured as acetic acid of 0.2 ± 0.11 g/L, and alcohol content of 11.2 ± 0.07%. The blueberry wine samples that were fermented at cold temperature (CT) had a pH of 3.8 ± 0.14, total acid content of 5.3 ± 0.18 g/L, malic acid content of 4.2 ± 0.18 g/L, volatile acid content measured as acetic acid of 0.3 ± 0.12 g/L, and alcohol content of 9.5 ± 0.32%. Comparing the temperature of fermentation, the RT samples had a slightly higher total acid content and higher alcohol content, indicating that CT fermentation of blueberries caused less acid and ethanol production than RT. Because the volatile acid content is actually lower for RT than CT and the malic acid contents varied only slightly, the larger acid content in the RT samples must be due to another acid present, most likely ellagic acid, a major sedimentation product formed during the production of berry wines.

Brix. °Brix was measured daily during the fermentation process as a measure of total sugar content; the fruit was pressed following a rapid decrease in Brix. As seen in Table 3, Brix for RT decreased by 16.1 °Brix from week 0 to the fermented wine stage, and for CT it decreased by 6.3 °Brix, showing a greater decrease in Brix during RT fermentation than during CT fermentation. A similar decrease in Brix was previously seen for fermented lowbush blueberries,[18] but the values of Brix for our highbush blueberry wines were much higher than the previously reported range. This difference could be due to higher initial sugar content in the blueberries used or an incomplete fermentation resulting in more sugar in our final fermented wine.

pH. pH was also measured daily throughout the fermentation process. Because of the addition of potassium bicarbonate to the blueberry lot, the initial pH was much higher than expected for blueberry juice. As seen in Table 3, the pH for RT samples decreased by 2.5 from week 0 to the fermented wine stage, and for CT the pH decreased only 0.8. Comparing the samples from the two fermentation temperatures, there was a greater decrease in pH for the RT samples than the CT samples, possibly due to a lower production of acids such as ellagic acid during CT

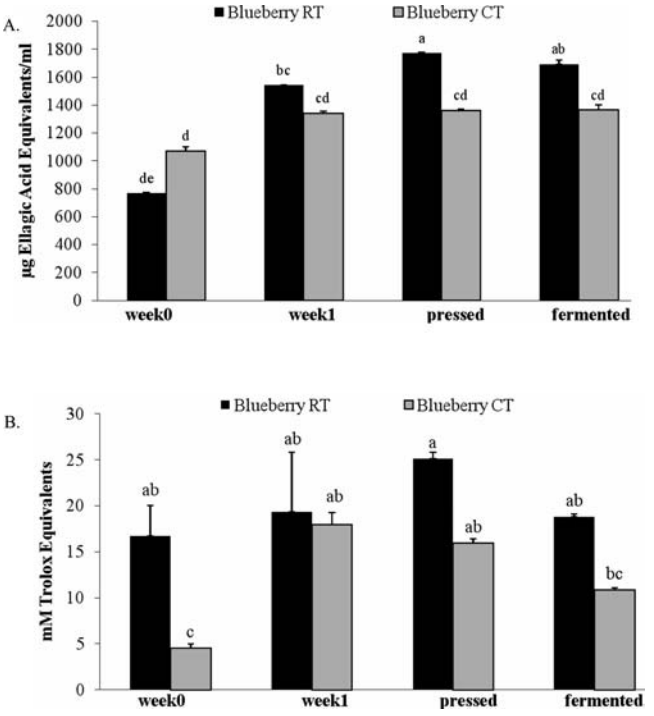

Figure 2. A. Total polyphenol trend throughout fermentation: room temperature vs cold temperature. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences ($p < 0.05$). B. Antioxidant capacity trend throughout fermentation: room temperature vs cold temperature. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences ($p < 0.05$). Time points and treatments: Week 0 = day yeast was added: no fermentation occurred. Week 1 = 7 days after yeast added. Pressed (skin removed): RT ready to press 14 days after yeast, CT ready 5 days later. Fermented = stored in cold room at 0 °C. Values are averages of independent duplicates for each temperature treatment: RT = room temperature (20−22 °C); CT = cold temperature (4 °C).

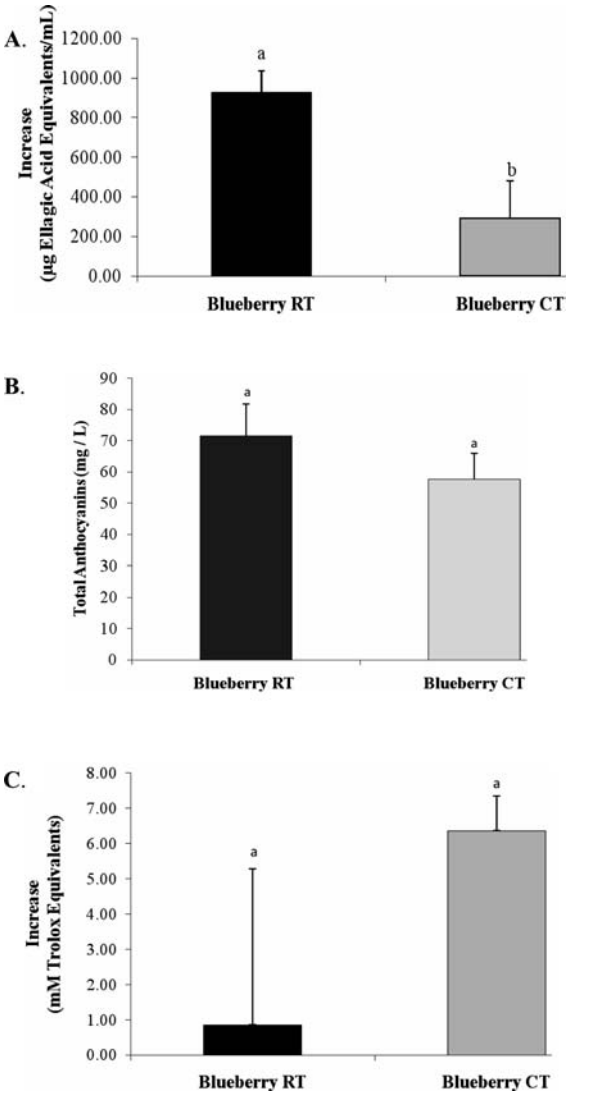

Figure 3. A. Changes due to fermentation in total polyphenols as ellagic acid equivalents for room temperature vs cold temperature. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences ($p < 0.05$). B. Total anthocyanins of fermented wines, room temperature (RT) vs cold temperature (CT). Results are expressed as monomeric anthocyanins as cyanidin-3-glucoside equivalents. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences ($p < 0.05$). C. Change in antioxidant capacity due to fermentation of blueberry wine at room temperature vs cold temperature. Bars represent means ± standard deviation based on data from independent experiments. Different letters indicate significant differences ($p < 0.05$). Blueberry wine was produced by fermentation of highbush blueberries (*Vaccinium corymbosum,* cultivars Blue Chip, Bluecrop, Blue Haven, Blue Jay, Blueray, Bluetta, Collins, Coville, Darrow, Earliblue, Elliot, Jersey, Late Blue, and Spartan) collected from Dixon Springs Agricultural Center in Simpson, IL, Summer 2010, at RT = room temperature (20−22 °C) or CT = cold temperature (4 °C).

fermentation, although both fermentation temperatures resulted in the same final pH of 3.8. There was no difference in final pH due to temperature of fermentation. Overall, this trend of pH change due to fermentation is consistent with that seen previously[18] in that there was an initial decrease of pH and then a gradual increase throughout fermentation.

Total Polyphenol Content. Figure 2A presents the TP content for the wines. TP increased from week 0 to the fermented wine by 228.0 μg GAE/mL (926.0 μg EAE/mL) for RT and increased by 65.9 μg GAE/mL (292.6 μg EAE/mL) for CT. The blueberry wine showed a significantly greater increase in TP from week 0 to fermented wine at RT than at CT fermentation ($p < 0.0023$) (Figure 3A). Since TP was measured in EAE, higher production of ellagic acid at RT could explain the higher total acid content measured in the finished wine for RT compared to CT. This indicates that the temperature of fermentation may affect the ellagic acid production in blueberry wine production. The TP results throughout time (Figure 2A) are consistent with those seen by Martin and Matar,[19] in that there was a significant increase in the total phenolic content initially. Results from this previous study on red wines made from grapes indicated lower concentrations of ellagic acid at higher temperature fermentation (37 °C compared to 20 °C), but our results indicated lower total polyphenols measured as ellagic acid equivalents at the cold temperature of 4 °C compared to 20−22 °C. Figure 3B presents the total anthocyanin content for the blueberry fermented wine at RT (71.4 ± 10.4 mg cyanidin-3-glucoside equivalents/L) and for the blueberry fermented wine at CT (57.7 ± 8.2 mg cyanidin-3-glucoside equivalents/L); these values were not statistically different.

Antioxidant Capacity. The AC trend is seen in Figure 2B, and the change from week 0 to the fermented wine is seen in Figure 3C. AC for RT samples increased by 0.86 mM TE from week 0 to the fermented wine stage, and for CT samples AC increased by 6.36 mM TE. RT fermentation showed a smaller increase in AC while CT fermentation showed a larger increase in AC for blueberry wine from the week 0 time point, although this was not significant between RT and CT ($p = 0.172$), as seen in Figure 3C. Increases in antioxidant activities during the beginning of fermentation are associated with the production of phenolic compounds having antioxidant capacity. A decrease in AC throughout fermentation could be due to oxidation or degradation of compounds with antioxidant activity. A loss of antioxidant activity toward the end of fermentation has been attributed to the alteration of phenolic compounds but not a loss in total phenolics,[18] which explains why we did not find a correlation between TP content and AC. The chemical profile of these wines will have to be further investigated in order to attribute the antioxidant activity to specific phenolic composition.

Berry Wine Enzyme Inhibitory Capacity. *α-Amylase.* Due to the fact that reducing sugars interfere with the accuracy of this assay, the wine samples were run through an Amberlite XAD-7 column to remove sugars along with pectins and phenolic acids. The crude Amberlite extract (CAE) was then tested for enzyme inhibition. Results, expressed as a percentage of inhibition compared to the positive control of acarbose at 100% inhibition, are presented in Table 3 for both temperatures of the initial samples and fermented wine samples. At week 0, the blueberry juice showed an averaged α-amylase inhibition between fermentation temperatures of 103.00%, and correcting for the TP content in the CAE used this was 4.27% inhibition/g GAE. The fermented wine, averaging RT with CT, showed 91.96% inhibition of α-amylase; correcting for the TP content in the CAE this was 3.77% inhibition/g GAE. The blueberry wines at both stages of fermentation showed inhibitory capacities that were similar to the positive control acarbose, although there was a significant decrease in inhibition from week 0 to fermented wine ($p < 0.0001$), which was not correlated to any measured specific fermentation changes.

α-Glucosidase. The blueberry juice before fermentation showed 314.3% inhibition of α-glucosidase compared to acarbose's 100% inhibition, and the fermented blueberry wine had 308.4% inhibition. Correcting for the TP content of the CAE used, this was 13.2%/g GAE and 12.7%/g GAE for the blueberry juice and fermented wine, respectively. There was no significant difference in α-glucosidase inhibition at any time point or temperature of fermentation. This shows that fermentation maintains the α-glucosidase inhibitory capacity of blueberry juice. When considering natural sources for inhibition of starch degrading enzymes, it is desirable to have high α-glucosidase inhibition and moderate α-amylase inhibition to avoid unwanted side effects from undigested starch.[8] Our results show almost twice as high inhibition of α-glucosidase as α-amylase for both unprocessed and fermented blueberry samples, indicating that both blueberry juice and fermented wines have desirable inhibition capacity for these two starch degrading enzymes. A previous study[31] found that wine had one of the highest α-glucosidase inhibitory activities and this activity was linked to specific phenols including rosmarinic acid and resveratrol. Wines high in these compounds may also have a high α-glucosidase inhibition relevant to diabetes management.

In summary, for the wine produced from blueberries at two different temperatures, we found that the cold temperature fermentation had significantly lower total polyphenolic content and no statistical difference in antioxidant capacity. It was found that fermentation retained the *in vitro* starch degrading enzyme inhibitory capacity of the blueberries. Exploring the phenolic profile of the blueberry wine would explain why different trends in activity were seen and is a goal for future studies. Blueberries and their fermented products are good natural sources of polyphenols, antioxidants, and *in vitro* inhibition of α-amylase and α-glucosidase.

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Funding Sources

This research was funded by the University of Illinois, ACES Office of Research.

ACKNOWLEDGMENT

Acknowledgment is made to Dixon Springs Agricultural Center especially to Jeff Kindhart and also to Bradley Beam, Enology Specialist.

ABBREVIATIONS USED

AAPH, 2,2-azobis 2-amidinopropane dihydrochloride; AC, antioxidant capacity; CAE, crude-Amberlite extract; CT, cold temperature (4 °C); EAE, ellagic acid equivalents; GAE, gallic acid equivalents; RT, room temperature (20−22 °C); TE, Trolox equivalents; TFA, trifluoroacetic acid; TP, total polyphenol; ORAC, oxygen radical absorbance capacity

REFERENCES

(1) Centers for Disease Control and Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011; U.S. Department of Health and Human Services, Centers for Disease Control and Prevention: Atlanta, GA, 2011.

(2) McDougall, G.; Shpiro, F.; Dobson, P.; Smith, P.; Blake, A.; Stewart, D. Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. *J. Agric. Food Chem.* **2005**, *53*, 2760–2766.

(3) Goncalves, A.; Lajolo, F.; Genovese, M. Chemical composition and antioxidant/antidiabetic potential of Brazilian native fruits and commercial frozen pulps. *J. Agric. Food Chem.* **2010**, *58*, 4666–4674.

(4) Pinto, M; de Carvalho, J.; Lajolo, F.; Genovese, M.; Shetty, K. Evaluation of antiproliferative, anti-type 2 diabetes and antihypertension potential of ellagitannins from strawberries (Fragaria x ananassa Duch.) using in vitro models. *J. Med. Food* **2010**, *5*, 1–9.

(5) Cheplick, S.; Kwon, Y.; Bhowmik, P.; Shetty, M. Clonal variation in raspberry fruit phenolics and relevance for diabetes and hypertension management. *J. Food Biochem.* **2007**, *31*, 656–679.

(6) Stull, A.; Cash, K.; Johnson, W.; Champagne, C.; Cefalu, W. Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. *J. Nutr.* **2010**, *140*, 1764–1768.

(7) Basu, A; Du, M.; Leyva, M.; Sanchez, K.; Betts, N.; Wu, M.; Aston, C.; Lyons, T. Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. *J. Nutr.* **2010**, *140*, 1582–1587.

(8) Rosenstock, J.; Brown, A.; Fischer, J.; Jain, A.; Littlejohn, T.; Nadeau, D.; Sussman, A.; Taylor, T.; Krol, A.; Magner, J. Efficacy and safety of acarbose in metformin-treated patients with type 2 diabetes. *Diabetes Care* **1998**, *21* (12), 2050–2055.

(9) Prior, R.; Wu, X.; Schaich, K. Standardized Methods for the Determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.

(10) Zafra-Stone, S.; Yasmin, T.; Bagchi, M.; Chatterjee, A.; Vinson, J.; Bagchi, D. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* **2007**, *51*, 675–683.

(11) McGhie, T.; Walton, M. The bioavailability and absorption of anthocyanins: Towards a better understanding. *Mol. Nutr. Food Res.* **2007**, *51*, 702–713.

(12) Castrejón, A.; Eichholz, I.; Rohn, S.; Kroh, L.; Huyskens-Keil, S. Phenolic profile and antioxidant activity of highbush blueberry (*Vaccinium corymbosum* L.) during fruit maturation and ripening. *Food Chem.* **2008**, *109*, 564–572.

(13) Ehlenfeldt, M.; Prior, R. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of Highbush blueberry. *J. Agric. Food Chem.* **2001**, *49*, 2222–2227.

(14) Zunino, S. Type 2 diabetes and glycemic response to grapes or grape products. *J. Nutr.* **2009**, *139*, 1794S–1800S.

(15) Kalt, W; Ryan, D.; Duy, J.; Prior, R.; Ehlenfeldt, M.; Kloet, S. Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of Highbush and Lowbush blueberries (*Vaccinium* Section *cyanococcus spp.*). *J. Agric. Food Chem.* **2001**, *49*, 4761–4767.

(16) Connor, A.; Luby, J.; Hancock, J.; Berkheimer, S.; Hanson, E. Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. *J. Agric. Food Chem.* **2002**, *50*, 893–898.

(17) Yang, M.; Koo, S.; Song, W.; Chun, O. Food matrix affecting anthocyanin bioavailability: Review. *Curr. Med. Chem.* **2010**, *18* (2), 291–300.

(18) Martin, L.; Matar, C. Increase of antioxidant capacity of the lowbush blueberry (*Vaccinium angustifolium*) during fermentation by a novel bacterium from the fruit microflora. *J. Sci. Food Agric.* **2005**, *85*, 1477–1484.

(19) Su, M.; Chien, P. Antioxidant activity, anthocyanins, and polyphenols of rabbiteye blueberry (*Vaccinium ashei*) fluid products as affected by fermentation. *Food Chem.* **2007**, *104*, 182–187.

(20) Talcott, S.; Lee, J. Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. *J. Agric. Food Chem.* **2002**, *50* (11), 3186–3192.

(21) Lindsay, H. A colorimetric estimation of reducing sugars in potatoes with 3,5-dinitrosalicylic acid. *Potato Res.* **1973**, *16*, 176–179.

(22) Grace, M.; Ribnicky, D.; Kuhn, P.; Poulev, A.; Logendra, S.; Yousef, G.; Raskin, I.; Lila, M. Hypoglycemic activity of a novel anthocyanin-rich formulation from lowbush blueberry, *Vaccinium angustifolium* Aiton. *Phytomedicine* **2009**, *16*, 406–415.

(23) Amerine, M.; Ough, C. *Wine and must analysis*; John Wiley and Sons: New York, 1974.

(24) Lee, J. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. *J. AOAC Int.* **2005**, *88*, 1269–1278.

(25) Prior, R.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodhill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydropilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. *J. Agric. Food Chem.* **2003**, *51*, 3273–3279.

(26) Apostolidis, E.; Kwon, Y.-I.; Ghaedian, R.; Shetty, K. Fermentation of milk and soymilk by *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* enhances functionality for potential dietary management of hyperglycemia and hypertension. *Food Biotechnol. (London)* **2007**, *21*, 217–136.

(27) Quality Protocol for Fresh Blueberries. Argentinean Secretariat of Agriculture, Livestock, Fishing and Food, Under secretariat of Agricultural Policy and Food. National Food Administration. 2007 Code: SAA006 Version: 08 04.20.2007.

(28) Highbush Blueberry Specifics. Blueberries: Bright, Bold, Beautiful. U.S. Highbush Blueberry Council: Folsom, CA, 2010.

(29) Matthews, R.; Pehrsson, P.; Farhat-Sabet, M. Sugar content of selected foods: individual and total sugars. United States Department of Agriculture: 1987.

(30) Dragović-Uzelac, V.; Savic, Z.; Brala, A.; Levaj, B.; Kovacevic, D.; Bisko, A. Evaluation of phenolic content and antioxidant capacity of blueberry cultivars (*Vaccinium corymbosum* L.) grown in the Northwest Croatia. *Food Technol. Biotechnol.* **2010**, *48* (2), 214–221.

(31) Kwon, I.; Apostolidis, E.; Shetty, K. Inhibitory potential of wine and tea against alpha-amylase and alpha-glucosidase for management of hyperglycemia linked to type-2 diabetes. *J. Food Biochem.* **2008**, *32* (1), 15–31.