JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Antioxidant and Enzyme Inhibitory Activities of Blueberry Anthocyanins Prepared Using Different Solvents

Floirendo P. Flores, Rakesh K. Singh, William L. Kerr, Ronald B. Pegg, and Fanbin Kong*

Department of Food Science and Technology, College of Agricultural and Environmental Sciences, The University of Georgia, 100 Cedar Street, Athens, Georgia 30602-2610, United States

ABSTRACT: We compared the biological activities of anthocyanins prepared from whole blueberries or pomace and extracted with acetone, ethanol, and methanol. Crude Amberlite extracts (CAE) and rehydrated powders of freeze-dried anthocyanins were used. Ethanolic CAE yielded the highest total monomeric anthocyanin content [TMAC] (160 ppm), ferric reducing antioxidant power [FRAP] (3.4 mM Fe^{2+}), total phenolics content [TPC] (382 ppm gallic acid equivalents [GAE]), and α-amylase inhibitory activity (36.8%). The rehydrated powder from acetonic extract gave the greatest FRAP (5.19) and TPC (422.7). α-Amylase (26.1%) and α-glucosidase (91.5%) inhibitory activities were also sustained. Methanolic CAE yielded values intermediate between ethanolic and acetonic extracts. Comparison of mass spectra between Amberlite extracts and rehydrated preparations revealed putative degradation and dimerization products in the rehydrated powders, which could account for loss in biological activities for rehydrated methanolic and ethanolic powders. Results of this study provide useful information in optimizing anthocyanin preparation methods for improved biological activity.

KEYWORDS: *blueberries, anthocyanin, solvents, antioxidant, α-glucosidase*

■ INTRODUCTION

There is a pressing need to address obesity and related metabolic disorders such as type 2 diabetes. The United States Centers for Disease Control and Prevention reported that in 2009 to 2010 over 78 million U.S. adults (35.7% of total) and 12.5 million U.S. children and adolescents (16.9%) were obese.[1] Evidence suggests a direct relationship between obesity and low-degree inflammation and oxidative stress, which may result in insulin resistance and the onset of type 2 diabetes.[2] Recent data reveal that 25.6 million (11.3%) adults over the age of 20 and 10.9 million (26.9%) adults over the age of 65 are afflicted with diabetes. Of these numbers, 16% do not take any form of medication,[3] implying that dietary intervention remains an option for management of diabetes.

Many studies report on the bariatric potential of bioactive compounds from blueberries (*Vaccinium* sp.). One of the major impacts of blueberry anthocyanins (ACNs) is postprandial glycemic control by enzymic inhibition of starch degradation, especially against α-glucosidase.[4−7] The possibility of ACN-mediated glucose absorption via the sodium glucose transporter in intestinal brush border membrane vesicles[8] and ACN upregulation of the insulin-dependent glucose transporter GLUT4 via activation of adenosine monophosphate-activated protein kinase[9] have also been reported. The high polyphenolic content of blueberries was also found to decrease systolic and diastolic blood pressure, possibly by decreasing vasoconstriction via the nitric oxide-mediated pathway.[10] Besides mediating oxidative stress,[2,11,12] polyphenols also prevented preadipocyte differentiation and adipogenesis.[13]

Theoretically, it is possible to achieve normoglycemic benefits by consuming a reasonable amount of fresh blueberries,[14] but berries are commonly processed before consumption. This could affect the amount of bioactives and concomitant bariatric potential.[15] Freeze-dried blueberry powder attenuated insulin resistance in a high-fat diet, but was not able to reverse hepatic insulin resistance.[14] Purified blueberry ACNs were more effective than whole blueberry powder in altering the development of obesity *in vivo*.[16] This shows that ACNs possess antioxidant and normoglycemic potential separate from other polyphenols present in blueberry. Most *in vitro* studies were performed with ACNs extracted using different solvents, but systematic optimization studies have yet to be reported.[17] The aim of this study was to compare the bariatric potential of blueberry extracts prepared using the three most commonly used solvents: methanol, ethanol, and acetone. The extracts were prepared from both whole berries and pomace.

■ MATERIALS AND METHODS

Materials. Fresh blueberries (Sunnyridge) were purchased from commercial supermarkets in Athens, Georgia, and were kept at −20 °C before use. Frozen blueberries (BB) were thawed between 4 and 6 °C and blanched for 3 min at 100 °C. For methods that require pomace, BB batches were subjected to juice extraction using a commercial 2500 mL centrifugal Kuvings NJ-9310U juicer (Elk Grove Village, IL) to separate the juice from the pomace. Both pomace and juice were dried using a VirTis Unitop-600L freeze drier (Gardiner, NY) and kept at −20 °C prior to use.

Methanol, ethanol, acetone, 2-propanol, acetic acid, hydrochloric acid, and sodium hydroxide were purchased from Fisher Scientific Ltd. (Suwanee, GA). Unless otherwise stated, all other materials were obtained from the Sigma-Aldrich (St. Louis, MO). A commercially available pectinase−arabinanase solution (Mzyme PearX) was a gift from J&H Biotechnology Co., Ltd. (Shanghai, China).

Received: January 28, 2013
Revised: April 8, 2013
Accepted: April 16, 2013
Published: April 16, 2013

 © 2013 American Chemical Society

Polyphenon 60 from green tea (Cat. No. P1204) and tannic acid (Cat. No. 403040) were used as controls in the antioxidant and enzyme inhibition studies.

Methods. *Preparation of Anthocyanin Extracts. Method 1.* The acetonic extraction is based on the method of Rodriguez-Saona and Wrolstad.[18] Briefly, the method involved extraction with 70% aqueous acetone acidified with 0.01% (v/v) HCl followed by filtration and solvent extraction with chloroform. The measured pH of the aqueous, acidified acetone was 2.4. The residual solvent of the aqueous layer was evaporated under vacuum at 40 °C (Rotavapor R-124, Büchi Corp., Newcastle, DE). Two batches (blanched whole BB and freeze-dried BB pomace) were individually ground for 10 min using the centrifugal juicer. Sufficient amount of 70% (v/v) aqueous acetone acidified with 0.01% (v/v) hydrochloric acid was added to facilitate trituration. More solvent was later added to achieve a 1:1 (sample:solvent) ratio, and the mixture was stirred for 1 h and filtered under vacuum. The residue was re-extracted with a proportional amount of the solvent (aqueous, acidified acetone) for another hour, and both filtrates were pooled prior to solvent extraction with chloroform and vacuum evaporation. Samples were obtained, and the resulting concentrates were labeled as acetonic extract of whole BB (AEW) and acetonic extract of BB pomace (AEP).

Method 2. As described previously, blanched whole BB were extracted with anhydrous methanol acidified with 0.01% (w/v) citric acid (1:2 sample:solvent). The measured pH of the solvent was 4.1. The mixture, however, did not undergo solvent extraction with chloroform. The filtrate was also evaporated under vacuum, and the concentrated liquid was labeled as methanolic extract of whole BB (MEW).

Method 3. Freeze-dried BB pomace was extracted for 24 h with 80% (v/v) aqueous ethanol (pH = 6.0) according to the method of Hogan and others.[19] The resulting pooled filtrate was evaporated under vacuum, and the concentrate labeled as ethanolic extract of BB pomace (EEP).

Method 4. Blanched whole BB were ground for 10 min with an equivalent amount of deionized water. The enzyme was added at 500 ppm and stirred for 2.5 h at room temperature. The juice was filtered under vacuum, placed in a sealed container fitted with a thermocouple and a data logger, and pasteurized at 63 °C in a water bath for 30 min. The concentrated sample was labeled enzymic extract of whole BB (ZEW) and kept at −20 °C prior to use.

Solid−Liquid Extraction and Powder Preparation. All extracts from the four methods described above were passed individually through an Amberlite XAD-16N column 20 cm high and 3 cm in diameter to concentrate the phenolic compounds.[20] Sugars and organic acids from the preparations were eluted with three column volumes of deionized water. The anthocyanin fraction was then collected by elution with anhydrous methanol. Aliquots (10 mL) were obtained and labeled as crude Amberlite extracts of acetone (CAW for whole BB, CAP for pomace), methanol (CMW), ethanol (CEP), and enzyme (CZW) and stored at −20 °C. Among these, CAW, CMW, and CEP were chosen for biological activity tests. The Amberlite extracts were then evaporated under vacuum, freeze-dried, and also kept at −20 °C prior to use. Of the resulting powder samples, four were selected for further tests: acetone−whole BB (AW), methanol−whole BB (MW), ethanol−pomace (EP), and enzyme−whole BB (ZW). Powder samples were later rehydrated (1 mg/mL) with deionized water prior to tests.

Total Monomeric Anthocyanin Concentration (TMAC) and Colorimetry. The pH differential method was used to determine the total monomeric anthocyanin (ACN) content of all crude extracts, Amberlite extracts, and ACN powders.[21] The absorbance at 510 nm (corresponding to cyanidin-3-*O*-glucoside, molar mass = 449.2, ε = 26 900) of aliquots was measured at pH values 1 and 4.5 using an Evolution 300 spectrophotometer (Thermo Scientific, Suwanee, GA).

As a rapid estimate of anthocyanin content, the color values (L^*, a^*, and b^*) of the rehydrated ACN powders were measured using HunterLab Miniscan EZ (Reston, VA). The colorimeter was calibrated with black and white tiles prior to use. Samples (4 mL) were added to clear plastic dishes (1 cm depth and 3.5 cm diameter) and placed against a white tile.

α-Amylase Inhibitory Assay. The method of Kwon et al.[22] was used with modifications. Crude Amberlite extracts (CAW, CMW, and CEP) and rehydrated ACN powders (AW, MW, EP, and ZW) were diluted 10-fold. Type VI-B porcine pancreatic α-amylase (500 μL of 1 U/mL in 100 mM sodium phosphate buffer with 6.7 mM sodium chloride, pH 6.9) was added to 500 μL of each sample in vented test tubes and incubated at 25 °C for 10 min. Next, 500 μL of 1% (w/v) potato starch (previously dissolved in 100 mM phosphate-buffered saline, pH 6.9, and boiled for 15 min) was added, and the mixture was incubated for 10 min. Finally, 1 mL of dinitrosalicylic acid (DNS) reagent (20 mL of 96 mM DNS mixed with 8 mL of 5.315 M sodium potassium tartrate tetrahydrate in 2 M sodium hydroxide and diluted with 12 mL of deionized water) was added. The tubes were placed in a boiling water bath for 5 min and afterward were diluted with 10 mL of deionized water. Absorbance was read at 520 nm. Results are presented as percent inhibition relative to a blank control.

α-Glucosidase Inhibitory Assay. The method of Hwang et al.[23] was used with modifications. Crude Amberlite extracts (CAW, CMW, and CEP) were diluted 2-fold, while rehydrated ACN powders (AW, MW, EP, and ZW) were diluted 5-fold. Type I α-glucosidase from *Saccharomyces cerevisiae* (100 μL of 0.35 U/mL in 100 mM phosphate-buffered saline, pH 6.9) was added to 50 μL of the diluted extracts and incubated at 37 °C for 10 min in a Boekel 290400S water bath (Boekel Scientific, Feasterville, PA). Next, 100 μL of 1.5 mM *p*-nitrophenyl-α-D-glucopyranoside (dissolved in 100 mM phosphate-buffered saline, pH 6.9) was added and incubated for 20 min. The reaction was terminated by the addition of 1 mL of 1 M sodium carbonate. Absorbance was read at 400 nm. Results are presented as percent inhibition relative to blank control.

Ferric Reducing Antioxidant Power (FRAP) Assay. The method of Benzie and Strain[24] was used with modifications. Crude Amberlite extracts (CAW, CMW, and CEP) were diluted 10-fold, while rehydrated ACN powders (AW, MW, EP, and ZW) were diluted 20-fold. The FRAP reagent was prepared fresh daily by adding 2.5 mL of 2,4,6-tri(2-pyridyl)-*s*-triazine solution (10 mM in 40 mM HCl), 2.5 mL of ferric chloride hexahydrate solution (20 mM), and 25 mL of acetate buffer (300 mM, pH 3.6). The resulting mixture was held at 37 °C. Briefly, 30 μL of the sample was pipetted in a cuvette, and 1 mL of the FRAP reagent was added. The contents were mixed using a pipet. After 4 min of reaction, the absorbance at 593 nm was measured. A standard curve was generated using 100 μL of various ferrous sulfate heptahydrate solutions (0.1 to 1 mM). Results are presented as mM Fe^{2+} equivalents.

Total Phenolics Content (TPC) Assay. A modified Folin−Ciocalteu method was employed.[20] Crude Amberlite extracts (CAW, CMW, and CEP) were diluted 10-fold, while rehydrated ACN powders (AW, MW, EP, and ZW) were diluted 20-fold. Briefly, 8 mL of deionized water, 0.5 mL of Folin−Ciocalteu's phenol reagent, and 1 mL of saturated sodium carbonate solution were added into each test tube that contained 0.5 mL of sample. The mixture was vortexed for 15 s followed by a 60 min resting period for optimum color development. Absorbance was measured at 765 nm. A standard curve was generated with gallic acid, and results are reported as ppm gallic acid equivalents.

Mass Spectrometry. Mass spectrometry was performed at the Proteomics and Mass Spectrometry Facility (University of Georgia) using a Bruker Daltonics Autoflex TOF mass analyzer with a nitrogen laser. Crude Amberlite extracts (CAW, CMW, and CEP) and rehydrated powders were applied at 1 μL on the target plate and dried. This was conducted twice. No matrix was added. The instrument was operated in reflectron mode, and 300 laser shots were averaged for one spectrum.

Statistical Analyses. Data were expressed as means of assays conducted in triplicates. The proc GLM function of SAS 9.2 (SAS Inst., Cary, NC) was used to analyze one-way design with Tukey's honestly significant difference (HSD) as *post hoc* test. Means were regarded as significantly different at $p < 0.05$.

Table 1. Anthocyanin Content (TMAC) and Color Properties of ACN Extracts[a]

sample	mg TMAC/L crude extract	yield: mg TMAC/100 g whole berries or dry pomace)	yield: mg TMAC/mg dry powder)	color measurement: L^*	a^*	b^*	chroma	hue
Whole Berries								
AEW	364.1 d	60.3	0.18	31.10 b	30.87 c	1.01 d	30.89 c	1.72 d
MEW	627.2 c	94.7	0.18	28.60 c	44.55 a	11.48 a	46.00 a	14.32 a
ZEW	197.4 e	30.8	0.09	37.42 a	34.85 b	4.33 c	35.12 b	6.88 c
Pomace								
AEP	982.2 a	436.9	0.14	37.29 a	28.37 d	6.20 b	29.04 d	12.60 b
EEP	813.7 b	490.0	0.19	26.13 d	35.30 b	−0.83 e	35.31 b	−1.15 e

[a]Columns with the same letter are not statistically different at $p < 0.05$. Abbreviations: TMAC, total monomeric anthocyanin content; AEW, acetonic extract of whole berries; MEW, methanolic extract of whole berries; ZEW, enzymic extract of whole berries; AEP, acetonic extract of berry pomace; EEP, ethanolic extract of berry pomace.

■ RESULTS

TMAC and Colorimetry. Table 1 shows that among different methods used to extract ACNs from whole BB, the methanol-based extraction generated the most ACNs, followed by the acetone-based extraction. For pomace, there were more ACNs in the acetonic extract than in the ethanolic extract, but on the basis of 100 g of freeze-dried pomace, the ethanol-based method extracted more ACNs than the acetone-based method. After purification and lyophilization, ACN yield per mg of dry powder was greatest in the order EEP, AEW, MEW, and AEP.

The same trend was observed in the average lightness (L^*). EEP was darkest, while ZEW and AEP were lightest. MEW was reddest (a^*), while AEP was least red. EEP was bluest (b^*), and MEW the least. Hue ($\arctan[b^*/a^*]$) values show that EEP lies between the blue and bluish-red region (270° to 360°). The rest of the values lie between the bluish-red and yellow region (0° to 90°), but are closer to the bluish-red spectrum.

ACN values of crude Amberlite extracts and rehydrated powders are given in Table 2. Expectedly, rehydrated powders contained more TMAC than Amberlite extracts due to concentration effects. The ethanol-based extraction method still extracted the most ACNs (CEP > CMW > CAW). The ethanolic extract (EP) possessed greater TMAC than the acetonic extract (AW), while TMAC of the methanolic extract (MW) was not statistically different from either AW or EP.

The correlation coefficients among the different tests are listed in Table 3. With crude Amberlite extracts, TMAC correlated strongly with α-glucosidase, FRAP, and TPC tests. However, upon freeze-drying, only the TMAC moderately correlated with FRAP.

Table 2. Biological Activity of Purified ACN Preparations[a]

sample	TMAC (mg/L)	enzyme inhibition (%): α-amylase	enzyme inhibition (%): α-glucosidase	FRAP (mM Fe^{2+})	TPC (ppm GAE)
Amberlite Extracts					
CAW	44.3 f	24.01 d	92.83 a	1.08 e	42.0 i
CMW	109.1 d	5.06 f	90.42 b	2.08 d	203.3 h
CEP	156.0 c	36.78 b	88.85 c	3.40 c	382.0 d
Rehydrated Powders					
AW	178.9 b	26.06 c	91.49 ab	5.19 b	422.7 c
MW	185.1 ab	2.29 g	n.d.	4.70 b	308.0 e
ZW	89.4 e	8.36 e	n.d.	3.64 c	214.7 g
EP	201.7 a	3.65 g	n.d.	4.30 bc	252.0 f
Control					
Polyphenon 60		24.80 cd	92.50a	9.99 a	682.7 b
tannic acid		67.34 a	92.92a	5.05 b	824.0 a

[a]Columns with the same letter are not statistically different at $p < 0.05$. Abbreviations: TMAC, total monomeric anthocyanin content; FRAP, ferric reducing antioxidant power; TPC, total phenolics content; GAE, gallic acid equivalents; CAW, crude Amberlite acetonic extract of whole berries; CMW, crude Amberlite methanolic extract of whole berries; CEP, crude Amberlite ethanolic extract of berry pomace; AW, acetonic extract of whole berries; MW, methanolic extract of whole berries; ZW, enzymic extract of whole berries; EP, ethanolic extract of berry pomace; n.d., not detected.

Table 3. Correlation Coefficients among Purified ACN Preparations[a]

	TMAC	α-amylase[b]	FRAP	TPC
Amberlite Extracts				
α-amylase[b]	0.335			
FRAP	0.989*	0.471		
TPC	0.995*	0.427	0.999*	
α-glucosidase[b]	−0.999*	−0.286	−0.980*	0.989*
Rehydrated Powders				
α-amylase[b]	−0.024			
FRAP	0.717*	0.566*		
TPC	0.477	0.789*	0.947*	0.947*

[a]Values with asterisks are statistically significant at $p < 0.05$. Abbreviations: TMAC, total monomeric anthocyanin content; FRAP, ferric reducing antioxidant power; TPC, total phenolics content. [b]Refers to enzyme inhibitory activity.

Biological Activities. Among rehydrated powders (Table 2), tannic acid exhibited the greatest inhibitory effect against α-amylase, followed by CEP. A slightly higher inhibitory effect was observed with the acetonic extract (AW) than the corresponding crude Amberlite extract (CAW). On a mass ratio, the inhibitory effect of Polyphenon 60 was not statistically different from AW. Low inhibitory effects were found with the remaining samples. Only the acetonic extract retained its relative inhibitory effect after lyophilization. The loss in inhibitory effect was most significant with the ethanolic extracts (CEP and EP).

All Amberlite extracts possessed a marked inhibitory activity against α-glucosidase in the order acetone (CAW) > methanol (CMW) > ethanol (CEP). The acetonic extract possessed a statistically-equivalent inhibitory effect to Polyphenon 60 and tannic acid on an equivalent mass basis. No inhibitory effect was found for rehydrated ACN powders prepared from

methanol, acetone, and Mzyme PearX. Similar to the α-amylase assay, only the acetonic extract retained its relative enzyme inhibitory activity.

The α-amylase inhibition did not correlate strongly with any of the tests that used the Amberlite extracts (Table 3). α-Glucosidase inhibition correlated strongly with TMAC, TPC, and FRAP. For tests that used rehydrated powders, the α-amylase inhibitory effect correlated moderately with TPC.

On a mass basis, Polyphenon 60 registered the highest ferric ion reducing ability followed by tannic acid, rehydrated acetonic (AW), methanolic (MW), and ethanolic (EP) extracts. Among crude Amberlite extracts, ferric reducing power was observed in the order ethanol (CEP) > methanol (CMW) > acetone (CAW). Tannic acid and Polyphenon 60 possessed the most phenolics (reported as ppm gallic acid equivalents). Among the rehydrated powders, the observed trend was acetone (AW) > methanol (MW) > ethanol (EP), while for Amberlite extracts, the trend was ethanol (CEP) > methanol (CMW) > acetone (CAW).

Among indexes of biological activities, there was strong correlation between antioxidant potential and α-glucosidase inhibitory activities for the Amberlite extracts and rehydrated powders. However, with rehydrated powders, only the correlation between FRAP and TPC was conserved (Table 3).

DISCUSSION

TMAC and Colorimetry. Blueberries are rich sources of anthocyanins, with the majority of the pigments accumulating in the peel/skin than in the flesh and seeds.[7,25] This, along with longer extraction time, would partially explain the slightly higher TMAC observed with the ethanolic pomace extract compared to the acetonic pomace extract or methods that used whole berries (Table 1). TMAC values from Table 2 do not show a statistical difference between EP and MW; nevertheless, a comparably high concentration of anthocyanins could be derived from pomace alone. Results confirm that the methanolic extracts (MEW and MW) possess more ACNs than acetonic extracts (AEW and AW).[26] We also observed higher TMAC (dry weight basis) for ethanolic pomace extracts (EEP) than acetonic pomace extracts (AEP). The relatively small contribution of BB juice toward total monomeric anthocyanins is seen from the difference of TMAC between AEP and AEW. This implies that BB byproducts, such as pomace, can be processed to recover bioactive compounds.[25]

As potential food colorants, anthocyanin color can be quantified using colorimetric scales, and changes in color parameters due to processing or degradation can be measured with ease.[27] Among the different parameters, the hue angle predicts whether a particular datum would be bluish, reddish, yellowish, or greenish, and 1° changes are perceptible by human vision. On the basis of our results, it did not appear to correlate well with TMAC. Lightness (L^*) was a better predictor of TMAC than hue angle.

Comparison of mass spectra of samples to published papers helps to identify putative peaks.[28,29] The peaks in the crude Amberlite extracts (Figure 1) corresponded to the m/z assignments for the major anthocyanidins malvidin (Mv), petunidin (Pt), delphinidin (Dp), cyanidin (Cy), and peonidin (Pn), with significantly fewer peaks likely to be the glycosylated forms (cyanidin+glucose, malvidin+glucose, peonidin+glucose). In all cases, peak intensities followed the trend Mv > Pt > Dp > Cy > Pn. When measured against malvidin intensity, we found that the ratio of intensities of extracted petunidin,

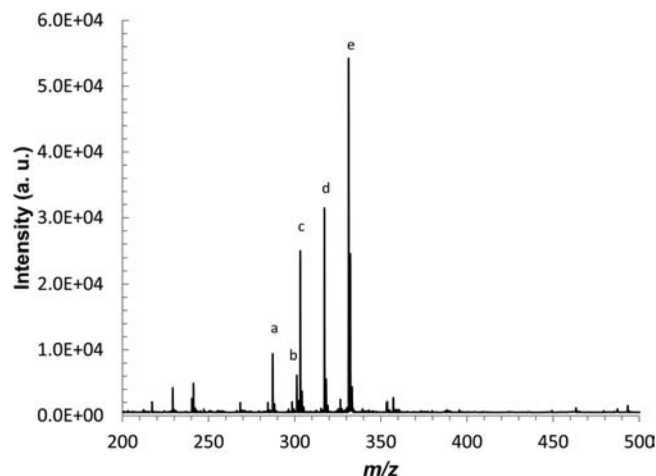

Figure 1. Mass spectra of crude Amberlite methanolic extract. Tentative peak assignments: a, cyanidin; b, peonidin; c, delphinidin; d, petunidin; e, malvidin.

delphinidin, cyanidin, and peonidin (Pt:Mv, Dp:Mv, Cy:Mv, and Pn:Mv, respectively) was highest with the ethanolic extract (0.80, 0.77, 0.46, 0.26), followed by the methanolic extract (0.52, 0.44, 0.17, 0.11), and lowest with the acetonic extract (0.21, 0.15, 0.07, 0.07). This means that the acetone-based extraction method (and solvent extraction with chloroform) tended to selectively extract Mv, while the methanol-based and ethanol-based methods extracted more Pt, Dp, Cy, and Pn. In a previous report, malvidin-3-*O*-galactoside was reported to be the most abundant anthocyanin in blueberries.[25] The relatively low concentration of glycosylated ACNs regardless of the extraction method might have been due to hydrolysis of the acyl groups from using acidified solvents, from blanching, or upon storage.[15,30]

In vivo tests using anthocyanins are usually conducted under neutral to slightly alkaline conditions, which could make identification of stable forms of ACNs difficult.[17,31] More peaks were observed when rehydrated powders were subjected to mass spectrometry. ACNs form the reactive quinoidal base under neutral media, leading to degradation products such as phenolic acids and aldehydes, or three-dimensional structures through self-association.[17,32] Glycosylation of the flavylium cation confers stability through steric hindrance,[32] and the absence of glycated forms in the spectra of the rehydrated powders implies that observed extraneous peaks were from degradation products. The identity and mass assignments of identified oligomers have already been reported.[28] Significantly intense peaks were observed at m/z = 633 for both the rehydrated methanolic and ethanolic extracts (Figure 2). On the basis of available mass assignments, this peak corresponds to a Mv-Dp dimer. Another peak (m/z = 661) was observed for the Mzyme extract (ZW), likely corresponding to an Mv−Mv dimer.

Biological Activities. Aside from anthocyanins, blueberries also contain phenolic acids (such as esters, glycosides, and amides of hydroxycinnamic and hydroxybenzoic acids) and flavanols.[33] Considering that the dry powders in Table 2 were composed of less than 50% monomeric anthocyanins, other phenolic compounds present likely contributed to the overall biological activity.[13] Several studies have concluded that the concentration of tannins is directly related to α-amylase inhibitory activity.[4,5,22] The mild inhibitory activity of the BB

 dx.doi.org/10.1021/jf400429f | J. Agric. Food Chem. 2013, 61, 4441−4447

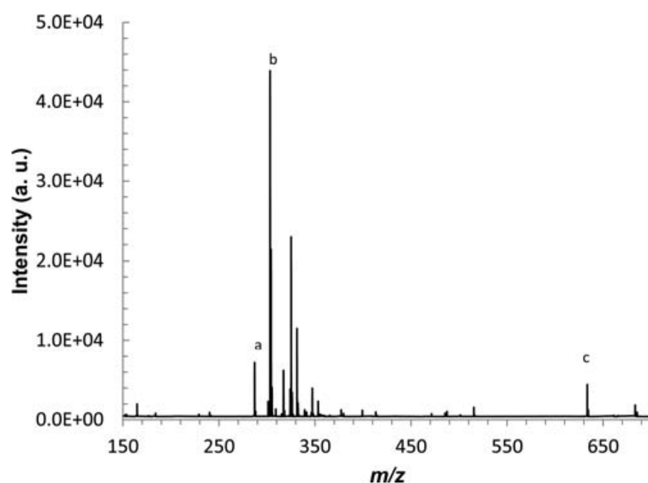

Figure 2. Mass spectra of rehydrated methanol extract. Tentative peak assignments: a, cyanidin; b, delphinidin; c, malvidin−delphinidin dimer (m/z = 633). Samples were spiked with quercetin hydrate, intensifying peak b.

extracts against α-amylase is indicative of the low ellagitannin content of blueberries. Our data agree with a previous report about low α-amylase inhibitory effect[5] but contradict the significantly higher inhibitory activity reported in another paper.[6] Attenuation of inhibitory activity for ethanolic and methanolic extracts could be due to significant dimerization. α-Amylase inhibition is dependent on stable binding between the flavylium cation and the enzyme. The presence of a double bond in the pyrone ring delocalizes electrons between the benzopyrone and benzene rings of the flavylium cation and confers binding stability.[34] Dimerization results in the loss of a double bond in the pyrone ring of the flavylium cation, as seen in proposed dimer structures,[28] and could thus explain reduction in α-amylase inhibition.

Inhibitory activity of BB extracts against α-glucosidase is more widely reported, and correlations with TMAC and TPC have been proposed.[5−7,22,35] It is currently understood that while glycosylated ACNs could competitively inhibit α-glucosidase, the anthocyanidin components also exert inhibitory activity,[4,35] as we have observed. Blueberries have greater ACN content and α-glucosidase inhibitory activity than strawberries, and it was proposed that the two parameters were directly related.[5,7] It was also reported that inhibitory activity varied directly with TPC and antioxidant activity.[22] However, with the Amberlite extracts we found a strong inverse relationship between inhibitory activity and TMAC, TPC, or FRAP. It was previously suggested that anthocyanidins could play a crucial part in α-glucosidase inhibition.[35] Among the Amberlite extracts, the acetone-based extraction method tended to extract more malvidin than any other anthocyanidin. Further tests can be done to determine if α-glucosidase inhibition is related to malvidin content of the extract.

The most surprising result in our work is the loss in α-glucosidase inhibitory activity of the rehydrated ethanolic and methanolic ACN powders (Table 2). A possible explanation may be that the formation of dimers (such as Mv−Dp and Mv−Mv) from the reactive quinoidal base could have attenuated enzyme inhibitory activity. However, rehydrated AW powder retained high α-glucosidase inhibitory activity, which may be due to degradation products under neutral pH resulting in compounds with inhibitory activity. Previously, the Cy/Mv ratio was lowest with the acetonic Amberlite extract. An examination of the mass spectra of rehydrated powders showed that the Cy/Mv ratio of methanolic and ethanolic extracts was practically unchanged, but a higher ratio was observed with the acetonic extract (approx 0.6). Under neutral media, aglycones degrade via the α-diketone pathway, resulting in free phenolic acids and aldehydes that could also possess biological activity.[32] Further, the effect of freeze-drying and subsequent rehydration on the stability of other phenolic compounds needs to be determined, as anthocyanins can undergo co-pigmentation with other flavonoid compounds.[26]

Regardless of the mild α-amylase inhibitory activity and instability in neutral media, BB extracts still have potential in mitigating postprandial hyperglycemia. Compared to α-amylase, α-glucosidases are key enzymes in starch digestion because they degrade oligosaccharides to monosaccharides prior to absorption in the intestinal epithelia.[4] Inhibition of α-glucosidases may slow down formation of glucose monomers and alleviate postprandial hyperglycemia, a risk factor for obesity and type 2 diabetes. Further studies can determine if anthocyanidins interact with glucose transporters in the intestinal epithelia to further delay glucose absorption. Our results confirm that ACNs possess biological activity even when prepared under neutral pH.[31]

Numerous papers have reported on the phenolics content and antioxidant potential of blueberries and processed products. However, it can be difficult to compare results because few papers reported using purification methods to remove sugars and other organic acids, which may interfere in the Folin-Ciocalteu assay.[6,20,25,36] It is generally held that TPC correlates better with antioxidant activity than TMAC.[37] In our results, we also report a slightly stronger positive correlation between FRAP and TPC than with TMAC among crude Amberlite extracts. A difference in correlation coefficients was more evident among rehydrated powders, where a strong correlation between FRAP and TPC (R = 0.95) was maintained, compared to FRAP and TMAC (R = 0.72).

Among crude Amberlite extracts, both TPC and FRAP correlated poorly with α-amylase inhibition. However, correlation between TPC and α-amylase inhibitory activity increased (R = 0.79) when rehydrated powders were used. Phenolic compounds that can form quinones, lactones, or a conserved 4-oxo-pyrane structure are associated with α-amylase inhibitory activity.[5] We surmise that ACNs could have either degraded under neutral pH or formed other phenolic structures, which is why acetonic extracts retained inhibitory action against α-amylase while ethanolic and methanolic extracts did not.

In conclusion, anthocyanin or anthocyanidin extracts that possess antioxidant and enzyme inhibitory activities could be prepared from both whole berries and pomace. Extraction with ethanol followed by solid-phase extraction was applied to pomace and found most effective at extracting the highest concentration of anthocyanins that possess mild α-amylase and high α-glucosidase inhibitory activities. The magnitude of biological activity of ethanolic extracts was comparable to that of methanolic and acetonic extracts of whole berries. However, after lyophilization and subsequent rehydration, the biological activities of the ethanolic and methanolic powders were either reduced or lost. The acetone-based extraction method was most effective at extracting anthocyanins that retain α-amylase and α-glucosidase inhibitory activities even after freeze-drying and reconstitution. The loss in biological activities of the

ethanolic and methanolic extracts under neutral media could be due to pigment degradation, dimerization, or co-pigmentation of anthocyanins with other flavonoids. Subsequent tests can be conducted to profile the phenolic compounds extracted with the acetone-based method and their biological activity as well as stability.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: fkong@uga.edu. Phone: (706) 542-7773. Fax: (706) 542-1050.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors wish to thank Dr. Dennis Phillips of the Proteomics and Mass Spectrometry Facility, The University of Georgia, for his assistance in analyzing the spectra.

■ REFERENCES

(1) Ogden, C. L.; Carroll, M. D.; Kit, B. K.; Flegal, K. M. *Prevalence of obesity in the United States, 2009−2010*; NCHS, Ed.; National Center for Health Statistics: Hyattsville, MD, 2012; pp 1−8.

(2) Hogan, S.; Canning, C.; Sun, S.; Sun, X. X.; Zhou, K. Q. Effects of grape pomace antioxidant extract on oxidative stress and inflammation in diet induced obese mice. *J. Agric. Food Chem.* **2010**, *58*, 11250−11256.

(3) CDC. *National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States*; Centers for Disease Control and Prevention: Atlanta, GA, 2011; pp 1−12.

(4) McDougall, G. J.; Stewart, D. The inhibitory effects of berry polyphenols on digestive enzymes. *Biofactors* **2005**, *23*, 189−195.

(5) Tundis, R.; Loizzo, M. R.; Menichini, F. Natural products as α-amylase and α-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: an update. *Mini-Rev. Med. Chem.* **2010**, *10*, 315−331.

(6) Johnson, M. H.; Lucius, A.; Meyer, T.; de Mejia, E. G. Cultivar evaluation and effect of fermentation on antioxidant capacity and in vitro inhibition of alpha-amylase and alpha-glucosidase by highbush blueberry (*Vaccinium corombosum*). *J. Agric. Food Chem.* **2011**, *59*, 8923−8930.

(7) Wang, S. O. Y.; Camp, M. J.; Ehlenfeldt, M. K. Antioxidant capacity and alpha-glucosidase inhibitory activity in peel and flesh of blueberry (*Vaccinium spp.*) cultivars. *Food Chem.* **2012**, *132*, 1759−1768.

(8) Cao, G. H.; Muccitelli, H. U.; Sanchez-Moreno, C.; Prior, R. L. Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. *Am. J. Clin. Nutr.* **2001**, *73*, 920−926.

(9) Takikawa, M.; Inoue, S.; Horio, F.; Tsuda, T. Dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity via activation of AMP-activated protein kinase in diabetic mice. *J. Nutr.* **2010**, *140*, 527−533.

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

(11) Kay, C. D.; Holub, B. J. The effect of wild blueberry (*Vaccinium angustifolium*) consumption on postprandial serum antioxidant status in human subjects. *Br. J. Nutr.* **2002**, *88*, 389−397.

(12) Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. Absorption of anthocyanins from blueberries and serum antioxidant status in human subjects. *J. Agric. Food Chem.* **2002**, *50*, 7731−7737.

(13) Moghe, S. S.; Juma, S.; Imrhan, V.; Vijayagopal, P. Effect of blueberry polyphenols on 3T3-F442A preadipocyte differentiation. *J. Med. Food* **2012**, *15*, 448−452.

(14) DeFuria, J.; Bennett, G.; Strissel, K. J.; Perfield, J. W.; Milbury, P. E.; Greenberg, A. S.; Obin, M. S. Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. *J. Nutr.* **2009**, *139*, 1510−1516.

(15) Howard, L. R.; Prior, R. L.; Liyanage, R.; Lay, J. O. Processing and storage effect on berry polyphenols: challenges and implications for bioactive properties. *J. Agric. Food Chem.* **2012**, *60*, 6678−6693.

(16) Prior, R. L.; Wilkes, S. E.; Rogers, T. R.; Khanal, R. C.; Wu, X. L.; Howard, L. R. Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet. *J. Agric. Food Chem.* **2010**, *58*, 3970−3976.

(17) Barnes, J. S.; Nguyen, H. P.; Shen, S.; Schug, K. A. General method for extraction of blueberry anthocyanins and identification using high performance liquid chromatography-electrospray ionization-ion trap-time of flight-mass spectrometry. *J. Chromatogr. A* **2009**, *1216*, 4728−4735.

(18) Rodriguez-Saona, L. E.; Wrolstad, R. E. Extraction, isolation, and purification of anthocyanins. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Acree, T. E., An, H., Decker, E. A., Pennere, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, C. F., Sporns, P., Eds.; John Wiley & Sons, Inc.: New York, 2001; pp F1.1.1−F1.1.11.

(19) Hogan, S.; Zhang, L.; Li, J. R.; Sun, S.; Canning, C.; Zhou, K. Q. Antioxidant rich grape pomace extract suppresses postprandial hyperglycemia in diabetic mice by specifically inhibiting alpha-glucosidase. *Nutr. Metab.* **2010**, *7*, 71−79.

(20) Srivastava, A.; Greenspan, P.; Hartle, D. K.; Hargrove, J. L.; Amarowicz, R.; Pegg, R. B. Antioxidant and anti-inflammatory activities of polyphenolics from Southeastern U.S. range blackberry cultivars. *J. Agric. Food Chem.* **2010**, *58*, 6102−6109.

(21) Giusti, M. M.; Wrolstad, R. E. Characterization and measurement of anthocyanins by UV-visible spectroscopy. In *Current Protocols in Food Analytical Chemistry*; Wrolstad, R. E., Acree, T. E., An, H., Decker, E. A., Pennere, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, C. F., Sporns, P., Eds.; John Wiley & Sons, Inc.: New York, 2001; pp F1.2.1−F1.2.13.

(22) Kwon, Y. 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*, 15−31.

(23) Hwang, I. G.; Kim, H. Y.; Woo, K. S.; Hong, J. T.; Hwang, B. Y.; Jung, J. K.; Lee, J.; Jeong, H. S. Isolation and characterisation of an α-glucosidase inhibitory substance from fructose−tyrosine Maillard reaction products. *Food Chem.* **2011**, *127*, 122−126.

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

(25) Lee, J.; Wrolstad, R. E. Extraction of anthocyanins and polyphenolics from blueberry-processing waste. *J. Food Sci.* **2004**, *69*, C564−C573.

(26) Castañeda-Ovando, A.; Pacheco-Hernández, M. d. L.; Páez-Hernández, M. E.; Rodríguez, J. A.; Galán-Vidal, C. A. Chemical studies of anthocyanins: a review. *Food Chem.* **2009**, *113*, 859−871.

(27) Wrolstad, R. E.; Durst, R. W.; Lee, J. Tracking color and pigment changes in anthocyanin products. *Trends Food Sci. Technol.* **2005**, *16*, 423−428.

(28) Vidal, S. M., E.; Cheynier, V.; Skouromounis, G.; Hayasaka, Y. Mass spectrometric evidence for the existence of oligomeric anthocyanins in grape skins. *J. Agric. Food Chem.* **2004**, *52*, 7144−7151.

(29) Giusti, M. M.; Rodriguez-Saona, L. E.; Griffin, D.; Wrolstad, R. E. Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. *J. Agric. Food Chem.* **1999**, *47*, 4657−4664.

(30) Revilla, E.; Ryan, J. M.; Martin-Ortega, G. Comparison of several procedures used for the extraction of anthocyanins from red grapes. *J. Agric. Food Chem.* **1998**, *46*, 4592−4597.

(31) Prior, R. L.; Wu, X. L.; Gu, L. W.; Hager, T. J.; Hager, A.; Howard, L. R. Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. *J. Agric. Food Chem.* **2008**, *56*, 647−653.

(32) Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S. E. Stability and biotransformation of various dietary anthocyanins in vitro. *Eur. J. Nutr.* **2006**, *45*, 7−18.
(33) Rodriguez-Mateos, A.; Cifuentes-Gomez, T.; Tabatabaee, S.; Lecras, C.; Spencer, J. P. E. Procyanidin, anthocyanin, and chlorogenic acid contents of highbush and lowbush blueberries. *J. Agric. Food Chem.* **2012**, *60*, 5772−5778.
(34) Lo Piparo, E.; Scheib, H.; Frei, N.; Williamson, G.; Grigorov, M.; Chou, C. J. Flavonoids for controlling starch digestion: structural requirements for inhibiting human alpha-amylase. *J. Med. Chem.* **2008**, *51*, 3555−3561.
(35) McDougall, G. J.; Kulkarni, N. N.; Stewart, D. Current developments on the inhibitory effects of berry polyphenols on digestive enzymes. *Biofactors* **2008**, *34*, 73−80.
(36) Schmidt, B. M.; Erdman, J. W.; Lila, M. A. Effects of food processing on blueberry antiproliferation and antioxidant activity. *J. Food Sci.* **2005**, *70*, S389−S394.
(37) Denev, P.; Ciz, M.; Ambrozova, G.; Lojek, A.; Yanakieva, I.; Kratchanova, M. Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties. *Food Chem.* **2010**, *123*, 1055−1061.