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Iron in Highbush Blueberries and It's Effect in Total Antioxidant Status (TAS) Assay

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ABSTRACT

Blueberries are well known for their antioxidant property, due to the polyphenolics present in these fruits. The antioxidant activity of blueberries is usually measured by colorimetric assays involving metals such as iron, copper. Total Antioxidant Status (TAS) assay was previously studied on blueberry fractions which has metmyoglobulin (protein bound iron) in its +3 oxidation state to assess the antioxidant activity of compounds. Studies showed that the amount of iron in highbush blueberries is too low to have a significant effect on the antioxidant activity measured by the TAS assay. A short comparative study was also performed using ascorbic acid in TAS assay and bathocuproine copper complex assay for a quick, inexpensive, rapid analysis of crude screening for antioxidant compounds.

Keywords: Antioxidant, Blueberries, Iron

INTRODUCTION

Most common edible berries such as blueberries, cranberries are well known as good sources of vitamins, minerals (iron, copper and other micronutrients), polyphenolic compounds and fiber (1). Of all the berries, blueberries have been studied extensively in human diet because of their nutraceutical value and biological activities such as anti-oxidative, anti-cancer (2) and urinary bacterial anti-adhesion properties (3). Recent advances in antioxidant assays led to the assessment of antioxidant activity of different berry fruits and the activity is attributed to the polyphenolic fraction of blueberries (4).

The polyphenolic compounds in blueberries are reported to suppress the effects of free radicals or mutagens in the human body reducing the amount of oxidative damage (5–6). Thus, determining antioxidant activity through screening studies for polyphenolics and phytochemicals has been in an increasing focus in recent years to maintain health and food stabilization processes (7). The most popular antioxidant assays use the abilities of phytochemicals in two major ways. The added phytochemicals quench stable colored radicals such as 2,2-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) / (Trolox®-equivalent antioxidant capacity (TEAC), 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) by transferring hydrogen atoms. These phytochemicals can also lead to the formation of colored complexes such as ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC) by transfer of electrons to reduce ions. Total antioxidant activities of phytochemicals and their antioxidant mechanisms may vary with a change in total antioxidant concentration, colloidal properties of substrates, targets of radicals, presence of competing metals such as iron and copper,

different temperatures, solvent used to dissolve antioxidant samples, localization of the antioxidant in different phases, pH of the samples, stages of oxidation, and presence of oxygen during assay (8). Of all the above factors, whole extracts of herbals often consists significant amounts of iron and are sources of minerals as part of nutraceutical significance. Therefore, Fe (II) and Cu (I) in samples may act as secondary antioxidants and may inhibit the rate of oxidation by inhibiting Fenton-type reactions that produce hydroxyl radicals in presence of hydrogen peroxide. The total antioxidant status (TAS) assay mechanism involves oxidation of protein bound iron from its +3 oxidation state (metmyoglobulin) to the +4 oxidation state (ferrylmyoglobulin) in the presence of hydrogen peroxide and the formation of ABTS stable radical ion from the reaction between ABTS and ferrylmyoglobulin. Hydrogen peroxide is used in the reaction to form a ferrylmyoglobulin intermediate and ABTS stable radical. It has been hypothesized based on the literature that a higher amount of iron in blueberries might react with hydrogen peroxide and lower the formation of ABTS radical which is essential in measuring the antioxidant activity of polyphenolics.

A copper based assay involving bathocuproine copper complex formation helps to analyze the antioxidant activity by estimating the reduction of copper by antioxidants. The decrease in the absorbance of bathocuproine copper complex formed as a result of the reduction of copper ions from +2 oxidation state to +1 oxidation state in the presence of ascorbic acid and a simultaneous chelation of reduced copper ions with bathocuproine sulphonic acid (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline). This procedure uses the same principle as that of CUPRAC except that CUPRAC has neocuproine instead of bathocuproine to form copper complex (9–11). This short comparative work was performed to analyze the amount of iron in highbush blueberries and to analyze its interference to the antioxidant activity reported in the TAS assay.

MATERIALS AND METHODS

Chemicals and reagents:

Fresh blueberries (*V. corymbosum* L.cv. Bluecrop) were obtained from Rutgers University Blueberry and Cranberry Research Center (Chatsworth, NJ). TAS assay was purchased from Randox Laboratories (Antrim, U.K); ferrous chloride tetrahydrate, Copper sulfate, 96-well plates were purchased from Sigma Aldrich (St. Louis, MO, U.S.A). Bathocuproine salt was purchased from GFS chemicals (Powell, OH, U.S.A).

Quantitative Iron Analysis:

Commercially available ferrous chloride tetrahydrate standard >98% (Sigma-Aldrich, St. Louis, MO) was dissolved in 10% 12M Hydrochloric acid to a concentration of 1.14 mg/mL containing 0.5 mg of elemental iron. This stock solution was then serially diluted to get the standard solutions of 1, 2, 3, 4, 5, 10, 15, and 20 ppm of elemental iron. The standard solutions were then analyzed at 248.3 nm using a Thermo Electron Corp. S Series AA Spectrometer (Shimadzu, Columbia, MD), consisting of a Stockdale Double Beam optics, 0.27 m Ebert type monochromator, Carousel 6 Lamp Coded, Photomultiplier Wide range (180 nm to 900 nm), Atomiser Universal system (uses 50mm Ti burner) flame, Atomiser option GFS97 combined module furnace, and a QuadLine deuterium system background correction. The analysis was controlled by SOLAAR PC Software version 1.26. The recorded values were used to generate a standard curve of ferrous chloride.

Sample Iron Preparation:

Different methods for the analysis of iron in blueberries were studied and a modified dry oxidation method was selected for quantitative iron analysis (12–14). This was done by heating the dried or lyophilized material to elevated temperatures at 900–1000°C and the ash was dissolved in a mixture of acids (HNO_3, H_2SO_4, HCl and aqua regia). Different quantities of lyophilized blueberries (3.78 g and 9.87 g) were placed in a crucible, burnt to ash at 900–1000°C for 24 h and 48 h. The burnt ash was dissolved in 5 mL of 10% 12M hydrochloric acid and filtered through Whatman no. 40 filter paper to remove the pulp or any kind of particulate matter from the solution. The solution was made up to a final volume of 25 mL with distilled water. Both the solutions were analyzed using Thermo Electron Corp. S Series AA Spectrometer (Shimadzu, Columbia, MD). The whole process was repeated in duplicates (n=2). The standard curve was used to quantitate the amount of iron in blueberry samples.

Total Antioxidant Status (TAS) assay:

Mechanism as described in the TAS kit. $HX\text{-}Fe^{III} + H_2O_2 = X\text{-}[Fe^{IV}{=}O] + H_2O$, $ABTS + X\text{-}[Fe^{IV}{=}O] = ABTS^{+\cdot} + HX\text{-}Fe^{III}$. A 20 µL of standard, sample and deionized water were added to three different cuvettes and an additional 1 mL of chromogen (ABTS) was added in each cuvette. These samples were mixed well and allowed to come to room temperature (25°C) using a water bath. Initial

absorbance (A_1) was read at 600 nm with UV/Visible spectrophotometer. Carefully a 200 µL of the substrate (hydrogen peroxide) was then added to the above mixture and mixed well. An absorbance was read exactly after 3 min (A_2) following the addition of substrate. The ΔA difference was calculated by subtracting A_1 from A_2. The factor was calculated using the formula (Factor = Conc of Standard/ ΔA). This factor was needed to calculate the mmol/L of total antioxidant activity of samples as described by the TAS Randox kit. The standard used in this assay was 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid and was lot specific.

Temperature Controlled TAS Assays in Presence of Iron:

Ferrous chloride tetrahydrate (295mg) was dissolved in 10 mL of distilled water. This was serially diluted to obtain 0.026 mg / 20 µL of elemental iron. The TAS assay was performed as explained in the earlier protocol. Temperature was manually controlled in TAS assay on a Genesys UV/Vis spectrophotometer (Thermo Electron Corp. Madison WI). The assay was also performed with an automatic temperature control on a Spectro UV/Vis Auto scanning spectrophotometer. Both the assays were repeated twice.

Bathocuproine Copper Antioxidant (BCA) Assay:

$Cu^{2+} + Ar(OH)_n \rightarrow n\,Cu^{1+} + Ar(=O)_n + n\,H^+$, $Cu^{1+} + Bc \rightarrow Cu(Bc)_2$. Bathocuproine copper complex has a maximum absorbance of 490 nm. Copper sulfate was dissolved in phosphate buffer at pH 7.4 and was made to a concentration of 10 mM. The bathocuproine sulfate salt was dissolved in phosphate buffer at pH 7.2 and made to a concentration of 30 mM (three times the concentration of copper ion). EDTA was dissolved in phosphate buffer at pH 7.4 and was made to a concentration of 30 mM. A standard solution of 2 mM uric acid was prepared as stock solution and serial dilutions were made. The assay was performed in 96-well plates with 100 µL of different concentrations of standard, sample and deionized water. Then 50 µL of copper sulfate was added to all the wells and mixed well on a rotary shaker for 1min. Then another 50 µL of Bathocuproine sulfate was added to all the wells and the absorbance was read at 490 nm on a Spectra max M2 UV/Vis 96 well plate reader with Spectra max Pro software as the initial absorbance (A_1). The wells were incubated at room temperature for 3 min and EDTA was added, and a second absorbance was recorded at 490 nm on a Spectra max M2 UV/Vis 96 well plate reader with Spectra max Pro software as the final absorbance (A_2). The difference between A_2 - A_1 absorbance were recorded and compared to the standard curve of uric acid. The samples were calculated in copper reducing equivalents to find the antioxidant activity.

Antioxidant Activity of Ascorbic Acid (Vitamin C):

Commercially available ascorbic acid was dissolved in distilled water to a concentration of 2 mM. This stock solution was serially diluted to get 0, 0.25, 0.5, 1, 2 mM. The solutions were then analyzed using the TAS and BCA assays.

Statistical Analysis:

The data are presented as mean ± SD (standard deviation). The correlation between methods and the regression analysis between the antioxidant capacity of a sample and its concentration were calculated by Microsoft excel 2007.

RESULTS AND DISCUSSION

Elemental Iron Analysis in Blueberries:

The ferrous ion is the most abundant ionic form chelated to different cellular components in nature (*14*). Thus, ferrous chloride was used as the standard to find the elemental iron. The amount of iron in ashed, lyophilized blueberry samples was quantitated using the standard ferrous chloride calibration curve. The standard curve was linear, with $R^2 \geq 0.99$. The samples to be analyzed fall within the range of the standard curve. The total amount of iron was calculated using the slope of the standard calibration curve. The amount of iron reported (USDA charts, 2008) for the highbush blueberry was found to be 280 µg/100 g. Our results of iron analysis carried out in duplicates of two different trials of ashing techniques were 221µg for 100 g of lyophilized blueberries at 24 h and 262µg for 100 g of lyophilized blueberries at 48 h, indicate that 100 g of lyophilized blueberries was found to contain 242 ± 23.7 µg (n=4 error bars=SD) of elemental iron.

Effect of iron on TAS Randox assay from blueberries:

By using the quantitative iron data from previous experiments on iron analysis in highbush blueberries, a higher concentration of elemental iron (26 µg present in 10 g of blueberries) was selected to analyze the effect of iron in antioxidant assays. The amount of iron used in the assay was 10 times the concentration of iron found in the samples of highbush blueberries. Iron in the natural biomass is in +2 oxidation state and easily gets oxidized to +3 oxidation state, this ferrous ion would react with hydrogen peroxide

(substrate) in the assay forming ferric ion (+3 oxidation state) reducing the availability of substrate hydrogen peroxide, leading to the reduction in hydrogen peroxide and increase in the ferric ion which might interfere in the formation of metmyoglobulin which would further lead to the formation of green colored complex ($ABTS^{+\cdot}$). The assay revealed that the total antioxidant activity of iron at 26 µg with manual and automatic temperature control of the TAS assay was ±0.07 antioxidant units. This matches with an error of ± 4% (As described in the TAS Randox kit). Contrary to the assumption, the amount of iron in blueberries at a concentration used in the TAS assay as shown in Figure 1. did not interfere with the antioxidant activity reported by TAS assay.

Antioxidant Activity of Ascorbic acid (Vitamin C):

Ascorbic acid at different concentrations was used to compare both TAS assay and BCA assay and its antioxidant activities were recorded. TAS assay measures

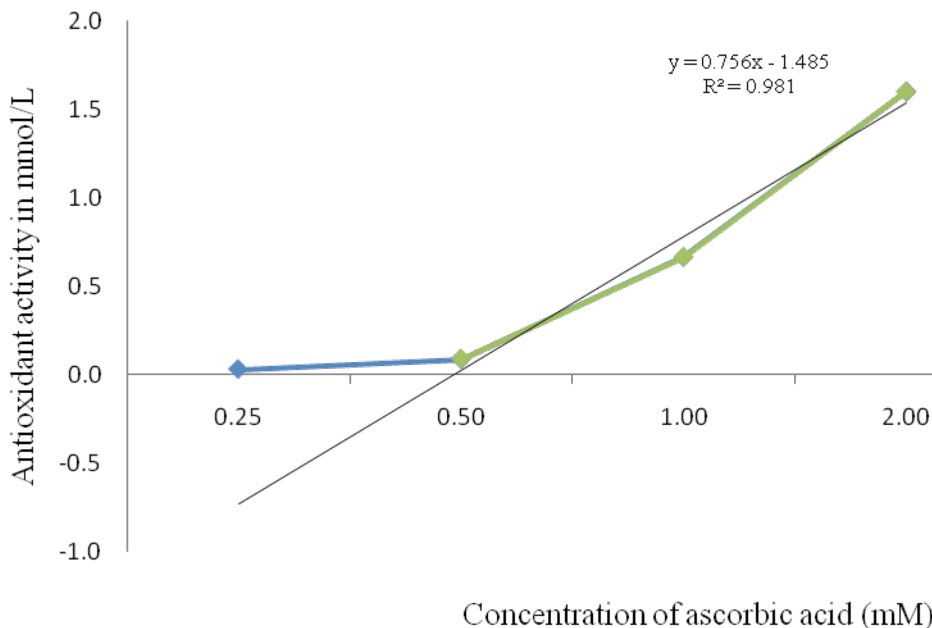

Figure 2: Antioxidant activity of ascorbic acid using TAS assay (♦)

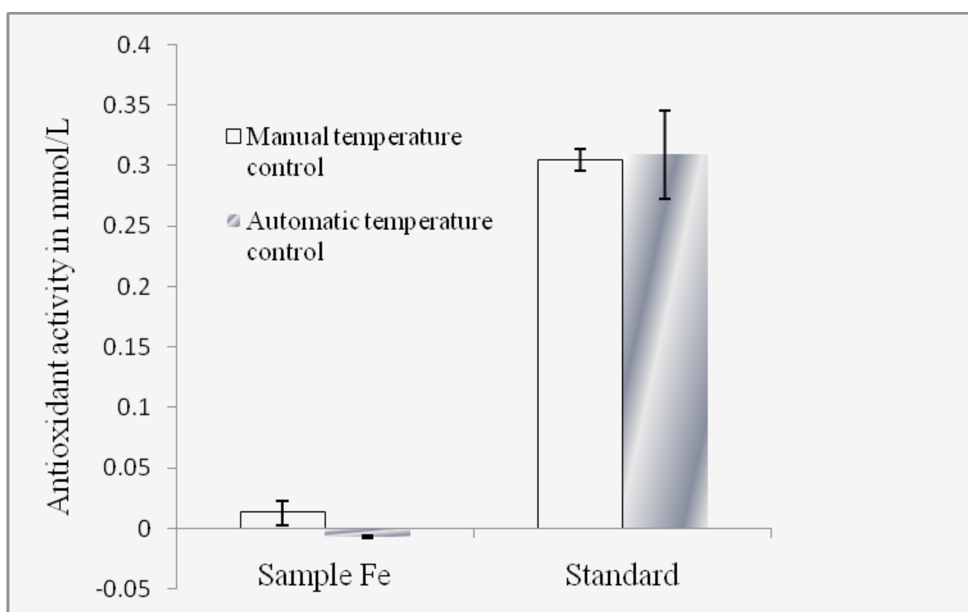

Figure 1: TAS antioxidant activity of sample with 26 µg of iron (n=3, error bars = SD).

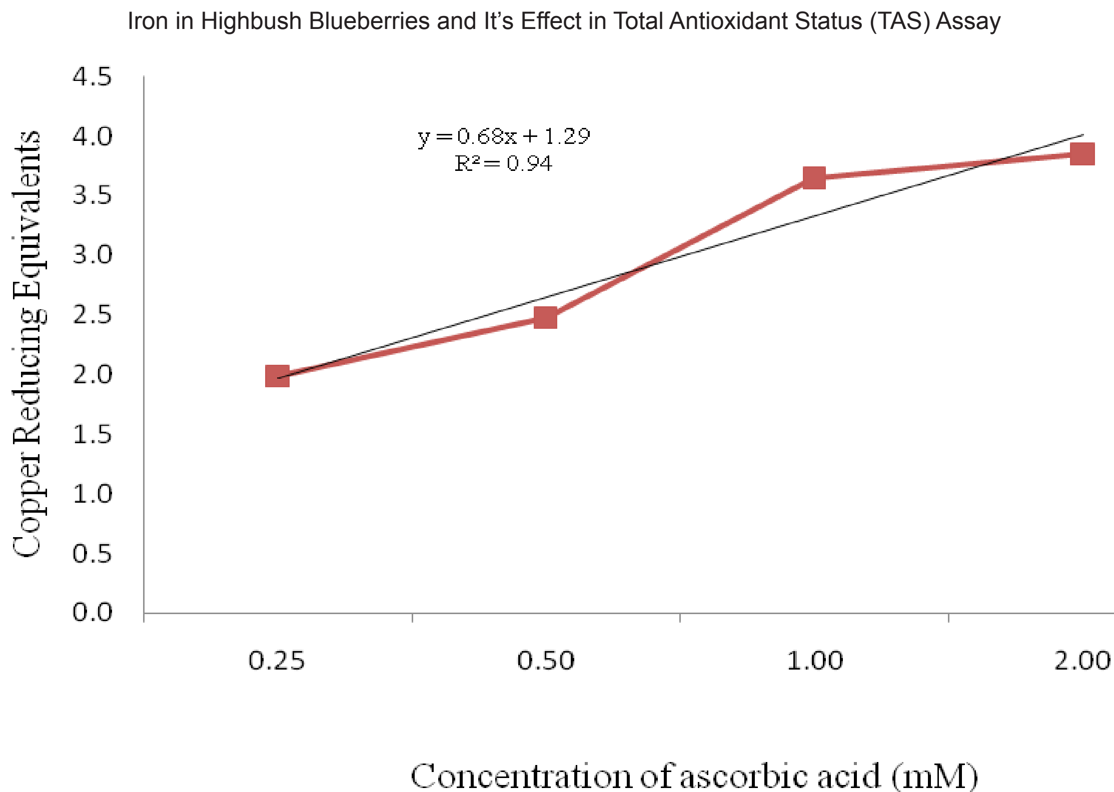

Figure 3: Antioxidant activity of ascorbic acid using BCA assay (■).

antioxidant activity in Randox antioxidant units and BCA assay measures antioxidant activity in copper reducing equivalents. In the procedure, TAS assay was performed in cuvettes individually and BCA assay was performed in 96-well plates. TAS was effective in evaluating antioxidant activity with $R^2 > 98$ (Figure 2). The initial concentration 0.25 mM was removed for the calculation of regression and trendline as it does not fall into the linearity of the curve and was completely flattened. This shows that samples with a lower concentration than 0.25 mM are void of antioxidant activity based on TAS assay. BCA assay was also effective in estimating antioxidant activity in copper reducing equivalents with $R^2 > 94$ (Figure 3). The graph shows high estimation of antioxidant activity at 0.25 mM of ascorbic acid and the graph obtains saturation at 2 mM concentration of ascorbic acid.

CONCLUSION

The amount of iron in blueberries did not produce any significant change in the antioxidant activity reported by TAS assay. Thus, the amount of iron in blueberries might be too low to have an effect on the total antioxidant activity and this amount can be ignored in the estimation of antioxidant activities of these extracts. However, if the samples have significantly high levels of these metals in the extracts the possible interaction should be ruled out before confirming the antioxidant activities of these crude extracts. BCA assay was found efficient and easy to use, requires lower sample size, less temperature sensitive, and can be performed in 96-well plates compared to TAS assay. Antioxidant activity reported from TAS assay was sensitive to temperature and requires higher concentration of ascorbic acid to obtain linearity in curve compared to BCA assay. BCA assay was simple, economic, requires lower sample for detection and easy to prepare in laboratory based on our observation and can be used for crude screening of antioxidants.

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