



Physico-chemical characterization and antioxidant capacity of the colombian berry (*Vaccinium meridionale* Swartz) with a high-polyphenol content: potential effects in people with metabolic syndrome

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Abstract

Colombian berry (agraz) has demonstrated a high antioxidant content *in vitro*. In the present study, it was elaborated and characterized an agraz nectar, designed a placebo with similar physicochemical and sensorial characteristics of the agraz nectar, but without polyphenols; and evaluated their antioxidant capacity both *in vitro* and *in vivo* in adults with cardiovascular risk factors. The polyphenolic concentration of the agraz nectar and its antioxidant capacity was superior than other reported for this fruit. A high content of total cyanidins was observed in the freeze-dried agraz, but not delphinin was detected by HPLC. For the intervention study, sixty-six subjects, with at least three cardiometabolic risk factors, consumed agraz nectar or placebo daily during 4 weeks each, separated by a 4-week washout period. Total phenol concentration and antioxidant capacity in the participants' serum were not statistically different after consuming agraz nectar and placebo. However, women increased significantly their serum DPPH scavenging capacity after consuming agraz nectar, compared to placebo. This is one of the first studies showing data about the effects of this colombian berry on the antioxidant capacity in people with metabolic syndrome.

Keywords: agraz; *Vaccinium meridionale*; antioxidant capacity; humans; metabolic syndrome.

Practical Application: antioxidant potential of this colombian fruit in human health.

1 Introduction

Blueberries belong to the genus *Vaccinium* and comprise about 400 species (Camp, 1945). *Vaccinium meridionale* Swartz, known as “agraz” or “mortiño”, is distributed in Venezuela, Colombia, Ecuador, Peru and Jamaica, and is located at the Andean (Escobar et al., 2009). A total of 20 populations and 100 plants of *V. meridionale* Swartz (Ligarreto et al., 2011) have been individually characterized in Colombia. The *V. meridionale* fruits are globose berries of 5-10 mm diameter, with purple-dark color at maturity, with persistent calyx at the apex, edible pulp with some acid, but nice flavor, and contain numerous small seeds (Ávila et al., 2007).

Studies evaluating the polyphenol content of *V. meridionale* have demonstrated its antioxidant properties *in vitro* with similar or even higher potency than other *Vaccinium* reported (Gaviria et al., 2009; Montoya et al., 2009). Also, it was showed that *V. meridionale* berries are an excellent source of dietary phytochemicals such as anthocyanins and polyphenolics, being comparable to *V. myrtillus* (Garzón et al., 2010) which is known by its nutraceutical properties including management of visual disorders and protection against cancer, age-related neurodegenerative conditions, and inflammatory responses

(Zhao et al., 2004; Bao et al., 2008; Yao et al., 2010). A more recent study reported for the first time that a non-alcoholic extract of this colombian blueberry (*V. meridionale*) exhibited cardioprotective effects against ischemia and reperfusion injury in isolated rat hearts (Lopera et al., 2013). Thus, *V. meridionale* is considered as a promissory fruit and a functional food for its high antioxidant content *in vitro* (Garzón et al., 2010) and although it has been included in the list of fruits with potential international market (Ligarreto, 2009), there is limited scientific information about the beneficial effects of the consumption of this colombian berry in humans.

People with metabolic syndrome (MetS), a group of interrelated risk factors, are at high risk of developing type 2 diabetes and cardiovascular disease (Grundy et al., 2005). It has been demonstrated that abdominal obesity, elevated blood pressure, impaired glucose tolerance, dyslipidemia, elevated oxidative stress, and inflammation, which are the prominent features of MetS, can be effectively modified with dietary interventions involving polyphenol-rich foods and beverages such as berries (Basu & Lyons, 2012; Lopera et al., 2013; Nile & Park, 2014). Studies have demonstrated that consumption of

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Vaccinium species is associated with a reduction in blood sugar levels (Grace et al., 2009), blood pressure (Basu et al., 2010) and low-density lipoprotein (LDL) oxidation (Basu et al., 2011; Shen et al., 2018). In addition, it was observed that after eating *V. myrtillus* L. resulted in significant increases in serum/plasma antioxidant capacity and this was associated to the activity of antioxidants present in this fruit, which may slow free-radical processes (Prior et al., 2007; Harasym & Oledzki, 2014). Thus, increases in this marker, which may be measured by different methods, could be associated to some beneficial effects of *Vaccinium* fruit consumption.

In fact, studies have demonstrated increases in plasma antioxidant capacity and improvements in cardiometabolic risk factors after berries consumption or its anthocyanins (Basu et al., 2011; Kuntz et al., 2014; Shen et al., 2018). However, there is limited information about the effects of *V. meridionale* consumption on serum antioxidant capacity in people with MetS. In this study, we characterized a freeze-dried and reconstituted nectar from agraz, designed a placebo and evaluated the antioxidant capacity both *in vitro* and *in vivo* in people at high risk of cardiovascular disease.

2 Materials and methods

2.1 Colombian freeze-dried berry / agraz preparation

Agraz fruits were obtained in the east of Antioquia (Colombia). Fruits between 0.5 ± 0.1 g of weight, soluble solids concentration of 13.9 ± 1.8 °Brix, pH of 2.4 ± 0.3 and percentage of humidity of $80.0 \pm 4.2\%$ were selected. Selected fruits were washed and disinfected with citosan at 30% (v/v) for 10 min. and blanched in boiling water for 5 min (Nurhuda et al., 2013).

The elaboration of the lyophilized agraz was made at the Pilot Plant of the Food Department of the University of Antioquia. The scalded fruit was processed with an industrial blender, then it was pressed to separate the extract No. 1 from

the solid residues (cake). The cake was mixed again with water to extract the greatest amount of polyphenolic compounds, and a second pressing was done to obtain the extract No. 2 from the solids of this cake. Both extracts (No. 1 and 2) were mixed with a maximum of 7°Brix, to avoid extract caramelization during the freeze-drying process.

The lyophilization process of the extract was done at 0.427 ± 0.5 mm Hg, temperature of -50 °C, and it was stored at -18 ± 2 °C protected from light (Figure 1). The freeze-dried (lyophilized) product of agraz was stored in refrigeration until use. The nectar of agraz provided for consumption in this study was prepared from the lyophilized product, 7.38 g were reconstituted in 200 mL of water to provide a daily dose equivalent to the phenols present in 200 g of fresh agraz fruits. The selection of this daily dose was based on beneficial effects observed in people with metabolic syndrome after consuming a polyphenol-rich beverage from grapes, using a similar protocol as this intervention (Barona et al., 2012).

2.2 Placebo design

The placebo was designed to match the sensorial and physicochemical characteristics and the calories contribution of 200 mL of agraz nectar, but without the polyphenolic compounds. The following food-grade ingredients were used to prepare 200 mL of placebo: carboxymethylcellulose, green food coloring, red mulberry food coloring, fructose, citric acid, and agraz flavoring. This last was design by *Casa del Sur S.A.S.*

2.3 Microbiologic and physicochemical characterization of the agraz nectar and placebo

The following parameters were analyzed to the freeze-dried and reconstituted agraz nectar, and the placebo: humidity, total protein, fat, ashes, pH, soluble solids, and titratable acidity.

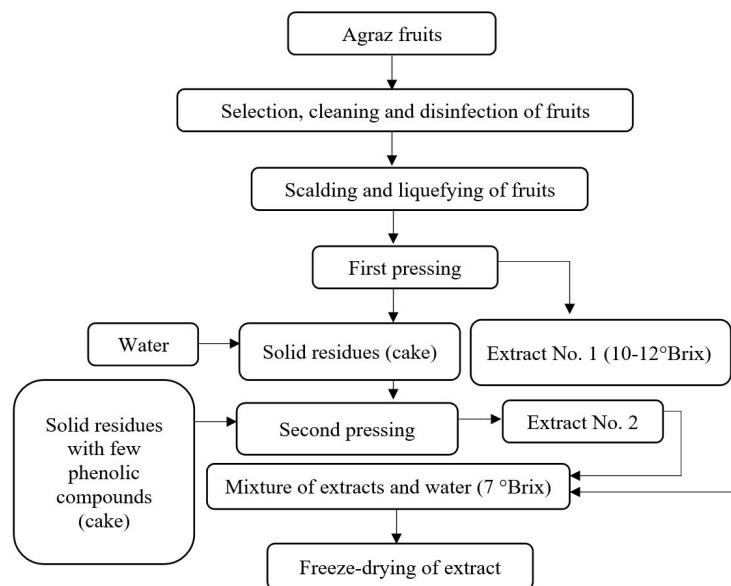


Figure 1. Process of obtaining the nectar of agraz with high content of polyphenols.

The methodologies used were recommended by AOAC (Association of Official Analytical Chemistry) (Horwitz et al., 1990).

The viscosity was measured with a needle viscometer (BROOKFIELD RV) and the color of the nectar and placebo samples was analyzed with a portable spectrophotometer (X-RITE model SP62, light D65, observer of 10°); it was obtained the following color parameters: luminosity (L^*), red-green color (a^*), yellow-blue color (b^*), chromaticity (c^*) and Hue angle (h_{ab}).

For the microbiologic analysis of the agraz nectar and placebo, the colombian technical standard 4092 (Instituto Colombiano de Normas Técnicas y Certificación, 2009) was followed for the counting of aerobic mesophiles, moulds and yeasts and the determination of the most probable number (MPN) of fecal and total coliforms.

2.4 Intervention in subjects with metabolic syndrome

A double-blind study with a crossover design during 12 weeks (wk) was carried out, in which 66 subjects (40 women and 26 men, 46.6 ± 10.4 years) with MetS, according to the ATP-III definition (Grundy et al., 2005), were included. The volunteers were randomly assigned to consume either agraz nectar (prepared daily from freeze-dried agraz) or placebo for 4 wk. After a washout period of 4 wk, participants were switched to the alternative treatment. Participants registered daily consumption of nectar, and a weekly questionnaire to assess adherence to the study. During the whole study, including the washout period, volunteers were asked to refrain from consuming foods rich in polyphenols such as fruits (grapes and other berries), wine and tea or derived products. At the end of each intervention period, measurements and blood samples were collected.

2.5 Ethical considerations

The protocol of the intervention study was approved by the Human Bioethical Committee of the *Sede de Investigación Universitaria*, Universidad de Antioquia, according to the Act. No. 14-35-558 (for men) and 15-35-558-02 (for women). All participants signed the informed consent form before entering the study. Participants' personal information was protected and not revealed.

2.6 Blood collection

Blood samples were collected from the antecubital vein using dry tubes after an overnight 12h fast. After 30 min, the tubes were centrifuged at $2000 \times g$ for 10 min to obtain serum, which was aliquoted and frozen at -80°C for further analysis.

2.7 Serum deproteinization

To remove protein interferences, the serum was deproteinized following the method by Serafini et al. (1998) with modifications. First, to separate the polyphenols from lipids, $300\mu\text{L}$ of 1.0 mol/L HCl was added to $150\mu\text{L}$ of serum. This mix was vortexed for 60s and incubated at 37°C for 30 min. Then, $300\mu\text{L}$ of 2 mol/L NaOH in 75% of methanol was added, vortexed for 3 min and incubated at 37°C for 30 min. After that, to precipitate de

proteins, $300\mu\text{L}$ of 0.75 mol/L metaphosphoric acid (MPA) was added, vortexed for 3 min and centrifuged at $1500 \times g$ for 10 min. The supernatant was removed and stored in refrigeration in darkness. In other vial, $300\mu\text{L}$ of a 1:1 (v/v) solution of acetone:water were added and centrifuged at $2700 \times g$ for 10 min. The two supernatants were combined and filtered through a HV 0.45-mm filter. The filtered serum was immediately used to measure total phenols and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging activity.

2.8 Total phenol concentration in agraz nectar, placebo and serum

Total phenolic concentration in agraz, placebo and deproteinized serum was measured by the Folin-Ciocalteu method (Swain & Hillis, 1959) modified to remove protein interferences (from serum) as previously described (Serafini et al., 1998). Twenty μL of sample or standard of gallic acid (GA) (standard curve of 0-1000 mg/L of GA) were diluted in $1580\mu\text{L}$ of distilled water. Then, $100\mu\text{L}$ of Folin-Ciocalteu reagent was added and mixed. After that, $300\mu\text{L}$ of 20% sodium carbonate were added and the color generated was read after 1h at 725nm in spectrophotometer (UV-1700, Shimadzu Europe®). During processing, the samples were protected from light. The experiments were performed in triplicate and the results were expressed as mg of gallic acid equivalents per liter (mg GAE/L).

2.9 ABTS assay in agraz nectar and placebo

The ABTS assay was performed following the method described by Contreras-Calderón et al. (2011). $100\mu\text{L}$ of test sample (diluted appropriately with water) or Trolox® standard was mixed with 1 mL of ABTS⁺ solution and incubated at 30°C for 30 min. Absorbance readings at 730 nm were taken using a spectrophotometer (UV-1700, Shimadzu Europe®). Aqueous solutions of Trolox®, at concentrations between 0 and $500\mu\text{mol}$, were used for calibration. Results were expressed as μmol of Trolox® equivalents per liter ($\mu\text{mol TE/L}$).

2.10 Ferric reducing ability of plasma/serum (FRAP) assay in agraz nectar, placebo and serum

FRAP was measured as previously described by Benzie & Strain (1996) with modifications. 90 mL of deionized water was mixed with $30\mu\text{L}$ of serum and added to $900\mu\text{L}$ of a FRAP reagent (pre-warmed at 37°C). The serum was incubated by 10 min and nectar and placebo were incubated for 30 min, respectively, at 37°C . Then, the sample absorbance at 593 nm using a spectrophotometer (UV-1700, Shimadzu Europe®) was obtained. A Trolox® calibration curve (0-400 μmol Trolox/L) was done for quantification purposes and the results were expressed as $\mu\text{mol TE/L}$.

2.11 DPPH assay in agraz nectar and placebo

The DPPH assay was performed as previously described (Nurhuda et al., 2013). 2 mL of DPPH reagent 0.5 mmol (containing DPPH and methanol) was mixed with 2 mL of methanol and 0.2 mL of diluted sample (dilution was performed as necessary

to ensure DPPH was not consumed before 30 min of reaction). Maximum absorbance values at 517 nm were recorded after 30 min of incubation in darkness (UV-1700, Shimadzu Europe®). Aqueous solution of Trolox® at concentrations between 0 and 2000 µmol were used for calibration purposes. Results were expressed as µmol TE/L.

2.12 Serum DPPH scavenging activity

DPPH scavenging activity was measured using a modification of the method described by Chrzczanowicz et al. (2008). Briefly, 5 µL of 10 mmol/L of DPPH radical in methanol was mixed with 970 µL of methanol. Then, 25µL of deproteinized serum with metaphosphoric acid or 25µL of the control (mix of the reagent to deproteinize without serum) was added, mixed and incubated by 30 min in dark. Finally, the decrease in the absorbance related to the DPPH radical decomposition in the samples and control was read at 517 nm using a spectrophotometer (UV-1700, Shimadzu Europe®). All experiments were performed in triplicate and the scavenging effect (Sc%) of DPPH was calculated using the following formula: $Sc\% = (1 - (A_{517} \text{ serum sample} / A_{517} \text{ control})) \times 100$.

2.13 ORAC assay in the freeze-dried agraz and placebo

The antioxidant capacity of the freeze-dried agraz and placebo was measured by the ORAC (Oxygen Radical Absorbance Capacity) method, following procedure as described previously by Ou et al. (2001) 2,2'-Azobis-(2-amidinopropane)-dihydrochloride (AAPH) was used as the peroxy radical generator, Trolox as the standard and fluorescein as the fluorescent probe. Fluorescein, AAPH and samples were prepared in 75 mmol buffer at pH 7.4. Samples (3 mg/mL) or Trolox standards (25 µL) were mixed with 150 µL of 1 µmol/L fluorescein and pre-incubated at 37 °C for 30 min before addition of 25 µL of AAPH solution (200 mmol/L). The fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 520 nm was measured every 2 min for 120 min using a Spectra Max Gemini EM (Molecular Devices, Orleans Dr, Sunnyvale, CA, USA). A calibration curve with Trolox (12.5 – 100 µmol/L) was performed for quantification purposes. The results are expressed as µmol TE/g of sample (Bravo et al., 2015).

2.14 Anthocyanin determination by HPLC (High performance liquid chromatography).

Freeze-dried agraz was analyzed using HPLC-DAD (diode array detection). The quantification of cyanidin and delphinidin and their glycosides was made by using the calibration curve of cyanidin and delphinidin standards (see complementary material). Anthocyanins in the freeze-dried agraz was reported as mg of cyanidin/delphinidin equivalents. These compounds are the most frequently reported for this fruit (Garzón et al., 2010).

2.15 Statistical Analysis

Results are presented as mean and standard deviation (SD), median and interquartile range, according to normality of data. The distribution of data was assessed by the Kolmogorov-Smirnov test with Lilliefors correction. A paired samples t-test

was conducted to analyze differences between the agraz and placebo consumption periods. To analyze differences in the physico-chemical characteristics between the freeze-dried agraz, nectar and placebo, it was used multiple range test. Differences with a $p < 0.05$ were considered significant. Analyses were done using Statgraphics centurion XVI and SPSS version 21 for Windows, 2012.

3 Results and discussion

Fruits belonging to *V. meridionale* have generated interest for their potential health benefits related to their high concentration of polyphenols with demonstrated antioxidant properties *in vitro* (Garzón et al., 2010).

Among the challenges the food industry has, it is to take advantage of the potentialities present in the fresh fruit and transform it into a product that potentialize and keep the natural bioactive properties of the primary source. During the process of fruit transformation, there is degradation of bioactive compounds and loss of the fruit natural color, due to the endogenous polyphenol oxidase (PPO) enzyme (Skrede et al., 2000), which oxidizes polyphenolic compounds to quinones -brown-colored substances (Lee et al., 2002). In this study, it was applied a thermic treatment to the agraz fruit, as an alternative to decrease its PPO activity. For this, the agraz fruits were submerged into hot water (80 °C) during 5 min (Figure 1). This process also allows polyphenols extraction from the skin of the fruit (Lee et al., 2002). However, one step of extraction was insufficient due to solvent (water) saturation. To guarantee a product with high concentration of polyphenolics, a second extraction step was performed to the presscake obtained during the first extraction.

Previous studies with blueberries have found the solid residue after anthocyanins extraction contains 55.3 to 71.8 mg of anthocyanins per 100 g of residue (Lee et al., 2002). These compounds can be easily extracted in a second stage of extraction. Finally, the filtrates obtained during the two steps of extraction were mixed until a 7 °Brix solution and adjusted to facilitate the lyophilization process. This freeze-drying process preserved the nectar for long periods of time, without losing its nutritious and sensorial characteristics (Zielinska et al., 2015). This preservation of the polyphenolic compounds present in the agraz nectar was important for the clinical study.

3.1 Microbiological analysis of the blueberry-agraz and placebo

The microbiological analysis of the freeze-dried and reconstituted agraz (nectar), and placebo showed they were innocuous. This was supported by the microbiological counts of < 1 UFC/mL for aerobic mesophylls and molds and yeasts, and negative of total and faecal coliforms by for the most probable numbers (MPN) when those products were analyzed. These results demonstrated that analyzed samples complied with the colombian standard technique (NTC, from the spanish: Norma Técnica Colombiana 4092) (Instituto Colombiano de Normas Técnicas y Certificación, 2009) and the products were safe for human consumption.

3.2 Physico-chemical characterization of the freeze-dried agraz and placebo.

For the evaluation of the agraz consumption effects in people with metabolic syndrome, the freeze-dried agraz was reconstituted in 200 mL of drinking water to provide a phenolic compound concentration equivalent to $1,027.97 \pm 41.99$ mg GA per L of agraz nectar.

Additionally, for the intervention study, it was also necessary to design a placebo with similar physicochemical and sensorial characteristics of the agraz nectar, but free of polyphenolics. The nectar color (a^* , b^* , L , c^* , h) and its rheological behavior (viscosity) determined the sensorial parameters perceived by the final consumer, such as the sensation in the mouth and visual appearance (Vahos et al., 2016). For this, it was used different food additives providing similar calories as the agraz nectar, but without polyphenol contribution.

The sensorial and physicochemical characteristics of the freeze-dried agraz, nectar and placebo are shown in Table 1. The freeze-dried product of agraz with high polyphenol content had the following physicochemical composition: $14.65 \pm 0.13\%$ humidity, $1.39 \pm 0.25\%$ protein, $3.48 \pm 0.24\%$ ether extract, $1.62 \pm 0.03\%$ ashes and $78.85 \pm 0.19\%$ carbohydrates. Additionally, the polyphenolic compound composition was 139.29 ± 5.69 mg of GA per g of freeze-dried product of agraz. These results differ from other reported by Tobón et al., (2016), who characterized physicochemically a freeze-dried product of agraz and reported the following composition: $5.28 \pm 0.01\%$ humidity, $4.70 \pm 1.09\%$

protein, $1.44 \pm 0.18\%$ ether extract, 1.75 ± 0.01 ashes, and a concentration of polyphenolic compounds of 12.00 mg GAE / g of product. This last result is 12 times lower than the concentration of phenolics reported in the present study. This might be explained by the thermic treatment to which the agraz fruit underwent to deactivate the endogenous enzymes that degrade polyphenolic compounds; and to the two stages of polyphenolic extraction applied in the process.

For the intervention, the physicochemical composition of the agraz nectar dose supplied daily for each volunteer is presented in Table 1.

The statistical analysis of the physicochemical properties (humidity, ether extract, titratable acidity, pH, carbohydrates, density and calories per dose) between the nectar and placebo showed no statistical differences ($p > 0.05$). Regarding the sensorial parameters of nectar and placebo, both samples had similar rheological behavior ($p > 0.05$). Thus, the agraz nectar viscosity (μ) varies with the revolutions per minute (N) of viscometer according to the following mathematical relation: $\mu = 0.0717N \pm 6.03$ cP, which is comparable to the placebo behavior ($\mu = 0.0598N \pm 6.51$ cP). Similarly, the color parameters did not differ between the nectar and placebo (luminosity: 11.39 ± 0.1 and 11.12 ± 0.01 , respectively).

On the other hand, the placebo was statistically different from the agraz nectar ($p < 0.05$) in the polyphenolic compound concentration (31.91 ± 3.15 mg GAE/L vs. $1,027.97 \pm 41.99$ mg GAE/L, respectively) and the antioxidant capacity measured by

Table 1. Physicochemical and nutritious properties of the freeze-dried agraz, nectar and placebo.

Physicochemical analysis	Freeze-dried agraz	Nectar	Placebo
		(reconstituted from freeze-dried agraz in 200 mL)	(200 mL)
Humidity (%)	14.65 ± 0.13^a	97.00 ± 0.23^b	97.47 ± 0.21^b
Protein (%)	1.39 ± 0.25^a	0.05 ± 0.01^b	0.1 ± 0.05^c
Ether extract (%)	3.48 ± 0.24^a	0.12 ± 0.01^b	0.01 ± 0.21^b
Ashes (%)	1.62 ± 0.03^a	0.06 ± 0.01^b	0.18 ± 0.03^c
Titratable acidity (%)	-----	2.53 ± 0.16^a	2.88 ± 0.24^a
pH	-----	2.56 ± 0.23^a	2.42 ± 0.29^a
Carbohydrates (%)	78.85 ± 0.19^a	2.08 ± 0.19^b	2.50 ± 0.19^b
Density (g/mL)	-----	0.999 ± 0.010^a	0.998 ± 0.01^a
Calories per dose (Kcal)	-----	26.00 ± 0.18^a	21.86 ± 0.21^a
Rheological behavior*	-----	$\mu = 0.07 N \pm 6.03^a$	$\mu = 0.06 N \pm 6.51^a$
Total phenols (mg GAE/g of freeze-dried or L)	139.29 ± 5.69^a	$1,027.97 \pm 41.99^b$	31.91 ± 3.15^c
DPPH ($\mu\text{mol TE/L}$)	-----	104.25 ± 15.14^a	0.43 ± 0.03^b
FRAP ($\mu\text{mol TE/L}$)	-----	664.87 ± 73.47^a	58.37 ± 3.78^b
ABTS ($\mu\text{mol TE/L}$)	-----	205.26 ± 9.99^a	83.39 ± 2.34^b
ORAC ($\mu\text{mol TE/g}$)	637.17 ± 13.03^a	$4,702.31 \pm 22.36^b$	0.44 ± 0.024^c
Color parameters			
hab	-----	4.66 ± 0.12^a	5.73 ± 0.08^a
a^*	-----	5.86 ± 0.03^a	5.85 ± 0.17^a
b^*	-----	0.50 ± 0.01^a	0.64 ± 0.02^a
c^*	-----	5.89 ± 0.09^a	6.09 ± 0.17^a
L	-----	11.39 ± 0.11^a	11.12 ± 0.01^a

Rheological behavior refers to: μ , viscosity (cP), N: velocity of agitation (rpm). Luminosity (L^), red-green color (a^*), yellow-blue color (b^*), chromaticity (c^*), Hue angle (h_{ab}). Different superscript letters for freeze-dried, nectar and placebo indicate significant differences ($p < 0.05$) according to Tukey's test. GAE: Gallic Acid Equivalents; TE: Trolox Equivalents.

DPPH, FRAP, ABTS and ORAC. Thus, the placebo achieved its goal, being a drink without significant antioxidant activity, but with similar sensorial characteristics as the agraz nectar. In fact, most of the participants did not recognize sensorial differences between the two beverages given in a blinded way.

The highest antioxidant capacity for the agraz nectar was found using the ORAC method, followed by FRAP, ABTS and DPPH (Table 1). As reported by others, the large difference found by ORAC, FRAP and ABTS may be indicative of the compounds present in blueberries that act stronger as a scavenger of peroxy radicals than as donors of electrons to the Fe^{3+} or ABTS^+ radical cation (Pertuzatti et al., 2014).

The antioxidant capacity of the freeze-dried agraz by the ORAC method was superior ($63,717 \pm 13.03 \mu\text{mol TE}/100 \text{ g}$ freeze-dried) than the reported ($41,775.2 \pm 6,168.2 \mu\text{mol TE}/100 \text{ g}$ lyophilized) by Maldonado-Celis et al. (2014) for the same fruit, which could be attributed to the thermic treatment applied to the agraz fruits, as it has been suggested previously, the thermic treatments favor the extraction of polyphenols present in the agraz fruits with the subsequent increase in their antioxidant capacity (Vahos et al., 2016). Regarding the ORAC value found for the freeze-dried agraz ($637.17 \mu\text{mol TE}/\text{g}$), this value is in the range reported by Pertuzatti et al. (2014) for 10 cultivars of the Brazilian *V. corymbosum* L. and *V. ashei* Reade ($533\text{--}1,028 \mu\text{mol TE}/\text{g}$ of dry weight).

The ABTS method measures the capacity of the antioxidant to react with the ABTS^+ radical (probably through electron transfer), while the FRAP assay measures the potential of an antioxidant to reduce the yellow-TPTZ ferric complex to a ferrous-TPTZ blue complex by electron donors in acidic conditions. Our results demonstrated the agraz nectar did not lose its *in vitro* capacity to donate electrons, with the ability to reduce the radical ABTS^+ and reduce the ferric complex to a ferrous complex (Garzón et al., 2010).

3.3 Anthocyanin determination by HPLC-DAD in the freeze-dried agraz

The HPLC analysis of the freeze-dried agraz showed a total cyanidin content of $4.66 \text{ mg cyanidin equivalents}/\text{g}$, which is higher than other results previously reported [$3.29 \text{ mg cyanidin equivalents}/\text{g}$ (Garzón et al., 2010) and $1.51 \text{ mg cyanidin equivalents}/\text{g}$ (Maldonado-Celis et al., 2014)]. Figure 2 shows 3 peaks, the peak of cyanidin (peak 3) and the peaks 1 and 2 correspond possibly to glucosides of cyanidin, which have been detected previously for this fruit using HPLC-MS with *in-house* protocol in which these peaks had a molecular ion value of 287.06 m/z corresponding to glucosides of cyanidin according to the literature (Garzón et al., 2010). Garzón et al., 2010 reported delphinidin glucoside peaks when analyzing the agraz fruit. However, in the freeze-dried agraz analyzed in this study, there was not detection of delphinidin given the sample did not evidence a peak in the retention time (7.7 min) corresponding to delphinidin standard (Figure 2). It is important to mention that the diversity in the content of anthocyanins in agraz fruits, it could be explained by multiple factors such as environmental conditions where the fruit grows, ripeness of the fruit, and storage conditions (Gaviria et al., 2009), and the increase in the content of anthocyanins reported in this study, it may be due to the previous treatment of the fruit, as described before for the characterization of the nectar.

3.4 Serum antioxidant capacity after consumption of agraz

A high polyphenol content and *in vitro* antioxidant capacity for agraz fruits have been previously reported (Gaviria et al., 2012). In addition, a meta-analysis showed that berries consumption significantly reduced several risk factors of cardiovascular disease (Huang et al., 2016), and these effects have been attributed in part to the antioxidant activity of polyphenols present in the fruits (Pérez-Jiménez et al., 2010). Therefore, we hypothesized that agraz consumption would increase serum total phenols and serum antioxidant capacity *in vivo*.

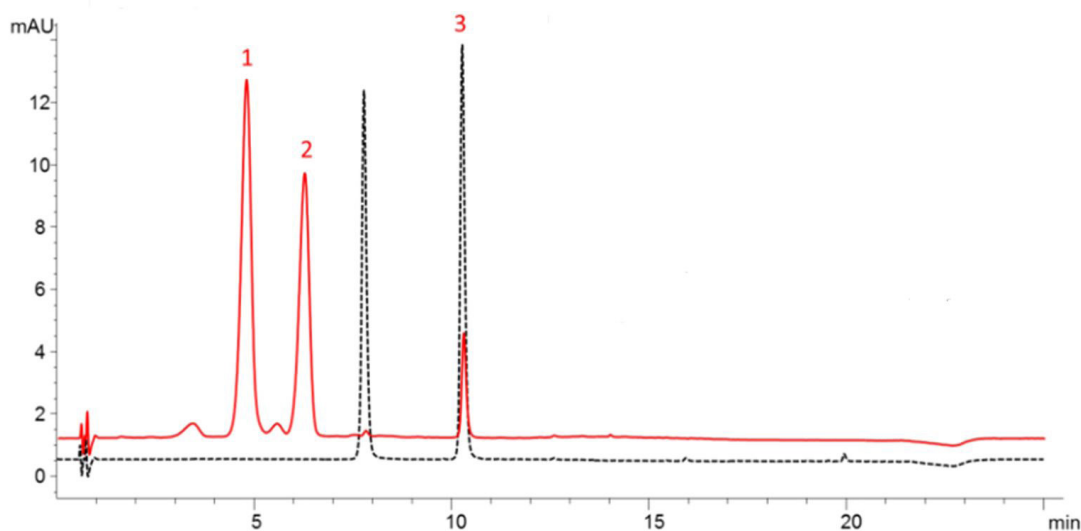


Figure 2. HPLC analysis of the freeze-dried agraz. Dotted line: Standards, Delphinidin (7.7 min), cyanidin (10.2 min). Red line. Peak 1: (4.8min) cyanidin glucoside 1, peak 2: (6.3 min) cyanidin glucoside 2, peak 3: (10.2 min) cyanidin.

Serum total phenols

After blueberry-agraz nectar consumption, compared to placebo (263.8 mgGAE/L), the serum content of total phenols (280.2 mgGAE/L) increased by 6%, although it was not significant ($p = 0.188$) (Figure 3). The same finding was reported by others evaluating blueberry juice supplementation in an acute study with 20 healthy female volunteers (Pedersen et al., 2000). Another acute study evaluating plasma antioxidant capacity after strawberries consumption, reported no increases in polyphenol levels. The authors stated that antioxidant activity may be confounded by selection of methods and time of blood sampling (Prymont-Przyminska et al., 2016). Volunteers in this study consumed the agraz nectar during 4 wk; thus, it was expected that with this chronic consumption the levels of polyphenols in serum and their antioxidant capacity would be higher, compared to the placebo period, regardless of time of blood sampling. Participants arrived for blood sampling after 12h of overnight fast.

Serum FRAP

There were no statistical differences ($p = 0.755$) in the antioxidant capacity measured by FRAP between the agraz (654.8 $\mu\text{molTE/L}$) and placebo (660.3 $\mu\text{molTE/L}$) periods (Figure 3). A similar result was found in a chronic intervention with cranberry, the antioxidant potential of the plasma measured by FRAP were unaffected by the intervention (Duthie et al., 2006). However, an acute intervention with cranberry juice showed a significant change in antioxidant capacity of plasma measured by the Fe^{3+} reduction (Pedersen et al., 2000). Interestingly, in the same study there were not significant increases in FRAP after acute blueberry juice consumption, which could be due to the composition of bioactive substances that varies from one fruit to another (Pedersen et al., 2000).

Serum DPPH. The DPPH radical scavenging capacity increased by 3.5% at the end of the agraz nectar consumption (17.7 Sc%), compared to placebo (17.1 Sc%), but this result was not statistically significant ($p = 0.227$) in the whole group (Figure 3). However, when analyzing by sex, women increased their serum DPPH scavenging capacity by 19% ($p = 0.045$) after consuming agraz nectar (12.1 Sc%), compared to placebo

(10.2 Sc%). This result differs from other studies done in healthy populations (Alvarez et al., 2015), in which the authors reported lower values of DPPH scavenging capacity in women compared to men; although the type of population and fruit consumed are different from this study. Another study evaluating the acute effects of apple juice in 12 healthy volunteers found a significant increment in serum DPPH radical scavenging activity 1h after consumption, after which the activity was reduced (Chrzczanowicz et al., 2008).

There are some aspects important to consider when analyzing the results of this study. First, the bioavailability of polyphenols, which is affected by the chemical structure of polyphenols and it determines the rate and extent of absorption and the nature of metabolites circulating in blood (D' Archivio et al., 2007). Thus, blood concentrations after polyphenol consumption vary highly depending on the structure of the polyphenol and food source (Manach et al., 2004). The maintenance of a high concentration of polyphenols in plasma was shown to require repeated ingestion overtime (van het Hof et al., 1999). Some studies in which polyphenol metabolites were present at much higher concentrations in specific tissues than in blood, suggest that circulating levels of polyphenols are not directly correlated with concentrations in target tissues, which implies that plasma metabolite concentration of polyphenols may not be the most appropriate marker of exposure (Ferruzzi et al., 2009). Second, it is possible that the metabolites produced by the action of gut microbiota or by enzymatic conjugation in different tissues, such as intestine and liver (Santo-Buelga et al., 2010), could not have been detected by the methods used in this study, which have been standardized or used preferably to measure native polyphenol compounds or the antioxidant capacity of fruits or food matrices, but not in human blood samples. Third, the dose used in the present study was to simulate the habitual consumption of this type of fruits; thus, studies with higher doses, and more time of intervention are warranted. However, this is one of the first studies showing data about the effects of this colombian agraz (*V. meridionale* Swartz) on the antioxidant capacity in people with metabolic syndrome, and about the potential of this fruit to be explore in human health conditions.

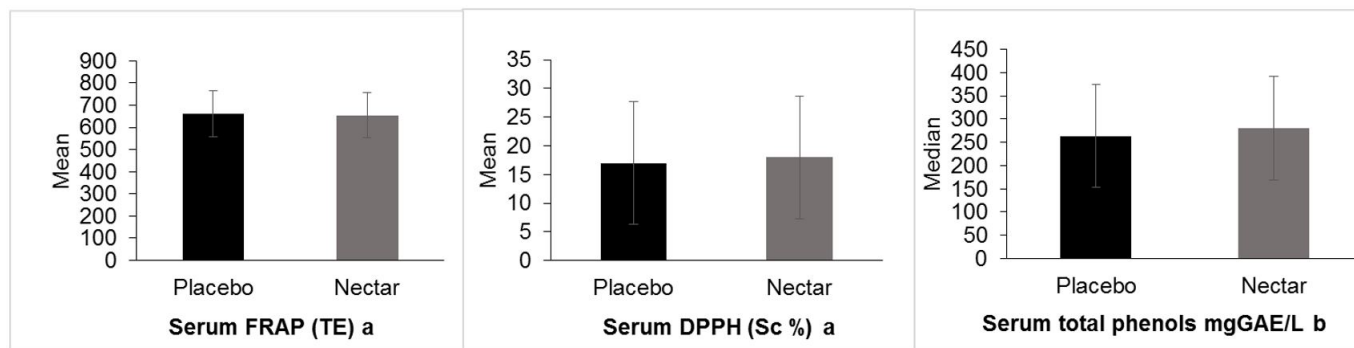


Figure 3. Total phenols and antioxidant capacity (measured by FRAP and DPPH) in serum after consumption of the colombian agraz nectar or placebo. FRAP, ferric-reducing antioxidant power; TE, Trolox equivalent ($\mu\text{mol TE/L}$ sample); DPPH, 2,2'-diphenyl-1-picrylhydrazyl; Sc, scavenging effect; GAE, Gallic Acid Equivalents; a. T paired; b. Wilcoxon.

4 Conclusions

The agraz nectar elaborated and characterized in this study showed a high polyphenolic content, which could be associated to the increase in participants' serum antioxidant capacity, measured by DPPH scavenging capacity, after consuming agraz nectar, compared to placebo. This is the first study showing initial data about the effects of this colombian berry on the antioxidant capacity in people at high risk for cardiovascular disease.

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