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Vitamin C content, anthocyanins and antioxidant capacity of fruits of *Rubus glaucus* Benth. (mora de Castilla) with and without prickles grown in Risaralda, Colombia

Gloria Edith Guerrero Álvarez[1], Sarah Muñoz Arias[1], Gustavo Alfonso Cifuentes Colorado[1]

1 Faculty of Technology, School of Chemistry, OLEOCHEMISTRY Research Group, Universidad Tecnológica de Pereira, Pereira, Risaralda, Colombia.
*Corresponding author: *gguerrero@utp.edu.co*

Abstract: "mora de Castilla" is a native shrub of the Andean tropics and can be found in Colombia, Ecuador, Mexico, and other countries of this region. In Colombia, this fruit is cultivated by small producers and is an important source of income and rural employment. "mora de Castilla" is in great demand for domestic consumption as well as for export because it can be consumed fresh or processed and because of its extraordinary organoleptic and nutritional attributes. Risaralda is one of the departments in which *Rubus glaucus* Benth. is cultivated; there, plants with and without prickles are widely distributed. Although some studies have been carried out on these cultivars, it is necessary to evaluate and compare some of their attributes as an approach to recognize potential uses in agribusiness. Therefore, a physicochemical characterization was performed, the vitamin C content was evaluated, the amount and profile of anthocyanins were determined, and the antioxidant capacity of fruits of "mora de Castilla" with and without prickles was analyzed. The physicochemical parameters evaluated were weight, pH, soluble solids and titratable acidity. The content of vitamin C was evaluated using the 2-nitroaniline spectrophotometric method, and anthocyanins were evaluated using 2 methods: spectrophotometry (pH differential) and chromatography (HPLC). Finally, the antioxidant capacity was determined using the FRAP and DPPH methods. Significant differences were found between the municipalities as well as between the materials with and without prickles with regard to antioxidant capacity and vitamin C and anthocyanin content, with the highest values for fruits grown in the municipality of Pereira, Risaralda. The results obtained indicate that "mora de Castilla" with and without prickles is a fruit of high quality and promise because of its high content of bioactive compounds (vitamin C and anthocyanins), its relevant function as an antioxidant and its valuable organoleptic attributes.

Key words: *Rubus glaucus* Benth, physicochemical characterization, antioxidant activity, anthocyanins, ascorbic acid.

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Teor de vitamina C, antocianinas e capacidade antioxidante de frutos de *Rubus glaucus* Benth. (mora de Castilla) com e sem espinhos cultivada em Risaralda, Colômbia

Resumo: A "mora de Castilla" é um arbusto nativo dos trópicos andinos que pode ser encontrado na Côlombia, Equador, México e em outros países desta região. Na Côlombia, esta fruta é cultivada por pequenos produtores e é uma importante fonte de renda e de empregos na zona rural. A "mora de Castilla" é muito procurada tanto para consumo interno, quanto para exportação, já que pode ser consumida fresca ou processada, devido aos seus extraordinários atributos organolépticos e nutritivos. Risaralda é um dos departamentos que cultiva *Rubus glaucus* Benth, sendo aí os materiais com e sem espinhos amplamente distribuídos. Embora alguns estudos tenham sido realizados com essas lavouras, é necessário avaliar e comparar alguns de seus atributos, como uma abordagem para reconhecer potenciais usos na agroindústria. Para isso, realizou-se uma caracterização físico-química, avaliou-se o teor de vitamina C, determinaram-se a quantidade e o perfil de antocianinas e analisou-se a capacidade antioxidante da "mora de Castilla" com e sem espinhos, cultivada nos municípios de Guática, Pereira e Santa Rosa, do departamento de Risaralda. Os parâmetros físico-químicos avaliados foram: peso, pH, sólidos solúveis e acidez titulável. O teor de vitamina C foi avaliado usando o método espectrofotométrico de 2-nitroanilina, e as antocianinas foram avaliadas por dois métodos: um espectrofotométrico (pH diferencial) e um cromatográfico (HPLC). Finalmente, a capacidade antioxidante foi determinada usando os métodos FRAP e DPPH. Foram encontradas diferenças significativas entre os municípios, assim como entre os materiais com e sem espinhos para a capacidade antioxidante e também para o teor de vitamina C e antocianinas, apresentando seus valores mais elevados para município de Pereira. Os resultados obtidos indicam que a "mora de Castilla", com e sem espinhos, é uma fruta de qualidade e promissora devido ao seu alto teor de compostos bioativos (vitamina C e antocianinas), sua relevante função como antioxidante e seus valiosos atributos organolépticos.

Termos de indexação: *Rubus glaucus* Benth, caracterização físico-química, atividade antioxidante, antocianinas, ácido ascórbico.

Introduction

The genus *Rubus*, of the Rosaceae family, includes approximately 750 species that are cultivated around the world, from temperate wooded areas to tropical areas. They are fruits of great interest because their consumption generates various health benefits (GUERRERO ÁLVAREZ et al., 2021; MORENO-MEDINA et al., 2018). These species include raspberry and blackberry, some of which have been domesticated and have become crops of great commercial importance (MARULANDA et al., 2012; MORENO-MEDINA et al., 2018).

In addition to its nutritional value, blackberry is rich in minerals, vitamins, fiber, and fatty acids and is an important source of bioactive compounds. Some studies have reported anti-inflammatory, antioxidant, antineurodegenerative and anticancer properties related to the consumption of these fruits and their high content of phenolic compounds, such as phenolic acids, tannins, stilbenes, ellagitannins, lignans and flavonoids (anthocyanins, catechins, quercetin, flavonols, flavanones, and isoflavonoids, among others) (GUERRERO ÁLVAREZ et al., 2021; MONROY CÁRDENAS et al., 2019; MORENO-MEDINA et al., 2018; SAMANIEGO et al., 2020).

Blackberry fruits can present great morphological and phytochemical variability depending on the agroclimatic conditions in which they are grown; agronomic management, temperature, solar radiation, and soil conditions, among others factors, can affect physicochemical characteristics such as soluble solids, titratable acidity and size, thus influencing the quality and flavor of the fruit. The presence of phenolic compounds can also be affected by various factors, such as light, temperature, and biotic and abiotic stress (MORAES et al.., 2020; MORENO-MEDINA et al., 2018; QUINTERO-CASTAÑO et al., 2021; SOLANILLA-DUQUE et al., 2020).

In Colombia, the most important commercial cultivar is blackberry (*Rubus glaucus* Benth) (MORENO–MEDINA; CASIERRA–POSADA, 2021; RINCÓN BONILLA et al., 2015), a perennial shrub native to the Andean region of South America. In addition to Colombia, it is cultivated in countries such as Ecuador, Costa Rica and Mexico (MARULANDA et al., 2012; MONROY CÁRDENAS et al., 2019; ODERAY MERINO PEÑAFIEL et al., 2018; SAMANIEGO et al., 2020). This crop is characterized by its versatility, low cost and nutritional attributes of the fruit, which has a dark red or violet color when ripe, a good aroma and a bittersweet flavor (QUINTERO-CASTAÑO et al., 2021; SAMANIEGO et al., 2020). Blackberry has, among others, antioxidant, anti-inflammatory and chemopreventive properties because of its high content of phenolic compounds (MONROY CÁRDENAS et al., 2019; QUINTERO-CASTAÑO et al., 2021). Due to the aforementioned attributes, the fruit is in great demand both for domestic consumption and in international markets (MONROY CÁRDENAS et al., 2019; MORENO-MEDINA et al., 2018) because blackberry can be used for the production of various products, such as juices, jams, pulp, wine, ice cream and yogurt; furthermore, it can also be marketed for fresh or frozen consumption (GUERRERO ÁLVAREZ et al., 2021; MONROY CÁRDENAS et al., 2019).

"mora de Castilla" is a traditional crop cultivated by small and medium farmers and is an important source of income and rural employment (MONROY CÁRDENAS, 2019; ODERAY MERINO PEÑAFIEL et al., 2018). National production for 2020 was 178,161.59 tons, with a planted area of 15967.41 ha (AGRONET, 2020). It is cultivated in 18 of the 32 departments of Colombia, with Santander being the main producer for the year 2020, with 25% of the volume of annual production, followed by Cundinamarca, with 24%, and Nariño, Boyacá, Antioquia and Caldas, each accounting for 7% (SIOC, 2021). Although Risaralda is not a high production department, it has the fifth highest crop yield (12.24 ton/ha) (AGRONET, 2020).

In Colombia, 2 types of "mora de Castilla" are cultivated, i.e., with prickles and without prickles. The latter is widely cultivated in the country because it generates advantages for agronomic management and is a fruit with interesting phenotypic characteristics (GUERRERO ÁLVAREZ et al., 2021; MARULANDA et al., 2012; ZARANTES et al., 2021). The cultivation of "mora de Castilla" without prickles has lower production costs and similar productivity compared to the cultivation of blackberry with prickles (MARULANDA et al., 2012).

Due to the high phenotypic and molecular variability of "mora de Castilla", it is necessary to physically and chemically characterize each cultivar (MARULANDA et al., 2012; MORAES et al., 2020; MORENO–MEDINA; CASIERRA–POSADA, 2021), with the purpose of identifying potential uses in agribusiness (for fresh consumption or as a processed product) as well as in other sectors where the demand for compounds of natural origin, such as vitamins, antioxidants, and dyes, among others, has increased. Consequently, the objective of this study was to physically characterize, quantify vitamin C and anthocyanins, and determine the antioxidant capacity of blackberry (*Rubus glaucus* Benth.) with and without prickles cultivated in 3 municipalities of Risaralda, Colombia.

Materials and methods

Origin and sampling of fruits

Fruits of *Rubus glaucus* Benth. (mora de Castilla) of 2 different varieties (with and without prickles) were collected in 3 municipalities of the department of Risaralda (Table 1). The fruits were collected in stages 4 and 5 of ripening in accordance with the classification established in the Colombian technical standard NTC 4106 (ICONTEC, 1997).

Table 1. Georeferencing of *Rubus glaucus* Benth. crops from which samples were taken, located in 3 municipalities of the department of Risaralda.

Municipality	Farm	Georeferencing
Guática	Santa Teresita	N: 05°19'7.1" W: 075°47'29.7" Altitude: 1880 ± 3 m
Pereira	Añoranzas	N: 4°44'33.0" W: 75°36'58.1" Altitude: 1749 ± 3 m
Santa Rosa	El Rubí	N: 04°54'24.1" W: 075°33'44" Altitude: 2013 ± 3 m

Sample treatment

The fruits of *Rubus glaucus* Benth. were transferred from the sampling site to the OLEOCHEMISTRY laboratory of the Technological University of Pereira. The physicochemical parameters were determined from the juice of freshly collected fruits. Another portion of the fruits was pulverized in liquid nitrogen in accordance with the methodology described by Rodriguez-Saona and Wrolstad (2001), stored at -20 °C and used to determine the antioxidant capacity and the content of vitamin C and anthocyanins.

Physicochemical characterization of the fruits of Rubus glaucus Benth.

The weight of the fruits was determined following the methodology described by Mazzoni, L. *et al*. (2020). pH, soluble solids (SS) and titratable acidity (TA) were determined in accordance with methods 10.041/84, 932.12/90 and 942.15/90, respectively, of the AOAC (BERNAL DE RAMÍREZ, 1998). The maturity index (MI) was calculated as indicated in NTC 4106 (ICONTEC, 1997).

Determination of the ascorbic acid (vitamin C) content

The pulverized sample was extracted with 80% methanol solution at a ratio of 1:40 and centrifuged at 4200 rpm in 5-min cycles for 15 min in accordance with the methodology described by Aguirre et al.(2019), with some modifications.

The 2-nitroaniline colorimetric method described by Bernal de Ramírez (1998) was employed. Briefly, in a 10-mL volumetric flask, 0.1 mL of 0.16% 2-nitroaniline solution, 0.1 mL of 0.08% sodium nitrite solution, 3.8 mL of absolute ethanol and 1 mL of extract were incubated for 5 minutes. Then, 1.2 mL of 10% NaOH solution was added and it was gauged to 10 mL with distilled water. The absorbance was measured at 540 nm, using a blank sample to calibrate the absorbance to zero.

A calibration curve was generated using ascorbic acid as the standard, and the results were expressed as mg of ascorbic acid per 100 g of sample.

Determination of anthocyanin content

Anthocyanin extraction

The pulverized sample was extracted with acidified methanol (0.01% of HCl) in accordance with the methodology described by Rodriguez-Saona and Wrolstad (2001), with some modifications. The extraction was performed by maceration for 1h, using a 1:10 sample to solvent ratio. The extract obtained was vacuum filtered, and the remaining sample was re-extracted 2 times under the same conditions. The filtrates were transferred to a flask to evaporate the solvent, and finally, the extract was brought to a normalized volume with acidified methanol (0.01% of HCl) for the determination of anthocyanins by the pH differential method. For the determination of anthocyanins by high-performance liquid chromatography (HPLC), a second extract of each sample was obtained under the same conditions, but the extract was brought to a normalized volume with acidified distilled water (0.01% of HCl).

Determination of the total monomeric anthocyanin content by spectrophotometry (pH differential method)

Initially, a spectral scan was performed for each methanolic extract to identify the characteristic absorption spectrum of the anthocyanins. The methodology described by Lee et al. (2005) was used for the quantification of anthocyanins. Two aliquots of each extract were taken, one of which was brought to volume with buffer pH 1 (potassium chloride, 0.025 M) and the other with buffer pH 4.5 (sodium acetate, 0.4 M), adding no more than 20% of the extract to each solution. Finally, the absorbance of both solutions was measured at 510 nm and at 700 nm using a Thermo Scientific GENESYS 10S UV–Vis spectrophotometer. The total anthocyanin content (TAC) was expressed as cyanidin-3-glucoside equivalents and was calculated using the following equation (1):

$$CAT\ (mg\ cyanidin-3-glycosid\,e/L)=\frac{A\,x\,PM\,x\,FD\,x\,1000}{\mathcal{E}\,x\,l}\quad(1)$$

where

$A = (A_{510\ nm} - A_{700\ nm})_{pH\ 1} - (A_{510\ nm} - A_{700\ nm})_{pH\ 4.5}$;

MW = molecular mass for cyanidin-3-glucoside (449.2 g/mol);

FD = dilution factor;

ε = molar extinction coefficient for cyanidin-3-glucoside (26900 L/mol.cm); and

ι = cell optical path in cm

Determination of anthocyanins by HPLC

The aqueous extracts were purified by solid phase extraction (SPE) using Strata™ C18-E cartridges (55 µm, 70 Å, 500 mg/3 mL), and then, the extracts were concentrated and brought to a normalized volume with acidified distilled water (0.01% of HCl) (RODRIGUEZ-SAONA; WROLSTAD, 2001). The purified aqueous extracts were analyzed in a Jasco 2000 plus HPLC system equipped with a quaternary gradient pump (PU-2089 Plus), intelligent autosampler (AS-2059Plus), column oven (CO-2065 Plus) and an intelligent diode array detector (MD-2015 Plus).

For separation, a Capital ODS2 Spherisil column (250 × 4.6 mm id, 5 µm) was used, and the mobile phases were 5% formic acid (v/v) in water (A) and 5% formic acid (v/v) in acetonitrile (B). The gradient conditions for phase B were 0-6 min, 14%; 6-16 min, 14-21%; 16-20 min, 21-23%; 20-22 min, 23-100%; and 22-33 min, 100-14%. Other chromatographic conditions were as follows: flow rate, 1 mL/min; column temperature, 25 °C; injection volume, 20 µL; and detection, 520 nm. To quantify anthocyanins, cyanidin-3-glucoside was used as an external standard, using the same conditions described above.

Determination of antioxidant capacity

The spectrophotometric method based on the absorption of the radical 2,2.diphenyl-1-picrylhydrazyl (DPPH) was used. Two milliliters of an ethanolic DPPH solution (20 mg L^{-1}) and 30 µL of methanolic extract were mixed. The solution was incubated for 30 min at room temperature. The absorbance was measured at 517 nm, using a blank to adjust the absorbance to zero. A calibration curve was generated using Trolox as a standard, and the results were expressed as micromoles of Trolox per g of sample.

The spectrophotometric method based on the reduction of ferric 2,4,6 tripyridyltriazine (TPTZ) and measurement of the absorbance of the colored reduction product was used. Briefly, 1.8 mL of FRAP working solution and 60 µL of methanolic extract were mixed. The solution was incubated at 37 °C for 30 min, and the absorbance was measured at 593 nm, using a blank to adjust the absorbance to zero. A calibration tion curve was generated using Trolox as a standard, and the results were expressed as mmol Trolox equivalent per 100 grams of sample.

Standardization of the methods used for the quantification of ascorbic acid and anthocyanins

Spectrophotometric method for the determination of ascorbic acid	A calibration curve was generated in duplicate with an ascorbic acid standard with concentrations ranging from 2 to 20 ppm, and the standards were prepared in accordance with the 2-nitroaniline method described above.
Spectrophotometric method (pH differential) for the determination of anthocyanins	A calibration curve was generated in triplicate with a cyanidin-3-glucoside standard, with concentrations ranging from 0.7 to 9.6 ppm. The standards were prepared in accordance with the pH differential method described above.
Standardization of the chromatographic method (HPLC) for the determination of anthocyanins	Initially, different gradient conditions were evaluated for the mobile phases until finding a method that would allow better separation of the components of the evaluated matrix and that was also suitable for the standard. Using this method, calibration curves were generated in triplicate using cyanidin-3-glucoside as a standard, with concentrations ranging from 2 to 50 ppm.

To verify the 3 methods to be implemented, parameters such as sensitivity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were determined.

Sensitivity was established as the slope of the linear regression curve, precision was evaluated with respect to repeatability using the relative standard deviation (RSD), the percentage relative error (% Er) was determined for accuracy, and the detection and quantification limits were calculated using equations 2 and 3, respectively.

$$LOD = \frac{3.3S_B}{m} \quad (2) \qquad LOQ = \frac{10S_B}{m} \quad (3)$$

where
S_B = standard deviation of blank; and
m = slope of the calibration curve

For the LOD and LOQ for the chromatographic method, S_B was taken as the standard deviation for a very low concentration (close to the experimentally observed LOD).

Statistical analysis

For all determinations, which were performed in triplicate, analysis of variance (ANOVA) was performed, followed by the Tukey test with a significance level of 5%. With respect to the standardization of the spectrophotometric and HPLC methods, the paired t test was used (with a significance level of 5%) between the theoretical concentrations and the concentrations obtained experimentally to validate the linear model. The analyses were performed using the statistical package InfoStat version 2020 (DI RIENZO et al., 2011). MANOVA was performed using IBM SPSS Statistics 21 (IBMCORP, 2012).

Results and discussion

Physicochemical characterization of *Rubus glaucus* Benth fruits

Table 2 provides the values obtained for the physicochemical parameters of the fruits evaluated. Regarding the weight of the fruits, no significant differences were found between the fruits with and without prickles, but there were differences for the fruits grown in Santa Rosa compared with those grown in Guática and Pereira. The values ranged from 4.65 to 8.63 g, within the ranges reported by previous studies (AYALA et al., 2013; GRIJALBA RATIVA et al., 2010; HORVITZ et al., 2017). Notably, the fruits grown in Santa Rosa had the lowest values, which could be due to the climate of this municipality; low temperatures result in smaller fruits but does not interfere with crop yield (GRIJALBA RATIVA et al., 2010). Some authors have found that there are slight differences in weight between plants with and without prickles (BERNAL ESTRADA; DÍAZ DIEZ, 2006; GRIJALBA RATIVA et al., 2010).

Table 2. Physicochemical parameters of fruits of *Rubus glaucus* Benth. with and without prickles grown in 3 municipalities of the department of Risaralda.

Municipality (Place of cultivation)	Prickles	Parameter evaluated				
		Weight (g)	pH	Soluble solids (° Brix)	Titratable acidity (g malic acid/100 g sample)	Maturity index (° Brix/% malic acid)
Guática	With	8.63 ± 0.85^{aA}	2.96 ± 0.07^{aA}	5.53 ± 0.05^{aA}	2.59 ± 0.04^{aA}	2.14 ± 0.02^{aA}
	Without	7.82 ± 1.34^{aA}	2.67 ± 0.08^{bA}	6.23 ± 0.19^{bA}	1.73 ± 0.06^{bA}	3.60 ± 0.15^{bA}
Pereira	With	6.48 ± 0.35^{aA}	2.72 ± 0.01^{aA}	5.30 ± 0.28^{aA}	2.82 ± 0.16^{aA}	1.88± 0.15^{aA}
	Without	8.16 ± 0.02^{aA}	2.98 ± 0.01^{bA}	6.60 ± 0.36^{bA}	1.78 ± 0.04^{bA}	3.71± 0.27^{bA}
Santa Rosa	With	5.21 ± 0.06^{aB}	2.89 ± 0.02^{aA}	7.27 ± 0.04^{aB}	2.66 ± 0.05^{aB}	2.78± 0.25^{aB}
	Without	4.65 ± 0.44^{aB}	2.87 ± 0.01^{aA}	8.30 ± 0.08^{bB}	2.31 ± 0.01^{bB}	3.60± 0.05^{bB}

Averages with a common letter in the same column are not significantly different according to the Tukey test ($p \geq 0.05$). Capital letters indicate differences among municipalities, and lowercase letters indicate differences between plant material with and without prickles.

Regarding the pH values, significant differences were found between the fruits with and without prickles for 2 cultivation locations (Guática and Pereira). The results obtained ranged from 2.67 to 2.98, within the range reported for “mora de Castilla” (AYALA et al., 2013; HORVITZ et al., 2017; MERTZ et al., 2007; MORENO-MEDINA et al., 2018). Some authors consider that this parameter is related to an increase in sugars and pigments in later stages of maturity because the pH can increase during enzymatic processes, favoring the accumulation of these compounds (AYALA et al., 2013).

Regarding soluble solids (SS), titratable acidity (TA) and maturity index (MI) results, there were significant differences between the plants with and without prickles for each cultivation location, and the fruits grown in the municipality of Santa Rosa were different from those grown in Guática and Pereira regarding these parameters.

The results obtained for soluble solids ranged from 5.3 to 8.3 °Brix, with the highest values for the fruits of Santa Rosa. For each cultivation site, the SS values for the plants without prickles were greater than those for plants with prickles. The SS values obtained are close to those reported in other studies (GRIJALBA RATIVA et al., 2010; GUERRERO ÁLVAREZ et al., 2021), but other authors have reported higher values (AYALA et al., 2013; HORVITZ et al., 2017; MERTZ et al., 2007; MORENO-MEDINA et al., 2018; RINCÓN BONILLA et al., 2015; SAMANIEGO et al., 2020).

The titratable acidity values ranged from 1.73 to 2.82 g of malic acid per 100 g of sample. The fruits from plants with prickles from the 3 cultivation zones presented the highest values, a finding that is consistent with observations by Guerrero Álvarez et al. (2021). The TA values for the material without prickles grown in Guática and Pereira were lower than those reported in the literature; for the other materials studied, the values were consistent with those reported previously (AYALA et al., 2013; GUERRERO ÁLVAREZ et al., 2021; RINCÓN BONILLA et al., 2015).

The maturity index values ranged from 1.88 to 3.7 °Brix/% malic acid and are similar to those reported by other authors (MORENO-MEDINA et al., 2018; RINCÓN BONILLA et al., 2015; SAMANIEGO et al., 2020). This parameter relates SS with TA and is used to determine both the organoleptic quality of fruit and its maturity; as fruit matures, SS increases, and TA decreases (AYALA et al., 2013; GUERRERO ÁLVAREZ et al., 2021; ICONTEC, 1997).

The soluble solids parameter is related to the amount of sugar in fruit (SCHULZ; CHIM, 2019). Therefore, it can be inferred that the fruits grown in Santa Rosa contained a greater amount of sugar. For certain markets, fruits with a high sugar content are important; however, TA is also relevant. Both parameters influence the overall flavor of fruit, and there must be a balance between sugars and acidity for fruit to have a pleasant flavor (SCHULZ; CHIM, 2019).

On the other hand, high acidity and low pH values allow the stability of some organoleptic attributes and decrease microbial proliferation (MORENO-MEDINA et al., 2018) Therefore, fruits with these characteristics can be used in certain agro-industrial processes, such as in the production of juices and jams.

Determination of the ascorbic acid (vitamin C) content

For this parameter, significant differences were found between the cultivation sites, and for fruits grown in Guática, there were differences between the material with and without prickles. The ascorbic acid in the analyzed fruits of "mora de Castilla" with and without prickles ranged from 21.8 to 38.7 mg of ascorbic acid per 100 g of fruit (Table 3). Fruits grown in Pereira had the highest vitamin C content, and fruits of plants with prickles from Guática had the lowest vitamin C content. The values obtained are higher than those reported by Garzón et al. (2009), Horvitz et al. (2017) and Vasco et al. (2008) for "mora de Castilla".

Table 3. The contents of ascorbic acid (vitamin C) and total anthocyanins (TAC) in fruits of *Rubus glaucus* Benth. with and without prickles grown in 3 municipalities of the department of Risaralda.

Municipality (Place of cultivation)	Prickles	Parameter evaluated	
		Vitamin C (mg ascorbic acid/100 g sample)	TAC (mg c and d-3-glu/100 g sample) (differential pH)
Guática	With	21.8 ± 0.02^{aA}	83.83 ± 0.02^{aA}
	Without	27.7 ± 0.12^{bA}	93.68 ± 0.03^{bA}
Pereira	With	38.7 ± 0.06^{aB}	113.97 ± 0.02^{aB}
	Without	36.0 ± 0.04^{aB}	120.45 ± 0.01^{bB}
Santa Rosa	With	31.63 ± 0.03^{aC}	75.83 ± 0.02^{aC}
	Without	29.97 ± 0.01^{aC}	35.33 ± 0.03^{bC}

Averages with a common letter in the same column are not significantly different according to the Tukey test ($p \geq 0.05$). Capital letters indicate differences among municipalities, and lowercase letters indicate differences between plant material with and without prickles.

The variation in the concentration of ascorbic acid among municipalities may be due to different factors, such as the state of maturity, storage or climatic conditions of the cultivation area (ALARCÓN-BARRERA et al., 2018). The fruits grown in Santa Rosa presented high SS values (Table 2), a parameter that is potentially related to vitamin C content, which was relatively high for the samples from this municipality.

Determination of anthocyanin content by the pH differential method

Figure 1 shows the absorption spectrum for one of the extracts evaluated and corresponds to the UV–Vis spectrum characteristic of anthocyanins; the other extracts presented similar spectra, thus establishing the presence of this type of compounds.

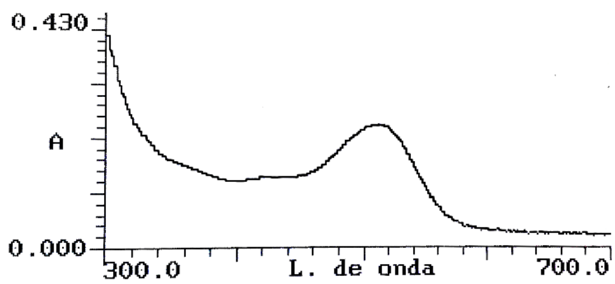

Figure 1. UV–Vis spectrum of an extract of *Rubus glaucus* Benth. analyzed by spectrophotometry. Similar spectra were obtained for each sample.

Table 3 provides the TAC results, for which significant differences were found between materials with and without prickles and among cultivation sites. The TAC values ranged from 35 to 120 mg cyanidin-3-glucoside/100 g sample. Five of the six samples presented relatively high values; only the sample from plants without prickles grown in Santa Rosa had a low TAC, close to half of the value obtained for fruit from plants with prickles grown in the same municipality. The fruits from plants with and without prickles from the municipality of Pereira had the highest anthocyanin content, and the fruits from Santa Rosa had the lowest content. The results obtained are within the range reported by Fan-Chiang and Wrolstad (2005) for 52 blackberry species and by Moyer et al.(2002) for 27 blackberry species.

The values obtained for fruits from plants with and without prickles from the municipality of Pereira, 114 and 120 mg c and d-3-glu/100 g, respectively, are slightly lower than those reported by Horvitz et al. (2017) for fruits of *Rubus glaucus* Benth. This difference could be explained by the degree of maturity at which the fruits were collected because some authors have observed that increasing the state of maturity increases the production and accumulation of anthocyanins. Similarly, several authors have reported that the amount of anthocyanins present in fruits depends on various factors, such as the type of cultivar, light, pH, biotic and abiotic stress, and other environmental factors (HORVITZ et al., 2017; ODERAY MERINO PEÑAFIEL et al., 2018).

On the other hand, except for the anthocyanin content in fruits from plants without prickles grown in Santa Rosa (35 mg c and d-3-glu/100 g), the TAC values obtained in this study are higher than those reported by other authors for fruits of *Rubus glaucus* Benth. (GARZÓN et al., 2009; SCHULZ; CHIM, 2019).

Determination of anthocyanin content by liquid chromatography (HPLC)

Initially, the presence of anthocyanin compounds in the samples was corroborated. The UV–Vis spectrum was analyzed to confirm that each peak detected presented the characteristic spectrum of anthocyanins, with absorption maxima around 250 and between 500-545 nm. Figure 2 shows one of the observed spectra.

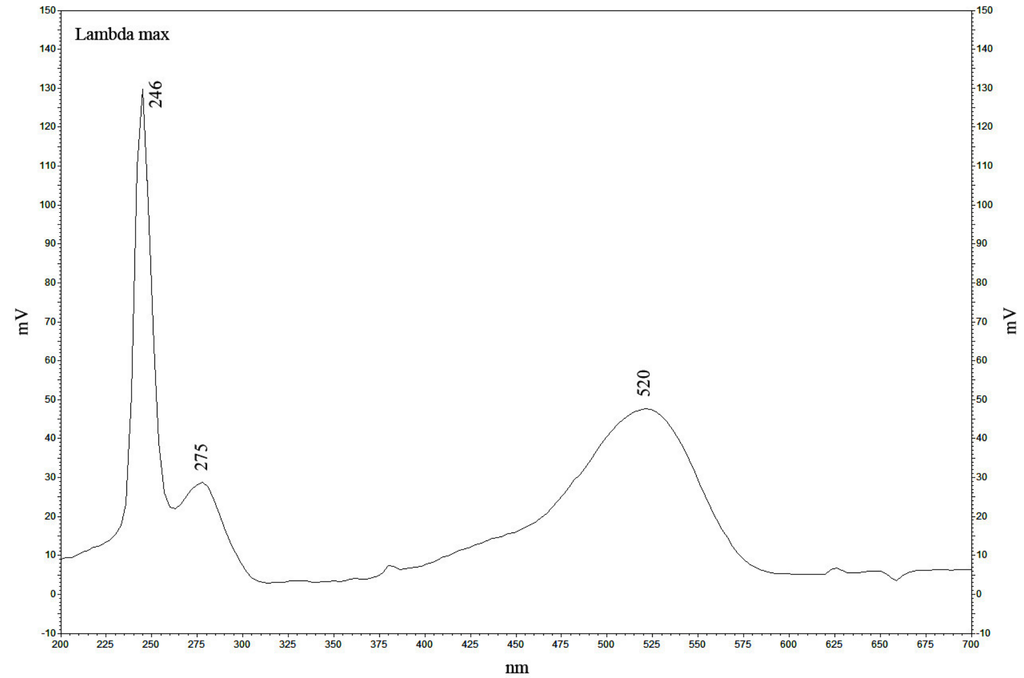

Figure 2. UV–Vis spectrum of an extract of *Rubus glaucus* Benth. analyzed by liquid chromatography. Similar spectra were obtained for each sample.

Subsequently, the chromatographic profile of the anthocyanins present in the fruits of *R. glaucus* was obtained (Figure 3). In these profile 5 peaks can be observed for each sample, which are common in all of them; their retention times (t_R) are presented in Table 4. These data indicate the presence of the same 5 anthocyanin compounds in the fruits from plants with and without prickles from the 3 municipalities evaluated.

Table 4. Retention time (t_R) for each anthocyanin peak detected.

Municipality (Place of cultivation)	Prickles	Retention time (min)				
		Peak number				
		1	2	3	4	5
Guática	With	13.85^{A}	15.11^{A}	16^{A}	18.88^{A}	19.38^{A}
	Without	13.83^{A}	15.1^{A}	15.98^{A}	18.86^{A}	19.37^{A}
Pereira	With	13.57^{A}	14.77^{A}	15.7^{A}	18.48^{B}	19.05^{A}
	Without	13.61^{A}	14.81^{A}	15.74^{A}	18.53^{AB}	19.09^{A}
Santa Rosa	With	13.58^{A}	14.81^{A}	15.72^{A}	18.53^{AB}	19.08^{A}
	Without	13.62^{A}	14.89^{A}	15.78^{A}	18.65^{AB}	19.17^{A}

Averages with a common letter in the same column are not significantly different according to the Tukey test ($p \geq 0.01$).

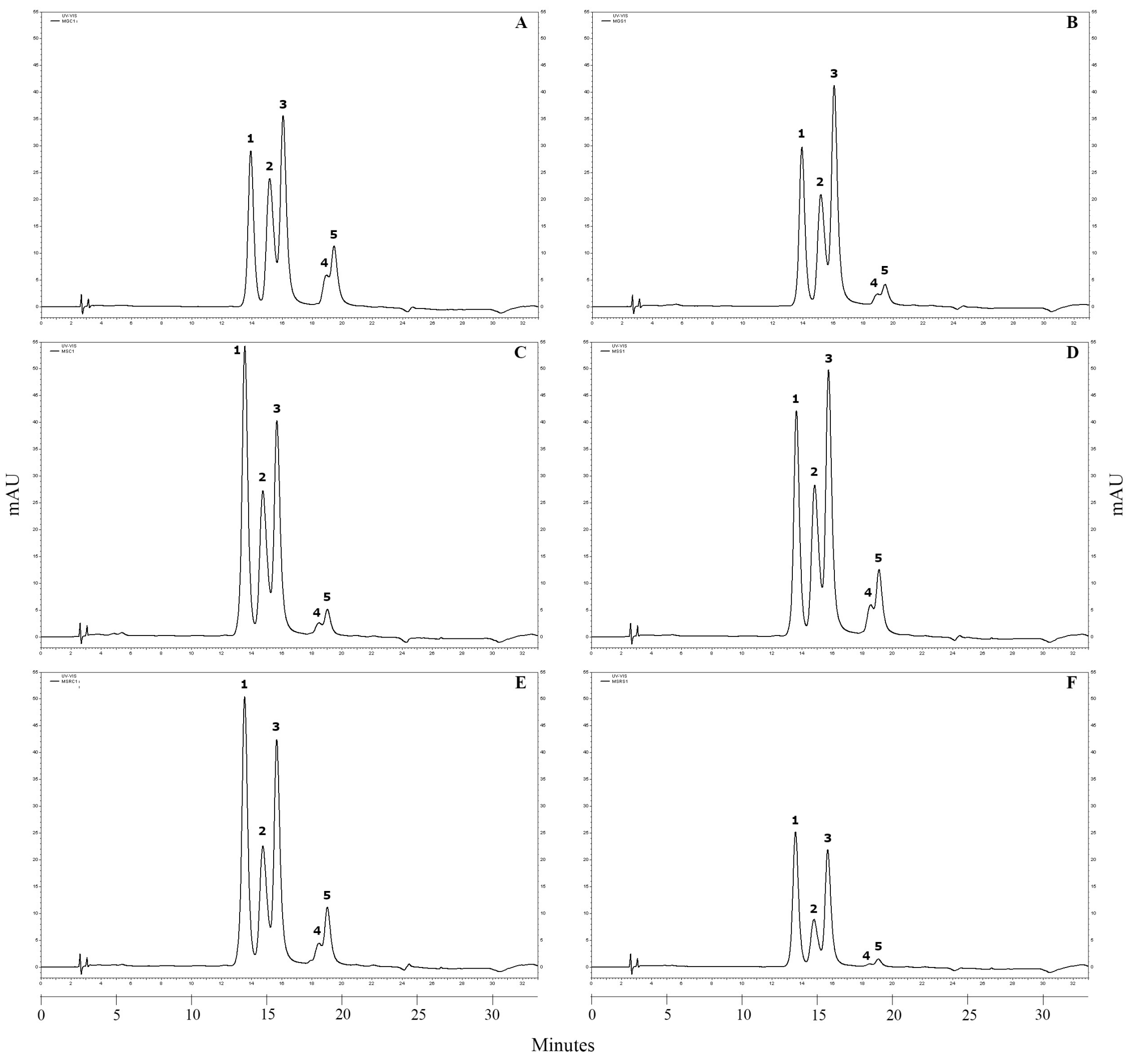

Figure 3. Chromatographic profile of anthocyanins present in fruits of *Rubus glaucus* Benth. with and without prickles from 3 municipalities of the department of Risaralda. A: Guática, with prickles; B: Guática, without prickles; C: Pereira, with prickles; D: Pereira, without prickles; E: Santa Rosa, with prickles; and F: Santa Rosa, without prickles.

The anthocyanins present in the samples were quantified using the external standard method with a cyanidin-3-glucoside standard (Table 5). Figure 4 shows the chromatogram and the UV–Vis spectrum corresponding to the cyanidin-3-glucoside standard, which has a retention time of 14.94 min.

Table 5. Anthocyanin content in fruits of *Rubus glaucus* Benth. with and without prickles from 3 municipalities of the department of Risaralda (determined by liquid chromatography (HPLC))

Municipality (Place of cultivation)	Prickles	Concentration of anthocyanins (mg cyanidin-3-glucoside/100 g sample)				
		Peak number				
		1	2	3	4	5
Guática	With	24.29 ± 0.22^{aA}	24.53 ± 0.68^{aA}	34.18 ± 0.51^{bA}	6.66 ± 0.09^{cA}	13.02 ± 0.12^{dA}
	Without	26.79 ± 0.83^{aB}	24.11 ± 1.84^{aA}	43.95 ± 3.95^{bB}	3.84 ± 0.24^{cB}	6.30 ± 0.69^{cB}
Pereira	With	38.88 ± 2.11^{aC}	25.94 ± 0.44^{bA} 7A	34.97 ± 1.15^{aA}	3.84 ± 0.33^{cB}	6.08 ± 0.70^{cB}
	Without	34.35 ± 0.32^{aD}	28.53 ± 0.33^{bB}	45.45 ± 0.45^{cB}	6.84 ± 0.12^{dA}	13.09 ± 0.13^{eA}
Santa Rosa	With	26.50 ± 0.31^{aAB}	14.73 ± 0.22^{bC}	24.71 ± 0.31^{cC}	3.62 ± 0.04^{dB}	7.46 ± 0.07^{eG}
	Without	13.71 ± 0.50^{aF}	6.29 ± 0.06^{bD}	12.60 ± 0.35^{cD}	1.34 ± 0.15^{dC}	1.71 ± 0.21^{dG}

Averages with a common letter in the same column (uppercase letters) or in the same row (lowercase letters) are not significantly different according to the Tukey test ($p \geq 0.05$).

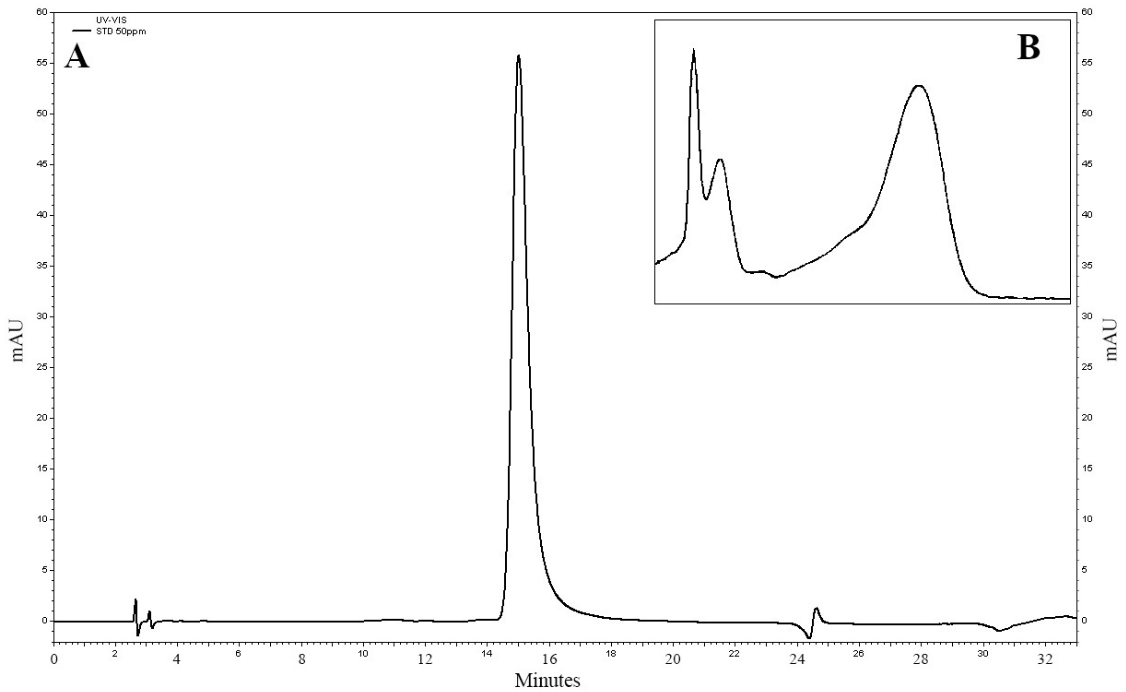

Figure 4. Chromatogram (A) and UV–Vis spectrum (B) for the cyanidin-3-glucoside standard.

As seen in Figure 3, the first 3 peaks are the majority in all samples, representing between 83 and 97% of the total area, and the last 2 peaks are minor, representing between 3 and 17% of the total area. The results obtained in this study are similar to those reported by Garzón et al. (2009), who found that the first 3 peaks of the profile corresponded to 40%, 10%, and 45% of the total area. For the evaluated cultivars, the third peak accounted for the majority in the samples from plants with and without prickles grown in Guática and in the samples from plants without prickles grown in Pereira, for the samples from Santa Rosa, the first peak accounted for the majority, and for the sample from plants with prickles grown in Pereira, it was not possible to establish whether peak 1 or 3 accounted for the majority because no significant differences were found between them. When comparing the retention time of the standard with those of the samples, there were no significant differences between the t_R of cyanidin-3-glucoside and the t_R of peak number 2 for all the samples, indicating that peak 2 corresponds to this compound, which represented between 17 and 24% of the total area calculated for each sample.

The anthocyanin profile obtained is similar to that observed by other authors; however, some differences were observed, possibly due to changes in the conditions of the equipment, the separation conditions and the mobile phases used. In other studies on fruits of *Rubus glaucus* Benth, compounds such as cyanidin-3-glucoside, cyanidin-3-xylorutinoside and cyanidin-3-rutinoside have been reported, which are present as major compounds, with minor compounds being pelargonidin derivatives (GARZÓN et al., 2009; HORVITZ et al., 2017; OSORIO et al., 2012). Garzón et al. (2009) identified 2 minor compounds, pelargonidin-3-glucoside and pelargonidin-3-rutinoside, and other authors indicated that these compounds may be derivatives of pelargonidin and cyanidin-3-O-malonyl glucoside (MERTZ et al., 2007; OSORIO et al., 2012; VASCO et al., 2009). Other compounds present in blackberry fruits of various cultivars are cyanidin-3-sambubioside, cyanidin-3-arabinoside, cyanidin-3-xyloside, malvidin-3-arabinoside and malvidin-3-galactoside (GARZÓN et al., 2009; MORAES et al., 2020; WU; PRIOR, 2005).

In several studies, the profile of “mora de Castilla” corresponds to compounds derived from cyanidin, with cyanidin-3-glucoside and cyanidin-3-rutinoside present in the greatest quantities (GARZÓN et al., 2009; MERTZ et al., 2007; OSORIO et al., 2012; VASCO et al., 2009). Some authors highlight the presence of cyanidin-3-rutinoside; it is the major compound in fruits of *Rubus glaucus* Benth., distinguishing itself from other blackberry cultivars in which the predominant anthocyanin is cyanidin-3-glucoside (HORVITZ et al., 2017; OSORIO et al., 2012).

As indicated above, the differences in the concentration of anthocyanins can be explained by the environmental conditions of the crop, in addition to other factors, and by the accumulation of anthocyanins with maturity (HORVITZ et al., 2017; ODERAY MERINO PEÑAFIEL et al., 2018). Osorio et al. (2012) found that peaks 1 and 2 increased as the fruit matured and that peaks 4 and 5 began to be detected in blackberry fruits with greater maturity. The sample from plants without prickles grown in Santa Rosa exhibited a profile with the lowest areas (Fig. 3), a finding that agrees with the TAC results, for which this sample had the lowest value (Table 3). In contrast, the highest amount of anthocyanins (both TAC and individual anthocyanins) was found in the samples from plants without prickles grown in the municipality of Pereira.

Determination of antioxidant capacity

The antioxidant activity of the analyzed blackberry fruits from plants with and without prickles was determined using DPPH and FRAP assays (Table 6). The antioxidant capacity obtained by the first method ranged from 46.7 to 178.4 µmol Trolox/g sample, with significant differences among the cultivation locations (samples from Pereira presented the highest values). The antioxidant capacity obtained by the FRAP method ranged from 2.2 to 8.6 mmol Trolox/100 g sample, and differences were found both among cultivation locations and between materials; the fruits from plants with prickles grown in Santa Rosa had the highest value. Differences were observed in the behavior of the antioxidant activity found by both methods, potentially indicating that the composition of phenolic compounds varied between the samples analyzed.

Table 6. Antioxidant activity in fruits of *Rubus glaucus* Benth. with and without prickles from 3 municipalities of the department of Risaralda (determined by spectrophotometry (DPPH and FRAP)).

Municipality (Place of cultivation)	Prickles	Parameter evaluated	
		DPPH (μmol Trolox/g sample)	FRAP (mmol eq Trolox/100 g sample)
Guática	With	139.18 ± 1.56^{aA}	2.16 ± 0.14^{aA}
	Without	114.01 ± 1.14^{bA}	5.24 ± 0.27^{bA}
Pereira	With	162.32 ± 2.97^{aB}	3.38 ± 0.31^{aB}
	Without	178.38 ± 3.35^{aB}	7.45 ± 0.93^{bB}
Santa Rosa	With	50.69 ± 2.10^{aC}	8.63 ± 0.58^{aC}
	Without	46.70 ± 1.68^{aC}	6.39 ± 0.28^{bC}

Averages with a common letter in the same column are not significantly different according to the Tukey test ($p \geq 0.05$). Capital letters indicate differences among municipalities, and lowercase letters indicate differences between plant material with and without prickles.

The antioxidant capacity values for fruits from Guática and Pereira found by the DPPH method agree with the values reported by Alarcón-Barrera et al. (2018). Although the values for fruits from the municipality of Santa Rosa were the lowest, i.e., less than half of those for fruits from the other 2 municipalities, the results obtained agree with those reported by Horvitz et al. (2017) and Vasco et al. (2008).

The values obtained with the FRAP test were slightly higher than those reported by Garzón et al. (2009) and similar to those observed by Vasco et al. (2008). The comparison of antioxidant activity is somewhat difficult due to the large number of techniques and options for reporting results; therefore, the values reported in the literature vary greatly.

The differences found between the fruits evaluated, similar to what was observed with the other organoleptic attributes and bioactive compounds, may be due to the growth conditions, the state of maturity, the climate, the geographical origin, the fertilizer, the type of soil, and the type of material, among other biotic and abiotic factors (ALFARSI et al., 2005; GARZÓN et al., 2009).

In general, fruits belonging to the genus *Rubus*, specifically *Rubus glaucus* Benth., have high phenolic compound contents and therefore have high antioxidant activity (ALARCÓN-BARRERA et al., 2018; SAMANIEGO et al., 2020; SIRIWOHARN; WROLSTAD, 2004; VASCO et al., 2008). The evaluated compounds (anthocyanins and ascorbic acid) contribute to this activity; however, other types of compounds, in particular ellagitannins and ellagic acid derivatives, can contribute to a great extent (HORVITZ et al., 2017; VASCO et al., 2009). Considering the above, the fruits evaluated in the present study had antioxidant potential, making them relevant as functional foods (GARZÓN et al., 2009; GUERRERO ÁLVAREZ et al., 2021).

As indicated above, when the maturity of the fruits increases, bioactive compounds, such as anthocyanins, accumulate, manifesting as an increase in the antioxidant activity of the fruits. This can be seen in the results obtained for fruits from the municipalities of Guática and Pereira. For each of these growing areas, fruits from plants without prickles had a higher MI, higher anthocyanin content and greater antioxidant capacity than did fruits from plants with prickles.

In the multivariate analysis of variance (MANOVA), $p < 0.05$ was obtained for the test statistics; the value of the Pillai trace statistic were close to 1, and that of the Wilks lambda was close to zero. The above allows us to conclude that both the place of cultivation and the type of material (fruits from plants with or without prickles) have an influence on all the parameters evaluated, with the place of cultivation having a greater impact.

All parameters, except pH, were affected and depended largely on the cultivation site ($p < 0.05$). The type of material (with or without prickles) influenced the parameters SS, TA, MI, TAC and antioxidant capacity, as determined by the FRAP test ($p < 0.05$).

All the differences found among the evaluated blackberry fruits correspond to those observed in multiple studies; there is evidence that various environmental and agronomic management factors greatly affect the quality and phytochemical composition of the fruits.

Standardization of the methods used for the quantification of ascorbic acid and anthocyanins

Verification of the spectrophotometric method for the determination of ascorbic acid

A calibration curve was obtained (Figure 5), yielding the equation y = 0.0238x - 0.0195, $R^2 > 0.99$, where y = absorbance and x = ascorbic acid concentration in mg/L. With the parameters obtained, presented in Table 7, it can be established that the method is adequate for the determination of ascorbic acid.

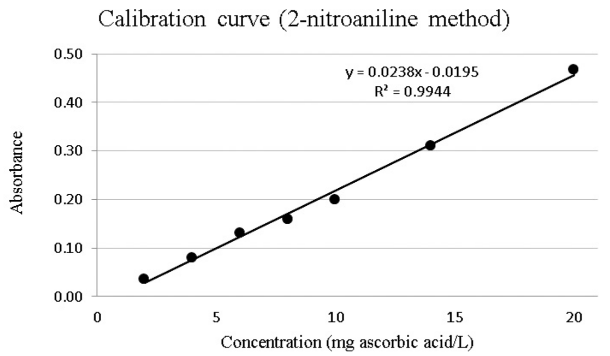

Figure 5. Calibration curve obtained for the spectrophotometry method (2-nitroaniline) employed for the determination of ascorbic acid.

Table 7. Statistical parameters for the verification of the 2-nitroaniline method to determine vitamin C content in *Rubus glaucus* Benth.

Parameter	Value
Sensitivity (absorbance/ppm)	0.0238
Precision (% RSD)	0.88
Accuracy (% Er)	5.95
LOD (mg ascorbic acid/L)	0.14
LOQ (mg ascorbic acid/L)	0.42

Verification of the spectrophotometry method (pH differential) for the determination of anthocyanins

A calibration curve was obtained (Figure 6), yielding the equation y = 0.0347x + 0.0033, $R^2 > 0.99$, where y = absorbance and x = concentration of cyanidin-3-glucoside in mg/L. Other parameters are presented in Table 8. With the results obtained, it can be established that the method is adequate for the determination of anthocyanins and that the method has good precision and accuracy.

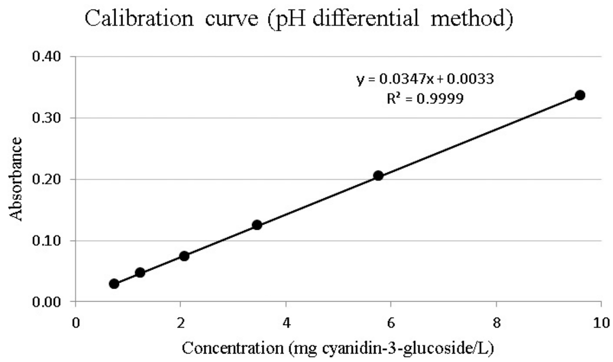

Figure 6. Calibration curve obtained for the spectrophotometry method (pH differential) employed for the determination of anthocyanins.

Table 8. Statistical parameters for the verification of the differential pH method for the determination of anthocyanins.

Parameter	Value
Sensitivity (absorbance/ppm)	0.0347
Precision (% RSD)	1.27
Accuracy (% Er)	1.84
LOD (mg cyanidin-3-glucoside/L)	0.09
LOQ (mg cyanidin-3-glucoside/L)	0.29

Standardization of the chromatographic method (HPLC) for the determination of anthocyanins.

A calibration curve was obtained (Figure 7), yielding the equation y = 184239x - 209309, $R^2 > 0.99$, where y = area and x = concentration of cyanidin-3-glucoside in mg/L.

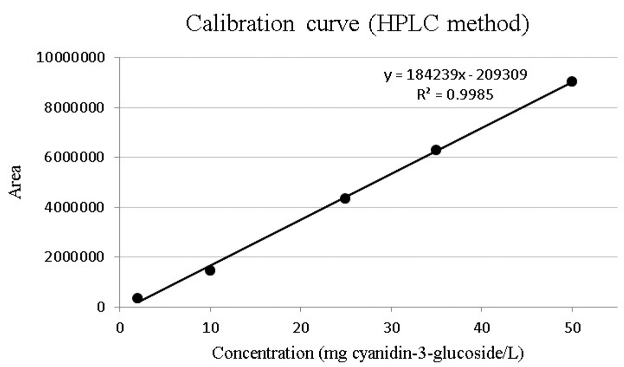

Figure 7. Calibration curve obtained for the chromatographic method (HPLC) for the determination of anthocyanins.

Precision and accuracy were evaluated using 3 different concentrations of the standard: low, medium and high (with respect to the range of the calibration curve). The RSD and Er values are shown in Table 9, as are other statistical parameters. The values obtained are within the ranges allowed for the analytical method chosen, indicating that the method is adequate for the determination of anthocyanins.

Table 9. Statistical parameters for the verification of the chromatography method (HPLC) for the determination of anthocyanins.

Parameter	Value
Sensitivity (area/ppm)	184239
Precision (% RSD)	4.95
Accuracy (% Er)	4.46
LOD (mg cyanidin-3-glucoside/L)	0.24
LOQ (mg cyanidin-3-glucoside/L)	0.73

Conclusions

The results obtained in this study allow us to establish the differences that exist between fruits of blackberry based on the type of material (with and without prickles) and on their place of cultivation. The parameters SS, TA and MI were different mainly with regard to the type of material. Some fruits had high SS (sugars) contents, and others had high acidity values. These characteristics allow the fruits to be used differently in various agroindustrial sectors.

Vitamin C content, TAC and antioxidant activity (DPPH and FRAP) were affected and depended on the place of cultivation. Additionally, the type of material (with or without prickles) influenced total anthocyanins and antioxidant capacity, as determined by the FRAP test. The fruits from one of the cultivation areas (Pereira) had a high bioactive compound content and antioxidant activity; therefore, these fruits stand out as a natural source of pigments and as a nutraceutical food with antioxidant potential. Regarding the observed anthocyanin profile, 5 compounds were detected, including cyanidin-3-glucoside, which was present in all the samples.

In general, the fruits of *Rubus glaucus* Benth. (“mora de Castilla”) with and without prickles grown in the studied areas of Risaralda, Colombia, are promising due to their bioactive compounds (vitamin C and anthocyanins) content, their high antioxidant activity and their valuable organoleptic attributes.

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