



Article

Phenolic Compound Content and the Antioxidant and Antimicrobial Activity of Wild Blueberries (*Vaccinium stenophyllum* Steud.) Fruits Extracts during Ripening

José O. Bernal-Gallardo ¹, Jorge Molina-Torres ^{2,*}, María V. Angoa-Pérez ¹, Jeanette G. Cárdenas-Valdovinos ¹, Ignacio García-Ruiz ¹, José A. Ceja-Díaz ¹ and Hortencia G. Mena-Violante ^{1,*}

¹ Instituto Politécnico Nacional, Department of Research, CIIDIR IPN Unidad Michoacán, Jiquilpan 59510, Mexico; jose.osvaldo.ber95@gmail.com (J.O.B.-G.); vangoa@ipn.mx (M.V.A.-P.); jcardenasv@ipn.mx (J.G.C.-V.); garinacho@gmail.com (I.G.-R.); jceja@ipn.mx (J.A.C.-D.)

² Laboratory of Phytobiochemistry, Department of Biotechnology and Biochemistry, Cinvestav IPN Unidad Irapuato, Irapuato 36821, Mexico

* Correspondence: jmolinat@cinvestav.mx (J.M.-T.); hmena@ipn.mx (H.G.M.-V.)

Abstract: The genus *Vaccinium* contains about 400 species distributed worldwide, but only a few species and varieties have had their phenolic composition and biological activity documented. In this study, phenols, flavonoids, anthocyanins, antioxidant and antimicrobial activity of methanolic extracts of *Vaccinium stenophyllum* Steud. fruits: 1-totally immature, 2-immature, 3-immature/close to maturity and 4-mature, were determined using UV-Visible spectrometry and HPTLC. The totally immature fruit extract showed the highest content of total phenols (19.153 ± 0.175 mg GAE/g DW), chlorogenic acid (20.867 ± 0.240 mg CAE/g DW), and the highest antioxidant activity by ABTS^{•+} (196.761 ± 0.641 μ M TE/g DW) and DPPH[•] (146.580 ± 6.466 μ M TE/g DW). Immature, immature/close to maturity and mature fruits extracts, exhibited the lowest MIC (9.37 mg/mL) and MBC (18.75 mg/mL) against *Escherichia coli*, *Salmonella choleraesuis*, and *Shigella flexneri*. The mature fruits extract exhibited the highest content of total anthocyanins (0.141 ± 0.004 mg CE/g DW) and cyanidin-3-glucoside (19.230 ± 0.309 mg CGE/g DW). The content of phenols, flavonoids and anthocyanins was higher than that reported for other *Vaccinium* species. These results showed the relevance of *Vaccinium stenophyllum* Steud. for breeding purposes to enhance the bioactivity of cultivars, or as a source of natural additives for the food industry, among others.

Keywords: phenols; flavonoids; anthocyanins; HPTLC analysis; antibacterial; antioxidants



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1. Introduction

The fruits of the genus *Vaccinium* are characterized by their red, blue, or almost black color, and are commonly known as blueberries, although they have other common names such as cranberry, bilberry, lingonberry, “ráspano”, “frutilla”, “madroño”, and “pingüica”, among others [1,2]. There are an estimated 450 species belonging to the genus *Vaccinium*, which have a wide geographical distribution comprising the northern hemisphere and the mountains of tropical Asia, Central America, South America, and southern Africa. About 35% of the species are native to the Americas, including 25% in North America and 10% in South and Central America [2,3].

Blueberries are widely cultivated and consumed, as there is a market demand for foods rich in secondary metabolites that aid in the prevention of chronic diseases [4,5]. The secondary metabolites reported in blueberries include flavonols (predominantly derived from quercetin), phenolic acids (caffeic, p-coumaric, ferulic, and chlorogenic), and anthocyanins, which are considered to be nutraceuticals with potential as both preventive agents

of diabetes, cancer, cardiovascular diseases and neurodegenerative diseases, and antimicrobial agents; this has been attributed to their biological activities, including antioxidant function [6–8].

For example, fruits of the variety ‘Blue Rose’ showed a trolox equivalent antioxidant capacity measured by the DPPH method of 2.11 mg of trolox equivalent per gram of fresh weight [9], while fruits of *Vaccinium meridionale* Sw., reached 143.68 µg of trolox equivalent per gram of dry weight [10]. On the other hand, studies on *Vaccinium floribundum* Kunth. Fruits, showed an inhibition percentage of 43% on *Escherichia coli* ATCC 25922 with an extract concentration of 10 mg of dry weight per milliliter [11]. In addition, studies on blueberries of the ‘Elliott’ variety showed a Minimum Inhibitory Concentration of 450 mg of dry weight per milliliter on *Salmonella enteritidis* CMCC50041 [12].

Moreover, it has been shown that there is a higher content of secondary metabolites in wild blueberries than in cultivated ones; for example, it was shown that the content of glycosylated peonidin and delphinidin in *Vaccinium myrtillus* L. (wild), was up to 3 times higher than that found in *Vaccinium corymbosum* L. (cultivated) [13].

Wild berry species, such as blackberry, strawberry, and blueberry, have been reported to possess greater biological activity as compared to cultivated species [14–16]. Therefore, one of the current methods of plant breeders is the identification of wild species, which are rich in secondary metabolites and have a greater biological activity, to be used as resources to achieve improved bioactivity in berries [17,18].

In Mexico, wild blueberry species are distributed in central and southeastern areas of the country. In the state of Michoacán, the native species *Vaccinium stenophyllum* Steud. has been reported upon, but there are no studies focused on its phytochemical or specific biological activity [19–21]. Therefore, the aim of the present work was to determine the phenolic content and the antioxidant and antimicrobial activity of *Vaccinium stenophyllum* Steud. fruit during ripening.

2. Materials and Methods

HPLC-grade methanol, hydrochloric acid (HCl), chlorogenic acid, and Trolox were obtained from Sigma-Aldrich® (St. Louis, MO, USA). All anthocyanin standards were obtained as HPLC-grade chloride salts. Cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-5-diglucoside, malvidin-3-glucoside and pelargonidin-3-glucoside were purchased from Sigma-Aldrich® (St. Louis, MO, USA). HPTLC Silica gel plates 60 F254 (20 × 10 cm, Art. 1.05729.0001) were supplied by Merck® (Darmstadt, Germany). For mobile phases, ethyl acetate, acetic acid, and formic acid purchased from Sigma-Aldrich® were used. Broth and Mueller–Hinton agar were purchased from BD Bioxon (Estado de Mexico, Mexico).

Certified strains of *Escherichia coli* ATCC 12792, *Salmonella choleraesuis* ATCC 12022, and *Shigella flexneri* ATCC 10708 were used.

Vaccinium stenophyllum Steud. fruits were collected in the locality of Los Gallineros, the Municipality of Cotija, Michoacán (19°40′16.0″ N, 102°41′47.5″ W at 1969 m.a.s.l.), in the autumn season of September 2020, from a total of 18 plants distributed in a triangular section, measuring 20, 29, and 35 m on each side; the plants were identified by M. en C. Ignacio García Ruíz (collection number 9971 and 9974). Specimens were deposited in the CIIDIR-MICHOACÁN Herbarium CIMI.

2.1. Fruit Preprocessing

The harvested fruits were classified according to their surface color in relation to their degree of ripeness, as reported by Arteaga Dalgo et al. [22], with some modifications. Color evaluation was performed using a colorimeter with a CIE L^* , a^* , b^* scale (HFBTE, NR100, China), lightness (L^*), and the color components a^* (green-red) and b^* (yellow-blue) were determined. In addition, the HUE-angle (h : hue) and chroma (C^* : color saturation) were calculated from the a^* and b^* values. The classification was in four maturity stages as shown in Table 1.

Table 1. *Vaccinium stenophyllum* Steud. fruit classification according to maturity stage.

Maturation Stage		Fruit Color
Stage 1	Totally immature	Green
Stage 2	Immature	green/red
Stage 3	Immature/close to maturity	Red
Stage 4	Mature	dark purple

2.2. Fruit Quality

The maturity index of each ripening stage was determined by means of the soluble solids/titratable acidity ratio based on the work of Santos et al. [23]. The test was carried out in triplicate.

2.3. Extract Preparation

The fruits were freeze dried (Lyophilizer, LABCON, Kansas City, MO, USA), and the freeze-dried tissue was pulverized with a pestle and mortar and stored at -20°C until use. Methanolic extracts from each stage were prepared as reported by Cretu et al. [24]. A total of 1 g of samples was extracted with 50 mL of absolute methanol, using an ultrasonic processor (ULTRASONIK, DENSTPLY, NEYTECH, Yucaipa, CA, USA) at 55 ± 5 Hz for 30 min at room temperature. The extracts were vacuum filtered through a $0.45\ \mu\text{m}$ cellulose filter, and 1.25 mL were stored in 40 1.5 mL microtubes at -20°C .

2.4. Total Phenol Quantification

The quantification of total phenols in the extracts was performed by UV-Visible spectrophotometry as reported by Spinardi et al. [25]. The absorbance was measured at 700 nm using a spectrophotometer (PowerWave HT, Biotek, Biotek Instruments, Vermont, USA). Total phenol content was quantified based on a calibration curve ($A_{700} = 0.6896 [\text{gallic acid}] + 0.0008$, $R^2 = 0.9956$) obtained from 10 concentrations (0.2–2.0 mg/mL) of gallic acid. The total content was expressed in milligram gallic acid equivalents per gram of dry weight (mg GAE/g DW). The assay was performed in triplicate.

2.5. Flavonoid Quantification

The quantification of the total flavonoids in the methanolic extracts was performed by UV-Visible spectrophotometry using the technique proposed by Woisky and Salatino [26]. The absorbance was measured at 425 nm using methanol as blank with a spectrophotometer PowerWave HT, Biotek, (Biotek Instruments, Vermont, VT, USA). Total flavonoid content was quantified based on the calibration curve ($A_{425} = 1.0443 [\text{quercetin}] - 0.048$, $R^2 = 0.9967$) obtained from 10 concentrations (0.2–2.0 mg/mL) of quercetin. The total content was expressed as milligram quercetin equivalents per gram dry weight (mg QE/g DW). The assay was performed in triplicate.

2.6. Total Anthocyanin Quantification

The total anthocyanin content in methanolic extracts was determined by UV-Visible spectrophotometry based on the work of Abdel-Aal and Hucl [27]. The absorbance of each extract was read at 535 nm using a spectrophotometer PowerWave HT, Biotek. The assay was performed in triplicate. The total anthocyanin content was expressed as cyanidin-3-glucoside equivalent (mg CGE/g DW), and was calculated by the following formula:

$$\text{Total anthocyanins content} = \frac{A_{535\text{ nm}}}{25,965} \times 449 \times \frac{1}{\text{extract concentration}} \times 10^6$$

2.7. General Chromatography Conditions

For the identification and quantification of chlorogenic acid using high-performance thin-layer chromatography (HPTLC); 10×20 cm silica gel plates 60 F 254 (Merck) were used. For pretreatment, the plates were activated in the plate heater (TLC Plate Heater

3, CAMAG, Muttenz, Switzerland) at 120 °C for 20 min. After cooling the plate, the samples were applied, and the plate was developed with the specific conditions for each compound to be quantified. Developed plates were derivatized with a 1% Natural Products (NP) methanolic solution (2-aminoethyl diphenyl borate, Sigma-Aldrich®, St. Louis, MO, USA) reagent, and the derivatization was performed in the immersion device (Chromatogram Immersion Device, CAMAG, Muttenz, Switzerland) using a vertical speed of 5 cm/s. After derivatization, the plate was dried under air flow for 3 min. Images of each plate in this study were documented with a TLC Visualizer (CAMAG, Muttenz, Switzerland), under visible light, at UV 254 nm and UV 366 nm. The obtained data were processed with the VisionCATS version 2.4 software (CAMAG, Muttenz, Switzerland).

2.7.1. Identification and Quantification of Chlorogenic Acid

In order to identify the chlorogenic acid in the methanolic extracts using HPTLC, we used the technique reported by Cretu et al. [24]. The reference solution was prepared at 1 mg/mL methanol. The extracts and the reference solution were tested with an automatic sampler (ATS 4, CAMAG, Muttenz, Switzerland) using a 25 µL syringe; the bands were applied with a length of 6 mm on the different lanes of the plates, with a distance between lanes of 8.3 mm, a distance from the bottom edge of 8 mm, and a distance from the left side of 21.1 mm. A total of 2.0 µL of the standard and of each extract were applied at a constant application rate (150 nL/s).

Chromatography was performed in an automatic development chamber ADC 2 (CAMAG, Switzerland) at a relative humidity of $25 \pm 2\%$ and a mixture of ethyl acetate (10): formic acid (1.1): acetic acid (1.1): water (2.3) (*v/v/v/v*) was used as solvent separation system. For standardized separation, the plate humidity control unit activity was exposed for 4 min and equilibrated with a saturated potassium acetate solution (257.6 g/100 g H₂O) to obtain a $37 \pm 2\%$ humidity content. The solvent front migration distance was 70 mm from the bottom edge of the plate and took 15 min. After development, the plate was dried under an air stream for 5 min.

After chromatography, the plate was heated on a TLC Plate Heater 3 (CAMAG, Muttenz, Switzerland) at 120 °C for 5 min and then derivatized. The images were documented and processed.

The chlorogenic acid content expressed as milligram chlorogenic acid equivalents per gram of dry weight (mg CAE/g DW) was obtained from the calibration curves ($y = 2.745 \times 10^{-4}x - 5.225 \times 10^{-2}$, $R^2 = 0.9900$). Five different volumes (3, 6, 9, 12 and 15 µL) of the reference solution were applied to the plate, and 2.0 µL of the methanolic extracts were applied in triplicate at a constant application rate (150 nL/s). The chromatogram was obtained, and the data were processed with the VisionCATS software (version 2.4, CAMAG, Muttenz, Switzerland).

2.7.2. Identification and Quantification of Anthocyanins

In order to identify anthocyanins using HPTLC, we used the technique reported by Cretu et al. [24] with some modifications. Reference solutions of cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-5-diglucoside, malvidin-3-glucoside, and pelargonidin-3-glucoside were prepared at a concentration of 1.1 mg/mL methanol. For the separation of anthocyanins from blueberry extracts, a mixture of ethyl acetate (10): formic acid (1.1): acetic acid (1.1): water (2.3) (*v/v/v/v*) was used as the mobile phase. Fruit methanolic extract at development stage 4, i.e., the stage with the highest total anthocyanin content, was used. The standard and stage 4 extracts were applied on the plate, with an automatic sampler ATS 4 (CAMAG, Muttenz, Switzerland) using a 25 µL syringe. Then, 4 mm length bands were applied on the plates, with a distance between lanes of 6.4 mm, a distance from the bottom edge of 8 mm, and a distance from the left side of 10.4 mm. A total of 2.0 µL of the extracts and standards were applied at a constant application rate (150 nL/s).

Chromatographic separation was performed in an automatic development chamber (ADC 2, CAMAG, Muttenz, Switzerland) with a relative humidity of $25 \pm 2\%$ and a mixture of ethyl acetate (10): formic acid (1.1): acetic acid (1.1): water (2.3) (*v/v/v/v*) solvent separation system. For standardized separation, the plate was exposed for 4 min using a saturated potassium acetate solution (257.6 g/100 g H₂O) in the humidity control unit at $37 \pm 2\%$. The solvent front migration distance was 70 mm from the bottom edge of the plate and took 20 min. After development, the plate was dried under an air stream for 5 min.

After chromatography, the plate was heated on a TLC Plate Heater 3 at 120 °C for 5 min and immediately derivatized. The images were documented and processed with the use of TLC Visualizer and the obtained data were processed using the Win-CATS software under visible light, at UV 254 nm and UV 366 nm.

The cyanidin-3-glucoside content was expressed as milligram cyanidin-3-glucoside equivalents per gram of dry weight (mg CE/g DW), which was obtained from the calibration curve ($y = -3.100 \times 10^{-3}x^2 + 6.82 \times 10^{-2}x - 1.480 \times 10^{-2}$, $R^2 = 0.9998$). Five volumes of the reference solution, i.e., 0.4, 1.0, 2.0, 4.0, and 5.5 µL, and 5.0 µL in triplicate of each methanolic extract were applied to each plate at a constant application rate (150 nL/s). The chromatogram was obtained, and the data were processed with the VisionCATS software.

2.8. Antioxidant Activity by ABTS Assay

The radical-scavenging capacity of the methanolic extracts was determined using the ABTS^{•+} method, as reported by Hosu et al. [28]. The ABTS solution was prepared by dissolving 360 mg of ABTS (2,2'-azinobis-(3-ethylbenzothiazdin-6-sulfonic acid) in 100 mL of distilled water, and 100 mL of ABTS solution was added to 100 mL of 2.45 mM potassium persulfate solution, for the activation of ABTS^{•+}. The mixture reaction was conserved in the dark for 24 h and refrigerated. The absorbance was measured at 734 nm using a spectrophotometer (PowerWave HT, Biotek, Vermont, VT, USA) and was adjusted to about 0.760 ± 0.001 by dilution with distilled water. A total of 280 µL of ABTS solution were added to 20 µL of methanolic extracts and the mixture was kept in the dark for 15 min. The absorbance of the sample and the blank was measured at 734 nm using the PowerWave HT spectrophotometer. The antioxidant capacity of the extracts was expressed as µmoles Trolox equivalent/g DW (µmol TE/g DW) using a calibration curve ($\mu\text{mol TE} = -5.4371 [A_{734\text{nm}}] + 1.4357$, $R^2 = 0.9852$) with five different concentrations (0–0.60 µmol Trolox). The assay was performed in triplicate.

2.9. Antioxidant Activity by DPPH Assay

The antioxidant activity of the methanolic extracts was determined using the microplate DPPH[•] method previously described by Untea et al. [29], with some modifications. In 96-well microplates, 20 µL of methanolic extracts were mixed with 200 µL of DPPH[•] (1,1-diphenyl-2-picrylhydrazyl) (150 mmol, ethanolic solution). The plate was kept in complete darkness for 30 min. After this time, the absorbance was measured at 515 nm using an PowerWave HT spectrophotometer (BioTek®, Vermont, VT, USA). The antioxidant capacity of the extracts was expressed as µmol TE/g DW using a calibration curve ($\mu\text{mol TE} = -2.8918 [A_{515\text{nm}}] + 1.4357$, $R^2 = 0.9849$) with five concentrations (0–0.60 µmol Trolox). The assay was performed in triplicate.

2.10. Antioxidant Activity by HPTLC-DPPH

Antioxidant activity was determined using the HPTLC-DPPH[•] method as described by Orisini et al. [30], with some modifications. The DPPH[•] reagent was prepared by dissolving 40 mg of DPPH in 200 mL of methanol. The developed plates, as described in Section 2.7.1, were used and derivatized with the DPPH reagent, in a Chromatogram Immersion Device (CAMAG, Muttenz, Switzerland) using a vertical speed of 5 cm/s, for 1 s. The plates were then dried in the same way as described in Section 2.7.1 at room temperature in the dark for 30 min. Images of the plate were documented with the TLC

Visualizer under visible light, at UV 254 nm and UV 366 nm. The obtained data were processed with the VisionCATS software.

2.11. Determination of Antimicrobial Activity

The antimicrobial activity was determined based on the method of Sun et al. [31]. The Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) were determined using the standard broth microdilution method. Thereafter, 100 µL aliquots of Mueller-Hinton broth were placed in sterile 96-well microtiter plates. Methanolic extracts were concentrated in a rotary evaporator (Rotavapor r-200, BUCHI, Zürich, Switzerland), and resuspended in sterile deionized water. Extracts, 50 µL each, were adjusted to 75, 37.5, 28.12, 18.75, 14.06, 9.37, 7.03, 4.68, 3.51, and 2.34 mg/mL and were added to the wells. The following controls were considered: positive control, broth + bacteria; negative control, broth only; and antibiotic control, broth + bacteria + antibiotic. In the negative control, 200 µL of broth were placed in the corresponding wells. Moreover, 20 µL of the inocula adjusted to a density of 1×10^7 CFU/mL were also placed in the corresponding wells. The plates were incubated at 37 °C for 19 h. The strains tested were *Escherichia coli* ATCC 12792, *Salmonella choleraesuis* ATCC 12022, and *Shigella flexneri* ATCC 10708. After incubation, 20 µL of MTT tetrazolium salt were added, and the incubation continued for 45 min at 37 °C. Finally, the MIC was recorded. Subsequently, an aliquot of 50 µL from the wells without bacterial growth was spread on plates with Mueller–Hinton agar medium, and the MBC was evaluated after 24 h of incubation at 37 °C. MIC was defined as the lowest concentration of the extracts without visible bacterial growth and MBC as the lowest concentration that killed 100% of the bacteria. For each of the dilutions and controls, three replicates were performed. The tests were performed in triplicate on different dates.

2.12. Statistical Analysis

The results of all the different determinations are reported as the means $\pm$ standard deviations. An analysis of variance (ANOVA) was also performed, the differences between means were evaluated using Tukey's test ($p < 0.05$), and Pearson's correlations were determined using the R Studio software version 4.0.3.

3. Results and Discussion

3.1. Fruit Color

Table 2 shows the values that were obtained for the color parameters L^* , a^* , b^* , h and C^* . The highest lightness value (L^*) was observed in stage 1, and it was between 0.21 and 2.63 times higher than those observed in the rest of the stages. In addition, stage 1 exhibited higher green (a^* : -7.28) and yellow (b^* : 44.44) coloring. Regarding the parameter a^* with positive values, the highest value was observed in stage 3 (a^* : 21.13), as this was where the red fruits were found. On the other hand, parameter b^* exhibited a decrease as ripening progressed, due to a decrease in the yellow and an increase in blue color. A similar behavior concerning the L^* , a^* , and b^* parameters was reported by Arteaga Dalgo et al. [22] in the ripening of *Vaccinium floribundum* fruit.

It should be noted that the color saturation (C^*) exhibited a decreasing trend as ripening progressed, as was reported in other *Vaccinium* species [22,32]. It is important to mention that h exhibited a descending trend as ripening progressed, since it started with a green fruit (stage 1) with a higher H than the red color present in the rest of the ripening stages. This was reported by Lin et al. [32] in the varieties 'Bluecrop' and 'Northblue'.

Table 2. Color parameters L^* , a^* , b^* , h and C^* for the four ripening stages of *Vaccinium stenophyllum* Steud. fruit.

Maturation Stage		Color Parameters				
		L^*	a^*	b^*	h	C^*
1	Totally immature	57.88 ± 0.55 ^a	−7.28 ± 0.35 ^d	44.44 ± 0.85 ^a	99.30 ± 0.38 ^a	5.03 ± 0.88 ^a
2	Immature	47.45 ± 0.64 ^b	3.38 ± 0.64 ^c	31.50 ± 1.05 ^b	83.85 ± 1.31 ^b	31.68 ± 0.99 ^b
3	Immature/close to maturity	27.50 ± 0.97 ^c	21.13 ± 0.76 ^a	8.82 ± 1.16 ^c	22.55 ± 2.26 ^c	22.92 ± 1.07 ^c
4	Mature	21.95 ± 0.53 ^d	5.64 ± 0.87 ^b	1.84 ± 0.43 ^d	17.81 ± 3.27 ^d	5.94 ± 0.93 ^d

L^* = luminance from 0–100; a^* = red (positive) and green (negative) colors; b^* = yellow (positive) and blue (negative) colors; h = HUE angle (hue); C^* = chroma (color saturation). Mean ± standard deviation is presented; different letters indicate statistically significant differences in the columns. Analysis of variance (ANOVA) and separation of means was performed by Tukey's test ($p < 0.05$). Different superscript letters within the same column indicate significant differences.

3.2. Quality Attributes

The maturity index that was obtained from the ratio of soluble solid content to titratable acidity is shown in Figure 1. Soluble solids increased from 9 to 18% from ripening stage 1 to 4. The maturity index increased 9.3 times from ripening stages 1 to 4. Previous studies have reported the content of soluble solids in *Vaccinium corymbosum* cv. 'Brigitta' (14.3%) and 'Rocio' as being 1.23 and 1.19 times lower than those found in stage 4 fruit, respectively. On the other hand, titratable acidity present in the fruits of *Vaccinium corymbosum* cv. 'Brigitta' (0.83%) and 'Rocio' (0.55%) resulted 2.28 and 3.65 times lower than that found in the fruit of stage 4, respectively [33,34].

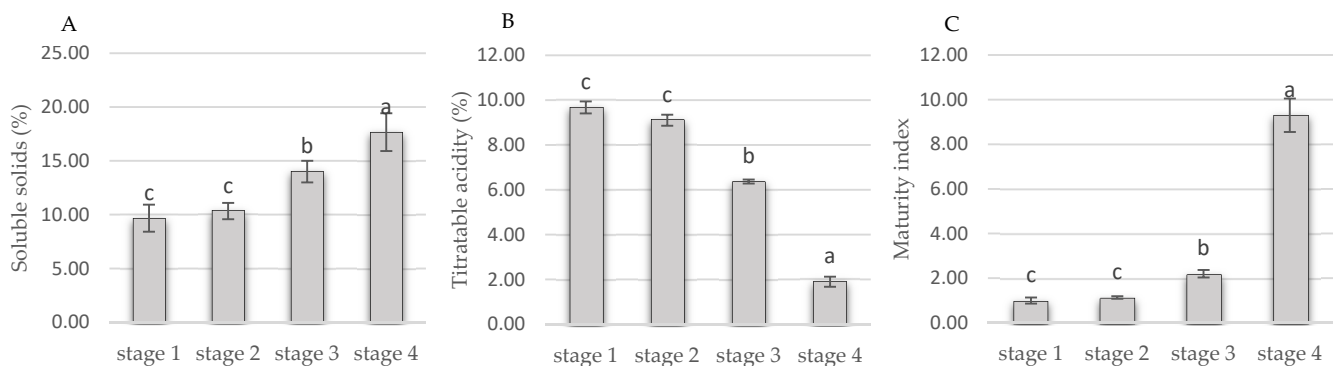


Figure 1. *Vaccinium stenophyllum* Steud. fruit quality attributes during ripening. (A) Soluble solids; (B) titratable acidity; (C) maturity index. The mean ± standard deviation is shown; different letters indicate significant statistical differences. An analysis of variance (ANOVA) was performed, and means were separated using the Tukey test ($p < 0.05$).

3.3. Total Phenols

The highest total phenol content was observed in the stage 1 extract (Table 3), and it was higher than those observed in the other stages by 13.06–28.56%. It was observed that the total phenol content decreased during ripening until the stage 3. This same trend was reported in fruits of the *Vaccinium corymbosum* L. [17,35,36], and could be due to a certain group of phenols requiring anthocyanin synthesis in the final stages of ripening [36]. Previous studies reported a total phenol content of 3.43 ± 0.07 mg GAE/g DW at stage 4 in *Vaccinium myrtillus* [37], which was 4.93 times lower than the content observed in this work. On the other hand, a content of 11.7 ± 2.44 mg GAE/g DW was previously reported in the variety 'Nelson' [35], which was 1.44 times lower than that found in stage 4 of *Vaccinium stenophyllum* Steud. fruits.

Table 3. Total phenolic, flavonoid, and anthocyanin contents of methanolic extracts of *Vaccinium stenophyllum* Steud. fruits during ripening.

Maturation Stage		Total Phenols (mg GAE/g DW)	Total Flavonoids (mg QE/g DW)	Total Anthocyanins (mg CGE/g DW)
1	Totally immature	19.153 ± 0.175 ^a	6.059 ± 0.185 ^a	0.032 ± 0.002 ^c
2	Immature	15.966 ± 0.105 ^c	5.299 ± 0.157 ^b	0.031 ± 0.001 ^c
3	Immature/close to maturity	14.898 ± 0.045 ^d	5.455 ± 0.176 ^b	0.047 ± 0.002 ^b
4	Mature	16.940 ± 0.130 ^b	5.660 ± 0.249 ^{ab}	0.141 ± 0.004 ^a

GAE = gallic acid equivalents; QE = quercetin equivalents; CGE = cyanidin-3-glucoside equivalents; DW = dry weight. The mean ± standard deviation is shown; different letters indicate significant statistical differences in the columns. An analysis of variance (ANOVA) was performed, and means were separated using the Tukey test ($p < 0.05$). Different superscript letters within the same column indicate significant differences.

3.4. Total Flavonoids

The highest total flavonoid content was observed in maturity stage 1 (6.059 ± 0.185 mg QE/g DW) (Table 3), and the value exceeded the values of the other stages by an average of 10.8%. Previous studies reported an increase in flavonoid content as ripening increases [25,38], however in this study, only an increasing trend was found from the maturity stage 2 to 4. This could be attributed to the solvent system, which in this case was not acidified. Among the flavonoid compounds that are extracted and preserved at acidic pH, anthocyanins represent a high proportion. This is why, in studies using acidified solvents, there is an increasing trend in total flavonoid content as ripening progresses [25,38]. On the other hand, in previous studies, a total flavonoid content of 0.916 ± 0.001 mg QE/g DW was reported in *Vaccinium arctostaphylos* fruits at the ripe stage and 0.840 ± 0.001 mg QE/g DW was reported in the ‘Bluegold’ variety [39], but both of these values are lower than that reported in this study. It has been reported by HPLC that the main flavonoids present in cultivated blueberries are quercetin and catechin, with values ranging from two $\mu\text{g/g}$ FW [40].

3.5. Total Anthocyanins

The highest total anthocyanin content was observed at stage 4 (Table 3), which was 4.5 times higher than that observed at stage 2. It was found that the total anthocyanin content increased as the ripening stage increased, which is a characteristic behavior of the genus *Vaccinium* during ripening [25,38]. On the other hand, previous studies reported 0.223 ± 0.003 mg CE/g DW at the mature stage in the variety ‘Misty’ and 0.916 ± 0.001 mg CGE/g DW in the species *Vaccinium arctostaphylos* [39], both values being higher than that observed at stage 4 in *Vaccinium stenophyllum* (by 1.58 and 6.49 times, respectively).

3.6. HPTLC

3.6.1. Identification and Quantification of Chlorogenic Acid by HPTLC

Chlorogenic acid was shown to be one of the most prevalent compounds in the extracts at an $R_f = 0.500$ (Figure 2). According to the quantification of chlorogenic acid, the highest chlorogenic acid content was observed at stage 1, i.e., 67% higher than in the rest of the ripening stages (Table 4). It should be noted that a decrease in chlorogenic acid as ripening increases was observed, as was the case for total phenol content (Table 3).

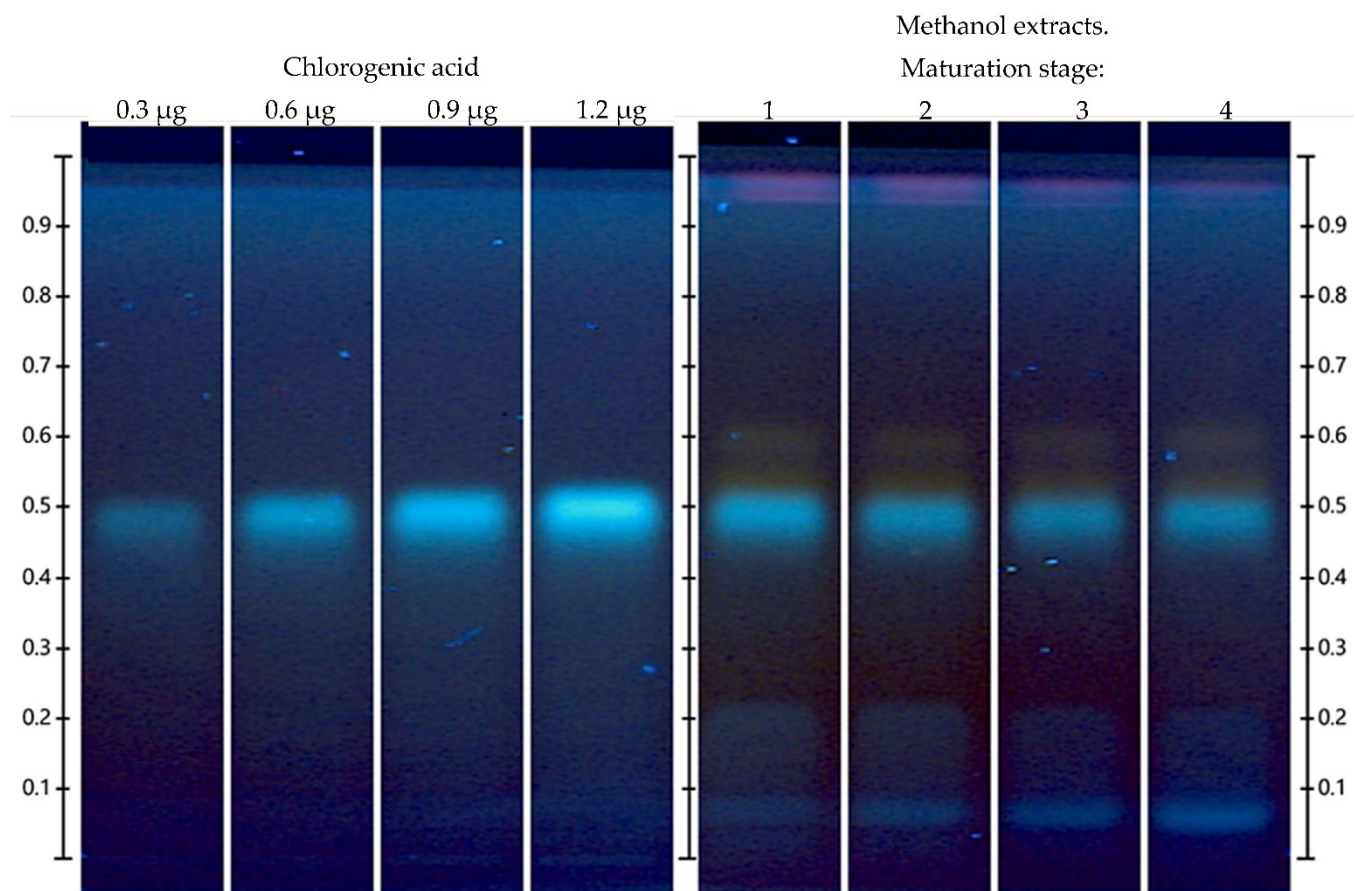


Figure 2. HPTLC chromatogram performed for the identification and quantification of chlorogenic acid from *Vaccinium stenophyllum* Steud. fruit extracts, taken under UV light (366 nm) after derivatization with NP reagent. The mobile phase was a mixture of ethyl acetate (10): formic acid (1.1): acetic acid (1.1): water (2.3) (v/v/v/v). Chlorogenic acid was applied on different volumes as a 4-point calibrated (3, 6, 9 and 12 µL, equivalent to 0.3, 0.6, 0.9 and 1.2 µg; $y = 2.745 \times 10^{-4}x - 5.225 \times 10^{-2}$, $R^2 = 0.9900$). *Vaccinium stenophyllum* Steud. fruit extract (stages 1–4) were applied in triplicate, volume = 2 µL (20 mg DW/mL).

Table 4. Quantification of chlorogenic acid by HPTLC in methanolic extracts of *Vaccinium stenophyllum* Steud. fruits during ripening.

Maturation Stage		Chlorogenic Acid (mg CAE/g DW)
1	Totally immature	20.867 ± 0.240^a
2	Immature	16.249 ± 0.361^b
3	Immature/close to maturity	13.270 ± 0.222^c
4	Totally mature	12.507 ± 0.289^d

CAE = Chlorogenic Acid Equivalents. The mean $\pm$ standard deviation is presented. An analysis of variance (ANOVA) was performed, and means were separated using the Tukey test ($p < 0.05$). Different superscript letters within the same column indicate significant differences.

It is important to mention that this behavior is opposite to that reported by Guofang et al. [38], who performed an HPLC analysis and reported that chlorogenic acid increased as ripening progressed in four cultivars of rabbit's eye blueberry (*Vaccinium ashei* Reade: 'Powderblue', 'Gardenblue', 'Baldwin', and 'Bright Well').

3.6.2. Identification and Quantification of Anthocyanins by HPTLC

With respect to the anthocyanin standards used, cyanidin-3-glucoside was found to be the most prevalent at stage 4 at $R_f = 0.34$ (Figure 3). Chung et al. [41] reported that the ripe fruit of *Vaccinium corymbosum* cv. Bluecrop contains cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-arabinoside, cyanidin-3-(malonyl) glucoside, cyanidin-3-(6''-acetyl) galactoside, and cyanidin-3-(6''-acetyl)glucoside according to HPLC. The bands located at $R_f = 0.44$ may be a glycosylated cyanidin other than cyanidin-3-glucoside or cyanidin-3-rutinoside.

On HPTLC plates developed for the quantification of cyanidin-3-glucoside, a sharp band separation of the cyanidin-3-glucoside standard was observed at a $R_f = 0.34$ and at similar R_f bands in the extracts from different maturity stages, which were found to be comparable with a calibration plate of cyanidin-3-glucoside standard (Figure 4). The highest cyanidin-3-glucoside content was observed at stage 4 (Table 5), which was higher than that in the other maturity stages (2.84–15.89%). It should be noted that the cyanidin-3-glucoside content increased as ripening progressed, as was the case with the quantification of total anthocyanins; see Table 3.

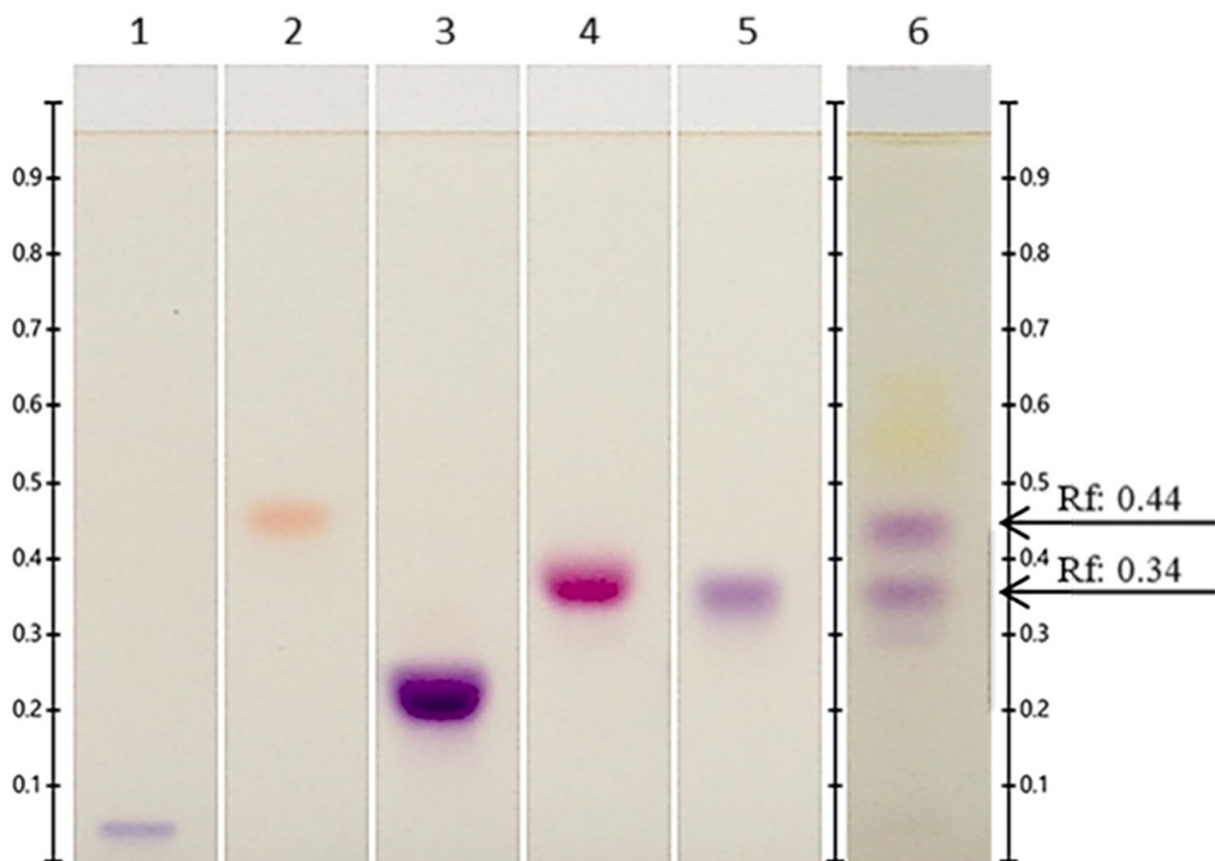


Figure 3. Identification of anthocyanins by HPTLC in extracts of *Vaccinium stenophyllum* Steud. fruits, taken under white light, after derivatization with NP reagent. Track 1: delphinidin-3-5-diglucoside; Track 2: pelargonidin-3-glucoside; Track 3: cyanidin-3-rutinoside; Track 4: malvidin-3-glucoside; Track 5: cyanidin-3-glucoside. Track 6: *Vaccinium stenophyllum* Steud. fruit extract (stage 4) was applied in triplicate, volume = 2 μ L (20 mg DW/mL). Band from extract not identified with standard (R_f : 0.44). Band from extract identified as cyanidin-3-glucoside (R_f : 0.34).

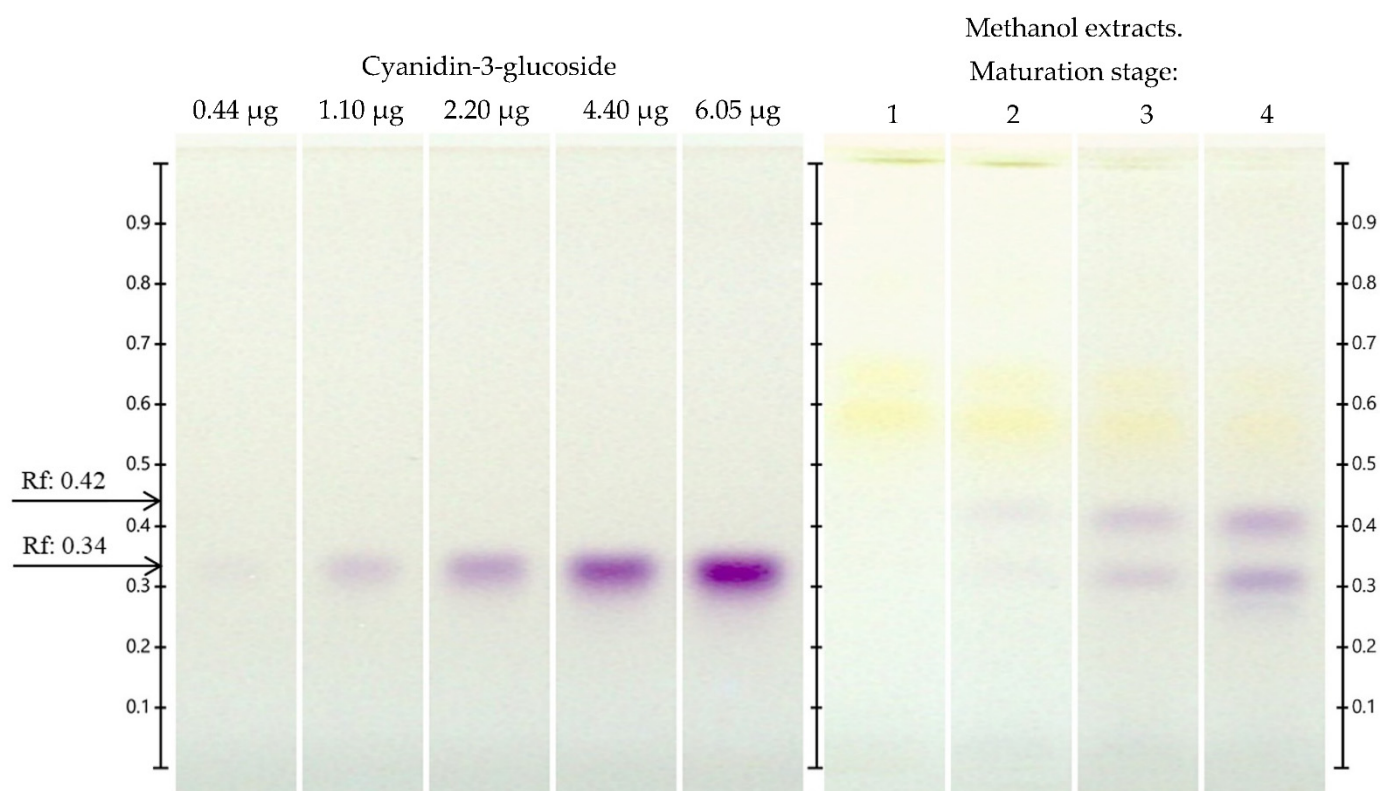


Figure 4. Quantification of cyanidin-3-glucoside by HPTLC in extracts of *Vaccinium stenophyllum* Steud. fruits, taken under white light, after derivatization with NP. Cyanidin 3-glucoside was applied on different volumes as a 5-point calibrated (0.4, 1.0, 2.0, 4.0, and 5.5 µL, equivalent to 0.44, 1.10, 2.20, 4.4 and 6.05 µg; $y = -3.100 \times 10^{-3}x^2 + 6.82 \times 10^{-2}x - 1.480 \times 10^{-2}$, $R^2 = 0.9998$). *Vaccinium stenophyllum* Steud. fruit extract (stages 1–4) were applied in triplicate, volume = 2 µL (20 mg DW/mL). Bands from extract not identified with standard (R_f : 0.42). Bands from extract identified as cyanidin-3-glucoside (R_f : 0.34).

As reported by Silva et al. [42], the cyanidin-3-glucoside content increased at the late ripening stages of *Vaccinium corymbosum* L. cultivars ('Duke', 'Bluecrop', 'Goldtraube' and 'Ozarkblue').

Table 5. HPTLC quantification of cyanidin-3-glucoside in methanolic extracts of *Vaccinium stenophyllum* Steud. fruits during ripening.

Maturity Stage		Cyanidin-3-Glucoside (mg CGE/g DW)
1	Totally immature	1.212 ± 0.059^d
2	Immature	1.788 ± 0.090^c
3	Immature/close to maturity	6.771 ± 0.103^b
4	Totally mature	19.230 ± 0.309^a

CE = cyanidin-3-glucoside equivalents. The mean $\pm$ standard deviation is shown; different letters indicate significant statistical differences in the columns. An analysis of variance (ANOVA) was performed, and means were separated using the Tukey test ($p < 0.05$). Different superscript letters indicate significant differences.

3.7. Antioxidant Activity

3.7.1. DPPH• and ABTS•⁺ Assay

The highest antioxidant activity according to the ABTS•⁺ method was observed at stage 1, i.e., 15.9–47.8% higher than at the other the stages (Table 6). On the other hand, previous studies reported a value of 170.68 µM TE/g DW in ripe *Vaccinium macrocarpon* Aiton fruits [43], which is only 0.5% higher than the antioxidant activity (ABTS) at stage 4 in

Vaccinium stenophyllum. Similarly, the highest antioxidant activity according to the DPPH• method was observed at stage 1, i.e., 15.4–19.55% higher than in the rest of the stages (Table 6). It should be noted that previous studies reported a value of 164.38 µmoles ET/g DW [44] in mature *Vaccinium myrtillus* fruits, which is 29.4% higher than the antioxidant activity (DPPH) of stage 4 *Vaccinium stenophyllum* Steud. The differences in results from the ABTS•+ and DPPH• methods are due to the fact that the ABTS•+ assay is applicable to hydrophilic and lipophilic antioxidant systems, whereas DPPH• is only applicable to hydrophobic systems [45].

Table 6. Antioxidant capacity of methanolic extracts of *Vaccinium stenophyllum* Steud. fruits during ripening.

Maturity Stage		ABTS•+ Method (µM TE/g DW)	DPPH• Method (µM TE/g DW)
1	Totally immature	196.761 ± 0.641 ^a	146.580 ± 6.466 ^a
2	Immature	133.056 ± 2.236 ^c	122.603 ± 1.837 ^b
3	Immature/close to maturity	137.775 ± 5.969 ^c	126.989 ± 4.369 ^b
4	Mature	169.676 ± 1.732 ^b	126.444 ± 5.486 ^b

TE = Trolox equivalents. The mean ± standard deviation is presented; different letters indicate statistically significant differences in the columns. An analysis of variance (ANOVA) was performed and means were separated using the Tukey test ($p < 0.05$). Different superscript letters within the same column indicate significant differences.

3.7.2. HPTLC-DPPH• Antioxidant Assay

The HPTLC-DPPH• technique allows one to observe which bands in the chromatogram have antioxidant potential and decolorizes DPPH• from purple to yellow (Figure 5). All the extracts exhibited antioxidant bands at $R_f = 0.80$, 0.60, and 0.50, with the stage 4 extract additionally showing antioxidant bands at $R_f = 0.44$ and 0.34. One of the main antioxidant bands in the chromatogram was that corresponding to chlorogenic acid ($R_f = 0.50$). There were also other bands with antioxidant power that could correspond to cyanidin-3-glucoside ($R_f = 0.34$), and one at $R_f = 0.42$ that probably corresponds to a glycosylated cyanidin other than cyanidin-3-glucoside or cyanidin-3-rutside. According to previous reports, the bands present at $R_f = 0.60$ correspond to hyperoside [24], which is another band with antioxidant activity. Hyperoside is one of the main flavonoids reported in species of the genus *Vaccinium* [43,46,47]. In addition, other antioxidant compounds were present in stage 4 extracts ($R_f = 0.42$) that were not identified.

Among the polyphenolic compounds, phenolic acids have the highest antioxidant capacity, which is greater than that of flavonoids; however, among the flavonoids, anthocyanins exhibited the highest antioxidant activity [17]. The antioxidant action of cyanidin-3-glucoside is usually attributed to its chemical structure, which is highly reactive because the electron deficiency of the C-ring is modulated by the pattern of hydroxyl (OH) substitutions, the degree of methylations on the aromatic β -ring, and the number of sugars attached to the molecule [48]. Chlorogenic acid is a potent electron-donating antioxidant, which is able to scavenge the nitrogen-centered stable free radical of DPPH• [49]. Previous studies on blueberries grown by liquid chromatography integrated mass spectrometry report *m-coumaric* as one of the antioxidant compounds in the fruit [50].

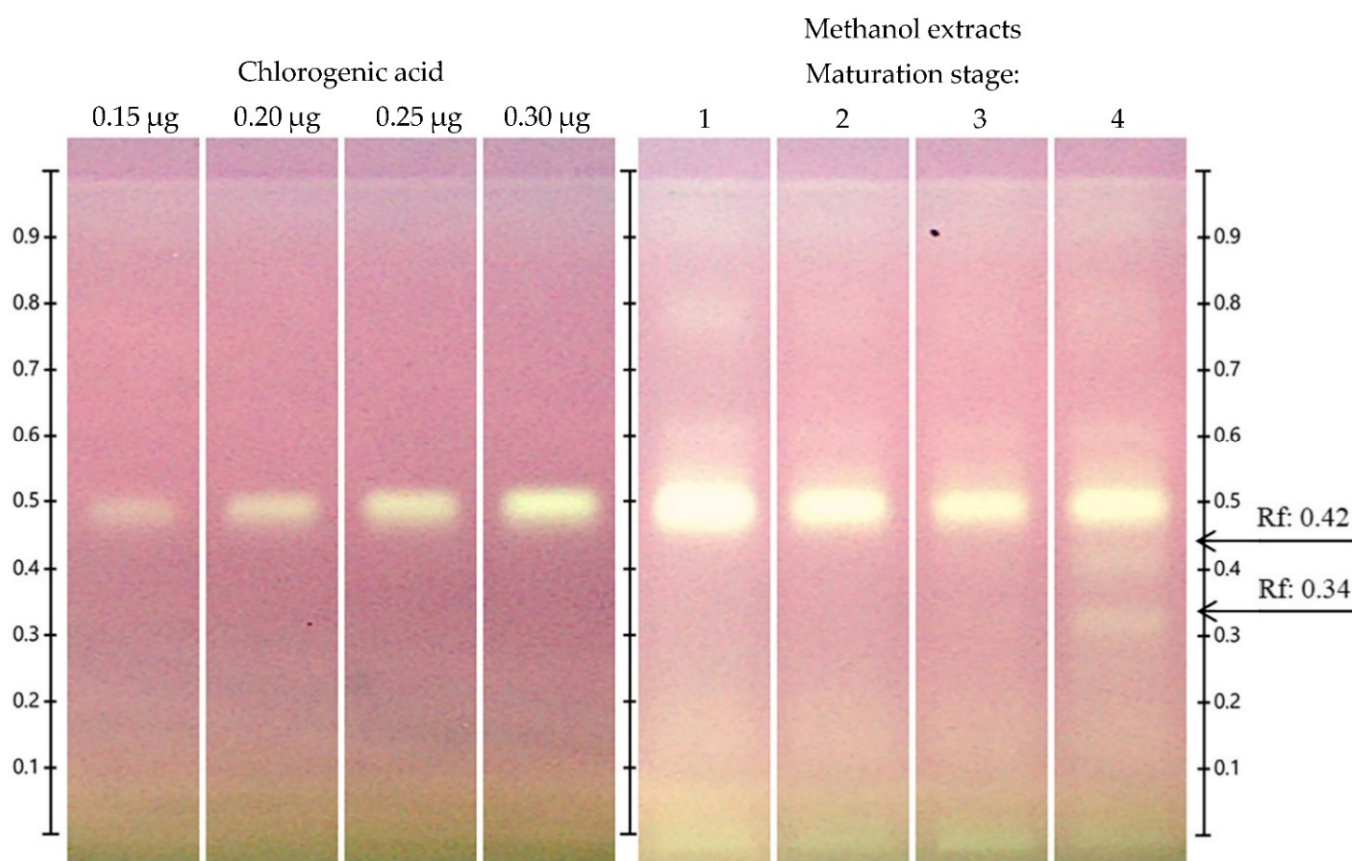


Figure 5. Detection of HPTLC-DPPH• antioxidant compounds in extracts of *Vaccinium stenophyllum* Steud. fruits under white light illumination, after derivatization with NP reagent. Chlorogenic acid was applied on different volumes as a 4-point calibrated (3, 6, 9 and 12 µL, equivalent to 0.3, 0.6, 0.9 and 1.2 µg; $y = 2.745 \times 10^{-4}x - 5.225 \times 10^{-2}$, $R^2 = 0.9900$). *Vaccinium stenophyllum* Steud. fruit extract (stages 1–4) were applied in triplicate, volume = 2 µL (20 mg DW/mL, *w/v*). Bands from extract not identified with standard (R_f : 0.42). Bands from extract identified as cyanidin-3-glucoside (R_f : 0.34).

3.8. MIC and MIB of *Vaccinium stenophyllum* Steud. Fruit Extracts

The highest MIC and MBC values (Table 7) were found in stage 1 extracts, being 1.5 times higher than the rest of the extracts. The MIC and MBC for *E. coli*, *S. flexneri*, and *S. choleraesuis* were identical. This phenomenon was previously reported in berry extracts of *Prunus spinosa* L., which exhibited the same MIC of 250 µg/mL for *Escherichia coli* ATCC 25922 and *Shigella sonnei* ATCC 25931 [51]. Contrarily, Cerezo et al. [52] found different MICs for *Escherichia coli* ATCC 25922 (3.74 mg/mL) and *Salmonella enteritidis* ATCC 13076 (2.16 mg/mL) in *Vaccinium corymbosum* extracts.

Notably, the methanol stage 1 extract had the highest chlorogenic acid content, but not the best MIC and MBC. However, the antimicrobial effect observed could be due to the toxicity of phenolic compounds to microorganisms. The mechanisms involved in this toxicity include enzyme inhibition by oxidized compounds, possibly through a reaction with sulfhydryl groups or through more nonspecific interactions with proteins that lead to their inactivation and loss of function [53,54].

Table 7. Antimicrobial activity of methanolic extracts of *Vaccinium stenophyllum* Steud. fruits during maturation for *E. coli*, *S. flexneri*, and *S. choleraesuis*.

Maturity Stage		<i>E. coli</i> ATCC 12792		<i>S. flexneri</i> ATCC 10708		<i>S. choleraesuis</i> ATCC 12022	
		MIC	MBC	MIC	MBC	MIC	MBC
1	Totally immature	14.06	28.12	14.06	28.12	14.06	28.12
2	Immature	9.37	18.75	9.37	18.75	9.37	18.75
3	Immature/close to maturity	9.37	18.75	9.37	18.75	9.37	18.75
4	Mature	9.37	18.75	9.37	18.75	9.37	18.75

Average values reported in mg dry weight/mL. MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration.

3.9. Correlation of the Variables under Study

Table 8 shows the correlation of the variables in this study. The minimum values of MIC and MBC were mainly correlated with the cyanidin-3-glucoside content. Furthermore, the best antioxidant capacity according to the DPPH• method was mainly correlated with the of chlorogenic acid, phenol, and total flavonoid contents. The ABTS•+ method's results were strongly correlated with the total phenol and flavonoid values.

Table 8. Correlation coefficient between quantifications and biological activity of *Vaccinium stenophyllum* Steud. fruit extracts.

	TPC	TFC	TAC	CAC	CC	ABTS•+	DPPH•	MIC	MBC
TPC	1	0.77 *	−0.02	0.77 *	−0.16	0.93 *	0.78 *	0.89 *	0.89 *
TFC		1	0.06	0.54	−0.03	0.84 *	0.90 *	0.78 *	0.78 *
TAC			1	−0.64 *	0.98 *	0.17	−0.26	−0.39	−0.39
CAC				1	−0.75 *	0.58	0.74 *	0.90 *	0.90 *
CC					1	0.07	−0.34	−0.48	−0.48
ABTS•+						1	0.78 *	0.83 *	0.83 *
DPPH•							1	0.91 *	0.91 *
MIC								1	1
MBC									1

TPC = total phenol content; TFC = total flavonoid content; TAC = total anthocyanin content; CAC = chlorogenic acid content; CC = cyanidin-3-glucoside content; ABTS•+ = antioxidant capacity ABTS•+ method; DPPH• = antioxidant capacity DPPH• method; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration. * Indicate significance at $p < 0.05$.

In *Vaccinium corymbosum* L. fruits, the content of total phenols, have been the main metabolites correlated with antioxidant activity [17], as it was observed in the present study. On the other hand, it has been reported a higher correlation of total anthocyanin content with antioxidant activity than that showed by total phenols content, contrary to what was found in this work [55]. Moreover, in fruits of *Vaccinium ashei* Reade cultivar, the best antioxidant activities obtained by the DPPH method were correlated with the content of chlorogenic acid, being similar to the results showed in this study. This could be attributed to the genetic load, which plays an important role both in the composition of phenols, flavonoids and anthocyanins, and in the biological activities of its extracts, as it has been reported for different blueberry varieties [38].

The fruits of *Vaccinium stenophyllum* Steud. showed a content of total phenols, total flavonoids, total anthocyanins, chlorogenic acid and cyanidin-3-glucoside close to that reported for other wild and cultivated species of *Vaccinium* [35,37,39,41,56]. Similarly, the antioxidant and antimicrobial activities showed values close to those reported in both wild and cultivated species [48,49,54,57]. It should be noted that the genetics of each species or variety of *Vaccinium* play an important role in its composition and content of bioactive compounds, which confer different potentials in biological activities [38].

This becomes important due to the growing interest in the use of natural antioxidants in food products [58], as well as natural and effective antimicrobial agents as an alternative to antibiotics or chemical additives [59].

4. Conclusions

This study is the first to report on the phenolic composition of *Vaccinium stenophyllum* Steud. fruits during ripening, and the antioxidant and antimicrobial potential of the fruits. Among the antioxidant compounds found in the extracts were chlorogenic acid and cyanidin-3-glucoside. In addition, the extracts exhibited an antimicrobial effect against enterobacteria of public health concern. Based on the results, *Vaccinium stenophyllum* Steud. could become a plant material of interest for plant breeding focused on fruit nutraceutical quality, in addition, the fruit has great potential as a natural source of metabolites with biological activities relevant for the development of nutritional supplements, food additives, among other products of plant origin that benefit health.

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