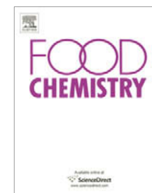
Food Chemistry 122 (2010) 980–986

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



# Chemical composition, anthocyanins, non-anthocyanin phenolics and antioxidant activity of wild bilberry (*Vaccinium meridionale* Swartz) from Colombia

G.A. Garzón [a,*], C.E. Narváez [a], K.M. Riedl [b], S.J. Schwartz [b]

[a] *Departamento de Química, Universidad Nacional de Colombia, AA 14490 Bogotá, Colombia*
[b] *Department of Food Science, 2015 Fyffe Road, Parker Food Science Building, The Ohio State University, Columbus, OH 43210, USA*

ARTICLE INFO

*Article history:*
Received 14 December 2009
Received in revised form 26 January 2010
Accepted 2 March 2010

*Keywords:*
*Vaccinium meridionale*
Anthocyanins
Phenolics
Antioxidant activity
Total phenolics

ABSTRACT

Berries of *Vaccinium meridionale* Swartz native to Colombia were analysed for chemical composition, total phenolic content, anthocyanin content, and antioxidant activity. In addition, high-performance liquid chromatography with photodiode array detection (HPLC–DAD) and HPLC-electrospray ionisation tandem mass spectrometry (ESI–MS/MS) were used to determine anthocyanin and phenolic composition. Anthocyanin content was 329.0 ± 28.0 mg cyanidin 3-glucoside equivalents/100 g (fresh weight) FW and total phenolic content was 758.6 ± 62.3 mg gallic acid equivalent/100 g FW. Cyanidin 3-galactoside was the major anthocyanin while the most abundant non-anthocyanin phenolic was chlorogenic acid.

The ABTS radical scavenging activity was 45.5 ± 2.3 μmol Trolox equivalents/g FW and the ferric reducing antioxidant potential (FRAP) value was 87.0 ± 17.8 μmol TE/g FW or 116.0 ± 23.7 μmol ferric iron reduced/g FW.

The unique anthocyanin composition of this fruit, as identified by classical techniques and ESI–MS/MS, can be differentiated from other bilberries and perhaps useful in authentification procedures. Overall, results from this study show that the fruit from Colombian wild bilberry has high antioxidant activity and potential applications as a source of phytochemicals in the nutraceutical and functional food market.

© 2010 Elsevier Ltd. All rights reserved.

## 1. Introduction

There is strong scientific evidence of the positive effect of dietary intake of berry fruits on human health, performance, and disease. Amongst berry fruits, bilberries (*Vaccinium myrtillus* L.) have received special attention due to their long history in folk medicinal uses. The fruit became widely known to herbalists in the 16th century, when it was used for treating bladder stones, biliary disorders, scurvy, coughs and lung tuberculosis (Valentová, Ulrichová, Cvak, & Šimánek, 2007). Later, clinical trials demonstrated the benefits of bilberries in the inhibition of cancer cell growth (Zhao, Giusti, Malik, Moyer, & Magnuson, 2004) and management of visual disorders (Canter & Ernst, 2004). Bao et al. (2008) reported that bilberry consumption triggers genetic signalling in disease prevention and promotes human health due to biomedical activities on conditions such cardiovascular disorders, advancing age-induced oxidative stress, inflammatory responses, and diverse degenerative diseases.

Phytochemical research on bilberry extracts has also shown that they prevent or control interstitial fluid formation, contribute to control the blood flow distribution in the microvascular network, modulate capillary resistance and permeability, and improve visual function (Morazzoni & Bombardelli, 1996). In addition, Martín-Aragón, Basabe, Benedi, & Villar (1998) and Valentová et al. (2007) reported antioxidative activity and cytoprotective effects against oxidative damage in various models in vitro.

*Vaccinium meridionale* Swartz is a wild shrub native to Andean South America, where it grows on open mountain slopes between 2200 and 3400 m above sea level. The fruit is a berry, 5–10 mm in diameter, dark redish, with a sour and tart character. In Colombia, wild bilberry is sold at the local markets and it is eaten fresh, dried, and cooked into sauces, jellies, and jams.

Although the anthocyanin and phenolic composition as well as the antioxidant activity of European and North American bilberry (*V. myrtillus*) have been extensively investigated (Mazza & Miniati, 1993; Moyer, Hummer, Finn, Frei, & Wrolstad, 2002; Prior et al., 1998), there are no reports documenting the composition, anthocyanin profile, phenolic profile or antioxidant activity of Colombian bilberry.

Considering the supporting information on the potential nutraceutical properties of bilberry, it is of interest to characterise the wild *Vaccinium* species that grow in Colombia. Accordingly, the objectives of this study were to determine the chemical composition, total anthocyanin and phenolic contents, anthocyanin profile, non-anthocyanin phenolic profile, and antioxidant activity of Andean bilberry (*V. meridionale*) and to compare them with reported compositions for other well-characterised *Vaccinium* species, specially (*V. myrtillus* L.).

* Corresponding author. Tel.: +571 316 5000x14457; fax: +571 3165220.
*E-mail address:* agarzonmo@unal.edu.co (G.A. Garzón).

0308-8146/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2010.03.017

## 2. Materials and methods

### 2.1. Plant material

Fresh, ripe berries of *V. meridionale* were harvested in Colombia in the native habitat of the species. Berries were picked randomly from different parts of unmanaged wild bushes on mountain slopes at an altitude between 2800 and 3000 m above sea level.

### 2.2. Reagents and standards

Chlorogenic acid (3-caffeoylquinic acid), quercetin, caffeic acid, ABTS (2,2′-azinobis(3-ethylbenzothiaoline-6-sulphonic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sodium carbonate, potassium persulphate, rutin, and Folin–Ciocalteau reagent were purchased from Merck® (Darmstadt, Germany). Gallic acid, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and TPTZ (2,4,6-tripyridyl-S-triazine) were from Sigma Aldrich (St. Louis, MO, USA). L-ascorbic acid, DTT (1,4-dithiothreitol), and $FeCl_3 \cdot 6H_2O$ were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Methanol and liquids were from Fisher Scientific. (Fair Lawn, NJ, USA).

Cyanidin 3-glucoside, cyanidin 3-galactoside and cyanidin 3-arabinoside were purchased from Indofine Chemical Co., Inc. (Somerville, NJ, USA). Concord grape juice (Welch Foods Inc., Concord, MA, USA) was purchased from a local supermarket.

### 2.3. Determination of titratable acidity (TA), pH, and Brix

Berry samples were selected according to colour and homogenised by a mortar and pestle for determination of TA, pH, and total soluble solids (TSS). TA was determined by titrating the sample (2 g of homogenate + 50 ml of $CO_2$-free distilled water) with standardized 0.1 N NaOH to pH 8.2 using a Schott Gerate® pH metre, model CG820 (Mainz, Germany). TA was expressed as grams citric acid per 100 g fruit. Measurement of the pH was done on 2 g of homogenate and TSS (Brix) was determined using a digital refractrometer Abbe II (Reichert-Jung, Leica Inc., Buffalo, NY, USA) on the supernatant after centrifugation of the homogenate at 4000*g*. Determinations were replicated four times.

### 2.4. Ascorbic acid analysis

For determination of ascorbic acid content, 10 g of fruit sample was processed in a Warring blender with 8 ml with deionized water. After centrifugation of the sample at 1500*g* at 4 °C for 10 min, the extract was collected and its pH was adjusted to 5–5.2 with 0.1 M NaOH. Ascorbic acid was kept in the reduced form by adding 0.1% 1,4-dithiothreitol (DTT). After 2 h-reaction with DTT, the sample was filtered through a 0.45-μm millipore filter (type HA) and analysed by HPLC.

HPLC analysis of total ascorbic acid was carried out using a Shimadzu LC-20AD/T HPLC equipped with a SPD-6AUV detector (Kyoto, Japan) and a LiChrosorb RP 18 column (5 μm) 250 × 4.6 mm (E Merck Inc., Darmstadt, Germany). The mobile phase consisted of 2% $KH_2PO_4$, pH 2.5 with 0.1% DTT and the running conditions were: Isocratic programme for 15 min, elution at 0.5 ml/min, injection volume 20 μl, detection at 254 nm.

For quantification of ascorbic acid in the samples, a standard curve of ascorbic acid (10–50 ppm) was developed. Linear regression analysis was applied to determine the ascorbic acid concentration in the extract.

### 2.5. Extraction of anthocyanins, and non-anthocyanin phenolics

Fresh berries were liquid-nitrogen powdered in a Waring blender and frozen at −70 °C until further analysis. The powder was extracted with 100% methanol, followed by three re-extractions with the same solvent until the solution became colourless. Methanol was evaporated with a Buchi Rotavapor at 40 °C and the extract was resolubilized in 50% aqueous methanol solution for subsequent analyses.

### 2.6. Determination of monomeric anthocyanin content and total phenolics

Monomeric anthocyanin pigment content was determined on the extract by the pH differential method as described by Giusti and Wrolstad (2001). Absorbencies were read at 510, and 700 nm. For comparison purposes, pigment content was calculated as cyanidin 3-glucoside using an extinction coefficient (∈) of 26900 L $cm^{-1}$ $mol^{-1}$ and molecular weight of 449.2. Measurements were replicated four times with means being reported.

Total phenolics were determined as gallic acid equivalents (GAE)/100 g FW using the method described by Waterhouse (2001). A 20-μl sample aliquot of extract or gallic acid standard (50–500 mg/L) was mixed with 1.58 ml water followed by 100 μl Folin–Ciocalteau's reagent. After vortexing and incubating at room temperature for 8 min, 300 μl of 20% aqueous sodium carbonate solution were added. Samples were vortexed and held at room temperature for 2 h. Absorbance of the blue-coloured solution was recorded at 765 nm on a Shimadzu UV–visible spectrophotometer, model UV 160 U (Kyoto, Japan), using 1-cm disposable cells. All measurements were replicated four times.

### 2.7. Acid hydrolysis of anthocyanins and solid-phase extraction (SPE)

For determination of anthocyanidins, acid hydrolysis of the anthocyanins was performed according to the method described by Durst and Wrolstad (2001). Approximately 1 mg of the pigment was mixed with 15 ml of 2 M HCl in a screw-cap test tube. The mix was flushed with nitrogen, capped and hydrolysed for 45 min at 100 °C, then cooled in an ice bath.

Cleaning of the hydrolysate containing the anthocyanidins was done with a C-18 cartridge (high load C-18 tube), 20 ml capacity (Alltech Association, Inc., Deerfield, IL, USA). The minicolumn was conditioned with methanol and upon loading with extract, washed with 0.01% HCl to remove sugars, acids, and other water-soluble compounds. Anthocyanidins were subsequently eluted with acidified methanol, which was removed from the fraction with the Buchi rotoevaporator at 40 °C.

### 2.8. Anthocyanidin, anthocyanin and non-anthocyanin phenolic determination by HPLC–DAD

HPLC–DAD analysis of anthocyanidins, anthocyanins and other phenolics were carried out on a Waters 2695 gradient HPLC separation module (Waters Corporation, Mildford, MA, USA) equipped with an autoinjector and a 996 photodiode array detector (PDA). Chromatographic separations were performed on two C18 Symmetry columns connected in series (each 75 × 4.6 mm i.d., 3.5 μm) (Waters Corporation, Mildford, MA, USA). The mobile phase consisted of 5% (v/v) formic acid in water (solvent A) and 5% (v/v) formic acid in acetonitrile (solvent B) at a flow rate of 1.0 ml/min. The gradient condition was 0–20 min 20% B and 20–30 min 20–58% B. Absorbance spectra was recorded every 1.2 nm from 200 to 700 nm.

Retention times and UV–Vis spectra of anthocyanidins were compared to known standards. Concord grape juice was used as

source of five anthocyanidins: cyanidin, delphinidin, petunidin, peonidin, and malvidin while strawberry juice was used as source of pelargonidin (Durst & Wrolstad, 2001). Co-chromatography was used when necessary for identification.

Peak identification of cyanidin-based anthocyanins was performed by comparison of retention times and diode array spectral characteristics with standards. Due to the lack of delphinidin standards, delphinidin anthocyanins were tentatively identified only by further HPLC–ESI/MS–MS analysis. All analyses were done in triplicate.

The non-anthocyanin phenolics were identified by their UV–Vis spectra, MS/MS spectra, and chromatographic retention time comparisons with standards (when available) and with the literature. Chlorogenic acid was adopted as the standard for identification and quantification of hydroxycinnamic acids at 320 nm while rutin was used as the standard for identification and quantification of flavonols at 355 nm. Results are expressed as mg/g FW.

### *2.9. HPLC–DAD/MS/MS of anthocyanins and polyphenolics*

Separation of compounds was conducted under the same conditions described above. Detection was performed using a quadrupole/time-of-flight mass spectrometer (QTof Premier, Micromass Limited, Manchester, UK) equipped with an electrospray ionisation (ESI) source operated in both positive and negative modes of polarity. ESI conditions included a capillary voltage of 3.2 kV for positive mode, 2.8 kV for negative, cone voltage of 35 V, ion guide at 1 V, source temperature of 100 °C, and nitrogen desolvation gas temperature of 400 °C flowing at 600 L/h. During experiments employing collisionally induced dissociation (CID), argon was held at a pressure of $3.0 \times 10^{-3}$ mbar.

To screen for potential parent > daughter ion relationships, two TOF-MS runs were conducted, in ESI positive and negative mode, respectively. Within each run, two functions run simultaneously. In the first, low collision energy (CE) was applied to allow transmission of intact parent ions while in the second high CE was applied to provide fragmentation data. For a given PDA peak (320, 355, 512 nm) the coincident MS spectra were inspected for possible parent and daughter ions. Presumed parent/daughter ions were extracted to give MS chromatograms. If the MS peak coincided with the PDA peak, then the match was inferred. Subsequently, MS/MS was performed to confirm this relationship in the appropriate mode (positive or negative).

### *2.10. Determination of antioxidant activity*

Antioxidant activity was determined by the ABTS and FRAP assays. ABTS radical cation scavenging activity was determined according to the method described by Re et al. (1999). $ABTS^{\cdot+}$ was produced by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate (final concentration). The solution was held at room temperature in the dark for 16 h before use. Once the radical was formed, the absorbance at 734 nm was adjusted to 0.7 by dilution with 95% ethanol. Fresh $ABTS^{\cdot+}$ solution was prepared for each analysis. $ABTS^{\cdot+}$ (1 ml) was added to 10 μl sample and the reaction mixture was allowed to stand at 30 °C for 6 min and the absorbance at 734 nm was immediately recorded. The percentage decrease of the absorbance at 734 nm was calculated by the formula $I = [(AB - AA)/AB] \times 100$; where, $I$ = $ABTS^{+}$ inhibition, %; $AB$ = absorbency of a blank sample ($t$ = 0 min); $AA$ = absorbency of a tested extract solution at the end of the reaction.

Standard curves using Trolox (0–0.25 mM) were run with each set of extracts. The reaction of the samples was followed until it reached the plateau. ABTS values were expressed as μmol Trolox equivalents/g FW.

The FRAP assay was performed according to Benzie and Strain (1999). FRAP reagent was prepared by mixing 2.5 ml TPTZ solution (10 mM in 40 mM HCl), 25 ml acetate buffer (300 mM, pH 3.6), and 2.5 ml of $FeCl_3.6H_2O$ solution (20 mM). After 4-min incubation at 37 °C, the reagent was used as a blank by determining the absorbance at 593 nm. FRAP solution (900 μL) was added to 90 μl of distilled water and 30 μl of standards or extracts. In addition to standard curves using trolox, ferrous sulphate standards (0–500 μM) were run with each set of extracts. The reaction of the samples was followed until it reached the plateau. Final results were expressed as μmol ferric iron reduced/100 g FW or as μmol TE/100 g FW. All measurements were replicated four times.

## 3. Statistical analysis

Quantitative data are presented as mean values with the respective standard deviation.

## 4. Results and discussion

### *4.1. Titratable acidity (TA), pH, Brix, and ascorbic acid*

The average pH of the fruit form *V. meridionale* was 2.85 ± 0.16 while the TA was 1.35 ± 0.20. The TA value is close to that one of *V. myrtillus* (1.52) found by Prior et al. (1998).

The TSS content was 7.00 ± 0.01. This value is in accordance to that one reported by Vasco, Riihinen, Ruales, and Kamal-Eldin (2009) for *Vaccinium floribundum* (7.00) from Ecuador and lower than the value for *V. myrtillus* (10.00) (Prior et al., 1998). The high TA of *V. meridionale* along with its low TSS seems to be the explanation for the low sugar/acid ratio (5.27 ± 0.70) of this fruit, which is lower than that one of *V. myrtillus* (6.60) reported by Prior et al. (1998).

The ascorbic acid content of *V. meridionale* was 8.10 ± 1.50 mg/100 g FW. This content is eight fold higher than the one reported by Prior et al. (1998) for *V. myrtillus* (1.30), but it is within the range of some *Vaccinium corymbosum* and *Vaccinium ashei* cultivars (6.20–14.60 mg/100 g FW) reported by the same author and close to the content in *V. floribundum* (9.00 mg/100 g FW) as reported by Vasco et al. (2009).

### *4.2. Total anthocyanin content and total phenolics*

The total monomeric anthocyanin content in Colombian bilberry was 329 ± 28 mg cyanidin 3-glucoside/100 g FW. This value is within the range (300–698 mg/100 g) reported for *V. myrtillus* native to parts of Europe and northern regions of Asia (Mazza & Miniati, 1993), comparable to the anthocyanin concentration (300 mg/100 g) found by Prior et al. (1998) for bilberry from northern United States and by Vasco et al. (2009) for *V. floribundum* form Ecuador (345 mg/100 g).

*V. meridionale* had a total phenolic content of 758.6 ± 62.3 mg GAE/100 g FW, which is lower than that one of *V. floribundum* from Ecuador (882 mg GAE/100 mg FW) (Vasco et al. 2009) and high compared to that one of other *Vaccinium* species. Prior et al. (1998) reported 525 mg GAE/100 g FW for North American *V. myrtillus* and a range between 190 and 473 mg GAE/100 g FW for several cultivars of *V. corymbosum*, *V. ashei* and *Vaccinium angustifolium*.

It has been reported that plant phenolics show qualitative and quantitative variation at different genetic levels (between and within species and clones) (Hakulinen, Julkunen-Titto, & Tahvanainen, 1995) and between different physiological and developmental stages (Kause et al., 1999). Phenolics also vary in response to environmental factors, such as light intensity and nutrient availability

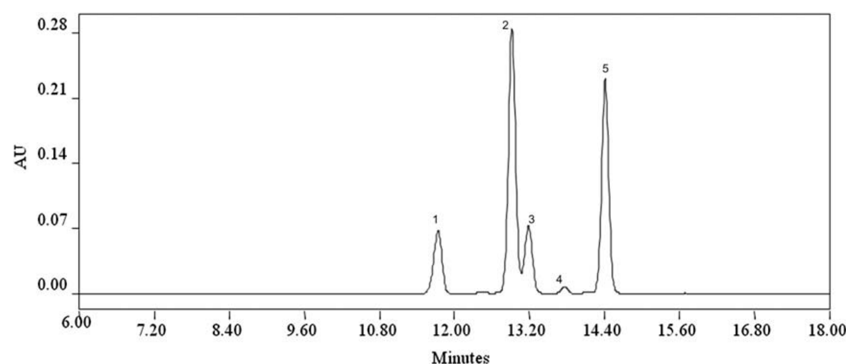



**Fig. 1.** HPLC profile of anthocyanins in Colombian bilberry (*V. meridionale*) detected at 512 nm. (1) Delphinidin 3-hexoside; (2) cyanidin 3-galactoside; (3) delphinidin 3-pentoside; (4) cyanidin 3-glucoside; (5) cyanidin 3-arabinoside.

(Herms & Mattson, 1992 and refs. therein). All these factors may contribute to the differences in anthocyanin and phenolic content between *V. meridionale* and other *Vaccinium* species previously studied.

*4.3. Identification and quantification of anthocyanidins, anthocyanins and non-anthocyanin phenolics*

Acid hydrolysis revealed the presence of two anthocyanidins, whose identity was confirmed by comparing retention times and UV/Vis data with Concord grape and strawberry anthocyanidins. Concord grape contains five of the six common anthocyanidins, while strawberry contains the remaining one, which is pelargonidin (Durst & Wrolstad, 2001). When comparing the HPLC profile and spectra of the aglycons from grape, strawberries and *V. meridionale*, there was match in retention times and $\lambda_{max}$ for cyanidin, and delphinidin. In addition, spiking of the berry extract with the Concord grape anthocyanidins resulted in coelution. Cyanidin represented 85% of the total peak area.

Fig. 1 and Table 1 show that five anthocyanins were found. Structural information was obtained from anthocyanin spectroscopic data. The $E_{440}/E\ \lambda_{max}$ calculated for each anthocyanin ranged from 29.7% to 31.6%. These values are typical of a glycosidic bond at position C-3 of the anthocyanidin (Hong & Wrolstad, 1990). The low absorbance observed at 310–320 nm for all peaks indicated that anthocyanins were not acylated with hydroxylated aromatic acids (Hong & Wrolstad, 1990). Peaks 1, 2, 3 and 5 represented 99% of the total area while peak 4 accounted for 1%. Cyanidin-based anthocyanins represented 77% of the total anthocyanin content, which is lower than the content found in *V. floribundum* from Ecuador (88% of the total anthocyanin content) (Vasco et al., 2009). A comparison of retention times and UV/Vis data with known standards revealed the presence of cyanidin 3-galactoside as the major anthocyanin representing 43% of the total peak area while cyanidin 3-glucoside and cyanidin 3-arabinoside were also found and the two remaining peaks were dephinidin-based anthocyanins according to UV/vis and MS/MS data.

Mass spectral data of the anthocyanins in Colombian bilberry (Table 1) showed that peak 1 revealed a molecular ion at *m/z* 465 and a major fragment at *m/z* 303, indicating it was a delphinidin derivative. The neutral loss of 162 mass units corresponded to one molecule of hexose. As a result, peak 1 was tentatively identified as delphinidin 3-hexoside.

Peaks 2 and 4 revealed a molecular ion at *m/z* 449 and a fragment ion at *m/z* 287 produced by the loss of a hexose unit. This, along with authentic standards confirms the HPLC–DAD identification of these peaks as cyanidin 3-galactoside and cyanidin 3-glucoside, respectively.

Peaks 3 and 5 produced molecular ions at *m/z* 435 and 419 and major fragments at *m/z* 303 and 287, which corresponds to the anthocyanidins delphinidin and cyanidin, respectively, after the loss of a pentoside. The fragmentation pattern, retention time and UV–Vis spectra of peak 5 matched that of the cyanidin 3-arabinoside standard. Accordingly, peak 3 was tentatively identified as a delphinidin 3-pentoside and peak 5 was labelled as cyanidin 3-arabinoside. The anthocyanin fragmentation pattern of molecular ions we found matched with those of previous studies in Andean blueberry (Vasco et al., 2009). However, these authors reported the presence of delphinidin and cyanidin aglycons, which could be present due to hydrolysis of the aglycon given the high acidic conditions they used for anthocyanin extraction.

In general, bilberry has been reported as a source of cyanidin, delphinidin, peonidin, petunidin and malvidin galactosides, with delphinidin and cyanidin glucosides and arabinosides as the most abundant anthocyanins (Määttä-Riihinen, Kamal-Eldin, Mattila, González-Paramás, and Törrönen, 2004; Lätti, Riihinen, & Kainulainen, 2008). In contrast, Colombian bilberry contained only cyanidin and delphinidin glycosides, which is in accordance to the anthocyanin composition found in Andean blueberry from Ecuador (Vasco et al., 2009).

Non-anthocyanin phenolics found in *V. meridionale* included hydroxycinnamic acids and flavonols (Table 2A). Six hydroxycinnamic acids were detected (Fig. 2) and quantified at 320 nm in equivalents of chlorogenic acid. All peaks presented a maximum

**Table 1**
Chromatographic and spectroscopic characteristics of anthocyanins detected in *V. meridionale* Swartz.

| Peak number | Peak area (%) HPLC–DAD | Max | Retention time (min) | Molecular ion $M^+$ *m/z* | Fragment ions in MS/MS *m/z* | Tentative peak assignment |
|---|---|---|---|---|---|---|
| 1 | 12 | 523 | 11.5 | 465 | 303 | Delphinidin 3-hexoside |
| 2 | 43 | 518 | 12.8 | 449 | 287 | Cyanidin 3-galactoside |
| 3 | 11 | 525 | 13.0 | 435 | 303 | Delphinidin 3-pentoside |
| 4 | 1 | 512 | 13.6 | 449 | 287 | Cyanidin 3-glucoside |
| 5 | 33 | 518 | 14.3 | 419 | 287 | Cyanidin 3-arabinoside |

absorption at 326–328 nm and 242 nm, and a shoulder at 290–300 nm, typical of hydroxycinnamic acids such as caffeic or ferulic (Sánchez-Rabaneda et al., 2003). Peaks 1, 2 and 3 represented 73.8% of the total nonanthocynin-phenolic peak area and were tentatively identified as members of the chlorogenic acid family (esters of trans-cinnamic acids and quinic acid). Peaks 1 and 2 had identical UV spectra and MS/MS fragmentation as the 3-caffeoylquinic acid standard, but peak 2 eluted 0.5 min later. HPLC-MS/MS in the negative mode revealed a molecular ion at *m/z* 353 for both peaks corresponding to a molecular formula $C_{16}H_{18}O_9$. The molecular ion was fragmented into four major fragments at *m/z* 191, 135, 161 and 179. Previous publications report that the fragment at *m/z* 191 corresponds to the loss of a hydrogen from quinic acid, fragment at *m/z* 179 corresponds to the $[\text{caffeoyl-H}^+]^-$ ion and the ion at *m/z* 135 corresponds to its decarboxylation product $[\text{caffeoyl-CO}_2\text{–H}^+]^-$ (Clifford, Johnston, Knight, & Kuhnert, 2003). The fragment at *m/z* 161 probably reveals the product of $[\text{caffeoyl-H}_2\text{O–H}^+]^-$. Accordingly, peaks 1 and 2 were identified as caffeoylquinic acid isomers.

Peak 3 represented 2.9% of the total non-anthocyanin-phenolic peak area. It produced a molecular ion at *m/z* 367 in accord with the $C_{17}H_{20}O_9$ and four major fragments at *m/z* 135, 179, 161, and

**Table 2**
Chromatographic and spectroscopic characteristics of nonanthocynin phenolics detected in *V. meridionale* Swartz.

| Peak number | Peak area (%) HPLC–DAD | λ Max | Retention time (min) | Molecular ion: $[M–H]^-$ *m/z* | Fragment ions in MS/MS *m/z* | Tentative peak assignment |
|---|---|---|---|---|---|---|
| 1 | 67.9 | 326, 242, 290 | 9.8 | 353 | 191, 135, 161, 179 | Caffeoylquinic acid isomer 1 |
| 2 | 3.0 | 326, 242, 290 | 10.3 | 353 | 191, 135, 161, 179 | Caffeoylquinic acid isomer 2 |
| 3 | 2.9 | 328, 242, 290 | 16.2 | 367 | 135, 179, 161, 191 | Caffeoyl methyl quinate |
| 4 | 5.6 | 328, 242, 290 | 17.4 | 433 | 161, 135, 179 | Caffeic acid derivative |
| 5 | 0.5 | 328, 242, 290 | 22.4 | 475 | 161, 135, 179 | Caffeic acid derivative isomer 1 |
| 6 | 1.9 | 328, 242, 290 | 23.1 | 475 | 161, 135, 179 | Caffeic acid derivative isomer 2 |
| 7 | 3.9 | 345, 255 | 18.4 | 463 | 300/301, 271, 255, 179, 151 | Quercetin hexoside |
| 8 | 0.7 | 345, 256 | 19.7 | 433 | 257, 300/301, 271 | Quercetin pentoside |
| 9 | 0.8 | 357, 256 | 20.1 | 433 | 300/301, 271, 255 | Quercetin pentoside |
| 10 | 2.2 | 353, 255 | 20.6 | 433 | 300/301, 271, 255 | Quercetin pentoside |
| 11 | 4.5 | 345, 255 | 21.0 | 447 | 300/301, 271, 255 | Quercetin rhamnoside |
| 12 | 6.1 | 349, 256 | 24.1 | 591 | 300/301, 447, 489, 529 | Quercetin hydroxylmethylglutaryl-α-rhamnoside |

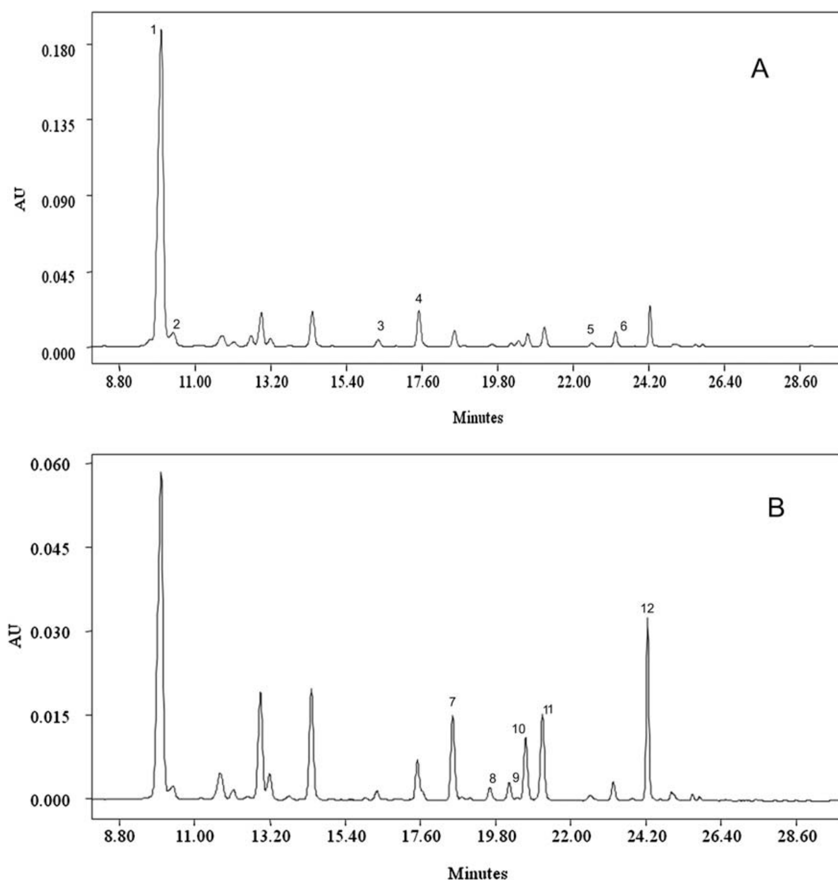



**Fig. 2.** HPLC profiles of non-anthocyanin phenolics in wild bilberry (*V. meridionale*) at 320 nm (A) and 355 nm (B). (1) Caffeoylquinic acid isomer 1; (2) caffeoylquinic acid isomer 2; (3) caffeoyl methyl quinate; (4) caffeic acid derivative; (5) caffeic acid derivative; (6) caffeic acid derivative; (7) quercetin hexoside; (8) quercetin pentoside; (9) quercetin pentoside; (10) quercetin pentoside; (11) quercetin rhamnoside; (12) quercetin hydroxylmethylglutaryl-α-rhamnoside. Unlabelled peaks are anthocyanins.

191. Shakya and Navarre (2006) found the same fragmentation pattern in potato phenolics. The parent mass corresponds to caffeoyl methyl quinate while the fragments at *m/z* 135, 179, and 191 are derivatives of caffeic acid as reported by Clifford et al. (2003).

Peaks 4, 5 and 6 were tentatively identified as caffeic acid derivatives since their molecular ions had the same UV–Vis spectra and MS/MS fragmentation as the caffeic acid standard (*m/z* 161, 135 and 179). However, while peak 4 yielded a molecular ion at *m/z* 433, peaks 5 and 6 gave a molecular ion at *m/z* 475, which suggests that these two compounds were isomers.

The total amount of hydroxycinnamic acids detected in *V. meridionale* was 99.2 ± 6.7 mg/100 g FW (Table 3), which is comparable to the amount of this compounds European bilberries (113–231 mg/100 g) (Kähkönen, Hopla, & Heinonen, 2001) and higher than the amount in Andean blueberry form Ecuador (33.7 mg/100 g FW) (Vasco et al., 2009). Chlorogenic acid accounted for 86.1 mg of the total hydroxycinnamic acids. This amount was five fold the amount of that of *V. floribundum* (17 mg/100 g FW) as reported by the same authors.

There were six major flavonols identified by LC-DAD (Fig. 2B), LC-MS and LC-MS/MS in the negative mode. All peaks and the standards quercetin 3-rhamnoglucoside or rutin generated the aglycone quercetin as fragment with *m/z* 300 (homolytic cleavage) and *m/z* 301 (heterolytic cleavage; Hokkanen, Mattila, Jaakola, Pirttila, & Tolonen, 2009).

Peaks 7, 8, 11, and 12 presented their maximum absorbance at 345–349 nm, characteristic of quercetin glycosides (Määttä, Kamal-Eldin, & Riitta-Törrönen, 2003) while peaks 9 and 10 exhibited a $\lambda_{max}$ in the 353–357 nm region. A shift to lower wavelengths is indication of additional methylation, glycosylation, or acylation of hydroxyl groups for quercetin derivatives (Markham, 1982).

Peak 7 revealed a molecular ion at *m/z* 463 corresponding to the $C_{21}H_{20}O_{12}$ formula of quercetin hexoside. Fragmentation of the molecular ion provided six fragment ions at *m/z* 300, 301, 271, 255, 179, and 151, which matched the fragmentation pattern of the rutin standard. The ion at *m/z* 301 is typical of the quercetin aglycon after a neutral loss of 162 amu corresponding to a hexose.

Peaks 8, 9 and 10 yielded a molecular ion at *m/z* 433 in accordance with the formula weight of quercetin-pentosides. Molecular ion from peak 8 fragmented into three ions at *m/z* 301, corresponding to the loss of 132 amu from a pentosyl residue, *m/z* 257 and *m/z* 271. Peaks 9 and 10 were considered isomers as they revealed identical UV spectra and fragment ions at *m/z* 301, 271 and 255.

**Table 3**
Content of non-anthocyanin phenolics in *V. meridionale* Swartz[a].

| Phenolic compounds | Content (mg/100 g FW) |
|---|---|
| Hydroxicinnamic acids | Chlorogenic acid equivalents |
| Chlorogenic isomer 1 | 82.4 ± 3.2 |
| Chlorogenic isomer 2 | 3.7 ± 0.5 |
| Caffeoyl methyl quinate | 3.4 ± 2.4 |
| Caffeic acid derivative | 6.7 ± 1.1 |
| Caffeic acid derivative isomer 1 | 0.7 ± 0.1 |
| Caffeic acid derivative isomer 2 | 2.3 ± 0.4 |
| Total | 99.2 ± 6.7 |
| Flavonols | Rutin equivalents |
| Quercetin hexoside | 9.0 ± 0.2 |
| Quercetin pentoside | 1.5 ± 0.3 |
| Quercetin pentoside | 1.5 ± 0.3 |
| Quercetin pentoside | 1.9 ± 0.1 |
| Quercetin pentoside | 5.2 ± 1.1 |
| Quercetin rhamnoside | 10.0 ± 2.6 |
| Quercetin hydroxylmethylglutaryl-α-rhamnoside | 14.0 ± 2.7 |
| Total | 41.9 ± 4.9 |
| Total nonanthocynin phenolics | 141.2 ± 11.9 |

[a] Results are expressed as mean ± SD (*n* = 3).

Peaks 11 and 12 were tentatively identified as quercetin rhamnoside and quercetin hydroxylmethylglutaryl-α-rhamnoside, respectively. This identification was done based on comparison to the identical fragmentation spectra for *Vaccinium vitis-idaea* L. as reported Hokkanen et al. (2009). Peak 11 revealed a molecular ion at *m/z* 447 and MS/MS ions *m/z* 301 due to the neutral loss of 146 amu, which is indicative of a deoxyhexosyl residue (such as rhamnose), as well as product ions of *m/z* 271 and 255. Peak 12 presented a molecular ion at *m/z* 591 and fragment ions at *m/z* 300/301, 447, 489, and 529.

Quercetin glycosides represented 100% of the total flavonoids in *V. meridionale* (41.9 ± 4.9 mg/100 g FW). Bilberries from Finland contain a lower amount of flavonoids (11.2 mg flavonoids/100 g FW) and only 72% or 8.1 mg/100 g of this total is represented by quercetin. The remaining amount is represented by myricetin (Määttä-Riihinen et al., 2004). Similarly, *V. floribundum* from Ecuador contains lower amount of flavonoids as compared to *V. meridionale*. In this case, 93% of total flavonoids (37.5 mg/100 g FW) are represented by quercetin and 7% by myricetin.

In general, the *Vaccinium* family is characteristic for the presence of quercetin glycosides and hydroxycinnamic acids. *V. myrtillus* specifically contains caffeic/ferulic acids, hexoses, deoxy hexoses and pentoses of quercetin as the most abundant flavonols, along with myricetin glycosides (Määttä-Riihinen et al., 2004).

The variation in the content and composition of phenolics within the *Vaccinium* varieties used as comparison may be dependent on the growth environment and genotype (Lätti et al., 2008). It has been reported that quercetin, cyanidin 3-glucosides and hydroxycinnamic acids are metabolized as a defense response against intense solar radiation (Jaakola, Määttä-Riihinen, Karenlampi, & Hohtola, 2004), which is common in tropical locations like Colombia. This may explain the difference in phenolic content and composition profile between bilberry from northern latitudes versus Colombian bilberry.

### 4.4. Antioxidant activity

Colombian bilberries showed high antioxidant activity as indicated by the ABTS and FRAP assays. The ABTS radical scavenging activity was 45.5 ± 2.3 μmol TE/g FW. This radical scavenging activity is close to that of *V. floribundum* from Ecuador (47.9 μmol TE/g FW) (Vasco et al., 2009) and comparable to that of blueberries (45.9 μmol TE/g), which constitutes one of the richest reported antioxidants (Kaur & Kapoor, 2001). Our value is also far above the range value for Rubus species (0–25.3 mmol TE/100 g FW) recommended for the improvement of nutritional value due to their high antioxidant activities (Deighton, Brennan, Finn, & Davies, 2000).

The contribution of ascorbic acid to the total antioxidant capacity of complex mixtures of antioxidants can be calculated from the ABTS assay (Deighton et al., 2000). Ascorbic acid has a molar activity equal to that of Trolox; thus, since the ascorbic acid content of Colombian bilberry corresponds to a concentration of 0.94 μmol Trolox/g FW, we can consider that it represents only 2.1% of the total observed TEAC (45.5 μmol TEAC/g).

Although the anthocyanin and non-anthocyanin phenolic contents and composition *V. meridionale* was somewhat different from that of *V. myrtillus* from the northern United States, *V. meridionale* provided similar iron reducing capacity. The FRAP value was 87.0 ± 17.8 μmol TE/g FW or 116.0 ± 23.7 μmol ferric iron reduced/g FW. Moyer et al. (2002) found 94.9 μmol ferric iron reduced/g FW for *V. myrtillus* harvested in the Pacific Northwest United States.

The high concentration and chemical structure of phenolic compounds in *V. meridionale* might be the explanation for its high antioxidant activity. It has been shown that monoglucosides of cyanidin and delphinidin as well as chlorogenic acid and quercetin posses high antioxidant activity and radical scavenging activity and that they compare well with the activity of the well-known antioxidants α-tocopherol and Trolox (Kähkönen & Heinonen, 2003).

The ABTS method measures the ability of the antioxidant to quench $ABTS^{\bullet+}$ radicals (probably by an electron transfer reaction) while the FRAP assay measures the potential of an antioxidant to reduce the yellow ferric-TPTZ complex to a blue ferrous-TPTZ complex by electron-donating substances under acidic conditions. According to our results, Colombian bilberry is a good electron donor as its extract was able to quench $ABTS^{\bullet+}$ radicals and reduce the ferric complex to a ferrous complex. The high ABTS and FRAP values of *V. meridionale* extract along with its low content of ascorbic acid, suggest that phenolic compounds are the main contributors to the high antioxidant activity of this fruit.

In conclusion, this study shows for the first time that the berries of *V. meridionale* can be differentiated from other bilberries by their unique anthocyanin pattern e.g., by the high proportions of both delphinidin and cyanidin. This information may be useful in the proper identification and authentication of products derived from this fruit. In addition, *V. meridionale* berries are an excellent source of dietary phytochemicals such as anthocyanins and polyphenolics, being comparable to *V. myrtillus*. The use of *V. meridionale* as source of natural antioxidants, natural colourants, and an ingredient of functional foods seems to be promising.

## References


Bao, L., Yao, X.-S., Yau, C.-C., Tsi, D., Chia, C.-S., Nagai, H., et al. (2008). Protective effects of bilberry (*Vaccinium myrtillus* L.) extract on restraint stress-induced liver damage in mice. *Journal of Agricultural and Food Chemistry, 56*, 7803–7807.

Benzie, I. F. F., & Strain, J. J. (1999). Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods of Enzymology, 299*, 15–27.

Canter, P. H., & Ernst, E. (2004). Anthocyanosides of *Vacinnium myrtillus* (bilberry) for night visions a systematic review of placebo controlled trials. *Survey of Ophthalmology, 49*, 38–50.

Clifford, M. N., Johnston, K. L., Knight, S., & Kuhnert, N. (2003). Hierarchical scheme for LC-$MS^n$ identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry, 51*, 2900–2911.

Deighton, N., Brennan, R., Finn, C., & Davies, H. V. (2000). Antioxidant properties of domesticated and wild Rubus species. *Journal of the Science of Food and Chemistry, 80*, 1307–1313.

Durst, R., & Wrolstad, R. E. (2001). Separation and characterization of anthocyanins by HPLC. In *Handbook of food analytical chemistry* (pp. 33–45). New Jersey: John Wiley and Sons.

Giusti, M. M., & Wrolstad, R. E. (2001). Characterization and measurement of anthocyanins by UV–visible spectroscopy. In *Handbook of food analytical chemistry* (pp. 19–31). New Jersey: John Wiley and Sons.

Hakulinen, J., Julkunen-Titto, R., & Tahvanainen, J. (1995). Does nitrogen fertilization have an impact on the trade-off between willow growth and defensive secondary metabolism? *Trees – Structure and Function, 9*, 235–240.

Herms, D. A., & Mattson, W. J. (1992). The dilemma of plants: To grow or defend. *Quarterly Review of Biology, 67*, 283–335.

Hokkanen, J., Mattila, S., Jaakola, L. J., Pirttila, A. M., & Tolonen, A. (2009). Identification of phenolic compounds from lingonberry (*Vaccinium vitis-idaea* L.), bilberry (*Vaccinium myrtillus* L.) and hybrid bilberry (*Vaccinium* × *intermedium Ruthe* L.) leaves. *Journal of Agricultural and Food Chemistry, 57*, 9437–9447.

Hong, V., & Wrolstad, R. E. (1990). Characterization of anthocyanin-containing colorants and fruit juices by HPLC/photodiode array detection. *Journal of Agricultural and Food Chemistry, 38*, 698–708.

Jaakola, L., Määttä-Riihinen, K. S., Karenlampi, S., & Hohtola, A. (2004). Activation of flavonoid biosynthesis by solar radiation in bilberry (*Vaccinium myrtillus* L.) leaves. *Planta, 218*, 721–728.

Kähkönen, M. P., & Heinonen, M. (2003). Antioxidant activity of anthocyanins and their aglycons. *Journal of Agricultural and Food Chemistry, 51*, 628–633.

Kähkönen, M. P., Hopla, A. I., & Heinonen, M. (2001). Berry phenolics and their antioxidant activity. *Journal of Agricultural and Food Chemistry, 49*, 4076–4082.

Kaur, C. K., & Kapoor, H. C. (2001). Antioxidants in fruits and vegetables-the millennium's health. *International Journal of Food Science and Technology, 36*, 703–725.

Kause, A., Ossipov, V., Haukioja, E., Lempa, K., Hanhimäki, S., & Ossipova, S. (1999). Multiplicity of biochemical factors determining quality of growing leaves. *Oecologia, 120*, 102–112.

Lätti, A. K., Riihinen, K. R., & Kainulainen, P. S. (2008). Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland. *Journal of Agricultural and Food Chemistry, 56*, 190–196.

Määttä, K. R., Kamal-Eldin, A., & Riitta-Törrönen, A. (2003). High performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: Ribes species. *Journal of Agricultural and Food Chemistry, 51*, 6736–6744.

Määttä-Riihinen, K. R., Kamal-Eldin, A., Mattila, P. H., González-Paramás, A. M., & Törrönen, R. (2004). Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *Journal of Agricultural and Food Chemistry, 52*, 4477–4486.

Markham, K. R. (1982). *Techniques of flavonoid identification*. New York: Academic Press. pp. 36–39.

Martín-Aragón, S., Basabe, B., Benedi, J. M., & Villar, A. M. (1998). Antioxidant action of *Vaccinium myrtillus* L. *Phytotherapy Research, 12*, S104–S106.

Mazza, G., & Miniati, E. (1993). *Anthocyanins in fruits, vegetables and grains*. Boca Raton, FL: CRC Press, Inc.

Morazzoni, P., & Bombardelli, E. (1996). *Vaccinium myrtillus* L. *Fitoterapia, 67*, 3–29.

Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, Rubus and Ribes. *Journal of Agricultural and Food Chemistry, 50*, 519–525.

Prior, R. L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., et al. (1998). Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity and variety of *Vaccinium* species. *Journal of Agricultural and Food Chemistry, 46*, 2686–2693.

Re, R., Pellegrinni, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical in Biology and Medicine, 26*, 1231–1237.

Sánchez-Rabaneda, F., Jáuregui, O., Casals, I., Andrés-Lacueva, C., Izquierdo-Pulido, M., & Lamuela-Raventós, R. M. (2003). Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *Journal of Mass Spectrometry, 38*, 35–42.

Shakya, R., & Navarre, D. A. (2006). Rapid screening of ascorbic acid, glycoalkaloids, and phenolics in potato using high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry, 54*, 5253–5260.

Valentová, K., Ulrichová, J., Cvak, L., & Šimánek, V. (2007). Cytoprotective effect of a bilberry extract against oxidative damage of rat hepatocytes. *Food Chemistry, 101*, 912–917.

Vasco, C., Riihinen, K., Ruales, J., & Kamal-Eldin, A. (2009). Chemical composition and phenolic compound profile of mortiño (*Vaccinium floribundum* Kunth). *Journal of Agricultural and Food Chemistry, 57*, 8274–8281.

Waterhouse, L. (2001). Determination of total phenolics. In *Handbook of food analytical chemistry* (pp. 463–470). New Jersey: John Wiley and Sons.

Zhao, C., Giusti, M. M., Malik, M., Moyer, M. P., & Magnuson, B. A. (2004). Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. *Journal of Agricultural and Food Chemistry, 52*, 6122–6128.