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Phenolic content and antioxidant capacity of tropical highland blackberry (*Rubus adenotrichus* Schltdl.) during three edible maturity stages

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ABSTRACT

Tropical highland blackberry (*Rubus adenotrichus* Schltdl.) is a good source of antioxidants and contains appreciable levels of phenolic compounds, mainly ellagitannins and anthocyanins. This study examined the influence of three ripening stages on phenolic contents. Major anthocyanin pigments increased from 0.20 (red fruit) to 1.34 mg g^{-1} fresh weight (FW) (fully ripe fruit), whereas ellagitannins and ellagic acid derivatives dropped from 3.8 to 2.2 mg ellagic acid equivalents g^{-1} (FW). Flavonols also dropped from 5.1 to 2.0 mg quercetin equivalents 100 g^{-1} (FW). Consequently, values for total phenolic compounds ranged from 5.8 to 5.2 mg gallic acid equivalents g^{-1} (FW), showing no specific trend. Antioxidant activity (H-ORAC) increased from 38.29 to 64.00 μmol of Trolox equivalents g^{-1} (FW) during ripening. When compared with other commercial cultivars, *R. adenotrichus* stands out for high H-ORAC value, although comparatively it possesses low anthocyanin content and average total phenolic content.

1. Introduction

Epidemiological studies show that diets rich in plant foods protect humans against degenerative diseases such as cancer and cardiovascular disease. Besides providing fibre, vitamins, phytosterols, sulphur compounds, carotenoids, and organic acids, plant foods contain a variety of phenolic compounds, which are increasingly being regarded as effective protective agents (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). Blackberries are also a good source of antioxidants, containing appreciable levels of phenolic compounds (Benvenuti, Pellati, Melegari, & Bertelli, 2004; Cho, Howard, Prior, & Clark, 2004; González, de Ancos, & Cano, 2003; Jiao, Liu, & Wang, 2005; Moyer, Hummer, Finn, Frei, & Wrolstad, 2002; Reyes-Carmona, Yousef, Martínez-Peniche, & Lila, 2005; Sellappan, Akoh, & Krewer, 2002; Siriwoharn & Wrolstad, 2004; Siriwoharn, Wrolstad, Finn, & Pereira, 2004; Wang & Lin, 2000).

Even so, most research has focused on commercial varieties grown in temperate climates, with little attention given to wild varieties grown extensively in tropical highlands. For example, in 2005, the production of wild blackberries, mainly *Rubus adenotrichus* and *Rubus glaucus* in Mexico, Central and South America, was estimated as being 38,531 ton. This corresponds to almost 25% of world blackberry production (Strik, Clark, Finn, & Bañados, 2007). These varieties are characterised by higher acidity and a distinctive flavour, which are used mainly by juice industries for blends.

Additionally, in a tropical climate stressful environmental changes such as drought during dry season, high relative humidity during rainy season, high irradiation levels, extreme temperatures, and attack by insects and pathogens, could enhance antioxidant production as plants use them to detoxify free radicals (Atkinson, Nestby, Ford, & Dodds, 2005). Recently, various phenolic compounds were identified and quantified in two tropical highland blackberries (*R. glaucus* and *R. adenotrichus*). The main classes were ellagitannins and anthocyanins (Mertz, Cheynier, Günata, & Brat, 2007). Ellagitannins and its derivative, ellagic acid have recently been receiving much attention because of their health benefits (Bakkalbaşi, Menteş, & Artik, 2009; Larrosa, Tomás-Barberán, & Espín, 2006; Losso, Bansode, Trappey, Bawadi, & Truax, 2004). They belong to the hydrolysable tannin class of phenolics and are complex derivatives of ellagic acid. They contain one or more hexahydroxydiphenic acid moieties, esterified to a polyol, most often to β-D-glucose. The occurrence of ellagitannins and its derivatives in dietary foodstuffs is rather uncommon. It occurs only in a few berry, fruit and nut species such as raspberry, blackberry,

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cloudberry, arctic bramble, strawberry, pomegranate, walnuts, and certain other nuts (Koponen, Happonen, Mattila, & Törrönen, 2007).

Anthocyanins (glycosides and acylglycosides of anthocyanidins) are important in the food industry, being regarded as potential replacements for synthetic food colourants, and in human nutrition, as protecting agent against some diseases (Wu & Prior, 2005). Previous studies have reported the influence of ripeness on total anthocyanins, phenolics, and antioxidant properties in blackberry fruits grown in temperate climates (Siriwoharn et al., 2004; Wang & Lin, 2000). Results showed that during ripening, total anthocyanin pigments and antioxidant capacity tend to increase. In contrast, total phenolics is claimed to not display a specific trend. A closer analysis of contents of specific phenolic compounds present during ripening has not been yet performed. This study aimed to evaluate the effect of ripening on major phenolic compounds and antioxidant capacity in tropical highland blackberry (*R. adenotrichus*).

2. Materials and methods

2.1. Collecting samples

Tropical highland blackberries (*R. adenotrichus*) were harvested from a 3-year-old plantation located in Santa María de Dota, San José, Costa Rica (2200 m above sea level). The berries were hand-picked from three separate plots in the same plantation. The plots were arranged in a randomised complete block design with about 30 plants per plot. Three composite samples (one from each plot) of about 1.5 kg of fruit were picked for each stage of ripeness, based on fruit surface colour: light red (grade 1), purple (grade 2), and dark bluish purple (grade 3).

Fruits were checked for apparent physical and microbiological damage at the plantation site. Fruits from composite samples were homogenised and some physicochemical analyses were performed (size, compressibility, colour, titratable acidity, pH, soluble solids, and moisture). The fruits were then frozen by immersion in liquid nitrogen, freeze-dried, ground, and finally stored at −20 °C in laminated bags until further analysis.

2.2. Chemicals

The following chemicals were used: fluorescein, gallic acid and total dietary fibre kit TDF-100A (heat-stable α-amylase, amyloglucosidase and protease) from Sigma Chemical Company (St. Louis, MO, USA); 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) from Wako Chemicals USA, Inc. (Richmond, VA, USA); Trolox (6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid) from Aldrich (Milwaukee, WI, USA); Folin–Ciocalteu's reagent and SigmaUltra standards for glucose, fructose and sucrose from Sigma–Aldrich (St. Louis, MO, USA); Kjeltabs Cu/3.5 from FOSS Analytical (Höganäs, Sweden); cyanidin-3-*O*-glucoside from Extrasynthèse (Genay, France); quercetin and ellagic acid from Sigma (l'Isle d'Abeau Chesnes, France). Other chemicals used were of analytical grade, and solvents of HPLC grade. These inputs were purchased from JT Baker Inc. (Phillipsburg, NJ, USA) or Carlo Erba France (Val-de-Reuil, France).

2.3. Physicochemical analyses

The weight and size (length and width) of the blackberries were determined by measuring a number of individual fruits from each composite sample. Weight was determined using a digital balance model GX-2000 (A&D Weighing, Milpitas, CA, USA), and size by measuring length and width with a vernier-type caliper (General Tools & Instruments Co., LLC, New York). Compressibility and colour were determined by measuring 30 individual fruits from each composite sample. Compressibility was determined by quantifying the necessary force to deform each fruit by 5 mm with a mount compression plate of 1.5 cm diameter, using a Chatillon pressure tester model LTC (John Chatillon & Sons, Inc., Kew Gardens, NY, USA).

Colour was measured with a Konica Minolta CR-300 Chroma Meter (Ramsey, NJ, USA), (2° standard observer angle and illuminant C). Colour was expressed as L^*, hue ($H^* = \arctan(b^*/a^*)$), and chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$). Total titratable acidity was determined, using standard AOAC method 942.15 (AOAC, 1999). Total soluble solids (expressed as °Brix) were measured on a digital refractometer with automatic temperature compensation (model "Palette" PR-100, Atago Co., Ltd., Tokyo, Japan).

Moisture, ash, protein and total dietary fibre contents were determined by using standard AOAC methods 920.151, 940.26, 920.152, and 985.29 respectively (AOAC, 1999). Total sugars (sucrose, glucose and fructose) were determined by HPLC, using a Shimadzu LC-6A (Kyoto, Japan) equipped with an Alltech Econosphere™ NH_2 column (Applied Science Labs, Deerfield, IL, USA), and with a Shimadzu RID-6A refractive index detector (Kyoto, Japan), using the conditions described previously (Acosta, Víquez, Cubero, & Morales, 2006). Antioxidant capacity was measured in terms of H-ORAC, following the method described previously (Ou, Hampsch-Woodill, & Prior, 2001).

Total anthocyanin content was determined by using a pH-differential method and results expressed as cyanidin-3-glucoside (Cy-3-glc) equivalents (Giusti & Wrolstad, 2001). Total phenolic compounds were assessed by using a modified Folin–Ciocalteu assay (Georgé, Brat, Alter, & Amiot, 2005), and results expressed as gallic acid (GA) equivalents.

2.4. HPLC analyses of phenolic compounds

Extraction procedures and analysis conditions were described previously in detail (Mertz et al., 2007). Briefly, lyophilised fruits were ground and two extractions for phenolic compounds made, each for 15 min, using 70% aqueous acetone with 2% formic acid. Once concentrated, samples were directly analysed by HPLC for ellagitannins and anthocyanins. Further extractions for other phenolics such as flavonols and ellagic acid derivatives were made with ethyl acetate solvent to yield ethyl acetate and aqueous extracts. The ethyl acetate extract was washed with water and directly analysed by HPLC. The HPLC quantitative analysis was carried out with a Dionex liquid chromatograph system that was equipped with P680 pumps, an ASI-100 autosampler and a UVD 340U photodiode array detector (Dionex Corporation, Sunnyvale, CA, USA) coupled to an HP ChemStation (Palo Alto, CA, USA), using an endcapped reversed-phase Lichrospher ODS-2 column (250 mm × 4.6 mm i.d., 5 μm) (Interchim, Montluçon, France).

Tentative identification of phenolic compounds had already been performed (Mertz et al., 2007) using HPLC–DAD–MS^n analysis. Quantification of both acetone and ethyl acetate extracts was achieved at 280 nm, using calibration curves established with authentic standards of ellagic acid (EA), cyanidin-3-glucoside (Cy-3-glc) and quercetin for, respectively, ellagitannins or ellagic acid derivatives, anthocyanins and flavonols. Correlation coefficients ranged from 0.994 to 0.999. All analyses were made in triplicate.

2.5. Statistical analysis

Values were given as means ± standard deviations. Statistical analysis was performed with JMP® v. 5.1 statistical software (SAS Institute, Inc., Cary, NC, USA). Analysis of variance was used to

determine significant differences ($P < 0.05$) between ripening stages. Means were further compared, using Tukey's test, and differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Influence of ripening on nutritional composition

Three different and relatively late developmental stages were chosen when the fruit is edible but present different colours. Indeed, fruits at these stages are generally picked and mixed for marketing. Fruits were first classified in three lots according to a homogeneous surface colour that ranges from light red (grade 1), through purple (grade 2) to dark bluish purple (grade 3). They were then characterised, using simple physicochemical analyses (Table 1). As for other blackberries (Perkins-Veazie, Clark, Huber, & Baldwin, 2000; Perkins-Veazie, Collins, & Clark, 1996), compressibility, titratable acidity, dietary fibre and protein content decreased significantly ($P < 0.05$) during ripening. In contrast, weight, size, moisture, total soluble solids and sugars increased significantly ($P < 0.05$). The maturity index (°Brix/acidity) also appeared to be a good indicator of fruit maturity as it increased during ripening as the fruits became darker, less red and bluer. Tropical highland blackberries such as *R. adenotrichus* exhibit noticeably higher acidity and much lower soluble solids content than do blackberry cultivars grown in temperate climates.

Table 1
Physical and chemical properties of blackberries at three maturity stages.[a]

Parameter	Maturity stage		
	Grade 1	Grade 2	Grade 3
Weight (g)[b]	1.8 ± 0.4 c	2.1 ± 0.6 b	2.7 ± 0.8 a
Length (cm)[b]	1.8 ± 0.2 b	1.8 ± 0.2 b	2.0 ± 0.3 a
Width (cm)[b]	1.3 ± 0.1 c	1.4 ± 0.1 b	1.5 ± 0.1 a
Compressibility (N)[c]	54 ± 9 a	34 ± 8 b	8 ± 5 c
Moisture (mg g^{-1} fruit FW)[d]	828 ± 5 c	841 ± 4 b	851 ± 3 a
Ash (mg g^{-1} fruit DW)[d]	35 ± 1 a	34 ± 3 a	31 ± 2 a
Protein (mg g^{-1} fruit DW)[d]	70 ± 2 a	63 ± 1 b	59 ± 3 b
Total dietary fibre (mg g^{-1} fruit DW)[d]	550 ± 46 a	521 ± 32 ab	432 ± 22 b
Total sugars (mg g^{-1} fruit DW)[d]	55 ± 5 c	131 ± 5 b	221 ± 15 a
Sucrose (mg g^{-1} fruit DW)[d]	ND[f]	ND	ND
Glucose (mg g^{-1} fruit DW)[d]	29 ± 2 b	92 ± 29 a	117 ± 7 a
Fructose (mg g^{-1} fruit DW)[d]	25 ± 2 b	81 ± 26 a	104 ± 8 a
L^*[c]	33 ± 3 a	21 ± 3 b	15 ± 1 c
H^*[c]	39 ± 3 a	21 ± 6 b	5 ± 3 c
C^*[c]	40 ± 3 a	25 ± 4 b	37 ± 1 c
pH[d]	2.26 ± 0.03 b	2.32 ± 0.01 b	2.51 ± 0.07 a
Total titratable acidity (mg malic acid equivalents g^{-1} fruit FW)[e]	28.5 ± 0.9 a	29.5 ± 0.6 a	24.0 ± 2.0 b
Total soluble solids (g 100 g^{-1} fruit FW)[e]	5.03 ± 0.05 c	6.40 ± 0.20 b	7.70 ± 0.60 a
Maturity index (total soluble solids/total titratable acidity)[e]	0.177 ± 0.007 b	0.220 ± 0.010 b	0.330 ± 0.060 a

[a] Mean ± standard deviation. Values within a row with similar letters are not significantly different (Tukey, $P > 0.05$).
[b] $n = 60$.
[c] $n = 30$.
[d] $n = 3$.
[e] $n = 6$.
[f] ND, not detected ($\leqslant 0.5$ mg g^{-1}).

3.2. Influence of ripening on phenolic compounds and antioxidant properties

On LC–DAD chromatograms of aqueous acetone extract of blackberry at the three stages of ripening, four peaks were identified using LC–MS and UV data published in a previous study (Mertz et al., 2007). They were assumed to be, the ellagitannins lambertianin C and sanguiin H-6, and the anthocyanins cyanidin-3-glucoside and cyanidin 3-(6′-malonyl) glucoside.

Also, minor phenolic compounds extracted with additional ethyl acetate fractionation (Mertz et al., 2007) were also identified and quantified generally as ellagic acid derivatives (acylated and/or glycosylated ellagic acid moieties) and flavonol glycosides. Contents of the different phenolic compounds found in the tropical highland blackberry are shown in Table 2.

Ellagitannins were the main phenolics, accounting for more than 92% (w/w) of phenolics in red fruit (grade 1) to almost 61% (w/w) in fully ripe fruit (grade 3) (Table 2). This finding agrees with those from previous studies (Kähkönen, Hopia, & Heinonen, 2001; Määttä-Riihinen, Kamal-Eldin, & Törrönen, 2004). Contents of the two main ellagitannins—lambertianin C and sanguiin H-6—ranged between 22 mg g^{-1} dry weight (DW) (378.4 mg 100 g^{-1} FW) for red fruits (grade 1) and 14.6 mg g^{-1} DW (217.5 mg 100 g^{-1} FW) for fully ripe fruits (grade 3). This range was higher than measurements reported previously for *R. adenotrichus* (Mertz et al., 2007), probably because analysis may have been performed on overripe fruits as a result of transport conditions.

The presence of lambertianin C and sanguiin H-6 in blackberries grown in USA was recently confirmed, although their respective concentrations have not been mentioned (Hager, Howard, Liyanage, Lay, & Prior, 2008). Ellagitannin contents in tropical highland blackberry are surprisingly high when compared with other blackberry cultivars such as "Marion" and "Evergreen" grown in temperate climates. In these cultivars, total ellagitannin content (expressed as ellagic acid) ranged only between 17.6 and 34.8 mg 100 g^{-1} FW (Siriwoharn et al., 2004). Although contents of ellagitannins decreased steadily during ripening, fully ripe fruits (grade 3) of *R. adenotrichus* appeared comparable only with some wild red raspberry cultivars (Kähkönen et al., 2001; Mullen, Yoko-

Table 2
Contents of main phenolic compounds of tropical highland blackberries at three maturity stages.[a]

Phenolic compounds	Maturity stage		
	Grade 1	Grade 2	Grade 3
Ellagitannins (mg EA equivalents g^{-1})			
Lambertianin C	12.0 ± 1.0 a	11.0 ± 1.0 a	8.0 ± 0.4 b
Sanguiin H-6	10.0 ± 0.5 a	9.0 ± 0.4 b	6.6 ± 0.3 c
Anthocyanins (mg Cy-3-glc equivalents g^{-1})			
Cyanidin 3-glucoside	1.03 ± 0.03 c	3.10 ± 0.20 b	8.30 ± 0.30 a
Cyanidin 3-(6′malonyl) glucoside	0.111 ± 0.002 c	0.391 ± 0.005 b	0.660 ± 0.040 a
Flavonols (mg quercetin equivalents g^{-1})	0.298 ± 0.002 a	0.231 ± 0.004 b	0.137 ± 0.002 c
Ellagic acid derivatives (mg EA equivalents g^{-1})	0.300 ± 0.003 a	0.216 ± 0.004 b	0.202 ± 0.004 c
Total (μmol g^{-1})[b]	77.0 ± 5.0 a	74.0 ± 2.0 ab	69.5 ± 0.3 b

[a] Mean ± standard deviation ($n = 3$). Values within a row with similar letters are not significantly different (Tukey, $P > 0.05$). Values are given on a dry matter basis for the weight of the standard.
[b] Sum of μmol of ellagic acid, quercetin and cyanidin-3-glucoside equivalents.

Table 3
H-ORAC values, total anthocynain and phenolic compounds of tropical highland blackberries at three maturity stages.[a]

Grade	Total anthocyanins (mg Cy-3-glc equivalents g^{-1})		Total phenolics (mg GA equivalents g^{-1})		H-ORAC (μmol TE g^{-1})	
	Fruit FW basis	Fruit DW basis	Fruit FW basis	Fruit DW basis	Fruit FW basis	Fruit DW basis
1	0.09 ± 0.02 c	0.5 ± 0.1 c	5.8 ± 0.2 a	33.7 ± 0.5 ab	38.29 ± 0.05 b	222 ± 6 b
2	0.28 ± 0.02 b	1.8 ± 0.1 b	4.6 ± 0.6 b	29.0 ± 3.0 b	43.00 ± 3.00 b	269 ± 20 b
3	0.77 ± 0.11 a	5.2 ± 0.6 a	5.2 ± 0.2 ab	35.0 ± 1.0 a	64.00 ± 4.00 a	432 ± 30 a

[a] Mean ± standard deviation ($n = 3$). Values within a column with similar letters are not significantly different (Tukey, $P > 0.05$).

ta, Lean, & Crozier, 2003; Mullen et al., 2002) and the Andean blackberry (*R. glaucus*). The latter shows a content of about 354 ± 60 mg ellagic acid equivalents 100 g^{-1} FW (Vasco, Riihinen, Ruales, & Kamal-Eldin, 2009). *R. adenotrichus* contains more ellagitannins than the pomegranate, even though this fruit is commercially known for this particular property (Bakkalbaşi et al., 2009).

Currently, ellagitannins receive increasing attention because of their supposed health benefits (Bakkalbaşi et al., 2009). Such high contents of this compound in a commercial blackberry grown on a large scale would be of considerable interest to the fruit-juice market. Additionally, red *R. adenotrichus* fruits present the highest amount of ellagitannins ever reported in any edible fruit at almost 3.8 g ellagic acid equivalents kg^{-1} of fresh fruits (grade 1). During the last stage of ripening, the two main ellagitannins (lambertianin C and sanguiin H-6), and ellagic acid derivatives present similar trends, each decreasing by about 33% from red fruits (grade 1) to fully ripe fruits (grade 3).

The second group of phenolic compounds present in the tropical highland blackberry comprises anthocyanins, more specifically, cyanidin-3-glucoside and cyanidin-3-(6′-malonyl) glucoside. In contrast with other phenolic compounds, anthocyanins increase significantly ($P < 0.05$), ranging from 1.14 to 8.96 mg g^{-1} DW, between grade 1 and fully ripe (grade 3) fruits, balancing the decrease of the other phenolics. In correlation, the hue value significantly decreases, indicating darker surface colours for fruits (Table 1). This fact explains why the total phenolic content determined by the Folin–Ciocalteu assay (Table 3) remained almost steady during ripening. This finding was also reported in another study (Siriwoharn et al., 2004), although other authors (Wang & Lin, 2000) reported a significant decrease in phenolics when compared with an earlier developmental stage of the fruit.

To permit comparisons with data found in the literature, Table 3 also shows antioxidant capacity (H-ORAC), as well as anthocyanin content (which was determined by the pH-differential method) and total phenolics (Folin–Ciocalteu assay). The H-ORAC value increased significantly during ripening, especially over the last two developmental stages. Similarly, Siriwoharn et al. (2004) reported an increase in H-ORAC values for two blackberry cultivars during ripening. For tropical highland blackberry, the antioxidant capacity increased almost two-fold between grades 1 and 3, from 38 to 64 μmol TE g^{-1} (FW), reaching, once fully ripe, a relatively high value comparable with that of fruits well-known for their high antioxidant capacity such as cranberry (Wolfe et al., 2008).

The values for H-ORAC in tropical highland blackberry are relatively high, when compared with those for fully ripe blackberries grown in temperate climates (Cho, Howard, Prior, & Clark, 2005; Cho et al., 2004; Reyes-Carmona et al., 2005). In contrast, anthocyanin content is low in comparison (Sellappan et al., 2002; Siriwoharn & Wrolstad, 2004; Siriwoharn et al., 2004; Wang & Lin, 2000).

Anthocyanin content may not have the predominant impact on antioxidant capacity (measured as H-ORAC) as expected. The relatively low anthocyanin content and average content of total phenolics (when compared with other values reported in the literature), but high antioxidant capacity in tropical highland blackberry may eventually be explained by its specific composition of other non-anthocyanin phenolic compounds such as ellagitannins.

4. Conclusions

Compared with blackberries grown in temperate climates, the tropical highland blackberry (*R. adenotrichus*) presents high contents of ellagitannins and low contents of anthocyanins. Even so, the fruit presents a relatively high antioxidant capacity, which may also be explained by synergistic effects between phenolic compounds. The tropical highland blackberry in this study is remarkable amongst the various blackberries and should be marketed with differentiation.

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