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Characterization of Anthocyanins and Proanthocyanidins in Wild and Domesticated Mexican Blackberries (*Rubus* spp.)

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This study was designed to characterize and compare wild, commercial, and noncommercial cultivated blackberry genotypes grown in Michoacán, Mexico. Six genotypes, including WB-3, WB-7, WB-10, and WB-11 (all wild blackberry types), Tupy (a commercial cultivar), and UM-601 (a cultivated breeding line), were selected and profiled for anthocyanins and proanthocyanidins by separating extracts over Amberlite XAD-7 resin and Sephadex LH-20 columns. Subsequent high-performance liquid chromatography (HPLC) and liquid chromatography−electrospray ionization−mass spectrometry (LC−ESI−MS) analyses revealed that the major anthocyanin for all genotypes was cyanidin 3-*O*-glucoside. The proanthocyanidins (condensed tannins) were present in mono- to hexamer forms. Also, hydrolyzable tannins, ellagitannins, were characterized in the blackberry fruits. The average anthocyanin concentration in Sephadex LH-20 fractions was 49.2 mg/g in the commercial cultivar Tupy, while in the wild genotypes and the breeding line, the range was 361.3−494.9 mg/g (cyanidin 3-*O*-glucoside equivalent). The proanthocyanidin concentration varied widely among wild genotypes (417.5−1343.6 mg/g, catechin equivalent). This study demonstrated that the use of Amberlite XAD-7 followed by Sephadex LH-20 chromatography, with subsequent HPLC and LC−ESI−MS analyses, was able to effectively separate and characterize the diverse polyphenolics in blackberry genotypes. These results suggest that recommendations for dietary intake of blackberries for human health benefits need to take into account the source, because of the wide inherent variation in bioactive polyphenolic content in different blackberry genotypes.

KEYWORDS: Anthocyanin; proanthocyanidin; ellagitannins; *Rubus*; HPLC−ESI−MS

INTRODUCTION

The *Rubus* genus (Rosaceae family), which includes the blackberry, is cultivated worldwide but primarily concentrated in the northern hemisphere. The blackberry was among the earliest fruits used for medicinal purposes; as early as the 16th century, blackberry juice was used in Europe to treat infections of the mouth and eyes (*1*). Recent studies have demonstrated very high antioxidant capacity in blackberries and their enhanced potential to reduce risks of cancer and cardiovascular diseases (*2*−*5*) compared to other fruits and vegetables (*6*). The genotype, species, environment, and cultivation conditions have all been shown to exert a profound influence on the content of bioactive compounds in berries (*7*−*9*). Wild berries may provide a preferred resource for investigating phytochemicals that can improve human health, because they contain natural inherent levels of compounds that have not been influenced by commercial breeding and selection (*10*).

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In addition to their fiber content, wild berries are rich in vitamins, minerals, various organic acids, and phenolic compounds (*11*). Because blackberries can contain a unique and intense complement of polyphenolic compounds, research on their composition has grown. The major polyphenolic compounds in berries are anthocyanins, hydrolizable tannins (gallo- and ellagitannins), flavonols, and flavan-3-ols, including proanthocyanidins (*1*, *3*). Anthocyanins are the predominant group of flavonoids present in berries (*11*, *12*). They are water-soluble glycosides and acylglycosides, which are polyhydroxy and polymethoxy derivatives of the 2-phenylbenzopyrylium (flavylium) cation (*13*). Flavan-3-ols, better known as proanthocyanidins or condensed tannins, are polymers of flavan-3-ols and/or flavan-3,4-diol mixtures (*14*). These compounds give a characteristic astringent or bitter taste to many berries (*11*). They are ubiquitous in nature and are the second most abundant group of natural phenolics after lignin (*15*). Anthocyanins and proanthocyanidins are found in a wide range of common foods, including cereals, legumes, fruits, vegetables, and wines, and affect texture, color, and taste. These polyphenolics are also of

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interest in nutrition and medicine because of their potent antioxidant capacity and human health-protective effects against chronic disease risks, including cardiovascular diseases and cancers (*15*, *16*).

Numerous analytical procedures have been employed to extract and characterize polyphenolics from different plant sources (*17*). Several polyphenolics have been detected in *Rubus* species, but the relative amounts reported were strongly influenced by the analytical conditions used by different researchers (*18*, *19*). Commonly, high-performance liquid chromatography (HPLC) is used for the separation and identification of polyphenolics. However, HPLC techniques are only suitable for fractionating relatively small quantities of plant extracts and can result in the loss of the biological activity of fractions (*20*). Adsorber technologies, using solid-phase resins, can concentrate, purify, and recover bioactive plant secondary metabolites (*21*). For example, anthocyanins were effectively separated from carbohydrates, salts, and other soluble compounds using Amberlite XAD-7 resin, which exhibited a high affinity for anthocyanins and proanthocyanidins. Amberlite XAD-7 resin was also found best suited for isolating anthocyanins from black chokeberry juice among 16 different solid-phase extractions (*22*). Both Amberlite XAD-7 and Sephadex LH-20 resins were previously used to isolate anthocyanins in red raspberry, but other polyphenolics were not taken into account (*21*).

The objectives of this study were (1) to establish an efficient isolation, separation, and characterization method for polyphenolics in blackberry using adsorber technology (with solid-phase columns, Amberlite XAD-7 and Sephadex LH-20) in combination with HPLC and liquid chromatography−electrospray ionization−mass spectrometry (LC−ESI−MS) techniques and (2) to assess the relative variations and characterize in depth the anthocyanin and proanthocyanidin content in six Mexican blackberries: four wild genotypes and two domesticated genotypes (both a commercial cultivar and a noncommercialized breeding line).

MATERIALS AND METHODS

Plant Material. Six blackberry genotypes (*Rubus* spp.), including four wild blackberries, one commercial cultivar, and a noncommercialized breeding line were used for this study. Fruits from the four Mexican wild blackberries (WB-3, WB-7, WB-10, and WB-11) were collected in the spring of 2008, in Uruapan, Michoacán, Mexico. The commercial blackberry (Tupy cultivar) fruits were collected in the winter of 2008, in Los Reyes, Michoacán, Mexico. This variety was originally developed by crossing the wild trailing blackberries of Uruguay with the commercial variety Comanche (*23*) and is widely grown by farmers in Mexico. Fruits from a cultivated/domesticated breeding line (UM-601) were provided by the Laboratorio de Agrobiologia of Universidad Michoacana de San Nicolás de Hidalgo, Uruapan, Michoacán, Mexico, in the winter of 2008. The UM-601 line was selected for its color and flavor through a breeding program aiming to develop superior cultivars adapted to the environmental conditions of Michoacán and other locations in central Mexico. Immediately after harvest, all fruits were washed, stored at −80 °C, lyophilized, and kept at −20 °C for further analysis.

Chemicals. Authentic commercial standards of anthocyanins, proanthocyanidins, and other flavonoids were used for polyphenolic characterization in blackberries. The anthocyanin standards (cyanidin, delphinidin, petunidin, and malvidin glycosides) were obtained from Polyphenols Laboratories (Sandnes, Norway) and proanthocyanidin monomers (including catechin and epicatechin) and dimers, kaemferol, quercetin, and myricetin glycosides were obtained from Chromadex (Laguna Hills, CA).

Preparation of the Polyphenolic Extract. Freeze-dried blackberries (100 g) were blended in a flask with 500 mL of acidified 80% methanol [0.3% trifluoroacetic acid (TFA)] (1:5, w/v) and filtered first through multiple layers of cotton 3 times to separate the purple pigment from the pulp. The resulting slurry was filtered through Whatman's filter paper number 4 and then number 1 (Florham Park, NJ) with the aid of light suction. The collected hydro-alcoholic extract was concentrated by rotary evaporation at < 40 °C to remove the methanol. The remaining aqueous concentrate (120 mL) was partitioned against ethyl acetate (4 × 120 mL). The aqueous fraction was loaded onto an Amberlite XAD-7 (Sigma Life Science, CO) column (30 × 10 cm), preconditioned with acidified water (0.3% TFA), where the polyphenolic mixture was adsorbed onto the Amberlite XAD-7 resin. The resin was then washed thoroughly with acidified water (0.3% TFA) to remove free sugars, pectins, and other impurities. The polyphenolic mixture was then eluted from the column with 1 L of methanol (0.3% TFA), and this post-Amberlite extract (PAE) was evaporated and freeze-dried to yield ~4 g of concentrated polyphenolic extract containing mainly anthocyanins and proanthocyanidins.

Isolation of Anthocyanins and Proanthocyanidins. The freeze-dried (2.0 g) polyphenolic extract (PAE) was completely dissolved in 5 mL of MeOH and applied to a column packed with Sephadex LH-20 (3 × 30 cm) preconditioned with 80:20 H_2O/MeOH (0.3% TFA). Two solvent systems were used to elute the polyphenolic compounds off the Sephadex LH-20 column: H_2O/MeOH (80:20) with 0.3% TFA and 70% acetone. A total of 12 fractions (50 mL each) were collected, starting when the colored material began to elute from the column. The first nine fractions were rich in anthocyanins and eluted with the H_2O/MeOH solvent. The last three fractions were eluted from the Sephadex LH-20 column with the 70% acetone solvent and mainly contained proanthocyanidins. Organic solvents for all fractions were evaporated (< 40 °C), and then the aqueous partition was immediately frozen at −80 °C and freeze-dried. A flow chart illustrating the procedures applied to extract and isolate anthocyanins and proanthocyanidins is shown in **Figure 1**. Polyphenolics in all extracts and fractions were identified and quantified at each step of the isolation procedures by HPLC and LC−ESI−MS systems (detailed below), compared to their commercial standards when available and also to published data.

HPLC−Diode Array Detector (DAD) Analysis. The HPLC analyses were conducted using 1100 HPLC (Agilent Technologies, Inc., Santa Clara, CA) with a DAD, an autosampler, a controlled-temperature column compartment, and Chemstation as a system controller and for data processing. Anthocyanin and proanthocyanidin separations were performed using a reversed-phase Supelcosil LC-18 column, 250 mm × 4.6 mm × 5 μm (Supelco, Bellefonte, PA). The mobile phase for anthocyanins consisted of 5% formic acid in H_2O (A) and 100% methanol (B). The flow rate was constant during HPLC analysis at 1 mL/min, with a step gradient of 10, 15, 20, 25, 30, 60, 10, and 10% of solvent B at 0, 5, 15, 20, 25, 45, 47, and 60 min, respectively, with a 25 °C constant column temperature. For proanthocyanidins, the mobile phase consisted of 5% acetonitrile and 0.1% formic acid in H_2O (A) and 5% H_2O and 0.1% formic acid in acetonitrile (B). The step gradient used was 0, 5, 30, 60, 90, 90, and 0% of solvent B at 0, 40, 45, 50, 55, 60, and 70 min, respectively. Samples were prepared by dissolving 5 mg in 1 mL of methanol and filtering through 0.22 μm nylon filters (Fisher Scientific, Pittsburgh, PA) before injecting 10 μL into the HPLC system. Three concentrations of cyanidin-3-*O*-glucoside or catechin were prepared as external standards for anthocyanins or proanthocyanidins, respectively, at 1.0, 0.5, and 0.25 mg/mL, where 10 μL was injected. Quantification of anthocyanins and proanthocyanidins was performed from the peak areas recorded at 520 and 280 nm, respectively. All blackberry extracts and fractions were analyzed in duplicates.

LC−ESI−MS Analysis. The LC−MS analyses were made with a LCQ Deca XP mass spectrometer (Thermo Finnigan Corp., San Jose, CA), MS version 1.3 SRI, ESI in the positive-ion mode (*m/z* 200−2000), with a photodiode array (PDA) detector (200−600 nm), version 1.2, autosampler, version 1.2, and Xcalibur software for data processing. The spray voltage was 10 kV, and the capillary temperature was 250 °C. The HPLC separations were carried out on a C-18 reversed-phase column (150 mm, 2.1 mm inner diameter, 5 μm particle size, 90 Å) (VYDAC, Western Analytical, Murrieta, CA). The LC−MS analysis was carried out using the same mobile phase and gradients described above (HPLC−DAD analyses), except with formic acid at 0.1%, the flow rate at 200 μL/min, and the injection volume at 5 μL per sample or standard. The column temperature and the samples were kept at 20 °C. The LC−MS/MS analysis was carried out using a LC−time-of-flight (TOF) Ultima mass spectrometer (Waters Corporation, Milford, MA). Acquisition of MS/MS data

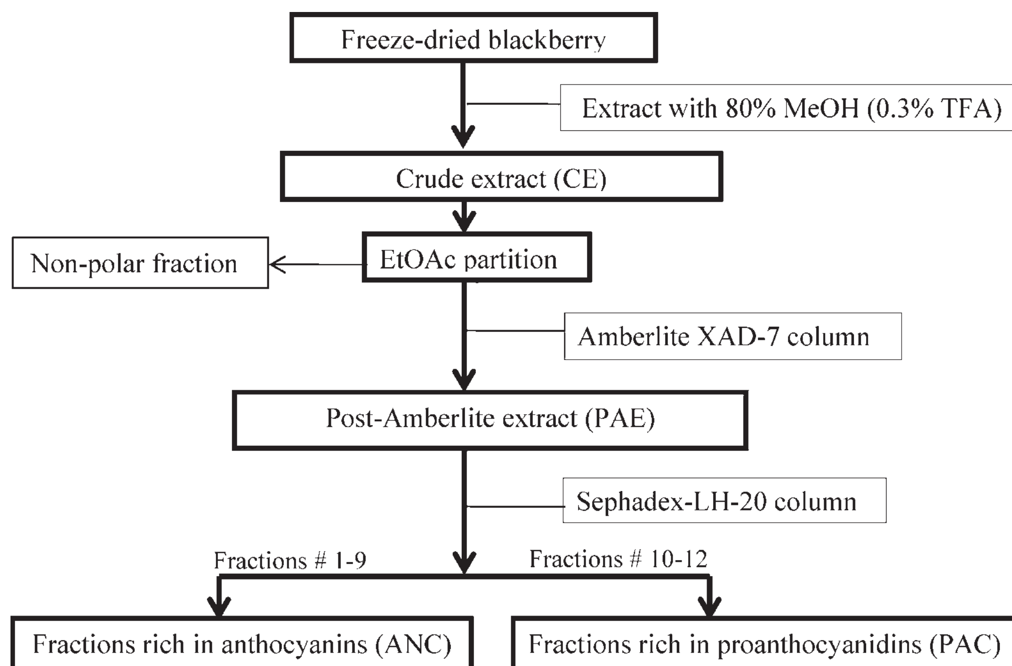


Figure 1. Flow chart illustrating the procedures applied to extract and isolate anthocyanins (ANC) and proanthocyanidins (PAC) in blackberry freeze-dried fruits. PAE contained a mixture of polyphenolics. ANC including fractions 1−9 mainly contained anthocyanins eluted with 20% MeOH. PAC including fractions 10−12 mainly contained proanthocyanidins eluted with 70% acetone.

was performed with MassLynx 4.0 software (Waters Corporation). Commercial standard references (0.25 mg/mL) and samples (5 mg/mL) were dissolved in 1 mL of 100% MeOH and filtered [0.22 μm polytetrafluoroethylene (PTFE)] before injection to compare retention time (Rt) and *m*/*z* values for polyphenolics in all extracts and fractions. The chemical structures for the basic units of anthocyanins, proanthocyanidins, and ellagitannins in blackberries are presented in **Figure 2**.

RESULTS AND DISCUSSION

Separation of Polyphenolics. The use of acidified methanolic solvent (0.3% TFA) facilitated the extraction of polyphenolics in blackberry and prevented the anthocyanins from degradation during the separation and subsequent analyses (*24*, *25*). The use of Amberlite XAD-7 resin polymer is typically used to remove sugars and pectins from phenolic compounds in plant natural product extracts (*24*), while Sephadex LH-20 resin, with appropriate solvent mixtures, can further separate anthocyanins and proanthocyanidins present in the PAE, facilitating HPLC identification of polyphenolics. The two steps of separation resulted in fractions that were enriched in either anthocyanins or proanthocyanidins, which greatly facilitated characterization and quantification (**Figure 1**).

Analyses of Blackberry Anthocyanins. HPLC analyses of crude extracts (CEs) revealed that all of the blackberry genotypes had characteristically complex mixtures of polyphenolics. Because the CEs contained both anthocyanins and proanthocyanidins, which both had UV absorption of 280 nm, further separation using Amberlite XAD-7 and Sephadex LH-20 columns was required before proanthocyanidins could be analyzed. The content of anthocyanins in the CE ranged from 3.4 to 9.2 mg/g of dry weight (cyanidin 3-*O*-glucoside equivalent) (**Table 1**). After the clean up procedure on Amberlite XAD-7 resin, the concentration of anthocyanins increased an average of 13 times across all blackberry genotypes. HPLC spectra for the commercial cultivar (Tupy) revealed a low anthocyanin concentration (3.4 mg/g) in the CE. The same general trend was observed in the nine Sephadex anthocyanin fractions (**Table 1**), where the concentration was elevated by about 4 times compared to PAE. The concentration was 49.2 mg/g with Tupy, while in the other genotypes, it ranged from 362.5 to 494.9 mg/g (**Table 1**). Some of the Sephadex LH-20 fractions contained concentrations of over 750 mg/g (75% anthocyanin-enriched fractions), which expedited their identification and characterization.

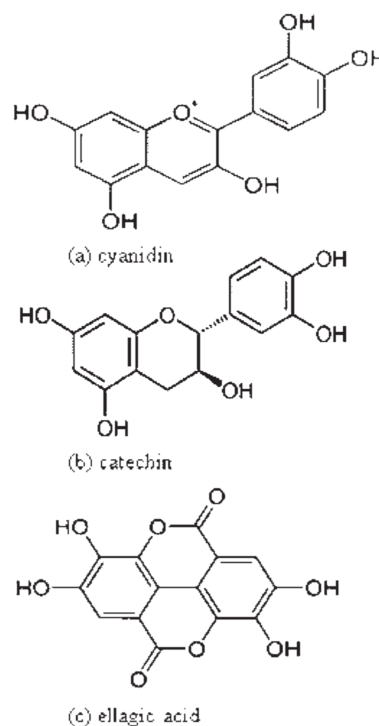
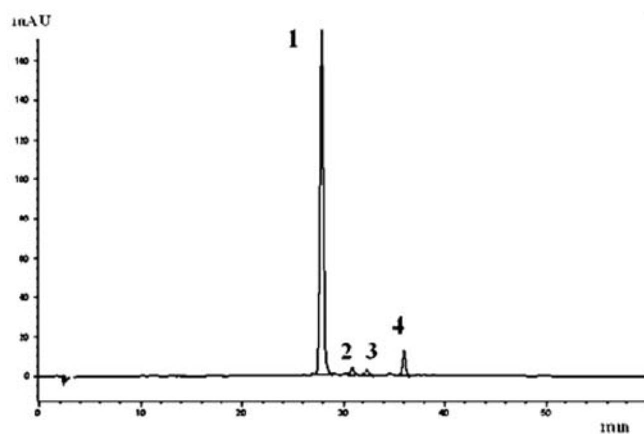


Figure 2. Basic chemical structures for anthocyanins, proanthocyanidins, and ellagitanins characterized in six Mexican blackberry fruits. (a) Cyanidin: anthocyanin. (b) Catechin: proanthocyanidin. (c) Ellagic acid: ellagitannin.

Table 1. Concentration of Anthocyanins in Freeze-Dried Samples in Different Extracts and Fractions from Six Mexican Blackberries

	anthocyanin concentration (mg/g of dry weight) (cyanidin 3-*O*-glucoside equivalent)					
sample/genotype	Tupy[a]	WB-3[b]	WB-7	WB-10	WB-11	UM-601[c]
CE	3.4	6.6	8.7	9.2	8.8	9.1
PAE	36.2	59.6	114.3	131.7	89.5	132.7
Sephadex LH-20 fractions[d]						
fraction 1	16.1	70.3	836.4	273.0	91.3	117.2
fraction 2	219.6	535.5	52.1	55.5	52.3	248.2
fraction 3	53.1	513.0	138.9	300.7	270.6	712.0
fraction 4	28.6	358.2	119.3	418.1	602.3	762.2
fraction 5	10.7	445.8	765.4	678.1	551.9	372.5
fraction 6	ND[e]	520.1	727.2	570.0	546.9	ND
fraction 7	ND	639.1	507.7	55.1	541.8	274.6
fraction 8	11.7	133.0	728.9	756.8	607.0	ND
fraction 9	4.97	104.5	577.9	155.1	131.1	37.4
average	49.2	368.8	494.9	362.5	377.2	361.3

[a] Tupy = commercial cultivar. [b] WB = wild blackberry genotypes. [c] UM-601 = cultivated noncommercial blackberry line. [d] Sephadex LH-20 fractions 1−9 were designated as anthocyanin-enriched fractions. [e] ND = not detected.



Anthocyanin ID	% individual anthocyanin within genotypes					
	Tupy	WB-3	WB-7	WB-10	WB-11	UM-601
1	34.6	79.4	77.0	79.2	77.3	71.1
2	26.7	9.1	6.2	6.1	5.7	19.6
3	17.7	11.5	10.2	5.8	5.7	9.4
4	20.9	NA	6.6	9.0	11.3	NA

Figure 3. Representative HPLC chromatogram showing identified anthocyanins and their relative distribution (%) within blackberry genotypes. Anthocyanin separation was performed using a LC-18 reversed-phase (250 mm × 4.6 mm × 5 μm) column, at 520 nm. Peak identification: (1) cyanidin 3-*O*-glucoside, (2) cyanidin 3-*O*-rutinoside, (3) cyanidin 3-*O*-arabinoside, and (4) cyanidin 3-*O*-(6-*O*-malonyl)glucoside. Tupy, commercial cultivar; UM-601, cultivated noncommercial blackberry line; WB, wild blackberry genotypes, ND; not detected. The percentage of individual anthocyanin values were calculated in the CE for each genotype.

Sephadex LH-20 fractions revealed the presence of four major anthocyanins (**Figure 3**). The HPLC chromatogram and LC−MS analysis of all extracts showed profiles typical for blackberries but with different relative distributions of individual anthocyanins. Data showed that each genotype contained a unique profile with compounds represented at different percentages for each individual anthocyanin among genotypes (**Figure 3**). The four major anthocyanins identified were cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-arabinoside, and cyanidin 3-*O*-(6-*O*-malonyl)glucoside, with the first compound as the most abundant; this pattern was consistent with previous reports on blackberry (*1*).

The major compounds were characterized according to their m/z value, UV spectrum absorbance characteristics, Rt as compared to commercial standards, and a comparison to published polyphenol data (*1*, *22*, *25*, *26*). Representative LC−ESI−MS spectra of anthocyanins from Sephadex LH-20 fraction 5 for the WB-11 genotype are shown in **Figure 4**. The spectra illustrated that the majority of the fraction was anthocyanins based on the total ion current (**Figure 4A**), which showed all compound ions in the fraction and the UV maximum absorption at 520 nm (**Figure 4B**). The individual anthocyanins were further verified on the basis of the LC−MS spectra (**Figure 4C**) based on the ion-charge ratio (m/z) and Rt. The cyanidin was present in the aglycone form (m/z 287), in addition to its glycosides: cyanidin 3-*O*-glucoside (m/z 449.02; Rt, 19.98), cyanidin 3-*O*-rutinoside (m/z 595.11; Rt, 23.58), cyanidin 3-*O*-arabinoside (m/z 419.02; Rt, 28.1), and cyanidin 3-*O*-(6-*O*-malonyl)glucoside (m/z 535.06; Rt, 29.3). The maximum wavelength absorption (**Figure 4B**, at 520 nm) demonstrated clearly that cyanidin-3-*O*-glucoside, eluted at Rt of 19.98 min, comprised the majority of the anthocyanins in the Sephadex LH-20 column fractions 1−9.

Analysis of Proanthocyanidins in Blackberries. HPLC and LC−MS analysis during the extraction and fractionation steps revealed the proanthocyanidin complexity in all six blackberry genotypes (**Figure 5**). In the earlier steps of extraction and fractionation procedures, the mixture of anthocyanins and proanthocyanidins was almost impossible to characterize; only after Sephadex LH-20 column chromatography could the proanthocaynidin compounds be separated and characterized using LC−MS, because of the dominance of co-occurring anthocyanins in the extracts and their mutual absorption of light wavelengths at both 280 and 520 nm. In the UV 280 nm chromatogram, proanthocyanidins were overshadowed by anthocyanins, and therefore, it was not feasible to have accurate estimates for them in the CE or PAE. In the Sephadex LH-20 fractions 10−12, proanthocyanidins were estimated as catechin equivalents and are presented in **Table 2**. The proanthocyanin estimates ranged from 118.3 to 1363.8 mg/g (catechin equivalent) in the individual fractions from the six blackberry genotypes. The average total proanthocyanidin content across all fractions and species ranged from 579.2 to 1343.6 mg/g, and cultivar Tupy was the lowest. The UM-601 (cultivated noncommercial line) had the highest proanthocyanidin content followed by WB-3 (1213.7 mg/g, catechin equivalent). While all four WB samples contained similar anthocyanin content (**Table 1**), they varied significantly in accumulated proanthocyanidins (**Table 2**). The WB-3 and UM-601 contained, on average, double the amount

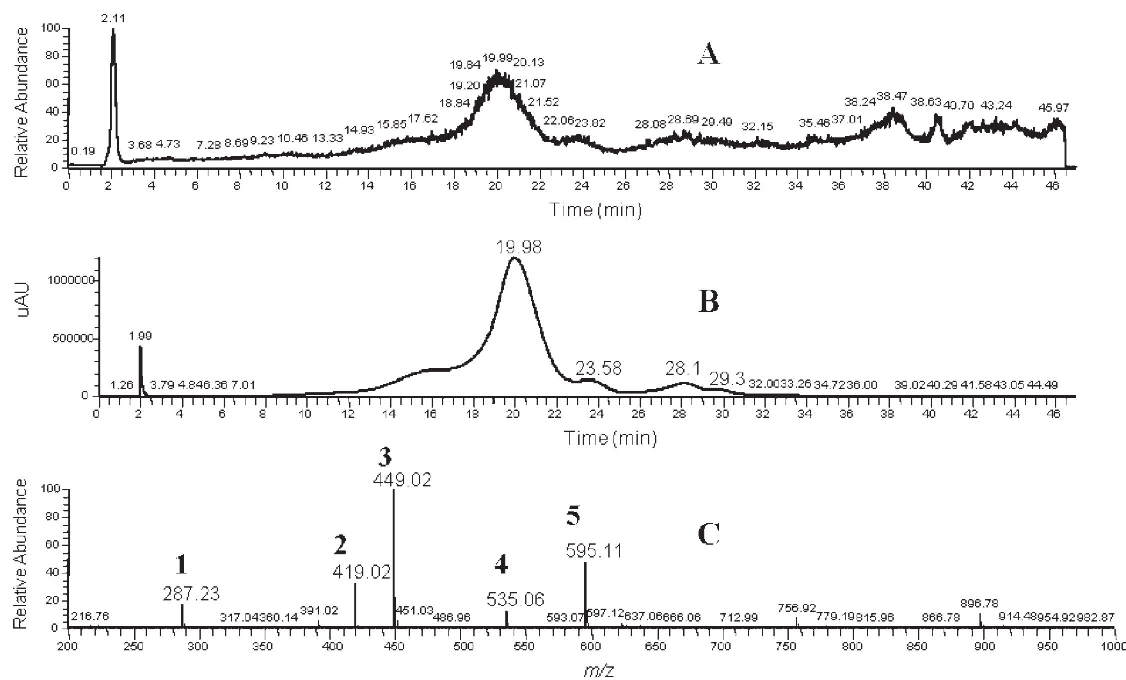


Figure 4. Representative LC−ESI−MS output spectrum, positive ion, of anthocyanins in Sephadex LH-20 fraction 5 for WB-11 genotype. (A) LC−ESI−MS output showing the total ion current, (B) photodiode array chromatograph at 520 nm wavelength, and (C) full mass spectrum, positive mode, *m*/*z* 200−1000. Anthocyanins were eluted before 35 min, as shown by UV maximum absorption, and included (1) cyanidin aglycone, (2) cyanidin 3-*O*-glucoside, (3) cyanidin 3-*O*-glucoside, (4) cyanidin 3-*O*-(6-*O*-malonyl)glucoside, and (5) cyanidin 3-*O*-rutinoside.

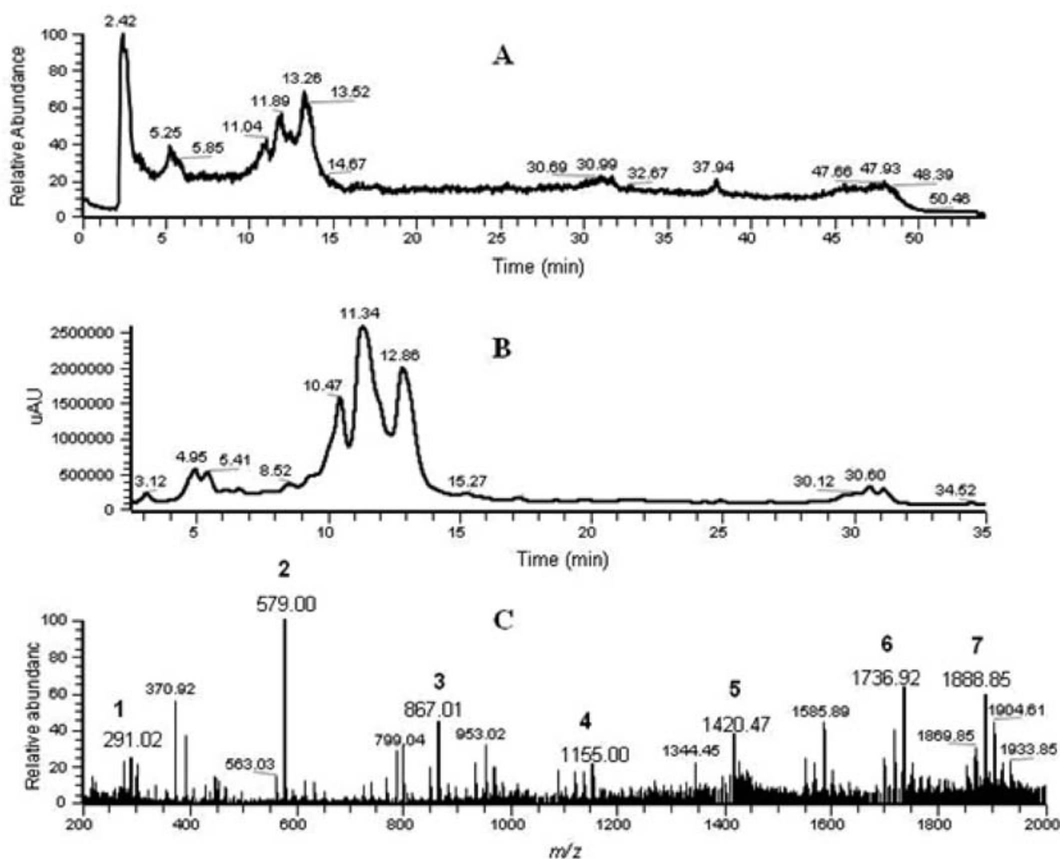


Figure 5. Representative LC−ESI−MS output spectrum, positive ion, of proanthocyanidins in Sephadex LH-20 fraction 11 for WB-11 genotype. (A) Total ion current, (B) photodiode array chromatogram, 280 nm, and (C) ESI full mass spectrum, positive mode, *m*/*z* 200−2000. Proanthocyanidins were eluted before 30 min as shown by UV maximum absorption: (1) monomer (catechin or epicatechin), (2) dimer, (3) trimer, (4) tetramer, (5) pentamer, (6) hexamer, and (7) ellagitannins.

of proanthocyanidins compared to WB-7, WB-10, and WB-11 blackberry genotypes.

The total ion current for Sephadex LH-20 fractions 10−12 showed that the proanthocyanidins constituted the majority of

Table 2. Concentration of Proanthocyanidins (mg/g, Catechin Equivalent) in Freeze-Dried Samples in Different Extracts and Fractions from Six Mexican Blackberries[a]

	proanthocyanidins (mg/g of dry weight) (catechin equivalent)					
sample/genotype	Tupy[b]	WB-3[c]	WB-7	WB-10	WB-11	UM-601[d]
CE	ND[e]	ND	ND	ND	ND	ND
PAE	ND	ND	ND	ND	ND	ND
Sephadex LH-20 fractions[f]						
fraction 10	510.2	1203.2	118.3	778.1	749.9	1323.4
fraction 11	919.8	1224.2	716.7	758.0	716.7	1363.8
fraction 12	307.6	ND	ND	ND	ND	ND
average	579.2	1213.7	417.5	768.0	733.3	1343.6

[a] Detection of proanthocyanidin in the CE and PAE was not possible because of interference of co-eluting anthocyanins, which had the same absorption in UV at 280 nm as proanthocyanidins. [b] Tupy = commercial cultivar. [c] WB = wild blackberry genotypes. [d] UM-601 = cultivated noncommercial blackberry line. [e] ND = not detected. [f] Sephadex LH-20 fractions 10−12 were designated as proanthocyanidin-enriched fractions.

the polyphenolics (**Figure 5A**), with the maximum absorption of UV at 280 nm (**Figure 5B**). Detailed LC−MS ESI mass spectra recorded at the m/z range from 200 to 2000 (**Figure 5C**) showed the presence of molecular ions for proanthocyanidin monomers, including catechin and/or epicatechin (m/z 291.02), dimers of catechin/epicatechin (m/z 579.00), and a series of higher degrees of polymerization, including trimers (m/z 867.01), tetramers (m/z 1155.00), pentamers (m/z 1420.47), and hexamers (m/z 1736.92). In addition, a series of ellagitannins were identified in the Sephadex LH-20 fractions for all blackberry species using LC−MS/MS. Very limited work characterizing proanthocyanidins in different blackberry species have been previously reported (*27*); however, recent studies have shown that blackberry and raspberry contain gallotannins and ellagitannins (hydrolyzable tannins) (*1*, *28*). Gallotannins are composed of gallic acid, whereas ellagitannins are polymers of hexahydroxydiphenic (HHDP) acid, which is a dimeric form of gallic acid that can spontaneously lactonize to form ellagic acid. For both gallotannins and ellagitannins, the gallic or HHDP acids are esterified to one or multiple glucopyranoses to form diversified polymers (*14*).

Characterization of ellagitannin compounds in blackberry genotypes was challenging because of their complexity, diversity, and large molecular size. Subsequent MS fragmentation procedures for the predominant positive ions in MS/MS were used to identify the molecular masses of ellagitannins and conjugates of ellagic acid in the samples. Whenever possible, chromatographic retention and literature data were used to support the identifications of compounds. In the analysis of MS/MS, several ellagitannins were identified in blackberry fractions obtained through Sephadex LH-20. The identified ellagitanins, in addition to the ellagic acid (m/z 303.01), included compounds described hereafter. Pedunculagin isomers had a $[M + H]^+$ at m/z 801.56, and the MS/MS spectrum showed fragments at m/z 766.01 (M − 35, loss of two water molecules) and m/z 464.56 (M − 302 loss of HHDP). This compound could be a series of structurally related compounds with the addition of a water molecule (*26*). Sanguiin H-10 had a $[M + H]^+$ at m/z 1586.32, and the major ions in MS/MS were at m/z 1568.15 (M − 18, loss of a water molecule), m/z 1398.23 (M − 170, loss of a gallate unit), m/z 1079.41 (M − 319, loss of a HHDP group and water molecule), m/z 917.10 (M − 162, loss of a glucosyl group), m/z 767.43 (M − 150, loss of a galloyl group), m/z 615.21 (M − 152 loss of a galloyl group), and m/z 453.12 (M − 162, loss of a glucosyl group). On the basis of the MS/MS spectra and published data, this peak was tentatively identified as Sanguiin H-10 (*28*) in raspberry fruits. Nobotanin A/Malabathrin B had a $[M + H]^+$ at m/z 1736.01; it produced fragments by MS/MS, including m/z 1720.02 (M −169, loss of a gallate group), m/z 1417.21 (M − 302, loss of a HDDP group), m/z 1085.22 (M − 332, loss of glucosyl and galloyl groups), and m/z 783.32 (M − 150, loss of a galloyl group). Similar conclusions for ellagitannin characterization have been previously reported (*28*, *29*). Sanguiin H-6/Lambertianin A had a $[M + H]^+$ at m/z 1888.85. The MS/MS fragmentation patterns of Sanguiin H-6 included m/z 1869.85 (M − 18, loss of a water molecule), m/z 1569.89 (M − 301, loss of a HHDP group), and m/z 1230.56 (M − 339, loss of glucosyl and galloyl groups). The remaining fragments were m/z 933.17 and 633.45. These findings were in agreement with earlier reports in *Rubus ideaus* (*28*, *30*). Ellagic acid isomers had a $[M + H]^+$ at m/z 479.22 with a fragmentation pattern of m/z 303.45 (M − 160, loss of a hexose), m/z 257.22, and m/z 229.25 and were tentatively identified as ellagic acid hexosides, previously detected in pomegranate juices (*31*). MS/MS on m/z 301.00 ion afforded ions at m/z 257.14 and 229.00, which have been established as ellagic-acid-like ions. On the basis of our spectral data and literature, doubly charged ions (half of the m/z value for the parent ion) were observed in Sephadex LH-20 fractions 10−12 spectra, contributing to the complexity in identifying ellagitannins in blackberry fruits.

To characterize the extracts from blackberries, it was necessary to repeatedly perform fractionation steps using Amberlite XAD-7 and Sephadex LH-20 resins. The use of HPLC−ESI−MS/MS facilitated the identification and quantification of the major polyphenolic groups in blackberry genotypes. The data indicated that the combination of Amberlite XAD-7 and Sephadex LH-20 column chromatography was a suitable method for the purification of anthocyanins, proanthocyanidins, and other flavonoids in the wild Mexican blackberries and provided reproducible and reliable quantification. Polyphenolic groups identified in this study, including anthocyanins and condensed and hydrolyzable tannins, varied widely among the six blackberry genotypes. The commercial Tupy cultivar had a relatively low anthocyanin content as compared to the wild genotypes and domesticated noncommercial line (UM-601). Considerable variability in the proanthocyanidin content was also observed in the HPLC spectra.

Previous reports have indicated difficulty in both achieving adequate separation of anthocyanin and proanthocyanidin classes of phytochemicals and accurate identification and quantification in general (*13*). In this study, the main polyphenolic groups in blackberry were isolated, categorized, and characterized separately, which permitted a more rigorous estimation of polyphenol content. Most previously published data have been reported on the basis of the estimation of the total phenolics in berries and other fruits using Folin−Ciocalteu and other colorimetric methods and have estimated the total phenolics in the range of 12.1−23.5 mg/g of dry weight (gallic acid equivalent) in commercial blackberry cultivars, such as Choktaw, Thornless, and Triple Crown (*4*, *12*, *32*, *33*). In another report (*26*), the anthocyanin content in the blackberry cultivar “Apache” was estimated at 2.6 mg/g, which was comparable to the Mexican cultivar “Tupy” evaluated in this study. However, the wild and cultivated Mexican berries (except WB-3) contained more than twice the anthocyanin content compared to the commercial cultivar (Tupy). Other researchers have reported anthocyanins in the range of 7.94−18.1 mg/g of dry weight (cyanidin 3-*O*-glucoside equivalent) in commercial blackberry fruits (*8*, *12*). Gu et al. (*27*) reported 0.27 mg/g of fresh weight (catechin equivalent) proanthocyanidins in blackberries. Proanthocyanidins were not quantified in CEs in this study but were easily characterized in the enriched fractions 10−12 at high concentrations.

Although blackberries as analyzed in this study contained mainly anthocyanins (cyanidin 3-*O*-glucoside), condensed

(proanthocyanidin) and hydrolyzable tannins were observed in appreciable amounts. To the best of our knowledge, this was the first report that details the characterization of polyphenolics in a number of Mexican blackberry genotypes, after separation into three main polyphenolic groups known for their potent *in vivo* and *in vitro* bioactivities. This study demonstrated that the four Mexican wild blackberry genotypes exhibit high polyphenolic concentrations and can be used as a plant source rich in polyphenolics for breeding programs aimed at developing new cultivars/varieties with elevated polyphenolic content.

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