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Analysis of Phenolic Compounds in Two Blackberry Species (*Rubus glaucus* and *Rubus adenotrichus*) by High-Performance Liquid Chromatography with Diode Array Detection and Electrospray Ion Trap Mass Spectrometry

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High-performance liquid chromatography with diode array (LC-DAD) and electrospray ionization mass spectrometric detection (ESI-MS) was used to analyze phenolic compounds of two blackberry species (*Rubus glaucus* Benth. and *Rubus adenotrichus* Schlech.) growing in South America. UV–visible spectrophotometry was a valuable tool for identifying the class of phenolic compound, whereas MS and MS^n fragmentation data were useful for their structural characterization. Ellagitannins were the major compounds, with sanguiin H-6 and lambertianin C being the predominant ones. The anthocyanin composition as well as the presence or absence of kaempferol glycosides can be used to distinguish the *Rubus* species studied. Flavonol hexoside-malonates were identified in both berries. Hydroxycinnamic acids were minor compounds and found as ferulic, caffeic, and *p*-coumaric acid esters. Similar contents were obtained by analysis of soluble ellagitannins and ellagic acid glycosides as ellagic acid equivalents and by analysis of ellagic acid equivalents released after acid hydrolysis.

KEYWORDS: Blackberry; phenolic compounds; HPLC; mass spectrometry; *Rubus glaucus*; *Rubus adenotrichus*

INTRODUCTION

The genus *Rubus* is worldwide, but absent from deserts and most well represented in the northern hemisphere. The important cultivated species are the European red raspberry (*Rubus idaeus* ssp. *vulgatus*), the North American red raspberry (*Rubus strigosus*), the eastern North American black raspberry (*Rubus occidentalis*), and the hybrid Andean blackberry (*Rubus glaucus*, *Rubus adenotrichus*). The Andean blackberries are native from Mexico to Ecuador and are widely cultivated in South America for their edible fruits, which are eaten fresh or consumed as juice, syrup, or desserts. Blackberries are currently promoted as being a rich source of polyphenols, which are compounds of interest because of their antioxidant activity as radical scavengers and possible beneficial roles in human health, such as reducing the risk of cancer, cardiovascular disease, and other pathologies (*1–5*).

Phenolic compounds include several classes such as hydroxybenzoic acids, hydroxycinnamic acids and flavonoids. The major phenolic compounds in berries are hydrolyzable tannins (gallo- and ellagitannins) and anthocyanins, hydroxycinnamic acids, flavonols, flavan-3-ols, including proanthocyanidins being present in lower amounts (*6, 7*). Major anthocyanins in red raspberry have been identified as cyanidin and pelargonidin glycosylated with rutinose and sophorose (*8, 9*), whereas cyanidin 3-glucoside and pelargonidin 3-glucoside were predominant in strawberry (*10*). Blackberry anthocyanins have been well-characterized and are only cyanidin-based compounds (*11, 12*). Ellagitannins and ellagic acid derivatives were detected in *Rubus* species, but amounts reported were closely dependent on the analytical conditions (*13–15*). Hydroxycinnamic acids are detected as glycosides and as esters with sugars or quinic acid. Flavan-3-ols are found as monomers as well as structural units in proanthocyanidin chains. To our knowledge, there is no report in the literature about the polyphenolic composition of the

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Andean blackberry. The objective of this study was therefore to tentatively identify and quantify phenolic compounds in extracts of *R. glaucus* and *R. adenotrichus*.

MATERIALS AND METHODS

Chemicals. All solvents were of HPLC grade, purchased from Carlo Erba (Val de Reuil, France). Orthophosphoric acid, glucose, galactose arabinose and rhamnose were purchased from Merck (Riom, France). Folin–Ciocalteu reagent and hydrochloric and formic acids were purchased from Carlo Erba. Chlorogenic, *p*-coumaric, caffeic, and *p*-hydroxybenzoic acids, cyanidin 3-*O*-glucoside, and kaempferol were from Extrasynthese (Genay, France). Ferulic acid, (+)-catechin, (−)-epicatechin, quercetin, ellagic acid, gallic acid, and sodium nitrite were from Sigma (L'isle d'Abeau, France). Sephadex LH-20 was a product of Pharmacia (Uppsala, Sweden).

Sample Collection. Ten kilograms of both species (*R. adenotrichus* and *R. glaucus*) was harvested at full-ripe stage in Costa Rica (La Trinidad: Copey, Santa María de Dota, Cartago) and Ecuador (Ambato), respectively. Fruits were ground during 3 min in a Waring blender (Vorwerk, Montpellier, France), and portions (500 g) of the resulting pulp were immediately freeze-dried. The moisture content was determined after freeze-drying. The powder was kept at −20 °C until analysis.

Total soluble solids were measured from pulp with an Atago refractometer (Japan) at 20 °C. Results were reported as Brix degree. Analysis was made in triplicate.

pH and Titratable Acidity. Ten grams of blackberry pulp was mixed with 90 mL of deionized water. The pH was measured with a Schott pH-meter. After determination of the pH, the solution was titrated with 0.1 N NaOH to pH 8.1. Results were expressed as citric acid percentage [grams of citric acid per 100 g of fresh weight (FW)]. Analysis was made in triplicate.

Extraction. The powder (2 g) was extracted twice for 15 min with 60 mL of 70% aqueous acetone containing 2% formic acid. The extracts were combined, filtered, and concentrated under vacuum to remove acetone (40 °C). The aqueous layer was concentrated to yield crude extract (80 mL). An aliquot was analyzed by LC-DAD/MS for ellagitannins and anthocyanins. For other phenolic compounds, two portions (20 mL each) were extracted with ethyl acetate to yield ethyl acetate and aqueous extracts. Ethyl acetate extract was washed with water (2 × 20 mL). The operation was repeated three times. Ethyl acetate extracts were combined, filtered, evaporated to dryness, and dissolved in methanol before LC-DAD/MS analysis. For analysis of sugar moieties of flavonoid and ellagic acid glycosides, both ethyl acetate and aqueous extracts were separately loaded onto a Sephadex LH-20 column (150 mm × 20 mm) packed in water. The column was washed with water to remove sugars and other non-phenolic compounds. Then, phenolic compounds were eluted with 300 mL of a $MeOH/H_2O$ (50:50, v/v) mixture and submitted to acid hydrolysis (MeOH/HCl, final concentration = 2 M, 2 h, 95 °C). Aglycones were analyzed by HPLC as described below.

Sugar Identification. Analysis of acid hydrolysates obtained as described above was performed on a Shimadzu apparatus, equipped with an LC-9A pump and an RID-6A refractometer (Shimadzu, Paris, France). A 300 mm × 7.8 mm i.d. HPX-87H column (Bio-Rad, Paris, France) was used with acidified water (H_2SO_4, 100 μL/L) as mobile phase, at a flow rate of 0.6 mL/min. Identification was achieved by comparison with authentic standards.

Alkaline Hydrolysis. Ten milliliters of crude extract was submitted to alkaline hydrolysis, which was carried out at room temperature in 2 M NaOH under nitrogen for 4 h and stopped by acidification to pH 1.5 with 2 M HCl. The solution was then evaporated to dryness and dissolved in 10 mL of $MeOH/H_2O$ (50:50, v/v) before HPLC analysis.

Total Phenolics. The total phenolic content was determined with Folin–Ciocalteu reagent according to the method optimized by George et al. (*16*). Fifty microliters of aqueous acetone extract was used for the quantification. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 g of dry matter (DM). Analysis was made in triplicate.

Table 1. Physicochemical Characteristics of Blackberry Species[a]

characteristic	*Rubus glaucus*	*Rubus adenotrichus*
soluble solids (°Brix)	10.2 ± 0.1	12.0 ± 0.1
moisture content (%/FW)	83.5 ± 0.5	81.5 ± 0.5
pH	2.98 ± 0.01	2.83 ± 0.01
titratable acidity (g of citric acid/100 g of FW)	2.55 ± 0.01	2.67 ± 0.01

[a] Values are the mean of three analyses.

Detection of Ellagitannins. Ellagitannins were analyzed as proposed by Bate-Smith (*17*). The esters from the hexahydroxydiphenic acid (HHDP) and the glucose are oxidized by nitrous acid under nitrogen. The reaction produces a blue coloration that could be measured at 600 nm. One milliliter of crude extract was mixed with 1 mL of methanol and 160 μL of 6% acetic acid, after which the oxygen was expelled by nitrogen sparging for 10 min; finally, 160 μL of 6% sodium nitrite was added followed by a brief sparging. The tube was hermetically sealed and the reaction developed within 60 min in a water bath at 30 °C.

Acid Hydrolysis. The method used was adapted from that described by Vrhovsek et al. (*18*). It is based on acid hydrolysis in an oil bath, followed by an HPLC quantitative analysis of free ellagic acid. Twenty milliliters of crude extract was evaporated to dryness and dissolved in 60 mL of MeOH/4 M HCl, and aliquots (5 mL) were subjected to hydrolysis for different lengths of time (1, 2, 3, 5, and 6 h) at 100 °C to determine the maximum ellagic acid recovery. The volume was then adjusted to 10 mL before HPLC analysis. Ellagic acid was quantified at 254 nm, using a calibration curve established with ellagic acid standard (concentration range of 25–300 mg/L, $R^2 = 0.991$). Analysis was made in triplicate.

HPLC-MS^n and DAD Analysis. Samples were filtered through a 0.45 μm filter (Millipore). The HPLC analysis was carried out on a Waters 2690 HPLC system equipped with a Waters 996 DAD (Waters Corp., Milford, MA) and Empower Software (Waters). The separation was performed at 30 °C using a 250 mm × 4.6 mm i.d., 5 μm, endcapped reversed-phase Lichrospher ODS-2 (Interchim, Montluçon, France). The solvents were 2% aqueous formic acid (solvent A) and acetonitrile/water/formic acid (80:18:2, v/v/v; solvent B). Anthocyanins and ellagitannins were analyzed using the following gradient: from 5 to 25% B in 50 min and from 25 to 100% B in 10 min, after which the column was washed during 5 min and equilibrated for 15 min. For other phenolic compounds, gradient conditions were as follows: from 5 to 10% B in 4 min, from 10 to 16% B in 4 min, from 16 to 25% B in 32 min, from 25 to 35% B in 15 min, from 35 to 80% B in 12 min, isocratically during 5 min, and then to 100% B in 3 min, at a flow rate of 0.5 mL/min. The injection volume was 10 μL, and detection was carried out between 200 and 600 nm. After passing through the flow cell of the diode array detector, the column eluate was split and 0.25 mL/min was directed to an LCQ ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan, San Jose, CA). Experiments were performed in both negative and positive ion modes. Scan range was 100–2000 and scan rate, 1 scan/sec. The desolvation temperatures were 250 and 300 °C in the positive and negative ion modes, respectively. High spray voltage was set at 5000 V. Nitrogen was used as the dry gas at a flow rate of 75 mL/min. MS/MS and MS^3 were carried out using helium as the target gas, and the collision energy was set at 30–35 and 50%, respectively. Identifications were achieved on the basis of the ion molecular mass, MS^n, and UV–visible spectra.

Quantitative Analysis. The HPLC analysis was carried on a Dionex liquid chromatograph equipped with model P680 pumps, an ASI 100 autosampler, and a UVD 340U diode array detector coupled to a HP ChemStation (Dionex, France), with the same column as described above. The injection volume was 20 μL. For total ellagic acid quantitative analysis, gradient elution was performed with solution A composed of water/orthophosphoric acid (99.9:0.1, v/v) and solution B composed of methanol/orthophosphoric acid (99.9/0.1, v/v) delivered at a flow rate of 1 mL/min as follows: from 0 to 30% B in 3 min, from 30 to 50% B in 5 min, from 50 to 70% B in 5 min, and finally from 70 to 80% B in 5 min. The column was washed with 100% of B for

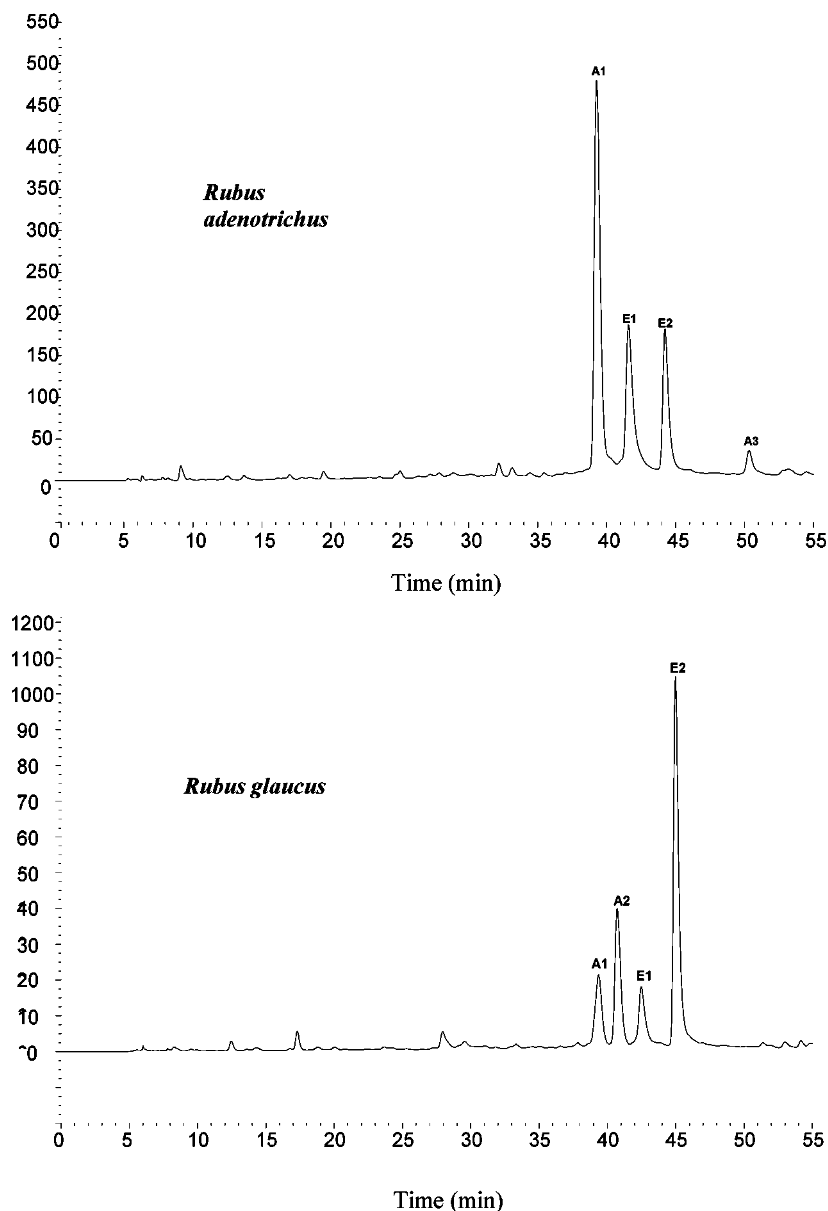

Figure 1. Segment from 0 to 55 min of LC-DAD chromatograms at 280 nm of aqueous acetone extracts of blackberries. Peak annotations refer to **Table 2**.

2 min and then equilibrated for 15 min prior to each analysis. Quantification was carried out at 254 nm. For other phenolics, gradient conditions were the same as used for identification. Quantification of both ethyl acetate and acetone extracts was achieved at 280 nm using calibration curves established with authentic standards. Free and conjugated forms of gallic acid were expressed as milligrams of gallic acid per 100 g of dry matter, free and conjugated forms of ellagic acid as well as ellagitannins as milligrams of ellagic acid, conjugated forms of hydroxycinnamic acids as milligrams of *p*-coumaric, caffeic, and ferulic acids, and quercetin and kaempferol glycosides as their corresponding aglycones. Epicatechin was quantified with commercial standard. For anthocyanins, quantification was made at 510 nm using cyanidin 3-*O*-glucoside standard. Correlation coefficients ranged from 0.994 to 0.999. Analysis was made in triplicate.

RESULTS AND DISCUSSION

Physicochemical Analysis. According to their respective Brix/acid ratios (4.0 and 4.5 for *R. glaucus* and *R. adenotrichus*, respectively), the collected berries were harvested at full-ripe stage (**Table 1**). It must be emphasized that blackberries from Costa Rica (*R. adenotrichus*) have a slightly higher soluble solids content and a lower pH than those from Ecuador (*R. glaucus*).

Identification of Chromatographic Peaks. Figure 1 shows the LC-DAD chromatograms of aqueous acetone extracts of blackberries (*Rubus glaucus*, *R. adenotrichus*). Peaks E1, E2, and A1 are common to both blackberries, whereas peak A2 was found in only *R. glaucus*. UV–visible characteristics and LC-MS data are given in **Table 2**. Glucose and rhamnose were tentatively identified by sugar analysis after acid hydrolysis. On the basis of LC-MS data and the identity of anthocyanins previously established in blackberry, peaks A1, A2, and A3 were identified as cyanidin glucoside, cyanidin rutinoside, and cyanidin malonyl-glucoside, respectively (*19*). Other major peaks in the chromatograms, E1 and E2, of blackberry aqueous acetone extracts had UV characteristics of ellagitannins. The negative ion mass spectra recorded from *m*/*z* 150 to 2000 showed molecular ions at *m*/*z* 1401 (peak E1) and *m*/*z* 1869 (peak E2). Zoom scan showed peak E1 to be doubly charged, giving a true mass of 2804 with another doubly charged fragment at *m*/*z* 1250 (M – 302, loss of HHDP unit). MS^2

Table 2. Identification of Free and Conjugated Forms of Phenolic Compounds in Extracts of Berries by Using Their Spectral Characteristics in LC-DAD, Positive or Negative Ions in LC-MS and MS^n, Respective Standards, and Previous Identification Data[a]

peak	LC-DAD t_R (min)	LC-DAD data (nm)	LC-MS data (m/z)[b] MS	LC-MS data (m/z)[b] MS^2/MS^3	tentative identification
gallic acid, galloyl esters					
1	12.3	232, 272	169 (−)		gallic acid (std)
3, 22	15.7, 41.8	236, 284	ND		galloyl esters
6, 26	19.2, 47.4	234, 286	ND		galloyl esters
13, 18	31.6, 36.8	238, 286	ND		galloyl esters
conjugated forms of hydroxycinnamic acids					
7	20.5	240, 300sh, 326	ND		caffeic acid ester
8	22.6	238, 300sh, 314	ND		*p*-coumaric acid ester
9	24.1	238, 300sh, 312	ND		*p*-coumaric acid ester
10A	26.5	238, 300sh, 314	ND		*p*-coumaric acid ester
12	29.3	238, 300sh, 328	ND		ferulic acid ester
15	33.3	238, 300sh, 328	ND		ferulic acid ester
19A	37.8	238, 300sh, 328	ND		ferulic acid ester
flavan-3-ols					
10B	27	278	91 (+)	273, 165, 139, 123	(−)epicatechin (std)
free and conjugated forms of ellagic acid					
19B, 21	37.1, 39.2	254, 362	433 (−)	301/257, 229	ellagic acid pentosides
24A	42.8	254, 368	301 (−)		ellagic acid (std)
24B	43.4	254, 362	463 (−)	301/257, 229	ellagic acid glucoside
32	54.4	254, 364	447 (−)	315/301	methyl-ellagic acid pentoside
33A	56.1	254, 368	ND		ellagic acid derivative
35	59	254, 364	463 (−)	301	ellagic acid glucoside
flavonols					
25A	45	256, 300sh, 354	477 (−)	301/179, 151	quercetin glucuronide
25B	45.2	256, 300sh, 354	463 (−)	301/179, 151	quercetin glucoside
28	50.3	256, 300sh, 354	505 (−), 551 (+)	301/179, 151	quercetin glucoside-malonate
29	52.2	256, 300sh, 356	491 (−)	463/301	quercetin derivative
30	53.6	266, 300sh, 348	447 (−)	285	kaempferol glucoside
31	54.2	266, 300sh, 348	461 (−)	285	kaempferol glucuronide
34	58.5	266, 300sh, 348	489 (−) 535 (+)	285	kaempferol glucoside-malonate
37	60.8	266, 300sh, 348	475 (−)	447/285	kaempferol derivative
anthocyanins[c]					
A1	39.3	280, 516	449 (+)	287	cyanidin-3-glucoside
A2	40.7	280, 516	595 (+)	449, 287	cyanidin-3-rutinoside
A3	50.3	280, 516	535 (+)	287	cyanidin-3-malonyl glucoside
ellagitannins[c]					
E1	42.1	240, 254sh	1401 (−)	1869, 1567, 1265, 633	lambertianin C
E2	44.6	240, 254sh	1869 (−)	1567, 1265, 633	sanguiin H-6
unknown compounds					
2	13.1	238, 296	ND		
4	16.4	234, 260	ND		
5, 17	17.6, 35.3	260, 296	ND		
11	28.9	263	ND		
14	32.5	266	ND		
27	49.2	292, 330	ND		
16	33.8	275	ND		
20, 23	39.7, 42.6	238, 260	ND		
33B	56.2	ND	ND		
36	60.2	266, 300sh, 348	ND		

[a] Abbreviations: ND, not detected; sh, maximum of the shoulder in the spectrum; std, standard. [b] In MS-MS, the most abundant parent ion in LC-MS is fragmented. [c] Retention times refer to **Figure 3**. (+), positive mode; (−), negative mode.

fragmentation, carried out with 30% energy, produced ions at *m/z* 1869, 1567 (1869 – 302, loss of HHDP), 1265, and 633. On the basis of these fragmentation patterns and literature data (*20, 21*), compound **E1** is tentatively identified as lambertianin C, a trimer of casuarictin/potentillin. This compound has been previously detected in leaves of *R. lambertianus* (*22*). MS full scan of compound **E2** showed an $[M - H]^-$ at *m/z* 1869 with an MS^2 fragmentation pattern matching that of lambertianin C. On the basis of the mass spectra and previously published data, peak E2 is tentatively identified as sanguiin H-6, the presence of which has been reported in *Ribes* species (*22, 23*), accompanied almost invariably by lambertianin C (**Figure 2**). However, only NMR experiments allow unambiguous identification.

To analyze minor compounds, aqueous acetone extracts of blackberries were partitioned with ethyl acetate. Thus, anthocyanins and ellagitannins, hydrophilic compounds, were separated from other phenolic compounds that are present in the ethyl acetate fraction. **Figure 3** shows the LC-DAD chromatograms of these ethyl acetate extracts. The peaks are identified with numbers (1–37) and letters for overlapping or coeluting peaks (e.g., 25A and 25B) following the elution order in LC-

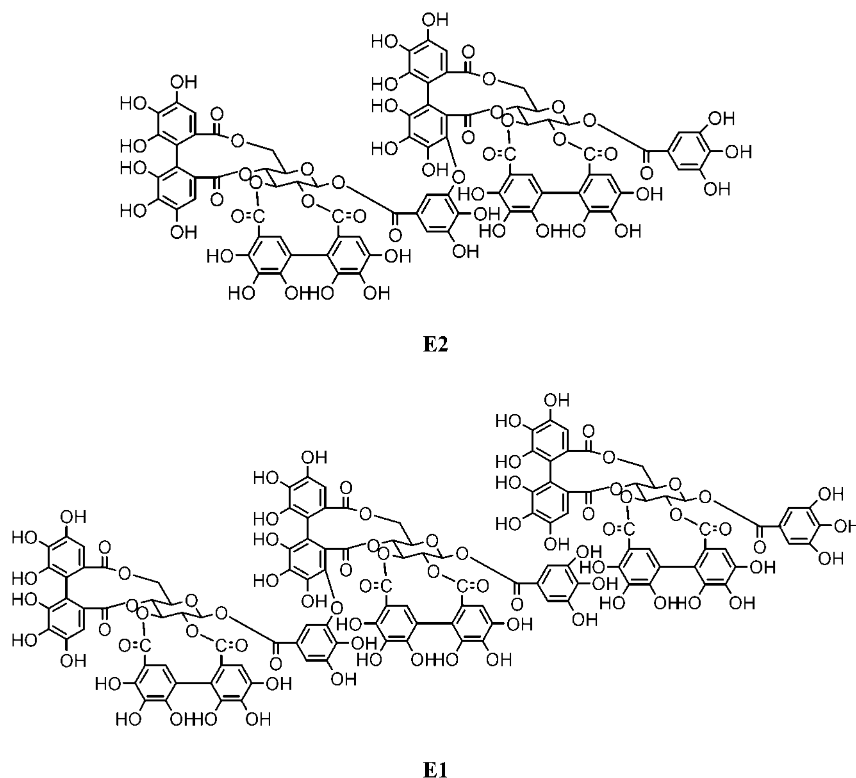

Figure 2. Structures of ellagitannins detected in blackberry extracts: lambertianin C (**E1**) and sanguiin H-6 (**E2**).

DAD and LC-MS chromatograms. Except for peaks 4, 22, 30, 31, 34, 36, and 37, which are absent in *R. glaucus*, and peaks 3, 21, 24B, and 26, which are more important in this variety, the profiles are similar. Other peaks are present in both blackberries with peaks 25A and 25B forming a part of major compounds.

Peaks in the chromatograms were classified into free and conjugated forms of hydroxycinnamic acids, gallic acid, ellagic acid, flavonols, and flavan-3-ols, by comparison of their UV–visible spectra with those of the corresponding available standard aglycones: gallic acid, ellagic acid, ferulic acid, caffeic acid, *p*-coumaric acid, (+)-catechin, (−)-epicatechin, quercetin, and kaempferol. Shifts in the UV–visible spectra, due to the esterification or the glycosylation of aglycones, were described previously (*24*).

LC-MS and the subsequent fragmentation of the predominant positive and negative ions in MS-MS were used to obtain more information about the molecular masses of conjugates and the structures of aglycones. MS^3 was used to differentiate ellagic acid and quercetin conjugates (*20*). Whenever possible, chromatographic retention and literature data were used to support the identification of the peaks. Characterization of the individual peaks was performed according to data presented in **Table 2**.

Free and Conjugated Forms of Gallic Acid. Peak 1 was identified as gallic acid, according to MS data in the negative mode (*m/z* 169) and cochromatography with an authentic standard (**Figure 3**). UV spectra features were used as described previously (*25*). In addition, after alkaline hydrolysis, these peaks completely disappeared, and an increase of gallic acid was observed (peak 1). No LC-MS data were obtained for the estimation of the molecular size of the galloyl esters. Similar results were obtained by Määttä et al. (*26*) and were supposed to be due to the strong background noise.

Conjugated Forms of Hydroxycinnamic Acids. No free hydroxycinnamic acid was found in the extracts by comparison with retention times of authentic standards. Free hydroxycinnamic acids are infrequently reported in fruits, and their occurrence is generally due to environmental stress factors (*27, 28*). Alkaline hydrolysis of extracts yielded caffeic acid (27.1 min), ferulic acid (45.6 min), and *p*-coumaric acid (39.1 min), whereas the esters were not detected in the chromatograms. Classification of peaks as esters of caffeic acid (peak 7), *p*-coumaric acid (peaks 8, 9, 10A), and ferulic acid (peaks 12, 15, 19A) was based on the retention times and the bathochromic shifts in their UV–visible maxima due to esterification. The signals for these esters were not clearly detected in LC-MS as previously mentioned (*26*), which hampered further identification of these conjugated forms. The composition of conjugated forms of hydroxycinnamic acids of these blackberries was consistent with those reported in previous studies (*3, 24, 29*).

Flavan-3-ols. Peak 10B was identified as (−)-epicatechin by its UV spectrum and LC-MS data, which are described in previous studies (*24, 30*) (**Table 2**). Berries are known to contain catechin and epicatechin with various amounts depending on *Rubus* species (*31*). Dimer B2 was also found in raspberry and cloudberry (*26*). In this study, only epicatechin was detected in our extracts.

Free and Conjugated Forms of Ellagic Acid. The presence of free ellagic acid (peak 24A) in samples was confirmed by its retention time and MS data (*m/z* 301). Peak 33A was distinguished as an ellagic acid derivative on the basis of its UV–visible spectrum being almost similar to that of ellagic acid (**Table 2**), but no further data were obtained for its tentative identification from LC-MS analysis. The hypsochromic shifts (4–6 nm) in UV–visible spectra of peaks 19B, 21, 24B, 32, and 35 suggested glycosylated forms of ellagic acid. Peaks 19B and 21 showed an $[M - H]^-$ at *m/z* 433 with MS-MS data at *m/z*301. The loss of 132 mass units (433 – 301) could be attributed to a pentose unit. Previously, ellagic acid arabinoside and xyloside have been identified in berries (*14*). The MS^2 ion at *m/z* 301 could either be ellagic acid or quercetin. MS^3 on *m/z* 301 afforded ions at *m/z* 257 and 229, which have been

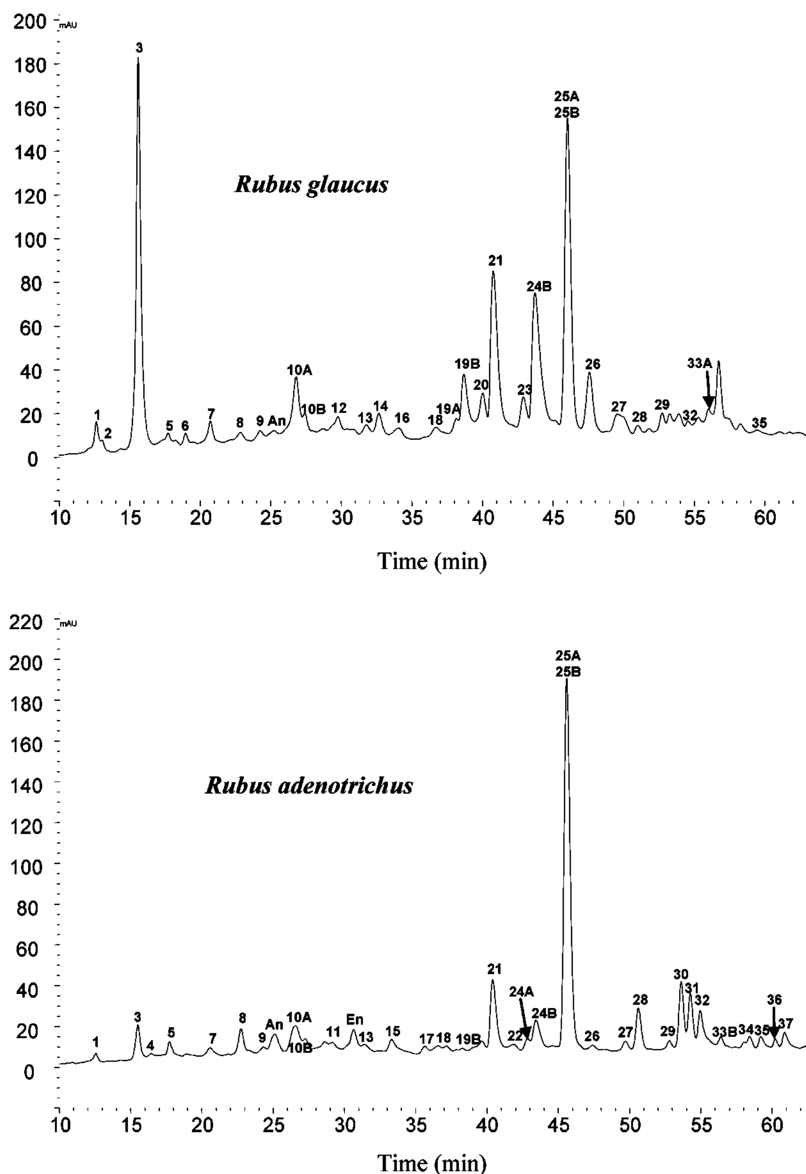

Figure 3. Segment from 10 to 63 min of LC-DAD chromatograms at 280 nm of ethyl acetate extracts of blackberries. Peak numbers refer to **Table 2**. An, anthocyanin residue; En, ellagitannin residue.

previously established as ellagic acid-like ions, whereas quercetin afforded ions at *m/z* 179 and 151 (*20*). Peaks 24B and 35 had an $[M - H]^-$ at *m/z* 463 with a fragmentation pattern of *m/z* 301 (loss of a hexose) and were tentatively identified as ellagic acid hexosides, previously detected in pomegranate juices (*32*). Peak 32 showed a molecular ion at *m/z* 447, which yielded MS^n fragments at *m/z* 315 (M – 132, loss of pentose) and 301. This peak could be a methyl ellagic acid pentose conjugate as described by Mullen et al. (*20*). After acid hydrolysis of ethyl acetate extract, only glucose was detected. Hexosides were therefore assumed to be glucosides of ellagic acid. Conversely, after hydrolysis no pentose could be identified because of lower concentrations.

Flavonol Glycosides. UV spectra of peaks 25A, 25B, 28, and 29 are similar to those of quercetin glycosides, whereas peaks 30, 31, 34, and 37 showed UV spectra similar to those of kaempferol derivatives (**Table 2**). After acid hydrolysis, only quercetin and kaempferol aglycones were observed, but none of them was detected in our extracts before hydrolysis. Although glucose is the most common sugar in flavonoid glycosides, flavonol galactosides and glucuronides were also identified in berries (*20, 21, 33*). However, as mentioned above, analysis of sugars revealed only glucose, suggesting the compounds as being flavonol glucosides. Further experiments are required for a more complete structural identification. Recently, flavonoid glycoside isomers have been differentiated by electrospray tandem mass spectrometry, by distinguishing the O- and C-glycosides, and by identifying the position of attachment of the glycoside (*34*), showing the difficulty of unequivocally identifying such compounds. On the basis of their fragmentation patterns (**Table 2**) and previously published data (*35*), peaks 25A and 25B could be identified as quercetin glucuronide and glucoside, respectively. In the same way, peaks 30 and 31 were identified as kaempferol glucoside and glucuronide, respectively. Peak 28 showed a molecular ion at *m/z* 505 in the negative ion mode and at *m/z* 551 in the positive ionization mode, respectively (**Table 2**). In the negative ionization mode, 44 mass units were lost from the pseudomolecular ion, which may be due to a loss of a carboxylic function. MS-MS in the positive ionization mode showed a loss of 248 mass units from the parent compound (peak at *m/z* 303).). As previously described, the loss of this mass unit and a UV–visible spectrum similar to those of other flavonol glycosides indicate the presence of a malonyl group attached to the glycosyl part of the molecule (*36*). In the

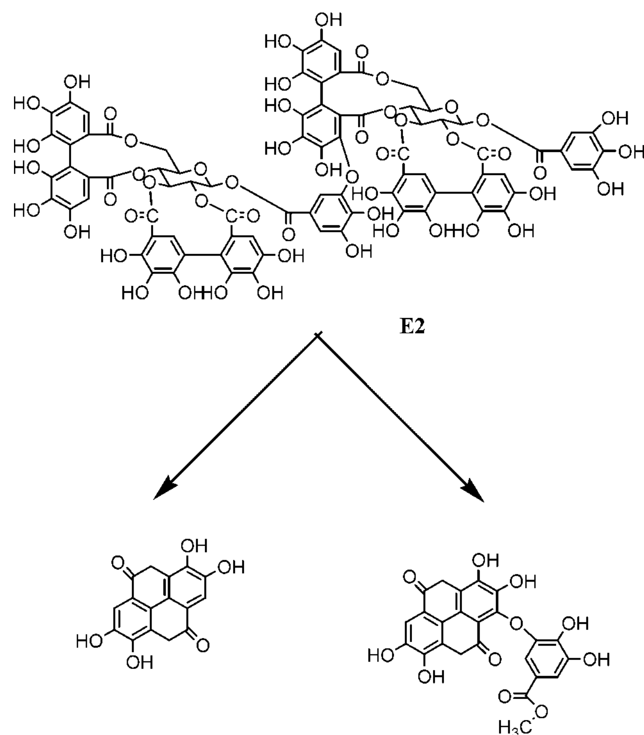

Figure 4. Schematic representation of *Rubus* spp. ellagitannin acid hydrolysis in methanol. Complex oligomeric ellagitannins such as sanguiin H-6 (**E2**) give rise to ellagic acid (**3**) and methyl sanguisorboate (**2**).

same way, peak 34 showed MS data characteristic of the corresponding kaempferol derivative. Peak 28 was therefore identified as quercetin glucoside malonate and peak 34 as kaempferol glucoside malonate. Although acylated flavonol glycosides are considered to occur rarely in berries (*37*), a study has shown the presence of quercetin and kaempferol hexoside malonates in black, green, and white currants (*24*). Peak 29 showed a UV spectrum similar to that of quercetin glycoside (254, 356 nm), with a molecular ion at *m/z* 491 and MS-MS fragments at *m/z* 463 and 301. This compound seems to be a derivative of quercetin glucoside, but further data are needed for a more complete structural identification. The corresponding kaempferol derivative was also observed (peak 37).

Unidentified Peaks in the Ethyl Acetate Extracts. Some peaks remained without identification according to LC-DAD and LC-MS data (**Table 2**). Peak 36 displayed a UV spectrum similar to that of kaempferol glycoside, but no MS data were obtained. Isolation and NMR identification are needed for an unequivocal structural elucidation of all unidentified peaks.

Identification of Hydrolytic Products of Ellagitannins. Following acid hydrolysis of red raspberry juices samples, Rommel and Wrolstad (*38*) reported the presence of two unidentified compounds, together with ellagic acid, with absorbance spectra very similar to that of ellagic acid. An unknown ellagic acid derivative was also observed by Määttä-Riihinen et al. (*26*), after acid hydrolysis of ellagitannins, and a similar observation was reported by Mattila and Kumpulainen (*39*) in strawberry samples. Recently, ellagic acid and two other compounds with DAD spectra similar to that of ellagic acid have been detected in hydrolyzed *Rubus* extracts, and one of them was identified as methyl sanguisorboate (**Figure 4**) (*18*).

The HPLC chromatogram at 254 nm of blackberry hydrolyzed extract shows three peaks with UV spectra similar to that of ellagic acid, which were not detected before hydrolysis (data not shown). In addition, no ellagitannin was found after hydrolysis. The major one was identified as ellagic acid on the basis of its retention time, UV–visible spectrum (λ_{max} at 254 and 366 nm), and MS data (molecular ion at *m/z* 301 in the negative ion mode), which were identical to those of the standard. The UV–visible absorption spectra of the two other peaks showed a bathochromic shift (λ_{max} = 370 and 369 nm, respectively) in comparison to ellagic acid. The MS full scan showed molecular ions at *m/z* 483 and 469, which fragmented to *m/z* 315, 301 and *m/z* 301 ions, respectively. These compounds were tentatively identified as methyl sanguisorboate and sanguisorbic acid (*40*). In a previous study, both methyl sanguisorboate and sanguisorbic acid concentrations increased rapidly and became constant after 6 and 4 h of hydrolysis, respectively, with a lower amount of sanguisorbic acid (*18*). In our case, when hydrolysis was carried out at 100 °C, sanguisorbic acid was predominant after 1 h of reaction, and its concentration continued to increase until 4 h while the amount of methyl sanguisorboate decreased. Afterward, the opposite phenomenon was observed, suggesting hydrolysis and methylation reactions. This is confirmed by the fact that the sum of peak area of corresponding compounds is quite constant during acid hydrolysis.

Total Polyphenols (TP). All flavonoids, anthocyanins, and nonflavonoid phenolic compounds were included in this determination. Blackberries had an average concentration of TP of 4250 mg/100 g of DM (±350 mg) and 6300 mg/100 g of DM (±100 mg) in *R. adenotrichus* and *R. glaucus*, respectively. It is difficult to compare the gallic acid content with results found in the literature because most of the authors did not use the same analytical method. Indeed, TP was generally overestimated because it included non-phenolic compounds, such as sugars or ascorbic acid, which interfere in the Folin–Ciocalteu reaction as described by George et al. (*16*). Nevertheless, TP content is higher than those mentioned in the literature (*3, 5, 41*), suggesting that these blackberries have a high antioxidant potential.

Total Soluble Ellagic Acid Content. Several methodologies for the acid hydrolysis of ellagitannins are described in the literature (*13, 42, 43*). In this study, the method used was adapted from that recently described by Vrhovsek et al. (*18*). The release of ellagic acid becomes constant after 5 h of hydrolysis. This is in agreement with results previously obtained (*18*). Total soluble ellagic acid content was significantly higher in *R. glaucus* (3.26 g/100 g of DM) than in *R. adenotrichus* (1.33 g/100 g of DM), and similar values were detected for individual ellagitannins and ellagic acid conjugates.

Contents of Phenolic Classes. The contents of selected phenolic classes of the blackberries studied are shown in **Table 3**. Results were expressed in milligrams per 100 g of dry matter for the weight of standard. In agreement with previous papers, ellagitannins are the major phenolic class that characterizes *Rubus* species (*26, 44*). Ellagitannins were analyzed with two different methods: in the soluble form and in the form of conversion products after acid hydrolysis (ellagic acid equivalents in aqueous/acetone extracts). In accordance with results obtained for total soluble ellagic acid content, ellagitannin amounts were higher in *R. glaucus*, due to the high amount of sanguiin H-6 (2450 mg/100 g of DM) (**Table 3**). Ellagic acid glycosides deconjugate similarly to ellagic acid in acid hydrolysis and thus participate in the total soluble ellagic acid content. Our results obtained after acid hydrolysis were similar to those when ellagitannins and conjugated forms of ellagic acid were extracted with 70% acetone and quantified using ellagic acid as a standard.

Table 3. Contents[a] of Phenolic Compounds in Blackberry[b]

phenolic compounds (peak)[c]	*Rubus adenotrichus*	*Rubus glaucus*
free and conjugated forms of gallic acid		
gallic acid (1)	0.46 ± 0.02	1.8 ± 0.1
galloyl esters (3, 6, 13, 18, 22, 26)	4.9 ± 0.4	31.5 ± 1.5
conjugated forms of hydroxycinnamic acids		
caffeoyl esters (7)	1 ± 0.1	2.6 ± 0.3
p-coumaroyl esters (8, 9, 10A)	4.2 ± 0.3	6.3 ± 0.1
feruloyl esters (12, 15, 19A)	1.54 ± 0.02	3.8 ± 0.1
flavan-3-ols		
(−)epicatechin (10B)	5.1 ± 0.3	6.3 ± 0.5
free and conjugated forms of ellagic acid		
ellagic acid pentosides (19B, 21)	13.5 ± 0.2	43.8 ± 0.2
methylellagic acid pentoside (32)	7.5 ± 0.2	2 ± 0.1
ellagic acid hexoside (24B, 35)	8.4 ± 0.3	33.4 ± 1
ellagic acid (24A)	2 ± 0.1	ND
ellagic acid derivative (33A)	ND	4.3 ± 0.3
flavonols		
quercetin glucuronide (25A) glucoside (25B)	57 ± 2	51 ± 2
quercetin glucoside-malonate (28)	6 ± 0.3	1.6 ± 0.1
quercetin derivative (29)	1.3 ± 0.1	3.2 ± 0.1
kaempferol glucoside (30)	7.3 ± 0.2	ND
kaempferol glucuronide (31)	5.6 ± 0.4	ND
kaempferol glucoside-malonate (34)	1.9 ± 0.1	ND
kaempferol derivative (37)	2.1 ± 0.1	ND
anthocyanins		
cyanidin-3-glucoside (A1)	680 ± 20	380 ± 20
cyanidin-3-rutinoside (A2)	ND	630 ± 20
cyanidin-3-malonyl glucoside (A3)	40 ± 3	ND
ellagitannins		
lambertianin C (E1)	598 ± 20	520 ± 30
sanguiin H-6 (E2)	420 ± 17	2450 ± 100

[a] Contents of ethyl acetate and aqueous acetone extractable phenolic compounds together are expressed in milligrams per 100 g of dry matter for the weight of standard. The mean values are for triplicate assays. [b] Abbreviations: ND, not detected. [c] Peak numbers refer to the identified phenolic compounds in our extracts (**Table 2**).

The second major phenolic class, anthocyanins, was consistent with the literature (*26, 44*). Anthocyanin content was higher for *R. glaucus* blackberry, approximately 1000 mg/100 g of DM (**Table 3**). Both blackberries contained cyanidin glucoside, which was major in *R. adenotrichus* (95%). Cyanidin rutinoside was major in *R. glaucus* (62%), and acylated cyanidin malonyl glucoside was not detected in this species.

Flavonols, conjugated forms of gallic and hydroxycinamic acid, were minor phenolic classes. We controlled by HPLC the full extraction by ethyl acetate of these phenolic compounds. The content of hydroxycinnamic acids (mg/100 g of DM) was 6.7 in *R. adenotrichus* and 12.7 in *R. glaucus*, in accordance with previously published studies on *Rubus* species (*39, 41*). In both blackberries, *p*-coumaric acid esters were the most abundant and caffeic acid ester the minor one (**Table 3**). Quercetin was found mainly as glucoside and glucuronide (88–91% of total quercetin glycoside content). Kaempferol was detected only in *R. adenotrichus* with glucoside and glucuronide being the predominant forms (41 and 31%, respectively). For flavan-3-ols, only epicatechin was detected (5–6 mg/100 g of DM).

The identification and quantification of phenolic compounds of blackberries (*R. glaucus* and *R. adenotrichus*) were achieved for the first time. Ellagitannins were the major compounds, which is typical of *Rubus* species. The content of ellagitannins estimated by acid hydrolysis was consistent with the values obtained for soluble ellagitannins. *p*-Coumaric acid was the predominant hydroxycinnamic acid esterified. Quercetin glycosides were the predominant flavonol glycosides. The lack of kaempferol glycosides in *R. glaucus* distinguished the two blackberry species studied. Flavonol glycoside malonates were also detected. Major anthocyanins were cyanidin glucoside and cyanidin rutinoside, which was present in *R. glaucus* only. Flavonol and anthocyanin composition could be used for distinguishing the two species.

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