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ARTICLE

Profiling and Accurate Quantification of *Rubus* Ellagitannins and Ellagic Acid Conjugates Using Direct UPLC-Q-TOF HDMS and HPLC-DAD Analysis

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Accurate quantification and structural characterization of ellagitannins and ellagic acid conjugates in food, beverages, and food supplements are essential starting points for studying their effect on human health. However, accuracy is hindered both by the lack of pure standard compounds and by methods that maintain the compounds in their native form, avoiding any chemical modification of the structure. The objective of this work was to develop a new method for the purification, chromatographic separation, and accurate quantification of ellagitannins and ellagic acid conjugates to provide thorough characterization of the diversity in composition of 11 *Rubus* cultivars grown in Trentino, Italy. As such, two major steps were required: (i) the isolation and purification (with associated detailed structural characterization and determination of their molar extinction coefficients) of sanguiin H-6 and lambertianin C, providing essential data for their use, together with ellagic acid, as external standards, and (ii) the determination of the chemical structure of 20 novel minor ellagitannins and 4 ellagic acid conjugates on the basis of their Q-TOF-HDMS and DAD spectra. This survey of ellagitannins and ellagic acid conjugates provides evidence for the existence of significant differences in the pattern between and within blackberry and raspberry cultivars. To our knowledge, this is the first paper that has combined detailed metabolite profiling with accurate quantification of the main ellagitannins in *Rubus* using their respective standards.

KEYWORDS: *Rubus*; ellagitannins; ellagic acid; sanguiin H-6; lambertianin C; NMR; mass spectrometry; molar extinction coefficient

INTRODUCTION

Rubus berries, raspberries and blackberries, are considered to be a rich source of dietary antioxidants due to their high content of phenolic compounds (*1*, *2*). In particular, research has focused on their high level of ellagitannins (ET) and ellagic acid conjugates (EAC), due to the fact that ellagitannins are relatively uncommon in fruit and vegetables in our diet, being found only in a few fruits (*3*, *4*), such as strawberries (*Fragaria x ananassa* D.) (*5*), pomegranates (*Punica granatum* L.), muscadine grapes (*Vitis rotundifolia*), (*6*) some nuts (*7*), and raspberries (*Rubus idaeus* L.) and blackberries (*Rubus* sp.).

Ellagic acid has been reported to have antiviral (*8*) and antioxidant properties (*9*). It may also protect against colon (*10*), lung, esophagus, and other types of cancer (*11*−*13*). Ellagitannins have been reported to strongly inhibit NO production *in vivo* (*14*), as well as having lipase (*15*) and α-amylase inhibitory activity (*14*).

Ellagic acid and ellagitannins can be considered as putative chemoprotective agents due to their interaction with cellular pathways (*16*). However, in *Rubus* berries, free ellagic acid represents only a small part of the total ellagic acid pool (*17*) and ellagitannins are the primary source of dietary ellagic acid. Indeed, there is evidence that ellagitannins are hydrolyzed in the gut to release ellagic acid (*18*), which is further metabolized by the intestinal microflora to yield urolithins (*19*, *20*). Urolithins are a family of compounds deriving from the metabolism of ellagic acid, characterized by a common dibenzopyran-6-one structure with different numbers of hydroxyl substituents (*21*). In a recent paper, the way urolithins modulate phase I and/or phase II enzymes was investigated in an in vitro model of human cancer colon cell, in Caco-2 cells, and in rat colon mucosa (*22*). Ellagitannins, ellagic acid, and urolithins are poorly absorbed (*21*, *23*, *24*), while they have been shown to have anticancer effects against some types of cancer in cells and animal models. Unfortunately, the molecular pathways are still unknown and only some mechanisms of cellular interaction have recently been described (*25*). In conclusion, ellagitannins are a very interesting group of phenolic compounds, although their bioavailability and metabolism by colonic microflora merits further study (*26*).

Accurate quantification and structural characterization of ellagitannins and ellagic acid conjugates in food, beverages, and food supplements are essential starting points for studying their effects on human health. In particular, knowledge of the real

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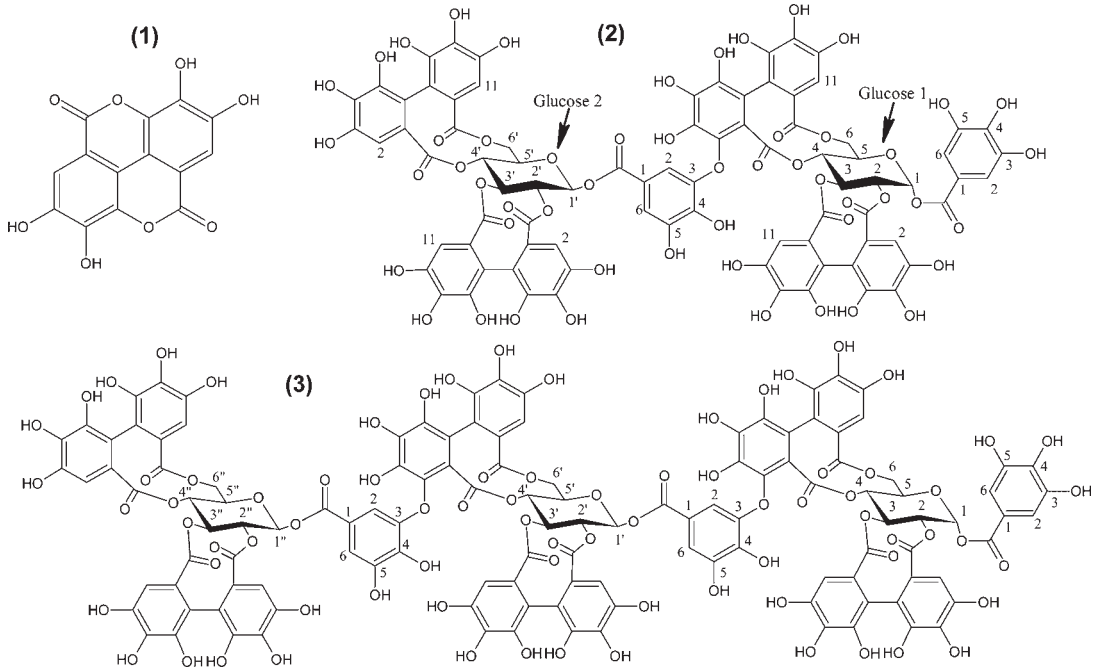

Figure 1. Structures of ellagic acid (**1**) and major *Rubus* ellagitannins, sanguiin H-6 (**2**), and lambertianin C (**3**).

amount of any single compound is essential to evaluate their biological and chemical effects in a given *in vivo* or *in vitro* experiment. However, high levels of accuracy are hindered by the lack of pure standard compounds and quantification methods that maintain the compounds in their native form (*27*), avoiding any chemical modification of the structure.

At the moment, the most widely used method for quantification of ET and EAC is HPLC analysis of free ellagic acid after chemical hydrolysis (*28*, *29*), which provides variable results depending on the conditions of extraction and acid hydrolysis (*30*) and loses information on the molecular structure of the native compound. An improvement on this method made it possible to include analysis of sanguisorboate and gallate moieties (*31*), thus allowing estimation of the mean degree of polymerization of *Rubus* ellagitannins. Such analytical approaches are relatively solid and adequate for routine quantification in agronomical studies, but obscure structural information which could be crucial for biological and nutritional studies. The structures of ellagitannins are completely different from that of free ellagic acid (**Figure 1**), and therefore, estimation on the basis of equivalents of ellagic acid (or indeed as sanguisorboyl esters) provides values that do not accurately describe the real concentration. The development and validation of a more solid and accurate method for direct quantification of ellagitannins, calibrated with appropriate standards, is essential (*32*).

This paper describes the development of a new method for the purification, chromatographic separation, and accurate quantification of ellagitannins and ellagic acid conjugates in raspberry and blackberry cultivars grown in Trentino, Italy.

MATERIALS AND METHODS

Standards and Solvents. All the chromatographic solvents were HPLC grade. Acetonitrile, methanol, and diethyl ether were purchased from VWR International (Milan, Italy). Hexane, formic acid, and ethyl ether were purchased from Carlo Erba (Milan, Italy). Ellagic acid standard (purity ≥96%) was purchased from Fluka (Steinheim, Germany).

Sampling. Blackberry (*Rubus fruticosus*) and raspberry (*Rubus idaeus* L.) berry samples of different cultivars were produced under standardized conditions (*33*) in a trial area in Vigolo Vattaro (Trentino, Italy) and sampled at berry maturity in three out of four consecutive years (from 2004, 2005, 2006, and 2007). The five varieties of blackberry, Apache, Chesapeake, Loch Ness, Thornfree, and Triple Crown, and six varieties of raspberry, two floricane fruiting (FC) raspberries (Octavia, Tulameen) and four primocanes (PC) (Himbotop, Pokusa, Polana, and Polka), were sampled, stored at 4 °C, and processed within 24 h.

Extraction of Polyphenols. Polyphenols were extracted following the method of Mattivi et al. (*34*). Before extraction, the fruit and extraction solution were cooled to 4 °C to limit enzymatic and chemical reactions. 60 g of fresh fruit was homogenized in a 847-86 model Osterizer blender at speed one, in 2 × 100 mL of an acetone/water (70/30 v/v) mixture for 1 min and made up to 250 mL with the same solvent. The centrifuged extracts were stored at −20 °C until analysis.

Isolation of Sanguiin H-6 and Lambertianin C from Cv. Polka Raspberry. Aqueous acetone raspberry extracts (200 mL) were evaporated until dryness in a pear-shaped flask, using rotary evaporation under reduced pressure at 40 °C. The sample was diluted to 100 mL with distilled water and filtered using a Durapore 0.22 μm filter (Millipore, Vimodrone, Italy). Isolation of sanguiin H-6 and lambertianin C was carried out in two consecutive chromatographic steps using a preparative HPLC Shimadzu SCL-10 AVP equipped with a Shimadzu SPD-10 AVP UV/vis detector, 8A pumps, and Class VP Software (Shimadzu Corp., Kyoto, Japan). The UV signal was recorded at 260 nm.

Step One. Flash chromatography was performed using ENV+ resin (IST, International Sorbent Technology, Hengoed Mid Glam, U.K.) to separate ellagitannins and their analogs from nonphenolic compounds and other polyphenols. A 150 mL Isolute SPE syringe column was packed with ENV+ resin (20 g) and activated with 300 mL of methanol and 400 mL of distilled water. One aliquot of extract (100 mL) was loaded onto the column using the solenoid valve at a flow rate of 10 mL/min, and the resin was washed with 300 mL of distilled water. Then the column was inserted in-line into the preparative HPLC system. The mobile phases used for flash chromatography were distilled water (solvent A) and methanol (solvent B). A linear gradient from 0% to 100% B in 60 min was performed. The flow rate was 10 mL/min. The main ellagitannins were eluted with 50% B while anthocyanins were eluted after 50% B. After this first separation, the partially purified fraction containing ellagitannins was collected, diluted to 50 mL with methanol/water (50/50 v/v), filtered using a Durapore 0.22 μm filter (Millipore, Vimodrone, Italy), and subjected to a second chromatographic step.

Step Two. The final separation of the ellagic acid conjugates was performed by preparative HPLC using a 250 mm × 50 mm 10 μm Discovery HS C18 column (Supelco, Bellefonte, PA). The column was protected by using a 2 μm PEEK filter (Gilson, Milano, Italy). The mobile

Table 1. NMR (400 MHz, 298 K) Assignments for Sanguiin H-6 in Hexadeuterated Acetone

moiety	signal	^{1}H NMR	^{13}C NMR (HSQC and HMBC)
α-glucose 1	1	6.52 (d, $J_{1,2}$ = 4.0)	90.70 d
OCO-1 (164.67 s)	2	5.26 (dd, $J_{1,2}$ = 4.0, $J_{3,2}$ = 9.4)	73.88 d
OCO-2 (168.12 s)	3	5.03 (dd, $J_{3,4}$ = 9.8, $J_{3,2}$ = 9.8)	75.49 d
OCO-3 (167.46 s)	4	5.01 (t, $J_{4,3} \approx J_{4,5}$= 9.8)	69.38 d
OCO-4 (167.40 s)	5	4.20 (dd, $J_{4,5}$ = 9.8, $J_{5,6}$ = 6.4)	71.23 d
OCO-6 (167.61 s)	6	3.88 (d, J_{gem} = 13.1)	63.27 t
		5.57 (dd, J_{gem} = 13.1, $J_{5,6}$ = 6.4)	
β-glucose 2	1′	6.16 (d, $J_{1',2'}$ = 8.5)	92.60 d
OCO-1′ (165.31 s)	2′	5.18 (t, $J_{1',2'} \approx J_{3',2'}$ = 8.5)	75.90 d
OCO-2′ (167.49 s)	3′	5.36 (dd, $J_{3',2'}$ = 8.5, $J_{4',3'}$ = 9.9)	77.39 d
OCO-3′ (168.96 s)	4′	5.10 (t, $J_{4',3'} \approx J_{4',5'}$ = 9.9)	69.04 d
OCO-4′ (167.48 s)	5′	4.35 (dd, $J_{6'5'}$ = 6.4, $J_{4',5'}$ = 9.9)	73.61 d
OCO-6′ (167.55 s)	6′	3.77 (d, J_{gem} = 12.9)	62.93 t
		5.25 (d, J_{gem} = 12.9, $J_{6'5'}$ = 6.4)	
central 3-O, 4,5-dihydroxybenzoate (sanguisorboyl)	1		118.28 s
	2	7.27 (d, $J_{2,6}$ = 2.0)	112.49 d
	3		147.93 s
	4		141.55 s
	5		147.93 s
	6	7.13 (d, $J_{2,6}$ = 2.0)	110.77 d
terminal 3,4,5-trihydroxybenzoate (galloyl)	1		120.03 s
	2 and 6	7.08 s, 2H	110.33 d
	3 and 5		145.77 s
	4		139.51 s
selected signals for 2,3 HHDP on glucose 1	2	6.37 s	107.18 d
	11	6.29 s	107.38 d
4,6HHDP on glucose 1	2	6.77 s	108.55 d
2′,3′HHDP on glucose 2	2	6.47 s	107.61 d
	11	6.30 s	108.21 d
4′,6′HHDP on glucose 2′	2	6.45 s	107.69 d
	11	6.78 s	108.57 d

phases were distilled water (solvent A) and acetonitrile (solvent B). The column was conditioned with 14% B. The sample (50 mL) was evaporated using rotary evaporation under reduced pressure at 40 °C, reconstituted with water, and loaded onto the column. Separation was achieved using a linear gradient from 14% B to 26.5% B in 50 min at a flow rate of 40 mL/min, and the elution was followed at 260 nm. After separation, sanguiin H-6 and lambertianin C were dried using rotary evaporation under reduced pressure and then dissolved in diethyl ether and crystallized in hexane. The pure sanguiin H-6 and lambertianin C were recovered by filtration as a pale-yellow powder which was further characterized by NMR, MS, CD, and UV spectroscopy.

NMR Measurements. NMR spectra (^{1}H NMR and gradient-enhanced COSY, NOESY, HSQC, and HMBC) for sanguiin H-6 and lambertianin C were recorded in hexadeuterated acetone (99.90% CD_3COCD_3) at 298 K on a Bruker-Avance 400 MHz NMR spectrometer by using a 5 mm BBI probe with a 90° proton pulse length of 8.7 μs at a transmission power of 0 db and equipped with a pulsed-gradient field utility. The chemical shift scale (δ) was calibrated on the residual proton signal of deuterated acetone at δ_H 2.040 ppm and δ_C 29.80 ppm.

Molecular mechanical calculations were carried out using the GMMX computer program as implemented in PCMODEL 7.0. [PCMOD 7.0/GMMX version 1.5, Serena Software, P.O. Box 3076, Bloomington, IN]. All the minimized structures falling within a strain-energy window of 3.0 kcal/mol were saved and finally minimized with MMX force fields, keeping only those falling within a 2.0 kcal/mol window. Experimental data are reported in **Tables 1** and **2**.

UV Measurements. The UV spectra of ellagic acid, sanguiin H-6, and lambertianin C were recorded in both methanol and ethanol, on a Hitachi U-2000 spectrometer (Tokyo, Japan). The following molar extinction coefficients were observed: (in methanol for ellagic acid) $\varepsilon_{254\ nm}$ = 40686 M^{-1} cm^{-1}, $\varepsilon_{260\ nm}$ = 32099 M^{-1} cm^{-1}; (in methanol for sanguiin H-6) $\varepsilon_{260\ nm}$ = 72070 M^{-1} cm^{-1}; (in methanol for lambertianin C) $\varepsilon_{260\ nm}$ = 104344 M^{-1} cm^{-1}; (in ethanol for ellagic acid) $\varepsilon_{254\ nm}$ = 38806 M^{-1} cm^{-1}; (in ethanol for sanguiin H-6) $\varepsilon_{260\ nm}$ = 80566 M^{-1} cm^{-1}; (in ethanol for lambertianin C) $\varepsilon_{260\ nm}$ = 109631 M^{-1} cm^{-1}. The molar extinction coefficients under the conditions suggested for HPLC analysis (solvent: 88% of acetonitrile and 12% of 1% formic acid in water; v/v) with UV detection were as follows: (ellagic acid) $\varepsilon_{260\ nm}$ = 28266 M^{-1} cm^{-1}; (sanguiin H-6) $\varepsilon_{260\ nm}$ = 63615 M^{-1} cm^{-1}; (lambertianin C) $\varepsilon_{260\ nm}$ = 95744 M^{-1} cm^{-1}. The molar extinction coefficient in ethanol for comparison with the published values for rubusuaviin C (*14*) was as follows: (lambertianin C) log $\varepsilon_{220\ nm}$ = 5.29, log $\varepsilon_{265\ nm}$ = 5.02.

Circular Dichroism Measurements. The CD spectra of sanguiin H-6 and lambertianin C were recorded in methanol on a Jasco J-40AS dichrograph. The following Cotton effects, as expressed in molar ellipticity Θ (mol^{-1} L cm^{-1}) at the corresponding wavelengths (λ in nm), were observed for sanguiin H-6 (4.4×10^{-6} M): $\Theta = +4.1 \times 10^5$ (240 nm), $\Theta = -1.1 \times 10^5$ (266 nm), $\Theta = +2.0 \times 10^4$ (286 nm), $\Theta = -5.0 \times 10^4$ (310 nm). CD data obtained for lambertianin C (2.5×10^{-6} M) were as follows: $\Theta = +6.0 \times 10^5$ (240 nm), $\Theta = -1.9 \times 10^5$ (266 nm), $\Theta = -2.8 \times 10^4$ (286 nm), $\Theta = -9.7 \times 10^4$ (310 nm).

Sephadex LH-20 Sample Purification. Anthocyanins are the main source of interference in HPLC analysis of ellagitannins, and their elimination is essential for obtaining high quality MS spectra of minor ellagitannins. The purification of ellagitannins from anthocyanins was performed following the slightly modified method of Hager et al. (*35*). An aliquot of 10 mL of the aqueous acetone berry extract was dried in a rotary evaporator to remove acetone, and the extract was rediluted in 40 mL of 30% MeOH in water. A column cartridge (6 cm × 1.5 cm) was packed with Sephadex LH-20 resin, connected to a vacuum line to speed up the elution, prewashed with MeOH, and equilibrated with 30% MeOH in water. The berry extract was loaded, and anthocyanins were washed off with 40 mL of 30% MeOH in water. The yellowish fraction containing the ellagitannins was eluted from the cartridge using 80 mL of 70% acetone in water, adjusted to a final volume of 100 mL using 70% acetone in water, and stored at −20 °C until analysis.

Quantitative Analysis Using HPLC-DAD. An aliquot (50 mL) of the purified extract was evaporated until dryness in a 100 mL pear-shaped flask using rotary evaporation under reduced pressure at 40 °C. Then the sample was diluted to 2.5 mL with 1% formic acid in water immediately

Table 2. NMR (400 MHz, 298 K) Assignments for Lambertianin C in Hexadeuterated Acetone

moiety	signal	^{1}H NMR	^{13}C NMR (HSQC and HMBC)
α-glucose1	1	6.51 (d, $J_{1,2}$ = 4.0)	90.70 d
OCO-1 (164.92 s)	2	5.27 (dd, $J_{1,2}$ = 4.0, $J_{3,2}$ = 9.4)	73.86 d
OCO-2 (168.35 s)	3	5.16 (dd, $J_{3,4}$ = 9.8, $J_{3,2}$ = 9.8)	75.26 d
OCO-3 (168.43 s)	4	5.01 (t, $J_{4,3} \approx J_{4,5}$ = 9.8)	69.37 d
OCO-4 (165.95 s)	5	4.28 (dd, $J_{4,5}$ = 9.8, $J_{5,6}$ = 6.4)	71.23 d
OCO-6 (167.70 s)	6	3.88 (d, J_{gem} = 13.1)	63.10 t
		5.47 (dd, J_{gem} = 13.1, $J_{5,6}$ = 6.4)	
β-glucose2	1′	6.00 (d, $J_{1',2'}$ = 8.5)	92.42 d
OCO-1′ (165.30 s)	2′	5.07 (t, $J_{1',2'} \approx J_{3',2'}$ = 8.5)	75.68 d
	3′	5.00 (dd, $J_{3',2'}$ = 8.5, $J_{4',3'}$ = 9.9)	76.87 d
	4′	4.93 (t, $J_{4',3'} \approx J_{4',5'}$ = 9.9)	69.32 d
	5′	4.05 (dd, $J_{6'5'}$ = 6.4, $J_{4',5'}$ = 9.9)	73.90 d
	6′	3.88 (d, J_{gem} = 12.9)	62.80 t
		5.57 (d, J_{gem} = 12.9, $J_{6'5'}$ = 6.4)	
β-glucose3	1″	6.15 (d, $J_{1',2'}$ = 8.5)	92.33 d
OCO-1″ (165.05 s)	2″	5.15 (t, $J_{1',2'} \approx J_{3',2'}$ = 8.5)	75.93 d
	3″	5.41 (dd, $J_{3',2'}$ = 8.5, $J_{4',3'}$ = 9.9)	77.36 d
	4″	5.09 (t, $J_{4',3'} \approx J_{4',5'}$ = 9.9)	69.08 d
	5″	4.43 (dd, $J_{6'5'}$ = 6.4, $J_{4',5'}$ = 9.9)	73.32 d
	6″	3.88 (d, J_{gem} = 12.9)	62.99 t
		5.30 (d, J_{gem} = 12.9, $J_{6'5'}$ = 6.4)	
central 3-O, 4,5-dihydroxybenzoate (sanguisorboyl)	1		118.28 s
	2	7.20 (d, $J_{2,6}$ = 2.0)	112.50 d
	3		147.93 s
	4		141.20 s
	5		147.93 s
	6	7.03 (d, $J_{2,6}$ = 2.0)	110.04 d
east 3,4,5-trihydroxybenzoate (galloyl)	1		120.03 s
	2 and 6	7.12 s, 2H	110.26 d
	3 and 5		145.77 s
	4		139.51 s
west 3-O, 4,5-dihydroxybenzoate (sanguisorboyl)	1		
	2 and 6	7.11 s, 2,H	110.97 d
	3 and 5		
	4		

prior to HPLC processing. HPLC analysis was carried out using a Waters 2690 HPLC system equipped with a Waters 996 DAD (Waters Corp., Milford, MA) and Empower Software (Waters). Separation was performed using a 150 mm × 2.00 mm i.d., 3 μm, end-capped reversed-phase Luna C18 (2) (Phenomenex) and a 4 mm × 2.00 mm Phenomenex precolumn. The solvents were A (1% formic acid in water) and B (acetonitrile). The gradients were as follows: from 5% to 16% B in 42 min; from 16% to 50% B in 10 min. The column was washed with 100% B for 2 min and then equilibrated for 7 min before each analysis. The flow rate was 0.4 mL/min, and the oven temperature was 40 °C. The injection volume was 20 μL. Ellagic acid and ellagitannins were quantified using UV detection at 260 nm. Ellagic acid and its conjugates were quantified following calibration with ellagic acid standard. Sanguiin H-6 and lambertianin C were quantified following calibration with the pure standard isolated as described above. Other ellagitannins were quantified as equivalents of sanguiin H-6.

Metabolite Profiling Using UPLC-Q-TOF-HDMS Analysis. Separation was carried out with a Waters Aquity UPLC system equipped with a UV−vis Waters PDA (Waters Corp., Milford, MA) under the same conditions described for HPLC analysis. The UV spectra, reported in the Supporting Information, were used to assign each peak to either the ellagitannin or ellagic acid conjugate family. Detailed compound characterization was carried out using a Waters HDMS-QTOF Synapt (Waters Corp.) mass spectrometer with an electrospray ionization system (ESI) and MassLynx Software 4.1 (Waters Corp.). HDMS analysis was performed in negative mode under the following conditions: capillary voltage 3 kV, sampling cone 40 V, extraction cone 3 V, source temperature 100 °C, desolvation temperature 350 °C, cone gas flow (N_2) 50 L/h, desolvation gas flow (N_2) 800 L/h. The m/z range was 50−3000 Da.

The MS was calibrated using sodium formate, and leucine enkephalin was used as the lock mass. In all the experiments, the experimental values were checked against the three available standards, to ensure that the m/z values (reported in **Tables 3** and **4**) for the standards were within ±3 ppm of the true value. The experimental m/z values reported in **Tables 3** and **4** for each unknown metabolite were within ±10 ppm of the monoisotopic m/z for the suggested structure. Fragmentation patterns were reconstructed with the aid of Mass Fragment version 2.0.w.15 (Waters Corp.).

Statistical Analysis. In order to distinguish major compounds that are ubiquitous components of berry extracts from minor compounds randomly/irregularly present in few extracts, only compounds which occurred in at least two-thirds of the raspberry or blackberry extracts or in all the repetitions of at least one variety were reported individually in **Tables 5** and **6**. Other compounds were grouped into two classes labeled "total minor ET", containing respectively the sum of peaks 3, 10, 12, and 20 for raspberry samples and peaks 4, 5, 14, and 15 for blackberry samples. Statistical analysis was carried out using STATISTICA data analysis software, version 8 (StatSoft, Tulsa, OK).

RESULTS AND DISCUSSION

Molar Extinction Coefficients for Ellagic Acid and *Rubus* Ellagitannins. Despite the fact that sanguiin H-6 and lambertianin C were among the first ellagitannins to be thoroughly characterized (*36*−*38*, *40*), their molar extinction coefficients have never been reported to date. Rubusuaviin C is a natural ellagitannin isolated in the leaves of Chinese sweet tea (*Rubus suavissimus* S. Lee), whose structure is very similar to that of lambertianin C, being its C(1) stereoisomer (*14*). The experimental values (log ε) obtained in ethanol at 220 and 265 nm for lambertianin C (5.29 and 5.02) were of the same order of magnitude as those reported for rubusuaviin C (5.38 at 200 nm and 5.07 at 265 nm). This result confirms the high degree of purity of our isolated standard.

Table 3. Characterization of Ellagitannins Using UPLC-Q-TOF_HDMS in Smoothstem (BB) Blackberry Cultivar and Polana (RB) Raspberry Cultivar Extracts. [a]

peak no.	R_t (min)	MS data		MS/MS value	MS/MS data		MM observed	tentative structural assignment	molecular formula	MM calculated	Δmass (ppm)	BB	RB
1	9.7	$[1567.1499]^{-1}$	$[1011.2841]^{-1}$	*783*	$[1235.0792]^{-1}$	$[935.0788]^{-1}$	1568.1577	sanguiin H-10 isomer	$C_{68}H_{48}O_{44}$	1568.1518	−3.7	X	X
		$[783.0630]^{-2}$	$[505.1343]^{-1}$		$[633.0684]^{-1}$	$[469.0041]^{-1}$							
					[300.9986]$^{-1}$								
2	12.7	$[858.0613]^{-2}$	$[577.1349]^{-1}$	*858*	$[1415.1243]^{-1}$	$[1235.0809]^{-1}$	1718.1364	sanguiin H-6 without gallic moiety	$C_{75}H_{50}O_{48}$	1718.1472	6.3	X	X
					$[1113.1176]^{-1}$	$[933.0700]^{-1}$							
					$[783.0651]^{-1}$	$[633.0653]^{-1}$							
					$[481.0608]^{-1}$	**[300.9994]$^{-1}$**							
3	13.2	$[1250.1001]^{-2}$	$[865.1954]^{-1}$	*1250*	$[2200.2434]^{-1}$	$[1867.1458]^{-1}$	2502.2158	lambertianin C without ellagic moiety	$C_{109}H_{74}O_{70}$	2502.2231	2.0	X	X
		$[589.1191]^{-1}$			$[1565.1946]^{-1}$	**[1250.1046]$^{-1}$**							
					$[933.0682]^{-1}$	$[633.0709]^{-1}$							
					$[301.0024]^{-1}$								
4	15.3	$[667.1877]^{-1}$	$[1335.3866]^{-1}$	*667*	**[609.1442]$^{-1}$**	$[284.0324]^{-1}$	1336.3944	unknown ellagitannin A					X
				1336	$[1335.4381]^{-1}$	**[667.1848]$^{-1}$**							
					$[609.1481]^{-1}$								
5	16.0	$[649.1779]^{-1}$	$[783.0672]^{-2}$	*783*	$[935.0713]^{-1}$	**[633.0828]$^{-1}$**	1568.1502	sanguiin H-10 isomer	$C_{68}H_{48}O_{44}$	1568.1518	1.2	X	
				649	$[300.9993]^{-1}$	**[325.0725]$^{-1}$**							
6	18.4	$[1250.1046]^{-2}$	$[933.0698]^{-1}$				2502.2248	lambertianin C without ellagic moiety	$C_{109}H_{74}O_{70}$	2502.2231	−0.7	X	
		$[633.0737]^{-1}$	$[301.0374]^{-1}$										
7	18.8	$[813.2470]^{-1}$	$[651.1939]^{-1}$	*813*	$[813.2494]^{-1}$	**[755.2055]$^{-1}$**	814.2548	unknown ellagitannin					X
				651	**[593.1560]$^{-1}$**	$[299.0577]^{-1}$							
					$[285.0408]^{-1}$								
8	19.4	$[1011.2817]^{-1}$	$[447.0933]^{-1}$	*505*	$[447.0928]^{-1}$	**[285.0397]$^{-1}$**	1012.2895	unknown ellagitannin B					X
		$[505.1350]^{-1}$		*1011*	$[505.1339]^{-1}$	**[447.0930]$^{-1}$**							
					$[285.0412]^{-1}$								
9	19.6	$[1517.4297]^{-1}$	$[1011.2806]^{-1}$	*1518*	**[1011.2841]$^{-1}$**	$[505.1359]^{-1}$	1518.4376	unknown ellagitannin C				X	
					$[447.0937]^{-1}$	$[285.0392]^{-1}$							
		$[447.0923]^{-1}$	$[505.1344]^{-1}$	*1011*	$[505.1417]^{-1}$	**[447.0924]$^{-1}$**							
					$[285.0420]^{-1}$								
				505	$[447.0944]^{-1}$	$[299.0580]^{-1}$							
					[285.0393]$^{-1}$								
10	20.1	$[783.0646]^{-2}$		*783*	$[935.0865]^{-1}$	$[633.0753]^{-1}$	1568.1448	sanguiin H-10 isomer	$C_{68}H_{48}O_{44}$	1568.1518	4.5	X	X
					$[469.0048]^{-1}$	$[314.9763]^{-1}$							
					[300.9986]$^{-1}$								
11	20.4	$[1335.3856]^{-1}$	$[667.1870]^{-1}$	*667*	**[609.1444]$^{-1}$**	$[284.0325]^{-1}$	1336.3934	unknown ellagitannin A					X
				1335	**[1335.3972]$^{-1}$**	$[667.1857]^{-1}$							
					$[609.1411]^{-1}$								
12	20.8	$[651.1946]^{-1}$	$[359.0785]^{-1}$	*651*	**[593.1517]$^{-1}$**	$[299.0569]^{-1}$	652.2024	unknown ellagitannin D					X
					$[285.0416]^{-1}$								
13	21.4	$[951.2570]^{-1}$	$[475.1226]^{-1}$				952.2649	unknown ellagitannin E				X	
		$[417.0819]^{-1}$											
14	23.0	$[1485.1060]^{-2}$	$[934.0706]^{-1}$				2972.2276	lambertianin C plus one gallic acid moiety	$C_{130}H_{84}O_{83}$	2972.2352	2.6	X	
		$[633.0517]^{-1}$	$[300.9991]^{-1}$										
15	23.4	$[1011.2755]^{-1}$	$[447.0936]^{-1}$	*505*	**[285.0371]$^{-1}$**	$[447.0935]^{-1}$	1012.2833	unknown ellagitannin B					X
		$[505.1349]^{-1}$	$[551.1396]^{-1}$		$[299.0594]^{-1}$								
				1011	**[447.0913]$^{-1}$**	$[505.1423]^{-1}$							
					$[285.0152]^{-1}$								

Table 3. Continued

peak no.	R_t (min)	MS data		MS/MS value	MS/MS data		MM observed	tentative structural assignment	molecular formula	MM calculated	Δmass (ppm)	BB	RB
16	23.6	$[1517.4308]^{-1}$	$[1011.2811]^{-1}$	*1518*	**[1011.2810]$^{-1}$**	$[505.1289]^{-1}$	1518.4386	unknown ellagitannin C				X	
		$[505.1346]^{-1}$	$[447.0927]^{-1}$		$[447.0905]^{-1}$								
				1011	$[505.1323]^{-1}$	**[447.0921]$^{-1}$**							
					$[285.0399]^{-1}$								
				505	$[447.0935]^{-1}$	$[299.0594]^{-1}$							
					[285.0394]$^{-1}$								
17	24.8	$[1250.1061]^{-2}$	$[733.1312]^{-1}$	*1250*	**[1250.1040]$^{-2}$**	$[1099.6100]^{-2}$	2502.2278	lambertianin C without ellagic moiety	$C_{109}H_{74}O_{70}$	2502.2231	−1.9	X	X
		$[649.1772]^{-1}$			$[933.0365]^{-1}$	$[300.9990]^{-1}$							
18	25.1	$[1018.0713]^{-2}$	$[417.0834]^{-1}$	*1018*	$[1567.1494]^{-1}$	$[933.0652]^{-1}$	2038.1582	sanguiin H-6 plus one gallic acid moiety	$C_{89}H_{58}O_{57}$	2038.1640	2.8	X	X
		$[359.0788]^{-1}$	$[475.1277]^{-1}$		$[633.0782]^{-1}$	$[469.0022]^{-1}$							
					$[300.9990]^{-1}$								
19	25.8	$[1250.1000]^{-2}$	$[359.0781]^{-1}$	*1250*	$[1565.1498]^{-1}$	**[1250.1130]$^{-2}$**	2502.2156	lambertianin C without ellagic moiety	$C_{109}H_{74}O_{70}$	2502.2231	3.0	X	X
					$[1099.1086]^{-1}$	$[933.0665]^{-1}$							
					$[633.0724]^{-1}$	$[301.0008]^{-1}$							
20	26.0	$[1103.0872]^{-1}$	$[551.0349]^{-2}$	*551*	**[300.9985]$^{-1}$**		1104.0952	sanguiin H-2	$C_{48}H_{32}O_{31}$	1104.0928	−2.0	X	X
		$[489.1395]^{-1}$	$[359.0804]^{-1}$										
21	27.1	$[1401.1038]^{-2}$	$[933.7272]^{-1}$	*1401*	**[1401.1074]$^{-2}$**	$[1250.1072]^{-2}$	2804.2234	lambertianin C (s)	$C_{123}H_{80}O_{78}$	2804.2293	2.1	X	X
					$[1235.0713]^{-1}$	$[935.0861]^{-1}$							
					$[633.0711]^{-1}$	$[300.9991]^{-1}$							
22	27.9	$[1869.1462]^{-1}$	$[934.0663]^{-2}$	*934*	$[1567.1520]^{-1}$	$[1235.0725]^{-1}$	1870.1540	sanguiin H-6 (s)	$C_{82}H_{54}O_{52}$	1870.1581	2.2	X	X
		$[433.0395]^{-1}$			$[935.0807]^{-1}$	$[633.0737]^{-1}$							
					$[469.0051]^{-1}$	**[300.9991]$^{-1}$**							

[a] The reported fragments were observed in the source and verified through MS/MS analysis of the parental ion (MS/MS value). MM, observed or calculated, molecular monoisotopic mass of the putative metabolite. Δmass (ppm), deviation of the observed ion mass from the corresponding calculated monoisotopic mass. (s) indicates identification based on the standard compound.

Table 4. Characterization of Ellagic Acid Conjugates by UPLC-Q-TOF_HDMS in Smoothstem (BB) Blackberry Cultivar and Polana (RB) Raspberry Cultivar Extracts[a]

peak no.	R_t (min)	MS-data			MM observed	tentative structural assignment	molecular formula	MM calculated	Δmass (ppm)	BB	RB
23	28.7	[**300.9985**]$^{-1}$			302.0062	ellagic acid (s)	$C_{14}H_6O_8$	302.0063	0.3	X	X
24	29.2	[**433.0404**]$^{-1}$	[301.0002]$^{-1}$		434.0518	ellagic acid pentose conjugate	$C_{19}H_{14}O_{12}$	434.0485	−7.6		X
25	30.1	[447.0579]$^{-1}$	[315.0069]$^{-1}$	[300.9998]$^{-1}$	448.0657	methylellagic acid pentose conjugate	$C_{20}H_{16}O_{12}$	448.0642	−3.3	X	
26	38.5	[**447.0581**]$^{-1}$	[315.0056]$^{-1}$		448.0659	methylellagic acid pentose conjugate	$C_{20}H_{16}O_{12}$	448.0642	−3.8	X	X
27	40.3	[**475.0555**]$^{-1}$	[300.9982]$^{-1}$		476.0633	ellagic acid acetilpentose conjugate	$C_{21}H_{16}O_{13}$	476.0591	−8.8	X	X

[a] MM, observed or calculated molecular monoisotopic mass of the putative metabolite. Δmass (ppm), deviation of the observed ion mass from the corresponding calculated monoisotopic mass. (s) indicates identification based on the standard compound.

Table 5. Quantification, Expressed in mg/kg, of Ellagitannins (ET) and Ellagic Acid Conjugates (EAC) in Raspberry Cultivar[a]

cultivar	**1**	**2**	**4**	**7**	**8**	**11**	**15**	**17**	**18**	**19**	lambertianin C (**21**)	sanguiin H-6 (**22**)	ellagic acid (**23**)	**24**	**26**	**27**	total minor ET (3.10.12.20)	total ET	total EAC
Himbotop	29.8	21.4	14.8	37.9	11.7	12.9	17.7	12.9	11.0	10.3	353.7	545.2	132.2	99.2	29.8	49.8	20.4	1099.8	310.9
s.d.	*3.4*	*6.1*	*1.1*	*3.1*	*0.9*	*12.6*	*8.8*	*3.7*	*9.6*	*0.7*	*65.8*	*90.6*	*10.8*	*36.3*	*2.3*	*2.8*	*9.2*	*153.5*	*29.9*
Octavia	48.2	36.2	15.6	49.3	7.4	15.4	21.1	17.8	14.8	15.0	627.1	747.2	164.7	116.1	31.3	48.8	22.1	1637.1	360.9
s.d.	*16.4*	*16.5*	*2.8*	*15.5*	*6.4*	*3.4*	*6.1*	*11.0*	*2.2*	*6.2*	*233.7*	*195.5*	*72.4*	*52.3*	*9.6*	*13.1*	*12.4*	*479.8*	*146.8*
Pokusa	21.0	5.2	19.1	22.5	10.7	22.3	20.0	9.2	11.1	10.9	284.8	427.4	47.4	58.1	24.8	36.7	26.0	890.2	167.0
s.d.	*5.6*	*9.0*	*10.1*	*4.5*	*9.4*	*12.7*	*14.9*	*8.9*	*10.0*	*1.6*	*92.1*	*105.2*	*10.4*	*17.6*	*3.5*	*9.0*	*24.4*	*196.3*	*29.1*
Polana	22.0	13.7	22.3	22.3	4.3	17.9	7.5	7.7	9.9	15.4	301.6	367.6	48.2	50.3	35.9	27.8	14.5	826.7	162.3
s.d.	*3.5*	*12.7*	*16.9*	*1.3*	*7.4*	*17.7*	*6.8*	*6.8*	*9.3*	*9.7*	*66.8*	*38.3*	*24.3*	*45.8*	*13.1*	*11.8*	*13.6*	*127.5*	*82.9*
Polka	23.4	23.6	20.5	30.1	11.4	19.1	13.2	8.4	17.5	11.5	373.3	420.8	135.9	68.5	24.8	26.2	9.3	982.0	255.4
s.d.	*2.7*	*1.5*	*5.8*	*9.6*	*6.9*	*11.9*	*1.6*	*8.3*	*12.1*	*2.1*	*30.6*	*49.7*	*40.8*	*10.9*	*2.0*	*11.0*	*9.1*	*79.4*	*40.5*
Tulameen	23.1	19.3	17.6	38.9	7.0	18.6	18.8	7.3	12.6	8.9	309.0	412.2	130.9	43.6	20.9	6.7	2.5	895.7	202.1
s.d.	*5.3*	*6.1*	*5.6*	*6.0*	*8.5*	*14.9*	*6.5*	*4.8*	*8.6*	*6.1*	*99.2*	*122.9*	*43.1*	*50.3*	*5.1*	*7.8*	*4.9*	*252.1*	*89.8*

[a] Average of three different years. Column header: peak number in agreement with **Figure 2** and **Tables 3** and **4**.

Table 6. Quantification, Expressed in mg/kg, of Ellagitannins (ET) and Ellagic Acid Conjugates (EAC) in Blackberry Cultivar[a]

cultivar	**1**	**2**	**3**	**6**	**9**	10	**13**	**16**	**17**	**18**	**19**	**20**	Lambertianin C (**21**)	sanguiin H-6 (**22**)	ellagic acid (**23**)	**25**	**26**	total minor ET (4.5.14.15)	total ET	total EAC
Apache	16.6	10.7	16.5	12.3	86.8	17.2	19.5	30.2	99.3	7.5	10.9	10.1	306.3	188.9	79.2	93.9	62.0	14.1	846.9	235.2
s.d.	*2.3*	*0.6*	*2.6*	*1.9*	*63.5*	*14.9*	*17.8*	*9.9*	*60.2*	*6.5*	*1.2*	*8.8*	*104.2*	*32.3*	*4.6*	*6.7*	*5.7*	*6.5*	*44.1*	*5.7*
Chesapeake	18.0	8.8	22.4	12.2	66.9	14.3	17.7	32.6	76.9	13.8	14.5	0.0	665.2	221.9	76.9	103.1	93.6	13.4	1198.5	273.6
s.d.	*3.8*	*7.8*	*5.6*	*1.8*	*29.8*	*14.2*	*4.7*	*14.1*	*24.7*	*2.8*	*2.8*	*0.0*	*291.2*	*114.1*	*18.0*	*40.5*	*26.0*	*13.0*	*404.0*	*80.7*
Loch Ness	17.3	9.0	17.2	12.8	94.6	17.5	12.4	19.6	99.3	8.4	11.3	3.6	315.1	330.0	45.6	68.7	14.0	23.6	991.5	128.3
s.d.	*2.1*	*0.6*	*1.3*	*2.3*	*55.6*	*18.1*	*11.1*	*6.0*	*51.3*	*7.3*	*1.6*	*6.2*	*34.3*	*39.8*	*18.7*	*4.2*	*24.3*	*25.0*	*173.5*	*5.4*
Thornfree	23.3	6.5	21.4	12.4	140.1	14.9	14.4	18.4	141.5	13.0	13.4	12.1	447.1	418.3	84.6	85.8	0.0	7.9	1304.5	170.4
s.d.	*2.4*	*5.6*	*2.4*	*0.9*	*75.6*	*2.3*	*12.7*	*2.8*	*68.4*	*1.5*	*1.3*	*1.7*	*26.5*	*52.5*	*15.0*	*14.5*	*0.0*	*7.1*	*229.5*	*28.1*
Triple Crown	16.1	7.3	18.8	10.6	80.3	18.9	0.0	46.3	86.2	6.0	13.1	7.1	492.1	243.7	66.7	96.0	29.4	9.7	1056.3	192.1
s.d.	*3.3*	*6.3*	*3.9*	*1.2*	*33.7*	*16.5*	*0.0*	*10.5*	*28.2*	*5.2*	*1.8*	*6.2*	*160.7*	*81.2*	*10.6*	*33.0*	*25.9*	*9.0*	*307.5*	*69.1*

[a] Average of three different years. Column header: peak number in agreement with **Figure 3** and **Tables 3** and **4**.

Circular dichroic (CD) spectra of sanguiin H-6 and lambertianin C, reported here for the first time, showed the same qualitative features as those of rubusuaviins, with a strong positive Cotton effect at 240 nm and a medium negative Cotton effect at 267 nm, thus confirming the S absolute configuration of atropisomeric biaryl HHDP groups of D-glucopyranose.

The molar extinction coefficients of sanguiin H-6 and lambertianin C were substantially in agreement. The UV signals at 260 nm divided by the formula weights are almost identical, as expected in light of their similar building units. This means that either the former or the latter could be used as the external standard for quantification of ellagitannins, giving substantially equivalent values.

On the contrary, the UV signal of ellagic acid at 260 nm, divided by its formula weight, measured under conditions reproducing the chromatographic solvent, showed that the signal of ellagic acid per unit of weight is about 2.5 higher than that of ellagitannins.

On the basis of these observations, we can conclude that ellagic acid is a convenient external standard for quantification of ellagic acid conjugates using HPLC-DAD or UV spectroscopy, while sanguiin H-6 or lambertianin C, rather than ellagic acid, should be used for accurate quantification of ellagitannins.

Structural Characterization of Sanguiin H-6 and Lambertianin C. Although the NMR spectra of sanguiin H-6 and lambertianin C have already been used to establish their structures (*36*, *37*), we report here for the first time a full NMR assignment of the most relevant ^{1}H and ^{13}C resonances of both metabolites by exploiting the most powerful capabilities of using inverse-detection and pulsed field gradient (PFG) 2D-NMR techniques. Our data for sanguiin H-6 (**Table 1**) and for lambertianin C thus represent a point of reference for NMR analysis of other structurally related ellagitannins. In particular, our assignments of the ^{1}H/^{13}C resonances of β-D-glucopyranose in sanguiin H-6 and lambertianin C (**Figure 1**, glucose 2) are in excellent agreement with those reported by Kouno et al. for rubusuaviins B and C (*17*) but not with those previously reported for sanguiin H-6 (*37*) where the ^{13}C signals for C2′, C3′, C4′, and C5′ appear to have been misassigned. Even the assignments (*37*)of the α-D-glucopyranosyl ^{13}C signals (**Figure 1**, glucose 1) of sanguiin H-6 must be corrected according to our data in **Table 1**.

Due to the intrinsic high capability of the NMR technique in quantitative analysis, the simple ^{1}H NMR measurement allows us to obtain a reliable estimate of their relative degree of purity. According to integration of the relative area of proton NMR signals for sanguiin H-6 as compared to signals of unidentified compounds present as impurities in our sample, the relative abundance of the latter must be lower than 3% . The same conclusion is also obtained from the ^{1}H-NMR spectrum of lambertianin C.

Molecular mechanics calculations carried out through extended geometry optimization of sanguiin H-6 and lambertianin C structures suggest that 10-membered rings (defined by 2,3 junctions of the DHHP moiety on glucose unities) adopt a twisted chairlike conformation, within which the two ester carbonyl groups are in *anti*-orientation (dihedral angle between the two ester C═O groups evaluated to be 175°); similarly, in 11-membered rings (defined by the 4,6 junction of the same moiety) the carbonyls occupy *anti*-positions (dihedral angle between the two ester C═O groups estimated to be 165°). In all the DHHP moieties, the phenyl rings are heavily twisted (by about 60° according to our calculations) around the connecting diphenyl bond, a structural need in order to minimize the strain interactions imposed not only by their hydroxyl-substituents but also by the steric requirements of the whole ring strains. As already noted (*36*), two sets of resonances for ester carbonyl groups are present in oligomeric ellagitannins, one at δ_C about 167−169 ppm for those linked to DHHP moieties and one at δ_C about 164−165 ppm for the sanguisorboyl and galloyl esters. The shielded values for the latter can be explained on the basis of our optimized geometry for sanguiin H-6 and lambertianin C, since they adopt a conformation within which the carbonyl groups are almost coplanar with the corresponding aromatic ring, allowing a better conjugation (upfield effect) than that for other DHHP carbonyl groups whose orientation is kept out of the plane of the corresponding phenyl rings by the conformational constraints of 10- and 11-membered rings.

Another structural feature of the oligomeric ellagitannins is the relative orientation of the galloyl aromatic ring and the glucose unit to which it is linked through the 4,6 DHHP moiety (sanguisorboyl moiety). Our calculations suggest that the former is almost parallel to the mean plane of the latter and is thus able to induce significant upfield shifts on all the glucose axial protons (H-3 and H-5 and also H-1 in β-galloyl substituted terminal glucose) lying in the shielding cone of the aromatic 2-O,3,4 dihydroxy benzoate ring. This outcome can be exploited for the assignment of all the signals belonging to the three different glucoses in lambertianin C, as reported in **Table 2**.

The possibility that sanguiin H-6 may form molecular aggregates in solution, at least in the range of concentration by us investigated (1−5 mM), seems to be ruled out by ^{1}H NMR detection of unvarying chemical shifts of their signals when acetone solutions containing increasing amounts of these metabolites were analyzed.

Method Validation. *Repeatability*. The repeatability of the method was calculated using a purified raspberry extract which was injected 20 times into the HPLC-DAD instrument. Repeatability was calculated on the basis of the chromatographic areas of three principal compounds, whose identity and purity were also confirmed by co-injection with the relative standard. Following 20 measurements, a relative standard deviation (RSD) of 1.6 was obtained for ellagic acid, and a RSD of 2.4 and 1.4 were obtained for lambertianin C and sanguiin H-6, respectively.

Linearity and Range. The calibration curves for standard ellagic acid, sanguiin H-6, and lambertianin C were found to be linear in their range of DAD detection. For ellagic acid, the range was 2.46−123 mg/L, with a coefficient of correlation (R^2) of 0.993. For sanguiin H-6, the range was 13.9−558.9 mg/L, with a coefficient of correlation (R^2) of 0.999. For lambertianin C, the range was 13.7−550.6 mg/L, with a coefficient of correlation (R^2) of 0.998. The range of concentration in the extracts of the other ellagitannins, expressed as equivalents of sanguiin H-6, was 0.27−27.9 mg/L, with a coefficient of correlation (R^2) of 0.997.

Limit of Detection (*LOD*) *and Limit of Quantification* (*LOQ*). The LOD and LOQ were experimentally estimated as three and ten times the signal-to-noise ratio (S/N), respectively. The LOD and LOQ in DAD were respectively 0.63 mg/L and 2.1 mg/L for ellagic acid, 0.07 mg/L and 0.24 mg/L for sanguiin H-6, and 0.09 mg/L and 0.34 mg/L for lambertianin C.

Metabolite Profiling Using UPLC-Q-TOF-HDMS Analysis. The purification step with Sephadex LH20 was included before HPLC analysis in order to remove other phenolics present in raspberry and blackberry extracts. In particular, ca. 95% of the anthocyanins were removed in a single step, thus preventing their interference. Chromatographic peak characterization and assignment of each peak to one of the two main chemical classes (ellagitannins and ellagic acid conjugates) were carried out on the basis of the UV spectrum. The two classes of compounds, according to the literature (*5*), have specific UV spectra in the 220−340 nm range. The most difficult problem for accurate quantification of ellagitannins is the lack of commercially available standards. In order to overcome this drawback, quantification of ellagitannins has usually been carried out after acid hydrolysis of the native compounds and subsequent quantification of the products of acid hydrolysis (*9*, *29*). At all events, this is an indirect method leading to loss of the ellagitannin profile and which does not achieve accurate quantification of native compounds. The central idea of this new method is to combine identification of the compounds in their native form with their quantification, using as external standards the main ellagitannins found in raspberries and blackberries: sanguiin H-6 and lambertianin C.

Ellagitannins and ellagic acid conjugates were identified in raspberries (Polana cultivar, **Figure 2**) and blackberries (Smoothstem cultivar, **Figure 3**). These two cultivars were chosen, as they had the highest number of peaks in DAD at 260 nm and were therefore considered to be the most representative samples for metabolite profiling with a mass spectrometer. A UPLC coupled to a Q-TOF-HDMS was used to analyze ET and EAC in raspberries and blackberries, applying the same chromatographic method already described for quantitative analysis using HPLC-DAD.

Structural Assignment of Ellagitannins (*see* ***Table*** **3**). *Peak 1* (R_t = 9.7) gave a major $[M - H]^-$ ion at m/z 1567.1499 and at m/z 783.0630 (doubly charged), giving a molecular mass of 1568 and 1577. MS^2 of the doubly charged ion produced a sequence of singly charged fragments. The singly charged ions at m/z 633.0684 and m/z 300.9986 were attributed to the sequential loss of ellagic units from the characteristic monomer of *Rubus* ellagitannins, galloyl-bis-HHDP glucoside (936 Da). Indeed, the *Rubus* ellagitannin monomer was confirmed by the presence at m/z 935.0788. The major MS^2 ions were at m/z 1235.0792, possibly resulting from the loss of galloylated glucose and a successive molecular rearrangement of the parental ions. The MS and MS/MS spectra of peak 1 were in agreement with a sanguiin H-10-like structure, that is a sanguiin H-6 with the loss of one of the three ellagic acid moieties. Three stereoisomers of this structure can be hypothesized, depending on which ellagic acid unit is lost (**Figure 4**). Two of these structures, named sanguiin H-3 and sanguiin H-10, were previously isolated from *Sanguisorba officinalis* (*37*).

Peak 2 (R_t = 12.7) gave a major $[M - H]^-$ ion at m/z 858.0613, which was shown to be doubly charged, giving an exact molecular

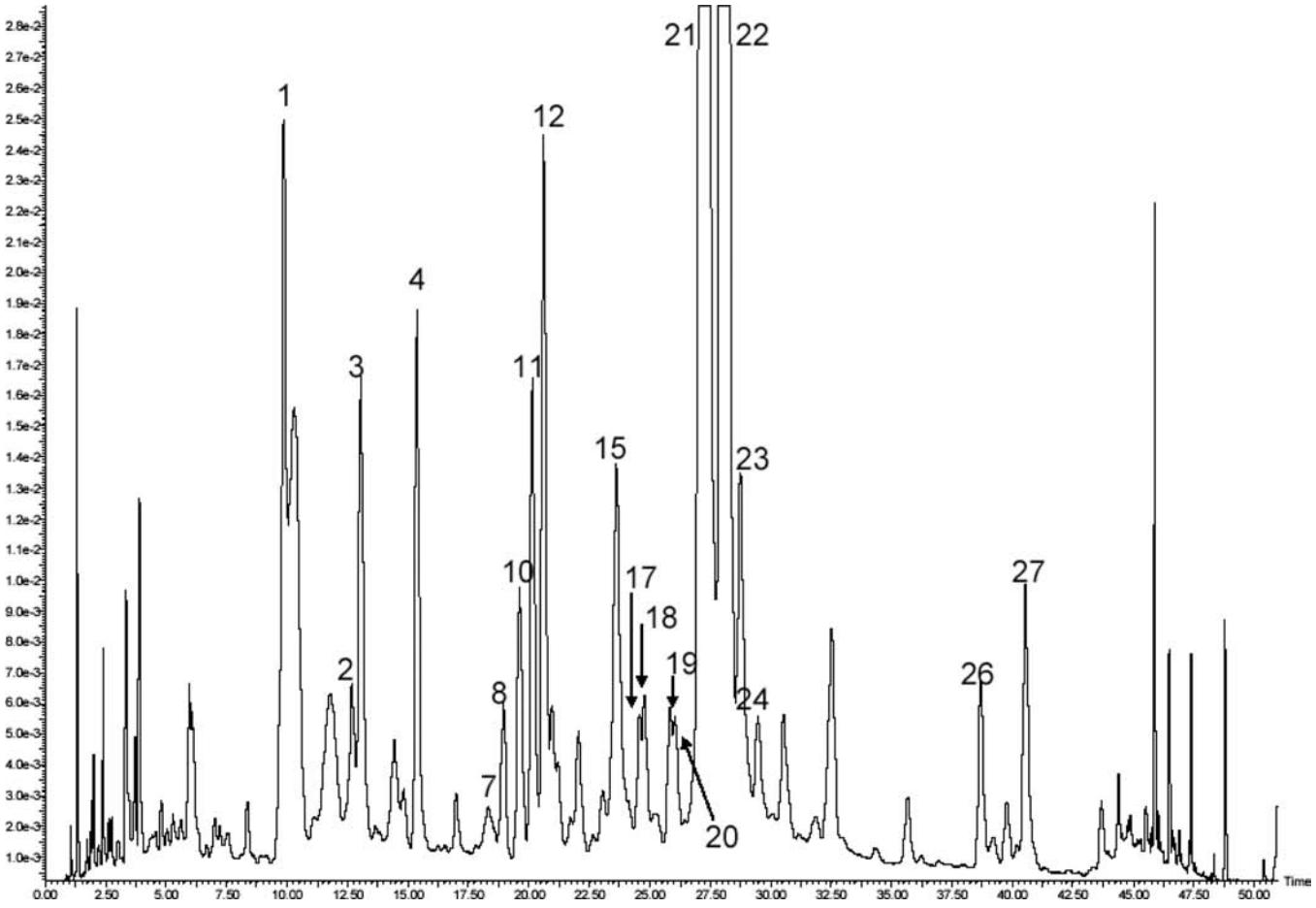

Figure 2. HPLC-DAD chromatogram showing the profile of ellagitannins (ET) and ellagic acid conjugates (EAC), detected at 260 nm, in the Polana raspberry cultivar. Peak key: see **Tables 3** and **4**.

mass of 1718.1364. MS/MS of the doubly charged ion produced singly charged fragments at *m*/*z* 1415.1243 (M − 302, loss of HHDP) and at *m*/*z* 783.0651 (1416 − 332 − 302, loss of a galloylglucosyl group and HHDP). The other fragments at *m*/*z* 1235.0809, *m*/*z* 933.0700, *m*/*z* 633.0653, and 300.9994 are presumed to derive from the *Rubus* monomer. On the basis of this information and in accordance with previous reports from Yoshida et al. (*39*), peak 2 is isobaric to roxbin A, a product of hydrolysis from the dimeric ellagitannins rugosin F found in *Rosa roxburghii* fruits. Roxbin A contains the valoneic acid moiety, while in *Rubus* genus only the sanguisorbic acid is expected. The suggested structure of peak 2 (**Figure 5**) could be easily produced from the partial hydrolysis of the gallic acid group from the dimer sanguiin H-6.

Peak 3 (R_t = 13.2) had a $[M - H]^-$ at *m*/*z* 1250.1001. It was doubly charged, giving an exact molecular mass of 2502.2158. MS/MS of the signal at 1250 produced singly charged fragments at *m*/*z* 2200.2434 (M − 302, loss of HHDP) and *m*/*z* 1867.1458 (2199−332, loss of glucosyl and galloyl groups). Singly charged fragments at *m*/*z* 1565.1946, *m*/*z* 933.0682, *m*/*z* 633.0709, and *m*/*z* 301.0024 came from the characteristic *Rubus* monomer described before. On the basis of mass spectral data, peak 3 was suggested to be a lambertianin C-like ellagitannin without an ellagic group, never reported to date in the literature. In this case, four isobaric structures can be hypothesized, depending on which ellagic acid unit is lost (**Figure 6**).

Peak 4 (R_t = 15.3) had a $[M - H]^-$ at *m*/*z* 1335.3866 and another signal at *m*/*z* 667.1877, both singly charged. MS/MS of the signal at 1335 produced singly charged fragments at *m*/*z* 1335.4381, *m*/*z* 667.1848, and *m*/*z* 609.1481. On the basis of this information, the unknown structure corresponding to peak 4 had a formula weight of 1336.3944.

Peak 5 (R_t = 16) had a $[M - H]^-$ at *m*/*z* 783.0672 (doubly charged), giving an exact molecular mass of 1568.1500. The MS and MS/MS fragmentation patterns were similar to those of peak 1. On the basis of this information, peak 5 was identified as a second isomeric form of sanguiin H-10 and sanguiin H-3, similar to peak 1.

Peak 6 (R_t = 18.4) had a $[M - H]^-$ at *m*/*z* 1250.1046 (doubly charged), giving an exact molecular mass of 2502.2248. Other signals were comparable with the fragmentation pattern of peak 3. Singly charged fragments at *m*/*z* 933.0698, *m*/*z* 633.0737, and *m*/*z* 301.0374 were found. These fragments matched the characteristic fragmentation of the *Rubus* monomer described in the previous peaks. On the basis of mass spectral data, peak 6 was tentatively identified as a second isomeric form of lambertianin C-like ellagitannin without an ellagic group, similar to peak 3 (**Figure 6**).

Peak 7 (R_t = 18.8) was not identified. Peak 7 had a $[M - H]^-$ at *m*/*z* 813.2470 and a main fragment at *m*/*z* 651.1939, corresponding to the loss of a glucose unit. MS/MS of the signal at 651 produced singly charged ions at *m*/*z* 593.1560 and *m*/*z* 299.0577. The tentatively assigned exact molecular mass was 814.2548, but no other structural information on this compound can be provided.

Peak 8 (R_t = 19.4) had a $[M - H]^-$ at *m*/*z* 1011.2817 and another intense signal at *m*/*z* 505.1350, both singly charged. MS/MS of the signal at 1011.2817 produced singly charged signals at *m*/*z* 505.1339 and 447.0930, and MS/MS of 505.1350 produced

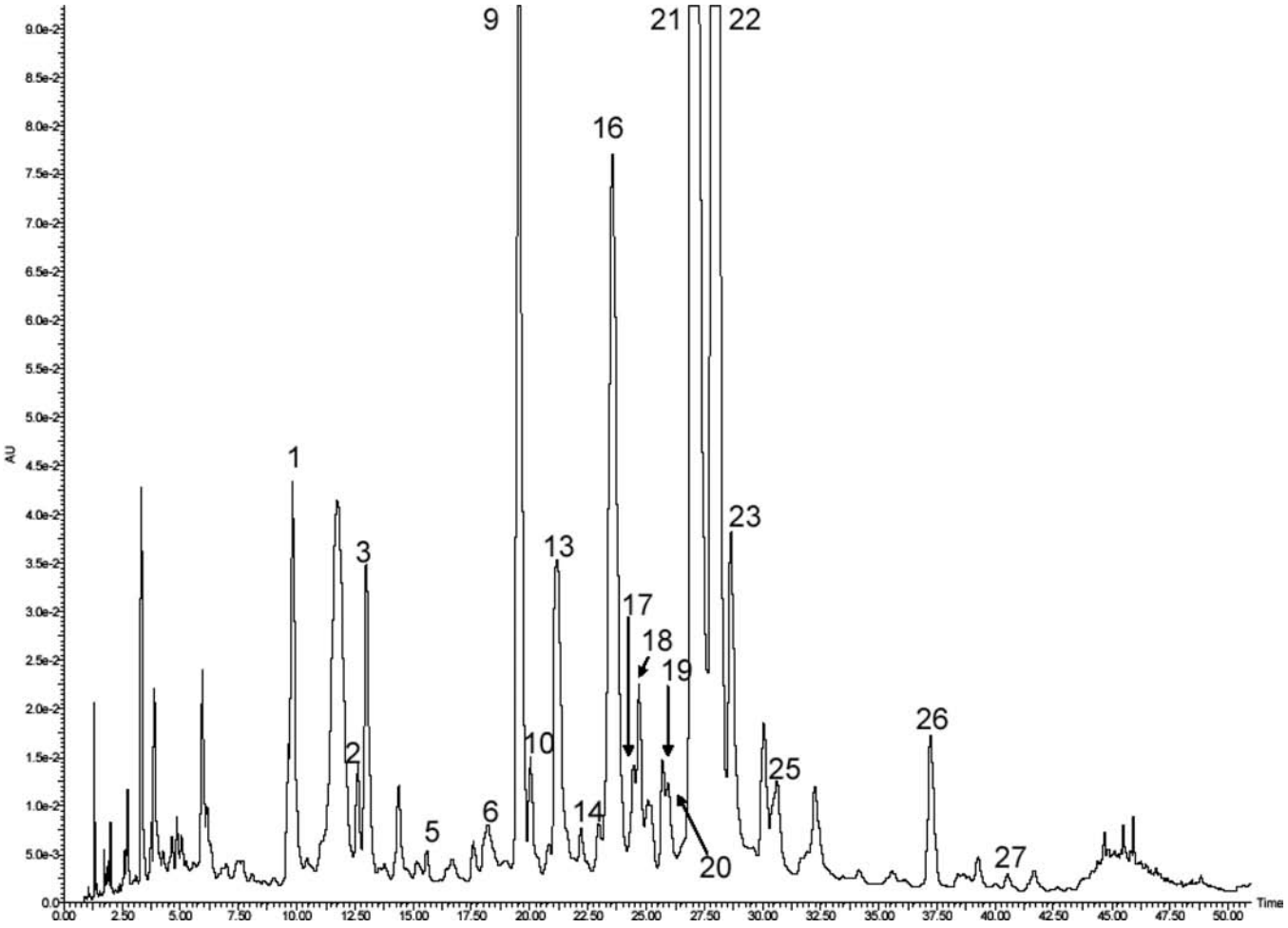

Figure 3. HPLC-DAD chromatogram showing the profile of ellagitannins (ET) and ellagic acid conjugates (EAC), detected at 260 nm, in the Smoothstem blackberry cultivar. Peak key: see **Tables 3** and **4**.

a signal at 447.0928. It behaves like a dimer of the unit at *m*/*z* 505, with a neutral loss of 58 amu followed by the loss of a glucose unit (162 amu). This dimer was characteristic for raspberry extracts only. The UV spectrum had the characteristic ellagitannin profile, but on the basis of mass spectral data and without any similitude with the fragmentation of the typical ellagitannin monomer, its structure remains unknown. The assigned molecular mass was 1012.2895.

Peak 9 (R_t = 19.6) had a $[M - H]^-$ at *m*/*z* 1517.4297, together with intense signals at *m*/*z* 1011.2806 and 505.1344. The MS spectrum was comparable with that of peak 8 and also the MS/MS data with the only additional signal at 1517. The MS and MS/MS pattern (**Table 3**) is in agreement with a trimer of the same building unit already found in peak 8. The MS/MS of 1517.4297 again showed signals at 1011.2841 and 505.1359. The UV spectrum produced the characteristic ellagitannin profile; therefore, the structure of peak 9 would appear to be related to that of peak 8, with a higher molecular mass (1518.4376). Peak 9 was found in blackberry extracts only.

Peak 10 (R_t = 20.1) had double charged signals at *m*/*z* 783.0646. MS/MS produced the fragmentation pattern of peaks 1 and 5. The true mass of the peak was 1568.1448, and on the basis of the fragment information, peak 10 was identified as a third isomeric form of sanguiin H-3 and sanguiin H-10.

Peak 11 (R_t = 20.4) had a $[M - H]^-$ at *m*/*z* 1335.3856, giving a true mass of 1336.3934. MS/MS showed the same singly charged fragments as for peak 4. The identity of peak 11 is therefore unknown, as for peak 4.

Peak 12 (R_t = 20.8) had a $[M - H]^-$ at *m*/*z* 651.1946, giving a true mass of 652.2024. MS/MS of *m*/*z* 651 produced a singly charged fragment at *m*/*z* 299.0569. On the basis of the mass spectra, the identity of peak 12 matches exactly the *m*/*z* and fragmentation pattern of the aglycon (*m*/*z* 651) of peak 7, to which it is possibly related. Its structure remains unknown.

Peak 13 (R_t = 21.4) had a $[M - H]^-$ at *m*/*z* 951.2570, giving a true mass of 952.2648, and *m*/*z* 475.1226. The *m*/*z* 951 has also been reported by Hager et al (*35*) as an unknown blackberry compound.

Peak 14 (R_t = 23) had a $[M - H]^-$ at *m*/*z* 1485.1060, which was shown to be doubly charged by zoom scan analysis, giving a true mass of 2972.2276. The other signals at *m*/*z* 934.0706, *m*/*z* 633.0517, and *m*/*z* 300.9991 showed the characteristic pattern fragmentation of the *Rubus* ellagitannin. On the basis of mass spectral data, peak 17 was tentatively surmised to be a lambertianin C-like ellagitannin with one additional gallic group (i.e., one additional sanguisorboyl group instead of the terminal ellagic moiety) (**Figure 5**). This trimer has not been reported, but it could be produced from partial hydrolysis of the tetramer lambertianin D.

Peak 15 (R_t = 23.4) had a $[M - H]^-$ at *m*/*z* 1011.2755 and another intense signal at *m*/*z* 505.1349, both singly charged. MS/MS of the signal at 1011.2755 produced singly charged signals at *m*/*z* 505.1423 and 447.0913, and MS/MS of 505.1349 produced a signal at 447.0935. The UV spectrum had the characteristic ellagitannin profile; on the basis of the mass and UV spectral data, it was suggested to be an isomer of peak 8. Like peak 8, also peak 15 was characteristic for raspberry extracts only.

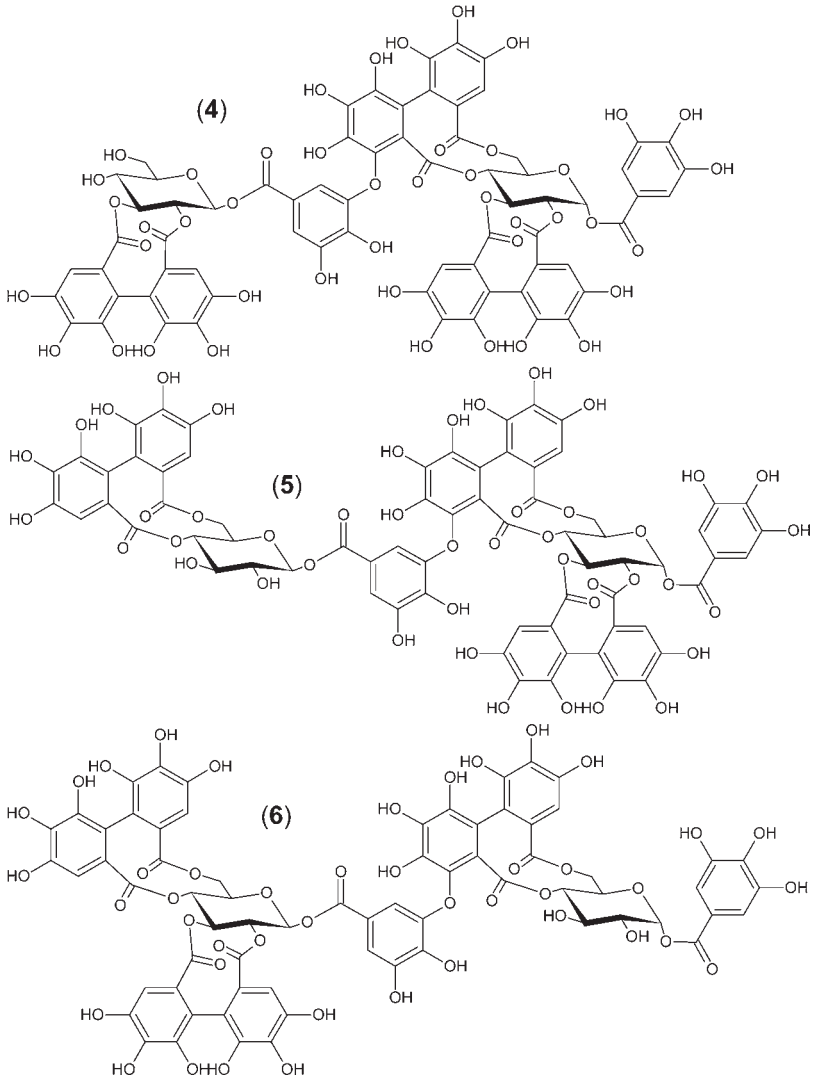

Figure 4. Structures of sanguiin H-3 (**4**), sanguiin H-10 (**5**), and a third new isomer (**6**). These three minor dimeric ellagitannins (corresponding to peaks 1, 5, and 10) derive from the loss of an ellagic acid moiety from sanguiin H-6 (**2**).

Peak 16 (R_t = 23.6) had a $[M - H]^-$ at *m*/*z* 1517.4308, together with intense signals at *m*/*z* 1011.2811 and 505.1346. The MS and MS/MS spectra are comparable with those of peaks 8 and 15 and, similar to peak 9, with the additional signal at *m*/*z* 1517. The MS/MS of 1517.4308 produced signals at 1011.2810 and 505.1289. The UV had the characteristic ellagitannin profile, so it was suggested to be an isomer of peak 9. Like peak 9, also this trimer was observed only in blackberry extracts.

Peak 17 (R_t = 24.8) had a $[M - H]^-$ at *m*/*z* 1250.1061 (doubly charged), giving an exact molecular mass of 2502.2278. MS/MS of the doubly charged ion produced a sequence of singly charged fragments comparable with the pattern of peaks 3 and 6 (*m*/*z* 1250.1040, *m*/*z* 933.0365, and *m*/*z* 300.9990). On the basis of spectral data and similarly to peaks 3 and 6, this peak was tentatively identified as a third isomeric form of lambertianin C-like ellagitannin without an ellagic group (**Figure 6**).

Peak 18 (R_t = 25.1) had a $[M - H]^-$ at *m*/*z* 1018.0713 (doubly charged), having an exact molecular mass of 2038.1582. MS/MS of the doubly charged ion produced singly charged fragments at *m*/*z* 1567.1494 (M − 169 − 302, loss of galloyl and HHDP groups) and the other characteristic fragments of the monomer galloyl-bis-HHDP glucoside (933.0652, 633.0782, 300.9990). On the basis of mass spectral data, peak 18 was tentatively identified as a sanguiin H-6-like ellagitannin with one additional gallic group (**Figure 5**). There have been no reports regarding this dimeric compound in the literature up to now. Similarly to peak 14, it could be produced by partial hydrolysis of the trimer lambertianin C.

Peak 19 (R_t = 25.8) had a $[M - H]^-$ at *m*/*z* 1250.1000 (doubly charged), giving an exact molecular mass of 2502.2156. MS of the doubly charged signal produced the same singly charged fragments as in the case of peaks 3, 6, and 17. Consequently, on the basis of the similar fragmentation pattern, peak 19 was identified as the fourth isomeric form of lambertianin C-like ellagitannin without an ellagic group (**Figure 6**).

Peak 20 (R_t = 26) had a $[M - H]^-$ at *m*/*z* 1103.0872 (singly charged) and at *m*/*z* 551.0349 which was doubly charged, and its exact molecular mass was 1104.0950. MS/MS of the doubly charged signal produced a singly charged fragment at *m*/*z* 300.9985 (HHDP group). On the basis of the mass spectral data, peak 20 was identified as sanguiin H-2 (**Figure 5**). Its monomer structure with the terminal sanguisorboyl suggests a similarity with the suggested structure of peaks 14 (trimer) and 18 (dimer).

Peak 21 (R_t = 27.1) had a $[M - H]^-$ at *m*/*z* 1401.1038, which was doubly charged, giving an exact molecular mass of 2804.2234. MS/MS of the doubly charged signal produced singly charged fragments at *m*/*z* 1235.0713 (1567−169−162, loss of galloylated glucose). The remaining fragments at *m*/*z* 935.0861,

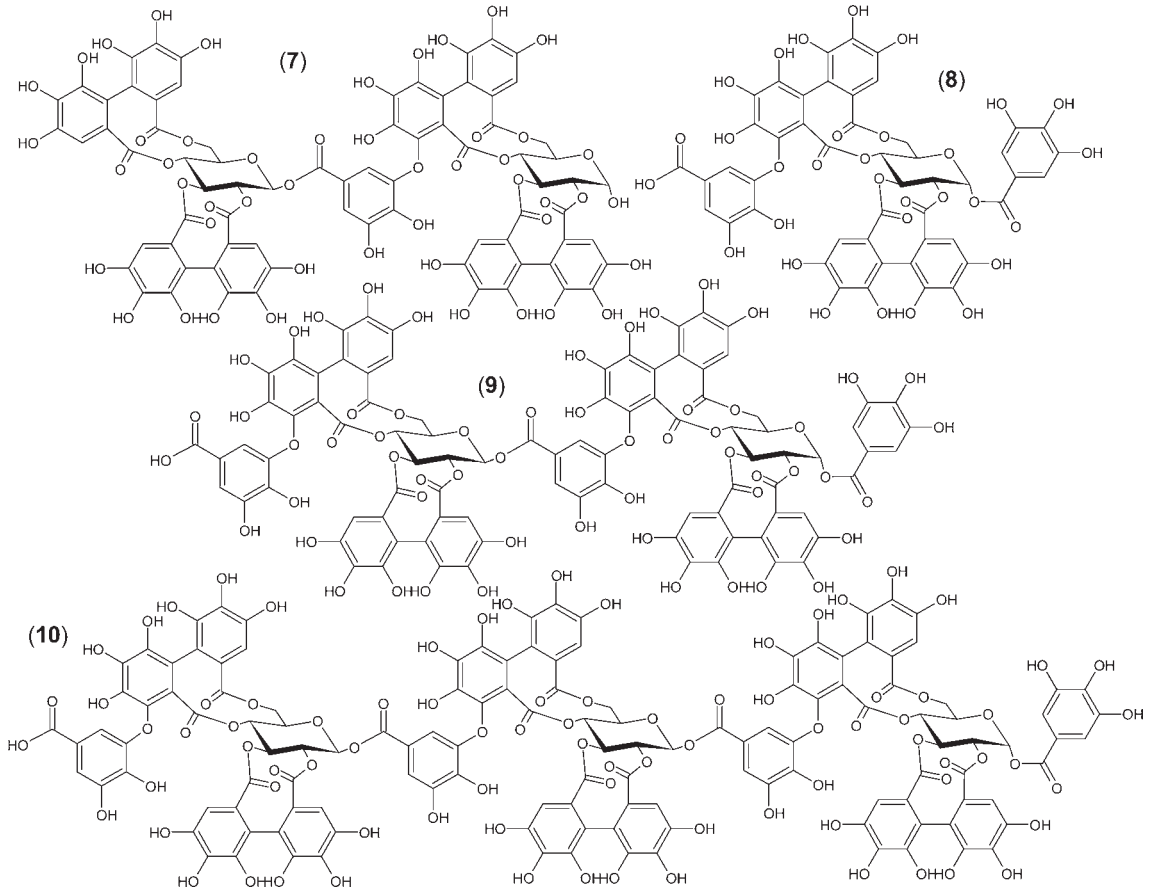

Figure 5. Structures tentatively suggested for peak 2 (**7**), peak 20—sanguiin H-2 (**8**), peak 18 (**9**), and peak 14 (**10**). The three latter minor ellagitannins are respectively a monomer, a dimer, and a trimer, bearing a terminal sanguisorboyl group.

m/*z* 633.0711, and *m*/*z* 300.9991 came from the *Rubus* monomeric structure of this compound. On the basis of MS and MS/MS information, peak 21 was identified as lambertianin C. The unambiguous identification of lambertianin C was supported by cochromatography with the isolated standard, which showed the same retention time and MS behavior.

Peak 22 (R_t = 27.9) had a $[M - H]^-$ at *m*/*z* 1869.1462, singly charged, and at *m*/*z* 934.0663, doubly charged, giving an exact molecular mass of 1870.1540. The MS/MS fragmentation pattern of the doubly charged signal showed the same signals as lambertianin C. In this case, on the basis of MS and MS/MS information, peak 22 was identified as sanguiin H-6. The unambiguous identification of sanguiin H-6 was supported by cochromatography with the isolated standard, which showed the same retention time and MS behavior.

In conclusion, a total of 22 structures were chromatographically separated. Besides the major components sanguiin H-6 and lambertianin C, a further 20 minor ellagitannins were preliminarily characterized. The 13 suggested structures (**Table 3** and **Figures 1**, **4**, and **5**) are in agreement with all the experimental data collected (retention time, UV spectrum, accurate MS and MS/MS) and with the assumption that *Rubus* oligomeric ellagitannins contain only the sanguisorboyl linking ester group (*31*), besides the well-known ellagic acid and gallic acid moieties. All known *Rubus* oligomeric ellagitannins share a common structure, originated via C−O oxidative coupling. More specifically, the linking unit in *Rubus* ellagitannins results from the donation of a galloyl hydroxyl oxygen to form an ether linkage to an HHDP group, which produces the class of GOD-type ellagitannins (*41*). Accurate MS and MS/MS experiments on a blackberry extract, hydrolyzed according to the literature (*31*), allowed us to confirm the presence of only the known products of *Rubus* ellagitannin and ellagic acid conjugate hydrolysis, i.e. ellagic acid, methyl sanguisorboate, methyl gallate, and sanguisorbic acid, which had been observed but not previously characterized (it was reported as "derivative 1" in ref *31*).

Thirteen compounds were structurally related to the main *Rubus* ellagitannins. We found, besides the well-known sanguiin H-6 and lambertianin C, the three expected isomers corresponding to the hydrolysis of a single ellagic unit from sanguiin H-6 (peaks 1, 5, and 10), together with the four expected isomers for hydrolysis of one ellagic unit from lambertianin C (peaks 3, 6, 17, and 19). A series of monomers (sanguiin H-2, peak 20), dimers (peak 18), and trimers (peak 14) of similar ellagitannins with one sanguisorboyl instead of the ellagic acid terminal unit were also observed. One structure (peak 2) could derive from the partial hydrolysis of the gallic acid group from sanguiin H-6. The origin of all these compounds could be justified by the hydrolysis of the ellagitannins, since both the 4,6-HHDP and 2,3-HHDP groups, as well as the 1-*O*-gallate ester, can be easily removed by hydrolysis in water (*36*−*38*). The presence of hydrolysis products could be expected, as a 5-fold increase of ellagic acid, probably due to hydrolysis, was observed to occur during the storage of red raspberries (*28*). On the other hand, we did not observe any significant change in the ellagitannin and ellagic acid conjugate amount and profile during the storage of one raspberry sample for 0−7 days at 4 °C or 0−6 days at 4 °C plus one day at laboratory temperature (data not shown). We cannot rule out also the possible contribution from some degradation of native ellagitannins during the sample manipulation prior to the preparative isolation of ellagitannins because, in spite of using only neutral solvents with our method, the final pH of the extracts,

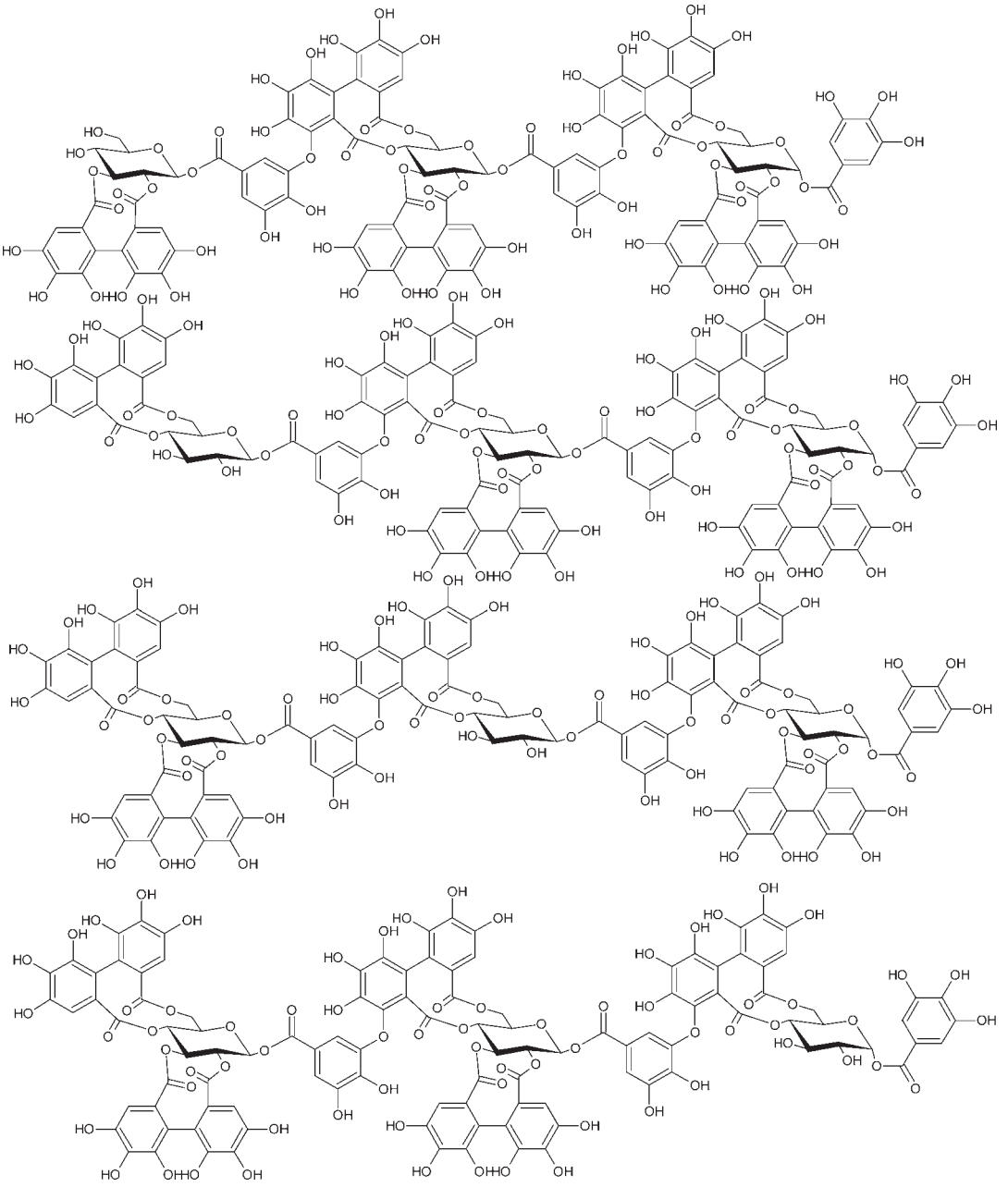

Figure 6. Structures of four minor trimeric ellagitannins (corresponding to peaks 3, 6, 17, and 19) which derive from the loss of an ellagic acid moiety from lambertianin C (**3**).

measured in water after removing acetone, was pH ~3.0 for raspberry extracts and pH ~3.5 for blackberry extracts. These values are due to the high content of organic acids in these berries (*33*). The widespread use of mineral acids (*9*,*35*) should be avoided in the extraction and analysis of ellagitannins, in order to prevent possible artifacts.

Some of the unknown ellagitannins (peaks 8 and 15 in raspberries only; peaks 9 and 16 in blackberries only) showed similar MS patterns of fragmentation and are probably closely related to one another. They are suggested to be respectively dimers (8 and 15) and trimers (9 and 16) of the same building unit, whose MS and MS/MS fragmentation pattern was described above.

Further research, including isolation and NMR characterization, is required for the complete structural characterization of minor ellagitannins described for the first time in our survey. This task can be easily accomplished by exploiting the materials and methods described in this paper.

Structural Assignation of Ellagic Acid Conjugates (***Table* 4**). *Peak 23* (R_t = 28.7) had a $[M - H]^-$ at *m*/*z* 300.9985 which was singly charged, giving an exact molecular mass of 302.0064. On the basis of UV data and cochromatography with the relative standard, this peak was identified as ellagic acid.

Peak 24 (R_t = 29.6) had a $[M - H]^-$ at *m*/*z* 433.0404 and at *m*/*z* at 301.0002 (M − 132 loss of a pentosyl unit), giving an exact mass of 434.0518. On the basis of the mass spectral data, peak 24 was identified as an ellagic acid pentose conjugate.

Peak 25 (R_t = 30.1) had a $[M - H]^-$ at *m*/*z* 447.0579, *m*/*z* 315.0069 (M − 132 loss of pentosyl group), and *m*/*z* 300.9998, giving an exact mass of 448.0657. On the basis of this spectral information, the peak was identified as a methylellagic acid pentose conjugate.

Peak 26 (R_t = 38.5) had a $[M - H]^-$ at *m*/*z* 447.0581 and *m*/*z* 315.0056 (M − 132 loss of a pentosyl group), giving an exact mass of 448.0659. On the basis of this spectral data and the similarity to

peak 25, peak 26 was identified as another methylellagic pentose conjugate.

Peak 27 (R_t = 40.6) had a $[M - H]^-$ at m/z 475.0555 and m/z 300.9982 (M − 132 − 42 loss of an acetylpentosyl group), giving an exact mass of 476.0633. In agreement with the previous assignation of Mullen et al (*42*), peak 27 was identified as ellagic acid acetylarabinoside.

In conclusion, besides free ellagic acid, four ellagic acid derivatives were found in *Rubus* extracts, basically being simply pentose conjugates of ellagic or methylellagic acid.

Survey of the Presence of ET and EAC in *Rubus* Berries. The new HPLC-DAD method was applied for the first time to investigate the presence of the 27 peaks previously characterized by UPLC-Q-TOF in five blackberry cultivars and six raspberry cultivars, grown in the same experimental field under standardized conditions during the years 2004−2007. The average ellagitannin and ellagic acid conjugate content in ripe blackberries and raspberries is reported in **Tables 5** and **6**.

The raspberries (**Table 5**) contained on average 1041 mg/kg of ET (range 662−2175 mg/kg) and 242 mg/kg of EAC (range 82−530 mg/kg). Sanguiin H-6 was the main ellagitannin, and the average ratio of lambertianin C/sanguiin H-6 was found to be 0.8 (range 0.6−1.0). The other ET were present as minor components in raspberry extracts, with their concentrations being at least 1 order of magnitude lower than those of sanguiin H-6 and lambertianin C. As far as EAC were concerned, peak 24 was present in raspberries at a concentration approaching that of free ellagic acid, while peaks 26−27 were at about one-half. The two isomer peaks 8 and 15, not observed in blackberries, were consistently found as minor components of the raspberry extracts. A factorial ANOVA for variety and year, performed on the data of the years 2005−2006−2007, suggested a significant role for the variety (p = 0.034) while the year-to-year variation and the interaction factor were not significant. The concentration of lambertianin C and of peaks 2, 8, 15, and 24 did significantly contribute to the model. The cultivar Octavia was in all three years the richest in ellagitannins.

Blackberries (**Table 6**) contained on average 1080 mg/kg of ET (range 704−1556 mg/kg) and 200 mg/kg of EAC (range 112−346 mg/kg). In contrast to raspberries, in blackberries, lambertianin C was the main ellagitannin, with an average lambertianin C/sanguiin H-6 ratio of 1.7 (range 0.9−3.4). The Chesapeake cultivar was particularly rich in lambertianin C, having a lambertianin C/sanguiin mean ratio of ∼3. Besides lambertianin C and sanguiin H-6, peaks 9 and 17 were the major ET in blackberry extracts, with concentrations of about half to one-third that of sanguiin H-6, while all other ET were at least 1 order of magnitude lower than sanguiin H-6. The two isomers peaks 9 and 16, not observed in raspberries, were consistently found in all the blackberry extracts. As far EAC were concerned, peak 25 was present in blackberries in a slightly higher concentration than free ellagic acid, while the concentration of peak 26 was on average the lowest. In the case of blackberries, it was not possible to perform a factorial ANOVA due to missing samples. A one-way ANOVA highlighted significant differences (p = 0.006) in the composition among the different cultivars. Sanguiin H-6 (higher in Thornfree cv.) and total ellagic acid conjugates (higher in Apache and Chesapeake cvs), as well as peaks 16 and 26, were significantly different among the cultivars investigated.

Altogether, the data of this survey confirmed that lambertianin C and sanguiin H-6 are by far the major ET of *Rubus* berries, with their sum representing respectively 81% of total ET (range 73−86%) in raspberries and 67% (range 41−83%) in blackberries. This study also gave the first accurate estimate of their presence in berries and demonstrated that lambertianin is the major ellagitannin in blackberries and sanguiin H-6 in raspberries. Besides these two compounds, another 20 minor ET were detected and preliminarily characterized in *Rubus* extracts. The free ellagic acid was present at a concentration slightly higher than its conjugates, representing respectively 46% (range 26−82%) of total EAC in raspberries and 37% (19−52%) in blackberries. The sum of the four conjugated forms of ellagic acid was present in *Rubus* at a concentration similar to that of free ellagic acid. The new method allowed us to compare the detailed composition of raspberry and blackberry cultivars, including 27 different structures belonging to the ellagitannin or ellagic acid conjugate classes. Beyond a general similarity in the composition of *Rubus* fruits, both qualitative and quantitative differences in the pattern were shown to exist between raspberry and blackberry ET and EAC. Importantly, it was also demonstrated that different raspberry cultivars can show quantitative differences consistent between growing seasons.

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Supporting Information Available: UV spectra of (a) ellagic acid, (b) sanguiin H-6, and (c) lambertianin C. This material is available free of charge via the Internet at http://pubs.acs.org.

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