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Ellagitannin Composition of Blackberry As Determined by HPLC-ESI-MS and MALDI-TOF-MS

TIFFANY J. HAGER,[†,§] LUKE R. HOWARD,*[,†] ROHANA LIYANAGE,[#] JACKSON O. LAY,[#] AND RONALD L. PRIOR[§,⊥]

Department of Food Science, University of Arkansas, 2650 North Young Avenue, Fayetteville, Arkansas 72704; Arkansas Children's Nutrition Center and Agricultural Research Service, U.S. Department of Agriculture, 1212 Marshall Street, Little Rock, Arkansas 72202; and University of Arkansas Statewide Mass Spectrometry Facility, 1260 West Maple Street, Fayetteville, Arkansas 72701

Blackberries (*Rubus* sp.) were evaluated by high-performance liquid chromatography−electrospray ionization−mass spectrometry (HPLC-ESI-MS) and matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry (MALDI-TOF-MS) to identify the ellagitannins present in flesh, torus (receptacle tissue), and seeds. Most ellagitannins were present (or detectable) only in seed tissues. Ellagitannins identified by HPLC-ESI-MS in the seeds included pedunculagin, casuarictin/potentillin, castalagin/vescalagin, lambertianin A/sanguiin H-6, lambertianin C, and lambertianin D. For several of the ellagitannins, isomeric separation was also obtained. The MALDI-TOF-MS analysis was primarily utilized to evaluate and identify high molecular mass (>1000 Da) ellagitannins. The MALDI analysis verified the presence of the ellagitannins identified by HPLC-ESI-MS including lambertianin A/sanguiin H-6, lambertianin C, and lambertianin D, but the analysis also indicated the presence of several other compounds that were most likely ellagitannins based on the patterns observed in the masses (i.e., loss or addition of a gallic acid moiety to a known ellagitannin). This study determined the presence of several possible isomeric forms of ellagitannins previously unidentified in fruit and presents a possible analytical HPLC method for the analysis of the major ellagitannins present in the fruit.

KEYWORDS: Ellagitannins; blackberry; HPLC-ESI-MS; MALDI-TOF-MS

INTRODUCTION

Over the past decade, there have been numerous epidemiological studies indicating that fruit and vegetable consumption may play a role in reducing the risks of various chronic diseases, including cardiovascular disease and cancer (*1, 2*). These health benefits have been widely attributed to the phenolic content of plant foods. Tannins are one group of phenolics that have been considered a potential source of significant health benefits (*3, 4*).

Tannins are oligomeric and polymeric forms of phenolics. They are divided into two classes: condensed and hydrolyzable tannins. Much research has been conducted on the structural classifications of condensed tannins or proanthocyanidins in fruits, but there has been limited work on hydrolyzable tannins, also known as gallotannins and ellagitannins. Gallotannins are composed of gallic acid (**Figure 1A**), whereas ellagitannins are polymers of hexahydroxydiphenic (HHDP) acid (**Figure 1B**), which is a dimeric form of gallic acid that can spontaneously lactonize to form ellagic acid (**Figure 1C**). For both gallotannins and ellagitannins, the gallic or HHDP acids are esterified to one or multiple glucopyranoses to form diversified polymers (*5*).

Ellagitannins are a very important class of phenolics that have received attention recently due to the purported health benefits of pomegranate fruit and juices (*6*). This has included bioavailability and metabolism studies of punicalagin, an ellagitannin found in pomegranate fruit (*7, 8*). However, in addition to pomegranate (*9*) there are other fruits that contain appreciable levels of ellagitannins, including strawberries (*10, 11*), raspberries (*10, 12–14*), and Muscadine grapes (*15*).

In *Rubus* (family Rosaceae) the primary unit of an ellagitannin is the bis-HHDP glucopyranose, which is commonly known as pedunculagin (**Figure 1E**), and the galloylated form, galloyl-bis-HHDP glucopyranose, or casuarictin/potentillin, which refers to specific isomeric forms [**Figure 1F** (*16, 17*)]. To complicate the matter structurally, these can be observed as α- or *β*-glucopyranosides (*16*) with the HHDP units in *R* or *S* configurations relative to the C4 and C1 of the glucose (*18*). Most of the ellagitannin structures that have been observed in *Rubus* fruit and leaves are polymerized forms of the galloyl-bis-HHDP. The names and structures of these can be observed in **Figure 2**. It

* Author to whom correspondence should be addressed (e-mail lukeh@uark.edu).
[†] University of Arkansas.
[§] Arkansas Children's Nutrition Center.
[#] University of Arkansas Statewide Mass Spectrometry Facility.
[⊥] U.S. Department of Agriculture.

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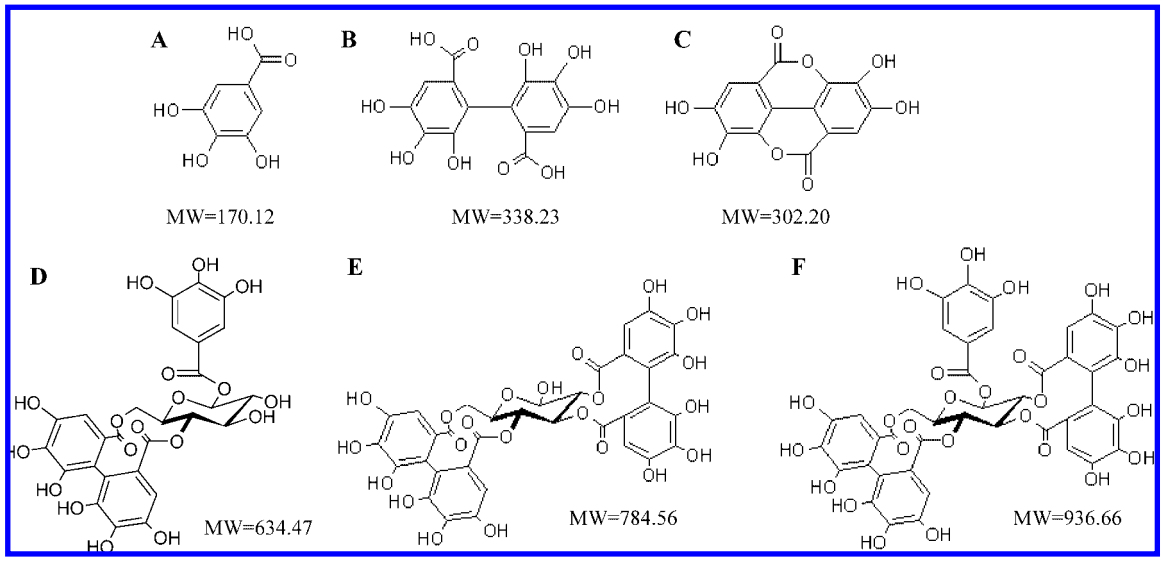

Figure 1. Structures of gallic acid (**A**), hexahydroxydiphenic acid (**B**), ellagic acid (**C**), and the basic monomeric units for the larger molecular mass ellagitannins: galloyl-HHDP glucose (**D**), pedunculagin (**E**), and galloyl-bis-HHDP glucose (**F**). Structures were adapted from refs *9, 15, 16*, and *32*.

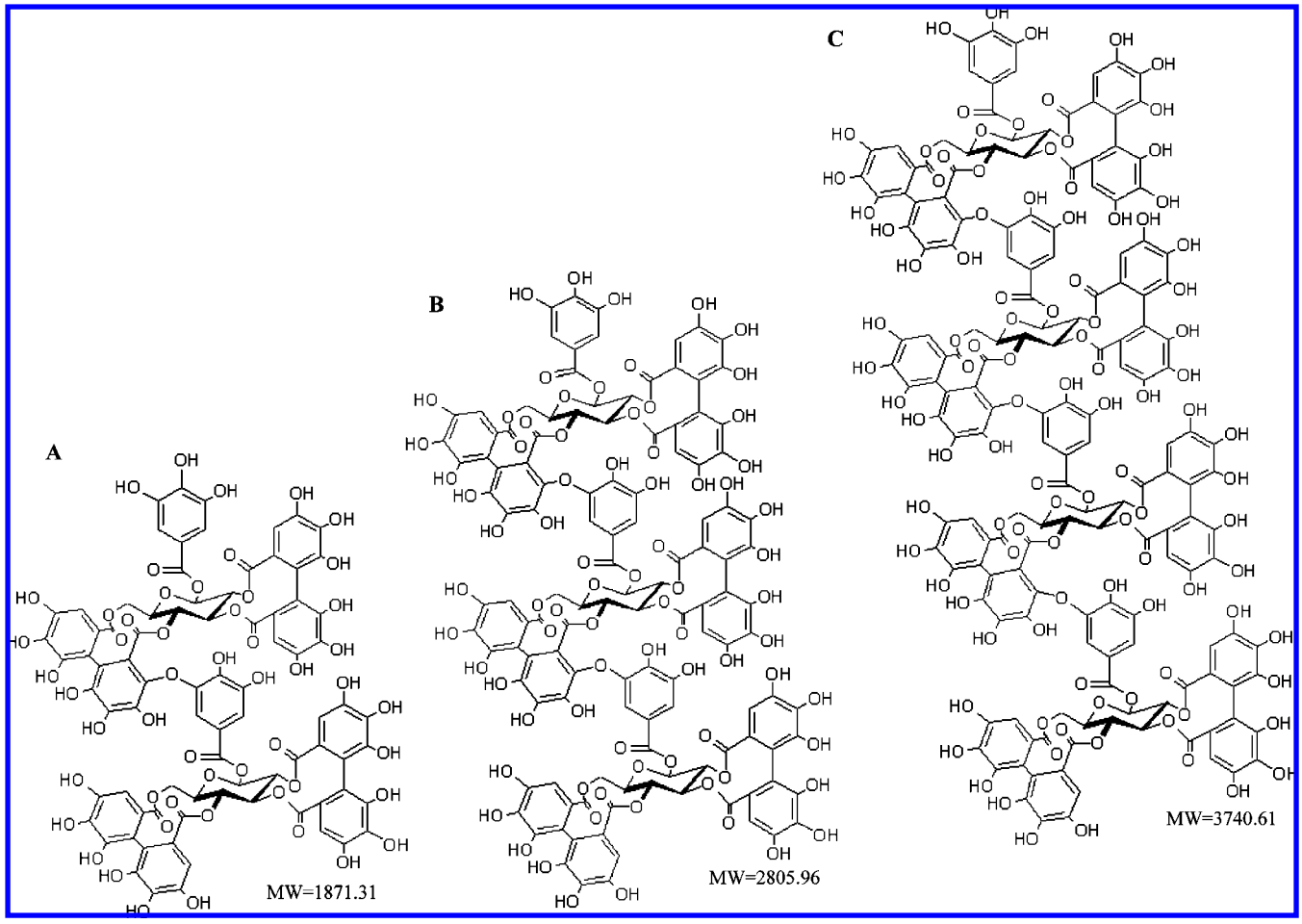

Figure 2. Common ellagitannin structures in *Rubus* formed from dimers (sanguiin H-6/lambertianin A) (**A**), trimers (lambertianin C) (**B**), and tetramers (lambertianin D) (**C**) of galloyl-bis-HHDP glucopyranose. Structures were adapted from ref *16*.

is important to note that many of the structures have multiple isomers that have not been evaluated in previous studies on *Rubus* fruit.

Sanguiin H-6, lambertianin C, and various ellagic acid derivatives (acylated and/or glycosylated ellagic acid moieties) have been identified in *Rubus* fruit, but in most of the studies there are still unidentified ellagitannins due to the diverse and complex nature of the structures. In addition, there is still very limited information on the ellagitannin composition of blackberries. In earlier studies, the ellagitannins were quantified by acid hydrolysis to form ellagic acid (*19, 20*), but the separation of individual ellagitannins was not conducted, and the structures were not determined. In later studies, the presence of multiple ellagitannins in their native state by HPLC analysis was

Table 1. Ellagitannin Identification in Apache Flesh, Torus, and Seed

peak	RT (min)	compound identification	MS data (fragments)	seeds	torus	flesh
1	8.0	pedunculagin isomer	783.2 (481.1, 301.0)	X	X	ND[a]
2	10.3	castalagin/vescalagin isomer	933.1 (783.1, 631.1, 451.1, 301.0)	X	ND	ND
3	10.8	castalagin/vescalagin isomer	933.1 (783.2, 631.0, 451.1, 301.0)	X	ND	ND
4	12.4	pedunculagin isomer	783.2 (481.1, 301.0)	X	X	ND
5	18.3	galloyl-HHDP glucose isomer	633.1 (300.8)	X	X	ND
6	22.5	lambertianin C isomer	$[1401.5]^{-2}$ (1567.4, 1235.0, 933.1, 783.2, 633.1, 301.0)	X	ND	ND
7	24.3	sanguiin H-6/lambertianin A	1870.0 (1567.2, 1235.1, 935.0, 633.2, 301.0)	X	X	X
8	26.6	lambertianin D isomer	$[1868.9]^{-2}$ (1235.1, 933.2, 633.2, 301.0)	X	X	ND
9	27.4	lambertianin C isomer	$[1401.5]^{-2}$ (1235.1, 935.2, 763.3, 633.1, 301.0)	X	X	X
10	29.3	sanguiin H-6/lambertianin A	1869.1 (1567.1, 1235.0, 935.0, 633.1, 301.0)	X	X	X
11	34.2	galloyl-bis-HHDP glucose isomer	935.2 (783.1, 433.1, 301.0)	X	X	ND
12	35.2	ellagic acid	301.0	X	ND	ND
A	9.4, 14.2, 16.5, 17.8	unknown compounds (*m*/*z* 951.0)	951.0 (907, 783, 633, 301)	X[b]	X[b]	ND
B	14.6, 17.8, 23.7, 25.6	unknown compounds (*m*/*z* 1718.1)	1718.1 (783, 631, 301)	X[b]	X[b]	ND

[a] ND, not detected. [b] At least one unknown was observed.

demonstrated, but again the structural composition was not reported (*21, 22*). In this study HPLC-ESI-MS and MALDI-TOF-MS were utilized to separate and elucidate the structural composition of several native ellagitannins in Apache blackberries.

MATERIALS AND METHODS

Reagents and Standards. Ellagic acid was purchased from Sigma Aldrich (St. Louis, MO). All solvents used in this study were of HPLC grade from EMD Biosciences (Madison, WI).

Materials. Blackberries (cv. Apache) were obtained from the University of Arkansas Agriculture Experiment Station (Clarksville, AR). Blackberries were frozen and stored at −20 °C until analysis.

Sample Preparation for HPLC-ESI-MS. Frozen blackberries were thawed to allow berries to be separated into fractions. The middle section, known as the torus (receptacle tissue), was removed from individual blackberries and prepared for freeze-drying. The remaining tissue (and seeds) was smashed by hand to ensure minimal seed disruption on a freeze-drying tray. The torus and blackberry mash samples were frozen overnight and subsequently lyophilized for 2 days in a Virtis model Genesis SQ freeze-dryer (Gardiner, NY). After complete drying, the mash was carefully broken and pressed through a fine mesh screen to separate the flesh from the seeds. The flesh, seeds, and torus were extracted according to the protocol below.

Extraction Protocol. Whole frozen blackberries and freeze-dried flesh, seeds, and torus were extracted. Frozen blackberry samples were blended to a puree, and 10 g samples were extracted with a small amount of acetone/water/acetic (70:29.5:0.5 v/v/v) with a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp., Mason, OH) to homogenize the tissue and seeds. The samples were filtered through Miracloth (CalBiochem, La Jolla, CA), and the filtrate was homogenized with additional extraction solvent. The samples were taken up to a final volume of 100 mL with extraction solvent and stored at −70 °C until further analysis. The same extraction protocol was utilized on the freeze-dried flesh, seeds, and torus with 2 g of seeds in a 50 mL final volume of extraction solvent and 1.5 g of flesh or 0.5 g of torus in a 25 mL final volume.

Sephadex LH-20 Sample Preparation. Preliminary analysis of the extracts indicated that there was significant interference with the matrix-assisted laser desorption/ionization analysis of the blackberry extract; therefore, a cleanup step was included to remove surfactants and other phenolics. The frozen extract of the whole berry was subjected to a Sephadex LH-20 cleanup (Sigma-Aldrich, St. Louis, MO) according to the methods for procyanidin cleanup in Gu et al. (*23*). Sephadex LH-20 (3 g) was equilibrated in water for 4 h, and the slurry was added to a 6 × 1.5 cm column. The column was attached to a Waters Sep-Pak vacuum manifold (Milford, MA) and a vacuum pump to facilitate the cleanup. The sample was prepared from 40 mL of the extract. The acetone was dried using a Thermo Savant SPD 1010 Speed Vac System (Holbrook, NY), and the sample was applied to the column. After loading, the sample was washed with 40 mL of 30% MeOH. The tannins were removed from the column with 80 mL of 70% acetone, and the recovered sample was dried by Speed Vac and resuspended in 5 mL of extraction solvent.

High-Performance Liquid Chromatography−Electrospray Ionization−Mass Spectrometry (HPLC-ESI-MS). The ellagitannins were evaluated by HPLC-ESI-MS using an HP 1000 series HPLC and a Bruker Esquire 2000 quadrapole ion trap mass spectrometer. The ellagitannins were separated using a Phenomenex (Torrance, CA) Aqua 5μm C_{18} (250 × 4.6 mm) column and a binary gradient (*24*) of 2% acetic acid for mobile phase A and 0.5% acetic acid in water/acetonitrile (1:1 v/v) for mobile phase B at a flow rate of 1.0 mL/min. The linear gradient was from 10 to 55% B from 0 to 50 min, from 55 to 100% B from 50 to 60 min, and from 100 to 10% B from 60 to 65 min. The mass spectrometry analysis was performed in negative ion mode under the following conditions: capillary voltage at +3 kV, nebulizer gas pressure at 32 psi, dry gas flow at 12 L/min, and skim voltage at −53.7.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). After Sephadex LH-20 cleanup, the ellagitannin extract was mixed with a 1 M solution of dihydroxybenzoic acid (DHB) in 90% methanol in a 1:1 ratio, and 1 μL of the mixture was spotted onto a ground stainless steel MALDI target for MALDI analysis using the dry droplet method. A Bruker Reflex III MALDI-TOF-MS (Billerica, MA) equipped with a N_2 laser (337 nm) was used in the MALDI analysis, and all the data were obtained in positive ion reflectron TOF mode.

RESULTS

Identification of Ellagitannins by HPLC-ESI-MS. Eleven ellagitannins were identified in the blackberry flesh, seeds, and torus; however, most were present only in the seeds with only a few present in all three regions of the fruit (**Table 1** and **Figure 3**). Using this method of separation, we also observed several other ellagitannins, based on mass spectral data, that remain unidentified, but they were minor contributors (<10%) to the level of ellagitannins (on an ellagic acid equivalent basis) present in the whole fruit (**Figure 3**).

Peaks 1 and 4 (Pedunculagin). Peaks 1 and 4 in **Figure 3A** displayed a parent peak at *m*/*z* 783.2, which was determined to be singly charged on the basis of observed isotopic pattern of the peak (1 *m*/*z* unit separation between isotopic peaks). For both peaks 1 and 4, a small signal was observed at *m*/*z* 1567.1, but this was presumed to be an artifact of the measurement due to the formation of gas phase non-covalent dimers. This is typically due to the use of a relatively low skimmer voltage. The peak at *m*/*z* 783.2 was determined to correspond to pedunculagin on the basis of the pattern of ellagitannins observed in a study on strawberries (*11*) with an actual molecular

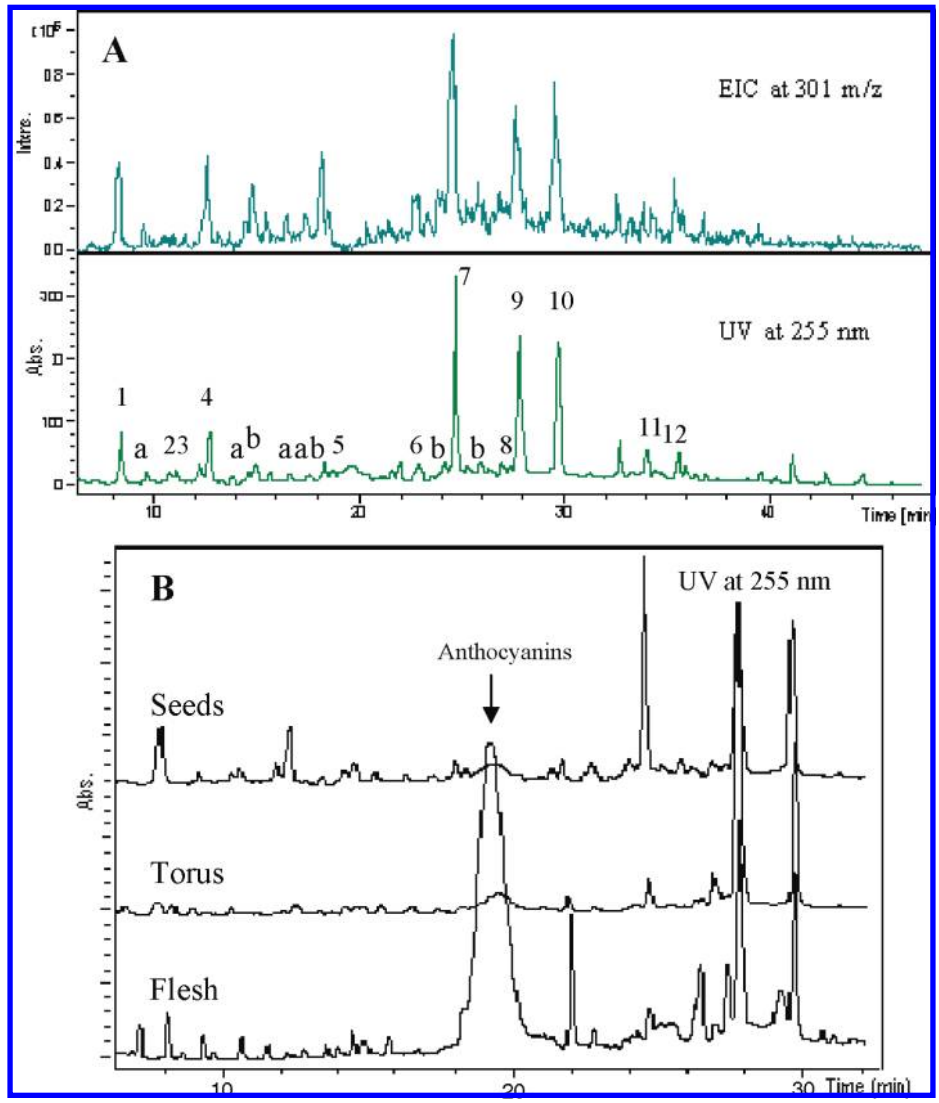

Figure 3. Apache seed chromatograms (**A**) including extracted ion chromatogram at *m/z* 301 (1) and UV detection at 255 nm (2) with the major ellagitannins indicated numerically corresponding to **Table 1**. Other possible ellagitannins (unidentified) are indicated by a or b. (**B**) Overlaid chromatogram of Apache seeds, flesh, and torus.

mass 784.56 (as observed in **Figure 1E**). Note that the observed *m/z* value is 1 unit less than the calculated molecular mass for the parent neutral molecule as expected. The observed *m/z* value in ESI-MS negative ion mode corresponds to the *m/z* value of the deprotonated $[M - H]^-$ ion, where M is the neutral parent molecule.

There also appeared to be a coeluting compound at 12.4 min, according to different temporal response of the mass spectral data on the front end of the peak. This was most likely an ellagitannin, but it had an unusual parent ion $[M - H]^-$ at *m/z* 951, but it seems to have fragments characteristic to ellagitannins (*m/z* 783 and 631) in addition to a fragment with a *m/z* of 907.

Peaks 2 and 3 (Castalagin/Vescalagin). On the basis of previous studies, compounds corresponding to peaks 2 and 3 were most likely isomeric forms of the structure commonly called castalagin/vescalagin (**Table 1**; **Figure 3A**). On the basis of observed similar mass spectra, both compounds were presumed be deprotonated ions with *m/z* 933.1. According to studies on hydrolyzable tannins in wood, pedunculagin or galloyl-bis-HHDP glucose may undergo enzymatic alterations that open the ring structure of the glucoside (and undergo other reactions) to form the compound castalagin or its isomer vescalagin (**Figure 4A**) having a molecular mass of 934 Da (*25, 26*).

The fragmentation patterns observed for compounds corresponding to peaks 2 and 3 were consistent with the loss of gallic acid units and, for the lower mass fragments, the loss of a glucosyl moiety. The observed fragments for both compounds were 783, 631.1, 451.1, and 301.0. Fragments at *m/z* 783.2 and 631.1 were attributed to the sequential loss of galloyl units from the parent molecule and the *m/z* of 301.1 to ellagic acid. The fragment consistent with the trigallic acid moiety of the castalagin/vescalagin structure was also consistent with the observed fragment at *m/z* 451.1 (**Figure 5**). The loss of the trigalloyl unit from the parent structure, if it undergoes lactonization, would result in a structure with a molecular mass of 452.3, consistent with the proposed structure for *m/z* 451.1.

Peak 5 (Galloyl-HHDP Glucose Isomer). The compound corresponding to peak 5 (**Table 1**; **Figure 3A**), with an *m/z* of 633.1, was presumed to be an isomer of galloyl-HHDP glucose. It is similar in structure to the galloyl-bis-HHDP glucose with only one hexahydroxydiphenolic group attached to the glucose moiety (**Figure 1D**). There was only one fragment ion observed

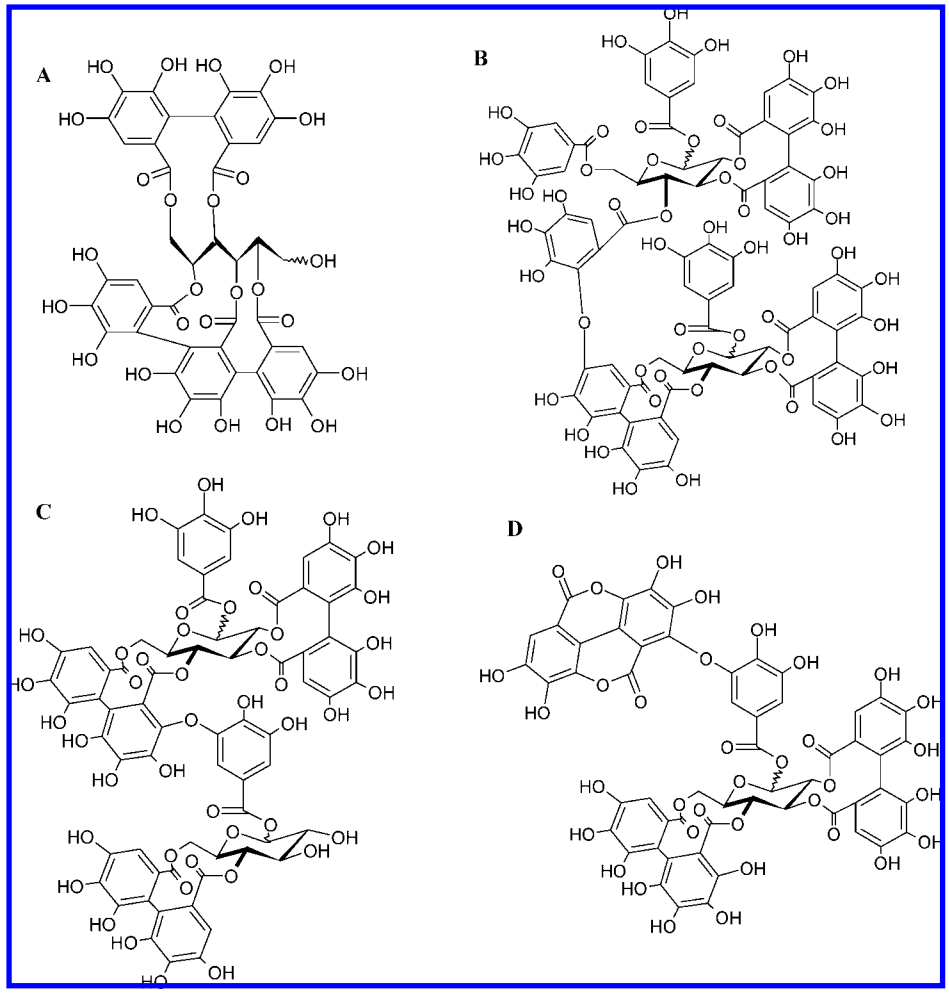

Figure 4. Structures of other ellagitannins: castalagin/vescalagin (**A**), nobotanin A (**B**), sanguiin H-10 (**C**) and lambertianin B (**D**). Structures were adapted from refs *14–16, 32*, and *33*.

at *m/z* 300.8, but it supports the proposed structure. This corresponded to the HHDP unit after lactonization (ellagic acid). This compound was difficult to identify due to the close proximity to anthocyanins in the chromatogram. The anthocyanins eluted between 18 and 20 min, resulting in interference in the mass spectral data and the UV trace at 255 nm. The compound was observed only in the seeds and torus, but we were unable to determine whether it was present in the flesh due to the interference of anthocyanins.

Peaks 6 and 9 (Lambertianin C Isomers). Lambertianin C (**Table 1**; **Figure 3A**) was one of the high-mass compounds identified in blackberry fruit. The molecular mass of this compound was 2805.9 Da, which is out of the normal *m/z* range of the Bruker-Esqire HPLC-ESI-MS; however, the doubly deprotonated ion observed (half of the *m/z* value for the parent deprotonated ion) allowed it to be detected in the normal *m/z* range of the instrument. The isotopic distribution (0.5 *m/z* unit separation between peaks) at *m/z* 1401.5 indicated that the actual mass was twice the observed *m/z* value, thereby confirming the molecular mass of this compound to be 2805 Da (**Figure 6**). This compound has been observed in other studies on raspberries (*12–14*) and *Rubus* leaves (*16*), but there was no evidence of multiple isomeric forms in the fruit. The observed fragmentation pattern, *m/z* 935.2, 783.2, 633.1, and 301.0, which is consistent with characteristic ellagitannins oberved in this and other studies on *Rubus* ellagitannins (*10, 12–14*), further supports the assignment. The structure of lambertianin C is shown in **Figure 2B**.

Peaks 7 and 10 (Lambertianin A/Sanguiin H-6 Isomers). Compounds corresponding to peaks 7 and 10 (**Table 1**; **Figure 3A**) were the predominant ellagitannins in the seeds. Lambertianin A and sanguiin H-6 are isomeric at C1 of one of the glucose moieties [(*16*); structure shown in **Figure 2A**]. In previous studies on *Rubus* fruits, only one isomer of lambertianin A/sanguiin H-6 was reported (*12–14*), but in our study we observed more than one isomer, and both contributed significantly to the ellagitannin composition of the whole fruit. These were identified on the basis of the relationship between the presumed parent deprotonated ion at *m/z* 1870 and fragments having *m/z* values corresponding to the losses of galloyl units, glucosyl units, and HHDP units. Whereas the fragment at *m/z* 1567.4 was attributed to the loss of an HHDP unit, the fragment at *m/z* 1235.0 was likely the result of the loss of galloylated glucose with an HHDP. The fact that subsequent spontaneous lactonization of the ellagic acid still bound to the gallic acid moiety of the other sugar results in a structure like that of deprotonated lambertianin B (see **Figure 4D** for structure) further supports this proposed structure. The fragment at *m/z* 935 was likely the result of the loss of the galloyl-bis-HHDP glucose moiety from the parent compound. The other fragments observed for lambertianin A/sanguiin H-6 were the same as the other compounds discussed previously.

Peak 8 (Lambertianin D Isomer). The compound corresponding to peak 8 (**Table 1**; **Figure 3A**) was a unique compound found in this study because it has not been previously reported in blackberry fruit, and it is the largest ellagitannin that has been

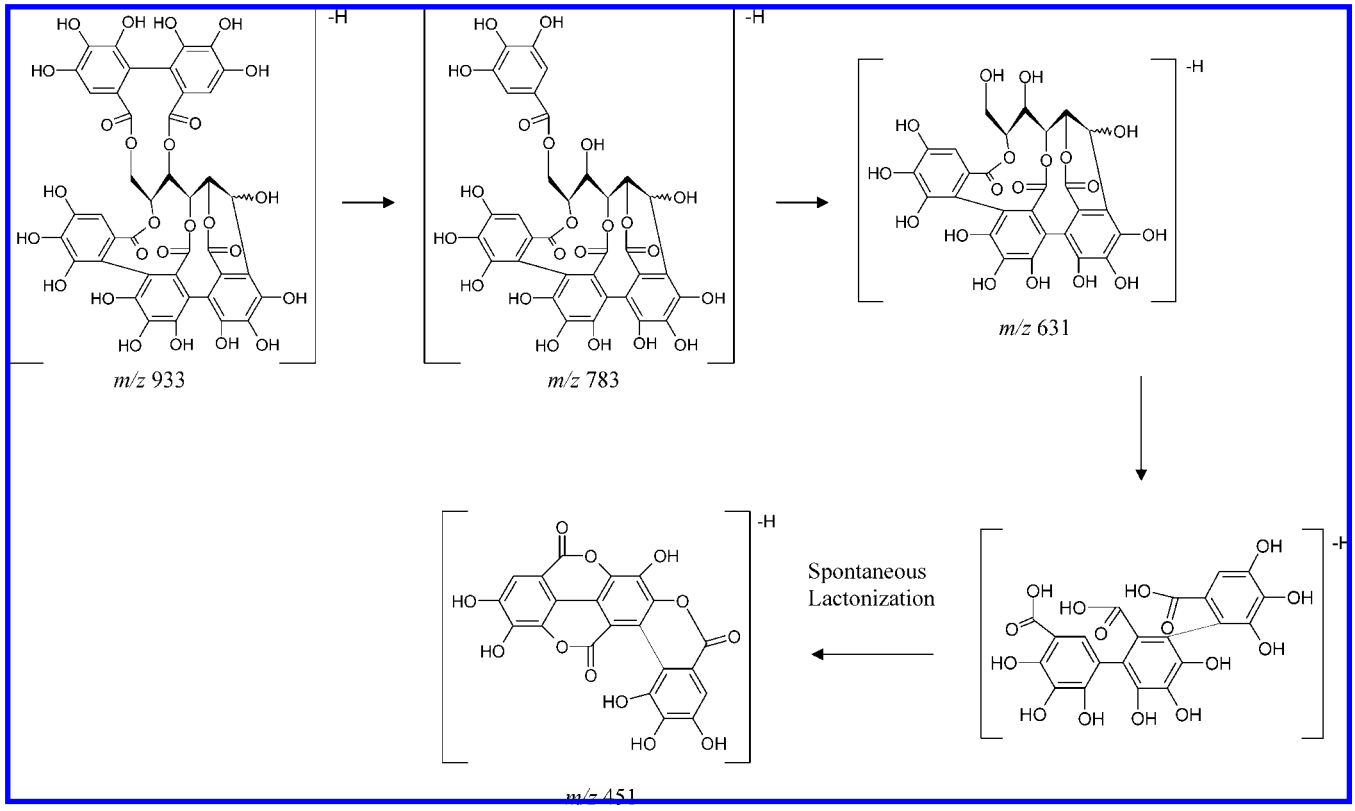

Figure 5. Proposed structure of oxidized galloyl-bis-HHDP glucose (with modification bolded) and the corresponding fragmentation.

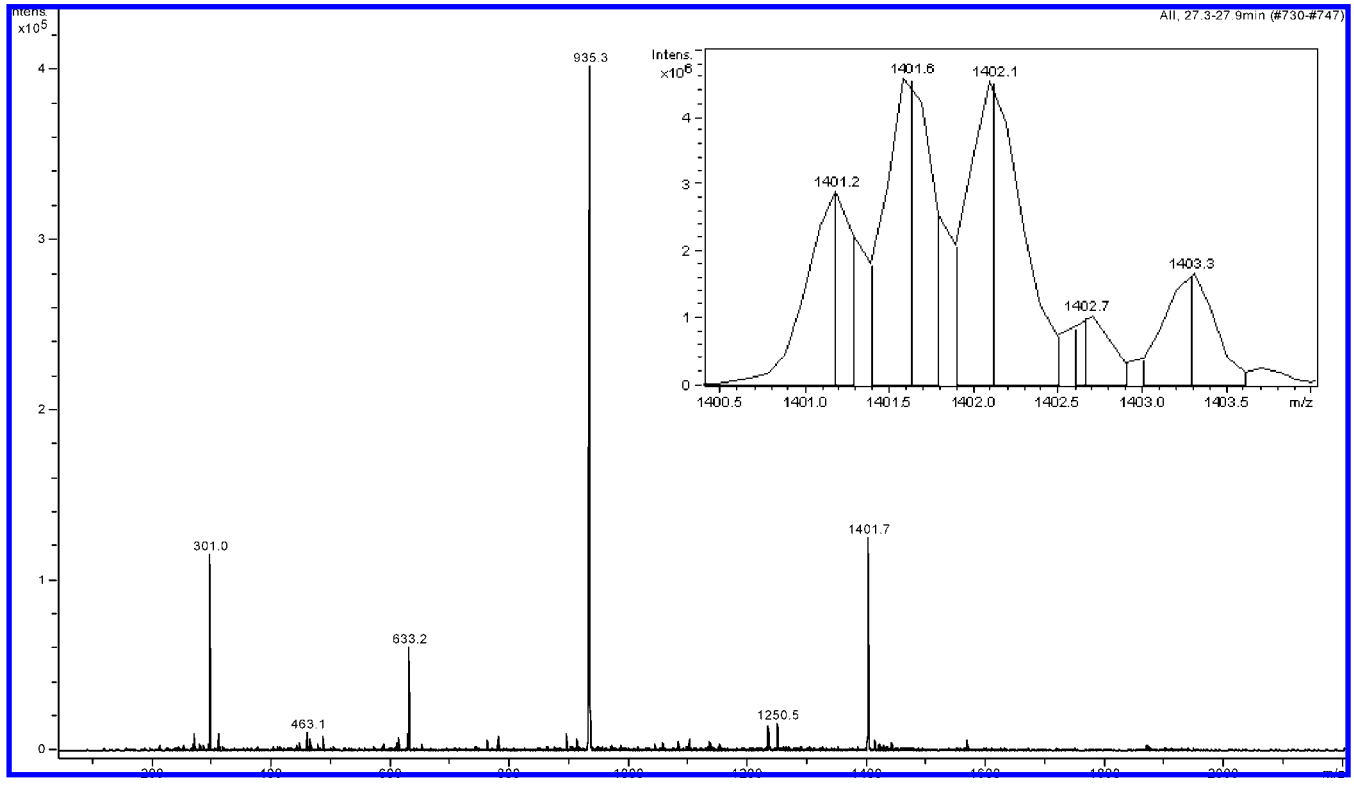

Figure 6. HPLC-ESI-MS data of a lambertianin C isomer (RT = 27.4 min) (**A**) and the enlarged view of the *m/z* 1401.7 (**B**), demonstrating the doubly charged peak, which verifies the presence of the large molecular mass compound at 2804.

identified in the fruit. On the basis of its mass spectrum (**Figure 7**), the parent peak at *m/z* 1868.9 has isotopic distribution consistent with a doubly deprotonated ion (0.5 *m/z* unit separation between isotopic peaks) and thus can be assigned to compound C in **Figure 2**, with a molecular mass of 3740 Da.

Peak 11 (Galloyl-bis-HHDP Glucose Isomer). On the basis of previous studies, the compound corresponding to peak 11 (**Table 1**; **Figure 3A**) was most likely an isomer of the structure commonly called galloyl-bis-HHDP glucose or casuarictin/potentillin (**Figure 1F**). The fragmentation pattern, including

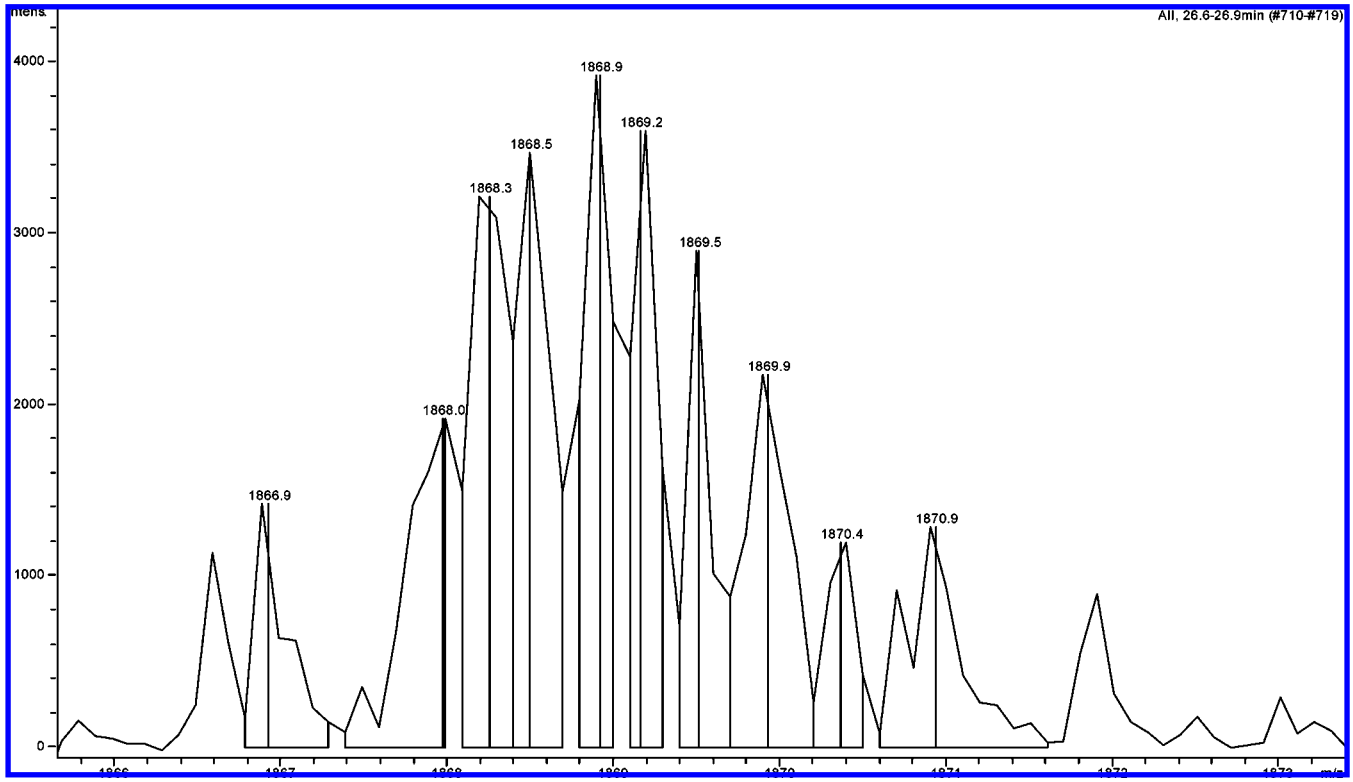

Figure 7. HPLC-ESI-MS data of a lambertianin D isomer (RT = 26.6 min) at *m/z* 1869.1 demonstrating the doubly charged peak (and fragment of the large molecular mass compound at 3740).

m/z 783.1 and 301.0, was likely due to the loss of HHDP moieties from the glucosyl unit, which was the typical fragmentation pattern observed in this study. This compound appeared to coelute with a flavonol, quercetin-3-rutinoside, with *m/z* 609.0, and an aglycone fragment of *m/z* 301.

Peak 12 (Ellagic Acid). On the basis of a pure standard and the *m/z* 301 ion observed at this retention time, compound **12** was identified as ellagic acid (**Table 1**; **Figure 3A**).

Other Peaks (Unknown Ellagitannins). The other compounds observed in the fruit (denoted by "a" or "b" in **Figure 3A**) were identified as ellagitannins due to the characteristic fragmentation patterns observed, but they had also demonstrated unique mass profiles that were difficult to discern due to interference or unknown parent ion masses. The unknown ellagitannins were identified as ellagitannins on the basis of several characteristic fragments that were consistently present in the profiles (i.e., *m/z* 783, 633, and 301). Several of the unknown ellagitannins remain unidentified because the parent masses were not discernible from fragments, but a few of the unknown compounds had deprotonated ions of 951.0 (denoted by "a" in **Figure 3A**). When fragmented by MS/MS (data not shown), the fragmentation pattern included *m/z* 907, 783, 633, and 301. Thus, several of the unknown ellagitannins may be isomers of this unique compound with a presumed molecular mass of 952, but further work is needed to verify the structure of this compound.

The compounds labeled "b" (**Figure 3A**) were also difficult to evaluate, but they were most likely degalloylated sanguiin H-6/lambertianin A isomers with presumed deprotonated molecules of 1718.1 or they may be structures similar to nobotanin A (**Figure 4B**) or malabathrin B. Nobotanin A/malabathrin B-like compounds were tentatively identified in a previous study with raspberries on the basis of a doubly deprotonated molecule of (*m/z* 859) and a fragmentation pattern indicative of an ellagitannin (*13*). Overall, the low molecular mass fragments observed in this study and in the previous study were similar (*m/z* 783, 631, and 301), but *m/z* 1567.0 was also observed in this study. The retention time at which this compound was observed is indicated by "b" in **Figure 3A**.

Identification of Ellagitannins by MALDI-TOF-MS. For the ellagitannins with higher molecular mass (>2000 Da), HPLC-ESI-MS has limited application because it is out of the regular instrument working range. Typically, analysis of high molecular mass compounds by HPLC-ESI-MS involves the analysis of a series of multiply charged compounds that enables the determination of the actual molecular mass. In our HPLC-ESI-MS analyses, we were able to identify several doubly charged compounds; however, to verify the presence of these higher molecular mass compounds that would correspond to the doubly charged *m/z* ratios observed by HPLC-ESI-MS, MALDI-TOF-MS analysis was performed on the blackberry fruit.

The MALDI-TOF-MS spectrum (**Figure 8**) verified the presence of several large molecular mass compounds that were observed by HPLC-ESI-MS analysis and indicated the presence of several ellagitannins that were not identified in the LC chromatogram. Compound **B** ([1741.5]+) appeared to be a sodium adduct of a degalloylated sanguiin H-6/lambertianin A with a molecular mass of 1719.2. Compounds **C**, **F**, and **G** ([1893.5]+, [2827.6]+, and [3763.8]+, respectively) were the sodium adduct peaks for the sanguiin H-6/lambertianin A, lambertianin C, and lambertianin D isomers, respectively. Thus, the MALDI-TOF-MS analysis verified the presence of the three major large molecular mass compounds identified in the LC chromatogram.

Compound **A** ([1591.5]+) was a sodium adduct of the ellagitannin sanguiin H-10 (sanguiin H-6 with one less HHDP moiety) with a molecular mass of 1569.1 (see **Figure 4C** for structure). Although this compound was not identified in the LC trace, it was present in many cases as a fragment of the

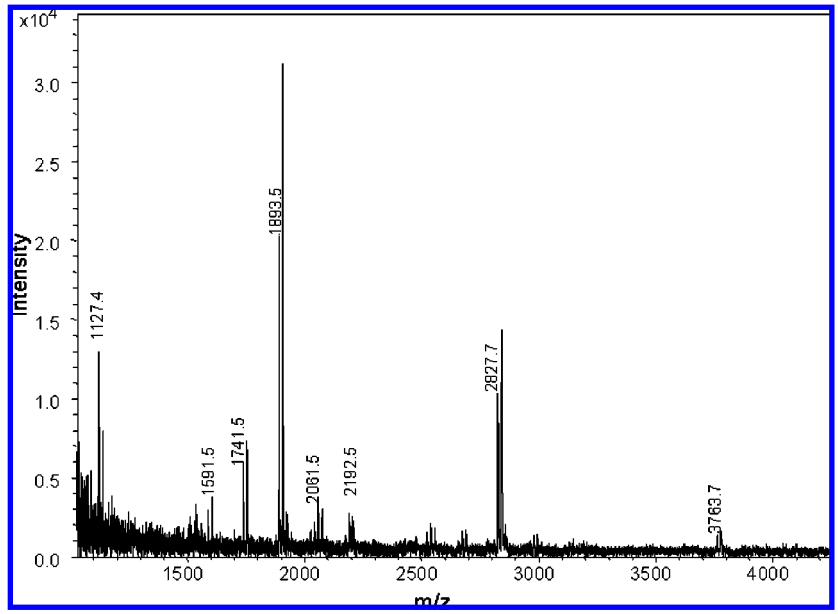

Figure 8. MALDI-TOF-MS spectrum (>1000 Da) of blackberry extract with Na^+ and K^+ adducts for each compound.

larger molecular mass compounds. In the MALDI-TOF-MS analysis, however, it is very unlikely that this was a fragment of a larger molecular mass compound; therefore, native sanguiin H-10 must also be present in the fruit (albeit unidentified in the LC trace). Compounds **D** and **E** were also not identified in the HPLC-ESI-MS analysis. Compound **D** ([2061.6]+) may be a sodium adduct of a galloylated lambertianin A with a molecular mass of 2039.4, whereas compound **E** ([2192.6]+) may be a sodium adduct of lambertianin A with an ellagic acid moiety attached that would result in a molecular mass of 2171.5. Although the addition of an ellagic acid to lambertianin A is speculative, the attachment of an ellagic acid moiety has been observed in previous ellagitannin research on *Rubus* by the presence of lambertianin B (**Figure 4D**) (*15*).

DISCUSSION

Characterization of ellagitannins in *Rubus* has been challenging due to the complexity, diversity, and large size of the compounds. There have been several papers published that have quantitatively evaluated ellagitannins in *Rubus* fruit, but they have not indicated the presence of isomeric forms in the chromatographic analyses. Furthermore, there has been much work on the structural characterization of ellagitannins (i.e., by NMR), but the studies on ellagitannin composition in specific fruits has been limited because the data are still primarily qualitative (*16, 17, 27*). This study showed that there are multiple isomers of most ellagitannins present in the fruit which can be separated by chromatography; however, each peak will require further evaluation by NMR of HPLC isolates to determine the elution order and structure of each isomer.

Verification of the chromatographic data by MALDI-TOF-MS was an invaluable tool, but it also proved to present additional information that was not available through HPLC-ESI-MS. We were able to determine that sanguiin H-10 was probably present in the fruit, despite not observing the compound in the HPLC-ESI-MS evaluation. Furthermore, the MALDI-TOF-MS data indicated that there were possibly distinct patterns that have not been observed in previous studies on *Rubus* fruit regarding the possible presence of compounds with additional gallic and ellagic acid moieties from the known dimeric and trimeric sanguisorbyl ellagitannins. Similar patterns in ellagitannin composition, based on MALDI-TOF-MS analysis, have been observed for chestnut ellagitannins (*28*) and pomegranate ellagitannins (*29, 30*), although with differing compounds and complexities.

Although ellagitannins are present in all parts of the fruit (seeds, torus, and flesh), the most abundant number of ellagitannins is located in the seeds. Siriwoharn and Wrolstad (*20*) observed more than a 50% loss in hydrolyzed ellagitannins (as ellagic acid) in fruit without seeds as compared to the seeded fruit. Interestingly, the fruit ellagitannins are the larger molecular mass ellagitannins, particularly sanguiin H-6/lambertianin A and lambertianin C. There do appear to be several ellagitannins in the middle region of the fruit, known as the torus, which would likely be consumed in processed jams and jellies, despite the exclusion of the seeds from these products.

Most quantitative evaluation of ellagitannins in *Rubus* fruit has been on the hydrolyzed ellagitannins as ellagic acid equivalents (*19, 20*). This has significant problems in terms of relating data to possible health effects, because there is significant evidence that larger molecular mass tannins (>1000 Da), including ellagitannins and procyanidins, are not absorbed to any appreciable extent in their native state (*8, 9, 31*). Knowledge of ellagitannin molecular structure, composition, and quantity is needed to understand their role in determining potential health effects. Using the HPLC method described in this paper, we will be able to further quantitatively evaluate blackberry ellagitannins on the basis of their native structures.

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