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Identification of Ellagic Acid Conjugates and Other Polyphenolics in Muscadine Grapes by HPLC-ESI-MS

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Ellagic acid, ellagic acid glycosides, and ellagitannins found in various fruits and nuts, including muscadine grape, are reported to have potential health-promoting benefits and antioxidant properties. This study isolated and identified several ellagic acid derivatives present in muscadine grapes and determined their relative antioxidant properties (AOX). Compounds were extracted from grape skins and pulp using methanol, and the solvent was evaporated. Isolates were dissolved in citric acid buffer (pH 3.5) and absorbed onto C18 cartridges. Nonretained polyphenolics were collected separately and again partitioned from Sephadex LH-20, whereas retained polyphenolics were first eluted with ethyl acetate followed by methanol. Ellagic acid derivatives were identified on the basis of UV and mass spectra, and the presence of ellagitannins was confirmed by a significant increase in free ellagic acid with HPLC followed by acid hydrolysis. Muscadine grapes contained phenolic acids, flavonols, anthocyanins, ellagic acid, and numerous ellagic acid derivatives. AOX varied in the order ethyl acetate > methanol > C18 nonretained fractions; each correlated to both total phenolics ($r = 0.90$) and total ellagic acid ($r = 0.99$) contents. Results of this study revealed previously unidentified ellagic acid derivatives in muscadine grapes.

KEYWORDS: Muscadine grape; ellagic acid; ellagic acid glycosides; ellagitannins; HPLC-ESI-MS

INTRODUCTION

Major polyphenolics present in muscadine grapes (*Vitis rotundifolia*) were previously reported, including their differentiation from *Vitis vinifera* with respect to anthocyanin content and the presence of ellagic acid. However, confirmatory analyses using advanced instrumentation have been lacking. Ellagic acid and its derivatives are known to be present in muscadine grapes and encompass a broad class of compounds that include the free acid state, those conjugated with various sugars, and the more complex ellagitannins (*1*). Ellagic acid derivatives are not necessarily uncommon in plants, being abundant in raspberry (*1*−*4*), pomegranate (*5*−*7*), oak (*8*), birch leaves (*9*), and many herbs, but their identification as well as that of common flavonoid glycosides has not been fully elucidated in the muscadine grape. Free ellagic acid is thought to form following hydrolytic release from ellagic acid derivatives including ellagic acid glycosides and ellagitannins. Concentrations of ellagic acid precursors and other hydrolyzable polyphenolics in plants are commonly quantified in terms of free ellagic acid following acid hydrolysis (*10*) for routine analysis, hindered by inadequate compound identification and the lack of chromatographic standards.

Ellagitannins are characterized as hydrolyzable conjugates containing one or more hexahydroxydiphenoyl (HHDP) groups esterified to a sugar, usually glucose. Ellagic acid glycosides contain sugar moieties linked to a hydroxyl group, usually the 4-position, that may include glucose, arabinose, xylose, or rhamnose as ester or acetyl linkages (*2*, *3*). Our previous study (*11*) tentatively quantified two ellagic acid glycosides using photodiode array (PDA) detection that differed from free ellagic acid by a 3−7 nm hypsochromic shift in UV spectra. Free ellagic acid and ellagic acid glycosides are partial contributors to total ellagic acid with the presence of ellagitannins inferred in muscadine grapes following acid hydrolysis. Specific information on ellagitannins is lacking for muscadine grapes, but their presence along with that of ellagic acid glycosides is important for marketability to the crop due to potential health benefits associated with these compounds (*12*−*16*). Structural diversity in ellagitannins originates from the number of HHDP units, the location of galloyl ester groups participating in biaryl linkage, and the conformation of the glucose ring (*17*). HPLC assisted by mass spectrometry and diode array detections are commonly employed to separate and identify ellagitannins from plant extracts. For example, raspberries have been identified as a rich source of the ellagitannins sanguiin H-10, lambertianin C, and sanguiin H-6 as well as free ellagic acid, flavonoid glycosides, and anthocyanins (*2*). The objective of this study was to elucidate and quantify previously unidentified polyphenolics in

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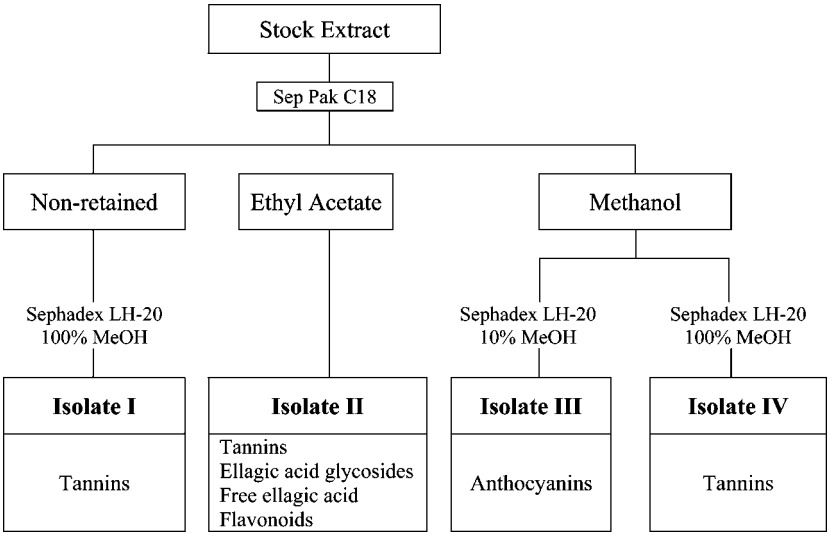

Figure 1. Flow diagram for the extraction and partition procedure utilized to isolate polyphenolic compounds present in muscadine grapes (cv. Doreen, Albemarle, and Noble).

muscadine grapes following solid-phase partitioning from methanolic extracts of muscadine grapes. By evaluating the polyphenolics and antioxidant capacity of the isolated fractions, a better understanding of their diversity, stability, and potential health benefits during processing and storage will be attained.

MATERIALS AND METHODS

Grape Processing. Muscadine grapes from the cultivars Doreen (white), Noble (red), and Albemarle (red) were donated from local growers in central Florida and polyphenolics extracted following seed removal with acidified methanol (0.01% of 12 N HCl). Extracts were filtered through Whatman no. 4 filter paper, solvent was removed at 40 °C under reduced pressure, and polyphenolics were dissolved in 0.1 M citric acid buffer at pH 3.5.

Polyphenolics were then fractionated on the basis of their affinity to Waters C18 Sep-Pak (5 g) cartridges and hand-packed Sephadex LH-20 (3 g) cartridges and partitioned on the basis of their affinity to ethyl acetate and methanol (**Figure 1**). Grape extracts were initially applied to a C18 cartridge, and the nonretained fraction (isolate I) was collected and subsequently partitioned from a Sephadex LH-20 cartridge with methanol. Polyphenolics retained on the C18 cartridges were eluted first with ethyl acetate followed by methanol into two distinct fractions. The ethyl acetate (isolate II) was evaporated at 40 °C under reduced pressure and dissolved in the pH 3.5 buffer for analyses. The methanol fraction was likewise evaporated, dissolved in pH 3.5 buffer, and partitioned from Sephadex LH-20 in two stages. Anthocyanins were first eluted from the Sephadex LH-20 with 10% (v/v) methanol in water (isolate III) followed by 100% methanol to elute the remaining polyphenolics (isolate IV). After solvent removal from each fraction, the polyphenolic residues were dissolved in a small amount of methanol (5% of final volume) and diluted to a known volume with the pH 3.5 buffer.

Chemical Analysis. Polyphenolics present in each isolate I−IV were initially evaluated by HPLC with a PDA detector to tentatively identify and quantify free ellagic acid and ellagic acid derivatives in each grape cultivar. Free ellagic acid and ellagic acid glycosides were quantified in ellagic acid equivalents, and total ellagic acid was quantified following acid hydrolysis in 50% (v/v) methanol containing 2 N HCl for 60 min at 95 °C as described by Lee and Talcott (*18*). Separations (50 μL) were conducted on a Dionex HPLC system using a PDA-100 detector and a 250 mm × 4.6 mm Acclaim 120 C18 column (Dionex, Sunnyvale, CA) with a C18 guard column. A gradient mobile phase of water (phase A) and 60% (v/v) methanol (phase B) both adjusted to pH 2.4 with orthophosphoric acid was run at 1.0 mL/min. Separation was conducted by increasing phase B from 0 to 60% in 30 min, from 60 to 80% in 10 min, from 80 to 100% in 10 min, and to 100% B in 10 min for a total run time of 60 min. Peaks were initially identified by spectral properties and retention time compared to authentic standards (Sigma Chemical Co., St. Louis, MO). Calibration curves ($R^2 = 0.99$) for ellagic acid dissolved in 50% methanol were used to quantify ellagic acid derivatives.

Total soluble phenolics were additionally analyzed using the Folin−Ciocalteu assay (*19*), and antioxidant capacity was measured using the oxygen radical absorbance capacity (ORAC) as described previously by Lee and Talcott (*18*) for muscadine grapes.

Mass spectrometric analyses (MS^n) were carried out to achieve structural information based on molecular masses and fragment ions present only in isolates I and II of Albemarle. Separations (20 μL) were conducted on an Agilent (Palo Alto, CA) HPLC system using an 1100 series binary pump and separated using the Phenomenex (Torrace, CA) Synergi 4μ Hydro-RP 80A (2 × 150 mm; 4 μm; S/N = 106273−106275) with a C18 guard column. Mobile phases consisted of 0.5% formic acid containing 5 mM ammonium formate in water (phase A) and 0.5% formic acid in methanol (phase B) run at 0.15 mL/min. Polyphenolics were separated with a gradient elution program in which phase B changed from 5 to 30% in 5 min, from 30 to 65% in 70 min, and from 65 to 95% in 30 min and was held isocratic for 20 min. Compounds were detected by UV at 280 nm (Applied Biosystems model 785A) and by an LCQ ion trap mass spectrometer equipped with an ESI ion source (ThermoFinnigan, San Jose, CA) conducted in both positive and negative ion modes. The following conditions were used in ESI-MS: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.3 kV; capillary temperature, 250 °C; capillary voltage, 1.5 V; tube lens offset, 0 V. Isolates were additionally separated on a Waters Alliance 2695 HPLC system with a 996 PDA detector under identical conditions to additionally obtain the UV−vis spectral properties of each compound. All solvents utilized for isolate preparation and chemical analyses were of HPLC grade.

Statistical Analysis. Data represent the mean duplicate analyses with analysis of variance conducted using JMP5 software (*20*); mean separation was conducted using the LSD test ($P < 0.05$).

RESULTS AND DISCUSSION

Polyphenolic Isolation and Quantification. Numerous polyphenolic compounds are present in muscadine grapes, and their identification was enhanced following isolation with solid-phase cartridges and solvent affinity characteristics. Isolate I contained only the most polar compounds, those with no affinity to a Waters Sep-Pak C18 cartridge, and when partitioned from Sephadex LH-20 was found to contain predominantly ellagitannins. Isolate II contained the majority of non-anthocyanin polyphenolics including phenolic acids, flavonoid glycosides,

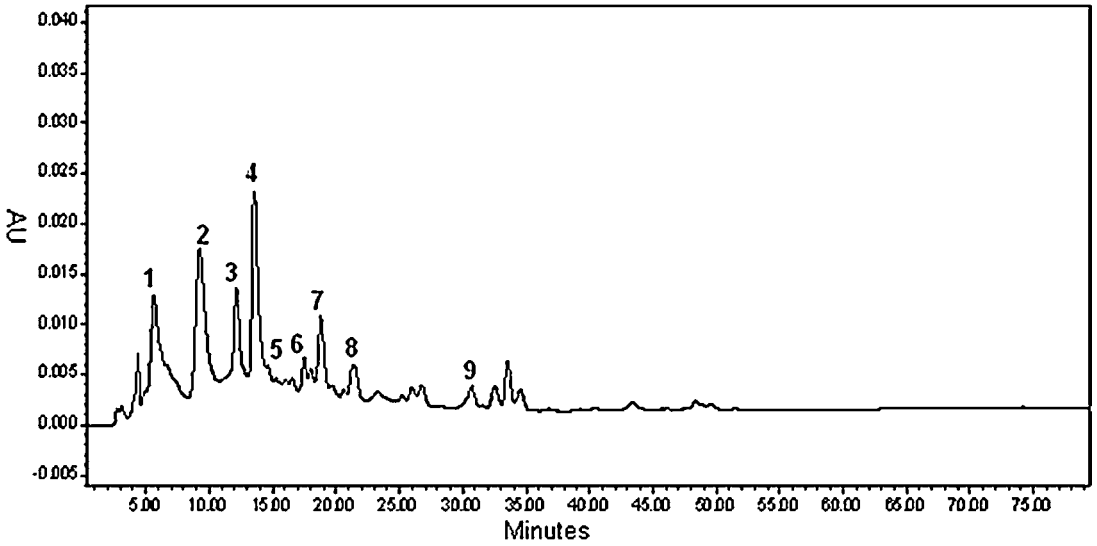

Figure 2. HPLC-PDA chromatogram (280 nm) of the predominant polyphenolic compounds present in a highly polar extract (isolate I) of muscadine grapes (cv. Albemarle).

Table 1. HPLC-ESI(±)-MS^n Analyses of Polyphenols in Isolate I from Muscadine Grapes

peak	RT	UV (nm)	compound	MW	ESI	BP (*m*/*z*); ID	MS^2 (*m*/*z*)[a]	MS^3 (*m*/*z*)[a]
1	5.7	228, 262sh	ellagitannin 1	802	(–)	801; $[M-H]^-$	757, **481**, 301, 275	**301**, 275
					(+)	820; $[M+NH_4]^+$	785, 483, 465, 447, 429, 411, 335, 321, **303**, 277	**285**, 275, 219, 191
2	9.2	268	HHDP-galloylglucose	634	(–)	633; $[M-H]^-$	481, **301**, 275, 249	**257**, 229, 211
					(+)	652; $[M+NH_4]^+$	635, 617, 465, 447, 353, 339, 321, 315, 303, **277**	**259**, 231
3	12.1	272	gallic acid	170	(–)	169; $[M-H]^-$	**125**	
					(+)	ND[b]		
4	13.6	265	HHDP-galloylglucose	634	(–)	633; $[M-H]^-$	**301**, 275, 249	285, **257**, 229
					(+)	652; $[M+NH_4]^+$	635, 617, 465, 447, 321, 315, 303, **277**	**259**, 231, 215
5	15.0	268	ellagitannin 2	834	(–)	833; $[M-H]^-$	789, 771, 745, 699; 469, 337, 319, **275**, 257, 247	NA[c]
					(+)	852; $[M+NH_4]^+$	817, 799, 321, **303**, 285, 277	**285**, 275, 259
6	17.5	291	epigallocatechin	306	(–)	305; $[M-H]^-$	287, 261, 247, 233, 221, 219, 201, **179**, 165, 151, 137, 125, 111	NA
					(+)	307; $[M+H]^+$	289, 257, 182, 151, **139**, 121	NA
7	18.8	295	digalloylglucose	484	(–)	483; $[M-H]^-$	**331**, 313, 295, 169, 151	295, 271, 211, **193**, 169, 126
					(+)	507; $[M+Na]^+$	NA	NA
8	21.4	276	ellagitannins	818	(–)	817; $[M-H]^-$	**799**, 755	**755**, 737, 711, 481, 479, 317, 301, 275
					(+)	836; $[M+NH_4]^+$	**801**, 783, 499, 481, 463, 337, 303, 285	499, 481, 445, 337, 319, **303**
				832	(–)	831; $[M-H]^-$	ND	
					(+)	833; $[M+H]^+$	481, 303	
9	30.7	272	digalloylglucose	484	(–)	483; $[M-H]^-$	331, **313**, 169	179, **169**, 151
					(+)	507; $[M+Na]^+$	NA	
			HHDP-diglucoside	626	(–)	625; $[M-H]^-$	NA	
					(+)	627; $[M+H]^+$	**465**, 303	**303**

[a] Ions in boldface indicate the most intense product ion, which was chosen for MS^3 in the MS/MS spectrum. [b] Not detected. [c] Not acquired.

free ellagic acid, ellagic acid glycosides, and ellagitannins due to their high affinity to ethyl acetate. Remaining compounds, still bound to the Sep-Pak C18 cartridges, were subsequently eluted with acidified methanol and were found to be predominantly anthocyanins and remaining ellagic acid derivatives. Subsequent partitioning from Sephadex LH-20 separated anthocyanins (isolate III) from these ellagic acid derivatives (isolate IV). These four fractions obtained from the extract of three muscadine grape cultivars were subsequently evaluated for polyphenolic content and antioxidant capacity.

Polyphenolic Identification: Isolate I. Only polyphenolics present in isolates I and II of Albemarle were selected for HPLC-PDA (280 nm) and HPLC-ESI (±) MS^n analysis due to the similarity of compounds present in each grape cultivar. By combining two detection methods, polyphenolics were tentatively characterized on the basis of spectroscopic properties of the parent compound, whereas sugar moieties were identified on the basis of retention time, molecular weight, and MS fragmentation characteristics. Molecular weights of each peak were determined after information from both positive and negative modes of ESI was compared. Nine polyphenolics were characterized in isolate I that included gallic acid, epigallocatechin, and other hydrolyzable tannins (**Figure 2**; **Table 1**). As mentioned above, isolate I was considered an ellagitannins fraction because no peaks were found at 360 nm, which is characteristic of an ellagic acid molecule. Acid hydrolysis of this fraction yielded free ellagic acid, confirming the presence of ellagitannins. Thus, resultant *m*/*z* 301 (−) and *m*/*z* 303 (+) ions from MS analysis are evidence for the presence of ellagic

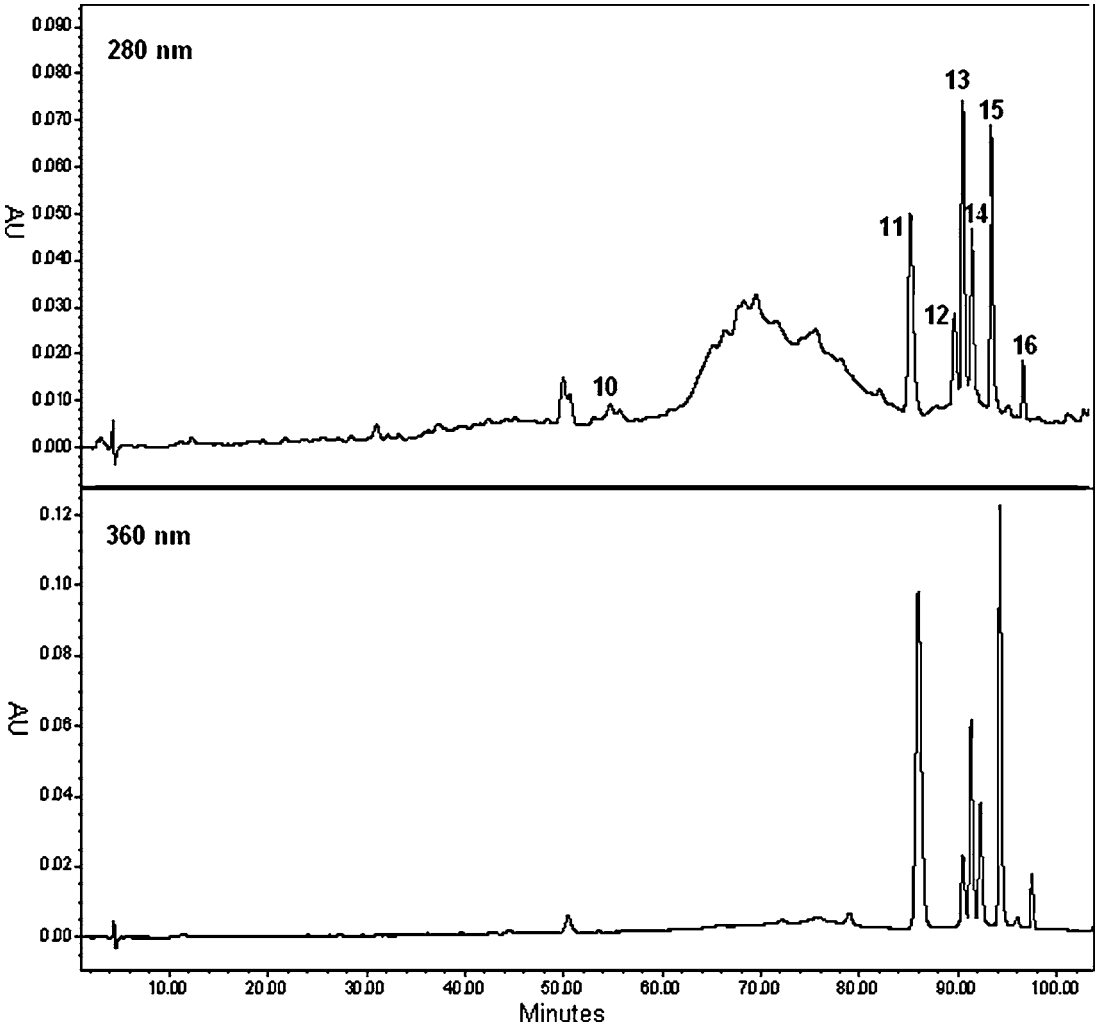

Figure 3. HPLC-PDA chromatogram (280 and 360 nm) of the predominant polyphenolic compounds present in an ethyl acetate extract (isolate II) of muscadine grapes (cv. Albemarle).

acid precursor, HHDP in the molecule, as reported in previous studies with fruit and plant materials (*2*, *3*, *8*, *9*, *21*, *22*, *24*).

Peak 1 was tentatively identified as an ellagitannin 1 (MW 802) according to its UV spectrum, which agreed with previous studies reported by Zafrilla et al. (*21*), and its ESI-MS^n spectra. The compound was considered to contain at least one HHDP unit esterified to glucose due to the evidence provided by MS analysis. The presence of HHDP was supported by the formation of *m*/*z* 301 (−) and *m*/*z* 303 (+) ions from the MS^n dissociation of the *m*/*z* 801 $[M - H]^-$ and *m*/*z* 820 $[M + NH_4]^+$ ions, respectively. The existence of glucose was verified indirectly as (−) ESI-MS^3 of the *m*/*z* 801 $[M - H]^-$ ion to form *m*/*z* 481, which was then dissociated to form the *m*/*z* 301 ion via consecutive losses of glucosyl (162 amu) and water (18 amu).

Peaks 2 and 4 exhibited the largest UV detector response, and each had similar UV spectroscopic properties and (−) ESI-MS spectra, which potentially indicated isomeric forms of an ellagitannin. With a molecular weight of 634 amu, these compounds likely corresponded to isomers of HHDP-galloyl-glucose, similar to that present in birch leaves (*22*). The most abundant ion was the *m*/*z* 633 $[M - H]^-$ ion, which dissociated to form *m*/*z* 301 (HHDP) via loss of 332 amu, which indicated the presence of a galloylglucose unit (332 amu). This compound, HHDP-galloylglucose (**Figure 4A**,**B**) is commonly referred to as sanguiin H4 or sanguiin H5 depending on the location of the galloyl group (*8*).

Peak 3 was identified as free gallic acid on the basis of its retention time, UV absorbance, and relation to an authentic standard. Identification was confirmed by its *m*/*z* 169 $[M - H]^-$ ion, which dissociated to form *m*/*z* 125 via loss of CO_2.

Peak 5 was tentatively identified as a molecular weight 834 amu ellagitannin 2 due to the presence of an *m*/*z* 303 ion resulting from the (+)ESI-MS/MS of the *m*/*z* 852 $[M + NH_4]^+$ ion.

Peak 6 was identified as epigallocatechin on the basis of retention time and UV absorbance spectrum in relation to an authentic standard. Its identity was confirmed by the formation of an *m*/*z* 305 $[M - H]^-$ ion, which underwent dissociation to yield *m*/*z* 261, 221, 219, and 179 ions.

Peak 7 was tentatively identified as an isomer of digalloyl glucose with its *m*/*z* 483 $[M - H]^-$ ion dissociating to yield an *m*/*z* 169 ion after sequential removal of a galloyl group (152 amu) and a glucosyl group (162 amu). Similar observations were reported with birch leaves (*22*).

Peak 8 contained more than one compound, and these were considered to be ellagitannins on the basis of the resultant product ions from MS^n analysis. Chemical identifications for these compounds were not resolved, and their molecular weights were determined to be 818 and 832. According to MS analysis, both ions likely contained HHDP-glucose in their makeup due to the presence of the positive product ions at *m*/*z* 481 (HHDP + glucose) and 303 (HHDP).

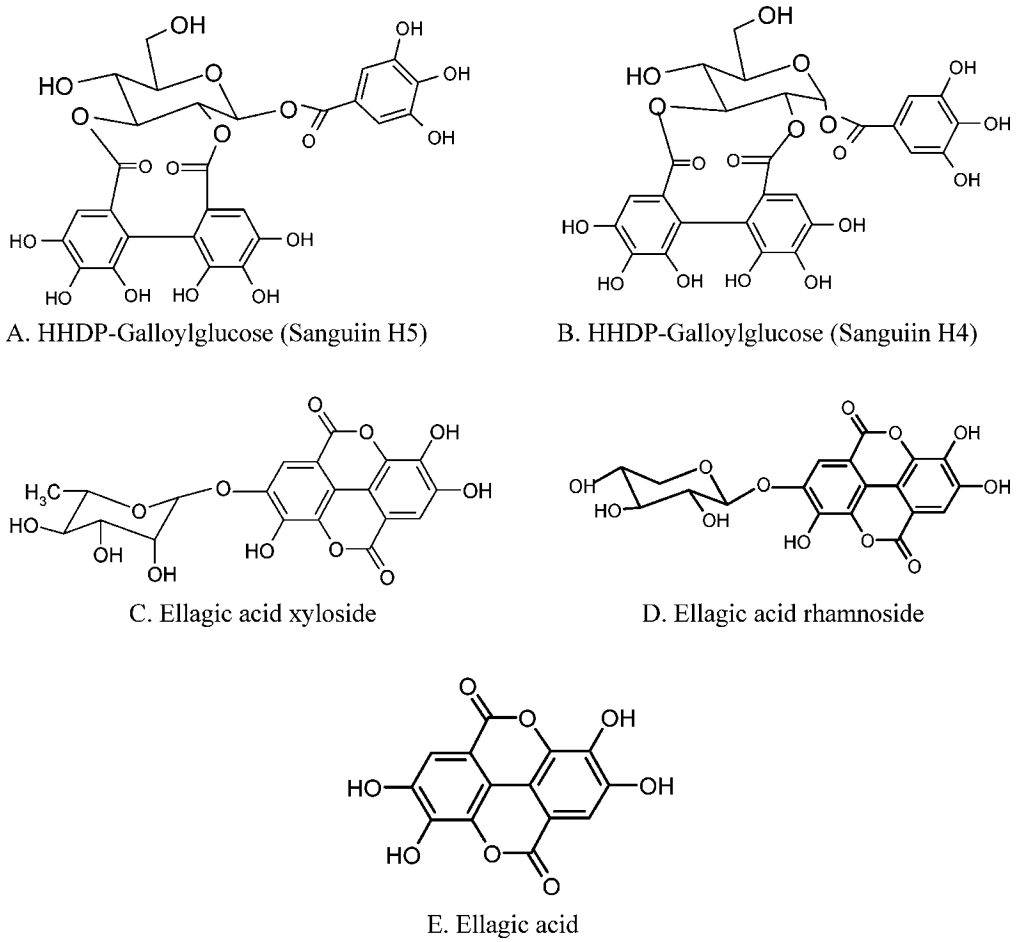

Figure 4. Structures of HHDP-galloylglucose (**A** and **B**), ellagic acid glycosides (**C** and **D**), and free ellagic acid (**E**) in muscadine grapes (cv. Albemarle).

Peak 9 was also coeluting MW 484 and 626 compounds corresponding to digalloyl glucose (similar to peak 7) and HHDP-diglucoside, respectively. The *m*/*z* 627 $[M + H]^+$ ion of the HHDP-diglucoside sequentially lost two glucosyl groups (*m*/*z* 162) to produce *m*/*z* 465 ($[M + H]^+ -$ glucosyl) and 303 ($[M + H]^+ -$ glucosyl $-$ glucosyl).

Polyphenolic Identification: Isolate II. Isolate II was prepared by eluting the Sep-Pak C18 cartridge with ethyl acetate to obtain a fraction enriched in ellagic acid derivatives and flavonoid glycosides, yet free of anthocyanins. Previous isolations using ethyl acetate revealed numerous compounds in muscadine grapes that yield free ellagic acid upon acid hydrolysis (*18*), whereas flavonoid aglycons were identified as myricetin, quercetin, and kaempferol (*12*). Ellagic acid precursors and flavonoid glycosides were monitored by HPLC-PDA at 280 and 360 nm with HPLC-MS^n analysis applied in both ($-$) and ($+$) ESI modes to help with compound elucidation. In addition to the seven compounds listed in **Table 1**, compounds were also present in isolate II, which were detected only with $[M + H]^+$ mode and not with the PDA (**Table 2**; **Figure 3**). These included trace amounts of HHDP-galloylglucose (*m*/*z* 635), myricetin glucoside (*m*/*z* 481), a flavonoid pentosyl conjugate (*m*/*z* 467), and unknown compounds containing galloyl or acetylrhamnosyl groups (*m*/*z* 923).

Peak 10 was detected only at 280 nm with a low detector response and exhibited characteristics similar to the ellagitannins observed in isolate I. MS^n analysis revealed that two compounds with molecular weights of 800 and 814 amu coeluted. These two compounds seemed to have similar chemical structures because MS^n analysis produced analogous fragmentation patterns and common major ions at *m*/*z* 781, 763, and 301 in negative mode and at *m*/*z* 447, 303, and 277 in positive mode. Although MS^n analysis did not clearly elucidate these compounds, the compounds were tentatively classified as ellagitannins on the basis of its UV absorbance spectrum and the presence of the *m*/*z* 303 ($+$) and *m*/*z* 301 ($-$) ions in the MS^n spectra.

Peak 11 was identified as myricetin rhamnoside on the basis of its characteristic spectroscopic properties for a flavonoid and its MS spectra. Myricetin was previously identified as the most abundant flavonoid in Noble muscadine grapes (*12*). The parent compound produced an *m*/*z* 465 $[M + H]^+$ ion, which produced a major *m*/*z* 319, indicative of the loss of a rhamnosyl unit (*m*/*z* 146). Further dissociation of the *m*/*z* 319 ion yielded typical myricetin ions at *m*/*z* 301, 273, 255, and 245 (*23*, *24*).

Peak 12 was identified as ellagic acid xyloside (**Figure 4C**) with spectroscopic properties similar to those of free ellagic acid as previously described by Lee and Talcott (*18*). The MW 434 compound produced an *m*/*z* 435 $[M + H]^+$ ion that lost 132 amu to form an *m*/*z* 303 product ion. The loss of 132 amu corresponded to a pentosyl unit and was tentatively identified as a xylose because similar compounds were found in other berries (*2*, *21*). The resultant *m*/*z* 303 ion underwent further dissociation to produce major ions at *m*/*z* 285 and 257 characteristic of ellagic acid (*2*, *3*). Two other compounds with molecular weights of 410 and 468 amu were also found; however, on the basis of their MS^n spectra, neither was considered an ellagic acid precursor.

Peak 13 was identified as ellagic acid rhamnoside (448 amu, **Figure 4D**) with its *m*/*z* 449 $[M + H]^+$ ion dissociating to form *m*/*z* 303 via loss of a rhamnosyl (146 amu) unit (*25*).

Table 2. HPLC-ESI(±)-MS^n Analyses of Ellagitannins, Glycosides of Ellagic Acid, and Flavonoids in Isolate II from Muscadine Grapes

peak	RT	UV (nm)	compound	MW	ESI	BP (*m*/*z*); ID	MS^2 (*m*/*z*)[a]	MS^3 (*m*/*z*)[a]
10	58–60	261, 280sh	ellagitannins	800	(–)	799; $[M-H]^-$	**781**, 763, 745, 735, 495, 481, 451, 317, 301, 273	**763**, 745, 735, 719, 479, 461, 301, 275, 247
					(+)	818; $[M+NH_4]^+$	801, 783, **447**, 429, 385, 357, 337, 303, 277, 259, 231	429, 411, 385, 357, 303, **277**
				814	(–)	813; $[M-H]^-$	**781**, 763, 753, 735, 301	**763**, 745, 419, 317, 301, 273, 229
					(+)	832; $[M+NH_4]^+$	797, 779, 461, **447**, 443, 397, 335, 317, 303, 277, 259, 241	427, 411, 385, 357, 335, 303, **277**
11	86.0	352	myricetin rhamnoside	464	(–)	463; $[M-H]^-$	359, 337, **317**	287, **271**, 179, 151
					(+)	465; $[M+H]^+$	447, 429, 361, **319**	301, 290, 283, **273**, 263, 255, 245, 165, 163, 137
12	90.5	360	ellagic acid xyloside	434	(–)	433; $[M-H]^-$	301	**257**, 229
					(+)	435; $[M+H]^+$	303	**285**, 275, 257, 247, 229, 165, 153, 137
			unknown	410	(–)	455; $[(M-H)+46]^-$	409	**263**, 161
					(+)	428; $[M+NH_4]^+$	411, **309**, 273, 255, 164, 147, 129	273, 269, 255, 243, 225, 165, **147**, 129
			unknown	468	(–)	513; $[(M-H)+46]^-$	467	**335**, 161
					(+)	486; $[M+NH_4]^+$	NA[b]	NA
13	91.3	361	ellagic acid rhamnoside	448	(–)	447; $[M-H]^-$	**300**, 301	272, **257**, 244, 229
					(+)	449; $[M+H]^+$	303	285, 275, 259
14	92.3	366	ellagic acid	302	(–)	301; $[M-H]^-$	301, 284, 257, 229, 185	NA
					(+)	ND[c]		
15	94.2	351	quercetin rhamnoside	448	(–)	447; $[M-H]^-$	301	283, 271, 255, 179, 169, 151, 121, 107
					(+)	449; $[M+H]^+$	431, 413, 303	303, 285, 275, 257, 247, 229, 165, 153, 137
16	97.5	344	kaempferol rhamnoside	432	(–)	431; $[M-H]^-$	327, 299, 285, 256	267, 257, 255, 241, 229, 213, 197, 163
					(+)	433; $[M+H]^+$	415, 397, 375, 287	287, 269, 241, 231, 213, 197, 183, 165, 153

[a] Ions in boldface indicate the most intense product ion, which was chosen for MS^3 in the MS/MS spectrum. [b] Not acquired. [c] Not detected.

Table 3. Concentration (Milligrams per Kilogram) and Antioxidant Capacity (Micromolar Trolox Equivalents per Milliliter) Present in Each of Four Polyphenolic Fractions Obtained from Three Muscadine Grape Cultivars (Doreen, Albemarle, and Noble)

cultivar	isolate[a]	free ellagic acid	ellagic acid glucoside	ellagic acid xyloside	ellagic acid rhamnoside	total ellagic acid	total soluble phenolics	antioxidant capacity
Doreen	initial	13.5 ± 1.30a[b]	1.60 ± 0.16a	19.5 ± 0.61a	22.5 ± 0.00a	360 ± 0.05a	899 ± 0.60a	4.35 ± 0.32a
	I	0.25 ± 0.05b	0.40 ± 0.05b	ND[c]	ND	13.1 ± 0.00c	142 ± 20.6c	0.75 ± 0.01c
	II	12.9 ± 0.2a	ND	9.15 ± 0.50b	9.70 ± 0.09b	58.9 ± 21.7b	263 ± 0.29b	1.80 ± 0.14b
	III	0.15 ± 0.05b	ND	ND	ND	ND	14.9 ± 0.12e	0.10 ± 0.01d
	IV	0.80 ± 0.19b	0.55 ± 0.08b	1.95 ± 0.62c	1.80 ± 0.28b	2.95 ± 0.35d	67.1 ± 4.34d	0.40 ± 0.00c
Albemarle	initial	32.9 ± 3.42a	7.80 ± 0.45a	20.0 ± 2.42a	37.6 ± 3.39a	912 ± 5.54a	1310 ± 21.34a	7.45 ± 0.04a
	I	0.50 ± 0.12c	ND	ND	ND	53.7 ± 1.27c	188 ± 4.19c	0.80 ± 0.02c
	II	27.2 ± 0.01b	ND	4.60 ± 0.01b	12.1 ± 0.01b	130 ± 2.75b	424 ± 49.0b	3.60 ± 0.16b
	III	1.15 ± 0.08c	ND	0.20 ± 0.01c	0.40 ± 0.04c	0.65 ± 0.08c	59.1 ± 17.2d	0.40 ± 0.01d
	IV	2.45 ± 0.13c	4.55 ± 0.11b	6.95 ± 0.65b	12.8 ± 1.57b	33.0 ± 0.05c	81.2 ± 5.52d	0.40 ± 0.00d
Noble	initial	49.7 ± 0.56a	6.05 ± 0.08a	31.4 ± 1.11a	49.4 ± 0.21a	686 ± 5.00a	2190 ± 16.3a	25.9 ± 1.98a
	I	0.35 ± 0.01d	ND	ND	ND	16.8 ± 4.36e	63.9 ± 2.02d	0.40 ± 0.00d
	II	11.5 ± 2.38b	ND	ND	1.40 ± 0.57d	102 ± 0.09b	415 ± 9.91c	4.00 ± 0.11c
	III	1.90 ± 0.00d	2.75 ± 0.18b	9.55 ± 0.65c	14.0 ± 0.48c	32.7 ± 0.01 d	723 ± 6.93b	10.5 ± 0.32b
	IV	5.55 ± 1.00c	2.20 ± 0.01c	13.5 ± 0.24b	24.0 ± 0.04b	63.2 ± 2.97c	401 ± 15.8c	1.80 ± 0.03c

[a] Isolates were prepared by using Sep-Pak C18 and Sephadex LH-20 cartridges and elution with either methanol or ethyl acetate (see Materials and Methods). [b] Values (mean ± SD, $n = 2$) within columns having similar letters for each cultivar isolate are not significantly different (LSD test, $P < 0.05$). [c] Concentrations below detection limit, 0.05 mg/kg.

Peak 14 was identified as free ellagic acid (302 amu, **Figure 4E**) on the basis of its retention time and spectroscopic properties as compared to those of an authentic standard. Ellagic acid was confirmed by its *m*/*z* 301 $[M-H]^-$ ion, yielding characteristic ions at *m*/*z* 257 and 229 upon dissociation (*2*, *3*).

Peak 15 was identified as quercetin rhamnoside (448 amu), which produced *m*/*z* 449 $[M+H]^+$ and *m*/*z* 447 $[M-H]^-$ ions as did peak 13. Dissociation of these ions produced *m*/*z* 303 and 301 product ions, respectively, via loss of rhamnosyl (146 amu). Both ellagic acid and quercetin and their glycosides produced positive *m*/*z* 303 and negative *m*/*z* 301 ions, making their distinction difficult without spectroscopic data and MS^n spectra of these ions. Further dissociation of the positive *m*/*z* 303 and negative *m*/*z* 301 ions resulted in product ions typical for a flavonoid. Due to its more rigid ring structure in relation to quercetin, ellagic acid's ions produced higher *m*/*z* product ions in the *m*/*z* 229−285 range. In contrast, quercetin's ions yielded ions in the *m*/*z* 100−200 range indicative of flavonoids (*2*). Additionally, the UV spectroscopic pattern was suggestive of a flavonoid.

Peak 16 was identified as kaempferol rhamnoside (432 amu), with its *m*/*z* 433 $[M+H]^+$ ion dissociating to form *m*/*z* 287, indicating the loss of a rhamnosyl (146 amu) unit (*24*, *25*).

Subsequent dissociation of the *m*/*z* 286 ion resulted in *m*/*z* 241, 213, 165, 133, and 121 ions indicative of kaempferol (*26*).

Muscadine Grape Cultivars. The isolation method described in **Figure 1** was applied to three muscadine grape cultivars and each analyzed for free ellagic acid, three ellagic acid glycosides, and total ellagic acid. Only two ellagic acid glycosides were identified in isolates I and II of Albemarle (xyloside and rhamnoside), but ellagic acid glucoside was also elucidated in the initial extracts of each cultivar (**Table 3**). Extracts from Noble had the highest concentrations of free ellagic acid (49.7 mg/kg) and total ellagic acid glycosides (86.9 mg/kg), yet following acid hydrolysis total ellagic acid was highest in Albemarle (912 mg/kg) compared to Noble and Doreen (686 and 360 mg/kg, respectively). These differences were due to the presence of ellagitannins. Although the isolation scheme was not 100% efficient, ellagitannins were successfully partitioned into isolate I as indicated by high concentrations of total ellagic acid in relation to free ellagic acid or ellagic acid glycosides.

Isolate II contained the majority of the free ellagic acid in Doreen (95%) and Albemarle (83%), but only 23% for Noble. Because Noble contains the highest concentration of anthocyanins followed by Albemarle and Doreen (*18*), there may have been interference from anthocyanins when partitioning from C18 Sep-Pak cartridges with ethyl acetate. Isolate II contained the majority of ellagic acid precursors, including ellagitannins, as indicated by the high total ellagic acid content of this fraction. Although isolate III contained predominantly anthocyanins, free ellagic acid and ellagic acid glycosides were also present, indicating their incomplete partitioning with ethyl acetate. Isolate IV exhibited only a slight increase in total ellagic acid in relation to concentrations of free ellagic acid and ellagic acid glycosides. This observation demonstrated the absence of ellagitannins in this fraction and that ellagic acid glycosides had similar detector response similar to that of free ellagic acid for quantification. Concentration differences observed among muscadine grape cultivars were previously noted (*18*), but variation among individual ellagic acid derivatives and other polyphenol contents was not investigated in detail, with concentrations additionally influenced by both preharvest and environmental conditions.

Additional analyses for total soluble phenolics and antioxidant capacity were conducted as a means to assess common characteristics used to assess the quality and potential health benefits of many fruit and vegetable products. Polyphenolics present in isolates I−IV contributed 70, 66, and 70% to the antioxidant capacity of the initial extract in Doreen, Noble, and Albemarle, respectively, and 54, 73, and 57% to the total soluble phenolics. Most of the antioxidant compounds present in Doreen and Albemarle were present in isolate II due to the abundance of non-anthocyanin polyphenolics, whereas isolate III of Noble was highest due to the abundance of anthocyanins. Total soluble phenolic concentrations correlated well to antioxidant capacity for each fraction ($r > 0.93$).

Conclusions. The major antioxidant polyphenolics present in three muscadine grape cultivars were identified by UV spectroscopic properties and tandem mass spectrometry (MS^n) analyses following extraction and elution from suitable solid phase supports. The application of tandem mass spectrometry produced ions consistent with known sugar moieties on three ellagic acid glycosides (rhamnoside, xyloside, and glucoside) and rhamnosides of quercetin, myricetin, and kaempferol in addition to at least four different ellagitannins containing HHDP units. In the case of ellagitannins, these methods were able to assess the molecular weights of the respective compounds, but not exact chemical identities due to diversity of ellagitannins present with varying positions of functional groups. Polyphenolics present in each isolate were found to contribute to the antioxidant capacity of each grape cultivar.

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