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Stilbenoid Profiles of Canes from *Vitis* and *Muscadinia* Species

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Supporting Information

ABSTRACT: We present stilbenoid profiles of canes from 16 grapevines. Fifteen stilbenoids were obtained through isolation and structure identification using MS, NMR, and $[\alpha]_D$ or as commercial standards. An HPLC−UV method for the simultaneous quantification of nine of these stilbenoids was developed and applied to canes of *Vitis amurensis*, *Vitis arizonica*, *Vitis berlandieri*, *Vitis betulifolia*, *Vitis cinerea*, *Vitis* × *champini*, *Vitis* × *doaniana*, *Vitis labrusca*, *Vitis candicans* (syn. *Vitis mustangensis*), *Vitis riparia*, *Vitis rupestris*, *Vitis vinifera*, *Muscadinia rotundifolia*, and a *V. vinifera* × *M. rotundifolia* hybrid. In these species, *E*-ampelopsin E, *E*-amurensin B, *E*-piceid, *E*-piceatannol, *E*-resveratrol, *E*-resveratroloside, *E*-ε-viniferin, *E*-ω-viniferin, and *E*-vitisin B were quantified, when found in sufficient amounts. Total concentrations ranged from ∼2.2 to 19.5 g/kg of dry weight. Additional stilbenoids, *E*-3,5,4′-trihydroxystilbene 2-C-glucoside, *Z*-ampelopsin E, *Z*-*trans*-miyabenol C, *E*-*trans*-miyabenol C, scirpusin A, and *Z*-vitisin B, were identified but not quantified. Our results indicate that canes, particularly those of non-*vinifera* species, have substantial quantities of valuable, health-promoting stilbenoids.

KEYWORDS: *grapevine cane, Muscadinia rotundifolia, quantification, stilbenoid, Vitis*

INTRODUCTION

A number of *Vitis* species are economically important because of their role in the production of wine, table grapes, and other grape-derived products. Of the approximately 60 species in the Vitaceae family, the most important of these is *Vitis vinifera* L., the main wine-producing grape. A number of other species are also economically important to the grape and wine industries, such as the Concord grape, *Vitis labrusca* L. cv. 'Concord', which is widely consumed in the United States in jams, jellies, and juice. Other species serve as rootstocks for *V. vinifera*, primarily cultivars or hybrids from different American *Vitis* species, such as *Vitis berlandieri* Planch., *Vitis riparia* Michx., and *Vitis rupestris* Scheele. These rootstocks were initially used because of their phylloxera resistance, but their use has been expanded to afford resistance to other diseases and specific advantages in challenging growth conditions.[1] In addition to their use as rootstocks, some of these species are also used for producing wine, albeit to a significantly lesser degree.[2] The highly disease resistant Muscadine grape [*Muscadinia rotundifolia* (Michx.) Small, syn. *Vitis rotundifolia*] is consumed as table grapes, in wines, and in jams in the southeastern United States. Because of its superior resistance to common grapevine diseases and pests, research into hybridization of *M. rotundifolia* with *Vitis* species has been, and continues to be, an active area of research.[3,4]

One well-characterized mechanism of disease resistance in *Vitis* species is the production of a group of phytoalexins, called stilbenoids. These compounds play an important role in the complex response to biotic and abiotic stresses in grapevines.[5] Within *Vitis*, stilbenoids are primarily represented by resveratrol and its derivatives, including glycosides and numerous oligomers.[6] A range of different stilbenoids are produced in the leaves, berries, cluster stems, and flowers of *Vitis* species, and their expression has been shown to be stronger in disease resistant species and cultivars than in susceptible *V. vinifera* cultivars.[7−10] Additional studies have substantiated these observations by showing that stilbene synthase and isoperoxidases, the enzymes currently understood to be responsible for stilbenoid oligomerization reactions, are more strongly upregulated in more resistant species.[11−15]

Stilbenoids are more constitutively expressed in the roots and stems, where they are believed to help in the prevention of wood rot.[16] This constitutive expression may lead to more consistent and greater levels of stilbenoids in these plant parts, in contrast to the leaves and berries in which stilbenoid production is mostly induced.[17] Currently, grape canes are pruned from the vines every year and are disposed of primarily via burning or composting. However, these grape canes have substantial potential economic value, as a significant source of resveratrol and other stilbenoids, because of the current interest in their health-promoting properties.[18] For example, the stilbenoid monomer, resveratrol, is currently in a number of clinical trials for a range of activities, including cardiovascular

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disease and cancer prevention.[19] Positive biological activities for the resveratrol metabolite, piceatannol, have also recently been discovered, including decreasing insulin resistance and prevention of adipogenesis.[20−22] These activities will certainly stimulate increased interest in this molecule. Furthermore, a number of stilbenoid oligomers are also showing promise as health-promoting compounds in humans. For instance, ampelopsin A, vitisin A, and vitisin B have shown substantial neuroprotective and anti-inflammatory activities in several experimental models.[23−26]

The potential value of plant material containing a high level of stilbenoids has prompted research into the chemical composition of *Vitis* canes. A 2006 study quantified *E*-resveratrol, *E*-ε-viniferin, and an unidentified trimer in three Estonian *V. vinifera* cultivar stems, finding levels as high as 3200, 1700, and 240 mg/kg of dry weight (dw), respectively.[27] A study by Vergara et al. determined the concentrations of *E*-piceatannol, *E*-resveratrol, and *E*-ε-viniferin in canes of different *V. vinifera* cultivars grown in Chile. A Gewürztraminer variety contained the greatest average levels of *E*-resveratrol (4628 mg/kg of dw), *E*-ε-viniferin (744 mg/kg of dw), and *E*-piceatannol (457 mg/kg of dw).[28] A third study examined the resveratrol content of canes from both cultivated and wild grapevine species, including a number of Asian and American species, grown across China. Of the species tested, the greatest resveratrol levels were found in the canes of *V. vinifera* wine grapes, with the highest levels found in Pinot noir (∼1700 mg/kg of fw) and the lowest concentrations found in *M. rotundifolia* canes.[29]

Currently, relatively little is known about the stilbenoid profile of economically important *Vitis* species, particularly highly disease resistant species, such as the American *Vitis* species. This information would provide a better understanding of stilbenoids, with regard to disease resistance in grapevine species. A comparison among species will also allow the determination of optimal stilbenoid source material. For this aim, we characterized the major stilbenoids in approximately a quarter of the known grapevines, including 11 American types, two Asian types, one European type, and one American−European hybrid.

MATERIALS AND METHODS

Chemicals and Standards. Pure grade acetone was obtained from Xilab (Bruges, France). HPLC grade ethyl acetate (EtOAc) (Scharlau, Barcelona, Spain) and methanol (MeOH) (Carlo Erba, Rodano, Italy) along with synthesis grade *n*-heptane were used for the centrifugal partition chromatography (CPC) fractionation. Water was purified using an Elga (Bucks, U.K.) water purification system for both the HPLC−MS and CPC experiments. For the LC−MS experiments, LC−MS grade acetonitrile (ACN) was obtained from Scharlau (Barcelona, Spain) and formic acid was obtained from Fischer Scientific (Loughborough, U.K.). Analytical standards of *E*-piceatannol (>98% purity), *E*-piceid (>98%), and *E*-resveratrol (99% purity) were obtained from Sigma-Aldrich (St. Louis, MO). Vitisin B for the quantification studies was obtained from the roots of *V. riparia* × *V. berlandieri* SO4 (Oppenheim selection no. 4) as previously described.[30] *E*-Ampelopsin E (>94% purity) and *E*-ε-viniferin (>95% purity) were obtained from *Vitis amurensis* as described in the isolation section. The percent purity of isolated compounds was calculated on the basis of the peak area obtained from HPLC−diode array detector (DAD) monitoring of the wavelength range (λ) of 190−600 nm. Sephadex LH-20 was obtained from Amersham Biosciences (Uppsala, Sweden). The d_6-acetone for the NMR experiments was obtained from Euriso-Top (Gif-sur-Yvette, France).

Plant Material. The canes were collected from the INRA germplasm collection at the field station in Villenave D'Ornon, France, in December 2011. Cane specimens were deposited in the Department of the Groupe d'Etude des Substances Végétales à Activité Biologique (GESVAB) at ISVV. The species names, cultivars, and accession numbers are listed in Table 1. *M. rotundifolia*, *V. cinerea* GH, and the hybrid species, *V. vinifera* × *M. rotundifolia* (VRH) 8771, were grown in 10 L containers in a greenhouse in primarily sandy soil, with a small amount of compost irrigated with a nutrient solution. The remaining species were planted outdoors and grafted onto Gravesac (*V. riparia* × *V. berlandieri* × *V. rupestris*) rootstock in slightly gravelly, sandy loam. All plants were planted in 2001, and grafting was performed to ensure more uniform growth among and between the different species. The plants were not treated with any fungicides or insecticides, and there was no observable disease on the canes when collected. Because of its susceptibility to frost, we were unable to obtain *M. rotundifolia* grown under the same conditions as the other species. To help control for potential differences between plants grown outdoors and indoors, a second species (*V. cinerea* GH), which was grown under identical conditions and collected from the same greenhouse as *M. rotundifolia*, was evaluated to compare with the same species, *V. cinerea*, grown outdoors. For each plant, the tendrils were removed and the 1-year-old canes, including nodes and internodes, were broken into smaller pieces with a Bosch AXT 22D shredder and dried in a 40 °C drying oven for approximately 2 weeks. The stems were then powderized and stored in the dark at 4 °C, in airtight containers. In conjunction, we placed the same quantity of powdered plant material in a 90 °C drying chamber for 72 h. No signficant change in weight was observed (<1%).

Table 1. Plants Used in This Study, Their Origin, and the Deposit Number of a Representative Sample

plant name	origin	deposit no.
M. rotundifolia (Michx.) small cultivars (Dulcet and Regale)	American	ISVV001 and ISVV002
V. vinifera × *M. rotundifolia* (Cabernet-Sauvignon × Alicante Bouschet) × (NC) North Carolina 184-4 (VRH 8771)	European−American hybrid	ISVV003
V. amurensis Rupr. (10151)	Asian	ISVV004
Vitis arizonica Engelm. (Wetmoore)	American	ISVV005
V. berlandieri Planch. (11124)	American	ISVV006
Vitis betulifolia Diels and Gilg 49G	Asian	ISVV007
Vitis × *champini* Planch. (*Vitis candicans* × *V. rupestris*) 10092	American	ISVV008
V. cinerea (Engelm.) Engelm. ex Millardet 10943		ISVV009
V. cinerea (greenhouse)	American	ISVV010
Vitis × *doaniana* Munson ex Viala (Doan's grape) (*Vitis mustangensis* × *Vitis acerifolia*)	American	ISVV011
V. labrusca L., cv. 'Concord' 10307	American	ISVV012
V. candicans Engelm. ex Durand 10096 (syn. *V. mustangensis* Buckley)	American	ISVV013
V. riparia Michaux cv. Pulliat *V. riparia* Michaux cv. Scribner	American	ISVV014 and ISVV015
V. rupestris Scheele	American	ISVV016
V. vinifera L., cv. Cabernet-Sauvignon	European	ISVV017

Equipment. The CPC fractionation was performed on a 200 mL capacity, Kromaton Technologies (Sainte-Gemmes-sur-Loire, France), FCPC200, CPC apparatus with a 20 mL injection loop. The binary high-pressure gradient pump was a Gilson model 321-H1 pump. The CPC fractions were monitored with a Varian (Victoria, Australia) Prostar 325 UV−visible detector, with wavelengths (λ) of 306 and 280 nm. HPLC semipreparative separations were conducted using a Varian Prostar 345 UV−visible detector and a binary pump with either a ProntoSIL C_{18} (5 μm, 250 mm × 8.0 mm, Bischoff Chromatography, Leonberg, Germany) column or a Varian Dynamax Microsorb 100-5 C_{18} (10 μm, 250 mm × 21.4 mm) column.

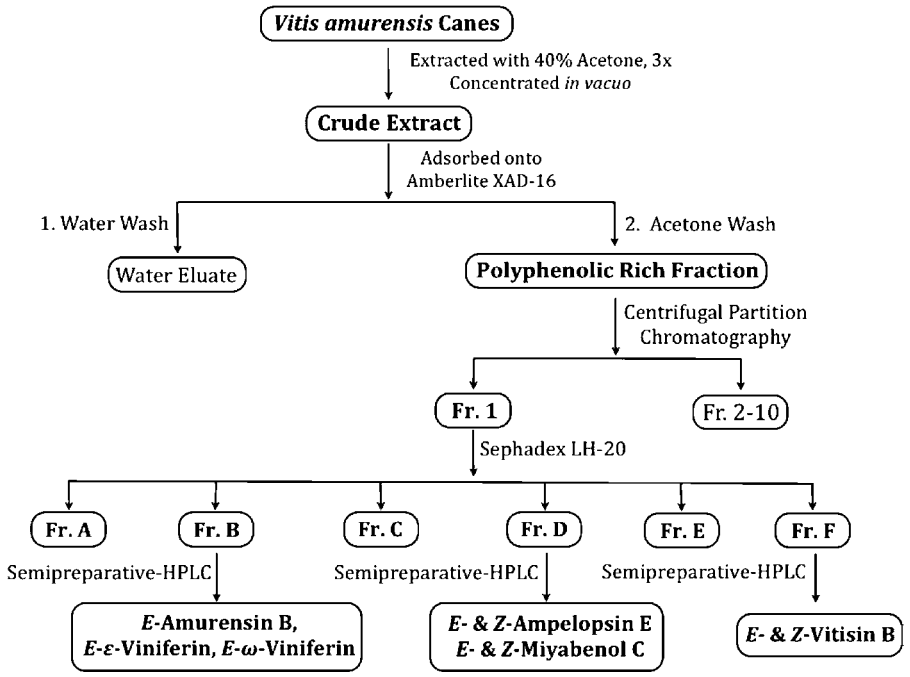

Figure 1. Flow diagram showing the isolation of stilbenoid oligomers from *V. amurensis*.

Quantification studies and HPLC−UV−MS analyses were performed on an Esquire 3000 Plus ion trap mass spectrometer (Bruker-Daltonics, Billerica, MA) using an ESI source (Agilent Technologies, Santa Clara, CA). The MS instrument was coupled to an Agilent 1200 (Agilent Technologies) binary pump with a degasser, DAD, an autosampler, a column heater, and a ProntosSIL C_{18} 250 mm × 4.0 mm, 5 μm column (Bischoff Chromatography) with a guard column. The column was kept at 25 °C with a flow rate of 1 mL/min. The HPLC output was split 1:10 into the MS detector. Total ion chromatograms were obtained using alternating positive and negative modes with a range of m/z 110−1500. Nitrogen was used as the drying gas at 5 L/min with a nebulizer pressure of 15 psi at 325 °C. For the negative ion mode, the capillary voltage was 3100 V, the capillary end voltage −127.7 V, the skimmer voltage −40 V, and the trap drive 71.0. For the positive ion mode, the capillary voltage was −3700 V, the capillary end voltage 127.7 V, the skimmer voltage 40 V, and the trap drive 68.7. Data analysis was performed with Bruker Data Analysis version 3.2. The extracted ion chromatogram MS data, presented here and in the Supporting Information, were made from internally developed software from mzXML converted files. The mzZML files were obtained using CompassXport (Bruker) from the acquisition files.

All ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 600 MHz NMR spectrometer and analyzed with Bruker Topspin version 2.0. Compounds were measured in 3 mm NMR tubes, with d_6-acetone as the solvent. The specific optical rotations were determined in methanol at 20 °C on a JASCO P-2000 polarimeter using the sodium emission wavelength (λ = 589 nm).

HPLC Gradients. For all analytical HPLC−MS and semiprepative HPLC gradients, the solvent systems consisted of solvent A (H_2O with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid). For the multiple different isolation and analytical experiments, five different gradient systems were used. All gradients had a 5 min wash with 100% solvent B and a 5 min reequilibration step. For simplification, we have numbered them 1−5 and refer to the gradients by these assigned numbers within the text. Gradient 1: 10 to 60% B (from 0 to 45 min), 60 to 100% B (from 45 to 46 min). Gradient 2: 20% B (from 0 to 10 min), 20 to 30% B (from 10 to 16 min), 30% B (from 16 to 25 min), 30 to 50% B (from 25 to 30 min), 50 to 100% B (from 30 to 31 min). Gradient 3: 10 to 30% B (from 0 to 10 min), 30 to 40% B (from 10 to 15 min), 40 to 42% B (from 15 to 40 min). Gradient 4: 10 to 30% B (from 0 to 10 min), 30 to 40% B (from 10 to 20 min), 40 to 42% B (from 30 to 32 min). Gradient 5: 10 to 20% B (from 0 to 10 min), 20% B (from 10 to 20 min), 20 to 30% B (from 20 to 25 min), 30 to 100% B (from 25 to 30 min).

Stilbenoid Purification and Identification from *V. amurensis*. The isolation and structure elucidation of all stilbenoids (Figures 1 and 2), except for resveratrol, piceid, resveratroloside, *E*-3,5,4′-trihydroxystilbene 2-*C*-glucoside, and scirpusin A, were performed with *V. amurensis*. For all *V. amurensis* stilbenoids, a total of 400 g of the dried and powderized stems was macerated with 2 L of acetone and water (4:6) three times at room temperature, for 1 day each. After each 24 h maceration period, the extract was filtered and reduced in vacuo at 32 °C to approximately 250 mL and immediately stored in the dark at 4 °C. After the third extraction, the crude extracts were combined and poured over an Amberlite XAD-16 column (500 g, 87 cm × 4.7 cm) and washed with 5 L of water. The column was then washed with a minimum of 3 L of acetone, and the compound was immediately concentrated to a syrup and combined to afford the polyphenol-rich fraction. This fraction was then diluted with approximately 100 mL of water and freeze-dried to afford 14 g of powder.

A portion of the freeze-dried powder (4.2 g) was then fractionated using a CPC. Solvent systems were chosen on the basis of results from previous optimization studies.[30] The ARIZONA L solvent system was used for the ascending mode, and ARIZONA system K was used for the mobile phase in the descending mode. For each injection, the CPC was first filled with 400 mL of stationary phase (aqueous phase of ARIZONA L) without rotation, introduced at 10 mL/min, and then incrementally increased to 1000 rpm at 4 mL/min, at three intervals (300, 600, and 1000 rpm) of 200 mL each. The mobile phase was then introduced, and the extract was injected immediately after the displacement of the stationary phase had been observed (∼70 mL). Prior to injection, 2.1 g of extract was dissolved in 15 mL of equal parts upper and lower phases and filtered with a Millipore (0.45 μm) syringe filter. The CPC was run in ascending mode for 180 min and then switched to descending mode, with the aqueous phase of ARIZONA K as the mobile phase, for an additional 120 min. Fractions were collected in round-bottom flasks based on the UV trace at 306 nm and immediately concentrated in vacuo at 32 °C, affording a total of 10 fractions, fractions 1−5 (Fr. 1−5) from the ascending mode and fractions 6−10 (Fr. 6−10) from the descending mode. The CPC experiment was repeated twice to avoid injection volume overloading.

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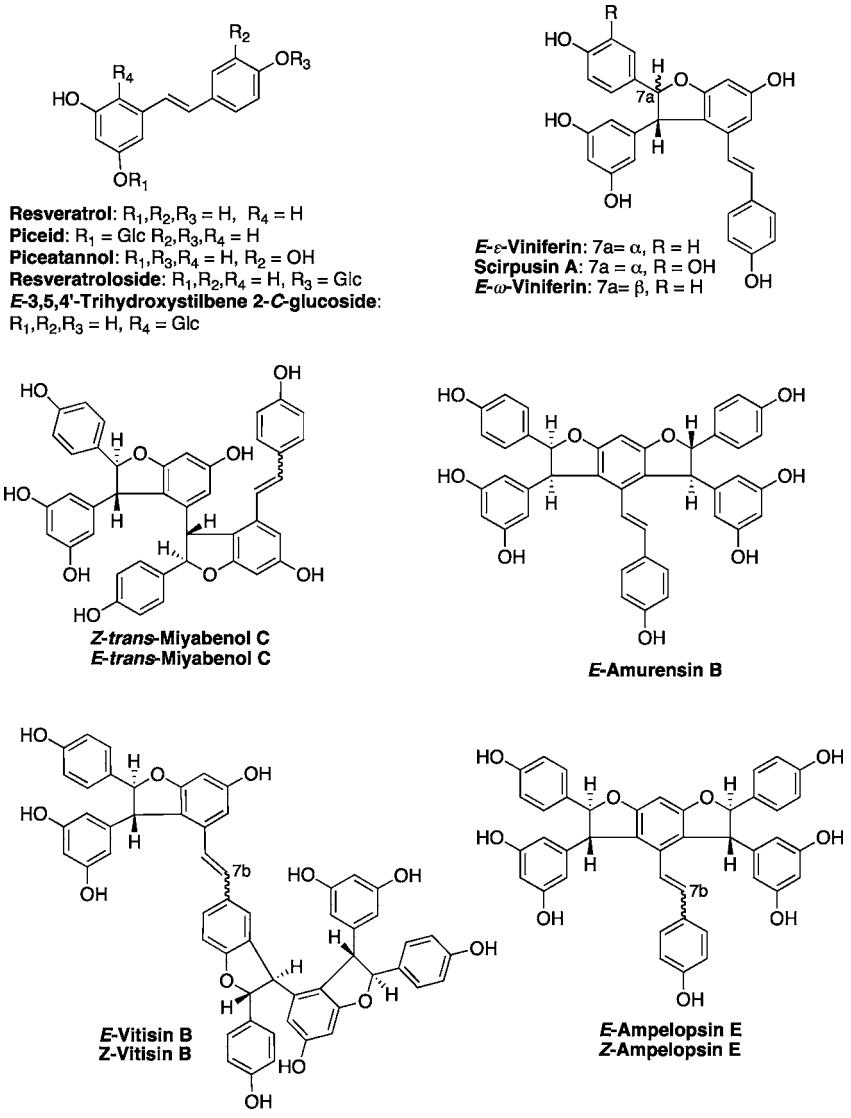

Figure 2. Structures of compounds isolated from *V. amurensis* or *M. rotundifolia*.

The concentrated fractions were then lyophilized and weighed, and a small aliquot (approximately 1 mg/mL) was analyzed via HPLC−DAD−ESI-MS with an injection volume of 10 μL. The presence of stilbenoid monomers, monomer glycosides, dimers, trimers, and tetramers was monitored by using the $[M + H]^+$ m/z values of 229, 245, 391, 455, 681, and 907 using gradient 1.

Fraction 1 (320 mg) was found to contain the stilbenoids of interest and was subjected to additional fractionation via Sephadex LH-20 gel filtration column chromatography (100 mg presoaked in MeOH). The column (99.5 cm × 2.1 cm) was connected to a peristaltic pump, which maintained a flow rate of 0.5 mL/min. A total of 27 fractions, of 15 mL each, were collected and monitored by thin layer chromatography (TLC). The TLC plates consisted of POLYGRAM SIL G/UV_{254} polyester sheets (Macherey-Nagel, Düren, Germany), developed with an 85:15:3 (v/v) $CHCl_3$/MeOH/acetic acid mixture, and sprayed with *p*-anisaldehyde sulfuric acid reagent, after they had been monitored with UV light at 254 and 365 nm. On the basis of the results from TLC plates, the 27 fractions were then combined to afford a total of six fractions (Fr. A−F), which were then analyzed by HPLC−DAD−MS. The stilbenoids of interest were detected in Fr. A (m/z 229 and 245), Fr. B (m/z 455 and 681 $[M + H]^+$), Fr. D (m/z 681 $[M + H]^+$), and Fr. F (m/z 907 $[M + H]^+$).

After the Sephadex LH-20 column, compounds from Fr. B (102.5 mg) and Fr. D (42.0 mg) were purified by semipreparative HPLC with the Varian Dynamax Microsorb column kept at room temperature using gradient 3 at a flow rate of 12 mL/min. Immediately prior to injection, the fractions were resolubilized to afford concentrations of 50 mg/mL (Fr. B) and 30 mg/mL (Fr. D), for 500 μL injections. *E*-Amurensin B (0.9 mg), *E*-ε-viniferin (12.1 mg), and *E*-ω-viniferin (6.6 mg) were isolated and identified from Fr. B. The semipreparative isolation of Fr. D yielded two pure compounds, *E*-ampelopsin E (14.6 mg) and *Z*-ampelopsin E (3.5 mg), and an additional fraction that contained two compounds. These two compounds were purified with a final semipreparative HPLC step with a ProntoSIL Bischoff, C_{18} (5 μm, 250 mm × 8.0 mm) column at room temperature. Using a flow rate of 3 mL/min and gradient 4 yielded two pure compounds, *E*-*trans*-miyabenol C (0.6 mg) and *Z*-*trans*-miyabenol C (0.4 mg). For the quantification studies, *E*-ampelopsin E and *E*-ε-viniferin were re-injected into the semipreparative HPLC system using this same gradient to remove any residual impurities.

The same method described for the purification of Fr. D was also used for the compounds in Fr. F (7.4 mg). Immediately prior to injection, the fractions were resolubilized to afford a concentration of 7.4 mg/mL, for 200 μL injections. This yielded two pure compounds, *Z*-vitisin B (0.6 mg) and *E*-vitisin B (0.4 mg). The ^{1}H NMR spectra of isolated compounds were identical to NMR data previously obtained in our laboratory and with the literature data, and their specific optical rotation, for >90% pure compounds, was recorded as follows: (+)-*E*-ε-viniferin[7] $[\alpha]_{589}$ (MeOH, *c* 0.13) = 41°, (−)-*E*-ω-viniferin[7] $[\alpha]_{589}$ (MeOH, *c* 0.025) = −2°, (−)-*E*-vitisin B[31] $[\alpha]_{589}$ (MeOH, *c* 0.02) =

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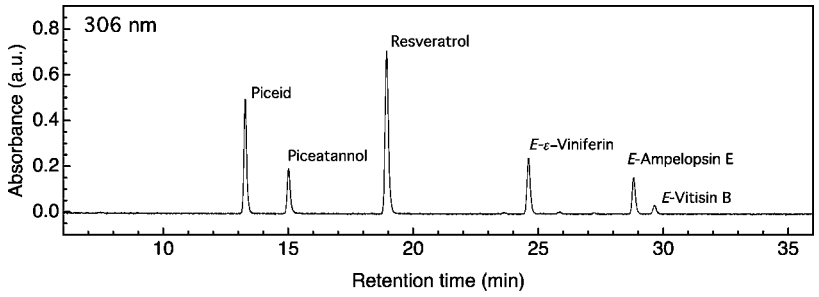

Figure 3. HPLC−UV spectra of the calibration curve at 12.5 μg/mL, with UV detection at 306 nm (λ).

−83°, *Z*-vitisin B[31] and (+)-*E*-amurensin B[32] $[\alpha]_{589}$ (MeOH, *c* 0.03) = 165°, (+)-*E*-ampelopsin E[33,34] $[\alpha]_{589}$ (MeOH, *c* 0.025) = 10°, and *Z*-ampelopsin E,[34] *Z*-*trans*-miyabenol C,[7] and (−)-*E*-*trans*-miyabenol C[7] $[\alpha]_{589}$ (MeOH, *c* 0.01) = −46°.

Purification and Identification of Compounds from *M. rotundifolia*. Preliminary HPLC−DAD−MS screening of all plants showed two unidentified compounds similar to piceid in LC−MS ($[M + H]^+$ *m/z* 391), but with different retention times (t_R), at 9.6 and 11.6 min, rather than the t_R of 13.3 min obtained for piceid. The MS analysis of the compounds, at a t_R of 11.6 min and piceid, demonstrated an $[M + H]^+$ peak at *m/z* 391 and a fragment ion at *m/z* 229. The first peak, with a t_R of 9.6 min, lacked this fragment ion, suggesting a possible *C*-glycoside monomer. Because the first and second compounds were major, previously unidentified, constituents in *M. rotundifolia*, we isolated them from this plant for identification. To achieve this, we extracted 50.6 g of dried powderized stems of *M. rotundifolia* cv. Dulcet three times with 500 mL of MeOH, which was then dried down, in vacuo, to a thick syrup (10.4 g). From this extract, 400 mg was redissolved in 2 mL of solvent and filtered with a 0.45 μm Millipore syringe filter. The compound was purified with repeat HPLC injections of 500 μL with a Varian Dynamax Microsorb 100-5 C_{18} (10 μm, 250 mm × 21.4 mm) column at a flow rate of 12 mL/min using gradient 5. The compounds eluted at 15.1 and 15.9 min and were determined to be resveratroloside (5.1 mg) and *E*-3,5,4′-trihydroxystilbene 2-*C*-glucoside (3.2 mg) by comparison of the ESI-MS and ^{1}H NMR data with the literature data and with the NMR spectra previously obtained in our laboratory.[35−37] During this fractionation, a third compound was isolated from a peak with a t_R of 31.9 min. This peak had an ESI-MS $[M + H]^+$ *m/z* value of 471 and ^{1}H and ^{13}C NMR spectra identical to the data reported for scirpusin A.[38]

Sample Preparation for Quantification Studies. For the quantification studies, 500 mg of powderized material from each species was weighed out into 15 mL sterile centrifuge tubes and 10 mL of an acetone/H_2O mixture (3:2) was added. The tubes were sonicated for 3 min and then gently agitated for an additional 12 min using a rotospin rotary mixer in the dark, at room temperature, for a total extraction time of 15 min. An aliquot of this extraction was immediately filtered (0.45 μm) and analyzed via HPLC−UV−MS. Each extraction was injected in triplicate and repeated twice on different days (n = 6). All injections were finished within 10 h of their extraction time.

Quantification was performed with UV data using external calibration curves of six compounds, *E*-piceid, *E*-piceatannol, *E*-resveratrol, *E*-ε-viniferin, *E*-ampelopsin E, and *E*-vitisin B, injected simultaneously at multiple concentrations, ranging from 0.098 to 300 μg/mL (Figure 3). The trimer, *E*-ampelopsin E, was also used for the quantification of its stereoisomer, *E*-amurensin B, and *E*-ε-viniferin was also used for the quantification of its stereoisomer, *E*-ω-viniferin. The pure compounds were tested for linearity and the limit of detection (LOD) and quantification (LOQ) using the peak area obtained at 306 nm (λ). The LOD was determined as a response of at least 3 times the level of noise, and the LOQ was determined to be at least 10 times the level of noise. The linearity of the response of the standards was evaluated by plotting the peak area versus the concentration of the compounds.

Identification of Compounds in Crude Extracts. Extracts of *V. amurensis*, 90 μL each, were individually spiked with 10 μL of each compound (approximately 1 mg/mL) and analyzed using HPLC−UV−MS using gradient 1. To confirm the compound, we repeated these experiments by changing the column, flow rate, and gradient. The column used for this second set of experiments was an Agilent Eclipse XDB C_{18} (5 μm, 4.6 mm × 150 mm) column with a flow rate of 2 mL/min, using gradient 2. These HPLC−UV−MS analyses were performed on each plant extract to confirm the identity of the compound. For each compound, a comparison of ESI-MS data, in both positive and negative mode, and two different retention times were made in conjunction with spiked samples of all known compounds.

RESULTS AND DISCUSSION

Isolation and Determination of Structures of Stilbenoids. To unambiguously identify and quantify the major stilbenoids in grapevine canes, we needed standards. A preliminary HPLC−MS analysis of extracts from all plant species demonstrated that *V. amurensis* had substantial concentrations of the majority of stilbenoids of interest. Therefore, this species was chosen for isolation work to allow the identification of its major stilbenoids. From this species, 11 compounds were identified using standards or from isolation and structure determination with ESI-MS and NMR. These included *E*-ampelopsin E, *Z*-ampelopsin E, *Z*-*trans*-miyabenol, *E*-*trans*-miyabenol C, *E*-piceid, *E*-piceatannol, *E*-resveratrol, *E*-ε-viniferin, *E*-ω-viniferin, *E*-vitisin B, and *Z*-vitisin B (Figure 2). To the best of our knowledge, four of these compounds, *Z*-ampelopsin E, *Z*-*trans*-miyabenol C, *E*-*trans*-miyabenol C, and *E*-ω-viniferin, are being reported from *V. amurensis* for the first time.

Two non-piceid glycoside monomers in high abundance in *M. rotundifolia* were isolated and identified as *E*-resveratroloside and *E*-3,5,4′-trihydroxystilbene 2-*C*-glucoside by MS and one- and two-dimensional NMR analysis. A third compound, scirpusin A, was also isolated and identified from *M. rotundifolia*. All three of these compounds are being reported for this species for the first time.

Identification of Compounds in Crude Extracts. We determined which compounds were present in detectable levels in all grapevine species prior to quantification. First, each extract, pure standard, and individually spiked extracts of *V. amurensis* were analyzed via HPLC−UV−MS using two different HPLC methods. A comparison of each compound's retention times, with MS confirmation in both positive and negative mode, with each extract was made. We used two different HPLC columns and gradients to reduce the chance of misidentifying a coeluting peak with the same MS data. The retention times are listed in Table 2.

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Table 2. Limits of Detection (LOD), Limits of Quantification (LOQ), and Retention Times (t_R) Using Two Different Gradients and HPLC Columns

compound	LOD ($S/N \geq 3$) (μg/mL)	LOQ ($S/N \geq 10$) (μg/mL)	t_R with gradient 1 (min)	t_R with gradient 2 (min)
E-resveratroloside (**1**)	ND[a]	ND[a]	12.0	6.4
E-piceid (**2**)	0.098	0.33	13.3	8.6
E-piceatannol (**3**)	0.098	0.33	15.1	10.5
E-resveratrol (**4**)	0.098	0.33	19.1	13.8
E-ε-viniferin (**5**)	0.195	0.65	24.7	19.3
E-*trans*-miyabenol C (**7**)	ND[a]	ND[a]	25.5	21.9
E-ω-viniferin (**6**)	ND[a]	ND[a]	27.0	23.8
E-ampelopsin E (**8**)	0.195	0.65	28.9	25.7
E-vitisin B (**10**)	0.78	2.6	29.7	28.9
E-amurensin B (**9**)	ND[a]	ND[a]	31.0	29.0

[a]ND stands for not determined. These compounds were not used for the quantification experiments.

During these spiking studies, we were unable to find UV or MS data for samples that corresponded to either *Z*-*trans*-miyabenol C or *Z*-vitisin B. These compounds are either minor constituents, unable to be identified in the crude plant samples with our methods, or extraction artifacts. It is well-known that UV light can convert the *trans* double bond in resveratrol to a *cis* bond,[39] and therefore, it is feasible that this isomerization may have occurred for these two compounds during the large-scale extraction and multiple fractionation steps. A MS method optimized for individual detection of these two compounds may help determine if they are minor constituents or extraction artifacts. A large number of stilbenoids were identified in the majority of species for the first time (Table 3).

Development of the Analytical Method. Because of the known instability of stilbenoids, we desired an extraction method with minimal sample handling for quantitation. The optimization of an extraction method was performed by first comparing 1 h extractions with several different solvent systems: acetone/H_2O (4:6 and 6:4), MeOH/H_2O (8:2), EtOH/H_2O (8:2), and 100% MeOH. These solvent systems were selected on the basis of previous experiences in our laboratory and reports in the literature.[28,40] The acetone/H_2O solvent system at 6:4 ratio gave the most efficient extraction of stilbenoids, determined by the peak area of stilbenoids obtained by HPLC−UV analysis at 306 nm (λ). The non-acetone-containing solvent systems had lower extraction efficiencies at this time point. We then tested the amount of solvent required versus the sample of powderized cane material and found that 10 mL per 500 mg of powder provided highly reproducible and quantifiable levels of the major stilbenoids with HPLC−UV.

Optimization of the extraction time was then performed using an acetone/H_2O mixture (6:4), in comparison with an EtOH/H_2O mixture (8:2) as a reference to ensure that increased time in this solvent system was not superior to that with the acetone/H_2O mixture. Using both *V. amurensis* and *V. vinifera*, plants containing high and low levels of stilbenoids, respectively, extractions of 5, 15, 30, and 60 min and 2, 4, 6, and 24 h, with gentle agitation in the dark, were compared. At 15 min, the maximal extraction of stilbenoids in an acetone/H_2O mixture was achieved in both plants, while an EtOH/H_2O mixture (8:2) had a lower extraction efficiency of stilbenoids, even after 24 h. A 3 min sonication time was added because sonication has been demonstrated to improve extraction efficiencies, particularly in harder material such as stems.[41] We did not find an increase in stilbenoid levels overall after sonication, compared with that of nonsonicated samples; however, sonication did increase the reproducibility between samples when it was performed in triplicate. The 15 min acetone/H_2O extraction was then tested for stability, after filtration and without agitation, by comparing peak areas of major stilbenoids at 0, 2, 6, 12, and 24 h in several *Vitis* species. The compound with the largest difference was resveratrol, which had a small (between 4.3 and 6.5%) decrease after sitting in closed HPLC vials at room temperature for 24 h. Because of the superior extraction levels, we chose a 15 min extraction time in an acetone/H_2O mixture for this study. All extractions were performed within 10 h of the final injection of each triplicate.

Stilbenoid quantification was performed with six compounds, *E*-piceid, *E*-piceatannol, *E*-resveratrol, *E*-ε-viniferin, *E*-ampelopsin E, and *E*-vitisin B (Figure 3). The trimer, *E*-ampelopsin E, was used for the quantification of its stereoisomer, *E*-amurensin B, because sufficient quantities of this molecule were not obtained for quantification studies. Likewise, *E*-ε-viniferin was used for the quantification of *E*-ω-viniferin, and piceid was used for the quantification of resveratroloside. Using HPLC−UV at 306 nm (λ), a linear response was found for all compounds in the range of 3.125−200 μg/mL with a correlation coefficient r of >0.998. The limits of detection (LOD) and quantification (LOQ) were also determined and are listed in Table 2.

In this study, the amount of *E*-resveratrol ranged from approximately 0.2 g/kg in *V. betulifolia* to 5.4 g/kg in *V. amurensis*. For *V. vinifera*, the amounts of resveratrol and piceatannol were 1.6 and 0.5 g/kg, respectively, consistent with previous studies of *V. vinifera* cultivars.[28] These results differ from the survey of different *Vitis* species grown throughout China, however, in that we found *V. vinifera* to be one of the species containing the lowest level of resveratrol.[29] Our data did correlate with this study, however, in that *M. rotundifolia* was found to have lower levels of resveratrol than the majority of species, including *V. vinifera*. Additionally, the amount of *E*-ε-viniferin in this study was approximately 3−4 times greater than in previous reports,[28] most likely due to the fact that it was reported previously as resveratrol units, which have a much greater UV absorbance than ε-viniferin. *V. riparia* cv. Scribner had the greatest total amount of stilbenoids measured (>19 g/kg). The amount of vitisin B is greatest in this plant, and it also had the second highest levels of resveratrol. The other *V. riparia* tested, the Pulliat cultivar, had substantially lower levels of both resveratrol and vitisin B.

The overall profiles of the plants are relatively similar, with respect to the fact that a majority of plants contain almost all of the compounds in detectable quantities via HPLC−UV at 306 nm (λ). However, the ratios of many compounds vary substantially between certain species. For example, *V. amurensis* differs from the other *Vitis* species in that it contains a substantial amount of the two trimers, *E*-ampelopsin E and *E*-amurensin B, in addition to vitisin B. For the majority of the plants, vitisin B is the major higher-order oligomer found. There is also a substantial amount of *E*-ω-viniferin in many species, which was not anticipated because this dimer was only recently discovered in the leaves of *V. vinifera*.[7]

Given the number of phytochemical investigations into *Vitis* species,[6] we also did not expect to find the piceid isomer,

Table 3. Concentrations of Stilbenoids from Individual Grapevine Species[a]

plant	*E*-resveratroloside[b]	*E*-piceid	*E*-piceatannol	*E*-resveratrol	*E*-ε-viniferin	*E*-ω-viniferin[c]	*E*-ampelopsin E	*E*-vitisin B	*E*-amurensin B[d]	total mg/kg of dw
V. amurensis	213.6 (7.2)[e]	NQ	1962.2 (122.4)	5432.9 (208.6)	4510.4 (20.9)	NQ[e]	1615.1 (35.4)	972.1 (48.9)[f]	567.1 (14.7)	15059.8
V. arizonica	NQ[e]	NQ[e]	1100.8 (21.5)[e]	2412.4 (7.5)[e]	3715.5 (11.5)[e]	122.2 (3.1)[e]	428.4 (3.5)[e]	4279.6 (62.5)[e]	NQ[e]	12058.9
V. berlandieri	ND	64.2 (2.5)[f]	894.4 (6.8)[e]	1951.2 (86.0)[e]	1264.5 (33.8)[f]	NQ[e]	NQ[f]	2038.0 (98.1)[f]	NQ[e]	6212.3
V. betulifolia	NQ[e]	101.0 (1.5)[e]	194.1 (13.6)[e]	191.0 (2.4)	1397.4 (16.9)	65.1 (2.6)[e]	149.6 (6.6)[e]	ND	55.9 (2.5)[e]	2154.2
V. × *champini*	NQ[e]	NQ[e]	599.7 (71.8)[e]	2534.9 (15.0)[e]	4054.9 (87.5)	365.9 (9.3)[e]	NQ[e]	5031.8 (95.2)	NQ[e]	12587.3
V. cinerea	NQ[e]	133.8 (8.6)[e]	1195.1 (73.6)[e]	3165.7 (131.3)[e]	1610.9 (29.0)[f]	NQ[e]	NQ[f]	2531.7 (69.4)[f]	NQ[e]	8503.3
V. cinerea (GH)	NQ[e]	188.6 (13.5)[e]	1558.1 (38.9)[e]	3651.0 (389.1)[e]	1092.5 (223.9)[f]	NQ[e]	NQ[f]	1259.3 (202.9)[f]	NQ[e]	7749.6
V. × *doaniana*	NQ[e]	NQ[e]	1151.1 (196.5)[e]	3572.2 (76.7)[e]	4352.5 (35.8)	288.3 (3.2)[e]	NQ[e]	2506.2 (12.0)[e]	NQ[e]	11870.3
V. labrusca	NQ[e]	NQ	377.0 (34.3)[e]	1028.2 (53.0)	4683.7 (99.7)[e]	214.2 (3.2)[e]	NQ[e]	5051.0 (237.7)[e]	NQ[e]	11354.0
V. mustangensis	NQ[e]	NQ[e]	199.6 (34.9)[e]	364.0 (2.1)[e]	3254.3 (68.5)[e]	270.5 (6.4)[e]	NQ[e]	6966.2 (69.2)[e]	NQ[e]	11054.5
V. riparia Pull.	NQ[e]	291.7 (11.6)[e]	615.4 (18.1)[e]	1666.1 (9.4)[f]	5739.0 (45.8)[f]	156.8 (3.2)[e]	575.6 (16.9)[f]	1950.7 (24.8)[f]	NQ[e]	10995.4
V. riparia Scrib.	NQ[e]	505.0 (26.6)[e]	1897.1 (154.3)[e]	4976.8 (139.4)[f]	4389.6 (28.6)[f]	132.6 (3.6)[e]	562.6 (9.7)[f]	7019.2 (155.9)[f]	NQ[e]	19482.9
V. rupestris	NQ[e]	257.0 (9.6)[e]	1182.7 (26.7)[e]	3966.5 (52.5)[e]	3912.6 (301.7)[f]	374.3 (26.9)[e]	NQ[f]	4916.1 (412.3)[f]	NQ[e]	14609.2
V. vinifera cv. Cab. Sav.	ND	NQ	573.1 (30.5)	1621.3 (14.2)	2584.9 (68.2)	NQ[f]	ND	2159.0 (80.5)	ND	6938.3
M. rotundifolia (Dulcet)	943.4 (35.9)[e]	ND	524.9 (31.1)[e]	1122.2 (19.2)[f]	728.1 (33.1)[f]	NQ[e]	216.1 (14.2)[e]	NQ[e]	103.1 (6.0)[e]	3637.8
VRH 8771	215.8 (10.8)[e]	NQ[e]	1393.4 (14.2)[e]	3323.3 (31.0)[e]	3585.1 (37.1)[f]	NQ[e]	594.6 (14.3)[e]	1113.3 (47.6)[e]	244.5 (10.2)[e]	10254.2

[a]Two sets of extractions were conducted in triplicate ($n = 6$) on separate days, and results are reported in milligrams per kilogram of dry weight. The standard deviation (SD) is given in parentheses. NQ means detected but not quantified because of low levels. ND means not detected in the crude plant extract via either UV or MS analysis. [b]Piceid was used for the quantitation of resveratroloside. [c]*E*-ε-Viniferin was used for the quantification of *E*-ω-viniferin. [d]*E*-Ampelopsin E was used for the quantification of *E*-amurensin B. [e]Compounds are identified in this plant species or hybrid for the first time. [f]Compounds were identified in this plant part for the first time.

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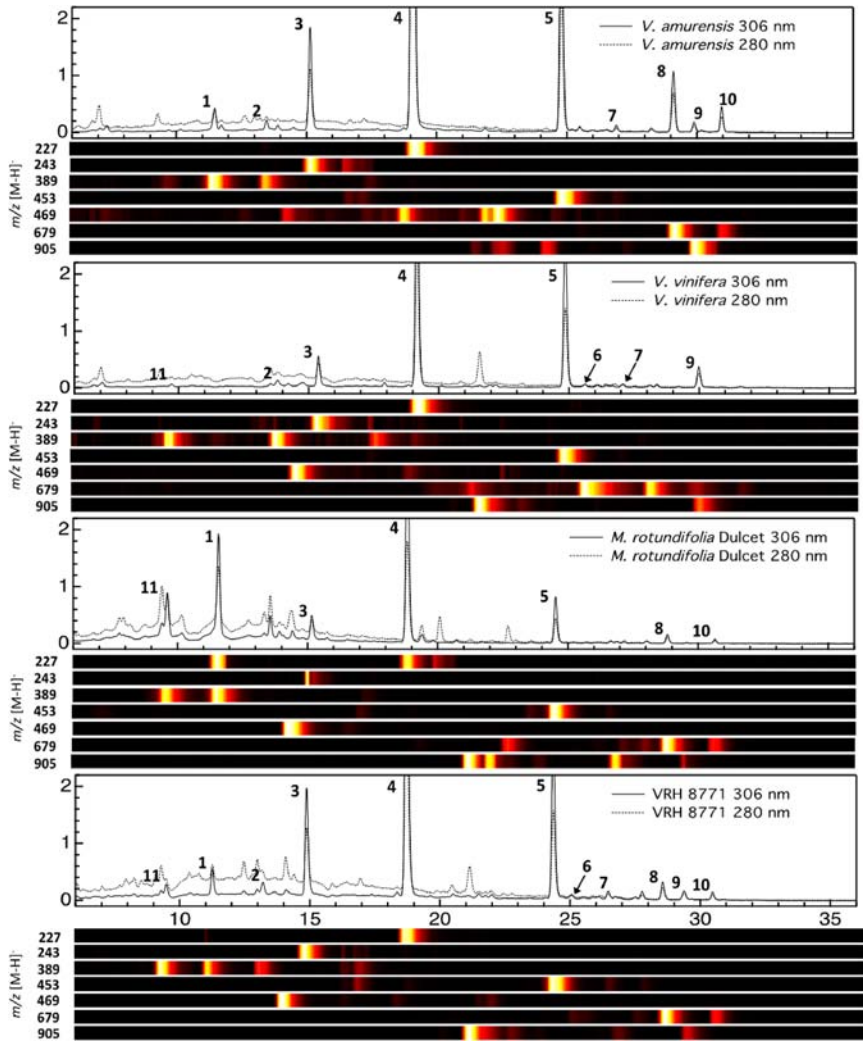

Figure 4. HPLC−UV chromatogram of *V. amurensis*, *V. vinifera*, *M. rotundifolia*, and *V. vinifera* × *M. rotundifolia* (VHR 8771) at 306 nm (λ). Compounds identified via NMR and ESI-MS: resveratroloside (**1**), piceid (**2**), piceatannol (**3**), resveratrol (**4**), *E*-ε-viniferin (**5**), *E*-*trans*-miyabenol C (**6**), *E*-ω-viniferin (**7**), *E*-ampelopsin E (**8**), *E*-vitisin B (**9**), *E*-amurensin B (**10**), and *E*-3,5,4′-trihydroxystilbene 2-*C*-glucoside (**11**). Bars below HPLC−UV traces represent MS data from individually extracted ion chromatograms. The m/z values correspond to common *Vitis* stilbenoids as described in the text. The relative intensities, from low to high, are darker to lighter, respectively.

resveratroloside, in the majority of species. Within the *Vitis* genera, this compound has only been previously reported in *V. vinifera* cell cultures.[36] Resveratroloside is a major constituent of the highly disease resistant muscadine canes. This species, *M. rotundifolia*, also has substantial amounts of *E*-3,5,4′-trihydroxystilbene 2-*C*-glucoside, with some of the lowest levels of the stilbenoid oligomers, as indicated by its relative UV absorbance at 306 nm (λ) in the HPLC chromatogram. It has been demonstrated that phenolic glycosides are more easily transported within a plant than their aglycones. Therefore, these relatively high levels of stored glycosides could confer an advantage in their rapid response to infections and stresses in other parts of the plant. However, it has been shown they lack antimicrobial activity on their own.[42] It would be interesting to investigate this as a potential mechanism of disease resistance in the leaves and stems of *M. rotundifolia*. Additionally, unlike the *Vitis* species, we were unable to detect any piceid in the crude extract of muscadine stems, via UV or MS analysis (see Figure 4). The differences in the placement of the glycoside, i.e., piceid has the glycoside at the 3-hydroxy (*meta*) position and resveratroloside has the glycoside at the 4′-hydroxy (*para*) position, may also have an impact on oxidation condensation reactions that lead to the formation of oligomers. This could be one reason for the smaller quantities of observed oligomers in the muscadine stems, because the 4′-hydroxy form is more reactive.[43] Given these observations, it would be interesting to verify a previous report of the presence of piceid as a major stilbenoid, rather than its isomer, resveratroloside, in the muscadine berries.[44]

Because of the unusual profile of *M. rotundifolia* cv. Dulcet, we compared the HPLC chromatograms of several additional *M. rotundifolia* cultivars grown in the INRA collection. Each overlapped closely with the Dulcet cultivar (see the Supporting Information for the HPLC−UV chromatogram of a second cultivar, Regale). We also analyzed a second *V. cinerea*, termed "*V. cinerea* GH" and grown in the same greenhouse with the muscadine grapes, and compared it with a *V. cinerea* grown in the field with the other *Vitis* species. This was done to determine if growing in the greenhouse could dramatically alter stilbenoid profiles and lead to our observed differences between the *Vitis* spp. and *M. rotundifolia*. We found these two *V. cinerea* species, one grown indoors and the other grown outdoors, to

have highly similar stilbenoid profiles. This helps rule out the possibility that the unusual profile of *M. rotundifolia* is an artifact caused by growing indoors (see the Supporting Information for HPLC−UV and MS profiles).

The wine-producing grapevine, *V. vinifera*, had the lowest levels of stilbenoids, with the exception of *V. berlandieri*, *V. betulifolia*, and *M. rotundifolia*. However, a close examination of the MS data shows that these three non-*vinifera* species contain potentially substantial amounts of nonquantified stilbenoids. This was determined by careful comparison of the relative intensities of nonquantified peaks, in the HPLC−UV and MS extracted ion chromatograms for m/z values of typical *Vitis* stilbenoids, of each species (see MS data in the Supporting Information). For example, *V. berlandieri* contains an unidentified trimer with a large $[M - H]^+$ signal, m/z 681, at 22.4 min, while *V. betulifolia* contains this same trimer in addition to a tetramer at 22.2 min. Another plant containing relatively low levels of stilbenoids, *V. cinerea*, also contained significant amounts of these two compounds, according to the MS response. Both *M. rotundifolia* cultivars, Dulcet and Regale, have two additional nonidentified tetramers, based on $[M - H]^-$ ions at m/z 905, at t_R values of 21.2 and 22.2 min. These compounds lack strong UV chromophores at 306 nm (λ), which suggests they lack the exocyclic double bonds present in compounds such as vitisin B and ampelopsin E.

The *V. vinifera* × *M. rotundifolia* hybrid (VRH 8771) is also highly noteworthy as it has substantial levels of all stilbenoids, unlike either of the parent species, which have some of the lowest overall levels of stilbenoids. The only other hybrid in which we also have data on both parent species, *V.* × *champini*, a cross between *V. candicans* and *V. rupestris*, has a more expected profile, similar to those of both parents.

There have been a number of studies showing a positive correlation between the rate and quantity of stilbenoids produced and disease resistance in leaves[8,45] and, to a lesser extent, berries.[46] Testing for the accumulation of stilbenoids after infection, however, requires many steps. The chemical profiling of constitutively expressed stilbenoid in stems, as a way to predict potential susceptibility or resistance during breeding programs, is a tempting alternative. Initial studies conducted by Pool et al., to correlate stilbenoid levels in leaves and xylem from canes with disease resistance as a screening method for disease resistance, were not successful. However, this study focused on the production of only two stilbenoids, resveratrol and *E*-ε-viniferin, postinfection and post-UV irradiation.[47] A more recent study showed that resveratrol and piceid levels in leaves are not indicative of disease resistance, but rather that accumulation of stilbenoid oligomers in the leaves was more strongly correlated with greater disease resistance.[48] The profiling method we present will allow rapid correlations between levels of constitutively expressed stilbenoids of potential biological importance in different species, hybrids, and cultivars, with their relative disease resistance.

This study demonstrates that grapevine canes are a rich source of multiple stilbenoid monomers, glycosides, and oligomers. This survey includes many economically important species, such as *V. riparia*, *V. rupestris*, and *V. labrusca*. We report the first observation of a number of stilbenoids in a majority of our screened species and also the first finding of any stilbenoids in the American species *V. arizonica* and *V. candicans*. We also present the first chemical study on the American−European hybrid, VRH 8771. We expect these results to stimulate more interest in the presence and profiling of stilbenoids across *Vitis* in disease resistance breeding programs and for using grapevine canes as a valuable source of many health-promoting stilbenoids.

■ ASSOCIATED CONTENT

Supporting Information

HPLC−UV chromatograms of all plants examined with corresponding one-dimensional plots of MS spectral data from the extracted ion chromatograms, with m/z values of common *Vitis* stilbenoids. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

EtOH, ethanol; NMR, nuclear magnetic resonance; t_R, retention time; ESI-MS, electrospray ionization mass spectrometry; UV, ultraviolet

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