DOI:10.1021/jf903732p

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

# Investigation into the Formation of Guaiacol Conjugates in Berries and Leaves of Grapevine *Vitis vinifera* L. Cv. Cabernet Sauvignon Using Stable Isotope Tracers Combined with HPLC-MS and MS/MS Analysis

Yoji Hayasaka,* Gayle A. Baldock, Kevin H. Pardon, David W. Jeffery, and Markus J. Herderich

The Australian Wine Research Institute, P.O. Box 197, Glen Osmond, South Australia 5064, Australia

Fermentation of grapes that had been exposed to bushfire smoke can potentially yield unpalatable, smoke-affected wine. Guaiacol and its glucoconjugate were previously found in smoke-affected grapes at an elevated concentration. To find and identify further guaiacol conjugates in smoke-affected grapes, a stable isotope feeding experiment combined with extensive HPLC-MS and MS/MS investigations was carried out. Leaves and berries of a potted grapevine were placed in contact with an aqueous mixture of $d_0$- and $d_3$-guaiacol for 1−2 days and collected 5 weeks later. Screening for potential guaiacol conjugates in the leaves and berries was facilitated by monitoring the unique mass spectrometric signature of an isotopic doublet separated by 3 Da. Seven different conjugates were detected in leaves and berries and were tentatively identified as mono- and diglycosides of guaiacol. Quantitative analysis demonstrated that the guaiacol conjugates were translocated between leaves and berries to a very limited extent and were also present as low-level natural compounds of untreated leaves and berries. The same guaiacol conjugates were also found at a considerably elevated concentration in leaves and berries obtained from grapevines exposed to bushfire smoke.

**KEYWORDS: Guaiacol; glycosides; guaiacol conjugates; smoke exposure; stable isotope tracer; grapes; bushfire; HPLC-MS; HPLC-MS/MS; *Vitis vinifera***

## INTRODUCTION

In 2003, the sensory effects of wine made from smoke-affected grapes was first noticed as a potential problem by Australian wine producers, after a large number of vineyards were heavily exposed to smoke from a series of bushfires (*1*). Since then, increasing occurrences of bushfires in the vicinity of grape-growing regions in Australia as well as overseas have led researchers and practitioners to investigate the effects of smoke on grapevine physiology, grape and wine composition, and wine sensory characteristics (*2*−*5*). The challenges are to quantify smoke exposure in grapes prior to winemaking and to develop winemaking practices that minimize the undesirable sensory properties of wine made from smoke-affected grapes.

Grapevine exposure to ash and smoke has resulted in some wines being smoke-affected and exhibiting "smoky", "burnt", "dirty", "earthy", "smoked foods", and "ashtray" characters (*1*−*3*). In some instances the smoke-related characters present in the juice or must have increased after fermentation and continued intensifying in the wine over time, even in the bottle. This anecdotal observation by winemakers was experimentally confirmed by Kennison et al. (*4*), who found that the smoky compounds guaiacol and 4-methylguaiacol were released throughout fermentation, resulting in elevated concentrations in wine. This study also demonstrated that enzyme and strong acid hydrolysates of the juice from model smoking experiments contained significantly elevated concentrations of these compounds, with acid hydrolysis releasing more of the volatiles. Subsequently, Hayasaka et al. (*6*) confirmed the presence of the β-D-glucopyranoside (glucoside) of guaiacol in grapes experimentally exposed to smoke, as well as those exposed to bushfire smoke. Although the guaiacol glucoside was found to be more susceptible to enzyme rather than acid hydrolysis, bushfire-smoked grapes released more guaiacol by acid treatment. These studies pointed out that in addition to guaiacol glucoside, other guaiacol conjugates were likely present in smoke-affected grapes that were less susceptible or even inert to enzyme hydrolysis.

This study was designed to investigate the formation of guaiacol conjugates in grapes and leaves that have been in contact with guaiacol in aqueous solution or as a result of vineyard smoke events. HPLC-MS and MS/MS analyses combined with a stable isotope tracer technique were used to identify and quantify previously unknown guaiacol conjugates present in leaves and grapes exposed to guaiacol.

## MATERIALS AND METHODS

**Materials.** All chromatographic solvents were of high-performance liquid chromatography (HPLC) grade, all chemicals were of analytical reagent grade unless otherwise stated, and water was obtained from a

*Corresponding author (telephone +61 8 8313 6637; fax +61 8 8313 6601; e-mail yoji.hayasaka@awri.com.au).

 Published on Web 01/21/2010 

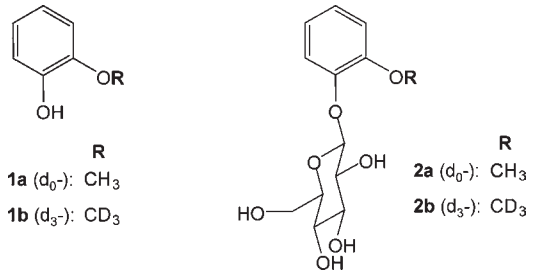


**Figure 1.** Chemical structures of $d_0$- and $d_3$-guaiacol and their glucosides.

Milli-Q purification system (Millipore, North Ryde, NSW, Australia). All prepared solutions were % v/v with the balance made up with Milli-Q water, unless otherwise specified. Merck solvents were purchased from Rowe Scientific (Lonsdale, SA, Australia). Unlabeled guaiacol ($d_0$-guaiacol, **1a** (**Figure 1**)) was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Labeled guaiacol ($d_3$-guaiacol, **1b**) was previously synthesized by Pollnitz et al. (*7*). The $d_0$- and $d_3$-guaiacol $\beta$-D-glucopyranosides (glucosides **2a** and **2b** (**Figure 1**)) were prepared according to a synthetic method closely based on that reported by Hayasaka et al. (*6*).

**Grapevine.** Two potted, own-rooted grapevines of *Vitis vinifera* L. cv. Cabernet Sauvignon were used. The plants were grown in 30 cm diameter pots and maintained at ca. 28 °C in a greenhouse.

**Smoke-Affected Leaves and Grapes.** Chardonnay and Shiraz leaves and grapes (nine samples of each) were collected from different vineyards located in Victoria (Australia) in a period from late February to late March 2009. The vineyards had been affected by smoke from a series of bushfires that occurred in the period February 7−March 14, 2009.

**Application of $d_0$- and $d_3$-Guaiacol to Leaves and Berries of the Potted Grapevine.** Aqueous guaiacol solutions (approximately 5 or 30 mg/L for low-guaiacol (LGu) and high-guaiacol (HGu) treatments, respectively) consisting of practically equal amounts of $d_0$- and $d_3$-guaiacol were prepared. For leaf application, the guaiacol solution (10 mL) was poured into a plastic sandwich bag with a quick seal (18 cm × 17 cm obtained from a local supermarket). A leaf was placed in the bag, which was then sealed and gently folded in half, allowing the leaf more direct contact with the guaiacol solution (**Figure 2A**). Similarly for berry application, the guaiacol solution (20 mL) was added to the plastic bag and a bunch of berries was placed in the bag (**Figure 2B**).

The guaiacol solutions were applied to leaves and berries of the same grapevine (treated grapevine) on December 17, 2008, when the grapevine was observed to be at an early stage of postveraison (more than half of berries were colored, **Figure 2B**). Forty leaves (leaf-LGu) and eight bunches of berries (berry-LGu) were randomly selected and treated with the 5 mg/L guaiacol solution for 2 days, and nine leaves (leaf-HGu) and three bunches of berries (berry-HGu) had the 30 mg/L guaiacol solution applied for 1 day. The remaining leaves (leaf-NoGu) and bunches of berries (berry-NoGu) on the treated grapevine were free of contact with the guaiacol solutions. All leaf and berry samples were collected on January 21, 2009, 35 days after the day of guaiacol application. Control leaf (leaf-Cont) and berry (berry-Cont) samples were also collected on the same day from the control potted grapevine, which completely lacked contact with any guaiacol solution. All samples were stored at −20 °C until analysis.

**Sample Preparation.** *Leaf Samples*. Five leaves were frozen and kept at −80 °C for at least 24 h prior to extraction. The frozen leaves were ground with a small coffee grinder. The ground leaves (ca. 1 g) were transferred into a 10 mL plastic tube, and 5 mL water was added, followed by vigorous shaking for 1 min and ultrasonication for 10 min. The supernatant was collected from the leaf/water sample after centrifugation at 4000 rpm for 5 min with a Thermo Electron Corp. IEX Micromax microcentrifuge (Biolab, Mulgrave, VIC, Australia). Subsequently, 5 mL of water was added to the pellet remaining in the tube, followed by the same procedures as described above. The supernatants were then combined for solid phase extraction (SPE).

*Berry Samples*. Randomly, eight berries were collected and placed in a 10 mL plastic tube. Berries were vigorously crushed and squeezed with a metal spatula and then centrifuged at 4000 rpm for 5 min, to obtain the supernatant (ca. 3 mL).

*Skin and Pulp Samples*. Skin and pulp portions were separated from 10 berries that had been stored at −80 °C for at least 24 h. Skins were peeled off from the frozen berries using a scalpel, and the remaining portion was collected as pulp. The skins were ground using a mortar and pestle under liquid nitrogen. The ground skins were transferred to a 10 mL plastic tube, and 5 mL of water was added, followed by vigorous shaking for 1 min and ultrasonication for 10 min. The supernatant was collected after centrifuging at 4000 rpm for 5 min. The pulp portion was transferred to a 10 mL plastic tube and treated in the same manner as the berry samples to collect the supernatant.

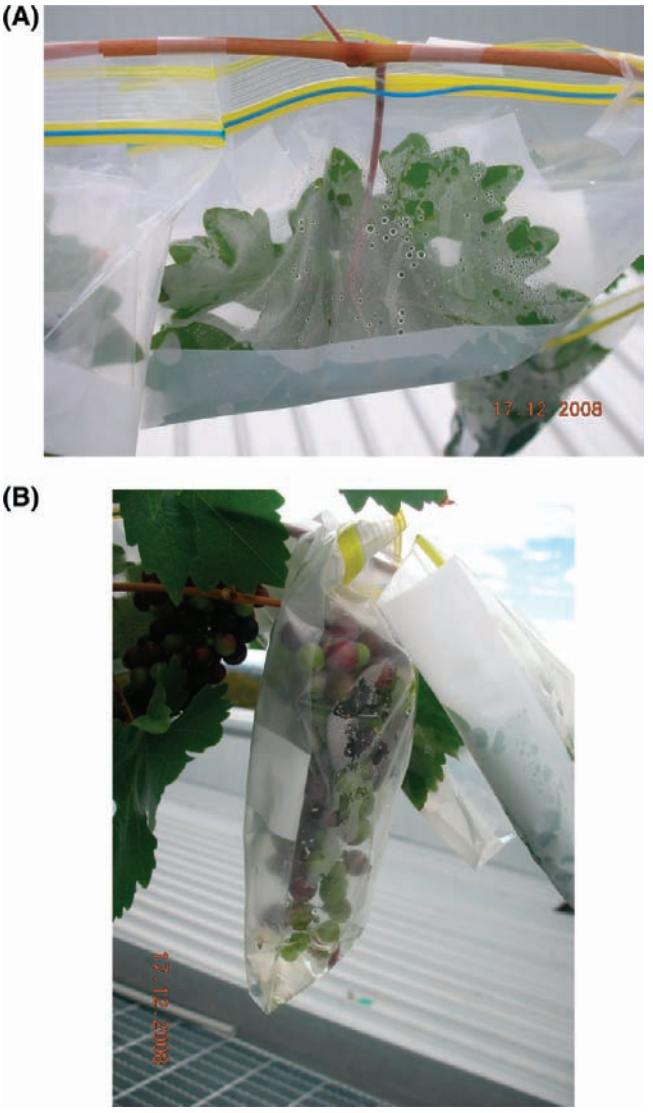


**Figure 2.** Application of the aqueous $d_0$- and $d_3$-guaiacol solution to (**A**) leaves and (**B**) a bunch of berries.

*Smoke-Affected Leaves and Grapes*. Leaves were treated with the same procedure as described above. Grapes were frozen at −20 °C and ground with a Grindomix grinder (Retsch, Germany). Approximately 25 g of the homogenate was centrifuged at 4000 rpm for 5 min to collect the supernatant.

*Solid Phase Extraction*. An aliquot of the supernatant (5 mL for leaves, 2 mL for berries, skins, and pulp, or 10 mL for smoke-affected leaves and grapes) was loaded onto an Extract-Clean C18-HF SPE cartridge (500 mg/4 mL, Grace Davison Discovery Sciences, Baulkham Hills, NSW, Australia) preconditioned according to the manufacturer's instructions. The SPE cartridge was washed twice with 10 mL of water, and the remaining materials were eluted with 2 mL of methanol. All extraction procedures were carried out using an SPE tube vacuum manifold (Grace Davison).

The methanol extract was concentrated to semidryness with a stream of nitrogen gas at 40 °C using a Zymark TurboVap LV evaporator (John Morris Scientific, Chatswood, NSW, Australia). The residue (extract) was reconstituted with 0.5 mL of water, filtered (Acrodisc Syringe Filters with 0.45 $\mu$m GHP membranes, PALL Life Science, Cheltenham, VIC,

Australia), and transferred to a vial ready for HPLC-MS or MS/MS analysis.

**High-Performance Liquid Chromatography−Mass Spectrometry and Tandem Mass Spectrometry Interfaced with Atmospheric Pressure Chemical Ionization (HPLC-APCI-MS and MS/MS).** A 4000 Q TRAP hybrid tandem mass spectrometer equipped with a TurboV ion source (Applied Biosystems/MDS Sciex, Concord, ON, Canada) combined with an Agilent 1200 HPLC system (Agilent Technologies, Forest Hill, VIC, Australia) equipped with a binary pump, degasser, autosampler, and column oven was used. Data acquisition and processing were performed using Analyst software version 1.5 (Applied Biosystems/MDS Sciex).

A 10 $\mu$L aliquot of the extract was injected and chromatographed on a 150 × 2 mm i.d., 3 $\mu$m Gemini C6-Phenyl 110 Å column combined with a 4 × 2 mm i.d. guard column packed with the same material (Phenomenex, Lane Cove, NSW, Australia). The column temperature was maintained at 25 °C during the HPLC run. A binary gradient with mobile phases consisting of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B) was used. The elution conditions were as follows: a flow rate of 300 $\mu$L/min, a linear gradient of solvent B from 10 to 30% in 10 min and from 30 to 70% in 5 min (15 min run and 10 min re-equilibration time). The effluent from the column was passed directly to the TurboV interface.

Mass spectra were recorded in negative ion mode. Nitrogen was used for the curtain, nebulizer, turbo, and collision gases. The ion source was used with an APCI probe, and the parameters were set at −4500 V for ion spray voltage, −10 V for entrance potential, −4 $\mu$A for nebulizer current, −40 V for declustering potential, 25 psi for gas 1, 50 psi for gas 2 (turbo), and 350 °C for gas 2 temperature. In scan mode, the first mass analyzer (Q1) was scanned from $m/z$ 100 to 1000 with a step size of 0.1 Da and scan time of 1.0 s. For selected ion monitoring (SIM), Q1 was scanned at appropriate masses with a dwell time of 50 ms. The tandem mass spectrometry (MS/MS) parameters were set at −15 or −18 V for collision potential, −5 V for collision cell exit potential, and high for collision gas pressure. For selected reaction monitoring (SRM), the mass transitions $m/z$ 507 → 447 and 323, $m/z$ 510 → 450 and 323, $m/z$ 345 → 285 and 161, $m/z$ 348 → 288 and 161, $m/z$ 477 → 417 and 293, $m/z$ 480 → 420 and 293, $m/z$ 491 → 431 and 307, and $m/z$ 494 → 434 and 307 were monitored with a dwell time of 50 ms.

**Mass Spectrometric Identification of Guaiacol Conjugates.** The leaf-HGu and berry-HGu samples were analyzed in the following order of HPLC-MS and MS/MS techniques to find and subsequently identify guaiacol-bound compounds (conjugates).

*HPLC-MS in Full Scan*. Isotopic doublets separated by 3 Da with similar ion intensities were used as mass spectrometric signatures (MS signature) for identification $d_0/d_3$-guaiacol conjugates. The MS signatures were searched in the full scan mass spectra obtained for 1 min retention time intervals across the entire HPLC run.

*HPLC-MS in SIM and HPLC-MS/MS in Product Ion Scan*. The doublets identified from the full scan data were examined to assess whether pairs of the doublets were isotopologues on the basis of their chromatographic behaviors and product ion spectra.

*HPLC-MS/MS with Selected Reaction Monitoring (SRM)*. A highly sensitive and compound-specific detection technique, SRM was used for confirmation of the absence or presence of the guaiacol conjugates in leaves and berries.

## RESULTS AND DISCUSSION

**Mass Spectrometric Strategy Validation.** The $\beta$-D-glucopyranoside (glucoside) of guaiacol, which can act as a guaiacol precursor, has previously been reported to be produced in grapes as a consequence of grapevine exposure to smoke (*6*). Accordingly, we used this glucoside to verify the mass spectrometric strategy for detection of guaiacol conjugates in leaves and grapes after direct contact with the $d_0$- and $d_3$-guaiacol solution.

When the leaf-HGu sample was analyzed by HPLC-MS in full scan mode (**Figure 3A**,**B**), the isotopic doublet, with $m/z$ 345 and 348 corresponding to $[M - H + CH_3COOH]^-$ ions of the $d_0$- and $d_3$-glucoside, respectively, was observed at approximately 5.8 min. The SIM chromatogram signals of ions $m/z$ 345 and 348 exhibited very similar intensities at close retention times of 5.83 and 5.74 min, respectively (**Figure 3C**) and showed good agreement with data for the respective reference glucosides (**Table 1**). The product ion spectra of the isotopologues were consistent with those obtained from the respective reference compounds (**Table 1**; **Figure 3D**,**E**). The same isotopic doublet was also found in the berry-HGu sample (**Table 1**). On the basis of the chromatographic and mass spectrometric agreement with the reference compounds, the isotopic doublet found in the leaf-HGu and berry-HGu samples was therefore identified to be the $d_0$- and $d_3$-guaiacol glucosides.

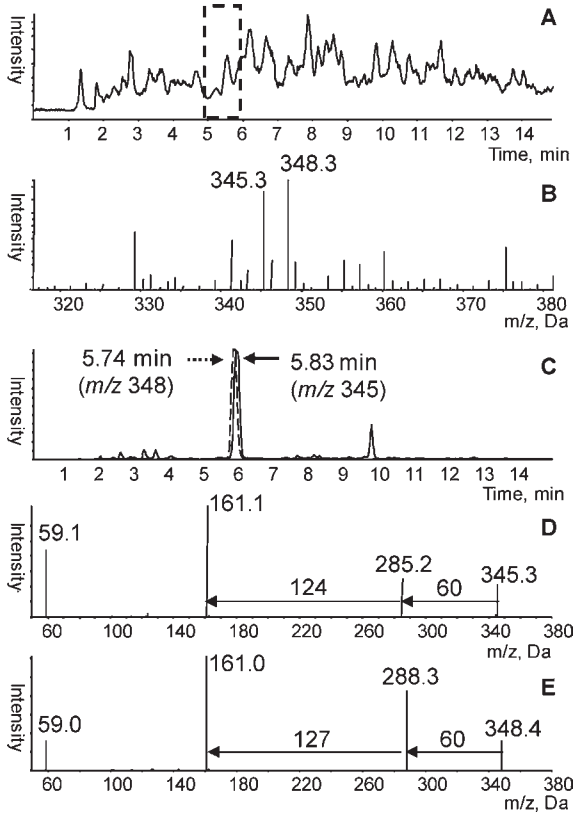


**Figure 3.** Mass spectrometric experiments to find guaiacol conjugates: (**A**) total ion chromatogram (TIC) of the leaf-HGu sample when analyzed by HPLC-MS in full scan; (**B**) mass spectrum obtained from retention times ranging from 5 to 6 min; (**C**) HPLC-SIM chromatograms of *m/z* 345 and 348; (**D**, **E**) product ion spectra of *m/z* 345 and 348, respectively.

**Searching Conjugates Based on the MS Signature.** On the basis of mass spectra obtained by HPLC-MS in full scan mode, the following doublets separated by 3 Da were found in the leaf-HGu and/or berry-HGu samples: $m/z$ 507/510, 477/480, 345/348, and 491/494 (data not shown). These doublets found in the leaf-HGu sample were examined by HPLC-MS in SIM. In addition to the ions $m/z$ 345/348 of the monoglucoside (**Figure 3C**), the doublets, $m/z$ 507/510, 477/480, and 491/494 were consistent with the MS signature of guaiacol-bound compounds (**Figure 4**). These isotopic doublets were also found in the berry-HGu sample (**Table 1**). The isotopic doublets identified as guaiacol conjugates were further examined by HPLC-MS/MS in product ion scan mode. The ions $m/z$ 507/510 and 491/494 were fragmented in the same way as those of the $d_0$- and $d_3$-glucosides, where the respective precursor ions fragmented with the sequential neutral loss of 60 Da (acetic acid) and either 124 or 127 Da ($d_0$- or $d_3$-guaiacol, respectively) (**Table 1**). Interestingly, the chromatogram for ions $m/z$ 477/480 gave four peaks with close retention times ranging from 5.4 to 6.6 min (**Figure 4B** and **Table 1**). The isotopic doublets of the four peaks showed the same product

**Table 1.** Isotopic Doublet Separated by 3 Da Found in Leaf-HGu and Berry-HGu

| | retention time (min) | | |
|---|---|---|---|
| doublet found | leaf-HGu | berry-HGu | product ions (neutral loss, Da) |
| 507 | 4.08 | 4.07 | 507 → 447 (−60) → 323 (−124) |
| 510 | 4.03 | 4.02 | 510 → 450 (−60) → 323 (−127) |
| 345 | 5.83 | 5.84 | 345 → 285 (−60) → 161 (−124) |
| 348 | 5.74 | 5.77 | 348 → 288 (−60) → 161 (−127) |
| 477 | 5.46, 5.91, 6.33, 6.57 | 5.43, 5.90, 6.33, 6.57 | 477 → 417 (−60) → 293 (−124) |
| 480 | 5.37, 5.85, 6.25, 6.51 | 5.37, 5.86, 6.27, 6.51 | 480 → 420 (−60) → 293 (−127) |
| 491 | 6.53 | 6.55 | 491 → 431 (−60) → 307 (−124) |
| 494 | 6.47 | 6.49 | 494 → 434 (−60) → 307 (−127) |
| reference $d_0$- and $d_3$-guaiacol glucosides | | | |
| $d_0$: 345 | 5.87 | | 345 → 285 (−60) → 161 (−124) |
| $d_3$: 348 | 5.75 | | 348 → 288 (−60) → 161 (−127) |

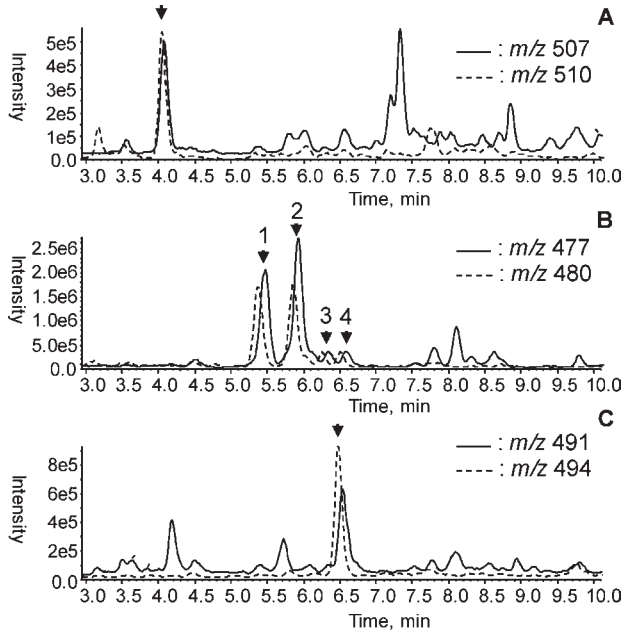


**Figure 4.** HPLC-SIM chromatograms of isotopic doublets found in the leaf-HGu sample: (**A**) *m*/*z* 507/510; (**B**) *m*/*z* 477/480; (**C**) *m*/*z* 491/494.

ion spectra and displayed the same fragmentation pattern as the other guaiacol conjugates (**Table 1**). These four sets of peaks were tentatively assigned as four isomeric guaiacol diglycosides of molecular mass 418/421 Da. In total, seven different guaiacol conjugates were found in the leaf- and berry-HGu samples.

**Identification of the Guaiacol Conjugates.** As confirmed by the reference compounds, the $d_0/d_3$-glucoside was detected as the acetic acid adduct ion $[M - H + CH_3COOH]^-$ (*m*/*z* 345/348) when analyzed by HPLC with APCI in negative ion mode (**Table 1**). This adduct ion is typically formed in the presence of acetic acid (molecular mass of 60 Da) in the mobile phases used. The fragment ions resulting from the neutral loss of 60 Da, equivalent to the deprotonated molecular ions $[M - H]^-$ (*m*/*z* 285/288), fragmented further to *m*/*z* 161 due to the neutral loss of either $d_0$-guaiacol (124 Da, **Figure 3D**) or $d_3$-guaiacol (127 Da, **Figure 3E**). The fragment ion *m*/*z* 161 was common to the isotopologues and represented the sugar moiety of the glucoside. This fragmentation pattern was also consistent with the product ion spectra observed from other isotopic doublets found in the leaf-HGu and berry-HGu samples (**Table 1**). Therefore, these isotopic doublets appeared to be derived from glycosidic forms of $d_0$- and $d_3$-guaiacol. The *m*/*z* 323, 293, and 307 ions fragmented from the respective isotopic doublets, *m*/*z* 507/510, 477/480, and 491/494, were consistent with masses of glycosides involving a hexose linked with either another hexose (162 + 162 Da), a pentose (162 + 132 Da), or a rhamnose (162 + 146 Da), respectively. Accordingly, these guaiacol-containing conjugates were tentatively identified as follows: *m*/*z* 507/510, dihexoside and most likely glucosylglucoside (either gentiobioside or sophoroside, GG); *m*/*z* 345/348, β-D-glucopyranoside (glucoside, MG); *m*/*z* 477/480, diglycosides (DGs) with terminal pentose units most likely linked to glucose, such as α-L-arabinofuranosyl-β-D-glucoside, α-L-arabinopyranosyl-β-D-glucoside, β-D-apiofuranosyl-β-D-glucoside, or β-D-xylopyranosyl-β-D-glucoside; *m*/*z* 491/494, α-L-rhamnopyranosyl-β-D-glucoside (rutinoside, RG). Glycosidically bound aroma compounds present in fruits and plants have been extensively studied and are mainly *O*-β-D-glucosides and *O*-diglycosides including the disaccharides GG, DGs, and RG tentatively identified by this study (*8*, *9*).

**Translocation of the Guaiacol Glycosides within a Vine.** The leaf and berry (HGu, LGu, NoGu, and Cont) samples were analyzed by HPLC-MS/MS in SRM mode for confirmation of the presence of the identified guaiacol conjugates. As expected, all leaves and berries in direct contact with the $d_0/d_3$-guaiacol solution (HGu and LGu) exhibited abundant peaks derived from the seven conjugates described above (**Figure 5A** for berry-LGu). In addition to the monoglucoside (MG), the mass transitions *m*/*z* 345/348 → 161 gave additional peaks eluting close to the MG peak around 6 min, suggesting that some guaiacol is likely glycosylated with other hexoses such as galactose. The leaf- and berry-NoGu samples, which had not been in contact with the guaiacol solution but were collected from the treated grapevine, showed only small peaks for all of the $d_0$- and $d_3$-conjugates (**Figure 6B**, in the case of rutinoside, *m*/*z* 491/494). These peaks observed in the NoGu samples were considerably smaller (up to only a few percent of the peak intensities) compared to those in the LGu samples (**Figure 6A**,**B**). Despite the abundance of the conjugates in the LGu samples, only trace levels were found in the NoGu samples, indicating that the translocation of these conjugates from leaf to leaf, berry bunch to berry bunch, or between leaf and berry bunch in the treated grapevine occurred to a very limited extent. On the other hand, only peaks derived from the $d_0$-conjugates were found in the leaf- and berry-Cont samples from the untreated grapevine, although the intensities were smaller than those in the NoGu samples (compare **Figure 6B**,**C**).

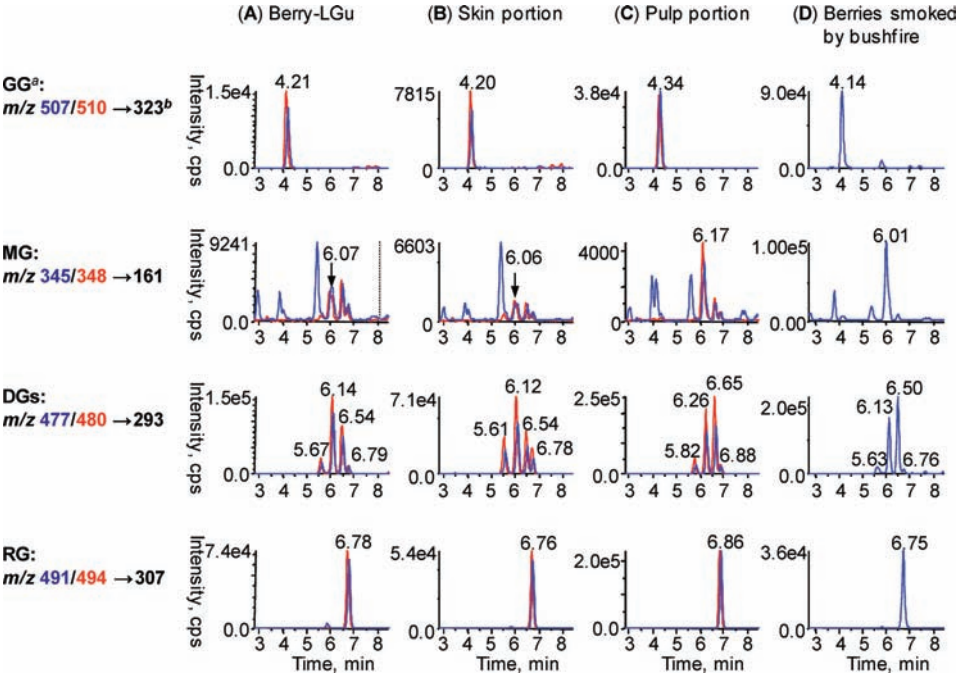


**Figure 5.** HPLC-SRM chromatograms of $d_0$- and $d_3$-guaiacol conjugates present in (**A**) the berry-LGu sample, (**B**) skin and (**C**) pulp portions of the berry-LGu sample, and (**D**) berries exposed to bushfire smoke.

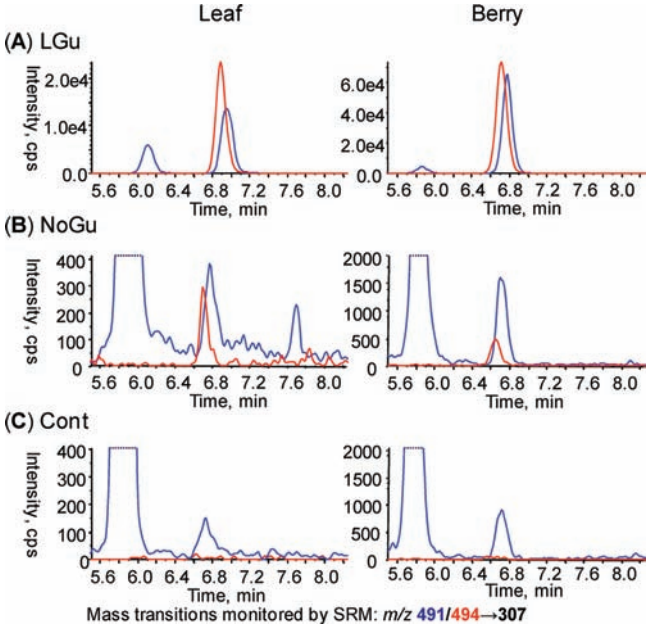


**Figure 6.** HPLC-SRM chromatograms of $d_0$- and $d_3$-guaiacol rutinoside present in the leaf and berry samples: (**A**) LGu; (**B**) NoGu; (**C**) Cont.

No guaiacol solution was applied to the control grapevine, indicating that these conjugates were likely present at trace concentrations as natural components of leaves and berries. This observation confirms a report by Sefton (*10*), who suggested glycoconjugated guaiacol exists as a natural component of juice, based on the detection of guaiacol in acid and enzyme hydrolysates of Merlot and Shiraz grapes.

**Localization of Guaiacol Glycosides in the Grape Berry.** Guaiacol was reported to be specifically localized in the skins of grapes following exposure of the grapevine to smoke (*1*). To examine this observation, localization of the conjugates within berries was investigated by analysis of the skins and pulp of the berry-LGu samples. Unlike the results reported previously for guaiacol, the seven $d_0$- and $d_3$-conjugates were present in both the skin and pulp portions (**Figure 5B**,**C**) and seemed to be nonspecifically distributed. This suggests that once guaiacol has entered the berry through the skin, glycosylation leads to relatively even distribution of conjugates between skin and pulp.

**Glycosides in Leaves and Berries Resulting from Bushfire Smoke.** To investigate whether the same or similar guaiacol glycoside patterns could be observed in leaves and grapes that had been exposed to bushfire smoke, Shiraz and Chardonnay leaves and berries collected from bushfire-affected areas were analyzed by HPLC-MS/MS in SRM mode.

All of the guaiacol glycosides identified through the isotope tracer experiment were monitored, and the relative abundance of the individual conjugates was estimated by expressing the ratio (%) of the peak area of GG, MG, DGs (as the sum of four diglycosides), or RG to the sum of all precursor peak areas (**Figure 7**). All smoke-affected leaf and berry samples contained all seven glycosides found in the stable isotope tracer experiment (**Figure 5D**). The intensity of the total conjugates varied considerably between the samples, probably due to the individual grapevines being affected by different degrees of intensity and/or duration of smoke exposure (data not shown). However, the relative abundance of the individual conjugates was consistent among leaves or berries of the same variety; also, the dominance of MG and DGs in the leaves and of DGs in the berries were the same for both varieties (**Figure 7**). This trend was also consistent with that observed for the stable isotope tracer experiment, with the exception of MG in the berry-HGu sample, which accounted for only 1% of the total conjugates (6% for Chardonnay and 14% for Shiraz from bushfire-affected grapes). Nevertheless, it was confirmed that biotransformation of guaiacol into its glycosides in the smoke-affected leaves and grapes occurred in a similar fashion to that observed in the stable isotope tracer experiment.

The use of a stable isotope tracer technique and extensive HPLC-MS investigations of $d_0$- and $d_3$-guaiacol conjugates enabled detection of characteristic MS signatures. Using these MS signatures, seven different guaiacol conjugates were found in grape leaves and berries that had been in contact with $d_0$- and $d_3$-guaiacol

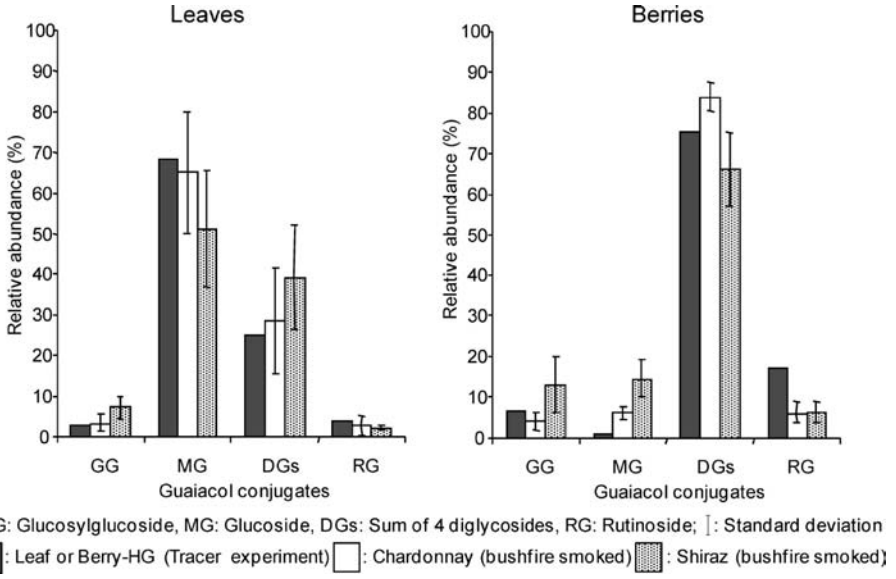


**Figure 7.** Relative abundance (%) of guaiacol glycosides present in the leaf- and berry-HGu samples and in Chardonnay and Shiraz leaves and berries exposed to bushfire smoke.

solution. These conjugates were identified to be mono- and diglycosides of guaiacol, translocated between leaves and berries to a very limited extent, and present as low-level natural compounds in leaves and berries and present in significant amounts in the leaves and berries following exposure of the grapevines to smoke derived from actual bushfires. Furthermore, the relative abundances of these conjugates were different between leaves and berries, regardless of the means of application of guaiacol (contact with an aqueous solution or from smoke).

By far the most abundant glycosidic conjugates were a group of four diglycosides that could be tentatively identified as guaiacol glucosides with a terminal pentose. This work highlights the need to evaluate all conjugates and not just guaiacol and/or guaiacol-$\beta$-D-glucoside when smoke-tainted juice is screened. It also provides opportunities to explore the impact of diglycosides on predictive assays and the potential for amelioration of smoke-affected juice through targeted removal of all guaiacol glycoconjugates.

## ABBREVIATIONS USED

HPLC-MS, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; APCI, atmospheric pressure chemical ionization; SIM, selected ion monitoring; SRM, selected reaction monitoring; SPE, solid phase extraction; MG, glucoside; DG, diglucosides; GG, glucosylglucoside; RG, rutinoside; HGu, high guaiacol; LGu, low guaiacol; NoGu, no guaiacol; Cont, control.

## ACKNOWLEDGMENT

We acknowledge Dr. Kerry Wilkinson of the University of Adelaide (UA), Dr. Alan Pollnitz of Forensic Science SA, and Con Simos and Randell Taylor of the Australian Wine Research Institute (AWRI) for valuable discussion. We also thank Prof. Steve Tyerman and Wendy Sullivan of UA for kindly providing the potted grapevines and Industry Development and Support and Commercial Services of AWRI, along with the Department of Primary Industries, Victoria, Australia, for collection and handling of the smoke-affected grapes and leaves.

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**Received for review October 25, 2009. Revised manuscript received December 20, 2009. Accepted December 22, 2009. The Australian Wine Research Institute, a member of the Wine Innovation Cluster in Adelaide, is supported by Australia's grapegrowers and winemakers through their investment body, the Grape and Wine Research Development Corporation, with matching funds from the Australian government.**