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Assessing the Impact of Smoke Exposure in Grapes: Development and Validation of a HPLC-MS/MS Method for the Quantitative Analysis of Smoke-Derived Phenolic Glycosides in Grapes and Wine

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Supporting Information

ABSTRACT: Bushfires occur frequently in the vicinity of grape growing regions, resulting in smoke drifting over the vineyards. Wine made from smoked grapes is often downgraded or unfit for sale due to negative sensory characters. To manage or avoid the risk of producing smoke-affected wine, a diagnostic assay was developed for assessing the extent of smoke exposure in grapes and the resulting wines. The method relies on the quantitation of the glycosidic grape metabolites that are formed from major volatile phenols present in smoke. Using HPLC-MS/MS with APCI, a quantitation method for phenolic glycosides as smoke marker compounds was developed and validated. The method was confirmed to be of sufficient sensitivity and reliability to use as a diagnostic assay. On the basis of phenolic glycoside concentrations, grapes or wine can be assessed as smoke exposed or not, and the relative intensity of smoke exposure can be determined.

KEYWORDS: *smoke taint, grapes, wine, phenolic glycosides, volatile phenols, HPLC-MS/MS, matrix effect*

INTRODUCTION

Increasing occurrences of bush and forest fires in the vicinity of grape growing regions in Australia as well as overseas has caused much concern among grapegrowers and winemakers. Wines made from grapes exposed to smoke in vineyards are often characterized by objectionable "smoky", "burnt", "ash", "ashtray", and "smoked salmon" aromas, with "an excessively drying" back-palate and a retronasal "ash" character.[1−3] Such sensory characteristics can result in significant quality downgrades in wine market value or even make the wine unfit for sale. To manage or avoid the risk of producing smoke-affected wine following a bushfire event, grapegrowers and winemakers need reliable diagnostic strategies to assess the impact of smoke exposure in grapes and the resulting wines as early as possible and ideally before harvest decisions are made. This could lead to considerable savings in time and money should the crop prove to be smoke-affected.

Measurements of guaiacol and 4-methylguaiacol in grapes have typically been carried out as a diagnostic assay for smoke exposure because these phenols are commonly found in smoke from wood fires. These compounds have a smoky aroma, and their concentrations in wine are reported to be strongly correlated with the overall sensory panel rating of the intensity of the smoke effect.[1] However, in some cases where the presence of guaiacol and 4-methylguaiacol in grapes was insignificant, the resulting wines exhibited smoke-related characteristics, which sometimes appeared to further increase after bottling.[4−7] Also, guaiacol in wine can originate from barrel aging and oak chips used in winemaking; this renders the measurement of free phenols problematic, especially in red wines. Such observations suggest the necessity for adopting more suitable smoke markers for a reliable diagnostic assay of smoke exposure.

Previous studies reported that smoke-derived volatile phenols including phenol, cresol isomers, guaiacol, 4-methylguaiacol, syringol, and 4-methylsyringol are metabolized into various glycoconjugated forms following their uptake by grapes.[6,8] With the aid of MS/MS experiments, the phenolic glycosides were tentatively identified to be β-D-glucosyl-β-D-glucosides (gentiobiosides), β-D-glucopyranosides (monoglucosides), and disaccharides with a terminal pentose unit (pentosylglucosides) such as α-L-arabinosyl-β-D-glucoside, β-D-apiosyl-β-D-glucoside or β-D-xylosyl-β-D-glucoside, and α-L-rhamnosyl-β-D-glucoside (rutinoside) (Figure 1). These phenolic glycosides were found in smoke-affected grapes and wines at significantly elevated levels, which led us to suggest the use of these glycosides as smoke markers.[5,6,8] This approach appeared promising due to the following observations: when a grapevine is exposed to smoke, the amount of the volatile phenols taken up by grapes can be related to the intensity and duration of smoke exposure;[2] once taken up by grapes, the volatile phenols are rapidly metabolized into their more stable and nonvolatile glycosidic forms;[6,9] the smoke induced glycosides persist and accumulate in grapes until the time of harvest,[9] so the amount of the grape phenol glycosides can be correlated to the intensity of smoke exposure; unlike free guaiacol, the concentrations of the phenolic glycosides are immaterial in nonsmoked grapes;[5,6,8] and the phenolic glycosides are easily extracted into wine and act as a pool of precursors which release volatile phenols during fermentations, aging, and storage.[4,6,7]

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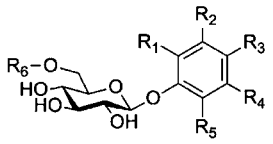

	R_1	R_2	R_3	R_4	R_5	R_6
1: Phenol gentiobioside	H	H	H	H	H	glucose
1a: Phenol pentosylglucosides	H	H	H	H	H	pentose
1b: Phenol rutinoside	H	H	H	H	H	rhamnose
2: Cresol gentiobioside		R_1, R_2 or R_3 = CH_3				glucose
2a: Cresol pentosylglucosides		R_1, R_2 or R_3 = CH_3				pentose
2b: Cresol rutinoside		R_1, R_2 or R_3 = CH_3				rhamnose
3: Guaiacol gentiobioside	OCH_3	H	H	H	H	glucose
3a: Guaiacol pentosylglucosides	OCH_3	H	H	H	H	pentose
3b: Guaiacol rutinoside	OCH_3	H	H	H	H	rhamnose
4: Methylguaiacol gentiobioside	OCH_3	H	CH_3	H	H	glucose
4a: Methylguaiacol pentosylglucosides	OCH_3	H	CH_3	H	H	pentose
4b: Methylguaiacol rutinoside	OCH_3	H	CH_3	H	H	rhamnose
5: Syringol gentiobioside	OCH_3	H	H	H	OCH_3	glucose
5a: Syringol pentosylglucosides	OCH_3	H	H	H	OCH_3	pentose
6: Methylsyringol gentiobioside	OCH_3	H	CH_3	H	OCH_3	glucose
6a: Methylsyringol pentosylglucosides	OCH_3	H	CH_3	H	OCH_3	pentose

Figure 1. Phenolic glycosides found at elevated concentrations in smoke-affected grape and wine samples.

On the basis of these observations, the aims of this study were to develop and validate a method for the quantitative analysis of phenolic glycosides in grapes and wines using HPLC-MS/MS with labeled syringol gentiobioside (d_3-**5**, Figure 2A) as internal standard, and to evaluate the capability of the method as a diagnostic assay for smoke exposure of grapes.

MATERIALS AND METHODS

Chemicals. All chromatographic solvents were HPLC grade, and all chemicals were analytical-reagent grade. Milli-Q water was used obtained from a Milli-Q purification system (Millipore, North Ryde, Australia). Merck solvents were purchased from Rowe Scientific (Lonsdale, Australia). All prepared solutions were % v/v with the balance made up with Milli-Q water, unless otherwise specified. d_3-Syringol was prepared as previously described in Hayasaka et al.[6]

Grape and Wine Samples. *Method Development and Validation.* Smoke-affected grapes (*Vitis vinifera* L. cv. Chardonnay and Shiraz) were collected from closely located vineyards in Victoria (Australia) in March 2009. The vineyards had been exposed to smoke generated by a series of bushfires that occurred in the period 7 February−14 March 2009. Control grapes with no history of smoke exposure were collected from various regions of South Australia, Victoria, and Tasmania.

Chardonnay and Cabernet Sauvignon smoke-affected wines were made from smoke-affected grapes from the same 2009 fire event in Victoria as described previously.[6] Control wines were commercially produced from grapes with no history of smoke exposure as judged by their vintage and harvest location.

Application of a HPLC-MS/MS Method to Analysis of Grapes Exposed to Bushfire Smoke. Shiraz, Cabernet Sauvignon, Pinot Noir, and Chardonnay grapes exposed to smoke in the same 2009 fire event in Victoria were used (three different samples for each variety).

Comparison between Smoke-Affected and Control Grape and Wine Samples. Smoke-affected grapes from the 2009 fire event containing guaiacol at concentrations less than 5 μg/kg were selected and control grapes used were described above.

Wines elaborated from grapes suspected of smoke exposure during the 2009 fire event were sourced from industry collaborators. Control wines were made from grapes with no history of smoke exposure by an industry collaborator according to the small-lot fermentation protocols as described elsewhere.[10] The prepared must was inoculated with 5 g/L of rehydrated EC1118 (Lallemand, Underdale, Australia) or Uvaferm 43 (Lallemand) and fermented to dryness. The ferment was centrifuged to obtain wine to which an addition of SO_2 was made (100 mg/L final concentration).

Synthesis of d_3-Syringyl-β-D-gentiobioside (d_3-5). *Synthetic Procedure.* Labeled syringol gentiobioside, d_3-**5** was synthesized using an improved procedure (Figure 2A) based on a method employed previously by our group to prepare unlabeled syringyl-β-D-gentiobioside (**5**).[6] The initial four steps to give 7-d_3-syringyl-2′,3′,4′-tri-*O*-acetyl-β-D-glucopyranoside were carried out in an identical fashion based on the method of Shao et al.[11] However, to decrease the formation of an orthoester byproduct, the disaccharide coupling step was modified to employ a glycosyl donor with isobutyryl groups (Figure 2B) in place of acetyl groups, thereby providing more sterically

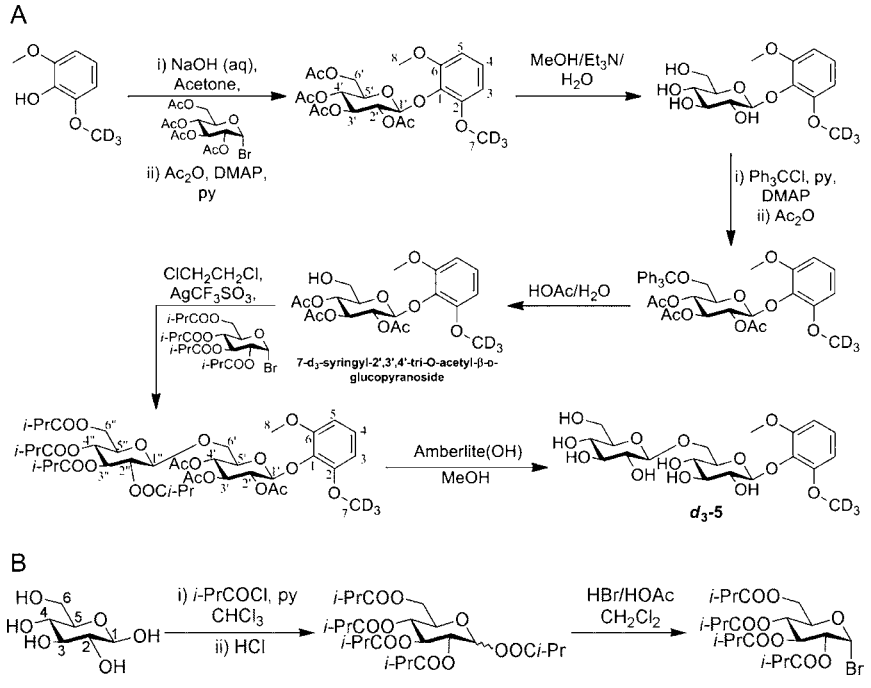

Figure 2. Syntheses of (A) 7-d_3-syringyl-β-D-gentiobioside (d_3-**5**) and (B) tetra-*O*-isobutyryl-α-D-glucopyranosyl bromide.

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hindered ester groups.[12] The method for the final deprotection to form d_3-**5** was adapted from Pathak.[13]

The purified reaction product was confirmed to be d_3-**5** as follows: R_f 0.06 (CH_2Cl_2/MeOH/HOAc 79.5:20:0.5); mp 112−114 °C; $[\alpha]_D$ −33.7 (*c* 0.3, H_2O). ^{1}H NMR (D_2O, ppm): δ 7.19 (1H, t, *J* = 8.5 Hz, H-4), 6.81−6.80 (2H, d, *J* = 8.5 Hz, H-3.5), 5.10 (1H, d, *J* = 7.5 Hz, H-1′), 4.31 (1H, d, *J* = 7.9 Hz, H-1″), 4.05 (1H, dd, *J* = 12.2, 1.2 Hz, H-6b′), 3.87 (1H, *J* = 12.4, 2.0 Hz, H-6b″), 3.86 (3H, s, H-8), 3.85 (1H, dd, *J* = 12.2, 5.3 Hz, H-6a′), 3.67 (1H, dd, *J* = 12.4, 6.0 Hz, H-6a″), 3.56 (1H, dd, *J* = 9.1, 7.5 Hz, H-2′), 3.54 (1H, app t, *J* = 9.2 Hz, H-3′), 3.51 (1H, ddd, *J* = 9.5, 5.3, 1.2 Hz, H-5′), 3.48 (1H, app t, *J* = 9.5 Hz, H-4′), 3.31 (1H, app t, *J* = 9.4 Hz, H-4″), 3.26 (1H, app t, *J* = 9.2 Hz, H-3″), 3.23 (1H, ddd, *J* = 9.4, 6.0, 2.0 Hz, H-5″), 3.16 (1H, dd, *J* = 9.2, 7.9 Hz, H-2″). ^{13}C NMR (D_2O, ppm): δ 153.23 (C-2.6), 133.66 (C-1), 126.22 (C-4), 106.82 (C-3,5), 102.86 (C-1′), 102.77 (C-1″), 76.76 (C-5′), 76.46 (C-5″), 76.31 (C-3′), 76.07 (C-3″), 74.17 (C-2′), 73.60 (C-2″), 70.16 (C-4″), 69.89 (C-4′), 68.13 (C-6′), 61.33 (C-6″), 56.79 (C-8), (C-7 not observed). ESI−HRMS (*m*/*z*): $[M + Na]^+$ Calcd for $C_{20}H_{27}D_3NaO_{13}^+$, 504.1769; Found, 504.1782.

Guaiacol Analysis by GC-MS. Guaiacol was quantitated by the stable isotope dilution assay method reported previously.[14] Analysis was performed by Commercial Services at the Australian Wine Research Institute (Adelaide, Australia) using an Agilent 6890N gas chromatograph coupled to a 5975 mass spectrometer.

Sample Preparations. Grape extract and wine samples for HPLC-MS/MS analysis were prepared according to the method described by Hayasaka et al.[6] with the exception of the use of d_3-**5** as internal standard instead of d_3-guaiacol monoglucoside. Briefly, a 5 g aliquot of thawed grape homogenate containing 500 μg/kg of d_3-**5** as internal standard was centrifuged at 4000 rpm for 5 min to collect the supernatant. A 2 mL aliquot of the supernatant was loaded onto an Extract Clean C18-HF SPE 500 mg/4 mL cartridge (Grace Davison Discovery Sciences, Australia). The cartridge was washed with 10 mL of water, and the remaining materials were eluted twice with 1 mL of methanol. After removal of methanol with a stream of nitrogen gas at 40 °C, the residue (grape extract) was reconstituted with 0.3 mL water, filtered (0.45 μm), and transferred to a HPLC sample vial ready for analysis.

A 1.00 mL aliquot of wine containing 1000 μg/L of d_3-**5** as internal standard was filtered (0.45 μm) and transferred to a HPLC sample vial ready for analysis.

HPLC-MS/MS Analysis. *Instrumentation.* Samples were analyzed using the same system as described previously.[6,8]

HPLC. The conditions described in the previous study[6] were used with the following column and mobile phase modifications: 150 mm × 2 mm id, 4 μm, 80 Å, Synergi Hydro-RP column (Phenomenex, Lane Cove, NSW, Australia); gradient for solvent B from 10 to 30% in 10 min, from 30 to 70% in 5 min, 70% held for 10 min, from 70 to 95% in 5 min and 95% held for 5 min.

Atmospheric Pressure Chemical Ionization (APCI) and Electrospray Ionization (ESI). Mass spectra were recorded in negative ion mode. Nitrogen was used for the curtain, nebulizer, turbo, and collision gases. The ion source was fitted with either an APCI or ESI probe and the parameters were set at −10 V for entrance potential, −45 V for declustering potential, and 60 psi for nebulizer gas for both probes. For APCI, −3 μA for corona discharge needle current and 450 °C for probe temperature were used, and for ESI, −3500 V for ionspray voltage, 55 psi for turbo gas, and 450 °C for turbo gas temperature were used.

Tandem Mass Spectrometry (MS/MS). Phenolic glycosides were detected by selected reaction monitoring (SRM). MS/MS parameters were set at −16 V for collision potential, −9 V for collision cell exit potential, and "High" for collision gas pressure. Mass transitions from acetic acid adduct ions of phenolic glycosides $[M + CH_3COO]^-$ to the fragment ions $[\text{glycosidic moiety}]^-$ resulting from the neutral loss of phenolic aglycone were monitored with dwell times of 50 ms for the following phenolic glycosides (Figure 1); *m*/*z* 477.2, 491.2, 507.2, 521.2, 537.2, and 551.2 → *m*/*z* 323.1 for the gentiobiosides, **1** (retention time, 2.9 min), **2** (6.4 min), **3** (4.3 min), **4** (7.6 min), **5** (5.3 min), and **6** (8.0 min), *m*/*z* 447.2, 461.2, 477.2, 491.2, 507.2, and 521.2 → *m*/*z* 293.1 for the pentosylglucosides, **1a** (4.0−5.5 min), **2a** (7.6−10.0 min), **3a** (5.5−7.5 min), **4a** (8.5−10.0 min), **5a** (6.2−8.7 min), and **6a** (8.8−10.5 min), *m*/*z* 461.2, 475.2, 491.2, and 505.2 → *m*/*z* 307.1 for the rutinosides, **1b** (5.8 min), **2b** (8.8 min), **3b** (7.2 min), and **4b** (9.5 min), and *m*/*z* 540.2 → 323.1 for the internal standard, d_3-**5** (5.3 min), respectively. Monoglucosides were comparatively less abundant in smoked grapes than disaccharides and were excluded from the experiment.[6]

Quantitation of Glycosides. *Calibration Function.* Control Cabernet Sauvignon and Chardonnay grapes and wines were used for preparing standard addition samples. The grape homogenate or wine was spiked with a constant amount of d_3-**5** (500 μg/kg for grape homogenate or 1000 μg/L for wine as a final concentration) and a known amount of **5** at 0, 10, 20, 50, 100, 500, or 1000 μg/kg for grape homogenate or μg/L for wine. Each standard addition sample was prepared in triplicate and subjected to HPLC-MS/MS analysis. A calibration function was constructed by plotting the peak area ratio of the mass transition of **5** (*m*/*z* 537.2 → 323.1) to that of d_3-**5** (*m*/*z* 540.2 → 323.1) against the known amounts of **5**.

Internal Standard. Labeled syringol gentiobioside, d_3-**5** was used as a single internal standard for all the phenolic glycosides monitored. Concentrations of all the phenolic glycosides expressed as **5** equivalents were determined using the calibration functions for **5** in the corresponding matrices.

Back Addition Experiment. *Preparation of Phenolic Glycoside Isolate.* Phenolic glycosides were extracted from smoke-affected Shiraz grapes. A 100 mL aliquot of juice was obtained from the grape homogenate by centrifugation at 4000 rpm for 5 min and loaded onto a column filled with 4 g of Amberlite FPX 66 resin (Rohm and Haas, Philadelphia, PA). The column was washed with 25 mL of water and dried with a stream of nitrogen gas. The resin was washed with 25 mL of pentane to remove nonpolar compounds, followed by elution of phenolic glycosides with 25 mL of ethanol. The ethanol extract was concentrated to semidryness under reduced pressure at 40 °C. The residue was reconstituted with 5 mL of water affording a solution of the isolate.

Isolate Back Addition. Control Chardonnay, Riesling, Cabernet Sauvignon, and Shiraz grape and wine samples described were used. A 1 mL aliquot of the isolate solution was mixed with 0.1 mL of d_3-**5** in 10% ethanol solution (100 μg/mL) and used as the isolate stock solution. The grape extract (0.300 mL) was prepared from a grape homogenate according to the procedures previously described without the addition of d_3-**5** internal standard. The control wines without the addition of d_3-**5** internal standard were also used. The isolate stock solution (10.0 μL) was added to 0.300 mL of the grape extract or wine sample and then analyzed for the quantitation of phenolic glycosides by HPLC-MS/MS with APCI or ESI. The same grape extracts and wines used for the isolate addition samples were also spiked with d_3-**5** only (i.e., approximately 300 μg/L final concentration) and analyzed for the measurement of pre-existing phenolic glycosides in the respective matrices. Subsequently, elevated amounts of phenolic glycosides in the grape and wine samples due to the isolate addition were determined by subtracting the concentration of the sample without the isolate addition from that of the isolate addition sample. Concentrations of the individual phenolic glycosides, obtained by measuring the peak area of a phenolic glycoside relative to the known amount of d_3-**5**, were expressed as d_3-**5** equivalents.

Quantitation Limits. Labeled syringol gentiobioside, d_3-**5** was used to determine the detection capability of the HPLC-MS/MS with APCI. Control Cabernet Sauvignon grape homogenates and Merlot wines (both in triplicate) were spiked with d_3-**5** at concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1, 2, and 10 μg/kg and 0, 0.1, 0.25, 0.5, 1, 5, or 10 μg/L, respectively, and treated for analysis as previously described without the addition of d_3-**5** internal standard.

Reproducibility and Recovery. Shiraz and Chardonnay grapes and Cabernet Sauvignon and Chardonnay wines (smoke-affected and control) were used for method validation. Smoke-affected, control, and their blended samples containing the smoke-affected sample at one (10%) or five (50%) out of 10 (v/v) portions were prepared as

triplicates. All samples were analyzed for the quantitation of phenolic glycosides by HPLC-MS/MS with APCI.

Reproducibility of quantitation values (n = 3) of the individual glycosides was evaluated based on a coefficient of variation (CV, %). Recovery of the 10% and 50% blended samples was obtained by dividing their mean concentration (Cm) by their expected concentration (Ce) calculated from mean concentrations of smoke-affected (Cs) and control (Cc) samples, e.g. recovery (%) = Cm/Ce × 100% = Cm/(0.5 × Cs + 0.5 × Cc) × 100% for the 50% blended sample.

Statistical Analysis. Statistical analysis was performed with JMP (SAS Institute, USA, version 5.0.1).

RESULTS AND DISCUSSION

Method Validation. *Calibration Functions.* The applicability of ESI and APCI to the analysis of phenolic glycosides in grape extracts and wine samples was initially investigated. When phenolic glycosides were analyzed under the HPLC-MS conditions used, both ESI and APCI similarly gave the dominant corresponding $[M + CH_3COO]^-$ ions together with the lesser abundant $[M - H]^-$ ions (data not shown). Calibration functions with d_3-**5** as internal standard and **5** ranging from 10 to 1000 μg/kg or μg/L in white and red grape extracts or wines, respectively, were obtained using ESI or APCI. All linear fitted calibration functions (R^2 > 0.999) of grape or wine matrices appeared to be consistent regardless of ionization technique and across red and white varieties (data not shown). Consequently, both ESI and APCI techniques seemed to be applicable to the quantitative analysis of phenolic glycosides in grape homogenate and wine samples.

Back Addition of the Phenolic Glycoside Isolate to Grape Extract or Wine Samples. The back addition experiment was carried out to investigate sample matrix dependency on the quantitation of individual glycosides (Figure 3). The same amount of the phenolic glycoside isolate was added to eight different matrices (grape extracts and wines of four different varieties: Shiraz, Cabernet Sauvignon, Chardonnay, and Riesling), followed by HPLC-MS/MS analysis with APCI or ESI. The increase in concentrations of the individual phenolic glycosides due to the isolate addition was expected to be constant across the eight different sample matrices. However, the average concentrations of the phenolic glycosides in the different matrices obtained by ESI were in poor agreement with those obtained by APCI. The concentrations of **1** and **2** after isolate addition were found to be very small, and were therefore not included in the evaluation of matrix effects. There were statistically significant differences (p < 0.05, ANOVA t-test) between the concentrations obtained by ESI and APCI for six (**3**, **4**, **6**, **3a**, **5a**, and **1b**) out of 14 glycosides. In addition, concentrations of phenolic glycosides determined by ESI varied considerably between the individual sample matrices, resulting in 11 out of 14 phenolic glycosides having a coefficient of variation (CV) of more than 40%. In contrast, the concentrations determined by APCI were consistent across the different sample matrices with a CV of nearly equal to or less than 10%, apart from **3a** (12.3%). Consequently, it was clearly demonstrated that the APCI method gave consistent quantitation results across grape and wine samples from four key varieties and accordingly was more robust and reliable for the quantitative analysis of phenolic glycosides in different matrices than the ESI method.

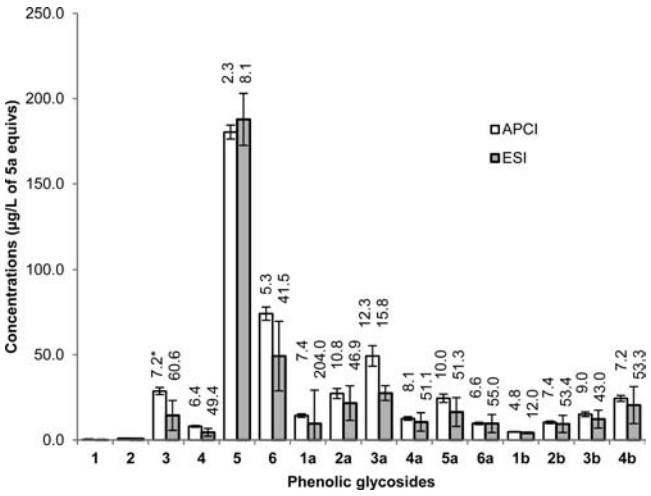

Figure 3. Increases in concentrations of phenolic glycosides measured by APCI and ESI after back addition of the phenol glycoside isolate to eight different sample matrices (Chardonnay, Riesling, Cabernet Sauvignon, and Shiraz grape homogenates and wines); *, coefficient of variation (CV, %); I, standard deviation (n = 8).

Sample matrix effect was considered to result in the discrepancy of the quantitation values obtained by ESI and APCI. Co-eluting grape extract or wine constituents could suppress or enhance the detection of phenolic glycosides. The matrix effect of coeluting materials on the detection of **5** was evaluated by the postcolumn infusion protocol.[15] The results showed that the APCI signal was comparably stable but the ESI signal varied considerably and irregularly due to the severe suppression by either grape extract or wine components (data not shown). This demonstrated that ESI was much more vulnerable to the sample matrices, which could be the reason for the unacceptable variation in the quantitation values observed in the back addition experiments.

The sample matrix can often affect the detection capability, precision, and/or accuracy for the analytes of interest. As observed in the present study, APCI is generally more robust to sample matrix effect than ESI.[16,17] Nevertheless, the confirmation of matrix effect should be considered to be an essential part of the validation protocol of HPLC-MS or MS/MS based quantitative analysis.[18] Where possible, the best option for either ionization technique to decrease the influence of matrix effect is the use of isotopically labeled internal standards for the respective analytes.[19,20] In our case, this would have involved an extensive amount of synthesis. Instead, a single labeled internal standard was chosen for quantitative analysis of all phenolic glycosides, requiring a consistent ion response for these types of compounds during an HPLC run. APCI gave stable signals of **5** within the retention time range in which phenolic glycosides eluted (data not shown). This steady response allowed for a reliable quantitative estimation of the individual phenolic glycosides in different sample matrices. Consequently, APCI was selected for further validation of the phenolic glycoside analysis method.

Quantitation Limit. Some phenolic glycosides are present as natural components in grapevines and berries,[5,6,8] and their natural abundance is likely to vary to some extent between samples from different grape varieties, growing regions, and/or climatic conditions. To avoid the interference from background levels of phenolic glycosides, d_3-**5** was used for the determination of limits of detection (LOD) and quantitation (LOQ). A series of d_3-**5** additions to red grape homogenate and wine samples was analyzed and showed linear fitted calibration

Table 1. Concentrations of Phenolic Glycosides in Control (Cc), Smoke-Affected (Cs), and Blended ($Cm_{10\%}$ and $Cm_{50\%}$) Samples Measured by HPLC-MS/MS with APCI

portion of smoke-affected sample (%)		5	6	1a	2a	3a	4a	5a	6a	1b	2b	3b	4b	Recovery mean[d]	Recovery Stdev[e]
							Shiraz Grape (μg/kg)								
0	mean[a] (Cc)	26.0	3.3	29.4	8.4	68.5	2.6	11.1	0.3	1.2	3.3	5.0	2.5		
	CV (%)[b]	2.4	5.1	3.1	5.1	2.4	12.2	5.5	8.9	9.6	0.5	0.9	6.0		
10	mean ($Cm_{10\%}$)	241.2	71.0	43.0	28.8	107.7	14.0	32.9	9.9	5.4	10.8	16.8	20.8		
	CV (%)	2.1	0.4	2.1	4.5	5.5	4.9	5.1	4.3	3.9	4.6	1.6	5.5		
	recovery (%)[c]	114.3	113.5	101.9	98.7	101.7	104.7	101.0	105.9	100.5	99.0	99.9	99.2	103.4	5.4
50	mean ($Cm_{50\%}$)	1023.9	321.3	92.9	108.6	255.1	55.7	117.7	44.6	21.3	39.9	64.9	94.4		
	CV (%)	0.9	8.1	2.5	4.1	1.0	2.8	5.5	2.7	2.0	5.3	3.2	5.4		
	recovery (%)	107.7	107.2	99.2	96.7	99.7	98.3	99.4	97.8	96.1	96.7	101.1	99.7	100.0	3.8
100	mean (Cs)	1875.2	596.0	157.9	216.2	443.3	110.8	225.8	90.8	43.0	79.2	123.4	186.7		
	CV (%)	1.5	8.0	2.7	0.9	2.0	2.0	1.4	1.6	1.1	3.2	4.0	0.1		
							Cabernet Sauvignon Wine ((μg/L)								
0	mean (Cc)	16.4	0.3	41.1	5.1	14.8	1.0	8.3	0.2	0.9	2.0	1.6	1.4		
	CV (%)	2.8	39.3	11.1	14.6	8.8	30.9	4.0	96.9	17.8	9.0	20.1	23.4		
10	mean ($Cm_{10\%}$)	156.4	50.1	46.8	20.2	45.7	8.6	31.4	7.5	5.4	11.3	9.4	13.8		
	CV (%)	7.4	11.7	9.2	8.2	11.0	11.3	13.0	3.5	14.1	8.8	18.7	7.7		
	recovery (%)	93.2	93.0	83.3	92.6	92.2	92.2	93.6	111.5	91.9	92.6	92.7	90.9	93.3	6.3
50	mean ($Cm_{50\%}$)	796.8	267.4	121.3	91.0	190.0	45.1	138.3	36.2	27.3	54.7	48.1	69.5		
	CV (%)	3.1	12.6	7.1	10.0	1.1	12.3	8.3	14.3	4.3	10.8	6.8	10.8		
	recovery (%)	103.0	99.9	104.0	103.1	100.6	106.1	102.8	110.9	105.6	103.6	108.4	99.2	103.9	3.4
100	mean (Cs)	1531.2	534.9	192.1	171.4	362.9	84.0	261.0	65.2	50.8	103.6	87.3	138.7		
	CV (%)	1.0	4.4	0.5	1.4	3.6	5.2	2.3	8.0	4.9	3.7	2.1	5.3		

[a] $n = 3$. [b] Coefficient of variation. [c] $= Cm_{10\%}/Ce \times 100\% = Cm_{10\%}/(0.1 \times Cs + 0.9 \times Cc) \times 100\%$ for the 10% blended sample, Cm = $Cm_{10\%}$ or $Cm_{50\%}$, Ce = expected concentration calculated by Cs and Cc. [d] Mean value of all glycoside recoveries. [e] Standard deviation.

functions ($R^2 > 0.999$) ranging from 0.05 to 10 μg/kg for grape homogenate and from 0.1 to 10 μg/L for wine. The LOD for d_3-**5** was estimated as low as 0.05 μg/kg for grape and 0.1 μg/L for wine, with S/N ratios of greater than three. The response factor (ratio of peak area to concentration) was comparably consistent in the range of 0.25−10 μg/kg for grape homogenate and 0.5−10 μg/L for wine, S/N ratios (mean value of triplicates) at 0.25 μg/kg for grape and 0.5 μg/L for wine were greater than 10, and the respective peak areas varied with CV values of approximately 10%. Accordingly, the LOQ of the HPLC-MS/MS method was determined to be 0.25 μg/kg for grape homogenate and 0.5 μg/L for wine.

Reproducibility and Recovery (Table 1). Previous studies had shown that the presence of some gentiobiosides (i.e., **1**, **2**, **3**, and **4**) in smoke-affected grapes and wines is relatively small in the varieties studied with the exception of Shiraz,[6] therefore these gentiobiosides were not included in this experiment. To make a sample set with a wide range of concentrations for the targeted phenolic glycosides, smoke-affected samples were blended with nonsmoked samples (controls) from the respective varieties with a portion of the smoked sample at 10% or 50% (by volume). Shiraz grape homogenate samples ($n = 3$) exhibited a small magnitude of variation between the concentration with a CV of less than 10%, apart from **4a** (12.2%) for the 0% sample (i.e., unblended control grape sample). In the Cabernet Sauvignon wine samples, 41 out of 48 concentrations measured had a CV of less than 15%; five data points (**6**, **4a**, **6a**, **3b**, and **4b**) in the 0% sample varied significantly with a CV of more than 20%. The Chardonnay grape homogenate and wine samples (data not shown) showed a similar trend to the red varieties, where most of the concentrations were relatively consistent with a CV of less than 15%, apart from data sets for the 0% grape and wine samples which had higher CV values. However, results with higher CV values were confined to the 0% samples (in other words, 100% nonsmoked control samples) which contained phenolic glycosides in very small amounts. For example, low concentrations of less than 2 μg/L for **6**, **4a**, **6a**, **3b**, and **4b** in the 0% Cabernet Sauvignon wine that were close to the LOQ determined using d_3-**5** had CV values of more than 20%. In

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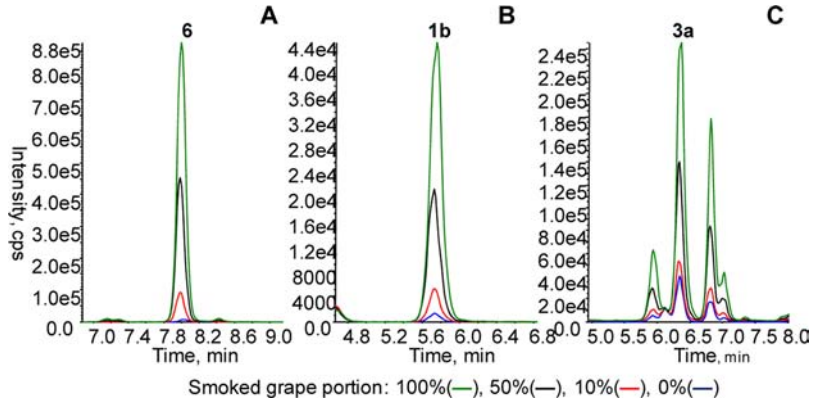

Figure 4. Overlaid SRM chromatograms of (A) **6**, (B) **1b**, and (C) **3a** in control, smoke-affected, and blended Shiraz grape homogenates analyzed by HPLC-MS/MS with APCI.

summary, the CV data determined with d_3-**5** as a single internal standard demonstrated good reproducibility for all phenolic glycosides across a wide range of concentrations that would be typical for smoke-affected grape or wine samples.

To assess the recovery across a range of concentrations, we compared control and smoke-affected grape or wine samples with composite samples generated through blending. The peak intensities of all phenolic glycosides showed a relative increase that reflects the portion of the smoked sample used for blending (Figure 4). The recoveries of the 12 phenolic glycosides in the 10% and 50% blended samples were 103.4 ± 5.4% (mean ± standard deviation) and 100.0 ± 3.8%, respectively, for Shiraz grape homogenate and 93.3 ± 6.3% and 103.9 ± 3.4%, respectively, for Cabernet Sauvignon wine. The Chardonnay 10% and 50% blended grape samples (data not shown) also showed good recoveries of 99.7 ± 12.9% and 106.6 ± 5.4%, respectively, for grape homogenate and 99.5 ± 3.9% and 104.2 ± 4.5%, respectively, for wine. Overall, when accounting for the respective concentrations in the blended proportions, the concentrations of all glycosides in the 10% and 50% blended grape and wine samples showed good agreement with those calculated for the 0% (control) and 100% (smoke-affected) samples.

Comparison to the Existing Methods. Basic differences between the present method and existing methods[6,8,9] were the use of labeled syringol gentiobioside (d_3-**5**) and guaiacol monoglucoside as internal standard and Synergi Hydro-RP and Gemini C6-Phenyl HPLC columns, respectively. The present method expresses the concentration of the most abundant phenolic glycoside **5**[6] found in smoke-affected grapes and wine as itself, instead of guaiacol monoglucoside equivalents using the existing methods. In addition, all target phenolic glycosides for quantitation were disaccharides (Figure 1), therefore the use of d_3-**5** was deemed more appropriate due to their similar molecular masses and structures compared to the labeled monoglucoside. In fact, disaccharide concentrations determined as **5** equivalent were 1.2−1.5 times higher than those expressed as guaiacol monoglucoside equivalent (data not shown), possibly resulting from differences in molecular mass ratio of **5** to guaiacol monoglucoside (478/286 = 1.67) and ion response factor between them. Consequently, **5** equivalents represent more closely actual concentrations of **5** as well as other disaccharides.

Both HPLC columns performed similarly, with the elution of phenolic glycosides in a period of approximately 3−10 min. The Synergi Hydro-RP column eluted phenolic glycosides up to 1.3 min for **4b** earlier than the Gemini C6-Phenyl column,[6] while their elution orders were virtually the same. In our experience, the Gemini C6-Phenyl seemed to be slightly more vulnerable to matrix effect (data not shown), so the Synergi Hydro-RP column was selected for the present study.

Application of a HPLC-MS/MS Method to Analysis of Phenolic Glycosides in Grapes Exposed to Bushfire Smoke. To investigate the composition of phenolic glycosides in a range of grape samples which were suspected of smoke exposure of different intensities and/or durations, Chardonnay, Pinot Noir, Cabernet Sauvignon, and Shiraz grapes (three different samples for each variety) which had been exposed to smoke from bushfires in 2009 were subjected to the validated phenolic glycoside analysis. The mean relative abundance (n = 3) of individual glycosides to the total phenolic glycoside pool (sum of all glycosides measured) is shown in Figure 5. The concentration of the glycosides varied considerably between the samples independent of variety, ranging from 330 to 4310 μg/kg as total glycosides (data not shown), most likely due to the individual grapevines being exposed to different intensities and/or durations of smoke. In spite of the wide range of concentrations and the likely variation in smoke exposure, the relative profile of the glycosides was consistent between the varieties with the exception of higher proportions of **6** in Cabernet Sauvignon, and **3** and **3a** in Shiraz. Syringol gentiobioside, **5**, was the most abundant glycoside, followed

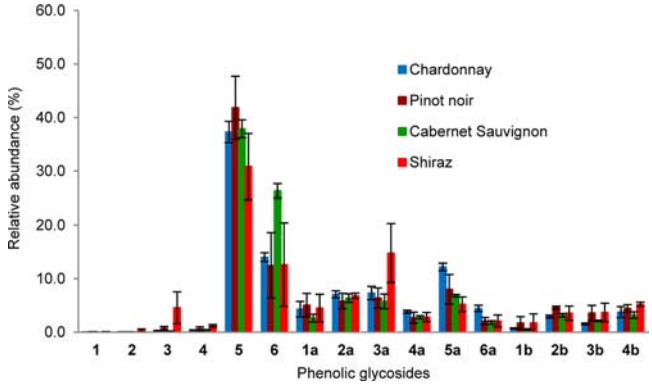

Figure 5. Relative abundance (%) of the individual phenolic glycosides to the total glycosides in smoke-affected grape homogenates; total glycosides, sum of all the glycosides measured; I, standard deviation (n = 3).

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Table 2. Correlation Matrix for Phenolic Glycoside Composition Data Obtained from 12 Different Smoke-Affected Grape Samples

phenolic glycosides	5	6	1a	2a	3a	4a	5a	6a	1b	2b	3b	4b	total[a]
5	1.000	0.846	0.848	0.943	0.758	0.911	0.860	0.800	0.579	0.963	0.841	0.906	0.989
6	0.846	1.000	0.495	0.845	0.597	0.821	0.702	0.623	0.166	0.805	0.556	0.691	0.872
1a	0.848	0.495	1.000	0.823	0.747	0.752	0.752	0.723	0.749	0.799	0.831	0.829	0.827
2a	0.943	0.845	0.823	1.000	0.846	0.975	0.876	0.873	0.388	0.863	0.709	0.911	0.975
3a	0.758	0.597	0.747	0.846	1.000	0.808	0.586	0.695	0.467	0.698	0.755	0.926	0.821
4a	0.911	0.821	0.752	0.975	0.808	1.000	0.921	0.943	0.299	0.828	0.624	0.896	0.947
5a	0.860	0.702	0.752	0.876	0.586	0.921	1.000	0.952	0.348	0.801	0.539	0.748	0.859
6a	0.800	0.623	0.723	0.873	0.695	0.943	0.952	1.000	0.268	0.711	0.495	0.809	0.827
1b	0.579	0.166	0.749	0.388	0.467	0.299	0.348	0.268	1.000	0.668	0.879	0.571	0.510
2b	0.963	0.805	0.799	0.863	0.698	0.828	0.801	0.711	0.668	1.000	0.872	0.854	0.941
3b	0.841	0.556	0.831	0.709	0.755	0.624	0.539	0.495	0.879	0.872	1.000	0.850	0.811
4b	0.906	0.691	0.829	0.911	0.926	0.896	0.748	0.809	0.571	0.854	0.850	1.000	0.930
total[a]	0.989	0.872	0.827	0.975	0.821	0.947	0.859	0.827	0.510	0.941	0.811	0.930	1.000

[a]Sum of all glycosides.

by **6** and the pentosylglucosides and the rutinosides to a lesser extent. The gentiobiosides other than **5** and **6** were either not detected or detected only in trace amounts, except for **3** in the Shiraz grape homogenates, as previously mentioned. Statistical analysis of compositional data obtained from the different smoked samples (n = 12) was used to generate a correlation matrix (Table 2). All the glycoside concentrations were highly correlated with those of the total glycosides, with a correlation coefficient of more than 0.8, apart from **1b** (0.5). The correlation between the individual phenolic glycosides was generally good, with a correlation coefficient of better than 0.7, with some exceptions, particularly **1b**, which was highly correlated with only **1a** (0.749) and **3b** (0.879). This indicates that the metabolic pathway(s), or its regulation, for phenol and/or **1b** may significantly differ from the glycosylation reactions that take place with the other phenolic substrates.

Together, the results demonstrated that the volatile phenols taken up by grapes following smoke exposure were generally metabolized in a similar fashion regardless of grape variety, and concentrations of individual phenolic glycosides were all related to the total pool of phenolic glycosides measured. This further corroborated the suitability of phenolic glycoside grape metabolites as markers for smoke exposure.

Smoke Diagnostic Assay. To further confirm the concept that these glycosidic grape metabolites can be used as diagnostic markers to detect smoke exposure, we compared results from analysis of the established marker, free guaiacol,[1,21] with concentrations of phenolic glycosides in grapes. Shiraz (n = 3), Cabernet Sauvignon (n = 2), Pinot Noir (n = 1), and Chardonnay (n = 1) grapes suspected of smoke exposure from the 2009 bushfires, and Shiraz (n = 2), Cabernet Sauvignon (n = 1), Pinot Noir (n = 2), Chardonnay (n = 2), and Riesling (n = 1) control grapes with no history of smoke exposure were analyzed for free guaiacol and phenolic glycosides. In control grapes (n = 8), free guaiacol was present at a mean concentration of 0.9 μg/kg with a standard deviation of ±1.2 μg/kg and an upper limit of guaiacol concentrations was statistically determined to be 3.4 μg/kg (i.e., the mean plus two standard deviations giving a 95% confidence). Grapes suspected of smoke exposure (n = 7) contained guaiacol at concentrations ranging from 0 to 5 μg/kg, and only two samples were higher than the upper limit of control grapes. Guaiacol concentrations of the remaining samples lay within 95% of a normal distribution of control grapes, therefore they could not be judged as smoke-affected based on guaiacol analysis alone. On the other hand, the phenolic glycoside analysis exhibited clear differences between the suspect and control grape samples, as shown in Figure 6A (using **5** and **6** as examples). In the cases of

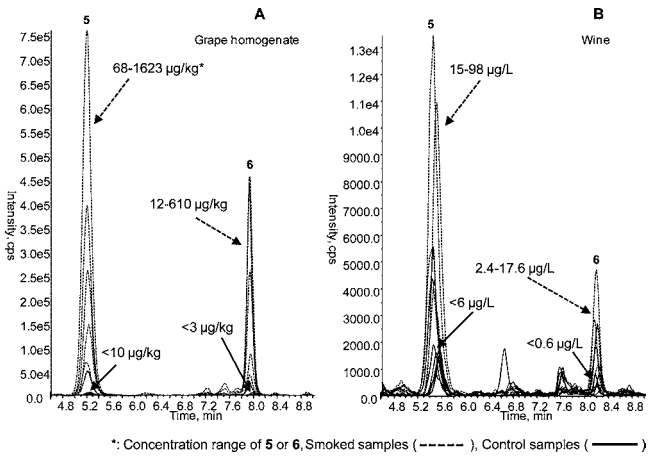

Figure 6. Overlaid SRM chromatograms of **5** and **6** in (A) smoke-affected and control grape homogenates and (B) wine samples monitored by HPLC-MS/MS with APCI.

5 and **6**, their mean concentrations and standard deviations (n = 6) in control samples were 3.6 ± 4.1 and 1.0 ± 1.1 μg/kg, respectively. Concentrations of **5** and **6** in the suspect samples varied considerably, ranging from 68 to 1623 μg/kg and from 12 to 610 μg/kg, respectively. These values were significantly higher than the upper limits with a 95% confidence for **5** (11.8 μg/kg) and **6** (3.2 μg/kg) in control grapes. Other phenolic glycosides were also found at elevated concentrations to various extents which were higher than the upper limits of the respective glycosides apart from two cases (data not shown). This demonstrated that the phenolic glycoside analysis was more capable than the guaiacol analysis as a diagnostic assay for the differentiation between smoke-exposed and control grapes.

To evaluate the effect of smoke exposure on wine, Shiraz (n = 4) and Cabernet Sauvignon (n = 2) wines made from grapes suspected of smoke exposure were selected and compared to the control wines from the same variety (n = 3 each) (Figure 6B). These suspected smoke-affected wines exhibited little or no smoke-related character assessed by informal bench tasting

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and contained guaiacol at concentrations ranging from 4 to 20 μg/L, and only three of the six wines had higher guaiacol concentrations than the upper limit of 14.7 μg/L for the control wines (6.5 ± 4.1 μg/L).

Phenolic glycoside analysis, however, showed that **5** and **6** were found in the suspected wines at concentrations ranging from 15 to 98 μg/L and from 2.4 to 17.6 μg/L, respectively, which were all greater than the upper limits of **5** (6.6 μg/L) and **6** (0.7 μg/L) in the control wines. Most of the pentosylglucosides and rutinosides in the suspect wines were also found at elevated concentrations higher than the upper limits of the respective glycosides in control wines (data not shown). It was therefore demonstrated that phenolic glycosides were also more suitable marker compounds in wine to indicate smoke exposure than guaiacol; judging these suspected smoke affected wines by considering the concentration of phenolic glycosides would suggest all six wines were smoke-affected, whereas considering the concentration of free guaiacol alone would suggest only three wines of the six were smoke-affected.

The quantitation of phenolic glycosides in grape homogenates and wines provided a significant improvement in the ability to distinguish between nonsmoked (clean) and smoke-exposed samples compared to the existing guaiacol (and 4-methylguaiacol) analysis. Accordingly, the method is more suitable as a diagnostic strategy to identify smoke exposure and to assess the likely impact of smoke exposure in grapes and the resulting wines.

Because concentrations of most glycosides were elevated and closely correlated following smoke exposure in all varieties studied so far, sufficient information on smoke exposure may be obtained by measuring only **5**, as the most relatively abundant glycoside, or from a small number of glycosides. The advantage of measuring a broad range of phenolic glycosides, however, is to give a more thorough picture of the smoke exposure. Also, more data from a broader range of smoke events would be required to narrow down smoke markers for routine diagnostic purposes.

Finally, a comprehensive survey (large sample numbers and a broad range of varieties and grape-growing regions) of the baseline concentrations of phenolic glycosides in control samples of nonsmoked grapes and wines is under way to improve the reliability of the phenolic glycoside analysis as a diagnostic tool.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods for synthesis of d_3-syringyl-β-D-gentiobioside (d_3-**5**) and matrix effect and figure showing matrix effect. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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