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Quantitating Volatile Phenols in Cabernet Franc Berries and Wine after On-Vine Exposure to Smoke from a Simulated Forest Fire

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Supporting Information

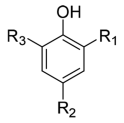
ABSTRACT: Smoke-taint is a wine defect linked to organoleptic volatile phenols (VPs) in *Vitis vinifera L.* berries that have been exposed to smoke from wildland fires. Herein, the levels of smoke-taint-associated VPs are reported in Cabernet Franc berries from veraison to commercial maturity and in wine after primary fermentation following on-vine exposure to simulated wildland fire smoke. VPs increased after smoke exposure were rapidly stored as acid-labile conjugates, and the levels of both free VPs and conjugated forms remained constant through ripening to commercial maturity. An increase in total VPs after primary fermentation suggested the existence of VP-conjugates other than the acid-labile VP-glycosides already reported. This conclusion was supported with base hydrolysis on the same samples. Relative to published results, the data suggested a multifactorial regional identity for smoke-taint and they inform efforts to produce a predictive model for perceptible smoke-taint in wine based on the chemical composition of smoke-exposed berries.

KEYWORDS: *Vitis vinifera, smoke, volatile phenol, wine, smoke taint, glycoside*

INTRODUCTION

Volatile phenols (VPs) are organoleptic compounds that are found in *Vitis vinifera L.* (*V. vinifera*) berries and wine, originating from both endogenous and/or exogenous sources. Endogenous VPs are plant secondary metabolites involved in host stress response and reproduction,[1,2] whereas exogenous VPs can originate from: (1) the barrel aging of wine,[3] where the increased VP concentrations impart desirable sensory attributes; (2) biosynthesis during fermentation[4] by *Brettanomyces bruxellensis*, which results in a wine defect known colloquially as "Brett-taint";[5] or (3) the exposure of *V. vinifera* berries to smoke from wildland fires or prescribed burns. When smoke-exposed berries are fermented, the resulting wine may possess negative sensory attributes (e.g., "smoky", "ashy", "burnt meat", and "Band-Aid" aromas) or a paucity of varietal characteristics that, collectively, comprise a wine defect known as smoke taint.[6−9] The impact of smoke-exposure to the wine industry is (and will remain) significant, as smoke-taint has been reported in North and South America, Australia, and South Africa, all of which are important wine growing regions where the frequency of wildland fires are projected to increase.[10]

The exogenous VPs observed in smoke-exposed berries and/or the wines produced from these berries have been hypothesized to originate from the thermal degradation of lignin during wildland fires. The three monolignol subunits (sinapyl, *p*-coumaryl, and coniferyl alcohols) can decompose to yield a variety of syringyl, alkylhydroxyphenyl, and guaiacyl VPs (Figure 1). Since the ratio of monolignols changes as a function of plant species, the combustion of different fuel sources will expose berries to different VP ratios. For example, Kelly et al. showed that the combustion of *Pinus radiata* yielded different VP ratios in smoke than *Eucalyptus* species.[11] This suggested that smoke-taint may possess a regional identity based in part on the flora available for combustion during a wildland fire.



compound	R_1	R_2	R_3
4-ethylguaiacol	OMe	Et	H
4-ethylphenol	H	Et	H
4-methylguaiacol	OMe	Me	H
guaiacol	OMe	H	H
eugenol	OMe	C_3H_5	H
o-cresol	Me	H	H
p-cresol	H	Me	H
syringol	OMe	H	OMe

Figure 1. A sample of the VPs reported in *V. vinifera* berries and wine after on-vine exposure of berries to wildland fire smoke.

Such regionality could impact the performance of algorithms used to predict the potential for smoke-exposed berries to produce smoke-tainted wine. As most of the published data on smoke-taint comes from Australia, where wildland fires generally consume grasses and *Eucalyptus* trees, an evaluation of fuel sources relevant to other wine growing regions is required. Additionally, while Kelly et al. looked at different fuel sources, they did not evaluate the composition of the smoke-

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exposed berries prior to fermentation.[11] This leaves a critical knowledge gap in the movement of VPs from smoke into berries and wine, especially since Kelly et al. observed VPs in wine that were not detected in their experimentally produced smoke.

In planta and in wine, VPs are present in their free, volatile forms, but more dominantly they have been reported as an array of glycoconjugates.[12] Kennison et al. analyzed VP concentrations in wine made from berries that were exposed to smoke at various times following the onset of veraison[13] and at different phenological stages.[14] However, they only looked at two or three VPs and did not look at the VP concentrations in berries prior to fermentation in either study. Indeed, with one exception,[6] most studies focus on the concentration of VPs in wine rather than in berries, since evaluating wine permits the direct correlation of VP concentrations with specific sensory characteristics.[13−15] Alternatively, Dungey et al. looked at the change in guaiacyl-glycosides from the time of smoke-exposure until harvest, but did not evaluate other VP-glycosides.[16] Since free VPs can be liberated during fermentation, with subsequent impact to the sensory characteristics of the resulting wine,[8] the "sensory potential" of glycosidically bound VPs is a critical component of smoke-taint. Accounting for all VP-glycosides is, therefore, necessary when assessing the potential for smoke-exposed berries to produce smoke-tainted wine.

Our research group recently published a validated analytical method to accurately quantitate free VPs and the VPs released following acid hydrolysis (e.g., VP-glycosides), with the goal of developing a model to predict the probability of producing smoke-tainted wines from smoke-exposed berries based on the chemical composition of the berries prior to fermentation.[17] In the present study, we demonstrate the application of these methods to the analysis of smoke-exposed Cabernet Franc berries and matched controls from the same vineyard, where the smoke was generated from a regionally relevant fuel source. We report the levels of free and bound VPs (as determined by acid hydrolysis) at multiple time points, including immediately preceding and postsmoke exposure, through to commercial maturity and to the end of primary fermentation. To the authors' knowledge this is the first study to address such temporal data with demonstrably accurate methods for assessing a wide range of VPs (free and acid-labile). We hypothesize that the detailed elucidation of the time-dependent VP metabolic flux will aid in the development of predictive risk-assessment models in the event of smoke exposure.

MATERIALS AND METHODS

Chemical and General Details. The following chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and used as received: HPLC-grade methanol (MeOH), isopropanol (IPA), acetonitrile (ACN), carbon disulfide, hexane, ethyl acetate (EtOAc), hydrochloric acid, 2-methoxyphenol (guaiacol), 2,6-dimethoxyphenol (syringol), 2-methoxy-4-methylphenol (4-methylguaiacol), 2-methoxy-4-ethylphenol (4-ethylguaiacol), 4-ethylphenol, 2-methoxy-4-allyl-guaiaicol (eugenol), 4-methylphenol (*p*-cresol), and 2-methylphenol (*o*-cresol). The internal standards d_7-*o*-cresol and d_7-*p*-cresol were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Details for the synthesis of d_3-syringol and the model glycosides (guaiacyl-*β*-*O*-D-glucopyranoside, *p*-cresyl-*β*-*O*-D-glucopyranoside, syringyl-*β*-*O*-D-glucopyranoside, syringyl-gentiobioside, 4-ethylphenyl-*β*-*O*-D-glucopyranoside, eugenyl-*β*-*O*-D-glucopyranoside) are reported elsewhere.[17] Synthetic details for d_3-guaiacol-*β*-D-glucopyranoside are summarized in the Supporting Information. Unless otherwise noted water was provided by a Barnstead E-Pure water purification system (Thermo Fisher Scientific; Waltham, MA, USA). Weighing was performed using an Adventure Pro AV264 analytical balance (Ohaus Corporation, Pinebrook, NJ, USA). A Mettler Toledo FE20 FiveEasy pH meter was used to measure pH. An Allegra X-12R centrifuge was used for sample preparation (Beckman Coulter, Mississauga, ON, Canada).

Stock and Calibration Solutions. Concentrated VP and isotopic internal standard (ISTD) stock solutions were prepared in IPA (d_3-syringol in MeOH) at 1.0−10.0 mg/mL. An ISTD intermediate stock solution was prepared at 5 μg/mL in IPA for free VPs and 30 μg/mL in IPA for VP-glycosides. These solutions were added to calibration samples and extracts with a 100-fold (v/v) dilution as required. A 100 μg/mL intermediate calibration stock solution for GC-MS/MS was prepared in IPA. Calibration samples from 1−1000 ng/mL were prepared fresh daily by diluting the intermediate stock solution into 1:1 hexane:ethyl acetate. ISTD was added to each vial and the calibration samples were analyzed without further workup. Model glycoside stock solutions were prepared in IPA at 1 mg/mL. All stock solutions were stored at −20 °C.

Study Design and Smoke Exposure. Ten Cabernet Franc vines (clone 214, rootstock 204, planted in 2005, located near 49.1233° N, 119.5509° W) were split into five control plants and five plants that were exposed to experimentally produced smoke (*vide infra*), with the two groups separated by one panel of vines. Two bunches of berries were collected per vine for 5 time points from immediately preceding smoke application (∼7 days post-veraison) until commercial maturity (23.5 ± 1.15°Bx; Table S1). Samples were stored in separate polyethylene bags on ice prior to processing. In each test group (control and smoke-exposed) only four of five Cabernet Franc vines yielded sufficient berries to permit vinification after completing the analysis of berries collected at each time-point following smoke exposure.

Fuel for the generation of smoke was collected from *Pinus ponderosa* (*P. ponderosa*) forests located near 49.9398° N, 119.3968° W and 49.8183° N, 119.4373° W. An amalgamated *P. ponderosa* fuel source was prepared by combining soil organic matter (SOM; 50% w/w), needles (20% w/w) and bark (30% w/w; cut into 3 cm pieces). This fuel mixture was stored in a polyethylene bag under ambient conditions.

Vines were exposed to smoke for 60 min using a purpose-built, modular enclosure comprised of PVC/steel structural elements and a translucent polyethylene cover (Figure S1). The enclosure was 18.3 m^3 (1.1 m × 1.8 m × 9.1 m [$w \times h \times l$]). Smoke from our amalgamated fuel source was produced in a Masterbuilt Pro commercial food smoker (Masterbuilt Manufacturing; Columbus, GA, USA) and directed from the smoker exhaust vent into the modular enclosure via flexible aluminum ducting containing an inline fan (0.003−0.005 m^3/s); the fan speed was adjusted as necessary to maintain consistent smoke density within the enclosure. Smoke density ($PM_{2.5}$), relative humidity and temperature in the enclosure were monitored using an AirBeam wearable air monitor (HabitatMap; NY, USA). Smoke was sampled in triplicate using Orbo 33 activated petroleum carbon (Sigma-Aldrich, 700 mg/390 mg, 8 × 150 mm), with the identical primary and secondary sorbent beds separated by a foam insert and each end of the sorbent beds contained with glass wool. Sampling was done at 250 mL/min for 60 min using a Gilian GilAir-3 sampling pump (Sensidyne, LP; FL, USA). Sorbent tubes were stored at 4 °C until analysis. The temperature inside the smoke enclosure did not exceed 30 °C and the relative humidity stayed above 25% (Figure S2). Smoking was done early in the day (∼6:00 am), approximately 7 days after the onset of *veraison*, which was determined by the vineyard. Control vines were not enclosed in the smoking structure (without smoke) due to a strong residual smoke aroma in the polyethylene cover after use.

Sample Preparation and Analysis. Samples were analyzed for free VPs using gas chromatography tandem-mass spectrometry (GC-MS/MS) and for intact VP-glycosides using ultrahigh performance liquid chromatography-quadrupole time-of-flight mass spectrometry (uHPLC-QToF). Method details are summarized in the (Supporting Information Tables S2−S5).

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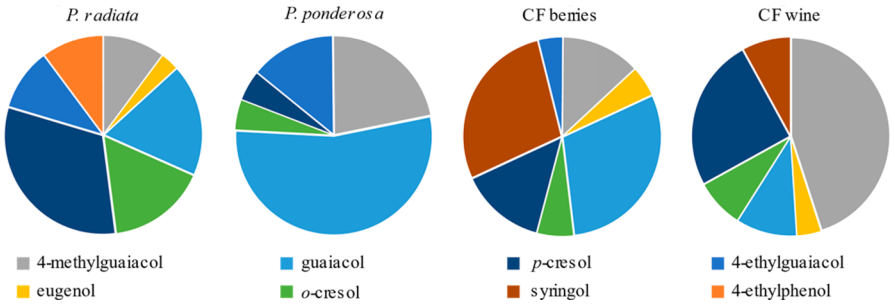

Figure 2. Relative levels of VPs identified in the smoke generated from the combustion of *P. radiata*,[11] the amalgamated *P. ponderosa* fuel source used herein, as well as smoke-exposed CF berries and wine. The *P. ponderosa* ratios were based on the raw instrument response as a fraction of the response for all detected VPs, while the CF data was taken from the total VPs quantified in smoke-exposed CF berries at commercial maturity and in wine made from the same berries.

On the same day as sample collection, berries were stemmed, a subset of 100 berries was weighed and the degrees brix (°Bx, n = 4 berries/vine) was recorded using an Atago PAL-1 refractometer (Atago USA, Inc.; Bellevue, WA, USA). Also on the same day as sample collection, berries were prepared as whole berry homogenate (HMG) by homogenizing whole berries in a Black & Decker BL2010BGC commercial blender (Stanley, Black & Decker; New Britain, CN, USA) for 60 s. Prior to preparation as HMG, samples from t_0 − t_2 (Table S1) were divided into subsets, with one set untreated and the other washed with gentle agitation in tap water for 30 s. All samples were stored at −20 °C in 50 mL polypropylene tubes (Sarstedt; Nümbrecht, Germany) after preparation for up to four months prior to analysis. Berries intended for vinification were stemmed and stored at −20 °C in polypropylene bags for up to 12 months.

The primary and secondary Orbo 33 sorbent beds were collected into separate 4 mL glass vials. Each vial received 2 mL of carbon disulfide and was incubated under ambient conditions for 60 min, with periodic agitation after the addition of 50 ng/g VP ISTD. The resulting extract was analyzed for VPs using targeted GC-MS/MS.

HMG extracts of free and acid-labile VPs for GC-MS/MS were prepared according to Noestheden et al.[17] Wine extracts were prepared the same way, except the extract was prepared in a 1:1 ratio (sample:extraction solvent) rather than a 5:2 ratio. The bound fraction of VPs was calculated from the difference between the free and total VPs (i.e., the VPs measured after acid hydrolysis) for all samples.

Base hydrolysis was performed on HMG, wine and 1 μg/mL model glucosides by adding 1 mL of 1 M NaOH to 5 mL of sample. After 4 h at 100 °C the base hydrolyzed samples were neutralized with 2 mL of 1 M NaH_2PO_4. Samples intended for stability studies had ISTD (VP or VP-glycoside) added after digestion. All other base hydrolyses were conducted with ISTDs added prior to digestion.

Yeast. Yeast cultures were prepared by streaking-plating yeast (strain EC1118) onto solid YEPD media (10% yeast extract, 10% bacterial peptone, 20% dextrose, 20% agar), followed by incubation at 28 °C for 48 h, then storage at 4 °C. A subsample of the yeast culture was collected with a sterile spatula and suspended in sterile water. The yeast culture was then adjusted to 2.0 absorbance units at 600 nm by dilution with sterile water.

Microvinification. Microscale wines for the control and smoke-exposed CF berries (n = 1) were prepared as follows. Erlenmeyer flasks (500 mL) were sanitized by exposure to 20 mL of 5% sodium metabisulfite solution for 1 h and rinsed thoroughly with water. For each of the berry samples from control and smoke-exposed vines, 100 g aliquots of frozen berries were weighed into a sanitized Erlenmeyer flask and 5 mL of a 1% sodium metabisulfite solution was added. The berries were thawed for 24 h at 4 °C, crushed with a spatula and, where necessary, the pH of the juice was adjusted to 3.6−3.8 by the addition of 10% aqueous tartaric acid (<500 μL). The resulting musts were inoculated with yeast culture (1 mL, adjusted to 2.0 AU at 600 nm). Flasks were then fitted with air locks and fermentations were allowed to proceed with twice daily agitation until mass loss stabilized (*ca.* 14 d). Fermentation was halted by removing yeast cells by centrifugation (3000*g* for 15 min). The clarified wines were then stored in glass at 4 °C under argon prior to analysis.

Method Performance. Calibration curves were fit using linear or quadratic regression and inverse concentration weighting. The limit of detection (LOD) and limit of quantitation (LOQ) calculations were adapted from Evard et al. as[18]

$$\mathrm{LOD}_i/\mathrm{LOQ}_i = k \times s_n \quad (1)$$

Where s_n was the standard deviation of n spike-recovery extracts for compound i, and k was a multiplier equal to 3.3 for the LOD and 10 for the LOQ. Accuracy and precision for the analysis of VPs in wine were evaluated by fortifying model wine (MW; for contents see Table S6) at 5, 20, and 200 ng/g (n = 5/concentration). Recoveries were corrected for a MW density of 0.98 g/mL (empirically determined).

Data Acquisition and Processing. GC-MS/MS data was processed using the Xcalibur (version 3.0.63) and TraceFinder (version 3.2.512.0) software packages (Thermo Scientific). The uHPLC-QToF data acquisition and processing were carried out using the MassHunter Workstation software suite (Agilent Technologies), with version numbers as follows: Data Acquisition Workstation (v B.08.00) and Qualitative Analysis (v B.07.00, Service Pack 2). Data reduction and statistical calculations were performed using Microsoft Excel (Microsoft Corporation; Redmond, WA, USA) and BoxPlotR.[19] Where relevant, data are displayed ±1 standard error of the mean (SEM). Unless noted, all statistical comparisons were done using a Mann−Whitney U test (α = 0.05).

RESULTS AND DISCUSSION

Simulating Wildland Fire Smoke. There are three types of wildland fires: (1) ground fire, where soil organic matter (SOM) is consumed; (2) surface fire, where short vegetation and SOM are consumed; and (3) crown fire, where tall trees and shrubs are consumed largely independently from SOM.[20,21] All three types may coexist during a given wildland fire and the occurrence of one over another is a complex interaction between weather patterns, moisture, ignition source, type of forest, proximity to urban centers, etc.[22] Given this complexity, the simulation of wildland fire smoke in the present study was achieved using an amalgamated fuel containing *P. ponderosa* bark (30% w/w), needles (20% w/w), and SOM (50% w/w). This approach deviated from the widely referenced study by Kennison et al., who selected barley straw to produce smoke with a consistent composition, relative to that produced during the combustion of native vegetation.[9] As the lignin composition of barley straw (17%)[23,24] is less than wood (23−45%),[11] and as the ratio of monolignol subunits in barley and wood differ, the VPs produced from each fuel source under a given set of combustion parameters are likely to change.[11] Given the prevalence and proximity of ponderosa pine forests to vineyards

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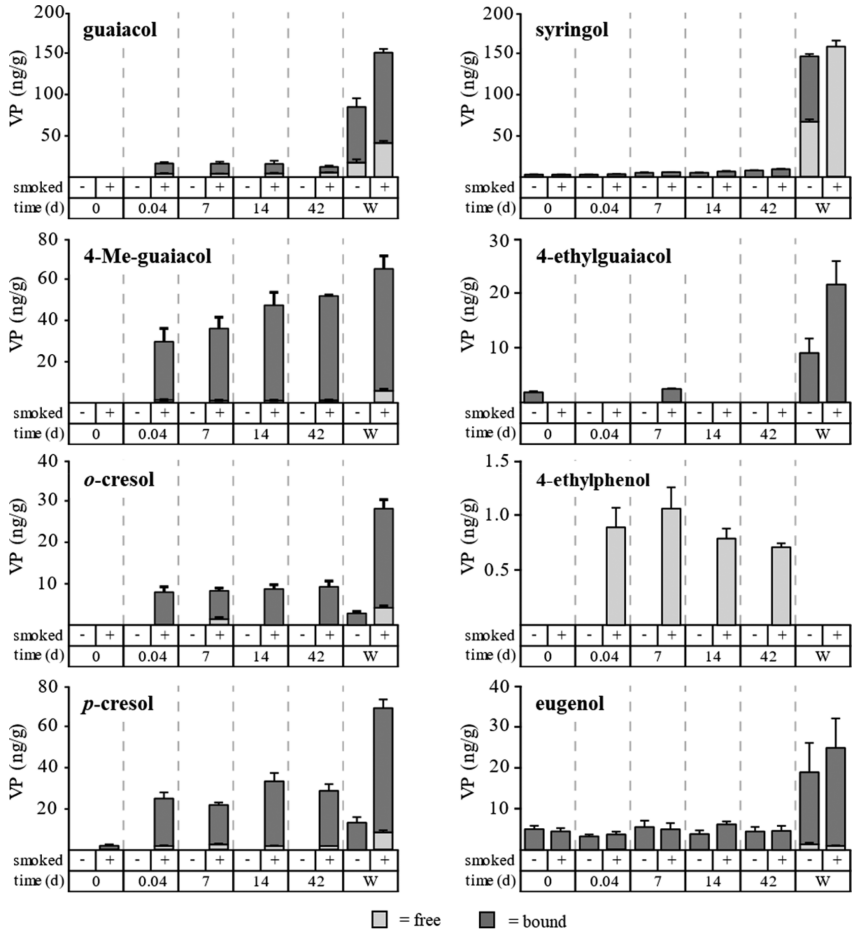

Figure 3. Total VPs expressed as free (light gray) and bound (dark gray, as determined by acid hydrolysis.) forms in Cabernet Franc berries from immediately preceding on-vine smoke exposure (0 d) until commercial maturity (42 d) and to the end of primary fermentation (W). Data are reported as the mean ±1 SEM and were tabulated from up to five biological replicates (see Table S8). With the exception of syringol, the levels of both free and bound VPs differed significantly (Mann−Whitney U test, $\alpha = 0.05$) between control (−) and smoke-exposed (+) groups.

in the western United States and Canadian wine growing regions, it was hypothesized that our amalgamated *P. ponderosa* fuel source would be more relevant to North America. Sheppard et al.[25] used *P. ponderosa* to study smoke-taint, but they used a chipped and homogenized fuel containing all parts of the tree and omitted SOM. In summary, an amalgamated fuel will provide a better approximation of the variability in VPs produced during a given wildland fire event, although, it should be noted that none of the approaches used to date (including the one used herein), fully account for environmental influences or the variability in the lignin composition of SOM[26−29] as they relate to the generation of VPs in smoke.

Targeted GC-MS/MS analysis of the smoke generated from our *P. ponderosa* fuel source showed the presence of *o*/*p*-cresol, guaiacol, 4-methylguaiacol, and 4-ethylguaiacol (Figure 2). While the VPs identified were consistent with the smoke generated upon combustion of *Pinus radiata (P. radiata)*,[11] the absence of several VPs and differences in the ratios of detected VPs supported the presence of regional differences in the presentation of smoke-taint. Further supporting this idea was the lack of 4-methylsyringol, which was identified as a major source of total VPs in the smoke, berries and wine of smoke-exposed Chardonnay and Cabernet Sauvignon.[30] No evidence of 4-methylysringol was found in the smoked-exposed CF berries used in this study, or in the experimental smoke (data not shown). It is not clear if these differences were a function of environmental, varietal, or experimental parameters (e.g., fuel source).

The reproducible application of smoke to *V. vinifera* berries on-vine is a logistical challenge, as controlling the volatile organic chemical composition (i.e., VPs) of smoke is multifactorial.[31,32] Nephelometry has previously been used as an aggregate measure of smoke exposure, with Kennison et al. demonstrating that a single 30 min exposure at 200 $\mu g/m^3$ (particulate matter ≤10 μm, PM_{10}) was sufficient to yield 90 $\mu g/L$ guaiacol in wine made from the smoke-exposed berries.[13] In the present study, $PM_{2.5}$ values were measured during smoke application, but high variance and frequent detector saturation limited the usefulness of the results (Figure S2). Given these results and the current lack of correlative studies between $PM_{2.5/10}$ values and the magnitude of VPs in smoke-exposed berries, it was decided that smoke application would best be reported as the amount of fuel consumed (500 g) over a set time (60 min) inside a given volume (18.3 m^3).

Cabernet Franc Field Trials. Cabernet Franc (CF) vines were exposed to simulated forest fire smoke approximately 7 days after the onset of veraison, as this is when the berries are most susceptible to increases in total VPs with smoke exposure.[13] To the best of our knowledge, this is the first report on the impact of smoke-exposure to CF berries and wine. Anecdotal evidence from growers in the Okanagan Valley of British Columbia, Canada, suggested that smoked-exposed

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CF berries were more likely to yield smoke-tainted wine when compared with other varietals exposed to similar smoke. For all samples, an average weight from 100 berries was recorded, as were the °Bx. When compared against the VP data no trends were observed between these metrics and the concentration of free or bound VPs, nor did the smoke-exposed berries differ significantly in °Bx or average berry mass from control berries (data not shown). These results were consistent with a study that evaluated the impact of smoke-exposure on vine physiology.[6]

Washed versus Unwashed Berries. It has been demonstrated that VPs can be translocated into berries and leaves when exposed directly to solutions of guaiacol.[33] Despite this evidence of direct uptake, it remains unclear if particulates from wildland fires act as vehicles to transport the VPs to the berry, or if the VPs are in their free form leading to direct uptake by the berry. Using smoke-exposed berries that were harvested immediately after smoke application, the effect of washing the berries was investigated as a model to test if overhead irrigation following smoke exposure could help mitigate the severity of smoke-taint, and as an indirect test of the role played by particulates (Figure S3). For VPs above their respective LOQs, the washed and unwashed berries showed the same concentrations of VPs. Evaluation of berries immediately after on-vine smoke-exposure has yet to be reported, so the kinetics of VP uptake have only been observed on the scale of days and weeks[6,13,14,25] rather than hours. While not definitive, these results suggested that regardless of how they arrive at the berry (particle-bound or free), VPs strongly adhere to and/or rapidly translocate into the berry. This indicated that overhead irrigation after a smoke-exposure event is unlikely to decrease the VPs that are concentrated in the berry.

Free versus Bound VPs. Several studies have examined how VPs are stored and/or are biochemically modified (e.g., glycosylated) in smoke-exposed berries and wine at different time-points between *veraison* and commercial maturity. Those studies looking at free VPs were from the infancy of smoke-taint research and only examined guaiacol, 4-methylguaiacol, and 4-ethylphenol.[13,14,25] Subsequent publications highlighted strong correlations between the intensity of typical smoke-taint characteristics in wine and the presence of syringol and cresols,[6,34,35] making their absence from these earlier studies noteworthy. Furthermore, with their focus on the impact of smoke at key developmental stages, these initial studies lack data on the evolution of VP storage forms during ripening and fermentation. Dungey et al. looked at the same vines over time following smoke-exposure, but only evaluated guaiacyl-glycosides.[16] To date, an evaluation of the time-dependent biochemical dynamics of VPs within the same smoke-exposed vines remains unexamined. This information is critical to the development of a robust smoke-taint risk-assessment model. Therefore, to fill this gap, the present study applied our recently developed analytical methods[17] to quantitate a broad range of free and bound VPs (as determined by acid hydrolysis) in the same control and smoke-exposed CF vines over a time span encompassing nearly all of the post-*veraison* ripening process until harvest and to the end of primary fermentation (Figure 3; see Table S7 for a numerical summary).

Examining these data broadly, it was intriguing that the ratio of VPs detected in the experimental smoke (Figure 2) were not reflected in the ratio of free, bound, or total VPs detected in smoke-exposed berries. This was most apparent for syringol, which was not detected in the smoke reported here, nor was it found in the work of Kelly et al.,[11] despite appearing at high concentrations in the wine produced for both studies. Moreover, analysis of VPs in berries and wines revealed that the relative VP ratios changed following primary fermentation. Upon comparing smoke-exposed berries with their development-matched controls, these results suggested differential uptake and/or metabolism of smoke-borne VPs. We observed that in the smoked-exposed berries there was a statistically significant increase in both free and bound guaiacol over control samples, with the total guaiacol levels remaining constant until commercial maturity (42 d after smoke exposure). Similar trends were observed for the cresols, although the free form of *o*-cresol was present below its' LOQ in all except the 7-day sample. 4-Methylguaiacol also displayed similar results for the free VP. Conversely, the bound form of 4-methylguaiacol trended upward until commercial maturity rather than holding constant, with statistical significance when comparing the 4-methylguaiacol levels at commercial maturity against those measured immediately post-smoke exposure (i.e. 0.04 d). With the exception of 4-ethylphenol, the levels of acid-labile VPs in berries exceed those of their free analogues. The in-berry levels of three other VPs, 4-ethylguaiacol, eugenol, and syringol did not permit the discrimination between smoke-exposed and control berries, with 4-ethylguaiacol being detected in only two of ten samples and eugenol/syringol displaying equal levels between all samples and time points.

The guaiacol and cresol data demonstrated that the ratio of bound-to-free VPs was established during smoke exposure and remained constant until commercial maturity. Assuming the acid-labile compounds (i.e., bound guaiacol) were simple glycosides, these results diverged from those previously reported for guaiacyl-glycosides, which were shown to increase in concentration for at least 2 weeks after smoke exposure in Merlot and Voignier berries.[16] In contrast to the trends observed for guaiacol and *o/p*-cresol, the bound-to-free VP ratio for 4-methylguaiacol increased following smoke exposure. Although this increase in the bound form of the VP was consistent with published guaiacyl-glycoside results,[16] the absence of a corresponding decrease in the levels of free VP indicated that at least some of the acid-labile 4-methylguaiacol conjugates observed in the berries at commercial maturity could be derived from an alternate pool of VP conjugates that was resistant to acid hydrolysis.

In addition to evaluating the free and bound VPs in CF berries, the levels of free and bound (acid-labile) VPs in wines (primary ferments) made from the control and smoke-exposed berries harvested at commercial maturity were also determined (Figure 3). This necessitated adaptation and validation of the GC-MS/MS methods developed for berries by Noestheden et al.[17] to include the analysis of VPs in wine (Table S9). Our analysis revealed that, with the exception of 4-ethylphenol, the levels of free VPs in wines made from smoke-exposed berries were statistically higher than the free VPs measured in the same berries prior to fermentation. The increase in free VP concentrations ranged from 4-fold for *o*-cresol to 150-fold for syringol. Surprisingly, in the case of guaiacol, syringol, and eugenol the levels of the free VPs in wines exceeded those of total VPs in the berries, an observation that intriguingly held for both the control and smoke-exposed berries. With the exception of eugenol, the levels of free VPs detected in wines produced from smoke-exposed berries were statistically higher than the corresponding control wines, with increases in free VP

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Table 1. Recovery of Free VPs and Model Glucosides from Acidic and Basic Digests[a,b]

	free VPs (%)		model glycosides (%)	
compound	acid[17]	base	acid[17]	base
4-ethylguaiacol	89 ± 1.9	81 ± 3.9	[c]	[c]
4-ethylphenol	97 ± 4.1	90 ± 1.8		118 ± 12.0
4-methylguaiacol	82 ± 3.9	93 ± 2.9	[c]	[c]
eugenol	92 ± 3.7	90 ± 1.7		102 ± 3.6
guaiacol	100 ± 1.4	88 ± 2.8		69 ± 5.4
o-cresol	103 ± 4.4	89 ± 1.9	[c]	[c]
p-cresol	92 ± 4.2	88 ± 2.4		96 ± 7.8
syringol	71 ± 2.3	100 ± 1.9		33 ± 11.1/25 ± 15.6[d]

[a]Acid and base digests were performed at 100 °C for 4 h, at pH 1.5 and 11.5, respectively. [b]Values are reported as mean ±1 standard error of the mean (n = 3). [c]Glucosides for these VPs were not synthesized. [d]Syringl-β-D-glucopyranoside/syringyl gentiobioside.

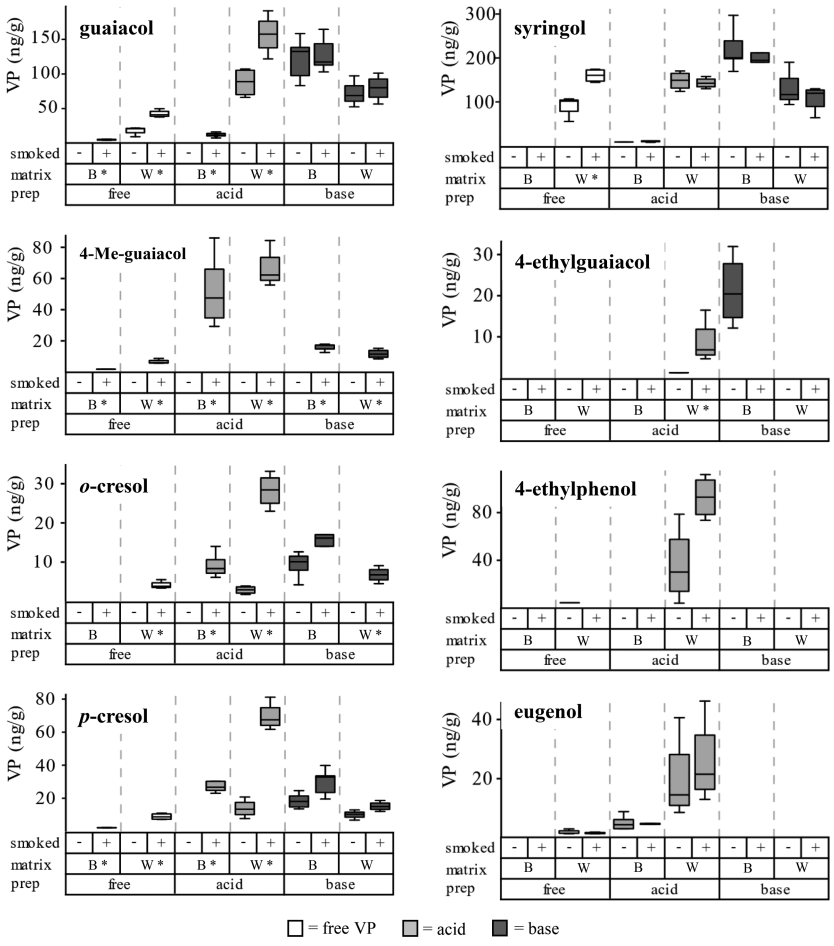

Figure 4. Box-whisker plots of free VPs (white), acid-labile VP-conjugates (light gray), and base-labile VP-conjugates (dark gray) in Cabernet Franc berries at commercial maturity (B) and after primary fermentation (W). Syringol results are scaled down 10-fold for clarity. Data were tabulated from up to five biological replicates (see Figure S8). Box plot center lines show medians and limits indicate the 25th and 75th percentiles. Whiskers extend to 1.5 times the interquartile range from the 25th and 75th percentiles, with points beyond these ranges assigned as outliers. * Statistical difference from respective control (−) samples (Mann−Whitney U test, α = 0.05).

concentrations ranging from 2.4-fold for guaiacol to 8.5-fold for *p*-cresol. While the present study did not focus on sensory analysis, the free guaiacol levels measured in wine made using the smoke-exposed CF berries relative to the reported aroma threshold of guaiacol in red wine suggested that a quantifiable sensory impact would be observed.[13]

Previous smoke-taint research suggested that free VPs detected in wine may be produced from glycosidically bound precursors by the action of microbial glycosidases[8] and/or acid hydrolysis during fermentation and aging.[15,36] Therefore, it was hypothesized that the increase in free VPs observed in our CF wines (relative to berries) should have been accompanied by a corresponding decrease in the levels of bound (acid-labile) VPs observed after fermentation. However, such a decrease was not observed in the smoke-exposed CF wines. For example, the in-wine levels of acid-labile forms of guaiacol, and *o*/*p*-cresol all significantly exceeded those observed in berries, while the levels of bound 4-methylguaiacol and eugenol remained effectively unchanged. As observed for the free VPs, in several instances (guaiacol, *o*/*p*-cresol, syringol, 4-ethylguaiacol, and eugenol)

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acid-labile VPs were also detected in control wines even though they were present below or just above their LOQs, in the same berries prior to fermentation. It is worth noting that 4-ethylguaiacol (Figure 3) showed a trend (albeit not statistically significant) in wine toward higher concentrations in the smoke-exposed berries, despite none of this VP being observed above the LOQ in either control or smoke-exposed berries. Note, however, that 4-ethylguaiacol was in the *P. ponderosa* fuel source (Figure 2).

Noestheden et al. previously demonstrated the quantitative recovery of free VPs following acid hydrolysis of VP-glycosides.[17] Therefore, the increase in total VPs (inclusive of acid-labile conjugates and free forms) following primary fermentation suggested the presence of VP storage forms other than simple acid-labile glycosides. In the present study, the data indicated that these alternate storage forms were more relevant to the final VP concentration in wines than VP-glycosides, given the large increase in bound VPs (up to 16-fold for guaiacol) in wine made from smoke-exposed CF berries. The increase in acid-labile VP-conjugates after primary fermentation suggested that microbial enzyme-catalyzed transformations not only increased the levels of free VPs (as previously demonstrated[8,34,37]), but that they also produce a larger pool of acid-labile VP-conjugates. It is plausible that this alternate VP storage form could also explain the increase in acid-labile 4-methylguaiacol conjugates observed in smoke-exposed berries during the ripening process (Figure 3), with differential metabolic flux between the two VP storage forms controlling the relative amounts of each observed during the ripening process.

Base Hydrolysis. As confirmation that the alternate VP-conjugates were not simple glycosides, the CF berries (control and smoke-exposed) and their corresponding wines were subjected to base hydrolysis (pH 11.5, 4 h, 100 °C). Prior to initiating this experiment, the stability of the free VPs and a set of model VP-glycosides under alkaline hydrolytic conditions were evaluated (Table 1). This experiment demonstrated that free VPs were stable to the basic hydrolysis conditions used and could be quantitatively recovered prior to GC-MS/MS analysis. The stability of the model glycosides under basic conditions depended on the nature of the VP aglycone. 4-Ethylphenyl, eugenyl, and *p*-cresyl glucosides were quantitatively recovered from basic digestion conditions, whereas the guaiacyl-glucoside and syringyl-glycosides displayed varying levels of base sensitivity, respectively exhibiting 30% and 70% loss of the glycosides as assessed by uHPLC-QToF. However, in these instances the loss of the glycosides did not correspond to a quantitative increase in free VPs, with only 39% of the lost guaiacyl-β-D-glucopyranoside, 5% of the syringl-β-D-glucopyranoside, and 55% of the syringl gentiobioside recovered as free VP (data not shown). This result was consistent with observations that the base-catalyzed hydrolysis of glycosidic bonds can require more aggressive conditions (170 °C, 2.5 M NaOH, > 101 kPa[38]) than employed in this study, depending on the nature of the glycone and aglycone.[39,40] The alkaline hydrolytic behavior of the VP-glycosides reported herein should be applicable to other matrices, as all reactions were performed at the same pH, regardless of matrix (e.g., berries, wine or model wine). It follows from these observations that, with an accommodation for the observed glycoside degradation and its subsequent impact on the recoveries of guaiacol and syringol in base, an increase in free VPs under these alkaline conditions would confirm the presence of a VP-conjugate other than a simple glycoside.

The base hydrolysis data for free guaiacol demonstrated that there were base-labile conjugates in berries and wine (Figure 4). For example, the statistically equivalent concentrations of guaiacol released after the acid hydrolysis of wine made using smoke-exposed berries (152 ± 13.7 ng/g) and the base hydrolysis of those berries (124 ± 10.9 ng/g) suggested that the base-labile pool of bound VPs was modified during fermentation to make them acid-labile. Similar results were obtained for syringol, although in this instance the syringol concentrations increased markedly (Figure 4; the syringol base hydrolysis data was scaled down 10-fold for readability). Again, in keeping with the trends observed for guaiacol, the syringol data showed the presence of a storage form that was not acid labile (i.e., not a simple glycoside) prior to fermentation. It is plausible that the mechanism responsible for these observations can also be used to explain why Kelly et al.[11] observed high levels of syringol in wine despite observing low syringol concentrations in smoke generated from *P. radiata*. The cresol data continued to provide evidence of base-labile VP-conjugates that were made accessible to acid hydrolysis during primary fermentation. In contrast, the 4-methylguaiacol results were inconclusive, as the base labile data was only marginally greater than the free VP concentrations. The base hydrolysis data did not yield significant results for eugenol, 4-ethylguaiacol, and 4-ethylphenol (Figure 4). Characterizing the identity of these abundant base-labile VP-conjugates is the subject of ongoing investigation in our research group.

Having more accurate and timely access to information regarding the potential of smoke-exposed berries to produce smoke-tainted wine will give growers and wineries time to plan suitable fermentation adjustments and/or viticultural changes to mitigate the impact of smoke exposure. The results presented herein suggested that correlations between total VP concentrations in berries and wine could be established immediately after smoke exposure. However, the base hydrolysis and fermentation data demonstrated the need to better elucidate the metabolic fate of VPs in smoke-exposed berries to yield more definitive predictive correlations between the VPs stored in smoke-exposed berries and the probability of producing smoke-tainted wines. Further influencing the generation of predictive correlations is the regionality of smoke-taint, with our data showing clear differences in VP profiles from those previously reported. Indeed, the results herein suggest that applying existing smoke-taint research, while an excellent starting point, may not be entirely applicable to the presentation of smoke-taint in all wine producing regions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b04946.

Synthetic details, analytical instrument parameters, field trial design and metrics, analytical results, and wine validation data (PDF)

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Notes
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