

Quantitating Organoleptic Volatile Phenols in Smoke-Exposed *Vitis vinifera* Berries

Matthew Noestheden,^{†,‡} Katelyn Thiessen,[†] Eric G. Dennis,[†] Ben Tiet,[†] and Wesley F. Zandberg^{*,†}

[†]University of British Columbia Okanagan, Kelowna, British Columbia, Canada

[‡]Supra Research & Development, Kelowna, British Columbia, Canada

S Supporting Information

ABSTRACT: Accurate methods for quantitating volatile phenols (i.e., guaiacol, syringol, 4-ethylphenol, etc.) in smoke-exposed *Vitis vinifera* berries prior to fermentation are needed to predict the likelihood of perceptible smoke taint following vinification. Reported here is a complete, cross-validated analytical workflow to accurately quantitate free and glycosidically bound volatile phenols in smoke-exposed berries using liquid–liquid extraction, acid-mediated hydrolysis, and gas chromatography–tandem mass spectrometry. The reported workflow addresses critical gaps in existing methods for volatile phenols that impact quantitative accuracy, most notably the effect of injection port temperature and the variability in acid-mediated hydrolytic procedures currently used. Addressing these deficiencies will help the wine industry make accurate, informed decisions when producing wines from smoke-exposed berries.

KEYWORDS: *Vitis vinifera*, smoke, volatile phenol, dansyl chloride, acid hydrolysis, GC–MS/MS, QToF

INTRODUCTION

In plants, volatile phenols (VPs) are secondary metabolites formed via the phenylpropanoid biosynthetic pathway that are primarily involved in host stress response and reproduction.^{1,2} Because of their organoleptic attributes, endogenous and exogenous VPs are important in a variety of consumer products, most notably in smoked foods, beer, whiskey, and wine, as well as in unprocessed foodstuffs like basil and tomatoes.^{3–7} In wine, the VP profile can be influenced by (1) endogenous VP sources related to the varietal of *Vitis vinifera* L. (*V. vinifera*), environmental conditions, and viticultural and vinification practices and (2) exogenous VP sources, including environmental factors (e.g., exposure of berries to smoke) and barrel/bottle aging.^{8–12} Understanding the influence of these variables is critical, as an imbalance in VPs can negatively impact wine quality. For example, when *V. vinifera* vines are exposed to smoke from wildland fires or prescribed burns at key phenological stages, VP concentrations in the berries increase. This change in VP concentrations has been correlated to the intensity of “smoky”, “ashy”, “burnt meat”, and “Band-Aid” sensory characteristics in wines made from smoke-exposed berries.^{12–15} Underscoring the importance of this “smoke taint” are the 2009 Australian wildland fires, which cost the local wine industry an estimated \$300 million.¹⁶ Given this economic impact, and with climate change models predicting an increase in the frequency of wildland fires in wine growing regions¹⁶ (i.e., California, British Columbia, Australia, and South Africa), smoke taint is an issue of increasing importance to wine producers.

An array of VPs correlate to the negative organoleptic attributes of wine made from smoke-exposed berries, not just the prototypical marker compounds guaiacol and 4-methylguaiacol.^{8,15,17} These VPs can be divided into three broad structure-driven categories: guaiacyls [guaiacol (5), 4-methylguaiacol (3), 4-ethylguaiacol (1), and eugenol (4)], syringyls

[syringol (9)], and alkyl-hydroxyphenyls [4-ethylphenol (2) and *o*-, *m*-, and *p*-cresols (6–8, respectively)]. Despite evidence supporting the involvement of such VPs in the perceived intensity of smoke taint in wine, the use of VP concentrations in berries to predict the sensory properties of wine remains a significant challenge, due in part to the fact that the VPs in smoke-exposed berries are found primarily as an array of nonvolatile glycosides.¹⁸ These VP-glycosides represent a sensory potential that can be metabolized during vinification to release free VPs, which will subsequently influence the sensory characteristics of the resulting wine.¹⁴ Indeed, Hayasaka et al. demonstrated as much when they showed that wine made from smoke-exposed berries could be identified by the concentration of VP-glycosides, even in the absence of perceptible smoke taint.¹⁸

Most studies focus on the concentration of free VPs and/or their glycosides in wine rather than in berries, because evaluating wine permits a correlation to the presence and intensity of specific sensory characteristics.^{17,19,20} For a winery, this requires the allocation of resources for fermentation at risk (i.e., there is no *a priori* knowledge regarding the potential for perceptible smoke taint following fermentation). To avoid this risk, the optimal situation is to correlate the concentration of total VPs in smoke-exposed berries (i.e., free and glycosidically bound VPs) to the chance of perceptible smoke taint in the resulting wine. This would remove the uncertainty and potentially wasted resources that come with assessing smoke taint after vinification. Accurate methods for quantitating the total VP load in smoke-exposed berries prior to and during fermentation are essential tools needed to achieve this goal.

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While several studies have demonstrated the quantitation of intact VP-glycosides using mass spectrometry,^{21,22} the known variability in response factors for VP-glycosides¹⁸ inherently limits the accuracy of this approach. An alternative method is the liberation of free VPs by hydrolysis because analytical standards and isotopic internal standards are available for most VPs of interest. The variation in VP-glycosides,¹⁸ as well as the presence of modified glycones (e.g., acetylated and malonylated),²³ presents a broad range of substrates that complicates the use of enzymatic hydrolysis. Acid-mediated hydrolysis of VP-glycosides in smoke-exposed berries and wine has been reported.^{21,24} However, there are no published, systematic optimizations of the hydrolytic conditions. Moreover, studies that attempted to correlate the loss of glycosides to the increase in the level of free VPs reported inconsistent results and low recoveries for the free VPs.^{19,24}

In light of the considerations described above, the goal of this study was the development and validation of a set of analytical methods for accurately quantitating total VP concentrations in smoke-exposed berries. To maximize the information content of each sample analysis and aid in the eventual development of a robust predictive model, nine VPs that were consistent with other reported analytical methods for quantitating VPs were evaluated.^{25,26} Two orthogonal analytical methods, one using gas chromatography–tandem mass spectrometry (GC–MS/MS) and the other using a novel ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (uHPLC–QToF), were developed and validated independently and against one another for the quantitation of VPs in smoke-exposed berries. In addition, a series of model VP-glycosides were synthesized and used to evaluate suitable conditions for the acid-mediated release and quantitation of glycosidically bound VPs.

MATERIALS AND METHODS

Chemicals and General Details. The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received: HPLC grade methanol (MeOH), isopropanol (IPA), acetonitrile (ACN), hexane, ethyl acetate (EtOAc), dichloromethane (CH₂Cl₂, reagent grade), diethyl ether (Et₂O, reagent grade), sodium bicarbonate, anhydrous sodium carbonate, hydrochloric acid, sulfuric acid, trifluoroacetic acid (TFA), 2-methoxyphenol [guaiacol (5)], 2,6-dimethoxyphenol [syringol (9)], 2-methoxy-4-methylphenol [4-methylguaiacol (3)], 2-methoxy-4-ethylphenol [4-ethylguaiacol (1)], 4-ethylphenol (2), 2-methoxy-4-allylguaiacol [eugenol (4)], 3-methylphenol [*m*-cresol (6)], 4-methylphenol [*p*-cresol (7)], 2-methylphenol [*o*-cresol (8)], *d*₃-guaiacol, *d*₄-4-ethylphenol, *d*₅-4-ethylguaiacol, CDCl₃, and *d*₄-MeOH. 2-Bromo-6-methoxyphenol was purchased from Alfa Aesar (Tewksbury, MA) and used as received. Type 1 water (16 MΩ) was provided by a Barnstead E-Pure water purification system (Thermo Fisher Scientific, Waltham, MA). Weighing was performed using a Mettler Toledo ME204E analytical balance (Thermo Fisher Scientific). 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). Supelclean ENVI-18 solid phase extraction cartridges (6 mL/500 mg) were purchased from Sigma-Aldrich. Unless noted, all analyses were conducted as outlined below.

Details regarding the synthesis of *d*₃-2,6-dimethoxyphenol (*d*₃-syringol), eugenyl-β-*O*-glucopyranoside, guaiacyl-β-*O*-glucopyranoside, syringyl-β-*O*-glucopyranoside, *p*-cresyl-β-*O*-glucopyranoside, 4-ethylphenyl-β-*O*-glucopyranoside, and syringyl-gentiobiose are described in the Supporting Information.

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 and stored at –20 °C. Sodium

carbonate buffers were prepared at 0.10–2.0 M. Dansyl chloride (2.5 mM) was prepared in acetone and stored protected from light at 4 °C. GC–MS/MS calibrators were prepared in a 1:1 hexane/EtOAc solvent with 50 ng of ISTDs/g. After ISTD had been added (50 ng/g), uHPLC–QToF calibration samples (in 20% ACN) were derivatized with dansyl chloride.²⁷ Briefly, 500 μL of the sample was mixed with 50 μL of 1.25 M carbonate buffer and 250 μL of dansyl chloride. After being vortexed, the reaction mixtures were incubated for 30 min at 75 °C and analyzed without further workup. Calibration samples for both methods were prepared fresh daily.

Plant Material. Method development and validation were performed using Cabernet Franc, Merlot, and Pinot Noir berries acquired from vineyards in the Okanagan Valley (British Columbia, Canada). The Cabernet Franc and Merlot berries were harvested at commercial maturity in 2016. Pinot Noir berries were harvested in 2015 at commercial maturity, after exposure to smoke from wild land fires (British Columbia, Canada). All samples were homogenized (HMG) in a commercial blender immediately after being harvested and stored at –20 °C.

Sample Preparation. For GC–MS/MS analyses, HMG samples were thawed, and 5 g was weighed into a 15 mL polypropylene centrifuge tube and fortified with ISTD (50 ng/g). Extraction protocols were adapted from published LLE procedures (Table 1).^{25,26,28,29} For all LLE, 2 mL of organic solvent was added per 5 g of

Table 1. Summary of the Extraction Conditions Reported for the Analysis of VPs in *V. vinifera* Berries and Wine^a

extraction solvent	NaCl _{sat}	matrix	injection temp (°C)
1:1 hexane/EtOAc ³⁵	yes	berry	240
2:1 pentane/ether ³⁰	–	wine	200
EtOAc	yes	berry	220
hexane ³⁸	yes	berry	250
pentane ^{b,9}	–	wine	200

^aThese solvent systems were adapted in this study for comparison of their ability to extract VPs from homogenized berries. ^bUsed liquid injection or headspace solid phase microextraction and matrix-matched calibrators. All others used liquid injection and solvent calibrators.

HMG. For the 1:1 hexane/EtOAc solvent, EtOAc, and hexane extraction systems, 1.5 g of NaCl (saturated solution, NaCl_{sat}) was also added. Extractions were vortexed for 30 s, mixed for 5 min, and centrifuged at 3000g for 3 min. An aliquot of the organic layer was transferred to a 2 mL glass vial and analyzed without further workup.

For derivatization with dansyl chloride, HMG was thawed and centrifuged for 30 min at 3500g. After ISTD had been added (50 ng/g), 5 mL of the supernatant was purified by solid phase extraction (SPE). SPE involved loading the sample onto a Supelclean ENVI-18 SPE cartridge (6 mL/0.5 g) that was conditioned with 2 × 5 mL of ACN and 2 × 5 mL of H₂O. After being washed with 5 mL of H₂O and dried, the extracts were eluted manually with 1 mL of ACN and diluted to 5 mL with H₂O. Conditioning, washing, and drying (5 min) were performed under a 15 mmHg vacuum. After SPE, samples were derivatized with dansyl chloride (*vide supra*).

Acid hydrolysis was performed in 4 mL borosilicate glass vials with polytetrafluoroethylene (PTFE)-lined caps or in 10 mL PTFE tubes with PTFE caps. Samples were adjusted to pH 1.5 with HCl, H₂SO₄, or TFA and heated at 100 °C for 1–24 h. After hydrolysis, samples were treated per their intended analysis: for free VPs, the LLE procedure described above was used, and for VP-glycosides, the acid digests were neutralized with 1 M NaOAc to pH 3.5 and analyzed by uHPLC–QToF without further workup.

GC–MS/MS. Analyses were performed on a TSQ 8000 Evo Triple Quadrupole GC–MS/MS instrument equipped with a TRACE 1310 gas chromatograph and a TriPlus RSH autosampler (Thermo Fisher Scientific). The carrier gas was helium (99.999%). The injection port temperature for quantitative analysis was 220 °C, whereas temper-

atures of 200–280 °C (20 °C steps) were used to evaluate the impact of injection port temperature. A 5 μ L injection volume was used for all analyses. Quantitative analyses were performed on an SLB-5 60 m \times 0.25 mm \times 0.25 μ m capillary column (Sigma-Aldrich) using a splitless single-taper liner (4.0 mm internal diameter, 6.2 \times 78.5 mm; Restek, Bellefonte, PA). Additional method development was performed on a ZB-WAX 30 m \times 0.32 mm \times 0.50 μ m capillary column (Phenomenex, Inc., Torrance, CA). The transfer line and source temperatures were 280 (230) °C and 300 (250) °C, respectively, for the SLB-5 (ZB-WAX) column. Temperature gradients are summarized in Figure S1, and MS/MS parameters are listed in Table S1.

uHPLC–QToF. uHPLC was performed using an Agilent 1290 Infinity system (Agilent Technologies, Santa Clara, CA) equipped with a model 1290 Infinity binary pump, a model 1290 Infinity autosampler, and a model 1290 Infinity thermostated column compartment. A 2:1:1 IPA/MeOH/H₂O needle wash solution was used, with a 3 s wash during each injection cycle. Dansylated VPs were analyzed using a 20 μ L injection on a Kinetex Biphenyl 100 mm \times 2.1 mm, 2.6 μ m column (40 °C). Model glucosides were analyzed using a 10 μ L injection on a Kinetex C18 50 mm \times 2.1 mm, 2.6 μ m column (40 °C). Gradient and mobile phase conditions are summarized in Figure S1.

QToF mass spectrometry was performed on an Agilent 6530 QToF instrument equipped with a Jet Stream electrospray ionization (ESI) source. Data were acquired in full scan mode (10000 resolution at m/z 118.0863; 20000 resolution at m/z 622.0287) from m/z 100 to 1000 at scan rate of 1 Hz. A summary of the formulas, retention times, and ions evaluated are provided in Table S2, and MS parameters are summarized in Table S3.

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.³⁰ as

$$\text{LOD}_i/\text{LOQ}_i = ks_n \quad (1)$$

where s_n is the standard deviation of n spike-recovery extracts for compound i and k is a multiplier equal to 3.3 for the LOD and 10 for the LOQ. Accuracy and precision ($n = 5$) were evaluated by fortifying Cabernet Franc (GC) HMG at 2 and 100 ng/g and Merlot (uHPLC) HMG at 5 and 200 ng/g prior to extraction. Recoveries were corrected for endogenous VP levels and use an HMG density of 1.06 g/mL (empirically determined). Cross validation was performed by comparing the quantitative results for a smoke-exposed Pinot Noir HMG ($n = 5$) with the Horwitz ratio.³¹

Data Acquisition and Processing. GC–MS/MS data were processed using the Xcalibur (version 3.0.63) and TraceFinder (version 3.2.512.0) software packages (Thermo Scientific). uHPLC–QToF data acquisition and processing were performed using the MassHunter Workstation software suite (Agilent Technologies), with version numbers as follows: Data Acquisition Workstation (version B.06.01, Service Pack 1), Qualitative Analysis (version B.07.00, Service Pack 2), and Quantitative Analysis (version B.07.00). Data reduction and statistical calculations were performed using Microsoft Excel (Microsoft Corp., Redmond, WA).

RESULTS AND DISCUSSION

GC–MS/MS Method Optimization. The quantitation of VPs prior to vinification is critical to developing an accurate risk assessment model when using smoke-exposed berries. Wilkinson et al.²⁵ summarized that free VPs in smoke-exposed berries were typically analyzed by GC–MS or GC–MS/MS, which was also the quantitative method of choice for the study presented here. Chromatographic resolution of all VPs was achieved on a ZB-WAX capillary GC column (Figure S2), which was used for method optimization experiments. While chromatographic resolution was important for method optimization, sample throughput was critical for routine quantitative analyses of smoke-exposed berries. Therefore, for method validation and subsequent quantitative analyses, a

second chromatographic method was developed on an SLB-5 capillary column (Figure 1). At 12.5 min, this separation was 25% faster than the ZB-WAX method, although the trade-off was co-elution of *m*-cresol and *p*-cresol.

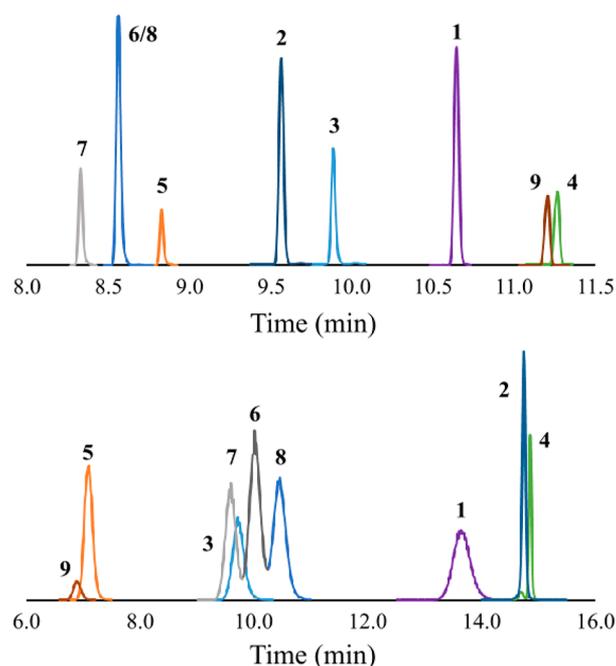


Figure 1. GC (top) and uHPLC (bottom) sample chromatograms for 50 ng/mL calibration standards: 4-ethylguaiaicol (1), 4-ethylphenol (2), 4-methylguaiaicol (3), eugenol (4), guaiaicol (5), *m*-cresol (6), *o*-cresol (7), *p*-cresol (8), and syringol (9).

VPs are generally isolated via liquid–liquid extraction (LLE) for GC analysis.^{25,26,28,32,33} However, only a minority of studies of smoke taint have evaluated a large panel of VPs,^{12,13,21,26,34} and there have been no rigorous and systematic optimizations of the extraction of VPs from smoke-exposed berries. This is a notable knowledge gap, as differences in extraction efficiencies between VPs could impact the risk assessment associated with using smoke-exposed berries. Therefore, a systematic comparison of the LLE procedures for the extraction of free VPs from *V. vinifera* berries was conducted. Adaptations of four published LLE methods and an aggregate condition (100% EtOAc with NaCl_{Sat}) were evaluated (Table 1).^{25,26,28,29} Previous research by Singh et al.^{26,29} used a 1:1 hexane/EtOAc mixture or 100% hexane as LLE extraction solvents but did not report an evaluation of 100% EtOAc; hence, 100% EtOAc was evaluated to complete the solvent polarity range of this binary mixture.

Using NaCl_{Sat} improved the absolute extraction efficiency for *p*-alkyl-substituted VPs (Figure S3) and the extraction precision for all VPs. Variations in absolute extraction efficiencies were observed as a function of VP and LLE system, irrespective of the use of NaCl_{Sat} (Figure 2). For instance, the single-solvent systems displayed lower yields, with pentane giving notably low extraction efficiencies for alkyl-substituted phenols and syringol, while the binary solvent systems yielded the highest absolute extraction efficiencies. The low syringol results for the pentane/ether system and the practical difficulties of working with ether supported the use of a 1:1 hexane/EtOAc solvent with NaCl_{Sat}. As such, an adaptation of the procedure of Singh et al.²⁶ was used to validate the GC–MS/MS method (*vide infra*). These results underscored the importance of this systematic

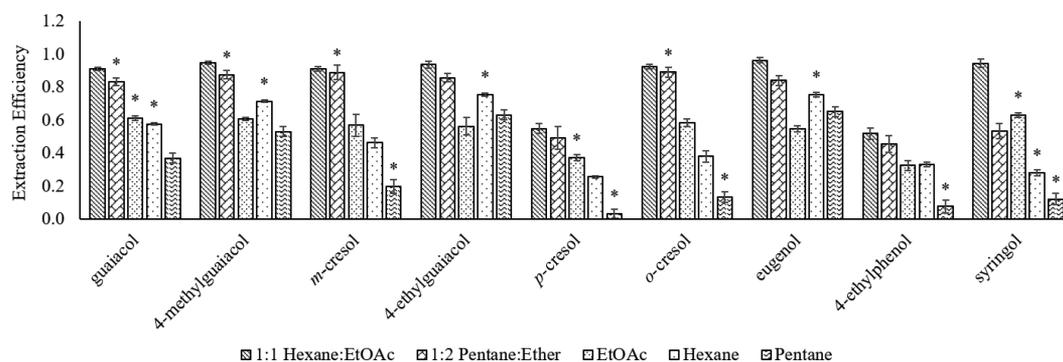


Figure 2. Mean ($n = 3$) VP extraction efficiency in berry HMG as a function of LLE solvent system. Each mean response was normalized against the mean ($n = 3$) of an unextracted control in the appropriate solvent system. An asterisk indicates a significant difference ($p < 0.05$) for a one-sided Student's t test with unequal variance, relative to the 1:1 hexane/EtOAc data. Data are shown ± 1 standard error of the mean.

evaluation, as the LLE procedure impacted the concentrations of VPs reported. While ISTD correction should account for these extraction losses, this was strictly true for only isotopically matched ISTDs [i.e., guaiacol, 4-ethylphenol, and 4-ethylguaiacol (Figure S4)]. For the cresols and syringol, which did not use matched ISTDs, the corrected extraction efficiencies were 48–66% in the hexane/EtOAc LLE system.

To the best of our knowledge, this is the first report of a triple-quadrupole MS method for the analysis of VPs in berries. The quantitation and qualifying transitions and the optimized MS voltages (Table S1) were consistent with those reported for single-quadrupole instruments.²⁶ Moving away from the MS to the GC parameters, we found the injection port temperature required evaluation based on a study by Pollnitz et al.,²⁸ who reported that higher-polarity LLE solvents and injection port temperatures of >200 °C resulted in an increased guaiacol response in oak extracts and wine. The authors hypothesized that co-extracted soluble lignin could thermally decompose in the injection port leading to the formation of guaiacol. It appears that several methods have not considered these important findings (Table 1), calling into question their quantitative accuracy. While this phenomenon has not been evaluated in berry extracts, a similar mechanism seemed plausible given the high guaiacyl content of *V. vinifera* lignin.³⁵ Given the nature of this effect, spike-recovery studies in a matrix would not reveal the problem, as the accuracy of such investigations is based on a difference between fortified and incurred levels. In addition, assuming the soluble lignin hypothesis is correct, the use of matched isotopic ISTDs would not help (*vide infra*). Because of the limited number of VPs in the study by Pollnitz and the potentially critical implications on quantitative accuracy it highlighted, the effect of injection port temperature on the nine VPs in the current study was evaluated (Figure 3).

The results for guaiacol and 4-methylguaiacol were consistent with the work of Pollnitz et al., although the magnitude of the increase in the guaiacol response was lower, with Pollnitz et al. reporting a ≤ 7 -fold increase in the response from 200 to 250 °C and this study showing a <3 -fold increase from 200 to 280 °C. There are numerous factors that could contribute to this difference, including varietal, extraction solvent, vinification practices, etc. The absence of a relationship between injection port temperature and the ISTD responses demonstrated that this phenomenon was specific to the target compounds. The observed 6-fold increase in syringol response and the modest increase in eugenol response as a function of

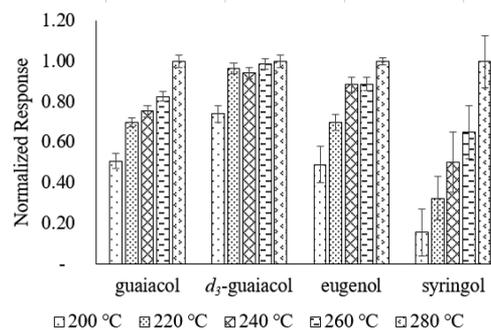


Figure 3. Mean ($n = 5$) VP peak area response as a function of GC injection port temperature for berry HMG extracts prepared using the optimized LLE system (1:1 hexane/EtOAc with NaCl_{sat}). Data were normalized to the largest area response for each VP and are shown ± 1 standard error of the mean. The consistently low response at 200 °C was attributed to a general injection issue, as a similar effect was observed in solvent extracts (data not shown). Only VPs that displayed an observable trend are shown.

injection port temperature were similar to the documented behavior of guaiacol in the study by Pollnitz. While the hypothesis of thermal decomposition of co-extracted soluble lignin as the source of these effects has not been conclusively proven, these data seem to support it given the lignin composition of *V. vinifera* (3:1 guaiacyl/syringyl units)³⁵ and the fact that the thermal decomposition of lignin is known to produce guaiacyl-type phenylpropanoids.^{36,37}

Taken together, the observed results for guaiacol, syringol, and eugenol demonstrate the importance of injection port temperature for the quantitation of VPs in berry extracts. Indeed, the fact that frequently referenced studies use high injection port temperatures calls into question their quantitative accuracy.^{21,24,26,29,34,38} The observations described above led to the selection of 220 °C as the injection port temperature to use for GC–MS/MS method validation.

uHPLC–QToF Method Development. Even though the injection port temperature evaluation supported the selection of 220 °C, the underlying data were based on spike-recovery studies that could not validate the quantitative accuracy of the developed GC–MS/MS method. Therefore, in addition to validation of the GC–MS/MS method, cross-validation using an alternative method was necessary. Pollnitz et al. showed that headspace solid phase microextraction (HS–SMPE) improved the quantitative accuracy of guaiacol compared to LLE.²⁸ However, in a separate study, Pollnitz et al. also showed that

Table 2. Validation Summary for the GC–MS/MS and uHPLC–QToF Methods^a

compound	GC–MS/MS				uHPLC–QToF				HOR _{RAT}
	LOD (ng/g)	LOQ (ng/g)	accuracy (ng/g) ^b	repeatability (ng/g) ^b	LOD (ng/g)	LOQ (ng/g)	accuracy (ng/g) ^c	repeatability (ng/g) ^c	
1 4-ethylguaiaicol	0.54	1.62	97/85	8/9	0.90	2.72	105/107	14/1	–
2 4-ethylphenol	0.18	0.55	95/82	5/5	1.12	3.39	90/100	17/2	–
3 4-methylguaiaicol	0.16	0.49	90/75	6/3	0.40	1.22	98/111	9/4	–
4 eugenol	0.35	1.05	113/106	13/3	0.40	1.22	112/94	6/5	1.5
5 guaiaicol	0.55	1.67	93/90	8/7	0.35	1.06	110/111	9/1	0.6
6 <i>m</i> -cresol	–	–	–	–	0.13	0.40	89/100	5/4	–
7 <i>o</i> -cresol	0.28	0.85	86/67	14/2	0.27	0.83	90/106	10/6	1.2
8 <i>p</i> -cresol	0.25	0.77	76/82	6/3	0.31	0.94	92/114	10/3	–
9 syringol	0.28	0.84	126/99	6/4	0.87	2.64	90/87	13/3	–

^aAccuracy and repeatability (reported as CV %) were calculated from five spike-recovery samples at each concentration. The Horwitz ratio (HOR_{RAT}) compared GC–MS/MS and uHPLC–QToF results above their respective LOQs for a sample of smoke-exposed Pinot Noir berries. The compound numbers correspond to those in Figure 1. ^bEvaluated at 2/100 ng/g. ^cEvaluated at 5/200 ng/g.

HS-SPME was not suitable for the analysis of 4-ethylguaiaicol with a matched isotopic internal standard (as used in the study presented here).³⁹ On-column injection may be useful to overcome this issue,⁴⁰ but for cross-validation of the GC–MS/MS method developed herein, including the extraction procedure, it was desirable to have a method with alternative sample preparation, separation, and detection procedures. Therefore, a novel method using uHPLC–QToF for the analysis of VPs in *V. vinifera* berries was developed and validated. The use of QToF has several advantages for this application. (1) The low precursor *m/z* of VPs leads to many types of isobaric interference using nominal mass instruments (even with MS/MS); QToF reduces these isobars. (2) QToF affords the possibility of retrospective analysis as new metabolites linked to smoke-exposure are discovered.

This method is also the first uHPLC separation of free VPs in smoke-exposed berries. It is likely that the use of uHPLC–MS for this application has been avoided because the direct analysis of simple phenolic compounds can suffer from ion suppression^{41,42} and poor ESI sensitivity, even in the presence of basic mobile phase modifiers. Sample cleanup (SPE) and derivatization can address these issues. Dansyl chloride is an excellent derivatization agent for mass spectrometry, as it contains a tertiary amine that imparts a high ionization efficiency in positive ESI with an acidic mobile phase modifier.²⁷ Indeed, several studies have used this approach to study eugenol and isoeugenol.^{43,44} To adapt these methods for the current application, it was necessary to have the pH be >10, as the pK_a values of the relevant VPs were approximately 10. To accomplish this, berry HMG was cleaned up via C18 SPE and the final eluent (20% ACN) buffered with 125 mM carbonate to pH 11. SPE was required to overcome the intrinsic buffering capacity of HMG (Figure S5). The concentration of carbonate was set at 125 mM to minimize MS source fouling and to avoid precipitation of the buffer during derivatization (in 30% acetone). While these conditions resulted in an absolute response from fortified berry extracts that was weaker than those of control reactions (Figure S6), method sensitivity was suitable for the analysis of VPs in smoke-exposed berries (*vide infra*).

Like the GC–MS/MS analysis of VPs, the developed uHPLC chromatographic method focused on obtaining adequate resolution of the cresol structural isomers, as all other dansylated VPs were resolved by *m/z*, so co-elution was

not expected to be an issue. While not baseline-resolved, the cresols were sufficiently separated using a biphenyl stationary phase (Figure 1). This chromatographic resolution would not have been possible without dansylation, as the naphthyl aromatic core of the dansyl group provided a large surface area for interaction with the biphenyl stationary phase that facilitated the separation.^{45,46}

Method Validation. Using the sample preparation, chromatography, and detector parameters optimized herein, the GC–MS/MS and uHPLC–QToF methods were validated to determine their accuracy, repeatability, LOD, and LOQ values (Table 2). For GC–MS/MS, the recovery and repeatability ranged from 67 to 124% and from 2 to 14%, respectively, across low (2 ng/g) and high (100 ng/g) spike-recovery samples. The low recoveries for *o*-cresol and *p*-cresol were attributed to two factors. (1) A co-eluting matrix interference impacted accurate quantitation of *o*-cresol, and (2) using *d*₄-4-ethylphenol as the ISTD for *o*-cresol had an impact (Figure S4). The reported LOD and LOQ values were consistent with those determined by similar methods in the literature and, on the basis of reported concentrations, were low enough to permit the analysis of free VPs in smoke-exposed berries.^{17,26,34} For the uHPLC–QToF method, the recovery and repeatability ranged from 87 to 114% and from 1 to 17%, respectively, across low (5 ng/g) and high (200 ng/g) spike-recovery samples (Table 2). The LOD and LOQ values were also adequate.

Cross-validation of the GC–MS/MS and uHPLC–QToF methods was performed to confirm quantitative accuracy. Pinot Noir berries that were exposed to wildland fire smoke in 2015 in the Okanagan valley of British Columbia, Canada, were evaluated to simulate a real-world sample, as might be encountered during routine testing. Using the Horwitz ratio [HOR_{RAT} (Table 2)] as a measure of equivalence, the results for samples above their respective LOQs showed that the methods yielded quantitative values that were within acceptable cross-validation variance (HOR_{RAT} = 0.5–2.0).³¹ More specifically, the results of the Horwitz test demonstrated that the selected GC–MS/MS injection port temperature of 220 °C yielded quantitatively accurate free VP concentrations, as did the novel uHPLC–QToF method developed herein.

Acid-Mediated Hydrolysis of VP-Glycosides. The majority of VPs in *V. vinifera* berries are sequestered as glycosides.²¹ Enzymatic activity during fermentation,¹⁴ which

can be influenced by vinification practices,¹¹ results in the hydrolysis of a fraction of the VP-glycosides, with a corresponding increase in the level of free VPs. Therefore, accurate quantitation of the VP-glycoside fraction in smoke-exposed berries was paramount, as this would provide information about the total VP load that can contribute to the expression of smoke taint after fermentation. In addition, while there are conflicting studies regarding the impact of bottle aging,^{19,29} there is strong anecdotal evidence that the intensity of smoke taint can increase with time. If we presume VPs are responsible for this phenomenon, an accurate assessment of the VP-glycoside concentration in berries may help predict the potential for this increase.

Methods using LC–MS to quantify intact VP-glycosides based on calibration against one isotopic internal standard have been reported.^{18,22} However, the broad range of VP-glycosides creates analytical challenges that can impact the quantitative accuracy of such methods. These challenges include the chromatographic resolution of isobars and, because of differences in response factors,¹⁸ the availability of analytical standards and isotopic standards for VP-glycosides. Because of these challenges, release of the VP and subsequent quantitation using the GC–MS/MS method reported herein were determined to be the best approach for assessing the total VP load in smoke-exposed berries.

The results of acid-mediated hydrolyses of VP-glycosides in smoke-exposed berries and wine are inconsistent in published studies, especially when the loss of the glycoside was compared with recovery of the liberated VPs. For instance, Hayasaka et al.²⁴ reported 5–37% recovery of VPs from acid digests in wine and Ristic et al.¹⁹ reported similarly low VP recoveries. Moreover, both studies quantified the intact glycosides using isotopic VP-glycosides with different response factors,¹⁸ making it inherently challenging to validate the hydrolytic procedures because the glycoside:VP mass balance would be difficult to determine. Given the importance of the glycoside:VP mass balance to the development of smoke taint, a series of model glucosides (Figure S7) were synthesized to facilitate the development and validation of an acid hydrolysis method. The glycosides were all β -D-glucosides, which is postulated to be the dominant anomeric form *in planta*.²¹

Existing methods use H₂SO₄ (pH 1) at 100 °C for 60 min to achieve VP-glycoside hydrolysis.^{14,21,24} While it was hypothesized that the oxidative character of H₂SO₄ and the antioxidant behavior of VPs were incompatible,⁴⁷ a series of control hydrolyses using H₂SO₄, HCl, and TFA (pH 1.3–1.5) demonstrated that free VPs were stable at 100 °C for 60 min regardless of the acid used (data not shown). Despite this equivalence and the published use of H₂SO₄, HCl was selected for the following reasons. (1) The developed hydrolytic procedure could be extended to other glycosidically bound secondary metabolites where the oxidative character of H₂SO₄ might be an issue. (2) Because of the high volatility, HCl can be removed *in vacuo* to facilitate additional sample processing. (3) TFA causes ion suppression issues in negative mode ESI.

With HCl as the selected acid, it was shown that 1 h was insufficient to quantitatively digest all of the model glucosides that were added to Merlot HMG (Figure S8). This suggested that existing methods underreport the total VP load in berries and wine, which may explain some of the difficulties encountered in predicting perceptible smoke taint based on VP concentrations.⁸ The observed differences in hydrolytic rates for the model glucosides led to 4 h at 100 °C and pH 1.5

being adopted as conditions that were likely to yield a quantitatively accurate assessment of the glycosidically bound VPs in smoke-exposed berries. It is worth noting that the syringol gentiobiose hydrolytic behavior is assumed to mirror the hydrolysis of other diglycoside VPs. The similarity between the syringol glucoside and gentiobiose hydrolyses (Figure S8) supports the validity of this extrapolation.

Also of interest was the observation that while these conditions were suitable for the liberation of VPs from their glycosides, control reactions in acid (borosilicate glass vials, pH 1.5) showed low recoveries for several compounds after 4 h at 100 °C in the matrix (Table 3), most notably for guaiacol

Table 3. Recoveries of Free VPs from Fortified Acid Digests in Glass and PTFE Reaction Vessels and Recoveries of VPs Liberated from Model Glucosides Fortified into Merlot HMG^a

compound	free VPs		glucosides
	glass (%)	PTFE (%)	Merlot HMG (%)
4-ethylguaiacol	55 ± 1.7	89 ± 1.9	– ^b
4-ethylphenol	96 ± 4.6	97 ± 4.1	113 ± 0.1
4-methylguaiacol	78 ± 9.2	82 ± 3.9	95 ± 0.6
eugenol	92 ± 3.9	92 ± 3.7	105 ± 0.2
guaiacol	28 ± 2.1	100 ± 1.4	– ^b
<i>m</i> -cresol	– ^c	– ^c	– ^{b,c}
<i>o</i> -cresol	98 ± 4.4	103 ± 4.4	– ^b
<i>p</i> -cresol	71 ± 1.0	92 ± 4.2	120 ± 0.2
syringol ^d	142 ± 7.2	71 ± 2.3	101 ± 3.1

^aAcid digests were performed at 100 °C for 4 h at pH 1.5. All values are means ±1 standard error of the mean (*n* = 3). ^bGlucosides for these VPs were not synthesized. ^c*m*-Cresol co-eluted with *p*-cresol on the SLB-5 column. ^dData are shown for syringol- β -D-glucopyranoside.

(28%) and 4-ethylguaiacol (55%). A subsequent change to PTFE reaction vessels corrected this issue, which suggested nonspecific surface effects were interfering with the assay when it was conducted in borosilicate glass. While it is unclear in published methods what type of reaction vessels were used for acid digests, these data demonstrated another key area that could skew the accurate quantitation of the total VP load in smoke-exposed berries.

A final test of the developed acid-mediated hydrolytic procedure was conducted by fortifying Merlot HMG with the model glucosides and correlating the loss of the glucosides with the recovery of free VPs (Table 3). These data showed quantitative recovery of VPs was achieved following digestion for 4 h at 100 °C in PTFE reaction vessels.

Accurate quantitation of the total VP load in smoke-exposed *V. vinifera* berries is likely to be a critical component of an effective risk assessment and mitigation program for vineyards and wineries. Herein, we report the development of a complete analytical workflow to accurately quantitate free and glycosidically bound VPs in smoke-exposed berries. Application of the demonstrated workflows should facilitate an accurate assessment of the potential correlation between perceptible smoke taint and total VP concentrations, which will help the wine industry make informed decisions regarding how to process smoke-exposed berries.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b03225.

Synthetic details, GC–MS/MS and uHPLC–QToF method and instrument details, sample preparation details, and model glucoside structures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Chemistry Department, University of British Columbia Okanagan, 1177 Research Rd., Kelowna, BC V1V 1V7, Canada. E-mail: wesley.zandberg@ubc.ca. Phone: 250-807-9821.

ORCID

Matthew Noestheden: 0000-0003-3187-2701

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Notes

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