Evaluating Diagnostics and Mitigation of Smoke Exposure in Grapes and Wine

A thesis presented in fulfillment of the requirement for the degree of Doctor of Philosophy

Colleen Szeto
B.A., M.P.S

School of Agriculture, Food, and Wine
The University of Adelaide

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Contents

Thesis Abstract

Smoke taint refers to the unpleasant smoky and ashy attributes that can characterise wines made from grapes exposed to bushfire smoke. As outlined in **Chapter 1**, there is an urgent need for strategies that detect smoke exposure in grapes and mitigate its effect on wine sensory profiles, especially given that climate change models predict more frequent bushfires in the future. Current diagnostics of smoke exposure in grapes involve the measurement of volatile phenols and their glycoconjugates by GC-MS and HPLC-MS/MS, respectively. While their presence is an essential indicator, their concentrations are inherently dynamic and must be contextualised with respect to their natural abundance in grapevines leaves and fruit, the timing, density, and duration of smoke exposure, the fuel from which smoke is derived, and the timing of sampling for smoke taint analysis of grapes. Critical gaps in these domains confound our interpretation of current diagnostics and to achieve clarity, they need to be addressed. **Chapter 1** concludes with a statement of research aims underpinning this thesis.

To begin, this thesis investigates the volatile phenol glycoconjugates that are most indicative of smoke exposure in grapes and wine across distinct wildfire seasons in California (**Chapter 2**). The complex biochemistry of current smoke taint markers prompted investigation into their accumulation in grapevines following smoke exposure. This thesis demonstrates a temporal gap between the depletion of smoke-derived volatile phenols and the subsequent appearance of glycoconjugates, as well as the potential use of particulate matter sensors to monitor vineyard smoke exposure (**Chapter 3**). In parallel, this thesis also responds to the challenges of high analytical demand during the peak of the 2019/2020 bushfire season in Australia. Measuring volatile phenols and glycoconjugates is a resource-intensive process, and this thesis explores fluorescence spectroscopy as a rapid detection tool (**Chapter 4**).

Beyond acquiring more knowledge of existing smoke taint markers and rapid methods of detecting them, further progress in smoke taint diagnostics may be achieved with the of alternative markers. Current diagnostics measure fewer than ten volatile phenols, but far more exist in smoke. In addition, smoke carries ozone, a known oxidant that induces a biochemical stress response in plants. Using an untargeted metabolomics workflow, this thesis unveils both endogenous and exogenous metabolites that differentiate smoke-affected grapevines at different time points, from 2 hours to 20 days, following smoke exposure (**Chapter 5**).

Fortifying the grape and wine industry against the risks of grapevine smoke exposure requires not only improved diagnostics, but also novel mitigation strategies. Current remediation practices involve reducing volatile phenols and their glycoconjugates from wine or masking their unpleasant smoky, ashy attributes. While essential, these approaches prolong mitigation efforts until wines are at or near completion. More importantly, they present risks of diminishing positive attributes in wine.

Ideally, preventative strategies should be employed in the vineyard. This thesis describes the development, evaluation, and application of a smoke box for conducting mitigation trials with improved efficiency and demonstrates the potential of activated carbon fabric to protect grapes from contamination by smoke (**Chapter 6**). To conclude, this thesis reflects on the enclosed chapters and other possible directions for future smoke taint research (**Chapter 7**).

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Colleen Szeto

Signature: Date: 14/9/2022

Publications

This thesis contains two manuscripts published in peer-reviewed scientific journals.

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Conferences

1. Overcoming smoke taint in the vineyard
Australian Research Council Training Centre for Innovative Wine Production Industry Seminar
Margaret River, Western Australia, 15 May 2019 (oral presentation)

2. Does in-canopy misting mitigate the intensity of smoke taint in grapes and wine?
17th Australian Wine Industry Technical Conference
Adelaide, Australia, 21-24 July 2019 (poster presentation)
3rd International Flavor and Fragrance Conference
Viña del Mar, Chile, 3 October 2019 (oral presentation)

3. Exploring regional, varietal, and temporal variation of naturally abundant volatile phenol glycoconjugates in grapes to improve evaluation of smoke taint risk
Crush 2021: The Grape and Wine Science Symposium
Adelaide, South Australia, 16 June 2020 (oral presentation)

4. Smoke taint amelioration
School of Agriculture, Food, and Wine Postgraduate Symposium
Adelaide, South Australia, 28 September 2020 (oral presentation)

5. Bridging grapevine smoke exposure to smoke taint in wine
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Coonawarra, South Australia, 9 November 2021 (oral presentation)

6. Thinking inside the box: a novel approach to smoke taint mitigation trials
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Acknowledgments

"Men exist for each other. Then either improve them, or put up with them."

—Marcus Aurelius

As my completion date approaches, I have realized that the journey of this doctorate has unexpectedly brought about great joy. Embarking on the pursuit of a PhD was a commitment to cultivating my thinking and skills as a research scientist. I chose oenology as my scaffold from the perspective that grapes and wine were not only interesting as chemical matrices, but also more appealing to work with than mice, bodily fluids, or little crystals in a flask. However, I grew up in a home squeezed between blocks of cranberry bogs in the Bay State, not amongst the sprawling vineyards of the Golden State, and while I acknowledge the privileges of having tasted many great wines and visiting many beautiful wine regions, I have never held a passion for making or drinking wine, and that has not budged. Thus, the joy from pursuing this doctorate was not a result of developing a niche understanding of a confronting problem in the grape and wine industry, nor did it emerge from sharing the findings enclosed in this thesis to those keen to learn about them. Instead, it has emerged from all of the people with whom I have been fortunate to live, play, and work with during my time in Adelaide.

I have always been comfortable working on my own, and this internal drive had never been questioned until my major review. After I gave my presentation, Kerry Wilkinson (my principal supervisor) told me that there was such a thing as being 'too independent' and that as part of a research team, it was my responsibility to keep everyone in the loop as the merry-go-round of research turned with each step forward. The completion of a doctorate is a massive team effort, but this is easy to forget when deep in the trenches of collecting, analyzing, and writing about data. Whenever I managed to drag myself out of these trenches, Kerry was always there to support me. She held me accountable on the occasions that I dropped the ball and encouraged me to take on a diverse array of challenges. She pointed me in the right direction when I asked for guidance, and knowing how I worked best, she left me to pursue it. I would like to express gratitude for her patience, humble leadership, honesty, and high standards. It has been a privilege to work with her and contribute to Australia's legacy as the pioneer in smoke taint research.

Once the concept of being part of a team had been planted, I had the opportunity to experience it through working with researchers from the Australian Wine Research Institute, Metabolomics SA, and E. & J. Gallo Winery. These experiences involved an Orbitrap mass spectrometer and regular team briefings, but most importantly, practice in the art of building a plane while flying it. Just 'doing' is always faster than 'learning while doing' but it takes considerable experience with the second to achieve the first. It would be easy for a senior team member to grab a hold of the reins and drop me off at my destination, but a strong team understands the value of teaching junior members how to map their course and drive.

From E. & J. Gallo Winery, I would like to thank Bruce Pan for also holding me to a high standard, offering support as I waded through the unfamiliar waters of method development, and teaching me to see how the results and implications from one trial transitioned into the hypothesis for the next. I would also like to thank Hui Feng, Bryant Blair, and Qiang Sui for being shepherds in the lab while I was in Modesto, polishing the developed method, analyzing additional samples on my behalf, and maintaining excellent communication, after I returned to Australia. I would also like to thank E. & J. Gallo Winery for their support and contribution as an industry partner of the ARC Training Centre for Innovative Wine Production.

From the Australian Wine Research Institute and Metabolomics SA, I would like to thank Natoiya Lloyd not only for her enthusiasm for all things mass spectrometry, but also for her encouragement and praise of each achievement that I made on the path from preparing homogenate samples to extracting a list of putative novel indicators of smoke taint. I would also like to thank Luca Nicolotti for his willingness to help me at any time regardless of what he was doing and ruffling the stillness of the lab with his humour. Lastly, I would like to thank Markus Herderich (one of my co-supervisors) for asking the tough questions, expecting nothing but well-reasoned responses, and providing access to the resources afforded by the Australian Wine Research Institute.

Not many PhD students get the chance to learn about teamwork through direct experience, and I would like to express gratitude to the Australian Research Council Training Centre for Innovative Wine Production for the opportunities to pursue these collaborations; to the Australian Research Council Industrial Transformation Research Program (project number ICI70100008) and Wine Australia (project number WAT 2005) for funding them; and to the University of Adelaide for waiving tuition fees. I also wish to extend my gratitude to my co-

authors and all those included in the acknowledgment sections within each thesis chapter for their respective contributions.

Being a part of the Training Centre required two presentations per year and a neat record of KPIs. In exchange, I was able to network and travel with other students to wine regions including the Adelaide Hills, McLaren Vale, Clare Valley, Coonawarra, and Margaret River. Even as someone that cannot differentiate a $30 bottle of wine from a $100 bottle of wine with confidence, these regions were beautiful, and I enjoyed the chance to get out of the office, learn a few things, share my knowledge with local growers and winemakers, and see more of Australia. The Training Centre also granted the opportunity to speak at the Crush Symposium held in Adelaide at the National Wine Centre and speak at the International Flavor and Fragrance Conference held in Viña del Mar, Chile. Throughout my candidature, cycling has been my primary mode of transportation, and these trips enabled me to explore Australia outside of my 30 km bubble. I wish to thank Vladimir Jiranek, Joanna Sundstrom, Nick van Holst, Vinay Pagay (one of my co-supervisors), Renata Ristic (my independent advisor), and Anne Auricht for their efforts to identify, organize, and deliver me to the doorsteps of these opportunities and making the most of our location in South Australia, particularly after Covid-19 rippled across the globe.

There was a delay between the first case of Covid-19 in Wuhan and the first case in South Australia, and I remember that between Sunday, 14 March 2020 in Port Elliot and Monday, 15 March 2020 in Cumberland Park, the toilet paper had vanished from the shelves. I am very fortunate that my work was not significantly impeded by the pandemic, but like everyone else, the combination of lacking certainty and control over basic aspects of living has at times, tipped my scales out of equilibrium. I would like to thank my colleagues for maintaining their focus and generosity over the difficult period of the last few years and keeping the Wine Innovation Central building a great place to work. There were moments when I believed it was ludicrous, and even selfish, to continue trying to think about smoky grapes and wine when people around the world were isolated, afraid, and dying from the coronavirus. I am not about to claim that the significance of the enclosed work in any way justified the privileges associated with completing it during this period. I only wish to express sincere gratitude that I was able to do so.

When I first landed in Adelaide, a person named Tim Wong picked me up in a hatchback. Based on evidence from a few phone conversations, a good feeling in my gut, and

a bit of luck, I moved into a home and a friendship with someone that has looked after me from the start. Tim emphasized that a home is a place to relax, retreat, and unwind. When I felt down and out, his kindness, generosity, and support always helped me to my feet. After moving in with Tim, my second order of business was to call my family and close friends from home. I would like to thank them for supporting my journey to Australia, a distant place they feared to be crawling with dangerous creatures poised to attack me upon landing. I would also like to thank them for their constant support of my endeavours, regardless of how unfamiliar they may seem. At times, I leave myself behind, and I wish to thank them for being both my gravity and my ladder to the stars. The final step to being sorted in Adelaide was to find a swimming club. I have always loved the water, but I had never learned to swim proper freestyle. After seeing the 50m pool at the Unley Swimming Centre for the first time, I was hooked. Learning how to move through the water rejuvenated my tired mind, relaxed my anxious body, and enabled me to focus on my work. Swimming was also a conduit to my friendship with Marc Loader, whom I would like to thank for always believing in me and reminding me to be (a little) less serious.

Chapter 1

Literature Review and Research Aims

Literature Review

1. Introduction

In December 2019, over 9,000 calls were made to the South Australian Country Fire Service bushfire hotline, relative to the rolling average of 3,198 from the preceding decade (CFS Annual Report 2019-2020). The severity of the 2019/2020 bushfire season served as a global reminder of the inextricable links between the extreme conditions of climate change and the risks of more extended, intense fire seasons ahead (Jolly *et al.* 2015). It also amplified concern regarding the risk of smoke taint, which refers to the 'smoked meat', 'campfire', and 'medicinal' aromas and flavours and a drying, ashy aftertaste that characterise wine made from grapes exposed to bushfire smoke (Parker *et al.* 2012). The awareness and concern regarding smoke taint has become ubiquitous across the grape and wine industry, a state primarily attributed to intense wildfires in or near major wine regions that is anticipated to increase in frequency and intensity due to climate change (Scholze *et al.* 2006). Between December 2019 and June 2020, the Australian Wine Research Institute's Commercial Services laboratory received over 4,600 grape and wine samples for smoke taint analysis, an increase from 600 samples in a "typical" year (AWRI Annual Report 2020). It is crucial that improved strategies for the detection and mitigation of grapevine smoke exposure are developed, especially given the predictions for increased fire activity and severity in the future and the ample concern for at-risk growing regions (Clarke *et al.* 2011; Krawchuk *et al.* 2009).

The occurrence of smoke taint in wine results from grapevine exposure to smoke, and its signature 'smoky', 'ashy' sensory profile is attributed to smoke-derived volatile phenols such as guaiacol, 4-methylguaiacol, *o*-cresol, and syringol (Parker *et al.* 2012). The risk of smoke taint following grapevine smoke exposure is driven by several factors, including the timing, duration, and frequency of smoke exposure (Kennison *et al.* 2009). Thus, this risk cannot be assessed by the mere presence or absence of volatile phenols in grapes. Some volatile phenols have a natural presence in specific grape cultivars (e.g. Shiraz), and they can be extracted from oak during wine aging (Pollnitz *et al.* 2004; Ristic *et al.* 2015). These confounding sources of volatile phenols introduce complexity to the identification of smoke-affected grapes and wines at risk of smoke taint. Moreover, volatile phenols are predominantly found in grapes and wine as an array of bound glycoconjugates, rather than their free analogues (Hayasaka *et al.* 2010b; Ristic *et al.* 2016). Glycoconjugates are non-volatile and relatively stable at pH levels in wine (Ristic *et al.* 2017; Whitmore *et al.* 2021), but they can contribute

to the perception of smoke taint if the bond between the sugar moiety and the volatile phenol is cleaved through enzymatic hydrolysis during fermentation or wine tasting (Kennison *et al.* 2008; Mayr *et al.* 2014).

The mechanisms that orchestrate the uptake and glycosylation of smoke-derived volatile phenols are not fully understood, but the apparent 'disappearance' of volatile phenols in grapes within 24 hours of smoke exposure has been attributed to glycosylation (van der Hulst *et al.* 2019). While the 'disappearance' of volatile phenols may be rapid, it can take up to two weeks for corresponding increases in glycoconjugate levels to reach a plateau (Dungey *et al.* 2011). Both leaves and grapes have the capacity to absorb smoke-derived volatile phenols, and while volatile phenols can be translocated between these sinks, this does not remain true for their glycosylated equivalents (Favell *et al.* 2021; Culbert *et al.* 2021a; Jiang *et al.* 2021; Hayasaka *et al.* 2010a). Thus, the prevailing hypothesis regarding the presence of smoke-derived volatile phenols and their glycoconjugates in grapes is that volatile phenols are absorbed by the skins of the berries and diffuse into the pulp once glycosylated (Krstic *et al.* 2015).

Glycosylation has been described as a detoxification strategy (Winterhalter and Skouroumounis 1997; Härtl *et al.* 2017), but it is not a response exclusive to grapevine exposure to abiotic stresses such as bushfire smoke. Research has also identified a natural presence of certain volatile phenol glycoconjugates in some grape varietals, independent of smoke exposure (Ristic *et al.* 2015; 2016). There is no doubt that volatile phenols in grapes increase following grapevine smoke exposure and contribute to the perception of smoke taint in wine, yet their presence in grapes and wine (independent of smoke exposure) and their conversion into glycoconjugates introduces complexity to establishing clear cut-off values that differentiate smoke-affected grapes and wine from those unaffected by smoke exposure.

Herein, this complexity is addressed by improving the speed and resolution of detection methods to enable earlier decision-making and developing proactive mitigation strategies. The first advantage of this approach is that earlier detection will capture chemical profiles with less interference from downstream sources accrued during winemaking. Concentrations of volatile phenols and glycoconjugates in grapes are influenced by baseline levels, grapevine smoke exposure, or both, whereas in wine, they are also influenced by winemaking decisions (e.g. yeast selection, duration of skin contact, oak contact). In other words, earlier detection provides a less filtered snapshot of grapevine smoke exposure. This paves the way towards the

establishment of objective cut-off values of smoke taint indicators that decisively differentiate smoke-affected fruit. The second advantage of this approach is that earlier detection streamlines decision-making for the management of smoke-affected fruit before it is even off the vine. While sensory analysis of wine is the ultimate evaluation of the intensity of smoke taint, it cannot be performed until fermentation is complete, and if remediation is required, winemakers are limited to a narrower range of options and time window within which to achieve it. By contrast, if grapevine smoke exposure detected early, mitigation can begin in the vineyard and proceed throughout the winemaking process. Alternatively, if the intensity of grapevine smoke exposure is beyond the capacity of remediation, it may be more cost-effective to leave the fruit on the vine than process it into unsaleable wine. Thus, earlier detection provides a more candid representation of smoke exposure and the options available to mitigate the risk of smoke taint in finished wine.

This thesis is devoted to evaluating and empowering the efficacy of a 'go hard and go early' approach towards the mitigation of smoke taint. To be successful, this approach requires not only targeted mitigation strategies, but also precise diagnostics that inform the severity of grapevine smoke exposure and the concomitant risk of smoke taint in grapes and wine. The prevailing aims of the enclosed work are towards improving the efficiency of smoke taint diagnostics and accelerating the development of novel mitigation strategies. These aims are pursued through developing an enhanced understanding of the current markers of smoke exposure and exploring rapid methods to detect them, investigating the potential for alternative markers, and evaluating vineyard-based mitigation strategies.

2. Detecting smoke exposure in grapes and wine

2.1 Quantitation of volatile phenols and their glycoconjugates in grapes and wine

The classical diagnostic of smoke exposure in grapes and wine is the quantitation of guaiacol and 4-methylguaiacol, two volatile phenols with low thresholds of sensory detection and high abundance in smoke (Maga *et al.* 1998). However, some volatile phenols have a natural presence in grapes that vary in concentration by grape variety (Ristic *et al.* 2016). Some volatile phenols are observed in wine due to extraction from toasted oak barrels during aging (Pollnitz *et al.* 2004). As a consequence of their shared formation pathway being lignin pyrolysis, some volatile phenols extracted from oak, including guaiacol, 4-methylguaiacol, phenol, syringol, and 4-methylsyringol (Ribéreau-Gayon et al. 2006), are also found in grapevine leaves and

fruit following smoke exposure (Kennison *et al.* 2007; Noestheden *et al.* 2018). However, when the source of the volatile phenols changes from oak barrels to bushfire smoke, the 'smoky' and 'smoked meat' attributes (Li *et al.* 2015) that once served to enhance wine complexity elicit the potential to suppress fruity attributes and transform wines to unidimensional 'cold ash', 'medicinal', and 'campfire' aromas and flavours. Thus, it is not the mere presence of volatile phenols, but the presence of sufficient volatile phenols derived from wildfire smoke exposure, that assembles the foundation for smoke taint perception.

In the early diagnostics of smoke taint, guaiacol and 4-methylguaicaol were the primary volatile phenols included due to their known association with smoky aromas and flavours (Maga *et al.* 1988). In a pioneering study by Kennison *et al.* (2007), these compounds were measured in wine using a stable isotope dilution assay (SIDA) method developed for gas chromatography-mass spectrometry (GC-MS) (Pollnitz *et al.* 2004; Hayasaka *et al.* 2010c). It was found that even in wines with guaiacol and 4-methylguaiacol concentrations below the referenced detection thresholds, smoke taint attributes could still be detected (Kennison *et al.*2007). The thresholds referenced by Kennison and colleagues were from Boidron *et al.* (1988), at 95 µg/L (for guaiacol) and 65 µg/L (for 4-methylguaiacol) in white wine. These thresholds were higher relative to those reported for guaiacol in later studies being 9.5 µg/L (Ferreira *et al.* 2000) and 23 µg/L (Parker *et al.* 2012) in red wine. Nonetheless, it was evident in the landmark study that, while useful indicators of grapevine smoke exposure, guaiacol and 4-methylguaiacol were not the only smoke-derived compounds driving the perception of smoke taint in wine (Kennison *et al.* 2007).

In a subsequent study, Kennison *et al.* (2008) monitored the evolution of volatile phenol concentrations in smoke-affected and unaffected Merlot must during fermentation. Volatile phenol concentrations increased throughout winemaking, a trend attributed to the acid- or enzyme-catalysed release (or both) of volatile phenols from the glycosylated precursors. Hayasaka *et al.* (2010b) confirmed this hypothesis through the identification of guaiacol β-D-glucopyranoside in smoke-affected Chardonnay and Sangiovese juice, and in a follow-up investigation, additional guaiacol glycoconjugates in smoke-affected Cabernet Sauvignon grapes (Hayasaka *et al.* 2010a).

The formation of volatile phenol glycoconjugates in grapes is achieved through the action of glucosyltransferases; however, the exact mechanism that underpins their formation remains unknown. Hartl *et al.* 2017 identified the UGT72B27 glucosyltransferase as a key

player due to its high gene expression in grapevines, specificity for volatile phenols, and rate of conversion of volatile phenols into glucosides. In a buffer environment, UGT72B27 glucosyltransferase yielded volatile phenol glucosides with no further substitution, which indicates regio-specificity. Glucosides of volatile phenols are found as natural constituents of grapes; however, volatile phenols are predominantly found in grapes as an array of disaccharides (van der Hulst *et al.* 2019). If disaccharides contain different sugar moieties, multiple enzymes are required to catalyse their donation, an exception being gentiobiosides, which contain two glucose moieties (Winterhalter and Skourimounis 1997). Additional work is required to identify other glucosyltransferases that contribute to the formation of glycoconjugates with greater complexity and the circumstances that prompt their formation.

Several studies have depended on the quantitation of guaiacol glycoconjugates in tandem with guaiacol and 4-methylguaiacol, to judge the efficacy of remediation trials (Ristic *et al.* 2011; 2013, Fudge *et al.* 2011; 2012a); however, a range of volatile phenols beyond guaiacol superseded this practice (Kelly *et al.* 2014; van der Hulst *et al.* 2019; Jiang *et al.* 2021). This change is attributed to studies that showed the synergistic effect of volatile phenols (including guaiacol, *o*-cresol, *p*-cresol, *m*-cresol, phenol, syringol, 4-methylsyringol, and others) on smoke taint intensity (Parker *et al.* 2012) and their ability to be hydrolysed from glycoconjugates during fermentation and wine tasting (Mayr *et al.* 2014). Thus, while glycoconjugates are non-volatile and relatively stable in wine during aging (Ristic *et al.* 2017; Whitmore *et al.* 2021), they are nevertheless volatile phenol reservoirs. Their inclusion in smoke taint analysis is paramount, particularly in grapes due to the rapid depletion of volatile phenols following grapevine smoke exposure and the potential risk of underestimating the severity of smoke exposure (van der Hulst *et al.* 2019; Jiang *et al.* 2021).

Glycoconjugates can be measured directly using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) (Dungey *et al.* 2011; Hayasaka *et al.* 2013). While also depending on a SIDA-based method, the absolute detection of glycoconjugates is hindered by the limited availability and expense of deuterium-labeled glycoconjugate standards. One strategy used to overcome these financial barriers is to quantify and report all volatile phenol glycoconjugates as syringol gentiobioside equivalents (Hayasaka *et al.* 2013); however, as the list and diversity of conjugated volatile phenol precursors continue to evolve (Caffrey *et al.* 2019), there are quantitative limitations associated with relying on a single chemical representative (Noestheden *et al.* 2018). Another strategy is to use acid hydrolysis as a proxy or indirect measurement of glycoconjugate concentrations (Wilkinson *et*

al. 2011). This approach involves measuring volatile phenol concentrations pre- and post-acid hydrolysis, and the difference between them is interpreted as the concentration of 'bound volatile phenols.' The bound volatile phenol fraction encapsulates the volatile phenol glycoconjugates, in addition to any other acid-labile volatile phenol conjugates that may have been excluded in the targeted, SIDA-based HPLC-MS/MS method. This analysis is readily performed by a GC-MS and more affordable deuterium-labelled and unlabelled standards, which in theory, makes it a more accessible and consistent method.

In practice, this strategy has been hindered by poor recovery of volatile phenols following acid hydrolysis. In a study conducted by Hayasaka *et al.* (2010c), volatile phenol concentrations were measured in grape homogenate and wine samples, after which they were acidified to pH 1 with sulfuric acid and heated to 100 °C for 1 hour. While 50% and 92% reductions in glycoconjugate concentrations were achieved in grapes and wine, respectively, the recovery of liberated volatile phenols was less than 10% in wine. Singh *et al.* 2011 modified this approach by including several sample preparation steps, such as sample purification by solid phase extraction prior to acid hydrolysis. Wilkinson *et al.* 2011 compared glycoconjugate quantitation results using the original acid hydrolysis approach (Kennison *et al.* 2008; Hayasaka *et al.* 2010c), the modified acid hydrolysis approach (Singh *et al.* 2011), and the HPLC-MS/MS approach (Dungey *et al.* 2011; Hayasaka *et al.* 2013). This work demonstrated that strong correlations existed between the acid hydrolysis approaches and the HPLC-MS/MS method; however, it also showed that the former were still impeded by low recovery values (i.e. 15-30% in grapes). Such a caveat indicates that, independent of the HPLC-MS/MS method, acid hydrolysis approaches may only serve as a preliminary screening tool.

To address these limitations, Noestheden *et al.* (2017) developed an optimised acid hydrolysis procedure for measuring bound volatile phenols in grapes and wine that resulted in recovery rates ranging from 71-103% for several key volatile phenols. Some implemented changes included the use of hydrochloric acid and a 4-hour incubation period in PTFE vessels, instead of the conventional sulfuric acid and a 1-hour incubation period in borosilicate glass tubes. Despite improved rates of recovery, subsequent work implementing this method did not resolve the mass balance discrepancy present between volatile phenol glycoconjugates and volatile phenols; in fact, the results inferred the presence of additional volatile phenol precursors resistant to acid hydrolysis (Noestheden *et al.* 2018).

At present, volatile phenols and their glycoconjugates remain the best chemical markers of grapevine smoke exposure. However, the significant cost, both the time and the resources required for their quantitation, warrant supplementation with rapid detection methods. This would improve the efficiency of current diagnostics by enabling grapes and wine samples to be categorised into different smoke taint risk profiles (e.g. low, medium, high) and reserve comprehensive analysis of volatile phenols and their glycoconjugates for samples that cannot be readily differentiated as low- or high-risk.

2.2 Approximation of grapevine smoke exposure with rapid, spectral analysis and chemometrics

Rapid spectral detection tools are an emerging diagnostic for grapevine smoke exposure. While GC-MS and HPLC-MS are essential analytical tools, these classical techniques have several disadvantages that are improved with the integration of spectral techniques into qualitative and quantitative analytical workflows. Classical techniques require time-intensive sample preparation, method development, and data acquisition and analysis. Additional disadvantages include the high costs incurred by instruments, internal standards, consumables, and skilled labour. On the contrary, spectroscopic techniques require minimal sample preparation and acquisition time, and many instruments used in spectral techniques are less expensive and readily adaptable to automation. Moreover, developments in sensor technology have led to wider availability of spectral techniques in hand-held formats. Relative to the classical equivalent, these characteristics reduce the skill level needed for labour associated with spectral data acquisition.

A critical difference between classical and spectral techniques is the specificity of data collected and the complexity of subsequent statistical analyses required to interpret the data. In general, classical techniques generate data that are more specific to analytes of interest and thus require less complex statistical analysis to extract key information. In classical techniques, the role of the analyst is to isolate analytes in a sample matrix, minimise interference from other constituents, and validate observed signals with a known standard or spectral fragmentation pattern. In spectral techniques, the opposite is true. The role of the analyst using a spectral technique is to extract portions of the holistic ‘fingerprint’ of the sample matrix that best correlate with the analytes of interest. As a result, a major obstacle to the implementation of

spectral techniques is the substantial skill level of labour required to develop and maintain data processing workflows that extract and model relevant information (Cozzolino 2014).

In the grape and wine industry, ultraviolet (UV), visible (Vis), mid infrared (MIR), near infrared (NIR), and fluorescence spectroscopy in tandem with multivariate statistical analysis have been investigated to facilitate quality control workflows and routine analyses; however, FTIR methods are the most common (Cozzolino and Dambergs 2010). Notably, Patz *et al.* 2004 demonstrated the ability of FTIR spectroscopy in combination with partial least squares regression to measure alcohol (% abv), fructose (g/L), glucose (g/L), total phenols (mg/L), and total acid (g/L) concentrations in wine, with correlation coefficients (R^2) in excess of 0.9734. Access to this information with a highly accurate, rapid method is invaluable to quality control of fermenting musts and finished wine, particularly at large-scale wineries and commercial laboratories; however, the adaptability of FTIR spectroscopy is limited to the quantitation of compounds with concentrations greater than 0.2 g/L (Bauer *et al.* 2008).

The limited sensitivity of FTIR spectroscopy was corroborated in a study that trialed the use of a hand-held NIR-Vis spectrometer to perform rapid grape quality assessment at receival (Porep *et al.* 2015). Among conventional quality assessment parameters (e.g. fructose, glucose, titratable acidity, pH, tartaric acid and malic acid), gluconic acid and acetic acid were also measured as indicators of grape rot, but their low concentrations and minimal impact on the spectra, led to poor prediction. This presents a challenge for the use of FTIR spectroscopy to detect smoke exposure in grapes and wine because volatile phenols, even summed, are at concentrations orders of magnitude lower than other constituents in the wine matrix such as sugars, acids, alcohols, polysaccharides, and phenolic compounds (Waterhouse *et al.* 2016). An early study reported a combined concentration of guaiacol and 4-methylguaiacol at approximately 1,800 µg/L (Kennison *et al.* 2007); however, this was in great excess of concentrations of guaiacol and 4-methylguaiacol typically reported in wines with perceivable smoke taint. For example, in a trial that examined the effects of grapevine defoliation on smoke taint intensity, the wine with elevated an ‘smoke’ aroma, ‘smoke’ flavour, and ‘ashy aftertaste’ contained a mere 3.3 µg/L of guaiacol and trace levels of 4-methylguaiacol (Ristic *et al.* 2013). Scrimgeour *et al.* 2021 investigated the feasibility of MIR spectroscopy to predict total volatile phenols, total volatile phenol glycoconjugates, and syringol gentiobioside concentrations in grapes and wine but found poor correlations between actual and predicted values in the test set.

Achieving adequate sensitivity in a complex matrix such as wine is also an obstacle in fluorescence spectroscopy. Many polyphenols in wine are natural fluorophores, including non-flavonoids (e.g. phenolic acids, phenolic aldehydes, and stilbene-like compounds) and flavonoids (e.g. flavanols, flavan-3-ols, and proanthocyanidins) (Airado-Rodríguez *et al.* 2011). These polyphenolic compounds contribute to wine astringency, flavour, and colour, and both their abundance and reflection of viticultural and winemaking practices enable them to discriminate region, variety, and vintage in wine (Waterhouse *et al.* 2016). Polyphenols present the largest obstacle to the discrimination of wine according to smoke-derived volatile phenols with fluorescence spectroscopy because they have a greater abundance and higher quantum yields due to their highly conjugated structure.

Another major challenge to successful smoke taint classification with spectral techniques is the diversity of volatile phenol profiles and their context- and concentration-dependent correlation to smoke taint intensity. These aspects complicate classification because spectral techniques rely on statistical analyses to sift through haystacks and identify needles that most consistently correlate with a property of interest. Unless measuring a pure standard, spectral data are correlative by default (Cozzolino *et al.* 2014). Thus, constructing a classification model for smoke taint is a matter of bridging two correlations, one between volatile phenol profiles and the sensory perception of smoke taint and the other between spectral data and volatile phenols in a complex matrix. The first is required to build representative calibration sets, and the second is required to identify the relevant spectral pattern.

Some of these challenges were encountered in a study by Fudge *et al.* 2012b, which assessed the feasibility of MIR spectroscopy data, interpreted via principal component analysis (PCA) and linear discriminant analysis (LDA), to classify wines as ‘control’ or ‘smoke-affected’. Wines were sourced from industry and previous experimental trials related to smoke-taint. For the wines from experimental trials, smoke-affected wines were accurately classified with 100% success; however, for wines sourced from industry, the accuracy of classification ranged from 62 to 87%. In contrast, only one experimental trial classified control wines with 100% accuracy, and the others ranged from 38 to 86%. In addition to smoke exposure, wines within each trial were characterised by additional factors such as grape varietal (Ristic *et al.* 2016), oak/tannin additions, yeast strain, or skin contact time (Ristic *et al.* 2011), and grapevine defoliation (Ristic *et al.* 2013). It is likely that the chemical consequences of these treatments had greater influence on the MIR spectra than smoke exposure. This was evident from the

classification of red cask wine as smoke-affected, regardless of whether it had been spiked with 30 mg/L of guaiacol, a concentration far exceeding the range observed in even the most severely smoke-tainted wine. The lack of evident differentiation—even between simplistic experimental wines made under similar conditions from grapes with comparable smoke exposure—indicated that the heterogeneity introduced by natural bushfire smoke and different winemaking practices would only complicate the classification task already inhibited by sensitivity.

Grapes used to make experimental wine are exposed to smoke at a controlled intensity for a set period, which offers the benefit of delineated 'smoke-affected' or 'control' groups, but for grapes used to make commercial wine, the timing, duration, and intensity of exposure to natural bushfire smoke are typically unknown. As a result, Fudge and colleagues measured guaiacol and 4-methylguaiacol concentrations in commercially-sourced wine. They also performed informal sensory analysis, but neither analysis brought clarity to the delineation of 'smoke-affected' and 'unaffected' wine. Guaiacol was ubiquitous across wines (range from 1 to 55 µg/L) and 4-methylguaiacol was only present in half of the wine samples (ranging in concentration from 3 to 33 µg/L). Informal sensory analysis highlighted the influence of the wine matrix on the correlation between the concentration of volatile phenols and the perceived intensity of smoke taint attributes. For example, in wines as chemically dissimilar as a red blend with 51 µg/L of guaiacol and 29 µg/L of 4-methylguaiacol and a Shiraz with 6 µg/L of guaiacol and undetected levels of 4-methylguaiacol, the presence of an ashy aftertaste—a hallmark of smoke taint—was noted in both. Regardless of the chemical and sensory data, all industry wines used to develop the classification model were considered to be 'smoke-affected'. The correct classification rate of 'smoke-affected' industry wine was reported as 68%, but this is misleading because not all wines demonstrated clear evidence of smoke taint; thus, not all should not have been treated as 'smoke-affected'.

Several changes are required to improve the correlation between chemical indicators of smoke exposure and spectral data. The first change is to frame the problem as a binary classification task to a non-binary classification or prediction task. Fudge *et al.* 2012b trialed the discrimination of wine into a 'control' or 'smoke-affected' binary classification using MIR spectroscopy. An alternative approach, as outlined by Scrimgeour *et al.* 2021, is to use a non-binary classification approach, which aims to group samples across a gradient of 'low', 'medium' or 'high' smoke exposure risk. This strategy acknowledges the diversity of smoke taint chemical profiles and in this work, afforded better success than the prediction of total

volatile phenols, total glycoconjugates, or syringol gentiobioside concentrations. This classification scheme would help streamline decision-making leading up to harvest or in the winery and offer a data-driven approach to deciding which samples require more extensive compositional analysis.

Another alternative is to use a non-binary prediction approach, which aims to approximate volatile phenol or volatile phenol glycoconjugate concentrations. If successful, this approach would enable faster and more economical quantitation of key smoke taint indicators relative to the pace of classical GC-MS and HPLC-MS/MS techniques. While smoke exposure is a necessary pre-requisite for smoke taint, predicting smoke exposure and predicting smoke taint are distinct problems. As mentioned previously, accurate classification of smoke taint relies on the convergence of two correlations—one between spectral signals and chemical indicators of smoke exposure and another between chemical indicators of smoke exposure and the sensory perception of smoke taint. Converting the task from a binary classification to a non-binary prediction focuses on the improvement of the former correlation and avoids being tangled by the latter correlation.

The second change is that volatile phenol glycoconjugates need to be included in the chemical profile because they are a critical sink of smoke-derived volatile phenols present at significantly higher concentrations than volatile phenols in grapes and wine. The inclusion of the more abundant glycoconjugates may be easier to detect by a range of different methods. For example, in a study that compared guaiacol and guaiacol glycoconjugates in several varieties, 1978 µg/L of guaiacol glycoconjugates were observed in smoke-tainted Shiraz wine, relative to just 26 µg/L of guaiacol in the same wine (Ristic *et al.* 2016). That being said, some volatile phenol glycoconjugates also have a natural presence that needs to be accounted for. In the same study, the corresponding control wine (made from Shiraz grapes unaffected by smoke exposure) contained 334 µg/L of guaiacol glycoconjugates (Ristic *et al.* 2016). Given this natural abundance and their relative stability through fermentation (Caffrey *et al.* 2019; Whitmore *al.* 2021) and bottle aging (Ristic *et al.* 2017), a more detailed glycoconjugate profile of unaffected grapes is warranted to define the boundaries of natural variation.

The third change is the inclusion of a greater proportion and richness of "real" samples from industry. In Fudge *et al.* 2012b, over 60% of the wines were sourced from experimental trials involving the application of smoke to grapevines using a model system. While of great value to the assessment of feasibility due to their simpler matrices, experimental wines may

not capture the compositional diversity in wines made from grapes exposed to "real" bushfire smoke, nor the winemaking practices implemented to produce them. As a result, the prediction performance of models calibrated with replicated experimental wines will not perform as well because they come from a distinct population (Cozzolino *et al.* 2014). Moreover, the inclusion of more diverse samples might reduce the risk of overfitting.

Increased smoke taint awareness and bushfire frequency have significantly increased the demand for smoke taint analysis by commercial laboratories. This prompts the need for reduced costs and sample turnaround times associated with the quantitation of volatile phenols and volatile phenol glycoconjugates. Spectral techniques are an appealing solution due to their rapid, non-destructive nature of analysis and minimal sample preparation. While they have been integrated as part of routine quality control operations, only a few studies have explored their adaptation for smoke taint detection. Developing the capacity to rapidly screen for volatile phenols and their glycoconjugates in grapes and wine would enable earlier, objective decision-making and enable the support of ongoing mitigation efforts.

2.3 Detection of smoke exposure in grapevines with alternative targets

2.3.1 Challenges associated with current targets of smoke exposure in grapevines

The preceding discussion addressed the aim to improve the efficiency of smoke taint diagnostics by developing strategies that rapidly detect volatile phenols and their glycoconjugates. There is no doubt that volatile phenols, in free and glycosylated forms, increase in grapes following smoke exposure and contribute to the perception of smoke taint in wine. However, as discussed above, the accurate prediction of smoke taint risk requires the convergence of two distinct correlations, the first between grapevine smoke exposure and volatile phenol/glycoconjugate concentrations and the second between volatile phenol/glycoconjugate concentrations and wine sensory perception. Spectral techniques may expedite the speed at which volatile phenols and glycoconjugates are quantified, but several challenges must be overcome to improve the practicality of these diagnostics in grape and wine samples.

The first challenge to establish is the natural presence of volatile phenols and glycoconjugates in grapes (Ristic *et al.* 2016). Based on research trials conducted in Australia, natural abundance of free and glycosylated volatile phenols in grapes at maturity seemingly

depends on variety (Ristic *et al.* 2015; Ristic *et al.* 2016). However, it is not known how these baseline levels change across other varieties and regions outside of Australia, nor how they interact with the developmental stage at which grapes are exposed to smoke and subsequently analysed. Many grape grower contracts contain stipulations for damage incurred by disease and spoilage, and as the concern for smoke exposure rises, a few are also beginning to implement acceptable tolerance levels for smoke marker compounds (Allied Grape Growers and California Association of Winegrape Growers, 2021). Comprehensive baseline data specific to grape varietal, region, and developmental stage are needed to ensure the accuracy and fairness of these tolerance levels and limit the risks of overestimating grapevine smoke exposure and thus, inadvertently rejecting unaffected fruit.

The second challenge relates to the temporal dependence of the uptake and subsequent glycosylation of volatile phenols. It has been demonstrated that the timing of smoke exposure affects the degree to which grapevines absorb volatile phenols (Kennison *et al.* 2009, 2011) and subsequently glycosylate them (Jiang *et al.* 2021). Kennison *et al.* (2009, 2011) exposed grapevines to single, 30 minute smoke treatments using a purpose-built tent at different time points, both pre- and post-véraison, and compositional analysis of resulting wine found guaiacol concentrations were highest when made from grapes exposed to smoke at 7 days post-véraison. In later work, Jiang *et al.* 2021 monitored grapevines that were continuously exposed to smoke from bushfires in the Hunter Valley from October 2019 to January 2020. Therein, it was observed that even when berries were green and hard at E-L stage 33, significant glycosylation of volatile phenols could occur. E-L stage 33 precedes the peak period of acute volatile phenol uptake as observed in work by Kennison and colleagues (2009, 2011). This demonstrates that prolonged smoke exposure during the early stages of grape development can elevate volatile phenols (and then glycoconjugates) to levels comparable to those from a single event of smoke exposure at peak sensitivity. The temporal dependence of the uptake of volatile phenols has been postulated to reflect changes in leaf or berry physiology and surface area. Subsequent studies have incorporated this finding regarding the temporal dependence of volatile phenols, as demonstrated by the execution of field trials within the prescribed 7-10 days post-véraison (Ristic *et al.* 2015; 2016, Noestheden *et al.* 2018, van der Hulst *et al.* 2019). On the other hand, the temporal dependence of glycosylation, as well as the practices needed to accommodate it, are still under investigation.

There is a delay between the uptake of volatile phenols and their subsequent glycosylation, and this creates a window post-smoke exposure in which the chemical profile

of grapes may depend on when samples are collected. Using HPLC-MS/MS methods, previous studies have reported minimal volatile phenol concentrations within 24 hours of smoke exposure and the significant accumulation and stabilisation of volatile phenol glycoconjugates within 2 weeks of smoke exposure (Dungey *et al.* 2011; van der Hulst *et al.* 2019). Contrarily, Noestheden *et al.* 2018 reported different accumulation patterns of bound volatile phenols using the combined acid hydrolysis and GC-MS method. Some bound volatile phenols were established very rapidly after smoke exposure and remained consistent over berry maturity, some steadily increased, and others were absent in grapes and only present in subsequent wines. Interestingly, increases in bound 4-methylguaiacol were not mirrored by a corresponding decrease in free 4-methylguaiacol, which suggested that there may be dynamic fractions of VP conjugates that become increasingly acid-labile over time. It is evident that elevated volatile phenol and volatile phenol glycoconjugate concentrations arise as a consequence of grapevine smoke exposure. However, the apparent lack of mass balance observed with free 4-methylguaiacol concentrations indicated that glycoconjugates might not be the only bound forms that are needed in a comprehensive smoke taint diagnostic, particularly at the early stages post-smoke exposure. If berry samples are collected for analysis at the minima between the rapid depletion of volatile phenols and the onset of glycosylation, there is a risk of underestimating the severity of grapevine smoke exposure. A better understanding of the timeline and mechanism of glycosylation is required to mitigate this risk and standardise not only the timing of field trials at 7-10 days post-véraison, but also the timing of sample collection.

The third challenge to consider is the dynamic nature of fermentation. Volatile phenol glycoconjugates are minimally affected by acid-catalysed hydrolysis at typical wine pH 3.4 (Ristic *et al.* 2017; Whitmore *et al.* 2021); however, they appear to be affected by enzyme-catalysed hydrolysis driven by yeast. Kennison *et al.* 2008 observed the steady increase of guaiacol and 4-methylguaiacol concentrations during fermentation, with the highest concentrations observed in finished wine. In contrast, Caffrey *et al.* 2019 indicated that volatile phenol glycoconjugates decreased by an average of 23% during the first half of fermentation, after which they were stable through to the end of fermentation. This difference between studies indicates that alternative volatile phenol storage forms may exist, a hypothesis supported by Noestheden *et al.* 2018, in which increased free volatile phenol levels in wine (relative to corresponding berries) were not accompanied by decreased bound volatile phenol levels in wine. Studies investigating the fate of volatile phenols and glycoconjugates over fermentation

have shown that enzymatic cleavage can hydrolyse volatile phenol glycoconjugates and that alternative storage forms likely exist, beyond conventional disaccharides. As a result, additional work is required to understand the fates of individual volatile phenol glycoconjugates during fermentation, identify whether the most indicative markers of smoke exposure in grapes carry over into wine, and elucidate alternative biochemical sinks.

The final challenge to examine is the dependence of sensory perception on the wine matrix. Some compounds exhibit changes in sensory attributes at different concentrations (Waterhouse *et al.* 2016), and sensory perception can be modified by the presence of other compounds in a mixture (Rochelle *et al.* 2018). For smoke taint, it is likely a combination of both. For example, guaiacol and 4-methylguaiacol have been observed higher concentrations in wines matured in oak barrels without imparting any smoke taint attributes (Kennison *et al.* 2007). Moreover, studies have shown that even at sub-threshold levels, smoke taint sensory attributes become evident (McKay *et al.* 2021) particularly when *o*-cresol, which is a volatile phenol not typically found in barrel-aged wines, is present with other volatile phenols (Favell *et al.* 2022; Parker *et al.* 2012). In conventional sensory analysis of smoke-affected wines, wines are screened for a discrete number of sensory attributes associated with smoke (e.g. 'smoky', 'cold ash', 'medicinal', 'ashy aftertaste', 'metallic'), oak ('woody'), spoilage ('earthy', 'moldy', 'barnyard', 'musty'), as well as overall fruit aroma/flavour intensity, basic taste ('acidity', 'bitterness') and mouthfeel attributes ('drying', 'astringent') (Ristic *et al.* 2016). However, there is evidence that volatile phenols and glycoconjugates interact with other aroma compounds in wine (McKay *et al.* 2021) and the current list of attributes would not enable an examination of these interactions.

The final quantity of volatile phenols and glycoconjugates in a wine depends on their natural abundance in grapes, the timing and duration of grapevine smoke exposure in the vineyard, and the conditions of fermentation, but the sensory implications of this quantity are dependent on the wine matrix. For example, in McKay *et al.* 2020, *o*-cresol was not described as 'ashy' unless it was present in wine with 2-isobutyl-3-methoxypyrazine (McKay *et al.* 2020). In another example, Ristic *et al.* 2011 demonstrated that the use of oak chips could lower the perceived intensity of smoke taint in wine, not through the removal of volatile phenols or glycoconjugates, but through increasing wine complexity and masking the severity of smoke taint. Furthermore, Favell *et al.* 2022 demonstrated that white wines with high levels of fruit could mask the intensity of smoke taint that would otherwise be predicted based on volatile phenol and glycoconjugate concentrations in isolation. It is evident that the perception of

smoke taint in wine is matrix-dependent and thus, other key aroma compounds in wine must be subject to chemical and sensory analysis to explore these interactions. This information will supplement the quantitation of volatile phenols, glycoconjugates, and sensory attributes associated with smoke taint.

Overcoming these challenges will improve the prediction of smoke taint risk in wine following grapevine smoke exposure and enable earlier, confident decision-making, but further research into volatile phenols and glycoconjugates may not be the only way to improve the efficiency of smoke taint diagnostics. Prior to the metabolism of volatile phenols in grapevines, many aspects of the smoke that carries them and the fire from which they are generated can be detected using appropriate sensors. If placed in the vineyard, sensors could be used to monitor vineyard exposure to bushfire smoke in real-time, well before grapes are off the vine. This would expand our attention to characterising the nature of smoke exposure in the vineyard and how it informs the risk of smoke taint, rather than exclusively focusing on the chemical consequences of its presence in grapevines. Another way to improve the efficiency of smoke taint diagnostics is to search for alternative chemical indicators of smoke exposure. As stationary organisms, plants have evolved different mechanisms to respond to their environment, one of which is the generation and emission of volatile compounds for signalling and communication (Dudavera *et al.* 2004). While the upregulation of plant-derived metabolites in response to smoke exposure may not have sensory relevance to wine, these metabolites could nonetheless be used as biochemical evidence of smoke exposure. The subsequent sections will describe these alternative strategies.

2.3.2 Approximation of grapevine smoke exposure with vineyard-based detection tools

Vineyard-based detection is an emerging diagnostic for grapevine smoke exposure. In domestic settings, smoke presents the risk of endangering occupants and so detectors are installed to prompt extinguishment or evacuation; however, the risks associated with smoke exposure in a vineyard are more nuanced. Smoke can disperse far from its original source, and its behaviour and composition depends on the type and extent of the fire, fuel type/load, prevailing wind conditions, and topography (Bell *et al.* 2013). Studies have demonstrated that the timing, frequency, and duration of grapevine smoke exposure affect the intensity of smoke taint in wine (Kennison *et al.* 2008; 2011). However, for more refined decision-making, data that show the density and duration of smoke exposure in a vineyard in real-time are required.

The properties and characteristics of fire such as heat, light, smoke, flicker, motion, and chemical by-products (e.g. particulate matter, carbon monoxide, and volatile organic compounds (VOCs)) present an assortment of material available for detection (Allison *et al.* 2016). Some conventional methods of fire detection include the use of satellite imagery, human observation from watchtowers, infrared (IR) sensors, visible spectrum cameras (Zhao *et al.* 2018), and multispectral cameras (Akloufi *et al.* 2021). More specifically to smoke, the feasibility of simple particulate matter sensors (Kelleher *et al.* 2018; Holder *et al.* 2020) and red, green, and blue (RGB) image analysis (Brunori *et al.* 2020) have been evaluated.

One approach to detecting vineyard smoke exposure is with a network of sensors. In addition to gases (e.g. NO_2, CO_2, SO_2, and O_3), smoke also carries particulate matter (PM) which can be detected by small, portable sensors (Radojevic 2003). Particulate matter ranges in size due to their relevance to human health, the diameters most often recorded are 1.0 µm, 2.5 µm, and 10 µm, abbreviated as $PM_{1.0}$, $PM_{2.5}$, and PM_{10}, respectively (Polichetti *et al.* 2009). Jiang *et al.* 2021 used PM_{10} concentrations collected over 8 weeks as a proxy of smoke exposure and compared them to volatile phenol and glycoconjugate concentrations in Chardonnay and Shiraz grapes. Two air stations, situated 25-30 km northwest of the vineyards, collected the PM_{10} data. Grapes were sampled at five time points and overall, the glycoconjugate levels were low at the first and second time points, after which they increased the most at the third time point, and plateaued through to the fifth time point. This accumulation pattern coincided with the PM_{10} data patterns from Station 1, but not Station 2. Station 1 was within 5 km northeast of the fire front and reported a southeast wind that would blow smoke toward the vineyards situated 25 km east of the fire front. On the other hand, Station 2 was 25 km northeast of the fire front and reported a northeast wind that would blow smoke away from the vineyards. Anecdotal evidence, including the presence of 'heavy haze and a strong smoky smell' during the period of increasing PM_{10} concentrations supported the data from Station 1; however, this study demonstrates the disagreement that can arise from two PM sensors, even under a simple situation characterised by a single fire front and clustered vineyards.

There are a few limitations with using PM sensors to detect vineyard smoke exposure. The first limitation is that a high density of PM sensors is required to ensure that all areas have sufficient cover. When incorporated as part of expensive, all-inclusive environmental sensors, this would be cost-prohibitive for many vineyards. However, PM sensors can also be found as stand-alone units at significantly lower price brackets and demonstrate comparable quality to units that are more expensive. One study compared the performance of $PM_{2.5}$ sensors

earmarked for citizen scientists and federal air quality agencies and found them to be comparable (Holder *et al.* 2020).

The second limitation is that particulate matter concentrations are only an indirect approximation of the volatile phenols present in smoke. They do not consider the dispersion and oxidation of volatile phenols in smoke over time nor the partition coefficients that govern the diffusion of volatile phenols to the berry surface (Krstic *et al.* 2015). More work is required to understand the correlation between PM concentrations and volatile phenol concentrations in grapes. Rather than use a PM sensor as an indirect approximation of volatile phenols, semi-conductor metal-oxide sensors have been trialed for the real-time measurement of volatiles emitted from (*Eucalyptus globulus*) wood and leaves during combustion (Paczkowski *et al.* 2018). This offers a more targeted measurement than particulate matter, but it would also require a large sensor density to be effective, and it is likely that the semi-conductor metal-oxide sensors would be more expensive than PM sensors. Despite these limitations, the sensitivity and low cost of PM sensors, as well as their ability to network and provide real-time information, present an opportunity for monitoring the presence of smoke in vineyards.

Another method for detecting vineyard smoke exposure is the deployment of unmanned aerial vehicles (UAVs), which would improve the range, resolution, and flexibility of vineyard smoke detection (Alkloufi *et al.* 2021). UAVs can be mounted with different types of sensors and when combined, provide a comprehensive assessment of the size and anticipated direction of the fire and smoke. Moreover, they are highly manoeuvrable and do not require extensive, premeditated deployment, both of which suit the dangerous and unpredictable nature of fire events. Brunori *et al.* 2020 tested the feasibility of an RGB camera mounted on a UAV to detect grapevine smoke exposure. Leaves can be damaged by the presence of gases in smoke (e.g. NO_2, CO_2, SO_2, and O_3) due to the formation of necrotic lesions and decline in photosynthetic activity (Ristic *et al.* 2016). Brunori and colleagues used this to build a Canopy Area Health Index (CAHI), which reflected the percentage of living and dead leaves relative to the total canopy area.

To test the feasibility of using the CAHI as an indicator of vineyard smoke exposure, Brunori and colleagues enclosed vines in a purpose-built tent and exposed them to smoke on two occasions. The occasions were spaced one week apart, and smoke exposure lasted for 60 minutes and 30 minutes, respectively, after which the UAV-mounted RGB camera scanned them immediately and then again 24 hours following smoke exposure. After generating maps

of damage attributed to smoke exposure, Brunori and colleagues found that significant leaf death occurred primarily after the second occasion of smoke exposure. This is corroborated by Kennison *et al.* 2009, in which only grapevines subject to multiple 30-minute smoke treatments developed necrotic lesions on their leaves. Assuming that the vineyard has no confounding sources of leaf necrosis, this rapid approach is useful to characterise grapevines exposed to bushfire smoke; however, this approach is limited because the absence of necrotic lesions does not preclude the occurrence of smoke exposure and thus any smoke taint risk.

Another approach to the detection of vineyard smoke exposure is the use of hand-held spectroscopic devices. In Summerson *et al.* 2020, the berries and leaves of smoke-affected grapevines were scanned with a hand-held NIR spectrometer. Spectral data were used to train artificial neural network models to classify grapevines into groups characterised by no smoke exposure, a low level of smoke exposure, or high levels of smoke exposure, and the accuracy of the developed models exceeded 90%. These are promising results, but as mentioned above, several challenges confront the widespread adaptation of spectral techniques. Primarily, there is considerable skill required to build models that robustly differentiate properties of interest across highly diverse matrices, and further work is required to assess the accuracy of developed models with different varietals, regions, and conditions of grapevine smoke exposure (e.g. timing, duration, frequency, and density). Moreover, for safety reasons, the use of handheld spectrometers may be hindered by limited access to vineyards following smoke exposure.

There are many challenges associated with detecting and monitoring smoke with sufficient resolution to characterise the degree of smoke exposure in a way that informs the risk of smoke taint on a vine-to-vine level. However, developing the capacity to screen for smoke exposure in the vineyard would enable earlier decision-making for smoke-affected grapes and potentially widen the range of available mitigation strategies to reduce the risk of developing smoke taint in subsequent wine.

2.3.3 Seeking alternative markers of grapevine smoke exposure with untargeted metabolomics

Volatile compounds are produced in plants for a range of reasons, such as to attract pollinators or seed dispersers, deter threatening herbivores, protect against abiotic stresses (e.g. ozone-induced oxidative stress, heat stress), and warn neighboring plants of incoming stress (Dudavera *et al.* 2006)**.** This “language” is comprised of at least 1700 volatiles (Knudsen and

Gershenzon 2006), and the composition and intensity of a given blend specifies not only the message but also the intended recipient (e.g. carnivores, herbivores, other plants) (Dudavera *et al.* 2006). The major volatiles in plants are terpenoids, fatty acid derivatives, amino acid derivatives, and phenylpropanoids/benzenoids (Dudavera *et al.* 2004). In grapes, the natural mechanisms that produce these volatiles are affected by vineyard practices, environmental conditions, grape varietal, and harvest date (Dunlevy *et al.* 2009).

The mechanisms that produce volatile phenols in grapes are not well understood, but based on studies in other plants, it is hypothesised that they are derivatives of phenylpropanoids and benzenoids (Dunlevy *et al.* 2009). Phenylpropanoids and benzenoids are produced by the shikimic acid pathway through multiple, interdependent routes. The shikimic acid pathway has roles in plant defense (Figueiredo *et al.* 2008) and reproduction (Dudavera *et al.* 2000). It is evident that compounds such as benzoic acid and salicylic acid could serve as molecular scaffolds for volatile phenols due to their separation by a few hydroxylation and methylation reactions. However, these carboxylic acids also serve as scaffolds for many other phenolic compounds including hydroxycinnamates, phenolic acids, stilbenoids, flavonoids, and anthocyanins (Noestheden *et al.* 2018). If the shikimic acid pathway is the primary source of naturally occurring volatile phenols in grapes as hypothesized, it is not known what factors favour the production of volatile phenols over that of other phenolic compounds, nor what purpose volatile phenols serve in grapevines.

Many volatiles in grapes exist as glycosidically bound forms because glycosylation reduces their chemical reactivity and increases their solubility in water to facilitate transport between organs, storage in the plant, or both (Winterhalter and Skouroumounis 1997; Piotrowska and Bajguz 2011). Across glycoconjugates, the direct link between the aglycone and the glycone unit is bridged by a β-D-glucose moiety. Glycoconjugate diversity stems from the range of volatiles that can be glycosylated in addition to whether and where the glucose moiety is further substituted with additional sugar moieties such as α-L-arabinofuranose, α-L-rhamnose, β-D-xylopyranose, β-D-apiofuranose, or β-D-glucose (Winterhalter and Skouroumounis 1997; Bowles *et al.* 2006). Endogenous aglycones relevant to wine sensory research include shikimic acid metabolites, monoterpenoids, norisoprenoids, and sesquiterpenoids (Winterhalter and Skouroumounis 1997), but more broadly, polyphenolic compounds (e.g. anthocyanidins), and hormones (e.g. auxins, salicylic acid, and abscisic acid) are found as conjugates (Bowles et al. 2006). Exogenous aglycones include pathogenic toxins and pollutants. As described previously, glycoconjugates can indirectly be quantified by

employing GC-MS analysis pre- and post- acid hydrolysis or directly by HPLC-MS/MS. The prevalence of glycosylation as a storage form across endogenous and exogenous volatiles suggests that alternative biochemical markers of smoke exposure would be best identified through the pursuit of an untargeted metabolomics workflow using UHPLC-MS/MS.

Volatile phenols served as the logical starting point of smoke taint diagnostics due to their sensory relevance and abundance in smoke; however, the seven to eight volatile phenols routinely quantified in smoke taint research (i.e. guaiacol, 4-methylguaiacol, phenol, *o*-cresol, *p*-cresol, *m*-cresol, syringol, and 4-methylsyringol) are far from being the only compounds in smoke. In a commercial liquid smoke product, Guillén *et al.* 1995 identified 67 compounds, 35 of which were phenol derivatives and the remainder comprised of furan, and pyran derivatives, ketones, lactones, diketones, alkyl aryl ethers, and hydrocarbons. In addition to the conventional guaiacol, syringol, and phenol, several other phenol derivatives were identified with different substitution patterns, a known influence of odour activity (Czerny *et al.* 2011) and chemical reactivity (Waterhouse *et al.* 2016). In later work that examined compounds found in an aqueous oak extract, Guillén and Manzanos (2002) identified 215 compounds, and in addition to the aforementioned classes, they reported aldehydes, alcohols, esters, acids, pyrocatechol derivatives, lignin dimers, and carbohydrate derivatives. The array of compounds found in smoke is attributed to the diversity of fuel composition (Maga *et al.* 1992; Kelly *et al.* 2014), region (Cadahia *et al.* 2003), and fire conditions, such as the temperature of combustion (Wittkowski *et al.* 1992), moisture content of fuel (Guillén *et al.* 1999), and duration of burning (Guillen *et al.* 1999). This presents a challenge for targeted, reductionist approaches, which evaluate one hypothesis at a time using highly customised acquisition methods. By contrast, metabolomics approaches examine samples from a holistic and unbiased perspective and deploy statistical techniques to identify the compounds that differentiate samples (Lloyd *et al.* 2015).

An untargeted metabolomics workflow was used to approach the chemical entity responsible for ‘pepperiness’ in wine (Parker *et al.* 2007). In this study, GC-MS data collected from grapes were subject to multivariate statistical techniques to identify areas of the spectra that demonstrated positive covariance with the ‘pepper’ aroma intensity of the samples. This led to the identification of four ions that accounted for greater than 99% of the ‘pepper’ variation in the grape samples, and the only compound with an abundance of all four ion was α-ylangene. This compound had no peppery sensory attributes and was absent in wine. Nonetheless, the authors emphasised its significance as a marker in grapes and drew the

distinction between markers and active aroma compounds. While less exciting, markers are also of great value to diagnostics, particularly if they are highly specific to the property of interest, abundant, stable, and easy to measure.

Untargeted metabolomics approaches have also led to the elucidation of many other markers and active aroma compounds in grapes and wine. In Kalua and Boss (2009), the volatile profiles of Cabernet Sauvignon grapes were analysed from fruit set to late ripening using HS-SPME-GC-MS to understand the evolution of key aroma compounds. GC-MS chromatograms demonstrated evident differences in peak size, distribution, and number as a function of developmental stage, but to quantify the degree of change and the compounds driving it, a combined univariate and multivariate statistical approach was employed. First, one-way ANOVA was used as a filter to identify compounds with statistical significance between groups. Second, the refined compound list was analysed with stepwise linear discriminant analysis to elucidate common developmental patterns and identify which compounds abided to each one. On a basic level, this study demonstrates the convenience afforded by metabolomics experiments, which enable researchers to capture a diverse array of compounds, i.e. aldehydes, alcohols, esters, terpenes, and benzene derivatives, using a single acquisition method. At a more fundamental level, this study highlights the unique capability of metabolomics experiments to monitor trends of that diverse array of compounds in a minimally processed, complex sample matrix. This is achieved primarily with a targeted statistical analysis workflow that enables identification of the metabolites driving differences between samples.

On the wine side, Rubert *et al.* 2014 used untargeted metabolomics to collected the 'fingerprints' of 343 wines of various geographical origin, vintage, and variety to evaluate their potential in wine varietal authentication. Between positive and negative ionisation mode, over 10,000 peaks were detected by UHPLC-HRMS, and when the unfiltered data from all samples were analysed by PCA, the primary mode of separation was by colour. As a result, the red and white wines were split into separate subsets, and a combination of t-tests and log-fold change calculations were used to select compounds that changed across (red or white) varietals and reduce the complexity of the dataset. In the last step, orthogonal partial least squares discriminant analysis (O-PLS-DA), a supervised technique, was performed with the selected list of compounds to develop models that differentiated between grape varietal, origin, and vintage, and assign tentative identities to markers responsible for those distinctions. This study demonstrates the effective use of data reduction strategies and supervised statistical methods

to extract relevant information. It also highlights a key limitation of PCA, which—due to its unsupervised nature—does not always partition a complex dataset in alignment with the variable of interest. More often, PCA is utilised to provide a preliminary overview of data structure and identify possible outliers (Theodoridis *et al.* 2011).

The enduring, exclusive focus on volatile phenols and their glycosylated equivalents despite the heterogeneity of smoke composition and the biochemical sensitivity of the grapevine warrant a search for additional compounds that define smoke-affected grapes and improve the efficiency of smoke taint diagnostics. Untargeted metabolomics workflows assess a holistic profile of samples and use multivariate statistical analysis to extract relevant information. These techniques enable the quantitation of a diverse array of compounds in a single analysis.

In addition to understanding how smoke-derived volatile phenols are metabolised by grapevines, improving the methods used to detect grapevine smoke exposure, and seeking alternative indicators of grapevine smoke exposure, practical strategies that mitigate the perceived intensity of smoke taint in wine are also required. As described in the next section, approaches to mitigating the perception of smoke taint in wine include those that prevent the uptake of smoke-derived volatile phenols by grapevines, reduce the levels of smoke-derived volatile phenols and glycoconjugates in wine, and mask the potency of smoke-derived volatile phenols and glycoconjugates in the perception of smoke-affected wine.

3. Mitigation of smoke taint in grapes and wine

3.1 Reduction strategies

Reduction strategies aim to remove volatile phenols and glycoconjugates from wine with high selectivity using fining agents and filtration or limit the amount of volatile phenols and glycoconjugates that are extracted during fermentation. In Fudge *et al.* 2011, reverse osmosis coupled with solid phase adsorption was trialled, and it was found that while effective at reducing volatile phenol concentrations, the glycoconjugates remained in the wine. Reverse osmosis was conducted with a membrane that had a molecular weight cut-off value of 150-200 amu. Size exclusion likely prohibited the passage of volatile phenol glycoconjugates, which range in mass from ~260 amu for monosaccharides (e.g. phenol glucoside) to >600 amu for triscaccharides (e.g. hexose-hexose-pentose-cresol) but not volatile phenols, which range from

94 (phenol) to 154 amu (syringol). To remove volatile phenols, the resulting permeate was pumped through a column lined with a polystyrene-based adsorbent resin, and this led to guaiacol reductions of 67% and 74% in Pinot Noir wines at pilot scale and commercial scale, respectively. However, there was no concomitant strategy deployed to reduce volatile phenol glycoconjugates from the retentate; thus, when the treated permeate was recombined with the untreated retentate, it was not surprising that the glycoconjugate concentrations were unchanged. In contrast to other filtration studies in which the targets of removal accumulate in the retentate or permeate, the diversity of characterising smoke-derived compounds regarding their size means that they will be found in both fractions. As a result, both the retentate and permeate require treatment prior to recombining them in order for filtration to be successful at the mitigation of smoke taint in wine.

One strategy for removing glycoconjugates from the retentate is to use yeast or enzymes to cleave the volatile phenol glycoconjugates, followed by solid phase adsorption to remove the liberated volatile phenols. Ristic *et al.* 2011 compared the effects of eight yeast strains on smoke taint intensity in wine, and there were some strain-specific reductions in guaiacol glycoconjugate concentrations—albeit, these reductions were not always mirrored by an increased guaiacol concentration. In a recent study that performed another yeast selection trial, six strains were screened for their capacity to liberate volatile phenols from different storage forms, including glycoconjugates (Whitmore *et al.* 2021). This trial involved the fermentation of Pinot Noir grapes with and without smoke exposure, and to assess the effect of regio-specificity of enzymatic hydrolysis, wines were spiked with 11 volatile phenol glycoconjugates at 200 µg/kg. Few significant differences in volatile phenol concentrations were observed in wines fermented with different yeast strains, with the exception of guaiacol and phenol. Smoke-affected wines without fortification showed narrow ranges of phenol and guaiacol across yeast strains, but their levels in smoke-affected wines with fortification were not only higher, but also characterised by greater variation across yeast strains. This work demonstrates that the efficacy of yeast-catalysed hydrolysis of volatile phenol glycoconjugates may be dictated by aglycone.

As an alternative to filtration and the treatment of separate fractions, the addition of fining agents has also been trialled as a reduction strategy. In Fudge *et al.* 2012a, thirteen fining agents were evaluated and based on their removal of volatile phenols, the most effective products were found to be an activated carbon and a synthetic mineral. The limitation of this work was that guaiacol glycoconjugates were not removed by the fining

agents, but this may have been a consequence of the type of activated carbon, the dose rate, and the matrix (Culbert *et al.* 2019). In a benchtop trial that screened the efficacy of several activated carbon products in smoke-affected juice and wine from red and white varietals, it was observed that some products were more effective at removing glycoconjugates than volatile phenols (Culbert *et al.* 2019). In this trial, the rates of glycoconjugate removal were higher in juice (relative to wine) and white varietals (relative to red varietals), and there was no evident interaction effect. Rates of volatile phenol removal were also higher in juice compared to wine. From this benchtop trial, two of the best performers were assessed on a larger scale in smoke-affected Chardonnay and Pinot Noir juice (Culbert *et al.* 2021b). The large-scale trial validated the affinity of activated carbon for volatile phenols and their glycosides with the caveat that excessive dose rates may sacrifice wine colour and desirable wine sensory attributes. To offset the risk of stripping desirable wine constituents in juice and wine severely affected by smoke exposure, it may be practical to use a lower dose rate of activated carbon and couple this addition with aforementioned mitigation strategies.

In addition to strategies that remove smoke-derived volatile phenols and glycoconjugates from wine, minimising skin contact can also be used to reduce the proportion that is extracted during fermentation. In Ristic *et al.* 2011, smoke-affected Grenache fruit was divided and processed as red and rosé wines. Red wine was fermented with skin contact for 7 days at 15 °C whereas rosé wine was cold soaked with skin contact for 3 days at 0 °C, prior to pressing and fermentation. The rosé wine made from smoke-affected Grenache contained a total of 204 µg/L guaiacol glycoconjugates whereas the red wine made from the same smoke-affected fruit contained a total of 290 µg/L guaiacol glycoconjugates, a statistically significant increase attributed directly to increased skin contact.

3.2 Masking strategies

Masking strategies aim to obscure the perceived intensity of smoke taint attributes in wine with more positive wine sensory attributes. Trials have explored the effects of defoliation, blending, yeast strain, and wine additives. Kennison *et al.* 2007 assessed the feasibility of masking the intensity of smoke taint in Verdelho wine by blending smoke-affected wine with unaffected wine. Difference testing indicated that perceivable smoke taint could still be reliably detected in samples that were diluted by over 98% with unaffected base wine; albeit, the smoke-affected wine had unprecedented levels of volatile phenols, with over 1470 µg/L of guaiacol and 326

µg/L of 4-methylguaiacol. The Verdelho wine in this study exceeded the capacity of amelioration, but more work is warranted to explore the boundaries of blending wine that has noticeable smoke taint but lower levels of volatile phenols. Ristic *et al.* 2011 examined the effects of oak tannin additives on the perception of smoke-affected Shiraz wines. The intensity of 'smoky', 'cold ash', and 'medicinal aroma' was reduced by the additives, achieved through increased wine complexity rather than the reduction of volatile phenols or glycoconjugates. The perceived intensity of smoke taint can be mitigated with the use of additives in the winery, but the complexity of wine can also be manipulated with the implementation of vineyard practices.

Defoliation increases the amount of light exposure on the berries, and this alters the profile of several key aroma compounds in grapes. For example, in a study that compared bunches with and without artificial shading, bunches with full sun exposure had higher bound terpenoids and bound C_{13}-norisoprenoids (Bureau *et al.* 2000). Ristic *et al.* 2013 examined the effect of defoliation on volatile phenols and total guaiacol glycoconjugate concentrations in Chardonnay juice and wine when conducted pre- and post-smoke exposure. When defoliation was performed pre-smoke exposure, there were increased guaiacol glycoconjugates (in juice) and volatile phenols (in wine), relative to levels in juice and wine from vines that had been exposed to smoke but were not defoliated. When defoliation was conducted post-smoke exposure, no significant differences were observed in volatile phenol or glycoconjugate concentrations of juice or wine relative to those made from vines with smoke exposure but no defoliation; however, it did lead to decreases in the 'cold ash' aroma and 'ashy aftertaste' in wine. Comparable levels of volatile phenols and guaiacol glycoconjugates between these treatments support previous work by Hayasaka *et al.* (2010a), which demonstrated minimal translocation of volatile phenols glycoconjugates between leaves and berries. Thus, the reduced intensity of smoke taint attributes in the wine made from vines defoliated post-smoke exposure is likely attributed to changes in other volatiles that interact with the perception of volatile phenols. Defoliation following smoke exposure may have increased levels of desirable aroma compounds in grapes promoted by sunlight and reduced the perceived intensity of smoke taint. Alternatively, studies have demonstrated that the uptake and glycosylation of volatile phenols can also occur in leaves (Jiang *et al.* 2021), and in tandem with handpicking bunches, defoliation following smoke exposure may reduce the amount of leaves that enter fermentation vessels.

Fruit aromas and flavours can mask low levels of smoke taint intensity to some degree and similarly, their absence can magnify the perceived intensity of smoke taint (Ristic *et al.* 2017). Subsequently, 'overall fruit aroma' and 'overall fruit flavour' are often included as attributes in the sensory analysis of smoke-affected wines. However, fruitiness and the compounds responsible for this attribute are not the only ones that interact with volatile phenols and smoke taint perception. McKay *et al.* (2021) spiked de-aromatised Shiraz wine with guaiacol, *o*-cresol, 4-ethylphenol, and 3-isobutyl-2-methoxypyrazine (IBMP) at or below the detection thresholds specific to each compound. Compounds were spiked individually and as binary mixtures. Clear distinctions were made between the sensory attributes of mixtures comprised of two volatile phenols and mixtures comprised of both a volatile phenol and IBMP. Individually, IBMP reduces the fruit intensity of wine and amplifies 'herbaceous', 'bell pepper', and 'cooked vegetable aromas'. However, when IBMP was coupled with any of the volatile phenols, the hallmark 'ashtray' attribute emerged, and this attribute was not observed when wine was spiked with volatile phenols alone. It is possible that the wines made from Chardonnay vines defoliated post-smoke exposure had decreased ashy attributes due to lower IBMP concentrations, given the sensitivity of IBMP to sunlight and temperature (Ryona *et al.* 2008; Scheiner *et al.* 2012). While more typical of Cabernet Sauvignon, Merlot, and Sauvignon Blanc, IBMP can also be found in Chardonnay (Hashizume and Samuta 1999).

A later study examined the effect of harvest date on smoke taint intensity (Ristic *et al.* 2015). When harvested at a later harvest date, Sauvignon Blanc wine had significantly reduced 'smoke aroma', 'cold ash aroma', 'smoky flavor', and 'ashy aftertaste'. The concentration of IBMP was not measured, nor was the intensity of 'green' character specifically rated, but *trans*-3-hexen-1-ol and *cis*-3-hexen-1-ol were measured, and these C_6-alcohols are likewise characterised by 'grassy' and 'green' sensory attributes. In smoke-affected Sauvignon Blanc wine, both C_6-alcohols were significantly higher when they were made from grapes harvested at an earlier date, a trend consistent with published research (Previtali *et al.* 2021). Smoke-affected Shiraz showed the same significant decrease in C_6-alcohols when harvested at a later date, but there was no corresponding decrease in smoke taint intensity, thus further study of the effects of other wine odorants on smoke taint perception is warranted.

3.3 Preventative strategies

Early prevention strategies aimed to treat grape bunches by harvesting and treating them immediately following smoke exposure, but later strategies have aimed to apply agrochemical sprays in advance of smoke exposure to limit the uptake of volatile phenols by grapevine leaves and fruit. The first reported incidents of smoke taint were brought to the AWRI Commercial Services in 2003 from growers and winemakers that were affected by bushfires in Victoria and New South Wales. To reduce guaiacol and 4-methylguaiacol concentrations in grapes and subsequent wines, several 'vineyard-washing' treatments were employed using water, 5% ethanol in water, 95% ethanol in water, and milk but no decreases were observed (AWRI Annual Report 2003). Washing was able to remove the ash and particulate matter from berry skins, but the lack of any significant change in volatile phenol concentrations was an early indication that volatile phenols readily adhere to berry cuticles and undergo rapid diffusion into the skins and pulp.

In another trial, smoke-affected grapes were treated with gaseous ozone immediately following harvest, and modest reductions in free and glycosylated volatile phenols concentrations were observed, depending on the dose of the ozone treatment and whether smoke exposure was administered to mature, excised bunches or to grapevines at 7 days post-véraison (Modesti *et al.* 2021). Therein, mature, excised bunches were exposed to smoke for 30 minutes and immediately treated with ozone. Volatile phenol concentrations were reduced by 40 to 100%. When grapevines were exposed to smoke for 60 minutes at 7 days post-véraison and fruit was subsequently treated with ozone 4 weeks later (i.e., at commercial maturity), volatile phenol concentrations were slightly reduced by 2-3 µg/L. However, the corresponding wines exhibited increased fruit intensity and decreased smoke attributes by sensory analysis. Thus, this work demonstrates the potential for ozone as a post-harvest mitigation treatment, particularly if is implemented soon after smoke exposure. Both the 'vineyard-washing' and ozone trials highlight the time-sensitive nature of treating smoke-affected fruit. This serves as the impetus to investigate preventative strategies that can be applied in the vineyard in advance of smoke exposure.

Throughout the growing season, viticulturists deploy a range of agrochemical sprays to protect developing berries from disease, pests, and conditions such as sunburn, splitting, and dehydration, and it is critical to understand how their application may affect the uptake of volatile phenols (as described in Culbert *et al.* 2021c). In the worst-case scenario, products may exacerbate the absorption of volatile phenols, but in the best-case scenario, products may decrease, delay, or prevent the uptake of volatile phenols. Understanding how agrochemical

sprays affect the intensity of smoke taint will enable viticulturists in fire-prone regions to balance risks associated with smoke taint against disease, pests, and climactic pressures.

In one trial, kaolin was evaluated as a protective barrier to limit the uptake of smoke-derived volatile phenols by grapevines (van der Hulst *et al.* 2019). Kaolin is a clay-based film that is used to mitigate the risk of berry sunburn, and in the published study, it was sprayed onto Merlot, Chardonnay, and Sauvignon Blanc fruit and foliage 24 hours before the vines were subjected to 1 hour of smoke exposure using purpose-built tents. Therein, the efficacy of kaolin as a mitigation strategy appeared to depend on the varietal, but it may have also been a result of variable spray coverage, smoke exposure, or a combination. Volatile phenol concentrations were minimal across smoke-affected fruit and as a result, quantitation of glycoconjugates was used to measure the efficacy of kaolin as a protective strategy. This study demonstrated minimal reductions in glycoconjugates measured in smoke-affected Sauvignon Blanc and Chardonnay grapes with or without kaolin, with the exception of a few glycoconjugates in the latter, which were reduced by up to 50% (van der Hulst *et al.* 2019). Contrarily, reductions were greater and more widespread in Merlot grapes, ranging from 58-92% across most of the glycoconjugates measured. This result was in contrast to earlier trials, in which kaolin appeared to increase the uptake of volatile phenols by grapevines exposed to straw-derived smoke (Kennison *et al.* 2009). It also contrasts recent trials in which kaolin did not demonstrate any significant effect on the concentrations of volatile phenols in excised bunches exposed to an aqueous solution of volatile phenols (Culbert *et al.* 2021c). Thus, the result observed in van der Hulst *et al.* 2019 warrants further research.

The varietal-dependent result evident in van der Hulst *et al.* (2019) may be attributed to spray coverage or variable smoke exposure. The issue of spray coverage highlights the potency of smoke-derived volatile phenols and will be a key limitation of any vineyard-based sprays i.e., it is difficult to achieve 100% coverage. This challenge was corroborated by Culbert *et al.* (2021c), in which 100% coverage was not attained for some coatings, even when excised bunches were fully dipped into them. If this effect was attributed to variable smoke exposure, it highlights a key challenge of vineyard-based mitigation trials, which is that highly reproducible applications of smoke are required. In van der Hulst *et al.* 2019, it is possible that the effect observed in Merlot was precluded in Chardonnay and Sauvignon Blanc grapevines by the comparatively low level of smoke exposure they received. It is essential that changes between smoke-affected vines with and without a mitigation treatment can be attributed to the

mitigation treatment rather than differences in smoke exposure. This is a key obstacle that continues to hinder the progress of vineyard mitigation trials.

Rather than act as a barrier, films may also be used to fortify the naturally protective properties of the berry cuticle. As shown in Kennison *et al.* 2009, it was observed that the post-harvest removal of wax blooms led to an increased uptake of guaiacol by excised grape bunches. A recent study by Favell *et al.* 2019 trialed three agrochemical sprays with high hydrophobicity, including a phospholipid-based biofilm and two commercial fungicides. While one fungicide had no effect and another had an exacerbating effect on the concentrations of free and bound volatile phenols in berries and cuticles, the biofilm appeared to have an ameliorative effect. The authors conducted a follow-up study to determine the duration of protection afforded by the biofilm on larger scale, and in stark contrast to their previous results, the authors observed the use of biofilm heightened free and bound volatile phenol concentrations in treated grapes (Favell *et al.* 2021).

The contradictory results characterising the trials may be attributed to differences in experimental design. In the first trial, fourteen vines were enclosed in a single tent and exposed to smoke for 1 hour on two occasions that were separated by 48 hours. Seven of the fourteen enclosed vines were also sprayed with biofilm, and there were no apparent experimental replicates. The average concentration of free guaiacol measured immediately after the first and second applications of smoke in grapes with biofilm were 9 and 21 ng/g, respectively. These levels were significantly lower than free guaiacol measured in smoke-affected fruit without biofilm, which averaged 48 ng/g (first application of smoke) and 34 ng/g (second application of smoke). In the second trial, sixteen vines were enclosed in four separate tents and exposed to smoke for 1 hour on a single occasion. Of the four vines enclosed in each tent, one vine was sprayed with water (as a control) whereas the other three vines were sprayed with biofilm at different times prior to smoke exposure (i.e. 1, 7, and 14 days). Unlike the first trial, the second trial was repeated in three different vineyards. The average concentration of free guaiacol measured 1 hour after smoke exposure in grapes without biofilm ranged from 4 to 20 ng/g, depending on the vineyard.

The second trial had lower levels of free guaiacol measured in smoke-affected grapes (without biofilm) than grapes in the first trial, which may reflect differences in smoke exposure between the two trials. This trend is supported by levels of total guaiacol in mature, smoke-affected berries (without biofilm). In the first trial, the total guaiacol concentration was 153

ng/g, whereas in the second trial, total guaiacol concentrations ranged from 17 to 24 ng/g, depending on the vineyard. The fact that the most promising results came from the first trial, in which vines were exposed to a heavier application of smoke, indicates that the authors' optimism may have been a product of the experimental design. In the first trial, all vines were enclosed in a single tent, and in the absence of experimental replication or details regarding whether the application of biofilm was randomised, it is not possible to discern whether the apparent effect of biofilm was a result of the treatment or a result of differences in smoke exposure. The ambiguity of these results is resolved by the design of the second trial, in which the consistency of trends across three vineyards indicates that the biofilm does not enhance the protective effect of wax blooms.

Research Aims

With longer, more intense fire seasons on the cards as a consequence of climate change wine regions around the world are at a heightened risk of exposure to bushfire smoke. It is critical to gain a stronger understanding of current markers and expedite their quantitation, to explore novel approaches and targets to detect grapevine smoke exposure, and to develop proactive mitigation strategies. The prevailing aims of this thesis are towards improving the efficiency of smoke taint diagnostics and accelerating the development of targeted mitigation strategies to enable earlier, confident, and objective decision-making in the vineyard and winery. To achieve these aims, project objectives were therefore to:

- Explore varietal, regional, and temporal variation of naturally occurring volatile phenol glycoconjugates in grapes in California
- Monitor temporal changes in volatile phenol and glycoconjugate concentrations in grapes following smoke exposure and their subsequent fate during winemaking
- Evaluate the suitability of an environmental sensor to provide real-time monitoring of grapevine smoke exposure
- Examine the feasibility of using fluorescence spectroscopy as a preliminary screening tool that approximates volatile phenol and glycoconjugates in wine
- Identify novel chemical indicators of grapevine smoke exposure using an untargeted metabolomics approach
- Design an apparatus to facilitate small-scale mitigation trials
- Assess the efficacy of in-canopy misting and agrochemical sprays as vineyard mitigation strategies

Chapter 2

Exploring Volatile Phenol Glycoconjugates as Markers of Smoke Taint in Grapes and Wine Affected by Wildfires in California

Preface

This chapter comprises research undertaken as part of an internship with E. & J. Gallo Winery between July and November 2019. The primary aim of the internship was to adapt an existing HPLC-MS/MS method for analysis of volatile phenol glycoconjugates in grapes and wine to an Orbitrap LC-MS configuration. This involved familiarisation of operation of an Orbitrap LC-MS, optimisation of sample preparation, interpretation of results generated with ThermoFisher™ software packages and development of workflows for streamlined data processing. The adaptation and optimisation of the method was not completed until late 2019. Collaboration with E. & J. Gallo continued following the internship, and the optimised method was utilised to analyse a wide variety of grape and wine samples. Data were collected by the E. & J. Gallo team and shared to achieve the following aims:

- To determine which volatile phenol glycoconjugates are most indicative of smoke exposure in Californian Cabernet Sauvignon grapes and wine
- To establish baseline levels of volatile phenol glycoconjugates in Californian Cabernet Sauvignon, Pinot noir and Chardonnay grapes grown in the Lodi/Delta region
- To assess the stability of baseline levels of volatile phenol glycoconjugates in grapes of several varieties, grown in different Californian regions sampled over three to seven weeks preceding harvest

To achieve the first aim, grape and wine samples were classified as ‘affected’ and ‘minimally affected’ groups based on operational thresholds employed by the E. & J. Gallo team, after which compositional data for samples were evaluated using descriptive and inferential statistics. Following identification of the volatile phenol glycoconjugates that were most indicative of smoke exposure, the second and third aims were achieved by evaluating varietal and temporal variation of grapes harvested in 2020.

A manuscript reporting the development and optimisation of an Orbitrap LC-MS method for quantitation of volatile phenol glycoconjugates, and its application to an investigation into the varietal, regional and temporal variation of naturally occurring volatile phenol glycoconjugates was subsequently submitted to the *American Journal of Enology and Viticulture*. Feedback received following review (and concurrent thesis examination) identified ambiguity around the thresholds used to classify samples as ‘affected’ vs ‘minimally affected’, and inadequate consideration for how different wildfire seasons might influence the chemical profiles of smoke-affected grapes, as

weaknesses of the original manuscript/thesis chapter. In response to this feedback, the manuscript has been significantly modified.

To address the ambiguity of the thresholds, further information was requested from the E. & J. Gallo team. However, this information could not be disclosed at a level of detail sufficient for publication due to proprietary interests. Thus, these thresholds were not used in the revised manuscript. Instead, descriptive statistics and thresholds adapted from recently published literature were used to identify the key glycoconjugates present in commercial wine from vintages with low vs. high fire activity, and to then examine their patterns of accumulation in grapes collected in 2018 and 2020.

Furthermore, to provide context regarding the 2018 and 2020 wildfire seasons, open-source spatial data obtained from the CAL FIRE website, the California Department of Forestry and Fire Protection FRAP (Fire and Resource Assessment Program), and the CAL FIRE Hub, were evaluated. Smoke plumes were also evaluated using open-source spatial data acquired from the National Oceanic and Atmospheric Administration/National Environmental Satellite Data and Information Service. Existing technology has yet to be adapted to ascertain the location, fuel source, and amassed duration/density of wildfire smoke exposure in vineyards and utilise this data together with volatile phenol glycoconjugate profiles to evaluate the risk of smoke taint in wine. Thus, bridging the smoke exposure that occurred in 2018 and 2020 with the observed grape volatile phenol glycoconjugate profiles was limited to the use of descriptive statistics and a discussion of the general trends that were observed.

Analysis of the spatial data collected from the 2020 wildfire season demonstrated a high probability that the grapes intended to fulfil the second and third research aims were exposed to low levels of wildfire smoke, and thus could not be categorically classified as ‘unaffected’. The potential for unprecedented levels of smoke exposure in California during 2020 to confound any observed differences in varietal or temporal trends was not considered at the time of original submission of the manuscript. This finding potentially compromises the validity of data as an accurate representation of baseline levels and their variation across varietals and time. Thus, only the differences between varietals have been included in the revised manuscript provided below, and analysis has been limited to the use descriptive statistics and discussion of the general trends that were observed, with these caveats hereby declared.

Introduction

Drier growing seasons and warmer temperatures arising from climate change have intensified the duration, frequency, and scale of wildfires around the world (Bowman et al. 2020). In California, seven of the ten largest wildfires (since 1932) occurred in 2017, 2018, and 2020, as reported on the CAL FIRE website (www.fire.ca.gov); the 2019/2020 Australian bushfire season was also one of the most devastating on record (Abram et al. 2021). When fires occur, the thermal degradation of lignin in plant material produces volatile phenols such as guaiacol and 4-methylguaiacol (Maga et al. 1988). Volatile phenols can also be found in wines due to their: (i) natural presence in grapes (Ristic et al. 2016); (ii) extraction from oak barrels during wine maturation (Pollnitz et al. 2004); and/or (iii) contamination from grapevine exposure to wildfire smoke (Kennison et al. 2007). The latter instigates risk of smoke taint, in which the sensory profiles of wines made from smoke-exposed grapes are characterized by unpleasant 'smoky', 'burnt', 'drying', 'cold ash', and 'medicinal' attributes (Kennison et al. 2007, Ristic et al. 2011, Parker et al. 2012).

The quantitation of volatile phenols using gas chromatography-mass spectrometry (GC-MS) is well-established (Pollnitz et al. 2004, Hayasaka et al. 2010a), but predicting the risk of smoke taint occurring in wine following processing and fermentation of potentially smoke-affected grapes based on this metric alone has been impeded by several factors. Firstly, the diversity of natural vegetation that fuels wildfires alters volatile phenol profiles in smoke-exposed grapes, which adds complexity to establishing thresholds for smoke taint risk (Kelly et al. 2012, Noestheden et al. 2018a). In addition, the rapid uptake and glycosylation of volatile phenols in grapes following smoke exposure hinders accurate risk assessment and increases the likelihood that smoke taint will be underestimated (Szeto et al. 2020).

This complexity arises because volatile phenols are metabolized by grapevines into a wide variety of non-volatile glycoconjugates including glucosides, gentiobiosides, diglycosides (with terminal pentose units) and rutinosides (Hayasaka et al. 2010b), with more recent work indicating the existence of trisaccharides (Caffrey et al. 2019). Volatile phenol glycoconjugates are quantitated by either direct analysis using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (Hayasaka et al. 2010b, Dungey et al. 2011, Hayasaka et al. 2013) or indirect analysis involving acid hydrolysis of volatile phenol glycoconjugates, followed by GC-MS (Noestheden et al. 2017). In situations where both volatile phenol and volatile phenol glycoconjugate concentrations are very high, the latter serves to validate the former. However, when concentrations of volatile phenols and their glycoconjugates are incongruent or in the low to medium range, their interpretation remains challenging due to their natural, varietal-dependent abundance (Ristic et al. 2016, van der Hulst et al. 2019; Coulter et al. 2022); delayed

accumulation following smoke exposure (Szeto et al. 2020); ability to be hydrolyzed during fermentation (Kennison et. al. 2008, Caffrey et al. 2019); and highly subjective, individualized perception thresholds (Parker et al. 2020) due to in-mouth hydrolysis by enzymes during wine tasting (Mayr et al. 2014).

The inclusion of both volatile phenol and volatile phenol glycoconjugate analysis has become standard practice for smoke taint risk assessment, despite the time- and resource-intensive process being burdensome during the demanding harvest and winemaking period of vintage. With longer, more intense fire seasons, the widespread concern regarding smoke taint requires innovative approaches to screening for grapevine smoke exposure (Fudge et al. 2012, Jiang et al. 2021) and ameliorating smoke taint in wine (Fudge et al. 2011, Modesti et al. 2021). However, it also requires a more contextualized interpretation of information acquired from current practice. Thus, the aims of this work were to develop a method for routine volatile phenol glycoconjugate analysis using high-resolution tandem mass spectrometry (HR-MS/MS) and utilize it to determine volatile phenol glycoconjugates that are: specifically elevated due to smoke exposure of grapevines; indicative of smoke exposure across distinct wildfire seasons (e.g., burn conditions, fuel type, age of smoke, etc.); and sensitive to low levels of smoke exposure.

Materials and Methods

Reagents. LC-MS grade solvents were purchased from Sigma-Aldrich (St Louis, MO). Milli-Q water was sourced from a Purelab Flex 2 system (ELGA LabWater NA, Woodridge, IL). Deuterium-labeled volatile phenol glycoconjugate standards (d_3-guaicaol glucoside and d_3-guaiacol gentiobioside), as well as their unlabeled equivalents (guaiacol glucoside and guaiacol gentiobioside), were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). β-Glucosidase from almonds (lyophilized, powder, ≥4 U/mg) was sourced from Sigma-Aldrich.

Quantitation of volatile phenol glycoconjugates in grapes and wine by LC-HRMS analysis.

LC-HRMS Method. A method to quantify volatile phenol glycoconjugates in grapes and wine was adapted to the LC-HRMS interface according to previously published SIDA methods (Dungey et al. 2011). Samples were analyzed with a Vanquish UHPLC system coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) configured with an Agilent ZORBAX RRHT Eclipse XDB-C18 column (2.1 mm x 100mm, 1.8µm particle size) and a heated electrospray ionization (H-ESI) source. Sample injection volume was 10 µL.

Column temperature was maintained at 40 °C throughout the run. A binary gradient was used, with mobile phases constituted of 0.1% v/v glacial acetic acid in LC-MS grade water (solvent A) and 0.1% v/v

glacial acetic acid in LC-MS grade acetonitrile (solvent B). The flow rate was set to 0.400 mL/min, with a linear gradient that began at 5% B for 4 min, after which it was increased to 35% B (over 12 min), increased to 100% B (over 1 min) and held for 3 min, and lastly, decreased to 5.0% B (over 0.5 min) and held for 3.5 min.

The Orbitrap was operated in negative ion mode with a capillary temperature of 350 °C and an auxiliary gas heater temperature of 425 °C. The sheath gas flow rate was 50 arbitrary units (au), the sweep gas flow was 3 au, and the auxiliary gas flow rate was 13 au. The spray voltage was set to -3 kV and the S-lens RF level at 55 au. Spectra were acquired from 100 to 1000 *m/z* in parallel reaction monitoring (PRM) mode at 5 ppm mass tolerance with 70,000 scan resolution, 1 microscan, 50 ms maximum injection time, and automatic gain control (AGC) of 1e5. Collision energy (CE) was set between 10 and 15 eV. Instrumental control and data acquisition were performed using XCalibur software (version 4.1.31.9) (Thermo Fisher Scientific). Included transitions corresponded to glucosides, gentiobiosides, pentosylglucosides, and rutinosides monitored in previous work (Hayasaka et al. 2013, Noestheden et al. 2018b) (see **Supplemental Table 1**).

Sample preparation. Grapes were homogenized, after which 10 g of each sample were spiked at 250 µg/kg of d_3-guaiacol glucoside and d_3-guaiacol gentiobioside as ISTD and prepared for solid phase extraction (SPE). Homogenate samples were centrifuged for 15 min at 7500 RPM using a Beckman Spinchron 15 (Beckman Coulter, Indianapolis, IN), adjusted to pH 13 (with 0.5 mL 10 N NaOH) and processed through a 0.45 mm PTFE syringe filter (Whatman, Buckinghamshire, UK). pH adjustments were made to lower the isobaric interference of phenolic compounds (Noestheden et al. 2018b). To prepare wine samples for SPE, 5 mL aliquots were spiked with 200 µg/L of d_3-guaiacol glucoside and d_3-guaiacol gentiobioside as ISTD and adjusted to pH 13 (with 0.5 mL of 10 N NaOH).

SPE is a time- and resource-intensive procedure and to reduce sample preparation time, automation was trialed with Oasis HLB 96-well SPE plates (30 mg, 30 µm; Waters, Milton, MA), hereafter referred to as 'Oasis HLB plates', prepared with a Hamilton Microlab STAR robotic liquid handler (Hamilton Robotics, Reno, NV). Wells were pre-conditioned with 0.5 mL of methanol followed by 0.5 mL of water, after which 1 mL of each sample (in triplicate) was loaded onto separate wells and rinsed with 1 mL of water, followed by 0.5 mL of dichloromethane. Plates were dried under vacuum and eluted with 0.5 mL of methanol. Eluates from the three wells corresponding to each sample were combined, transferred into 1.5 mL micro-centrifuge tubes and evaporated to dryness under vacuum in an Eppendorf Vacufuge

(Eppendorf, Enfield, CT, USA) at room temperature. Lastly, samples were reconstituted in 0.4 mL of water and transferred into a syringeless 0.45 µm filter vial (Agilent Technologies) for LC-HRMS analysis.

As a comparison to high-throughput Oasis HLB plates, wine samples were also prepared with conventional LiChrolut® EN cartridges (100 mg, 40-120 µm) configured to a 20-position manifold (Waters, Milton, MA). Cartridges were pre-conditioned with 2 mL of methanol followed by 2 mL of water, after which 5 mL of each sample were loaded onto individual cartridges and rinsed with 2 mL of water. Samples were air dried for 25 min and eluted with 1 mL of methanol. Eluates were dried, reconstituted, and transferred through filter vials for LC-HRMS analysis, as outlined above for samples prepared with Oasis HLB plates.

Wine matrix effects. To investigate the impact of matrix effects on calibration linearity and signal intensity in wine samples, model wine and "bag-in-box" dry red wine were prepared with differing concentrations of ethanol. Model wine was prepared with 0 to 20% ethanol (at 5% increments) in water with 7 g/L of tartaric acid, adjusted to pH 3.4 (with 10 N NaOH). The dry red wine (pH 3.4) was analyzed without alcohol adjustment (13.5% ABV), and as its dealcoholized equivalent (0% ABV) following lyophilization. To remove alcohol from the dry red wine, 40 mL aliquots were poured into 120 mL plastic tubes (Sarstedt Pty. Ltd., Nümbrecht, Germany), weighed, and frozen overnight at -80 °C. Caps were replaced with a layer of tightly wrapped, perforated Parafilm, before samples were transferred into purpose-built flasks attached to a freeze dryer (Kinetics FTS FD-3-85A-MP Flexi Dry Microprocessor Freeze Dry Lyophilizer) and kept under 97 mT vacuum and -88 °C conditions until a powder was obtained. Samples were then reconstituted with water to their original recorded mass.

Method validation. Method validation in grapes was conducted with frozen, mature Chardonnay grapes, whereas in wines validation was conducted with model wine (0% ABV) and dealcoholized dry red wine (0% ABV). All grape and wine samples received an addition of ISTD and an adjustment to pH 13 (as above), except model wine samples, which were not pH adjusted due to the simplicity of their matrix. Model wine and grape homogenate samples were prepared with Oasis HLB plates, whereas dealcoholized dry red wines were prepared with both Oasis HLB and LiChrolut EN® cartridges. A series of standard additions of guaiacol glucoside and guaiacol gentiobioside led to eight calibration levels in grapes (at 0, 10, 20, 30, 50, 100, 200, and 500 µg/kg) and seven calibration levels in (model and red) wines (at 0, 5, 10, 25, 50, 100, and 250 µg/L). Method precision (reported as percentage residual standard deviation (%RSD) values) and accuracy (reported as percentage recovery values) were examined with ten replicate samples spiked at 30 µg/kg each of guaiacol glucoside and guaiacol gentiobioside (grapes) or

four replicate samples spiked at 10 and 25 µg/L each of guaiacol glucoside and guaiacol gentiobioside (0% ABV model wine) or 5 and 25 µg/L each of guaiacol glucoside and guaiacol gentiobioside (dealcoholized dry red wine).

Samples. To identify volatile phenol glycoconjugates that were elevated specifically due to smoke exposure in grapevines, Cabernet Sauvignon wine samples (n=43) were sourced from commercial wineries across several regions and vintages in California. Samples from 2011 and 2012 were commercial wines with no known presence of smoke taint and were classified as 'minimally affected' by smoke exposure. Samples from 2015, 2018 and 2020 were research wines made from growing regions proximal to wildfires and thus, were classified as 'affected'. This distinction was supported with historical wildfire data and an adapted classification scheme (Crews et al. 2022). Additional details regarding the sources of wine can be found in **Supplemental Table 2**. Wine samples were used instead of grapes due to the availability of wine from years with low wildfire activity. The glycoconjugates identified as being important in wine were then used to examine grape samples. Previous work has demonstrated that volatile phenol glycoconjugates are affected by yeast-catalyzed hydrolysis in primary fermentation, with lower levels reported in finished wine compared to levels in juice at crush; however, the average decrease across glycoconjugates did not exceed 25% (Caffrey et al. 2019). Previous work has also shown that this substantial reservoir of volatile phenol glycoconjugates in wine is stable, even after years of bottle aging (Ristic et al. 2017).

The glycoconjugates deemed to be most indicative of smoke exposure in wine were subsequently quantified in grape samples to investigate their consistency across distinct wildfire seasons and their sensitivity to low levels of smoke exposure. Cabernet Sauvignon grapes (n=108 total) were sourced from commercial vineyards (at maturity), comprising n=61 samples in 2018 (78% from Lake County) and n=47 samples in 2020 (74% from Napa Valley). Samples in 2018 were collected between 8/30 and 10/11, whereas samples in 2020 were collected between 9/22 and 10/8. Based on their total glycoconjugate concentrations, these grapes were classified as having had low to extreme levels of smoke exposure (Crews et al. 2022). Mature Chardonnay (n=52), Cabernet Sauvignon (n=40) and Pinot noir (n=20) grapes were also sourced from commercial vineyards in the Central Valley (n=112 total, 83% from the Lodi/Delta AVA) in 2020. Based on their total glycoconjugate concentrations, these grapes were classified as having had low to modest levels of smoke exposure (Crews et al. 2022).

Spatial data. *Fire perimeters.* Fire perimeters from the 2018 and 2020 wildfire seasons in California were sourced from the CAL FIRE Fire and Resource Assessment Program (FRAP) Geospatial

Information System (GIS) database (https://frap.fire.ca.gov/mapping/gis-data). Several statewide agencies contribute to the database, and for a fire to be recorded, it must have burned an area of at least 10 acres for timber fires, 30 acres for brush fires and 300 acres for grass fires.

Acres burned. For each wildfire season from 2008 to 2021, the total acres burned, number of megafires (i.e., fires with > 100,000 acres burned) and size of the largest wildfire were identified using the Cal Fire historical wildfire activity statistics (i.e., Redbooks) (https://www.fire.ca.gov/stats-events).

Smoke plumes. Shapefiles of smoke plumes from the 2018 and 2020 Californian wildfire seasons were sourced from the Hazard Mapping System (HMS) Fire and Smoke Analysis, a product offered by the National Oceanic and Atmospheric Administration (NOAA)/National Environmental Satellite Data and Information Service (https://www.ospo.noaa.gov/Products/land/hms.html). Smoke plumes were identified by classifying imagery acquired by two NOAA-NASA (National Aeronautics and Space Administration) satellites from the Geostationary Operational Environmental Satellites (GOES) fleet, GOES-16 and GOES-17. Smoke plumes were generated using a sequence of satellite images collected over a period of 1 to 3 hours of daylight. The geographic domain of the HMS is focused on North America, stretching from 14.6 °N to 72 °N and from 50 °W to 170 °W. In addition to the location and outline of each smoke plume, aerosol optical depth (AOD) data can be used to estimate the density of each plume, being light (up to 10 $\mu g/m^3$), medium (10 up to 21 $\mu g/m^3$) and heavy (21 up to 32 $\mu g/m^3$).

Polygons of daily smoke plumes with light, medium and heavy density were examined over approximately 80 days in 2018 (7/24 to 9/30) and 2020 (7/26 to 10/11). This range of dates was relevant to grape sampling dates within periods of significant wildfire activity. The boundaries of Lake County, Napa County, and Sonoma County (in 2018) and those of Napa County, Sonoma County, and the Lodi/Delta region (in 2020) were used as a base layer. The boundaries for Napa, Sonoma and Lake County were sourced from the California Open Data Portal, which is procured by the California Department of Technology (https://data.ca.gov/dataset/ca-geographic-boundaries). The boundary for the Lodi/Delta American Viticultural Area (AVA) was sourced from the American Viticultural Areas Digitizing Project Team (https://github.com/UCDavisLibrary/ava). The polygons of smoke plumes were visualized over these perimeters and given a value of 0 if they did not intersect with at least 30% of a region and a value of 1 if they intersected with at least 30% of a region. This visual analysis was performed for each day, region, and smoke density level.

Vegetation types. A raster layer showing the spatial distribution of California Wildlife Habitat Relationship (WHR) classes was sourced from the CAL FIRE Hub (https://hub-calfire-

forestry.hub.arcgis.com). California WHR classes are grouped into thirteen major land cover types, being agriculture, barren (or other), conifer forest, conifer woodland, desert shrub, desert woodland, hardwood forest, hardwood woodland, herbaceous, shrub, urban, water and wetland. In this raster layer, desert shrub and desert woodland were combined into a single 'desert' class and wetland areas were not included. The most acreage within the fire perimeters identified by the CAL FIRE FRAP GIS database in 2018 and 2020 was accounted for by conifer forests, hardwood forests, hardwood woodlands, herbaceous and shrub land cover. Zonal histograms were used to calculate the number of pixels corresponding to unique types of land cover within each fire perimeter. The raw raster data had a distance resolution of 50 m and to improve computational efficiency, the raw raster data were exported as a GeoTiff image with a larger pixel size of 100 m x 100 m.

Data analysis. Spatial data were analyzed and visualized in QGIS (version 3.22.8) and projected using the North American Datum of 1983 (NAD83) / California Albers (EPSG:3310) as a coordinate reference system. Chemical data were analyzed in RStudio (RStudio Inc., Boston, MA) version 4.1.1 and visualized using the *ggplot2* package.

Results and Discussion

Solid phase extraction (SPE) is a common sample preparation technique employed prior to analysis that serves to purify and concentrate analytes of interest; however, the increased prevalence of samples submitted to commercial laboratories for smoke taint analysis—and the time-sensitive decisions resting on the results—would benefit from faster sample processing times than can be achieved with vacuum extraction manifolds. Standard practice analyzes volatile phenol glycoconjugates following sample extraction with C18 cartridges and/or filtration through a 0.45 µm filter (Hayasaka et al. 2010a); however, a recent study demonstrated improved recovery of glycoconjugates with the inclusion of an alkaline wash step (0.1 N NaOH, pH 13) to limit interference presented by polyphenolic flavonoids and their corresponding glycoconjugates (Noestheden et al. 2018b). The inclusion of this step requires the replacement of the C18 sorbent, which would not retain the volatile phenol glycoconjugates under such an extreme pH, with a polymeric equivalent (Noestheden et al. 2018b). Oasis HLB 96-well plates were selected in the present study because they offer the dual benefits of being stable under extremely high pH conditions and adaptable to automation, and their performance was compared to LiChrolut® EN, a highly selective, choice product in conventional SPE workflows. The sorbents of both Oasis HLB and LiChrolut cartridges are lined with copolymer substrates.

Red wine and model wine samples were spiked with guaiacol glucoside and guaiacol gentiobioside (0 to 250 µg/L) and d_3-guaiacol glucoside and d_3-guaiacol gentiobioside as ISTD (200 µg/L) and analyzed by LC-HRMS in PRM mode. Model wine was prepared with 0% ABV and red wine was dealcoholized to assess cartridge performance in the absence of competing matrix effects. As shown in **Supplemental Table 3**, standard curves demonstrated high linearity over the calibration range, having high coefficient of determination (R^2) values across both SPE cartridges and wine matrices (R^2 range = 0.9995 to 0.9999). Precision values in dealcoholized red wine for guaiacol gentiobioside (at spiking levels 5 and 25 µg/L) were 7% and 4%, respectively (LiChrolut® EN) and 3% and 2%, respectively (Oasis HLB plates). Precision values in dealcoholized red wine for guaiacol glucoside (at spiking levels 5 and 25 µg/L) were 2% and 1%, respectively (LiChrolut® EN) and 2% and 4%, respectively (Oasis HLB). Accuracy levels for both compounds (at spiking levels 5 and 25 µg/L) were between 94 to 104% (LiChrolut® EN) and 91 to 101% (Oasis HLB plates).

Due to the time-intensive process of dealcoholization, its impact on calibration linearity and peak area intensity were quantified in model wines (containing 0, 5, 10, 15, and 20% ABV) dry red wine (0 and 13.5% ABV) prepared with Oasis HLB plates. In model wine, peak areas corresponding to guaiacol glucoside and guaiacol gentiobioside decreased with increasing %ABV, indicating that as matrix polarity decreased, fewer glycoconjugates partitioned to the SPE sorbent; however, as expected, this change was mirrored by the internal standards, thus conserving the analyte/ISTD ratio (data not shown). Calibration linearity of guaiacol gentiobioside quantitation in model wine was high, with R^2 values $\geq$0.9992 across the range of model wine. On the contrary, calibration linearity of guaiacol glucoside quantitation in model wine demonstrated higher sensitivity to matrix effects, with R^2 values decreasing from 0.9994 in 0% ABV to 0.9942 and 0.9988 in 15% ABV and 20% ABV matrices (**Supplemental Table 4**).

A comparison of red wine without alcohol adjustment (13.5% ABV) and its dealcoholized equivalent demonstrated a very similar trend, with lower absolute guaiacol glucoside and guaiacol gentiobioside peak areas in the presence of alcohol, albeit this was accounted for with an ISTD. Regardless of dealcoholization of the dry red wine, high calibration linearity was maintained for both guaiacol glucoside and guaiacol gentiobioside, with R^2 values from 0.9994 to 0.9998. This work highlights the impact of %ABV on the absolute peak area of volatile phenol glycoconjugates. While an ISTD can account for these differences, the absence of deuterated standards corresponding to the full suite of volatile phenol glycoconjugates elevated by grapevine smoke exposure prompted incorporation of a dealcoholization step

into the sample preparation procedure. Reducing the time to achieve dealcoholization will be the subject of a future study.

Following method validation in wines, which demonstrated the comparable performance of Oasis HLB plates and LiChrolut EN cartridges, validation in grapes was carried out using Oasis HLB plates only. White grape homogenate was spiked with guaiacol glucoside and guaiacol gentiobioside (0 to 500 µg/kg) and d_3-guaiacol glucoside and d_3-guaiacol gentiobioside (250 µg/kg) as ISTD. Guaiacol glucoside and guaiacol gentiobioside, respectively, demonstrated high linearity (R^2 values = 0.9994 and 0.9995), with good precision (6% and 4% RSD) and accuracy (108% and 118% at 30 µg/kg spike level). Further details regarding method validation in wine and grape matrices can be found in **Supplemental Table 3.**

Previous studies have shown the effects of timing, duration, and density of smoke exposure on the abundance of volatile phenols and their glycoconjugates in grapes and wine (Kennison et al. 2009, 2011, Szeto et al. 2020). Yet the potential effects of these factors on the compositional profile of volatile phenol glycoconjugates in grapes have yet to be explored in great depth (Jiang et al. 2021). The Australian Wine Research Institute's (AWRI) Commercial Services laboratory currently measures six volatile phenol glycoconjugates (in tandem with free volatile phenols) to inform smoke taint analysis; being cresol rutinoside, guaiacol rutinoside, 4-methylguaiacol rutinoside, phenol rutinoside, syringol gentiobioside, and 4-methylsyringol gentiobioside. These glycoconjugates are a subset selected from over twenty volatile phenol glycoconjugates that have been reported in the literature (Hayasaka et al. 2013; Noestheden et al. 2018b; Caffrey et al. 2019; van der Hulst et al. 2019). Few studies have assessed whether this subset of glycoconjugates is relevant to grapevines exposed to wildfire smoke in regions outside of Australia (Noestheden et al. 2018a; Crews et al. 2022); thus, Cabernet Sauvignon wines made from grapes affected by a range of smoke exposure in California were examined.

There are several methods to classify samples affected by different levels of smoke exposure. They are not grounded in wine sensory studies and remain coarse indications of smoke exposure only. While it is known that in general, a higher intensity of smoke exposure in grapevines increases the risk that smoke taint will be perceived in subsequent wine, this relationship is not linear (Kennison et al. 2009; Parker et al. 2012; Szeto et al. 2020). The first option is to use varietal-specific baseline levels established in grape and wine samples collected from over several regions and vintages in Australia (Coulter et al. 2022). This system provides a conservative classification which attests the presence or absence of smoke exposure. It is critical for decisions involving samples with lower levels of smoke exposure but for samples with

glycoconjugates at levels above baselines, an alternative option involves use of 'total' volatile phenol glycoconjugate values.

Total volatile phenol glycoconjugate values correspond to the sum of the six glycoconjugates included in the smoke taint analysis performed by AWRI Commercial Services. In a recent study, samples were classified into seven levels of smoke exposure based on quantitation of total volatile phenol glycoconjugates, comprising: baseline (less than 6 ppb total), light (6 to 30 ppb total), modest (31 to 100 ppb total), significant (101 to 200 ppb total), elevated (201 to 300 ppb total), substantial (301 to 400 ppb total), and severe (greater than 400 ppb total) (Crews et al. 2022). In the present study, wines were classified as 'minimally affected' and 'affected' based on the severity of relevant wildfire seasons and this latter classification scheme.

In each wildfire season, total acres burned was used to approximate the prevalence of wildfires and associated risk that wines were made from smoke-affected grapes. For wildfire seasons with a comparable number of total acres burned, the specific location and duration of wildfires relative to the appellation of wine samples were examined in greater detail. Between 2011 and 2021, the second lowest value for total acres burned (228,559 acres) occurred in 2011, whereas the areas burned in 2018 and 2020 were amongst the top five wildfire seasons, with 1.9 and 4.3 million total acres burned, respectively (CAL FIRE Redbooks). With such contrasting values in the range of total acres burned over the last twenty years, wines from 2011 and 2020 were considered 'minimally affected' and 'affected', respectively.

The 2012 and 2015 wildfire seasons had comparable number of total acres burned, being 829,224 and 880,899, respectively (CAL FIRE Redbooks). However, closer examination indicated that the 2012 commercial wines (primarily from Napa Valley, Sonoma County, and Central Coast) and the 2015 research wines (from Lake County) were likely made from grapes with different amounts of smoke exposure. The CAL FIRE Redbooks considers fires to be 'large' if the total area burned exceeds 300 acres. During 2012, most large fires that occurred across California burned less than 5,000 acres each and were contained in under four days (CAL FIRE Redbook 2012); Napa Valley and Sonoma reported a combined 359 total acres burned, while on the Central Coast, Monterey and San Luis Obispo counties each recorded less than 3,000 total acres burned (CAL FIRE Redbook 2012). On the other hand, Lake County alone recorded 171,849 total acres burned in 2015, due in part to the Rocky Fire (which burned 69,438 acres) and the Jerusalem Fire (which burned 25,1118 acres), and it took around two weeks to contain each of these fires (CAL FIRE Redbook 2015). Wine from 2012 was therefore classified as 'minimally affected', whereas wine from 2015 was classified as 'affected'.

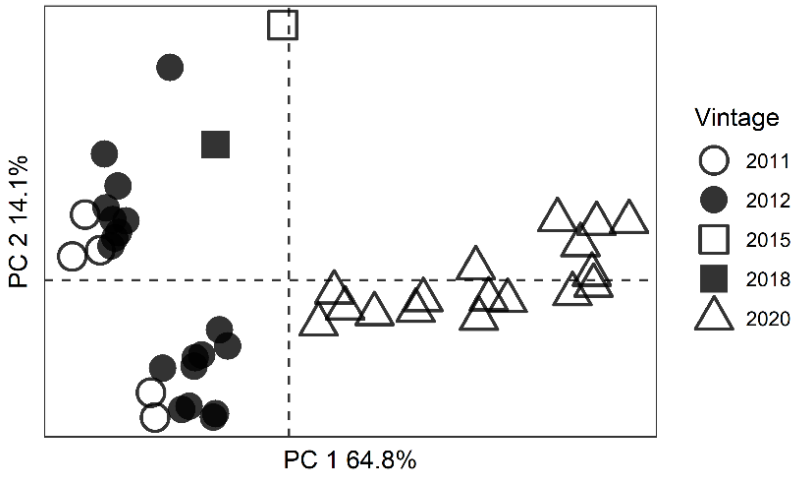
Table 1. Range and mean values of total volatile phenol glycoconjugate concentrations (μg/L) in minimally affected wines (from 2011 and 2012) and affected wines (from 2015, 2018 and 2020). Total volatile phenol glycoconjugates refer to the total concentrations of of cresol, phenol, guaiacol and 4-methylguaiacol rutinoside and syringol and 4-methylsyringol gentiobioside.

Classification	Vintage	N	Minimum	Maximum	Mean
Minimally affected	2011	5	3	15	9
	2012	19	12	60	27
Affected	2015	1	-	-	396
	2018	1	-	-	240
	2020	17	155	871	522

The total volatile phenol glycoconjugate levels measured in wines supported the distinction between samples from 2011 and 2012 vintages, and those from 2015, 2018 and 2020 vintages (**Table 1**). Wine from 2011 and 2012 had a mean total volatile phenol glycoconjugate value of 23 μg/L, with 17 samples containing less than 30 μg/L and 7 containing less than or equal to 60 μg/L total volatile phenol glycoconjugates. This suggested that wine from 2011 and 2012 were made from grapes with low (6 to 30 μg/L total volatile phenol glycoconjugates) to modest (31 to 100 μg/L total volatile phenol glycoconjugates) amounts of smoke exposure. Cresol, phenol and guaiacol rutinoside were the most abundant glycoconjugates observed in wine from 2011 and 2012, with mean values 8, 5 and 5 μg/L. For wines from 2015, 2018 and 2020, total volatile phenol glycoconjugate levels confirmed they were likely made from grapes with significant (101 to 200 ppb total) to severe (greater than 400 ppb total) levels of smoke exposure; total volatile phenol glycoconjugate concentrations ranged from 155 to 871 μg/L (with a mean value of 501 μg/L). Cresol rutinoside, syringol gentiobioside, and phenol rutinoside were the most abundant volatile phenol glycoconjugates measured in affected wines, with mean values of 162, 123 and 108 μg/L, respectively. Interestingly, guaiacol rutinoside had a mean value of only 40 μg/L in the affected wine. Relative to the affected wines from the 2020 vintage, the most notable difference in affected wines from 2015 and 2018 was the lower levels of guaiacol rutinoside (which were less than 10 μg/L). The affected wine from 2020 had a mean guaiacol rutinoside concentration of 44 μg/L.

To identify additional markers that could differentiate minimally affected and affected wines, principal component analysis (PCA) was performed on the concentrations of all 24 volatile phenol glycoconjugates (**Figure 1**). The first two components captured 78.9% of total variance, with 64.8% explained by PC 1. Samples with the lowest and highest total volatile phenol glycoconjugates were positioned on opposite ends of PC 1, which indicates that separation along this axis is strongly influenced by differences in smoke exposure. However, many glycoconjugates contributed to variance captured by PC 1, including: phenol, guaiacol and cresol pentosylglucosides; phenol, syringol, 4-methylguaiacol and guaiacol glucosides; and phenol gentiobioside. As an unsupervised data reduction strategy, it is uncertain whether these additional glycoconjugates are relevant to differentiating samples with different levels of smoke exposure.

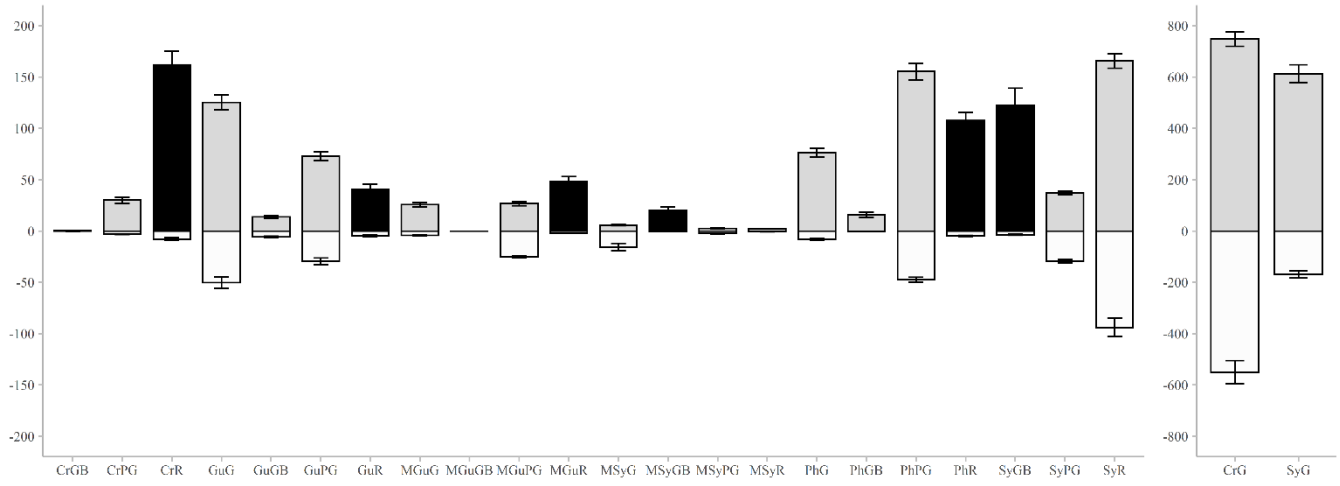
Figure 1. PCA scores plot of volatile phenol glycoconjugate concentrations measured in wines from vintages with low (2011, 2012) and high (2015, 2018, 2020) wildfire activity.



As a consequence, glycoconjugate levels were compared between minimally affected and affected wines **(Figure 2**). The most indicative markers were identified as those with low concentrations in minimally affected wines and elevated concentrations in affected wines. To compare groups, absolute and relative differences in means were referenced. Among the six established markers of smoke taint, 4-methylsyringol gentiobioside had the lowest mean concentration in affected wine, being 20 μg/L, and guaiacol rutinoside had the lowest relative difference between affected and minimally affected groups, being 8.4. Using these numbers as a reference, glycoconjugates were not further examined if they had a

mean concentration less than 20 µg/L in the affected group, a relative difference between groups of less than eight, or both.

Figure 2. Bar chart of mean volatile phenol glycoconjugate concentrations (µg/L) in minimally affected wines (from 2011, 2012) (white bars) and affected wines (from 2015, 2018 and 2020) (grey bars). The mean concentrations of established markers of smoke taint in affected wine are shaded in black (i.e., cresol rutinoside, guaiacol rutinoside, 4-methylguaiacol rutinoside, phenol rutinoside, syringol gentiobioside, 4-methylsyringol gentiobioside). Values are reported as guaiacol gentiobioside equivalents (for rutinosides, gentiobiosides and pentosylglucosides) and guaiacol glucoside equivalents (for glucosides). Error bars represent standard error of the mean. Minimally affected values are plotted as negative for visualization purposes only—all concentration values are positive. Cr = cresol; Gu = guaiacol; MGu = 4-methylguaiacol; MSy = 4-methylsyringol; Ph = phenol, and Sy = Syringol; GB = gentiobioside; PG = pentosylglucoside; R = rutinoside; G = glucoside.



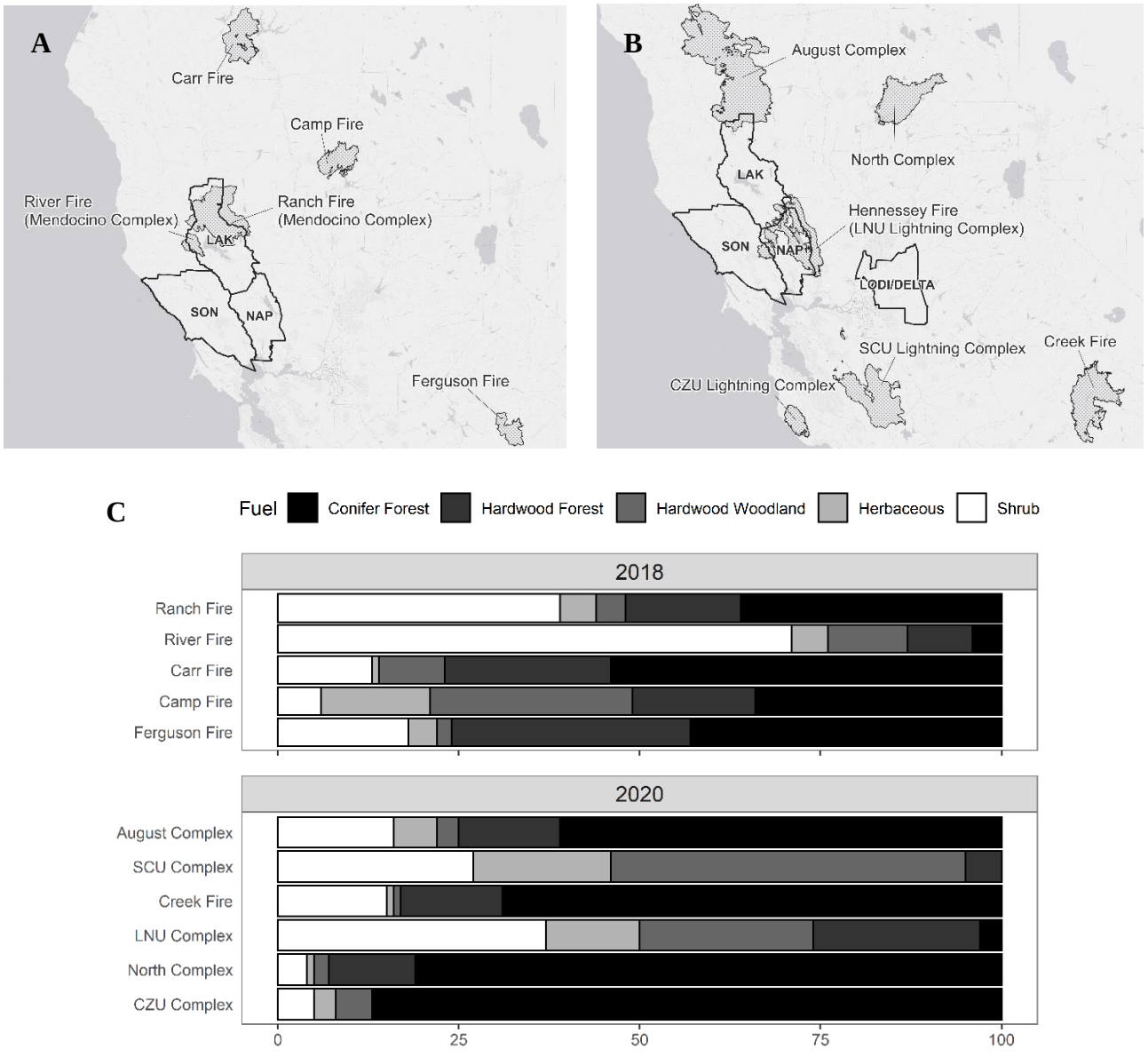
Several volatile phenol glycoconjugates had mean concentrations less than 10 µg/L in affected wine, including: cresol, phenol, guaiacol and 4-methylguaiacol gentiobiosides, 4-methylsyringol pentosylglucoside and 4-methylsyringol rutinoside. The remaining glycoconjugates had elevated mean concentrations in minimally affected wines, ranging from 16 µg/L (4-methylsyringol glucoside), to between 25 and 50 µg/L (guaiacol glucoside, and pentosylglucosides of guaiacol, 4-methylguaiacol, phenol and syringol) and in excess of 90 µg/L (syringol glucoside, cresol glucoside and syringol

rutinoside). Elevated means of volatile phenol glycoconjugates in minimally affected wines reduced the relative difference to means in affected wines to less than eight. For example, while 4-methylguaiacol glucoside had a mean concentration of 4 μg/L in minimally affected wines, it was only elevated to a mean concentration of 25 μg/L in affected wines; thus, the relative difference between means was less than eight.

Each of the established markers of smoke taint exceeded these criteria, i.e., cresol, phenol, guaiacol and 4-methylguaiacol rutinosides, and syringol and 4-methylsyringol gentiobiosides. Additional glycoconjugates that met these criteria included cresol pentosylglucoside and phenol glucoside. The potential for pentosylglucosides to be included as additional markers of smoke taint has been supported by several other studies (Caffrey et al. 2019; van der Hulst et al. 2019; Szeto et al. 2020; Crews et al. 2022). On the other hand, there has been little evidence for the use of phenol glucoside as an additional marker of smoke taint. Previous studies conducted in Australia have shown levels of phenol glucoside lower than 5 μg/kg in smoke-affected grape and juice samples (Culbert et al. 2021; Jiang et al. 2021); thus, the potential for phenol glucoside to be used as a marker of smoke taint may be specific to California. The markers identified as being most important to wine were considered to also be representative of smoke exposure in Cabernet Sauvignon grapes grown in vineyards in Californian wine regions. This is supported by studies showing that a large proportion of volatile phenol glycoconjugates in smoke-affected grapes are stable through fermentation (Caffrey et al. 2019) and in finished wine, even after several years of bottle aging (Ristic et al. 2017). Thus, the occurrence of these markers was examined further for grapes collected from distinct wildfire seasons.

Previous studies have suggested that the combustion of different fuel sources may lead to differences in exogenous volatile phenol loads (Kelly et al. 2012; Noestheden et al. 2018b); however, the extension of this effect to the profile of volatile phenol glycoconjugates in grapes has not been thoroughly evaluated. The results presented above indicate that phenol glucoside may be an example of a regiospecific marker of smoke taint. Herein, mature grapes were sampled from 2018 and 2020, two of the largest wildfire seasons in the modern history of California (since 1932), with 1.9 million and 4.3 million total acres burned in these years, respectively (CAL FIRE Redbooks).

Figure 3. Perimeters of major California wildfires near key winegrowing regions in 2018 (A) and 2020 (B) and the most abundant vegetation types found within the perimeter of each fire (C). Key regions include Sonoma County (SON), Lake County (LAK), Napa County (NAP) and the Lodi AVA (LODI/DELTA).

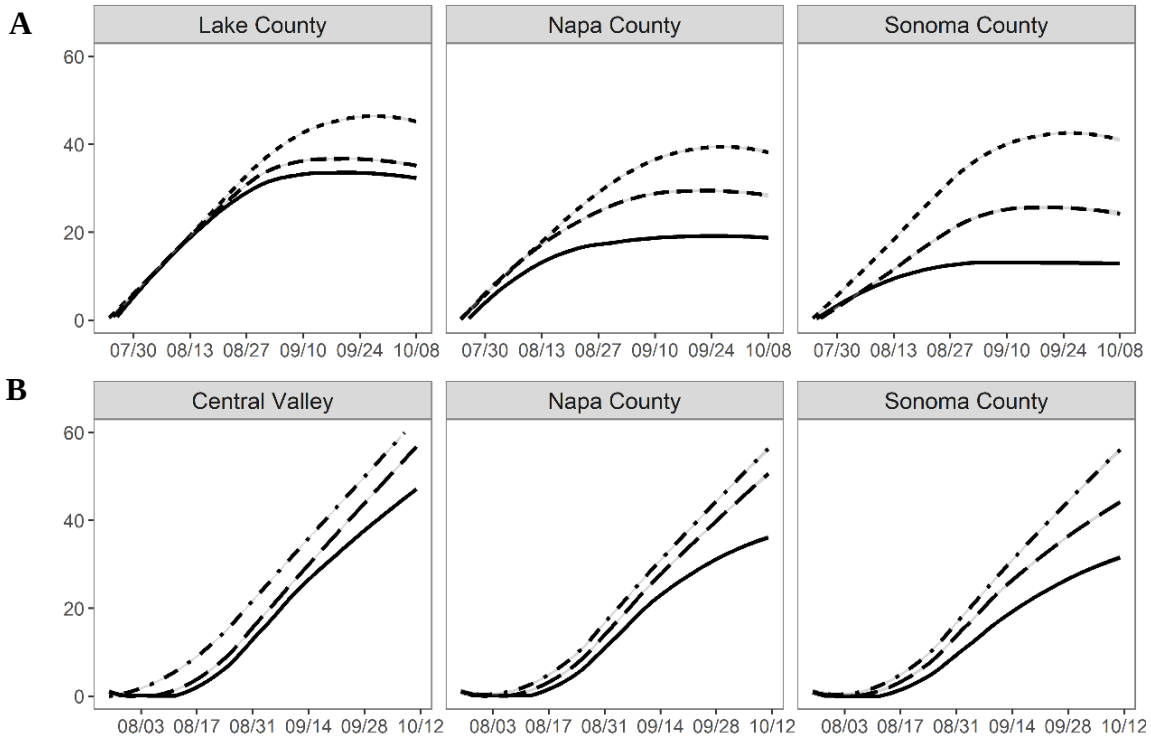


In 2018, grapes were primarily collected from Lake County, and as shown in **Figure 3,** the wildfire closest to vineyards in Lake County was the Mendocino Complex. The complex formed through the convergence of the Ranch and River Fires. This was the largest wildfire of 2018, and from its 7/27 start date, it spread 459,123 acres over Colusa, Glenn, Lake, and Mendocino County and burned 38% of the total area in Lake County before it was contained two months later, on 9/27. The area burned by the Ranch Fire (410,203 acres) comprised 36% conifer forest, 29% shrub, and 16% hardwood forest land cover, while the smaller River Fire (48,920 acres) was fueled by 71% shrub and 11% hardwood woodland land cover (**Figure 3**).

In 2020, grapes were primarily sourced from Napa County, and several megafires burned in proximity to this region, including the August Complex, LNU Lighting Complex and the SCU Lightning Complex **(Figure 3**). All three complexes began within a day of each other on either 8/16 or 8/17. The LNU and SCU Lightning Complex were contained on 9/22 and 9/15 respectively, while the August Complex was not contained until a month later, on 10/15. The closest wildfire to vineyards in Napa County was the LNU Lightning Complex, which spread 363,220 acres and burned 41% of the total area in Napa County and 2% of the total area in Lake County. The August Complex burned 1,032,648 acres and affected 5% of the total area in Lake County. Although the perimeter of the August Complex did not intersect with that of Napa County, the probability of smoke exposure from this fire affecting vineyards in Napa County cannot be discounted, given its unprecedented magnitude. Similar reasoning justified the inclusion of details regarding the SCU Complex, a megafire south of Napa County that burned 396,624 acres and burned key regions in the Central Valley, such as San Joaquin and Stanislaus County. The land cover defining each of the megafires was distinct, with predominant fuel types in each megafire being conifer forest (August Complex), hardwood woodland (SCU Lightning Complex) and shrub (LNU Complex) (**Figure 3**).

In addition to differences in the scale and fuel load characterizing the 2018 and 2020 wildfire seasons, satellite imagery suggests that there were also differences in the duration and density of smoke exposure. In 2018, there was a steep, four-week accumulation of days with light, medium and heavy smoke exposure in Lake County that spanned from late July until the end of August (**Figure 4**), after which medium and heavy smoke exposure plateaued. Smoke with lighter density continued to linger until the end of September.

Figure 4. Line graphs depicting the cumulative number of days (y-axis) on which smoke plumes covered at least 30% of a key winegrowing area on a given day (x-axis), over 80 days from early ripening to harvest in 2018 (A) and 2020 (B). Plume density varies from light (- - -), medium (-- -- --) and heavy (—).

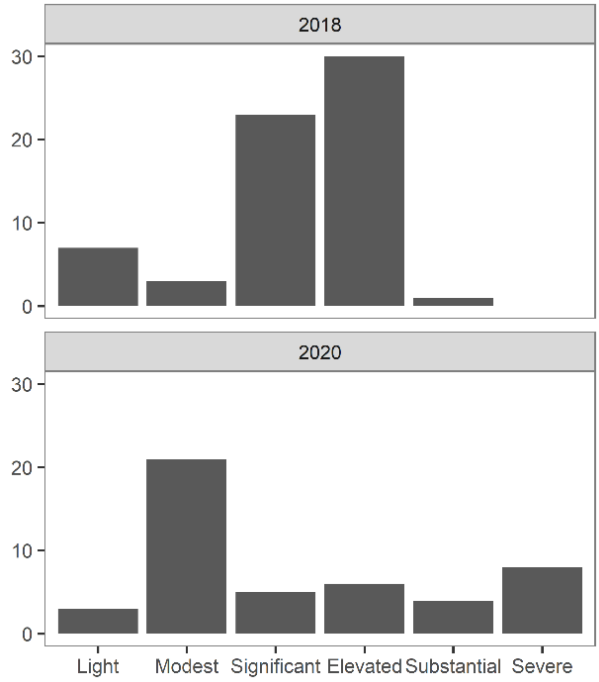


The observed plateau of medium and heavy smoke plumes may reflect the containment of the Ranch Fire on 8/17 (CAL FIRE Redbook 2018). In contrast, a gradual accumulation of light, medium, and heavy smoke exposure occurred in 2020, and persisted from the middle of August, when the three megafires ignited, until the end of the wildfire season. The rate at which days of heavier smoke exposure accumulated showed a slight reduction from mid-September, which coincided with the suppression and containment (on 9/22) of the LNU Complex. Nevertheless, the cumulative number of days during which smoke plumes (across density levels) were observed, continued to rise. From the perspectives of scale, fuel load and burn conditions, it is evident that 2018 and 2020 were two distinct wildfire seasons.

As shown in **Figure 5**, the distribution of total volatile phenol glycoconjugates measured in (mature) grape samples harvested in 2018 was narrow, with 78% of samples classified as having 'significant' (101 to 200 ppb total volatile phenol glycoconjugates) or 'elevated' (201 to 300 ppb total volatile phenol glycoconjugates) categories of smoke exposure. Only five samples were found to have less

than 100 µg/L of total volatile phenol glycoconjugates and a single sample had an excess of 300 µg/L of total volatile phenol glycoconjugates. Grape samples harvested in 2020 were seemingly exposed to a much wider distribution of smoke, with 35% classified as having had 'modest' smoke exposure (31 to 100 ppb total volatile phenol glycoconjugates), 12.5% as 'significant' (101 to 200 ppb total volatile phenol glycoconjugates), 15% as 'elevated' (201 to 300 ppb total glycoconjugates), 10% as 'substantial' (301 to 400 ppb total volatile phenol glycoconjugates) and 20% as 'severe' (greater than 400 ppb total volatile phenol glycoconjugates).

Figure 5. Bar graph showing the number of grape samples exposed to different levels of smoke during 2018 and 2020, as indicated by total glycoconjugates concentrations; i.e., the sum of cresol rutinoside, phenol rutinoside, guaiacol rutinoside, 4-methylguaiacol rutinoside, syringol gentiobioside and 4-methylsyringol gentiobioside concentrations, reported as guaiacol gentiobioside equivalents. Categories refer to light (6 to 30 ppb), modest (31 to 100 ppb), significant (101 to 200 ppb), elevated (201 to 300 ppb), substantial (301 to 400 ppb) and severe (greater than 400 ppb) smoke exposure (Crews et al. 2022).



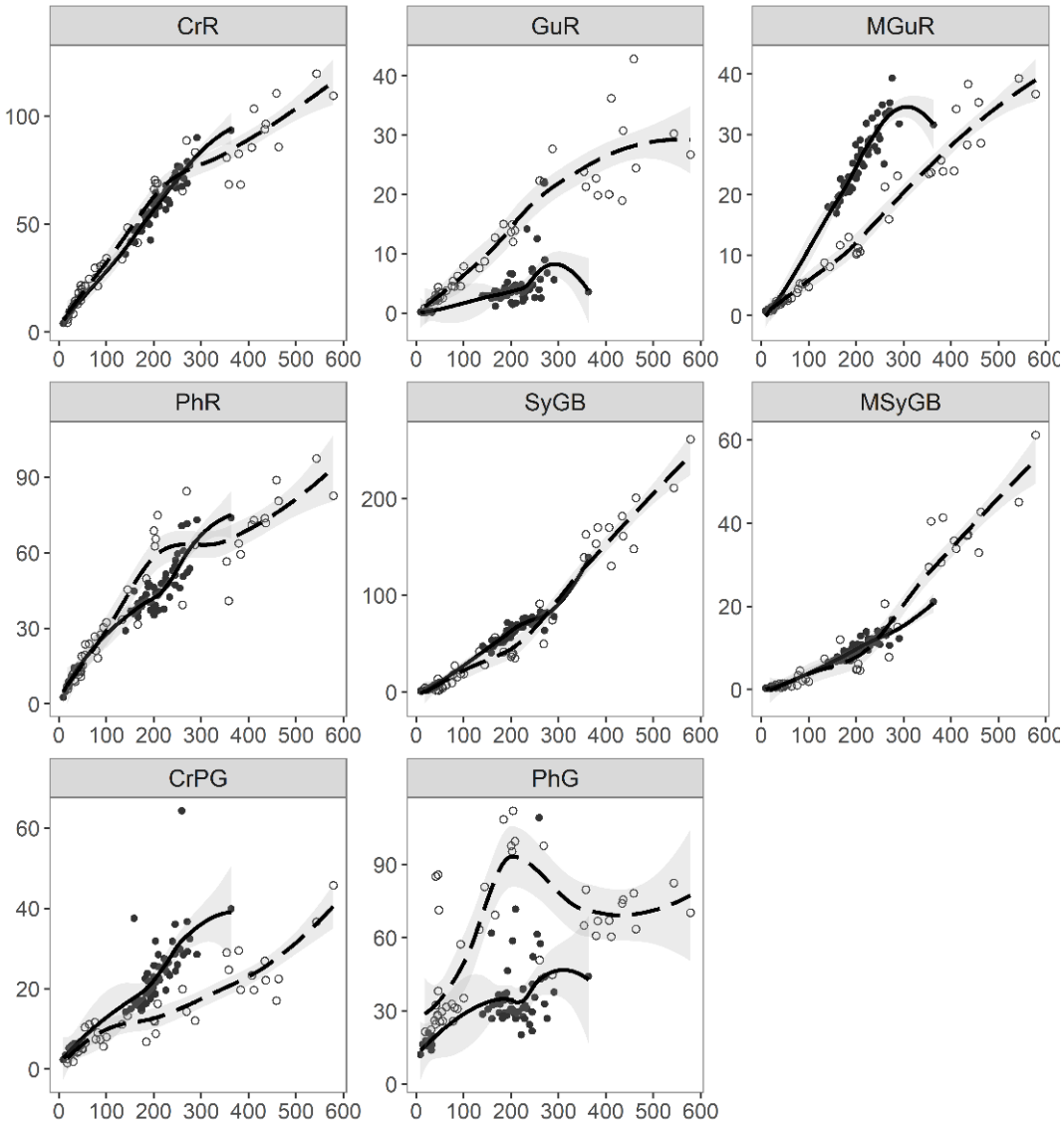
It was unexpected that grapes with modest smoke exposure would be account for a higher proportion of samples collected in 2020 than in 2018. In 2020, wildfires were more prevalent (**Figure 3**) and sampled regions were affected by a higher number of days with smoke exposure, across levels of

smoke density (**Figure 4**). This may reflect sampling that did not capture the topographical (e.g., aspect, elevation) and environmental factors (e.g., wind speed, wind direction, humidity) that affect the dispersion of smoke exposure. However, it may also be explained by differences in the timing of sampling and the nature of smoke exposure (i.e., acute or chronic). In addition to the duration and density of smoke exposure, volatile phenol glycoconjugates are also affected by the timing of smoke exposure (Kennison et al. 2011) and the relative date(s) of sampling (Szeto et al. 2020; Jiang et al. 2021).

Samples from 2018 were primarily collected in Lake County from late August to mid-October, a period following the observed plateau in the cumulative number of days with medium and heavy density plumes of smoke. The type of acute smoke exposure that affected grapes in 2018 has been modelled in conventional field trials, in which grapevines are enclosed in purpose-built tents and exposed to a singular dose of straw-derived smoke for one hour (Kennison et al. 2009). Field trials have shown that while the uptake of smoke-derived volatile phenols is immediate, there is a delay in the subsequent accumulation of volatile phenol glycoconjugates, which can take several weeks (Dungey et al. 2011; van der Hulst 2019; Szeto et al. 2020). The period of sample collection in 2018 granted sufficient time for glycoconjugates to accumulate. By contrast, samples from 2020 were primarily collected in Napa County, over a period defined by persistent smoke exposure. No studies have modelled this type of chronic smoke exposure; thus, it is not known how it may influence the rate of glycosylation or abundance of volatile phenol glycoconjugates in affected grapes.

In the current study, the eight volatile phenol glycoconjugates measured as key smoke taint markers (cresol, guaiacol, 4-methylguaiacol and phenol rutinosides; syringol and 4-methylsyringol gentiobiosides; cresol pentosylglucoside and phenol glucoside) all demonstrated positive correlations with the intensity of smoke exposure, measured as total grape glycoconjugates; albeit maximum levels varied from approximately 40 μg/L of 4-methylguaiacol rutinoside and guaiacol rutinoside, to more than 90 μg/L for cresol rutinoside, phenol rutinoside and syringol gentiobioside (**Figure 6**).

Figure 6. Trendlines showing correlations between total volatile phenol glycoconjugates (µg/kg) (x-axis) and concentrations of individual volatile phenol glycoconjugates identified as being most indicative of smoke exposure (µg/kg) (y-axis). Closed circles ( ) fitted with a solid line (—) describe samples from 2018. Open circles ( ) fitted with a dashed line (- - -) describe samples from 2020. Total glycoconjugates refer to the sum of cresol rutinoside, phenol rutinoside, guaiacol rutinoside, 4-methylguaiacol rutinoside, syringol gentiobioside and 4-methylsyringol gentiobioside. All values reported as guaiacol gentiobioside equivalents. Cr = cresol; Gu = guaiacol; MGu = 4-methylguaiacol; MSy = 4-methylsyringol; Ph = phenol, and Sy = Syringol; GB = gentiobioside; PG = pentosylglucoside; R = rutinoside; G = glucoside.



The observed trendlines suggest that cresol rutinoside, phenol rutinoside and syringol gentiobioside consistently accounted for high proportions of the total glycoconjugates measured, irrespective of vintages; whereas 4-methylsyringol gentiobioside consistently accounted for a lower share, with a maximum concentration of 60 μg/L, even in grapes subjected to severe smoke exposure (i.e., where total volatile phenol glycoconjugates were greater than 400 ppb) (**Figure 6**).

Differences were observed between vintages for cresol pentosylglucoside and 4-methylguaiacol rutinoside concentrations, with greater accumulation of these glycoconjugates evident in 2018. The opposite effect was observed for phenol glucoside and guaiacol rutinoside, with these glycoconjugates contributing a higher proportion of total glycoconjugates in 2020, than 2018. These differences might be attributable to the burn conditions or the types of vegetation that fueled the wildfires that burned during each vintage. The flattest curve corresponded to guaiacol rutinoside in 2018, with concentrations that ranged from 1 to 22 μg/L across grape samples with significant (101 to 200 ppb total glycoconjugates) and elevated (201 to 300 ppb total glycoconjugates) levels of smoke exposure.

The unpredictable nature of wildfires makes it challenging to acquire information needed to inform decision-making in vineyards potentially affected by smoke exposure. The best markers of smoke exposure are volatile phenol glycoconjugates that demonstrate strong correlations to total grape glycoconjugates, independent of burn conditions or fuel type; herein identified as cresol rutinoside, phenol rutinoside, syringol gentiobioside and 4-methylsyringol gentiobioside. However, it does not necessarily follow that glycoconjugates that demonstrate variation between vintages or that occur at lower concentrations ought to be removed from smoke taint diagnostics. Prior to their removal, further research is necessary to understand to what extent different profiles of volatile phenol glycoconjugates contribute to the sensory perception of smoke taint in wine.

In addition to being consistently indicative of smoke exposure across vintages, it is also critical for key markers to be sensitive to low levels of smoke exposure. As shown in **Table 2**, total volatile phenol glycoconjugate levels indicate that Cabernet Sauvignon, Pinot noir and Chardonnay grapes from the Lodi/Delta AVA were affected by low levels of smoke exposure, being light (6 to 30 ppb total glycoconjugates) in Cabernet Sauvignon and Chardonnay, and modest (31 to 100 ppb total glycoconjugates) in Pinot noir. This assessment is supported by a recent study, in which 21, 15, and 9 μg/kg corresponded to the 99th percentile of total glycoconjugate values in non-smoke-exposed Cabernet Sauvignon, Pinot noir and Chardonnay grapes (Coulter et al. 2022).

Table 2. Concentration of key volatile phenol glycoconjugates (µg/kg) in Cabernet Sauvignon (n=40), Chardonnay (n=52), and Pinot noir (n=20) grapes collected at maturity in 2020 from the Lodi/Delta regions of California.

Varietal	CrR	GuR	MGuR	MSyGB	PhR	SyGB	Total
Cabernet Sauvignon	11	1	1	0.7	10	5	29
Chardonnay	4	0.3	1	0.4	3	0.4	9
Pinot noir	13	2	4	0.9	7	8	35

Cresol rutinoside and phenol rutinoside were present at the highest levels, regardless of variety, ranging from 7 to 13 µg/kg in the red varieties and 3 to 4 µg/kg in Chardonnay (**Table 2**). This may indicate that cresol rutinoside and phenol rutinoside are the glycoconjugates most sensitive to small amounts of smoke exposure. The levels of the remaining glycoconjugates were less than 2 µg/kg in Chardonnay and less than 9 µg/kg in Cabernet Sauvignon and Pinot noir. Concentrations of additional glycoconjugates are reported in **Supplemental Table 5.** Decisions regarding harvest and processing of grapes affected by low levels of smoke exposure are challenging without robust baselines. It is critical to continue expanding knowledge of naturally occurring volatile phenol glycoconjugate concentrations across a range of varieties and regions to create a comprehensive, accessible resource for growers and wineries to use to make informed decisions regarding price adjustments and/or rejection criteria that fairly and accurately reflect vineyard smoke exposure.

The lower levels of smoke exposure observed in grapes collected during 2020 were unexpected, given the unprecedented scale of wildfire activity. In fact, the Lodi/Delta AVA and Napa County appeared to experience comparable levels of smoke exposure (**Figure 4**). Nonetheless, as previously described, the samples from the Lodi/Delta AVA were characterized by light (6 to 30 ppb total) to modest (31 to 100 ppb total glycoconjugates) smoke exposure, whereas fruit from Napa County were affected by light (6 to 30 ppb total glycoconjugates) to severe (greater than 400 ppb total glycoconjugates) smoke exposure. It is worth noting that the polygons generated for each smoke plume are two-dimensional, static representations of three-dimensional, dynamic phenomena, and they may not accurately depict the density and/or duration of smoke exposure at ground level. This limitation could be overcome through the use of sensors that monitor particulate matter (PM). Recent studies have employed PM sensors to monitor the temporal changes in the density and location of smoke plumes (Jiang et al. 2021; Wilkinson et al. 2021).

The combined use of satellite imagery, particulate matter sensors, and GIS software could lead to customized sampling protocols for affected vineyards and significantly enhance our ability to contextualize wildfire events and the risk of smoke taint.

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Supplemental Data

Supplemental Table 1. List of exact masses corresponding to volatile phenol glycoconjugates featured in the PRM inclusion list.

Volatile phenol glycoconjugate	Abbreviation	Precursor (*m/z*)
cresol glucoside	CrG	329.12364
cresol gentiobioside	CrGB	491.17646
cresol pentose glucoside	CrPG	461.16590
cresol rutinoside	CrR	475.18155
guaiacol glucoside	GuG	345.11855
d_3-guaiacol glucoside	d_3-GuG	348.11855
guaiacol gentiobioside	GuGB	507.17138
d_3-guaiacol gentiobioside	d_3-GuGB	510.17138
guaiacol pentose glucoside	GuPG	477.16081
guaiacol rutinoside	GuR	491.17646
4-methylguaicol glucoside	MGuG	359.13420
4-methylguaiacol gentiobioside	MGuGB	521.18703
4-methylguaiacol pentose glucoside	MGuPG	491.17646
4-methylguaiacol rutinoside	MGuR	505.19211
4-methylsyringol glucoside	MSyG	389.14477
4-methylsyringol gentiobioside	MSyGB	551.19759
4-methylsyringol pentose glucoside	MSyPG	521.18703
4-methylsyringol rutinoside	MSyR	535.20268
phenol glucoside	PhG	315.10799
phenol gentiobioside	PhGB	477.16136
phenol pentose glucoside	PhPG	447.15025
phenol rutinoside	PhR	461.16590
syringol glucoside	SyG	375.12912
syringol gentiobioside	SyGB	537.18194
syringol pentose glucoside	SyPG	507.17138
syringol rutinoside	SyR	521.18703

Supplemental Table 2. Source, vintage and regional details of Cabernet Sauvignon wines collected to determine the volatile phenol glycoconjugates most indicative of smoke exposure in wine.

Total	Vintage	Region	Smoke-affected
3	2012	California	-
2	2012	Central Coast	-
17	2020	Central Coast	14
1	2012	Lodi	-
5	2011	Napa Valley	-
3	2012	Napa Valley	-
2	2012	North Coast	-
3	2012	Paso Robles	-
1	2015	Snows Lake	1
1	2018	Snows Lake	1
5	2012	Sonoma	-

Supplemental Table 3. Figures of merit for the quantitation of guaiacol glucoside (GuG) and guaiacol gentiobioside (GuGB) in dealcoholized model wine, red wine and white grape matrices via LC-HRMS.

		SPE cartridge	**Lichrolut EN**			**Oasis HLB**		
Matrix	Compound	Expected (ppb)	R^2	RSD (%)	Recovery (%)	R^2	RSD (%)	Recovery (%)
Dealcoholized model wine	GuGB	10	0.9999	5	96	0.9999	7	95
		25		3	88		2	104
	GuG	10	0.9995	4	101	0.9997	7	102
		25		3	99		5	100
Dealcoholized red wine	GuGB	5	0.9999	7	97	0.9995	3	101
		25		4	94		2	97
	GuG	5	0.9996	2	98	0.9996	2	99
		25		1	104		4	91
White grapes	GuGB	30	-	-	-	0.9995	4	118
	GuG	30	-	-	-	0.9994	6	108

Supplemental Table 4. Effects of alcohol by volume (%ABV) on the linearity of calibration (R^2) and RSD (%) of guaiacol gentiobioside (GuGB) and guaiacol glucoside (GuG) in model and red wine matrices at different concentrations, as determined by LC-HRMS.

	% RSD													
Concentration (µg/kg)	5		10		25		50		100		250		**R^2**	
Matrix	GuGB	GuG	GuGB	GuG	GuGB	GuG	GuGB	GuG	GuGB	GuG	GuGB	GuG	GuGB	GuG
0% ABV model wine	107	96	96	82	103	98	103	109	98	100	101	99	0.9998	0.9994
5% ABV model wine	80	91	96	96	105	106	96	104	102	97	100	101	0.9997	0.9995
10% ABV model wine	100	36	100	71	103	101	103	111	94	106	100	98	0.9992	0.9978
15% ABV model wine	96	52	87	62	94	92	103	104	103	113	100	97	0.9997	0.9942
20% ABV model wine	103	55	106	84	100	96	98	105	94	106	100	99	0.9993	0.9988
0% ABV red wine	93	-97	85	-10	103	53	98	78	100	89	100	91	0.9998	0.9994
14% ABV red wine	129	362	100	167	102	139	94	127	101	117	101	109	0.9996	0.9996

Supplemental Table 5. Concentration of additional volatile phenol glycoconjugates (µg/kg) in mature, Cabernet Sauvignon (n=40), Chardonnay (n=52), and Pinot noir (n=20) grapes from the 2020 Lodi/Delta AVA vintage affected by light to modest levels of smoke exposure.

Grape Varietal	GuG	GuGB	GuPG	MGuG	MGuGB	MGuPG	CrG	CrGB	CrPG
Cabernet Sauvignon	31	0.3	12	50	0.1	14	369	0.3	5
Chardonnay	12	0.1	16	19	0.2	23	205	0.1	3
Pinot noir	48	0.7	18	45	0.2	35	443	0.4	5
Grape Varietal	PhG	PhGB	PhPG	SyG	SyPG	SyR	MSyG	MSyPG	MSyR
Cabernet Sauvignon	22	0.2	0.2	498	44	85	31	0.2	0.3
Chardonnay	10	0	0	191	51	68	93	0.1	0.1
Pinot noir	26	0.4	0	462	41	123	227	0.1	2

Values are mean concentrations reported as guaiacol gentiobioside equivalents (for volatile phenol rutinosides, gentiobiosides, and pentose glucosides) and guaiacol glucoside equivalents (for volatile phenol glucosides). Key smoke taint indicators are highlighted in **bold**. Cr = cresol; Gu = guaiacol; MGu = 4-methylguaiacol; MSy = 4-methylsyringol, Ph = phenol, and Sy = syringol; GB = gentiobioside, PG = pentose glucoside; R = rutinoside; G = glucoside.

Chapter 3

Uptake and Glycosylation of Smoke-Derived Volatile Phenols by Cabernet Sauvignon Grapevines and their Subsequent Fate during Winemaking

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Colleen Szeto
Contribution to the Paper	Conceptualization of the work / experimental design Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing
Overall percentage (%)	70
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third [illegible]on in this thesis. I am the primary author of this paper.
Signature	Date: 1/12/2021

Co-Author Contrib

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Renata Ristic
Contribution to the Paper	Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing
Signature	Date: 1/12/2021

Name of Co-Author	Dimitra Capone
Contribution to the Paper	Conceptualization of the work / experimental design Data organization, interpretation, and analysis Manuscript preparation and editing
Signature	Date: 02/12/2021

Name of Co-Author	Carolyn Puglisi		
Contribution to the Paper	Conceptualization of the work / experimental design Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	1/12/21

Name of Co-Author	Vinay Pagay		
Contribution to the Paper	Conceptualization of the work / experimental design Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	14/12/2021

Name of Co-Author	Julie Culbert		
Contribution to the Paper	Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	6/12/2021

Name of Co-Author	WenWen Jiang		
Contribution to the Paper	Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	6/12/2021

Name of Co-Author	Markus Herderich		
Contribution to the Paper	Conceptualization of the work / experimental design Manuscript preparation and editing		
Signature		Date	1/12/2021

Name of Co-Author	Jonathon Tuke		
Contribution to the Paper	Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	14/12/21

Name of Co-Author	Kerry Wilkinson		
Contribution to the Paper	Conceptualization of the work / experimental design Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	1/12/21

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Article

Uptake and Glycosylation of Smoke-Derived Volatile Phenols by Cabernet Sauvignon Grapes and Their Subsequent Fate during Winemaking

Colleen Szeto [1,2], Renata Ristic [1,2], Dimitra Capone [1,2], Carolyn Puglisi [1], Vinay Pagay [1,2], Julie Culbert [3], WenWen Jiang [3], Markus Herderich [1,2,3], Jonathan Tuke [4] and Kerry Wilkinson [1,2,*]

1 School of Agriculture, Food and Wine, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia; colleen.szeto@adelaide.edu.au (C.S.); renata.ristic@adelaide.edu.au (R.R.); dimitra.capone@adelaide.edu.au (D.C.); carolyn.puglisi@adelaide.edu.au (C.P.); vinay.pagay@adelaide.edu.au (V.P.); markus.herderich@awri.com.au (M.H.)
2 The Australian Research Council Training Centre for Innovative Wine Production, PMB 1, Glen Osmond, SA 5064, Australia
3 The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA 5064, Australia; julie.culbert@awri.com.au (J.C.); maddy.jiang@awri.com.au (W.J.)
4 School of Mathematical Sciences, The University of Adelaide, SA 5000, Australia; simon.tuke@adelaide.edu.au
* Correspondence: kerry.wilkinson@adelaide.edu.au; Tel.: +61-8-8313-7360

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Abstract: Wine made from grapes exposed to bushfire smoke can exhibit unpleasant smoky, ashy characters, which have been attributed to the presence of smoke-derived volatile phenols, in free or glycosylated forms. Here we report the uptake and glycosylation of volatile phenols by grapes following exposure of Cabernet Sauvignon vines to smoke, and their fate during winemaking. A significant delay was observed in the conversion of volatile phenols to their corresponding glycoconjugates, which suggests sequestration, the presence of intermediates within the glycosylation pathway and/or other volatile phenol storage forms. This finding has implications for industry in terms of detecting smoke-affected grapes following vineyard smoke exposure. The potential for an in-canopy sprinkler system to mitigate the uptake of smoke-derived volatile phenols by grapes, by spraying grapevines with water during smoke exposure, was also evaluated. While "misting" appeared to partially mitigate the uptake of volatile phenols by grapes during grapevine exposure to smoke, it did not readily influence the concentration of volatile phenols or the sensory perception of smoke taint in wine. Commercial sensors were used to monitor the concentration of smoke particulate matter (PM) during grapevine exposure to low and high density smoke. Similar PM profiles were observed, irrespective of smoke density, such that PM concentrations did not reflect the extent of smoke exposure by grapes or risk of taint in wine. The sensors could nevertheless be used to monitor the presence of smoke in vineyards during bushfires, and hence, the need for compositional analysis of grapes to quantify smoke taint marker compounds.

Keywords: acid hydrolysis; cresols; guaiacol; particulate matter; rate-all-that-apply; sensors; smoke taint; syringol; volatile phenol glycosides; wine

1. Introduction

"Smoke taint" describes unpleasant smoky, medicinal and ashy characters that can arise in wine following grapevine exposure to bushfire smoke [1,2]. The intensity of smoke taint depends on the timing

and duration of smoke exposure [3,4], grape variety [5], fruit maturity at harvest [6] and winemaking practices (e.g., skin contact time during fermentation) [7,8]. The presence of smoke taint can be determined in wine by sensory analysis and/or by measuring the concentrations of smoke-derived volatile phenols, including guaiacol, 4-methylguaiacol, *o*-, *m*- and *p*-cresol, syringol and 4-methylsyringol [5,9–11]; but in grapes, volatile phenols accumulate in glycoconjugate forms (i.e., as mono-, di- and even trisaccharides [12–17]), complicating their detection. Analytical methods have been developed to measure volatile phenol glycoconjugates, either directly by liquid chromatography-tandem mass spectrometry [11,18], or indirectly, by quantifying the volatile phenols released following acid or enzyme hydrolysis [19–21]. However, to date, few studies have monitored temporal changes in grape volatile phenol glycoconjugates following grapevine exposure to smoke or the fate of volatile phenol glycoconjugates during winemaking.

Dungey and coworkers reported the accumulation of guaiacol glycoconjugates in smoke-affected Merlot and Viognier grapes, and while glycoconjugates were detected 3–5 days post-smoke exposure, significant increases were observed in grapes sampled 12 or more days after smoke exposure, leading the authors to conclude that glycosylation occurred over 10 to 14 days [18]. In a more recent study, van der Hulst and colleagues found low levels of volatile phenols (≤4 µg/L) in grapes sampled one day after grapevine exposure to smoke, but the concentrations of key volatile phenol glycoconjugates increased significantly between 1 and 7 days post-smoke exposure [16]. Unfortunately, neither of these studies measured grape volatile phenols immediately after smoke exposure, nor did they involve winemaking, so questions remain regarding the uptake of smoke-derived volatile phenols by grapes and how glycoconjugate profiles change as grapes are processed into wine. This study sought to address these knowledge gaps by measuring grape volatile phenols, in free and glycosylated forms, following grapevine exposure to low and high density smoke, and then in corresponding wines. The study also included preliminary evaluations of: (i) in-canopy misting as a strategy for mitigating the uptake of volatile phenols during grapevine smoke exposure; and (ii) a commercial sensor for monitoring vineyard exposure to smoke during a bushfire.

A recent study used in-canopy sprinklers to mitigate the effects of heat stress in Cabernet Sauvignon berries during ripening, by spraying water within the bunch zone (for 20 s/10 min when air temperature exceeded 38 °C) to cool the vine microclimate by 3–5 °C [22]. Attempts to "wash" grapevines/fruit following exposure to smoke (using water, 5% aqueous ethanol or milk) did not reduce the guaiacol concentration of grapes or juice [23,24], which might reflect rapid diffusion of smoke-derived volatile phenols into berries. The in-canopy sprinkler system could instead be used to "wash" grape bunches during smoke exposure, i.e., to potentially mitigate the uptake of smoke-derived volatile compounds through the removal of smoke particles in a manner similar to the way in which rain cleanses the atmosphere by capturing aerosols [25]. This study therefore included an investigation into the impact of in-canopy misting during grapevine exposure to smoke on the concentration of smoke taint marker compounds in grapes and wine, and the perception of smoke taint in wine. Additionally, commercial sensors were deployed during field trials to measure the concentration of particulate matter, in order to determine their suitability for monitoring smoke from bushfires, with different densities of smoke achieved by burning different amounts of fuel.

2. Results and Discussion

The composition of grapes from control and smoke-exposed grapevines were determined just prior to smoke exposure (i.e., at t = 0, being approximately 7 days post-veraison); then, at 1 h, 1 day and 1 week post-smoke exposure (hereafter t = 1, t = 2 and t = 3, respectively), and again at harvest/maturity (hereafter t = 4, being 4 weeks post-smoke exposure). The composition and sensory profiles of control and smoke-affected wines were also determined. This enabled investigation of: (i) the uptake and in vivo glycosylation of smoke-derived volatile phenols by grapes; and (ii) the subsequent fate of volatile phenols (and their glycoconjugates) during winemaking.

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2.1. Uptake and Glycosylation of Smoke-Derived Volatile Phenols by Grapes

Prior to smoke exposure (i.e., at t = 0), grape juice volatile phenol concentrations were ≤3.6 μg/L, with the exception of syringol which ranged from 6.2 to 12 μg/L (Table 1). Elevated volatile phenol levels were detected in juice from grapes sampled 1 h after exposure to smoke (i.e., at t = 1), irrespective of smoke density, albeit only phenol and cresol concentrations of grapes exposed to low density smoke ("LS") were significantly different (P = 0.034 and 0.033, respectively) from their corresponding control grapes (i.e., "C" at t = 1). Guaiacol and syringol were detected at the highest concentrations, being 108 and 126 μg/L in juice from grapes exposed to high density smoke ("HS") respectively, followed by cresols, phenol, 4-methylguaiacol and 4-methylsyringol, which were detected at 83, 55, 20 and 17 μg/L respectively (Table 1). Within 24 h of smoke exposure, the elevated volatile phenol levels observed in LS and HS grapes had decreased by as much as 75%, such that syringol, 4-methylguaiacol, phenol and 4-methylsyringol levels were not significantly different from those detected in control grapes (Table 1). Guaiacol and cresol concentrations similarly decreased, but remained significantly higher in HS grapes (than in control grapes) until one and four weeks after smoke exposure (i.e., until t = 3 and t = 4), respectively (Table 1).

These results demonstrate the rapid uptake of volatile phenols from smoke by grapes during grapevine exposure to smoke, and as reported in previous studies [12–18], their subsequent in vivo glycosylation. However, while some volatile phenol glycoconjugates (measured as syringol glucose-glucoside equivalents) were observed at significantly elevated concentrations in HS grapes 24 h after smoke exposure (i.e., at t = 2), namely, syringol glucose glucoside (gentiobioside), cresol glucoside and cresol rutinoside (Table S1), accumulation of other volatile phenol glycoconjugates seemingly occurred one to four weeks after smoke exposure (i.e., between t = 3 and t = 4) (Table S1). By harvest (i.e., at t = 4), the concentrations of guaiacol pentose glucoside, phenol pentose glucoside, cresol pentose glucoside and syringol glucose glucoside in HS grapes were 803, 576, 988 and 535 μg/kg respectively (Table S1); meanwhile, pentose glucosides of 4-methylguaiacol and syringol, 4-methylsyringol glucose glucoside, and rutinosides of 4-methylguaiacol, phenol and cresols ranged from 98 to 258 μg/kg (Table S2). Other volatile phenol glycoconjugates, including glucosides, were detected at ≤50 μg/kg.

The glycoconjugate profiles observed for LS and HS grapes were similar to those reported in previous studies involving the application of smoke to grapevines of different varieties [11,13,16], in that pentose glucosides of guaiacol, 4-methylguaiacol, cresol, phenol and syringol, and glucose glucosides (gentiobiosides) of syringol and 4-methylsyringol were most abundant in smoke-exposed grapes (at harvest). In the latter study, van der Hulst and colleagues reported a similar (albeit shorter) delay in the accumulation of volatile phenol glycoconjugates. Volatile phenols were detected at ≤4 μg/L in grapes sampled 1 d after grapevine exposure to smoke, but the concentrations of several volatile phenol glycoconjugates increased significantly between one and seven days post-smoke exposure, especially in Merlot vines; glycoconjugate levels then remained relatively constant until harvest [16].

The apparent delay between the "disappearance" of volatile phenols and "appearance" of their glycoconjugates might be explained by sequestration of volatile phenols in plant cell walls or vacuoles, the presence of "intermediates" within the glycosylation pathway and/or other volatile phenol metabolites, as suggested by Noestheden and colleagues [24]. Nevertheless, this delay has important implications for the wine industry, since it suggests that analysis of volatile phenols and/or their glycoconjugates in grapes sampled between one and seven days after smoke exposure (and possibly longer in some grape varieties), might underestimate the levels that are subsequently detected in mature grapes and/or wine, i.e., there is potential for the level of smoke taint to be underestimated.

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Table 1. Concentrations of volatile phenols in juice (μg/L) and volatile phenol glycosides in homogenate (μg/kg) from control and smoke-exposed grapes sampled from pre-smoke exposure (t = 0) to maturity (t = 4), and in corresponding wines (μg/L); different densities of smoke were achieved by burning different amounts of fuel.

Treatment/ Timepoint		Guaiacol	4-Methyl Guaiacol	Phenol	Cresols	Syringol	4-Methyl Syringol	Guaiacol Glycosides	4-Methyl Guaiacol Glycosides	Phenol Glycosides	Cresol Glycosides	Syringol Glycosides	4-Methyl Syringol Glycosides
C	t = 0	1.9 b	3.6	1.5	2.6	12 b	2.5	3.9 b	1.5 b	3.1 b	12 b	4.1 b	nd
	t = 1	9.5 a	4.1	2.6	5.1	21 a	3.0	5.5 b	2.1 b	3.8 b	16 b	5.9 b	1.1 b
	t = 2	2.4 b	3.6	1.6	2.7	8.4 b	2.0	8.4 b	3.0 b	4.7 b	26 b	14 b	2.2 b
	t = 3	1.9 b	3.6	1.6	2.4	7.9 b	1.8	13 b	4.6 b	8.0 b	31 b	30 ab	3.6 b
	t = 4	2.2 b	3.6	1.6	2.4	13 b	1.8	44 a	22 a	45 a	83 a	44 a	13 a
	P	0.033	ns	ns	ns	0.017	ns	0.002	<0.001	<0.001	<0.001	0.037	0.011
LS	t = 0	1.7 b	3.5 b	1.4 c	2.5 c	6.2 c	2.0 b	3.5 b	1.1 b	3.6 b	9.0 c	3.1 d	nd
	t = 1	12 a	4.1 a	6.9 a	12 a	25 a	2.9 a	6.4 b	2.1 b	5.3 b	20 bc	12 cd	1.5 b
	t = 2	2.8 b	3.6 b	4.7 b	4.9 b	6.0 c	1.9 b	14 b	4.8 b	16 b	46 b	27 bc	3.6 b
	t = 3	2.6 b	3.6 b	5.1 ab	4.8 b	13 b	1.8 b	16 b	6.3 b	26 b	47 b	42 b	4.4 b
	t = 4	3.1 b	3.6 b	6.3 ab	5.0 b	11 bc	1.8 b	73 a	38 a	121 a	154 a	77 a	18 a
	P	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
HS	t = 0	1.8 c	3.5 b	1.8 b	2.7 b	7.8 b	1.9 b	3.2 b	1.4 b	3.3 b	10 b	3.5 c	nd
	t = 1	108 a	20 a	55 a	83 a	126 a	17 a	45 b	14 b	22 b	98 b	71 c	11 b
	t = 2	25 b	5.1 b	12 b	23 b	24 b	2.7 b	158 b	51 b	69 b	263 b	310 bc	48 b
	t = 3	12 c	4.6 b	17 b	18 b	12 b	1.9 b	229 b	70 b	144 b	316 b	526 ab	69 b
	t = 4	10 c	4.2 b	21 b	13 b	12 b	1.8 b	894 a	297 a	745 a	1118 a	843 a	248 a
	P	<0.001	<0.001	0.012	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.001	<0.001
P [1]		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD [1]		15.6	3.7	14.2	15.0	24.6	3.3	182.0	52.6	112.6	61.1	172.8	36.0
C	wine	1.7 b	nd	–	nd	1.7 b	nd	19 b	4.2 b	6.2 b	7.5 b	30 b	1.4 b
LS	wine	4.3 b	nd	–	5.9 b	2.7 b	nd	30 b	7.7 b	17 b	15 b	53 b	2.3 b
HS	wine	29 a	4.0	–	28 a	4.7 a	nd	283 a	68 a	112 a	115 a	501 a	30 a
P		<0.001	–	–	<0.001	0.011	–	0.002	0.001	<0.001	<0.001	0.002	<0.001

C = control (no smoke exposure); LS = low density smoke exposure; HS = high density smoke exposure. Values are means of three replicates (n = 3); nd = not detected. Different letters (within columns) indicate statistical significance (P = 0.05, one way ANOVA) amongst: (i) time points (i.e., immediately prior to smoke exposure (t = 0); 1 h after smoke exposure (t = 1); 1 day after smoke exposure (t = 2); 7 days after smoke exposure (t = 3); and 4 weeks after smoke exposure (t = 4) being maturity) for grape data; and (ii) wines; ns = not significant. [1] P and LSD values for two-way ANOVA of grape data, by treatment and time. Phenol was not measured in wines.

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Several previous studies employed enzyme, acid and/or base hydrolysis of grape homogenate, juice or wine to facilitate quantification of glycoconjugate forms of volatile phenols [1,15,19–21,24,26]. Noestheden and colleagues optimized a method for measuring glycosidically-bound volatile phenols in smoke-exposed grapes using acid-mediated hydrolysis [15], key recommendations being the use of Strata X solid phase extraction (SPE) cartridges for isolation of glycoconjugates and PTFE tubes for acid digestion (instead of borosilicate glass vials, which seem to interfere with the assay yielding low recoveries for some volatile phenols). In the current study, a similar method was applied to HS grapes (at each time point) to further investigate the accumulation of volatile phenols in bound forms (Figure 1). The acid hydrolysate from HS grapes sampled at t = 0 contained low levels of volatile phenols (i.e., 0–25 µg/L; Figure 1). Acid hydrolysis of smoke-affected grape samples liberated ≤13 µg/L of 4-methylguaiacol and 4-methylsyringol, irrespective of sampling time, but released 50–90, 70–100 and 120–260 µg/L of guaiacol, cresols and syringol, respectively (Figure 1). These results were consistent with: 4-methylguaiacol and 4-methylsyringol being the least abundant volatile phenols in HS grapes, especially in free form at t = 1 (Table 1) and in glycosylated forms at t = 4 (Table 1 and Table S1); and guaiacol, cresols and syringol being the most abundant volatile phenols, in both free and glycosylated forms (Table 1 and Table S1).

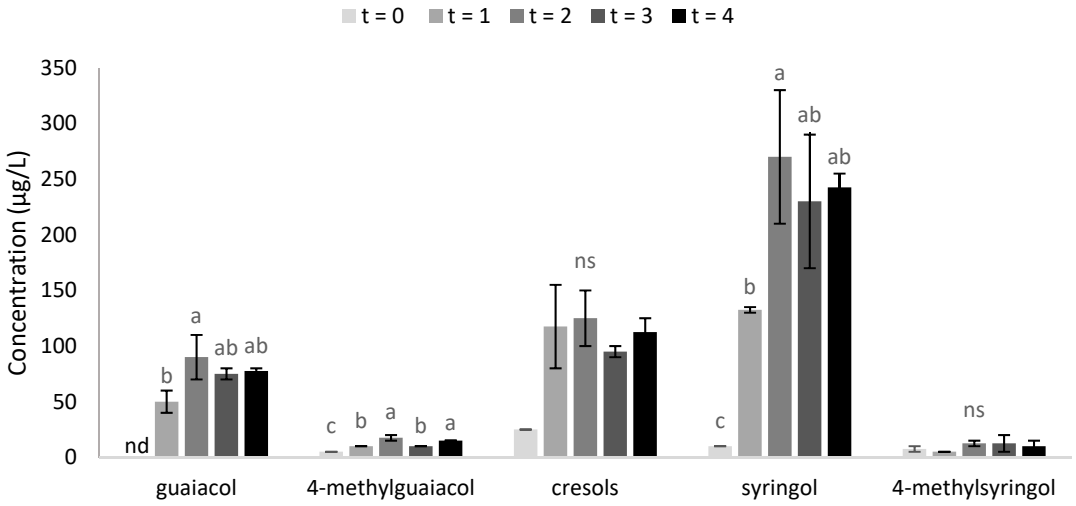

Figure 1. Volatile phenol concentrations in acid hydrolysates derived from HS grapes sampled at different time points, i.e., immediately prior to smoke exposure (t = 0); 1 h after smoke exposure (t = 1); 1 day after smoke exposure (t = 2); 7 days after smoke exposure (t = 3); and 4 weeks after smoke exposure (t = 4) being maturity. Values are means of two replicates (n = 2) ± standard errors. Different letters indicate statistical significance (P = 0.05, one-way ANOVA); ns = not significant; nd = not detected.

The elevated concentrations of guaiacol, cresols and syringol in the t = 1 hydrolysate (compared with the t = 0 hydrolysate) suggest these compounds were being metabolized within the berry during and/or immediately after smoke exposure. The significant increase in guaiacol and syringol concentrations observed for hydrolysates between t = 1 and t = 2 might reflect conversion of free forms of these volatile phenols into bound forms. However, the glycoconjugate levels at t = 2 do not account for the quantities of guaiacol and syringol observed in t = 2 hydrolysate, suggesting the presence of intermediates (or other metabolites) of these volatile phenols. The guaiacol and syringol levels in t = 3 and t = 4 hydrolysates were not significantly different from those in either t = 1 or t = 2 hydrolysates; surprisingly, there was also no statistical significance amongst cresol concentrations in acid hydrolysates, at any time point (Figure 1). These results are particularly interesting given that by t = 4, substantial quantities of guaiacol, cresol and syringol glycoconjugates had accumulated in HS grapes (Table 1 and Table S1) and high performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) analysis of hydrolysates confirmed there were no volatile phenol glycoconjugates remaining after acid hydrolysis (data not shown). Based on the concentrations of guaiacol and cresol

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pentose glucosides and syringol glucose glucoside found in mature HS grapes (i.e., the most abundant glycoconjugates for each of these volatile phenols), complete hydrolysis would be expected to yield guaiacol, cresol and syringol concentrations of at least 238, 265 and 172 μg/L, respectively; i.e., mass balance could not be achieved. Whereas Noestheden and colleagues reported quantitative recovery of free volatile phenols following acid hydrolysis of their corresponding glucosides [21], similar results were not obtained in the current study, for the glycoconjugates measured.

The acid hydrolysis experiments performed as part of the current study are considered preliminary only. More detailed studies could not be pursued because very limited quantities of samples remained after completion of other analyses, but will instead be undertaken as part of ongoing smoke taint research. Nevertheless, these findings further support the existence of other intermediates and/or storage forms of volatile phenols. They also demonstrate the potential for acid hydrolysis to be used to further investigate the uptake and accumulation of smoke-derived volatile phenols by grapes, and to detect smoke taint in grapes, especially when direct analysis of volatile phenols and/or their glycoconjugates might not account for the presence of all forms of volatile phenols (e.g., in the days immediately after smoke exposure).

2.2. Comparison of Smoke Taint Markers in Grapes vs. Wine

The chemical analyses performed in the current study enabled comparisons to be made between the compositions of control and smoke-exposed grapes, and their corresponding wines, to determine the fate of volatile phenols (both free and glycosylated forms) during winemaking.

Prior to smoke exposure, the background ("natural") levels of volatile phenols present in LS and HS grapes were ≤7.8 μg/L (Table 1), with syringol being the most abundant volatile phenol. After smoke exposure (i.e., at t = 1), guaiacol and syringol were the most abundant volatile phenols (in both LS and HS grapes), followed by cresols, phenol, 4-methylguaiacol and 4-methylsyringol. However, by maturity, there were no significant differences in the syringol or 4-methylsyringol levels of C, LS and HS grapes (Table 1). Elevated levels of guaiacol, phenol and cresols in HS grapes (and of phenol and cresols in LS grapes, but to a lesser extent) provided the only evidence of smoke exposure at maturity (i.e., at t = 4). As outlined above, this was because volatile phenols were predominantly present in conjugate forms. In LS and HS wines, guaiacol was the most abundant volatile phenol, albeit combined, cresols were present at similar levels (Table 1); syringol, 4-methylguaiacol and 4-methylsyringol concentrations were ≤5 μg/L (and phenol was not measured in wines). These results show good agreement with compositional analyses of Cabernet Sauvignon grapes and/or wines reported in several previous studies on smoke taint [5,20,26].

Several studies have shown that a significant pool of volatile phenol glycoconjugates remains in wine after fermentation [5–7,17,19,20,27,28]. However, to date, the changes in glycoconjugate concentrations during fermentation have not been extensively studied. In this study, the relative abundance of volatile phenol glycoconjugates observed in control and smoke-exposed grapes at maturity (Table 1 and Table S1) were not preserved during fermentation (Table 1 and Table S2). The most abundant glycoconjugates, cresol, guaiacol and phenol pentose glucosides and syringol glucose glucosides, were detected in control grapes at 73, 38, 35 and 23 μg/kg, and in HS grapes at 988, 803, 576 and 535 μg/kg, respectively (Table S1). In contrast, guaiacol pentose glucoside and syringol glucose glucoside were present in control wines at 15 and 24 ug/L, while all other glycoconjugates were ≤6.3 μg/L (Table S2). Elevated levels of guaiacol pentose glucoside and syringol glucose glucoside (234 and 413 μg/L) remained in HS wines after fermentation, but the most abundant glycoconjugates of phenol and cresol were then rutinosides, at 59 and 102 μg/L, respectively (Table S2). The metabolic fates of cresol and phenol pentose glucosides are unclear, given the low cresol and cresol glucoside concentrations in HS wine do not support significant hydrolysis (unless cresols are further metabolized), and phenol was not measured in any of the wines.

To date, Caffrey and colleagues have published the only other study that measures changes in volatile phenol glycoconjugates during winemaking [17]. Of the 31 glycoconjugates measured (a mix

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of mono-, di- and trisaccharides), 20 decreased in concentration, five increased in concentration and six were not significantly different in concentration, from juice after pressing to wine after bottling; the largest changes in glycoconjugate concentrations were observed during the first half of primary fermentation, when glycosidase activities of *Saccharomyces* yeast were highest. However, it is difficult to make direct comparisons with the current study, given few glycoconjugates of guaiacol or syringol were detected, and the scope of the project was limited to analysis of glycoconjugates (i.e., no volatile phenol or sensory data were reported for finished wine) [17]. As smoke taint research has progressed over the past ~15 years, the suite of volatile phenols used as smoke taint markers has evolved, with new methods for direct and indirect measurement of free and bound volatile phenols being developed. Differences in both the volatile phenols measured and the methods used to measure them (in free and/or bound forms) complicate comparisons amongst the scientific literature on this topic. This is likely to continue in the short–medium term, as analytical methods for smoke taint are refined, particularly if new marker compounds and/or alternate storage forms of marker compounds are identified.

2.3. Influences of in-Canopy Misting and Smoke Density on the Degree of Smoke Taint in Grapes and Wine

As expected, few compositional differences were observed between control grapes with and without in-canopy misting (i.e., "CM" and "C" grapes), irrespective of sampling time (Table 2, Tables S1 and S3). While there were no significant differences in the volatile phenol concentrations of smoke-exposed grapes with and without misting (i.e., "HSM" and "HS" grapes) at maturity (i.e., at t = 4, Table 2), HSM grapes sampled 1 h after smoke exposure (i.e., at t = 1) comprised significantly lower volatile phenol levels than HS grapes (Table S3). Furthermore, at t = 4, the concentrations of several volatile phenol glycoconjugates were significantly lower in HSM grapes (than HS grapes), including pentose glucosides of guaiacol, 4-methylguaiacol, phenol and syringol (Table S1). These findings suggest in-canopy misting partially mitigated the uptake of volatile phenols by grapes during grapevine smoke exposure.

Table 2. Concentrations of volatile phenols in juice (μg/L) from control and smoke-exposed grapes at maturity (t = 4), and in corresponding wines (μg/L), with and without in-canopy misting; different densities of smoke were achieved by burning different amounts of fuel.

	Volatile Phenols	C	CM	LS	HS	HSM	P
juice	guaiacol	2.2 ± 0.1 b	2.4 ± 0.1 b	3.1 ± 0.1 b	10 ± 1.2 a	7.6 ± 1.9 a	<0.001
	4-methylguaiacol	3.6 ± 0.1 b	3.5 ± 0.1 b	3.6 ± 0.1 b	4.2 ± 0.1 a	4.0 ± 0.2 a	0.003
	phenol	1.6 ± 0.3 b	1.9 ± 0.2 b	6.3 ± 0.9 b	21 ± 4.1 a	17 ± 2.9 a	<0.001
	cresols	2.4 ± 0.1 b	2.7 ± 0.1 b	5.0 ± 0.7 b	13 ± 2.1 a	12 ± 1.5 a	<0.001
	syringol	13 ± 0.6	12 ± 1.1	11 ± 0.7	12 ± 0.9	13 ± 0.7	ns
	4-methylsyringol	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	ns
wine	guaiacol	1.7 ± 1.0 b	1.0 ± 0.7 b	4.3 ± 0.1 b	29 ± 0.3 a	23 ± 4.9 a	<0.001
	4-methylguaiacol	nd	nd	nd	4.0 ± 0.1 a	3.0 ± 0.6 b	<0.001
	o-cresol	nd	nd	2.7 ± 0.1 b	11 ± 0.3 a	11 ± 1.7 a	<0.001
	m-cresol	nd	nd	1.9 ± 0.1 b	10 ± 0.1 a	10 ± 1.9 a	<0.001
	p-cresol	nd	nd	1.3 ± 0.1 b	6.7 ± 0.3 a	5.0 ± 1.2 a	<0.001
	syringol	1.7 ± 1.0 b	2.0 ± 0.1 b	2.7 ± 0.1 b	4.7 ± 0.3 a	4.7 ± 0.7 a	<0.001
	4-methylsyringol	nd	nd	nd	nd	nd	–

C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure; HS = high density smoke exposure; HSM = high density smoke exposure with misting. Values are means of three replicates (n = 3) ± standard errors. Different letters (within rows) indicate statistical significance (P = 0.05, one-way ANOVA); ns = not significant.

Excluding 4-methylsyringol, which was not detected in any of the wines, the concentrations of volatile phenols were significantly higher in HS and HSM wines than in C and CM wines (Table 2). However, the only significant difference observed amongst the volatile phenol levels of HS and HSM wines was for 4-methylguaiacol, being 4 vs. 3 μg/L, respectively. With the exception of guaiacol glucose glucoside, the concentrations of volatile phenol glycoconjugates were again found to be significantly

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lower in HSM wines compared to HS wines (Table S2). This provides further evidence that in-canopy misting partially mitigated the uptake of volatile phenols during grapevine smoke exposure, but as discussed below, did not affect the sensory perception of smoke taint in HSM wines.

The difference in smoke density achieved during LS and HS treatments significantly influenced the compositions of grapes and wines. LS grape juice contained far lower levels of volatile phenols than HS grape juice at t = 1, and in some cases at t = 2 and t = 3 also (Table 1), as well as lower volatile phenol glycoconjugate levels, especially at t = 4 (Table 1 and Table S1). However, as indicated above, only the phenol and cresol concentrations of LS grapes were significantly different from control grapes at t = 1 (Table 1). One-way ANOVA confirmed significant (albeit relatively small) differences in the glycoconjugate content of LS and C grapes (Table S1), specifically: guaiacol rutinoside (P = 0.015), 4-methylguaiacol rutinoside (P = 0.019), phenol pentose glucoside (P = 0.025), phenol rutinoside (P = 0.009) and cresol rutinoside (P = 0.004). HS wines contained significantly higher levels of volatile phenols and volatile phenol glycoconjugates than LS wines (Table 1 and Table S2), whereas the presence of low levels of cresols (1–3 µg/L) in LS wines differentiated them from control wines (Table 2). One-way ANOVA also confirmed the concentration of 13 of the 17 glycoconjugates reported in Table S2 were significantly higher in LS wines than in control wines, including: guaiacol pentose glucoside (P = 0.047), phenol rutinoside (P = 0.007), cresol rutinoside (P = 0.005), syringol glucose glucoside (P = 0.032) and syringol pentose glucoside (P = 0.017). Collectively, these results provide compositional evidence of low level smoke taint in LS wine, in agreement with sensory analysis results (Figure 2).

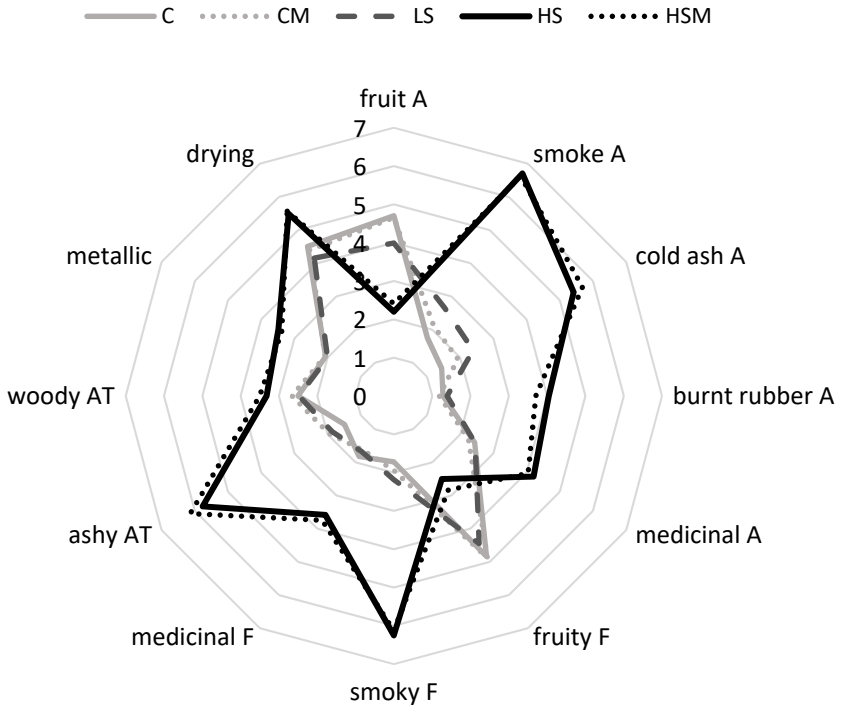

Figure 2. Sensory profiles of control and smoke-affected wines; A = aroma; F = flavor; AT = aftertaste. C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure; HS = high density smoke exposure; HSM = high density smoke exposure with misting. Values are mean intensity ratings of one wine per treatment, presented to 50 judges; ratings for each attribute were statistically significant (P = 0.05, one-way ANOVA).

The sensory profiles of wines made from control (C and CM) and LS grapes were quite similar and comprised the most intense fruit aromas and flavors, and least intense smoke-related attributes (Figure 2). Few significant differences were perceived amongst these wines. The intensity of smoke aroma in the LS wine was slightly higher than in the control wine (Figure 2, Table S4), and provided the only sensory evidence of smoke taint, but was not significantly different from the CM wine and

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was considerably lower than for HS and HSM wines. The CM wine was also found to exhibit increased hotness (Table S4), due to the higher alcohol content of this wine (Table S5). In contrast to C, CM and LS wines, the sensory profiles of HS and HSM wines were dominated by smoke-related aromas and flavors, an ashy aftertaste and drying finish, which significantly diminished fruit intensity (Figure 2, Table S4); i.e., these wines were noticeably tainted by smoke, in agreement with wine volatile phenol data (Table 2). The only significant sensory difference observed between HS and HSM wines was the perception of hotness (Table S4), which was rated lower in HS wine compared to HSM wine, reflecting the lower alcohol content of HS wine (Table S5). As such, despite appearing to partially mitigate the uptake of smoke-derived volatile phenols by grapes, misting did not significantly influence the sensory perception of smoke taint in wine. Further research is needed to determine whether or not optimization of other factors, such as water droplet size and/or flow rate, might improve the efficacy of misting.

2.4. Concentration of Particulate Matter During Grapevine Exposure to Smoke

Particulate matter (PM) concentrations were measured during field trials to monitor smoke emission and exposure (Figure 3), using two commercial sensors: one positioned amongst control vines and one within the smoke tent. In the two days prior to field trials, $PM_{1.0}$, $PM_{2.5}$ and PM_{10} levels were ≤6.2, 13.6 and 89.7 μg/m^3 respectively (data not shown). During field trials, PM levels detected by sensors positioned amongst control vines were typically <100 μg/m^3 (e.g., Figure 3a,b), reflecting occasional, but minimal smoke drift. In contrast, elevated PM levels were detected by sensors positioned inside the smoke tent, for the duration of smoke treatments (Figure 3c–f). PM levels increased as soon as fuel was combusted to produce smoke, with PM_{10} > $PM_{2.5}$ > $PM_{1.0}$, in agreement with particle size distributions previously reported for smoke from domestic wood fires [29]. As expected, PM levels then decreased when smoke production stopped. The occasional PM signals (especially PM_{10}) that were observed either before or after smoke treatments (e.g., as seen in Figure 3b,e,f) can be attributed to the movement of either smoke tents or sensors. During HS treatments (with or without misting), $PM_{2.5}$ and PM_{10} levels approximated 1000 and 2000–2500 μg/m^3, respectively (Figure 3c–e), but considerable signal variation was observed, which likely reflects a combination of the recurring combustion of fuel, detector saturation and the algorithm behind data acquisition. Detector saturation has been reported in previous smoke taint research [24], and in the current study, one of the sensors stopped acquiring PM data when its detector became fouled during the second HSM treatment (Figure 3e). Interestingly, the $PM_{2.5}$ and PM_{10} levels detected during the LS treatment (Figure 3f) were similar to those from the HS and HSM treatments (Figure 3c–e); the increased fluctuation observed in the PM_{10} signal again reflects the recurring combustion of fuel (and smaller amounts of fuel compared with that combusted during HS and HSM treatments). These results suggest the levels of PM generated during LS treatments were still at (for $PM_{2.5}$), or near (for PM_{10}), the detector saturation levels, such that the sensors did not differentiate low and high smoke treatments as readily as was expected given the obvious visual differences in smoke density. The density of smoke applied to grapevines during HS and HSM treatments was likely far higher than would occur in vineyards during most bushfires, and where similar levels of smoke exposure do occur, the resulting taint should be easy to detect, either by chemical or sensory analysis. In contrast, the density of smoke applied to LS grapevines was readily detected by the environmental sensors, despite yielding wine in which the presence of smoke taint was difficult to detect by chemical or sensory analysis. The sensors could therefore be used to monitor the presence of smoke in vineyards during bushfires, and where vineyard exposure to smoke is detected, the need for grape compositional analysis to determine the presence of volatile phenols (and/or their glycoconjugates) as markers of smoke taint, based on both the duration and density of smoke exposure.

The environmental sensors also recorded temperature and relative humidity during the field trials (data not shown). Temperatures within the smoke tents increased by ~10 °C (relative to ambient temperature) during smoke treatments. Relative humidity differed more between the two days during which field trials were undertaken (i.e., from ~25–30% to ~40–55%) than between treatments, with the exception of the HSM treatment, during which the relative humidity in the smoke tent increased from

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25–30% to 40%, due to the in-canopy misting. However, the differences observed in microclimate conditions were not expected to have significant or lasting effects on grapevine physiology, especially relative to the known effects of smoke on grapevine physiology [5].

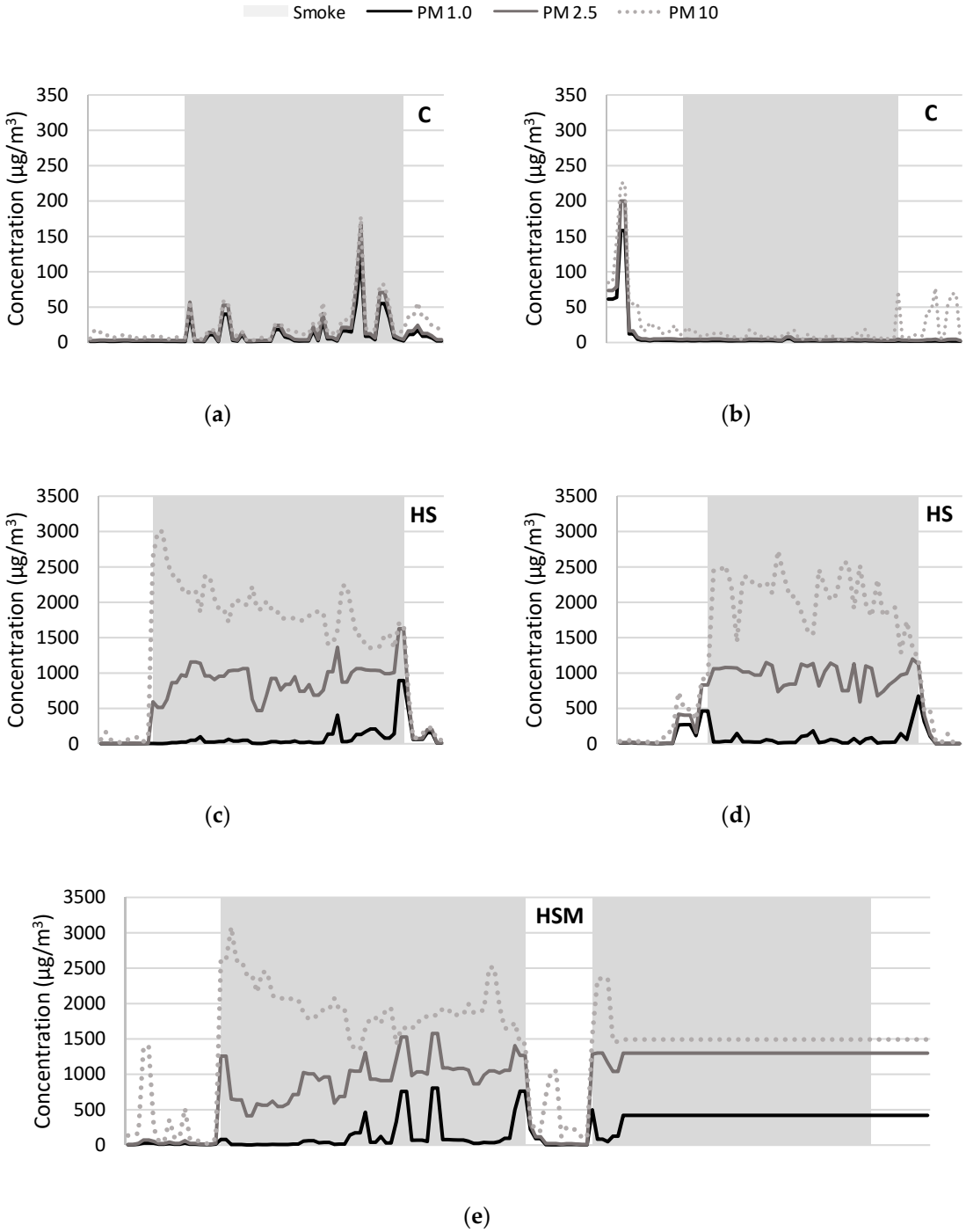

Figure 3. *Cont.*

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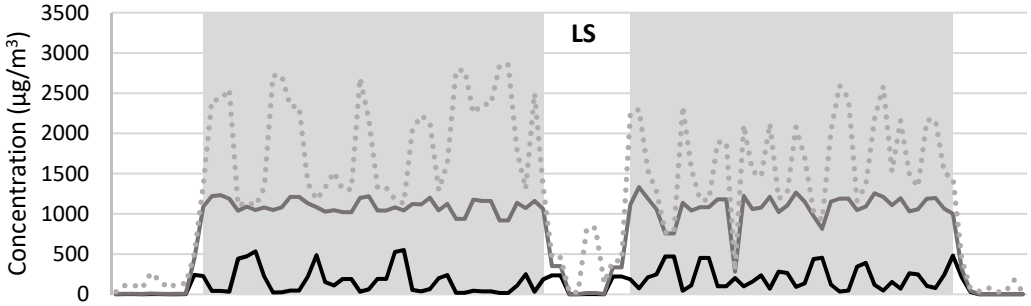

(**f**)

Figure 3. Particulate matter ($PM_{1.0}$, $PM_{2.5}$ and PM_{10}) concentrations measured during field trials. The x axes reflect time, with shading indicating the 1 h window of each smoke treatment: (**a**–**d**) show PM data recorded during the high density smoke exposure (HS) treatments, with sensors positioned amongst the control vines and within the smoke tent, respectively (sensor positions were swapped between the two duplicate HS treatments); (**e**,**f**) show PM data recorded during the duplicate high smoke with misting (HSM) and low smoke (LS) treatments, respectively (with sensors again positioned within the smoke tent).

3. Materials and Methods

3.1. Chemicals

Chemicals (analytical grade) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Solvents (HPLC grade) were sourced from Sigma Aldrich or Merck (Darmstadt, Germany). Deuterium-labelled internal standards (d_3-guaiacol, d_4-guaiacol, d_3-4-methylguaiacol, d_7-*o*-cresol, d_3-syringol, d_3-syringol gentiobioside) were synthesized in house, as previously reported [11,13,18,30].

3.2. Field Trials

Field trials involved the application of smoke (with or without in-canopy misting) to Cabernet Sauvignon grapevines (*Vitis vinifera*) growing in two adjacent rows of a vineyard located at the University of Adelaide's Waite Campus in Urrbrae, South Australia (34°58′ S, 138°38′ E). Grapevines were planted (in 1998) in north-south aligned rows on their own roots (at 2.0 and 3.3 m vine and row spacings, respectively), trained to a bilateral cordon-vertical shoot positioned trellis system, hand-pruned to a two-node spur system and drip-irrigated twice weekly from fruit set to pre-harvest. Treatments comprised: (i) a control (C), i.e., no smoke exposure; (ii) a low smoke treatment (LS), i.e., exposure to low density smoke; (iii) a high smoke treatment (HS), i.e., exposure to high density smoke; (iv) a control with misting (CM), i.e., in-canopy misting but no smoke exposure; and (v) a high smoke treatment with misting (HSM), i.e., exposure to high density smoke with in-canopy misting.

Smoke treatments involved grapevines being exposed to smoke for 1 h (at approximately 7 d post-veraison), using purpose-built smoke tents (6.0 × 2.5 × 2.0 m) and experimental conditions similar to those described previously [3–5]; except that barley straw was combusted in two commercial fire box smokers, positioned at each end of the smoke tent. Low and high density smoke treatments were achieved by burning approximately 1.5 and 5 kg of barley straw respectively, with fuel added at regular intervals (i.e., every ~10 min) to ensure smoke production throughout the duration of treatment. Control and smoke-exposed vines were separated by at least one buffer vine. In-canopy misting treatments involved the continuous application of fine (65 µm) water droplets to the bunch zone of six adjacent vines, using a purpose-built sprinkler system (comprising two CoolNet Pro "tee" configuration sprinklers (Netafim Australia, Adelaide, SA, Australia) per vine, suspended 30 cm above the cordon, each delivering water at a rate of 11 L/h) supplied with mains water pumped from a 1000 L plastic tank, as described previously [22].

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Whereas smoke treatments were applied to panels of vines in triplicate in previous trials [5,6,16,27], fire bans imposed by the state government (due to increased fire danger ratings associated with hot, dry and/or windy weather conditions) limited the number of smoke treatments that could be applied in the current study. Field trials were also constrained by where the in-canopy sprinkler systems were installed. As such, each treatment was applied to six adjacent grapevines, as depicted in Figure 4. LS, HS and HSM treatments comprised duplicate applications of smoke to three adjacent vines at a time, with the in-canopy sprinkler system turned on 5 min before the first HSM treatment was applied, and off 15 min after the second HSM treatment was completed, such that CM and HSM grapevines were misted for approximately 2.5 h in total. Three vine replicates per treatment were subsequently selected for berry sampling and winemaking (with vine replicates becoming wine replicates, for each treatment). With the exception of CM, three adjacent vines were chosen as vine replicates; for LS, HS and HSM, vine replicates were from the same smoke application. In the case of CM, the selection of non-adjacent vine replicates accounted for missing sprinklers.

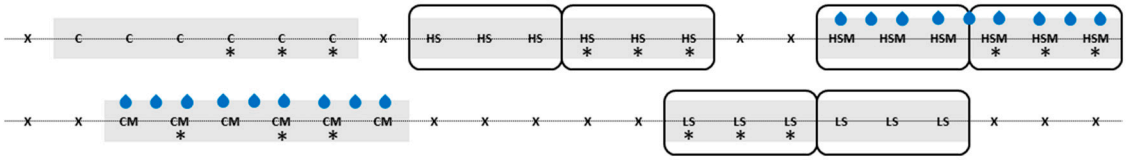

Figure 4. Schematic diagram of treatments (C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure; HS = high density smoke exposure; HSM = high density smoke exposure with misting), showing the positioning of smoke tents, in-canopy sprinklers (💧), vine replicates (*) and buffer vines (×), within the two adjacent rows of Cabernet Sauvignon vines.

Two portable environmental sensors (R9 series, Attentis Pty. Ltd., Cheltenham, Vic., Australia) were used to monitor temperature, relative humidity, and the concentration of particulate matter ($PM_{1.0}$, $PM_{2.5}$ and PM_{10}) during field trials. One sensor was positioned inside the smoke tent during each smoke treatment, while the other sensor was positioned mid-row, amongst control (C and CM) vines. Environmental data were captured continuously (typically at 1–2 min intervals) and uploaded to the manufacturer's network via an internal Wi-Fi connection. Data were subsequently exported from the network as Excel files.

Samples (50 berries, from each of the three vine replicates per treatment, chosen randomly according to a previously published sampling protocol [31]) were collected at five time points: (i) immediately prior to smoke exposure (t = 0); (ii) 1 h after smoke exposure (t = 1); (iii) 1 day after smoke exposure, (t = 2); (iv) 7 days after smoke exposure, (t = 3); and (v) 4 weeks after smoke exposure (t = 4) at maturity. Samples were homogenized (T18 Ultra Turrax, IKA, Staufen, Germany) and frozen at –4 °C until quantitation of volatile phenols and volatile phenol glycoconjugates (approximately 1 month after sampling). The remaining control and smoke-exposed fruit were harvested (4 weeks after smoke exposure) for winemaking, with the vine replicates from each treatment becoming wine replicates. Grapes were intended to be harvested when TSS levels were approximately 24 °Brix, with maturity sampling performed on samples (50 berries) collected from buffer vines. However, analysis of juice following harvest and crushing of grapes indicated significant variation in maturity amongst vine replicates, with average TSS levels ranging from 19.6 to 22.3 °Brix (Table S6). Viticultural data were collected to evaluate variation in vine physiology and while significant differences were not observed for TSS, bunch number, yield, shoot number or pruning weight between treatments (Table S6), this was attributed to the large relative standard errors (i.e., 10–25.9%) associated with one or more treatments, for each measurement. Vine variation likely explains the different TSS levels observed amongst juice samples, and therefore the differences in wine alcohol content, which were perceived by the sensory panel (Figure 2, Table S4). Nevertheless, the differences in the intensity of hotness between wines were not considered to have significantly affected the panel's perception of smoke taint. Other significant differences in basic wine composition (i.e., titratable acidity (TA) and

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color; Table S5) did not significantly affect the panel's rating of acidity (Table S4) or were addressed by presenting wines to panelists monadically.

3.3. Preparation of Acid Hydrolysates

Juices from HS grape homogenate samples (two replicates from each time point) were subjected to strong acid hydrolysis, using methodology similar to that reported by Noestheden and colleagues [15]. Briefly, aliquots of homogenate (10 g) were centrifuged for 30 min at 3500× *g* (Universal 320R centrifuge, Andreas Hettich GmBH and Co. KG, Tuttlingen, Germany), and 2 mL of the resulting juice was purified by solid phase extraction (using Strata X 33 µm polymeric reversed phase cartridges, 200 mg/3 mL; Phenomenex, Lane Cove, NSW, Australia). Samples were eluted with 40% acetonitrile in water (2 mL), dried (under nitrogen at 35 °C), reconstituted in 2 mL water and acidified to pH ~1 (via dropwise addition of 1 M hydrochloric acid), before being heated at 100 °C for 4 h in 8 mL PTFE tubes (SPI Supplies, West Chester, PA, USA). Hydrolysates were subsequently cooled to ambient temperature, pH adjusted back to wine pH (i.e., pH 3.0–3.5, via dropwise addition of 1 M aqueous sodium hydroxide) and frozen prior to chemical analysis.

3.4. Winemaking

Bunches (5 kg per replicate, per treatment, chosen randomly) were crushed and de-stemmed, with the addition of 50 mg/L sulfur dioxide (added as an 8% solution of potassium metabisulphite). Tartaric acid was added to adjust the pH of must to 3.5, prior to inoculation with 150 mg/L of PDM yeast (Maurivin, AB Biotek, Sydney, NSW, Australia) and the addition of diammonium phosphate (100 mg/L). Musts were fermented on skins at ambient temperature (25–27 °C), with the cap plunged twice daily. When wines approached dryness (2 g/L residual sugar), they were pressed and held at 25 °C until completion of fermentation (i.e., until residual sugars approached 0 g/L), after which they were racked from gross lees and cold stabilized (at 0 °C for 4 weeks). No wines underwent malolactic fermentation. Wine pH and free SO_2 were adjusted to 3.5 and 30 mg/L respectively, before bottling (in 375 mL glass bottles, with screw cap closures). Bottles were stored at 15 °C for two months prior to sensory analysis. Prior to bottling, wines were sampled for chemical analysis.

3.5. Chemical Analysis of Grapes, Wine and Acid Hydrolysates

Residual sugars were measured enzymatically (using a glucose/fructose enzymatic test kit from Vintessential Laboratories Pty. Ltd., Dromana, Vic., Australia) using a Chemwell 2910 automated analyzer (Awareness Technology Inc., Palm City, FL, USA). pH and titratable acidity (TA, expressed as g/L tartaric acid) were measured using a Mettler Toledo T50 autotitrator coupled to a Mettler Toledo InMotion Flex autosampler (Port Melbourne, Vic., Australia). Ethanol content (% alcohol by volume, abv) was measured with an alcolyzer (Anton Paar, Graz, Austria). Wine color density, wine hue and total phenolics were determined by the modified Somers color assay [32] using an Infinite® 200 PRO spectrophotometer (Tecan, Männedorf, Switzerland).

3.5.1. Determination of Volatile Phenols

The concentrations of volatile phenols (guaiacol, 4-methylguaiacol, phenol, *o*-, *m*- and *p*-cresol, syringol and 4-methylsyringol) were measured in grape juice and wine samples, using stable isotope dilution analysis (SIDA) methods described previously [13,18,30], with the method developed for analysis of wine also used for acid hydrolysates. These publications describe the preparation of isotopically labelled standards (d_4-guaiacol and d_3-syringol for analysis of grape juice performed at the University of Adelaide and d_3-guaiacol, d_3-4-methylguaiacol, d_7-*o*-cresol and d_3-syringol for analysis of wine and acid hydrolysates, performed by the Australian Wine Research Institute's (AWRI) Commercial Services Laboratory, Adelaide, Australia), and method validation and instrumental operating conditions. All measurements were performed using an Agilent 6890 gas chromatograph

coupled to a 5973 mass spectrometer (Agilent Technologies, Forest Hill, Vic., Australia). The limit of quantitation for volatile phenols was 1–2 µg/L.

3.5.2. Determination of Volatile Phenol Glycosides

The concentrations of volatile phenol glycosides were measured in grape (homogenate), wine and acid hydrolysate samples, as syringol glucose glucoside (gentiobioside) equivalents, using liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) according to previously published SIDA methods [11,13]; the method developed for analysis of wine was also used for acid hydrolysates. Glycoconjugate analyses were performed on an Agilent 1200 high performance liquid chromatograph (HPLC) equipped with a 1290 binary pump, coupled to an AB SCIEX Triple Quad^{TM} 4500 tandem mass spectrometer, with a Turbo V^{TM} ion source (Framingham, MA, USA). Data acquisition and processing were performed using Analyst software (version 1.7 AB SCIEX). The preparation of the isotopically labelled internal standard (d_3-syringol gentiobioside), method validation and instrumental operating conditions were as previously reported [11,13]. The limit of quantitation for volatile phenol glycosides was 1 µg/kg (as syringol glucose glucoside equivalents).

3.6. Sensory Analysis of Wine

The replicate wines from each treatment were assessed by a group of sensory experts from the University of Adelaide (for evidence of faults or obvious differences between replicates), before replicates were blended. The sensory profiles of wines (as one wine per treatment) were then determined using the rate-all-that-apply (RATA) method [33] and a panel comprising staff and students from the University of Adelaide and AWRI, and regular wine consumers (n = 50, 12 male and 38 female, aged between 20 and 74 years). Prior to wine evaluation, panelists completed a brief induction, during which they were familiarized with both the RATA procedure and a list of attributes and their definitions (Table S7), which were adapted from previous studies [5,26]. RATA assessments were conducted in sensory booths at 22–23 °C under sodium lights, with wine aliquots (30 mL) presented monadically, in a randomized order, in covered, 3-digit coded 215 mL stemmed International Organization for Standardization wine glasses. Panelists rated the intensity of each sensory attribute using line scales (where 0 = "not perceived", 1 = "extremely low" and 9 = "extremely high"). Panelists rinsed thoroughly with pectin solution (1 g/L) and rested for at least 1 min between samples, with water and plain crackers provided as palate cleansers. Data were acquired with Red Jade software (Redwood Shores, CA, USA).

3.7. Data Analysis

Chemical data were analyzed by one and two-way analysis of variance (ANOVA) using GenStat (19th Edition, VSN International Limited, Herts, UK). Sensory data were analyzed using SenPAQ (version 5.01, Qi Statistics, Reading, UK) and XLSTAT (version 2018.1.1, Addinsoft, New York, NY, USA). Mean comparisons were performed by Fisher's least significant difference (LSD) multiple comparison test at $P < 0.05$.

Supplementary Materials: The following are available online: Table S1: Concentrations (µg/kg) of volatile phenol glycoconjugates in control and smoke-exposed grapes sampled from pre-smoke exposure (t = 0) to maturity (t = 4). Table S2: Concentrations (µg/L) of volatile phenol glycoconjugates in wines made from control and smoke-exposed grapes. Table S3: Concentrations (µg/L) of volatile phenols in juice from control and smoke-exposed grapes sampled from pre-smoke exposure (t = 0) to maturity (t = 4). Table S4: Mean intensity ratings for sensory attributes of control and smoke-affected wines. Table S5: Basic composition of control and smoke-affected wines. Table S6: Viticultural measurements for control and smoke-affected grapevines. Table S7: Aroma and palate attributes used in sensory analysis of wines.

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Supplementary Materials

Uptake and Glycosylation of Smoke-Derived Volatile Phenols by Cabernet Sauvignon Grapes and their Subsequent Fate during Winemaking

Colleen Szeto [1,2], Renata Ristic [1,2], Dimitra Capone [1,2], Carolyn Puglisi [1], Vinay Pagay [1,2], Julie Culbert [3], WenWen Jiang [3], Markus Herderich [1,2,3], Jonathan Tuke [4] and Kerry Wilkinson [1,2,*]

[1] The University of Adelaide, School of Agriculture, Food and Wine, PMB 1, Glen Osmond, SA, 5064, Australia; colleen.szeto@adelaide.edu.au (C.S.); renata.ristic@adelaide.edu.au (R.R.); dimitra.capone@adelaide.edu.au (D.C.); carolyn.puglisi@adelaide.edu.au (C.P.); vinay.pagay@adelaide.edu.au (V.P.)

[2] The Australian Research Council Training Centre for Innovative Wine Production, PMB 1, Glen Osmond, SA, 5064, Australia

[3] The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA, 5064, Australia julie.culbert@awri.com.au (J.C.); maddy.jiang@awri.com.au (W.J.); markus.herderich@awri.com.au (M.H.);

[4] School of Mathematical Sciences, The University of Adelaide, SA 5000, Australia; simon.tuke@adelaide.edu.au

* Correspondence: kerry.wilkinson@adelaide.edu.au; Tel.: +61-8-8313-7360

Table S1. Concentrations (μg/kg) of volatile phenol glycoconjugates in control and smoke-exposed grapes sampled from pre-smoke exposure (t = 0) to maturity (t = 4).

Treatment/ Timepoint		GuG	GuGG	GuPG	GuR	4MGuG	4MGuPG	4MGuR	PhG	PhGG	PhPG	PhR	CrG	CrGG	CrPG	CrR	SyG	SyGG	SyPG	MSyGG	MSyPG
C	t = 0	nd	nd	3.7 b	nd	nd	tr	tr	nd	nd	2.5 b	tr	nd	tr	9.2 b	2.0	tr	2.7	tr	tr	nd
	t = 1	tr	nd	5.0 b	nd	nd	1.2 b	tr	tr	nd	3.0 b	tr	1.1	tr	12 b	2.7	1.1 b	3.7	1.1 b	1.0 b	nd
	t = 2	tr	tr	7.3 b	nd	nd	1.7 b	1.3 b	nd	nd	3.7 b	tr	1.0	tr	19 b	4.9	1.6 b	11	2.0 b	2.1 b	nd
	t = 3	tr	tr	11 b	tr	nd	2.3 b	2.2 b	nd	tr	6.3 b	1.3 b	tr	tr	23 b	7.0	1.5 b	24	3.9 b	3.3 b	tr
	t = 4	1.8	1.9	38 a	2.2 a	1.8	10 a	10 a	1.6	1.0	35 a	7.3 a	nd	3.0	73 a	6.3	4.8 a	23	16 a	10 a	2.8
	P	–	–	<0.003	–	–	<0.001	<0.001	–	–	<0.001	<0.001	ns	–	<0.001	ns	0.003	ns	0.003	0.026	–
CM	t = 0	nd	nd	3.1 b	nd	nd	tr	1.0 b	tr	nd	2.6 b	tr	nd	tr	8.7 d	2.1	tr	2.3	tr	tr	nd
	t = 1	nd	nd	2.7 b	nd	nd	tr	tr	nd	nd	1.6 b	tr	nd	tr	8.4 d	1.8	tr	2.5	tr	tr	nd
	t = 2	nd	nd	3.7 b	nd	nd	tr	tr	nd	nd	3.5 b	tr	nd	tr	12 c	2.3	tr	3.6	1.2 b	tr	nd
	t = 3	nd	nd	3.7 b	tr	nd	tr	tr	nd	nd	3.9 b	tr	nd	tr	14 b	2.4	tr	4.4	1.3 b	tr	nd
	t = 4	1.9	0.7	17 a	1.2	1.6	6.9	7.8 a	1.4	nd	20 a	4.4	nd	2.9	43 a	3.3	2.7	6.7	6.8 a	2.7	1.7
	P	–	–	<0.001	–	–	–	<0.001	–	–	<0.001	–	–	–	<0.001	ns	–	ns	<0.001	–	–
LS	t = 0	nd	nd	3.4 b	nd	nd	tr	tr	tr	nd	2.8 b	tr	nd	tr	6.7 b	1.7 d	tr	2.3 c	tr	tr	nd
	t = 1	1.5 c	nd	4.0 b	nd	nd	1.0 b	1.0 b	tr	nd	4.0 b	1.0 b	3.0 b	nd	13 b	3.2 d	1.3 c	9.0 c	1.4 b	1.5 b	nd
	t = 2	3.0 a	tr	9.2 b	nd	nd	2.5 b	2.3 b	0.6 b	tr	13 b	2.3 b	10 a	tr	27 b	9.2 c	3.2 b	21 b	3.4 b	3.2 b	tr
	t = 3	1.2 c	tr	13 b	1.5 b	nd	3.0 b	3.3 b	0.5 b	tr	21 b	4.3 b	0.6 c	tr	33 b	12.6 b	1.4 c	35 a	5.3 b	4.0 b	tr
	t = 4	2.3 b	3.6	63 a	4.3 a	2.3	16 a	20 a	3.0 a	1.3	94 a	23 a	nd	2.2	136 a	15.0 a	6.5 a	43 a	27 a	14.5 a	3.7
	P	<0.001	–	<0.001	<0.001	–	<0.001	<0.001	ns	–	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	–
HS	t = 0	nd	nd	3.1 b	nd	nd	tr	tr	tr	nd	2.6 b	tr	nd	tr	7.8 b	1.8 c	tr	2.3 c	tr	tr	nd
	t = 1	24	1.1 b	19 b	1.0 c	nd	9.2 b	4.5 b	1.0 b	tr	18 b	3.0 b	51 ab	tr	36 b	11 c	23 b	44 c	3.6 b	11 b	nd
	t = 2	35	5.7 b	115 b	2.7 c	nd	36 b	15 b	2.6 b	2.4 b	55 b	9.8 b	70 a	1.0 b	137 b	56 b	43 ab	248 b	19 b	44 b	4.2 b
	t = 3	23	10 b	185 b	11 b	nd	46 b	25 b	2.2 b	5.4 b	115 b	21 b	9.4 c	1.2 b	217 b	89 ab	22 b	455 a	49 b	62 b	7.1 b
	t = 4	20	45 a	803 a	25 a	8.4	171 a	118 a	14 a	20 a	576 a	135 a	27 bc	5.5 a	988 a	98 a	50 a	535 a	258 a	220 a	27 a
	P	ns	<0.001	<0.001	<0.001	–	0.002	<0.001	0.006	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.005	0.002	<0.001	<0.001	<0.001
HSM	t = 0	nd	nd	3.4 c	nd	nd	tr	tr	nd	nd	2.5 d	tr	nd	tr	9.6 c	2.3 c	tr	2.3 d	tr	tr	nd
	t = 1	9.0 b	tr	7.5 c	tr	nd	4.5 c	2.3 d	1.6 c	tr	8.7 cd	1.3 b	27 b	tr	22 c	5.9 c	6.6 c	18 d	2.0 d	4.0 c	tr
	t = 2	15 a	3.7 b	55 bc	1.9 c	nd	18 b	9.6 c	1.2 c	1.3 b	38 c	6.8 b	43 a	tr	96 bc	42 b	19 b	129 c	11 c	21 bc	2.4 b
	t = 3	15 a	6.2 b	87 b	8.1 b	nd	22 b	16 b	4.3 b	2.7 b	86 b	17 b	8.5 bc	tr	147 b	71 a	11 c	238 b	26 b	30 b	3.8 b
	t = 4	9.0 b	30 a	421 a	17 a	5.4	83 a	66 a	8.8 a	11 a	351 a	97 a	17 bc	4.2	673 a	69 a	32 a	325 a	136 a	115 a	16 a
	P	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
P[1]		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.007	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD[1]		7.2	16	307	5.0	2.4	68	27	6.5	6.6	173	34	6.6	1.2	303	20	14	172	80	62	5.9

C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure; HS = high density smoke exposure; HSM = high density smoke exposure with misting. Values are means of three replicates (n = 3) measured as syringol glucose-glucoside equivalents; nd = not detected; tr = trace (i.e. 0.5–1 μg/kg). Different letters (within columns, by treatment) indicate statistical significance (P = 0.05, one way ANOVA) amongst time points, i.e.: immediately prior to smoke exposure (t = 0); 1 hour after smoke exposure (t = 1); 1 day after smoke exposure (t = 2); 7 days after smoke exposure (t = 3); and 4 weeks after smoke exposure (t = 4) being maturity; ns = not significant. Gu = guaiacol; Cr = cresol; Ph = phenol; Sy = syringol; 4MG = 4-methylguaiacol; MSy = 4-methylsyringol; G = glucoside; GG = glucose-glucoside; PG = pentose-glucoside; R = rutinoside. [1] *P* and LSD values for one way ANOVA of data by treatment at maturity (t = 4).

Table S2. Concentrations (μg/L) of volatile phenol glycoconjugates in wines made from control and smoke-exposed grapes.

Treatment	GuG	GuGG	GuPG	GuR	4MGuPG	4MGuR	PhG	PhGG	PhPG	PhR	CrPG	CrR	SyG	SyGG	SyPG	MSyGG	MSyPG
C	tr	tr	15 c	3.3 c	2.3 c	2.0 c	1.8 c	tr	1.1 c	3.1 c	1.1 c	6.3 c	tr	24 c	4.8 c	tr	tr
CM	nd	tr	6.1 c	1.2 c	1.5 c	tr	1.0 c	nd	tr	1.7 c	tr	3.2 c	tr	11 c	1.8 c	tr	tr
LS	1.0 c	tr	21 c	6.6 c	3.3 c	4.4 c	3.9 c	tr	2.6 c	9.8 c	1.5 c	14 c	1.4 c	43 c	8.8 c	1.5 c	tr
HS	9.4 a	2.1	234 a	37 a	37 a	31 a	31 a	4.7 a	17 a	59 a	14 a	102 a	11 a	413 a	77 a	23 a	6.6 a
HSM	6.2 b	1.6	126 b	28 b	19 b	21 b	21 b	3.0 b	10 b	43 b	10 b	78 b	7.4 b	272 b	47 b	12 b	4.1 b
P	<0.001	ns	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure ; HS = high density smoke exposure; HSM = high density smoke exposure with misting. Values are means of three replicates (n = 3) measured as syringol glucose-glucoside equivalents; nd = not detected; tr = trace (i.e. 0.5–1 μg/L). Different letters (within columns) indicate statistical significance (P = 0.05, one way ANOVA); ns = not significant. Gu = guaiacol; Cr = cresol; Ph = phenol; Sy = syringol; 4MG = 4-methylguaiacol; MSy = 4-methylsyringol; G = glucoside; GG = glucose-glucoside; PG = pentose-glucoside; R = rutinoside. 4-Methylguaicol glucoside (4MGuG), cresol glucoside (CrG) and cresol glucose glucoside (CrGG) were not detected in any wine.

Table S3. Concentrations (μg/L) of volatile phenols in juice from control and smoke-exposed grapes sampled from pre-smoke exposure (t = 0) to maturity (t = 4).

Treatment/ Timepoint		Guaiacol	4-Methyl Guaiacol	Phenol	Cresols	Syringol	4-Methyl Syringol
C	t = 0	1.9 b	3.6	1.5	2.6	12 b	2.5
	t = 1	9.5 a	4.1	2.6	5.1	21 a	3.0
	t = 2	2.4 b	3.6	1.6	2.7	8.4 b	2.0
	t = 3	1.9 b	3.6	1.6	2.4	7.9 b	1.8
	t = 4	2.2 b	3.6	1.6	2.4	13 b	1.8
	P	0.033	ns	ns	ns	0.017	ns
CM	t = 0	1.7 b	3.5	1.5 b	3.2	8.6	2.0
	t = 1	2.6 a	3.5	1.5 b	2.8	8.0	1.9
	t = 2	1.9 b	3.5	3.4 a	2.8	21	1.9
	t = 3	1.9 b	3.5	1.8 b	2.8	8.8	1.8
	t = 4	2.4 a	3.5	1.9 b	2.7	12	1.8
	P	<0.001	ns	0.006	ns	ns	ns
LS	t = 0	1.7 b	3.5 b	1.4 b	2.5 c	6.2 c	2.0 b
	t = 1	12 a	4.1 a	6.9 a	12 a	25 a	2.9 a
	t = 2	2.8 b	3.6 b	4.7 a	4.9 b	6.0 c	1.9 b
	t = 3	2.6 b	3.6 b	5.1 a	4.8 b	13 b	1.8 b
	t = 4	3.1 b	3.6 b	6.3 a	5.0 b	11 bc	1.8 b
	P	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
HS	t = 0	1.8 c	3.5 b	1.8 c	2.7 c	7.8 b	1.9 b
	t = 1	108 a	20 a	55 a	83 a	126 a	17 a
	t = 2	25 b	5.1 b	12 b	23 b	24 b	2.7 b
	t = 3	12 c	4.6 b	17 b	18 b	12 b	1.9 b
	t = 4	10 c	4.2 b	21 b	13 b	12 b	1.8 b
	P	<0.001	<0.001	0.012	<0.001	<0.001	<0.001
HSM	t = 0	1.7 d	3.5 b	1.8 c	2.5 c	8.0 d	1.9 b
	t = 1	76 a	14 a	40 a	59 a	59 a	8.6 a
	t = 2	17 b	4.7 b	11 b	21 b	21 b	2.2 b
	t = 3	7.4 c	4.1 b	12 b	13 b	15 c	2.0 b
	t = 4	7.6 c	4.0 b	17 b	12 b	13 c	1.9 b
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
P[1]		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
LSD[1]		12.1	2.8	11.4	12.2	19.9	2.5

C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure; HS = high density smoke exposure; HSM = high density smoke exposure with misting. Values are means of three replicates (*n* = 3). Different letters (within columns, by treatment) indicate statistical significance (P = 0.05, one way ANOVA) amongst time points, i.e.: immediately prior to smoke exposure (t = 0); 1 hour after smoke exposure (t = 1); 1 day after smoke exposure (t = 2); 7 days after smoke exposure (t = 3); and 4 weeks after smoke exposure (t = 4) being maturity; ns = not significant. [1] P and LSD values for two way ANOVA of data by treatment and time.

Table S4. Mean intensity ratings for sensory attributes of control and smoke-affected wines.

Attribute	C	CM	LS	HS	HSM	P
fruit aroma	4.7 a	4.7 a	4.0 a	2.2 b	2.4 b	<0.0001
smoke aroma	1.8 c	2.1 bc	2.7 b	6.7 a	6.6 a	<0.0001
cold ash aroma	1.4 b	1.9 b	2.4 b	5.4 a	5.7 a	<0.0001
earthy aroma	2.6	2.5	2.9	3.1	2.9	ns
medicinal aroma	2.4 b	2.3 b	2.5 b	4.2 a	4.0 a	0.0001
burnt rubber aroma	1.3 b	1.2 b	1.4 b	4.0 a	3.7 a	<0.0001
fruit flavor	4.8 a	4.9 a	4.5 a	2.5 b	2.8 b	<0.0001
smoky flavor	1.7 b	1.9 b	2.2 b	6.3 a	6.1 a	<0.0001
medicinal flavor	1.8 b	1.6 b	1.7 b	3.6 a	3.8 a	<0.0001
ashy aftertaste	1.5 b	2.0 b	1.9 b	5.8 a	6.1 a	<0.0001
woody aftertaste	2.5 b	2.7 b	2.5 b	3.3 a	3.5 a	0.0025
metallic	2.1 b	2.1 b	2.0 b	3.5 a	3.4 a	0.0003
acidity	5.0	5.4	4.9	5.3	5.2	ns
hotness	3.3 bc	4.2 a	3.7 b	3.1 c	3.7 b	0.0002
bitterness	2.3 b	2.1 b	2.3 b	3.4 a	3.4 a	0.0004
drying	4.5 b	4.4 b	4.2 b	5.5 a	5.6 a	0.0025

C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure; HS = high density smoke exposure; HSM = high density smoke exposure with misting. Values are means for one wine per treatment presented to 50 judges. Different letters (within rows) indicate statistical significance (P = 0.05, one way ANOVA); ns = not significant.

Table S5. Basic composition of control and smoke-affected wines.

Measurement	C	CM	LS	HS	HSM	P
pH	3.7	3.7	3.7	3.6	3.6	ns
TA (g/L)	6.6 b	7.1 a	7.1 a	6.4 b	7.0 a	<0.001
alcohol (% abv)	11.5	12.9	11.9	10.6	12.5	ns
wine color density	6.7 bc	7.9 a	7.4 ab	6.5 c	8.2 a	0.005
wine color hue	0.89 a	0.82 b	0.83 b	0.87 a	0.82 b	0.002
total phenolics	137.7	137.0	137.2	138.1	141.0	ns

C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure; HS = high density smoke exposure; HSM = high density smoke exposure with misting. Values are means of three wine replicates. Different letters (within rows) indicate statistical significance (P = 0.05, one way ANOVA); ns = not significant.

Table S6. Viticultural measurements for control and smoke-affected grapevines.

Measurement	C	CM	LS	HS	HSM	P
TSS (° Brix)	20.8 ± 1.7%	22.3 ± 3.7%	19.6 ± 17.1%	19.6 ± 1.6%	21.7 ± 1.2%	ns
bunch number	67.3 ± 18.2%	58.0 ± 8.8%	59.0 ± 14.5%	71.7 ± 12.9%	56.3 ± 6.8%	ns
yield (kg)	6.7 ± 22.8%	5.4 ± 14.5%	5.4 ± 9.1%	6.9 ± 20.4%	4.7 ± 4.0%	ns
shoot number	45.3 ± 9.9%	42.3 ± 21.6%	42.3 ± 5.2%	51.0 ± 14.5%	37.3 ± 6.3%	ns
pruning weight (kg)	2.6 ± 9.4%	2.7 ± 25.9%	1.9 ± 1.9%	2.1 ± 17.8%	1.6 ± 5.9%	ns

C = control (no smoke exposure); CM = control with misting; LS = low density smoke exposure; HS = high density smoke exposure; HSM = high density smoke exposure with misting. Values are means of three replicates ± relative standard error. No statistical significance observed amongst treatments (P = 0.05, one way ANOVA); ns = not significant.

Table S7. Aroma and palate attributes used in sensory analysis of wines.

Attributes	Definition
Aroma	
fruit	intensity of the overall fruit aroma
smoke	perception of any type of smoke aroma, including smoked meat/bacon, toasty, charry, cigar-box, estery
cold ash	burnt aroma associate with ashes, including ashtray, tarry, campfire
earthy	any aroma associated with musty, dusty, wet-wood, barnyard, mushroom-like, dank, moldy, stagnant, stale
medicinal	aromatic characteristic of Band-Aids, disinfectant-like, including cleaning products, solvents, chemicals
burnt rubber	perception of burnt rubber-like aromas
Palate	
fruit	intensity of the overall fruit flavor
smoky	perception of smoke flavor, including bacon and smoked meat
ashy aftertaste	length of taste associated with residue of ashtray perceived in the mouth after expectorating, including coal ash, ashtray, tarry, acrid, campfire
woody aftertaste	length of taste associated with woody residue, includes wood, oak, pencil shavings
metallic	the 'tinny' flavor associated with metals
acidity	intensity of sour/acid taste
hotness	intensity of warmth/heat due to ethanol
bitterness	intensity of bitter taste, bitter aftertaste
drying	intensity of drying, puckering mouthfeel

Chapter 4

Evaluation of Fluorescence Spectroscopy as a Rapid Diagnostic for Predicting the Risk of Smoke Taint in Wine

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Colleen Szeto
Contribution to the Paper	Conceptualization of the work / experimental design Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing
Overall percentage (%)	70
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third this thesis. I am the primary author of this paper.
Signature	Date 20/12/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Adam Gilmore		
Contribution to the Paper	Conceptualization of the work / experimental design Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	2021/12/21

Name of Co-Author	Neil Scrimgeour		
Contribution to the Paper	Experimental work / data collection Manuscript preparation and editing		
Signature		Date	21/12/21

Name of Co-Author	Eric Wilkes
Contribution to the Paper	Experimental work / data collection Manuscript preparation and editing
Signature	Date 20/12/21

Name of Co-Author	David Jeffery
Contribution to the Paper	Conceptualization of the work / experimental design Data organization, interpretation, and analysis Manuscript preparation and editing
Signature	Date 21/12/21

Name of Co-Author	Kerry Wilkinson
Contribution to the Paper	Conceptualization of the work / experimental design Data organization, interpretation, and analysis Manuscript preparation and editing
Signature	Date 21/12/21

Name of Co-Author	
Contribution to the Paper	
Signature	Date

Name of Co-Author	
Contribution to the Paper	
Signature	Date

Name of Co-Author	
Contribution to the Paper	
Signature	Date

Name of Co-Author	
Contribution to the Paper	
Signature	Date

Evaluation of fluorescence spectroscopy as a rapid diagnostic for predicting the risk of smoke taint in wine

Colleen Szeto[1,2], Adam Gilmore[3], Neil Scrimgeour[4], Eric Wilkes[4], David Jeffery[1,2], and Kerry Wilkinson[1,2*]

[1]Department of Wine Science and Waite Research Institute, The University of Adelaide, Glen Osmond, SA 5064, Australia
[2]Australian Research Council Training Centre for Innovative Wine Production, Glen Osmond, SA 5064, Australia
[3]HORIBA Instruments Inc., 20 Knightsbridge Rd., Piscataway, NJ 08854, United States
[4]The Australian Wine Research Institute, Glen Osmond, SA 5064, Australia

*****Corresponding author: Prof. Kerry Wilkinson, telephone: +61 8 8313 7360, email: kerry.wilkinson@adelaide.edu.au

Abstract

When bushfires occur in proximity to vineyards, there is a risk that generated smoke will leave behind not only chemical traces of its presence in grapevines, but also the potential for the forthcoming wine to be characterised by an undesirable smoky, burnt and ashy sensory profile known as smoke taint. Smoke exposure in grapevines is determined via measuring volatile phenols (including guaiacol, 4-methylguaiacol, *o*-cresol and syringol) with gas chromatography mass spectrometry and their glycosylated equivalents with high-performance liquid chromatography tandem mass spectrometry. These methods are resource-intensive and require highly trained personnel to perform. A bottleneck has resulted from a big demand for smoke taint analysis and a small number of accredited laboratories offering the service. This pressure is intensified by the need for quick sample turnover to drive time-sensitive decisions made by growers and winemakers who are affected by vineyard smoke exposure, especially during the compressed period of vintage. The confluence of these factors urges the development of techniques to rapidly detect smoke exposure in grapes and wine.

In this work, a diverse array of experimental, research and commercial wine samples (n=158) was analysed by fluorescence spectroscopy using the absorbance-transmittance excitation emission matrix (A-TEEM) technique. Extreme gradient boosting (XGB) algorithms and partial least squares (PLS) methods aimed to classify wine samples with low, medium and high risk of smoke exposure and predict concentrations of volatile phenols and glycoconjugates; however, limited success was achieved. Initial models built with experimental wines were promising, the XGB regression models predicting volatile phenols with R^2 levels ranging from 0.78to 0.96. But in models built with commercial wines, performance was neither high nor reproducible. For example, in XGB and PLS regression models, the R^2 coefficient ranged from <0.02 to 0.86, depending on how the same data were divided into calibration and validation sets. On a similar note, the XGB discriminant analysis methods classified samples as low, medium and high risk with 33%, 22% and 50% accuracy, respectively. While the technique was unsuccessful, these findings prompted a discussion of the key challenges associated with using spectral detection methods to predict levels of smoke exposure in grape and wine matrices.

1. Introduction

Smoke taint refers to the presence of smoky, burnt, and medicinal aromas and flavours, and an ashy, drying aftertaste, that define wine made from grapes exposed to bushfire smoke (Kennison *et al.* 2007). Smoke taint analysis has become a year-round challenge for the global wine industry, due to prolonged droughts and increased temperatures associated attributed to climate change, and the consequent increases in the duration and intensity of fire seasons (Clarke *et al.* 2011; Dowdy 2018). To assess smoke taint, current practice involves measuring volatile phenols (e.g. guaiacol, 4-methylguaiacol, syringol, and *o*-cresol) by gas chromatography-mass spectrometry (GC-MS) (Hayasaka *et al.* 2010c), and their glycosylated equivalents by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (Dungey *et al.* 2011; Haysaka *et al.* 2013). The necessity of these specialised, expensive resources has conditioned industry for dependence on a small subset of fee-for-service providers and increased demand for smoke taint analysis following fire events saw significant bottlenecks occur, resulting in lengthy delays in data becoming available to inform decision-making.

The resolution of this bottleneck has been approached in different ways. In Szeto *et al.* 2021, particulate matter sensors were used to monitor grapevine smoke exposure. This approach aims to reduce the number of samples submitted for analysis that are minimally affected by smoke exposure. In Noestheden *et al.* 2017, a method was developed to measure both volatile phenols and volatile phenol glycoconjugates via GC-MS. This aims to streamline data collection by eliminating the need to develop a separate analytical method for the glycoconjugates, which are conventionally measured with an HPLC-MS/MS. It also enables more commercial laboratories to offer the full suite of smoke taint diagnostics at a reduced cost. In Fudge *et al.* 2012, mid-infrared (MIR) spectroscopy with multivariate chemometrics was evaluated as a rapid method for classifying smoke-affected wine. By differentiating wines as ‘smoke-affected’ or ‘unaffected’, this type of approach aims to prioritise comprehensive quantitation of volatile phenols and glycoconjugates for smoke-affected samples only, thereby streamlining decisions regarding unaffected samples. As a complement to these ongoing approaches, herein we evaluate the ability of fluorescence spectroscopy to be used as a rapid screening tool to identify wines with low-, medium-, and high-risk of smoke taint, based on volatile phenol and glycoconjugate levels. This type of approach aims to build on work by Fudge *et al.* 2012 and reduce the incidence of minimally affected samples submitted for conventional smoke taint analysis.

Fluorescence spectroscopy involves the light-induced excitation of electrons and the subsequent detection of emitted light as the excited electrons of certain molecules return to the ground state. The ability to fluoresce and the wavelength at which it occurs are specific to conjugated compounds, and in wine, this includes phenolic acids, anthocyanins, stilbenes, and flavan-3-ols (Airado-Rodríguez *et al.* 2009). The excitation-emission matrix (EEM) generated is essentially a fingerprint of optically active components in a sample, and the exclusivity of this physical property limits background interference and improves sensitivity (Coelho *et al.* 2015). Volatile phenols are less abundant than other naturally occurring phenolic compounds in wine, and their emission will have lower quantum yields than polyphenolic compounds with more extensive π- π conjugation (e.g. anthocyanins). As a result, the impact of volatile phenols on the EEM fingerprint is expected to be minimal. To assess the feasibility of fluorescence spectroscopy to differentiate smoke-affected wine, chemometrics is required to extract the relevant information.

In previous work that aimed to classify wines as 'smoke-affected' or 'unaffected', MIR spectral data was analysed by linear discriminant analysis (LDA) of scores from principal component analysis (PCA); however classification accuracy was hindered by varietal differences and oak treatment (Fudge *et al.* 2012), which seemingly impacted wine composition to a greater degree than fruit exposure to smoke. LDA aims to identify the linear combination of predictors that maximises between-group variance relative to the within-group variance of different groups (Kuhn and Johnson 2013). For LDA, the predictors must be independent and exceed the number of samples, the opposite of spectral data sets, which encapsulate a large range of wavelengths characterised by high degrees of collinearity. As demonstrated therein, one strategy is to reduce data dimensionality with PCA and perform LDA using the principal component scores (James *et al.* 2021); however, there are several limitations associated with this approach.

PCA generates linear combinations of predictors in the absence of any information about the class structure, modeling objective, or scale of responses (Kuhn and Johnson 2013). Thus, it is unlikely that PCA scores will enable LDA to find the optimal discriminant function relevant to the desired classification—particularly when the variable of interest is neither isolated (via sample preparation or a targeted acquisition method) nor the primary source of variation between samples. In wine, some competing sources of variation include grape varietal, oak maturation, region of origin (terroir), production technique, and age. Another strategy to reduce data dimensionality is the use of partial least squares (PLS), which aims to

generate linear combinations of predictors that reduce data dimensionality with respect to a response (e.g. class membership, concentration) (Kuhn and Johnson 2013).

LDA seeks to divide classes using linear decision boundaries, and its classification performance will be sub-optimal if the true nature of the decision boundary is non-linear (James *et al.* 2021). As an alternative, decision trees split the predictor space into non-overlapping regions based on a set of rules (Boehmke and Greenwell, 2020). The structure and performance of single decision trees are highly variable and as a result, it is common to use an ensemble method (e.g. boosting, bagging, random forests) that combines many simple trees into one predictive model with improved robustness and accuracy (James *et al.* 2021). In boosting, simple trees are sequentially grown, fitted to the residuals of the previous iteration, and added to the model, after which classification or regression can be conducted (Boehmke and Greenwell, 2020). In particular, extreme gradient boosting (XGB) has garnered attention across disciplines due to its speed, high accuracy, and improvements over other machine learning techniques, such as mitigating the risk of overfitting and enhancing model generalizability (Goodin *et al.* 2021; Ranaweera *et al.* 2021; Chen and Guestrin 2016).

To resolve the bottleneck in smoke taint diagnostics, it would be of great benefit to develop a preliminary screening tool that identifies low- and high-risk samples, to streamline time-sensitive winemaking decisions. This would reserve extensive commercial analysis for medium-risk samples. We hypothesise that nonlinear regression models, coupled with a supervised data reduction technique, can be developed to predict volatile phenol and volatile phenol glycoconjugate concentrations in wine using fluorescence spectroscopy. We also explore the feasibility of using fluorescence data to classify wines made from grapes with low-, medium-, and high-risk levels of smoke taint.

2. Materials and Methods

2.1 Chemicals

Ethanol (LC-grade) and 37% hydrochloric acid (HCl, analytical grade) were sourced from Merck (Castle Hill, NSW, Australia) and water was purified through a Milli-Q purification system (Millipore, North Ryde, NSW, Australia).

2.2 Wine samples

Wines (n=158) were collected, consisting of commercial wines (n=121, 1000 L+ scale), research wines (n=10, ~200 L scale), and experimental wines (n=27, ~5 L scale). Experimental wines were sourced from two trials focused on the mitigation of smoke taint. Both trials involved the application of straw-derived smoke to Cabernet Sauvignon grapevines for 1 hour under conditions described previously (Kennison *et al.* 2007). For the mitigation of smoke taint, Trial 1 evaluated the efficacy of post-harvest ozonation of grapes, whereas Trial 2 evaluated in-canopy misting (Szeto *et al.* 2020; Modesti *et al.* 2021). These wines were produced at the University of Adelaide in a benchtop fermentation facility. Research wines were made from grapes exposed to smoke in the Adelaide Hills over the 2019/2020 bushfire season. These wines were produced at the University of Adelaide winery.

Commercial wines were collected from several sources. The majority of commercial wine (n=102) was collected from several wineries located in growing regions impacted by the 2019/2020 bushfire season around Australia (e.g. Adelaide Hills and Barossa Valley (SA), Hunter Valley (NSW), and Rutherglen (VIC)). These wines were submitted to the Australian Wine Research Institute Commercial Services laboratory for smoke taint analysis and were donated to this study. Another set of commercial wine (n=9) was sourced from wineries in the Okanagan Valley, British Columbia. These wines were collected from a study that compared the reproducibility of volatile phenol quantitation across several commercial and research laboratories (Favell *et al.* 2022). The third set of commercial wine (n=10) was collected from wineries in Port Macquarie, NSW.

2.3 Sample preparation and A-TEEM data collection.

Wine samples were prepared using methods similar to those outlined previously (Ranaweera *et al.* 2021). Briefly, wine samples (1 mL) were centrifuged in 1.5 mL micro-centrifuge tubes for 10 min. at 9300 × *g*. Samples were then diluted (150-fold) with 50% aqueous ethanol that had been adjusted to pH 2 with 1.0 M hydrochloric acid and vacuum-filtered through a 0.45 µm PTFE filter (Rowe Scientific). Samples were sonicated for 10 min. and transferred to a Hellma high performance fluorescence cuvette (Hellma GmbH & Co. KG, Mullheim, Germany) for spectral analysis.

A-TEEM data were recorded for each sample using a Horiba Scientific Aqualog® spectrometer (version 4.2, Quark Photonics, Adelaide, SA, Australia) operating with Origin software (version 8.6, OriginLab Corporation, Massachusetts, USA). All wines were scanned in duplicate, with the exception of experimental wines, which were scanned in triplicate. After 2 min. stirring, samples were analysed under medium gain with a 0.2 s integration time for excitation wavelengths from 240-750 nm (5 nm increments) and emission wavelengths from 242-824 nm (4.66 nm increments). All EEMs were normalised according to the water Raman scattering unit factor and corrected for Rayleigh masking and inner filter effects (IFEs) prior to multivariate statistical analyses in Solo software (ver. 8.8.1, Eigenvector Research Inc., Manson, WA, USA). EEM data were transformed from a three-way to a two-way data set using the transform unfold multiway tool in Solo software prior to statistical analysis.

2.4 Chemical analysis of wines.

Volatile phenols were analysed in wines via stable isotope dilution analysis (SIDA) using gas chromatography-mass spectrometry (GC-MS), according to methods published previously (Pollnitz *et al.* 2004; Hayasaka *et al.* 2010). Volatile phenol glycoconjugates were analysed in wines via a SIDA-based high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method, as outlined elsewhere (Dungey *et al.* 2011; Hayasaka *et al.* 2013). All research and commercial wines were analysed by the AWRI Commercial Services laboratory. For experimental wines, volatile phenols were analysed at the University of Adelaide and volatile phenol glycoconjugates were measured at the AWRI. In a recent study, volatile phenols in wine samples were analysed by several commercial laboratories conducting smoke taint analysis, and the results indicated a high level of consistency across labs for this method. Thus, the effect of institution on the levels of volatile phenols reported in the wines herein was considered negligible.

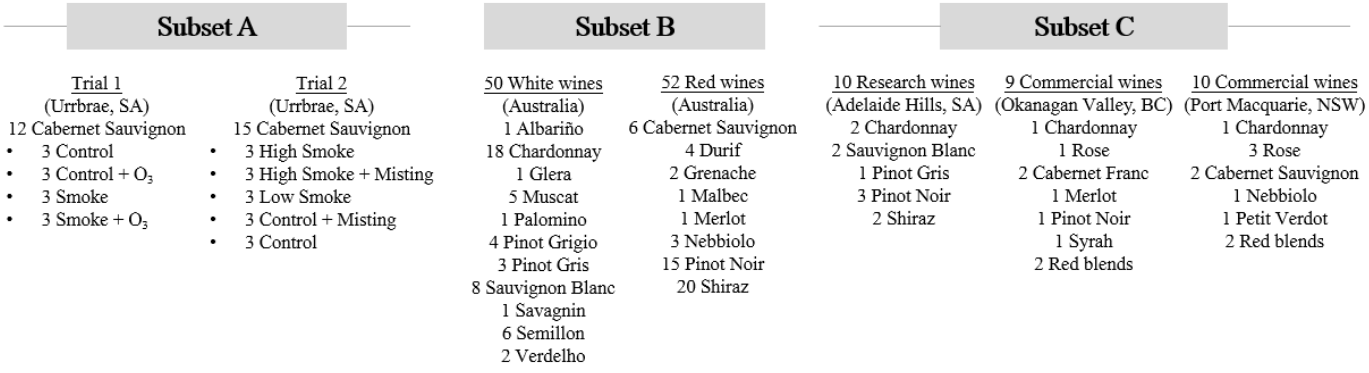
2.5 Data analysis

Samples were split into Subset A, Subset B, and Subset C (**Figure 1**) in order of increasing complexity, which refers to the diversity of variables that drive potential differences between the spectra of each sample. In Subset A, all wines were of the same varietal and made with identical winemaking techniques; thus, any differences between them reflected experimental

treatments or smoke exposure. On the contrary, Subset B and Subset C were considered more complex because they included wines of different varietals that were sourced from several regions. But most importantly, all wines in Subset B and Subset C were commercial wines made from grapes exposed to real bushfire smoke. Subset C was considered more complex than Subset B due to its inclusion of wines from outside of Australia. Collinearity between variables was explored within each subset, using Pearson's correlation analysis. Regression models were developed to assess the feasibility of spectral data to predict the concentrations of key indicators of smoke taint in each subset.

For Subset A wine, extreme gradient boosting regression (XGBR) models were used to predict volatile phenol concentrations. Boosting parameters including tree depth, learning rate, and the number of rounds, were optimised, whereas regularisation parameters (i.e. alpha, lambda, and gamma) were held constant. Boosting works by converting weak learners into strong ones by building them in succession, each one learning from the mistakes of the previous tree. Tree depth must match the complexity of the dataset because if trees are too deep or too shallow, it increases the risks of overfitting or underfitting, respectively. The number of rounds refers to the number of grow-and-boost cycles, and the learning rate dictates the speed at which the cycles occur.

Figure 1. Partition of wine into subsets (in order of increased complexity) used to develop extreme gradient boosting regression (XBGR) and partial least squares regression (PLSR) models that assess the feasibility of fluorescence spectroscopy as a rapid tool to predict volatile phenol and glycoconjugate concentrations in wine



Raw spectra from Subset A wine were compressed with partial least squares (20 latent variables) and pre-processed with different combinations of decluttering methods, centring (class centroid, multiway, median, mean), and scaling (Poisson or Pareto) (**Supplemental Table 1**). Compression can be performed with principal component analysis or partial least squares, and this technique reduces data dimensionality to improve model robustness and computational efficiency. Mean centring provides each variable with an average value of zero to emphasise differences between samples rather than their similarities (van den Berg *et al.* 2006). Scaling divides all values with a constant factor to grant each variable a more equitable chance of influencing the model, regardless of its absolute intensity (van den Berg *et al.* 2006). Decluttering methods such as general least squares weighting, external parameter orthogonalisation, or external mixture model filtering operate on the premise that samples with similar y-block data (e.g. concentration) should have similar spectra. Depending on the algorithm, clutter is either weighted to reduce its impact on the model (as in generalised least squares weighting) or removed from spectra (as in external parameter orthogonalisation and extended mixture model filtering), and the degree of decluttering must be balanced by the risk of removing variance related to the signal of interest (Eigenvector Research, 2021).

The pre-processing strategies for each regression model were determined with a combined visual inspection and trial-and-error approach (Engel *et al.* 2013). The effects of pre-processed spectra were first visualised alongside the raw spectra, and a pre-processing step was chosen if it appeared to improve the normality of signal distribution, reduce noise, or both. Once a strategy was chosen, calibration models were built and cross-validation was performed. The success of a strategy was evaluated with root mean square error values during the cross-validation stage.

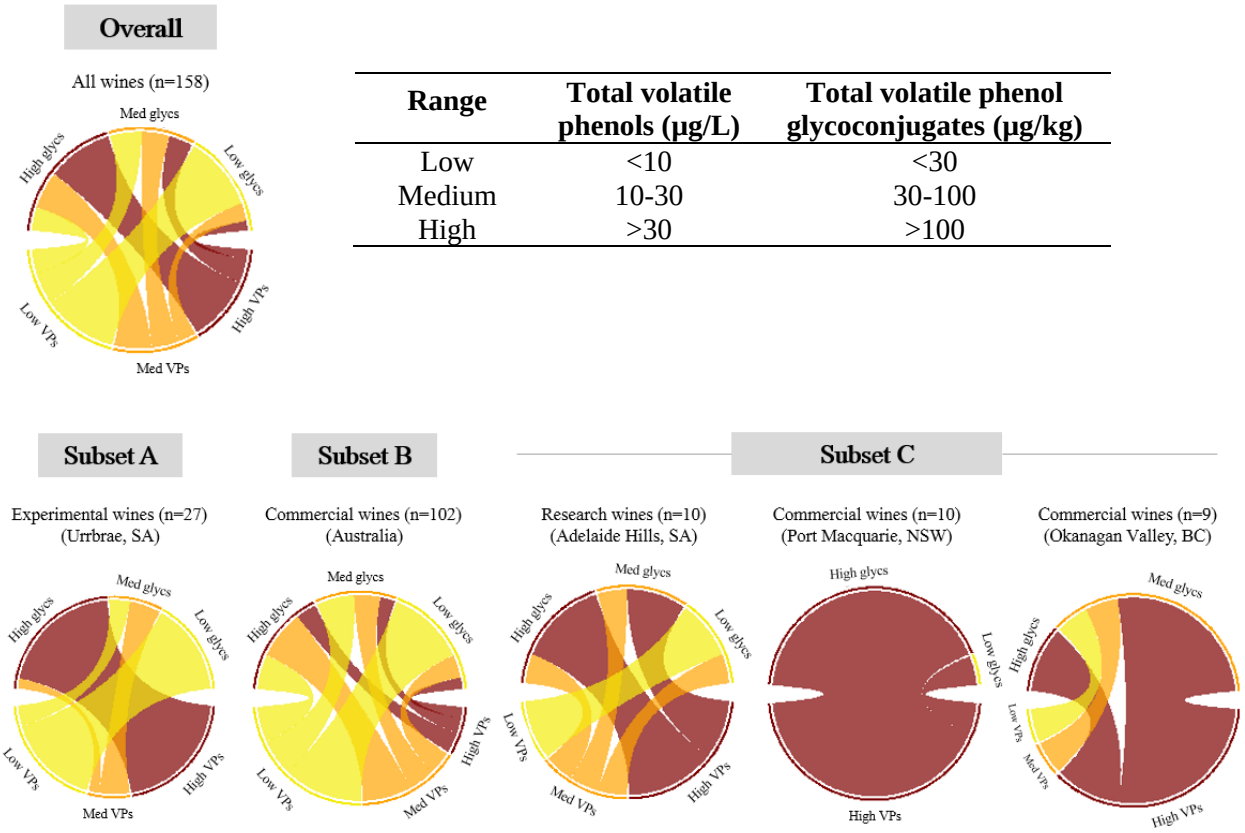
Cross-validation of the XGBR model was performed with *k*-fold cross validation was performed using Venetian blinds (10 splits, blind thickness = 1). In *k*-fold cross-validation, the data are divided into *k* subsets, and the model is calibrated with *k*-1 subsets, after which it is validated with the subset not involved in model building. This process is reiterated until all subsets have been held out (Kuhn and Johnson 2013). Leave-one-out-cross-validation is a special case of *k*-fold cross-validation that operates by calibrating the model with all but a single sample and using the excluded sample as the validation set. Leave-one-out-cross-validation runs until each sample has been excluded once, and it has been used in previous work (Fudge *et al.* 2012). However, we selected *k*-fold cross-validation over leave-one-out-cross-validation because the former is more suitable to a dataset with over 50 samples

(Hawkins 2004). Moreover, both methods generate comparable assessments of model performance and *k*-fold cross-validation conducts the assessment with greater computational efficiency (Kuhn and Johnson 2013).

Prediction accuracy of the XGBR model was assessed using a test set, comprised of one replicate of each experimental wine. These samples were held out from the calibration and cross-validation stages of modelling. Accuracy was evaluated with the root mean square error and the correlation coefficient values. These parameters, in addition to the bias of the model, were used to assess overfitting. Bias refers to the difference between true and predicted values. Over-fit models closely approximate the relationship between the predictors and the outcomes of the samples used to train them, and they are unable to generalise beyond these samples because their performance is unstable beyond the recognition pattern developed during model training (Kuhn and Johnson 2013). Over-fit models are characterised by poor prediction accuracy and very low bias.

For Subset B wine, an extreme gradient boosting regression (XGBR) model was used to predict volatile phenol and glycoconjugate concentrations. This model was defined by a learning rate of 0.5, a maximum depth of 3, and 300 rounds. Raw spectra compressed with PLS (25 latent variables) and pre-processed with EMM filtering and mean centring. Model performance was assessed using *k*-fold cross validation with Venetian blinds (10 splits, blind thickness = 1) and evaluated with RMSE and R^2 values at the cross-validation stage, rather than a single test set. The choice of a representative test set for the (Subset B) XGBR models was complicated by the contrasting diversity (from a regional and varietal perspective) and uniformity (from a volatile phenol/glycoconjugate perspective) that defined the samples. Many wines were defined by low concentrations of volatile phenols (0-10 µg/L) and low glycoconjugates (0-30 µg/kg) (**Figure 2**), and if split, it would not be reasonable to expect a model primarily trained with minimally affected wines to extrapolate to heavily smoke-affected wines. As a result, the Subset B model was evaluated with a resampling technique, *k*-fold cross-validation.

Figure 2. Chord diagrams depicting the diversity of volatile phenol ("VP") and glycoconjugate ("Glyc") profiles that constitute the wines within each subset. The nodes correspond to volatile phenol and glycoconjugate concentrations categorised into low, medium ("Med") and high ranges. The thickness of the bands in each diagram demonstrate the frequency of the pairings within each group of wine.



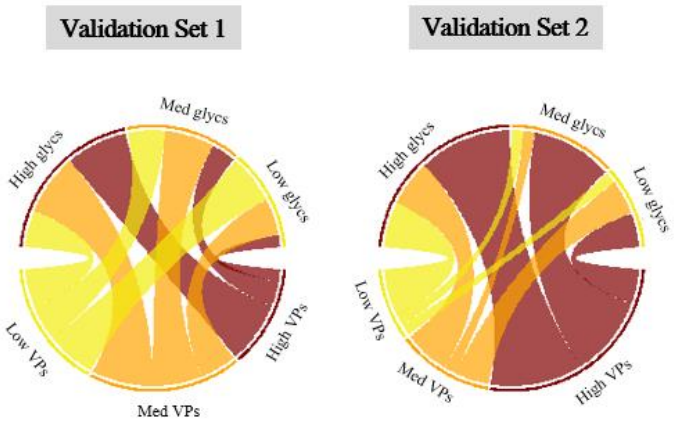
Range	Total volatile phenols (µg/L)	Total volatile phenol glycoconjugates (µg/kg)
Low	<10	<30
Medium	10-30	30-100
High	>30	>100

Subset C wines were modelled together with some Subset A wine (i.e. Trial 2) and Subset B wine, comprising of 146 wines. For this group of wines, an XGBR model and a PLSR model were used to predict guaiacol concentrations. The XGBR model (eta = 0.1, max depth = 6, number of rounds = 300) was constructed with raw spectra compressed with PLS (5 latent variables) and pre-processed with mean centring and Pareto scaling. The PLSR model was built as a conventional linear comparison, with 5 latent variables. The tuning parameters and pre-processing steps for the XGBR and PLSR models were held constant across the various iterations of each model. These models were built to evaluate the performance of XGBR relative to PLSR and the effect of different data splits and cross-validation strategies on critical indicators of model performance. Models were limited to guaiacol to prioritise the depth of our assessment over its breadth.

To assess how much model performance depended on how data were split, three splits were examined. In the first way, the data were not split into calibration and validation sets and all samples were included in model calibration and evaluated with *k*-fold cross-validation. These models were evaluated with the RMSE and R^2 values from the cross-validations stage. In the second way, samples were manually split into 232 calibration and 60 validation samples, and in the third way, samples were manually split into 228 calibration and 64 validation samples. These models were evaluated with the RMSE and R^2 values of the test set.

Figure 3. Chord diagrams depicting the diversity of volatile phenol and glycoconjugate profiles that constitute the validation wines used to assess the performance of (Subset C) XGBR and PLSR models. The nodes correspond to volatile phenol and glycoconjugate concentrations categorised into low, medium ("Med") and high ranges. The thickness of the bands in each diagram demonstrate the frequency of the pairings within each validation set.

Range	Total volatile phenols (µg/L)	Total volatile phenol glycoconjugates (µg/kg)
Low	0-10	0-30
Medium	10-30	30-100
High	30+	100+



Volatile phenol and glycoconjugate profiles (**Figure 2**) were used as the central metrics to decide which samples were included in each test set. The first test set was built to mirror the composition of the calibration set. This first set was defined by an equal distribution of samples across volatile phenol and glycoconjugate concentration ranges, and constituted of 8, 12, and 10 wines with high, medium, and low volatile phenol concentrations, respectively (**Figure 3**).

The second test set was defined by wines with high volatile phenols and glycoconjugates, including 18, 8, and 6 wines with high, medium, and low volatile phenol concentrations, respectively.

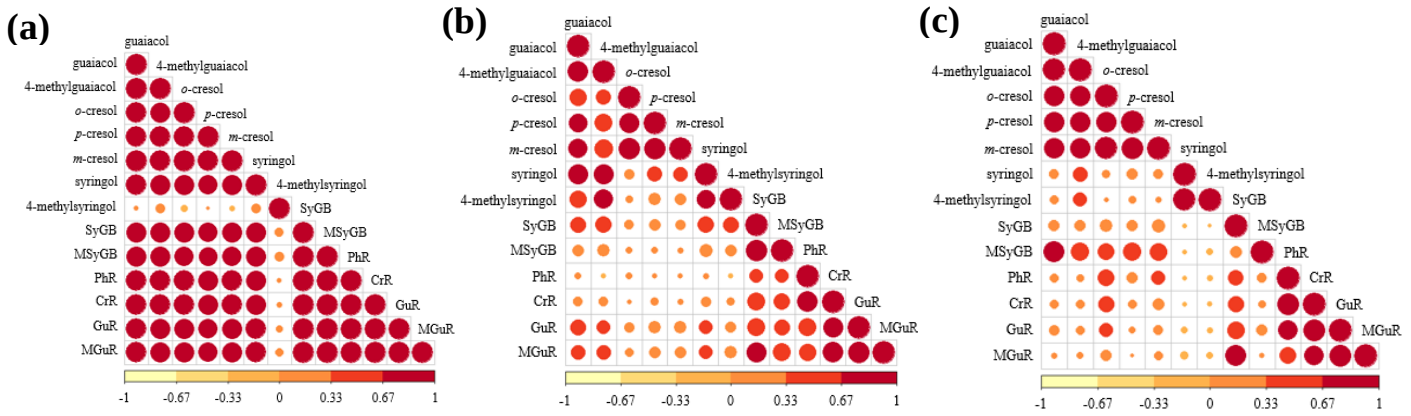
An extreme gradient boosting discriminant analysis (XGBDA) model was also built to predict the classification of wines into low-, medium-, and high-risk groups based on ranges established previously (Scrimgeour *et al.* 2021). Wine samples were categorised based on total volatile phenol glycoconjugate concentrations, in which the range of <30 µg/L was low-risk, 30-100 µg/L was medium-risk, and >100 µg/L was high-risk. The XGBDA model was defined by a learning rate of 0.1, 500 rounds, and a tree depth of 3. Raw spectral data were compressed with PLS (5 latent variables) and pre-processed with mean centring and Pareto scaling. The XGBDA model was built and evaluated by dividing data into the same 228 calibration and 64 validation split (as above). This split led to 76, 70, and 82 samples classified as low-, medium-, and high-risk, respectively in the calibration set and 14 (low-risk), 18 (medium-risk), and 32 (high-risk) samples in the validation set.

3. Results

Regression models were built to assess the feasibility of fluorescence spectroscopy as a tool to predict volatile phenol and glycoconjugate concentrations in a diverse array of experimental, research, and commercial wines. Subset A comprised the experimental wines, all of which were Cabernet Sauvignon wines that differed primarily as a function of grapevine smoke exposure. Subset A wines were characterised by samples with either high total volatile phenols and high total glycoconjugates or with low total volatile phenols and low total glycoconjugates (**Figure 2**). Thus, it was not surprising that volatile phenol and glycoconjugate concentrations in Subset A wines demonstrated high collinearity. The 25th percentile of Pearson correlation scores was 0.85, indicating that only 25% of correlations fell below this score. This represents a strong positive relationship between variables. The correlation plot in **Figure 4** demonstrates that compounds were highly correlated within and between volatile phenols and glycoconjugates. The only exception to this trend was 4-methylsyringol, which did not correlate well with any other compounds due to its trace levels (<1 µg/L) across wines, even in those wines made from heavily smoke-affected grapes.

Figure 4. Collinearity between volatile phenols and glycoconjugates in (a) Subset A, (b) Subset B, and (c) Subset C wine, as demonstrated by correlation diagrams and Pearson's coefficient values. Pearson's correlation coefficient values were computed with volatile phenol and glycoconjugate concentrations in Subset A (n=27), Subset B (n=102) and Subset C wines (n=29). Data are presented as percentiles, in which the n^{th} percentile corresponds to the value at which *n* percent of values are below it.

Subset	25th Percentile	50th Percentile	75th Percentile	100th Percentile
A	0.85	0.90	0.93	0.99
B	0.18	0.37	0.61	0.88
C	0.11	0.24	0.62	0.94

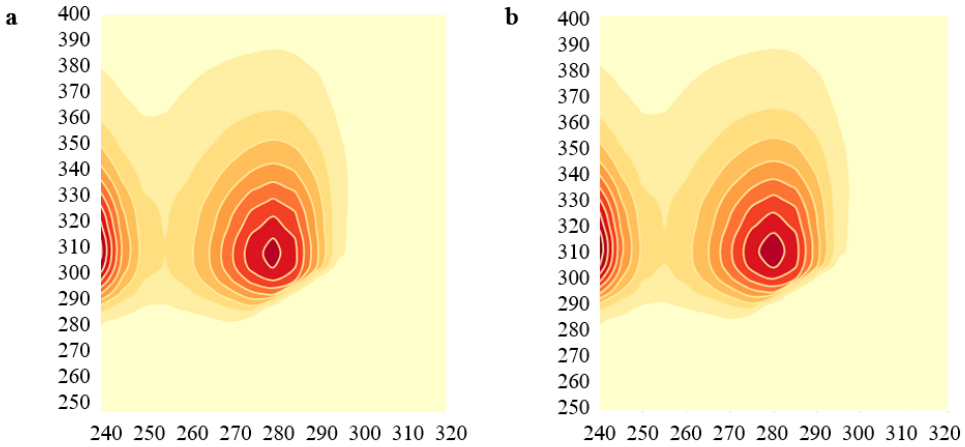


Subset B comprised the commercial wines from Australia, and they were more diverse than the Subset A wines with respect to wine varietal and region, as well as their levels of smoke taint. Subset B wines had volatile phenol and glycoconjugate profiles that tended to be skewed toward the low to middle ranges **(Figure 2)**. A large share of the Subset B wine (n=52 out of 102) was characterised by low total volatile phenols (0-10 µg/L), half of which were further defined by low total volatile phenol glycoconjugates (0-30 µg/kg). Volatile phenol and glycoconjugate concentrations in Subset B wines did not show as much collinearity as observed in those in Subset A wines. In Subset B, the 75th percentile of Pearson correlation scores was 0.61, indicating that the vast majority of scores fell below r=0.61, a moderate positive relationship between variables. This can be attributed to the poor correlations between volatile phenols and glycoconjugates (**Figure 4**). The highest correlation between a volatile phenol and glycoconjugate was r=0.50 (guaiacol and guaiacol rutinoside), but the average coefficient was 0.22 ± 0.02 (SEM).

Subset C comprised the commercial and research wines, and they were characterised by medium (10-30 µg/L) and high (>30 µg/L) volatile phenol concentrations (**Figure 2**). Volatile phenol and glycoconjugate concentrations in Subset C wines demonstrated high collinearity among volatile phenols, with the exception of syringol and 4-methylsyringol, which did not correlate well with any other compounds. The glycoconjugates also demonstrated high collinearity, with the exception of MSyGB, which correlated well with volatile phenols rather than other glycoconjugates. The highest coefficients achieved between volatile phenols and glycoconjugates ranged from r=0.57 to r=0.81), which corresponded to MSyGB and guaiacol, 4-methylguaiacol, *o*-cresol, *m*-cresol, and *p*-cresol.

The Subset A and Subset B XGBR models were built to explore the feasibility of fluorescence spectroscopy as a tool to predict volatile phenol and volatile phenol glycoconjugate concentrations in increasingly complex wine matrices. As a preliminary step, the excitation-emission matrices of Subset A wine were averaged as a function of smoke exposure and plotted. As expected, minimal differences were observed in the fluorescence spectra (**Figure 5**), and the primary activity, at 280 nm (excitation) and 315 nm (emission) can be attributed to flavan-3-ols such as catechin and epicatechin (Airado-Rodríguez *et al.* 2009; Ranaweera *et al.* 2021). This justifies the need for multivariate analysis to extract the data from the spectra relevant to smoke-derived volatile phenols and glycoconjugates.

Figure 5 Averaged excitation-emission matrix (EEM) fingerprints corresponding to experimental wines affected (**a**) and unaffected (**b**) by smoke exposure. The axes correspond to excitation (x-axis) and emission (y-axis) wavelengths (nm).



The Subset A XGBR models were built to predict volatile phenol concentrations in experimental wines, and they were assessed by the RMSE and R^2 generated from an independent test set. The calibration and cross-validation stages demonstrated promising results, with high R^2 values, low RMSE, and low model bias (**Table 1**). At the prediction stage, R^2 values demonstrated a slight decrease from nearly R^2=1 to R^2 = 0.86, but order-of-magnitude increases were observed in RMSE and bias values.

A similar pattern was observed with the Subset B XGBR model, which was built to predict volatile phenols and glycoconjugates in a set of commercial wines. The Subset B XGBR model was assessed by the R^2 and RMSE values generated from *k*-fold cross-validation. At the calibration stage, R^2 =1, which indicates a perfect correlation between the spectral data and chemical concentrations. The RMSE values ranged from 5.8E-4 to 9.6E-4 and the model bias was even lower, in the range of 10^{-6} to 10^{-8}. At the cross-validation stage, correlation coefficients remained high, ranging from R^2 = 0.9634 to R^2 = 0.9997, yet the RMSE and model bias also demonstrated order-of-magnitude increases (**Table 2**).

Table 1. Results of extreme gradient boosting regression (XGBR) models built to predict **volatile phenol** concentrations in Subset A wines[1]. Model performance measures included R^2 (coefficient of correlation), RMSE (root mean square error), and bias (difference between actual and observed values) at the calibration (Cal), cross-validation (CV) and prediction (P) stages of development. Refer to **Supplementary Table 1** for pre-processing options and XGBR tuning parameters used for each model.

	R^2			**RMSE**			**Bias**		
Compound	**Cal**	**CV**	**P**	**Cal**	**CV**	**P**	**Cal**	**CV**	**P**
guaiacol	1	1	0.78	1.8E-4	2.2E-4	6.24	-1.6E-7	1.7E-5	0.81
4-methylguaiacol	1	0.92	0.94	1.9E-7	5.8E-1	0.54	8.1E-8	2.9E-2	0.22
o-cresol	1	1	0.87	4.4E-3	4.3E-2	1.57	-4.1E-7	4.8E-3	-0.45
p-cresol	1	1	0.88	1.6E-4	2.0E-4	1.03	-2.3E-7	-1.3E-5	0.70
m-cresol	1	1	0.96	1.8E-4	2.8E-4	0.98	-4.0E-7	-8.3E-5	0.13
syringol	1	0.93	0.86	1.4E-3	5.0E-1	0.69	7.7E-8	6.9E-2	-0.34

Table 2. Results of an extreme gradient boosting regression (XGBR) model[1] built to predict **volatile phenols** and **glycoconjugate** concentrations in Subset B wines[2]. Model performance measures included R^2 (coefficient of correlation), RMSE (root mean square error), and bias (difference between actual and observed values) at the model calibration (Cal) and cross-validation (CV) stages of development.

	R^2		RMSE		Bias	
Compound	**Cal**	**CV**	**Cal**	**CV**	**Cal**	**CV**
guaiacol	1	0.996	7.2E-4	0.68	-1.2E-7	-0.15
4-methylguaiacol	1	0.998	5.9E-4	0.12	-3.5E-7	-0.02
o-cresol	1	0.990	6.1E-4	0.11	1.5E-7	-0.02
p-cresol	1	0.963	6.3E-4	0.33	-1.9E-8	-0.11
m-cresol	1	0.991	6.2E-4	0.15	-6.4E-8	-0.04
syringol	1	0.993	9.6E-4	1.95	-1.3E-6	0.18
4-methylsyringol	1	1.000	6.2E-4	0.15	6.1E-8	0.01
CrR	1	0.980	5.8E-4	1.48	1.3E-8	-0.42
GuR	1	0.989	7.0E-4	1.27	-2.9E-8	-0.34
MGuR	1	0.983	6.9E-4	1.55	-3.1E-7	-0.46
MSyGB	1	0.996	7.0E-4	0.78	-3.6E-7	-0.16
PhR	1	0.991	6.6E-4	0.86	-1.6E-7	-0.23
SyGB	1	0.9848	6.84E-4	9.03	5.4E-8	-0.75

The Subset C XGBR and PLSR models were built to predict guaiacol concentrations across a diverse set of commercial, experimental, and research wines. These models were first evaluated using *k*-fold cross-validation and no data spilt, after which they were evaluated with independent test sets derived from different data splits (**Table 3**). When the models were evaluated with *k*-fold cross-validation, the XGBR model (R^2=0.95) performed better than the PLSR model (R^2=0.84) with respect to R^2 values, but RMSE was 3.79 for both models. When the models were evaluated with independent test sets, the predictive accuracy of the XGBR and PLSR models depended on how the data were split.

Using the first data split, the XGBR model demonstrated higher R^2 values and lower RMSE values than the PLSR model during the calibration and cross-validation stages. However, these promising results were negated by the prediction stage, at which both models deteriorated, with R^2 values <0.1 and high RMSE values of 16.07 and 18.43 for XGBR and PLSR, respectively. Using the second data split, high, comparable R^2 of prediction values were achieved for both models, with XGBR at $R^2 = 0.86$ and PLSR at $R^2 = 0.85$. RMSE of prediction values were at 4.84 and 5.42 for XGBR and PLSR, respectively. These values were significantly different to those observed with the previous (232/60) split.

Table 3. Comparison of results from extreme gradient boosting regression (XGBR) and partial least squares regression (PLSR) models for predicting **guaiacol** concentration in a diverse array of wines (n=146), using different data splits (Split 0 = no split, Split 1 = 232 calibration/60 validation, Split 2 = 228 calibration/64 validation). The wines included in each model were derived from Subset C (n=29), Subset B (n=102) and Subset A (n=15). Model performance measures include R^2 (coefficient of correlation), RMSE (root mean square error), and bias (difference between actual and observed values) at the model calibration (Cal), cross-validation (CV), and prediction (P) stages of development. All models were compressed with PLS (5 latent variables) and pre-processed with mean centring and Pareto scaling.

		R^2			RMSE			Bias		
Split	**Type**	**Cal**	**CV**	**P**	**Cal**	**CV**	**P**	**Cal**	**CV**	**P**
0	XGBR	1	0.95	-	0.017	3.79	-	2.5E-5	-0.06	-
1	XGBR	1	0.84	1.2E-7	0.009	7.65	16.07	-4.1E-6	-0.42	3.05
2	XGBR	1	0.98	0.86	0.002	2.17	4.84	1.7E-6	0.01	1.78
0	PLSR	0.87	0.84	-	5.10	3.79	-	-5.3E-15	0.15	-
1	PLSR	0.66	0.55	0.02	11.09	12.91	18.43	0.00	0.04	3.45
2	PLSR	0.89	0.87	0.85	4.87	5.39	5.42	3.6E-15	0.06	0.31

Table 4. Confusion matrices for Subset C XGBDA model built to predict whether a wine has high, medium, or low risk of smoke taint, based on total glycoconjugate concentrations, using the prediction rule=most probable. Low <30 µg/L, medium = 30-100 µg/L, and high = >100 µg/L of total glycoconjugates, evaluated at the (a) cross-validation and (b) prediction stages.

(a) Cross-validation stage

	Predicted				
Actual	High	Medium	Low	Total	Correct
High	75	2	5	82	91%
Medium	3	58	7	68	85%
Low	5	3	66	74	89%

(b) Prediction stage

	Predicted				
Actual	High	Medium	Low	Total	Correct
High	12	6	6	24	50%
Medium	12	4	2	18	22%
Low	6	2	4	12	33%

The Subset C XGBDA model was built to predict the classification of wines into high-, medium-, or low-risk groups, based on their total volatile phenol glycoconjugate concentrations. The XGBDA model was evaluated using an independent test set, with the data split into 228 calibration and 64 validation samples. Like previous regression models, the results at the cross-validation stage were promising, as shown by high classification accuracy, which ranged from 85% to 91% across classes (**Table 4**). At the cross-validation stage of the Subset C XGBDA model, both false negatives and false positives were present, but the rate of the former was higher than the latter. False negatives were identified for 9-15% of samples, whereas false positives were identified for 6-8% (**Supplemental Table 2**). Weaknesses in the model evident at the cross-validation stage were exacerbated when the model was evaluated with the test set, in which classification accuracy fell to 50% (high-risk), 22% (medium-risk), or 33% (low-risk) (**Table 4**). Overlap was anticipated between neighbouring classes, as reflected by 67% of medium-risk samples being misclassified as high-risk and 25% of high-risk samples being misclassified as medium-risk. However, there were also high-risk wines that were misclassified as low-risk wines and vice versa.

4. Discussion

Non-linear regression models were developed to predict volatile phenols and glycoconjugates in wine using fluorescence spectroscopy and classify wines made from grapes with high, medium, and low levels of smoke exposure. For regression, the preliminary Subset A and Subset B XGBR models showed encouraging results, in contrast to the final Subset C XGBR

model, the performance of which depended strongly on how it was evaluated. For classification, the Subset C XGBDA model demonstrated promising results at the cross-validation stage, but performance declined when evaluated with an independent test set. These results highlight the challenges associated with extracting variation in the spectral data relevant to smoke exposure and balancing the trade-offs between model accuracy and generalizability.

Different levels of collinearity were observed in each subset of wine, with Subset A demonstrating the highest levels (**Figure 4**). Volatile phenols and glycoconjugates were present in Subset A wine in an 'all-or-nothing' fashion due to the experimental conditions used to produce them. The Cabernet Sauvignon grapes used to make the Subset A wines were sourced from neighbouring vines, defined by a low natural abundance of volatile phenols and glycoconjugates, and exposed to an intense, hour-long dose of smoke from a consistent fuel source at ~7 days post-véraison (Modesti *et al.* 2021; Szeto *et al.* 2020). Subsequent wines were then bottled without any oak maturation.

On the other hand, the grapes used to make Subset B and Subset C wine were sourced from different vineyards, comprising unknown natural levels of volatile phenols and glycoconjugates (prior to smoke exposure), and exposed to unknown levels of bushfire smoke (as a function of smoke density and duration of exposure). Moreover, red wine from Subset B and Subset C may have received oak treatment during fermentation or aging; however, maturation details were not specified. Previous work has also shown that aside from grapevine smoke exposure, some volatile phenols can also be present in grapes as natural metabolites (at different levels depending on variety) and in wines following extraction from oak barrels during aging (Pollnitz *et al.* 2004; Ristic *et al.* 2015).

The exact mechanisms underpinning the formation of volatile phenols in grapevines are not known, but the current hypothesis is that they are produced as artefacts of the shikimic acid pathway (Dunlevy *et al.* 2009; as described in Noestheden *et al.* 2018). When volatile phenols accumulate in grapes and wine under these circumstances (independent of smoke exposure), the biochemical drive to glycosylate them may be reduced, relative to when grapevines are imbued with volatile phenols during high levels of dense smoke exposure. It is well established that when plants are exposed to an abiotic stress, glycosylation is stimulated as a detoxification response (Dudavera *et al.* 2004); albeit only a few studies have examined the specific effects of smoke exposure on grapevines at the transcriptomic (van der Hulst 2018) and physiological levels (Bell *et al.* 2013; Ristic *et al.* 2016). The experimental conditions used to produce the

smoke-affected Subset A wine might have limited the confounding sources of volatile phenols and better preserved the link between smoke-derived volatile phenols and the glycosylation response connected with it, whereas in Subset B and Subset C wine, this relationship may have been influenced by other confounding sources of volatile phenols.

The higher levels of collinearity between volatile phenols and glycoconjugates in Subset A wine may also have been influenced by the timing, high density, and acute nature of smoke exposure that characterised experimental conditions. If grapevines are exposed to smoke towards maturity and immediately processed, there may not be sufficient time for the accumulation of glycoconjugates, which can take 2 weeks or longer prior to stabilisation (van der Hulst *et al.* 2019; Szeto *et al.* 2020). Further examination of the temporal dependence of key smoke taint markers is required to optimise the timing of diagnostic assessments relative to vineyard smoke exposure.

The results from the Subset A XGBR models demonstrated the potential for fluorescence spectroscopy to be used to predict volatile phenol concentrations with some accuracy (**Table 1**); however, the high degree of similarity between the training set used to build the model and the test set used to validate it, suggest that the results may be overoptimistic. Subset A comprised 9 Cabernet Sauvignon wines sourced from grapes in the same vineyard row, fermented (in triplicate) under minimalistic conditions (i.e. no oak or malolactic fermentation), and scanned in triplicate. The ensuing Subset A XGBR models were built with 81 EEMs. The three scans corresponding to each wine were not separated across training and test sets, but this minor precaution did not compensate for the considerable similarity between wines nor the subsequent inadequacy of the test set as an indication of model performance. An alternative approach would have been to average the values corresponding to replicate scans before performing data splits (Logan *et al.* 2020); however, if this approach had been used, then sample number would have become a limiting factor to model building. While training and test samples must be from the same population, it is critical that the test set presents a sufficient challenge to the calibrated model. The unchallenging nature of the test set may have generated overoptimistic R^2 and RMSE values (**Table 1**).

The results of the Subset B XGBR models were also promising for the prediction of volatile phenols and glycoconjugates (**Table 2**); however, differences in model bias and RMSE values between the calibration and cross-validation stages suggest that these results may have been attributed to overfitting. Over-fit models closely approximate the relationship between

the predictors and the outcomes of the samples used to train them, but they are unable to generalise beyond these samples because their performance is unstable beyond the recognition pattern developed during the training phase (Kuhn and Johnson 2013). Overfitting is quantified with bias, which reflects the differences between true values and values predicted by the model. When a model is over-fit, model bias is extremely low. At the calibration stage, the Subset B XGBR models had extremely low bias levels in ranges from 10^{-6} to 10^{-8}.

Overfitting was likely attributed to retaining 25 latent variables from the compression of the spectral data with partial least squares. Rather than fit a model to raw data, compression is used as a tool to reduce data dimensionality and improve model robustness and computational efficiency. If a greater number of latent variables (or components) is retained, data with greater complexity will be generated. In a conventional regression technique such as PLSR, the number of latent variables is the principle tuning parameter. The number of latent variables that should be included in a model is based on the proportion of variance that is accounted for by each cumulative component, relative to RMSE values during cross-validation. The evident criteria used to choose the number of latent variables in a PLSR setting is in stark contrast to performing the same task in an XGBR setting.

In XGBR, each latent variable is given a variable importance score that describes its significance to the constructed ensemble of trees. This significance is quantified as the gain, and it reflects how much the model residuals were reduced due to the inclusion of that latent variable. The reduction is calculated across all of the trees in the ensemble. As the number of latent variables increases, the maximum significance attributed to the most important variable decreases, but unlike PLSR, the variable that contributes the most gain is not necessarily the first latent variable, nor is it consistent across XGBR models built with a different number of variables. The algorithms that optimise the complexity of XGBR models in Solo consider the learning rate, number of iterations, tree depth, and XGBR regularisation parameters; however, the algorithms do not consider the complexity of the incoming data used to train the trees. Short of an exhaustive manual evaluation, it was not clear how to find the optimal number of latent variables to retain in an XGBR model with respect to the complexities defining the XGBR model and the compressed spectral data used to train it. Herein, the optimal number of latent variables chosen for the Subset A and Subset B models was selected using R^2 and RMSE values at the cross-validation stage, but these metrics provide minimal insight on the degree of model generalizability. As such, it is possible that the optimal combination of PLS components and XGBR tuning parameters was not obtained.

For the Subset C wines, the predictive performance of XGBR and PLSR models were compared. The principle of parsimony prompts the selection of models that feature only what is necessary. If a relationship can be adequately captured with a linear model (e.g. PLSR), this principle obliges us to choose it over a more complex nonlinear model (e.g. XGBR), which can be too flexible, include irrelevant variation, or both (Hawkins 2004). The results of the Subset C XGBR and PLSR models demonstrated that performance was driven primarily by how the data were split and evaluated rather than the complexity of the multivariate approach.

When no split was performed and the model was evaluated with *k*-fold cross-validation, R^2 values of 0.95 and 0.84 were achieved for the Subset C XGBR and PLSR models, respectively (**Table 3**). Considering our aim to develop fluorescence spectroscopy as a screening tool, these values would be acceptable. When the data were evaluated using independent splits of the data, the first split (232 calibration/60 validation) suggested a weak relationship between spectral data and guaiacol concentrations (R^2 = 1.2E-7 (XGBR), R^2=0.02 (PLSR)), whereas the second split (228 calibration/64 validation) indicated the opposite (R^2=0.85 (PLSR), R^2=0.86 (XGBR)). The performance of a model can vary substantially when assessed by an independent data set due to its dependence on what samples constitute the set (Kuhn and Johnson 2013; Lia *et al.* 2018). However, the overt differences observed herein as a function of data split (**Table 3**) suggest that the Subset C models did not adequately capture the relationship between guaiacol and fluorescence spectral data.

The work conducted in the present study cannot reconcile the inconsistent results observed in the Subset C XGBR and PLSR models, but it is likely a consequence of the larger influence of other fluorophores within the spectra. This obstacle has been encountered in previous work which trialled the use of MIR spectra to classify smoke-affected wine (Fudge *et al.* 2012). Therein, the classification accuracy of an LDA model built with MIR data was compromised when evaluating wines sourced from a trial that featured seven varietals. This was not a surprising result because the PCA scores used to train the model reflected varietal-driven differences. In the present work, we used PLS as a data reduction strategy, but this measure may not have been sufficient to overcome the minimal impact that the volatile phenols and glycoconjugates had on the fluorescence data (**Figure 5**).

The diversity associated with region and grape variety was acknowledged, but it was perceived as a challenge that could be overcome with a non-linear method coupled to a supervised data reduction method. In fact, regional and varietal diversity was viewed as a

means of improving the robustness of the developed models, but the primary fluorophores in wine are also sensitive to grape varietal and region, to the extent that they can be used for authentication (Airado-Rodríguez et al. 2011; Versari *et al.* 2014; Ranaweera *et al.* 2021). Moreover, other fluorophores in wine have higher quantum yields, concentrations, and variability across wine samples relative to volatile phenols and glycoconjugates. Despite awareness of this challenge *a priori*, this work did not address it with a proof-of-concept spiking study.

A proof-of-concept spiking study would involve spiking volatile phenols and glycoconjugates into increasingly complex wine matrices to enable the identification of regions associated with volatile phenols and glycoconjugates in the fluorescence spectra. This proof-of-concept spiking study would offer guidance regarding the efficacy of data reduction techniques and pre-processing strategies on the preservation of those regions in the increasingly complex matrices. Introducing varietal and regional variation into models would be performed only after demonstrating that the quantitation of volatile phenols and glycoconjugates with spectral data could be performed with high linearity and reproducibility in model wine and cask wine. The absence of a proof-of-concept spiking study is a limitation of the present study, and will be addressed in future work.

For the Subset C XGBDA models, wines were divided into low-, medium-, and high-risk categories based on total volatile phenol glycoconjugate concentrations rather than total volatile phenol concentrations. These criteria were adopted from a previous study involving the classification of smoke-affected wines based on MIR data (Scrimgeour *et al.* 2021). Therein, accuracy of the model was higher when predicting classification based on total glycoconjugate concentrations than when they were categorised by total volatile phenols, albeit the highest accuracy was 69%, which was achieved for the prediction of low-risk wines. Volatile phenols have a natural abundance in grapevines that is varietal dependent and a significant abundance in oak, some of which can be extracted into wine during fermentation, aging, or both (Pollnitz *et al.* 2004; Ristic *et al.* 2016). While glycoconjugates also have a natural abundance in grapevines, there is greater confidence that elevated levels can be attributed to smoke exposure (Ristic *et al.* 2016). Thus, total glycoconjugates were used as a basis to classify wines into low-risk (<30 µg/L), medium-risk (30-100 µg/L) and high-risk (>100 µg/L) categories.

Given the variable results from the Subset C regression models, strong performance from the Subset C XGBDA model was not anticipated. The data split used to train and evaluate the model (228 calibration/64 validation) corresponded to the split (Split #2) that yielded optimistic Subset C XGBR and PLSR results (**Table 3**). While results from the Subset C XGBDA model were promising at the cross-validation stage, with accuracy levels >84% across classes, performance declined when the model was evaluated with an independent test set, as indicated by accuracy levels at 50% or lower (**Table 4**). The model had the lowest prediction accuracy for medium-risk wines, at 22%. The difficulty of accurately classifying medium-risk wines is corroborated by Scrimgeour *et al.* 2021, but in contrast to the present study, medium-risk wines were more likely to be misclassified as low-risk wines. In the context of smoke taint diagnostics, there are costs associated with both false positives and false negatives, but the relative cost of a false negative (e.g. misclassifying a high-/medium-risk sample as a low-risk sample) is greater than the cost of a false positive (e.g. misclassifying a low-risk sample for a high-/medium-risk sample). As suggested in Scrimgeour *et al.* 2021, using a two-class model (i.e. high-risk and low-risk) and adjusting the cut-off criteria may improve model accuracy and provides a basis for future work.

5. Conclusion

There is an urgent need to develop rapid screening methods to facilitate time-sensitive decision-making in regards to smoke-affected grapes and wine. This work investigated the hypothesis that nonlinear models of fluorescence data could be used to: i) predict smoke-derived volatile phenols and glycoconjugates in wine and ii) classify smoke-affected wines into low-risk, medium-risk, and high-risk groups when coupled with a supervised data reduction technique. A diverse array of experimental, research, and commercial wines were collected and split into different subsets, for which various models were built. XGBR models built for Subset A and Subset B XGBR wines demonstrated promising results that fluorescence spectroscopy could be used to predict volatile phenols and glycoconjugates in wine. This was in contrast to the Subset C XGBR and PLSR models, which had variable performance that was strongly dependent on the samples used to train and evaluate them. Subset C XGBDA model demonstrated potential at the calibration and cross-validation stages, but its performance was not as strong at the prediction stage.

Based on the present results, the null hypothesis, which is that there are no differences in fluorescence spectra between wines with low, medium, and high levels of volatile phenols and glycoconjugates, cannot be rejected. Additional work is required to discern whether these results reflect the pre-processing or modelling strategies used to extract relevant information from the spectral data or the methods utilised to evaluate the performance of each model. It may also be that the effect of the volatile phenols and glycoconjugates on the fluorescence spectra is so minor relative to other more intense and variable fluorophores in wine, that no amount of pre-processing could isolate the relevant information without additional sample preparation prior to when samples are screened with fluorescence spectroscopy. This work highlights many of the challenges associated with this task and recommends that future studies perform a proof-of-concept spiking study prior to further investigation.

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Supplemental Data

Evaluation of fluorescence spectroscopy as a rapid diagnostic for predicting the risk of smoke taint in wine.

Colleen Szeto[1,2], Adam Gilmore[3], Neil Scrimgeour[4], Eric Wilkes[4], David Jeffery[1,2], and Kerry Wilkinson[1,2*]

[1]Department of Wine Science and Waite Research Institute, The University of Adelaide, Glen Osmond, SA 5064, Australia
[2]Australian Research Council Training Centre for Innovative Wine Production, Glen Osmond, SA 5064, Australia
[3]HORIBA Instruments Inc., 20 Knightsbridge Rd., Piscataway, NJ 08854, United States
[4]The Australian Wine Research Institute, Glen Osmond, SA 5064, Australia

***Corresponding author**: Prof. Kerry Wilkinson, telephone: +61 8 8313 7360, email: kerry.wilkinson@adelaide.edu.au

Supplemental Table 1. Pre-processing options and extreme gradient boosting regression (XGBR) model tuning parameters selected to predict **volatile phenol** concentrations in Subset A wines.

Volatile phenol	Spectral data		XGBR tuning parameters		
	PLS compression (Latent variables)	Pre-processing	eta	max depth	number of rounds
guaiacol	17	GLS weighting (α = 1e-07) Median centre	0.5	5	100
4-methylguaiacol	20	GLS weighting (α = 0.02) Poisson scaling	0.5	2	300
o-cresol	6	EMM filter (full rank) Class centroid centring Pareto scaling	0.5	2	300
p-cresol	2	EMM filter (full rank) Multiway centre	0.3	5	50
m-cresol	4	GLS weighting (alpha = 0.02) Multiway centre Pareto scaling	0.5	4	100
syringol	4	EPO/EMM Filter (20 PCs) Median centre Pareto scaling	0.05	4	500

Supplemental Table 2. Performance metrics of Subset C XGBDA model built to predict whether a wine has high, medium, and low risk of smoke taint, based on total glycoconjugate concentrations, using the prediction rule=most probable. Low <30 µg/L, medium = 30-100 µg/L, and high = >100 µg/L of total glycoconjugates, evaluated at the **(a)** cross-validation and **(b)** prediction stages. TPR = True positive rate, FPR = false positive rate, TNR = true negative rate, FNR = false negative rate, N = number of samples belonging to each class, Err = Misclassification error, P = precision, and F1 = F1 score. The calculations behind each value are shown below the tables.

(a) Cross-validation stage

Class	TPR (sensitivity)	FPR	TNR (specificity)	FNR	N	Err	P	F1
High	0.91463	0.05634	0.94366	0.08537	82	0.6696	0.90361	0.90909
Medium	0.85294	0.03205	0.96795	0.14706	68	0.06696	0.92063	0.88550
Low	0.89189	0.08000	0.92000	0.10811	74	0.08929	0.84615	0.86842

(b) Prediction stage

Class	TPR (sensitivity)	FPR	TNR (specificity)	FNR	N	Err	P	F1
High	0.50000	0.60000	0.40000	0.50000	24	0.55556	0.40000	0.44444
Medium	0.2222	0.2222	0.7778	0.7778	18	0.40741	0.33333	0.266667
Low	0.3333	0.19048	0.80592	0.6667	12	0.29630	0.33333	0.33333

$$True\ positive\ rate\ (TPR) = \frac{TP}{(TP + FN)}$$

$$False\ positive\ rate\ (FPR) = \frac{FP}{(FP + TN)}$$

$$True\ negative\ rate\ (TNR) = \frac{TN}{(TN + FP)}$$

$$False\ negative\ rate\ (FNR) = \frac{FN}{(FN + TP)}$$

$$Misclassification\ error\ (Err) = \frac{(FP + FN)}{(TP + TN + FP + FN)}$$

$$Precision\ (P) = \frac{TP}{(TP + FP)}$$

$$F1\ Score\ (F1) = \frac{(2 \times TP)}{(2\ \times TP + FP + FN)}$$

Chapter 5

Beyond Volatile Phenols: Revealing Additional Markers of Smoke Taint in Grapevines (*Vitis Vinifera* L.) cv. Merlot

Statement of Authorship

Title of Paper	Beyond volatile phenols: revealing additional markers of smoke taint in grapevines (*Vitis vinifera* L.) cv. Merlot
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	We aim to submit this manuscript for publication in *Analytica Chimica Acta* or *Food Chemistry*.

Principal Author

Name of Principal Author (Candidate)	Colleen Szeto
Contribution to the Paper	Conceptualization of the work / experimental design Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing
Overall percentage (%)	75
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third I this thesis. I am the primary author of this paper.
Signature	Date 3/3/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Natoiya Lloyd
Contribution to the Paper	Conceptualization of the work / experimental design Data organization, interpretation, and analysis Manuscript preparation and editing
Signature	Date 3/3/2022

Name of Co-Author	Luca Nicolotti
Contribution to the Paper	Conceptualization of the work / experimental design Data organization, interpretation, and analysis Manuscript preparation and editing
Signature	Date 03-03-2022

Name of Co-Author	Markus Herderich
Contribution to the Paper	Conceptualization of the work / experimental design Manuscript preparation and editing
Signature	Date 11/3/2022

Name of Co-Author	Kerry Wilkinson
Contribution to the Paper	Conceptualization of the work / experimental design Manuscript preparation and editing
Signature	Date 3/3/21

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Name of Co-Author			
Contribution to the Paper			
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Contribution to the Paper			
Signature		Date	

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Beyond volatile phenols: revealing additional markers of smoke taint in grapevines (*Vitis vinifera* L.) cv. Merlot

Colleen Szeto[1,2], Natoiya Lloyd[3,4], Luca Nicolotti[3,4], Markus Herderich[2,3,4], and Kerry Wilkinson[1,2]

[1]Department of Wine Science and Waite Research Institute, The University of Adelaide, Glen Osmond, SA 5064, Australia
[2]Australian Research Council Training Centre for Innovative Wine Production, Glen Osmond, SA 5064, Australia
[3]Metabolomics Australia, The Australian Wine Research Institute, Glen Osmond, SA 5064, Australia
[4]The Australian Wine Research Institute, Glen Osmond, SA 5064, Australia

*Corresponding author: Prof. Kerry Wilkinson, telephone +61 8 8313 7360, email: kerry.wilkinson@adelaide.edu.au

Abstract

When bushfires occur in proximity to vineyards, there is a risk that grapevines may be exposed to smoke, which can negatively affect fruit, and therefore wine. When evaluating the severity of smoke exposure, volatile phenols (and their glycosides) are quantified as chemical markers of smoke taint. This is due to the abundance of volatile phenols in wood smoke and their known contribution to the smoky, burnt and medicinal sensory characters associated with smoke-tainted wine. While critical to smoke taint diagnostics, few studies have leveraged the chemical complexity of smoke or its physiological effects on grapevines as the bases for identification of additional markers of smoke exposure in affected grapes.

In this study, Merlot vines were exposed to smoke for 1 hour at approximately 7-10 days post-véraison. Grape samples were collected before and after smoke exposure (i.e. at t=0 days, t=2 hours, t=24 hours, t=6 days, and t=20 days), and analysed with liquid chromatography Fourier transform high-resolution mass spectrometry (LC-FT-HRMS). The metabolic profiles of control and smoke-affected grapes were then compared using an untargeted metabolomics approach. Nine and twenty-one compounds readily differentiated control and smoke-affected grapes sampled from 2 hours to 20 days post-smoke exposure, in negative and positive ion modes, respectively. Tentative identifications suggested the presence of novel volatile phenol glycoconjugate moieties and stress-related metabolites. These findings support the expansion of smoke taint diagnostics beyond existing volatile phenol markers and demonstrate a need for further investigations into the impact of grapevine smoke exposure from the perspectives of abiotic stress and plant defence mechanisms.

1. Introduction

When bushfires occur in proximity to vineyards, there is a risk that grapes will be exposed to and tainted by the resulting smoke. If grapes are exposed to sufficient smoke, the ensuing wine can exhibit 'smoky', 'medicinal', and 'cold ash' aromas and flavours and elicit a drying, ashy aftertaste, an array of sensory characteristics otherwise known as 'smoke taint' (Kennison *et al.* 2007). Smoke taint is chemically assessed via the quantitation of volatile phenols—such as guaiacol, 4-methylguaiacol, *o*-cresol, and syringol—in both free and bound, (i.e., glycosylated) forms (Hayasaka *et al.* 2010a; Dungey *et al.* 2011). Volatile phenols are degradation products of lignin pyrolysis, and they impart characteristic 'smoky', 'woody', 'burnt' sensory properties with relatively low sensory detection thresholds (Maga *et al.* 1992). While their contributions to the sensory perception of smoke taint have been well established, the perceived intensity of smoke taint in wine remains a challenge to predict if based on the concentrations of volatile phenols and volatile phenol glycoconjugates in grapes (Parker *et al.* 2012). Several strategies could be undertaken to improve the diagnostics of grapevine smoke exposure and smoke taint in grapes and wine.

The first strategy is to consider that smoke taint intensity may not be a phenomenon exclusively attributable to a small number of volatile phenols and their glycosylated derivatives, given the chemical complexity of smoke. When wood burns, the composition of the smoke depends on species and origin, moisture content, and the temperature and duration of combustion (Cadahía *et al.* 2003; Guillén *et al.* 1999). The critical role of combustion temperature was demonstrated in a study that monitored volatile emissions derived from pyrolysis of ferulic acid at temperatures ranging from 50 to 500 °C (Wittkowski *et al.* 1992). Ferulic acid is a precursor to guaiacol (Maga *et al.* 1992) and a highly abundant structural component in plant cell walls (Wallace and Fry 1994). While guaiacol and its derivatives dominated emissions from pyrolysis at 230 to 260 °C, they became intermediates in the formation of pyrocatechol and dialkylphenols once the pyrolysis temperature range reached 360 to 410 °C. Wotton *et al.* (2012) established that prescribed burns achieve a maximum temperature of 1100 °C at the flame base, with temperature decreasing exponentially to ~300 °C at the flame tips. The temperatures generated in a bushfire encapsulate both phases of ferulic acid thermal degradation and thus, the distinct degradation products associated with them. There are a myriad of other phenol derivatives generated by lignin pyrolysis under the diverse range of conditions unique to each bushfire (Guillén and Manzanos 2002). To date, no studies

have screened grapes for alternative smoke-derived volatiles despite the array of other compounds in smoke.

The second strategy is to consider that grapevines are not passive recipients of volatile phenols during smoke exposure. In addition to the uptake of volatile phenols and other products of lignin degradation, primary pollutants derived from wildfires include nitrogen oxides, volatile organic compounds (e.g. methanol, acetaldehyde, acetone, and benzene), and polycyclic aromatic hydrocarbons (e.g. acenaphthene, acenaphthylene, fluorine, and phenanthrene) (Wentworth *et al.* 2018). Moreover, primary pollutants can be transformed into secondary pollutants, such as when volatile organic compounds react with nitrogen oxides in the presence of sunlight to form ozone (Lindaas *et al.* 2017). The uptake and degradation of ozone in plant cells produces reactive oxygen species, which in turn, initiate plant defensive mechanisms, including the synthesis of hormones, metabolites from the phenylpropanoid pathway, products from the ascorbate/glutathione cycle, and polyamines (Ludwikow and Sadowski 2008). Ozone has been used in the grape and wine industry for sanitation, preservation, and aroma enhancement (Segade *et al.* 2017; Pazarlar *et al.* 2017), and in the context of smoke taint, it has recently demonstrated potential as a mitigation agent when applied to smoke-affected grapes post-harvest (Modesti *et al.* 2021). Ozone is a powerful oxidant and little work has explored the feasibility of using grapevine secondary metabolites related to defence to detect smoke exposure in grapes.

Taken together, we hypothesised that endogenous or exogenous compounds may accrue in grapes over time, enabling them to be distinguished from grapes unaffected by smoke. We chose to focus on non-volatile metabolites due to the rapid uptake and metabolism of volatile phenols in grapes following smoke exposure (van der Hulst *et al.* 2019; Szeto *et al.* 2020; Jiang *et al.* 2021) and the tendency for grapes to store small, lipophilic volatile compounds as glycoconjugates (Winterhalter and Skouroumounis 1997). To test this, we analysed the composition of Merlot grapes over a three-week period following smoke exposure (approximately 7-10 days post-véraison until commercial maturity) using an untargeted metabolomics workflow that employed ultrahigh-performance liquid chromatography coupled with Orbitrap mass spectrometry.

2. Materials and Methods

2.1 Chemicals, Reagents, and Reference Compounds.

The following chemicals were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia): 2-methoxy-4-methylphenol (4-methylguaiacol), *o*-cresol, *p*-cresol, *m*-cresol, phenol, 2,6-dimethoxyphenol (syringol) and 4-methyl-2,6-dimethoxyphenol (4-methylsyringol). Guaiacol was purchased from Scharlau (Port Adelaide, SA, Australia). Formic acid for LC-MS analyses was purchased from Rowe Scientific (Lonsdale, SA, Australia). The internal standards d_4-guaiacol and d_3-syringol gentiobioside were synthesised in-house, using methods described previously (Pinchbeck, 2011; Hayasaka *et al.* 2013), while d_3-syringol was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). Solvents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) classified as hyper-grade for LC-MS (LiChrosolv®) and included: ethanol, methanol, acetonitrile, ethyl acetate, and chloroform. GC-grade pentane was also purchased from Sigma-Aldrich.

2.2 Field trials

Field trials involved the application of smoke to Merlot grapevines growing in a single row within a vineyard located at the University of Adelaide's Waite campus in Urrbrae, South Australia (34°57'59.2"S 138°37'59.0"E). Vines were planted in north-south aligned rows (in 1998) and grown on their own roots, trained to a bilateral cordon, vertical shoot positioned trellis system, hand-pruned to a two-node spur system, and drip irrigated.

Smoke was administered to vines for 1 hour at approximately 7-10 days post-véraison using a purpose-built tent described elsewhere (Kennison *et al.* 2009). Control grapevines were not exposed to any smoke. The application of smoke was conducted in duplicate, with three vines contained within each experimental replicate. For each replicate smoke treatment, 2.4 kg of barley straw was combusted in fireboxes positioned at opposing ends of the smoke tent. The fuel allocated to each firebox (1.2 kg) was added in 10 min increments over the course of the trial to ensure that grapevines were exposed to smoke throughout the hour-long treatment. Smoke entered the tent via an aluminum tube connected to the exhaust of each firebox. To prevent control vines from being contaminated by smoke, at least nine buffer vines separated them from the smoke-exposed vines. To prevent smoke-exposed vines in the first treatment from being exposed to smoke from the second smoke treatment, six buffer vines separated the two replicates of smoke-exposed vines.

Fifty berries were collected from each vine within each smoke replicate, using an established sampling procedure (Sala *et al.* 2004). Samples were collected at the following time points (relative to smoke exposure): immediately prior to smoke exposure, 2 and 24 hours post-smoke exposure, and then 3, 6, 8, 10, 13, 15, 17, and 20 days post-smoke exposure, where 20 days corresponded to commercial maturity (i.e. total soluble solids (TSS) of 25 °Brix). These time points were selected to build on previous work that highlighted the delay between the depletion of smoke-derived volatile phenols and the subsequent accumulation of their glycoconjugates (Szeto *et al.* 2020). Herein, a similar timeframe was used to confirm previous trends and enable an untargeted approach to offer insight on additional markers that may also reflect the immediate- and long-term effects of grapevine smoke exposure.

Following collection, berries were homogenised (T18 Ultra Turrax, IKA, Safen, Germany and frozen at -4 °C in 120 mL polypropylene tubes (Sarstedt, Nümbrecht, Germany) until needed for chemical analyses. Samples were prepared for analysis of volatile phenols and volatile phenol glycoconjugates, approximately two months after harvest. Samples were then refrozen (at -4 °C) until needed for the untargeted metabolomics study, which commenced approximately nine months after harvest.

2.3 GC-MS analysis of grape homogenate

Volatile phenols (guaiacol, 4-methylguaiacol, phenol, *o*-, *m*-, and *p*-cresol, syringol, and 4-methylsyringol) were measured in grape homogenate samples using SIDA-based methods established in previous studies (Pollnitz *et al.* 2004; Hayasaka *et al.* 2010a). The internal standards used for quantitation were d_3-syringol and d_4-guaiacol. Analysis was conducted with an Agilent 6890 gas chromatograph coupled to a 5973 mass spectrometer (Agilent Technologies, Forest Hill, Vic., Australia). With sample preparation, method validation, and instrumental parameters as previously reported (Hayasaka *et al.* 2010a). Data acquisition was conducted with ChemStation and data processing was carried out using MassHunter Quantitative Analysis software.

2.4 HPLC-MS/MS analysis of grape homogenate

Volatile phenol glycoconjugates were measured in grape homogenate using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The internal standard was d_3-syringol gentiobioside, and all measured glycosides were quantified as syringol gentiobioside equivalents. Analysis was conducted with an Agilent 1200 HPLC equipped with a 1290 binary pump, coupled to an AB SCIEX Triple Quad^{TM} 4500 tandem mass spectrometer

with a Turbo V^{TM} ion source (Framingham, MA, USA). Sample preparation, method validation, internal standard preparation, and instrumental parameters were performed as previously reported (Hayasaka *et al.* 2013). Both data acquisition and processing were conducted using Analyst software (version 1.7, AB SCIEX).

2.5 Sample preparation for untargeted LC-FT-HRMS analysis

Liquid chromatography Fourier transform high-resolution mass spectrometry (LC-FT-HRMS) was used for the untargeted metabolomics study. A total of 60 frozen homogenate samples were prepared in four, randomly selected batches of 12-15 samples. Aliquots of grape homogenate (2 g) were weighed into 10 mL centrifuge tubes and extracted with 1 mL of MilliQ water, 2 mL of methanol, and 2 mL of chloroform. Samples were then spiked with 20 µL of d_3-syringol gentiobioside (20 µg/mL) as internal standard, to achieve a concentration of 200 µg/kg. Next, they were vortexed for 1 min, shaken gently on a rotary tube mixer (Rate Instruments Pty Ltd, Boronia, VIC, Australia) for 15 min (at 25 °C and 25 RPM) and centrifuged for 25 min at 4 °C and a relative centrifugal force of 3270 × g using an Allegra X-12R Centrifuge (Beckman-Coulter, Lane Cove, NSW, Australia).

Supernatant from each sample (4 mL) was collected and transferred into borosilicate culture tubes. Extraction was repeated on the residual grape homogenate, using 1 mL of water and 2 mL of methanol, after which extracts were vortexed, shaken, and centrifuged under conditions described above for the first extraction. Supernatant (3 mL) from the second extraction was combined with the 4 mL collected from the first extraction. The samples were dried overnight (i.e., at least 15 h at 30 °C under a flow of 3.5 L/min of N_2) using a TurboVap (Biotage, Rydalmere, NSW, Australia).

Once dry, extracts were reconstituted in 1 mL of mobile phase, comprising 75% solvent A (0.1% formic acid, 0.5% methanol in Milli-Q water) and 25% solvent B (0.1% formic acid, 2% Milli-Q water, 40% acetonitrile in methanol). Samples were vortexed, transferred into 1.5 mL Eppendorf tubes, and centrifuged for 15 min at 4 °C and a relative centrifugal force of 23,907 × g with a Universal 32R centrifuge (Hettich, Thebarton, SA, Australia). Lastly, samples were transferred into 2 mL amber vials for chemical analysis. For quality control and compound identification purposes, a pooled biological quality control (PBQC) sample was prepared by combining 20 µL of each reconstituted sample into a single vial. To assess any background contamination accrued from the solvents, reagents, or materials used, solvent blanks (comprised of solvent A) were also prepared and analysed in duplicate. Procedural

blanks were prepared following the same protocol as described for samples, but in the absence of grape homogenate material.

2.6 LC-FT-HRMS method.

Metabolites were separated with a Kinetex PFP LC column (150 mm x 2.1 mm; 2.7 µm, Phenomenex) held at 30 °C with a binary gradient, consisting of 0.1% formic acid and 0.5% methanol in Milli-Q water (solvent A) and 0.1% formic acid, 2% Milli-Q water, and 40% acetonitrile in methanol (solvent B). All solvents were gradient grade for liquid chromatography from Supelco (Bellefonte, PA, USA). The flow rate was 0.40 mL/min and the gradient progressed with linear increases from 0 to 1% B over 6.25 min, to 7.5 %B over 13.75 min, to 60% B over 10 min and to 90% B over 3 min. The gradient was held at 90% B held for 5 min, after which the column was washed and re-equilibrated. The injection volume was 1 µL for samples acquired in MS1 mode and 3 µL for samples acquired in MS/MS mode.

Mass spectral data were collected using an Thermo Fisher Orbitrap-IDX Tribrid mass spectrometer fitted with a heated electrospray ionisation source and a Vanquish™ Horizon uHPLC system via electrospray ionisation using the following conditions: sweep gas 1 (arbitrary units, au), sheath gas 25 au, auxiliary gas 7 au, vaporiser temperature 300 °C, and ion transfer tube temperature 275 °C. The RF lens value was 35%. Data were collected in positive and negative ion mode with spray voltages of 3500 V and 3400 V, respectively.

MS1 data were collected with a 60,000 scan resolution, 1 microscan, 110 ms maximum injection time and automatic gain control (AGC) of 600,000 over a mass range of 100-2000 *m/z*. Data-dependent MS2 scans were collected with a 15,000 scan resolution, 1 microscan, 22 ms maximum injection time, AGC of 75,000, isolation window of 1.5 *m/z*, and dynamic exclusion of 2.5 s. The collision energies of high-energy collisional dissociation (HCD) were stepped from 20, 35, and 50%.

Prior to the acquisition of sample data, a solvent blank (i.e. solvent A) was run to prepare the column, followed by a procedural blank (run in duplicate). The latter was used to perform blank subtraction in the data processing step. MS2 data of the PBQC sample was then collected (in triplicate) for identification purposes. Lastly, MS1 data were acquired via five replicate injections of the PBQC sample to equilibrate the column. Following the study setup, the PBQC sample was injected once every seven samples to enable correction for potential batch effects via QC-based normalization.

2.7 LC-FT-HRMS method validation

To determine the reproducibility of the sample preparation method, seven replicates of a single grape homogenate sample (a control sample from t=15 days) were prepared (as described above). To assess the reproducibility of the instrumental analysis, 100 µL of the final extract from the seven replicates were pooled to create a PBQC sample. Five replicate injections of the PBQC sample were conducted, from which MS1 data were collected. The coefficient of variance was calculated across the replicate grape homogenate samples and across three out of five replicate injections of the PBQC sample to assess the reproducibility of the sample preparation method and instrument, respectively.

2.8 Data processing

Raw data files were processed through the "Untargeted Metabolomics with Statistics Detect Unknowns with ID using Online Databases and mzLogic" workflow available in Compound Discoverer (version 3.1, Thermo-Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia). As shown in **Supplemental Figure 1**, the workflow aligned retention times, detected extracted ion chromatogram traces from MS1 scans, and grouped compounds across all files if they shared molecular weight and retention time. After grouping the compounds, QC-based area correction was performed and background compounds were identified. Simultaneously, unknown compounds were tentatively identified by name, chemical formula, and/or structure through searching ChemSpider, mzCloud, mzVault, and mass lists developed in-house. Search results from mass lists and ChemSpider libraries were ranked by mzLogic, which analysed MSn fragmentation patterns and used them to calculate a similarity score to potential structures of each compound. A detailed overview of specific settings utilised in each node of the workflow can be found in **Supplemental Table 1**.

When raw files were imported into Compound Discoverer, they were assigned a classification and (if appropriate) a group. Classifications dictate how samples are processed through the workflow, and groups outline how samples are evaluated with statistical analysis. Two Compound Discoverer studies were conducted using the previously described processing workflow, and samples were classified as 'Blank', 'Identification only', 'Quality control', or 'Sample', depending on the sample and purpose of the study. The 'Blank' classification was assigned to procedural blank samples. Any compounds identified in these samples were assumed traces of the solvents/materials used during sample preparation and labelled as background compounds by the workflow. The 'Identification only' classification was reserved for the PBQC sample when it was collected in MS/MS mode. This information enabled the

annotation of candidate compounds. The 'Quality control' classification was assigned to the PBQC sample when it was collected in MS1 mode. With the 'Quality control' classification, the MS1 data from PBQC sample was used to minimise batch effects by fitting a cubic spline regression model to the area of each compound across the replication injections of the PBQC sample with respect to time (Dunn *et al.* 2011). Alternatively, when given a 'Sample' classification, the MS1 data from the PBQC sample was adapted to assessing method reproducibility. By convention, the 'Sample' classification was applied to experimental samples that were collected to evaluate the effects of variables under study, i.e. factors. Factors split the data into groups, and ratios between pairs of groups designated the comparisons for subsequent statistical analysis.

The first study was conducted to validate the reproducibility of the method and instrument. Files corresponding to the seven replicates of a grape homogenate sample and the five replicate injections of the PBQC sample (collected in MS1 mode) were classified as 'Samples'. The second study was performed to assess the effects of smoke exposure and time on the metabolic profiles of grape homogenate samples. In this study, the procedural blank was classified as 'Blank', the PBQC sample was collected in both MS1 (i.e. as 'Quality control') and MS/MS modes (i.e. as 'Identification only'), and the experimental samples were classified as 'Samples'.

In the first study, samples were processed through the workflow without study factors, but in the second study, the experimental samples were sorted by two study factors, 'Treatment' and 'Time'. Experimental samples that shared both factors constituted a group. The 'Treatment' factor had two categorical levels, corresponding to the samples collected from smoke-affected vines (labelled as 'S' i.e. smoke) and the samples collected from vines without smoke exposure (labelled as 'C' i.e. control). The 'Time' factor had five numerical levels, corresponding to the samples collected at each time point (t=0 days, t=2 hours, t=24 hours, t=6 days, t=20 days). Thus, ten groups were created, being: C/S (0 days), C/S (2 hours), C/S (24 hours), C/S (6 days), and C/S (20 days). To indicate which groups to compare in statistical analyses, five ratios were set up to compare the effects of treatment at each time point. In addition, eight ratios were created to explore the effects of time within each treatment by comparing each group C/S (t=2 hours, 24 hours, 6 days, or 20 days) to its respective C/S (t=0 days) group. For each ratio, the Log2 fold change, area ratio, and one-way ANOVA were calculated. To adjust for multiple comparisons, p-values were corrected for the false discovery rate using the Benjamini-Hochberg algorithm.

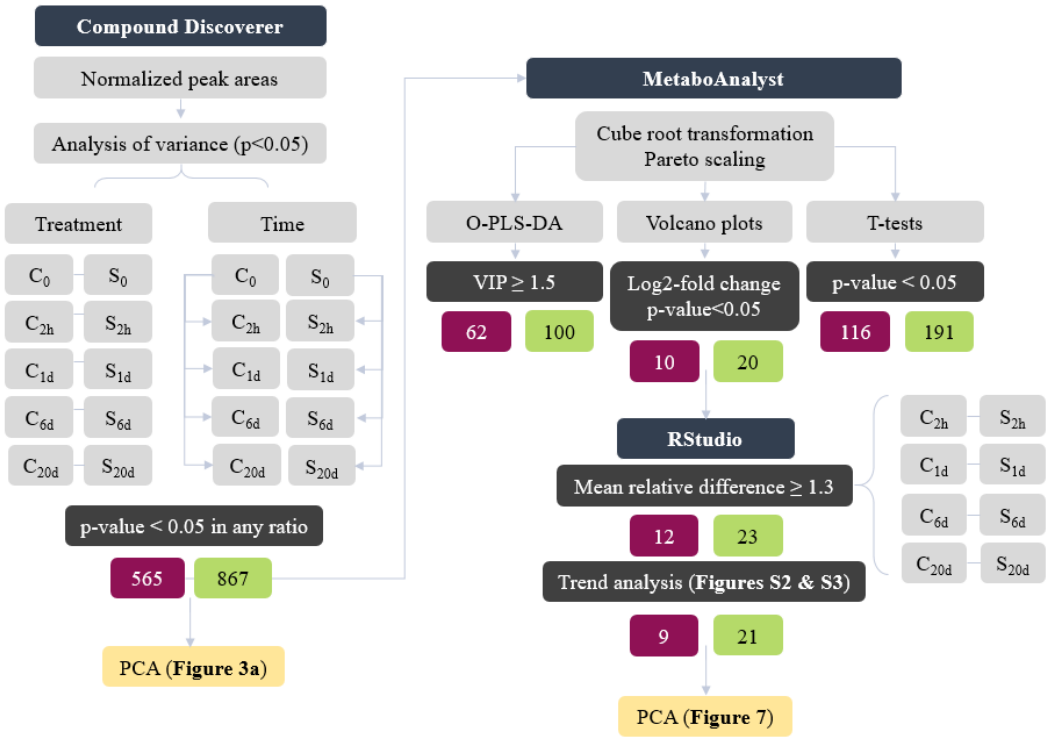
Resultant compound tables were filtered to reduce the incidence of compounds defined by low-quality peaks (e.g. high baselines, non-Gaussian distributions, low signal to noise ratios, etc.). In the first study, compounds were removed from the table if identified in the procedural blank (i.e. 'Background is false'), characterised by a peak area less than 20 million in the PBQC sample (i.e. 'Area ≥ 20 million in file PBQC'), or lacking a proposed chemical formula (i.e. 'Formula is not blank'). This set of filters was followed by a manual inspection of peak quality, and the resulting compound table was used to assess reproducibility. In the second study, compounds were filtered from the table if they were present in procedural blank samples (i.e. 'Background is false'). Compounds also needed to demonstrate statistical significance in at least one ratio (i.e. 'Adjusted p-value ≤0.05 in any ratio'), and detectable MS/MS data (i.e. 'MS2 is not equal to 'no MS2'). Finally, compounds were required to meet the criteria for QC-based normalisation (i.e. 'Normalised area has any value in any file', 'Number of usable quality control samples = 14').

In this study, the maximum number of usable quality control samples corresponded to the number of PBQC sample injections performed during the sequence, being 14 in each ionisation mode. QC-based normalisation occurs for a particular compound only if certain conditions are met. First, the gap between PBQC sample injections must not exceed 15 experimental samples. In this study, an injection of the PBQC sample was scheduled to occur once following every seven experimental samples. A gap exceeding 15 experimental samples would indicate a problem with injection, a compromised PBQC sample, or both. Second, compounds must be detected in at least 50% of PBQC sample injections. Third, the peak areas of compounds across PBQC injections are required to have a residual standard deviation value at or below 30%. The 'Normalised area has any value in any file' filter selected compounds that met these three criteria. The 'Number of usable quality control samples = 14' is a more restrictive extension and further limited selection to compounds that were detected in 100% of PBQC sample injections. The resultant list of compounds was manually inspected for peak quality and exported for further analysis.

2.9 Data analysis.

The workflow used to explore and analyse the data is shown in **Figure 1**. Normalised peak areas were exported from Compound Discoverer and uploaded to RStudio and the data were initially explored with principal component analysis (PCA) using the FactoMineR (version 2.4) and ggplot2 (version 3.3.5) packages in RStudio (version 4.0.3). Data were centred and scaled prior to plotting.

Figure 1. Data processing workflow for control and smoke-affected Merlot grapes collected over five time points, analysed by uHPLC-Orbitrap IDX MS in positive and negative ionisation modes. Following the application of a **filter**, the resultant number of compounds in **negative** and **positive** ionisation modes are shown. The degree of separation achieved following the application of key filters are visualized in **PCA plots**.



It was anticipated that compounds driven by temporal change (e.g. sugars, anthocyanins) would be the predominant influence on the overall variation in the metabolic profile (rather than smoke exposure). In part, this expectation was driven by the high abundance of sugars, anthocyanins, and other aroma compounds relative to smoke-derived metabolites (e.g. volatile phenols and their glycoconjugates), as well as the timing of sample collection (i.e. three weeks following véraison) and magnitude of biochemical changes associated with this period (Kalua and Boss 2009). To account for this, several statistical techniques were explored in MetaboAnalyst (version 5.0, www.metaboanalyst.ca).

Normalised peak areas were pre-processed with Pareto scaling and a cube root transformation to achieve a normal distribution. Data were analysed using a combination of univariate and multivariate analyses. As a supervised alternative to PCA, orthogonal partial least squares discriminant analysis (OPLS-DA) was performed. To evaluate model quality and predictive ability, R^2Y and Q^2 metrics were used. To test the significance of the classification,

samples were permuted 1000 times. Critical compounds from the OPLS-DA model were identified as those with variable importance on projection scores ≥1.5.

The multivariate strategy was complemented with independent t-tests and volcano plots. The t-tests were corrected for the false discovery rate, and critical compounds were identified as those with an adjusted p-value <0.05. Volcano plots identify compounds with high relative differences between groups (as a log 2-fold-change) and quantify the statistical significance of that difference (as $\log_{10}$ p-value of the fold change). Critical compounds were identified as those with a log 2-fold change >2| or <-2 and a p-value <0.05. For the analyses conducted in MetaboAnalyst, all time points within each treatment were pooled and treated as members of the 'control' or 'smoke' group (with the exception of t=0 hours hours); thus, any compounds identified through this workflow are not specific to a point in time.

The compounds identified through the MetaboAnalyst workflow were uploaded into RStudio. Mean concentrations and relative mean differences between control and smoke groups (at t=2 hours, t=1 day, t=6 days, and t=20 days) were calculated. Compounds were further filtered according to whether they had relative mean differences between smoke-exposed and control groups higher than a factor of 1.3. Compound trends were visualised using box-and-whisker plots drawn with the ggplot2 package. The final list of compounds selected for annotation were those with evident elevation in the smoke-exposed group. This degree of separation in the data achieved with this list was also visualised with PCA.

Tentative identifications were carried out through a manual search of the *m*/*z* Cloud Library, ChemSpider, PubChem, METLIN, and PlantCyc for putative compounds by mass, structure, and formula. Fragment Ion Search (FISh) scores were calculated in Compound Discoverer software. FISh scores reflect the degree to which a proposed structure can account for the fragmentation pattern of an unknown peak. Manual review of MS2 spectra was also conducted using a combination of Compound Discoverer and Freestyle (version 3.4, Thermo-Fisher Scientific Australia Pty. Ltd., Scoresby, VIC, Australia).

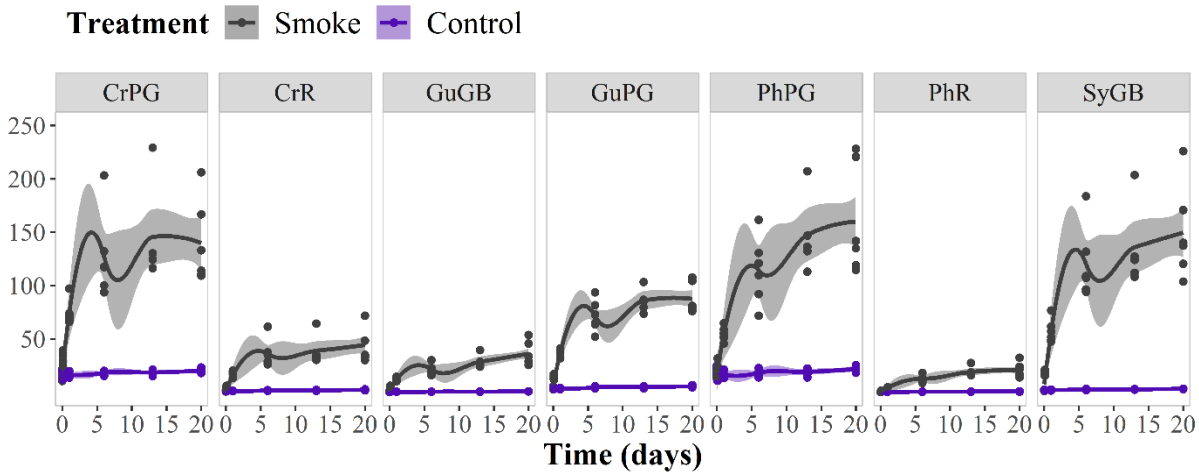
3. Results and discussion

Six Merlot grapevines were exposed to straw-derived smoke for 1 hour at 7-10 days post-véraison. Following this event, the six smoke-affected vines and six corresponding control vines (without smoke exposure) were sampled at several time points over three weeks. Volatile phenols and volatile phenol glycoconjugate concentrations were analysed in both control and smoke-affected grapes at t=0 hours (immediately before smoke exposure) and then at t=2 hours, 24 hours, 6 days, and 20 days post-smoke exposure. Volatile phenol glycoconjugates

were also measured in grapes collected at t=13 days. As expected, there were no statistically significant differences in either volatile phenol or glycoconjugate concentrations between control and smoke-affected grapes prior to smoke exposure (i.e., t=0 hours). Control grapes contained volatile phenol concentrations below established limits of detection across all time points. Smoke-affected grapes had significantly elevated volatile phenols only at t=2 hours post-smoke exposure, having 17, 20, 5, and 61 µg/L for guaiacol, phenol, *o*-cresol, and syringol, respectively ($p<0.05$, Mann-Whitney U-Test). At all other time points, the volatile phenol concentrations of smoke-affected fruit were also below their limits of detection (**Supplemental Table 2**).

Contrarily, the majority of volatile phenol glycoconjugate concentrations were significantly elevated in smoke-affected grapes relative to control grapes ($p<0.05$, Mann-Whitney U-Test), at every time point except t=0 hours. The accumulation patterns of the most abundant glycoconjugates followed a similar trend, in which concentrations rapidly increased within the first 24 hours and remained relatively stable thereafter (**Figure 2**).

Figure 2. Trends in volatile phenol glycoconjugates in Merlot grape homogenate (µg/kg) from **control** and **smoke-exposed** grapes sampled from pre-smoke exposure (t=0 hours) to commercial maturity (t=20 days), fitted using the smoothed conditional means plotting method. Each point represents a single observation from each treatment (at t=0 hours, 2 hours, 24 hours, and 6, 13, and 20 days). Cr = cresol, Gu = guaiacol, Ph = phenol, Sy = syringol, PG = pentose glucoside, R = rutinoside, and GB = gentiobioside.



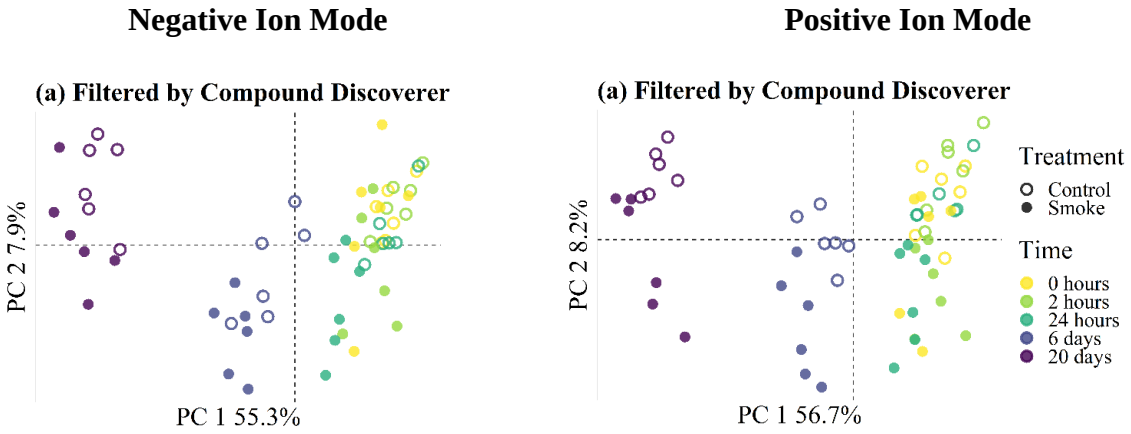
The most abundant glycoconjugates in smoke-affected fruit were cresol pentose glucoside, phenol pentose glucoside, and syringol gentiobioside, at 140, 160, and 150 µg/kg respectively

at t=20 days (harvest) (**Supplemental Table 3**). In control fruit, the most abundant glycoconjugates were similarly recorded at t=20 days and corresponded to the pentose glucosides of phenol, cresol, and guaiacol, at 22, 20, and 6 μg/kg respectively. The remaining glycoconjugates were at levels ≤ 3 μg/kg. These results indicated that grapes were exposed to levels of smoke sufficient to induce known compositional effects (i.e. increased levels of volatile phenols and their glycoconjugates), a pre-requisite to searching for additional markers of smoke exposure.

Prior to analyzing homogenate samples via LC-FT-HRMS, the reproducibility of the methods used for homogenate preparation and data acquisition with the Orbitrap-IDX were determined via the analysis of a grape homogenate sample (prepared seven times) and several injections of the PBQC sample, respectively. Reproducibility was assessed by calculating the coefficient of variation across the normalised peak areas of compounds identified by the Compound Discoverer workflow. After filtering criteria were applied in Compound Discoverer, 135 compounds remained in the table and the coefficient of variation was calculated across replicate homogenate samples and injections of the PBQC sample. When the coefficient of variation was calculated across the replicate grape samples, the value of the 95th percentile was 18.58%, and when it was calculated across the third, fourth, and fifth replicate injections of the master mix, the value of 95th percentile was 17.06%. These low values indicated sufficiently low variation in the samples associated with their method of preparation.

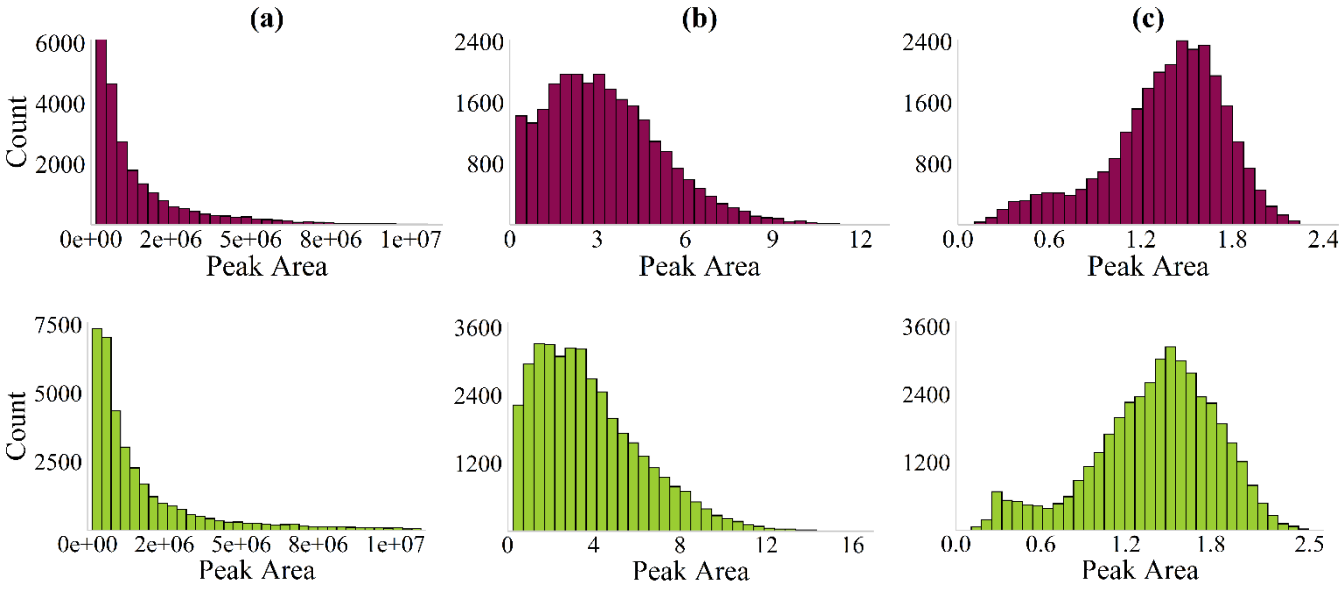
Once satisfied with the quality of control and smoke-affected samples and the validity of the LC-FT-HRMS method, experimental samples were analysed and data were processed through the workflow. The resultant compound tables were explored with PCA (**Figure 3**). This PCA included 565 and 867 compounds in negative and positive ion modes, respectively. Across positive and negative ion modes, ~59% of total variation was accounted for by PC1 and PC2, of which the first PC accounted for ~56%. As expected, PCA primarily separated samples according to grape maturity rather than smoke exposure. Samples that were collected within 24 hours of each other (i.e., at t=0 hours, t=2 hours, and t=24 hours) were positioned on the right side of the plot, whereas samples collected 6 days and 20 days following the first time point were positioned further left. There was some separation achieved along PC2 that reflected differences in smoke exposure, with most control samples positioned in the upper quadrants. Supervised data analysis techniques were used to take the directionality of change into account and focus on compounds that were significantly elevated in smoke-affected grapes relative to control grapes.

Figure 3. Principal components analysis of normalised peak areas of compounds measured in Merlot grapes with and without smoke exposure in negative and positive ionisation mode. Each plot is inclusive of all samples at every time point. Colours correspond to different time points (t=**0 hours**, **2 hours**, **24 hours**, **6 days**, and **20 days**), and the open or filled status of each data point represent control and smoke-affected treatments, respectively.



Following exploration of the data using PCA, histograms were plotted, and data from both positive and negative ion modes were skewed (**Figure 4**). A right skew indicates that the mean peak area is strongly influenced by a minority of compounds with significantly higher peak areas. In the positive and negative ion data, most compounds have a peak area <2.5 million; however, there were some samples with peak areas of >10 million. Absolute peak area does not necessarily indicate that compounds are relevant to the hypothesis. Thus, Pareto scaling was used to reduce the influence on the distribution that was wielded by compounds with high peak areas by dividing the peak area of each compound by the square root of its standard deviation across samples (van den Berg *et al.* 2006) (**Figure 4**). Following Pareto scaling, the skew was corrected with a cube root transformation (**Figure 4**). A normal distribution is required to obtain valid results from hypothesis testing and while log and power transformations were explored (data not shown), the cube root transformation resulted in the best distribution.

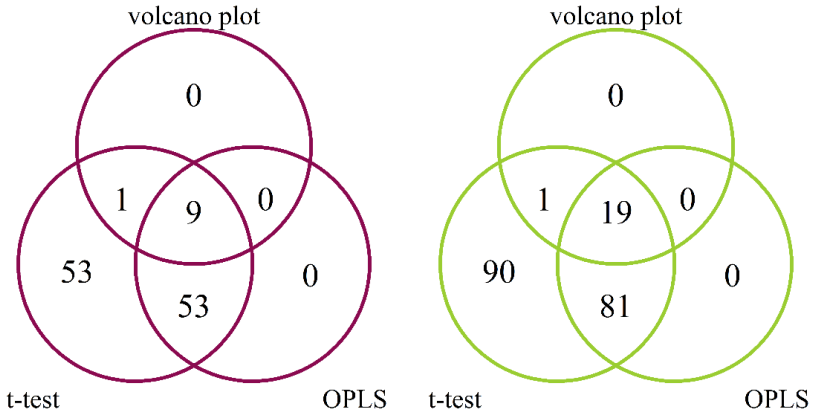
Figure 4. Histograms depicting the distribution of the raw feature data (a) prior to pre-processing and then with (b) Pareto scaling and (c) Pareto scaling with a cube root transformation. Different colours represent data collected in **positive** and **negative** ionisation mode.



Once data were pre-processed, a combination of t-tests, volcano plots, and supervised OPLS-DA were used to examine the compounds that differentiated control and smoke-affected grapes over time. Rather than examine differences between the smoke-affected and control grapes at each time point, samples were pooled and analysed together, with the exception of samples collected at t=0 hours. The number of compounds identified by each method are shown in **Figure 5**.

The t-tests identified 116 and 191 compounds as statistically significant in the negative and positive ion modes respectively. This is a much higher number of significant compounds than the 23 and 70 compounds that were identified when samples were explored at each time point using one-way ANOVA. ANOVA and t-tests operate by comparing the variation between groups relative to the variation within groups. For the ANOVA, comparisons were conducted at each time point, whereas for the t-tests, comparisons were conducted across time points. The pooled data included samples collected from over the duration of 3 weeks, and from the PCA analysis, it was clear that the effect of time had a greater contribution to the overall compositional variance than that of smoke exposure. Nonetheless, the use of Pareto scaling and cube root transformation enabled greater differences to be captured between the two groups that were likely obscured by the range of peak areas observed across compounds and the skewed distribution.

Figure 5. Venn diagrams showing the number of potential markers that may be used to detect smoke exposure in grapes, extracted from compound lists using t-tests, volcano plots, and OPLS-DA. Different colours represent data collected in and **negative** and **positive** ionisation modes.



Hypothesis testing is critical to quantify the probability that identified differences between groups are due to the intended treatment rather than chance, but statistical significance is not always sufficient to demonstrate real-world relevance to the biological impact of smoke exposure on grapevines. Thus, volcano plots were also generated to identify compounds that not only demonstrated statistical significance ($p<0.05$) but also exhibited a log 2-fold change >2 or <-2. In negative ion mode, only 10 compounds met these criteria, whereas 20 compounds met these criteria in positive ion mode. The majority of the compounds identified in the volcano plot (70% in negative ion mode and 75% in positive ion mode) had also shown statistical significance at multiple time points when the data was analysed by one-way ANOVA at each time point. This indicates that the low number of compounds identified by the volcano plot can be attributed to the restrictive log 2-fold criteria.

Univariate analyses offer a snapshot of each feature in isolation, but to examine how groups of compounds might interact in a matrix, multivariate analyses was required. Partial least squares (PLS) is a data reduction technique that seeks linear combinations of raw predictor data that capture the maximum amount of variation with respect to a response variable (Kuhn and Johnson 2013). If the response variable is continuous, then PLS regression is performed to predict the value of a variable, whereas if the response variable is categorical, then partial least discriminant analysis is performed to predict a class assignment. Although PLS models are built with respect to the variation related to the response variable, the variance in the predictor data that is unrelated to the response variable is still included in the model when it is built. On

the other hand, orthogonal partial least squares discriminant analysis (OPLS-DA) separates the variation from the raw predictor data that is unrelated to the response variable prior to data modeling, a step which enhances the interpretability and robustness of PLS models (Trygg and Wold 2002).

OPLS-DA was performed to extract differences in grapes that were specifically related to smoke exposure rather than other variables, such as maturation. The percentage of predictor variance explained by the model (R^2Y value) was 98% for both ionisation modes. The predictive performance of the model estimated by cross-validation, given by Q^2 values, was 0.903 and 0.959 for negative and positive ion modes, respectively. The percentage of predictor variance and the performance of the models were significantly different to randomly permuted models ($p<0.001$), the values of which were calculated over 1000 permutations. These values indicate the robustness of the OPLS-DA model. To identify the most important features, variable importance in projection (VIP) scores were used as a filter. VIP scores are often used for features selection because they reflect how much a variable influences a given PLS component and the share of the total variance that is accounted for by that component (Thévenot *et al.* 2015).

In negative and positive ion modes respectively, 62 and 100 compounds analysed by OPLS-DA had a VIP threshold greater than 1.5. All of the compounds with a VIP score >1.5 in OPLS-DA were also found using t-tests, volcano plots, or both. In fact, 9 compounds in negative ion mode and 19 compounds in positive ion mode were deemed as important distinctions between the control and smoke-affected grapes by all three methods. While all of the compounds with high VIP scores in the OPLS-DA model also demonstrated statistical significance when analysed via t-test, the reverse was not true. In both ionisation modes, ~46% of total compounds had only showed significance when analysed using t-tests. As mentioned previously, t-tests are an important first step to revealing compounds that are significantly different between groups, but OPLS-DA takes this further through the removal of variance in the data that is unrelated to smoke exposure and the calculation of latent variables that characterise how entire groups of compounds change between control and smoke-affected grapes across time. Across the three methods of analysis, 116 compounds in negative ion mode and 191 compounds in positive ion mode were revealed as tentative markers of smoke exposure.

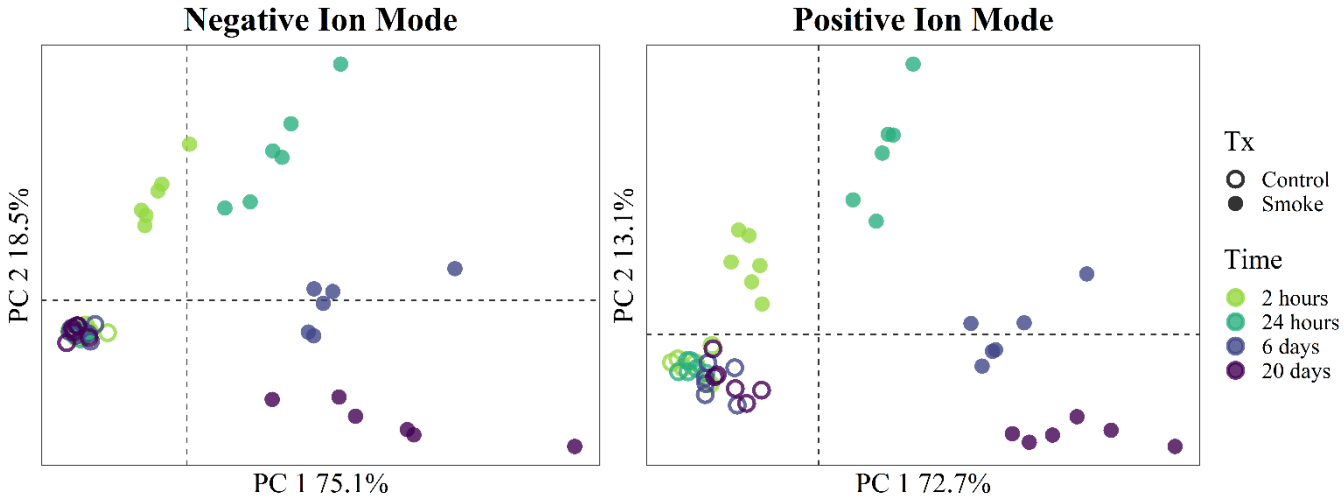
In the next phase of data analysis, relative differences in peak area each compound were calculated between the mean values for control and smoke-affected grapes to identify compounds that were elevated due to smoke exposure by a factor of at least 1.3. This is distinct

to the volcano plots, which not only had more restrictive change criteria, but also included compounds regardless of the directionality of the change. The relative differences were calculated by dividing the smoke-affected mean values by the control mean values to focus on compounds that increased due to grapevine smoke exposure. These criteria resulted in 12 (for negative ion mode) and 23 (for positive ion mode) compounds that were associated with smoke exposure of grapes. As an alternative, the relative differences at each time point could have been calculated, but it was important to focus on markers that persist in grapes over time. Previous work has highlighted the rapid depletion of volatile phenols in grapes after smoke exposure (Szeto *et al.* 2020; Jiang *et al.* 2021), and the time-sensitive nature of acquiring a representative sample that accurately reflects the degree of grapevine smoke exposure. While it would be interesting to identify compounds that accumulate within a few hours of smoke exposure, identification of markers with higher diagnostic potential were prioritised. Following a bushfire event, vineyard access can be limited due to safety restrictions, and it is critical to ensure that markers are stable in grapes to enable accurate quantitation, independent of when samples can be collected for analysis.

Box-and-whisker plots were created to examine the distribution of each variable within the distinct populations. The centre line demarcates the median and the edges of the box correspond to the 25^{th} and 75^{th} percentiles. The interquartile range is the difference between the two percentiles and this value is used to generate the whiskers. The value obtained by multiplying the interquartile range by 1.5 is added to the highest observation and subtracted from the lowest to generate the upper and lower whiskers, respectively. Observations that lie outside the range of the whiskers are plotted as outliers. Compound identities were coded with an arbitrary number preceded with the prefix 'V' for 'variable'. Based on the box plots made with the negative ion mode data, compounds V_42 and V_44 were removed due to the degree of overlap between the control and smoke-affected populations (**Supplemental Figure 2**). While compound V_513 demonstrated an 8-fold difference between smoke-affected and control groups, its box-and-whisker plot shows that this difference was driven by several outlying points. After compound V_513 was removed, 9 final candidates in negative ion mode remained for annotation. Based on the box plots made with the positive ion mode data, compound V_650 was removed due to a high level of overlap between compounds and compound V_781 was removed due to the influence of outliers (**Supplemental Figure 3**). Once these compounds were removed, 21 final candidates in positive ion mode remained for annotation.

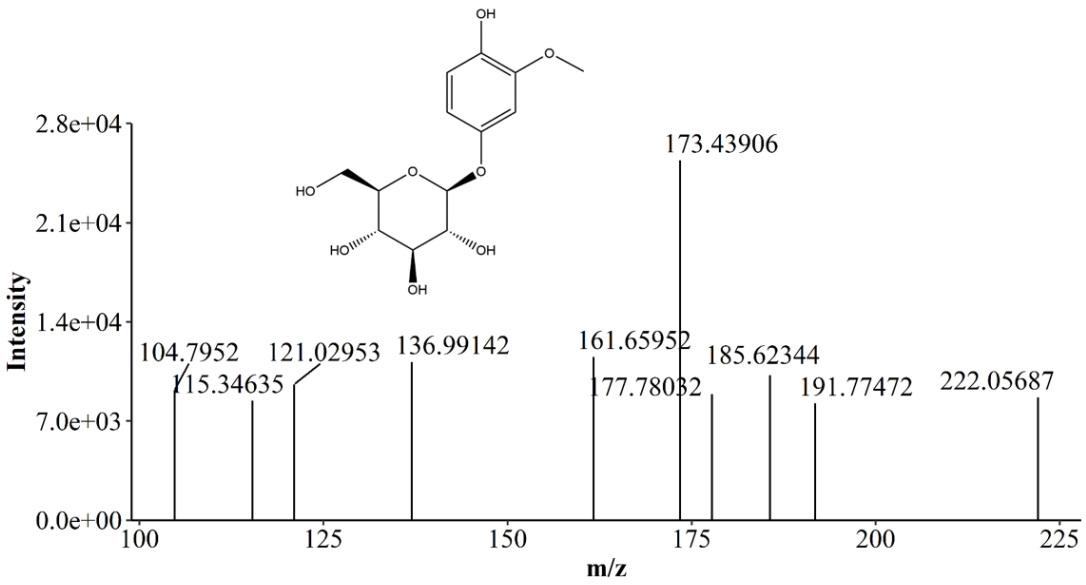
When these final subsets of compounds were analysed by PCA, a remarkable 75.1% and 72.7% of total variance was accounted for by PC1 in negative and positive ion modes, respectively (**Figure 6**). In both PCA scores plots, all control samples were tightly clustered across time and the smoke-affected samples fanned out by time point. These plots demonstrate that the selected compounds distinguished smoke-affected grapes from control grapes and that the distinction between the two populations grew over time. This is the reverse of the trend suggested by earlier PCAs, in which the differences between populations decreased over time. Using search-by-formula or search-by-mass functions in several databases, candidate compounds were tentatively identified based on FISh scores computed in Compound Discoverer. FISh scores reflect the fraction of MS2 data that can be explained by the fragmentation of a proposed structure. Compounds were also assessed for their feasibility as grapevine metabolites based on a search of the literature. Details regarding the RT, chemical formula, molecular weight, and key MS2 ions pertaining to each compound in the final list can be found in **Supplemental Table 4** (negative ionisation mode) and **Supplemental Table 5** (positive ionisation mode).

Figure 6. Principal components analysis of normalised peak areas of compounds measured in Merlot grapes with and without smoke exposure in **negative** and **positive** ionisation mode following OPLS-DA, volcano plots, and t-tests analysis. Each plot is inclusive of all samples at every time point. Colours correspond to different time points and the open or filled status of each data point represent control and smoke-affected treatments, respectively.



Several compounds were tentatively identified as endogenous plant metabolites in plants that were thought to be upregulated in berries in response to smoke exposure. This supports the hypothesis that grapevines may activate the phenylpropanoid pathway as a defense mechanism against smoke exposure. **Compound 2** (negative ion mode, V_86) was tentatively identified as 4-hydroxy-3-methoxyphenyl-β-D-glucopyranoside (tachioside) based on a search-by-mass in the *m/z* Cloud online database. As shown in **Figure 7**, there were no matching fragments detected in the MS/MS data; tachioside is nonetheless labelled as a tentative candidate because of the proximity of its exact mass to that of **Compound 2**, as well as its known existence in plants. In Spreng and Hofmann (2014), tachioside was identified as a key antioxidant in a pilsner-type beer. Tachioside has also been found as a natural substance in other plants, including the bark of birch trees (*Betula pendula*) (Šmite, *et al.* 1995) and the leaves of a Balinese long pepper (*Piper retrofractum*) (Luyen *et al.* 2014). To our knowledge, tachioside has not been identified as a naturally occurring compound in *Vitis vinifera*.

Figure 7. Structure and MS/MS spectra of **Compound 2**, tentatively identified as tachioside. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.

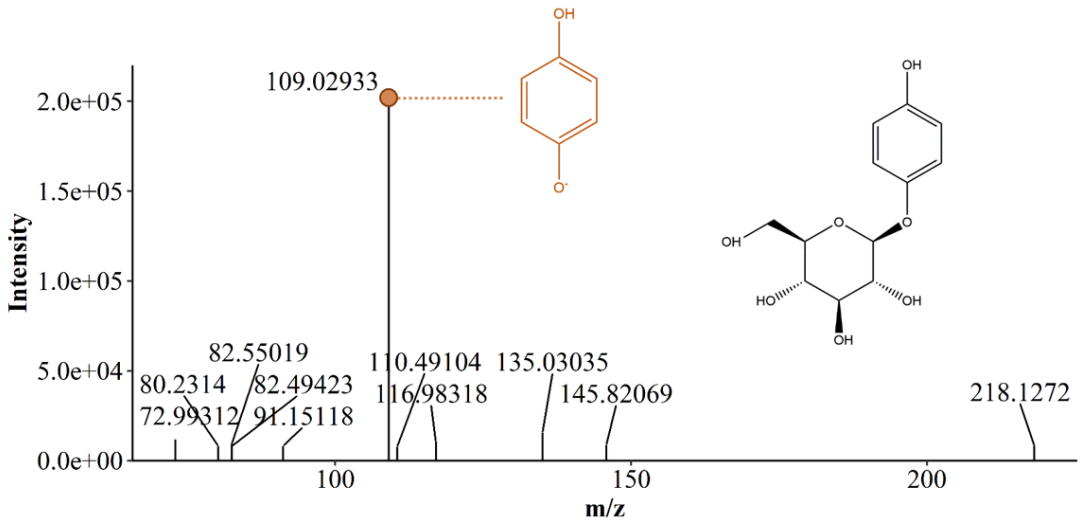


Compound 3 (negative ion mode, V_90) was putatively identified as arbutin, based on a search-by-formula in the PlantCyc database. MS/MS spectra demonstrated evidence of the deprotonated hydroquinone (*m/z* 109) (see **Figure 8**). In the cosmetics industry, arbutin serves as a key ingredient in skin whitening products due to its ability to inhibit tyrosinase, the enzyme that catalyses the synthesis of melanin (Ortiz-Ruiz *et al.* 2015). In fruit, arbutin is a naturally

occurring antioxidant in pears and lingonberries (Cui *et al.* 2015; Liu *et al*. 2014). While arbutin has not been reported in grapes, arbutin synthase (therein referred to as hydroquinone glucosyltransferase) has demonstrated high upregulation in smoke-affected grapevines (van der Hulst 2018). As expected, this enzyme demonstrates the highest specificity for hydroquinones; however it is capable of glycosylating other phenolic substrates (Hefner *et al.* 2002).

In theory, the upregulation of arbutin synthase could act as a defence mechanism in response to smoke exposure in two ways. First, the enzyme might glycosylate smoke-derived volatile phenols; second, the enzyme could produce arbutin, a compound with antioxidant properties that could defend the plant against damage incurred by reactive oxygen species. **Compound 3** might also have been furaneol 4-glucoside, which generated the same FISh score of 40 (see **Supplemental Figure 4)**.

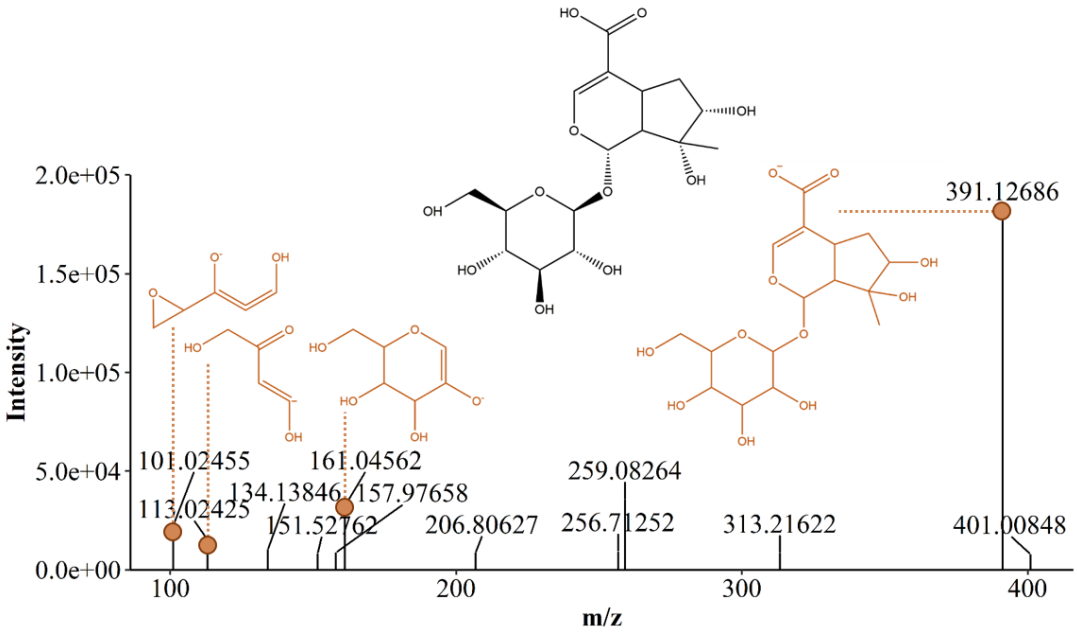
Figure 8. Structure and MS/MS spectra of **Compound 3**, tentatively identified as arbutin. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.



Compound 4 (negative ion mode, V_98) was annotated as caryoptosidic acid based on a search-by-formula in PubChem. Caryoptosidic acid (*m/z* 392) was predominantly detected as the formic acid adduct ion $[M\text{-}FA\text{-}H]^-$ (*m/z* 437) (**Supplemental Table 4**); however, in the MS/MS spectra, the deprotonated acid $[M\text{-}H]^-$ was the most prevalent fragment (see **Figure 9**). Other fragments, including *m*/z 161, *m/z* 113, and *m/z* 101, were characteristic of a glucoside (Noestheden *et al.* 2018).

Caryoptosidic acid belongs to a class of monoterpenoids called iridoids, which are known for their antioxidant properties (as reported in Heffels *et al.* 2017). While monoterpenoids, norisoprenoids, and sequiterpenoids are known contributors to grape and wine aroma (Winterhalter and Skouroumounis 1997), iridoids have yet to be reported. However, work by van der Hulst (2018) has demonstrated that grapevine smoke exposure elevates the activity of 7-deoxyloganetic acid glucosyltransferase, a key enzyme in the biosynthesis of secologanin, an iridoid in periwinkle (Asada *et al.* 2013). The upregulation of 7-deoxyloganetic acid glucosyltransferase may facilitate the production of iridoids to defend the plant against the reactive oxygen species present in smoke.

Figure 9. Structure and MS/MS spectra of **Compound 4**, tentatively identified as caryoptosidic acid. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.

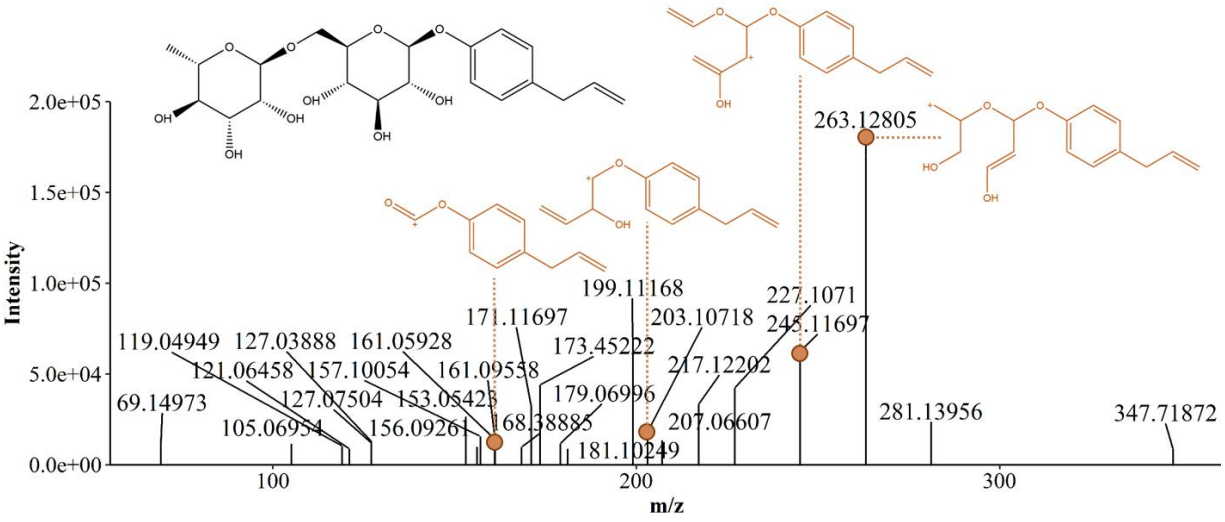


Compound 19 (positive ion mode, V_285) was tentatively annotated as lusitanicoside, based on a search-by-formula in *m/z* Cloud. This compound was detected primarily as a protonated $[M+H]^+$ ion (*m/z* 463) (**Supplemental Table 5**). The MS/MS spectra showed various fragments associated with the phenylpropene group attached to the central glucose ring (see **Figure 10**). This compound is also known as chavicol β-D-glucoside, and its free form, chavicol, has been found in numerous fruits including apples, wild musk, and melon (as

reported in Atkinson 2018). Chavicol is a naturally occurring phenylpropanoid and may reflect upregulated plant defence in response to smoke exposure.

Tentative assignments of an array of novel volatile phenol glycoconjugates were also made, supporting the hypothesis that other smoke-derived compounds are present and might further distinguish smoke-affected grapes. **Compound 6** (negative ion mode, V_137) was tentatively identified as 2-(3,4-dihydroxyphenyl)ethyl 6-*O*-β-D-glucopyranoside via a search-by-structure in ChemSpider. This compound was detected as an $[M\text{-}FA\text{-}H]^-$ adduct (*m/z* 523) (**Supplemental Table 4**) and it is an isomer of syringol gentiobioside ($C_{20}H_{30}O_{13}$). As shown in **Figure 11**, MS/MS spectra supported the presence of a gentiobioside moiety, characterized by *m*/z 323, as reported previously (Hayasaka *et al.* 2010b).

Figure 10. Structure and MS/MS spectra of **Compound 19**, tentatively identified as lusitanicoside. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.



Compound 13 (positive ion mode, V_214) is likely a rutinoside. Characterised by an *m*/z ratio of 496 (**Supplemental Table 5**), its size surpassed that of 4-methylguaiacol rutinoside (*m/z* 446), the largest rutinoside currently included in commercial analysis of smoke-affected grape and wine. To assess the similarity of the observed MS/MS spectra and that of a rutinoside, 4-methylsyringol rutinoside (*m/z* 476) was used as a theoretical template (see **Figure 12**). 4-methylsyringol was used as the aglycone in place of 4-methylguaiacol because it was supported by *m/z* 155. Several fragments were associated with the fragmentation of a rutinoside, including *m/z* 163, 145, and 85 (Noestheden *et al.* 2018).

Figure 11. Structure and MS/MS spectra of **Compound 6**, tentatively identified as lusitanicoside. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.

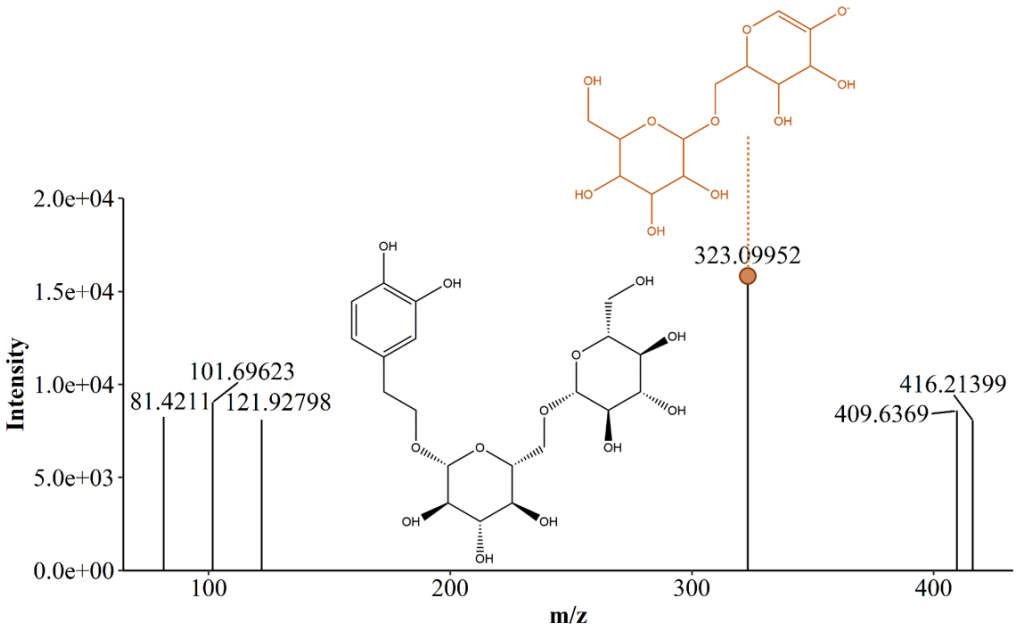
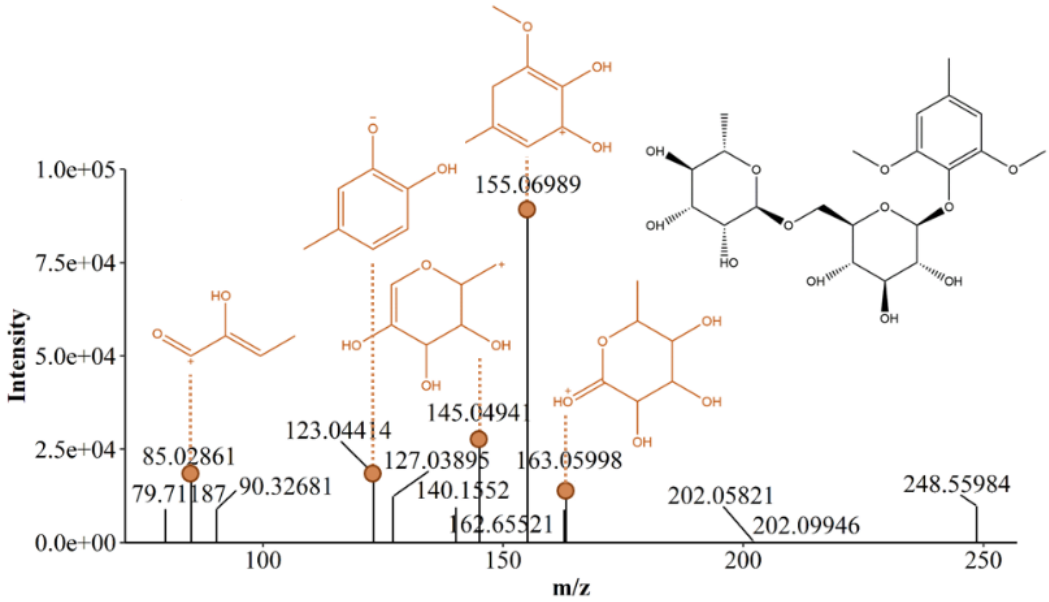
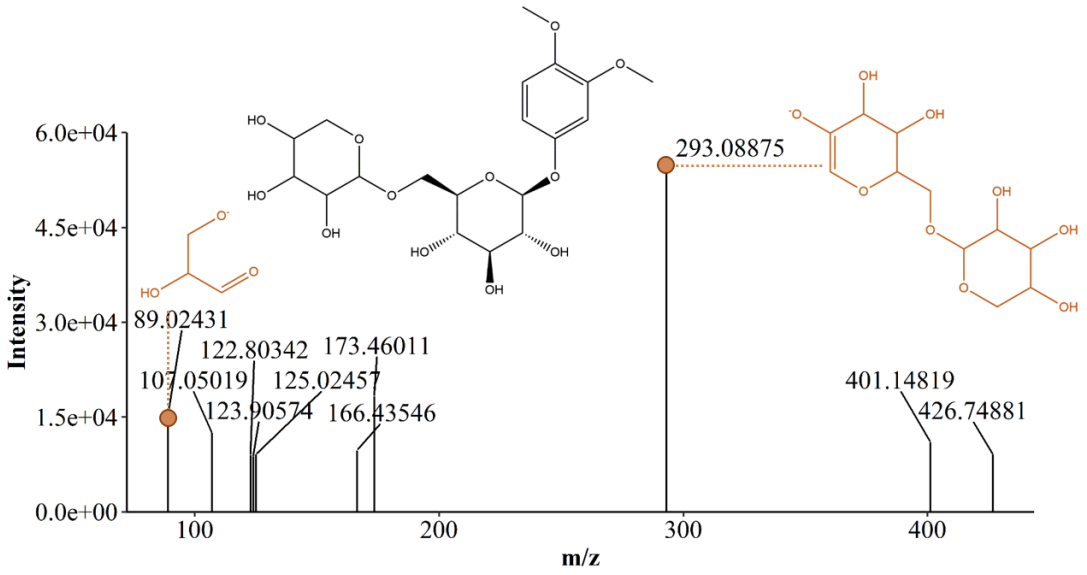


Figure 12. Structure and MS/MS spectra of **Compound 13**, tentatively identified as a novel rutinoside. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.



Compound 8 (negative ion mode, V_150) was detected as an $[M-H]^-$ ion (*m/z* 448) and tentatively identified as 3,4-dimethoxyphenyl 6-*O*-pentopyranosylhexopyranoside (**Supplemental Table 4**). MS/MS spectra showed a large *m/z* 293 fragment, which has been attributed to diglycosides with terminal pentose units in previous literature (Hayasaka *et al.* 2010b) (see **Figure 13**). The sugar moiety is a unique combination comprised of a pentopyranose (or arabinopyranose) with a hexose moiety. Previous research has demonstrated the natural presence of disaccharides in grapes comprising glucose with xylopyranose (primverosides), rhamnose (rutinosides), and glucose (gentiobiosides) (Noestheden *et al.* 2018, Hayasaka *et al.* 2010b); however, the combination of hexose and pentopyranose has not previously been reported. The aglycone 3,4-dimethoxyphenol has been observed as a constituent of liquid smoke (Guillén *et al.* 1995), but it is not a volatile phenol that is currently measured in the analysis of smoke-affected grapes and wine. In addition to the selected annotation, other possible included benzyl β-primeveroside and benzyl *O*-[arabinofuranosyl-(1→6)-glucoside] (see **Supplemental Figure 5**).

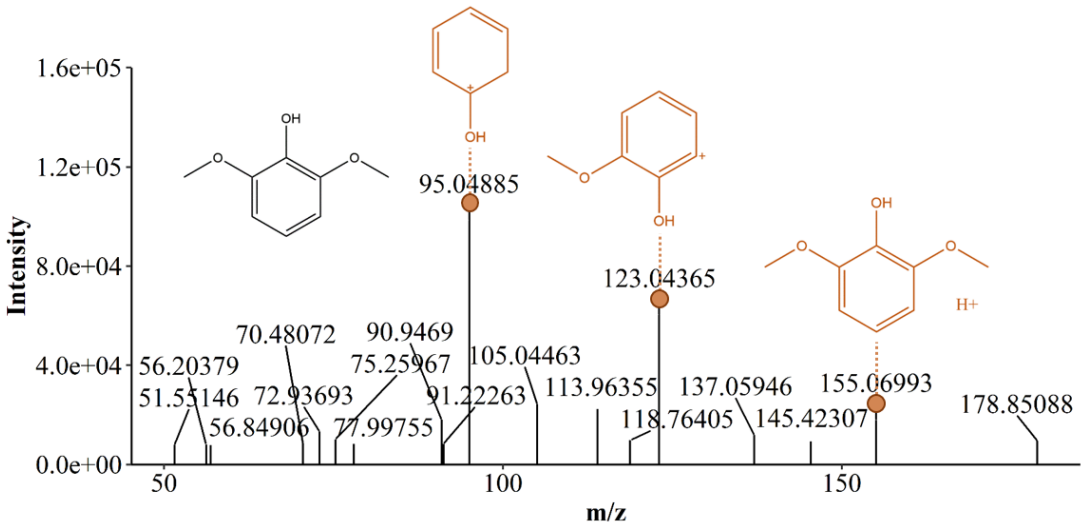
Figure 13. MS/MS spectra of **Compound 8**, tentatively identified as 3,4-dimethoxyphenol-6-*O*-pentopyranosylhexopyranoside. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.



Compound 17 (positive ion mode, V_230) is likely a volatile phenol based on a search-by-formula in ChemSpider, detected as an $[M+H]^+$ adduct (*m/z* 155) (**Supplemental Table 5**). In addition to syringol, many other volatile phenols with the same chemical formula were

found, including vanillyl alcohol, 3,5-dimethoxyphenol, 2,4-dimethoxyphenol, and 3,4-dihydroxyphenyl ethanol (see **Supplementary Figure 6**). As demonstrated by **Figure 14**, MS/MS spectra supported the loss of the first and second methoxy (i.e. $–OCH_3$) groups (*m/z* 123 and 95, respectively). It was surprising to find a free volatile phenol, not only because sampled were analysed by liquid chromatography, but also due to the time at which the compound eluted from the reverse phase column, being 21.145 min. Thus, it is likely that the putative phenol moiety is a part of a larger molecule.

Figure 14. MS/MS spectra of **Compound 17**, tentatively identified as syringol. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.



In smoke taint diagnostics, the differentiation of grapes and wine affected by smoke exposure is typically conducted by chemical and sensory analysis. The former has been restricted to volatile phenols and their glycoconjugate as markers of smoke taint, but the complex composition of smoke and the biochemically responsive nature of the grapevine during an abiotic stress event such as smoke exposure prompted us to hypothesise that additional chemical indicators may be present in grapes that could further distinguish smoke-affected grapes. Using a combination of univariate and multivariate approaches and selective filtering criteria, we were able to reveal compounds that can differentiate control and smoke-affected grapes in the weeks following grapevine smoke exposure. Additional work is required to isolate and confirm the identity of the compounds generated through the present workflow.

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Supplemental Data

Beyond volatile phenols: revealing additional markers of smoke taint in grapevines (*Vitis vinifera* L.) cv. Merlot

Colleen Szeto[1,2], Natoiya Lloyd[3,4], Luca Nicolotti[3,4], Markus Herderich[2,3,4], and Kerry Wilkinson[1,2]

[1]Department of Wine Science and Waite Research Institute, The University of Adelaide, Glen Osmond, SA 5064, Australia
[2]Australian Research Council Training Centre for Innovative Wine Production, Glen Osmond, SA 5064, Australia
[3]Metabolomics Australia, The Australian Wine Research Institute, Glen Osmond, SA 5064, Australia
[4]The Australian Wine Research Institute, Glen Osmond, SA 5064, Australia

***Corresponding author**: Prof. Kerry Wilkinson, telephone: +61 8 8313 7360, email: kerry.wilkinson@adelaide.edu.au

Supplementary Table 1. List of all settings pertaining to each node of the modified "Untargeted Metabolomics with Statistics Detect Unknowns with ID using Online Databases and mzLogic" workflow in Compound Discoverer.

Node	*Settings*
Select Spectra	**General settings** • Precursor Selection: Use MS(n - 1) Precursor • Use Isotope Pattern in Precursor Re-evaluation: True • Provide Profile Spectra: Automatic • Store Chromatograms: False **Spectrum Properties Filter** • Lower RT Limit: 0 • Upper RT Limit: 0 • First Scan: 0 • Last Scan: 0 • Ignore Specified Scans: (not specified) • Lowest Charge State: 0 • Highest Charge State: 0s • Min. Precursor Mass: 0 Da • Max. Precursor Mass: 5000 Da • Total Intensity Threshold: 0 • Minimum Peak Count: 1 **Scan Event Filters** • Mass Analyzer: (not specified) • MS Order: Any • Activation Type: (not specified) • Min. Collision Energy: 0 • Max. Collision Energy: 1000 • Scan Type: Any • Polarity Mode: (not specified) **Peak Filters** • S/N Threshold (FT-only): 1.5 **Replacements for Unrecognized Properties** • Unrecognized Charge Replacements: 1 • Unrecognized Mass Analyzer Replacements: ITMS • Unrecognized MS Order Replacements: MS2 • Unrecognized Activation Type Replacements: CID • Unrecognized Polarity Replacements: + • Unrecognized MS Resolution@200 Replacements: 60000 • Unrecognized MSn Resolution@200 Replacements: 30000
Align Retention Times	**General Settings** • Alignment Model: Adaptive curve • Alignment Fallback: Use Linear Model • Maximum Shift [min]: 2 • Shift Reference File: True • Mass Tolerance: 5 ppm Remove Outlier: True
Detect Compounds	**General Settings** • Mass Tolerance [ppm]: 5 ppm • Intensity Tolerance [%]: 30

	• S/N Threshold: 3 • Min. Peak Intensity: 100000 • Ions: [2M+H]+1; [M+ACN+H]+1; [M+H]+1; [M+H+MeOH]+1; [M+H-H2O]+1 • Base Ions: [M+H]+1; [M-H]-1 • Min. Element Counts: C H • Max. Element Counts: C90 H190 N5 O40 S5 **Peak Detection** • Filter Peaks: True • Max. Peak Width [min]: 0.5 • Remove Singlets: True • Min. # Scans per Peak: 5 • Min. # Isotopes: 1
Group Compounds	**Compound Consolidation** • Mass Tolerance: 5 ppm • RT Tolerance [min]: 0.2 **Fragment Data Selection** • Preferred Ions**:** [M+H]+1
Fill Gaps	**General Settings** • Mass Tolerance: 5 ppm • S/N Threshold: 1. • Use Real Peak Detection: True
Normalize Areas	**QC-based Area Correction** • Regression Model: Cubic Spline • Min. QC Coverage [%]: 50 • Max. QC Area RSD [%]: 30 • Max. # Files Between QC Files: 15 **Area Normalization** • Normalization Type: [None] • Exclude Blanks: True **Scaling Factor** • Study Factor Name: (not specified)
Mark Background Compounds	**General Settings** • Max. Sample/Blank: 10 • Max. Blank/Sample: 0 • Hide Background: True
Assign Compound Annotations	**General Settings** • Mass Tolerance: 5 ppm **Data Sources** • Data Source #1: MassList Search • Data Source #2: Predicted Compositions • Data Source #3: mzCloud Search • Data Source #4: mzVault Search • Data Source #5: Metabolika Search • Data Source #6: ChemSpider Search • Data Source #7: (not specified) **Scoring Rules** • Use mzLogic: True • Use Spectral Distance: True • SFit Threshold: 20 • SFit Range: 20
Search mzCloud	**General Settings**

	• Compound Classes: All • Precursor Mass Tolerance: 10 ppm\ • FT Fragment Mass Tolerance: 10 ppm • IT Fragment Mass Tolerance: 0.4 Da • Library: Autoprocessed; Reference • Post Processing: Recalibrated • Max. # Results: 10 • Annotate Matching Fragments: True **DIA Search** • Use DIA Scans for Search: False • Max. Isolation Width [Da]: 500 • Match Activation Type: False • Match Activation Energy: Any • Activation Energy Tolerance: 10 • Apply Intensity Threshold: False • Match Factor Threshold: 20
Search mzVault	**Search Settings** • mzVault Library: RP_Phenolics.db; StdMix1.db; StdMix2.db • Max. # Results: 10 • Match Factor Threshold: 50 • Search Algorithm: HighChem HighRes • Match Analyzer Type: True • IT Fragment Mass Tolerance: 0.4 Da • FT Fragment Mass Tolerance: 10 ppm • Use Retention Time: False • Precursor Mass Tolerance: 10 ppm • Apply Intensity Threshold: True • Match Ionization Method: True • Ion Activation Energy Tolerance: 20 • Match Ion Activation Energy: Match with Tolerance • Match Ion Activation Type: True • Compound Classes: All • Remove Precursor Ion: True • RT Tolerance [min]: 2
Predict Compositions	**Prediction Settings** • Mass Tolerance: 5 ppm • Min. Element Counts: C H • Max. Element Counts: C90 H190 N5 O40 S5 • Min. RDBE: 0 • Max. RDBE: 40 • Min. H/C: 0. • Max. H/C: 4 • Max. # Candidates: 10 • Max. # Internal Candidates: 200 **Pattern Matching** • Intensity Tolerance [%]: 30 • Intensity Threshold [%]: 0.1 • S/N Threshold: 3 • Min. Spectral Fit [%]: 3 • Min. Pattern Cov. [%]: 9 • Use Dynamic Recalibration: True

	Fragments Matching • Use Fragments Matching: True • Mass Tolerance: 5 ppm • S/N Threshold: 3
Search Mass Lists	**Search Settings** • Mass Lists: Arita Lab 6549 Flavonoid Structure Database.masslist; Smoke taint.massList; 20210203_smoke_taint_positive_ion_mode_V3.massList • Mass Tolerance: 5 ppm • Use Retention Time: True • RT Tolerance [min]: 2
Search ChemSpider	**Search Settings** • Databases: Food and Agriculture Organization of the United Nations; Nature Chemistry; PubMed; Royal Society of Chemistry; Springer Nature; Toronto Research Chemicals; UsefulChem • Search Mode: By Formula or Mass • Mass Tolerance: 5 ppm • Max. # of results per compound: 50 • Max. # of Predicted Compositions to be searched per Compound: 5 • Result Order (for Max. # of results per compound): Order By Reference Count (DESC) **Predicted Composition Annotation** • Check All Predicted Compositions: False
Map to Metabolika Pathways	**Search Settings** • Metabolika Pathways: Cellulose and hemicellulose degradation (cellulolosome); Meta cleavage pathway of aromatic compounds; Reactive oxygen species degradation; Sucrose biosynthesis I (from photosynthesis); Superpathway of anthocyanin biosynthesis (from cyanidin and cyanidin 3-O-glucoside); Superpathway of anthocyanin biosynthesis (from delphinidin 3-O-glucoside); Superpathway of anthocyanin biosynthesis (from pelargonidin 3-O-glucoside); Superpathway of carotenoid biosynthesis • Search Mode: By Formula or Mass **By Mass Search Settings** • Mass Tolerance: 5 ppm **By Formula Search Settings** • Max. # of Predicted Compositions to be searched per Compound: 3 **Display Setting** • Max. # Pathways in 'Pathways' column: 20
Apply mzLogic	**Search Settings** • FT Fragment Mass Tolerance: 10 ppm • IT Fragment Mass Tolerance: 0.4 Da • Max. # Compounds: 0 • Max. # mzCloud Similarity Results to consider per Compound: • Match Factor Threshold: 60

Supplemental Table 2. Concentrations of volatile phenols in Merlot grape juice from control and smoke-exposed grapes sampled from pre-smoke exposure (t=0 hours) to commercial maturity (t=20 days). Values represent means with standard errors from six biological replicates. **Bold numbers** indicate a statistically significant difference between control and smoke-affected grapes at each time point (Mann-Whitney U Test, $p<0.05$). Blank values indicate that volatile phenols were either not detected or below their LOQ.

Treatment	**Time point**	guaiacol	4-methylguaiacol	phenol	*o*-cresol	*p*-cresol	*m*-cresol	syringol	4-methylsyringol
Control	0 hours	-	-	-	-	-	-	-	-
	2 hours	-	-	-	-	-	-	-	-
	24 hours	-	-	-	-	-	-	-	-
	6 days	-	-	-	-	-	-	-	-
	20 days	-	-	-	-	-	-	-	-
Smoke	0 hours	-	-	-	-	-	-	-	-
	2 hours	**17 ± 2**	-	**20 ± 2**	**5 ± 0.6**	-	-	**61 ± 5**	-
	24 hours	-	-	-	-	-	-	-	-
	6 days	-	-	-	-	-	-	-	-
	20 days	-	-	-	-	-	-	-	-

Supplemental Table 3. Concentrations of volatile phenol glycoconjugates in Merlot grape homogenate (μg/kg) from control and smoke-exposed grapes sampled from pre-smoke exposure (t=0 hours hours) to commercial maturity (t=20 days). Values represent means and standard error of the mean (as syringol gentiobioside equivalents) from up to six biological replicates. **Bold numbers** indicate a statistically significant difference between control and smoke-affected grapes at each time point (Mann-Whitney U Test, $p<0.05$). Blank values indicate that glycoconjugates were either not detected or below their LOQ. Gu = guaiacol, 4MGu = 4-methylguaiacol, Ph = phenol, G = glucoside, GB = gentiobioside, PG = pentose glucoside, and R = rutinoside.

Time point	Treatment	GuG	GuGB	GuPG	GuR	4MGuG	4MGuPG	4MGuR	PhG	PhGB	PhPG	PhR
0 hours	Control	-	-	4.1 ± 0.3	-	-	2.4 ± 0.2	1.1 ± 0.1	-	-	21 ± 2	-
	Smoke	-	-	3.4 ± 0.3	-	-	2.0 ± 0.1	1.0 ± 0.1	-	-	17 ± 1	-
2 hours	Control	-	-	3.4 ± 0.1	-	-	2.2 ± 0.1	1.0 ± 0.1	-	-	15 ± 1	-
	Smoke	**4.6 ± 0.7**	**4.7 ± 0.3**	**14 ± 0.8**	**1.4 ± 0.1**	**1.6 ± 0.1**	**5.5 ± 0.3**	**2.9 ± 0.2**	**1.5 ± 0.5**	**-**	**23 ± 2**	**1.1 ± 0.1**
24 hours	Control	-	-	3.6 ± 0.2	-	-	2.3 ± 0.1	1.1 ± 0.1	-	-	17 ± 1	-
	Smoke	**5.1 ± 0.6**	**12 ± 0.6**	**37 ± 2**	**3.2 ± 0.0**	**1.5 ± 0.1**	**11 ± 0.5**	**6.2 ± 0.5**	**2.4 ± 0.9**	**-**	**55 ± 3**	**4.3 ± 0.1**
6 days	Control	-	-	4.6 ± 0.3	-	-	2.5 ± 0.1	1.4 ± 0.1	-	-	18 ± 1	-
	Smoke	**3.3 ± 0.4**	**21 ± 2**	**71 ± 6**	**8.0 ± 0.6**	-	**16 ± 2**	**11 ± 1**	**11 ± 3**	**1.4 ± 0.2**	**114 ± 13**	**13 ± 1**
13 days	Control	-	-	5.1 ± 0.3	-	-	2.6 ± 0.1	1.9 ± 0.2	-	-	19 ± 1	-
	Smoke	**1.9 ± 0.1**	**28 ± 3**	**86 ± 5**	**11 ± 0.4**	-	**17 ± 2**	**12 ± 1**	**4.0 ± 0.5**	**1.7 ± 0.2**	**147 ± 16**	**19 ± 2**
20 days	Control	-	-	5.6 ± 0.3	-	-	2.7 ± 0.1	2.2 ± 0.1	-	-	22 ± 1	-
	Smoke	**1.7 ± 0.1**	**36 ± 5**	**88 ± 6**	**12 ± 0.5**	-	**17 ± 2**	**12 ± 1**	**5.6 ± 1**	**1.8 ± 0.2**	**160 ± 21**	**21 ± 3**

Table continues onto next page.

Supplemental Table 3 (continued). Concentrations of volatile phenol glycoconjugates in Merlot grape homogenate (μg/kg) from control and smoke-exposed grapes sampled from pre-smoke exposure (t=0 hours hours) to commercial maturity (t=20 days). Values represent means and standard error of the mean (as syringol gentiobioside equivalents) from up to six biological replicates. **Bold numbers** indicate a statistically significant difference between control and smoke-affected grapes at each time point (Mann-Whitney U Test, $p<0.05$). Blank values indicate that glycoconjugates were either not detected or below their LOQ. Cr = cresol, Sy = syringol, 4MSy = 4-methylsyringol, G = glucoside, GB = gentiobioside, PG = pentose glucoside, and R = rutinoside.

Time point	Treatment	CrG	CrGB	CrPG	CrR	SyG	SyGB	SyPG	4MSyGB	4MSyPG
0 hours	Control	-	1.0 ± 0.1	18 ± 0.9	1.4 ± 0.1	1.5 ± 0.5	2.1 ± 0.2	1.3 ± 0.1	-	1.4 ± 0.1
	Smoke	-	-	15 ± 1	1.2 ± 0.1	0.9 ± 0.2	1.8 ± 0.2	1.2 ± 0.1	-	1.2 ± 0.1
2 hours	Control	-	1.3 ± 0.1	16 ± 0.9	1.2 ± 0.1	1.0 ± 0.1	2.1 ± 0.1	1.2 ± 0.0	-	1.2 ± 0.1
	Smoke	**31 ± 3**	-	**32 ± 2**	**4.4 ± 0.4**	**4.0 ± 0.2**	**18 ± 0.8**	**1.9 ± 0.1**	**1.9 ± 0.1**	1.3 ± 0.1
24 hours	Control	-	1.1 ± 0.0	17 ± 0.8	1.2 ± 0.1	-	2.2 ± 0.1	1.2 ± 0.0	-	1.2 ± 0.1
	Smoke	**41 ± 4**	1.0 ± 0.1	**74 ± 5**	**16 ± 1**	**6.9 ± 0.7**	**57 ± 4**	**5.1 ± 0.2**	**5.0 ± 0.4**	**2.2 ± 0.2**
6 days	Control	-	1.6 ± 0.1	18 ± 0.7	1.7 ± 0.1	1.4 ± 0.2	2.6 ± 0.2	1.4 ± 0.1	-	1.1 ± 0.1
	Smoke	**12 ± 2**	**1.3 ± 0.1**	**127 ± 16**	**36 ± 5**	**4.5 ± 0.5**	**120 ± 14**	**9.7 ± 1**	**8.2 ± 0.9**	**2.7 ± 0.3**
13 days	Control	-	1.6 ± 0.1	19 ± 1	1.9 ± 0.1	1.4 ± 0.3	2.8 ± 0.1	1.5 ± 0.1	-	-
	Smoke	**4.9 ± 0.7**	1.4 ± 0.1	**145 ± 21**	**39 ± 6**	2.8 ± 0.3	**135 ± 18**	**11 ± 1**	**7.9 ± 1**	**2.7 ± 0.3**
20 days	Control	-	1.9 ± 0.1	20 ± 0.8	2.4 ± 0.2	1.8 ± 0.3	3.3 ± 0.1	1.7 ± 0.1	-	1.0 ± 0.0
	Smoke	**3.7 ± 0.3**	1.6 ± 0.1	**140 ± 16**	**44 ± 6**	**1.8 ± 0.2**	**150 ± 18**	**12 ± 1**	**8.6 ± 0.6**	**2.6 ± 0.2**

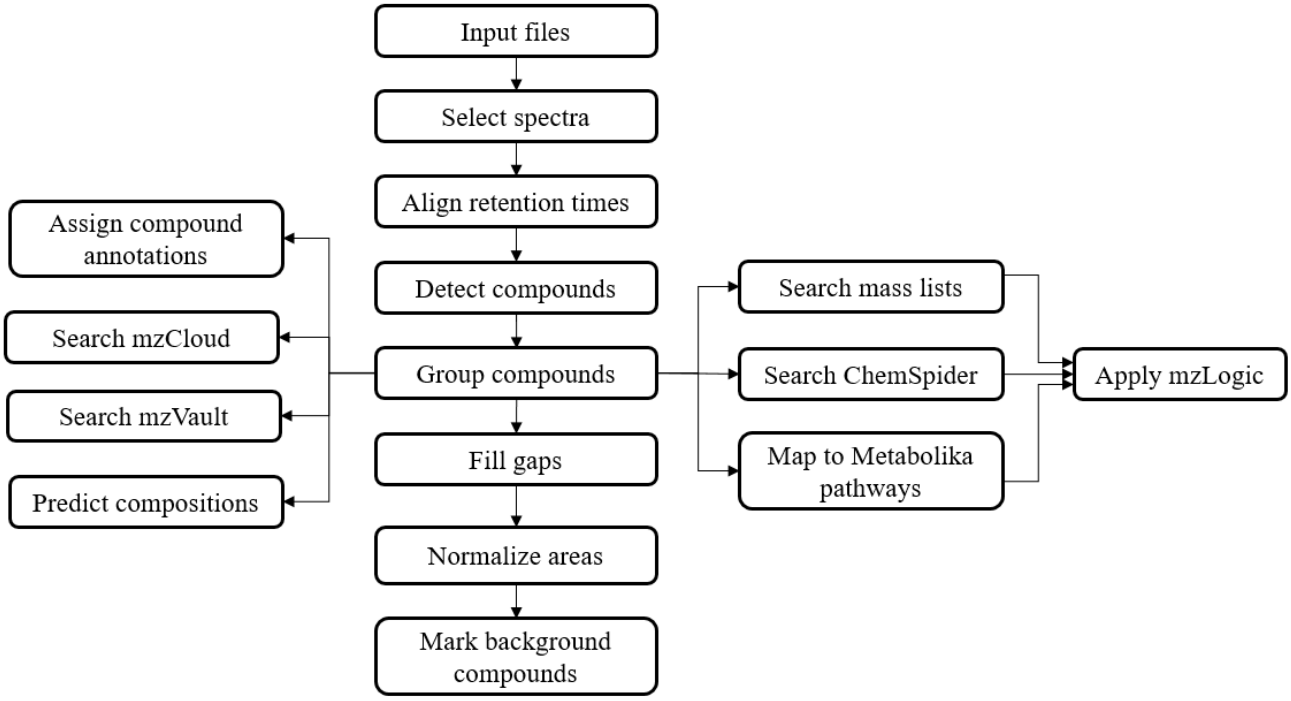
Supplemental Table 4. Tentatively identified compounds in negative ion mode that differentiate control and smoke-affected Merlot grapes. Name, chemical formula, exact mass, adduct, Fragment Ion Search (FISh scores), and MS2 product ions are shown where available. Formulas were generated in Compound Discoverer and possible solutions were limited to the following elements: C, H, N, O, P, and S. Exact masses were calculated from the most abundant isotopes of the elements comprising each compound. FISh scores were computed in Compound Discoverer, based on how much a proposed structure could account for the fragmentation pattern in an unknown peak. Product ions in **boldface** type were the most abundant in the selected spectra.

Compound ID	Name	Formula	RT (min)	Exact mass	Adduct	m/z	FISh Score	MS2 Product ions
1 (V_65)		$C_{13}H_{20}O_{10}$	4.358	336.105516	[M-H]	335.09824	-	222.06807, 154.99892, 144.92371, 98.46259, 85.79871
2 (V_86)	4-hydroxy-3-methoxyphenyl-β-D-glucopyranoside (Tachioside)	$C_{13}H_{18}O_8$	7.111	302.099496	[M-H]	301.09222	0	222.05687, 185.62344, 177.78032, **173.43906**, 161.65952, 136.99142, 121.02953, 115.34635, 104.79520
3 (V_90)	arbutin	$C_{12}H_{16}O_7$	8.639	272.088846	[M-H]	271.08157	40	218.1270, 145.82069, 135.03035, 116.98318, **109.02933,** 82.55019, 80.23140, 72.99312
4 (V_98)	caryoptosidic acid	$C_{16}H_{24}O_{11}$	10.063	392.13186	[M+FA-H]	437.12848	72	401.00848, **391.12686**, 313.21622, 259.08264, 256.71252, 161.04562, 134.13846, 113.0242, 101.0245
5 (V_115)		-	14.158	-	[M-H]	433.13617	-	324.81854, **293.08890**, 174.83430, 128.97581, 116.38879
6 (V_137)	2-(3,4-dihydroxyphenyl)ethyl 6-O-β-D-glucopyranoside	$C_{20}H_{30}O_{13}$	19.659	478.16864	[M+FA-H]	523.16644	100	416.21399, 406.63690, **323.09952,** 121.92798, 101.69623, 81.42110
7 (V_141)		$C_{30}H_{40}O_{14}S$	20.446	656.21376	[M-H]	655.20654	-	637.05676, 455.14655, 235.34605, 118.68836, 83.70055
8 (V_150)	3,4-dimethoxyphenyl 6-O-pentopyranosylhexopyranoside	$C_{19}H_{28}O_{12}$	22.160	448.15715	[M-H]	447.14966	50	426.74881, 401.14819, **293.08875**, 173.46011, 166.43546, 89.02431

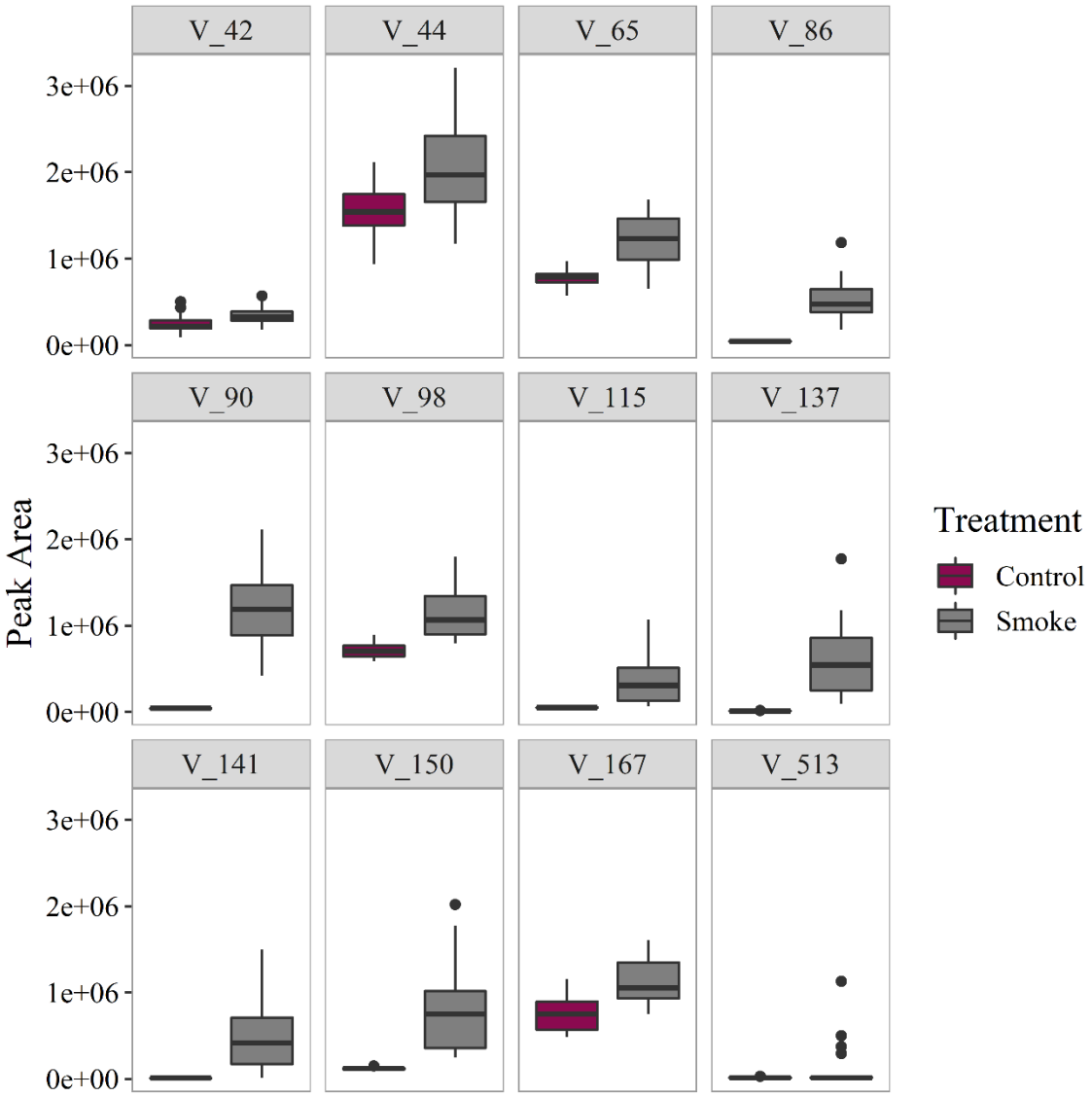
Supplemental Table 5. Tentatively identified compounds in positive ion mode that differentiate control and smoke-affected Merlot grapes. Name, chemical formula, exact mass, adduct, Fragment Ion Search (FISh scores), and MS2 product ions are shown where available. Formulas were generated in Compound Discoverer and possible solutions were limited to the following elements: C, H, N, O, P, and S. Exact masses were calculated from the most abundant isotopes of the elements comprising each compound. FISh scores were computed in Compound Discoverer, based on how much a proposed structure could account for the fragmentation pattern in an unknown peak. Product ions in **boldface** type were the most abundant in the selected spectra.

Compound ID	Name	Formula	RT (min)	Mass	A	m/z	FISh Score	MS/MS Product ions
1 (V_23)		$C_{22}H_{17}NO_6$	2.883	391.10586	[M+H]	392.11319	-	389.84644, 252.72415, 234.93306, 161.76767, 149.58134, 121.50095, 101.17762, 67.73016
2 (V_54)		$C_{14}H_{29}NO_8$	4.223	339.18960	[M+H]	340.19684	-	324.47116, 209.60641, 140.301212, 88.25010, 70.23579, 65.89900
3 (V_58)		$C_{13}H_{17}N_5O_4$	4.337	307.12704	[M+H]	308.13431	-	308.17093, 214.73059, 211.97791, 201.01224, 131.26486, **111.04372**, 83.04934
4 (V_59)		$C_{14}H_{25}NO_8$	4.338	335.15835	[M+H]	336.16556	-	241.47081, 202.05797, 138.06606, 132.49782
5 (V_60)		$C_{13}H_{20}O_7$	4.338	312.08243	[M+H]	313.08975	-	**313.09018**, 244.40137, 210.18295, 200.03398, 113.70119, 118.25055, 89.34587, 83.54485
6 (V_87)		$C_{13}H_{27}NO_7$	5.195	309.17904	[M+H]	310.18634	-	179.09314, 103.50321, 75.67420, 59.23890
7 V_95)		$C_{18}H_{31}NO_{12}$	6.746	453.18512	[M+H]	454.19238	-	440.53296, 269.80685, 257.11310, 172.03996, 150.94844, 138.52840, **113.05936**
8 (V_96)		$C_{20}H_{35}NO_{12}$	6.748	481.21648	[M+H]	482.22375	-	344.09787, 330.16290, 232.44911, 145.04950, **113.05947**, 104.49859, 88.64746
9 (V_122)		$C_{18}H_{31}NO_{11}$	10.045	437.19023	[M+H]	438.19748	-	325.08737, 296.16986, 286.44464, 274.92377, 84.69194, 69.90922
10 (V_123)		$C_{17}H_{23}N_5O_7$	10.046	409.15903	[M+H]	410.16641	-	395.84903, 281.81772, 213.07617, 197.54613, **133.04909**, **123.04343**, **115.03847**, 97.0287, **81.03310**
11 (V_164)		$C_{18}H_{23}N_5O_6$	14.138	405.16392	[M+H]	406.17136	-	412.22498, 280.30133, 197.03401, 194.69070, 166.27464, 145.0495, 143.04051, 133.04991, 66.28886
12 (V_213)		$C_{22}H_{37}NO_{13}$	19.650	523.22709	[M+H]	524.23438	-	**524.233358**, 349.43805, 331.60492, 248.24800, 235.56512, 233.75864, 163.67529, 126.81951, 85.13928
13 (V_214)	rutinoside	-	19.659	495.19580	-	496.20309	67	248.55984, **163.05998**, **155.06989**, **145.04941**, **123.04414**, 85.02861, 90.32681
14 (V_220)		$C_{29}H_{38}O_{15}$	20.454	627.23771	[M+H]	628.24536	-	461.40399, 358.328.7712, 163.06082, **155.06990**, 127.0384, 115.95625, 87.91180
15 (V_226)		$C_{19}H_{25}N_5O_7$	20.785	435.17443	[M+H]	436.18176	-	202.05746, 133.04985, 127.03884, 97.02799
16 (V_229)		$C_{16}H_{27}NO_8$	21.131	361.17391	[M+H]	362.18121	-	323.46265, 168.33810, 133.18292, 115.71628, 104.78747
17 (V_230)	syringol	$C_8H_{10}O_3$	21.145	154.06284	[M+H]	155.07011	36	202.05739, 175.12941, **123.04371**, 113.96336**,** 105.04426, **95.04879**, 93.22483, 72.93705
18 (V_284)		$C_{15}H_{18}O_4$	23.020	262.12048	[M+H]	263.12772	-	221.43633, 199.11153, 189.93918, 171.11774, 153.05406, 156.09245, 141.98993, 57.60071
19 (V_285)	lusitanicoside	$C_{21}H_{30}O_{10}$	23.021	442.18434	[M+H]	443.19165	33	347.71872, 281.13956, **263.12805, 245.10710**, **199.11168**, **173.45222**, 171.11697, 161.09558, 153.05423, 127.03888
20 (V_479)		$C_{14}H_{22}O_3$	25.090	238.15701	[M+H]	239.16426	-	206.24719, 129.77292, 109.10078, 97.87645
21 (V_481)		$C_{20}H_{32}O_8$	25.100	400.21009	[M+H]	401.21741	-	376.76575, 277.54495, **239.16483**, 153.09033, 108.05788, 68.94369

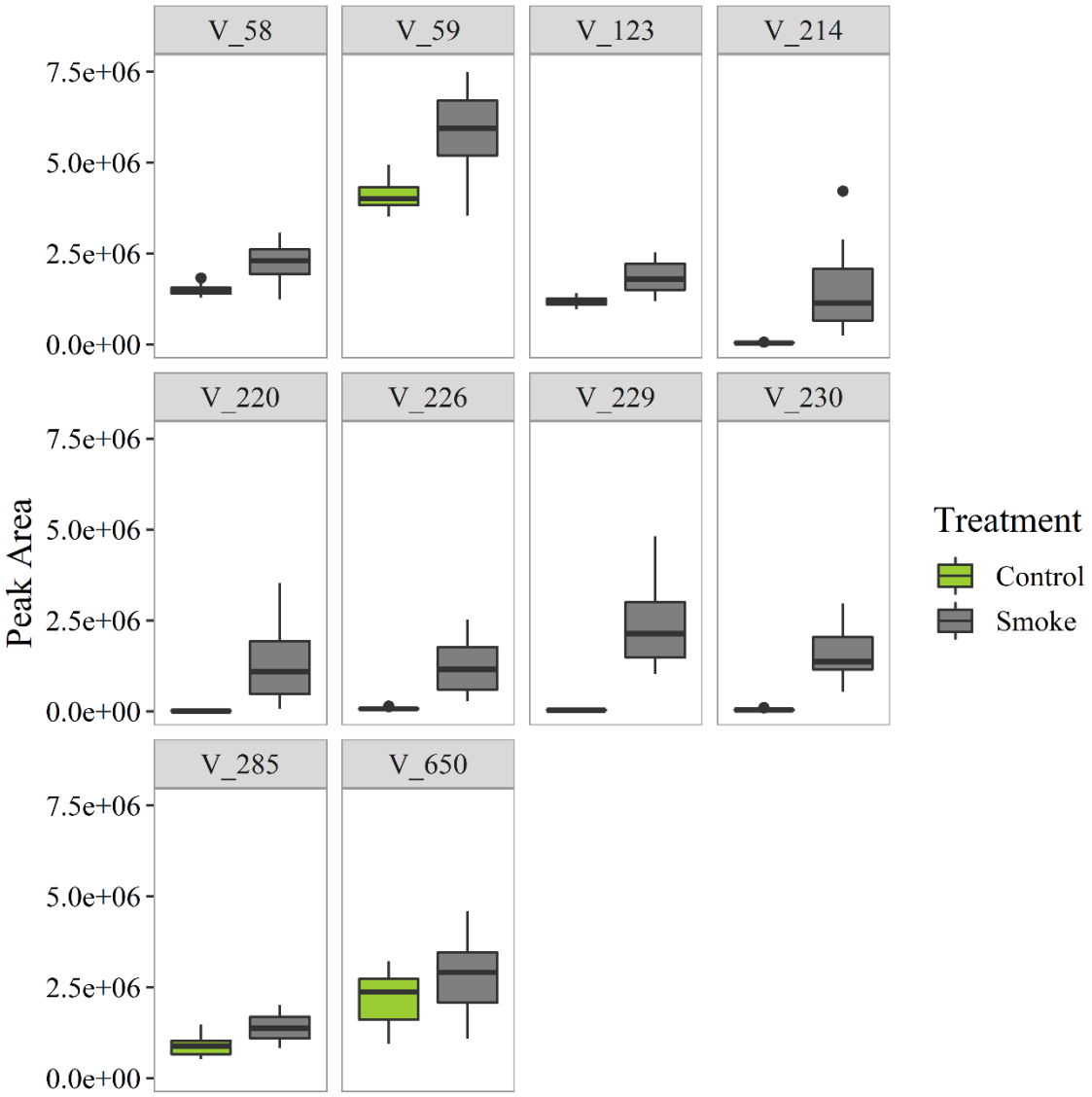
Supplemental Figure 1. Modified data processing workflow, based on the "Untargeted Metabolomics with Statistics Detect Unknowns with ID using Online Databases and mzLogic" template in Compound Discoverer.



Supplemental Figure 2. Box-and-whisker plots demonstrating mean differences in pre-processed peak areas differentiating features detected in control and smoke-affected Merlot grapes when analysed using **negative** ionisation mode.



Supplemental Figure 3. Box-and-whisker plots demonstrating mean differences in pre-processed peak areas differentiating features detected in control and smoke-affected Merlot grapes when analysed using **positive** ionisation mode.



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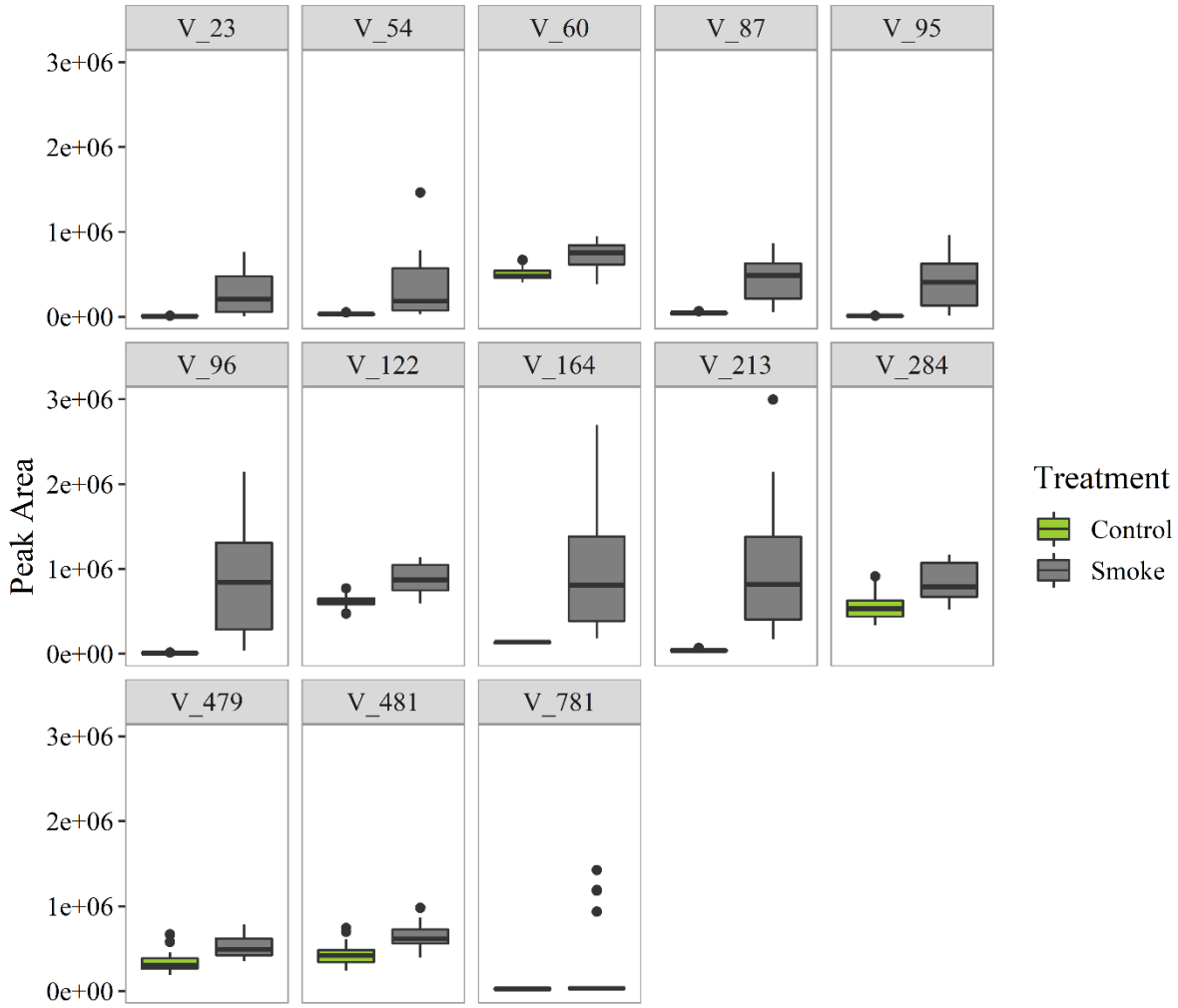
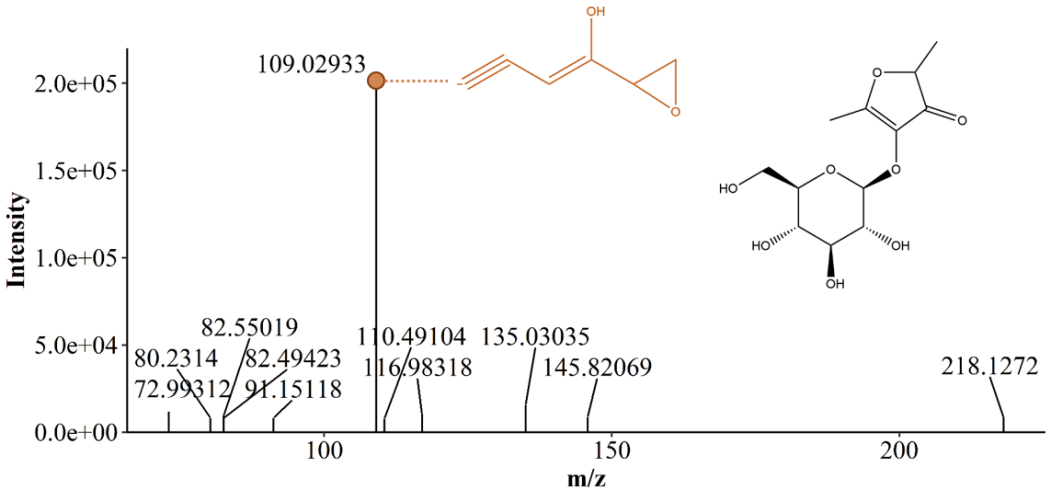
Supplemental Figure 3 (continued)**.** Box-and-whisker plots demonstrating mean differences in pre-processed peak areas differentiating features detected in control and smoke-affected Merlot grapes when analysed using **positive** ionisation mode.

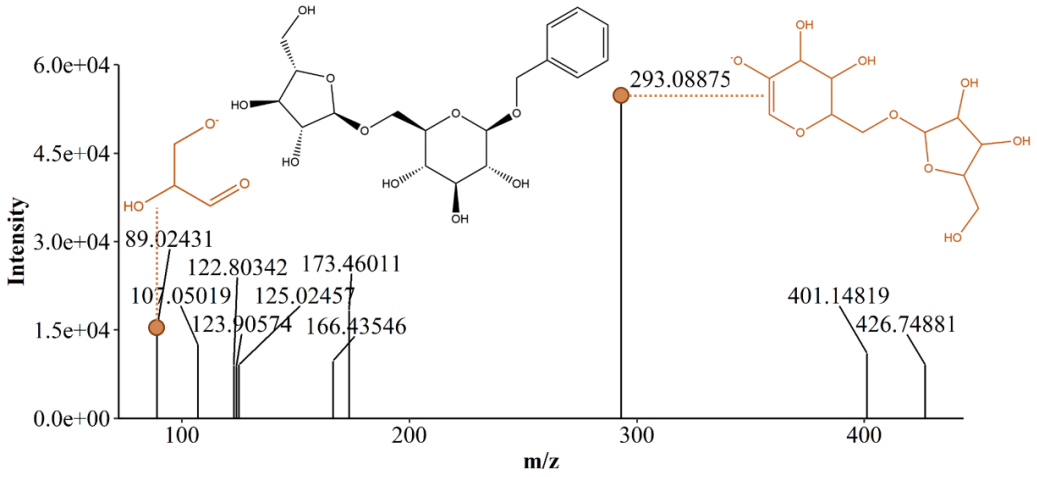


Supplemental Figure 4. MS/MS spectra for **Compound 3**, tentatively identified as 4-furaneol glucoside. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.

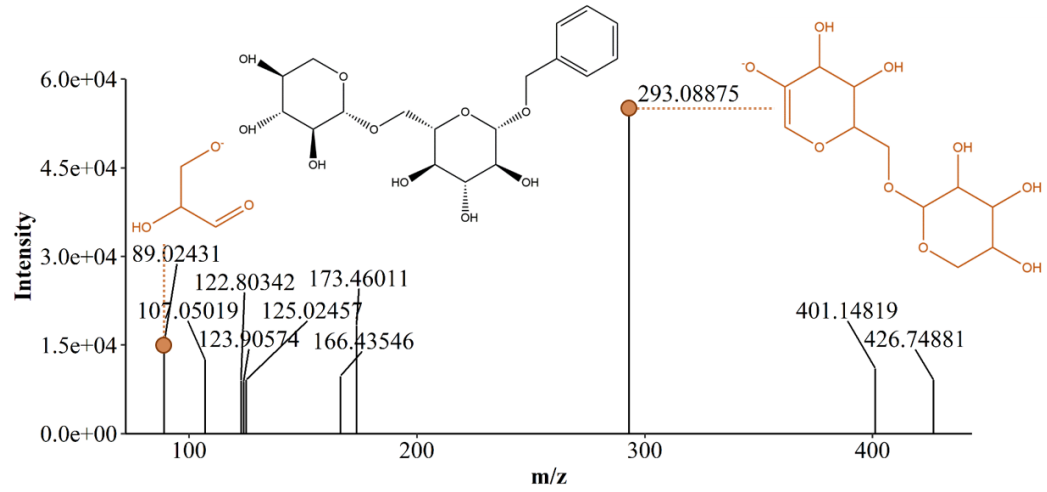


Supplemental Figure 5. MS/MS spectra for **Compound 6**, tentatively identified as (a) benzyl-*O*-[arabinofuranosyl-1->6] glucoside and (b) benzyl primeveroside. The complete structures of the proposed compounds are drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.

(a) benzyl-O-[arabinofuranosyl-1->6] glucoside

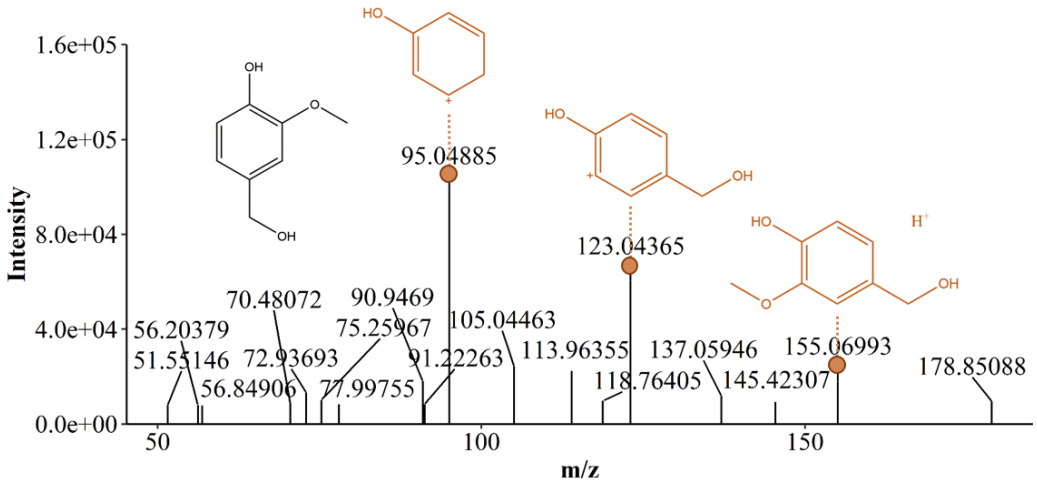


(b) benzyl-β-primeveroside

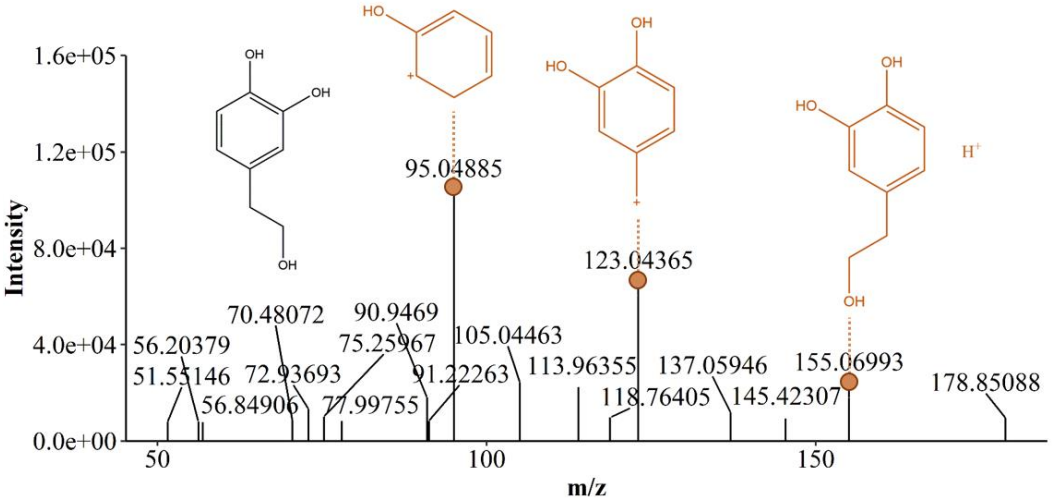


Supplemental Figure 6. MS/MS spectra of **Compound 17**, tentatively identified as (a) vanillyl alcohol, (b) 3,4-dihydroxyphenyl ethanol, (c) 2,4-dimethoxyphenol, and (d) 3,5-dimethoxyphenol. The complete structure of the proposed compound is drawn in **black**. Ions with *m/z* values matching a theoretical fragment ion are highlighted and their structures are drawn in **orange**.

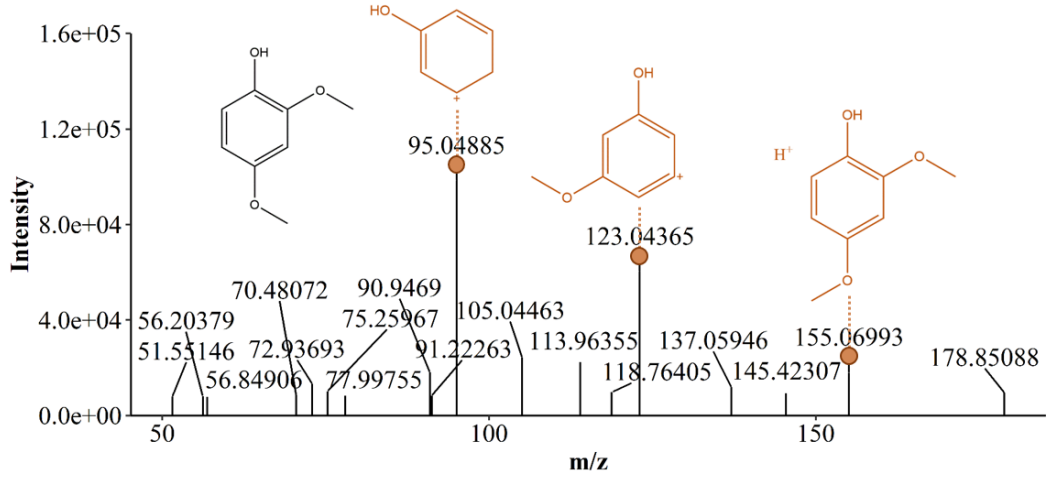
(a) vanillyl alcohol



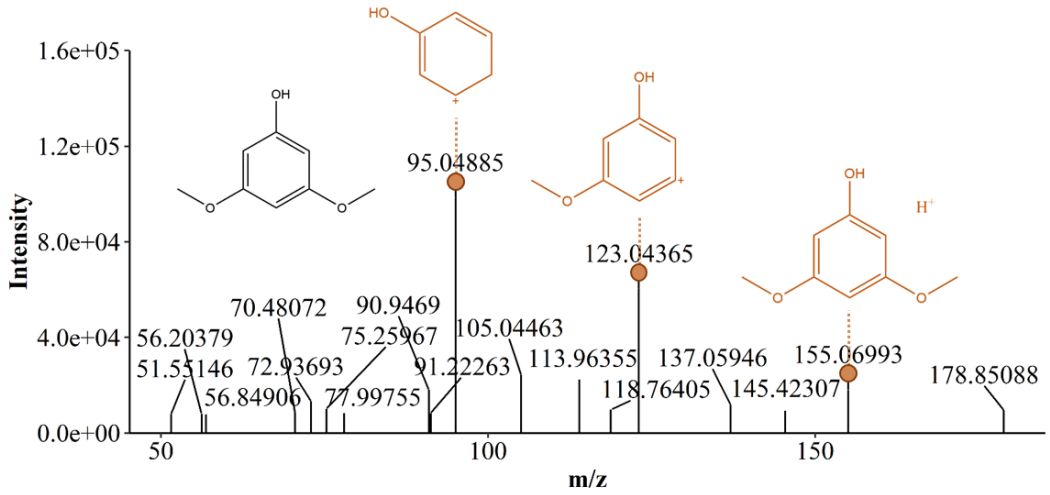
(b) 3,4-dihydroxyphenyl ethanol



(c) 2,4-dimethoxyphenol



(d) 3,5-dimethoxyphenol



Chapter 6

Thinking Inside the Box: A Novel Approach to Smoke Taint Mitigation Trials

Statement of Authorship

Title of Paper	Thinking inside the box: a novel approach to smoke taint mitigation trials
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Colleen Szeto		
Contribution to the Paper	Conceptualization of the work / experimental design Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing		
Overall percentage (%)	70		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a th on in this thesis. I am the primary author of this paper.		
Signature		Date	14/9/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Renata Ristic		
Contribution to the Paper	Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	14/9/2022

Name of Co-Author	Kerry Wilkinson		
Contribution to the Paper	Conceptualization of the work / experimental design Experimental work / data collection Data organization, interpretation, and analysis Manuscript preparation and editing		
Signature		Date	14/9/2022

Please cut and paste additional co-author panels here as required.

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Article

Thinking Inside the Box: A Novel Approach to Smoke Taint Mitigation Trials

Colleen Szeto [1,2], Renata Ristic [1] and Kerry Wilkinson [1,2,*]

[1] Department of Wine Science, Waite Research Institute, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia; colleen.szeto@adelaide.edu.au (C.S.); renata.ristic@adelaide.edu.au (R.R.)
[2] The Australian Research Council Training Centre for Innovative Wine Production, PMB 1, Glen Osmond, SA 5064, Australia
* Correspondence: kerry.wilkinson@adelaide.edu.au; Tel.: +61-8-8313-7360

Abstract: When bushfires occur near wine regions, grapevine exposure to smoke can taint grapes due to the uptake of smoke-derived volatile compounds that can subsequently impart unpleasant smoky, medicinal, burnt rubber and ashy characters to wine. Whereas early research sought to understand the effects of smoke on grapevine physiology, and grape and wine chemistry, research efforts have shifted towards the strategic imperative for effective mitigation strategies. This study evaluated the extent to which excised grape bunches could be reproducibly tainted during smoke exposure in a purpose-built 'smoke box'. The volatile phenol composition of grapes exposed to smoke for 30 min was similar to that of smoke-affected grapes from field trials involving grapevine exposure to smoke. Some variation was observed between replicate smoke treatments, but implementing appropriate controls and experimental replication enabled the smoke box to be used to successfully evaluate the efficacy of several agrochemical sprays and protective coverings as methods for mitigating the smoke exposure of grapes. Whereas the agrochemical sprays did not provide effective protection from smoke, enclosing grape bunches in activated carbon fabric prevented the uptake of up to 98% of the smoke-derived volatile phenols observed in smoke-affected grapes. As such, the study demonstrated not only a convenient, efficient approach to smoke taint research that overcomes the constraints associated with vineyard-based field trials, but also a promising new strategy for preventing smoke taint.

Keywords: activated carbon fabric; anti-transpirant; bushfires; grapes; guaiacol; kaolin; volatile phenols; volatile phenol glycoconjugates; wine

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1. Introduction

Grape growers and winemakers are keenly aware of the impacts of climate change on grape production [1] and have already begun adapting viticultural practices in response to warmer and drier growing conditions, for example, through the use of heat- and drought-tolerant cultivars and rootstocks [2], in-canopy sprinkler systems to mitigate heat stress [3], manipulation of crop load and water status to slow ripening [4] and delayed pruning to counter vintage compression [5]. Wine regions around the world are also being challenged by wildfires (or bushfires) which are occurring with increased frequency and severity [6]. Vineyard exposure to smoke can taint grapes due to the absorption of smoke-derived volatile compounds, including volatile phenols [7–9], which can impart smoky, medicinal, burnt rubber and ashy characters to wine [8,10,11]. In the last 5 years, fires have affected one or more vintages in prominent wine regions in Australia, Canada, Chile, New Zealand, South Africa and the USA [12,13], and revenue losses arising from 'smoke taint' are thought to be in the hundreds of millions of dollars [14,15]. Strategies that mitigate or ameliorate the effects of vineyard smoke exposure are therefore needed.

Early research found that smoke-derived volatile phenols could be removed from wine either by the direct addition of activated carbon [16] or solid phase adsorption following

nanofiltration [17], and these methods are still being used by industry to ameliorate smoke-tainted wine. However, ideally, preventative strategies should mitigate smoke taint in the vineyard. To date, few mitigation studies have been performed on grapes or grapevines. Washing grapes (during or after smoke exposure) does not prevent the uptake of smoke-derived volatile phenols by grapes or the perception of smoke attributes in wine [18,19], nor does partial defoliation of grapevines [20]. Several recent studies have evaluated the application of agrochemicals such as kaolin, biofilm, anti-transpirants and activated carbon to grapes or vines as protective sprays [21–24]. In some instances, promising results were obtained, although the efficacy of the treatment depended on spray coverage [21,22], but some treatments seemed to exacerbate the adsorption of smoke volatiles [22,24]. As such, an effective vineyard-based strategy for preventing smoke taint is yet to be found.

A key challenge associated with field trials evaluating the mitigation of smoke taint is the logistics of achieving reproducible experimental treatments with appropriate controls, both grapevines/grapes which are not exposed to smoke (i.e., negative controls) and grapevines/grapes which are exposed to smoke but without the mitigation treatment(s) (i.e., positive controls). The need for reproducible smoke treatments precludes mitigation trials involving grapevine exposure to wildfire/bushfire smoke, because the occurrence of fires cannot be predicted, the density and duration of smoke exposure is often unknown (and is likely to be highly variable, even within a single vineyard) and there are usually no appropriate controls. Model systems have therefore been developed to overcome these limitations. In the vineyard, purpose-built smoke tents (ranging from ~18 to 60 m^3) facilitate grapevine exposure to smoke [8,10,18,19,25,26]. This approach enables smoke and mitigation treatments to be applied at different phenological stages during the growing season [25,26], and the intensity of the taint can be influenced according to the duration of smoke exposure [10] and the density of the smoke (i.e., the mass of fuel that is burned) [18]. Several of these studies have attempted to monitor/qualify smoke density using air quality monitors or particulate matter sensors [18,19,25], but the density of smoke achieved in the smoke tents resulted in detector saturation [18,19].

More recently, model systems involving the exposure of excised grape bunches to either smoke (in smoke tents) [23,27] or gaseous volatile phenols (in closed systems, ranging from 6 to 156 L) [24,27] have been used. Surprisingly, the glycosylation of volatile phenols observed in grapes following grapevine exposure to smoke or guaiacol [8,18–21,28,29] was also found to occur in excised bunches [23,24,27], even in table grapes purchased from retail stores [23,24]. These approaches can therefore be used to generate grapes with elevated concentrations of volatile phenols, in both free and bound (glycosylated) forms. The use of smoke tents and excised bunches provides access to smoke-affected grapes in quantities that allow for winemaking (and therefore chemical and sensory analyses of wine), but smoke treatments need to be applied at or near commercial maturity, because grapes are non-climacteric (i.e., they do not continue to ripen post-harvest). Excised bunches can instead be exposed to different concentrations and/or combinations of gaseous volatile phenols at different phenological stages to simulate smoke exposure (e.g., to study the kinetics of absorption), but the scale of this approach is less suitable for winemaking and sensory analysis (because a 156 L glass tank can only accommodate so many excised bunches).

Collectively, the model systems described above have enabled researchers to undertake controlled and replicated smoke taint experiments with fewer logistical challenges such as seasonal or environmental constraints and/or restrictions related to safety, the occurrence of fires, and vineyard access [23]. Researchers can simulate smoke exposure to screen prospective mitigation strategies before pursuing more time- and resource-intensive field trials with the most promising strategies. Nevertheless, the extent to which model systems can replicate smoke treatments, and therefore mitigation trials, needs to be validated. This study describes the evaluation of a 'smoke box' designed specifically as a model system for exposing grapes to smoke with improved efficiency, flexibility and convenience (relative to field trials involving the use of smoke tents). Importantly, the study sought to evaluate how reproducibly excised bunches could be tainted by smoke, not only between replicate

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smoke treatments but also within smoke treatments (i.e., taking the potential for spatial variation in the density of smoke into account). The smoke box was subsequently used to compare the efficacy of several agrochemical sprays and protective coverings as methods for mitigating the uptake of smoke-derived volatile phenols by grapes, i.e., the risk of smoke taint.

2. Results and Discussion

2.1. Evaluation of the Purpose-Built Smoke Box (Repeatability Trial)

To evaluate the reproducibility of smoke treatments (both the spatial variation in smoke density during individual treatments and the variation among three replicate smoke treatments), mature bunches of Semillon grapes were suspended in the smoke box depicted in Figure 1 (and described in more detail in Section 3.1) in a 3 × 3 array (i.e., evenly spaced both horizontally (left, centre and right) and vertically (top, middle and bottom)) and exposed to smoke. Preliminary experiments confirmed that the smoke density in the box depended on the mass of fuel burned and the duration of smoke exposure (data not shown). Smoke treatments were therefore standardized in the current study by combusting set quantities of fuel (100 g of barley straw) and removing grape bunches from the smoke box after a set time (30 min).

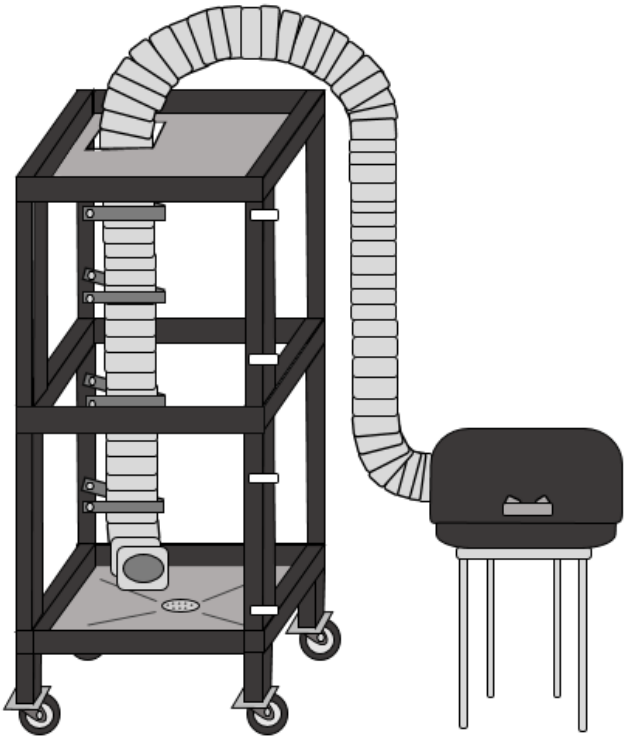

Figure 1. Schematic of the purpose-built smoke box.

Whereas the volatile phenols measured as markers of smoke taint (i.e., guaiacol, 4-methylguaiacol, phenol, cresols, syringol and 4-methylsyringol) were not detected in the control (unsmoked) grapes, they were found at elevated concentrations in grapes following smoke exposure. The heat maps shown in Figure 2 visualize the variation in guaiacol concentration, both by bunch position and by replicate smoke treatment. Bunches positioned in the top left and bottom right of the box tended to have higher guaiacol concentrations, whereas bunches at the bottom left of the box had the lowest guaiacol concentrations. This likely reflects the initial anticlockwise trajectory of smoke as it entered the box, after which the smoke dispersed to fill the box; nevertheless, the ~1 min required to achieve complete obscuration may account for the observed spatial variation in volatile phenol concentrations (Figure 2). Similar results were observed for the other smoke-derived volatile phenols that were measured (Figure S1).

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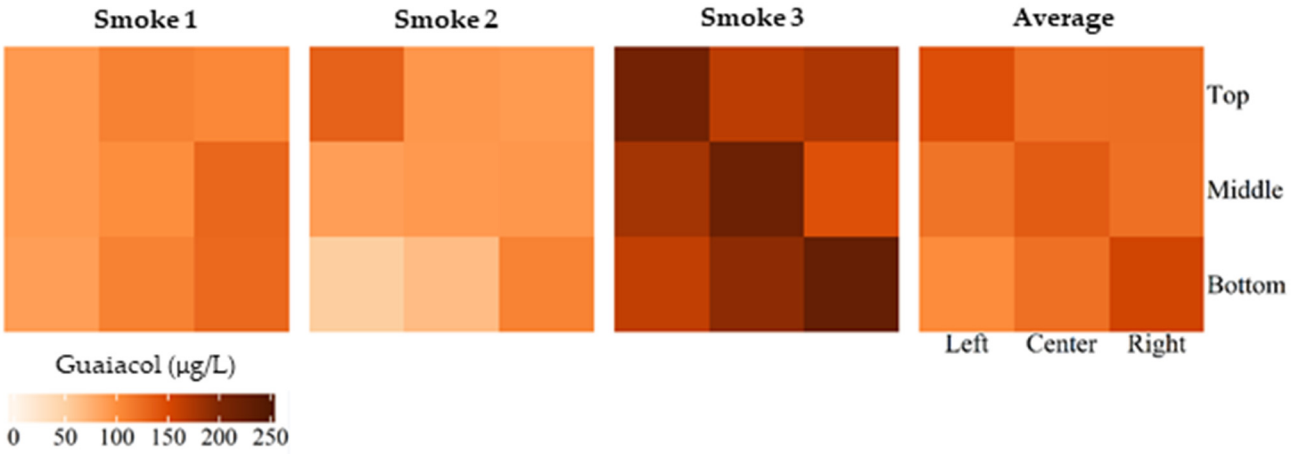

Figure 2. Heat maps depicting spatial variation in the guaiacol concentration of grapes exposed to smoke post-harvest, using the purpose-built smoke box, in replicate smoke treatments and as an average across the three smoke treatments.

Guaiacol, phenol, *o*-cresol, *m*-cresol and syringol were the most abundant grape volatile phenols (Table 1), at 90–187, 121–220, 38–66, 30–53 and 18–71 µg/L, respectively, in agreement with previous research [18]. When volatile phenol concentrations were compared as means of each replicate smoke treatment (i.e., irrespective of bunch position), statistically significant differences in the composition of smoke-exposed grapes were apparent. Volatile phenols were significantly higher in grapes from the third smoke treatment, while significant differences in phenol, *o*-cresol, syringol and 4-methylsyringol were also observed between the first two smoke treatments (Table 1). This demonstrates the difficulty of exactly replicating smoke treatments. The variation observed in grape volatile phenols reflected the variation in smoke density attributable to a combination of incomplete combustion of fuel and the inevitable loss of some smoke from the smoker (which did not fully seal to give a closed system) and, to a lesser extent, from the fitting connecting the smoker and the exhaust ducting. It was slightly cooler (22–24 °C) during the first two smoke treatments, with a slight breeze that may have contributed to some loss of smoke, whereas during the third treatment, it was slightly warmer (25 °C) but still (i.e., there was no wind). During windy conditions, greater smoke loss might occur, potentially resulting in greater variation in smoke density between smoke replicates.

Table 1. Concentration of volatile phenols (µg/L) in juice from control grapes and grapes exposed to smoke (or smoke residue).

Treatment	Guaiacol	4-Methyl Guaiacol	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Syringol	4-Methyl Syringol
Control	nd	nd	na	nd	nd	nd	nd	nd
Smoke 1	105 ± 5 b	17 ± 0.8 b	121 ± 7 c	38 ± 2 c	30 ± 2 b	6 ± 0.5 b	50 ± 2 b	7 ± 0.2 b
Smoke 2	90 ± 8 bc	14 ± 1.4 b	176 ± 15 ab	52 ± 5 b	36 ± 3 b	8 ± 1 b	18 ± 2 c	2 ± 0.1 c
Smoke 3	187 ± 9 a	31 ± 1.6 a	220 ± 10 a	66 ± 3 a	53 ± 3 a	13 ± 1 a	71 ± 3 a	9 ± 0.3 a
Smoke Residue	55 ± 2 c	7 ± 0.7 c	149 ± 18 bc	46 ± 1 bc	28 ± 2 b	8 ± 3 b	15 ± 2 c	2 ± 0.1 c
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values are means of three replicates ($n = 3$) ± standard error for control and residual smoke samples or and nine replicates ($n = 9$) ± standard error for replicate smoke samples. nd = not detected; na = not available. Different letters (within columns) indicate statistical significance ($p = 0.05$, one-way ANOVA).

When grape volatile phenol concentrations were instead compared according to the position of the excised bunches during the smoke treatment (i.e., as the means of each bunch position across replicate smoke treatments), differences in the composition of smoke-exposed grapes were again apparent (e.g., guaiacol concentrations ranged from 100 to 154 µg/L). However, differences were not statistically significant, due to the variation

between replicate smoke treatments (Table S1). This variation has also been encountered with field trials involving the application of smoke to grapevines using smoke tents, and can result in elevated standard deviation/error values for smoke taint marker concentrations [18]. Nevertheless, the use of the smoke box reduces the likelihood that insufficient smoke will be applied to achieve detectable levels of smoke taint. Mitigation trials need to account for the possibility of variation between replicate smoke treatments; i.e., by ensuring experimental treatments are adequately controlled and replicated across smoke treatments. Where mitigation strategies are effective, changes in volatile phenol concentrations will easily exceed any variation observed between replicate smoke treatments, but where the variation is such that the mitigating effect is difficult to ascertain, it likely suggests that the strategy is not capable of providing meaningful protection from smoke exposure.

Upon completion of the replicate smoke treatments, three excised bunches of Semillon grapes were suspended in the box for 48 h to evaluate the potential for grapes to absorb volatile phenols from the smoke residue that remained. Elevated concentrations of volatile phenols were detected following exposure to smoke residue (Table 1), with guaiacol, phenol, *o*-cresol, *m*-cresol and syringol again being the most abundant at 55, 149, 46, 28 and 15 µg/L, respectively, which were ~40–90% of the average concentrations observed for grapes exposed to smoke in the box (i.e., concentrations averaged across both bunch position and smoke treatment). While residual smoke was not expected to meaningfully contribute to the uptake of volatile phenols by grapes during the 30 min smoke treatments used in the current study (i.e., due to carryover), the results highlight the need for/importance of adequately cleaning/airing the box between experimental trials.

Previous research has demonstrated the glycosylation of volatile phenols in fruit and/or leaves following grapevine exposure to either smoke or volatile phenols [8,18–21,28,29], and likely occurs (through the action of glucosyltransferase enzymes) to mitigate the risk of cellular damage [30]. More recent studies have shown that glycosylation also occurs following post-harvest exposure of grapes to smoke or volatile phenols, i.e., in excised bunches [23,24,27], including in table grapes [23,24]. Similar results were obtained in the current study. Control grapes comprised $\leq$ 7 µg/kg of the volatile phenol glycoconjugates that were measured, but substantial quantities of several glycoconjugates accumulated in the week after the grapes were exposed to smoke in the box (Table 2); in particular, the pentose-glucosides of guaiacol, phenol and cresols were quantitated (as syringol glucose-glucoside (gentiobioside) equivalents) at 242, 216 and 213 µg/kg, respectively. Again, this was in agreement with the results from previous research [18,21,27]; however, some important differences were observed in the volatile phenol glycoside profiles reported in these studies.

Table 2. Concentration of volatile phenol glycoconjugates (µg/kg) in control grapes and grapes exposed to smoke post-harvest, using the purpose-built smoke box, analysed 7 days after smoke exposure.

Treatment	GuPG	GuR	4MGPG	4MGR	PhPG	PhR	CrPG	CrR	SyrGG	4MSGG
Control	2.4 ± 0.1 b	nd	nd	nd	3.7 ± 0.1 b	nd	7.1 ± 0.5 b	1.4 ± 0.1 b	1.0 ± 0.1 b	nd
Smoke	242 ± 27 a	110 ± 10	22 ± 3	27 ± 3	216 ± 31 a	89 ± 11	213 ± 25 a	114 ± 10 a	55 ± 7 a	9 ± 1
p	<0.001	-	-	-	0.003	-	<0.001	<0.001	0.001	-

Values are means of three replicates (n = 3) ± standard error, measured as syringol glucose-glucoside equivalents, for control grapes or nine replicates (n = 9) ± standard error for smoke-affected grapes. nd = not detected. Different letters (within columns) indicate statistical significance (p = 0.05, one-way ANOVA). Gu, guaiacol; 4MG, 4-methylguaiacol; Ph, phenol; Cr, cresol; Syr, syringol; 4MS, 4-methylsyringol; PG, pentose-glucoside; GG, glucose-glucoside; R, rutinoside.

Table S2 presents a cross-study comparison of volatile phenol glycoconjugate concentrations observed in grapes following exposure to either smoke or gaseous volatile phenols under different experimental conditions. One of these studies monitored the accumulation of volatile phenol glycoconjugates in smoke-affected Cabernet Sauvignon grapes [18] and reported glycoconjugate concentrations (i.e., 89–217 µg/kg) 1 week after smoke exposure

that were comparable to those observed in excised Semillon bunches in the current study; the notable exceptions being rutinosides of guaiacol and phenol, which were <30 μg/kg in the Cabernet Sauvignon grapes. These results were also in agreement with an earlier study that monitored glycoconjugate accumulation in smoke-affected Merlot grapes [21] and reported rutinosides of guaiacol and phenol at 22 and 26 μg/kg, respectively, relative to the aforementioned volatile phenol pentose glucosides, which were present at 113–300 μg/kg. In contrast, cresol rutinoside concentrations were surprisingly consistent across these three studies, at 114, 89 and 113 μg/kg for Semillon, Cabernet Sauvignon [18], and Merlot [21] grapes, respectively. This suggests that the enzymes responsible for transforming volatile phenols into rutinosides are not inhibited by bunch excision, although glycosylation might be influenced by substrate substitution patterns. This is an important consideration, given that gentiobiosides and rutinosides represent the key glycoconjugates monitored by some commercial laboratories for screening grapes (and wine) for smoke taint, based on their strong association with smoke taint's sensory attributes [11]. The abundance of pentose glucosides following smoke exposure of excised bunches may influence the perceived efficacy of mitigation trials and/or the intensity of smoke-related sensory attributes in wines, and warrants further investigation.

Another notable difference in the glycoconjugate profiles was that of syringol gentiobioside concentrations. In previous studies involving the application of smoke to Cabernet Sauvignon and Merlot grapevines, syringol glucose glucosides (gentiobiosides) were amongst the most abundant glycoconjugates observed in grapes (7 days after smoke exposure and at harvest). However, in the current study, and in studies involving the exposure of excised bunches to gaseous phenols [24,27], the concentrations of both syringol and its glucose glucoside (gentiobioside) were comparatively lower than those observed in grapes harvested from smoke-exposed grapevines [18,21]. It is not clear if this reflects the use of excised bunches or other experimental conditions, e.g., fruit maturity at the time of exposure, grape variety or the relative volatility of different phenols.

Although the application of smoke to grapevines or grape bunches presents inherent logistical challenges, a major benefit compared with the exposure of grape bunches to gaseous volatile phenols [24,27] as an alternate model system is that smoke-exposed grapes can be taken through to a winemaking outcome for sensory analysis; however, in the case of excised bunches, smoke exposure would need to occur at or near maturity. The use of gaseous volatile phenols offers the benefit of regulating the quantity of volatile phenols being applied to grapes, which may afford opportunities to investigate the kinetics of uptake and/or biochemical metabolism of volatile phenols (e.g., to resolve knowledge gaps relating to volatile phenol/glycoconjugate mass balance) [18]. Each of the model systems described above afford different advantages and disadvantages, and the most suitable option will depend on the research aim(s) to be investigated (e.g., Figure S2). The smoke box serves as a compromise between the use of smoke and the convenience inherent to its smaller scale.

2.2. Application of the Purpose-Built Smoke Box (Mitigation Trial)

The second aim of this study was to demonstrate the potential for the smoke box to be used to evaluate novel strategies for mitigating the risk of smoke taint in grapes.

In a preliminary field trial involving the application of smoke to Semillon grapevines (using smoke tents), the extent to which an activated carbon (AC) fabric could protect grapes from exposure to smoke was evaluated. Immediately prior to smoke exposure, a number of grape bunches were individually enclosed in bags made from the AC fabric and, for comparative purposes, adjacent grape bunches were similarly enclosed in plastic and paper bags (Figure S3). Following smoke exposure, grape volatile phenol concentrations were compared in the control, smoke-affected and bagged/smoke-affected bunches.

Control grapes did not contain detectable levels of volatile phenols, but smoke exposure resulted in grapes with guaiacol, syringol, *o*- and *m*-cresol and 4-methylguaiacol concentrations of 21, 16, 8.7, 7.0 and 4.3 μg/kg, respectively (Table 3). In contrast, grapes

that were enclosed in plastic or paper bags contained significantly lower volatile phenol concentrations. Guaiacol levels were approximately 50% lower, while other volatile phenol concentrations were ~48–88% lower. Food packaging (including plastic and paper bags) are known to be permeable to small molecules, including aroma volatiles, to different degrees [31]. It is therefore not surprising that in the current study, smoke-derived volatile phenols were detected in grape bunches enclosed in plastic and paper bags. Previous studies that sought to investigate the uptake and glycosylation of exogenous oak volatile compounds by grapevine leaves and fruit reported similar results [28,32], e.g., the presence of the analytes of interest in grapes that were enclosed in plastic bags (as protective barriers) prior to foliar applications of oak extracts or oak volatiles, due to their permeation through the packaging [31]. The AC fabric seemingly provided superior protection, resulting in grapes that contained just 1.3 µg/L of guaiacol and no other detectable smoke-derived volatile phenols (Table 3). These results suggested that the AC fabric adsorbed the vast majority of volatile smoke compounds, preventing their permeation and thus, contamination of the enclosed grapes.

Table 3. Concentrations of volatile phenols (µg/kg) in control grapes, smoke-exposed grapes, and grapes enclosed in paper, plastic or activated carbon fabric bags (as protective coverings) during grapevine exposure to smoke.

Treatment	Guaiacol	4-Methyl Guaiacol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Syringol	4-Methyl Syringol
Control	nd	nd	nd	nd	nd	nd	nd
Smoke	21 ± 2.9 a	4.3 ± 0.9 a	8.7 ± 0.9 a	7.0 ± 1.2 a	nd	16 ± 1.7 a	nd
Paper Bag + Smoke	10 ± 1.0 b	1.5 ± 0.5 b	4.5 ± 0.5 b	3.0 ± 0.1 b	nd	2.0 ± 0.1 b	nd
Plastic Bag + Smoke	11 ± 3.3 b	1.7 ± 0.9 b	3.0 ± 1.0 b	2.0 ± 0.6 b	nd	5.3 ± 1.5 b	nd
AC Fabric Bag + Smoke	1.3 ± 0.7 c	nd	nd	nd	nd	nd	nd
p	<0.001	0.004	<0.001	<0.001	-	<0.001	-

Values are means of three replicates (n = 3) ± standard error. nd = not detected. Different letters (within columns) indicate statistical significance (p = 0.05, one-way ANOVA).

The smoke box was subsequently used to further validate the potential of the AC fabric to mitigate the uptake of smoke-derived volatile phenols by grapes, alongside two agrochemical sprays, an anti-transpirant and kaolin (a clay-based barrier coating, typically used to protect grapes from sun damage [21]). Paper bags were again included for comparison, but not plastic bags, given their propensity for condensation, which promotes microbial spoilage. The same experimental conditions used in the repeatability trial (i.e., 100 g of straw as fuel and 30 min exposure of excised bunches to smoke) were again used in the mitigation trial, ensuring dense smoke treatments and thus testing the efficacy of each mitigation strategy. To overcome potential variation in smoke density between treatments (as occurred in the repeatability trial), each mitigation treatment was undertaken in triplicate, both within and between three replicate smoke treatments (i.e., n = 9 in total).

Smoke exposure again resulted in Semillon grape bunches with significantly elevated volatile phenol concentrations: i.e., 231 µg/L of guaiacol, 354 µg/L of phenol, 103 µg/L of *o*-cresol and 78 µg/L of syringol (Table 4). Volatile phenol concentrations were several times higher than those observed in the preliminary field trial (Table 3) because the smoke box enabled applications of smoke that were much denser than achieved in the field using the smoke tent. Some variation was again observed between replicate smoke treatments, with the second replicate resulting in significantly higher grape volatile phenol concentrations than the first and third smoke replicates; for example, guaiacol concentrations were 291 µg/L, compared with 186 and 215 µg/L, respectively (data not shown). However, this variation was accounted for by replicating mitigation treatments across replicate smoke treatments, with treatment replicates (excluding the control) positioned randomly within the box during each smoke replicate.

Table 4. Concentrations of volatile phenols (μg/L) in juice from control grapes, smoke-exposed grapes, and grapes treated with anti-transpirant or kaolin (as protective sprays) or enclosed in paper or activated carbon (AC) fabric bags (as protective coverings) during smoke exposure.

Treatment	Guaiacol	4-Methyl Guaiacol	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Syringol	4-Methyl Syringol
Control	nd	nd	na	nd	nd	nd	nd	nd
Smoke	231 ± 16 ab	39 ± 3 ab	354 ± 15 a	103 ± 5 ab	81 ± 4 a	10 ± 1 a	78 ± 10 ab	7.3 ± 1 ab
Anti-transpirant	239 ± 24 a	42 ± 4 a	406 ± 26 a	119 ± 9 a	92 ± 7 a	13 ± 3 a	88 ± 13 a	9.0 ± 2 a
Kaolin	183 ± 19 b	29 ± 4 b	286 ± 27 b	81 ± 8 b	64 ± 7 b	13 ± 0.8 a	58 ± 9 b	5.6 ± 1 b
Paper Bag + Smoke	75 ± 9 c	10 ± 1 c	81 ± 9 c	29 ± 3 c	15 ± 2 c	3.9 ± 0.1 b	nd	1.5 ± 0.0 c
AC Fabric Bag + Smoke	4.5 ± 1 d	2.0 ± 0.1 d	7 ± 2 d	2 ± 0.4 d	2 ± 0.3 c	nd	nd	1.4 ± 0.0 c
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Values are means of three replicates (n = 3). nd, not detected; na, not available. Different letters (within columns) indicate statistical significance (p = 0.05, one-way ANOVA).

Of the four mitigation strategies that were evaluated, the AC fabric was by far the most effective: enclosing grape bunches in activated carbon fabric prevented the uptake of up to 98% of the smoke-derived volatile phenols that were observed in the smoke-affected grapes (Table 4). Indeed, the volatile phenol levels in grapes enclosed in AC fabric were only 1–7 μg/L. Activated carbon fabrics are used as adsorbents in various industries [33], and activated carbon is routinely used as a fining agent in the wine industry, including for remediating smoke tainted wine [16]. However, this is the first study to demonstrate the capacity of AC fabric to mitigate the risk of smoke taint. The application of bags to individual grape bunches is neither practical nor financially viable, so further research and development is needed, but these results demonstrate proof-of-concept.

The paper bags again afforded the excised bunches reasonable protection from smoke exposure, and, with the exception of *p*-cresol (which was present at very low levels even in smoke-affected grapes), enclosing bunches in paper bags prevented the uptake of ~68 to 81% of each volatile phenol and, seemingly, 100% of syringol (Table 4). The apparent selectivity of protection from different volatile phenols might reflect the molecular size and/or the paper bag's porosity and surface chemistry [31]. The interior of the paper bag was coated with a wax layer to inhibit moisture loss, and it is possible that following diffusion through the paper layer, syringol and 4-methylsyringol were retained by the hydrophobic wax, such that they did not permeate into the bag and the grapes within.

Of the two agrochemicals applied to bunches prior to smoke exposure, neither provided meaningful protection. Significantly lower levels of phenol and *m*-cresol were detected in kaolin pre-treated grapes compared with smoke-affected grapes, while the anti-transpirant treatment typically yielded the highest grape volatile phenol concentrations, suggesting this mitigation strategy may actually have facilitated the uptake of smoke-derived volatile phenols by grapes. This is reasonable, given that the active ingredient in the anti-transpirant is a carboxylated hydrophilic polymer, which may well have affinity for smoke-derived volatile compounds.

Importantly, these results were in agreement with findings from recent studies that evaluated various agrochemicals as protective sprays for the mitigation of smoke taint [21–24]. Foliar applications of kaolin prior to smoke exposure achieved an 80% reduction in guaiacol glycoconjugates in Merlot grapes at harvest (relative to the corresponding smoke-affected Merlot grapes), but only 40% reductions were achieved when kaolin was applied to Chardonnay grapes and no significant differences were observed following kaolin applications to Sauvignon Blanc grapes [21]. The same anti-transpirant was evaluated as a smoke taint mitigation treatment in a recently published trial [24], albeit under different experimental conditions. In that study, there was no significant difference in the composition (i.e., free or bound volatile phenol concentrations) of Muscat Gordo or Shiraz grapes exposed to gaseous volatile phenols, with or without prior treatment with the anti-transpirant. However, it was observed that the application of other hydrophobic products (e.g., Biopest® Paraffinic Oil, Victoria Fruit Drying Oil, and Parka Plus) significantly in-

creased the concentration of volatile phenols (and their glycoconjugates) in both varieties, a finding consistent with earlier studies that evaluated the influence of lipid-based fungicides on the uptake of volatile phenols [22,23]. Promising results were initially obtained through the application of a synthetic grape cuticle [22], but the outcome could not be replicated in a subsequent growing season. These results reflect the challenge in achieving effective spray coverage, but also suggest that some viticultural practices, e.g., the use of fungicides to manage disease pressure in cooler, wetter areas or anti-transpirants to mitigate water stress in hotter, drier areas, might exacerbate the risk of smoke taint in the event of a nearby bushfire/wildfire.

In conclusion, the results presented herein demonstrate the potential for the smoke box to be used as a rapid, convenient approach to smoke taint mitigation research, overcoming the logistical constraints associated with vineyard-based field trials, as well as a very promising strategy for preventing smoke taint, i.e., activated carbon fabric.

3. Materials and Methods

3.1. Purpose-Built Smoke Box

A purpose-built smoke box (0.8 m × 0.8 m × 1.5 m, 0.96 m^3, Figure 1) comprising a steel frame fitted with glass panes (as walls) and aluminium sheeting (as the ceiling and floor), and sealed with silicone rubber was constructed by the University of Adelaide's School of Physical Sciences mechanical workshop. One wall was lined with a self-adhesive weather strip and mounted as a door, fitted with four metal latches, while the floor was angled downwards to a centrally positioned drain to facilitate cleaning. The smoke box was also fitted with swivel plate castors to allow it to be easily moved. Flexible aluminium exhaust ducting (125 mm × 3 m) was mounted in the left rear corner of the box, running from the floor and out via the ceiling for connection to a commercial fire box smoker (CharGriller, www.chargrilleraustralia.com.au (accessed on 21 February 2022)). This enabled fuel to be combusted in the smoker and the resulting smoke to be carried into the smoke box.

3.2. Field Trial

A preliminary field trial involving the exposure of Semillon grapevines to smoke (for 1 h, approximately 2 days before maturity when TSS was ~21 °Brix) was conducted in a vineyard at the University of Adelaide's Waite Campus in Urrbrae, South Australia (34°58′ S, 138°38′ E). Three adjacent vines were enclosed in a purpose-built smoke tent (2.0 m × 6.0 m × 2.5 m) and barley straw (~2 kg) combusted portionwise (i.e., over the hour) in two commercial smokers (as described previously [18]) to maintain smoke production. Prior to the smoke treatment, grape bunches (one per vine, per treatment) were enclosed in plastic, paper or activated carbon (AC) felt bags (approximately 25 cm × 20 cm each). The plastic and paper bags were purchased from a supermarket, while the AC felt bags were made in-house from a commercial AC fibre felt (Nature Technologies, Hangzhou, China). The bagged bunches were harvested immediately after smoke exposure, together with the smoke-exposed bunches (one per vine) and control bunches (three from a Semillon vine that had not been exposed to smoke). Grapes were separated from the rachis and homogenized with a T18 Ultra Turrax (IKA, Saufen, Germany). The resulting grape homogenate was frozen at −4 °C until needed for volatile phenol analysis.

3.3. Box Trials

3.3.1. Repeatability Trial

A trial involving the exposure of excised bunches of grapes to smoke using the purpose-built smoke box was performed in triplicate to test both the repeatability of the smoke treatments and the extent to which the position of grape bunches within the smoke box influenced their level of taint. Grape bunches (30 in total) were harvested (at maturity, when TSS was ~22–23 °Brix) from (control) Semillon grapevines from the field trial described in Section 3.2. Three replicated smoke treatments were performed, each involving the exposure of 9 grape bunches to smoke (for 30 min), with the bunches positioned in the

smoke box in a 3 × 3 array: top left, top centre, top right, middle left, middle centre, middle right, bottom left, bottom centre and bottom right. Barley straw (~100 g per treatment) was combusted in the fire box (as above) and the duration of smoke exposure commenced when smoke was first observed in the box (i.e., exiting the exhaust duct). Grape samples (50 berries per bunch per trial, chosen randomly) were collected immediately after smoke exposure and homogenized (as above), and the resulting grape homogenate was frozen at −4 °C until needed for volatile phenol analysis. After sampling, the 9 bunches remaining from the third replicate box trial were stored in darkness at 21 °C for 1 week. Grape samples (50 berries per bunch, chosen randomly) were again collected, homogenized and frozen at −4 °C until needed for volatile phenol glycoconjugate analysis. Volatile phenol glycoconjugate concentrations were also quantified in the control grape homogenate (i.e., homogenate derived from control grapes from the field trial described in Section 3.2). Upon completion of the smoke treatments, the three remaining grape bunches were suspended in the box for 48 h to evaluate the potential uptake of volatile phenols from residual smoke. Grapes were again sampled and homogenized for chemical analysis, as above.

3.3.2. Mitigation Trial

A separate trial involving the exposure of excised bunches of grapes to smoke using the purpose-built smoke box was performed (also in triplicate) to evaluate the efficacy of four strategies for mitigating the compositional effects of smoke on grapes: the use of powdered kaolin (a clay-based sunscreen, trade name Surround, sourced from AgNova Technologies; Box Hill, VIC, Australia) and an anti-transpirant (trade name Envy®, sourced from AgroBest Nutritional Systems; Nerang, QLD, Australia) as protective sprays, and the use of paper and AC felt bags (as described in Section 3.2) as protective coverings. Grape bunches (45 in total) were again harvested from (control) Semillon grapevines from the field trial described in Section 3.2 (at maturity when TSS was ~22–23 °Brix). Kaolin (prepared as a 50 g/L aqueous solution) and Envy (prepared as a 50 mL/L aqueous solution) were applied liberally to the grape bunches (using hand-held pump-action spray bottles) 24 h prior to harvest and smoke exposure. Three replicated smoke treatments were performed (as described in Section 3.3.1), each involving the exposure of 15 grape bunches (i.e., three replicates per treatment, including a smoke-only treatment) to smoke (for 30 min), with the bunches randomly positioned in the smoke box in a 5 × 3 array, positioned at the same height, in the centre of the box. Grape samples (50 berries per bunch per treatment per replicate, chosen randomly) were again collected immediately after smoke exposure and homogenized (as above), and the resulting grape homogenate was frozen at −4 °C until needed for volatile phenol analysis.

3.4. Chemical Analysis

The concentrations of smoke-derived volatile phenols (guaiacol; 4-methylguaiacol; phenol; *o*-, *m*- and *p*-cresol; syringol and 4-methylsyringol) were measured in grape juice or homogenate using an Agilent 6890 gas chromatograph coupled to a 5973 mass selective detector (Agilent Technologies, Forest Hill, VIC, Australia) according to previously published stable isotope dilution analysis (SIDA) methods [29,34], using d_4-guaiacol (synthesized in-house, as described previously [35]) and d_3-syringol (CDN Isotopes, Pointe-Claire, QC, Canada) as internal standards. Data acquisition and processing were performed using ChemStation (version B.04.03, Agilent Technologies) and MassHunter software. Field trial samples were analysed by the Australian Wine Research Institute's (AWRI) Commercial Services Laboratory (Adelaide, SA, Australia). Volatile phenol glycoconjugate concentrations (measured as syringol gentiobioside equivalents) were also measured in grape homogenate using an Agilent 1200 high-performance liquid chromatograph equipped with a 1290 binary pump coupled to an AB SCIEX Triple Quad™ 4500 tandem mass spectrometer, with a Turbo V™ ion source (Framingham, MA, USA), and previously published SIDA methods [29]; d_3-syringol gentiobioside (Toronto Research Chemicals, Toronto, ON, Canada) was used as the internal standard. Data acquisition and processing were performed using

Analyst software (version 1.7 AB SCIEX). The limits of quantitation for volatile phenols and volatile phenol glycoconjugates were 1–2 and 1 µg/L, respectively.

3.5. Data Analysis and Visualization

Compositional data were analysed by one-way analysis of variance using R statistical software (version 4.0.3, Cambridge, MA, USA), with mean comparisons performed by Tukey's honest significant difference test at a significance level of $\alpha < 0.05$. Heatmaps were generated using the "Complex Heatmap" package in R.

Supplementary Materials: The following supporting information can be downloaded online. Figure S1: Heat maps depicting spatial variation in the (a) phenol, (b) *o*-cresol, (c) *m*-cresol, and (d) syringol concentrations of grapes exposed to smoke post-harvest using the purpose-built smoke box, by replicate smoke treatments and as an average across the three smoke treatments. Figure S2: Photograph showing Semillon grape bunches enclosed in plastic, paper and activated carbon fabric bags prior to treatments involving grapevine exposure to smoke. Figure S3: Comparison of natural, experimental and model smoke exposure as tools to pursue different smoke taint research aims, and their relative advantages and limitations. Table S1: Concentrations of volatile phenols (µg/L) in grapes exposed to smoke post-harvest, according to the spatial position of bunches within the smoke box. Table S2: Cross-study comparison of volatile phenol glycoconjugate concentrations (µg/kg) measured in grapes following exposure to smoke or gaseous volatile phenols, under different experimental conditions.

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Supplementary Materials

Thinking Inside the Box: a Novel Approach to Smoke Taint Mitigation Trials

Colleen Szeto [1,2], Renata Ristic [1], and Kerry Wilkinson [1,2,*]

[1] Department of Wine Science, Waite Research Institute, The University of Adelaide, PMB 1, Glen Osmond, SA 5064, Australia; colleen.szeto@adelaide.edu.au (C.S.); renata.ristic@adelaide.edu.au (R.R.)
[2] The Australian Research Council Training Centre for Innovative Wine Production, PMB1, Glen Osmond, SA 5064, Australia
* Correspondence: kerry.wilkinson@adelaide.edu.au (K.W.); Tel: +61-8-8313-7360

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Table S1. Concentrations of volatile phenols (μg/L) in grapes exposed to smoke post-harvest, according to spatial position of bunches within the smoke box.

Vertical	Horizontal	Guaiacol	4-Methyl Guaiacol	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Syringol	4-Methyl Syringol
Top	Left	145 ± 36	25 ± 7	199 ± 48	61 ± 15	46 ± 11	12 ± 3	51 ± 13	6 ± 2
Top	Center	122 ± 22	20 ± 4	170 ± 19	49 ± 5	39 ± 4	11 ± 1	47 ± 15	6 ± 2
Top	Right	123 ± 22	22 ± 4	173 ± 20	54 ± 6	40 ± 5	9 ± 2	45 ± 10	6 ± 1
Middle	Left	119 ± 30	20 ± 6	156 ± 28	49 ± 10	37 ± 7	8 ± 3	45 ± 16	6 ± 2
Middle	Center	137 ± 42	22 ± 7	183 ± 43	56 ± 12	43 ± 10	8 ± 2	49 ± 17	6 ± 2
Middle	Right	122 ± 15	20 ± 3	178 ± 13	52 ± 2	39 ± 2	9 ± 3	45 ± 14	6 ± 2
Bottom	Left	100 ± 33	17 ± 6	130 ± 30	39 ± 9	30 ± 8	8 ± 2	41 ± 17	6 ± 2
Bottom	Center	122 ± 37	20 ± 6	152 ± 30	47 ± 9	36 ± 8	6 ± 1	45 ± 17	6 ± 2
Bottom	Right	154 ± 37	25 ± 6	218 ± 37	64 ± 10	50 ± 8	11 ± 4	55 ± 17	7 ± 2
p		0.976	0.981	0.724	0.750	0.773	0.801	0.999	1.000

Values are means of three replicates (n = 3) ± standard error. No statistical significance was detected as a function of bunch position during smoke treatment (p = 0.05, one way ANOVA).

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Table S2. Cross-study comparison of volatile phenol glycoconjugate concentrations (μg/kg) measured in grapes following exposure to smoke or gaseous volatile phenols, under different experimental conditions.

Variety	Model System	Timing and Duration of Smoke Exposure	Sampling Time	Sample Density	GuR	CrR	SyGG	MSyGB	CrPG	GuPG	References
Semillon	smoke box	0.5 h, post-harvest	7 d post-smoke	9 bunches/0.96 m^3	110 ± 10	114 ± 10	55 ± 7	9 ± 1	213 ± 25	242 ± 27	current study
Viognier	smoke tent	1 h, 1 week pre-harvest	7 d post-smoke	3 vines/30 m^3	tr	3 ± 0.3	39 ± 9	8 ± 2	21 ± 3	40 ± 2	[27]
Viognier	smoke tent	1 h, post-harvest	7 d post-smoke	3 vines/30 m^3	tr	3 ± 0.3	40 ± 8	9 ± 1	23 ± 0.5	43 ± 2	[27]
Cabernet Sauvignon	smoke tent	1 h, 1 week pre-harvest	7 d post-smoke	3 vines/30 m^3	tr	3 ± 0.5	12 ± 4	2 ± 0.5	3 ± 0.4	3 ± 0.4	[27]
Cabernet Sauvignon	smoke tent	1 h, post-harvest	7 d post-smoke	3 vines/30 m^3	tr	3 ± 0.5	9 ± 2	1 ± 0.3	3 ± 0.2	3 ± 0.3	[27]
Viognier	gaseous phenols	60 h, post-harvest	immediate	3 bunches/0.16 m^3	25 ± 4	205 ± 36	45 ± 5	17 ± 3	2114 ± 135	1111 ± 104	[27]
Cabernet Sauvignon	gaseous phenols	60 h, post-harvest	immediate	3 bunches/0.16 m^3	90 ± 14	571 ± 74	102 ± 17	26 ± 5	1196 ± 223	482 ± 122	[27]
Muscat Gordo[1]	gaseous phenols	60 h, post-harvest	immediate	3 bunches/0.16 m^3	10 ± 4	67 ± 23	88 ± 37	-	299 ± 91	197 ± 54	[24]
Shiraz	gaseous phenols	60 h, post-harvest	immediate	3 bunches/0.16 m^3	24 ± 6	218 ± 51	6 ± 0.5	-	197 ± 19	105 ± 6	[24]
Cabernet Sauvignon	smoke tent	1 h, 7-10 days post-veraison	7 d post-smoke	3 vines/30 m^3	11 ± 2	89 ± 12	455 ± 86	62 ± 10	217 ± 34	185 ± 33	[18]
Merlot	smoke tent	1 h, 14 days post-veraison	7 d post-smoke	3 vines/30 m^3	22 ± 12	113 ± 64	176 ± 96	-	300 ± 156	283 ± 189	[21]

Data are reported as means ± standard error values; tr = trace (i.e., 0.5–1 μg/kg).
Gu = guaiacol; Cr = cresol; Sy = syringol; MSy = 4-methylsyringol; PG = pentose-glucoside; GG = glucose-glucoside (gentiobioside); R = rutinoside.

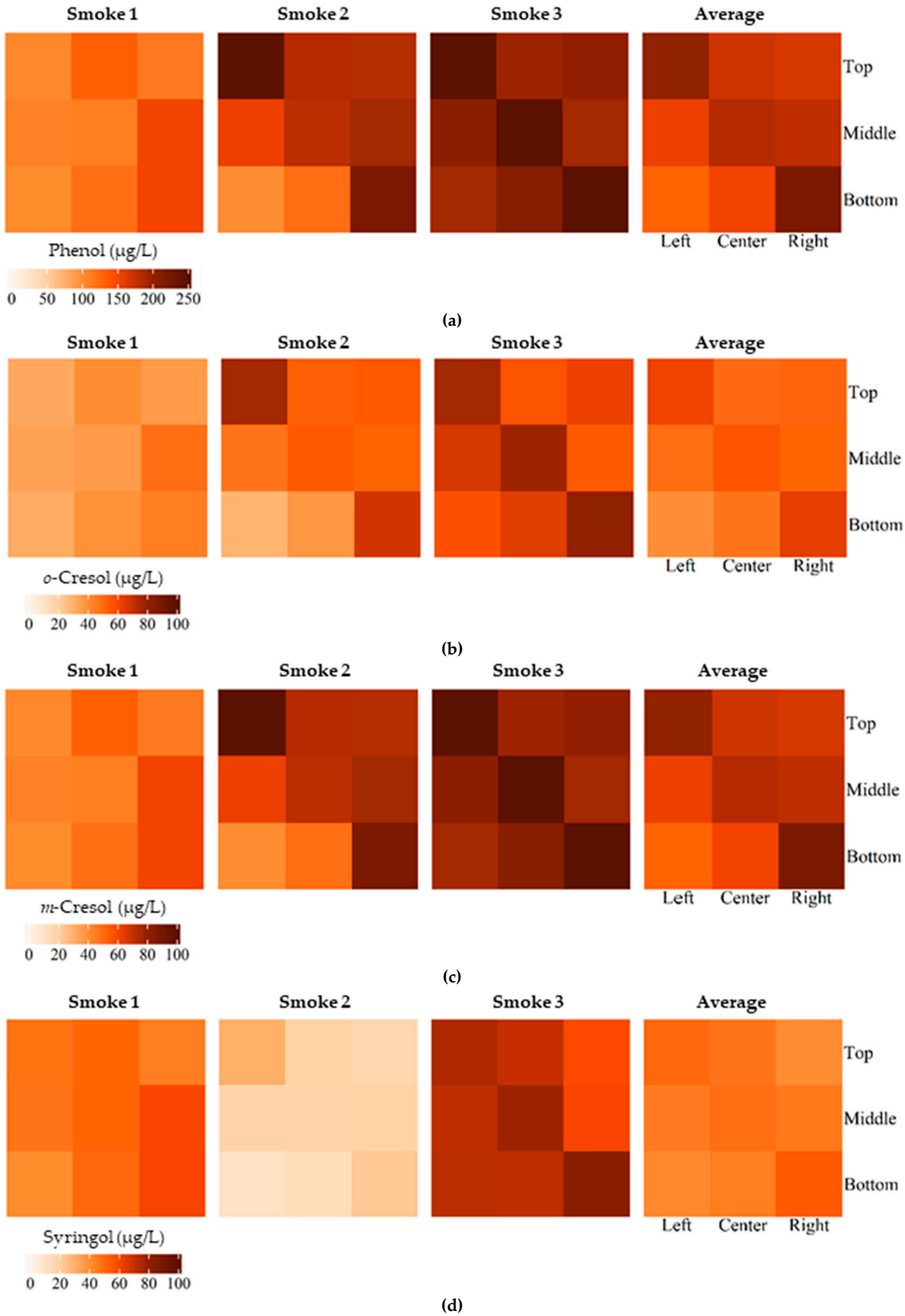

Figure S1. Heat maps depicting spatial variation in (**a**) phenol, (**b**) *o*-cresol, (**c**) *m*-cresol, and (**d**) syringol concentrations of grapes exposed to smoke post-harvest, using the purpose-built smoke box, by replicate smoke treatments and as an average across the three smoke treatments.

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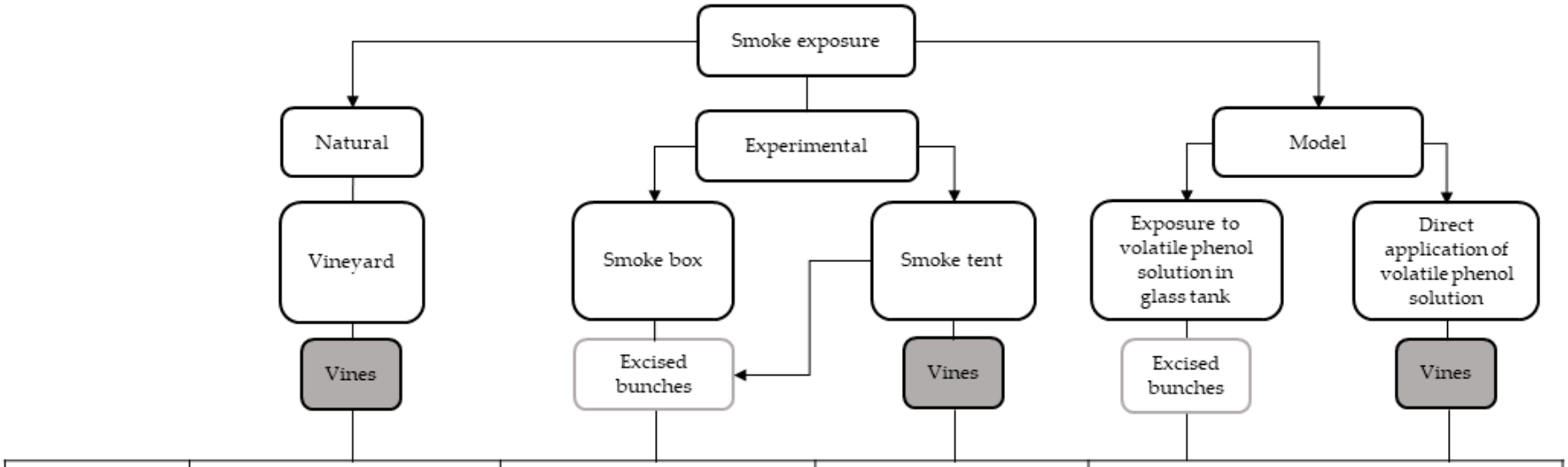

Resources	Low	Low-Medium	High	Minimal
Flexibility	Low	Medium-High	Medium	High
Advantages	Provides unique opportunities to observe impacts of actual bushfire smoke on grape/wine chemical and wine sensory assessments and practical, industry-oriented data interpretation	Provides benefit of actual smoke exposure to a high volume of fruit in less time, enables pursuit of preliminary trials prior to scale-up in the vineyard	Provides most accurate model of actual bushfire smoke, enables pursuit of most diverse research aims, e.g. impacts of grape maturity, vineyard mitigation, and timing of smoke exposure	Provides least accurate model of actual bushfire smoke, but enables pursuit of unique basic research aims, e.g. volatile phenol translocation between leaves and fruit and exposure of vines/bunches to known quantities of volatile phenols
Limitations	Limited by vineyard access, uncertainty of bushfire occurrence, and inconsistent/unknown levels of grapevine smoke exposure	Limited by bunch stability post-excision, variable bunch maturity/size/density, and weather	Limited by fruit volume, time-sensitive experimental window, time-intensive experimental Smokes, and weather	Limited by variable bunch size/density, simplification of smoke to a few volatile phenols, and minimal industry relevance

Figure S2. Comparison of natural, experimental and model smoke exposure as tools to pursue different smoke taint research aims, and their relative advantages and limitations.

Figure S3. Photograph showing Semillon grape bunches enclosed in plastic, paper and activated carbon fabric bags prior to treatments involving grapevine exposure to smoke.

Chapter 7

Conclusions and Future Directions

Conclusions

Global warming modelling indicates more frequent and intense bushfires are likely in the future. The confronting reality of these trends was manifested during the 2019/2020 bushfire season in Australia, and it strengthened the imperative for the development of innovative strategies to mitigate the effects of grapevine smoke exposure. Conventional mitigation trials investigate to what extent a proposed strategy can reduce the presence of smoke-derived volatile phenols (and their glycoconjugates) in grapes and wine, or mask their sensory perception in wine. In addition to developing effective mitigation treatments, it is just as crucial to ensure that rapid diagnostics are available detect smoke taint, thereby informing management/remediation strategies. Current chemical diagnostics of grapevine smoke exposure involve the measurement of volatile phenols and their glycoconjugates in grapes and wine by conventional GC-MS and HPLC-MS/MS, respectively; however, interpretation of results depends on contextual factors that require further elucidation. The research described in this thesis responds to the urgent need for smoke taint mitigation with a 'go hard, go early' approach, which includes:

i) Developing vineyard-based detection and mitigation strategies
ii) Investigating the contextual factors that influence volatile phenol (and their glycoconjugate levels) in grapes (both naturally occurring and smoke-derived)
iii) Assessing rapid methods for detecting smoke taint in grapes and wine
iv) Identifying novel chemical indicators of smoke exposure in grapes

7.1 Detection and mitigation of smoke taint in the vineyard

Field trials offer the certainty of knowing which grapevines are exposed to smoke because its administration can be controlled and replicated. Contrarily, in "real" bushfire scenarios, the consistency, intensity, and frequency at which vineyards are exposed to smoke is highly variable. This limits the ability to pinpoint smoke-affected areas of the vineyard and collect representative samples for the analysis of volatile phenols and glycoconjugates. **Chapter 3** explored the potential for environmental sensors to be used to detect and monitor real-time smoke exposure based on particulate matter concentrations. Different densities of smoke exposure were achieved by burning different fuel loads (i.e., high and low fuel mass), and as expected, grapes contained significantly less volatile phenols (and glycoconjugates) if they

were exposed to low density smoke, relative to high density smoke. However, the observed difference in smoke density was not paralleled by particulate matter concentration; the levels of particulate matter detected in each scenario were sufficient to saturate the sensor. Saturation in the experimental trials was likely attributed to the high density of smoke in purpose-built tents, relative to levels present in a vineyard from a real bushfire. This is supported by a recent study demonstrating that the same particulate matter sensors could be used to successfully monitor smoke derived from stubble burns (Wilkinson *et al.* 2021).

Ideally, a vineyard-based method that detects smoke exposure would enable implementation of a mitigation strategy. For the first time, **Chapter 3** explored the efficacy of using a real-time preventative strategy in which bunches were exposed to smoke and simultaneously sprayed with mist using in-canopy sprinklers. While this strategy did not show strong potential, the trial highlighted several challenges of conducting smoke taint mitigation trials in the field. Relative to smoke from "real" bushfire scenarios, the level of grapevine smoke exposure is more consistent, but prevailing weather conditions can make it challenging to maintain consistency across replicate smoke treatments. Moreover, the opportunity to conduct smoke taint trials is limited to the growing season, and in some cases, the scale at which treatments can be applied.

These limitations were addressed in **Chapter 6** by building a smoke box, which enabled small-scale mitigation trials to occur with greater efficiency and convenience. Smoke exposure within the box had some variation, which could largely be overcome by appropriate replication of experiments, i.e., the inclusion of experimental treatments across replicate smoke treatments with adequate randomisation of bunches. To achieve this in field trials, randomised block designs and significantly more labour would be required. In a subsequent box mitigation trial, activated carbon fabric was identified as a promising candidate for mitigation. Activated carbon has shown great efficacy as a fining for removal of volatile phenols (and their glycoconjugates) from juice and wine (Fudge *et al.* 2012a; Culbert *et al.* 2021b), but this is the first study demonstrating its ability to directly shield grapes from smoke-derived volatile phenols in the vineyard. In its current form, the activated fabric would not be practical to implement at a large scale, but these preliminary results serve as a proof-of-concept and justify future research and development.

7.2 Contextual factors that influence volatile phenols and glycoconjugates

Levels of volatile phenols (in free and glycosylated forms) in grapes are influenced by several factors. As shown in **Chapter 3**, volatile phenols are rapidly metabolised in grapes within 24 hours of smoke exposure, but there can be a delay in their accumulation as glycoconjugates. Thus, observing low levels of volatile phenols in grapes is not sufficient to rule out high levels of smoke exposure, a critical finding that has since been validated in other studies (Jiang *et al.*2021). These findings further instil the limitations of relying on guaiacol and 4-methylguaiacol alone. Many commercial laboratories offering smoke taint diagnostics have expanded the suit of volatile phenols measured in their analysis, but there remains a persistent myth that smoke taint risk can be sufficiently predicted without consideration of their glycoconjugates, and this needs to be addressed through educational workshops and seminars.

In addition to a delay in the accumulation of glycoconjugates, there was also a discrepancy between expected levels (based on volatile phenol concentrations measured 1 hour post-smoke exposure) and the significantly elevated glycoconjugate levels observed at harvest. These findings suggest that measuring volatile phenol glycoconjugates at maturity would provide the most accurate assessment of any smoke taint. However, from an industry perspective, prolonging analysis until the apex of the season equates to compressed decision-making and minimal time to implement mitigation strategies. Further work is required to understand the cause of this delay and determine the earliest point at which an accurate assessment of smoke exposure can be made to optimise and standardise the timing of sample collection for analysis.

The concentrations of volatile phenols and their glycoconjugates in grapes may also be influenced by regional fuel types, burn conditions unique to each bushfire (Kelly *et al.* 2012; Noestheden *et al.* 2018), and for some varieties, natural abundance (Ristic *et al.* 2016). **Chapter 2** explores the profiles of volatile phenol glycoconjugates in grapes and wine affected by wildfire smoke in California and compares results to data from analyses currently used for smoke taint diagnostics in Australia. It was demonstrated that key markers in California were consistent with those in Australia using a method developed for an uHPLC-Orbitrap MS. The same six volatile phenol glycoconjugates (plus phenol glucoside and cresol pentosylglucoside) were identified as being the most indicative of smoke exposure in wine. Further examination of grapes affected by smoke from distinct wildfire seasons highlighted the need to develop infrastructure that will enable better characterisation of the density, duration, timing, and fuel

source of smoke exposure in real wildfire events. It is apparent from experimental trials that these factors matter, thus, it is necessary to integrate them into real world diagnostics of smoke taint.

7.3 Rapid methods to detect smoke exposure in grapes and wine

The quantitation of volatile phenols and glycoconjugates is a time- and resource-intensive procedure, and when operating under the constraints of harvest, it is critical to acquire results on a timely basis. **Chapter 4** evaluated fluorescence spectroscopy as a rapid method of classifying wine into categories of low-, medium-, and high-risk of smoke taint, based on their volatile phenol and glycoconjugate concentrations. These smoke taint markers could be detected in less complex wines derived from experimental trials, but as the sample set was expanded to include a greater diversity of commercial wines (sourced from different varietals and regions), prediction accuracy declined.

In most classification studies, the true class of every sample is known, and the objective is to acquire a representative training set to develop a model that predicts the classes of test samples that were held out from the calibration and cross-validation stages of model building. However, aside from wine samples with very low or very high levels of volatile phenols and their glycoconjugates across the board, the true class of potentially smoke-affected wine samples is not obvious. This limits confidence in the representativeness of training sets and complicates assessments of model performance. In the presented study, there were several wine samples with very high levels of volatile phenols but low levels of volatile phenol glycoconjugates (and vice versa) but it is not possible to interpret the significance of these levels in the absence of known baselines in equivalent wines.

7.4 Novel chemical indicators of smoke exposure in grapes

Smoke taint diagnostics have evolved from measuring guaiacol and 4-methylguaiacol alone to a broader suite of volatile phenols and their glycoconjugates, and as outlined in **Chapters 2-4** there is considerable work ahead to understand their accumulation in smoke-affected grapes, establish representative baselines, and develop faster methods of detection. **Chapter 5** moved beyond volatile phenols and their glycoconjugates to deploy an untargeted metabolomics approach to the discovery of additional chemical indicators of smoke exposure. Along with

many other volatile compounds (Guillén *et al.* 1995), smoke also contains ozone, a known oxidant that may induce stress responses in plants, one of which is the production of antioxidants that can scavenge highly reactive free radicals.

Tentative identifications were made for several compounds that were significantly elevated in smoke-affected Merlot grapes relative to control grapes. While the MS2 data suggested that some elevated compounds may have been exogenously derived volatile phenol glycoconjugates, others appeared to be endogenous metabolites, potentially released as part of a stress response. **Chapter 3** outlines the temporal dependence of detecting diagnosis of smoke exposure in grapes based on volatile phenol and glycoconjugate concentrations. A key advantage of the compounds in **Chapter 5** is that they were able to differentiate control and smoke-affected grapes just 2 hours after smoke exposure, and this discrimination persisted through to commercial maturity some 20 days later. Identification of these compounds using authentic standards may facilitate development of new analytical methods that complement existing smoke taint diagnostics. A limitation of measuring stress-related metabolites as markers is that smoke may not be the only source of stress that leads to their production. Moreover, their production may vary depending on the underlying health of the vine.

Future Directions

The intense wildfire seasons experienced in the last few years saw prominent wine regions in Australia, California, British Columbia in blanketed smoke, and the impacts of smoke taint on wine quality have therefore been brought to the forefront of research supporting the grape and wine industry. This thesis presents significant progress in understanding the impact of smoke, and in developing proactive mitigation strategies and diagnostics of smoke exposure in grapes and wine. Nevertheless, several research questions are still to be resolved.

7.5 Incorporating baseline levels into grower contracts

The severity of risk associated with vineyard smoke exposure and the prevalence of bushfires during the last decade have led to conflicts over the quality of grapes and wine in regions affected by smoke exposure. Few grower contracts include accurate tolerance levels for volatile phenols and their glycoconjugates (if any), which is partially attributed to knowledge gaps regarding the natural abundance of volatile phenols and their glycoconjugates in grapes. This underscores the importance of future work that establishes baseline marker concentrations for additional regions and varietals, which will serve as the foundation of fair and accurate benchmarks for naturally occurring levels of smoke taint markers in grapes and wine.

7.6 Analysis of volatile phenols and their glycoconjugates

Following unprecedented bushfires in 2020, commercial laboratories specific to the grape and wine industry sought to meet the increased demand for smoke taint diagnostics; however, the list of target compounds being measured varied considerably across laboratories. The most comprehensive analyses measure free guaiacol, 4-methylguaiacol, *o*-cresol, *p*-cresol, *m*-cresol, syringol, and 4-methylsyringol, as well as various volatile phenol glycoconjugates (e.g., guaiacol, 4-methylguaiacol, phenol, and cresol rutinosides as well as syringol and 4-methylsyringol gentiobioside). Interestingly, very few commercial labs measure phenol, despite results from **Chapter 6** suggesting phenol is the most abundant volatile phenol observed in smoke-affected grapes. This finding may be attributed to the use of barley straw as a fuel source in field experiments, but its abundance warrants further assessment of its contribution to the sensory profiles of smoke tainted wines. There were also several

commercial laboratories who took advantage of the demand for smoke taint diagnostics and started to offer analyses that comprised only quantitation of guaiacol and 4-methylguaiacol. While guaiacol and 4-methylguaiacol are a piece of the puzzle, it is well established that reliance on these compounds can present a significant risk of underestimating smoke exposure when considered in isolation. Increasing demand for smoke taint analysis needs to be addressed via a combination of rapid methods of detection, such as highlighted in **Chapter 4**, and increasing the capacity of commercial laboratories that offer comparable services and interpretation of results that reflect the current smoke taint literature.

7.7 Additional markers in leaves

In addition to volatile phenols (in free and glycosylated forms), work from **Chapter 5** indicates that additional markers present in Merlot grapes could be used to identify smoke exposure. While some markers appeared to be novel, exogenous volatile phenol glycoconjugates, others were tentatively identified as endogenous, stress-related metabolites. To build on these findings, the developed metabolomics workflow could be used to identify additional markers of smoke exposure in grapevine leaves. Relative to grapes, leaves are more abundant, have a greater surface area, and are present on the vine earlier in the season. As such, they might offer a more appropriate substrate for detection and quantitation of grapevine smoke exposure.

7.8 Mitigation of smoke taint in wine

Previous mitigation research has focused on the removal of volatile phenols and their glycoconjugates from wine, suppressing their contribution to wine sensory profiles, or both. As described in **Chapter 3** and **Chapter 6,** there are several advantages to vineyard-based mitigation strategies. However, the unpredictable nature of smoke exposure and the safety concerns of working in the vineyard during a bushfire event require preventative strategies that have enduring effects no matter the prevailing weather conditions. This would enable their application early in the growing season, in case of a bushfire event. That being said, it is unlikely that any singular strategy will be able to eliminate the risk of smoke taint. Rather than viewing vineyard-based mitigation strategies as ‘silver bullets’, they should be viewed as part of a workflow that—in tandem with other remediation practices in the winery—can be used to mitigate the risk of smoke taint in wine.

7.9 Summary

It is fair to say that our collective understanding of the chemical and sensory impacts of grapevine smoke exposure have increased significantly over the last few decades. Nonetheless, smoke taint remains a major challenge in the grape and wine industry. This project evaluated and empowered the efficacy of a ‘go hard and go early’ approach towards the mitigation of smoke taint by improving the efficiency of current diagnostics and accelerating the development of remediation strategies. As we continue to expand our knowledge of the chemical and biochemical responses of grapevines to smoke exposure, we increase the likelihood that effective solutions for the detection and mitigation of smoke taint in grapes and wine will be found.

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Appendix

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