The Analysis of Grapevine Response to Smoke Exposure

A thesis presented in fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Smoke taint is a fault found in wines made from grapes exposed to bushfire smoke. It is characterised by objectionable smoky and ashy aromas and flavours, which have been attributed to the presence of smoke derived volatile phenols, in free and glycoconjugate forms. Chapter 1 comprises a summary of the impact of bushfires on the wine industry and a review of previous smoke taint research, which includes many investigations into the composition of wine produced from smoke-affected fruit. Gaps of knowledge are identified in Chapter 1, and the issues addressed in this thesis are identified and summarised in the research aims.

Chapter 2 describes a field trial that investigated the accumulation of smoke taint precursors in three *Vitis vinifera* cultivars, Sauvignon Blanc, Chardonnay and Merlot, at different time points, following grapevine exposure to smoke under experimental conditions. Varietal differences in volatile phenol glycoconjugate profiles were observed; interestingly, these profiles also differed between samples harvested 1 day after smoke exposure and samples harvested at maturity. An evaluation of the effect of an agrichemical applied to grapevine fruit and foliage as a physical barrier to prevent the uptake of smoke is also reported; together with the results of an investigation into the potential for reflectance spectroscopy, measured using a handheld spectrometer, to detect smoke-affected fruit. A subsequent field trial sought to further verify the use of a second agrichemical to mitigate the impacts of grapevine exposure to smoke; and reflectance spectroscopy to evaluate smoke exposure in the vineyard and is also included in Chapter 2.

Whereas the glycosylation of smoke derived volatile phenols in grapevine fruit and leaves following exposure to smoke is reasonably well understood, the biochemical and

molecular consequences of grapevine smoke exposure have received comparatively little consideration. The research described in Chapter 3 endeavours to address this knowledge gap through investigations into the expression of grapevine glycosyltransferases (GTs) following smoke exposure. Higher expression profiles of certain sets of genes (including heat shock proteins and putative GTs) were identified through RNA sequencing of two grape cultivars grown as potted grapevines in a growth room. Selected GT candidates were analysed in a subsequent field trial, in which Q-PCR expression analysis showed higher expression of two GT1 family genes at specific time points; with differential expression found to be highest in skin, rather than pulp, fractions following smoke exposure.

To date, the occurrence of smoke taint has not been reported in crops other than grapes, despite the proximity of bushfires in regions comprising broader agricultural production. The final chapter of experimental work in this thesis, Chapter 4, describes analysis of a field trial involving the application of smoke to apple trees, to investigate whether or not apples can be similarly affected by smoke.

Chapter 5 reflects on the experimental work described in this thesis, including a discussion towards challenges and future directions in the research of smoke taint.

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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Lieke van der Hulst

Date: 17/11/2017

Publications

This thesis comprises a collection of manuscripts prepared for submission to peer-reviewed scientific journals. Authorship statements are included in chapters which incorporate a manuscript.

In chapter 2: **Accumulation of volatile phenol glycoconjugates in grapes, following the application of kaolin and/or smoke to grapevines (*Vitis vinifera* cv Sauvignon Blanc, Chardonnay and Merlot).**; prepared for submission to Frontiers in Plant Science.

In chapter 3: **Transcription profiling of glycosyltransferases in *Vitis Vinifera* cultivars following smoke exposure.**; prepared for submission to Frontiers in Plant Science.

Chapter 4: **Smoke exposure influences the composition of apples (*Malus domestica* Borkh cv Sundowner).**; prepared for submission to the Journal of Agricultural and Food Chemistry.

An additional paper, published in the Journal of Agricultural and Food Chemistry, concerning the stability of smoke derived volatile phenols and their precursors, and the persistence of smoke taint in wine following bottle aging, is included in the appendices:

Ristic, R., van der Hulst, L., Capone, D.L., Wilkinson, K.L., *Impact of Bottle Aging on Smoke Tainted Wines from Different Grape Cultivars.* Journal of Agricultural and Food Chemistry, 2017.

Symposia

Scientific conferences

Ristic, R., **van der Hulst, L.**, Wilkinson, K. (2016) *Stability of smoke taint during the aging of smoke-affected wine*, 252nd ACS National Meeting, Philadelphia (PA), US (oral presentation)

Van der Hulst, L., Ford, C., Burton, R., Lloyd, N., Wilkinson, K. (2016), *Potential for kaolin application to grapevines to mitigate smoke taint,* 16th Australian Wine Industry Technical Conference, Adelaide, Australia (poster presentation)

Van der Hulst, L., Ford, C., Burton, R., Lloyd, N., Wilkinson, K. (2016), *Impact of smoke exposure on the chemical composition of grapes,* Macrowine 2016, Changins (Nyon), Switzerland (oral presentation)

Van der Hulst, L., Ford, C., Burton, R., Lloyd, N., Wilkinson, K. (2016) *Impact of smoke exposure on the composition of different fruit,* 11th Wartburg Symposium on Flavor Chemistry & Biology, Eisenach, Germany (oral presentation)

Van der Hulst, L., Ford, C., Burton, R., Lloyd, N., Wilkinson, K. (2015) *Uptake and glycosylation of smoke-derived volatile compounds in grapevines*, Crush Grape and Wine Symposium, Adelaide (oral presentation)

Industry workshops

Van der Hulst, L., Ristic, R. (2017) *The chemical markers of smoke taint,* Smoke taint symposium – Vinos de Chile / Wines of Chile, Mar 2017

Van der Hulst, L., Ford, C., Burton, R., Wilkinson, K. (2016) *Biochemical response of grapevines to smoke exposure*, ARC Training Centre industry visit - Limestone Coast Grape and Wine Council, Coonawarra, Oct 2016

Van der Hulst, L., Ford, C., Burton, R., Ristic, R., Wilkinson, K. (2016) *Biochemical response of grapevines to smoke exposure*, ARC Training Centre Workshop – Charles Sturt University, Wagga Wagga, May 2016

Van der Hulst, L., Ford, C., Burton, R., Lloyd, N., Wilkinson, K. (2015) *Biochemical response of grapevines to smoke exposure*, ARC Training Centre Workshop – Launceston, Tasmania

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CHAPTER 1

Literature review and introduction

Literature review and introduction

Changes in the environment have led to an increase in temperature and a higher prevalence of dry and hot summers all over the world [1]. As a consequence, many countries have seen bushfires occurring more frequently due to warmer, drier climatic conditions and it is expected that global warming will lead to a further increase in air and land temperatures of between 1.8 and 4.0°C by the end of this century [1]. Wine producing countries, including Australia, Canada, South Africa, Chile, New Zealand, Spain, France and the US (Table 1) have felt the economic impact of bushfires in various ways: fire damage to vineyard planting damage, reduced visitor rates to cellar doors and most importantly and the risk of taint in smoke affected grapes [2, 3].

Table 1 Overview of years in which bushfires affected wine regions leading up to or during vintage in top producing wine countries since 2003. Production based on 2016 year report of the International Organisation of Vine and Wine (OIV).

Country	2016 production (MhL)	Bushfire events
Italy	48.8	2015, 2016, 2017
France	42.2	2015, 2016, 2017
Spain	37.8	2015, 2016, 2017
USA	22.5	2008, 2015, 2016, 2017
- Napa valley, Sonoma valley		
Australia	12.5	2003, 2005, 2009, 2015, 2016, 2017
- Yarra valley, Adelaide Hills, Canberra, Alpine valley, Tasmania		
China	11.5	No data available
South Africa	10.5	2013, 2016, 2017
- Stellenbosch		
Chile	10.1	2017
- Colchagua, Valparaíso, Maule		
Argentina	8.8	No recent data, extreme fires predicted for coming years [i]
Germany	8.4	No data available
Portugal	5.6	2015, 2016, 2017
New Zealand	3.1	2014, 2015, 2016, 2017

[i] www.winerisk.com

Smoke taint is now a well-established wine fault, characterised by aroma and flavour profiles dominated by ashy, smoky and medicinal notes [4, 5]. The chemical composition of smoke tainted wines has been investigated extensively and several smoke-derived volatile phenols such as guaiacol, 4-methylguaiacol, syringol and cresol have been identified as smoke taint marker compounds [6]. Several other smoke-derived compounds have been reported, such as 4-ethylguaiacol and 4-ethylphenol, albeit usually at much lower concentrations [7]. These volatile phenols are derived from the thermal degradation of lignin, which occurs during combustion of plant material in bushfires [8, 9]. However, some of these compounds, for example guaiacol, occur naturally in the berries of *Vitis vinifera* cv Shiraz and Merlot. Hydrolysis of the juice of these cultivars led to the identification of several volatile phenols, some of which are associated with smoke taint [10, 11]. Furthermore, ageing wines in oak barrels can also introduce volatile compounds into wine, as a result of the toasting process employed in barrel cooperage [12-14].

Even though smoke taint related compounds are naturally present in some grapes and wine, smoke tainted wines often have a combination of these compounds present, leading to the unpalatable smoke taint aroma and flavour. Consumer acceptance for the detractive descriptors that come with elevated levels of these smoke-derived compounds is low and because of this smoke taint poses an important challenge for winemakers and grape growers worldwide [9].

The occurrence of bushfires and prescribed burning

Globally, fire events occur naturally, or as part of landscaping and maintenance of rural areas and as a means of land management via prescribed burning [15]. Major fire events have significant impacts on not only the local vegetation and environment, but also have social and economic effects within the affected areas. Consequently, problems associated

with bushfires and prescribed burning are complex, as there are many positive and negative implications [16].

Fire has been of great importance for the development of terrestrial ecosystems around the world as an initiator for regeneration. Not only do fires clear areas for new growth, but smoke acts as a germination signal for seeds and has been used as a pre-treatment for enhancing conservation, and to stimulate plant growth on reclaimed mine spoils and disturbed land [17]. However, with the settling of humans and the establishment of agriculture in bushfire prone areas there is a need to better control these fires and mitigate their negative effects [16]. Scientific literature on bushfires and their consequences most commonly either has a focus on the worldwide problem of increasing fire danger days, or is looking at the socioeconomic impact of a larger fire in a specific area [16, 18]. Most examples come from industrialised countries such as Australia (more specifically the south and southeast of the country) and the United States. However, over the past 20 years larger fires with highly negative impacts on social, economic and environmental assets have been seen all over the world [15, 19].

The occurrence of bushfires over the last decade has increased, as both changes in climate and land use have made bushfire conditions more favourable. Climate and environmental changes have affected global temperatures, reflected in the significant increase of the mean annual maximum temperature as well as the length of the annual fire weather season [20, 21]. Future fire activity is difficult to predict, and globally, fire frequency will increase in some areas and decrease in others [22]. However, the general trends for many fire activity metrics, such as fire weather occurrence, seasonality and intensity, as well as area burned, in wine producing countries like the USA, Canada and Australia, indicate an increase in future fire activity [22]. Many countries have their own index to predict bushfires, based on factors such as drought, temperature, fuel load, wind speeds and humidity [23]. In Australia the Forest Fire Danger Index is used, developed in 1960 by

CSIRO, which predicts and helps to track the amount of fire danger days over the years. Since 1973 a clear increase in the number of fire danger days has been observed across Australia, particularly in the south and southeast regions, where significant vineyard plantings are found (Figure 1) [23].

Worldwide, most fire events occur in equatorial and subtropical regions [20]. Deforestation and grassland management are considered to be the cause of many fires in South America, sub-Saharan Africa and Southeast Asia [20, 22]. However, most of these fire events are intentional. In contrast, unplanned bushfires have occurred in many agricultural areas in the US, Australia and South America [22].

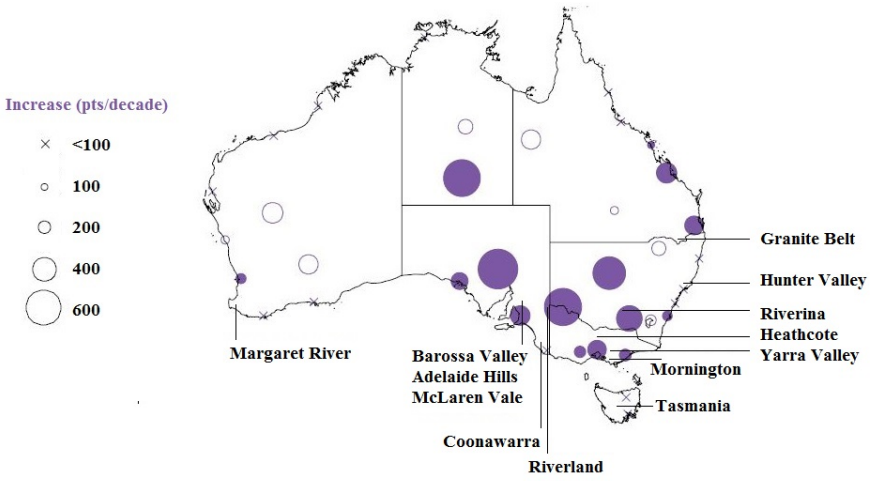


Figure 1 Map of trends in the magnitude of annual cumulative FFDI in Australia from 1973 to 2010. Indicated on the map are wine regions in proximity to fire prone areas. Marker size is proportional to the magnitude of trend, with reference sizes shown in the legend. Filled markers represent trends that are statistically significant.

Economic impact of bushfires

It is difficult to estimate the full economic impact of bushfires. Direct costs based on fire damage to infrastructure and property can be valued more easily than loss of productivity and certainly loss of life. The 2009 Black Saturday bushfires in Victoria (Australia) were estimated at a total economic cost for the country of $4.4 billion, and the total annual cost for fires in Australia has been estimated to be $8.5 billion [16, 24].

These numbers represent economic cost alone and do not reflect loss of historical landmarks, long-term environmental impacts and the cost of psychological damage to residents and support networks [16].

The cost of bushfires for the wine industry is based on loss of physical assets, for example the loss of vineyards, cellar door and production facilities. But, grape growers and wine makers in bushfire prone areas also have to take into consideration the effects of smoke on crop quality, as fruit can be exposed to smoke even when bushfires have occurred some distance away. Economic loss caused by smoke taint is determined by the amount of fruit and wine that are affected, usually due to quality being downgraded or to fruit or wine being discarded. The estimated loss for 2006-2007 in the Victorian Alps (Australia) was $75-90 million for wine not produced due to crop loss, and $7.5 million for the Pemberton region (in Western Austria) in 2004 due to unmarketable grapes [4, 25]. Other examples of economic loss can be indicated by the number of cellar door visits during bushfire season or the decision not to release vintage wines in these specifically bad years [4].

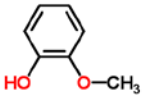
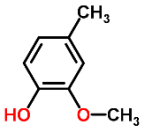
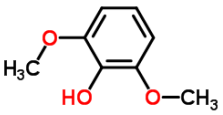
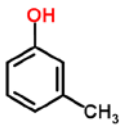
Smoke-derived volatile compounds

The smoke taint sensory profile is mainly caused by the presence of smoke-derived volatile phenols, but following uptake by the grape these compounds are subsequently glycoconjugated [26, 27]. These glycoconjugate compounds are thought to be less active and odour- and flavourless. However, recent work has indicated that these compounds can be broken down by enzymes in human saliva, and in this way contribute to a specific smoke taint aftertaste and mouth feel [28, 29].

A wide range of smoke-derived volatile compounds have been identified in grapes affected by smoke, including (amongst others) phenols, furans and aldehydes [3, 30]. These compounds are pyrolysis products, i.e. volatile compounds formed during combustion of vegetative material, and the thermal degradation of lignin [30, 31]. Many of these compounds are commonly found in smoked foods, such as smoked meat, fish and cheese [32-34]. Investigation into the different fuel sources for bushfires affecting vineyards found differences in the intensity and character of the smoke taint sensory profile, as well as differences in the concentrations of phenols found in these smoke tainted wines [9, 30]. Regardless of the fuel type however, about 20 % of the total phenols found in wine made from smoke affected fruit seem to be the guaiacyl lignin products, guaiacol and 4-methylguaiacol. Syringyl derivatives were also present, and at much higher quantities, but these are likely to have less impact on aroma and flavour due to higher sensory thresholds [35]. Interestingly, it has been reported that specific lignin derivatives such as syringol were present in pine smoke tainted wines, even though the associated syringyl compound was not found in the smoke produced from burning pine wood [30]. This might indicate that phenyl metabolic pathways are affected by smoke exposure. Grape variety and wine making practice can also influence the composition and sensory profiles of smoke tainted wines [9, 36]. From the list of smoke-derived volatiles

detected in smoke and smoke tainted wines guaiacol, syringol and *o*-, *m*-, and *p*-cresols are currently considered the key marker compounds (Table 2).

Table 1 Volatile smoke taint marker compounds and their detection thresholds in (a) model wine or (b) 10% aqueous alcohol solution [28].

Compound	CAS number	Detection threshold (µg/L)	Sensory descriptors	Structure
guaiacol	90-05-1	9.5 a	phenolic smoky bitter	
4-methylguaiacol	93-51-6	30 a	solvent ash dry	
syringol	91-10-1	570 a	smoky medicinal	
cresol	108-39-4	10-70 b	phenolic plastic Band-Aid bitter	

These compounds have been found in elevated levels in smoke affected fruit, and are also responsible for more intense smoke flavour and aroma [7, 32]. Compounds included in initial smoke taint research are 4-ethylguaiacol, 4-ethylphenol, eugenol, furfural, 5-methylfurfural and vanillin, either for their roles in other off flavours, as spoilage markers or oak aroma and flavour compounds [3].

However, research has shown that the highest concentration of a specific volatile compound does not necessarily lead to the biggest influence on the sensory perception of smoke taint. Odour activity values depend on the detection threshold of a compound, and as shown in Table 2, these are quite different for the smoke taint marker compounds.

Research into the set group of smoke taint-causing volatiles is ongoing, as there is no straight-forward correlation found between the observation of smoke taint sensory profiles by experts and the level of volatile phenols in grapes and wine [28]. At the moment both *m*-cresol and guaiacol are thought to be the biggest smoke taint contributors [28]. Other volatile compounds may have a supportive role in the development of the sensory profile, even though these compounds are often found below detection threshold concentrations [28]. For example, compounds such as 4-methylsyringol and syringol are often found in smoke tainted wines in low concentrations and have high detection thresholds, but may still contribute to the overall perception of taint [28]. More importantly, it has become increasingly clear that quantification of volatile compounds alone does not adequately predict the likelihood or intensity of smoke taint in wine. Increasingly, determination of glycosylated volatile phenols has been used to assess smoke affected fruit and wine – either directly via liquid chromatography tandem mass spectrometry (LC-MSMS) analysis or indirectly via gas chromatography mass spectrometry (GC-MS) analysis following treatment with glucosidase or acid [37, 38].

Volatile compounds in wine

Unfortunately, the volatile compounds found in wine associated with smoke taint are not unique smoke taint markers. Compounds that can contribute to a smoky aroma and flavour such as guaiacol and 4-methylguaiacol are also commonly present in oak-aged wines [39, 40]. Moreover, guaiacol can also be present as an endogenous product (albeit at relatively low concentrations) in grape varieties including Merlot and Shiraz [26, 41]. The metabolome of fruits and plants harbours a wide range of organic volatile compounds and low molecular weight molecules responsible for a multitude of sensory characteristics. In grapes a range of volatile compounds are responsible for mechanisms involved in, for example, UV-B protection and the build-up of colour and aroma to attract

seed dispersers, as well as imparting these sensory characteristics on wine produced from said grapes [42].

Glycosylation of volatile phenols in grapes

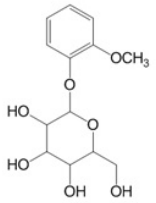
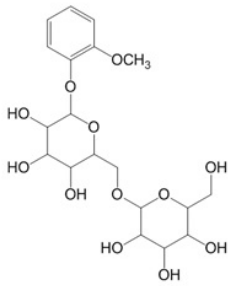
During grape development many volatile compounds responsible for grape and wine aroma are metabolised, including endogenous and exogenous volatile phenols. Monoterpenes and norisoprenoids are important aroma compounds in many grape cultivars, and are most commonly found in a glycoconjugated state in grape berries [43-45]. Upon hydrolysis these compounds will release their flavour-active aglycone, and so many of these free compounds are responsible for the varietal aroma and flavour profiles of different wines [44].

Glycosylation is one of the many enzymatic reactions involved in the ripening process and glycosylation activity changes over the different stages of phenological development [43]. The attachment of an activated sugar moiety to a small molecule is a common process in all organisms, and employed by a wide range of plants in order to facilitate transport, solubility and storage of endogenous and exogenous compounds [46, 47]. Consequently, volatile compounds in plants often accumulate as non-volatile (odour inactive) 'bound' or precursor forms [48]. It has been proposed that glycosyltransferases are located in the cytosol of plant cells, where they are part of multi-enzyme complexes [49].

Smoke taint research has uncovered a small range of glycoconjugates to be present in grapes and wine following smoke exposure, including seven forms of conjugated guaiacol [27]. Grape glycoconjugates identified so far only include mono- and diglycosides and all of them include at least a direct linkage to a β-D-glucose moiety [48]. Rhamnose and arabinose (a sugar of the pentose class) have been identified in grapes to be the preferred terminal sugars in disaccharide glycosides [45]. This was confirmed by identification of

guaiacol glucoconjugates in smoke affected juice samples in the work by Hayasaka et al., which includes monoglucosides and diglycosides formed by linkage between a hexose and a hexose, a pentose or a rhamnose (Table 3) [26].

Table 2 Chemical structures of putative glycoconjugates of guaiacol identified in smoke tainted grapes and wines [26, 50].

Glycoside	Putative compound identification	Structure
Glucoside	*β*-D-glucopyranoside	
Glucosylglucoside	Gentiobioside Sophoroside	
Diglycoside	α-L-arabinosyl-*β*-D-glucoside *β*-D-apiosyl- *β*-D-glucoside *β*-D-xylosyl- *β*-D-glucoside	
Rutinoside	α-L-rhamnosyl-*β*-D-glucoside	

Higher order glycosides however, e.g. those containing more than two sugar compounds attached to an aglycone, have been identified in other types of fruit such as apples and tomatoes [51]. In apples and tomatoes, triglycosides are commonly found to be formed during fruit development. Interestingly, these compounds are present as diglycosides for some time before further fruit development leads to the formation of non-aromatic triglycosides compounds closer to maturation [48, 51, 52]. Not all glycoconjugates form odour-active compounds directly after cleavage during hydrolysis, as often further modification is needed [53]. Because of this, not all glycosides present in grapes can easily be linked to a volatile aglycone. It is unclear if certain sets of volatile aroma compounds that have been commonly identified in fruit from other plants, but not in grapes, are simply not present in *Vitis vinifera*, or might not be characterised just yet [48]. Recent research has also uncovered another reason for the need to analyse both volatile

as well as non-volatile smoke taint markers, as glycoconjugate precursors have been found to significantly impact flavour and aroma upon tasting smoke affected wine. Glycoconjugates can be broken down by hydrolase enzymes present in human saliva, upon which volatiles can be released, thereby imparting a sensory response [28, 29].

Previous smoke taint research

Research into smoke taint began in 2003, when objectionable smoky aromas and flavours were discovered in wines made from fruit harvested in Australian wine regions in close proximity to bushfires in the Alpine Valley in north east Victoria and in the Canberra wine regions [2, 4]. Even though smoke exposure at this time was thought to contribute to the smoky aroma and flavour of wines from smoke affected regions, a direct link had not been scientifically established. Investigations on Verdelho grapes exposed to smoke post-harvest established the presence of smoke-derived volatile phenols, including guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol, eugenol and furfural, quantified via stable isotope dilution assays [3]. A distinguishable smoky sensory profile in juice and wine was established by means of difference tests [3].

Subsequent research demonstrated that the intensity of smoke taint in wine made from smoke exposed grapes is highly dependent on timing of smoke exposure at certain phenological stages of grapevine development [7]. Early in grapevine development, up to full bloom, smoke exposure resulted in relatively low concentrations of guaiacol and 4-methylguaiacol in smoke tainted wine, whereas at about a week after veraison until harvest, smoke exposure caused high levels of these smoke-derived volatile phenols [7, 54].

This research also indicated that carry-over of smoke taint into the next season is unlikely. Yield from smoke affected vines in the following season was decreased, but no smoke taint compounds were detected in wine produced a year later [54]. Not only the timing of

smoke exposure, but also the duration of exposure impacts the severity of smoke taint [7]. Both longer and repeated smoke exposure produced wines with higher levels of guaiacol and 4-methylguaiacol, as well as higher degrees of unpalatable sensory characteristics [7]. The duration of smoke exposure depends in part on how long a fire event lasts. In some cases, smoke exposure might only last a few hours, but where bushfires have burned for prolonged periods of time, e.g. the 2009 Black Saturday bushfires in Victoria, smoke exposure can occur over several days. Smoke density can also be affected by climatic conditions, e.g. wind speed and direction, which will not only determine for how long smoke lingers in the vineyard, but the density of smoke [4, 38]. Furthermore, even within a single vineyard block, the extent to which individual vines are exposed to smoke can vary considerably. In contrast, experiments that involve the application of smoke to vines endeavour to standardise both the duration, density and timing of smoke exposure; e.g. the application of smoke to grapevines for 1 hour at approximately 7 days post-veraison [5, 7, 14, 25, 37, 80]. Attempts have been made to monitor smoke intensity during field trials, based on measurements of particle size using portable dust trackers and nephelometers, in order to characterise the amount of smoke applied to grapevines [30, 54]. However, the success of these efforts has been limited due to the rapid fouling of instruments by smoke, and to date, these methods haven't been used to monitor smoke exposure in the vineyard during a bushfire event.

Preliminary studies conducted by the Australian Wine Research Institute (AWRI), published in the 2003 AWRI year report, confirmed the presence of smoky sensory attributes which were thought to be due to the presence of guaiacol and 4-methylguaiacol [2]. Their trials on smoke taint reduction at the time suggested leaf plucking vines, hand harvesting and whole bunch pressing of fruit were the best strategies for reducing the level of smoke taint in finished wine. Most of these recommendations have been

supported by subsequent research and are employed by industry following a smoke event [37].

The release of smoke-derived volatile phenols during winemaking indicated the presence of smoke taint precursors; hydrolysis by strong acid and β-glucosidase enzyme assays verified guaiacol and 4-methylguaiacol to be present as glucoconjugates [5]. Subsequent research confirmed guaiacol to be present as β-D-glucopyranoside in smoke affected juice and fruit, as opposed to unaffected grapes [50]. Investigations of the response to hydrolysis of the precursor showed it to be highly susceptible to enzyme hydrolysis, but to only be partially affected by strong acid. Glucoside precursors were not detected by HPLC-MSMS in enzyme hydrolysates but small amounts of the guaiacol precursor were still present in the acid treated samples [50]. GC-MS quantification of guaiacol in acid hydrolysates of Sangiovese grapes yielded higher amounts than in enzyme hydrolysates. This suggested the presence of other smoke taint precursors, besides β-D-glucopyranoside, as a source of released guaiacol [50]. A stable isotope feeder experiment confirmed the presence, as mentioned before, of seven different guaiacol glycoconjugates in vines treated with aqueous solutions of d_0- and d_3-guaiacol [26]. Subsequent research confirmed these glycoconjugated precursors to be formed not only with guaiacol as aglycone, but also phenol, *p*-, *m*- and *o*-cresols, methylguaiacol, syringol and methylsyringol [27].

Over the years, subsequent research has added to the list of preventative and amelioration techniques through suggestions to minimise skin contact, enhance wine complexity and use a range of fining agents to reduce the presence of smoke-derived volatiles [55-57]. Most amelioration techniques used in the winery however, also diminish desired aroma and flavour profiles, making it difficult to produce acceptable quality wine from smoke affected grapes. One of these amelioration techniques involves the treatment of smoke tainted wine by reverse osmosis (RO) coupled to solid phase adsorption [55]. This

treatment was shown to be successful in removing smoke-derived volatile phenols but taint returned over time, possibly by the breakdown of glycoconjugates [55].

Aside from trying to remove smoke-derived compounds, winemakers can decide to increase the wine complexity in a way to cover up smoke taint by the use of for example oak compounds as an addition to finished wine [14]. Recent work on the stability of smoke taint precursors confirmed that a more complex wine will cover smoke taint more easily [58]. Wine from grape varieties that produce more complex wines, such as Shiraz, over a period of six years also seemed better at hiding the negative smoky flavour and aroma in smoke tainted wines [58]. This same research also indicated that, even though some additional smoke-derived volatile compounds will be released over time, smoke taint precursors are relatively stable in bottle aged wines [58]. Acid hydrolysis indicated more precursors to be present still, and only small additional quantities of guaiacol, 4-methylguaiacol and syringol were released after 6 years in bottle [58].

Reporting on smoke taint is complex as there is no one minimum certain set or combination of glycoconjugated and volatile compounds that will indicate a certainty of developing smoke taint aroma and flavour in wine, as the presence of certain levels of these compounds in grape is not always directly correlated with the perceived sensory profile [59]. Furthermore, quantifying smoke taint by HPLC-MSMS is highly dependent on a specific internal standard, as all phenolic glycosides will be expressed as equivalents to the calibration function for each particular internal standard [59].

Glycosyltransferases

As mentioned before, glycosylation is the process of attachment of an activated sugar to a small molecule (an aglycone). This process is guided by glycosyltransferases (GTs) which are ubiquitous enzymes present as plant secondary metabolite modifiers [60]. GTs play an important role in many processes, as these enzymes regulate hormone levels,

compartmentalisation of secondary metabolites and accumulation of both endogenous and potentially toxic exogenous compounds. Full sequencing and phylogenetic profiling of the plant GT families has been conducted for several economically important crops such as tobacco, maize, tomato and the model plant *Arabidopsis thaliana* [51, 61]. Currently, 94 GT families have been identified in plants and enzymes from most of these families have been identified in *Vitis vinifera* based on sequence homology [47, 62]. These GT families are large and typically contain tens to hundreds of genes encoding glycosyltransferase enzymes [62]. Most of the GTs identified in *Vitis vinifera* are part of GT Family 1 and are small (45-60 kD), soluble enzymes [61]. A common motif found in GT enzymes concerned with secondary metabolism is the Plant Secondary Product GT (PSPG) box, found at the C-terminal region of the enzyme (Figure 2) [63]. This well-conserved domain is found across a range of plants, and is thought to be the region concerned with the nucleotide-diphosphate-sugar binding, as well as maintenance of the C-terminal fold of the enzyme [64].

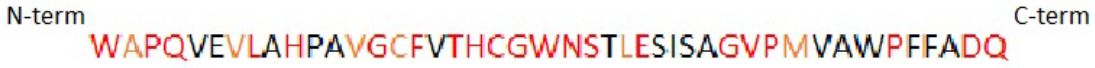


Figure 2 Conserved consensus sequence of the PSPG-box. Highly conserved amino acids are shaded orange (identity >50%) and red (identity >80%) [64]

So far, the PSPG box seems to be the only common region found with highly conserved amino acid motifs in the GT family. Because of the high sequence variability, it is believed that the N- and C-terminal regions have specific roles, in which the N-terminal is responsible for acceptor interactions, while the C-terminus binds donor substrates [63]. Fewer than 10 GTs have been functionally identified in *Vitis vinifera*, and the main enzymatic roles are associated with the metabolism of terpene alcohols and flavonoid

metabolism [65, 66]. The lack of a direct link between genomic information and the structure of these enzymes makes it difficult to elucidate their molecular mechanisms [67]. It has been suggested that the selectivity of GTs can be different for *in vivo* vs. *in vitro* reactions – where *in vitro* GTs can accept a broader range of acceptors with less regio-specificity [68]. Because of this, functional identification of these enzymes based on sequence information is difficult; i.e. GTs seem to be promiscuous in their choice of donor, acceptor and therefore subsequent product [69, 70]. Glycosyl transfer can occur on the nucleophilic oxygen of the hydroxyl substituent of an acceptor molecule (most often), but can also take place on sulphur, nitrogen and carbon nucleophiles [71, 72].

Regioselective glycosylation is quite common, and results in different GTs recognising the same substrate, but glycosylation of the acceptor can occur at various positions – this in turn results in different products for each GT [73]. GTs that can glycosylate different linkages – depending on the substrate they are working on – have also been identified [47]. This means that the designation of GTs as O-, S-, N-, or C-glycosyltransferases is not as strict as initially thought. Furthermore, not every GT uses the same sugar-donor for specific acceptors – leading to a range of glycoconjugate products of an acceptor molecule, depending on the GT that catalyses the reaction and the preference of this GT for specific sugars [73]. Lastly, GTs seem to be able to distinguish between enantiomers [73]. A GT found in *Arabidopsis* favours the glycosylation of only one of the enantiomers of abscisic acid, indicating the possibility of using GT enzymes as means of performing chiral separation [73]. These regio- and stereo-selective traits of GTs indicate the difficulty in predicting their glycoconjugate products, as well as the problems with obtaining these complexes using chemical *in vitro* synthesis. Classification of GTs also differs from most enzymes because of this promiscuity and so GT families are also classified on the basis of their 3D structure (GT -A, -B and predicted -C) and mechanism (either inverting or retaining) [69, 74]. Because of the complexity of GT classification, it

is not surprising that recently a promiscuous glucosyltransferase has been identified as having a possible role in smoke taint precursor formation [75]. Gene expression analysis of a subset of naturally occurring GTs in grapevines, followed by kinetic studies of recombinant candidate GTs showed a resveratrol GT (UGT72B27) to be well adapted for glucosylation of smoke-derived volatile phenols. Compounds such as guaiacol, syringol and 4-methylguaiacol were successfully transformed into their corresponding glucoconjugate precursor forms, even though *trans*-resveratrol is the putative substrate for UGT72B27. This research is an important first step in understanding the role of multiple GTs in *V. vinifera*. However, this study only identified glucosides that were present, i.e. precursor compounds with a single sugar moiety added, whereas it is well known that smoke taint precursors exist as diglycosides as well [26, 75]. Hence, it is still unclear what other GTs might play a role in the development of smoke taint precursors. Furthermore, even though the transcript profiles of glucosyltransferases were measured at several stages of grapevine development, this study did not involve the application of smoke to vines. Therefore other sets of GTs could play a role in glycoconjugation depending on differential gene expression following smoke exposure or the even higher temperatures associated with bushfires.

In tomatoes for example, differential gene expression for certain glycosyltransferases occur during development of the fruit [51, 52]. Some tomato cultivars synthesise guaiacol naturally, which is released as a free volatile compound upon tissue disruption of unripe fruit [51]. Glycoconjugated guaiacol is present in unripe tomato fruit as a diglycoside – and upon fruit tissue damage this disaccharide is cleaved causing the aglycone to be released. Tomatoes can be differentiated as ‘non-smoky’ and ‘smoky’ cultivars – depending upon their GT action during maturation of the fruit and subsequent metabolism of guaiacol disaccharides. ‘Non-smoky’ tomato cultivars express a gene during fruit ripening, encoding a GT which converts guaiacol diglycosides into triglycosides,

resulting in an un-cleavable unit [51]. In contrast, ‘smoky’ tomatoes do not express this gene and as a consequence release guaiacol, and therefore a smoky aroma, upon tissue disruption of mature fruit [76]. This implies that the addition of extra sugar units produces a glycoconjugate form that is resistant to hydrolysis. This could be of interest for the potential amelioration of smoke taint in smoke affected grapes.

Research aims

In the last 10 years, fires have occurred in close proximity to wine regions in countries including Australia, Canada, South Africa and the USA, resulting in vineyard exposure to smoke, and as a consequence, leading to the production of smoke tainted wine. Globally, taint as a consequence of smoke exposure has resulted in significant financial losses for both grape and wine producers. Unfortunately, the incidence of vineyard smoke exposure is expected to increase due to the warmer, drier conditions associated with climate change. Considerable research has been undertaken to investigate the impact of grapevine exposure to smoke on the composition and quality of wine. However, several key research questions remain, and as yet there is no ‘silver bullet’ available for the industry.

The research described in this thesis aimed to improve the current understanding of smoke taint better and to identify novel methods preventing and mitigating smoke taint in the earlier stages of wine production.

The key aims of the research in this thesis were therefore:

- To investigate the molecular response of grapevines to smoke exposure using a range of *Vitis vinifera* cultivars growth room and field trials by analysing the expression of several glycosyltransferases (chapter 3) and glycosylation patterns (chapter 2) occurring in fruit following the application of smoke to grapevines.

- To investigate the capacity of a hand-held spectrophotometer to identify smoke affected fruit (chapter 2).
- To evaluate the potential for agrichemicals (a silicate clay, kaolin and a polymer suspension, *Envy*) to provide a physical barrier, mitigating the effects of grapevine exposure to smoke.

Additionally, investigations were also done towards:

- The susceptibility of apples to taint from smoke.

CHAPTER 2

Detection and mitigation of smoke taint

in the vineyard

Statement of Authorship

Title of Paper	Accumulation of volatile phenol glycoconjugates in grapes, following the application of kaolin and/or smoke to grapevines (Vitis vinifera cv Sauvignon Blanc, Chardonnay and Merlot).
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Principal Author

Name of Principal Author (Candidate)	Lieke van der Hulst
Contribution to the Paper	Designed and conducted experiments; collected, processed, analysed and interpreted compositional and spectral data; drafted and edited the manuscript
Overall percentage (%)	70
Certification:	This paper reports on original research conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 15/11/17

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.

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Chapter 2: Detection and mitigation of smoke taint in the vineyard

Introduction

Over the years, several studies have attempted to identify and evaluate techniques for preventing and/or ameliorating smoke taint in grapes and wine. In the vineyard, hand-harvesting fruit not only reduces the risk of smoke tainted leaves being processed, but also minimises damage to skins, which reduces the extraction of smoke-derived volatile compounds (in free or bound form) [2]. Defoliation, either before or after smoke exposure, has also been trialled, but with limited success [37]. During winemaking, the use of specific yeast strains, the duration of skin contact and the addition of oak or tannin can mitigate the development of smoke aromas and flavours [14]. Adjustments can also be made to finished wine, either by the addition of processing aids (e.g. fining agents) [57] or by reverse osmosis and adsorbent treatment [55], to reduce the concentration of smoke-derived volatile phenols, and thus the intensity of smoke taint. However, ideally detection of smoke taint should occur in the vineyard prior to harvest, to avoid incurring unnecessary costs associated with harvesting smoke tainted fruit. Techniques that mitigate the impact of smoke exposure in the vineyard would similarly benefit grape and wine producers. The work described in this chapter therefore describes a series of field trials intended to evaluate methods for the detection and amelioration of smoke taint in the vineyard.

Manuscript 1 reports field trials conducted in 2016, which involved the application of kaolin (a clay-based particulate film) to grapevine fruit and foliage, as a physical barrier to mitigate the uptake of smoke-derived volatile compounds. The capacity of kaolin to lessen the effects of smoke was determined by comparing the accumulation of volatile phenols, and their glycoconjugates, in control and smoke affected grapes. Concurrently, reflectance spectroscopy was evaluated as a rapid method for differentiating control and

smoke affected grapes. Spectral methods have previously been adopted for the analysis of a wide array of crops, for example for the detection of leafroll-associated viruses in grapevine leaf samples and determination of ripening quality in apples [77, 78]. New applications of spectroscopy continue to be investigated, including the use of reflectance spectroscopy to study the impact of environmental conditions on grapes and grapevines [79]. The current study sought to determine to what extent grapevine exposure to smoke influenced berry spectral reflectance.

Field trials were repeated in 2017, and additionally involved: (i) Part A: the application of Envy (a polymer concentrate, with anti-transpiration properties) to grapevine fruit and foliage (of different cultivars than those studied in 2016); and (ii) Part B: berry spectral reflectance measurements from control and smoke-affected grapevines representing a broader range of cultivars.

Accumulation of volatile phenol glycoconjugates in grapes, following the application of kaolin and/or smoke to grapevines (*Vitis vinifera* cv Sauvignon Blanc, Chardonnay and Merlot).

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Abstract

Smoke taint is a fault found in wines made from grapes exposed to smoke from bushfires or prescribed burns. It is characterized by objectionable smoky and ashy aromas and flavors, which have been attributed to the presence of smoke-derived volatile phenols, in free and glycoconjugate forms. This study investigated the accumulation of volatile phenol glycoconjugates in grapes, following the application of kaolin (a clay-based protective film) and/or smoke to Sauvignon Blanc, Chardonnay and Merlot grapevines, at approximately 10 days post-veraison. Varietal differences were observed in the glycoconjugate profiles of smoke-affected grapes; the highest glycoconjugate levels were found in Merlot grapes, being pentose-glucosides of guaiacol, cresol, and phenol, and gentiobiosides of guaiacol and syringol. Changes in volatile phenol glycoconjugate profiles were also observed with time, i.e. between fruit sampled 1 day after smoke exposure and at maturity.

The application of kaolin did not significantly affect the glycoconjugate profiles of Sauvignon Blanc and Chardonnay grapes, but significantly lower volatile phenol glycoconjugate levels were observed in Merlot fruit that was treated with kaolin prior to smoke exposure. The potential for control and smoke-affected grapes to be differentiated by measurement of spectral reflectance with a handheld spectrometer, was also demonstrated.

Keywords

Cultivars, Glycoconjugates, Kaolin, Smoke taint, Spectroscopy, Volatile phenols

1. Introduction

The impact of grapevine exposure to smoke, i.e. as a fault characterized by objectionable smoky and ashy notes in wines made from smoke-affected grapes, has been well documented over the past decade (e.g. Kennison, Wilkinson, Williams, Smith, & Gibberd, 2007; Sheppard, Dhesi, & Eggers, 2009; Krstic, Johnson, & Herderich, 2015). The occurrence of bushfires or prescribed burns during the grape growing season can result in the uptake of smoke constituents by grapevines, in particular, volatile phenols such as guaiacols, cresols and syringols (Kennison, Gibberd, Pollnitz, & Wilkinson, 2008; Hayasaka et al., 2010a). Several studies have demonstrated the accumulation of volatile phenols in glycoconjugate forms, i.e. as glucosides, pentose-glucosides, gentiobiosides and rutinosides, in fruit and/or leaves following grapevine exposure to either smoke (Hayasaka et al., 2010a; Hayasaka, Dungey, Baldock, Kennison, & Wilkinson, 2010; Dungey, Hayasaka, & Wilkinson, 2011) or volatile phenols (Hayasaka, Baldock, Pardon, Jeffery, & Herderich, 2010; Pardo-Garcia et al., 2017). During fermentation, hydrolysis of glycoconjugates results in the volatile phenols being released (Kennison et al., 2008; Ristic et al., 2011), albeit a significant pool of volatile phenol glycoconjugates remains in finished wine (Ristic et al., 2016), even after prolonged bottle-aging (Ristic et al., 2017).

Both the volatile phenols and their glycoconjugates are thought to contribute to the sensory attributes associated with smoke taint (Kennison et al., 2007; Mayr et al., 2014; Ristic et al., 2016). As such, a range of analytical methods have been developed to facilitate their quantification, enabling the extent to which grapes and wine are affected by smoke to be assessed. Smoke-derived volatile phenols can be measured by gas chromatography-mass spectrometry (GC-MS) (Pollnitz, Pardon, Sykes, & Sefton, 2004; Hayasaka et al., 2010a); volatile phenol glycoconjugates can be measured either directly, by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Dungey et al. 2011;

Hayasaka et al., 2013), or indirectly by quantification of volatile phenols following enzyme or acid hydrolysis of juice, homogenate or wine (Wilkinson et al. 2011; Singh, Chong, Pitt, Cleary, Dokoozlian, & Downey, 2011; Noestheden, Thiessen, Dennis, Tiet, & Zandberg, 2017). Mid-infrared spectroscopy has also been evaluated as a rapid analytical method for detecting smoke tainted wines (Fudge, Wilkinson, Ristic, & Cozzolino, 2012; Fudge, Wilkinson, Ristic, & Cozzolino, 2013). However, ideally, screening of fruit for evidence of smoke taint should occur in the vineyard, i.e. before costs associated with harvesting and winemaking are incurred. As such, one aim of this study was to investigate whether or not measurement of spectral reflectance, measured using a simple, handheld spectrometer, can be used to differentiate control and smoke-affected grapes, prior to harvest.

The accumulation of volatile phenol glycoconjugates in grapes following grapevine exposure to smoke has not been extensively studied. Dungey and colleagues found guaiacol glycoconjugates accumulated in smoke-affected Merlot and Viognier fruit within a few days of smoke exposure (Dungey et al. 2011), but most studies report glycoconjugate concentrations in fruit at harvest (i.e. at commercial maturity) and/or in wine. The main aims of this study were therefore: (i) to evaluate temporal changes in the volatile phenol glycoconjugate profiles of smoke-affected grapes from different cultivars, following grapevine exposure to smoke; and (ii) to determine to what extent, if any, the application of kaolin, a particulate film used to mitigate light and heat stress in grapevines (Dinis et al., 2016), might provide a physical barrier that protects grapevine leaves and fruit against the uptake of smoke constituents.

Considerable progress has been made towards understanding the chemical and sensory impacts of smoke taint. Factors such as the timing and duration of smoke exposure

(Kennison, Wilkinson, Pollnitz, Williams, & Gibberd, 2009; Kennison, Wilkinson, Pollnitz, Williams, & Gibberd, 2011), grape cultivar (Ristic et al., 2016), vineyard management practices (Ristic, Pinchbeck, Fudge, Hayasaka, & Wilkinson, 2013), fruit maturity at harvest (Ristic, Boss, & Wilkinson, 2015) and winemaking techniques (Ristic et al, 2011; Kelly, Zerihun, Hayasaka, & Gibberd, 2014), in particular the duration of skin contact time during fermentation, are known to influence the intensity of smoke taint in finished wine. Two methods for amelioration of smoke taint have been identified, being the treatment of wine either by reverse osmosis and solid phase adsorption (Fudge, Ristic, Wollan, & Wilkinson, 2011) or with activated carbon (Fudge, Schiettecatte, Ristic, Hayasaka, & Wilkinson, 2012). Smoke taint nevertheless remains a significant challenge for grape and wine producers worldwide and additional insight into the compositional consequences of grapevine exposure to smoke, together with improved methods for detecting and ameliorating smoke taint, are required.

2. Materials and methods

2.1. Chemicals

Chemicals (analytical grade) and solvents (HPLC grade) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia) and Merck (Damstadt, Germany), respectively. Deuterium labelled internal standards were synthesized according to previously published methodology (Pollnitz, Pardon, Sykes, & Sefton, 2004; Hayasaka et al., 2010a). Powdered kaolin (trade name Surround) was sourced from AgNova Technologies (Box Hill North, Vic., Australia).

2.2. Application of kaolin and/or smoke to grapevines

Field trials involved the application of kaolin and/or smoke to Sauvignon Blanc, Chardonnay and Merlot grapevines growing at the University of Adelaide's Waite

Campus in Urrbrae, South Australia (latitude 34°58'S, longitude 138°38'E). Vines were planted in north-south aligned rows (in 1998), and were 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. Treatments comprised: (i) neither kaolin or smoke application, i.e. a control; (ii) the application of smoke, but not kaolin; and (iii) the application of kaolin, and then 24 h later, the application of smoke. Kaolin was prepared according to the manufacturer's recommendations and applied as an aqueous suspension to fruit and foliage (5 L per panel of three vines). Grapevines were exposed to smoke for 1 h, at approximately 10 d post-veraison, using purpose-built smoke tents (6 m x 2.5 m x 2 m), according to methodology described previously (Kennison et al., 2008; Ristic et al., 2011). Air temperature was monitored during smoke exposure by placing thermocouples (HOBO, Onset Computer Corporation, Bourne, MA, USA) in the grapevine canopy, but only small increases in temperature (i.e. $\leq$ 2 ºC) were observed relative to control vines (data not shown). Treatments were conducted in triplicate, with each experimental replicate comprising a panel of three vines; at least one buffer panel was retained between treatments.

Samples (approximately 500 berries per replicate per treatment) were collected at three time points: (i) 1 day after smoke exposure, (t = 1); (ii) 7 days after smoke exposure, (t = 7); and (iii) maturity, being 15, 12 and 30 days after smoke exposure for Sauvignon Blanc, Chardonnay and Merlot respectively, (t = 15, t = 12 and t = 30). Total soluble solids (TSS, as ºBrix) and berry weight were measured at each timepoint. The concentration of volatile phenols was determined in samples collected at t = 1 and t = 7; while berry homogenate was prepared from samples collected at t = 1, t = 7 and maturity, for determination of volatile phenol glycoconjugates.

2.3. Determination of volatile phenols by gas chromatography-mass spectrometry (GC-MS)

The concentrations of guaiacol, 4-methylguaiacol, *m*-, *o*-, and *p*-cresol, and syringol were determined in grapes according to stable isotope dilution assay methods reported previously (Pollnitz, Pardon, Sykes, & Sefton, 2004; Hayasaka et al., 2010a). These publications describe the preparation of internal standards (d_3-guaiacol, d_3-4-methylguaiacol, d_7-*o*-cresol, and d_3-syringol), method validation and instrumental operating conditions. Analyses were performed by the Australian Wine Research Institute's Commercial Services Laboratory (Adelaide, Australia), using an Agilent 6890 gas chromatograph coupled to a 5973 mass spectrometer (Agilent Technologies, Forest Hill, Vic., Australia).

2.4. Determination of volatile phenol glycoconjugates by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The concentrations of glucosides, pentose-glucosides, gentiobiosides and/or rutinosides of guaiacol, 4-methylguaiacol, *m*-, *o*-, and *p*-cresol, syringol and phenol were determined in berry homogenate according to stable isotope dilution assay methods reported previously (Hayasaka et al., 2010a). This publication describes the preparation of the internal standard (*d3*-syringol gentiobioside), method validation and instrumental operating conditions. Analyses were performed by the Australian Wine Research Institute's Commercial Services Laboratory (Adelaide, Australia), using an Agilent 1200 high performance liquid chromatograph (HPLC) coupled to an Applied Biosystems 4000 QTrap hybrid tandem mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA, USA).

2.5. Spectral reflectance measurements

The spectral reflectance of control and smoke-affected grapes were measured (relative to a white Spectralon standard) using a Jaz spectrometer (Ocean Optics Inc., Dunedin, FL, USA). Reflectance measurements were performed at three time points: (i) 1 day prior to smoke exposure, (t = –1); (ii) 1 day after smoke exposure, (t = 1); and (iii) 7 days after smoke exposure, (t = 7); with 15 measurements taken (in triplicate) per treatment, comprising 3 grapes from each of 5 bunches (chosen randomly).

2.6. Data analysis

Berry weight and compositional data were analyzed by one- or two- way analysis of variance (ANOVA) using GenStat (15th Edition, VSN, International Limited, Hemel Hempstead, UK). Mean comparisons were performed by least significant difference (LSD) multiple comparison test at $P < 0.05$. Spectral data were exported to The Unscrambler (Edition 10.2, CAMO ASA, Oslo, Norway) and pre-processed using the second-derivative transformation, the Savitzky-Golay derivation and smoothing (20-point and 2nd-order filtering operation), prior to principal component analysis (PCA).

3. Results and discussion

3.1. Influence of kaolin and/or smoke applications on berry ripening and composition.

The application of kaolin and/or smoke had no impact on berry development or ripening; i.e. there were no significant differences in either the sugar accumulation or berry weight of fruit harvested from control vs. treated grapevines (Table 1), irrespective of sampling time. This was in agreement with previous research reporting the influence of either kaolin treatment (Conde et al., 2016) or smoke exposure (Ristic et al., 2016) on berry development and ripening; albeit, repeated applications of smoke to Merlot grapevines between veraison and harvest yielded fruit with significantly lower sugar levels than

control fruit (Kennison et al., 2009). Nevertheless, in the current study, significant differences in TSS and berry weight were only observed between varieties, which is not unexpected.

Surprisingly, smoke exposure did not yield fruit with substantial levels of (free) volatile phenols, even at t = 1 (Supplementary Table 1). None of the volatile phenols measured, i.e. guaiacol, 4-methylguaiacol, *m*-, *o*- and *p*-cresol or syringol, were detected in control fruit from any of the grape varieties studied and only low levels, i.e. ≤ 4 µg/kg, were observed in kaolin and/or smoke-affected fruit. Whereas cresols were found (at 2 µg/kg) in Sauvignon Blanc grapes at both t = 1 and t = 7 (following kaolin and/or smoke applications), guaiacol and syringol were only detected at t = 1. Guaiacol was found in both Sauvignon Blanc and Merlot fruit, but syringol was only observed in Merlot; 4-methylguaiacol was not detected in any samples. Significant quantities of smoke-derived volatile phenols were not expected to be present at t = 7, given they have previously been shown to accumulate in grapevine leaves and/or fruit in glycoconjugate forms (Hayasaka et al., 2010a; Hayasaka et al., 2010c; Dungey et al., 2011; Pardo-Garcia et al., 2017). However, since it is not clear how quickly glycosylation occurs following smoke exposure, free volatile phenols were expected to have been detectable at t = 1 (i.e. 1 day after the application of smoke). These results suggest glycosylation begins soon after smoke exposure.

The concentrations of glucoside, pentose-glucoside, gentiobioside and/or rutinoside precursors of guaiacol, 4-methylguaiacol, cresol, syringol and phenol were measured (as syringol gentiobioside equivalents) at t = 1, t = 7 and at maturity, and in contrast to volatile phenol concentrations, significant differences were observed in the glycoconjugate profiles of fruit from control vs. treated grapevines (Table 2). Compositional differences

were readily observed not only by variety, but in some cases, by sample time as well. With the exception of syringol gentiobioside and phenol pentose-glucoside, which were detected at concentrations up to 18.3 µg/kg, only low levels (i.e. < 10 µg/kg) of each of the glycoconjugates measured were found in fruit from control grapevines, irrespective of sample time (Table 2). Syringol gentiobioside was the most abundant glycoconjugate present in control Sauvignon Blanc grapes, but for control Chardonnay and Merlot grapes, the phenol pentose-glucoside was most abundant.

Significantly higher glycoconjugate levels were observed in smoke-affected fruit (relative to control fruit), but interestingly, the glycoconjugate profiles differed between varieties. For Sauvignon Blanc, the most abundant glycoconjugates were the rutinosides of cresol and phenol, and the syringol gentiobioside, which were present at concentrations up to 70, 35 and 70 µg/kg, respectively. In contrast, the pentose-glucosides of guaiacol, cresol and phenol, and the syringol gentiobioside were most abundant in Chardonnay fruit, at concentrations between 25 and 60 µg/kg. Glycoconjugate concentrations tended to be several fold higher in smoke-affected Merlot fruit, compared to that of the white grape varieties. The most abundant glycoconjugates in Merlot included the gentiobioside of guaiacol, the pentose-glucosides of guaiacol, cresol and phenol, and the rutinoside of cresol, for which concentrations exceeded 100 µg/kg, at one or more time points. The concentrations of pentose-glucosides of guaiacol and cresol were the highest, at 283 and 300 µg/kg, respectively.

Some glycoconjugate levels remained consistently low irrespective of experimental treatment, variety or timepoint; for example the glucoside of guaiacol and gentiobioside of cresol. However, in some instances, significant changes in glycoconjugate levels were observed between sampling times. Certainly the concentration of cresol glucoside in

smoke-affected fruit decreased with ripening, which might reflect further glycosylation, i.e. conversion from a glucoside to a disaccharide. Whereas the concentration of cresol pentose-glucoside increased in smoke-affected Merlot grapes with maturity (i.e. between t = 1 and t = 7), a decrease was observed in smoke-affected Sauvignon Blanc grapes. This provides further evidence to support the suggestion that the uptake of smoke-derived volatile compounds and/or biochemical response of grapevines to smoke exposure may be influenced by grape variety (Ristic et al., 2016). Certainly consistent trends in the accumulation of volatile phenol glycoconjugates were not observed amongst varieties in the current study. The factors responsible for these differences remain unclear, however, the uptake of smoke-derived volatile phenols may be affected by varietal differences in berry physiology, such as skin thickness and the presence (or absence) of cuticular waxes. Variation in the expression of enzymes responsible for the glycosylation of volatile phenols might also account for the different volatile phenol glycoconjugate profiles observed by variety. A recent study employed gene expression analysis to identify glucosyltransferases capable of glycosylating smoke-derived volatile compounds (Härtl et al. 2017), but glucosyltransferase expression following grapevine exposure to smoke has not otherwise been investigated.

The application of kaolin to the fruit and foliage of Sauvignon Blanc and Chardonnay grapevines did not seem to strongly influence the uptake of smoke-derived volatile compounds, such that few meaningful differences were observed between the volatile phenol glycoconjugate profiles of grapevines treated with smoke vs. kaolin and smoke, at maturity (Table 2). However, moderate reductions in cresol rutinoside, cresol pentose-glucoside, phenol rutinoside and phenol gentiobioside levels were observed when Chardonnay grapevines were treated with kaolin prior to smoke exposure. The kaolin treatment was considerably more effective at mitigating the impact of smoke exposure

for Merlot, with reductions of 58 to 92 % achieved for most of the volatile phenol glycoconjugates measured, at maturity (Table 2). The efficacy of kaolin as a physical barrier to smoke would be expected to be influenced by the degree of coverage. In the current study, kaolin applications were intended to achieve coverage of both fruit and foliage, but kaolin may have been applied more evenly to Chardonnay and Merlot fruit, than to Sauvignon Blanc fruit; i.e. upon drying, the kaolin suspension formed spots on Sauvignon Blanc berries. This may have indicated only partial coverage and might therefore account for the similarity in glycoconjugate profiles observed for smoke vs. kaolin and smoke treatments in Sauvignon Blanc fruit. Repeated applications of kaolin could potentially resolve this issue. However, the reductions achieved for Merlot nevertheless demonstrate the potential for kaolin to moderate the uptake of volatile phenols during grapevine exposure to smoke.

3.2. Influence of smoke exposure on berry spectral reflectance.

Reflectance spectroscopy has been used to measure compositional changes in plant tissues (particularly in chlorophyll, carotenoid, anthocyanin and flavonol composition) associated with fruit development and/or in response to environmental conditions (Merzlyak, Gitelson, Chivkunova, & Rakitin, 1999; Solovchenko, Merzlyak, & Pogosyan, 2010; Rustioni, Rocchi, Guffanti, Cola, & Failla, 2014). In the current study, spectral reflectance was found to be capable of differentiating control and smoke-affected grapes, but the timing of spectral measurements influenced how readily smoke exposure could be detected for each grape variety (Figures 1 and 2).

At t = 1, there were clear differences in the reflectance spectra for control and smoke-affected Sauvignon Blanc grapes (Figure 1a), but these differences were less apparent at t = 7 (Figure 1b). In contrast, differences in spectral reflectance were not only observed

for control and smoke-affected Chardonnay at each time point, but also between time points (Figures 1c and 1d); i.e. percentage reflectance was considerably higher at t = 1, compared with t = 7. Reflectance was also higher at t = 1 relative to t = 7, for Merlot grapes (Figures 1e and 1f), but similar reflectance spectra were observed for control and smoke-affected Merlot grapes, at each time point.

Principal component analysis (PCA) was subsequently performed on spectral reflectance data for each variety, at each time point, and the resulting PCA biplots are shown in Figure 2. The first two principal components (PCs) derived from reflectance spectra explained between 66 and 97% of the variation observed in the PCA biplots; with PC1 explaining 40 to 88% of variation and PC2 explaining a further 8 to 26% of variation. Partial separation of control and smoke-affected Sauvignon Blanc fruit was observed at t = 1 (Figure 1a): control samples tended to cluster in quadrants on the left, whereas smoke-affected samples tended to cluster on the right, with only a few control and smoke-affected samples over-lapping. However, by t = 7 there was no longer clear separation, such that control and smoke-affected samples were located across all four quadrants (Figure 2b). The most apparent separation was observed for Chardonnay fruit: control and smoke-affected Chardonnay samples were located on opposite sides of the PCA biplot at both t = 1 (Figure 2c) and t = 7 (Figure 2d). This suggests the compositional and/or physiological consequences of grapevine exposure to smoke were not only readily detected, but persisted, in Chardonnay fruit. In the case of Merlot, there was no separation of samples at t = 1 (Figure 2e); but partial separation was evident at t = 7 (Figure 2f), with most of the control samples located in quadrants on the right, and all but one of the smoke-affected samples located in quadrants on the left. The lack of differentiation of Merlot samples at t = 1 might be attributable to variation in berry color, at this time point. At t = 1 (~11 days post-veraison), bunches still comprised both green and red colored berries,

and the inherent differences in anthocyanin levels alone, would undoubtedly influence berry spectral properties (Rustioni, Di Meo, Guillaume, Failla, & Trouillas, 2013). At t = 7 (~18 days post-veraison), fruit ripening had progressed such that berry color was far more consistent, so positioning of samples on the PCA plot now reflects the influence of smoke exposure.

PCA was repeated on spectral data from both t = 1 and t = 7 (for each variety, Supplementary Figure 1) and clear separation of control and smoke-affected fruit was observed for both Chardonnay and Merlot, irrespective of the timing of spectral measurements (Supplementary Figures 1b and 1c, respectively). For these varieties, clustering of samples by time point was also observed; separation was most apparent for control Chardonnay grapes, but to a lesser degree, for smoke-affected Merlot grapes also. However, similar separation patterns were not observed for Sauvignon Blanc (Supplementary Figure 1a), with control and smoke-affected samples from each time point were observed in multiple quadrants of the PCA biplot.

Standard practice for assessing smoke taint in grapes following vineyard exposure to smoke typically involves sampling fruit prior to maturity for chemical analysis (Ristic et al. 2015), either before or after fermentation. Few grape and wine producers have the expertise and instrumentation required for determination of volatile phenols or their glycoconjugates, so analyses are usually out-sourced to commercial laboratories. For some producers this is prohibitively expensive and often results are not available in time to inform decisions regarding harvest. Improved methods for detecting smoke taint in the vineyard, within the time constraints of harvest are therefore required. Results from the current study suggest reflectance spectroscopy may offer a rapid, cost-effective method by which smoke exposure can be detected in the vineyard. Spectral measurements of

control and smoke-affected fruit (and perhaps leaves) from a broader range of grape varieties and phenological growth stages could be used to develop a predictive model for detecting smoke taint based on spectral properties.

4. Conclusions

Temporal changes in the volatile phenol glycoconjugate profiles of three grape varieties were measured following grapevine exposure to smoke, for the first time. Varietal differences were observed in glycoconjugate profiles; with the highest glycoconjugate levels, being pentose-glucosides of guaiacol, cresol, and phenol, and gentiobiosides of guaiacol and syringol, observed in Merlot grapes. Changes in glycoconjugate profiles observed with time, i.e. between fruit sampled 1 day after smoke exposure and at maturity, suggest conversion of glycoconjugates; from glucosides to disaccharides, for example. The application of kaolin to fruit and foliage prior to smoke exposure did not significantly affect the glycoconjugate profiles of Sauvignon Blanc and Chardonnay grapes, but did result in significantly lower glycoconjugate levels in Merlot fruit. These results suggest kaolin might provide a protective barrier against the uptake of smoke, depending on the rate of application and extent of coverage. Spectral reflectance measurements enabled differentiation of control and smoke-affected fruit and might therefore offer a rapid method for detecting smoke taint in the vineyard; albeit the timing of measurements influenced how readily smoke exposure could be detected for each of the grape varieties studied.

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Supplementary material

Supplementary Table 1 Concentrations of volatile phenols (µg/kg) in grapes harvested from control (C), smoke-affected (S) and kaolin treated (K) grapevines, at different time points.

Supplementary Figure 1 PCA biplots generated from spectral data for control (●/■) and smoke-affected (○/□) Sauvignon Blanc (a), Chardonnay (b) and Merlot (c) grapes, at t = 1 (●/○) and t = 7 (■/□).

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Figure captions

Figure 1 Reflectance spectra for control (solid line) and smoke-affected (dotted line) Sauvignon Blanc (a,b), Chardonnay (c,d) and Merlot (e,f) grapes at t = 1 (a,c,e) and t = 7 (b,d,f). Values are means of 45 replicate measurements (per treatment per variety).

Figure 2 PCA biplots generated from spectral data for control (●) and smoke-affected (○) Sauvignon Blanc (a,b), Chardonnay (c,d) and Merlot (e,f) grapes at t = 1 (a,c,e) and t = 7 (b,d,f).

Table 1 Total soluble solids (TSS) and weight of grapes sampled from control (C), smoke-affected (S) and kaolin treated (K) grapevines, at different time points.

Treatment		TSS (°Brix)			Berry weight (g)		
		t = 1	t = 7	maturity[†]	t = 1	t = 7	maturity[†]
Sauvignon Blanc	C	17.2	18.9	22.3	1.1	1.2	1.2
	S	17.9	20.5	23.6	1.1	1.4	1.4
	K+S	16.3	18.3	22.7	1.1	1.4	1.4
Chardonnay	C	21.7	21.0	23.4	1.3	1.3	1.5
	S	21.2	20.6	22.9	1.3	1.3	1.4
	K+S	20.2	20.3	23.1	1.2	1.4	1.4
Merlot	C	17.5	20.4	24.1	0.9	1.0	1.0
	S	15.7	18.1	24.0	0.9	1.1	1.1
	K+S	16.5	19.3	24.5	0.8	1.0	1.1

Sample times are reported as days after kaolin and/or smoke applications.
[†]maturity corresponded to t = 15, t = 12 and t = 30 days for Sauvignon Blanc, Chardonnay and Merlot respectively.
Values represent the mean of three replicates.
Values within columns (i.e. by treatment, for each variety) were not significantly different ($P = 0.05$, one-way ANOVA).

Table 2 Concentrations of volatile phenol glycoconjugates (µg/kg) in grapes harvested from control (C), smoke-affected (S) and kaolin treated (K) grapevines, at different time points.

Treatment		Sample time	Gu-R	Gu-GB	Gu-PG	Gu-G	Cr-R	Cr-GB	Cr-PG	Cr-G	Ph-R	Ph-GB	Ph-PG	Syr-GB	Syr-G	4MG-R	4MG-PG
Sauvignon Blanc	C	t = 1	0.5 f	0.4 c	2.2 c	tr	3.4 d	tr	5.5 c	1.3 d	1.0 e	tr	5.6 d	0.9 e	0.6 c	0.7 c	nd
		t = 7	2.2 e	2.7 c	3.6 c	tr	9.3 d	0.6	8.8 c	4.2 cd	4.7 e	3.3 d	7.3 d	17.5 d	1.9 c	2.7 c	0.7 c
		t = 15†	3.5 e	2.8 c	2.5 c	3.4	8.8 d	0.5	5.5 c	nd	7.0 de	3.4 d	8.3 d	18.3 d	0.8 c	2.2 c	1.1 bc
	S	t = 1	13.6 d	14.5 b	16.0 ab	2.3	68.6 b	1.5 bc	34.8 a	48.8 a	14.5 c	5.1 cd	14.3 b	28.0 cd	11.5 b	13.8 b	3.8 a
		t = 7	17.6 c	16.0 b	11.2 b	0.7	61.5 b	1.3 c	19.0 b	10.3 c	33.3 b	7.1 cd	6.5 d	39.3 cd	4.4 c	13.0 b	1.7 b
		t = 15†	21.4 b	17.6 b	12.3 b	3.0	41.4 c	1.8 bc	17.3 b	3.7 cd	35.2 b	12.5 b	24.2 a	68.7 b	1.8 c	12.9 b	3.1
	K+S	t = 1	12.8 d	15.3 b	17.1 a	3.4	46.7 c	2.1 b	32.3 a	44.6 a	10.4 cd	7.6 c	12.5 b	43.2 bc	19.0 a	14.5 b	3.7 a
		t = 7	25.7 a	27.3 a	19.9 a	nd	75.7 a	3.3 a	34.1 a	19.7 b	42.0 a	19.2 a	11.2 b	110.1 a	13.7 b	21.6 a	3.0 a
		t = 15†	23.7 ab	17.3 b	11.4 b	3.0	48.7 c	1.4 c	18.0 b	4.2 cd	43.5 a	11.9 b	23.5 a	65.3 b	1.7 c	12.1 b	2.8 a
	P	*treatment*	*<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*	*<0.001*	*<0.001*
		treatment x time	*0.02*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*<0.001*	*0.001*	*ns*	*<0.001*	*ns*	*ns*	*ns*	*ns*
Chardonnay	C	t = 1	tr	tr	5.4 c	tr	0.6 c	tr	6.9 c	nd	tr	tr	12.6 d	0.5 e	tr	tr	0.8 c
		t = 7	tr	0.5 c	5.6 c	nd	0.6 c	tr	6.2 c	nd	tr	tr	13.0 d	0.6 e	tr	tr	0.8 c
		t = 12†	tr	0.6 c	3.8 c	4.7 a	0.6 c	tr	4.2 c	tr	tr	nd	11.5 d	0.6 e	tr	tr	nd
	S	t = 1	2.2 c	1.7 b	29.1 b	1.8 c	10.2 ab	0.5 c	29.5 ab	10.3 a	4.5 c	4.9 c	24.7 c	26.1 d	3.1 ab	1.9 b	4.5 ab
		t = 7	3.3 abc	1.9 b	34.1 ab	1.1 c	14.2 a	0.7 ab	33.6 ab	3.6 bc	7.2 ab	8.1 ab	29.5 bc	45.2 ab	3.0 ab	2.5 ab	5.2 ab
		t = 12†	4.4 a	2.5 ab	39.8 ab	5.5 a	16.1 a	0.8 a	40.4 a	1.3 c	8.6 a	10.3 a	58.2 a	55.5 a	0.6 c	2.6 ab	6.0 a
	K+S	t = 1	3.1 abc	2.7 ab	37.5 ab	3.4 b	13.1 ab	0.6 bc	40.9 a	12.0 a	4.8 bc	7.0 bc	29.6 bc	38.9 bcd	4.0 a	2.5 ab	5.4 ab
		t = 7	3.7 ab	3.3 a	50.0 a	1.5 c	16.1 a	0.6 bc	40.4 a	4.5 b	7.6 a	7.4 bc	40.0 b	42.3 abc	2.6 b	3.1 a	6.9 a
		t = 12†	2.3 bc	1.6 b	23.1 b	5.0 a	7.3 b	0.4 c	21.8 b	tr	4.4 c	5.4 c	36.3 b	29.8 cd	0.5 c	1.6 b	3.2 bc
	P	*treatment*	*<0.001*	*0.003*	*0.002*	*0.004*	*<0.001*	*<0.001*	*<0.001*	*0.005*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*0.004*	*0.002*
		treatment x time	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Merlot	C	t = 1	tr	0.6 b	3.8 b	tr	0.8 b	tr	9.0 b	11.5 b	tr	tr	11.7 c	0.8 b	0.9 c	tr	0.8 b
		t = 7	tr	1.0 b	5.1 b	tr	1.5 b	0.6 b	9.3 b	0.9 b	tr	tr	11.2 c	2.0 b	0.6 c	tr	1.0 b
		t = 30†	1.4 b	1.8 b	4.0 b	6.8 b	2.6 b	1.0 b	9.8 b	nd	0.9 c	1.2 b	13.9 c	5.2 b	0.6 c	1.2 b	1.2 b
	S	t = 1	1.8 b	10.3 b	21.4 b	5.9 b	6.3 b	0.9 b	28.1 b	37.0 a	0.9 c	3.3 b	15.0 c	17.5 b	8.4 b	3.0 b	4.9 b
		t = 7	21.7 a	118.5 a	283.1 a	15.5 a	112.9 a	4.0 a	300.0 a	49.1 a	25.9 b	30.2 a	143.2 ab	175.6 a	16.3 a	26.6 a	40.1 a
		t = 30†	34.2 a	137.9 a	278.7 a	8.8 b	110.8 a	3.9 a	243.4 a	17.0 b	54.3 a	46.6 a	233.2 a	211.0 a	1.9 c	22.2 a	36.6 a
	K+S	t = 1	2.0 b	13.8 b	21.6 b	4.8 b	9.5 b	0.7 b	35.5 b	47.3 a	1.2 c	3.0 b	18.6 c	14.8 b	4.8 bc	3.2 b	5.3 b
		t = 7	6.1 b	21.0 b	51.7 b	3.1 b	27.9 b	1.1 b	76.9 b	15.8 b	7.6 bc	8.4 b	40.7 bc	45.5 b	3.0 c	6.1 b	7.5 b
		t = 30†	7.6 b	19.9 b	43.9 b	7.6 b	23.3 b	1.5 b	51.4 b	1.3 b	11.5 bc	9.5 b	57.9 bc	48.8 b	0.8 c	4.7 b	6.3 b
	P	*treatment*	*0.024*	*0.040*	*0.042*	*0.054*	*0.030*	*0.001*	*0.024*	*0.004*	*0.063*	*0.017*	*0.078*	*0.011*	*0.002*	*0.009*	*0.025*
		treatment x time	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Sample times are reported as days after kaolin and/or smoke applications; †designates sampling times corresponding to maturity.
Values are means of three replicates (n = 3); nd = not detected; tr = trace (i.e. < 0.5 µg/kg).
Different letters within a column (for each variety) indicate statistical significance (*P* = 0.05, two-way ANOVA); ns = not significant.
Gu = guaiacol; Cr = cresol; Ph = phenol; Syr = syringol; 4MG = 4-methylguaiacol; R = rutinoside; GB = gentiobioside; PG = pentose glucoside; G = glucoside.

Figure 1

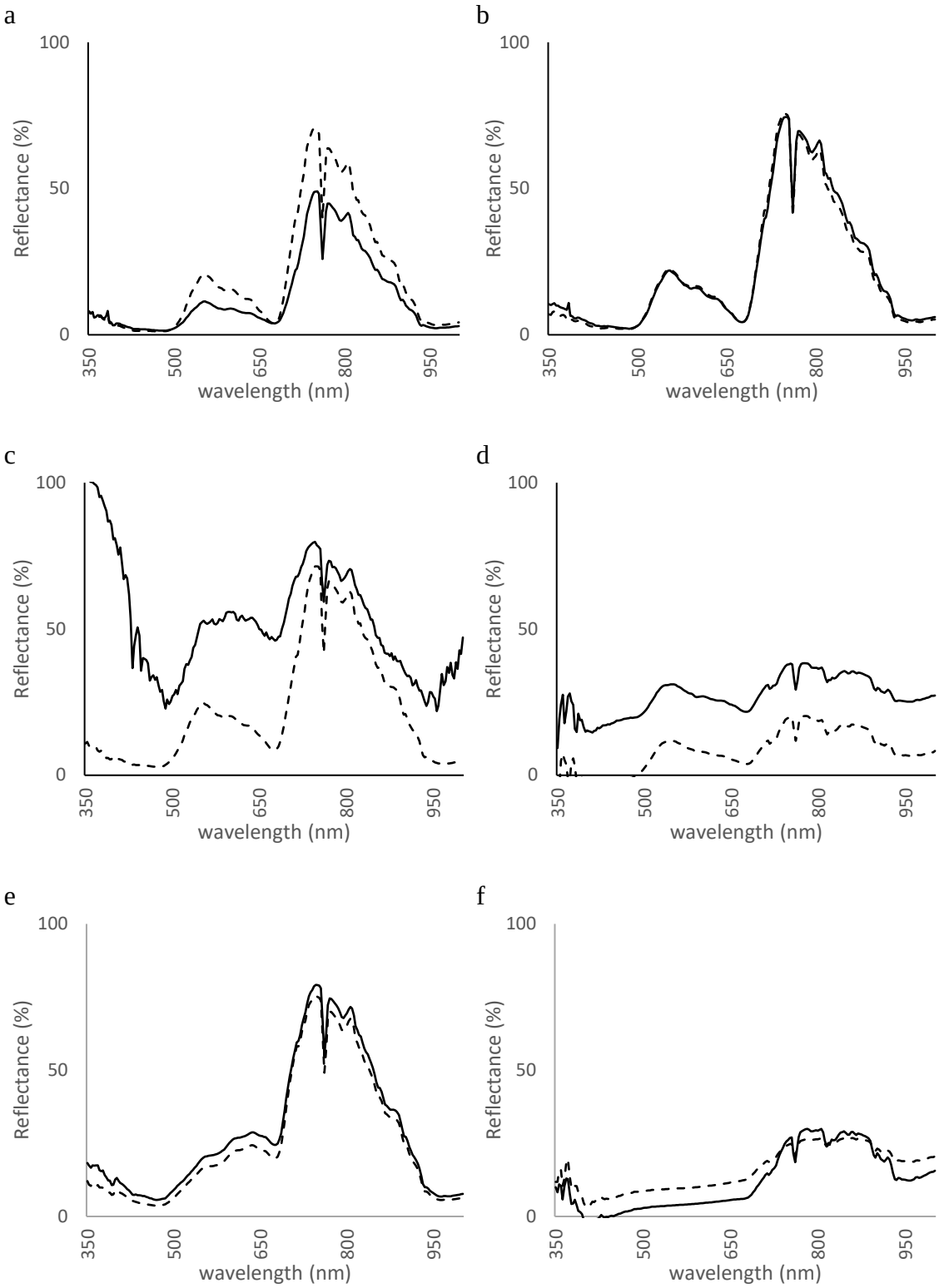
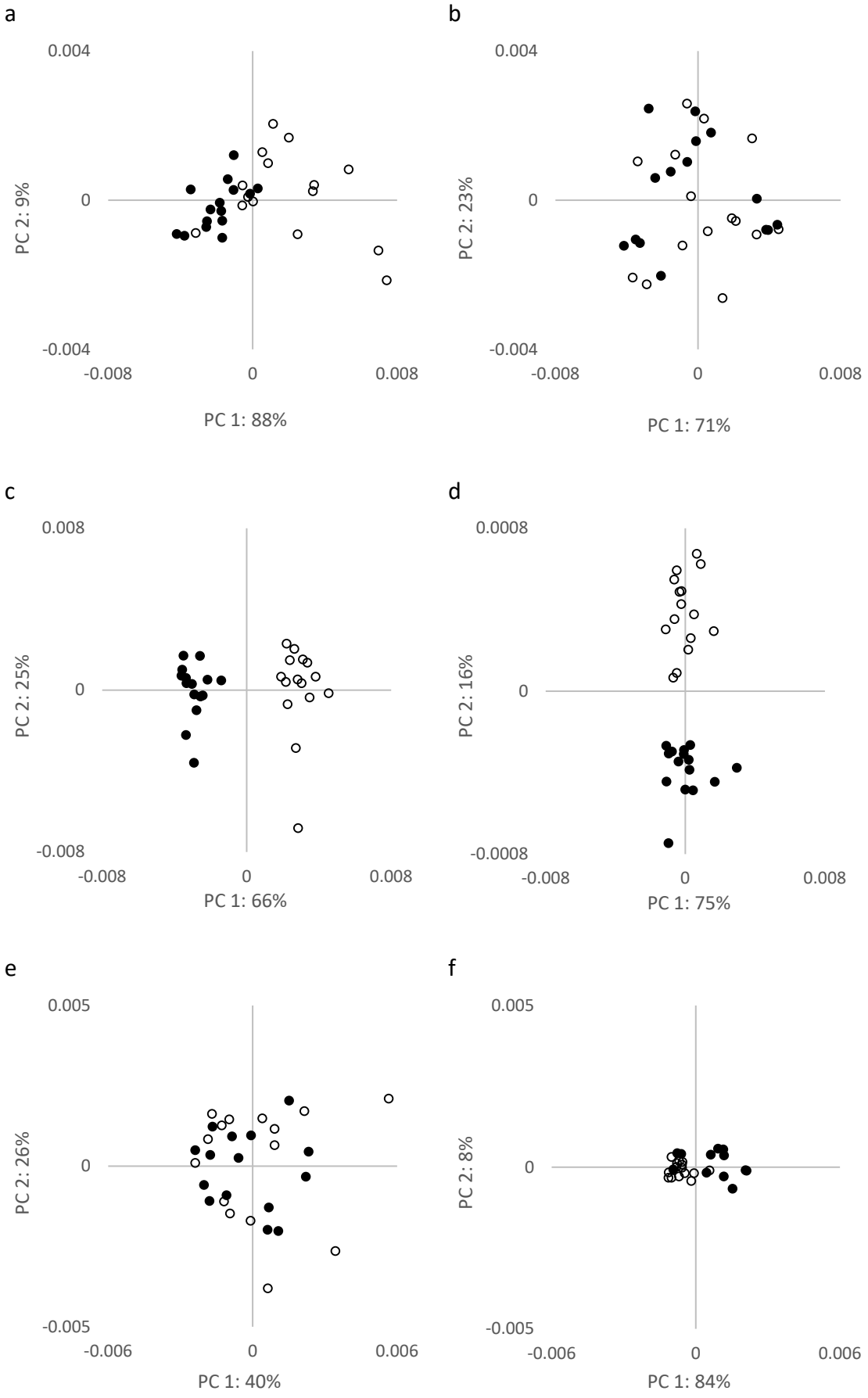

a
b
c
d
e
f
100
50
0
Reflectance (%)
350
500
650
800
950
wavelength (nm)

Figure 2

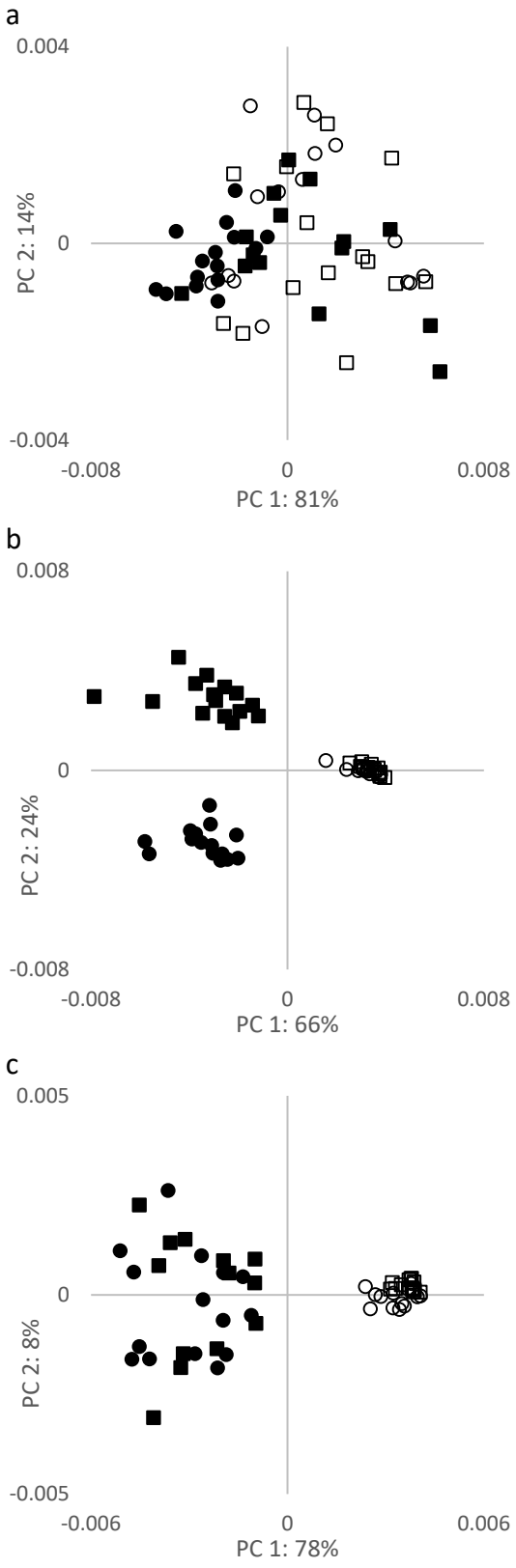

a
0.004
0
-0.004
PC 2: 9%
-0.008
0
0.008
PC 1: 88%
b
0.004
0
-0.004
PC 2: 23%
-0.008
0
0.008
PC 1: 71%
c
0.008
0
-0.008
PC 2: 25%
-0.008
0
0.008
PC 1: 66%
d
0.0008
0
-0.0008
PC 2: 16%
-0.008
0
0.008
PC 1: 75%
e
0.005
0
-0.005
PC 2: 26%
-0.006
0
0.006
PC 1: 40%
f
0.005
0
-0.005
PC 2: 8%
-0.006
0
0.006
PC 1: 84%

Supplementary Table 1 Concentrations of volatile phenols (μg/kg) in grapes harvested from control (C), smoke-affected (S) and kaolin treated (K) grapevines, at different time points.

Treatment		Sample time	guaiacol	4-methyl guaiacol	total cresols	syringol
Sauvignon Blanc	C	t = 1	nd	nd	nd	nd
		t = 7	nd	nd	nd	nd
	S	t = 1	1	nd	2	nd
		t = 7	tr	nd	2	nd
	K+S	t = 1	1	nd	2	nd
		t = 7	nd	nd	2	nd
Chardonnay	C	t = 1	nd	nd	nd	nd
		t = 7	nd	nd	nd	nd
	S	t = 1	nd	nd	tr	nd
		t = 7	nd	nd	nd	nd
	K+S	t = 1	nd	nd	nd	nd
		t = 7	nd	nd	nd	nd
Merlot	C	t = 1	nd	nd	nd	nd
		t = 7	nd	nd	nd	nd
	S	t = 1	4	nd	nd	1
		t = 7	tr	nd	tr	nd
	K+S	t = 1	3	nd	tr	2
		t = 7	nd	nd	nd	nd

Sample times are reported as days after kaolin and/or smoke applications.
Values represent the mean of three replicates; nd = not detected; tr = trace (i.e., ≤ 1 μg/L).

Supplementary Figure 1 PCA biplots generated from spectral data for control (●/■) and smoke-affected (○/□) Sauvignon Blanc (a), Chardonnay (b) and Merlot (c) grapes, at t = 1 (●/○) and t = 7 (■/□).



Further investigation into methods for the detection and mitigation of smoke taint in the vineyard

Part A: The potential for an agrichemical to inhibit the uptake of smoke-derived volatile phenols.

Following the field trial in 2016, in which the agrichemical kaolin was applied to grapevine fruit and foliage prior to smoke exposure (described in manuscript 1), a similar trial evaluating an anti-transpirant polymer concentrate, was performed in 2017. Two grape varieties, Chardonnay and Cabernet Sauvignon, were treated with the polymer concentrate (trade name Envy), 24 hours prior to smoke exposure. As in 2016, volatile phenol glycoconjugates were measured at maturity to determine the extent to which Envy inhibited the uptake of smoke volatiles.

Part B: Detection of smoke-affected grapes by reflectance spectroscopy.

To further investigate the potential for reflectance spectroscopy to be used to detect smoke taint in the vineyard, spectral reflectance measurements were acquired for control and smoke-affected grapes from four different cultivars, Chardonnay, Sauvignon Blanc, Merlot and Cabernet Sauvignon, at 1 and 7 days post-smoke exposure. Principal component analysis was performed on spectral data to investigate separation of control and smoke-affected samples; i.e. differentiation of samples based on smoke exposure.

Materials and methods

Chemicals

Chemicals (analytical grade) and solvents (HPLC grade) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia) and Merck (Damstadt, Germany), respectively. Deuterium labelled internal standards were synthesised according to previously published methodology [27]. The polymer concentrate Envy was sourced from Agrobest (Nerang, Qld, Australia).

Field trials

Part A: Application of Envy and/or smoke to grapevines

The protocol for this experiment was similar to that employed in 2016, however, whereas a single application of kaolin was used, two applications of Envy were made to ensure full coverage of the fruit and foliage. Chardonnay and Cabernet Sauvignon grapevines (grown in a vineyard located at the University of Adelaide's Waite Campus) were exposed to smoke (in triplicate) for 1 hour at approximately 10 days post veraison, using experimental conditions described previously [54]. Air temperature was monitored during smoke exposure by placing a temperature tracker (model number 010-11092-30, Garmin International Inc., Kansas, USA) within the vine canopy. As in 2016, only small increases in temperature (i.e. $\leq$ 2 ºC) were observed relative to ambient temperature (data not shown).

Following smoke exposure, samples were collected at regular time points to monitor fruit ripening, based on TSS measurements; berry weight was also determined at maturity. Samples (approximately 200 berries per replicate per treatment) were collected at

maturity for precursor analysis. Preparation of berry homogenate for LC-MS/MS was as described in manuscript 1.

Part B: Spectral reflectance measurements

Reflectance measurements were performed at two time points: 1 day after smoke exposure, (t = 1); and 7 days after smoke exposure, (t = 7); with 27 measurements taken (in triplicate) per treatment, comprising 9 grapes from each of 3 bunches (chosen randomly). Spectral data were pre-processed and analysed in The Unscrambler as described in manuscript 1.

Determination of volatile phenol glycoconjugates by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The concentrations of glucosides, pentose-glucosides, gentiobiosides and/or rutinosides of guaiacol, 4-methylguaiacol, *m*-, *o*-, and *p*-cresol, syringol and phenol were determined in berry homogenate (derived from fruit samples collected from field trials in both Part A and Part B), according to SIDA methods reported previously [27]. This publication describes the preparation of the internal standard (*d3*-syringol gentiobioside), method validation and instrumental operating conditions. Analyses were performed by the AWRI's Commercial Services Laboratory (Adelaide, Australia), using an Agilent 1200 high performance liquid chromatography (HPLC) coupled to an Applied Biosystems 4000 QTrap hybrid tandem mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA, USA).

Data analysis

Statistical analysis of chemical data was performed by one-way ANOVA using GenStat (15th Edition, VSN, International Limited, Hemel Hempstead, UK), and verified through

one-way ANOVA combined with a Tukey test through XLSTAT (version 2015.1, Addinsoft, NY, USA). Mean comparisons were performed by least significant difference (LSD) multiple comparison test at $P < 0.05$.

Results and discussion

Part A: Influence of Envy and/or smoke applications on berry ripening and composition

The influence of Envy and/or smoke applications on berry ripening were investigated by comparing the berry weight and TSS content of control and treated fruit (Table 1). Small differences were observed between the weights of control and treated Chardonnay grapes, but were attributed to natural variability, rather than treatment with Envy or smoke exposure, and were not considered to be meaningful differences. Significant differences were not observed in the weight of Cabernet Sauvignon grapes, for the different treatments. Although a significant difference in the TSS content of Chardonnay fruit was observed at t = 5, this difference was no longer evident at t = 8, or at maturity. However, in the case of Cabernet Sauvignon, differences were observed between treatments, both at t = 5 and at maturity (t = 16). Nevertheless, these differences were quite small, and as such, were again considered to reflect natural variability, rather than a treatment effect.

Table 2 TSS and berry weight for Chardonnay and Cabernet Sauvignon grapes sampled from control (C), smoke-affected (S) and Envy treated grapevines (E+S).

Treatment		TSS (°Brix)				Berry weight (g)
		t = 5	t = 8	t = 13	t = 16	maturity
Chardonnay	C	21.9 a	21.2	23.0	-	1.4 b
	S	20.9 a	21.6	22.7	-	1.5 ab
	E+S	17.3 b	22.7	23.3	-	1.6 a
Cabernet Sauvignon	C	19.2 ab	18.5	20.7	24.3 a	1.2
	S	18.8 b	19.7	20.6	23.9 a	1.1
	E+S	20.6 a	18.7	20.5	22.2 b	1.1

Sample times are reported as days after smoke exposure. Berry weight measured at maturity i.e. at t = 13 and t = 16 for Chardonnay and Cabernet Sauvignon, respectively. Values represent the mean of three replicates. Different letters within a column (for each variety) indicate statistical significance (P = 0.05, one-way ANOVA).

The volatile phenol glycoconjugate levels of control and treated grapes are reported in Table 2. Most glycoconjugate precursors were either not detected or were present at low levels (i.e. ≤ 2 µg/kg) in control grapes; only the pentose-glucosides of cresol, phenol and 4-methylguaiacol were present at higher concentrations, being 3 to 6 µg/kg. The concentrations of volatile phenol glycoconjugates present in smoke-affected Chardonnay and Cabernet Sauvignon grapes ranged from 1–24 and 2–59 µg/kg, respectively. These concentrations indicate the grapes were indeed smoke tainted, but interestingly, levels were lower than seen previously; i.e. than levels reported in manuscript 1. Compared with glycoconjugate levels observed in 2016, the overall precursor concentrations for 2017 were significantly lower. For example, syringol gentiobioside levels in smoke-affected Chardonnay grapes decreased from 55 to 26 µg/L, while cresol rutinoside levels decreased from 16 to 2 µg/L. However, precursor levels detected in Cabernet Sauvignon tended to be higher than for Chardonnay, which was in agreement with the levels of

precursors found in red and white wine in previous work [80]. The precursor profile obtained for Chardonnay showed the pentose glucosides of guaiacol, cresol and phenol, together with the syringol gentiobioside, as the most abundant precursors; this was similar to results reported in manuscript 1.

In the case of smoke-affected Cabernet Sauvignon grapes, the most abundant precursor was the gentiobioside of syringol, which was present at 59 µg/kg; i.e. at several fold higher concentrations than for other precursors, including the pentose glucosides of guaiacol, cresol and phenol, as well as the gentiobioside of 4-methylsyringol. Where grapevines were treated with Envy prior to smoke exposure, fruit glycoconjugate levels ranged from 1 to 26 µg/kg for Chardonnay and up to 139 µg/kg for Cabernet Sauvignon. Only some precursors were present at significantly different concentrations following Envy treatment, but these included the syringol gentiobioside, which increased from 59 to 139 µg/kg, for Cabernet Sauvignon. This might suggest Envy actually enhanced the uptake of smoke-derived volatile compounds. However, it should be noted that glycoconjugate data for one replicate was consistently higher than for the other two replicates (Supplementary Table 1). This might reflect uneven exposure of grapevine replicates to smoke; albeit this has not been experienced in previous experiments using the same experimental protocol for exposing grapevines to smoke [5, 7]. A comparison of the concentrations of pentose glucosides of guaiacol and cresol provides a case in point: large differences were observed between the levels of these glycoconjugates present in fruit from smoke exposed and Envy treated grapevines, yet ANOVA found no statistical significance (Table 2). This might be attributable to variation amongst concentrations observed for individual replicates (Supplementary Table 1). Data were subjected to a one-way ANOVA combined with the Tukey test, but this did not yield statistically significant differences either (data not shown).

Table 1 Concentrations of volatile phenol glycoconjugates (µg/kg) in grapes harvested from control (C), smoke-affected (S) and Envy treated (ES) grapevines.

Treatment		Gu-R	Gu-GB	Gu-PG	Gu-G	Cr-R	Cr-PG	Ph-R	Ph-GB	Ph-PG	Syr-GB	4MG-R	4MG-PG	4MS-GB	4MS-G
Chardonnay	C	nd	tr	2	1	tr	4	tr	nd	6	1	tr	3 b	tr	tr
	S	1	tr	12	2	2	9	1	1	13	24	2	3 b	2	2
	E+S	tr	tr	9	1	3	8	2	1	11	26	2	6 a	3	1
	P	–	–	*ns*	*ns*	–	*ns*	–	–	*ns*	*ns*	*ns*	*<0.001*	–	–
Cabernet Sauvignon	C	tr	nd	2	1	tr	2	tr	tr	4 b	2 b	tr	2	tr	tr
	S	7	4	27	3	14	13	7	3 b	26 a	59 ab	5	2	5	2
	E+S	5	5	12	3	19	11	8	6 a	24 a	139 a	6	3	13	3
	P	–	*ns*	*ns*	*ns*	–	*ns*	–	*0.004*	*0.013*	*0.009*	–	*ns*	–	–

Values are means of three replicates (n = 3); nd = not detected; tr = trace (i.e. < 0.5 µg/kg), limit of quantification was 1.0 µg/kg. Different letters within a column (for each variety) indicate statistical significance (P = 0.05, one-way ANOVA); ns = not significant.
Gu = guaiacol; Cr = cresol; Ph = phenol; Syr = syringol; 4MG = 4-methylguaiacol; 4MS = 4-methylsyringol; R = rutinoside; GB = gentiobioside; PG = pentose glucoside; G = glucoside.

Part B: Influence of smoke exposure on berry spectral reflectance

Chemical composition of smoke-affected grapes

The influence of smoke exposure on berry ripening was determined for each of the varieties for which spectral measurements were taken, i.e. Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Merlot, by measuring TSS and berry weight (Table 3) and volatile phenol glycoconjugate concentrations (Table 4).

Few statistically significant differences in TSS were observed between control and smoke affected fruit during ripening; with no significant differences in TSS or berry weight found at maturity. These results were in agreement with results reported above in Part A, and results obtained from the 2016 field trial (manuscript 1).

Table 3 TSS and berry weight of grapes sampled from control (C) and smoke-affected (S) grapevines, at different time points.

Treatment		TSS (in °Brix)				Berry weight (g)
		t = 0	t = 1	t = 7	maturity†	maturity†
Chardonnay	C	13.0	14.6	15.6 b	23.2	1.4
	S	13.5	15.7	17.1 a	22.7	1.5
	P	*ns*	*ns*	*0.031*	*ns*	*ns*
Sauvignon Blanc	C	13.5	10.9	16.7	23.6	1.2
	S	13.0	12.9	17.9	23.7	1.3
	P	*ns*	*ns*	*ns*	*ns*	*ns*
Cabernet Sauvignon	C	12.9	13.4	14.5	24.0	1.1
	S	13.6	12.7	14.7	23.9	1.1
	P	*ns*	*ns*	*ns*	*ns*	*ns*
Merlot	C	16.4	15.8	17.5 b	23.7	1.7
	S	15.3	15.3	16.4 a	23.2	1.7
	P	*ns*	*ns*	*0.037*	*ns*	*ns*

Sample times are reported as days after smoke exposure.
†Maturity corresponds to t = 17, t = 28, t = 28 and t = 22 days for Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Merlot respectively.

Values represent the mean of three replicates (n = 3). Different letters within columns (for each variety) indicate statistical significance (P = 0.05, one-way ANOVA); ns = not significant.

As expected, most volatile phenol glycoconjugates were found at significantly higher concentrations in smoke-affected fruit, compared with control fruit (Table 4). Clear varietal differences were also seen in glycoconjugate profiles. For example, fruit from the red grape varieties, Cabernet Sauvignon and Merlot, contained the highest concentrations of volatile phenol glycoconjugates; levels were certainly higher than for the white varieties, Sauvignon Blanc and Chardonnay. The pentose glucosides of guaiacol, cresol and phenol, together with the gentiobioside of syringol, were again the most abundant precursors, i.e. in agreement with smoke taint profiles reported in manuscript 1. However, surprisingly, precursor levels were several fold lower than reported in other smoke taint research [80]. As indicated above (in Part A), one replicate gave glycoconjugate levels that were consistently higher than the other two replicates (Supplementary Table 2) which affected ANOVA such that results were not statistically significant.

Table 4 Concentrations of volatile phenol glycoconjugates (µg/kg) in grapes harvested from control (C) and smoke-affected (S) grapevines.

Treatment		Gu-R	Gu-GB	Gu-PG	Gu-G	Cr-R	Cr-PG	Ph-R	Ph-GB	Ph-PG	Syr-GB	4MG-R	4MG-PG	4MS-GB
Chardonnay	C	tr	tr	2 b	1	tr	4 b	tr	tr	6 b	1 b	tr	3	tr
	S	tr	tr	12 a	2	2	9 a	1 a	1 a	13 a	24 a	2	3	2
	P	*ns*	*ns*	*0.016*	*ns*	–	*0.038*	–	–	*0.005*	*0.033*	–	*ns*	–
Sauvignon Blanc	C	tr	tr	1	1	tr	2	tr	tr	3	1	tr	tr	tr
	S	10	23	13	4	25	11	11	1	24	33	10	tr	6
	P	–	–	*0.003*	*0.002*	–	*0.004*	–	–	*< 0.001*	*< 0.001*	–	*ns*	–
Cabernet Sauvignon	C	tr	tr	2	1	1	2	tr	tr	4	2	tr	2	tr
	S	7	4	27	3	14	13	7	3	26	59	5	2	5
	P	–	–	*ns*	*ns*	*ns*	*ns*	–	–	*ns*	*ns*	–	*ns*	–
Merlot	C	tr	tr	3	1	tr	4	tr	tr	7	2	tr	3	tr
	S	3	24	77	10	9	43	4	3	56	66	5	4	9
	P	–	–	*ns*	*ns*	–	*ns*	–	–	*ns*	*ns*	–	*ns*	–

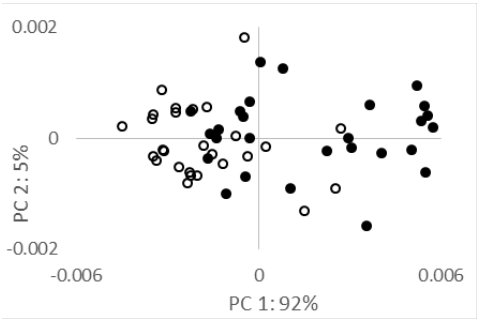
Values represent the mean of three replicates; nd = not detected; tr = trace (i.e., ≤ 1 µg/L).
Different letters within columns (for each variety) indicate statistical significance (P = 0.05, one-way ANOVA); ns = not significant.
Gu = guaiacol; Cr = cresol; Ph = phenol; Syr = syringol; 4MG = 4-methylguaiacol; 4MS = 4-methylsyringol; R = rutinoside; GB = gentiobioside; PG = pentose glucoside; G = glucoside.

Spectral reflectance measures

Spectral reflectance measurements were performed on berries from control and smoke affected grapevines, to verify results from the field trial conducted in 2016 (manuscript 1). Principal component analysis (PCA) of spectral data for each variety, at t = 1 and t = 7, gave the biplots shown in Figure 1. The first two principal components (PCs) explained 81 to 97% of the variation seen between samples, with the first PC again accounting for much of the variation (i.e. 66 to 92%).

Principal component analysis of the obtained spectral reflectance showed no trend to be identified for Sauvignon Blanc (Figure 1 a,b) or Cabernet Sauvignon (Figure 1 e,f) for day 1 or day 7. For Chardonnay a clear trend indicated the differentiation between control and smoke affected samples on day 1 (Figure 1 c). Clusters of control and smoke affected samples are clearly identified for both data obtained at day 1 and day 7 (Figure 1c, d). Merlot shows partial separation on day 1 with smoke affected samples being respectively either more concentrated on the left hand side and bottom of the PCA biplot quadrants (Figure 1g, h) on day one. However, for both varieties, measurements taken on day 7 do not show the same separation of spectral measurements in control and smoke affected fruit.

a



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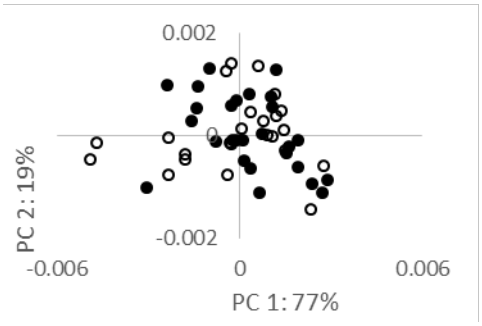

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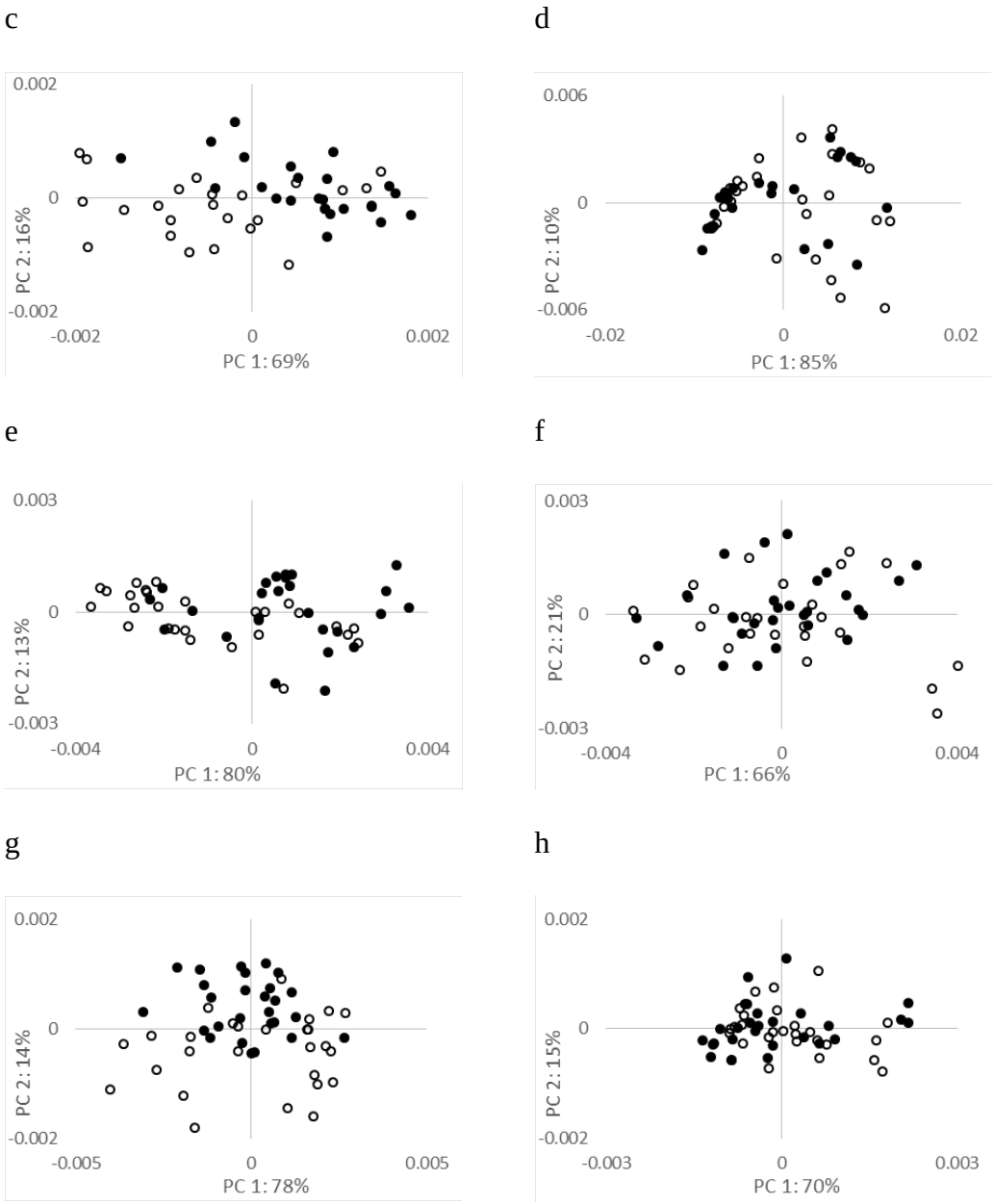
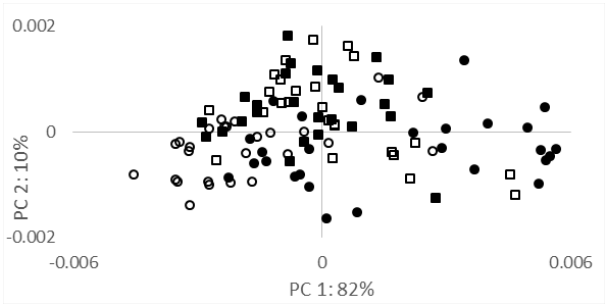


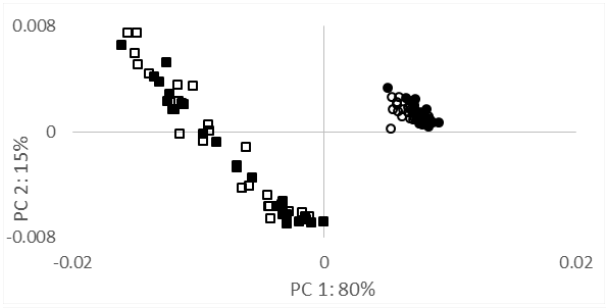
Figure 1 PCA biplots generated from spectral data for control (●) and smoke-affected (○) Sauvignon Blanc (a,b), Chardonnay (c,d), Cabernet Sauvignon (e,f) and Merlot (g.h) grapes at t = 1 (a,c,e,g) and t = 7 (b,d,f,h).

PCA was repeated for all varieties on spectral data obtained both at t = 1 and t = 7, resulting in the figures taken up in Figure 2a-d.

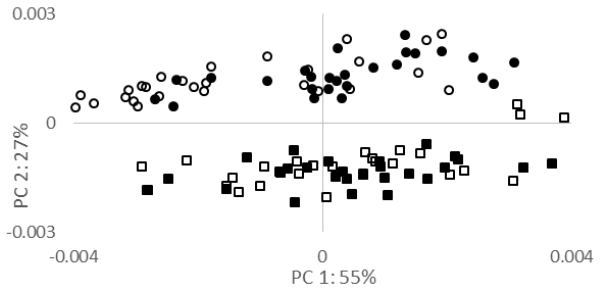
a



b



c



d

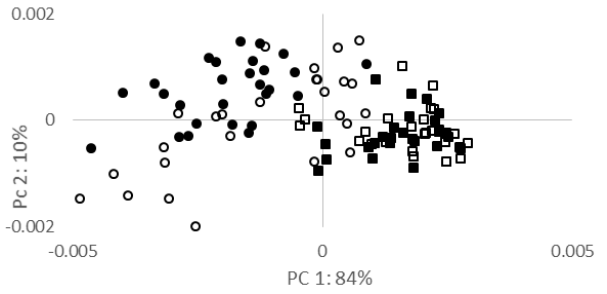


Figure 2 PCA biplots generated from spectral data for control (● at t = 1, ■ at t = 7) and smoke-affected (○ at t = 1, □ at t = 7) Sauvignon Blanc (a), Chardonnay (b), Cabernet Sauvignon (c) and Merlot (d) grapes.

The separation of control and smoke exposed fruit is still observed for Chardonnay at t = 1 (Figure 2b), however, not as clear as when the data is separated. For both Cabernet Sauvignon and Merlot the sampling time had the largest impact on variation of the data (Figure 2c-d). Especially for Cabernet Sauvignon, the accumulation of spectral data obtained at day 1 (circles in Figure 2C) in the top quadrants of the PCA biplot indicates physiological changes in the berries over time to affect the data. Identification of Chardonnay samples as either control or smoke affected also seems to be highly impacted by the sampling time as well, as separation is a lot more clear between t = 1 and t = 7 than based on treatment.

When comparing data from the 2016 field trial with smoke affected fruit, the overall amounts of precursors found in 2017 was significantly lower. Comparing the amounts of precursors found in Chardonnay in 2016 and 2017, the amount of syringol gentiobioside dropped from 55 to 24 μg/L in smoke exposed fruit, or 16 to 2 μg/L for cresol rutinoside. The smoke exposure of both years might not have been as intense in 2017 as it was in 2016, causing a lower uptake of smoke-derived volatile phenols and lower amounts of precursors in the berries.

Overall, the separation of control and smoke affected samples in PCA biplots was identified for Chardonnay and Merlot on day 1, in agreement with the results obtained in 2016. For Sauvignon Blanc no clear identification of smoke affected fruit could be made based on the spectral measurements performed in either year. The results of spectral measurements were less pronounced in 2017, but the amount of smoke taint precursors identified in the fruit was also found in lower quantities. Further work could potentially identify if more heavily smoke-affected fruit would be more readily identified by these spectral measures.

Conclusion

The field trials conducted in 2016 and 2017 have evaluated the potential for selected agrichemicals, i.e. kaolin and Envy, to be applied to grapevines prior to smoke exposure to mitigate the uptake of smoke-derived volatile compounds. Whereas the application of kaolin to Merlot grapevines resulted in significantly lower volatile phenol glycoconjugates following smoke exposure, similar results were not achieved for Sauvignon Blanc and Chardonnay. The use of the polymer concentrate Envy had little effect on the accumulation of smoke taint precursors, with the exception of the gentiobioside of syringol, for which the concentration was elevated from 59 to 139 µg/kg, i.e. more than a two-fold increase, in Cabernet Sauvignon fruit. In this instance, Envy actually exacerbated the impact of grapevine exposure to smoke.

Reflectance spectroscopy was also evaluated as a rapid method for detecting smoke-affected grapes in the vineyard, using a handheld spectrometer. For some varieties, i.e. Chardonnay, Cabernet Sauvignon and Merlot, discernible differences were found in spectral reflectance 1 day after smoke exposure; but these differences were no longer apparent 1 week after smoke exposure. In some instances, physiological characteristics (e.g. berry colour) may have influenced reflectance spectra more strongly than smoke exposure, such that control and smoke-affected fruit could not be readily differentiated.

Given the unpredictable nature of bushfire events and the difficulty in safely accessing affected areas, there may be limitations in both the application of agrichemicals to vineyards prior to a fire, and the collection of spectral data after a fire. Nevertheless, results from the current study suggest further research is warranted to optimise these methods for the detection and amelioration of smoke taint in the vineyard.

Supplementary Table 1 Concentrations of volatile phenol glycoconjugate replicates (µg/kg) in grapes harvested from smoke-affected (S) and Envy treated (E+S) grapevines.

Treatment		Replicate	Gu-R	Gu-GB	Gu-PG	Gu-G	Cr-R	Cr-PG	Ph-R	Ph-GB	Ph-PG	Syr-GB	4MG-R	4MG-PG	4MS-GB	4MS-G
Chardonnay	S	1	1.7	1.3	16.2	2.6	2.9	10.1	1.6	1.4	15.2	37.9	1.6	2.8	2.9	0.2
		2	0.6	0.9	11.7	1.1	2.1	10.0	1.0	0.9	12.3	20.8	1.4	2.8	2.1	0.0
		3	0.6	0.5	7.6	1.9	1.9	5.5	0.6	0.6	11.4	13.5	1.5	3.2	1.4	0.4
	E+S	1	1.8	1.4	21.0	1.3	6.5	15.1	3.7	2.7	18.2	53.3	2.5	6.5	5.5	2.5
		2	0.5	0.4	3.7	0.6	2.4	3.5	1.2	0.9	7.3	17.4	1.8	6.3	1.3	1.3
		3	0.2	0.1	3.1	1.5	1.4	5.3	0.6	0.5	7.4	6.4	0.7	6.4	0.9	0.9
Cabernet Sauvignon	S	1	4.0	2.1	16.3	2.0	8.9	9.2	4.2	1.9	17.2	44.9	3.1	2.2	3.1	1.9
		2	12.4	8.2	50.0	5.1	19.9	21.9	11.1	4.1	37.5	95.3	8.5	2.3	7.9	3.6
		3	4.0	2.3	14.9	2.1	14.3	7.4	5.6	1.6	21.9	37.6	4.7	2.4	4.0	1.4
	E+S	1	4.1	5.3	6.5	2.0	12.2	7.8	5.3	4.2	18.4	106.4	4.9	4.3	9.1	1.5
		2	5.5	5.1	12.9	3.7	24.0	12.5	9.9	7.5	27.9	199.8	8.6	2.6	20.8	3.8
		3	6.5	4.7	15.1	3.5	20.6	11.3	9.0	5.4	25.4	109.6	5.4	2.7	9.4	2.4

Supplementary Table 2 Concentrations of volatile phenol glycoconjugate replicates (µg/kg) in grapes harvested from smoke-affected (S) grapevines.

Treatment		Replicate	Gu-R	Gu-GB	Gu-PG	Gu-G	Cr-R	Cr-PG	Ph-R	Ph-GB	Ph-PG	Syr-GB	4MG-R	4MG-PG	4MS-GB
Cabernet Sauvignon	S	1	4.0	2.1	16.2	2.0	8.9	9.2	4.2	1.9	17.2	44.9	3.1	2.2	3.1
		2	12.4	8.2	50.0	5.1	19.9	21.9	11.1	4.1	37.5	95.2	8.5	2.3	7.9
		3	4.0	2.3	14.9	2.1	14.3	7.3	5.6	1.6	21.9	37.6	4.7	2.4	4.0
Merlot	S	1	2.1	9.1	33.3	2.3	5.7	26.0	2.8	1.2	31.1	37.5	3.5	4.2	5.6
		2	6.1	55.2	174.3	25.1	18.4	85.4	7.9	5.6	113.1	138.9	10.5	3.1	16.5
		3	1.1	7.9	22.0	1.6	3.4	18.8	1.1	1.2	23.6	23.0	2.5	4.0	3.4

CHAPTER 3

Transcriptomic analysis of smoke affected grapes

Statement of Authorship

Title of Paper	Transcription profiling of glycosyltransferases in *Vitis Vinifera* cultivars following smoke exposure
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	To be submitted to Frontiers in Plant Science – Plant Physiology

Principal Author

Name of Principal Author (Candidate)	Lieke van der Hulst
Contribution to the Paper	Designed and conducted experiments; collected, processed, analysed and interpreted compositional and molecular data; drafted and edited manuscript.
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 party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16/11/17

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.

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Contribution to the Paper	Performed Q-PCR experiments; analysed sequencing and expression data; edited the manuscript; co-author.
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Name of Co-Author	Julian Schwerdt
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Signature		Date	17-NOV-17

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Signature		Date	17/11/17

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

Chapter 3: Transcriptomic analysis of smoke affected grapes

Introduction

Until recently, the focus of smoke taint research has largely concerned the chemical and/or sensory consequences of grapevine exposure to smoke but now the molecular aspects of smoke taint are receiving attention. The glycosylation of smoke derived volatile phenols and the downstream effects this has on the detection, amelioration and perception of smoke taint in grapes and wine have become increasingly clear, and hence there is a need to better understand the role of glycosyltransferases in these processes [28, 29].

Glycosyltransferases (GT) are not easily identified by donor and substrate preference since they tend to be promiscuous enzymes with broad stereo- and regio-selectivity [69, 70]. To date, over 90 GT families have been identified based on sequence homology and function, with most plant GTs classified as family 1 (GT1) [62]. Recently, a promiscuous glucosyltransferase has been identified, with a possible role in smoke taint precursor formation [75]. Transcript analysis of a subset of GTs in grapevines, followed by kinetic studies of recombinant candidate GTs showed a resveratrol GT to be well adapted for glucosylation of smoke derived volatile phenols. Compounds such as guaiacol, syringol and 4-methylguaiacol were successfully transformed into their corresponding glucoconjugate precursor forms, even though *trans*-resveratrol is the putative substrate for this gene candidate [75].

The manuscript presented in this chapter describes the analysis of the transcriptomic response of berry tissues harvested from grapevines exposed to smoke. Two distinct trials were conducted; (i) an initial trial using potted grapevines involving RNA sequencing of control and smoke-affected berries, and (ii) a subsequent field trial involving Q-PCR analysis of a subset of genes (associated with glycosylation and stress-responses) in control and smoke affected fruit from several grape varieties.

Transcription Profiling of Glycosyltransferases in *Vitis Vinifera* Cultivars Following Smoke Exposure

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Abstract

Smoke taint is a fault found in wines produced from smoke affected grapevines. Research into smoke taint has commonly been based on the chemical and sensory profile of affected grapes and wine and little is known about changes in gene expression. This study analyzed transcript patterns in five cultivars of *Vitis vinifera*, in two separate studies where plants were exposed to smoke. To identify broad patterns of transcript changes, RNA sequencing was initially performed on berries from control and smoke affected potted grapevines grown under controlled environmental conditions (cultivar (cv)s Shiraz and Chardonnay). Significant increases in transcript levels of predominantly heat shock genes and several glycosyltransferases were observed in smoked versus control fruit. Phylogenetic trees were built for GT1 and GT8 families for functional identification of candidate genes and were combined with the RNAseq results to identify target glucosyltransferase genes for Q-PCR analysis in a field trial comprises of control and smoke affected grapes from vines of cvs Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Merlot, which were separated into skin and pulp fractions prior to RNA extraction. Five glucosyltransferase candidates were profiled and higher transcript levels of a hydroquinone glucosyltransferase (HqGT), a crocetin glucosyltransferase (CrocGT) and a 7-deoxyloganetic acid glucosyltransferase (7DaGT) were found in smoke affected fruit, particularly at specific time points. Differential expression seemed to be higher in the skin fractions, especially for Cabernet Sauvignon and Merlot.

Key words

Smoke taint, grapes, glycosyltransferases, phylogenetic analysis, GT1, GT8, volatile phenols

Abbreviations

CAZy - Carbohydrate-Active enZYmes Database, Q-PCR – quantitative real time PCR, RPKM - Reads Per Kilobase of transcript per Million mapped reads, TPM - Transcripts Per Kilobase Million, GT – Glycosyltransferase, cv- cultivar, GolS - galactinol synthase HqGT1 - hydroquinone glucosyltransferase, CrocGT1 - crocetin glucosyltransferase, 7DaGT - 7-deoxyloganetic acid glucosyltransferase, UGT92G6 - UDP-glycosyltransferase 92A1

Introduction

Over the past decade an increase of hotter and drier conditions has led to a higher incidence of bushfires all over the world (IPCC 2014). Because of this, countries such as Australia, Canada, South Africa and the US have also seen an increase in bushfires in wine regions (Høj, Pretorius et al. 2003, Kennison, Wilkinson et al. 2007, Whiting and Krstic 2007). Not only do these fires pose a high economic stress on grape growers and wine makers in terms of vineyard management, grape production and cellar door visits, but the development of smoke taint in wine produced from smoke affected grapes is also a very pressing issue (Whiting and Krstic 2007, Kennison 2009). Due to the undesirable acrid and smoky sensory profile seen in smoke tainted wine the end product is unsellable (Kelly and Zerihun 2015). The chemical and sensory profiles occurring after a smoke event in vineyards have been well documented, but the molecular and biochemical pathways of smoke taint development in grapes are not yet sufficiently investigated (Krstic, Johnson et al. 2015).

The chemical profile of smoke taint is caused by the uptake of smoke derived volatiles, predominantly by the fruit and to a lesser extent the leaves, of grapevines, leading to a pool of lignin derivatives such as guaiacol, syringol and cresols which bring out smoky, ashy and fishy flavours and aromas (Kennison, Wilkinson et al. 2007, Kennison, Gibberd et al. 2008, Hayasaka, Baldock et al. 2010). Besides the accumulation of these volatiles forming the basis of the aroma profile, a pool of glycoconjugate precursors builds up. These precursors are broken down by hydrolysis during wine making, causing release of flavour and aroma compounds, but a significant part of the pool stays intact in this process (Fudge, Schiettecatte et al. 2012). Mildly acidic conditions found in wine can lead to further hydrolysis of precursor compounds, but smoke taint glycoconjugates have been shown to be relatively stable over time (Ristic, van der Hulst et al. 2017). Even though glycoconjugates are usually not associated with aroma and flavour, the glycosylated

precursors are thought to influence the flavour of smoke affected wine since they can be broken down by enzymes found in human saliva. Specifically, it is thought that the rutinoside forms of cresol, guaiacol and phenol contribute to the excessively drying mouthfeel of smoke taint, as well as the overall ashy flavour (Mayr, Parker et al. 2014).

Altogether, the formation of these precursors is an important aspect of smoke taint development in grapes. However, this is the first research to investigate this process from a transcriptomic point of view. The formation of glycoconjugates of compounds absorbed from the environment in plants is a frequently occurring response to increase solubility and ease of transport of these compounds, as well as provide a convenient form of storage for small, lipophilic molecules in the cytosol of plant cells (Bowles, Isayenkova et al. 2005, Bowles and Lim 2010). Apart from glycoconjugation of foreign compounds, grapes and other types of crops and vegetation form precursors of endogenous compounds throughout development (Williams, Strauss et al. 1982, Sefton, Francis et al. 1993, Martinez-Gil, Angenieux et al. 2013, Tikunov, Molthoff et al. 2013). Many important grape-derived volatile compounds, e.g. monoterpenoids and norisoprenoids, are known to accumulate as glycosides during grape development and/or ripening, contributing to the non-aromatic pool of flavour and aroma compounds (Kuhn et al. 2013). These secondary metabolites are often involved in stress responses, interactions with pollinators and/or general plant defense, in their aglycone forms (Bonisch et al 2014). In grapevines alone, over 200 volatile aglycone glycoside acceptors have been identified, with monoglucosides and diglycosides being the most commonly described bound forms present in all grape varieties (Hjelmeland and Ebeler 2014, Schwab, Fisher et al. 2015). Higher order glycosides, containing more than two forms of sugar attached to an aglycone, have not been identified for grapes, but have been determined in other types of fruit such as apple and tomatoes (Hjelmeland and Ebeler 2014). In these crops higher

order glycoconjugation leads to the formation of sets of non-aromatic compounds in maturing fruit (Tikunov, de Vos et al. 2010, Tikunov, Molthoff et al. 2013).

Glycosyltransferase activity can vary depending on many biotic and abiotic influences, such as grape variety, phase of fruit development and circumstances that might promote or stunt development overall. Because of this, this study started with an initial analysis to investigate the activity of these genes upon contact with smoke. Following the outcomes of this work a larger trial was set up in the vineyard during the subsequent vintage in which Q-PCR was used to investigate the activity of a subset of genes in separated fractions of skin and pulp at several time points following smoke exposure. The candidates selected for Q-PCR were either differentially expressed genes from the growth chamber smoke exposure experiment, or were chosen based on the putative activity of the proteins they encode towards smoke-derived volatile compounds. Recently published research indicated the presence of already highly expressed GTs to be potentially responsible for the glucoconjugation of smoke taint marker compounds into monosaccharide glucosides (Härtl, Huang et al. 2017). From the published work, the protein encoded by the UGT72B27 gene was identified as having the highest glucoconjugating activity of smoke taint marker compounds, and so was also included as a candidate in this study (Härtl, Huang et al. 2017). As smoke taint markers and precursors are mainly thought to accumulate in the skins of grape berries the glycosyltransferase profiles in skins and pulp were also investigated here to confirm possible differences (Dungey, Hayasaka et al. 2011).

Material and Methods

2.1 Grapevine samples – potted *Vitis vinifera* (cv Shiraz, Chardonnay)

Two *Vitis vinifera* cultivars, cv Chardonnay and cv Shiraz, were grown as potted plants in a controlled environment from cuttings taken from a South Australian vineyard (Coombe, Waite campus, research vineyard) over the period May – December 2015, as described previously (Baby, Hocking et al. 2014). A diurnal rhythm was mimicked by creating 'days' with 16 hours of artificial daylight (intensity 400 µmol photons/m^2/s) at 27°C and 8 'night' hours without lighting at 22°C. Humidity in the growth room was maintained at 35% using a dehumidifier (SECCO ULTRA, Applied Climate Control Pty Ltd, Sydney, Australia). Potted grapevines were moved to an area outside the growth chamber to be exposed to smoke so that control vines were not affected. At the time of smoke exposure the temperature in the growth room was 27°C and outside was 28°C. Grapevines were exposed to smoke for 1 h, approximately two weeks after veraison, using purpose-built smoke tents (6 m x 2.5 m x 2 m), according to methodology described previously (Ristic, Osidacz et al. 2011). Air temperature was monitored during smoke exposure by placing a temperature tracker in the middle of the set up on a vine (Garmin temperature tracker, 010-11092-30), but only small increases in temperature (i.e. ≤ 2 ºC) were observed relative to the outside temperature (data not shown). The treated vines were returned to the controlled environment of the growth chamber directly after the treatment and sampled an hour after exposure (time point day 0). Treatments were conducted in triplicate, with each experimental replicate comprising 3 separate potted grapevines. Per sample 10 to 15 berries were picked from each replicate and flash frozen in liquid nitrogen for storage at -80°C.

2.2 Total RNA extraction for RNAseq – potted grapevines

Per replicate 10 whole berries (skin, pulp and seeds) were ground under liquid nitrogen. An amount of 100 mg per sample was used for RNA extraction using the Spectrum plant total RNA kit (Sigma Aldrich), following the manufacturer's instructions for samples with high water and sugar content. Samples were eluted from the supplied binding column in a single step, and multiple eluents of the same replicate were pooled following quality control on a 1% agarose gel (clear bands for both 18S and 28S RNA) as well as being measured on the Qubit (Invitrogen Qubit 1.0 Fluorometer Q32857, Turner Biosystems) for RNA concentration.

2.3 Illumina RNA sequencing

Extracted RNA from grape samples was delivered to the Australian Genome Research Facility (AGRF), Adelaide, South Australia, for RNA sequencing. Prior to sequencing quality control was performed to determine RNA Integrity Numbers (RIN) and concentration per sample. All samples presented a RIN of 9 or higher. Stranded RNA libraries were constructed per biological replicate and next generation Illumina sequencing was performed using HiSeq chemistry with single end reads (Supplementary Table 1). An average number of 18,138,277 reads per sample was obtained.

2.4 Sequencing data

Sequences were trimmed and assembled for analysis using CLC Workbench (version 9.5.2, QIAGEN Aarhus A/S), and 26,340 genes were annotated based on the existing 12x *Vitis vinifera* genome (Jaillon, Aury et al. 2007). Fold changes were determined by calculating RPKM and TPM for both varieties for comparison of control and smoke affected samples. This data was analysed to identify the top ten differentially expressed genes in the sample set, as well as the top five genes annotated as glycosyltransferases in the CAZy database (Table 1, Table 2) (Lombard, Golaconda Ramulu et al. 2014).

2.5 Coombe vineyard samples (*Vitis vinifera* cv Chardonnay, Sauvignon Blanc, Cabernet Sauvignon, Merlot)

Grape berry samples were collected from four different *Vitis vinifera* cultivars Sauvignon Blanc, Chardonnay, Cabernet Sauvignon and Merlot, growing at the University of Adelaide's Waite Campus (latitude 34°58'S, longitude 138°38'E). Vines were planted in north-south aligned rows (in 1998), and were 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 treatment took place approximately 7 days to 2 weeks post-veraison in a purpose built smoke tent (dimensions: 6m long x 2.5m high x 2m wide) during the months of February and March 2017 (Kennison, Wilkinson et al. 2009). Smoke treatment was the same as used for the potted grapevines in December 2015. For each variety samples (triplicates, approximately 200 berries per replicate) were taken at maturity for precursor analysis by HPLC-MSMS. Samples for gene expression studies were taken at 3 distinct time points, respectively an hour after smoke exposure (day 0), 24 hours after smoke exposure (day 1) and 7 days after smoke exposure (day 7). These samples were immediately separated into skin and pulp fractions from approximately 50 berries per sample, to obtain 10 gram of wet tissue per sample. These fractions were flash frozen in liquid nitrogen for storage at -80°C.

2.6 Total RNA extraction and cDNA synthesis

Extraction of total RNA was carried out following the same protocol as for RNAseq, with the exception of the addition of a DNAse digestion on the column, as per the manufacturer's protocol (Sigma Aldrich). For cDNA synthesis 2 independent reactions were undertaken for each sample. In the first step 2 to 11 µl of RNA (depending on concentration) was mixed with 1 µL of 50 µM oligo-dT primer, 2 µL of 5 mM dNTP mix and sterile water to a volume of 14.75 µL. The mixture was heated to 65°C for 5 minutes

and immediately cooled on ice. A master mix containing 4 μL 5 x First Strand Buffer, 1 μL dithiothreitol, and 0.25 μL SuperScript III was added to each sample for a total volume of 20 μL before incubating the reaction at 50°C for 70 minutes, followed by inactivation at 70°C for 15 minutes. cDNA was stored at -20°C for further verification and analysis (Burton, Jobling et al. 2008).

2.7 Real-time Q-PCR

Real-time Q-PCR was performed on a single bulked replicate (containing tissue from many different berries) of each skin and pulp field sample for all four cultivars, due to cost and time constraints. Primers were designed using Primer3 (Supplementary Table 3) and real-time Q-PCR was performed as previously described (Burton, Shirley et al. 2004). The following modifications to the method were made. To provide a template for the standard curve, between four and six 20-μL PCR reaction mixtures were combined for purification by HPLC using a HELIX DNA DVB 50- 3 3.0-mm monolithic polymer reversed-phase column (Varian). Chromatography was performed using buffer A (100 mM triethylammonium acetate [Applied Biosystems] and 0.1 mM EDTA) and buffer B (100 mM triethylammonium acetate, 0.1 mM EDTA and 75% acetonitrile). The gradient was as follows: time 0 min, 10% buffer B; time 6 min, 21.5% buffer B; time 7 min, 21.5% buffer B; time 8 min, 10% buffer B; time 12 min, 10% buffer B. The flow rate was 0.45 mL/min and the temperature was 50℃. Three replicates of each of the seven standard concentrations were included with every Q-PCR experiment together with a minimum of three no-template controls. Q-PCR experiments were assembled by the liquid-handling CAS-1200 robot (Corbett Robotics). Three replicate PCRs for each of the cDNAs were included in every run containing: 2 μL of cDNA solution, the diluted standard, or water was used in a reaction containing 5 μL of IQ SYBR Green PCR reagent (Bio-rad Laboratories), 1.2 μL of each of the forward and reverse primers at 4 mM, 0.3 μL of 103

SYBR Green in water, and 0.3 µL of water. The total volume of the PCR reactions was 10 µL. Reactions were performed in an RG 6000 Rotor-Gene real-time thermal cycler (Corbett Research): 3min at 95_C followed by 45 cycles of 1 s at 95_C, 1 s at 55_C, 30 s at 72_C, and 15 s at the optimal acquisition temperature. Normalization was carried out using the control genes for *Vitis vinifera* actin, ubiquitin, tubulin and malate dehydrogenase (primers found in Supplementary table 3) and the final concentrations of mRNAs of the genes of interest are expressed as arbitrary units that represent the numbers of copies per microliter of cDNA, normalized against the geometric means of the three control genes that vary the least with respect to each other (Burton, Jobling et al. 2008).

2.8 Phylogenetic trees for GT1 and GT8 families

2.8.1 Tree construction

Vitis vinifera, *Solanum lycopersicum*, *Arabidopsis thaliana* and *Medicago trunculata* were sampled for amino acid sequences with assignments to the PF00201 (GT1) and PF01501 (GT8) PFAM hidden markov models (HMM) and retrieved from Phytozome ((Goodstein, Shu et al. 2011) http://www.phytozome.net). Selected sequences were aligned using default parameters that are tuned for accuracy. To account for mis-alignment and excessive sequence divergence we used BMGE to reduce the alignment to biological meaningful positions using a permitted gap rate of 0.7, the BLOSUM30 substitution matrix and a block size of 2 (Criscuolo and Gribaldo 2010). The final alignments were 222 and 373 positions long for GT1 and GT8, respectively.

2.8.2 Phylogenetic analyses

The RAxML auto model selection was used (-m PROTGAMMAAUTO) with AIC, BIC and AICc criteria to select the substitutional model with the highest likelihood (Stamatakis 2014). Final model selected was VT (GT8) and GTR (GT1) with gamma rate variation and estimation of amino acid frequencies. Phylogenies of PF00201 and PF0150

sequences were reconstructed using amino acid data, under maximum likelihood (ML) using RAxML version 8.2 (Stamatakis, 2014) and the PROTGAMMAVTX and PROTGAMMAGTRX evolutionary models for GT8 and GT1, respectively. RAxML analyses began with three independent autoMRE rapid bootstrap analysis. The tree with the highest likelihood was used as the starting tree for an additional 1000 rapid hill-climb ML tree searches and 1000 randomised tree searches. The tree with the highest GAMMA-based likelihood was selected as the final output.

2.9 Chemical analysis of grape samples

Analysis of the glycoconjugate smoke taint precursor pool by HPLC-MSMS was performed by commercial services of the Australian Wine Research Institute (Hayasaka, Baldock et al. 2010, Hayasaka, Baldock et al. 2010). An aliquot of 5 g of the berry homogenate was spiked with d_3-syringol-gentiobioside as an internal standard. This sample was spun down and a 2 mL aliquot of the supernatant was applied to an Extract Clean C18-HF SPE cartridge (500 mg/4mL, Grace Davison Discovery Sciences, Australia). The methanol extract was dried and subsequently reconstituted with 0.5 mL water prior to running the sample. A 4000 Q TRAP hybrid tandem mass spectrometer with a TurboV ion source (Applied Biosystems/MDS Sciex, Concord, ON, Canada) combined with an Agilent 1200 HPLC system (Agilent Technologies, Forest Hill, VIC, Australia) was used. Aliquots of 10 µL of the extracted samples were injected and a 3 µm Gemini C6-Phenyl 110 Å column was used for chromatographic analysis. The mobile phases consisted of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B), with a linear elution gradient at a flow rate of 300 µL/min. Mass spectra were recorded in negative ion mode and acquisition and processing of the obtained data was performed using Analyst software version 1.5 (Applied Biosystems/MDS Sciex).

Total soluble solids (TSS in °Brix) were determined for control and smoke samples obtained in the field trial of 2017 by a handheld refractometer (Atago, Tokyo, Japan).

Results

3.1 Differentially expressed genes in control and smoke affected potted Chardonnay and Shiraz vines

Control and smoke-exposed *Vitis vinifera* cvs Chardonnay and Shiraz were analyzed using RNA sequencing to profile differential gene expression and identify candidates for Q-PCR. These samples were obtained from potted grapevines cultivated in a controlled growth room environment and sampled at t =0, meaning an hour after smoke exposure.

RPKM was calculated from the RNAseq data sets and used to calculate fold changes and *p*-values for all gene identifiers (data not shown). Limiting the data-set to only gene identifiers with a fold change larger than 2 as well as a *p*-value less than 0.05 produced 1338 and 880 out of 26346 genes with an interesting differential response for Chardonnay and Shiraz respectively. TPM was calculated from raw data to verify fold change for a top ten list of highest upregulated genes following smoke exposure (Table 1).

Table 1 Collection of the ten highest differentially expressed genes in smoked affected versus control grapes (based on fold change, FC) in Chardonnay and Shiraz from RNA sequencing data analysis.

Gene identifier	*Vv*PFAM	*At* description protein family	FC Chardonnay	FC Shiraz
GSVIVG01035432001	At2g40230	HXXXD-type acyl-transferase family protein	2318	686
GSVIVG01035433001	PF00011	Gamma interferon responsive lysosomal thiol reductase family protein	1989	448
GSVIVG01016428001	PF00011	HSP20-like chaperones superfamily protein	1215	266
GSVIVG01016429001	PF00011	HSP20-like chaperones superfamily protein	1154	2262
GSVIVG01035429001	PF00011	Heat shock protein 17.6A	946	322
GSVIVG01035434001	PF00011	Heat shock protein 17.6A	751	257
GSVIVG01016426001	At2g29500	HSP20-like chaperones superfamily protein	652	522
GSVIVG01035428001	PF00011	Heat shock protein 17.6A	559	392
GSVIVG01030320001	PF00011	Heat shock protein 18.2	377	388
GSVIVG01035430001	PF00011	Heat shock protein 17.6A	336	2503

The highest differentially expressed genes were generally found to be annotated as heat shock proteins, as well as being members of a heat shock protein chaperone superfamily based on both the PFAM *V. vitis* annotation (PF00011) and the *Arabidopsis* description.

Sorting the data in similar fashion produced 523 out of 26346 genes for Shiraz which seemed to have lower expression following smoke exposure, with fold changes ranging from approximately -2 to -39, and 222 gene identifiers with lower expression and fold changes down to -31 for Chardonnay. TPM calculations and fold change verification yielded a short-list of seven clearly downregulated genes (Table 2).

Table 2 Collection of the seven most downregulated genes in smoke affected versus control grapes (based on fold change, FC) in Chardonnay and Shiraz from RNA sequencing data analysis.

Gene identifier	*Vv*PFAM	*Vv*Cazy	*At* description protein family	FC Chardonnay	FC Shiraz
GSVIVG01011437001	PF01357 PF03330	Expansin	Expansin A10	-31	-16
GSVIVG01028766001	PF00560 PF08263 PF13855	At5g46620	Unknown protein	-39	-13
GSVIVG01008003001	PF02704	At1g74670	Gibberellin-regulated family protein	-23	-8
GSVIVG01009962001	PF00657	At1g75900	GDSL-like Lipase / Acylhydrolase superfamily protein	-19	-
GSVIVG01021779001	PF02309	At5g43700	AUX/IAA transcriptional regulator family protein	-12	-25
GSVIVG01009961001	PF00657	At1g75900	GDSL-like Lipase / Acylhydrolase superfamily protein	-5	-17
GSVIVG01013913001	PF00847	At4g17490	Ethylene responsive element binding factor 6	-3	-13

3.2 Identification of responsive glycosyltransferase genes

Mining of the contigs assembled from the RNA sequencing data to obtain genes annotated in CAZy as members of a GT family provided 27 results for Chardonnay and 25 results for Shiraz. After comparisons of their RPKM and TPM values to determine fold change in expression, four gene candidates were identified for further Q-PCR analysis (Table 3). These candidates included galactinol synthase 2 (GSVIVG01028176001, *GolS2*), hydroquinone glucosyltransferase UGT72B27 (GSVIVG01027064001, *HqGT1*), crocetin glucosyltransferase UGT75L6 (GSVIVG01031580001, *CrocGT1*), and 7-deoxyloganetic acid glucosyltransferase (GSVIVG01016417001, *7DaGT*). A fifth candidate, UDP-glycosyltransferase 92A1 (GSVIVT01031678001, *UGT92G6*) did not show higher expression in either Shiraz or Chardonnay following smoke exposure, but

was selected since its corresponding protein has recently been reported to have moderate activity towards smoke derived volatile compounds to form smoke taint glucosides (Härtl, Huang et al. 2017). HqGT1 was also identified by the same research group to have preferential activity towards these smoke taint volatile phenols (Härtl, Huang et al. 2017).

Table 3 Collection of the five highest expressed glycosyltransferase genes (based on fold change, FC, Supplementary table 2) from Chardonnay and Shiraz, as selected from the Cazy database using CLC workbench. Their *Vitis vinifera* protein families (VvPFAM), *Vitis vinifera* CAZy descriptor (all GTs) and *Arabidopsis thaliana* protein family description (GTs and heat shock protein) are provided. Additionally, the table also includes the candidate GSVIVG01031678001, which did not show higher expression following smoke exposure but has medium activity towards glucoconjugating smoke derived volatile phenols.

Gene identifier	*Vv*PFAM	*Vv*Cazy	*At* description protein family	FC Chardonnay	FC Shiraz
GSVIVG01028176001	PF01501	GT8	Galactinol synthase 1	134	110
GSVIVG01031580001	PF00201	GT1	UDP-glucosyltransferase 75B1	78	40
GSVIVG01027064001	PF00201	GT1	UDP-glucosyl transferase 72B3	29	28
GSVIVG01015859001	PF00201	GT1	UDP-glucosyl transferase 71B5	20	10
GSVIVG01016417001	PF00201	GT1	Heat shock protein 18.2	13	12
GSVIVG01031678001	PF00201	GT1	UDP-glycosyltransferase superfamily protein	1	1

3.1 Transcript changes in non-smoked white and red grape varieties over time

Transcript levels of the five selected glycosyltransferases *GolS2, HqGT1*, *CrocGT*, *UGT92G6* and *7DaGT* were examined using Q-PCR on field samples collected at days 0, 1 and 7 post smoke exposure for the cultivars Chardonnay, Sauvignon Blanc, Cabernet

Sauvignon and Merlot. Transcript levels were determined for separated skin and pulp fractions from smoked and control berries for all samples at all time points (Supplementary table 4).

The first set of differentially expressed genes to be defined were those that naturally change in red versus white unsmoked control berries as they develop. These genes are likely to be part of the berry development or ripening process. For all cultivars *UGT92G6* was highly expressed in both skin and pulp samples, at all time points sampled (Table 4 and Table 5) whilst levels of HqGT1 were lower but reasonably consistent. Differences in transcript levels were observed between varieties, but these were not consistently associated with either white or red cultivars. For example, *GolS2* had relatively low transcript numbers in the skin of Chardonnay, a white variety, showing a 2 fold change between t = 0 and t = 7 (fold change based on transcript numbers in Table 4). In contrast, for both Sauvignon Blanc (white) and Cabernet Sauvignon (red) *GolS2* transcripts increased over time, showing changes of 28 and 80-fold in levels in skin samples respectively between t = 0 and t = 7. For the same gene in Merlot tissues, a red variety, levels of *GolS2* increased 54 fold between t = 0 and t = 1, but decreased 28 fold between t = 1 and t = 7. Therefore, for both skin and pulp the biggest consistent differentiator between white and red grapes was levels of *HqGT1* transcript, which was abundant at 1401 - 2256 units in white grape berries, but consistently lower, at ranging from 270 to 836, in red varieties (Table 4 and 5)

Table 4 Heatmap of transcript levels of the five candidate genes in skin fractions of control samples of Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Merlot. Highest amounts in green and lowest amounts in red and 50th percentile of the data in yellow.

Cultivar	Sample time	*GolS2*	*HqGT1*	*CrocGT1*	*UGT92G6*	*7DaGT1*
Chardonnay	t = 0	325	1912	173	7555	238
	t = 1	240	2256	79	8954	109
	t = 7	645	1423	161	9374	235
Sauvignon Blanc	t = 0	177	4608	171	5098	57
	t = 1	148	1235	95	7411	183
	t = 7	4984	1401	132	3338	377
Cabernet Sauvignon	t = 0	254	398	107	4344	107
	t = 1	892	479	57	4589	87
	t = 7	20353	489	80	5123	181
Merlot	t = 0	182	836	84	5955	104
	t = 1	9993	459	86	3323	160
	t = 7	354	270	100	4915	44

Table 5 Heatmap of transcript levels of the five candidate genes in pulp fractions of control samples of Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Merlot. Highest amounts in green and lowest amounts in red and 50th percentile of the data in yellow.

Cultivar	Sample time	GolS2	HqGT1	CrocGT1	UGT92G6	7DaGT1
Chardonnay	t = 0	687	2926	104	6652	62
	t = 1	180	1707	30	9315	8
	t = 7	194	991	24	9731	14
Sauvignon Blanc	t = 0	191	2095	38	4927	70
	t = 1	100	777	63	4838	36
	t = 7	1231	763	47	3074	67
Cabernet Sauvignon	t = 0	108	311	23	4798	27
	t = 1	436	271	14	4749	43
	t = 7	11781	272	40	6632	63
Merlot	t = 0	86	515	17	5023	30
	t = 1	8174	329	24	4035	34
	t = 7	257	120	13	6588	10

The overall transcript levels of the candidate genes were compared in control pulp versus skin samples of all varieties (Table 4 and 5). Three gene candidates have slightly lower transcript levels in pulp samples, *UGT92G6* had relatively similar levels in both skin and pulp and a bigger difference was seen for *GolS2*, where levels in skin tissue were generally higher than in pulp, especially for Cabernet Sauvignon at t = 7.

3.2 Differentially expressed genes in control versus smoke affected berries

Differences in transcript levels in response to smoke exposure were calculated as fold changes of each transcript between control versus smoke affected samples (Figure 1a-d, Supplementary table 4). Transcript levels of *UGT92G6* were relatively stable for all varieties for all sampling points, with a fold change never exceeding 2 for either up- or downregulation. However, large differences were seen in varietal response to smoke exposure as well as more generally between red and white grapes.

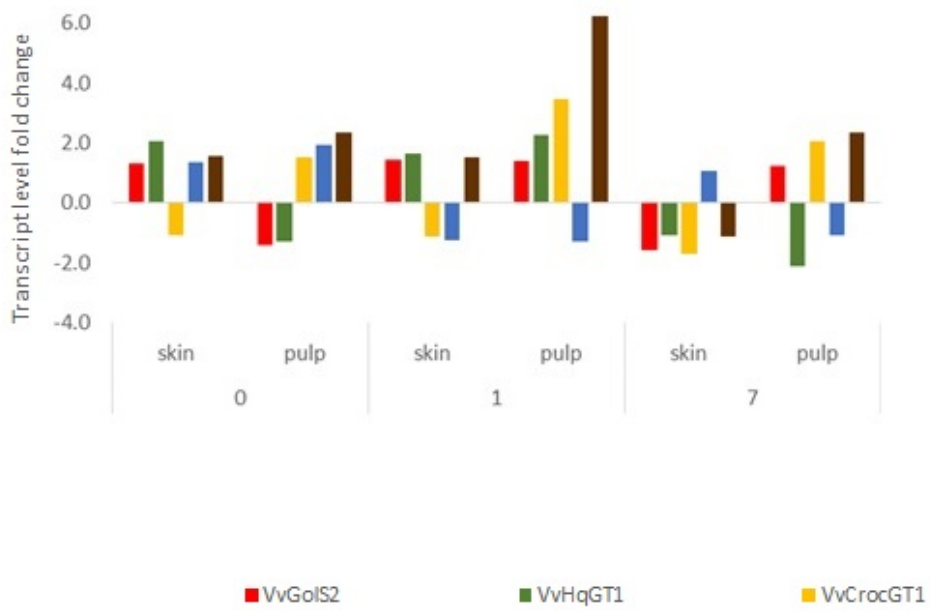
White grape varieties: Chardonnay and Sauvignon Blanc

Chardonnay showed significant upregulation for *7DaGT* at all three time points in pulp samples (Figure 1a). At t = 1 there was also upregulation of *HqGT1* and *CrocGT1*. Sauvignon Blanc was the only variety that showed downregulation of transcripts to be the major response following smoke exposure. For example transcript levels of *7DaGT* in Sauvignon Blanc (Figure 1b) were highly downregulated with a fold change of -7 in pulp samples at t = 0, and levels of this gene were still lower in smoke affected samples at t = 1 and t = 7. This trend was not observed in Chardonnay grapes, nor in the red varieties.

Red grape varieties: Cabernet Sauvignon and Merlot

In contrast to the white varieties, both Cabernet Sauvignon and Merlot show clear responses at t = 0 in the berry skin. There was an upregulation of *HqGT1* by almost 7 fold in Cabernet Sauvignon and *CrocGT1* increased by 7 fold in the same cultivar but by 15 fold in Merlot, which was by far the most significant increase in the whole data set.. The low, and stable, levels of *CrocGT* found for control samples of all varieties at all time points (Table 4 and 5) indicate that the 15 fold increase represents a specific response to smoke for the red varieties, Merlot in particular, as opposed to being related to berry development over time.

a



b

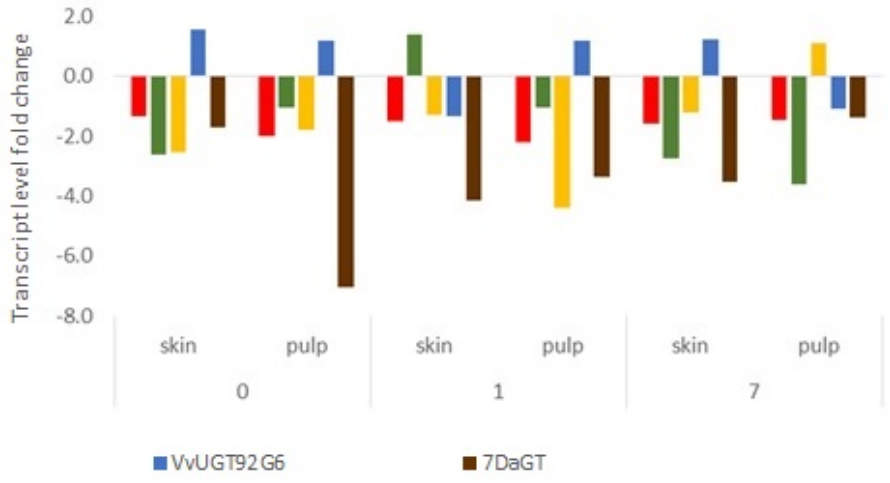
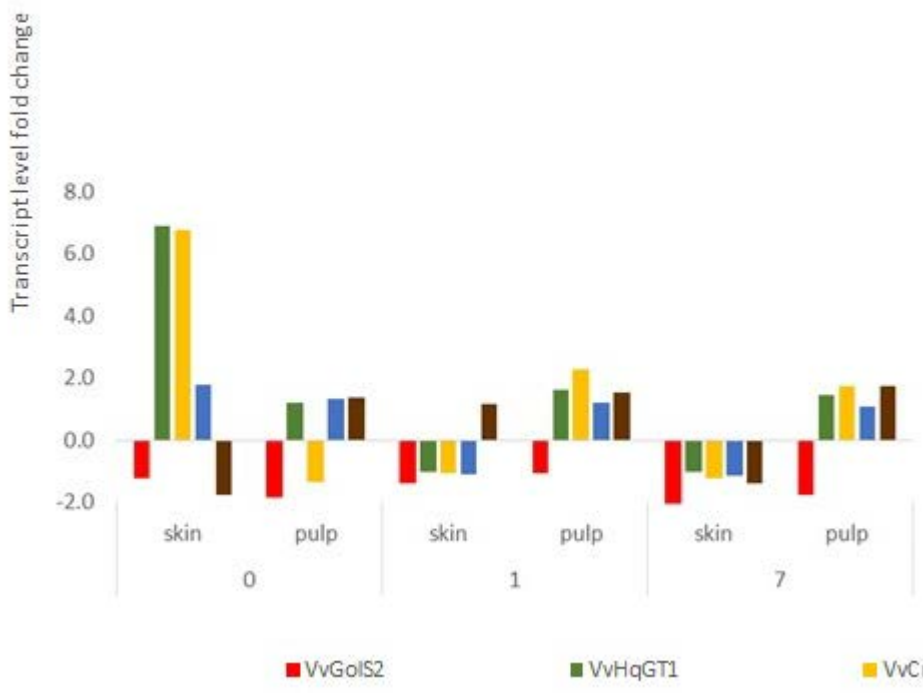


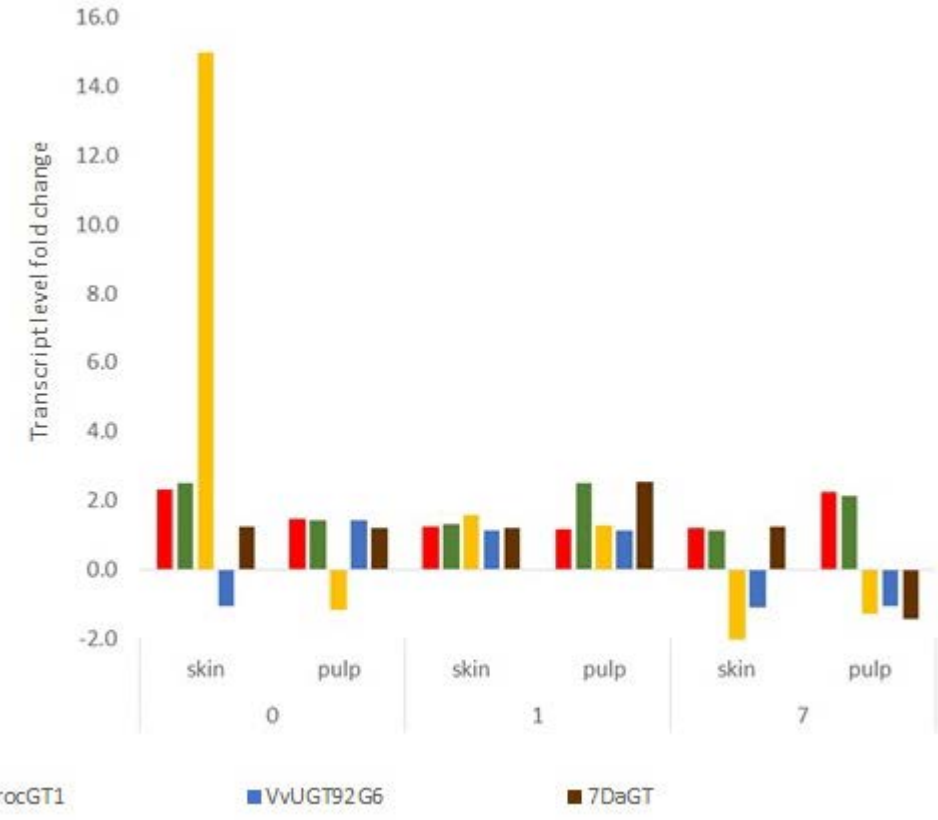
FIGURE CONTINUES ON NEXT PAGE

Figure 1 Transcript level fold changes between smoke-exposed and control berries of target genes *GolS2* (red), *HqGT1* (green), *CrocGT1*(yellow), *UGT92G6* (blue) and *7DaGT* (brown) over time for *Vitis vinifera* cv Chardonnay (a), Sauvignon Blanc (b), Cabernet Sauvignon (c) and Merlot (d)

c


Transcript level fold change
8.0
6.0
4.0
2.0
0.0
-2.0
skin
pulp
skin
pulp
skin
pulp
0
1
7
VvGolS2
VvHqGT1
VvCrocGT1

d


Transcript level fold change
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
skin
pulp
skin
pulp
skin
pulp
0
1
7
VvUGT92G6
7DaGT

3.4 Phylogenetic tree

Phylogenetic trees were constructed for the candidate gene families GT1 and GT8 for *Vitis vinifera, Arabidopsis thaliana, Medicago truncatula* and *Solanum lycopersicum* in order to investigate predicted function (Figure 2-3, 5).

Analysis of the constructed unrooted tree for GT8 indicated clear separation in five functional gene groups, including GAUT (Galacturonosyl transferase1), GATL (GAUT-like), PGSIP (plant glycogenin - like starch initiation proteins), GUX (GlcA substitution of xylan 1) and GOLS (galactinol synthase) (Figure 2). Arrangement of the GOLS group of the tree, shows that the candidate *GolS2* is clustered together with its paralogue (GSVIVG01028174001), which was not previously identified in the RNAseq data and is closely related to the *Medicago* and *Arabidopsis* GolS1 sequences (Figure 2) (Gelineo-Albersheim, Xu et al. 2011). In this research, *GolS2* was initially identified as *GolS1*, and GSVIVG01028176001 and GSVIVG01028174001 were thought to be the same gene but potentially badly annotated. However, upon closer inspection of the GT8 tree in the GolS family clade, both GSVIVG01028176001 and GSVIVG01028174001 were found in the same location (Figure 3), as duplicated but separate sequences.

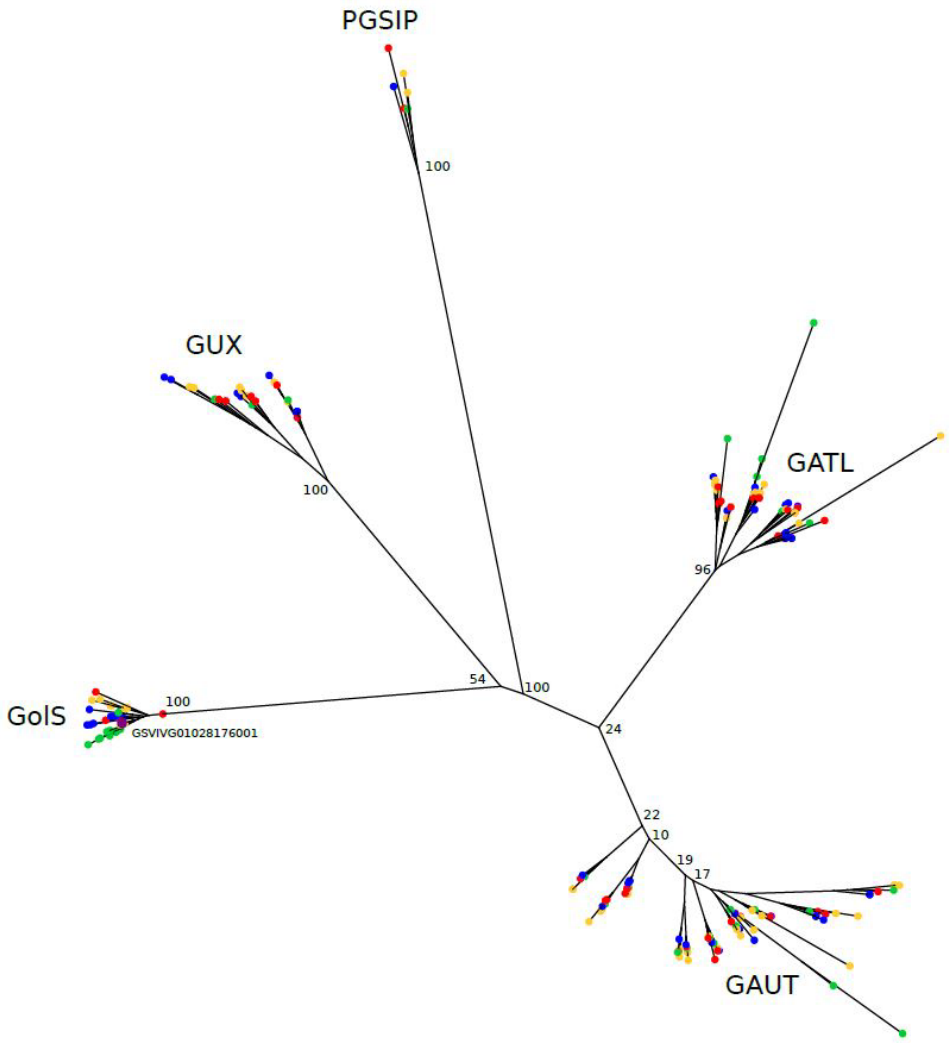


Figure 1 Best-known maximum likelihood RAxML tree for GT8 sequences in sampled species. Species origin is indicated by coloured dots with green representing *Vitis vinifera*, red *Solanum lycopersicum*, blue *Arabidopsis thaliana* and yellow *Medicago trunculata*. Bootstrap support values are annotated on deep nodes in black.

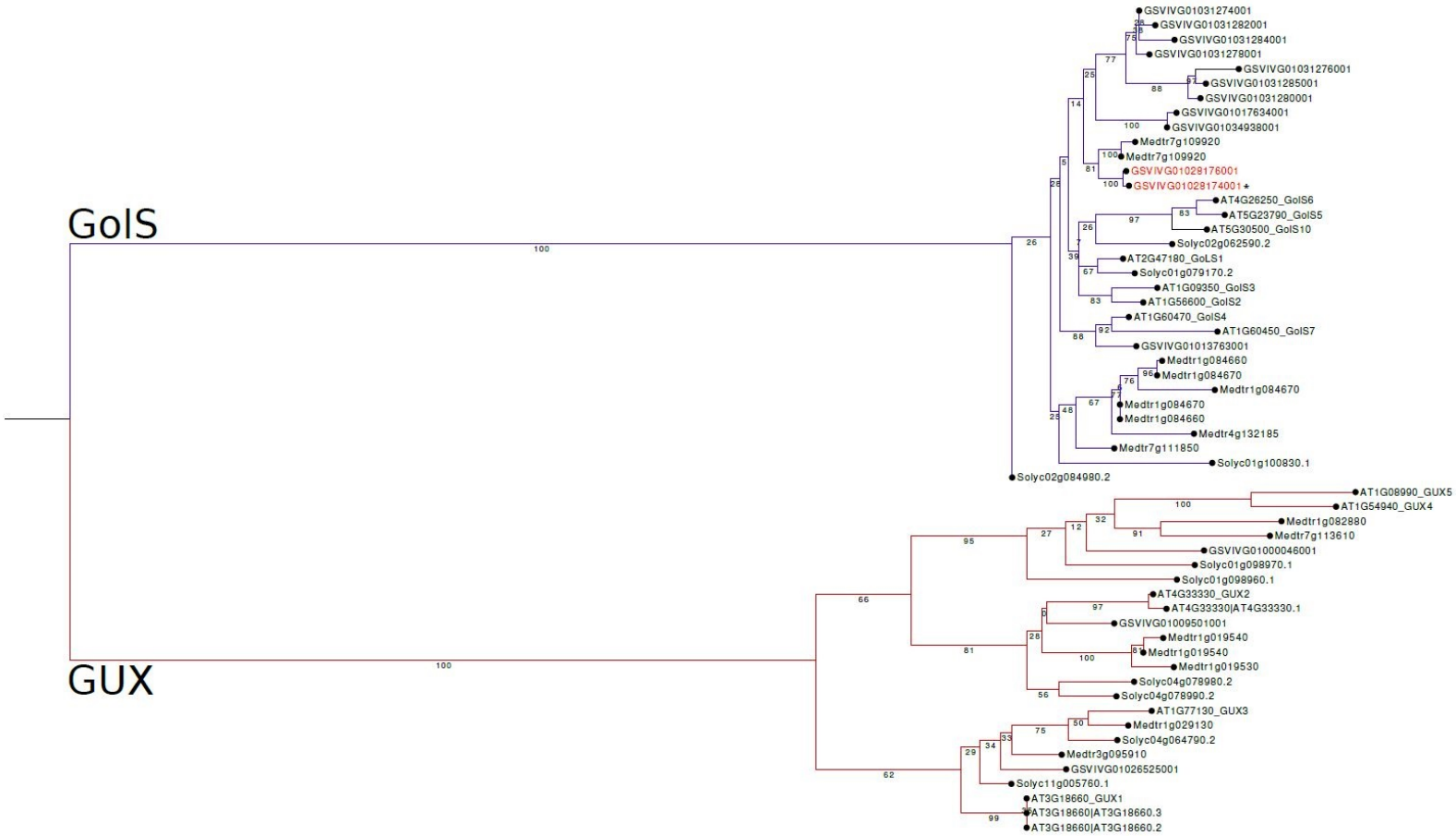


Figure 1 Sub-tree of best-known maximum likelihood RAxML tree for GolS (blue) and GUX (red) sequences in sampled species. Bootstrap support values are annotated on branches in black. Candidate *Vitis* GolS sequence is highlighted in red whilst the duplicate *Vitis* candidate is highlighted in red with an asterisk (*).

Finding both GolS candidates in the GT8 tree warranted further examination of the reads obtained through RNA sequencing (Figure 4). Closer inspection of the sequences of both candidates identified them to be tandem repeats on chromosome 7, respectively *GolS1* and *GolS2*. Furthermore, both *GolS1* and *GolS2* were found to have significantly higher transcription profiles for smoke affected Chardonnay and Shiraz (Figure 4).

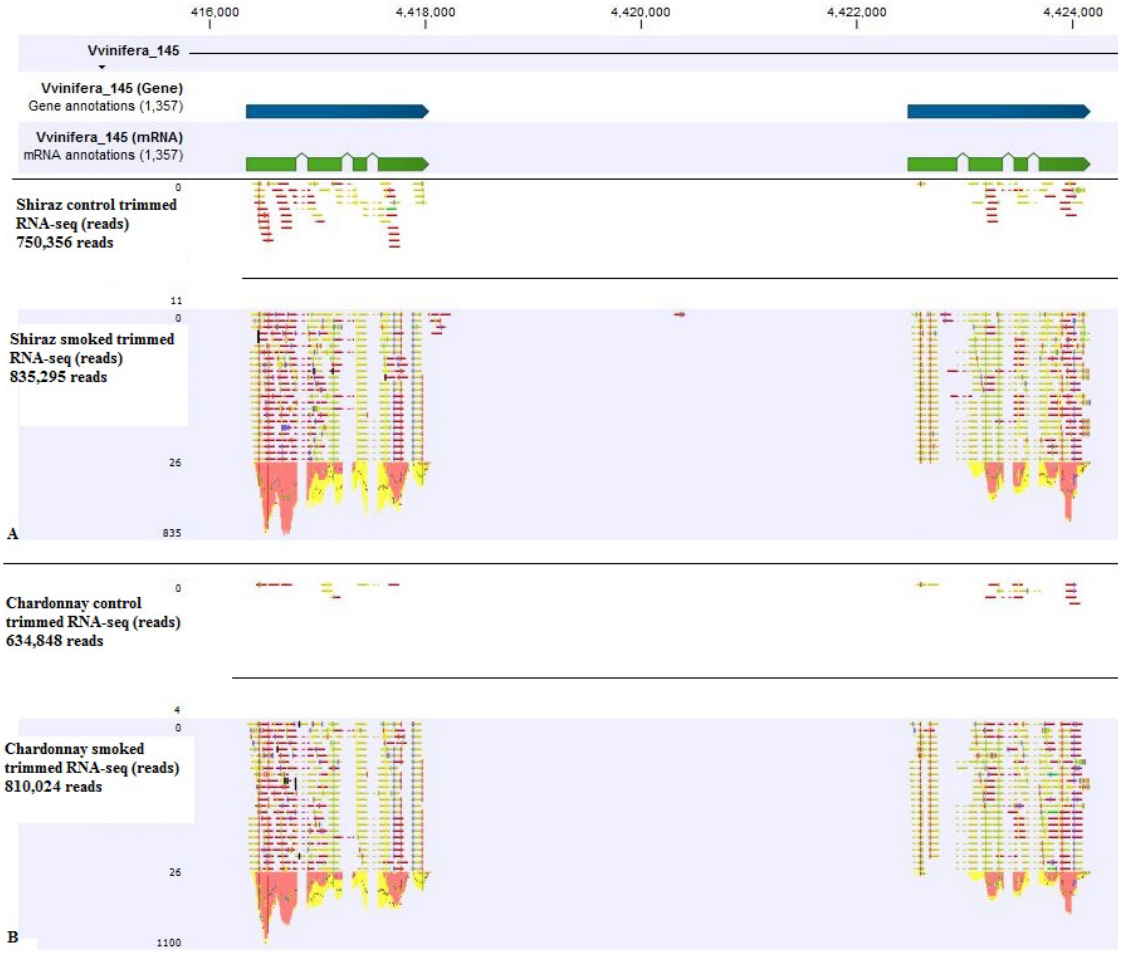


Figure 4 Reads obtained through RNA sequencing for control (top) and smoke-exposed (bottom) Chardonnay (A) and Shiraz (B) for *GolS1* and *GolS2* as tandem repeats on chromosome 7.

Analysis of the GT1 tree assembled based on PFAM 00201 showed 25 different groups (A-Y) , with 3 major groupings (A to I, J to O and P to Y) (Figure 5). Candidates *HqGT*, *CrocGT*, *UGT92G6* and *7DaGT* are all found in separate clusters across the tree, which

are respectively clusters B, L, T and H. Potentially assigning a functional annotation based on the GT1 phylogenetic tree was not possible, as many of the proteins found in this family have proven to be promiscuous in their choice of donor and acceptor (Jones, Messner et al. 2003).

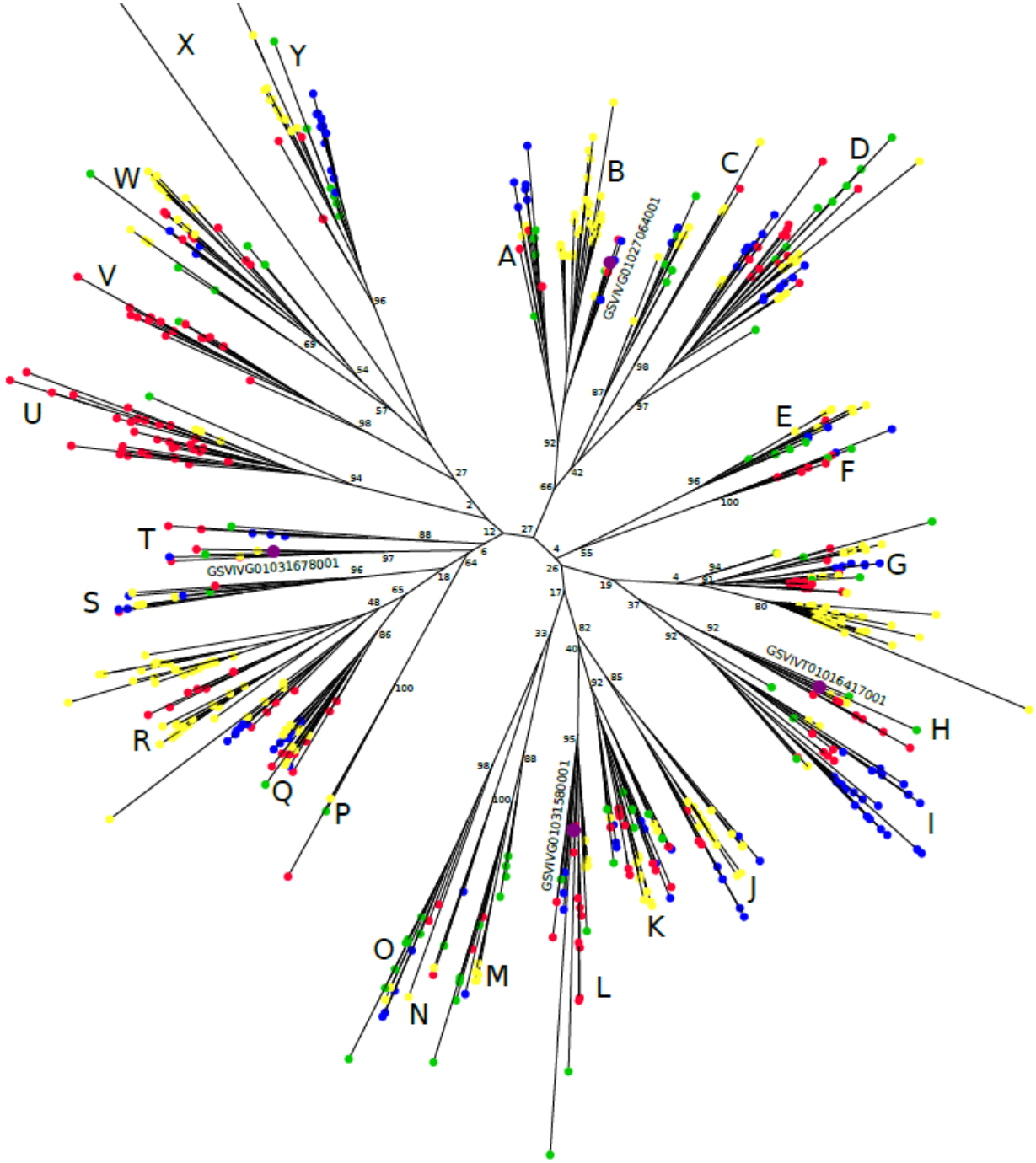


Figure 5 Best-known maximum likelihood RAxML tree for GT1-PF00201 sequences in sampled species. Species are indicated by coloured dots with green representing *Vitis vinifera*, red *Solanum lycopersicum*, blue *Arabidopsis* thaliana and yellow *Medicago truncula*. This tree is only a partial representation of the entire GT1 family and is joined to the remainder via the branch labelled X.

3.5 Chemical composition of smoke-exposed field grown vines

The influence of smoke exposure on berry ripening was determined for each of the varieties for which spectral measurements were taken, i.e. Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Merlot, by measuring total soluble solids (TSS) and berry weight (Supplementary table 5). Few statistically significant differences in TSS were observed between control and smoke affected fruit during ripening; with no significant differences in TSS or berry weight found at maturity. These differences were attributed to natural variability and not to the impact of smoke exposure, as in agreement with reports in the literature (Ristic, Fudge et al. 2016).

The concentration of a range of smoke taint precursors was quantified by measuring as syringol gentiobioside equivalents at commercial maturity of the grape berries. Higher concentrations of most glycoconjugate precursors were observed for smoke-exposed fruit (Table 6). However, due to the large variability in amounts of the compounds detected in the smoke-exposed samples, no significant differences were found. Clear varietal differences were seen in the amount of glycoconjugates present, with for example the red varieties Cabernet Sauvignon and Merlot containing higher concentrations of most precursors than the white varieties Sauvignon Blanc and Chardonnay. The pentose glucosides of guaiacol, cresol and phenol, together with the gentiobioside of syringol were the most abundant precursors. Surprisingly, the levels of precursors identified were several fold lower than has been seen in other smoke taint research (Ristic, Fudge et al. 2016).

Table 6 *Concentrations of volatile phenol glycoconjugates (µg/kg) in grapes harvested from control (C) and smoke-affected (S) grapevines.*

Treatment		Gu-R	Gu-GB	Gu-PG	Gu-G	Cr-R	Cr-PG	Ph-R	Ph-GB	Ph-PG	Syr-GB	4MG-R	4MG-PG	4MS-GB
Chardonnay	C	tr	tr	2 b	1	tr	4 b	tr	tr	6 b	1 b	tr	3	tr
	S	tr	tr	12 a	2	2	9 a	1 a	1 a	13 a	24 a	2	3	2
	P	*ns*	*ns*	*0.016*	*ns*	-	*0.038*	-	-	*0.005*	*0.033*	-	*ns*	-
Sauvignon Blanc	C	tr	tr	1	1	tr	2	tr	tr	3	1	tr	tr	tr
	S	10	23	13	4	25	11	11	1	24	33	10	tr	6
	P	-	-	*0.003*	*0.002*	-	*0.004*	-	-	*< 0.001*	*< 0.001*	-	*ns*	-
Cabernet Sauvignon	C	tr	tr	2	1	1	2	tr	tr	4	2	tr	2	tr
	S	7	4	27	3	14	13	7	3	26	59	5	2	5
	P	-	-	*ns*	*ns*	*ns*	*ns*	-	-	*ns*	*ns*	-	*ns*	-
Merlot	C	tr	tr	3	1	tr	4	tr	tr	7	2	tr	3	tr
	S	3	24	77	10	9	43	4	3	56	66	5	4	9
	P	-	-	*ns*	*ns*	-	*ns*	-	-	*ns*	*ns*	-	*ns*	-

Values represent the mean of three replicates; nd = not detected; tr = trace (i.e., ≤ 1 µg/L).
Different letters within columns (for each variety) indicate statistical significance (P = 0.05, one-way ANOVA); ns = not significant.
Gu = guaiacol; Cr = cresol; Ph = phenol; Syr = syringol; 4MG = 4-methylguaiacol; 4MS = 4-methylsyringol; R = rutinoside; GB = gentiobioside; PG = pentose glucoside; G = glucoside.

4. Discussion

Smoke exposure of grapevines can lead to the development of smoke taint in affected fruit. The uptake of smoke derived volatile phenols including guaiacol, syringol and cresol is followed by glucosylation, causing the accumulation of a smoke taint precursor pool. To investigate the molecular effects of smoke exposure on grapevines, this investigation sought to provide a general profile of the genes upregulated in response to smoke exposure and more specifically to identify glycosyltransferase genes that may encode the proteins responsible for modifying the volatile phenols which contribute to the smoke taint.

Initial RNA sequencing of smoke-exposed potted grapevines indicated higher transcripts of genes annotated as heat shock proteins (PFAM 00011) and a fall in expression of a more diverse range of genes, including genes linked to hormone sensing (Table 1 and 2). The change in temperature for untreated and treated grapes would not necessarily be associated with heat stress (less than 2°C, with an ambient temperature of approximately 27°C). However, upregulation of genes annotated in the PFAM 00011 group has been associated with other forms of abiotic stress that the grapevine has responded to (Liu, Wang et al. 2012). In contrast to the upregulated genes all being part of one PFAM, the downregulated genes showed high variability in predicted function. The most downregulated gene for both Chardonnay and Shiraz is predicted to be from the expansin family. Expansins are proteins implicated in cell wall expansion, and are highly regulated during berry development, with different expansins being expressed at different developmental stages (Dal Santo, Vannozzi et al. 2013). These proteins are thought to be down regulated as a response to abiotic stress such as heat and drought, in order to halt cell division and growth (Baena-González 2010). For two grapevine cultivars (Touriga Nacional and Trincadeira) β-expansin has been shown to be downregulated when exposed

to heat and light radiation stress (Rocheta, Coito et al. 2016). This could potentially be linked to a temporary halt in berry expansion in response to a number of stresses.

Analysis of RNAseq data identified a set of glycosyltransferase genes in whole berry samples from Chardonnay and Shiraz, which increase just an hour following smoke exposure. For both cultivars *GolS1* and *GolS2* were most abundant. The higher transcription profile of these two genes is not surprising, as both *GolS1* and *GolS2* encode galactinol synthases, and their proteins catalyse the formation of raffinose, an osmoprotectant. Accumulation of this trisaccharide in grapevines has been known to occur after several types of abiotic and biotic stress, but has not been identified in earlier smoke taint research (Pillet, Egert et al. 2012, Agudelo-Romero, Erban et al. 2015). Earlier work found only *GolS1* to be upregulated after heat stress (Pillet, Egert et al. 2012), but here we clearly showed the higher expression of both genes. Although the enzymes encoded by *GolS1* and *GolS2* are closely related as galactinol synthases further work should identify the role of these genes in *Vitis vinifera* after smoke exposure; either as general stress-related genes due to the smoke exposure, or potentially as functional enzymes producing smoke taint precursors. The latter might not be the expected role for either *GolS1* or *GolS2* however, as the proteins in this family seem not to be as promiscuous in their choice of aglycone and glycone as other GTs (Gelineo-Albersheim, Xu et al. 2011).

The upregulation of *HqGT1* was not expected, but is potentially the best candidate in this group, as it has already been identified to preferentially glucosylate smoke-derived volatile phenols into monosaccharidic smoke taint precursors. The candidates *CrocGT1* and *7DaGT* are less defined for their roles in *Vitis vinifera*, but are important in crocus (*Crocus sativus*) and Madagascar periwinkle (*Catharanthus roseus*). Both catalyze important steps during development of these plants, as crocetin GT glucosylates the insoluble carotenoid crocetin and *7DaGT* was identified to glucosylate the cyclic

monoterpene 7-deoxyloganetic acid (Asada et al. 2013, Moraga, Nohales et al. 2004). For these genes, only glucosylation functionality has been ascribed, and so these candidates may only be involved in the addition of the first sugar unit to the aglycone (Asada et al. 2013, Moraga, Nohales et al. 2004). Further work to biochemically define their activity, with a specific focus on smoke-derived volatile phenols in grapes, is needed to identify the specific steps in glucosyltransferase that these candidates are involved in

The differential response of grapevines to smoke exposure might possibly also explain the varietal precursor profiles presented in Table 6. However, kinetic studies are needed to identify if for example *CrocGT* makes specific contributions to precursor profiles of red grape varieties, directly after smoke exposure. Similarly, the change of volatile phenol precursor profiles over time, i.e. between fruit sampled 1 day after smoke exposure and at maturity (manuscript 1, this thesis), might be explained by the higher expression of *7DaGT* in Chardonnay and Merlot at t = 1. However, as it is not known whether the candidate genes encode proteins that can catalyze the formation of volatile phenol diglycosides, it is not possible to conclusively link the varietal precursor profiles to specific gene activity patterns. It may be necessary to heterologously express these GT genes and test their protein products *in vitro* against a broad range of substrates to start to define their various activities.and potential products *in vivo*.

All gene candidates identified through RNA sequencing of smoke-exposed potted grapevines were found to be expressed in field grown samples in both control and smoke affected fruit. Due to the large sample set, with four varieties, two tissue types, and three sampling points only one biological replicate was used for this initial study. However, differences in expression were identified based on variety, tissue type and treatment. Notable increases in transcript levels in the skin were not unexpected since this tissue usually accumulates a higher amount of glycosidic compounds than others (Cabrita, Freitas et al. 2006).

In conclusion, it is likely that smoke exposure can regulate the expression of glucosyltransferases in grape berries at certain time points following exposure. Still, glycosyltransferases are highly regulated during development of the grapevine, including the fruit, and so it is difficult to identify the impact of the exposure from only three separate sampling time points. At the moment it is challenging to link the precursor profiles obtained in this work to the activity of one of the genes investigated due to lack of proper functional identification of the candidates. *HqGT1* and *UGT92G6* have been identified in earlier work as glucosyltransferases with an affinity for smoke-derived volatile phenols, however, only monoglucosides were identified as potential products. The profiles obtained in this work clearly indicate a preference for diglycosides to be formed following smoke exposure, in the form of pentose glucose or gentiobioside glycoconjugates.

Conflict of interest

The authors state no conflict of interest.

Author contributions

LH, CF, RB and KW designed the experiments. LH and NS performed the experiments. LH, CF, RB, KW, NS and JS analyzed the data. LH, CF, RB, KW, NS and JS wrote and edited the article. All authors read and approved the manuscript.

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Supplementary figures / tables

Supplementary table 1 RNA sequencing results as supplied by AGRF

100bp Single End - Flowcell ID: C9P2AANXX			
Lane	Sample Name	Single Reads	Data Yield (bp)
3	10_Ch_C1	17,912,594	1.79 Gb
	11_Ch_C2	21,945,092	2.19 Gb
	12_Ch_C3	19,172,783	1.92 Gb
	1_Sh_C1	18,108,940	1.81 Gb
	2_Sh_C2	19,071,909	1.91 Gb
	3_Sh_C3	18,275,556	1.83 Gb
	4_Sh_S1	20,413,483	2.04 Gb
	5_Sh_S2	16,620,576	1.66 Gb
	6_Sh_S3	18,166,211	1.82 Gb
	7_Ch_C1	14,864,484	1.49 Gb
	8_Ch_C2	14,260,737	1.43 Gb
	9_Ch_C3	18,846,957	1.88 Gb
Total		217,659,322	21.77 Gb

Supplementary table 2 Triplicate TPM data calculated from RNA sequencing reads for Shiraz and Chardonnay

	Gene identifier	Shiraz						Chardonnay					
		C			S			C			S		
Upregulated	GSVIVG01035432001	0.0	0.0	0.7	188.8	174.5	132.2	0.0	0.0	0.0	121.5	87.9	118.8
	GSVIVG01035433001	2.2	2.4	0.2	730.6	844.6	559.2	0.0	0.3	0.4	551.9	356.0	367.7
	GSVIVG01016428001	1.5	1.5	1.3	391.0	375.0	375.8	0.1	0.2	0.1	219.9	134.4	169.5
	GSVIVG01016429001	0.3	0.1	0.0	398.9	259.9	213.2	0.1	0.1	0.2	183.9	88.3	130.4
	GSVIVG01035429001	9.2	4.6	5.3	2445.4	1880.8	1841.1	0.6	0.4	0.6	668.1	350.5	538.2
	GSVIVG01035434001	1.2	2.1	2.1	521.9	510.5	359.4	1.1	1.2	1.5	1252.7	732.5	865.2
	GSVIVG01016426001	1.5	1.4	1.0	819.6	616.5	595.3	0.2	0.3	0.4	227.9	131.5	195.0
	GSVIVG01035428001	1.8	0.7	1.9	619.1	698.6	434.3	0.2	0.8	0.6	396.5	244.5	246.1
	GSVIVG01030320001	0.2	0.0	0.2	51.6	74.7	29.9	0.0	0.0	0.4	63.5	40.1	45.4
	GSVIVG01035430001	0.1	0.1	0.0	164.5	126.6	86.2	0.1	0.1	0.1	41.3	21.6	28.5

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Downregulated	GSVIVG010114 37001	8.4	39.3	7.1	1.2	0.3	1.8	7.3	0.5	12.7	0.2	0.3	0.1
	GSVIVG010287 66001	1.4	3.4	1.3	0.2	0.1	0.2	0.8	0.2	0.5	0.0	0.0	0.0
	GSVIVG010080 03001	2.1	6.4	1.5	0.4	0.2	0.6	4.9	2.1	3.2	0.1	0.3	0.1
	GSVIVG010099 62001	0.1	0.2	0.0	0.0	0.0	0.0	8.1	9.4	7.3	0.3	0.6	0.4
	GSVIVG010217 79001	2.4	4.5	1.2	0.1	0.1	0.1	0.5	0.1	2.3	0.0	0.2	0.1
	GSVIVG010099 61001	46.5	81.9	18.8	2.5	1.2	5.1	29.1	20.4	43.3	2.6	9.5	7.0
	GSVIVG010139 13001	3.1	5.9	3.0	0.7	0.2	0.1	1.5	0.4	0.7	0.0	0.7	0.2
Candidates	GSVIVG010281 74001	2.7	1.5	1.9	253.4	343.9	172.2	0.5	1.8	1.1	320.2	190.0	205.2
	GSVIVG010281 76001	1.4	0.7	1.2	125.5	157.7	83.7	0.8	0.9	0.7	134.6	85.2	96.4
	GSVIVG010315 80001	0.2	0.2	0.2	13.5	8.8	0.7	1.0	0.8	0.6	110.5	52.9	18.0
	GSVIVG010270 64001	1.1	1.1	1.2	55.5	29.2	11.9	1.6	4.3	1.9	99.3	64.9	61.6
	GSVIVG010158 59001	0.2	0.2	0.2	2.3	2.5	0.6	0.2	0.2	0.1	3.5	4.1	2.3
	GSVIVG010164 17001	20.2	16.1	24.3	288.1	249.9	174.9	7.9	21.9	9.8	233.4	124.3	150.4
	GSVIVG010316 78001	87.3	107.0	95.6	116.9	100.8	121.5	67.2	40.8	104.4	94.2	89.3	100.5

Supplementary table 3 Q-PCR primers used. Tubulin, MDH, actin and ubiquitin are positive controls. UGT92G6* awaits sequencing.

Locus	Gene Name	Fwd Primer	Rev Primer	Size (bp)	Acquisition temperature
GSVIVT01004414001	*VvTubulin*	CAGCCAGATCTTCACGAGCTT	GTTCTCGCGCATTGACCATA	119	79
GSVIVT01028330001	*VvMDH*	CCATGCATCATCACCCACAA	GTCAACCATGCTACTGTCAAAACC	72	80
GSVIVT01026580001	*VvActin*	GCATCCCTCAGCACCTTCC	AACCCCACCTCAACACATCTCC	203	80
GSVIVT01038617001	*VvUbiquitin*	AGTAGATGACTGGATTGGAGGT	GAGTATCAAAACAAAAGCATCG	177	80
GSVIVG01028176001	*VvGolS2*	TGAATTTGGAGCCTTTCATTG	ACCCCCAAACCACAAAAATAA	187	79
GSVIVG01027064001	*VvHqGT1*	GAAAGGATGTACGCAGTCGAA	TGTGAGCCTTCCATTTTTGAG	117	79
GSVIVT01031580001	*VvCrocGT*	AAGAGGTGCTTGGAATTGGTC	TCAGATGATCCACCTTCCATC	115	78
GSVIVG01031678001	*UGT92G6**	TTTCCTGATTCCTGCCGCTT	GGAAAGCGCTATCTGAGGCT	108	80
GSVIVT01016417001	*7DaGT*	TGAAAGACACTTGCGATAGGG	AACACACCACACACGTTTCAA	233	79

Supplementary table 4 Heatmap of xpression of candidate genes in control (c) and smoke-exposed (s) grape berries. Highest amounts in green and lowest amounts in red and 50th percentile of the data in yellow.

Cultivar	Tissue	Treatment	Sample time	GolS2	HqGT1	CrocGT1	UGT92G6	7DaGT
Chardonnay	skin	c	t = 0	325	1912	173	7555	238
			t = 1	240	2256	79	8954	109
			t = 7	645	1423	161	9374	235
		s	t = 0	429	3917	161	10151	377
			t = 1	347	3676	70	7220	168
			t = 7	407	1317	95	9963	211
	pulp	c	t = 0	687	2926	104	6652	62
			t = 1	180	1707	30	9315	8
			t = 7	194	991	24	9731	14
		s	t = 0	491	2280	159	13009	145
			t = 1	256	3892	104	7244	52
			t = 7	240	469	49	9051	34
Sauvignon Blanc	skin	c	t = 0	177	4608	171	5098	57
			t = 1	148	1235	95	7411	183
			t = 7	4984	1401	132	3338	377
		s	t = 0	133	1761	67	7906	33
			t = 1	99	1700	74	5539	44
			t = 7	3120	512	108	4023	107
	pulp	c	t = 0	191	2095	38	4927	70
			t = 1	100	777	63	4838	36
			t = 7	1231	763	47	3074	67
		s	t = 0	96	2009	21	5744	10
			t = 1	45	753	14	5758	11
			t = 7	829	212	51	2847	49
Cabernet Sauvignon	skin	c	t = 0	254	398	107	4344	107
			t = 1	892	479	57	4589	87
			t = 7	20353	489	80	5123	181
		s	t = 0	208	2745	727	7716	61
			t = 1	638	463	55	4206	102
			t = 7	8972	483	66	4528	129
	pulp	c	t = 0	108	311	23	4798	27
			t = 1	436	271	14	4749	43
			t = 7	11781	272	40	6632	63
		s	t = 0	58	378	17	6424	36
			t = 1	405	437	31	5695	65
			t = 7	6650	395	69	7222	110

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Merlot	skin	c	t = 0	182	836	84	5955	104
			t = 1	9993	459	86	3323	160
			t = 7	354	270	100	4915	44
		s	t = 0	421	2095	1265	5652	129
			t = 1	12204	594	137	3734	195
			t = 7	423	304	49	4515	54
	pulp	c	t = 0	86	515	17	5023	30
			t = 1	8174	329	24	4035	34
			t = 7	257	120	13	6588	10
		s	t = 0	125	739	14	7079	37
			t = 1	9396	824	30	4541	85
			t = 7	581	255	10	6092	7

Supplementary table 5 *TSS and berry weight of grapes sampled from control (C) and smoke-affected (S) grapevines, at different time points.*

Treatment		TSS (in °Brix)				Berry weight (g)
		t = 0	t = 1	t = 7	maturity†	maturity†
Chardonnay	C	13.0	14.6	15.6 b	23.2	1.4
	S	13.5	15.7	17.1 a	22.7	1.5
	P	*ns*	*ns*	*0.031 (1.3)*	*ns*	*ns*
Sauvignon Blanc	C	13.5	10.9	16.7	23.6	1.2
	S	13.0	12.9	17.9	23.7	1.3
	P	*ns*	*ns*	*ns*	*ns*	*ns*
Cabernet Sauvignon	C	12.9	13.4	14.5	24.0	1.1
	S	13.6	12.7	14.7	23.9	1.1
	P	*ns*	*ns*	*ns*	*ns*	*ns*
Merlot	C	16.4	15.8	17.5 b	23.7	1.7
	S	15.3	15.3	16.4 a	23.2	1.7
	P	*ns*	*ns*	*0.037 (1.02)*	*ns*	*ns*

Sample times are reported as days after smoke exposure.
†*Maturity corresponds to t = 17, t = 28, t = 28 and t = 22 days for Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Merlot respectively.*
Values represent the mean of three replicates (n = 3).
Different letters within columns (for each variety) indicate statistical significance (P = 0.05, one-way ANOVA); ns = not significant

CHAPTER 4

The effect of smoke exposure to apple

Statement of Authorship

Title of Paper	Smoke Exposure Influences the Composition of Apples (*Malus domestica* Borkh cv Sundowner).
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Contribution to the Paper	Designed and conducted experiments; collected, processed, analysed and interpreted compositional and molecular data; drafted and edited manuscript.
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 party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 16/11/17

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.

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Chapter 4: The effect of smoke exposure on apples

Introduction

Vineyard smoke exposure has caused financial losses for grape growers and winemakers, where fruit has been downgraded, or even discarded, due to smoke taint. To date, the occurrence of smoke taint has not been reported in other fruit crops, despite the proximity of for example, orchards, to wine regions affected by smoke from bushfires or prescribed burns. This chapter describes a preliminary study which sought to investigate the composition of apples (*Malus domestica* Borkh cv 'Sundowner') following exposure to smoke during ripening. The starch pattern index and total soluble solids content of apples were measured to determine any effect of smoke on ripening of fruit. The concentrations of volatile phenol glycoconjugates were measured in control and smoke-affected apples to determine whether or not apples accumulate smoke derived volatile phenols in glycoconjugate forms, in a similar fashion to that observed in wine grapes.

Smoke Exposure Influences the Composition of Apples (*Malus domestica* Borkh cv Sundowner).

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Abstract

The occurrence of smoke taint in grapes and wine following vineyard exposure to smoke from bushfires or prescribed burns has received considerable attention over the last decade.

Smoke taint is a fault found in wines produced from smoke affected vines. No reports from industry have arisen of smoke taint in other types of produce, even though bushfires commonly affect large agricultural areas. This research investigated the effect of smoke exposure on apples as the main crop to be used in cider production. Experimental smoke exposure of an hour applied to apples led to the formation of smoke taint precursors and significant differences in brown pigments and color density in early harvest fruit, but not in mature fruit. This outcome indicates that apples are susceptible to taking up and glycosylating smoke derived volatile compounds, but not, however, in quantities commonly found in smoke affected grapes. Further research is needed to analyse the effect of using smoke exposed apples in the production of cider to identify the effect on the sensory profile of this beverage.

Keywords: apple, glycoconjugates, guaiacol, smoke taint, volatile phenols

INTRODUCTION

Vineyard exposure to smoke from either bushfires or prescribed burns can result in smoke tainted wines, i.e. wines which exhibit unpleasant smoky, ashy characters[1-3]. As a consequence, considerable research has been undertaken: to understand the impact of smoke on grape and wine composition[1, 3-5], to develop analytical methods for detecting and quantifying smoke taint [6-8], and to identify methods of amelioration that can mitigate the financial losses incurred by grape and wine producers[9-11]. Volatile phenols, including guaiacol, 4-methylguaiacol, cresol and syringol, have been identified as constituents of both smoke[12] and wines made from smoke-affected grapes[1]. However, quantification of smoke derived volatile phenols, as markers of smoke taint, is complicated by their *in vivo* glycosylation following grapevine smoke exposure[7,13]. Increases in volatile phenol concentrations observed during winemaking have been attributed to hydrolysis of glycoconjugate precursors[1]; but a significant proportion of the glycoconjugate pool remains in the finished wine[3], even after bottle aging[14].

To date, the occurrence of smoke taint has not been reported in other fruit crops, despite the prevalence of fruit production in close proximity to wine regions. This may be explained by the timing of fire events, i.e. the risk of bushfires may be low during the growing season of other fruit crops. It could also reflect the accumulation of smoke derived volatile compounds in glycoconjugate forms, in a similar manner to that which occurs in grapes, such that there is a less apparent sensory impact. Apples are used in the production of cider, and apple juice undergoes fermentation in a manner similar to that of white wine production. Apples are crushed and the resulting juice is fermented to obtain cider[15]. It is therefore reasonable to assume that apples (and cider) might also be tainted by smoke, and the accumulation of smoke-derived volatile phenols in apples in glycoconjugate forms might still result in the release of volatile phenolsduring

fermentation. As such, this study sought to investigate the composition of apples following smoke exposure, to determine the potential for smoke taint to occur in a fashion similar to that observed in wine grapes.

MATERIALS AND METHODS

Chemicals. Chemicals (analytical grade) and solvents (HPLC grade) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) and Merck (Damstadt, Germany). The deuterated internal standard, *d3*-syringol gentiobioside, was synthesized in house, as previously described[5]

Field Trials. Field trials involved the application of smoke to apple trees (*Malus domestica* Borkh cv. Sundowner) grown in an orchard at the University of Adelaide's Waite Campus in Urrbrae, South Australia (latitude 34°58'S, longitude 138°38'E). Apple trees were enclosed in purpose-built smoke tents (approximately 6 m x 2.5 m x 2 m) and exposed to smoke for 1 hour (in duplicate) at the start of fruit ripening, using experimental conditions previously employed for the application of smoke to grapevines[1-2]. Apples (3 control and 3 smoke-affected, chosen randomly) were sampled before smoke exposure (i.e. at t = 0) and at t = 1, 7, 14 and 28 days after smoke exposure, to enable determination of the starch pattern index [16] and the total soluble solids (TSS) content of juice (measured by refractometry), as measures of fruit maturity. A range of color and phenolic measurements were also performed on juice samples (at t = 1, 14 and 28 days after smoke exposure), using a spectrophotometer (GBC Scientific Equipment, Melbourne, Vic, Australia).

Determination of Volatile Phenol Glycoconjugates by Liquid Chromatography-Tandem Mass Spectrometry. Extracts of skins from control and smoke-affected apples

were prepared for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the concentration of a range of glycoconjugates of smoke derived volatile phenols. Apples (3 control and 3 smoke-affected, chosen randomly) were peeled and a sub-sample of the resulting skins (20 g, of approximately 2 mm thickness) homogenized with water (20 g). Aliquots (5 g) of apple homogenate were spiked with d_3-syringol gentiobioside as an internal standard, centrifuged (4,300 x g for 5 min) and a portion of the resulting supernatant (2 mL) loaded onto an Extract Clean C18-HF solid phase extraction cartridge (Grace Davison, Australia). The C18 cartridge was washed with Milli-Q water (3 mL, in duplicate), and then eluted with methanol (3 mL). The methanol extract was concentrated and reconstituted in water (0.5 mL) prior to LC-MS/MS analysis, which was performed by the Australia Wine Research Institute's Commercial Services Laboratory (Adelaide, Australia) using an Agilent 1200 high performance liquid chromatograph (HPLC) coupled to an Applied Biosystems 4000 QTrap hybrid tandem mass spectrometer (Applied Biosystems, MDS Sciex, Foster City, CA, USA). The concentrations of glycoconjugate forms of guaiacol, 4-methylguaiacol, *m*-, *o*-, and *p*-cresol, syringol and phenol (as syringol gentiobioside equivalents) were determined using a stable isotope dilution assay method reported previously[5].

Data Analysis. Data were analyzed by one-way analysis of variance (ANOVA) using GenStat (15th Edition, VSN International Limited, Herts, UK). Mean comparisons were performed by least significant differences (LSD) multiple comparison test at $P<0.05$.

RESULTS AND DISCUSSION

The composition of control and smoke-affected apples was compared to determine the potential for apples to be tainted by smoke, in a similar manner to that observed in grapes and wine. No significant differences were observed in either the starch pattern index (Figure 1) or TSS content (Table 1) of apples during the 4 weeks between smoke exposure and maturity. Smoke exposure therefore had no apparent impact on fruit ripening, in agreement with results obtained for the ripening of smoke-affected wine grapes[17, 3]. With the exception of color density and brown pigments, no significant differences were observed in colour and phenolic measurements of control and smoke-affected apple juice either (Table 2). Where significant differences were observed, i.e. in color density at t = 14, and brown pigments at t =1 and t = 14, these differences were no longer significant at maturity (i.e. at t = 28). Differences in color, in the concentration of brown pigments in particular, have been observed in wine made from grapes exposed to smoke post-harvest[18]. In the current study, natural variation in apple color and phenolic content was greater than any impact resulting from smoke exposure.

The volatile phenol glycoconjugate profiles of control and smoke-affected apples were compared to determine whether or not smoke derived volatile phenols were adsorbed and glycosylated following smoke exposure (Table 3). Control and smoke-affected fruit contained similar levels of some glycoconjugates, i.e. the glucoside of guaiacol and pentose glucoside of 4-methylguaiacol; but significantly higher levels of rutinosides of cresol and phenol, and the gentiobioside of syringol were observed in smoke-affected fruit. These glycoconjugates are often the most abundant precursors observed in smoke-affected grapes (manuscript 1 in this thesis). Importantly, these results suggest apples adsorb and glycosylate smoke derived volatile phenols in the same way as has been shown to occur in grapes.

CONCLUSION

Smoke exposure of apples leads to the formation of smoke taint associated precursors. Higher amounts of smoke taint precursors such as syringol gentiobioside, cresol rutinoside and phenol rutinoside were found in smoke affected samples than in control apple samples following smoke exposure of an hour. The amount of glycoconjugate precursors was lower than usually found in smoke affected grapes, as well as in experimentally smoke exposed grapes. Because precursor analysis was only performed for partial pomace and skin per apple it is unclear if the lower concentration of precursors is an indication of apples being more resistant to smoke exposure than grapes. Further work should include the analysis of juice, as well as the investigation into smoke derived volatiles present in the apples. Furthermore, a longer smoke exposure time could potentially lead to higher uptake and glycosylation of smoke derived compounds.

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FIGURE CAPTIONS

Figure 1. Starch conversion numbers for control (in black) and smoke-affected (in greay) apples during ripening.

Table 1. Total Soluble Solids (ºBrix) of Control and Smoke-Affected Apples, at Different Time Points Following Smoke Exposure.

treatment	TSS (ºBrix)				
	t = 0	t = 1	t = 7	t = 14	t = 28
control	9.8	10.0	10.6	11.3	12.2
smoke	9.4	9.5	10.4	11.2	11.8

Sample times are reported as days after smoke application; t = 28 represents commercial maturity.
Values are means of three replicates (n = 3).
Values within columns were not significantly different (*P* = 0.05, one-way ANOVA).

Table 2. Color and Phenolic Measurements for Juice from Control and Smoke-Affected Apples.

Treatment		color density (au)	color hue	total phenolics (au)	flavonoids (au)	brown pigments (au)
t = 1	control	0.4	2.2	14.5	8.8	0.3 b
	smoke	1.0	4.4	13.0	8.1	0.8 a
	P	*ns*	*ns*	*ns*	*ns*	*0.03*
t = 14	control	0.4 b	2.1	15.3	8.4	0.3 b
	smoke	0.6 a	2.2	13.8	8.1	0.4 a
	P	*0.03*	*ns*	*ns*	*ns*	*0.05*
t = 28	control	0.6	1.9	13.3	7.2	0.4
	smoke	0.4	1.6	14.7	8.3	0.3
	P	*ns*	*ns*	*ns*	*ns*	*ns*

Sample times are reported as days after smoke application; t = 28 represents commercial maturity.
Values are means of three replicates (n = 3).
Different letters within a column (for each time point) are statistically significance (*P* = 0.05, one-way ANOVA); ns = not significant.

Table 1. Concentrations of Volatile Phenol Glycoconjugates (µg/kg) Present in Control and Smoke Affected Apples (at Maturity).

	Gu-R	Gu-PG	Gu-G	Cr-R	Cr-PG	Ph-R	Ph-GB	Ph-PG	Syr-GB	4MG-R	4MG-PG	MS-GB
control	nd	nd	7	2 b	nd	1 b	nd	1	2 b	nd	6	nd
smoke	1	1	8	6 a	2	7a	1	4	18 a	1	6	1
P	–	–	*ns*	*0.04*	–	*0.04*	–	*ns*	*0.003*	–	*ns*	–

Values are means of three replicates (n = 3); nd = not detected; limit of quantification = 1.0 µg/kg.
Different letters within a column indicate statistical significance (*P* = 0.05, one-way ANOVA); ns = not significant.
Gu = guaiacol; Cr = cresol; Ph = phenol; Syr = syringol; 4MG = 4-methylguaiacol; R = rutinoside; GB = gentiobioside; PG = pentose glucoside; G = glucoside.

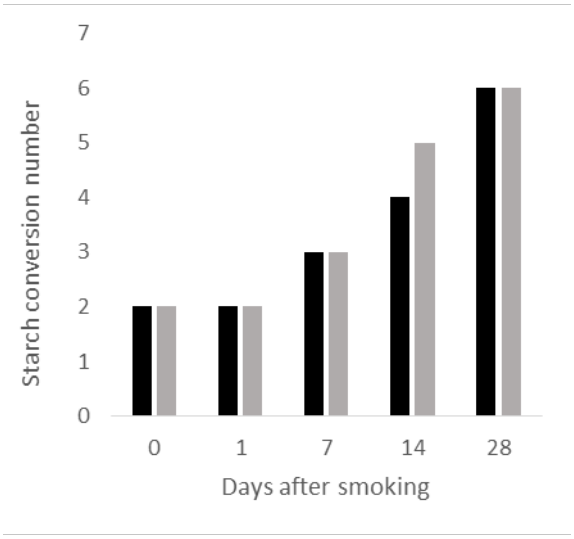
Starch conversion number
7
6
5
4
3
2
1
0
0
1
7
14
28
Days after smoking

Figure 1

CHAPTER 5

Conclusions and future directions

Conclusions and future directions

Conclusions

Climate change predictions indicate bushfires are likely to occur more frequently and last longer, due to the drier, hotter days forecast globally. As a consequence, vineyard exposure to smoke, and therefore smoke tainted grapes and wine, will continue to be a significant challenge for the wine industry. Previous research on smoke taint has helped understand the chemical and sensory profiles of smoke tainted wine. The research described in this thesis adds to the body of knowledge concerning the impact of smoke on grapes and wine, in particular (i) the accumulation of glycoconjugate precursors in grapes, and (ii) expression of glucosyltransferase enzymes following grapevine exposure to smoke.

Investigations identified changes in the accumulation of volatile phenol glycoconjugates in smoke-exposed grapes over time. Smoke exposure of *Vitis vinifera* cvs Sauvignon Blanc, Chardonnay and Merlot at approximately 10 days post-veraison showed varietal differences in the glycoconjugate profiles of smoke-affected grapes. Merlot grapes showed the highest levels of glycoconjugates present, of the three varieties studied, with the most abundant precursors being pentose-glucosides of guaiacol and cresol. For Sauvignon Blanc however, rutinosides of cresol and phenol were most abundant, while for Chardonnay, pentose-glucosides of guaiacol, cresol and phenol, as well as syringol gentiobioside, were observed at the highest levels. Similar trends in glycoconjugate profiles were seen throughout the experimental work described in this thesis. Furthermore, changes in volatile phenol glycoconjugate profiles were observed over time, i.e. for fruit sampled 1 day after smoke exposure compared with fruit sampled at maturity.

The application of agrichemicals (i.e. kaolin, a particulate clay and Envy, a polymer-based anti-transpirant), prior to smoke exposure did not significantly affect the volatile

phenol glycoconjugate profiles in Sauvignon Blanc, Chardonnay and Cabernet Sauvignon; indeed, some precursor levels were higher in grapes following the application of Envy. However, significantly lower levels of glycoconjugate precursors were identified in Merlot grapes after treatment with kaolin, suggesting kaolin may afford some protection from smoke exposure, depending on the level of coverage.

Field trials were also undertaken in an attempt to identify smoke-affected fruit in the vineyard, using a handheld spectrometer to measure berry reflectance. Whilst it was not possible to identify smoke-affected Sauvignon Blanc fruit, significant differences were observed in the spectral reflectance of control and smoke-affected Chardonnay, Cabernet Sauvignon and Merlot fruit. PCA of reflectance spectra measured at both 1 and 7 days after grapevine exposure to smoke enabled differentiation of control and smoke-affected fruit particularly at day 1 and to a lesser extent at day 7. Whilst this finding suggests reflectance spectroscopy might represent a rapid tool for evaluating smoke exposure in the vineyard, practically, it might be difficult to implement where bushfire affected regions cannot be safely accessed shortly after a fire event.

To date, smoke taint research has largely focused on the chemical and sensory consequences of vineyard exposure to smoke; investigations employing molecular and biochemical approaches to understanding the development of smoke taint in grapes have received little attention in the literature. A study concerning the role of glucosyltransferases in the conjugation of smoke derived volatile phenols was published in early 2017 [75]. The work described in this thesis builds on that study, to improve the current understanding of the metabolic pathways involved in the accumulation of glycoconjugate forms of smoke-derived volatile phenols. Grapevines grown in both a controlled growth room environment and in the field, were exposed to smoke under experimental conditions, and their transcriptional response determined. RNA sequencing of control and smoke-affected grapes from potted Shiraz and Chardonnay indicated

higher expression of heat shock proteins and glucosyltransferases following smoke exposure. Six glucosyltransferases yielded higher expression in both Chardonnay and Shiraz, and four of these were selected as candidates for further investigation in the subsequent field trials. One additional GT was included in this investigation as it has been reported to show preferential activity towards smoke derived volatile phenols, and has a high overall abundance in grapevines (ref). Real time quantitative PCR of Chardonnay, Sauvignon Blanc, Cabernet Sauvignon and Merlot fruit indicated a putative hydroquinone glucosyltransferase, crocetin glucosyltransferase and 7-deoxyloganetic acid glucosyltransferase were more highly expressed in smoke-affected grapes at specific time points; with differences observed in relative expression in skin and pulp fractions also.

A final investigation involving the application of smoke to apples was performed to determine the potential for smoke taint to occur in a crop other than wine grapes. Low levels of volatile phenol glycoconjugates were observed in apples exposed to smoke for an hour, but smoke exposure did not affect the development and maturation of apples.

Future directions

Smoke taint is likely to remain an issue for the wine industry over the years to come, with climate change continuing to exacerbate conditions conducive to bushfires. As research into smoke taint continues, many of the early knowledge gaps have been addressed. Nevertheless, important questions remain unanswered, and so future research directions might include:

1. *Identification of volatile phenol glycoconjugate profiles for a broader range of grape cultivars.* The work described in this thesis included the determination of volatile phenol glycoconjugate profiles for Sauvignon Blanc, Chardonnay, Merlot and Cabernet Sauvignon. However, bushfires occur in regions in which other grape cultivars are grown. For example, in 2017, grape-growing areas of Chile were affected by significant bushfires, where the most prominent grape varieties include Pais, Merlot and Malbec. Given the variability in precursor profiles found in several varieties in the present work, further investigation is needed to identify specific profiles possibly found in these other varieties. The provision of benchmarking data to establish the glycoconjugate profiles of a broader range of grape varieties, both naturally occurring (i.e. the glycoconjugate levels present in control fruit) and smoke derived (i.e. the distribution and levels present in smoke-affected fruit), would enable industry to determine levels of smoke taint in fruit following vineyard smoke exposure. Furthermore, the occurrence of more highly conjugated precursors, e.g. trisaccharides, could be investigated, as to date, only glucosides and disaccharides have been identified.

2. *Identification of the pathway for uptake of smoke derived volatile phenols.* The mechanism by which smoke derived volatile phenols are taken up by grapevine leaves and fruit has not been adequately investigated. In the current study, the application of kaolin to Merlot grapevines mitigated the impact of subsequent smoke exposure, giving

fruit with lower levels of volatile phenol glycoconjugates (compared with smoke-affected fruit from grapevines which were not treated with kaolin). Identification of pathways by which the constituents of smoke are taken up by grapevines would help to establish more effective preventative measures, and therefore warrants further research.

3. *Identification of genes that respond to smoke exposure.* The transcriptomic analysis of smoke-affected grapevine tissue identified upregulation of heat shock proteins associated with abiotic stress. Further investigation into the functionality of these genes is warranted. Given gene transcription is often influenced by multiple factors, the contribution of other abiotic factors towards the development of smoke taint could also be studied.

4. Investigation towards other factors that affect the magnitude of smoke taint. The variation observed in the accumulation of smoke taint precursors in 2016 and 2017 might reflect the influence of environmental factors, e.g. ambient temperatures, on the intensity of smoke taint. The 2016 and 2017 growing seasons differed significantly, with 2016 having a dry summer leading to an early vintage, whereas 2017 had a slower, cooler start to the season, followed by rapid ripening due to a sudden spike in temperature. Future research could investigate other abiotic factors that might affect the uptake of smoke-derived volatiles, including ambient temperature and humidity.

4. *Investigation into the activity of GolS1, GolS2, CrocGT and 7DaGT towards smoke derived volatile phenols.* The upregulation of genes associated with glycosylation observed in potted vines in the growth room experiment, together with the higher expression observed for these candidates in the field, suggests their possible involvement in the glycosylation of smoke derived volatile phenols. Therefore, testing these GT candidate proteins with the key smoke taint marker compounds, i.e. guaiacol, cresol, syringol and phenol, would enable determination of their catalytic activity towards such compounds. It may be possible to heterologously express the GT proteins in E.coli or

yeast and test the various substrates against purified protein. It may also be feasible to use CRISPR/Cas9 to edit GT genes in grapevines to turn them off, which would facilitate the study of the effects of removing this gene in the downstream pathways as related to the appearance of the glycoconjugates and the sensory effects caused by their absence in both juice and wine.

Despite an extensive body of knowledge having been accumulated on the topic of smoke taint in recent years, there is still scope for further research; particularly given improved methods for preventing and/or ameliorating smoke taint are still required.

APPENDIX

Ristic, R., **van der Hulst, L.,** Capone, D. L., Wilkinson, K. L. (2017)

Impact of Bottle Aging on Smoke Tainted Wines from Different Grape Cultivars.

Journal of Agricultural and Food Chemistry

Ristic, R., van der Hulst, L., Capone, D. L. & Wilkinson, K. L. (2017). Impact of Bottle Aging on Smoke Tainted Wines from Different Grape Cultivars. *Journal of Agricultural and Food Chemistry*, *65*(20), 4146-4152.

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