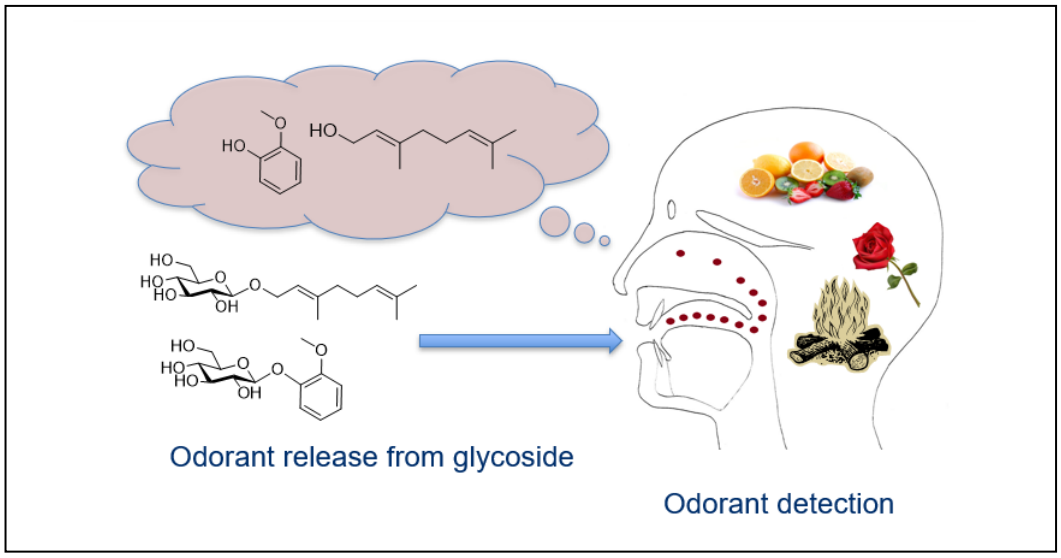
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AWRI Australian Wine Research Institute

Using glycosides and other flavour precursors for improved wine flavour



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1. Abstract

Much of the flavour in wine comes from non-flavour-active precursors that have to be broken down to provide known varietal attributes. Through chemical, sensory and winemaking experiments, the potential of different unrealised flavour precursors to improve wine flavour was determined. Additives prepared from grape marc were effective at increasing flavour of wine and no- and low-alcohol products. Flavour formation was not reliant on fermentation but largely dependent on wine pH. Additionally, flavour evolves from precursors in the mouths of tasters, although their response to precursors depends largely on their individual sensitivity to the odorant released.

2. Executive summary

The route from grape to wine flavour can occur via many different pathways. Many classes of compounds are present in the grape in a non-volatile form, bound to sugars or amino acids. For them to be perceived as flavour or aroma, they must firstly be broken down and the volatile portion released. While this release of flavour into wine can happen either during winemaking or wine storage, there is a portion of these precursors that is not extracted into wine and can be found in grape marc. Additionally, monoterpene glycosides present a novel opportunity to add flavour via in-mouth breakdown during wine consumption.

Using previously prepared Chardonnay and Riesling wines with added Gewürztraminer marc extract, analysis after six months of storage showed marked changes. The extract that was rich in monoterpene glycosides provided no added bitterness in the wines, confirming adequate removal of phenolics from the isolation procedure. All wines showed increased 'floral' attributes which resulted from the hydrolysis of monoterpene glycosides to yield the aroma-active free monoterpenes. Adding the Gewürztraminer marc extract before or after fermentation made little difference to the outcome, showing that chemical hydrolysis was the driver for volatile release and not enzymatic hydrolysis during fermentation. As such, marc extracts can be added post-fermentation once the wine can be assessed for flavour intensity.

Importantly, when tasted, there were observed increases in 'floral aftertaste' for the wines with added Gewürztraminer marc extract. However, this observation was largely from those sensory panellists who had previously been shown to perceive flavour from glycosides via in-mouth release of the volatiles (deemed 'tasters'). This suggests that a major factor in 'floral aftertaste' attribute rating was the in-mouth hydrolysis of monoterpene glycosides present in the wines.

The underlying reasons for panellists' different responses to glycosides were investigated. While the microbial populations in-mouth were linked to the breakdown of glycosides when in contact with saliva samples, these did not relate to the ability of the panellists to detect aroma from the glycosides. Instead, the main factor in determining the response to glycosides was the sensitivity to the released volatile. It was also shown that individual thresholds for glycosides and the released volatiles were wide-ranging. The classification of the panellists into either 'tasters' or 'non-tasters' was a function of their threshold being above or below the concentration used in the test. Regardless, it was shown that glycoside additions to wines produce flavour via two mechanisms:

- in-mouth hydrolysis of the glycosides themselves during tasting and retronasal aroma detection of the released volatile
- slow chemical hydrolysis due to the acidic nature of wine to yield the volatile compound (and further rearrangement products) in wine.

The Chardonnay and Riesling wines with added Gewürztraminer marc extract were monitored chemically during three years of storage. The low pH Riesling (pH 3.1) showed a more rapid hydrolysis of geraniol glucoside, with key monoterpenes also reducing in concentration from the six-month analysis timepoint. For the Chardonnay wines (pH 3.4), the concentration of linalool was still increasing at the three-year analysis point. The 0.3 difference in pH created significant differences in the evolution of flavour as the wines aged. Adjusting for the speed of breakdown at different pH and comparing the Riesling wines after 11 months with the Chardonnay wines at 31 months showed a

better alignment of the volatile profiles. While there were a number of chemical reactions taking place in these wines, the hydrolysis step of geraniol glucoside alone appears useful in understanding shelf-life characteristics of the wines.

The winery-scale production of glycoside-rich extracts was further optimised using cross-flow filtration from a 300 kg parcel of Muscat Gordo Blanco marc. This produced a similar extract to the Gewürztraminer extraction but with much faster throughput in the winery. Additionally, an in-depth review of the FSANZ regulations against the protocol for extraction indicated that these marc extracts were permissible additives for winemaking.

The Gordo extract was used to understand the first six months of storage in wine. The initial winemaking trials showed an increase in geraniol glucoside from what was in the extract to what was observed at the first timepoint after six months. When the Gordo extract was added to a commercial Chardonnay and monitored monthly, there was a continual increase in the concentration of geraniol glucoside. However, the quantification of disaccharide analogues did not account for the increases seen. As such, there must be another contributor to geraniol glucoside that is yet to be identified. Regardless, the concentration of geraniol glucoside in the extracts provided a good predictor for the evolution of volatile monoterpenes in wine.

As the bound monoterpenes are not odour-active and can largely be disposed of with marc, there was potential that traditionally 'non-floral' varieties might be hiding a pool of flavour precursors that were never realised. However, when marc from a number of floral and non-floral varieties was used to create extracts, this was shown to be incorrect. When stored in commercial wine for six months, the floral varieties produced much higher concentrations of volatiles, especially the Muscat varieties. Therefore, to produce potent glycoside-rich extracts from grape marc it would be beneficial to keep Muscat varieties separated from others, although this would present logistical hurdles.

From industry consultation on the use of glycoside-rich extracts in winemaking, it was highlighted that there was strong interest in using them to improve the flavour outcomes of no- and low-alcohol (NOLO) products. When the Gordo extract was tested in NOLO matrices, it was observed that the aggregated knowledge on the use of glycosides in winemaking is transferable. Initial tastings of Gordo marc extract in NOLO products showed potential to increase 'wine-likeness'.

When a similar isolation protocol was performed on Sauvignon Blanc juice to obtain extracts rich in tropical thiol precursors, there was less success. Firstly, the requirement to use juice created extraction and filterability issues due to the high viscosity. Secondly, when the extracts were used in informal tastings, extremely high concentrations were required for only a portion of the panel to perceive flavour. It was determined that this process is unlikely to be applicable, for a number of reasons.

This project has delivered an extraction method for the isolation of purified glycosidic material that can be added to wine or wine-like products. The method has been developed specifically to be used in a winery setting and involves materials that are accessible to a winery. The development of the glycosidic extract has undergone significant industry consultation, specifically looking at segments of the wine industry that would be amenable to integrating it into their usual practices, with a number of large wine companies indicating they would use such an extract if it were available. Furthermore, there appears to be a natural fit between glycoside-rich extracts and a desire to achieve greater flavour outcomes in NOLO products.

3. Acknowledgements

This work was supported by Australia's grapegrowers and winemakers through their investment body Wine Australia with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster in Adelaide, SA.

This work would not have progressed without the input of many winemakers and wine sector members into the ON Prime pre-commercialisation process, as well as a select group of winemakers from Pernod Ricard Winemakers, Treasury Wine Estates and Accolade Wines, who attended tastings. The project team also recognises invaluable input to the ON Prime program from Paul Smith (Wine Australia), Mark Krstic, Neil Scrimgeour and Kieran Hirlam (AWRI).

Production of monoterpene-rich extracts required grape marc, which was kindly provided by Accolade Wines, SARDI/PIRSA and K1 Wines (Geoff Hardy), often in the form of grapes. Equipment required for large-scale extractions and purification was obtained from Memstar/VA Filtration.

Many of the project outcomes were a result of the PhD work of Mango Parker, which was supported by the University of South Australia and Miguel De Barros Lopez. All work conducted at the AWRI relied on the input of many staff members as well as the AWRI sensory panel and WIC Winemaking Services.

4. Background

The route from grape to wine flavour can occur via many different pathways. Many classes of compounds are present in the grape in a non-volatile form, bound to sugars or amino acids. For them to be perceived as flavour or aroma, they must firstly be broken down and the volatile portion released. Traditionally, this occurs via the action of yeast during alcoholic fermentation or via slow chemical hydrolysis during wine storage.

However, there is a possibility to isolate 'flavour precursors' from juice or wine and use these as flavour supplements. It has previously been demonstrated that purified (i.e. phenolic-free) monoterpene glycosides present a valuable, novel opportunity to add flavour, through enzymatic release during winemaking but also via in-mouth breakdown during wine consumption, contributing flavour intensity and flavour persistence (Parker et al. 2017). This work showed that when a panel of tasters was provided with different glycosides, they varied in their ability to perceive flavour (Figure 1). Additionally, an ability to perceive flavour from one glycoside did not relate to the perception of flavour from others.

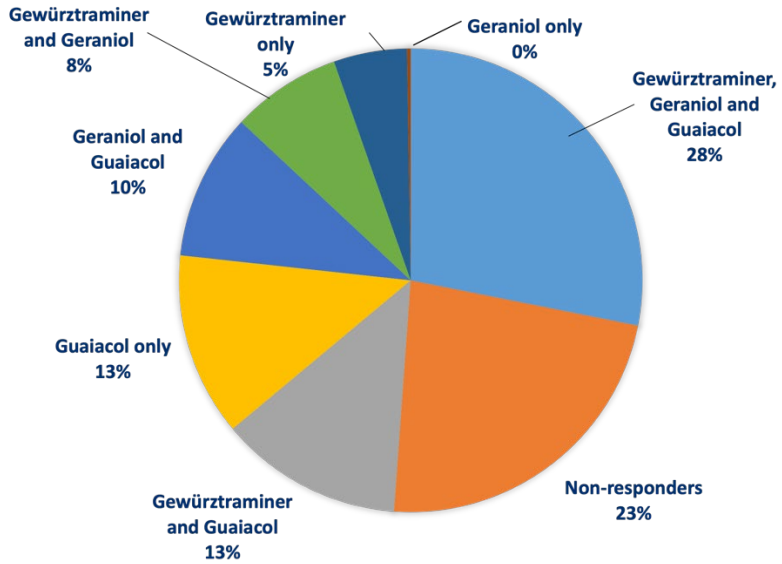


Figure 1. The perception of flavour via in-mouth release of volatiles from different glycosides, showing the inter-individual variation in the response. Percentages refer to the percentage of the sensory panel that perceived flavour from various different glycosides. Created using the data in Parker et al. (2017).

5. Project aims

This project aimed to investigate grape glycosides as a potential wine additive, including timing of additions and dose rates to achieve measurable sensory effects during wine consumption. Characterisation of glycoside extracts from various grape varieties was achieved using non-targeted LC-MS and GC-MS analysis. Glycosides from different varieties were extracted and partially purified, their stability investigated during fermentation and wine ageing, and their sensory impact assessed. The concentration of residual precursors in wines following fermentation was measured, to determine whether these may act as quality markers.

Additional precursor systems such as thiol precursors were also assessed, providing a complementary avenue for building additional flavour during wine consumption.

A better understanding of the factors underlying individual variability in sensory response to in-mouth release of precursors was to be obtained.

Year 3 deliverables (June 2020)

- Isolation procedures for phenol-free monoterpene glycosides from floral varieties and knowledge about their sensory significance in winemaking applications.
- Knowledge about the sensory effect of glycosides from non-floral varieties and their flavour contributions after storage in-bottle.

Year 5 deliverables (June 2022)

- Understanding of the effects of glycosides in alternative products (e.g. low- or no-alcohol products).
- Understanding of the sensory significance of thiol precursors and processing options.
- Practical advice and extension materials for winemakers.

Year 3 deliverables and associated activities for the year 2017-18

	Deliverables	Activities
A	Isolation procedures for phenol-free monoterpene glycosides from floral varieties and knowledge about their sensory significance in winemaking applications (2019)	• Investigate the distribution of free and bound monoterpenes through isolation and characterisation of glycosides from at least five grape lots from floral varieties. • Determine the glycoside composition, of juices and wines from the Gewürztraminer glycoside addition experiment, expanding analytical capabilities to allow compositional determination of glycosides above currently measured monoterpene glycosides. • Assess the practical value of glycoside addition pre- or post- fermentation in a production context by consulting with a group of winemakers, including presentation of wines from the recent Gewürztraminer glycoside addition experiment. • Assess factors contributing to individual differences in sensory response to glycosides by assessing orthonasal response to monoterpenes and retronasal response to glycosides, and saliva glycoside hydrolysis, of at least ten individuals.

B	Knowledge about the sensory effect of glycosides from non-floral varieties and their flavour contributions after storage in-bottle (2020)	• Obtain grape material from at least five sources from non-floral varieties such as Sauvignon Blanc, Semillon, Shiraz and Chardonnay to allow the subsequent investigation of free and bound aroma compounds through isolation and characterisation of glycosides.

Year 3 deliverables and associated activities for the year 2018-19

	Deliverables	Activities
A	Isolation procedures for phenol-free monoterpene glycosides from floral varieties and knowledge about their sensory significance in winemaking applications	1. Investigate optimised and efficient means of isolating phenol-free glycosides in consultation with a commercial provider with expertise in production. 2. Prepare study to allow changes in volatile and glycoside composition of a wine with added phenol-free glycosides to be determined over time. 3. Determine structural requirements for glycoside release during winemaking and in bottle. 4. Prepare research paper on saliva release experiment.
B	Knowledge about the sensory effect of glycosides from non-floral varieties and their flavour contributions after storage in-bottle	1. Isolate and characterise glycosides from at least five grape lots from non-floral varieties in preparation for a sensory and compositional study in 2019-20.

Year 3 deliverables and associated activities for the year 2019-20

	Deliverables	Activities
A	Isolation procedures for phenol-free monoterpene glycosides from floral varieties and knowledge about their sensory significance in winemaking applications.	1. Isolate and purify a pool of glycosides from Muscat of Alexandria marc. 2. Study changes in volatile and glycoside composition of a wine with added phenol-free glycosides over time. 3. Profile the glycoside material in a number of floral varieties using LC-MS.
B	Knowledge about the sensory effect of glycosides from non-floral varieties and their flavour contributions after storage in-bottle.	1. Conduct a sensory study on the aroma impact of glycosides isolated from grape marc of non-floral varieties.

Year 5 deliverables and associated activities for the year 2020-21

	Deliverables	Activities
A	Understanding of the effects of glycosides in alternative products (e.g. low or no alcohol products).	1. Expand investigations of glycoside structure-dependent hydrolysis, including into matrices with low and no alcohol. 2. Complete a laboratory-scale sensory addition study involving wines spiked with glycosidic

		material extracted from marc of different grape varieties to investigate source-dependence.
B	Understanding of the sensory significance of thiol precursors and processing options.	1. Review isolation protocols and key factors for consideration when assessing the potential of thiol precursors in in-mouth release and in-bottle release of thiol aromas/flavours. 2. Investigate methods for isolating thiol precursors for use in flavour precursor-type applications and conduct a preliminary sensory assessment on extracts.
C	Practical advice and extension materials for winemakers.	1. Compile information to date into an industry article and webinar/roadshow talks.

Year 5 deliverables and associated activities for the year 2021-22

	Deliverables	Activities
A	Understanding of the effects of glycosides in alternative products (e.g. low or no alcohol products).	1. Complete a sensory addition study using Gordo marc-derived glycoside-rich extract at alcohol concentrations of relevance for Project 3.1.10, and move towards an understanding of their usefulness in formulating low- or no-alcohol products. 2. Summarise research into glycoside hydrolysis in bottle and the kinetics of flavour evolution in a manuscript for a peer-reviewed publication.
B	Understanding of the sensory significance of thiol precursors and processing options.	1. Complete a sensory addition study and saliva hydrolysis analytical study, to demonstrate impacts of thiol precursors. 2. Assess extraction procedures for thiol precursors that are amenable to scale-up, if warranted by sensory results.
C	Practical advice and extension materials for winemakers.	1. Prepare extension materials for the 18th AWITC. Depending on outcomes, summarise results from the thiol work into an industry journal article. 2. Support Projects 2.1.1 (Extension) and 2.1.2 (Communication and awareness-raising activities) through identifying topics and preparing materials for awareness-raising and practice change activities.

6. Methods

6.1 Material available from prior work

6.1.1 Synthetic glycosides

Pure glycosides used and described in this work were synthesised in-house prior to the initiation of this project. The synthesis of geraniol glucoside and its deuterated analogue, guaiacol glucoside and its deuterated analogue, and syringol gentiobioside and its analogue have been described in Parker et al. (2017).

6.1.2 Grape marc derived glycosides

A monoterpene glucoside-rich extract was prepared at winery scale in 2016 from Gewürztraminer marc. Briefly, 300 kg of marc was extracted with water in a rotary fermenter, and the aqueous extract was filtered and extracted as outlined by Parker et al. (2019). This extract was used throughout this project.

6.1.3 Wines with added glycoside-rich extracts

The above-mentioned Gewürztraminer marc extract was added pre- and/or post-fermentation to Chardonnay or Riesling. The winemaking protocol was conducted in 2016, as outlined in Parker et al. (2019), although the first wine analyses (chemical and sensory) were performed during the scope of this project work. These wines were followed chemically for three years throughout this project.

6.2 Grape marc extractions

6.2.1 Winery-scale extractions of glycosides from grape marc

In addition to the Gewürztraminer extract described above (section 6.1.2), a 300 kg parcel of Gordo marc was extracted in a similar manner, as described in Pisaniello et al. (2022). Gordo Muscat marc was collected from a commercial winery in the 2019 vintage (Riverland, South Australia). The marc was pressed off at the winery and within five hours was stored at 0°C. The following day the marc was shovelled into 3 x 240 L drums and stored at -18°C for 6 months. Before extraction, the marc was allowed to thaw and warm to room temperature before being split into two parcels (150 kg each) and each parcel was mixed with 300 L of water in an upright half-tonne fermenter with the temperature held between 10 - 15°C. The marc/water was left overnight to hydrate, then received regular plunging the following day before being left overnight. Each fermenter was drained and pressed, and the resulting liquid was passed through a cross-flow filter to yield the aqueous extract. This extract was purified in the same manner as described previously (Parker et al. 2019). In brief, 50 L aliquots of the extract were loaded onto 3 kg of FPX66 resin in a 6 L column housing, rinsed with caustic solution (20 L, 25 mL of liquid concentrate into 20 L water – theoretically pH 12.5), water (20 L) and eluted with ethanol (20 L, Tarac, food grade, spirit neutral). The combined ethanol extracts were concentrated using a high vacuum distillation (VA Filtration/Memstar) to 20 L before being further concentrated using a laboratory rotary evaporator and lyophilised to dryness. This yielded 145.55 g of extract.

6.2.2 Laboratory-scale extractions of glycosides from grape marc

Grape varieties classified as either floral or non-floral based on the monoterpene characterisation of Strauss et al. (1986) were hand-picked at approximately commercial maturity (20-24 Brix for whites, 23-26 Brix for reds). In 2017 several Muscat and non-Muscat floral varieties were collected from different locations (Muscat à Petits Grains Blanc and Viognier from McLaren Vale, South Australia; Riesling, Muscat Gordo and Gewürztraminer from the Barossa Valley, South Australia) and in 2018 several non-floral varieties were collected from the Barossa Valley (Chardonnay, Semillon, Verdelho, Sauvignon Blanc, Shiraz and Cabernet Sauvignon). Approximately 10 kg of grapes were hand-picked at commercial harvest and stored at 4°C for a maximum of two days before being processed. A known mass of grapes was divided into 2 kg parcels and each parcel separately crushed and pressed in a 2 kg capacity stainless steel hand press. Each parcel was pressed three times to 20 Nm using a torque wrench, and between press cycles the press cake was broken up and mixed to ensure even pressing of all bunches. The juice and marc from the entire 10 kg sample were pooled and weighed, then stored at -18°C and retained for later analysis. For each variety, the marc was defrosted and extracted in water (1.5 L per kg of marc) containing potassium metabisulfite (150 mg/L) with constant stirring for 24 hours at ambient temperature. The extract was strained to remove the marc then centrifuged (Beckman, 9000 RPM, 10 minutes) before being purified. The extract was loaded onto FPX66 resin, washed with caustic solution (2 L, 1.26 g/L), then water (2 L), and eluted with ethanol (2 L). The ethanolic extract was concentrated using a rotary evaporator then lyophilised to yield the purified marc extract.

6.3 Winemaking and storage trials

6.3.1 Assessment and monitoring of 2016 winemaking trials

The duplicate Chardonnay and Riesling wines described above (Section 6.1.3) were monitored periodically for three years. Analysis was conducted by GC-MS for volatiles and LC-MS for non-volatiles. Here, duplicates with the same extract concentration but added at different times were grouped into quadruplicates for simplicity.

6.3.2 2019 Gordo marc extract storage trials – six months

A commercially available Chardonnay wine(Yalumba Wine Smiths, 14 x 2L casks) was mixed in a 30L stainless steel keg under an inert atmosphere. Approximately 6 L of wine (5.93 kg of 0.9885 g/mL wine) was dispensed into a 9.5 L stainless steel keg pre-filled with carbon dioxide. For the glycoside addition, 2.4 g of marc extract, pre-dissolved in the wine was added and gently mixed (0.4 g/L in wine). The wine was dispensed into 375 mL brown glass bottles and sealed with crown seals. The control was handled in the same way, without the glycoside addition. The wines were stored at 15°C for five months, with three bottles opened every month for analysis.

6.3.3 Storage trials for floral and non-floral marc varieties

The storage trial was conducted using the same 30 L keg of commercial wine as described above. For each treatment approximately 1.2 L of wine (1.186 kg of 0.9885 g/mL wine) was dispensed into a 2 L stainless steel keg followed by the addition of 0.4 g/L of marc extract, pre-dissolved in a small volume of the wine. After gentle mixing, the wine was dispensed in triplicate into 375 mL brown glass bottles and sealed with crown seals. The wines were stored at 15°C for five months before being assessed chemically and using rapid sensory methodology.

6.4 Chemical analysis

6.4.1 LC-MS analysis for glycosides

Quantitation of geraniol glucoside by LC-MS/MS using a d_2-geraniol glucoside internal standard and d_3-syringol gentiobioside for disaccharides was as previously described by Pisaniello et al. (2022). Wines were analysed neat and grape marc extracts were dissolved in water, followed by the addition of the internal standards (d_2-geraniol glucoside for quantification of geraniol glucoside, and d_3-syringol gentiobioside for disaccharide bound monoterpenes) prior to instrumental analysis. Transitions monitored were: syringol gentiobioside (537 → 323, 537 → 477), d_3-syringol gentiobioside (540 → 323, 540 → 341), monoterpene pentosyl-glucose (507 → 293, 507 → 311, 507 → 477) and monoterpene rhamnosyl-glucose (521 → 307, 521 → 325, 521 → 461).

6.4.2 GC-MS analysis for volatiles

Volatiles were quantified from extract hydrolysates or wine samples for a range of monoterpenes and norisoprenoids by GC-MS, as described by Parker et al. (2019). During this work, the analytical method was improved by the inclusion of membrane assisted solvent extraction (MASE, below).

6.4.3 MASE-GC-MS analysis for volatiles

The improved analytical method provided improved sensitivity and sample preparation. The method is described in full in Pisaniello et al. (2022).

6.5 Sensory analysis

6.5.1 Glycoside response phenotype identification

Forty-one individuals (mean age 42, range 24-62 years, 25 males) were screened for their flavour response to geranyl glucoside tested at 3.0 mg/L, and guaiacyl glucoside tested at 0.50 mg/L, dissolved in water, with a water blank, using the method as described in Parker at al. (2017). The full procedure is outlined in Parker et al. (2020). From this test, a subset of individuals representing the four phenotypes (tasters of both glycosides, tasters of neither glycoside, tasters of one or the other glycoside) was selected for threshold testing.

6.5.2 Glycoside and aglycone threshold testing

Retronasal detection thresholds (n=23, 14 males, age 24-58, mean age 43 years) were determined for four compounds: geraniol, guaiacol, geranyl glucoside and guaiacyl glucoside. Geranyl glucoside

and guaiacyl glucoside were tested in pure water. A three-alternative forced choice (3-AFC) test was carried out based on the procedure described in ASTM method E 679-04, for each of the four compounds. The full procedure is outlined in Parker et al. (2020).

6.5.3 Sensitivity to geraniol glucoside prior to oral microbial experiments

The 11 panellists used in the descriptive analysis study were screened for their ability to perceive flavour from synthetic geranyl glucoside (3.0 mg/L) in water, as described in Parker et al. (2020). Panellists were classified as tasters of geranyl glucoside if they had a significant response to the geranyl glucoside, above the water control, with $p < 0.15$.

6.5.4 Descriptive analysis of wines with added Gewürztraminer extract

A panel of 11 assessors (six males, five females) with an average age of 42 years (SD = 9.6) was convened for a consensus-based descriptive methodology. The full methodology and outcomes are detailed in Parker at al. (2019).

6.5.5 Consumer hedonic testing for wines with added Gewürztraminer extract

Six experimental wines were selected for the consumer liking test based on diversity of sensory properties as characterised by the above descriptive analysis. A Central Location Test (CLT) was conducted in Sydney, NSW, Australia with 127 white wine consumers, recruited on the basis of drinking white wine at least once per fortnight. Recruitment of the consumers was completed by a contract sensory research company. The full methodology and outcomes are detailed in Parker at al. (2019).

6.5.6 Rapid sensory profiling of wines with added varietal marc extracts

On opening the storage trials of the 2019 Gordo marc extract, the triplicate wines were mixed and presented to the sensory panel in singlicate to limit sample numbers. The wines were assessed by a rapid screening method. The projective mapping sensory method of Napping (N) was used to holistically evaluate the wines based on their perceived similarities and differences. Ultra-Flash Profiling (UFP) was also employed to provide indicative sensory descriptors of the sample set and groupings (Pages 2005). Panellists assessed the set of 13 wines (one control and 12 samples) on the same day and were allowed as much time as they required to complete their projective maps and to add descriptive attributes. Samples were provided to panellists in 30 mL aliquots, in 3-digit-coded, covered, ISO standard wine glasses at 22-24°C, in a randomised presentation order, and panellists were allowed to revisit samples as needed. Formal evaluation was completed in one two-hour session. Each sample was presented to assessors once in a randomised William's block design generated by Compusense Cloud sensory evaluation software (Compusense Inc., Guelph, Canada). The samples were assessed by each panellist and responses transferred directly onto a 1.5:1 digital sensory space via a tablet, with panellists able to select comments from the provided list or enter free choice comments for each sample or group of samples. Multiple factor analysis (MFA) of the sample coordinates of all panellists' maps was conducted to produce a group consensus map for the 13 wines, which included 95% confidence ellipses calculated using the bootstrapping function. Greatly overlapping ellipses suggest similarities in sensory properties while separated ellipses signal clear differences. Secondarily, UFP descriptors were incorporated as supplementary variables to the MFA consensus map through the ratio scaling of the scores of the correlations between variables and factors to the range of the group factor scores of the wines.

6.6 Drivers of in-mouth flavour release

6.6.1 *In vitro* saliva hydrolysis

Whole fresh saliva was incubated with glucosides as reported by Parker et al. (2020). Briefly, 10 mL of saliva was collected from sensory panellists immediately prior to undertaking sensory experiments. Saliva (3 mL) was incubated with 5 µg each of guaiacyl glucoside and geranyl glucoside for 30 min at 37°C. The reaction was stopped by adding 2 mL saturated $CaCl_2$; the samples were then mixed and analysed by LC-MS for remaining glucosides and by GC-MS for volatiles.

6.6.2 Oral microbiological metagenomics

DNA from saliva samples was extracted using a PureLink™ Microbiome DNA Purification Kit following the manufacturer's instructions. Samples were sequenced and the data processed as described in Parker at al. (2020).

6.7 Industry consultation for glycoside use in winemaking

An initial tasting was conducted with staff from Treasury Wine Estates (TWE), Pernod Ricard Winemakers (PRW) and Accolade Wines. They were presented with wines from the 2016 Chardonnay and Riesling addition trial, approximately one year after bottling. The potential of glycosides in winemaking and future directions were discussed.

Subsequently, the concept of marc-derived glycoside extracts in winemaking was workshopped via the CSIRO *ON Prime* program. *ON Prime* is targeted at a pre-commercialisation stage of research and teaches participants to understand where their scientific discovery or product fits with respect to potential end-users or purchasers. It uses several tools to re-orient thinking away from a solution-focus (a solution looking for a problem) and towards the potential end-user and which aspects of their business would provide an incentive to employ the potential solution.

To do this, a potential end-user group is hypothesised, which can be as broad as 'grape grower' or as specific as 'new product developer at large wine company'. An assumed understanding of their role is mapped, including the parts of their job with inherent inefficiencies (or 'pains') as well as their ultimate outcomes in each task (referred to as 'gains'). Ideally, the scientific solution can be linked with providing or increasing an assumed 'gain' and/or reducing or removing 'pain' in the end-user's business, which would go hand-in-hand with the incentive for embracing the solution.

Regardless of how much confidence there is in the understanding of an end-user's potential application, the assumptions made about their role, pains or gains, must be tested (confirmed or invalidated) via consultation with a number of people in that end-user group. This is ideally done without talking about the solution directly, to avoid creating confirmation bias. This is generally approached using open questions that do not reference the discovery or 'lead' the interviewee.

6.8 Glycoside hydrolysis in no- and low-alcohol products

Model wine was made at three different ethanol concentrations (12.0%, 5.0%, 0.5%) using a saturated potassium bitartrate solution. The model wines were acidified to pH 3.2 using tartaric acid and 0.2 g/L of Gordo marc extract was added. Simultaneously, two bottles of a commercial Chardonnay (<0.5% alcohol) were combined and then divided in half. One aliquot remained as the control wine, while the other had 0.2 g/L of Gordo marc extract added. All wines were sealed into glass ampoules and stored at 15°C with triplicate ampoules for each timepoint. The model wines were opened monthly for six months, and then at nine months, while the commercial wines were opened at five months of storage. All wines will also undergo analysis at a 12-month timepoint after the conclusion of this project. The opened ampoules underwent volatile analysis by MASE-GC-MS and glycoside analysis by LC-MS, as outlined above.

6.9 Tropical thiol precursors

6.9.1 Isolation of juice extracts rich in thiol precursors

Sauvignon Blanc juice was obtained from a commercial winery in the Coonawarra region, with one parcel having been grown under standard practices and a second with additional sulfur and urea foliar sprays to increase thiol concentration. Isolation procedures were based on previous work or Starkenmann et al. (2008), Capone et al. (2011) and Parker et al. (2019). The final extraction protocol after improvements involved multiple laboratory-scale batch extractions using XAD-4 resin. Unsuccessful extractions are detailed in the Results and Discussion section of this report. Sauvignon Blanc juice was cold settled and the clarified juice was decanted from gross lees. Pre-wetted XAD-4 resin (50 g) was added to an aliquot of juice (700 mL) and stirred for one hour. The resin was removed by vacuum filtration and washed by stirring with water (200 mL) for 30 minutes. The

washed and recovered resin (via vacuum filtration) was stirred with ethanol (200 mL, spirit neutral, Tarac Technologies) for 30 minutes. The slurry was filtered and the remaining resin washed with ethanol. The aggregated ethanol washes were concentrated via rotary evaporator and then freeze-dried. Analysis of juice and extracts for amino acid-bound thiol precursors was performed using an in-house method.

6.9.2 Preliminary tasting of Sauvignon Blanc juice extracts

Model wine (12% ethanol in water, saturated with potassium hydrogen tartrate and cold-settled) was acidified to pH 3.36 with tartaric acid. The 1.93 g portion of extract was dissolved in Milli-Q water (100 mL, 19.3 g/L), and aliquots of this were used to make the tasting solution. A 30 mL volume of the 19.3g/L solution was added to Milli-Q water and model wine (270 mL, 1.93 g/L) and 10 mL of this solution and 20 mL of Milli-Q water were added to Milli-Q water and model wine (270 mL, 0.64 g/L). In addition, the remaining 20 mL of the 19.3 g/L solution was diluted with Milli-Q water to give 100 mL of a 3.86 g/L solution – above the extract equivalent extracted from juice. An informal bench tasting with AWRI staff was used to determine the potential of the extracts for in-mouth release of flavour.

7. Results and discussion

7.1 Use of glycoside-rich extracts during winemaking

The entirety of the work in section 7.1 has been detailed in Parker et al. (2019).

7.1.1 Gewürztraminer marc extract – six months in wine

Prior to the commencement of this project, Chardonnay and Riesling wines were made with additions of a Gewürztraminer marc extract. The marc extract was characterised in that earlier work and was found to be rich in monoterpene glycosides, specifically geraniol glucoside. The first analysis timepoint after six months of storage was captured in this work. In short, the wines were supplemented with the extract at rates of 0.4 g/L, with single additions made either pre-or post-fermentation and double rates (0.8 g/L) being added either to juice alone pre-fermentation, or split between pre- and post-fermentation. After six months in bottle, the wines were assessed chemically for volatiles by GC-MS and for glycosides by LC-MS, and underwent sensory analysis.

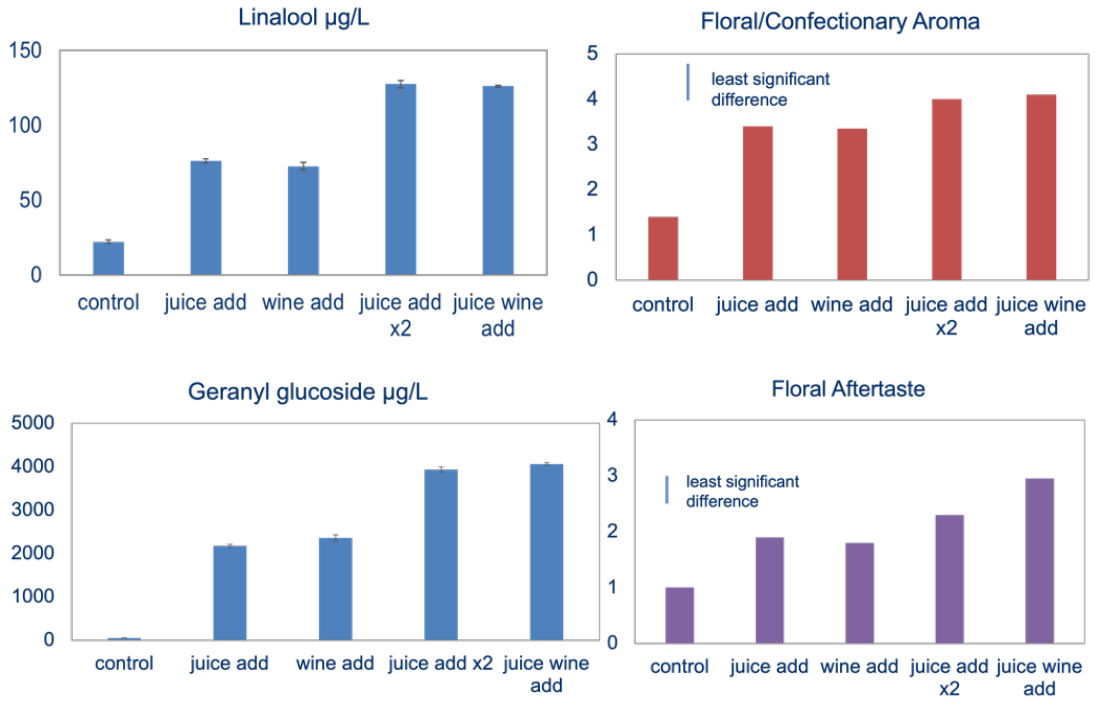


Figure 2. Chemical and sensory analysis outcomes in Riesling wines with added Gewürztraminer marc extracts after six months of storage in bottle. Top left, linalool concentration determined by GC-MS; top right, 'floral/confectionary aroma' attribute rating determined by sensory descriptive analysis; bottom left, geraniol

glucoside remaining in the wines as determined by LC-MS; bottom right, 'floral aftertaste' attribute rating determined by sensory descriptive analysis.

Wines with added marc extract showed increases in the concentration of linalool and associated higher attribute ratings for 'floral/confectionary aroma'. Similarly, the pool of remaining geraniol glucoside was also increased in wines with added extract, as was the 'floral aftertaste' rating. For the wines that had a single addition (0.4 g/L), the timing of the addition did not have a significant impact on the composition or sensory rating of the wines. As such, the extracts could be added to a wine post-fermentation in response to an under performing fermentation outcome.

The trend of increased monoterpenes, free or bound, and the associated sensory attributes were also observed for the Chardonnay wines. When the sensory descriptors and ratings were visualised following Principal Component Analysis, the trend for increased 'floral' attributes is clear with larger doses of the marc extract (Figure 3).

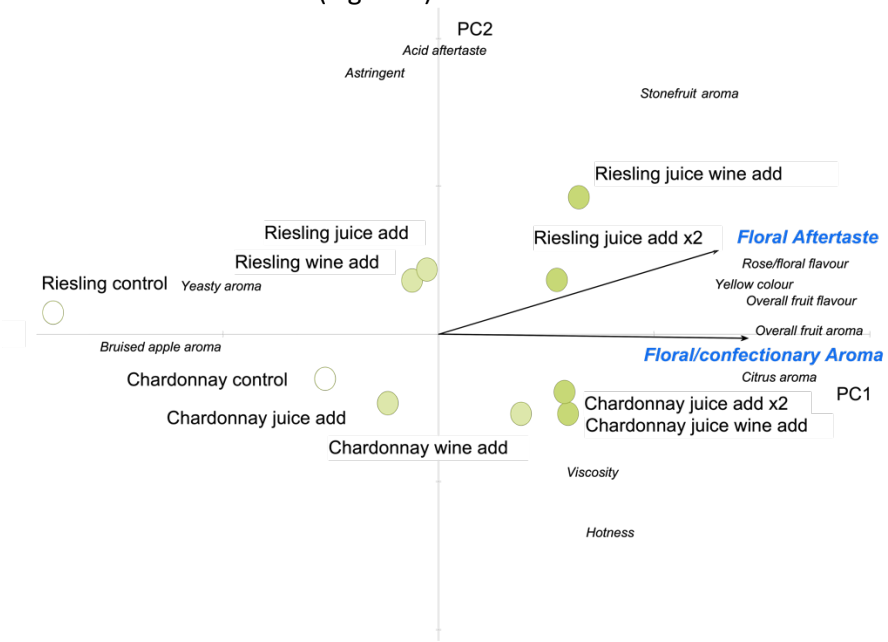


Figure 3. Principal Component Analysis of the sensory attributes and panel mean ratings for Chardonnay and Riesling wines with 0.4 g/L or 0.8 g/L additions of Gewürztraminer marc extract, added at different stages of winemaking.

The use of marc extracts to modulatieflavour proved successful in increasing floral attributes without adding bitterness that could be imparted by phenolics present in grape marc. This work showed that the extracts could be added post-fermentation to adulterate a wine once the outcome of fermentation is known, providing a reactive mechanism for flavour adjustment. Additionally, the pool of geraniol glucoside remaining after six months suggested that the wines would continue to evolve free monoterpene and increase in flavour. One factor that was not well understood in this experiment was that the geraniol glucoside concentration present in the marc extract would have equated to around 1,000 mg/L with a 0.4 g/L addition of the extract. However, after six months the wines that had received 0.4 g/L additions had geraniol glucoside concentrations of around 2,000 mg/L, or around twice the expected concentration.

Furthermore, while the addition of the marc extracts was successful in providing increased wine flavour and aroma via hydrolysis and release of the free monoterpenes, there was also an increase in the 'floral aftertaste'. Previous work has focused on the role of glycosides in 'in-mouth release', where the compound is broken down in the oral cavity releasing the volatile aglycone, which is detected retronasally (Parker et al. 2017). As such, there are two mechanisms in which the monoterpene glycoside-rich extracts impart flavour in wine.

7.1.2 Glycoside response and aftertaste in wine

To better understand the role of in-mouth glycoside breakdown on the ability to perceive aftertaste, the sensory panellists were tested for their ability to perceive flavour from Gewürztraminer glycosides. They were deemed to be either 'tasters' (T) or 'non-tasters' (NT), depending on whether they could detect floral character from the extracts or not.

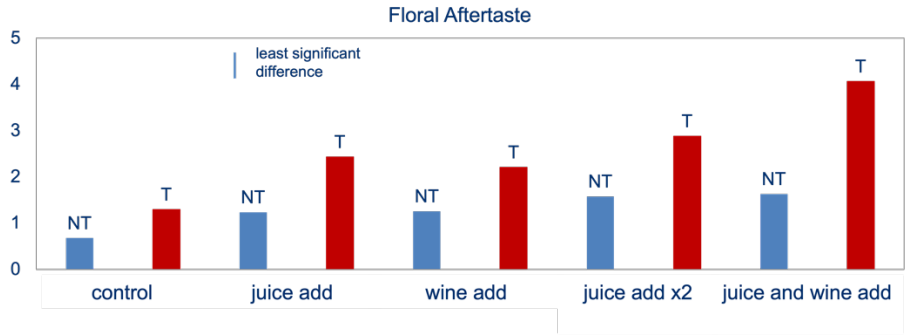


Figure 4. 'Floral aftertaste' attribute rating determined during sensory descriptive analysis, divided into panellists that were glycoside 'tasters' (T) or 'non-tasters' (NT)

It was immediately clear that the panellists who could perceive flavour from the Gewürztraminer marc extract were rating significantly higher 'floral aftertaste' than those that were 'non-tasters'. As such, wines with added monoterpene glycosides could provide flavour firstly via chemical hydrolysis in wine during storage and release of volatiles, and secondly via in-mouth release albeit for only a portion of the population. However, it was not clear as to what separated people as either 'tasters' or 'non-tasters'.

7.2 Drivers of in-mouth flavour release from glycosides

The entirety of the work in section 7.2 has been detailed in Parker et al. (2020).

7.2.1 Inter-individual response in perception of flavour from glycosides

As determined prior to this project and detailed in Parker et al. (2017), within the population there is a range of responses to different glycosides, or phenotypes. Some people can detect flavour from many glycosides, or from no glycosides, with just about every combination in between. Here, the drivers for these differences in response were investigated using threshold testing, saliva collection and hydrolysis assays, and oral microbial screening.

To ensure that there was representation from the different phenotypes of tasters, 41 people were screened for sensitivity to guaiacol and geraniol glucosides. Most (85%) had a significant response to at least one of the glycosides, with 49% responding to both, 34% responding to guaiacol glucoside only, 2% responding to geraniol glucoside only and 15% not having a significant response to either glycoside. Twenty-three people were selected from this cohort for further testing. Of this 23, they were split between those that could or could not perceive geraniol glucoside (n=11 and 12, respectively), as well those that could or could not perceive guaiacol glucoside (n=16 and 7, respectively). Overall, six of the panel did not perceive either glucoside, six detected guaiacol glucoside only, one detected geraniol glucoside only, and 10 detected both glucosides.

7.2.2 Factors contributing to inter-individual responses

The 23 panellists underwent a series of experiments to undercover the drivers of interindividual response to glycosides. Initially, saliva was obtained from all panellists which was used for *in vitro* hydrolysis assays as well as for microbial screening. The saliva of each individual was incubated in the presence of geraniol and guaiacol glucoside and the percentage release of the volatile determined by GC-MS (Figure 5). No significant difference was observed in the release of volatiles in saliva from people deemed as either 'taster' or 'non-tasters' of the associated glycoside.

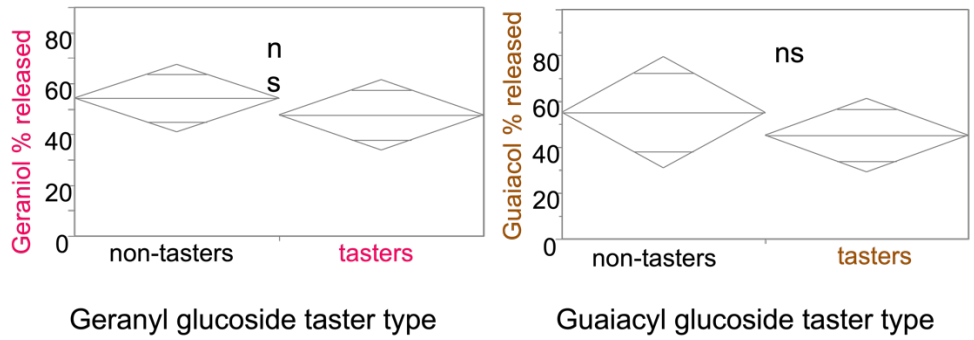


Figure 5. Percentage of volatile released from the associated glucoside during *in vitro* saliva hydrolysis assays, with panellists grouped by their ability to perceive flavour from each glycoside during screening.

Subsequently the relationship between oral microbiota, as characterised by metagenomics techniques and *in vitro* saliva hydrolysis was determined. Analysis of metagenomic data on saliva samples used in *in vitro* assays showed two main clusters among sensory panel members of microbial communities, with *Streptococcus* species dominating one cluster and *Prevotella* and *Veillonella* species dominating the other (Figure 6). Comparison of the metagenomic data with chemical data showed a strong correlation between the microbial community and *in vitro* saliva hydrolysis of glycosides. However, neither *in vitro* saliva assays nor microbial community could properly explain the differences observed between 'tasters' and 'non-tasters' in the sensory booths.

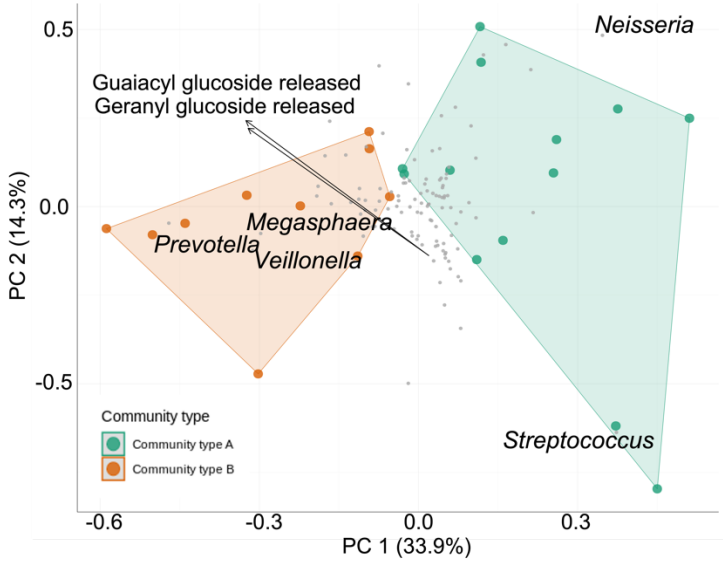


Figure 6. Principal Component Analysis biplot of genus classification for oral microbial metagenomic data (light grey dots) and panellists (orange or green dots), showing separation of microbial community types and volatile release during *in vitro* assays overlayed.

Previous classifications of people as either 'tasters' or 'non-tasters' were conducted at a single concentration of glycoside. Here, the threshold of individual panellists to the two glycosides and associated volatiles was determined to investigate drivers for the inter-individual responses.

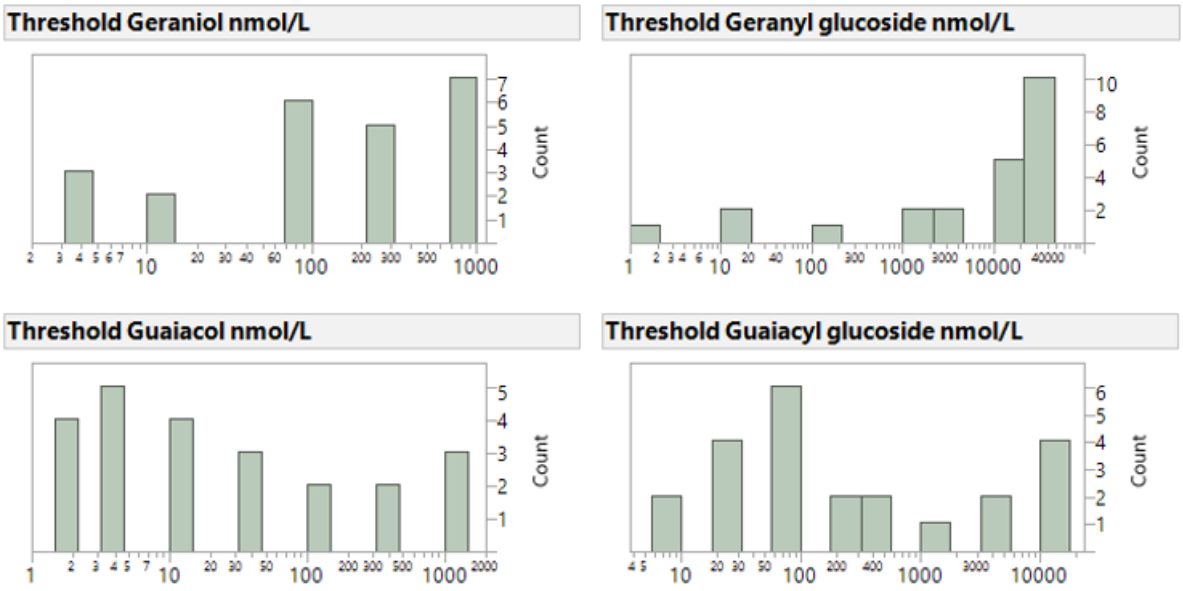


Figure 7. Detection thresholds for individual panellists as determined by a series of three alternative forced choice tests. Top left, geraniol; top right, geraniol glucoside; bottom left, guaiacol; bottom right, guaiacol glucoside.

Surprisingly, several panellists could detect the glucosides at the lowest concentrations presented. This outcome suggested that there was not a phenotype of 'tasters' and 'non-tasters' but that this was a result of the chosen concentration for the test. Some of the differences observed for glycoside perception could be explained by each individual's ability to respond to the free odorant (geraniol or guaiacol).

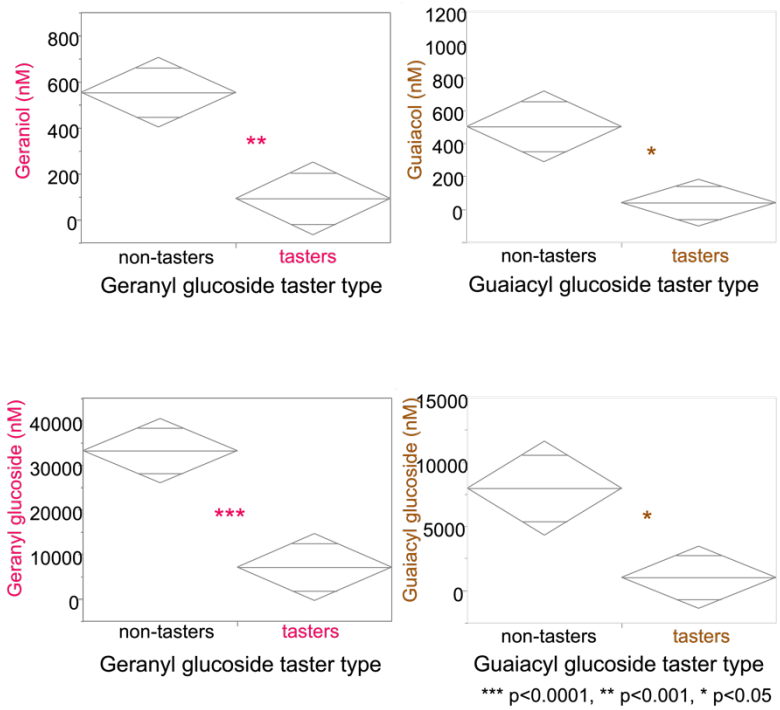


Figure 8. Best estimate thresholds for glucosides and associated volatiles, grouped by 'tasters' or 'non-tasters' of each glucoside.

7.3 Glycoside extracts during wine storage

Sections 7.3.1 through to 7.3.3 of this work have largely been detailed in Pisaniello et al. (2022). Sections 7.3.4 and 7.3.5 have been captured in Hixson et al. (2021).

7.3.1 Exploring increases in geraniol glucoside

Gordo marc collected in vintage 2019 was extracted using similar conditions to the 2016 Gewürztraminer marc, albeit with slightly less water. After an identical purification procedure, the resulting Gordo marc extract was analysed for geraniol glucoside content and then added to a commercial Chardonnay wine and stored for five months. Triplicate bottles were opened approximately every month to track the degradation of geraniol glucoside, or otherwise, and to quantify the evolution of volatile monoterpenes. As shown below, the mean concentration of geraniol glucoside in the spiked Chardonnay wines continued to increase across the first four months, before a slight decrease by the end of month five (Figure 9). The initially spiked amount of geraniol glucoside, based on the quantity in the extract, was 380 µg/L, showing an approximate increase of 56% (to 591 µg/L), slightly less than the increase observed for the 2016 Gewürztraminer extract. However, this finding supports the previous observation of an increase in geraniol glucoside in the initial months of storage, potentially being derived from a disaccharide-bound analogue.

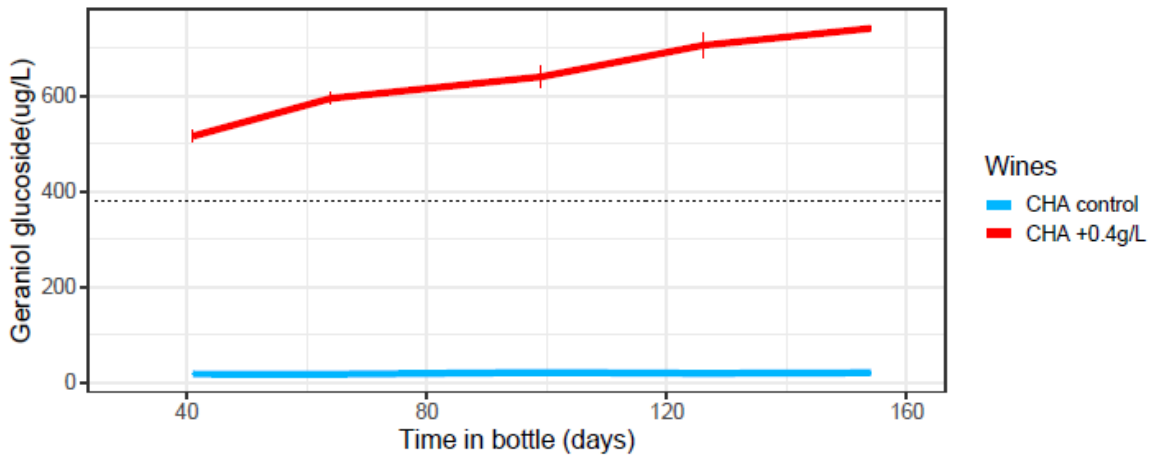


Figure 9. Geraniol glucoside concentration in Chardonnay wines over six months of storage in bottle, with and without 0.4 g/L addition of Gordo marc extract. Error bars represent standard deviation of three wines.

Monoterpenes can be found bound not just to glucose (i.e. geraniol glucoside) but also to disaccharides. Predominantly these are pentosyl glucose (PG) analogues or rhamnosyl glucose (RG) analogues. Potentially, the elimination of the pentosyl or rhamnosyl group, respectively, from these disaccharides will yield geraniol glucoside. While the increase in geraniol glucoside across the storage period was approximately 200 µg/L, this would have been accompanied by a concomitant hydrolysis, as such the increase would be significantly greater than 200 µg/L disregarding any hydrolysis. However, the analysis of the disaccharide classes of monoterpenes shows only a very minor hydrolysis of around 40 µg/L for PGs and less than 5 µg/L for RGs (Figure 10). The significant increase in geraniol glucoside with respect to the small decrease in disaccharide species can be explained by either generation of geraniol glucoside from a non-disaccharide source (i.e. a malonated species), or that the observed disaccharide classes are also being simultaneously formed and degraded to yield a much higher net hydrolysis rate than what is apparent.

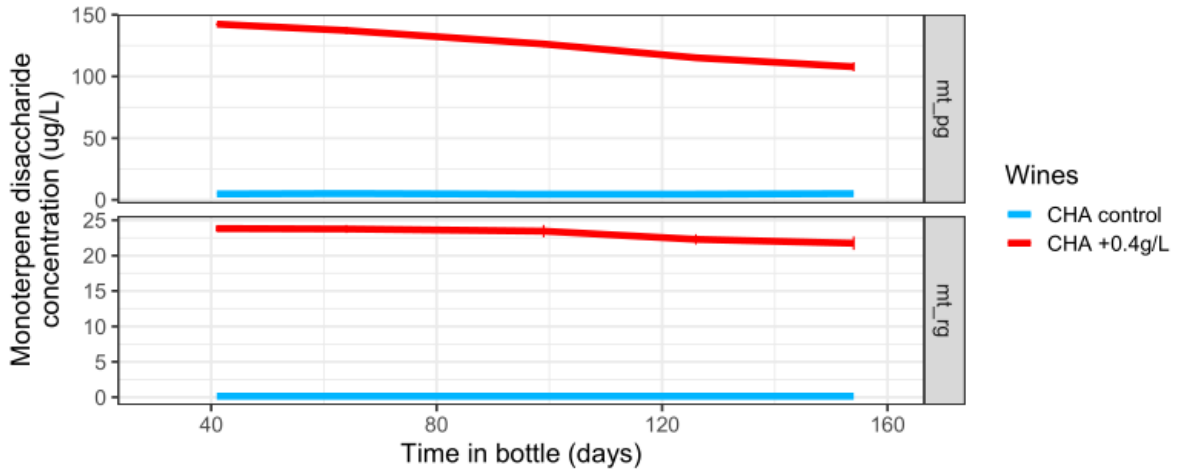


Figure 10. Monoterpene disaccharide concentration in Chardonnay wines over six months of storage in bottle, with and without 0.4 g/L addition of Gordo marc extract. Error bars represent standard deviation of three wines.

With respect to the free monoterpenes, there is a continued increase in geraniol, linalool and α-terpineol across the initial five months of storage, which aligns with the results in the 2016 storage trial, where increases continued for the first 18 months for geraniol, and across all 36 months for linalool and α-terpineol. However, in this case, the concentrations of released monoterpenes were greatly in excess of what was observed for the previous Chardonnay, although the wines only differed in pH slightly (pH 3.4 in 2016 vs pH 3.37 in 2019), and the theoretical starting concentration of geraniol glucoside was much higher in the 2016 Chardonnay (1,000 µg/L in 2016 versus 380 µg/L in 2019). Potentially the impact of spiking with an extract that originates from different varieties is causing this effect, and suggests that the forms of bound monoterpene that are currently being analysed for are not adequate for determining the aromatic potential of the extract when hydrolysed under wine conditions.

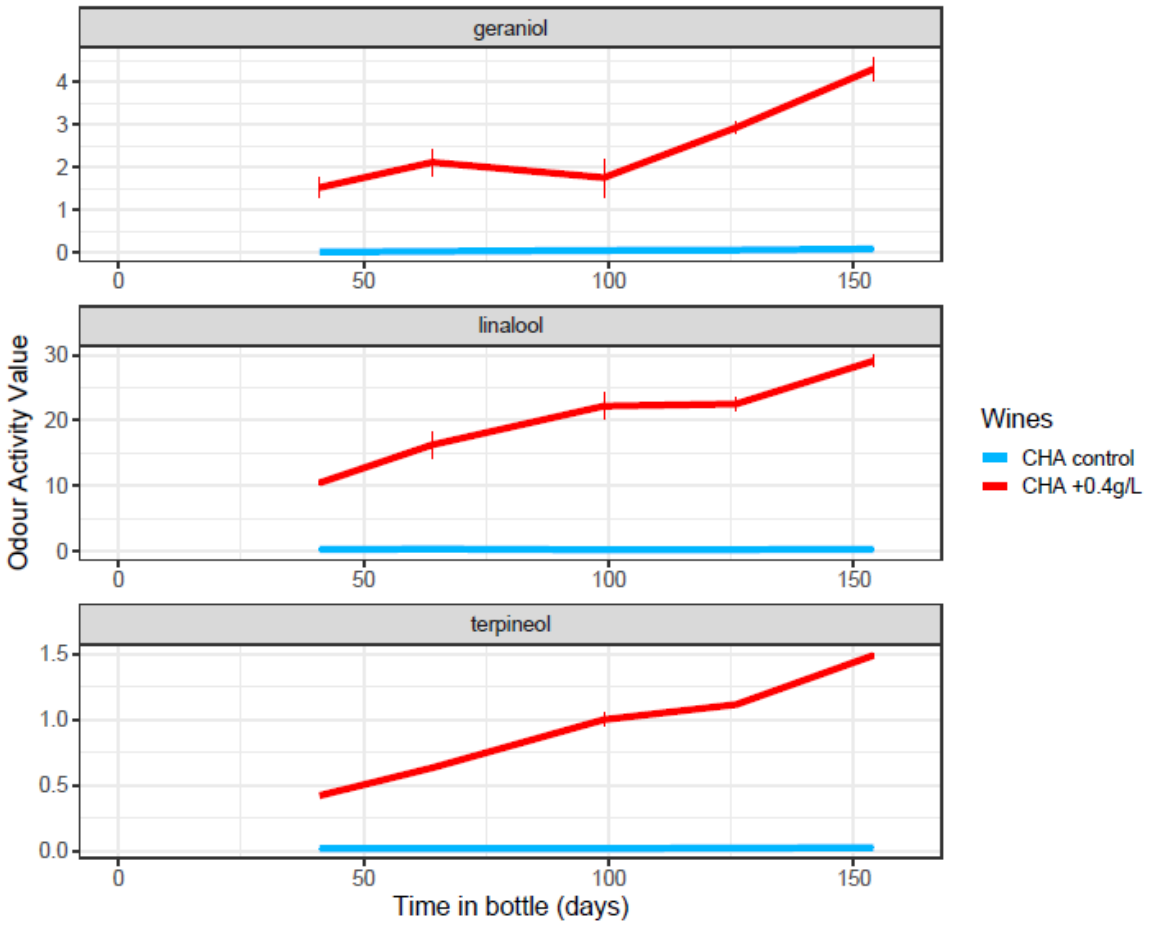


Figure 11. Odour activity values (OAVs) of key monoterpenes in Chardonnay wines over six months of storage in bottle, with and without 0.4 g/L addition of Gordo marc extract. Error bars represent standard deviation of three wines.

7.3.2 Laboratory-scale extraction of marc varieties

In vintage 2017, a number of grape samples were collected that represented floral varieties, and in the 2018 vintage samples were collected that represented non-floral varieties, according to expected monoterpene content. These samples were used to understand the impact of variety on marc extracts to be used for boosting floral aroma in winemaking. All samples were crushed and pressed within a few days of harvest, and the resulting marc and juice frozen for later analysis. The grape varieties collected were:

Floral

- Muscat à Petits Grains Blanc (White Frontignac)
- Viognier
- Riesling
- Muscat Gordo (Muscat of Alexandria)
- Gewürztraminer
- Additionally, the Gewürztraminer extract obtained in 2016 was also used.

Non-floral

- Chardonnay
- Semillon

- Verdelho
- Sauvignon Blanc
- Shiraz
- Cabernet Sauvignon

The grape marc samples were extracted in the laboratory using a standard phenol-free glycoside isolation procedure. The marc extracts and the grape juice were analysed for geraniol glucoside content, with an aim to confirm that a floral variety also contains a higher content of bound monoterpene and is not just proportionally higher in the free form.

With respect to both the extract yield per marc weight (Figure 12A) and the extract yield by grape weight (B), there was little difference. In both cases, a number of non-floral samples provided an extract yield within the 95% confidence bars of the floral varieties. However, when the extracts were analysed for geraniol glucoside, the floral varieties showed a significantly higher amount per mass of extract than the non-floral varieties (C). With respect to the ratio of geraniol glucoside in marc versus the geraniol glucoside in the juice, there appears to be a weak correlation (D).

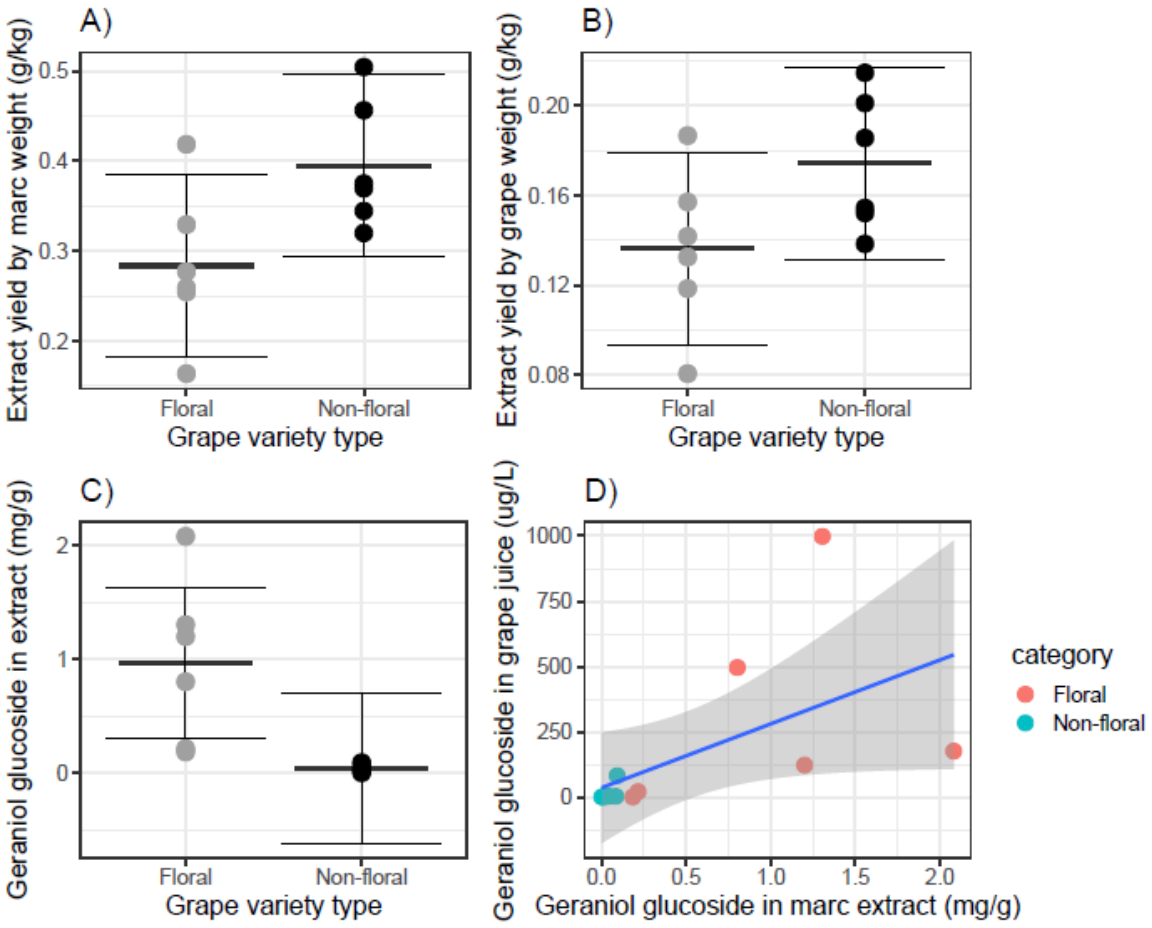


Figure 12. A) Extract yield from marc by grape type, error bars show mean +/- Tukey HSD at 95%. B) Extract yield from grapes by grape type, error bars show mean +/- Tukey HSD at 95%. C) Geranyl glucoside in marc extract by grape type, error bars show mean +/- Tukey HSD at 95%. D) Geranyl glucoside in marc extract versus in grape juice, coloured by grape type, Shading shows linear regression CI at 95%.

The above comparison between marc and juice content of geraniol glucoside does not take into consideration the minor differences in juice yield during crushing and pressing. To better account for these changes, the juice and marc yields were converted back to a total mass of geraniol glucoside in

the juice or in the extract, assuming that the geraniol glucoside present in the extract accounts for all the geraniol glucoside that was present in the marc (i.e. does not account for differences in marc extract recovery). For some grape/marc parcels, the concentration of geraniol glucoside in marc and/or juice was below the limit of detection, so calculating the ratio of marc to juice content was not possible. In the Figure 13, three samples had geraniol glucoside concentrations below the limit of detection in marc (Chardonnay) or juice (Riesling, Chardonnay and Cabernet Sauvignon). The ratio of geraniol glucoside in marc to juice varied greatly from 0.4 to 5.4, with no trends within the floral or non-floral categories, and this supports the hypothesis that the floral varieties are not categorised by simply having a higher proportion of the free form of monoterpenes over the bound form.

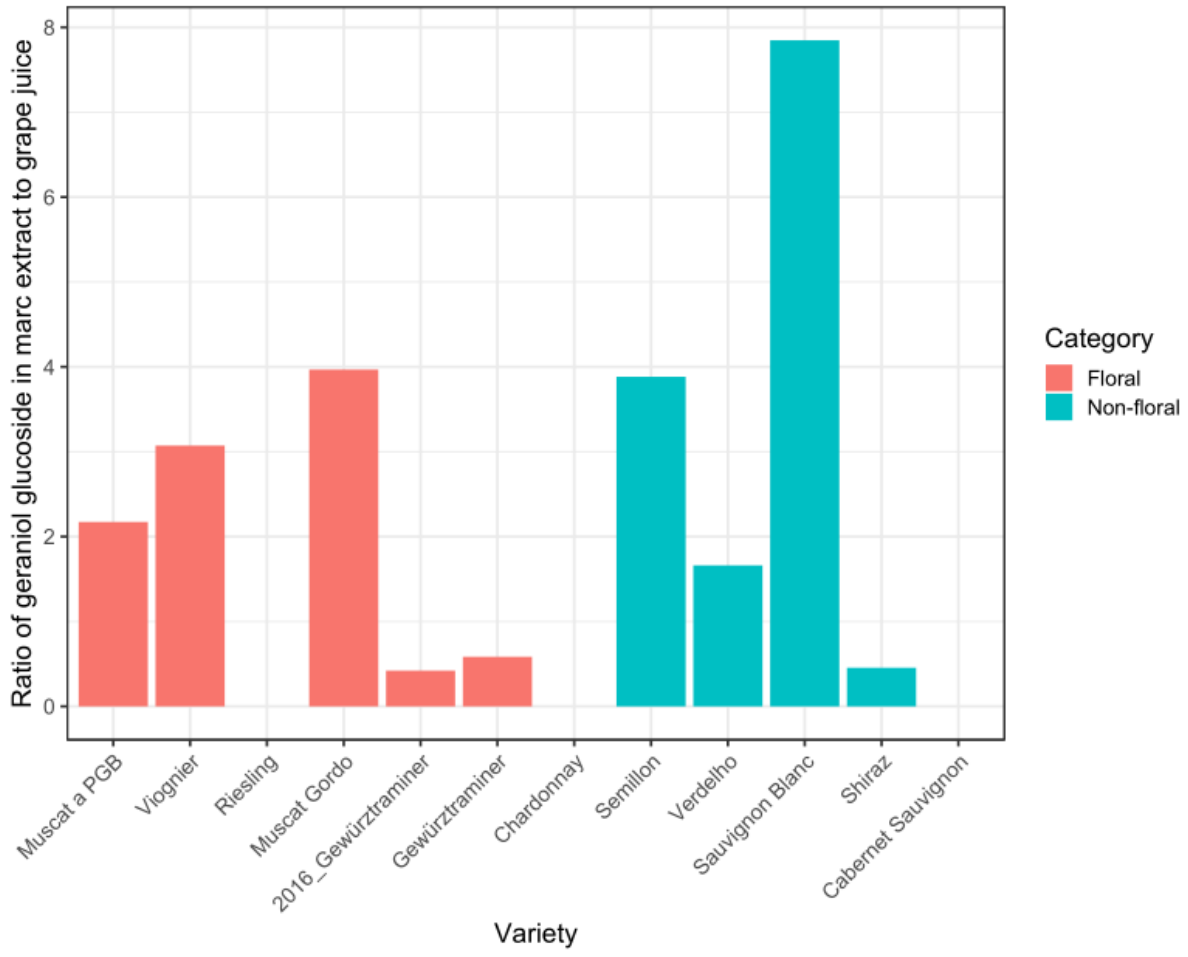


Figure 13. Ratio of total geraniol glucoside in grape marc extract (mg) to total gerianol glucoside in juice (mg), coloured by grape type (red: floral, blue: non-floral). Varieties with no bar had at least one value below the limit of detection.

With respect to the composition of the marc extracts versus that of the juices, the extracts are over-represented in geraniol glucoside when compared to the juices, apart from for the Chardonnay marc extract where geraniol glucoside was not detected (Figure 14). It appears geraniol glucoside is preferentially maintained in the grape marc when compared to the disaccharide classes, or that the extraction and isolation process which is designed to remove sugars, also acts to eliminate the higher order monoterpene-sugar analogues and effectively enriches the geraniol glucoside concentration.

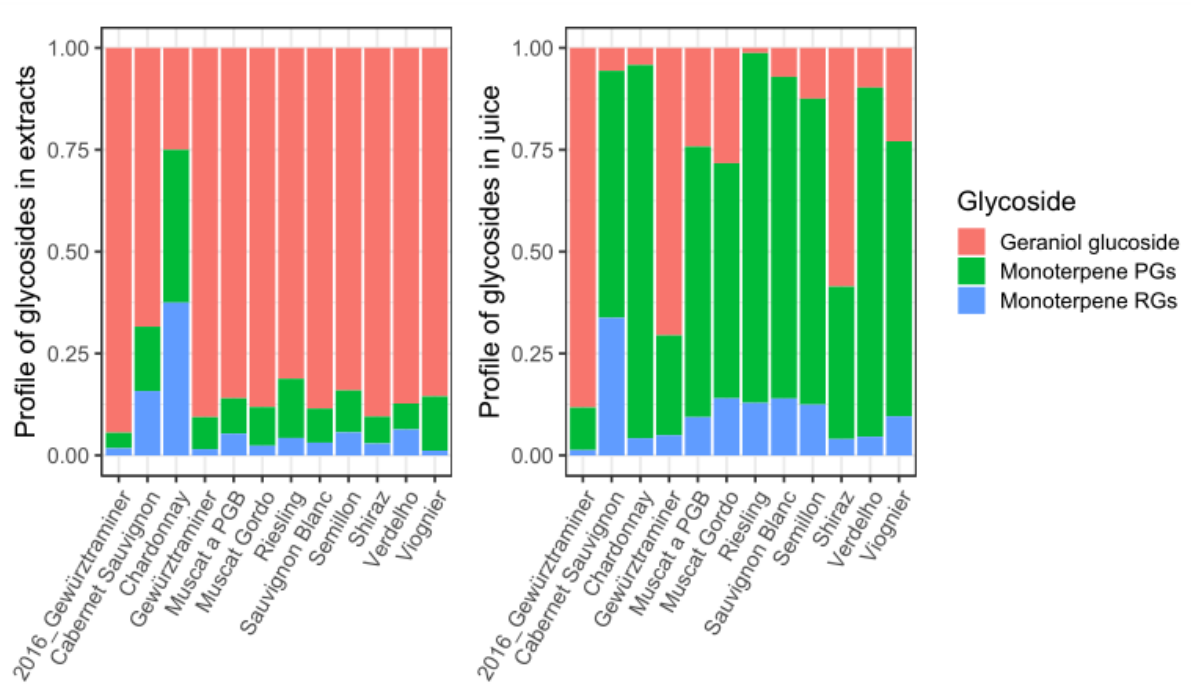


Figure 14. Profiles of glycosides in grape marc extracts and in the juice of each variety.

To better understand the aromatic potential of the marc extracts, they were subjected to acid hydrolysis followed by GC-MS analysis for a suite of monoterpenes, norisoprenoids and other volatiles of note. The outcomes were visualised via Principal Component Analysis, which showed that the Muscat Gordo extract highly influenced the model, and was dominated by the evolution of monoterpenes (linalool and α-terpineol) and some C_6-compounds (Figure 15). Interestingly, there was a grouping of samples that correlated with higher concentrations of phenolic volatiles, including 2-phenylethanol, as well as a strong influence of β-damascenone.

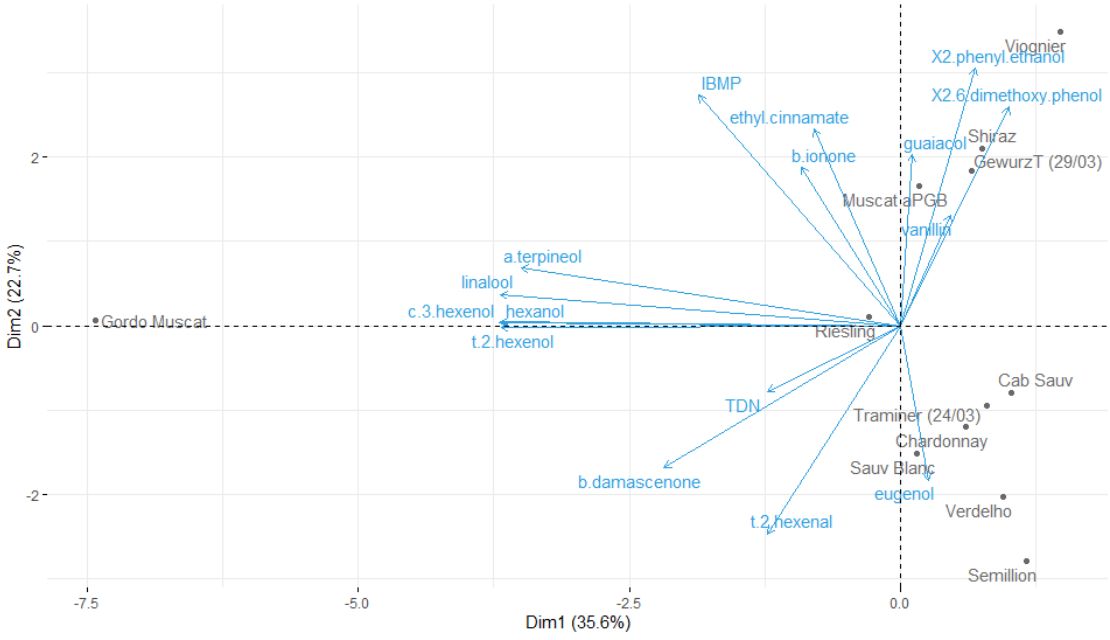


Figure 15. PCA biplot of volatiles created via hydrolysis of marc extracts from different grape varieties.

When the scores plot is visualised with sample type (floral or non-floral), including the 95% confidence ellipses, there is some separation of type, but with some overlap of the floral and non-floral ellipses (Figure 16). However, the use of acid hydrolysis is known to induce changes in the volatile profile that are not representative of the final wine concentrations. As such, this is simply a guide to the classes of compounds that could be generated during storage in wine. It was performed here to provide a comparison with future wine storage to better understand how acid hydrolysis could be used to predict volatile evolution.

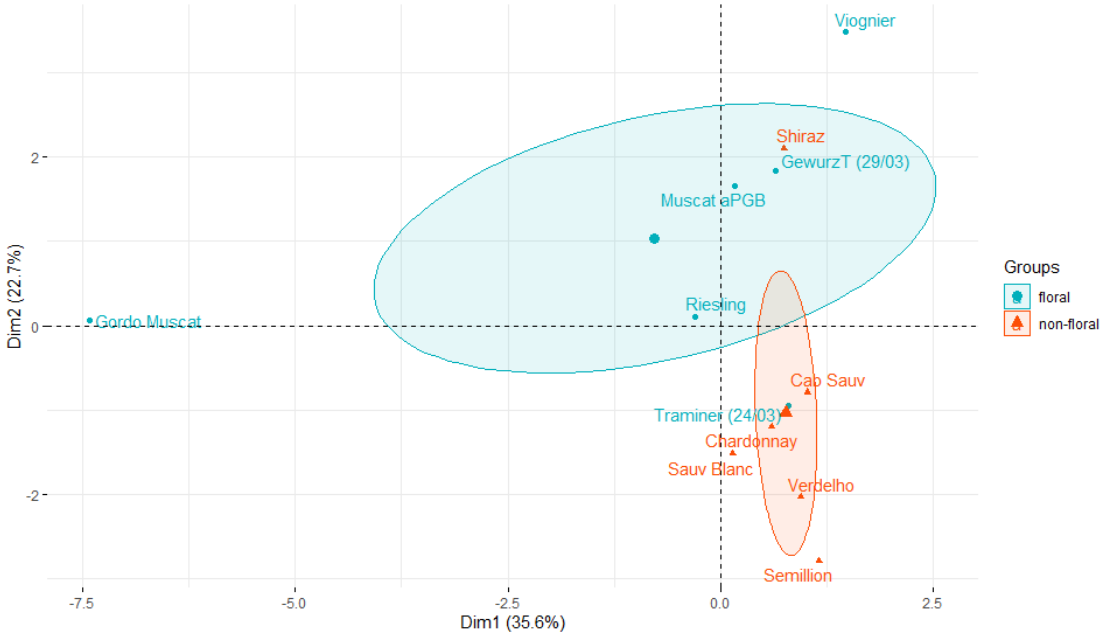


Figure 16. PCA scores plot of marc extracts volatile compound production, showing 95% confidence ellipses for floral varieties versus non-floral varieties.

With respect to the absolute values of volatiles produced from hydrolysis of the grape marc extracts, the floral varieties produced 500 – 5,000 µg/L of the analysed volatiles, largely dominated by 2-phenylethanol, α-terpineol and linalool (Figure 17). For the non-floral varieties, there was a notable decrease in the evolution of volatiles ranging from around 400-650 µg/L and showing a larger proportion of β-damascenone and TDN.

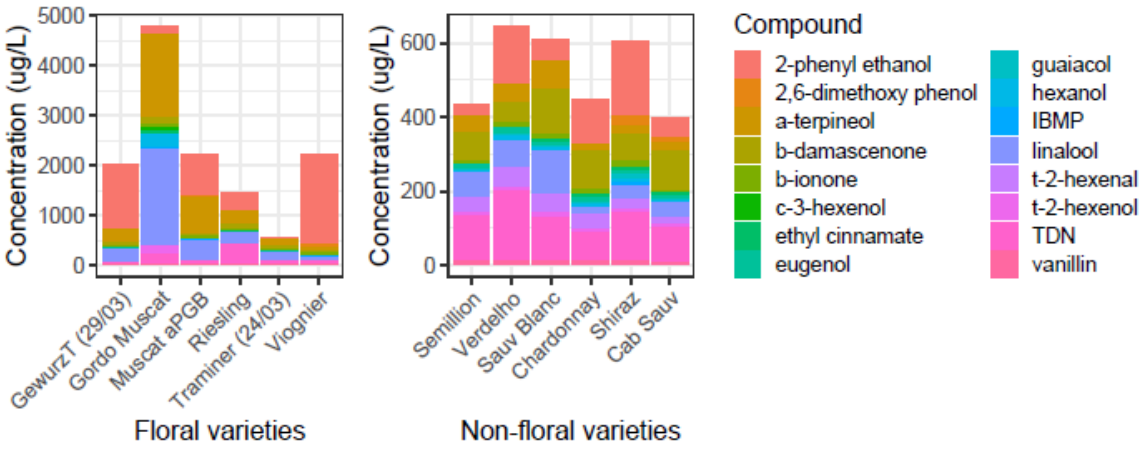


Figure 17. Concentration of volatiles in hydrolysates of grape marc extracts of different varieties.

7.3.3 Varietal marc extracts in wine after six months

The marc extracts were spiked into a commercially available Chardonnay wine, with all extracts spiked at 0.4 g/L regardless of aromatic potential. The spiked wines were bottled in triplicate and stored for six months to assess the evolution of volatiles from different marc varieties. Firstly, the wines were assessed for geraniol glucoside content remaining after the six months of storage to observe how these wines aligned with previous observations of increasing geraniol glucoside content after the first few months in wine (Figure 18). The absolute geraniol glucoside concentration in the wines was much higher for the floral varieties, which was expected based on the previous analysis of content in the extract. However, all wines showed a higher geraniol glucoside concentration than the expected spiked amount (indicated by a black point).

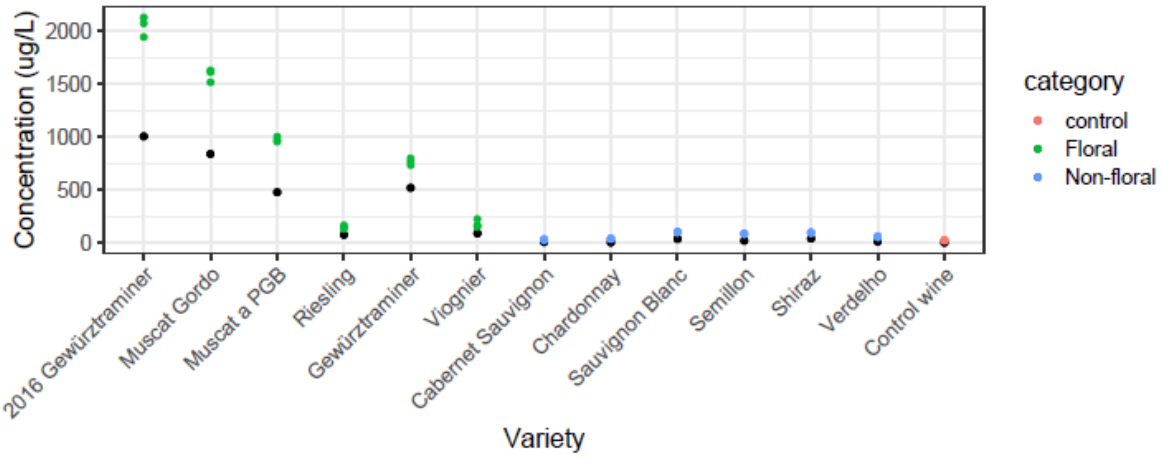


Figure 18. Concentration of geraniol glucoside in marc extract spiked wines after six months of storage. Black points show the theoretical spiked amount.

With respect to the volatile evolution over six months of storage, the Gordo extract and the Muscat à Petits Grains Blanc (PGB) extract provided a much larger increase in total volatile concentration, with an aggregated volatile concentration of over 3,500 µg/L and 1,000 µg/L, respectively (Figure 19). As expected from the acid hydrolysis, where the Gordo extract produced significant quantities of monoterpenes, after storage the volatile profile of the wines spiked with Gordo (and Muscat PGB) were dominated by geraniol, linalool and α-terpineol. All wines spiked with floral varieties had a final volatile concentration of above 100 µg/L (Viognier, 416; Riesling 350; Gewürztraminer 102 µg/L), except for Gewurz_2 (average of 53 µg/L). For the non-floral varieties, only the volatile evolution of the Verdelho spiked wine exceeded the lowest floral sample (62 versus 53 µg/L), with the remainder of the wines at or below 40 µg/L. As such, the volatile evolution did follow the expectations created by the designation of varieties into floral and non-floral, to some extent, especially with respect to the relative proportions of volatiles produced (Figure 20). This also implied that an extract produced from an aggregated source would dilute out the benefits of the highly floral varieties, and any efforts to obtain large quantities of marc extract for flavour additions should be made from varietally pure sources.

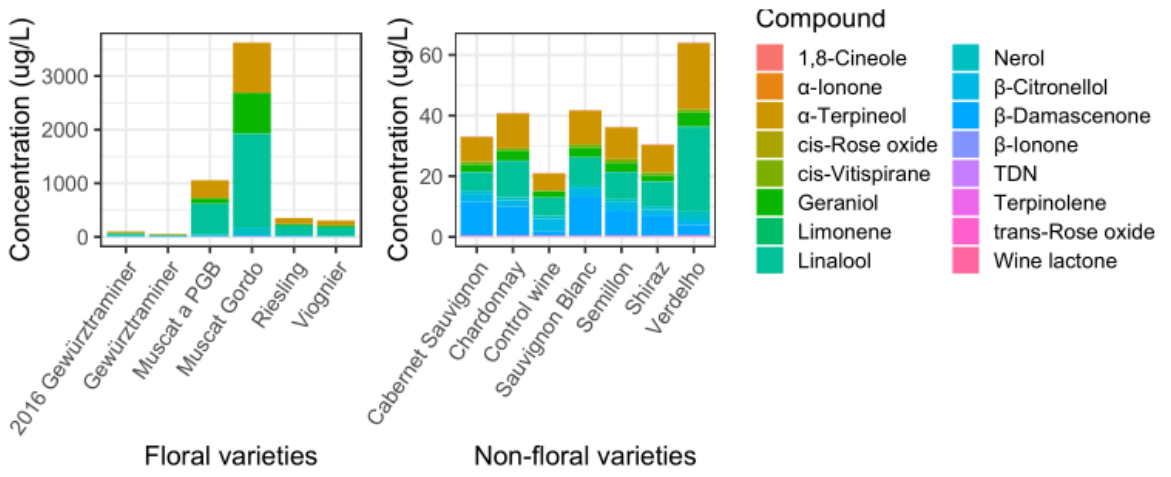


Figure 19. Concentration of selected volatiles in Chardonnay wines spiked with 0.4 g/L of extract from marc of different varieties, after six months of storage in bottle.

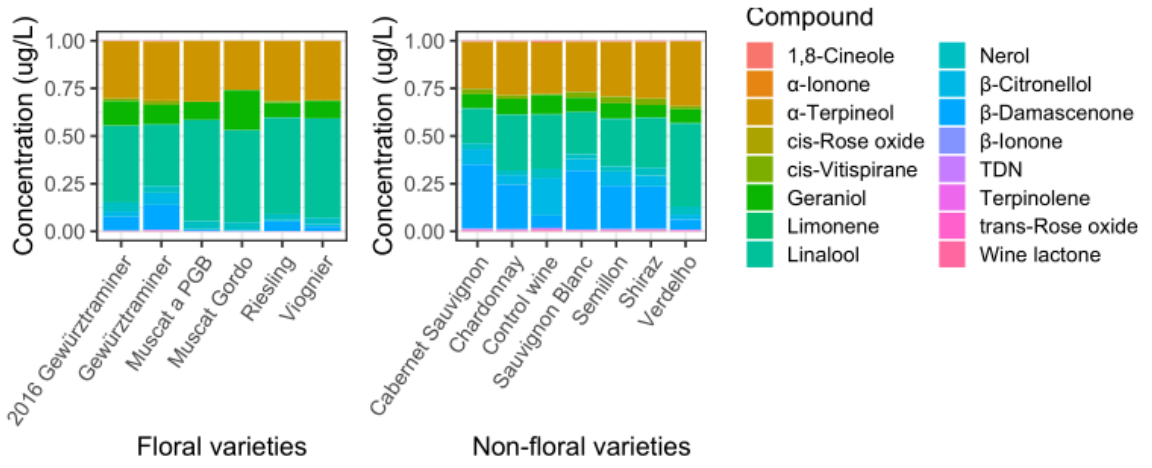


Figure 20. Percentage profile of selected volatiles in Chardonnay wines spiked with 0.4 g/L of extract from marc of different varieties, after six months of storage in bottle.

Mirroring the Principal Component Analysis of the acid hydrolysis outcomes, the volatile evolution during storage was influenced strongly by the wine spiked with Muscat Gordo extract, and the release of monoterpenes (Figure 21). However, in this instance, the wines spiked with Riesling extract and Muscat PGB extract were dominant in PC2, being correlated with norisoprenoid formation and rose oxide formation, respectively. Furthermore, all wines were separated from the control wine to some extent, so even if the addition of an extract had minimal impact, it still provided some chemical adulteration to the wine.

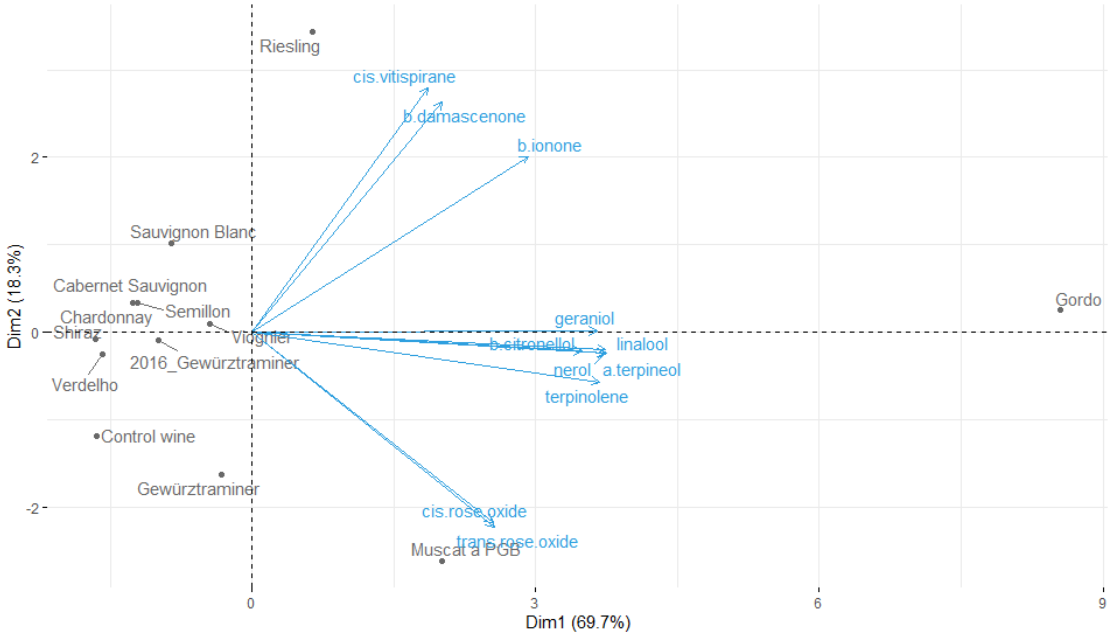


Figure 21. PCA biplot of volatile compound profile in Chardonnay wines spiked with marc extracts from different varieties, after six months of storage

Figure 21 includes the amount of volatiles produced as well as the composition. In addition, the relative proportions of volatiles have been calculated and analysed by PCA (Figure 22). This was performed to see if bulking grape marc types together might be a useful mechanism to provide similar aroma profiles, regardless of the magnitude of flavour provided.

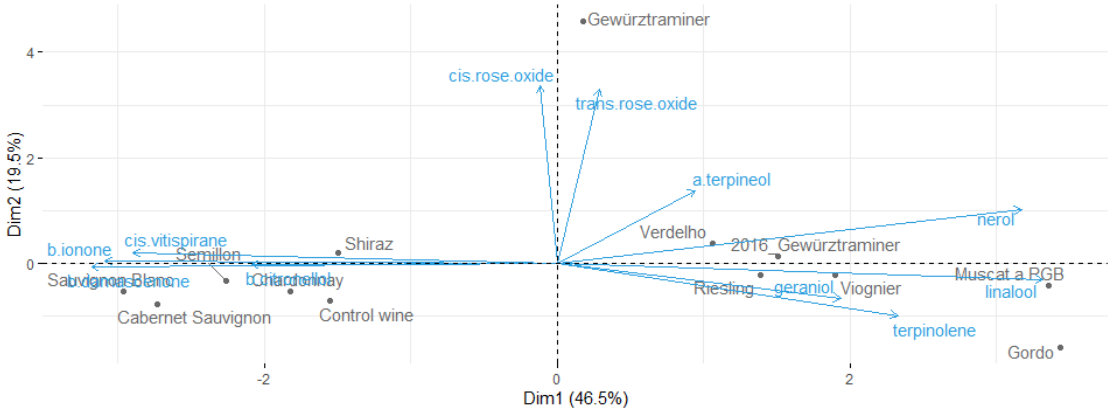


Figure 22. Compositional profile of the proportions of selected volatiles in Chardonnay wines spiked with 0.4 g/L of extract from marc of different varieties, after six months of storage in bottle.

In this plot, PC1 accounted for nearly 50% of the variation in the compositional profiles and was the main driver of separation between the floral varieties on one side, and the non-floral on the other. This was largely due to correlations with monoterpenes, nerol and linalool, at one end, and higher proportions of norisoprenoids at the other end.

To assess the sensory impact of the marc extracts on the base Chardonnay wine, the three storage replicates were combined into a single wine to limit sample numbers, and the wines assessed via 'Napping', a rapid method whereby wines are placed on a two-dimensional space based on perceived similarities or differences. While the sensory outcomes were not definitive, they did

support the increased 'floral' character in the two wines that contained either Muscat Gordo or Muscat PGB extracts.

Importantly, these wines were assessed holistically in clear glasses, with comments relating to the colour or opacity of the wines being quite important. Notably, the wines treated with extracts from red varieties (Shiraz and Cabernet Sauvignon) possessed a light red colour, while other extracts resulted in a hazy appearance of the wines.

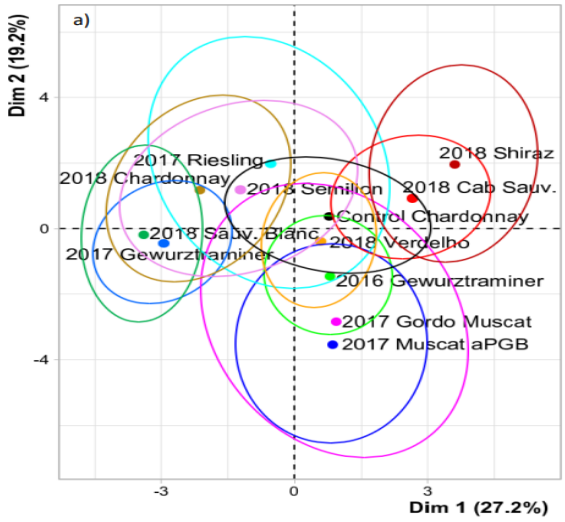


Figure 23. Consensus multiple factor analysis (MFA) map for dimensions 1 and 2, with the 95% confidence ellipse for each wine shown. The 2017 Viognier 95% confidence ellipse covered a large area of and was removed to provide a clearer figure.

Dimensions 1 and 2 of the consensus map explained 46.4% of the variance in the data (Figure 23). Given many overlapping ellipses, the sensory differences between the wines were relatively small. However, there were sufficiently large distances between some of the wines to show that there was a sensory effect. The 2017 Muscat Gordo and 2017 Muscat PGB samples were mapped closely together, vertically separated from the control Chardonnay to the bottom right of Figure 24, and were mapped close to the 2016 Gewürztraminer. Considering the descriptors used compared with the control Chardonnay, these samples were described more frequently as citrus, stone fruit, floral, rose, herbal and honey. Additionally, the 2017 Muscat PGB was noted less frequently for acidity but more for sweetness. The most obvious separation of the samples was on the basis of appearance, with the wines treated with Cabernet Sauvignon and Shiraz extracts plotted to the upper right section of Figure 24 and the samples described as 'cloudy' or 'yellow' positioned to the middle left of the figure.

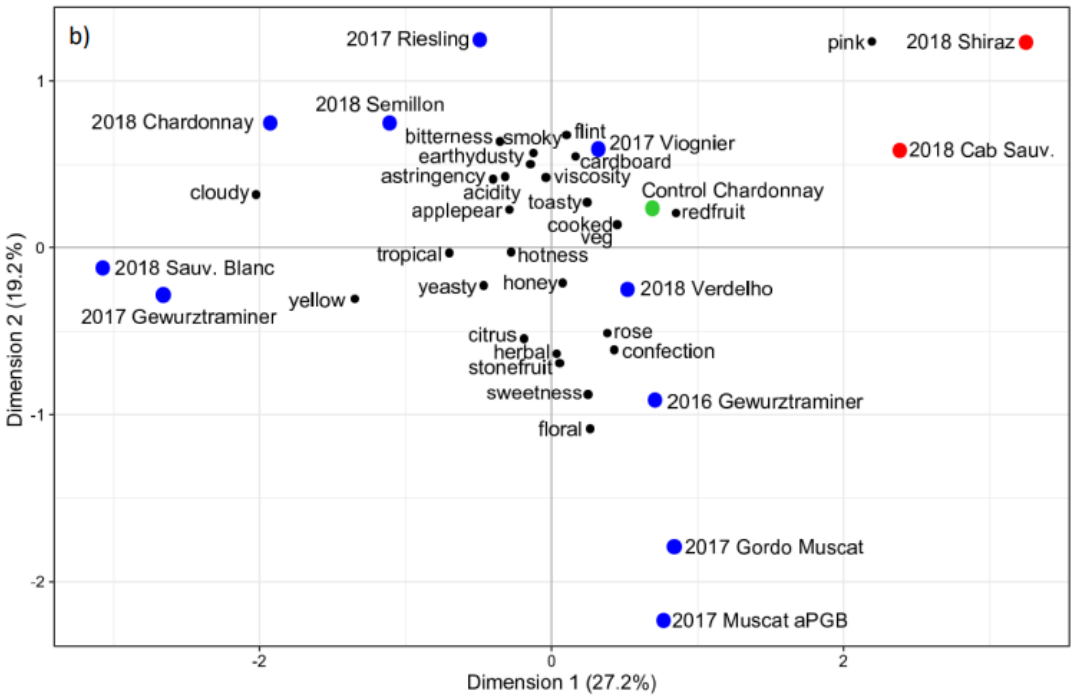


Figure 24. Consensus MFA map for dimensions 1 and 2, with the descriptors from ultra-flash profiling (UFP) superimposed

The samples treated with 2018 Shiraz and 2018 Cabernet Sauvignon marc extracts were closely mapped together at the top right of the figure, both vertically and horizontally separated from the control Chardonnay which was centrally positioned. Considering the descriptors used compared with the control Chardonnay, these samples were rated more frequently for pink (colour), cardboard, cooked vegetal, earthy/dusty and red fruit characters as well as bitterness. The wine treated with 2018 Shiraz marc extract was also described with a higher frequency for flint and viscosity and lower for tropical and confection. The wine treated with 2018 Cabernet Sauvignon marc extract was noted frequently for floral and astringency. The wine treated with 2017 Riesling extract was mapped at the top middle of the figure, vertically separated from the control Chardonnay. Considering the descriptors, this sample was associated with citrus, yeasty, earthy/dusty, smoky, bitterness and viscosity descriptors.

The samples treated with 2016 and 2017 Gewürztraminer extracts were not mapped closely together, with the samples being vertically and horizontally separated from the control Chardonnay, respectively. The 2016 Gewürztraminer extract-treated sample was associated with stone fruit, confection, yeasty, bitterness, sweetness and viscosity while the 2017 Gewürztraminer extract-treated sample was described more similarly to the control Chardonnay, whilst additionally being noted more frequently for yellow (colour), cloudy, tropical, earthy/dusty, acidity and viscosity. The samples treated with 2018 Chardonnay and 2018 Sauvignon Blanc extracts were also closely mapped with the 2017 Gewürztraminer extract-treated sample but were noted more frequently as floral, yeasty, cardboard, astringency and bitterness.

In conclusion, the additions of Muscat Gordo and Muscat PGB marc extracts had significant impacts on the volatile composition of the wines, including the desired increase in monoterpenes. This chemical change was related to a tendency for the sensory panel to note an increase in fruity/floral characters in these wines, again supporting the need to create marc extracts from a varietally uniform source, rather than a homogeneous aggregated marc pile.

7.3.4 Longevity of flavour in wines

In the initial analysis of these wines, the additions of glycoside extract had similar effects regardless of whether the addition was made pre- or post-fermentation, only really differing by amount/dose of addition (0.4 versus 0.8 g/L) and variety (Chardonnay versus Riesling, Figure 25). As such, within each dose rate and variety, the duplicate treatments from each timing were aggregated across addition timing. Each analysis point shown is the combination of four single analyses across duplicated treatments and duplicated addition doses. Furthermore, the volatile analysis outcomes have been converted to odour activity values (OAVs) to better compare across the vastly different thresholds of the key monoterpenes.

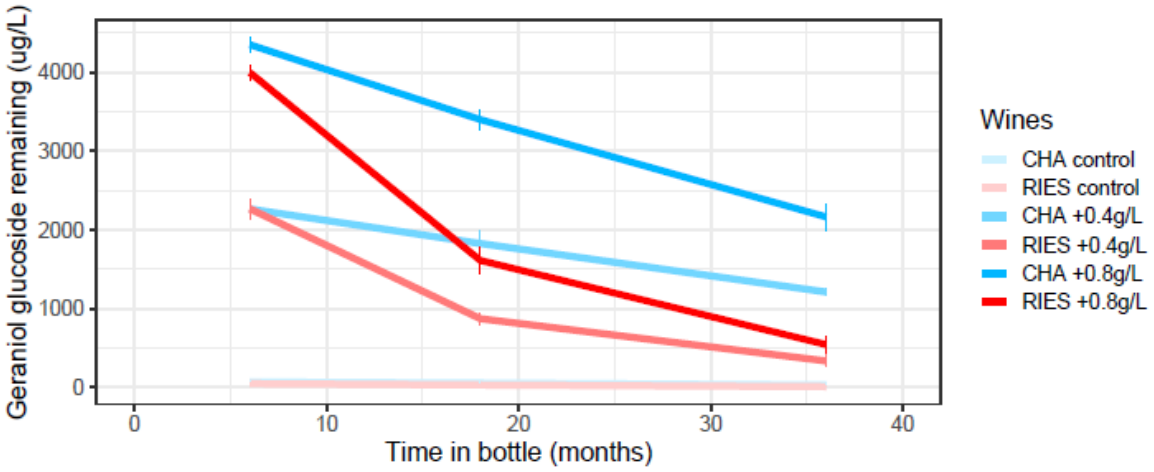


Figure 25. Geraniol glucoside concentration in Chardonnay (pH 3.4, blue lines) and Riesling wines (pH 3.1, red lines) over three years of storage in bottle, with either 0.4 g/L or 0.8 g/L addition of Gewürztraminer marc extract. Error bars represent standard the deviation of four wines.

Within each variety, the pattern of geraniol glucoside degradation across three years of storage was nearly identical regardless of the amount added to each wine type. For the higher pH Chardonnay (pH 3.4), there was a slow and even degradation from the four month time point until the 36 month time point, with the 0.4 g/L and 0.8 g/L additions both containing about 50% of the total geraniol glucoside compared to what was present at the first analysis time point. However, the Riesling wine (pH 3.1) showed a much more rapid degradation in geraniol glucoside, with the concentration reducing by over 50% from month 4 to month 18, and then a slower reduction to the 36 month time point.

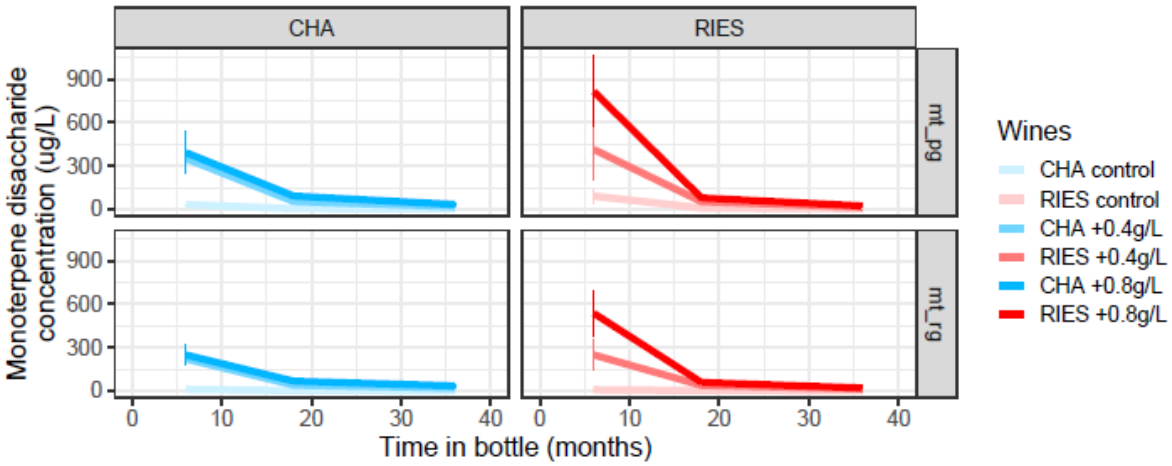


Figure 26. Monoterpene disaccharide (PGs or RGs) concentration in Chardonnay (pH 3.4, blue lines) and Riesling wines (pH 3.1, red lines) over three years of storage in bottle, with either 0.4 g/L or 0.8 g/L addition of Gewürztraminer marc extract. Error bars represent standard the deviation of four wines.

For the Riesling wines, the concentration of monoterpene PGs or RGs at the first analysis timepoint (six months) followed the expected pattern of being twice as high in the 0.8 g/L addition as in the 0.4 g/L addition (Figure 26). For both classes of compounds, a rapid reduction in concentration was observable for the time period from six to 18 months. The same decrease was observed in the Chardonnay wines, but in this case, the concentrations at the first analysis point did not reflect the differences in spiked extract amounts.

With respect to the volatile evolution across those 36 months, the pattern of monoterpene evolution between the 0.4 g/L and 0.8 g/L additions was very similar, mimicking that of the geraniol glucoside degradation (Figure 27). As such, only the 0.4 g/L addition is shown to simply the interpretation of the volatile data. The pH differences between the two sets of wines appeared again to be driving the main differences in compositional changes. The Riesling wines (red lines) showed a continued degradation of geraniol from the first analysis time point, whereas the higher pH Chardonnay wines increased in geraniol for up to 28 months, before degrading. Likewise for linalool, there was a constant degradation in the Riesling wines, while the Chardonnay wines appeared to still be accumulating linalool after 36 months, albeit very slowly. The α-terpineol concentrations continually increased for both sets of wines, although this occurred much more rapidly in the lower pH Riesling wines during the initial months of storage.

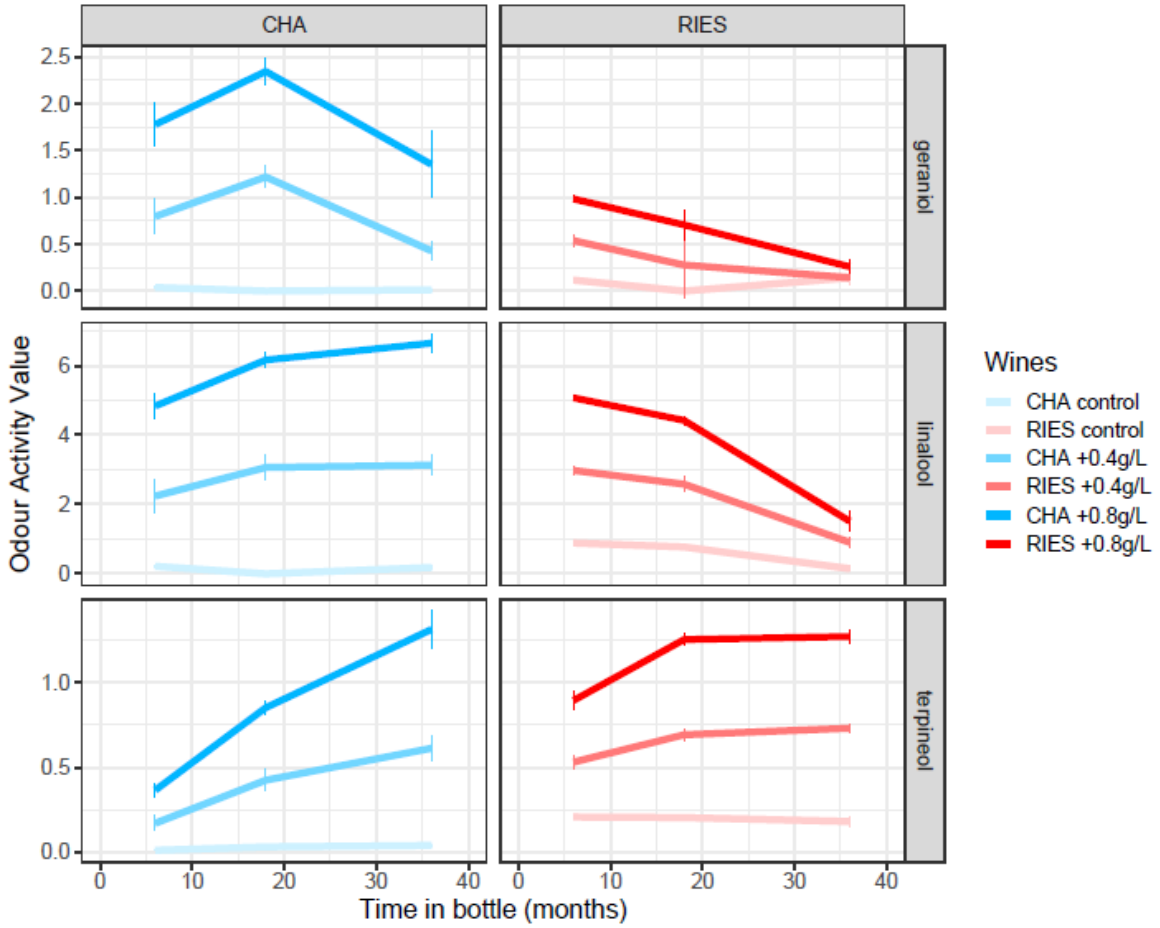


Figure 27. Odour activity values (OAVs) of key monoterpenes in Chardonnay (pH 3.4, blue lines) and Riesling wines (pH 3.1, red lines) over three years of storage in bottle, with and without 0.4 g/L addition of Gewürztraminer marc extract. Error bars represent standard deviation of four wines.

7.3.5 Structure and breakdown kinetics

**Figures in this section have been reproduced from Hixson et al. (2021) under a CC-BY license.*

For the addition of glycoside-rich extracts to be useful in winemaking, the evolution of flavour (or shelf-life) needs to be predictable. As the above trial showed a distinct difference in the evolution of flavour with a pH difference of 0.3 over three years of storage, more needed to be done to understand the speed of flavour evolution.

Glycoside breakdown is reported to occur via a first-order reaction. In support of this, the natural logarithm of geraniol glucoside concentration in the wines over three years follows the expected linear trend (Figure 28). However, the rate constant given at each pH (by the y-intercept) differed, suggesting that a first-order breakdown reliant on concentration of reactant alone was not sufficient, but also relied on pH.

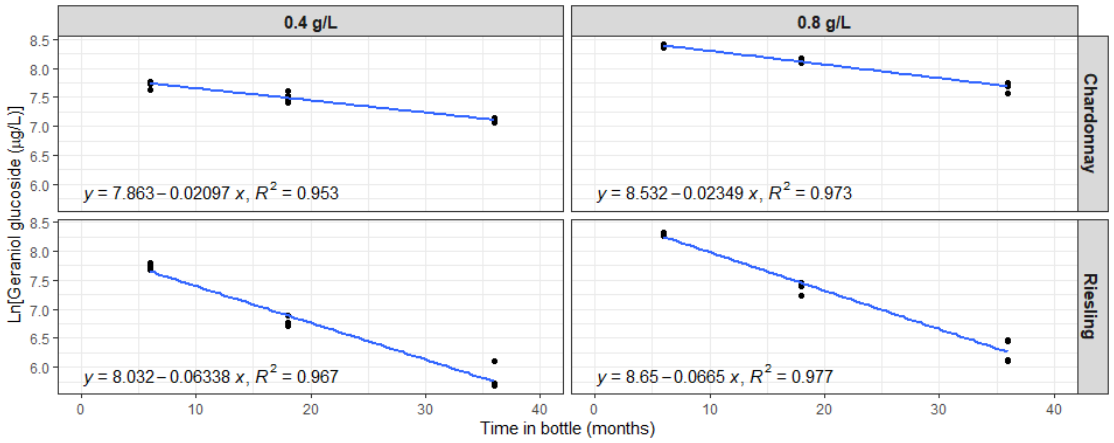


Figure 28. Natural logarithm of geraniol glucoside concentration in Chardonnay (pH 3.4) or Riesling wines (pH 3.1) over three years of storage in bottle, with either 0.4 g/L or 0.8 g/L addition of Gewürztraminer marc extract.

A mechanistic investigation of monoterpene glycoside breakdown and reaction intermediate stability via computational chemistry provided evidence that there is an equilibrium between the neutral and protonated form of the glucoside. This equilibrium is pH-dependent and determines the concentration of the true reactant, protonated geraniol glucoside (see Hixson et al. 2021 for full mechanistic details). As such, future kinetic investigations into this breakdown should first determine the equilibrium constant of protonation to predict geraniol glucoside breakdown at different pH values. However, given the rate constants shown above, the half-life of reaction was estimated to be 11 or 31 months at pH 3.1 or 3.4. At those given half-lives, the wines possessed more similar volatile profiles, suggesting that reaction kinetics could be a useful tool in shelf-life determination (Figure 29).

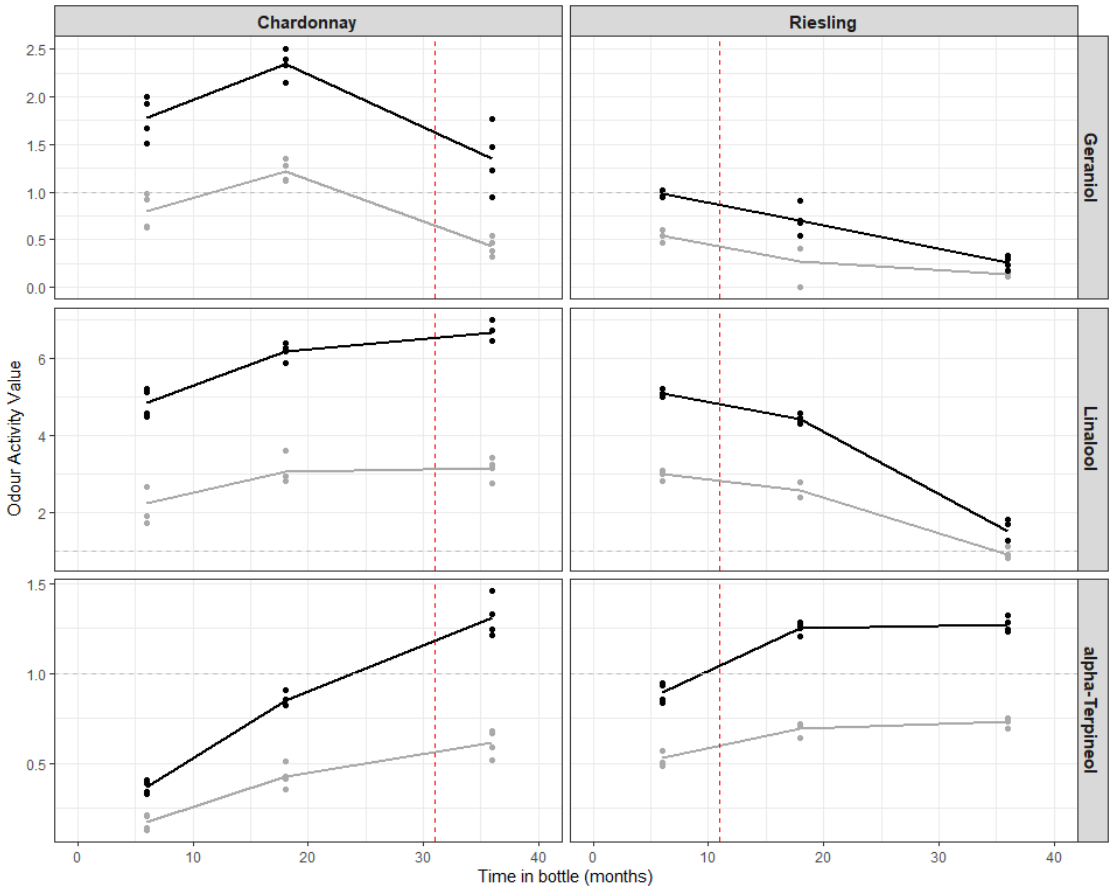


Figure 29. Odour activity values (OAVs) of key monoterpenes in Chardonnay (pH 3.4) and Riesling wines (pH 3.1) over three years of storage in bottle, with either 0.4 g/L (grey lines) or 0.8 g/L (black lines) addition of Gewürztraminer marc extract. Points represent the value of the four individual wines. Dotted red lines represent the reaction half-life at each pH.

7.4 Industry consultation for glycoside use in winemaking

Using the Chardonnay and Riesling wine that had additions of Gewürztraminer marc extracts, a workshop was conducted with staff from Treasury Wine Estates (TWE), Pernod Ricard Winemakers (PRW) and Accolade Wines. The wines presented had been approximately 12 months in bottle at the time of the tasting. The project outcomes to date were presented followed by a tasting of research wines with added glycosides. The response from industry personnel was positive, with interest in commercial use of grape marc-derived flavour additives if it were commercially available.

Subsequently, the potential use of marc-derived flavour extracts was taken through the pre-commercialisation program, CSIRO *ON Prime*. Project team members, along with additional AWRI and Wine Australia staff, studied barriers to uptake of glycoside extracts as flavour agents and where they fit into the life of a winemaker.

In conversations with the rather broad 'wine producer' group it became clear very quickly, that although the extraction and purification of marc extracts was designed to be achieved in a winery setting, that there was no interest from winemakers to perform this additional task. Immediately this suggested that the process of extract production could be performed on an aggregated marc feedstock at a centralised location, rather than in-house at a winery from marc that they created. However, it was also obvious that regardless of the segment that the winery or winemaker was in within the sector (small, medium, large, winery owned vineyards, grape purchaser etc.), that the use of additives was acceptable and regularly used, even if simply for 'finishing' wines. Most had no significant aversion to using additives.

From there, the 'wine producer' end-user group was broken down to into segments to obtain clarity on the information that was being gathered. This could be done by size, distribution channels, relationship to the grapes (own vineyard or purchased grapes), philosophy, or combinations of these. For example, when it came to the question of modulating wine flavour to meet a certain style, there was a segment that was more likely to prefer to let the wine reflect the vineyard and the given vintage conditions. For these people, wine with vintage-to-vintage consistency was not a goal, and as such they would not be compelled to use flavour enhancement simply to meet stylistic targets. However, even within this segment there were a few comments regarding the influence of distribution avenues, with wines going through a distributor needing to be more consistent with a promised style, while those with direct-to-customer avenues appreciated being able to tell the story of the vintage through the wine.

Those more likely to desire flavour modulation to meet stylistic targets were large, commercial winemakers. The segment set to gain the most from flavour modulation was the small but rapidly growing low- and no-alcohol production space. However, when it came to modulating flavour in commercial wine, producers expressed very strict upper limits on additive costs. While the extraction of grape marc and purification of the liquid extract was simple and imposed a relatively low additional cost, the energy-intensive mode of concentrating the extract meant that the cost of production exceeded that upper limit by a significant amount.

7.5 Glycosides in no- and low-alcohol matrices

The outcomes of sector consultation, especially the *ON Prime* program, highlighted the growing need for flavour manipulation in no- and low-alcohol wine products (NOLO). Glycoside extracts have been shown to provide flavour via two mechanisms, firstly via in-mouth release and immediate perception when tasting, and secondly via slow hydrolysis and volatile generation in bottle.

To examine the first of these possibilities, the Gordo marc extract was used in NOLO product tastings alongside and/or combined with other suspected enhancers. The entirety of these tastings is reported under Project 3.1.10, but in short, the Gordo marc glycosides were considered useful in providing a more wine-like product, especially when used alongside proline additions. The slow release of flavour was noted to add to the length of flavour of the product being tasted.

For the second of these mechanisms, the biggest question was whether the aggregated knowledge on glycoside use in winemaking could transfer directly into NOLO products. For this, the Gordo marc extract was added to wines and model wines determined to be of interest to NOLO production and stored for several months to understand any difference in hydrolysis and volatile production when ethanol concentrations are altered.

The wines were analysed for volatile and non-volatile monoterpenes, and in all cases, the concentration of volatile monoterpenes showed an expected increase (Figure 30). This was consistent with the findings for full-strength wines, and the model wines appeared to perform similarly to the commercial Chardonnay. However, the concentration of geraniol glucoside in the model wines did not perform as expected, showing a gentle decline in all model wines regardless of alcohol concentration. The commercial Chardonnay showed an analogous increase in geraniol glucoside to that previously noted in full-strength wines.

The inconsistency between what was observed in full-strength wines and in the <0.5% ABV Chardonnay versus that in the model wines suggests that model systems may not be suitable for understanding glycoside transformations, even though the volatile profile appeared to be consistent.

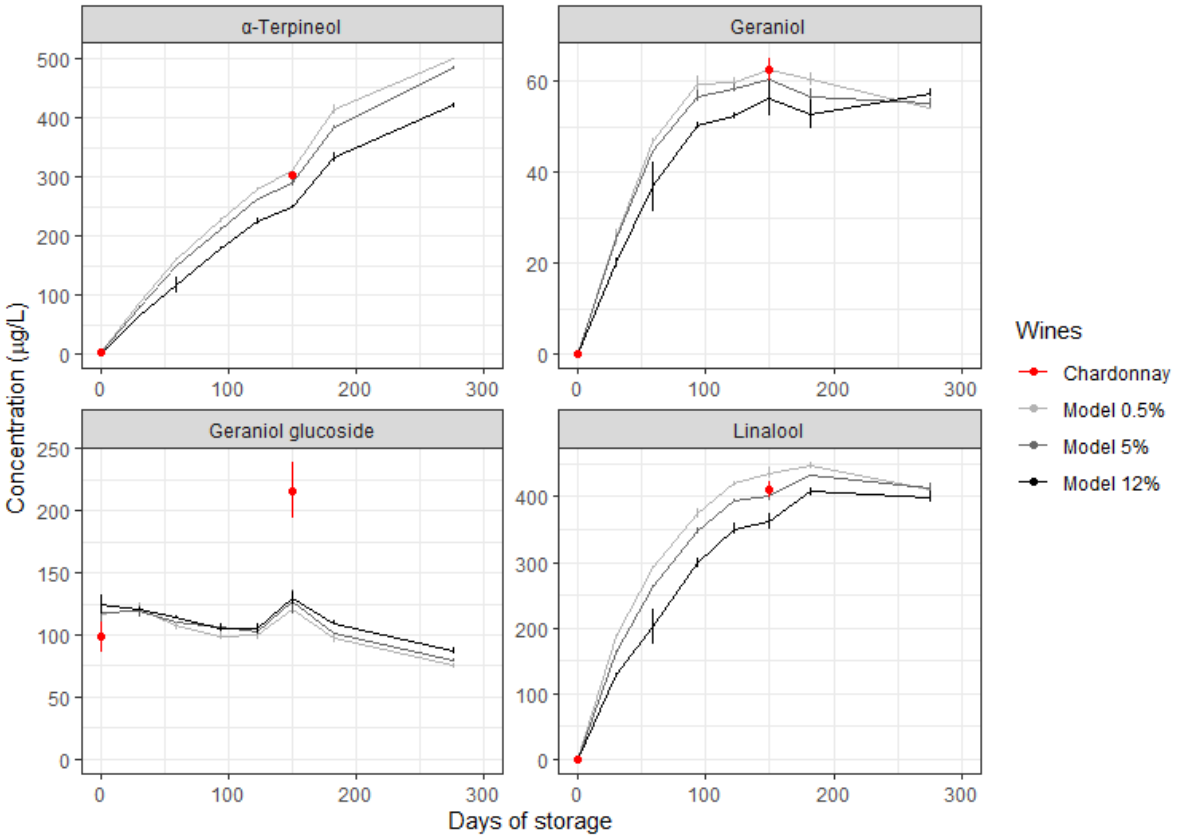


Figure 30. Concentration of key compounds in low alcohol matrices with added Gordo marc extract after storage at 15°C in ampoules. Data represent mean ± standard deviation of triplicate wines. Grey and black lines represent model wine matrices, red points commercial <0.5% alcohol Chardonnay.

Initially, there was a trend for the lower-alcohol model wines to provide a greater formation of volatile monoterpenes, however, this converged as the storage trial continued. It appears as though the aggregated knowledge from this project on glycoside usage in wines with traditional levels of alcohol is also applicable to NOLO products.

7.6 Thiol precursors

7.6.1 Extraction options and isolation procedure

A literature investigation of extraction and isolation options for thiol precursors was conducted. While the aforementioned glycoside extractions were conducted using a food-grade FPX66 resin, this differed significantly from most other reported isolation procedures. Most procedures used a styrene-divinylbenzene-based resin, with or without additional sulfonic acid functionality, while the FPX66 is an aromatic polymer-based resin. However, given that the glycosides were isolated using FPX66 and it is food grade, it was considered an option for extraction in case the glycosides and thiol precursors could use the same procedure. Alternatively, XAD-4 was chosen as a representative of the styrene-divinylbenzene resin family.

Initially, the extraction was attempted in an analogous manner to the glycoside extractions where the XAD-4 resin was put into a housing or column and all loading, washing and elution steps were pumped through the housing. Unfortunately, due to the viscous nature of juice (rather than aqueous extracts) the column became quickly clogged and the resin could not be loaded. As such, the batch procedure as listed in the materials and methods was developed to avoid clogging a column, but also meant that the extraction was much longer and involved more manual handling. It is unlikely that an extraction performed in this way would become a useful industrial-scale process.

Lastly, the batch procedure was attempted with FPX66 instead of XAD-4 resin. However, the resin particle size of FPX66 is much smaller and again caused the filtration equipment to clog. So, while

the FPX66 resin was useful in the extraction of glycosides at winery- and laboratory-scale in a housing, it was not amenable to a batch system using the available filtration equipment.

7.6.2 Recovery of thiol precursors from juice to extracts

For each extraction, two separate batches of juice were extracted and then the two extracts combined. Each juice contained thiol precursors at the concentrations listed below (Table 1). From each juice, the extraction produced a similar mass of extract per volume extracted (2.99 g of extract per L extracted for the foliar treated juice and 2.82 g/L for the control juice), and the extracts were proven to retain the thiol precursors.

Table 1.Concentrations of known thiol precursors in juice and extracts of two separate Sauvignon Blanc parcels.

	Thiol precursor	Control juice	Foliar juice
Concentration in juice (µg/L)	**Cysteine-3MH**	20.11	34.13
	Glutathione-3MH	239.34	301.58
	CysGlycine-3MH	9.08	10.76
Concentration in extract (µg/g)	**Cysteine-3MH**	5.01	16.63
	Glutathione-3MH	126.62	271.06
	CysGlycine-3MH	1.47	3.02

7.6.3 Preliminary tasting

The preliminary tasting of the thiol precursors was performed using the foliar spray-treated extract to maximise the chance of perception. However, apart from one taster, nobody perceived an aftertaste from the initial solutions (1.93 g/L or 0.64 g/L in Milli-Q water or model wine). At these concentrations of extract, the concentration of glutathione-bound 3MH in the tasting solutions was approximately 520 µg/L and 170 µg/L.

When the concentration of the extract was increased to 3.86 g/L, this solution could only be perceived by roughly half of the panel (described as passionfruit seeds). This equates to a glutathione-bound 3MH concentration of approximately 1,040 µg/L, or approximately four times what was in the control juice. If the extract from the control juice was used, the extract would need to be added at approximately 8 g/L to obtain a glutathione-bound 3MH concentration of 1,000 µg/L, equivalent of extracting 3 L of juice to supplement 1 L.

It is unlikely that Sauvignon Blanc juice extracts would be useful as a flavour enhancer, especially given that without microbial fermentation, the precursors present are limited to the evolution of the less potent thiol 3MH, and not 3MHA. Secondly, to create this extract, a winemaking input had to be redirected (Sauvignon Blanc juice) rather than a waste stream. As such, thiol precursors were deemed to be of low relevance to flavour production due to their need to be used either pre-fermentation, when Sauvignon Blanc juice could simply be used, or due to a lack of in-mouth perception from bound analogues.

8. Outcome and conclusion

This project had five overarching deliverables (as listed in Section 5) covering knowledge generation for glycosides and other known flavour precursors:

- Isolation procedures for phenol-free monoterpene glycosides from floral varieties and knowledge about their sensory significance in winemaking applications.
- Knowledge about the sensory effect of glycosides from non-floral varieties and their flavour contributions after storage in-bottle.
- Understanding of the effects of glycosides in alternative products (e.g. low- or no-alcohol products).

- Understanding of the sensory significance of thiol precursors and processing options.
- Practical advice and extension materials for winemakers.

The outputs aligned with sector engagement are evidenced in Section 7.4, where consultation helped to guide the direction of the project, and in Appendix 1 (below) detailing the communication outputs. Additionally, the potential for the extraction to be performed at an existing aggregation facility was discussed, with little traction due to unknown market demand for the product.

Scientifically, this project has provided knowledge on two main fronts: the in-mouth release of flavour from precursors during tasting, and the extraction and use of precursors in winemaking.

The first of these relates to the role of glycosidically bound aroma compounds in generating flavour (detected retronasally) during wine tasting or consumption. Initially, structurally different glycosides were assessed across a population and time to understand the variation among individuals' ability to release volatile compounds in-mouth, and how stable this ability was over time. This work showed that the ability to perceive retronasal flavour from glycosidically bound aroma compounds was dependent on the individual, but also the glycoside structure, with geraniol glucoside, guaiacol glucoside and a glycoside mix isolated from Gewürztraminer marc eliciting every possibly combination of response phenotype. Previously, glycosides of volatile phenols from smoke were shown to contribute to flavour via retronasal perception of the odorant during tasting, but it was not known whether other types of glycosides formed in berries without smoke exposure could also produce flavour by the same mechanism.

The reasons behind the inter-individual differences were investigated via individual threshold testing of different glucosides against their respective free aroma compound, by collecting saliva from individuals and performing *in vitro* hydrolysis assays with spiked glucosides, and by microbial genetic screening of participants' saliva. Overall, the microbial populations related to the release of volatiles from glucosides during *in vitro* saliva assays, but neither factor related to the inter-individual variation in flavour perception on a sensory timescale. An individual's ability to perceive retronasal flavour from glycosides was found to be closely related to their individual threshold for perceiving the aglycone.

The role of thiol precursors in generating flavour via in-mouth release was less obvious. While the extracts that were prepared from Sauvignon Blanc juice could be perceived by a portion of the tasting panel, it was at high concentrations that would render the process inefficient. Furthermore, use of such extracts would require redirecting winemaking inputs to isolate the precursors, unlike the glycosides which could be obtained from waste streams. Secondly, the most potent thiol compound is 3MHA, which only evolves from acetylation of 3MH by yeast during winemaking. As such, the source of the precursor (juice) is not amenable to producing flavour extracts with a high potential potency.

The second front relates to providing industrially relevant mechanisms to extract glycosidically bound aroma compounds from grape marc and use these to impart flavour into wine via hydrolysis during fermentation and ageing. Chardonnay and Riesling wines were spiked with phenol-free glycosidic extract originating from Gewürztraminer marc, showing the potential flavour benefit currently discarded in the wine industry. These wines were analysed after four months in bottle and showed an increase in 'fruity' and 'floral' ratings without increases in bitterness, with concomitant increases in monoterpenes. Interestingly, there was no significant difference between adding the glycosides pre- or post-fermentation, deviating from the expectation that fermentation-related glycosidases would be a major driver in releasing flavour from the glycosides. Over three years, the wines were monitored and showed a dramatic deviation due to the pH difference between the Riesling (pH 3.1) and the Chardonnay (pH 3.4), not only in the hydrolysis of the glycosides, but also the re-arrangement of released monoterpenes.

The extraction of a second pool of grape marc was conducted using a slightly altered procedure. The use of rotary fermenters for agitation was replaced by plunging and instead of attempting to cold settle the extract, it was immediately pressed and cross-flow filtered. This produced a similar yield of extract, but with a much quicker turn-around time. This extract was added to commercial Chardonnay wine to investigate the first six months of storage and confirmed that geraniol glucoside concentrations increased initially. This same outcome was also observed when a number of extracts from different varieties were stored in the same commercial Chardonnay for six months.

Laboratory-scale extractions of numerous marc parcels and additions of the resulting extracts into wines showed that the optimum source of flavour precursors is from Muscat varieties. Other floral varieties produced a smaller amount of flavour but with a similar relative profile of volatiles, while non-floral varieties yielded much lower proportions of monoterpenes. This suggests that practically, marc should be extracted for flavour precursors from specific varieties prior to aggregation, to maintain a potent product.

The addition of glycoside-rich marc extracts to NOLO media showed that changes in ethanol concentrations had little impact on the evolution of volatiles. However, the model wine experiments showed a different geraniol glucoside profile than in all other wine storage trials, including in the <0. 5% ABV Chardonnay. There may be some solubility differences between wines and model wines that have contributed to this outcome. Otherwise, the knowledge generated in this project appears to be directly transferable to NOLO products.

This project has delivered an extraction method for the isolation of purified glycosidic material that can be added to wine or wine-like products. The method has been developed specifically to be used in a winery setting, and involves materials that are accessible to a winery. The two extraction trials to date (both on 300 kg of marc) have used winery infrastructure to obtain the aqueous extract, and commercially available products to purify it. The process has been considered from a regulatory point of view and is considered to be applicable without the need to amend FSANZ regulations. All purification solvents used in the extraction method are already used in wineries (water, neutral spirit and caustic solution).

The development of the glycosidic extract has undergone significant industry consultation, specifically looking at segments of the wine industry that would be amenable to integrating it into their usual practices, with a number of large wine companies indicating that they would use such an extract if it were available. A number of possible avenues for development of a larger-scale extraction capability have been explored, but no suitable development partners are engaged at this stage. At this stage, the cost of concentrating the aqueous marc extract is the prohibitive factor in creating these extracts.

9. Recommendations

9.1 Sector context of project outcomes

Inter-individual variation was observed in the response to glycosides, which was largely dependent on the individual's sensitivity to the aglycone. This has previously been shown for smoke-related glycosides, and now for varietal aromas. This adds further weight to the narrative that tasting panels within wine companies should be as large and diverse as possible to account for this variation between tasters.

The extraction and purification protocol for glycoside-rich marc extracts is applicable to wineries, but the sector consultation indicated that wineries would prefer to simply purchase the extract rather than make it. As such, a central facility or aggregation point is the most likely option. However, the findings on the varietal source of marc have shown that floral varieties should remain separate from non-floral, and ideally use only Muscat varieties. This introduces logistical hurdles to keep the desired marc parcels separate to retain the potency of the extracts.

The winemaking trial of Chardonnay and Riesling wines with added Gewürztraminer marc extract showed that additions to must (pre-fermentation) or to wine (post-fermentation) made little difference. As such, the success of the addition did not rely on the action of enzymes during alcoholic fermentation and allows for reactive modulation of flavour once the outcome of fermentation is known. However, the fact that chemical hydrolysis is the main mechanism of flavour release, rather than enzymatic, means that the nature of the wine to which it is added affects the evolution of flavour.

The Riesling wines (pH 3.1) showed a much more rapid breakdown of geraniol glucoside and formation of volatile monoterpenes than the Chardonnay wines (3.4). This means that the shelf-life of products with added marc extracts will depend on the pH of the medium. Fortunately, the kinetics for the hydrolysis of geraniol glucoside appears to be useful for understanding shelf-life, even though there are other reactions also taking place.

When the glycoside-rich extracts were stored in model wines of differing ethanol concentration, the evolution of monoterpenes followed a similar trend in all media. When this extract was added to a commercial NOLO product, the evolution of volatiles matched that of the model systems, and the changes in geraniol glucoside concentration were similar to what had been observed in full-strength wines. As such, our aggregated knowledge on glycosides in winemaking appears to be directly transferable to NOLO products.

While the isolation and purification methods used in this work were determined to meet the FSANZ requirements for a wine additive, the regulatory framework around the use of alternative products in winemaking has the potential to hinder similar works. For this type of research to be as efficiently moved to industry adoption as possible, regulatory barriers should be as low as possible. Furthermore, the initial work on NOLO products has further highlighted a need for a tidier regulatory landscape that allows for product innovation in a timely manner.

9.2 Future research directions

The following directions are recommended for future research stemming from this project:

- Studies on the kinetics of flavour evolution from glycosides should be extended to analogous systems such as smoke taint glycosides. This should include equilibrium constants of protonation and other key temporal processes that determine product acceptability.
- Procedures to recover the glycosides of other useful compounds from grape marc should be investigated, with a view to overcoming economic barriers to their use as winemaking additives.
- With an increasing focus on NOLO products, there is a need to further sense check what is known about flavour evolution in wine and understand the transferability of existing knowledge to NOLO matrices.
- Given the evolution of geraniol glucoside in the first six months of storage, there is a need to obtain pure forms of other monoterpene precursors (disaccharides or malonic acid esters) to understand formation pathways. This includes developing quantitative methods for analysing these species.
- In the preparation of marc extracts, the concentration and drying steps were the most energy-intensive and expensive. Alternative drying technologies should be investigated to reduce cost of preparing flavour extracts.

10. Appendix 1: Communication

Industry-directed communication

Technical Notes, AWRI Technical Review:

- 'Boosting floral aroma of wine with aromaless grape marc extracts'. Issue 240, June 2019.

- 'From product to purpose: creating value from flavour glycosides'. Issue 257, April 2022.
- 'The flavour hiding in different marc varieties'. Issue 248, June 2022.

Australian Wine Industry Technical Conference:

- 'Phenolic-free glycosides from grape marc can increase wine flavour'. Poster presentation, 17th AWITC July 2019.
- 'Glycoside flavour release in-mouth: the role of oral bacterial populations'. Poster presentation, 17th AWITC July 2019.
- 'Why do some people experience flavour from glucosides while others don't?'. Poster presentation, 17th AWITC July 2019.
- 'Modulating monoterpenes and glycosides for wine style and flavour'. Workshop presentation, in Workshop 28: *What makes a wine tick: new advances in understanding flavour compounds*. 17th AWITC July 2019.
- 'Phenolic-free glycosides from grape marc can increase wine flavour'. In the Wine Light, 3-minute presentation. 17th AWITC July 2019.
- 'Exploring the varietal effect of flavour precursors derived from grape marc on wine flavour'. Poster presentation, 18th AWITC June 2022.

Roadshow seminar program:

- 'Increasing wine flavour with glycoside additions'. Griffith seminar, November 2020.
- 'Increasing wine flavour with glycoside additions'. Stanthorpe seminar, May 2022.

AWRI Webinar + YouTube video:

- 'Increasing wine flavour by adding glycosides from grape marc'. November 2018.

AWRI decanted podcast:

- 'Why are winemakers throwing away flavour?'. September 2021

Research-directed communication

Peer-reviewed publications:

- Parker, M., Onetto, C., Hixson, J., Bilogrevic, E., Schueth, L., Pisaniello, L., Borneman, A., Herderich, M., de Barros Lopes, M., Francis, L. 2020. Factors contributing to interindividual variation in retronasal odor perception from aroma glycosides: the role of odorant sensory detection threshold, oral microbiota, and hydrolysis in saliva. *J. Agric. Food Chem.* 68(38): 10299-10309.
- Parker, M., Barker, A., Black, C. A., Hixson, J., Williamson, P., Francis, I. L. 2019. Don't miss the marc: phenolic-free glycosides from white grape marc increase flavour of wine. *Aust. J. Grape Wine Res.* 25(2): 212-223.
- Pisaniello, L., Watson, F., Siebert, T., Francis, L., Hixson, J.L. 2022. The varietal influence of flavour precursors from grape marc on monoterpene and C13-norisoprenoid profiles in wine as determined by membrane-assisted solvent extraction (MASE) GC-MS. *Molecules*. 27(7): 2046.
- Delgado, L., Parker, M., Fisk, I., Paradisi, F. 2020. Performance of the extremophilic enzyme BglA in the hydrolysis of two aroma glucosides in a range of model and real wines and juices. *Food Chem.* 323: 126825.

Conference proceedings:

- Parker, M., Barker, A., Pearson, W., Hayasaka, Y., Hixson, J., Francis, L. 2018. Flavour release from wine glycosides during tasting. Siegmund, B., Leitner, E. (eds.) *Flavour Science: Proceedings of the XV Weurman Flavour Research Symposium, 18-22 September, 2017, Graz University of Technology, Austria*. Graz, Austria: Verlag der Technischen Universität Graz: 31-36.

- Hixson, J., Pisaniello, L., Parker, M., Grebneva, Y., Bilogrevic, E., Stegmann, R., Francis, L. 2021. Methods for predicting and assessing flavour evolution during white wine ageing. Guichard, E., Le Quéré (eds) *Proceedings of the 16th Weurman Flavour Research Symposium, May 4-6, 2021*. doi: 10.5281/zenodo.5346589: 1-6.

11. Appendix 2: Intellectual property

Program IP has been communicated through presentations and publications and resides in the public domain. Copyright in any reports, posters or presentations is held by the AWRI. Copyright in publications remains with the publisher. No inventions or patentable discoveries have been identified from the work. No Program IP has been commercialised or identified for commercialisation at the time of writing this report.

This project was workshopped in a pre-commercialisation setting, specifically looking at potential customers for a glycosidic flavour enhancer. While a number of non-wine industry applications were investigated, the novelty of the glycoside extract preparation was deemed to be not worthy of protection.

12. Appendix 3: References

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13. Appendix 4: Staff

Project staff that contributed to this work: Leigh Francis, Mango Parker, Josh Hixson, Lisa Pisaniello.

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