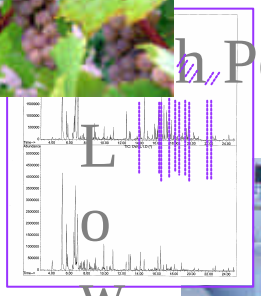
VITICULTURAL CONTROL OF FLAVOUR COMPOUNDS IN WINE.







Pepper - C3
Low Pepper C1

INTERIM FINAL REPORT to

GRAPE AND WINE RESEARCH & DEVELOPMENT CORPORATION

Project Number: **CRV 6AWR (CRCV 1.3)**

Project leader (Principal Investigator): **Mark Sefton**

Research Organisation: **Cooperative Research Centre for Viticulture**

Date: **November 15, 2003**

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Executive Summary

- The project is on track to achieve our aim of having rapid and accurate analytical methods for determining 95% of known important flavour compounds in wine, as well as methods to determine at least several flavour precursors in grapes. So far, rapid and accurate analytical methods have been developed for more than fifty volatile wine components that are likely to be important to wine aroma and flavour. Of these compounds, 48 are derived directly or indirectly from the grape. Development of analytical methods for wine lactone, two grape-derived wine lactone precursors, three important grape derived thiols (mercaptans) which confer various fruit characters to a wide range of wines, and several grape-derived precursors for important volatile wine phenols is in progress

- A hitherto unknown wine flavour compound, derived from grape glycoconjugates, has been identified as 2,3,6-trimethylbutadiene (TPB). The threshold of TPB in a white wine was determined to be 40 ng/L. This makes it one of the post potent wine flavour compounds known. TPB was most commonly found in Semillon, and to a lesser extent, Chardonnay wines.

- A sensory descriptive analysis study on a set of 20 commercial Riesling wines and 20 one to two-year old commercial unwooded Chardonnay wines has been completed. The data set should enable insights to be made into the relationships between the concentration of particular volatile compounds and the intensity of Riesling and Chardonnay wine flavour attributes once all chemical analyses have been completed.

- Several potential damascenone precursors have been synthesised and all of these give damascenone in high yield and extremely rapidly. These findings have led us to re-evaluate the mechanism by which damascenone is formed in young wine. The information obtained from this work will be used to devise methods for measuring the damascenone potential of grapes. Similarly, we intend to use the sulphur compounds we have made to develop methods for measuring this aspect of flavour potential in grapes. We see these two areas as having the highest priority over the next twelve months.

Introduction and Project Objectives

- To develop relatively simple precise analytical methods for quantification of key volatiles
- To develop specifications for flavour constituents of wine grapes.
- To determine the influence of viticultural practices on the levels of specified grape flavour constituents

Volatile aroma and flavour compounds in wines can be classified according to their origin. Some are formed microbiologically, others are derived from the grape, either directly, or indirectly by chemical or biological transformations of involatile flavour precursors. Further aroma and flavour modification can result during wine oxidation and oak barrel maturation. All of these aspects of wine aroma and flavour are inextricably linked with each other. For example, the concentration of many important products of fermentation is determined by must composition. In particular, several thiols, which impart fruit characters to many red and white wine styles, are released by some, but not all yeast strains from grape derived precursors. Similarly, the concentration of vinylphenols in wines is determined by both the concentration of their grape-derived precursors and the metabolic activity of fermenting yeasts. The modifying effects of oxidation on wine composition can occur on wine components of diverse origin. The influence of oak maturation on wine composition is partly through the extraction

of flavour volatiles directly from oak, but other modifications of compounds derived from grapes and wine microorganisms can take place during barrel maturation.

For these reasons, and despite research efforts by a number of groups over the last decade, the relationships among viticultural parameters, grape composition and subsequent wine sensory properties have remained poorly understood. In particular, despite the success of project AWR 6V in generating information about the importance of the total glycoside pool to wine flavour, we continue to have limited knowledge of the identity of individual wine flavour components derived from the grape berry that are basic and essential to wine quality, and of the concentration of such compounds in grapes that give rise to particular flavours in wine.

One factor which has limited our understanding of these issues has been the lack of availability of suitable analytical methods for important volatile wine aroma and flavour compounds, and authentic samples of known wine aroma and flavour compounds for carrying out sensory studies. We therefore intended to make a concerted effort over the lifetime of the CRCV to develop such analytical methods. We intend to bring this program to near-completion during 2006 and to then continue to develop analytical methods for new volatile wine aroma and flavour compounds ad hoc, as and when they are discovered. The production of reference compounds for analytical methods has, and will continue to be achieved through our collaboration with Flinders University. Such compounds will also enable us to carry out sensory studies that were hitherto not possible.

With such compounds in hand, we would then be more readily able to achieve our other objectives – to identify the aroma and flavour components derived from the grape berry that are basic and essential to wine quality, knowledge of the concentration of such compounds in grapes that give rise to particular flavours in wine, and an understanding of the processes that lead to the presence of these compounds at particular concentrations in wine, and finally to determine the influence of viticultural practices on the levels of specified grape-derived wine flavour constituents and/or their precursors.

Results

The following is a chronological list of papers which describe the results of this project. The full text of these papers are in the appendix

1. Pollnitz, A.P.; Pardon, K.H.; Sefton, M.A. Quantitative analysis of 4-ethylphenol and 4-ethylguaiacol in red wine. J. Chromatography A. (874): 101-109; 2000.
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3. Skouroumounis, G.K.; Sefton, M.A. Acid catalysed hydrolysis of alcohols and their β-D-glucopyranosides. J. Agric. Food Chem. (48); 2033-2039; 2000.
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5. Williams, P.J.; Francis, I.L. Wine flavor research-experiences from the past offer a guide to the future. Proceedings of the ASEV 50th Anniversary Annual Meeting; June 19-23, 2000, Ed J. M. Rantz, American Society for Enology and Viticulture, Davis, California, 2001; pp 191-195
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9. Elsey, G.M., Francis, I.L., Sefton, M.A. Sensory properties of some grape and wine aroma compounds. Proceedings of the eleventh Australian wine industry technical conference; 7-11 October 2001; Adelaide, SA., Eds Blair, R.J., Williams, P.J., H¢j, P.B., Australian Wine Industry Technical Conference Inc., Adelaide, South Australia 2002; p266.
10. Y. Hayasaka, K. MacNamara, G.A. Baldock, R.L. Taylor, A.P. Pollnitz (2003) Application of stir bar sorptive extraction for wine analysis. Analytical Bioanalytical Chemistry (published on line 15 March 2003)
11. Janusz, A. The Separation of Glycosylated Flavour Precursors from Grape Leaves with Multi-Layer Counter Current Chromatography (MLCCC). Aust. J. Chem. (55); 745, 2002
12. M.A. Smith, G.M. Elsey, D.L. Capone and M.A. Sefton (2002) The effect of glycosylation on the formation of 1,1,6-trimethyl-1,2-dihydronaphthalene in wine. Presented at the 21st International Carbohydrate symposium, 7-12 July, Cairns, Australia.
13. Janusz, G.M. Elsey, M.V. Perkins, G.K. Skouroumounis, and M.A. Sefton (2002) The use of multi-layer counter-current chromatography (MLCCC) for the separation of glycosidically bound aroma precursors from grapes. Presented at the 21st International Carbohydrate symposium, 7-12 July, Cairns, Australia.
14. Gordon Elsey. The development of stable isotope dilution assays for the quantification of the important aroma compounds citronellol, 4-vinylphenol and 4-vinylguaiacol in wine. AWRI Technical Review (142) 2002; 4-5.
15. Leigh Francis, Kate Lattey, Heather Smythe (2002). 'Reduced' aroma in screw-cap bottled white wines. AWRI Technical Review (142) 2002; 51-53.
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17. On the Formation of Naturally Occurring Damascenone: C.J.Puglisi, G.M.Elsey, R.H.Prager, and M.A.Sefton, Presented at the 19thRoyal Australian Chemical Institute Organic Chemistry Division conference, 6-11 July, Lorne, Australia., 2003.
18. The Consumption of Damascenone in Wine: M.A.Daniel, G.M.Elsey, and M.A.Sefton: Presented at the 19thRoyal Australian Chemical Institute Organic Chemistry Division conference, 6-11 July, Lorne, Australia., 2003.

Discussion

Analysis of known wine flavour compounds and their grape-derived precursors.

Rapid and accurate analytical methods have been developed for more than fifty volatile wine components that are likely to be important to wine aroma and flavour. Of these compounds, 48 are derived directly or indirectly from the grape. With just two exceptions (analyses for TDN and cis-rose oxide) these methods all employ gas chromatography coupled with mass spectrometry (GC/MS), using a technique known as stable isotope dilution analysis (SIDA).

This technique makes use of internal standards that are stable isotope (usually deuterium) labelled analogues of the analytes. In most cases, the most time-consuming part of the development of such methods is the synthesis of such labelled analogues. Occasionally, the analytes themselves also have to be synthesised if they are not commercially available. Details of the synthesis of the labelled standards and of their use in developing the analytical methods is given in Section 4 for most compounds. In addition to those described in Section 4, labelled damascenone, beta-ionone and isobutylmethoxypyrazine were prepared using modifications of published methods.

Syntheses of authentic optically pure wine lactone (the enantiomer reported to be present in wine), and a deuterium labelled analogue have been completed. These syntheses were modifications of published methods. The use of these samples to develop analytical methods for wine lactone is in progress. Two precursors of wine lactone and their deuterated analogues have also been synthesised by methods developed in-house. These compounds will be used to develop methods for measuring these precursors in grapes, and will also be used to study the rate of formation and yield of wine lactone from a given quantity of precursor material. The wine lactone work will only be written up for publication once all of this work has been completed.

A program to synthesise three important grape-derived sulphur compounds, 3-mercaptohexanol, 3-mercaptohexyl acetate and 4-mercapto-4-methyl-2-pentanone, their cysteine conjugates and the deuterated analogues of these compounds is in progress. The synthesis of several of these compounds has been completed, and two are already being used in a collaborative study of the capacity of yeast to release these important thiols during fermentation. Once the synthetic program is complete, these compounds will also be used for analytical method development and sensory assessments.

A synthesis of deuterium-labelled analogues of coumaric and ferulic acids, plus their ethyl and glucose esters is near completion. These fruit-derived compounds are important to the formation of odorous volatile phenols in wine, and the analytical methods will facilitate our understanding of the link between the vineyard and phenolic wine aroma and flavour.

Wine composition and sensory characteristics.

Hydrolysates of a grape-derived glucoconjugate purified by MLCCC have been analysed by GC/olfactometry/mass spectrometry and these chromatograms compared with those of hydrolysates of the total glycoside pools isolated from grapes. The former are much simpler than the latter, and it has been possible to assign structures to five out of the six odorants detected in the chromatogram. Four of the odorants have previously been reported as wine components, and we have so far been unable to identify the fifth. The sixth component, which was also the most potent of the six in the GC/olfactometry assessments, was tentatively identified as 2,3,6-trimethylphenylbutadiene (TPB) from its mass spectrum. An authentic sample of TPB was synthesised and this has confirmed the assignment. TPB has not been previously reported as a grape or wine component. The threshold of TPB in a white wine was determined to be 40 ng/L. This makes it one of the post potent wine flavour compounds known. An analytical method for TPB using d_8-naphthalene as internal standard was developed and several wine samples were analysed for TPB, which was observed in four out of the five white wines examined at a concentration of 50-210 ng/L. It was not observed in the four red wines examined. A more sensitive and accurate analytical method for TPB using stable isotope dilution analysis is well advanced – a deuterium labelled analogue has already been successfully synthesised in a multi-step pathway.

A sensory descriptive analysis study on a set of 20 commercial Riesling wines and 20 one to two-year old commercial unwooded Chardonnay wines has been completed. The sensory data indicate that, for the Riesling wines, there were three main groups of samples: those young

wines which were dominated by floral and lemon aromas, those that were tropical fruit/passionfruit-like, and older wines with lime, kerosene and toasty attributes. Within each broad group there were wines with varied intensities of specific attributes, such as samples with a relatively strong floral perfumed aroma compared to those with a dried rose character, and wines with stronger honey-like aroma. For the Chardonnay wines, honey, butterscotch, woody and spicy descriptors were prominent in the two year old wines, while the one year old wines were dominated by estery, citrus and fruity notes. A subset of these younger wines also showed prominant passionfruit and herbaceous characters. The data set should enable insights to be made into the relationships between the concentration of particular volatile compounds and the intensity of Riesling and Chardonnay wine flavour attributes once all chemical analyses have been completed. For the Riesling wines, the concentration of esters, alcohols and acids have already been quantified, as well as the major monoterpenes and nor-isoprenoids. The compositional analyses of all of these wines has been largely completed, and some preliminary data analysis has already been undertaken.

Early work on black pepper aroma in Shiraz wines had shown that the black pepper character can be tasted and scored in Shiraz grapes from the 1999 vintage. Analytical data had indicated that certain compounds, that are reported to be involved in the aroma of peppercorns, occur in higher concentration in the high-pepper Shiraz samples. Further grape lots were obtained from vineyards identified from historical data as producing wines with peppery characteristics. Fruit from the 2000 season from four separate vineyards in Coonawarra, and two lots of grapes from a central Victorian vineyard, have been studied. Following informal tasting of the berry samples, only one lot of fruit (from the Central Victoria vineyard) was characterised as high in pepper flavour, and this plus a low pepper sample has been analysed by GC/MS. From data collected from the 1999 samples analysed, it was intended to confirm whether the compounds identified previously were also present in the 2000 season samples, and carry out more detailed investigations The project was then placed on hold as a result of the key researcher having taken maternity leave and subsequently resigned. The project was reactivated recently following the appointment of a new researcher to work in this area.

A program to determine the flavour impact of a group of megastigmatrienones (norisoprenoids) and maleimides (chlorophyll degradation compounds) has been wound up. Although the patent literature indicated that these wine components were potentially important odorants, the samples synthesised in our laboratory had weak aromas only at the concentration normally found in wine.

Formation of wine flavour compounds from grape derived precursors and the subsequent degradation of these compounds.

Damascenone is one of the most important flavour compounds known to science; it is found in many fruit and vegetable products, including wine, and tonne quantities are produced commercially for the perfume and flavouring industries. Recent studies by us have indicated that damascenone in wine results from the hydrolytic breakdown of complex grape-derived secondary metabolites formed from carotenoids such as neoxanthin. Two synthetic sequences towards an acetylenic damascenone precursor were developed in parallel, and the target compound has now been obtained. Consequently, we have been able to identify this precursor as an intermediate in the conversion of a grape-derived norisoprenoid to damascenone in fruit, and a preliminary hydrolytic study in model wine has shown that this precursor gives damascenone in high yield. Two isomeric glucoside derivatives of this acetylenic precursor have also been synthesised, and a preliminary hydrolytic study has indicated that these too are converted to damascenone in high yield. The student undertaking this work is currently on maternity leave and is expected to return in January 2004 to complete the project.

Four isomeric allenic diols, all potential damascenone precursors, have also been synthesised and purified. The availability of these authentic samples allowed us to prove that they were intermediates in the formation of damascenone, as had been postulated previously by us. Hydrolytic studies into the formation of damascenone have been conducted for all four isomers. At room temperature and pH 3.0, each isomer gave damascenone in high yield within a few days. Surprisingly, no trace of 3-hydroxydamascone was formed from these compounds. 3-Hydroxydamascone is normally found in grapes and wines at much higher concentration than is damascenone. These findings have led us to reevaluate the mechanism by which damascenone is formed in young wine. Analysis by chiral gas chromatography of the intermediates formed in the hydrolytic studies has given us a valuable insight into the relative reactivity of the functional groups within the precursor molecule, and this has helped clarify our understanding of the formation of damascenone in nature.

A preliminary study concerning the reaction of damascenone with common wine components (ie. the loss of damascenone as wines age) has been completed. Ten potentially reactive compounds were examined. The decrease in damascenone concentration in these reactions was less than expected, and alone cannot account for the more rapid decrease in damascenone concentration normally seen in ageing wine. The expected products of these reactions were synthesised, and one of these has been identified as a minor byproduct of damascenone formation from the allenic precursors discussed above.

Riesling Acetal is a relatively weak-smelling grape-derived norisoprenoid that is formed along with the more potent 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) from grape derived precursors. In the short term, these two products are formed in competition, and we considered that factors such as the position of glycosylation on the polyfunctional precursors could steer the conversion towards or away from TDN formation Recent hydrolytic studies by us of some of these precursors, synthesised in our laboratory, have shown that Riesling Acetal can in fact be completely converted to TDN at wine pH, probably over a period of several years, and that this will contribute to a gradual increase in TDN in bottled wines over this period. As expected for acid catalysed transformations, the formation of TDN from Riesling Acetal was approximately twice as fast at pH 3.0 as at pH 3.2. This indicates that the position of glycosylation in the grape-derived precursors is probably immaterial in determining the TDN potential of grapes.

Other lines of research on the formation of flavour compounds from grape-derived precursors, ie wine lactone and volatile thiols, have already been mentioned above. In addition, the analytical methods we have developed for wine flavour compounds derived from fermentation are being used to by the Institute's microbiological group to investigate the influence of grape composition (eg amino acids) on this aspect of wine quality.

Conclusions and recommendations

- The project is on track to achieve our aim of having rapid and accurate analytical methods for determining 95% of known important flavour compounds in wine, as well as methods to determine at least several flavour precursors in grapes. To help achieve this aim it will be necessary to find a suitable candidate for the new Analysts position which has been funded from January 2004. As well as assisting with method development, the new Analyst will also undertake a screening program of Australian wines from different regions to determine which compounds should be focussed on during analysis of samples from viticultural trials.

- In the area of relating wine composition to wine flavour, we expect that the project on Chardonnay and Riesling flavour will be completed within 12 months, and much useful

information on the flavour impact of specific compounds will be available as a result. We see this avenue of research as being of great importance, but will not be able to extend it further to other varieties without the additional in-kind funding sought by the AWRI in its recent seven year application to the GWRDC. We also intend to place a special focus on identifying the compounds responsible for the peppery aroma in Shiraz wines. The sensory work we have carried out so far indicates that these compounds are present in grapes in free form. This is in contrast to most wine flavour compounds which are formed from involatile and flavourless grape components, and therefore the measurement of peppery compounds in grapes should relate to the final concentration of these compounds in wine.

- Our work on damascenone precursors will be completed by 2006. The information obtained from this work will be used to devise methods for measuring damascenone in the vineyard. Similarly, we intend to use the sulphur compounds we have made to develop methods for measuring this aspect of flavour potential in grapes. We see these two areas as having the highest priority over the next twelve months.

Industry Summary

- The project is on track to achieve our aim of having rapid and accurate analytical methods for determining 95% of known important flavour compounds in wine, as well as methods to determine at least several flavour precursors in grapes. So far, rapid and accurate analytical methods have been developed for more than fifty volatile wine components that are likely to be important to wine aroma and flavour. Of these compounds, 48 are derived directly or indirectly from the grape. With just two exceptions (analyses for TDN and cis-rose oxide) these methods all employ gas chromatography coupled with mass spectrometry (GC/MS), using a technique known as stable isotope dilution analysis (SIDA). This technique makes use of internal standards that are stable isotope-labelled (usually with deuterium) analogues of the analytes. In most cases, the most time-consuming part of the development of such methods is the synthesis of such labelled analogues. Occasionally, the analytes themselves also have to be synthesised if they are not commercially available.

- Analytical methods for wine lactone, two grape-derived wine lactone precursors, three important grape derived thiols (mercaptans) which confer various fruit characters to a wide range of wines, and several grape-derived precursors for important volatile wine phenols are under active investigation. The syntheses of the compounds needed for the wine lactone and volatile phenol work have been completed, and preparation of the sulphur compounds and their deuterium-labelled analogues is well advanced.

- A hitherto unknown wine flavour compound, derived from grape glycoconjugates, has been identified as 2,3,6-trimethylbutadiene (TPB). The threshold of TPB in a white wine was determined to be 40 ng/L. This makes it one of the post potent wine flavour compounds known. An analytical method for TPB was developed and a number of wine samples analysed. TPB was most commonly found in Semillon, and to a lesser extent, Chardonnay wines. So far, TPB has not been found in any red wine.

- A sensory descriptive analysis study on a set of 20 commercial Riesling wines and 20 one to two-year old commercial unwooded Chardonnay wines has been completed. The sensory data indicate that, for the Riesling wines, there were three main groups of samples: those young wines which were dominated by floral and lemon aromas, those that were tropical fruit/passionfruit-like, and older wines with lime, kerosene and toasty attributes. For the Chardonnay wines, honey, butterscotch, woody and spicy descriptors were prominent in the two year old wines, while the one year old wines were dominated by estery, citrus and fruity notes. A subset of these younger wines also showed prominant passionfruit and herbaceous characters. The data set should enable insights to be made into the relationships between the concentration of particular volatile compounds and the intensity of Riesling and Chardonnay wine flavour attributes once all chemical analyses have been completed.

- β-Damascenone is one of the most important flavour compounds known to science; it is found in many fruit and vegetable products, including wine, and tonne quantities are produced commercially for the perfume and flavouring industries. Recent studies by us have indicated that β-damascenone in wine results from the hydrolytic breakdown of complex grape-derived secondary metabolites formed from carotenoids such as neoxanthin. Several potential damascenone precursors have been synthesised and all of these give damascenone in high yield and extremely rapidly. These findings have led us to reevaluate the mechanism by which damascenone is formed in young wine. The information obtained from this work will be used to devise methods for measuring damascenone potential in grapes. Similarly, we intend to use the sulphur compounds we

have made to develop methods for measuring this aspect of flavour potential in grapes. We see these two areas as having the highest priority over the next twelve months.

Appendix (published papers available in electronic form)

Note the following is the submitted version of the papers. The published papers cannot be reproduced here without contravening copyright

Quantitative Analysis of 4-Ethylphenol and 4-Ethylguaiacol in Red Wine.

Alan P. Pollnitz*, Kevin H. Pardon and Mark A. Sefton.

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Abstract
2,3,5,6-[$^2H_4$]-4-Ethylphenol (d_4-4-ethylphenol) was synthesised for use as an internal standard in a new, rapid and accurate analytical method, employing gas chromatography - mass spectrometry to determine the concentration of the important aroma compounds 4-ethylphenol and 4-ethylguaiacol in red wine. The concentrations of both compounds in wine stored in 44 American and 47 French new and used oak barrels from several suppliers were measured. Wine stored in shaved and refired oak barrels contained up to 85% less 4-ethylphenol and 4-ethylguaiacol than wine stored in normal barrels of the same age that were not shaved.
The concentration of 4-ethylphenol found in 61 bottled commercial Australian red wines of various ages ranged from 2 µg/L in a Merlot up to 2660 µg/L in a Shiraz, with a mean concentration of 795 µg/L. 4-Ethylguaiacol was also detected in every red wine analysed, ranging in concentration from 1 µg/L (in a Pinot Noir) up to 437 µg/L (in a Merlot) with a mean concentration of 99 µg/L.

Keywords: 4-ethylphenol, 4-ethylguaiacol, stable isotope dilution, analysis, gas chromatography, mass spectrometry, aroma, flavour, wine, oak.

1. Introduction.

The yeast *Brettanomyces / Dekkera* has been described as "one of the most complex and controversial issues encountered in the making of red wine" [1]. Among the aroma compounds produced in red wines by these yeasts are the volatile phenols, 4-ethylphenol and 4-ethylguaiacol (Fig. 1), which are formed from grape-derived *p*-coumaric acid and ferulic acid respectively [2-5]. The aroma associated with 4-ethylphenol in red wine has been varyingly described as 'horsy', 'leather', 'medicinal', 'smoky', 'barnyard', 'animal' and 'sweaty saddle'-like [1, 2, 6, 7]. 4–Ethylguaiacol in wine has a smoky, spicy, clove-like aroma [1, 2, 6, 8-10]. At higher concentrations these odours can be undesirable, especially those from 4-ethylphenol [1, 2, 6]. While 4-ethylphenol and 4-ethylguaiacol are apparently ubiquitous in red wine, the concentration found can vary considerably [2, 3, 11]. This paper describes a new method, which is both fast and accurate, for the analysis of 4-ethylphenol and 4-ethylguaiacol in wines to assist winemakers in determining how winemaking and storage processes affect the concentration of these compounds.

2. Experimental

Nmr spectra were of deuterochloroform solutions and were obtained with a Jeol FX90Q spectrometer operating at 90 MHz.

2.1 Synthesis of 2,3,5,6-[$^2H_4$]-4-ethylphenol (Scheme 1)

Aluminium trichloride (15.73 g) was added to a carbon disulphide (15.7 mL) solution of [$^2H_5$]-phenylacetate (7.87 g, prepared in 96% yield from [$^2H_6$]-phenol, Aldrich, 98% $^2H_6$, in pyridine and acetic anhydride at room temperature), and the mixture was stirred at reflux for 70 min. The solvent was distilled off, and the residue was then stirred at 130°C for 3h. The product was cooled, hydrochloric acid (6 M, 8 mL) then water (100 mL) were added and the mixture left to stand overnight. The aqueous solution was extracted with diethyl ether-pentane (1:4, 4 x 50 mL) to remove 2-hydroxyacetophenone. Sodium chloride (40 g) was then added to the aqueous solution, and the crude 4-hydroxyacetophenone (4.43 g) was recovered with diethyl ether (4 x 50 mL). The crude product was purified by flash chromatography under standard conditions [12] followed by recrystallization from diethyl ether-pentane (1:1) to give 2,3,5,6-[$^2H_4$]-4-hydroxyacetophenone (**4**, 3.72 g); nmr (δ) 2.55 (s); m/z 140 (M^+, 37%), 139 (6%), 126 (9%), 125 (100%), 124 (14%), 97 (26%), 96 (6%), 69 (16%).

Potassium hydroxide (2.93 g), followed by 2,3,5,6-[$^2H_4$]-4-hydroxyacetophenone (**4**, 1.22 g), then hydrazine hydrate (>99 %, 1.27 mL) was added to diethylene glycol (15 mL) under nitrogen at room temperature. The reaction mixture was stirred for 15 min. at room temperature, then at 130°C for 1 h. The temperature was further raised to 200°C over 30 min. and maintained for 3 h. The reaction mixture was then cooled in an ice bath, acidified with hydrochloric acid (0.1 M) to pH < 3. The volume was adjusted to 500 mL by the addition of water, and the product (**5**, 0.92g) was recovered with *n*-pentane; nmr (δ) 1.20 (t, J=7Hz, 3H), 2.58 (q, J=7Hz, 2H), 6.73 (s, 2H); m/z 124 (M^+, 35%), 123 (5%), 110 (21%), 109 (100%), 108 (9%), 79 (9%), 78 (8%). A portion (852 mg) of this product was placed in a 60 mL capacity thick-walled glass ampoule. Deuterium oxide (50 mL), then thionyl chloride (1.7 mL) were added, the ampoule was sealed, and the contents heated to 100°C for 5 days. The solution was neutralised with potassium carbonate (4.2 g), the product (830 mg) isolated with pentane and redistilled *in vacuo* to yield pure 2,3,5,6-[$^2H_4$]-4-ethylphenol (**2**) as a clear liquid; m/z 126 (M^+, 32%), 125 (4%), 112 (10%), 111 (100%), 110 (8%), 81 (4%), 80 (8%), 79 (5%). When unlabelled 4-ethylphenol (**1**) was treated with deuterium oxide and thionyl chloride under identical conditions, the $^2H_2$ analogue (**6**) was obtained; m/z 124 (M^+, 37%), 123 (5%), 110 (20%), 109 (100%), 108 (8%), 79 (8%), 78 (5%); nmr (δ) 1.20 (t, J=7Hz, 3H), 2.58 (q, J=7Hz, 2H), 7.02 (s, 2H); cf unlabelled 4-ethylphenol (**1**), 1.20 (t, J=7Hz, 3H), 2.58 (q, J=7Hz, 2H), 6.73 (AA' of AA'BB', 2H), 7.05 (BB' of AA'BB', 2H).

2.2 Preparation of samples for analysis

A solution of [$^2H_4$]-4-ethylphenol (2.34 μg) in ethanol (100 μL) was added to the wine sample (5 mL) in a screw cap vial using a glass syringe (100 μL Hamilton). Diethyl ether-pentane (1:2, 2 mL) was added and the mixture was shaken briefly. A portion of the organic layer was then transferred to a vial ready for instrumental analysis.

2.3 Instrumental Analyses

The organic extracts were analysed by gas chromatography - mass spectrometry as described previously [13]. The unlabelled 4-ethylphenol and 4-ethylguaiacol used as standards were purchased from Aldrich. For quantification of 4-ethylphenol and 4-ethylguaiacol, mass spectra were recorded in the Selective Ion Monitoring (SIM) mode. The ions monitored in SIM runs were: *m/z* 111 and 126 for [$^2H_4$]-4-ethylphenol (internal standard), *m/z* 107 and 122 for 4-ethylphenol and *m/z* 122, 137 and 152 for 4-ethylguaiacol. Selected fragment ions were monitored for 40 ms each. The ions used for quantitation were: *m/z* 126 for [$^2H_4$]-4-

ethylphenol (internal standard), *m/z* 122 for 4-ethylphenol and *m/z* 152 for 4-ethylguaiacol. The other ions were used as qualifiers.

2.4 Validation

The method was validated by a series of duplicate standard additions (0 to 5000 μg/L 4-ethylphenol and 4-ethylguaiacol, N=10 x 2 for both compounds) to model wine (10.0% ethanol, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid), white wine (1996 Chardonnay, 13.1% ethanol, pH 3.4) and red wine (1994 Shiraz, 12.6% ethanol, pH 3.4). The standard addition curves obtained were linear throughout the concentration range, with the following coefficients of determination and linear regression equations: $r^2 = 0.999$ for 4-ethylphenol in red ($y = 1.07x + 0.02$) and white ($y = 1.07x$) wines, $r^2 = 1.000$ ($y = 1.18x$) for 4-ethylphenol in model wine, $r^2 = 1.000$ ($y = 1.21x + 0.004$) for 4-ethylguaiacol in red wine and $r^2 = 0.999$ for 4-ethylguaiacol in white ($y = 1.19x$) and model ($y = 1.18x$) wines. Because all red wines apparently contain 4-ethylphenol (the lowest level of 4-ethylphenol found in a bulk red wine was 55 μg/L), the repeatability of the analysis at the lower end of the concentration range (10 μg/L) was determined by spiking seven replicate aliquots of model wine. The coefficient of variance in the model wine was 2.73% for 4-ethylphenol and 1.27% for 4-ethylguaiacol. For seven replicate analyses of the bulk red wine (1995, 10.0% ethanol, pH 3.05, mean concentration of 4-ethylphenol and 4-ethylguaiacol of 55 μg/L and 10 μg/L respectively), the coefficient of variance was 1.35% for 4-ethylphenol and 1.31% for 4-ethylguaiacol. The repeatability of the analysis was also determined at 2000 μg/L by spiking a separate bottled red wine in septuplicate, giving a coefficient of variance of 0.75% for 4-ethylphenol and 0.63% for 4-ethylguaiacol. The same red wine was also used for the standard addition curves above and contained 65 μg/L 4-ethylphenol and 23 μg/L 4-ethylguaiacol.

2.5 Investigation into deuterium exchange during chromatography.

Separate solutions (diluted to *ca* 0.5 mg/mL in dichloromethane) of [$^2H_4$]-4-ethylphenol (**2**) and unlabelled 4-ethylphenol (**1**) were analysed at the start, middle and end of a 200 run sequence of wine extracts. They were injected with the injector block temperature set at 200°C, 220°C and 250°C on each occasion (GC conditions were as above). The ions monitored were *m/z* 118, 119, 120, 121, 122 for 4-ethylphenol (**1**) and *m/z* 122, 123, 124, 125 and 126 for [$^2H_4$]-4-ethylphenol (**2**).

2.6 Determination of 4-ethylphenol and 4-ethylguaiacol in red wine aged in French and American oak barrels of different ages.

All new oak barrels used in the trial, were 'medium toast'. The main 1998 Shiraz red wine had pH = 3.53, titratable acidity = 6.7 g/L (expressed as tartaric acid), alcohol = 13.7%, total SO_2 approx. 40 ppm, (no SO_2 added pre fermentation). Details of other wines are given in the text or in table I.

3. Results and discussion

3.1 Synthesis of 2,3,5,6-[$^2H_4$]-4-ethylphenol (2).

2,3,5,6-[$^2H_4$]-4-Ethylphenol (**2**) was prepared from the acetate of polydeuterated phenol (**3**) via the Fries rearrangement followed by Wolff Kischner reduction (Scheme 1). The formation of deuterium-labelled 4-hydroxyacetophenone (**4**) was accompanied by a loss of slightly more than one deuterium ($^2H_4$:$^2H_3$, 4:1). Small singlets in the nmr spectrum of **4** at δ 6.92 and 7.87 in a ratio of 3:1 indicated that more deuterium was lost from the position *ortho* to the phenol group than from the *meta*-position. Reduction of the side chain of [$^2H_4$]-4-hydroxyacetophenone (**4**) to give labelled 4-ethylphenol (**5**) was accompanied by complete exchange of two of the deuterium atoms which were easily reintroduced by back-exchange with deuterium oxide under strongly acidic conditions. The [$^2H_4$]-analogue (**2**) could not be prepared from unlabelled 4-ethylphenol (**1**) directly, as only two of the aromatic protons (presumably those *ortho* to the phenol group, as shown in Scheme 1) were exchangeable under strongly acidic conditions.

3.2 Investigation into deuterium exchange during sample preparation and chromatography.

The ratios of the molecular ions, m/z 126, 125, 124, 123, 122 of [$^2H_4$]-4-ethylphenol (internal standard) remained the same before and after extraction; thus there was no back exchange into the wine during sample preparation. Indeed, during the synthesis, strong acid and high temperature for a prolonged period was required for exchange *ortho* to the phenol group, and the other hydrogens were not exchanged at all under these conditions. Solutions of [$^2H_4$]-4-ethylphenol and unlabelled 4-ethylphenol were also analysed separately at the start, middle and end of a 200 run sequence of wine extracts. They were injected with the injector block temperature set at 200°C, 220°C and 250°C on each occasion. For all the analyses there was no significant difference in the ratios of the ions monitored for either compound at all injection temperatures. Thus no significant deuterium exchange occurred during sample preparation, injection or chromatography.

3.3 The analytical method

Using [$^2H_4$]-4-ethylphenol as internal standard, 4-ethylphenol and 4-ethylguaiacol in wine could be quantified in pentane-diethyl ether (2:1) extracts at concentrations down to 1 μg/L and often lower. The internal standard [$^2H_4$]-4-ethylphenol differs from 4-ethylguaiacol in volatility and in solubility in non-polar solvents, as evidenced by experiments using solid-phase microextraction (SPME) for the analysis or employing pentane as an extracting solvent (data not shown). Nevertheless, under the conditions described in this paper (extraction with pentane-diethyl ether (2:1)), the similarity in behaviour between 4-ethylguaiacol and [$^2H_4$]-4-ethylphenol was such that the precision and accuracy for determining 4-ethylguaiacol were as good as those for 4-ethylphenol. Under conditions recently developed for oak lactone analysis [13], 4-ethylphenol could be determined with equal sensitivity by SPME of the headspace above the wine. However, because of differences in volatility between analyte and standard, the SPME method was less reliable for 4-ethylguaiacol.

The relative intensity of mass spectral fragments for fixed concentrations of [$^2H_4$]-4-ethylphenol, unlabelled 4-ethylphenol and 4-ethylguaiacol varied according to the instrumental operating conditions. It is therefore important to determine the relative molar ion response factors for standard solutions of all three compounds under the same instrumental conditions as employed for the analyses of each set of wine samples.

There are several published methods for determining volatile phenols in wine [eg. 2, 3, 7, 11, 14], but the advantages of the method described in this paper are that it is precise, accurate,

has low detection limits, uses only 5 mL of wine, and sample preparation takes just a few minutes.

Furthermore the analyses for 4-ethylphenol and 4-ethylguaiacol can be combined with those for other oak volatile compounds, eg. *cis*- and *trans*-oak lactone [13], vanillin [15], vanillyl ethyl ether [16], guaiacol and 4-methylguaiacol (unpublished data), ie the compounds analysed in this manner need not be of similar chemical structures, providing that isotopically labelled analogues are used as standards.

3.4 Determination of 4-ethylphenol and 4-ethylguaiacol in red wine aged in French and American oak barrels of different ages.

The concentrations of 4-ethylphenol and 4-ethylguaiacol in red wine aged in new and used French and American oak barrels are shown in Table I. Coopers were instructed to give all these barrels a medium toast at the time of firing. All the red wines were fermented on skins for 5-7 days and went through partial malolactic fermentation prior to going into the barrel. It is not certain that all the 4-ethylphenol was generated only in the barrel, as measurements of the wine were not taken prior to storage. Nevertheless, some differences between groups of barrels were observed.
A Cabernet Sauvignon / Shiraz red wine blend matured in new American oak had significantly less 4-ethylphenol and 4-ethylguaiacol (201 µg/L and 24 µg/L respectively) than the same blend aged in barrels previously used once (391 µg/L and 35 µg/L respectively). A Shiraz red wine stored in American oak barrels, previously used two to four times, had a mean 4-ethylphenol concentration of 541 µg/L, with no significant difference in the level of 4-ethylphenol between the barrels previously used two, three or four times. However, shaving and firing the barrels previously used four times resulted in a substantial decrease (of over 80%) in the concentration of 4-ethylphenol and 4-ethylguaiacol in the stored wine.

For the French oak, the previous usage of the barrels had no significant effect on the amount of 4-ethylphenol or 4-ethylguaiacol found in the wine. However shaving and re-firing the barrels previously used three times resulted in a reduction of the mean 4-ethylphenol concentration by over 20% (from 514 µg/L to 401 µg/L).
The effects of shaving and refiring old French and American oak barrels can be attributed to a reduction of the microbiological load on the inner surface of the barrel, prior to use [2, 3].

As a separate trial, the same 1998 Shiraz red wine was aged in 19 different types of new 300 L oak barrels of either French or American origin, made by 11 different cooperages. Two barrels of each type were analysed by the method. There were similar amounts of 4-ethylphenol (range 385 - 680 µg/L) and 4-ethylguaiacol (range 28 - 45 µg/L) found in all of the barrels. The mean 4-ethylphenol concentration was 496 µg/L with a 21% coefficient of variance (cv). The mean 4-ethylguaiacol concentration was 31 µg/L with a 5% cv. There was no significant difference in 4-ethylphenol and 4-ethylguaiacol content between wine aged in French compared to American oak, nor was there any significant difference between wines aged in fine or medium grained oak.

Chatonnet *et al* [2], using the methodology of Boidron *et al* [6], determined detection thresholds of 605 µg/L and 110 µg/L for 4-ethylphenol and 4-ethylguaiacol respectively in a red wine. The aroma of the wines in all of the barrels are therefore unlikely to be strongly affected by these two compounds.

3.5 Analysis of 61 Commercial Australian Red Wines

Sixty one bottles of different commercially available single variety Australian red wines were analysed by the method. The results are shown in Table II. It must be stressed that these wines are not necessarily representative of Australian red wines as too few samples were selected and there may well be some regional biases (ie some regions over-represented and many regions not represented at all).

4-Ethylphenol was detected in every red wine. The concentrations found in the wines varied between 2 μg/L in a Merlot and 2660 μg/L in a Shiraz, with a mean concentration of 795 μg/L. (The highest level of 4-ethylphenol we have observed whilst analysing "problem wines" is 4500 μg/L.) 4-Ethylguaiacol was also found in every red wine analysed, varying in concentration from 1 μg/L (in a Pinot Noir) up to 437 μg/L (in a Merlot) with a mean concentration of 99 μg/L. Within the wines of each variety, a wide range of concentrations was observed, consistent with the results of Chatonnet *et al* [2, 3] who have demonstrated the importance of winemaking practices on the formation of 4-ethylphenol and 4-ethylguaiacol. Although not enough wines were analysed to make a comprehensive investigation into the relationship between variety and 4-ethylphenol concentration, some trends were nevertheless observed. The mean concentration of 4-ethylphenol found in the Cabernet Sauvignon wines (1250 μg/L) was greater than that of Shiraz (605 μg/L) with 95% confidence and Pinot Noir (338 μg/L) with 99% confidence. Too few Merlots were analysed to make any significant comparisons, especially considering that the level of 4-ethylphenol found in the nine Merlots analysed ranged from 2 μg/L up to 2200 μg/L. There was no significant difference in 4-ethylguaiacol concentration between varieties.

The ratio of 4-ethylphenol to 4-ethylguaiacol also varied from wine to wine. The average ratio was approximately 10:1 for Cabernet Sauvignon, 9:1 for Shiraz, 8:1 for Merlot and 3.5:1 for Pinot Noir. The ratio difference between Shiraz and Pinot Noir was significant with 95% confidence.
Apart from a Merlot which had 2 μg/L of each compound, the lowest ratio of 4-ethylphenol to 4-ethylguaiacol observed was 193:121 = 1.6:1 in a 1995 Pinot Noir. The highest ratio was 709:31 = 23:1 in a 1995 Shiraz.

Goldberg *et al* [14] measured the concentration of *p*-coumaric acid, the precursor to 4-ethylphenol, in single-variety red wines from various countries. The range in *p*-coumaric acid concentration can be compared to that observed for 4-ethylphenol in the bottled red wines studied here. Among the Australian wines, *p*-coumaric acid was lowest in Pinot Noir as compared to Shiraz and Cabernet Sauvignon. In Californian wines, Pinot Noir was also equally lowest in *p*-coumaric acid (along with Zinfandel) and in South African wines, Pinot Noir had the lowest *p*-coumaric acid of the six varieties assayed. Indeed, Goldberg *et. al* [14] found Pinot Noir generally had low levels of *p*-coumaric acid across all varieties and countries.

Thus, although wine maturation conditions are paramount in determining the concentration of 4-ethylphenol and 4-ethylguaiacol to be found in commercial wines [2, 3], genetic or cultural factors may also be influential. Further experiments are necessary to test this hypothesis.

4. Conclusions

The analytical method described here is fast, precise, accurate and reliable. Combined with automated instrumental analysis, the method has enabled our laboratory to measure 4-ethylphenol and 4-ethylguaiacol, along with other oak volatiles in the same analysis, in wines in large numbers. The method is now widely used in problem solving and is used in a commercial analytical service to support oak-barrel quality trials and evaluation of cellar practices throughout the Australian wine industry.

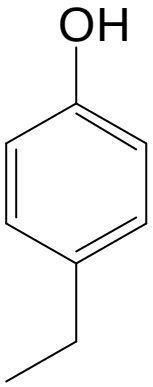
5. Acknowledgments

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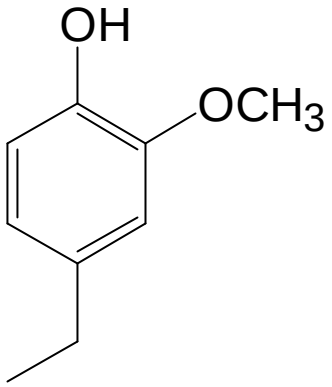
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Figure 1. Structures of 4-ethylphenol and 4-ethylguaiacol.

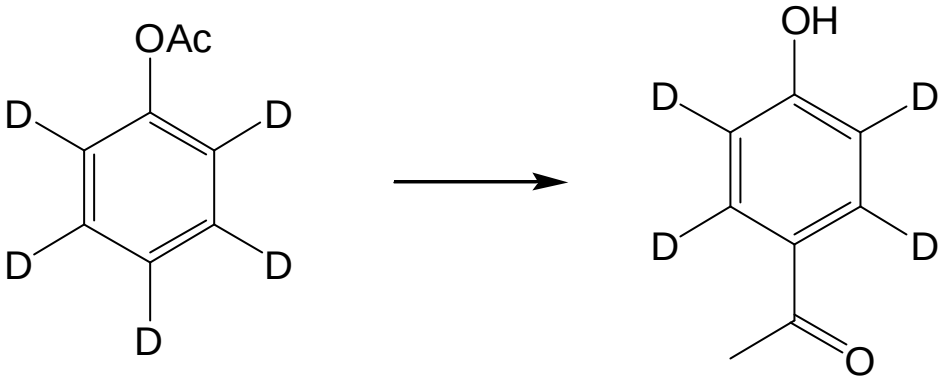


4-Ethylphenol

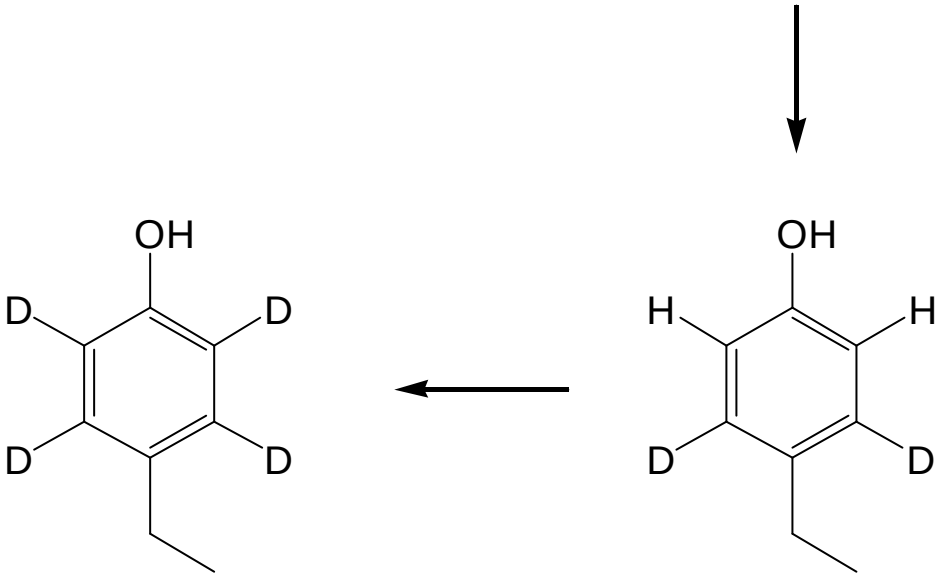


4-Ethylguaiacol

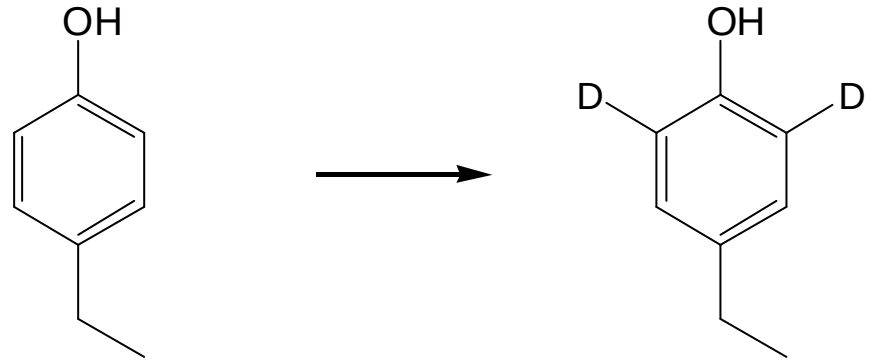
Scheme 1. Formation of deuterium-labelled analogues of 4-ethylphenol (1)



(3) **(4)**



(2) **(5)**



(1) **(6)**

Table I Determination of 4-ethylphenol and 4-ethylguaiacol in red wines aged in French and American oak barrels of different ages.

1998 Cabernet Sauvignon / Shiraz, pH=3.52, titratable acidity = 7.2 g/L (expressed as tartaric acid), alcohol = 14.3%, total SO_2 approx 35 ppm (no SO_2 added pre fermentation).

Oak Origin	**Previous Barrel Use**	**4-Ethylphenol Conc. / Coeff. of var.**		**4-Ethylguaiacol Conc. / Coeff. of var.**	
American Oak	New	201 µg/L	6%	24 µg/L	4%
American Oak	used once	391 µg/L	8%	35 µg/L	10%

1998 Shiraz, pH=3.55, titratable acidity = 6.8 g/L (expressed as tartaric acid), alcohol = 14.3%, total SO_2 approx 40 ppm (no SO_2 added pre fermentation).

Oak Origin	**Previous Barrel Use**	**4-Ethylphenol Conc. / Coeff. of var.**		**4-Ethylguaiacol Conc. / Coeff. of var.**	
American Oak	used twice	563 µg/L	5%	31 µg/L	3%
American Oak	used 3 times	505 µg/L	9%	31 µg/L	10%
American Oak	used 4 times	555 µg/L	11%	31 µg/L	13%
American Oak (re-shaved and fired 1999)	used 4 times	95 µg/L	29%	5 µg/L	30%

1998 Shiraz, pH=3.53, titratable acidity = 6.7 g/L (expressed as tartaric acid), alcohol = 13.7%, total SO_2 approx 40 ppm (no SO_2 added pre fermentation).

Oak Origin	**Previous Barrel Use**	**4-Ethylphenol Conc. / Coeff. of var.**		**4-Ethylguaiacol Conc. / Coeff. of var.**	
French Oak	New	540 µg/L	24%	34 µg/L	19%
French Oak	used once	500 µg/L	8%	28 µg/L	6%
French Oak	used twice	499 µg/L	15%	33 µg/L	14%
French Oak	used 3 times	514 µg/L	8%	24 µg/L	6%
French Oak (re-shaved and fired 1999)	used 3 times	401 µg/L	14%	23 µg/L	10%

Concentrations shown are the mean from the analysis of five barrels and are expressed in µg/L of red wine. All wines had matured in the barrels for approximately 8 months. Wine samples (5 mL) were taken and the internal standard added immediately on site.

Table II Determination of 4-ethylphenol and 4-ethylguaiacol in 61 different bottles of commercial Australian red wines.

Cabernet Sauvignon

Vintage	4-Ethylphenol (mg/L)	4-Ethylguaiacol (mg/L)	Ratio 4-EP/4-EG
1986	2060	240	8.6
1992	267	45	5.9
1992	2450	141	17.4
1992	851	61	14.0
1993	2150	226	9.5
1994	594	47	12.6
1994	697	69	10.1
1994	1840	187	9.8
1994	1530	104	14.7
1994	683	72	9.5
1994	688	96	7.2
1994	518	45	11.5
1994	1130	145	7.8
1994	1870	115	16.3
1995	1130	295	3.8
1995	1240	134	9.3
1995	834	73	11.4
1997	1910	129	14.8
Mean	**1250**	**124**	**10.1**

Merlot

Vintage	4-Ethylphenol (mg/L)	4-Ethylguaiacol (mg/L)	Ratio 4-EP/4-EG
1988	604	62	9.7
1991	2200	437	5.0
1994	1820	165	11.0
1995	528	61	8.7
1995	2100	232	9.1
1995	1280	113	11.3
1995	23	6	3.8
1996	2	2	1.0
1996	324	72	4.5
Mean	**987**	**128**	**7.7**

Pinot Noir

Vintage	4-Ethylphenol (mg/L)	4-Ethylguaiacol (mg/L)	Ratio 4-EP/4-EG
1986	3	1	3.0
1991	51	21	2.4
1992	114	28	4.1
1994	1240	421	2.9
1995	58	13	4.5
1995	202	98	2.1
1995	1560	311	5.0
1995	32	23	1.4
1995	83	35	2.4
1995	193	121	1.6
1996	197	39	5.1
1996	498	126	4.0
1996	169	28	6.0
Mean	**338**	**97**	**3.5**

Shiraz			
Vintage	**4-Ethylphenol (mg/L)**	**4-Ethylguaiacol (mg/L)**	**Ratio 4-EP/4-EG**
1987	633	75	8.4
1987	275	75	3.7
1988	186	51	3.6
1989	82	12	6.8
1993	115	12	9.6
1994	1310	84	16.0
1994	282	66	4.3
1994	1580	99	16.0
1995	232	38	6.1
1995	407	67	6.1
1995	113	6	18.8
1995	709	31	22.9
1995	844	57	14.8
1995	258	29	8.9
1995	524	78	6.7
1995	283	28	10.1
1996	169	14	12.1
1996	572	44	13.0
1996	1390	161	8.6
1996	72	9	8.0
1996	2660	350	7.6
Mean	**605**	**66**	**9.2**
All varieties			
Mean	**795**	**99**	**8.0**

4-EP = 4-ethylphenol
4-EG = 4-ethylguaiacol

4-Ethylphenol, 4-ethylguaiacol and oak lactones in Australian red wines.

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The origins of aroma and flavour in wine are diverse and far from completely understood. Much, of course, is derived from the grape, although not all such grape-derived flavour is evident in the fruit prior to vinification. Some flavour characteristics, especially those associated with bottle-aging are released as a result of the transformation of involatile, flavourless grape metabolites into volatile aroma compounds, catalysed by the acidic medium of the wine. The formation, in old Riesling wines, of 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), which has a kerosene-like aroma, is one example of a particular wine character formed in this way (Winterhalter et al. 1990).

It is becoming increasingly recognised that micro-organisms are also able to generate flavour from flavourless grape-derived precursors. Thus, during alcoholic fermentation, yeasts can not only produce a variety of esters which may contribute to a wine's fruity character, but are also capable of releasing several sulphur compounds, probably from cysteine conjugates (Tominaga et al. 1998). Among the most important of these is the recently identified 4-methyl-4-mercapto-2-pentanone which contributes tropical fruit/catty-like odours to white wine, especially Sauvignon Blanc (Darriet et al. 1995).

Several important volatile phenols found in wine are formed by microbiological transformations of cinnamic acid derivatives which also have their origin in the grape. Two of these volatile phenols are 4-ethylphenol and 4-ethylguaiacol which nearly always occur together, and which are formed in red (but not white) wine from *p*-coumaric acid and ferulic acid respectively by yeasts of the *Brettanomyces / Dekkera* genus (Chatonnet et al. 1992, 1995). In grapes, these acids are mainly bound up as tartrate esters. The aroma associated with 4-ethylphenol in red wine has been varyingly described as 'horsy', 'leather', 'medicinal', 'smoky', 'barnyard', 'animal' and 'sweaty saddle'-like, while 4-ethylguaiacol in wine has a smoky, spicy, clove-like aroma (Boidron et al. 1988, Chatonnet et al. 1992, Olsen 1994). At higher concentrations these odours can be undesirable, especially those from 4-ethylphenol. While 4-ethylphenol and 4-ethylguaiacol are apparently ubiquitous in red wine, the concentration found can vary considerably (Chatonnet et al. 1992, 1995).

We have recently synthesised d_4-4-ethylphenol for use as an internal standard in a new, rapid and accurate analytical method, employing gas chromatography - mass spectrometry to determine the concentration of 4-ethylphenol and 4-ethylguaiacol in red wine (Pollnitz et al. 2000). Combined with automated instrumental analysis, the method has enabled our laboratory to measure these compounds in a large number of wines, along with other oak-derived volatile compounds. The method is now widely used in problem solving investigations and is offered as a commercial analytical service to support oak-barrel quality trials and evaluation of cellar practices throughout the Australian wine industry (see http://winetitles.com.au/awri/oak.html).

As an example of this, the concentration of 4-ethylphenol and 4-ethylguaiacol in red wine stored in a local winery in 44 American and 47 French new and used oak barrels from several suppliers was recently measured. In general, it was found that wine stored in new oak contained less of these two compounds than did wine stored in used barrels, while wine stored in shaved and refired oak barrels contained up to 85% less 4-ethylphenol and 4-ethylguaiacol than wine stored in barrels of the same age that had not been shaved. This latter observation can most likely be attributed to a reduction of the microbiological load on the inner surface of

the barrel, prior to use as a result of the shaving and refiring process (Chatonnet et al. 1992, 1995).

As a separate trial, a 1998 Shiraz red wine was aged in 19 different types of new 300 L oak barrels of either French or American origin, made by 11 different cooperages. Two barrels of each type were analysed. There were similar amounts of 4-ethylphenol (range 385 - 680 µg/L) and 4-ethylguaiacol (range 28 - 45 µg/L) in red wine sampled from all of the barrels after 8 months storage. There was no statistically significant difference in 4-ethylphenol and 4-ethylguaiacol content between wine aged in French compared to American oak, nor was there any significant difference between wines aged in fine or medium grained oak.

Chatonnet *et al* (1992) determined individual detection thresholds of 605 µg/L and 110 µg/L for 4-ethylphenol and 4-ethylguaiacol respectively in a red wine. However, the sensory impact of these two compounds is additive, and for a mixture of 4-ethylphenol and 4-ethylguaiacol in a ratio of 10:1 (typical of the ratio commonly found in red wine), the detection threshold was 335 µg/L of 4-ethylphenol plus 34 µg/L of 4-ethylguaiacol. The aroma of the wines stored in each of the barrels is therefore likely to be only moderately affected by these two compounds.

This is certainly not the case with all Australian red wines however. We have also analysed sixty-one bottles of different commercially available single variety Australian red wines that had been cellar-stored at the AWRI. The wine vintages ranged from 1986 to 1997.

4-Ethylphenol was detected in every red wine (Table 1). The concentrations found in the wines varied between 2 µg/L in a Merlot and 2660 µg/L in a Shiraz, with a mean concentration of 795 µg/L. A third of the bottles contained in excess of 1000 µg/L of 4-ethylphenol, while one in ten had over 2000 µg/L (the highest level of 4-ethylphenol we have observed whilst analysing "problem wines" now stands at 8000 µg/L). 4-Ethylguaiacol was also found in every red wine analysed, varying in concentration from 1 µg/L (in a Pinot Noir) up to 437 µg/L (in a Merlot) with a mean concentration of 99 µg/L. Within the wines of each variety, a wide range of concentrations was observed, consistent with the results of Chatonnet *et al* (1992, 1995) who have demonstrated the importance of winemaking practices on the formation of 4-ethylphenol and 4-ethylguaiacol.

Although not enough wines were analysed to draw firm conclusions about the relationship between variety and 4-ethylphenol/4-ethylguaiacol concentration, some trends were nevertheless observed. A high concentration of 4-ethylphenol was particularly evident with many of the Cabernet Sauvignon wines. A larger proportion of Shiraz and Pinot Noir wines contained sub-threshold amounts of 4-ethylphenol, as indicated by the median values, although high concentrations (> 1000 µg/L) were still detected in some of these wines.

The ratio of 4-ethylphenol to 4-ethylguaiacol also varied from wine to wine (Table 1). The average ratio was approximately 10:1 for Cabernet Sauvignon, 9:1 for Shiraz, 8:1 for Merlot and 3.5:1 for Pinot Noir. The ratio difference between Shiraz and Pinot Noir was significant with 95% confidence. Apart from a Merlot which had only 2 µg/L of each compound, the lowest ratio of 4-ethylphenol to 4-ethylguaiacol observed was 32:23 = 1.4:1 in a 1995 Pinot Noir. The highest ratio was 709:31 = 23:1 in a 1995 Shiraz.

Several factors are likely to be conducive to the formation of excessive amounts of 4-ethylphenol and 4-ethylguaiacol in red wines. These include inadequate sanitation , the use of oak cooperage, especially used barrels, high wine pH and maturation temperature, and low SO_2 concentration, conditions which are likely to encourage the growth of *Brettanomyces* yeasts (Olsen 1994). The concentration of *p*-coumaric and ferulic acid, the precursors to 4-ethylphenol and 4-ethylguaiacol, in the wine could also be a determining factor. Additional

amounts of these two compounds can result from the use of certain pectolytic enzyme preparations or fermentation yeast strains which have esterase activity and can generate *p*-coumaric and ferulic acid by hydrolysis of the corresponding tartrate esters.

Goldberg et al. (1998) measured the concentration of *p*-coumaric acid in single-variety red wines from various countries. Among the Australian wines, *p*-coumaric acid was lowest in Pinot Noir as compared to Shiraz and Cabernet Sauvignon wines. This compares with the generally low amounts of 4-ethylphenol found in the 13 bottles of Pinot Noir wine analysed to give the data in Table 1. Thus, although wine maturation conditions are paramount in determining the concentration of 4-ethylphenol and 4-ethylguaiacol to be found in commercial wines (Boidron et al. 1988, Chatonnet et al. 1992, Olsen 1994), genetic or cultural factors may also be influential. Further experiments are necessary to test this hypothesis.

Oak-derived wine components

Because some oak-derived wine components,namely guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone (Pollnitz et al. 1999) and vanillin (Spillman et al. 1997a) can be analysed in wines along with 4-ethylphenol and 4-ethylguaiacol in a single analysis, the concentration of these oak-derived compounds was also determined in all of the 61 commercial bottles of wine, as well as the wines in the barrel trials referred to above. There was no significant difference between the varieties in the amount of vanillin or 4-methylguaiacol in the bottled wines. The concentration of vanillin in these wines ranged from 34 to 364 µg/L while that of 4-methylguaiacol varied between 1 and 26 µg/L.

The concentration of guaiacol, which is formed during barrel toasting, varied from 10 to 72 µg/L. There was significantly more guaiacol in the Shiraz wines (mean concentration 38 µg/L) than the other varieties (mean concentrations of 20-23 µg/L). This is unlikely to be due to the use of more heavily toasted barrels in Shiraz winemaking, as the mean concentration of 4-methylguaiacol, which is also formed during barrel toasting, was virtually identical across the varieties. Guaiacol has been previously identified in hydrolysates of glycosidic fractions from Cabernet Sauvignon and Merlot grape samples (Sefton, 1998), so it is possible that some of the guaiacol in the wines, particularly the Shiraz wines, was also derived from the grape.

Sensory thresholds for vanillin, 4-methylguaiacol and guaiacol in red wine have been determined by Boidron et al (1988) as 320, 65 and 75 µg/L respectively. The determination of sensory thresholds of wine components can vary substantially, depending on wine type, panel accuity, and experimental methodology, and individuals can vary enormously in their sensitivity to such components. Any threshold determination should therefore always be interpreted with caution. On the basis of the above figures, we might expect these three compounds to have no great impact on the aroma and flavour of the wines in Table 1 for a majority of consumers.

In sensory terms the most important oak-derived compound is generally assumed to be the *cis*-oak lactone (e.g. Boidron et al. 1988, Spillman et al. 1997b). Despite its apparent importance, however, the sensory threshold in wine of the naturally occuring form of *cis*-oak lactone (which is not commercially available) has never been determined. Chatonnet et al (1990) reported thresholds for a commercial mixture of racemic *cis* plus *trans* isomers to be 80 µg/L in a red wine.

The concentration of *cis*-oak lactone and the ratio of the *cis*/*trans* isomers is also shown in Table 1. As oak lactones have only been detected in wines that have been matured in oak barrels or treated with other oak products. it would appear that virtually all of the wines in

Table 1 have also had some oak contact during their production. Both the absolute amount of *cis*-oak lactone and the *cis*/*trans* ratio varied widely in the bottled wines.

Waterhouse and Towey (1994) have found that the ratio of the *cis*/*trans* oak lactones in wines stored in eight lots of European oak (French and Hungarian) varied from 1 to 1.6, while for wines in seven lots of American oak (excluding one lot of Oregan oak) the ratio ranged from 5 to 8. These data are similar to those obtained by Guichard et al (1995) who observed ratios between 0.3 and 2 for brandies stored in French oak and from 7 to 10 for those stored in American oak. In these and similar studies, the *cis*/*trans*-isomer ratio is likely to be slightly underestimated (by as much as 20%) as the analytical methods used do not take into consideration the different volatilities of the two isomers. In the red wines stored in 44 American and 47 French new and used oak barrels from several suppliers, referred to earlier, we observed ratios of between 1 and 2 for the wines matured in the French oak while for those kept in American oak the ratio varied from 8 to 10.

Based on these observations, there appears to be some differences in oak usage between the wines analysed to give the data in Table 1, with French (or other European) oak apparently favoured for the production of the Pinot Noir wines, while American oak seems to have been predominantly used in the maturation of the Shiraz wines. Some products, of course, may have been aged in a mixture of both oak types. In several of the wines the ratio of *cis*- to *trans*-oak lactones is much higher than 10. This may simply indicate that the range of ratios in oak products is somewhat higher than indicated by previous trials. Alternatively, it is possible that, during bottle maturation, *cis*-oak lactone is generated faster than the *trans*-isomer from precursor forms that have also been extracted from the oak. However, such a mechanism for enhancing oak lactone concentration in wine has not so far been demonstrated.

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Footnote: Sections of this article are reprinted from Pollnitz et al. (2000), Use of Oak Barrels in Winemaking - Proceedings of an oenology seminar held in Adelaide, October 1999, M. Allen, G. wall (Eds), Winetitles, Adelaide, pp10-11., with permission from Australian Society of Viticulture and Oenology. Full details of the work on 4-ethylphenol and 4-ethylguaiacol described here are given in Pollnitz, A.P.; Pardon, K.H.; Sefton, M.A. (2000) Quantitative analysis of 4-ethylphenol and 4-ethylguaiacol in red wine. J. Chromatography A. 874: 101-109. Copies of the latter paper are available from:

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Table I Concentration (mg/L) of 4-ethylphenol and *cis* oak lactone, and the ratio of 4-ethylphenol: 4-ethylguaiacol and cis-:trans oak lactone in 61 different bottles of commercial Australian red wines.

Cabernet Sauvignon

Vintage	4-Ethylphenol	4-EP/4-EG	*cis*-Oak lactone	*cis/trans*
1986	2060	8.6	178	1.9
1992	267	5.9	320	1.7
1992	2450	17.4	192	3.6
1992	851	14.0	183	2.4
1993	2150	9.5	157	1.7
1994	594	12.6	410	13.3
1994	697	10.1	434	13.6
1994	1840	9.8	417	4.5
1994	1530	14.7	206	2.0
1994	683	9.5	232	2.8
1994	688	7.2	779	11.1
1994	518	11.5	213	10.3
1994	1130	7.8	428	5.8
1994	1870	16.3	149	8.0
1995	1130	3.8	214	9.3
1995	1240	9.3	143	1.9
1995	834	11.4	887	18.5
1997	1910	14.8	183	1.4

Merlot

Vintage	4-Ethylphenol	4-EP/4-EG	*cis*-Oak lactone	*cis/trans*
1988	604	9.7	118	2.7
1991	2200	5.0	160	1.6
1994	1820	11.0	267	3.0
1995	528	8.7	136	2.0
1995	2100	9.1	263	7.3
1995	1280	11.3	395	10.7
1995	23	3.8	tr	n.d.
1996	2	1.0	74	2.8
1996	324	4.5	197	3.9

Pinot Noir

Vintage	4-Ethylphenol	4-EP/4-EG	*cis*-Oak lactone	*cis/trans*
1986	3	3.0	80	1.5
1991	51	2.4	266	2.2
1992	114	4.1	191	1.7
1994	1240	2.9	103	2.2
1995	58	4.5	34	17.0
1995	202	2.1	186	1.5
1995	1560	5.0	221	1.6
1995	32	1.4	159	1.6
1995	83	2.4	80	1.7
1995	193	1.6	306	1.9
1996	197	5.1	71	4.4
1996	498	4.0	83	13.8
1996	169	6.0	104	1.6

Shiraz

Vintage	4-Ethylphenol	4-EP/4-EG	*cis*-Oak lactone	*cis/trans*

1987	633	8.4	828	9.2
1987	275	3.7	794	11.3
1988	186	3.6	750	11.0
1989	82	6.8	90	1.7
1993	115	9.6	621	12.9
1994	1310	16.0	103	1.8
1994	282	4.3	533	5.4
1994	1580	16.0	630	8.2
1995	232	6.1	153	3.8
1995	407	6.1	622	6.3
1995	113	18.8	79	19.8
1995	709	22.9	78	15.6
1995	844	14.8	102	>100
1995	258	8.9	295	7.8
1995	524	6.7	219	7.6
1995	283	10.1	15	>15
1996	169	12.1	39	>39
1996	572	13.0	298	11.5
1996	1390	8.6	462	11.6
1996	72	8.0	404	12.8
1996	2660	7.6	553	9.4

4-EP = 4-ethylphenol; 4-EG = 4-ethylguaiacol; *cis* = *cis*-oak lactone; *trans* = *trans*-oak lactone; tr = trace; n.d. = not determined.

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Acid catalysed hydrolysis of alcohols and their beta D glucopyranosides

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Dedicated by the authors to the memory of the late Evangelos Cotsaris, a fellow scientist.

Keywords : β-Damascenone, geraniol, monoterpenes, norisoprenoids, hydrolysis, glycosides, precursors.

ABSTRACT

The hydrolysis, in model wine at pH 3, of the allylic, homoallylic and propargylic glycosides, geranyl-β-D-glucopyranoside, [3'-(1''-cyclohexenyl)-1'-methyl-2'-propynyl]-β-D-glucopyranoside, (3'*RS*,9'*SR*)-(3'-hydroxy-5'-megastigmen-7-yn-9-yl)-β-D-glucopyranoside, (3',5',5'-trimethyl-3'-cyclohexenyl)-β-D-glucopyranoside, *E*-(7'-oxo-5',8'-megastigmadien-3'-yl)-β-D-glucopyranoside (3-hydroxy-β-damascone-β-D-glucopyranoside), and their corresponding aglycons have been studied. In general, aglycons were more rapidly converted to transformation products than were the corresponding glucosides. Glycoconjugation of geraniol in grapes is a process that reduces the flavor impact of this compound in wine, not only because geraniol is an important flavor component of some wines but also because the rate of formation of other flavor compounds from geraniol during bottle-ageing is reduced. However, where flavor compounds such as β-damascenone are formed in competition with flavorless by-products, such as 3-hydroxy-β-damascone, by acid catalysed hydrolytic reactions of polyols, then glycoconjugation is a process that could enhance as well as suppress the formation of flavor, depending on the position of glycosylation. At 50 °C, (3'*RS*,9'*SR*)-(3'-Hydroxy-5'-megastigmen-7'-yn-9'-yl)-β-D-glucopyranoside hydrolysed more slowly, but gave a higher proportion of β-damascenone in the products than did the aglycon.

INTRODUCTION

In wines and other beverages, the development of flavor is of great importance to the consumer. Flavor is derived from a range of compounds, differing in chemical functionality. These compounds, which originate from primary or secondary metabolic pathways in grape berries or other fruits, can be formed by many routes such as the mevalonic acid, shikimate and polyketide pathways as well as from carotenoid breakdown products (Ohloff et al., 1973, Sefton et al., 1989, Williams et al., 1992; Razungles et al., 1993). Many compounds apparently important to wine flavor such as β-damascenone (**1**) are thought to be derived from the hydrolytic breakdometabolites wn of grape-derived conjugates (mainly glycoconjugates) of complex secondary (Francis et al., 1992, 1999; Sefton et al., 1993; Sefton et al., 1994, Winterhalter and Skouroumounis, 1996), yet little is known about the comparative hydrolytic behaviour of such conjugates and their corresponding aglycons.

This paper reports the mild acid catalysed hydrolyses of some model and naturally occurring allylic, homoallylic and propargylic alcohols and their β-D-glucopyranosides. The synthesis

of these β-D-glucopyranosides has been described previously (Skouroumounis et al., 1995). Some of the hydrolytic data have also been reported in a preliminary form (Skouroumounis et al., 1992; 1993).

EXPERIMENTAL PROCEDURES

Preparation of Substrates. The alcohols (**2-6)** and the corresponding β-D-glucopyranosides (**2a-6a**), figure 1, were obtained or synthesized as described previously Skouroumounis et al., (1995) except that 3-hydroxy-β-damascone (**6**) was synthesized by refluxing the acetylenic triol (**26**), (Massy-Westropp et al., 1992), in 0.1N HCl for 1hour. The reaction mixture was cooled and extracted with dichloromethane. The aqueous layer was then salted out (sodium chloride), unreacted acetylenic triol was extracted with ethyl acetate, dried and the above procedure repeated until only trace amounts of acetylenic triol (**26**) was left. The dichloromethane extracts were combined, evaporated and the oil directly chromatographed under standard conditions, (Still et al., 1978) using diethyl ether as solvent giving 3-hydroxy-β-damascone (**6**) as a clear oil (70%) and β-damascenone (**1**), (16%). 1-(1'-Cyclohexenyl)-2-buten-1-one **(21)** was synthesized by the method of (Richter and Otto, 1990). All chiral substrates used in the hydrolytic studies were racemates.

Preparation of Hydrolysates : Hydrolytic studies of substrates were carried out with 10% aqueous ethanolic solutions (model wines). The hydrolyses were conducted at pH 1.0, 1.1 or 3.0, in sealed ampoules. Before sealing the ampoules, the solutions were deoxygenated with a stream of oxygen-free dry nitrogen, (Shriver, 1969), for 15 seconds. All hydrolyses were done in duplicate. A constant temperature oven was used for experiments conducted at 50°C over extended periods (greater than 2 days). Constant temperature oil baths were used at 50, 80 or 100°C for hydrolyses carried out over shorter periods. The hydrolytic media were made up as follows: model wines at pH=1.0, (+)- Tartaric acid was dissolved in 10% aqueous ethanol and the pH adjusted with sulphuric acid (1M). pH=1.1, 0.1N HCl solution. pH=3.0, 10% aqueous ethanol was saturated with potassium hydrogen tartrate and the pH adjusted with sulphuric acid (1M); pH was determined with a pH meter, standardized with a reference solution (Weast, 1979-1980). D_2O experiments, pH =1.0 or 3.0, tartaric acid was dissolved in D_2O and the water was evaporated under reduced pressure. This was repeated twice. Finally, the tartaric acid (d_4) was dissolved in D_2O and the pH adjusted with D_2SO_4 for pH =1.0 and with anhydrous potassium carbonate for pH=3.0. The aglycon (**6**) used for hydrolytic studies in D_2O was dissolved in deuterated ethanol (EtOD) and solvent was evaporated. This was repeated three times to ensure complete deuterium exchange. Finally, hydrolysis was carried out under model wine conditions.

The following amounts of aglycon or glycoside (mg/ml of hydrolytic medium) and internal standard (I.S.) were used: Geraniol (**2**) or its β-D-glucopyranoside (**2a**) (each 1 mg/ml) with 2-phenylethanol (I.S., 1 mg/ml); enyne alcohol (**3**), the β-D-glucopyranoside (**3a**), 3,5,5-trimethyl-3-cyclohexen-ol (**5**) or the β-D-glucopyranoside (**5a**) (each 1 mg/ml, with n-butyl benzene [I.S., 1 mg/ml]); enynediol (**4**) (0.5 mg/ml) the β-D-glucopyranoside (**4a**) (2 mg/ml), or the β-D-glucopyranoside (**6a**) (1 mg/ml) each with octadecanol (I.S., 1 mg/ml). All internal standards were added as dichloromethane solutions after hydrolysis but before work-up.

Analysis of Hydrolysates.

Hydrolysates were worked up by cooling the ampoules, adding the internal standard, then extracting with dichloromethane (2x1ml). The dichloromethane layer was washed with saturated sodium hydrogen carbonate (1ml), dried ($MgSO_4$), filtered and injected directly into the GC for analysis. For the hydrolyses of enynediol (**4**) the dichloromethane extract was concentrated by distillation through a column of Fenske's helices prior to analysis. Analyses were performed with a Varian 3300 gas chromatograph coupled to either a Finnigan TSQ 70 mass spectrometer or a flame ionisation detector. The column used was a 30m J &

W DB 1701 fused silica column (0.25 mm i.d. and 0.25 µm film thickness) with helium carrier gas at a linear velocity of 40 cm/sec. Injections were made with the injector at 200°C, and the splitter, with a split ratio of 1:10, was opened after 0.3 minutes. Flame ionisation detection was used for compound quantification with a Hewlett Packard auto integration system. For identification, hydrolysates were analysed by GC/MS under the following conditions: The column was held for one minute at 50°C, or (100°C for compounds (**4)**, (**4a)**, (**6)** and (**6a**), then programmed at 4°C / min to 250°C and held at that temperature for 20 minutes. Electron impact spectra were taken at 70 eV.

Identification of Hydrolysis Products

^{1}H nmr spectra were recorded with a Jeol JNM-PMX (60 MHz) or a Bruker CXP300 (300 MHz) spectrometer. All compounds in Table 1 and 2, (figure 2) have been identified previously by Strauss (1983). 1-(1-Cyclohexenyl)-2-buten-1-one **(21)** was compared with the synthetic sample, ^{1}H nmr (60MHz / $CDCl_3$); δ: 7.1-6.6, m, 3H; 2.4-2.2, m, 4H; 1.9, d, *J* =5Hz, 3H; 1.8-1.6, m, 4H. m/z; 151 (8), 150 (46), 149 (8), 136 (9), 135 (100), 122 (6), 121 (11), 117 (11), 109 (9), 108 (6), 107 (17), 93 (6), 91 (7), 81 (19), 79 (24), 77 (6), 69 (45), 53 (5), 41 (38), 39 (14). Compounds (**29**) and (**30**) were identified by comparison with authentic materials, (Sefton et al., 1989). Compounds (**22**), (**24a**), (**25a**), (**27a-d**), and (**28**) were tentatively assigned based on interpretation of their mass spectral data. Compound (**22**): m/z; 168 (23), 153 (5), 150 (8), 126 (4), 125 (36), 124 (7), 99 (7), 98 (100), 83 (25), 81 (3), 79 (5), 77 (3), 71 (3), 70 (37), 69 (9), 67 (4), 56 (3), 55 (15), 53 (3). Ethyl ether (**24a**). Diastereoisomer 1, m.z; 254 (8), 239 (8), 208 (13), 195 (11), 193 (6), 175 (5), 168 (13), 167 (100), 166 (8), 153 (6), 151 (18), 149(10), 123 (8), 122 (9), 121 (34), 113 (7), 95 (7), 93 (9), 86 (8), 79 (7), 73 (35), 69 (14), 67 (7), 55 (14), 45 (51), 43 (20), 41 (14). Diastereoisomer 2, m.z; 254 (7), 239 (8), 209, (9), 208 (20), 195 (10), 193 (13), 175 (8), 168 (13), 167 (100), 166 (9), 153 (8), 151 (20), 149(11), 140 (12), 139 (11), 138 (7), 126 (15), 125 (9), 123 (14), 122 (10), 121 (39), 113 (7), 107 (6), 95 (10), 93 (10), 91 (6), 81 (6), 79 (11), 77 (8), 73 (33), 69 (13), 67 (9), 55 (17), 45 (48), 43 (26), 41 (23). Ethyl ether (**25a**) m.z; 236 (7), 221 (7),192 (3), 175 (5), 150 (5), 149 (51), 134 (3), 133 (36), 131 (3), 123 (11), 122 (100), 121 (59), 120 (8), 119 (9), 113 (6), 107 (25), 106 (4), 105 (19), 103 (5), 93 (4), 91 (14), 83 (7), 79 (10), 78 (3), 77 (9), 73 (44), 69 (5), 65 (4), 55 (3), 49 (4), 45 (57), 43 (8), 41 (9). Compound (**27a**): m/z; 122 (37), 107 (100), 91 (50), 79 (14), 49 (17). (**27b**): m/z; 122 (38), 107 (100), 105 (18), 91 (52), 84 (25), 79 (17), 77 (12), 51 (12). (**27c**): m/z; 122 (40), 107 (100), 93 (47), 91 (58), 86 (45), 79 (85), 77 (35), 66 (30), 49 (58). (**27d**): m/z; 122 (15), 107 (100), 105 (33), 92 (16), 91 (65), 86 (18), 84 (33), 79 (16), 77 (15), 68 (17), 49 (25). (**28**): m/z; 140 (8), 125 (32), 109 (8), 107 (18), 97 (25), 82 (100), 69 (22), 55 (25). Compounds (**24**), (**25**), have been tentatively identified previously, (Sefton et al., 1989). 3-Hydroxy-β-damascone (**6**) at pH 1.0 for 8 hours at 100°C in D_2O/10% EtOD, ^{1}H nmr (300MHz / $CDCl_3$); δ: 6.75-6.65, b, 1H; 6.14, d, *J* =15.8Hz, 1H; 4.1, m, 1H; 2.34, ddd, *J* =16.6, 5.9, 1Hz, 1H; 2.0, dd, *J* =16.6, 9.9Hz, 1H; 1.72, m, 1H; 1.55, s, 3H; 1.48-1.44, m, 1H; 1.14, s, 3H; 0.97, s, 3H. The C10 methyl doublet at δ 1.72 was replaced by a complex multiplet resulting from partial deuterium incorporation at C10. m/z; 211 (7), 210 (27), 209 (38), 208 (16), 195 (15), 194 (32), 193 (42), 177 (29), 176 (54), 175 (39), 150 (25), 149 (27), 122 (29), 121 (100), 107 (19), 105 (30), 93 (27), 91 (20), 79 (19), 72 (13) 71 (62), 70 (95), 69 (54), 55 (31). The above compounds are represented in Figure 3.

RESULTS AND DISCUSSION

Hydrolysis of geraniol (2) and its glucopyranoside (2a).

Hydrolysis of the simple allylic alcohol geraniol (**2**) and its glucopyranoside (**2a**) were carried out at a typical wine pH (3.0) at 50°C and 80°C. The results are shown in Tables 1 and 2.

The hydrolysis of geraniol led ultimately to 14 products observed by gas chromatography (figure 2). These all increased in concentration with increasing temperature and reaction time, except for linalool (**13**) which is an intermediate in the formation of α-terpeniol (**14**) and other products from geraniol (**2**) (Baxter et al., 1978). No ethyl ethers, including geraniol ethyl ether were observed in the hydrolysates, even though the hydrolysis medium contained ethanol. The hydrolysis of many monoterpenes under a variety of acid conditions have previously been studied and reviewed by Baxter et al., (1978), Strauss and Williams, (1983). The pattern of products shown in Table 1 is consistent with results previously reported, (Baxter et al., 1978; Ohta et al., 1991).

As a control for purity of starting material and for possible artifacts of analysis, geranyl-β-D-glucopyranoside (**2a**) was dissolved in 10% ethanol and the solution extracted with dichloromethane (2ml). Analyses by GC/MS showed that 0.16% free geraniol (**2**) was present. Hydrolysis of the glucoside (**2a**) at 50°C, gave three products only (Table 2), linalool (**13**), a larger amount of α-terpineol (**14**) and 2,6-dimethyl-7-octene-2,6-diol (**16**), compared to the thirteen compounds identified in the aglycon hydrolysis at this temperature. No additional geraniol (**2**) was observed other than that present in the control run. At 80°C additional products were seen, along with linalool (**13**) and α-terpineol (**14**) as the major products. Again no additional geraniol (**2**) other than that in the control was observed suggesting that under these conditions primary allylic glucosides cleave at the ether linkage and not at the glycosidic bond (figure 4).

Earlier studies reported by Williams et al., (1982) on the hydrolysis of geranyl-β-D-glucopyranoside (**2a**), under more vigorous conditions, suggested that a substantial amount of geraniol (**2**) was formed, although no quantitative data was reported in that study. Their results indicate that breakage of both the C1-O and the glycosidic bond may have occurred at the higher temperature. Strauss and Williams, (1983) noted that when glucopyranoside (**2a**) was hydrolysed (for 30 min. at an unspecified temperature), the proportion of total monoterpene ethers to alcohols was approximately equal to the molar ratio of ethanol to water present in the reaction mixture. No ethyl ethers, in particular geranyl ethyl ether, were detected in our study.

From the amounts of linalool (**13**) plus α-terpineol (**14**) formed at 80°C after one hour from geraniol (**2**) or its glucoside (**2a**), the aglycon geraniol (**2**) reacts at a rate approximately ten times faster than its glucoside (**2a**). (Tables 1 and 2). At the lower temperature 50°C the differences were even greater. These results are in good agreement with the recently published results of Ohta et al., (1991) who reported that the conversion of geraniol-β-D-glucoside (**2a**) to linalool (**13**) at 100°C was approximately eight times slower than the corresponding hydrolytic conversion of geraniol (**2**). The reactivity of the glucoside (**2a**) is contrary to the belief expressed earlier (Williams et al., 1982, Williams et al., 1989) that glycosides of allylic alcohols (eg geraniol) form the allylic cation more readily than do the corresponding aglycons and that geraniol (**2**) is a major product of the hydrolysis of geranyl glucoside (**2a**).

Hydrolysis of alcohols and glycoconjugates related to b**-damascenone (1) formation.**

Ohloff et al., (1973) have shown that the acetylenic triol (**26**) gave β-damascenone (**1**) and 3-hydroxy-β-damascone (**6**) upon strong acid hydrolysis and suggested that the enyne diol (**4**) was an intermediate in this conversion. Subsequently, the enyne diol (**4**) was confirmed as a grape/wine constituent both in free and glycoconjugated forms (Sefton et al., 1989; Baderschneider et al., 1997). Preliminary studies by us showed that both the aglycon (**4**) and the glucoside (**4a**) give β-damascenone (**1**) and 3-hydroxy-β-damascone (**6**) on mild

hydrolysis (Skouroumounis et al., 1993). In the study reported here, the role of glycoconjugation in modifying the hydrolytic behaviour of the enyne diol (**4**) was first examined by studying the hydrolysis of the model propargyl alcohol (**3**) and the corresponding glucoside (**3a**). The results are shown in Table 3.

At pH 3 and 100°C the reactivity of compound (**3**) was low. After forty-six hours, approximately 26% of compound (**3**) had reacted to give two major products, ketone (**21**), formally derived via a Meyer Schuster rearrangement, (Swaminathan and Narayanan, 1971; Edens et al., 1977) and a second product, tentatively identified from its mass spectrum as the hydrate (**22**). The mass spectrum of compound (**22**) included a molecular ion at m/z 168, no characteristic m/z 109 ion peak via loss of 59 ($C_3H_7O\cdot$), expected for the C9 hydroxy ketone (**23**), but had a base peak of 98, a strong m/z 70 (37%) and a significant m/z 69 (9%) ion (Scheme 1).

The hydrolysis of the C9 glucoside (**3a**) was found to be slower than that of the aglycon (**3**). After forty-six hours the products were detected in 14% yield only, compared to 26% for the aglycon. The major products were the aglycon (**3**), ketone (**21**) and β-hydroxyketone (**22**). It is uncertain whether the formation of the aglycon (**3**) arose via hydrolysis of the glycosidic or the ether linkage. Thus the magnitude of the reduction in the rate of breakage of the ether bond, associated with glycosylation of aglycon (**3**) could not be determined. However the formation of rearrangement products (**21**) and (**22**) was approximately three times faster with the aglycon (**3**) than with the glucoside (**3a**) (Table 3). The hydrolytic studies clearly indicate that the model acetylenic alcohol (**3**) and its β-glucoside (**3a**) undergo relatively slow hydrolysis, and that the propargylic glucoside reacts slower than its corresponding aglycon.

The yields of products of the hydrolyses of the enyne diol (**4**) and its glucoside (**4a**) under various conditions are shown in Table 4.

Hydrolysis of the enyne diol (**4**) at pH 3 and 100°C gave β-damascenone (**1**) and 3-hydroxy-β-damascone (**6**) as the major products. The latter is not converted to the former under these conditions (see below), and so at any given pH and reaction temperature the ratio of compounds (**1**): (**6**) remained constant, regardless of reaction time. Small quantities of the hydrates (**24**) and (**25**) (tentatively identified only) were observed with prolonged hydrolysis. This indicates that, in the absence of significant generation of β-damascenone (**1**) from one or more precursors during ageing, β-damascenone (**1**) (and 3-hydroxy-β-damascone (**6**)) already present in wine can decrease in concentration through formation of this hydrate.

β-Damascenone (**1**) and 3-hydroxy-β-damascone (**6**) were formed approximately eight times slower from the glucoside (**4a**) than from the aglycon (**4**) (Table 4). However, at the lower temperature (50°C) the proportion of β-damascenone (**1**) formed was greater from the glucoside (**4a**) than from the aglycon (**4**). It appears that by slowing the acid-catalysed ionization of the propargyl alcohol function, the presence of the glycopyranosyl moiety promotes other competing transformations within the molecule. The side chain of **4a** and can either react directly to give Meyer-Schuster rearrangement products, or indirectly via the aglycon (**4**).

As previously reported (Skouroumounis et al., **1993**), the ratio of β-damascenone (**1**)**:**3-hydroxy-β-damascone (**6**) in the products of the hydrolysis of compounds (**4)** or (**4a**) is dependent on the reaction temperature. The ratio is a reflection of the selectivity of ionization exhibited at the different temperatures, with the proportion of β-damascenone (**1**) in the products from both the aglycon (**4**) and the glycoside (**4a**) being greater at higher temperature. Thus, heating wine (or juice) is not only likely to accelerate the generation of β-damascenone

(**1**), but could ultimately lead to a higher yield of this compound than could be obtained by natural wine ageing.

The reactivity of compounds (**4**) and (**4a**) is significantly greater than that of the analogues (**3**) and (**3a**), and this is attributed to the extra methyl substituents which can stabilise incipient carbocations via the inductive effect.

Hydrolysis of 3,5,5-trimethylcyclo-3-hexen-1-ol (5), 3-hydroxy-b-damascone (6) and their b-D-glucopyranosides (5a) and (6a). It has been suggested that 3-hydroxy-β-damascone (**6**) acts as a precursor of β-damascenone (**1**) in nature (Ohloff, 1978). However, Ohloff et al., (1973) have also demonstrated that 3-hydroxy-β-damascone (**6**) does not give β-damascenone (**1**) under strongly acidic conditions but rather products derived by Nazarov type cyclization (Rouvier and Santelli, 1983). Nevertheless, nothing is known about the behaviour of the corresponding glucoside (**6a**) under mild acid conditions. Before studying this hydrolysis we examined the hydrolysis of 3,5,5-trimethylcyclo-3-hexen-1-ol (**5**) and its glucoside (**5a**), as a model for homoallylic cyclohexenols at wine pH, as these functional groups are common to many C13 norisoprenoids. Recently the glucoside (**5a**) has been identified as a natural product in Saffron (Straubinger et al., 1998).

At pH 3 the aglycon (**5**) was relatively unreactive and even after 24 hours at 100°C it was found that only 17% had reacted. The main products, identified from their mass spectra, were trace amounts of the isomeric dienes of undetermined structure (**27a-d**), the isomeric alcohol (**28**) and small quantities of oxidation products (**29**) and (**30**), (Table 5). The β-glucoside (**5a**) gave approximately, 13% of the aglycon (**5**) under these conditions. Under more forcing conditions (pH 1.0), various products were observed (Table 5).

At 80°C, 24 hours, the aglycon (**5**) underwent dehydration yielding a total of 48% diene formation, whereas the glycoside (**5a**) yielded trace amounts of dienes and aglycon (**5**), 24%.

At pH 1 and 100°C, virtually all of the aglycon (**5**) had reacted after eight hours with multiple product formation. Only 3.2% of the aglycon remained, and **27a-d** accounted for 82% of the products. The glycoside (**5a**) under the same conditions, yielded 80% dienes (**27a-d**) and 10% aglycon (**5**). It is evident that at the lower temperatures there are differences between the reactivity of the glycoside and aglycon but at the highest temperature (100°C) the products were formed in similar yields after 8 hrs, with dehydration product predominant.

Although these results indicate that simple homoallylic cyclohexenols and their glucosides are likely to have low reactivity at wine pH it is conceivable that the additional conjugation with the enone side chain of β-D-glucopyranoside (**6a**) could enhance the reactivity of the homoallylic glycosidic function. However, when the hydrolysis of β-D-glucopyranoside (**6a**) was carried out at pH 3.0 and 100°C for twenty-four hours, 16% of aglycon, 3-hydroxy-β-damascone (**6**) was obtained but no trace of β-damascenone (**1**) could be detected. At pH 1.1 and 100°C for eight hours only 3-hydroxy-β-damascone (**6**) was obtained in 35% yield, (Table 5). No elimination products were seen with the hydrolysis of the glycoside (**6a**) even under forcing conditions. The reason for these differences is unclear. It is possible that the side chain is easily enolized and that this favours the formation of glycoconjugates of Nazarov cyclisation products which are known to prevail under even more forcing conditions (Rouvier and Santelli, 1983). To check this hypothesis, hydrolysis of 3-hydroxy-β-damascone (**6**) was carried out in deuterated solvents. After four hours at 100°C and at pH 3.0, 3-hydroxy-β-damascone (**6**) was found to be stable and there was no deuterium incorporation. However, at pH 1.0 and 100°C for 80 minutes Nazarov cyclization products were evident in trace amounts

and the 3-hydroxy-β-damascone (**6**) incorporated deuterium, albeit only in the side chain methyl and not in the ring. This was demonstrated by high field ^{1}H nmr spectroscopy where the side chain vinyl methyl doublet and the vinyl proton at C8 both collapsed to complex multiplets. The C4 multiplets remained unchanged. This suggests that the enone side chain is rotated out of conjugation with the ring double bond, presumably to relieve steric interaction with the geminal dimethyl groups. Thus the hydrolytic behaviour of 3-hydroxy-β-damascone glucoside (**6a**) was unlikely to be influenced by enolization in the cyclohexenol ring.

Implications for b-damascenone (1) formation from the allenic triol (31).

The multifunctional allenic triol (**31**) (scheme 2) is known to have a half life of less than 24 hrs at pH 3 and room temperature (Skouroumounis et al., 1992; Skouroumounis et al., 1993) yet a glycoconjugate has been islolated from *Lyium halifmifolium.* Mil. (Naf et al., 1990), at the natural plant pH of 4.0 as well as from apples *Malus sylvestris* Mill., cv. Jonathan (Winterhalter et al., 1993). The allenic triol (**31**) has also been observed in enzyme hydrolysates of glycosidic fractions obtained from Merlot grapes (Sefton, 1998). The allenic triol (**31**) has been postulated as the natural precursor to β-damascenone (**1**) (Ohloff et al., 1973; Isoe et al., 1973), and recent studies in our laboratories (Skouroumounis et al., 1992) have shown the allenic triol (**31**) does give β-damascenone (**1**) in small amounts, less than 1%, after 24 hours at 24°C, at pH 3.0. The two major products however were 3-hydroxy-β-damascone (**6**) and enynediol (**4**). Trace amounts of the allenic diol (**32**) and the dienealkyne (**33**) were tentatively identified as intermediates in this transformation (Skouroumounis et al., 1992).

It was evident from these studies that the three end products of allene triol (**31**) hydrolysis were formed from several competing pathways. Glycosylation (which is thought to be the terminal step in the biotransformations of secondary metabolites) is likely to influence the relative importance of these pathways (e.g. scheme 2). Thus, the tentatively identified bifunctional intermediate (**32**) might eliminate a hydroxyl function, under acid conditions, to form either the C3 or C9 carbocation (megastigmane numbering, Aasen et al., 1972), the former leading to the dienealkyne (**33**) and ultimately to β-damascenone (**1**), and the latter process leading mostly to 3-hydroxy-β-damascone (**6**) (Skouroumounis et al., 1992). If either the C3 or C9 position is stabilized by glycosylation, then ionization of the underivatised hydroxyl can be favoured. In the case of C9 glycosylation, this would be expected to enhance β-damascenone (**1**) formation.

Conclusion.

Allylic, propargylic and homoallylic alcohols are more reactive than their corresponding β-D-glucopyranosides. Although the rates of hydrolysis of such glucosides are significantly lower than for the aglycons (possibly orders of magnitudes lower in some cases), this may nevertheless have an important impact on the direction of favour release. In the case of floral grape varieties, where geraniol (**2**) is concerned, glycosylation is a process that lowers the flavor of the grape juice / wine, not only because geraniol (**2**) itself is an important flavorant in some wines but because the rate of formation of other flavor compounds from geraniol (**2**) is also reduced. However, where flavor compounds in wines are formed from polyfunctional aglycons in competition with flavorless by-products by hydrolytic reactions, then glycoconjugation, through its influence on the reactivity of alcoholic functional groups can influence the fate of the aglycon and thus either enhance or suppress the formation of flavor.

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Table 1.1 : Hydrolysis of Geraniol (**2**) at 50°C and 80°C, pH 3 in 10% Aqueous Ethanol

	Products[a]	Yields (%) **50°C**				Yields (%) **80°C**	
Entry		1h	4h	8h	24h	1h	
1	2,2,6-Trimethyl-2-vinyl-tetrahydropyran (**7**)[b]	n.d.	n.d.	n.d.	+	0.1	
2	Myrcene (**8**)[b]	+	0.1	0.1	0.2	0.2	
3	Limonene (**9**)[b]	n.d.	n.d.	n.d.	+	0.1	
4	Z-Ocimene (**10**)[b]	n.d.	+	0.1	0.2	0.3	
5	E-Ocimene (**11**)[b]	n.d.	0.1	0.1	0.2	0.3	
6	Terpinolene (**12**)[b]	n.d.	n.d.	n.d.	+	0.1	
7	Linalool (**13**)	2.5	6.2	12.5	27.5	25.0	4
8	α-Terpineol (**14**)	0.25	0.4	0.5	1.4	1.1	
9	Nerol (**15**)[b]	+	0.1	0.4	0.7	0.8	
10	Geraniol (**2**)	97	97.2	85	67	71.0	3
11	2,6-dimethyl-7-octene-2,6-diol (**16**)[b]	+	0.1	0.2	0.6	0.3	
12	Cis-1,8-Terpin (**18**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n
13	Trans-1,8-Terpin (**17**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n
14	(Z)-3,7-dimethyl-2-octene-1,7-diol (**19**)[b]	+	+	0.1	0.1	+	
15	(E)-3,7-dimethyl-2-octene-1,7-diol (**20**)[b]	n.d.	n.d.	0.7	1.6	0.6	

a Assignments of all compounds have been established previously in this laboratory by (Strauss, 1983) b co
assumption that components give the same GC / FID peak area / mg as the internal standard.
n.d., not detected + less than 0.1%

Table 1.2 : Hydrolysis of Geranyl-β-D-glucopyranoside (**2**) at 50°C and 80°C, pH 3 in 10% Aqueous Ethanol

	Products[a]	Yields (%) **50°C**				Yields (%) **80°C**	
Entry		1h	4h	8h	24h	1h	
1	2,2,6-Trimethyl-2-vinyl-tetrahydropyran (**7**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n
2	Myrcene (**8**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n
3	Limonene (**9**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n
4	Z-Ocimene (**10**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n
5	E-Ocimene (**11**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	
6	Terpinolene (**12**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n
7	Linalool (**13**)	0.1	0.2	0.5	0.5	1.5	
8	α-Terpineol (**14**)	0.4	0.4	0.5	0.5	1.0	
9	Nerol (**15**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	
10	Geraniol (**2**)	0.15	0.15	0.1	n.d.	0.2	n
11	2,6-dimethyl-7-octene-2,6-diol (**16**)[b]	n.d.	0.4	0.3	0.2	n.d.	n
12	Cis-1,8-Terpin (**18**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n
13	Trans-1,8-Terpin (**17**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n

14	(Z)-3,7-dimethyl-2-octene-1,7-diol (**19**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	
15	(E)-3,7-dimethyl-2-octene-1,7-diol (**20**)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	

a Assignments of all compounds have been established previously in this laboratory by (Strauss, 1983) b co assumption that components give the same GC / FID peak area / mg as the internal standard.
n.d., not detected

Table 2 Aglycone (**3**) and β-D-glucoside (**3a**) at pH 3 and 100°C

Conditions[a] Aglycone	(**3**)	(**21**)	(**22**)	β-D-glucoside (**3a**)	(**3**)
4h	98.5	1.0	0.5	4h	0.9
8h	94.8	3.9	1.3	8h	1.9
24h	83.5	13.6	2.9	24h	5.3
46h	74.0	21.5	4.8	46h	5.9

[a] 100°C, pH 3

Table 3 Hydrolysis of Enynediol (4) and its C-9 glucopyranoside (**4a**) at pH 3

Conditions Aglycone (**4**)	% Conversion	(**1**)	(**6**)	(**4**)	(**25**)	(**24**)	β-D-glucoside (**4a**)
pH=3, 100°C,							pH=3, 100°C
4h	23	2.8	20	77	n.d[a].	n.d.	4h
1d[b]	81	10	71	19	trace	trace	
2d	97.5	14	83	2.5	trace	trace	2d
pH=3, 50°C							pH=3, 50°C
1d	2	0.1	1.8	98	n.d.	n.d.	
7d	13	0.8	12.3	87	n.d.	n.d.	7d
28d	44	2.3	41	56	n.d.	n.d.	28d
pH=3, 20°C							
28d	2.6	trace	2.6	97	n.d.	n.d.	
90d	6.4	0.18	6.2	93.6	n.d.	n.d.	
1year	22	0.9	21	78	trace	trace	
6.3years[c]	81.8	2.6	60.5	18.2	1.8.	11.5.	

[a] n.d. not detected [b] d, day [c] Ethylether of compounds (**24**) 5.0% and (**25**) 0.4% at C-9 were dete

Table 4.. Aglycone (**5**) and Glucoside (**5a**) at varying pH and temperature

Aglycone (**5**)	(**28a**,**b**,**c**,**d**)	(**5**)	(**29a**,**b**)	(**30**)	(**31**)
pH 3 100°C,24h	+ : + : + : +	87	0.4 : 1.4	1	+
pH 1 50°C	1.4: 0.4: 0.7: n.d.	82	0.8: 2.6	7.9	0.3
pH 1 80°C	1.4: 0.4: 0.7: +	64	0.5: 1.7	4.3	0.5
pH 1 100°C	34.6: 13: 17.3: 8.7	8.4	3.1: 3.7	7.7	1.1
Glucoside (**5a**)					
pH 3 100°C,24h	+ : n.d. : n.d. : n.d	0.8	n.d	n.d	n.d
pH 1 50°C	n.d.	+	n.d	n.d	n.d
pH 1 80°C	1 : n.d. : 0.4 : n.d	1.3	0.3 : n.d	n.d	n.d
pH 1 100°C	39: 13: 19.3: 10.8	9.7	3.5: 4.3	n.d	n.d

n.d. not detected

Table 5.0 Hydrolysis of β-D-glucopyranoside (**6a**) at varying pH

Conditions[a]	% (**6**)
pH=3, 24h	16
pH=1.1, 8h	35
[a] 100°C	

Identification of a Precursor to Naturally Occurring β-Damascenone.

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Keywords: *b-damascenone, flavour precursors, wine, carotenoid metabolites.*

ABSTRACT

9-Hydroxymegastigma-3,5-dien-7-yne** 8a **was synthesised and shown to be identical to an intermediate found in the acid catalysed conversion of 3,5,9-trihydroxymegastigma-6,7-diene** 4 **to** b**-damascenone** 1**, 3-hydroxydamascone** 5 **and megastigm-5-en-7-yne-3,9-diol** 6**. When subjected to acid hydrolysis,** 8a **produced** b**-damascenone** 1**, in high yield. Importantly, the hydrolysate was completely free of 3-hydroxydamascone** 5**.

RESULTS AND DISCUSSION

β-Damascenone **1** is one of the most important flavour compounds known to science;[1] it is found in many fruit and vegetable products, and tonne quantities are produced commercially for the perfume and flavouring industries. It is believed to be formed in nature from the hydrolytic breakdown of complex secondary metabolites[2-6] derived from carotenoids such as neoxanthin **2**. Recent studies of wine grapes have indicated that β-damascenone **1** can be formed *in vivo* as shown in Scheme 1.

Scheme 1 and Structures 7-8 here

Both the so-called 'grasshopper ketone' **3**[7] and the allenic triol **4**[6] have been identified as components of wine grape extracts treated with a glycosidase enzyme preparation. Hydrolytic studies[8] have shown that triol **4** is converted rapidly to the products **1**, **5** and **6** at room temperature and pH 3.0 in an aqueous environment. Furthermore, the ratio of these three products in at least some grape samples[9] was broadly similar to that found in the hydrolysates of the triol **4**. β-Damascenone **1**, 3-hydroxydamascone **5** and enyne diol **6** are apparently formed by competing pathways from the triol **4**, as no significant interconversion of these products takes place at room temperature. The diol **6** (and also its C_9 glucopyranoside)[ξ] will form both **1** and **5** extremely slowly under these conditions, and therefore may generate β-damascenone **1** in wine over several years of bottle ageing, but is not a significant precursor to **1** in either grapes or young wines.

In the early stages of hydrolysis, two intermediates in the conversion of **4** to **1**, **5** and **6** were observed by GC/MS and tentatively identified as **7** and **8a**. The latter has also been reported (without presentation of evidence) as a constituent of rum.[10] We now wish to report the successful synthesis and characterisation of **8a** and its role as an intermediate in the formation of β-damascenone.

Scheme 2 here

The synthesis of **8a** was accomplished in six steps (Scheme 2) from 2,6-dimethylcyclohexanone **9**. Methylation, then bromination followed by dehydrobromination gave the enone **10** which underwent reaction with the dilithio derivative[11] of 2-hydroxybut-3-yne. Acetylation of the secondary hydroxy group in **11a** gave **11b**, which was dehydrated with P_2O_5 on celite.[12] Treatment of **8b**[13] with potassium hydroxide under mild conditions produced the free alcohol **8a**[14] which was shown by GC/MS (retention time, fragmentation pattern and co-injection) to be identical with one of the two compounds observed as intermediates in the hydrolysis of the triol **4**.

Hydrolysis of **8a** was effected in model wine,[15] and produced β-damascenone **1** as the major product (>90%) by GC/MS. The absence of 3-hydroxydamascone **5** and the enyne diol **6** in the hydrolysate confirms the hypothesis that **1**, **5** and **6** are formed from the triol **4** by competing pathways.

The compounds **3**-**6** are known to accumulate in grapes and other fruits as glycoconjugates. Studies of the reactivity of allylic and propargyllic alcohols and their corresponding β-D-glucopyranosides have shown that the glucopyranosyl moiety retards the acid-catalysed hydrolysis of these compounds. Thus, in plant products containing glycoconjugated forms of the allenic triol **4**, the position of glycoconjugation has the capacity to steer hydrolysis either towards or away from β-damascenone formation by promoting or suppressing formation of the intermediate **8a** in competition with other products.[4,5]

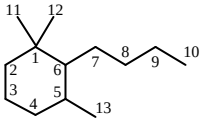
ACKNOWLEDGEMENTS

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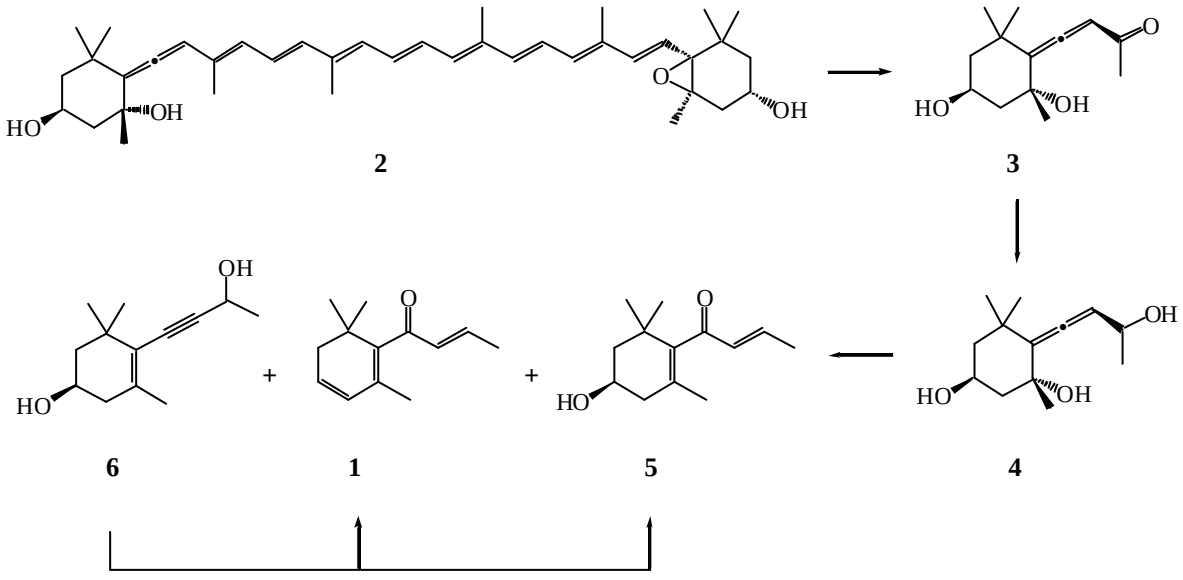
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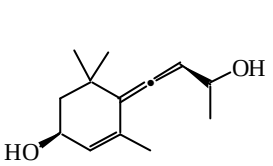
[ξ] The numbering scheme is that employed for the megastigmane skeleton:



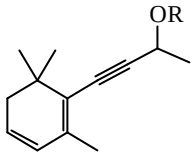
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13. Selected spectral data for (**8b**): ^{1}H NMR ($CDCl_3$, 300 MHz), 5.88-5.76 (2H, m, H_3, H_4); 5.66 (1H, q, *J*=6.7 Hz, H_9); 2.10-2.07 (2H, m, H_2); 2.08 (3H, s, OAc); 1.92 (3H, br s, H_{13}); 1.54 (3H, d, *J*=6.7 Hz, H_{10}); 1.06 (3H, s, H_{11}); 1.05 (3H, s, H_{12}). ^{13}C NMR ($CDCl_3$, 75.5 MHz), 169.9 (CO); 137.4, 127.6, 126.8, 122.6 (C_3, C_4, C_5, C_6); 94.7, 83.2 (C_7, C_8); 61.3 (C_9); 38.2 (C_2); 32.7 (C_1); 26.9, 26.9 (C_{11}, C_{12}); 21.8 (C_{10}); 21.2 (CH_3CO); 20.5 (C_{13}).
14. Selected spectral data for (**8a**): 5.86-5.76 (2H, m, H_3, H_4); 4.75 (1H, q, *J*=6.6 Hz, H_9); 2.11-2.07 (2H, m, H_2); 1.93 (3H, br s, H_{13}); 1.52 (3H, d, *J*=6.6 Hz, H_{10}); 1.06 (6H, s, H_{11}, H_{12}). ^{13}C NMR ($CDCl_3$, 75.5 MHz), 136.5, 127.6, 126.4, 122.9 (C_3, C_4, C_5, C_6); 98.5, 82.1 (C_7, C_8); 58.8 (C_9); 38.2 (C_2); 32.6 (C_1); 26.8, 26.8 (C_{11}, C_{12}); 24.7 (C_{10}); 20.3 (C_{13}).
15. Model wine: 10% (v/v) EtOH in H_2O, buffered at pH 3.0. Hydrolysis was conducted at 45°C overnight.



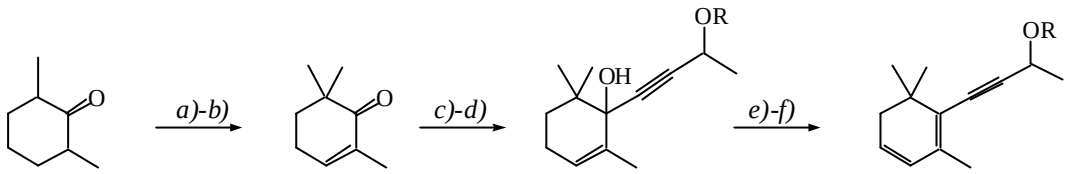
Scheme 1



7



8a, R=H
8b, R=Ac



9 **10** **11a**, R=H **11b**, R=Ac **8a**, R=H **8b**, R=Ac

Scheme 2

a) LDA, MeI (84%) *b)* i) Br_2 ii) pyr, Δ (79%) *c)* LiC≡CCH(CH_3)OLi (99%)

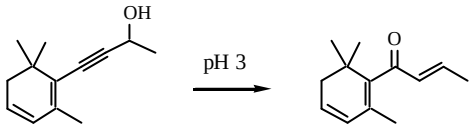
d) Ac_2O, THF, Δ (88%) *e)* P_2O_5/celite, $PhCH_3$, Δ (54%) *f)* KOH (78%)

Identification of a Precursor to Naturally Occurring b-Damascenone.

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Dienyne alcohol **8a** has been synthesised and shown to produce β-damascenone **1** under acidic hydrolysis conditions.



8a **1**

'Reduced' aroma in screw-cap bottled white wines

Screw cap closures are an increasingly popular means of sealing wines in Australia, especially white wines. Wine producers and consumers recognise that this type of closure largely avoids problems with cork taint and oxidation, which can seriously affect some individual bottles of wine sealed with traditional natural cork closures.

In the Institute closure study (Godden et al 2001a,b, Godden et al 2002), it was found that the roll on tamper evident (ROTE) closure studied provided a seal that allowed the sulfur dioxide concentration in the Semillon wine used to remain higher than all of the other closures following storage, and the wine to be least brown as measured by absorbance at 420 nm. From sensory assessment the wine stored in bottles sealed with this closure was consistently rated as high in fruit descriptors and did not show any significant oxidised or musty TCA-like aroma. However, as discussed in the original paper (Godden et al 2001), after 18 months of storage, the wine bottled with this closure was rated as significantly higher in a character that was defined as 'reduced' or 'rubber'. This aroma has remained evident in the wine at each of the subsequent sensory evaluation sessions (see 11AWITC paper) and has continued to be rated by the sensory panel as highest in the ROTE wine. In recent assessments, most recently in June 2002 when the wine had been stored for 36 months, the character has been described as 'struck flint' by some of the panel. At this assessment period, a single bottle of one of the technical cork closed wines was rated relatively highly in reduced aroma, as were two bottles of the reference 3 natural cork closed wine.

During panel discussions regarding the character observed in the ROTE sealed closure trial wine there was some debate about whether it was a true reduced sulfur aroma or whether it might be a varietal aroma of older Semillon. Following the last sensory rating sessions of the closure trial wine, a simple sensory trial was carried out on the ROTE closed Semillon wine to assess the presence of sulfide and mercaptan compounds. The diagnostic procedure followed is detailed in Zoecklein et al (1995) and requires three separate additions to aliquots of the wine: copper sulfate, cadmium sulfate, and an addition of ascorbic acid together with copper sulfate. The additions were made and five of the most experienced panellists from the closure trial sensory assessments were asked to smell the samples independently. The untreated ROTE closed control was identified to the tasters and the treated samples were presented as blind coded glasses, together with a coded glass of the untreated control. The tasters agreed that the copper treated sample, while still showing a rubbery, reduced aroma, was improved as a result of the treatment, while the cadmium treatment had little or no effect. This indicates that hydrogen sulfide or mercaptans are likely to be responsible for the aroma. The ascorbic/copper treatment had the greatest effect with the aroma no longer being evident. This strongly suggests that the main compounds in the ROTE closed wine giving rise to the rubbery/struck flint aroma are mercaptans. This test provides confirmation that the rubbery aroma that developed over time in the ROTE closed wine is due to chemical reactions of sulfur compounds in the relatively anaerobic environment of the ROTE seal. As an aside, it does not appear that the reduced/rubbery aroma diminishes in intensity with decanting or with swirling in the glass over time.

It should be noted that cadmium is not a prescribed additive or processing aid in wine according to the specifications of The Australian Food Standards Code part P4 (Food Liaison Pty Limited 2001). It is considered useful as a diagnostic tool, however, to provide some indication of the types of sulfur compound present in the wine. Assessment of aliquots of wine to which cadmium salts have been added is limited to aroma only, due to its toxicity when ingested. It should be noted that the concentration of copper used in the diagnostic test also makes the samples unsuitable for tasting.

Having been exposed to this rubber-like aroma during the closure trial sensory training process and evaluations, it has become more and more apparent to Institute tasters that, after a

period in the bottle, some commercial wines can develop this aroma, and it seems most common in wines bottled under screw cap closures. In recent months it has been observed on occasions by a panel that has been assessing a number of commercial Rieslings as part of a sensory/volatile compositional study being carried out by Heather Smyth for her PhD research. We thus have a small amount of data available to us regarding the incidence of this character.

To select wines for the study, 55 commercial Rieslings were tasted over nine sessions in October 2002. The wines ranged from the 2002 vintage to 1993, although most were from the years 2002 (12) and 2001 (28), and were from regions/wineries from all states of Australia, and were obtained from retail outlets at prices ranging from $7 to $33. The wines were assessed by a group of 7 to 11 tasters over the nine sessions, in the Institute tasting booths. Many of these tasters had previously participated on the closure trial panel. Coded glasses were presented on a tray ie there was no identification of the wines to the tasters at all, and the bottles hidden so no information was available to the tasters as to whether cork or screw cap wines were being tasted. Being a preliminary sensory session, the tasters were simply asked to comment on the wine's sensory characteristics and give a quality score. No instruction was given regarding reduced or rubbery aroma. Following independent tasting, the panel convened to discuss the wines after each session.

Twenty three of the 55 wines tasted were closed with a screw cap. Of these, four were considered by the majority of tasters to be substantially affected by a strong rubbery/reduced character. Three of these wines were from the 2001 vintage, and one from the 1999 vintage. They were all from relatively small wineries, and all from relatively cool viticultural regions. Another two screw cap wines were less obviously affected, with only a few tasters noting the character: these two wines were a 2001 wine and a 1995 wine, both from large companies. It should be noted that some cork sealed wines were also considered by some tasters to be slightly rubbery, and as expected, a number of the cork sealed wines were found to be affected by a musty TCA cork taint character. The formal sensory analyses of the 20 selected Riesling wines have recently been completed, and while all the wines were rated as quite low in 'rubbery' aroma, two wines were higher than the rest. The wine with the highest score was a 1999 cork closed wine, while the second highest was a 2001 screw cap closed wine. Both of these wines were produced by large wine companies.

In considering this reduced aroma character, it may be that the use of screw cap closures necessitates more careful avoidance by winemakers of sulfide compounds in the wine at bottling. The wine used in the closure trial was not copper treated before bottling. It is also likely that for the closure trial wine several factors combined with the inherent sealing properties of the screw cap closure to be particularly conducive to strongly reductive conditions: ascorbic acid was used; conventional sulfur dioxide additions were made; the wine was of low pH, higher than recommended fill heights at bottling were achieved, so there was minimal headspace above the wine; and the bottles were 'snow-dropped' with solid carbon dioxide before sealing.

Of course, it is still not certain that there is a generally greater incidence of reduced aroma in screw cap wines compared to cork closed wines. For winemakers using screw cap closures for white wines intended for early consumption, ie where all stocks of the wine would be sold in the year of release, it is unlikely that reduced aroma would be an issue. Because the use of screw caps is still a relatively new technology for many wineries, it is understandable that there may be a period during which the industry develops experience with the closure, and fine tunes its practices to ensure the optimal 'shelf-life' of the wine.

Leigh Francis, Senior Research Chemist and Kate Lattey, Chemist/Sensory Analyst

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*We wish to make it clear that any reference to Tage closures in these publications is a reference to closures supplied by Esvin Wine Resources in New Zealand in May 1999, and which, we are informed, had been made by a Californian manufacturer, and not Novembal. We have been informed by Novembal that these closures are not the same as Tage closures now made by Novembal. We take no position on Esvin's right or that of the Californian manufacturer to make or supply closures under the name Tage. The Tage closures that were tested were not supplied by Novembal

Williams and Francis 2001, Puglisi et al 2002, Hakansson et al 2002 and Elsey 2002 are not available in electronic form.

Application of Stir Bar Sorptive Extraction for Wine Analysis

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Abstract
Stir bar sorptive extraction (SBSE) coupled with gas chromatography/mass spectrometry (GC/MS) was used to analyse wine samples for three applications: flavour and compositional analysis; 2,4,6-trichloroanisole (TCA), a common off-aroma in wine; and agrochemicals. SBSE was found to be orders of magnitude more sensitive than modern conventional methodology, allowing for lower detection and quantitation levels, and improved confirmation of identity, SBSE often giving better signal to noise in scan mode than other methods in selective ion monitoring (SIM) mode. With the help of their characteristic mass spectra all agrochemicals could be identified unambiguously at concentrations of 10 µg/L in wine and a further 100 constituents were detected in a Cabernet Sauvignon sample. Thus it is now possible to analyse complex samples such as wine by scan mode, with better confirmation of identity, and without sacrificing sensitivity, where previously SIM methodology had to be used.

Keywords GC/MS · SBSE · Wine · TCA · Agrochemical · Flavour

Introduction

The identification and quantification of trace organic compounds in complex mixtures can be achieved by combining efficient concentration and powerful separation procedures with highly selective and sensitive detection techniques. Such strategies are inevitably important in the analysis of volatile constituents, agrochemicals and taint compounds in wine, which can sometimes be likened to "finding a needle in a haystack". The individual compounds responsible for flavour or off-flavour of wine are often present at extremely low concentration, eg. less than 1 ? g/L [1, 2, 3, 4, 5]. Therefore the analytical methods for detecting these compounds in wine are required to be at least as sensitive as the human nose, and sometimes as selective as the human nose. The control of agrochemicals is of considerable importance to consumers as well as producers. Wine is no exception due to the public interest in the levels of residual agrochemicals in food and drink samples. Multiresidue analysis for pesticides and fungicides is essential to monitor and control the use of agrochemicals in vineyards and subsequently to protect consumers by determining withholding periods, maximum residue limits (MRLs) etc [6].
Prior to GC/MS analysis, the analyte enrichment from matrix was traditionally carried out by liquid/liquid and solid phase extractions [7, 8, 9, 10, 11, 12, 13, 14, 15] or headspace and purge-trap techniques [16, 17, 18, 19, 20, 21]. Recently solid phase microextraction (SPME) [22, 23, 24, 25, 26, 27, 28, 29] has become more frequently used because of advantages due to rapid and solvent-free extraction.
Recently, stir bar sorptive extraction (SBSE) has been introduced and evaluated both practically [30, 31, 32, 33, 34, 35, 36, 37] and theoretically [38]. SBSE uses a stir bar (typically 10 mm length) incorporated in a glass tube and coated with polydimethylsiloxane (PDMS). Upon stirring in a liquid sample matrix, the analytes are partitioned between the matrix and the PDMS phase on the stir bar according to their partitioning coefficients. Subsequently the stir bar is transferred from the sample to a compact thermal desorption unit mounted on a Programmable Temperature Vaporization (PTV) injector of a gas chromatograph (GC) where the analytes are thermally desorbed and delivered to a GC column. The extraction theory of SBSE is the same as for SPME with PDMS fibre coating but

the volume of the PDMS phase is significantly different with typically 55 µL for SBSE (ranges from 25 to 125? L) and 0.6 µL for SPME (100 ? m fibre) [30, 38]. This affects directly the enrichment of analytes since their recoveries from liquid samples increases with the volume ratio of the PDMS phase to the sample matrix.
The aim of this study was to evaluate the capability of the SBSE technique to analyse flavour and off-flavour compounds as well as agrochemicals in wine.

Experimental

Chemicals

2,4,6-Trichloroanisole (TCA) was purchased from Sigma-Aldrich Australia. The agrochemicals chlorothalonil, ethion, fenthion, propiconazole and flusilazole were purchased from Chem. Service (West Chester, PA, USA), chlorpyrifos and dicofol op from Novachem (South Yarra, Vic., Australia), metalaxyl and penconazole from Ciba Geigy (Basel, Switzerland), fenarimol and chlorpyrifos methyl from DowElanco (Letcomb, UK), dicofol pp and procymidone from Dr. Ehrenstorfer (Augsburg, Germany), benalaxyl from Hoechst Schering AgrEvo (Frankfurt, Germany), methidathion from AGAL (Pymble, NSW, Australia), myclobutanil from Rohm & Haas (Paris, France), parathion methyl from Riedel-de Haen (Seelze, Germany) and triadimefon from Bayer (Monheim, Germany).

Sample preparation

Wine analysis: The red wine analysed was made from Cabernet Sauvignon grapes (Vitis vinifera) harvested from Coonawarra, South Australia in 2000. For quantification of wine components, dimethylphenol and butylated hydroxytoluene were added to the wine as internal standards at concentrations of 1.2 µg/L and 100.5 µg/L, respectively.
This red wine was also used as a base wine for TCA and agrochemical analysis.
TCA analysis: The red wine was spiked with TCA at concentrations of 10 ng/L or 1 ng/L.
Agrochemical analysis: The red wine was spiked with the eighteen agrochemicals listed above at the concentrations of 0.5 µg/L or 10 µg/L. The reference agrochemicals were dissolved in 10% methanol in water at a concentration of 10 µg/L.

Extraction procedure

SBSE

A stir bar (0.5 mm film thickness, 10 mm length, Gerstel GmbH, Mülheim an der Ruhr, Germany) coated with PDMS was used. The wine sample (10 mL) and stir bar were placed in a 10 mL glass headspace vial. The vial was sealed with a Teflon coated crimp cap. The stir bar was stirred at 700 rpm at 25°C for 90 min. After removal from the wine sample, the stir bar was gently dried with a lint free tissue and then transferred into a glass thermal desorption tube for GC/MS analysis.

SPME

The SPME fibre, 100 µm PDMS (Supelco) was exposed to the headspace above 8 mL of the wine in a 15 mL glass vial with a Teflon coated septum after the addition of 2 g of NaCl. The extraction was carried out for 30 min at 25°C with stirring (700 rpm).

Pentane/diethyl ether liquid/liquid extraction

Wine (50 mL) was extracted three times with 100 mL of pentane/diethyl ether (1:1, v/v). The combined extracts were concentrated to *ca.* 0.5 mL by fractional distillation with a glass Vigreux column packed with Fenske helices.

GC/MS analysis

Analyses were carried out with Agilent 6890/5973 GC/MS (Agilent technologies, Palo Alto, CA).

SBSE

The GC/MS equipped with a Gerstel TDS 2 thermodesorption system (Gerstel GmbH) was used. The glass thermal desorption tube was introduced into the thermodesorption unit where the stir bar was heated to release and transfer the extracts into a cooled injection system / programmed temperature vaporizer (CIS 4 PTV) via a liner packed with deactivated quartz wool (Gerstel GmbH). The thermal desorption was carried out with a temperature program from 20°C held for 1 min, ramped at 60°C /min to 250°C and held for 5 min, the helium flow rate was 150 mL/min. The PTV injector was held at -150°C for the total desorption time and then ramped at 10°C/sec in splitless mode to 350°C and held for 5 min. The pneumatics configuration during desorption allows a combination of splitless desorption and high flow which gives optimum transfer of compounds from the stir bar to the GC/MS system. The GC was fitted with a HP-5MS capillary column (approx. 30 m x 0.25 mm i.d., 0.25 µm film thickness, Agilent Technologies). Helium was used as carrier gas with a column flow rate of 1.2 mL/min. The MS transfer line and ion source temperatures were set at 280°C and 230°C, respectively. The MS was operated in EI mode and positive ions at 70 eV were recorded with a scan range from *m/z* 35 to *m/z* 350 at 2 scans/sec for the compositional wine analysis and from *m/z* 50 to *m/z* 350 at 2 scans/sec for the TCA and agrochemical analyses.
The GC oven temperature was programmed as follows: for wine analysis, 40°C for 1 min, ramped at 5°C/min to 300°C held for 10 min; for TCA analysis, 40°C for 2 min, ramped at 5°C/min to 180°C, followed by 25°C/min to 300°C held for 10 min; for agrochemical analysis, 40°C for 1 min, ramped at 5°C/min to 250°C followed by 10°C/min to 300°C held for 7 min.
The ions recorded in selected ion monitoring (SIM) mode for TCA analysis were *m/z* 167, 169, 195, 197, 210 and 212 with a dwell time of 20 msec each.
For the multi-residue analysis of 25 agrochemicals, including the 18 agrochemicals listed above, the ions monitored (with a dwell time of 20 msec each), and their elution time windows, are listed in Table 1. The isomers of dicofol were analysed as their corresponding acetophenone derivatives, as the isomers of dicofol are quantitatively converted into these derivatives when vaporized.

SPME and pentane/diethyl ether extraction
The samples were injected to the GC/MS in the splitless mode held for 0.5 min. The injector temperature was 250°C, and the solvent delay time was set at 4 min for the liquid extract. Other parameters were as detailed above.

Results and discussion

Analysis of the composition of a Cabernet Sauvignon wine
Subsamples of the same Cabernet Sauvignon wine were extracted by either SBSE, SPME or pentane/diethyl ether liquid/liquid extraction, then analysed by GC/MS in order to compare the three extraction techniques. Their three total ion chromatograms are shown in Figure 1. With SBSE the same compounds could be extracted as by SPME, although the recovery was much higher, especially for minor components. Although the solid phase absorbent was the same (PDMS) there are also differences due to differences in volatility of compounds such as ethyl succinate, *8* and ethyl octanoate, *9* since SPME was used as a headspace technique whereas the stirring bar used for sorptive extraction was immersed in the wine. Greater differences in selectivity are seen for the liquid/liquid extract where more polar hydrophilic components such as phenyl ethanol, *7* can be detected. Analysis of peaks eluting before four minutes was not plausible for liquid/liquid extraction, as the solvent delay was set at the four minute mark in order to protect the MS from large early-eluting peaks (eg ethanol). Other solvent systems used for liquid/liquid extraction would be expected to give different profiles of extracted compounds.

The total ion chromatogram (TIC) for the wine extracted by the SBSE method is shown in Figure 2. Peak numbers were assigned in order of retention time, and identifications were

based on comparison with mass spectra in the NBS75000, Wiley 275, NIST 98 and Australian Wine Research Institute spectral libraries, and established rules for the fragmentation of organic substances upon EI. Table 2 summarises the identified compounds. Approximations of the relative levels of these compounds were estimated from the ratios of their areas to that of the relevant internal standard. However many factors can affect the peak size (such as affinity for the PDMS, volatility, ease and extent of fragmentation and stability of fragment ions). And the fact that two different compounds of the same concentration do not necessarily yield similar signals by MS is well known. Thus the data represents comparison of relative abundances between samples prepared by different extraction techniques rather than absolute quantitative values.

The sensitivity we obtained using the SBSE methodology varied somewhat from compound to compound but was much greater than that seen in previous scan runs of wine using conventional methods in favour of SBSE, in the approximate range of 10 to 100 times. Accordingly compounds previously detected in only trace amounts were identifiable by clearly resolved EI mass spectra, for example the odd-numbered acids eg nonanoic (*41*) and pentadecanoic (*89*) and aliphatic ethyl esters {eg pentanoate (*4*), heptanoate (*23*), nonanoate (*44*), pentadecanoate (*91*)} as well as trace norisoprenoids such as ? -ionone (*67*). Likewise both the *cis*- and *trans*- isomers of oak lactone (*47* and *43* respectively) were clearly detected in scan mode following extraction with SBSE. These must be present at quite low levels, likely less than 1 ? g/L, as this wine did not have any contact with oak throughout all stages of winemaking [39, 40].

Baltussen et al. [38] studied polycyclic aromatic hydrocarbons to study how SBSE compared to SPME, and similarly to our results found that the difference was compound dependent. For very apolar compounds SPME is good but SBSE is better because of the much higher amount of PDMS available. As the polarity increases this difference becomes very accentuated and then SBSE is very much superior to SPME.
Their data was also useful for showing that SBSE can be compared with SPME as the comparative extractions were done from aqueous medium.

TCA analysis
TCA is one of the most studied off-flavour compounds in wines [1, 41, 42, 43] and has low olfactory thresholds ranging from 1.4 ng/L [44] up to 4.6 ng/L depending on the age and variety of the wine, and the sensory panel [42]. The analytical detection limits of TCA (using SIM) in wine have been reported to be at or below 0.5 ng/L determined by liquid/liquid extraction combined with GC/MS [1] and ranging from 2.9 ng/L to 5 ng/L by GC/MS with SPME [24, 25]. In the case of SBSE, concentrations of TCA at 1 ng/L and 10 ng/L in the wine were easily detected by SIM (Figure 3a and 3b). The relative ion abundance between *m/z* 167, 195 and 210 were consistent at the two different concentrations and good signal to noise ratios of the individual ions were exhibited even at the concentration of 1 ng/L. It is important to note that such excellent signal to noise could be achieved without attempting to optimise the SBSE and GC/MS conditions.

Agrochemical analysis
Reverse phase solid phase extraction (SPE) has become commonly used for the enrichment of agrochemicals from the wine matrix. The combination of SPE with GC/MS in SIM mode allowed for detection limits ranging from 10 to 100 µg/L for 48 pesticides [45] and from 2 to 5 µg/L for seventeen pesticides [46].
Using SBSE with GC/MS in scan mode, all the agrochemicals at concentrations of 10 µg/L in wine exhibited unambiguous mass spectra, typical or better than as shown in Figure 4 (for fenarimol). It was interesting to note that the detection limits by SBSE with GC/MS in scan mode were as low as those by SPE with GC/MS in SIM mode. In GC/MS in SIM mode, the

reconstructed ion chromatograms of 10.0 µg/L and 0.5 µg/L in wine are shown in Figure 5a and 5b for SBSE. The extracted ion chromatograms of procymidone and myclobutanil at the concentration of 0.5 µg/L are shown in Figure 6a and 6b. Procymidone exhibited one of the strongest ion responses, and myclobutanil one of the weakest. The ion abundance of *m/z* 96, 283 and 285 derived from procymidone at 0.5 µg/L exhibited approximately at 100000, 50000 and 30000 (Figure 6a), respectively. Therefore it may be estimated that the detection limit for procymidone by SBSE with GC/MS in SIM mode would be down to low ng/L levels. This estimation has good agreement with that reported by Sandra et al. [33]. The detection limit of myclobutanil via SIM (Figure 6b) was approximately 0.5 µg/L.

Conclusions

SBSE combined with GC/MS is a very promising tool for the elucidation of new wine compounds qualitatively, and following proper validation – quantitatively, at levels orders of magnitude below those previously obtained by conventional methods. Thus it is now possible to analyse complex samples such as wine by scan mode, with better confirmation of identity, and without sacrificing sensitivity, where previously SIM methodology had to be used.

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Captions to the Figures

Figure 1. Comparison of Cabernet Sauvignon wine components extracted by SBSE, SPME and liquid/liquid extraction with GC/MS techniques. Peak no. *1*; ethanol, *2*; ethyl acetate, *3*; isoamyl alcohol, *4*; isoamyl acetate, *5*; ethyl hexanoate, *6*; phenylethyl alcohol, *7*; ethyl succinate, *8*; ethyl octanoate, *9*; ethyl decanoate, *10*; ethyl isoamyl succinate, *11*; butyl hydroxytoluene (IS), *12*; phenylethyl octanoate, *13*; ethyl-9-octadecenoate.

Figure 2.GC/MS analysis of Cabernet Sauvignon wine extracted by SBSE. Identification of individual peaks is summarised in Table 2.

Figure 3. Selected ion chromatograms obtained by SBSE-GC/MS of wine spiked with trichloroanisole at concentrations of (a) 10 ng/L and (b) 1 ng/L.

Figure 4. Mass spectra of fenarimol at the concentration of 10 μg/L in (a) wine and (b) 10% aqueous methanol obtained by SBSE with GC/MS in scan mode.

Figure 5. Reconstructed ion chromatograms of SBSE with GC/MS SIM analysis of wines spiked with eighteen agrochemicals at the concentration of (a) 10.0 μg/L and (b) 0.5 μg/L. Identification of peaks shows in Table 1, numbering corresponds to the SIM group.

Figure 6. Selected ion chromatograms of (a) procymidone and (b) myclobutanil at the concentrations of 0.5 μg/L in wine analyzed by SBSE with GC/MS SIM mode.

Figure 1

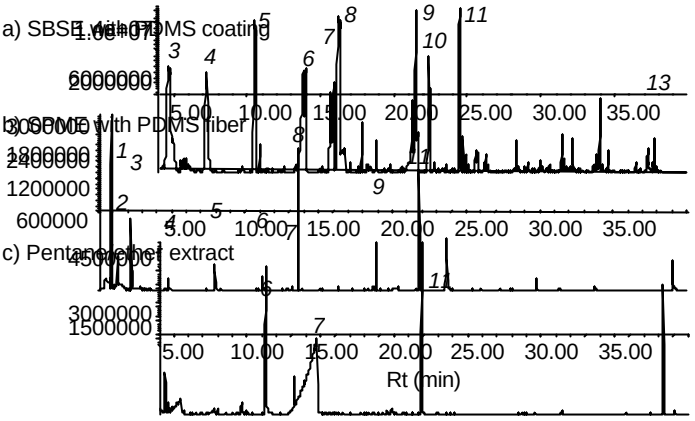

a) SBSE with PDMS coating
b) CDME with PDMS fiber
c) Pentane ether extract
Rt (min)

Figure 2

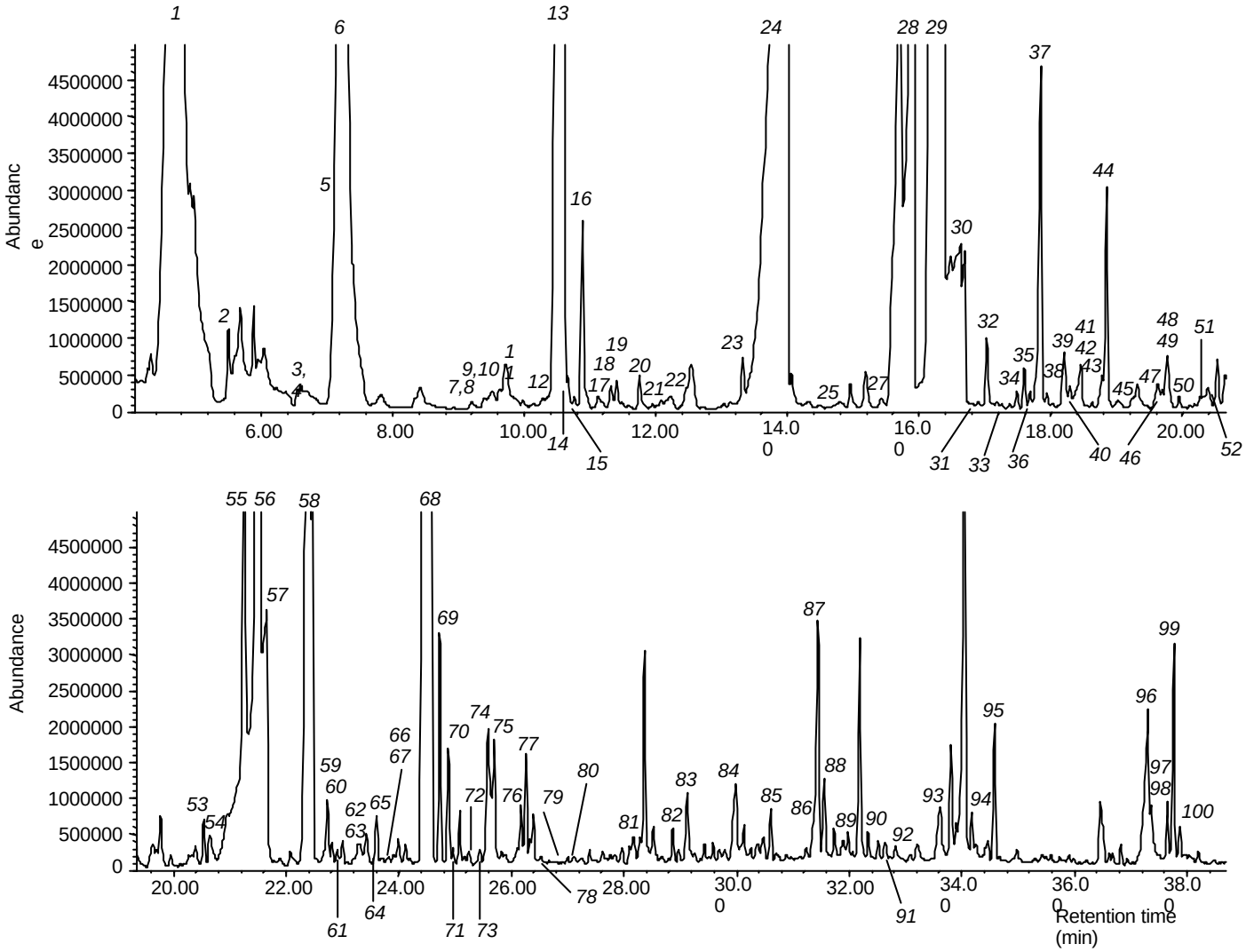


Figure 3

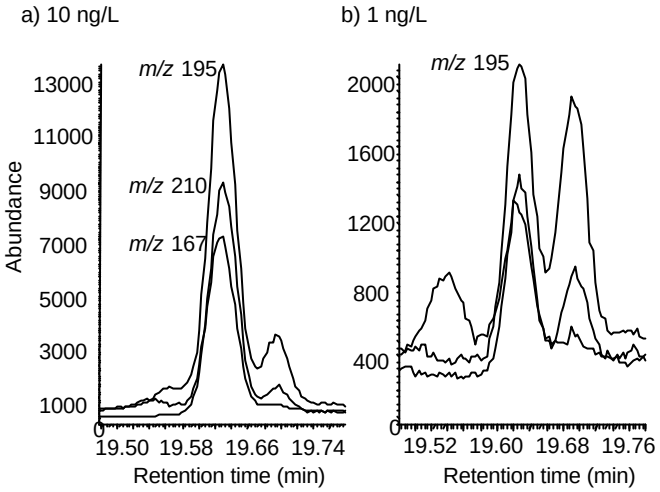

a) 10 ng/L
b) 1 ng/L
m/z 195
m/z 210
m/z 167
Abundance
13000
11000
9000
7000
5000
3000
1000
0
2000
1600
1200
800
400
19.50 19.58 19.66 19.74
19.52 19.60 19.68 19.76
Retention time (min)
Retention time (min)

Figure 4

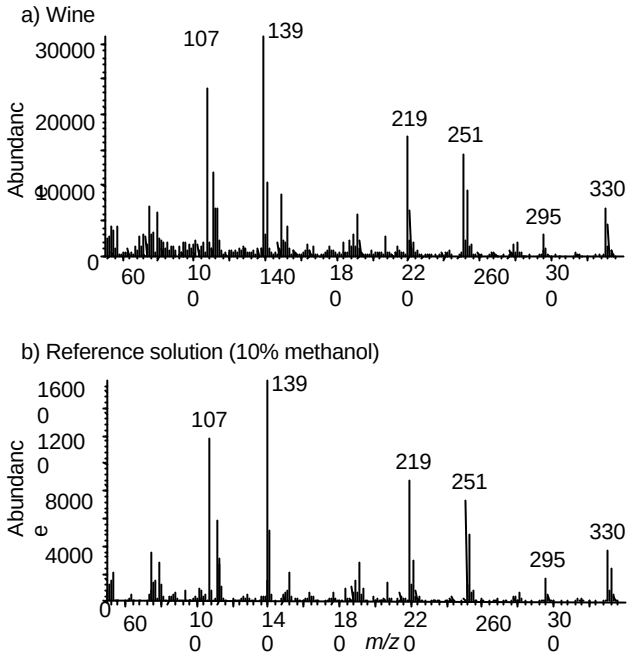

a) Wine
Abundanc
30000
20000
10000
0
107
139
219
251
295
330
60
10 0
140
18 0
22 0
260
30 0
b) Reference solution (10% methanol)
Abundanc e
16000
12000
8000
4000
0
107
139
219
251
295
330
60
10 0
14 0
18 0
m/z
22 0
260
30 0

Figure 5

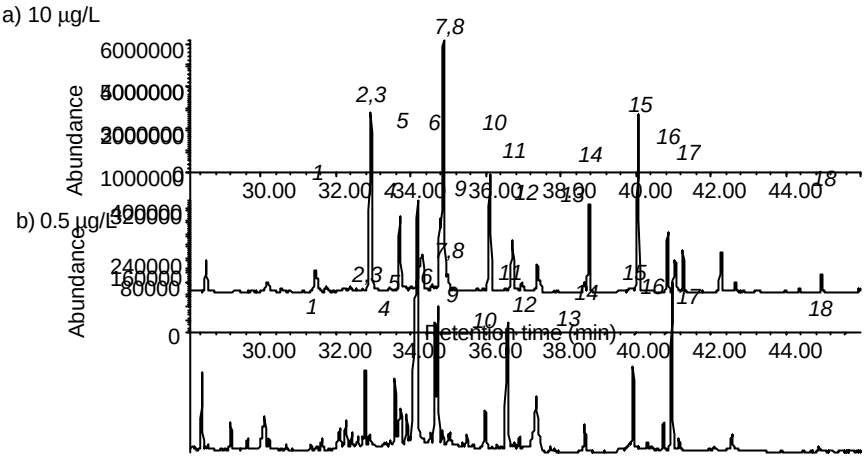

a) 10 µg/L
b) 0.5 µg/L
Abundance
Retention time (min)
30.00
32.00
34.00
36.00
38.00
40.00
42.00
44.00
1
2,3
4
5
6
7,8
9
10
11
12
13
14
15
16
17
18

Figure 6

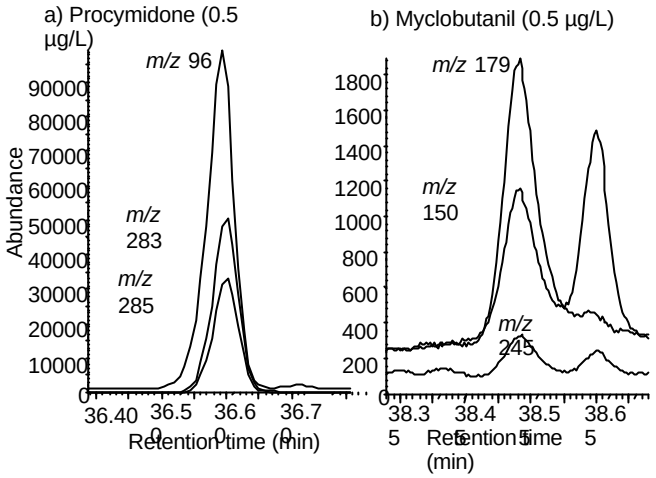

a) Procymidone (0.5 µg/L)
b) Myclobutanil (0.5 µg/L)
Abundance
m/z 96
m/z 283
m/z 285
m/z 179
m/z 150
m/z 245
Retention time (min)
Retention time (min)

Table 1. Group of ions monitored in SIM for agrochemical analysis

Group	Time (min)	Ions (*m/z*)	Compounds
1	28.0	87, 93, 125, 173, 186, 200, 201, 215, 229	Atrazine, Simazine
2	30.4	137, 152, 179, 199, 264, 266, 268	Chlorothalonil, Diazinon
3	32.0	109, 115, 116, 125, 132, 139, 141, 144, 146, 160, 201, 206, 250, 252, 263, 286, 288	Carbaryl, Chlorpyrifos Methyl, Metalaxyl, Parathion Methyl
4	34.0	57, 85, 109, 125, 139, 141, 181, 197, 199, 208, 250, 252, 258, 278, 314	Chlorpyrifos, Dicofol pp, Dicofol op, Fenthion, Triadimefon
5	35.5	85, 96, 112, 128, 145, 149, 159, 161, 168, 248, 250, 264, 283, 285	Methidathion, Penconazole, Procymidone, Triadimenol
6	37.5	150, 179, 206, 233, 245	Flusilazole, Myclobutanil
7	39.5	97, 105, 120, 125, 132, 153, 163, 187, 189, 231, 234, 244, 246	Ethion, Iprodione, Oxadixyl
8	40.5	91, 148, 173, 175, 187, 206, 245, 259, 314, 316	Benalaxyl, Propiconazole
9	43.5	139, 219, 251, 253	Fenarimol

Table 2. Compounds found in Cabernet Sauvignon wine by SBSE with GC/MS

Peak no.	Rt (min)	*Estimated quantity	Proposed compounds
Acids			
14	10.7	+	Hexanoic acid
30	16.7	++	Octanoic acid
41	18.4	+	Nonanoic acid
57	21.7	+++	Decanoic acid
75	25.7	++	Dodecanoic acid
84	30.0	+	Tetradecanoic acid
89	32.0	+	Pentadecanoic acid
93	33.6	+	9-Hexadecenoic acid
96	37.3	++	9-Octadecenoic acid
97	37.7	+	Octadecanoic acid
Alphatic ethyl esters			
2	5.5	++	Ethyl butyrate
3	6.6	+	Ethyl 2-methylbutyrate
4	6.6	+	Ethyl pentanoate
13	10.6	+++	Ethyl hexanoate
15	10.8	+	Ethyl 3-hexenoate
20	11.8	+	Ethyl 2-hexenoate
22	12.3	+	Ethyl 4-hydroxybutanoate
23	13.3	+	Ethyl heptanoate
28	15.7	+++	Ethyl succinate
29	16.2	+++	Ethyl octanoate
44	18.9	++	Ethyl nonanoate
48	19.8	+	Ethyl 3-hydroxyoctanoate
56	21.5	++	Ethyl decanoate
62	23.3	+	Ethyl cinnamate
70	24.9	+	Ethyl 3-hydroxy tridecanoate
77	26.3	+	Ethyl dodecanoate
85	30.6	+	Ethyl tetradecanoate
91	32.6	+	Ethyl pentadecanoate
94	34.2	+	Ethyl 9-hexadecanoate
95	34.6	+	Ethyl hexadecanoate
98	37.7	+	Ethyl 9,11-octadecadienoate
99	37.8	++	Ethyl 9-octadecenoate
100	37.9	+	Ethyl 9-octadecenoate isomer
Other esters			
10	9.6	+	Isoamyl propionate
21	12.1	+	Isoamylbutyrate
35	17.6	+	Isoamyl hexanoate
36	17.7	+	2-Methylbutyl hexanoate
40	18.3	+	Ethyl-n-propyl succinate
46	19.6	+	Methyl decanoate
49	19.8	+	Diisopropyl succinate
58	22.3	++	Ethyl isoamyl succinate
60	22.7	+	Isoamyl octanoate
74	25.5	+	Diisobutyl succinate
Alcohols			
1	4.7	+++	Isoamyl alcohol
5	7.1	++	1-Hexanol

24	13.8	+++	Phenylethyl alcohol
39	18.2	+	1-Decanol

Acetates

6	7.3	+++	Isoamyl acetate
16	10.9	+	Hexylacetate
34	17.5	+	Ethylphenyl acetate
37	17.8	++	Phenethyl acetate
92	32.8	+	Indole-3-ethanol acetate

Phenol and benzene derivatives

7	9.2	+	1-Propylbenzene
9	9.4	+	Benzaldehyde
12	10.3	+	Trimethylbenzene
25	14.8	+	4-Ethyl phenol
27	15.4	+	Ethyl benzoate
51	20.3	+	Ethyl hydrocinnamate
52	20.5	+	Eugenol
53	20.5	+	4,7-Dimethyl-3-benzofuranone
59	22.7	+	Ethyl 2-hydroxy-3-phenylpropanoate
69	24.7	++	Ethyl 4-ethoxy benzoate
76	26.2	+	Ethyl vanillate
78	26.5	+	Methoxyeugenol
79	27.1	+	Benzophenone
80	27.2	+	2,3-Dimethyl naphthoquinone
86	31.3	+	Ethyl 3-(4-hydroxyphenyl)-propenoate
87	31.5	++	Phenylethyl octanoate
88	31.6	+	Syringic acid ethyl ester

Terpenes and norisoprenoids

17	11.2	+	*p*-Cymene
18	11.3	+	Limonene
19	11.4	+	Eucalyptol
31	16.9	+	*beta*-Cyclocitral
32	17.0	+	*beta*-Citronellol
33	17.4	+	*alpha*-Terpinene
38	17.9	+	*alpha*-Terpinolene
42	18.5	+	Vitispirane
50	20.0	+	*cis*-Damascenone
55	21.3	++	*trans*-Damascenone
67	23.8	+	*beta*-Ionone
71	25.0	+	Dihydroactinidiolide
73	25.4	+	*alpha*-Megastigmatrienone

Lactones

43	18.8	+	*trans*-Oaklactone
45	19.0	+	*gamma*-Heptalactone
47	19.6	+	*cis*-Oaklactone
54	20.7	+	Octalactone
63	23.4	+	Nonalactone
81	28.2	+	*gamma*-Decalactone
82	28.9	+	*delta*-Decalactone

Miscellaneous

8	9.3	+	1,1-Diethoxy-3-methylbutane
11	9.7	+	Acetaldehyde ethyl amyl acetal
61	22.9	+	6,7-Dihydro-3-hydroxy isoquinolinedione

64	23.4	+	Cadina-1,6,8-triene
65	23.6	+	2,6-di-t-Butyl-4-methylene-2,5-cyclohexadiene-1-one
66	23.7	+	Methylphenylfulvene
72	25.3	+	Isocoumarin
83	29.1	+	Farnesol
90	32.4	+	1-Hexadecene

Internal Standards

26	15.2	1.2	Dimethylphenol (IS-1)
68	24.5	100.5	Butyl hydroxy toluene (IS-2)

*: The quantity of each compound was estimated by dimethyl phenol (IS-1)
'+' (<5x10^{-6} g/L), '++' (>5, <50x10^{-6} g/L), '+++' (>50x10^{-6} g/L)

The Separation of Glycosylated Flavour Precursors from Grape Leaves with Multi-Layer Counter Current Chromatography (MLCCC)

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BACKGROUND

Countercurrent chromatography (CCC) is a low-pressure, all-liquid chromatographic technique which requires no solid support.[1] It utilises two immiscible solvent phases, plus an auxiliary solvent which is miscible in both phases. In this technique the mobile phase is pumped through the stationary phase, causing the compounds to partition between two immiscible solvents, based on the partition coefficients of the solutes. MLCCC is an advanced form of CCC, and employs teflon tubing wound onto a drum, which is centrifugally rotated in a planetary fashion.[2] (fig. 1.1) The planetary motion sets up alternating zones of mixing and settling which travel along the length of the coil and result in the partitioning of the components.

The main advantages of CCC over conventional chromatography techniques are the absence of a solid support, the use of inexpensive solvents and complete recovery of highly functional and reactive compounds. In contrast to other forms of CCC, MLCCC can be used on both analytical and preparative scale. Due to high centrifugal forces, an increase in the number of theoretical plates is achieved, thereby allowing up to 50,000 mixing zones to occur per hour. This results in efficient partitioning and separation of the components in a complex mixture. Hence MLCCC is beginning to be more widely accepted as a method of choice for the separation and isolation of highly polar natural products.

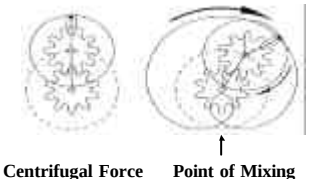


Figure 1.1

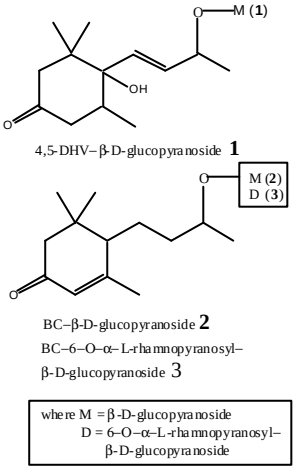


Figure 1.2

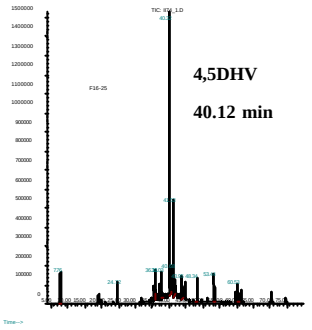


Figure 1.3

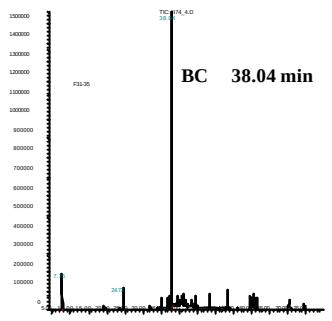


Figure 1.4

Figures: 1.1) Planetary motion of the MLCCC coils, 1.2) Target compounds, 1.3) Gas chromatograph (GC) of the enzyme hydrolysates of fractions 16-25, where main component is 4,5-dihydrovomifoliol (4,5DHV) **1**, 1.4) GC of the enzyme hydrolysates of fractions 31-35, where main component is Blumenol C (BC) **2**.

APPLICATION

Among the most successful applications of MLCCC has been the separation of complex mixtures of glycosylated secondary metabolites from a variety of plant products, including grapes (and wine). An example from our laboratories involves the separation of two compounds (4,5-diydrovomifoliol and Blumenol C β-D-glucopyranosides, **1** and **2** respectively, fig. 1.2) from Riesling leaf glycosides. Although glycosides from Riesling leaves have previously been separated with the MLCCC using an isocratic solvent system [3,4], this separation was achieved using a gradient solvent system, which involved varying methanol concentrations in a chloroform/water system. Both target glycosides were of very similar polarity and chemical structure and their separation by conventional chromatography would be both difficult and low yielding. However, both glycosides have been successfully separated with the use of the Quattro MLCCC. The separation of the glycosides in the MLCCC fractions was monitored by GC-MS, after the removal of the sugar moiety with a fungal glycosidase enzyme. Compound **1,** which was more polar than compound **2** eluted earlier (fraction 16-25 cf. fraction 31-35, fig 1.3 and 1.4, respectively). In addition, Blumenol C-β-D-glucopyranoside **1** and Blumenol C-6-O-α-L-rhamnopyranosyl-β-D-glucospyranoside **3** were separated with the same solvent system. The disaccharide, which was more polar than the monosaccharide, eluted earlier in the MLCCC separation. In each case, the existence and identity of the sugar moiety on the glycoconjugate was tentatively identified by the GC-MS analysis of the trifluoroacetylated glycoconjugates.

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Agnieszka Janusz completed her undergraduate and honours degrees at Flinders University (F.U.), before commencing, in 2001, her Ph.D. with the Cooperative Research Centre for Viticulture, in collaboration with the Australian Wine Research Institute (AWRI) and F.U. She is supervised by Dr. Gordon Elsey (AWRI) and Dr. Michael Perkins (F.U.). The MLCCC work is conducted at AWRI under the guidance of Dr. George Skouroumounis and Dr. Mark Sefton.

The Development of Stable Isotope Dilution Assays for the Quantificationof the Important Aroma Compounds Citronellol, 4-Vinylguaiacol and 4-Vinylphenol in Wine.

The ability to measure accurately aroma compounds which may be present only in extraordinarily minute quantities in grapes and wine is an important part of understanding and controlling wine flavour. In order to achieve this aim, stable isotope dilution analysis (SIDA) has proven itself to be the method of choice in our laboratory, and has been used in the quantitative analysis of a wide variety of aroma compounds. Some of these analyses, such as for TCA (cork taint) and 4-ethylphenol (Brettanomyces character) are now offered as an analytical service to industry. The procedure involves the addition of a precisely measured amount of an appropriate standard for the analyte in question to the wine, and subsequent analysis by Gas Chromatography-Mass Spectrometry (GC-MS). The choice of standard is critical, as a standard which does not mirror the physical characteristics - particularly the solubility and volatility - of the analyte can lead to erroneous results, due mainly to inadequate extraction of the standard and/or analyte, as well as through disproportionate chemical losses.

For this reason the use of a standard which differs only in the isotope pattern is ideal; isotopomers, as these isomeric compounds are sometimes called display essentially identical physical properties. One consequence of choosing a standard which is virtually identical to the analyte is that one requires an analytical tool which is able to distinguish between the two, which is why mass spectrometry has proven invaluable.

Name	4-vinyl guaiacol	4-vinyl phenol	citronellol
Aroma Descriptors	smoky, spicy, clove-like, medicinal	medicinal, elastoplast, horsey	spicy, floral
Reported concentration range (micrograms/litre)	1.4 - 710	0.8 - 450	15 - 42
r^2 obtained	0.998	0.998	0.999

As part of the Institute's collaboration with Flinders University through the CRCV, Ms Katryna van Leeuwen, working under the supervision of Dr Gordon Elsey, has recently prepared stable isotope-labelled forms of 4-vinylguaiacol, 4-vinylphenol and citronellol. These particular compounds are found in varying concentrations in wine, and are associated with a variety of aroma descriptors (see table above). Citronellol is a monoterpene found mainly in wines made from floral/muscat varieties, while 4-vinylguaiacol and 4-vinylphenol are produced during fermentation. The assays were then developed in collaboration with Ms Dimi Capone at the Institute, and provided excellent linear correlations, as evidenced by the high r^2 values obtained (table, above). Finally, the assays were verified by measurement of samples to which precisely measured amounts of the analyte had been added; these yielded results with typically no more than 2-2.5% error.

These analyses will now be used in our studies of the relationship between composition and flavour of Chardonnay and Riesling wines. 4-Vinylguaiacol and 4-vinylphenol are also intermediates in the formation of ethylphenols by Brettanomyces and in the formation of stable pigments in red wines, and the analyses will also be used to further the aims of our research in those areas.

Gordon .M. Elsey

Quantitative Analysis of Geraniol, Nerol, Linalool, and α-Terpineol in Wine.

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Abstract
A mixture of [$^2H_7$]-geraniol, [$^2H_7$]-nerol, [$^2H_7$]-linalool and [$^2H_7$]-? -terpineol was prepared for use as internal standards in a rapid and accurate analytical method, employing gas chromatography - mass spectrometry (GC/MS), to determine the concentration of geraniol, nerol, linalool and ? -terpineol in wine. The method avoids the possible formation, degradation and interconversion of these compounds during their analysis.

Keywords: wine, analysis, geraniol, nerol, linalool, ? -terpineol, artefacts.

1. Introduction

Of all alcoholic beverages, wine displays the greatest variation in flavour. Subtle nuances of colour, aroma and flavour create a unique character for each wine. Several hundred volatile components have been identified in grapes and wine and many of these are important to wine aroma and flavour [1-2]. Among these, the monoterpene alcohols geraniol (**1**), nerol (**2**), linalool (**3**) and ? -terpineol (**4**) are important to the aroma and flavour of young wines made from Muscat varieties of grapes [3-9] such as Traminer, whereas juice from non-Muscat grape varieties contain low levels only of these monoterpenes [3,10-13]. When present in young wines, geraniol (**1**) and nerol (**2**) decrease in concentration as wine is aged [3, 14-19] and are usually present in no more than trace amounts after two to three years in the bottle. The concentration of linalool (**3**) and ? -terpineol (**4**) can initially increase during bottle storage, but decreases afterwards, as they are converted mainly to terpin hydrate [12, 20]. Traces of linalool (**3**) and ? -terpineol (**4**) are still detectable in aged Riesling wine [21]. Geraniol (**1**) and linalool (**3**) are considered to be the most important of the monoterpene alcohols as they are present in greater concentrations, and have lower flavour thresholds than other major wine monoterpenes [22]. Geraniol (**1**), nerol (**2**), linalool (**3**), ? -terpineol (**4**) and related compounds are also considered to be important to the flavour of other alcoholic beverages [23-24], soft drink [20] and are widely used in the perfume and flavour industries [25].
This paper describes a new method for the analysis of these compounds in wine, which is both fast and accurate, and accounts for the possible formation and degradation of these monoterpene alcohols during the analysis. The method can assist researchers and winemakers in determining how winemaking and storage processes affect the concentration of these compounds.

2. Experimental

Nmr spectra were of deuterochloroform solutions and were obtained with a 200 MHz Varian Gemini-2000 system.

All solvents were Mallinckrodt nanopure grade, and verified for purity by GC/MS prior to use. All reagents and standards were purchased from Sigma-Aldrich Australia Pty. Ltd. Model wine used for the stability studies was 10% aqueous ethanol, buffered with potassium hydrogen tartrate and sulfuric acid (5M) to pH 2.9.

2.1 Preparation of [$^2H_7$]-geraniol (7), [$^2H_7$]-nerol (8), [$^2H_7$]-linalool (9) and [$^2H_7$]-? -terpineol (10) (Scheme 1)

2.1.1 (E)- and (Z)-4-[$^2H_2$]-7-methyl-3-([$^2H_3$]-methyl)-2,6-octadienoic acid ethyl ester (6)

NaOD in D_2O (ca 40%, 15 drops) was added to a stirred solution of 6-methyl-5-hepten-2-one (10.5 mL) in dry pyridine (90 mL) and D_2O (40 mL) under nitrogen. After 2h., the reaction was quenched with NH_4Cl and extracted with CH_2Cl_2 (3 x 200 mL). The combined organic layers were washed with saturated aqueous $CuSO_4$ (until the pyridine was removed as indicated by the absence of any darkening of the $CuSO_4$ solution), then water (3 x 200 mL), dried (Na_2SO_4) and the solvent was evaporated, yielding 1,3-[$^2H_5$]-6-methyl-5-hepten-2-one (**5**) as a colourless oil (6.4 g) which was used in the next step without further purification. Sodium hydride (95%, 1.29 g) was added to a cooled (~5°C) solution of triethyl phosphonoacetate [26] (10.65 mL) in diethyl ether (60 mL) and the solution was left stirring at room temperature for 60 min. A solution of **5** (6.4 g) in diethyl ether (60 mL) was then added over 30 min. and the reaction mixture was stirred for a further 44h. The reaction was quenched with D_2O (5 mL), then water (500 mL) and the product, 4-[$^2H_2$]-7-methyl-3-([$^2H_3$]-methyl)-2,6-octadienoic acid ethyl ester (**6**, (*E*):(*Z*) 4:1 by GC/MS), was isolated with diethyl ether as a yellow liquid (8.8 g), ^{1}H nmr, *(E)*-isomer: δ 5.66 (s, *C2*-H), 5.2-5.0 (m, *C6*-H), 4.15 (q, J=7.4 Hz, $OC\underline{H_2}CH_3$), 2.14 (br d, J=6.2 Hz, *C5*-H_2), 1.68 and 1.61 (2 x br s, *C8*-H_3 and *C7*-CH_3), 1.28 (t, J=7.1 Hz, $OCH_2C\underline{H_3}$); *(Z)*-isomer; δ 5.65 (s, *C2*-H), 4.14 (q, J=7.3 Hz, $OC\underline{H_2}CH_3$), 1.27 (t, J=7.1 Hz, $OCH_2C\underline{H_3}$). A portion (3 g) of the ester (**6**) in dry diethyl ether (40 mL) at ~5°C was treated with $LiAlD_4$ (0.6 g) under dry nitrogen. After 10 min., the mixture was warmed to room temperature, stirred for 4h., cooled to ~5°C and treated with acetone (5 mL), followed by aqueous sodium hydroxide (1M, 3 mL) then sodium sulfate. The ether solution was filtered and the solvent evaporated, leaving a clear oil that was further purified by distillation *in vacuo*, giving 2,4-[$^2H_4$]-7-methyl-3-([$^2H_3$]-methyl)-2,6-octadienol (**7**):(**8**) = 4:1, 1.97 g, by GC/MS). ^{1}H nmr (both isomers) δ 5.40 (s, *C2*-H), 5.2-5.0 (m, *C6*-H), 2.08 (br d, *C5*-H_2), 1.99 (br s, -OH), 1.68 and 1.60 (2 x br s, *C8*-H_3 and *C7*-CH_3). ^{13}C-nmr (**7** only) δ 139.4, 131.6, 123.9, 123.3, 58.5 (qn), 39.1 (m), 26.2, 25.6, 17.6, 15.6 (m).

2.1.2 Preparation of a mixture of [$^2H_7$]-geraniol, [$^2H_7$]-nerol [$^2H_7$]-linalool and [$^2H_7$]-? -terpineol for use as internal standards

A solution of [$^2H_7$]-geraniol (**7**) and [$^2H_7$]-nerol (**8**) (4:1, 5.24 mg) in ethanol (20 mL) was added to water (80 mL) saturated with potassium hydrogen tartrate, adjusted to pH 3.0 with sulfuric acid (5M) and prewashed with dichloromethane prior to use. The mixture was heated to 80°C for 1 h., then cooled and placed in the refrigerator. This solution, containing a mixture of (**7**), (**8**), [$^2H_7$]-linalool (**9**) and [$^2H_7$]-? -terpineol (**10**), was used directly as an internal standard for the analyses. *m/z* (%); [$^2H_7$]-geraniol (**7**), 161 (M^+, 1), 143 (2), 128 (10), 127 (5), 100 (5), 99 (6), 75 (11), 74 (6), 70 (8), 69 (100), 68 (8), 67 (6), 53 (5); [$^2H_7$]-nerol (**8**), 161 (M^+, 1), 143 (3), 128 (8), 100 (11), 99 (12), 86 (10), 75 (11), 70 (9), 69 (100), 68 (9), 67 (8), 53 (6); [$^2H_7$]-linalool (**9**), 161 (M^+, 1), 143 (7), 128 (14), 100 (48), 99 (48), 98 (28), 86 (32), 85 (30), 76 (100), 75 (40), 71 (20), 69 (55), 57 (37), 56 (48); [$^2H_7$]-? -terpineol (**10**), 142 (M-HOD, 27), 141 (13), 127 (26), 126 (14), 99 (30), 98 (25), 85 (24), 71 (14), 59 (100).

2.2 Preparation of samples for analysis

An aliquot (100 ? L) of the solution of (**7**)-(**10**), prepared as above, was added to the wine sample (5 mL) in a screw cap vial using a glass syringe (100? L SGE). Pentane:diethyl ether (2:1, 3 mL) was added and the mixture was shaken briefly. A portion of the organic layer was then transferred to a vial for GC/MS analysis. For calculating the concentration of the analytes in the wine samples, replicate standards were prepared at the same time as the wine samples, by adding the same amount of internal standard as above (100 ? L) to a 100 ? L solution containing 2.50 ? g each of unlabelled geraniol (**1**), nerol (**2**), linalool (**3**) and ? -terpineol (**4**) in ethanol, diluted with pentane:diethyl ether (2:1, 1800 ? L) and analysed by the GC/MS method (see below) to calculate the relative response factors.

2.3 Instrumental Analyses

Samples were analysed with a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a Gerstel MPS2 autosampler and coupled to a HP 5973N mass spectrometer. The Gerstel MPS2 was operated in fast liquid injection mode with a 10 ? L syringe (SGE, Australia) fitted. The gas chromatograph was fitted with an approx. 30 m x 0.25 mm Phenomenex fused silica capillary column ZB-Wax, 0.25 ? m film thickness. The carrier gas was helium (BOC gases, Ultra High Purity), flow rate 1.2 mL/min. The oven temperature was started at 50? C, held at this temperature for 1 min. then increased to 220? C at 10? C/min and held at this temperature for 10 min. The injector was held at 200? C and the transfer line at 250? C. For liquid injections, the sample volume injected was 2 ? L and the splitter, at 30:1, was opened after 36 sec. Fast injection was done in purge splitless mode with an inlet pressure of 25.0 psi maintained until splitting. The glass liner (Agilent Technologies) for liquid injections was borosilicate glass with a plug of resilanised glass wool (2-4 mm) at the tapered end to the column. For SPME, a direct borosilicate glass liner with an ID of 1.5 mm (Agilent Technologies) was used and the Gerstel MPS2 was operated in SPME mode with either a 100 ? m polydimethylsiloxane (PDMS) or 85 ? m polyacrylate/divinylbenzene(DVB) fibre fitted (fibres sourced from Supelco). Positive ion electron impact spectra at 70 eV were recorded in the range *m/z* 35-350 for scan runs. For quantification of the monoterpene alcohols, mass spectra were recorded in the Selective Ion Monitoring (SIM) mode. The ions monitored in SIM runs were: *m/z* 99, <u>127</u>, 128 and 142 for [$^2H_7$]-geraniol (**7**), *m/z* <u>121</u>, 136 and 154 for geraniol (**1**), *m/z* 69, <u>127</u> and 142 for [$^2H_7$]-nerol (**8**), *m/z* 69, <u>121</u>, 139 and 154 for nerol (**2**) *m/z* 76, <u>100</u>, 128, and 142 for [$^2H_7$]-linalool (**9**), *m/z* <u>121</u>, 136 and 154 for linalool (**3**), *m/z* 69, 99, 127 and <u>142</u> for [$^2H_7$]-? -terpineol (**10**), *m/z* 121, 136 and <u>139</u> for ? -terpineol (**4**). Selected fragment ions were monitored for 20 ms each. The underlined ion for each compound was the ion typically used for quantitation, having the best signal to noise and the least interference from other wine components. The other ions were used as qualifiers.

2.4 Validation

The method was validated by a series of duplicate standard additions of unlabelled geraniol (**1**), nerol (**2**), linalool (**3**), and ? -terpineol (**4**) (0 to 1000? g/L, n = 9 x 2 for all compounds) to white wine (Australian Chenin Blanc, 11.5% ethanol, pH 3.4).

The standard addition curves obtained were linear throughout the concentration range, with the following coefficients of determination and linear regression equations: $r^2 = 0.999$ ($y = 2.16x + 0.05$) for geraniol (**1**), $r^2 = 1.000$ ($y = 56.5x + 2.09$) for nerol (**2**), $r^2 = 0.999$ ($y = 4.08x + 0.06$) for linalool (**3**), and $r^2 = 1.000$ ($y = 4.31x + 0.06$) for ? -terpineol (**4**).

The repeatability of the analysis was determined at two concentrations (10 ? g/L and 500 ? g/L) by spiking several replicate aliquots of the same white wine with (**1**)-(**4**). For seven replicate analyses of the wine spiked at the 10 ? g/L level, the coefficient of variance (or relative standard deviation) was 5.16% for geraniol (**1**), 2.74% for nerol (**2**), 2.23% for linalool (**3**), and 1.90% for ? -terpineol (**4**). For eight replicate analyses of the wine spiked at the 500 ? g/L level, the coefficient of variance (or relative standard deviation) was 0.56% for geraniol (**1**), 0.68% for nerol (**2**), 0.27% for linalool (**3**), and 0.27% for ? -terpineol (**4**). To ensure that the accuracy of the analysis was maintained, duplicate control wines, each with and without spiked standard addition of 300 ? g of the analytes (**1-4**) per litre of wine, were analysed with every set of wine samples over a period of several months. The difference in concentration of **1-4** between the spiked and unspiked controls deviated from the expected value of 300 ? g by less than 2%.

2.5 Stability studies – Robustness of the analytical method

Geraniol (**1**, 20.6 mg), nerol (**2**, 20.0 mg), linalool (**3**, 21.3 mg) and ? -terpineol (**4**, 27.4 mg) were each dissolved in separate aliquots of model wine (pH 2.9, 50 mL). All flasks were left at 25? C. After 1, 2, 4, 8, 24, and 120 hours, 5 mL aliquots were extracted with pentane : diethylether (2:1) (3 mL) and analysed by GC/MS, with the MS in scan mode.

For studying the stability of the analytes in the organic phase, geraniol (**1**, 20.9 mg), nerol (**2**, 19.3 mg), linalool (**3**, 24.7 mg), and ? -terpineol (**4**, 21.0 mg) were added to separate 50 mL volumetric flasks, which were made up with model wine to the mark, and shaken thoroughly. Pentane : diethylether (2:1) (3 mL) was added, and the flasks stoppered, inverted and shaken. 1 drop subsamples were taken immediately and diluted with pentane : diethylether (2:1) (1 mL) and analysed by GC/MS, using scan mode. Similar subsamples were analysed after five and 24 hours. The flasks were left stoppered at 25? C apart from the few moments when subsamples were being taken.

3. RESULTS AND DISCUSSION

3.1 The analytical method

A 4:1 mixture of [$^2H_7$]-geraniol (**7**) and [$^2H_7$]-nerol (**8**) was synthesised as shown in Scheme 1. Partial hydrolysis of a sample of (**7**) and (**8**) in a model wine solution then gave a solution of the standards (**7-10**) which was used directly in the analytical method. Knowledge of the exact concentration of the individual standards (**7-10**) was unnecessary (see below).

Geraniol (**1**), nerol (**2**), linalool (**3**), and ? -terpineol (**4**) could be accurately and precisely quantified in pentane-diethyl ether (2:1) extracts of 5 mL of wine at concentrations down to 1 ? g/L, where signal/noise was > 10:1 for all ions of all compounds, using their [$^2H_7$]-analogues (**7-10**) as internal standards and the analytical method described above. As there were seven deuterium atoms in each labelled standard, baseline resolution of each analyte from its standard was achieved with relatively short GC run times. While baseline resolution is not essential for analysis by GC/MS as extracted ion chromatograms of compound-specific ions can be used, the baseline separation of analytes and labelled standards allowed measurement of abundant common ions and confirmed peak homogeneity. In this method, the concentration of the individual standards (**7-10**) was not determined. It was therefore necessary to measure the relative ion response factors for standard solutions prepared by mixing known quantities of unlabelled reference standards of **1-4** with the same solution of labelled standards (**7-10**) used to spike the wines being analysed. This mixture of standards was then analysed under the same instrumental conditions as employed for the analyses of each set of wine samples. Even if the concentration of the labelled standards (**7-10**) were to be known, the relative intensity of mass spectral fragments for all eight of these compounds varied according to the instrumental operating conditions, thus still necessitating analysis of the mixture of labelled and unlabelled standards along with wine samples.

Adequate sensitivity and limits of quantitation down to 1 ? g/L and below were also obtained for analysis by headspace SPME using either 100 ? m polydimethylsiloxane (PDMS) or 85 ? m polyacrylate fibres (data not shown). However, the liquid-liquid extraction and analysis method described in this paper gave better signal to noise, especially at lower concentrations.

Compared to already published methods for determining monoterpene alcohols in grape juice or wine [3, 27-30], the advantages of the method described in this paper are that it is precise, accurate, has low detection limits, uses only 5 mL of wine, and sample preparation takes just a few minutes. It does not require salting out to improve precision [3], or further concentration [3, 19, 21, 31] to attain good reporting limits. The chromatographic conditions, combined with SIM EI/MS employed in this method limit the problems of non-baseline resolution evident in other published methods [3, 7, 19, 21, 27-28, 32-33].

3.2 Stability studies – Robustness of the analytical method

Geraniol (**1**), nerol (**2**), linalool (**3**), and ? -terpineol (**4**) undergo a variety of transformations, even in dilute acidic media [12, 20, 23, 27, 34-36]. Thus, Skouroumounis [34] hydrolysed geraniol (**1**) in 10% ethanol / water at pH 3.0 at 50? C. After 24 hours, 67% of geraniol (**1**) remained, and 27.5% linalool (**3**), 1.4% ? -terpineol (**4**) and 0.7% nerol (**2**) had been formed, plus trace amounts of 11 other compounds. In *shochu* (alcoholic beverage distilled from various materials eg rice, barley, sweet potato), the main product from acid catalysed transformation of geraniol (**1**) was linalool (**3**) [23]. These authors also state that the main products from nerol (**2**) were linalool (**3**) and ? -terpineol (**4**). To our knowledge, the reactivities and transformations of these monoterpene alcohols under the conditions of extraction or analysis have not been investigated previously.

To test the stability of the analytes, model solutions of individual monoterpene alcohols at pH 2.9 (the lower end of the pH range for commercial wines) were prepared and analysed after standing from one hour to five days at room temperature. The products observed (Table 1) were those expected (see discussion above). From the data shown in table 1, it is evident that wine samples need to be extracted within a few hours after spiking with the labelled standards, or else transformations of the compounds could occur.

Solutions of geraniol (**1**), nerol (**2**), linalool (**3**) and ? -terpineol (**4**) in model wine (pH 2.9) were also shaken with pentane : diethylether (2:1) and the two phase system left sitting stoppered at 25? C for 24 hours, to gauge the extent of interconversion in the organic phase. Subsamples of the organic layer were taken immediately after extraction, after 5 hours and after 24 hours, and analysed by GC/MS. No compositional

changes were observed after 5 hours, and even after 24 hours less than 0.5% conversion to other products was observed. The same results were obtained for extracts of a white wine (1996 Australian Semillon, pH 3.1). We conclude that our liquid-liquid extracts were sufficiently stable, provided that they were not kept at room temperature for more than 24 hours. Another study (data not shown) demonstrated that standard solutions of the compounds (**1**-**4**) in ethanol were stable for up to six months storage at -20? C. Over longer time periods, however, or at higher temperatures (eg room temperature for several days), some conversion could be seen.

Apart from ambient SPME, liquid-liquid extraction at room temperature as applied by us is one of the mildest extraction methods possible. Even so, some interconversions still took place if wines were allowed to stand for more than a few hours. Thus methods eg [7, 23] involving more exhaustive extractions, concerning high temperatures or harsh pH conditions, without appropriate robustness validation, have limited value. Many authors [3, 19, 21, 27, 29, 30-33] have observed linalool (**3**) and ? -terpineol (**4**) in Muscat or Riesling grape juice. Some of these [6, 19, 28-30], however, did not detect significant quantities of geraniol (**1**) and nerol (**2**) in their samples. It is possible that this is due, at least in part to the analytical methodology, which involved elevated temperature or acid in the extraction, thus allowing the transformation of geraniol (**1**) and nerol (**2**) to linalool (**3**), ? -terpineol (**4**) and other products.

4. Conclusions

The analytical method described here is fast, precise, accurate and reliable. No significant generation, degradation or interconversion of the analytes occurred during the extraction and analysis, provided that the wines were extracted within a few hours of addition of the standards and these extracts were then analysed within 24 hours. Furthermore the analyses for linalool, ? -terpineol, nerol and geraniol (**1**-**4**) can be combined with methods for other volatile compounds found in wine, eg. guaiacol and 4-methylguaiacol [37], 4-ethylphenol and 4-ethylguaiacol [37-38], *cis*- and *trans*-oak lactone [37, 39], vanillin [37, 40], vanillyl ethyl ether [41], ? -damascenone [42-43], ? -ionone [43-44], isobutylmethoxypyrazine [45] and the rose oxides, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and various ethyl esters [43]. The compounds analysed in this manner need not be of similar chemical structures, providing that isotopically labelled analogues are used as standards, and that the conditions of extraction and analysis are suitably robust to ensure that the analytes and standards do not interconvert or form artefactually as a result of the extraction or analysis.

5. Acknowledgments

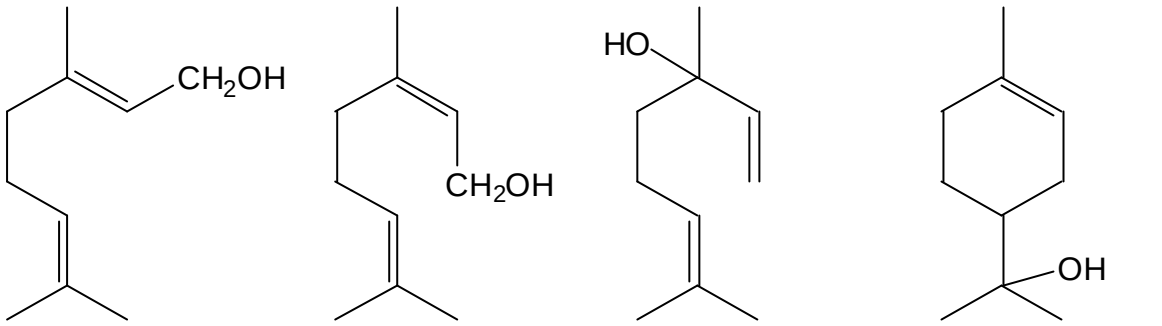
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Fig 1. Chemical structures of geraniol (1), nerol (2), linalool (3) and α-terpineol (4).



geraniol (**1**) nerol (**2**) linalool (**3**) α-terpineol (**4**)

Scheme 1

Preparation of [$^2H_7$]-geraniol (7), [$^2H_7$]-nerol (8), [$^2H_7$]-linalool (9) and [$^2H_7$]-α-terpineol (10)

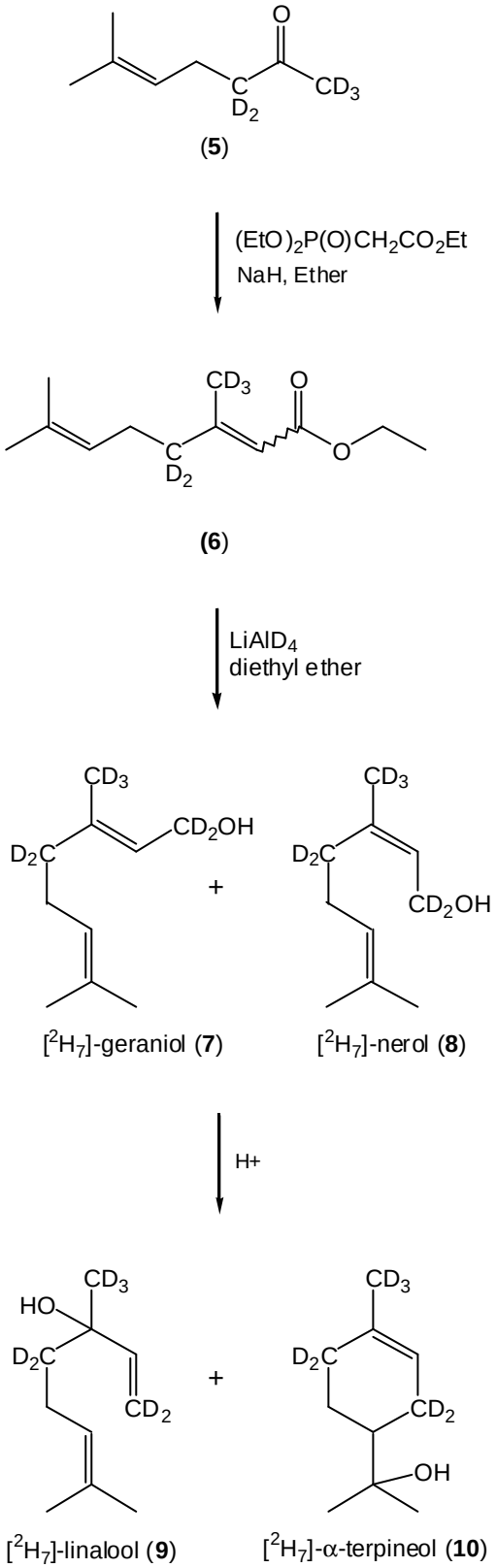


Table 1.

Stability of linalool, nerol and geraniol in pH 2.9 model wine [1] at room temperature over time

Starting compound(s) (purity %)	% Compounds after 1 hour at 25°C	% Compounds after 2 hours at 25°C	% Compounds after 4 hours at 25°C	% Compounds after 8 hours at 25°C	% Compounds after 24 hours at 25°C	% Compounds after 5 days at 25°C

Linalool (~97%)	97% linalool nd α-terpineol nd nerol nd geraniol	97% linalool <0.5% α-terpineol nd nerol nd geraniol	97% linalool <0.5% α-terpineol nd nerol nd geraniol	97% linalool <0.5% α-terpineol nd nerol nd geraniol	95% linalool 2% α-terpineol <0.5% nerol trace geraniol	85% linaloo1 8% α-terpineol 1% nerol 3% geraniol
Nerol (~93%)	92% nerol nd linalool <0.5% α-terpineol <0.5% geraniol	91% nerol <0.5% linalool <0.5% α-terpineol 1% geraniol	91% nerol <0.5% linalool 1% α-terpineol 1% geraniol	90% nerol 1.5% linalool 2.5% α-terpineol 1% geraniol	85% nerol 4% linalool 7% α-terpineol 1% geraniol	64% nerol 12% linalool 21% α-terpineol 0.5% geraniol
Geraniol (94%) Nerol (2%)	94% geraniol <0.5% linalool nd α-terpineol 2.5% nerol	94% geraniol 0.5% linalool nd α-terpineol 2.5% nerol	94% geraniol 1% linalool trace α-terpineol 2.5% nerol	93% geraniol 2% linalool trace α-terpineol 2.5% nerol	90% geraniol 7% linalool trace α-terpineol 2.5% nerol	72% geraniol 22% linalool 0.5% α-terpineol 2% nerol
α-Terpineol (96%)	96% α-terpineol nd linalool nd nerol nd geraniol	96% α-terpineol nd linalool nd nerol nd geraniol	96% α-terpineol nd linalool nd nerol nd geraniol	96% α-terpineol nd linalool nd nerol nd geraniol	96% α-terpineol nd linalool nd nerol nd geraniol	96% α-terpineol nd linalool nd nerol nd geraniol

1. Model wine was 10% ethanol, saturated with potassium hydrogen tartrate and pH adjusted to 2.9 by addition of sulfuric acid (5M). No analytes were detected in any of the control samples of this model wine before or after standing for up to 5 days. "nd" means compound not detected above an approximate upper limit of 0.1%.

On the formation of naturally occurring damascenone

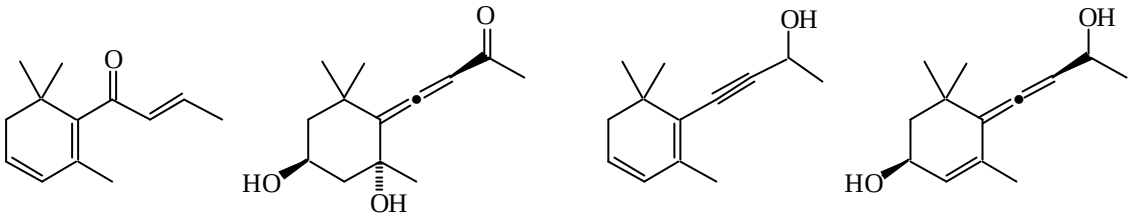
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Damascenone (formerly β-damascenone, **1**) is one of the most potent aroma compounds known to science. It is found in virtually all fruit and vegetable products, and has long been employed by the perfume industry. Despite this importance, the biogenetic pathway to naturally occurring damascenone has received comparatively little attention. The current thinking is that this formation occurs *via* degradation of carotenoids such as neoxanthin, through intermediates including "grasshopper ketone" **2**. We recently showed[1] that the dienyne species **3** is the immediate precursor to damascenone.



1 **2** **3** **4**

This paper concerns the synthesis of allenic diol **4** (and isomers) and its role as a precursor to damascenone.

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(*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene – a potent grape-derived odorant in wine.

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Running title header: 2,3,6-Trimethylphenylbutadiene

ABSTRACT

(*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (TPB) was identified as a potent odorant in acid hydrolysates of crude glycoconjugate fractions isolated from grapes and grape vine leaves. TPB was also identified in a Semillon wine, using gas chromatography/mass spectrometry, by co-injection with an authentic sample. TPB had an aroma detection threshold of 40 ng/L in a neutral white wine and the concentration of TPB in four out of five white wines analysed ranged from 50 ng/L to 210 ng/L.

Keywords*: Wine; grape; odorant; glycoside; 2,3,6-trimethylphenylbutadiene.

INTRODUCTION

Various sensory investigations [*1-5*] have indicated that volatile compounds, generated by wine acid-catalysed hydrolysis of grape-derived involatile precursor forms (mostly glycoconjugates), have a significant impact on the aroma of wines. Whereas enzyme preparations with glycosidase activity form aglycones from their glycoconjugate forms by breaking the glycosidic (i.e. acetal) linkage, the liberation of volatile compounds from glycoconjugates of alcohols by acid catalysis during wine maturation and conservation, apparently only takes place with glycosides of activated (e.g. allylic) alcohols [*6-9*]. This presumably entails cleavage of the ether bond rather than the glycosidic bond between glucose and the aglycone. Under acid conditions, this process can be accompanied by other transformations, including dehydration, cyclisation and rearrangement reactions.

Several studies [*10-14*] have been conducted on the composition of grape-derived volatile compounds, liberated from crude glycoconjugate fractions by hydrolytic means. Although the composition of such hydrolysates is highly complex, the vast majority of the components of these hydrolysates are either odorless or have weak aromas only, and probably contribute little or nothing to wine aroma and flavour. The acid hydrolysis products 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), which can impart a kerosene-like character to aged Riesling wines [*15, 16*], and β-damascenone, which has an intense fruity aroma [*8, 16*], are likely to be contributors to the aroma and flavor of some wines. However, the majority of such hydrolysis products that contribute to wine aroma and flavor are probably yet to be identified.

We report here, the identification of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB, **1**) as a potent odorant in pH 3 hydrolysates of crude glycosidic extracts of grapes, and its subsequent identification and quantification in several white wines.

MATERIALS AND METHODS

Materials. The chemicals used were of the highest purity commercially available. All solvents were Mallinckrodt nanopure grade, and verified for purity by GC/MS prior to use. Wine samples were obtained from retail outlets. Grape and grape vine leaf samples (Shiraz and Cabernet Sauvignon) were obtained from Nuriootpa in the Barossa Valley, South Australia.

Analysis by GC/O/MS. Gas chromatography/olfactometry/mass spectrometry (GC/O/MS) was carried out with a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a Gerstel MPS2 autosampler, a Gerstal thermal desorption unit (TDU) and Gerstel programmed temperature vaporisation (PTV) inlet (CIS-4). The GC was coupled to a HP 5973N mass spectrometer and Gerstel olfactory detector port (ODP2). The Gerstel MPS2 was fitted with a 70µm carbowax/divinylbenzene stable flex (CW/DVB) fibre (Supelco, USA, Cat-No. 57337-U) and was operated in automated solid phase microextraction (SPME) mode. The gas chromatograph was fitted with an approx. 30 m x 0.25 mm Phenomenex fused silica capillary column ZB-Wax, 0.25 µm film thickness. A crosspiece was installed at the end of the analytical column, approximately 2.2 m of 220 µm ID/320 µm OD of deactivated fused silica tubing (SGE, Melbourne Australia) was connected from the crosspiece to the ODP and approximately 2m of 110µm ID/320µm OD of deactivated fused silica tubing was connected from the crosspiece to the mass spectrometer, giving a split ratio of approximately 2:1 in favour of the ODP. The carrier gas was helium (BOC gases, Ultra High Purity) with a flow rate of 1.8 mL/min. For SPME, the headspace of the sample was extracted at 55°C for 5 min and desorbed in the injector for 10 min. A direct SPME Injection Sleeve with an ID of 0.75 mm (Supelco, USA) was installed in the inlet and the splitter, at 28:1, was opened after 60 sec. Injection was done in pulse splitless mode with an inlet pressure of 25.0 psi maintained until splitting. The oven temperature was started at 80°C, held at this temperature for 1 min., increased to 170°C at 4°C/min then to 250°C at 50°C/min and held at this upper temperature for 10 min. The injector was held at 220°C and the transfer line at 260°C. Analysis by stir bar sorptive extraction (SBSE), was performed by placing a Gerstel Twister stir bar (10mm long, coated with PDMS, 0.5mm film thickness) into a 15 ml glass sample tube with an aluminium-lined cap (Supelco, USA) with 10ml of wine. The samples were stirred at room temperature for 3 hours, the Twister was then removed, dried on a lint free tissue, then inserted into a glass desorption tube. The stir bar was thermally desorbed in the splitless mode using the following desorption temperature program: 20 °C, 60 °C/min to 180 °C and held there for 5 minutes. The desorbed solutes were cryofocused in the PTV inlet at -50 °C. The PTV inlet was then programmed to 250 °C at 12 °C/sec and held for 5 minutes. Injection was done in solvent vent mode. The PTV was fitted with a glass liner with silanized glass wool (GERSTEL). The compounds were separated on the column described above, using helium (BOC gases, Ultra High Purity) carrier gas at 1.2 ml/min (constant flow). The oven temperature was started at 50°C, held at this temperature for 1 min. then increased to 150°C at 10°C/min then increased to 250°C at 25°C/min and held at this temperature for 30 min. The transfer line was at 250°C. Positive ion electron impact spectra at 70 eV were recorded in the range *m/z* 35-350 (cycle time 1.01 sec). Quantitative analysis of TPB in wines was carried out using instruments and operating conditions as described above (except that the GC column was connected to the MS only) with the following modifications – a HP 6890 series liquid injector was used and operated in fast liquid injection mode with a 10 µL syringe (SGE, Australia) fitted. The injector liner used was of deactivated borosilicate glass, 4 mm I.D., with a plug of silanised glass wool (2-4 mm), tapered at the column end. Wine samples were prepared for quantitative analysis by adding a solution of [$^2H_8$]-naphthalene (500 ng) in ethanol (50 µL) as internal standard to the wine (50 mL), which was then extracted with *n*-pentane (3 mL). A portion (2 µL) of the extract was then injected onto the GC. The splitter, at 42:1, was opened after 36 sec. Fast injection was done in pulse splitless mode with an inlet pressure of 25.0 psi maintained until splitting. The carrier gas flow rate was 1.2 mL/min and the GC column was connected to a HP 5973 mass spectrometer. Positive ion electron impact spectra at 70 eV were recorded in the range *m/z* 35-350 for scan runs. For quantification of TPB (**1**), the mass spectrometer was operated in selected ion monitoring (SIM) mode The ions monitored in SIM runs were: *m/z 108 and* <u>136</u> for [$^2H_8$]-naphthalene and <u>157</u>*, 142, 172* for TPB (**1**). Selected fragment ions were monitored for 30 ms each. The underlined ion for each compound was the ion typically used for quantitation, having the best signal to noise and the least

interference from other wine components. The other ions were used as qualifiers. The analytical method was validated by a series of duplicate standard additions of unlabelled TPB (**1**, 0.1 to 15.36 µg/L, n = 9 x 2 for the analyte) to a dry red wine (12.5% ethanol, pH 3.6). The standard addition curves obtained were linear throughout the concentration range, with a coefficient of determination (r^2) of 0.997 and linear regression equation $y = 0.293x + 0.005$. The repeatability of the analysis was determined at two concentrations (0.5 µg/L and 10 µg/L) by spiking seven replicate aliquots of the same red wine with TPB (**1**). For seven replicate analyses of the wine spiked at the 0.5 µg/L level, the coefficient of variance (or relative standard deviation) was 8.5%. For seven replicate analyses of the wine spiked at the 10 µg/L level, the coefficient of variance (or relative standard deviation) was 8.4%. The limit of detection was 10 ng/L and the limit of quantitation was 50 ng/L.

Preparation of acid hydrolysates of crude glycoconjugate fractions from grapes and grape vine leaves. Cabernet Sauvignon or Shiraz grapes (1 kg) were crushed by hand, sulphur dioxide (200 mg/L) was added and the juice (600 mL) was clarified by centrifugation (8000 *g*, 20 min.). The juice was then stirred overnight with polyvinylpolypyrrolidine (PVPP, 30 g), further clarified by centrifugation, then passed down a column packed with XAD2 resin (added as 100 mL of aqueous slurry). The column was washed with water (400 mL), then dichloromethane (400 mL), then eluted with methanol (1 L). The methanol eluate was concentrated to dryness *in vacuo* to yield the crude glycosidic fraction. Cabernet Sauvignon and Shiraz leaves (1.5 kg) were frozen with liquid nitrogen, powdered in a mortar and pestle, and extracted with methanol (5 L) over three days. The filtrate was then concentrated *in vacuo*, redissolved in distilled water (1.5 L) and stirred with PVPP (150 g) overnight. The PVPP was removed by centrifugation and the juice passed twice through a column packed with XAD-2 (approx 600mL aqueous slurry) and then through a second XAD-2 (200 mL slurry) column. The crude glycosidic fraction was then recovered from the columns as described above. For preparation of acid hydrolysates, crude XAD-2 extracts (10-30 mg) of grape juice were dissolved in model wine (10 mL, 10% aqueous ethanol, buffered to pH 3.2 with potassium hydrogen tartrate and tartaric acid) and the solution was heated to 100 °C overnight. Leaf glycosides (10-30 mg) were heated to this temperature in the model wine (10 mL) for three days.

Synthesis of (E)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (TPB, 1). Megastigm-4-en-7-yne-6,9-diol (**2**) was prepared as described previously [*17*]. Para-toluene sulphonic acid (approx 2-3eq) was added to a solution of the diol (**2**, 0.2g) in $CDCl_3$ (14ml). The reaction mixture was heated to 50°C overnight (nmr monitoring), then quenched with aqueous sodium hydroxide (10%, a few drops), washed with brine, dried with sodium sulphate and concentrated in vacuo to yield the crude product as a clear orange oil. This was subjected to column chromatography on silica (eluant X4) and the main product distilled at 90-100°C at 2.8mm mercury, yielding (E)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (**1**, 10%). *m/z* (%), 172 (M^+, 35), 158 (13), 157 (100), 156 (10), 143 (15), 142 (85), 141 (30), 129 (13), 128 (15), 127 (8), 115 (20), 91 (5), 77 (6). Kovats GC retention indices, 1486 (DB-1701) and 1830 (ZB-Wax). For nmr data, see **Table 1**

Identification of TPB (1) in a Semillon wine . A 1996 commercially produced Semillon wine (50 mL) was extracted with 3ml pentane. Isooctane (3 drops) was added to approximately 1ml of this extract which was then concentrated with a gentle stream of nitrogen to approximately 0.5mL. This concentrate was loaded onto a preparative TLC plate (Merck 20 x 20 cm silica gel 60, F 254nm) which had been prewashed with ethanol and air dried. The TLC plate was developed in pentane. The fraction from the plate corresponding to TPB (**1**) was isolated with pentane (5 x 3mL - the Rf of TPB (**1**) was determined on a separate plate, developed in another tank to avoid cross-contamination). Isooctane (3 drops) was added to this pentane extract which was then concentrated under nitrogen to approx 50uL and analysed by GC/MS in scan mode as described above.

Aroma threshold of TPB (1) The aroma threshold of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB, **1**) in a young (<12 months old) neutral dry white wine (Chenin Blanc, pH 3.5, 11.5% ethanol) and in unbuffered 10% aqueous ethanol was determined according to the American Society for Testing and Materials (ASTM) method E 679-79, using 24 judges. The judges were of European origin, aged between 20 and 50, with similar numbers of males and females. The white wine had a free and total sulfur dioxide

content of ca 20 mg/L and 180 mg/L respectively. Wines were presented in ascending order of TPB (**1**) concentration, at 10, 30, 90, 270, 810, and 2300 ng/L for both the white wine and the unbuffered model wine. Panellists who could detect the spiked wines at all of these concentrations were then tested at lower concentrations of TPB (**1**). The same panellists were used for threshold determinations in both media.

RESULTS AND DISCUSSION

As part of our continuing investigations into the contribution of grape glycoconjugates to wine flavor, we recently conducted GC/Olfactometry/Mass Spectrometry (GC/O/MS) assessment of pH3 hydrolysates of fractions isolated by XAD2 resin from crude extracts of grapes and grape vine leaves. In every hydrolysate examined by GC/O/MS, we observed a strongly odorous zone with an aroma varyingly described by several assessors as 'green', 'cut-grass', 'tobacco', 'insecticide', 'chemical' and 'pungent'. This strong aroma was detected just before elution of β-damascenone on a DB 1701 column and just after this compound, which was easily recognised by its familiar stewed apple/quince-like odor, on a Carbowax column. In some GC/O runs, there was an overlap between the two aromas, giving rise to many complex odor descriptors.

In GC/O/MS of diluted samples, this 'green', 'cut-grass' aroma coincided with a peak with a mass spectrum (**Figure 1**) that was very similar to that of 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN, *15*), but was clearly not this compound as an authentic sample of the latter eluted at an earlier retention time on both columns. TDN was also seen as a much larger but much more weakly smelling peak in all chromatograms. The mass spectrum of the highly odiferous peak did not appear to match that of any other known wine component.

The mass spectrum of this unknown component of the hydrolysates was essentially the same as that of a by-product we had previously encountered during our synthesis of the damascenone precursor (**3**, **Figure 2**, *17*). Initial attempts to produce (**3**) by acid-catalysed dehydration of (**2**) with p-toluenesulfonic acid had resulted in over-reaction and the production of predominantly butadiene (**1**). The structure of **1** was unambiguously established as (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB **1**) by its proton and carbon nmr spectra (**Table 1**). The geometry of the C7-C8 olefinic linkage is assigned the *E*-configuration on the basis of the magnitude of the H7-H8 coupling constant (16.1 Hz). The arrangement of the three methyl groups on the aromatic ring was suggested on mechanistic grounds and confirmed by a HMBC nmr experiment; long-range correlations between H13 and C6/C5 as well as correlations between H12 and C3/C4 are only possible if the methyl substitution pattern is as indicated. Were the three methyl substituents actually contiguous, then we would expect only one long range ring-carbon/methyl-hydrogen correlation. That the four substituents are themselves contiguous (as in **1**) is evident from the size of the coupling between H4 and H5. This authentic sample of TPB (**1**) had an identical mass spectrum, retention time and GC/O profile to the unknown component of the hydrolysates on both the DB1701 and Carbowax columns.

TPB (**1**) was also detected in a commercial Semillon using stir bar sorptive extraction [*18*], although this compound co-eluted with other wine components desorbed from the stir bar. When a pentane extract of the same wine was concentrated and subjected to fractionation by preparative thin layer chromatography, TPB (**1**) could be clearly identified by GC/MS in the isolated fraction. The TPB peak in this fraction was symmetrically enhanced by co-injection with an authentic sample, and both the authentic sample and the wine component had identical mass spectra. To our knowledge, this is the first time TPB (**1**) has been observed as a wine component.

TPB (**1**) was subsequently quantified in several wines by GC/MS using selected ion monitoring and d_8-naphthalene as an internal standard. TPB (**1**) was found at a concentration ranging from 50-210 ng/L in four of the five white wines examined. No trace (< approx. 10 ng/L) was detected in the six red wines analysed (**Table 2**), nor in the young white wine used for aroma threshold determination (below).

The aroma detection threshold of the authentic TPB (**1**) was determined for both a neutral white wine and unbuffered 10% aqueous ethanol (model wine). In determining the sensory properties of reference compounds, it is important to ensure that they do not contain impurities which could modulate their sensory impact. The reference sample of TPB (**1**), which was > 95% pure by nmr and GC, gave only a single aroma-active region, coinciding with the dominant peak, during GC/O on both the DB1701 and Carbowax columns.

Importantly, the sample of TPB (**1**) contained no trace of damascenone detectable by GC/O or selected ion chromatograms.

The aroma detection threshold of TPB (**1**) in a neutral white wine was 40 ng/L. The distribution of best-estimate thresholds is shown in **Figure 3**. This makes it among the most potent of volatile wine components, comparable to β-damascenone, β-ionone and wine lactone [*19, 20*]. Informal descriptors used by the sensory panel to distinguish spiked from unspiked white wines ranged from 'floral', 'geranium' and 'tobacco' at the lower concentrations to 'pungent', 'very green', 'unpleasant', 'plastic', and 'insecticide' at concentrations of 270 ng/L and above. Surprisingly, despite using the same panellists and stock solutions, the aroma detection threshold of TPB (**1**) in the model wine was an order of magnitude higher – 430ng/L. Informal comments from the sensory panel indicated that the harsh aroma of the ethanol in the model wine (but not the white wine) had a masking effect on TPB (**1**). Such observations show the need for caution in extrapolating sensory data from model wines to real wines or, indeed, from one wine to another (other examples of sensory detection thresholds varying in different media are given in *21*).

TPB (**1**) has been previously reported as a potential staling compound in beer, having a leather/geranium odor [*22*], although no structural or formal sensory information was provided to support this statement. Kleipool et al [*23*] demonstrated the presence of TPB (**1**) in rum and cognac by comparison with an authentic sample synthesised by a different route to the one we report here. They described TPB (**1**) as having a "harsh and metallic character - - -remarkably strong for a hydrocarbon". De Rijke and Ter Heide [*24*] also included TPB (**1**) in a list of compounds isolated from rum and cognac but did not discuss this compound.

Not withstanding the small number of samples analysed, the presence of TPB (**1**) in four out of five white wines and in all of the hydrolysates (which were from berry and leaf samples obtained from Cabernet Sauvignon and Shiraz vines) but in none of the red wines examined is intriguing. While it is plausible that the samples analysed may not be typical of these varieties, it is also possible that the absence of TPB (**1**) in the red wines is because this highly conjugated molecule can be easily protonated and the resulting cation is able to react with compounds (e.g. nucleophilic polyphenols) that are present in red wines at higher concentration than in white wines or model wine hydrolysate solutions. Whether or not this is the case, such considerations illustrate the inadequacy of relying solely on compositional or sensory analysis of hydrolysates formed in model wines to predict the composition or sensory properties of real wines, in which biological and chemical transformations of hydrolysis products can compete with their generation. Nevertheless, such model studies can still provide pointers towards possible important wine flavour compounds, as has clearly been the case in this study.

The structure of TPB (**1**) and its facile formation from the diol (**2**) indicate that it may belong to the class of compounds known as C_{13} norisoprenoids. These compounds are presumed to be formed by oxidative biodegradation of plant carotenoids followed by further enzyme mediated transformations and, in some cases, by mild acid-catalysed dehydration and rearrangement reactions [*25*]. While grapes and grape vine leaves are known to yield a rich variety of such compounds, only three - β-damascenone, TDN and β-ionone - have hitherto been implicated in wine flavour [*16, 19, 20*]. Studies on potential precursors to TPB (**1**), currently underway in our laboratory, should indicate whether TPB (**1**) also belongs to this group.

Abbreviations used : TPB, (*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene; GC/O/MS, gas chromatography/olfactometry, mass spectrometry; TDU, thermal desorption unit; PTV, programmed temperature vaporisation; SPME, solid phase microextraction; SBSE, stir bas sorptive extraction.

Acknowledgments

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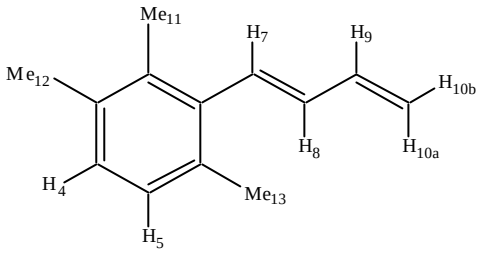
Figure captions

Figure 1. Mass spectrum of a) (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB, 1) and b) TDN.

Figure 2. Synthesis of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB, 1) and megastigma-3,5-dien-7-yn-9-ol (3).

Figure 3 Best-estimate aroma thresholds of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB, 1) in a neutral dry white wine.

Table 1. NMR parameters for TPB (1) (300 MHz (^{1}H), 75 MHz (^{13}C)., acetone-d_6).



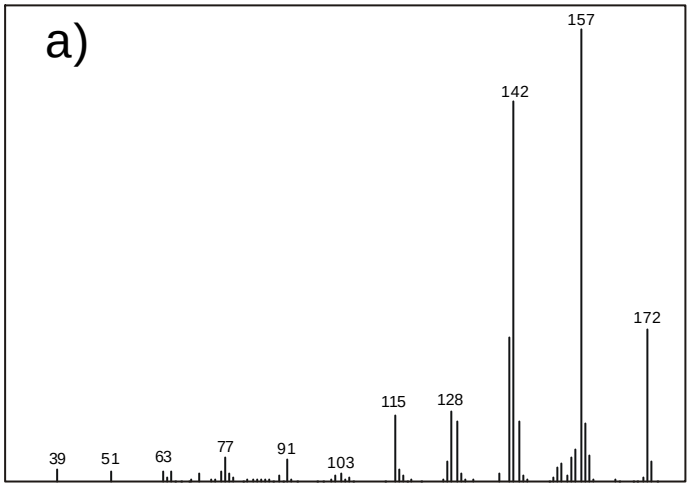
Signal[a]	δ[b] (Multiplicity[c])	Signal[a]	δ
		C_1	138.3
		C_2	135.6
		C_3	135.6
H_4	6.98 (d) A part of AB quartet	C_4	129.9
H_5	6.96 (d) B part of AB quartet	C_5	128.8
		C_6	134.8
H_7	6.71 (br d)	C_7	133.4
H_8	6.28 (dddd)	C_8	136.8
H_9	6.64 (dddd)	C_9	139.2
H_{10a}	5.31 (dddd)	C_{10}	117.9
H_{10b}	5.18 (dddd)		
H_{11}	2.19, s	C_{11}	17.8
H_{12}	2.22, s	C_{12}	21.2
H_{13}	2.22, s	C_{13}	21.8

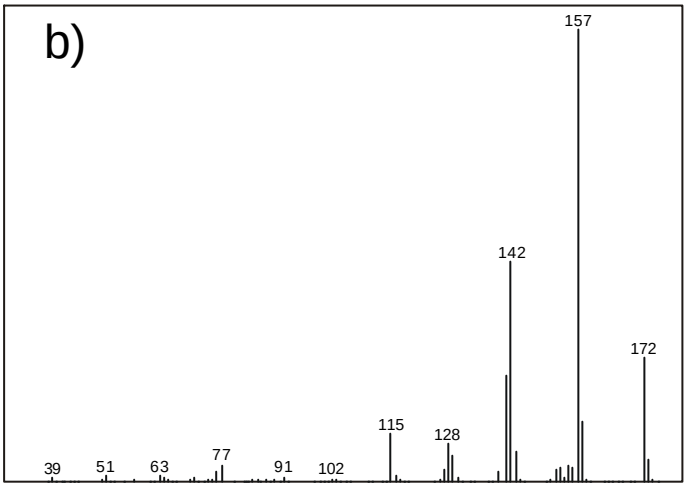
a) Assignments were made with the aid of COSY HETCOR and HMBC[d] experiments
b) δ values in ppm
c) $J_{9,10a}$=16.9 Hz; $J_{7,8}$=16.1 Hz; $J_{9,10b}$=$J_{8,9}$=10.4 Hz; $J_{4,5}$=7.8 Hz; $J_{10a,10b}$=1.9 Hz; $J_{7,9}$= $J_{7,10a}$= $J_{7,10b}$= $J_{8,10a}$= $J_{8,10b}$=0.8 Hz.
d) 600 MHz (^{1}H), 150 MHz (^{13}C).

Table 2. Concentration of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB, **1**) in commercial wines.

Wine	Concentration (ng/L)
1997 Riesling	60
1996 Chardonnay	100
1997 Chardonnay	nd
1996 Semillon	210
1994 Chardonnay	50
1997 Cabernet Sauvignon	nd
1996 Cabernet Sauvignon	nd
1988 Shiraz	nd
1996 Merlot	nd
1995 Cabernet Sauvignon	nd
1995 Shiraz	nd

nd not detected.


a)
157
142
172
115
128
39
51
63
77
91
103


b)
157
142
172
115
128
39
51
63
77
91
102

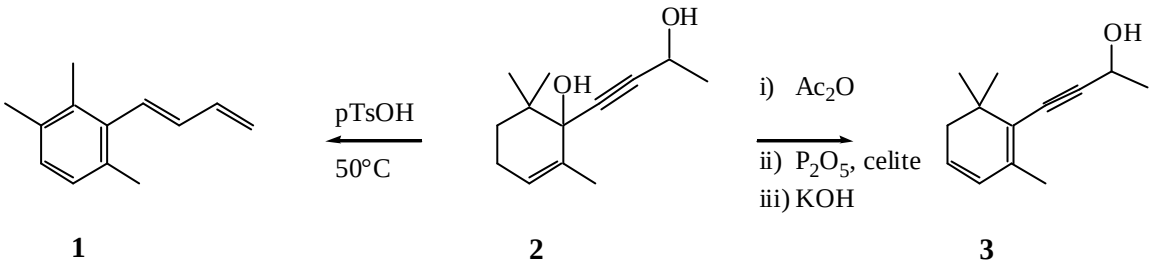


Figure 2. Synthesis of (*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (TPB, **1**) and megastigma-3,5-dien-7-yn-9-ol (**3**, R=H)

2,2,6,8-Tetramethyl-7,11-dioxatricyclo[6.2.1.0^{1,6}]undec-4-ene (Riesling Acetal) is a Precursor to 1,1,6-Trimethyl-1,2-dihydronapthalene (TDN) in Wine

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ABSTRACT

2,2,6,8-Tetramethyl-7,11-dioxatricyclo[6.2.1.0^{1,6}]undec-4-ene (Riesling Acetal) has been synthesised and shown to be a precursor of 1,1,6-trimethyl-1,2-dihydronapthalene (TDN) in wine. Riesling Acetal is completely converted to TDN at wine pH.

INTRODUCTION

1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN **1**) is found in a variety of wines. When present at a concentration of several hundred micrograms per litre as is sometimes observed in bottle-aged Riesling wines, **1** can impart an undesirable petroleum or kerosene note to the beverage (Simpson, 1978; Simpson and Miller, 1983; Winterhalter, 1991)

Like TDN **1**, Riesling Acetal **2**, is thought to be formed in wine by acid hydrolysis of grape-derived glycoconjugated precursors (Winterhalter et al, 1990a, 1990b). Winterhalter (1991) obtained both **1** and **2** as acid hydrolysis products from the hemiacetal **7**, which is the stable form of the diol **6** in solution. In addition to the major products TDN **1** and Riesling Acetal **2**, the hydrolysate contained small amounts of several other products, one of which was tentatively assigned the structure **5**. The assignment of the structure **5** was based on the mass spectrum and an IR spectrum obtained by GC/FTIR. Finally, upon standing at −18°C, this compound was observed to dehydrate over a few days.

Subsequently, Humpf et al (1991) isolated compound **4** from red currant (*Ribes rubrum L.*) leaves and showed that it produced, *inter alia*, TDN **1** and Riesling Acetal **2** when heated at pH 2.5 under simultaneous distillation-extraction (SDE) conditions. A proposed mechanism, based on that suggested by Humpf et al (1991), for formation of **1** and **2** from aglycone **3** is outlined in figure 2. Initial allylic rearrangement produces **6** which can then either eliminate water to produce, ultimately, TDN **1**, or undergo ring-closure to give the hemiacetal **7** and then Riesling Acetal **2**.

We were interested in synthesising glycoconjugates of diols **3**, **6** and **7** to determine whether the position of glycoconjugation had an effect on the ratio of TDN **1** to Riesling Acetal **2** formed in wine. At an early stage of this research, we obtained the acetal **10**. This paper describes the hydrolysis of the acetal **10** at wine pH, and the consequent observation that Riesling Acetal **2** is in fact an intermediate in the formation of TDN **1**.

MATERIALS AND METHODS

The chemicals used were the highest purity commercially available. Solvents were distilled prior to use as described (Perrin et al., 1966). NMR spectra were run on a Varian Gemini Spectrometer operating at either 300 MHz (^{1}H) or 75.5 MHz (^{13}C). Unless otherwise indicated, all spectra were of $CDCl_3$ solutions.

Preparation of samples for analysis of TDN 1

An aliquot (100 µL) of a solution of $^{2}H_8$ naphthalene in ethanol (0.5 mg/100ml) was added to the hydrolysate samples (8 mL) in a 15 mL glass screw cap vial with an aluminium-lined cap(Supelco) using a glass syringe (100 µL SGE). Pentane:diethyl ether (2:1, 3 mL) was added and the mixture was shaken briefly. A portion of the organic layer was then transferred to a vial for GC/MS analysis. For calculating the concentration of the analytes in the wine samples, replicate standards were prepared at the same time as the hydrolysate samples, by adding the same amount of internal standard as above (100 µL) to a 100 µL of a solution of

TDN **1** in ethanol (0.5 mg/10-0 mL.), diluted with dichloromethane (1800 µL) and analysed by the GC/MS method (see below) to calculate the relative response factors.

GC/MS analysis

All solvents used were Mallinckrodt nanopure grade, and verified for purity by GC/MS prior to use. Samples were analysed with a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a liquid HP 6890 series injector and coupled to a HP 5973 mass spectrometer. The liquid injector was operated in fast liquid injection mode with a 10 µL syringe (SGE, Australia) fitted. The gas chromatograph was fitted with an approx. 30 m x 0.25 mm I.D. Phenomenex fused silica capillary column ZB-Wax, 0.25 µm film thickness. The carrier gas was helium (BOC gases, Ultra High Purity), and the flow rate was 1.2mL/min. The oven temperature started at 50°C, was held at this temperature for 1 min., then increased to 220°C at 10°C/min and held at this temperature for 10 min. The injector was held at 200°C and the transfer line at 250°C. The sample volume injected was 2µL and the splitter, at 42:1, was opened after 36 sec. Fast injection was done in pulse splitless mode with an inlet pressure of 25.0 psi maintained until splitting. The glass liner (Agilent Technologies) was borosilicate glass with a plug of resilanised glass wool (2-4 mm) at the tapered end to the column. Positive ion electron impact spectra at 70 eV were recorded in the range *m/z* 35-350 for scan runs. For quantification of TDN **1**, mass spectra were recorded in the Selective Ion Monitoring (SIM) mode. The ions monitored in SIM runs were: *m/z 108 and* <u>*136*</u> for [$^2H_8$]-naphthalene and *142, <u>157</u>, 172 for TDN*. Selected fragment ions were monitored for 20 ms each. The underlined ion for each compound was the ion typically used for quantitation, having the best signal to noise and the least interference from other wine components. The other ions were used as qualifiers.

Validation

The method was validated by a series of duplicate standard additions of unlabelled TDN (1.0 to 200 µg/L, n = 9 x 2 for compound) to a dry white wine (Australian Chenin Blanc, 11.5 % alc/vol, pH =3.40). The standard addition curves obtained were linear throughout the concentration range, with a coefficients of determination (r^2) = 0.998 and a linear regression equations: $y = 0.428x + 0.002$. To ensure that the accuracy of the analysis was maintained, duplicate control wines, each with and without spiked standard addition of 100 µg of TDN **1** per litre of wine, were analysed with every set of hydrolysate samples

2,2-Ethylenedioxy-4-bromobutane

To a solution of methyl vinyl ketone (23.75 mL, 0.285 mol) in benzene (200 mL) was added at 0°C HBr (20 mL 45% w/v in acetic acid, 0.113 mol). The solution was stirred at 0°C for 30 min, then ethylene glycol (47.7 mL, 0.855 mol) and trimethyl orthoformate (62.4 mL, 0.57 mol) were added, followed by stirring for a further 2 hours at 0°C. The reaction was then washed with water (3x50 mL), $NaHCO_3$ (50 mL) and brine (50 mL), dried (Na_2SO_4) and the solvent evaporated. The compound was purified by column chromatography (20% ether/X4) yielding 2,2-ethylenedioxy-4-bromobutane (13.1g, 24%) as a colourless oil. ^{1}H NMR δ: 3.93 (4H, m, OCH_2CH_2O); 3.42 (2H, t J=8.1 Hz, H_3); 2.27 (2H, t J=8.1 Hz, H_2); 1.33 (3H, s, H_1); ^{13}C NMR δ: 109.0 (C_2); 64.7 (C_5); 42.8 (C_4); 26.8 (C_3); 24.0 (C_1).

1,1,5-trimethyl-3,6-dihydroxy-9,9-ethylenedioxymegastigm-4-ene 10.

Commercially available 4-oxoisophorone **8** was regiospecifically reduced according to the method of Ishihara et al. (1986). After chromatography the desired isomer (45% yield) was silylated with TMSCl/pyridine. 2,2-Ethylenedioxy-4-bromobutane (0.33g) was added to lithium metal (0.04g) in THF (3 mL) and to this was added the silylated ketone (**9**, 0.5g). After stirring overnight the reaction was quenched with ammonium chloride solution, and the reaction mixture diluted with ether and washed successively with water, bicarbonate solution and brine. After drying, the solution was concentrated and the product subjected to column chromatography on silica (15% EtOAc/petrol) to give the silylated analogue of **10** as a viscous oil (237mg, 29%). ^{1}H NMR δ: 5.35 (1H, m, H_4); 4.26-4.16 (1H, m, H_3); 3.94 (4H, m, OCH_2CH_2O); 1.83-1.69 (4H, m, $H_{7,8}$); 1.68-1.55 (2H, m, H_2); 1.72 (3H, dd J=1.4, 0.6 Hz, H_{13}) 1.29 (3H, s, H_{10}); 0.97, 0.96 (6H, 2s, H_{12}); 0.11 (9H, s, TMS). ^{13}C NMR δ: 140.2; 127.3; 110.1; 76.8; 66.2; 64.6, 64.5; 44.9; 40.1; 34.4, 30.8; 25.0, 23.8, 23.5, 18.6; 0.3. The silyl group was then removed by treatment with TBAF in THF to give the ketal **10**. ^{1}H NMR δ: 5.45 (1H, m, H_4); 4.22-4.12 (1H, m, H_3); 3.93 (4H, m, OCH_2CH_2O); 1.82-1.69 (4H, m, $H_{7,8}$); 1.72 (3H, m, H_{13}); 1.66-1.51 (2H, m, H_2); 1.28 (3H, s, H_{10}); 0.97, 0.94 (6H, s, $H_{11,12}$). ^{13}C NMR δ: 140.7 (C_5); 126.8 (C_4); 109.9 (C_9); 76.9 (C_6); 65.6 (C_3); 64.6, 64.5 (OCH_2CH_2O); 44.6 (C_2); 40.1 (C_1); 34.7, 30.8 ($C_{7,8}$); 24.8, 23.7, 23.4, 18.5 ($C_{10,11,12,13}$).

Hydrolysis of 10 in model wine.

Ketal 10 **(2.5mg) was dissolved in 50 mL of aqueous 12% EtOH, buffered to pH 3.0 with potassium hydrogen tartrate/tartaric acid. Portions (1 mL) of the solution were sealed in glass ampoules and heated at the temperatures and times indicated in Table 1. The reactions**

were quenched by immersion in ice-water, and the hydrolysates were extracted with pentane (2x0.5 mL) and ethyl ether (2x0.5 mL). GC-MS analysis showed varying amounts (see table 1) of three major products: Riesling Acetal 2**, m/z 208 (M^+), 193, 180, 166, 165, 148, 138, 133, 125, 123, 119, 109, 95, 91, 77, 67, 55, 43, 41; TDN** 1**, m/z 172 (M^+), 157, 142, 141, 128, 115, 91, 77, 71, 63, 51; tentative structure** 5**, m/z 190 (M^+), 172, 157, 147, 142, 133, 132, 128, 120, 115, 105, 91, 79, 77, 71, 57,43.**

2,2,6,8-Tetramethyl-7,11-dioxatricyclo[6.2.1.$0^{1,6}$]undec-4-ene (Riesling Acetal) 2.

Ketal **10** (54 mg) in pH 3.0 buffer (60 mL) was heated in an SDE apparatus with ether/pentane (30 mL each) for 90 minutes. After this time the organic extracts were concentrated and chromatographed on silica (230-400 mesh, 25% EtOAc/Petrol) to give pure fractions of TDN (Rf 0.78) and Riesling Acetal (15 mg, Rf 0.58). The spectral properties of **2** thus obtained were in accord with those previously reported (Winterhalter et al., 1990b).

Hydrolytic Study of 2.

Rieling Acetal (c. 1250 ppb) was dissolved in model wine (10% EtOH) at either pH 3.0 or 3.2 and this solution was then sealed in ampoules and heated at 45°C. At set times, ampoules were removed and quenched in ice-water. An aliquot (100 µL) of the solution 2, described as above, was added to the hydrolysate samples (8mL) in a screw cap vial using a glass syringe (100µL SGE). Pentane:diethyl ether (2:1, 3mL) was added and the mixture was shaken briefly. A portion of the organic layer was then transferred to a vial for GC/MS analysis.

RESULTS AND DISCUSSION

The synthetic scheme employed for the synthesis of **10** is outlined in figure 3. Reduction of oxoisophorone **8** with sodium borohydride in the presence of cerium(III)chloride (Ishihara et al., 1986) produced mainly the 3-hydroxy compound,[ξ] which was converted into the corresponding silyl ether **9**. Treatment of **9** with the lithio derivative of 2,2-ethylenedioxy-4-bromobutane (Ponaras, 1976), followed by desilylation provided the ketal **10**.

We decided to examine the susceptibility of **10** to weak acid. The ketal **10** was dissolved in pH 3.0 buffer and sealed in ampoules, which were then heated at either 50°C or 100°C. After set times the ampoules were opened, the contents extracted with organic solvent, and the extracts subjected to GC-MS analysis. The results are collected in Table 1.

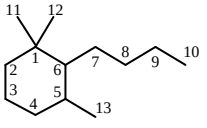
At the lower temperature, it is clear that there is formation of all three main products, in agreement with the previous work of Winterhalter (1991). However, whereas their study suggested that **1** and **2** were competing end products in the sequence outlined in figure 2, our data indicated that Riesling Acetal **2** is converted into TDN **1** upon extended hydrolysis - probably via the intermediacy of the compound tentatively identified by Winterhalter (1991) as **5**. Indeed, at the higher temperature there was no Riesling Acetal **2** present, with the hydrolysate containing almost exclusively TDN **1**. The discrepency between the results obtained in this study and by Winterhalter (1991) for the observed products at 100°C can be ascribed to the longer reaction time employed here, and also to the fact that under SDE conditions the volatiles are extracted into ether as they are formed, thus preventing any further acid catalysed transformations.

To test this hypothesis it was necessary to prepare some pure Riesling Acetal **2** and subject it to acidic hydrolysis. The ketal **10** was heated under SDE conditions, as described by Winterhalter (1991), and pure Riesling Acetal **2** was obtained by column chromatography of the organic fraction on silica gel. This pure Riesling Acetal **2** was dissolved in model wine, sealed in ampoules at either pH 3.0 or pH 3.2 and heated at 45°C. The TDN formed was quantified by GC-MS with the commercially available d_8-naphthalene as the internal standard.

The evolution of TDN **1** in the hydrolysates is shown in Figure 4. As expected for acid-catalysed transformations, the rate of TDN **1** formation was greater at the lower pH. Following prolonged hydrolysis at the lower pH (achieved by heating at 100°C for 7 days) the concentration of TDN was 1088 ppb, close to

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[ξ] The numbering scheme is that employed for the megastigmane skeleton:



the theoretical maximum that could be formed from the Riesling Acetal **2**. These prolonged hydrolyses also demonstrated the stability of TDN **1** under the reaction conditions.

These data indicate that the formation of Riesling Acetal **2** from precursors such as **3**, **6** and **7** is reversible, and that Riesling Acetal **2** (and possibly also compound **5**) is an intermediate in the formation of TDN. TDN presumably has the greatest thermodynamic stability of the hydrolysis products of these precursors. Thus, while glycoconjugation of the polyols **3**, **6** and **7** may be important to the rate of formation of TDN, it is unlikely to have any bearing on the amount of TDN that can ultimately be formed in wine.

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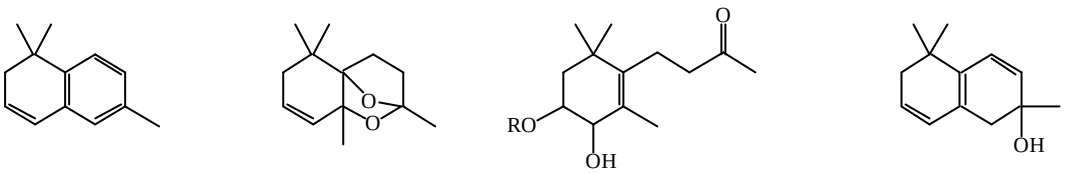
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1 **2** **3**, R=H **5**
4, R=β-D-Glucose

Figure 1. Structures of TDN **1**, Riesling Acetal **2**, and compounds **3**-**5**.

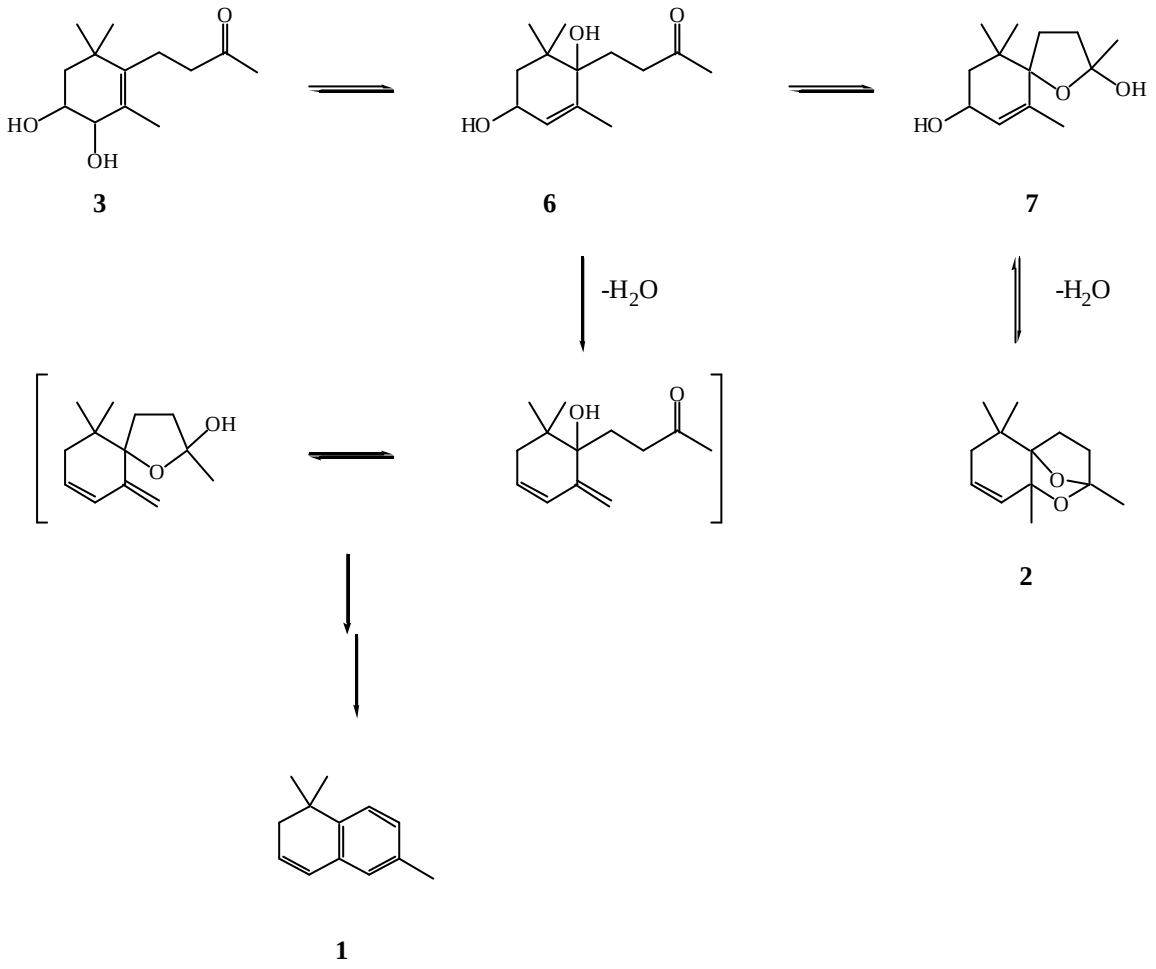
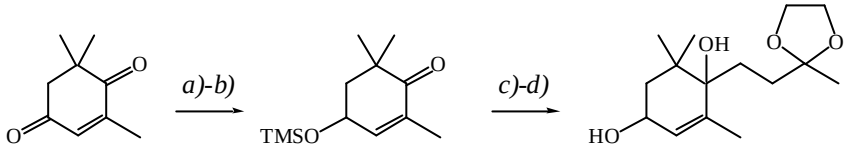
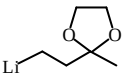


Figure 2. Proposed mechanism of formation of both **1** and **2** from aglycone **3**



8 **9** **10**

a) $NaBH_4$, $CeCl_3$ *b)* KH, TMSCl *c)* 

d) TBAF

Figure 3. Synthesis of ketal **10** from commercially available **8**.

Temp (°C)	Time (hr)	1	2	5
50	48	23	65	12
50	96	67	1	32
100	24	>99	n.d.	<1

Table 1: Relative proportions of **1**, **2** and **5** formed in the hydrolysis of **10**. (Trace hydrolysis products not included; n.d. not detected)

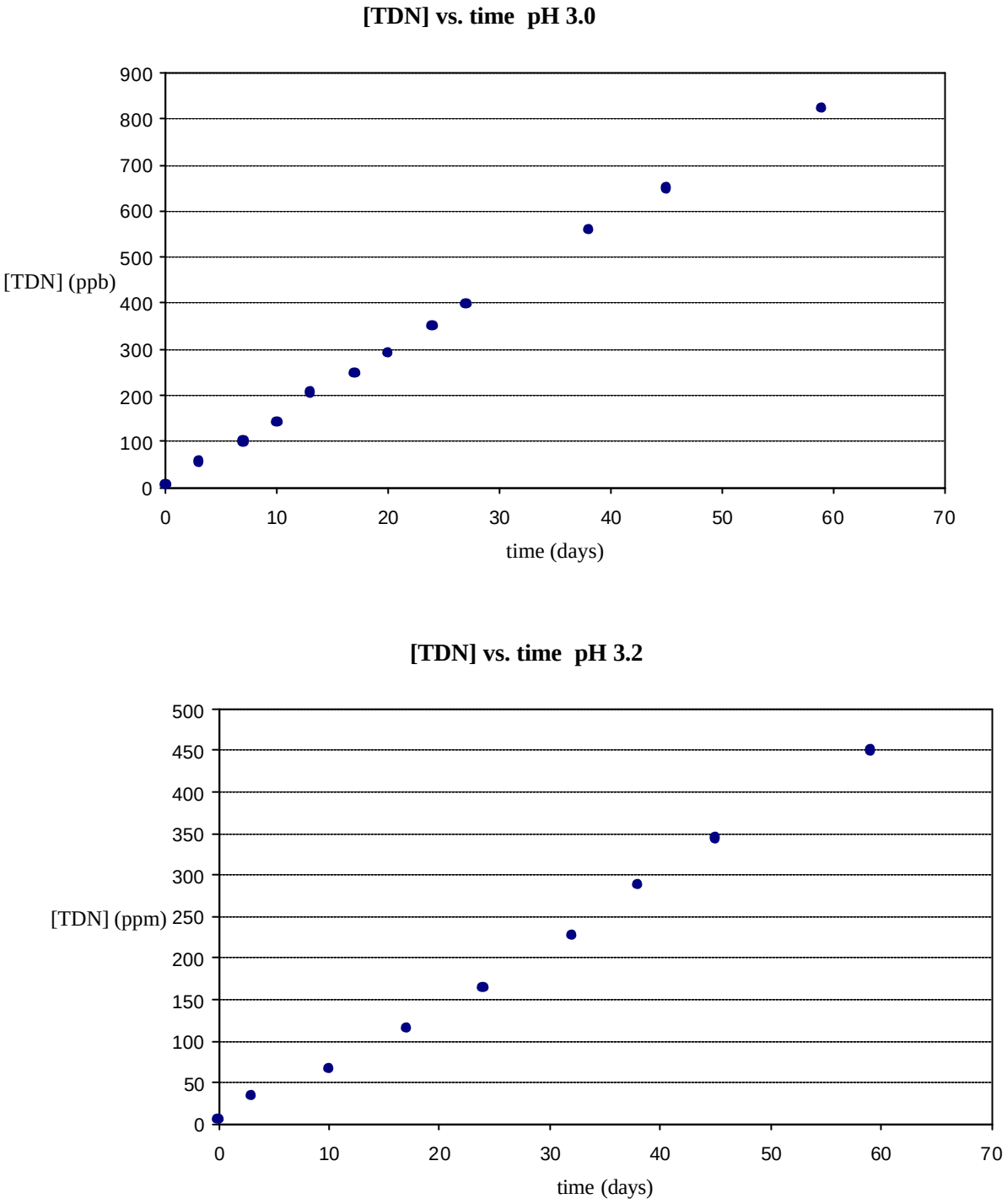


Figure 4. Concentration of TDN vs. time in the hydrolysis of **2** at pH 3.0, 45°C (upper) and pH 3.2, 45°C (lower).

The effects of sample preparation and gas chromatograph (GC) injector techniques on the accuracy of measuring guaiacol, 4-methylguaiacol and other volatile oak compounds in oak extracts by stable isotope dilution analyses (SIDA).

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Abstract

The deuterium-labelled standards, [$^2H_3$]-guaiacol and [$^2H_3$]-4-methylguaiacol were synthesized and utilised in a method employing gas chromatography-mass spectrometry to determine the concentration of guaiacol and 4-methylguaiacol in wine or extracts of oak shavings. The method was combined with previously published methods for 4-ethylphenol, 4-ethylguaiacol, *cis*- and *trans*-oak lactone and vanillin, so that all these compounds could be determined in a single analysis. The method can employ either liquid-liquid extraction or headspace solid-phase microextraction (SPME), and is rapid, robust, precise and accurate. Under certain conditions there was artefactual generation, to varying degrees, of guaiacol, 4-methylguaiacol, *cis*-oak lactone and vanillin during the analysis of oak extracts, especially when diethyl ether extraction and injector block temperatures at or above 225°C were employed. The most substantial effects were observed for guaiacol, in which results could be exaggerated by over ten times. These artefacts could be avoided by using headspace SPME or by using liquid-liquid extracts with pentane or pentane:diethyl ether (2:1) injected at 200°C providing spot checks using headspace SPME were done. Data obtained for previously published 'quantitative' analyses of guaiacol in oak extracts should be considered to have questionable value.

KEYWORDS: oak, wine, analysis, artefacts, precursors, guaiacol

INTRODUCTION

Although barrels were originally regarded as little more than inert containers they are now used primarily to enhance wine flavour. Wine is often stored in oak barrels or in contact with oak chips, staves of oak wood, or other oak products, which are all sources of additional wine aroma and flavour components. After the value of the grapes, the cost of the oak is the second greatest expense in the production of many wines. It

has been estimated that oak costs the Australian wine industry in the range of 89 to 108 million Australian dollars annually (*1*).

The oak composition and the condition in which wine maturation takes place is of primary importance (e.g. *2, 3*) to the impact of oak on wine quality. Several aroma compounds identified in wine are derived principally during the process of oak barrel maturation of the wine. Some volatile oak derived compounds are formed from thermal degradation of lignin or cellulose and hemicellulose (*4-10*) and extracted during wine storage. Others are derived from the charring or toasting of oak barrels, which transforms some of the lignin into forms that react more readily with wine than does unheated lignin.

Hundreds of oak compounds have been identified in the literature (*8, 11-18*), but only several are considered to have a significant effect on wine quality and flavour. The relative concentrations of these important compounds, and the effect on wine quality and flavour, due to variations in oak origin, seasoning, coopering, microbial interaction (such as in malolactic fermentation) and other industry practices have been studied by many authors (*4-5, 8, 14, 16, 19-23*). Seven of the most important flavour compounds in wine, associated with maturation in contact with oak barrels, are guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone, vanillin, 4-ethylphenol and 4-ethylguaiacol, the last two compounds as products of *Brettanomyces / Dekkera* yeast which can flourish during barrel maturation. The structures of these seven compounds are shown in Figure 1.
Guaiacol (2-methoxyphenol) has a smoky, phenolic, aromatic, burnt, burnt bacon aroma (*19, 24-26*). The aroma threshold of guaiacol in a dry white table wine of unspecified variety was 20 µg/L (*27*). Boidron et al. obtained aroma thresholds for guaiacol at 95 µg/L in an Ugni-blanc white wine and 75 µg/L in a Merlot red wine (*19*). 4-Methylguaiacol has a smoky aroma and a reported threshold of 65 µg/L in both the Ugni-blanc and Merlot wines (*19*). In hundreds of analyses done at this laboratory we have observed that wine matured in oak typically contains between 10 and 100 µg/L of guaiacol and between 1 and 20 µg/L of 4-methylguaiacol, although much higher values have occasionally been determined. Both guaiacol and 4-methylguaiacol are formed by the pyrolysis of lignin (*6, 9, 28*) during the toasting of oak barrels with more guaiacol and 4-methylguaiacol produced at higher temperatures. Chatonnet observed that, of the oak-derived volatile phenols, only these two compounds and vanillin were present in wine at concentrations above their individual aroma thresholds (*14-15, 29*). Above 80 µg/L Rapp and Versini (*30*) found guaiacol to have a negative effect on wine aroma. In a study of the aroma of barrel aged wine, Spillman (*31*) found that the concentration of guaiacol was negatively correlated with the perceived aroma intensity of 'green apple', and positively correlated with 'smoky' character in a Chardonnay wine. Other authors have discussed the importance of oak lactone (*32-34*), vanillin (*14-15, 29, 35*), and 4-ethylphenol and 4-ethylguaiacol (*32, 36, 37*) to wine flavour.

In any analysis, the internal standard used should be as similar as possible to the analyte measured, without being present in the matrix to begin with. Isotopically labelled standards are ideal as they have virtually identical physical and chemical properties to their unlabelled analogues. The principle way to discriminate between the two compounds, and measure their quantities, is by mass spectrometry. As the labelled internal standard is virtually identical chemically to the substrate being assayed, the accuracy of the analysis is not reduced by inefficiency in isolation or by analyte decomposition. An advantage of this is that complete extraction of the analyte of interest from the matrix is no longer a necessity. Another advantage is that methods can be streamlined so that several components can be analysed in the one extraction and GC/MS run (as done in this paper), even though they have different chemical structures. Stable isotope dilution analysis (SIDA) has been used for over a decade to determine the concentration of methoxypyrazines in grapes and in wine (*38-44*). Recently SIDA techniques have been described for the quantitation of trichloroanisole (*45*), vanillin (*35*), ethyl dihydrocinnamate, ethyl cinnamate, methyl anthranilate and ethyl anthranilate (*46*), β-damascenone, α-ionone and β-ionone (*44*), diacetyl (*47*), oak lactone (*33*), 4-ethylphenol (*36*) and linalool, geraniol, nerol and α-terpineol (*48*) in wine.

Even the use of SIDA, however, does not guarantee accuracy of measurement. The generation of an analyte as an artefact of the analytical conditions is one potential source of error, as we have already described for the analysis of vanillin (*35*). This paper describes the susceptibility of the analysis of other oak components to such problems, and the need for extra caution in determining the concentration of guaiacol in oak extracts.

Materials and methods

Materials

All solvents were Mallinckrodt nanopure grade, and verified for purity by GC/MS prior to use. Unless stated otherwise, all model wine was 10% ethanol in water, adjusted to pH 3.4 with potassium hydrogen tartrate and tartaric acid.

Unlabelled guaiacol and 4-methylguaiacol were purchased from Sigma-Aldrich Australia. Purity of all standards was verified by GC/MS prior to use, and periodically prior to making stock standard solutions.

We have already published analytical methods for 4-ethylphenol and 4-ethylguaiacol (*36*), *trans*-oak lactone and *cis*-oak lactone (*33*) and vanillin (*35*). These publications include details of the syntheses of the internal standards and acquisition of the unlabelled reference materials for these compounds. The structures of the deuterated standards we used can be seen in Figure 2.

2-[$^2H_3$]-Methoxyphenol (i.e. d_3-guaiacol)

4-Methyl-2,6-di-*t*-butylphenol (250 mg), followed by 1,2-dihydroxybenzene (catechol) (4.97 g), then [$^2H_3$]-methyl iodide (2.8 mL), was added to a solution of sodium isopropoxide (generated with 2.52 g of sodium hydride) in isopropanol (320 mL) under nitrogen. The mixture was stirred at 25°C overnight, then acidified with conc. aqueous hydrogen chloride to pH < 4 and evaporated to dryness *in vacuo*. Water (50 mL) was added to the product, which was then extracted with pentane (5 x 50 mL). The combined pentane extracts were dried (sodium sulfate) and the pentane evaporated *in vacuo* leaving crude product (2.94 g, 54% yield), which was purified by dry column chromatography using acidic alumina (activity IV) as the stationary phase and dichloromethane:pentane (1:1) as the eluant. Column fractions were washed with aqueous sodium bisulfite to remove traces of iodine and dried (sodium sulfate). The solvent was removed *in vacuo* and the product purified by distillation under reduced pressure with a Kugelrohr microdistillation apparatus to yield d_3-guaiacol as clear oil, which crystallized at low temperature (1.62 g, 30% yield, 100% pure by GC/MS). Mass spectrum: *m/z* 128 (7%), 127 (M+, 93%), 110 (9%), 109 (100%), 81 (56%), 53 (13%), 52 (10%), 51 (8%) similar to that reported by Cerny and Grosch (*49*).

2-[$^2H_3$]-Methoxy-4-methylphenol (i.e. d_3-4-methylguaiacol)

A solution of [$^2H_3$]-vanillin (500 mg, as made by Spillman et al. *35*), potassium hydroxide (1.24 g, 15 molar eq.) and hydrazine hydrate (>99% pure, 0.54 mL, 7.6 molar eq.) in diethylene glycol (20 mL) was heated under nitrogen to 130°C for 1 h., then 190°C for 3 h. The solution was cooled, hydrochloric acid (0.3 M, 73.5 mL) was added, and the d_3-4-methylguaiacol isolated with *n*-pentane (6 x 30 mL). The product was purified by distillation under reduced pressure with a Kugelrohr microdistillation apparatus to yield d_3-4-methylguaiacol as clear crystals. (419 mg, 92% yield, 100% pure by GC/MS). Mass spectrum: *m/z* 142 (10%), 141 (M+, 100%), 140 (9%), 123 (77%), 95 (19%), 77 (7%), 67 (9%), 55 (5%).

Quantitative analysis of guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, *trans* and *cis*-oak lactone and vanillin in one assay

Preparation of samples for analysis

For analysis of liquid-liquid extracts of wine or model wine oak extracts, a solution of [$^2H_3$]-guaiacol (0.500 μg), [$^2H_3$]-4-methylguaiacol (0.500 μg), [$^2H_4$]-4-ethylphenol (2.34 μg), [$^2H_4$]-*trans*-oak lactone (3.36 μg), [$^2H_4$]-*cis*-oak lactone (1.90 μg) and [$^2H_3$]-vanillin (2.50 μg) in ethanol (100 μL) was added to the sample (5.000 mL) in a screw cap vial using a glass syringe (100 μL Hamilton). The organic solvent (*circa* 2 mL), was added and the mixture was shaken briefly. A portion of the organic layer was then placed in a vial ready for instrumental analysis.

Reference standards containing 100 μL of deuterated internal standards ethanolic solution (as described above) and 100 μL of normal analytes ethanolic solution (guaiacol (0.200 μg), 4-

methylguaiacol (0.200 µg), *cis*-oak lactone (0.500 µg), *trans*-oak lactone (0.500 µg) and vanillin (0.500 µg)) in diethyl ether:*n*-pentane (1:2, approximately 1 mL) were used. It is important that these reference calibrants are concocted on the same day and using the same mix of deuterated standards as that used to spike the wine extracts for analysis on that day, with the same glass syringe as that used to add the internal standards to the wine, and a separate syringe (preferably of the same type and size) to measure the solution of unlabelled compounds used to make up the reference mix.

For analysis by solid-phase microextraction (SPME) the deuterated standards solution (as described previously) and sodium chloride (*circa* 1 g) were added to the wine (5.000 mL) in a screw cap vial with a Teflon seal. A SPME portable field sampler (Supelco, Bellefonte, USA) fitted with either a 65 µm Carbowax-DVB or a 100 µm PDMS (polydimethylsiloxane) fibre assembly (Supelco, Bellefonte, USA) was used to sample the headspace above the stirred wine sample for 20 minutes at room temperature, immediately prior to instrumental analysis.

Instrumental Analyses

Samples were analysed with either a Hewlett-Packard (HP) 5890A Series II gas chromatograph coupled to a HP 5971 mass spectrometer or with a HP 6890 gas chromatograph coupled to a HP 5973 mass spectrometer. The analytical method was the same for both gas chromatograph / mass spectrometer systems, except that the 6890 gas chromatograph was run in the pulse splitless mode. The gas chromatograph was fitted with an approx. 30 m x 0.25 mm J&W fused silica capillary column DB-1701, 0.25 µm film thickness. The carrier gas was helium (Air Liquide or BOC Gases, high purity). On the 5890A Series II, linear velocity was 31 cm/sec, flow rate was 0.72 mL/min. On the 6890, linear velocity was 50 cm/sec; flow rate was 1.2 mL/min. All flow rates were vacuum compensated at the mass spectrometer interface. The oven temperature was started at 50°C, held at this temperature for 1 min. then increased to 250°C at 10°C/min and held at this temperature for 20 min. The injector temperature was varied (see text) and the transfer line held at 280°C. For liquid injections, the sample volume injected was 2 µL, the splitter, at 30:1, was opened after 36 sec. and the liner used was resilanised borosilicate glass, tapered, with a plug (2-4 mm) of resilanised glass wool at the column interface. The residence time for the needle in the injector block was approximately 100 ms. Positive ion electron impact spectra at 70 eV were recorded in the range *m/z* 50-350 for scan runs.

For quantification of the oak volatiles, mass spectra were recorded in Selective Ion Monitoring (SIM) mode. The ions monitored in SIM runs were: *m/z* 81, 109, <u>127</u> for d_3-guaiacol; *m/z* 81, 109, <u>124</u> for guaiacol; *m/z* 95, 123, <u>141</u> for d_3-4-methylguaiacol and *m/z* 95, 123, <u>138</u> for 4-methylguaiacol. MS conditions for the analysis of *trans*- and *cis*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*) and vanillin (*35*) are described elsewhere. The underlined ions were the ones used for quantitation (by peak area). Selected fragment ions were monitored for 50 ms each.

The analyses by SPME were done in the same manner, except as follows; the fibre was baked for at least 10 min. before retraction; the liner was resilanised borosilicate glass, direct injection (1.5 mm ID); the oven temperature was started at 50°C, held at this temperature for 1 min. then increased to 250°C at 10°C / min. and held at this temperature for 10 min.; the injector was held at 220°C and the transfer line at 280°C.

Validation of the analytical method

The precision of the assay was validated by a series of standard addition experiments to red, model and white wine matrices. Red and white wines were spiked at 0, 1, 2, 5, 10, 25, 50 and 250 µg/L with guaiacol and 4-methylguaiacol. Another red wine and a model wine were spiked at 0, 1, 2, 5, 10, 25, 50, 250, 500 and 1000 µg/L with guaiacol and 4-methylguaiacol and the other oak volatiles. Validation of the other oak volatiles was as described previously: *trans*- and *cis*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*) and vanillin (*35*).

Studies on the artefactual generation of oak volatiles during the analysis of extracts of oak shavings – injector and solvent effects

Fine shavings (1 mm thickness) were taken from six oak wood samples, two staves of *Quercus alba*-fine grain (A & B), two of *Quercus alba*-medium grain (A & B), and two of Chestnut Oak (*Q prinus*) (A & B) all supplied by a local supplier in South Australia. The species of the oak samples was assessed by the suppliers, but has not been independently confirmed by us. About half of the shavings from each oak sample were heated in a Carbolite constant temperature (+/- 1°C) oven fitted with a Eurotherm digital controller (Medos, Adelaide, Australia) at 175 +/- 1°C for 2 h and then allowed to cool. The shavings were heated in the same Schott bottle in which they were later immersed in model wine. During heating, the bottle openings were wrapped with alfoil, as best as possible, to minimize the loss of any escaping volatiles. All twelve samples of shavings (*circa* 100 g each, but each weighed exactly) were soaked in 1 L of model wine at 25°C for 1 week, after which time the shavings were removed by filtration through glass wool. Separate 5 mL aliquots of six of the extracts (*Q. alba* fine grain B and Chestnut Oak A & B, heated and unheated) were extracted and analysed in triplicate by headspace SPME, and also by liquid-liquid extraction with pentane (~2 mL), pentane-diethyl ether (2:1) (~2 mL) and diethyl ether (~2 mL) and each liquid-liquid extract was analysed by the method at three different injector block temperatures; 200°C, 225°C and 250°C, in triplicate, giving a total of thirty determinations for each model wine solution of *Q. alba* fine grain B and Chestnut Oak A & B, heated and unheated. Separate 5.000 mL aliquots from the remaining six solutions were extracted and analysed in triplicate by headspace SPME and also by liquid-liquid extraction with pentane-diethyl ether (2:1) (~2 mL) and injected at two different injector block temperatures; 200°C and 250°C, in triplicate, giving a total of nine determinations for each model wine solution of *Q. Alba* fine grain A and *Q. Alba* medium grain A & B, heated and unheated.

At the same time that the extractions and analyses of the twelve oak extracts above were performed, model wines were spiked in triplicate with the unlabelled oak compounds (to concentrations of 200 µg/L guaiacol, 200 µg/L 4-methylguaiacol, 500 µg/L 4-ethylphenol, 500 µg/L 4-ethylguaiacol, 500 µg/L *trans*-oak lactone, 500 µg/L *cis*-oak lactone and 500 µg/L vanillin) and extracted with 2:1 pentane:diethyl ether and also with diethyl ether and analysed by the method at three injector temperatures, 200°C, 225°C and 250°C, giving a total of six determinations for each of the three model wine solutions.

Results and discussion

Quantitative analysis of guaiacol and 4-methylguaiacol

The precision of the multi-component analysis for guaiacol and 4-methylguaiacol was validated by duplicate spiked standard additions to red, white and model wine matrices (details in materials and methods above). All of the calibration functions obtained were linear throughout the concentration range. Table 1 shows the correlations for the compounds in model, white and red wine matrices. The method was also precise and repeatable, as shown by the data in Tables 2 and 3.
Although DB-1701 was the GC column of choice, the method worked equally well on DB-5 and Carbowax columns, although 4-methylguaiacol had similar retention to *trans*-oak lactone on Carbowax, and sometimes co-eluted.
The analysis could be performed equally well by headspace SPME. Good results were obtained for both types of fibres trialled (65 µm Carbowax-DVB and 100 µm PDMS). Optimum conditions were 100 µm PDMS fibre, 1 g sodium chloride per 5 mL wine sample, 20 minutes extraction time at 25°C (data not shown).

Relative intensity of mass spectral fragments and ratio drift

The relative intensity of mass spectral fragments for fixed concentrations for each internal standard versus its corresponding analyte (e.g. d_3-guaiacol vs. unlabelled guaiacol) varied slightly (up to +/- 10% over 12 months) according to the instrumental operating conditions. It is therefore important to determine the relative molar ion response factors for standard solutions of all compounds under the same instrumental conditions as employed for the analyses of each set of wine samples. Thus every time a batch of wine or oak extracts was analysed, replicate reference standards were made containing known concentrations of each deuterium labelled IS and each unlabelled analyte. Also, with each batch, a reference standard was run

which contained just the isotopically labelled standards portion, to account for ions common to both labelled and unlabelled compounds. This is especially relevant to d_3-guaiacol and d_3-4-methylguaiacol as the isotopic labels are the three deuteriums on their methyl groups. The major cleavage is the loss of this methyl group, hence the labelled standard and the unlabelled analyte give subsequent fragments of the same mass to charge ratio (i.e. guaiacol and d_3-guaiacol have the same qualifier ions of *m/z* 81 and 109). These qualifier ions can nevertheless be useful in confirming the identity of guaiacol and 4-methylguaiacol in wine and oak extracts, but only the molecular ions can be appropriately used for quantification, unless impractically slow GC programs are used to fully resolve the labelled IS and unlabelled analyte peaks.

As $\frac{m/z\,124}{m/z\,81}$ and $\frac{m/z\,127}{m/z\,81}$ are similar $\Rightarrow \frac{m/z\,124 + m/z\,127}{m/z\,81} = k$, was one of the factors used to ensure peak homogeneity was checked thoroughly.

Studies on the artefactual generation of oak volatiles during the analysis of oak extracts – injector and solvent effects.

Guaiacol and 4-methylguaiacol are known to be formed by the pyrolysis of lignin (*6, 9, 31*). Therefore, to test the possibility that oak volatiles were released from bound forms by thermal degradation of soluble lignin in the gas chromatograph injector block during the analysis, duplicate model wine extracts of untoasted and toasted oak shavings were analysed under a variety of conditions. As controls, model wine (with no contact with oak shavings) was spiked in triplicate with the unlabelled oak compounds of interest and extracted in triplicate for each extraction condition tested, at the same time the extractions and analyses of the oak extracts were performed. Under all analysis conditions, these controls gave the expected values with < 4% relative standard deviation (RSD).

If the same concentration of an oak volatile is measured, independent of whether the wine is extracted by headspace SPME or liquid / liquid extraction and independent of injector block temperature, then it can be concluded that artefactual formation of the compound of interest from precursors as a result of the analysis is highly unlikely. This was the case for the spiked model wine controls (with no oak wood extraction) and for some of the real oak extracts but not all. Results are shown in Tables 4 to 7.

The effect of artefactual oak volatile formation varied according to the analyte. Guaiacol shows the largest effects, with more than ten-fold increases observed when higher injector temperature (250°C) and the polar solvent diethyl ether are involved (Figures 3 and 4). In general, there was much more guaiacol found in the heated wood as compared to the unheated, which is in agreement with published data and our own observations. In contrast to guaiacol, 4-methylguaiacol usually had low 0-6% (but statistically significant) artefact formation when diethyl ether was the extracting solvent at higher injector temperatures.

Significant (> 99.9%) artefactual generation of guaiacol for diethyl ether extractions of this model wine extract of heated Chestnut oak, especially at higher injector block temperatures, can be seen in Figure 3. The result (0.53 µg/g guaiacol) obtained by headspace SPME can be exaggerated by more than an order of magnitude (e.g. mean of 5.89 µg/g guaiacol, a 1110% factor of increase, with 4-methylguaiacol only increasing 4% to 1.04 µg/g mean, for diethyl ether injections at 250°C). These artefacts could be avoided by using headspace SPME, or minimized in most cases by using liquid-liquid extracts with pentane or pentane:diethyl ether (2:1) injected at 200°C providing spot checks using headspace SPME were done.

Note that in Figure 3, the precision of the replicates is good for all conditions except the injection at 250°C. Even for injections at 225°C, the precision (repeatability) of the analysis is excellent though the accuracy is poor, with the result exaggerated by more than 450%! The precision of the analyses are good for most of the other oak samples as can be seen from the statistical significance of the results in Tables 4 to 6, although the accuracy of results varied considerably. It is worth emphasising here that the accuracy of analyses is commonly presumed from standard curve data, obtained by spiking matrices with pure samples of analytes. It is clear that had the standards shown in Figure 3 been analysed by diethyl ether extraction and injection at 225°C that a high correlation coefficient (r^2) and good precision would have been obtained, giving no indication of the inherent inaccuracy of the method.

As reported previously, although the artefactual formation of *cis*-oak lactone observed was often extremely significant statistically, the total increase was only 0-12%, and was highest when more polar solvents (diethyl ether), higher injector temperature (250°C) and unheated wood are factors (*33*). No 4-ethylphenol and very little 4-ethylguaiacol were formed by heating the oak; hence, it is unsurprising that there is no significant artefact formation for these compounds.

Vanillin shows only slight (up to 7%) artefactual generation, even for concentrated oak shaving extracts. Headspace SPME analyses of vanillin generally had poor precision and sensitivity, presumably due to the relatively low volatility of vanillin and its preference for the liquid phase. Thus quantitative comparisons of vanillin obtained from liquid-liquid extracts with SPME were uninformative. However, comparisons of liquid-liquid extracts injected at 200°C versus injection at 225°C and 250°C, gave some significant results, as shown in Table 7. The only significant effects were observed for untoasted oak samples, with the largest artefactual generation of vanillin being 7% for an untoasted American oak sample. However, when oak extracts are pH adjusted, much greater artefactual generation can be obtained (*35*).
It must be stressed that these results are only for the handful of American oak samples that we studied in detail. It is not known whether other samples, species, seasoning factors, etc. would increase or decrease the propensity for artefactual generation of guaiacol and other oak volatiles as a result of the analysis. It is entirely possible that much greater artefactual generation of analytes could occur for some samples.

Alternative methods of analysis for guaiacol, 4-methylguaiacol, 4-ethylphenol, 4-ethylguaiacol, oak lactone and vanillin

In the literature, there is an abundance of analytical methods for the oak volatiles discussed here. For the most part the analysis for these compounds is carried out in concert with the analysis of other oak volatiles. The following observations are provided to illustrate comparison of the method developed here with other methods previously recorded.

Some authors (e.g. *2, 50, 51*) use only one ion for the identification and quantitation of their analytes and internal standards, thus no additional confirmation of identity apart from retention time is possible, and co-eluting peaks could remain undetected (*33*). Other authors use flame ionization detection (FID) (*29, 52*). FID also gives no further confirmation of identity, although some authors use FID to quantitate, but confirm identities via GC/MS (e.g. *4, 53*). Some authors (e.g. *19, 21, 50, 51, 53, 54, 55, 56, 57, 58, 59*) don't determine recoveries, and use inappropriate internal standards, which are quite different in solubility and other chemical properties to their analytes of interest, and yet assume they are extracted at the same rate from the matrix for all the matrices they analyse. Pérez-Coello et al. (*54*) show one chromatogram (presumably their best) with many co-eluting peaks, and no description of how they estimated areas of these co-eluting peaks.

Generally, authors demonstrate no awareness that some methods of extraction increase the risk of artefact formation as a result of their analyses. The relatively polar solvent (e.g. dichloromethane) used by some authors (e.g. *51, 53, 54, 56, 59)* could well extract precursors to the volatiles of interest that may generate those volatiles during the analysis. Hale et al. (*50*) extracted oak chips with toluene / ethanol (2:1) to measure oak lactone isomers. Ethanol is relatively polar, significantly more polar than diethyl ether, and thus conducive to the extraction of precursors, which can form artefacts of the analytes of interest during analysis.

Despite earlier reports by us demonstrating the potential for artefactual formation as a result of the analysis of vanillin (*35*), other authors persist in using conditions conducive to artefact formation, such as high pH (Cutzach et al. (*60*) use initial adjustment of the pH to 8.5 and several washes at pHs from 8.5 to 13!), polar solvent systems for extraction (e.g. *57-58*), and inappropriate internal standards such as 3,4-dimethylphenol (*19, 53*), or even a primary alcohol (1-octanol) or secondary alcohol (3-octanol) as "internal standard" (e.g. *21, 57-58*) and presume it extracts and oxidizes at the same relative rate as the phenolic aldehyde vanillin in all their sample matrices! Even when higher variance for vanillin is reported (e.g. *58*) it is still not attributed to possible problems with the analytical methodology. The earlier paper by Boidron et al. (*19*) does not mention any quantitative results for vanillin, despite stating its importance to oak-derived wine flavour. Related papers (*4, 5, 29, 60*) also omit data on vanillin. This implies that at least some of these authors had problems in measuring vanillin due to artefactual formation effects and oxidative or acid-catalysed conversions of vanillin not accounted for by an isotopically labelled standard and robust analytical methodology (*35*).

In summary, many methods in the literature for analysis of the volatile oak-derived aroma and flavour compounds guaiacol, 4-methylguaiacol, *cis*- and *trans*-oak lactone, and vanillin involve steps, such as polar extracting solvents, pH adjustment and high injector block temperatures, conducive to the possible formation of artefacts. Furthermore, these methods give inadequate consideration to other factors (e.g. inappropriate internal standards, no confirmation of peak homogeneity, no robustness validation in different matrices). Quantitatively, these methods are questionable.
As demonstrated here, no analysis is foolproof, even SIDA – and constant vigilance is necessary to eliminate as many potential sources of error as possible.

ABBREVIATIONS USED

GC gas chromatography, **MS** mass spectrometry, **SPME** solid-phase microextraction, **SIDA** stable isotope dilution analysis

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FIGURE CAPTIONS

Figure 1. Structures of the compounds discussed in this work.

Figure 2. Structures of the isotopically labelled standards used.

Figure 3. Guaiacol and 4-methylguaiacol (4-MeG) concentrations measured in a model wine extract of toasted Chestnut oak – SPME and diethyl ether liquid-liquid extraction injected at different injector block temperatures

All determinations were done in triplicate.

Figure 4. Guaiacol and 4-methylguaiacol (4-MeG) concentrations measured in a model wine extract of toasted Chestnut oak – SPME and liquid-liquid extraction with different solvent systems injected at 250°C.

All determinations were done in triplicate.

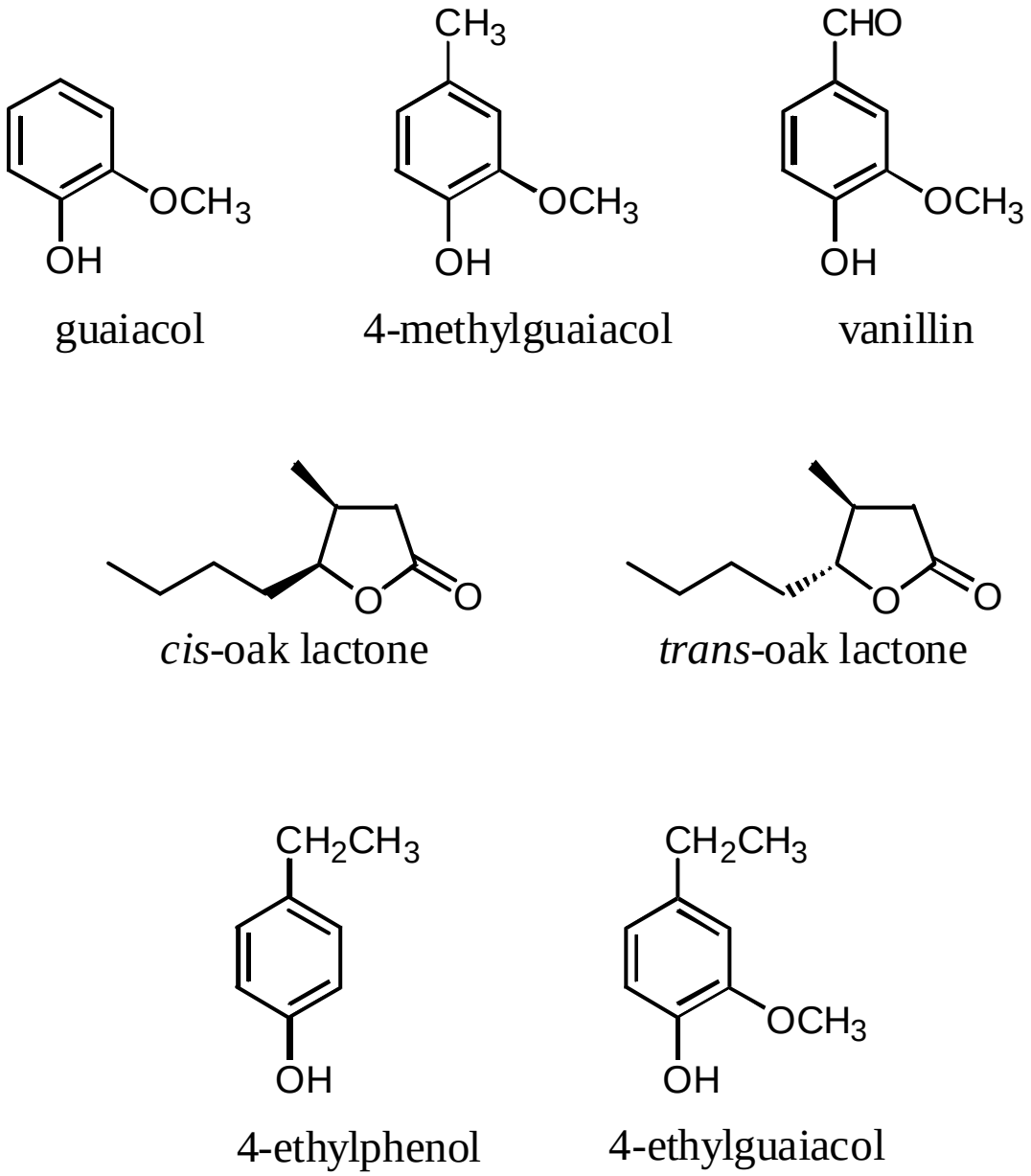


Figure 1. Structures of the compounds discussed in this work.

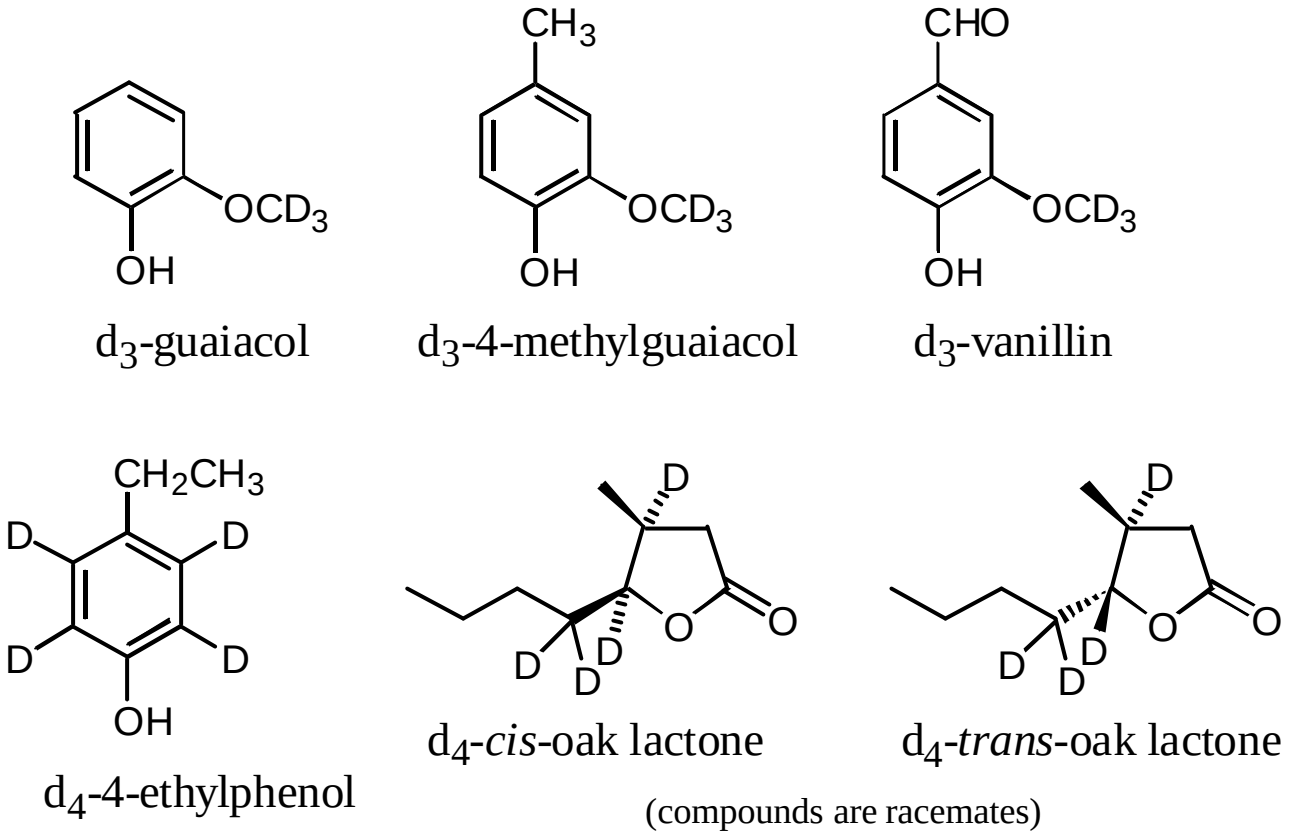
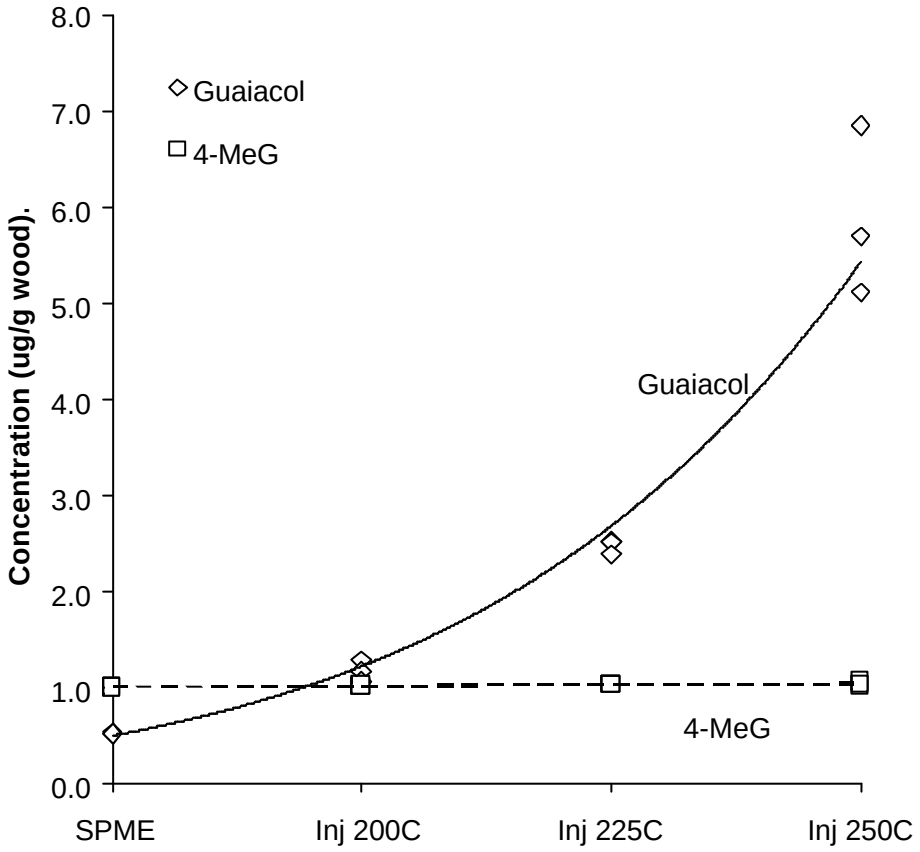


Figure 2. Structures of the isotopically labelled standards used.

Figure 3. Guaiacol and 4-methylguaiacol (4-MeG) concentrations measured in a model wine



extract of toasted Chestnut oak – SPME and diethyl ether liquid-liquid extraction injected at different injector block temperatures.

All determinations were done in triplicate.

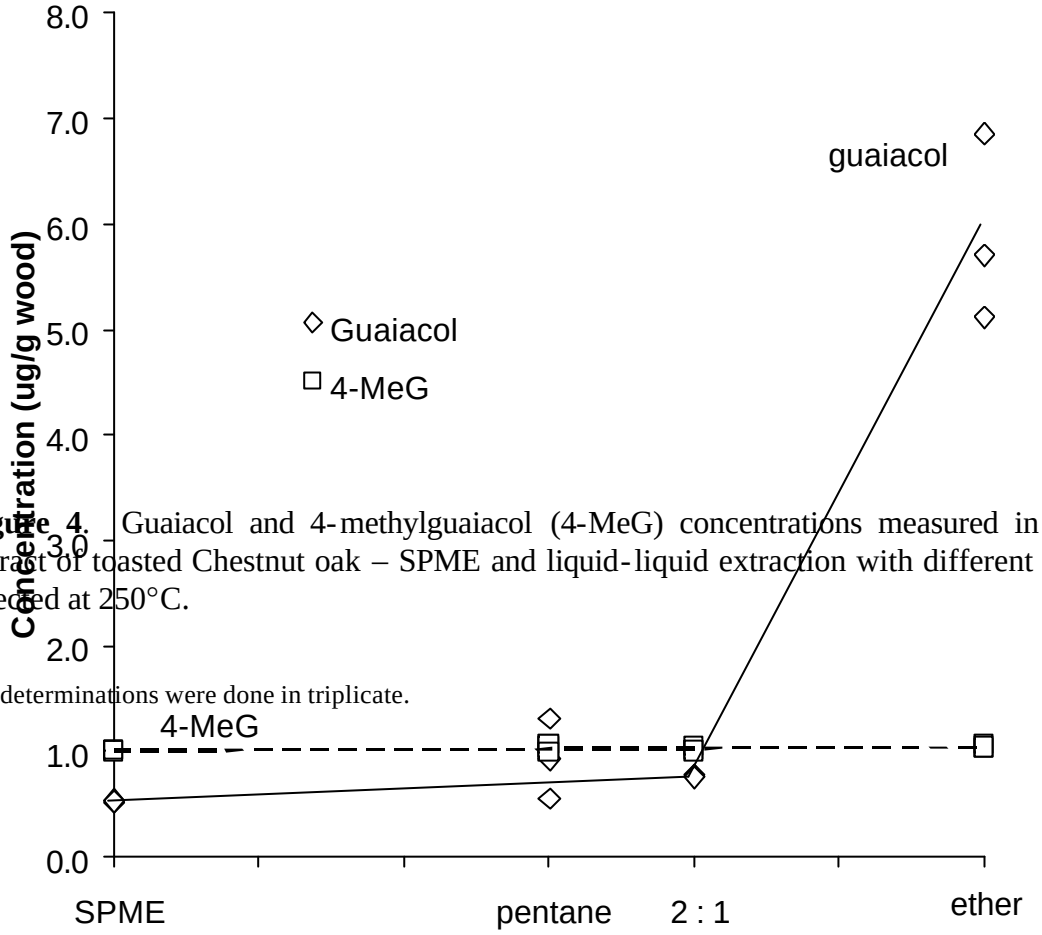


Figure 4. Guaiacol and 4-methylguaiacol (4-MeG) concentrations measured in a model wine extract of toasted Chestnut oak – SPME and liquid-liquid extraction with different solvent systems injected at 250°C.

All determinations were done in triplicate.

Table 1. Correlation coefficients (r^2) of standard additions of guaiacol and 4-methylguaiacol to red, white and model wine matrices

	r^2		
Compound	**Red wine**	**White wine**	**Model wine**
guaiacol	0.999 [1,2]	1.000 [1]	1.000 [1,2]
4-methylguaiacol	1.000 [1,2]	1.000 [1]	1.000 [1,2]

Validation data for *cis*- and *trans*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*) and vanillin (*35*) have already been published.

The data shown in the above table is for injections at 200°C. The same pentane:diethyl ether (2:1) extracts were also run at 220°C and 250°C giving similar results. Thus, there were no injector block artefact generation effects observed for these wine extracts. This is not the case, however, for all extracts of oak shavings as explained in detail elsewhere in this paper.

[1] Range 0–250 µg/L, n = 8 x 2
[2] Range 0–1000 µg/L, n = 10 x 2

Table 2. Repeatability of the oak volatiles assay at higher concentrations

Amounts measured in mg/L – septuplicate determinations

Model wine

	spike level	mean	st dev	st dev / mean
guaiacol	200	204	3	1.6%
4-methylguaiacol	200	194	10	5.4%

Oaked white wine (1996 Semillon)

	spike level	mean	st dev	st dev / mean
guaiacol	200	212	6	2.8%
4-methylguaiacol	200	203	6	2.9%

Oaked red wine (1995 Cabernet Sauvignon / Shiraz / Malbec)

	spike level	mean	st dev	st dev / mean
guaiacol	200	229	6	2.5%
4-methylguaiacol	200	201	8	3.8%

Model, white and red wines were spiked and analysed in septuplicate to show the repeatability of the extraction and analysis. Extracts were all run on the HP 5890 II / 5971 GC/MS.
st dev = standard deviation, st dev / mean = coefficient of variance = relative standard deviation
Validation data for *cis*- and *trans*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*) and vanillin (*35*) have already been published.
The red and white wines give higher values than that added because they contained unlabelled analytes of interest prior to spiking (the wines were made in contact with oak).

Table 3. Repeatability of the oak volatiles assay at lower concentrations

Amounts measured in mg/L – septuplicate determinations

Model wine

	spike level	mean	st dev	st dev / mean
guaiacol	10	9.7	0.1	1.3%
4-methylguaiacol	10	9.7	0.2	1.7%

Bag-in-box dry white wine

	spike level	mean	st dev	st dev / mean
guaiacol	10	10.6	0.1	1.3%
4-methylguaiacol	10	9.9	0.1	1.3%

Bag-in-box red wine (Claret)

	spike level	mean	st dev	st dev / mean
guaiacol	10	14.8	0.5	3.2%
4-methylguaiacol	10	10.7	0.2	1.9%

Model, white and red wines were spiked and analysed in septuplicate to show the repeatability of the extraction and analysis. Extracts were all run on the HP 5890 II / 5971 GC/MS.
st dev = standard deviation, st dev / mean = coefficient of variance = relative standard deviation
Validation data for *cis*- and *trans*-oak lactone (*33*), 4-ethylphenol and 4-ethylguaiacol (*36*) and vanillin (*35*) have already been published.
The red and white wines gave higher values than that added because they contained unlabelled analytes of interest prior to spiking, indicating that possibly some contact with oak had occurred during winemaking, or that a small amount of guaiacol and 4-methylguaiacol came from the fruit, as guaiacol has been observed in glycoside hydrolysates of red grape juice (*61*).

Table 4 Summary - significant effects on guaiacol concentration of liquid injection at 200°C vs SPME

Oak Sample	Solvent System	L/L : SPME	Significance
Q. Alba Fine Grain B - Raw	2:1 P:E	115%	***
Q. Alba Fine Grain B - Raw	Ether	144%	***
Chestnut Oak A - Raw	2:1 P:E	104%	***
Chestnut Oak A - Raw	Ether	107%	***
Chestnut Oak B - Raw	2:1 P:E	107%	***
Chestnut Oak B - Raw	Ether	113%	***
Q. Alba Fine Grain B - Heated	Ether	139%	***
Q. Alba Med. Grain A - Heated	*2:1 P:E*	*110%*	***
Q. Alba Med. Grain B - Heated	*2:1 P:E*	*117%*	***
Chestnut Oak A - Heated	2:1 P:E	104%	**
Chestnut Oak A - Heated	Ether	221%	***
Chestnut Oak B - Heated	Pentane	130%	***
Chestnut Oak B - Heated	Ether	178%	***

Guaiacol was the only analyte that gave significant artefactual formation at 200°C.

L/L : SPME denotes the ratio of the amount of the analyte measured by liquid-liquid extraction (using the solvent system in the second column) over the amount of the analyte measured by SPME, expressed as a percentage.

Italics mean that the oak wood extract was only analysed by 2:1 pentane:diethyl ether (2:1 P:E) liquid-liquid extraction and SPME.

****** ($p < 0.01$), ******* ($p < 0.001$)

Table 5 Summary - significant effects of liquid injection at 225°C vs SPME

Oak Sample	Solvent System	Analyte	L/L : SPME	Significance
Q. Alba Fine Grain B - Raw	2:1 P:E	Guaiacol	116%	***
Q. Alba Fine Grain B - Raw	Ether	Guaiacol	287%	***
Q. Alba Fine Grain B - Raw	Ether	4-MeG	103%	***
Q. Alba Fine Grain B - Raw	Ether	cis-OL	106%	***
Chestnut Oak A - Raw	2:1 P:E	Guaiacol	109%	***
Chestnut Oak A - Raw	Ether	Guaiacol	135%	***
Chestnut Oak A - Raw	Ether	4-MeG	150%	***
Chestnut Oak B - Raw	2:1 P:E	Guaiacol	107%	*
Chestnut Oak B - Raw	Ether	Guaiacol	147%	***
Chestnut Oak B - Raw	Ether	4-MeG	136%	***
Q. Alba Fine Grain B - Heated	Ether	Guaiacol	315%	***
Chestnut Oak A - Heated	2:1 P:E	Guaiacol	122%	*
Chestnut Oak A - Heated	Ether	Guaiacol	467%	***
Chestnut Oak A - Heated	Ether	4-MeG	103%	***
Chestnut Oak B - Heated	Pentane	Guaiacol	164%	**
Chestnut Oak B - Heated	2:1 P:E	Guaiacol	113%	**
Chestnut Oak B - Heated	Ether	Guaiacol	321%	***

L/L : SPME denotes the ratio of the amount of the analyte measured by liquid-liquid extraction (using the solvent system in the second column) over the amount of the analyte measured by SPME, expressed as a percentage.

***** ($p < 0.05$), ****** ($p < 0.01$), ******* ($p < 0.001$)

2:1 P:E = 2:1 pentane:diethyl ether, 4-MeG = 4-methylguaiacol, cis-OL = *cis*-oak lactone

Table 6 Summary - significant effects of liquid injection at 250°C vs SPME

Oak Sample	Solvent System	Analyte	L/L : SPME	Significance
Q. Alba Fine Grain A - Raw	*2:1 P:E*	*Guaiacol*	*242%*	***
Q. Alba Fine Grain B - Raw	2:1 P:E	Guaiacol	128%	***
Q. Alba Fine Grain B - Raw	2:1 P:E	cis-OL	107%	***
Q. Alba Fine Grain B - Raw	Ether	Guaiacol	768%	***
Q. Alba Fine Grain B - Raw	Ether	4-MeG	106%	***
Q. Alba Fine Grain B - Raw	Ether	cis-OL	112%	***
Q. Alba Med. Grain A - Raw	*2:1 P:E*	*Guaiacol*	*271%*	*
Q. Alba Med. Grain B - Raw	*2:1 P:E*	*Guaiacol*	*193%*	*
Chestnut Oak A - Raw	2:1 P:E	Guaiacol	115%	**
Chestnut Oak A - Raw	Ether	Guaiacol	227%	***
Chestnut Oak A - Raw	Ether	4-MeG	188%	***
Chestnut Oak A - Raw	Ether	cis-OL	103%	***
Chestnut Oak B - Raw	2:1 P:E	Guaiacol	110%	***
Chestnut Oak B - Raw	Ether	Guaiacol	234%	***
Chestnut Oak B - Raw	Ether	4-MeG	164%	***
Chestnut Oak B - Raw	Ether	cis-OL	103%	***
Q. Alba Fine Grain A - Heated	*2:1 P:E*	*Guaiacol*	*155%*	***
Q. Alba Fine Grain B - Heated	Pentane	Guaiacol	108%	***
Q. Alba Fine Grain B - Heated	Ether	Guaiacol	750%	***
Q. Alba Med. Grain A - Heated	*2:1 P:E*	*Guaiacol*	*128%*	***
Q. Alba Med. Grain B - Heated	*2:1 P:E*	*Guaiacol*	*126%*	***
Chestnut Oak A - Heated	2:1 P:E	Guaiacol	145%	***
Chestnut Oak A - Heated	Ether	Guaiacol	1110%	***
Chestnut Oak A - Heated	Ether	4-MeG	104%	***
Chestnut Oak A - Heated	Ether	cis-OL	104%	***
Chestnut Oak B - Heated	Pentane	Guaiacol	183%	*
Chestnut Oak B - Heated	2:1 P:E	Guaiacol	137%	**
Chestnut Oak B - Heated	Ether	Guaiacol	818%	***
Chestnut Oak B - Heated	Ether	4-MeG	106%	**

L/L : SPME denotes the ratio of the amount of the analyte measured by liquid-liquid extraction (using the solvent system in the second column) over the amount of the analyte measured by SPME, expressed as a percentage.
4-MeG = 4-methylguaiacol, cis-OL = *cis*-oak lactone
Italics mean that the oak wood extract was only analysed by 2:1 pentane:diethyl ether (2:1 P:E) and SPME.
***** ($p < 0.05$), ****** ($p < 0.01$), ******* ($p < 0.001$)

Table 7 Summary - significant effects of liquid injection at 225°C and 250°C vs 200°C on the measured concentration of vanillin in oak extracts

Oak Sample	Solvent System	Injection Temp. (°C)	Ratio vs Inj 200°C	Significance
Q. Alba Med. Grain B - Raw	*2:1 P:E*	*250*	*107%*	**
Chestnut Oak B - Raw	2:1 P:E	225	104%	***
Chestnut Oak B - Raw	2:1 P:E	250	104%	***

Italics mean that the oak extract was only analysed by 2:1 pentane:ether (2:1 P:E) liquid-liquid extraction and SPME.
"Ratio vs Inj 200°C" denotes the ratio of the amount of vanillin measured by liquid-liquid extraction at the injection temperature shown in the middle column over the amount of vanillin measured for the same extract injected at 200°C, expressed as a percentage. As discussed in the text, SPME gave poor levels of detection for vanillin, and thus were not very accurate for vanillin quantitation due to signal to noise interference. Liquid – liquid extraction with pentane also gave low sensitivity for vanillin, but was better than SPME analyses of vanillin.

****** ($p < 0.01$), ******* ($p < 0.001$)

Identification of key aroma compounds in Australian Riesling wines

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Introduction

The main objective of wine flavour research is to improve wine quality. To do so requires identification of the compounds responsible for the aroma characteristics of a wine (Schreier 1979). There are hundreds of volatile organic compounds present in wine, of which only a small portion actively contribute to wine aroma. The compounds in wine found to be responsible for aroma are minor constituents, ranging in concentration from several mg/L to just a few ng/L (Schreier 1979, Rapp and Mandery 1986). Volatile aroma compounds comprise many different chemical classes and differ in both volatility and sensory potency. Ultimately, it would be of great benefit to be able to analyse just a small number of the most important aroma compounds in wine and use these chemical data to explain the aroma and flavour of a wine.

Recent advances in both instrumentation and analytical technique have made it possible to measure volatile aroma compounds in wine accurately and precisely. However, quantitative chemical data, without reference to sensory data, cannot be used to describe wine aroma. Only through pairing chemical with rigorous sensory data is it possible to define the importance and contribution of particular compounds to the aroma of wine.

The work described here aims to integrate, through multivariate statistical methods, both highly accurate and precise analytical techniques with quality quantitative sensory data to elucidate which aroma compounds are most important to Riesling wine.

Wine selection

For the purpose of this study, it was important to carefully choose 20 Riesling wines of varied aroma characteristics to be quantified by a sensory panel and analysed for aroma compounds using gas chromatography / mass spectrometry. For the selection of the wines, an experienced panel of tasters evaluated more than 60 commercially available Australian Riesling wines over a number of sessions. Table 1 provides some information regarding the 20 wines selected as a result of this process.

Table 1 Riesling wines selected for sensory and chemical analysis

Sensory analysis

The aroma and flavour properties of the 20 Riesling wines were quantified in early 2003 using a technique known as quantitative sensory descriptive analysis (Lawless and Heymann 1998, Meilgaard et al. 1999). This method was used to describe and quantify the sensory differences within the set of wines using 17 aroma terms and six taste terms, which were developed by the sensory panel. The aroma terms included *estery* (or bubblegum), *perfumed floral, dried rose*, *lemon*, *grapefruit, lime* (or lime cordial), *lychee*, *pineapple*, *passion fruit, herbaceous* (or green capsicum), *stewed apple*, *honey*, *toasty*, *caramel, kerosene* and *rubber/plastic*. Following an extensive training period, the 16 member sensory panel rated the intensity of each of the defined terms on a 0-9 scale under controlled conditions with triplicate assessments.

All of the aroma attributes rated by the panel were found by analysis of variance to be significantly different among wines (ANOVA; $p<0.05$), with the exception of *apricot.* Principal Component Analysis (PCA) was used to interpret the sensory data and the results are shown in Figure 1. The PCA plot shows the major differences and similarities among the Riesling wines. Figure 1 also shows the relationships among the attributes, showing for example that those wines rated as high in *honey* were also rated highly in *caramel, stewed apple*, *kerosene*, *toasty*, and *lime*. Three major groups of wines were observed from the PCA plot. These include the wines situated in quadrant D on Figure 1, which were rated highly in *perfumed floral, estery*, *lemon, dried rose* and *pineapple*, wines in quadrant A and C, predominantly from older vintages, that were rated as higher in *caramel, honey, stewed apple*, *kerosene*, *toasty* and *lime*, and wines in quadrant B which were more intense in *herbaceous passionfruit*, *grapefruit* and *lychee* characters. Significant differences in intensity ratings were also observed within groups.

Figure 1 – PCA plot of aroma attributes for 20 Riesling wines

Chemical analysis

A listing of the recognised most important white wine aroma compounds was developed from a review of the literature as a target for analysis in the present study (Etievant 1991, Guth 1997, Ferreira et al. 2000). The list included many different classes of compounds including fermentation-derived ethyl esters, acetates, acids and alcohols; carbonyl compounds and lactones; grape derived monoterpenes and norisoprenoids; and nitrogen and sulfur-containing compounds. Analytical methods were developed for many of these compounds, but for several compounds methods are yet to be completed. The concentration of 45 compounds was determined for each of the 20 Riesling wines using gas-chromatography mass-spectroscopy (GC/MS) coupled with stable isotope dilution analysis (SIDA) (Aubry et al. 1997, Guth 1997, Kotseridis et al. 1999, Allen and Lacey 1999, Pollnitz et al. 1999). Of the 45 volatile compounds measured in the wines, 18 compounds were found on average above their sensory threshold and 29 compounds were measured above threshold in at least one of the wines in the study. Across the wines, 39 of the compounds measured were found at significantly different concentrations (ANOVA; $p<0.05$).

Investigation of the relationships between the sensory and chemical data

There are a number of possible approaches to relate sensory and chemical composition data. A particularly useful and powerful method is partial least squares (PLS) regression, which has been successfully used in wine studies (Francis et al. 1998, Guth and Sies 2001, Noble and Ebeler 2002, Aznar et al. 2003). With PLS, regression models can be developed from compositional data so that aroma attribute scores can be predicted. The prediction ability of the PLS models is then assessed to find how practically useful such relationships may be. The parameters used to evaluate the prediction ability of the PLS models in the present study were the coefficient of determination R^2 (where an $R^2=1$ means an excellent model is generated, so that 100% of variation is accounted for), the standard error of cross validation (SECV, where the lower the number the more accurate the prediction) and the F value, which indicates the statistical significance of the regression model. Table 2 provides a summary of the predictive models generated for each of the aroma attributes for the wines, using the analytical chemical data obtained to date. Note that these models will likely be modified when data from further volatile compounds is included. All of the attributes except *grapefruit*, *lime*, *passionfruit* and *herbaceous* were able to be predicted relatively well by regression models incorporating from three to 14 aroma compounds. For example, with the term *estery*, three compounds were found, from the 45 aroma compounds analysed, to provide the best predictive power. In the PLS model two compounds were found to strongly contribute positively to the prediction of this aroma: the ester ethyl-3-methyl butanoate and the monoterpene geraniol. On the other hand it was found that the presence of the norisoprenoid TDN strongly contributed in a negative manner to the *estery* regression model.

The use of PLS modelling allows the identification of a small number of aroma compounds that are most likely to be responsible for or contribute towards the perception of particular aroma nuances in wine. Overall, the following compounds have been found to be of greatest importance to the models generated: the yeast fermentation-derived ethyl esters ethyl-3-methyl butanoate, ethyl propanoate, ethyl-2-methyl propanoate, the acetates 3-methylbutyl acetate and 2-phenylethyl acetate, the alcohol 2-phenylethanol; and the grape-derived compounds linalool and geraniol (monoterpenes) and TDN and β-damascenone (norisoprenoids). This preliminary data indicates that these compounds are candidates for further investigation.

There are several possible explanations why particular aroma terms were not well predicted by the regression models. One explanation is that the aroma compounds responsible for these attributes may not have been measured in the wine. After a more complete suite of compounds have been analysed, finalised models will be developed relating the sensory attributes and the chemical data. The new models will then be tested and validated with further sensory spiking and reconstitution experiments to determine whether the relationship between composition and aroma is one of cause and effect. Essentially, a spiking experiment would involve taking a wine from the study with low concentrations of target compounds, add to the wine specific compounds at appropriate concentrations, and use a sensory panel to evaluate the change in the sensory properties of the wine.

Table 2 Summary of PLS prediction models for Riesling wine attributes

Conclusion

Understanding the chemical basis of wine aroma has the potential to become a powerful tool for the wine industry. The use of quantitative sensory and chemical analytical methods, together with the application of PLS regression models, have allowed the identification of aroma compounds likely to contribute most strongly to the aroma properties of Riesling wine. The aroma compounds identified include a small number of yeast fermentation-derived compounds, and grape-derived monoterpenes and norisoprenoids. The ability to objectively assess flavour through instrumental measurements of a relatively small number of constituents, will surely assist the wine industry to continue to improve the quality of its products.

Improved understanding regarding key quality determining flavour compounds would have many ramifications for future research projects and industry trials. As a result of this work, in the future it may be possible to manipulate viticultural and wine-making processes to favour the production of compounds related to desirable aroma characteristics, and to analyse grapes for their 'potential' to form particular aroma compounds.

Acknowledgements

Heather Smyth wishes to thank Mark Sefton and Markus Herderich for their supervision. The authors wish to thank Kate Lattey and the panellists from the AWRI for their assistance with sensory analysis; Alan Pollnitz, Tracey Siebert, Kate Howell, Dimitra Capone for assistance with chemical analysis. The wineries and winemakers who supported this project with information and wine are gratefully acknowledged. This work was financially supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Cooperation, with matching funds from the Federal Government, and by the Commonwealth Cooperative Research Centres Program.

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Vintage	*Viticultural Region*	*Alcohol (%v/v)*	*Indicative Retail Price (AUD)*
2002	Central Vic	12.7	$ 19
2002	Clare Valley, SA	13.5	$ 33
2002	blend, SA	12.5	$ 10
2002	Canberra	13.7	$ 18
2002	blend, SA	11.8	$ 10
2002	Barossa Valley, SA	12.5	$ 9
2002	Northern Tas	12.5	$ 25
2002	Mount Barker, WA	12.8	$ 15
2002	blend, WA	12.0	$ 18
2002	Eden Valley, SA	12.8	$ 11
2002	Eden Valley, SA	12.6	$ 17
2001	Clare / WA blend	12.5	$ 7
2001	Clare Valley, SA	10.8	$ 20
2001	Coonawarra, SA	12.2	$ 16
2000	Central Vic	13.0	$ 17
1996	Coonawarra, SA	11.4	$ 26
1997	Eden Valley, SA	13.0	$ 35
1997	Clare Valley, SA	12.5	$ 24
1999	Western Vic	12.6	$ 30
1993	Eden Valley, SA	12.1	$ 35

Table 3 Riesling wines selected for sensory and chemical analysis.

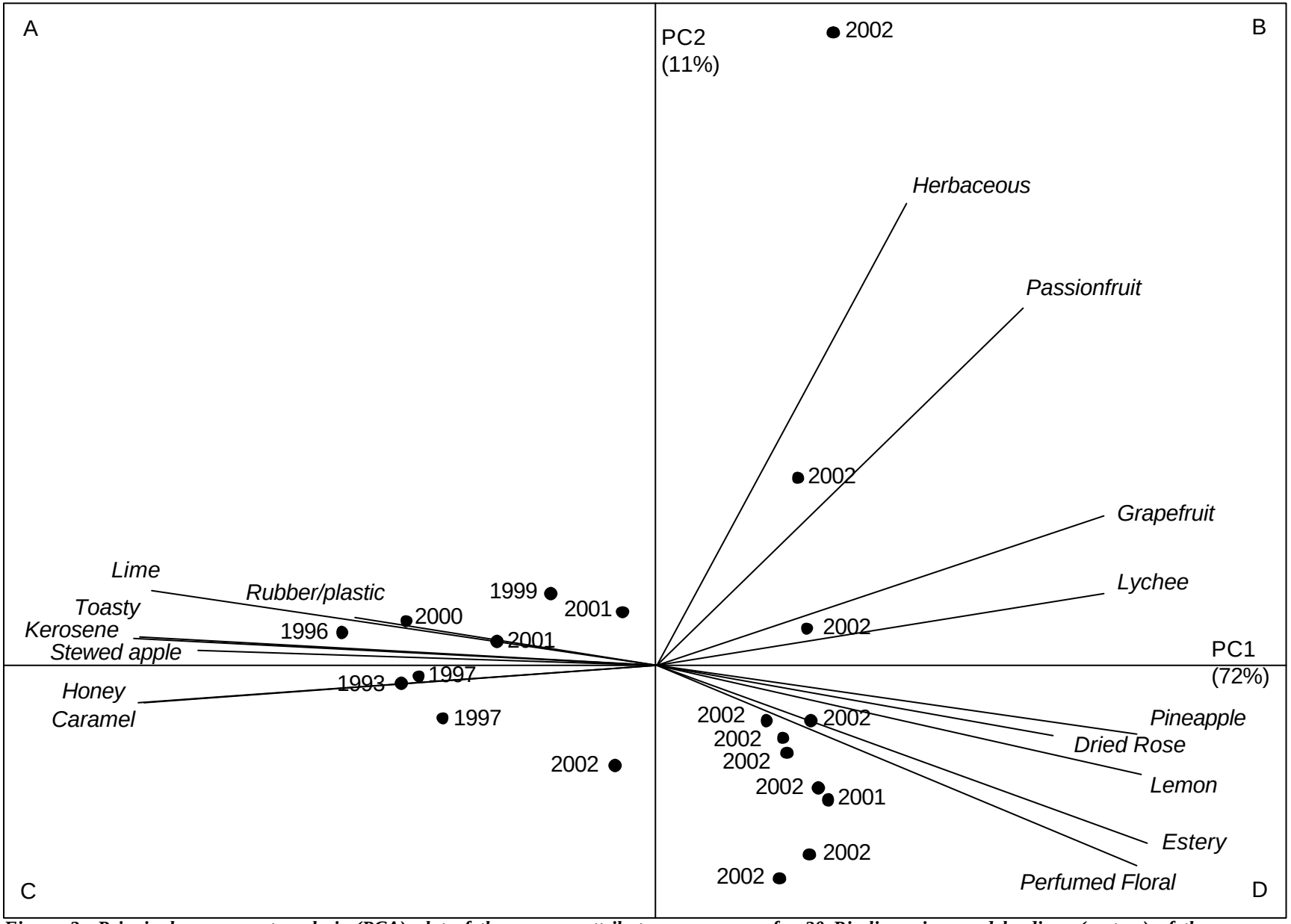


Figure 2 Principal component analysis (PCA) plot of the sensory attribute mean scores for 20 Riesling wines and loadings (vectors) of the sensory attributes (n=16 by 3 reps). Each wine is represented by a black dot and the labels refer to year of vintage. The four quadrants of the plot are labelled A, B, C and D as referred to in the text.

Attribute	*No. compounds*	*SECV*	R^2	*F value*	
Estery	3	0.32	0.87	36	**
Perfumed Floral	4	0.40	0.90	34	**
Dried Rose	3	0.22	0.76	17	*
Lemon	2	0.31	0.81	36	*
Grapefruit	13	0.16	0.84	2	ns
Lime	14	0.29	0.86	2	ns
Lychee	5	0.18	0.77	9	*
Pineapple	5	0.19	0.85	16	**
Passionfruit	28	0.77	0.44	0	ns
Herbaceous	6	0.45	0.41	2	ns
Stewed Apple	3	0.17	0.69	12	*
Honey	3	0.31	0.91	54	**
Toasty	14	0.30	0.97	12	**
Caramel	6	0.15	0.96	52	**
Kerosene	8	0.23	0.97	44	**
Rubber/Plastic	12	0.08	0.95	11	**

Table 4 Summary of PLS prediction models for each of the Riesling wine attributes for each sensory attribute providing the number of compounds used in the model, the standard error of cross validation (SECV), the coefficient of determination (R^2), and the F value (**P<0.01, *P< 0.05, ns=not significant)

Use of Multilayer counter current chromatography (MLCCC) in the separation of glycosidically bound aroma precursors from grapes

USE OF MULTILAYER COUNTER CURRENT CHROMATOGRAPHY (MLCCC) IN THE SEPARATION OF GLYCOSIDICALLY BOUND AROMA PRECURSORS FROM GRAPES.

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INTRODUCTION

GLYCOSIDES AND THEIR IMPORTANCE IN WINE

The aroma compounds responsible for grape derived flavour fall into two categories: *free volatiles* (aglycons) which are responsible for perceived aroma, and *glycosides* (Fig. 1) which are a potential source of aroma. Although glycosides are non-volatile and odourless, they function as *flavour precursors* because the sugar unit can be readily removed by either acidic or enzymatic hydrolysis. Sensory studies in the past have demonstrated that volatile compounds generated from such grape-derived precursors can have a significant impact on the aroma of wines.[1]

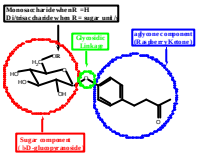
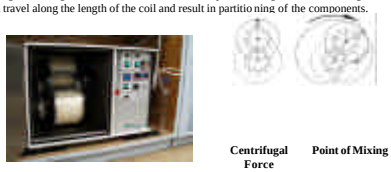


Figure 1: Example of a glycosidic aroma precursor in grapes.

MULTI-LAYER COUNTER CURRENT CHROMATOGRAPHY (MLCCC)

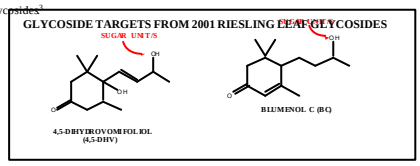
CCC is a low-pressure, liquid-liquid chromatography technique which requires no solid support. It utilises two immiscible solvent phases, plus an auxillary solvent which is miscible in both phases. In this technique, one phase (mobile phase, MP) is pumped through the second phase (stationary phase, SP) causing the compounds to partition between the two immiscible solvents, based on the partition coefficients of the solutes. MLCCC is an advanced form of CCC consisting of teflon tubing wound onto a drum (ie, the coil) which is centrifugally rotated in a planetary fashion. (Fig. 2) A given point on the coil will experience fluctuating force fields from high to low gravitational forces. This sets up alternating zones of mixing and settling which travel along the length of the coil and result in partitioning of the components.



Centrifugal Force **Point of Mixing**

Figure 2: a) Inside the Quattro MLCCC; b) The theory of partitioning in the MLCCC

Conventional chromatography methods are rarely successful for the purification of glycosides due to their highly polar nature, and their solubility in water. MLCCC is a particularly good technique for this purpose because it needs no solid support and can be used for both analytical (mg) and preparative (g) scale. For a successful MLCCC separation the selection of an appropriate solvent system is crucial.[2] The chloroform/methanol/water system has previously been used in the purification of five major glycosylated C_{13} norisoprenoids from Riesling leaf glycosides.[3]



RESULTS AND DISCUSSION

ISOLATION OF THE TOTAL GLYCOSIDE POOL

The glycosides were isolated from fruit and leaves by selective retention on Amberlite XAD-2 resin, followed by desorption of the retained glycosides with methanol [4]. The glycosides were hydrolysed with AR 2000 (enzyme preparation) in pH 5.0 buffer at 40ºC for 16 hours. The hydrolysates were extracted with DCM:pentane (1:2) followed by diethyl ether:DCM (3:1) and analysed by GGMS.

SEPARATION OF INDIVIDUAL GLYCOSIDES

SEPARATION 1: 7 $CHCl_3$: 1 → 13 MeOH : 8 H_2O **(GRADIENT)**

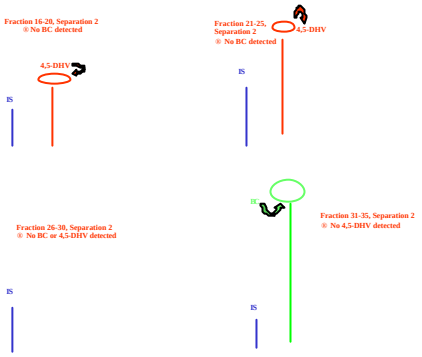
***Conditions*:** 100ml coil (2 individual runs)
SP - upper phase & MP lower phase @ 2 ml/min (1 → 3 → 5 → 8 → 13 MeOH)
- MeOH with run time \ increase polarity of MP
~ 250mg RLG 2001; 800rpm; 35ºC & 25ºC; 75 x 4ml fractions

***Result*:** A representative amount (10%) of the fractions was pooled into groups of 20. The pooled fractions were screened by enzyme hydrolysis for aglycons. No partitioning of the glycosides was observed. All glycosidic precursors were left in the SP. The MP was not polar enough to pull the glycosides from the SP.

SEPARATION 2: 7 $CHCl_3$: 13 → 1 MEOH : 8 H_2O **(GRADIENT)**

***Conditions*:** 100ml coil (2 individual runs)
SP - lower phase & **MP** - upper phase @1.5ml/min (13 → 10 → 7 → 4 MeOH)
- MeOH with run time \ increase polarity of MP
~400mg RLG 2001; 800rpm; 25ºC; 80 x 3mL fractions

***Result*:** A representative amount (10%) of the fractions was initially pooled into groups of 20 & and then into groups of 5. The pooled fractions were screened by enzyme hydrolysis for aglycons. Initial results showed that 4,5-DHV was present in F1-40 and BC in F21-40. Further results showed that **4,5-DHV was present in F16-25** and **BC in F31-35**. No aglycons, hence glycosidic precursors, were detected after F40.



SUMMARY AND FUTURE WORK

- Two glycosides of similar chemical structure were separated from one another with the $CHCl_3$/MeOH/H_2O solvent system.
- Another solvent system of different polarities will be used to further purify the individual glycosides

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Australian grapegrowers and winemakers are key stakeholders in the CRCV, contributing levies matched by the Commonwealth Government and invested by the Grape and Wine Research and Development Corporation in the Centre.

GWRDC

THE EFFECT OF GLYCOSYLATION ON THE FORMATION OF TDN IN WINE.

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Introduction



1

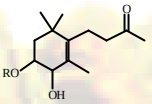


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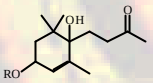
1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN), **1** is an aroma compound found in wines made from certain floral varieties of grapes (eg. riesling), which in high concentrations can impart a petrol or kerosene note to the beverage, but at lower concentrations adds to the complexity of the perceived aroma.[1] **1** is normally associated with wines which have been bottle-aged for several years, and is invariably found along with Riesling Acetal **2**, which led to the suggestion that both compounds share a common source.

Compound **4** has previously been isolated from red currant (*Ribes rubrum L.*) leaves[2] and both it and the corresponding aglycone **3** have been shown to produce, *inter alia*, TDN and Riesling Acetal when heated in an acidic medium (pH 3.2 for **3**, and pH 2.5 for **4**) under simultaneous distillation-extraction (SDE) conditions.[3] The proposed mechanism for their formation (from **3**) involves initial allylic rearrangement to give **5** which can then either eliminate water to produce, ultimately, TDN, or undergo ring-closure to give a hemiacetal which can then further ring-close to produce Riesling Acetal.

According to this mechanism, positioning of a glycoside unit on **5** should have a major effect on the product distribution: a glycoside at C_4 (ie. **6**) would be expected to produce enhanced amounts of Riesling Acetal, whereas a glycoside at C_6 would produce TDN preferentially.



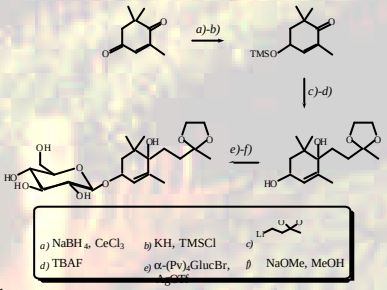
3, R=H
4, R=β-D-Glucose



5, R=H
6, R=β-D-Glucose

Synthesis

The synthetic scheme employed for the synthesis of both ketal-protected **5** and **6** is outlined in scheme 1.



Scheme 1

Hydrolytic Studies

All hydrolyses were performed in model wine [10% ethanol (v/v) in water] buffered at either pH 3.0 or pH 3.2, using KHT and citric acid. Initial results (Fig. 1) showed that **2**, far from being a competitive product, was actually an intermediate in the eventual formation of TDN.

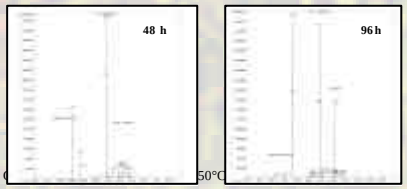


Fig. 1: 50°C

This was confirmed when an authentic sample of **2** was prepared and subjected to acid hydrolysis conditions. (Fig. 2)

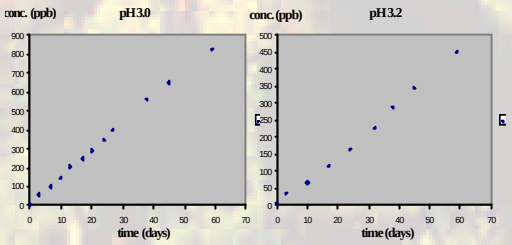


Fig. 2: Results of hydrolysis of **2** at 45°C

Glycoside **6** was itself subjected to acid hydrolysis and the results are contained in Table 1.

t (h)	T (°C)	TDN (ppb)	t (h)	T (°C)	TDN (ppb)
45	100	1395	45	100	1257
103	100	1423	103	100	1411
45	45	151	45	45	47

Table 1: Results of hydrolysis of **6**

Conclusion

Contrary to our initial hypothesis, it would appear that the position of glycosylation has little effect on the course of the hydrolysis given that **2** will ultimately be converted into **1** over time.

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1. Simpson, R.F. *Chem. Ind.* **1978**, 37.
2. Humpf, H.; Winterhalter, P.; Schreier, P. *J. Agric. Food Chem.* **1991**, *39*, 1833-1835.
3. Winterhalter, P. *J. Agric. Food Chem.* **1991**, *39*, 1825-1829.

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Consumption of Damascenone in Wine

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b) Flinders University of South Australia, P.O. Box 2100, Adelaide, South Australia, 5001.

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Introduction:

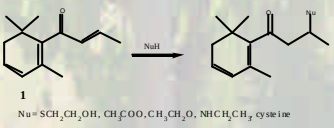
?-damascenone (**1**) is an important flavour and aroma compound found in a wide variety of foods and beverages, including wine and beer. It has a very low threshold (2 ng/L in water and 50 ng/L in model wine), and a pleasant aroma of stewed apples. Damascenone has been seen to be formed from several precursors in grapes and wine. Concentrations of damascenone are quite high in young wine, post fermentation, and are then seen to decrease as the wine ages, followed by a gradual rise in levels as precursors are hydrolysed to form damascenone. It is likely that these initial decreases are due to the consumption of damascenone by reactions with nucleophiles that are commonly present in wine.

Aim:

The aim of this study was to investigate the extent of consumption of damascenone by reaction with common nucleophiles in model wine. Ampoules containing damascenone (100 ?g/L) in model wine (10% ethanol) and one of acetate (2g/L), tartrate (satd), alanine (100 mg/L), cysteine (2 mg/L), NaSH (100 ?g/L), mercaptoethanol (200 ?g/L) or ethyl amine (100 mg/L) were heated at 45°C for 60 days. Each combination was reacted at pH 3.0, 38 and 4.5.

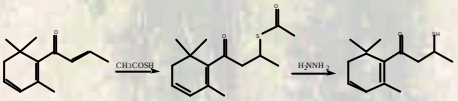
Synthesis:

Each of the expected nucleophilic addition products had to be synthesised for identification purposes. The general procedure was to react damascenone with a concentrated sample of the nucleophile (Scheme 1).



Scheme 1.

The exception was the thiol derivative, which was synthesised in 2 steps using thiolacetic acid (Scheme 2).



Scheme 2.

Results:

The samples were analysed by SIM GC-MS, and the damascenone quantified. The concentrations of damascenone were seen to decrease at pH 3.0, remain constant at pH 3.8 and decrease slightly at pH 4.5 (Fig. 1).

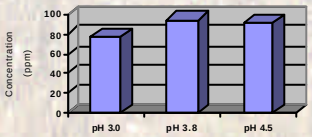
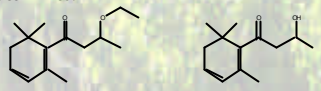


Figure 1.

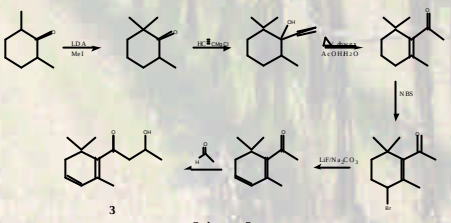
There were no nucleophilic addition products detected at pH 3.0, and concentrations of products at pH 4.5 were too low for identification.

These results indicate that at pH 3.0 the method of consumption of damascenone is not through nucleophilic addition, but rather by acid-catalysed decomposition. At pH 3.8 it is no longer acidic enough for these processes, while at pH 4.5 the nucleophilic addition reactions become important as alkalinity increases.

In each of the samples there was a small amount of the ethanol adduct (**2**) detected. Skouroumounis and Sefton[1] noted two minor products from the hydrolysis of damascenone in model wine at pH 3.0, which were assigned as the ethanol derivative (**2**) and the hydroxy derivative (**3**), but not confirmed.



The hydroxy derivative was synthesised (Scheme 3) and the identification of **2** and **3** in the hydrolysates was confirmed.



Scheme 3.

This led to the investigation of **2** as a possible consumption product of damascenone. Samples of damascenone in model wine (A) and **2** in model wine (B) were heated at 100°C and their consumption monitored. The results are seen in Figure 2.

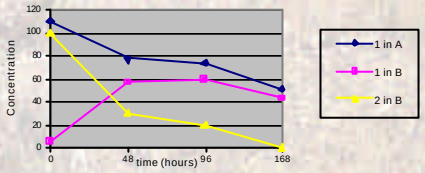


Figure 2.

Conclusion:

These results show that damascenone is being consumed in the hydrolysate by reaction or decomposition due to heat and aqueous acid conditions. The ethoxy derivative is completely converted to damascenone, which decomposes as it is formed.

The results concerning the formation of **2** are not consistent, and temperature and other effects need to be considered.

These results are not conclusive and further studies need to be carried out. The acid catalysed decomposition of damascenone will be investigated, as will the nucleophilic addition reactions at higher pH.

Reference: [1] Skouroumounis, G.K. and Sefton, M.A. *J. Agric. Food Chem.* **2000**, 48, 2033-2039

* The Cooperative Research Centre for Viticulture is a joint-venture between Australia's viticulture industry and leading research and education organisations. It promotes cooperative scientific research to accelerate quality viticultural management from vine to palate. Australian grapegrowers and winemakers are key stakeholders in the CRCV, contributing levies matched by the Commonwealth Government and invested by the Grape and Wine Research and Development Corporation in the Centre.