SYNTHESIS OF THE ONGOING WORKS ON ROTUNDONE, AN AROMATIC COMPOUND RESPONSIBLE OF THE PEPPERY NOTES IN WINES

M.J. Herderich, T.E. Siebert, M. Parker, D.L. Capone, C. Mayr, P. Zhang[1], O. Geffroy[2], P. Williamson, I.L. Francis
The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA 5064, Australia.
[1] School of Land and Environment, University of Melbourne, Grattan Street, Parkville, Vic 3010, Australia.
[2]Institut Français de la Vigne et du Vin Pôle Sud-Ouest, 81 310 Lisle Sur Tarn, France.
Email: markus.herderich@awri.com.au

Oral paper presented at "Colloques Internationaux sur les Arômes du Vin" (VINAROMAS Project), held on 20 and 22 November 2012 in Toulouse (France) and Zaragoza (Spain)

Summary
Shiraz is Australia's most important red grape variety, and is essential for producing a unique diversity of red wine styles, including some of Australia's 'icon' wines. Anecdotal evidence suggests that a spicy, 'pepper' aroma is important to some high quality Australian Shiraz wines. Despite the significance of Shiraz to the wine sector, little is known about the aroma compounds that are the key contributors to the perceived aroma and flavour of premium quality Shiraz wine. Furthermore, the compound responsible for this distinctive 'pepper' aroma in wine had eluded identification until recently. In this paper we summarise the experiments employed for the identification of key Shiraz grape and wine sesquiterpenes, α-ylangene (Parker et al. J. Agric. Food Chem., 2007, 55, 5948–5955) and rotundone (Wood et al. J. Agric. Food Chem., 2008, 56, 3738–3744). The relatively unknown sesquiterpene rotundone was identified as an important aroma impact compound with a strong spicy, 'pepper' aroma in grapes and wine from a number of varieties including Shiraz and Duras. An aroma detection threshold of 16 ng/L in red wine indicates that rotundone is a major contributor to peppery characters in Shiraz grapes and wine, and also in wine of other varieties; rotundone is also found in a number of common spices. Rotundone is quite unusual for a wine aroma compound as it comes directly from grapes, has a very low sensory threshold, and is relatively stable in wine. This opens opportunities to influence the level of rotundone in wine, 'pepper' aroma and flavour, and wine style and consumer preferences, through clonal selection, viticultural practices or by varying winemaking procedures.

Introduction
Syrah is one of the world's top six grape varieties along with Merlot, Cabernet Sauvignon, Pinot Noir, Sauvignon Blanc and Chardonnay. The vineyard area planted to Syrah vines has grown from less than 10,000 hectares in the early 1980s to more than 140,000 hectares in 2004/2005. About 50% of Syrah is grown in France, and 25% in Australia, with Argentina, South Africa, California, Chile, USA, Italy, New Zealand, Greece, Spain, Switzerland and other smaller producing countries accounting for the remainder. Shiraz is Australia's favourite red wine variety, accounting for 380,000 tonnes or 45% of red and 22.9% of total wine grape production of 1.660 million tonnes in 2011/12.

Shiraz (the name used by many New World producers for the grapevine variety known as Syrah in France) is an ancient variety and is thought to have emerged from Mondeuse blanche and Dureza in the northern Rhône Valley, ca. 100 AD (*1*); it was also one of the first vine varieties to arrive in Australia in 1832. To date, grapes are still used for winemaking from own rooted Shiraz vines that have been planted in Australia more than 120 to 160 years ago in the Hunter Valley, Victoria and the Barossa Valley. Shiraz wines have interesting and diverse aromas ranging from plum, berries and chocolate to liquorice and spice, depending on the regions. Shiraz is a very versatile variety and is used on its own or in blends with Cabernet Sauvignon, with Grenache and Mourvedre, or Viognier. Prominent Australian Shiraz styles include elegant, peppery cool-climate wines (for example from the Adelaide Hills, or the Grampians); more intensely flavoured, spicy and sometimes minty styles of Margaret River, Coonawarra or Clare Valley; sweet chocolaty and ripe-fruited wines (Barossa Valley, McLaren Vale), and leathery and rich wines (Hunter Valley).

Despite the importance of Shiraz to the Australian wine industry, little was known until recently about the aroma compounds that are the key contributors to the perceived aroma and flavour of premium quality Shiraz wine. Anecdotal evidence, tasting notes, and the backlabels of Australian Shiraz wine bottles suggested that a 'spicy', 'pepper' aroma is important to some high quality Australian Shiraz wines. The pepper character could be thought of as quintessentially Australian and possibly may even form part of the 'terroir' for a particular wine, yet the compound(s) responsible for this distinctive aroma in Shiraz had not been identified. Thus it was important to isolate and gain a greater understanding of such a powerful odorant that is present in grapes and wine in our own backyard.

Identification of rotundone as key peppery aroma compound in grapes and wine

In early experiments, many extracts of Shiraz grapes were investigated by gas chromatography with olfactory detection (GC-O) and gas chromatography-mass spectrometry (GC-MS), but no single region or known compound corresponding to a distinctive 'spicy' or 'pepper' aroma could be found. However, the 'black pepper' flavour could be perceived in individual berries and deseeded Shiraz grape berry homogenates. Based on anecdotal evidence that there are 'peppery' vineyards that consistently produce 'peppery' wines, especially in cooler years, a large sample set of potentially 'peppery' grapes was sourced from 12 vineyards in South Australia and Victoria. The important sensory attributes of 18 grape samples, including the aroma descriptor 'pepper', were rated by sensory descriptive analysis (*2*). *This 'black pepper' attribute was independent of the 'green', 'grassy' and 'raisin' attributes also present.* The sensory study revealed a strong correlation between the intensity of 'pepper' aroma and the intensity of 'pepper' flavour on the palate and enabled us to concentrate on grape volatiles for further experiments. Chemical analyses of these grape samples were carried out for pH, TA, and TSS. *However, there were no significant trends relating any of these standard maturity and quality measures of the grapes to their sensory 'pepper' scores.*

To study all grape volatile metabolites in a comprehensive, nontargeted fashion, grape homogenate samples were analyzed by static headspace GC-MS. For the metabolomics experiments a cool inlet system was used, we achieved enrichment of trace volatile aroma compounds for improved limits of detection in the low ppb-range, and avoided undesirable discrimination and matrix effects from sampling techniques such as SPME. This GC-MS analysis yielded over 13000 individual mass spectra per grape sample. Prior to multivariate data analysis the data were preprocessed using smoothing and mean normalisation procedures. To explain the intensity of the rating of the 'pepper' character, principal component analysis and partial least-squares regression were then used to develop multivariate models based on mass spectra and aroma descriptors. Optimisation of the methodology enabled selection of *a single region of the GC-MS chromatogram that allowed prediction of 'pepper' aroma intensity* with a correlation coefficient >0.98. This led to the identification of α-ylangene, a tricyclic sesquiterpene, which was confirmed through co-injection with an authentic reference compound. Although not a significant aroma compound by itself, α-ylangene was a very good marker for the 'pepper' aroma, and its concentration showed similar discrimination between 'peppery' vineyards and vintages as that obtained using the multivariate models (*2*).

At the same time we missed out on detecting the key aroma impact compound due to its very low odour threshold and concentration. The subsequent *identification of rotundone, the 'peppery' key aroma impact compound in extracts from Piper nigrum and Shiraz berries*, required traditional GC-MS-O experiments, and succeeded only after sensory-guided, elaborate optimisation of sample preparation and enrichment (*3*). It was further complicated by the unusally late elution time of rotundone towards the end of the GC-MS-O analysis. Finally, the presence of rotundone was confirmed in the enriched pepper and grape extracts by GC-MS-O and co-injections with increasing amounts of the synthesised compound, which gave symmetrical peak enhancement, a matching mass spectrum, and the distinctive pepper aroma on three GC column phases.

Sensory properties of rotundone

Once the identification of the sesquiterpene rotundone as aroma compound had been verified, we developed a method to robustly quantify rotundone by stable isotope dilution analysis (SIDA) and GC-MS (*4*). More recently, the quantitative analysis of rotundone was further refined through adopting a two-dimensional GC-separation that aids with resolving co-eluting compounds. This enabled us to conduct sensory experiments to better understand rotundone's aroma properties, and to compare these sensory results with the very low concentrations at which rotundone is typically found in grapes and wine. Excellent correlations were observed between the concentration of rotundone and the mean 'black pepper' aroma intensity rated by sensory panels for both grape and wine samples, indicating that rotundone - at very low ng/kg concentrations - is a major contributor to peppery characters in Shiraz grapes and wine. *Furthermore, sensory thresholds for rotundone were determined to be 8 ng/L in water and 16 ng/L in red wine (3).*

Notably, approximately 20% of sensory panellists could not detect rotundone during the threshold testing even at 500 times above the best estimate detection threshold in water (3). Thus, the sensory experiences of two consumers enjoying the same glass of Shiraz wine might be very different. To follow on from this observation, a sensory study assessed the effect of rotundone (black pepper), along with eucalyptol (mint, camphor, eucalyptus) and guaiacol (smoky) when added at moderate and high levels to a red wine. This study explored consumer preferences and tolerances to naturally occurring flavour components in wines normally described as peppery, eucalyptus and smoky to understand desirable levels of these compounds in wines. The sensory properties were determined by a sensory descriptive panel, and 104 Adelaide consumers tasted the wines and gave liking scores. Through the descriptive study it was demonstrated that the attributes 'red berry', 'dark berry', 'vanilla', 'smoky', 'pepper', 'mint/eucalyptus', 'vanilla palate', 'smoky palate', 'mint/eucalyptus palate', and 'pepper palate' were significantly different among the samples. From the liking scores three groups of consumers with similar preferences could be identified by cluster analysis, and rotundone addition was positive for a third of the consumers and fairly neutral to the rest. To assess the effects of rotundone on quality as perceived by consumers further work is required with other base wines and, for rotundone, in the presence of additional compounds that influence 'acidity', 'green', 'berry' and 'overall fruit' flavours.

Ocurrence of rotundone in commercial wine

With the identification and analytical method development hurdles overcome, we started testing some of the factors that may contribute to pepperiness, such as grape variety, cultivar, clone type and region. To assess the distribution of rotundone and to help guide further studies rotundone analyses were undertaken of a large range of commercially available Australian wines (137 predominantly red wines obtained from local retailers) of different varieties and vintages from various regions (*5*). The majority were bottled either under screwcap or natural cork and included Shiraz, Merlot, Durif, Pinot Noir, Cabernet Sauvignon and several other interesting wines from popular winegrowing regions from the early 1990s until 2006. Figure 1 shows the amounts of rotundone encountered and wine variety/region in samples where the compound was present. The vast majority (81%) of the wines had no detectable rotundone, and of the wines that contained rotundone, 62% were Shiraz. From Figure 1 *it is apparent that above-threshold levels of rotundone (>16 ng/L) are often encountered in wines originating from cool climate regions and/or colder vintages, and are not limited to Shiraz.* The wide-spread occurrence of rotundone in wine is in agreement with previous observations (*3, 6*) and recent results obtained by Mattivi's group for Schioppettino, Vespolina and Grüner Veltliner wines (*7, 8*). In collaboration with IFV Sud-Ouest rotundone was demonstrated to be a key peppery aroma compound in Duras and Pineau d'Aunis wine; it was also present in Graciano and Gamay wine albeit only at concentrations around its sensory threshold. Beyond grapes and wine, rotundone was found in much higher amounts in other common herbs and spices, especially black and white peppercorns, where it was present at approximately 10000 times the level found in very 'peppery' wine (*3*).

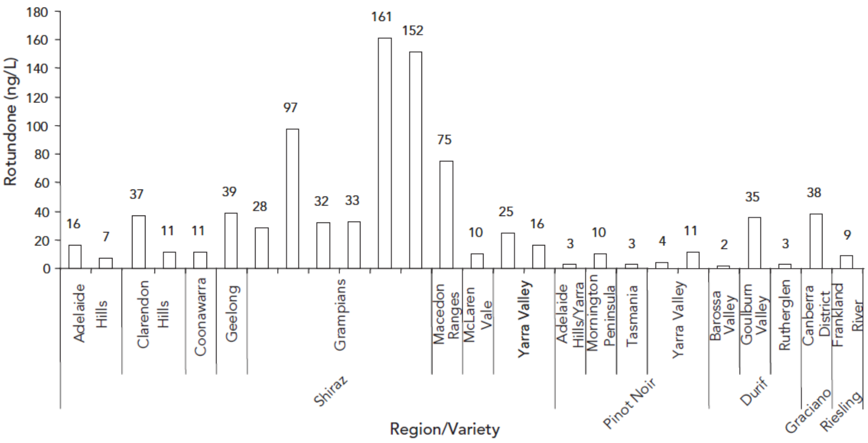

Figure 1. Rotundone concentration in commercial Australian wine (5).

To characterise the stability of rotundone in wine during ageing, we studied the effects of several closures on rotundone levels in bottled wine (*5*). To determine whether the compound is 'scalped' by the closure, as is the case with other aroma compounds, Shiraz wine was spiked with rotundone at approximately 100 ng/L. Bottles (750 mL; 24 for each closure) were sealed with either natural cork, synthetic cork, or screw cap and sealed glass ampoules were prepared as controls at the time of bottling. Triplicate samples were analysed for rotundone after 0, 6, 12 and 39 months. There was no change in rotundone levels until 39 months, whereupon minimal scalping by the synthetic closure was observed (~6% reduction based on the original concentration). *The stability of rotundone under wine-like conditions and the relative lack of scalping of the compound* indicate that the pepper characteristics of a particular wine at bottling are unlikely to change drastically over time with proper storage conditions. Indeed, a Shiraz wine from the Grampians region with the highest level of rotundone (161 ng/L) appearing in Figure 1 was from the 2002 vintage, while another Grampians region Shiraz from 1999 still had 152 ng/L present some 10 years after bottling. These examples indicate the relative stability of the compound over many years.

Factors influencing the concentration of rotundone in grapes and wines

Rotundone is quite unusual for a wine aroma compound as it is one of a small group of important impact aromas (such as isobutyl-methoxypyrazine or some monoterpenes) that come directly from grapes. We assume that rotundone present in a wine would have been extracted without any further chemical or biochemical transformation during winemaking. In contrast it is much more common that volatile wine aroma compounds are released from their odourless precursors (such as glycosides, or cysteine-*S*-conjugates) or that they are formed by the yeast entirely during fermentation. Based on the direct grape-to-wine relationship for rotundone (*3,8)*, and given the low sensory threshold for rotundone (*3*) and its apparent stability in wine (*5*), this opens opportunities to influence the level of rotundone, and hence 'pepper' aroma and flavour in wine through either clonal selection, appropriate viticultural practices or by varying winemaking procedures.

First we needed to determine when rotundone develops in the berries, where it is localised and how much is extracted from berries during winemaking. To study formation, localisation and extraction of rotundone, and as climate is known to impact on grape and wine rotundone concentrations (*3,5*), an Adelaide Hills vineyard planted with Shiraz clones 1127 and 2626 was selected because of its cool climate and regular production of moderately 'peppery' Shiraz grapes. To monitor rotundone levels in the berries during ripening, bunch samples were taken from comparable rows of both Shiraz clones at veraison, 50% red colouring midway between veraison and harvest, and one day before commercial harvest. At early ripening stages we measured only low levels of rotundone in the berries (typically below 5 pg/berry) until well after veraison, with most of the rotundone accumulating in the last six weeks of ripening. At harvest, a higher rotundone concentration of 20 pg/berry was found in Shiraz clone 2626, which is in agreement with the anecdotal belief that 2626 is a 'spicier' Shiraz clone (*9*). In subsequent studies in a number of Australian regions and New Zealand on Shiraz, and in France on Duras, it was confirmed that *rotundone concentration in grapes increases significantly at a time relatively close to harvest* and remains then at a plateau or slightly declines (unpublished data).

To investigate the *location of rotundone in Shiraz grapes*, we analysed fresh harvest samples, skins separated from pulp, juice and seeds, and pulp and juice with seeds removed. *Rotundone was only found in the skin of the Shiraz berries and not detected in the pulp, juice or seeds after separation.* While this experiment involved only a limited sample set, and more work is required before general conclusions can be drawn, the finding that rotundone is located in berry skins is consistent with research by others (*8*).

The extraction of rotundone from the berries into the wine was explored by measuring the concentration of rotundone in samples taken daily during the commercial fermentation of the two clones, from the initial must to the pressed wine. Grapes from Shiraz clones 1127 and 2626 were commercially picked on the same day at similar ripeness and the winemaking parameters were the same for all ferments apart from the day of pressing. In this fermentation trial *most of the rotundone was extracted from the berries* between days 2 and 5, and *rotundone concentrations reached a plateau in all fermentations prior to pressing*. Overall, the data are consistent with rapid extraction of rotundone from the skins during fermentation; the lag phase between crushing at day 0 and day 2 (day 3 for the fermentation of grapes from clone 1127) indicates that ethanol concentration and/or other yeast-related effects are likely involved in facilitating extraction of rotundone.

In the skins of Shiraz clone 1127, rotundone was quantified at 24.7 ng/kg, and at 49.5 ng/kg in clone 2626. Hence, clonal effects may play a role, with a higher level of rotundone found in the Shiraz 2626 clone (*9*). More recently, some clonal effects in Shiraz were apparent in a New Zealand study; however in a comprehensive analysis of rotundone in Shiraz grapes from 35 clones grown in the SARDI germplasm collection in the Barossa Valley in Australia only small concentrations of rotundone and no obvious clonal effects could be observed (unpublished data). The latter results might reflect the particularly hot and dry conditions of the 2008 vintage in the Barossa Valley that were not conducive of rotundone formation; in any case grapes from warm to hot climates have generally been observed to contain only very low low levels of the compound. *The incoherent results from the studies of Shiraz clones* so far certainly point towards vintage conditions, and potentially vineyard site, as interacting factors that may influence rotundone biosynsthesis and 'peppery'aroma in wine.

While clonal effects may play some role for influencing rotundone concentration in Shiraz grapes, the data obtained so far indicate that rotundone biosynthesis is likely to be associated with an interaction of the grapevine genome with its environment: This hypothesis is based on the propensity of rotundone to be predominantly present in the variety Shiraz (ie this point towards a genetic factor related to the variety), with significantly elevated concentrations typically observed in some vintages, vineyards and for grapes grown in cooler climates. Also, in other plant species it has been demonstrated that induction of biosynthesis of structurally related sesquiterpene is a common plant response to environmental pressures (*10*). Obviously, there is much scope for more detailed research until grapegrowers and winemakers fully understand how we can best manage rotundone in grapes and may take advantage of its sensory effects in wine. To achieve this objective the studies that are currently underway in Australia to characterise climatic effects and variation in rotundone concentration within a grapevine and between Shiraz grapevines, and the research by French (on Duras) and New Zealand groups (on Shiraz) into the impact of viticultural management practices such as leaf removal, bunch exposure, grape thinning or irrigation, are very important starting points.

Acknowledgements
We thank all AWRI colleagues and sensory panellists, past and present, and our partners in Australia, New Zealand and France who contributed to the research into rotundone. We very much appreciate the ongoing support and interest by many Australian wine companies and their supply of 'peppery' grape and wine samples, and especially thank the staff of Mount Langi Ghiran and Shaw and Smith Wines. We acknowledge valuable discussions and reference materials provided by Symrise and Charles Cornwell of Australian Botanical Products. This research by AWRI, a member of the Wine Innovation Cluster in Adelaide, Australia, was financially supported by Australia's grapegrowers and winemakers through their investment body, the Grape and Wine Research and Development Corporation, with matching funds from the Australian Government.

References

(1) Bowers, J. E.; Siret, R.; Meredith, C. P.; This, P.; Boursiquot, J.-M. *Acta Hort. (ISHS),* **2000**, 528, 129-132.

(2) Parker, M.; Pollnitz, A. P.; Cozzolino, D.; Francis, I. L.; Herderich, M. J. *J. Agric. Food Chem.,* **2007,** 55, 5948–5955.

(3) Wood, C.; Siebert, T. E.; Parker, M.; Capone, D. L.; Elsey, G. M.; Pollnitz, A. P.; Eggers, M.; Meier, M.; Vossing, T.; Widder, S.; Krammer, G.; Sefton, M. A.; Herderich, M. J. *J. Agric. Food Chem.*, **2008,** 56, 3738–3744.

(4) Siebert, T. E.; Wood, C.; Elsey, G. M.; Pollnitz, A. P. *J. Agric. Food Chem.*, **2008**, 56, 3745–3748.

(5) Jeffery, D. W.; Siebert, T. E.; Capone, D. L.; Pardon, K. H.; Van Leeuwen, K. A.; Solomon, M. R. *Technical Review No. 180* (2009), pp 11-16.

(6) Iland, P.; Gago, P. *Discovering Australian Wine - A Taster's Guide;* Patrick Iland Wine Promotions: Adelaide, Australia,1995.

(7) Mattivi, F.; Caputi, L.; Carlin, S.; Lanza, T.; Minozzi, M.; Nanni, D.; Valenti, L.; Vrhovsek, U. *Rapid Commun. Mass Spectrom.,* **2011**, 25, 483-488.

(8) Caputi, L.; Carlin, S.; Ghiglieno, I.; Stefanini, M.; Valenti, L.; Vrhovsek, U.; Mattivi, F. *J. Agric. Food Chem.*, **2011**, 59, 5565-5571.

(9) Siebert, T.; Solomon, M. R. In *Proceedings of the 14th Australian Wine Industry Technical Conference;* Blair, R., Lee, T., Pretorius, S., Eds.; 2011; pp 307-308.

(10) Chen, F.; Tholl, D.; Bohlmann, J.; Pichersky, E. *The Plant Journal*, 2011, 66, 212–229.