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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Vineyard and Fermentation Studies To Elucidate the Origin of 1,8-Cineole in Australian Red Wine

Dimitra L. Capone,*[,†,‡] David W. Jeffery,[‡] and Mark A. Sefton[‡]

[†]The Australian Wine Research Institute, P.O. Box 197, Glen Osmond, South Australia 5064, Australia

[‡]School of Agriculture, Food and Wine, Waite Research Institute, The University of Adelaide, PMB 1, Glen Osmond, South Australia 5064, Australia

Supporting Information

ABSTRACT: Preliminary investigations revealed that the proximity of *Eucalyptus* trees to grapevines can directly influence the concentration of the aroma compound 1,8-cineole present in the corresponding red wines. For two different vineyards, the closer the grapevines were to the trees, the greater was the amount of 1,8-cineole in the wines elaborated from those grapes. This led us to carry out further studies to quantify the levels of 1,8-cineole found in grape berries, leaves, and stems at set distances from *Eucalyptus* trees over multiple vintages. Generally, the highest concentration of 1,8-cineole was found in the grapevine leaves, followed by grape stems and then grapes. In each sample type, we observed greater concentrations of 1,8-cineole in samples closer to the trees. Various fermentation treatments carried out with Shiraz grapes showed that matter other than grapes (MOG, e.g., *Eucalyptus* or grape leaves) could contribute significant amounts of 1,8-cineole to the finished wines. These studies confirmed that vineyard position and winemaking conditions can determine the 1,8-cineole concentration in red wine. The fermentation study also showed for the first time that the concentration of rotundone in red wine can be strongly influenced by grapevine leaves and stems in the ferment.

KEYWORDS: *1,8-cineole, GC–MS, Eucalyptus trees, rotundone, wine aroma, red wine*

INTRODUCTION

Australia is the native habitat of the *Eucalyptus* genus, but its home has expanded to many countries around the world, including China, India, and Brazil. Every continent apart from Antarctica has been populated by *Eucalyptus* trees.[1] There are over 850 species of *Eucalyptus* grown around the world, and they can prosper in diverse climates.[1] *Eucalyptus* trees have a multitude of uses in industries including cultivation of timber for construction, pulp, fuel, and essential oil production.[1] Most species of *Eucalyptus* contain volatile essential oils in their leaves, although the bulk of the world's *Eucalyptus* oil production comes from only six species.[2] Depending on the species, the main component (60–90%) of the oil from most of these *Eucalyptus* trees is 1,8-cineole, commonly known as eucalyptol.[2] *Eucalyptus* oils are present in numerous consumer goods, and 1,8-cineole has also been found as a component of red wine, where it has been described as "fresh", "cool", "medicinal", and "camphoraceous".[3]

The origin of 1,8-cineole in wine has not been verified, but several theories have been reported. Herve et al. proposed that the "eucalyptus" character in wines occurs when vineyards are adjacent to *Eucalyptus* trees,[3] whereas Farina et al. used hydrolytic studies to propose that terpene compounds such as α-terpineol and limonene were precursors of 1,8-cineole.[4] More recently, we showed that hydrolysis of limonene and α-terpineol at wine pH gave very low molar conversions into 1,8-cineole (<0.6%) over a 2-year period, which does not account for the concentration of 1,8-cineole in many young red wines.[5]

A study by Kalua and Boss[6] suggested that Cabernet Sauvignon grapes have a tendency to form 1,8-cineole, which was the major monoterpene found early in berry development but which decreased during ripening. This was contrary to the observations of Farina et al., who reported an increase in 1,8-cineole toward the end of berry ripening.[4] Kalua and Boss also found that 1,8-cineole was detected at similar levels in berries situated adjacent to *Eucalyptus* trees as at some distance from the trees,[7] which is in contrast with the proposal of Herve et al.[3] Kalua and Boss suggested that the existence of 1,8-cineole in berries may be attributable to the persistence of the compound from floral tissues, or, alternatively, the production of 1,8-cineole may be promoted by herbivore predation, as reported for other plant species (ref 7 and refs therein).

We recently conducted a survey of 190 commercially available Australian wines of mixed varieties, highlighting that 1,8-cineole was found in significant concentrations in red wines only.[5] We also showed that a continuous increase in the concentration of 1,8-cineole occurred during red wine fermentation but ceased once the wine was drained from the skins, indicating that the compound was extracted from the grape skins and/or matter other than grapes (MOG).[5] It was reasoned that the differences in winemaking techniques between red and white wines explained the absence of 1,8-cineole in the latter.[5]

A study by Saliba et al. indicated a consumer rejection threshold of 27.5 μg/L for 1,8-cineole in a red wine, and levels below this were deemed to be acceptable to consumers.[8] Another survey of consumers showed that on average the

Received: November 4, 2011
Revised: January 31, 2012
Accepted: February 4, 2012
Published: February 23, 2012

participants may have had a slight preference for a wine spiked with 1,8-cineole at 4 and 30 μg/L as compared to the unspiked wine containing 0.18 μg/L, with one cluster of consumers (38%) strongly preferring the wine spiked with 30 μg/L of 1,8-cineole.[9] Of more than 150 commercially available Australian red wines we had previously analyzed,[5] only two contained 1,8-cineole above 28 μg/L. Because 1,8-cineole is extremely stable in wine and is barely scalped by synthetic closures,[5] it would be advantageous to be able to clarify how this character arises in wine so 1,8-cineole profiles can be tailored to meet consumer demands.

Our previous results were the first demonstration of when 1,8-cineole was evolved during red winemaking,[5] but we also wanted to confirm the impact of vineyard variables to elucidate the origin of 1,8-cineole in red wine. Therefore, a detailed study of the relationship between grape composition and proximity to *Eucalyptus* trees was conducted over three vintages, and the impact of grape leaves/stems and *Eucalyptus* leaves contained in red wine fermentations was also investigated.

MATERIALS AND METHODS

Materials. Wine samples from Western Australia, Victoria, and Coonawarra were supplied by the producers. 1,8-Cineole was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia), and $^2H_6$-1,8-cineole was synthesized as described in Capone et al.[5] Rotundone ((3*S*,5*R*,8*S*)-3,4,5,6,7,8-hexahydro-3,8-dimethyl-5-(prop-1-en-2-yl)-1(2*H*)-azulenone) was synthesized as described in Wood et al.,[10] and $^2H_5$-rotundone was synthesized as outlined in Siebert et al.[11] Stock solutions of standards were prepared volumetrically in redistilled ethanol and stored at −20 °C, and working solutions were stored at 4 °C until required. All chemicals were analytical reagent grade unless otherwise stated, and water was obtained from a Milli-Q purification system (Millipore, North Ryde, NSW, Australia). Merck solvents and sodium chloride (NaCl) were purchased from Rowe Scientific (Lonsdale, SA, Australia), and other chemicals were obtained from either Sigma-Aldrich (Castle Hill, NSW, Australia) or BDH (Kilsyth, VIC, Australia).

Grape Samples for Vineyard Study. Healthy Shiraz grapes (basic chemical data for each vintage appear in Supporting Information Table 1) were hand-harvested from the Padthaway region of South Australia one day prior to commercial harvest. This study was conducted over three vintages (2008, 2009, and 2011), in the same locations each year (±4 m using GPS). Triplicate samples were taken from three locations within four rows (i.e., 3 × 3 × 4 = 36 samples). Rows 1, 10, 20, and 60 were chosen, with row 1 being within about 5 m of a group of *Eucalyptus* trees and row 60 being the furthest away, around 125 m from the trees. Grape leaves were also collected from the same positions in 2009 and 2011, and *Eucalyptus* leaves were also taken in 2011 from the grapevine canopy in the first row for analysis and addition to ferment treatments. Polyethylene traps were installed in the vineyard in the same row sampling locations in 2008 and 2009.

Fermentation Treatments and Winemaking. Shiraz wines were prepared by a contracted research winemaker from grapes harvested from the first two rows (i.e., within 10−15 m of *Eucalyptus* trees) from the Padthaway vineyard. Hand-harvested fruit (approximately 550 kg) was collected and delivered to the winemaking facility and stored at 0 °C in a coldroom for 24 h. Fruit was randomized into 9 × 50 kg lots. Seven of these lots were crushed and destemmed, and duplicate batches were pressed to juice immediately through a 50 kg bag press under CO_2 (rosé treatment). The other five batches were used for the treatments with the addition of grapevine leaves and stems or *Eucalyptus* leaves and bark. Berries from the remaining two 50 kg lots were hand plucked from the stems and crushed to serve as duplicate controls (control). Each treatment replicate had 50 mg/L of SO_2 added as potassium metabisulfite (PMS) when crushed. The rosé treatment juices were transferred into 50 L stainless steel vessels in a 20 °C temperature controlled room, and the other 50 kg lots were transferred into 50 kg plastic drums with their skins. The duplicate control samples had no further additions prior to inoculation. Triplicate treatments had 500 g of grapevine leaves, which were obtained from the first row, and approximately 1.3 kg of grape stem (from the destemming process) added back into the ferments (grape leaf/stem treatment). The final duplicate treatments contained four *Eucalyptus* leaves (1 g total) and a small piece of *Eucalyptus* bark (3.5 g total) that were collected from within the grapevine canopy (eucalypt treatment). Because of the potential for *Botrytis* activity in vintage 2011, 200 mg/L of VR Supra tannin was added to the ferments (excluding rosé), and they were all supplemented with 100 mg/L diammonium phosphate and inoculated with 300 mg/L Maurivin EC1118 (PDM) wine yeast (Mauri Yeast Australia). All ferments were pressed and racked 4 days after inoculation and then put through malolactic fermentation (MLF, except the rosé). When MLF was complete the wine was racked off gross lees, 60 mg/L SO_2 was added as PMS, and the wines were cold stabilized at 0 °C in a coldroom for 72 h. The stable wine was adjusted to 80 mg/L of total SO_2 added as PMS and passed through a Z6 grade filter (polishing, nonsterile), then a 0.45 μm sterile membrane, and bottled under ROTE screwcap closures in 375 mL bottles (basic chemical data obtained after bottling can be found in Supporting Information Table 2).

Preparation of Samples for 1,8-Cineole Analysis. *Wines and Ferments.* An aliquot (50 μL) of an ethanol solution containing $^2H_6$-1,8-cineole (5.12 μg/mL) was added to the sample (10 mL) in a 22 mL amber glass screw cap SPME vial. A 5 mL aliquot of the sample was removed, and 5 mL of Milli-Q water was added to the vial. The sample was mixed, 2 g of NaCl was added, and the contents were shaken by hand, then sealed and kept at 4 °C until GC−MS analysis. The ferment samples were placed in a water bath at 65 °C for 15 min before storage at 4 °C until GC−MS analysis.

Grapes. Approximately 1 kg of Shiraz grape berries from each replicate position was plucked from their stems and randomized into triplicate 200 berry lots, which were weighed and homogenized with a household stab mixer (Breville Wizz Stick). The homogenate was weighed out into 8 g lots in 22 mL glass screw cap vials with aluminum lined lids (Supelco, Australia). A 1 mL aliquot of redistilled ethanol was added to each vial along with an aliquot (50 μL) of an ethanol solution containing $^2H_6$-1,8-cineole (5.12 μg/mL), and vials were agitated on a shaker for up to 7 days (length of extraction time was found not to be critical). After shaking was complete, 9 mL of Milli-Q water was added to each vial, and shaking was continued for a further 3 h. Approximately 10 mL of the extract was removed into an amber 20 mL SPME vial, 2 g of NaCl was added, and samples were heated in a water bath at 65 °C for 15 min before storage at 4 °C until GC−MS analysis.

Grape Stems. The stems from the destemmed grapes were weighed into approximately 50 g lots. The stems were finely cut using both secateurs and scissors and weighed out in triplicate 8 g lots in 22 mL glass screw cap vials with aluminum lined lids. A 1 mL aliquot of redistilled ethanol was added to each vial along with an aliquot (50 μL) of an ethanol solution containing $^2H_6$-1,8-cineole (5.12 μg/mL), and vials were agitated on a shaker for between 5 and 7 days. When shaking was complete, 9 mL of Milli-Q water was added to each vial, and shaking was repeated for 3 h. A 5 mL aliquot of the sample was removed into a 20 mL amber screw cap SPME vial, and 5 mL of Milli-Q water was added. The sample was mixed, 2 g of NaCl was added, and the contents were shaken by hand, then sealed and stored at 4 °C, ready for GC−MS analysis.

Grape Leaves. Samples were collected at each row and position in the vineyard. Approximately 30 g of grape leaves was weighed out from each of the three positions within a row. The leaves were finely cut using both secateurs and scissors, and triplicate 8 g lots from each position were weighed into 22 mL glass screw cap vials with aluminum lined lids. A 2 mL aliquot of redistilled ethanol was added to each vial along with an aliquot (50 μL) of an ethanol solution containing $^2H_6$-1,8-cineole (5.12 μg/mL) and agitated on a shaker for between 5 and 7 days. When shaking was complete, the samples were transferred into 40 mL glass screw cap vials, an aliquot of Milli-Q water (18 mL) was

added to each vial, and shaking was repeated for 3 h. A 5 mL aliquot of the sample was removed into a 20 mL amber screw cap SPME vial, and 5 mL of Milli-Q water was added. The sample was mixed, 2 g of NaCl was added, and the contents were shaken by hand, then sealed and stored at 4 °C, ready for GC–MS analysis.

Polyethylene Traps. Food grade polyethylene sheets were cut into 20 cm × 30 cm rectangles and placed between wire mesh and sewn in place using fine wire (Figure 1A). A handle was fashioned using wire, and the traps were installed in the vineyard (triplicate positions in rows 1, 10, 20, and 60 at each of the grape sampling positions) in a vertical configuration (vintage 2008) and both a vertical and a horizontal direction (vintage 2009, Figure 1B). The traps were erected in early January and removed approximately 3 months later, one day prior to commercial harvest. The polyethylene sheets were removed from the wire mesh, carefully rolled, and placed into measuring cylinders equipped with glass stoppers. Redistilled ethanol was added to each cylinder to allow complete immersion of polyethylene sheet (130 mL), which was soaked for 4 days. A 1 mL aliquot of the ethanol extract was placed into an amber 20 mL screw cap SPME vial, and 9 mL of Milli-Q water was added along with 50 μL of $^2H_6$-1,8-cineole (5.12 μg/mL). After the sample was shaken, 5 mL was removed, and 5 mL of Milli-Q water was added. The sample was mixed, 2 g of NaCl was added, and the contents were shaken by hand, then sealed ready for GC–MS analysis.

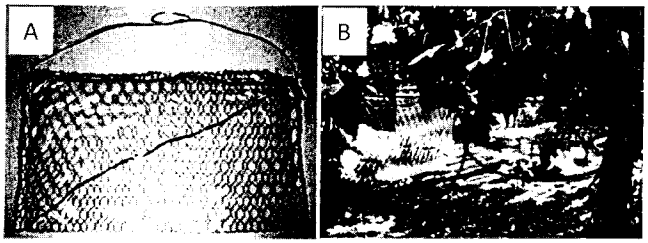

Figure 1. Vineyard trap for airborne 1,8-cineole fashioned out of 20 cm × 30 cm polypropylene sheet and wire mesh (A) and traps installed in the grapevine canopy (B) in vertical (left) and horizontal (right) positions.

Skin and Flesh. Approximately 1 kg of Shiraz fruit from row 1 of the Padthaway vineyard was plucked and randomized, and triplicate 200 berry lots were weighed out. Each grape berry was individually squashed, and the pulp and the skins were separated. The seeds were removed from the pulp and discarded, and the skin and flesh samples were weighed. The separate samples were homogenized with a stab mixer, and then triplicate 8 g lots of both skin and pulp were weighed separately into 22 mL glass screw cap vials with aluminum lined lids. A 1 mL aliquot of redistilled ethanol was added to each vial along with an aliquot (50 μL) of an ethanol solution containing $^2H_6$-1,8-cineole (5.12 μg/mL), and the samples were then agitated on a shaker for 6 days. When shaking was complete, 9 mL of Milli-Q water was added, and samples were shaken for a further 3 h. A 5 mL aliquot of the sample was removed into a 20 mL amber screw cap SPME vial, and 5 mL of Milli-Q water was added, together with 2 g of NaCl, and the contents were shaken by hand, then sealed ready for GC–MS analysis.

GC/MS Analysis of 1,8-Cineole. Quantitative analysis of 1,8-cineole was carried out as described in Capone et al.[5]

Preparation of Samples and GC/MS Analysis of Rotundone. Wine samples were prepared for rotundone analysis using the same parameters as described in Siebert et al.,[11] except a Varian Factor Four VF-35 ms, 60 m × 0.25 mm × 0.25 μm film thickness fused silica capillary column (Agilent Technologies Australia, Forest Hill, VIC, Australia) was used. Grape leaf and grape stem extractions were carried out as detailed in Wood et al.[10] with the following modifications: finely cut up grape leaf and grape stem (2 g) from the vintage 2011 investigations were weighed into 20 mL glass screw cap vials with aluminum lined lids and soaked in 20 mL of redistilled ethanol for 48 h. The samples were filtered, and 10 mL of the ethanolic extracts was placed in a 100 mL volumetric flask and topped up to the mark with Milli-Q water and prepared for rotundone analysis.

Identification of *Eucalyptus* Species. The species of *Eucalyptus* located in the vicinity of the grapevines in Padthaway was identified by a botanist as *Eucalyptus leucoxylon* subsp. *pruinosa* (South Australian Blue Gum).

Statistical Analysis. The effects of the various treatments were analyzed using one-way analysis of variance (ANOVA) and Student's *t* comparison of means using unequal variance (JMP 5.0.1a, SAS Institute Inc., Cary, NC). Two-way ANOVAs with interactions with year and row number as factors were also conducted for grape berries, grape leaves, and grape stems. Other statistical data were obtained using Microsoft Excel 2007.

■ RESULTS AND DISCUSSION

Preliminary investigations were carried out on wines derived from three different regions of Australia. Wine producers provided the wine samples after conducting fermentations on separate parcels of fruit from the associated vineyards. In the first two investigations, wines were made from batches of grapes harvested at set distances from *Eucalyptus* trees in single vineyards in Western Australia and Victoria. The results in Figure 2 clearly show that the greatest amount of 1,8-cineole was found in wines elaborated from grapes obtained from the rows closest to the *Eucalyptus* trees. For the wines from Western Australia, the highest concentration of 1,8-cineole (9.5 μg/L) was derived from fruit harvested within 20 m of the trees. The concentration of 1,8-cineole in the corresponding wines was lower the further away the fruit was harvested, and was almost negligible when fruit was obtained at 230–430 m away from trees (0.4 μg/L, Figure 2). The same trend was observed in the investigation of wines from Victoria, where grapes harvested within 50 m of the *Eucalyptus* trees afforded a wine 1,8-cineole concentration of 15.5 μg/L, and those harvested further away produced a wine with 0.1 μg/L (Figure 2). From these results, it appeared that harvesting fruit a distance of approximately 50 m from *Eucalyptus* trees was sufficient to minimize the concentration of 1,8-cineole in the corresponding wine. In a third investigation, wines from consecutive vintages were provided from the Coonawarra region where the vineyard was in close proximity to well-

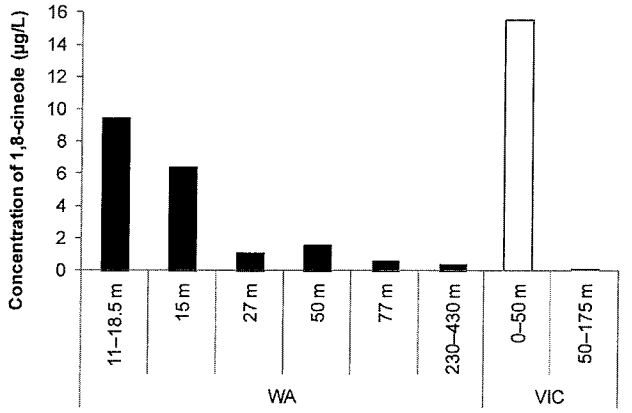

Figure 2. Concentration (μg/L) of 1,8-cineole in wines arising from single vineyards in Western Australia and Victoria located in close proximity to *Eucalyptus* trees. The *x*-axis indicates the distance of the grapevine rows to the *Eucalyptus* trees. For the WA wine, the 15 m samples were picked from row 2 and the samples 11–19 m were picked from rows 1, 3, and 4. The remaining WA samples were from individual rows at the distances specified in the figure.

dx.doi.org/10.1021/jf204499h | J. Agric. Food Chem. 2012, 60, 2281–2287

established *Eucalyptus* trees. The wines contained relatively high amounts of 1,8-cineole, at 47 μg/L (2006 vintage) and 81.5 μg/L (2007 vintage), and were considered by the winemaker to display an obvious "eucalyptus" character. These wines were not sold commercially and were blended with wine made from other fruit, which is common practice to moderate and refine wine sensory characters. These preliminary investigations supported the theory by Herve et al. that the presence of 1,8-cineole is likely to be related to *Eucalyptus* trees.[3] Additional vineyard studies were therefore undertaken to examine possible modes of transmission of 1,8-cineole from *Eucalyptus* trees to the grapes and subsequently into the wine.

Vineyard Study. The relationship between grape composition and proximity to *Eucalyptus* trees was investigated, including evaluation of grape bunches, stems, and leaves. A vineyard was selected that had *Eucalyptus* trees in the vicinity of the vines and a history of producing wines with 1,8-cineole concentrations well above the recognition threshold of 3.2 μg/L in a red wine reported for this compound.[3] As part of these investigations, the location of 1,8-cineole within the grape berry was determined (Figure 3), using fruit collected from the row

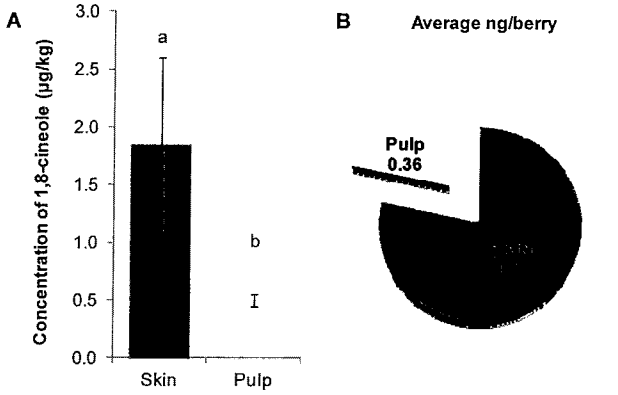

Figure 3. Concentration of 1,8-cineole in grape skin and grape pulp (A) as μg/kg and (B) as μg/berry. Error bars represent the standard deviation of three replicates. Different letters indicate significant differences between the means ($p < 0.05$).

closest to the *Eucalyptus* trees (within 5 m). There was a statistically significant difference between grape components ($p = 0.0403$), with skin containing approximately 4 times as much 1,8-cineole on a per kilogram basis as compared to the pulp (Figure 3A). As expected on the basis of its extraction during winemaking,[5] most of the 1,8-cineole was contained in the skin (approximately 80%) on a per berry basis (Figure 3B).

Additionally, four grapevine rows were selected in the same vineyard at set distances from the *Eucalyptus* trees, and grapes were sampled over three vintages. Row 1, 10, 20, and 60 were chosen, with row 60 being the furthest from the trees (around 125 m) and selected as a control row, because it was presumed to be far enough from the *Eucalyptus* trees to be unaffected by them. Triplicate sampling was conducted at each of the three positions within each row, with the results presented in Figure 4. We again observed a clear trend with greater concentrations of 1,8-cineole found for grapes in the rows closest to the *Eucalyptus* trees. Fruit sampled from row 1 had 1,8-cineole concentrations that were 2–10 times higher than fruit from row 10. A significant interaction between year and row number was found ($p = 0.015$). However, 1,8-cineole concentration decreased monotonically with row number in all three years,

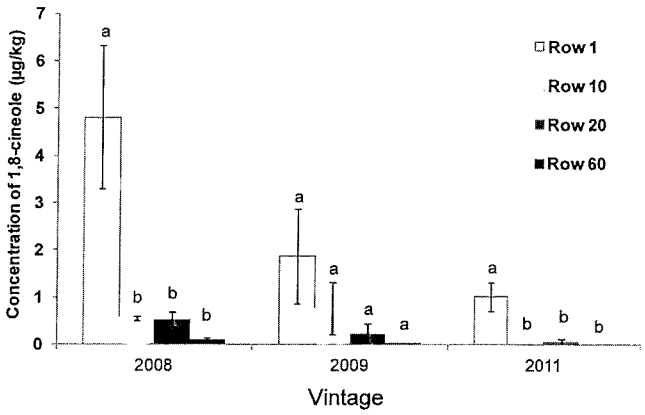

Figure 4. Concentration of 1,8-cineole (μg/kg) in grapes from different rows at set distances from the *Eucalyptus* trees over three vintages. Error bars represent the standard error of the mean for three replicates. Different letters indicate significant differences between the means ($p < 0.05$).

and the interaction was due to a much larger decrease from row 1 to 10 in 2008 as compared to the other years. There were vintage variations in overall 1,8-cineole concentration, with 2008 being the highest and 2011 the lowest. The error bars (standard error of the mean) in Figure 4 show that large variation exists within rows, and there may be a number of factors that could influence this variability including the vigor of the canopy, degree of grape exposure, or position and size of the berries. These results tended to indicate the airborne transfer of 1,8-cineole, which was pronounced within 5 m of the *Eucalyptus* trees and was seemingly limited to within 20 m of them. This concept of airborne transfer of volatile organic compounds is not surprising as it has been shown to occur in other studies, including those involving plants.[12−15]

The concentration of 1,8-cineole was much greater on a per weight basis in the grape leaf and stem samples taken from the same position as the berries. This is possibly due to the large surface area of the grape leaf or composition of leaf or stem epidermis in comparison to the grape berry or the fact that leaves can obscure the grape bunches, minimizing airborne transfer onto berries. Figure 5 presents the concentration of 1,8-cineole in the grape leaves over vintage 2009 and 2011, showing higher levels in rows closer to the trees. Grape leaves were not analyzed in vintage 2008 but were included in subsequent vintages after we observed a large number of grape leaves and stems in a commercial fermentation. The implication of grape leaves being able to affect the concentration of 1,8-cineole in wine was therefore considered, particularly for machine-harvested fruit. The row position for the grape leaves at set distances from the *Eucalyptus* trees had a significant effect in each vintage ($p < 0.0001$ across both vintages), again indicating the possibility of airborne transfer. Interestingly, while the impact on grape berries was restricted to rows that were close to the trees, grape leaves as far away as row 60 revealed measurable 1,8-cineole concentrations. Figure 5 also shows that grape stem 1,8-cineole concentrations were similar to those obtained for the grape leaves, and also followed the same trend, with greater concentrations of 1,8-cineole found in grape stems harvested closest to the *Eucalyptus* trees. We again found that row position was highly significant ($p < 0.0001$ across both vintage 2009 and 2011). In the grape stem, there was greater variability within rows, particularly in vintage 2011, but similar to the results for grape leaves, 1,8-cineole could be

dx.doi.org/10.1021/jf204499h | J. Agric. Food Chem. 2012, 60, 2281–2287

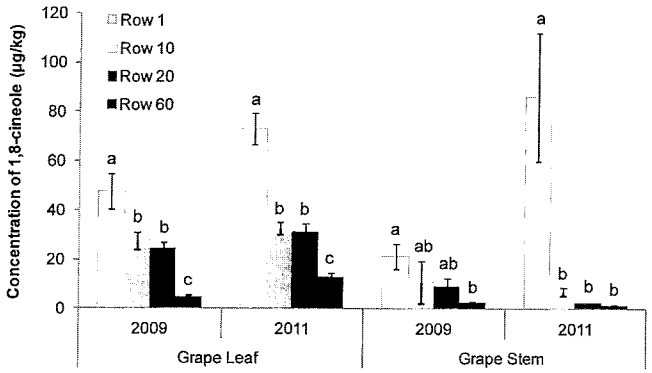

Figure 5. Concentration of 1,8-cineole (μg/kg) in grape leaves and stems from different rows at set distances from the *Eucalyptus* trees over two vintages. Error bars represent the standard error of the mean of three replicates. There were statistically significant differences ($p < 0.0001$) for the grape leaves in the various rows across both 2009 and 2011 vintages. There were statistically significant differences ($p < 0.0001$) for the grape stems in the various rows across both 2009 and 2011 vintages. Different letters indicate significant differences between the means ($p < 0.05$).

found at greater distances from the trees as compared to grape berries.

To verify that airborne transmission of 1,8-cineole was possible, as first suggested by Herve et al.,[3] we designed volatile traps for use in the vineyard (Figure 1A). The traps comprised polyethylene sheets sewn between wire mesh and installed in the same rows as the fruit collected for the study. Polyethylene sheets were chosen because our previous work on 2,4,6-trichloroanisole[16] and flavor scalping[17] showed polyethylene to be a good adsorber of nonpolar volatile compounds. We confirmed that 1,8-cineole could be adsorbed onto the polyethylene prior to installation of the traps in the vineyard (data not shown). In vintage 2008, the traps were installed in a vertical position only, whereas in vintage 2009, they were installed in both horizontal and vertical positions (Figure 1B). The traps installed in the vertical orientation in both vintages showed similar trends, where the greater concentrations of 1,8-cineole were found in the samples closest to the *Eucalyptus* trees (Table 1). This highlighted that aerial transfer of 1,8-cineole was possible and distance was a potential factor. The traps installed in the horizontal position showed a similar trend after the first row. In general, the horizontal traps adsorbed more 1,8-cineole than the vertical traps despite the greater exposure of the former to sunlight. This indicated the possibility that 1,8-cineole can be transferred as an aerosol as well as or instead of in the vapor phase. The anomalous result for row 1 may result from greater exposure of the horizontal traps to sunlight as the canopy growth was visibly less vigorous in this row.

Table 1. Average Concentration of 1,8-Cineole (μg/trap) and Standard Deviation (SD) Determined for Triplicate Polyethylene Traps Suspended in the Grapevine Canopy in Different Rows, Which Were at Increasing Distance from *Eucalyptus* Trees

	trap position and vintage					
row position	vertical 2008	SD	vertical 2009	SD	horizontal 2009	SD
row 1	1.0	0.3	0.65	0.3	0.9	0.3
row 10	0.6	0.2	nd[a]		2.3	0.1
row 20	0.5	0.1	nd		1.7	0.9
row 60	0.4		nd		nd	

[a]nd < 0.05 μg/trap.

During collection of vineyard samples, we noticed *Eucalyptus* twigs, bark, and leaves lodged within the grapevine canopy. We collected and analyzed some of this material and determined that if the 67.5 g collected from the canopy was harvested and totally extracted in a 1 tonne fermenter, it could contribute around 210 μg/L of 1,8-cineole in the corresponding wine. This theoretical amount, being considerably higher than in any wine we have so far analyzed, led us to carry out a range of fermentation experiments that included the addition of *Eucalyptus* material.

Determination of the Effect of MOG in Ferments. Grape leaves and stems can be found in fermentations, and *Eucalyptus* leaves and twigs can lodge in the grapevine canopy in the vicinity of the trees. While at least some 1,8-cineole in wine can arise from aerial transfer to grapes, there could be an even more important contribution from MOG (i.e., *Eucalyptus* and grape leaves). We therefore performed a study on the effect of MOG using grapes picked from the Padthaway vineyard. Hand-harvested fruit from the first two rows closest to a stand of *Eucalyptus* trees was collected. The fruit was delivered to the winery where it was completely randomized and sorted into multiple 50 kg lots for replicate fermentations. One treatment (rosé) involved first crushing and destemming grapes and then immediately subjecting them to a bag press, so that skin contact was minimized. This wine was then made similar to a rosé style. Another treatment (control) that involved hand plucked grape berries was chosen to eliminate any traces of MOG in the ferment. A third treatment (grape leaf/stem) involved passing the grapes through the crusher/destemmer and adding back the stems into the ferments along with grape leaves collected from row 1 to create a grapevine-based MOG effect. A final treatment (eucalypt) was performed by passing the grape bunches through the crusher/destemmer and adding a mix of *Eucalyptus* leaves and bark into the ferments to create a *Eucalytpus*-based MOG effect.

Each of these treatments was analyzed daily throughout fermentation to determine the evolution of 1,8-cineole (Figure 6). The rosé style wine was not included in Figure 6 as the concentration of 1,8-cineole was $\leq$0.4 μg/L and did not change throughout fermentation. This verified that maceration with skins and/or MOG is needed to contribute to 1,8-cineole concentration in wine, and further explains why we did not find 1,8-cineole in a range of white wines,[5] because these are generally made without skin contact. The evolution of 1,8-cineole during fermentation of the other treatments was consistent with the commercial scale fermentations assessed previously.[5] The controls exhibited a small increase in the concentration of 1,8-cineole (to 1.8 μg/L), which provided confirmation that 1,8-cineole is extracted from grape skins and can increase in concentration during fermentation[5] (when compared to the rosé treatment). For grape leaf/stem treatments, the concentration of 1,8-cineole (around 6.0 μg/L) can be seen to increase several-fold relative to the control samples, finishing with levels above the reported odor difference threshold of 1.1 μg/L for 1,8-cineole.[3] This was consistent with the higher amounts of 1,8-cineole determined in grape leaves and stems as compared to the berries. The eucalypt treatments were even more informative, revealing

dx.doi.org/10.1021/jf204499h | J. Agric. Food Chem. 2012, 60, 2281–2287

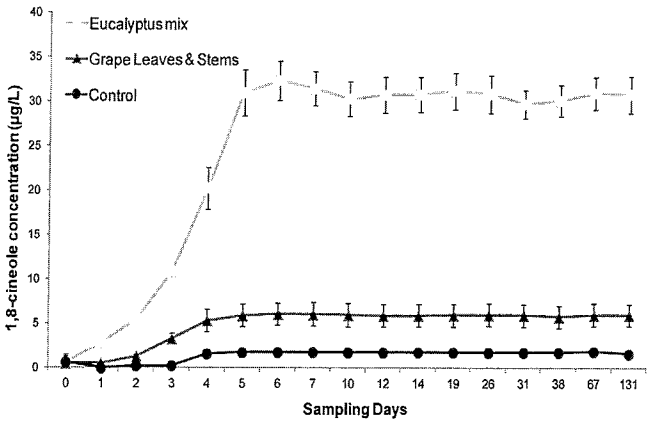

Figure 6. Mean concentrations of 1,8-cineole (μg/L) for the various replicated treatments determined during fermentation and storage. Error bars represent the 95% confidence interval (i.e., 2 × standard error of the mean) of the replicate ferments. Where error bars are not visible, the standard error was zero. Day 0 = crushed and cold soaked, day 1 = inoculated with yeast, day 6 = pressed, day 8 = racked, day 12 = inoculated for MLF, day 38 = racked, day 67 = prior to bottling, and day 131 = 64 days postbottling.

substantially increased concentrations of 1,8-cineole (approximately 30 μg/L). These relatively high levels could easily be achieved, depending on how the vineyard parcels are allocated, as in our study we removed 33 *Eucalyptus* leaves lodged within the grape bunches during hand sorting and randomizing of the 550 kg of hand-harvested fruit. Considering this vineyard and many others are normally harvested by machine, it would be reasonable to expect there would be a noticeable contribution to 1,8-cineole concentration in the wine as a result of such MOG beyond what is extracted solely from the grapes.

In our experiments, the presence of *Eucalyptus* leaves and to a lesser extent grapevine leaves and stems in the harvested grapes was determined to be the main contributor to 1,8-cineole concentrations in the wine. While there were apparent differences between vintages for the grapevine material examined, there was a clear effect of proximity to *Eucalyptus* trees, and the impact of MOG was obvious. Winemakers can heed these results and base decisions on them, fermenting fruit that grows near *Eucalyptus* trees separately and using it for blending, or ensuring minimal MOG is included from grapes that are machine-harvested in the vicinity of *Eucalyptus* trees. Such an effect from 1,8-cineole may also be evident in other viticultural regions around the world where *Eucalyptus* trees are a part of the natural landscape.

Following the production of these experimental fermentation treatments, six assessors informally evaluated the finished wines to assess their 1,8-cineole aroma. These rudimentary assessments revealed an obvious "eucalyptus" aroma to all of the assessors for the wines produced with the addition of *Eucalyptus* leaves. Surprisingly, the wines with the addition of the grape leaves and stems seemed to exhibit a strong "peppery" aroma that was less evident in the other treatments. This raised our curiosity about the nature of the compound(s) responsible for this character.

Effect of MOG on Wine Rotundone Concentrations. The sesquiterpene rotundone, previously identified as being responsible for giving wine a pepper aroma, has an extremely low aroma detection threshold of 16 ng/L in red wine.[10] We therefore analyzed all of the finished wines from the MOG study to determine rotundone concentrations. Rotundone was found in high concentrations, above 200 ng/L in the grape leaf/stem treatments (Table 2), where it was about 13 times above the reported aroma detection threshold of this compound. This particular treatment exhibited up to 6 times more rotundone than the controls (around 36 ng/L) and contained the highest concentration of rotundone of all of the treatments. The fermentation with the addition of *Eucalyptus* leaves had slightly higher amounts of rotundone (around 54 ng/L) than the controls, most likely due to small amounts of stems that would have been present as the grapes were passed through a crusher/destemmer and not all of the stems were removed. As expected, the rosé style wine contained much lower concentrations of rotundone (around 7 ng/L), which is below its aroma detection threshold. The rosé and control results were in accord with the findings of Caputi et al.,[18] highlighting that the largest proportion of rotundone in grape berries is located in the skins.

Table 2. Concentration of Rotundone from Duplicate Measurements (Means ± SD) of Wines Arising from the 1,8-Cineole Investigations and Duplicate Grape Leaf and Stem Extractions

samples	rotundone
Fermentation Treatments	
rosé style 1	8.5 ± 0.7 ng/L
rosé style 2	≤5 ng/L
control (hand plucked) 1	34.5 ± 2.1 ng/L
control (hand plucked) 2	38 ± 0 ng/L
grape leaf and stem 1	221 ± 1.5 ng/L
grape leaf and stem 2	213.5 ± 0.7 ng/L
grape leaf and stem 3	205.5 ± 2.1 ng/L
eucalyptus mix 1	58 ± 0 ng/L
eucalyptus mix 2	49.5 ± 0.7 ng/L
Ethanolic Extracts	
grape leaf row 1	4.8 ± 2.7 μg/kg
grape leaf row 20	0.9 ± 0.3 μg/kg
grape stem row 1	12.4 ± 0.5 μg/kg
grape stem row 20	0.65 ± 0.5 μg/kg

To confirm the impact of MOG on wine rotundone concentrations, we also determined the amount of rotundone in both grape leaf and grape stems extracts, thereby showing these were the main contributors to elevated wine rotundone levels. Grape leaves contained an average of 2.8 μg/kg, whereas grape stems contained an average of 6.5 μg/kg (Table 2). We observed large variability between row positions, but this is not surprising as large vineyard variability has been previously shown for other volatile compounds.[19] This vineyard variability may be attributed to factors such as vine vigor, size of the canopy, or health status of the vines.

If we consider the average concentration of rotundone found in the stem sample (6.5 μg/kg) and assumed complete extraction into a 50 kg ferment, this could equate to approximately 280 ng/L of rotundone in the finished wine. Similarly, if we consider the leaf extracts (average 2.9 μg/kg), total extraction into the ferment could equate to approximately 50 ng/L of rotundone in the finished wine. As such, if there was complete extraction into the ferment from both the grape leaves and the stems, this would contribute approximately 330 ng/L of rotundone to the wine. This is consistent with the greater amounts in the finished grape leaf/stem wines as compared to the controls.

dx.doi.org/10.1021/jf204499h | J. Agric. Food Chem. 2012, 60, 2281–2287

In addition to our findings regarding 1,8-cineole, we have also shown through our fermentation treatments that the presence of grape leaf and grape stem can considerably enhance the concentration of rotundone in a finished wine much more than the grapes themselves. This serendipitous result could provide an avenue for manipulating rotundone concentration in wine, which hitherto has eluded winemakers. This could be particularly important for red wines made with whole bunch pressing or for ferments containing grape leaf and stem. Overall, the results give winemakers practical options for having a level of control over both 1,8-cineole and rotundone concentrations through vineyard and winery operations. The proximity of grapevines to *Eucalyptus* trees has a conclusive effect on 1,8-cineole concentrations in wine, while the presence of MOG can not only impact 1,8-cineole levels, but also wine rotundone concentrations. These factors can lead to altered wine sensory characteristics and highlight that there is more to consider than grape composition alone when investigating vineyard effects of wine aroma.

■ ASSOCIATED CONTENT

Supporting Information

Tables displaying basic analytical data for Shiraz juices and wines. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: +61 8 8313 6689. Fax: +61 8 8313 6601. E-mail: dimitra.capone@awri.com.au.

Funding

The Australian Wine Research Institute (AWRI) and The University of Adelaide are members of the Wine Innovation Cluster in Adelaide. The work was performed at AWRI and supported by Australia's grapegrowers and winemakers through their investment body, the Grape and Wine Research and Development Corp., with matching funds from the Australian Government, and partial funding from the School of Agriculture Food and Wine, University of Adelaide.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Samantha Anderson, Katryna van Leeuwen, and Natoiya Lloyd for technical support, Gemma West for conducting the winemaking, and Richard Gawel for statistical advice. We also thank Leigh Francis and Dennis Taylor for encouragement and manuscript evaluation and Nick Bruer and Tim McCarthy of Orlando Wines for valuable discussions. We are grateful to members of the Australian wine industry for their continued support and provision of numerous juice and wine samples.

■ ABBREVIATIONS USED

GC–MS, gas chromatography–mass spectrometry; MOG, matter other than grapes; MLF, malolactic fermentation

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