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Identification and Quantification of a Marker Compound for 'Pepper' Aroma and Flavor in Shiraz Grape Berries by Combination of Chemometrics and Gas Chromatography–Mass Spectrometry

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'Black pepper' aroma and flavor is important to some Australian Shiraz red wine styles but the aroma compounds involved have yet to be identified, and no objective analytical method to assess 'pepper' grape aromas is available to date. Samples of potentially 'spicy'/'peppery' grapes were obtained from vineyards in South Australia and Victoria over two vintages. The important sensory attributes of the grapes, including the aroma descriptor 'pepper', were rated by a sensory panel. The sensory study revealed a strong correlation between the intensity of 'pepper' aroma and the intensity of 'pepper' flavor perceived on the palate. The grape homogenates were analyzed by static headspace GC-MS using a cool inlet system. Vectors obtained by analysis of over 13 000 individual mass spectra per grape sample were then subjected to multivariate analyses. Both principal component analysis and partial least-squares regression were used to develop multivariate models based on mass spectra and aroma descriptors to explain the intensity of the rating of the 'pepper' character. Corresponding differences in mass spectra and aroma were observed among vineyards and from the same vineyards in different years. Additional optimization 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 and led to the identification of α-ylangene, a tricyclic sesquiterpene. To assess the potential of α-ylangene as a marker for this sensory characteristic, a method for α-ylangene analysis of grapes and wine using HS-SPME-GC-MS was developed. Although not a significant aroma compound by itself, α-ylangene was a satisfactory marker for the 'pepper' aroma in grapes and wine, and its concentration showed similar discrimination between 'peppery' vineyards and vintages as that obtained using the multivariate models. Despite its presence in grapes, we could not detect α-ylangene in wine.

KEYWORDS: GC-MS; SPME; grape; wine; aroma; multivariate analysis; α-ylangene; sesquiterpenes

INTRODUCTION

Shiraz, also known as Syrah or Hermitage, is one of Australia's most popular red wine varieties. Various studies on the compositional and sensory analysis of Shiraz have been published (*1*−*8*). Some Australian red wines have a distinctive 'black pepper' aroma and flavor that are regarded as positive attributes (*9*, *10*). The 'black pepper' flavor can be perceived in individual 'spicy'/'peppery' berries and deseeded Shiraz grape berry homogenates (*9*). There are 'peppery' vineyards that consistently produce 'peppery' wines, especially in cooler years (*10*). Anecdotal evidence implies that the compound or compounds of interest are located primarily in the grape berry skins rather than the pulp of the berry (*11*). Brightman (*9*) evaluated the effect of fermentation on skins compared to fermentation without skin contact and showed that there was no difference in the 'black pepper' aroma perceived in the finished wine. As this work was done with 'low pepper' fruit, it can be speculated that the 'black pepper' compound or compounds are inherent within 'high pepper' grapes to begin with and that fermentation alone is not responsible for the formation of 'black pepper' character, unlike many other compounds important to the aroma of wine (*12*).

Black peppercorns from *Piper nigrum* are known to have a complex flavor based on more than a dozen identified compounds (*13*−*17*). In an early study of black pepper oil, Pangborn et al. (*18*) found three gas chromatography (GC) elution

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windows that had a distinct aroma of ground black pepper and noted that these areas also contained α-*trans*-bergamotene, α-*cis*-bergamotene, and santalene, which are sesquiterpenes that can be commonly found among plant volatiles (*19*−*22*). However, to date the role of these compounds from peppercorns for the 'black pepper' aroma in wine remains to be established.

In our laboratory, Kassara (unpublished data) and Brightman (*9*) investigated many extracts of Shiraz grapes by gas chromatography-olfactory detection (GC-O) and gas chromatography-mass spectrometry (GC-MS) and could not find a single region or compound corresponding to a distinctive 'spicy' or 'pepper' aroma (*9*). This outcome of our initial experiments was not surprising as there are over several hundred volatile components that have been identified in grapes and wine; a large proportion of the compounds are not aroma-active and the compounds of most interest are typically present at only trace levels (*23*−*26*). Although valuable, traditional approaches (*27*−*29*) to identify new aroma compounds can be tedious and time-consuming, and advantages and limitations of GC-O for wine aroma analysis have been discussed in the literature (*23*, *24*).

As our preliminary work investigating the compounds that cause pepper flavor in grapes had not yielded a lead of immediate relevance, we adopted a different approach. A sensory study was designed to allow identification of batches of 'peppery' grapes and to confirm the volatile character of the target compound(s). Next, we developed a method for static headspace analysis to capture all volatile components of the selected Australian Shiraz grapes by GC-MS in a nontargeted fashion. We then aimed to evaluate the GC-MS data with multivariate analysis techniques to elucidate factors related to the 'peppery'/'spicy' aroma of these grapes and to develop models that predicted the 'pepper' aroma intensity of grape samples based on their GC-MS data.

MATERIALS AND METHODS

Materials. All solvents were Mallinckrodt nanopure grade. Unless stated otherwise, all water was purified by the Milli-Q system. *n*-Alkane solutions and neat standards used for retention time markers were purchased from Adelab Scientific (Adelaide, Australia) and Sigma-Aldrich (Castle Hill, New South Wales (NSW), Australia). Reference standards of α-copaene, α-cubebene, *β*-caryophyllene, caryophyllene oxide, calarene, carvone, 1,8-cineole, geraniol, limonene, longifolene, myrcene, α-pinene, *β*-pinene, *cis*-rose oxide, sotolon, and terpinen-4-ol were supplied by Sigma-Aldrich. All solvents and analytical standards were verified for purity by GC-MS prior to use. Essential oils were sourced from Australian Botanical Products (ABP) (Hallam, Victoria, Australia): basil, black pepper (*Piper nigrum*), Cananga java (*Cananga odorata*) (ylang ylang), cubeb (*Piper cubeba*), lime terpenes (Peru), muhuhu (*Brachylaena hutchinsii*), sandalwood (*Santalum album*), Sandalwood (*Santalum spicatum*). Jurlique (Mount Barker, South Australia, Australia): basil (*Ocimum basilicum*), black pepper (*Piper nigrum*), sandalwood. Thursday Plantation (Ballina, NSW, Australia): tea tree (*Melaleuca*). Where stated, the species of the sample was determined by the suppliers, but was not confirmed by us independently.

Methods. *Grape Sampling and Subsampling.* Samples were taken during the 2002 and 2003 vintages. After picking, grape samples were immediately frozen and kept below −18 °C for a minimum of 24 h prior to transport on dry ice (or refrigerated transport) to the Australian Wine Research Institute. These larger samples (5−200 kg each) were each further randomized prior to taking smaller subsamples (typically 250 g). Grape samples were frozen and kept at −20 °C until immediately prior to extraction and analysis.

pH, Titratable Acidity (TA), and Total Soluble Solids (TSS) Analyses. The pH value and TA of the grape samples were determined on free run juice from crushed grapes (250 g subsamples). The pH was determined using a calibrated pH meter and combination Orion electrode. Titratable acidity was determined in tartaric acid equivalents by titration to an end point of pH 8.2. TSS was determined using a digital refractometer. pH, TA and TSS analyses were performed by Analytical Services, the Australian Wine Research Institute.

Sample Preparation for Static Headspace (HS) Analyses: Scan Runs. Grape subsamples (250 ± 1 g each) were allowed to thaw partially and then were blended using a hand-held blender (Breville wizz stick). The blender has stainless steel blades, and grapes were blended in a glass beaker to avoid contact with plastic and potential "scalping" of volatiles of interest (*26*). Internal standard (d_{13}-hexanol) was added (250 μL of 1 mg/mL solution in ethanol) to give a concentration of 1 mg/kg grapes and was thoroughly mixed with the blender. Aliquots (5.0 g) and sodium chloride (2.0 g) were placed in 20 mL HS vials. The HS vials were sealed with magnetic, Teflon-lined rubber septum crimp-caps and were analyzed by HS-GC-MS as described below. A preliminary study (data not shown) was conducted investigating whole grape samples versus homogenates, different sample sizes (5 to 20 g), and different levels of sodium chloride addition (0, 1, 2 g) to the HS vials prior to analysis. As expected, homogenates were found to be more representative of the larger subsamples and gave more accurate and precise results. There was little improvement upon addition of sodium chloride, however; a 2 g addition was found to be optimal. No significant differences in the signal-to-noise of the resultant chromatograms were observed for different sample sizes.

Sample Preparation for Analyses of Monoterpenes and Sesquiterpenes. Grapes (500 g blended homogenate) were extracted by continuous liquid−liquid extraction using dichloromethane for 23 h, followed by column chromatography with Merck silica gel 60 (70−230 mesh) using pentane (*25*, *27*, *28*). Eluent fractions were concentrated by fractional distillation through a Vigreux column packed with Fenske helices, prior to analysis by GC-MS.

Sample Preparation for Determination of α*-Ylangene by Headspace Solid-Phase Microextraction (HS-SPME).* For determination of α-ylangene by HS-SPME, grape subsamples (250 g each) were allowed to half thaw and then were blended, as described above. Internal standard (α-copaene, 3.125 μg in ethanol, 100 μL) was added. Aliquots (5.0 g) and sodium chloride (2.0 g) were placed in 20 mL HS vials and were analyzed by HS-SPME-GC-MS as described below.

The calibration function was established as follows. In addition to unspiked controls, spiked replicate standard additions of α-copaene were made at levels of 0.246, 2.46, 4.93, and 24.6 μg/kg to separate 250 g subsamples of grapes from the same larger source. Five subsamples (5.0 g each) at each standard addition level were placed in 20 mL HS vials and were analyzed by HS-SPME-GC-MS as described below.

Sensory Analyses. Grape subsamples (approximately 250 g each) were allowed to thaw partially and then were blended using a hand-held blender (Breville wizz stick). Aliquots of the homogenate were transferred to coded, covered ISO standard wine tasting glasses (approximately 30 mL grape homogenate per glass). A panel of 10 judges decided on terms to describe the aroma and flavor of the samples during several training discussion and practice rating sessions. The panel consensus arrived at several aroma and flavor descriptors including 'black pepper'. The 10 judges rated the 18 berry homogenate samples presented in duplicate with six samples presented per session and a forced rest between samples. Each attribute was rated for each sample using a 15 cm unstructured line scale from 0 to 10 with anchors of "weak" and "strong" placed at 10 and 90% of the line, respectively. Data acquisition was obtained using FIZZ software (Biosystemes, Couternon, France). The 'black pepper' attribute was rated significantly differently among the samples ($P < 0.001$) treating judges as a random effect. The 'black pepper' attribute was independent of other attributes rated, such as 'green apple', 'grassy', 'tobacco', 'compost', 'red berry', and 'raisin' attributes, and was correlated with the 'spicy' attribute. There was good correlation between the intensity of 'black pepper' aroma and the same character on the palate. The panel mean for aroma was used to calculate a 'pepper' intensity score.

Instrumental Analyses. An Agilent Technologies 6890 GC was equipped with a Gerstel MPS2 multipurpose sampler and was coupled to an Agilent 5973N mass selective detector. In addition to the standard Agilent GC inlet, the GC was also equipped with a Gerstel cool inlet system (CIS-4) (liquid nitrogen cooling facility) with programmed temperature vaporizing injector (PTV). The GC was also fitted with a

Gerstel olfactory detection port. The instrument was controlled with Agilent G1701CA ChemStation software in conjunction with the Gerstel MASter software (version 1.81). The data was analyzed with Agilent G1701CA ChemStation software.

For the static HS analyses of grape samples, analytes in the headspace (2.5 mL, injected at 500 μL/s) were cryofocused in the Gerstel CIS-4, held at −50 °C prior to injection. A resilanized borosilicate glass liner with glass wool insert was used in the PTV. The temperature of the PTV was then ramped at 10 °C/s to 240 °C transferring the previously trapped analytes onto the GC column in a sharp band. The PTV was then held at 240 °C for 20 min ensuring no analyte carryover to the next sample, as verified by the analysis of blanks and negative control samples. The gas chromatograph was fitted with an approximately 60 m × 0.25 mm, 0.25 μm, ZB-Wax-fused silica capillary column (Phenomenex, Pennant Hills, NSW, Australia). The carrier gas was helium (Ultrahigh Purity), linear velocity was 36 cm/s, and initial flow rate was 2.0 mL/min in constant-pressure mode. The oven temperature was started at 40 °C, held at this temperature for 1 min, then was increased to 240 °C at 5 °C/min, and held at this temperature for 10 min. The MS transfer line was held at 250 °C.

For identification of the monoterpenes and sesquiterpenes, the gas chromatograph was fitted with an approximately 30 m × 0.25 mm, 0.25 μm ValcoBond VB-5 mass spectrometry grade capillary column (Chromalytic Technology, Boronia, Victoria, Australia). The carrier gas was helium (Ultrahigh Purity), and initial flow rate was 1.2 mL/min in constant flow mode. The Agilent inlet was fitted with a resilanized borosilicate glass liner, 6.5 mm od, 4 mm id, 78.5 mm long, and was tapered at the column interface with a plug (2−4 mm) of resilanized glass wool. Injector temperature was 220 °C. The sample (2 μL) was injected in pulse splitless mode. The splitter, at 30:1, was opened after 36 s. The oven temperature was started at 50 °C, held at this temperature for 1 min, then was increased to 250 °C at 3 °C/min, held at 250 °C for 10 min, then was ramped to 280 °C at 20 °C/min, and held at 280 °C for 5 min. The MS transfer line was held at 260 °C. The *n*-alkane series used to benchmark retention indices was run in the same manner in accordance with best practice (*30*). Our methodology for GC-MS in combination with an olfactory (sniff) detector (GC-MS-O) was as described in previous publications (*24*, *25*, *29*). The identity of these compounds was verified by comparison of Kovats retention indices and mass spectra with mass spectrometric databases (NBS, Wiley, MassFinder 3 including the Terpenoids library (Dr. Detlev Hochmuth, Hamburg, Germany), and the AWRI GC-MS database).

α-Ylangene, Kovats retention indices (KI) 1375 (DB-5), 1480−1490 (Carbowax); *m*/*z* 204 (M^+, 24%), 189 (12%), 161 (67%), 120 (68%), 119 (93%), 105 (100%), 93 (63%), 92 (37%), 91 (51%), 77 (24%).

α-Copaene, Kovats retention indices (KI) 1382 (DB-5), 1490−1500 (Carbowax); *m*/*z* 204 (M^+, 19%), 189 (5%), 161 (100%), 120 (22%), 119 (94%), 105 (85%), 93 (40%), 92 (24%), 91 (41%), 77 (19%).

For SPME, a Supelco polydimethylsiloxane 100 μm fiber was exposed to the headspace of the sample for 10 min at 25 °C. The Agilent GC inlet was fitted with a resilanized borosilicate glass SPME inlet liner (6.5 mm o.d., 0.75 mm i.d., 78.5 mm long) and was held at 220 °C. The SPME fiber was desorbed in the pulsed splitless mode and the splitter, at 50:1, was opened after 30 s. The fiber was allowed to bake in the inlet for 10 min. The remaining GC conditions were the same as described above for the analysis of the static HS of the grape homogenates.

The mass spectrometer quadrupole temperature was set at 150 °C, and the source was set at 260 °C. Positive ion electron impact spectra at 70 eV were recorded in the range of *m*/*z* 35 to 350 for scan runs. The ions monitored via selected ion monitoring for determination of α-ylangene with α-copaene as internal standard (IS) were: *m*/*z* 93, 105, 119, 161, 189, and 204, dwell time 25 ms. The target ion was typically *m*/*z* 161 with the other ions used as qualifiers.

The SPME method was validated by a series of standard additions of α-copaene (0 to 25 μg/kg, $n = 5 \times 5$) to subsamples of the same blended grapes. The calibration function obtained was linear throughout the concentration range with coefficient of determination (R^2) = 0.99 and linear regression equation, $y = 1.30x + 0.0003$ when *m*/*z* 161, was used as the target ion for both α-copaene and α-ylangene. Similar linearity across the concentration range and good R^2 were also observed when calibration functions were constructed using either *m*/*z* 189 or *m*/*z* 204 as the target ion for both α-copaene and α-ylangene, although the slopes differed on the linear regression equation (0.42*x* and 0.63*x*, respectively), as one would expect from the relative ratios of these ions in the mass spectra of α-copaene and α-ylangene (**Figure 1**). Blank SPME runs and negative controls were checked regularly.

Multivariate Analyses. The GC-MS data used for multivariate analyses did not include scans after 35 min as there were no real peaks, but significant column bleed and noise evidence. Data from the GC-MS scan runs were exported into comma-separated value file format, creating large files (approximately 9 MB). These could be viewed as tables in Microsoft Excel with abundances for each integer value of mass-to-charge ratio (*m*/*z* 35, 36, 37,...220) versus the abundance at each scan number (1, 2,...7978). We chose to limit the scan range exported to *m*/*z* 35−220 because ions with *m*/*z* less than 35 or greater than 220 were noisy and provided limited information of value. As this data was deemed too complex to analyze, vectors (i.e., composite mass spectra) were constructed by summing the individual integer values of the mass to charge ratio (*m*/*z* 35, 36, 37,...220) from selected scans. The advantage of this approach is that either the whole run time of 35 min or any range of scan values (i.e., any time window) and scan range (e.g., *m*/*z* 35−220) can be chosen for any number of samples. Likewise, ions or groups of ions (i.e., sets of individual *m*/*z* integer values) can be included or excluded from the multivariate analyses. In this iterative process, it was important to exclude ions of less importance to structural elucidation such as major ions related to ethanol, which was used as a solvent for the IS.

To confirm the usefulness of the α-ylangene related region as a marker for 'pepper' in a second series of multivariate analyses, vectors were constructed from 100 scans around the ylangene midpoint (scans 5400−5500) for integers in the scan range of *m*/*z* 35−220. All the GC-MS data vectors were exported to *The Unscrambler* software (version 9.1, CAMO ASA, Norway) for chemometric analysis. Before performing principal component analysis, GC-MS data were preprocessed to avoid baseline shifts, retention time drifts, variations in peak shapes, and differences in recovery between the analyzed samples. Smoothing (moving average on each of seven data points) and normalization (mean normalization) provided by *The Unscrambler* software were used as preprocessing methods. The moving average reduced the noise and made it easier to observe the start and end of peaks in the spectra of each grape sample. Mean normalization consisted of dividing each row of a data matrix by its average, thus neutralizing the influence of the hidden factor, which is equivalent to replacing the original variables by a profile centered on one. Only the relative values of the variables were used to describe the sample, and the information carried by their absolute level dropped. This is appropriate in the specific case where all variables are measured in the same unit, and their values are assumed to be proportional to a factor that cannot be directly taken into account in the analysis (*31*). Such transformation enabled us to express the results in the same units for all samples. Multivariate models of GC-MS data were constructed to relate the mass spectra based vectors to the 'pepper' sensory data.

Principal component analysis (PCA) is a well-known technique used for reducing the dimensionality of data, detecting the number of components, and visualizing the outliers. PCA is one of the most commonly applied techniques in multivariate data analysis (*31*−*35*). PCA is a mathematical procedure for resolving sets of data into orthogonal components whose linear combinations approximate the original data to any desired degree of accuracy. PCA was used to derive the first principal components from the GC-MS data and was used to examine the groupings of the 18 samples.

Calibration models between 'pepper' aroma intensity and GS-MS spectra were developed using partial least-squares (PLS) regression with full cross validation (*31*, *32*, *34*). The optimum number of terms in the PLS calibration models were defined by the prediction residual error sum of squares function to avoid over fitting of the models. The calibration models were evaluated by the coefficient of correlation in calibration (*r*) and the standard error of cross validation. The sensory rating scores were standardized using the (1/STD) option in *The Unscrambler* before PLS calibration models were developed. Full

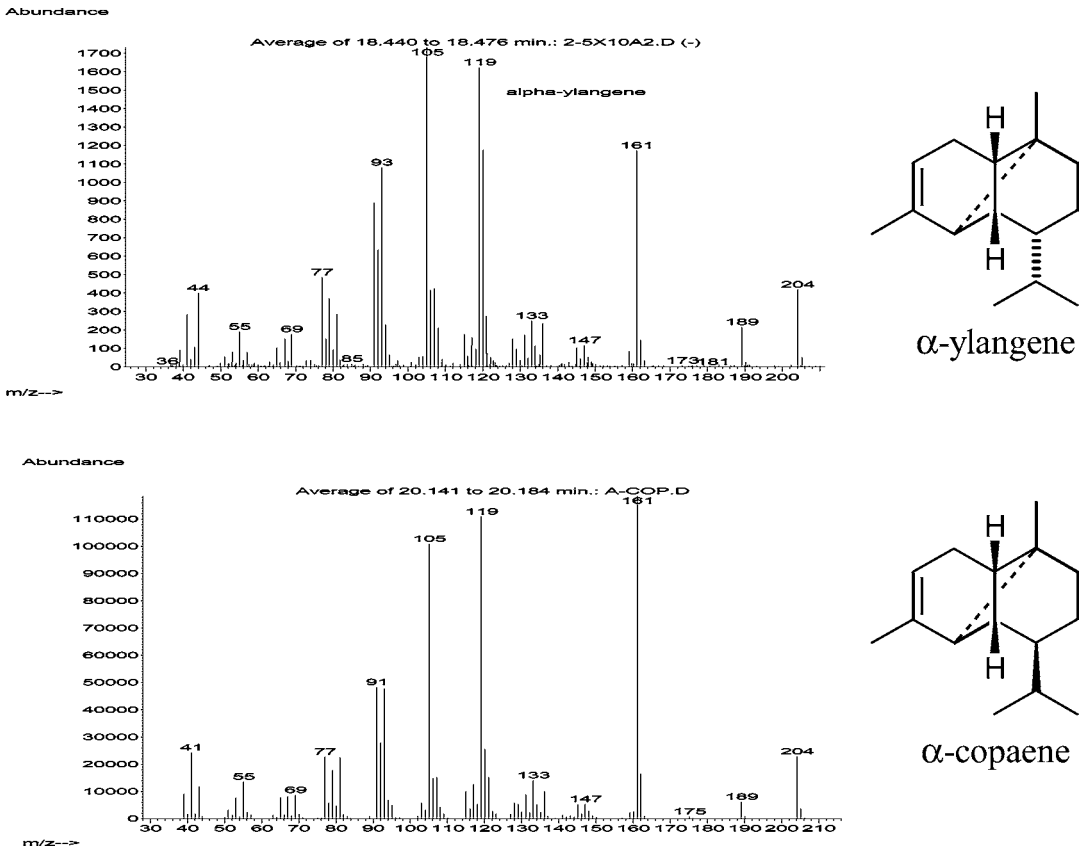

Figure 1. Positive ion electron impact mass spectra at 70 eV and chemical structures of α-ylangene and α-copaene.

internal cross validation (leave one out) was used when PCA and PLS models were developed.

RESULTS AND DISCUSSION

To investigate 'black pepper' flavor in Australian Shiraz wines, we have conducted studies to characterize the volatile compounds related to this sensory character. On the basis of advice from industry collaborators, in 2002 and 2003 we obtained large sets of potentially 'peppery' grapes 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. 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' flavor 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. The pH of the grape samples ranged from 3.48 to 4.13, mean 3.79, standard deviation 0.23. TA of the grape samples ranged from 2.7 to 5.3 g/L, mean 4.3 g/L, standard deviation 0.7 g/L. TSS (measured in °Brix) of the grape samples ranged from 21.2 to 28.2°, mean 24.5°, standard deviation 2.2°. There were no significant trends relating any of these standard parameters in the grapes to their sensory 'pepper' scores.

To study all grape volatiles in a comprehensive, nontargeted fashion, grape homogenate samples were analyzed by static HS-GC-MS using a CIS coupled to GC-MS. Compared to conventional inlets, the CIS allows more sensitive analysis of all volatile trace aroma compounds and enables the analysis of thermally labile compounds. While GC-MS (and GC-MS-O) analysis yielded no direct answers as to the cause of the difference in perceived 'spicy'/'pepper' characters, qualitative analyses by visual

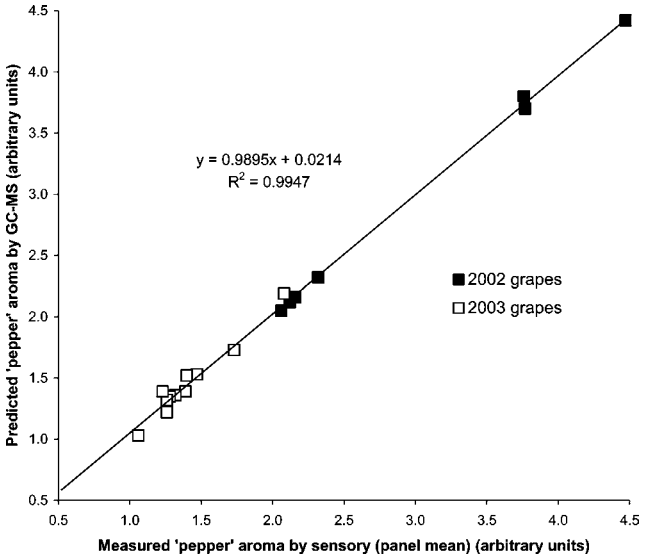

Figure 2. Relationship between predicted 'pepper' aroma by GC-MS by a PLS regression model with the measured aroma by the sensory panel.

inspection of chromatograms showed that the grape samples were clearly different between regions and vintage years.

Vectors obtained by acquisition of over 13 000 individual mass spectra per grape homogenate sample were then subjected to multivariate analyses. PCA in combination with PLS was used to develop a model that could predict 'pepperiness' (**Figure 2**). Furthermore, differences were observed among vineyards and of the same vineyards in different years with 2002 generally being more 'peppery' than 2003 for most of the vineyards studied. Also, similar vineyard specific trends were observed in both vintages in agreement with the observations of Iland and Gago (*10*) who reported that some 'peppery' vineyards consistently

Table 1. Mass-to-Charge Ratio Values Used for PLS Loadings and Corresponding Linear Correlations of the PLS Model

mass-to-charge ratio (*m*/*z*) values	coefficient of determination (R^2)
m/*z* 35–80	0.5471
m/*z* 35–220 (excluding *m*/*z* 45 and 149)	0.9998
m/*z* 51, 60, 74, 85, 92	0.9947
m/*z* 100–180	0.9999
m/*z* 150–190	0.9978
m/*z* 160–220	0.9997
m/*z* 180–220	0.9995

produce 'peppery' wines, especially in cooler years. The precision of the GC-MS multivariate models for predicting the 'pepperiness' level was dependent upon the *m*/*z* range or values chosen for the PLS loadings used in the model (**Table 1**). For example, the model using *m*/*z* range 35−80 showed poor predictive ability ($R^2 = 0.5471$), presumably due to poor discrimination, because the vast majority of volatile compounds fragment to give ions in this *m*/*z* range, although there are specific *m*/*z* values that give good discrimination. For the other *m*/*z* ranges investigated, correlation (R^2) between the predicted and measured berry 'pepper' character was generally very good and ranged from 0.9995 up to 0.9999 (**Table 1**). This result demonstrated that GC-MS scan data could be used in multivariate analyses to construct models that successfully predicted the 'spicy'/'peppery' aroma of grapes based on differences formerly hidden in the GC-MS data and without further knowledge of the nature of the key impact aroma compound(s). The correlation was very good ($R^2 = 0.9998$) for the model using the entire scan range *m*/*z* 35−220 when ions *m*/*z* 45 (ethanol was used as solvent for the internal standard) and *m*/*z* 149 (phthalates) were excluded. However the best correlation was observed for *m*/*z* range 100−180 ($R^2 = 0.9999$) although PLS loadings in this range did not yield any revelations. Conversely, the PLS loadings in the range of *m*/*z* 50−100 revealed five *m*/*z* integer values (ions), four (*m*/*z* 51, 60, 74, and 92) that gave strong positive influences on the predictive model for 'pepper' aroma and one (*m*/*z* 85) that showed a negative influence. These five ions explained >99% of the 'pepper' variation for these 18 grape samples (**Table 1**, **Figure 2**) and ranked the samples in the same 'pepper' intensity order as that obtained from using the full scan range or sensory analysis.

Upon reinvestigating the static headspace GC-MS data files, targeted mass spectrometric data mining using extracted ion chromatograms for compounds that contained relatively high amounts of the four positive contributing ions, but not the negative-loading ion, revealed only one single compound, α-ylangene, identified by its mass spectrum and Kovats retention indices, even though the relative intensities of the four positively influencing ions from the PLS model were relatively weak in the mass spectrum of α-ylangene. Identification of α-ylangene was based on its retention time and mass spectrum and confirmed by analysis of Cananga java (*Cananga odorata*) (ylang ylang) and muhuhu (*Brachylaena hutchinsii*) essential oils, that are established sources of α-ylangene and by co-chromatography of essential oil fractions with grape fractions, resulting in the symmetrical enhancement of the α-ylangene peak, on both DB-5 and Carbowax stationary phases.

To confirm the relevance of the α-ylangene region to contain the marker for 'pepper' aroma in Shiraz grapes, vectors were also constructed from 100 scans around the ylangene peak maximum around scans 5400−5500 using integers in the scan range of *m*/*z* 35−220. This region predicted the 'pepper' intensity with very good correlation, $R^2 > 0.98$, thus confirming the relevance of α-ylangene for the predictive models and indicating the absence of major marker compounds in other parts of the chromatogram. We call α-ylangene a marker, not an active aroma compound, as it does not have a strong aroma and certainly not a strong 'spicy' or 'peppery' aroma by GC-MS-O. Ylang ylang oil rich in ylangene is not spicy and ylangene has not been described as 'spicy'/'peppery' in the literature. As a marker, no direct relationship with the unknown 'peppery' compound(s) is required.

Table 2. Major Monoterpenes and Sesquiterpenes Identified in Australian Shiraz Grapes

compound	KI on DB-5
p-cymene	1025
limonene	1029
β-phellandrene	1030
1,8-cineole	1034
cis-rose oxide	1110
camphor	1146
cis-linalool oxide (pyran)	1167
trans-linalool oxide (pyran)	1171
carvone	1240
geraniol	1252
cyclosativene	1371
α-ylangene	1375
β-bourbonene	1388
β-ylangene	1421
β-copaene	1432
guaia-6,9-diene	1447
selina-3,7-diene	1455
γ-muurolene	1465
bicyclosesquiphellandrene	1481
α-amorphene	1485
epizonarene	1498
zonarene	1520
γ-cadinene	1514
isocalamenene	1524
α-cadinene	1540
α-calacorene	1553
β-calacorene	1566
cadalene	1663

To explore the role of terpenoids in 'peppery' Shiraz grapes further, the major monoterpenes and sesquiterpenes found in the grapes were identified by GC-MS analysis as shown in **Table 2**. The identity of these compounds was verified by comparison of Kovats retention indices and mass spectra with mass spectrometric databases and the relevant literature (*27*, *30*, *36*−*38*). Reference standards were also used to confirm identity. This is by no means a definitive list of all the monoterpenes and sesquiterpenes in the Shiraz grape samples studied but represents the major compounds that could be confidently identified and verified. α-Ylangene was the most abundant sesquiterpene in the Shiraz grape samples, even in those of lower 'pepper' intensity. However, none of the compounds listed in **Table 2** gave a 'spicy' or 'peppery' aroma by GC-MS-O.

The identification of a substantial number of terpenoids in 'peppery' grapes is of interest as it allows comparison of sesquiterpene profiles between grapes and other plant products such as peppercorns that are used in spices. According to Pino et al. (*14*), pepper oil is composed of monoterpenes (70−80%) and sesquiterpenes (20−30%). Sesquiterpenes identified in black peppercorns include α-copaene, β-caryophyllene, α-cubebene, α-humulene, α-guaiene, and many more (*13*−*15*). In grapes, numerous sesquiterpenes have previously been identified as well. Schreier et al. (*27*) extracted seven different German white grape

varieties and described many compounds for the first time in grapes, including the sesquiterpenes β-caryophyllene, α-humulene, calamenene, δ-cadinene, γ-cadinene, β-bourbonene, α-muurolene, γ-muurolene, β-selinene, α-copaene, β-ylangene, α-guaiene, and farnesene. Vernin et al. (*1*) extracted Syrah (Shiraz) grape skins and identified sesquiterpenes including: α-copaene, β-bourbonene, calarene, β-caryophyllene, α-muurolene, α-humulene, δ-cadinene and γ-cadinene. Recently, α-ylangene was tentatively identified in Baga grapes (*Vitis vinifera* L. *cv* Baga) along with β-bourbonene, β-caryophyllene, α-guaiene, 3,7-guaiadiene, (+)-aromadendrene, γ-cadinene, (−)-δ-selinene, germacrene D, epizonarene, β-cadinene, δ-cadinene, α-muurolene, γ-elemene, germacrene B, α-calacorene, valencene, and manoyl oxide (*43*). In this study, α-ylangene concentration was related to the ripening of Baga grapes (*43*); however, Baga grapes are not renowned for making 'spicy'/'peppery' wine.

To robustly discriminate between α-copaene and α-ylangene in the absence of a pure reference compound for α-ylangene, we coinjected equal volumes of the same grape extract, rich in naturally occurring α-ylangene, with increasing concentrations of pure α-copaene, resulting in corresponding symmetrical enhancement of the α-copaene peak, on both DB-5 and Carbowax stationary phases. We also verified that there was no detectable α-copaene present in these and many other grape extracts prior to standard addition. As all of the Shiraz grape samples we analyzed contained α-ylangene and none of our grape samples contained a detectable quantity of α-copaene, it is possible that previous researchers might have misidentified α-ylangene as α-copaene in grapes (*1*, *9*, *27*). As can be seen in **Figure 1**, the two compounds are stereoisomers, and the mass spectra are similar but show some differences such as relative abundance of *m*/*z* 119, 161, and 189 compared with the molecular ion *m*/*z* 204. Hence, identification through matching purely on the basis of mass spectra fit can be misleading. In addition, the KI of α-ylangene and α-copaene are very close on DB-5 (∼1375 and ∼1382, respectively) and on Carbowax columns (α-ylangene, KI 1480−1490 and α-copaene, KI 1490−1500), although we had only a pure authentic sample of α-copaene and essential oils rich in α-ylangene available to confirm this.

When we analyzed sesquiterpene profiles from essential oils of black and white pepper, sandalwood, basil, geraniums, cubeb, and tea tree, it became clear that grapes were unusual in that they did not possess any detectable quantity of α-copaene, but rather contained α-ylangene. Similarly, numerous reports list other plant-derived products where α-copaene and numerous other sesquiterpenes have been identified, but not α-ylangene (e.g., basil (*39*), black and white pepper (*40*), mango (*41*), and cotimajo (*42*)).

It is possible that α-ylangene is not directly related to the still unknown 'spicy'/'pepper' flavor compound(s) in Shiraz grapes but rather reflects other factors that could be responsible for the development of the 'spicy'/'pepper' character in grapes such as cool climate, grape variety, cultivar or possibly clone, grapegrowing location or region, or other factors. To further explore the relationship between α-ylangene and the 'spicy'/'pepper' character, we decided to quantify α-ylangene in Shiraz grapes. As in any quantitative analysis, the internal standard used should be as similar as possible to the analyte measured without being present in the original matrix (*24*, *44*). Thus, in the absence of stable isotope labeled α-ylangene, α-copaene is a very suitable internal standard for the determination of α-ylangene in grapes. As part of method validation, it is best practice to demonstrate the linearity of the calibration function by a series of spiked standard additions of the analyte of interest to the matrix at a constant amount of internal standard (*12*, *38*, *44*−*47*). However, in this case we did not have access to pure α-ylangene but only to α-copaene. So we adopted the reverse approach by making replicates of the same grape extract, rich in naturally occurring α-ylangene, and spiked it with increasing standard additions of α-copaene, which demonstrated that the analytical method to quantify α-ylangene was indeed linear across the calibration range with good correlation ($R^2 > 0.99$).

The concentration of α-ylangene in the Shiraz grape samples ranged from undetectable (<0.2 μg/kg) in one sample, then from 0.9 up to 15.2 μg/kg. The correlation of α-ylangene concentration versus 'pepper' sensory scores was $R^2 = 0.65$. The most 'peppery' grapes had correspondingly high relative concentrations (10 to 15 μg/kg) of α-ylangene, which means α-ylangene is a good marker for the grapes high in 'peppery' character. At the same time, the correlation of mid to low 'peppery' levels (by sensory) with α-ylangene was not always good (i.e., some samples with low pepper had moderate levels (around 5 μg/kg) of α-ylangene and vice versa). However, the correlations of peppery sensory scores with total concentration of all sesquiterpenes (with *m*/*z* 119, 161, 204) other than α-ylangene, or with selected other sesquiterpenes, gave poorer fits. Thus, of the specific analytes detectable by the analytical methodology employed, α-ylangene was the best indicator for the 'pepper' aroma and flavor of the Shiraz grape samples. Still, the multivariate method (focusing on either mass spectra across the whole chromatogram or just the α-ylangene region) gave much better correlation with the 'pepper' sensory scores with $R^2 > 0.98$. It is therefore likely that the relationship between α-ylangene and the grape 'pepper' aroma is not linear or that other compounds are involved.

Finally, we analyzed several 'peppery' wines for α-ylangene, using the HS-SPME-GC-MS method described above for grapes, with α-copaene as the internal standard. No α-ylangene was detected in any of the wines at concentrations above the estimated detection limit of 0.2 μg/L. This led us to conclude that although the 'spicy'/'peppery' aroma and flavor (and thus the 'spicy'/'peppery' aroma and flavor compound(s)) were present in these wines, the marker compound α-ylangene was suitable only for the prediction of the 'peppery' character in grapes but not wine. As a marker, no direct relationship with the unknown 'pepper' compound(s) is required. It is possible that α-ylangene is a precursor that has to be transformed into a derivative to be efficient. More likely, α-ylangene is retained by solid-phase ("chapeau de marc") interactions during winemaking because of the potentially strong interaction of this relatively nonpolar compound with lipophilic molecules or polymers. This is well known for orange juice where monoterpenes and sesquiterpenes are strongly stabilized by pulp (peptic, hemicellulosic, cellulosic fractions). Current studies continue regarding the exact nature of the compound or compounds directly responsible for the 'spicy'/'peppery' aroma and flavor in Shiraz grape and wine.

As the vectors used in the multivariate analyses of GC-MS data can use selected scan ranges, groups of ions, or retention time windows, this technique is potentially useful for other nontargeted metabolomics applications (e.g., in the discovery of new aroma impact compounds or bioactive compounds) and for mining data from related analytical techniques such as high-performance liquid chromatography (HPLC)-MS.

Unlike many other natural products, Shiraz grapes did not contain the sesquiterpene α-copaene at detectable concentration. Rather, grapes contained α-ylangene, an epimer of α-copaene. We developed and applied a novel, rapid method for quantitative

α-ylangene analysis of grapes and wine using HS-SPME-GC-MS and α-copaene as the internal standard. Although not a significant aroma compound by itself, α-ylangene was a good marker compound for high-'pepper' aroma intensity in Australian Shiraz grapes. However, in wine α-ylangene has not been found so far. Whether α-ylangene is a marker for other factors anecdotally related to 'spicy' or 'black pepper' aroma notes in Shiraz grapes (e.g., cool climate, location, variety, or cultivar) or is related to the biosynthesis of the yet to be identified 'peppery' aroma compounds has yet to be demonstrated.

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