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ORIGINAL PAPER

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Quantitative analysis of geraniol, nerol, linalool, and α-terpineol in wine

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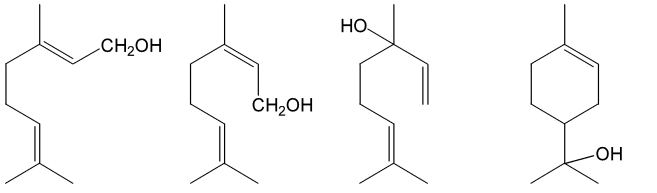
Abstract A mixture of [$^2H_7$]-geraniol, [$^2H_7$]-nerol, [$^2H_7$]-linalool and [$^2H_7$]-α-terpineol was prepared for use as internal standards in a rapid and accurate analytical method, employing gas chromatography–mass spectrometry (GC/MS), to determine the concentration of geraniol, nerol, linalool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

Keywords GC/MS · Wine · Geraniol · Nerol · Linalool · α-Terpineol · Artefacts

Introduction

Of all alcoholic beverages, wine displays the greatest variation in flavour. Subtle nuances of colour, aroma and flavour create a unique character for each wine. Several hundred volatile components have been identified in grapes and wine and many of these are important to wine aroma and flavour [1, 2]. Among these, the monoterpene alcohols geraniol (**1**), nerol (**2**), linalool (**3**) and α-terpineol (**4**) (Fig. 1) are important to the aroma and flavour of young wines made from Muscat varieties of grapes [3, 4, 5, 6, 7, 8, 9] such as Traminer, whereas juice from non-Muscat grape varieties contain low levels only of these monoterpenes [3, 10, 11, 12, 13]. When present in young wines, geraniol (**1**) and nerol (**2**) decrease in concentration as wine is aged [3, 14, 15, 16, 17, 18, 19] and are usually present in no more than trace amounts after two to three years in the bottle. The concentration of linalool (**3**) and α-terpineol (**4**) can initially increase during bottle storage, but decreases afterwards, as they are converted mainly to terpin hydrate [12, 20]. Traces of linalool (**3**) and α-terpineol (**4**) are still detectable in aged Riesling wine [21]. Geraniol (**1**) and linalool (**3**) are considered to be the most important of the monoterpene alcohols as they are present in greater concentrations, and have lower flavour thresholds than other major wine monoterpenes [22]. Geraniol (**1**), nerol (**2**), linalool (**3**), α-terpineol (**4**) and related compounds are also considered to be important to the flavour of other alcoholic beverages [23, 24], soft drinks [20] and are widely used in the perfume and flavour industries [25].



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

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

This paper describes a new method for the analysis of these compounds in wine, which is both fast and accurate, and accounts for the possible formation and degradation of these monoterpene alcohols during the analysis. The method can assist researchers and winemakers in determining how winemaking and storage processes affect the concentration of these compounds.

Experimental

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

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All solvents were Mallinckrodt nanopure grade, and verified for purity by GC/MS prior to use. All reagents and standards were purchased from Sigma–Aldrich. Model wine used for the stability studies was 10% aqueous ethanol, buffered with potassium hydrogen tartrate and sulfuric acid (5 mol L^{-1}) to pH 2.9.

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

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

NaOD in D_2O (ca 40%, 15 drops) was added at room temperature to a stirred solution of 6-methyl-5-hepten-2-one (10.5 mL) in dry pyridine (90 mL) and D_2O (40 mL) under nitrogen. After 2 h the reaction was quenched with NH_4Cl and extracted with CH_2Cl_2 (3×200 mL). The combined organic layers were washed with saturated aqueous $CuSO_4$ (until the pyridine was removed as indicated by the absence of any darkening of the $CuSO_4$ solution), then water (3×200 mL), dried (Na_2SO_4), and the solvent was evaporated, yielding 1,3-[$^2H_5$]-6-methyl-5-hepten-2-one (**5**) as a colourless oil (6.4 g) which was used in the next step without further purification.

Sodium hydride (95%, 1.29 g) was added to a cooled (~5 °C) solution of triethyl phosphonoacetate [26] (10.65 mL) in diethyl ether (60 mL) and the solution was left stirring at room temperature for 60 min. A solution of **5** (6.4 g) in diethyl ether (60 mL) was then added over 30 min and the reaction mixture was stirred for a further 44 h. The reaction was quenched with D_2O (5 mL), then water (500 mL) and the product, 4-[$^2H_2$]-7-methyl-3-([$^2H_3$]-methyl)-2,6-octadienoic acid ethyl ester (**6**, (*E*):(*Z*) 4:1 by GC/MS), was isolated with diethyl ether as a yellow liquid (8.8 g), ^{1}H NMR, (*E*) isomer: δ 5.66 (s, C2-H), 5.2–5.0 (m, C6-H), 4.15 (q, J=7.4 Hz, OC$\underline{H}_2$CH$_3$), 2.14 (br d, J=6.2 Hz, C5-H$_2$), 1.68 and 1.61 (2×br s, C8-H$_3$ and C7-CH$_3$), 1.28 (t, J=7.1 Hz, OC$\underline{H}_2$CH$_3$); (*Z*)-isomer; δ 5.65 (s, C2-H), 4.14 (q, J=7.3 Hz, OC$\underline{H}_2$CH$_3$), 1.27 (t, J=7.1 Hz, OC$\underline{H}_2$CH$_3$). A portion (3 g) of the ester (**6**) in dry diethyl ether (40 mL) at ~5 °C was treated with $LiAlD_4$ (0.6 g) under dry nitrogen. After 10 min the mixture was warmed to room temperature, stirred for 4 h, cooled to ~5 °C and treated with acetone (5 mL), followed by aqueous sodium hydroxide (1 mol L^{-1}, 3 mL) then sodium sulfate. The ether solution was filtered and the solvent evaporated, leaving a clear oil that was further purified by distillation in vacuo, giving 2,4-[$^2H_4$]-7-methyl-3-([$^2H_3$]-methyl)-2,6-octadienol (**7**):(**8**)=4:1, 1.97 g, by GC/MS). ^{1}H NMR (both isomers) δ 5.40 (s, C2-H), 5.2–5.0 (m, C6-H), 2.08 (br d, C5-H$_2$), 1.99 (br s, -OH), 1.68 and 1.60 (2×br s, C8-H$_3$ and C7-CH$_3$). ^{13}C-nmr δ 139.4, 131.6, 123.9, 123.3, 58.5 (qn), 39.1 (m), 26.2, 25.6, 17.6, 15.6 (m).

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

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

Preparation of samples for analysis

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

GC/MS analysis

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

Validation

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

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

The repeatability of the analysis was determined at two concentrations (10 µg L^{-1} and 500 µg L^{-1}) by spiking several replicate aliquots of the same white wine with (**1**)–(**4**). For seven replicate analyses of the wine spiked at the 10 µg L^{-1} level, the coefficient of variance (or relative standard deviation) was 5.16% for geraniol (**1**),

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

Stability studies—robustness of the analytical method

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

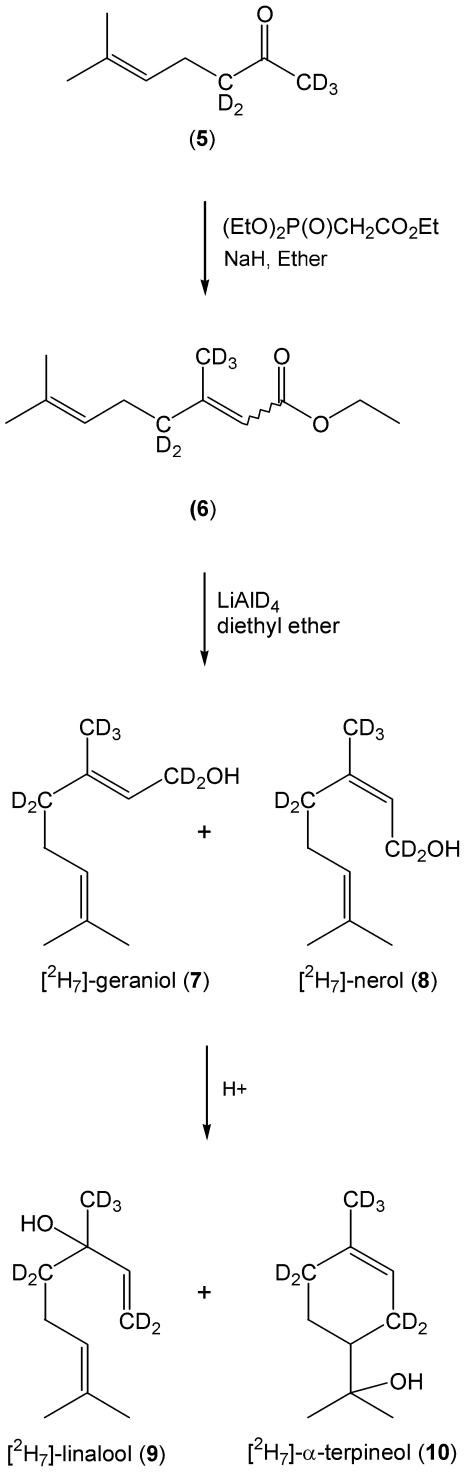
For studying the stability of the analytes in the organic phase, geraniol (**1**, 20.9 mg), nerol (**2**, 19.3 mg), linalool (**3**, 24.7 mg), and α-terpineol (**4**, 21.0 mg) were added to separate 50 mL volumetric flasks, which were made up with model wine to the mark, and shaken thoroughly. Pentane:diethylether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

Results and discussion

The analytical method

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

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



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

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

Starting compound(s) (purity)	% Compounds after 1 h at 25 °C	% Compounds after 2 h at 25 °C	% Compounds after 4 h at 25 °C	% Compounds after 8 h at 25 °C	% Compounds after 24 h at 25 °C	% Compounds after 5 days at 25 °C
Linalool (~97%)	97% linalool	97% linalool	97% linalool	97% linalool	95% linalool	85% linalool
	nd α-terpineol	<0.5% α-terpineol	<0.5% α-terpineol	<0.5% α-terpineol	2% α-terpineol	8% α-terpineol
	nd nerol	nd nerol	nd nerol	nd nerol	<0.5% nerol	1% nerol
	nd geraniol	nd geraniol	nd geraniol	nd geraniol	trace geraniol	3% geraniol
Nerol (~93%)	92% nerol	91% nerol	91% nerol	90% nerol	85% nerol	64% nerol
	nd linalool	<0.5% linalool	<0.5% linalool	1.5% linalool	4% linalool	12% linalool
	<0.5% α-terpineol	<0.5% α-terpineol	1% α-terpineol	2.5% α-terpineol	7% α-terpineol	21% α-terpineol
	<0.5% geraniol	1% geraniol	1% geraniol	1% geraniol	1% geraniol	0.5% geraniol
Geraniol (94%) Nerol (2%)	94% geraniol	94% geraniol	94% geraniol	93% geraniol	90% geraniol	72% geraniol
	<0.5% linalool	0.5% linalool	1% linalool	2% linalool	7% linalool	22% linalool
	nd α-terpineol	nd α-terpineol	trace α-terpineol	trace α-terpineol	trace α-terpineol	0.5% α-terpineol
	2.5% nerol	2.5% nerol	2.5% nerol	2.5% nerol	2.5% nerol	2% nerol
α-Terpineol (96%)	96% α-terpineol	96% α-terpineol	96% α-terpineol	96% α-terpineol	96% α-terpineol	96% α-terpineol
	nd linalool	nd linalool	nd linalool	nd linalool	nd linalool	nd linalool
	nd nerol	nd nerol	nd nerol	nd nerol	nd nerol	nd nerol
	nd geraniol	nd geraniol	nd geraniol	nd geraniol	nd geraniol	nd geraniol

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

to the instrumental operating conditions, thus still necessitating analysis of the mixture of labelled and unlabelled standards along with wine samples.

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

Compared to already published methods for determining monoterpene alcohols in grape juice or wine [3, 27, 28, 29, 30], the advantages of the method described in this paper are that it is precise, accurate, has low detection limits, uses only 5 mL of wine, and sample preparation takes just a few minutes. It does not require salting out to improve precision [3], or further concentration [3, 19, 21, 31] to attain good reporting limits. The chromatographic conditions, combined with SIM EI/MS employed in this method limit the problems of non-baseline resolution evident in other published methods [3, 7, 19, 21, 27, 28, 32, 33].

Stability studies – Robustness of the analytical method

Geraniol (**1**), nerol (**2**), linalool (**3**), and α-terpineol (**4**) undergo a variety of transformations, even in dilute acidic media [12, 20, 23, 27, 34, 35, 36]. Thus, Skouroumounis and Sefton [34] hydrolysed geraniol (**1**) in 10% ethanol/water at pH 3.0 at 50 °C. After 24 h, 67% of geraniol (**1**) remained, and 27.5% linalool (**3**), 1.4% α-terpineol (**4**) and 0.7% nerol (**2**) had been formed, plus trace amounts of 11 other compounds. In shochu (alcoholic beverage distilled from various materials, e.g. rice, barley, sweet potato), the main product from acid-catalysed transformation of geraniol (**1**) was linalool (**3**) [23]. These authors also state that the main products from nerol (**2**) were linalool (**3**) and α-terpineol (**4**). To our knowledge, the reactivities and transformations of these monoterpene alcohols under the conditions of extraction or analysis have not been investigated previously.

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

Solutions of geraniol (**1**), nerol (**2**), linalool (**3**) and α-terpineol (**4**) in model wine (pH 2.9) were also shaken with pentane:diethylether (2:1) and the two-phase system left sitting stoppered at 25 °C for 24 h, to gauge the extent of interconversion in the organic phase. Subsamples of the organic layer were taken immediately after extraction, after 5 h and after 24 h, and analysed by GC/MS. No compositional changes were observed after 5 h, and even after 24 h less than 0.5% conversion to other products was ob-

served. The same results were obtained for extracts of a white wine (1996 Australian Semillon, pH 3.1). We conclude that our liquid–liquid extracts were sufficiently stable, provided that they were not kept at room temperature for more than 24 h. Another study (data not shown) demonstrated that standard solutions of the compounds (**1**–**4**) in ethanol were stable for up to six months storage at –20 °C. Over longer time periods, however, or at higher temperatures (e.g. room temperature for several days), some conversion could be seen.

Apart from ambient SPME, liquid–liquid extraction at room temperature as applied by us is one of the mildest extraction methods possible. Even so, some interconversions still took place if wines were allowed to stand for more than a few hours. Thus methods, e.g. Refs. [7, 23], involving more exhaustive extractions, concerning high temperatures or harsh pH conditions, without appropriate robustness validation, have limited value. Many authors [3, 19, 21, 27, 29, 30, 31, 32, 33] have observed linalool (**3**) and α-terpineol (**4**) in Muscat or Riesling grape juice. Some of these [6, 19, 28, 29, 30], however, did not detect significant quantities of geraniol (**1**) and nerol (**2**) in their samples. It is possible that this is due, at least in part, to the analytical methodology, which involved elevated temperature or acid in the extraction, thus allowing the transformation of geraniol (**1**) and nerol (**2**) to linalool (**3**), α-terpineol (**4**) and other products.

Conclusions

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

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