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Quantitative Analysis, Occurrence, and Stability of (*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene in Wine

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A stable isotope dilution assay has been developed for quantification of (*E*)-1-(2,3,6-trimethylphenyl)-buta-1,3-diene (**4**) in wine using a [$^2H_6$]-analogue. Using this method, **4** was found in 96 out of 97 white wines, but in none of 12 red wines analyzed. **4** was found to be most prevalent in Semillon wines, followed by Chardonnay, with Riesling showing the least amount of **4** among these three varieties. **4**, like 1,1,6-trimethyldihydronaphthalene (TDN, **3**), appears to be formed during the aging process. **4** was found to be unstable in model wine, and in both white and red wine, with the order of stability being model > white > red. In a PVPP-stripped red wine, the rate of degradation of **4** was substantially lessened, with the final concentrations very close to those observed in model wine. When treated with either grape or wine tannin extracts in model wine, the concentration of **4** was found to decrease to levels very close to those observed with an untreated red wine. When white wine was heated at 45 °C, **4** was formed, indicating the presence of precursor forms. The amounts formed were much higher than those found in a commercial white wine. **4** was also observed in red wine heated to 45 °C, but the concentration produced was much less than that with white wine.

KEYWORDS: TPB; SIDA; polyphenols; grapes; precursors; C_{13}-norisoprenoids

INTRODUCTION

The C_{13}-norisoprenoids are found in wine and are believed to be degradation products of carotenoids (*1*). Important members of this group of aroma compounds (**Figure 1**) include damascenone (**1**), β-ionone (**2**), and the hydrocarbon TDN (**3**). Damascenone is a ubiquitous compound characterized by a very pleasant "stewed apple" or "quince" aroma (*2*), while β-ionone is characterized by a "violet" aroma (*3*). TDN is typically found in aged Riesling wines and is responsible for the "kerosene" aroma often associated with Riesling wines which have undergone bottle maturation (*4*, *5*). Until recently, these three compounds were the only members of this class to have been demonstrated to contribute to wine aroma (*6*−*8*).

(*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (**4**) is a new aroma compound we reported recently (*9*). This compound, which is likely to also be a C_{13}-norisoprenoid, was found in several white wines, but was absent in six red wines studied. At low concentrations, its aroma was described as "green" or "cut-grass", while at higher concentrations less favorable descriptors such as "pungent" or "chemical" were common. The aroma detection threshold was measured in both white wine and model wine and was found to be 40 and 430 ng/L, respectively.

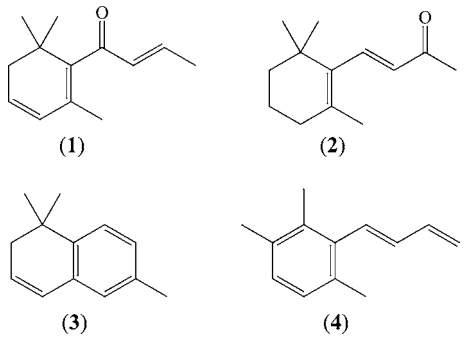

Figure 1. Structures of some potent C_{13} norisoprenoid aroma constituents found in wine.

Compound **4** was identified and characterized from the hydrolysates of glycosidic extracts sourced from a selection of grape varieties, including Cabernet Sauvignon and Shiraz (*9*). Although it is possible that the grape samples used to generate the hydrolysates may not be typical of these varieties, the question remains as to whether there is a chemical basis for its presence in glycoside hydrolysates from red varieties but not in the red wines themselves. Red wines differ from white wines

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in several key areas, among them the relative polyphenol content (*10*). **4** is a highly conjugated molecule and could easily be protonated, and the resulting cation may then react with nucleophilic species, such as polyphenols. Adducts between grape and wine anthocyanins and activated styrene derivatives have been implicated in the stabilization of red wine color (*11*−*14*). We were thus keen to investigate whether the polyphenol content of red wines could play a role in the removal of **4** from the matrix.

In the initial reporting of **4**, the levels of this compound were measured by gas chromatography−mass spectrometry (GC−MS) using [$^2H_8$]-naphthalene as the standard for quantification (*9*). This procedure and standard had previously given reliable results for the quantification of TDN. However, as **4** is much more potent than TDN, and as submicrogram levels of this compound would be expected to impact strongly on a wine's perceived aroma, it was felt that a stable isotope dilution assay (SIDA) method specific to the quantification of **4** was required. This study was undertaken to develop such a method and to assess its application to wine analysis.

MATERIALS AND METHODS

Materials. NMR spectra were recorded as solutions in $CDCl_3$ and were obtained on a Varian Gemini spectrometer operating at either 300 MHz (^{1}H) or 75.5 MHz (^{13}C). Centrifugation was performed using a Beckmann L7-55 Ultracentrifuge. All reagents were purchased from Sigma-Aldrich. All solvents were of the highest commercial grade available. Diethyl ether and THF were distilled from sodium/benzophenone immediately prior to use. All organic solutions were dried over anhydrous sodium sulfate prior to filtration. Unlabeled **4** was prepared as indicated in Janusz et al. (*9*). Details are given below for the labeled analogue. Buffered model wine solutions were prepared by saturating a 10% solution of ethanol in water with potassium hydrogen tartrate, and adjusting the pH to the desired value with 10% aqueous tartaric acid solution. Mass spectra were recorded as described previously (*9*).

Methods. *[$^2H_3$]-2,6,6-Trimethylcyclohex-2-enone (6).* *n*-Butyllithium (44.0 mL of 1.6 M) was added dropwise to a solution of diisopropylamine (7.24 g) in THF (100 mL) at −30 °C. After being stirred for 30 min, the solution was cooled to −78 °C and 2,6-dimethylcyclohexanone (**5**) (9.8 g) was added dropwise. The solution was stirred for 1 h before [$^2H_3$]-methyl iodide (9.4 g) was added, and the mixture was allowed to warm to room temperature and stirred overnight. Saturated aqueous ammonium chloride solution (20 mL) was added, and the layers were separated. The organic layer was dried, the solvent evaporated, and the residue dissolved in chloroform (50 mL). A solution of bromine (16 g) in $CHCl_3$ (20 mL) was added dropwise until the red color of bromine persisted. Residual bromine was removed by washing with 5% thiosulfate, and the organic solution was dried and filtered. Pyridine (39.2 g) and chloroform (total volume 100 mL) were added, and the mixture was heated at a gentle reflux for 48 h. After cooling, the solution was washed with saturated aqueous $CuSO_4$ solution (until no darkening occurred), water, and brine. After drying and concentrating, the residue was distilled (90 °C at 31 mm) to give **6** as a clear liquid (4.25 g, 39%).

δ_H ($CDCl_3$): 6.62 (1H, m, H_3), 2.31 (2H, m, H_4), 1.80 (2H, t J = 6.1 Hz, H_5), 1.75 (3H, m, H_9), 1.09 (3H, s, $H_{7,8}$).

δ_C ($CDCl_3$): 204.3, 143.2, 133.4, 40.7, 36.3, 23.9, 22.7, 16.1.

[$^2H_7$]-2-Hydroxy-4-(1-hydroxy-2,6,6-trimethylcyclohex-2-enyl)-but-3-yne (8). To a solution of (**6**) (1.0 g) in THF (20 mL) was added a solution of ethynylmagnesium chloride (70 mL of 0.5 M) at 0 °C. The reaction was allowed to warm to room temperature and stirred overnight. The solution was washed with saturated aqueous ammonium chloride solution (2 × 50 mL). The aqueous layer was back-extracted with diethyl ether (50 mL), and the combined organic extracts were washed with brine (2 × 50 mL) and dried. After concentration, the residue was distilled (90−100 °C at 19 mm) to give [$^2H_3$]-1-hydroxy-1-ethynyl-2,6,6-trimethylcyclohex-2-ene (**7**) as a clear pale orange liquid (0.82 g, 69%).

δ_H ($CDCl_3$): 5.51 (1H, m, H_3, 2.49 (1H, s, ≡CH), 2.01 (2H, m, H_4), 1.70−1.44 (2H, m, H_5), 1.89 (3H, m, C_2−Me), 1.09 (3H, s, C_6−Me).

δ_C ($CDCl_3$): 134.8, 125.0, 85.7, 74.8, 74.1, 37.4, 32.1, 24.6, 23.1, 19.7.

A portion of this product (200 mg) in diethyl ether (1 mL) was treated with *n*-butyllithium (1.5 mL of 1.6 M) at −78 °C. The solution was allowed to warm to room temperature and left to stir for 2 h, after which time [$^2H_4$]-acetaldehyde (60 μL) was added and the reaction was stirred overnight. The mixture was diluted with diethyl ether (1 mL) and washed with saturated ammonium chloride solution (2 × 3 mL). The aqueous layer was back-extracted with diethyl ether (2 mL), and the combined organic extracts were dried and concentrated. The crude product was chromatographed on silica gel (20% ethyl acetate in hexanes) to provide recovered alkyne (**7**) (72 mg) and the desired diol (**8**) (114 mg, 69%).

δ_H ($CDCl_3$): 5.46 (1H, m, H_3), 2.76, 2.35 (2H, 2 × s, OH), 1.95 (2H, m, H_4), 1.85 (3H, m, C_2−Me), 1.60−1.40 (2H, m, H_5), 1.03, 1.02 (3H, s, C_6−Me). (Both signals were present, but gave a combined integral of three protons.)

δ_C ($CDCl_3$): 134.4, 124.2, 87.5, 85.1, 74.3, 57.8, 37.0, 31.6, 24.1, 23.4, 22.7, 22.5, 19.4.

[$^2H_6$]-(E)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene [$^2H_6$]-(4). To a solution of (**8**) (171 mg) in chloroform (12 mL) was added *p*-toluenesulfonic acid (500 mg). The reaction was heated to 50−55 °C for a total of 18 h. The reaction mixture was diluted with diethyl ether (30 mL) and washed with 10% NaOH solution (2 × 40 mL), water (2 × 40 mL), and brine (2 × 40 mL). After drying and concentrating, the residue was chromatographed on silica (hexanes) three times to afford pure [$^2H_6$]-(**4**) (18 mg, 13%).

δ_H ($CDCl_3$): 6.99 (1H, d J 7.8 Hz, $H_{4'}$), 6.96 (1H, d J 7.8 Hz, $H_{5'}$), 6.61 (1H, d J 15.9 Hz, H_1), 6.22 (1H, d J 15.9 Hz, H_2), 2.28 (3H, s, $C_{6'}$−Me), 2.27 (3H, s, $C_{3'}$−Me), 2.24 (3H, s, $C_{2'}$−Me). (Both signals were present, as a consequence of the mechanism of formation of TPB, but gave a combined integral of three protons.)

MS m/z (%): 178 (40), 177 (51), 176 (11), 163 (71), 162 (100), 161 (40), 160 (45), 159 (51), 157 (7), 148 (31), 147 (51), 146 (44), 145 (73), 144 (89), 143 (47), 142 (15), 132 (16), 131 (22), 130 (20), 118 (13), 117 (15), 116 (9), 93 (7), 79 (8), 65 (5).

Sample Preparation for GC−MS Analysis. 10 mL of wine was transferred to a 22 mL SPME glass vial containing NaCl (2 g). The wine was spiked with [$^2H_6$]-**4** (100 μL of 10 μg/L) (followed by **4** for standard addition curves), the vial was capped, and the headspace was extracted and analyzed by GC−MS. For calculating the concentration of **4** in the wines, replicate standards were prepared at the same time as the wine samples, by adding the same amount of internal standard as above to pH 3.2 model wine (10 mL) containing **4** at 0 or 96 ng/L. These were then used to calculate the relative response factors for **4** and [$^2H_6$]-**4**.

GC−MS Analysis. This was carried out with a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a Gerstel MPS2 autosampler, a Gerstel thermal desorption unit (TDU), and Gerstel programmed temperature vaporization (PTV) inlet (CIS-4). The GC was coupled to a HP 5973N mass spectrometer. The GC was fitted with a 30 m × 0.25 mm i.d., 0.25 μm, ZB-Wax fused silica capillary column (Phenomenex, Sydney, Australia). The carrier gas was helium (BOC Gases, Ultrahigh Purity), flow rate 1.2 mL/min (constant flow). The oven was held at an initial temperature of 80 °C for 1 min, then increased to 170 °C at 4 °C/min, followed by an increase to 250 °C at 50 °C/min, and held at this temperature for 10 min. The Gerstel MPS2 was operated in SPME mode with a 100 μm poly(dimethylsiloxane) (PDMS, red) fiber fitted (Supelco, USA). The sample was extracted for 5 min at 30 °C before being desorbed in the injector for 10 min at 220 °C. For quantification of **4**, mass spectra were recorded in Selected Ion Monitoring (SIM) mode. The ions monitored in SIM runs were: *m*/*z* 128, 142, 157, and 172 for **4** and *m*/*z* 131, 144, 145, 162, 163, 177, and 178 for [$^2H_6$]-**4**. Selected fragment ions were monitored for 25 ms each (dwell time). The ions at *m*/*z* 157 or 172 from the unlabeled compound, and *m*/*z* 178 from the labeled compound, were used for quantification. The other ions were used as qualifiers.

Standard Addition Curves. The method was validated by a series of duplicate standard additions of unlabeled **4** (0, 9.6, 19.2, 48, 96, 192, 403, 806, and 1152 ng/L) to a red wine (bag-in-box dry red wine, 12.5% alcohol, pH 3.47) or a dry white wine (Chenin Blanc, 2002, 11.5% alcohol, pH 3.41) containing 100 ng/L [$^2H_6$]-**4** as the internal standard. Additional data points were used for the white wine standard addition curve at 307, 499, 595, 691, and 960 ng/L. The reproducibility of the analyses was determined at two concentrations: 48 and 499 ng/L for the white wine and 19.2 and 403 ng/L for the red wine, by spiking five replicate aliquots of the same wine with unlabeled **4**. No **4** was detected in the red or white wine prior to spiking.

Stability of Solutions of 4 at −18 °C. Several solutions of unlabeled **4** (9.6 μg/ L in ethanol), prepared over a 5-month period and stored at −18 °C in volumetric glassware, in the absence of light for various times, were checked at one time against the same labeled [$^2H_6$]-**4** solution (10 μg/L in ethanol) in pH 3.2 model wine (by the comparison of the ion ratios). No significant loss of **4** was observed during storage.

Solutions of 4 in Model, White, and Red Wines. The pH of all red wines was adjusted with 30% aqueous (+)-L-tartaric acid to that of a Chenin Blanc (2002, 11.5% alcohol, pH 3.41). 500 ng/L stock solutions (26 μL of 9600 μg/L solution of **4**, diluted to 500 mL with the appropriate medium) were prepared in model wines (buffered at pH 3.0, 3.2, and 3.4), Chenin Blanc (2002, 11.5% alcohol, pH 3.41), bag-in-box dry red wine (2003, 12.5% alcohol, initial pH 3.48), and Cabernet Sauvignon (2001, 13.5% alcohol, initial pH 3.58). Aliquots (15 mL) of these solutions were sealed under nitrogen in 20 mL glass ampules and heated in the absence of light at either 25 or 45 °C. Unspiked samples of the white wine were similarly heated. At various times, ampules (two or three replicates) were removed and extracted and analyzed on the same day by one of the two methods below, depending on the absolute concentration of **4**. Blank samples of all media were analyzed for **4** on the day of preparation, and zero time points were analyzed within a few hours of preparation of the stock solutions.

Extraction of 4 from Hydrolysates and Quantitative GC−MS Analysis. The heated wine samples were analyzed with one or both of the two methods below, depending on the absolute concentration of **4** in the sample, determined by Method 1.

Method 1 (for samples not requiring dilution). [$^2H_6$]-**4** was added to the opened ampule (100 μL or 150 μL of 10 μg/L [$^2H_6$]-**4**), and the contents were stirred well with a Pasteur pipet. The ampule was then covered with aluminum foil and sonicated for 20 min in a waterbath at room temperature, after which time a 10 mL aliquot of the hydrolysate was transferred into a SPME vial containing 2.0 g of NaCl, and the headspace was analyzed by GC−MS.

Method 2 (for samples requiring dilution). [$^2H_6$]-**4** was added to the opened ampule (100 μL or 150 μL of 100 μg/L [$^2H_6$]-**4**), and the contents were stirred well with a Pasteur pipet. The ampule was covered with aluminum foil and sonicated for 20 min in a waterbath at room temperature, after which time the required aliquot of the sample (x mL) was transferred into a SPME vial containing NaCl (2.0 g). The liquid volume in the SPME vial was made up to 10 mL with pH 3.2 model wine ((10 − x) mL), and the headspace was analyzed by GC−MS.

Consumption of 4 in the Presence of Polyphenols. *Red Wine Media.* The pH of a Cabernet Sauvignon wine (2001, 13.5% alcohol, initial pH = 3.58) was adjusted to 3.40 with (+)-L-tartaric acid (30% aqueous solution). A portion of this wine (450 mL) was then stirred with polyvinylpolypyrrolidone (PVPP) (26 g) overnight at room temperature, under nitrogen. The PVPP was removed by centrifugation (8000 rpm, 5 °C for 45 min), and the clear wine was decanted into a fresh container.

Model Polyphenol Systems. Two model polyphenol solutions were prepared in pH 3.4 model wine containing either 0.96 g/L of a grape seed extract (250 mg in 260 mL model wine) (Advanced Grape GrapeEx Technology, extract rich in monomers) or 0.99 g/L of a grape extract (250 mg in 252 mL model wine) (SA Ferco Development Ferco Tannin Vinification, extract rich in tannin polymers). A model wine control (pH 3.4) was also prepared.

Preparation of Solutions of 4. The above media were each spiked with approximately 500 ng/L of **4** (499 ng/L for the red, PVPP-stripped, and model wine; 481 ng/L for grape seed tannin media; 494 ng/L for grape tannin media). Aliquots (15 mL) of these solutions were sealed under nitrogen in 20 mL ampules and heated at either 25 or 45 °C in the absence of light. At various time points, duplicate ampules were removed, extracted, and analyzed by GC−MS, as per Method 1.

RESULTS AND DISCUSSION

Synthesis of Labeled Standard. Initially it was anticipated that a [$^2H_3$]-analogue of **4** would suffice as an internal standard for quantification in wine by GC−MS. However, [$^2H_3$]-**4** was found to be unsuitable as a standard because it did not contain sufficient unique ions that could be used for quantification. Also, it suffered from the interference of coeluting compounds, which contained several of the same ions. Hence, an alternative labeled standard was required; [$^2H_6$]-**4** proved to be an ideal substitute. The synthesis of d_6-**4** is based on the synthesis of unlabeled **4** (*9*) and is outlined in **Figure 2**.

The first three deuterium atoms were incorporated by alkylation of 2,6-dimethylcyclohexanone (**5**) with [$^2H_3$]-methyl iodide. After bromination and elimination, the [$^2H_3$]-enone **6** was treated with ethynylmagnesium chloride to furnish the [$^2H_3$]-alkyne **7**. The remaining deuterium atoms were added in the form of [$^2H_4$]-acetaldehyde to produce the [$^2H_7$]-diol **8**. Treatment of [$^2H_7$]-**8** with *para*-toluenesulfonic acid in chloroform afforded the desired [$^2H_6$]-TPB (d_6-**4**). Because the mechanism of formation of **4** from **8** requires migration of one of the geminal methyl groups, and because each of the two would be expected to migrate with almost equal facility, the final product d_6-**4** is necessarily a mixture (∼50:50) of two isomers, differing in the position of the labeled methyl substituent (**Figure 2**).

Method Development. Previously **4** was quantified using a liquid−liquid extraction technique coupled to GC−MS analysis with [$^2H_8$]-naphthalene as internal standard. While this method was adequate, it suffered from the fact that **4** could only be quantified accurately above 50 ng/L. This is slightly higher than the measured aroma detection threshold in white wine (40 ng/L) (*9*). Consequently, the development of a more sensitive method was deemed appropriate. A poly(dimethylsiloxane) (PDMS) SPME fiber had a high affinity for **4** and proved extremely efficient at extracting this compound via the headspace, so much so that if an ampule that had been previously quantified for **4** was reanalyzed, the residual **4** was close to zero (*15*). This more efficient extraction of **4** translated into higher sensitivity and a significantly lower limit of detection. In contrast to the previously reported liquid extraction method (*9*), the SIDA method developed especially for this compound allowed its accurate quantification with a limit of detection of approximately 10 ng/L. Below this concentration, the presence of **4** could be inferred from GC−MS analysis, but accurate quantification could not be achieved.

Standard addition functions were constructed in both red and white wines. In a young, neutral, white wine (Chenin Blanc, 2002), the ions used to construct the function were m/z 172 for **4** and m/z 178 for [$^2H_6$]-**4**. These ions were chosen based on consideration of their abundance, lack of interference with similar ions belonging to other compounds, and signal-to-noise ratio. Other prominent ions in the mass spectra (m/z 128, 157 for **4**, and m/z 131, 163 for [$^2H_6$]-**4**) were used as qualifiers.

The standard addition function in the young white wine was near-linear over the concentration employed (0−960 ng/L). There was, however, a noticeable, although minor, upward deviation of the slope as the concentration increased. This behavior was also observed when analysis of separately spiked samples was conducted on a subsequent occasion. The final

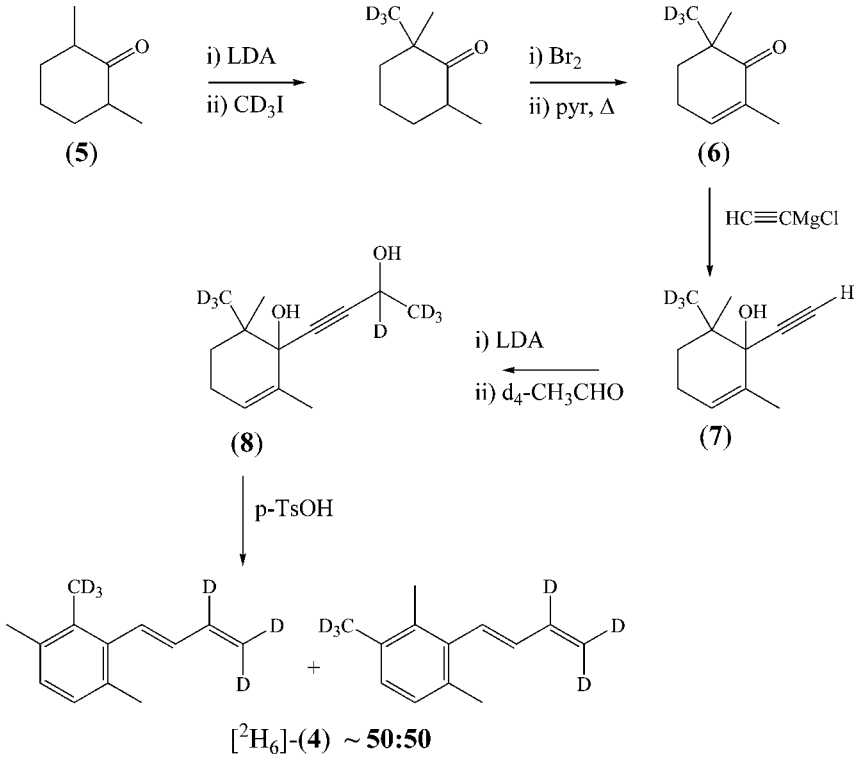

Figure 2. Synthesis of $[^2H_6]$-**4**.

Table 1. Effect of Calibration Concentration Range on Replicate Precision and Accuracy in a Neutral White Wine

concentration range[a]	added [**4**] (ng/L)	measured [**4**] (ng/L)[b]	standard deviation (ng/L)	difference (%)[c]
0–960 ng/L	48.0	43.0	0.5	–10.4
	499	483	18	–3.2
0–499 ng/L	48.0	46.6	0.6	–2.9
	499	523	20	4.8
0–96 ng/L	48.0	48.6	0.6	1.2
	499	546	21	9.4

[a] Quantified with *m*/*z* 172 for **4**, and *m*/*z* 178 for d_6-**4**. [b] Average of five replicates. [c] $[(\mathbf{4}_{measured} - \mathbf{4}_{added})/\mathbf{4}_{added}] \times 100$.

values of the slope and coefficient of determination (R^2) were (slightly) influenced by the choice of concentration range: for the concentration range 0−96 ng/L, these values were $y = 2.22x$, $R^2 = 0.996$; for the range 0−499 ng/L, the values were $y = 2.32x$, $R^2 = 0.994$; and for the full range (0−960 ng/L), the values were $y = 2.51x$, $R^2 = 0.992$. The reasons for this slight deviation from linearity are unknown.

To assess the ramifications of this small deviation from linearity, two spiked solutions of **4** were quantified using all three concentration ranges; the results are collected in **Table 1**. It can be seen that all of the concentrations were measured with a high degree of precision, as evidenced by the small standard deviations. However, in terms of accuracy, the least accurate results were obtained when the spiked concentration and the concentration range employed were disparate. The 48 ng/L spike was underquantified (by 10.4%) when the full (0−960 ng/L) calibration range was employed. Similarly, the 499 ng/L spike was overquantified (by 9.4%) when the narrower (0−96 ng/L) calibration range was employed. The most accurate results were obtained when the calibration range and spiked concentration were comparable; the 48 ng/L spike was quantified with an error of only 1.2% using the 0−96 ng/L range, whereas the 499 ng/L spike was almost equally well quantified using either the 0−960 ng/L range (−3.2% error) or the 0−499 ng/L range (4.8% error). However, the magnitude of these differences ($\leq \pm 10\%$) is not huge and would not be expected to impact significantly. A plot of the logarithm of the concentrations employed against the logarithm of the signal response over the full range gave a slightly better fit ($R^2 = 0.994$) to a linear function. Nevertheless, in the hydrolytic experiments discussed later, it was decided to match the calibration function with the amount of **4** added or expected. In those experiments spiked at 500 ng/L, the calibration range (0−499 ng/L) was employed, whereas those that produced large (>1000 ng/L) amounts of **4** were handled using the full calibration range.

Table 2. Effect of Calibration Concentration Range on Replicate Precision and Accuracy in a Neutral Red Wine

concentration range	added [**4**] (ng/L)	measured [**4**] (ng/L)[c]	standard deviation (ng/L)	difference (%)[d]
0–1152 ng/L[a]	19.2	24.8	0.8	29.2
	403	456	20	13.1
0–403 ng/L[a]	19.2	18.9	0.9	–1.6
	403	431	31	6.9
0–1152 ng/L[b]	19.2	19.6	1.4	2.0
	403	413	19	2.5
0–403 ng/L[b]	19.2	20.2	1.2	5.2
	403	402	21	–0.2

[a] Quantified with *m*/*z* 172 for **4**, and *m*/*z* 178 for d_6-**4**. [b] Quantified with *m*/*z* 157 for **4**, and *m*/*z* 178 for d_6-**4**. [c] Average of five replicates. [d] $[(\mathbf{4}_{measured} - \mathbf{4}_{added})/\mathbf{4}_{added}] \times 100$.

A similar state of affairs was observed for the red wine calibration also (**Table 2**). In addition to the calibration function constructed using the *m*/*z* 172 ion, a second was constructed using the *m*/*z* 157 ion. It was found that this latter ion gave a better signal-to-noise ratio and was not subject to interference with coeluting compounds containing the same ion, as was sometimes encountered with the *m*/*z* 172 ion in this particular medium. In both cases, as was observed in white wine, the lower

concentration spike was best quantified with the lower calibration range, and the higher concentration spike with the higher range.

Occurrence of 4. In our initial description of **4** (*9*), we reported that this compound was found, at various levels, in several white wines, but was absent in six red wines examined. In that initial study, **4** was quantified using [$^2H_8$]-naphthalene as internal standard. We have now applied the SIDA methodology described above for the quantification of **4** in a total of 97 white wines and 12 red wines. As was the case with the earlier quantifications (*9*), **4** could not be detected in any of the red wines analyzed. It was, however, found in all but one of the white wines analyzed. The wines chosen covered a wide range of regions (the wines were exclusively Australian, but sourced from all of the major viticultural regions) and vintages (1993−2003). The white wines studied consisted of Chardonnay (43 wines), Semillon (24 wines), Riesling (23 wines), and miscellaneous varieties (7 wines).

The aroma detection threshold (defined as the geometric mean of the individual best estimate thresholds for 24 panelists) for **4** in a white wine was 40 ng/L, although there was some variation in the sensitivity of individual panelists toward this compound (*9*). **4** was present in all of the Chardonnay wines analyzed, ranging from just detectable (<10 ng/L) to 86 ng/L in a 1997 South Australian Chardonnay. In all, six out of 43 Chardonnay wines (14%) showed levels of **4** at or above 40 ng/L. It appeared to be less prevalent in Riesling wines, with only two out of 23 wines (9%) showing levels at or above 40 ng/L. In contrast to these two varieties, **4** was present, at or above this concentration, in 11 out of 24 Semillon wines (46%) with the highest amount being 233 ng/L in a 2000 South Australian Semillon. **4** was also present in six out of seven miscellaneous varieties, but at low levels. The one wine in which it appeared to be absent, a young bag-in-box Chenin Blanc, was chosen as the white wine medium for stability studies.

4 is an isomer of TDN (**3**), both having the molecular formula $C_{13}H_{16}$. TDN has been shown to be an aged wine component in Riesling wines (*5*), imparting a distinct "kerosene" aroma. It is likely that the presence in TDN of the aromatic ring, which would impart considerable thermodynamic stability, results in this species being an end-product in the hydrolysis of precursor forms (*16*). For similar reasons, **4** might also be expected to be formed during the aging process. To test this hypothesis, two young wines (2002 Chardonnay, 2002 Semillon) were selected for analysis. Both wines (in duplicate) were quantified to give a time zero concentration before aliquots of the wines were sealed in glass ampules and kept in a wine cellar for 6 months before being requantified. In both cases, the concentration of **4** in the ampules had increased over the time of cellaring. For the Chardonnay sample, a zero time concentration of 10 (±1) ng/L had increased to 21 (±1) ng/L, while the Semillon sample showed an increase from 43 (±2) ng/L to 108 (±2) ng/L. These results are consistent with the proposition that **4**, like its isomer TDN, is formed during the aging process. However, it is unclear whether **4** is important to the aroma of very old wines.

Stability of TPB in Various Wine Media. Our initial experiments into the stability of **4** revealed a curious observation. Measurements from wine stored in glass ampules showed that the measured levels of **4** were significantly lower (in one case by as much as 36%) than the spiked values, even at time zero points, for all media employed. Further experiments (*15*) indicated that **4** was being adsorbed onto the glass surface and could be recovered by rinsing the glassware with ethanol. We found that the best results were achieved when labeled standard

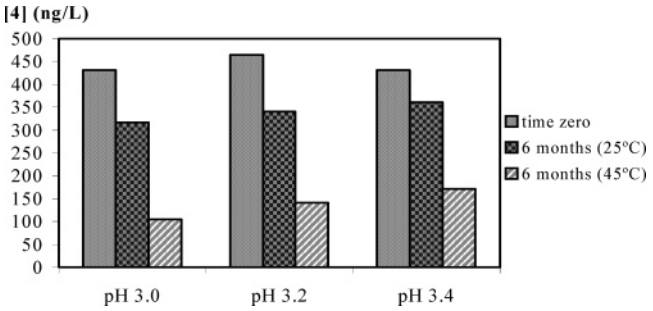

Figure 3. Mean ($n = 2$) **4** remaining in 10% model wine (pH 3.0, 3.2, and 3.4) at 25 and 45 °C after 6 months. All samples were spiked with **4** (500 ng/L) at zero time. All values measured were within ±10 ng/L of the averages shown, except for the 6 month point at 25 °C (pH 3.4), which was within ±20 ng/L.

was added to the ampule under investigation and the whole was sonicated at room temperature for 20 min. Under these conditions, values much closer (mostly to within 10%) to the spiked value were routinely obtained. It is possible that sonication does not necessarily desorb all of the **4** from the glass, but it is likely that sonication results in a distribution of normal and labeled **4** between the free and adsorbed phases, in similar amounts. These discrepancies are substantially smaller than the changes in concentration of **4** experienced over the duration of the hydrolyses. Some of these discrepancies might also result, in part, from rapid reaction with wine components.

The stability of **4** was investigated in three related media: model wine (10% ethanol in water, pH 3.0, 3.2, or 3.4), white wine (dry cask white wine, pH 3.41), and red wine (Cabernet Sauvignon and a bag-in-box red wine, both pH adjusted to 3.41). Studies were conducted at both room temperature (25 °C) and elevated temperature (45 °C).

(a) Model Wine. In model wine, the concentration of **4** (spiked at 500 ng/L) declined at all pH values to give the final values shown in **Figure 3**. As expected, the decrease in concentration was slightly more pronounced at the lower pH values, at both temperatures. At 25 °C, the proportions of **4** remaining after 6 months were 62%, 68%, and 72% of the spiked value at pH 3.0, 3.2, and 3.4, respectively, while at the higher temperature these proportions were 21%, 28%, and 34%, respectively. **4** is a substituted styrene and, in keeping with the chemistry of such compounds, would be expected to display a sensitivity toward acidic conditions. However, other forms of chemical degradation, such as oxidation, cannot be excluded as contributing to its loss in model systems.

(b) White Wine. The results in white wine (**Figure 4**) were not as clear-cut as those in model wine; in this medium, temperature played a critical role in affecting the concentration of **4**. At 25 °C, there was a gradual decrease in the level of **4** (spiked at 500 ng/L) to 47% of its initial value. This value is significantly lower than that observed for model wine (72%) at essentially the same pH. In this latter medium, decreases in concentration were assumed to be a result of acid-catalyzed and/or oxidative degradation of **4**, whereas in a real white wine the loss of **4** would be a combination of such processes plus losses through interactions between **4** and wine specific components. However, it was at the higher temperature that the most interesting observations were made. When heated at 45 °C in white wine, the concentration of **4** increased to high levels (>2000 ng/L) for both the spiked and the unspiked samples. In the spiked sample, the concentration of **4** decreased slightly (minimum value 364 ng/L after 8 days) before increasing rapidly to its final value of 2205 ng/L after 6 months. Indeed, after the

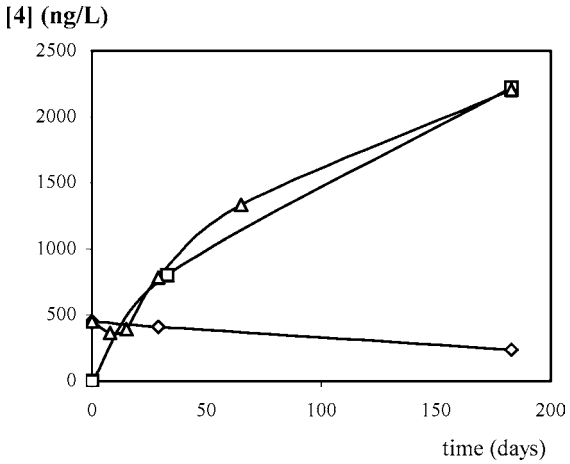

Figure 4. Stability of **4** in a neutral white wine, at 25 and 45 °C. Those samples labeled as spiked were spiked with **4** (500 ng/L) at zero time. All values measured were within ±25 ng/L of the averages shown. Duplicate samples were analyzed for each time point [◇ spiked, 25 °C; □ unspiked, 25 °C; △ spiked, 45 °C].

first month, the increase in concentration of **4** in both the spiked and the unspiked samples was virtually identical. These data clearly indicate the presence of precursor forms of **4**, which under elevated temperatures can realize large amounts of this odorant. They also demonstrate that the observed concentration of **4** is the result of a fine balance between its generation (from precursor or precursors unknown) and its consumption (by acid-catalyzed degradation and interaction with other wine components). At lower temperatures, consumption prevails, but at higher temperatures the generation of **4** completely dominates.

(c) Red Wine. Over 6 months at 25 °C, the level of **4** (spiked at 500 ng/L) decreased (**Figure 5A**) to just 3% of its initial value in a pH adjusted Cabernet wine, and to 5% in a bag-in-box red wine. After 6 months at 45 °C, the levels were 14% and 18%, respectively. This is in contrast to the results in white wine (**Figure 4**), where after 6 months at the lower temperature, levels of **4** had decreased to 43% of the initial value, and at 45 °C the concentration of **4** had actually increased approximately 5-fold. It is clear that either red wine is a much more destructive environment toward this compound than white wine, or it contains lower amounts of precursors. It is important to point out that both red wines employed in this study were pH adjusted to the natural level of the white wine, pH 3.41. **Figure 5B** shows the detailed change in the concentration of **4** in a spiked red wine at 45 °C. There is a rapid decrease in concentration (to ∼10% of the original value) followed by a slow increase in the concentration of **4**. In white wines, the rates of these two processes were the converse. This is also consistent with the proposition that either certain red wine specific components are responsible for the rapid consumption of **4** in red wines, or it contains lower amounts of precursor forms, or a combination of both.

Effect of Polyphenol Content on TPB Concentration. To test the hypothesis that the much higher polyphenol content was a major contributor to the reduction in concentration of **4** in red wines, the stability of **4** was examined in both a natural red wine and the same red wine that had been stripped of its polyphenols by treatment with PVPP (*17*, *18*). The red wine used in this experiment was the same Cabernet Sauvignon used for the red wine stability experiments. After 25 days at 45 °C, the concentration of TPB in the untreated red wine (**Figure 6**) had fallen to approximately 12% of its original value. In contrast

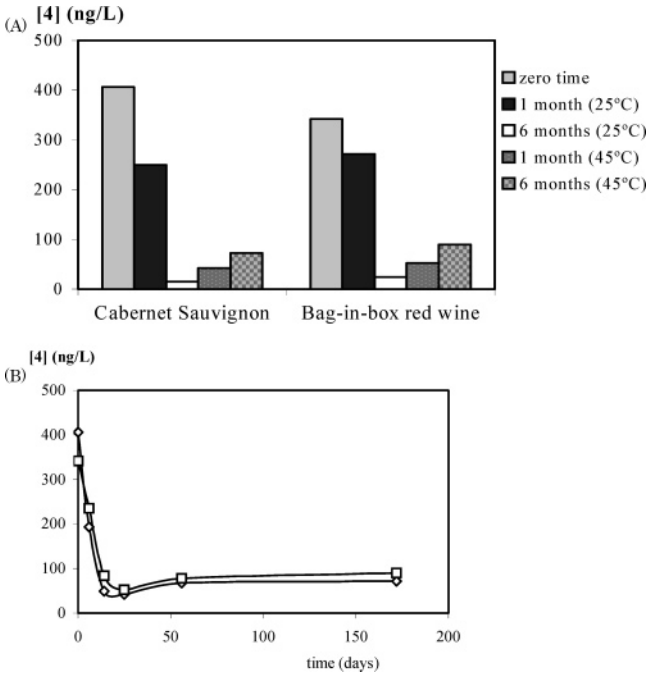

Figure 5. (**A**) Mean ($n = 2$) **4** remaining in red wine at 25 and 45 °C after 6 months. All samples were spiked with **4** (500 ng/L) at zero time. All values measured were within ±10 ng/L of the averages shown, except for the zero point (bag-in-box) which was within ±50 ng/L. (**B**) Stability of **4** in a neutral red wine at 45 °C. All samples were spiked with **4** (500 ng/L) at zero time. All values measured were within ±10 ng/L of the averages shown, except for the zero point (bag-in-box) which was within ±50 ng/L. Duplicate samples were analyzed for each time point [◇ Cabernet Sauvignon; □ bag-in-box red wine].

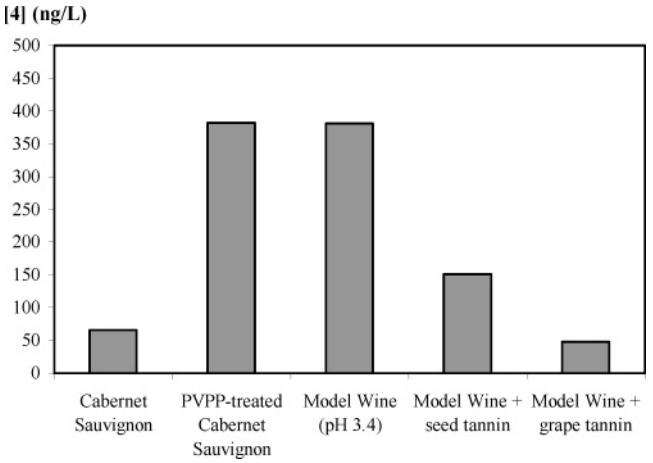

Figure 6. Mean ($n = 2$) **4** remaining in Cabernet Sauvignon, PVPP-stripped Cabernet Sauvignon, and in model wine containing either seed tannins (0.96 g/L) or grape tannins (0.99 g/L) after 25 days at 45 °C. All samples were spiked with **4** (∼500 ng/L) at zero time. All values measured were within ±20 ng/L of the averages shown.

to this result, the wine that had been stripped of its polyphenol content proved much less deleterious toward **4**.

The stability of **4** was also investigated in two model polyphenol systems, both of grape origin. These were a seed extract, rich in monomeric polyphenol flavanols, and a grape extract, rich in higher molecular weight polymeric tannins. Both of these groups of polyphenols are found at high levels in red wines due to fermentation on skins and seeds, in comparison to white wines where in general skin contact is avoided and only the clear juice is fermented.

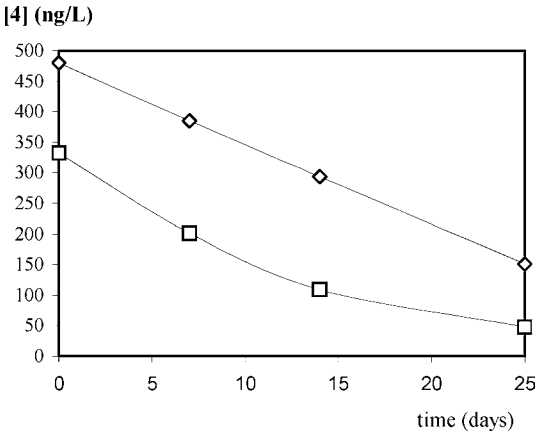

Figure 7. Stability of **4** in model wine (pH 3.4) containing either seed tannins (0.96 g/L) or grape tannins (0.99 g/L) over 25 days at 45°C. Both samples were spiked with **4** (~500 ng/L) at zero time. All values measured were within ±20 ng/L of the averages shown. Duplicate samples were analyzed for each time point [◇ seed tannin extract; □ grape tannin extract].

Model wine systems containing either grape tannin extract or seed tannin extract (both at ~1 g/L) were spiked with **4** (~500 ng/L) and heated at 45 °C (**Figure 7**). The system containing the grape tannins (approximately 10% remaining) showed a greater consumption of **4** than did the system containing seed tannins (approximately 30% remaining) after 25 days. However, the rate of consumption was very similar for both systems (**Figure 7**). The discrepancy in absolute amounts occurred in the time between preparation of the ampules and quantification of the zero time point, suggesting the presence in the grape tannin extract of a component or components that react immediately and rapidly with **4**. We feel that the main effect on the concentration of **4** in red wines is likely to be one of increased reaction, relative to the white wines, rather than a manifestation of lower precursor concentrations. In support of this, the original isolation and characterization of **4** was performed on bulk glycoside extracts sourced from either Cabernet Sauvignon or Shiraz fruit or leaves, which produced levels of **4** comparable to those produced from similar extracts from white varieties (*15*).

The amount of **4** present in wines is a result of various concurrent chemical transformations. It has been shown that **4** is unstable at all temperatures under acidic conditions, being more unstable at lower pH. Some wine components contribute to the consumption of **4**, primarily polyphenols as demonstrated here for red wine. This means that model systems, where only acid-catalyzed and/or oxidative degradation contributes to the consumption of **4**, do not sufficiently represent its fate in wine. The amount of **4** found in wines is actually the result of a balance between its formation from certain precursors, its consumption by other wine components, its consumption by the packaging (glass and/or closure, (*15*)), and its degradation. In addition, the accelerated hydrolysis conditions have also been shown to misrepresent the amount of **4** that could be obtained in wine under natural maturation conditions. This is because the rates of formation and consumption of **4** can be affected in different ways by increases in temperature. For example, the concentration of **4** only decreased in red and white wines at room temperature, with no evidence of any production over 6 months, as was observed at 45 °C. In addition, the accelerated hydrolysis conditions generated amounts of **4** far in excess of those found in wine, especially when conducted in the absence of wine polyphenols. The observations emphasize the importance of temperature in obtaining realistic results and evaluating the actual role of **4** as a contributor to the aged-wine aroma under normal wine aging conditions.

The formation of **4** in hydrolysates of reverse phase extracts of red grapes is a case in point. Sensory studies on red fruit hydrolysates including those of Abbott et al. (*19*) and Francis et al. (*20*), which used crude glycosidic extracts, employ solutions in which not all wine polyphenols are necessarily present. Such hydrolysates can contain **4** at concentrations above 1000 ng/L (*15*), whereas commercial red wine appears to contain no detectable **4** at all. The interpretation of such sensory data should be conducted with caution.

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