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# Synthesis of labelled [$^2H_4$]β-damascenone, [$^2H_2$]2-methoxy-3-isobutylpyrazine, [$^2H_3$]α-ionone, and [$^2H_3$]β-ionone, for quantification in grapes, juices and wines[1]

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**Abstract**

The syntheses of four isotopically labelled aroma compounds, [$^2H_4$]β-damascenone, [$^2H_2$]2-methoxy-3-isobutylpyrazine, [$^2H_3$]α-ionone, and [$^2H_3$]β-ionone, whose natural analogues are commonly found in grapes and wines, have each been achieved in a one step reaction. These compounds were used to establish analytical procedures to quantify the natural analogues in grape juices and wines, by gas chromatography–mass spectroscopy. The concentration of β-damascenone, 2-methoxy-3-isobutylpyrazine, α-ionone and β-ionone in a 1996 St-Emilion Merlot wine was, 2.9 μg $l^{-1}$, 11.4 ng $l^{-1}$, 27.9 ng $l^{-1}$ and 84.1 ng $l^{-1}$ respectively, with a coefficient of variance below 5% for each analyte. 

*Keywords:* Grape juice; Wine; Stable isotope dilution; β-Damascenone; 2-Methoxy-3-isobutylpyrazine; Ionone

## 1. Introduction.

The important odiferous compound β-damascenone, was first isolated from Bulgarian rose oil by Demole et al. in 1973 [1] and first identified in grape and wine by Schreier and Drawert [2]. This compound belongs to a family called the rose ketones, whose importance has seen a ten tonnes annual production in the flavour and fragrance industry [3]. It has since been detected in many other fruits and foods from the plant kingdom. β-Damascenone is believed to originate from the breakdown of the carotenoid neoxanthin by a complex pathway [4,5]. It has an odour threshold of 0.002 ppb in water [6] and has been described as flowery and ionone like [7] although concentration plays an important role in its perceived character [7]. 2-Methoxy-3-isobutylpyrazine was first detected in wines and identified in our laboratories [8]. It has an odour threshold of 0.002 ppb in water and has been described as the principal compound responsible for the odour of bell peppers [9]. α- and β-Ionone are also commonly found as secondary metabolites from enzymatic oxidation of their respective carotenoids [10], and have significant uses in the flavour field [11]. α-Ionone has multiple descriptors of violets, floral, woody and fruity whereas β-ionone has been described as woody, dry fruit and raspberry-like [12].

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[1]Dedicated to Dr. Claude Bayonove on the occasion of his retirement.

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α- and β-Ionone have odour thresholds of 0.4 ppb [13] and 0.007 ppb in water [7] respectively.

The measurement of flavour development during grape maturation, is important to the wine maker, especially during the period of harvest. Such measurement can be used to map seasonal differences (from a year to year basis), regional differences, soil differences and enable viticulturists to determine why certain trends have occurred and plan accordingly. However, β-damascenone, 2-methoxy-3-isobutylpyrazine, α-ionone, and β-ionone occur in grape juices (and young wines), at low concentrations compared to other volatiles making quantitative analysis difficult.

An increase in the use of stable isotope labelled aroma compounds, as analytical standards, for quantification purposes, has been seen in the last decade eg. [14–19]. The simplicity and accuracy of this method in the determination of trace amounts of compound is its major attraction to analytical scientists. We report here the synthesis of stable isotope labelled analogues, [$^2H_4$]β-damascenone, [$^2H_2$]2-methoxy3-isobutylpyrazine, [$^2H_3$]α-ionone and [$^2H_3$]β-ionone and their use in quantitative measurement of their natural analogues.

## 2. Experimental

### 2.1. Instrumental analysis

GC/MS analysis was carried out using a Hewlett Packard HP gas chromatograph 5890 series II fitted with a 50 m fused-silica column (0.25 mm i. d. and 0.2 μm film thickness), coated with Carbowax 20 M. The splitless/split injection port was heated to 200°C. The split vent was opened after 30 s. The carrier gas was Helium 55 Norme Aga ®, and the pressure was 170 kPa with a linear velocity 40 cm $s^{-1}$. at 40°C. The temperature program was 60°C (for 1 min), then increased at 4°C $min^{-1}$ to 220°C and held at this temperature for a further 20 min. The GC instrument was coupled to a 5970 B mass selective detector and a 5990 A MS chemstation (HP-UX) The electron impact (EI) energy was 70 eV. The quadrupole temperature was set at 250°C.

For the determination of β-damascenone, 2-methoxy-3-isobutylpyrazine, α- and β-ionone using selective ion monitoring scans (SIMS) the following ions were monitored: β-damascenone, $m/z$=69, 175, 190 and for [$^2H_4$]β-damascenone, $m/z$=73, 179, 193, 194; ions 69, 73 were used for quantification and ions 175, 179, 190, 193, 194 as qualifiers; 2-Methoxy-3-isobutylpyrazine, $m/z$=124, 151, 166 and for [$^2H_2$]2-methoxy-3-isobutyl-pyrazine $m/z$= 126, 153 and 168; ions 124, 126 were used for quantification and ions 151, 153, 166, 168 as qualifiers; β-Ionone, $m/z$=177, 192 and [$^2H_3$]β-ionone $m/z$=180, 195; ions 177, 180 were used for quantification and ions 192, 195 as qualifiers; α-Ionone, $m/z$=136, 192 and [$^2H_3$]α-ionone $m/z$=139, 195; ions 136, 139 were used for quantification and ions 192, 195, as qualifiers.

A Varian 400 MHz NMR spectrometer was used to determine proton spectra. Sample spectra were for deuterated chloroform solutions and C*H*$Cl_3$ (7.25 ppm $\delta$) was used as reference. Boiling points were determined using a Kugelrohr apparatus (Buchi GKR 51) and refer to the oven temperatures of their equipment.

### 2.2. Reagents and other materials

β-Damascenone was obtained from Firmenich, Switzerland, and was further purified by flash chromatography [20] using silica gel 60 (Aldrich 230–400 mesh, gradient of pentane/diethyl ether as solvent, followed by microdistillation (Kugelrohr) prior to use. α-Ionone, β-ionone, 2-methoxy-3-isobutylpyrazine, deuterium oxide ($D_2O$), dry tetrahydrofuran, deuterated sulphuric acid ($D_2SO_4$, 98 wt.% solution in $D_2O$), lithium diisopropylamide mono-(tetrahydrofuran) complex solution, 1.5 *M* in cyclohexane, sodium hydride 60% dispersion in mineral oil, *n*-butyllithium 2.0 *M* solution in cyclohexane, were all purchased from Aldrich Chemical Company Inc. Diethyl ether, pentane and dichloromethane were ultra-pure grade, obtained from SDS, 13124, Peypin, France. All glassware was meticulously cleaned by washing several times with dichloromethane, absolute alcohol and then high purity Milli-Q water, followed by oven baking at 100°C prior to use.

*2.2.1. Preparation of [$^2H_4$]β-damascenone*

In a flame dried ampoule under $N_2$, β-damascenone (26 mg, 1.3 mmol) in dry tetrahydrofuran (2.8 ml),) was added to a mixture of deuterium oxide (0.25 ml, 13.9 mmol), catalytic amount of *n*-butyllithium in cyclohexane, with cautious addition, (2 *M*, 0.05 ml, 0.1 mmol) and the ampoule was sealed. The reaction mixture was heated at 60°C for 4 days, was then extracted with diethyl ether (2×30 ml), the ethereal layer was dried over magnesium sulphate, and evaporated to yield a yellow oil (20 mg). The oil was directly chromatographed using flash chromatography (2% diethylether/pentane), followed by microdistillation using Kugelrohr, (90°C at 0.01 mm Hg) to yield β-damascenone-[$^2H_4$], (6 mg, 23%); $^1$H NMR $\delta$ 1.05 (s, C-1–$CH_3$), 1.64 (s, C-5–$CH_3$), 2.11 (dd, $J$=4.1, 1.7 Hz, (H-2)$_2$), 5.81 (d, $J$=9.3 Hz, H-4), 5.85 (dt, $J$=9.3, 4.1 Hz, H-3), 6.83 (βr s, H-9). Mass spectrum see Fig. 1a. A

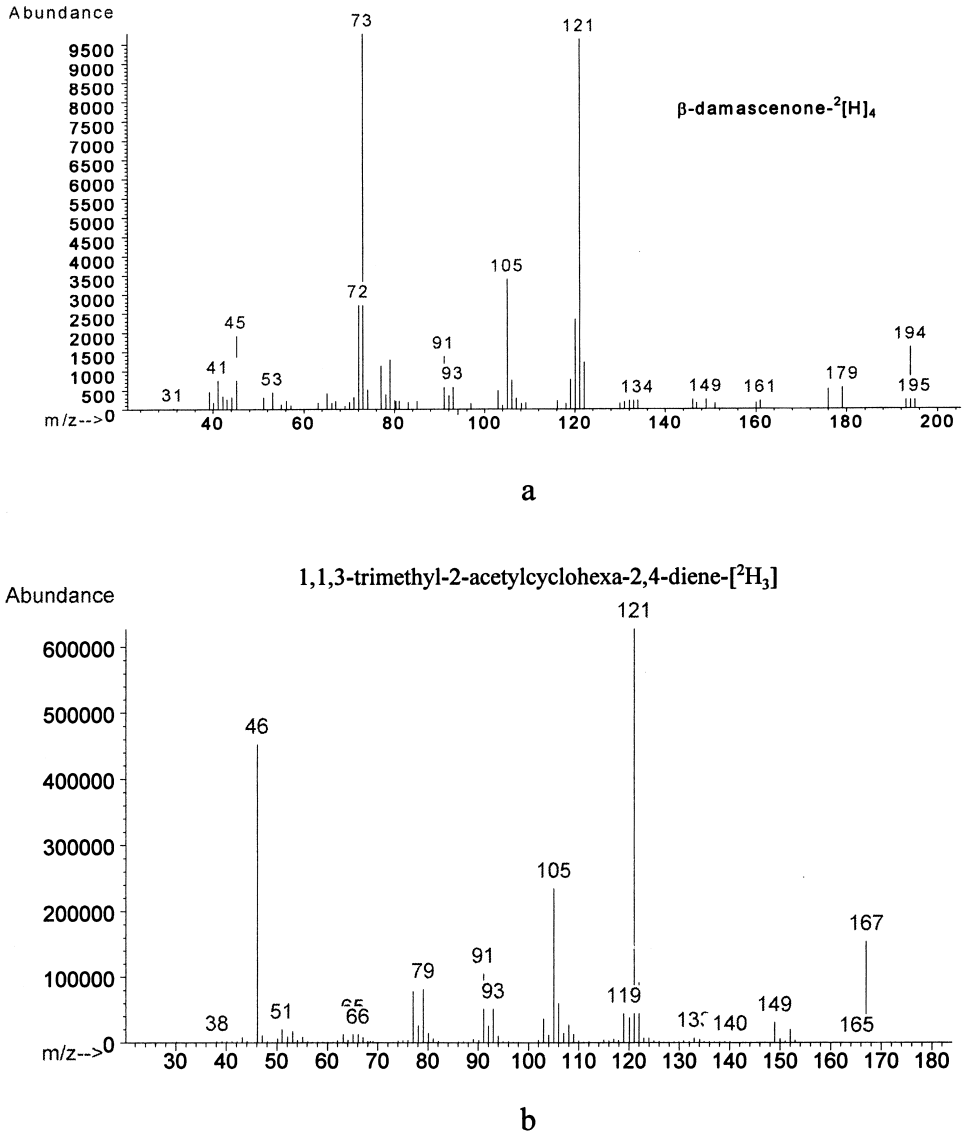


Fig. 1. Mass spectra of (a) [$^2H_4$]β-damascenone and (b) [$^2H_3$]1,1,3-trimethyl-2-acetylcyclohexa-2,4-diene.

by-product was separated and tentatively identified by mass spectrometry as [$^2H_3$]1,1,3-trimethyl-2-acetylcyclohexa-2,4-diene (Fig. 1b).

*2.2.2. Preparation of [$^2H_2$]2-methoxy-3-isobutylpyrazine*

In a flame dried ampoule under $N_2$, 2-methoxy-3-isobutylpyrazine (100 mg, 0.6 mmol) was added to solution of deuterated sulphuric acid (30 μl) in deuterium oxide (3 ml, 0.17 mol), the ampoule was sealed and the mixture heated at 120°C for 24 h. Upon heating and after a short period, a homogeneous solution was formed. The slightly pink reaction mixture was then extracted with pentane, the pentane layer was dried over magnesium sulphate, and evaporated to yield 100 mg of a pale pink oil. The oil was distilled, (Kugelrohr 80–85°C at 0.01 mm Hg) to yield 86 mg of [$^2H_2$]2-methoxy-3-isobutylpyrazine, 85%; $^1$H NMR $\delta$ 0.93, 0.94 (s, geminal $CH_3$), 2.15 (br septet, $J$=6.8 Hz, CH), 3.96 (s, $OCH_3$), 7.92(d, $J$=2.4 Hz, H-5), 8.03 (d, $J$=2.4 Hz, H-6). Mass Spectrum see Fig. 3.

*2.2.3. Synthesis of [$^2H_3$]β-ionone*

Under strictly anhydrous conditions and under $N_2$, β-ionone (40 mg, 0.21 mmol) in dry tetrahydrofuran (1 ml) was added to a mixture of deuterium oxide (1 ml, 0.056 mol), catalytic amount of sodium hydride (10 mg, 0.4 mmol) and was stirred at room temperature overnight (24 h). The solution was extracted with diethyl ether (1×20 ml), the ethereal layer was dried over magnesium sulphate, filtered and evaporated to yield 20 mg of β-ionone-[$^2H_3$]. Microdistillation (Kugelrohr, 135°C/13 mm Hg) yielded 12 mg of β-ionone-[$^2H_3$] (29%) with deuterium incorporation at the C-10; $^1$H-NMR $\delta$ 1.07 (s, C-1–$CH_3$), 1.48 (m, (H-2)$_2$), 1.62 (m, (H-3)$_2$), 1.76 (s, C-5–$CH_3$), 2.07 (br t, $J$=6.2 Hz, (H-4)$_2$), 6.11 ($\delta$, $J$=16.5 Hz, H-8), 7.27 (d, $J$=16.5 Hz, H-7). For large scale preparation, the amount of deuterium oxide ($D_2O$) used could be reduced by conducting the reaction at 50°C for longer periods.

The mass spectra of the compound is the same with that showed by Thomas et al. [21].

*2.2.4. Synthesis of [$^2H_3$]α-ionone*

In a flame dried ampoule under $N_2$, α-ionone (300 mg, 1.56 mmol) in dry tetrahydrofuran (1 ml) was added to a mixture of deuterium oxide (5 ml, 0.28 mol), a catalytic amount of *n*-butyllithium in cyclohexane (2 *M*, 0.2 ml, 0.4 mmol). The ampoule was sealed and allowed to stand at 60°C for 4 days. The mixture was then extracted with diethyl ether (2×20 ml), the ethereal layer was washed with dilute acid (tartaric acid solution adjusted to pH 2.8, 10 ml), separated, dried over magnesium sulphate, filtered and evaporated to yield 100 mg of labelled α-ionone-[$^2H_3$]. Purification by flash chromatography, (2% diethyl ether/pentane) followed by microdistillation (Kugelrohr, 140°C/13 mm Hg) yielded 35 mg (11%); $^1$H-NMR $\delta$ 0.86, 0.93 (s, C-1–$CH_3$), 1.22, 1.45 (m, (H-2)$_2$), 1.56 (s, C-5–$CH_3$), 2.05 (m, (H-3)$_2$), 2.29 (d, $J$=9.9 Hz, H-6), 5.51 (m, H-4), 6.05 (d, $J$=15.7 Hz, H-8), 6.62 (dd, $J$=15.7, 9.3 Hz, H-7).

The mass spectra of the compound is the same with that showed by Thomas et al. [21].

*2.2.5. Stability of internal standards.*

General procedure: Internal standards were dissolved in 12% ethanol/water and pH adjusted to 2.8 (lowest likely wine pH) and allowed to stir at room temperature for 24 h. Extraction with diethyl ether, followed by concentration and GC–MS analysis was carried out to check if any exchange had occurred.

*2.3. Linearity and limit of quantification*

Calibration curves were generated for each of β-damascenone, 2-methoxy-3-isobutylpyrazine, α-ionone, and β-ionone. A standard solution of the analyte was dissolved in 100% ethanol, and subsequent serial dilutions were done in an ethereal solution followed by addition of the internal standard.

β-Damascenone: Integrated peak area ratios (peak area of β-damascenone 69/peak area of I.S. 73) were calculated and plotted against the concentration ratios (ng β-damascenone/5.8 μg I.S). The resultant curve was linear [response ratio=(0.3604× concentration ratio)−0.0022] with a coefficient of determination ($r^2$) of 1.000 at the following concentrations: 15.25, 30.5, 61, 122, 213.5 and 300.5 ng (three replicate analyses at each concentration). Quantification was reliable down to 10 ng $l^{-1}$ with

an estimated signal to noise ratio of 3:1 for a red 1996 Bordeaux wine.

2-Methoxy-3-isobutylpyrazine: Integrated peak area ratios (peak area of 2-methoxy-3-isobutylpyrazine 124/peak area of I.S. 126) were calculated and plotted against the concentration ratios (ng 2-methoxy-3-isobutylpyrazine/500 ng I.S). The resultant curve was linear [response ratio= (0.6988×concentration ratio)−0.0207] with a coefficient of determination ($r^2$) of 0.999 at the following concentrations: 6.04, 12.08, 24.16, 48.32, 84.56 and 120.8 ng (three replicate analyses at each concentration). Quantification was reliable down to 2 ng $l^{-1}$ with an estimated signal to noise ratio of 3:1 for a red 1996 Bordeaux wine.

β-Ionone : Integrated peak area ratios (peak area of β ionone 177 /peak area of I.S. 180) were calculated and plotted against the concentration ratios (ng β-ionone/285 ng I.S). The resultant curve was linear [response ratio=(0.2477×concentration ratio)−0.0031] with a coefficient of determination ($r^2$) of 1.000 at the following concentrations: 10.05, 20.11, 40.22, 80.44, 140.77 and 201.10 ng (three replicate analyses at each concentration). Quantification was reliable down to 10 ng $l^{-1}$ with an estimated signal to noise ratio of 3:1 for a red 1996 Bordeaux wine.

α-Ionone: Integrated peak area ratios (peak area of α ionone 136/peak area of I.S. 139) were calculated and plotted against the concentration ratios (ng β-ionone/2.025 μg I.S). The resultant curve was linear [response ratio=(1.626×concentration ratio)+ 0.0116] with a coefficient of determination ($r^2$) of 0.999 at the following concentrations: 7.65, 15.3, 30.6, 61.20, 107.11 and 153.01 ng (three replicate analyses at each concentration). Quantification was reliable down to 5 ng $l^{-1}$ with an estimated signal to noise ratio of 3:1 for a red 1996 Bordeaux wine.

### 2.4. Preparation of sample for analysis of wines

Two hundred milliliters of a wine was spiked with 500 μl of an anhydrous alcohol solution containing the internal standards [$^2H_4$]β-damascenone (11.6 μg $ml^{-1}$), [$^2H_2$]2-methoxy-3-isobutyl pyrazine (1μg $ml^{-1}$), [$^2H_3$]β-ionone (0.57 μg $ml^{-1}$) and [$^2H_3$]α-ionone (4.05 μg $l^{-1}$) by calibrated microliter syringe (SGE, 500 μl). The wine sample was placed in a flask, stoppered and stirred for 10 min allowing equilibration of the medium. This was then placed in a 500 ml flask and was stirred with ether (3×25 ml) for 5 min. The ethereal layers were separated, combined, centrifuged, dried over $Na_2SO_4$ and concentrated to a volume of 5 ml by rotary evaporation. The sample was further concentrated under a nitrogen stream to approximately 200 μl. The whole procedure was repeated three times to check the coefficient of variance (C.V.) of the analytical method.

## 3. Results and discussion

Literature procedures exist for the synthesis of labelled [$^2H_6$]β-damascenone [22], and [$^2H_3$]2-methoxy-3-isobutylpyrazine [15,16]. These procedures however are complex, and not suitable for many laboratories and so alternative syntheses were sought. For α- and β-ionone, syntheses of the labelled analogues have been documented and modified in this work [21,23].

### 3.1. [$^2H_4$]β-Damascenone

[$^2H_4$]β-Damascenone was prepared by equilibrating β-damascenone in a two-phase solvent system in deuterium oxide/tetrahydrofuran under basic conditions at 60°C. The reaction was monitored by mass spectrometry and found to be near-complete in 4 days. The incorporation of four deuterium atoms is seen in the side chain of the megastigmane skeleton. In the $^1H$ NMR spectrum, the signals for H-8 ($\delta$= 6.19 ppm, dq, $J$=15.7, 1.7 Hz) and H-10 ($\delta$=1.93 ppm, dd, $J$=7, 1.7 Hz), (in the megastigmane numbering system [24]), were completely removed and the multiplet for H-9 collapsed to a broad singlet, the deuterium coupling being less than the corresponding hydrogen coupling by a factor of about 6.5. The mass spectrum (Fig. 1a), showed a base peak of $m/z$ 73 consistent with fragmentation of the side arm.

The reaction can be monitored and the level of deuterium incorporation regulated with time. At elevated temperatures, we found that many by-products were formed including a retroaldol product

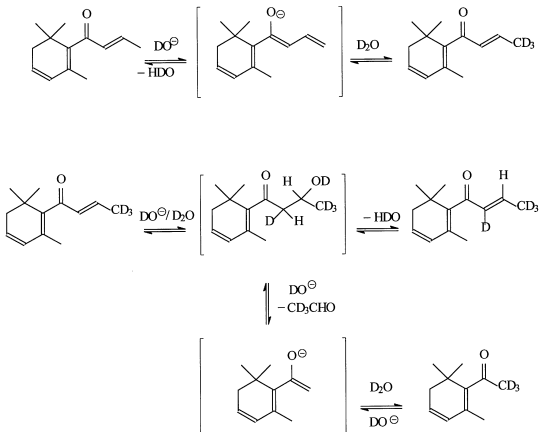

Fig. 2. Synthesis of [$^2H_4$]β-damascenone and of its by-product [$^2H_3$]1,1,3-trimethyl-2-acetylcyclohexa-2,4-diene.

tentatively identified by mass spectroscopy (Fig. 1b) as [$^2H_3$]1,1,3-trimethyl-2-acetylcyclohexa-2,4-diene, labelled on the side chain (fragment ion $CD_3C{\equiv}O^+$ at $m/z=46$ and trimethylcyclohexadiene fragment ion at $m/z=121$). This compound exhibited a powerful eucalyptus/menthol odour. The first step of this side-reaction, addition of $D_2O$ on the double bond of β-damascenone [25,26], explained the labelling on the C8 (Fig. 2).

### *3.2. [$^2H_2$]2-Methoxy-3-isobutylpyrazine*

Imine–enamine chemistry under acidic conditions was employed in order to synthesise [$^2H_2$]2-methoxy-3-isobutylpyrazine. This is a relatively clean, high yielding reaction with only one product being obtained. Surprisingly, at room temperature two layers were formed, 2-methoxy-3-isobutylpyrazine being insoluble in aqueous acid under these conditions, but upon heating a homogeneous solution was formed. After workup, complete incorporation of two deuteriums in the isobutyl chain occurred (Fig. 3). The $^1H$ NMR showed the disappearance of the doublet corresponding to the benzylic protons at 2.68 ppm ($J=7$ Hz) and the collapse of the methine multiplet ($J=2.16$ ppm) to a septet. Indeed the methine multiplet of the unlabelled pyrazine, *M* part of the $A_6MX_2$ system, with nine lines involving the similar coupling constants $J_{AM}{\sim}J_{XM}{\sim}7$ Hz, was changed to a septet with broad lines due to the residual coupling of the methine proton with the benzylic deuteriums.

Analytical protocols proposed for the analysis of 2-methoxy-3-isobutylpyrazine in wine or grape media, by SIDA, are complex [15]. One method requires the use of ion-exchange resin to trap 2-methoxy-3-isobutylpyrazine (which was expected to act as a volatile base) overnight in the wine distillate,

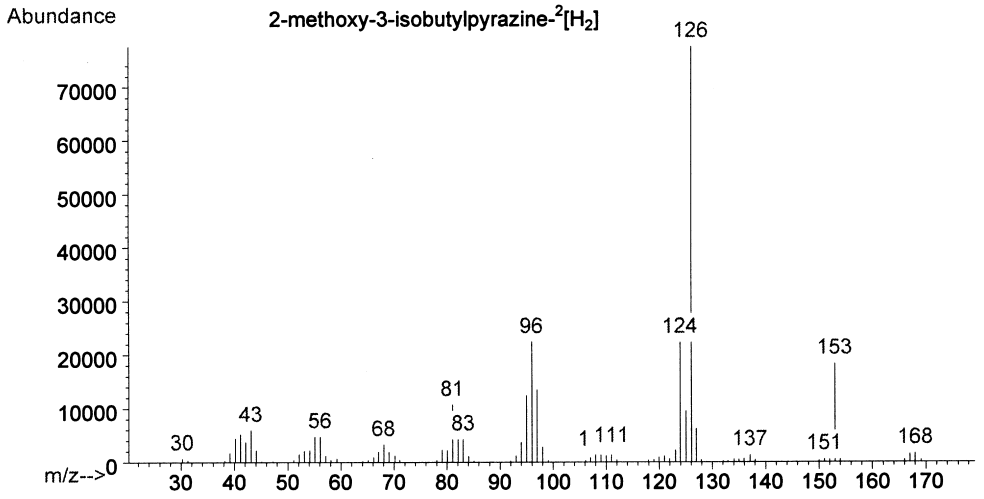


Fig. 3. Mass spectra of [$^2H_2$]2-methoxy-3-isobutylpyrazine.

followed by extraction of this compound with water at high pH (10), saturation of the aqueous layer with sodium chloride and extraction with dichloromethane. The dichloromethane layer was then concentrated down under a flow of nitrogen for 6–7 h to a final volume of 10–15 μl. An alternative procedure employed head space analysis with the effluent filtered through the ion-exchange resin before repetition of the treatment to resin was performed as described above. In both cases, analysis takes approximately two days.

In our hands, a simple diethyl ether extraction at wine pH followed by concentration which can be performed in less than one hour was adequate. Indeed, as shown by the work up of the deuterium exchange reaction, it was not necessary to basify the wine prior to the extraction of the pyrazine. It is important to recognise that analytical methods for 2-methoxy-3-isobutylpyrazine should treat the compound as neutral rather than as a base.

### *3.3. [$^2H_3$]β-ionone and [$^2H_3$]α-ionone*

Treatment of β-ionone in a two phase system incorporating 0.7% sodium deuteroxide in deuterium oxide/tetrahydrofuran mixture (1:1) overnight at room temperature yielded the β-ionone-[$^2H_3$], labelled at the C10 position in the side chain, (megastigmane numbering system [22]), as confirmed by $^1$H nmr (disappearance of the singlet for C10–$CH_3$ at 2.3 ppm). When α-ionone was treated under the same conditions, however, only the C6 hydrogen was exchanged. The deuterium readily back exchanged under acidic conditions to regenerate the unlabelled α-ionone. The lability of the allylic C6 hydrogen is known, as optically active α-ionone can be racemized under these conditions [27]. However, at 60°C and after four days, complete deuterium exchange was seen to give [$^2H_4$]α-ionone. Under acidic conditions the C6 deuterium readily back exchanged giving rise to the corresponding (and stable) $^2H_3$ analogue ([$^2H_3$]α-ionone at the C10 position) as confirmed by both $^1$H nmr (disappearance of the singlet at 2.25 ppm) and mass spectroscopy. The $^2H_3$ analogue was preferred to the $^2H_4$ as an internal standard, as there was less likely of back exchange during wine analysis.

### *3.4. Stability of labelled internal standards at wine pH*

All of the labelled aroma compounds synthesised were treated in a model wine consisting of 12% ethanol in water at pH 2.8 (lowest likely pH in wine) for 24 h, at room temperature and were found to be completely stable without undergoing proton exchange.

### *3.5. Analysis of 1996 St Emilion Merlot*

Application of the analytical method to a Merlot wine gave the data shown in Table 1. The coefficient of variance in β-damascenone concentration over three replicates was 0.8%. Similarly the coefficient of variance was below 5%, for each of the other compounds.

## 4. Conclusion

Equilibration of acidic protons under basic conditions is the simplest way of preparing deuterated labelled analogues of aroma active ketones such as β-damascenone and the ionones (α- and β-). The synthesis of labelled 2-methoxy-3-isobutylpyrazine-[$^2H_2$] by imine–enamine chemistry under acidic conditions is a new procedure which can be applied to all pyrazines which have benzylic hydrogens.

Table 1
Concentrations (ng $l^{-1}$) of the four volatile compounds in a St Emilion Merlot wine (vintage 1996)

| | β-Damascenone | α-Ionone | β-Ionone | 2-Methoxy-3-isobutyl-pyrazine |
|---|---|---|---|---|
| Mean Value of 3 replicates | 2863 | 28 | 84 | 11 |
| C.V. (%) | 0.8 | 4.8 | 4.1 | 3.4 |

No sophisticated laboratory materials are required for these simple one step reactions and these can be performed in any reasonably equipped laboratory. The labelled products are stable at wine pH. Application of SIDA with GC–MS in SIMS mode to a Merlot wine has illustrated the versatility in measuring minute quantities accurately in wine. The application of the method in measuring these aroma compounds during grape development will be discussed in subsequent publications [28].

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