

Supporting Information for

Simple quantitative determination of potent thiols at ultratrace levels in wine by derivatization and HPLC-MS/MS analysis

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SUPPLEMENTARY EXPERIMENTAL SECTION

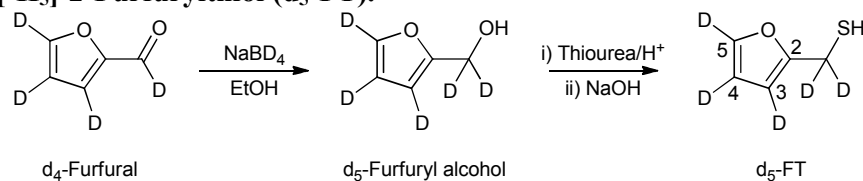
Chemicals and Materials. Chromatographic solvents were HPLC grade (Merck, Rowe Scientific, Lonsdale, SA, Australia), chemicals and reagents were analytical reagent grade (Sigma-Aldrich, Castle Hill, NSW, Australia), and water was obtained from a Milli-Q purification system. 4,4'-Dithiodipyridine (DTDP) was purchased from Sigma-Aldrich. Bag-in-box Sauvignon Blanc wine (10.5% v/v ethanol, pH 3.4, 15 mg/L free SO₂, 50 mg/L total SO₂) was purchased from a local retail outlet. [²H₅]-Benzyl chloride (d₅-BnCl) and 2-furfurylthiol were purchased from Sigma-Aldrich and [²H₄]-furfural (d₄-furfural) was obtained from C/D/N Isotopes Inc. (Pointe-Claire, QC, Canada). The following SPE cartridges (500 mg, 6 mL) were sourced: LMS, PPL, C18, ENV, (Varian Bond Elut); Lichrolut EN (Merck); Supelclean ENVI 18 (Supelco); and Strata SDB-L and Strata-X (Phenomenex). Stock solutions of standards were prepared volumetrically in absolute ethanol and stored at -20 °C, and working solutions were stored separately at -20 °C until required. All other prepared solutions were % v/v with the balance made up with Milli-Q water, unless specified otherwise.

NMR Analysis. Proton (¹H) and carbon (¹³C) nuclear magnetic resonance (NMR) spectra were recorded with Bruker spectrometers operating at 400 or 600 MHz for proton and 100 or 150 MHz for carbon nuclei. Chemical shifts were recorded as δ values in parts per million (ppm). Spectra were acquired in chloroform-*d* at ambient temperature, and resonances were assigned by routine 2D correlation experiments. For ¹H and ¹³C NMR spectra, the residual CHCl₃ peak (δ 7.26) and the central peak of the chloroform-*d* triplet (δ 77.16) were used as the internal references, respectively.

High Resolution Mass Spectrometry. Spectra were obtained on a Bruker micrOTOF-Q II with electrospray ionization (ESI) in negative mode. Samples were dissolved in ethanol/10% aqueous NH₄OH (4:1, v/v) and analyzed by flow injection.

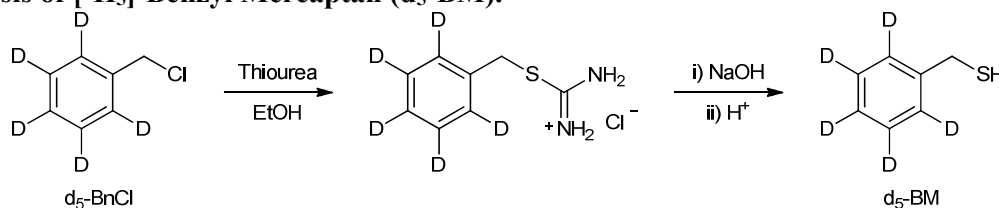
GC-MS Instrumentation. Synthesized compounds were analyzed with an Agilent 6890 gas chromatograph equipped with a Gerstel MPS2 multi-purpose sampler and coupled to an Agilent 5973N mass spectrometer. The gas chromatograph was fitted with a 30 m J&W DB-5ms fused silica capillary column (0.25 mm i.d., 0.25 μ m film thickness). The carrier gas was helium (Ultra High Purity) in constant flow mode at approximately 1.1 mL/min. The oven temperature started at 50 °C, was held at this temperature for 1 min, then increased to 260 °C at 10 °C/min, and held at this temperature for 10 minutes. The injection volume was 2 μ L and the injector was held at 200 °C throughout the run. Positive ion electron ionization (EI) spectra at 70 eV were recorded in the range *m/z* 35-350 for scan runs.

Synthesis of [²H₅]-2-Furfurylthiol (d₅-FT).



Preparation of this compound was based on the methods reported by Sen and Grosch¹. Briefly, d₄-furfural (1.02 g, 10.19 mmol) was reduced with NaBD₄ (0.172 g, 4.11 mmol) in ethanol (10 mL). The isolated d₅-furfuryl alcohol (1.04 g, 10.08 mmol, 99%) was treated with thiourea² (0.820 g, 10.77 mmol) and HCl (2 mL, 32% w/w) in water (23.5 mL) and ethanol (1 mL), yielding the title compound (0.49 g, 4.13 mmol, 41%) as a colorless oil after isolation, which was >95% pure by ¹H NMR.

¹H NMR (400 MHz, CDCl₃): δ 1.88 (1H, br s, SH); ¹³C NMR (100 MHz, CDCl₃): δ 153.4 (C₂), 141.8 (t, *J* = 31.4 Hz, C₅), 110.1 (t, *J* = 26.6 Hz, C₄), 106.1 (t, *J* = 26.6 Hz, C₃), 20.7 (quin, *J* = 21.6 Hz, CD₂); HRMS (*m/z*): [M-H]⁻ calcd. for C₅D₅OS⁻, 118.0380; found, 118.0381; GC-MS (*m/z*): 119 (34), 90 (2), 87 (8), 86 (100), 58 (40), 56 (7), 54 (6), 46 (6), 42 (4).

Synthesis of [$^2\text{H}_5$]-Benzyl Mercaptan (d_5 -BM).

This compound was prepared by adapting the method of Urquhart et al.³ Thiourea (0.580 g, 7.6 mmol) was dissolved in warm absolute ethanol (12 mL), and the solution was cooled to room temperature and added to d_5 -BnCl (1.0 g, 7.6 mmol) under nitrogen. The mixture was stirred overnight and the resultant suspension was filtered, and the solid was dried under high vacuum to give the isothiuronium salt (1.4 g, 6.7 mmol, 89 %). The crude salt was added under nitrogen to degassed aqueous NaOH (1.3 M, 20 mL) and the solution was stirred for 2 h before being adjusted to pH 3 with aqueous HCl (10%). The solution was saturated with NaCl, filtered and extracted with CH_2Cl_2 under nitrogen, and the organic extract was washed with brine and dried (MgSO_4). The solvent was removed by distillation under nitrogen to give the title compound (0.65 g, 5.0 mmol, 66 %) as a colourless oil, which was >95% pure by ^1H NMR.

^1H NMR (600 MHz, CDCl_3): δ 3.78 (2H, d, $J = 7.6$ Hz, CH_2), 1.81 (1H, t, $J = 7.6$ Hz, SH); ^{13}C NMR (150 MHz, CDCl_3): δ 141.0 (*ipso* C), 129.5–126.1 ($5 \times \text{CD}$), 28.9 (CH_2); HRMS (m/z): [M-H] $^-$ calcd for $\text{C}_7\text{H}_2\text{D}_5\text{S}^-$, 128.0588; found, 128.0580. GC-MS (m/z): 129 (27), 97 (8), 96 (100), 95 (5), 69 (7), 68 (7), 65 (4), 54 (4), 45 (8), 41 (4).

Preparation of Buffer, Model Wine and Derivatizing Reagent. Model wine consisted of 10% aqueous ethanol saturated with potassium hydrogen tartrate and pH adjusted to 3.2 or 3.6 with tartaric acid. Sørensen's phosphate buffer was prepared from sodium phosphate monobasic (0.2 M, pH 4.6) and sodium phosphate dibasic heptahydrate (0.2 M, pH 9.2); 0.1 M buffer at pH 7 was achieved by mixing 117 mL of monobasic stock with 183 mL dibasic stock, and adding Milli-Q water to give a final volume of 600 mL. DTDP reagent (4 mM or 10 mM) was prepared by stirring 88 mg (or 220 mg) of DTDP in a mixture of 3 mL water and 100 μL of concentrated HCl (37% w/w). After dissolution, Milli-Q water was added to give a final volume of 100 mL and aliquots of DTDP solution were stored at -20°C until required⁴.

HPLC-MS Instrumentation. Method development was conducted on either a ThermoFinnigan Surveyor HPLC connected to a ThermoFinnigan LCQ Deca XP Plus mass spectrometer or an Agilent 1200 HPLC connected to an Applied Biosystems 4000 QTrap hybrid tandem mass spectrometer. Electrospray ionization in positive ion mode was used and data acquisition and processing were performed using Xcalibur software (version 1.3) for the LCQ and Analyst software (version 1.5) for the 4000 QTrap. Method validation and quantitative analyses were conducted with the 4000 QTrap instrument.

HPLC Conditions. Separation was performed with a 250×2.1 mm i.d., 5 μm , 100 Å Alltima C18 column operated at 25°C and protected by a 7.5×2.1 mm i.d. guard cartridge of the same material. The solvents were 0.5% aqueous formic acid (solvent A) and 0.5% formic acid in acetonitrile (solvent B), with a flow rate of 0.200 mL/min. A range of linear gradients were assessed, with initial compositions of 5, 10, 15, 20, and 30% B, and run times of 30 and 36 min. The optimized linear gradient for solvent B was: 0 min, 20%; 10 min, 50%; 15 min, 80%, 20min, 80%, 21 min, 20% followed by 15 min of column equilibration with 20% B. An injection volume of 10 μL was used.

Mass Spectrometer Conditions. The LCQ had the following conditions: nitrogen was used for sheath gas, 35 arbitrary units and auxiliary gas, 20 arbitrary units; the ion spray voltage, capillary voltage, tube lens offset voltage and capillary temperature were set at 4500 V, 20 V, 35 V and 250°C , respectively; helium was used as the collision gas, and normalized collision energy, activation Q, activation time and isolation width were 34–38%, 0.250, 30 ms and m/z 1.4–1.7, respectively.

The 4000 Q Trap had the following conditions: nitrogen was used for curtain gas, 103.4 kPa; nebulizing gas, 344.7 kPa; drying gas, 344.7 kPa; and collision gas, high; the ion spray voltage, declustering potential, source temperature, and collision energy were set at 5500 V, 45 V, 500 °C, and 20 eV, respectively; for multiple reaction monitoring (MRM), Q1 and Q3 had unit resolution and the transitions chosen had a dwell time of 100 ms each.

Infusion MS. Mass spectrometer parameters were optimized with infusion MS/MS experiments of compounds in model wine (pH 3.2 or 3.6), derivatized as described below under *HPLC Gradient Optimization*, using an infusion pump operating at 5 µL/min. The mass transitions chosen for MRM experiments are listed in Table 1 of the main paper.

Derivative Stability. Samples prepared for infusion MS experiments and stored at 4 °C were repeatedly analyzed over several weeks without detrimental effects. The same applies to the stability of other samples in various matrices, which could be reanalyzed over a matter of months without obvious degradation.

HPLC Gradient Optimization. In duplicate, an aliquot (50 µL) of an ethanolic solution containing 4-MMP, 3-MH and 3-MHA (final concentrations of 10 mg/L of each analyte) was added to 10 mL of 0.1 M phosphate buffer (pH 4.6 or 7.0) containing EDTA 2Na (10 mg). Freshly prepared DTDP reagent (4 mM, 125 µL) was added, and the samples were thoroughly mixed and left at room temperature for 30 min prior to HPLC-MS/MS analysis.

Derivatization Time. In duplicate, an aliquot (50 µL) of an ethanolic solution containing 4-MMP, 3-MH and 3-MHA (final concentrations of 10 mg/L of each analyte) was added to 10 mL of model wine (pH 3.6) or Sauvignon Blanc wine (adjusted to pH 3.6 with 1 M NaOH) containing EDTA 2Na (10 mg). Freshly prepared DTDP reagent (4 mM, 125 µL) was added, the samples were thoroughly mixed and left at room temperature for 30 or 60 min before 150 µL of concentrated HCl was added prior to HPLC-MS/MS analysis.

Acetaldehyde Addition. In duplicate, an aliquot (50 µL) of an ethanolic solution containing 4-MMP, 3-MH and 3-MHA (final concentrations of 10 mg/L of each analyte) was added to 10 mL of Sauvignon Blanc wine (pH 3.4, and the same wine and adjusted to pH 4.5 with 10 M NaOH) containing EDTA 2Na (10 mg). Freshly prepared DTDP reagent (4 mM, 250 µL) was added, followed by 50% acetaldehyde (10, 20, 30, 40, 50 and 100 µL) and the samples were thoroughly mixed and left at room temperature for 30 min prior to HPLC-MS/MS analysis.

SPE Cartridge Selection. Eight cartridges (listed under *Chemicals and Materials*) were evaluated in duplicate after being conditioned with 6 mL of methanol followed by 6 mL of water. A batch (500 mL) of model wine (pH 3.2) or Sauvignon Blanc wine (pH 3.4), containing EDTA 2Na (500 mg), was spiked with ethanolic solutions of unlabeled 4-MMP, 3-MH and 3-MHA and their labeled analogues (final concentrations of 500 ng/L of each compound), and freshly thawed DTDP reagent (10 mM, 2.5 mL) was added, followed by 50% acetaldehyde (500 µL). After 30 min, aliquots (10 mL) were loaded onto the various cartridges, and the cartridges were washed with 5% MeCN (6 mL) and then dried under air for 5 min or dried without washing, before each sample was eluted with methanol (3 mL). The eluates were collected, concentrated to dryness with a gentle stream of nitrogen at 25 °C using a TurboVap LV evaporator, and reconstituted with 10% ethanol (200 µL) for HPLC-MS/MS analysis.

Optimization with Bond Elut C18 Cartridge. Duplicate samples with analytes at 0, 5 and 50 ng/L were prepared as described under *SPE Cartridge Selection* for an assessment of the following conditions: 10 mL or 20 mL of wine; 100 and 200 µL of 10 mM DTDP reagent; 20, 40 and 80 µL of 50% acetaldehyde; washing the loaded cartridge with 6 or 12 mL of 1, 5, 10, 20, 30, 40 and 50% methanol prior to drying the cartridge under air for 5 min.

Analytical Method Validation. Linearity. The analytical method was validated by a duplicate series of standard additions⁵ of unlabeled 4-MMP (0, 6.3, 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and 100 ng/L), 3-MH (0, 312, 625, 1250, 1875, 2500, 3125, 3750, 4375 and 5000 ng/L), 3-MHA (0, 63, 125, 250, 375, 500, 625, 750, 875 and 1000 ng/L), FT and BM (0, 6.3, 12.5, 25, 37.5, 50, 100, 200, 300 and 400 ng/L) to: (1) a model wine (9.5% v/v ethanol, pH 3.25, titratable acidity [TA] 3 g/L, SO₂ [free] < 4 mg/L, SO₂ [total] < 4 mg/L); (2) a Sauvignon Blanc wine (10.7% ethanol, pH

3.33, TA 6.1 g/L, SO₂ [free] 49 mg/L, SO₂ [total] 148 mg/L); (3) a 2010 rosé wine (12.6% ethanol, pH 3.28, TA 6.5 g/L, SO₂ [free] 14 mg/L, SO₂ [total] 87 mg/L); (4) a 2007 Chardonnay wine (13.4% ethanol, pH 3.25, TA 6.4 g/L, SO₂ [free] 14 mg/L, SO₂ [total] 156 mg/L); (5) a 2010 red wine blend (Shiraz, Cabernet Sauvignon and Merlot) (12.6% ethanol, pH 3.5, TA 6.2 g/L, SO₂ [free] 25 mg/L, SO₂ [total] 71 mg/L).

Precision and Recovery. To determine the precision and recovery of the analysis for each matrix, seven replicate samples were spiked at two different concentrations: 12.5 ng/L and 62.5 ng/L for 4-MMP, 625 ng/L and 3125 ng/L for 3-MH, 125 ng/L and 625 ng/L for 3-MHA and 12.5 ng/L and 100 ng/L for both FT and BM.

Quantitation of Samples. For quantifying the analytes in batches of unknown samples, duplicate standards in model wine matrix were prepared at the same time as the wine samples, with 4-MMP at concentrations of 0, 12.5, 37.5, 50, 75 and 100 ng/L; 3-MH at 0, 625, 1875, 2500, 3750 and 5000 ng/L; 3-MHA at 0, 125, 375, 500, 750 and 1000 ng/L; FT and BM at 0, 12.5, 37.5, 50, 200 and 400 ng/L.

Control. To ensure that the accuracy of the analysis was maintained, duplicate control samples, consisting of randomly selected wines spiked with 4-MMP (0 ng/L and 25 ng/L), 3-MH (0 ng/L and 1250 ng/L), 3-MHA (0 ng/L and 250 ng/L), and FT and BM (0 ng/L and 25 ng/L), were included with every set of samples to be quantified.

All validation and calibration samples were prepared and analyzed according to the optimized method and the performance parameters for each analyte appear in Table S-1. Naturally-occurring levels of analytes in the wines used for validation are shown in Table S-2.

The limit of detection (LOD) was determined based on visual evaluation of the chromatograms by establishing the minimum level at which the analytes could be reliably detected from the analysis of samples with known concentrations of analyte (i.e., signal/noise ratio of 3). LOQ was determined by multiplying LOD by 3.3 (i.e., signal/noise ratio of 10)⁵. Linearity was evaluated for the different matrices based on coefficient of determination (R^2)⁶ and D'Agostino-Pearson omnibus K^2 normality test of the residuals⁷.

Table S-1. Analytical method validation parameters in different wine matrices.

Wine Matrix	4-MMP					3-MH					3-MHA					FT					BM				
	% Recovery (RSD) ^a					% Recovery (RSD) ^a					% Recovery (RSD) ^a					% Recovery (RSD) ^a					% Recovery (RSD) ^a				
	Curve R ²	12.5 ng/L	62.5 ng/L	LOD ^b	LOQ ^c	Curve R ²	625 ng/L	3125 ng/L	LOD ^b	LOQ ^c	Curve R ²	125 ng/L	625 ng/L	LOD ^b	LOQ ^c	Curve R ²	12.5 ng/L	100 ng/L	LOD ^b	LOQ ^c	Curve R ²	12.5 ng/L	100 ng/L	LOD ^b	LOQ ^c
Model Wine	0.9924	85.2 (4.1)	104.4 (4.2)	0.8	2.6	0.9969	93.7 (4.4)	106.2 (4.9)	6.4	21.0	0.9977	94.9 (5.6)	93.3 (4.0)	2.2	7.4	0.9924	104.8 (4.3)	107.4 (2.9)	0.7	2.3	0.9964	103.4 (4.8)	97.1 (2.1)	0.3	1.1
Sauvignon Blanc	0.9992	99.8 (2.3)	97.1 (2.5)	0.9	3.1	0.9937	100.1 (4.2)	99.1 (2.4)	8.3	27.5	0.9994	97.6 (2.5)	101.3 (2.4)	1.3	4.3	0.9998	98.2 (1.9)	99.8 (1.1)	1.0	3.3	0.9997	97.5 (2.7)	100.2 (1.5)	0.5	1.6
Chardonnay	0.9981	98.1 (4.8)	102.8 (3.8)	1.1	3.6	0.9901	101.2 (1.2)	94.0 (2.9)	6.7	22.5	0.9982	97.8 (1.6)	99.9 (1.8)	1.2	3.9	0.9989	96.5 (3.6)	97.4 (7.3)	1.4	4.5	0.9988	96.4 (4.3)	97.2 (1.5)	0.8	2.8
Rosé	0.9978	100.8 (4.2)	98.4 (4.3)	1.4	4.6	0.9950	100.1 (5.6)	96.6 (3.4)	5.4	17.8	0.9973	95.7 (3.5)	99.2 (3.3)	4.3	14.2	0.9992	96.7 (3.0)	99.5 (2.5)	1.3	4.4	0.9998	96.2 (4.0)	100.9 (0.9)	1.1	3.7
Red Blend	0.9993	100.8 (1.6)	101.8 (3.6)	1.6	5.3	0.9981	100.4 (0.4)	97.6 (2.7)	10.6	35.4	0.9986	96.4 (1.5)	99.3 (1.3)	2.2	7.2	0.9993	100.3 (6.2)	99.6 (8.5)	1.5	5.0	0.9985	96.2 (5.0)	93.7 (3.0)	0.6	1.9

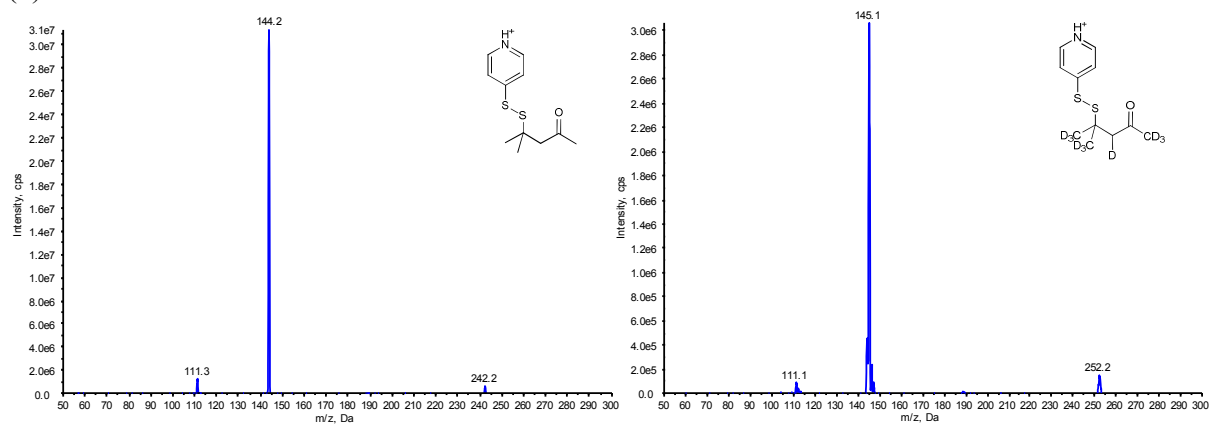
^a RSD, relative standard deviation of the mean (ng/L) for repeatability (N = 7). ^b LOD, limit of detection (ng/L). ^c LOQ, limit of quantitation (ng/L).

Table S-2. Existing analyte concentrations (ng/L) in the commercial wines used for method validation.

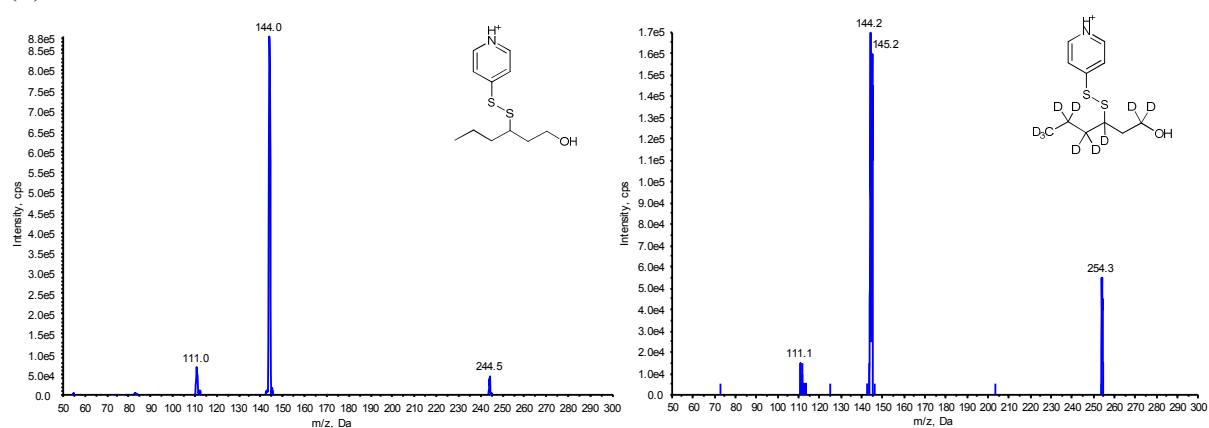
Wine Matrix	4-MMP	3-MH	3-MHA	FT	BM
Sauvignon Blanc	3.2	1138.3	54.2	ND ^a	ND
Chardonnay	ND	381.9	1.9	7.8	ND
Rosé	NQ ^b	310.2	2.0	NQ	ND
Red Blend	ND	503.2	2.9	NQ	ND

^a ND, not detected; analyte concentration <LOD. ^b NQ, not quantified; analyte concentration <LOQ.

(a)



(b)



(c)

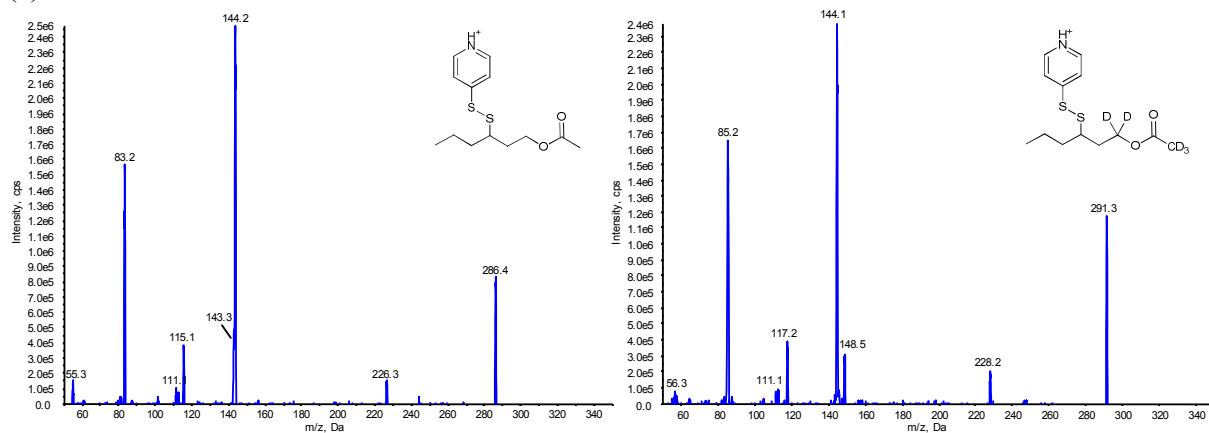
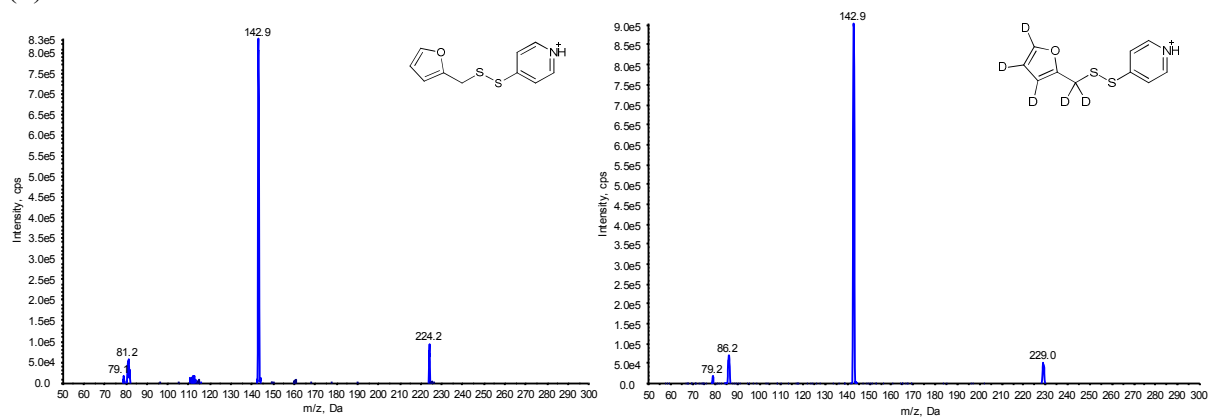


Figure S-1. Infusion MS/MS analysis showing unlabeled (left panel) and deuterium-labeled (right panel) derivative fragmentation patterns for (a) 4-MMP, (b) 3-MH and (c) 3-MHA.

(d)



(e)

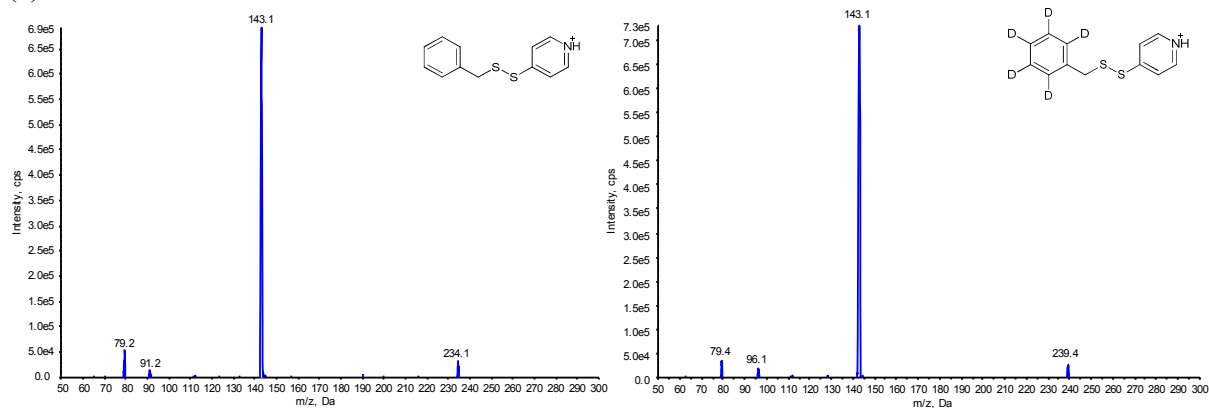


Figure S-1 contd. Infusion MS/MS analysis showing unlabeled (left panel) and deuterium-labeled (right panel) derivative fragmentation patterns for (d) FT and (e) BM.

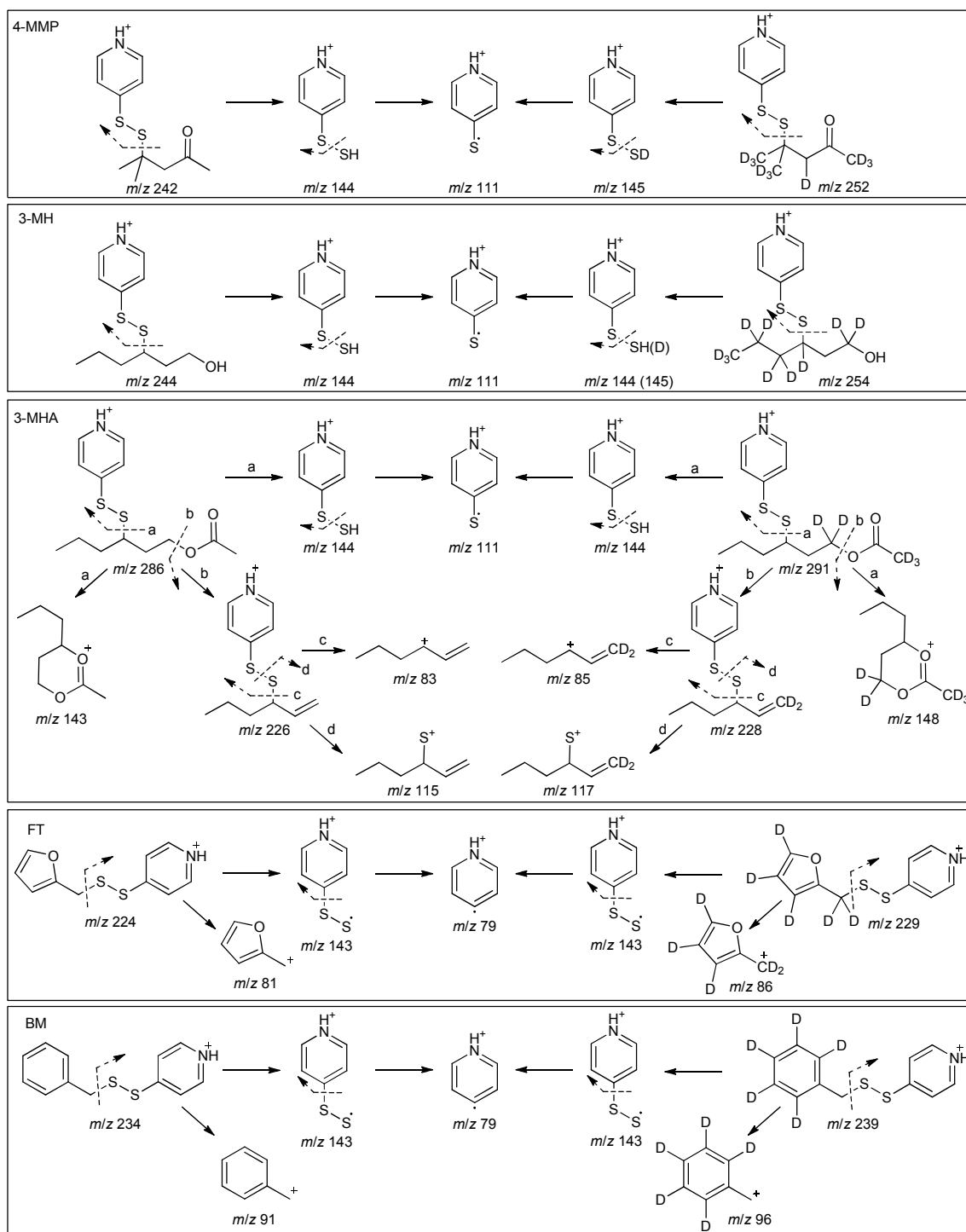


Figure S-2. Postulated fragmentation pathways of unlabeled and deuterium-labeled thiol derivatives based on MS/MS analysis.

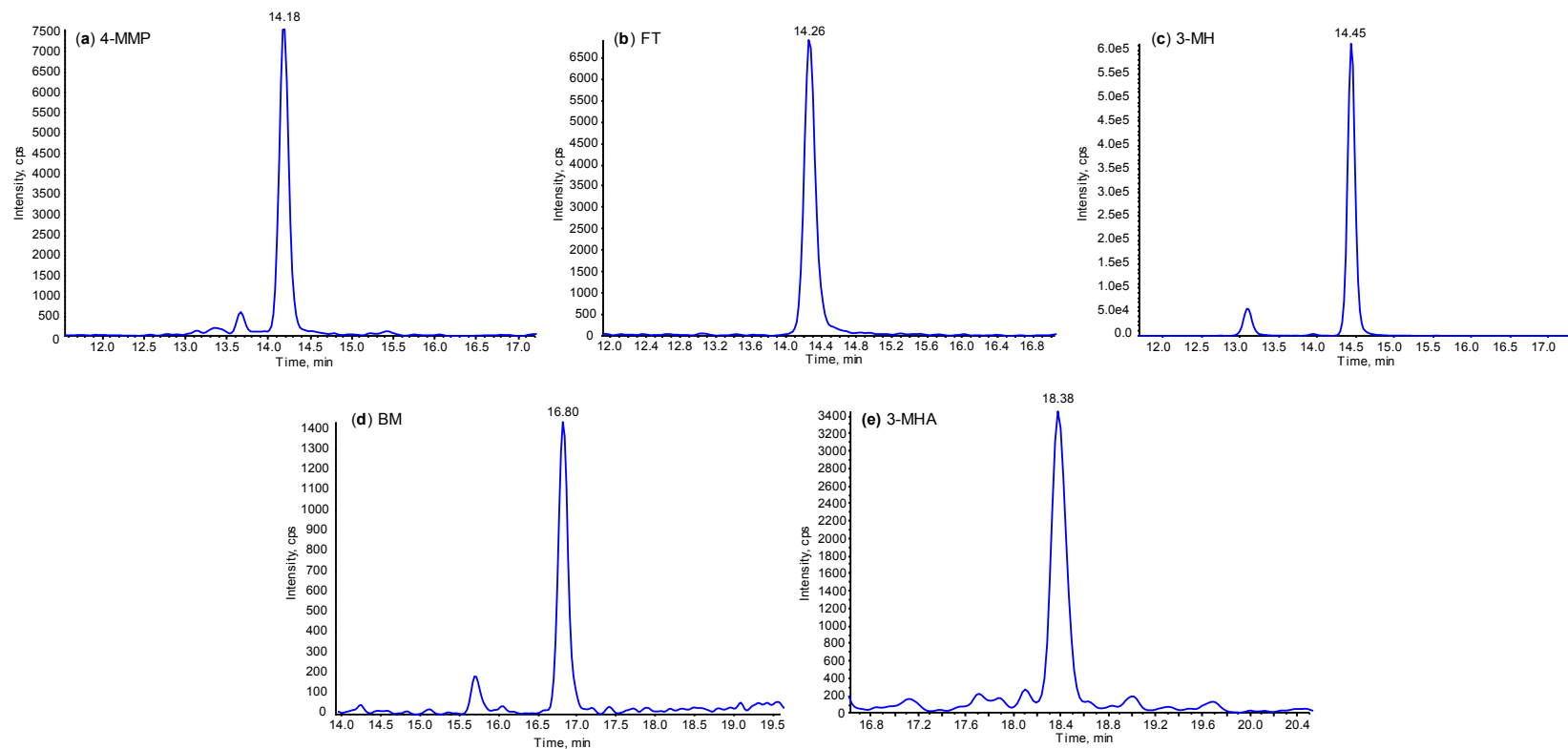


Figure S-3. Example MRM chromatograms (expanded time segments) of analytes in real wine samples. Transitions shown are (a) 4-MMP, m/z 242.2 \rightarrow 144.2, (b) FT, m/z 224.3 \rightarrow 143.0, (c) 3-MH, m/z 244.5 \rightarrow 144.1, (d) BM, m/z 234.3 \rightarrow 143.0, and (e) 3-MHA, m/z 286.4 \rightarrow 144.2.

REFERENCES

- (1) Sen, A.; Grosch, W. Z. *Lebensm. Unters. Forsch.* **1991**, *192*, 541-547.
- (2) Kofod, H. *Org. Synth.* **1955**, *35*, 66.
- (3) Urquhart, G. G.; Gates, J. W.; Connor, R. *Org. Synth.* **1941**, *21*, 36.
- (4) Riener, C. K.; Kada, G.; Gruber, H. J. *Anal. Bioanal. Chem.* **2002**, *373*, 266-276.
- (5) Thompson, M.; Ellison, S. L. R.; Wood, R. *Pure Appl. Chem.* **2002**, *74*, 835-855.
- (6) Ellison, S. *Accred. Qual. Assur.* **2006**, *11*, 146-152.
- (7) D'Agostino, R. B.; Belanger, A.; D'Agostino Jr., R. B. *Am. Stat.* **1990**, *44*, 316-321.