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Simple Quantitative Determination of Potent Thiols at Ultratrace Levels in Wine by Derivatization and High-Performance Liquid Chromatography−Tandem Mass Spectrometry (HPLC-MS/MS) Analysis

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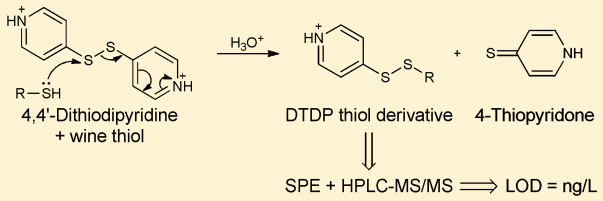
Supporting Information

ABSTRACT: Volatile sulfur compounds contribute characteristic aromas to foods and beverages and are widely studied, because of their impact on sensory properties. Certain thiols are particularly important to the aromas of roasted coffee, cooked meat, passion fruit, grapefruit, and guava. These same thiols enhance the aroma profiles of different wine styles, imparting pleasant aromas reminiscent of citrus and tropical fruits (due to 3-mercaptohexan-1-ol, 3-mercaptohexyl acetate, 4-mercapto-4-methylpentan-2-one), roasted coffee (2-furfurylthiol), and struck flint (benzyl mercaptan), at nanogram-per-liter levels. In contrast to the usual gas chromatography (GC) approaches, a simple and unique high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) method was developed for routine analysis of five wine thiols, using 4,4′-dithiodipyridine (DTDP) as a derivatizing agent and polydeuterated internal standards for maximum accuracy and precision. DTDP reacted rapidly with thiols at wine pH and provided stable derivatives, which were enriched by solid-phase extraction (SPE) prior to analysis by HPLC-MS/MS. All steps were optimized and the method was validated in different wine matrices, with method performance being comparable to a well-optimized but more cumbersome gas chromatography−mass spectrometry (GC-MS) method. A range of commercial wines was analyzed with the new method, revealing the distribution of the five thiols in white, red, rosé, and sparkling wine styles.



Among the many scientific fields requiring investigation, foods and beverages certainly garner a fair share of the attention. Apart from the obvious importance of the nutritional and commercial value of these everyday commodities, there is also an array of fascinating natural products that stimulate the interest of scientists. Flavor chemistry has a prominent role in food and beverage research and, more specifically, the study of aroma volatiles spans across various products, such as fruit, cheese, cooked meat, vegetables, bread, coffee, beer, and wine.[1]

Enjoyed by consumers on an increasing international scale in a global market worth many billions of dollars,[2] wine is somewhat unique, because of the complex combination of hundreds of volatile compounds of varying importance to the overall aroma of the different styles.[3] These compounds arise from the grapes, fermentation processes, oak contact, and aging, and although not necessarily limited to wine, there are several components that are especially impactful, despite being found at remarkably low concentrations. Volatile sulfur compounds are one potent class of molecules occurring in a range of foods, which contribute desirable aromas to wine when present at nanogram-per-liter levels.[4] Of particular importance are the polyfunctional thiols 4-mercapto-4-methylpentan-2-one (4-MMP, **1**), 3-mercaptohexan-1-ol (3-MH, **2**), 3-mercaptohexyl acetate (3-MHA, **3**), benzyl mercaptan (BM, **4**), and 2-furfurylthiol (FT, **5**) (see Figure 1). These impact compounds (present as pairs of enantiomers in the case of chiral 3-MH and 3-MHA) are among the most potent food odorants known, with extremely low aroma detection thresholds and aromas reminiscent of boxwood, passion fruit, grapefruit, smoke, and roasted coffee.[5]

Driven by the needs for basic understanding and ways to assess quality, analytical chemistry provides the key to investigating the impacts of grape variety and growing conditions, yeast and bacterial effects during fermentation, changes during storage, and impacts of winemaking operations on polyfunctional thiols. However, the low concentrations of these thiols, coupled with their reactivity (e.g., susceptible to oxidation, disulfide formation, and adsorption on surfaces) and complexity of the wine matrix, poses particular analytical challenges. Many specialized methods have been proposed for the analysis of these compounds in wine, with common techniques involving mercury complexes (due to thiol affinity

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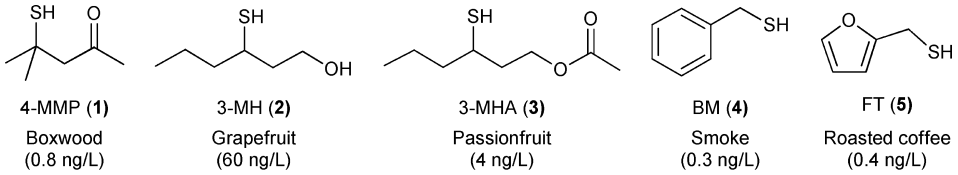

Figure 1. Polyfunctional thiols present as impact odorants in wine (with aroma detection thresholds given in parentheses, as determined in hydroalcoholic or model solutions).

for this metal), and liquid–liquid or solid-phase extraction to isolate and concentrate the thiols.[6] Extracts are then analyzed using gas chromatography–mass spectrometry (GC-MS), either as the free thiol or after derivatization (an excellent summary, including method limitations, can be found in a recent review[6]), but the most advanced procedure is far from straightforward, despite several iterations and optimizations.[7] Nonetheless, methods such as these have been adopted for analysis of polyfunctional thiols in matrices such as beer,[8,9] cheese,[10] boiled meats,[11] and fruit extracts.[12,13]

Consideration of the critical aspects involved with extraction and quantitative analysis of polyfunctional thiols found at ultratrace concentrations in wine prompted further investigation to develop a method that was simple, robust, and amenable to large numbers of samples. One approach that has been well-exploited for the spectrophotometric determination of biologically relevant thiols such as glutathione, cysteine, and proteins involves appending (and releasing) a chromophore through derivatization of sulfhydryl functional groups. Ellman's reagent (i.e., 5,5′-dithiobis(2-nitrobenzoic acid))[14] still proves to be a common choice, but many other reagents are available, including those used in conjunction with separation by high-performance liquid chromatography (HPLC) and ultraviolet (UV) detection.[15] Typically, derivatization is undertaken at alkaline pH, which is appropriate for biological matrices but less applicable for an acidic matrix such as wine. As an alternative, we considered the use of 4,4′-dithiodipyridine (DTDP, **6**; see Figure 2), which reacts by thiol–disulfide exchange in the same

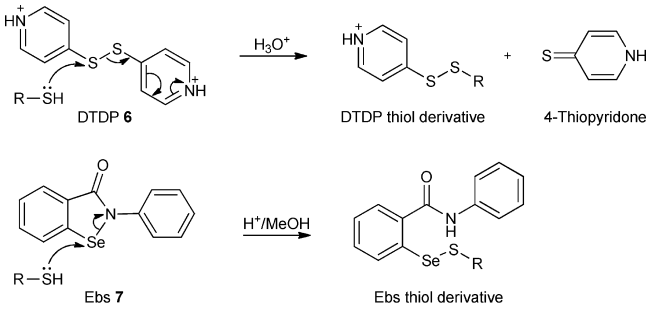

Figure 2. Derivatization reaction of a thiol (R-SH) with DTDP or Ebs under acidic conditions.

manner as Ellman's reagent, but is effective at pH ≥3.4 and reacts rapidly and completely.[16] Moreover, the use of an acidified eluent for high-performance liquid chromatography–mass spectrometry (HPLC-MS) analysis would yield positively charged pyridyl derivatives, thereby improving sensitivity with electrospray ionization in positive ion mode; attaching a chromophore was of little importance in this instance. A recent report[17] on the analysis of volatile thiols in lipid matrices by HPLC with high-resolution mass spectrometry (MS) detailed a similar approach, employing ebselen (Ebs, i.e., 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one, **7**) for derivatization, yielding a selenenylsulfide linkage (see Figure 2).

In stark contrast to the usual GC-MS approaches, our unique solution to simplify the challenging analysis of volatile thiols in wine involved HPLC-MS/MS analysis of DTDP derivatives isolated by solid-phase extraction (SPE). We describe the optimization and validation of the method, which employed stable deuterium-labeled internal standards for maximum accuracy and precision, and its application to a selection of wines. The performance characteristics of the new method are at least equivalent—and, in many instances, superior—to other reported approaches for thiol analysis.

EXPERIMENTAL SECTION

Chemicals and Materials. All prepared solutions were expressed in terms of volume percent (%, v/v), with the balance composed of Milli-Q water, unless specified otherwise. 4,4′-Dithiodipyridine (DTDP) was purchased from Sigma–Aldrich (Castle Hill, NSW, Australia). Unlabeled and isotopically labeled standards previously synthesized included 3-mercaptohexan-1-ol (3-MH), [$^2H_{10}$]-3-mercaptohexan-1-ol (d_{10}-3-MH), 3-mercaptohexyl acetate (3-MHA), [$^2H_5$]-3-mercaptohexyl acetate (d_5-3-MHA), 4-mercapto-4-methylpentan-2-one (4-MMP), and [$^2H_{10}$]-4-mercapto-4-methylpentan-2-one (d_{10}-4-MMP).[18−20] Additional information on the materials, standard solutions, and synthesis of [$^2H_5$]-2-furfurylthiol (d_5-FT) and [$^2H_5$]-benzyl mercaptan (d_5-BM) appears in the Supporting Information. All wine samples were commercially available and obtained from retail outlets.

Assessment of Derivatization Parameters. Details of the conditions trialed for derivatization and isolation of derivatives by SPE are provided in the Supporting Information.

Derivatizing Reagent. A solution (10 mM) was prepared by sonicating 220 mg of DTDP in a mixture of 20 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 required.[21]

Preparation of Wine Samples for Analysis. An aliquot (50 μL) of an ethanolic solution containing d_{10}-4-MMP, d_{10}-3-MH, d_5-3-MHA, d_5-FT, and d_5-BM (final concentrations of 500 ng/L of each internal standard) was added to 20 mL of wine. This was followed by the addition of EDTA 2Na (20 mg), 50% acetaldehyde (80 μL), and freshly thawed DTDP reagent (10 mM, 200 μL). After 30 min, the sample was passed through a 6-mL, 500-mg Bond Elut C18 cartridge, previously conditioned with 6 mL of methanol, followed by 6 mL of water. The cartridge was washed with 12 mL of 50% methanol, dried under air for 5 min, and eluted with 3 mL of methanol. The eluate was 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.

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HPLC-MS Instrumentation and Conditions. Full details of instrument parameters are provided in the Supporting Information. Quantitative analyses were performed with an Agilent 1200 HPLC, using a 250 mm × 2.1 mm i.d., 5 μm, 100 Å Alltima C18 column operated at 25 °C and protected by a 7.5 mm × 2.1 mm i.d. guard cartridge of the same material, connected to an Applied Biosystems 4000 QTrap hybrid tandem mass spectrometer with electrospray ionization in positive ion mode. 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. The linear gradient for solvent B was as follows: 0 min, 20%; 10 min, 50%; 15 min, 80%; 20 min, 80%; 21 min, 20%; followed by 15 min of column equilibration with 20% B. An injection volume of 10 μL was used. Infusion MS/MS experiments were used to obtain the mass transitions (see Table 1) for multiple reaction monitoring (MRM) of derivatized thiols based on their fragmentation patterns (see Figures S-1 and S-2 in the Supporting Information).

Table 1. Mass Transitions and Analyte Retention Times for HPLC-MS/MS Analysis of Derivatized Thiols Using Multiple Reaction Monitoring (MRM)

internal standard	MRM pairs	analyte	MRM pairs	analyte RT (RSD)[a]
d_{10}-4-MMP	252.5 → 145.1	4-MMP	242.2 → 144.2	13.85 (0.7)
	252.5 → 111.1		242.2 → 111.3	
d_5-FT	229.1 → 143.1	FT	224.3 → 143.0	14.03 (1.1)
	229.1 → 86.4		224.3 → 81.3	
d_{10}-3-MH	254.3 → 144.9	3-MH	244.5 → 144.1	14.29 (0.7)
	254.3 → 144.1		244.5 → 111.3	
	254.3 → 111.1			
d_5-BM	239.3 → 143.1	BM	234.3 → 143.1	16.68 (0.8)
	239.3 → 79.4		234.3 → 79.2	
d_5-3-MHA	291.3 → 144.1	3-MHA	286.4 → 144.2	18.27 (0.5)
	291.3 → 85.2		286.4 → 83.2	

[a]RT, average retention time (in minutes) and relative standard deviation (RSD) of wine samples (N = 24).

Analytical Method Validation. Full details appear in the Supporting Information.

Statistical Analysis. The results reported for the calibration of the method were derived from the average of two replicate measurements for each concentration of analyte (and seven replicates for repeatability samples). Statistical analyses were performed with GraphPad Prism 6 and Microsoft Excel 2010.

RESULTS AND DISCUSSION

Thiol Derivatization and Isolation. Derivatizing volatile thiols in wine with DTDP was without precedent, so we began our investigations by reacting 4-MMP, 3-MH, and 3-MHA with DTDP in phosphate buffer (see the Supporting Information) at typical pH values used with this reagent (pH 4.6 and 7.0).[21−23] EDTA was included to prevent thiol oxidation and reactions were assessed by HPLC-MS/MS to identify the derivatives and optimize the chromatography. Reversed-phase mobile and stationary phases were typical of those used for analysis of wine by HPLC-MS, and gradient modifications were undertaken to derive a suitable run time. MS/MS provided confirmation of the derivatization procedure and fragmentation data for 4-MMP, 3-MH, and 3-MHA derivatives, with an intense peak at m/z 144 and a much smaller one at m/z 111, arising from the derivatized portion of the parent ions (see Figures S-1 and S-2 in the Supporting Information). 3-MHA yielded additional fragmentation pathways, because of the acetate group, producing peaks at m/z 226, 143, 115, and 83. We also identified the presence of excess DTDP at this stage. This observation became important when moving to wine matrices, because there is a need to ensure complete derivatization of volatile thiols among the other sulfhydryl-possessing compounds.

The reaction of thiols with DTDP is known to be rapid and occurs over a wide pH range,[16] so only a brief assessment of derivatization time was undertaken at wine pH using model and Sauvignon Blanc wines (see the Supporting Information). The reaction was quenched after 30 or 60 min with HCl,[23] and the samples were analyzed by HPLC-MS/MS. Model wine reacted smoothly and showed no effect from the increased reaction time; a period of 30 min was chosen for the remainder of the method development. In contrast, some inconsistencies arose from the Sauvignon Blanc matrix, where an excess of reagent was no longer evident during analysis. The Sauvignon Blanc wine contained bisulfite (HSO_3^-, used as a preservative in most wines) and DTDP (and Ellman's reagent) reacts quantitatively with sulfite ions (which occur in pH-dependent equilibrium with HSO_3^- and SO_2) in aqueous systems at room temperature.[24]

The interference from bisulfite was overcome by the addition of acetaldehyde in excess. Acetaldehyde, naturally present in wine, is commonly added during analytical procedures to bind interfering bisulfite, and has also been used effectively when derivatizing glutathione for analysis[25] or determining low-molecular-weight sulfur compounds.[26] As well as doubling the amount of DTDP reagent, different volumes of acetaldehyde were added to determine the amount required to overcome interference from bisulfite (see the Supporting Information). With the increased amount of DTDP, ∼40 μL of acetaldehyde was sufficient to eliminate the effects of bisulfite, leaving a large excess of derivatizing agent, as determined by HPLC-MS/MS (data not shown).

With the derivatization step optimized, the ideal situation would have been to simply undertake the direct analysis of volatile thiol derivatives in wine. So far, we had been working with relatively high concentrations of analytes and it soon became clear that direct analysis would not be possible (perhaps except for samples containing high levels of 3-MH). As with other volatile thiol methods, a concentration step was required in order to determine these compounds at levels near the sensory detection threshold where they start to become important to wine aroma (i.e., low ng/L, see Figure 1). Concentration of derivatives and cleanup of the samples was achieved by SPE.

Various reversed-phase SPE cartridges (C18 and polymeric) were examined (see the Supporting Information) for their ability to retain the derivatized analytes from wine samples, enabling their concentration prior to analysis. The cartridges performed similarly and adding a wash step after sample loading led to an improvement in sensitivity through increased peak heights (data not shown). Bond Elut C18 was chosen for

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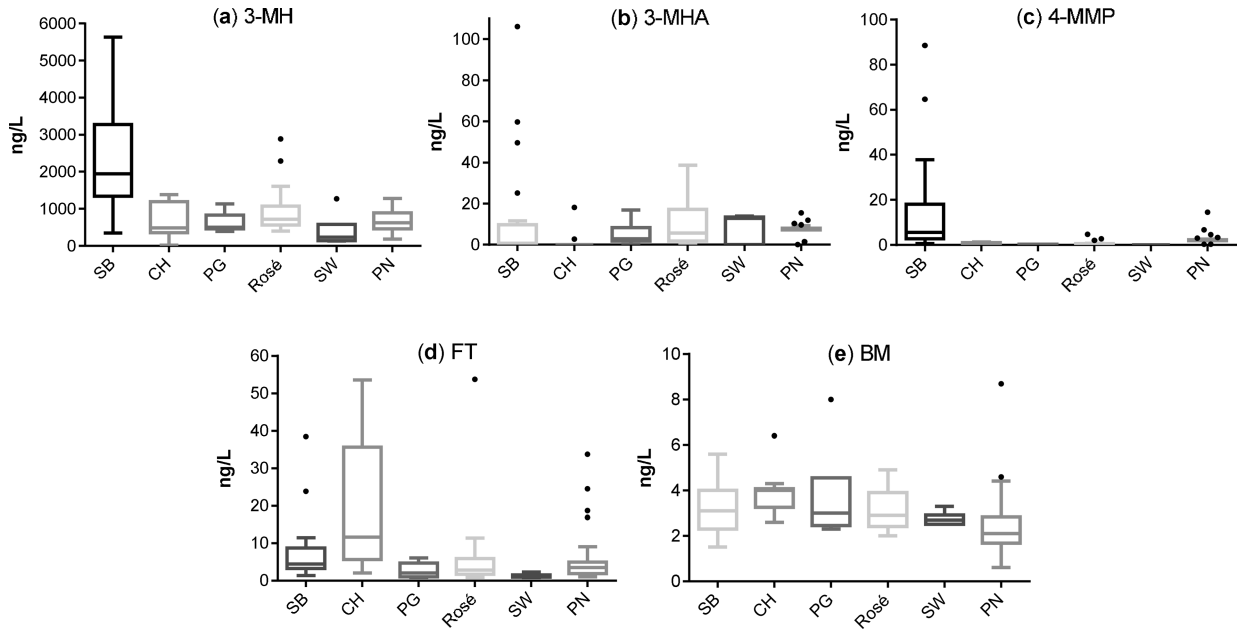

Figure 3. Tukey box plots showing distribution of concentrations (ng/L) for (a) 3-MH, (b) 3-MHA, (c) 4-MMP, (d) FT, and (e) BM in a range of commercial Sauvignon Blanc (SB), Chardonnay (CH), Pinot Gris (PG), rosé, sparkling white (SW), and Pinot Noir (PN) wines. Note the different *y*-axis scales for each analyte.

further optimization (see the Supporting Information), because of its common availability in our laboratory. Optimization was primarily related to improving sensitivity with lower levels of analytes. Assessments of larger volumes of wine, derivatizing agent, and acetaldehyde provided only incremental improvements at best, with the greatest gains in sensitivity arising from a wash step after sample loading (data not shown). Increasing concentrations of methanol in the wash solution yielded improvements through cleaner chromatograms, and a wash with 50% aqueous methanol was included in the optimized procedure. Interestingly, despite the derivatives potentially being ionized due to the pyridine moiety, the ability to tolerate a wash with 50% aqueous methanol highlights their hydrophobic nature. The optimized derivatization and extraction procedure was subsequently applied to a thorough validation of the method, and two additional analytes (FT and BM) and their deuterated analogues (as internal standards) were included at this stage.

Method Validation. Polydeuterated analogues of 4-MMP, 3-MH, and 3-MHA required for stable isotope dilution analysis (SIDA) were already on hand, and deuterium-labeled FT and BM were synthesized from commercially available starting materials and fully characterized for this study (see the Supporting Information). Infusion MS/MS experiments were used to determine the mass transitions for the analytical method using multiple reaction monitoring (MRM) (recall Table 1, which includes analyte retention times). Deuterium-containing 4-MMP, 3-MH, and 3-MHA fragmented analogously to their unlabeled isotopologues (see Figures S-1 and S-2 in the Supporting Information); however, for aromatic compounds FT and BM (and their labeled counterparts), the most abundant fragment appeared at m/z 143. This unusual odd-electron ion was proposed to be a stable cationic pyridyl disulfide bearing a sulfur-centered radical (see Figures S-1 and S-2 in the Supporting Information), arising from the derivatized portion of the parent ions due to the neutral loss of stabilized benzyl and furyl radicals.[27−29] Small peaks were also identified for the aryl cations resulting from neutral loss of the derivatized portion of the parent ions (i.e., benzyl cation from BM, and furfuryl cation from FT).

Validation of the analytical method[30] was conducted with analyte concentrations spanning the range typical for these compounds in wine and was undertaken in model, red, rosé, and white wine matrices (recall the Supporting Information). The usual performance parameters of linearity, recovery, precision, and limits of detection and quantitation (LOD and LOQ) were determined (see Table S-1 in the Supporting Information). Ten-point calibration functions were linear throughout the concentration ranges (R^2 > 0.99) in each matrix with average slopes of 1.759 for 3-MH, 1.264 for 3-MHA, 11.580 for 4-MMP, 2.084 for FT, and 1.025 for BM. Recoveries at low and high levels of analyte were excellent (94%−103%) in real wines, with the majority of repeatability values being less than 5% RSD, and none greater than 8.5%. Overall, model wine was consistent with these recovery and repeatability values, although recoveries for 4-MMP and FT at 12.5 ng/L were 85% and 105%, respectively, and 107% for FT at 100 ng/L. Depending on the wine matrix, LOD values (see Table S-1 in the Supporting Information) were mostly at or below the aroma detection thresholds for each analyte (see Figure 1), and were in the range of 0.8−1.6 ng/L for 4-MMP, 6.4−10.6 ng/L for 3-MH, 1.2−4.3 ng/L for 3-MHA, 0.7−1.5 ng/L for FT, and 1.1−3.7 for BM. These performance characteristics are at least equivalent—and, in many instances, superior—to those reported for more-laborious methods that are employed for the routine analysis of these thiols in wine.[7,31,32] Importantly, the low LOD values mean that small but significant changes in analyte concentrations, around the level of human perception of the aromas, could be detected. In considering this statement, it is important to realize that aroma detection thresholds determined in simple hydroalcoholic or model solutions, as presented in Figure 1, merely act as a guide. In simple solutions, aroma detection thresholds are commonly lower than would be expected for complex matrices such as

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wine that contribute great variability to the determinations. For instance, the aroma detection threshold for 4-MMP has been determined at ∼0.1 ng/L in distilled water, 1 ng/L in 10% aqueous ethanol, 0.8 ng/L in model wine solution, or 10% ethanol containing 100 g/L sucrose, and 3.3 ng/L in neutral red and white wines.[33]

Quantitative Analysis. The validated method was applied to a range of commercially available wines (Figure S-3 in the Supporting Information shows examples of MRM chromatograms), encompassing a selection of still and sparkling wine varieties. For quantification, multipoint calibration functions in model wine matrix were run with every batch of wine samples, and quality control samples were prepared from randomly selected wines (see the Supporting Information). Commercial samples comprised Sauvignon Blanc (SB, $N = 25$), Chardonnay (CH, $N = 12$), Pinot Gris (PG, $N = 6$), Pinot Noir (PN, $N = 34$), rosé ($N = 21$), and sparkling white (SW, $N = 6$) (see Figure 3). The Tukey box plots show the 25th and 75th percentiles as the bottom and top of the box, respectively, and the median values as a line bisecting the box. The minimum and maximum values are represented by the lower and upper whiskers, respectively, for those data points that fall within 1.5 times the interquartile range (i.e., the difference between the 75th and 25th percentiles) above and below the box. Individual data points are shown where they fall outside of the whiskers as defined above. Three data points for 3-MH were above 5000 ng/L (i.e., the highest point in the method calibration), so these values were extrapolated using the calibration function.

Compared to the other samples, Sauvignon Blanc wines were characterized by higher concentrations of 4-MMP, 3-MH, and 3-MHA (Figure 3), in accord with the varietal nature of these three compounds and the abundance of nonvolatile thiol precursors in Sauvignon Blanc grapes.[6,34] The other wine styles had 3-MH and 3-MHA concentrations frequently above their detection thresholds (see Figure 1), meaning these thiols are likely to contribute characteristic tropical and citrus notes to those wines. The exception to this was for 3-MHA in Chardonnay, where only two wines were found to contain suprathreshold concentrations. With respect to 4-MMP, aside from Sauvignon Blanc, only a small number of rosé and the majority of Pinot Noir wines in this study contained quantities which might be relevant to sensory profiles. A previous study, limited to two Oregon Pinot Noir wines, did not find 4-MMP to be an important aroma contributor,[35] so this current finding opens the pathway for further investigation of the impact of 4-MMP on the aroma of Pinot Noir wines.

The remaining two thiols, FT and BM, were often above their respective aroma detection thresholds (Figure 3, and see Figure 1), which accords well with results from a previous study.[7] Higher concentrations of FT, which is a Maillard reaction product associated with contact of wine with toasted oak,[36] were generally found in the Chardonnay wines, and this variety is often aged in oak. In the case of the other wines, apart from Pinot Noir, oak contact is not a likely contributor but acid-catalyzed sugar degradation may provide sources of furanyl precursors,[37] which produce FT by combining with the H_2S present in wine.[38] Although the formation pathway for BM is inconclusive (it may involve H_2S and benzaldehyde in a similar manner to FT formation), it can be found in white and red wines,[39] and it has been associated with the extent of bottle aging for Champagne.[40] The results in Figure 3 show that some examples of red and sparkling wines had a tendency to have lower concentrations of BM, whereas Sauvignon Blanc, Pinot Gris, Chardonnay, and rosé wines generally had slightly higher amounts. To some extent, these wines may exhibit aromas reminiscent of smoke and struck flint, which can both be attributable to BM.[39] The suprathreshold occurrence of BM in Sauvignon Blanc, Chardonnay, and rosé wines was not surprising,[7] although further studies of the prevalence of BM and its contribution to aroma in Pinot Gris are warranted, as suggested above with 4-MMP in Pinot Noir wines.

CONCLUSIONS

A novel polyfunctional thiol derivatization and SIDA quantification method has been developed, validated, and applied to a range of wine samples. Thiols and deuterated standards were derivatized with DTDP in wine without prior pH adjustment, isolated and concentrated with SPE, and analyzed by HPLC-MS/MS in MRM mode. Method performance was directly comparable to other more-laborious methods and the procedure allowed for simpler determination of wine thiols at concentrations close to their aroma detection thresholds. Importantly, DTDP is classified as "not dangerous goods", according to dangerous goods regulations, in comparison to other reagents currently used for thiol extraction or derivatization such as mercury complexes, or 2,3,4,5,6-pentafluorobenzyl bromide and 1,8-diazabicyclo[5.4.0]undec-7-ene, respectively.

Analysis of commercial wines revealed the presence of 3-MH, 3-MHA, 4-MMP, FT, and BM at concentrations that are related to grape variety and winemaking practices. The elevated concentrations of 4-MMP in Pinot Noir and BM in Pinot Gris were of particular interest, and these findings pave the way for further studies into the importance of these potent aroma compounds to these grape varieties. Furthermore, the method may find application in other aqueous matrices, particularly where the presence of thiols is important to the aroma characteristics and quality of the products.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org/.

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Author Contributions

D.L.C. performed the design of experiments, optimization of SPE and MS parameters, method validation, wine survey, data analysis, manuscript preparation, and editing; R.R. performed the design of experiments, optimization of derivatization procedure and HPLC parameters, data analysis, and manuscript editing; K.H.P. performed the synthesis and characterization of standards, and manuscript editing; and D.W.J. performed the conception of study, design of experiments, optimization of HPLC and MS parameters, data analysis, and manuscript preparation and editing.

Notes

The authors declare no competing financial interest.

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