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Highlights

- High throughput derivatization and isolation of thiols from wine.
- Highly selective and sensitive MS detection of thiol derivatives by Orbitrap HRMS.
- Parallel reaction monitoring (PRM) suitable for quantitation of trace thiols.
- First quantitative datasets reported for several recently identified thiols.

Journal Pre-proof

Quantitation of trace polyfunctional thiols in wine by liquid chromatography quadrupole Orbitrap high-resolution mass spectrometry in parallel reaction monitoring

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Abstract: Polyfunctional thiols are key contributors to wine aroma due to their extremely low odor thresholds, and their quantitative analysis remains challenging as a result of their ultra-trace concentrations and high reactivity. This work presents the first method based on Orbitrap high-resolution mass spectrometry (HRMS) in parallel reaction monitoring (PRM) mode for quantifying thiols at ng/L levels in wine. Thiols in wine were derivatized using 4,4'-dithiodipyridine and isolated by liquid-liquid extraction. This protocol allowed rapid sample preparation with minimum labor input and low consumable expenses. Instrumental analysis was conducted using ultra-high-performance liquid chromatography (UHPLC) coupled to quadrupole Orbitrap HRMS in PRM mode. Twenty thiol analytes, including literature-known, recently identified, and novel thiols were selected and validated by the optimized method in three wine matrices. The overall analytical performances demonstrated by this method were equivalent, and in most cases, greater than many previously developed GC–MS or LC–MS methods. The validated method was applied to analyze a selection of wines in which 12 thiols were quantified.

Keywords: Wine, Thiols, Quantitation, Orbitrap, Parallel reaction monitoring.

1. Introduction

Polyfunctional thiols, also known as varietal thiols, are a subclass of volatile sulfur compounds that have critical organoleptic impacts on the aroma quality of wine [1,2]. With odor detection thresholds in the range of ng/L, thiols are some of the most potent volatile compounds ever found in nature. The ongoing scientific interests surrounding thiols in wine arise from their odor potency and quality. A number of polyfunctional thiols contribute to typical nuances of wine aroma, including the pleasant notes such as “grapefruit,” “tropical fruit,” “coffee,” and “empyreumatic.” [3,4].

In wine, these sensorially impactful thiols usually occur at trace to ultra-trace levels (~ ng/L). As a result of such low abundance and the high reactivity of sulfhydryl group (–SH), the analysis of polyfunctional thiols has been challenging. Over the past three decades, a sizeable collection of analytical methods for thiol isolation and quantitation in wine became available [1]. As thiols are low-molecular-weight volatile analytes, many first developed quantitation methods were constructed using classic single dimension gas chromatography (GC) with mass spectrometry (MS) [5–8], and gradually, expanded on to more advanced GC–MS systems such as GC–MS/MS [9,10] and GC×GC–TOF/MS [11]. However, the sample preparation (e.g., affinity chromatography, derivatization, or solid-phase extraction) and instrumental analysis for most GC–MS methods can be lengthy and laborious, although a few automated protocols were proposed [12,13]. GC–MS had dominated the field of thiol analysis until liquid chromatography (LC) MS emerged as an alternative platform for thiol quantitation [14–17]. Not only demanding less sample preparation input but also offering superior analytical performances over GC–MS approaches, LC–MS based methods have been quickly catapulted to the frontline of routine analysis of thiols in wine.

To facilitate LC–MS analysis of thiols, suitable chemical derivatization is prerequisite for sample preparation. The derivatization usually involves a selected derivatization reagent reacting with –SH of thiols and forming the derivatives that are more suited for liquid chromatograph separation, electrospray ionization (ESI), and MS detection. Among many proposed derivatization reagents, 2-phenyl-1,2-benzoselenazol-3-one (ebselen) [18] and 4,4'-dithiodipyridine (DTDP) [14], likely due to their immediate availability and ease of derivatization, have been widely used for derivatizing thiols in wine (Figure 1A).

Ebselen was initially reported for derivatizing biological thiols (e.g., amino acids, peptides, and proteins) by reacting with –SH to form a selenosulfide bond [19]. Several sample preparation and LC–MS quantitation methods based this principle were subsequently developed for quantifying thiols in olive oil [18], coffee [20,21], and alcoholic beverages including beer and wine [16]. The simultaneous derivatization and extraction sample preparation protocols [16,18,20] were quick and simple and the following instrumental analysis was performed using Orbitrap high-resolution MS (HRMS) [16,18,20], reporting limit of quantitation (LOQ) as low as 0.01 ng/L. Several recently developed LC tandem MS (MS/MS) methods adapted ebselen in QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) workflow for only a few (≤ 3) thiols in wine [17,22–24] and beer [25], with [17,22,25] or without [23,24] targeting conjugated thiol precursors in a single analytical run. These demonstrated QuEChERS methods were generally lengthier than the simultaneous derivatization and extraction methods. As no enrichment step was included in QuEChERS workflows, higher LOQs from these methods were unsurprisingly observed.

DTDP was specifically proposed for varietal thiol analysis in wine [14]. It reacts rapidly with thiols through thiol-disulfide exchange at wine-pH to form pyridyl derivatives that were analyzed by LC–ESI–MS/MS in multiple reaction monitoring (MRM) mode [14]. The originally reported

sample preparation protocol consisted of chemical derivatization and SPE in a stable isotope dilution assay (SIDA) format. This SIDA SPE LC–MS/MS MRM method gave great analytical performances [14] and has since been widely adapted for both thiol quantitation and screening [15,26–28]. In a recent work, derivatization by DTDP was incorporated into QuEChERS for simultaneous extraction of three varietal thiols and three thiol precursors from Sauvignon blanc wines [22]. This QuEChERS method [22] required 2 hours waiting time at 4 °C after extraction, likely to promote the separation of organic layers from the formed emulsions, prior to the derivatization by DTDP.

The current LC–MS quantitation methods mentioned earlier, using either DTDP or ebselen as derivatization reagents, cover a few thiol analytes (≤ 5), require manual SPE cleanup or moderate waiting time, and some still struggled to provide sufficient repeatability, linearity, or LOQs.

In this study, we propose an ultra-high-performance liquid chromatography (UHPLC) quadrupole Orbitrap HRMS Parallel Reaction Monitoring (PRM) method for the quantitation of an expanded range of thiols in wine. Twenty selected thiol analytes included literature-known, newly identified, and previously unknown thiols in wine. First described is a simplified and cost-effective sample preparation procedure based on thiol derivatization using DTDP [14]. Next, UHPLC gradient was optimized for selected thiol analytes. Afterwards, quadrupole Orbitrap HRMS was compared for three acquisition modes and PRM that provided the highest selectivity was optimized for the final quantitation method. Method validation conducted in three wine matrices demonstrated great analytical performances. To our knowledge, this is the first time that an UHPLC quadrupole Orbitrap HRMS PRM quantitation method was reported for thiol quantitation in wine. The validated method was employed to screen a set of commercial wines in

which 12 thiols were detected. The findings presented in this study offer new advances and opportunities for thiol research in wine and beyond.

2. Materials and Methods

2.1. Chemicals.

Organic solvents (Fisher Scientific, Illkirch-Graffenstaden, France) used were of HPLC grade or higher: ethanol (EtOH), methanol, dichloromethane (CH₂Cl₂), acetonitrile (ACN, LC/MS grade). Chemicals used were of analytical reagent grade or higher. Formic acid (LC/MS grade), 4,4'-dithiodipyridine (98%), and acetaldehyde (99%) were obtained from Fisher Scientific. Water was purified through a Milli-Q purification system (Merck Millipore, Guyancourt, France). Ethylenediaminetetraacetic acid disodium salt (EDTA 2Na, ultra-pure) was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Model wine solution consisted of 12% ethanol and 5 g/L of (L)-tartaric acid in Milli-Q water with pH adjusted to 3.2 using a 5M sodium hydroxide solution.

2.2. Reference standards and standard solutions.

Analytical reference standards of thiols (**1–20**, **Figure 1B**) were purchased from commercial suppliers: 2-methyl-3-tetrahydrofuranthiol (*cis*-**1**, **1a**; *trans*-**1**, **1b**; ≥97%, racemic mixture, Sigma-Aldrich), 2-furanmethanethiol (**2**, ≥98%, Sigma-Aldrich), ethyl 3-sulfanylpropanoate (**3**, >98%, TCI, Zwijndrecht, Belgium), 4-methyl-4-sulfanylpentan-2-one (**4**, ≥95%, Sigma-Aldrich), ethyl 2-mercaptopropionate (**5**, ≥97%, TCI), 3-sulfanylhexasan-1-ol (**6**, ≥95 %, Sigma-Aldrich), thiophen-2-ylmethanethiol (**7**, ≥95%, TCI), 2-(1-mercaptoethyl)furan (**8**, ≥95%, Enamine, Kyiv, Ukraine), 2-methyl-3-furanthiol (**9**, ≥95%, TCI), phenylmethanethiol (**10**, ≥99%, Sigma-Aldrich), 3-sulfanyl-1-heptanol (**11**, ≥95%, Eptes, Vevey, Switzerland), 1-phenylethanethiol (**12**, ≥98%,

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Thermo Scientific Chemicals), 3-sulfanylhexyl acetate (**13**, $\geq 98\%$, Sigma-Aldrich), 2-phenylethanethiol (**14**, $\geq 97\%$, TCI), *m*-thiocresol (**15**, $\geq 98\%$, TCI), *o*-thiocresol (**16**, $\geq 97\%$, TCI), *p*-mentha-8-thiol-3-one (*cis*-**17**, **17a**; *trans*-**17**, **17b**; $\geq 97\%$, racemic mixture, TCI), 1-octanethiol (**18**, $\geq 98\%$, TCI), 4-methoxy-2-methyl-2-butanethiol (**19**, $\geq 98\%$, Sigma-Aldrich), and 2,5-dimethylfuran-3-thiol (**20**) (BLDpharm, Namiki Shoji Co., Tokyo, Japan). Purity of the thiol reference standards was independently verified by GC-MS/O analyses. The p*K*_a of thiol analyte was estimated using MarvinSketch software package (version 24.1.2, ChemAxon, Budapest, Hungary) [29].

Stock solutions of individual reference standard and the internal standard were prepared by weighing pure compound on a balance and then dissolving them in EtOH to reach to desired concentration (~ 1 g/L). Stock solutions were derived from stock solutions by mixing and diluting to afford standard working solutions of desired concentrations. All solutions were stored in glass vials filled with N₂ and kept at -20 °C. After 30 days of preparation, all solutions were discarded, and a new set of solutions was re-prepared to ensure the integrity of standard solutions.

2.3. Wine samples.

A selection of commercial wine samples ($n = 27$), consisting of red ($n = 12$, red blend, Point Noir, Syrah) white ($n = 15$, Sauvignon blanc, Sauvignon blanc/Sémillon blend, Riesling) from France, New Zealand, South Africa, and Germany, was purchased from local retailers in Bordeaux, France. The selection of these wine was based on their variety, region, vintage, and their availability in local retailers. Basic sample information including variety, vintage, alcohol content (abv, %), and origin of these wines is provided in **Table S1**.

2.4. Sample preparation.

2.4.1. *Initial investigation of two ebselen methods.*

Two previously published sample preparation methods using ebselen [16,25] were tested. In duplicate or triplicate, a model wine/dry Riesling/red blend bag-in-box wine was spiked with known amounts of a mixture of thiol standards to reach one or multiple levels of standard additions. The fortified wines were extracted.

For the simultaneous derivatization/extraction method tested [16], 20 mL of wine sample was spiked with a 100 μ L of internal standard solution, 400 μ L of EDTA solution (6 g/L in H₂O), and 6 mL of 0.1 mM ebselen in CH₂Cl₂. The mixture was vortexed for 1 min and centrifuged. One mL of the organic phase was pooled and dried under N₂. The dried residues were redissolved in 100 μ L of LC-MS grade MeOH and stored at -20 °C pending analyses. The second protocol assessed was based on QuEChERS sample preparation [25]. Briefly, wine sample (25 mL) was transferred to a 50 mL centrifuge tube. A 6 mL of ebselen (0.6 g/L in 1:1 EtOH/ACN) was added. The mixture was vortexed for 1 min. QuEChERS salts (12 g of magnesium sulfate, 3 g of sodium chloride, 3 g of sodium citrate tribasic dehydrate, and 1.5 g of sodium citrate dibasic sesquihydrate) were added, and the mixture was vortexed for 1 minute. The mixture was centrifuged and left standing at room temperature for the organic phase to separate. An aliquot organic phase was collected, filtered (0.22 μ m), and kept at -20 °C. The final extracts prepared from either protocol were analyzed by a workable UHPLC quadrupole Orbitrap HRMS methods operated in Full Scan/t-SIM/PRM modes.

2.4.2. *Proposed sample preparation.*

The sample preparation method consisted of chemical derivatization and liquid-liquid extraction cleanup. The derivatization followed a previously described protocol [14] with

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modifications. Briefly, an aliquot of 20 mL of wine was transferred into a 50 mL centrifuge tube. To the wine sample were added with 100 μ L of internal standard solution (**19**), 100 μ L of 6 g/L aq. EDTA solution, 80 μ L of ice-cold acetaldehyde/H₂O (50/50, v/v) solution, and 400 μ L of 10 mM DTDP solution. The sample was briefly vortexed and left to stand at room temperature for 30 min. Afterwards, an aliquot of CH₂Cl₂ (5 mL) was added to the sample mixture and was thoroughly vortex-mixed for 1 min and centrifuged. The organic phase (1 mL) was pooled and evaporated in a vacuum evaporator (Labconco, Kansas City, MO) to dryness. The dried residual was redissolved in 100 μ L of LC–MS grade MeOH and stored at –20 °C pending UHPLC quadrupole Orbitrap HRMS analysis. Three organic solvents (CH₂Cl₂, diethyl ether, and n-pentane) and five different solvent volumes (2, 3, 5, 6, 9 mL) were tested and the optimal parameters selected for the final protocol are underlined.

2.5. Liquid chromatography.

UHPLC was performed using a Vanquish system (Thermo Fisher Scientific, Bremen, Germany) consisted of a binary pump, an autosampler, and a column chamber. An ACQUITY premier BEH C18 column (1.7 μ m, 130 Å, 2.1 \times 150 mm, Waters, Guyancourt, France) with a VanGuard FIT cartridge (Waters) was used for chromatographic separation. Column temperature was maintained at 30 °C. The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Flow rate was set at 0.3 mL/min. The gradient for mobile phase B was as follows: 0 min, 15%; 10 min, 20%, 18 min, 35%, 25.1 min, 98%; 31 min, 98%; 31.1 min, 15%; 35 min, 15%. The injection volume was 2 μ L. The autosampler tray was maintained at 10 °C.

2.6. Quadrupole Orbitrap high resolution mass spectrometry (HRMS).

HRMS analyses were performed using a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization source (HESI-II) probe operated in positive-ion mode. Key ionization and spectrometric parameters were as follows: spray voltage, +3 kV; capillary temperature, 290 °C; sheath gas, flow rate: 60 arbitrary unit; auxiliary gas flow, 20 arbitrary unit; spare gas, 0 arbitrary unit; HESI-II probe temperature, 290 °C; S-Lens RF level, 45. Mass spectrometer was calibrated with a PierceTM Ion calibration solution (Thermo Fisher Scientific). Instrument control and data acquisition were conducted using Thermo Fisher Scientific software Xcalibur (version 4.3) and Tune Plus (version 2.9).

2.7. *Quadrupole Orbitrap HRMS acquisition.*

Full Scan, t-SIM, t-SIM/dd-MS², and PRM acquisition modes were evaluated. A summary of the major parameters tested for each acquisition mode are listed and the final parameters for PRM quantitative method are underlined in bold: (1) Full Scan, resolution (full width at half maximum, FWHM, at m/z 200) 35,000 / 70,000 / 140,000, automatic gain control (AGC) target $3e^5$ / $1e^6$ / $5e^6$, maximum injection time (IT) 200 ms, scan range 50–350 m/z ; (2) t-SIM, resolution 35,000 / 70,000 / 140,000, AGC target $5e^4$ / $2e^5$ / $5e^6$, maximum IT 50 ms / 100 ms / 200 ms, isolation window 1.0 / 2.0 / 4.0 m/z ; (3) t-SIM/dd-MS², for SIM: resolution 70,000 / 140,000, AGC target $5e^4$ / $2e^4$ / $2e^5$, maximum IT: 100 ms, isolation window: 4.0 m/z , for dd-MS²: resolution 35,000 / 70,000, AGC target $2e^5$ / $1e^6$, maximum IT 100 ms, loop count 5, top N 5, isolation window 4 m/z , NCE 15 / 25 / 35 / 45 / 55 / 65; (4) **PRM**, resolution **17,500** / 35,000 / 70,000 / 140,000, AGC target $5e^4$ / **$2e^5$** / $1e^6$ / $5e^6$, maximum IT **100 ms**, isolation window: **1 m/z** / 2 m/z / 4 m/z , normalized collision energy (NCE) 10 / 20 / 30 / 35 / 40 / 50. The optimization of NCE were performed with UHPLC–HRMS injections. **Table 1** summarizes thiol analyte information, exact

masses, accurate masses, retention times, precursor ions, and selected product ions of the derivatized thiols.

2.8. Data processing.

Raw MS data was processed using Xcalibur software with a mass extraction window of 5 ppm, a Gaussian smoothing of 7 points, and Genesis peak algorithm. Peak areas were used for integration. Thiol **9** encountered persistent and visible baseline noises across all runs during its elution region (Figure 2), so only the peak areas detected above noises were integrated. Qualifier and quantifier ions used for each analyte are listed in **Table 1**.

2.9. Analytical method validation.

The validation of the analytical method was conducted in model wine, a 2022 bag-in-box red wine, and a 2021 dry Riesling wine. In triplicate, a series of standard addition samples was prepared to reach 9 levels of concentrations for each thiol in three wine matrices. The levels of standard additions spanned corresponded to 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, and 1-fold of the maximum calibration concentration of each thiol analyte. A plot of peak area ratio (y) between analyte/internal standard against analyte concentration (x) was constructed. Simple linear regression functions were derived from triplicate 9-point standard addition curves to obtain slope, intercept, and coefficient of determination (R^2). D'Agostino-Pearson normality test (omnibus K_2 , $\alpha = 0.05$) was calculated to examine the linearity of calibration curves. Method trueness was assessed by recovery experiment conducted at three different concentrations of each analyte (0.1, 0.3, and 0.5-fold of its upper calibration range limit, respectively) in triplicate in separate wine matrices. Recovery was calculated as the percentage (%) of found concentration/added concentration of each analyte. Precision (repeatability) was determined based on relative standard

deviation (% RSD) of five replicates of samples spiked at two different concentration levels (0.1 and 0.3-fold of the upper calibration range limit for each analyte). Method limit of detection (LOD) and limit of quantitation (LOQ) were estimated by visual inspection of the chromatograms of wine samples supplemented with thiol reference standards at the lowest calibration concentrations. Method LOD and LOQ were calculated as 3 and 10 times of the signal-to-noise ratio S/N, respectively. Progressively lower levels (final concentrations at 0.02 ng/L, 0.1 ng/L, 0.5 ng/L, and 2.5 ng/L) of each analyte were prepared in three matrices (model wine, a dearomatized Riesling wine, and a dearomatized red wine) for method LOD and LOQ S/N verification. The two dearomatized wines were prepared by adding charcoal (10 g/L) to wine, and stirring for 1 hour, followed by 3 weeks of cold settle at 4 °C in dark. Spiking of thiol standards to the dearomatized wines was done within 1 hour prior to the sample preparation.

2.10. Quantitation of thiols in wines.

In triplicate, a set of calibration samples including quality control (QC) samples was prepared in matching wine matrix within each batch of samples to be quantitated.

2.11. Statistical analyses.

Initial data reduction and sorting, the calculation of mean values, standard deviations (SD), relative standard deviations (RSD), and linear regressions were performed in Microsoft Excel.

3. Results and Discussion.

3.1. Analyte selection.

Figure 1 presents the chemical structures of 19 thiols included in this study. The analyte range covered five most well-known wine thiols (i.e., **3**, **4**, **9**, **10**, **13**), several recently identified

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thiols (e.g., **1b**, **7**, **12**, **14**, **15**, **16**) in wine, and some previously unknown thiols in wine of similar structural and odor properties previously reported in fruits (**14** [30]) or model systems (**8** [31]). To our knowledge, this accounts the largest coverage of thiols quantitatively analyzed in wine.

3.2. Sample preparation.

For quantitation of thiols in wine by LC–MS, most methods rely on the use of ebselen or DTDP as derivatization reagent (Figure 1A). We initially tested a simultaneous derivatization/extraction procedure that utilized ebselen to derivatize volatile thiols in wine [16]. A series of calibration samples in model or real wines were prepared and analyzed, but poor repeatability (and therefore accuracy and linearity) was observed for several analytes, particularly for 2-methyl-3-furanthiol (**9**) (quantifier m/z 390.0050, data not shown), reflecting the previously reported validation data [16] showing 4 to 38% intra-day repeatability variations and modest linearity values ($R^2 > 0.977$). Similarly, the linearity ($R^2 > 0.96$) and repeatability variations derived from another ebselen-based protocol also appeared to be unsatisfactory [22]. However, neither work [16,22] analyzed **9** or its structural analogues. According to our observations, the poor repeatability was unrelated to the internal standard (**19**) which had consistent peak areas across the samples/runs. The issue was apparently to be analyte-dependent (i.e., only for **9**). In addition, unsatisfactory results were observed when the attempts were made to analyze another structurally similar thiol **20** (data not shown) further indicating a likely limited reactivity between furan-3-thiols **9**, **20** and ebselen. Ebselen is a selenium-nitrogen bond containing heterocycle and when reacting with thiols, it undergoes ring opening to furnish selenosulfide (Figure 1A), which was described as the nucleophilic attack of the thiolate at the electrophilic selenium center leading to ring opening [32]. In another study on the reactivity between alkanethiols and 2-(4-methylphenyl)-

1,2-benzisothiazol-3(2*H*)-one (structurally similar to ebselen) [33,34], the reaction first proceeded through the nucleophilic attack of the sulfhydryl hydrogen atom by the carbonyl oxygen atom to form a thiolate anion. The nucleophilicity of carbonyl oxygen atom was due to the electron donation from the nitrogen lone pair. The thiolate anion subsequently attacked electrophilic selenium atom resulting ring opening. The intermediate formed underwent tautomerization to form the final product. However, the reaction conditions in these studies (e.g., physiological pH >7, CH₂Cl₂) were different from wine-like condition (i.e., aq. solution with pH 3–4). The reactivity of thiols was mostly determined by two opposing factors: the nucleophilicity and basicity of thiol group [35]. To gain mechanistic insights, we therefore calculated the p*K*_a of all thiol analytes (**Figure 1**). The p*K*_a of the two furan-3-thiols **9** and **20** were at 4.81 and 4.46, respectively, significantly lower than that of all other thiols usually ranging from 7 to 10, including tetrahydrofuranthiol **1** at 9.79. The low p*K*_a of furan-3-thiols is likely to be a result of electron delocalization and resonance stabilization of the conjugate base that contains an aromatic furan ring. In wine-like condition, it was probable that the nucleophilicity was the predominant factor as the thiols with a high p*K*_a (stronger nucleophile) was favored whereas the thiols with a low p*K*_a (weaker nucleophile) appeared to be unfavored for reacting with ebselen.

A recently reported QuEChERS protocol [25] based on the same ebselen derivatization principle was also evaluated. After QuEChERS extraction, the resulting extracts from the tested red wine sample (2022 vintage, red blend, oaked, bag-in-box) exhibited pink color. This indicated a certain level of colored components being coextracted by the QuEChERS protocol, which was not observed with the first tested simultaneous derivatization/extraction protocol [16] nor in our later proposed protocol. The coextractions of interferences during QuEChERS led to elevated instrument baseline noises (data not shown). Moreover, as no enrichment step employed in the

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QuEChERS protocol, detection of some ultra-trace thiols (e.g., 2-furfuryl thiol **2**, phenylmethanethiol **10**) in red wines became increasingly difficult. This was mirrored by the higher LOQs reported in several studies using similar QuEChERS protocols for the sample preparation and thiol quantitation [22]. Given the observations detailed above, efforts to further optimize QuEChERS or any other ebselen-based sample preparation were no longer pursued.

Sample preparation method development using DTDP as thiol derivatization reagent was explored. After reviewing the originally developed protocol [14] and its various successful adaptations [15,22,28,36], we demonstrate a simplified sample preparation protocol consisting of thiol derivatization by DTDP and a one-minute liquid-liquid extraction (LLE) cleanup. Readers are referred to the section **2.4.2. in Materials and Methods** for a complete description of the method.

In our proposed method, a slightly increased amount of DTDP was used to ensure a complete derivatization of a larger set of thiol analytes. After derivatization, an optimized simple LLE cleanup was introduced. Three organic solvents CH₂Cl₂, diethyl ether, and *n*-pentane were compared. Higher peak areas of analytes were observed in extracts prepared from using CH₂Cl₂ than those from using *n*-pentane or diethyl ether. Different volumes (2 mL, 3 mL, 5 mL, 6 mL, and 9 mL) of CH₂Cl₂ were also assessed. Neither 2 mL nor 3 mL of CH₂Cl₂ was sufficient in yielding 1 mL of organic layers from the formed emulsions despite the higher peak areas detected. Increasing CH₂Cl₂ volume (5 mL, 6 mL, and 9 mL) resolved the issue of emulsions, but larger volumes led to a decrease in the peak areas of thiol-DTDP derivatives. Compared with the analyte peak areas obtained from using 2 mL CH₂Cl₂, an average 77% peak area decrease was observed when 9 mL of CH₂Cl₂ was utilized. A scatter plot depicting the analyte peak areas in response to different CH₂Cl₂ volumes is given in Figure S1. A moderate volume of CH₂Cl₂ (5 mL) was selected

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for a total of 20 mL of wine, in order to easily pool enough organic layers from the sample that can yield sufficient peak detection. This solvent-to-sample ratio was mirrored by a similar protocol (6 mL vs. 20 mL) mentioned previously [16].

Compared with previously reported sample preparation methods, this proposed protocol offered the following advantages. First, using a quick LLE (1 min) to replace manual SPE clean-up [14], the sample preparation became more simplified and more cost-effective (no SPE consumables required). Extracting derivatives with CH_2Cl_2 achieved good analyte yield from complex wine matrix (e.g., no coextractions of colored components). The non-water miscible CH_2Cl_2 gave instant separation of organic phase after centrifugation, without the need to wait for phase separation. Moreover, recovering the analytes by evaporating CH_2Cl_2 extracts either using a nitrogen flow or a vacuum evaporator (~ minutes) was much faster than that for MeOH or acetonitrile, therefore significantly increasing the sample preparation throughput. More importantly, the proposed sample preparation enabled the subsequent chromatographic and mass spectrometry analysis of thiol analytes investigated. The resulting method merits were at least equal, and in many cases, superior to those of many previous methods. The relevant detail is presented in the following discussions.

3.3. UHPLC

A recently reported UHPLC gradient [36] was modified for the chromatograph separation of derivatized thiols of interests. After optimization, baseline separation for 20 thiol analytes (including two pair of diastereomers of **1** and **17**) was achieved within a 35 min run (including equilibrium time). **Figure 2** depicts the chromatographic separation of thiol analytes using the final UHPLC program on a C18 column. The retention time and quantifier ions are given in **Table 1**.

3.4. UHPLC quadrupole Orbitrap HRMS method development.

Liquid chromatography hyphenated system for quantifying of thiols in foods and beverages was dominated by conventional tandem quadrupole (QqQ) MS or time-of-flight (qTOF) MS instrumentation (as reviewed in reference [1]). In comparison, Orbitrap HRMS is not frequently utilized. Regardless, the Exactive Orbitrap HRMS analyzers offer several acquisition modes resembling those by QqQ or qTOF MS instrument but significant technicality differences exist among different types of MS instruments [37].

Exactive Orbitrap HRMS analyzer was only reported for detecting thiols in the form of their thiol-ethylselen derivatives in a few limited cases [16,18,21] and had not been applied for assaying thiol-DTDP derivatives. Our investigation began by derivatizing each thiol analyte (final concentration ~2,000 ng/L) with DTDP (200 μ L, 10 mM) in a model wine solution (20 mL). The prepared thiol-DTDP derivatives were assessed by UHPLC quadrupole Orbitrap HRMS in full MS scan mode. **Table 1** presents the retention time and accurate mass measurements of the protonated precursor ions $[M+H]^+$. The observed mass errors for protonated thiol-DTDP derivatives were between -3.78 to -0.41 ppm (**Table 1**). Although full MS scan is the most universal acquisition mode for Exactive Orbitrap (and other types) MS analyzer, other available acquisition modes might be better suited for quantification tasks in complex matrix [38,39]. All previously demonstrated Exactive Orbitrap HRMS quantifications of thiols (as thiol-ethylselen derivatives) were operated in full MS scan in conjunction with all ion fragmentation (AIF) scan [16,18,21], an acquisition mode consisted of precursor ion scans followed by product ions scans from all incoming precursors ions [38]. However, the exact reason for choosing full MS/AIF acquisition mode for targeted thiol quantitation was not elaborated [16,18,21]. Regardless, full MS/AIF

acquisition mode could be viewed as a form of data independent acquisition (DIA) and has shown promising capabilities in nontargeted profiling in proteomics [40] and in metabolomics [41]. In this study, we evaluated the following acquisition modes t-SIM, t-SIM/ddMS², and PRM.

3.4.1. t-SIM for sensitivity, PRM for selectivity.

Three acquisition strategies (t-SIM, t-SIM/ddMS², and PRM) of higher selectivity than full MS scan were evaluated. The retention time and accurate mass of protonated thiol-DTDP derivatives acquired by full MS scan were used to build the mandatory *inclusion list* for t-SIM, t-SIM/dd-MS² and PRM. The comparison of three acquisition modes was conducted in three wine matrices (model wine, a 2021 unoaked Riesling wine, and a 2022 oaked bag-in-box red blend). The wines were supplemented with known amounts of reference thiol standards. Thiols were isolated with the proposed sample preparation method and analyzed. The collected quadrupole Orbitrap HRMS data were examined for selectivity (coelutions) and sensitivity (peak intensity of targeted analytes).

In the example of **1a** and **1b** analyzed by three acquisition modes, shown in **Figure 3A**, both t-SIM and t-SIM/dd-MS² yielded substantial higher sensitivity than PRM. The HRMS signal intensity for the precursor ion m/z 228.0506 was approximately five-fold higher for t-SIM or t-SIM/ddMS² than that for PRM. This observation was not surprising, because PRM is a fragmentation-based acquisition mode. Therefore, when a precursor ion was used as quantifier ion, it would usually result in a lower intensity in PRM data than that from t-SIM acquisition using the same precursor ion as quantifier ion.

By contrast, PRM offered greater selectivity than both t-SIM and t-SIM/ddMS². As indicated in **Figure 3A**, t-SIM and t-SIM/ddMS² XICs of **1a** and **1b** displayed visible baseline

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noises and shouldering peaks which were completely absent in PRM data. This was a crucial benefit for analyzing trace analytes such as thiols, because their chromatographic peaks were usually small and therefore are more susceptible to interfering ions causing peak deformation. More importantly, as PRM employs higher-energy collisional dissociation (HCD) to induce fragmentation to be scanned by Orbitrap analyzer for the entire m/z range, the resulting mass spectra gave enhanced selectivity through flexible data processing. This is clearly exemplified for two isomers **2** and **9** (**Figure 3B**). When extracting chromatogram using the precursor ion (m/z 224.0192), baseline signals were evidently observed. When the product ion (m/z 143.9936) was applied to the same chromatogram as the quantifier ion, no signal was recorded (data not shown). XICs of m/z 142.9860 showed a clean peak for **2**. The odd-electron ions for aromatic thiols (**2** and **10**) were previously reported and postulated [14]. However, this was not applicable for **9** which showed no detection of m/z 142.9860 according to our data. In addition, we did not observe the furfuryl cation (m/z 81.0335) for **3** as reported previously [14]. Additional potential quantifier ions (m/z 112.0220, 111.0142) were attempted (**Figure 3B**). The final selected quantifier ions are summarized in **Table 1**. Both examples in **Figure 3A** and **3B**, one with precursor ion and the other one with product ion(s) as quantifiers, highlighted superior selectivity through PRM acquisition.

3.4.2. PRM

Once PRM was decided for HRMS acquisition, further efforts were directed to optimize parameters for PRM. To facilitate readability, parameters termed in Thermo Fisher *Xcalibur* software suite (including *Free Style*) are *italicized*. The primary aim for our optimization was to achieve unequivocal identification and accurate quantitation of analytes. As for identification, collecting both characteristic product ion(s) and unfragmented precursor ion $[M+H]^+$ was critical

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and this was of particular interests for two considerations. The first reason was the sample preparation involving chemical derivatization. In the case of thiol derivatization by DTDP, most formed derivatives yielded several identical and abundant diagnostic product ions (e.g., m/z 143.9936) and this may pose identification issues in some extreme situations where precursor ions would be required as additional identification criteria. The second consideration was that the quadrupole in the Exactive Orbitrap has its limit in filtering precursor ions (isolation width). For instance, the exact mass of 3-sulfanylhhexan-1-ol (**6**) and ethyl 2/3-sulfanylpropanoate (**3** and **5**) differed only by 0.0364 m/z , and the same mass differences of their formed derivatives were under the isolation width limit of the quadrupole ($\pm 0.2 m/z$) of the instrument used. The derivatives of **6** (m/z 244.0824), **3** (m/z 244.0461), and **5** (m/z 244.0461) were resolved chromatographically at baseline level (**Figure 2**). Their respective PRM acquisition windows slightly overlapped. Surprisingly, we noticed that in the obtained *.RAW* file which was supposed to contain all scan *Filters* of the pre-defined precursor ions from the *Inclusion List*, the *Filter* for **3** was not computerized as a separate *Filter*. Instead, the scans of precursor ion **3** was merged under the header of the *Filter* for **6** (**Figure 3C**), as if that the *Xcalibur* software was merging *Scan spectrum* from two very similar precursor ions into one *Filter*. When mass spectrum of both peaks was examined, the header of the *Spectrum* correctly displayed the predefined precursor ions (**Figure 3C**) again. The noted PRM data computerization discrepancy would not prevent quantitative data processing, but this could be useful information for further refinement of software packages. The NCE regimes were individually optimized to yield both precursor ion and product ion(s) in the MS2 spectrum for each analyte, as shown in **Table 1**.

3.5. Method validation.

The optimized UHPLC quadrupole Orbitrap HRMS PRM method was validated according to the International Union of Pure and Applied Chemistry (IUPAC) guidelines for single laboratory validation of method analysis [42] for calibration range, linearity, trueness, precision, limit of detection (LOD), LOQ, and matrix effects. **Table 2** summarizes the method validation results.

The selected calibration ranges of known thiols covered their realistic concentrations in wines of interest. Simple linear regression was used to derive the calibration functions, which afforded R^2 values greater than 0.99 for all analytes in three wine matrices. Normality tests of residuals ($\alpha=0.05$) gave P values 0.16–0.43, passing the normality test, which further indicated that the fitting linear model was appropriate. The excellent linearity values for analyte concentrations spanning 3 orders of magnitudes derived by using just a commercially available non-stable isotopically labelled internal standard clearly outperformed those utilizing other non-SIDA internal standards [16,17,22,25], and surprisingly in some cases, were at a comparable [14] or better [15,22] quality than SIDA approaches.

Matrix effect was accessed by comparing the slope of the calibration equation obtained from real wine matrices to that from model wine. Soft matrix effect (–10% to 10%) was seen for 3 thiols (**1a**, **1b**, and **4**) in white wine matrix and 6 thiols (**1a**, **1b**, **3**, **4**, **10**, **12** and **17**) in red wine matrix, respectively. Strong matrix effect (<–50% or >50%) was observed only for **9**, as a result of elevated MS baseline (**Figure 2**). The efficiency of LLE clean-up, as well as the high selectivity of quadrupole Orbitrap HRMS operated in PRM mode could attribute to the performance of matrix effects.

Recoveries at three levels in three matrices ranged from 86.6% to 123.6% with most values fitting within $100 \pm 20\%$. One extreme recovery of 123.6% was observed for the last eluting

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analyte **18** in a more challenging red wine matrix. Better recoveries were shown in several SIDA-based methods [14,15,43]. Precision of the method expressed as the repeatability was less than 9.5% with a mean RSD at 4.1% across 19 analytes in three matrices. These values were at least comparable to, if not better than, previously developed HPLC–MS/MS [14,15,22,43,44] or LC–Orbitrap HRMS protocols [16]. Sensitivity was not estimated by experimental LOD and LOQ results other than by statistical approach using slope and standard deviation of the calibration curves. This was because the Fourier transformation process in the Orbitrap can lead to a “a detect or a not detect situation” [39] when analyte concentrations are exceedingly low. From the four spiking concentrations (0.02, 0.1, 0.5, 2.5 ng/L) and the lowest calibration level spiking tested for each analyte, method LOD was calculated as 3 times of the signal-to-noise ratio. The obtained method LODs ranged from 0.02 to 4.03 ng/L, comparable to previously reported data from Orbitrap results [16] and much lower than previously LC–QQQ–MS/MS results [14,15,22,27]. A compilation of chromatograms for method LOD in model wine is given in Figure S2 and S3 and the clean chromatograms (except for **9**) demonstrated the significant superior selectivity offered by Orbitrap HRMS PRM.

3.6. Thiols in wine by UHPLC–Quadrupole Orbitrap HRMS PRM.

To further assess the suitability of the method, a selection of commercial wines (n=27, **Table S1**) of various varieties, vintages, and regions was analyzed for thiol composition. The detected thiols are visualized as aligned dot plot and violin plot in **Figure 4**. Out of 20 thiol analytes validated for the method, 12 were detected at above LOQ levels in the selected wine samples. The most abundant thiol detected was 3-sulfanylhhexan-1ol (**6**). The highest level (2826 ng/L) of **6** was observed in a 2020 Sauvignon blanc wine from New Zealand. The average concentration of **6** in

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the Riesling and red wine samples were 919 and 388 ng/L, respectively. In all wines, **6** presented at concentration at least several fold higher than its odor threshold hold (60 ng/L in model wine [7]), confirming a rather universal and significant sensory contribution of **6** to the aroma of many varieties as previously demonstrated for Sauvignon blanc [7], Riesling [45], Chardonnay [46], and Cabernet Sauvignon [47]. Surprisingly, high concentrations (1424 ng/L) of **6** were noted in three aged dry Riesling wines from 2008 or 2009 vintage from Mosel. The preserve of thiols during white winemaking has been of high interests and as indicated by the data of **6** in aged Riesling wines, more attention is warranted for further investigation. The next most abundant thiol was 3-sulfanylpropanoate (**3**) that was previously identified in Concord grapes [48] and aged Champagne wines [49]. Again, the concentration of **3** was much higher in Sauvignon blanc (avg. 529 ng/L) or Riesling wines (avg. 583 ng/L) than that in the red wines (182 ng/L). The reported olfactory detection threshold of **3** was at 200 ng/L in water [48]. According to the observation that **3** exhibited a pleasant fruit aroma but changed to skunky or foxy odors at high concentrations [48], the aroma impacts of **3** on white and red wines could be different. 2-Methyl-3-furanthiol (**9**) was measured at an average concentration of 56 ng/L in all the wines, relatively similar among red wines (avg. 54 ng/L) and white wines (51 ng/L in Sauvignon blanc and 64 ng/L in Riesling). Comparing to its detection threshold at 4 ng/L in diluted alcohol solution [50], **9** could be estimated to readily contribute to the overall “grilled meat,” “toasty” aroma attributes in these wines. However, cautions should be exercised when interpret the quantitative results of **9**, considering the earlier note that peak area integration of **9** was performed with baseline correction to eliminate the visible noises. Other common thiols such as the potent 2-furanmethanethiol (**2**) usually related to ageing wine in new oak barrel, phenylmethanethiol (**10**), 3-sulfanyl-1-heptanol (**11**), 4-methyl-4-sulfanylpentan-2-one (**4**), ethyl 2-mercaptopropionate (**5**), 3-sulfanylhexyl acetate (**13**) were found

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at much lower concentrations than the major thiols. Amongst the wines, **4** was in higher concentrations in Sauvignon blanc, whereas **11** was more predominant in Riesling wines. The recently identified *trans*-2-methyl-3-tetrahydrofuranthiol (**1b**) [51] was found to range from 1.4 to 23.6 ng/L in the analyzed wine samples, still under its reported detection threshold at 55 ng/L [51]. Unexpectedly, higher concentrations (avg. 9.5 ng/L) were noticed in Riesling wines other than in Sauvignon blanc (avg. 4.9 ng/L) or red wines (avg. 5.8 ng/L). Out of two thiophenols, *m*-thiocresol (**15**) and *o*-thiocresol (**16**), **16** was quantified in most wine samples at extremely low concentrations (avg. 1.8 ng/L). **15** was not detected in any of the wine samples but occurred at ultra-trace levels in two Bordeaux red wine samples (vintage 2008, Merlot) which also contained another recently identified 2,5-dimethyl-3-furanthiol (**20**) [52] in ultra-low abundance (data not shown). The variation of thiol contents in the wines analyzed could raise from a number of factors, such as grape variety, grape growing condition, yeast strains, winemaking practices and ageing modalities (see review in reference [3]). The quantitative dataset obtained using the UHPLC–Orbitrap HRMS PRM method not only captured the well-known information of the obvious variety difference of common thiols (e.g., **4** and **6** rich in Sauvignon blanc), but also provided the first look of the content of some thiols (e.g., **1b** and **16**) that were only discovered in wine very recently. The coverage of the novel thiols that were usually at exceedingly low concentrations was attributed to the superior sensitivity and selectivity of Orbitrap HRMS. The quantitative data shown here would be beneficial for further research to understand the sensory impacts and formation pathways of these novel thiols in wine.

Other thiols thiophen-2-ylmethanethiol (**7**), 2-(1-mercaptoethyl)furan (**8**), 1-phenylethanethiol (**12**), 2-phenylethanethiol (**14**), and *p*-mentha-8-thiol-3-one (**17a** and **17b**) were not detected at above LOD levels in our wine samples. A few recent studies have identified **7**

(tentative identification) [51] and **12** [53] in aged red wines, but no quantitative data was reported so far. The absence of both thiols in our samples may indicate their likely occurrence in more aged wines. Less common thiols **8**, **14**, and **17** were not yet been mentioned in wine, so their non-detection in our wine samples is not surprising. Nevertheless, research efforts in our laboratory are ongoing for the identification of thiols. The quantitation of either recently identified and novel thiols may be readily to achieved upon screening a larger set of wine samples or upon their potential discovery in wine in the future.

4. Conclusion.

This work presents an UHPLC quadrupole Orbitrap HRMS PRM quantitation method for a wide range of ultra-trace thiol analytes in wine. The proposed sample preparation was simple and cost-effective. Orbitrap HRMS PRM demonstrated superior sensitivity and selectivity. The method was validated in model and real wine matrices, demonstrating good analytical performances. It was applied for the determinations of thiols in a set of commercial wine samples, providing more comprehensive thiol coverage. Limitations of this work include a relative long chromatograph run, a small set of wine samples screened, and the limited availability of Orbitrap HRMS in laboratories. Future work could shorten chromatograph, incorporate stable isotope labelled internal standards for a stable isotope dilution assay, include a more exhaustive list of known thiols for more comprehensive quantitation, or leverage the capability of Orbitrap HRMS to explore the presence of other novel thiol candidates in wine.

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CRedit Authorship Contribution Statement

L.C.: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Supervision, Project administration, Writing – Original draft. Writing – Review & Editing. **E.D.L.:** Methodology, Formal analysis, Investigation, Writing – Review & Editing. **M.G.:** Methodology, Writing – Review & Editing. **A.M.:** Resources, Writing – Review & Editing. **P.D.:** Conceptualization, Funding acquisition, Writing – Review & Editing.

Declaration of Interest

The authors declare no competing interests.

References

- [1] L. Chen, D.L. Capone, D.W. Jeffery, Analysis of potent odour-active volatile thiols in foods and beverages with a focus on wine, *Molecules* 24 (2019) 2472.
- [2] D.W. Jeffery, Spotlight on varietal thiols and precursors in grapes and wines, *Aust. J. Chem.* 69 (2016) 1323–1330. <https://doi.org/10.1071/CH16296>.

- [3] A. Roland, R. Schneider, A. Razungles, F. Cavelier, Varietal thiols in wine: Discovery, analysis and applications, *Chemical Reviews* 111 (2011) 7355–76. <https://doi.org/10.1021/cr100205b>.
- [4] D. Dubourdieu, T. Tominaga, Polyfunctional Thiol Compounds, in: M.V. Moreno-Arribas, M.C. Polo (Eds.), *Wine Chemistry and Biochemistry*, Springer New York, New York, NY, 2009: pp. 275–293. https://doi.org/10.1007/978-0-387-74118-5_15.
- [5] P. Darriet, T. Tominaga, V. Lavigne, J. Boidron, D. Dubourdieu, Identification of a powerful aromatic component of *Vitis vinifera* L. var. Sauvignon wines: 4-mercapto-4-methylpentan-2-one, *Flavour and Fragrance Journal* 10 (1995) 385–392.
- [6] T. Tominaga, L. Blanchard, P. Darriet, D. Dubourdieu, A Powerful Aromatic Volatile Thiol, 2-Furanmethanethiol, Exhibiting Roast Coffee Aroma in Wines Made from Several *Vitis vinifera* Grape Varieties, *Journal of Agricultural and Food Chemistry* 48 (2000) 1799–1802. <https://doi.org/10.1021/jf9906660r>.
- [7] T. Tominaga, M.-L. Murat, D. Dubourdieu, Development of a Method for Analyzing the Volatile Thiols Involved in the Characteristic Aroma of Wines Made from *Vitis vinifera* L. Cv. Sauvignon Blanc, *J. Agric. Food Chem.* 46 (1998) 1044–1048. <https://doi.org/10.1021/jf970782o>.
- [8] D.L. Capone, M.A. Sefton, D.W. Jeffery, Application of a Modified Method for 3-Mercaptohexan-1-ol Determination To Investigate the Relationship between Free Thiol and Related Conjugates in Grape Juice and Wine, *J. Agric. Food Chem.* 59 (2011) 4649–4658. <https://doi.org/10.1021/jf200116q>.
- [9] K. Takazumi, K. Takoi, K. Koie, Y. Tuchiya, Quantitation Method for Polyfunctional Thiols in Hops (*Humulus lupulus* L.) and Beer Using Specific Extraction of Thiols and Gas

Submitted Manuscript: Confidential

- Chromatography–Tandem Mass Spectrometry, *Anal. Chem.* 89 (2017) 11598–11604.
<https://doi.org/10.1021/acs.analchem.7b02996>.
- [10] N. Ochiai, K. Sasamoto, F. David, P. Sandra, Dual stir bar sorptive extraction using conventional and solvent-assisted approaches including in-situ derivatization followed by thermal desorption and gas chromatography–tandem mass spectrometry for analysis of polyfunctional thiols in white wine samples, *Advances in Sample Preparation* 3 (2022) 100034. <https://doi.org/10.1016/j.sampre.2022.100034>.
- [11] K. Reglitz, M. Steinhaus, Quantitation of 4-Methyl-4-sulfanylpentan-2-one (4MSP) in Hops by a Stable Isotope Dilution Assay in Combination with GC×GC-TOFMS: Method Development and Application To Study the Influence of Variety, Provenance, Harvest Year, and Processing on 4MSP Concentrations, *J. Agric. Food Chem.* 65 (2017) 2364–2372.
<https://doi.org/10.1021/acs.jafc.7b00455>.
- [12] L. Mateo-Vivaracho, V. Ferreira, J. Cacho, Automated analysis of 2-methyl-3-furanthiol and 3-mercaptohexyl acetate at ngL⁻¹ level by headspace solid-phase microextracion with on-fibre derivatisation and gas chromatography–negative chemical ionization mass spectrometric determination, *Journal of Chromatography A* 1121 (2006) 1–9.
<https://doi.org/10.1016/j.chroma.2006.04.029>.
- [13] L. Dagan, F. Reillon, A. Roland, R. Schneider, Development of a routine analysis of 4-mercapto-4-methylpentan-2-one in wine by stable isotope dilution assay and mass tandem spectrometry, *Analytica Chimica Acta* 821 (2014) 48–53.
<https://doi.org/10.1016/j.aca.2014.03.004>.
- [14] D.L. Capone, R. Ristic, K.H. Pardon, D.W. Jeffery, Simple Quantitative Determination of Potent Thiols at Ultratrace Levels in Wine by Derivatization and High-Performance Liquid

Submitted Manuscript: Confidential

- Chromatography–Tandem Mass Spectrometry (HPLC-MS/MS) Analysis, *Anal. Chem.* 87 (2015) 1226–1231. <https://doi.org/10.1021/ac503883s>.
- [15] L. Chen, D.L. Capone, D.W. Jeffery, Chiral analysis of 3-sulfanylhexan-1-ol and 3-sulfanylhexyl acetate in wine by high-performance liquid chromatography–tandem mass spectrometry, *Analytica Chimica Acta* 998 (2018) 83–92. <https://doi.org/10.1016/j.aca.2017.10.031>.
- [16] S. Vichi, N. Cortés-Francisco, J. Caixach, Analysis of volatile thiols in alcoholic beverages by simultaneous derivatization/extraction and liquid chromatography-high resolution mass spectrometry, *Food Chemistry* 175 (2015) 401–408.
- [17] L. Tonidandel, R. Larcher, A. Barbero, R.E. Jelley, B. Fedrizzi, A single run liquid chromatography-tandem mass spectrometry method for the analysis of varietal thiols and their precursors in wine, *Journal of Chromatography A* 1658 (2021) 462603. <https://doi.org/10.1016/j.chroma.2021.462603>.
- [18] S. Vichi, N. Cortés-Francisco, J. Caixach, Determination of volatile thiols in lipid matrix by simultaneous derivatization/extraction and liquid chromatography–high resolution mass spectrometric analysis. Application to virgin olive oil, *Journal of Chromatography A* 1318 (2013) 180–188.
- [19] K. Xu, Y. Zhang, B. Tang, J. Laskin, P.J. Roach, H. Chen, Study of highly selective and efficient thiol derivatization using selenium reagents by mass spectrometry, *Analytical Chemistry* 82 (2010) 6926–6932.
- [20] S. Vichi, Y. Jerí, N. Cortés-Francisco, O. Palacios, J. Caixach, Determination of volatile thiols in roasted coffee by derivatization and liquid chromatography–high resolution mass spectrometric analysis, *Food Research International* 64 (2014) 610–617.

- [21] B. Quintanilla-Casas, N. Dulsat-Serra, N. Cortés-Francisco, J. Caixach, S. Vichi, Thiols in brewed coffee: Assessment by fast derivatization and liquid chromatography–high resolution mass spectrometry, *LWT - Food Science and Technology* 64 (2015) 1085–1090. <https://doi.org/10.1016/j.lwt.2015.07.010>.
- [22] J.R. Muhl, M. Derycke, L.I. Pilkington, B. Fedrizzi, R.C. Deed, A green liquid chromatography-tandem mass spectrometry method for the simultaneous analysis of volatile thiols and their precursors in oenological samples, *Journal of Chromatography A* 1707 (2023) 464273. <https://doi.org/10.1016/j.chroma.2023.464273>.
- [23] S. Carlin, M. Piergiovanni, E. Pittari, M. Tiziana Lisanti, L. Moio, P. Piombino, M. Marangon, A. Curioni, L. Rolle, S. Río Segade, A. Versari, A. Ricci, G.P. Parpinello, G. Luzzini, M. Ugliano, D. Perenzoni, U. Vrhovsek, F. Mattivi, The contribution of varietal thiols in the diverse aroma of Italian monovarietal white wines, *Food Research International* 157 (2022) 111404. <https://doi.org/10.1016/j.foodres.2022.111404>.
- [24] T. Román, T. Tonidandel, R. Larcher, E. Celotti, G. Nicolini, Importance of polyfunctional thiols on semi-industrial Gewürztraminer wines and the correlation to technological treatments, *Eur Food Res Technol* 244 (2018) 379–386. <https://doi.org/10.1007/s00217-017-2963-6>.
- [25] R.E. Jelley, H. Jones-Moore, A. Guan, C.Z.-J. Ren, J.L.-Y. Chen, L. Tonidandel, R. Larcher, B. Fedrizzi, Simultaneous extraction, derivatisation and analysis of varietal thiols and their non-volatile precursors from beer, *LWT* 164 (2022) 113563. <https://doi.org/10.1016/j.lwt.2022.113563>.
- [26] Y. Yan, J. Lu, Y. Nie, C. Li, S. Chen, Y. Xu, Characterization of volatile thiols in Chinese liquor (Baijiu) by ultraperformance liquid chromatography–mass spectrometry and

Submitted Manuscript: Confidential

- ultraperformance liquid chromatography–quadrupole-time-of-flight mass spectrometry, *Front. Nutr.* 9 (2022) 1022600. <https://doi.org/10.3389/fnut.2022.1022600>.
- [27] Z. Wu, D. Qin, J. Duan, H. Li, J. Sun, M. Huang, B. Sun, Characterization of benzenemethanethiol in sesame-flavour baijiu by high-performance liquid chromatography-mass spectrometry and sensory science, *Food Chemistry* 364 (2021) 130345. <https://doi.org/10.1016/j.foodchem.2021.130345>.
- [28] M. Mafata, M.A. Stander, B. Thomachot, A. Buica, Measuring Thiols in Single Cultivar South African Red Wines Using 4,4-Dithiodipyridine (DTDP) Derivatization and Ultraperformance Convergence Chromatography-Tandem Mass Spectrometry, *Foods* 7 (2018). <https://doi.org/10.3390/foods7090138>.
- [29] J. Szegezdi, F. Csizmadia, Method for calculating the pKa values of small and large molecules, in: *Abstracts of Papers of The American Chemical Society*, Amer Chemical Soc 1155 16TH ST, NW, WASHINGTON, DC 20036 USA, 2007.
- [30] S. Schoenauer, P. Schieberle, Screening for novel mercaptans in 26 fruits and 20 wines using a thiol-selective isolation procedure in combination with three detection methods, *Journal of Agricultural and Food Chemistry* 67 (2019) 4553–4559. <https://doi.org/10.1021/acs.jafc.9b01242>.
- [31] T. Hofmann, P. Schieberle, Identification of Potent Aroma Compounds in Thermally Treated Mixtures of Glucose/Cysteine and Rhamnose/Cysteine Using Aroma Extract Dilution Techniques, *J. Agric. Food Chem.* 45 (1997) 898–906. <https://doi.org/10.1021/jf960456t>.
- [32] K. Amporndanai, X. Meng, W. Shang, Z. Jin, M. Rogers, Y. Zhao, Z. Rao, Z.-J. Liu, H. Yang, L. Zhang, P.M. O'Neill, S. Samar Hasnain, Inhibition mechanism of SARS-CoV-2

Submitted Manuscript: Confidential

- main protease by ebselen and its derivatives, *Nat Commun* 12 (2021) 3061.
<https://doi.org/10.1038/s41467-021-23313-7>.
- [33] N. Kamigata, M. Takata, H. Matsuyama, M. Kobayashi, Novel ring opening reaction of 2-aryl-1, 2-benzisoxazol-3 (2H)-one with thiols, *Heterocycles (Sendai)* 24 (1986) 3027–3030.
- [34] P.C. Bulman Page, R.D. Wilkes, D. Reynolds, 2.03 - Alkyl Chalcogenides: Sulfur-based Functional Groups, in: A.R. Katritzky, O. Meth-Cohn, C.W. Rees (Eds.), *Comprehensive Organic Functional Group Transformations*, Elsevier Science, Oxford, 1995: pp. 113–275.
<https://doi.org/10.1016/B0-08-044705-8/00156-4>.
- [35] P.A. Fernandes, M.J. Ramos, Theoretical Insights into the Mechanism for Thiol/Disulfide Exchange, *Chemistry – A European Journal* 10 (2004) 257–266.
<https://doi.org/10.1002/chem.200305343>.
- [36] Y. Yan, J. Lu, Y. Nie, C. Li, S. Chen, Y. Xu, Characterization of volatile thiols in Chinese liquor (Baijiu) by ultraperformance liquid chromatography–mass spectrometry and ultraperformance liquid chromatography–quadrupole-time-of-flight mass spectrometry, *Frontiers in Nutrition* 9 (2022).
<https://www.frontiersin.org/articles/10.3389/fnut.2022.1022600>.
- [37] A. Kaufmann, Analytical performance of the various acquisition modes in Orbitrap MS and MS/MS, *Journal of Mass Spectrometry* 53 (2018) 725–738.
<https://doi.org/10.1002/jms.4195>.
- [38] A. Kaufmann, M. Bromirski, Selecting the best Q Exactive Orbitrap mass spectrometer scan mode for your application, ThermoScientific. White Paper 65147 (2018).

- [39] A. Kaufmann, Analytical performance of the various acquisition modes in Orbitrap MS and MS/MS, *Journal of Mass Spectrometry* 53 (2018) 725–738. <https://doi.org/10.1002/jms.4195>.
- [40] T. Geiger, J. Cox, M. Mann, Proteomics on an Orbitrap Benchtop Mass Spectrometer Using All-ion Fragmentation *, *Molecular & Cellular Proteomics* 9 (2010) 2252–2261. <https://doi.org/10.1074/mcp.M110.001537>.
- [41] J. Zhou, Y. Li, X. Chen, L. Zhong, Y. Yin, Development of data-independent acquisition workflows for metabolomic analysis on a quadrupole-orbitrap platform, *Talanta* 164 (2017) 128–136. <https://doi.org/10.1016/j.talanta.2016.11.048>.
- [42] M. Thompson, S.L. Ellison, R. Wood, Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report), *Pure and Applied Chemistry* 74 (2002) 835–855.
- [43] A. Roland, S. Delpech, L. Dagan, M.-A. Ducasse, F. Cavelier, R. Schneider, Innovative analysis of 3-mercaptohexan-1-ol, 3-mercaptohexylacetate and their corresponding disulfides in wine by stable isotope dilution assay and nano-liquid chromatography tandem mass spectrometry, *Journal of Chromatography A* 1468 (2016) 154–163. <https://doi.org/10.1016/j.chroma.2016.09.043>.
- [44] L. Tonidandel, R. Larcher, A. Barbero, R.E. Jelley, B. Fedrizzi, A single run liquid chromatography-tandem mass spectrometry method for the analysis of varietal thiols and their precursors in wine, *Journal of Chromatography A* 1658 (2021) 462603. <https://doi.org/10.1016/j.chroma.2021.462603>.

- [45] A. Schüttler, M. Friedel, R. Jung, D. Rauhut, P. Darriet, Characterizing aromatic typicality of Riesling wines: merging volatile compositional and sensory aspects, *Food Research International* 69 (2015) 26–37. <https://doi.org/10.1016/j.foodres.2014.12.010>.
- [46] D. I. Capone, A. Barker, P. o. Williamson, I. I. Francis, The role of potent thiols in Chardonnay wine aroma, *Australian Journal of Grape and Wine Research* 24 (2018) 38–50. <https://doi.org/10.1111/ajgw.12294>.
- [47] M. Picard, C. Thibon, P. Redon, P. Darriet, G. de Revel, S. Marchand, Involvement of Dimethyl Sulfide and Several Polyfunctional Thiols in the Aromatic Expression of the Aging Bouquet of Red Bordeaux Wines, *J. Agric. Food Chem.* 63 (2015) 8879–8889. <https://doi.org/10.1021/acs.jafc.5b03977>.
- [48] M.G. Kolor, Identification of an important new flavor compound in Concord grape: ethyl-3-mercaptopropionate, *ACS Publications* (1983) 1125–1127. <https://doi.org/10.1021/jf00119a052>.
- [49] T. Tominaga, G. Guimbartau, D. Dubourdieu, Role of Certain Volatile Thiols in the Bouquet of Aged Champagne Wines, *J. Agric. Food Chem.* 51 (2003) 1016–1020. <https://doi.org/10.1021/jf020755k>.
- [50] T. Tominaga, D. Dubourdieu, A Novel Method for Quantification of 2-Methyl-3-furanthiol and 2-Furanmethanethiol in Wines Made from *Vitis vinifera* Grape Varieties, *J. Agric. Food Chem.* 54 (2006) 29–33. <https://doi.org/10.1021/jf050970b>.
- [51] L. Chen, E. De Longhi, A. Pons, T. Buffeteau, N. Daugey, P. Redon, S. Shinkaruk, P. Darriet, Identification, Quantitation, and Sensory Evaluation of Thiols in Bordeaux Red Wine with Characteristic Aging Bouquet, *J. Agric. Food Chem.* (2023). <https://doi.org/10.1021/acs.jafc.3c05854>.

- [52] E. Suhas, S. Shinkaruk, A. Pons, Optimizing the identification of thiols in red wines using new oak-wood accelerated reductive treatment, *Food Chemistry* 437 (2024) 137859. <https://doi.org/10.1016/j.foodchem.2023.137859>.
- [53] E. Suhas, Molecular and sensory characterization of the aging bouquet of Bordeaux red wines: influence of the stopper, *Enoforum.Eu* (2024). <https://enoforum.eu/en/zaragoza-2024-en/molecular-and-sensory-characterization-of-the-aging-bouquet-of-bordeaux-red-wines-influence-of-the-stopper/> (accessed August 12, 2024).

Tables

Table 1. Retention time (min), monoisotopic mass, precursor ion (m/z), mass error^a, Normalized Collision Energy (NCE, %) regime, and quantifier ion (m/z) for the thiol analytes^b investigated.

| | RT (min) | Theoretical [M+H] ⁺ | Prec. Ion (m/z) | Δ ppm | NCE | Quantifier ion (m/z) |
|-----------|----------|-----------------------------------|------------------------|--------------|-----|-----------------------------|
| 1a | 4.02 | 228.0511 | 228.0506 | -2.19 | 30 | 143.9936 |
| 1b | 4.43 | 228.0511 | 228.0506 | -2.19 | 30 | 112.0220 |
| 2 | 7.14 | 224.0198 | 224.0194 | -1.79 | 30 | 142.9860 |
| 3 | 7.42 | 244.0461 | 244.0460 | -0.41 | 30 | 244.0460 |
| 4 | 7.71 | 242.0668 | 242.0665 | -1.24 | 30 | 143.9936 |
| 5 | 8.40 | 244.0461 | 244.0457 | -1.23 | 35 | 142.9860 |
| 6 | 10.72 | 244.0824 | 244.0820 | -1.64 | 30 | 143.9936 |
| 7 | 12.30 | 239.9970 | 239.9969 | -0.42 | 30 | 142.9860 |
| 8 | 13.36 | 238.0354 | 238.0345 | -3.78 | 10 | 143.9936 |
| 9 | 14.62 | 224.0198 | 224.0194 | -1.79 | 30 | 111.0142 |

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|------------|-------|----------|----------|-------|----|----------|
| 10 | 14.93 | 234.0406 | 234.0403 | -1.28 | 30 | 142.9860 |
| 11 | 16.52 | 258.0981 | 258.0977 | -1.55 | 30 | 143.9936 |
| 12 | 19.02 | 248.0562 | 248.0559 | -1.21 | 30 | 143.9936 |
| 13 | 19.80 | 286.0930 | 286.0925 | -1.75 | 30 | 143.9936 |
| 14 | 20.05 | 248.0562 | 248.0559 | -1.21 | 30 | 248.0562 |
| 15 | 20.49 | 234.0406 | 234.0403 | -1.28 | 30 | 111.0142 |
| 16 | 20.77 | 234.0406 | 234.0403 | -1.28 | 30 | 111.0142 |
| 17a | 20.93 | 296.1137 | 296.1133 | -1.35 | 30 | 143.9936 |
| 17b | 21.64 | 296.1137 | 296.1133 | -1.35 | 20 | 143.9936 |
| 18 | 25.10 | 256.1188 | 256.1185 | -1.17 | 40 | 143.9936 |
| 19 | 11.90 | 244.0824 | 244.0820 | -1.64 | 30 | 143.9936 |

^a Δ ppm = (observed m/z - theoretical m/z)/theoretical m/z * 10^6 .

^b Numberings of the analytes are referred to **Figure 1B**.

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Table 2. Method Validation Results for the UHPLC quadrupole Orbitrap HRMS PRM method developed for quantitation of thiols in wine.

| Analyte | Range (ng/L) | Matrix ^a | ME (%) | R ² | P value | Turness (recovery, %) | | | Precision (%) | | Method | Method |
|-----------|-----------------|---------------------|------------------|----------------|---------|-----------------------|---------------------|-------------------|------------------|-------------------|---------------|---------------|
| | | | | | | low ^b | medium ^c | high ^d | low ^b | high ^d | LOD (ng/L) | LOQ (ng/L) |
| 1a | 12–240 | MW | N/A ^e | 0.9991 | 0.19 | N/A | N/A | N/A | 2.8 | 2.2 | 0.50 | 1.66 |
| | | White | 1.1 | 0.9980 | 0.21 | 115.2 ± 2.5 | 115.1 ± 1.7 | 111.4 ± 1.6 | 3.4 | 4.3 | 0.46 | 1.54 |
| | | Red | 2.2 | 0.9993 | 0.19 | 111.0 ± 2.6 | 112.9 ± 1.2 | 117.8 ± 3.6 | 2.9 | 2.1 | 0.62 | 2.07 |
| 1b | 3–60 | MW | N/A | 0.9989 | 0.20 | N/A | N/A | N/A | 2.5 | 1.9 | 0.34 | 1.13 |
| | | White | -7.7 | 0.9987 | 0.20 | 116.3 ± 0.5 | 114.9 ± 1.2 | 108.6 ± 1.5 | 5.0 | 3.9 | 0.23 | 0.77 |
| | | Red | 0.0 | 0.9991 | 0.19 | 112.9 ± 4.7 | 113.2 ± 2.9 | 118.4 ± 3.4 | 4.0 | 0.8 | 0.17 | 0.56 |
| 2 | 5–100 | MW | N/A | 0.9993 | 0.20 | N/A | N/A | N/A | 4.1 | 2.6 | 0.28 | 0.94 |
| | | White | -10.2 | 0.9982 | 0.18 | 112.3 ± 3.7 | 111.9 ± 3.3 | 105.6 ± 1.6 | 6.6 | 4.7 | 0.25 | 0.82 |
| | | Red | 10.2 | 0.9990 | 0.16 | 97.8 ± 0.4 | 106.6 ± 11.2 | 96.0 ± 2.4 | 6.4 | 6.0 | 0.31 | 1.02 |
| 3 | 100–2000 | MW | N/A | 0.9991 | 0.20 | N/A | N/A | N/A | 3.5 | 1.7 | 0.05 | 0.17 |
| | | White | 11.9 | 0.9984 | 0.22 | 109.0 ± 0.5 | 106.9 ± 0.3 | 98.9 ± 3.1 | 4.3 | 3.7 | 0.59 | 1.98 |
| | | Red | 2.8 | 0.9989 | 0.18 | 111.5 ± 6.1 | 111.5 ± 5.5 | 115.1 ± 2.5 | 3.2 | 2.4 | 0.59 | 1.96 |
| 4 | 5–100 | MW | N/A | 0.9985 | 0.19 | N/A | N/A | N/A | 2.9 | 2.2 | 0.15 | 0.49 |
| | | White | -6.0 | 0.9961 | 0.20 | 101.8 ± 1.7 | 106.2 ± 1.7 | 103.6 ± 2.4 | 3.5 | 4.7 | 0.11 | 0.38 |

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|-----------|----------|-------|-------|--------|------|--------------|-------------|--------------|-----|-----|------|------|
| | | Red | -2.3 | 0.9996 | 0.20 | 107.9 ± 1.0 | 106.9 ± 0.4 | 108.5 ± 1.4 | 1.9 | 1.8 | 0.16 | 0.54 |
| 5 | 50-1000 | MW | N/A | 0.9986 | 0.19 | N/A | N/A | N/A | 2.7 | 2.0 | 0.38 | 1.27 |
| | | White | -33.0 | 0.9979 | 0.22 | 116.6 ± 2.8 | 117.0 ± 2.1 | 112.4 ± 0.7 | 4.0 | 4.8 | 0.48 | 1.61 |
| | | Red | -18.4 | 0.9995 | 0.19 | 104.2 ± 2.7 | 103.4 ± 1.7 | 106.4 ± 2.2 | 4.0 | 3.2 | 0.42 | 1.40 |
| 6 | 150-3000 | MW | N/A | 0.9981 | 0.20 | N/A | N/A | N/A | 3.5 | 1.9 | 0.94 | 3.15 |
| | | White | -22.1 | 0.9984 | 0.19 | 104.3 ± 5.8 | 107.7 ± 5.7 | 99.0 ± 1.0 | 4.3 | 5.4 | 1.21 | 4.02 |
| | | Red | -24.2 | 0.9993 | 0.19 | 109.3 ± 4.9 | 110.2 ± 2.0 | 113.6 ± 0.8 | 2.7 | 2.6 | 1.24 | 4.13 |
| 7 | 5-100 | MW | N/A | 0.9948 | 0.18 | N/A | N/A | N/A | 4.6 | 4.2 | 0.28 | 0.92 |
| | | White | -22.1 | 0.9933 | 0.23 | 117.8 ± 1.7 | 117.4 ± 1.1 | 107.3 ± 9.3 | 5.2 | 3.6 | 0.29 | 0.97 |
| | | Red | -24.3 | 0.9990 | 0.21 | 112.6 ± 3.3 | 113.3 ± 1.9 | 116.9 ± 2.3 | 4.7 | 3.8 | 0.25 | 0.82 |
| 8 | 2.5-50 | MW | N/A | 0.9990 | 0.19 | N/A | N/A | N/A | 5.4 | 2.4 | 0.22 | 0.75 |
| | | White | -18.0 | 0.9981 | 0.26 | 111.6 ± 2.9 | 110.8 ± 2.9 | 105.3 ± 0.3 | 4.6 | 4.7 | 0.12 | 0.41 |
| | | Red | -11.8 | 0.9987 | 0.17 | 91.0 ± 1.1 | 97.1 ± 4.5 | 92.6 ± 2.4 | 5.7 | 8.2 | 0.28 | 0.93 |
| 9 | 5-100 | MW | N/A | 0.9979 | 0.29 | N/A | N/A | N/A | 7.5 | 4.1 | 1.21 | 4.03 |
| | | White | -77.8 | 0.9921 | 0.43 | 104.3 ± 15.7 | 101.7 ± 8.8 | 104.4 ± 11.5 | 6.7 | 7.0 | 0.21 | 0.70 |
| | | Red | 18.5 | 0.9961 | 0.21 | 97.1 ± 11.9 | 110.0 ± 4.1 | 89.8 ± 10.7 | 9.5 | 9.4 | 0.42 | 1.41 |
| 10 | 5-100 | MW | N/A | 0.9986 | 0.20 | N/A | N/A | N/A | 6.4 | 1.9 | 0.25 | 0.84 |
| | | White | -21.0 | 0.9969 | 0.21 | 112.3 ± 2.6 | 112.5 ± 3.4 | 108.1 ± 1.1 | 4.9 | 7.7 | 0.28 | 0.93 |
| | | Red | -9.1 | 0.9991 | 0.21 | 99.3 ± 2.4 | 111.0 ± 1.7 | 112.7 ± 4.0 | 3.0 | 3.0 | 0.13 | 0.43 |

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|------------|--------|-------|-------|--------|------|--------------|-------------|-------------|-----|-----|------|------|
| 11 | 15–300 | MW | N/A | 0.9982 | 0.19 | N/A | N/A | N/A | 3.7 | 1.7 | 0.10 | 0.35 |
| | | White | -27.1 | 0.9980 | 0.20 | 103.4 ± 1.9 | 107.9 ± 2.9 | 102.9 ± 1.8 | 4.3 | 4.9 | 0.39 | 1.31 |
| | | Red | -18.1 | 0.9994 | 0.19 | 109.4 ± 1.0 | 113.6 ± 0.9 | 118.1 ± 1.4 | 2.3 | 2.2 | 0.15 | 0.51 |
| 12 | 2.5–50 | MW | N/A | 0.9991 | 0.19 | N/A | N/A | N/A | 3.9 | 1.4 | 0.09 | 0.30 |
| | | White | -20.6 | 0.9979 | 0.21 | 110.7 ± 2.1 | 110.8 ± 2.9 | 107.8 ± 1.1 | 5.0 | 5.3 | 0.07 | 0.23 |
| | | Red | -4.7 | 0.9994 | 0.19 | 118.6 ± 0.2 | 117.2 ± 1.7 | 118.8 ± 0.1 | 3.3 | 2.9 | 0.08 | 0.26 |
| 13 | 5–100 | MW | N/A | 0.9977 | 0.20 | N/A | N/A | N/A | 3.9 | 1.4 | 0.20 | 0.68 |
| | | White | -35.6 | 0.9983 | 0.18 | 105.4 ± 1.8 | 109.1 ± 5.4 | 103.0 ± 4.2 | 5.9 | 5.8 | 0.18 | 0.60 |
| | | Red | -15.4 | 0.9983 | 0.19 | 98.3 ± 0.5 | 107.6 ± 4.3 | 113.3 ± 1.4 | 5.3 | 3.9 | 0.15 | 0.49 |
| 14 | 2.5–50 | MW | N/A | 0.9991 | 0.19 | N/A | N/A | N/A | 4.1 | 1.4 | 0.15 | 0.50 |
| | | White | -12.2 | 0.9970 | 0.26 | 109.3 ± 3.3 | 109.6 ± 2.1 | 104.7 ± 1.7 | 3.5 | 7.0 | 0.11 | 0.38 |
| | | Red | 15.6 | 0.9985 | 0.19 | 119.4 ± 11.5 | 113.5 ± 2.6 | 116.7 ± 1.8 | 4.5 | 4.9 | 0.11 | 0.36 |
| 15 | 5–100 | MW | N/A | 0.9993 | 0.19 | N/A | N/A | N/A | 5.3 | 1.7 | 0.41 | 1.37 |
| | | White | -19.8 | 0.9977 | 0.21 | 101.2 ± 5.4 | 100.1 ± 2.9 | 95.8 ± 4.4 | 2.9 | 5.2 | 0.26 | 0.86 |
| | | Red | 18.8 | 0.9972 | 0.17 | 105.4 ± 0.9 | 117.0 ± 1.4 | 116.8 ± 2.8 | 6.6 | 5.8 | 0.32 | 1.08 |
| 16 | 5–100 | MW | N/A | 0.9987 | 0.28 | N/A | N/A | N/A | 2.7 | 1.3 | 0.37 | 1.23 |
| | | White | -30.4 | 0.9976 | 0.21 | 110.5 ± 3.2 | 109.1 ± 3.5 | 104.1 ± 2.1 | 4.5 | 6.1 | 0.23 | 0.77 |
| | | Red | 10.8 | 0.9981 | 0.19 | 112.6 ± 7.6 | 117.7 ± 2.7 | 115.4 ± 7.0 | 4.8 | 5.2 | 0.24 | 0.80 |
| 17a | 2.5–50 | MW | N/A | 0.9994 | 0.13 | N/A | N/A | N/A | 3.1 | 0.7 | 0.09 | 0.29 |

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|------------|----------|-------|-------|--------|------|-------------|-------------|-------------|-----|-----|------|------|
| | | White | -26.7 | 0.9970 | 0.21 | 105.8 ± 1.5 | 102.8 ± 5.7 | 90.3 ± 10.8 | 6.4 | 5.3 | 0.07 | 0.22 |
| | | Red | -11.1 | 0.9982 | 0.17 | 108.6 ± 2.0 | 104.9 ± 1.9 | 101.9 ± 1.6 | 3.4 | 2.7 | 0.08 | 0.25 |
| 17b | 12.5–250 | MW | N/A | 0.9990 | 0.18 | N/A | N/A | N/A | 3.3 | 1.3 | 0.14 | 0.47 |
| | | White | -18.3 | 0.9964 | 0.22 | 97.6 ± 2.1 | 98.3 ± 5.1 | 88.8 ± 11.1 | 7.7 | 4.9 | 0.08 | 0.25 |
| | | Red | -0.0 | 0.9978 | 0.16 | 105.3 ± 0.3 | 102.0 ± 4.4 | 98.2 ± 2.3 | 2.8 | 2.9 | 0.09 | 0.29 |
| 18 | 2.5–50 | MW | N/A | 0.9951 | 0.17 | N/A | N/A | N/A | 5.1 | 2.3 | 0.09 | 0.31 |
| | | White | -35.7 | 0.9905 | 0.24 | 87.5 ± 3.8 | 91.6 ± 2.5 | 104.2 ± 7.7 | 7.2 | 6.6 | 0.02 | 0.06 |
| | | Red | 23.1 | 0.9930 | 0.16 | 86.6 ± 18.3 | 114.2 ± 8.4 | 123.6 ± 0.9 | 6.1 | 5.9 | 0.04 | 0.15 |

^a MW, model wine. ^b Low, low-level standard spiked sample. ^c Medium, medium-level standard spiked sample. ^d High, high-level standard spiked sample. ^e N/A, not applicable. Please refer to **Materials and Methods** for spiking levels.

Figures

Figure 1

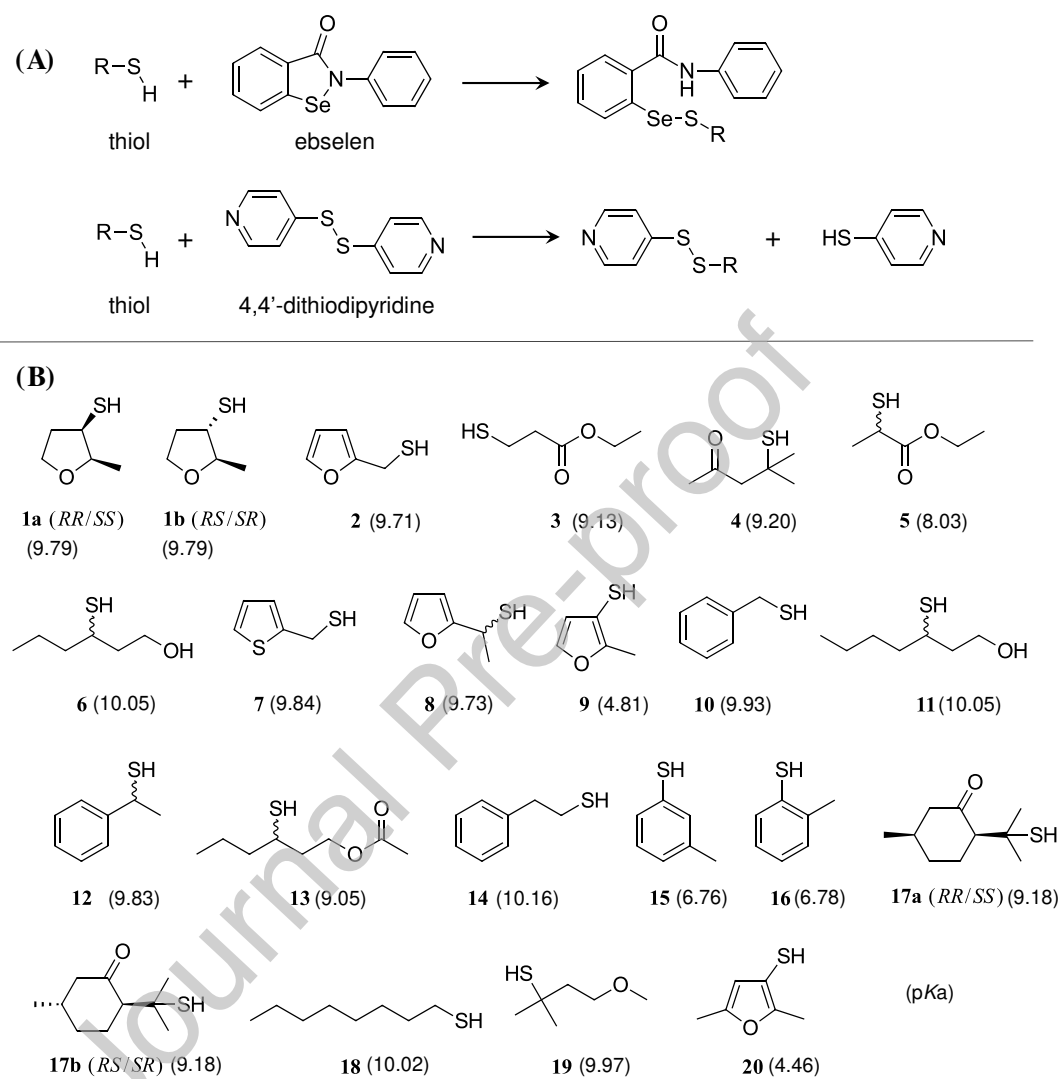


Figure 2

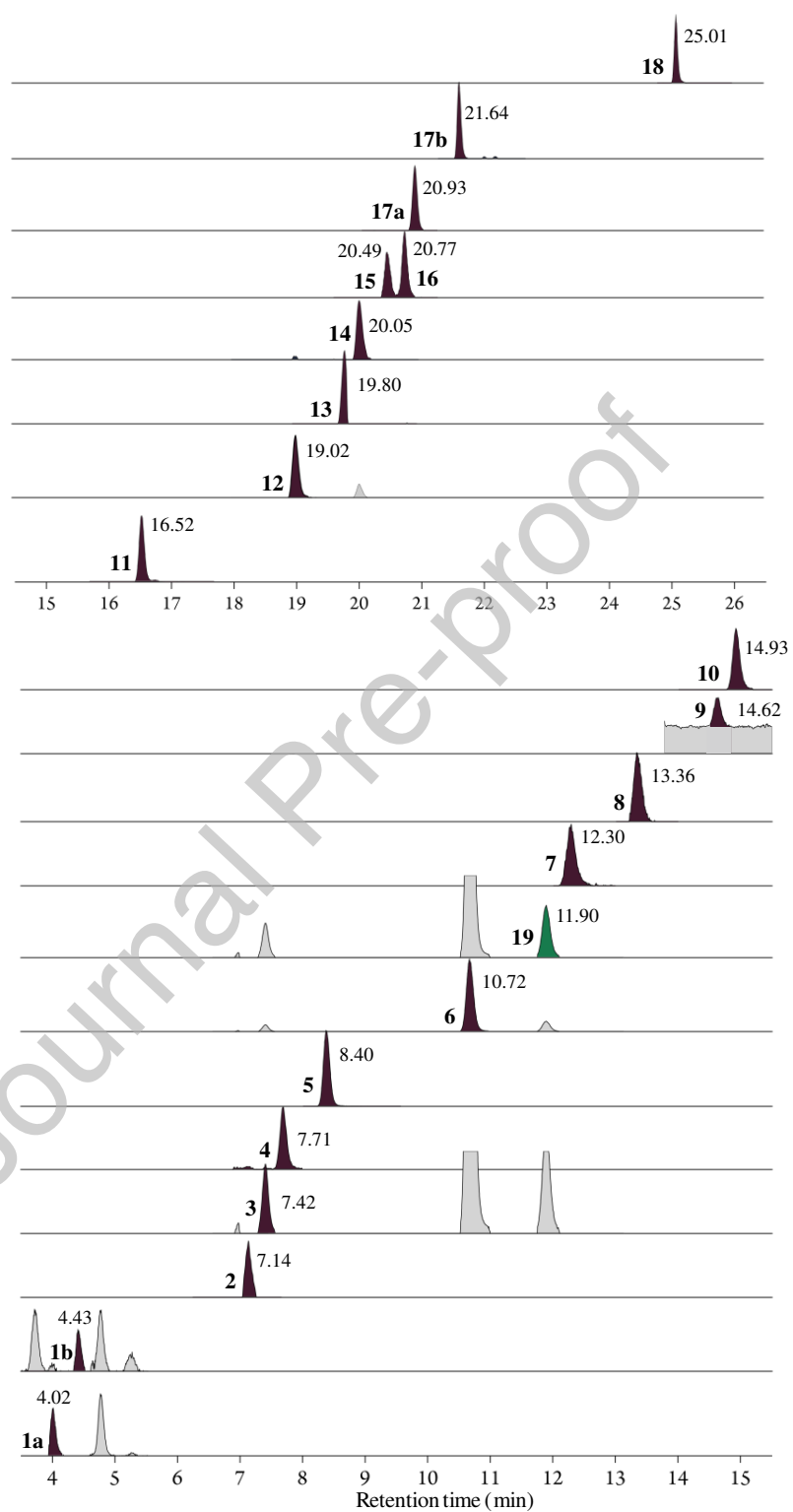


Figure 3

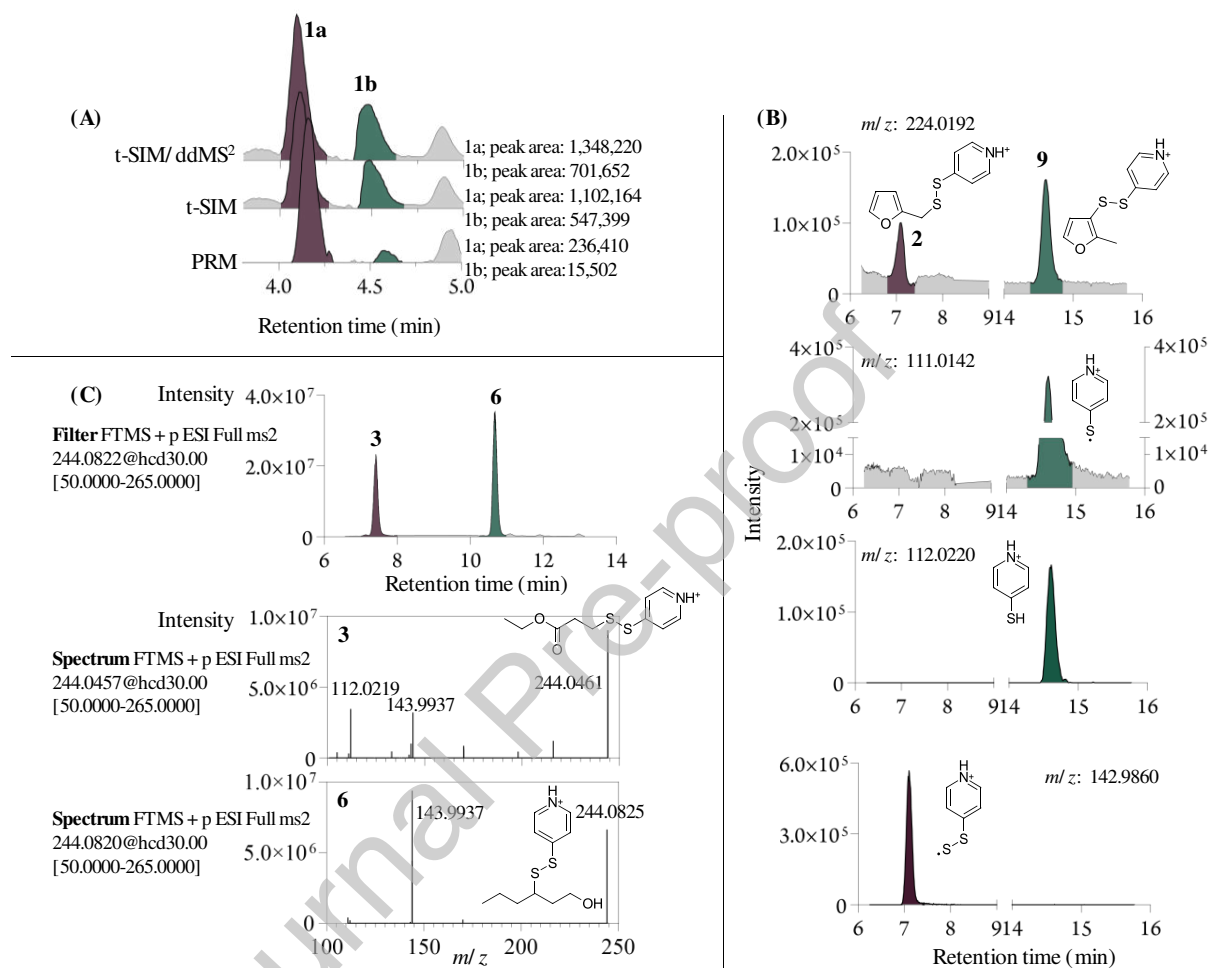


Figure 4

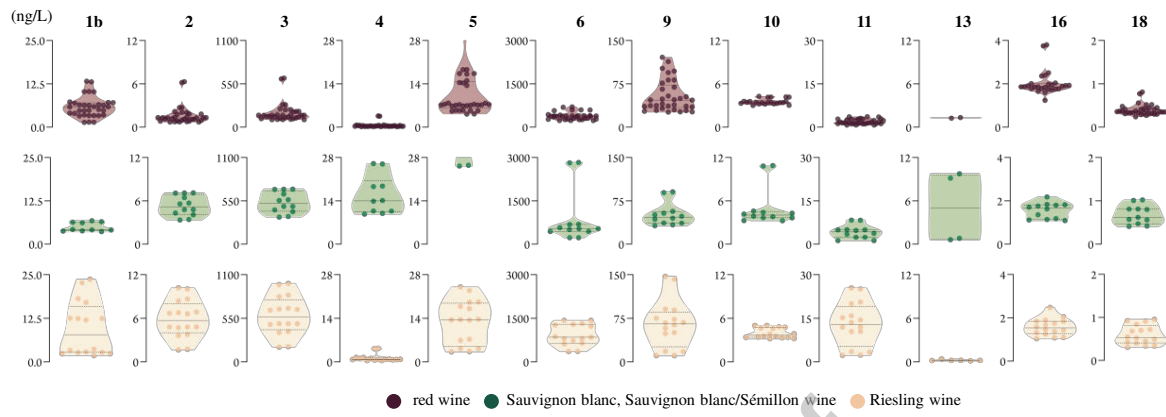


Figure captions

Figure 1. (A) Chemical derivatization of thiols using ebselen and 4,4'-dithiodipyridine (B) Chemical structures of thiol analytes (**1–18**) and internal standard (**19**) covered by the proposed method.

Figure 2. UHPLC quadrupole Orbitrap HRMS PRM extract ion chromatograms (XICs) of representative thiol analytes (as their derivatives) in a red wine sample fortified with the second lowest fortification level (see method validation experiments). The targeted analytes are colored to distinguish from the interferences indicated in gray. All chromatograms were obtained with a mass tolerance of 5 ppm. Numberings of compounds are referred to **Figure 1B**. See **Table 1** for the quantifier ions.

Figure 3. (A) Representative XICs (m/z 228.0506) of t-SIM, t-SIM/ddMS², and PRM results of *cis*-2-methyl-3-tetrahydrofuranthiol (**1a**, 240 ng/L) and *trans*-2-methyl-3-tetrahydrofuranthiol (**1b**, 60 ng/L) isolated from a 2022 bag-in-box red wine spiked with racemic 2-methyl-3-tetrahydrofuranthiol at 300 ng/L. (B) XICs (m/z 224.0192, 12.0220, 142.9860, and 111.0142) of PRM results of 2-furfuryl thiol (**2**, 100 ng/L) and 2-methyl-3-furanthiol (**9**, 100 ng/L) isolated from a 2021 Riesling wine spiked with both thiols at 100 ng/L. (C) Full MS² PRM chromatograms (filter 244.0822@hcd 30) and the corresponding mass spectra for peak **3** and peak **6**. All chromatograms were obtained with a mass tolerance of 5 ppm.

Figure 4. Concentrations (ng/L) of thiol analytes measured in selected wine samples by the validated UHPLC quadrupole Orbitrap HRMS PRM method, as shown as staggered dot plot and violin plot overlapped. The middle line in represents median value and the top and bottom dash lines represent quartiles. Note the different scales for the y-axis. Refer to **Figure 1B** for compound numbering.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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