Development of a novel liquid/liquid extraction and ultra-performance liquid chromatography tandem mass spectrometry method for the assessment of thiols in South African Sauvignon Blanc wines

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Abstract

Background and Aims: The thiol compounds, 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), are important, pleasant volatile thiols conferring fruity notes in wines. The analytical determination of these thiols in wine remains problematic due to their trace concentration and instability. The main aim of this study was to develop a liquid/liquid extraction and ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the determination of 3MH and 3MHA concentration in Sauvignon Blanc wines.

Methods and Results: A novel sample preparation based on a liquid/liquid extraction was developed. Thiols were quantified by UPLC-MS/MS after derivatisation with *o*-phthaldialdehyde (OPA). Good results were obtained with the method in terms of limit of detection and of quantification, accuracy and repeatability. Average concentration of 3MH in 18 South African wines was 1320.32 and and of 3MHA 313.48 ng/L.

Conclusions: The analytical method proposed allows for the detection of 3MH and 3MHA by liquid chromatography at a concentration lower than that of their respective sensory thresholds.

Significance of the Study: The analytical method described is the first that allows for liquid/liquid extraction of thiols from wine, followed by detection and quantification by UPLC-MS/MS.

Key words*: derivatisation, liquid/liquid extraction, Sauvignon Blanc wine, thiols, UPLC-MS/MS*

Introduction

Sulfur-derived aroma compounds are often characterised by strong odours, which can have different origins in wine. These compounds can originate from grapes as non-volatile precursors, or be released through microbial fermentation or chemical reactions taking place in wine during ageing. Many volatile sulfur compounds, such as ethanethiol, methanethiol and hydrogen sulfide, are responsible for olfactory defects in wine (Bartowsky and Pretorius 2009), however, certain long-chain volatile sulfur compounds can contribute to a large extent to the pleasant tropical aromatic profile of certain wines. In particular, 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyl acetate (3MHA) and 4-mercapto-4methylpentan-2-one (4MMP) are regarded as the most important, pleasant volatile thiols in wines (Tominaga et al. 1998, Roland et al. 2011). They are released from their non-volatile S-glutathionylated and S-cysteinylated precursors by yeast activity (Peyrot des Gachons et al. 2002, Fedrizzi et al. 2009, Capone et al. 2011a). These precursors, however, normally account for only a fraction of the 3MH and 3MHA present in white wine, and the reaction between (E)-2-hexen-1-ol and H_2S may also yield a large amount of 3MH (Harsch et al. 2013). 3-Methyl-3-mercaptobutanal and 2-methylfuran-3-thiol, together with 3-mercaptopropyl acetate, 3-MH and 3-mercaptoheptanal, play a key role in Sauternes wine (Bailly et al. 2009), while the latter two compounds and 4MMP play a crucial role in the passionfruit and guava aroma of Sauvignon Blanc wines (Coetzee and Du Toit 2013, Van Wyngaard et al. 2014). The perception threshold for 4MMP, 3MHA and 3MH in model wine has been shown to be 0.8, 4.2 and 60 ng/L, respectively (Tominaga et al. 1996, 1998, Dubourdieu et al. 2006). This means that these compounds can influence the aromatic profile of wine even when present at extremely

low concentration. As a consequence, they are one of the most widely studied molecules within the different classes of wine aroma compounds.

Despite their importance, the analytical determination of thiols in wine remains difficult due to their trace concentration (Roland et al. 2011) and instability (Nikilantonaki et al. 2012). Gas chromatography is generally an excellent analytical approach for aroma compound analysis. In several methods, mercuric compounds (*p*-hydroxymercuribenzoate and *p*-aminophenylmercuric acetate) have been demonstrated to be effective for thiol determination (Tominaga et al. 1998, Schneider et al. 2003, Tominaga and Dubourdieu 2006). Although these methods are powerful for obtaining purified thiol extracts, the employment of mercury compounds constitutes a hazard for health and for the environment. Methods based on mercury salts are also time consuming, and an accurate quantification can be achieved only by using isotopically labelled internal standards (Schneider et al. 2003).

Analysis of thiols as their derivatives can improve detectability in mass spectrometry. Analytical approaches employ pentafluorobenzyl bromide as the derivatising agent, which transforms thiols into their corresponding pentafluorobenzyl derivatives (Capone et al. 2011b, Mateo-Vivaracho et al. 2006, 2007, 2008). The derivatising reaction is normally carried out in a purified extract (i.e. water) (Capone et al. 2011b), organic solvent (Mateo-Vivaracho et al. 2007), in-cartridge (Mateo-Vivaracho et al. 2008), or in-fibre (Mateo-Vivaracho et al. 2006), as phenols can react with thiols under the conditions required for derivatisation (high concentration of alkali). The main advantage with this derivatising agent is related to increased sensitivity due to pentafluoro adducts. In fact, these derivatives show excellent electron-capturing

93 properties, which are valuable for negative ion chemical ionisation mass spectrometry
94 or electron-capturing detectors (Mateo-Vivaracho et al. 2007). Such detector systems
95 are not as common in laboratories as electron impact spectrometers.

96 Another promising derivatising agent in gas chromatography analysis of thiols is
97 ethyl propiolate, which is able to derivatise thiols directly in the wine matrix and is a
98 suitable derivatising reagent for the electron impact mass spectrometry detection
99 system (Herbst-Johnston et al. 2013).

100 The fragmentation patterns of un-derivatised thiols in mass spectrometry lack
101 intensity and specific m/z ions for these compounds (Mateo-Vivaracho et al. 2007).
102 When either pentafluorobenzyl derivatives are used with chemical ionisation, or
103 ethylpropiolate derivatives with electron impact ionisation, specific and abundant
104 fragments are obtained. The sensitivity of the detection is improved when these
105 fragments are used in selected ion-monitoring mode (Mateo-Vivaracho et al. 2007,
106 Herbst-Johnston et al. 2013).

107 Several liquid chromatography approaches to assess sulfur compounds in grape
108 juices and wines have been reported (Park et al. 2000, Fracassetti et al. 2011). Their
109 effectiveness relies on the generation of highly absorbent UV or fluorescent active
110 species. To our knowledge, this is the first reported method for the determination of
111 volatile thiols in wine by liquid chromatography. With this method we determined the
112 concentration of 3MH and 3MHA in several South African Sauvignon Blanc wines.

113 **Materials and methods**

114 *Materials*

Dichloromethane (DCM) (≥ 99.8%), sodium chloride (≥ 99.5%), methanol (≥ 99.9%), acetonitrile LC-MS CHROMASOLV (≥ 99.0%), iso-propanol LC-MS CHROMASOLV (≥ 99.0%), potassium metabisulfite, sodium borohydride, ethanolamine (EA), *o*-phthaldialdehyde (OPA), 6-mercaptohexanol (6MH) and anhydrous sodium sulfate (≥ 99.0%) were purchased from Sigma-Aldrich (St Louis, MO, USA). Calcium carbonate and boric acid were purchased from Merck (Merck Millipore, Modderfontein, South Africa). Water for UPLC was obtained from a Milli-Q filtration system (EMD Millipore, Bedford, MA, USA). Polyvinylpolypyrrolidone (PVPP) resin was purchased from Dal Cin Gildo Spa (Milan, Italy). The model wine contained 12% (v/v) ethanol and 5 g/L of tartaric acid, and the pH was adjusted to 3.5 with sodium hydroxide (Sigma-Aldrich).

3-Mercaptohexan-1-ol (3MH) was purchased from Acros Organics (Geel, Belgium) and 3-mercaptohexyl acetate (3MHA) from Oxford Chemical (Hartlepool, England). The deuterated internal standards d2-3-mercaptohexan-1-ol (d2-3MH) and d2-3-mercaptohexyl acetate (d2-3MHA) were generously donated by the University of Auckland.

Samples

All samples were Sauvignon Blanc wines, bottled or tank samples, from the 2012 and 2013 vintages. All samples were extracted in duplicate. The concentration of the thiols in the wine samples was quantified by means of internal standard calibration.

Sample preparation method

Sample preparation was optimised by several assays in order to detect the compounds of interest, as well as to improve the sensitivity and the extraction yield. These assays

included sample preparation, which was undertaken in synthetic wine (tartaric acid 5 g/L, ethanol 10% v/v, pH 3.5) spiked with standard solutions and white wine.

Potassium metabisulfite (6 g/L) and PVPP (5 g/L) were added to the wine sample (180 mL) containing the deuterated internal standards and stirred for 10 min. After centrifugation at 6200 x *g* for 10 min, sodium chloride was added (50 g/L) and the wine sample was again stirred until the salt had dissolved completely. The pH was adjusted to 5.0 with calcium carbonate, followed by sodium borohydride addition (3.84 g/L) with stirring. The wine sample was extracted by shaking with 110 mL of DCM for 20 min at room temperature, after which the organic phase was recovered. IIf emulsion formed, the phases were centrifuged at 6200 x g for 5 min and the organic phase was recovered. The extract was washed with 100 mL Milli-Q water. Anhydrous sodium sulfate (2 g) was added to the DCM extract to remove water traces before transferring the extract into hermetically sealed bottles. The bottles were stored at -20°C until solvent evaporation. Solvent was evaporated under vacuum after the addition of another 2 g of anhydrous sodium sulfate. The final volume was approximately 6 mL. The concentrated extract was transferred to a tube, evaporated under a gentle nitrogen flow to approximately 1 mL, after which methanol (300 µL) was added. The evaporation was continued until the final sample volume was approximately 200 µL. The extracted wine sample in methanol (50 µL) was derivatised with 5 µL OPA (5 g/L 5 µL in methanol) and 5 µL ethanolamine (10 g/L in borate buffer, 80 mmol at pH 7.3). The derivatised sample was held room temperature for 5 min and injected into the ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) system.

Instrumental conditions for ultra-performance liquid chromatography-fluorescence detection

The liquid chromatography system was an Acquity UHPLC coupled with a multi λ fluorescence detector 2475 (Waters Corporation, Milford, MA, USA). The thiols were separated on a Kinetex phenyl-hexyl column (150 × 4.6 mm, 2.6 µm, 100 Å) (Phenomenex, Torrance, CA, USA). The column temperature was 28°C and the temperature of the samples 15°C. The injection volume was 10 µL and the flow rate was 0.8 mL/min. The thiols were separated in a gradient (Table 1) using 30 mmol citrate buffer at pH 6.0 (A) and methanol (B) for a running time of 16 min. The wavelength was set at 330 nm for excitation and 440 nm for emission.

Instrumental conditions for ultra-performance liquid chromatography-tandem mass spectrometry method

Thiols were separated with a Waters Acquity UPLC system fitted to a Waters Xevo triple quadrupole mass spectrometer (MS/MS) (Waters Corporation). Data were acquired and processed with MassLynx version 4.1 software (Waters Corporation).

The thiols were separated on a Acquity UPLC BEH C18 2.1 x 100 mm, 1.7 µm particle column, fitted with a guard cartridge (VanGuard C18 2.1 x 5 mm, 1.7 µm particle size) (Waters Corporation). The column was thermostated at 50°C. The injection volume was 5 µL. The thiols were eluted in gradient mode, using 10 mmol ammonium acetate (mobile phase A) and methanol:acetonitrile:i-propanol 49:49:2 (mobile phase B). The gradient program is shown in Table 2.

Thiols were detected in multiple reaction mode (MRM). The optimised parameters for the electrospray source (positive mode) were as follows: capillary voltage, 3.5 kV; cone voltage, 20 V; source, 140°C; desolvation temperature, 400°C; desolvation gas, N_2, 900 L/h; and cone gas, 50 L/h. The remaining MS settings were optimised for the best sensitivity and resolution. The monitored MRM transitions are shown in Table 3.

Methodology for evaluating method performance

The qualitative and quantitative performance of the chromatographic method was evaluated. Selectivity of the method was evaluated through direct injections of the mixture of standards and internal standards and comparing the results to those obtained from extracts of wine spiked with the mixture. Linearity was evaluated for the range used, 25–500 ng/L for 3MHA and 50–2500 ng/L for 3MH at six calibration points. The limit of quantitation was calculated for a signal-to-noise ratio (S/N) of 10.

The matrix effect was evaluated through recovery assays at all levels of calibration. Briefly, a non-aromatic wine was spiked at each calibration level (sample referred to as spiked wine). The original wine with no spiking constituted the blank sample. All the spiked and unspiked wines were subjected to the sample preparation procedure. The extractions were done in duplicate. The recovery values were obtained by comparing concentration values from direct injection of standards (no extraction) to

values obtained after the extraction of wine samples. The values from extracted wine samples were corrected if the unspiked wine contained thiols. The results are expressed as a proportion (%).

Precision was measured for the extraction step (three extractions on the same spiked wine sample), for the derivatisation step (three derivatisations on the same extracted spiked wine sample), and for the instrumental analysis (by injecting the same wine sample in triplicate). Precision was measured at two concentration values, 50 ng/L 3MHA and 100 ng/L 3MH (medium–low), and 250 ng/L 3MHA and 1000 ng/L 3MH (medium–high).

Stability was evaluated for the standards and extracts. Concentration for the standard stock solutions in methanol was determined with Ellman's reagent (Eyer et al. 2003). The stability of extracts was evaluated by UPLC-MS/MS after 1 week of storage at two stages of the sample preparation.

Results and discussion

Method optimisation

The method developed for thiol quantification in wine consisted of a liquid-liquid extraction in an organic solvent, followed by thiol re-dissolution in methanol, the medium in which the thiols were derivatised with OPA in the presence of excess amino ethanol. The OPA derivatives were separated by UPLC coupled with mass spectrometry. The major issues in method optimisation were thiol reactivity towards several wine constituents that also were extracted, thiol loss during the concentration step, and the

derivatisation yield obtained for different solvents in which the thiols were dissolved at the end of the sample preparation. This method allowed the quantification of 3MH and 3MHA in white wine, but not of 4MMP, as this compound was not derivatised. 6-Mercaptohexan-1-ol (6MH) could be also derivatised and detected and therefore was used as the model compound during method optimisation.

Optimisation of extraction procedure. Different solvents can be used to extract volatiles from wines. Dichloromethane (Tominaga et al. 1998) and, more recently, pentane (Capone et al. 2011b), have been proposed as solvents for thiol extraction from wine.

Synthetic wine was used for a preliminary investigation of the composition of the extraction solvent. The characteristic hydrophobicity of the analytes in the presence of sodium chloride was also assessed during the same assay. Extraction with DCM when using 50 g/L NaCl was identified as the most suitable. This is due to the partition coefficient ($K_{d(\mathrm{DCM/wine})}$) calculated for the liquid-liquid extraction of 3MH, the most hydrophilic thiol of interest. The value of the partition coefficient was double when 50 g/L NaCl was added compared to no NaCl addition, while a higher concentration of salt did not further affect the extraction yield. Dichloromethane is highly effective for the extraction of un-dissociated thiols (Tominaga et al. 1998). The volume of DCM and the extraction steps were established using the partition coefficient: from the theoretical values, a single extraction step with 110 mL of DCM allowed the complete extraction of thiols from 180 mL of both the synthetic wine and from the white wine.

Dichloromethane is incompatible with reversed phase separations in liquid chromatography. In contrast, water is an appropriate solvent for liquid chromatography.

252 Moreover, water has been reported as a suitable solvent for the derivatisation of thiols
253 with OPA (Molnár-Perl 2001).

254 The back-extraction of thiols from an organic solvent (pentane) to water has
255 already been reported (Capone et al. 2011b). In alkaline solution, these compounds are
256 present in both dissociated and un-dissociated forms. The ratio between the two forms
257 is pH dependent and the two forms have a different affinity towards water and DCM. As
258 a consequence, adjusting the pH influences the degree of dissociation and, further, the
259 presence of thiols in water can be favoured, leading to a higher extraction yield from the
260 DCM (Yabroff 1940). On this basis, partition coefficients between DCM and 10 mmol
261 sodium hydroxide ($K_{d\ (NaOH/DCM)}$ pH 12.0) were calculated to be 0.61, not detected and
262 0.91 for 3MH, 3MHA and 6MH respectively. The volatile thiols dissolved in sodium
263 hydroxide solution were determined as indole derivatives obtained after alkaline pH
264 adjustment of this solution.

265 As the calculation shows, the partition coefficient allowed for poor thiol
266 extraction from DCM using 10 mmol NaOH as back-extraction solvent. Even after
267 multiple extractions, a large volume of alkaline solution was needed to obtain a high
268 back-extraction yield from DCM. Higher alkaline concentration has been suggested to
269 improve the back-extraction of thiols from oil phases (Yabroff 1940). At any rate, a high
270 concentration of NaOH is not suitable for the back-extraction of thiols from DCM and
271 3MHA hydrolysis could also occur.

272 Good yields were achieved with water (Table 4, but the use of methanol allows
273 a faster evaporation step, limiting thiol loss during the sample preparation. In a DCM
274 and methanol mixture, DCM is the first solvent to evaporate, since it boils at a lower

temperature (39.6°C) than that of methanol (64.7°C). Thus, the proposed method consists of a solvent switch between DCM and methanol by removing DCM first under vacuum and then under nitrogen flow in the presence of methanol.

Optimisation of derivatisation procedure. Free thiol compounds cannot be detected by UPLC coupled with either fluorescence or MS detectors, thus the final step of the sample preparation entailed thiol derivatisation. Among the derivatising reagents employed for thiol groups, OPA is of interest because the resulting derivatives have fluorescent properties, allowing for the quantification of primary amines and thiols at trace concentration with good derivatisation yield (Kutlán and Molnár-Perl 2003). This characteristic of OPA was taken into account, since the thiol determination was initially made by UPLC coupled to a fluorescence detector. The reaction of OPA with a primary amino group [i.e. ethanol amine (EA)] and a thiol (RSH) leads to the formation of an indole (OPA-EA-SR) (Simons and Johnson 1978). Besides the fluorescent properties of indoles (Park et al. 2000), the derivatisation improves the detection of these compounds in mass spectrometry. For these reasons, this compound was chosen as it allowed the quantification of wine thiols at ng/L concentration (Figure 1).

The influence of both water pH and methanol on the derivatisation reaction was evaluated. It has been reported that the derivatisation yield is strongly affected by the thiolate form of thiols, while the protonation of the amino groups showed a negligible effect (Nakamura and Tamura 1982). The yield of the derivatives formation was evaluated in water for a pH range of 5.0 to 9.0 and in methanol. Figure 2 shows the derivatisation yields obtained in water.

When the pH ranged from 6.5 to 9.0, the derivatisation yield significantly increased only at pH 9.0. Under this condition of neutral–basic pH, no significant degradation of 3MHA to 3MH was observed, as previously reported (Herbst-Johnstone et al. 2013). For pH lower than 6.5, the indole formation could not take place, probably due to low thiolate concentration as well as to a high content of protonated EA. Derivatisation yield in methanol was comparable to that obtained in water at pH 6.5 to 9.0. Independent of the solvent used, the derivatisation of 4MMP did not occur. The formation of the OPA derivative of 4MMP was probably prevented by the hydrogen bonding between the thiol group and the carbonyl moiety within the compound itself, and its steric hindrance. Derivatisation of 4MMP was an issue when other derivatising reagents were used (Mateo-Vivaracho et al. 2008).

Minimising matrix components affecting thiol determination in white wines. Dichloromethane is a suitable solvent for the extraction of volatile thiols (Tominaga et al. 1998), as well as several other compounds from wine (Ortega-Heras et al. 2002), such as acids, alcohols, carbonyl compounds, esters, volatile phenols, lactones and terpenes (Hernanz et al. 2008). Many of these compounds can react with volatile thiols in both water and organic solvent, and thereby can influence the derivatisation yield.

The extraction of certain acids, including hexanoic, octanoic, decanoic, hydroxybenzoic and hydroxycinnamic acids, could modify the final sample pH, altering the derivatisation yield. The phenolic substances and their corresponding quinones could also be extracted and react with thiols, both in water (Nikolantonaki et al. 2012) and in methanol under certain conditions (Yadav et al. 2007). Moreover, thiols are

319 strong nucleophilic compounds and their reaction with phenols and quinones based on
320 Michael-type mechanism is pH dependent.

321 The pH of the back-extraction water after DCM evaporation was determined to
322 be 4.56 (average of two replicates), meaning that the derivatisation will not be effective.
323 To limit the extraction of organic acids, the dissociation of carboxylic functions and the
324 formation of the corresponding salts were necessary. The total dissociation of organic
325 acids present in wine can be obtained at high pH. Calcium carbonate was used to
326 transform the carboxylic acids into the corresponding calcium salts. The adjustment of
327 wine pH up to 5.0 before the liquid extraction led to an increase in the pH of the back-
328 extraction water to more than 6.0, thus allowing the derivatisation of thiols.

329 Nevertheless, the high pH has the disadvantage of promoting the formation of
330 both quinones and thiolates (Danilewicz et al. 2008)**,** thereby increasing the rate of
331 nucleophile additions between the thiols and quinones. This reaction has been reported
332 as the major cause of thiol aroma loss in wine (Nikolantonaki et al. 2010) and it can also
333 take place in water (Yadav et al. 2007). Both phenols and quinones could be extracted
334 by DCM, causing a loss of thiols during sample preparation. The qualitative evaluation
335 of phenols in the back-extraction water was carried out by the ferric chloride test (Wesp
336 and Brode 1934). This assay confirmed that phenols had been extracted from the wine.
337 For this reason, the DCM washing step with water was included to partially remove the
338 extracted phenols. At the same time, the treatment with PVPP was carried out as the
339 first step of the sample preparation in order to decrease the phenolic substances
340 content of wine from the beginning. Both treatments limited the amount of phenolic
341 substances in the back-extraction water and methanol, while not affecting the recovery

342 of thiols in the synthetic wine. Extraction yields of 99.1 ± 10.1, 95.6 ± 8.4and 88.3 ± 7.9
343 % were found for 3MH, 3MHA and 6MH respectively after $CaCO_3$ and PVPP treatment
344 in synthetic wine. In contrast, thiol detection was not possible without these steps
345 during the wine sample preparation, even when the wine was spiked with thiols.
346
347 *Choice of internal standard*
348 A suitable internal standard is crucial for analysis methods based on extensive sample
349 preparation procedures. With the present study, deuterated standards were available.
350 6-Mercaptohexan-1-ol was used for the sample preparation development and
351 fluorescence detection, while deuterated standards were chosen for the MS work.
352 Deuterated standards are ideal, as the chemical structures are identical to those of the
353 compounds of interest and therefore their behaviour during the various steps of sample
354 preparation would mimic that of the analytes. For the chromatographic analysis, the
355 retention of a deuterated standard is expected to be similar to that of the compounds
356 of interest due to the identical chemical character. In this case, the use of MS detection
357 is necessary.
358
359 *Method performance*
360 **Selectivity of the chromatographic method.** As can be seen from Figure 3, the
361 chromatographic method achieved the separation of the compounds of interest. The
362 MS/MS detection provided the additional selectivity necessary to distinguish between
363 the analytes and their deuterated equivalents used as internal standards.
364

Calibration and quantitation limits. The linearity of the detector response was evaluated over the concentration range 25–500 ng/L for 3MHA and 50–2500 ng/L for 3MH. These concentration values were chosen in accordance with the previously reported thiol concentration found in white wine and their threshold values (Lund et al. 2009, Mateo-Vivaracho et al. 2010, Benkwitz et al. 2012, Van Wyngaard 2013).

Calibration curves were constructed with two approaches: direct injection of standards and injection of extracted, spiked non-aromatic wines at the same concentration. Direct injection of standards has the advantage of not requiring sample preparation. The presence of the matrix in the ionisation source, however, could have a great impact on the ionisation (Trufelli et al. 2011). Therefore the linearity study had to be repeated using wine spiked at the same calibration concentration, doing the complete sample preparation procedure and comparing the results of the two approaches. Both methodologies included a blank where only internal standards were added, and six calibration points in the range mentioned above. For the calibration with extraction, the sample preparation was done in duplicate and the response was averaged. The results are shown in Table 5 For 3MH there was negligible difference in the two calibration equations. For 3MHA the matrix had an impact on the detector response, and a matrix signal enhancement could be observed. This phenomenon has been reported previously for MS detection (Trufelli et al. 2011). For both compounds and calibration approaches, the correlation coefficient was higher than 0.99. As the detector response was similar for both types of calibration and the differences in intercept value were minor, the direct calibration was preferred for further analyses.

388

389 The limit of quantitation values was calculated from the signal-to-noise ratio
390 obtained for samples extracted from spiked model wine. Even though the model wine
391 is a non-interfering matrix, and therefore matrix effects cannot be accounted for, using
392 a standardised medium is common practice for these types of determinations. As can
393 be seen from Table 7, the limit of quantification (LOQ) values were lower than the
394 perception threshold of the respective compounds (4.2 ng/L for 3MHA and 60 ng/L for
395 3MH) in the same media (model wine). This is extremely important in the case of
396 combined wine chemical and sensory analyses. Moreover, to our knowledge, the LOD
397 for 3MH is the lowest reported in the literature: 0.07 ng/L compared to 1 ng/L by gas
398 chromatography ion trap (GCIT)-MS/MS (Schneider et al. 2003). For 3MHA, the LOD was
399 found to be 1.68 ng/L and the lowest value reported in the literature was 0.3 ng/L by
400 gas chromatography negative chemical ionisation (GCNCI)-MS (Mateo-Vivaracho et al.
401 2008). Both these values show that the method is suitable for the analysis of 3MH and
402 3MHA in white wine.

403

404 **Recovery.** As mentioned in the Materials and methods section, the recovery was
405 calculated as proportion of 'practical' as compared to 'theoretical' value. This was done
406 for the entire calibration range (six values). For the 'practical' values, the matrix and the
407 extraction will play a role. A matrix blank (no additions except for IS) was also considered
408 to account for the possible presence of analytes in the base wine. To obtain the
409 'theoretical' values, standards were directly injected. This implies no matrix effect and
410 no loss due to extraction.

411

412 For 3MHA, the average recovery was 128.36% [6.36% relative standard deviation
413 (%RSD)] and for 3MH it was 98.06% (4.04% RSD) (Table 6. The RSD values are excellent
414 for such a wide concentration range tested and extensive sample preparation; they
415 indicate the consistency of the method over the tested range. The difference in recovery
416 values can be an indication of matrix effects manifesting stronger for 3MHA than for
417 3MH. The matrix effect could take place during sample preparation (different extraction
418 yield for the analyte and its corresponding IS or during the instrumental analysis,
419 especially the detection (signal enhancement or suppression). As the IS is chemically
420 identical to the analyte in this case, the recovery result could rather be explained by
421 matrix signal enhancement. Therefore the level of recovery of over 100% for 3MHA is
422 most probably due to the detection and not to the disproportionate extraction of the
423 analyte compared to its corresponding IS.

424

425 **Precision.** Precision was evaluated with repeatability tests. The repeatability of the
426 extraction was measured in spiked wine at two concentration values and in the blank.
427 The extractions were done in triplicate and over 2 days. The repeatability of the
428 derivatisation was tested at the same two concentration values, in triplicate, for 3MH
429 and 3MHA. The results are shown in Table 7 and are calculated for retention factor
430 values. The values of the %RSD are acceptable for both extraction and derivatisation.
431 The variability was higher for 3MHA.

432 The suitability of the instrumental method was also assessed through
433 repeatability for response factor (RF) and retention times (RT). For RF, three injections

434 were done from the same vial for the two concentration values indicated in Table 7. For
435 RT, an average was measured over 24 injections. For 3MHA and 3MH, the RSD for the
436 retention times was 0.22 and 0.27%, respectively. The variability levels were considered
437 acceptable.
438
439 **Stability of analytes and samples.** The stability of the analytes and samples was also
440 assessed. The standards and stock solutions (in methanol) were stored at -80°C and were
441 found to be stable over a period of 2 years. The concentration of these analytes and
442 samples was determined with Ellman's reagent (Eyer et al. 2003).
443
444 Extracted samples in DCM were stable for up to a week when stored at -20°C
445 (results not shown). Extracted samples for injection (in methanol) were stored at -80°C.
446 Injection of the same samples a week apart indicated rapid degradation. For three
447 concentration values, the decrease in both peak areas and RF was calculated. There was
448 a significant decrease in peak areas (between 4.6 and 81.8%) for analytes and IS. This
449 would ultimately lead to the peak areas falling below the limit of quantitation. Taking
450 into account the RF values (compound peak area/IS peak area), the analytes and their
451 respective deuterated forms did not degrade at the same rate, indicating that a delay in
452 analysis would lead to inaccurate quantification of thiols.
453
454 *Volatile thiol concentration of South African Sauvignon Blanc wines*
455 Volatile thiols, such as 3MH and 3MHA, play an integral role in the passionfruit, grape
456 fruit and guava aroma of Sauvignon Blanc wines (Coetzee and Du Toit 2012, Van

457 Wyngaard et al. 2014). It therefore is important for wine producers and researchers of
458 Sauvignon Blanc wines to be able to assess the concentration of these compounds in
459 wines. Several publications have reported the concentration of 3MH and 3MHA in
460 Sauvignon Blanc wines, especially from France and New Zealand. Concentration in
461 Sauvignon Blanc wines from these countries ranged from 688 to 18 681 ng/L for 3MH
462 and up to 2507 ng/L for 3MHA (Lund et al. 2009, Benkwitz et al. 2012). There has not
463 been a concerted effort before, however, to assess the concentration of 3MH and 3MHA
464 in South African Sauvignon Blanc wines.

465 Van Wyngaard (2013) found an average concentration of 970 ng/L for 3MH and
466 158 ng/L for 3MHA, in 27 South African Sauvignon Blanc wines, while Benkwitz et al.
467 (2012) and Lund et al. (2009) reported a concentration in the same range for a few South
468 African Sauvignon Blanc wines. Ranges for those values reported by Van Wyngaard
469 (2013) were 10 to 720 ng/L for 3MHA and 500 to 3500 ng/L for 3MH. This concentration
470 was determined using a GC-MS method (Suklje et al. 2014), which was adapted from a
471 method originally developed by Tominaga et al. (1998). Average concentration obtained
472 in our study was 313.48 ng/L (range of 18.98 to 1028.70 ng/L) for 3MHA and 1320.32
473 ng/L for 3MH (range of 717.92 to 2262.22 ng/L). Such concentration was thus in the
474 same range as that found by Van Wyngaard (2013) for 3MH, but higher than that found
475 for 3MHA. The reasons for this difference in 3MHA concentration could be due to
476 vintage effects, the ability of different yeast strains to convert 3MH into 3MHA (Coetzee
477 and Du Toit 2012), as well as different acid hydrolysis rates of 3MHA to 3MH and acetic
478 acid during bottle ageing (Makhotkina et al. 2012).

Our study, however, revealed that 3MH and 3MHA in South African Sauvignon Blanc wines occur at a concentration higher than their respective perception thresholds (Table 8. These compounds thus probably play an important role in the perception of tropical aromas in these wines. The aroma descriptors associated with 3MH have been found to change in number and intensity depending on the level of this compound (Van Wyngaard et al. 2014).

Conclusions

In this paper a novel sample preparation based on a liquid/liquid extraction is presented. Thiols were quantified by UPLC-MS/MS after their derivatisation with OPA. The analytical method described is the first method allowing the liquid/liquid extraction of thiols from wine, followed by detection and quantification by UPLC-MS/MS. The method was successfully validated and applied to thiol quantification in 18 South African Sauvignon Blanc wines. The average 3MH content found in these South African wines was in accordance with previous findings, while 3MHA was higher in the present study. The development of methods to determine the concentration of 3MH and 3MHA in Sauvignon Blanc wines could therefore assist wine producers in expecting a wine with certain sensorial characteristics if their chemical composition is known.

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638

639 Figure legends:

640 **Figure 1.** Ultra-performance liquid chromatography with fluorescence detection (UPLC-
641 FLD) chromatogram of the *o*-phthaldialdehyde derivatives of (a) 6-mercaptohexan-1-ol
642 (retention time 4.9 min) and of (b) 3-mercaptohexan-1-ol (retention time 5.1 min) and
643 3-mercaptohexyl acetate (retention time 8.3 min) in wine.

644 **Figure 2**. Effect of pH on the formation of OPA derivatives of 6-mercaptohexan-1-ol
645 (■)3-mercaptohexan-1-ol (■), and 3-mercaptohexyl acetate (■)

646 **Figure 3.** Selectivity of the ultra-performance liquid chromatography tandem mass
647 spectrometry method for the separation of: (a) 3-mercaptohexyl acetate (retention time
648 10.99 min); (b) deuterated 3-mercaptohexyl acetate (retention time 11.03 min); (c) 3-
649 mercaptohexan-1-ol (retention time 8.47 min) ; and (d) deuterated 3-mercaptohexan-
650 1-ol (retention time 8.48 min).

651

652

653 **Table 1**. Gradient program for the ultra-performance liquid chromatography with

654 fluorescence detection.

Time (min)	Flow rate (mL/min)	A (%)†	B (%)‡	Curve
0	0.800	30	70	-
0.50	0.800	30	70	6
8.30	0.800	20	80	6
8.42	0.800	0	100	6
9.92	0.800	0	100	6
10.42	0.800	30	70	6
16.00	0.800	30	70	6

655 †A, 30 mmol citrate, pH 6; ‡B, methanol.

656

657 **Table 2.** Gradient programme for the ultra-performance liquid chromatography tandem

658 mass spectrometry method

Time (min)	Flow rate (mL/min)	A (%)†	B (%)‡	Curve
0	0.350	70	30	-
1.00	0.350	70	30	6
12.00	0.350	30	70	6
13.00	0.400	0	100	6
14.00	0.400	0	100	6
14.10	0.350	70	30	6
17.00	0.350	70	30	6

659 †A, 10 mmol ammonium acetate; ‡B, methanol:acetonitrile:i-propanol at 49:49:2.

660

Table 3. Multiple reaction mode (MRM) transitions monitored for the mass spectrometric detection of the thiol derivatives.

Compound name	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone (V)	Collision (eV)
3MH	294.2	176.2	15	30
		194.1	15	15
d3MH	296.2	176.2	20	30
		194.2	20	15
3MHA	337.4	83.2	15	15
		177.2	15	30
		195.2	15	15
d3MHA	338.1	85.3	20	15
		145.3	20	15

3MH, 3-mercaptohexan-1-ol; 3MHA, 3-mercaptohexyl acetate; d3MH, deuterated 3-mercaptohexan-1-ol; d3MHA, deuterated 3-mercaptohexyl acetate.

667

668 **Table 4.** Extraction yield of thiols during back-extraction (from dichloromethane to
669 water) or solvent switch (from dichloromethane to methanol) under reduced pressure.

	Extraction yield (%)	
Analyte	**Water**	**Methanol**
3MH	104.6 ± 5.6	103.1 ± 3.2
3MHA	90.33 ± 6.1	58.8 ± 4.7
6MH	103.3 ± 4.8	94.9 ± 1.3

670 Data reported as mean values ± SD (n=3). 3MH, 3-mercaptohexan-1-ol; 3MHA, 3-
671 mercaptohexyl acetate; 6MH, 6-mercaptohexan-1-ol.

672

673

674 **Table 5**. Figures of merit for the method performance.

Compound	Calibration equation†	R^2	LOQ (ng/L)
3MH	0.6173*conc + 20.296‡	0.9987	0.07
	0.6084*conc + 23.283§	0.9979	
3MHA	0.2580*conc + 4.851‡	0.9933	1.68
	0.3656*conc + 2.9729§	0.9951	

675 †The equation is for RF x 1000; ‡direct injection of standards; §calibration in base
676 wine, with extraction. LOQ, limit of quantitation; 3MH, 3-mercaptohexan-1-ol; 3MHA,
677 3-mercaptohexyl acetate.

678

679

680 **Table 6**. Recovery of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate .

	3MH		**3MHA**
Concentration (ng/L)	**Recovery (%)**	**Concentration (ng/L)**	**Recovery (%)**
50	93.8	25	133.1
100	100.6	50	122.9
250	93.2	100	125.1
500	96.5	200	129.0
1000	99.1	250	130.5
2500	99.7	500	139.4

681 Average of two determinations. 3MH, 3-mercaptohexan-1-ol; 3MHA, 3-mercaptohexyl

682 acetate.

683

684 **Table7**. Figures of merit for sample preparation and instrumental repeatability.

Compound	Concentration (ng/L)	Extraction (%RSD)	Derivatisation (%RSD)	Instrumental RF (%RSD)
3MHA	50	11.87	6.38	1.43
	250	8.33	3.07	2.45
3MH	100	2.09	2.77	2.72
	1000	4.16	0.56	1.59

685 3MH, 3-mercaptohexan-1-ol; 3MHA; 3-mercaptohexyl acetate; RF, retention factor.

686

687 **Table 8**. Concentration of 3-mercaptohexan-1-ol and 3-mercaptohexyl acetate in

688 commercial South African Sauvignon Blanc wines.

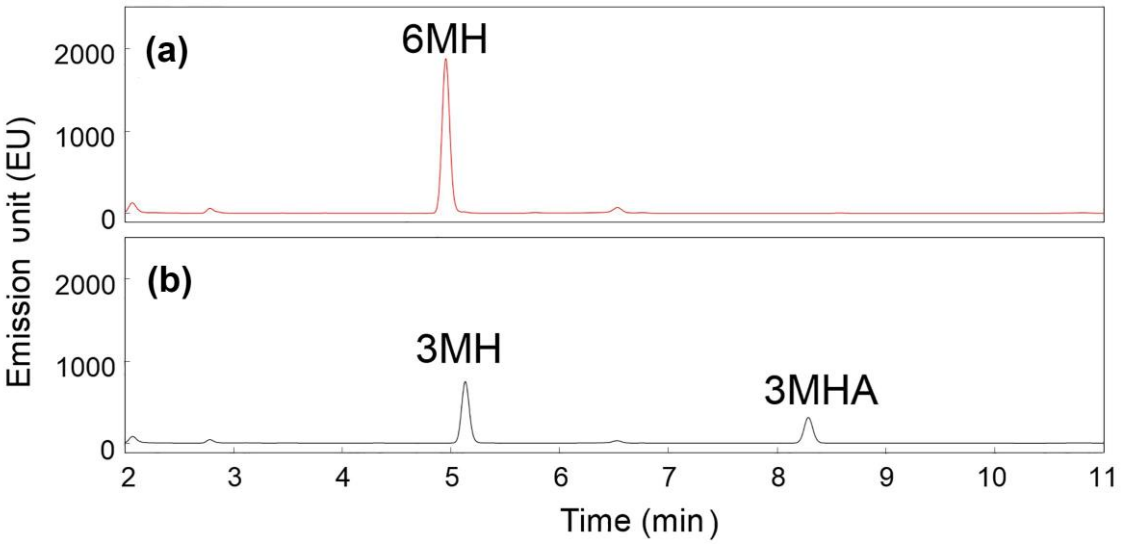
Name	Vintage	Type of sample	3MHA (ng/L)	3MH (ng/L)
Cellar 1	2012	Bottle	83	893
Cellar 1	2013	Bottle	572	1646
Cellar 2	2013	Bottle	553	3137
Cellar 3	2012	Bottle	231	1891
Cellar 3	2013	Bottle	300	825
Cellar 4	2013	Tank	112	365
Cellar 4	2013	Bottle	89	754
Cellar 5	2013	Bottle	323	820
Cellar 6	2013	Tank	676	1289
Cellar 6	2013	Tank	457	1154
Cellar 7	2012	Bottle	236	1802
Cellar 7	2013	Bottle	1029	2262
Cellar 8	2012	Bottle	53	1460
Cellar 8	2013	Bottle	184	1469
Cellar 9	2013	Tank	219	1001
Cellar 9	2013	Tank	277	1065
Cellar 10	2012	Bottle	19	718
Cellar 11	2013	Bottle	231	1216

689 Average of two extractions. 3MH, 3-mercaptohexan-1-ol; 3MHA, 3-mercaptohexyl

690 acetate.

691

692 Figure 1



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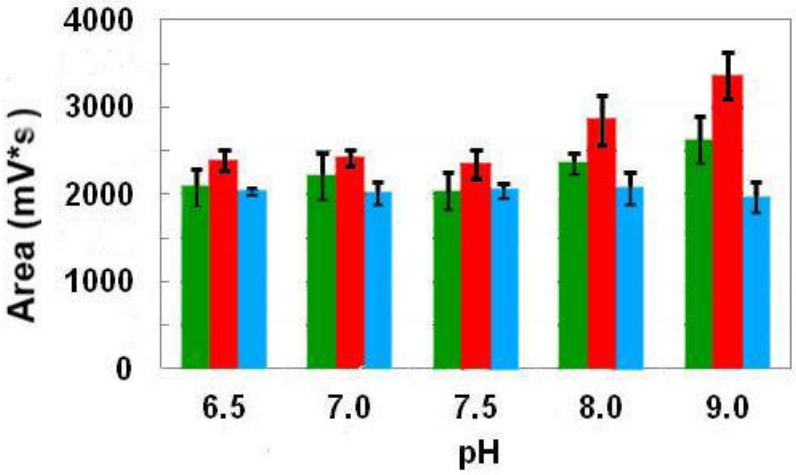
695 Figure 2



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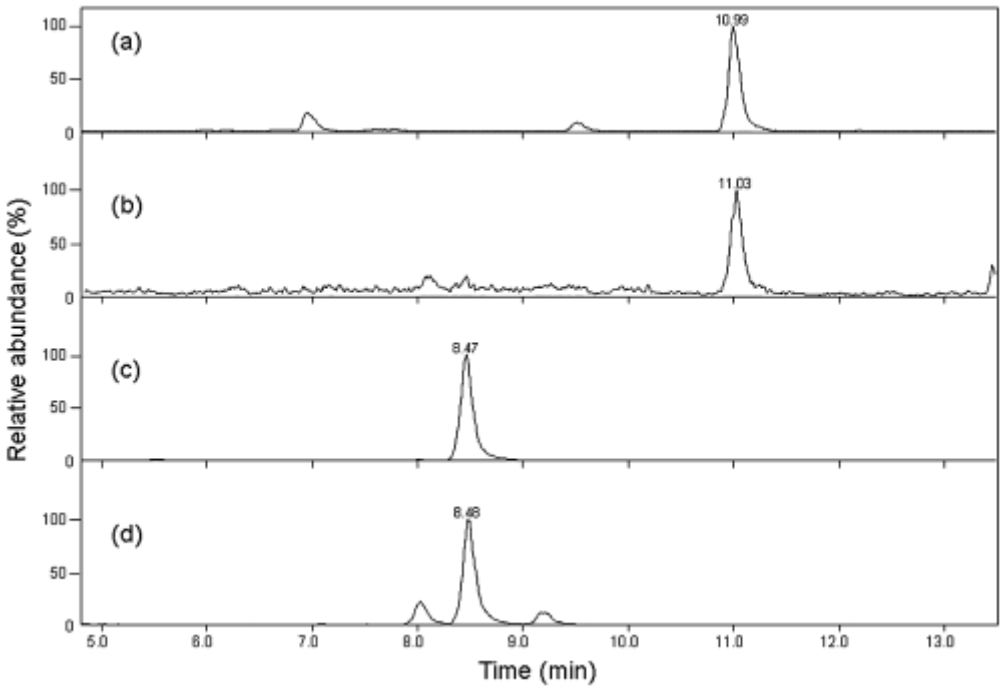
698 Figure 3



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