Quantitative determination of varietal disulfides in wine and their behavior during alcoholic fermentation

J.J J Bencomo-Rodriguez, J. Gambetta, P. Rigou, N. Canelo, Aurélie Roland, J.-M. Salmon, N Bouvier

Quantitative determination of varietal disulfides in wine and their behavior during alcoholic fermentation

J. J. BENCOMO-RODRIGUEZ, J. GAMBETTA, P. RIGOU, N. CANELO, N. BOUVIER, A. ROLAND, J.-M. SALMON

INRA, UMR 1083 « Sciences pour l'œnologie », 2, place Viala, F-34060 Montpellier, France

Mots clés : Varietal thiols, wine, oxidation, disulfides

Introduction

Varietal volatile thiols, that is to say the 3-mercaptohexan-1-ol (3MH), its acetate (3MHA) and the 4-mercapto-4-methylpentan-2-one (4MMP), are among some of the most important odorant molecules in wine, being responsible for the typicity of certain grape varieties as Sauvignon Blanc, Colombard, Merlot and Cabernet Sauvignon cultivars, and are responsible for box tree, exotic fruit and grapefruit aromas (1, 2). Varietal thiols content in wines can vary with the type and composition of must (origin and grape varieties, nitrogen content, polyphenol content) as well as with the oenological conditions such as fermentation temperature, presence of additives, dissolved oxygen content in the must, or the type of yeast used (3-9). But it was demonstrated that although all cysteinylated and glutathionylated precursors of varietal thiols totally disappear by the end of fermentation, thiols content in wine only represents a 10% conversion rate of the precursors (10-12). In addition, it was demonstrated that thiols content can significantly vary during fermentation or wine storage and that such variation is dependent on the type of yeast strains being used (10, 13, 14). Because varietal thiols are important compounds for the aromatic distinctiveness and complexity of white wine, it is therefore important to understand the chemical mechanisms responsible for their disappearance. Oxidation phenomena occurring in wine can be partly responsible for thiols disappearance. Indeed, it was demonstrated that oxidative conditions can lead to the formation of quinones from phenolic compounds present in wine that can chemically react with the sulfur compounds to form new adducts (1, 15, 16). Nikolantonaki et al. (16) showed in a wine-model medium that in the presence of catechin or epicatechin and in oxidative conditions, up to 50% of 3MH content disappeared. Thiols are also are highly reactive and easily oxidized into their corresponding symmetric and mixed disulfides in the presence of oxygen or trace levels of metal ions such as Cu and Fe (17, 18). However, the presence of disulfides of the varietal thiols 4MMP, 3MHA and 3MH and their impact on the wine aroma have never been evidenced and could be one hypothesis to explain part of thiols disappearance during or after fermentation. We developed a stable isotope dilution assay (SIDA) method for the determination and quantification of symmetric disulfides of varietal thiols in wine, based on GC-MS analysis, after a Solid Phase Extraction (SPE) of the disulfides from the wine followed by a Solid Phase Micro Extraction (SPME) of the dried extract.

Material and Methods

Synthesis of disulfides

The following disulfides 4MMP-4MMP, 3MHA-3MHA, 3MH-3MH, 4MMP-3MHA, 4MMP-3MH and 3MHA-3MH were obtained by oxidation of the pure thiols in aqueous solution using $NaIO_4$ as an oxidant for 1 hour at room temperature. The nature of the disulfide obtained depended on the pH, and oxidations were performed at pH 4, 7 and 12 in order to obtain all combinations of disulfides. All oxidation products were characterized by GC/MS by liquid injection. The concentration of each disulfide was determined by GC-AED using ethoxythiazole as external standard. The deuterated internal standards 4MMPd10-4MMPd10, 3MHd2-3MHd2 and 3MHAd5-3MHAd5 were obtained following the same procedure.

Analytical methods for the determination and quantification of disulfides

Disulfides were extracted three times with dichloromethane from a wine sample previously spiked with 4MMP-d10-4MMP-d10, 3MHAd5-3MHAd, and 3MHd2-3MHd2. The extract was evaporated to dryness under nitrogen in a SPME vial. SPME extraction on the dry extract was performed using an automatic combi-PAL system (CTC Analytics) for 45 minutes at 127ºC using a DVB/ PDMS fiber. The compounds were desorbed from the fiber directly into the GC-MS injector in splitless mode for 5 min at 250ºC. Chromatographic analysis was performed using a Shimadzu GC-MS QP5050 coupled to a quadrupole mass spectrometer detector and equipped with an automatic CombiPal system. The capillary column was a DB-WAXETR (30 m x 0.25 mm x 0.25 mm, J&W

Scientific). Helium was used as carrier gas and injector temperature was set at 250ºC. Detection was made in negative chemical ionization (NCI) with methane and acquisition made in selective ion monitoring mode. Quantification of thiols was performed using the method published previously (19).

Fermentation conditions

An industrial *Saccharomyces cerevisiae* strain, commercialized as ADY for winemaking, was used: Levuline ALS (Lallemand, Montréal, Canada). All media were heat-sterilized (110°C, 20 min). Unless otherwise specified, the yeast strain was cultivated in a synthetic fermentation medium (SM) strongly buffered to pH 3.3 (20). Ergosterol (15 mg L^{-1}) and oleic acid (5 mg L^{-1}) dissolved in 1 ml Tween 80 / pure ethanol (50:50, v/v) at 70°C were also added to the medium after sterilization. SM culture medium was spiked before inoculation with 623 ng L^{-1} 4MMP, 2.09 µg L^{-1} 3MH and 580 ng L^{-1} 3MHA.

Results and Discussion

Optimization and validation of the analytical method for the determination of disulfides

Deuterated disulfides were synthesized from the deuterated thiols (chromatograms in SIM mode on Figure 1) and the repeatability was assessed on 5 repetitions of extractions on a same white wine spiked with different concentrations of disulfides. For each natural mixed disulfide, we considered two related deuterated analogues disulfides and selected the one that gave the best % RSD. Selected deuterated analogues and % RSD obtained are reported in Table 1. Finally, all RSD values are below or equal to 10%, showing a very good repeatability of the method. Calibrations were built on three different white wines in order to assess the influence of the matrix on the method repeatability. The average of the slopes and the %RSD of the three calibrations are reported in Table 2. In the range of concentrations studied, calibrations on different wine matrices showed R^2 near 0.99 for all compounds. The method was very repeatable on a same wine matrix with RSD about 10% for all symmetric disulfides.

Influence of yeast on the formation of disulfide from varietal thiols during alcoholic fermentation

In a synthetic medium spiked with a mix of varietal thiols (4MMP, 3MH and 3MHA), degradation of these compounds was observed during the lag phase and before the maximum CO_2 fermentation rate was reached, in the presence of fermenting yeasts (Figure 2). The final disulfide content in the medium was generally lower than in the absence of yeasts (Table 3), suggesting an eventual role of oxygen transfer during the lag phase or an enzymatic contribution of yeasts in the oxidation process. The molar conversion yields of disulfides from varietal thiols along the fermentation were on the whole higher than those obtained in the absence of yeast (Table 4). This latter observation reinforced the second hypothesis dealing with the enzymatic oxidation by yeast of thiols into disulfides during the lag phase.

Yeast-dependent disulfide degradation at the end of stationary phase

A significant degradation of all detected disulfides was observed at the end of the stationary phase (Figure 3), with different molar conversion yields into the corresponding thiols (Table5). Unlike the redox potential of the culture medium at the end of alcoholic fermentation did not reach a sufficient value to chemically reduce disulfides, it seemed that yeasts were therefore able to partially catalyze this reaction.

Conclusion

The analytical method developed for the determination and quantification of disulfides of varietal thiols is based on isotopic dilution and has proved to be sufficiently sensitive for the investigation of the formation of disulfides of varietal thiols and to obtain an accurate quantification of their concentrations during fermentation and/or during wine storage.

Behavior of varietal thiols 4MMP, 3MHA and 3MH and their corresponding disulfides was studied during alcoholic fermentation in a spiked synthetic culture medium simulating wine conditions. This medium was free of complex phenolic compounds, which are well known to interact with thiols (16). As observed by many other groups, it clearly appeared that the decrease of volatil thiols in sterile conditions is a consequence of their strong reactivity with oxygen, as previously observed by other groups (1, 14). However, the formation of disulfides during alcoholic fermentation had never been proved. The precise detection of the corresponding putative disulfides showed that in presence of a *S. cerevisiae* yeast strain, the formation of disulfides can only explain 25 to 42 % of the volatile thiols disappearance from the medium. In the presence of fermenting yeasts, although oxygen content in the fermentation medium rapidly decreases, 3MH- and 3MHA-containing disulfides are

formed at higher molar conversion yields than those observed in sterile conditions. In addition, the 4MMP-3MH disulfide formation is very strongly favored by the presence of the yeast strain with a ten-fold factor effect.
Volatile thiol degradation and formation of the corresponding disulfides are mainly observed during the lag- and early yeast growth phases, although 4MMP-disulfides formation keeps on occurring later along the fermentation. One important result obtained from these experiments is the ability of the yeast strain to degrade thiol disulfides to a significant extent at the end of the fermentation. In the case of 4MMP-containing disulfides, the degradation leads only to a recovery of about 20% of the original 4MMP but for 3MH and 3MHA-containing disulfides, it reaches conversion yields up to 90%. The final behaviour of the rest of the degraded 4MMP-containing disulfides remains an open research question. However, disulfide formation could partly explain the results previously obtained and that concern unexplained loss of 4MMP at the end of alcoholic fermentation (9). Concerning 3MH-containing disulfides, a strong degradation is also observed after a fermentation progress of 0.8, but the observed conversion into 3MH and 3MHA is almost total with a recovery close to 100%.
This study is the first evidence for the formation of thiol disulfides from volatile thiols during alcoholic fermentation, and more peculiarly during the lag-and early-growth phases. Although oxygen transfer to the culture medium is thought to be reduced to a minimum (Argon-flushed medium, and CO_2 bubbling outlets), formation of disulfides occurs. On the contrary, during yeast growth and stationary phases, the disulfide formation seems to occur at a lower rate. This observation can be the result of the complete oxygen depletion from the medium by growing yeasts (21, 22). This means that even a very low oxygen concentration (less than 1 mg $L^{-}1$ at the onset of fermentation) is able to promote the formation of thiol disulfides.
A very surprising result is the ability of yeast to break disulfides bridges. This result is even more surprising since thiol disulfides are known to be quite stable molecules, that need very low redox potential to be hydrolyzed into their respective volatile thiols (17, 18). It is therefore of great importance to check the behavior of different yeast strains towards this potentiality of thiol disulfide hydrolysis, and to identify the mechanisms involved in such hydrolysis (effect of redox potential or enzymatic activity). Furthermore, the knowledge of these mechanisms would give leads for the understanding of their real sensory impact on the final wines.

References

1. Blanchard L., Darriet P., Dubourdieu D. 2004, Reactivity of 3-Mercaptohexanol in red wine: impact of oxygen, phenolic fractions, and sulfur dioxide. *Am J Enol Vitic,* 55, 115-120.
2. Kotseridis Y., Baumes R. 2000. Identification of impact odorants in Bordeaux red grape juice, in the commercial yeast used for its fermentation, and in the produced wine. *J Agric Food Chem*, 48, 400-406.
3. Dufourq T. et al. 2008. Contribution des facteurs viticoles et oenologiques au potentiel aromatique des vins blancs de Colombard en Gascogne. VIIe Congrès International des terroirs viticoles, Changins (Suisse), 530-535.
4. Subileau M. et al. 2008. Precursor uptake modulates the production of aromatic thiols during fermentation. *FEMS Yeast Res*, 8, 771–780.
5. Howell K. et al. 2004. Variation in 4-mercapto-4-methyl-pentan-2-one release by *Saccharomyces cerevisiae* commercial wine strains. *FEMS Microbiol Letters*, 240, 125-129.
6. Dubourdieu D. et al. 2006. The role of yeasts in grape flavor development during fermentation: the example of Sauvignon blanc *Am J Enol Vitic*, 57, 81-88.
7. Swiegers J. et al. 2006. Modulation of volatile thiol and ester aromas in wine by modified wine yeast. *Dev Food Sci*, 43, 113-116.
8. Masneuf-Pomarède I. et al. 2006. Influence of fermentation temperature on volatile thiols concentrations in Sauvignon blanc wines. *Int J Food Microbiol*, 108, 385-390.
9. Roland A. et al. 2010. Evolution of S-cysteinylated and S-glutathionylated thiol precursors during oxidation of Melon B. and Sauvignon blanc musts. *J Agric Food Chem*, 58, 4406-4413.
10. Subileau M. et al. 2008. New insights on 3-mercaptohexanol (3MH) biogenesis in Sauvignon Blanc wines: Cys-3MH and (E)-hexen-2-al are not the major precursors. *J Agric Food Chem*, 56, 9230- 9235.
11. Fedrizzi B. et al. 2009. First identification of 4-S-glutathionyl-4-methylpentan-2-one, a potential precursor of 4-mercapto-4-methylpentan-2-one, in Sauvignon Blanc juice. *J Agric Food Chem*, 57, 991-995.
12. Roland A. et al. 2010. Identification and quantification by LC-MS/MS of a new precursor of 3-mercaptohexan-1-ol (3MH) using stable isotope dilution assay : Elements for understanding the 3MH production in wine. *Food Chem*, 121, 847-855.
13. Murat M.L. et al. 2003. Effect of anthocyanins on stability of a key odorous compound, 3-Mercaptohexan-1-ol, in Bordeaux rosé wines. *Am J Enol Vitic*, 54, 135-138.
14. Brajkovich M. et al. 2005. Effect of screwcap and cork closures on SO_2 levels and aromas in a Sauvignon blanc wine. *J Agric Food Chem*, 53, 10006-10011.
15. Cheynier V.F. et al. 1986. Characterization of 2-S-glutathionyl caftaric acid and its hydrolysis in relation to grape wines. *J Agric Food Chem*, 34, 217-223.

16. Nikolantonaki M. et al. 2009. Reactivity of volatile thiols with polyphenols in a wine-model medium: Impact of oxygen, iron, and sulfur dioxide. Anal Chim Acta, 660, 102-109.
17. Guth H. et al. 1995. Model reactions on the stability of disulfides in heated foods. *J Agric Food Chem*, 43, 2199-2203.
18. Hofmann T. et al. 1996. Model studies on the oxidative stability of odor-active thiols occurring in food flavors. *J Agric Food Chem*, 44, 251-255.
19. Rodriguez-Bencomo J..J. et al. 2009. Improved method to quantitatively determine powerful odorant volatile thiols in wine by headspace solid-phase microextraction after derivatization. *J Chrom A*, 1216, 5640-5646.
20. Bely M., Sablayrolles J.M., Barre P. 1990. Description of alcoholic fermentation kinetics: its variability and significance. *Am J Enol Vitic²*, 40, 319-324.
21. Rosenfeld E. et al. 2003. Oxygen consumption by anaerobic *Saccharomyces cerevisiae* under enological conditions: effect on fermentation kinetics. *Appl Env Microbiol*, 69, 113-121.
22. Salmon J.M. 2006. Interactions between yeast, oxygen and polyphenols during alcoholic fermentations : practical implications. *LWT-Food Sci Technol*, 39, 959-965.

Table 1. Internal standard chosen for each compound and relative standard deviation.

Compound	Internal Standard	RSD (%) [a]
4MMP-4MMP	4MMPd10-4MMPd10	8.1
4MMP-3MHA	3MHAd5-3MHAd5	6.2
4MMP-3MH	4MMPd10-4MMPd10	10.6
3MHA-3MHA	3MHAd5-3MHAd5	5.6
3MHA-3MH	3MHd2-3MHd2	10.6
3MH-3MH	3MHd2-3MHd2	4.6

[a] Relative standard deviation of 5 repetitions using the internal standard chosen.

Table 2. Validation of the method and limits of quantification (LOQ) and detection (LOD).

Compound	Average slope [a]	SD slope [a]	RSD slope [a] (%)	Average R^2 [a]	LOD [b] (ng L^{-1})	LOQ [b] (ng L^{-1})
4MMP-4MMP	0.06243	0.01711	27.4	0.993	0.71	2.4
4MMP-3MHA	0.00131	0.00014	10.8	0.990	12	39
4MMP-3MH	0.00698	0.00050	7.2	0.981	5.7	19
3MHA-3MHA	0.00111	0.00008	7.4	0.989	16	54
3MHA-3MH	0.00095	0.00011	11.8	0.993	6.0	20
3MH-3MH	0.00064	0.00014	21.2	0.996	39	129

[a] Obtained from three calibrations curves built with three different white wines. [b] Calculated on chromatograms obtained from real white wine samples spiked at low level.

Table 3: Effect of the presence of a yeast strain on the formation of disulfides from varietal thiols.

		Initial contents ($nmol\ L^{-1}$)	Without yeast (165 h)	With yeast End of fermentation (165 h)
Varietal thiols ($nmol\ L^{-1}$)	4MMP	4.68	0.07 ± 0.01	1.45 ± 0.23 (***)
	3MHA	3.29	0.25 ± 0.01	1.86 ± 0.53 (**)
	3MH	15.36	1.78 ± 0.04	10.21 ± 0.08 (***)
Thiol disulfides ($nmol\ L^{-1}$)	4MMP - 4MMP	n.d.	0.15 ± 0.01	0.03 ± 0.01 (*)
	4MMP - 3MHA	n.d.	1.55 ± 0.09	0.36 ± 0.08 (**)
	4MMP - 3MH	n.d.	0.07 ± 0.007	0.87 ± 0.31 (**)
	3MHA - 3MHA	n.d.	0.92 ± 0.05	0.93 ± 0.14 (NS)
	3MHA – 3MH	n.d.	n.d.	0.12 ± 0.17 (NS)
	3MH – 3MH	n.d.	0.27 ± 0.01	0.05 ± 0.06 (**)

Mean and standard deviation of duplicates. n.d. : not detectable. The differences between each modality and the control (without yeast) were tested by Tukey statistical test. NS: no significant,*: significant differences at a level of $P \leq 0.05$, **: significant differences at a level of $P \leq 0.01$, ***: significant differences at a level of $P \leq 0.005$.

Table 4: Effect of the presence of a yeast strain on the molar conversion yields of disulfides from the corresponding varietal thiols.

		Without yeast (165 h)	With yeast End of fermentation (165 h)
Thiol disulfides (%)	from 3MHA and 3MH	24.1 ± 0.03	50.2 ± 1.5 (***)
	from 4MMP	41.6 ± 0.05	39.6 ± 4.9 (NS)

Mean and standard deviation of duplicates. The differences between each modality and the control (without yeast) were tested by Tukey statistical test. NS: no significant,*: significant differences at a level of $P \leq 0.05$, **: significant differences at a level of $P \leq 0.01$, ***: significant differences at a level of $P \leq 0.005$.

Table 6: Effect of the presence of a yeast strain on the molar conversion yields of varietal thiols from the corresponding disulfides at the end of alcoholic fermentation between fermentation progress of 0.8 and 1.0.

Varietal thiols	**Molar conversion yields (%)**	
4MMP	from 4MMP- containing disulfides	20.8
3MHA	from 3MHA- containing disulfides	8.9
3MH	from 3MH- containing disulfides	n.c.
3MH + 3MHA	from 3MH- and 3MHA- containing disulfides	93.2

n.c.: not calculable.