HYDROGEN SULFIDE DEVELOPMENT IN WINE DURING ANOXIC STORAGE

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Sulfur-like off-aromas (SLOs) are reportedly responsible for nearly 30% of the faults identified in premium wines in competition. Hydrogen sulfide (H_2S, "rotten egg aroma") is most frequently reported in excess of its sensory threshold (~ 1 µg/L) in wines with SLOs. H_2S can be produced during fermentation through several pathways but is sufficiently volatile such that the majority formed during fermentation will be lost to CO_2 entrainment. After fermentation, winemakers may attempt to remove H_2S by inert gas sparging, aeration to oxidize H_2S or other VSCs, or addition of cupric (Cu[II]) salts to form non-volatile complexes.

A convenient and inexpensive approach for analysis of H_2S in wine samples was developed using common winery laboratory glassware and disposable, colorimetric, gas detection tubes. Excellent linearity is achieved using both proposed methods of operation, the N_2 Method and the Aspiration Method. Limits of detection are comparable to those achieved using conventional analytical techniques.

Recent work has further established that soluble copper-sulfhydryl complexes can serve as precursors for SLO development during wine storage. Copper-sulfhydryl complexes are disrupted in the presence of strong NaCl brine. The quantity of H_2S released in this manner is correlated with H_2S formation during bottle storage. The

factors affecting the stability of these copper-sulfhydryl complexes and the release of H_2S during storage are explored in this work and a brine dilution assay has been optimized for releasing H_2S from copper-sulfhydryl complexes. Model and commercial wines were treated with copper, sulfide, and glutathione to form metastable copper-sulfhydryl complexes. In wines prepared with the addition of glutathione along with copper and H_2S, up to 4-fold increase in recovery of H_2S by brine dilution was achieved, compared to the control. Only a small portion of added H_2S could by detected following addition of disulfide bond reducing agent (TCEP) suggesting that most of the unrecovered H_2S likely formed more stable copper-sulfhydryl complexes.

A growing concern for H_2S formation is in canned wines and the phenomenon is credited to the reaction of SO_2 in wine with aluminum metal. Evidence suggests this can occur even in the presence of a polymeric liner in the can. Considerable variation is observed in H_2S production among canned wines with similar free SO_2 concentrations, such that predicting the suitability of a given wine for aluminum packaging remains challenging. The initial development of an accelerated bench-test for predicting H_2S formation is described, as well as its validation against real canned wine storage for up to eight months. In accelerated aging, negligible formation of H_2S was observed in red wines (<10 µg/L), and up to 65 µg/L of H_2S was observed in white and rosé wines, even with the best performing liner. In initial experiments, H_2S is best correlated with molecular SO_2, but the effects of ethanol content and pH cannot yet be fully decoupled to determine the relative roles of different SO_2 species.

BIOGRAPHICAL SKETCH

Rachel Allison first joined Dr. Gavin Sacks' lab as an undergraduate Summer Scholar in 2010. She received her undergraduate degrees in Chemical Engineering and Economics from Queen's University in Canada and returned to Cornell University to pursue a PhD in Food Science with a focus on wine flavor chemistry. Her research is concerned with the development of reductive off-aromas in wine during storage, particularly those aromas resulting from the instability of copper fining treatments and the interaction between wine and aluminum can packaging. She received the 2020 President's Award for Scholarship and several presentation awards from the American Society for Enology and Viticulture. Outside of academic work, Rachel baked an unprecedented number of cakes and served in leadership roles across campus, most significantly as President of the Food Science Graduate Students' Organization, Captain of the Cuvée Blind Tasting Team and Wine Education Society, and as Co-Founder/President of the Graduate Wine Society.

To all the generations of my family,

especially those who were denied the opportunity to live as

the scholars and visionaries that they were.

Those whose risks, sacrifice, and perseverance gave me

the incomparable gift to pursue knowledge.

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CHAPTER 1

INTRODUCTION

Overview

With hundreds of possible aroma compounds,[1] enjoying wine is a complex sensory experience. Many factors can contribute to the perceived characteristics of a wine, including geographical and geological place of origin, viticultural practices, winemaking techniques, climate, and aging conditions. Wine is a complex chemical system where flavor, aroma, and textural compounds participate in myriad interdependent chemical reactions and create unique and changeable organoleptic characteristics over the lifespan of the product. Unfortunately, even the most conscientious winemaking can still result in the development of undesirable characteristics and off-aromas are a regular issue in wine, even at the highest price and quality levels.[2] Among the most common faults are those classified as “reduced”, characterized by the presence of unpleasant volatile sulfur compounds (VSCs) in excess of their odor thresholds.

There are different types of VSCs present in wine. Some VSCs can contribute desirable aromas at appropriate concentrations and are considered integral to the varietal character of a wine. For example, varietal thiols like 3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpetan-2-one (4MMP) are part of the typical aroma of Sauvignon blanc wines.[3] However, other VSCs contribute to sulfur-like off-aromas (SLOs), or “reduced aromas”. SLOs are reportedly responsible for upwards of 25% of the faults identified in premium wines in competition.[2] Hydrogen sulfide (H_2S) is the

most common VSC associated with SLOs and it produces a characteristic aroma of rotten eggs. With its low aroma detection threshold (~ 1 µg/L) in wine-like matrices, it usually presents at suprathreshold levels in wine (Siebert et al., 2010).[4] H_2S is useful as a marker for SLOs due to its ubiquity in reduced wines and its potential to interact with wine components and produce additional SLOs. Other VSCs that contribute to SLOs in wine include various low molecular weight sulfhydryls and disulfides, producing a range of aromas from onion, cooked cabbage, and garlic to burned rubber, sewage, and putrescence.[5] While SLOs can be remediated when they are formed during winemaking and prior to bottling, these aromas can also reappear in bottle during wine storage, posing a challenge to winemakers.

Copper fining is a well-established and widely used technique which has only recently been identified as a source of the very problem it is intended to remediate. Though there are other recommended approaches to remediate high levels of H_2S,[6] they are not practical substitutions for copper fining and many wines will still develop commercially unacceptable levels of reduced aromas.[5] There is a need for mechanistic understanding of H_2S release from copper-sulfhydryl complexes, shown to account for the majority of bound H_2S in wines,[7] to develop more effective remediation techniques. Quality control for wine as it relates to interactions with aluminum cans is not widely addressed outside the patent literature, though mechanical quality control of metal beverage cans has been demonstrated based on their widespread use for many products. With the use of aluminum cans for wine increasing and expectations of continued category growth, coupled with wine's unique chemical properties,

winemaking parameters must be adapted to the specific chemical environment of cans to provide appropriate quality control.

A growing area of concern for H_2S formation in wine is in the use of aluminum cans for packaging, and the tendency for canned wines to develop SLOs. Records of commercial canned wines date back to the mid-1930s[8] and though canned wines make up less than 1% of the market today, the growth of this packaging vastly outpaces traditional glass and other alternative packaging (Tetra Pak, bag-in-box) in recent years.[9] Aluminum cans offer convenience to consumers (portable, different serving sizes), inexpensive lightweight shipping, and low-cost materials. Additionally, the relatively high rate of recycling for aluminum, and the rising costs and challenges of paper and plastic recycling,[10] offers significant sustainability advantages compared to other alternative packaging. The global canned wine market is predicted to reach $350 M by 2025;[11] again, there is a need for a stronger mechanistic understanding of H_2S formation in canned wines so that appropriate preventative measures can be identified.

The goal of this dissertation is to shed light on certain mechanisms of H_2S formation in wines during storage, all towards the goal of improving quality control tools for winemakers.

H_2S Analysis

H_2S is challenging to measure in wine due to its low concentration and high reactivity and volatility. H_2S is reported to be in the range of 1-20 µg/L in wines at the end of fermentation,[12] the lower end of which is around the odor detection threshold,[5] depending on the matrix. In addition to research applications, enologists in the winery may wish to quantify H_2S not only to determine its potential contribution to faulty

wines, but also to evaluate different winemaking parameters like yeast selection, fermentation conditions, remediation treatments, and packaging options. These evaluations would be facilitated by convenient, quantitative approaches for sensitive H_2S analysis.

Conventional approaches for H_2S analysis require the use of specialized analytical approaches. Early reports on H_2S quantitation in wines mainly relied on wet-chemical approaches, e.g. capturing sparged H_2S with a $Cd(OH)_2$ solution, followed by redox titration with methylene blue.[13] Modern approaches use gas chromatography (GC) coupled with a range of specialized detectors, including pulsed-flame photometric detection (PFPD)[14] and sulfur chemiluminescence detection (SCD)[5]. While these methods offer excellent detection limits (< 1 µg/L) and high selectivity, the equipment and skilled operation is costly, and the chromatography step can be time-consuming. These drawbacks make conventional approaches inappropriate for use in any modest commercial winery setting. Historically, wineries who wished to quantify H_2S have had to adopt these cumbersome approaches or send their samples for analysis by an external lab (which at the time of this work was >$100/sample for a sulfide panel analysis). An additional consideration with external analysis is that as H_2S is highly volatile and easily oxidized, there can be significant risk of losses during sample preparation and handling.

A modern version of classic colorimetric approaches utilizes gas detection tubes (GDT) for selective H_2S quantification. Originally developed for the mining industries, GDTs for H_2S are composed of glass tubes filled with an inert packing coated with an appropriate indicator compound, e.g., lead acetate. As H_2S flows

through the tube, it reacts irreversibly and causes a discoloration such that the length of the stain is proportional to the mass of H_2S passing through the tube. The use of GDTs for measurement of H_2S in enological studies was first reported for measurement of total H_2S produced by yeast strains during small-scale fermentations.[12, 15] These reports use the CO_2 produced during fermentation to force H_2S through the GDT, an approach that is not viable for post-fermentation wines without CO_2. For post-fermentation H_2S analysis, we previously reported that CO_2 could be generated *in situ* through addition of carbonate-containing antacid tablets to a flask containing a wine sample and fitted with a GDT.[16] However, this approach results in a shift of the pH to ~6, which could potentially release H_2S from known precursors.[17]

Since various winemaking decisions can influence the development of H_2S during and after fermentation, a simple approach suitable for both research and industry would be useful. Winery labs are often equipped with modest glassware, which when coupled with adapted GDT approaches, have been developed into a rapid H_2S analysis method for wine samples, as described in Chapter 2.

Development of H_2S and related reductive off-aromas in wine

H_2S is produced by yeast during fermentation as an intermediary step in amino acid synthesis, particularly in fermentations with insufficient yeast assimilable nitrogen (YAN).[18] Naturally occurring sulfates and added sulfites are converted to sulfide as part of the sulfate reduction pathway, but in the low-pH environment of wine, excess sulfide can be converted to H_2S.[19] Yeast can also form H_2S from S^0

fungicide residues.[16, 20] Degradation of amino acids, such as cysteine and methionine, have also been identified as a source of various SLOs in wine.[21]

The phenomenon of reappearing SLOs has led to various theories on the precursors and formation of H_2S and related VSCs during storage. Wines stored under low-oxygen or anoxic conditions produce more H_2S during storage than those stored under higher oxygen conditions.[22-23] The total content of H_2S and methanethiol (MeSH) is strongly correlated to the amino acid and metal content of wines.[24] Glutathione, particularly in combination with copper, induces conditions favorable to the accumulation of H_2S and MeSH in storage.[23] Sulfhydryl concentration is further affected by quinones,[23] which have been demonstrated to be responsible for VSC loss, including loss of varietal thiols.[25] Various transition metals, notably Cu(II), can form complexes with H_2S and MeSH, which can subsequently be released when the complexes are disrupted by dilution with NaCl brine.[7] The release of H_2S and MeSH from complexes can be promoted under accelerated anoxic storage at 50°C, where over 90% of the increase in free H_2S was attributable to release from bound precursors in red wines, with a 58% increase observed in white/rosé wines.[24] The correlation is much weaker between brine dilution and 1-year of room temperature storage,[26] suggesting that both brine dilution and accelerated anoxic are more forcing conditions that typical wine storage. The mechanism of brine dilution is not fully understood, and Chapter 3 will evaluate some of the factors that determine the effectiveness of different brine dilution conditions to release bound H_2S, indirectly comparing the stability of copper-sulfhydryl complexes.

Wines packaged aluminum cans can also release H_2S from bound copper-sulfhydryl precursors,[27] but it appears that other chemical pathways may be involved. Aluminum is a highly reducing metal and forms an aluminum oxide coating (Al_2O_3, "alumina") on surfaces exposed to oxygen, and in addition to a polymer liner, this is supposed to prevent wine from coming into direct contact with metal beverage cans. However, if the liner and alumina layer can be permeated or damaged, undesirable interactions between the metal and wine components can occur. Evidence suggests that this is likely the case. Alumina is susceptible to dissolution in aqueous media of pH < 4.5.[28-29] Corrosion can be further facilitated in the presence of halide, sulfate, and copper ions.[30-33] The polymer liner, typically epoxy-based resin, is also susceptible to degradation from acid[34] and ethanol.[35] There are well-established reports demonstrating that H_2S is generated when wine is exposed to aluminum alloy turnings, but not when exposed to pure aluminum,[36] suggesting that non-Al components either interact directly with wine or facilitate metal/wine interactions. Several transition metals, including Cu, Fe, Mn, Zn, and Al, have been shown to have synergistic effects on VSC evolution.[37] Beverage cans are typically manufactured from aluminum alloys of the 3xxx series for the body and the 5xxx series for the lid.[38] The main alloying metals are Mn and Mg, respectively, but other trace metals relevant to wine systems may also be present.[29] Impurities in aluminum can affect the integrity of the oxide coating and the interaction of the metal with the bulk solution.[39] In terms of damage to the aluminum can by pitting and corrosion, in a model beverage solution, several wine components are known to contribute, including copper, chloride and bisulfite.[31] Chapter 4 will address the need for a better understanding of how some characteristic

chemical properties of wine (low pH, presence of SO_2 and Cu) in the presence of aluminum, have the potential to create new pathways for H_2S formation during anoxic storage.

Effect of copper fining on H_2S formation in wine

Prior to bottling, winemakers have several options for remediating SLOs, with the two most common being aeration and copper fining. Aeration involves techniques such as splash racking and sparging to volatilize and oxidize malodorous VSCs. However, oxidation of VSCs can lead to the formation of non-volatile polysulfides/polysulfanes, precursors which have been shown to release H_2S by chemical reducing agents and under reductive storage.[20, 40] In copper fining, Cu^{2+} in the form of $CuSO_4$ is added to wine to produce non-volatile and odorless complexes with H_2S and other thiols. The reaction between copper and free sulfhydryl groups is rapid, though not selective.[40] Due to the low solubility of these complexes, the added copper was previously presumed to form a precipitate that could be removed by racking or filtration,[19] as copper-sulfhydryl precipitation occurs readily in other aqueous model solutions.[41] In the United States, up to 6.0 ppm of copper can be added to wine, but the residual copper at bottling cannot exceed 0.5 ppm, or 8.3% of the maximum addition.[42] However, there is now clear evidence to suggest that only a small fraction of the copper added to wines is precipitated and removed as copper-sulfhydryl complexes. Over a range of sulfide to copper ratios, it has been shown that 79% or more of the added copper remains in the wine following racking/filtration, including filtration at pore sizes smaller than those typically use in wine making.[43] The

ineffectiveness of racking and filtration for removing copper-sulfhydryl complexes highlights a need for improved remediation techniques for SLOs prior to bottling.

Interestingly, in model systems, only ~1% of added copper remained following racking/filtration using high sulfide: copper ratios.[43] The authors suggest that rather than a precipitate, copper-sulfhydryl nanoclusters are formed in real wine, and do not necessarily aggregate or grow to a sufficient size for precipitation.[43] Further, aggregation in real wines is thought to be inhibited by competing copper complexing ligands or other crystal growth inhibitors.[43] More recently, it has been suggested that these inhibitors may be organic thiols native to the wine, such as cysteine, which interrupt the regular polymerization and condensation of the bulk copper sulfide.[40] Chapter 3 describes differences in the stability of copper-sulfhydryl complexes during brine dilution between different commercial and model wines, using different brine solutions, and under varying temperature and incubation times. Brine dilution was most effective with a halide salt brine, though some variation was observed for wine analysis, addition experiments yielded no significant differences between brines. H_2S release also increased at higher temperatures. Notably, copper-sulfhydryl complexes in model wine appear to be more stable than those formed in real wine, after just 20 minutes of incubation. If particle size is analogous to its stability during brine dilution, this is consistent with relatively rapid rate of precipitate formation in model wine solutions containing a single sulfhydryl (H_2S or Cysteine), compared to mixed sulfhydryls.[40] The brine dilution results for model wine could be made to more closely resemble those for real wine when model wine was first treated with an addition of glutathione. From this, I hypothesized that the incorporation of organic sulfhydryls

into copper-sulfhydryl complexes is related to the observed decrease in their stability during brine dilution, both in real wine and glutathione-enhanced model wine. To investigate this hypothesis, model wine-derived copper-sulfhydryl complexes were produced, with different ratios of copper, sulfide, and glutathione. Validation was also carried out against commercial wines, which were evaluated for free sulfhydryls and copper content, and then analyzed by brine dilution, to see how native copper and organic sulfhydryl content influence the stability of copper-sulfhydryl complexes.

Currently, there are general recommendations to address the problem of H_2S formation during storage. Recent work has shown the effectiveness of (1) aerative instead of reductive winemaking techniques, (2) careful yeast nutrient and fermentation management to avoid stuck and sluggish fermentations that are prone to excess H_2S production, and (3) early rather than late copper treatments for limiting H_2S reappearance.[6] The current recommendations mediate the severity SLO issues but do not solve them completely or address the individual qualities of different wines. While fermentation and nutrient management is generally recommended in winemaking (excluding natural fermentations and other minimal intervention approaches), the timing of copper additions depends on the timing of SLO development, and aerative winemaking techniques are not a realistic option for all wine styles. The latter is particularly true when it is desirable to preserve varietal thiols and other favorable qualities of reductive winemaking. Thus, current recommendations are not practical substitutions for copper fining and many wines can develop commercially unacceptable levels of reduced aromas.[5]

Effect of aluminum cans on H_2S formation in wine

When H_2S is formed in canned wines, we observe that is can appear more quickly and at higher levels during storage than in bottled wines. When screw cap closures were first introduced, more reduction issues were initially noticed with the lower O_2 ingress closures, compared to cork. By comparison, cans have a theoretically lower O_2 ingress rate than bottle closures or alternative packaging,[44] due to the effectively hermetic double seam. While this anoxic environment undoubtedly contributes to the incidence of reduction in canned wines, we hypothesize that there are mechanisms of H_2S formation unique to canned wines. In the patent literature, H_2S generation in canned wines reportedly correlates with SO_2 content.[45-46] Total SO_2 in wine consists of bound forms and free forms, the latter of which further comprises dissolved bisulfite (HSO_3^-) and molecular SO_2 fractions. While HSO_3^- is about 95% of the free SO_2 in solution at wine pH, molecular SO_2 can permeate polymer films [47] and potentially participate in reactions at the alumina or aluminum surfaces. The maximum levels of SO_2 are regulated by the TTB, and general guidelines are used by winemakers to achieve the desired level of antioxidant and antimicrobial power. Aluminum can producers also provide general guidelines for SO_2 in canned wines but fail to distinguish between recommended levels within the free SO_2 pool. Quality control measures for wine packaged in cans has not kept pace with the growing use of this packaging and there is a need for more specific SO_2 limits, recognizing the different roles that played by the HSO_3^- and molecular SO_2 fractions. In Chapter 5, we have begun to evaluate the correlation between different SO_2 fractions and the generation of H_2S in canned wine model systems, and the hypothesis that despite the

relative abundance of HSO_3^-, the molecular SO_2 fraction could be most strongly predictive of H_2S due to the former's transport limitations.

The discussion in Chapter 4 provides the basis for several subsequent experiments on the factors influencing H_2S formation in canned wines, some of which is presented in Chapter 5. In summary, this work aims to identify the factors driving H_2S appearance in wines stored in a variety of real and model anoxic environments, provide a basis for precise and individualized component limits (SO_2, Cu) for canned wines and, ultimately, refine predictive tests for assessing the risk of H_2S formation during storage.

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CHAPTER 2

ANALYSIS OF FREE HYDROGEN SULFIDE IN WINES USING GAS DETECTION TUBES

Abstract

Reduction in wines, characterized by sulfur-like off-aromas (SLOs), is a commonly reported fault. In such wines, hydrogen sulfide (H_2S) is likely to be present above its odor detection threshold (~ 1 µg/L), presenting a marker for reductive character. H_2S formation in wines during fermentation and post-packaging can be influenced by various winemaking decisions, including yeast strain selection, nutrient additions, remediation treatments, packaging type, and storage conditions. Currently, winemakers do not have convenient methods of measuring H_2S in the winery, to assist with these winemaking decisions. Conventional methods for H_2S analysis involve the use of gas chromatography coupled with specialized detectors and are inappropriate for most wineries. We propose a new approach using selective, colorimetric gas detection tubes (GDTs) for quantification of free H_2S in still wines. The approach has been developed by adapting common winery glassware from an Aeration-Oxidation unit for an apparatus, through which a gas stream is used to force H_2S in the wine sample through the GDT. The approach has been validated using either N_2 gas to push the gas stream (N_2 Method), or vacuum-generated air to pull the gas stream (Aspiration Method). Excellent linearity and in-lab reproducibility were achieved

using both methods ($r^2 > 0.99$; mean %CV < 5%), and limits of detection are comparable to more expensive and cumbersome conventional approaches.

Summary

Goals: Hydrogen sulfide (H_2S, "rotten egg" aroma) concerns winemakers due to its contribution to sulfur-like off-aromas (SLOs). However, there are a lack of inexpensive, convenient methods for quantitation of H_2S in wines at or below its reported odor threshold. The use of selective, colorimetric gas detection tubes (GDT) for measurement of H_2S during fermentation has been previously described, but this approach has not been adapted and validated for finished wines. We developed and validated protocols for rapid, inexpensive analysis of H_2S using GDTs and Aeration-Oxidation (A-O) glassware commonly available in wineries. Video demonstration of the approaches is also provided.

Key Findings

- Two approaches were validated for GDT-based quantitation of H_2S in wine. In the first approach, H_2S was sparged from the sample with N_2 gas, analogous to Monier-Williams analyses of SO_2. In the second approach, H_2S was sparged by a vacuum-generated air stream, analogous to A-O analyses of SO_2.
- Both approaches require <15 min/sample and achieve excellent linearity. The calibration curve for the N_2 Method was identical to the curve predicted from the manufacturer's markings. The Aspiration Method was less sensitive, likely because of oxidative losses. However, the Aspiration Method was simpler to set up, operate, and adapt to higher concentration samples.

- The limits of detection were 12-13 ng H_2S for the methods, or ~0.2 µg/L using a 60 mL sample. The mean coefficients of variance (%CV) were <5% for both approaches.
- Using the new method, we observed that commercially purchased wines stored in aluminum cans have significantly higher H_2S than commercial wines in glass packaging.

Significance

The novel methods can be used for routine H_2S analysis in wineries without the need for significant investment in new equipment. In addition to cost savings, the ability to test H_2S onsite rather than send samples to an external lab decreases the risk of H_2S losses through oxidation or volatilization. These new analytical tools can be used for benchmarking, diagnosing faulty wines, or evaluating the effects of winemaking parameters, such as yeast selection, remediation treatments, and packaging options on H_2S.

Overview

Sulfur-like off-aromas (SLOs) are one of the most common faults observed in commercial wines[5]. Although several sulfhydryls can contribute to SLOs, hydrogen sulfide (H_2S) is reported to be the *S*-compound most frequently in excess of its sensory threshold (~1 µg/L)[5] in wines with this fault. H_2S may contribute directly to SLOs due to its rotten egg aroma and/or its presence could serve as a marker for other related malodorous sulfhydryls. H_2S can be produced during fermentation through several pathways, including as an intermediary step in *S*-amino acid biosynthesis;[18] as a degradation product of S^0 fungicide residues;[16, 20] and through catabolism of *S*-amino acids, especially cysteine[21]. H_2S formed during fermentation will be partially lost due to CO_2 entrainment[48] and can be further diminished post-fermentation by winemaking approaches like copper addition or aeration[49]. However, these approaches can generate precursor compounds (copper sulfide complexes; organopolysulfanes) capable of releasing H_2S during the reductive storage conditions typical of bottled wine, i.e. low oxygen in the presence of SO_2[7, 23, 50]. Finally, H_2S is anecdotally reported to form through storage of wine in aluminum cans, possibly due to reaction of SO_2 with the aluminum metal as well as the previously described precursors. Thus, enologists may wish to quantify H_2S not only to determine its potential contribution to faulty wines, but also to evaluate winemaking parameters such as yeast selection, fermentation conditions, remediation treatments, and packaging options.

H_2S is reported to be in the range of 1-20 µg/L in wines at the end of fermentation[51], and its low concentration and high reactivity requires the use of specialized analytical

approaches. Early reports on H_2S quantification in wines generally relied on laborious wet-chemical approaches, e.g. capturing sparged H_2S with a $Cd(OH)_2$ solution, followed by redox titration with methylene blue[13]. More recent reports utilize gas chromatography (GC) coupled with a range of detectors, including pulsed-flame photometric detection (PFPD)[14] and sulfur chemiluminescence detection (SCD)[5]. These methods offer excellent detection limits (< 1 μg/L) and high selectivity but are inappropriate for use in most commercial wineries due to the expense of the equipment and specialized skill necessary for their operation. Additionally, H_2S is highly volatile and readily oxidized, which necessitates considerable precautions during sample handling.

A modern version of classic colorimetric approaches utilizes gas detection tubes (GDT) for selective H_2S quantification. Originally developed for the mining industries, GDTs for H_2S are composed of glass tubes filled with an inert packing coated with an appropriate indicator compound, e.g., lead acetate. As H_2S flows through the tube, it reacts irreversibly and causes a discoloration such that the length of the stain is proportional to the mass of H_2S passing through the tube. An example of discoloration in different GDTs is provided in the Supplemental Information.

The use of GDTs for measurement of H_2S in enological studies was first reported for measurement of total H_2S produced by yeast strains during small-scale fermentations[12, 15]. These reports use the CO_2 produced during fermentation to force H_2S through the GDT, an approach that is not viable for post-fermentation wines without CO_2. For post-fermentation H_2S analysis, our group previously reported that CO_2 could be generated *in situ* through addition of carbonate-containing antacid

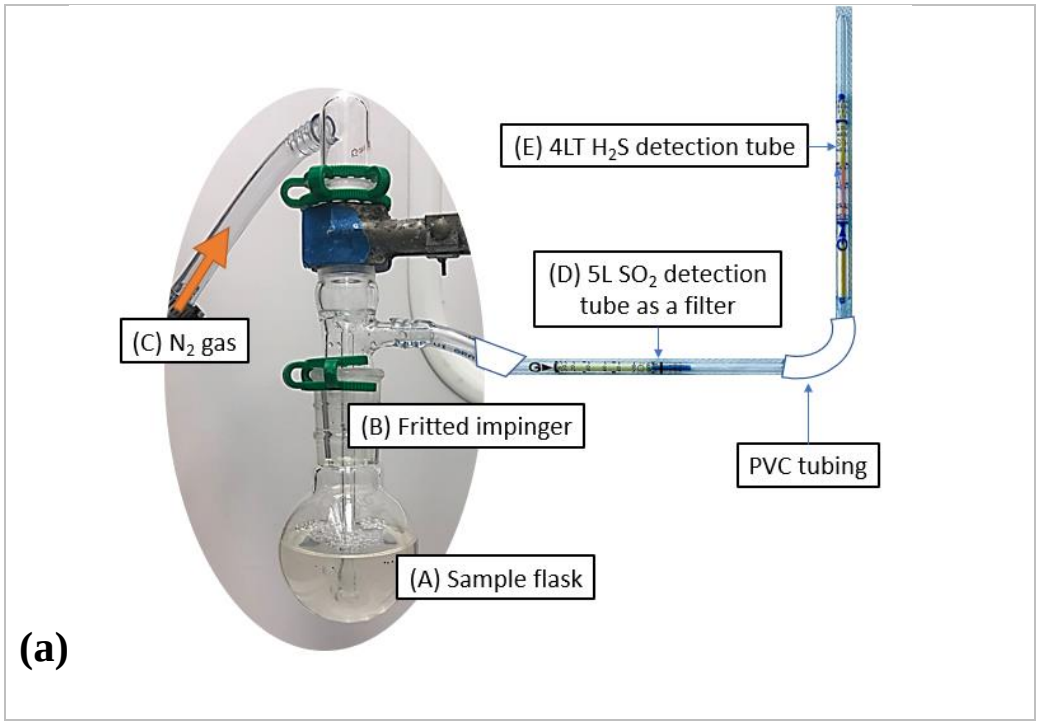
tablets to a flask containing a wine sample and fitted with a GDT. However, this approach results in a shift of the pH to ~6, which could potentially release H_2S from known precursors[17]. Additionally, the rapid generation of CO_2 gas can occasionally result in dislodging the detection tube or another connection.

In this work, we describe a rapid, inexpensive method for detecting and quantifying H_2S in still wine samples using a GDT and the widely available Aeration-Oxidation (A-O) apparatus. We report figures of merit, apply the assay to commercial wines, and provide a detailed video description of the protocol.

Major Observations and Interpretations

Apparatus and Materials. The principle for the proposed H_2S method is based on the GDT protocols previously developed for measurement of S^0 residues in grape must[16] or H_2S formed following release from wine precursors or during the course of fermentation[12, 52]. The current approach used either inert gas (N_2 Method) or vacuum aspiration (Aspiration Method) to sparge H_2S from the sample and through the GDT (Figs 1a and 1b). The flask and tubes are connected in series through PVC tubing. A sufficient length of PVC tubing (15 cm or more) between sample flask and the first GDT is recommended to prevent splashing of water droplets and fouling of the GDT inlet. A demonstration of these approaches can be found in the accompanying video (see Supplementary Video). In contrast to the earlier works which relied on antacid tablets to evolve CO_2 gas *in situ*, these new approaches did not cause pH changes or

dilution of the sample which could have risked release of H_2S from precursors like copper-sulfhydryl complexes[17]. The antacid-based approaches also required the opening and resealing of the apparatus to sequentially add tablets, which risked losses of H_2S due to volatilization.



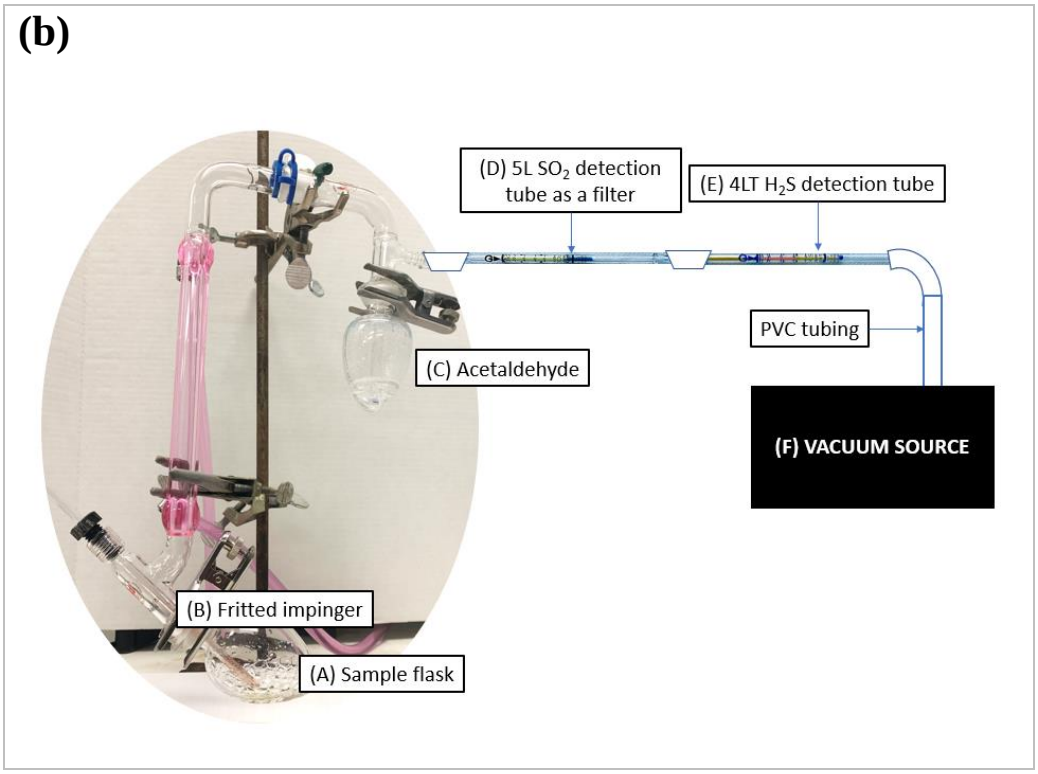


Figure 2.1. Apparatus for measuring H_2S using (a) inert gas (N_2) sparging and (b) vacuum aspiration. The sample flask is connected to an impinger and the gas detection tubes (GDTs) are connected to the outlet, in series.

As described in more detail below, both the N_2 and Aspiration Methods yield satisfactory results. However, the Aspiration Method is easier to use in practice, as the N_2 Method can overpressure and dislodge the GDT or other components, resulting in leaks. Additionally, the Aspiration Method allows easy replacement of the GDT if the tube becomes saturated during a run. However, the N_2 Method produced a calibration curve identical to the curve calculated from the manufacturer's GDT markings, and thus may require less frequent calibration (Fig 2).

GDT selection and interferences from SO_2 and other sources: The Gastec 4LL and 4LT GDTs are not susceptible to interferences from most wine components, including sulfate, acetic acid, or water vapor [16]. Several other compounds listed by the

specification sheets for these GDTs (e.g. ozone, nitrogen dioxide, nitric acid, hydrogen chloride, hydrogen fluoride, ammonia)[53] are unlikely to be important to wine analyses. However, there are two potentially relevant interferences: thiols (mercaptans) and SO_2. Thiols are reported to be an interference for the more sensitive 4LT tubes (which contain $HgCl_2$) and not for the 4LL tubes (which contain $Pb(CH_3COO)_2$). Although the response of 4LT tubes to thiols is greater than for H_2S[12], thiols are also less volatile and are typically at lower concentrations in wine[5]. We had previously estimated that interference from methanethiol (the thiol of greatest concern due to volatility and concentration) in a typical wine would be only 25% of the H_2S signal[52]. However, in situations where interferences from methanethiol or other thiols are of concern, 4LL tubes can substituted at the expense of ~3-fold lower sensitivity[16].

SO_2 is described as an interference for both 4LL and 4LT tubes[53]. Earlier reports by our group using GDTs relied on antacid tablets to generate a gas stream, which buffered the pH to ~6 and strongly favored non-volatile forms of SO_2 (i.e. bisulfite, sulfite). However, in initial studies at native pH, we observed considerable interferences from model solutions containing SO_2 (data not shown). To remove SO_2 from the gas stream, we investigated three approaches – addition of H_2O_2 or acetaldehyde directly to the sample; using an inline SO_2 GDT to scrub the gas stream; and using an inline acetaldehyde solution.

Direct addition of acetaldehyde was not effective at eliminating the interference (data not shown). Addition of H_2O_2 to the wine sample was effective at removing the SO_2 interference for additions of 0.18% and 0.35% by volume but was not pursued further due to concerns about H_2S oxidation.

SO_2 GDTs were effective in eliminating interferences from SO_2. The Gastec 5Lb and 5L SO_2 tubes were both tested, and the 5L was ultimately selected for its higher capacity. We also evaluated the use of an inline solution of acetaldehyde (1.5% v/v; Fig 1(b), Item C). By itself, the inline acetaldehyde solution was unable to fully remove SO_2 interferences but including the acetaldehyde trap before the SO_2 GDT had the advantage of preserving the lifetime of the SO_2 GDT. If this approach is used, then the acetaldehyde solution should be replenished when the SO_2 GDT is replaced.

Calibration and Figures of Merit for H_2S GDTs. Figures of merit for the N_2 and Aspiration Methods are summarized in Table 1. We observed a linear relationship ($r^2 > 0.99$) between the length of color change on the H_2S GDT and the nominal concentration of the calibration standards for both methods (Fig 2).

(i) **N_2 Method** Length of color change (mm) = 0.100 x H_2S (in ng)

(ii) **Aspiration Method** Length of color change (mm) = 0.068 x H_2S (in ng)

Theoretical calibration curves based on the manufacturer-provided markings (see Supplementary Information) were also plotted, along with a curve from a previous report which used antacid tablets as an *in situ* gas source (Fig 2)[52]. The slope of the theoretical curve based on manufacturers markings (dotted line) was identical to the slope observed for the N_2 Method. However, the slope of the Aspiration Method was about 30% lower than the theoretical slope, with a similar value reported for the earlier antacid tablet method (dashed line).

Table 2.1. Figures of merit for H_2S GDT N_2 and Aspiration Methods

	Mean %CV[a]	Linear range (ng H_2S)	r^2	LOD [b] (ng H_2S)	LOD [b] (µg/L H_2S for 60 mL sample)
N_2 Method	4.5	0 - 307	0.9991	13	0.2
Aspiration Method	4.1	0 - 875	0.9993	12	0.2

(a) Mean and range for % coefficient of variation (CV), where %CV for each wine was calculated as (standard deviation / mean).

(b) Limit of detection (LOD) is calculated as 3.3 × standard deviation of the lowest concentration standard divided by the slope.

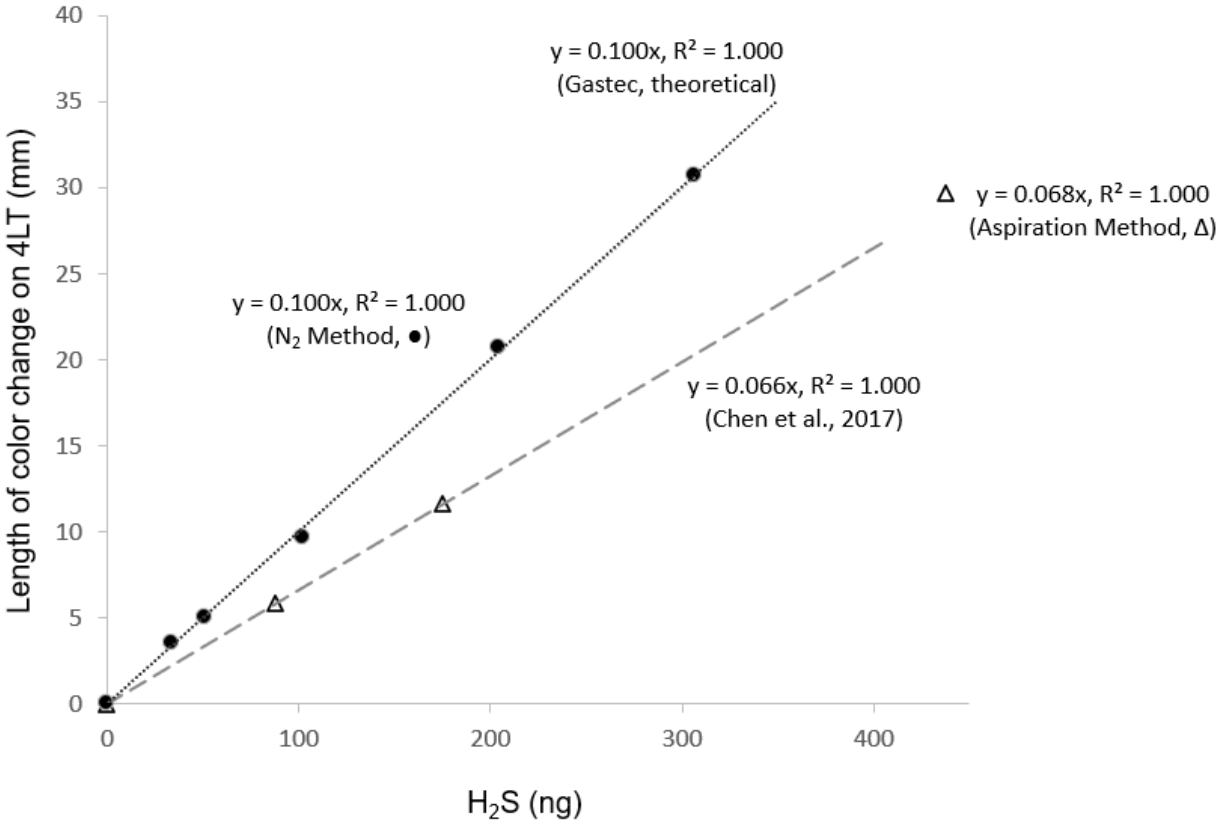


Figure 2.2. Calibration curves for H_2S [mass of H_2S (y, ng) vs. stain length (x, mm)] on Gastec 4LT gas detection tubes: (i) Aspiration Method from the current study, regression equation is y = 14.7x, (ii) N_2 Method from the current study, y = 10.0x, (iii) Alka-Seltzer tablet sparging, with regression equation calculated from reported values (Chen et al, 2017), y = 14.9x. The Manufacturer's theoretical regression equation (H_2S vs. stain length) calculated from tube markings, y = 10.0x, labeled as (iv). For all regressions, the intercept was not significantly different from zero and was omitted.

The close agreement between the N_2 Method slope and the theoretical slope based on manufacturer markings is a potential advantage for this method over the Aspiration Method, as the N_2 method could potentially be used with less extensive calibration. The lower sensitivity of the Aspiration Method could be due to partial losses from oxidation of H_2S during analyses, as the sample is not protected from air in this analysis. The reason for the lower-than-theoretical sensitivity of the antacid method is less clear, as the evolved CO_2 gas should have created an anoxic environment. However, the earlier method required the opening and closing of the reaction flask to add additional tablets, which could have introduced air.

Coefficients of variance (%CV) were calculated for each of the five calibration standards for each method (n=3 replicates for each standard), and the repeatability calculated as the mean %CV. We observed excellent repeatability (%CV <5%; Table 1). We also observed excellent within-lab reproducibility (Fig 3). Two standard solutions (n=16 total) were run at regular intervals over a four-week period without recalibrating the tubes. Data were normalized to the expected value. We observed 95% confidence interval of 92-97% recovery (ideal = 100%) of the expected signal over the four week experiment.

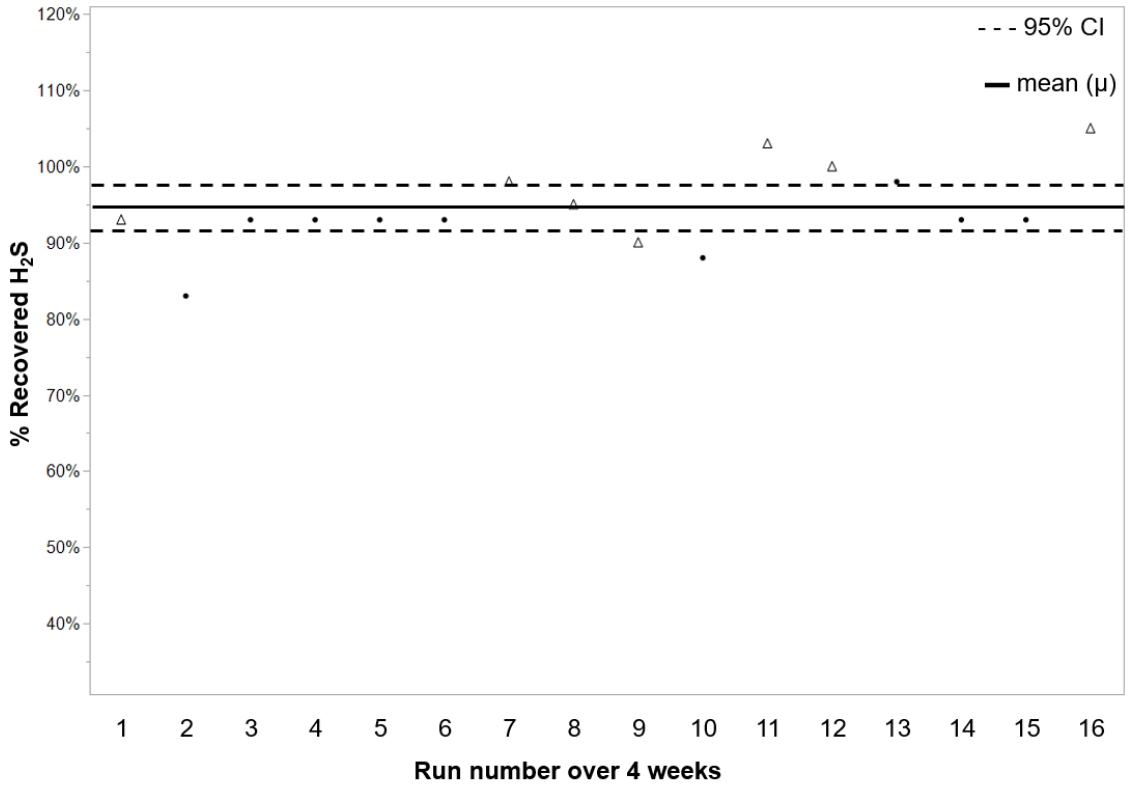


Figure 2.3**.** In-lab reproducibility of N_2 Method measured over 4 weeks with higher concentration (Δ) = 3.10 µg/L (n=7) or lower concentration (●) = 1.55 µg/L ng (n=9) standards. Sample measurements are normalized to the expected value (% Recovery H_2S = H_2S measured /H_2S added). The 95% CI is shown as µ = 94.4 ± 2.9%.

Based on noise calculations for the lowest calibration standard on the 4LT GDTs, we calculated that a limit of detection of 13 ng H_2S for the N_2 Method and 12 ng H_2S for the Aspiration Method. As previously reported for GDT methods, the limit of detection is primarily determined by the smallest observable change (0.5-1.0 mm) in stain length[16]. This can be more challenging for the 4LT tubes than other tubes due to the subtle color change (yellow to pink). GDTs respond to the initial mass of H_2S, and not the initial concentration, and we were able to vary sample volume of model wine or Milli-Q water over a wide range (5 to 66 mL) without affecting the relative response (see Supplemental Information). Initially, calibration solutions of H_2S were

measured in sample volumes of 6, 10, and 50 mL, using the N_2 Method and 5 min of sparging with inert gas. When lower relative signal was observed for the 50 mL samples, analysis time was increased to 10 min. Sample volumes of 30, 60, and 66 mL were measured for 10 min using the N_2 Method, and sample volume of 5 mL was measured for 10 min using the Aspiration Method. The fractional recovery (Free H_2S/Added H_2S) was not significantly different between the sample volumes and analysis time combinations listed (Tukey's test, α=0.05), with the 50 mL/ 5 min sample excluded. From this, we recommend using a 10 min analysis time for both methods for sample volumes in the reported range. A shorter analysis time of 5 min may be appropriate for sample volumes of 10 mL or less. Using a 60 mL sample volume, we could achieve a detection limit of 0.2 µg/L. This value is below the reported odor threshold for H_2S in wine[5] and compares favorably to detection limits reported with more expensive technologies. For example, an LOD of 0.2 µg/L has been reported for H_2S using GC-SCD[5], while quantitation limits of 1.0 and 1.7 µg/L were reported using GC-PFPD[14, 54].

As mentioned above, 4LT tubes achieve the best sensitivity and detection limits but suffer from interferences from thiols[12], and the use of the less sensitive Gastec 4LL tube may be prudent when thiol interferences are suspected. Furthermore, the cost of a box of 10 GDTs is ~ $70 USD, and tubes cannot be regenerated. Although multiple samples can be analyzed on one GDT until the tube is exhausted, high H_2S concentration samples can quickly exhaust the capacity of a Gastec 4LT (~500 ng of H_2S). In practice, we typically used a GDT only once before disposal, particularly when handling samples with unknown H_2S concentration when we did not want to

risk saturating the GDT. However, reuse of tubes that are not yet saturated was practiced when the expected concentration is known (such as with calibration standards). In general, we recommend using the 4LT tube and 60 mL sample size for low concentration samples (less than ~5 µg/L), and to use a 4LL tube when measuring higher concentration samples or when high levels of thiol interferences are expected.

Analysis of commercial wines. A convenient sample of twelve commercial wines (6 bottled, 6 canned) were purchased from local stores, and were evaluated using the GDT methods (Fig 4). We observed an average H_2S concentration in the bottled wines of 1.1 ± 0.9 µg/L, a range consistent with values reported elsewhere[5]. The mean H_2S in the canned wines was 13.5 ± 9.9 µg/L significantly higher than the bottled wines ($p<0.05$). The highest concentration of H_2S in the canned wines was nearly 30 µg/L, comparable to the highest values observed in a survey of commercial wines described as "reduced"[5]. These observations concur with recent anecdotal observations that wines stored in aluminum cans will develop H_2S during storage due to i) anoxic conditions and degradation of H_2S precursors, and ii) reaction of SO_2 with the aluminum metal to form H_2S[55]. As a caveat, the effects of packaging may have been confounded with other variables, e.g. production practices used for wines destined for cans, and the higher levels of H_2S in canned wine are not necessarily due to interactions between the can and wine.

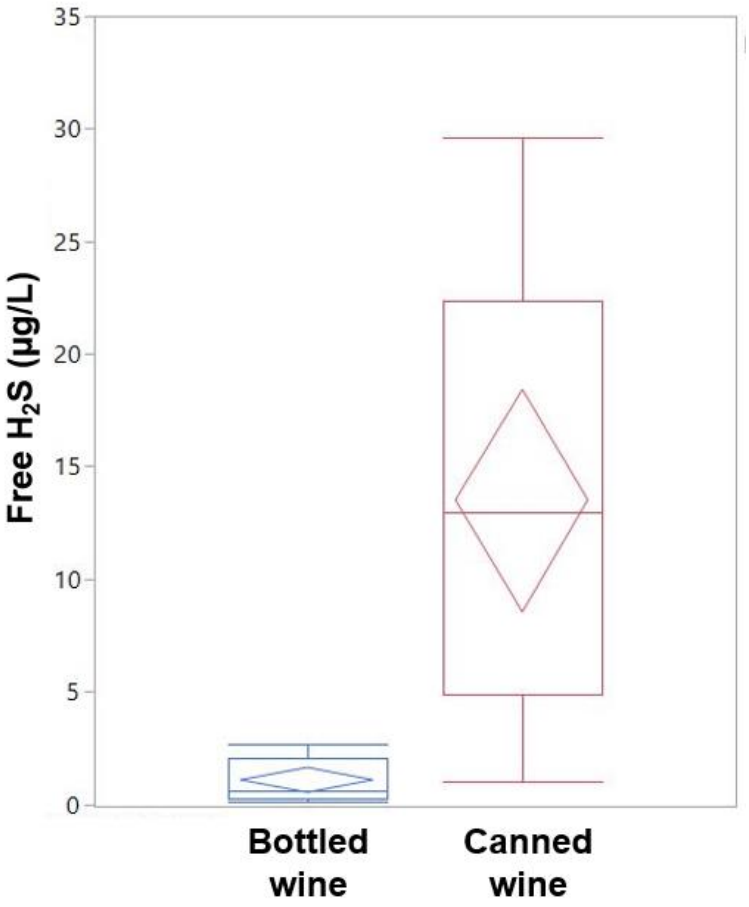


Figure 2.4. Quantitation of free H_2S in a selection of commercial bottled (n=6) and canned wines (n=6).

Broader Impact

We have described and validated two convenient, inexpensive approaches to measuring free H_2S in commercial wine samples using selective GDTs and glassware from an Aeration-Oxidation unit. With 60 mL sample volumes, limits of detection below the sensory threshold in wine could be achieved. The calibration produced using the N_2 Method is identical to the calibration calculated from the manufacturer's markings on the 4LT GDT, suggesting that the method could be used without the need for regular calibration. The alternative Aspiration Method was easier to operate,

making it more practical in winery settings, but should be regularly calibrated with standard solutions. By allowing for onsite measurement with minimal resources, both methods avoid risks of H_2S loss associated with sending samples to offsite labs. In addition to diagnosing faulty wines, these analytical tools can be used for benchmarking and evaluating the effects of winemaking choices (e.g. packaging, fining trials). Finally, although not the subject of the current investigation, the method could also likely be adapted for analysis of H_2S precursor forms by appropriate pre-treatment of the wine, e.g. addition of brine to release H_2S from copper-sulfhydryl complexes[17].

Experimental Design

Chemical Reagents. Ethanol (EtOH) at 140- and 190-proof was from Koptec (King of Prussia, PA). L(+)-tartaric acid (99%) and hydrogen peroxide (H_2O_2, 35% w/w) were purchased from Acros Organics (Morris Plains, NJ). Sodium hydroxide solution (NaOH, 50% w/w) was purchased from Fisher Chemical (Fair Lawn, NJ). Sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$, 98%) was purchased from Beantown Chemical (Hudson, NH). Silicone oil was purchased from Sigma-Aldrich (St. Louis, MO). Acetaldehyde (CH_3CHO, 99%) was purchased from Alfa Aesar (Ward Hill, MA). Deionized, distilled water with a resistance of 18.2 MΩ × cm at 25 °C was provided by a Milli-Q system (Millipore Sigma; Burlington, MA) was used for all experiments. Nitrogen gas (N_2, UHP) cylinders were supplied from Airgas USA LLC (Elmira, NY). Samples were held in a temperature-controlled incubator at 10 °C until use.

Gas detection tubes (GDT). Commercially available gas detection tubes (Gastec International, San Diego, CA) used for analyses of H_2S (Gastec 4LT) and SO_2 (Gastec 5L) were purchased from Airgas (Radnor, PA) and W. W. Grainger (Lake Forest, IL), respectively. The H_2S 4LT tubes rely on the reaction of H_2S with an $HgCl_2$, resulting in a color change from yellow to pink. The SO_2 5L tubes rely on the reaction of SO_2 with $BaCl_2$ to generate HCl, resulting in the appearance of a yellow color. In this method, SO_2 GDTs are used only for filtering the gas stream of any volatilized SO_2, to prevent interferences in the H_2S tube. Gastec 5L GDT in this method are not appropriate for quantification of SO_2. GDTs should be stored at cool temperature, refrigerated at 10 °C or below, or as indicated on the package. After opening, GDTs may be used reliably for several sequential analyses within one day (data not shown) but should be replaced with a new tube each day.

Quantitation of free H_2S in wines using GDT with N_2 sparging (N_2 Method). The N_2 Method is depicted in Figure 1(a). For an analysis, a volume of wine sample (up to 60 mL) is added to a 100 mL round-bottom flask (A). For red wines, 4-5 drops of silicone oil were added to decrease foaming. One neck of the flask was fitted with a fritted impinger (B) from an Aeration-Oxidation unit (Adams & Chittenden Scientific Glass Coop, Berkeley, CA) and the impinger connected to an N_2 cylinder on the inlet side (C). The outlet side was connected by PVC tubing to an SO_2 scrubber (see next sub-section), e,g. an SO_2 GDT (D), followed by an H_2S GDT (E). The sample was sparged with N_2 for 10 min at ambient temperature (~ 20 °C), and the length of color

change on the 4LT tube measured. The flow rate was ~100 mL/min, as faster flow rates would occasionally dislodge the GDT. Under these conditions, negligible change to the GDT stain length was observed after 10 min.

Quantitation of free H_2S in wines using GDT with vacuum aspiration (Aspiration Method). In the Aspiration Method [Fig 1(b)], wine samples are added to a 100 mL pear shaped flask (A) and attached to a fritted impinger (B) left open to atmosphere. The outlet side was connected by PVC tubing to an SO_2 scrubber (see next sub-section), e,g. a flask containing a 1.5% by volume acetaldehyde solution (C) and SO_2 GDT (D), followed by an H_2S GDT (E). The outlet of the H_2S GDT is attached by PVC tubing to a vacuum source (F). The sample is vacuum aspirated for 10 min and analyses were carried out at room temperature (~ 20 °C), and the length of color change on the 4LT tube measured. Unlike the N_2 method, we encountered no issues with dislodging the tubes, as the highest gas flow rate we could achieve was < 100 mL/min.

Removal of SO_2 interferences in N_2 and Aspiration Methods. Three strategies were evaluated for preventing interferences of SO_2 on the Gastec 4LL and 4LT tubes: i) inserting an SO_2 GDT in series before the H_2S GDT, as shown in Fig 1(a), Item D or Fig 1(b), Item D; ii) pre-treating the sample with hydrogen peroxide or acetaldehyde; iii) inserting a 100-mL pear-shaped flask containing 10 mL of 1.5% by volume acetaldehyde solution between the sample flask and the GDT, as shown in Fig 1(b), Item C. In evaluations, i) and iii) were carried out as described in the methods above.

For pre-treatment of the samples in ii), an aliquot of the oxidizing agent was added directly to 60 mL of a commercial white wine which was previously observed to cause an interference in the 4LT GDT. H_2O_2 solution was prepared at 3.5% and added at 1, 3, and 6 mL, corresponding to 0.06 - 0.35% by volume. Acetaldehyde was added at 5 µL, 25 µL, 50 µL and 100 µL, corresponding to 0.01 - 0.17% by volume.

Effects of sample volume and analysis time. The sample volume was varied from 5 mL to 66 mL, to accommodate for higher and lower concentration samples, respectively. With high concentration samples, lower sample volumes helped to avoid saturating the GDT before the analysis was complete. Analysis time was initially determined by recording the point when the color change ceased on the H_2S GDT for 6 and 10 mL samples of model wine or Milli-Q water spiked with H_2S. Aliquots of H_2S standard solutions were added to the selected sample volume and the analysis of Free H_2S was carried out as previously described. When sample volumes were increased to 60 or 66 mL, and when the sample matrix was changed to real or treated wines of greater density, the analysis time was increased to 10 min. Analysis time up to 15 min was recorded but no additional color change was observed (data not shown), so analysis time was standardized to 10 min for all sample volumes. The recorded response on the GDTs in mm was compared to the mass of H_2S added, and four replicates were carried out for each sample volume (see Supplemental Information).

Method Calibration. $Na_2S \cdot 9H_2O$ working solution (50 µM) was freshly diluted from a standardized stock solution (5 mM) every 48 h, and the stock solution was

newly prepared every 2 weeks. Stock solutions were stored in the refrigerator when not in use. Calibration standards of 0.017, 0.025, 0.050, 0.100, and 0.150 µM as H_2S were prepared in model wine (12% ABV, 5 g/L tartaric acid, pH adjusted to 3.5 by dropwise addition of NaOH). Calibration curves were prepared by plotting "length of stain (mm)" vs. "mass H_2S (ng)".

The observed slopes from each approach were compared against the slopes indicated by the manufacturer's markings on the GDT tubes. Because the GDT markings are reported in units of ppm (v/v), the values were converted to units of "ng H_2S" using the Ideal Gas Law and the manufacturer's suggested 100 mL gas volume for air sampling[53]. Details of the conversion calculation are provided in the Supplementary Information.

Reproducibility. In-lab reproducibility was assessed for the N_2 Method where an addition of $Na_2S \cdot 9H_2O$ working solution (50 µM) was measured in Milli-Q water at two concentrations, 1.55 µg/L (n=9) and 3.10 µg/L (n=7), over the course of 4 weeks.

Figures of Merit

Calibration curves were used to determine the linear range. The limit of detection (LOD) was calculated as 3.3 × standard deviation for the lowest concentration standard (0.6 µg/L or 34 ng using 60 mL sample volume) within the linear range (0 – 5.1 µg/L, or 0 – 307 ng using 60 mL sample volume). Standard deviations were determined for each calibration standards, and the coefficient of variance was calculated as the mean of these values.

Evaluation of Commercial Wines. A convenient sample of commercial bottled (n=6, 2 red, 4 white) and canned (n=6, 2 white, 2 rosé, 2 rosé sparkling) wines was purchased from local retailers (Ithaca, NY) and represented a range of regions and cultivars. Vintages ranged from 1-3 years old, although a few products were labeled as "non-vintage". Bottled samples were analyzed for H_2S in duplicate and canned samples were analyzed in triplicate.

Statistical Analysis. JMP Pro 14 (SAS Institute Inc., Cary, NC) was used for statistical analysis.

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CHAPTER 3

BRINE-RELEASABLE HYDROGEN SULFIDE IN WINE: MECHANISM OF RELEASE FROM COPPER COMPLEXES AND EFFECTS OF GLUTATHIONE

Abstract

Copper-sulfhydryl complexes in wine can be disrupted by addition of brine to release free hydrogen sulfide (H_2S), and the resulting "brine-releasable H_2S" is reported to correlate with formation of H_2S during bottle storage. However, both the mechanism of the brine-release assay and factors affecting the stability of copper-sulfhydryls under brine release conditions are not well understood. By varying brine composition and concentration, it is shown that release of copper-complexed H_2S requires the presence of a halide (Cl^-, Br^-), and is not due to a general "salting-out" effect. Release of copper-complexed H_2S by the brine dilution assay is highly temperature dependent. When H_2S and Cu(II) are added to a model wine, brine-releasable H_2S decreases markedly (~10-fold) after a 20 min incubation period prior to performing the brine-release assay. In commercial wines, the fraction of added H_2S recovered through the brine-release assay was correlated with the initial GSH concentration ($r^2 = 0.58$), but not with initial Cu. Negligible additional release of H_2S from organopolysulfanes was observed following addition of a disulfide reducing agent (TCEP). As previous studies have reported a correlation between H_2S formed through brine-release conditions and normal storage, these results suggest that the

susceptibility of a wine to forming latent copper-sulfhydryl precursors of H_2S following copper addition is dependent on the concentration of sulfhydryls like GSH.

Introduction

Sulfur-like off-aromas (SLOs) are reportedly responsible for just under 30% of the faults identified in premium wines in competition.[2] Of the many volatile sulfur compounds (VSC) reported in wine, hydrogen sulfide (H_2S, "rotten egg aroma") is most frequently reported to be in excess of its sensory threshold (~ 1 µg/L) in wines with SLOs.[5] H_2S can be produced during fermentation through several pathways, including: an intermediary step in *S*-amino acid biosynthesis,[18] a degradation product of S^0 fungicide residues,[16, 20] and through catabolism of *S*-amino acids, especially cysteine.[21] H_2S is sufficiently volatile that the majority formed during fermentation will be lost to CO_2 entrainment.[48] After fermentation, winemakers may attempt to remove H_2S by inert gas sparging, by aeration to oxidize H_2S or other VSCs,[56] or by addition of Cu(II) salts to form non-volatile complexes.[43] As discussed below, these last two approaches (aeration, copper addition) may yield products capable of reforming H_2S during storage.

Recent reviews have highlighted that sulfhydryls – particularly H_2S – can increase during abiotic storage, especially in wines stored in near-anoxic conditions (e.g. under a screwcap).[17, 22-23, 57-59] There is considerable interest in determining the likely precursors of latent H_2S in wines and developing appropriate strategies for their control.[60] Several classes of latent H_2S precursors have been suggested,[17, 61] including i) polysulfides and organopolysulfanes, which form by reaction of S^0-residues with glutathione (GSH) or oxidation of H_2S and other sulfhydryls in the presence of

[Cu(II)];[20, 50] ii) degradation of cysteine, GSH, or related aminothiols,[49] possibly catalyzed by [Cu(II)] or other transition metals; and (iii) reduction of transition metal-sulfhydryl complexes, especially those containing copper.[7]

Evidence supporting copper-sulfhydryl complexes as an important latent source of H_2S (and possibly other VSCs, such as methanethiol) during wine storage is indirect but convincing. Cu is often present at high concentrations (> 1 mg/L) in grape musts but will decrease markedly (to <0.1 mg/L) following fermentation due to lees binding.[62] Cu concentrations in finished wine can be higher (up to 0.8 mg/L) due to leaching from brass fittings or by intentional addition of Cu(II) salts to wine to remediate SLOs,[43, 63] as mentioned earlier. Historically, it was believed that Cu(II) addition would result in formation of insoluble precipitates with H_2S and other sulfhydryls which would largely be removed from wine by filtration or racking.[19] However, recent work demonstrated that following Cu(II) addition to wines with free H_2S, no more than 21% of the Cu could be removed using standard practices, including sterile filtration.[43] In contrast, a visible and readily removable precipitate was formed following Cu(II) addition to model wine solutions containing H_2S.[43] Electron paramagnetic resonance (EPR) studies have demonstrated that Cu(II) is rapidly reduced to Cu(I) following addition to sulfhydryl-containing model wines, and stable [$Cu(I)_xS_y$] complexes of varying stoichiometries may remain dispersed in solution.[40-41] Several wine components can reportedly contribute in preventing copper sulfide precipitation, supporting the persistence of complexes in solution.[17] Although Cu(II) salt addition results in an immediate decrease in free H_2S, it may also result in greater formation of H_2S during storage,[64] again suggesting that copper-sulfhydryl

complexes may be a source of H_2S. The extent to which complexed H_2S is reformed during storage may be further affected by other wine compositional parameters including GSH,[23] SO_2[65] and elevated pH.[6]

Copper-complexed H_2S can be recovered through addition of concentrated NaCl brine (so-called "brine-releasable H_2S").[7, 52] Although other transition metals (e.g. Zn) can also form brine-releasable complexes with H_2S, the concentration of brine-releasable H_2S (BR H_2S) in commercial wines is best correlated with their concentration of Cu.[7] BR H_2S is also correlated with free H_2S formation following accelerated anoxic storage at 50 °C and, to a lesser extent, following extended room temperature storage in bottle.[24, 26] The mechanism through which H_2S is released from copper-sulfhydryl complexes during wine storage is unknown; however, it has been demonstrated that reducing agents like dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) will partially release H_2S from copper-sulfhydryl complexes,[52] and anoxically stored wine is characterized by a low redox potential and the presence of many reducing species.[61]

One challenge with understanding factors affecting formation and stability of copper-sulfhydryl complexes is that their size (10-200 nm)[43] and low concentrations make them inappropriate for direct chemical analysis by techniques like mass spectrometry, and instead are typically only characterized by size using physical techniques, e.g. Nanoparticle tracking analysis.[66] Knowledge of the chemical composition of these copper-sulfhydryl complexes is typically from measurement of H_2S and other species following their disruption with brine addition.[16, 52] However, there is little understanding of the mechanism of brine release and what chemical

factors would affect the stability of complexes under brine-release (and, by extension, wine storage) conditions.

In this work, it is demonstrated that brine-release of H_2S from copper-sulfhydryl complexes in wine is due to the halide group. It is also shown that the stability of copper-sulfhydryl complexes in the presence of brine varies considerably among wines, and that the presence of GSH will encourage formation of less stable and more brine-releasable copper-sulfhydryl complexes.

Materials and Methods

Materials and Chemical Reagents.

Gastec gas detection tubes (Gastec International, San Diego, CA) were used for analyses of H_2S (Gastec 4LT) and SO_2 (Gastec 5L) were purchased from Airgas (Radnor, PA) and W. W. Grainger (Lake Forest, IL), respectively. Ethanol (EtOH) at 140- and 190-proof was from Koptec (King of Prussia, PA). L-(+)-tartaric acid (99%) was purchased from Acros Organics (Morris Plains, NJ). Sodium hydroxide solution (NaOH, 50% w/w) was purchased from Fisher Chemical (Fair Lawn, NJ). Sodium sulfide nonahydrate ($Na_2S \cdot 9H_2O$, 98%) was purchased from Beantown Chemical (Hudson, NH). Sodium chloride (NaCl) and ammonium sulfate ($(NH_4)_2SO_4$) were purchased from VWR (Solon, OH). L-glutathione (GSH; 97%) was purchased from Alfa Aesar (Tewksbury, MA). Isotopically labeled glutathione (glycine-$^{13}C_2$, 98%; ^{15}N, 96-99%; 65-70% net peptide) (($^{13}C_2$,^{15}N)-GSH) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Sodium bromide (NaBr), anhydrous copper sulfate ($CuSO_4$, 99%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%), N-ethylmaleimide (NEM, >99%), glacial acetic acid (>99.7%), ammonium bicarbonate

(NH_4HCO_3, >99%), and silicone oil were purchased from Sigma-Aldrich (St. Louis, MO). Calcium chloride ($CaCl_2$) was purchased from Allied Chemicals (Morristown, NJ). Ammonium chloride (NH_4Cl) was purchased from Macron Fine Chemicals (Center Valley, PA) and ammonium nitrate (NH_4NO_3) from Merck (Darmstadt, Germany). Deionized, distilled water with a resistance of 18.2 MΩ × cm at 25 °C was provided by a Milli-Q system (Millipore Sigma; Burlington, MA) and used for all experiments. Nitrogen gas (N_2, Ultra High Purity) cylinders were supplied from Airgas USA LLC (Elmira, NY).

Commercial wines and initial chemical analysis.

Wines representing a range of styles were purchased from a local retailer in Ithaca, NY (USA). All wines had screw cap closures. Subsequently, the wines will be designated PG (Pinot Grigio), SB (Sauvignon Blanc), VV (Vinho Verde), MA (Malbec), PR (Primitivo), RO (Rosé), and FS (Fino Sherry). Once opened and samples removed, wines were sparged with N_2 and placed in refrigerated storage for up to one week for re-use, after which they were discarded and replaced with a fresh bottle. Details on provenance, wine style, vintage, and basic wine chemistry analyses are listed in Table 1**.** Basic wine chemistry was determined by established methods at the Cornell Craft Beverage Analytical Laboratory (Geneva, NY): alcohol by volume (ABV) was analyzed using the Foss OenoFoss (Hilleroaed, Denmark), free and total SO_2 analyses were carried out by flow injection analysis on the Foss FIAstar 5000 Analyzer (Hilleroaed, Denmark), titratable acidity (TA) was measured by titration with the Metrohm 862 Compact Titrator (Herisau, Switzerland), pH was measured on the Fisher Scientific Accumet Excel XL25 Dual Channel pH/ ion meter (Pittsburgh,

PA), and glucose and fructose (Glu/Fru) were measured enzymatically (Randox RX Monaco; Crumlin, UK). Copper was analyzed at a local facility (DairyOne Forage Laboratory, Ithaca, NY) using a Thermo iCAP 6300 ICP Radial Spectrometer (Waltham, MA).

Table 3.1. Wine provenance and chemistry for commercial wines used in this study.

Code	**Region (style)**	**Vintage**	**ABV (%)**	**Free SO_2 (mg/L)**	**Total SO_2 (mg/L)**	**TA**	**pH**	**Glu/Fru (g/L)**	**Cu (mg/L)**	**GSH (µM)**
PG	Italy (white)	2018[a]	12.34	25	104	6.26	3.15	1.77	0.22	0.74
SB	Australia (white)	NV	11.72	25	135	7.46	3.35	4.75	0.62	2.21
VV	Portugal (white)	NV	9.19	26	158	8.41	3.28	11.52	0.14	0.67
MA	Argentina (red)	2019	13.49	20	71	6.18	3.46	1.57	0.13	0.94
PR	Italy (red)	2018	13.60	14	90	5.97	3.51	3.15	0.37	0.47
RO	France (rosé)	2018	12.29	16	79	5.65	3.47	0.69	0.30	0.27
FS	Spain (fortified)	NV	15.60	<5	12	4.89	3.21	<0.09	0.15	0.25

[a]The 2017 vintage was used in initial exploratory experiments, as described in the text.

The derivatization and measurement of GSH was adapted from a protocol described by Roland and Schneider.[67] Calibration standards for GSH (0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 8.0, 10 µM) were prepared in model wine. For derivatization, samples and standards (5 mL) were adjusted to neutral pH (6.5 – 7) with NaOH, dropwise, and vortexed. Labeled GSH (20 µM) was added as an internal standard. The sample was then derivatized with buffered NEM solution (0.5 mM), stirred with a magnetic stir bar at room temperature for 15 min, and the reaction quenched with glacial acetic acid (5 µL).

LC-MS/MS analyses were carried out at the Proteomics and Metabolomics Facility (Cornell University, Ithaca, NY). The instrument used was a Sciex X500B QTOF mass spectrometer (Sciex, Framingham, MA), coupled to an ExionLC HPLC system (Sciex), operated in positive ion mode. The sample injection volume was 5 µL. The column was a Luna HILIC 3 µm, 200 Å column (100 mm x 2 mm inside diameter, Phenomenex, Inc., Torrance, CA). Mobile phase A was 0.1% formic acid in 5 mM aqueous ammonium formate and mobile phase B was 0.1% formic acid in methanol. The flow rate was 200 µL/min. The gradient was as follows: starting solvent 90% B, held for 1 min, decreased to 50% B over 6 min, held at 50% B for 1 min, and returned to 90% B, followed by a 1.5 min equilibration. The MS was operated in ESI positive ion mode, scanning from *m/z* 100 to 1000, followed by MRM scans. Conditions were optimized using a 1000 mg/L GSH standard. The following optimized operating conditions were used: voltage of 5.5 kV, nebulizer gas and heater gas of 20 psi, curtain gas 20, collision gas 7, source temperature of 325 °C, DP 20 V, and accumulation time 0.15 sec. For NEM-derivatized samples, the MS/MS transitions used for quantitation were *m/z* 433 → 304 and 436 → 307 for derivatized GSH and derivatized labeled internal standard, respectively.

Quantitation of free and BR H_2S in wines using gas detection tubes (GDT).

Free H_2S in model and real wines were quantitated using a GDT method described elsewhere.[68] Briefly, a 60 mL wine sample was added to a 100 mL round-bottom flask, and free H_2S is sparged from the flask into commercial colorimetric gas detection tubes for quantitation.

Measurement of BR H_2S involved modification of the free H_2S protocol. A sample (6 mL) was diluted with 60 mL of deaerated brine (35% w/v NaCl, unless otherwise specified) in a 100 mL round-bottom flask. The 1:10 dilution ratio was selected based on an earlier study.[52] Immediately after brine addition, the BR H_2S was sparged into the GDT for 10 min, where signal is observed to plateau, as described elsewhere.[68] Reagents were held at 10 °C until immediately prior to use and the brine-release analysis was carried out at room temperature (≈ 21 °C).

In experiments involving addition of copper and H_2S to commercial wines, it was necessary to distinguish the "Initial" Free and BR H_2S present in the unadjusted wine from the "Final" Free and BR H_2S present after Cu(II) and H_2S additions, as described in equations 1-3.

(Eq 1) Total Initial H_2S = Initial Free H_2S + Initial BR H_2S

(Eq 2) Total Final H_2S = Final Free H_2S + Final BR H_2S

Upon treatment with Cu(II) in excess, no detectable Final Free H_2S was observed in any sample (data not shown) and the Total Final H_2S is equal to the Final BR H_2S. One assumption is that the contribution of Initial Free H_2S which could be converted to BR H_2S is negligible, based on the incomplete conversion of bound H_2S and Cu to brine-releasable forms. The increase in brine-releasable (Δ BR H_2S) can then be calculated as follows:

(Eq 3) Δ BR H_2S = Final BR H_2S – Initial BR H_2S

The steps used to calculate Δ BR H_2S are depicted in Figure 1. Recovery of added H_2S present in the brine releasable form was calculated as follows:

(Eq 4) % BR H_2S Recovered = (Δ BR H_2S / Added H_2S) x 100%

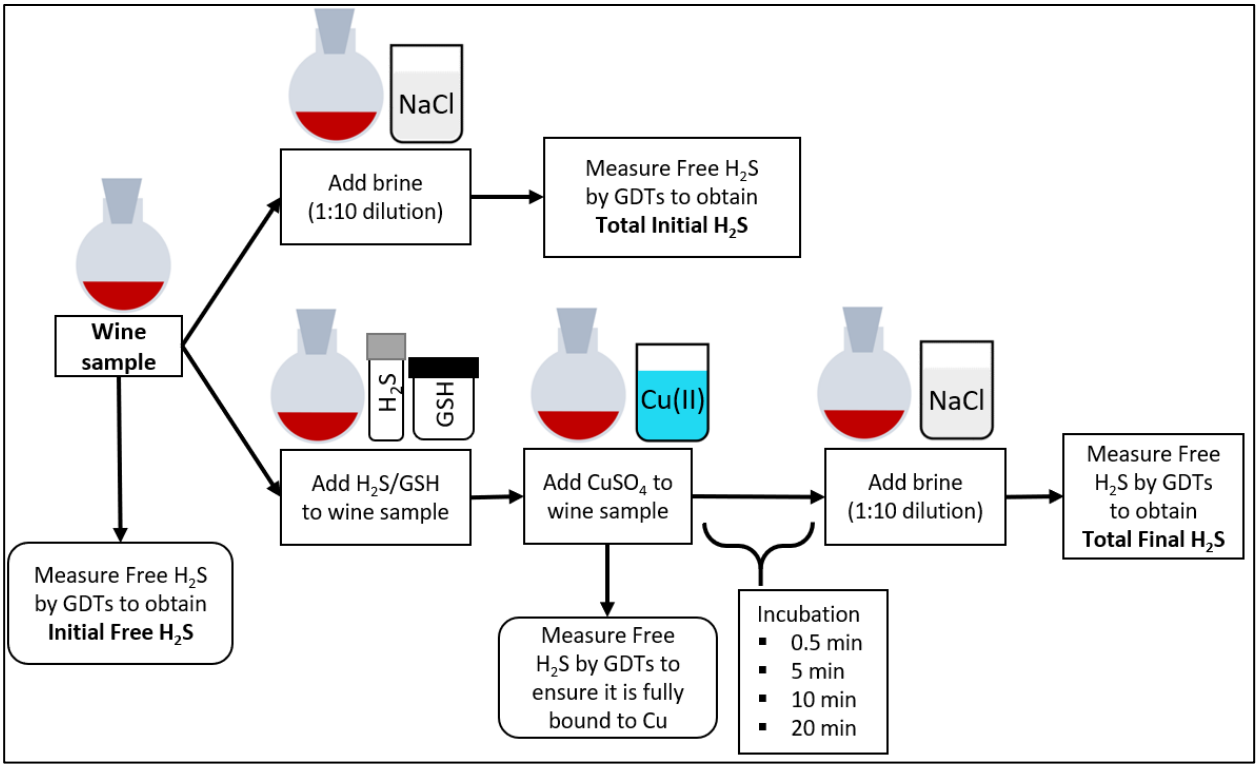

Figure 3.1. Outline of method for Free and Brine Releasable (BR) H_2S measurements.

Formation of copper-complexed H_2S in model and commercial wines.

Copper-sulfhydryl complexes were prepared in model and real wines by adding H_2S and Cu(II) prior to brine dilution. Cu(II) was added as $CuSO_4$ solution (1 mM) freshly prepared every 48 h. H_2S standards were first prepared as a $Na_2S{\cdot}9H_2O$ working solution (50 µM), freshly diluted every 48 h from a stock solution (5 mM). The 5 mM stock solution of $Na_2S{\cdot}9H_2O$ was discarded and prepared fresh every 2 weeks. Stock solutions were stored in the refrigerator when not in use.

Effects of salt type, ionic strength, and temperature on brine-release of H_2S from copper-sulfhydryl complexes.

Effect of Salt Type and Ionic Strength: The effects of changing the composition of the salt solution used for the brine-release protocol was investigated by comparing H_2S released from wine (PG 2017) with varying salt solution composition. Eight different salt solutions were evaluated, including four halide salts (NaCl, NaBr, $CaCl_2$, NH_4Cl) and two non-halide salts (NH_4NO_3 and $(NH_4)_2SO_4$). $CaCl_2$ and $(NH_4)_2SO_4$ solutions were also prepared at different molarities to allow for comparison of different salts at the same ionic strength. Detailed information on brine composition is provided in Supporting Information. The different salt solutions were used with the brine-release protocol on wine (PG 2017) with added H_2S and Cu(II) (final concentrations = 1 µM and 10 µM, respectively) to determine Initial Total H_2S and Δ BR H_2S. Each treatment was prepared and measured in triplicate.

Effect of Temperature: A 35% w/v NaCl brine was held at one of three temperatures (21 °C (room temperature), 35 °C, 50 °C) prior to addition to the wine (PG 2017) sample to which H_2S and Cu(II) had been added. The temperature of the sample and brine was maintained by submerging the flask in a water bath during the H_2S analysis. The brine dilution assay was otherwise carried out as described above using wine (PG 2017), with each condition prepared and measured in triplicate.

Recovery of copper-complexed H_2S under brine-release conditions in model and commercial wines

BR H_2S recovery experiments were performed using model wine (MW) (12% ABV, 5 g/L tartaric acid, pH to 3.5 with NaOH) and commercial wines (n=7, Table 1) at room temperature (21 °C). An outline of the recovery experiments is shown in **Error! Reference source not found.**. Briefly, a 6 mL wine sample was added to a 100 mL round-bottom flask followed by sequential additions of H_2S and $CuSO_4$ to final concentrations of 1 µM and 10 µM, respectively. The flask was then lightly swirled and analyzed for free or BR H_2S near-immediately (t = 0.5 min) following addition of reagents, or after stoppering and incubation at 10 °C for a time. In preliminary experiments with MW and PG 2017 samples, the complex-forming reagents were incubated for t = 0.5, 5, 10, and 20 min prior to analysis. Based on preliminary observations, the wines (PG, SB, VV, MA, PR, RO, FS) were incubated for 0.5 or 20 min, only. All analyses were performed in triplicate.

Recovery of copper-complexed H_2S under brine-release conditions in the presence of added GSH.

GSH stock solution (19.5 mM) was prepared fresh every 2 weeks and stored in the refrigerator when not in use. Wine samples were prepared as described for the previous H_2S recovery experiments, except that for each wine (model and seven commercial) an aliquot of GSH (final concentration = 300 µM) was added immediately after H_2S addition and prior to $CuSO_4$ addition. BR H_2S was then measured as described earlier following incubation times of t=0.5 and 20 min.

Quantitation of TCEP-releasable precursors of H_2S in commercial wines remaining following the brine-release assay.

TCEP solution (0.9 M) was prepared and refrigerated until use. After a wine sample underwent the previously described brine dilution assay, the round bottom flask was disconnected from the apparatus and an aliquot of TCEP was added (final concentration = 1.5 mM, and H_2S quantitated by the free H_2S protocol.

Statistical analyses.

JMP Pro 14 (SAS Institute Inc., Cary, NC) was used for statistical analysis. ANOVA (α=0.05) was used to compare BR H2S under different treatments. A linear mixed effects model was used to evaluate % BR H2S Recovered data (fixed effects of GSH Addition, Incubation Time and their cross-term; random effect of Wine Type; random coefficients of GSH Addition, Incubation Time, and their cross-term; Native GSH as a covariant).

Results and Discussion

Measurement of BR H_2S in wine samples.

The approach for measuring BR H_2S in wines with commercially available gas detection tubes was adapted from a method recently reported for measurement of free H_2S in wines.[68] The previous method was validated for sample sizes up to 66 mL, and could achieve limits of detection (LOD) of ~0.2 µg/L. The brine dilution assay requires at least a 1:10 dilution ratio to maximize recovery (>90% of maximum

value),[52] necessitating a smaller sample size (6 mL), and resulting in a proportionally higher LOD (2.7 µg/L, data not shown). Otherwise, calibration and reproducibility of the BR H_2S assay were comparable to the free H_2S assay.

Brine-release of H_2S from wine is a function of halide concentration rather than ionic strength.

The initial discovery of the BR H_2S fraction in wines was serendipitous; the authors observed that H_2S measured by solid-phase microextraction (SPME) prior to chromatography yielded consistently higher H_2S values than measurements by static headspace (SHS) sampling.[7, 14] The authors also presented evidence that these BR H_2S precursors were primarily composed of H_2S complexed to copper. A key difference between the SPME and SHS extraction methods is that the SPME approach involved the addition of saturated NaCl brine, which is commonly used to increase the ionic strength of the sample and the volatility of the analytes ("salting out"). However, in the case of BR H_2S, it is unclear if the release of H_2S from copper complexes was related to the increase in ionic strength, or if a specific mechanism related to NaCl was involved.

To understand if brine-release of H_2S is solely due to the ionic strength of the salt, the extent of H_2S release following dilution of a commercial wine (PG 2017) was compared among brine solutions containing different salts at different ionic strengths. Results are shown in **Error! Reference source not found.**a. Significant differences in H_2S release were observed among different salts' ionic strengths (Tukey test, α=0.05). For example, addition of concentrated NaCl brine (6M) generated BR H_2S of 3.9 µg/L, comparable to values previously reported in real wine.[52] Comparable

concentrations of BR H_2S were observed when using halide salts at approximately the same ionic strength ($CaCl_2$, NaBr, NH_4Cl), but nearly undetectable H_2S was observed when using brines with non-halide anions (NO_3^-, SO_4^{2-}). A subsequent *post hoc* analysis determined that halide salts released significantly more H_2S than non-halide salts (ANOVA, $p<0.05$). Interestingly, higher BR H_2S (9.2 µg/L vs. 5.8 µg/L) was observed for 3M $CaCl_2$ than for 6M $CaCl_2$, but the reason for this was unclear.

To confirm that the greater BR H_2S release with halide vs. non-halide salts was due to copper-sulfide complexes as opposed to another precursor, the same brine dilution trials were performed on wine (PG 2017) with added H_2S (34 µg/L) and $CuSO_4$. Again, significantly higher concentrations of Total Final BR H_2S was observed when the assay was performed with halide salts (ANOVA, $\alpha=0.05$; Figure 2b). Average Total Final BR H_2S was 10.2 µg/L for the halide salts, and 1.3 µg/L for the non-halide salts. The limited ability of a non-halide brine to release H_2S again indicates that the halide (and not ionic strength) is responsible for H_2S release from copper-sulfhydryl complexes. Interestingly, incomplete H_2S recovery was observed for all brine release assays following addition of H_2S and Cu(II) for both halide and non-halide salts. The highest recovery (~7 µg/L, or 20% of added H_2S) was observed for 6M NaCl, and lower recoveries observed for other salts. Similar behavior was observed for other wines, as discussed in later sections. Additionally, the presence of BR H_2S in the non-halide treatments in wine with added Cu(II) and H_2S but not in the original wine suggests that despite their incomplete recovery, at least some of the newly formed complexes are less stable than those in the original wine.

These results indicate that brine-release of H_2S from copper-sulfhydryl complexes is a result of displacement of sulfides by halide ions rather than a general "salting out" mechanism. Similar displacement reactions have been reported in the mining literature, where Cl- can displace sulfhydryls in copper sulfides to yield $CuCl_4^{2-}$ and related species.[69] Additionally, all halide brines released similar amounts of H_2S, suggesting an upper limit to the amount of H_2S that can be recovered by brine dilution in this wine sample. This further suggests that the ratio of halide ions to copper-sulfhydryl complexes may have a limit past which H_2S released by brine dilution is no longer proportional to latent H_2S.

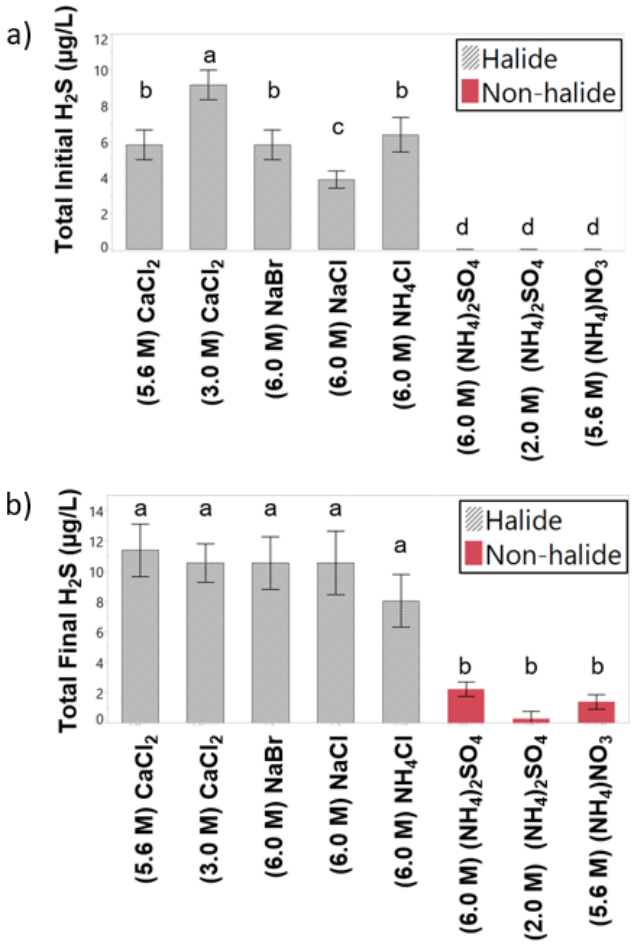

Figure 3.2. BR H_2S measured (µg/L) from brine dilution of wine (PG 2017) at room temperature (21 °C), using various salts for a) untreated wine and b) wine spiked with 1 µM H_2S (34 µg/L) and 10 µM $CuSO_4$. Error bars represent 1 standard deviation from the mean. The brine concentrations and ionic strengths are summarized in the supplementary material.

Brine-release of H_2S from wine is temperature dependent.

Initial reports on brine-release with SPME extraction were performed at 35 °C.[7] A GDT based approach that was performed at room temperature was subsequently described.[52] To determine if temperature affected BR H_2S, brine release experiments using NaCl were performed at three temperatures (21, 35 and 50 °C) on wine (PG 2017). It was observed that temperature had a significant effect (ANOVA, $p<0.05$), with a 2-fold increase in H_2S released at 50 °C as compared to 21 °C (Figure 3). This greater release is presumably due to thermodynamic effects, i.e. at higher temperatures, copper-sulfhydryl complexes are less stable and more susceptible to halide displacement. Kinetic effects are a less likely explanation because increasing sparging time did not further increase H_2S release (data not shown). The effect of temperature may explain why BR H_2S values measured in one report at room temperature (3.4 – 4.9 µg/L, n=3 red wines)[52] were generally lower than values measured elsewhere at elevated temperatures (9.2 – 41.5 µg/L, n=16 red wines).[24] A kinetic effect could theoretically accelerate release of H_2S from other putative precursors, such as disulfides, polysulfanes, di-organopolysulfanes, but this seems unlikely to occur within the course of this rapid analysis.[17, 49] Based on this result, caution is recommended when comparing BR H_2S values among protocols that use different temperatures during analysis. For further experiments, 21 °C was selected for the assay temperature to better mimic ordinary wine storage conditions.

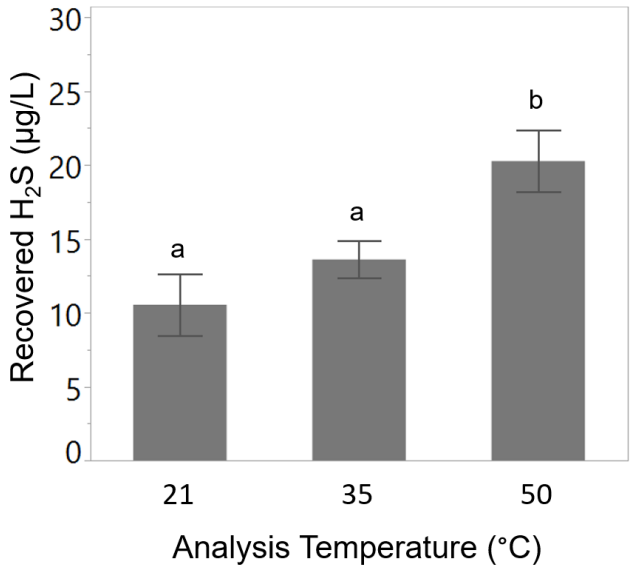

Figure 3.3. BR H_2S recovered (µg/L) by brine dilution of wine (PG 2017) with NaCl brine at room temperature = 21 °C, 35 °C and 50 °C. Error bars represent 1 standard deviation from the mean.

Following addition of Cu(II) and sulfide to wines the recovery of BR H_2S decreases within minutes, but recovery can be increased if GSH is present.

Using a commercial wine (PG 2017) and a model wine (MW), the time dependence of BR H_2S recovery following addition of Cu(II) (at 10 µM) and H_2S (at 1 µM) was evaluated. After addition, the brine dilution assay was performed at 0.5 (immediately after sample preparation), 5, 10, and 20 min. Recent work has demonstrated that Cu(II) addition to model wine solutions containing H_2S both with and without cysteine (as a model thiol) results in rapid reduction of Cu(II) to Cu(I), with concurrent oxidation of H_2S to yield putative copper-sulfhydryl complexes; in experiments where cysteine was added, H_2S could also form organopolysulfanes (R-S-S_n-S-R).[40] Experiments to determine the extent to which organopolysulfane formation could account for releasable H_2S are described in more detail below.

Measurements of BR H_2S for both samples after different incubation times were also performed. In MW, a decrease of 95% in BR H_2S was observed between 0.5 and 20 min. A likely interpretation is that the copper-sulfhydryl complexes in model wine rapidly increased in stability, possibly through coalescence of smaller nuclei,[70] and thus became less amenable to disruption by addition of halide salts (**Error! Reference source not found.**). However, no significant change in BR H_2S was observed between 0.5 and 20 min for the PG 2017 wine, suggesting that the components of real wines inhibited stabilization of copper-sulfhydryl complexes (**Error! Reference source not found.**).

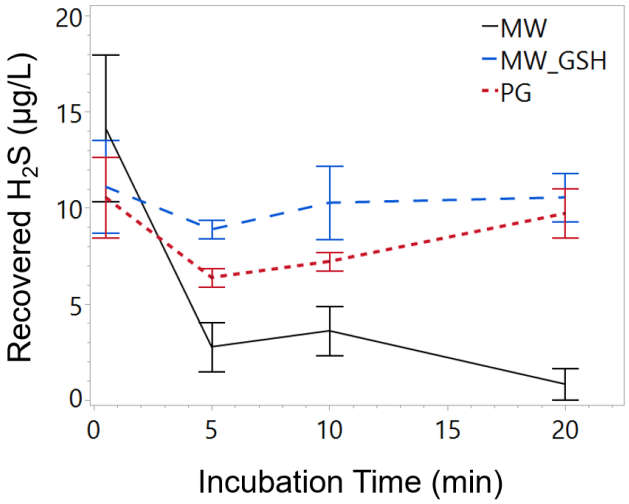

Figure 3.4. BR H_2S released by brine dilution (temperature = 21 °C) after t=0.5, 5, 10, 20 min pre-dilution incubation at 10 °C. Solutions investigated were wine (PG 2017), model wine (MW) and model wine with 325 µM GSH (MW-GSH), all spiked with 1 µM H_2S and 10 µM $CuSO_4$. Error bars represent 1 standard deviation from the mean.

Interestingly, when the MW was prepared with 325 µM of GSH added (MW-GSH), no significant decrease in BR H_2S was observed between 0.5 and 20 min, comparable to real wine (Figure 4). Brine dilution controls of MW-GSH without H_2S

or Cu added produced no detectable H_2S (data not shown), making GSH itself an unlikely source of H_2S. Although copper sulfide will readily precipitate from simple model wine systems once Cu(II) and S^{2-} exceed their solubility product, soluble copper sulfide complexes can exist in wine at concentrations well in excess of these maximum Cu(II) and S^{2-} concentrations, and the presence of thiols like GSH in real wines are hypothesized to contribute to this phenomenon.[40] Recent studies of nanoscale particle distributions in wine following Cu(II) addition have reported larger particles in real wines, which the authors hypothesize is a result of incorporation of thiols ("end-capping") into the growing copper-sulfhydryl complexes.[66] Presumably, the presence of thiols increases the critical nuclei radii of the copper-sulfhydryl complexes and allows them to grow without precipitation.

In real wine, it was hypothesized that the amount of BR H_2S would correlate with the native GSH content of the wine, as GSH is the most abundant sulfhydryl present in wine. Further, GSH was used as a proxy for investigating the effect of adding a high level of free sulfhydryls, along with the H_2S, during the formation and subsequent brine release of copper-sulfhydryl complexes in different real wines.

To confirm that effect of GSH in promoting formation of brine-releasable copper-sulfhydryl complexes, H_2S, GSH, and Cu(II) spiking experiments were performed on additional commercial wines at two incubation times (t=0.5, t=20 min) and two GSH levels (0, 300 µM) for each wine (Figure 5). Because wines started with different levels of Initial Free and BR H_2S (see Supporting Information Table S2), values for BR H_2S are reported as "% BR H_2S Recovered", to account for background concentrations. The PG wine used in this study was of a different vintage than for the

initial experiments shown in Figure 4. In all wines, no free H_2S was detected following Cu(II) addition. Using a mixed effects model, it was determined that GSH Addition and Incubation Time significantly affected % BR H_2S Recovered. Notably, in 7 of 8 wines (FS, MA, MW, RO, PG, PR, VV) lower % BR H_2S Recovered at t=20 min was observed in the GSH addition treatment, including a 12-fold difference in BR H_2S in MW-GSH vs. MW. Only the SB wine did not show a significant increase in BR H_2S with GSH addition at either t=0.5 or 20 min. As discussed in the next paragraph, SB has the highest concentration of native GSH, which may have limited the impact of an exogenous GSH addition. On the whole, these results support the previous observation that the concentration of brine-releasable complexes following Cu(II) and H_2S addition decreases with longer incubation time, but higher concentrations of brine-releasable forms can be preserved by GSH.

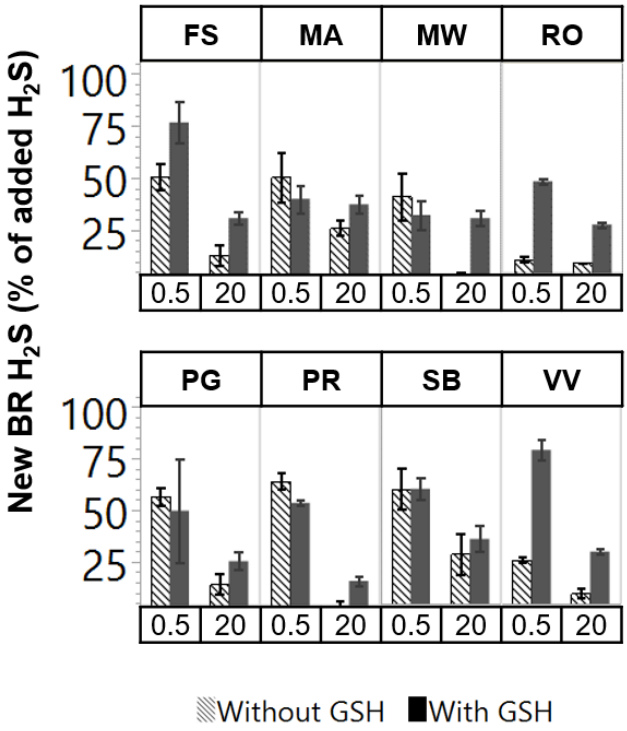

Figure 3.5. BR H_2S released by brine dilution in wine and MW (temperature = 21 °C) after t=0.5 and 20 min pre-dilution incubation at 10 °C, with and without an addition of [GSH]=300 µM (for MW, [GSH] = 325 µM). All wines are spiked with 1 µM H_2S and 10 µM $CuSO_4$. Error bars represent 1 standard deviation from the mean.

Significant variation ($p<0.05$, 1-way ANOVA) was also observed in % BR H_2S Recovered at t=20 min among the original commercial wines in the absence of added GSH, ranging from 3.6% (in the PR) to 28.6% (in the SB). The lowest % BR H_2S Recovered for any experiment was in model wine. These differences were not easily explained by differences in wine style (e.g. red vs. white wines). It was hypothesized that higher % BR H_2S Recovered in the original wines was likely due to differences in native concentrations of GSH, cysteine, and other sulfhydryls among the wines. Measurement of GSH by LC-MS-MS was selected as a proxy for total

sulfhydryl content. The native concentration of GSH in the commercial wines ranged from 0.25 – 2.21 µM, within the range previously reported for wines.[71] As a caveat, these GSH concentrations are ~100-fold lower than the GSH concentration added in the earlier model system study. However, GSH is expected to serve as a proxy for total sulfhydryl content, which would also include species such as cysteine and homocysteine, either free or as part of soluble proteins. In the absence of added GSH, the native GSH concentration is correlated ($R^2 = 0.58$, $p<0.001$) with % Recovered BR H_2S at t=20 min (**Error! Reference source not found.**a)**.** This is consistent with the observation that added GSH will reduce the stability of newly formed copper-sulfhydryl complexes, resulting in more BR H_2S.

Initial copper content is not significantly correlated with formation of brine-releasable copper-sulfhydryl complexes.

The concentration of Cu in the commercial wines (0.13 to 0.62 mg/L) was within the range typically reported in wines.[72] However, in contrast to native GSH, the concentration of native Cu was not significantly correlated with the concentration of % BR H_2S Recovered (**Error! Reference source not found.**b). This was surprising, as it was expected that wines with high concentrations of native Cu could more readily form stable complexes with H_2S. However, recent work has observed that the majority of Cu in wines (in contrast to exogenously added Cu) exists in a "non-labile" form, presumably as copper-sulfhydryl complexes, and thus would not be readily available to bind free H_2S.[72]

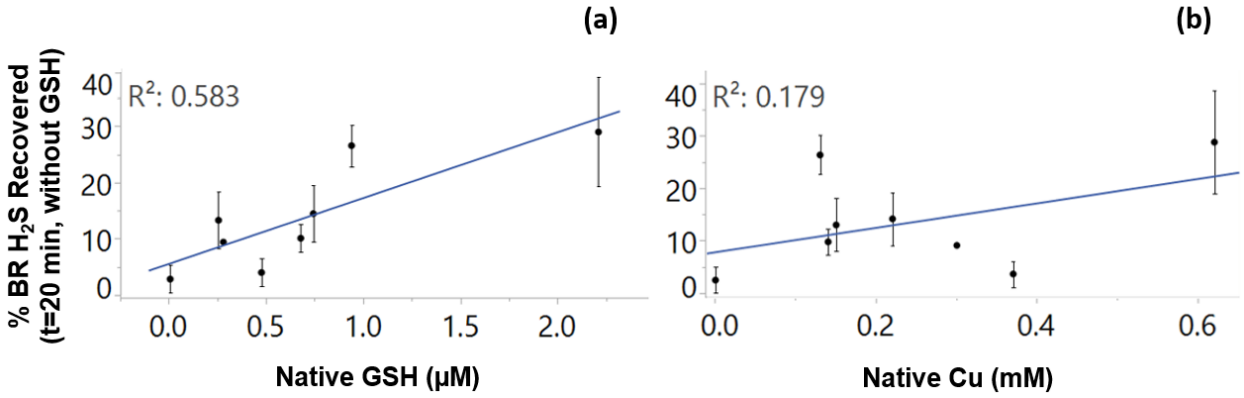

Figure 3.6. Correlation of BR H_2S and (a) Native GSH and (b) Native Cu among wines. Error bars represent 1 standard deviation from the mean.

Organopolysulfanes account for only a small fraction of "missing" H_2S following addition of Cu(II) and H_2S to commercial wines.

Even in the presence of added GSH, incomplete recoveries of H_2S (54-79%) by the brine-release assay immediately following Cu(II) addition were observed in commercial wines, and even lower recoveries following 20 min incubation (**Error! Reference source not found.**). An explanation for this incomplete recovery presented in the previous section is that some copper-sulfhydryl complexes are not amenable to brine release, but this phenomenon could also be because H_2S is lost through other oxidative reactions. Recent work demonstrated that incubation of Cu(II) with cysteine and H_2S in model systems produced not only copper-sulfhydryl complexes, but also di- and mono-organopolysulfanes (RS-S_n-SR and RS-S_n-SH) through Cu(II)-mediated oxidation.[50] H_2S can be released from these precursors by TCEP or other disulfide bond reducing agents,[16] but not by brine. To determine if the missing H_2S could be accounted for by organopolysulfanes, a subset of commercial wines was selected (2 white, 1 red, 1 fortified) for treatment with TCEP following H_2S/GSH/$CuSO_4$ addition

and the brine dilution assay. In the original wines, prior to treatment, little endogenous TCEP-releasable H_2S was observed (n.d. – 1.5 µg/L, data not shown). BR H_2S and subsequent TCEP-releasable H_2S are presented in **Error! Reference source not found.**. In wines with added GSH, TCEP-releasable H_2S accounted for only a small portion of added H_2S immediately after addition (t=0.5 min),1.0 to 4.7 µg/L, or 1 – 14% of added H_2S. After 20 min incubation, TCEP-releasable H_2S could only account for 0 – 5%. These values were over 5-fold lower than those observed for BR H_2S. Potentially, this is because of the high ratio of GSH as compared to Cu(II) and H_2S in the current study (300:10:1 molar ratio), which would favor formation of glutathione disulfide over organopolysulfanes. Previous work on reactions of Cu(II), cysteine, and H_2S in model wine systems had all components at roughly equimolar concentrations (50 or 100 µM for Cu(II), 300 µM for H_2S and other sulfhydryls),[40] which would favor greater formation of organopolysulfanes. Wines are typically reported to have higher thiol concentrations than copper or H_2S.[73] Therefore, these experimental results, which show low levels of organopolysulfanes following Cu(II) addition to a wine with free H_2S, are more likely to occur in real wines.

An additional alternative hypothesis that was not tested is if H_2S is lost not only by reaction with Cu(II), but also by reaction with *o*-quinones or H_2O_2 formed through Fe-catalyzed oxidation of *o*-diphenols.[25] If substantial H_2S was lost via this "iron-phenolic" pathway rather than the copper-mediated pathway, the role of GSH or other sulfhydryls could be to react preferentially with *o*-quinones or H_2O_2, allowing H_2S to be preserved for reaction with Cu(II). However, the consumption of oxygen through the iron-phenolic pathway is very slow (on the order of hours or days) as compared to

the reaction of Cu(II) with sulfhydryls (on the order of seconds),[17] and thus this scenario is unlikely. Thus, the most likely explanation for "missing" H_2S following Cu(II) addition is that incorporation of GSH or other sulfhydryls into complexes of H_2S and copper makes these complexes more amenable to brine-release.

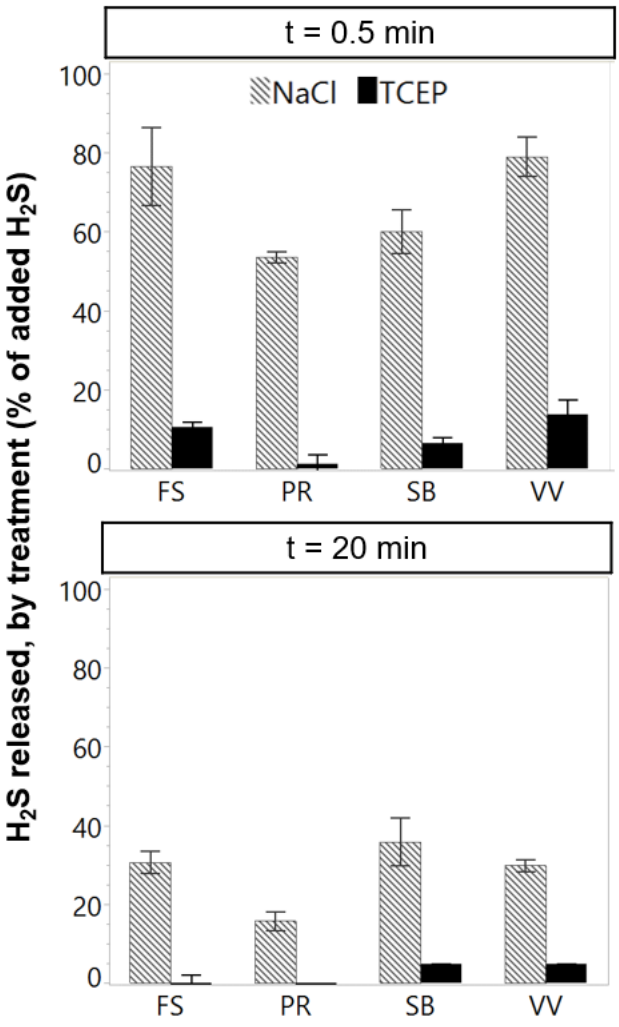

Figure 3.7. H_2S released by brine dilution (temperature = 21 °C), followed by TCEP addition. Wines (n=4) were commercial wines with added GSH (300 µM). Error bars represent 1 standard deviation from the mean.

One question that cannot be answered by the current work is if variation in H_2S brine release after 20 min is reflected in variation in H_2S release for these same wines during long term storage. However, other authors have reported that BR H_2S in wines is reported to correlate strongly with free H_2S formation under accelerated aging conditions, and moderately with free H_2S during long-term room-temperature storage.[24, 26] The pathway by which H_2S would be released from copper-sulfhydryl complexes in real wine is unclear. H_2S release by nucleophilic substitution as occurs with halides seems unlikely, but reduction of Cu_xS_y complexes by HSO_3^- to yield new complexes with lower Cu:S stoichiometry along with free H_2S seems plausible.[17]

In conclusion, it has been demonstrated that brine treatment of wines results in release of H_2S from copper-sulfhydryl complexes due to halide displacement, and not due to the increase in ionic strength. Addition of Cu(II) and H_2S to wines forms brine-releasable complexes, as previously reported, but recovery of H_2S is incomplete and decreases with incubation times. BR H_2S recovery was greater in wines with higher native GSH or in wines with added GSH, and was lowest in a model wine lacking GSH. These results suggest that thiols like GSH are important for the formation of BR H_2S complexes. Other work has shown that BR H_2S correlates with H_2S release during long term storage.[26] However, the pathways for H_2S release under brine-release and ordinary bottle storage may differ. Future studies can evaluate the hypothesis that the presence of GSH (or related sulfhydryls) will not only increase BR H_2S but will also increase the risk of H_2S formation during long-term storage. If the hypothesis is correct, it would indicate the BR-H2S assay is not only useful for winemakers

predicting in-bottle H2S formation, but also can be used to evaluate the role of GSH and other sulfhydryls in forming these complexes.

Abbreviations Used

BR H_2S, brine-releasable hydrogen sulfide; GDT, gas detection tube; GSH, glutathione; H_2S, hydrogen sulfide; MW, model wine; MW-GSH, model wine with added glutathione; $Na_2S \cdot 9H_2O$, sodium sulfide nonahydrate; NEM, *N*-ethylmaleimide; QTOF, quadrupole time-of-flight; SLO, sulfur-like off-aroma; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; VSCs, volatile sulfur compounds; wine sample names: PG, Pinot Grigio; SB, Sauvignon Blanc; VV, Vinho Verde; MA, Malbec; PR, Primitivo; RO, Rosé; FS, Fino Sherry.

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CHAPTER 4

THE CHEMISTRY OF CANNED WINES

Abstract

An emerging area of concern for wine quality is in wine packaged in aluminum beverage cans ("canned wines"), a category whose growth has eclipsed other wine packaging formats in recent years. Sales of canned wines have grown significantly over the last decade, likely due to considerable advantages in their consumer appeal, light weight, strength, and recyclability. Canned wines consist of a metal can with a polymeric liner on the inside surface, to protect the wine from the metal. In terms of packaging effects on wine quality, these can generally be classified as either flavor degradation, scalping, or tainting. While the likelihood of the first and second are negligible, given the sealing capability and materials present in a standard beverage can, issues of tainting have been reported. In particular, hydrogen sulfide ("rotten egg" aroma) is reported to develop in canned wines, especially whites and roses, resulting is unacceptable short shelf-lives. This phenomenon has been nominally attributed to reaction of sulfites in wine with aluminum metal, but little work has been done to elucidate the factors that influence this interaction in canned wines. In this work we discuss the current understanding of the canned wine system, and present hypotheses and preliminary evidence of the role of SO_2 and can liners in the formation of hydrogen sulfide.

Canned wine - Moving fast and not breaking things

Commercial examples of wine in metal cans dates to attempts with tinplate steel in 1930s [74], but the modern history of wine in aluminum beverage dates only to the last two decades [75]. In the US, the value of wines packaged in aluminum cans grew from $2 million in 2012 to $69 million in 2018 [76]. The appeal of canned wines has several explanations [77]. For consumers, cans are more convenient than conventional 750 mL glass packaging – no corkscrews required, single portion (187 mL, 250 mL) or double portion (375 mL) packaging is common, and there are no worries about broken glass at pools, concerts, or backpacking trips. Cans also lend themselves to distinctive designs and are less expensive to ship due to their light weight and ruggedness. Finally, from an environmental perspective, wine in cans are an attractive alternative to glass as well as less conventional plastic packaging like bag-in-box, due the well-established domestic recycling stream for aluminum.

The challenges of packaging wines in cans – especially in contrast to packaging in glass bottles – have been recently reported [77-78]. These articles highlight important considerations surrounding logistical aspects of canning wines (e.g. label design, headspace and fill level control, can sizes), yet only briefly mention the importance of wine chemistry in determining the shelf life of a canned wine. Here, we summarize our state of knowledge regarding the chemistry of wine in cans, and to identify opportunities and needs for future research.

What is an aluminum beverage can?

A cartoon depiction of an aluminum beverage can cross-section is shown in Figure 1a. Bare aluminum metal is highly reactive but will also rapidly form a very thin (nanometer scale) ***passive layer*** of alumina oxide when exposed to air or water [79]. The low reactivity of the passive layer is the reason why aluminum foil and other common aluminum-based materials are relatively inert. Even with the passive layer, exposed aluminum will corrode slowly in acidic media. Therefore, the can interior must be protected from direct contact with wine and other low pH beverages by coating it with a thin layer (typically 1-10 μm) of a polymer, referred to as a ***coating, liner or lacquer*** [80]. The liner is typically invisible to the consumer but can be seen by chemically etching away the outer aluminum layer (e.g. by dissolving it in a caustic solution, see Figure 1). From this perspective, an aluminum can is better thought of as a plastic bottle, with an aluminum can surrounding the bottle for mechanical support – however, the plastic liner of a can is about 1/100th the thickness of a typical plastic bottle, and thus creates much less waste.

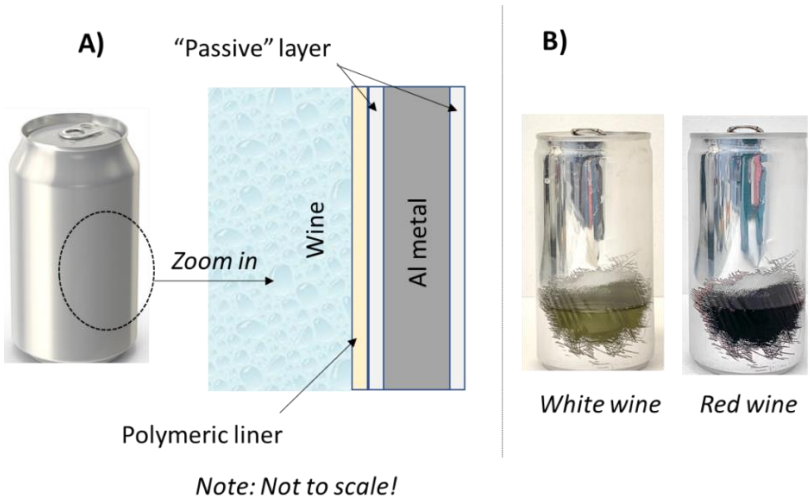

Figure 4.1. (A) Anatomy of an aluminum beverage can. The body and lid are formed from aluminum alloys. The aluminum metal surface rapidly and spontaneously oxidizes in the presence of O_2 and/or H_2O to produce a less reactive 'passive layer' of aluminum

Can liners are considered food contact substances, and their composition must be approved by the FDA [81]. In the US, most beverage can liners were historically composed of bisphenol A (BPA) based epoxy resins. However, due to the introduction of California's Prop 65, which requires the labeling of BPA-based packaging materials as suspected endocrine disruptors, many producers are utilizing alternative liner materials, including non-BPA epoxies, acrylic and polyester [82].

Finally, cans are not produced from pure aluminum, but rather from ***alloys*** of aluminum mixed with small amounts of other metals. For example, can bodies are usually produced from aluminum alloy 3004, which contains ~1% each of Mn and Mg [79]. The use of alloy improves the formability and strength of the metal for the rolling and extrusion steps necessary for can body production, as well increasing resistance to corrosion [79].

The Three Demons of Storage - Degradation, Scalping, Tainting

In the absence of microbial spoilage, the detrimental sensory changes that occur during the shelf storage of foods and beverages can be classified as either flavor degradation, scalping, or tainting [83].

Degradation refers to ordinary chemical processes that occur during product storage that result in a loss of quality, e.g. the staling of bread as moisture is lost. In the case of wine, the most important staling reaction is typically oxidation, and most table wines are packaged with 20-40 mg/L free sulfur dioxide (SO_2) to limit the effects of oxidation. Exposure of wines to oxygen results in formation of oxidation products (quinones and hydrogen peroxide), which will react with total sulfur dioxide (SO_2) at a ratio of 4:1 on a weight-by-weight basis. Oxidized aromas, brown color formation and other signs of quality deterioration are reported to start appearing at free SO_2 < 10 mg/L. Oxygen introduced during or after the packaging step may arise from several sources: oxygen initially present in the wine or headspace (also called total package oxygen, TPO), external diffusion from the environment, and diffusion from the packaging material [84].

i) O_2 may ingress into the package from the external environment. In a sound aluminum can with a well-formed double seam, **external oxygen ingress should be negligible**. Although no data exist for ingress into canned wine, research on 12-oz (355 mL) beer cans reported 0.04 mL of air ingress over a 12-week period[85]. This equates to 0.1 mg O_2/L per year, comparable of less than what has been reported for wines stored in glass bottles under screwcaps [86]. Using the 4:1 ratio described above, this amount of O_2 would result in the

loss of 0.4 mg SO_2/L per year. Considering that most canned wines are consumed within a year of release, this amount of SO_2 loss is unlikely to affect quality.

ii) O_2 may diffuse from the packaging materials into the wine. This effect can be important to traditional cork-finished wines due to the presence of voids in the cork. However, even for a relatively thick can liner (10 µm) and an implausibly high void percentage (10%) the can liner could contain only trace amounts of O_2 (<.1 mg per can). Thus, diffusion of O_2 from the can materials is likely to be negligible.

iii) O_2 may be present in the headspace or dissolved in the wine at the time of packaging. The beer industry standard for fill height of a 355 mL (12 oz) can is 12 mm of headspace, which equates to about 10 mL. If this headspace is composed entirely of air, the oxygen present in this headspace of 375 mL can (~5 mg O_2/L) would be enough to consume 20 mg/L of SO_2 (!). During canning of beer and sparkling wines, this headspace oxygen is largely limited by the CO_2 gas expelled following filling, and in still wines can be controlled with inert gases like nitrogen. Canning operators typically target < 1 mg/L total package oxygen (TPO) for 375 mL cans, which would result in a loss of 4 mg/L SO_2.

In the authors' experiences, SO_2 in canned wine will decrease in the 2 weeks following canning as oxygen present at packaging is consumed, after which total SO_2 typically decreases <5 mg/L over the subsequent year. Although this indicates that SO_2 loss due to reaction with the can liner or ingressing O_2 is negligible, we have

occasionally worked with winemakers who have observed dramatic losses of SO_2 in canned wines (up to 70 mg/L over several months) during long-term storage for unknown reasons. Potentially, this SO_2 loss could be due to its reaction with the can liner, although further investigation is warranted.

Scalping refers to the loss of compounds from a food or beverage by its migration into the packaging material. Typically, the compounds of greatest concern for scalping are non-polar flavors and odorants, which can be absorbed into non-polar polymer packaging materials. Scalping has not been studied in canned wine but has been studied with hop constituents in canned beer [87]. In this work, scalping was only detected for highly non-polar odorants, like limonene. Similar observations have been made for wines stored in the presence of other polymeric packaging materials, such as synthetic corks [88], although these would have much greater absorptive volumes than can liners. Although limonene is found in sub-sensory thresholds in wine, there are other odorants with similar or greater log P values to limonene (approximate log P of 4) [1]. Using log P > 4 as a criterion, the impact odorants in wine at risk for scalping in wine include:

- 1,1,6-trimethyldihydronaphthalene (TDN, "petrol" odor of aged Riesling)
- Rotundone ("black pepper" odor of Syrah and other varietal wines)
- 2,4,6-trichloroanisole (TCA, "cork taint") and related haloanisoles.

Presumably, winemakers would not be opposed to the scalping of TCA from their wines, if present! Many other important wine odorants like linalool/geraniol, most

[1] Log P is a measure of the relative solubility of a compound in water vs. a non-polar solvent. A higher log P indicates a more non-polar compound

esters, oak-derived volatiles, and volatile thiols have log P values < 4 and their scalping is expected to be negligible in canned wines.

Tainting refers to introduction of undesirable flavor compounds into the food or beverage due to the packaging. Often, tainting is due to the migration of odorants from the packaging into the product, as can occur in the well-known phenomenon of TCA introduction from contaminated corks.

A less common type of tainting involves the reaction of foodstuff components with the packaging to produce tainting compounds, but examples exist in the literature. For example, coatings on steel food cans may contain trace amounts of mesityl oxide impurities, which can react with hydrogen sulfide (H_2S) naturally present in foods to generate the potent 4-mercapto-4-methyl-pentan-2-one (4-MMP, "catty taint") [89]. These types of taints (which we will refer to as "secondary taints") are more challenging to predict because they will form only with certain combinations of foods (or beverages) and packaging materials, and therefore may be overlooked during initial testing with simple models like water.

In canned wine, an occasional but important taint appears to be a "rotten egg, reduced" odor brought about by H_2S. H_2S has a sensory threshold of around 1 ng/mL (1 ppb) in wine [4], and is most often experienced by winemakers during fermentation as a byproduct of yeast metabolism . The formation of H_2S during storage of wines in aluminum cans has not yet been described in peer-reviewed papers, but has been reported in multiple patents [90-91], and more recently at conferences [92-94]. In these reports and in conversations with winemakers, the time necessary to form detectable H_2S or reduced aromas can vary considerably among both wines and can types – many

canned wines experience no issues after a year of storage, while others develop detectable H_2S within several months. Importantly, the existing reports all point to SO_2 as a likely source of H_2S, thus tainting presents a potential technical challenge to canned wine quality.

SO_2 as an H_2S precursor - Not the Usual H_2S-uspect

The phenomenon of wines developing sulfurous off-aromas (also called "reduced" aromas) during anoxic storage has been well reported in recent years. At the International Wine Challenge in London, a competition that draws over 10,000 entries from prestigious wineries from around the world, reductive off-aromas accounted for 25-30% of all reported wine faults, comparable to the incidence of cork taint and oxidation [95]. A survey of commercial wines with reduced aromas reported that H_2S was the volatile sulfur compounds most often found in excess of its sensory threshold [96]. Several latent precursors of H_2S in finished wine have been identified, including copper sulfide complexes (Cu_xS_y) and organopolysulfanes ($RS\text{-}S_x\text{-}SR'$) [17].

Could these same H_2S precursors be responsible for forming H_2S in canned wines, too? As mentioned above, cans allow less oxygen ingress than screwcaps, and the resulting anoxic environment will certainly favor formation and preservation of H_2S from any precursors in the wine. However, the anoxic environment appears to be only a partial explanation for the incidence of H_2S in canned wines. Anecdotally, winemakers have reported that wines stored in aluminum cans are more likely to produce detectable H_2S than the same wines stored under screwcap. Beyond this, there

are several pieces of evidence that point to SO_2 as a key source of H_2S during canned wine storage.

- Model wines composed of SO_2 in buffered solutions of 10% ethanol in water will produce detectable H_2S within weeks when stored in commercial, lined aluminum cans (Figure 2). These model solutions generate H_2S in the absence of any wine-derived latent H_2S precursors, and H_2S is not observed when SO_2 is omitted from the model solution.
- Real wines (as well as model SO_2 containing solutions) will rapidly generate H_2S when exposed to aluminum and its alloys. Formation of H_2S is reported to increase in dose-dependent manner with increasing SO_2.[2]

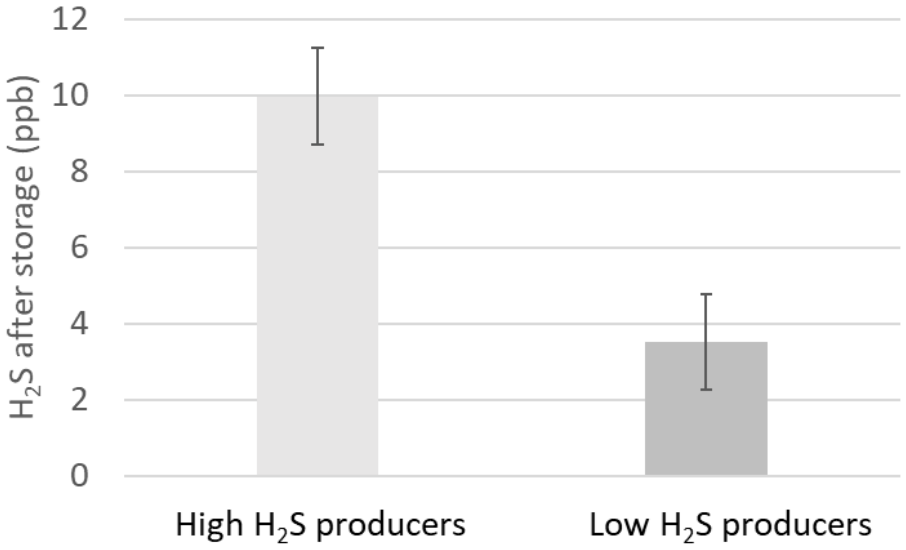

Figure 4.2. H_2S formed in model wine (pH 3.5, 50 mg/L free SO2, 12% EtOH) following 2 weeks of storage at room temperature. Cans were classified as high or low H_2S producers (n=4 for each) based on previous observations with commercial wines. No H_2S was observed in cans stored without SO_2.

[2] An additional piece of circumstantial evidence is that canned beer (which usually has no added SO_2) is not reported to develop H_2S during storage, but canned cider (which does have added SO_2) will occasionally develop H_2S.

With respect to this second point, the observation that wines will generate H_2S when brought into contact with aluminum alloys dates back over 80 years [973]. The noted enologist Bryan Rankine later reported that both wine and SO_2 solutions would produce H_2S when they are exposed to aluminum surfaces [98]. Rankine, along with other authors [90, 93], hypothesized that H_2S formation could be explained by a redox reaction between aluminum and SO_2 under acidic conditions:

$$2\ Al^0_{(s)} + SO_{2\ (aq)} + 6\ H^+_{\ (aq)} \rightarrow 2\ Al^{3+}_{\ (aq)} + H_2S_{\ (g)} + 2\ H_2O$$

This reaction is thermodynamically favorable at wine pH. In our own experience, we observe production of > 10 µg/L H_2S (well in excess of sensory threshold) after incubating an aluminum coupon in a 500 mg/L SO_2 solution for a couple of days at room temperature. At neutral pH, we observe white, pustule-like blisters of aluminum hydroxide on the aluminum surface (Figure 2). The blisters are not visible in wine, possibly because they dissolve at wine pH, but will sometimes be observed in can headspace. H_2 (from reduction of H^+) is also expected to be formed [79]. These conditions are extreme as compared to what would be observed in a lined can with a typical wine, where detectable H_2S is expected to take months to be detected.

The importance of SO_2 is appreciated by can manufacturers, who will provide recommendations for maximum free SO_2 at packaging (e.g., <35 mg/L) [91]. Maximum molecular SO_2 recommendations are not always provided, although for reasons described below it may be prudent to do so.

[3] The paper lists Lyman Cash as a middle author. Mr. Cash was a long-time enologist for E&J Gallo and is best known for his eponymous still for measurement of volatile acidity.

What about the liner? Shouldn't that stop SO_2?

In principle, the polymer liner is supposed to prevent contact between the beverage and the metal surface. However, as mentioned earlier, neutral compounds can diffuse into and through components of packaging, a phenomenon responsible for "flavor scalping" of odorants. Volatile species like molecular SO_2 are also reported to be able diffuse through coatings and accelerate corrosion in industrial settings [99]. In a 2006 patent, Daiwa Corp proposed that molecular SO_2 diffuses through the liner material to reach the passive layer [90]. An alternative mechanism could involve imperfections (pores) in liner material which allow free SO_2 (primarily in the form of bisulfite) to reach the metal surface.

With either diffusion of molecular SO_2 through the liner, or passage of free SO_2 through pores, we expect the liner to have a strong effect on performance. In our lab, we have observed considerable variation in H_2S produced during storage of model wines in cans with different liners (Figure 3). Anecdotally, winemakers report less incidence of H_2S with BPA epoxy based liners, although these liners have been declining in usage for reasons previously described [82]. The first generation of BPA – non-intent (BPA-NI) liners for wine cans was reported to have more problems with H_2S formation. However, more recent generations of liners are reported to have better performance.

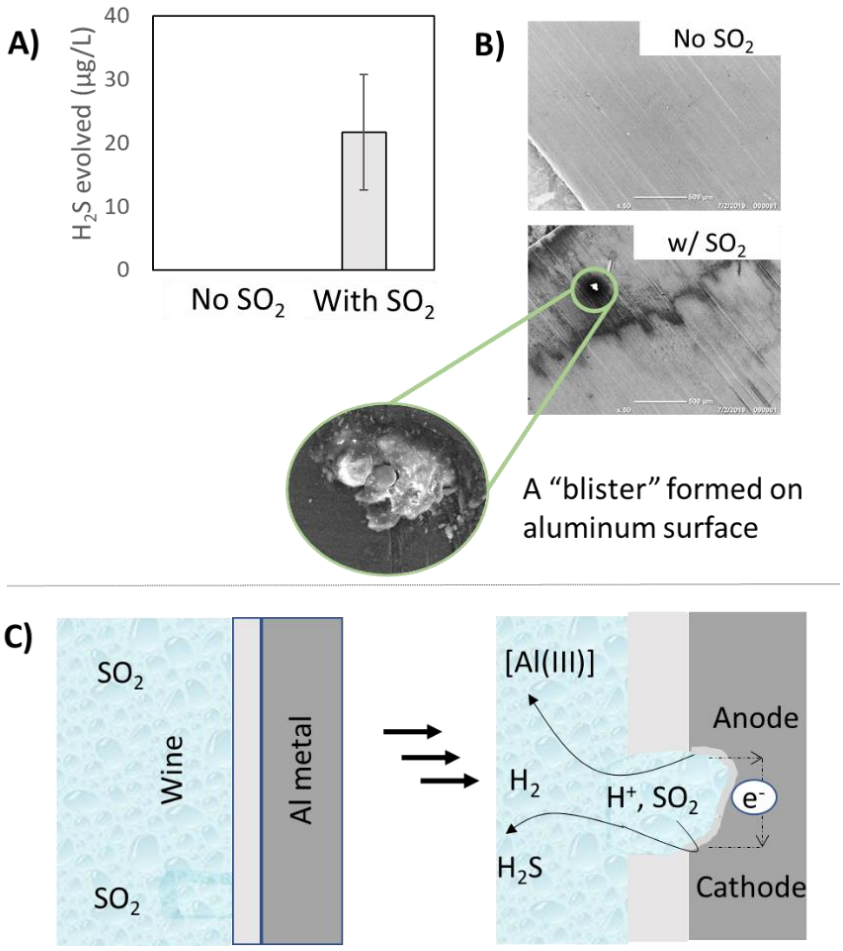

Figure 4.3. (A) H_2S production following incubation of aluminum coupon in aqueous ethanol solutions w/ or w/o SO_2. B) Scanning electron microscope (SEM) images of pit and "blister" on aluminum surface following exposure to SO_2, from previous experiment. C) Cartoon of hypothesized redox reaction between SO_2 and aluminum, leading to H_2S and pitting corrosion

The potential role of other wine components

Wine is acidic, in a range (pH 3-4) typical of other canned beverages (e.g. juice, carbonated soft drinks). By itself, pH is not expected to cause storage problems due to the protective action of the can liner. For some perspective, many carbonated soft drinks have a pH of <2.5. However, lower pH will favor higher proportions of molecular SO_2, which may be the more active form of sulfur dioxide. Additionally,

protons (H^+) will directly participate in the reaction of SO_2 with Al. Other trace components, such as chloride (Cl^-) and copper (mostly in the form of Cu[I] in wine) are known to participate in or accelerate aluminum corrosion, although existing data is for non-wine systems [79]. Thus, in addition to recommendations for SO_2, can manufacturers typically provide recommendations for Cu, Cl, and pH, along with several other compositional parameters [91]. Two caveats should be considered with these recommendations. First, the rationale behind restricting all listed components is not always evident based on existing literature. Second, these recommendations consider each component separately, even though they likely interact synergistically in accelerating corrosion reactions between the can and the beverage components.

Questions, questions, questions

The recent growth in canned wines is exciting for both wine consumers and producers. Beyond their obvious advantages in convenience, light weight, strength, and visual appeal, the inherent recyclability of aluminum cans makes them an attractive alternative to both glass and plastic (or multilaminate) packaging.

For producers concerned about the potential for H_2S formation during canned wine storage, there is good circumstantial evidence that SO_2 is a key source, although it is unclear if molecular SO_2 or bisulfite is the more important species. For wines where microbial spoilage is of low concern, i.e. sterile filtered wines without residual sugar, the primary role of SO_2 is as free SO_2, to prevent oxidative spoilage. Because cans allow little oxygen to enter a wine during storage, it is likely appropriate to use lower free SO_2 (15-20 mg/L) in cans than what is typically recommended for table wines at

bottling, as long as best practices are used to ensure low total package oxygen. Also, most red wines have low free and molecular SO_2 due to binding by anthocyanins [1004] – this factor may explain why winemakers rarely report H_2S formation during storage of red wines in cans. Beyond SO_2 concentrations, there are many other questions surrounding H_2S formation to be answered (Figure 4)

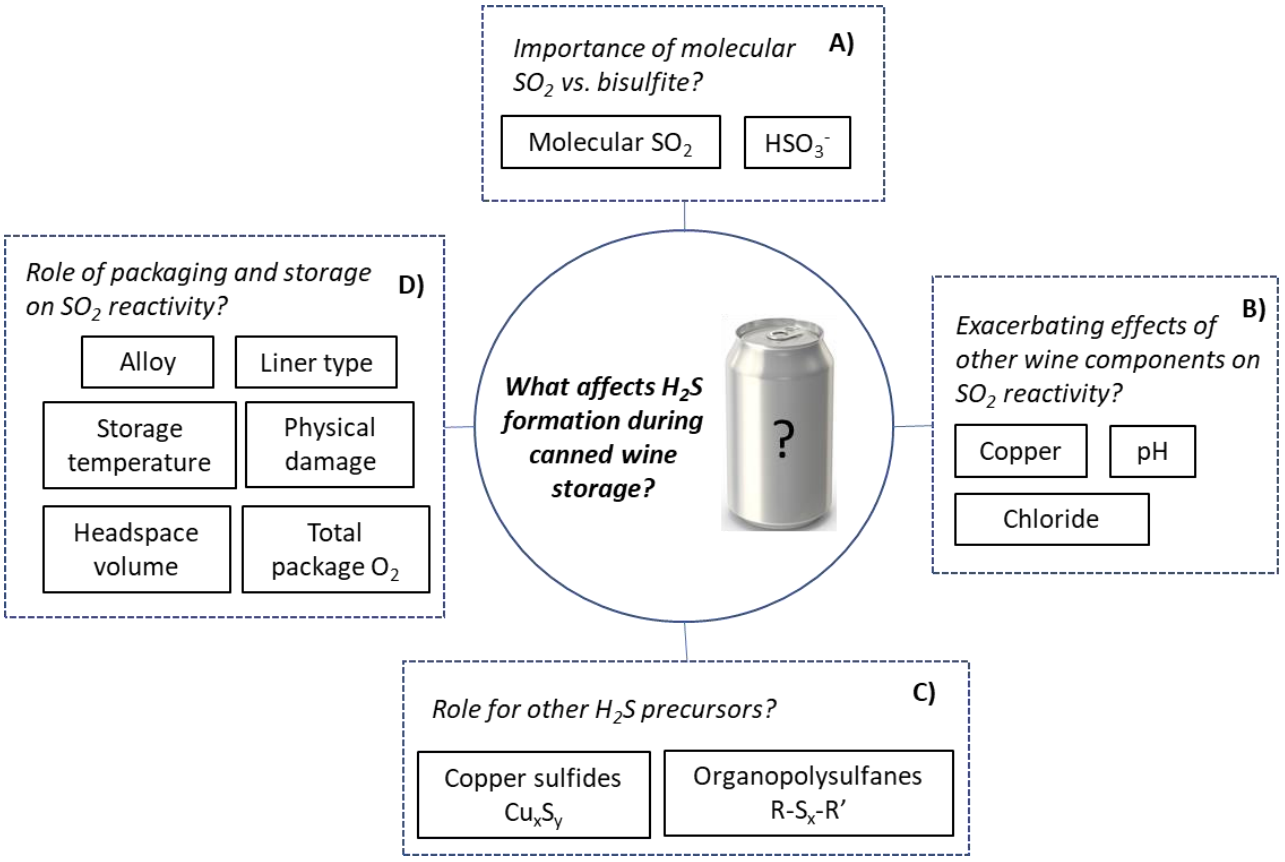

Figure 4.4. Formation of H_2S in canned wine – questions to be investigated

In the longer term, there is a need for validated tools for predicting H_2S formation during canned wine storage. Ideally, this will be more sophisticated than existing tools

[4] The actual free and molecular SO_2 of a red wine is typically only 30% of what is measured by aeration-oxidation and related techniques which disrupt anthocyanin-bisulfite complexes during measurement

provided by can manufacturers, which are based on limits for individual wine components (which ignores potential synergies) or corrosion potentials (which assumes that the corrosivity of a wine will be correlated with its ability to form H_2S). Instead, there is a need to develop and validate accelerated tests, whether they are based on wine composition or on short-term, high temperature studies. Ideally, this should allow winemakers to make statements along the lines of "Based on accelerated testing, this wine in this can liner has a 0.1% chance of forming detectable levels of H_2S after 6 months storage at room temperature, or after 2 months at 35 °C". Developing such evidence-based tools to predict quality and shelf-life of canned wines will help producers manage changes in product or package composition, and expand opportunities for canned wine producers.

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CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

In Chapter 2, we described a convenient colorimetric approach for H_2S analysis in still wines using commercially available gas detection tubes (GDTs). The proposed apparatus repurposes the glassware from an Aeration-Oxidation unit, making it appropriate for use in the winery. The limits of detection for both the N_2 and the Aspiration Methods were comparable to conventional analytical methods, and excellent linearity was achieved. The linear ranges were 0 – 307 ng and 0 – 875 ng, for N_2 and the Aspiration Methods respectively. When minimum sample volumes were used, this corresponded to a concentration range of up to 50 µg/L (N_2 Method) and 175 µg/L (Aspiration Method) on the 4LT GDT, which easily accommodates the H_2S concentrations typically reported in wines.[12] The major advantage of the simple approach is also a limitation of the method, in that it is designed for manual operation and to handle one sample at a time, making it challenging to scale up.

Therefore, future considerations in this work are to:

1) Investigate high throughput and automated alternatives
2) Validate different capacity H_2S GDTs to expand the analytical range and increase sample volumes to improve sensitivity of the method
3) Expand this approach for other wine-relevant sulfur compounds based on selective GDTs

For 1) while a simple-to-operate format is likely sufficient in most winery needs, increased throughput and automation could be of interest for larger wineries, research applications, or analytical service providers. There are two approaches which can be considered. The first would be to attach a multi-apparatus array, running samples in parallel. This would maintain the

simplicity of the approach, and still use common glassware, though it is not suitable for automation. Also, flow regulation difficulties (back pressure for N_2 Method, insufficient vacuum for the Aspiration Method) could limit the extent and practicality of this approach.

The second approach is also colorimetric but does not utilize GDTs; the colorimetric reaction for the GDTs relies on reaction of sulfide with lead or mercury acetate,[16] neither of which are the most desirable reagents for handling. The alternate approach relies instead on the specific reaction of dimethyl-*p*-phenylenediamine with sulfide in acidified solution, in the presence of an oxidizer such as Fe(III), to produce methylene blue. This analysis has been reported using flow-injection analysis (FIA) and GD (gas diffusion) techniques in non-wine samples,[101-102] with LODs approaching those of the GDT method in Chapter 2. Notably, it is reported that LODs lower than 0.2 µM could be achieved by slowing the donor and acceptor stream flowrates for GD.[102] There is a precedent for this technique in wine analyses as automated and high throughput measurements for various wine components, notably SO_2, are often conducted using FIA with an autosampler. As previously noted, H_2S analyses are not standard practice in the winery, but adapting existing analytical tools with automated FIA to accommodate H_2S analysis could be useful.

Regarding 2) and 3), we propose improving the versatility of this approach by expanding the analytical range. For 2), we have reported mainly on the use of 4LT GDTs, which has the smallest capacity and limits of detection. Other GDTs with higher capacity are also commercially available at a similar cost and make it possible to use this approach to measure H_2S in other (non-wine) liquid samples. We report on the use of the 4LL GDT, but can also point to the 4D, 4H, 4HM, 4L, 4LK, 4M and 4S as H_2S GDTs with no reported mercaptan interferences.[103] In addition, we have measured samples volumes of 6 – 66 mL with a 100-mL

sample flask. The sensitivity of the method can be improved by increasing the sample size, allowing more analyte to be introduced when analyzing low concentration or diluted samples (such as the brine dilution assay in Chapter 3). Larger volumes with the current flask, or larger flask sizes, can be investigated but the potential mass transfer limitations must be considered when using larger volumes, and may require longer analysis times.

For 3) we propose to investigate the effectiveness of this approach for other VSCs associated with reduction. Though VSCs are not typically quantified in the winery, an external sulfides panel may include 10 relevant VSCs (including H_2S). GDTs for general mercaptans (No. 70LN), including ethyl (EtSH) and methyl mercaptan (MeSH), are commercially available and include a H_2S pre-treatment layer at the beginning of the tube to prevent interferences.[103] Recent work identified relevant bound fractions of MeSH in wines,[7] and in conjunction with H_2S analyses, this could give wineries a more comprehensive picture of the extent of reductive issues. As with any GDTs that are not designed specifically for wine samples, a potential pitfall is interferences, so this should be rigorously checked.

In Chapter 3 we described a brine dilution assay for releasing H_2S from copper-sulfhydryl complexes and demonstrated that the free sulfhydryls content, represented by GSH, affects the amount of H_2S released from complexes. We observed significant differences among wines regarding the extent of this effect and we observe no significant grouping by wine style. The native GSH concentration correlates ($r^2 = 0.58$) with brine-releasable H_2S (BR-H_2S) in wines (n=8) and the addition of 300 µM endogenous GSH increased BR-H_2S after a brief incubation for most wines. The implication of this increase in BR-H_2S is that copper-sulfhydryl complexes are less stable and more susceptible to disruption by brine dilution when formed in the wines with higher free sulfhydryls content, or with a large excess of added free sulfhydryls.

For future work, the brine dilution assay could be refined into a predictive test for the likelihood of wines developing H_2S issues from copper-sulfhydryls during storage. This would be of interest to winemakers in choosing an appropriate remediation strategy for wines with excessive H_2S levels prior to bottling. Validation of a predictive test would require a comprehensive survey of chemically differentiated wines for post-hoc analysis. We propose a sample size of 24 wines (12 red, 12 white) per a recent study of copper-sulfhydryl complexes,[24] and with a more targeted selection of wines. Accounted for as a random effect in the statistical analysis, the variance observed between wines was significant compared to the change in BR-H_2S within each wine. To address this, the future selection of wines should consider the factors which affect GSH content in the final wine. This would require considerably more detail on the viticultural and fermentation conditions for each wine, though there are mixed reports on changes in GSH concentration during winemaking.[73] Alternately, a large number of wines could first be screened for GSH content. Then, a wider range of GSH concentrations, similar to that reported in the literature,[71] could be used to better understand the observed correlation. As GSH is not a standard metric reported for wine, and relies on HPLC-MS techniques similar to what we described in Chapter 3, the cost of screening could be a limitation. There are two questions from Chapter 3 that should be addressed to develop the predictive assay:

1) The tendency of brine dilution to overestimate H_2S formation

2) The absence of correlation with Cu content

For 1), though brine dilution has previously been shown to overestimate the amount of H_2S produced during normal storage,[26] work in our group showed that lower brine dilution ratios released less H_2S from complexes.[52] Further, in Chapter 3 we discuss the effect of temperature on the brine dilution assay, with lower temperatures resulting in less H_2S released during brine

dilution; it is plausible that cooling the reaction conditions below ambient conditions could further suppress H_2S release. This suggests the potential for refining the brine dilution conditions to be less forcing, and more representative of real aging.

For 2), it would be ideal to quantify both total copper as well as the different fractions, as described in recent work.[72] Interestingly, we found no significant effect of copper content, native or added, with BR-H_2S. Considering recent work highlighting that most copper in wine is bound to organic acids,[104] segmentation of the copper analysis could produce a more meaningful correlation.

In summary, for copper fining to be a universally appropriate remediation strategy for reduction in wines, the copper-sulfhydryl complexes either need to be much more stable or effectively removed after formation. As we have shown that the stability of the complexes is decreased by higher levels of free sulhydryls, this factor would have to be carefully controlled and reduced. This is not practical within conventional winemaking; sulfhydryl compounds like GSH persist throughout fermentation in most wine styles and GSH content evolves based on many factors, not all of which are understood.[73] Further, there are currently no convenient analytical techniques for quantifying and tracking GSH in the winery. Therefore, removal of copper-sulfhydryl complexes is the more practical approach, and while conventional mechanical filtrations are ineffective at removing copper-sulfhydryl nanoclusters in wine,[43] several recent studies have demonstrated that certain polymers can significantly decrease these precursors.[66, 105]

In Chapter 4 we have discussed the advantages and limitations of aluminum beverage cans as an alternative packaging for wine. We have highlighted the issue of tainting and the development of H_2S due to the proposed interaction between Al and SO_2 in the wine. Current canned wine guidelines in the industry and patent literature suggest limits for free SO_2, as well as other wine

components that could allegedly contribute to deterioration of the can/ liner.[45-46] The potential for other VSCs to develop in canned wines is also of concern, due to the high levels of H_2S observed in our recent work in model and real wine systems.[68] Based on the severe reduction observed in some commercial canned wines, it is clear that the polymer liner in the can is not universally effective at preventing interaction between the metal and wine components.

The future direction of this work is to mitigate the risk of H_2S development in canned wines so that the packaging type can become attractive for wider adoption. There are two approaches that can be explored:

1) Prevent the initial formation of H_2S in cans by identifying which wine chemistry parameters are driving formation

2) Remediate H_2S after formation, within the can

To determine which wine chemistries influence H_2S formation, we have begun investigating this question using both commercial and model wines in accelerated and real storage trials. Ultimately, this work will support improved recommended chemistry metrics for wines going into cans.

Accelerated bench-tests for predicting H_2S formation in cans

First, we will develop and validate an accelerated aging method for formation of H_2S using commercially coated aluminum coupons as a proxy for the can. We are investigating three different can liner chemistries: epoxy, epoxy acrylate, and acrylic. Since H_2S formation has typically been reported in whites and rosés, and rarely in reds, 10 commercial wines (6 whites, 2 reds, 2 rosés) representing different chemistry were selected for use in both real can storage and accelerated bench-tests.

In accelerated aging, a small coupon of Al, coated in one of the three liners, is incubated in a vial of each of the 10 different wines. The edges of the coupon are sealed with an ethylene vinyl acetate “hot melt glue” to prevent the exposed Al from interacting with the wine. The wine is degassed prior to incubation and dissolved O_2 is minimized, to represent canning best practices. The vials are stored at 50 °C for 3 days, then analyzed for H_2S. Sample vials containing epoxy coupons are shown in Figure 5.1.

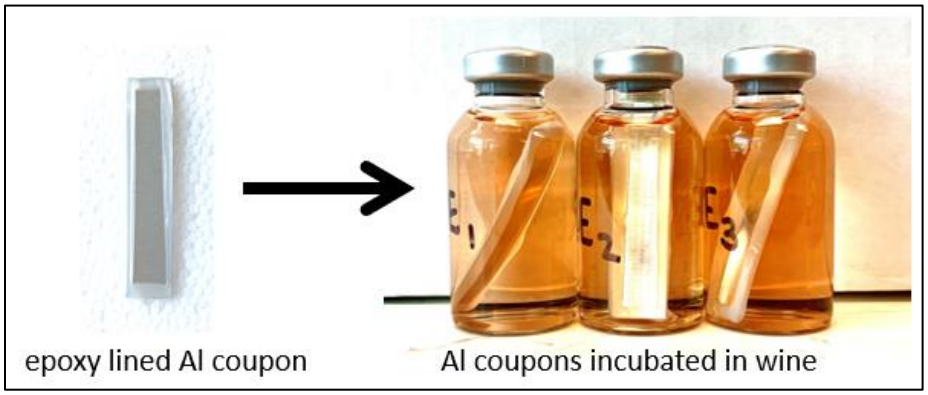

Figure 5.1. Example of an epoxy lined Al coupon with sealed edges (left) and accelerated bench-test sample vials containing degassed wine and an Al coupon. The vials are sealed with a butyl rubber stopper and metal crimp cap.

For comparative normal storage, standard 355 mL aluminum beverage cans with the same three liners are filled and manually seamed, stored at room temperature, and analyzed for H_2S at 1, 2, 4, and 8 months. The Aspiration Method described in Chapter 2 is used for quantitation of H_2S. Among the different liners, epoxy is the best performing, followed by the epoxy acrylate. The acrylic liner performs very poorly in the wine system, with some wines producing 50-fold higher H_2S than is typically reported in wines. For this discussion, the focus will be on the potential of the epoxy lined coupons and cans. The H_2S production in canned wines after each storage period (Figure 5.2) can be compared to the H_2S produced using the current accelerated aging conditions. The best correlation occurs at 8 months of Real Storage, shown in Figure 3.

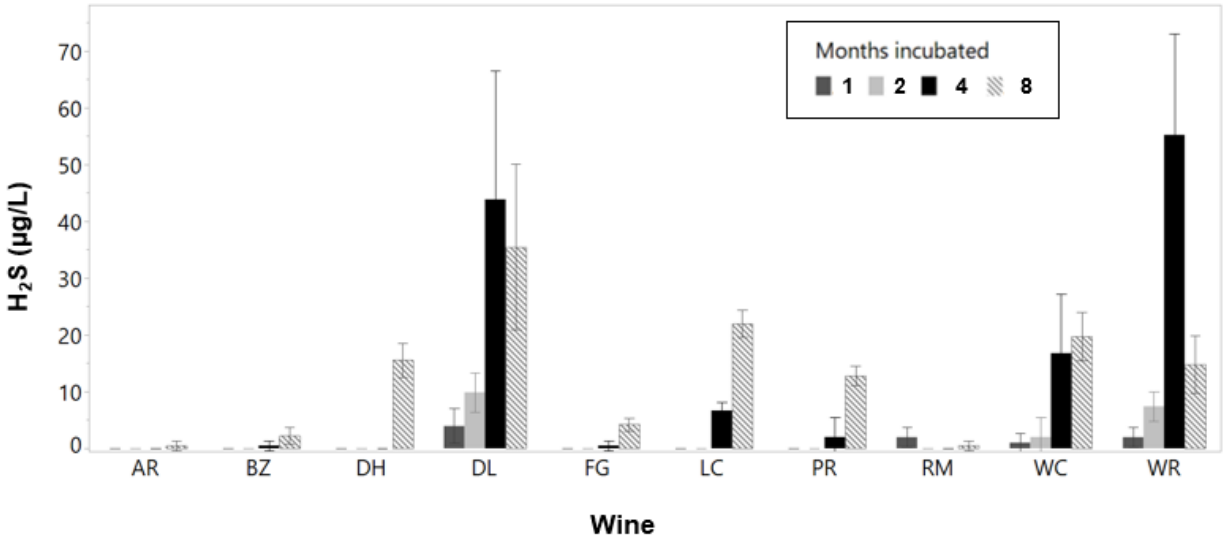

Figure 5.2. H_2S formation (µg/L) in wines (n=10) packaged in epoxy lined aluminum cans and stored at room temperature for 1, 2, 4, and 8 months. Error bars represent 1 standard deviation from the mean.

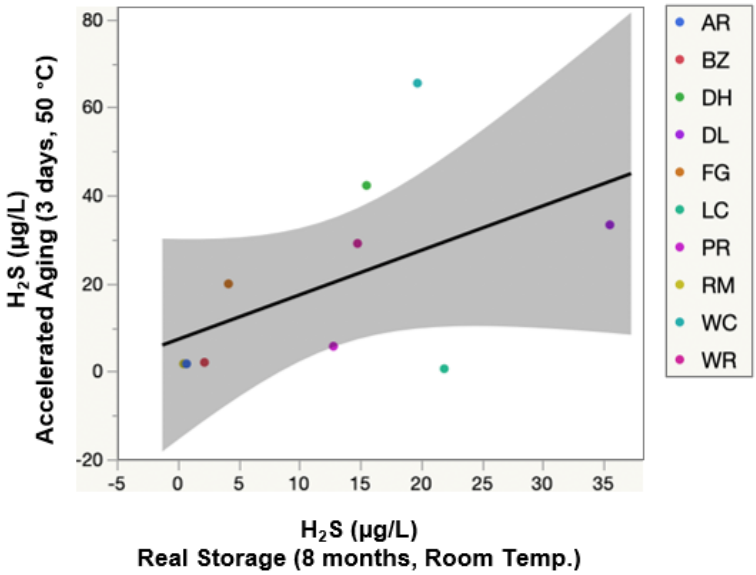

Figure 5.3. Comparison of H_2S from Accelerated Aging to H_2S formed during Real canned wine storage for 8 months.

While the fit of the real can storage to the accelerated aging results improved with later timepoints, the accelerated model reasonably predicts only 8 of 10 wines for H_2S. The two wines (PR, LC) which are not well fit are false negatives, which would be a major concern for

producers. The next step is to refine the assay such that these wines produce more H_2S under accelerated conditions, without seriously impacting the other wines. Longer accelerated storage times of 7 and 14 days have been investigated for PR and LC, as well as two additional wines (DL, BZ) at the higher and lower end of H_2S production. Results from the 14-day accelerated storage are thus far promising for PR and LC (Figure 5.4), but from the change in H_2S for DL, there is a clear need to validate the remainder of the wines using the modified accelerated conditions. If further adjustments are needed for the accelerated bench-test, adjusting the amount/surface area of Al coupons, pre-treating Al coupons to accelerate liner degradation, and varying the incubation temperature could subsequently be explored.

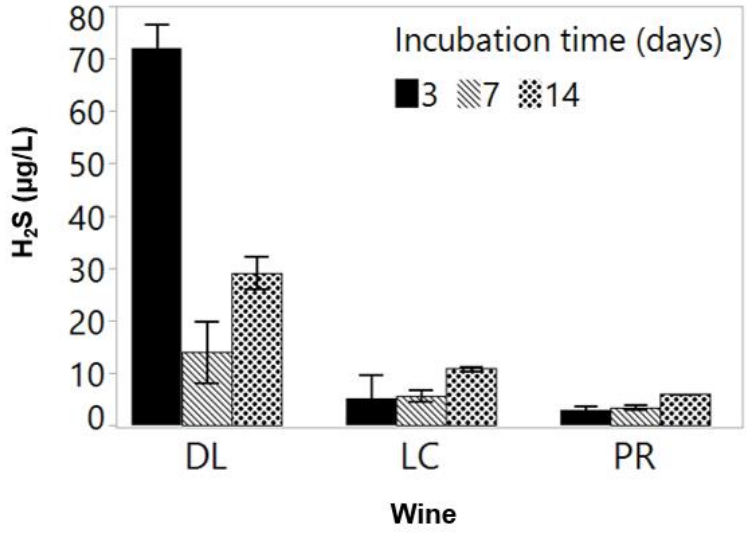

Figure 5.4. Effect of different incubation times (3, 7, and 14 days) on H_2S formed (µg/L) during accelerated aging with epoxy lined Al coupons in wines. Error bars represent 1 standard deviation from the mean.

Effects of pH, ethanol, and free and molecular SO_2 on H_2S formation in model systems

While free SO_2 has been identified as the likely source of H_2S in canned wines, this consists of two chemical forms in wine. The major fraction is bisulfite (HSO_3^-), which has antioxidant activity, and the minor fraction is molecular SO_2 which has antimicrobial activity. Both fractions

are integral to the stylistic integrity of many wines. We have hypothesized that molecular SO_2, though only ~5% of the sulfite, is a better predictor of H_2S than either free SO_2 or bisulfite, because molecular SO_2 can theoretically permeate through polymer films.[47] In our preliminary work with epoxy coupons incubated in model wine (prepared using a base of commercial wine), we have shown that when free SO_2 is held constant ([free SO_2] = 50 ppm), lowering the pH (and thus, increasing the molecular SO_2) significantly increased the H_2S produced in the accelerated bench-test for canned wine (Figure 5.5). However, while [H+] and molecular SO_2 only vary by a factor 5, H_2S increases by a factor of 30. In a complementary experiment, free SO_2 was adjusted to give constant molecular SO_2, for 2 pH levels and 2 ethanol levels. Despite the constant molecular SO_2, we still observe differences in H_2S formation, with lower pH and higher ethanol content both corresponding to higher H_2S formation. Interestingly, when these same conditions were tested with uncoated Al coupons, the opposite trends were observed for H_2S production (Figure 5.6). This suggests that pH, ethanol, and free SO_2 have some independent activity in H_2S formation, and this depends on the presence of a liner in the model. In future investigations, the use of liner coated coupons is recommended as the best representation of the system.

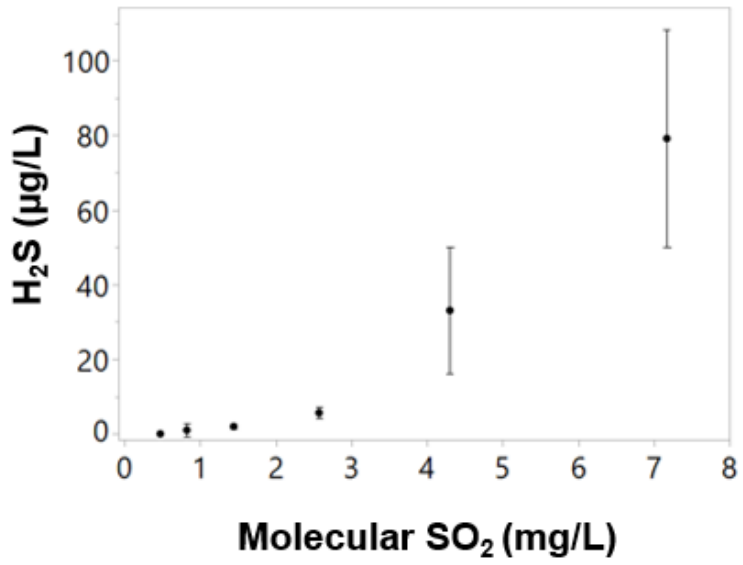

Figure 5.5. H_2S formed in modified wine solutions during accelerated aging (3 days at 50 °C) with constant free SO_2 (50 mg/L) and ethanol (12.5%) and variable pH/molecular SO_2. Error bars represent 1 standard deviation from the mean.

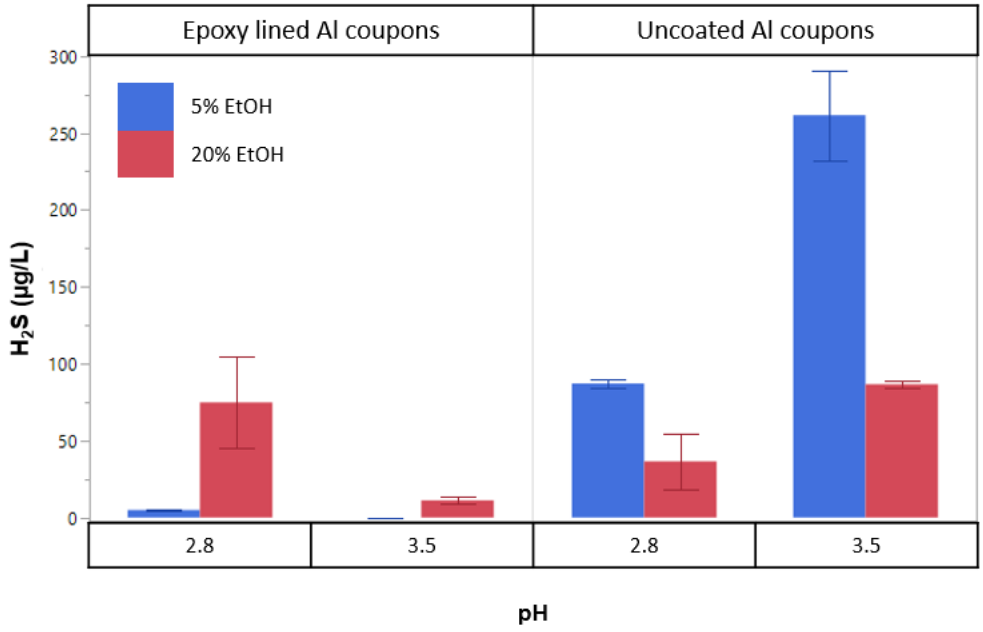

Figure 5.6. Effect of variable pH (2.8, 3.5) and ethanol (5%, 20%) on H2S formation in Accelerated Aging (3 days at 50 °C) using epoxy lined Al coupons (left) and Uncoated Al coupons (right). Error bars represent 1 standard deviation from the mean.

As the equilibrium between molecular SO_2 and bisulfite is affected by pH and ethanol content, and there is evidence of the latter two's independent roles in facilitating H_2S formation in canned wine, further work is needed to decouple the relative role of these factors. From studies in other industries, we speculate that [H+] contributes to liner degradation[34] and ethanol to liner swelling,[35] jointly enabling the interaction between SO_2 fractions and the Al metal.

In terms of 2), remediating H_2S after packaging bottling is challenging for producers across packaging types. The main approaches for remediation would be through additives which react with H_2S to form non-odor active products or using active packaging to sequester H_2S. Regarding additives, there is an opportunity that in better understanding the mechanism of H_2S formation, the apparent absence of this phenomenon in red wines could be extended to white wines by imitating the relevant chemistry. We speculate that SO_2 is less available in red wines because the anthocyanins present tend to form weakly bound adducts with SO_2, leaving very little effective free or molecular SO_2 to even form H_2S. The advantage of using red wine chemistry as the target system is that legally permitted wine additives could potentially be identified. The main obstacles with this approach are the limited number of allowed, food-safe additives which could be used prophylactically, the undesirability of labelling such additives, and their potential effect on the other organoleptic properties of the wine. A more promising future direction is in the development of non-migratory active packaging, where active compounds only need to be regulated as packaging, not as food additives. For example, beverage containers functionalized with oxygen absorbers to remove residual oxygen.[106] In canned wines, the polymer liner would be the best candidate for incorporating H_2S-trapping functionality. While previous work has largely focused on the scalping loss of desirable volatiles in plastic packaging, this phenomenon could potentially be used in a controlled way to target H_2S. H_2S is highly

volatile and relatively non-polar, making it a good candidate for scalping. The scalping capacity of different bottle closure materials on light VSCs in model wines has been previously assessed, with synthetic, natural cork, microagglomerate cork, and Saranex screwcap resulting in H_2S scalping after 25 days of soaking.[107] Further investigation into the scalping capabilities of approved packaging materials, as well as their impact on the overall wine quality, could provide a useful basis for future packaging design.

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SUPPORTING INFORMATION

Chapter 2

Figure A. Example of the color change in Gastec 4LT (a, b) and 5L (c, d) gas detection tubes, with tubes before and after use.

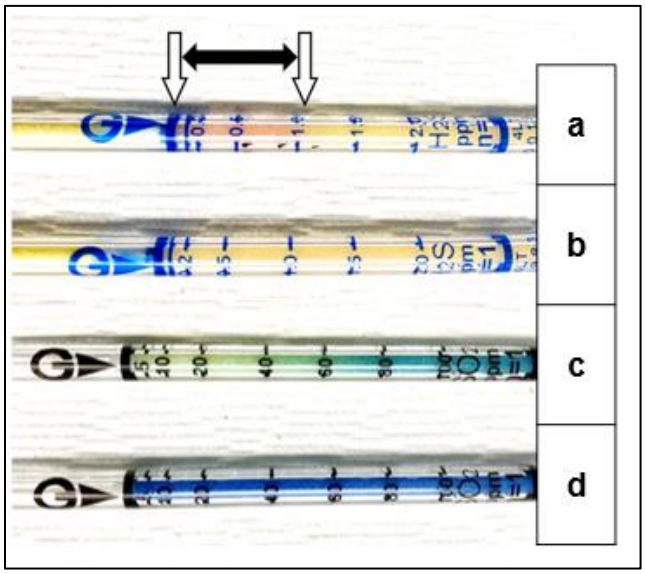
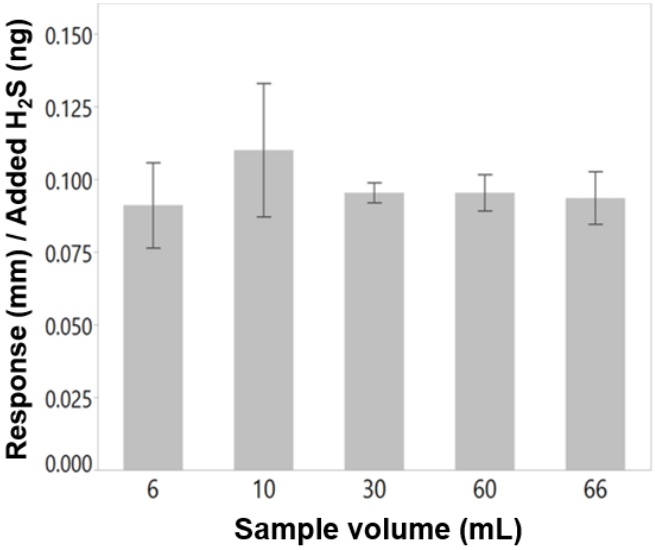


Figure B. The response on the GDTs using different sample volumes is shown, with Response (mm) normalized to the amount of Added H_2S, from standard solution additions. The samples are prepared in both in model wine and water and measured using the N_2 Method. The fractional response is not significantly different across sample volumes (Tukey's test, α=0.05), indicating that the response is independent of the sample volume used. Four replicates are calculated for each sample volume except 30 mL samples, which were in duplicate.



Chapter 3

Table S1. Summary of different brine conditions investigated.

Brine Salt	Brine Concentration (M)	Halide Concentration (M)	Ionic Strength (M)	Halide/ Non-Halide
NaCl	6.0	6.0	6.0	Halide
NaBr	6.0	6.0	6.0	Halide
$CaCl_2$ - low	3.0	6.0	9.0	Halide
$CaCl_2$ - high	5.6	11.2	16.8	Halide
NH_4Cl	6.0	6.0	6.0	Halide
NH_4NO_3	6.0	0.0	6.0	Non-halide
$(NH_4)_2SO_4$ -low	2.0	0.0	6.0	Non-halide
$(NH_4)_2SO_4$ -high	5.6	0.0	16.8	Non-halide

Table S2. Summary of measured Total Initial H_2S by brine dilution with GDTs, Initial Free H_2S with GDTs, and the resultant Initial BR H_2S. All analyses are measured in duplicate.

Wine	Total Initial H_2S (µg/L)	Initial Free H_2S (µg/L)	Initial BR H_2S (µg/L)	Total Final H_2S[b] (µg/L)
FS	0.0±0.0	0.0±0.0	0.0	4.4±1.7
MA	2.1±0.6	1.5±0.1	0.6	11.1±1.3
RO	4.4±0.5	1.2[c]	3.2	7.5±0.0
PG	3.7±0.6	2.0±0.1	1.7	8.6±1.7
PR	0.4±0.6	0.2±0.1	0.2	1.7±.0.9
SB	2.9±0.6	2.4±0.4	0.5	12.8±3.4
VV	0.4±0.0	0.5±0.1	-0.1[a]	3.3±0.9

[a] Initial BR H_2S for VV is taken to be not detectable, within error.

[b] Total Final H_2S at t=20 min, GSH=0, H_2S=1 µM, $CuSO_4$=10 µM

[c] No replicate measurements were taken