RE-THINKING FREE AND MOLECULAR SULFUR DIOXIDE MEASUREMENTS IN WINE

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RE-THINKING FREE AND MOLECULAR SULFUR DIOXIDE MEASUREMENTS IN WINE

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Data from thirteen years of wine industry laboratory proficiency testing were reviewed. Reproducibility was evaluated with Horwitz Ratios (HorRat); only alcohol, titratable acid, and total SO_2 had acceptable values (mean HorRat < 2). Reproducibility and repeatability imprecision generally increased with analyte concentration, with notable exceptions of alcohol (both), volatile acidity (reproducibility), and total SO_2 (repeatability). The methods or instruments for alcohol, titratable acidity, free and total SO_2, and volatile acidity changed significantly over the time period. Results demonstrate the need for industry-wide improvement in analytical performance for some assays, and the potential benefit of adopting criteria guidelines for method performance.

Accurate measurements of the major active sulfur dioxide species in wine (HSO_3^- and SO_2) are important to studies of wine oxidation chemistry and microbial stability. These so-called "free SO_2" forms are traditionally measured by either iodometric titration or by aeration-oxidation (A-O), or by comparable modern variants. These standard approaches require sample dilution and/or pH shifts, which perturb the equilibrium between bound and free forms, resulting in overestimation of free SO_2 species and the reporting of “apparent” SO_2.

We describe a simple, non-perturbing headspace gas detection tube (HS-GDT) method for measurement of molecular and free sulfur dioxide (SO_2) in wine using commercial industrial safety colorimetric tubes. A syringe is used to sample a wine and to create a closed headspace, which is expelled through the GDT after equilibrium is obtained. Henry's coefficients were constant over ethanol concentrations of 0-17% v/v. The HS-GDT method limit of detection in a model wine (pH 3.56, 12% v/v ethanol) was 0.21 mg/L molecular SO_2, and was linear over 0.29-1.13 mg/L. Good agreement was observed between HS-GDT and A-O for white and blush wines, but molecular SO_2 in red wines averaged 2-fold lower by HS-GDT. The difference in molecular SO_2 values by A-O and HS-GDT was well correlated with estimated molecular SO_2 decrease due to anthocyanin-bisulfite adduct formation ($r^2 = 0.936$), supporting the hypothesis that dissolution of anthocyanin-bisulfite adducts occurs during A-O analysis.

Future work on the relative impact of this molecular SO_2 value relative to the commonly used “apparent” value on wine microbes is recommended.

BIOGRAPHICAL SKETCH

Patricia Howe earned a BS in Fermentation Science from the University of California, Davis (1983) and a MS in Food Science (sensory science emphasis) also from UC Davis (2004). In the times between her educational phases she has worked in the California wine industry, in both production and technical roles.

For Dave, since it was his bright idea.

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PREFACE

A version of Chapter 1 has been accepted for publication on 2/13/2015 as: Patricia A. Howe, Susan E. Ebeler, and Gavin L. Sacks. A review of thirteen years of CTS Winery Laboratory Collaborative Data. Am. J. Enol. Vitic. 66: *In Press.* I was the primary investigator, responsible for all major areas of concept formation, data collection and analysis, as well as manuscript composition. SE Ebeler was involved in the early stages of concept formation and contributed to manuscript edits. GL Sacks was involved throughout the later stages of the project and contributed to data analysis, manuscript development and edits.

A version of Chapter 2 has been accepted for publication on 2/24/2015 as: Coelho, J.M., P.A. Howe, and G.L. Sacks. 2015. A headspace gas detection tube method for measurement of SO_2 in wine without disruption of sulfur dioxide equilibria. Am. J. Enol. Vitic. 66: *In Press.* I was a primary co-investigator, responsible for major areas of concept formation, data analysis and manuscript edits. The apparatus for headspace sampling described on pp 65-66 is my work. JM Coelho was primary co-investigator, responsible for concept formation, data collection, analysis, and primary manuscript composition. GL Sacks was supervisory author, involved throughout the project and contributed to concept formation, analysis, manuscript development and edits.

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Chapter 4 is an original, unpublished work of mine, with guidance from my committee. GL Sacks and RW Worobo contributed to concept formation and manuscript edits; R Dando contributed to manuscript edits.

CHAPTER 1: A REVIEW OF THIRTEEN YEARS OF CTS WINERY LABORATORY COLLABORATIVE DATA

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Winery Laboratory Analytical Data

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Abstract

Data from thirteen years (78 wines) of wine industry laboratory proficiency testing were reviewed. After outlier removal, within-laboratory precision (repeatability) and across-laboratory precision (reproducibility) were determined for measurements of alcohol, titratable acidity, volatile acidity, total SO_2, free SO_2, malic acid, specific gravity, pH, residual sugar, glucose plus fructose, and absorbance at 420 and 520 nm. Reproducibility was 3.6 to 57.8 times higher than repeatability.

Reproducibility was evaluated with Horwitz Ratios (HorRat); only alcohol, titratable acid, and total SO_2 had acceptable values (mean HorRat < 2). Measurement z scores demonstrated non-normal distributions, particularly specific gravity, likely due to confounding with density. Reproducibility did not vary significantly over the time period studied, with exceptions: imprecision of ethanol measurements decreased (improved) by 0.0084% v/v per year, while the imprecision of titratable acidity, pH and malic acid measurements increased by 0.0089 g/L as tartaric, 0.0008 pH units, and 0.13 g/L per year, respectively. Reproducibility and repeatability imprecision generally increased with analyte concentration, with notable exceptions of alcohol (both), volatile acidity (reproducibility), and total SO_2 (repeatability). The methods or instruments for alcohol, titratable acidity, free and total SO_2, and volatile acidity changed significantly over the time period. Significant differences were observed among techniques for many analytes, which can be rationalized by attribution to well-known matrix effects manageable in a properly run method; e.g. higher apparent concentrations of alcohol by boiling point methods in high sugar matrices. Evaluation of method accuracy was not possible due to a lack of wine reference materials with known true values. Results demonstrate the need for industry-wide improvement in analytical performance for some assays, and the potential benefit of adopting criteria guidelines for method performance.

Key words: Wine analysis, winery laboratory, proficiency testing, performance criteria, method validation, wine quality, HorRat.

Introduction

Chemical analysis in the wine industry

Winery laboratories run analysis to comply with regulations and to improve or ensure product quality (Amerine and Ough 1980). In the United States, the Department of the Treasury Alcohol and Tobacco Tax and Trade Bureau (TTB) requires that alcohol, total SO_2, and volatile acid levels are within specified limits (Federal Alcohol Administration Act, Internal Revenue Code), and specific gravity may be run as a means to check bottle fill level (Jacobson 2006). However, most routine wine analyses are performed to evaluate wine quality through measurement of compounds associated with spoilage, stability, or sensory properties (Amerine and Ough 1980). These parameters are measured using analytical methods that are identical to or derived from published methods in the AOAC official methods (AOAC 2012), many of which are described in popular wine analysis texts (Amerine and Ough 1980, Iland et al. 2004, Jacobson 2006, Zoecklein et al. 1994). The TTB recommends but does not require that wineries use either the AOAC methods or the methods used by their laboratory (Alcohol and Tobacco Tax and Trade Bureau 2010), and the wine industry has thus operated under the "results driven" or "fitness for purpose" principle rather than on dictated methods. "Fitness for purpose" requires that a method has accuracy and precision appropriate to the application; one method for determining precision is to test similar samples across multiple labs to evaluate both across-method and across-laboratory errors (Garfield et al. 2000, Wernimont and Spendley 1985). Collaborative testing also offers an opportunity to evaluate the analytical proficiency

of a laboratory or individual, regardless of the method used (ISO 2005), which is a necessary aspect of a multi-faceted laboratory quality program (Butzke and Ebeler 1999).

Analytical performance terminology

Several references (Butzke and Ebeler 1999, Garfield et al. 2000, Horwitz and Albert 2006) discuss the terminology of analytical performance. The collection of data to evaluate the analytical performance of individuals, laboratories, methods, or equipment is referred to as *validation*. Validation studies can potentially provide information about the *accuracy* – the closeness to the true value; *precision*- the expected and normal scatter of results around the target; *linearity* – the accuracy and precision change with increased concentrations; *range* – the concentrations of analyte which can be properly tested; *matrix effects* – the presence of other sample components that alter the sensitivity of the method to the analyte; *limit of detection* – the smallest concentration or amount of the analyte that can be detected; and *limit of quantification* – the minimum analyte concentration or amount that can be accurately quantified. Of these, *precision* – the agreement of a set of results – is of particular importance for evaluating the performance of analytical methods or individuals. As *precision* describes agreement, it is inversely related to the standard deviation (SD). The term *imprecision* is used to describe clearly how an increase in standard deviation impacts this agreement; *imprecision* increases as standard deviation increases, while *precision* decreases. Precision measurements can be classified as follows: *repeatability*– the variation seen from the same analyst running the same sample

within a short time frame using the same method/equipment/material, which should have the lowest variation; *replicatability* or *within-lab reproducibility* –changing at least one of these previously controlled variables, and *reproducibility* –altering many or all of these variables, resulting in what should be the most inconsistent situation and the maximum variation in precision (Butzke and Ebeler 1999, Garfield et al. 2000, Horwitz and Albert 2006). *Reproducibility* and across-*laboratory precision* are interchangeable terms (Garfield et al. 2000). A useful and common expression of the precision is the *coefficient of variation* (CV), which is the standard deviation divided by the average value, or the *relative standard deviation* (RSD), which is the CV expressed as a percentage (Garfield et al. 2000). Additionally, there is a concept of analytical "ruggedness" or "robustness", that is, the ability of a method to tolerate common variations in technique, materials, or operating conditions both within and between laboratories, and still deliver good precision (Garfield et al. 2000).

Collaborative laboratory data in the wine industry

Early collaborative wine analysis studies were primarily done in conjunction with the Association of Official Analytical Chemists (AOAC) (Caputi and Wright 1969, Vahl and Converse 1980) or by researchers evaluating specific analytes. These studies were short term programs aimed at validating new analytical methods prior to industry acceptance or adoption by the AOAC as Official Methods. Two multi-analyte collaborative proficiency studies were conducted in 1965 and 1975; relevant results include RSDs for alcohol (1.2% in 1965, 5.1% in 1975), total (titratable) acid (1.9% and 7.0%), volatile acid (18.2% and 29.2%), total SO_2 (16.3% and 12.2%), free

SO_2 (45.8% and 20.1%), and reducing sugar (4.5% and 19.6%). pH performance was expressed as standard deviation (0.2 and 0.1 pH units) (Wildenradt and Caputi 1977). Although improvements were observed for some analytes due to implementation of new technology, the authors bemoaned the deteriorating performance for many of the analytes over the 10 year period between studies, which they attributed to sloppy analytical technique.

The Horwitz Ratio (HorRat) for evaluating analytical performance

Beyond providing insight into current analytical performance, data from proficiency testing can also indicate potential room for improvement in across-laboratory reproducibility (AOAC 2012). It is well established that reproducibility (expressed as RSD) for a given analyte increases with decreasing analyte concentration and that this relationship can be empirically described by the Horwitz equation. If the concentration (C) of the analyte is expressed as a dimensionless mass fraction, with aqueous solutions substituting the acceptable concentration factor of g/mL, then the empirically predicted RSD is equal to $2 \times C^{-0.1505}$ (Horwitz and Albert 2006). The Horwitz ratio (HorRat) is the ratio of the observed RSD to the predicted RSD. The use of HorRat values has been adopted by the Association of Analytical Chemists (AOAC), and is in use by agencies such as the US Department of Agriculture and is being evaluated in the European Union. Generally, HorRat values between 0.5 and 2 are indicative of satisfactory industry-wide performance, but the decision on which value to accept can be influenced by regulations, industry

standards, financial or legal risks, or analytical costs needed to improve the ratio (Horwitz and Albert 2006).

History and description of the Collaborative Testing Services (CTS) program

The CTS program has evolved over the years in response to technical and consumer feedback, but the overall concept has remained constant: two different wines of similar analytical composition are sent to subscribing laboratories, and each laboratory analyzes the two wines using the procedures that are in use at that facility; each laboratory reports their results within a specified time period in duplicate (to capture within-laboratory variation). Coded results are returned to laboratories; each one can identify their own results but the data from other laboratories remains anonymous. Reports contain Comparative Performance Values (CPVs), which are z-scores of individual laboratory results as compared to the overall mean ("Grand Mean") of all non-outlier results. For each analyte, results from the two wines are graphed on a two sample Youden plot, which allows laboratories to readily evaluate systematic and random errors for each analytical measure; tracking that error over multiple cycles can facilitate identification of sources of analytical errors. Data are classified as outliers and excluded from statistics when > 3 standard deviations (σ) from the Grand Mean for a given sample. Data are flagged as warnings when they are > 2σ from the Grand Mean for a sample or if a laboratory has exceeded what other laboratories have determined is an acceptable difference in values for the two samples. After a delay of some months, the coded results from each round are accessible to the public on the CTS website (www.collaborativetesting.com). As of 2014, routine

testing parameters include alcohol, titratable acidity, pH, specific gravity, volatile acidity, free SO_2, total SO_2, residual sugar, glucose plus fructose, malic acid, absorbance at 420 and 520 nm, and copper.

Some of the changes in the CTS program since its beginning in 1999 include reducing the number of annual cycles from four to three in 2001, reclassifying the "residual sugar" measurement into two separate tests based on significant differences in target analyte ("residual sugar" and "glucose plus fructose") in Cycle 19 in 2005, adopting recommended units (Burns and Caputi 2002) in Cycle 22 in 2006, establishing a pattern of red, wine, and blush wines as matrices in Cycle 23 in 2006, and addition of standard parameters of absorbance at 420 and 520 nm in 2009 and of copper in 2011.

This publicly available CTS data provides a good opportunity for evaluating industry and methodological performance, but only a few testing cycles have been evaluated. A thorough review of approaches to laboratory quality and an introduction to the first cycle of results (Butzke and Ebeler 1999) also set a high analytical performance goal, stating that commercial wine production should target a reproducibility (across-laboratory RSD) of 1% following outlier removal, as described above. These authors note that only pH and alcohol methods approached this criterion. Little improvement was noted after the first six cycles of the program (Butzke 2002), with only alcohol, specific gravity, and titratable acidity measurements deemed adequately reproducible. Poor reproducibility was noted for residual sugar, volatile acidity, malic acid, pH, and free and total SO_2 analyses. The author also noted impact of the combined errors of pH and free SO_2 on the calculated value for molecular SO_2.

Unlike earlier researchers, no explanations for poor reproducibility were suggested, although improved performance in titratable acidity as compared to earlier collaborative testing was attributed to increased use of autotitrators.

In this report, we review the first 13 years of data from the CTS program. As with earlier reports on collaborative testing, we present the overall performance for specific analytes and the bias and reproducibility of individual methods. By normalizing individual analytical results for each individual wine sample we are also able to compare results across all wines (and thus, all years), providing a more complete picture of ongoing performance despite individual wine sample chemistries over the span of more than a decade. We also evaluate potential space for analytical improvements based on HorRat ratios. Finally, the large data set and extended time period of the study allows us to evaluate the relative impact of method selection on analytical performance

Materials and Methods

The raw analytical data on each of the two wines from the CTS program Cycle 2 to Cycle 40 (Spring 1999 to Spring 2012) were entered in Microsoft Excel for eleven parameters (alcohol, titratable acidity, pH, free SO_2, total SO_2, malic acid, volatile acidity, residual sugar (combined methods), residual sugar (post method separation), glucose plus fructose, specific gravity, and absorbance at 420 nm and 520 nm). When the CTS raw data included laboratory-specified method or instrument information, it was preserved for each retained data point, as was the wine color (red, white, or blush) and grape variety (when specified). Additionally, information obtained from the

industry supplier of the wines provided data on the use of sorbate in some wine samples, primarily blush wines. These data (red, white, blush, and sorbate-positive) were classified as dummy variables.

Laboratories were asked to report results in duplicate; averages of the two values are considered one data point, and the standard deviation of the two replicates was preserved for later within-lab standard deviation (repeatability) calculations, unless the original data was deemed an "outlier", in which case it was removed.

Data were expressed in recommended standard units (Burns and Caputi 2002), with data from cycles prior to 2006 being converted to these standard units when necessary. In the 2005 cycle, residual sugar was divided into residual sugar and glucose plus fructose, with the glucose plus fructose data being analyzed separately after this time. pH results were converted to molar activities of hydrogen ions and analyzed both as pH values and as hydrogen ion activity values, as logarithmic values are not well suited to statistical operations.

Outliers were removed by repeated application of the four-sigma rule. Within each cycle and for each wine, mean values and standard deviations (σ) for each analyte (across all methods) were calculated and results that differed from the mean by more than four sigma ($|\text{observed} - \text{mean}| > 4\sigma$) were removed. This process was iterated until no values were $> 4\sigma$ from the mean. This criterion was less stringent than the three-sigma criterion used by CTS for outlier detection for the reason that we wanted to better characterize existing analytical variation. The primary effect of outlier removal in our current study was to eliminate gross errors arising from outside

of the analytical method such as those due to errors in data entry, unit conversion, "powers of ten", or related transcription issues.

Following outlier removal, descriptive statistics: mean, standard deviation (within-laboratory and across-laboratory), and relative standard deviation were calculated for each for each analyte of the 78 individual wine samples.

Mean values for each data point for each individual wine were used to calculate z scores for all individual analyses of that wine, rather than using a grand mean across all 78 wines. The resulting individual wine-weighted analyte z scores were compared across all wine samples to give the complete distribution of the targeted analyte results across all wines used in the 39 testing cycles for a maximum *n* of 78. The purpose of this approach was to find the variation of the imprecision of the analyses, rather than to find the variation of wine composition. These data were intended to show the overall industry performance for each analyte regardless of method or instrument used.

Histograms and linear regression of these individual-wine z score values of analyte means and their corresponding within- and across-laboratory standard deviations were made for comparison of imprecision. Additionally, both within-laboratory and across-laboratory SDs and RSDs were compared to the analyte concentration to provide information about the concentration dependence of the imprecision for each analyte.

Concentration factors for the Horwitz equation were calculated using mean analyte concentration values for each individual wine, and HorRats determined for titratable acidity, alcohol, free and total SO_2, volatile acidity, malic acid, and the sugar

measurements: residual sugar (prior to 2005, when all sugar methods were not distinguished), glucose plus fructose (after methods were split in 2005), and residual sugar (after methods were split in 2005).

To characterize the wine sample matrices, one-way ANOVA was performed with dummy variables (red, white, and blush) against analyte mean values. To represent the covariance of the analytes and the matrices, principle component analysis (PCA) of the analyte z scores and a dummy variable of sorbate addition was performed. To evaluate if wine matrix or composition contributed to imprecision, Pearson's correlations were run using average analyte values and dummy variables (red, white, blush, and sorbate-positive) against the normalized across-laboratory standard deviations. Absorbance at 420 and 520 nm were excluded from the correlations due to their limited data set.

Lastly, to determine if method or instrumentation choices affected reproducibility, individual z scores for every data point, along with self-reported information on method and/or instrumentation, were generated. These data also allowed evaluation of changes in method or instrumentation use over the course of the study. Histograms, linear regression, and ANOVA were made on these individual-analysis data. When needed, the z scores were converted back to analyte concentration equivalents using the appropriate sample mean and standard deviations.

Minitab statistical software was used for chi square analysis, regression analysis, correlations, ANOVA, PCA, Tukey's significant difference, and for plotting histograms (Minitab® 16.2.4, Minitab Inc. State College, Pennsylvania)

Results and Discussion

Participation

Participation in the CTS wine analysis proficiency testing program has ranged from 30 to 77 participants per cycle, with an average of 55 contributing results per cycle. During the first four years of the program, the number of reporting laboratories ranged from 30 to 60; during the past four years this figure has grown to between 60 and 77. The identities of the participating winery laboratories are secret and protected, but it can be assumed that there are three subsets of participating laboratories: those with formally accredited quality control systems (or those with at least sound laboratory quality systems concepts in place), those with few or no laboratory quality systems in place (notwithstanding experienced personnel), and those laboratories with neither training, experience, or knowledge of laboratory quality systems. Anecdotal observations by administrators of the program suggest that some of the latter are occasional "visitors" to the program who participate randomly; this is based on the higher number of outliers seen in the infrequent participants relative to the ongoing participants.

Outlier removal and distribution of remaining data

As expected, the repeated four-sigma outlier removal method identified fewer outliers (2.9% of data) than the three-sigma method used by CTS (10.8%). Malic acid, glucose plus fructose, and specific gravity had relatively higher numbers of outliers, while pH and free SO2 had fewer (χ^2-test, $p<0.05$). No significant correlation was observed between analytical method or instrumentation and likelihood of outlier

removal (χ^2-test, $p<0.05$) for any given parameter. Outliers largely appeared to be due to clerical, mathematical, or unit conversion errors, e.g. a factor of 10 differences between mean and outlier, rather than methodology-specific issues.

Following outlier removal, histograms of the individual-wine weighted z scores for each analyte typically appeared unimodal, although most of the distributions were leptokurtic and many showed left or right skews (Figure 1). The higher kurtosis may be naturally expected from distributions made with 4-sigma data selection. Most striking was a deviation from the unimodal pattern suggesting some potential problems with analytical nomenclature. The bimodal distribution for specific gravity (Figure 1-G) may arise from operator confusion between specific gravity and density; the location of the smaller mode is 0.0018 specific gravity units lower than the mode of the larger distribution, or approximately the error expected if density values were reported rather than specific gravity.

Other deviations from symmetrical distributions may arise from methodological biases in cases where multiple methods exist. For example, the positive skew for the residual sugar (prior to the analytical separation of cycle 19 in 2005) (Figure 1-J) may be because the two primary analytical methods – copper reduction assays and enzymatic assays – have different selectivities, with the former measuring other reducing compounds in addition to fructose and glucose (Amerine and Ough 1980, Iland et al. 2004). This would result in two overlapping distributions, with the enzymatic methods having a slightly lower mean.

Table 1 Average wine analyte values, within-laboratory and across-laboratory SD and RSD, and the ration of the SC sources for 39 cycles of available collaborative testing data.

Analyte	Unit	Total analyses	Number of wines	Mean analyte value	Within-laboratory analyte SD	Within-laboratory analyte RSD	Across-laboratory analyte SD	Across-laboratory analyte RSD	Ratio of across-laboratory analyte SD to within-laboratory analyte SD
Alcohol	% v/v	4228	78	12.07	0.03	0.2%	0.16	1.4%	5.8
Titratable Acid	g/L as tartaric	4162	78	6.08	0.04	0.7%	0.26	4.2%	6.0
Volatile Acid	g/L	4053	78	0.425	0.01	2.9%	0.07	17.5%	5.9
Total SO_2	mg/L	4123	78	86.2	2.05	2.4%	9.14	10.6%	4.4
Free SO_2	mg/L	4225	78	20.0	1.09	5.4%	3.88	19.4%	3.6
Malic Acid	g/L	3172	78	1.27	0.03	2.0%	0.21	16.5%	8.2
Specific Gravity	(20°/20°)	3537	78	0.99922	0.00010	0.01%	0.0012	0.12%	12.6
Residual Sugar (before split)	g/L	1342	34	9.24	0.18	1.9%	2.12	22.9%	11.8
Glucose plus Fructose	g/L	2096	44	18.52	0.25	1.4%	2.69	14.5%	10.6
Residual sugar (after split)	g/L	927	44	19.56	0.28	1.4%	3.58	18.3%	13.0
A 520	AU (1 cm)	708	18	1.433	0.012	0.8%	0.543	37.9%	45.2
A 420	AU (1 cm)	636	18	1.301	0.008	0.6%	0.461	35.4%	57.8
pH		4191	78	3.431	0.007	0.2%	0.042	1.2%	5.9
[H+a]	mole/L	4124	78	4.02E-04	3.83E-05	na	7.04E-06	1.8%	na

This issue resulted in separating residual sugar (Figure 1-K) and glucose and fructose (Figure 1-L) to CTS into different reporting categories after 2005 (hereafter referred to as pre- and post-split). As shown in Table 1, the average residual sugar

concentration was ~ 1 g/L higher than glucose plus fructose concentrations in post-2005 measurements. Both of these post-2005 categories analytes demonstrate more symmetrical distributions than the earlier date, but residual sugar now shows a negative skew, indicating that this terminology is still used inconsistently among some laboratories. Other positively skewed distributions, such as for titratable acidity, volatile acidity, and free SO_2 (Figure 1B, 1C and 1E), likely arise from biases among methodologies, and are discussed in more detail later.

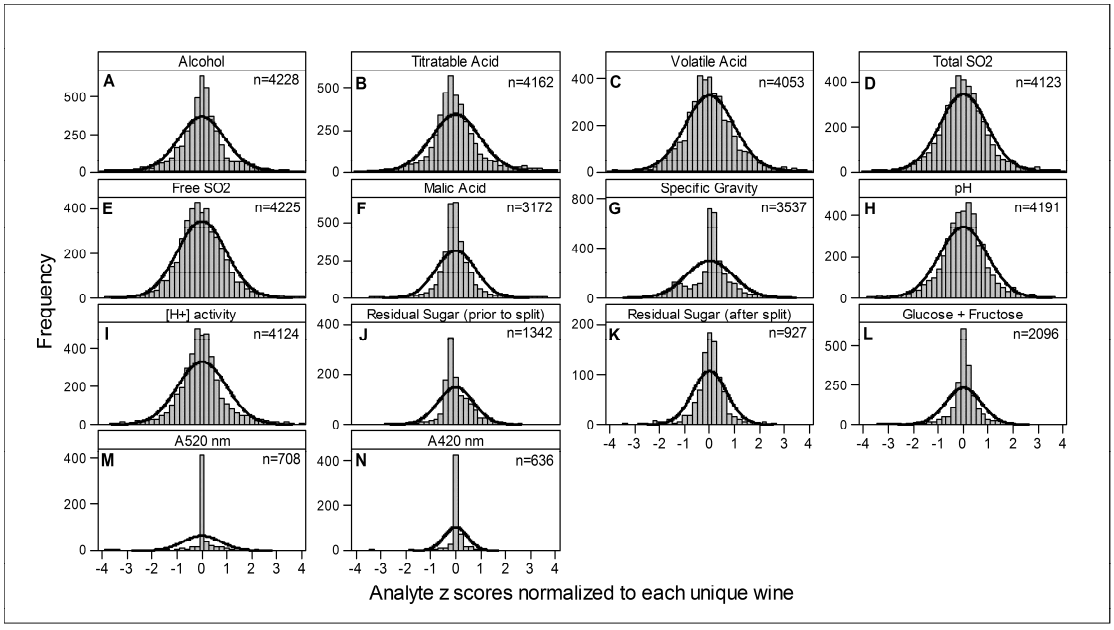

Figure 1 Frequency distribution of individual-wine weighted z-scores for the analysis parameters: A= Alcohol, B= Titratable Acid, C= Volatile Acid, D= Total Sulfur Dioxide, E= Free Sulfur Dioxide, F= Malic Acid, G= Specific Gravity, H= pH, I= Hydrogen Activity Concentration, J= Residual Sugar (all methods prior to split), K= Residual sugar (after split), L= Glucose plus Fructose, M= A520, N=A420

Overall Performance: Standard Deviation and Relative Standard Deviation

To estimate the overall performance for the time course of the study, the individual wine values for both the mean (μ) and the standard deviations (σ) of the analytes were averaged across all wines, and a calculation of reproducibility was expressed as a percentage (σ/ μ), the across-laboratory relative standard deviation, RSD. Within-laboratory precision data was calculated by CTS from duplicate analyses for each wine and also reported as SD and RSD (Table 1). Generally, reproducibility was comparable to results from collaborative studies of earlier decades: alcohol RSDs of 1.2 % and 5.1 % (earlier studies) vs 1.4 % (current study); titratable RSDs of 1.9% and 7.0% vs 4.2 %; volatile acid RSD's of 18.2% and 29.2% vs 17.5 % ; reducing sugar RSDs of 4.5% and 19.6% vs 18.3%; and pH standard deviations of 0.2 and 0.1 vs 0.042 (for the 1965, 1975, and current study, respectively)(Wildenradt and Caputi 1977). CTS testing is open to any interested laboratory, while these earlier collaborative studies used specific, pre-selected participants (Wildenradt and Caputi 1977). One possible explanation for the apparent lack of improvement in reproducibility could be from the less restricted approach to winery laboratory inclusion.

The across-laboratory reproducibility can be compared with the within-laboratory repeatability to show the relative impact of typical sources of imprecision. In every case the across-laboratory values are, as expected, higher, ranging from approximately three to eight times (for most analytes) to greater than ten times the within-laboratory variation (Table 1). The very high relative variations in the sugar, malic acid, and absorbance at 420 and 520 nm results, all of which are primarily (but

not exclusively) tested using spectrophotometric methods, indicate the possibility that spectrophotometer calibration might be responsible for some of these across-laboratory performance issues; laboratory-to-laboratory variation in these instrument calibrations could explain the lower within-laboratory error and the very high across-laboratory error. Other instrument-based errors related to these analytes could include issues with pipet calibrations, and selection or availability of different cell path lengths, or calibration data set of infrared spectrophotometers. Method-based errors involving incomplete or slow enzymatic reactions, or impact of dilutions, may also be a factor. Calibration of equipment, use of standards and blanks, and testing the method using different samples sizes/concentrations are all ways to locate these systematic errors (Skoog et al. 1992).

Horwitz Ratios applied to wine collaborative data

Horwitz ratios (HorRat) represent the ratio of observed to empirically predicted precision, and may be calculated from the analyte concentration. Mean and standard deviation HorRat values across all individual wine samples for the various analyses are summarized in Table 2. Typical targets for mean HorRat values recommended by international analytical organizations range from 0.5 to 2.0, and in our study only three parameters achieved this criterion when averaged across all samples: alcohol, titratable acidity, and total SO_2. All other analytes had overall average HorRat values >2.0: free SO_2, malic acid, volatile acid, and all the analytical methods for sugar measurements. It is not possible to calculate the Horwitz value for pH, specific gravity, or absorbance because the values are not concentrations.

Individual HorRat values for each individual wine over the course of the 39 cycles show that HorRat values are not consistent, and may vary considerably; even most analytes with average HorRat values of <2.0 have occasional samples above this value, represented by the percentage of times the HorRat value is >2.0 for each analyte (Table 2). Only titratable acidity had a HorRat value consistently <2.0 over the 13 year period; other analytes have HorRat values >2.0 more that 79% of the time (volatile acid, malic acid, and all the analytical methods for sugar). Actual HorRat values for analyses of particular economic or regulatory importance can be well below the maximum recommended level (2.0); in the measurement of milk fat and solids in the dairy industry, inter-laboratory testing has resulted in HorRat values of 0.1-0.4, possibly reflecting the great economic importance of these analytes (Horwitz and Albert 2006). For the wine industry, the analyte with the greatest economic impact may be alcohol, due to the critical maximum value of 14.04% for the lower "table wine" tax class (Alcohol, Tobacco Products, and Firearms) of $1.07 per gallon versus $1.57 per gallon if between 14.05% and 21% alcohol (Internal Revenue Code). Although alcohol is one of the three analytes with an average HorRat across all 78 wine samples of <2.0, it nevertheless had 10% of wine samples with HorRat values above the maximum recommended acceptable HorRat value. Total SO_2, another regulated analyte, had only 8% of the 78 wines with HorRat values >2.0. Volatile acidity, although also regulated, had an average HorRat of 3.0 and 79% of the individual wine samples with values above this. The highest HorRat values were found with malic acid and with all the methods for analyzing sugar; although these are not regulated components, they have significant impact on the taste and stability of the

wines, and improvement would be recommended. To our knowledge, this is the first time that the HorRat concept has been applied to wine analysis, and yields interesting insights for the potential for further improvements.

Table 2. HorRat values for wine collaborative testing from 1999-2013

Analyte	n	HorRat Average	HorRat SD	% samples with HorRat>2.0
Titratable Acid	78	1.0	0.3	0%
Total SO2	78	1.4	0.5	8%
Alcohol	78	1.4	0.5	10%
Free SO2	78	2.0	0.8	36%
Volatile acid	78	3.0	1.2	79%
Malic acid	78	3.8	2.0	86%
Glucose plus fructose	44	3.9	2.0	91%
Residual sugar (all methods before split)	34	5.4	3.0	98%
Residual sugar (after split)	44	7.4	4.1	100%

Analytical methods used

Wine laboratories self-reported the methods or instruments used beginning at the eighth cycle. The percentage of data points used for each technique demonstrates the range of methods and instruments used over the study (Table 3). Most of the methods, techniques, or instruments listed are mentioned or described can be found in common texts (Amerine and Ough 1980, AOAC 2012, Iland et al. 2004, Jacobson 2006, OIV 2004, Zoecklein et al. 1994). Although the versions published in these texts are similar, they are rarely if ever identical; therefore, use of any of these self-reported method descriptors does not imply that a specific protocol or instrument model was used.

Table 3. Percentage of self-reported methods or instruments used for each analyte.

Target Analyte	Self-reported method/instrument	Total % reported
Alcohol	Near Infrared	36.5%
	Ebulliometer	14.8%
	Gas Chromatography	12.1%
	FTIR	11.6%
	Unassigned	11.6%
	Distillation/density	11.2%
	Other	2.1%
	Dichromate	0.2%
Malic Acid	Enzymatic	78.3%
	Unassigned	12.4%
	FTIR	5.2%
	Capillary Electrophoresis	1.6%
	HPLC	1.5%
	Segmented Flow	0.9%
pH	Unassigned	100.0%
	Manual pH Meter	0.0%
	Automated pH Meter	0.0%
	FTIR	0.0%
	Other	0.0%
Free SO2	Aeration Oxidation	43.6%
	Ripper	26.6%
	Unassigned	13.3%
	Colorimetric	6.4%
	Segmented Flow	5.1%
	Flow injection analysis	3.8%
	Enzymatic	1.1%
Total SO2	Ripper	45.6%
	Aeration Oxidation	23.3%
	Unassigned	13.2%
	Colorimetric	6.6%
	Segmented Flow	5.4%
	Flow injection analysis	4.4%
	Enzymatic	1.2%
	FTIR	0.4%
Titratable Acid	Autotitration	46.9%
	Manual titration	33.0%
	Unassigned	11.8%
	FTIR	7.8%
	Segmented Flow	0.6%
Volatile acid	Cash still	46.3%
	Enzymatic	21.1%

Wine matrix composition

To characterize the typical composition of the sample matrix (red, white, or blush) for the wines used over the course of the study, the three matrices were classed as dummy variables and analyzed by one-way ANOVA for each analyte and for the known addition of sorbate (as a dummy variable). Tukey's differences demonstrate statistically significantly differences for each analyte, and provide a view of matrix typicity for this set of wines (Table 4). Red wines had significantly higher (and blush wines significantly lower) levels of volatile acid, pH, and free SO_2, while red and white wines were significantly higher than blush wines for alcohol. Red wines were significantly higher in absorbance at 420 and 520 nm than white or blush wines. Blush wines were significantly higher (and red wines significantly lower) for total SO_2, malic acid, hydrogen ion activity, residual sugars (after the split), and glucose plus fructose. Blush wines were significantly higher than both red and white wines for titratable acid, specific gravity, and residual sugars (prior to split). These data characterize the composition of the wines used in this study and may or may not be typical of these matrices in the broader sense. Principle component analysis of the matrices using the z scores of means of these analytes (excluding the absorbance values due to incomplete data, and using a combined before/after split of sugar analyses to achieve coverage of all samples) plus a dummy variable of sorbate addition were also performed (Figure 2), and indicate the covariance of many of the analytes, as might be expected. The primary separation is of red wines and blush wines on the first component, explaining 64% of the variation; red wines are associated with higher volatile acid, pH, and alcohol; blush wines are associated with

higher titratable acid, malic acid, total SO_2, specific gravity, sugar (by combined methods) and the addition of sorbate. The second component explained 11% of the variation and was primarily associated with free SO_2. Neither component was able to separate the white wines from the red and blush wines; presumably this would occur if the absorbance at 420 and 520 nm data could be included, as color may be the primary analytical difference between white and non-white wines in this group of samples.

Table 4. Wine matrix (red, white or blush) average composition for the 78 wines tested. Tukey's significant differences from one-way ANOVA of analyte mean values and dummy variables ($p<0.05$)

	Analyte/units	Grand mean	Matrix	n	Mean matrix value and Tukey's significance level groupings	
Red>White>Blush	Volatile Acid (g/L)	0.43	red	29	0.54	A
			white	32	0.41	B
			blush	17	0.26	C
	pH	3.43	red	29	3.56	A
			white	32	3.44	B
			blush	17	3.18	C
	Alcohol (%v/v)	12.1	red	29	13.0	A
			white	32	12.5	A
			blush	17	9.7	B
	Free SO_2 (mg/L)	20	red	29	22	A
			white	32	20	A B
			blush	17	16	B
Red>Blush>White	A520 (1 cm)	1.43	red	6	3.98	A
			blush	8	0.22	B
			white	4	0.02	B
	A420 (1 cm)	1.30	red	6	3.45	A
			blush	8	0.29	B
			white	4	0.09	B
Blush>White>Red	Total SO_2 (mg/L)	86	blush	17	108	A
			white	32	88	B
			red	29	71	C
	Malic acid (g/L)	1.27	blush	17	2.4	A
			white	32	1.6	B
			red	29	0.2	C
	$[H^+]_a$ (m/L)	4.0×10^{-4}	blush	17	6.6×10^{-4}	A
			white	32	3.8×10^{-4}	B
			red	29	2.8×10^{-4}	C
	Residual sugars after split (g/L)	19.6	blush	14	32.1	A
			white	14	21.4	A
			red	16	7.0	B
	Glucose + fructose (g/L)	18.5	blush	14	31.5	A
			white	14	20.1	A
			red	16	5.0	B
	Titratable Acid (g/L)	6.1	blush	17	6.7	A
			white	32	5.9	B
			red	29	5.9	B
	Specific gravity (20°/20°)	0.999	blush	17	1.008	A
			white	32	0.998	B
			red	29	0.996	B
	Residual Sugars prior to		blush	3	34.1	A

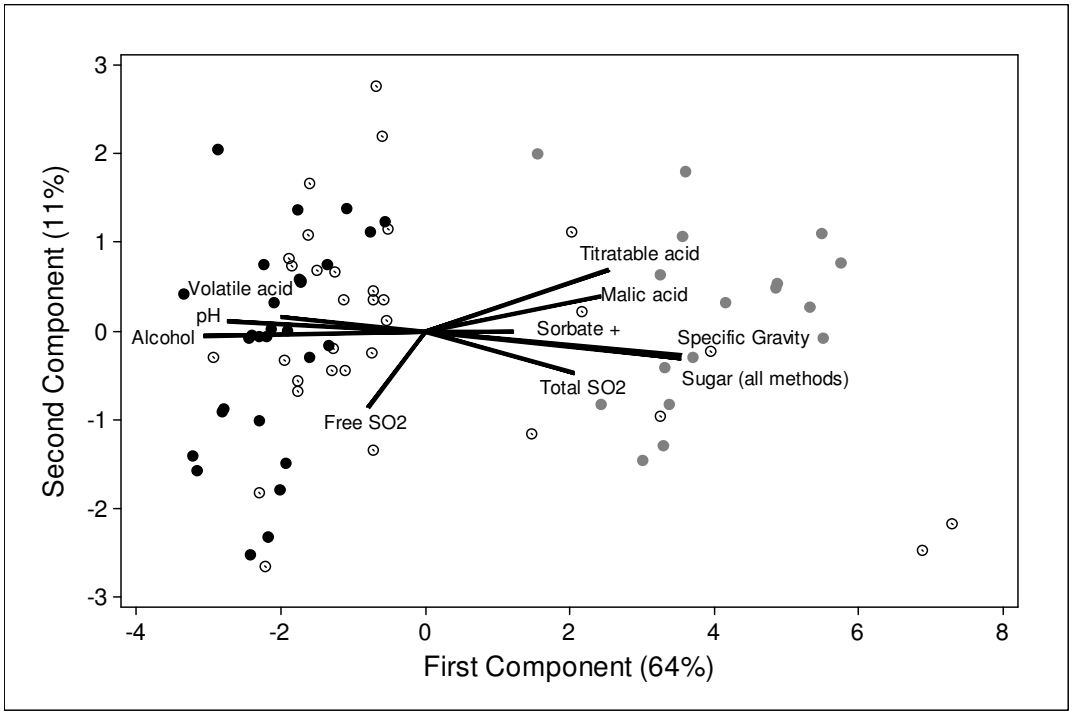

Figure 2 Principal components of wine matrix using mean analyte composition for the 78 wines tested (○=white wines, ●= blush wines, ●= red wines)

Wine matrix and analytical composition effects on imprecision

To evaluate the impact of wine matrix/composition on analytical performance, the dummy variables of wine matrix (red, white, and blush) along with the z scores of the analyte composition were compared against the across-laboratory imprecision using Pearson's correlation (Table 5). The matrices of red and blush wines had significant impact on the precision for malic acid, hydrogen ion activity, and all measures of sugar with red wines decreasing (improving) the imprecision for these analytes while blush wines increased (worsened) the industry performance. The blush wine matrix increased (worsened) the imprecision for volatile acid and titratable acid. Notably, the white matrix had no impact on any analytical imprecision. Individual

analytical components also had significant correlation with reproducibility for all the analytes, with the notable exception of alcohol. As alcohol is the analyte with the best performance characteristics as discussed above, this is not unexpected. Several analytes (titratable acid, free SO_2, total SO_2, malic acid, hydrogen ion activity, and all the sugar analyses) showed concentration effects and will be discussed separately. Specific method bias will also be discussed later. In some cases, the correlation between matrix composition and precision for an analyte may indicate a causative effect, although in other cases the correlation may be due to covariance. For example, across-laboratory imprecision of volatile acidity analysis increased with increasing specific gravity, malic acid, total SO_2, and titratable acid levels. Volatile acidity imprecision also increased in blush wines and those with added sorbate; imprecision decreased as alcohol levels increased; there was no significant impact of volatile acid concentration. Of these, sorbate and total SO_2 are well known to interfere in the determination of volatile acidity using the Cash steam still method (Zoecklein et al. 1994). This factor is discussed more in the section on methodological bias. Because the IR spectra of acids and sugars are similar, volatile acid analysis by IR methods can be affected by any other acid and by sugars, especially if the target analyte concentration is approaching the IR limit of detection of 0.2 g/L (Bauer et al. 2008). Titratable acidity imprecision increased with sugar levels (using any parameter), specific gravity, total SO_2, malic acid, hydrogen ion activity. Titratable acid imprecision also increased in blush wines and those with added sorbate; imprecision decreased with volatile acidity, alcohol, free SO_2, and pH. Known interferences with the titratable acid methods include the endpoint determination and the presence of

carbon dioxide for titration based methods (Guymon 1963). While it is possible that these parameters are interferences that somehow increase or decrease imprecision, it appears more likely that these parameters co-vary with titratable acid (Figure 2) and error in titratable acidity measurements by titration increases with concentration. IR based methods for titratable acid have similar issues, as samples must be degassed, and both sugar and other acids can interfere with the measurements (Bauer et al. 2008). Industry imprecision for free SO_2 increased with total SO_2 and with free SO_2, showing an effect of concentration; total SO_2 imprecision increased with total SO_2, and also with volatile acidity. Volatile acidity is known as a potential interference when determining total SO_2 with the aeration- oxidation method (Rankine and Pocock 1970). Malic acid industry imprecision increased in blush wines and with sorbate additions, and with increasing levels of titratable acid, total SO_2, specific gravity, all measures of sugar, hydrogen ion activity, and with malic acid (showing a concentration effect); all these factors are typical of wines which do not undergo malic conversion as discussed earlier in regard to the matrix (Table 4); further, malic acid imprecision decreased with increasing pH, free SO_2, alcohol, volatile acid, and in the red wine matrix; as all these are typical characteristics of wines which undergo a malolactic conversion, their correlation with malic acid imprecision is more likely due to covariance rather a direct causative effect. The industry imprecision of pH and of the related hydrogen ion activity is interesting, as the only significant correlation with increasing pH imprecision is with a decrease in volatile acid. Yet, hydrogen ion activity imprecision increased with increasing titratable acidity, total SO_2, malic acid, specific gravity and all the measures of sugar, in addition to increasing hydrogen ion

activity (a concentration effect). Hydrogen ion activity imprecision also increased in blush wines and with added sorbate; the imprecision decreased in red wines and with increasing volatile acidity and alcohol (and pH). These correlations suggest that hydrogen ion measurement precision is concentration dependent, and that the analyses performed by the industry are less precise at lower pH values (i.e., higher hydrogen ion activities). This result is discussed further below. The imprecision experienced by the industry for the specific gravity measurements increased in wines with sorbate added and decreased in wines with higher volatile acid, possibly indicating that the sweeter blush wines introduced more imprecision than did red wines. Finally, all industry measurements of sugar showed the same correlations with imprecision: all sugar imprecision increased with increasing titratable acid, total SO_2, hydrogen ion activity, and specific gravity; all showed increasing imprecision with increasing concentration of sugar (a concentration effect), and all had imprecision increase with blush wines and with sorbate additions. All sugar measurements saw decreases (improvements) in imprecision with increases in pH, free SO_2, volatile acidity, and in the red wine matrix; all these factors may indicate that concentration is a primary factor and these other parameters are covariates with the sweeter blush wines. All data are shown in Table 5.

Table 5. Pearson Correlation of wine matrix and composition effects on across-laboratory imprecision (inclusive of all methods) for the individual wines. Coefficients shown are significant at p<0.05. A420 and A520 excluded due to smaller data set. N= 78 for all except residual sugar before split (n=34), residual sugar after split and glucose plus fructose (n=44).

		Normalized Across-Laboratory Standard Deviations											
		Volatile acid SD	Alcohol SD	Titratable acid SD	Free SO_2 SD	Total SO_2 SD	Malic acid SD	pH SD	$[H^+]_a$ SD	Specific Gravity SD	Residual sugars (before split) SD	Residual sugars (after split) SD	Glucose plus fructose SD
Dummies	Red						-0.59		-0.58		-0.33	-0.42	-0.60
	Blush	0.32		0.37			0.59		0.73		0.49	0.51	0.56
	White												
Normalized Average Levels for all wines	Sorbate positive	0.31		0.33			0.50		0.74	0.26	0.65	0.61	0.83
	Volatile acid			-0.26		0.41	-0.51	-0.28	-0.68	-0.31	-0.46	-0.45	-0.59
	Alcohol	-0.39		-0.44			-0.63		-0.72		-0.56	-0.49	-0.71
	Titratable acid	0.31		0.41			0.46		0.49		0.27	0.26	0.46
	Free SO_2			-0.26	0.31		-0.33				-0.26	-0.46	-0.49
	Total SO_2	0.43		0.35	0.28	0.27	0.54		0.56		0.31	0.27	0.43
	Malic acid	0.26		0.31			0.81		0.54				0.52
	pH			-0.30			-0.52		-0.92		-0.65	-0.59	-0.64
	$[H^+]_a$			0.31			0.50		0.91		0.71	0.66	0.66
	Specific Gravity	0.26		0.36			0.40		0.69		0.66	0.59	0.74
	Residual sugars (before split)			0.29			0.39		0.72		0.67	0.60	0.75
	Residual sugars (after split)			0.36			0.52		0.67		0.60	0.60	0.75
	Glucose plus fructose			0.36			0.53		0.68		0.60	0.60	0.75

Performance over time

Changes over time in the analytical performance of the subscribing labs show a variety of historical trends. Over the course of the 13 years of data, alcohol analysis results have significantly ($p<0.01$) improved in precision, with an average decrease in standard deviation of 0.0028 %v/v per cycle (Figure 3a); alcohol RSDs also showed significant improvement (Figure 3e). As described later, this increased precision in alcohol analysis may be due to the adoption of infrared based methods for alcohol measurements. In contrast with the improved precision of alcohol analysis, when regressing the standard deviation or the RSD per wine over the 39 cycles, significant ($p<0.01$) loss in performance (increased imprecision) was found for both titratable acidity and pH (Figure 3b, 3c, 3f, 3g) with the average increase in standard deviation of 0.0027 g/L as tartaric or 0.0003 pH units per cycle, respectively. Malic acid imprecision (as standard deviation) also increased significantly ($p<0.05$), at a rate of 0.0044 g/L malic per cycle, yet the RSD for malic did not change significantly over the same period (Figure 3d), indicating that the loss in precision may be a proportional error. This loss in performance for titratable acidity, pH, and malic acid is surprising, as increases in technology (specifically, the increase in the use of autotitrators) have previously been assigned responsibility for increased precision (Butzke 2002). No other significant improvements or loss in performance were found. However, the variation in reproducibility for the same parameter across multiple cycles was striking. For example, reproducibility for titratable acidity varied from 0.15 - 0.5 g/L across all cycles (RSD range, 2-7%). Similar effects were observed in other parameters (supplementary material). In some cases, cycle-to-cycle variation may arise from wine

matrix effects as discussed earlier, but could also be due to variability in participating wineries. Regardless, this observation cautions against using the results of a single testing cycle to draw conclusions about methodological or laboratory performance.

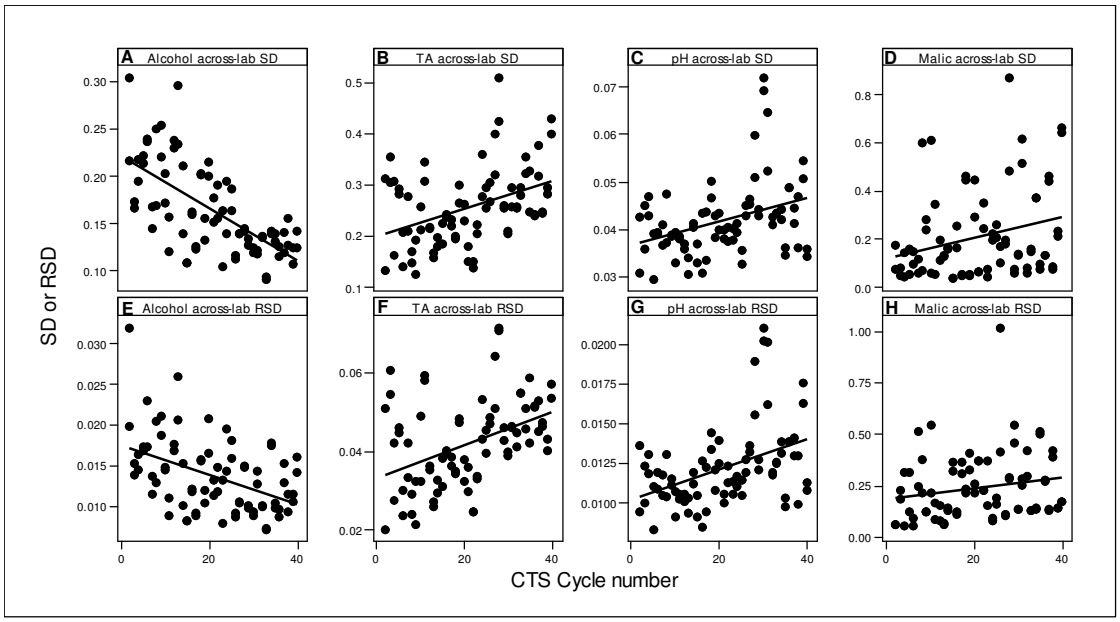

Figure 3 Across-laboratory precision (reproducibility) over the time course of the thirty nine cycles for alcohol, titratable acid, pH, and malic acid. (A= alcohol standard deviation (SD, %v/v), B= titratable acid SD (g/L), C= pH SD, D= Malic acid SD (g/L), E=alcohol relative standard deviation (RSD = SD/concentration), F= titratable acid RSD, G= pH RSD, H= malic acid RSD). Alcohol SD = 0.22-0.0028x; R^2 =0.44 ($p<0.01$); Alcohol RSD = 0.018-0.0002x; R^2 =0.20 ($p<0.01$). Titratable acidity SD =0.20+0.0027x, R^2 = 0.1642 ($p<0.01$); Titratable acidity RSD = 0.03+.0004x, R^2 = 0.1772 ($p<0.01$). pH SD= 0.037+0.0003x, R^2 =0.13 ($p<0.01$): pH RSD=0.01+0.0001x, R^2=.179 ($p<0.01$); Malic SD= 0.12+0.0044x, R^2= .074 ($p<0.05$): Malic RSD= 0.19+0.003x, R^2=0.03 (ns).

Concentration dependence of imprecision

Evaluating the across-laboratory imprecision against analyte concentration provides information about relative concentration dependence of the errors, that is, whether they are constant or proportional errors, which may indicate how to find and control the sources of variation. For example, systemic errors such as those introduced from titration endpoints have an error which is constant, but have a relative

error which varies when sample size is changed. In these cases, the SD/concentration curve would be constant with increasing concentration, while the RSD/concentration curve would reflect this constant error by decreasing with increasing concentration; thus, these constant errors can become problematic with very low concentrations. Similarly, interfering substances in IR measurements would behave as a constant, compounded at lower target analyte concentrations as the levels approach the lower limit of the methods (Bauer et al. 2008). If the imprecision is a proportional error, then errors introduced by multipliers are likely sources of the error (Skoog et al. 1992), e.g. dilution steps, volume measurements, enzymatic reaction times, or interfering contaminants. In these cases of proportional errors, relative error (RSD) would remain constant with increasing concentration, while the absolute error (SD) would increase with concentration. Alternatively, upper and lower limits of the methods used may have been exceeded without operator knowledge. Within- and across-laboratory standard deviations and relative standard deviations for each wine were plotted against the analyte mean values to determine if measurement imprecision expressed as SD or RSD (absolute or relative error) was concentration dependent. Summary data for slope and correlation coefficients of within- laboratory (repeatability) (Table 6) and across-laboratory (reproducibility) (Table 7) vs. concentration are provided. Several parameters had errors that were correlated with concentration ($p<0.05$ and $R^2>0.7$); correlations were strongest for malic acid and glucose plus fructose (Figures 4 and 5). Both analytes show an increasing SD with increasing concentration indicating a proportional error (Figures 4a, 4b, 5a, and 5b). However, the sharp increase in RSD vs. concentrations at low concentrations indicate

the presence of a constant, low level source of error in malic acid analyses at < 0.5 g/L (Figure 4c and 4d) and to a lesser extent with glucose plus fructose (Figures 5c and 5d). The concentration dependent error can be explained by the dilution steps typically necessary for enzymatic/spectrophotometric analysis of malic acid and glucose plus fructose, or, alternatively, by interferences of acids and sugars in IR methods, along with loss of precision as the malic acid approaches the lower limits of detection for this method. The poor reproducibility (average RSD = 35%, max of 100%) for malic acid at concentrations <0.5 g/L may reflect the noise limit of typical methods, and is problematic since malic acid measurements at these concentrations are often necessary to evaluate if malo-lactic fermentation is completed (Butzke 2010). This reduced performance at low malic acid concentrations indicates a need among wineries to review protocols used in the commonly employed enzymatic analysis method (dilution protocols, sample sizes, and enzyme concentrations and reaction times, in addition to checking instrument calibrations), or to be aware of when IR methods are at lower limits. These issues may have been less apparent for glucose plus fructose analyses because no wines under study had concentrations < 2.5 g/L (as compared to the minimum malic concentrations of <0.1 g/L). In contrast, standard deviations for many other parameters were weakly or un-correlated with concentration, such as volatile acidity, titratable acidity, specific gravity, alcohol, and total and free SO_2 measurements. In some of these cases, the results had negative correlations of concentration with RSD, indicative of constant sources of error (Tables 5 and 6). Notably, parameters that display concentration-independent errors were typically analyzed by methods that do not require sample dilution steps, and the critical source

of error may be either constant interferences, challenges in defining the endpoint, or other issues typical of a consistent systematic error. Finally, pH measurement, when evaluated as hydrogen ion activity, shows a concentration dependent error. Speculatively, this may arise from wineries calibrating their pH meters with pH 4 and 7 solutions as typically recommended in wine texts (Iland et al. 2004, Zoecklein et al. 1994)) rather than over the typical pH range of wine (3 to 4), and would provide an interesting avenue for recommended improvements to the method in future work.

Table 6 Concentration dependence of within-laboratory (repeatability) precision. Regression of standard deviation (SD) and relative standard deviation (RSD) against average analyte concentration for individual wines.

			Within Laboratory (repeatability)					
			SD			RSD		
Analyte	unit	n	slope (SD unit/unit change)	R^2	Significance	slope (RSD unit/unit change)	R^2	Significance
Residual sugar (combined)	g/L	42	0.016	0.96	p<.01	ns	ns	ns
Glucose plus fructose	g/L	42	0.012	0.96	p<.01	0.000	0.19	p<.01
A520	1 cm	18	0.009	0.83	p<.01	ns	ns	ns
Malic acid	g/L	78	0.014	0.74	p<.01	-0.019	0.51	p<.01
A420	1 cm	18	0.005	0.72	p<.01	ns	ns	ns
[H+]a	mole/L	78	0.099	0.57	p<.01	ns	ns	ns
Residual sugar (post-split)	g/L	36	0.009	0.53	p<.01	0.000	0.14	p<.05
Volatile acidity	g/L	78	0.012	0.30	p<.01	-0.051	0.55	p<.01
TA	g/L	78	0.010	0.16	p<.01	ns	ns	ns
Free SO2	mg/L	78	0.008	0.06	p<.05	-0.003	0.62	p<.01
Total SO2	mg/L	78	ns	ns	ns	0.000	0.55	p<.01
Alcohol	%v/v	78	ns	ns	ns	ns	ns	ns
pH	pH unit	78	ns	ns	ns	ns	ns	ns
Specific Gravity	(20°/20°)	78	ns	ns	ns	ns	ns	ns

Table 7 Concentration dependence of across-laboratory (reproducibility) precision. Regression of standard deviation (SD) and relative standard deviation (RSD) against average analyte concentration for individual wines.

			Across Laboratory (reproducibility)					
			SD			RSD		
Analyte	unit	n	slope (SD unit/unit change)	R^2	Significance	slope (RSD unit/unit change)	R^2	Significance
A520	1 cm	18	0.396	0.99	p<.01	ns	ns	ns
A420	1 cm	18	0.394	0.96	p<.01	0.060	0.53	p<.01
Malic acid	g/L	78	0.142	0.65	p<.01	-0.091	0.36	p<.01
Residual sugar (combined)	g/L	42	0.186	0.62	p<.01	-0.008	0.17	p<.05
Glucose plus fructose	g/L	42	0.110	0.56	p<.01	ns	ns	ns
[H+]a	mol/L	78	0.014	0.47	p<.01	ns	ns	ns
Residual sugar (post-split)	g/L	36	0.129	0.36	p<.01	ns	ns	ns
TA	g/L	78	0.049	0.17	p<.01	ns	ns	ns
Free SO2	mg/L	78	0.049	0.10	p<.01	-0.010	0.40	p<.01
Total SO2	mg/L	78	0.029	0.07	p<.05	-0.001	0.32	p<.01
Volatile acidity	g/L	78	ns	ns	ns	-0.474	0.58	p<.01
Alcohol	%v/v	78	ns	ns	ns	-0.001	0.23	p<.01
pH	pH unit	78	ns	ns	ns	-0.006	0.17	p<.01
Specific Gravity	(20°/20°)	78	ns	ns	ns	ns	ns	ns

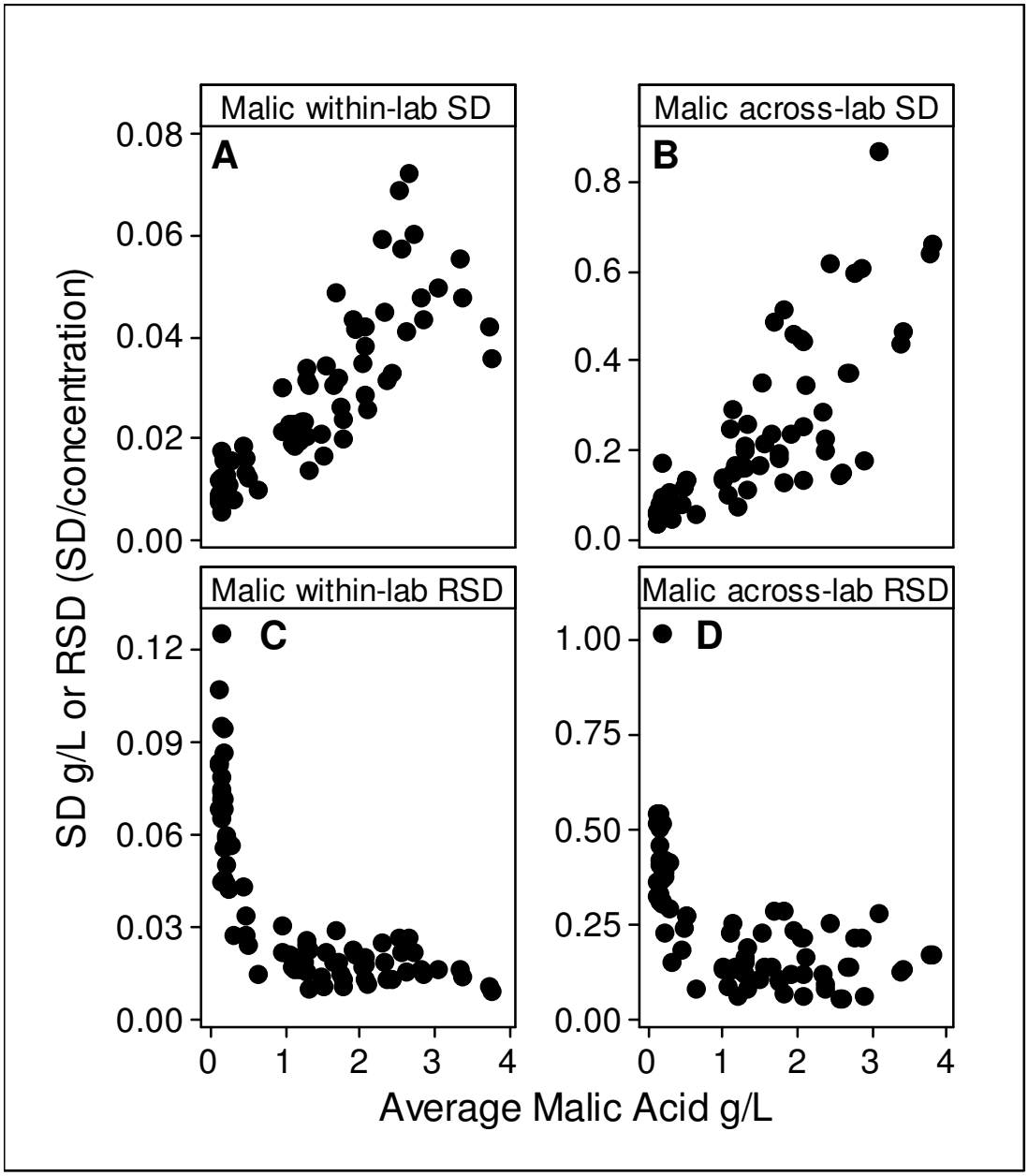

Figure 4 Within-lab and Across-lab Standard Deviation (SD) and Relative Standard Deviation (RSD) for Malic Acid concentration (n=78). A= Within-lab SD, B= Across-lab SD, C= Within-lab RSD and D= Across-lab RSD. SD units are in g/L, RSD is unitless (SD/concentration).

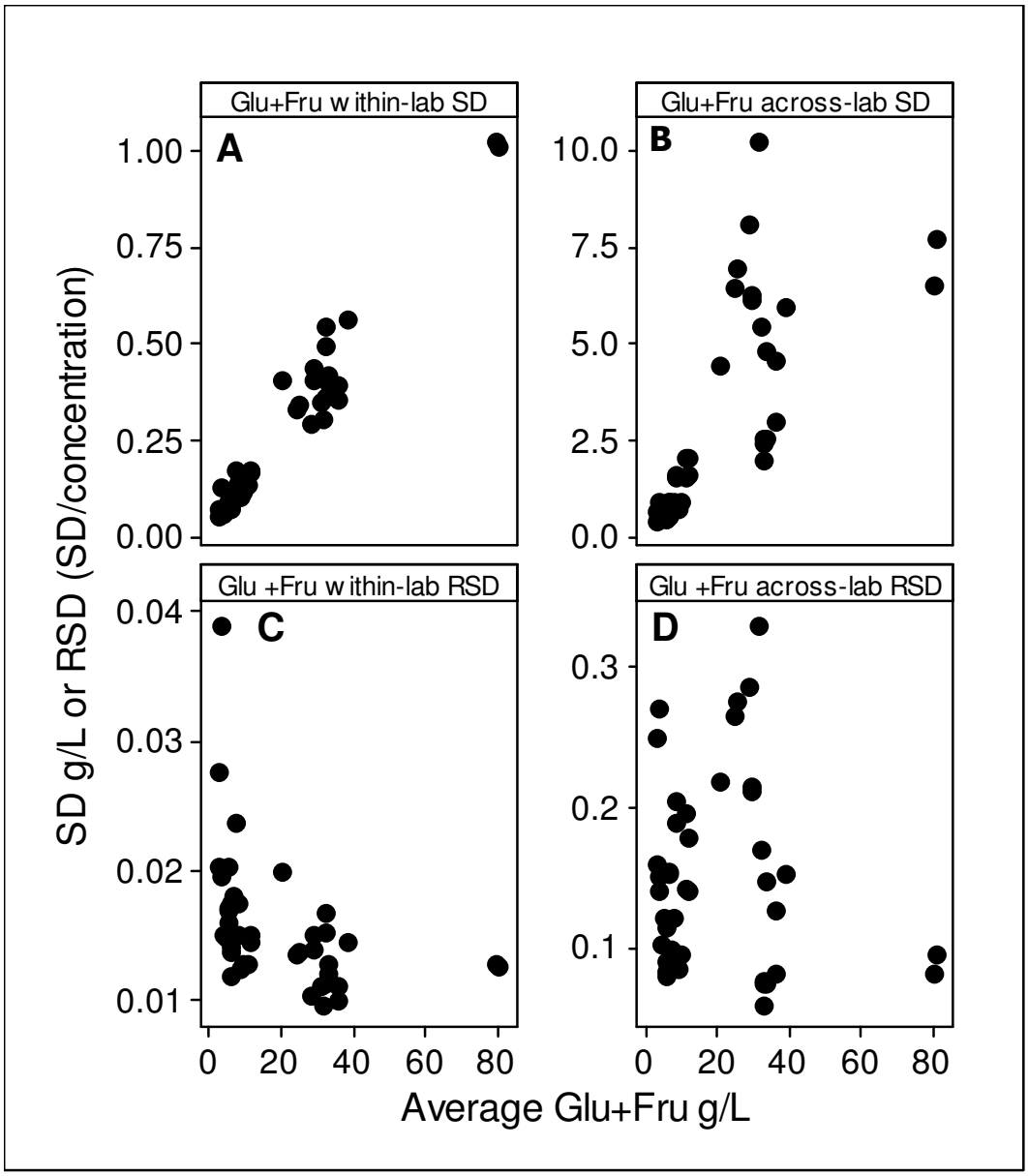

Figure 5 Within-lab and Across-lab Standard Deviation (SD) and Relative Standard Deviation (RSD) for Glucose plus Fructose concentration (n=42). A= Within-lab SD, B= Across-lab SD, C= Within-lab RSD and D= Across-lab RSD. SD units are in g/L, RSD is unitless (SD/concentration).

Changes in method usage over time

Several analytical parameters were analyzed by multiple methods across wineries as mentioned earlier (Table 3). To evaluate if the method usage frequency changed over the course of the study, the percent usage of each method (with usage greater than 2%) was plotted against cycle and trend analysis parameters calculated (Table 8) using least squares regression. Method self-reporting did not begin until Cycle 8. Five analytes showed significant changes in method/instruments over the course of the study: alcohol, titratable acid, volatile acid, free SO_2, and total SO_2. For alcohol analyses, infrared equipment (NIR and FTIR) and distillation/density increased (+0.73, +0.54, and +0.42 % per cycle) at the expense of ebulliometry and gas chromatography (-0.91 and -0.73 % per cycle). For volatile acidity analysis, Cash still usage declined by 0.95% per cycle, as enzymatic methods (for acetic acid), segmented flow, and FTIR use increased (+0.26, +0.26, and +0.47% per cycle). Manual titrations for titratable acid declined (-0.36 % per cycle) as FTIR usage increased (+0.35 % per cycle). Segmented flow, flow injection, and other colorimetric methods increased for free SO_2 analysis (+0.12, +0.36, and +0.19% per cycle) at the expense of aeration-oxidation (-0.42% per cycle). For total SO_2 analyses, segmented flow, flow injection, and other colorimetric methods increased (+0.24, +0.31, and +0.31% per cycle) at the expense of Ripper (-1.0% per cycle). Despite the increase in automation and technology over the course of the study, no increases in precision have been found (except as noted for alcohol analysis).

Table 8 Changes in self-reported method use over cycle, for cycles 8-40, for methods with >2% average reported use. Regression analysis of percentage reported use of method by cycle number.

Analyte	Self-Reported method used	initial % reported use	average % use change per cycle	R^2	Significance	average % change in reported use over course of study
Free SO2	Aeration Oxidation	51%	-0.30%	0.12	p<.05	-10%
	Ripper	39%	-0.42%	0.29	p<.01	-14%
	Colorimetric	6%	0.19%	0.19	p<.01	6%
	Segmented Flow	3%	0.12%	0.32	p<.01	4%
	Flow injection analysis	0%	0.36%	0.41	p<.01	12%
Total SO2	Ripper	74%	-1.00%	0.81	p<.01	-33%
	Aeration Oxidation	20%	0.08%	0.03	ns	3%
	Colorimetric	6%	0.25%	0.46	p<.01	8%
	Segmented Flow	0%	0.24%	0.63	p<.01	8%
	Flow injection analysis	0%	0.31%	0.76	p<.01	10%
Titratable Acid	Autotitration	59%	-0.01%	0.00	ns	0%
	Manual titration	41%	-0.36%	0.52	p<.01	-12%
	FTIR	0%	0.35%	0.54	p<.01	11%
Alcohol	Ebulliometer	34%	-0.91%	0.90	p<.01	-30%
	Near Infrared	31%	0.73%	0.66	p<.01	24%
	Gas Chromatography	28%	-0.73%	0.87	p<.01	-24%
	Distillation/density	6%	0.42%	0.64	p<.01	14%
	FTIR	0%	0.54%	0.75	p<.01	18%
Volatile acid	Cash still	71%	-0.95%	0.83	p<.01	-31%
	Enzymatic	19%	0.26%	0.31	p<.01	8%
	GC	3%	-0.73%	0.19	p<.05	-24%
	Segmented flow/colorimetric	3%	0.26%	0.49	p<.01	9%
	FTIR	0%	0.47%	0.81	p<.01	15%

Estimation of relative method accuracy and method bias

Although the CTS proficiency scheme does not have the ability to provide "true" values due to the nature of the samples, it is desirous to provide some information on the accuracy of the methods used. Mean z scores by method, standard deviations, and a conversion value for z score into analyte unit value are provided in Table 9. The methods for absorbance at 420 and 520 nm were not analyzed because

of the small sample size. Glucose plus fructose methods and residual sugar methods showed no significant differences among methods used (excluding the "other" or "unassigned" methods), which is in part a consequence of the poor reproducibility of the methods (HorRat > 2 for >90% of samples) and also because of the diminished statistical power due to the splitting of the methods at cycle 19. One exception to this statement is that prior to the split of methods, the enzymatic method gave significantly lower (-0.35 g/L) residual sugar results than the HPLC, FTIR, and copper reduction methods.

To evaluate if differences existed among methods for a given analyte, distributions of analyte z-scores for each method were plotted as histograms showing the deviation from the overall (combined methods) analyte mean (Figures 6-12). While these results are interesting for discussion of differences among methods, we caution that they provide **no certain** information on which methods are the most accurate because the samples were not reference materials with known values. Because the method with the most analyses will dictate the mean value for a given analyte, one method will usually dominate the data; proximity to the mean is thus not an indication of superior accuracy.

For alcohol measurements, we observed that the distillation/density method yielded values of -0.63 z (about 0.10 %v/v lower), as compared to the average. Potentially, this is due to incomplete recovery of ethanol during the distillation procedure, errors in the attempering of samples, or issues with determination of mass (AOAC 2012); alternatively, these values could be the true results and the other methods are delivering high values, as explained earlier. Ebulliometry had a higher

standard deviation than other methods and the mean z value was + 0.40 (about+ 0.06%) higher than the overall z value; descriptive statistics of the data show a second mode at +1.87 z (+0.3% v/v); the difference of this mode from the mean is approximately +0.24% alcohol, and may be explained by the effect of sugar on ebulliometer boiling points (Figure 6). One recommended sugar correction is to subtract 0.05 times the percent reducing sugar in the wine from the apparent ebulliometer alcohol level, and that this correction factor is only relevant to perform when reducing sugars are > 20 g/L (Zoecklein et al. 1994). Over the period studied, 23 wines had sugar concentrations >20 g/L, with an average value of 36 g/L. Using the formula above, an uncorrected ebulliometer alcohol measurement for these higher sugar wines would be 0.2 %v/v high, which accounts for most of the difference seen between the second mode and the mean. Finally, a regression of the residual sugar mean values (using "residual sugar" prior to cycle 19 and "glucose plus fructose" afterwards) against the alcohol across-lab z-scores from the self-reported ebulliometry methods gave a good fit: across-laboratory z score = 0.039 × g/L sugar – 0.0092 (n=78, p<.01, R^2=0.64). Since the intercept is negligible, this expression converts to: %v/v alcohol error = 0.06 × %RS, very close to the Zoecklein recommendation, and seeming to indicate that users of the ebulliometer method are unaware of the need for sugar correction (Figure 6 and Table 9).

Total SO_2 method comparisons show that Ripper is biased higher with respect to aeration-oxidation (2.3 mg/L) and flow injection (0.3 mg/L). However, other methods with fewer data points such as enzymatic methods (biased 8.8 mg/L higher than average) and FTIR (biased 7 mg/L lower than the average) are very indicative of

the variation in methods. The skew and multimodal distributions of the segmented flow and flow injected methods make direct comparisons even more challenging (Figure 7 and Table 9). Total SO_2 results are expected to be independent of the method used, and aside from the challenges of oxidative stability, manufacturing a certified reference material in a wine matrix may be appropriate for evaluating methodological accuracy. In any total SO_2 method, there will be a balance between maximizing the dissociation of the carbonyl-bisulfite adducts (usually done at a high pH or with heat) and minimizing the oxidation of the sulfites (which occurs more readily at these higher pH values) during the analysis (Joslyn 1955). In addition to these considerations, method specific issues, such as the ability of iodine to react with non-sulfite reducing agents, can affect accuracy (Joslyn 1955). The AOAC reference method for total SO_2 in wine is the Monier-Williams method, which is similar to the more commonly used aeration-oxidation method, with the primary differences in the glassware design, sample volume, gas flow rate, and selection of acidifying agent (AOAC 2012, Williams et al. 1992). Finally, the aeration-oxidation method for total SO_2 must be optimized to dissociate the bound adducts with acid and heat while minimizing the potential carryover of volatile acid, and also balance the gas flow rate to allow complete carryover of the gaseous SO_2 while allowing adequate time for reaction with H_2O_2 in the receiver flask (Rankine and Pocock 1970). These competing reactions and method limitations may explain why some authors have reported lower values with iodometric titration as compared to aeration-oxidation for total SO_2 (Buechsenstein and Ough 1978). While this type of bias may occur in some wine analytical labs, our large data set of 78 wines reveals that across-laboratory

reproducibility for individual methods is much poorer than within-laboratory repeatability, and that singular within-laboratory comparisons are not necessarily appropriate for broader statements about methodological bias.

Free SO_2 methods show aeration oxidation, flow injection, and segmented flow methods have similar distributions and means. As with the total SO_2, the Ripper method (iodometric titration) results in significantly higher values above flow injection (3.7 mg/L) and aeration-oxidation (2.7 mg/L), although all of these methods are within one standard deviation (Figure 8 and Table 9). Again, as with the total SO_2, some of the distributions appear to be skewed, which indicate additional bias. The higher value by Ripper as compared to aeration-oxidation is comparable to the bias observed previously in an intra-laboratory comparison (Buechsenstein and Ough 1978), potentially due to titration of other reducing species. Although many of the challenges in free SO_2 analyses are similar to those encountered in total SO_2, free SO_2 analyses must also minimize bisulfite adduct dissolution. In most cases, this is considered impossible; early researchers specifically warned against using aeration-oxidation for free SO_2 in red wines (Rankine and Pocock 1970) and carefully noted the correct conditions for using the Ripper for free SO_2 on wines with carbonyl-bisulfite adducts (Joslyn 1955). In addition, temperature can have an impact on the analysis, as increased temperature increases the dissociation rate of the bisulfite adducts and also impacts the equilibrium of the sulfurous acid species (Rankine and Pocock 1970, Usseglio-Tomasset and Bosia 1984). In summary, it is inappropriate to discuss which method for free SO_2 analysis yields the most accurate results, since it is not clear that any of the widely used methods accounts for these factors.

There is great overlap in the results of the volatile acid methods, but the Cash still is biased significantly higher than most of the other methods (capillary electrophoresis, enzymatic, FTIR, GC, HPLC, segmented flow) by 0.04-0.05 g/L as acetic. One potential issue is the target analytes of these methods; volatile acidity encompasses all the volatile short chain fatty acids (formic, acetic, propionic, etc.)(Amerine and Ough 1980), while CE, enzymatic, GC and HPLC methods are specifically targeting acetic acid. When the volatile acidity distillation is properly controlled, the correlation with acetic acid in wines is 1:1, which should indicate a false dichotomy between these two terms (Dubernet and Peraldi 2006). In practice, the Cash still method can suffer from several interferences, which include sorbate, sulfur dioxide, lactic acid, and carbon dioxide (Cottrell et al. 1985, Dubernet and Peraldi 2006, Gowans 1964, Pilone 1967), which could account for the higher observed values if not well controlled. It is interesting to note the discrepancy between the Cash still method and the segmented flow method, as one of the segmented flow instruments contain a miniaturized distillation apparatus, making that method more comparable with the Cash still method. This may indicate differences in method application between the two devices. The FTIR distribution is multimodal with a second mode at -0.06 g/L from the mean (Figure 9 and Table 9). Part of this disparity may be due the interchangeable use of the term “volatile acid” with the primary target analyte, acetic acid, especially with the FTIR, as the calibrations may be based on different primary methods. Secondarily, IR methods are not suitable for analyte concentrations less than 0.2 g/L due to, and because of the similarity in the chemical structure, acids and sugars can mutually interfere with quantification, especially if approaching the detection limit

(Bauer et al. 2008). This would indicate that wines with higher sugar and higher acids could affect the precision of IR results, especially at lower volatile acidity levels.

The FTIR multimodality appears again with the titratable acid values, with three modes at -0.03, -0.01, and +0.13 g/L from the mean (Figure 10 and Table 9). Unlike volatile acidity (which is theoretically different from acetic acid), the choice of primary method used for calibration seems less likely as an explanation. Some other factor, such as interferences or improper calibrations, might explain the distribution; again, because of the similar chemical structure of acids and sugars, IR methods are sensitive to interferences from these analytes. The manual titration shows a skew and a significantly higher result (+0.02 g/L higher than average) compared to autotitration, which is significantly lower (0.01 g/L compared to average) (Figure 10 and Table 9). Common interferences in titratable acid include endpoint definition and interference of carbon dioxide, and perhaps these are affecting the manual results (Guymon 1963).

Malic acid data shows a significantly higher values by FTIR (+0.14 g/L as compared to overall mean), capillary electrophoresis (+0.12 g/L) and HPLC (+0.08 g/L) as compared to enzymatic methods (-0.1 g/L) (Figure 11 and Table 9). While it is not possible to determine which approaches are more accurate, most enzymatic methods have a large number of potential systemic errors in both instrumental variability (calibration of spectrophotometers and pipets) and methodological variability (reaction times, enzyme activity) (Henniger and Mascaro 1985), which could be contributing to a consistent low result. As was mentioned earlier, the reproducibility of the malic acid enzymatic methods is most compromised at low concentrations, <0.5 g/L. And, as mentioned before, IR methods for malic acid,

especially at lower levels approaching the limits of the methods (0.2g/L) are particularly sensitive to interferences from other acids and from sugars (Bauer et al. 2008)

Finally, the distributions for the different specific gravity methods show bimodality in all methods except those with small samples sizes (Figure 12 and Table 9). This bimodality was first evident in the histograms of all the data combined (discussed earlier). The average difference between the two major modes in every method was between 0.0012 and 0.0024 specific gravity units. Since the specific gravity of water at 20 °C is 1.000 and the density of water at 20 °C is 0.9982 (a difference of 0.0018), the most likely explanation is human operator error in confusing density and specific gravity. The mass/known volume method is biased significantly lower than the other methods (-0.0032 specific gravity units, Table 9). This method requires proper calibration of balances, thermometers, and volumetric containers, along with proper determination of the density of water, and it is not surprising that a systematic error might be involved (AOAC 2012).

From discussions with both CTS and the ASEV lab proficiency ad hoc committee, along with unnamed participants, it seems that many laboratories are using the collaborative service without having their own in-house quality control program in place. Such a quality control program would include method validation, use of certified reference standards, ongoing analyst training, and control samples (ISO 2005). The lack of such quality control programs industry-wide undoubtedly has a negative effect on the overall results of the CTS results, most likely inflating the

values for repeatability and reproducibility, but having an unknown impact on the overall performance.

Table 9 Z scores and standard deviations for self-reported methods for each analyte. Within an analyte category, shared letters within a Tukey grouping indicate that the methods were not significantly different (p<0.05).

Method	N	Mean z score	SD of z score	Tukey's Grouping p<.05	z value (standard units)	Average method offset (standard units)
Alcohol-all methods	4225	0.00	0.93		0.16	
Ebulliometer	625	0.40	1.40	A		0.06
Dichromate	8	0.39	0.60	A B C		0.06
Gas Chromatography	512	0.12	0.74	B		0.02
FTIR	490	0.06	0.77	B C		0.01
Unassigned	489	-0.01	1.14	B C		0.00
Near Infrared	1541	-0.02	0.54	C		0.00
Other	88	-0.03	0.77	B C		0.00
Dist./Density	472	-0.63	0.90	D		-0.10
Titratable Acid-all methods	7789	0.00	0.97		0.26	
Manual Titration	1370	0.06	1.09	A		0.02
FTIR	323	0.04	1.29	A B		0.01
Segmented Flow	24	0.04	1.43	A B		0.01
Auto titration	1953	-0.04	0.82	B		-0.01
Unassigned	489	-0.05	0.91	A B		-0.01
Specific Gravity-all methods	3535	0.00	0.95		0.0012	
FTIR	95	0.16	0.98	A		0.00020
Hydrometer	562	0.03	1.08	A		0.00003
Unassigned	411	0.01	0.98	A		0.00002
Densitometer	2423	0.00	0.89	A		0.00000
Pycnometer	35	-0.31	0.74	A		-0.00037
Mass/Known volume	9	-2.65	0.90	B		-0.00318
Free SO2-all methods	4213	0.00	0.98		3.9	
Ripper	1118	0.39	0.93	A		1.5
Colorimetric	269	0.29	1.27	A B		1.1
Enzymatic	48	0.25	1.41	A B C		1.0
Segmented Flow	216	0.06	0.84	B C		0.2
Unassigned	562	0.00	0.96	C		0.0
Aeration Oxidation	1839	-0.25	0.89	D		-1.0
Flow Injection	161	-0.56	0.71	E		-2.2
Total SO2-all methods	4120	0.00	0.95		9.1	
Enzymatic	51	0.96	1.21	A		8.8
Flow Injection	181	0.34	1.05	B		3.1
Colorimetric	272	0.26	0.93	B		2.4
Segmented Flow	223	0.14	0.87	B C		1.3
Ripper	1876	0.02	0.92	C		0.2
Unassigned	543	-0.05	0.97	C D		-0.5
Aeration Oxidation	959	-0.23	0.92	E		-2.1
FTIR	15	-0.77	1.21	D E		-7.0
Volatile Acid-all methods	3193	0.00	0.99		0.07	
Cash Still	1877	0.31	1.01	A		0.02
Colorimetric	8	0.16	0.92	A B C D E		0.01
Segmented Flow	400	0.12	0.65	B		0.01
Unassigned	487	0.00	0.98	B D E		0.00

Table 9 con't.. Z scores and standard deviations for self-reported methods for each analyte. Within an analyte category, shared letters within a Tukey grouping indicate that the methods were not significantly different ($p<0.05$).

FTIR	288	-0.10	0.97	E		-0.01
GC	103	-0.29	0.74	D E		-0.02
HPLC	14	-0.32	1.13	A B C D E		-0.02
Capillary Electrophoresis	16	-0.46	1.62	B C D E		-0.03
Enzymatic	856	-0.66	0.73	C		-0.05
Glucose plus Fructose-all methods	5703	0.00	0.71		2.7	
Unassigned	6	0.38	0.91	A B C		1.0
Segmented Flow	32	0.26	0.65	A		0.7
HPLC	95	0.23	0.47	A		0.6
FTIR	164	0.00	0.58	A B C		0.0
Enzymatic/Spectrophotometric	1713	-0.01	0.73	A C		0.0
Other	84	-0.26	0.72	B C		-0.7
Residual Sugar (after split)-all methods	3609	0.00	0.69		3.6	
FTIR	108	0.24	0.50	A		0.86
HPLC	8	0.15	0.51	A B C		0.53
Cu Reduction	617	0.01	0.65	B		0.05
Other	112	-0.09	0.64	B C		-0.32
Another	70	-0.27	1.09	C		-0.98
Unassigned	10	-0.33	0.24	A B C		-1.20
Residual Sugar (before split)-all methods	1342	0.00	0.71		2.1	
another	10	0.78	1.07	A		1.61
HPLC	44	0.51	0.83	A		1.06
FTIR	46	0.51	0.62	A		1.06
Cu Reduction	125	0.50	0.69	A		1.03
Unassigned	432	-0.01	0.81	B		-0.01
Enz/segmented flow	661	-0.17	0.53	C		-0.35
other	24	-0.25	0.78	BC		-0.51
Malic acid-all methods	0	0.00	0.80		0.21	
FTIR	166	0.64	1.32	A		0.14
Capillary Electrophoresis	50	0.57	1.10	A		0.12
HPLC	49	0.40	1.18	A		0.08
Segmented Flow	30	0.30	0.60	A B		0.06
Unassigned	393	0.00	0.54	B		0.00
Enzymatic	2482	-0.06	0.74	B		-0.01

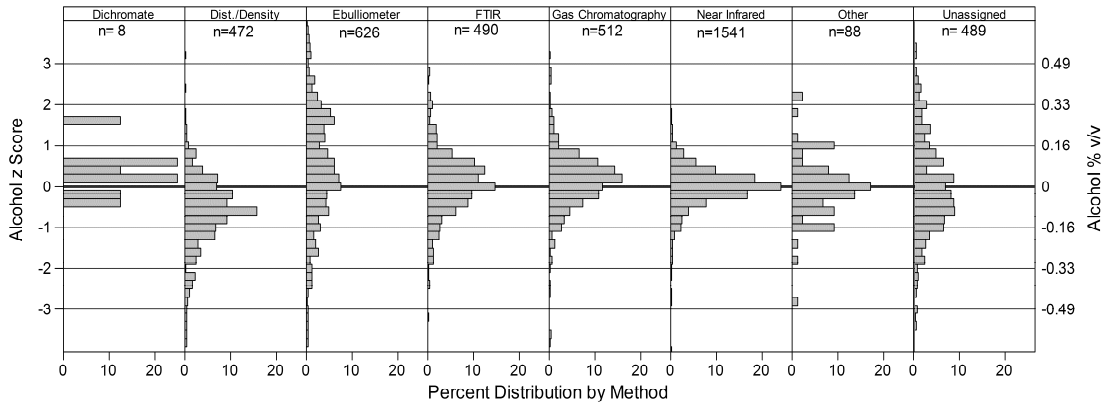

Figure 6 Distributions of alcohol analyses z-scores by self-reported method over time course of study. Left axis is z score, right axis is equivalent units. Scores were normalized by individual wine results to allow analytical comparison across all wines.

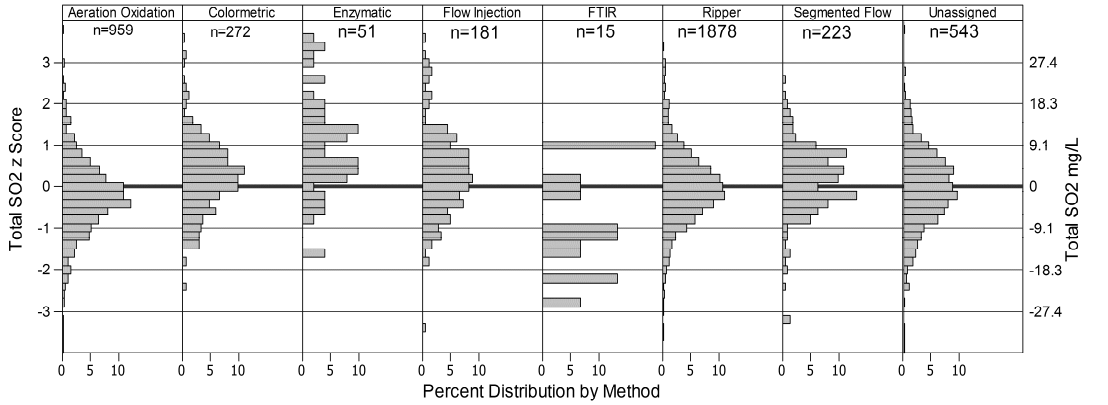

Figure 7 Distributions of total SO_2 analyses z-scores by self-reported method over time course of study. Left axis is z score, right axis is equivalent units. Scores were normalized by individual wine results to allow analytical comparison across all wines.

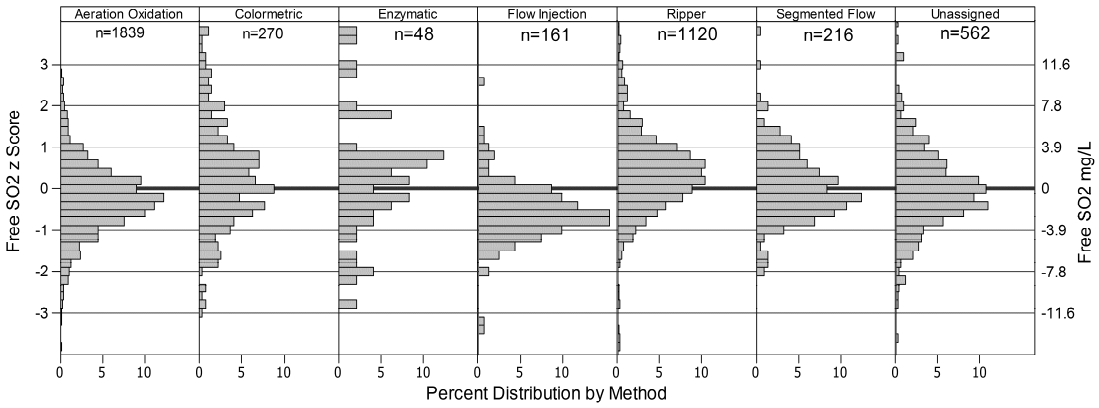

Figure 8 Distributions of free SO_2 analyses z-scores by self-reported method over time course of study. Left axis is z score, right axis is equivalent units. Scores were normalized by individual wine results to allow analytical comparison across all wines.

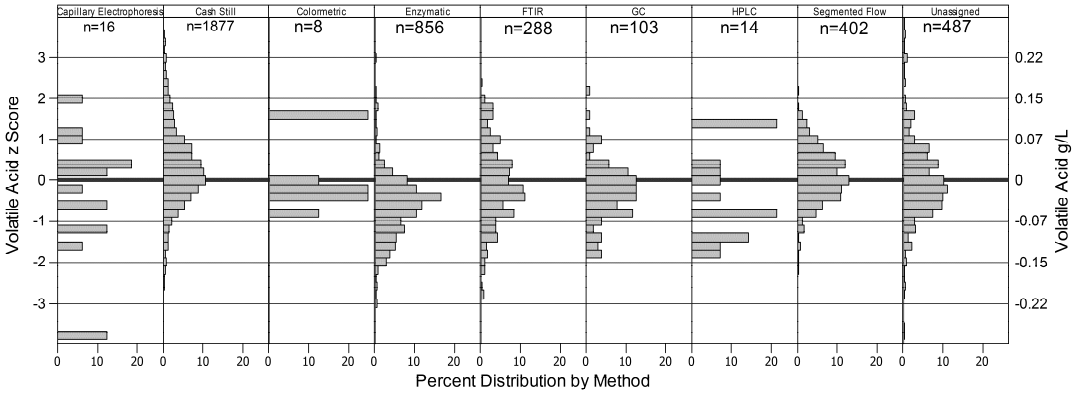

Figure 9 Distributions of volatile acidity analyses z-scores by self-reported method over time course of study. Left axis is z score, right axis is equivalent units. Scores were normalized by individual wine results to allow analytical comparison across all wines.

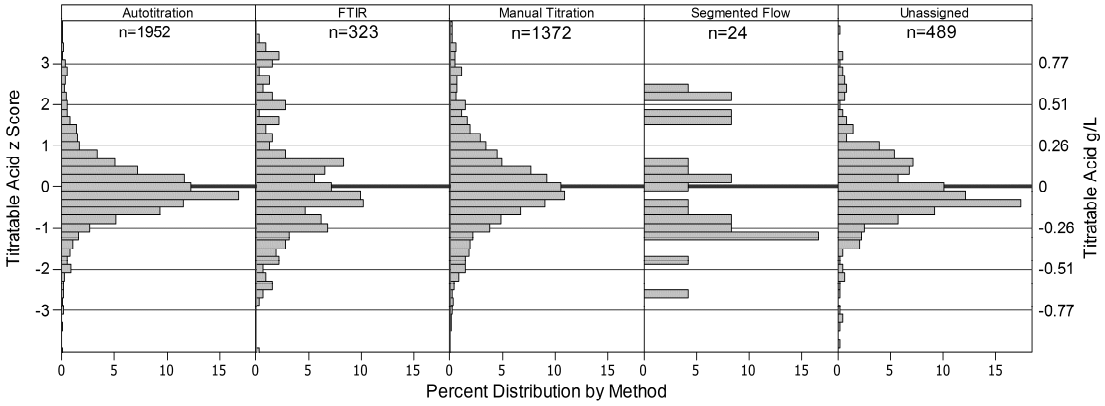

Figure 10 Distributions of titratable acidity analyses z-scores by self-reported method over time course of study. Left axis is z score, right axis is equivalent units. Scores were normalized by individual wine results to allow analytical comparison across all wines

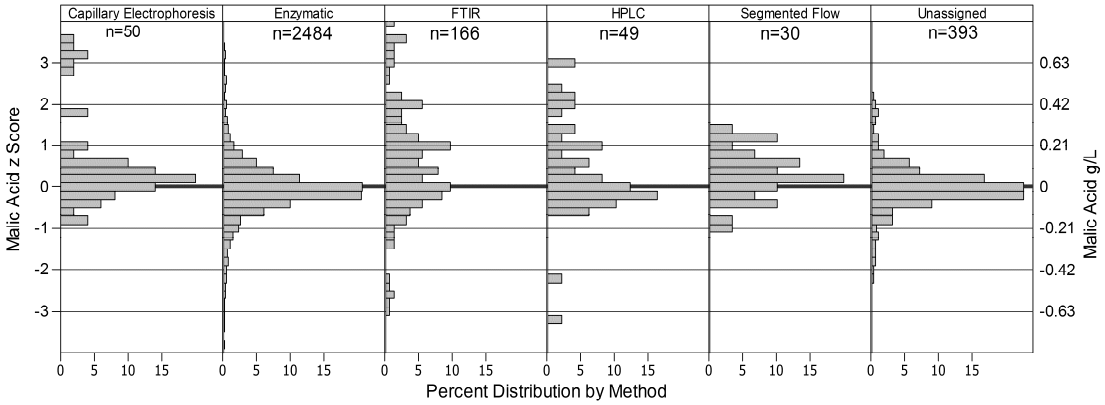

Figure 11 Distributions of individual-wine weighted malic acid analyses z-scores by self-reported method over time course of study. Left axis is z score, right axis is equivalent units. Scores were normalized by individual wine results to allow analytical comparison across all wines.

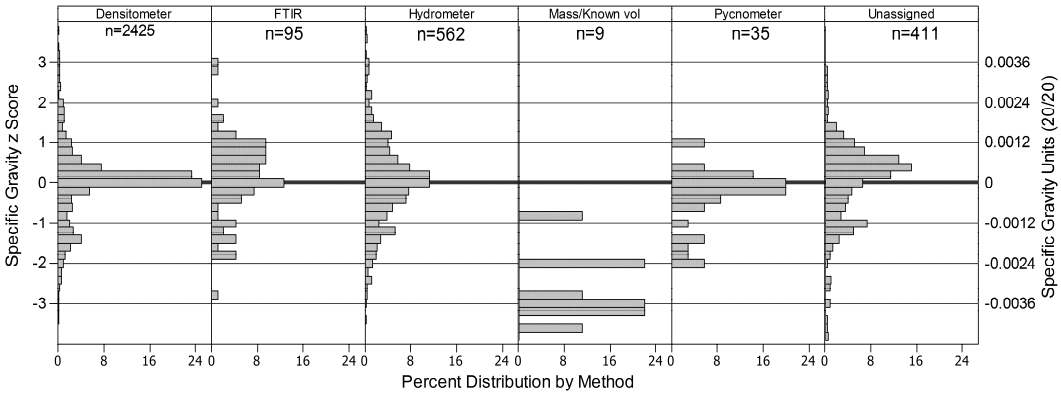

Figure 12 Distributions of individual-wine weighted specific gravity analyses z-scores by self-reported method over time course of study. Left axis is z score, right axis is equivalent units. Scores were normalized by individual wine results to allow analytical comparison across all wines.

Conclusion

Thirteen years of collaborative testing data indicate that many wine industry performance issues could likely be addressed by simple laboratory quality control systems, including in-house method validation, calibration schedules, and ongoing checks for volumetric and quantitative equipment (such as spectrophotometers, balances, and pH meters); all of this involves increased training and education of laboratory technicians and managers. Other data, specifically for alcohol analysis, indicate that improvements in technology can have an impact regardless of other quality considerations. Ironically, introduction of similarly new technologies for measurement of titratable acidity, pH, and malic acid has coincided with worsening precision for these analyses. Across-laboratory industry precision (reproducibility) is currently 3.6 to 57 times worse than within-laboratory precision (repeatability), depending on analyte. Some regulated analytes, such as SO_2 and volatile acidity, could

benefit from an improvement in methods. Specific gravity imprecision would undoubtedly be reduced by ensuring that specific gravity rather than density is reported. Application of the Horwitz ratio (HorRat) to this data indicates that three analytes are within or near the upper limits of internationally acceptable precision levels (alcohol, titratable acidity, and total SO_2), while other analytes have not met this precision standard (free SO_2, malic acid, volatile acid, and any of the sugar measurements). Of these, analytical performance for malic acid at low concentrations (<0.5 g/L) appears to be particularly problematic as measurements at these levels are critical for evaluating completion of malolactic fermentation. Finally, although some methods yield significantly different mean values and precisions for the same analyte, it is still challenging to evaluate the relative accuracy of methods. Certified reference materials containing these analytes, as have been developed for other foodstuffs, will be necessary to determine the accuracy of individual methods or laboratories. Further analysis of this data, particularly looking at the correlation between individual method precision and wine matrix, may contribute to a more thorough understanding of the strengths or limitations of specific methods.

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CHAPTER 2: A HEADSPACE GAS DETECTION TUBE METHOD FOR MEASUREMENT OF SO_2 IN WINE WITHOUT DISRUPTION OF SULFUR DIOXIDE EQUILIBRIA

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Short version of title: **Molecular and Free SO_2 by HS-GDT**

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Abstract:

The headspace gas detection tube (HS-GDT) method for measurement of molecular and free sulfur dioxide (SO_2) in wine is a simple and low cost procedure using commercial industrial safety colorimetric tubes. A syringe is used to sample a wine

and to create a closed headspace, which is expelled through the GDT after equilibrium is obtained. The vapor-phase concentration of SO_2 (P_{SO2}) is determined from the manufacturer's printed markings, and then related to the molecular SO_2 concentration based on Henry's law coefficients or, more accurately, on calibration curves. Typical wine ethanol concentrations had a significant effect on the pK_a of SO_2, as has been previously reported, but no effect on Henry's law coefficients; calibration curves in model wine and aqueous buffer yielded indistinguishable results when appropriate pK_a values were used. Best results for calibration curves were achieved with 200 mL headspace of model wines (5-40 mg/L free SO_2, equivalent to 0.14 to 1.12 mg/L molecular SO_2), which yielded satisfactory linearity (r^2 = .99), reproducibility (mean CV = 8% for molecular SO_2 > 0.4 mg/L), and detection limits (0.21 mg/L molecular SO_2). Molecular SO_2 measured by HS-GDT and by aeration-oxidation (A-O) showed a strong correlation between methods for white and blush wines (r^2 = 0.97) and a poor correlation for red wines (r^2 = 0.72). A-O values averaged 2-fold higher than HS-GDT values in red wines. The difference in molecular SO_2 values by A-O and HS-GDT was well correlated with estimated molecular SO_2 decrease due to anthocyanin-bisulfite adduct formation (r^2 = 0.936), supporting the hypothesis that dissociation of anthocyanin-bisulfite adducts occurs during A-O analysis. By not perturbing this equilibrium, HS-GDT accurately reports free and molecular SO_2 values.

Key words: Sulfur dioxide, wine analysis, headspace, method validation, SO_2

Introduction

Sulfur dioxide (SO_2) is widely used as a preservative in winemaking due to its anti-microbial and anti-oxidant properties (Boulton et al. 1996, Ribéreau-Gayon et al. 2007). At wine pH (3-4), the majority of SO_2 (> 95%) exists in the form of bisulfite (HSO_3^-) with the remainder existing as neutral "molecular" SO_2 and a negligible portion existing as sulfite (SO_3^2) (Zoecklein et al. 1995, Boulton et al. 1996). The sum of these forms (molecular SO_2, HSO_3^-, and SO_3^{2-}) is referred to as "free SO_2". Additionally, HSO_3^- may form covalent adducts with electrophilic compounds in wine, including ketonic acids, sugars, quinones and anthocyanins (Burroughs and Sparks 1973, Beech et al. 1979). This covalently bound HSO_3^- is referred to as "bound SO_2" and has lower antimicrobial and antioxidant activity than free SO_2 forms (Boulton et al. 1996). "Total SO_2" is the sum of the "free" and "bound" SO_2, and is the form subject to regulatory limitations in most wine producing countries (Zoecklein et al. 1995).

Of the forms described above, the molecular SO_2 and HSO_3^- species are reportedly the most important for the germicidal and antioxidant properties of SO_2, respectively (Danilewicz 2011, Divol et al. 2012). For dry table wines, winemakers will typically aim to keep free SO_2 (predominantly HSO_3^- for reasons described earlier) at more than a minimum of 10 mg/L (Godden et al. 2001) or at a target of 30 mg/L (Waterhouse and Elias 2010) to mitigate or slow the appearance of oxidative effects such as aldehyde formation and browning. Recommended molecular SO_2 levels in wine production depend on the target microorganism, but the most commonly cited values range from 0.5 to 0.8 mg/L molecular SO_2 (Beech et al. 1979, Boulton et

al. 1996). Free SO_2 concentrations can decrease during wine production and storage through binding, volatilization, or, more often, oxidation reactions. Thus, winemakers must routinely assess wines for free SO_2 to ensure stability (Zoecklein et al. 1995).

In most production wineries and research laboratories, free SO_2 rather than molecular SO_2 is measured, and the latter calculated via a modified Henderson-Hasselbalch equation following measurement of pH. Common approaches for measurement of free SO_2 fall into two categories: first, titrimetric and colorimetric methods that rely on direct addition of an oxidizing reagent to the wine, e.g. by iodometry ("Ripper" method) or addition of p-rosaniline (Joslyn 1955, Zoecklein et al. 1995), and second, methods that separate free SO_2 from the wine followed by quantification by titrimetry, colorimetry, or other means (Zoecklein et al. 1995, Pundir and Rawal 2013). In modestly equipped wineries, the most common variant of this "separation-first" approach is the aeration-oxidation (A-O) method, in which an acidified wine sample is stripped by stream of air and SO_2 captured by a hydrogen peroxide solution to generate H_2SO_4, which can be quantified by titration (Rankine and Pocock 1970). Automated variants of both direct and separation-first methods also exist (Claudia and Francisco 2009, Pundir and Rawal 2013).

Beyond these methods, the Association of Analytical Communities (AOAC) lists several methods for *total* sulfites in wine (Monier-Williams, flow injection/chromatography-spectrophotometry, and ion exclusion chromatography), and a method for *free* sulfites for white wines only (flow injection/ chromatography-spectrophotometry) (AOAC 2015). These methods give comparable values to A-O,

and the A-O method is the one used by the US Alcohol and Tobacco Tax and Trade Bureau due to its relative simplicity (http://www.ttb.gov/ssd/pdf/tm500.pdf).

A well-known shortcoming of the direct titrimetric or colorimetric approaches is the measurement of reducing compounds other than free SO_2, resulting in overestimation of the true value (Buechsenstein and Ough 1978). However, a less-appreciated shortcoming of all aforementioned standard methods for SO_2 measurement is the dissociation of weak SO_2 adducts following changes to wine equilibrium during the measurement. These perturbations to equilibrium may result from acidification, temperature change, dilution, and/or consumption of the free SO_2 by reagents over the measurement timecourse, which will result in overestimation of free and molecular SO_2 (Rankine and Pocock 1970, Burroughs 1975, Boulton et al. 1996). This overestimation is more pronounced in red wines, likely due primarily to dissociation of the anthocyanin-bisulfite adducts; more modest discrepancies are observed for white wines (Burroughs 1975, Bogren 1996).

This overestimation of free and molecular SO_2 in wine by standard techniques is of potential concern to researchers or winemakers interested in a mechanistic understanding of oxidation or microbial growth in wine (Boulton et al. 1996). However, few papers report using techniques that avoid pH shifts, sample dilution, and/or temperature changes, and thus avoid disturbance of SO_2 equilibria in wine. One such approach is capillary electrophoresis (CE), which can be used to quantify bisulfite with minimal contributions from artifactual dissociation of bound SO_2 forms (Boulton et al., 1996, Collins and Boulton, 1996). Results from CE indicate that free SO_2 may be overestimated by up to an order of magnitude in red wines. Similar

observations were reported using a colorimetric method based on comparison of absorbance values at 520 nm for acetaldehyde- and SO_2-treated wines to an original wine sample, although this method relied on several assumptions regarding anthocyanin spectral behavior, was developed before an appreciation of copigmentation effects had been described, and would only be appropriate for detecting overestimation due to anthocyanins and not to other weak binders (Burroughs 1975).

Alternatively, the headspace SO_2 concentration of an equilibrated sample can be measured, and the partial pressure related to the molecular SO_2 concentration in wine via Henry's Law. Using headspace gas chromatography (HS-GC) coupled to an electrolytic conductivity detector (Davis et al. 1983), good agreement was observed between A-O and HS-GC approaches for a white wine and juice, but A-O yielded 45% higher values for the red wine. Headspace infrared (HS-IR) spectroscopy can also reportedly be used for SO_2 detection from unadjusted wine samples (Henningsen and Hald 2003). Inductively-coupled plasma optical-emission spectrometry (ICP-OES) (Čmelík et al. 2005) and flame molecular absorption spectrometry (Huang et al. 2008) have also been used for measurement of vapor-phase SO_2 from wine, but wine samples were acidified and gas-purged during analysis, which was expected to yield results comparable to classic methods. None of these approaches to measuring vapor-phase SO_2 have been widely adopted in either wineries or research labs, possibly due to the cost and complexity of the techniques.

An unexplored approach to measuring wine headspace SO_2 concentrations are colorimetric gas detection tubes (GDTs). GDTs were originally developed for

industrial safety in the early 1900's (Haag 2001) and consist of a glass tube packed with color-sensitive reagent, such that the length of tube undergoing color change is proportional to the analyte concentration. Commercial SO_2 gas detection tubes have been used for measurement of free SO_2 in wines using a modified A-O protocol (Pegram et al. 2013) , adapted from a related method for quantifying H_2S in wines (Park 2008, Ugliano and Henschke 2010). Similar to A-O, this earlier use of GDT for free SO_2 measurement relied on acidification of the sample and sparging prior to SO_2 detection, and thus likely also experienced dissociation of weak adducts and overestimation of free SO_2.

In this paper, we report on the development of a headspace gas detection tube (HS-GDT) method for determination of molecular and free SO_2, both calculated from the measurement of the headspace gas concentration. This method does not require sample preparation and thus avoids disturbance of SO_2 equilibria and in combination with its low cost and simplicity should facilitate investigations into the antimicrobial and antioxidative properties of SO_2.

Materials and Methods

Chemicals:

Potassium metabisulfite (97% w/w) and ethanol (95% v/v) were obtained from Acros Organics (Geel, Germany). Lactic acid (85% w/w), potassium bitartrate (99% w/v), hydrogen peroxide (30% w/v), sodium hydroxide (0.01 N) and *o*-phosphoric acid (85% w/w) were obtained from Fisher Scientific (Waltham, MA). A nominally 25% phosphoric acid solution was prepared as a 2.38:1 dilution of 294 mL phosphoric acid

(85%) with 700 mL DI water. Potassium acetate (99% w/w) and potassium sorbate (98% w/w) were obtained from J. T. Baker (Phillipsburg, New Jersey). Hydrochloric acid (36.5% w/w) was obtained from BDH Merck (Poole Dorset, United Kingdom).

SO_2 Working Standards:

SO_2 stock solutions at nominal concentrations of 1000 mg/L as SO_2 were prepared weekly by dissolution of potassium metabisulfite in a solution of methanol in water (10% v/v) to avoid SO_2 auto-oxidation. Working standards were then prepared as needed by addition of an appropriate volume of a stock SO_2 solution to saturated potassium bitartrate buffer (pH 3.58). Iodometric titrations were used to determine the actual concentration of the stock and working solutions.

SO_2 Gas Detection Tubes:

Three commercial SO_2 detection tubes were initially evaluated: i) Gastec 5Lb (Gastec Corporation, Fukayanaka, Japan), ii) Sensidyne/Kitigawa 103SE SO_2 tubes (Sensidyne LP, St Petersburg, FL, USA), and iii) Draeger 0.1/a SO_2 tubes (Drägerwerk AG & Co. KGaA, Lübeck, Germany).

SO_2 Measurements by Aeration-Oxidation (A-O):

A-O was performed according to a protocol described elsewhere (Iland et al. 2004,).

Protocol for SO_2 Measurement by Headspace Gas Detection Tube (HS-GDT):

The HS-GDT apparatus was assembled as depicted in Figure 13. It consists of a 60 mL Becton Dickinson syringe (polypropylene barrel, polypropylene plunger, latex-free polyisoprene rubber tip treated with Dow 360 medical grade silicone) with a luer-

lock tip, fitted to a two-way polycarbonate male luer stopcock. A short piece of silicone tubing connects a GDT to the top of the stopcock. The syringe is fitted with a 3 cm plastic dispensing stop, used to prevent accidental expulsion of liquid during syringe depression, made by cutting a second 60 mL Becton Dickinson syringe barrel to the correct height. If the GDT had been previously used and the "end" marking from previous runs was unclear or had shifted, the "start" point of color transition was marked on the tube with a fine point permanent marker.

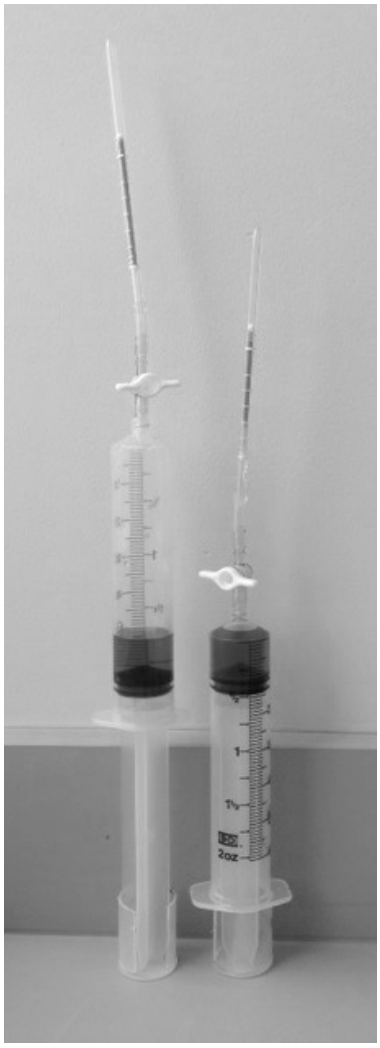

Figure 13: The HS-GDT apparatus prior to (left) and after (right) headspace expulsion through the gas detection tube.

For each analysis, the syringe was used to aspirate 10 mL of wine or working standard, the syringe inverted and then withdrawn further to create 50 mL of headspace. The stopcock was closed, the syringe was placed nose-up, the syringe stop put in place, and the sample allowed to equilibrate for 5 min. The stopcock was then connected to a GDT via the short piece of silicone tubing, the stopcock opened, and the syringe depressed at a constant rate to the stop, such that 50 mL of headspace were expelled through the GDT over a 10 second period. Following gas expulsion, the colorimetric reaction was allowed to stabilize for 1 min. For some calibration curve experiments and for analyses of wines, this process would be repeated up to four times for one sample, resulting in up to 200 mL of headspace through the GDT. For the wine analyses, the limitation of the number of repetitions was based on those that could be quantified on one tube. The final color transition point was marked with a fine-tip marker, and the locations of the stain startpoint and endpoint were measured in millimeters with a ruler.

Determination of PSO_2, Molecular SO_2, and Free SO_2 from raw GDT measurements:

Gas detection tubes have printed scales showing the vapor-phase concentration of SO_2 (P_{SO2}) in ppm (µL/L) as a non-linear function of stain distance, based on a brand-dependent standard volume (200 mL for the Gastec and Sensidyne/Kitigawa tubes, 100 mL for the Draeger) of headspace sampled. The scale resolution was insufficient for repeated high-precision measurements. To facilitate interpolation, a cubic function, f(x), was determined to relate SO_2 vapor pressure (P_{SO2}) based on the manufacturer's

markings as a function of distance along the GDT in mm. The best-fit equations were calculated using Microsoft Excel. Because the spacing of markings varied among GDT lots, cubic interpolation functions were determined for each lot of tubes. For each sample, P_{SO2} was calculated from the location of the startpoint (x_{start}) and the endpoint (x_{end}) measurements in mm, shown in Equation 1, and adjusted based on the actual volume of headspace gas sampled, shown here for the Gastec or Sensidyne tubes.

Eq 1: $$P_{SO2} = (f(xend) - f(xstart)) \times \frac{200\ mL}{Headspace\ sample\ (mL)}$$

Molecular SO_2 (mg/L) was calculated from P_{SO2} using from Henry's Law and the SO_2 molecular mass, as shown in Equation 2. A Henry's Law coefficient (K_H) of 0.38 Atm M^{-1} at 294 K was determined as part of this work.

Eq 2: $$Molecular\ SO_2\ (mg/L) = \frac{P_{SO2}}{K_H} \times 0.064$$

A literature value of 3100 K was used as the temperature dependence constant (Sander, 2015). Equation 3 reports the temperature corrected Henry's Law coefficient, $K_{H(T)}$, used in this work, where T is the temperature during the analysis.

Eq 3: $$K_{H(T)} = 0.38\ Atm\ M^{-1} \times \exp^{(3100 \times (\frac{1}{294} - \frac{1}{T}))}$$

Free SO_2 was calculated from molecular SO_2, the sample pH, and the pK_a of SO_2 by Equation 4. Literature values for pK_a as a function of ethanol were used in this calculation (Usseglio-Tomasset and Bosia 1984) and were corroborated as part of this work.

Eq 4: $$[Free\ SO_2] = (1 + 10^{(pH - pKa)})[Molecular\ SO_2]$$

Evaluation of Interferences:

The potential of ethanol, lactic acid, sorbic acid and acetic acid to act as interferences were evaluated by preparing solutions containing either 14% ethanol, 5 g/L lactic acid, 300 mg/L sorbic acid or 1.4 g/L acetic acid in a potassium bitartrate buffer. The HS-GDT responses for these solutions were compared against a blank potassium bitartrate buffer. Analyses were done in triplicate using 50 mL headspace samples. For these evaluations, a measurable discoloration of the GDT was taken as evidence of interference.

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Determining Optimal Equilibration Time:

SO_2 solutions (1 mg/L) were prepared with varying ethanol concentrations (0, 8, 11, 14 and 17% v/v) at pH 0 by addition of SO_2 stock directly into the syringe containing 10 mL of appropriate ethanol/HCl solutions. At this pH, SO_2 was predominantly in the molecular SO_2 form, and care was taken keep the syringe closed except during sampling. The samples were analyzed by the HS-GDT method in replicate (n=8) for each ethanol concentration. The Henry's coefficient (K_H) of SO_2 was then calculated in units of Atm L mol^{-1} for each ethanol concentration as $K_H = P_{SO2} / [SO_2]$.

Determination of pKa of SO_2 as a Function of Ethanol:

SO_2 solutions (50 mg/L) were prepared in a potassium bitartrate buffer with one of four ethanol concentrations (0, 7, 14 or 20 % v/v) and one of four pH values (3.1, 3.3, 3.5, or 3.7), for a total of 16 solutions. pH values were adjusted by dropwise addition

of either 25% phosphoric acid or 1N sodium hydroxide. The HS-GDT protocol was then used to determine P_{SO2}, which was then converted to molecular SO_2. The apparent pK_a for each solution was calculated from the measured molecular SO_2 and known free SO_2 concentration using the Henderson-Hasselbalch equation. The room temperature at the time of the experiment was 21.9°C.

Figures of Merit for HS-GDT method – Linearity, %CV, Detection Limit:

Standards solutions at nominal concentrations of 5, 10, 15, 20, 30 and 40 mg/L as SO_2 were made in a potassium bitartrate buffer (pH 3.56) and ethanol 12% v/v, and at nominal concentrations of 5, 10, 25, 50 and 75 mg/L as SO_2 made in aqueous potassium bitartrate buffer (pH 3.56). Actual free SO_2 concentrations were determined by the Ripper method (Iland et al. 2004). Each sample was analyzed by the HS-GDT method in replicate (n=12, total of 72 analyses). The coefficient of variation for each standard concentration was calculated as the standard deviation divided by the mean and expressed as a percentage. Linearity was evaluated by linear regression of measured P_{SO2} vs. expected P_{SO2}. The detection limit (LOD) was calculated as $3 \times \sigma$, where σ is the signal-independent noise calculated by Pallesen's method (Berthouex and Brown 2002).

Comparison of A-O and HS-GDT for SO_2 in Commercial Wines:

Twenty seven commercial wines (9 red, 14 white, and 4 blush) were evaluated by both the A-O and HS-GDT methods on site. The red wines were from the 2009-2012 vintages and the white wines were from the 2011, 2012 and 2013 vintages. The wines were from Australia, Argentina, and the United States (California, New York, Oregon,

and Washington). Composition of the commercial wines was determined by ETS Laboratories (St Helena, CA), using accredited methods for pH (by meter), alcohol (FTIR), and glucose plus fructose (enzymatic); additionally tannins, polymeric and total anthocyanins were measured by HPLC (Waterhouse et al. 1999). Similar to standard curves, the detection limit (LOD) for molecular SO_2 was calculated as $3 \times \sigma$, where σ is the signal-independent noise calculated by Pallesen's method (Berthouex and Brown 2002).

To evaluate if discrepancies between A-O and HS-GDT measurements could be explained by the presence of labile anthocyanin-bisulfite adducts, the concentration of anthocyanin-bisulfite adducts, [Flav-Bisulfite], was estimated by solving a system of equilibria equations:

Eq 5: $K_D = [Flav]*[Bisulfite_{HS}] / [Flav\text{-}Bisulfite]$

Eq 6: $K_A = [Quin][H^+] / [Flav]$

Eq 7: $[Total\ Anth] = [Flav] + [Quin] + [Flav\text{-}Bisulfite]$

Eq 8: $[Bisulfite_{AO}] = [Bisulfite_{HS}] + [Flav\text{-}Bisulfite]$

[Flav] and [Quin] are the concentrations of flavylium and quinoid forms of anthocyanins. [Flav-Bisulfite] is the concentration of anthocyanin bisulfite adducts, and the free SO_2 concentration measured by A-O, $[Bisulfite_{AO}]$, was assumed to equal the sum of [Flav-Bisulfite] and $[Bisulfite_{HS}]$, i.e. all of the difference between A-O and HS-GST could be explained by binding to flavylium ions. Acid dissociation constants (pK_a) for anthocyanins were based on literature values and assumed to be 2.94 for *V. vinifera* wines and 2.6 for diglucoside-containing hybrid-based wines (Brouillard and El Hage Chahine 1980,Francis and Markakis 1989, Ribéreau-Gayon et al. 2007). The

dissociation constant (K_d) for Flav-Bisulfite adducts was assumed to be 1×10^{-5} (Burroughs 1975, Timberlake and Bridle 1976). [Total Anth] was the total anthocyanin concentration, and $[H^+]$ for each wine was derived from the measured pH value. For each wine, K_D, K_A, [Total Anth], $[H^+]$, and $[Bisulfite_{AO}]$ were independent variables and the equations were solved for [Flav], [Quin], [Flav-Bisulfite] and $[Bisulfite_{HS}]$ using R v3.1.2 (The R Foundation for Statistical Computing, Vienna, Austria) and the "nleqslv" v2.5 add-in package.

Statistical Analyses:

JMP Pro 11.0.0 (SAS Institute, Cary, NC) was used for statistical analyses. Comparison of means tests for the effects of ethanol on pK_a and K_H were performed by 1-way ANOVA followed by a Tukey test if the effect was significant. Linear regression analyses for calibration curves comparison of A-O vs. HS-GDT data were performed with 1/x weighting factors. Linear regressions of wine composition parameters and the difference between A-O and HS-GDT SO_2 values were performed without weighting.

Results and Discussion

Apparatus and Materials for SO_2 Measurements by HS-GDT:

The apparatus, shown in Figure 13, was used in the protocol as described above..

Three different gas detector tubes were initially investigated: i) Gastec 5Lb, which rely on the reaction of SO_2 with $BaCl_2$ to generate HCl, resulting in the color change of a pH sensitive dye; ii) Sensidyne/Kitigawa 103SE SO_2 tubes, which rely on

the colorimetric reaction of SO_2 with NaOH to generate Na_2SO_3; iii) Draeger 0.1/a tubes, which rely on reaction of SO_2 with $NaHgCl_4$ to generate HCl, whose evolution is detected by a pH sensitive indicator. Initial trials with alcoholic buffer solutions containing SO_2 resulted in no detectable signal for the Draeger 0.1/a tubes, and thus these were not further considered. We observed inconsistent results with Sensidyne tubes, with coefficients of variance (CV) > 20% in the length of tube darkened, while Gastec 5Lb tubes yielded reproducible results (CV < 10%). The reason for the poor performance of some of the tubes is unclear: however, commercial tubes require that gas sampling occur at precise, non-linear, and proprietary rates that are unique to each manufacturer (Haag 2001) and the poor performance may reflect incompatibility of some brands of tubes with this method's syringe-depression sampling rate. The Gastec 5Lb tubes were selected for further study, as they performed best with the current syringe apparatus sampling protocol.

Evaluation of Interferences:

Gastec GDT exhibited uniform discoloration from green to light blue even when exposed to 'blank' 14% ethanol buffer solutions (data not shown). However, because this color change was distinct from the color change associated with SO_2 (green to yellow), it did not interfere with measurements.

Because the reaction chemistry of the GDT employed will respond to all volatile acids, we investigated the susceptibility of the HS-GDT method to interferences from acetic (1.4 g/L), lactic (5 g/L) and sorbic acid (300 mg/L). A small signal, equivalent to < 0.1 mg/L molecular SO_2, was observed at these concentrations

(data not shown). Since these levels represent the upper end of concentrations expected to be found in wines (Zoecklein et al. 1995), the susceptibility of the method to these volatile acid interferences is low.

Interpolation of Manufacturer Markings on GDT:

The manufacturer-provided markings on the GDT were used for quantification of the SO_2 vapor pressure, P_{SO2}. To facilitate interpolation of markings, best-fit cubic functions relating P_{SO2} to distance along the tube were generated for each lot of Gastec GDT. P_{SO2} for each sample could then be calculated using Equation 1, which in turn could be used to calculate molecular SO_2 and free SO_2. Tube-to-tube variation in marker spacing within the same lot number was imperceptible and not a source of error.

Effect of equilibrium time on HS-GDT measurements:

Fifty mg/L SO_2 solutions in pH 3.56 buffer were analyzed by the HS-GDT apparatus using varying static equilibration times (1, 5 and 10 min) at room temperature. No significant differences were observed, indicating that headspace and liquid-phase SO_2 equilibrate quickly. Five minutes was selected as the optimal equilibrium time for the HS-GDT method because it yielded the best precision (Table 10).

Effects of Ethanol on Henry's Coefficient of SO_2:

Using the HS-GDT assay, Henry's coefficient (K_H) was determined for 1 mg/L SO_2 solutions at 21 °C over varying ethanol concentrations. Samples were acidified with HCl to pH ~0 to favor the molecular SO_2 form and eliminate any confounding ethanol-

induced effects on the pK_a of SO_2. K_H was independent of ethanol concentration over the range of 0-17% v/v ethanol (Figure 14), and was calculated to be 0.38 Atm M^{-1} at 21 °C, comparable to a recent previous report of 0.28 Atm M^{-1} for SO_2 in water at 25 °C (Zhang et al. 2013). The lack of dependence of SO_2 volatility on ethanol concentration is in contrast to other wine volatiles; for example, the volatility of many wine esters is about two-fold lower in model wine than in a model juice solution (Mouret et al. 2012). The minimal effect of ethanol on SO_2 volatility may result from its greater polarity as compared to many other wine volatiles. Regardless of the explanation, these results indicate that it is not necessary to control for ethanol concentration prior to HS-GDT analyses of wine.

Table 10 Effect of equilibrium time on SO2 vapor pressure by HS-GDT method

	Equilibration Time		
	1 min	**5 min**	**10 min**
P_{SO2} (µL/L)	1.18	1.19	1.21
Standard deviation	0.16	0.11	0.23
Coefficient of variation (%)	13.28	9.37	19.29

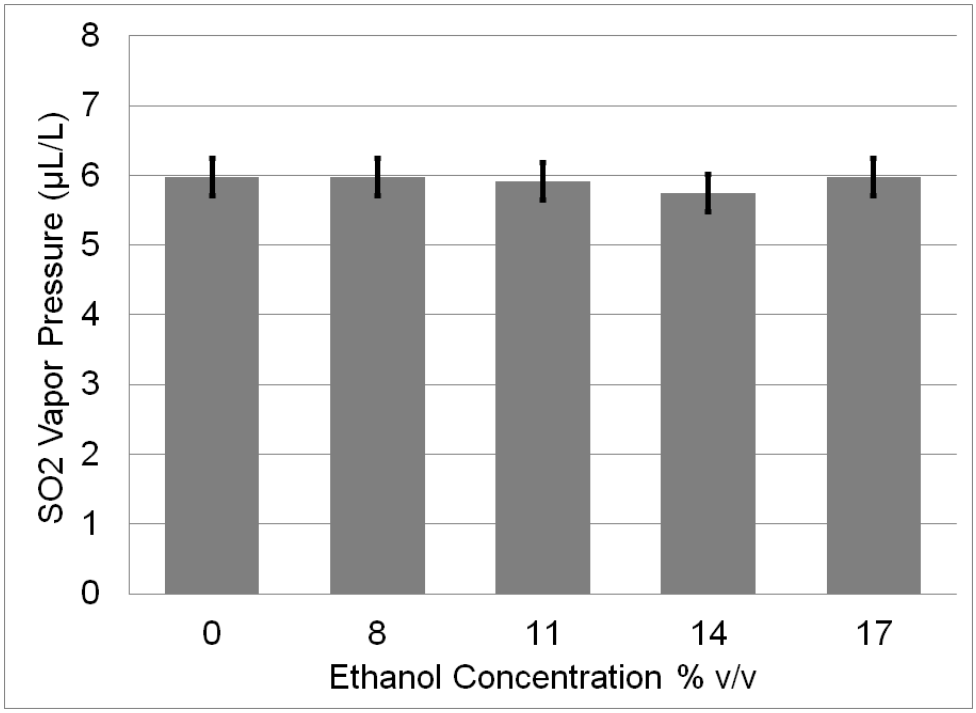

Figure 14: SO_2 vapor pressure as a function of ethanol concentration. Samples were prepared at pH ~0. Error bars represent standard errors (n=8).

Effect of Ethanol Concentration on the pKa for SO_2:

P_{SO2} was determined by HS-GDT for model wine solutions containing different ethanol concentrations and pH values, and the P_{SO2} and pH values subsequently used to calculate pK_a as a function of ethanol concentration (Figure 15). Because K_H had previously been determined to be independent of ethanol concentration, changes in P_{SO2} could be assigned solely to effects on pK_a. The best fit line ($y = 0.0137x + 1.83$, $r^2 = 0.97$) yielded predicted acid-dissociation constants of $pK_a = 1.83$ at 0% ethanol and $pK_a = 1.96$ at 10% alcohol. These values are comparable to pK_a values determined by titrimetry in model solutions for 0 % and 10% ethanol solutions (1.81 and 2.00, respectively) (Usseglio-Tomasset and Bosia 1984) (Table 11). Interestingly, the pK_a of SO_2 in water (1.81) is widely recommended in calculations of molecular

SO_2 from free SO_2 in wine regardless of the ethanol concentration, which is expected to lead to 25-50% underestimations of the actual molecular SO_2 concentration in wines, depending on the pH. Temperature and ionic strength can, in lesser degree, also impact pK_a, and should be taken into consideration (Usseglio-Tomasset and Bosia 1984). In this paper, all conversions between free and molecular SO_2 are based on ethanol-corrected pK_a values at specific room temperature and 50 m*M* ionic strength unless otherwise specified.

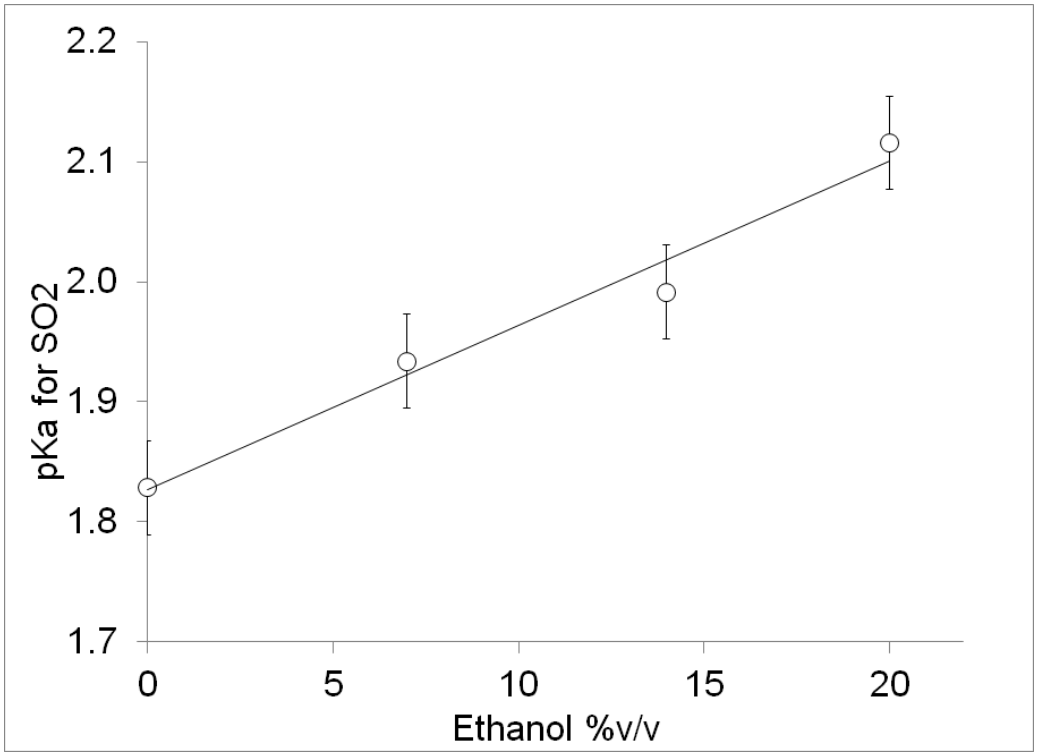

Figure 15: Experimentally determined pK_1 values for SO_2 in a buffered standard solution as a function of alcohol concentration. Error bars represent standard deviations (n=4).

Table 11: Comparison of experimentally determined pK_1 values to literature pK_1 values for SO_2.

Alcohol	pK_1 (observed) [a]	pK_1 (literature) [b]
0%	1.83	1.80
5%	1.90	1.90
10%	1.96	2.00
15%	2.03	2.10
20%	2.10	2.20
[a] Experimentally derived, T = 21.8°C		
[b] Calculated based on formulae from Usseglio-Tomasset using T = 21.8°C and ionic strength = 0.056M		

Figures of Merit for the HS-GDT Method:

Calibration curves were analyzed by the HS-GDT method using SO_2 standards prepared at pH 3.56 in either aqueous buffer (0.09 to 1.28 mg/L molecular SO_2) or model wine (0.14 to 1.12 mg/L molecular SO_2) using either a 50 mL or 200 mL (4 × 50 mL) headspace sample. Limits of detection and % CV for the analyses of the commercial wines were also calculated. The figures of merit for these data are reported in Table 12. The slope and intercept of all methods were near identical (<5% difference for each parameter across the four curves), indicating that the method was scalable with headspace volume size and the use of Equation 1 was valid. As expected from our observations that wine-like concentrations of ethanol do not affect Henry's

coefficient, the best fit lines were near identical for the aqueous and model wine calibrations (y = 5.92x – 0.48 vs. y = 5.99x – 0.55 for 200 mL sample sizes).

Although reasonable detection limits (0.21 mg/L molecular SO_2) could be achieved for 50 mL sample sizes in the aqueous standards, detection limits were considerably higher for model wine (0.59 mg/L molecular SO_2). Additionally, linearity was unacceptable for the 50 mL samples from model wine (r^2 = .75), and precision for range of molecular SO_2 typically targeted in wine (>0.4 mg/L) was also mediocre (mean CV = 18%). In the model wine system, the use of repeat sampling improved limits of detection from 0.59 mg/L for a 50 mL headspace sample to 0.21 mg/L for a 200 mL sample. The use of repeat sampling also improved the precision (mean CV = 8% for standards with >0.4 mg/L molecular SO_2). Based on these results, 200 mL headspace sample sizes (i.e. repeated sampling) were used for later analyses on commercial wines.

Unexpectedly, the y-intercept was negative for all calibration curves, indicating that a minimal amount of SO_2 (approximately 0.07 mg/L molecular SO_2) was necessary to effect a color change. An explanation for this phenomenon is not clear, but may be related to differences in the manufacturer's optimized flow rate through the GDT versus the flow rate used in our experiments (Haag 2001). Regardless, the offset is well below typical recommendations for molecular SO_2 necessary to prevent spoilage, and thus the method should still be useful for most wines.

Table 12: Figures of merit for the HS-GDT method, based on analysis of calibration standards in either aqueous or 10% ethanol (model wine) solutions, adjusted to pH 3.56

HS-GDT conditions	Intercept [a]	Slope [a]	r^2 [a]	Mean CV for molecular SO_2 > 0.4 mg/L (%) [b]	Detection limit for molecular SO_2 (mg/L) [c]
50 mL headspace sample, aqueous [d]	-0.48	5.84	0.99	15%	0.21
50 mL headspace sample, model wine [d]	-0.60	5.96	0.75	18%	0.59
200 mL headspace sample, aqueous	-0.48	5.92	0.97	9%	0.29
200 mL headspace sample, model wine	-0.55	5.99	0.99	8%	0.21

[a] Intercept, slope, and r^2 are based on 1/x weighted best-fit lines for plots of P_{SO2} (μL/L) vs. molecular SO_2 (mg/L)
[b] CV calculated as (standard deviation/mean).
[c] Calculated as 3 × signal independent noise, σ
[d] Responses for 50 mL sample sizes were scaled by a factor of 4, as described in Methods

Comparison of HS-GDT and A-O Methods:

Twenty seven commercial wines (9 red, 14 white, and 4 blush) were evaluated by both HS-GDT and A-O methods. HS-GDT measurements in P_{SO2} were converted to free SO_2 values. The wines covered a wide range of free SO_2 concentrations (4-51 mg/L by A-O). The average precision for HS-GDT measurements of wines with > 0.4 mg/L molecular SO_2 (mean CV = 17%) was different than values achieved with calibration standards, and the limit of detection (0.06 mg/L) was 3-fold lower than the values achieved for calibration standards (Table 12). The reason for the reduced precision in real wines may be due to limiting the number of repetitions to those that would fit on one tube, thus increasing the variability on the wines with higher levels of SO_2.

The lower limit of detection for HS-GDT in real wines as opposed to calibration standards may is also unclear. Potentially, the imprecision observed in model wine for low concentration standards (<0.2 mg/L) arises in part from variable degrees of oxidation. In real wines, these losses may be buffered by dissociation of the bound SO_2 pool or minimized by the presence of the other anti-oxidant wine components

A very good correlation was observed between the HS-GDT and A-O methods in white and blush wines (r^2 = 0.97, Figure 16), but much weaker correlation was observed for red wines (r^2 = 0.72, Figure 16). HS-GDT values for red wines were on average only 49% (range = 24-76%) of the A-O value recorded for the same wine, with absolute differences approaching 25 mg/L for some wines.

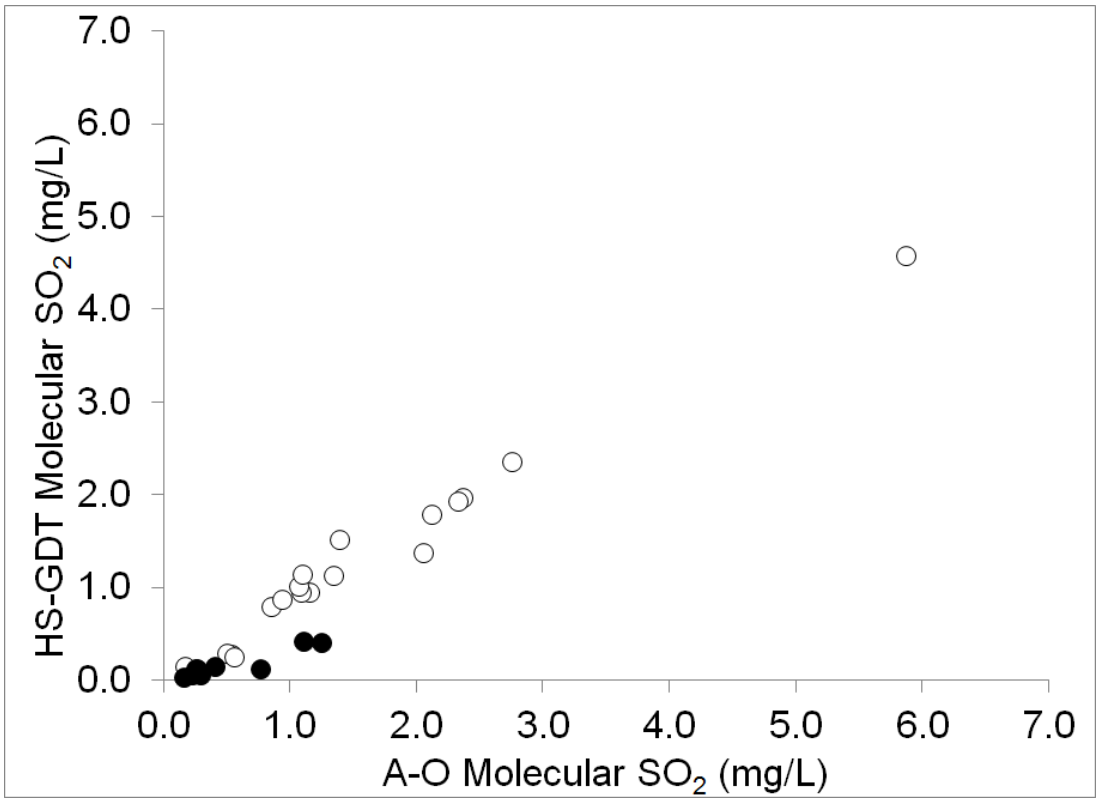

Figure 16 Plot of molecular SO_2 measured by the headspace gas detection tube (HS-GDT) method vs. an Aeration-Oxidation (A-O) reference method for 9 red wines, 14 white wines and 4 blush wines. Each data point represents the SO_2 concentration of an individual wine (mean of n=3). Best fit-lines equations determined with 1/x weighting were: $y = 0.82x + 0.05$, $r^2 = 0.97$ (white and blush wines, open circles) and $y = 0.32x + 0.05$, $r^2 = 0.72$ (red wines, solid circles).

This large discrepancy between the two methods for red wines is likely an artifact of A-O sample preparation steps (acidification, dilution) and its long sampling time (10-15 minutes) resulting in dissociation of weakly bound anthocyanin-bisulfite adducts (Burroughs 1975) and erroneously high measurements of free SO_2. Although equilibrating the 10 mL sample in contact with 50 mL headspace will slightly decrease wine SO_2 concentrations due to volatilization in the HS-GDT method, the effect will be minimal (<1% change in bisulfite or molecular SO_2 assuming equilibrium) because the pH is not changed. Similar results have been observed in the limited number of other studies that have used non-perturbing methods to quantify free SO_2 in red wines.

For example, free SO_2 values were up to an order of magnitude higher by Ripper and A-O as compared to measurement by CE where equilibria were not perturbed (Bogren 1996). A group using a non-perturbing headspace GC method reported a 45% higher free SO_2 value in a red wine as compared to A-O, although this report used only a single wine adjusted to different pH values rather than multiple wines (Davis et al. 1983). The HS-GDT and A-O methods were in better agreement for white and blush wines. Based on the slope of the regression analysis (Figure 16), free SO_2 values by HS-GDT were 87% that of the A-O method. This slight discrepancy (13% lower by HS-GDT) is comparable to what was observed in a previous report comparing CE and A-O (Bogren 1996). This previous work suggested that any discrepancy could be explained by dissociation of weakly bound adducts of bisulfite and diacetyl, pyruvate, and other carbonyl species during A-O analyses. A separate report comparing HS-GC and A-O methods for a single white wine reported differences of <5% (Davis et al. 1983), but this work used a pK_a value for SO_2 (1.81) that was uncorrected for the effects of ethanol, which would have resulted in a higher free SO_2 value based on measured headspace SO_2. In contrast, a good correlation was observed between A-O and a modified A-O approach using GDT for both red and white wines (Pegram et al 2013); in that approach, the wine pH was decreased to <2 to favor SO_2 volatilization, resulting in perturbation of equilibrium conditions and measurement of weak bisulfite adducts.

Predicted vs. Actual Discrepancies in Molecular SO_2 in Red Wine – the Role of Anthocyanin Bisulfite Adducts:

To evaluate the hypothesis that the large differences between A-O and HS-GDT methods for red wines could be explained by dissociation of anthocyanin-bisulfite adducts, the concentration of anthocyanin-bisulfite adducts was estimated by solving a system of non-linear equations involving the equilibria of true free HSO_3^- and flavylium, quinoid, and bisulfite adduct forms of anthocyanins. Measured values of total anthocyanins, pH, and free SO_2 by A-O (assumed to be true free SO_2 + anthocyanin-bound adducts) were used in these calculations; literature value for the anthocyanin hydration constant (pK_h) of 2.94 was used for anthocyanins from *V. vinifera* wines (Brouillard and El Hage Chahine 1980) and a value of $pK_h = 2.6$ was used for the diglucoside containing hybrid-based wines (Francis and Markakis 1989). Only adducts of flavylium ion forms and bisulfite were considered in the model, as the other anthocyanin forms do not bind bisulfite (Timberlake and Bridle 1967). The effects of self-aggregation on flavylium cations were ignored in the model. The apparent equilibrium constant for dimerization, K_d, was calculated to be no greater than 6000 for all red wine samples (estimated from Eq A11 in Houbiers, et al 1998), which would have resulted in only a small overestimation (less than 10%) in flavylium ion concentrations used in our model. The observed discrepancy in molecular SO_2 between the A-O and HS-GDT methods was plotted against the estimated concentration of anthocyanin-bisulfite adducts, expressed as SO_2 equivalents (Figure 17a); it exhibits a strong positive correlation ($r^2 = 0.936$), and has a slope near unity (0.94), supporting our hypothesis. Similarly, molecular SO_2 by HS-GDT plotted

against A-O values which were corrected for the calculated dissociation (Figure 17b) yielded a modest r^2 of 0.755 and a slope of 0.765, very similar to the curve for the white and blush wines as shown in Figure 16.

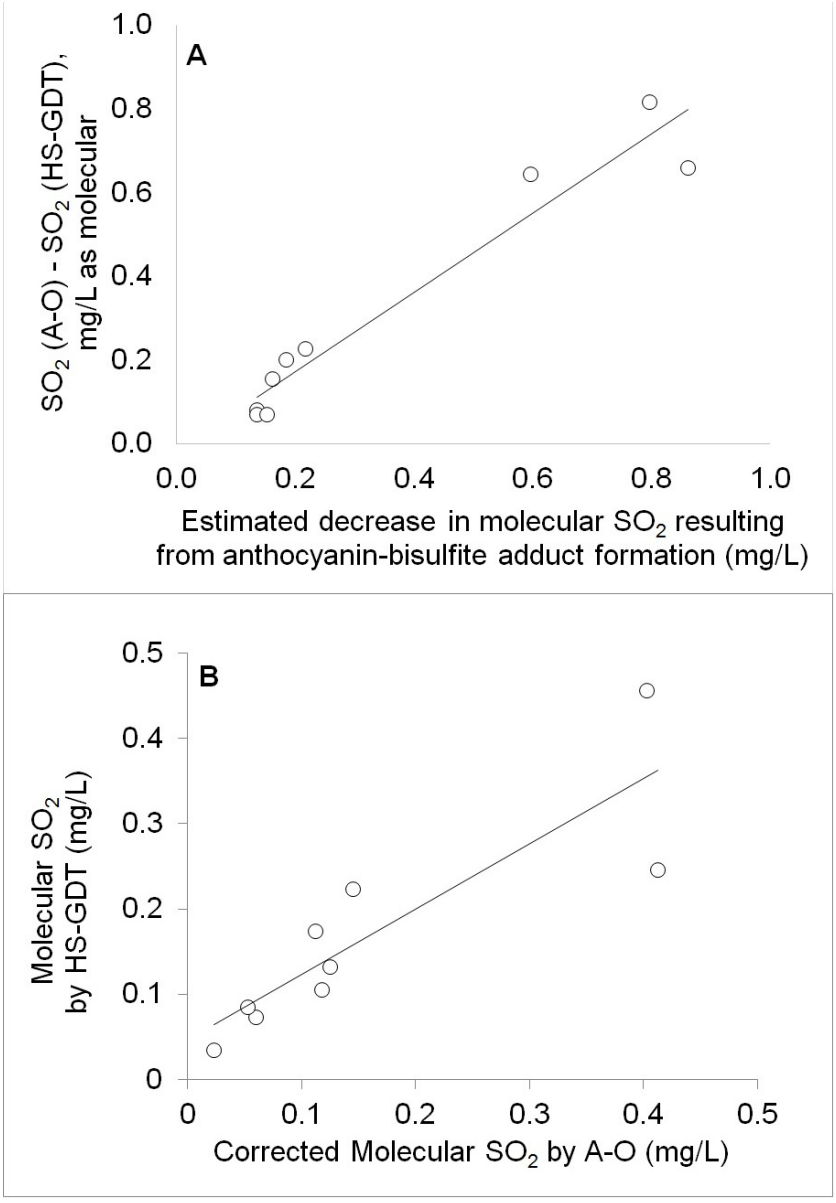

Figure 17: A: Plot of differences in molecular SO_2 by A-O and HS-GDT methods vs. estimated decrease in molecular SO_2 resulting from anthocyanin-bisulfite adduct formation. Best fit-lines equations determined with 1/x weighting were y = 0.9436x - 0.0161, r^2= 0.9362. B: Plot of molecular SO_2 measured by the headspace gas detection tube (HS-GDT) method vs. the Aeration-Oxidation (A-O) reference method corrected for theoretical anthocyanin-bisulfite adduct formation for 9 red wines. Each data point represents the SO_2 concentration of an individual wine (mean of n=3). Best fit-lines equation determined with 1/x weighting were: y = 0.7652x + 0.0461, r^2 = 0.76.

Based on linear regression analyses, other measured wine components (tannins, glucose + fructose, ethanol) were uncorrelated with the percentage difference between A-O and HS-GDT methods (data not shown) in either red or white wines. The lack of correlation of method difference with glucose concentration is not surprising. Dissociation of glucose-bisulfite complexes has been suggested as a potential problem to standard approaches to SO_2 measurement (Burroughs and Sparks 1964), and several wines included in the study had fructose + glucose concentrations > 50 g/L. However, the first order rate constant for the dissociation of the glucose-bisulfite complex is reportedly 3.7×10^{-4} min^{-1} at pH~1 (Vas 1949), which is slow as compared to the time necessary for A-O or Ripper analysis, and considerably slower than the first order rate constant for the dissociation of anthocyanin-bisulfite adducts (0.2 min^{-1}) (Brouillard and El Hage Chahine 1980).

Conclusion

The HS-GDT method described here provides a convenient and inexpensive means for determining molecular and free SO_2 concentrations typically found in wines without perturbing equilibria of free and bound SO_2 forms. Our work also supports previous (and often overlooked) observations that standard approaches to SO_2 measurement overestimate free and molecular SO_2 due to dissociation of weakly bound bisulfite adducts. This is particularly notable in red wines where discrepancies up to 5-fold were noted, and is also a minor issue in white and blush wines. Thus, the HS-GDT method may be of use to researchers interested in determining the mechanisms of wine oxidation or better establishing microbial tolerance to SO_2.

Because of the low requirements for consumables and fast analysis time (~ 5min), HS-GDT could also be adopted for use by winemakers for quantifying SO_2 without the need for a specialized lab space.

Although additional validation of the HS-GDT method is desriable, traditional approaches to method validation, such as standard addition and percent recovery calculations, are particularly challenging when working with free (or molecular) SO_2 in wine matrices. Unlike the validation of a total SO_2 method, when any addition of SO_2 to a wine matrix would be reflected in the same increase in the total SO_2 level, addition of SO_2 will result in variable increases the free or molecular SO_2 level because of varying concentrations of SO_2 binders across wines. Standard addition could potentially be done by measurements of major SO_2 binders in wine to calculate the expected increase in free SO_2 following SO_2 addition. Because of the challenges of using standard addition, further validation of the HS-GDT method could be done by comparison with free (or molecular) SO_2 values determined by other non-perturbing techniques, such as headspace GC, headspace IR, and CE.

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CHAPTER 3: GAS DETECTION TUBES FOR MEASUREMENT OF MOLECULAR AND FREE SO_2 IN WINE

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Abstract:

Accurate measurements of the major active sulfur dioxide species in wine (HSO_3^- and SO_2) are important to studies of wine oxidation chemistry and microbial stability. These so-called "free SO_2" forms are traditionally measured by iodometric titration, aeration-oxidation (A-O) or by comparable modern variants. These standard approaches require sample dilution and/or pH shifts. We describe a simple headspace method for quantifying either molecular or free SO_2 in wine utilizing colorimetric gas detection tubes (HS-GDT) that avoids perturbation of equilibria. Henry's coefficients were constant over ethanol concentrations of 0-17% v/v. The HS-GDT method limit of detection in a model wine (pH 3.56, 12% v/v ethanol) was 0.21 mg/L molecular SO_2, and was linear over 0.29-1.13 mg/L. Good agreement was observed between HS-GDT and A-O for white and blush wines, but molecular SO_2 in red wines averaged 2-fold

lower by HS-GDT, likely because the standard A-O approach results in dissolution of weakly bound bisulfite-anthocyanin adducts.

Introduction

The first written description of using sulfur dioxide (SO_2) as a wine preservative dates to 1670 (McGovern 2003), in which it is suggested to add wine or other alcoholic beverages to a container filled with fumes from burnt sulfur. In modern winemaking, SO_2 is a near ubiquitous addition for prevention of wine oxidation and microbial spoilage (Boulton et al. 1999). While low levels of SO_2 are formed endogenously by yeast metabolism during fermentation, typically <10 mg/L (Suzzi et al. 1985), larger amounts of SO_2 are typically added following alcoholic and/or malolactic fermentation and then at regular intervals throughout storage, in the form of compressed SO_2 gas, liquid SO_2 solutions, or potassium metabisulfite (Boulton et al. 1999).

SO_2 Species in Solution.

In wine and other food systems, SO_2 exists as multiple species with different activities, sensory effects, and regulatory requirements. The roles and typical targets or constraints for these species are summarized in Table 13.

Because SO_2 is a weak acid (pK_{a1} in water at 20 °C = 1.81), the predominant species at wine pH (3-4) is bisulfite (HSO_3^-), with minor concentrations of the free acid SO_2 species (<5%) and negligible concentrations of sulfite (SO_3^{2-}, pK_{a2} = 7.2) (Figure 18). The sum of these species (molecular, bisulfite, sulfite) is referred to as "free SO_2", and as described later, free SO_2 (mostly bisulfite) is the primary species involved in wine redox chemistry.

Table 13 - Properties of different species of SO_2 in wine

SO_2 Fraction	*Major Role*	*Typical target or constraint (as SO_2 equivalents)*
Molecular SO_2 (free acid form)	Antimicrobial	Microbial stability: typically, 0.5-0.8 mg/L. Sensory threshold (irritation) for irritation: 2 mg/L
Bisulfite (HSO_3)	Antioxidant, accounts for >95% of Free SO_2 (see below)	
Sulfite (SO_3^{2-})	Negligible (<0.01% of bisulfite at wine pH)	
Free SO_2 (Sum of Molecular, HSO_3^-, SO_3^{2-})	Antioxidant	Preventing wine oxidation: typically, 20-40 mg/L
Bound Bisulfite Adducts	Contribute to total SO_2; May have minor antimicrobial activity. Weakly bound can dissociate and add to free SO_2 pool following loss of free bisulfite	
Total (Free + Bound SO_2	Regulatory	Regulated in most countries: In US, must be < 350 mg/L for all wines

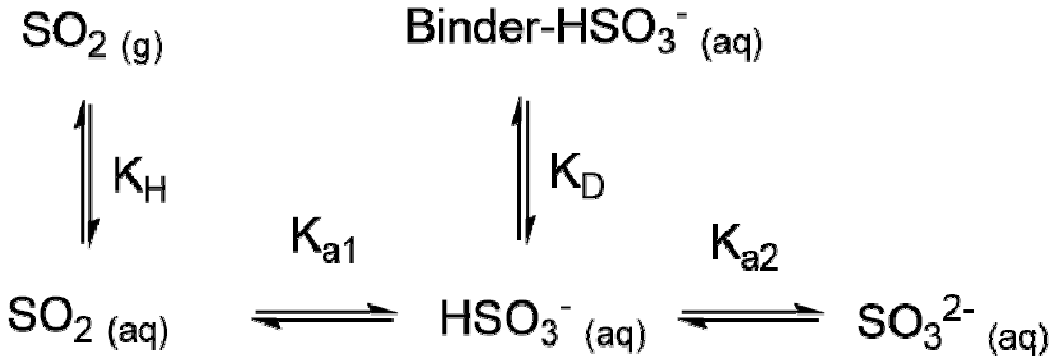


Figure 18 Equilibrium relationships among SO_2 species in wine. K_{a1}, K_{a2}: First and second acid-dissociation constants; K_H: Henry's Coefficient; K_D: Dissociation constant of a sulfonate adduct ("bound SO_2" species)

HSO_3^- may covalently bind with electrophiles to form the fraction referred to as "bound SO_2". In most wines, the major contributor to bound SO_2 is acetaldehyde, which will form a *strongly bound* α-hydroxysulfonic acid adduct with bisulfite. Because of the low dissociation constant ($K_d = 1\times10^{-6}$) of the acetaldehyde-bisulfite adduct, >99% of acetaldehyde will be bound in a wine with a typical free SO_2 concentrations of 20-40 mg/L (Boulton et al. 1999). Other carbonyl species, particularly α-keto acids from fermentation (e.g. pyruvate, α-ketoglutarate) or oxo-acids from spoilage organisms (e.g. glucuronic acid) may form *weakly bound* adducts, ($K_d > 1\times10^{-5}$) (Leonard F. Burroughs and Arthur H. Sparks 1973). In sweet wines, binding by glucose may represent a significant pool of bound SO_2, in spite of its relatively low affinity for bisulfite ($K_d = 0.6$) (Leonard F. Burroughs and Arthur H. Sparks 1973). In red wines, anthocyanins can also represent a major sink of bound SO_2 due to their relatively high binding constants ($K_d = 1\times10^{-5}$) (R. Brouillard and J. M. El Hage Chahine 1980). Over a range of free SO_2 concentrations (30 - 90 mg/L – on the high end for commercial wines) and pH values (3.0-3.8) approximately 70-85% of monomeric anthocyanins are reported to be bound (Usseglio-Tomasset et al. 1982a). Finally, total SO_2, refers to the sum of free and bound forms of SO_2 species and is regulated in many countries due to health concerns (Margalit and Crum 2004).

Activity of SO_2 Species - Antimicrobial and Antioxidant:

SO_2 may be added to grapes or must prior to fermentation to inhibit spoilage micro-organism activity, favor *S. cerevesiae* growth, or inhibit polyphenol oxidase and slow enzymatic browning (Boulton et al. 1999). However, SO_2 is more widely used as an antimicrobial and antioxidant after fermentation. In spite of its low concentration

(<1% of free SO_2), the molecular SO_2 species has been demonstrated to be the species responsible for antimicrobial activity in juices and wine (Macris and Markakis 1974b). The mechanism of action is believed to involve the passive diffusion of neutral molecular SO_2 across the cell membrane, followed by formation of HSO_3^- at intracellular pH (Divol et al. 2012). Several mechanisms for toxicity are then available to HSO_3^-, including nucleophilic addition to key metabolites, damage to DNA, and reduction of protein disulfide bridges. Typical recommendations for preventing microbial spoilage are in the range of 0.5-0.8 mg/L molecular SO_2 for dry wines, with higher concentrations sometimes recommended for sweet wines (Boulton et al. 1999; Zoecklein et al. 1999).

As mentioned previously, free HSO_3^- is the major species involved in redox reactions. The primary pathway by which O_2 is consumed in wine under abiotic conditions involves initial activation of triplet O_2 to a reactive oxygen species (ROS, singlet O_2) by transition metal catalysts (particularly Fe^{2+}) (Danilewicz 2007). The ROS can then react with 1,2-diphenol moieties in wine to yield 1,2-quinones and H_2O_2, which can participate in further oxidative reactions with wine components, e.g. by coupling with nucleophiles or oxidizing alcohols to aldehydes (Ugliano 2013). HSO_3^- can exert its antioxidant influence by rapidly reacting with 1,2-quinones and H_2O_2 (Ugliano 2013), although it may also accelerate the rate of O_2 consumption by facilitating regeneration of transition metal catalysts and reacting with quinones (Danilewicz 2012). The many potential roles of HSO_3^- in wine oxidation are summarized in Figure 19.

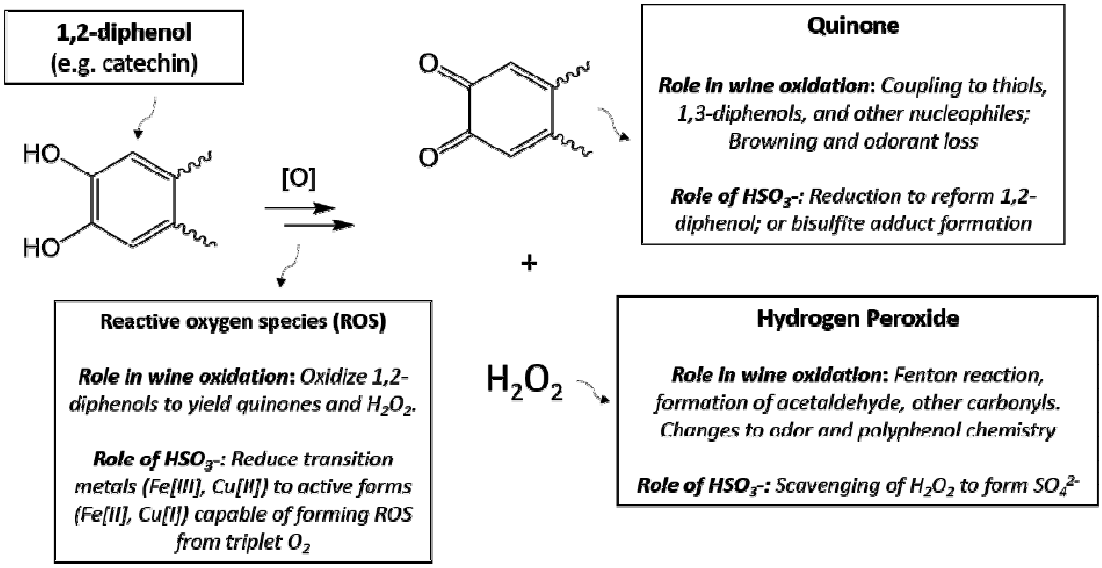


Figure 19 Overview of role of HSO_3^- during wine oxidation

The bound SO_2 fraction is thought to have weaker anti-microbial and anti-oxidant activity than free SO_2, but some forms (particularly acetaldehyde-bisulfite adducts) appear to have a weak inhibitory effect against lactic acid bacteria (Wells and Osborne 2012). Weakly bound bisulfite adducts do not appear to directly participate in oxidation reactions, but may dissociate to partially replenish free SO_2 following loss of the latter.

Current methods for measurement of molecular and free SO_2

As compared to other SO_2 species, winemakers are generally most concerned with measurement of free and molecular SO_2 (Zoecklein et al. 1999), as bisulfite is the major species responsible for preventing wine oxidation and molecular SO_2 is the main species responsible for preventing microbial growth. If the pH is known, the free SO_2 can be used to calculate the molecular SO_2 concentration via the Henderson-

Hasselbalch equation (Boulton et al. 1999). Common methods for measuring free SO_2 in wines can be classified into two categories:

Approach 1, Direct colorimetric or titrimetric measurement:

These methods utilize an oxidizing reagent, e.g. iodine in the "Ripper" titration of an acidified wine sample (Iland 2004) and *p*-rosaniline in the "fuschin" colorimetric assay (Joslyn 1955c). While these assays are relatively straightforward to perform, the absence of a separation step results in interferences from other reducing compounds and suspect interlaboratory reproducibility (Joslyn 1955b, 1955c; Vahl and Converse 1980b).

Approach 2, Isolation of molecular SO_2 following sample acidification and prior to quantification:

Improved selectivity and reproducibility for free SO_2 measurements can be achieved by initially acidifying the sample and then separating molecular SO_2. In many wineries, a modified version of the classic Monier-Williams method is used, often referred to as 'aeration-oxidation' or A-O (Buechsenstein and Ough 1978a; Rankine and Pocock 1970a). Using an aspirator, SO_2 is swept from the acidified sample by a gas stream into a receiving flask containing H_2O_2. Reaction of H_2O_2 and SO_2 yields H_2SO_4 which can then be titrated to calculate the original concentration of SO_2. A similar but more readily automated approach to distillation is to use a gas-permeable membrane to separate SO_2 from the acidified wine sample prior to quantification of SO_2 by some means, e.g. colorimetric (Bartroli et al. 1991) or electrochemical (Ruiz-Capillas and Jiménez-Colmenero 2009). More recently, the use of colorimetric gas detection tubes for quantification of SO_2 has been described, and is

reported to yield near-identical results with lesser requirements in time and cost (Pegram et al. 2013).

Problems with classic approaches, and alternate approaches to artifact-free measurements of free and molecular SO_2

As has been pointed out by multiple authors, the acidification and dilution steps inherent to the approaches described above can result in the dissolution of sulfonate adducts and overestimation of free and molecular SO_2 (Boulton et al. 1999; Burroughs 1975b; Rankine and Pocock 1970a). This is less of a concern for more stable sulfonate adducts like acetaldehyde-bisulfite, but is of greater concern for less stable bound forms such as anthocyanin-bisulfite adducts with dissociation rate constants on the order of a 0.2 min^{-1} under acidic conditions (Raymond Brouillard and Jean Michel El Hage Chahine 1980) and thus may dissociate over the time frame of classical SO_2 analyses. The degree to which the standard approaches overestimate HSO_3^- is not well established, but has been reported to be up to an order of magnitude too high for red wines (L.N.H. Bogren 1996).

Despite this recognized shortcoming of classic approaches, only few papers report using techniques which would avoid disturbance of SO_2 wine equilibria. Capillary electrophoresis (CE) can be used to measure bisulfite without artifactual dissolution of bound SO_2 forms (L.N.H. Bogren 1996), but this approach has not been widely adopted, likely due to the technical complexity of the analysis. Ion chromatography (IC) has been used for measurement of total SO_2 in wine following pH adjustment to 7.5 or greater (Kim 1990), but it is unclear if this approach would be appropriate for free SO_2, i.e. measurement of HSO_3^- without pH adjustment. Alternatively, the

molecular SO_2 concentration can be calculated from the headspace SO_2 concentration of an equilibrated sample, assuming the Henry's coefficient is known (see Figure 18). HSO_3^- and free SO_2 can then be calculated from molecular SO_2 via the Henderson-Hasselbalch equation. Good agreement was observed between A-O and a headspace gas chromatography (HS-GC) method for white wines (Davis et al. 1983b) but A-O yielded two-fold higher values for a red wine, presumably due to less dissolution of weak anthocyanin-bisulfite adducts. Infrared spectroscopy (Henningsen 2003), inductively-coupled plasma optical-emission spectrometry (ICP-OES) (Cmelik et al. 2005b) and flame molecular absorption spectrometry (Huang et al. 2008b) can also be used for measurement of vapor-phase SO_2 from wine. However, to our knowledge, artifact-free approaches to measurement of SO_2 (e.g. headspace methods, CE) have not been widely adopted in either wineries or research labs, possibly due to the cost and complexity of the techniques. This will be a constraint on future studies intending to define minimal SO_2 concentrations necessary to inhibit microbial spoilage in real wines, or in understanding wine oxidation mechanisms.

Gas detection tubes (GDT) were originally developed for the mining industry and consist of a glass tube packed with color-sensitive reagent that stains following exposure to a target analyte. GDT have been used for several wine applications including quantification of H_2S during fermentation (Park 2008) and more recently for measurement of free SO_2 in a modified A-O protocol (Pegram et al. 2013). This latter application involved initial acidification and dilution of the sample, and thus would be expected to suffer from similar artifacts as reported for other standard approaches. We

chose here to evaluate the use of GDT for quantifying headspace SO_2 above an unaltered wine sample, and using this information to calculate molecular and free SO_2 in the wine.

Materials and Methods

Chemicals:

Potassium metabisulfite (97% w/w) and ethanol (95% v/v) were obtained from Acros Organics (Geel, Germany). Potassium bitartrate (99% w/v), hydrogen peroxide (30% w/v), sodium hydroxide (0.01 N) and *o*-phosphoric acid (85% w/w) were obtained from Fisher Scientific (Waltham, MA). A nominally 25% phosphoric acid solution was prepared as a 2.38:1 dilution of 294 mL phosphoric acid (85%) with 700 mL DI water. Hydrochloric acid (36.5% w/w) was obtained from BDH Merck (Poole Dorset, United Kingdom).

SO_2 working standards:

SO_2 stock solutions at nominal concentrations of 1000 and 10000 mg/L as SO_2 were prepared weekly by dissolution of potassium metabisulfite in a solution of methanol in water (10% v/v) to avoid SO_2 auto-oxidation. Working standards for analysis were prepared as needed by addition of an appropriate volume of a stock solution to 100 mL of a saturated potassium bitartrate buffer (pH 3.56). Iodometric titrations of the Ripper method were used to determine the actual SO_2 concentration of the stock and working solutions.

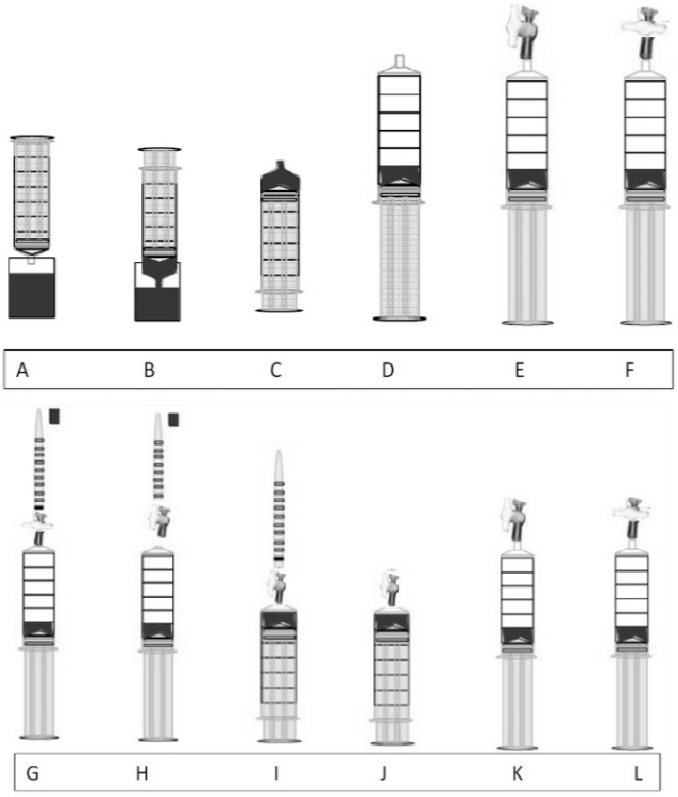
SO_2 Measurements by Aeration-Oxidation (A-O):

A-O was performed according to established protocols to determine free SO_2 (Iland 2004). Molecular SO_2 concentration was then determined by rearrangement of the Henderson-Hasselbalch equation (Equation 1) using ethanol-, ionic strength-, and temperature- corrected pK_{a1} values from the literature (Usseglio Tomasset and Bosia 1984). Ionic strength was estimated to be constant at 0.50M based on typical literature values (Prenesti et al. 2004).

Equation 1: [Molecular SO_2] = [Free SO_2] / $(1+10^{pH-pKa})$

Protocol for SO_2 Measurement by Headspace Gas Detection Tube (HS GDT):

The HS-GDT protocol is summarized in Figure 20. The apparatus consists of a 60 mL Becton Dickinson polypropylene syringe with a Luer tip, and a polypropylene plunger with a polyisoprene latex-free plunger tip. A customized dispensing stop was constructed from an additional syringe body. The Luer tip was fitting to a two- way polycarbonate male Luer stopcock, and a short piece of silicone tubing connected the other end of the stopcock to a Gastec 5Lb GDT (Gastec Corporation; Fukayanaka, Japan). If the GDT had been previously used, the "start" point of color transition was marked on the tube with a fine point permanent marker. If the tube had not been used, the beginning of the packing material indicated the run "start".

1) 

2) *Figure 20 Schematic of HS-GDT measurement. Step A: Prepare syringe and sample; B: Sample 10 mL of wine into syringe; C: Invert syringe; D: Pull plunger to create 50 mL of headspace in syringe; E: Place valve on syringe; F: Close valve and equilibrate syringe for 5 min; G: Connect Gas Detection Tube (GDT) with stain location marked by felt tip marker; H: Open valve; I: Quickly (10 sec) depress syringe to expel headspace; J: Remove GDT, mark new stain location; K, L: Repeat starting with Step D.*

For each analysis, the syringe was used to sample 10 mL of a wine or working standard and the syringe barrel then withdrawn further to create an additional 50 mL of headspace. Once the stopcock was closed and the syringe stop set at 10 mL, the syringe was placed nose-up and allowed to equilibrate for 5 min. A GDT was then connected, the stopcock was opened, and the syringe was depressed at a constant rate to the stop over a 10 sec period. Following expulsion of headspace through the GDT,

the colorimetric reaction was allowed to stabilize for 1 min. This action was performed four times, such that a total volume of 200 mL was sampled through the tube. The distance from the stain startpoint to endpoint were measured in millimeters using a ruler.

Determination of P_{SO2}, Molecular SO_2, and Free SO_2 from raw GDT measurements:

The commercial GDT have printed scales in units of SO_2 partial pressure (P_{SO2}) in ppm (μL/L). To overcome the poor resolution of the scale, a best fit cubic interpolation function, f(x) was determined for each lot of tubes. To achieve this, the distance from the origin (in mm) of each printed P_{SO2} value (in μL/L) was recorded, and the best fit function, f(x), was calculated using Microsoft Excel 2010 (Redmond, WA).

Following a sample analysis, P_{SO2} was calculated from the locations of the startpoint (x_{start}) and the endpoint (x_{end}) measurements in mm using the aforementioned cubic interpolation function, f(x).

Equation 2: $P_{SO2} = f(x_{end}) - f(x_{start})$

The molar concentration of molecular SO_2 can then be calculated by dividing P_{SO2} by either an experimentally determined or literature Henry's coefficient. In practice, we achieved better results by utilizing calibration curves to relate P_{SO2} to molecular SO_2, due to a consistent non-zero intercept associated with the HS-GDT.

Ethanol Dependence of Henry's Coefficient for SO_2:

SO_2 solutions (1 mg/L) were prepared from a 100 mg/L SO_2 stock solution in 10 mL of 1M HCl (pH 0) to favor the molecular SO_2 form and isolate the effect of ethanol on SO_2 volatility. These solutions were prepared with varying ethanol concentration (0, 8,

11, 14 and 17% v/v) by addition of the stock solution directly to syringes containing the hydroalcoholic solutions to minimize losses due to volatization. The samples were analyzed by the HS-GDT method (n=8 for each ethanol concentration). The Henry's coefficient (K_H) of SO_2 was determined from P_{SO2} and the molecular SO_2 concentration.

Equation 3: $K_H = P_{SO2}$ / [Molecular SO_2]

Calibration Curve and Figures of Merit for HS-GDT Method:

Standards solutions at nominal concentrations of 5, 10, 15, 20, 30 and 40 mg/L as free SO_2 were made in a potassium bitartrate buffer with ethanol 12% v/v. Molecular SO_2 concentration was then determined from the Henderson-Hasselbalch equation using ethanol-, ionic strength-, and temperature- corrected pK_{a1} values from the literature (Usseglio Tomasset and Bosia 1984).

Standards were then analyzed by the HS-GDT method in replicate (n=12 per standard, total of 72 analyses). The coefficient of variation for each standard concentration was calculated as the standard deviation divided by the mean. Linearity was evaluated by linear regression of measured P_{SO2} vs. expected P_{SO2}. The concentration independent noise (σ) was estimated by Pallesen's method (Mac Berthouex and Brown 2002), and the detection limit (LOD) was calculated as $3 \times \sigma$.

pK_a of SO_2 as a Function of Ethanol Concentration:

SO_2 solutions (50 mg/L) were prepared in a potassium bitartate buffer with varying ethanol concentrations (0, 7, 14 or 20% v/v) and pH values (3.1, 3.3, 3.5, and 3.7). The HS-GDT protocol was then used to determine P_{SO2} which was then converted to

molecular SO_2. The apparent pK_a for each solution was calculated from the measured molecular SO_2 and known free SO_2 concentration using the Henderson-Hasselbalch equation. The room temperature at the time of the experiment was 21.9°C.

A-O vs. HS-GDT: Comparison with Commercial Wines:

Commercial wines (10 red, 12 white, 5 blush) from the 2009-2013 vintages and representing multiple countries of origin (US, Australia, Argentina) were purchased at local stores. Molecular SO_2 was determined for each wine by both A-O and HS-GDT approaches. Additional wine parameters were analyzed by ETS Laboratories (St Helena, CA): pH (by meter), alcohol (FTIR), volatile acidity (as acetic acid, measured by enzymatic assay), malic acid (enzymatic) and glucose plus fructose (enzymatic) were measured by accredited methods; quercetin glucosides, catechin, tannins, polymeric anthocyanins and total anthocyanins were measured by HPLC (Waterhouse et al. 1999a). Monomeric anthocyanins were calculated as the difference in concentration between total and polymeric anthocyanins.

Statistical Analyses:

SAS 9.0 was used for statistical analysis.

Results and Discussion

Effects of Ethanol on Henry's Coefficient (K_H) of SO_2:

Using the HS-GDT assay, P_{SO2} was measured for 1 mg/L SO_2 solutions (pH 0, 21 °C) over a range of ethanol concentrations. Samples were acidified to well below the pK_{a1} of SO_2 to eliminate any confounding effects from ethanol-induced changes to pK_{a1}. The mean value for P_{SO2} based on the manufacturer markings was 5.92 µL/L, which equates to a K_H = 0.38 Atm/M at 21 °C. This is highly comparable to a recent

previous report of 0.28 Atm/M for SO_2 in water at 25 °C (Na Zhang et al. 2013). Furthermore, P_{SO2} (and thus K_H) were ethanol independent over the range 0-17% v/v ethanol (Table 14).

Table 14 Effects of ethanol on SO_2 partial pressures (P_{SO2}) in a pH 0 matrix (>95% Molecular SO2 form). Lack of significant differences indicates that Henry's coefficient is ethanol-independent over the range 0-17% abv

Ethanol (%v/v)	*P_{SO2} (µL/L)*	*Standard error*
0	5.98	0.19
8	5.99	0.18
11	5.91	0.14
14	5.75	0.17
17	5.96	0.27

The independence of K_H from ethanol concentration is somewhat surprising, since the volatility of wine components such as esters and higher alcohols are well known to decrease in real or model wines as compared to 100% aqueous systems (Mouret et al. 2012b). This lesser effect may arise from the greater polarity of SO_2 as compared to other wine volatiles, which diminishes its ability to participate in hydrophobic interactions with ethanol.

Effects of Ethanol Concentration on pK_{a1} of SO_2:

P_{SO2} was determined by HS-GDT for standard solutions containing a range of ethanol and SO_2. pK_a values were then calculated for each ethanol concentration, and these values then used to determine a best-fit line ($r^2 = 0.97$) for pK_a as a function of ethanol concentration

Equation 4 $pK_a = 0.0137 \times (\text{Ethanol \%v/v}) + 1.83$

Eq 4 yields predicted acid-dissociation constants of pK_a = 1.83 at 0% ethanol and pK_a = 1.96 at 10% alcohol, comparable to pK_a values determined elsewhere by titrimetry: 1.81 and 2.00, respectively *(Usseglio Tomasset and Bosia 1984)*. Some wine texts recommend using the pK_a of SO_2 in water at 20 °C (1.81) for calculations of molecular SO_2 from free SO_2 in wine regardless of the ethanol concentration *(Jacobson 2006b; Margalit and Crum 2004)*, which would lead to a non-trivial underestimation of the actual molecular SO2 concentration in typical wines: A wine with an alcohol content of 14.6% v/v and analyzed at 23.5 °C, as was done in our experiments, has a predicted pK_a value of 2.15, which would lead to over a 2-fold error in calculated molecular SO_2. In any case, our ability to match values observed with other methods suggests that HS-GDT is an appropriate method for measurement of molecular SO_2 and further estimates of molecular SO_2 in this paper are based on ethanol-, temperature-, and ionic strength- corrected pK_a values.

Figures of Merit for the HS-GDT Method:

Figures of merit were determined for the HS-GDT method using SO_2 standards (0.14 to 1.13 mg/L molecular SO_2) in a model wine containing 12% v/v ethanol. Results are shown below in Table15. Linearity (r^2 = .97) was satisfactory, and reproducibility was good (coefficient of variation < 10%) for concentrations at or above typical target values for molecular SO_2 in wine, 0.5 mg/L. The limit of detection was determined to be P_{SO2} = 0.74 μL/L, which equates to a molecular SO_2 of 0.21 mg/L at pH 3.56. Predicted molecular SO_2 concentrations were within ±0.06 mg/L of the expected values. Since the pK_{a1} of SO_2 in wine is typically around 2.1 following

corrections for temperature, ionic strength, and ethanol (Usseglio Tomasset and Bosia 1984), this equates to free SO_2 within ±2 mg/L of expected values – comparable to the reproducibility expected for conventional methods like A-O (Pegram et al. 2013).

Interestingly, the y-intercept was negative, and negligible signal could be observed for standards with $P_{SO2} < 0.25$ μL/L (~.07 mg/L molecular SO_2 for the system tested). This reason for this effect is unknown, but higher vapor-phase concentrations yielded a linear response, and the method is still useful.

The detection limit of the HS-GDT method in model wine (0.21 mg/L as molecular SO_2) is above what is typically reported for the A-O method. However, it is below typical target molecular SO_2 levels for wine. These targets vary among source and wine type, but typical values recommended to inhibit microbial spoilage range from 0.5 to 0.8 mg/L molecular SO_2 (Boulton et al. 1999).

Table 15 SO_2 partial pressures, (P_{SO2}), calculated molecular SO_2, and coefficients of variation (%CV) for calibration standards in model wine using the HS-GDT approach

Molecular SO_2 of Standard (mg/L) [a]	*P_{SO2} by HS-GDT (μL/L)*	*Molecular SO_2 by HS-GDT (mg/L)* [b]	*Molecular SO_2 by HS-GDT, CV (%)*
0.14	0.45	<LOD	<LOD
0.29	0.84	0.23	30%
0.46	1.91	0.41	14%
0.55	2.94	0.58	6%
0.84	4.85	0.90	8%
1.13	6.15	1.12	3%

[a] Standards prepared in potassium bitartrate buffer, 12% ethanol, pH = 3.56, T = 21 °C. Molecular SO_2 calculated from Henderson-Hasselbalch equation using $pK_a = 1.99$.
[b] Molecular SO_2 concentration predicted from P_{SO2} by inversion of calibration curve, $P_{SO2} = 5.99$ [Molecular SO_2] + 0.55[c] Abbreviations: CV = coefficient of variance, LOD = Limit of detection

A-O vs. HS-GDT: Comparison with Commercial Wines

Twenty seven commercial wines were evaluated for molecular SO_2 by both HS-GDT and A-O methods. The wines covered a wide range of styles as well as free SO_2 concentrations (4-51 mg/L by A-O). A good correlation was observed between the two methods in white and blush wines (r^2 = 0.89, plot not shown), and on average HS-GDT values were 85% that of A-O values. However, a much weaker correlation was observed for red wines (r^2 = 0.46, plot not shown), and HS-GDT values averaged 50% lower than A-O values (Figure 21).

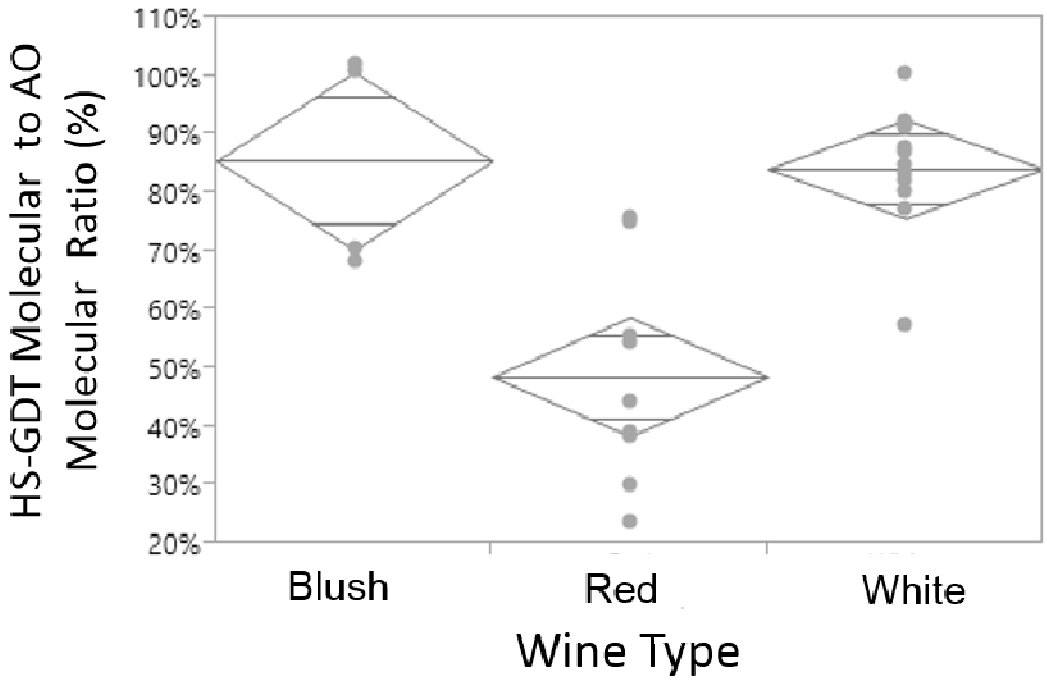


Figure 21 Ratio of 'Molecular SO_2 measured by HS-GDT' to 'Molecular SO_2 by A-O', grouped by wine type. Plots depict individual wine values (circles), mean values (center lines), overlap regions among wine types ($p<0.05$), and 95% confidence intervals (diamond tips)

Regression analysis was then performed to determine which wine components were correlated with a lower ratio of HS-GDT / A-O measurements. As shown in

Table 16, most wine components were uncorrelated with this ratio, with the exception of total anthocyanins (range = 36-221 mg/L; median = 146), and to lesser extent monomeric anthocyanins. Thus, the large discrepancy between the two methods for red wines is likely due to artifactual dissolution of weakly bound anthocyanin-bisulfite adducts during A-O sample preparation steps (acidification, dilution) and the long subsequent sampling time (15 minutes) (Rankine and Pocock 1970a). A similar overestimation would be expected with other "standard" approaches such as the Ripper iodometric titration. A limited number of previous studies that have used non-perturbing methods to quantify SO_2 in red wines, and have observed similar results. A group using a non-perturbing headspace GC method reported a 45% higher free SO_2 value in a single red wine as compared to A-O (Davis et al. 1983b). Thesis work by Bogren reported free SO_2 values up to an order of magnitude higher by Ripper and A-O as compared to the non-perturbing CE method (L.N.H. Bogren 1996), and another report using a colorimetric method observed similar degree of over-estimation (Burroughs 1975b). Interestingly, in our work, polymeric anthocyanins (defined by HPLC elution time) did not correlate with the HS-GDT / A-O ratio, likely because components of this late eluting peak are known to be less susceptible to SO_2 bleaching (Versari et al. 2008).

Table 16 Correlation coefficients between wine GDT/AO ratios and wine chemical parameters

Correlation	*Correlation coeff., R*	*P value*
GDT/AO Ratio . . . × Ethanol (red or white wines) × Malic acid (red or white) × residual sugars (red or white) × volatile acidity (red or white) × catechin (red) × quercetin glycosides (red) × tannin (red) × polymeric anthocyanins (red)	n.s.	n.s.
GDT/AO Ratio × monomeric anthocyanins (red)	0.67	0.047
GDT/AO Ratio × total anthocyanins (red)	0.80	0.011

The slightly lower values (85%) in white and blush wines by HS-GDT as compared to A-O are likely due to dissolution of other weakly bound adducts, such as those formed by pyruvate, diacetyl, or α-ketoglutarate (Leonard F. Burroughs and Arthur H. Sparks 1973). Several sweet wines were included in the study (up to 220 g/L fructose + glucose; median for all wines = 19 g/L). The lack of correlation of between methodological differences and residual sugars was somewhat surprising, since glucose can act as important sink of bound SO_2 in sweet wines and the dissolution of glucose-bisulfite complexes has been suggested as a potential cause of free SO_2 overestimation (Boulton et al. 1999). However, the first order rate constant for the dissolution of the glucose-bisulfite complex is reportedly 3.7×10^{-4} min^{-1} at pH~1 (Vas 1949b), and thus this complex is expected to be stable during the time of an A-O analysis. By comparison, the first order rate constant for the dissolution of

anthocyanin-bisulfite adducts is approximately 2×10^{-1} min^{-1} (Raymond Brouillard and Jean Michel El Hage Chahine 1980).

While additional validation of the HS-GDT method would be desirable, traditional approaches to method validation, e.g. standard addition, are not appropriate for free and molecular SO_2 in wine matrices. SO_2 additions result in variable increases in free or molecular SO_2 because of varying concentrations of SO_2 binders across wines. Further validation of the HS-GDT method could be done by comparison with free (or molecular) SO_2 values determined by other techniques that preserve equilibria, such as headspace GC or CE.

Conclusion

These preliminary results appear to confirm the occasionally noted (and often overlooked) observation that standard approaches for measurement of free and molecular SO_2 result in overestimation of both due to dissolution of weakly bound bisulfite adducts. This issue is of particular concern in red wines due to the high concentration of labile anthocyanin-bisulfite adducts. Additionally, common estimates for pK_a used in wine analysis are often incorrectly low because of the significant and frequently overlooked effects of temperature, ionic strength, and ethanol on this value. However, accurate measurements of both molecular and free SO_2 are expected to be critical for future work on the mechanisms of wine oxidation or detailed investigations of microbiological tolerance to SO_2. The HS-GDT approach described here may be a useful, inexpensive tool for these investigations, by providing a rapid measurement of the accurate molecular and free SO_2 concentration of a wine.

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CHAPTER 4: FUTURE DIRECTIONS: THE IMPACT OF TRUE VS APPARENT MOLECULAR SULFUR DIOXIDE ON VIABILITY OF WINE MICROBES

Introduction

Spoilage faults in commercial wines are tracked each year at the International Wine Challenge in London, and have held steady over the years at between 6 and 7% (Goode and Harrop 2008). The specific faults are somewhat dynamic and responsive to changes in wine production; cork taints have decreased and reduction/sulfides have increased as the industry shifted to screw caps. However, the faults directly attributed to microbial spoilage (volatile acidity, *Brettanomyces*, and biogenic amines) along with sulfur dioxide faults have remained steady at about 19-20% of all faults (excluding the data on "oxidation"). As sulfur dioxide (SO_2) is the primary antimicrobial agent used in winemaking, this data demonstrates that SO_2 mismanagement, resulting in either direct SO_2 faults or indirect faults from undesired microbial spoilage, is a problem for about 1.3% of commercial wines.

Molecular SO_2 is typically determined by measuring the free SO_2 and the pH of the wine, and mathematically calculating the percentage of the free SO_2 in the molecular form at the sample pH using a modified Henderson Hasselbalch equation (Vas 1949). Unfortunately, the most commonly used analytical methods are inappropriate for determining free SO_2 in wine, due to the disruption of the target analyte equilibrium during analyses (Burroughs 1975; Rankine and Pocock 1970). Additionally, the influence of alcohol, temperature, and ionic strength of the wine on

the acid dissociation constants of sulfur dioxide is not accounted for during the calculations, and the impact can be significant (Usseglio-Tomasset 1984). With limited accuracy for determining the molecular SO_2, most previous work on levels needed for controlling wine spoilage organisms should be questioned. Industry proficiency data also reveals that current analytical methods are imprecise (Butzke 2002; Butzke and Ebeler 1999). Thus, current best practice is to use imprecise and inaccurate analytical methods to match antimicrobial molecular sulfur dioxide levels determined by studies using equally erroneous methods.

Sulfur dioxide has been used as an antimicrobial agent (and an antioxidant) in winemaking since at least the end of the 18^{th} century; burning elemental sulfur, which produces SO_2 gas, has been used even longer for preserving wine containers (Boulton et al. 1995) . Although it was shown that the antimicrobial activity (anti-fungal, specifically) was related to free rather than total SO_2 (Ingram 1948), it wasn't until the 1970's that it was demonstrated that the antimicrobial action was based on the concentration of molecular SO_2, thus explaining the pH dependence. Radioactive sulfur dioxide was used to determine that molecular SO_2 was the form taken up by *Saccharomyces cerevisiae*, and uptake rates were quite rapid, reaching maximum at about two minutes (Macris and Markakis 1974).

Sulfur dioxide as wine organism antimicrobial in non-wine media:

Molecular SO_2 decimal reduction times (the time required to kill 90% of the population) were calculated for *Saccharomyces cerevisiae* in growth media at 20 °C as

19 min for 2.56 mg/L, 25 min for 1.28 mg/L, and 83 min for 0.64 mg/L (Macris and Markakis 1974).

Saccharomyces cerevisiae germicidal levels (concentrations required to get to one or fewer viable cells/mL) and decimal reduction concentrations after 24 hours at "room temperature" in growth media with initial cell counts of 5 x 10^4 cfu/mL were found to be and 1.41, 1.74 and 1.54 mg/L molecular SO_2 and 0.30, 0.24, and 0.32 mg/L molecular SO_2, respectively. *Hansenula anomala* kill levels and decimal reduction concentrations after 24 hours at "room temperature" in growth media with initial cell counts of 7 x 10^3 cfu/mL were found to be and 1.10, 1.01 and 1.04 mg/L molecular SO_2 and 0.17, 0.11, and 0.09 mg/L molecular SO_2, respectively (King et al. 1981).

The Long Ashton Research Station, working with a synthetic wine (10% ethanol) calculated the concentration of free and molecular SO_2 needed to reduce the microbial of population by 10^4 in 24 hours for *Lactobacillus plantarum* (4 mg/L molecular), *Saccharomyces bailii* (2.5 mg/L molecular), *Brettanomyces* species (0.625 mg/L molecular), and *Pichia membranefaciens* (0.25 mg/L molecular) (Beech et al. 1979).

Eighty one strains of *Leuconostoc oenos*, (now *Oenococcus oeni*), 23 strains of *Pediococcus parvulus*, and 22 strains of *Lactobacillus* species were subjected to various total SO_2 levels in a Mann Rogosa Sharp plus tomato juice broth (MRS-TJ) at pH 4.5 and incubated at 30 °C. Percentages of the numbers of strains with growth after 14 days were reported. As typical in many of these studies, free and molecular SO_2 values were not reported. Calculations made assuming no bound SO_2 were used to

generate presumptive molecular values from the author's data; the higher temperature effects on the pK_1 value are also shown (Davis et al. 1988a).

Although not a viability study, Carreté et al reported that in a buffered media at pH 4 incubated at 27 °C, a control of *Oenococcus oeni* took 2 days (at 0 % ethanol) and 3 days (at 7% ethanol) to metabolize 3 g/L of malic acid. The same culture with 2 mg/L or 5 mg/L added SO_2 required 2 days or 45 days (at 0 % ethanol) and 15 or 45 days (at 7% ethanol), respectively to metabolize 3 g/L of malic acid. Note that this is a very low level of added SO_2, and indicates that the *Oenococcus* malolactic function may be very sensitive to SO_2. When five strains of *Oenococcus* were subjected to 20 and 40 mg/L SO_2 additions (pH 5 and 27 °C), their ATPase activity levels became less than 50% of that of a control. As ATPase has been proposed as the control for intracellular pH and proton transport, and because malolactic conversion has been described as generating a proton motive force for ATPase activity, these authors proposed that any loss in cell viability and malolactic ability in *Oenococcus* from SO_2 additions may be due to the inhibition of ATPase (Carreté et al. 2002).

Yeast sulfur dioxide studies in wine and juice

In 1979, the International Organization of Wine and Vine (OIV) presented a review of the role of sulfur dioxide in winemaking; it recommended between 0.54 and 0.9 mg/L molecular SO_2 in must prior to fermentation to suppress aerobic spoilage yeasts such as *Hanseniaspora*, *Hansenula*, *Pichia* and *Kloeckera*; a table is provided of suggested total SO_2 additions by pH required to obtain these molecular levels 24 hours later based on observed SO_2 binding capacities (Beech et al. 1979).

A dry white wine was centrifuged and supplemented with 36 g/L equimolar glucose/fructose and adjusted to give a pH of 3.6 and 3.3 and an alcohol of 11 and 12.5% and inoculated with *Saccharomyces bayanus* in a declined phase (unknown inoculum level); half of these were supplemented with 80 mg/L total SO_2 to give a free SO_2 of 30 mg/L, and the other half supplemented to 120 mg/L total SO_2 to give a free SO_2 of 50 mg/L. The combination of pH and SO_2 addition levels resulted in wines with 0.5 mg/L, 0.8 mg/L, 0.95 mg/L, and 1.55 mg/L molecular SO_2. These four molecular SO_2 and two alcohol levels were then stored at both 10 and 19 °C, and viable cells enumerated after 16 days. At 0.5 mg/L molecular SO_2, the warmer temperatures resulted in 10^7 cfu/ml decrease in cell counts, and the colder temperatures in 10^2 *Saccharomyces bayanus* cells/mL. No viable cells were found in any of the 1.55 mg/L molecular treatments. The in-between levels of 0.8 and 0.95 mg/L molecular SO_2 were less definitive. No cells were found at 0.8 mg/L at the colder temperature and higher alcohol, but between 10^2 and 10^5 cfu/mL in the other treatments. No cells were found at the 0.95 mg/L molecular at the colder temperature at the lower alcohol, and between 10^1 and 10^5 cfu/mL in the other treatments (Sudraud and Chauvet 1985).

A fermentation from botrytised grapes at different alcohol levels (11.8, 12.8 and 13.6 %) was adjusted to three pH levels (3.45, 3.6, and 3.8); 240 mg/L and 300 mg/L SO_2 added, the free SO_2 measured and the molecular calculated, and viable yeast cells (genera not described) enumerated 15 days later. All wines with molecular SO_2 levels below 0.9 mg/L contained viable yeast cells, while 8 of the 9 wines at 0.9 mg/L or above contained no viable yeast cells (Sudraud and Chauvet 1985). However,

molecular SO_2 was calculated from free SO_2 (method not stated) and pH while using a pKa of 1.81 with alcohol levels ranged from 11.8 to 13.6 %. These factors alone will result in a pKa values between 1.4 and 2.0; the resulting inaccuracy make these conclusions hard to support.

A survey of 21 sweet and semisweet wines at risk for refermentation were sampled from a cellar and stored for one week at 25 °C and then viable yeast cells (genera not described) enumerated. All 10 wines with less than 0.9 mg/L molecular SO_2 had viable yeast cells; 8 of the 11 wines above 0.9 mg/L molecular SO_2 had no viable yeast cells (Sudraud and Chauvet 1985).

Yeasts (genera not described) in Sauterne wines were counted using direct epifluorescence method (DEFT) and plating methods before and after a "standard" sulfur dioxide addition, ranging from 200 to 250 mg/L total. Virtually no difference was seen in the cell counts from the two methods prior to the SO_2 addition; after the addition, the plating counts dropped to zero cells/mL within 12 days and stayed at zero until monitoring stopped at 34 days after the addition. DEFT counts dropped only a small percentage during the same time period. No other details on the pH or free SO_2 were available. The same authors repeated the situation on two different *Saccharomyces cerevisiae* strains, a *Zygosaccharomyces bailii*, and a *Candida stellata*, and then added a recovery step at 96 hours after the 250 mg/L total SO_2 addition. The recovery step was a 1/5 dilution step with 50% unsulfured must. The recoveries were measured by the relative intensity of the fluorescent stain; the *Zygosaccharomyces bailii* yeast, known for being resistant to sulfur dioxide, had the strongest recovery, while the *Candida stellata* showed virtually no recovery. The two

Saccharomyces strains were intermediate to the two other yeasts. Recovery using acetaldehyde to bind the free SO_2 was not found to be effective by these authors (Divol and Lonvaud-Funel 2005).

In a thorough study of *Brettanomyces bruxellensis* B3a viability (by DEFT) and culturability (by plate counts), no changes in DEFT viability counts were observed after 2 days exposure to 0 mg/L, 0.1 mg/L, 0.25 mg/L, and 0.8 mg/L molecular SO_2, but the corresponding plate counts for the 0.25 and 0.8 mg/L molecular SO_2 were 0 cells/mL for the same time period; initial counts for all methods were approximately 5 x 10^5 cells/mL. Short term exposure of *Brettanomyces bruxellensis* B3a to 0.64 mg/L molecular SO_2 resulted in zero cells/mL after 330 minutes, along with a drop in the epifluorescence intensity, but no change in the total DEFT viability counts for the same time frame; initial cell counts for all methods was approximately 1 x 10^5 cells/mL. Exposure to 0.64 mg/L molecular for times as short as 5 minutes resulted in a drop from 1 x 10^6 cells/mL to 1 x 10^4 cells/mL per the plating viabilities and intensity of the epifluorescence while still having no effect on the DEFT viability cell counts; after 45 minutes continued exposure, plating and epifluorescence intensity indicators had dropped to 0 cells/mL while still not change in the DEFT cell counts. Sulfur dioxide for this study was measured by the Ripper method (du Toit et al. 2005).

Lactic acid bacterial sulfur dioxide studies in wine and juice

Lactic acid bacteria (specific genera not described) populations in a wine with a of pH of 3.6, an alcohol of 11.25%, a free SO_2 of 0 mg/L and an total SO_2 of 200 mg/L declined when pH was dropped to 3.3, alcohol raised to 12.5, or SO_2 added at 20

and 40 mg/L - but the populations made an unexplained recovery after 100 days with the SO_2 ; no corresponding measurements on the sulfur dioxide levels were provided (Lafon-Lafourcade et al. 1983).

A red wine which had just completed malo-lactic fermentation, with bacterial (presumably lactic acid bacteria, but specific genera not described) plating cell counts of 1.2×10^6 cells/mL and direct epifluorescence method (DEFT) cell counts of 3×10^6 cells/mL was treated with an addition of 30 and 50 mg/L SO_2. Within two days, the plating counts were showing less than 1 cell/mL, while the DEFT viability counts were at 44×10^5 for the 30 mg/L addition and 44×10^4 for the 50 mg/L addition. No values were provided for temperature, alcohol, pH, or pre- or post- free SO_2 levels for this study. However, this was an early mention of possible VBNC states in wine bacteria, or at least a mention of the discrepancy between the direct cell counts and the plating methods. No recovery or resuscitation steps were applied to this wine to validate that conclusion with the SO_2 treated wine; in a separate trial the authors applied a recovery step to another discrepant condition and showed removal of the discrepancy in the two counting methods, claiming enough justification to note the existence of a VBNC state in wine lactic acid bacteria (Millet and Lonvaud-Funel 2000).

Bacterial (presumably lactic acid bacteria but specific genera not described) cell counts and malic acid conversion were monitored from the beginning of an inoculated and a wild red wine alcoholic fermentation for 70 days at two SO_2 addition levels (40 mg/L and 100 mg/L). Final wine pH was 3.6-3.7 and final alcohol was 12.5-12.8%. Free SO_2 levels at the end of the fermentation were circa 5 mg/L for the

40 mg/L addition and circa 10 mg/L for the 100 mg/L addition. All treatments except the inoculated and 100 mg/L addition level completed malolactic conversion within 50 days; this treatment showed no change in malic acid, and never exceeded 10^3 cells/mL. Again, inadequate information was provided to understand the molecular SO_2 levels in this study. Although not discussed, it is presumed that the wild fermentations might have produced additional aldehydes, resulting in lower free SO_2, which could explain the higher cell counts and faster malic acid conversions in both SO_2 levels with the uninoculated alcoholic fermentation (Reguant et al. 2005).

Acetic acid bacterial sulfur dioxide studies in wine and juice

Du Toit et al (2005) make reference to earlier studies which found strain dependent sensitivity of *Acetobacter pasteurianus* to molecular SO_2 concentration was found; levels needed to prevent growth ranged from 0.05 mg/L to 0.6 mg/L, while levels needed to prevent the growth of *Gluconobacter hansenii*, another acetic acid bacteria, was 0.8 mg/L molecular; it is not known what analytical method was used to determine the sulfur dioxide levels; the culture matrix is also not stated (du Toit et al. 2005).

Exposure of *Acetobacter pasteurianus* A8 to 0 mg/L, 0.35 mg/L, 0.8mg/L and 1.2 mg/L molecular SO_2 resulted in viability vs culturability differences. After a small drop after the first day, no changes in the DEFT viable cell counts occurred at any level, although the intensity of the dye did drop. However, *Acetobacter pasteurianus* A8 plate counts dropped to 0 cells/mL for the 0.8 mg/L and the 1.2 mg/L molecular

SO_2 after 2 days. Starting cell counts by all methods were approximately 1.2 x 10^5 cells/mL (du Toit et al. 2005).

A higher toxicity of molecular SO_2 at higher temperatures (30 °C) was rationalized as being due to a higher metabolic rate, and thus a faster accumulation and metabolization by Acetobacter species versus as 15 °C and 22 °C (du Toit and Lambrechts 2002).

Bound sulfur dioxide as antimicrobial agent

Two strains of heterofermentative lactic acid wine bacteria (*Lactobacillus hilgardii* and *Leuconostoc mesenteroides*) were found to rapidly attack the aldehyde in the bisulfite-acetaldehyde adduct and liberate enough free sulfur dioxide to prevent further growth, while a homofermentative wine bacteria (*Lactobacillus arabinosus*) consumed much less aldehyde than the others, but also failed to grow in the 100 mg/L bound SO_2 levels. The authors did not know if carbonyl bisulfite adducts would have the same effect (Fornachon 1963).

The anthocyanin binding of 30 mg/L SO_2 delayed the start of fermentations by *Saccharomyces cerevisiae*, *Saccharomyces bayanus, Saccharomyces uvarum,* and *Saccharomycodes ludwigii* by less than predicted, based on its effect on the available molecular SO_2; the authors conclude that this bound fraction only slightly affects the power of the molecular SO_2 to penetrate the cell wall and maintain its antiseptic ability (Usseglio-Tomasset et al. 1982). It was not clear how the sulfur dioxide was measured.

Acetaldehyde-bound SO_2 had no effect on *Lactobacillus plantarum*, a homo-fermentative lactic acid bacterium; some researchers believe the hetero-fermentative cocci are more sensitive to SO_2 than the homo- or hetero-fermentative rods, since they were affected by the acetaldehyde-bisulfite complex (Vivas et al. 1997).

Acetobacter pasteurianus A8 was also insensitive (by DEFT, epifluorescence intensity, or plate counts) to 0 mg/L, 80 mg/L, or 160 mg/L of bound SO_2 when no free was present, being deliberately bound by the addition of acetaldehyde (du Toit et al. 2005).

Brettanomyces bruxellensis B3a viability (by DEFT) and culturability (by plate counts), showed the yeast to be completely indifferent to 160 mg/L of bound SO_2 in a red wine (with no measurable free SO_2 due to binding with added acetaldehyde) over a two day period as measured by epifluorescence intensity (du Toit et al. 2005).

Acetaldehyde is normally metabolized by *Oenococcus oeni* during the malo-lactic conversion, but it was shown that when the aldehyde is bound to SO_2, even when no measurable free SO_2 is found by Ripper analysis, the growth of the bacteria *Oenococcus oeni* EQ54 and *Oenococcus oeni* VFQ is adversely affected. The two theories used to explain this are, first, that there is enough free SO_2 (sub-measurement) to impact the bacteria or, second, that the acetaldehyde bisulfite complex is in itself inhibitory (Osborne et al. 2006).

Difficulty in interpreting existing studies

As it is clear from the literature review, much of the work on the effect of SO_2 on wine microbes has been lacking adequate control of many factors; additionally,

none of these studies had access to any method able to show the true molecular SO_2 at the onset of the trial, much less at later stages. We have shown that the traditional molecular SO_2 method can result in a higher molecular SO_2 value relative to the new method, at least in red wines, and now we need to see which value is a better indicator of antimicrobial action Although a simple observational study will not advance the understanding of the complexity and dynamics of the chemistry and microbiology of sulfur dioxide as an antimicrobial in wine, we can at least determine which method is a better analytical tool for future studies. Several recent works have demonstrated the value of modeling and factorial design on multiple variables in wine and sulfur dioxide microbiological experimentation (Sturm et al. 2014; Zuehlke and Edwards 2013). These approaches, in combination with more traditional log reduction information typically used for the effects of temperature might provide some helpful direction and examples.

The concept of "True" molecular SO_2 vs "Apparent" molecular SO_2

Microbiologists frequently choose an exposure temperature conducive to microbial activity, such as 25 or even 30 °C; if molecular SO_2 is reported in these studies, this temperature impact on the pK_1 value and on the resulting molecular SO_2 level is not considered. It is not clear in any of the reviewed articles if temperature, alcohol, or ionic strength effects were considered in their calculations of molecular SO_2 levels, and thus perhaps all values should be noted as "apparent" molecular SO_2 values. An example of how the "apparent" and "working" values can be critical is shown in the early work of Macris and Markakis. These authors may not realize have

been aware that the "true" SO_2 the higher temperatures was much greater than the "apparent", resulting in a proposal of a mediated yeast cell membrane transport system to explain the faster-than-diffusion exchange rate (Macris and Markakis 1974). Microbiology studies also have not accounted for the errors in measurement of the free SO_2 to begin with, which would overestimate the free SO_2. In a convenient but misleading way, these two sets of errors may have canceled each other out, resulting in "apparent" molecular values which may have approached the "true" molecular values. Until this is controlled or standardized, the reported levels of molecular SO_2 that are published as effective at 25 °C cannot be applied at cellar (10-15 °C) temperature without the potential for failure. To confuse this further, microbial growth itself is modulated by temperature (Fugelsang and Edwards 2007).

True addition levels of SO_2 and their maintenance during time course of experiments

Several recurring issues are observed in the microbiology experiments involving the amounts of sulfur dioxide which make the global picture difficult to grasp. Additions of sulfur dioxide, usually made from potassium metabisulfite liquid solutions and sterilized by filtration, are assumed to accurately meet of the target value. Chemists are more familiar with the instability of these solutions (Green and Hine 1974) and the potential for rapid degradation, possibly made worse by the introduction of oxygen during a filtration step. Additions using these untrustworthy solutions into culture media are rarely confirmed for the true total and free levels. Total and free SO_2 levels are not checked during the course of the test to determine if loss from oxidation, volatilization, or binding might be occurring during the course of

the test or if levels might be increasing due to the productions by yeast. Changes in sulfur dioxide levels and equilibrium due to production of binding compounds such as aldehydes and ketones or the production of sulfur dioxide may not be controllable but they should at least be monitored.

Future Directions

We would propose for a future direction that, by using the new method, determine molecular sulfur dioxide decimal reduction values and kill rates for wine spoilage microbes. Variables to control include (in addition to the selected microbe): molecular SO_2 concentration, cell concentration, temperature, pH, alcohol level, exposure time, initial oxygen level, and bound SO_2. Traditional molecular SO_2 measurements (determined using free SO_2 and pH) will be also performed to provide a comparison. Viability will be determined using several methods (direct cell counts with staining, traditional plating, and quantitative RT-PCR (Scorpions)) to determine if cell death, cell injury, or VBNC states are induced. Target genera may include one or more of the following: the yeasts *Saccharomyces*, *Brettanomyces*, and *Zygosaccharomyces*, and the bacteria *Acetobacter*, *Gluconobacter*, *Pediococcus*, *Oenococcus*, and *Lactobacillus*. We would hypothesize that the use of headspace molecular SO_2 analysis will yield microbial decimal reduction values different from those determined using existing analytical methods and commonly referenced in winemaking literature, when available, particularly in red wines and sweet wines with high SO_2 binding capacities.

We would further propose to verify the induction of VBNC states by sub-lethal molecular SO_2 levels in wine microbes by observing differences in viability measurements and by a successful resuscitation or recovery step. VBNC status is defined by the inability to complete cell division and grow on solid media. Quantitative RT PCR (Scorpions) cell counts are based on intact RNA; exposure of RNA to the acidic alcoholic wine matrix precludes. We would hypothesize that: Sub-lethal molecular SO_2 can induce VBNC states in wine yeast and bacteria which will be evident by differences in viable counts compared to cultured counts.

Additional considerations for future wine microbial work involving SO_2

Sample preparation and sterile filtration

Some work documenting possible VBNC states induced by sulfite has shown that much smaller cell size is typical of the VBNC condition, possibly as a mode of energy conservation (Serpaggi et al. 2012). These smaller cells can pass through pore sized of filters generally regarded as adequate for healthy cells; the wine industry generally works with the assumption of 0.45 microns as adequate to remove all wine microbes; if cells are in the VBNC state a pore size of 0.20 microns would be necessary.

Cleavage by sulfur dioxide of molecules critical to microbial growth

Bisulfite ions can cleave thiamin, which has been reported as a critical nutrient for *Brettanomyces* and some lactobacilli (König 2009); however, others report that the half time for 10 mg/L SO_2 concentration is 319 days at pH 3 and 72 days at pH 3.5, too slow to prevent thiamin dependent microorganisms in the first months of aging. It might, however, contribute to thiamin deficient musts or wines after longer term storage (Boulton et al. 1995); a similar cleavage has been reported for NAD and folic acid.

Oxygen as an additional variable

Oxygen levels can affect the viability or growth rates of yeast and aerobic bacteria and could contribute to sulfite oxidation and loss (Aceituno et al. 2012; Danilewicz and Wallbridge 2010; Davis et al. 1988b; du Toit and Pretorius 2002; Fugelsang and Edwards 2007).

Decimal/log reduction calculations and starting cell concentrations

Decimal or log reduction graphs have been used by several early researchers in molecular SO_2 (Beech et al. 1979; King et al. 1981) ; it seems more common with other antimicrobial agents such as sodium hypochlortie and hydrogen peroxide (Malik et al. 2013; Winniczuk and Parish 1997). It is expected that initial cell concentrations will have an impact on these decimal recution values as seen in earlier work (King et al. 1981) .It will be important to stress the impact and ramifications of different starting inoculum levels on the effectiveness of the same treatment.

Microbe culture preparation

Pure cultures of wine yeast and bacteria can be obtained from commercial sources (http://www.laffort.com/ , http://www.lallemand.com/ http://www.chr-hansen.com/) or public collections such as the UC Davis wine culture collection (http://wineserver.ucdavis.edu/collection/index.php) via a materials transfer agreement, or isolated, purified and identified from local sources. Conditioning and culturing purified cultures for inoculation can be performed in a variety of ways (Monk and Storer 1986). For this experiment, the first stage media will be a 50% wine (fined with active charcoal to remove phenolics and SO_2, then 0.22 micron sterile filtered) with 50% organism-specific heat sterilized culture broth (such as yeast extract-peptone-dextrose (YPD) for yeast, Mann Rosa Sharpe with tomato juice (MRS-TJ) for lactobacilli (OIV 2010) . One colony of pure culture will be used to inoculate the stage one media, and then grown at 25 °C for 2 days or until a high cell density is obtained. This culture will be used in the second stage to inoculate the undiluted fined and 0.22 µ filtered wine and grown until an acceptably high optical density at 600 nM (i.e., a value of 1 for a 10 mm path), and then inoculated into the wine chosen for the experiment (du Toit et al. 2005).

Staining and counts

Viability stains indicate the cell's capacity to reduce a dye from colored to clear (methylene blue) or hydrolyze fluorescent chemical such as acridine orange or fluorescein diacetate (Fugelsang and Edwards 2007). Methylene blue is the most common for wine microorganism; the toxicity of the dye necessitates a relatively short

window for counting (Fugelsang and Edwards 2007); other viability stains include the LIVE/DEAD yeast viability kits with a proprietary FUN-1 stain, or bacterial LIVE/DEAD kits, which utilize propidium iodide and SYTO 9 nucleic acid stains (Molecular Probes, Eugene, OR).

Cell counting chambers are (using Levy Hauser chambers for yeast, and the smaller volume Petroff Hauser for bacteria) and are usually used for total counts; viable counts can be determined by first determining the relative percentage of live and dead cells on a regular slide and then applying that percentage to the counts determined with the chambers (Fugelsang and Edwards 2007; OIV 2010; Mills and Howe 2004).

Plating and plate cell counting

Current recommendations of solid media for wine yeast and bacteria, with mention of additional selectivity with the use of specific antibiotics such as natamycin (pimaricin) to inhibit yeast, chloramphenicol to suppress bacteria, biphenyl to suppress mold, and cycloheximide to inhibit specific yeasts, are nicely summarized in the OIV methods (OIV 2010); if pure cultures are used the selectivity will not be needed. Cell counting by the drop plate method is an efficient and accurate method and can save on media and space (Herigstad et al. 2001; Hoben and Somasegaran 1982).

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